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# Flavor analysis of heritage apple varieties

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"Wenn du Apfel-Samen säst, erntest du Äpfel. Wenn du positive Gedanken in deinem Geist säst, erntest du positive Ergebnisse in deinem Leben."

Denis Herger

# Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material, which has been quoted either literally or by content from the used sources.

17.10.2019

Date

Signature

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Am Ende wird alles gut.
Wenn es nicht gut wird,
ist es noch nicht das Ende.

# Abstract

Fruit cultivation, especially the cultivation of apples, has had a long tradition in the southern regions of Austria. An annual apple harvest of about 200 000 tons, which represents about 85% of the overall Austrian apple harvest, demonstrates the importance of the crop for this geographical region. 75% are cultivated and harvested in "intensive farming" of only a few apple varieties, such as Golden Delicious, Gala or Braeburn. These three apple varieties can be found throughout the world on the apple market. The remaining 25% have been cultivated for several hundred years in traditional meadow orchards, which are characterized by the presence of very old trees as well as the co-existence with diverse flora and fauna. These orchards represent an enormous pool of mainly heritage apple varieties, for example Kronprinz Rudolf, Ilzer Rose, and Schafsnase. Many of the heritage varieties have not been cultivated in intensive farming. They often do not fulfill marketing requirements in terms of shape and appearance, crisp texture, good storage abilities. Recent developments regarding, for example, apple prices on the international market as well as the general trend among consumer towards a preference of regional products, have heritage apple varieties back on the map. Investigations of the intrinsic, as well as extrinsic factors of the flavor formation of the apple variety require a basic characterization of the fruit. Therefore, it was the aim of this study to perform a characterization of the flavor of heritage apple varieties from Styria. The impact of ripeness and different growing conditions (plantation or meadow orchards) was also analyzed. In addition, the formation of the volatile compounds was determined in intact apples, and also differences of the volatiles between apple peel and flesh were analyzed. The apple flavor/aroma is very complex, as a result different analytical methods have been proposed to isolate, identify and quantify different compounds that characterize apples. For flavor characterization, two different complementary approaches were used: (i) complete sensory characterization with the use of an expert panel of the intact apples and sliced apples after enzyme inactivation, (ii) identification of the volatile and odor active compounds by using gas chromatography-mass spectrometry (GC-MS) and gas chromatography – olfactometry (GC-O) using headspace solid-phase microextraction (HS-SPME) for the extraction and enrichment of the volatiles. In addition, the enormous capacity regarding separation as well as the sensitivity of comprehensive GC×GC-MS allowed a deeper insight into the flavor of the heritage apple varieties.

The flavor profiles of the heritage apple varieties were characterized by the important groups of alcohols (e.g. 2-butanol and 1-hexanol), aldehydes (e.g. (E)-2-hexenal and hexanal), esters (e.g. ethyl butanoate and methyl butanoate), and terpenes (e.g. α-farnesene and α-bergamotene). Additionally, high concentrations of terpenes were found in the peel of the variety Ilzer Rose. It was demonstrated, that the impact of ripeness is high for secondary flavor compounds which increased after the ripeness process. In combination with high concentrations of different alcohols, this leads to a shift in the distribution of volatile compounds in the apple peel in favor of terpenes and aldehydes.

# Kurzfassung

Der Apfelanbau hat speziell in den südöstlichen Regionen von Österreich eine lange Tradition. Eine jährliche Apfelernte von ungefähr 200 000 Tonnen, ergeben ca. 85% der österreichischen Gesamtapfelernte, kommen aus dieser Region und zeigen dadurch den enormen Wirtschaftsfaktor. Der Großteil der Apfelernte (75%) werden in intensiven Plantagenanbau kultiviert und geerntet, vor allem Apfelsorten wie Golden Delicious, Gala oder Braeburn. Diese drei Apfelsorten sind am weltweiten Apfelmarkt hauptsächlich zu finden. Die restlichen 25% der österreichischen Apfelernte werden aus den traditionellen Streuobstwiesen (extensiver Anbau) geerntet, die durch eine enorme Vielfalt an alten Obstbäumen aufweist und durch eine einzigartige Flora und Fauna charakterisiert wird. Diese Streuobstwiesen bieten einen großen Pool an alten Apfelsorten, Sorten wie Ilzer Rose, Kronprinz Rudolf und Schafsnase. Viele dieser Streuobstapfelsorten werden noch nicht im intensiven Plantagenanbau kultiviert. Die alten Apfelsorten erfüllen oft nicht die Kriterien für den Verkauf/Vermarktung in Bezug auf einheitliche Größe und Aussehen, Textur und gute Lagerstabilität. Jüngste Studien zeigen, dass zum Beispiel die Apfelpreise am weltweiten Markt großen Schwankungen unterliegen und auch der Trend von den Konsumenten wieder mehr auf regionale Produkte zurückzugreifen, die alten Apfelsorten vermehrt gefordert werden. Untersuchungen zu den unterschiedlichen Einflussfaktoren auf die Aromastoffbildung im Apfel und eine Basischarakterisierung der alten Sorten sind notwendig.

Das Ziel dieser Studie war es, eine umfassende Charakterisierung der alten Apfelsorten in Bezug auf das Aroma durchzuführen. In weiterer Folge wurde auch der Einfluss durch den Reifeprozess und der Kultivierungsart (Plantage oder Streuobst) des Apfels untersucht. Außerdem wurde die Aromastoffentwicklung von unversehrten Äpfeln analysiert, und auch die Unterschiede zwischen Apfelschale und dem Fruchtfleisch wurden genauer untersucht. Das Apfelaroma ist sehr komplex, dadurch wurden unterschiedliche analytische und sensorische Methoden eingesetzt um die unterschiedlichen Aromastoffe zu isolieren, identifizieren und quantifizieren.

Für die Charakterisierung der Aromastoffe, wurden zwei sich ergänzende Methoden eingesetzt: (i) eine komplette sensorische Evaluierung durch den Einsatz eines Expertenpanels von den unversehrten Äpfeln, aber auch von den enzym-inaktivierten Apfelstücken, (ii) Identifizierung von den flüchtigen und geruchsaktiven Verbindungen mit dem Einsatz von Gaschromatographie-Massenspektrometrie und Gaschromatographie-Olfaktometrie nach Anreicherung der flüchtigen Verbindungen durch Festphasenmikroextraktion (SPME). Zusätzlich wurden Analysen mit dem System der Comprehensive GC×GC-MS durchgeführt, die eine enorme Kapazität in Bezug auf Trennleistung und Empfindlichkeit aufweist. Das Aroma Profil der alten Apfelsorten wurde durch die chemischen Gruppen, Alkohole (z.B. 2-Butanol und 1-Hexanol), Aldehyde (z.B. (E)-Hexenal und Hexanal), Ester (z.B. Ethylbutanoat und Methylbutanoat) und Terpene (α-Farnesene und α-Bergamotene), geprägt. Des Weiteren konnten hohe Konzentrationen der Gruppe von Terpenen in der Apfelschale quantifiziert werden. Es konnte gezeigt werden, dass sekundäre Aromastoffe, die

beim Zerstören/Zerkleinern von den Apfelfrüchten entstehen, im Laufe des Reifeprozesses an Konzentrationen deutlich zunehmen. In Verbindung mit erhöhten Konzentrationen an Alkoholen führte dies in den Apfelschalen zu einer Verschiebung der flüchtigen Verbindungen zugunsten der Aldehyde und Terpene.

# Content

# Introduction

# 1. Scope and Outline of the Thesis

The specific objective of this study was to characterize selected heritage apple varieties of Austria and, in particular, of Styria. This research examines the emerging role of heritage apple varieties in context of the production of high-quality products such as cider and apple juice, as the knowledge about the flavor has been missing to date. Thus, one main focus of this dissertation is the identification and characterization of the flavor of heritage apple varieties. For the production of outstanding products from these varieties, the knowledge of the flavor properties is a prerequisite for the producers. Thus, this work aims to close this gap and to improve the understanding of the flavor of heritage apple varieties.

In addition to the interest of high-quality products, local products, such as authentic apple juices and ciders, dried apple slices or apple chutneys from Styria, have gained in significance over the years. Therefore, in order to enable the production of Austrian high-quality products, flavor identification and feasible process improvement of apple cider/juice production are important. Over the past years, studies have provided important information on the flavor formation in apple fruits. However, these studies did not include heritage apple varieties from Austria, nor the difference of flavor from apples grown in plantations or meadow orchards. Facing this incompleteness, this thesis focuses on the flavor analysis by the use of sensory evaluation and analytic techniques, such as GC-MS and comprehensive GC×GC-MS analysis. The overall structure of the thesis takes the form of six chapters, including the scope of the thesis, the introduction, the required theoretical background, material and methods, results and discussion, a conclusion and the future perspectives.

The first section of this dissertation will examine the important general facts about the apple fruit, also the apple cultivation, the production worldwide with a special focus on Austria, the difficulties in recent harvest years will also be discussed. Chapter 2 introduces the necessary theoretical background, it is based on a brief overview in the field of flavor science, but also a brief introduction of the important techniques of flavor analysis, sensory evaluation and analytical techniques. Chapter 0 presents the used Material and Methods for this study. Chapter 4 is dedicated to the outcome of the project, while the last part of this study, provides a comprehensive conclusion of the dissertation.

# 2. Theoretical background

This chapter introduces the necessary theoretical background of apple production, the botanical classification, the anatomy and the morphology of the apple fruit, as well as the history of apples.

## 2.1. General facts of the apple fruit

### 2.1.1. History of Apples

'From the Garden of Eden to the Big Apple, from William Tell to Johnny Appleseed, to Paris of Troy who gave the troublesome golden apple to Aphrodite which started the Trojan War. The apple has occupied a very special place in our affections both as a symbol and as one of the simplest and most delicious of Nature's gifts', written by Rosanne Sanders, a prize-winning apple artist from England (Way et al., 1991), is the perfect description of the most popular fruit worldwide.

The apple is a fruit with a long history, the origins of the apple fruit trees are found in Asia, specifically in the region of the central west corresponding with modern-day Kazakhstan, and archeological evidence locates the Tien Shan mountain forests as the probable origin of all known apple cultivators. Around 10.000 years ago, the first human settlements started to form across the near east, India and further east in China. The apple passed through Persia and Greece, and by the time of the Romans, the cultivated apple was transported throughout the empire and Europe (Janick, 2005). Prior to the good storage, the apples come into vogue in the whole continent of Europe. Besides, apples are also juiced and often used in recipes for many other products, for example as sweet additive or in jam. Apple technology, including grafting, pruning, and storage, dates back to the time of the Romans. Grafting has probably played a key role in the worldwide spread of apples. The Romans learned this practice from the Greeks and brought not only the grafting technique, but also apple cultivation, harvesting and storage technology to the rest of the Roman empire (Cornille, Giraud, Smulders, Roldán-Ruiz, & Gladieux, 2014). With the discovery of these grafting techniques, the cultivation of apples increased as well. The second center of diversification was established in the United States and most of the new apple varieties derive from American and Canadian selections. The contemporary apple is the result of interspecific hybridization and the number of species in the genus *Malus* is not completely defined (Musacchi & Serra, 2018). In 1826, the Royal Horticultural Society of England records 1200 different apple cultivars (Howes, 2016). The fixing of genotypes had a long-lasting effect on apple production, enabling varieties to be grown in orchards and providing horticulturalists with the possibility of selecting the best varieties (Gardiner, Bus, Rusholme, Chagné, & Rikkerink, 2007).

At the beginning of apple farming, the target was different than today: the taste and the healthiness were of primary objectives. Mendel's laws provided key findings, and after that, crossbreeding increased. In the 19$^{th}$ century, the East Malling Research Station in England created a standardized

collection of ten rootstocks with new names, two of them (M9 – Paradis Jaune de Metz and M7 – Doucin Reinette) are still used by horticulturists worldwide (Cornille et al., 2014).
The majority of heritage apple varieties is grown in so-called meadow orchards. These meadow orchards are a typical cultural landscape in Austria, especially in Styria. The local clones and rootstocks are performed to yield valuable information to help local apple farmers for growing high-value apple fruits. The rootstocks have different functions such as fixation, support, absorption, secretion, synthesis, effects on growth and developments of scions, fruit yield and quality, and the resistance to biotic and abiotic stress (Y. Wang et al., 2019). The East Malling Station in the United Kingdom started in 1917 the first apple rootstock breeding and application program in the world. This station screened 16 types of apple rootstocks, named from M1 to M16. After that, many countries created a hybrid breeding program of apple rootstocks by using the M series rootstocks. The worldwide cultivation system has changed since then because of this successful breeding program. M9 is the most commonly used rootstock in Western Europe (Samuolienė, Viškelienė, Sirtautas, & Kviklys, 2016) and was the first standardized rootstock available on a large scale in Europe. A huge number of M9 clones exist with varying qualities and seedlot vigors. One big disadvantage is that it is very susceptible to fireblight (Rühmer, 2009). But M9 rootstocks have good performance in terms of production, fruit size and quality control (Y. Wang et al., 2019).

### 2.1.1. Apple Cultivars and forms of cultivation

The domesticated apple has a very long history. At the beginning of the 13$^{th}$ century, the apples were grown in gardens throughout Europe and became more and more popular by the royalties and by the commoners. The consumption of apples ranged from the raw apple to ones cooked with spices, sugar and honey, and also to fermented juice and cider (Ferree & Warrington, 2003). The apple production was growing fast and the process for apple products was increasing across Northern and Eastern Europe. In the 16$^{th}$ century, the first apple varieties were described in German-speaking countries, varieties such as Goldparmäne, Weißer Winterkalvill, Roter Eisenapfel and Backapfel (Richter, 2013). The different varieties were very important for the farmers, as they had few trees with different picking dates and storage times in their gardens. Thus, due to the temporal offset, they could use the apples from early summer until the next spring.

At the end of the 17$^{th}$ century, a noticeable collection of different apple varieties such as Maschanzker, Großer und Kleiner Brünnerling and Limoniapfel (Grüll, 1949), existed in an Austrian cloister (Windhaag bei Perg). At that time, the worldwide center of pomology was in France, at the Carthusian cloister in Paris. The gardens of this cloister had a huge area with over a thousand different varieties and the pomologists throughout Europe obtained their basic material for new crossbreeding's from this cloister. The Austrian pomologists (Kraft, Märter, Moscon, and Schmidberger), directly or indirectly, received a variety of their material from Paris. The first important Austrian pomologist Johann Kraft (1738-1808) was the director of the fruit growing School in Vienna and wrote the fruit variety book "Pomona austriaca". Another very important Austrian pomologist was Dr. Rudolf Stoll (1847-1913), he was a co-founder of the Austrian pomology association (1881) and wrote the outstanding collected works "Österreichische-

Ungarische Pomologie" (Stoll, 1888). Heinrich Graf Attems-Petzenstein (1834-1909) was the president of the k.k. Styrian garden association and chairman of the k.k. Austrian pomology association. Under the guidance of Attems-Petzenstein, the k.k. Austrian pomology association took part in many European fruit exhibitions. Heinrich Graf Attems-Petzenstein himself organized a lot of fruit exhibitions, especially noteworthy the Vienna fruit exhibition in 1888. His extraordinary work of various descriptions and watercolor pictures of the apples was integrated into the book "Empfehlenswerte Obstsorten" of Löschnig, Müller, & Pfeiffer, 1914. With the end of the Austrian monarchy time also expired for the pomology association. After the First World War, the pomiculture association was established in Austria and each year, the Austrian pomologists held a meeting for representatives of farmers, the provincial governments, the fruit growing schools and the federal ministry of the agricultural economy and university of natural resources and life sciences. After the Second World War, the federal fruit growing association was established, which mainly represented farmers using plantation growing (intensive farming). This establishment was a huge structural change in the Austrian agriculture after the world war, mainly induced by the migration of farmers and the decreasing personal requirements of apple products.

Plantation growing or intensive farming started rapidly and the traditional form of apple farming, the meadow orchards or extensive farming, ran out. Intensive farming was easier for apple farmers because new agricultural tractors meant collecting more apples with less effort. In addition, the consumption behavior of the population had changed. Thus, the amount of the apple production of meadow orchards was halved between 1968 to 1988 (Bernkopf, 1994). By the end of the 1980s, the extensive apple farming slowly reemerged in peoples' minds and the apple consumption for this type of farming regained its significance. The definition of extensive farming[1] in Austria is "meadow orchards have consisted of different large fruit trees with big crowns, the trees should have fruits for consuming or for fruit processing products and the freestanding fruit trees are unassisted without any synthetic agricultural pesticides. This form of farming is an ecologically sustainable farming type by the location."

This cultivation form - meadow orchards (extensive farming) - represents an important cultural landscape not only in Austria, but also in other parts of the apple zone in Europe, such as Germany, Switzerland, and France. The definition of the traditional form of apple farming in Austria, the meadow orchards or extensive farming, was defined on 16.02.2017 by Arche Noah[2] and ARGE Streuobst[3]. Before that, there was no clear differentiation between intensive and extensive farming in Austria. This regulation was necessary for the distribution of aid money and a clear boundary between intensive and extensive farming. The ecologic significance of extensive farming is considerably higher than the value of intensive farming.

[1] Definition of Austrian extensive farming/meadow orchards: https://www.arche-noah.at/files/streuobst_definition_oesterreich_arche_noah_und_arge_streuobst_1.pdf
[2] Arche Noah: Seed Savers Association in Central Europe; https://www.arche-noah.at/
[3] ARGE Streuobst: Austrian working partnership for the conservation of fruit growing in extensive farming; http://www.arge-streuobst.at/

Heritage apple varieties are typically grown in meadow orchards and the amount of different apple varieties is huge in the extensive farming. Furthermore, these cultural landscapes are rich in microstructures, blooms, and fruits, which offer an outstanding habitat for birds (Richter, 2013).

For this thesis typical heritage apple varieties from Styria are used, but also typical varieties of the worldwide apple market which have been grown in Austria for decades. The variety Golden Delicious (Figure 1) is the most important apple variety in the world trade. It is a chance seedling from the Clay County farm, West Virginia (USA) of Anderson H. Mullins and was first discovered around 1890. This variety is a yellow-green apple with a light-yellow flesh and has a mild sweet flavor. The variety Kronprinz Rudolf (Figure 1) is the most famous Styrian apple. It was first discovered around 1873 as a chance seedling from Gleisdorf, Styria (Austria). The variety is named in honor of Rudolf, Crown Prince of Austria. The variety has a characteristic peel color with green as the base color, blood-red pigments The flesh is light yellow-white and juicy. The flavor of this variety is sweet, fruity and lightly spicy. One of the most interesting apple varieties is the Ilzer Rose (Figure 2). It is a chance seedling from Ilzberg bei Puch, Styria (Austria). Apples of this variety are rather small with intense red peel and white flesh. The flavor is sweet with floral notes and has favorable characteristics for producing juice and must/cider.

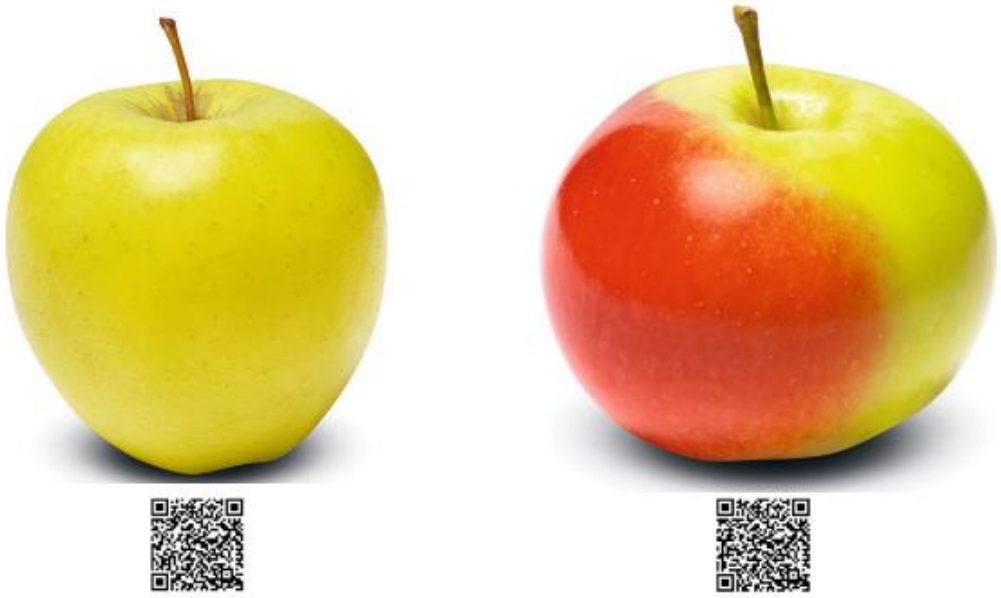

**Figure 1: The varieties Golden Delicious (left) and the variety of Kronprinz Rudolf (right).** The QR Code marked the source of the picture.

The apple variety Krummstiel (Figure 2) probably descended from the Rheinischer Krummstiel (Stoll, 1888) which was discovered about two hundred years ago in the area of Cologne (Germany). This variety is light orange-yellow in color and up to half is often striped in carmine red. The flavor is sweet-sour with spicy notes. It is well-known that this variety is suitable for storage and also resistant to frost and fungal diseases.

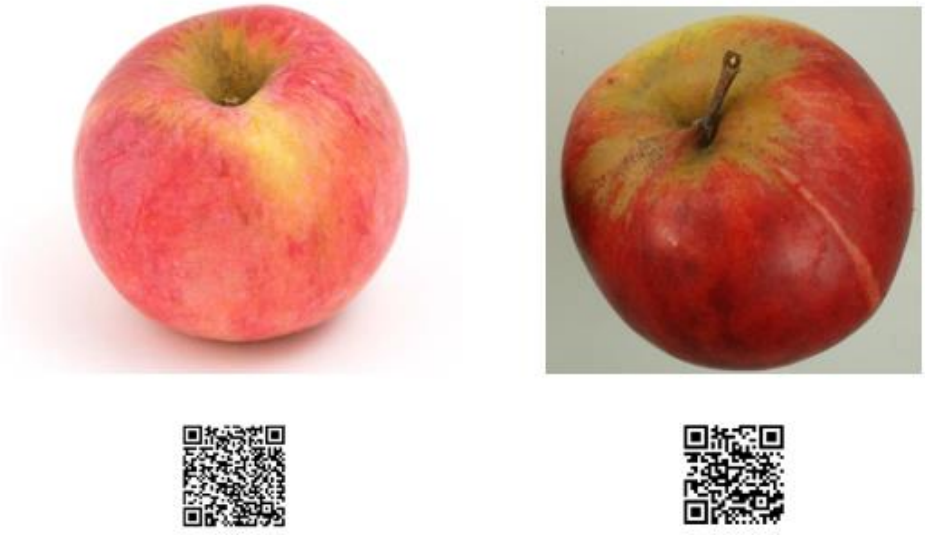

**Figure 2: The apple varieties Ilzer Rose (left) and Krummstiel (right):** The QR Code marked the source of the picture.

Another special apple variety is the Steirische Schafnase (Figure 3). It is named for its cylindrical, tall-conical fruit. The origin of this variety is located on Krim (Ukraine)[4], and the pomologists Liegel and Schmid brought it to Austria. In the first description it was named Ochsennase, but later renamed to Steirische Schafsnase. The peel of the apple is yellow-orange colored and flamed red, its mouthfeel is juicy, its texture medium-hard and its taste is sour-sweet. A very common heritage apple variety is the variety of Cox Orange (Figure 3). This variety was first discovered in 1830 by M.R. Cox in Colnbrocklawn (Great Britain). It descended from the variety Ribston Pepping[5]. This apple variety has a yellow base color and is covered in speckles that turn from lighter red to brown-red. It is a sweet apple with juicy flesh.

[4] Arche Noah apple description https://www.arche-noah.at/files/obstsortenblatt_2016_steir_schafnase_web.pdf
[5] Cox Orange description https://austria-forum.org/af/Heimatlexikon/Cox_Orangenrenette

**Figure 3: The apple varieties Schafsnase (left) and Cox Orange (right):** The QR Code marked the source of the picture.

The apple variety Goldrenette or Goldrenette von Blenheim is a chance seedling and was first discovered around 1740 in Woodstock, Oxfordshire (Great Britain). The name Blenheim is derived from the Palace of Blendheim, one of the biggest Palaces in England. This variety has a greenish-yellow to orange base color streaked with red and the flesh is yellow-white. The flavor is spicy and tart with a slightly juicy, sweet-sour taste[6].

## 2.1.2. Botanical classification, anatomy, and morphology of Apples

With respect to the botanical classification, the apple belongs to the Rosaceae family with the subfamily Maloidae, which includes a variable number of species depending on the different studies and different views on taxonomy (Phipps, R. Robertson, G. Smith, & R. Rohrer, 1990; Harris, P Robinson, & E Juniper, 2002; Juniper, 2007; Cornille et al., 2012). The apples are pomme fruits such as pears or quince. Apple trees with hermaphroditic flowers are self-incompatible plants (Miller & Gross, 2011).

The genus and the section are *Malus*, and the section consists of series *Malus,* including European and Asian species (*Malus sieversii* and *Malus x domestica*) (Phipps et al., 1990). There are many synonyms of the cultivates (or 'sweet') apple, but the most widely used one is *Malus domestica*. In the review of Cornille, Giraud, Smulders, Roldán-Ruiz, & Gladieux, 2014, four wild apple species (*Malus sylvestris*, *Malus sieversii*, *Malus orientalis*, and *Malus baccata*) are described, which are mostly pollinated by bees and flies. The presumed wild relative of the *Malus x domestica* Borkh is *Malus sieversii*, a crab apple from modern-day Kazakhstan. However, the precise origin of today's' apple is not entirely clear (Ferree & Warrington, 2003). The extraordinary diversity of wild apples in Kazakhstan and the morphological similarity between *Malus domestica* and *Malus sieversii* were

[6] Arche Noah apple description https://www.arche-noah.at/files/goldrenette_von_blenheim.pdf

first reported by Vavilov and then by Ponomarenko (Vavilov & Freier, 1951; Luby, Alspach, Bus, & Oraguzie, 2002). The modern domesticated apple *Malus domestica* diversified through cultivation and further hybridization over the past two millennia. As a result of hundreds of thousands of years of genetic isolation, the hybrids expressed larger fruits (Spengler, 2019).

Apples belong to the healthiest foods a person can eat. Apples contain important nutrients, in particular, polyphenols, vitamin C (ascorbic acid), B-complex vitamins, dietary fiber, and minerals, such as calcium, potassium, and phosphorus. Vitamin C has an important role as biosynthetic enzyme. The loss of vitamin through processing of apples is as high as in fresh apples; it is around 18% for vitamin C and around 37% for vitamin A (Belitz, Grosch, & Schieberle, 2009). Carbohydrates, such as glucose, fructose and saccharose, and polysaccharides, in particular cellulose, are also resorbed by apples. The apple cell wall consists of pectins, celluloses, and hemicellulose. Pectins are abundant in apples. They are a group of polymers rich in galacturonic acid. Cellulose is a cell wall polymer of β-1,4-linked glucose units and hemicellulose is a crosslinking glycan (García-Solís & Celis, 2019).

Sorbitol, a sugar alcohol, is also found in apples and has a unique role as an end product of photosynthesis (Litz, 2005). The lipid content of apples is highly correlated with the water content, meaning that because apples have a high water content (more than 80 g of 100 g fruit) the lipid content is low (under 1 g of 100 g fruit) (Domínguez-Avila & González-Aguilar, 2019). The lipids are mostly structural lipids, like those of the cell membrane. Water is the main component of apples and the metabolic cost to generate the structure of the apples is low (around 80 kcal per 100 g)[7]. Most of the fatty acids are found esterified to polar lipids, a modest portion in neutral lipids. Free fatty acids comprise very small portion (Contreras, Tjellström, & Beaudry, 2016). The lipid composition is dominated by glyco- and phospholipid in apple peel and flesh (S. Y. Wang & Faust, 1992). Glycolipids consist of a monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG), while the main phospholipids in apples are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylinositol (PI). Paillard, 1990 found that the most abundant saturated and unsaturated fatty acids in the polar lipid fraction are palmitic (C16:0) and linoleic acid (C18:2). Linoleic acid is a common constituent in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Linolenic acid is the predominant fatty acid in MGDG and DGDG, and palmitic acid is dominant in PG (S. Y. Wang & Faust, 1992).

Genetically, apples are mostly diploids with 2n=34 and a genome of moderate size (1C = 2.25 pg which corresponds to approximately 1.5 x $10^9$ bp) (Janssen et al., 2008). However, there are also some triploids (3x = 51) and a few tetraploids (4x = 68) (Podwyszyńska, Kruczynska, Machlańska, Dyki, & Sowik, 2016). The study of Podwyszyńska et al. indicated that around 10% of the investigated Polish apple cultivars are triploids and that the higher ploidy level of triploids is

[7] United States Department of Agriculture, National Nutrient Database for Standard Reference Legacy Release https://ndb.nal.usda.gov/ndb/foods/show?ndbno=09500&fg=9&man=&lfacet=&format=Abridged&count=&max=25&offset=125&sort=c&qlookup=&rptfrm=nl&nutrient1=204&nutrient2=&nutrient3=&subset=0&totCount=345&measureby=g

generally associated with increased sizes of stomata, leaves, flowers and/or fruits like in many other plant genera (such as tea, banana, mulberry).

The apple is structured as follows: the peel represents the outer surface of the apple fruit and consists of the cuticle as well as epidermal, subepidermal and a multi-layered hypodermis (Lara, Belge, & Goulao, 2014; Konarska, 2013). The apple features a central placenta that contains the pips, and only when the placenta is broken and the pips are dispersed and apple seedlings emerge (Juniper, 2007). Figure 4 shows a cross-section of fleshy apple fruit (variety Ilzer Rose). The fruit consists of the stalk that attaches the fruit to the stem. The exocarp, mesocarp, and endocarp form the apple's core (pericarp), the hypanthium is the fruit flesh and the endocarp surrounds the ovary with the seeds or pips (Carrillo-López, 2019).

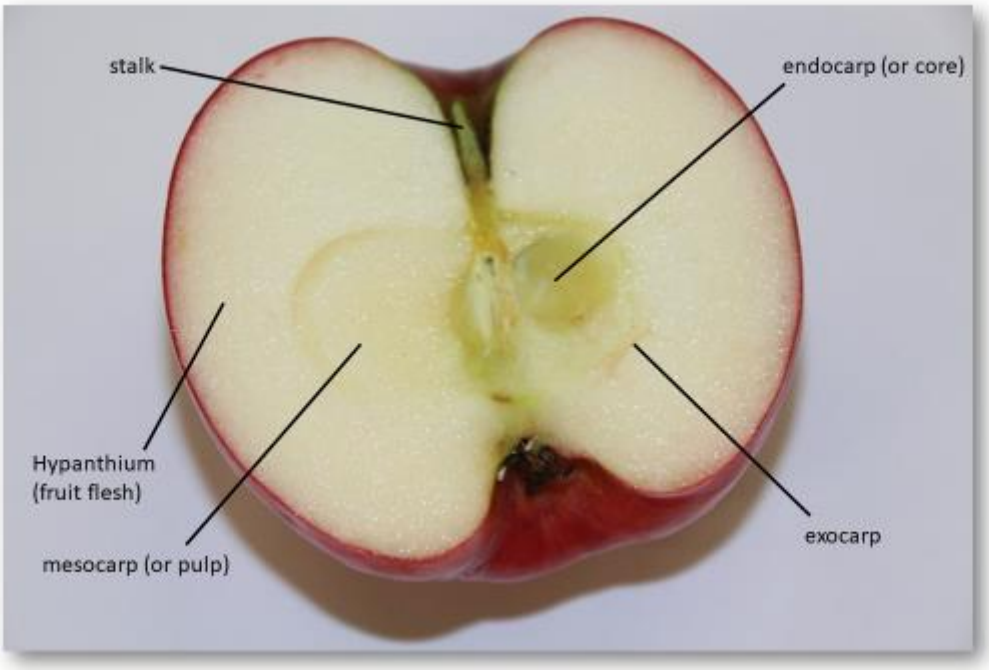


**Figure 4: Cross-section of a fleshy apple fruit:** stalk (part of the fruit attached to the stem), endocarp or core (central part of the fruit which contains the pips), exocarp or peel (plant tissue covering the fruit), mesocarp or pulp (part of the apple between the peel and the core)

The cuticle internally adheres to the polysaccharides of the epidermal cell walls, while externally it presents a series of lipids. The cuticular layer is composed of a hydrophobic structure mainly comprised of cutin, covalently linked to a scaffold of long-chain fatty acid, and wax, formed by a very-long-chain fatty acid and their derivate, such as alcohols, aldehydes, and alkenes (Pollard, Beisson, Li, & Ohlrogge, 2008). The cuticle had an important role as the primary barrier of aerial organs, protection against abiotic and biotic stress, which can affect the postharvest performance of fruits (Costa, 2016). Various studies were published about the peel structure of apples assessed by histological approaches and different instrumental approaches, such as electron microscope (Konarska, 2013) and chromatography (Belding, Blankenship, Young, & Leidy, 1998).

The characteristics and composition of apple cuticular wax change in response to environmental stresses such as rain acidity, temperature, and radiation (Konarska, 2013). However, the sensory

properties also change from the starting fruit with juicy and crisp notes to the matured fruit with mealy and dry notes. The apple fruit develops from pollination to the full tree ripeness in over 150 days (Janssen et al., 2008).

Apples are primarily consumed as raw fruit or in processed form (dried, deep-frozen or cooked). Besides, apples are also used to produce juice, cider, vinegar, liqueur or jams. Moreover, dried apples are used for special tea blends for health reasons. The high content of pectin in apples (10-20% of apple pomace) shows liquid-binding and stabilizing characteristics for the human gut (Belitz et al., 2009).

### 2.1.3. Apple Cultivation and the difficulties of recent apple harvest years

The worldwide apple production has been rising since the second world war and in particular in countries like China, Turkey, Iran, India, and Pakistan (Ferree & Warrington, 2003). China (39.000.000 t/year) is now the leading country for apple production, followed by the United States (5.000.000 t/year), Italy and New Zealand (USDA Foreign Agricultural Service, 2018). The global apple production is dominated by a few cultivars including McIntosh, Jonathan, Cox's Orange Pippin, Granny Smith, Delicious, Golden Delicious, and Braeburn. All these varieties were mostly selected from chance seedlings over 100 years ago (Gardiner et al., 2007).

The environmental conditions play an increasingly important role in worldwide apple cultivation. The climate is changing and influences tree physiology and therefore also the fruit quality. Apples are grown in many countries all over the world, but not all areas have optimal conditions. There are four climate types that influence the general physiology of the tree (Musacchi & Serra, 2018); the first is identified by cool days and cool nights, especially in countries like England, the Netherlands, Germany, Austria, and South Tyrol. Apple trees in these areas exhibit slow growth and the fruit quality is good. The second has warm days and cool nights, and these conditions are typical for countries (or areas) like Washington state (USA), central Italy, New Zealand, and Australia. In these regions, the apple trees are very productive and have optimal conditions to yield high quality apples and high productivity. The third climate zone is a variation of the first and second type, with warm days and with moderate temperatures of the nights. This climate is typical for areas like California, central Chile, southern Australia and areas around the Mediterranean Sea. The trees have a moderate size and regrowth after pruning is moderate. The fourth climate type is described by warm days and warm nights and is typical for regions like China, Japan, southern France and in the Mid Atlantic area of the US. The apple trees here have the lowest productivity among all four climate zones and the trees also loose over 40% of the daily produced carbohydrates through nighttime respiration.

The apple is the most famous fruit in Austria with a per-capita consumption of 19 kg per year[8]. More than 80% of the Austrian apple harvest (plantation growing and growing in meadow orchards) are harvested in Styria. Apples are cultivated on approximately 6.000 hectares of apple plantation

[8] https://de.statista.com/statistik/daten/studie/348617/umfrage/pro-kopf-konsum-von-aepfeln-in-oesterreich/

growing area with approximately 200.000 t apple harvest per year (Statistik Austria, 2018). The apple production and area under cultivation of Austria and Styria are shown in Table 1. Styria is located in the apple zone of Europe and has particularly favorable conditions for apple production. Nevertheless, in the last years a lot of the Austrian apple farmers stopped farming apples and production, which puts a lot of pressure on the remaining farmers as they have to compensate for this decrease in apple cultivating farms. Climate change (with the very late onset of winter in May during which the apple trees are in full bloom or the dry/tropical heat with low amounts of precipitation) and especially the fluctuating apple price force small apple farmers to abandonment. The apple harvest in the year 2016 had a catastrophic failure of 90% in some regions of Austria, especially in Styria. The difficulties started with the late onset of winter at the end of April 2016 (with frost and snow), when the apple trees were in full bloom. The next challenge for apple farmers occurred in August 2016, where hail and heavy rainfalls destroyed big apple cultivations areas. The Austria meteorological network (ZAMG – Zentralanstalt für Meterologie und Geodynamik[9]) reported a resulting damage of 215 million Euros and an apple crop failure of over 70% for this harvest year.

Table 1: Fruit cultivation in Austria and Styria in the last four years[10]

| year | Area [ha] | | Production [t] | |
|---|---|---|---|---|
| | Austria | Styria | Austria | Styria |
| 2015 | 6.700 | 5.200 | 216.000 | 177.000 |
| 2016 | 6.700 | 5.200 | 61.000 | 35.000 |
| 2017 | 6.700 | 5.200 | 130.000 | 94.000 |
| 2018 | 6.700 | 5.200 | 240.000 | 188.000 |

In Austria alone, there are more than 2.000 different apple varieties (März, 2011), but only a few dozens of these are grown commercially on a worldwide scale (O'Rourke, 2003). At the start of the 1990s, the apple variety Golden Delicious was the most widely grown cultivar worldwide, nowadays the cultivars like Elstar, Fuji and Gala have become very popular. Golden Delicious makes up more than 20% of the European apple market, followed by the varieties Gala, Idared, Red Delicious, and Shampion[11]. The majority of the apples in Austria, mainly new apple varieties like Golden Delicious, Gala or Idared, are cultivated in plantations. A smaller amount, about 25% of the apple harvest, are grown in so-called meadow orchards. These meadow orchards represent an enormous pool of apple varieties, especially old apple varieties as Maschanzker, Kronprinz Rudolf, Bohnapfel, Ilzer Rose and many more that have been traditionally grown there. In many cases, these varieties show

[9] https://www.zamg.ac.at/cms/de/klima/klima-aktuell/unwetterchronik?jahr=2016&monat=4 and https://www.zamg.ac.at/cms/de/klima/klima-aktuell/unwetterchronik?jahr=2016&monat=8

[10] https://www.statistik.at/web_de/statistiken/wirtschaft/land_und_forstwirtschaft/agrarstruktur_flaechen_ertraege/obst/index.html

[11] https://de.statista.com/statistik/daten/studie/29114/umfrage/bedeutende-apfelsorten-in-der-eu-nach-sortenanteil/

completely different properties than fruits from new varieties in terms of flavor, and texture, but also of storage conditions, and processing abilities.

## 2.2. Flavor Science – a brief overview

This chapter begins with laying out the theoretical dimensions of the research and explains how flavors are described and analyzed. The application of the flavor science and sensory science does reinforce the Styrian fruit cultivation. The results of the heritage apple varieties highlight the specific flavor of each cultivar. The Styrian cider culture is more in common in recent years and the producers revert to the heritage apple varieties, because of their specific flavor.

### 2.2.1. Elements of Flavor

In general, flavor is a combined perception of aroma and taste sensations and plays an important role in consumers' acceptance of food. Flavor is the sensory impression of food or other substances. It is formed by the complex interaction between all sensory impressions (Siegmund, 2015). There are three elements of flavor (seen in Figure 5), including taste (soluble substances perceived by the gustatory system in the mouth), odor (or aroma) and trigeminal sensation (produced by chemical irritants that stimulate trigeminal nerve ends; for example, astringency or pungency).

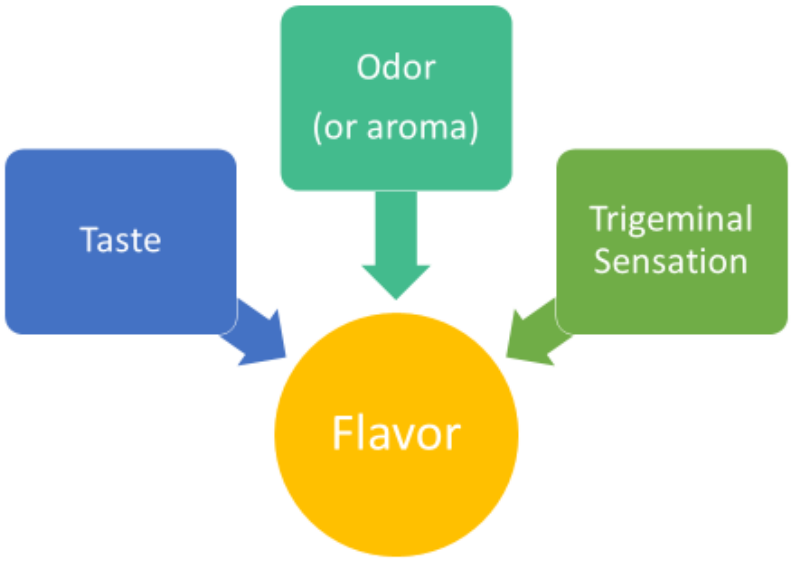


**Figure 5: Elements of Flavor.** The flavor consists of three elements, the taste, the odor (or aroma) and the trigeminal sensation.

Taste is one of the five traditional senses and consists of five established basic tastes: sweetness, sourness, saltiness, bitterness, and umami. The compounds which are responsible for taste are generally non-volatile compounds at room temperature. These compounds can interact only with taste receptors located in the taste buds of the tongue (Belitz et al., 2009). The odor (or aroma), perceived by the smell organ, has up to 8.000 different odor impressions, mainly volatile compounds or aroma substances (Buettner, 2017). The perception of the volatile compounds over

the olfactory system can happen in two ways: the direct odor with the breath, named nasal odor, and the indirect odor, called retro-nasal odor. The odor receptor site in the olfactory epithelium is located in the upper part of the nose (Berger, 2007). After a volatile compound is received by a receptor, a cascade of biochemical reactions amplifies and transmits these signals to the brain where they can be analyzed as such. The definition of an odor consists of three conditions: to bind an odorant receptor, to result in the odorant receptor transmitting to the brain and the brain has to recognize it as a signal that can be interpreted (Buettner, 2017).

In general, the amount of volatile compounds present in food is very low (up to approx. 10 to 15 $mgkg^{-1}$) and they comprise a large number of different compounds. A huge variety of volatile compounds is often present in fruits, but only a limited number are important for their flavor. The compounds which are primarily present in food in concentrations higher than the odor thresholds are calculated with the odor activity value (OAV) and they are really important for a specific flavor of the food. The odor threshold (OTV) (or recognition threshold) is the lowest concentration of a compound that is just enough for the recognition of its odor. The detection threshold is lower and defined as the concentration at which the compound is detectable without complete recognition. These two values are determined by smelling and by tasting the sample (Belitz et al., 2009). The OAV is calculated as the ratio between the concentration of an individual compound in the sample and the OTV concentration of this compound. When the odor activity value is higher than 1 than the compound is considered to have an important odor contribution (Buettner, 2017).

### 2.2.2. Flavor compounds of fruits

The flavor and aroma of fruits are the main part of the consumers' acceptance and the volatile compounds are responsible for specific fruity characters. It is the interaction of volatiles with non-volatile compounds that defines the sensory perception of food. In fruits, the common basic tastes are sweet, sour and bitter. The main compounds responsible for the sweet and sour tastes of fruits are soluble sugars and organic acids, while others such as phenolic compounds, triterpenes, or some aldehydes provide bitterness (Sánchez-Rodríguez et al., 2019). The composition and content of sugars depend on factors such as the cultivar and the fruit maturity stage at harvest. The sweet taste is directly related to the sugar content and depends on the activity of the main metabolic pathways (such as glycolysis and respiration). As reported for sugars, the acid profile is also changing depending on these factors. Malic acid is the main compound responsible for the sour taste in apples. This compound is an intermediate in the metabolic pathway of the tricarboxylic acid cycle. The two enzymes phosphoenolpyruvate carboxylase and NAD (nicotinamide adenine dinucleotide) –malate dehydrogenase are involved in malic acid synthesis, and the NADP (nicotinamide adenine dinucleotide phosphate) - the malic enzyme is responsible for the malic acid degradation. The bitterness of apples is related to the content of polyphenolic compounds.

Apples are known to have high concentrations of polyphenols (it depends on the variety, maturity of the fruit, conditions on cultivations, harvest, storage) with antioxidant capacity. A review by Kalinowska, Bielawska, Lewandowska-Siwkiewicz, Priebe, & Lewandowski, (2014) discussed reports on apple phenolic compounds and their biological properties. Generally, it is reported that

apple peel is richer in total phenolic compounds, total procyanidins and total flavonoids than apple flesh. The difference of individual phenolic compounds in apple peel and apple flesh is explainable by the biosynthesis pathway of these compounds. The biosynthesis pathway starts with the Shikimate pathway and aromatic acids (phenylalanine) biosynthesis, the enzyme phenylalanine ammonialyase is catalyzed the next pathway (phenylpropanoid pathway). Next enzyme (chalcone synthase) is catalyzed the flavonoid pathway and the end-products of this pathway are anthocyanins (Kalinowska et al., 2014; X. Wang, Wei, & Ma, 2015). Anthocyanins are pigments and are responsible for the color of the apple skin. Ganai et al., 2015 have reported that the content of anthocyanins varies between different harvest dates (late harvested apple fruits showed a content of 33 mg/100 g and early harvested showed a content of 20 mg/ 100 g). There are two possible regulators for anthocyanin accumulation during the different ripening stages, first is the enzyme phenylalanine ammonia-lyase and second, it is the hormone ethylene (Dar et al., 2019).

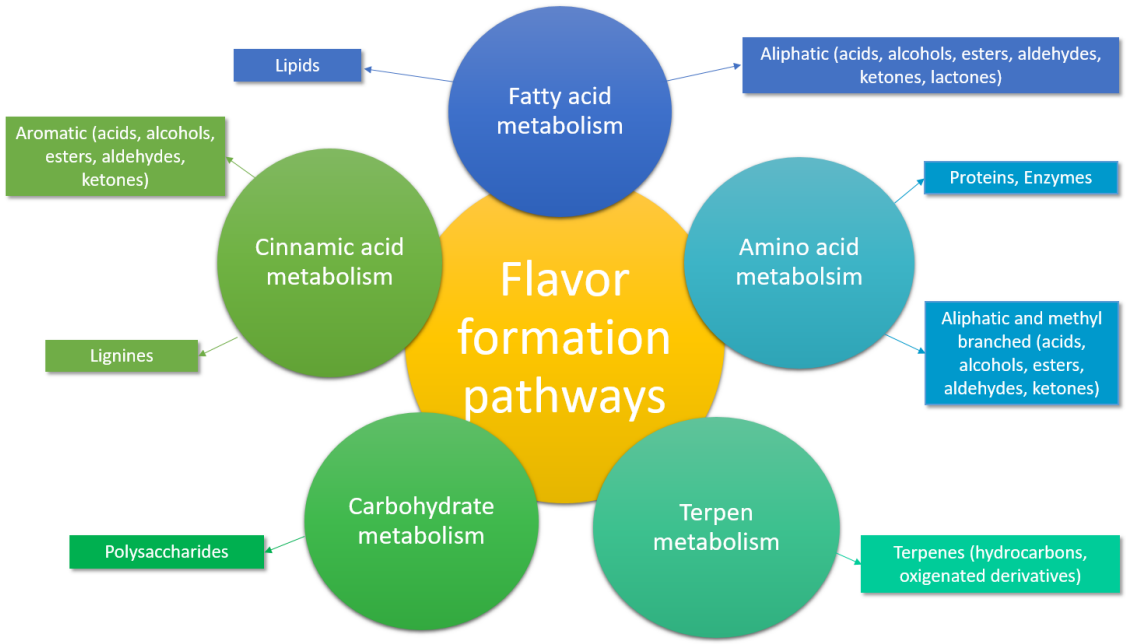


**Figure 6: Main flavor formation pathways in fruits**. Adaptations based on Reineccius, 2006.

Fruit flavor is a complex mixture of a large number of volatile compounds including different chemical classes: esters, aldehydes, alcohols, ketones, terpenes and furanones (seen in Figure 6). The main flavor pathways are the metabolism of fatty acids, amino acids, cinnamic acids, carbohydrates. The volatile compounds can be classified as primary and secondary flavor compounds (among release of enzyme systems from the cells and contact with oxygen) (seen in Figure 7), depending on whether they were present in intact fruit or produced as a result of tissue disruption (F. Drawert, Heimann, Emberger, & Tressl, 1969; Siegmund, 2015). The maturation and ripening stages of the apple fruit influence the sensory quality and the eating quality (Paul & Pandey, 2014). Fruit volatiles generally form during growth, maturation and ripening of the fruit over the course of the plant's metabolic or anabolic pathway (formation of primary flavor compounds) as well as during the preparation of the fruit prior to consumption or processing (formation of secondary flavor compounds)(Siegmund, 2015).

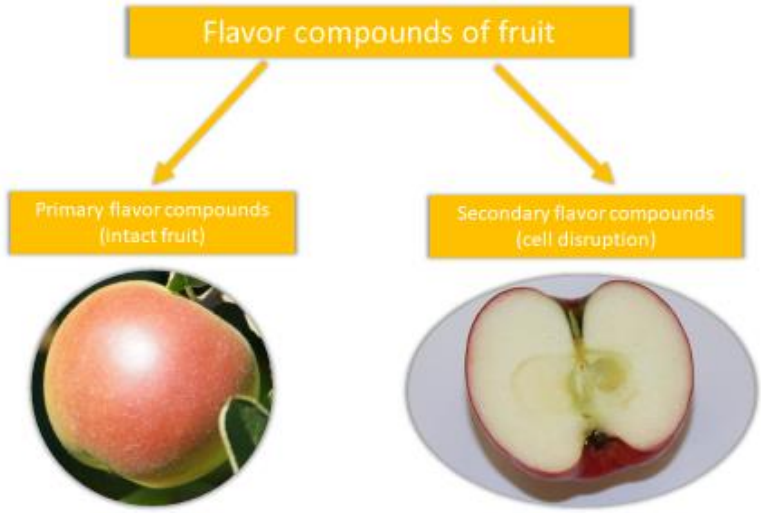


**Figure 7: Flavor compounds of fruit:** the primary compounds are present in intact fruits and the secondary compounds are produced while cell disruption

## 2.2.3. Ripening and Maturation of Apples

Apple quality is very important for consumers, they select apples by the general human senses: sight, touch, taste and smell (Cárdenas-Pérez et al., 2017). Apples are harvested at complete maturity; at this point the fruits are self-sufficient with their own catalytic system without the parent plant (Prasanna, Prabha, & Tharanathan, 2007). At maturity stages, the apple fruits have a minimal respiration rate and ethylene formation, and raise to climacteric peak at the onset of ripening (Prasanna et al., 2007). After the fruit development is completed, the ripening process starts a series of transformations. The change is characterized in color, texture, aroma, and nutrients (Alós, Rodrigo, & Zacarias, 2019). Generally, fruits are classified into two groups (climacteric and non-climacteric type) based on the distinctive respiratory pattern during ripening. Apples are climacteric fruits. They show a dramatic increase in respiration rate during ripening, this is called a climacteric rise (Paul & Pandey, 2014). It is reported that the rise in respiration is either simultaneous or it is immediately follows the rise in the rate of ethylene production (Paul & Pandey, 2014).

The ripening stages of apples are very important for the determination of the best harvest time and sensory quality. Over process of photosynthesis, starch accumulates in apple fruits. There is an association between ethylene and aroma production, which has been shown through the use of both ethylene action and ethylene biosynthesis inhibitors that result in a reduction in levels of volatiles in apple fruit (Harb, Lara, Saleh, Streif, & Khraiwesh, 2011). A mature apple has maximum starch accumulation, has finished enlarging, and can continue to ripen after being removed from the tree.

Generally, the ripening process changes the content of sugar and organic acids, influence the softening and color changes and the synthesis of volatile compounds. At the beginning of ripening, there is usually an increase in the total soluble solids (TSS), whereas the content of sugars rises by

hydrolysis of starch. There is a decrease in organic acids during ripening that is converted into sugars during respiration. The decrease of total acidity (TA) and the increase of the total soluble solids (TSS) during ripening are responsible for the ripe organoleptic sensory attributes. Malic acid, the major organic acid in apples, accumulates in the young fruit and decreases again during growth and ripening (Berüter, 2004).

Early studies of V. F. Drawert, Heimann, & Emberger, 1966 have shown that volatiles primarily formed during storage and after 70 days of storage, the amount of the volatiles decreased. Song & Bangerth, 1996 have also established that the ripening is directly correlated with the amount of volatile compounds. Most volatiles formed by the intact fruit is under the control of ethylene production. Ethylene is emitted as an enzymatically catalyzed degradation product of methionine and acts as a phytohormone for the fruits. Its binding to the ethylene receptor sites in the cell membranes induces an autocatalytic process triggering the ripening processes in the fruit leading to a rapid increase in the formation of volatile compounds (Botton, Tonutti, & Ruperti, 2019). The binding of ethylene to membrane receptors starts many genetic reactions in the apple fruit. Ethylene accelerates the ripening and increases ethylene synthesis. Ethylene action is required for the synthesis of ripening-related esters in the apple fruit (Defilippi, Dandekar, & Kader, 2005). There is also a change in the apple flavor from aldehydes (green notes) to esters (fruity notes) during ripening (Contreras & Beaudry, 2013). Contreras & Beaudry, 2013 presented in their studies that hexanal synthesis by intact fruit is dependent on ripening. Also important is the activity of alcohol dehydrogenase (ADH), which is responsible for the conversion of aldehydes to alcohols. The ADH activity declines or remains steady during ripening (Echeverría, Graell, López, & Lara, 2004b). Ester biosynthesis, C6 aldehydes, and alcohols in apple fruits are limited by the level of alcohol acyltransferase (AAT), ADH and lipoxygenase (LOX) enzymes. The activity of these enzymes is connected with ethylene regulation (Schaffer et al., 2007). After apples were exposed to ethylene, the ADH expression decreases (Defilippi et al., 2005). A study about the genomics of aroma production in apples (Schaffer et al., 2007) showed that the volatile compounds butyl and hexyl acetate (these two were the major compounds) and the two alcohol precursors for these compounds (butanol and hexanol) were not exposed expressed by ethylene.

Also important for the quality of apples and their storage life are the postharvest metabolic changes which lead to increased respiratory activity and transpirational loss of water. The application of different storage conditions (controlled atmosphere =CA, modified atmosphere = MA, modified atmosphere packaging = MAP) modify the internal gaseous atmosphere of the fruits in terms of low $O_2$ to $CO_2$ ratio, related humidity, the temperature, but also regulate the ethylene production and its response. A decrease of ethylene in the atmosphere decelerates the ripening and softening of apples. This process occurs either by disabling the ethylene formation or by the displacement of ethylene through diffusion, ventilation or absorption (Buchner, 2012).

The phytohormone ethylene has a major role in the ripening process of apple fruits and is additionally responsible for the expression of ripening-related genes even at advanced stages of fruit ripening (seen in the Figure 8). Ethylene biosynthesis originates from S-adenosyl-ʟ-Met (SAM) and

consists of two steps catalyzed by the enzymes 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO). The enzyme ACO converts 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Yang & Hoffman, 1984). Ethylene receptor proteins, located in the endoplasmatic reticulum, bind ethylene. After an ethylene signaling pathway, the ethylene transcription factors are active and bind to the ethylene response elements and the ripening-associated genes are activated for expression (M. Liu, Pirrello, Chervin, Roustan, & Bouzayen, 2015).

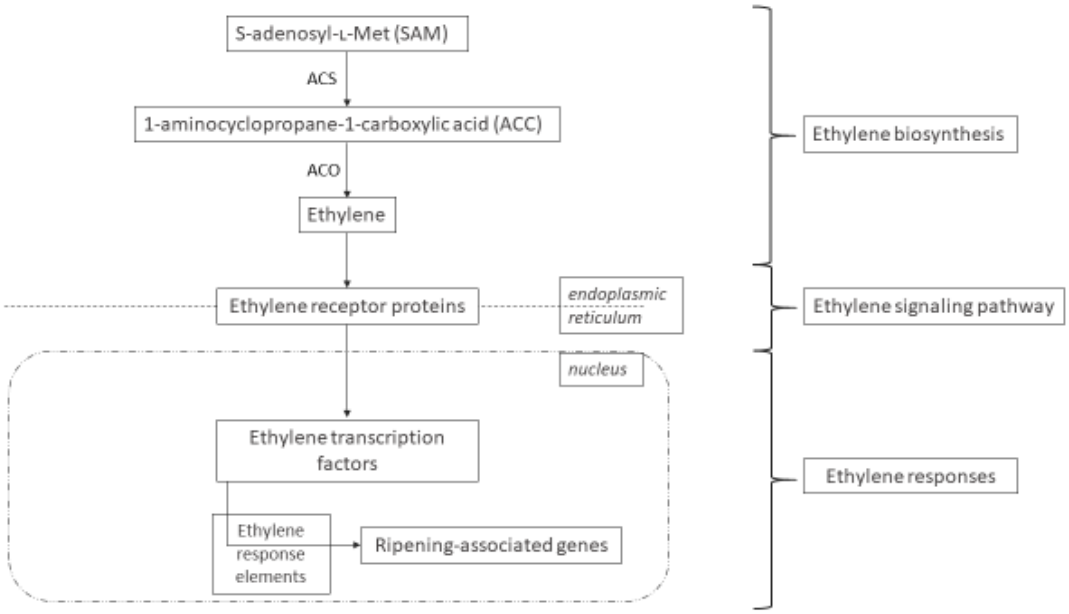


**Figure 8: Simplified scheme showing ethylene synthesis in apples.** The synthesis of ethylene results from the activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO). Adaptations based on Liu, Pirrello, Chervin, Roustan, & Bouzayen, 2015

There are two ethylene-producing systems (referred to as System 1 and System 2) that the climacteric process induced (Giné-Bordonaba, Echeverria, Duaigües, Bobo, & Larrigaudière, 2019a). In the immature fruit, System 1 operates and it is non-autocatalytic. System 2 operates during ripening and induces the autocatalytic ethylene production and the climacteric burst. The rate of respiration during ripening shows a strong increase. This respiration rise is immediately followed or simultaneous to the rise of the ethylene production. During the ripening process, there are changes in the taste compounds, especially the levels of sugar and organic acids are important factors. Sugar accumulates during growth and imports sorbitol and sucrose from photosynthetic leaves (Berüter, 2004).

### 2.2.4. Biosynthesis of volatile compounds in Apples

The apple flavor is a typical example where the final flavor is the combination of many volatiles without specific character impact compounds (Buettner, 2017). In the past, the volatile profiles of apples have been analyzed extensively (Fellman, Miller, Mattinson, & Mattheis, 2000; Fuhrmann & Grosch, 2002; Berger, 2007; Christensen, Edelenbos, & Kreutzmann, 2007) and more than 370 different volatile compounds have been reported. The volatile profiles of apples are complex and

vary depending on cultivar, ripeness, pre-and post-harvested environmental conditions (El Hadi, Zhang, Wu, Zhou, & Tao, 2013a). Esters make up a large, diverse group of aroma compounds in apples. In early studies, more than 100 of them have been described in different cultivars (Paillard, 1990; Yahia, 1994). Other identified volatile compounds in apples are alcohols, aldehydes, ketones and ethers (Berger, 2007).

Volatile compounds in apples form biosynthetically through the metabolism of fatty acids, amino acids, and carbohydrates. The most important pathways are the following (Meigh, 1957, Bartley, 1985; Paillard, 1990; Rowan, Lane, Allen, Fielder, & Hunt, 1996; Espino-Diaz, Sepulveda, Gonzalez-Aguilar, & Olivas, 2016):

a. straight-chain aldehydes, alcohols, and esters are synthesized from lipids, mainly linolenic and linoleic acids, through β-oxidation and lipoxygenase (LOX) activity
b. branched-chain aldehydes, alcohols, and esters are derived from the amino acid isoleucine
c. terpenoids are synthesized via the mevalonate pathway and deoxyxylulose phosphate pathway
d. phenylpropanoids are synthesized in the phenylpropanoid pathway.

Researchers have started to clone and characterize gene products to understand the postharvest disorder superficial scald, for example, the regulation of the production of α-farnesene in apple peel (Litz, 2005).

The following section describes the main pathways involved in the biosynthesis of volatile compounds in apples.

The fatty acid metabolism in apples has two different pathways, first, β-oxidation is important in intact fruit for degradation of fatty acids and second, the lipoxygenase (LOX) pathway is involved after cell disruption (cut fruit) for the oxygenation of polyunsaturated fatty acids. The β-oxidation takes place in peroxisomes and consists of a four-step reaction sequence (seen in Figure 9). The four-step enzymatic reaction includes three proteins (Espino-Diaz et al., 2016): (a) acyl-CoA oxidase which transforms acyl-CoA into trans-2-enoyl-CoA, (b) a multifunctional protein which is responsible for four enzymatic activities (including the enzymes trans-2-enoyl-CoA hydratase and ι-3-hydroxy acyl-CoA dehydrogenase), (c) 3-keto-thiolase which catalyzes the cleavage of the thiol end of 3-ketoacyl-CoA resulting in one molecule of acetyl-CoA and one of acyl-CoA. The β-oxidation cycle occurs by cleaving two carbons in each cycle to form acetyl-CoA. The enzyme alcohol transferase (AAT) is responsible for the esterification of alcohols and acyl-CoA after the β-oxidation cycle. Most of the resulting esters are acetate esters (Dixon & Hewett, 2000).

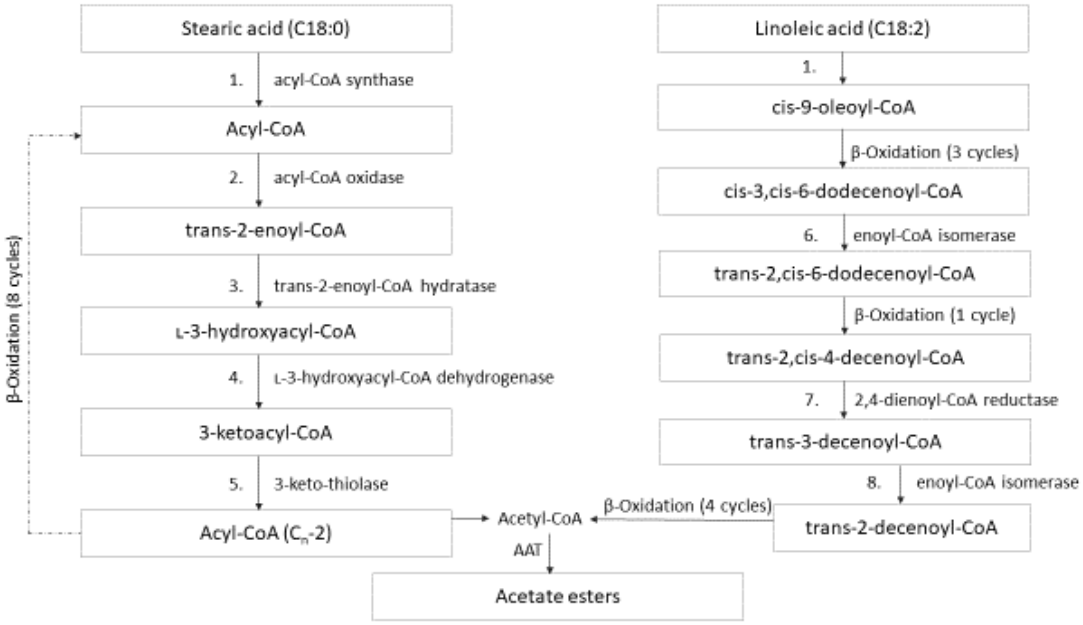


**Figure 9: Fatty acid metabolism via the β-oxidation pathway (for example two C18 fatty acids).** Stearic acid (C18:0) is a saturated fatty acid and Linoleic acid (C18:2) is an unsaturated acid with two double bonds in *the cis* configuration. The β-oxidation pathway includes four enzymatic reactions (2.-5.) performed by three proteins. Alcohol acyltransferase (AAT) enzyme esterified the reducing alcohols with acyl-CoA. Adaptations based on Pérez & Sanz, 2008, Espino-Diaz et al., 2016.

The lipoxygenase pathway (LOX) is the main pathway in disrupted cell tissues of fruits, additionally it is an alternative route to the β-oxidation pathway of the whole fruit during maturation (Dixon & Hewett, 2000). Higher cell membrane fluidity and the resulting increased permeability to different substrates during ripening activates the LOX pathway (Echeverría et al., 2004b). LOX is a dioxygenase that catalyzes the hydroperoxidation of polyunsaturated fatty acids (seen in Figure 10). The main substrates of LOX are linoleic acid (18:2) and Linolenic acid (18:3), which are released from triacylglycerol's, phospholipids (main components of cell membranes), and glycolipids and the insertion of the oxygen takes place to generate the corresponding 13-hydroperoxides (Espino-Diaz et al., 2016; Domínguez-Avila & González-Aguilar, 2019). The enzyme hydroperoxide lyase (HPL) acts on hydroperoxides to form short-chained aldehydes (6 or 9 carbons). The C6 or C9 aldehydes are reduced to their corresponding alcohols by the enzyme alcohol dehydrogenase (ADH). ADH forms a wide range of linear, branched and cyclic alcohols. The enzyme AAT acts upon the alcohol pool to produce esters. The diversity of esters can be influenced by substrate availability or the specificity of the enzyme (Espino-Diaz et al., 2016).

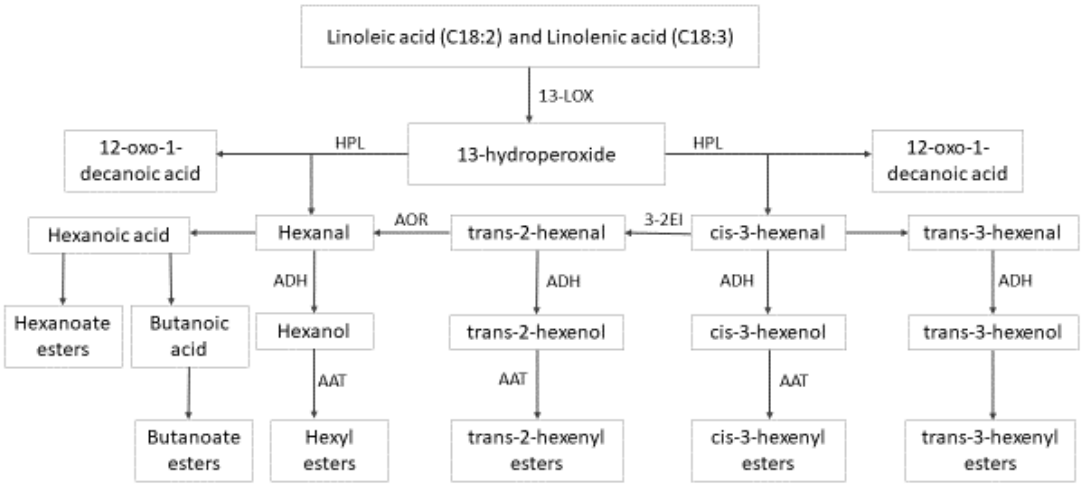


**Figure 10: Fatty acid metabolism via the Lipoxygenase (LOX) pathway (catabolism of linoleic acid and linolenic acid).** LOX=lipoxygenase, HPL=hydroperoxide lyase, ADH=alcohol dehydrogenase, AOR=alquenal oxidoreductase, 3-2EI=*cis*-3: *trans*-2-enal isomerase, AAT=alcohol acyltransferase. Adaptations based on Pérez & Sanz (2008) and Espino-Diaz et al. (2016).

The amino acid pathway (see in Figure 11) is also involved in flavor formation metabolism for apple volatiles. The amino acids isoleucine, leucine and valine are the most important precursors for the production of branched-chain alcohols, aldehydes, and esters (El Hadi, Zhang, Wu, Zhou, & Tao, 2013b). This metabolism starts via the enzyme branched-chain aminotransferase (BCAT), it catalyzes the synthesis of branched amino acids (for example isoleucine) and later, their degradation (Espino-Diaz et al., 2016). The removal of the amino group by BCAT forms α-keto acids, which can be metabolized via three different pathways: (a) pyruvate decarboxylase (PDC), (b) branched-chain α-keto acid dehydrogenase (BCKDH) (c) hydroxy acid dehydrogenase (HDH) (Smit, Engels, & Smit, 2009). The PDC enzyme (a) converts the keto acids into branched-chain aldehydes. These aldehydes can be reduced via ADH to their corresponding branched-chain alcohol. Then, the BCKDH enzyme (b) catalyzes the conversion of α-keto acid in to a branched-chain acyl-CoA. The resulting acyl-CoA can be converted to a fatty acid by phosphate acetyltransferase (PAT), these branched-chain fatty acids are further transformed into alcohols and esters. Finally, the enzyme HDH (c) reduces the acids to hydroxyl acids, but these hydroxyl acids are irrelevant for the flavor.

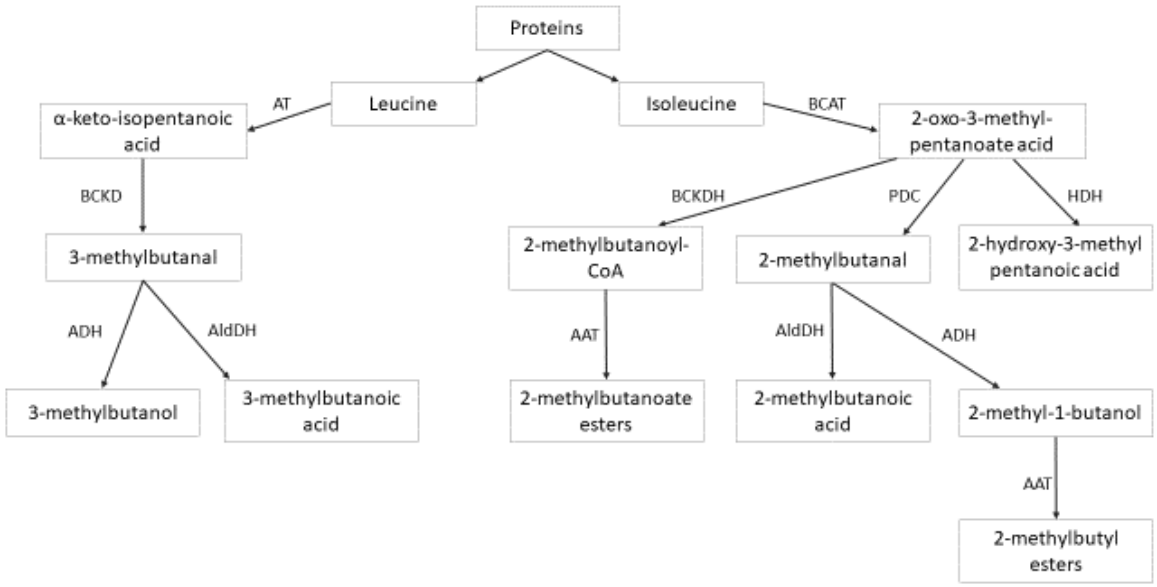


**Figure 11: Amino acid pathways demonstrated at the degradation of leucine and isoleucine.** AT=aminotransferase, BCKD=branched-chain α-keto acid-decarboxylase, ADH=alcohol dehydrogenase, AldDH=aldehyde dehydrogenase, BCAT=branched-chain amino acid transferase, PDC=pyruvate decarboxylase, HDH= hydroxy acid dehydrogenase

Carbohydrate metabolism is another important pathway for the metabolism of apple compounds and for the sugar composition in apple plants. The carbohydrate metabolism can link to terpene metabolism or amino acid metabolism (Siegmund, 2015). The photosynthetic products, sucrose, and sorbitol are the main forms of assimilated carbohydrate transported from apple leave to fruit (Zhang, Yan, Fu, Li, & Wang, 2016). Sorbitol in the leaves is biosynthesized from glucose-6-phosphate and converted into fructose and glucose once it is translocated to the fruits. Sucrose is biosynthesized from glucose and fructose-6-phosphate in apple tissue. In the early 1990s, Fuleki, Pelayo, & Palabay described that sorbitol only made up 2.7% of total soluble carbohydrates in 100 mL apple juice and, thus, the sorbitol level in apples was quite low. After the starch breakdown, the main carbohydrates are fructose, sucrose, and glucose. Horikawa, Hirama, Shimura, Jitsuyama, & Suzuki (2019) showed that the distribution of sucrose and sorbitol in apple tissue can be visualized using Maldi-TOF MSI and this method will be useful for examining carbohydrate metabolism during the maturing of apple fruit.

Terpenes can be synthesized via two pathways: (a) mevalonate (MVA) pathway and (b) 2-methyl-D-erythritol-4-phosphate (MEP) pathway (Figure 12). Acetyl-CoA is the starting molecule of the MVA pathway. This pathway is active in the cytosol of the cell and forms the precursors for sesquiterpenes (such as α-farnesene and β-caryophyllene). The starting molecule of the MEP pathway is pyruvate. The MEP pathway forms the precursors for the monoterpenes (such as α-pinene and sabinene) in the plastid. The key building block for both pathways is the isopentenyl pyrophosphate (IPP), which is isomerized via the enzyme isopentenyl diphosphate isomerase (IDI) to dimethylallyl pyrophosphate (DMAPP). These molecules (IPP and DMAPP) are converted to geranyl pyrophosphate (GPP) and to farnesyl diphosphate (FPP), respectively, which are then utilized by various terpene synthases resulting in monoterpenes and sesquiterpenes.

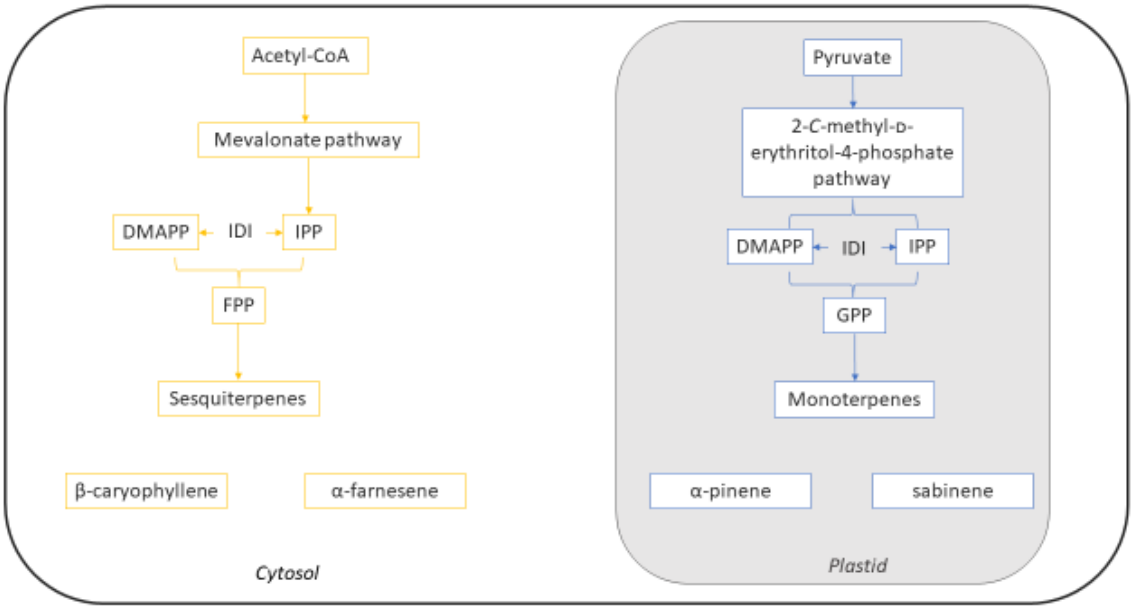


**Figure 12: Biosynthetic pathways for terpenoids.** Sesquiterpenes are formed via the mevalonate (MVA) pathway and the MVA occurs in the cytosol of the cell. The 2-methyl-D-erythritol-4-phosphate (MEP) pathway produces the precursors of the monoterpenes and is active in the plastid. DMAPP=dimethylallyl pyrophosphate; IPP=isopentenyl diphosphate, IDI=isopentenyl diphosphate isomerase; FPP=farnesyl diphosphate; GPP=geranyl pyrophosphate. Adaptations based on Siegmund (2015) and Meena et al., (2017).

## 2.2.5. Flavor Analysis – a brief introduction

Apple fruit research has a long history. Historically, research focused on the factors associated with apple storage, apple fruit quality, and new apple varieties. Measurements of volatile compounds are complex. Qualitative and quantitative information is required to characterize flavor- and aroma-related compounds. Volatile compounds are present in low amounts (from $ngkg^{-1}$ to $mgkg^{-1}$) in food matrices. The combination of analytical techniques and sensory analysis provides a deeper insight into the flavor of apples. Research on flavor analysis has increased enormously during the last decades and the development of methodologies of analytical techniques and human sensory analysis has developed considerably. Over the past century, there has been a big change in our society, the accession of wealth and with this the access for better and healthier food increased. Fruit quality and the acceptance by the consumers have become more and more important for the worldwide food market. The ability to identify the flavor of food and the use of this knowledge, raise us to a better food level/standard. The isolation and analysis of the different flavor compounds are difficult, particularly the fact that these compounds comprise a large number of chemical classes. Food matrices are a challenge in terms of isolation and concentration processes of the different flavor compounds. To separate the non-volatiles from the volatiles was a bigger problem in the early days. Nowadays, with novel analytical techniques and sample preparations it has become easier. Apple flavor analysis started in the 1920s. The first described flavor compounds were acetaldehyde, methyl methanoate, ethyl acetate and ethyl hexanoate (Power & Chesnut, 1920). The separation and identification of volatile compounds were performed via column chromatographic with UV/VIS spectroscopy until gas chromatography was established. Previous studies of Meigh (1956, 1957) have dealt with collecting and analyzing volatile substances from the air of a store of apples held in

conditions of a good commercial gas store and using methods such as chromatography on paper or columns. The results of their work showed that the main constituent of the volatile matter from apples is acetone, with smaller amounts of acetaldehyde, n-butanal, propanal, 2-butanone, 2-methylpropanal, 3-methylbutanal, and 2-pentanone. Alcohols like ethanol, 2-methyl-1-butanol, 2-methyl propanol, methanol, 2-propanol, and esterified acids from $C_1$ to $C_6$ were also identified. However, the research on the apple flavor has consistently grown with further development of gas chromatographical systems.

Drawert et al. (1966 and 1969) were the first to describe volatile compounds, like (E)-2-hexenal and hexanal, which are formed after cell disruption (see LOX pathway in 2.2.4) and also that the inactivation of apple enzymes with an antioxidant solution is a better way before GC analysis. In 1971, Guadagni, Bomben, & Hudson observed that the rate and extent of apple volatile production were several times greater in peels than in the flesh of whole apples by gas chromatography coupled with olfactory assessment and $H_2$ flame detector and a gas-liquid chromatography system. The first review on the apple flavor was published in 1983 and described 266 volatile components isolated from apples and included a sensory evaluation with analytical methods (gas chromatography – olfactometry) (Dimick & Hoskin, 1983). Paillard (1990) showed that the volatile profile of apples has a great range of compounds, the majority of which are esters and alcohols. In this study, the apple volatile production was categorized according to type and quantity of esters or alcohols, skin color or C6 aldehydes and it was demonstrated that differences in proportions of volatile compounds exist between cultivars.

In this chapter, sensory and analytical analysis are briefly introduced and the methods and principles which are used for this work are clarified.

#### 2.2.5.1. Sensory analysis

The International Organization of Standardization (ISO 8402, 1986) outlined the important word quality as “the sum of all characteristics, properties, and attributes of a product or commodity which is aimed at fulfilling the established or presumed customer requirements”. Quality has different meanings and there are many definitions. The first book about fruit quality was published by Schuphan, in 1961. He defined quality as a set of factors consisting of internal (nutritional value) and external (market value) characteristics, technological characteristics, the value of an image, and sensory value. All different characteristics of fruit quality can be measured nowadays, which is reasonable considering the big research sector of fruit and for the fruit industry. Commercial standards define the quality of apples. The most important aspects of the apple quality are size, color, integrity, and the presence of some damage effects of the skin or diseases. The European Union (EU) defined a Regulation CE No 1238/2005 about apple classification (Fischer Boel, 2005). One important practice to get information about (apple) quality is sensory science.

The early history of the sensory evaluation is not really specified, because the senses of taste and smell have influenced the human behavior for millennia. The Egyptians used important odors like myrrh or balsam. In India, sandalwood oil has been used for hundreds of years. A lot of different spices, fruits, and other food materials were brought from India and America to Europe by

Portuguese and British sailors. Also very important for the beginnings of the sensory evaluation were the Romans and the Greeks. The Romans made perfumes a world-wide industry and the Greeks cultivated flowers for esthetic olfactory pleasures. The sense of smell is also a common topic in former literature. Amerine, Pangborn, & Roessler (1965) of the Food Science Department at the University of California at Davis had big influence on the development of sensory testing and traced the history of systematic sensory analysis.

The modern sensory analysis has developed mainly due to the expansion of processed food and consumer product industries in the second half of the twentieth century (Lawless & Heymann, 2010). Sensory analysis is used in the fields of quality control, product development, and research. The definition of sensory evaluation as a scientific method is the usage to evoke, measure, analyze, and to interpret those responses to products as perceived through the senses of sight, smell, touch, taste, and hearing (Stone, Bleibaum, & Thomas, 2012). The definition is not limited to food, it is also applicable for non-food products because sensory evaluation includes the measurement and evaluation of the sensory properties of food and other materials. The human subject is used as an instrument. The "original" five senses are vision, audition, taste, smell, and touch, and the "new" ones include the senses of temperature, pain, and pressure.

Generally, sensory analysis can be separate in two categories: (1) analytical methodology (objective), which concerns perception of stimuli and the panelists are being used as an instrument in order to gather measurements of perceptual product differences or attribute magnitude; and (2) affective methodology (subjective), which concerns with subjectivity such as consumer's hedonic judgement of products (Methven, 2015). Analytical or objective testing are evaluated by a selected or trained panel, whereas in affective (hedonic) or subjective testing the reactions of consumers to the sensory properties are measured (S E Kemp, Hollowood, & Hort, 2011). There are three types of test methods in sensory evaluation, (1) discrimination class, which screens for sensory acuity, oriented to test method, sometimes trained panel (analytic); (2) descriptive class, which screens for sensory acuity and motivation, trained or highly trained panel (analytic); (3) affective class, which screens for product use, untrained (hedonic) (Lawless & Heymann, 2010). Discrimination testing, like triangle, duo-trio, or paired comparison procedures, is a simple and often used application. Descriptive testing, like Quantitative Descriptive Analysis QDA® or the Texture Profile method, quantifies the perceived intensities of the sensory characteristics of a product, and has proven to be the most comprehensive and informative sensory evaluation tool (Lawless & Heymann, 2010). Hedonic or affective testing, like 9-point hedonic scale testing, is used to quantify the degree of liking or disliking of a product by consumers. For the analytic methodologies, a trained panel is needed and each panelist is selected based on having average to good sensory acuity for the critical characteristics of products to be evaluated.

Sensory analysis is important for food industry/consumer products is order to get an interpretation of sensory experience by the human brain prior to responding which is missing in instrumental assessments (Lawless & Heymann, 2010).

In the 1990s, sensory analysis was applied for the classification and evaluation of fruit cultivars (Daillant-Spinnler, MacFie, Beyts, & Hedderley, 1996; Zerbini, Pianezzola, & Grassi, 1999). The apple industry reacted to the "new" consumer preference to get high-quality apples on the worldwide market and also the willingness to pay more for a better quality of apples (Jaeger, Andani, Wakeling, & MacFie, 1998). The sensory evaluation of 13 different apple varieties and their volatile composition was conducted by Karlsen, Aaby, Sivertsen, Baardseth, & Ellekjaer (1999). In this sensory study the attributes: odor intensity, acidic odor, grassy odor, honey odor, fruity odor, chemical odor, almond odor, flavor intensity, acidic flavor, grassy flavor, honey flavor, fruity flavor, sour flavor, sweet flavor, bitter flavor, chemical flavor, almond flavor, hardness, chewiness, crispiness, mushiness, juiciness, and after taste were chosen for apple profiling. The used method for evaluation was a continuous non-structured scale, corresponding to the lowest and highest intensity. The results showed a significant difference of attributes for different apple varieties.

The issue of food waste has become more important in the last years and the change in consumption, meaning that the apple does not have to be perfect in shape, size, and color, have influenced the research of apples (de Hooge et al., 2017; Jaeger et al., 2018).

The principles and practices of sensory evaluation follow guidelines for the preparation and serving of samples under controlled conditions (Lawless & Heymann, 2010).

Descriptive methods (and variants) are used for the main description of the sensory characteristics of products in sensory science. One of the most common descriptive methods is the Quantitative Descriptive Analysis (QDA®), a conventional profiling method, to get the profile of all the perceived sensory characteristics of a product (Ng et al., 2012; Albert, Varela, Salvador, Hough, & Fiszman, 2011). This conventional technique makes single point evaluations of sensory properties with an overall impression of attribute maximum intensity. Several new methodologies for sensory profiling have developed as an alternative to conventional profiling (like QDA®), including sorting (Rao & Katz, 1971), similarity scaling (Schiffman, Schiffman, Reynolds, & Young, 1981), projective mapping (Risvik, McEwan, Colwill, Rogers, & Lyon, 1994), free choice profile (Williams & Langron, 1984) and flash profile (Dairou & Sieffermann, 2002). Flash profiling combines two sensory principles like free-choice terms selection with a ranking method and the method is previously run to describe commercial apple purees (Tarea, Cuvelier, & Sieffermann, 2007).

Check-all-that-apply (CATA) questions are one of the most commonly used methods for sensory product characterization (Adams, Williams, Lancaster, & Foley, 2007) because the task is easy as the panelists simply select from a predefined list of attributes describing each focal sample (Ares et al., 2013).

#### 2.2.5.2. Analytical Techniques

Since the development of gas chromatography (GC) in the early 1960s, flavor research has developed enormously. In the beginnings, with the use of this technique, more than 8.000 flavor compounds could be identified to date in various food matrices (Heredia, González-Miret, Meléndez-Martínez, & Vicario, 2013). After the development of the GC, the flavor analysis of apples

had a scientific breakthrough. The application of olfactory detection (sniffing port) was established in 1966. This was the beginning of the measurements and identification of odor-active compounds in different food matrices (Stephan, Bücking, & Steinhart, 2000). Flath, Black, Guadagni, McFadden, & Schultz, 1967 were the first to apply the coupling of a GC with a mass selective detector (MS) for their research on apple flavor compounds. The two most common detectors in flavor analysis are the mass spectrometer (MS) and the flame ionization detector (FID) and after GC separation, the olfactory detection is also used. To detect trace levels of sulfur or nitrogen, selective detectors, such as nitrogen phosphorus detector (NPD), are used.

The basic principle of gas chromatography (GC) is the gaseous transport (mobile phase) through a column and the separation of components as vapors. The affinity for the stationary phase, which is coated in a column, is important for the separation of the different components. The mobile phase transports the sample components through a separation column and after separation, each component enters the detector. Important for a GC measurement is the volatility of compounds and their thermal stability. The elementary parts of a GC instrument are shown in Figure 13. The GC system consists of a carrier gas, an injector, a separation (capillary) column in a heated oven, and a detector. The carrier gas supply unit generates an optimized gas flow through the column and is connected to a gas cylinder (for hydrogen $H_2$, helium He, and nitrogen $N_2$) (Schomburg, 1990).

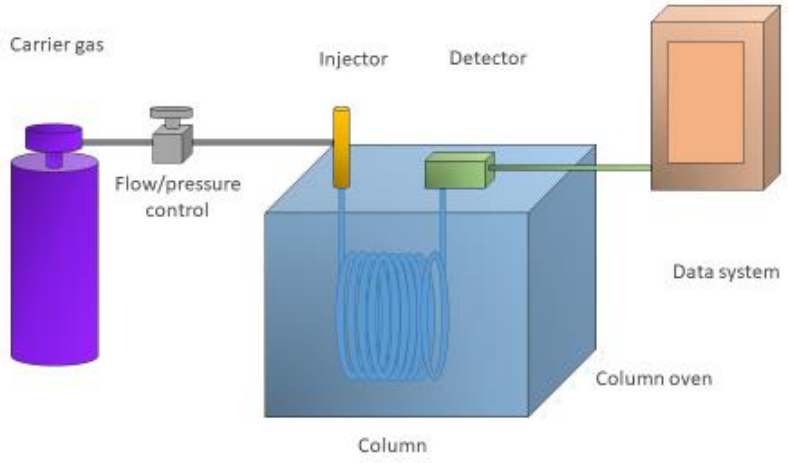


**Figure 13: Basic components of a gas chromatography system.** It consists of carrier gas, flow/pressure control, injector, capillary column, detector, and a data system.

The GC is usually coupled to a mass selective detector (MS) or a flame ionization detector (FID). The FID is a universal detector for carbon-containing compounds with mass flow-dependent response behavior. This universal detector monitors the analytes via the differing electrical conductivity of a flame into which the analyte is introduced.

The coupling of GC with MS has developed to be one of the most sensitive and selective analytical methods for flavor analysis. These techniques employ the sample in the gas phase, giving a 2-dimensional identification consisting of a GC retention time and a mass spectrum for each

component of the sample mix (Worsfold, Townshend, Poole, & Miró, 2019). Mass spectrometry is generally used in flavor science to identify unknown compounds and/or to act as a mass selective GC detector (G. Reineccius & Peterson, 2013). First, the sample reaches the ion source, in which an electron impact (EI) ionizes the sample. The EI gives extensive fragmentation and produces spectra suitable for library searching. The most favored MS analyzer is a quadrupole instrument, in which ion separation is achieved by allowing the ions to drift between four concentric rods carrying DC and AC potentials. The data system (computer) digitalizes the analog output from the MS detector and calculates the peak centroid to produce the *m/z* value. This data can then be processed by the data system to produce spectral plots, various chromatograms or library searches (Worsfold, Poole, et al., 2019).

The GC-MS has limitations like peak overlapping (which means that a complex sample mixture cannot be separated into its single components). The coupling of two or more chromatographic systems is defined as multidimensional chromatography (two separated GC oven) or as comprehensive technique (two columns in the same GC oven). In comprehensive separation systems, two-dimensions are often considered to be the practical limit of such a system. Comprehensive GC×GC was invented by Z. Liu & Phillips, 1991 and they used a thermal modulator to interface two serially coupled GC columns (shown in Figure 14). The first column (for example nonpolar) and the second column (for example polar) are connected in series through the thermal modulator. On the first column (usually 20-30 m length) the sample mix is separated by the boiling points of the compounds and by the interaction with the stationary phase, and the analysis is slow. On the second column (usually 2-5 m length) the compounds are separated by polarity and the analysis is fast.

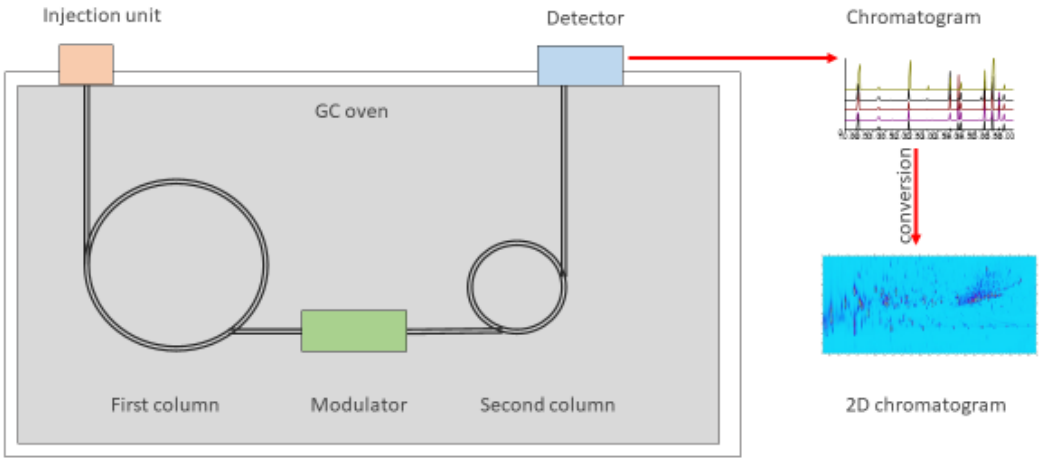


**Figure 14: Overview of Comprehensive GC×GC/MS System.** In the GC oven, there are two columns, the first column (nonpolar) separates by boiling point and the second column (polar) separates by polarity, between them is a thermal modulator which is connected in series. Adaptations based on Shimadzu Corporation.

For the detection of odor-active compounds, gas chromatography coupled with a sniffing device was invented in 1964 (Fuller, Steltenkamp, & Tisserand, 1964). Gas chromatography-olfactometry (GCO) analysis is needed to detect components responsible for the smell of an apple. The sniffing

device is simple (Figure 15). The flow of the gas eluting from the analytical column is split 1:1 to an FID and led to a sniffing port, which is operated by a human. The GCO experiments were combined with traditional threshold analysis to give a value called the aroma value (van Ruth, 2001). The aroma value is defined as the ratio of the concentration of an odor active compound to its odor threshold (van Ruth, 2001). The technique of dilution sniffing analysis determines the relative odor potency of compounds present in an extract. The aroma extraction dilution analysis (AEDA) (Grosch, 1994) is based on the odor detection threshold principle and the flavor dilution factor (FD value) is the highest dilution at which an odor active compound is detected (van Ruth, 2001). The results of GC-olfactory measurements can describe the odor of each peak corresponding to an odor-active component (Heredia et al., 2013, Worsfold et al., 2019). Acree, Barnard, & Cunningham, 1984 developed a dilution analysis technique named CharmAnalysis. This technique has been used to determine the potency of odor-active compounds in foods, also in apples (Acree, Barnard, & Cunningham, 1984b, Cunningham, Acree, Barnard, Butts, & Braell, 1986). Detection frequency methods are based on recording detected odors over a group of assessors and the number of assessors detecting an odor (detection frequency) is used as an estimate of the odors' intensity (van Ruth, 2001).

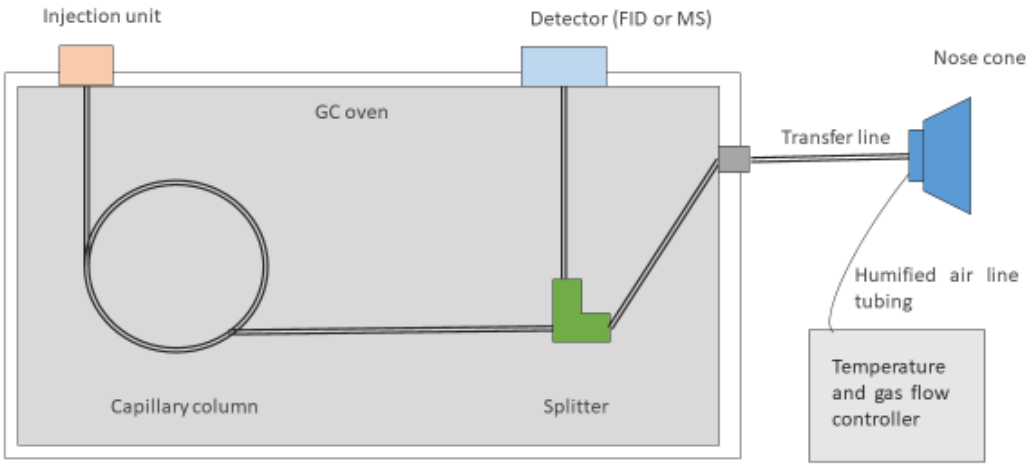


**Figure 15: Gas chromatography-olfactometry system setup.** The column effluent is split 1:1 so that a portion of the effluent goes to a sniffing port and the remainder goes to a GC detector. A stream of air saturated with water is sent to the sniffing port to avoid dehydration of the nasal tissues.

Solid-phase microextraction (SPME) is an effective sample preparation technique in flavor analysis and was first established in the work of Arthur & Pawliszyn, 1990. This sample preparation technique integrates the sample extraction, analyte enrichment and isolation from different sample matrices. The process consists of two basic steps: first, the partitioning of analytes between the extraction phase and the sample matrix and, as second step, the subsequent desorption of analytes into an analytical instrument (Worsfold, Townshend, et al., 2019). The SPME technology is an adaptation of solid-phase extraction (SPE). Its advantages are that it is simple to perform, lowered costs, applicability to small sample sizes, rapid extraction of volatile compounds and a solvent-free extraction (Arthur & Pawliszyn, 1990; Razzazi-Fazeli & Reiter, 2011; Lappas & Lappas, 2016). An SPME device consists of a fiber inside a retractable needle (the retractable feature provides fiber

protection) and an assembly holder (shown in Figure 16). There are two types of SPME fibers, one are fused-silica fiber which are coated on their outer surface with a thin film of extraction phase (liquid polymer and/or a solid sorbent) and the other type are stable flex fibers that consist of a flexible fused-silica core (Worsfold, Townshend, et al., 2019).

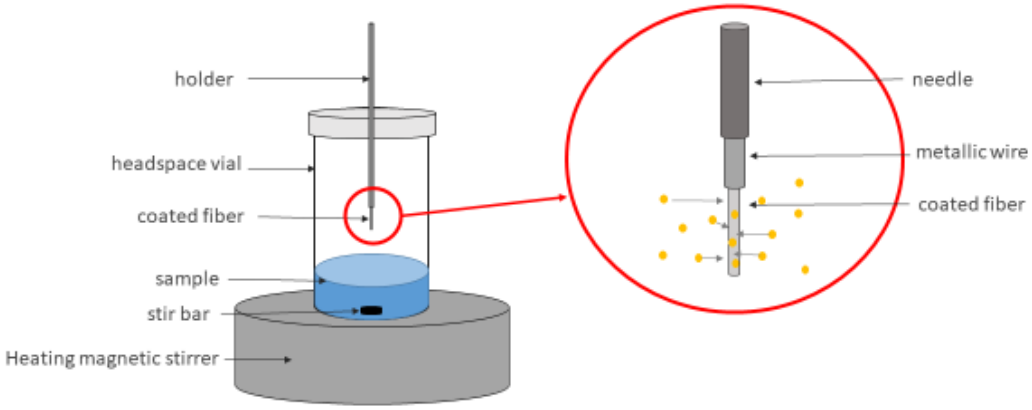


**Figure 16: Schematic representation of headspace solid-phase microextraction.** The set-up of the HS SPME process is simple, a coated fiber is connected to a retractable needle and an assembly holder. The coated fiber, which is placed in the headspace of a sample, adsorbed different classes of volatile compounds as long as equilibrium is reached. Adaptations based on Schmidt & Podmore, 2015 and Farhadi, Tahmasebi, Biparva, & Maleki, 2015

The use of headspace solid-phase microextraction (HS-SPME) for fruit flavor analysis was first demonstrated in the work of Ibáñez, López-Sebastián, Ramos, Tabera, & Reglero, 1998. This method is based on the partitioning of the volatile compounds between the sample headspace and a polymer-coated fiber. The HS-SPME technique depends on the equilibrium of experimental conditions like heating temperature, extraction time, sample volume, the concentration of volatiles, and sample matrix, and also on significant reproducible measurements (Ma, Hamid, Bekhit, Robertson, & Law, 2013). The ad- or absorbent-coated fiber, which can be coated with different coating materials, is placed over the headspace of a sample mix. The fiber can ad- or arb different volatiles. After enrichment, the volatiles are thermally desorbed in the injection system of the GC. The different choices for the ad-/absorbent-coated fiber are Carboxen (CAR), polyacrylate (PA), Carbowax (PEG), polydimethylsiloxane (PDMS) and divinylbenzene (DVB). The choice of the coating material depends on the investigated volatiles, the best to cover an entire range of compounds is a DVB/CAR/PDMS fiber (Gherghel et al., 2018). After the enrichment of the volatiles on an HS SPME fiber, the volatiles are thermally desorbed into an inlet of gas chromatography (GC).

### 2.2.5.3. Identification of the compounds

The identification of the compounds accomplishes to these points:

- the mass spectra of the relevant compound correspond to the relevant reference substance, literature, or mass spectra database
- the retention indices correspond to those listed in in-house database or with relevant reference substances which are measured on two columns with different polarity

- the quality of the odor of the relevant compound match with relevant substances, literature or databases

Retention indices can assist with a clear identification of compounds by measurement and comparison with available retention data collections (Babushok, Linstrom, & Zenkevich, 2011). The system of retention indices (RI) and its modification to temperature programming conditions were first described by Kováts, 1958 and allow the identification of compounds. The identification of the compounds is based on the index of an analyte's relative elution order between the nearest n-alkanes which elute immediately before and after a target analyte (Babushok, 2015). The linear temperature programmed retention indices (RI) were first calculated of van Den Dool & Dec. Kratz, 1963. The equation for the calculation of the linear-temperature programmed retention indices is defined by the following equation:

$$RI = 100\frac{(t_x - t_n)}{(t_{n+1} - t_n)} + 100n \qquad (1)$$

where $t_n$ and $t_{n+1}$ are the retention times of the reference n-alkane hydrocarbons eluting before and after the target analyte x, and $t_x$ is the retention time of analyte x. The retention index of the target analyte x represents one hundred times the number of carbon atoms in the molecule of a hypothetical hydrocarbon, which has the same retention as the target analyte x.

A large number of retention indices data libraries are available in literature sources and many of them are web-based collections (for example NIST Chemistry WebBook[12] or flavornet[13]).

[12] NIST (National Institute of Standards and Technology) Chemistry WebBook https://webbook.nist.gov/chemistry/
[13] Flavornet and human odor space http://www.flavornet.org/flavornet.html

# 3. Material and Methods

The fourth section describes the material and methodology used for this study. The methodological approach taken in this study is a combined methodology based on flavor analysis.

## 3.1. Material

### 3.1.1. Plant materials

The different apple varieties were either purchased from local farmers' market Kaiser Josef Markt in Graz or obtained directly from farmer or School for Fruit Growing and Viticulture Silberberg. The origin of every single batch is listed in Table 2, together with date of harvest or date of purchase, respectively. The Ilzer Rose apple fruits were obtained (a representative sample size of 15 kg per picking date) from a local apple farmer (Krispel, Markt Hartmannsdorf, Styria, Austria) and the fruits were collected from the same apple trees at four different ripening stages in two successive years (2017 and 2018). The selected trees were marked for the harvest. The Kronprinz Rudolf apple fruits (a representative sample size of 7 kg per picking date) were obtained by apple experts of School for Fruit Growing and Viticulture Silberberg. A representative sample of 3 to 5 kg of each investigated apple variety (except Ilzer Rose and Kronprinz Rudolf) was purchased at the Kaiser Josef farmer market and then divided into parts for the sensory and analytical analysis. The identification and description of the apple varieties was performed by an apple expert of the Agricultural Research Center Styria; identification was cross-checked and confirmed using two databases specially designed for the identification of heritage fruit varieties (database 1 by 'Arche Noah' and database 2 by the School for Fruit Growing and Viticulture, Klosterneuburg, Austria). All investigated apple varieties were brought to the lab and were stored at 6°C for a maximum of ten days until the analysis was performed. During this storage period, the storage space was aerated once a day to avoid ethylene accumulation and, consequently, prevent the accelerated ripening of the fruits. Prior analyses, the intact apples have to be taken out of the storage space to reach room temperature.

Table 2: List of analyzed apple varieties

| cultivar | date of harvest | date of purchase | harvested at/ purchased from |
|---|---|---|---|
| Ilzer Rose | - | 04/11/2015 | Kaiser Josef Markt |
| Krummstiel | - | 04/11/2015 | Kaiser Josef Markt |
| Kronprinz Rudolf | - | 04/11/2015 | Kaiser Josef Markt |

| | | | |
|---|---|---|---|
| Schafsnase | - | 04/11/2015 | Kaiser Josef Markt |
| Cox Orange | - | 04/11/2015 | Kaiser Josef Markt |
| Herbstkalvil | - | 04/11/2015 | Kaiser Josef Markt |
| Goldrenette | - | 04/11/2015 | Kaiser Josef Markt |
| Golden Delicious | - | 24/10/2016 | Kaiser Josef Markt |
| Kronprinz Rudolf | - | 24/10/2016 | Kaiser Josef Markt |
| Goldrenette | - | 24/10/2016 | Kaiser Josef Markt |
| Ilzer Rose | - | 24/10/2016 | Kaiser Josef Markt |
| Schafsnase | - | 24/10/2016 | Kaiser Josef Markt |
| Krummstiel | - | 24/10/2016 | Kaiser Josef Markt |
| Kronprinz Rudolf | 18/09/2017 | - | Haidegg, plantation |
| Kronprinz Rudolf | 28/09/2017 | - | Wagersbach, meadow orchard |
| Kronprinz Rudolf | 04/10/2017 | - | Wagersbach, meadow orchard |
| Kronprinz Rudolf | 05/10/2017 | - | Silberberg, plantation |
| Kronprinz Rudolf | 05/10/2017 | - | Hatzendorf, organic, plantation |
| Ilzer Rose | 09/10/2017 | - | M.Hartmannsdorf, Krispel |
| Ilzer Rose | 18/10/2017 | - | M.Hartmannsdorf, Krispel |
| Ilzer Rose | 23/10/2017 | - | M.Hartmannsdorf, Krispel |
| Ilzer Rose | 24/09/2018 | - | M.Hartmannsdorf, Krispel |
| Ilzer Rose | 01/10/2018 | - | M.Hartmannsdorf, Krispel |
| Ilzer Rose | 08/10/2018 | - | M.Hartmannsdorf, Krispel |
| Ilzer Rose | 15/10/2018 | - | M.Hartmannsdorf, Krispel |

An overview of the investigated apple varieties of the harvest year 2015 is presented in Figure 17. The intact apple fruits were stored at 6°C, some of the apple fruits were additionally stored for different time scales and some of them were directly prepared and stored at-20°C before analysis.


Figure 17: The different investigated apple varieties of the harvest 2015

The investigated apple varieties of the harvest 2016 were the same as the year before, such as Ilzer Rose, Schafsnase, Herbstkalvill, Goldrenette, Kronprinz Rudolf, and Krummstiel. In the harvest year 2017 the investigated apple varieties were Ilzer Rose and Kronprinz Rudolf only, but more samples of different plantation growing and locations of the apple farmers were taken. For the harvest in the year 2018 only Ilzer Rose apple variety was investigated, but with differences of ripening stages.

### 3.1.2. Chemicals

The antioxidant solution for the sensory evaluations was prepared by dissolving citric acid (purity ≥ 99.0%; Sigma-Aldrich, Vienna/Austria), ascorbic acid (purity ≥ 99.0%; Sigma-Aldrich, Vienna/Austria) and calcium chloride dehydrate (purity 98%; VWR, Vienna/Austria) in tap water.

The antioxidant mix for the GC analysis was prepared by sodium chloride (purity 99.9%), ascorbic acid (purity 99.0%) and citric acid (purity ≥ 99.0%) (all purchased from Sigma-Aldrich, Vienna/Austria) in tap water. The internal standard 2-octanol (97%) was purchased from Sigma-Aldrich, Vienna/Austria. Additional chemicals used are listed in Table 3.

Table 3: List of used chemicals

| substance | supplier |
|---|---|
| Methanol | VWR, Vienna/Austria |
| NaOH (purity ≥ 98%) | Carl Roth, Karlsruhe/Germany |
| n-alkane (purity ≥ 99.9%) | VWR, Vienna/Austria |

## 3.2. Methods

The analysis of an apple is complex because the sample cannot be simply put into the instrument and the flavor profile is instantly available. The flavor must first be extracted from the apple and then prepared for analysis.

### 3.2.1. Basic parameters

#### 3.2.1.1. Titratable acidity (TA)

An aliquot of the harvested apple samples (Ilzer Rose 2017 and 2018 of the apple farmer Krispel) was taken and juiced for determination of the titratable acidity. It was performed by the titration of 25 ml apple juice with 0.1 M NaOH solution up to pH 9.1 by using an auto titrator (Metrohm 785 DMP Titrino). NaOH (purity ≥ 98%) was obtained from Roth (Carl Roth, Karlsruhe/Germany). The results were expressed as percentage (%) of 0.1 M NaOH used for titration.

#### 3.2.1.2. Determination of soluble solids content (SSC)

From the same sample of juice that was used to determine titratable acidity, the soluble solids content by refractometry (Kern ORD 1RS) was assessed. 1 mL from the apple juice was obtained for the measurement and the results were expressed in °Brix. The conversion of the measured °Brix to the SI- unit $gL^{-1}$ is defined by the following equation:

$$\left[\frac{g}{L}\right] = [°\text{Brix}] \times 10 \qquad (2)$$

### 3.2.2. Sample preparation

#### 3.2.2.1. Sensory analysis

For sensory evaluation of the sliced apples, the apple samples were washed and cut into small slices without the core of the fruits. Immediately after cutting, the apple slices were treated with an antioxidant solution (the composition of this solution is listed in Table 4) according to Corollaro et al., 2013 to avoid browning of the apple pieces and excessive formation of secondary flavor compounds. Once dipped in the solution, apple slices were put into three coded plastic cups and covered with a plastic lid. The samples were presented in a random order for each panelist. Sample preparation was done about one hour before the sensory evaluation.

Table 4: Composition of an antioxidation solution used for the sensory evaluation of apples.

| substance | amount (g per liter) |
|---|---|
| CaCl2 | 5 |
| ascorbic acid | 2 |
| citric acid | 2 |

### 3.2.2.2. Analytique techniques

About 15 kg of each investigated apple variety and each ripening stage of Ilzer Rose apple fruits were picked and transported immediately to the University. The apple fruits were measured in different ways: as intact fruit, the skin only, the flesh only and also the homogenized apple. The intact fruits were washed, weighed, and placed in a 2L glass jar.

At the picking day, a selection of the apple samples was washed and then the apple skin was carefully separated from the flesh. To inactivate apple enzymes as far as possible, apple flesh and skin were treated separately with an antioxidant solution (the composition is described in Table 5) before being used for the subsequent GC analysis (Aprea et al., 2011). For the homogenization of the apples with the added antioxidants, the solution was mixed with a hand blender (Philips blender HR1600/00). The water plays an important role for the dissolving of the acids. The acids and sodium chloride were used for inhibition of the browning enzymatic reactions. Sodium chloride also supported the efficiency of the extraction for the volatile compounds due to the resulting salting-out effect. After homogenization of the apples with the antioxidant solution, the mix was portioned in small screw cap glass vials and stored at -20°C before further analysis.

Table 5: Composition of an antioxidation solution used for the analytical techniques for 75 g of apples.

| substance | amount |
|---|---|
| H2O | 75 mL |
| NaCl | 30 g |
| ascorbic acid | 250 mg |
| citric acid | 250 mg |

Before analysis, the stored apple mix was thawed, 250 mg of the apple mix (50 mg for the comprehensive GC×GC-MS measurements) was weighed into a headspace vial, an internal standard and a glass magnet stirrer were added. 2-Octanol was used as internal standard (50 ng absolute dissolved in methanol for GC-MS and 10 ng absolute dissolved in methanol for comprehensive GC×GC-MS measurements).

Before GC-O analysis, the stored apple mix was thawed and the melted apple mix was weighed into glass vials (1 g/ 500 mg/ 250 mg). After that, a glass magnet stirrer was added and the vial was put on a heating block.

### 3.2.2.3. Enrichment of the apple volatiles with Headspace - Solid Phase Microextraction (SPME)

To enrich the volatile compounds from the intact apples, headspace solid-phase microextraction (HS-SPME) was used. Approximately 1 kg of each variety of apple (corresponding to 10 to 15 apples, depending on their size) was placed into a glass jar that had been washed and heated to 250°C prior to its use to avoid contamination from previous measurements. The glass jar was closed with a closely fitting Teflon lid. The Teflon lid was punctured with four holes, which were then equipped with GC-injector seals, allowing the accurate positioning of the SPME fibers (see in Figure 18). Three SPME fibers were exposed simultaneously into the headspace above the apples. 2 cm stable flex SPME fibers, (DVB/CAR/PDMS, 50/30 µm, Supelco, Bellefonte, PA, USA) mounted on manual sampling devices, were used. Prior to fiber exposure, the apple material was allowed to equilibrate in the glass jar for 20 minutes at room temperature. The three fibers per jar were exposed simultaneously into the headspace above the apples for 60 minutes. After the volatiles had been enriched, the SPME fibers were transferred into the GC injection system for thermal desorption of the volatiles. This procedure resulted in a three-fold repetition per sample.

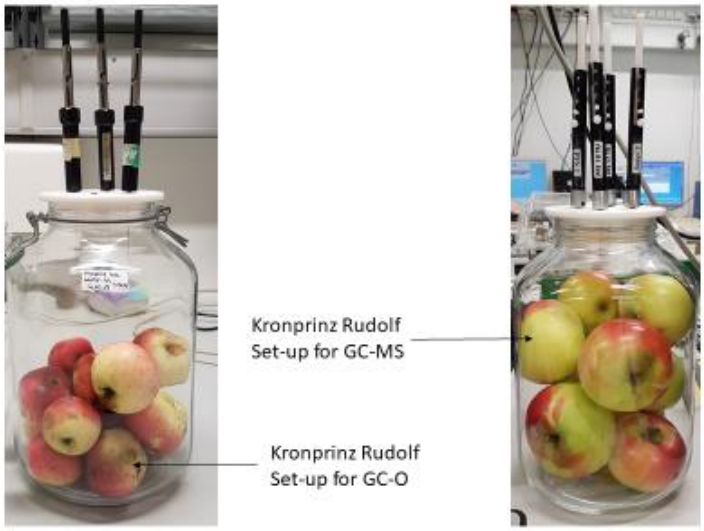


**Figure 18: Set-up for the measurement of the intact apples –** Portable Field Sampler (PFS) were used for the enrichment of the volatiles

The volatile compounds of the different picking dates were extracted by headspace solid-phase micro-extraction (HS-SPME). Aliquots of the homogenized apple samples (250 mg each for 1-dimensional GC-MS and 50 mg for comprehensive GC×GC-MS) were transferred into 20 mL headspace glass vials and an internal standard (2-octanol) was added (50 ng absolute for the GC-MS and 10 ng absolute for the comprehensive GC×GC-MS). After enrichment of the volatiles by HS-SPME at 30°C for 20 min with a 50/30 µm divinylbenzene-carboxen-polydimethylsiloxane DVB/CAR/PDMS 2 cm stable flex fiber (Supelco CO., Ltd, Bellefonte, PA, USA) analyses were performed with GC-MS.

Before GC-O analysis, the volatiles were enriched with SPME and the fibers were 2 cm 50/30 µm DVB/Carboxen/PDMS stable flex.

## 3.2.3. Identification and Quantification

### 3.2.3.1. Sensory evaluation of volatile constituents

The sensory evaluation was conducted in the sensory laboratory of the Graz University of Technology under standardized conditions. An in-house trained panel containing between 12 to 15 assessors (four males, and eleven females, age from 25 to 53 years) from Graz performed the sensory profiling of apples. These individuals had gathered vast sensory experience (i.e., between 5 and 15 years conducting sensory evaluations) prior to the start of this study. Each of the panelists fulfilled the basic requirements set by the DIN EN ISO 8586 (ISO, 2012) standard, including the requirements for the recognition of the basic tastes as well as the ability to recognize a set of odors and describe them using proper descriptors. However, training sessions specific to apple odors were carried out prior to the study to sensitize the panelists to the sensory impressions they would perceive from the investigated samples. During this training period, special emphasis was put on the perception, recognition, and description of compounds that were expected to be present in the apples. Solutions of the compounds were prepared in ethanol in adequate concentrations (1-2%). Filter strips were dipped into the solutions and used as such for the sensory evaluation. The following compounds were used: ethyl propanoate, propyl acetate, ethyl butanoate, ethyl-2-methyl butanoate, propyl butanoate, butyl acetate, butyl butanoate, ethyl hexanoate, hexyl acetate, (E) 2-hexenyl acetate, (Z) 3-hexenyl acetate, butyl hexanoate, 2,3-butandione, n-hexanal, (E) 2-hexenal, (Z) 3-hexenal, (E,E) 2,4-hexadienal, octanal, (E) 2-octenal, (E) 2-decenal, 1-butanol, 2-methyl butanol, 3-methyl butanol, hexanol, (E) 2-hexenol, (Z) 3-hexenol, heptanol, octanol, β-damascenone, linalool. At the time of the investigations, all compounds were registered in the European Union as flavoring compounds and were authorized to be used in flavored foods according to regulation (EU) No 872/2012. The compounds were purchased from Sigma-Aldrich (Vienna, Austria) and had a purity of ≥ 96%. The final selection of the panelists was made according to the ability of the individuals to describe the odour of the samples.

The panelists were selected and trained over weeks according to ISO standards 13299 (ISO, 2010) with different sensory methods to evaluate apples. They had been trained in pre-sessions to study an apple specific knowledge.

For the sensory evaluation of the intact apples, two to three apples (depending on the size of the apples) were placed into a 1-L glass jar equipped with a glass lid. Only apples of high quality without any visible damage were used. The samples were prepared about 30 minutes before the sensory evaluation to allow the system to equilibrate. All samples were marked with three-digit random numbers. To characterize the odor of the apple samples descriptive analysis in the form of 'open-ended questioning' (Piqueras-Fiszman, 2015, Muñoz, Kemp, Hollowood, & Hort, 2018) was applied. The descriptors given were collected and transformed into a list of accurate descriptors by grouping terms according to their meaning. Similar descriptors such as 'apple' and 'fresh apple' were summarized as 'apple.' Descriptors that were named fewer than five times and that could not

be associated with any other descriptors were eliminated. Results are presented in terms of a contingency table.

### 3.2.3.2. Determination of odor thresholds

The general guidance for measuring odor, flavor, and taste detection thresholds by a three-alternative forced-choice (3-AFC) procedure is regulated by the international standard ISO 13301:2002. The determination of the odor threshold was based on ASTM (E679-04) (2011) and previous research with minor modification.

Before the determination of odor thresholds were conducted, the expert panel was trained with sniffing sticks spiked with relevant compounds. Each relevant compound was dissolved in an aqueous ethanol solution and was presented to the expert panel for describing. This training program was important to get information about the odor descriptors of the interesting compounds.

The determination of the odor thresholds was replicated in duplicate by the expert panel. For each compound of interest (i.e., 3-methylbutyl propanoate, propyl hexanoate, hexyl 2-methyl butanoate, ethyl-2-butenoate, 2-methylpropyl butanoate, octyl-2-methyl butanoate, 2-methylpropyl octanoate, 2-methylpropyl propanoate, butyl 3-methylbutanoate, and 5-hexenyl acetate) five 3-AFC tests were carried out. The odor thresholds were determined in an aqueous ethanol solution and performed by using a three-alternative, forced-choice test (3-AFC). The 3-AFC test was conducted with two samples of water spiked with ethanol to compensate for the perception of ethanol and one sample with the investigated compound diluted in ethanol. The concentration of the investigated compound was increased stepwise in each of the five 3-AFC tests in three coded plastic cups covered with a plastic lid. Each panelist started with the lowest concentration followed by the next concentration and selected one sample that was different from the other two. In addition to the 3-AFC test, the expert panel was informed about the odor signature of the investigated compound. This information was important for the expert panel because their perception was more sensitive as in a triangle test. This point is the main difference between these two tests. First, the results of the 3-AFC tests were evaluated separately for each panelist and the individual best estimate threshold was calculated as the geometric mean of the last missed concentration and the next higher concentration. Then the average of each calculated threshold was generated and the group threshold was identified. All sensory tests were carried out with Compusense (Compusense Inc., Guelph, Canada) in computerized individual booths located in a sensory laboratory.

### 3.2.3.3. Gas chromatography-mass spectrometry

The separation and the identification of the volatile compounds were performed by means of gas chromatography–mass spectrometry (GC-MS). One dimensional GC-MS analysis with was performed on two analytical columns of different polarities to confirm the identity of the volatile compounds.

Gas chromatography-mass spectrometry (GC-MS) conditions:

| | |
|---|---|
| Instrument: | Agilent Technologies 7890 MS 5975c VL MSD, Santa Clara, CA, USA |
| Capillary columns: | Rxi-5MS (30 m x 0.25 mm i.d., 0.25 µm film thickness; Restek Corporation U.S.) |
| Temperature program: | -10°C (1 min) - 12°C/min - 280°C (3 min) |
| Carrier gas: | helium (5.0); constant flow; |
| | Flow rate: 31 cm/s |
| | Column head pressure: 133.07 kPa |
| Injector: | split/splitless injector: 270°C; |
| | Splitless mode; SPME Liner; |
| Detector: | MSD; triple-axis detector; |
| | quadrupole mass filters in EI-mode; |
| | Ionization energy: 70 eV; |
| | Source Temperature: 280°C |
| Mass spectrum: | Scan mode; 35-300 amu |

Data acquisition was done with Agilent ChemStation version B.04.03 (Agilent Technologies, Santa Clara, CA, USA).

Gas chromatography-mass spectrometry (GC-MS) conditions:

| | |
|---|---|
| Instrument: | Shimadzu GC-2010 Plus; GCMS-QP 2020; |
| | Shimadzu Europa GmbH |
| Capillary columns: | Rxi-5MS (30 m x 0.25 mm i.d., 1 µm film thickness; Restek Corporation U.S.) |
| Temperature program: | -10°C (1 min) - 12°C/min - 280°C (8 min) |
| Carrier gas: | helium (5.0); constant flow; |
| | Flow rate: 31 cm/s |
| | Column head pressure: 133.07 kPa |
| Injector: | split/splitless injector: 270°C; |
| | Splitless mode; SPME Liner; |
| Detector: | quadrupole mass filters in EI-mode; |
| | Ionization energy: 70 eV; |
| | Ion source Temperature: 230°C |
| | Interface Temperature: 270°C |
| Mass spectrum: | Scan mode; 35-300 amu |

Data acquisition was done with Shimadzu GCMSsolution Workstation version 2.5 (Shimadzu Europe GmbH).

Comprehensive GC×GC-MS analysis was performed on a Shimadzu GC-2010 plus coupled with a fast-scanning quadrupole mass selective detector (for more details – see the following comprehensive GC×GC-MS conditions).

Comprehensive GC×GC-MS conditions:

| | |
|---|---|
| Instrument: | Shimadzu GC-2010 Plus; GCMS-QP 2010 Ultra; |
| | Shimadzu Europa GmbH |
| Modulator: | Thermal Cryo modulator ZOEX ZX-1; |
| | Modulation time: 5 s; Hot jet: 280°C, |
| | Pulse time: 350 msec |
| Capillary columns: | Rxi-1HT (30 m x 0.25 mm i.d., 0.25 µm film thickness; Restek Corporation U.S.) |
| | BPX 50 (2 m x 0.15 mm i.d., 0.15 µm film thickness; SGE GmbH, Germany) |
| Temperature program: | 35°C (1 min) - 3°C/min - 210°C - 20°C/min - 280°C (3 min) |
| Carrier gas: | helium (5.0), constant flow; |
| | Pressure: 35 kPa |
| | Total flow: 12.9 mL/min |
| | Linear velocity: 24.7 cm/sec |
| Injector: | split/splitless injector: 270°C; |
| | Splitless mode; SPME Liner; |
| Detector: | quadrupole mass filters in EI-mode; |
| | Ionization energy: 70 eV; |
| | Source Temperature: 200°C; |
| | Interface: 220°C |
| Mass spectrum: | Scan mode; 35-300 amu |
| | Scan Speed: 20 000 |

Data acquisition was done with GC image version 2.7 (Shimadzu Europe GmbH).

### 3.2.3.4. Gas chromatography – olfactometry (GC-O)

A Hewlett-Packard 5890 series II (Agilent, Santa Clara, CA, USA) was equipped with a FID detector and a sniffing port. During the GC-O analysis, the panelists sniffed the column effluent and reported each detected odorant. For more details, see the following conditions:

Gas chromatography-olfactometry (GC-O) conditions:

| | |
|---|---|
| Instrument: | Hewlett-Packard 5890 series II; FID |
| Capillary columns: | HP-5 (30 m x 0.32 mm i.d., 0.25 µm film thickness; Restek Corporation U.S.) |
| Temperature program: | 35°C (1 min) - 10°C/min - 270°C |

| | |
|---|---|
| Carrier gas: | helium (5.0); constant pressure; |
| | Pressure: 50 kPa |
| Injector: | split/splitless injector: 270°C; |
| | Splitless mode; SPME Liner; |
| Detector: | Flame ionization detector (FID); |
| | Detector Temperature: 300°C |
| Sniffing port: | Gerstel ODP; sniffing port (200°C) eluent split FID/ODP 1:1 |

### 3.2.3.5. Determination of retention indices (RIs)

The volatile compounds were analyzed by GC-O, GC-MS, and comprehensive GC×GC-MS. The identifications were carried out by comparison of mass spectrometric and retention indices to those of authentic reference compounds. For determination of RIs on all systems used, the homologous series of n-alkanes (C5 to C20) were used as standard compounds. RIs were calculated according to equation (1).

### 3.2.3.6. Statistical analysis

Multivariate statistical data analyses were performed to identify correlations between the investigated apple varieties and the results obtained from the gas chromatographic analysis of volatile compounds. Each experiment was performed in triplicate and results were presented as means ± standard deviations. Multivariate statistical data analysis was performed by the use of XLSTAT Software (version 2018.5; Addinsoft, New York, NY; USA). Principal component analysis (PCA) using Pearson correlation was conducted. Cluster analysis of the investigated apple varieties was performed by the use of the relative concentrations of the volatile compounds.

# Results

# 4. Results and Discussion

While volatile compounds were described previously for the most frequently commercially available apple varieties such as Golden Delicious (De Pooter, Dirinck, Willaert, & Schamp, 1981; Salas et al., 2011), Gala (Both et al., 2016), Elstar (Fuhrmann & Grosch, 2002), Topaz (Giannetti, Boccacci Mariani, Mannino, & Marini, 2017), Braeburn (Aprea et al., 2012) and Cox Orange (Fuhrmann & Grosch, 2002), volatile compounds identified for heritage Austrian apple varieties Herbstkalvil, Krummstiel, Kronprinz Rudolf, Goldrenette, Ilzer Rose and Schafsnase have not been reported so far. Therefore, one of the objectives of the present work was to identify and quantify volatile compounds in heritage apple varieties from Styria, applying a gentle extraction method, such as Headspace-Solid Phase Microextraction (HS-SPME), and to illustrate the impact of the volatiles on the flavor profile. In addition, sensory evaluation was used to characterize the overall flavor properties. The influence of the different growing conditions (see section 4.3) and the ripeness (see section 4.4) was additionally investigated.

## 4.1. Flavor profiles of heritage apple varieties from Styria

The odor and the primary flavor compounds emitted from intact apples (harvest year 2015) and enzyme-inactivated apples were investigated by sensory evaluation. In contrast to other studies, in this work the emitted compounds were not analyzed via gas chromatographic techniques alone, also the odor of the intact apples and enzyme-inactivated apple slices were determined by a sensory expert panel.

As described in Table 8, a several number of volatile compounds were identified in the seven investigated apple varieties (Ilzer Rose, Schafsnase, Goldrenette, Herbstkalvil, Krummstiel, Cox Orange, Kronprinz Rudolf). Thus, the investigated heritage apple varieties were evaluated by an expert panel under standardized conditions in a sensory lab. The apple fruits were evaluated in a two-step procedure: first, the intact apple samples were investigated by their odor and their primary flavor compounds that were emitted from them, and second, the enzyme-inactivated apple slices were analyzed. To the best of our knowledge, this approach for the evaluation of apple odor has not been used in previous studies. The sample preparation for the intact apples was performed analogously to the sample preparation for the gas chromatographic analysis. By providing a standardized amount of apples of each variety in standardized glass jars, standardized conditions were established for each panelist. It was necessary to allow the samples to equilibrate in the glass jars for the distinct apple odor to develop. The sample preparation for the enzyme-inactivated apple slices was performed according to Corollaro et al., (2013) to avoid browning of the apple pieces and excessive formation of secondary flavor compounds. As the panelists had been specifically trained to describe the perceived odors, 'open-ended questioning' (Piqueras-Fiszman, 2015; Muñoz et al., 2018) was selected as the method of choice. Through this approach the panelists had as much

freedom as possible while describing the perceived odor. The results obtained from the open-ended questioning session can be seen in Table 6.

Table 6: Contingency table as result from open-ended questioning of the intact apples and enzyme-inactivated apple slices; n=9

| Attribute | Ilzer Rose | Schafs-nase | Gold-renette | Herbst-kalvil | Krumm-stiel | Cox Orange | Kronprinz Rudolf |
|---|---|---|---|---|---|---|---|
| sweet | 2 | 1 | 5 | 0 | 1 | 3 | 3 |
| sour | 5 | 0 | 0 | 3 | 0 | 0 | 3 |
| astringent | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| crisp | 6 | 0 | 2 | 2 | 0 | 1 | 1 |
| mealy | 0 | 4 | 2 | 3 | 5 | 2 | 3 |
| juiciness | 3 | 1 | 2 | 0 | 0 | 0 | 2 |
| fruity | 5 | 2 | 4 | 1 | 2 | 4 | 3 |
| banana | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| citrus | 0 | 0 | 0 | 1 | 1 | 1 | 2 |
| red berries | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| flower | 2 | 1 | 2 | 0 | 1 | 1 | 0 |
| apple flavor | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| green | 2 | 1 | 1 | 1 | 2 | 0 | 2 |
| Spicy | 0 | 3 | 1 | 1 | 1 | 2 | 1 |
| fermentation notes | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Fusty | 0 | 1 | 0 | 1 | 1 | 0 | 0 |

Clear differences were observed within odor descriptors as well as in frequencies of the given descriptors, depending on the investigated apple variety. Interestingly, the descriptor with the highest overall numbers of entries was general ‘fruitiness’ followed by ‘crispness’ and ‘sweet odor’. Ilzer Rose was given the highest ratings for ‘fruitiness’ and ‘crispness’, whereas Schafsnase was only evaluated as ‘mealy’ with minor nominations in spicy and fruity attributes. In contrast, Goldrenette was described with distinct ‘sweet odor’ and ‘fruitiness’ odor.

The detailed results of the descriptive evaluation of each investigated heritage apple variety are listed in Table 7. The odor of the intact apples is generally different to the odor of the enzyme-inactivated apple slices. These results confirm that the odor of intact apples formed mainly by primary flavor compounds and the odor of enzyme-inactivated apple slices formed also by secondary flavor compounds (after cell disruption). Furthermore, the description of the flavor of the apple slices also varies from the odor.

Table 7: Results of the sensory evaluation of the intact apples and enzyme-inactivated apple slices from heritage apple varieties.

| Variety | Odor – intact apples | Odor – apple slices | Flavor – apple slices |
|---|---|---|---|
| Krumm-stiel | sweet, slightly fruity and banana notes, mature | woody, herb, sweet, green and grassy notes | mealy, sweet, slightly fruity and spicy, slightly banana and citrus notes, hardly sour |
| Gold-renette | mature, sweet, fruity, earthy | sweet, banana, citrus, fusty | sweet, hardly sour, fruity, floral notes, green and grassy notes, juicy |
| Ilzer Rose | sweet, floral, fruity | floral, mature, sweet, apple flavor, red berry notes | sour, juicy, fruity, crisp, slightly sweetness |
| Kron-prinz Rudolf | green and grassy notes, citrus, fruity, sweet, berry | sweet, sour, apple flavor, slightly green and grassy notes | juicy, sweet, moderate sourness, crispy peel, mealy flesh, spicy, berry |
| Cox Orange | sweet, herb, mature, fruity | fruity, banana notes, mature, green and grassy notes | juicy, sweet, slight sourness, crisp |
| Herbst-kalvil | sweet, slightly fruity, mature, herb, fermentation notes | sweet, slightly fruity, green and grass notes | sour, crisp, citrus, juicy |
| Schafs-nase | sweet, mature, spicy, herb | sweet, mature, citrus, green and grassy notes, banana | mealy, slightly sourness and sweetness, herb, woody notes, hardly apple flavor |

Volatile compounds of seven heritage Austrian apple varieties (Herbstkalvil, Krummstiel, Kronprinz Rudolf, Goldrenette, Cox orange, Ilzer Rose, and Schafsnase) were isolated via Headspace-solid phase microextraction (HS-SPME). HS-SPME coupled to gas chromatography-mass spectrometry (GC-MS) was selected for the enrichment, separation, and identification of volatile compounds emitted from the enzyme-inactivated apple samples. Through the set of analyses of the harvest 2015 the impact of the several amount of the different volatile compounds on each investigated heritage apple variety was investigated. The investigated apples (without the core) were mixed with an antioxidation solution and subsequently measured via GC-MS. The addition of the antioxidation solution was necessary for the inhibition of enzymatic reactions and consequent oxidation due to polyphenol oxidases (Aprea et al., 2011). A summary of the identified compounds, including mean value and standard deviation (quadruplicate analysis), are given in Table 8. In total, 46 compounds were identified in seven different heritage apple varieties. In agreement with previous studies, the major class of volatile compounds isolated from apple fruits are the class of esters. However, the rather high concentrations of C6-compounds, a typical class of the secondary flavor compounds, are reported in data from previous studies (Meigh, 1957; Lopez, Lavilla, Riba, & Vendrell, 1998; Dixon & Hewett, 2000; Pérez & Sanz, 2008). Table 8 lists the volatile compounds obtained from the analysis of the investigated seven apple varieties of the harvest year 2015. In total, 46 compounds were identified in the seven apple samples.

Table 8: Volatile compounds obtained from HS-SPME GC-MS analysis from seven heritage apple varieties of the harvest 2015 expressed as relative concentration to the internal standard 2-octanol in µgkg$^{-1}$ +/i RSd (%) (n=4);. TIC total ion chromatogram;

| Compounds | RT | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | Odor description[a] | Ilzer Rose ± RSD (%) | Cox Orange ± RSD (%) | Goldrenette ± RSD (%) | Herbstkalvil ± RSD (%) | Kronprinz Rudolf ± RSD (%) | Krummstiel ± RSD (%) | Schafsnase ± RSD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Butanal | 6.87 | | 596 | green, pungent | 1 ± 0.05 | 4 ± 0.01 | 12 ± 0.52 | 1 ± 0.03 | 9 ± 0.16 | 5 ± 0.13 | 19 ± 0.47 |
| Hexane[t] | 6.99 | 601 | 600 | alkane | 55 ± 0.23 | 56 ± 0.39 | 54 ± 0.21 | 50 ± 0.18 | 46 ± 0.50 | 41 ± 0.35 | 43 ± 0.29 |
| Ethyl acetate[t] | 7.31 | 617 | 612 | pineapple | 461 ± 1.48 | 432 ± 1.23 | 418 ± 0.03 | 407 ± 1.35 | 364 ± 1.55 | 381 ± 2.24 | 380 ± 1.30 |
| (E) - 2-Butenal | 7.99 | 653 | 648 | flower | n.d. | n.d. | n.d. | n.d. | 15 ± 2.17 | n.d. | 14 ± 1.91 |
| 2-Methyl-1-propanol | 7.58 | 631 | 629 | ethereal, winey | 6 ± 0.90 | n.d. | 25 ± 1.46 | 9 ± 0.71 | 44 ± 0.56 | 66 ± 0.11 | 26 ± 1.85 |
| 1-Butanol | 8.24 | 666 | 675 | medicine, fruit | 426 ± 9.36 | n.d. | 957 ± 16.61 | 828 ± 10.04 | 923 ± 14.25 | 1148 ± 11.06 | 2300 ± 5.31 |
| 2-Pentanone | 8.66 | 688 | 685 | fruit, pungent | 53 ± 2.40 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| n-Pentanal | 8.84 | 698 | 699 | fermented, bready, fruity | 9 ± 0.24 | 148 ± 0.43 | 18 ± 0.40 | 14 ± 0.23 | 29 ± 0.64 | 9 ± 0.09 | 24 ± 0.77 |
| Heptane[t] | 8.90 | 701 | 700 | alkane | 1 ± 0.14 | 55 ± 0.39 | 2 ± 0.15 | 2 ± 0.14 | 4 ± 0.39 | 1 ± 0.11 | 1 ± 0.14 |
| Ethyl propanoate | 9.10 | 712 | 713 | fruit | n.d. | n.d. | n.d. | 8 ± 0.54 | 2 ± 0.09 | 3 ± 0.20 | n.d. |
| Propyl acetate | 9.14 | 715 | 713 | celery, floral, pear, red fruit | 14 ± 1.11 | 3 ± 0.18 | 1 ± 0.05 | 1 ± 0.03 | n.d. | n.d. | n.d. |
| Methyl butanoate | 9.30 | 724 | 723 | ether, fruit, sweet | 2 ± 0.02 | 2 ± 0.10 | 2 ± 0.01 | 7 ± 0.32 | 3 ± 0.05 | 6 ± 0.31 | 2 ± 0.01 |
| 2-Methyl-1-butanol | 9.58 | 740 | 739 | malt | 495 ± 7.62 | 31 ± 1.29 | 200 ± 7.65 | 391 ± 5.81 | 320 ± 5.32 | 217 ± 5.31 | 871 ± 6.42 |
| 1-Pentanol | 10.06 | 767 | 766 | fruity, balsamic | 7 ± 1.01 | 6 ± 0.16 | 32 ± 1.27 | 54 ± 1.32 | 47 ± 0.69 | 59 ± 0.87 | 82 ± 3.40 |
| Methyl 2-methylbutanoate | 10.27 | 779 | 776 | apple | n.d. | n.d. | 6 ± 0.10 | n.d. | 9 ± 1.21 | n.d. | 4 ± 0.27 |
| Hexanal/Ethyl butanoate* | 10.64 | 800 | 801/ 803 | grass, tallow /apple | 13 ± 0.27 | 42 ± 1.16 | 52 ± 1.46 | 292 ± 2.14 | 264 ± 9.33 | 144 ± 8.64 | 61 ± 1.97 |
| Butyl acetate | 10.84 | 812 | 812 | pear | 115 ± 6.67 | 195 ± 10.69 | 17 ± 1.75 | n.d. | n.d. | 6 ± 0.43 | 2 ± 0.15 |
| 2-Methylbutanoic acid | 11.25 | 837 | 846 | cheese, sweat | 7 ± 0.77 | 4 ± 0.19 | 9 ± 0.13 | 2 ± 0.03 | 9 ± 1.26 | n.d. | 174 ± 2.45 |
| Ethyl 2-methylbutanoate | 11.45 | 850 | 848 | apple | n.d. | n.d. | n.d. | 51 ± 3.65 | 9 ± 0.64 | 8 ± 0.65 | n.d. |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (E)-2-Hexenal | 11.54 | 855 | 854 | apple, green | 80 ± 0.79 | 55 ± 0.03 | 143 ± 0.81 | 97 ± 0.44 | 53 ± 0.20 | 146 ± 0.49 | 354 ± 0.71 |
| 1-Hexanol | 11.73 | 867 | 867 | resin, flower, green | 1887 ± 7.39 | 1575 ± 5.12 | 3122 ± 10.39 | 1543 ± 14.56 | 1944 ± 8.42 | 1599 ± 1.84 | 2488 ± 2.56 |
| 2-Methylbutyl acetate | 11.90 | 878 | 880 | fruit | 238 ± 9.44 | 41 ± 1.60 | n.d. | n.d. | n.d. | n.d. | n.d. |
| Pentyl acetate | 11.90 | 910 | 912 | ethereal, fruity, banana, pear | 9 ± 0.48 | 7 ± 0.18 | n.d. | n.d. | n.d. | n.d. | n.d. |
| (E)-2-Heptenal | 12.43 | 960 | 957 | green | 28 ± 1.15 | 51 ± 5.14 | 116 ± 1.36 | 96 ± 2.90 | 245 ± 1.14 | 69 ± 1.91 | 199 ± 10.82 |
| 1-Octen-3-one/ 1-Octen-3-ol* | 13.15 | 979 | 975/ 977 | earthy/ mushroom | 15 ± 1.18 | 448 ± 3.85 | 89 ± 0.64 | 56 ± 1.35 | 209 ± 9.82 | 45 ± 0.99 | 136 ± 5.93 |
| 1-Heptanol | 13.28 | 968 | 970 | mushroom | n.d. | n.d. | n.d. | 5 ± 0.17 | n.d. | 9 ± 0.22 | 16 ± 0.41 |
| Benzaldehyde | 13.35 | 973 | 971 | fruity, almond, cherry | n.d. | n.d. | n.d. | 2 ± 0.02 | 2 ± 0.15 | 1 ± 0.01 | 3 ± 0.10 |
| 6-Methyl-5-hepten-2-one | 13.44 | 979 | 986 | pungent | n.d. | 15 ± 2.15 | 9 ± 0.95 | n.d. | n.d. | n.d. | n.d. |
| Butyl butanoate | 13.64 | 992 | 996 | fruity, banana, pineapple, sweet | 39 ± 4.52 | n.d. | n.d. | 19 ± 1.42 | 29 ± 0.37 | 32 ± 2.53 | n.d. |
| Ethyl hexanoate | 13.69 | 996 | 1002 | apple peel, fruit | n.d. | n.d. | n.d. | 46 ± 2.52 | 36 ± 0.63 | n.d. | n.d. |
| Octanal | 13.81 | 1004 | 1002 | aldehydic, citrus, orange | n.d. | n.d. | 3 ± 0.24 | 3 ± 0.05 | 9 ± 0.37 | 4 ± 0.09 | 12 ± 0.46 |
| Hexyl acetate | 13.88 | 1009 | 1008 | fruit, herb | 119 ± 6.15 | 432 ± 10.51 | n.d. | n.d. | n.d. | n.d. | n.d. |
| (E,E)-2,4-Heptadienal | 13.97 | 1016 | 1015 | fatty, green, vegetable | n.d. | 1 ± 0.14 | 3 ± 0.01 | 3 ± 0.10 | 10 ± 0.47 | 2 ± 0.06 | 9 ± 0.61 |
| Butyl 2-methylbutanoate | 14.32 | 1041 | 1041 | green | 16 ± 0.72 | 4 ± 0.12 | 3 ± 0.16 | 16 ± 0.59 | 4 ± 0.10 | 7 ± 0.57 | 2 ± 0.15 |
| (E)-2-Octenal | 14.62 | 1063 | 1060 | fatty, green, herbal | 16 ± 0.38 | 24 ± 1.85 | 51 ± 0.49 | 44 ± 1.04 | 143 ± 5.28 | 34 ± 0.64 | 89 ± 4.06 |
| 1-Octanol | 14.72 | 1070 | 1074 | waxy, green, orange | 7 ± 0.11 | 4 ± 0.16 | 4 ± 0.49 | 17 ± 0.13 | 13 ± 1.02 | 20 ± 1.44 | n.d. |
| (Z)- Linalool oxide[t] | 14.92 | 1085 | 1066 | earthy, floral, sweet, woody | 9 ± 0.27 | 11 ± 0.22 | 8 ± 0.12 | 7 ± 0.11 | 6 ± 0.12 | 6 ± 0.07 | 11 ± 0.20 |
| (E)- Linalool oxide[t] | 15.12 | 1099 | 1088 | floral, green, earthy | n.d. | 5 ± 0.18 | 5 ± 0.03 | 3 ± 0.06 | 3 ± 0.12 | 3 ± 0.04 | 5 ± 0.04 |
| Hexyl butanoate | 16.29 | 1190 | 1192 | apple peel | 8 ± 0.24 | 74 ± 1.12 | n.d. | 102 ± 2.36 | 35 ± 0.42 | 171 ± 4.94 | n.d. |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Hexyl 2-methylbutanoate | 16.88 | 1237 | 1237 | green, waxy, fruity | 54 ± 1.50 | 48 ± 0.72 | n.d. | 73 ± 2.36 | 15 ± 0.33 | 43 ± 0.49 | 30 ± 0.21 |
| Hexyl hexanoate | 18.57 | 1377 | 1385 | apple peel, peach | 5 ± 0.31 | 24 ± 0.52 | n.d. | 83 ± 3.49 | 16 ± 1.11 | 58 ± 1.31 | n.d. |
| Ethyl decanoate | 18.68 | 1393 | 1392 | sweet, fruity, apple, waxy | n.d. | 7 ± 0.21 | n.d. | n.d. | n.d. | n.d. | n.d. |
| β Damascenone | 18.85 | 1404 | 1397 | sweet, fruity, floral | 2 ± 0.05 | 3 ± 0.02 | 3 ± 0.04 | 2 ± 0.03 | 3 ± 0.02 | 2 ± 0.02 | 1 ± 0.05 |
| (E)-α-Geranyl acetone | 19.42 | 1460 | 1451 | fresh, rose, leafy, floral | 5 ± 0.17 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| (E)-α-Bergamotene[t] | 19.81 | 1498 | - | woody | 4 ± 0.27 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| α- Farnesene | 19.99 | 1517 | 1509 | woody, sweet | 53 ± 1.08 | 121 ± 1.36 | 55 ± 2.66 | 72 ± 0.72 | 159 ± 5.49 | 117 ± 2.17 | 316 ± 7.47 |

RT – retention time; $RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; a - Odor descriptions are based on Flavornet (www.flavornet.org), femaflavor (www.femaflavor.org) and Pherobase (www.pherobase.com) online databases; n.d. – not detectable or below limit of detection; t - tentatively identified; * - coelution by these compounds, not clearly detected on this column; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org)

Multivariate data analysis (i.e., principal component analysis PCA) was conducted to identify correlations between the volatiles and the seven investigated heritage apple varieties. The PCA was performed using the seven investigated apple varieties and their concentrations of the 48 compounds of the total ion chromatograms (TIC). Results from multivariate data analysis are presented in Figure 19 (observations) and Figure 20 (corresponding biplot).

As can be seen from Figure 19, the investigated apple varieties can be differentiated based on their volatile compounds. High correlations can be observed between the apple varieties Herbstkalvil and Krummstiel that are located in quadrant I, as well as Ilzer Rose, Cox Orange, and Goldrenette, which are located in quadrant II. The two heritage apple varieties Kronprinz Rudolf (quadrant III) and Schafsnase (quadrant IV), do not show any correlations with any of the other apple varieties under investigation.

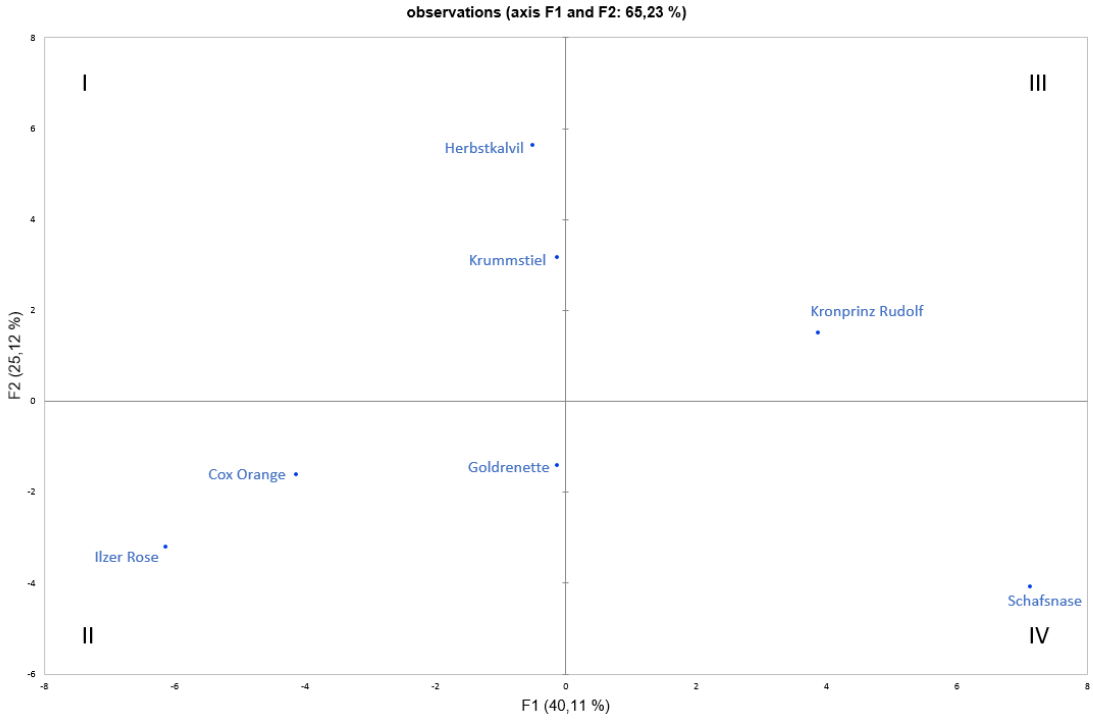


**Figure 19: Harvest 2015 -** Observations obtained by PCA based on the HS-SPME-GC-MS results from the investigated seven apple varieties of Styria (Ilzer Rose, Cox Orange, Goldrenette, Schafsnase, Kronprinz Rudolf, Krummstiel, Herbstkalvil).

The biplot (Figure 20) of the seven investigated heritage apple varieties from the harvest year 2015 presents the correlations between the concentrations of the analysed volatile compounds and the different varieties. The apple varieties Krummstiel and Herbstkalvil have a high correlation with esters such as hexyl butanoate, methyl butanoate, hexyl hexanoate, ethyl propanoate, butyl butanoate, and ethyl hexanoate. Also, the alcohol 1-octanol is highly correlated with these two varieties. The apple varieties Cox Orange and Ilzer Rose are highly correlated with the acetates such as butyl acetate, propyl acetate, 2-methylbutyl acetate, and with other compounds like 6-methyl-5-hepten-2-one, (E)-α-bergamotene, (E)-α-geranyl acetone, and 1-heptanol. It seems that the heritage apple variety Goldrenette has no clear correlation with such volatile compounds. The

resemblance between the heritage apple varieties Kronprinz Rudolf and Schafsnase is also confirmable through the high correlations of the volatile compounds such as 1-pentanol, 1-hexanol, 2-octenal, 1-butanol, (E)-2-heptenal, octanal, 2-methyl-1-propanol, pentanal, α-farnesene, (E)-2-hexenal, butanal, methyl-2-methylbutanoate, 1-propanol, and 2-methyl-1-butanol. The compounds 1-octen-3-one and 1-octen-3-ol could not be separated, these two compounds were co-eluting. Another co-elution appeared with the compounds hexanal and ethyl butanoate.

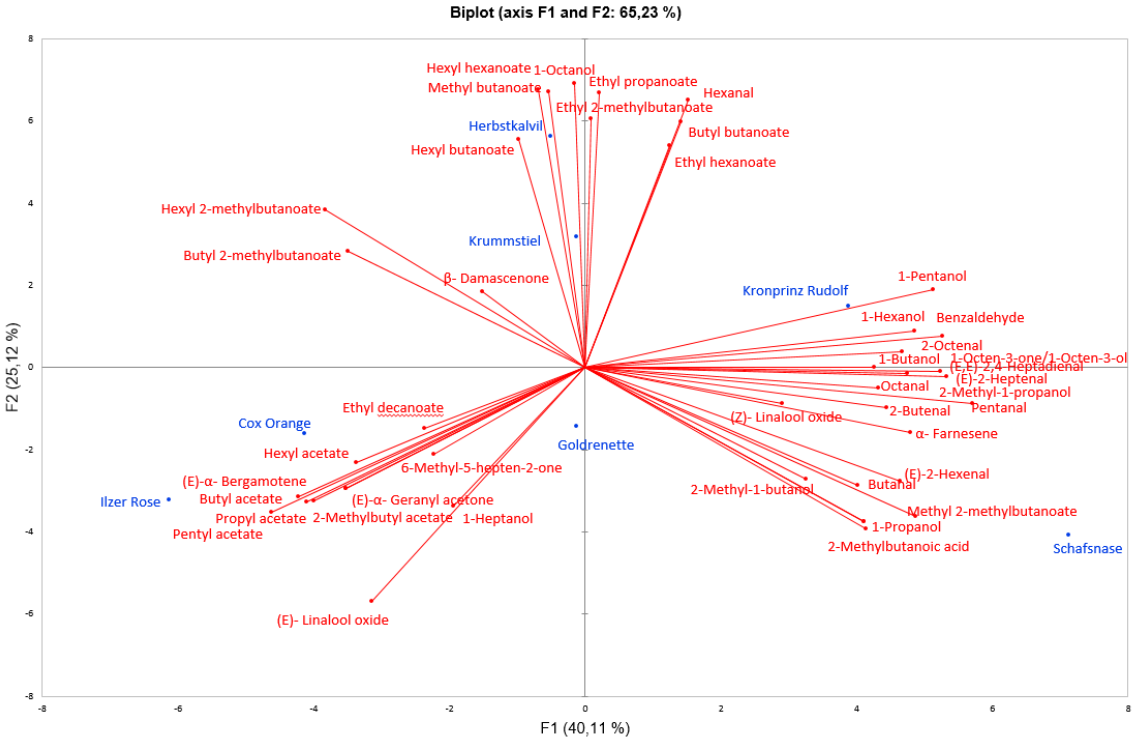


**Figure 20: Harvest 2015** - Biplot scores and factor loadings obtained by PCA based on the HS-SPME-GC-MS results from the investigated seven apple varieties (Ilzer Rose, Cox Orange, Goldrenette, Schafsnase, Kronprinz Rudolf, Krummstiel, Herbstkalvil)

The sensory properties of the apple varieties can be explained by examining their correlation with the relative concentrations of the volatiles (see also odor descriptions in Table 8). The dominated alcohols, such as 1-pentanol, 1-hexanol, 1-butanol, are correlated with the apple-fruity odor of the apple variety Kronprinz Rudolf. The odor of the apple varieties Cox Orange and Ilzer Rose are dominated by the presence of the acetate esters hexyl acetate, butyl acetate, propyl acetate, pentyl acetate, and 2-methylbutyl acetate. Acetate esters are generally known for their fruity, banana- and pear-like odor, and all these attributes were displayed by these two apple varieties. These findings are in accordance with the results of previous investigations, in which a correlation between the banana notes in apples and the presence of acetate esters was shown (Aprea et al., 2012). The odor of Schafsnase is dominated by (E)-2-hexenal, butanal, methyl 2-methyl butanoate, 1-propanol, 2-methylbutanoic acid and 2-methyl-1-butanol. These compounds are expressed with a green, apple flavor (Carrapiso, Jurado, Timon, & Garcia, 2002). A correlation was found between the volatiles hexyl butanoate, hexyl hexanoate, methyl butanoate, and the apple varieties Krummstiel and Herbstkalvil. These compounds are described with fruity and apple peel notes.

According to these data sets of the harvest 2015, it can be concluded that the heritage apple varieties have significant amounts of different volatiles which are important for their specific flavor. Most of these apple varieties were reported for the first time on the flavor composition. The fact that the flavor composition of each investigated apple variety was different, prompted to specifically address the impact of a variety-specific spectrum of volatile compounds.

## 4.2. Differences in the compositions of volatile compounds determined from peel and flesh of heritage apple varieties

Because of the climatic fluctuations, 2016 was a very difficult harvest year for the apple farmers; the spring frost in April and the heavy rain days in August risked the whole agricultural production in Styria (Unterberger et al., 2018). The sensitive apple blossoms were damaged by spring frost and as consequence, the Austrian apple production (in special, the main apple-producing region Styria) dropped down to only 60.000 t harvest in this year (Statistik Austria, 2016). As a result, the implementation of the planned measurements for the seven different heritage apple varieties were not possible in reproducible quality (not 25 kg per variety with different growing conditions and different ripening stages). Subsequently, another approach was taken for this part of the thesis: the biosynthesis of the volatile compounds in the apple peel and flesh (seen in Figure 21).

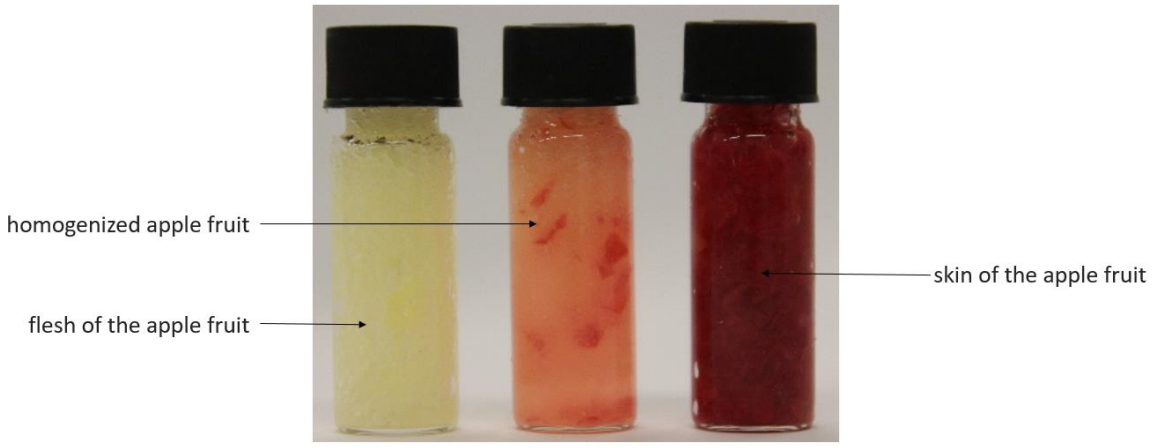


**Figure 21: Example for homogenized apple fruit, only flesh and peel of the fruit;** The apple variety Ilzer Rose is shown.

As mentioned in section 2.2.4 the significant functional differences between the peel and the flesh of apples indicate that the composition of the volatiles may also differ. However, the biosynthesis of the volatile compounds in apples is a very complex system, due to the fact, that the different pathways of the compounds are linked together. In general, the production of volatiles, but also the enzyme activity levels, are higher in the apple peel than in the apple flesh. Also important to know

is, that the availability of primary precursor substances is highly regulated in terms of their amount and composition during fruit development (Ackermann, Fischer, & Amado, 1992).

Small amounts (such as 5-8 kg) of apple varieties Golden Delicious, Kronprinz Rudolf, Goldrenette, Ilzer Rose, Schafsnase, and Krummstiel were purchased at local farmer's market Kaiser-Josef Platz. Prior to the GC analysis, the apple were prepared and inactivated with an antioxidation solution according to 3.2.2. In total, 60 volatile compounds were identified in the apple peel samples (see Table 9) and 48 compounds in the apple flesh samples (see Table 10). Eleven alcohols, 23 esters, twelve aldehydes, twelve other compounds such as ketones and terpenes, and two co-elutioning compound pairs (hexanal/ethyl butanoate and 1-octen-2-one/1-octen-3-ol) were determined. Figure 22 shows the principal component analysis (PCA) based on the correlation of the HS-SPME-GC-MS results from the six investigated apple varieties. The separation of the apple peel from the flesh is therefore substantial. Nevertheless, all apple flesh samples are show a similar correlation of the volatiles.

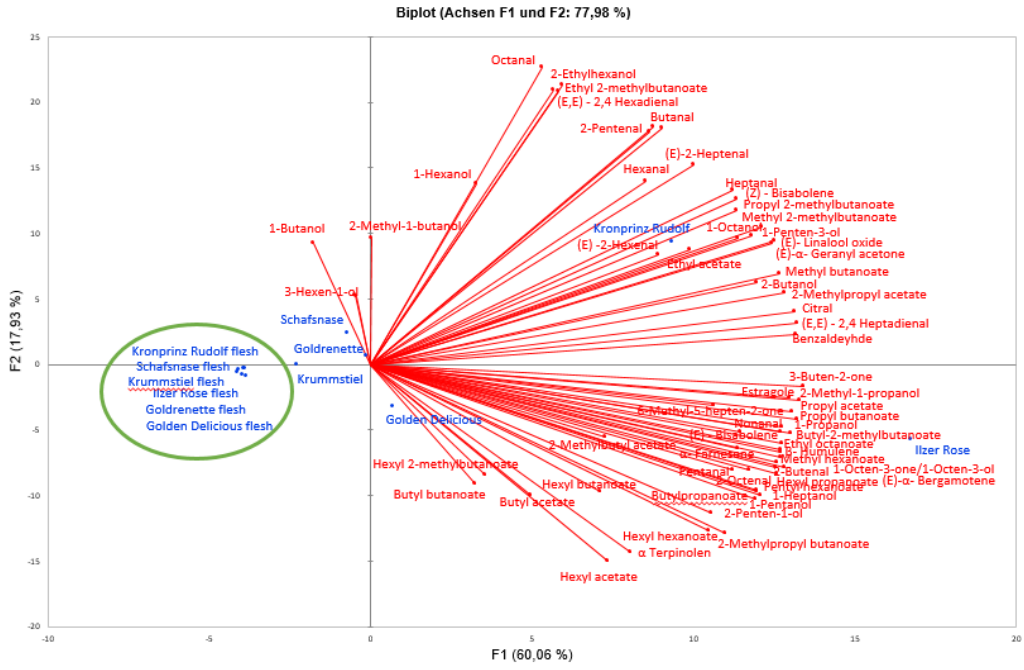


**Figure 22: Harvest 2016 –** biplot scores and factor loadings obtained by PCA based on the HS-SPME-GC-MS results from the investigated apple varieties with the separation of apple peel and apple flesh of each variety and the correlation with the volatile compounds.

Interestingly, all six apple varieties of this study have another peel color (see in Figure 23). Although it is known that the anthocyanin's are responsible for the red color in apple peels, it seems possible that these different colors also have an impact on the composition of the volatile compounds within each apple variety. As mentioned Dixon & Hewett (2000) in their literature review, the yellow-skinned apple varieties have been reported to produce mainly acetate esters, while the red-skinned varieties have mostly butyl esters (Dixon & Hewett, 2000). The results of this study confirm the color association with each different apple variety. The yellow-skinned apple variety Golden Delicious has had the highest amounts of the acetate esters in the peel compared with the other

investigated apple varieties. Also, the red-skinned apple variety Ilzer Rose shows the highest concentrations of the butyl esters such as butyl propanoate, butyl butanoate and butyl -2-methylbutanoate in comparison to the other varieties.

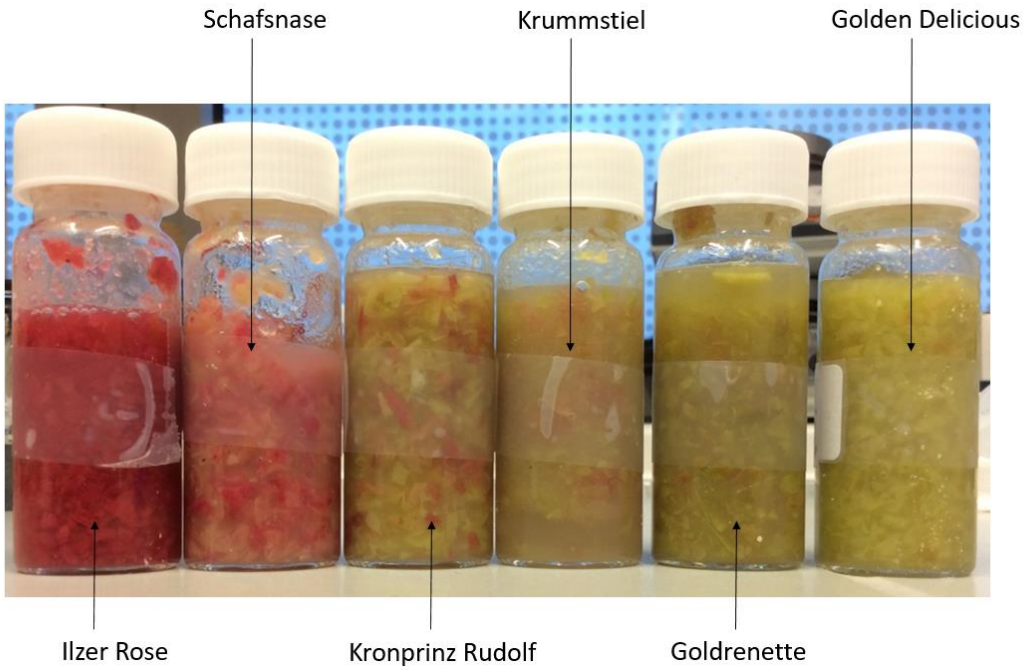


**Figure 23: Harvest 2016 - only the peel of the investigated apple varieties** (left to right: Ilzer Rose, Schafsnase, Kronprinz Rudolf, Krummstiel, Goldrenette, Golden Delicious)

The most obvious finding to emerge from the results of these analysis is that the two apple varieties Kronprinz Rudolf and Ilzer Rose shown a significant different composition of the volatile compounds than the other investigated apple varieties. Both varieties have a variety-specific flavor, the apple variety Kronprinz Rudolf is described with fruity, berry notes, while the variety Ilzer Rose is reported fruity in flavor and with floral notes (described in section 4.1). Due to the results (Figure 22 and Figure 24), these two varieties were picked for analysis of their specific flavor influenced by growing conditions and ripening stages.

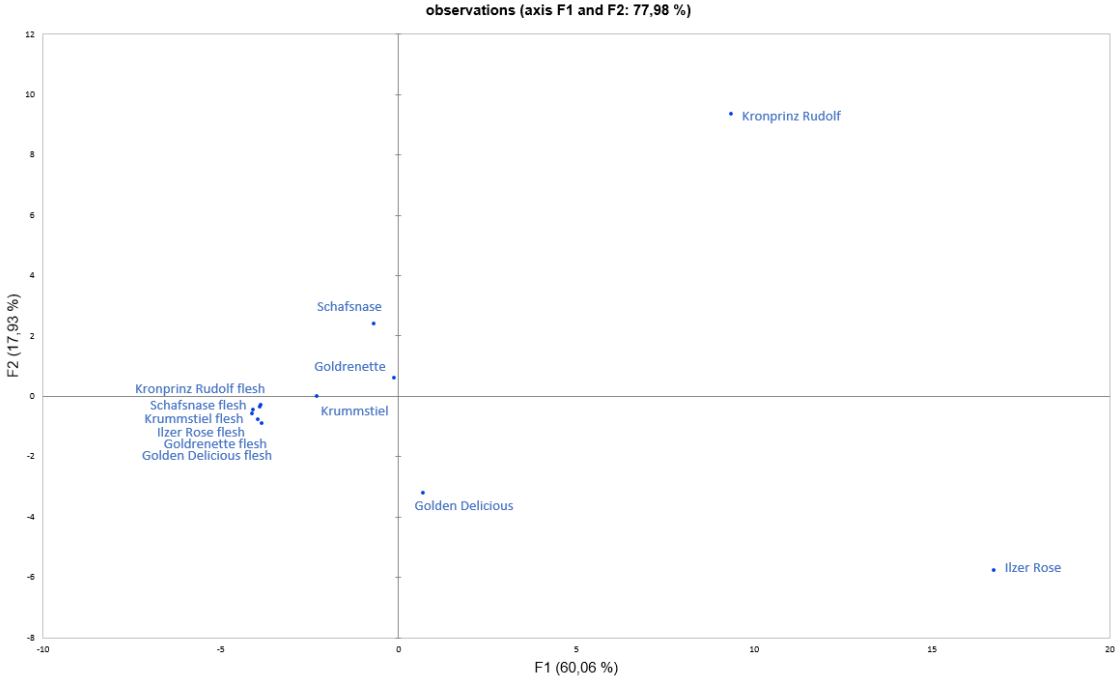


**Figure 24: Harvest 2016 –** observations plot obtained by PCA based on the HS-SPME-GC-MS results from the investigated apple varieties with the separation of apple peel and apple flesh of each variety.

Table 9: Volatile compounds obtained from HS-SPME GC-MS analysis from the apple peel samples of six apple varieties expressed as average peak areas (n=4); estimated quantities in µgkg$^{-1}$ +/i RSd (%) of the investigated apples calculated by comparison with internal standard (2-Octanol); TIC total ion chromatogram;

| Compound | RT | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | Goldrenette ± RSD (%) | Golden Delicious ± RSD (%) | Ilzer Rose ± RSD (%) | Kronprinz Rudolf ± RSD (%) | Krummstiel ± RSD (%) | Schafsnase ± RSD (%) |
|---|---|---|---|---|---|---|---|---|---|
| 2-Butenone | 6.66 | 599 | 601 | 77 ± 2.90 | 33 ± 2.65 | 712 ± 6.88 | 378 ± 12.01 | n.d. | 30 ± 2.45 |
| Butanal | 6.76 | 600 | 596 | 77 ± 1.40 | 14 ± 1.26 | 56 ± 0.84 | 204 ± 3.33 | 17 ± 0.62 | 32 ± 1.35 |
| 2-Butanol | 6.95 | 602 | 603 | 86 ± 2.20 | n.d. | 119 ± 0.81 | 116 ± 6.75 | n.d. | 11 ± 0.49 |
| Ethyl acetate | 7.22 | 616 | 612 | 385 ± 4.48 | 284 ± 4.26 | 410 ± 5.10 | 500 ± 7.45 | 53 ± 1.32 | 423 ± 11.41 |
| 2-Methyl-1-propanol | 7.47 | 629 | 628 | 30 ± 4.76 | 23 ± 2.14 | 171 ± 4.77 | 87 ± 11.46 | 22 ± 0.53 | 40 ± 0.99 |
| 1-Butanol | 8.17 | 666 | 675 | 606 ± 4.14 | 711 ± 1.61 | 191 ± 0.54 | 752 ± 4.81 | 1306 ± 3.11 | 1690 ± 3.85 |
| 1-Penten-3-ol | 8.49 | 683 | 678 | 43 ± 4.61 | 41 ± 1.58 | 347 ± 4.42 | 450 ± 7.81 | 21 ± 1.02 | 58 ± 1.55 |
| n-Pentanal | 8.74 | 696 | 699 | 564 ± 9.85 | n.d. | 1147 ± 5.54 | 172 ± 1.17 | 39 ± 1.57 | 154 ± 8.94 |
| Propyl acetate | 9.05 | 713 | 713 | n.d. | 9 ± 0.71 | 259 ± 6.14 | 117 ± 2.63 | n.d. | 7 ± 0.79 |
| Methyl butanoate | 9.21 | 722 | 723 | n.d. | n.d. | 107 ± 3.88 | 112 ± 4.21 | 13 ± 0.58 | 10 ± 0.41 |
| 2-Methyl-1-butanol | 9.50 | 739 | 739 | 96 ± 6.59 | 464 ± 14.32 | 28 ± 7.19 | 526 ± 7.55 | 137 ± 4.44 | 1690 ± 17.76 |
| (E)-2-Pentenal | 9.78 | 755 | 754 | 19 ± 6.96 | 14 ± 1.24 | 93 ± 0.82 | 94 ± 2.41 | 12 ± 1.68 | 46 ± 2.55 |
| 1-Pentanol | 9.97 | 766 | 766 | 45 ± 6.91 | 58 ± 3.08 | 789 ± 3.10 | 46 ± 7.24 | 68 ± 1.89 | 125 ± 3.86 |
| (Z)-2-Penten-1-ol | 10.03 | 769 | 769 | 28 ± 8.03 | 37 ± 2.13 | 396 ± 8.16 | 46 ± 3.59 | 19 ± 0.49 | 73 ± 1.05 |
| 2-Methylpropyl acetate | 10.09 | 772 | 771 | n.d. | 31 ± 0.64 | 248 ± 7.74 | 253 ± 9.51 | n.d. | n.d. |
| Methyl 2-methylbutanoate | 10.17 | 777 | 776 | 37 ± 8.97 | n.d. | 107 ± 0.89 | 148 ± 1.38 | n.d. | 12 ± 0.39 |
| Hexanal / Ethyl butanoate | 10.55 | 799 | 801/803 | 201 ± 1.76 | 738 ± 18.76 | 630 ± 5.29 | 1511 ± 8.82 | 353 ± 16.55 | 1075 ± 14.95 |
| Butyl acetate | 10.75 | 811 | 812 | 82 ± 2.51 | 845 ± 9.57 | 501 ± 11.03 | 201 ± 2.09 | 150 ± 7.45 | n.d. |
| Ethyl 2-methylbutanoate | 11.37 | 849 | 846 | n.d. | n.d. | n.d. | 459 ± 9.00 | 37 ± 1.19 | n.d. |
| 3-(Z)-Hexenol | 11.40 | 851 | 853 | 13 ± 1.23 | n.d. | n.d. | n.d. | n.d. | 15 ± 0.68 |
| (E) -2-Hexenal | 11.46 | 855 | 854 | 940 ± 9.88 | 2260 ± 4.26 | 1706 ± 4.13 | 2713 ± 10.96 | 1550 ± 6.72 | 2219 ± 14.86 |
| 1-Hexanol | 11.67 | 868 | 867 | 1082 ± 6.40 | 1478 ± 4.37 | 649 ± 2.35 | 2441 ± 3.43 | 1475 ± 4.59 | 3742 ± 8.81 |
| 2-Methylbutyl acetate | 11.81 | 877 | 880 | 439 ± 1.39 | 1087 ± 2.70 | 567 ± 2.53 | 406 ± 4.04 | 51 ± 1.72 | 52 ± 1.52 |
| Propyl butanoate | 12.10 | 894 | 900 | 73 ± 7.28 | 44 ± 0.33 | 543 ± 8.22 | 233 ± 1.90 | 56 ± 2.51 | n.d. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Heptanal | 12.20 | 901 | 903 | 47 ± 1.10 | n.d. | 106 ± 8.94 | 195 ± 1.51 | n.d. | 12 ± 0.77 |
| Butyl propanoate | 12.25 | 904 | 910 | 41 ± 1.79 | 123 ± 2.68 | 229 ± 5.48 | 62 ± 8.20 | 5 ± 0.21 | 8 ± 0.41 |
| (E,E)-2,4-Hexadienal | 12.35 | 911 | 910 | 201 ± 1.13 | n.d. | n.d. | 460 ± 10.81 | 33 ± 1.26 | 36 ± 1.82 |
| Methyl hexanoate | 12.51 | 922 | 924 | 22 ± 1.43 | n.d. | 614 ± 5.41 | 175 ± 6.31 | n.d. | 13 ± 1.05 |
| Propyl 2-methylbutanoate | 12.86 | 945 | 944 | n.d. | 9 ± 0.27 | 44 ± 1.29 | 80 ± 1.45 | 3 ± 0.11 | n.d. |
| 2-Methylpropyl butanoate | 12.98 | 953 | 953 | 24 ± 1.52 | 8 ± 0.22 | 54 ± 0.83 | n.d. | n.d. | n.d. |
| (E)-2-Heptenal | 13.06 | 958 | 957 | 51 ± 1.38 | 93 ± 4.23 | 158 ± 1.65 | 340 ± 2.86 | 62 ± 1.87 | 177 ± 1.17 |
| 1-Heptanol | 13.18 | 966 | 969 | 81 ± 8.64 | n.d. | 680 ± 10.58 | 58 ± 9.32 | n.d. | 22 ± 0.93 |
| Benzaldeyhde | 13.25 | 971 | 971 | 93 ± 1.05 | 13 ± 0.49 | 238 ± 3.92 | 172 ± 5.96 | n.d. | 16 ± 0.55 |
| 1-Octen-3-one/1-Octen-3-ol | 13.35 | 978 | 975/977 | 26 ± 1.13 | 37 ± 1.37 | 767 ± 11.62 | 191 ± 2.69 | 27 ± 0.81 | 90 ± 5.33 |
| 6-Methyl-5-hepten-2-one | 13.47 | 986 | 986 | 111 ± 1.07 | 382 ± 6.43 | 666 ± 18.84 | 350 ± 2.24 | 521 ± 4.51 | 255 ± 1.93 |
| Butyl butanoate | 13.55 | 991 | 996 | 132 ± 1.84 | 191 ± 13.25 | 286 ± 3.02 | 67 ± 8.62 | 225 ± 10.66 | 66 ± 1.73 |
| Octanal | 13.72 | 1003 | 1002 | 11 ± 1.25 | n.d. | n.d. | 57 ± 11.14 | 7 ± 0.20 | 26 ± 1.17 |
| Hexyl acetate | 13.79 | 1008 | 1008 | 72 ± 2.99 | 3116 ± 13.93 | 2750 ± 2.35 | 151 ± 9.21 | 110 ± 6.08 | 72 ± 2.23 |
| (E,E) - 2,4 Heptadienal | 13.87 | 1013 | 1015 | n.d. | 16 ± 0.24 | 220 ± 1.57 | 178 ± 3.54 | 12 ± 1.10 | 14 ± 0.78 |
| 2-Ethylhexanol | 14.06 | 1027 | 1028 | 21 ± 0.85 | n.d. | n.d. | 68 ± 0.32 | n.d. | 5 ± 0.25 |
| Butyl-2-methylbutanoate | 14.24 | 1040 | 1041 | 15 ± 1.68 | 454 ± 13.63 | 830 ± 12.81 | 427 ± 1.50 | 29 ± 1.11 | 43 ± 1.69 |
| (E)-2-Octenal | 14.52 | 1061 | 1060 | 229 ± 11.19 | 29 ± 0.93 | 532 ± 11.32 | 96 ± 2.70 | 34 ± 1.09 | 76 ± 6.52 |
| 1-Octanol | 14.62 | 1068 | 1074 | 51 ± 9.63 | 5 ± 0.78 | 201 ± 4.53 | 218 ± 4.89 | 14 ± 0.34 | 173 ± 5.05 |
| (E)- Linalool oxide | 15.04 | 1098 | 1088 | 200 ± 0.71 | n.d. | 327 ± 4.50 | 442 ± 1.51 | 9 ± 0.28 | n.d. |
| Hexyl propanoate | 15.07 | 1100 | 1108 | 111 ± 1.63 | 247 ± 4.34 | 620 ± 9.55 | 204 ± 7.26 | n.d. | 51 ± 1.61 |
| α Terpinolen | 15.09 | 1102 | 1104 | n.d. | n.d. | 23 ± 1.74 | n.d. | 18 ± 0.82 | n.d. |
| Nonanal | 15.13 | 1105 | 1104 | 12 ± 5.05 | n.d. | 157 ± 9.27 | 52 ± 4.73 | 6 ± 0.15 | 35 ± 1.13 |
| Hexyl butanoate | 16.20 | 1188 | 1192 | 446 ± 16.03 | 2392 ± 6.44 | 1435 ± 1.63 | 365 ± 5.32 | 278 ± 1.65 | 566 ± 2.68 |
| Ethyl octanoate | 16.26 | 1192 | 1193 | n.d. | n.d. | 140 ± 3.95 | 45 ± 7.88 | n.d. | n.d. |
| Estragole | 16.50 | 1212 | 1201 | 73 ± 7.13 | 65 ± 2.37 | 232 ± 11.82 | 128 ± 2.37 | n.d. | n.d. |
| Hexyl 2-methylbutanoate | 16.79 | 1236 | 1237 | 102 ± 14.33 | 3890 ± 2.21 | 1081 ± 3.09 | 176 ± 10.12 | 250 ± 10.90 | 991 ± 6.07 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| α-Citral | 17.30 | 1278 | 1271 | 53 ± 1.89 | 8 ± 0.30 | 173 ± 8.86 | 148 ± 13.48 | 14 ± 0.74 | n.d. |
| Pentyl hexanoate | 17.39 | 1285 | 1286 | 21 ± 1.84 | 81 ± 3.04 | 1148 ± 5.17 | 270 ± 3.26 | 14 ± 0.76 | 29 ± 1.32 |
| Hexyl hexanoate | 18.51 | 1384 | 1385 | 378 ± 1.66 | 1592 ± 19.15 | 2078 ± 11.76 | 351 ± 6.59 | 205 ± 1.47 | 582 ± 2.81 |
| (E)-α- Geranyl acetone | 19.37 | 1463 | 1451 | 13 ± 1.93 | 30 ± 7.32 | 391 ± 7.01 | 497 ± 9.59 | 36 ± 1.41 | 63 ± 3.46 |
| cis-ß-Farnesene | 19.42 | 1468 | 1458 | 25 ± 1.50 | 54 ± 7.72 | 143 ± 12.61 | 49 ± 9.87 | 65 ± 1.71 | 73 ± 3.08 |
| α-Caryophyllene[t] | 19.53 | 1479 | 1457 | 12 ± 1.52 | 12 ± 0.02 | 181 ± 14.34 | 346 ± 6.09 | 40 ± 1.57 | 45 ± 2.23 |
| cis-α-Bisabolene | 19.70 | 1495 | 1498 | 53 ± 1.93 | 190 ± 5.65 | 464 ± 1.73 | 864 ± 11.33 | 132 ± 3.23 | 151 ± 12.87 |
| (E)-α- Bergamotene[t] | 19.74 | 1499 | 1441 | n.d. | 160 ± 4.54 | 958 ± 14.73 | 415 ± 11.28 | 121 ± 7.52 | 146 ± 7.65 |
| α- Farnesene | 19.92 | 1516 | 1509 | 1989 ± 13.55 | 6044 ± 15.34 | 10263 ± 10.19 | 3571 ± 12.73 | 4130 ± 24.63 | 4676 ± 12.15 |

RT – retention time; $RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; t – tentatively identified; n.d. – not detectable or below limit of detection; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org)

**Table 10:** Volatile compounds obtained from HS-SPME GC-MS analysis from the apple flesh samples of six apple varieties expressed as average peak areas (n=4); estimated quantities in µgkg$^{-1}$ +/i RSd (%) of the investigated apples calculated by comparison with internal standard (2-Octanol); TIC total ion chromatogram;

| Compound | RT | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | Goldrenette ± RSD (%) | Golden Delicious ± RSD (%) | Ilzer Rose ± RSD (%) | Kronprinz Rudolf ± RSD (%) | Krummstiel ± RSD (%) | Schafsnase ± RSD (%) |
|---|---|---|---|---|---|---|---|---|---|
| Butanal | 6.76 | 600 | 596 | n.d. | 10 ± 0.68 | 11 ± 0.18 | 2 ± 0.89 | 9 ± 0.12 | 4 ± 0.08 |
| 2-Butanol | 6.95 | 602 | 603 | n.d. | n.d. | 4 ± 0.52 | n.d. | n.d. | 4 ± 0.05 |
| Ethyl acetate | 7.22 | 616 | 612 | 157 ± 4.55 | 18 ± 1.48 | 156 ± 5.45 | 143 ± 2.79 | 23 ± 0.27 | 135 ± 0.89 |
| 2-Methyl-1-propanol | 7.47 | 629 | 628 | 23 ± 1.98 | 12 ± 0.49 | 7 ± 1.33 | 46 ± 2.83 | 13 ± 0.22 | 18 ± 0.28 |
| 1-Butanol | 8.17 | 666 | 675 | 473 ± 1.73 | 669 ± 14.12 | 113 ± 5.88 | 340 ± 4.10 | 776 ± 12.89 | 305 ± 6.81 |
| 1-Penten-3-ol | 8.49 | 683 | 678 | n.d. | n.d. | n.d. | n.d. | n.d. | 1 ± 0.04 |
| n-Pentanal | 8.74 | 696 | 699 | n.d. | 6 ± 0.10 | n.d. | 41 ± 1.07 | 8 ± 0.16 | 14 ± 0.66 |
| Propyl acetate | 9.05 | 713 | 713 | n.d. | 12 ± 0.54 | 7 ± 1.45 | n.d. | n.d. | 1 ± 0.02 |
| Methyl butanoate | 9.21 | 722 | 723 | n.d. | n.d. | n.d. | n.d. | 4 ± 0.08 | 3 ± 0.02 |
| 2-Methyl-1-butanol | 9.50 | 739 | 739 | 132 ± 1.12 | 137 ± 4.95 | 100 ± 5.58 | 325 ± 3.82 | 74 ± 1.25 | 281 ± 6.15 |
| (E)-2-Pentenal | 9.78 | 755 | 754 | n.d. | n.d. | n.d. | n.d. | n.d. | 4 ± 0.30 |
| 1-Pentanol | 9.97 | 766 | 766 | 18 ± 1.84 | 15 ± 0.46 | 8 ± 3.74 | 23 ± 5.00 | 34 ± 0.77 | 22 ± 0.50 |
| (Z)-2-Penten-1-ol | 10.03 | 769 | 769 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 2-Methylpropyl acetate | 10.09 | 772 | 771 | n.d. | 8 ± 0.16 | 8 ± 1.97 | n.d. | n.d. | 7 ± 0.37 |
| Methyl 2-methylbutanoate | 10.17 | 777 | 776 | n.d. | n.d. | n.d. | n.d. | n.d. | 1 ± 0.02 |
| Hexanal / Ethyl butanoate | 10.55 | 799 | 801 / 803 | 13 ± 0.81 | 33 ± 1.11 | 19 ± 1.79 | 347 ± 1.58 | 48 ± 1.12 | 23 ± 0.52 |
| Butyl acetate | 10.75 | 811 | 812 | 14 ± 1.85 | 619 ± 11.01 | 143 ± 11.92 | n.d. | 47 ± 1.32 | 2 ± 0.07 |
| Ethyl 2-methylbutanoate | 11.37 | 849 | 846 | n.d. | n.d. | n.d. | 53 ± 1.68 | n.d. | n.d. |
| 3-(Z)-Hexenol | 11.40 | 851 | 853 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| (E) -2-Hexenal | 11.46 | 855 | 854 | 6 ± 0.72 | 10 ± 0.35 | 19 ± 1.79 | 8 ± 4.03 | 19 ± 0.21 | 10 ± 0.25 |
| 1-Hexanol | 11.67 | 868 | 867 | 335 ± 0.74 | 513 ± 12.37 | 125 ± 4.38 | 430 ± 3.64 | 843 ± 14.13 | 460 ± 8.91 |
| 2-Methylbutyl acetate | 11.81 | 877 | 880 | 6 ± 1.84 | 162 ± 5.24 | 151 ± 1.36 | n.d. | 8 ± 0.17 | n.d. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Propyl butanoate | 12.10 | 894 | 900 | n.d. | 5 ± 0.11 | n.d. | n.d. | 9 ± 0.18 | n.d. |
| Heptanal | 12.20 | 901 | 903 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Butyl propanoate | 12.25 | 904 | 910 | n.d. | 7 ± 0.24 | n.d. | n.d. | n.d. | n.d. |
| (E,E)-2,4-Hexadienal | 12.35 | 911 | 910 | n.d. | 27 ± 0.84 | n.d. | n.d. | n.d. | n.d. |
| Methyl hexanoate | 12.51 | 922 | 924 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Propyl 2-methylbutanoate | 12.86 | 945 | 944 | n.d. | n.d. | n.d. | n.d. | n.d. | 1 ± 0.03 |
| 2-Methylpropyl butanoate | 12.98 | 953 | 953 | n.d. | n.d. | n.d. | n.d. | 2 ± 0.03 | 1 ± 0.04 |
| (E)-2-Heptenal | 13.06 | 958 | 957 | n.d. | 4 ± 0.14 | 37 ± 0.31 | 9 ± 2.20 | 5 ± 0.42 | 43 ± 2.61 |
| 1-Heptanol | 13.18 | 966 | 969 | n.d. | 1 ± 0.01 | n.d. | n.d. | 3 ± 0.07 | 3 ± 0.06 |
| Benzaldeyhde | 13.25 | 971 | 971 | n.d. | n.d. | n.d. | n.d. | 1 ± 0.06 | n.d. |
| 1-Octen-3-one/1-Octen-3-ol | 13.35 | 978 | 975/977 | n.d. | 3 ± 0.11 | 9 ± 2.36 | 16 ± 1.46 | 5 ± 0.24 | 21 ± 0.85 |
| 6-Methyl-5-hepten-2-one | 13.47 | 986 | 986 | n.d. | 3 ± 0.03 | 5 ± 0.97 | 6 ± 1.54 | 6 ± 0.18 | 7 ± 0.15 |
| Butyl butanoate | 13.55 | 991 | 996 | 4 ± 1.95 | 37 ± 0.90 | 9 ± 0.78 | 5 ± 1.23 | 43 ± 1.02 | 5 ± 0.04 |
| Octanal | 13.72 | 1003 | 1002 | n.d. | 2 ± 0.07 | 0 | 0 | 2 ± 0.09 | 3 ± 0.09 |
| Hexyl acetate | 13.79 | 1008 | 1008 | 14 ± 3.66 | 420 ± 13.62 | 189 ± 5.44 | 20 ± 1.46 | 9 ± 0.26 | 1 ± 0.03 |
| (E,E) - 2,4 Heptadienal | 13.87 | 1013 | 1015 | n.d. | n.d. | n.d. | n.d. | 1 ± 0.03 | 3 ± 0.15 |
| 2-Ethylhexanol | 14.06 | 1027 | 1028 | n.d. | 1 ± 0.05 | n.d. | n.d. | 1 ± 0.03 | 1 ± 0.02 |
| Butyl-2-methylbutanoate | 14.24 | 1040 | 1041 | n.d. | 22 ± 0.61 | 3 ± 0.39 | n.d. | 2 ± 0.04 | 1 ± 0.02 |
| (E)-2-Octenal | 14.52 | 1061 | 1060 | n.d. | 5 ± 0.10 | n.d. | n.d. | 9 ± 0.30 | 21 ± 0.92 |
| 1-Octanol | 14.62 | 1068 | 1074 | n.d. | 2 ± 0.11 | n.d. | n.d. | 6 ± 0.19 | 21 ± 0.31 |
| (E)- Linalool oxide | 15.04 | 1098 | 1088 | n.d. | n.d. | n.d. | n.d. | n.d. | 8 ± 0.37 |
| Hexyl propanoate | 15.07 | 1100 | 1108 | n.d. | 2 ± 0.06 | n.d. | n.d. | n.d. | n.d. |
| α Terpinolen | 15.09 | 1102 | 1104 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Nonanal | 15.13 | 1105 | 1104 | n.d. | n.d. | 3 ± 1.32 | n.d. | n.d. | 3 ± 0.22 |
| Hexyl butanoate | 16.20 | 1188 | 1192 | 66 ± 4.89 | 55 ± 2.38 | n.d. | n.d. | 78 ± 2.84 | n.d. |
| Ethyl octanoate | 16.26 | 1192 | 1193 | n.d. | n.d. | n.d. | n.d. | 1 ± 0.23 | n.d. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Estragole | 16.50 | 1212 | 1201 | n.d. | 4 ± 0.12 | n.d. | n.d. | 1 ± 0.21 | n.d. |
| Hexyl 2-methylbutanoate | 16.79 | 1236 | 1237 | 10 ± 3.55 | 59 ± 1.99 | 10 ± 3.15 | n.d. | 3 ± 0.07 | n.d. |
| α-Citral | 17.30 | 1278 | 1271 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Pentyl hexanoate | 17.39 | 1285 | 1286 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Hexyl hexanoate | 18.51 | 1384 | 1385 | 27 ± 0.74 | 3 ± 0.12 | n.d. | n.d. | 6 ± 0.27 | 1 ± 0.31 |
| (E)-α- Geranyl acetone | 19.37 | 1463 | 1451 | n.d. | n.d. | n.d. | n.d. | 2 ± 0.07 | 3 ± 0.14 |
| cis-ß-Farnesene | 19.42 | 1468 | 1458 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| α-Caryophyllene[t] | 19.53 | 1479 | 1457 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| cis-α-Bisabolene | 19.70 | 1495 | 1498 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| (E)-α- Bergamotene[t] | 19.74 | 1499 | 1441 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| α- Farnesene | 19.92 | 1516 | 1509 | 64 ± 0.71 | 16 ± 1.23 | 11 ± 2.30 | 6 ± 2.03 | 7 ± 0.67 | 7 ± 0.22 |

RT – retention time; $RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; t – tentatively identified; n.d. – not detectable or below limit of detection; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org)

## 4.3. Difference of volatile compounds identified from Kronprinz Rudolf apples depending on location and growing conditions

In this section, the difference of volatile compounds was identified from Kronprinz Rudolf apples depending on location and growing conditions (harvest 2017). In addition, the basic parameters (SSC and TA) were also measured for the Kronprinz Rudolf apple samples of the harvest year 2017.

The sugar content can fluctuate from year to year and between harvest dates, as a result there is no absolute value for the apple sugar content. The main sugar type in apple are fructose, sucrose, and glucose and they are expressed in Brix degree (°Brix) or percentage, where 1°brix corresponds to 1 g of sugar in 100 g of aqueous solution representing a strength in percentage based on the mass at 17.5°C (Kingston, 2010; Iwanami et al., 2017). The most broadly used method to quickly determine sugars is the refractometer since fruit soluble solid content (SSC) is made of sugars followed by organic acids and inorganic salts (Musacchi & Serra, 2018). If apple fruits are picked at a ripe stage, the soluble solids increase in concentration after storage since starch is converted via hydrolysis into sugars over time (Visser, Schaap, & De Vries, 1968). In this study, the SSC was measured in the Kronprinz Rudolf apple samples from the harvest years 2017. The results are shown as mean value ± standard deviation (triplicated analysis).

The content of total sugars in Kronprinz Rudolf apple samples from different growing conditions and locations is shown in Figure 25. The plantation grown apples from Silberberg, Hatzendorf, Haidegg were different in their SSC content, the samples from Haidegg showed the highest SSC content in contrast to the samples from Silberberg which showed the lowest SSC content. The apples, which were harvested from meadow orchards of Wagersbach at two different picking dates (P1: 28.09. and P2: 04.10.), had both a similar SSC content in comparison to the plantation Silberberg apple samples. Interestingly, the Wagersbach P1 samples showed higher SSC content than the second picking date samples. This result is somewhat counterintuitive because P2 apple samples were picked at a higher degree of ripeness and they were expected to have a higher SSC content. However, the opposite results were found. No significant differences were found between the different growing conditions (plantation and meadow orchards) of the Kronprinz Rudolf apple samples.

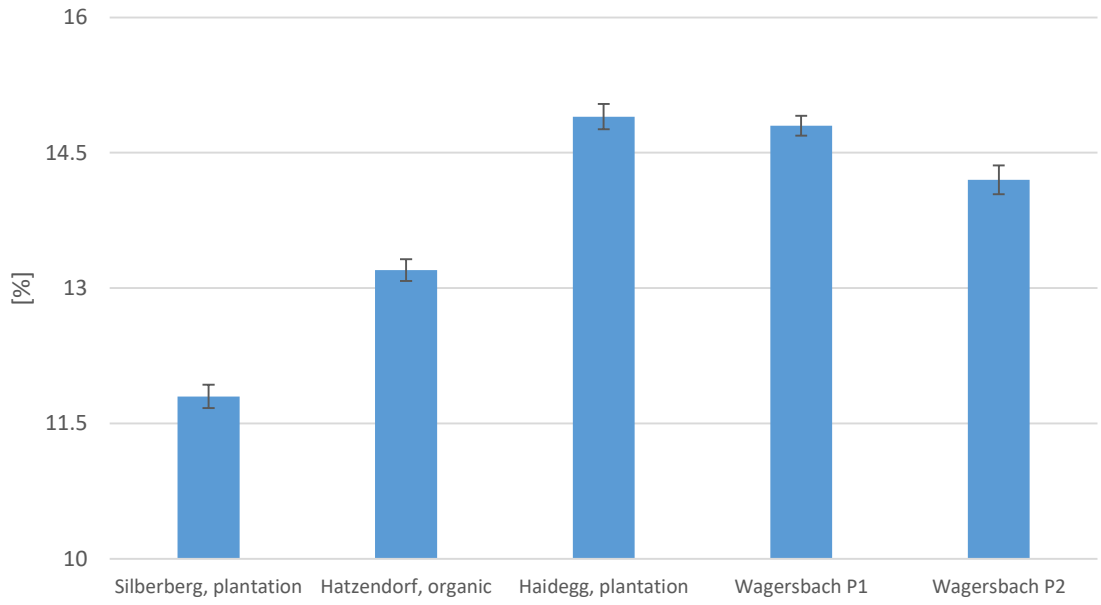


**Figure 25: Soluble Solids content [%] in Kronprinz Rudolf apple samples from different growing conditions and locations;** Plantation growing: Silberberg, Hatzendorf (organic, plantation), Haidegg; Growing in meadow orchards: Wagersbach P1 (picking date 1: 28.09), Wagersbach P2 (picking date 2: 04.10.)

The acid content in apples is a very important parameter for the fruit-eating quality and the sweet-sour balance is essential for consumers. When the apple fruit is in cell expansion in summer, the total acid content usually increases and decreases with the advance of ripening (Nybom, 1959). The most relevant compound accounting for 90% of organic acids in apple is malic acid (Musacchi & Serra, 2018). Malic acid is the main substrate for the respiration of fruit in postharvest for about 90% of the acid content in apples (Ackermann et al., 1992).

Titratable acidity (TA) is measured starting from the juice by titration (neutralization) of hydrogen ions present in the juice with NaOH. TA is generally expressed for apples as g $L^{-1}$ of malic acid or %, being 1% TA equivalent to 10 g $L^{-1}$ of malic acid (Musacchi & Serra, 2018). TA can range between 1 – 0.4% for unripe to ripe apples (after harvest – after storage) and this value is accepted as the "normal acidity" apple category (Ackermann et al., 1992).

In this study, the total acid content [%] was determined from the apple varieties Kronprinz Rudolf (harvest 2017). The results are shown as the mean value ± standard deviation (triplicated analysis).

The TA of the Kronprinz Rudolf apple samples is presented in Figure 26. Interestingly, the plantation growing apple samples show a similar trend compared to the SSC content. The Haidegg apple samples showed the highest TA and the Silberberg samples the lowest. Comparing the two results of the samples harvested in Wagersbach, it can be seen that the samples from the second picking date showed a higher TA content than the samples from the first picking date, and this result is contrary to information given in most literature on this subject (Ackermann et al., 1992). According to the results presented in Figure 26, no significant difference between the two different growing conditions is clear.

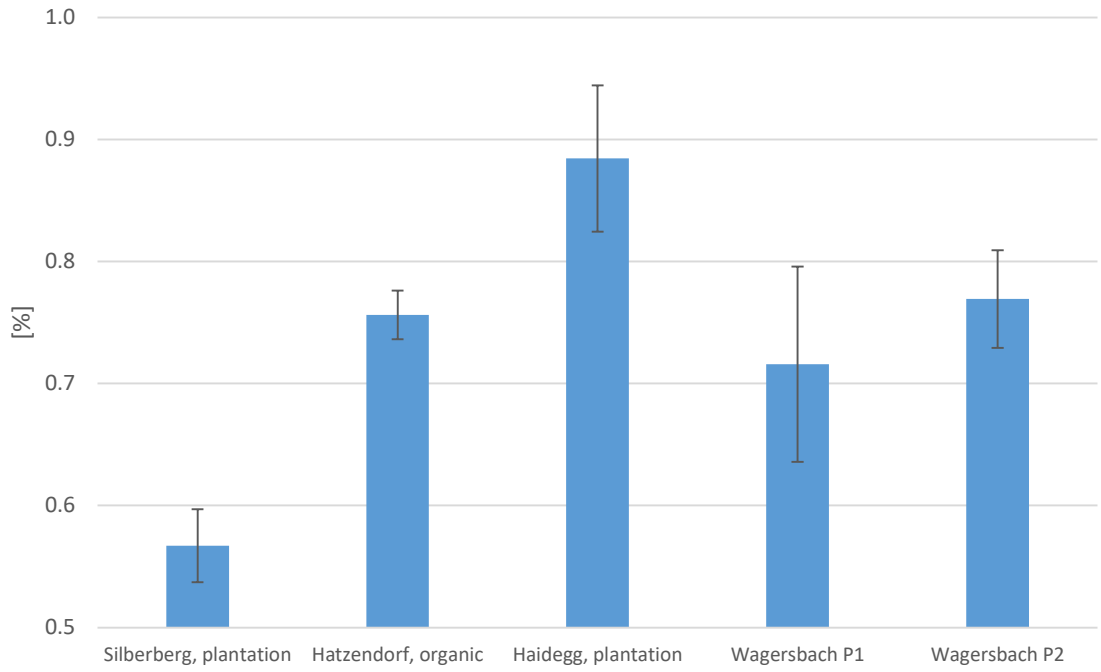


**Figure 26: Total Acid Content [%] in Kronprinz Rudolf apple samples from different growing conditions and locations;** Plantation growing: Silberberg, Hatzendorf (organic), Haidegg; Growing in meadow orchards: Wagersbach P1 (picking date 1: 28.09), Wagersbach P2 (picking date 2: 04.10.)

Headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) was selected for the enrichment, separation, and identification of primary flavor compounds emitted from the intact apples of the apple variety Kronprinz Rudolf. The different growing conditions of the Kronprinz Rudolf apple samples, plantation growing and growing in meadow orchards, were investigated and compared with each other (shown in Figure 27). The identification of the compounds was based on the comparison between the obtained mass spectra and mass spectra of authentic reference compounds, mass spectra from the literature, or from the NIST11 mass spectra library. As a further criterion for identification, the linear temperature programmed retention indices were calculated according to van Den Dool & Dec. Kratz (1963) and Farkas, Le Quere, Maarse, & Kovac (1994) respectively. Additionally, the obtained retention indices were compared to those obtained from authentic reference compounds and data from the literature as well as from RI databases. The relative concentrations are given in % area of the total ion chromatograms.


**Figure 27: Harvest 2017;** An overview of the Kronprinz Rudolf apple samples from different locations and growing conditions.

A summary of the relative concentrations of the primary flavor compounds that were emitted from the intact apples is shown in Table 11. The results are given in % areas of the total ion chromatograms identified in the respective investigated Kronprinz Rudolf apple samples. Linear and methyl or ethyl branched-chain esters are the most important primary flavor compounds in apples; these are mainly formed via β-oxidation. Their formation is dependent on the availability of C2-C8 acids and alcohols (Dixon & Hewett, 2000; De Pooter et al., 1981b; Paillard, 1990). As they have mainly fruity sensory characters, members of this compound group play important roles in the formation of apple flavor.

In total, 35 individual compounds were identified in the five different Kronprinz Rudolf growing condition samples (see Table 11). 24 esters, four alcohols, two acetates, and five other compounds were determined. The 24 esters range from esters with five C-atoms such as ethyl propanoate to esters with 12 C-atoms such as hexyl hexanoate as the longest straight chain ester. Significant differences in the numbers and relative amounts of the identified esters were detected between the investigated samples. In general, ethyl esters are known to have a large impact on apple aroma as many of these possess distinct apple flavor properties and low odor thresholds. Ethyl butanoate was identified in all five Kronprinz Rudolf samples with the highest relative concentration. The presence of ethyl hexanoate and ethyl-2-methylbutanote were also observed in all five apple samples and were detected with the second-highest levels. Both compounds have distinct apple notes and low odor thresholds. The impact of ethyl 2-methylbutanoate on the Kronprinz Rudolf aroma was described previously by our group (Ragger, Heil, Innerhofer, Leitner, & Siegmund, 2015). The four methyl esters 3-methylbutyl butanoate, 2-methylbutyl-2-methylbutanoate, 2-methylpropyl hexanoate, and 2-methylbutyl hexanoate were found in the plantation growing samples of Silberberg and the apple samples of the meadow orchards Wagersbach, but not in the plantation growing samples of Haidegg and Hatzendorf. Several hexanoates and octanoate esters were identified in the volatile fractions of the investigated apple samples. In general, hexanoate and

octanoate esters are formed from longer chain free fatty acids via β-oxidation or oxidation of hexanal (Paillard, 1990; Contreras et al., 2016). However, the sensory impact of these esters is considered low, as the compounds have high odor thresholds. Acetate esters are formed from reactions of acetyl-CoA with higher alcohols; the latter, in turn, is formed from the degradation of amino acids or carbohydrates (Espino-Diaz et al., 2016). Butyl acetate and hexyl acetate were identified in relatively high amounts in the five different samples (except for Wagersbach P1). The low odor thresholds and the fruity notes of the acetates indicate that they have a high impact on the apple flavor. These results are in agreement with those of previous studies, which reported that these esters are found in high concentrations in apples and, thus, have impact on apple flavor (Dimick & Hoskin, 1983; Dunemann et al., 2012).

Alcohols are important compounds for the characterization of apple aroma and more than 40 of them have been reported for apples (Dimick & Hoskin, 1983). Alcohols are produced by the reduction of the corresponding aldehydes (Espino-Diaz et al., 2016), linear alcohols are derived from fatty acid catabolism, whereas branched-chain alcohols are formed from amino acid metabolism (Contreras & Beaudry, 2013). In this study, four alcohols (1-butanol, 1-pentanol, 1-hexanol, and 2-methyl-1-butanol) were identified in the volatile fraction of the intact apples. Among these, 1-hexanol was identified as the dominant compound in this group in every investigated apple sample. The fact that the formation of 1-hexanol in the intact fruit clearly depends on the fruit ripeness was described already by Contreras & Beaudry, (2013). Alcohols are direct precursors of esters, which are synthesized by the action of esterase enzymes (Knee & Hatfield, 1981).

6-Methyl-5-hepten-2-one was a carbonyl compound identified in the Wagersbach P1 apple sample. Unlike other carbonyls, 6-methyl-5-hepten-2-one is not formed from lipid degradation, but instead via the oxidative degradation of carotenoids during ripening and senescence of the fruits (Siegmund, 2015).

Thousands of different terpenoid compounds are found in nature as secondary metabolites in plant material with different physiological roles. However, four terpenes (D-Limonene, α-farnesene, α-curcumene, and cis-γ-bisabolene) were identified in this study. But only α-farnesene, which is associated with apple fruit ripening (Ju & Curry, 2000, Whitaker, 2004, Nieuwenhuizen et al., 2013), was found in comparable amounts in each of the five investigated samples. Due to their low concentrations and their potential localization in apple peel, further investigations were required to study the contributions of terpenes to apple flavor using techniques with higher sensitivity (see section 4.5).

To compare the difference between the flavor compounds of intact apples and flavor of the apple peel, the samples of the Kronprinz Rudolf were also prepared for the analysis of the peel only. The enzyme-inactivated (as far as possible) apple peel samples of the different growing conditions were analyzed. Table 12 presents the HS-SPME-GC-MS results from the five investigated different apple peel samples. In total, 62 volatile compounds were identified in the five apple peel samples of the

variety Kronprinz Rudolf. Nine alcohols, 26 esters, three acetates, 14 aldehydes, and ten other substances were determined.

The most surprising aspect of the comparison between the results of the intact apples and the apple peel is the rather high concentrations of the chemical group of aldehydes. This chemical group is not determined in intact apples. The aldehydes are mainly formed via the lipoxygenase (LOX) pathway and so these compounds are secondary flavor compounds. After the cell disruption, the enzyme alcohol dehydrogenase (ADH), an oxidoreductase, is catalyzing the reduction of aldehydes to alcohols (Espino-Diaz et al., 2016). The LOX activity is associated with higher emission of 1-hexanol (Altisent, Echeverría, Graell, López, & Lara, 2009), which was identified with the highest concentration in the apple peel. The branched-chain aldehydes, alcohols, and esters are derived from the amino acid pathway (Rowan et al., 1996), this pathway is responsible for the formation of compounds such as methyl-2-methylbutanoate and 2-methylpropyl-butanoate, which were not determined in intact apples.

These results confirmed that primary flavor compounds are mostly determined in the intact apples, whereas the secondary flavor compounds are mainly formed after cell disruption within the apple peel. Low levels of secondary flavor compounds are also found in intact apples. Another major finding is that there is no correlation between apples which are growing in meadow orchards and plantation.

Table 11: Relative concentrations of volatile compounds determined in the investigated plantation growing and growing in meadow orchards Kronprinz Rudolf apple samples are given in % area of the total ion chromatograms determined on one-dimensional GC-MS on the non-polar stationary phase; expressed as average peak areas (n = 4) obtained from HS-SPME GC-MS analysis of the intact apples.

| Volatile Compounds | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | Haidegg plantation | Hatzendorf organic | Silberberg plantation | Wagersbach P1 | Wagersbach P2 |
|---|---|---|---|---|---|---|---|
| **Total peak area** | | | 296631767 ± 2 | 493200543 ± 3 | 468652295 ± 8 | 308725119 ± 6 | 466997138 ± 4 |
| 1-Butanol | 657 | 675 | 1.94 | n.d. | 1.24 | 2.56 | 3.18 |
| Ethyl propanoate | 711 | 713 | 0.92 | 0.94 | 1.48 | 0.64 | 0.33 |
| 2-Methyl-1-butanol | 739 | 736 | 0.44 | 0.24 | n.d. | 0.78 | 1.61 |
| Ethyl 2-methylpropanoate | 757 | 756 | n.d. | 0.33 | 0.44 | 0.39 | n.d. |
| 1-Pentanol | 763 | 769 | n.d. | 0.25 | n.d. | n.d. | n.d. |
| Ethyl butanoate | 797 | 804 | 46.82 | 41.16 | 45.89 | 39.88 | 33.70 |
| Butyl acetate | 812 | 812 | 0.38 | 0.19 | 0.30 | n.d. | 0.22 |
| Ethyl 2-methylbutanoate | 848 | 846 | 9.42 | 5.90 | 8.88 | 13.46 | 7.30 |
| 1-Hexanol | 866 | 867 | 4.08 | 4.95 | 3.48 | 6.54 | 10.06 |
| Propyl butanoate | 896 | 900 | 0.19 | 0.20 | n.d. | 0.25 | 0.47 |
| Ethyl pentanoate | 897 | 900 | 0.59 | 0.78 | 1.26 | 0.54 | 0.76 |
| Propyl 2-methylbutanoate | 946 | 943 | n.d. | n.d. | n.d. | 0.33 | 0.37 |
| 6-Methyl-5-hepten-2-one | 987 | 986 | n.d. | n.d. | n.d. | 0.32 | n.d. |
| Butyl butanoate | 993 | 994 | 1.38 | 0.98 | n.d. | 0.91 | 2.75 |
| Ethyl hexanoate | 996 | 1002 | 10.73 | 21.78 | 18.18 | 11.22 | 8.18 |
| Hexyl acetate | 1009 | 1008 | 3.19 | 2.13 | 2.61 | 0.55 | 1.04 |
| D-Limonene | 1043 | 1033 | 0.60 | 0.28 | n.d. | 0.39 | n.d. |
| Butyl 2-methylbutanoate | 1041 | 1041 | 0.13 | 0.14 | 0.54 | 0.73 | 2.02 |
| 3-Methylbutyl butanoate | 1058 | 1061 | n.d. | n.d. | n.d. | 0.17 | 0.39 |
| Propyl hexanoate | 1092 | 1097 | n.d. | 0.33 | 0.21 | 0.25 | 0.44 |
| Ethyl heptanoate | 1095 | 1100 | n.d. | 0.22 | 0.14 | 0.07 | n.d. |
| 2-Methylbutyl 2-methylbutanoate | 1105 | 1103 | n.d. | n.d. | 3.81 | 0.10 | 0.24 |
| Hexyl propanoate | 1103 | 1108 | n.d. | n.d. | 0.21 | 0.13 | 0.51 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Pentyl 2-methylbutanoate | 1139 | 1140 | n.d. | n.d. | n.d. | n.d. | 0.21 |
| 2-Methylpropyl hexanoate | 1150 | 1149 | n.d. | n.d. | n.d. | n.d. | 0.11 |
| Hexyl 2-methylpropanoate | 1147 | 1151 | n.d. | n.d. | n.d. | n.d. | 0.38 |
| Butyl hexanoate | 1190 | 1192 | 4.84 | 5.12 | n.d. | 5.22 | 7.00 |
| Ethyl octanoate | 1194 | 1193 | 1.03 | 2.06 | 1.42 | 0.84 | 0.71 |
| Hexyl 2-methylbutanoate | 1238 | 1237 | 2.00 | 0.92 | 1.00 | 4.03 | 9.75 |
| 2-Methylbutyl hexanoate | 1253 | 1251 | n.d. | n.d. | n.d. | 0.27 | 0.37 |
| Pentyl hexanoate | 1288 | 1282 | n.d. | n.d. | n.d. | 0.19 | 0.27 |
| Hexyl hexanoate | 1386 | 1379 | 1.60 | 2.09 | 1.24 | 1.44 | 1.86 |
| α-Farnesene | 1517 | 1509 | 4.94 | 4.46 | 2.60 | 2.13 | 2.34 |
| α-Curcumene | 1506 | 1516 | 0.47 | 0.46 | 0.33 | 0.29 | 0.26 |
| cis-γ-Bisabolene | 1536 | 1540 | n.d. | n.d. | n.d. | 0.17 | n.d. |

$RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; n.d. – not detectable or below limit of detection; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org)

Table 12: Volatile compounds obtained from HS-SPME GC-MS analysis from the apple peel samples of five different Kronprinz Rudolf apple samples expressed as average peak areas (n=4); estimated quantities in $\mu g kg^{-1}$ +/i RSd (%) of the investigated apples calculated by comparison with internal standard (2-Octanol); TIC total ion chromatogram;

| Volatile Compounds | RT | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | Hatzendorf organic ± RSD (%) | Silberberg plantation ± RSD (%) | Haidegg plantation ± RSD (%) | Wagersbach P1 ± RSD (%) | Wagersbach P2 ± RSD (%) |
|---|---|---|---|---|---|---|---|---|
| ***Alcohols*** | | | | | | | | |
| 1-Butanol | 8.49 | 654 | 675 | 58 ± 1.40 | 47 ± 0.63 | 74 ± 0.53 | 109 ± 1.00 | 35 ± 0.22 |
| 1-Penten-3-ol | 8.86 | 669 | 678 | 32 ± 0.85 | 22 ± 0.32 | 39 ± 0.40 | n.d. | n.d. |
| 2-Methyl-1-butanol | 9.91 | 720 | 739 | 47 ± 1.14 | 53 ± 0.64 | 28 ± 0.14 | 172 ± 1.23 | 72 ± 0.41 |
| 1-Pentanol | 10.43 | 756 | 766 | 34 ± 0.79 | 31 ± 0.29 | 47 ± 0.66 | 45 ± 0.37 | 44 ± 0.05 |
| (Z)-2-Penten-1-ol | 10.50 | 761 | 769 | 36 ± 0.65 | 34 ± 0.35 | 45 ± 0.36 | 17 ± 0.28 | 17 ± 0.05 |
| 3-(Z)-Hexenol | 11.96 | 852 | 853 | 2 ± 0.25 | n.d. | 23 ± 0.42 | n.d. | n.d. |
| 1-Hexanol | 12.22 | 867 | 867 | 658 ± 9.45 | 693 ± 4.68 | 995 ± 10.02 | 1081 ± 11.88 | 739 ± 3.21 |
| (Z)-2-Octen-1-ol | 15.05 | 1047 | 1054 | 28 ± 2.01 | n.d. | n.d. | n.d. | n.d. |
| 1-Octanol | 15.38 | 1070 | 1074 | 34 ± 0.95 | 7 ± 0.14 | 9 ± 0.37 | 19 ± 0.12 | 17 ± 0.18 |
| ***Acetates*** | | | | | | | | |
| Ethyl acetate | 7.51 | 614 | 612 | 75 ± 0.59 | 84 ± 0.64 | 145 ± 1.39 | 147 ± 1.47 | 116 ± 0.58 |
| Butyl acetate | 11.25 | 811 | 812 | 12 ± 0.15 | 90 ± 0.03 | 64 ± 0.78 | 38 ± 0.40 | 44 ± 0.23 |
| Hexyl acetate | 14.49 | 1009 | 1008 | 218 ± 2.78 | 220 ± 2.92 | 592 ± 4.52 | 133 ± 0.33 | 123 ± 0.85 |
| ***Aldehydes*** | | | | | | | | |
| n-Pentanal | 9.14 | 680 | 699 | 91 ± 2.55 | 82 ± 0.89 | 108 ± 2.36 | 91 ± 0.79 | 112 ± 0.22 |
| (E)-2-Pentenal | 10.25 | 743 | 754 | 49 ± 1.28 | 25 ± 0.41 | 41 ± 0.91 | 18 ± 1.28 | 41 ± 0.13 |
| Hexanal | 11.07 | 800 | 801 | 172 ± 7.69 | 106 ± 1.64 | 125 ± 5.10 | 248 ± 1.38 | 193 ± 1.10 |
| (E) -2-Hexenal | 12.12 | 861 | 854 | 56 ± 1.45 | 15 ± 0.29 | 16 ± 0.46 | 40 ± 0.26 | n.d. |
| Heptanal | 12.82 | 903 | 903 | 16 ± 0.56 | 9 ± 0.22 | 11 ± 0.29 | 14 ± 0.08 | 23 ± 0.26 |
| (E)-2-Heptenal | 13.74 | 961 | 957 | 175 ± 2.90 | 129 ± 1.87 | 149 ± 3.29 | 232 ± 1.31 | n.d. |
| Octanal | 14.44 | 1005 | 1002 | 103 ± 6.02 | 134 ± 1.95 | 399 ± 15.40 | 98 ± 4.12 | 72 ± 4.51 |
| (E,E) - 2,4 Heptadienal | 14.60 | 1017 | 1015 | 27 ± 0.49 | 16 ± 0.31 | 25 ± 0.56 | 17 ± 0.03 | 30 ± 0.09 |
| (E)-2-Octenal | 15.29 | 1064 | 1060 | 57 ± 1.07 | 31 ± 0.61 | 41 ± 1.01 | 61 ± 0.49 | 85 ± 0.59 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Nonanal | 15.93 | 1108 | 1104 | 80 ± 2.12 | 28 ± 0.52 | 17 ± 0.27 | 41 ± 2.47 | 31 ± 0.55 |
| (E)-2-Nonenal | 16.73 | 1167 | 1166 | 9 ± 0.28 | 1 ± 0.13 | n.d. | 3 ± 0.02 | 5 ± 0.07 |
| Decanal | 17.31 | 1211 | 1209 | 81 ± 11.37 | 2 ± 0.27 | n.d. | n.d. | n.d. |
| 2(E)-Decenal | 18.07 | 1271 | 1262 | 10 ± 0.13 | 8 ± 0.20 | 9 ± 0.32 | 10 ± 0.22 | 20 ± 0.09 |
| ***Esters*** | | | | | | | | |
| Methyl butanoate | 9.64 | 700 | 723 | 22 ± 0.52 | 19 ± 0.26 | 26 ± 0.50 | 35 ± 0.45 | 35 ± 0.70 |
| Methyl 2-methylbutanoate | 10.66 | 772 | 776 | 6 ± 0.11 | 11 ± 0.14 | 6 ± 0.22 | 16 ± 0.18 | 13 ± 0.03 |
| Ethyl butanoate | 11.11 | 801 | 803 | 111 ± 0.21 | 94 ± 1.97 | 148 ± 1.42 | 99 ± 0.34 | 103 ± 0.72 |
| Ethyl 2-methylbutanoate | 11.92 | 850 | 846 | 29 ± 0.63 | 95 ± 1.86 | n.d. | 30 ± 0.25 | 23 ± 0.18 |
| Propyl butanoate | 12.73 | 897 | 900 | 27 ± 0.53 | 20 ± 0.32 | 44 ± 1.02 | n.d. | 37 ± 0.20 |
| Methyl hexanoate | 13.14 | 923 | 924 | 60 ± 1.54 | 42 ± 0.84 | 46 ± 1.25 | 440 ± 1.38 | 275 ± 1.15 |
| 2-Methylpropyl butanoate | 13.51 | 946 | 953 | n.d. | 5 ± 0.21 | n.d. | 7 ± 0.05 | n.d. |
| Butyl butanoate | 14.25 | 993 | 996 | 63 ± 1.08 | 31 ± 0.46 | 109 ± 1.06 | 145 ± 0.47 | 59 ± 0.40 |
| Ethyl hexanoate | 14.29 | 996 | 1002 | 81 ± 1.29 | 159 ± 1.53 | 72 ± 1.55 | 56 ± 0.33 | 96 ± 0.70 |
| Methyl heptanoate | 14.69 | 1023 | 1021 | n.d. | n.d. | n.d. | 9 ± 0.08 | 10 ± 0.16 |
| Butyl-2-methylbutanoate | 14.97 | 1042 | 1041 | 25 ± 0.45 | 27 ± 0.32 | 23 ± 0.33 | 123 ± 0.35 | 71 ± 0.47 |
| 2-Methylbutyl butanoate | 15.21 | 1058 | 1056 | 172 ± 1.85 | 125 ± 1.38 | 178 ± 2.30 | 134 ± 0.64 | 143 ± 0.86 |
| Pentyl butanoate | 15.69 | 1091 | 1094 | 12 ± 0.18 | 6 ± 0.10 | 20 ± 0.35 | 23 ± 0.21 | 19 ± 0.20 |
| Hexyl propanoate | 15.85 | 1102 | 1108 | 36 ± 2.58 | 34 ± 0.36 | 43 ± 0.66 | 71 ± 0.35 | n.d. |
| Methyl octanoate | 16.13 | 1123 | 1126 | 27 ± 0.62 | 31 ± 0.51 | 28 ± 0.93 | 65 ± 0.46 | 56 ± 0.86 |
| Pentyl 2-methylbutanoate | 16.35 | 1139 | 1140 | n.d. | n.d. | n.d. | 37 ± 0.43 | 41 ± 0.46 |
| 2-Methyl propylhexanoate | 16.46 | 1147 | 1149 | 16 ± 0.14 | 19 ± 0.34 | 31 ± 0.67 | 48 ± 0.16 | 59 ± 0.45 |
| (E)-2-Hexenyl butanoate | 16.95 | 1183 | 1193 | 10 ± 0.07 | n.d. | 10 ± 0.21 | n.d. | 6 ± 0.07 |
| Hexyl butanoate | 17.04 | 1190 | 1192 | 385 ± 6.55 | 195 ± 3.93 | 417 ± 6.78 | 468 ± 2.02 | 336 ± 2.47 |
| Ethyl octanoate | 17.10 | 1194 | 1193 | n.d. | 44 ± 0.70 | n.d. | n.d. | n.d. |
| Hexyl 2-methylbutanoate | 17.67 | 1238 | 1237 | 172 ± 12.19 | 308 ± 5.66 | 353 ± 6.32 | 973 ± 6.47 | 816 ± 1.57 |
| 2-Methylbutyl hexanoate | 17.86 | 1254 | 1251 | 12 ± 0.11 | 18 ± 0.41 | 10 ± 0.25 | 90 ± 0.42 | 52 ± 0.39 |
| Pentyl hexanoate | 18.29 | 1288 | 1286 | 13 ± 0.09 | 12 ± 0.20 | 18 ± 0.71 | 36 ± 0.22 | 25 ± 0.33 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Heptyl 2-methylbutanoate | 18.89 | 1337 | 1336 | n.d. | n.d. | n.d. | 12 ± 0.25 | 22 ± 0.37 |
| Hexyl hexanoate | 19.47 | 1387 | 1385 | n.d. | 172 ± 2.83 | 292 ± 4.55 | 307 ± 1.07 | 241 ± 2.84 |
| 2-Methylbutyl octanoate | 20.21 | 1452 | 1453 | n.d. | 15 ± 0.21 | n.d. | 12 ± 0.16 | 6 ± 0.41 |
| ***Others*** | | | | n.d. | | n.d. | | |
| Acetoin (3-Hydroxy-2-butanone) | 9.46 | 693 | 721 | n.d. | 19 ± 0.15 | n.d. | n.d. | 51 ± 0.24 |
| 2-Methylbutanoic acid | 11.72 | 838 | 846 | 53 ± 0.66 | 12 ± 0.15 | n.d. | 43 ± 0.65 | 97 ± 0.49 |
| Hexanal dimethyl acetal | 14.00 | 977 | 980 | 260 ± 7.64 | n.d. | 274 ± 4.90 | n.d. | n.d. |
| 1-Octen-3-one | 14.05 | 980 | 975 | 167 ± 3.69 | 123 ± 1.74 | 135 ± 3.12 | 202 ± 1.52 | 260 ± 1.00 |
| 6-Methyl-5-hepten-2-one | 14.17 | 988 | 986 | 251 ± 4.39 | 179 ± 3.45 | 415 ± 12.59 | 245 ± 4.09 | 221 ± 0.44 |
| D-Limonene | 15.01 | 1045 | 1033 | n.d. | 6 ± 0.16 | n.d. | n.d. | n.d. |
| Geranial (α-Citral) | 18.22 | 1282 | 1271 | 11 ± 0.12 | 9 ± 0.07 | 18 ± 0.41 | n.d. | 6 ± 0.45 |
| (E)-α- Geranyl acetone | 20.35 | 1464 | 1451 | 16 ± 0.16 | 11 ± 0.12 | 14 ± 0.30 | 11 ± 0.20 | 13 ± 0.13 |
| (E)-α- Bergamotene[t] | 20.77 | 1502 | 1441 | 1 ± 0.14 | 6 ± 0.19 | 11 ± 0.75 | 5 ± 0.08 | 14 ± 0.20 |
| α- Farnesene | 20.94 | 1518 | 1509 | 185 ± 2.32 | 386 ± 6.54 | 656 ± 2.47 | 483 ± 5.08 | 120 ± 1.98 |

$RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; t – tentatively identified; n.d. – not detectable or below limit of detection; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org)

To identify the odor-active compounds in intact Kronprinz Rudolf samples and the impact of the different growing conditions on this variety, gas chromatography – olfactometry (GCO) as method of choice was applied. The odor description of the volatile compounds of the five panelists was collected, as well as the nasal impact frequency (NIF) was calculated. As described in Table 11, 35 of individual volatile compounds were identified in the intact Kronprinz Rudolf samples of the different growing conditions. However, it was shown that only a limited number of volatile constituents may be sufficient for the specific aroma profile of foods (Gary Reineccius, 2006, Dunkel et al., 2014). Twelve out of fifteen odor-active volatile compounds were identified by comparison of chromatographic, sensory data, and the retention indices on the non-polar column (see Table 13).

Table 13: Aroma compounds identified in intact Kronprinz Rudolf apple samples from the meadow orchards with the retention indices (RI) on a non-polar HP5 column and the sensory data

| Aroma compound | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | NIF [%] | Odor Quality$^{lit}$ | Odor Description$^{exp}$ |
|---|---|---|---|---|---|
| Butyl acetate | 812 | 812 | 93 | sweet, ripe banana, tutti frutti, tropical, candy, green | sweet, cotton candy |
| Ethyl 2-methylbutanoate | 856 | 854 | 86 | fruity, apple, berry, estery, tropical | berry, fruity |
| Propyl-2-methylpropanoate | 860 | 855 | 93 | sweet, fruity, estery, ripe, tropical, berry, tutti frutti | sweet, fruity |
| 1-Hexanol | 865 | 867 | 26 | ethereal, pungent, alcoholic, sweet, green | fusel, oily, green |
| 3-Methyl butylacetate* | 876 | 876 | 53 | sweet, fruity, banana, ripe | banana, sweet |
| n.i. | 882 | - | 33 | | acidic apple, fresh, green |
| Ethyl pentanoate | 907 | 902 | 26 | sweet, fruity, green | sweet, fruity |
| Methional | 909 | 909 | 33 | potato | potato |
| n.i. | 951 | - | 73 | | fruity |
| 1-Octen-3-ol/1-Octen-3-one* | 984 | 982 | 73 | mushroom, earthy, green, fungal | fungal, mushroom |
| Ethyl hexanoate | 1003 | 1002 | 66 | sweet, fruity, pineapple, green, banana | fruity, sweet |
| n.i. | 1009 | - | 73 | | metallic, sweet |
| Pentyl 2-methylbutanoate | 1140 | 1140 | 26 | fruity, apple, ethereal, pear | fruity, apple |
| Ethyl octanoate | 1197 | 1198 | 26 | fruity, sweet, pear, banana, brandy | sweet, citrus notes |
| Butyl hexanoate | 1203 | 1192 | 26 | fruity, berry, apple, green | musty, apple, floral notes |

$RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; $RI_{ref}$ – reference RI obtained from authentic standard compounds or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org); NIF – nasal impact frequency in %; lit – odor quality obtained from databases (http://www.thegoodscentscompany.com/); exp – odor description as collected in the experiments; * tentatively identified, the similarity from 1-octen-3-ol to 1-Octen-3-one (herbal, mushroom, earthy, musty) is high and a clear detection is not possible by this column.

The important compounds influencing the odor of the intact Kronprinz Rudolf apple samples from the meadow orchards are butyl acetate, ethyl 2-methylbutanoate and propyl-2-methylpropanoate (see in Figure 28). These three compounds are given the highest relative nasal impact frequency (NIF) between 86 and 93%.

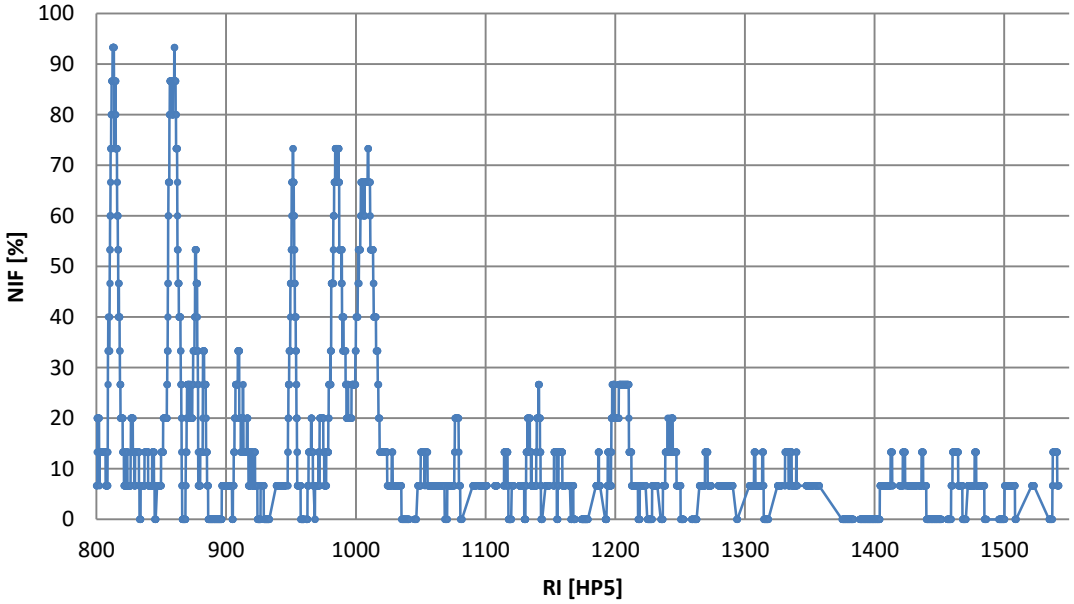


**Figure 28: Olfactogram:** example of an intact Kronprinz Rudolf from the meadow orchards; Retention indices (RI) are given on x-axis and the relative nasal impact frequency (NIF) on the y-axis.

The results of the GC-O experiments of the intact Kronprinz Rudolf samples of plantation growing are presented in Table 14. In total, eighteen odor-active compounds were determined and seventeen of them were identified by comparison of chromatographic and sensory data, and the retention indices of the non-polar column. Compounds with the highest NIF (between 80 – 86%) are butyl acetate, methyl pentanoate, (E)-2-hexenal, and ethyl hexanoate (Figure 29).

**Table 14:** Aroma compounds of the intact Kronprinz Rudolf apple samples of the plantation growing with the retention indices (RI) and the sensory data

| Aroma Compound | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | NIF [%] | Odor Quality$^{lit}$ | Odor Description$^{exp}$ |
|---|---|---|---|---|---|
| Butyl acetate | 811 | 812 | 86 | sweet, ripe banana, tutti frutti, tropical, candy, green | sweet, candy, berry |
| Methyl pentanoate | 817 | 820 | 86 | sweet, green, fruity, apple, nutty, sweaty | cheesy, green |
| Ethyl 2-methylbutanoate | 855 | 846 | 73 | fruity, apple, berry, estery, tropical | fruity, berry, apple |
| (E) -2-Hexenal | 857 | 854 | 80 | green, banana, fresh, leafy, fruity | apple, green |
| 1-Hexanol | 864 | 867 | 60 | ethereal, pungent, alcoholic, sweet, green | fruity, green, apple |
| 3-Methyl butylacetate* | 872 | 876 | 26 | sweet, fruity, banana, ripe | sweet, candy, fruity |
| Methional | 912 | 909 | 40 | potato | potato |
| Methyl hexanoate | 929 | 924 | 26 | fruity, pineapple, fatty, ethereal | herb, fatty |
| 2-Methylpropyl butanoate | 952 | - | 60 | fruity, green, ethereal, sweet | slightly acid, yogurt, sweet |
| 1-Octen-3-ol/1-Octen-3-one* | 984 | 982 | 73 | mushroom, earthy, green, fungal | fungal, mushroom |

| | | | | | |
|---|---|---|---|---|---|
| Propyl pentanoate | 987 | 988 | 53 | metallic, ethereal, fruity, pineapple | metallic, ethereal |
| Butyl butanoate | 999 | 996 | 33 | fruity, banana, pineapple, green, cherry | fruity, sweet, ripe fruit |
| Ethyl hexanoate | 1003 | 1002 | 80 | sweet, fruity, pineapple, green, banana | fruity, sweet |
| 2-Methylpropyl-2-methylbutanoate | 1008 | 1004 | 66 | sweet, fruity, citrus, melon | citrus, fruity |
| n.i. | 1011 | - | 60 | | spicy, slightly acid |
| Hexyl acetate | 1014 | 1014 | 60 | fruity, herb | herb, slightly acid |
| Limonene[t] | 1027 | 1027 | 33 | citrus | citrus, floral |
| Ethyl octanoate | 1201 | 1198 | 26 | fruity, sweet, pear, banana, brandy | sweet, citrus notes |

$RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; $RI_{ref}$ – reference RI obtained from authentic standard compounds or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org); NIF – nasal impact frequency in %; lit – odor quality obtained from databases (http://www.thegoodscentscompany.com/); exp – odor description as collected in the experiments; * the similarity from 1-octen-3-ol to 1-Octen-3-one (herbal, mushroom, earthy, musty) is high and a clear detection is not possible by this column.

The findings of this research suggest that there are differences between the apple samples of the two growing conditions. The apple samples of the plantation growing are given more odor than the apple samples of the meadow orchards. Although this study focuses on intact apples, the findings may well have a bearing on the growing conditions. Further studies need to be carried out in order to calculate the odor activity value (OAV) to check the impact of each compound in the samples of the different growing conditions.

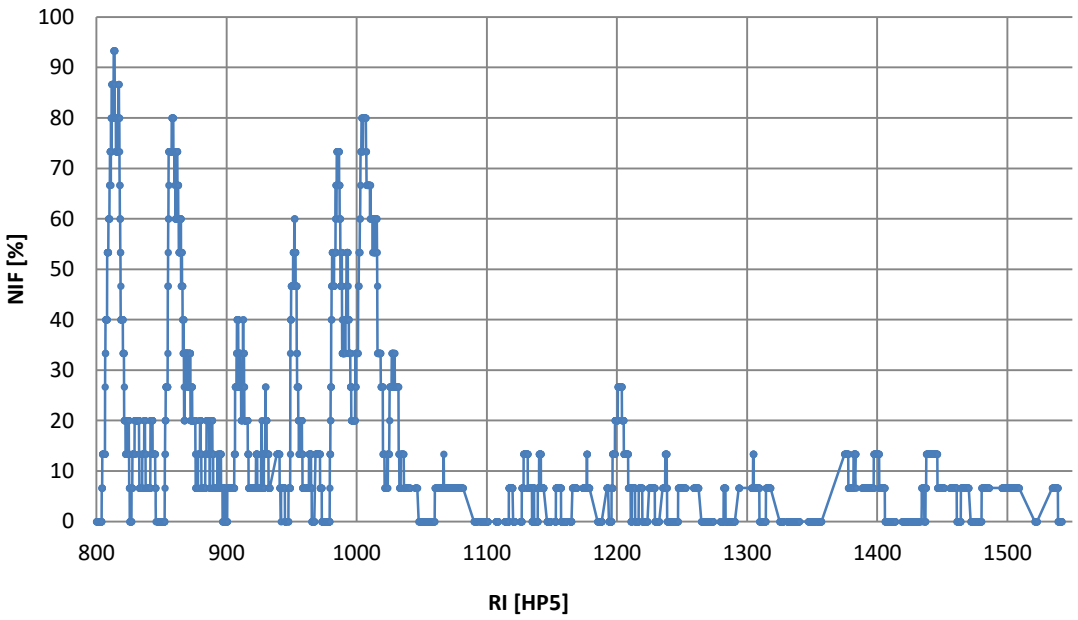


**Figure 29: Olfactogram:** example of an intact Kronprinz Rudolf from plantation growing; Retention indices (RI) are given on x-axis and nasal impact frequency (NIF) on the y-axis.

## 4.4. Impact of ripening stages on the volatile compositions of the peel from Ilzer Rose apples

In this section, the impact of four ripening stages (of two harvest years 2017 and 2018) was investigated of the volatile compositions of the apple peel from the Ilzer Rose apples. In addition, the basic parameters (SSC and TA) were also measured for the Ilzer Rose apple samples of both investigated harvest years.

First, the SSC was measured in the Ilzer Rose apple samples from the harvest years 2017 and 2018. The results are shown as mean value ± standard deviation (triplicated analysis). The content of sugars in Ilzer Rose apple fruits of four different ripening stages (2017 P1, 2017 P2, 2017 P3, 2017 P4, 2018 P1, 2018 P2, 2018 P3, 2018 P4) from two harvest years are presented in Figure 30. The SSC ranged from 12.5 to 14.7 %. In the first investigated year 2017, the SSC showed an increasing tendency during ripening, but at the fourth picking date the SSC decreased, this is explainable with the drastic change after the third picking date (heavy rain, cold temperature, and the climatologic frost)(Unterberger et al., 2018). The highest SSC was reached around the perfect harvest/picking date (P3). The second investigated year 2018 presented an increasing tendency of SSC after the first picking date during ripening. This result was unexpected; in general, the soluble solids content is increasing between the ripening stages. Except for the first picking date in 2018, the results correlated with the trend of SSC increase with the ripening stages described in literature. Nevertheless, the high SSC at 2018 P1 remains inexplicable (Ackermann, Fischer, & Amado, 1992; Musacchi & Serra, 2018).

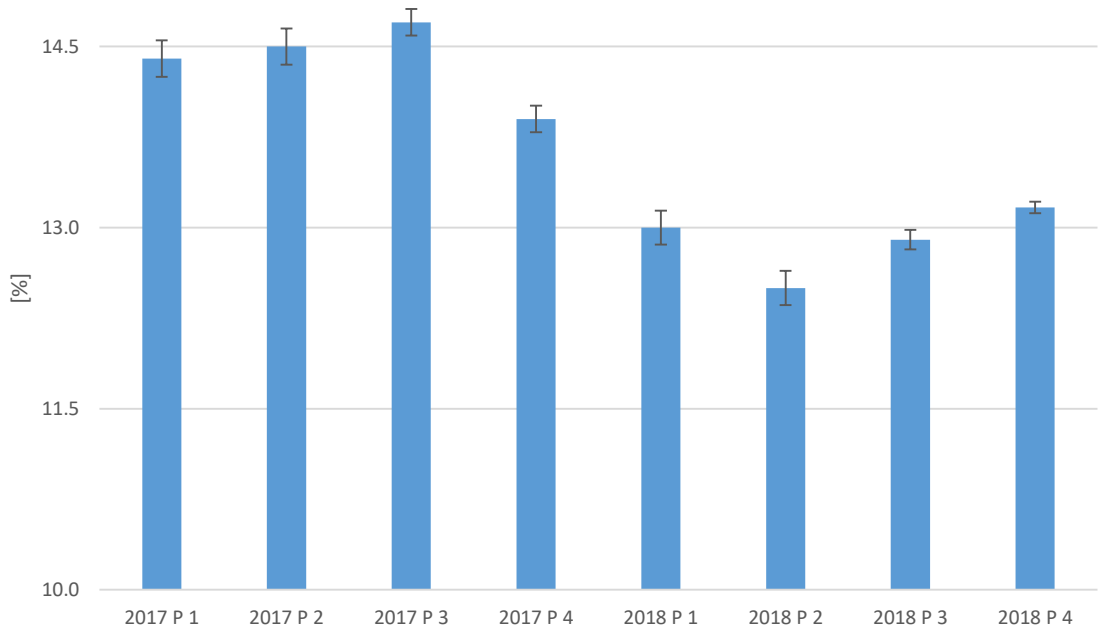


**Figure 30: Soluble Solids content [%] in Ilzer Rose apples at different ripening stages from two harvest years;** 2017 P1 (picking date 1: 03.10), 2017 P2 (picking date 2: 09.10.), 2017 P3 (picking date 3: 18.10.), 2017 P4 (picking date 4: 23.10.), 2018 P1 (picking date: 24.09.), 2018 P2 (picking date 2: 01.10.), 2018 P3 (picking date 3: 08.10.), 2018 P4 (picking date 4: 15.10.).

Second, the total acid content [%] was determined from the Ilzer Rose samples (harvest 2017 and 2018). The results are shown as the mean value ± standard deviation (triplicated analysis). The total acid content [%] in Ilzer Rose apples at different ripening stages is presented in Figure 31. The highest TA is shown at the first harvest date of the year 2017 and the third harvest date of 2018. TA varied in the first investigating year 2017 within the four different picking dates. There was no clear trend in TA changes during the first investigating year. The acid concentration declined after 2018 P3, this decrease can be attributed to a dilution effect caused by a mass increase during the cell growth phase (Ackermann et al., 1992). TA often varies more between seasons and orchards than between harvest dates (Kingston, 2010), as a result it is not properly explainable why the TA fluctuated in the year 2017.

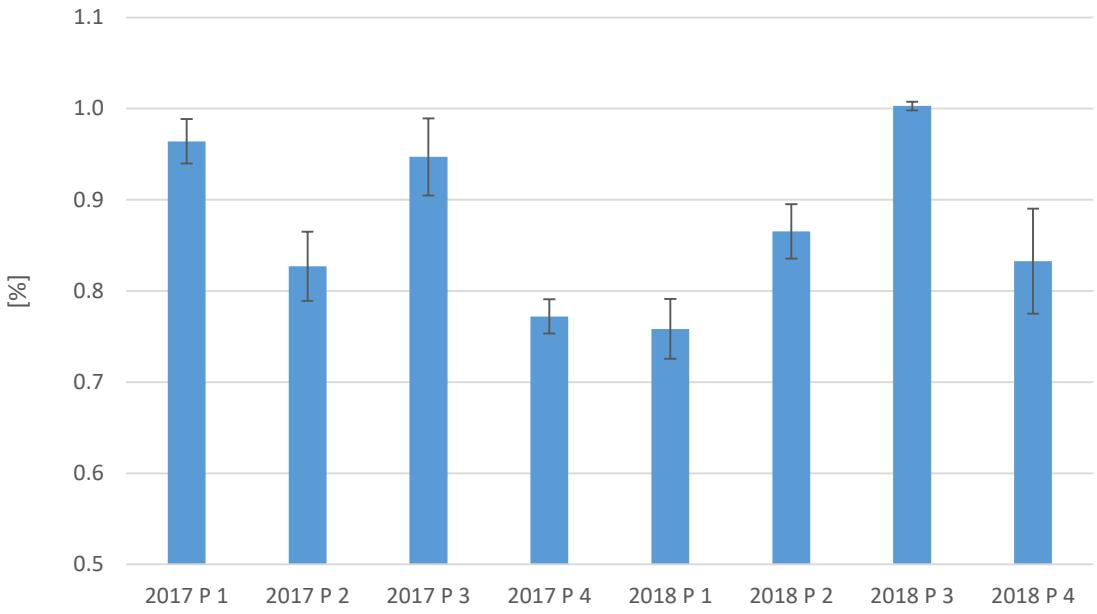


**Figure 31: Total Acid Content [%] in Ilzer Rose apples at different ripening stages from two harvest years;** 2017 P1 (picking date 1: 03.10), 2017 P2 (picking date 2: 09.10.), 2017 P3 (picking date 3: 18.10.), 2017 P4 (picking date 4: 23.10.), 2018 P1 (picking date: 24.09.), 2018 P2 (picking date 2: 01.10.), 2018 P3 (picking date 3: 08.10.), 2018 P4 (picking date 4: 15.10.).

An expert panel, well-trained and experienced in the sensory assessment of apples, evaluated the Ilzer Rose apples of all four different ripening stages from two successive years (2017 and 2018). The sample preparation for both harvest years was conducted according to the description in section 3.2.2.1.

In 2017, the expert panel investigated the Ilzer Rose apples by a Quantitative Descriptive Analysis QDA®. The QDA® methodology was used to get detailed information about the differences between the different ripening stages and not only the descriptive evaluation. The similarity/dissimilarity of the apple samples was evaluated by calculating a principal component analysis (PCA). The results from QDA®, presented in Figure 32, is shown as a biplot of a PCA Correlation between the four ripening stages of the harvest year 2017 (picking date 1 – Krispel 1; picking date 2 – Krispel 2, picking date 3 – Krispel 3, picking date 4 – Krispel 4). The most interesting aspect of Figure 32 is that the samples of the picking date 3 (Krispel 3) are highly correlated with the attributes floral notes, sweetness, fruity and apple odor. The sensory evaluation

revealed clearly that the fourth picking date of the year 2017 is completely different. This effect is explainable because of the sudden change in climate after the third picking date. The ripening of the apples on the tree is significantly affected by the lower temperatures and heavy rainy weather. The third picking date appeared to be the perfect date for the Ilzer Rose apples in the year 2017, they showed an intensely floral, good sweet/sour balance flavor. The sensory attributes of apple odor, sweet, floral and fruity increased with later picking dates, in contrast, the attributes sour, grassy/green and citrus flavor decreased.

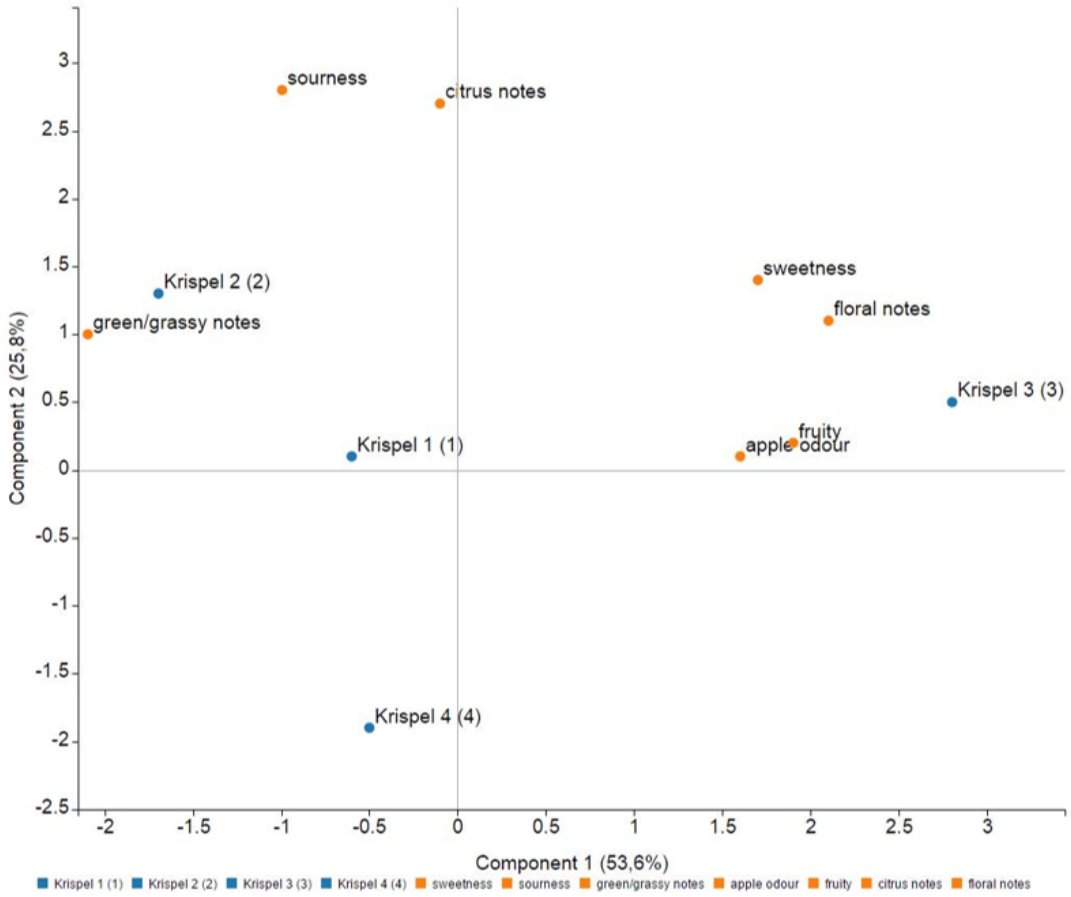


**Figure 32: PCA Correlation of the QDA® of the four different ripening stages of Ilzer Rose apple samples of the harvest 2017;** Krispel 1 (picking date 1), Krispel 2 (picking date 2), Krispel 3 (picking date 3) Krispel 4 (picking date 4).

In 2018, the check-all-that-apply (CATA) questions were applied to get more focus on the differences from the ripening stages. The main advantage of CATA questioning is the easy catch of the attributes. In several pre-sessions with apple samples, the expert panel were trained to find the right attributes for the CATA questioning. The structured question format of CATA can provide a complete description of the sensory characteristics of the investigated apple samples. The similarity/dissimilarity of sample configurations was evaluated by calculating a principal component analysis (PCA). Figure 33 presents the PCA correlation results of the CATA questioning from the four different ripening stages of the harvest year 2018 (IR P1 – picking date 1, IR P2 – picking date 2, IR P3 – picking date 3, IR P4 – picking date 4). The interesting point about the data in Figure 33 is that the samples of the fourth picking date are positioned further away from the

other three picking dates. The samples of the fourth picking date are in the same quadrant with the attributes sourly, ripe, juicy, crisp and apple odor. The samples of the first picking date are correlated with the attributes honey, flavorless, soft, very sweet, green/grassy notes, and well-balanced sweetness-sourness. The samples of the second and third picking dates are positioned in the same quadrant with the attributes mealy, astringent, overripe, bitter, citrus notes, and freshly squeezed apple juice. The correlation between the floral notes and the third picking date is not as obvious as in the harvest year before. However, the results of both harvest years show a similar correlation regarding the fourth picking date. This is an interesting outcome because the apples of the fourth picking dates were overripe at the time of harvest in 2018. Nevertheless, the sensory evaluation of the expert panel showed no correlation with the attribute 'overripe'.

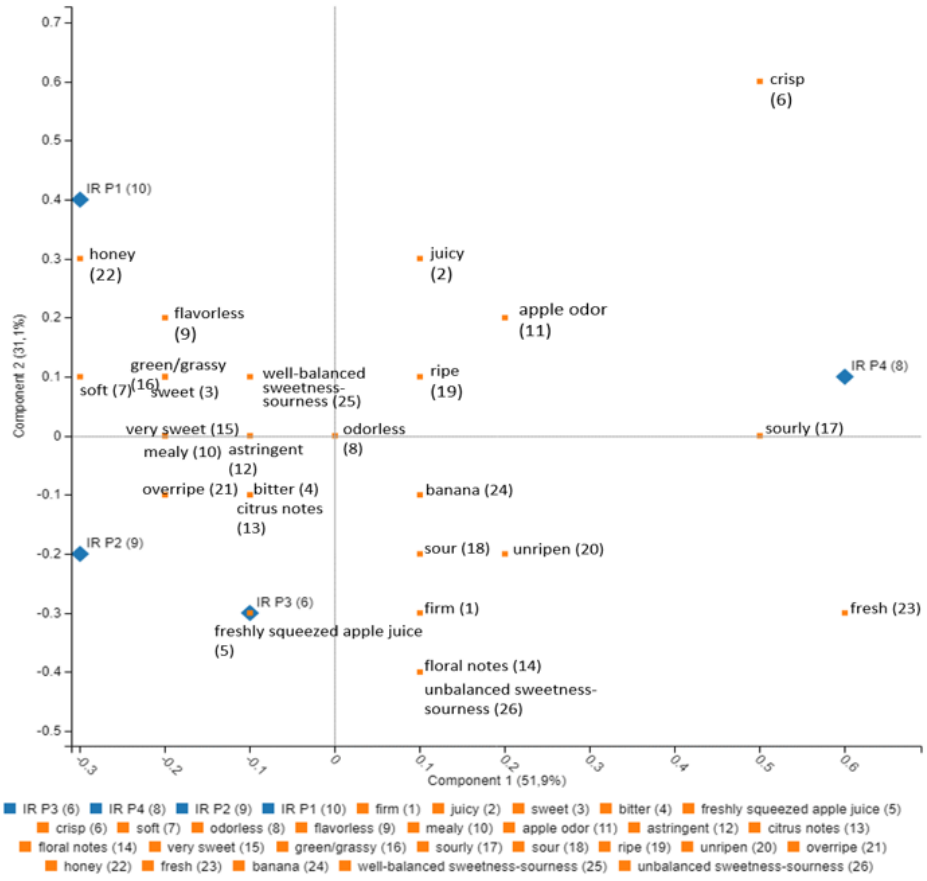


**Figure 33: PCA Correlation obtained from CATA questioning of the four different ripening stages of Ilzer Rose apple samples of the harvest 2018;** IR 1 (picking date 1), IR 2 (picking date 2), IR 3 (picking date 3), IR 4 (picking date 4).

Changes during ripening and maturation were already observed in previous studies (Prasanna, Prabha, & Tharanathan, 2007, Paul & Pandey, 2014, Alós, Rodrigo, & Zacarias, 2019; Giné-Bordonaba, Echeverria, Duaigües, Bobo, & Larrigaudière, 2019b), and by the known effects of ripening, the impact of ripeness on the formation of volatile compounds in Ilzer Rose apples was investigated. The ripening stages of apples are very important to find the best harvest time and sensory quality. There is a correlation between ethylene and aroma production, which was shown through the use of both ethylene action and ethylene biosynthesis inhibitors that result in a reduction in levels of volatiles in apple fruit (Harb et al., 2011). At the beginning of many of the

aroma pathways, ethylene plays an important role in their regulation. The process of photosynthesis accumulates starch production in apple fruits. A mature apple has maximum starch accumulation, has finished enlarging, and can ripen after being removed from the tree. A ripe apple has an acceptable eating quality (good or desired flavor and texture). There is also a change in the apple flavor from aldehydes (green notes) to esters (fruity notes) during ripening (Contreras & Beaudry, 2013). Contreras & Beaudry, 2013 presented in their studies that hexanal synthesis by intact fruit is dependent on ripening. Also important is the activity of alcohol dehydrogenase (ADH), which is responsible for the conversion of aldehydes to alcohols. The ADH activity declines or remains steady during ripening (Echeverría, Graell, López, & Lara, 2004a).

There are significant differences observed between peel and flesh in the levels of volatiles, precursors, and enzymes in the mechanism of the regulation. The alcohol acyltransferase (AAT) enzyme is in the flesh a more active biochemical step than in the peel, in which the metabolism of amino acids and fatty acids affected more critical (Defilippi et al., 2005). However, up until now no influence of the ripening stages and on the harvest year on the distribution of single compounds in the apple peel were evaluated. Within this work, the profiles of volatile compounds were analyzed in the peel of Ilzer Rose apple fruits of two consecutive harvest years (2017 and 2018) at four different ripening stages. The volatile compounds of the different apple peel samples of the Ilzer Rose apple variety were enriched from the headspace using headspace SPME and were (semi-) quantified after the 1-dimensional GC-MS separation (on nonpolar capillary column). The concentrations of volatile compounds were expressed as relative concentrations to the internal standard 2-octanol. By analysis of GC-MS measurements were identified 81 volatile compounds, these compounds were identified in all apple peel samples of the two successive years 2017 and 2018 (with some exceptions). The volatile compounds were (semi-) quantified and grouped into chemical classes, including alcohols, aldehydes, esters, acetates, ketones, and terpenes. Volatile compounds were identified in the investigated 2017 and 2018 apple samples including 7 alcohols, 16 aldehydes, 36 esters, 9 terpenes, 5 acetates and 6 ketones (Table 15). The results show clearly that the concentrations varied with throughout picking date/year and ripening stages. Additionally, they showed that esters were the predominant volatile class in all apple peel samples and they played an important role in the apple characteristic fruity flavor (Mehinagic, Royer, Symoneaux, Jourjon, & Prost, 2006; Thewes et al., 2017).

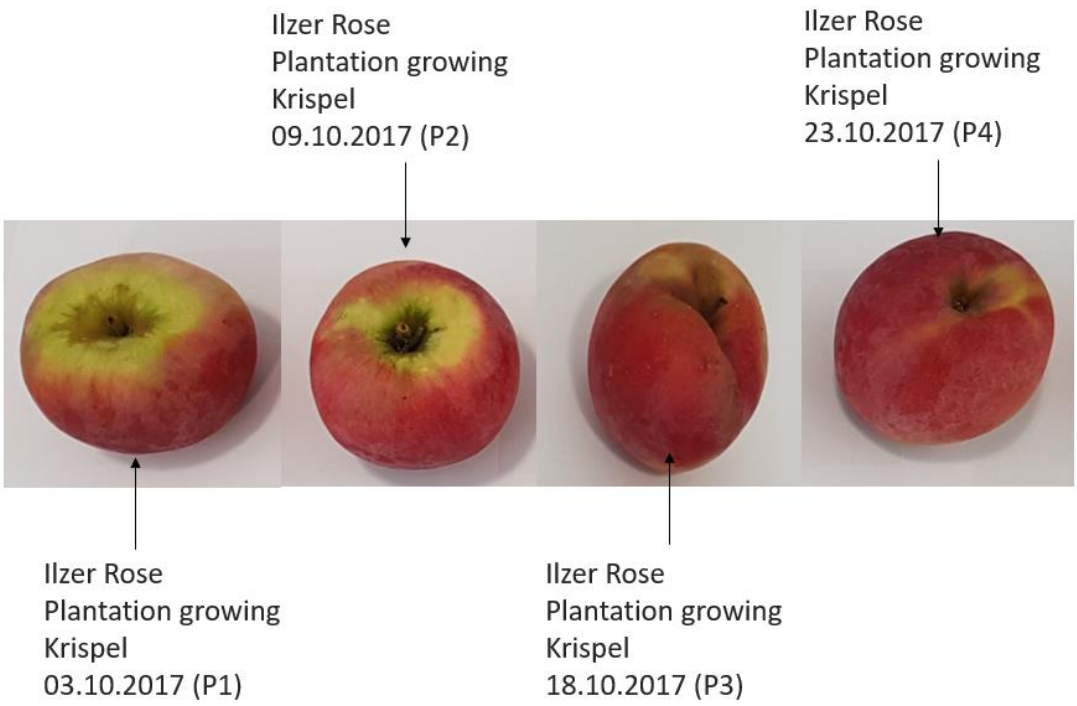


**Figure 34: Harvest 2017:** An Overview of the Ilzer Rose apple samples from the four different picking dates.

Fellman et al., (2000) described changes in the profile of volatile compounds by maturation: at the beginning aldehydes were predominating, then the content of alcohol starts to increase and as the final step, the esters dominate. The formation of alcohols occurs by the reduction of corresponding aldehydes, by the action of the enzyme ADH. Alcohols are synthesized by two different pathways, while linear alcohols are produced by the fatty acid catabolism, the branched-chain alcohols are derived by the metabolism of branched-chain amino acids (Contreras & Beaudry, 2013, Espino-Diaz et al., 2016).

Previous research showed that more than 60 alcohols are synthesized by apples (Dimick & Hoskin, 1983). In this study, seven alcohols (1-butanol, 1-penten-3-ol, 2-methyl-1-butanol, (*Z*)-2-penten-1-ol, 1-pentanol, (*Z*)-2-Hexen-1-ol and 1-hexanol) were identified in the volatile fraction of the apple peel of the eight different picking samples (Figure 35). Alcohols are direct precursors of esters, which suggests that the ester biosynthesis in apples is limited by the alcohol concentration (Defilippi et al., 2005). The content of the investigated alcohol in the apple peel samples is not constant. It depends on the maturity of the fruit, picking date and harvest year. The harvest year 2017 shows that the concentration of (*Z*)-2-Hexen-1-ol, 1-pentanol and (*Z*)-2-penten-1-ol decreased in the first three picking dates, but after the third picking date, the concentration of all three alcohols increased. Typically, the amount of the alcohols decreases over time, because the apples ripen and thereby the alcohols are converted to esters, aldehydes or ketones. The harvest year 2018 shows higher alcohol concentrations than the harvest year 2017 and a completely different profile of the alcohol content. However, the concentration of the seven different alcohols increased over time and show the highest amount at the fourth picking date of the harvest year 2018 (excluded (*Z*)-

2-Hexen-1-ol, 1-pentanol and 1-penten-3-ol; they have their highest amount at the third picking date).

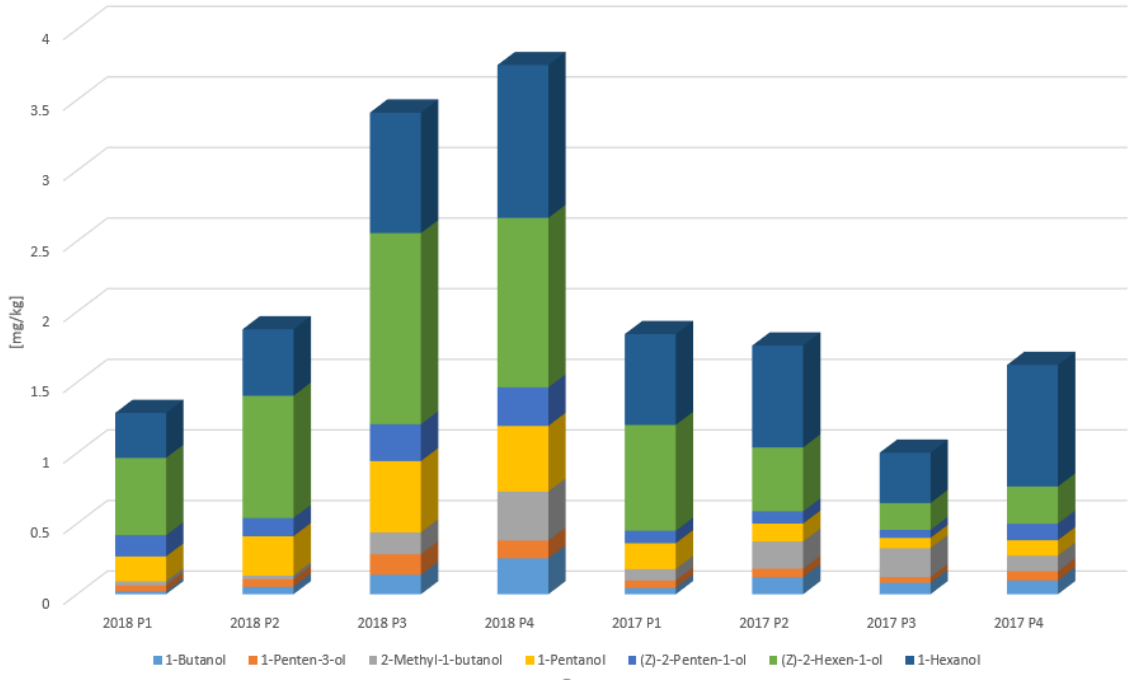


**Figure 35: Content of alcohols (calculated in mgkg$^{-1}$) in apple peels of each picking date in the harvest years 2017 and 2018;** 2018 P1 = picking date 1; 2018 P2 = picking date 2; 2018 P3 = picking date 3; 2018 P4 = picking date 4; 2017 P1 = picking date 1; 2017 P2 = picking date 2; 2017 P3 = picking date 3; 2017 P4 = picking date 4.

Esters are the most important volatile compound group in apples, not only the numbers of the investigated esters are higher (36), but also because of the content of some of the esters and their aroma activity. The content of the esters is depending on the availability of precursors (such as alcohols) and the selectivity as well as the activity of the enzymes involved (Espino-Diaz et al., 2016). In this study, 36 individual esters were identified in the eight different peel samples of the two harvest years 2017 and 2018. Esters can have linear or branched chains and synthesized from lipids via fatty acid metabolism (β-oxidation and lipoxygenase activity) or derived from the amino acid catabolism (leucine, isoleucine and valine pathway). The amino acids leucine, isoleucine, and valine are branched compounds of aliphatic nature and are synthesized in chloroplasts (Rowan et al., 1996). Due to the synthesis and metabolism of proteins, the content of amino acids decreases with the maturation of apples (Espino-Diaz et al., 2016). When apples mature, the reaction rates of the two lipid synthesis, β-oxidation pathway and lipoxygenase activity, increase, because of the change in membrane fluidity. This effect can be seen clearly in the content of esters of the harvest year 2018. The concentration of the esters hexyl hexanoate, butyl hexanoate, and hexyl-2-methyl butanoate is highest at the fourth picking date (see in Figure 36 esters with the highest concentrations). Hexyl butanoate decreased from the third to the fourth picking date.

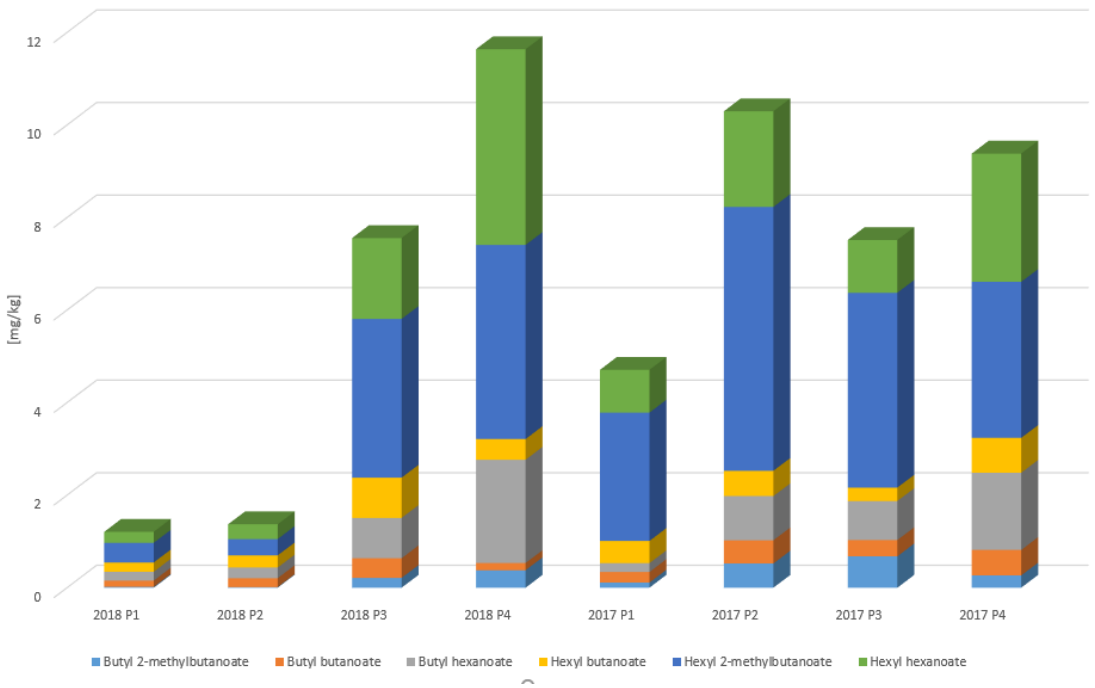


**Figure 36: Content of esters (with highest concentrations in mgkg$^{-1}$) in apple peels of each picking date in the harvest years 2017 and 2018;** 2018 P1 = picking date 1; 2018 P2 = picking date 2; 2018 P3 = picking date 3; 2018 P4 = picking date 4; 2017 P1 = picking date 1; 2017 P2 = picking date 2; 2017 P3 = picking date 3; 2017 P4 = picking date 4.

In this study, five acetates are identified (butyl acetate, 2-methyl butyl acetate, pentyl acetate, hexyl acetate, (*Z*)-2-Hexen-1-ol acetate) in the eight different apple peel samples of the harvest years 2017 and 2018 (Figure 37). The harvest year 2018 presents an increase in the amount of the five acetates, but the harvest year 2017 shows a completely different acetate proportion.

Aldehydes are also an important volatile compound group for the apple flavor profile (Figure 38). Thirteen different aldehydes ((*E*)-2-butenal, n-pentanal, (*E*)-2-pentenal, 3-methyl-2-butenal, hexanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-hexadienal, (*E*)-2-heptenal, 2,4-heptadienal, (*E*)-2-octenal, nonanal, decanal, and 2-(*E*)-decenal) are determined in this study. All aldehydes increased in concentration over time in the harvest year 2018, but in the harvest year 2017, they all have fluctuations with no trend.

Seven ketones, such as 2-butanone, 2-pentanone, 3-pentanone, 1-penten-3-one, 1-octen-3-one, 6-methyl-5-hepten-2-one, acetoin (3-hydroxy-2-butanone), were identified in this work. There are three ketones that could be identified in both harvest years (2-butanone, acetoin, and 6-methyl-5-hepten-2-one). Interestingly, 2-pentanone and 3-pentanone were only present in the harvest year 2017 and 1-penten-3-one and 1-octen-3-one were only identified in the harvest year 2018. The ketones do not show an important impact on the apple flavor.

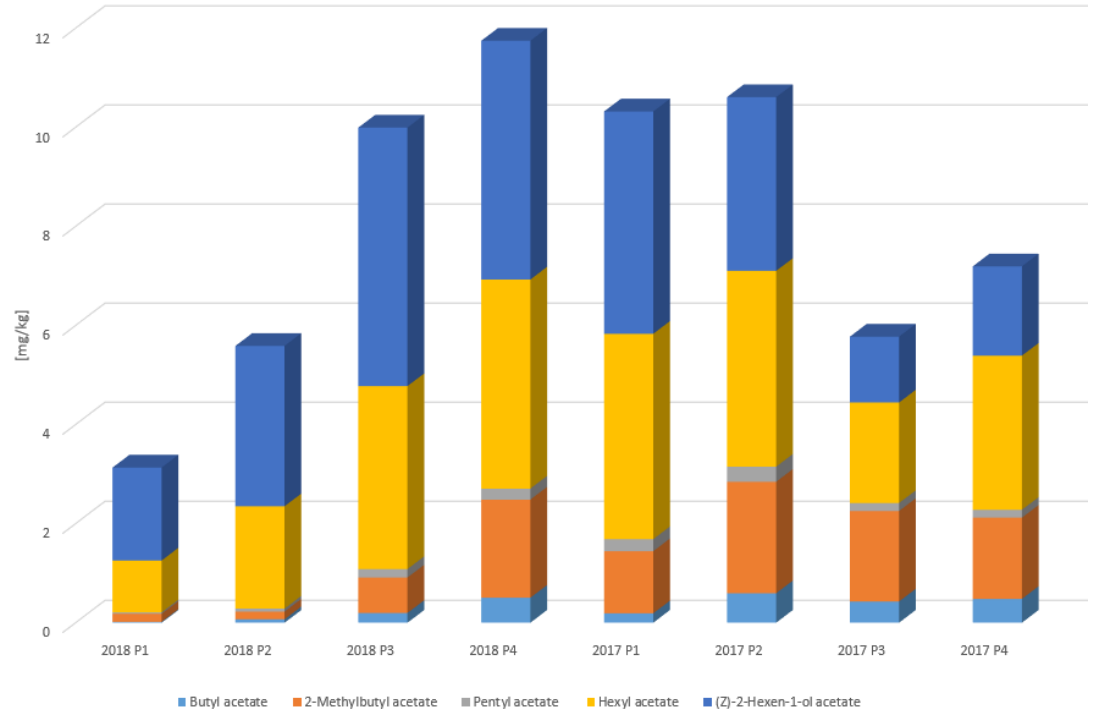


**Figure 37: Content of acetates (calculated in mgkg$^{-1}$) in apple peels of each picking date in the harvest years 2017 and 2018;** 2018 P1 = picking date 1; 2018 P2 = picking date 2; 2018 P3 = picking date 3; 2018 P4 = picking date 4; 2017 P1 = picking date 1; 2017 P2 = picking date 2; 2017 P3 = picking date 3; 2017 P4 = picking date 4.

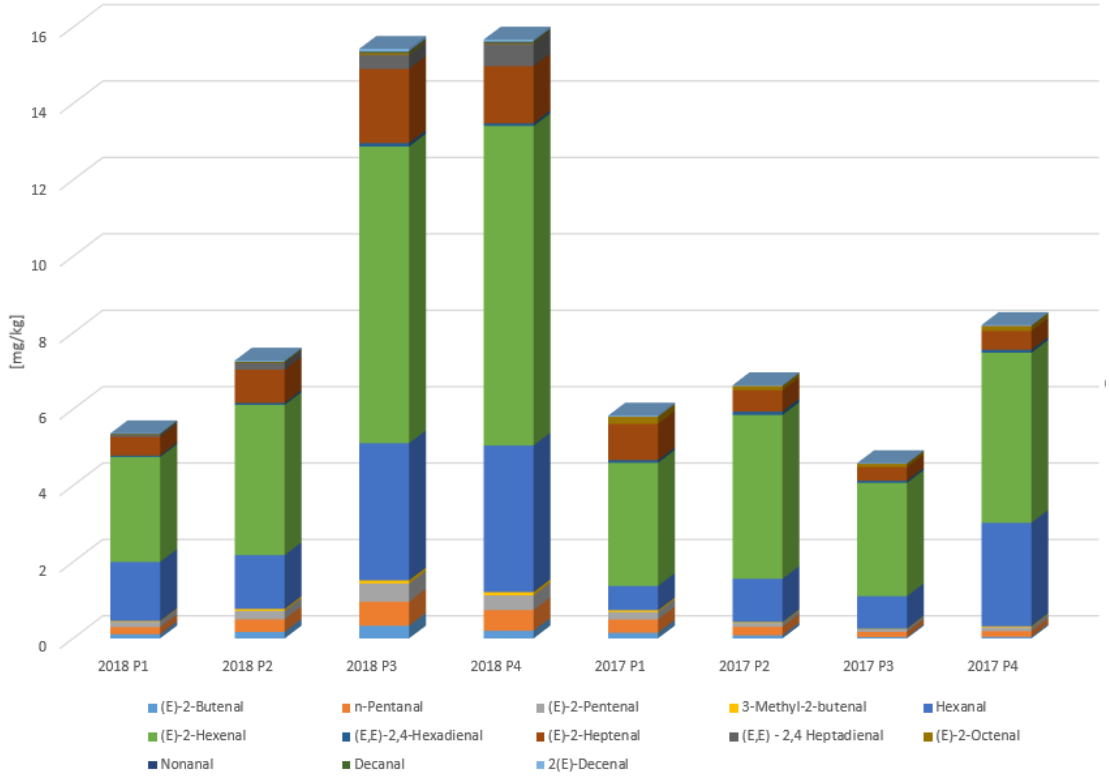


**Figure 38: Content of aldehydes (calculated in mgkg$^{-1}$) in apple peels of each picking date in the harvest years 2017 and 2018;** 2018 P1 = picking date 1; 2018 P2 = picking date 2; 2018 P3 = picking date 3; 2018 P4 = picking date 4; 2017 P1 = picking date 1; 2017 P2 = picking date 2; 2017 P3 = picking date 3; 2017 P4 = picking date 4.

In total, twelve terpenes were identified in the investigated eight apple peel samples, most of them the first time in apples (Figure 39). The terpenes, that could be identified with the use of 1-dimensional GC-MS, are limonene, (E)-linalool oxide, linalool, neral, geranial, geranyl acetone, β-farnesene, bisabolene, zingiberene, α-farnesene, α-bergamotene, and α-caryophyllene. However, in apple peel from the harvest 2018 twelve terpenes were identified, whereas samples from the harvest year 2017 only five terpenes (i.e., (E)-linalool oxide, geranial, geranyl acetone, α-bergamotene, and α-farnesene) could be found.

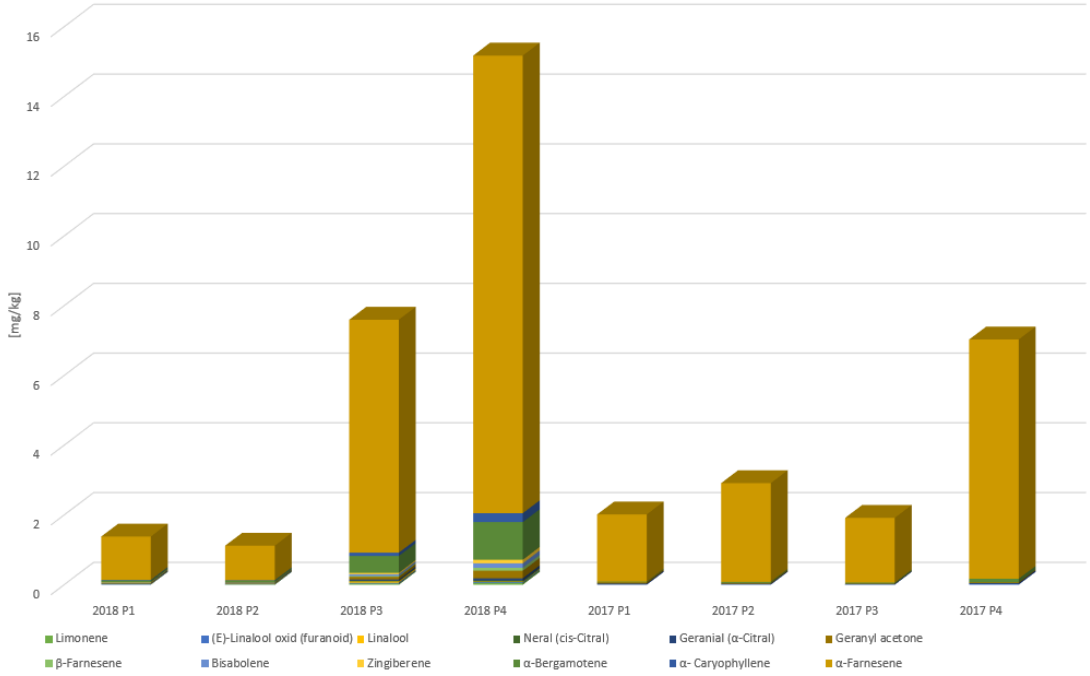


**Figure 39: Content of terpenes (calculated in mgkg$^{-1}$) in apple peels of each picking date in the harvest years 2017 and 2018;** 2018 P1 = picking date 1; 2018 P2 = picking date 2; 2018 P3 = picking date 3; 2018 P4 = picking date 4; 2017 P1 = picking date 1; 2017 P2 = picking date 2; 2017 P3 = picking date 3; 2017 P4 = picking date

Table 15: Volatile compounds obtained from headspace solid-phase microextraction (HS-SPME) GC-MS analysis from the Ilzer Rose apple peel samples of two harvest years (2017 and 2018) expressed as relative concentrations (n=3); estimated quantities in µgkg-1 +/i RSd (%) of the investigated apples calculated by comparison with internal standard (2-Octanol); TIC total ion chromatogram;

| volatile compounds | RI $[HP5]_{exp}$ | RI $[HP5]_{lit}$ | 2018 Krispel P1 ± RSD (%) | 2018 Krispel P2 ± RSD (%) | 2018 Krispel P3 ± RSD (%) | 2018 Krispel P4 ± RSD (%) | 2017 Krispel P1 ± RSD (%) | 2017 Krispel P2 ± RSD (%) | 2017 Krispel P3 ± RSD (%) | 2017 Krispel P4 ± RSD (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Alcohols*** | | | | | | | | | | |
| 1-Butanol | 665 | 675 | 21 ± 0.66 | 53 ± 3.32 | 137 ± 11.01 | 254 ± 0.78 | 46 ± 0.26 | 120 ± 0.82 | 81 ± 0.58 | 98 ± 0.39 |
| 1-Penten-3-ol | 684 | 678 | 42 ± 0.46 | 54 ± 3.97 | 146 ± 20.76 | 126 ± 12.05 | 52 ± 0.3 | 61 ± 0.44 | 40 ± 0.29 | 65 ± 0.23 |
| 2-Methyl-1-butanol | 739 | 739 | 28 ± 3.19 | 25 ± 2.10 | 155± 13.04 | 347 ± 17.81 | 80 ± 0.55 | 192 ± 1.32 | 205 ± 1.56 | 110 ± 0.39 |
| 1-Pentanol | 766 | 766 | 177 ± 1.70 | 279 ± 8.11 | 505 ± 4.27 | 465 ± 9.80 | 183 ± 0.91 | 128 ± 1.31 | 73 ± 0.99 | 110 ± 0.45 |
| (Z)-2-Penten-1-ol | 770 | 769 | 150 ± 0.88 | 129 ± 5.73 | 261 ± 19.48 | 273 ± 9.50 | 89 ± 0.55 | 86 ± 0.23 | 57 ± 0.31 | 117 ± 0.18 |
| (Z)-2-Hexen-1-ol | 866 | 864 | 548 ± 11.01 | 866 ± 35.21 | 1353 ± 11.42 | 1200 ± 17.19 | 749 ± 12.67 | 453 ± 3.13 | 190± 2.48 | 263 ± 0.84 |
| 1-Hexanol | 868 | 867 | 318 ± 10.66 | 470 ± 23.91 | 854 ± 7.20 | 1082 ± 29.65 | 643 ± 6.43 | 721 ± 2.58 | 356 ± 4.58 | 861 ± 5.03 |
| ***Esters*** | | | | | | | | | | |
| Ethyl propanoate | 711 | 713 | n.d. | 2 ± 0.48 | 4 ± 0.98 | 5 ± 1.68 | 1 ± 0.01 | 5 ± 0.10 | 10 ± 0.04 | 3 ± 0.02 |
| Ethyl 2-methylbutanoate | 850 | 846 | n.d. | n.d. | n.d. | n.d. | 16± 0.07 | 40 ± 0.28 | 53 ± 0.77 | 44 ± 0.38 |
| Ethyl hexanoate | 996 | 1002 | n.d. | n.d. | n.d. | n.d. | 309 ± 4.89 | 422 ± 4.89 | 317 ± 6.67 | 579 ± 6.90 |
| Butyl 2-methylbutanoate | 1043 | 1041 | 19 ± 0.62 | 13 ± 0.08 | 213 ± 15.58 | 377 ± 18.68 | 110 ± 0.44 | 524 ± 10.75 | 678 ± 13.65 | 265 ± 3.19 |
| Methyl propanoate | 634 | 626 | n.d. | n.d. | n.d. | n.d. | 19 ± 0.08 | 19 ± 0.12 | 14 ± 0.09 | 16 ± 0.03 |
| Methyl butanoate | 723 | 723 | n.d. | n.d. | n.d. | n.d. | 73 ± 0.22 | 71 ± 0.27 | 54 ± 0.37 | 62 ± 0.27 |
| Methyl 2-methylbutanoate | 778 | 776 | n.d. | n.d. | n.d. | n.d. | 42 ± 0.96 | 41 ± 0.62 | 32 ± 0.69 | 39 ± 0.80 |
| Propyl butanoate | 896 | 900 | 13± 0.28 | 23 ± 1.82 | 72 ± 5.21 | 200 ± 6.63 | 19 ± 0.12 | 19 ± 0.56 | 14 ± 1.15 | 16 ± 0.48 |
| 2-Hexenyl propanoate | 1106 | 1111 | 24 ± 0.88 | 54 ± 2.73 | 205 ± 13.06 | 153 ± 5.24 | n.d. | n.d. | n.d. | n.d. |
| Butyl propanoate | 906 | 910 | 44 ± 19.86 | 59 ± 33.29 | 24 ± 14.28 | 10 ± 1.77 | 21 ± 0.11 | 64 ± 0.70 | 78 ± 1.15 | 37 ± 0.37 |
| Methyl hexanoate | 924 | 924 | 8 ± 0.70 | 14 ± 0.78 | 59 ± 9.91 | 51 ± 2.50 | 31 ± 1.01 | 44 ± 0.54 | 37 ± 1.41 | 68 ± 2.23 |
| Propyl 2-methylbutanoate | 947 | 953 | 15 ± 0.37 | 25 ± 4.93 | 63 ± 6.23 | 15 ± 0.99 | 4 ± 0.13 | 26 ± 0.62 | 81 ± 1.53 | 15 ± 0.17 |
| Pentyl propanoate | 971 | 972 | 14 ± 6.44 | 20 ± 7.86 | 41 ± 4.71 | 9 ± 0.32 | 15 ± 0.13 | 31 ± 0.55 | 51 ± 0.75 | 20 ± 0.20 |
| Butyl butanoate | 993 | 996 | 136 ± 4.88 | 193 ± 10.05 | 423 ± 2.99 | 156 ± 15.63 | 230 ± 0.99 | 500 ± 5.79 | 352 ± 6.91 | 552 ± 7.23 |
| 2-Methylbutylbutanoate | 1059 | 1056 | n.d. | n.d. | n.d. | n.d. | 64 ± 0.31 | 116 ± 2.06 | 96 ± 1.88 | 96 ± 1.05 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Pentyl butanoate | 1092 | 1094 | 13 ± 0.28 | 23 ± 1.82 | 72 ± 5.21 | 200 ± 6.63 | 62 ± 0.77 | 64 ± 1.07 | 37 ± 0.85 | 55 ± 0.74 |
| n-Propyl hexanoate | 1093 | 1097 | 15 ± 1.97 | 24 ± 2.38 | 47 ± 3.83 | 22 ± 3.37 | 10 ± 0.48 | 47 ± 1.80 | 89 ± 2.78 | 88 ± 1.62 |
| Hexyl propanoate | 1103 | 1108 | 28 ± 1.72 | 58 ± 19.79 | 194 ± 13.8 | 35 ± 16.52 | 114 ± 0.80 | 135 ± 1.58 | 115 ± 2.18 | 100 ± 1.26 |
| 2-Methylbutyl 2-methylbutanoate | 1106 | 1106 | 70 ± 28.92 | 97 ± 19.19 | 292 ± 24.3 | 107 ± 2.71 | 70 ± 0.67 | 192 ± 4.75 | 293 ± 6.95 | 115 ± 1.39 |
| Methyl octanoate | 1123 | 1126 | 4 ± 0.21 | 11 ± 0.70 | 25 ± 2.46 | 30 ± 1.76 | 14 ± 0.40 | 16 ± 0.11 | 12 ± 0.48 | 25 ± 0.75 |
| Pentyl 2-methylbutanoate | 1140 | 1140 | 13 ± 0.54 | 16 ± 0.50 | 117 ± 7.98 | 149 ± 5.27 | 62 ± 0.25 | 187 ± 4.83 | 175 ± 4.31 | 69 ± 0.88 |
| Hexyl 2-methylpropanoate | 1148 | 1150 | 4 ± 1.60 | 71 ± 3.21 | 35 ± 1.96 | 9 ± 0.70 | 49 ± 0.36 | 43 ± 0.87 | 27 ± 0.57 | 54 ± 0.62 |
| 2-Methyl propylhexanoate | 1151 | 1149 | 4 ± 1.94 | 6 ± 1.12 | 12 ± 9.91 | 3 ± 1.02 | 7 ± 0.13 | 16 ± 0.25 | 12 ± 0.12 | 30 ± 0.13 |
| Hexyl butanoate | 1192 | 1192 | 201 ± 16.37 | 261 ± 15.75 | 869 ± 6.17 | 446 ± 4.73 | 480 ± 3.73 | 546 ± 10.60 | 290 ± 4.67 | 752 ± 11.55 |
| Butyl hexanoate | 1190 | 1190 | 189 ± 13.83 | 231 ± 14.67 | 873 ± 6.55 | 2233 ± 8.36 | 191 ± 2.45 | 956 ± 30.18 | 843 ± 24.60 | 1669 ± 22.43 |
| Hexyl 2-methylbutanoate | 1240 | 1237 | 425 ± 19.41 | 353 ± 13.00 | 3430 ± 22.23 | 4196 ± 19.51 | 2773 ± 9.77 | 5704 ± 8.62 | 4211 ± 7.46 | 3372 ± 3.55 |
| 3-Methylbutyl hexanoate | 1255 | 1251 | 16 ± 0.56 | 16 ± 0.82 | 126 ± 9.30 | 447 ± 23.16 | 75 ± 0.68 | 288 ± 8.14 | 261 ± 6.98 | 263 ± 2.20 |
| Pentyl hexanoate | 1288 | 1286 | 18 ± 1.09 | 25 ± 1.41 | 124 ± 10.07 | 328 ± 14.49 | 58 ± 0.60 | 139 ± 3.99 | 82 ± 2.19 | 136 ± 1.51 |
| Butyl 2-methylbutanoate | 1043 | 1041 | 4 ± 0.11 | 5 ± 0.25 | 33 ± 3.26 | 44 ± 2.68 | 110 ± 0.44 | 525 ± 10.75 | 678 ± 13.65 | 265 ± 3.19 |
| Propyl octanoate | 1291 | 1290 | 12 ± 1.59 | 5 ± 2.99 | 17 ± 12.70 | 10 ± 4.06 | 8 ± 0.18 | 25 ± 0.83 | 33 ± 1.08 | 46 ± 0.58 |
| Butyl heptanoate | 1289 | 1289 | n.d. | n.d. | n.d. | n.d. | 25 ± 0.23 | 51 ± 1.43 | 32 ± 0.71 | 17 ± 0.51 |
| Heptyl 2-methylbutanoate | 1338 | 1336 | 2 ± 0.54 | 5 ± 0.50 | 14 ± 7.98 | 21 ± 5.27 | 9 ± 0.20 | 43 ± 2.86 | 32 ± 1.96 | 17 ± 0.25 |
| Hexyl hexanoate | 1388 | 1385 | 238 ± 15.77 | 320 ± 27.28 | 1748 ± 15.17 | 4229 ± 20.20 | 924 ± 14.71 | 2066 ± 4.86 | 1141 ± 28.35 | 2772 ± 14.35 |
| 2-Methylbutyl octanoate | 1453 | 1453 | 6 ± 0.23 | 8 ± 0.17 | 64 ± 5.35 | 191 ± 15.80 | 23 ± 0.21 | 92 ± 1.76 | 55 ± 1.48 | 81 ± 1.22 |
| Pentyl octanoate | 1485 | 1490 | 5 ± 0.29 | 9 ± 0.13 | 44 ± 3.82 | 114 ± 5.74 | n.d. | n.d. | n.d. | n.d. |
| Hexyl octanoate | 1585 | 1584 | 10 ± 0.28 | 19 ± 0.64 | 102 ± 8.04 | 249 ± 15.86 | 37 ± 0.22 | 69 ± 0.51 | 29 ± 0.49 | 83 ± 1.21 |
| ***Acetates*** | | | | | | | | | | |
| Butyl acetate | 812 | 812 | 18 ± 0.66 | 69 ± 4.63 | 197 ± 12.68 | 505 ± 29.43 | 190 ± 1.75 | 594 ± 6.71 | 427 ± 4.27 | 481 ± 3.22 |
| 2-Methylbutyl acetate | 878 | 880 | 161 ± 6.15 | 155 ± 7.93 | 716 ± 4.91 | 1979 ± 9.11 | 1254 ± 8.50 | 2251 ± 16.30 | 1827 ± 24.71 | 1640 ± 10.71 |
| Pentyl acetate | 911 | 912 | 29 ± 0.30 | 61 ± 4.32 | 169 ± 12.79 | 222 ± 11.10 | 242 ± 2.15 | 304 ± 2.70 | 162 ± 2.79 | 159 ± 1.64 |
| Hexyl acetate | 1010 | 1014 | 1047 ± 3.63 | 2064 ± 10.84 | 3694 ± 2.51 | 4220 ± 15.55 | 4146 ± 19.51 | 3953 ± 27.17 | 2028 ± 28.04 | 3110 ± 17.51 |
| (Z)-2-Hexen-1-ol acetate | 1012 | 1014 | 1876 ± 5.37 | 3238 ± 16.37 | 5217 ± 3.85 | 4820 ± 14.05 | 4487 ± 27.52 | 3505 ± 13.37 | 1329 ± 16.32 | 1802 ± 10.26 |
| ***Aldehydes*** | | | | | | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (E)-2-Butenal | 653 | 648 | 111 ± 7.35 | 172 ± 16.25 | 331 ± 4.41 | 201 ± 6.68 | 147 ± 0.92 | 80 ± 0.31 | 37 ± 1.03 | 43 ± 0.44 |
| n-Pentanal | 697 | 699 | 181 ± 4.21 | 327 ± 7.87 | 630 ± 5.57 | 540 ± 16.68 | 349 ± 1.99 | 222 ± 3.81 | 133 ± 2.79 | 150 ± 2.12 |
| (E)-2-Pentenal | 757 | 754 | 142 ± 5.93 | 223 ± 12.66 | 476 ± 4.74 | 387 ± 3.07 | 195 ± 1.48 | 104 ± 0.13 | 70 ± 1.06 | 97 ± 0.84 |
| 3-Methyl-2-butenal | 785 | 783 | 22 ± 1.50 | 48 ± 2.81 | 83 ± 9.21 | 84 ± 0.34 | 45 ± 0.32 | 20 ± 0.09 | 13 ± 0.25 | 26 ± 0.20 |
| Hexanal | 801 | 801 | 1547 ± 5.27 | 1409 ± 6.65 | 3588 ± 25.46 | 3837 ± 13.24 | 637 ± 3.98 | 1134 ± 8.29 | 852 ± 7.14 | 2709 ± 25.83 |
| (E)-2-Hexenal | 857 | 854 | 2746 ± 8.07 | 3933 ± 23.42 | 7766 ± 5.14 | 8363 ± 4.19 | 3224 ± 31.91 | 4289 ± 35.68 | 2965 ± 21.97 | 4454 ± 12.97 |
| (E,E)-2,4-Hexadienal | 913 | 910 | 38 ± 1.35 | 53 ± 3.73 | 91 ± 7.32 | 71 ± 4.97 | 68 ± 0.60 | 88 ± 1.04 | 58 ± 0.08 | 76 ± 0.41 |
| (E)-2-Heptenal | 961 | 957 | 488 ± 2.94 | 872 ± 5.94 | 1942 ± 2.03 | 1496 ± 3.71 | 950 ± 11.46 | 560 ± 0.88 | 354 ± 8.14 | 490 ± 6.77 |
| (E,E) - 2,4 Heptadienal | 913 | 960 | 55 ± 2.39 | 168 ± 5.16 | 367 ± 3.46 | 578 ± 2.26 | n.d. | n.d. | n.d. | n.d. |
| (E)-2-Octenal | 1064 | 1060 | 13 ± 0.90 | 22 ± 1.18 | 51 ± 6.28 | 31 ± 2.29 | 186 ± 2.26 | 111 ± 0.45 | 90 ± 2.11 | 136 ± 2.18 |
| Nonanal | 1108 | 1104 | 4 ± 0.18 | 7 ± 0.12 | 15 ± 1.46 | 18 ± 0.45 | n.d. | n.d. | n.d. | n.d. |
| Decanal | 1207 | 1209 | 11 ± 0.21 | 11 ± 0.23 | 20 ± 1.61 | 16 ± 0.61 | n.d. | n.d. | n.d. | n.d. |
| 2(E)-Decenal | 1271 | 1262 | 11 ± 1.39 | 28 ± 1.81 | 73 ± 7.58 | 54 ± 2.27 | 32 ± 0.64 | 22± 0.31 | 17 ± 0.47 | 22 ± 0.34 |
| ***Ketons*** | | | | | | | | | | |
| 2-Butanone | 604 | 601 | 10 ± 0.32 | 12 ± 0.81 | 15 ± 1.49 | 18 ± 0.78 | 171 ± 0.66 | 164 ± 1.44 | 117 ± 0.54 | 141 ± 0.14 |
| 2-Pentanone | 688 | 685 | n.d. | n.d. | n.d. | n.d. | 46 ± 1.19 | 37 ± 2.21 | 29 ± 1.03 | 39 ± 0.74 |
| 3-Pentanone | 698 | 700 | n.d. | n.d. | n.d. | n.d. | 138 ± 0.23 | 141 ± 1.18 | 106 ± 0.39 | 137 ± 0.45 |
| 1-Penten-3-one | 688 | 680 | 159 ± 4.14 | 241 ± 10.93 | 424 ± 4.72 | 374 ± 8.19 | n.d. | n.d. | n.d. | n.d. |
| Acetoin (3-Hydroxy-2-butanone) | 716 | 721 | 4 ± 0.56 | 11 ± 0.62 | 14 ± 1.67 | 12 ± 2.25 | 9 ± 0.17 | 12 ± 0.07 | 9 ± 0.12 | 18 ± 0.16 |
| 1-Octen-3-one[t] | 979 | 975 | 244 ± 15.21 | 444 ± 3.17 | 845 ± 8.93 | 771 ± 3.05 | n.d. | n.d. | n.d. | n.d. |
| 6-Methyl-5-hepten-2-one | 989 | 986 | 378 ± 14.05 | 620 ± 24.73 | 1364 ± 14.82 | 1986 ± 9.60 | 1024 ± 10.52 | 613 ± 5.29 | 573 ± 8.08 | 1282 ± 14.49 |
| ***Terpenes*** | | | | | | | | | | |
| Limonene | 1036 | 1033 | 7 ± 0.98 | 12 ± 1.37 | 31 ± 5.45 | 56 ± 7.09 | n.d. | n.d. | n.d. | n.d. |
| (E)-Linalool oxid (furanoid) | 1087 | 1088 | 13 ± 12.61 | 13 ± 8.84 | 27 ± 4.37 | 22 ± 5.75 | 11 ± 0.29 | 13 ± 0.26 | 10 ± 0.27 | 20 ± 0.21 |
| Linalool | 1105 | 1100 | 10 ± 2.61 | 11 ± 3.46 | 39 ± 4.37 | 13 ± 2.74 | n.d. | n.d. | n.d. | n.d. |
| Neral (cis-Citral) | 1275 | 1248 | 4 ± 0.33 | 5 ± 0.02 | 22 ± 0.85 | 32 ± 0.92 | n.d. | n.d. | n.d. | n.d. |
| Geranial (α-Citral) | 1283 | 1271 | 10 ± 0.87 | 14± 1.16 | 37 ± 4.54 | 64 ± 0.43 | 24 ± 0.12 | 16 ± 0.08 | 14 ± 0.09 | 30 ± 0.26 |
| Geranyl acetone | 1465 | 1451 | 13 ± 3.16 | 9 ± 0.60 | 73 ± 3.82 | 216 ± 5.75 | 37 ± 0.30 | 22 ± 0.11 | 13 ± 0.09 | 21 ± 0.32 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| β-Farnesene | 1462 | 1449 | 3 ± 0.31 | 3 ± 0.27 | 25 ± 2.81 | 83 ± 3.55 | n.d. | n.d. | n.d. | n.d. |
| Bisabolene[t] | 1470 | 1514 | 7 ± 0.29 | 5 ± 0.13 | 50 ± 4.64 | 129 ± 6.52 | n.d. | n.d. | n.d. | n.d. |
| Zingiberene | 1506 | 1493 | 8 ± 0.66 | 4 ± 0.30 | 39 ± 3.06 | 102 ± 6.09 | n.d. | n.d. | n.d. | n.d. |
| α-Bergamotene[t] | 1501 | 1431 | 48 ± 8.50 | 43 ± 1.86 | 483 ± 7.50 | 1081 ± 13.15 | 18 ± 0.39 | 34 ± 0.66 | 26 ± 0.72 | 102 ± 1.99 |
| α- Caryophyllene[t] | 1532 | 1457 | 15 ± 0.95 | 12 ± 0.33 | 94 ± 6.87 | 254 ± 15.20 | n.d. | n.d. | n.d. | n.d. |
| α-Farnesene | 1520 | 1509 | 1245 ± 9.76 | 991 ± 20.04 | 6681 ± 5.45 | 13124 ± 5.61 | 1928 ± 26.59 | 2830 ± 53.72 | 1856 ± 32.36 | 6861 ± 8.07 |

$RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; t - tentatively identified - coelution by 1-octen-3-ol and 1-octen-3-one, not clearly detected on this column; n.d. – not detectable or below limit of detection; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org)

Due to the huge amount of volatile compounds identified in the peel, multivariate statistical data analysis (PCA) was carried out, to give a correlation of the different ripening stages of the two successive harvest years on the flavor profile (Figure 40). As presented in Figure 40, there is a significant difference between the two harvest years and the different picking dates. The most surprising aspect of the data is that the picking dates 2017 P1, 2018 P1 and 2018 P2 are in the same quadrant. The correlation between 2017 P2 and 2017 P4 is also interesting because of the climate change after the 2017 P3, and that not the 2017 P2 is similar to 2017 P3. This correlation within the PCA is an interesting outcome, because it shows that the harvest year has not an immense impact on the volatile composition, more important is the picking date with the different ripening stages and naturally, the climate in each year.

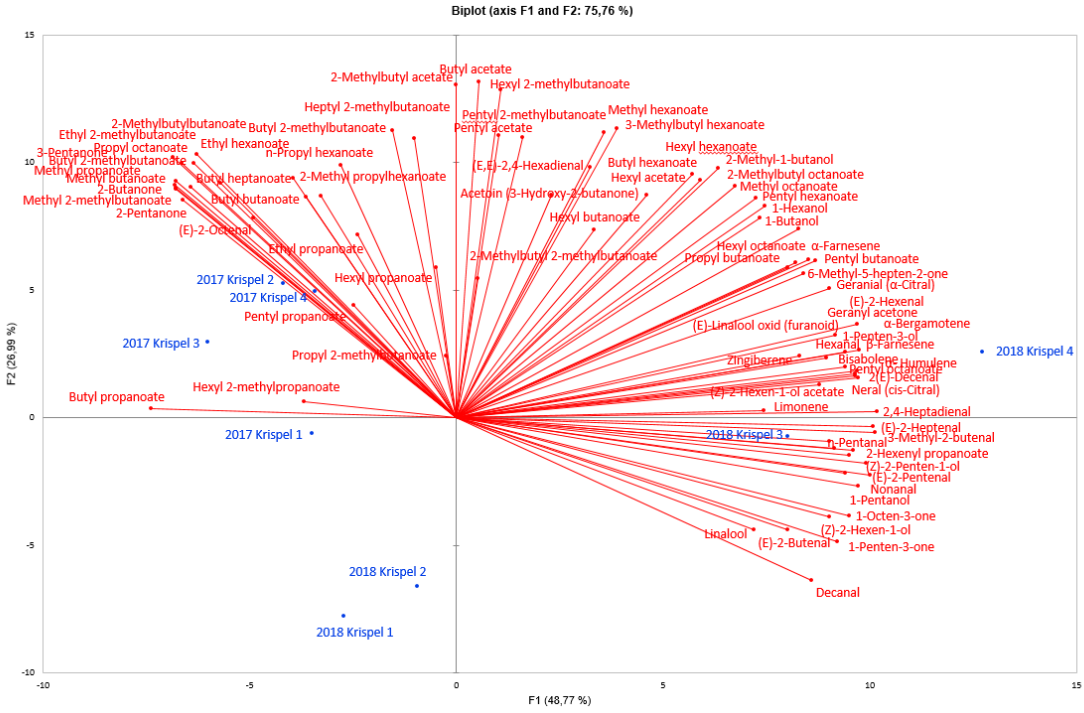


**Figure 40: Harvest 2017 and 2018 -** Biplot scores and factor loadings obtained by PCA based on the HS-SPME-GC-MS results from the volatile compounds in apple peel samples of the harvest 2017 and 2018 with the different picking dates (2017 Krispel 1, 2017 Krispel 2, 2017 Krispel 3, 2017 Krispel 4, 2018 Krispel 1, 2018 Krispel 2, 2018 Krispel 3, 2018 Krispel 4).

Until now, no sensory evaluation of volatile compounds in Austrian heritage apple varieties from the peel has been conducted. Therefore, the objectives of this part of the work was to identify the odor-active volatiles in the peel of the apple variety Ilzer Rose of two picking dates (P1 and P4). The evaluation of the odor-active volatiles in the apple peel was performed with five trained panelists. Twenty-one odor-active compounds were determined and fourteen of them were identified by comparison of chromatographic, mass spectrometric, and sensory data (Table 16). The odor-active compounds with the highest nasal impact frequency (NIF) are ethyl butanoate, (E)-hexenal, and heptanal. The difference between the picking dates is clear, the P4 showed higher intensity for most of the odor-active compounds than P1. Also, seven compounds were determined but were not identified. These compounds showed a fruity, sweet, apple odor.

**Table 16:** Aroma compounds of the Ilzer Rose peel samples (picking date 1 and 4) with the retention indices (RI) and the sensory data

| Aroma compound | RI $[HP5]_{exp}$ | RI $[HP5]_{ref}$ | NIF [%] P1 | NIF [%] P4 | Odor Quality$^{lit}$ | Odor Description$^{exp}$ |
|---|---|---|---|---|---|---|
| Ethyl butanoate | 807 | 804 | 75 | 70 | apple | apple, fruity, sweet |
| n.i. | 830 | | 17 | 50 | | fruity, sweet |
| (E)-2-Hexenal | 857 | 854 | 75 | 80 | green, banana, fresh, leavy, fruity | apple, green |
| 3-Methyl butylacetate | 872 | 876 | 17 | 30 | sweet, fruity, banana, ripe | sweet, fruity |
| Ethyl pentanoate | 893 | 902 | 8 | 30 | sweet, fruity, green | sweet, fruity |
| Heptanal | 905 | 903 | 42 | 70 | fat, citrus, rancid | musty, foul-smelling |
| 1-Octen-3-one* | 981 | 975 | 75 | 70 | herbal, mushroom, earthy, musty | mushroom |
| 1-Octen-3-ol* | 982 | 977 | 67 | 80 | mushroom, earthy, green, fungal | fungal, earthy |
| n.i. | 989 | | 67 | 60 | | fungal, mushroom |
| Methyl hexanoate | 994 | 1000 | 42 | 30 | fruit, fresh, sweet | sweet, fruity |
| Ethyl hexanoate | 1005 | 1002 | 67 | 40 | sweet, fruity, pineapple, green, banana | citrus, sweet, fruity |
| (E)-2-Octenal | 1054 | 1060 | 8 | 20 | green, nut, fat | leafy, green |
| n.i. | 1102 | | 17 | 30 | | fruity, apple |
| n.i. | 1105 | | 8 | 30 | | fruit, sweet |
| n.i. | 1130 | | 25 | 20 | | fruity, citrus |
| Pentyl 2-methylbutyrate | 1141 | 1140 | 33 | 30 | apple | fruity, apple, sweet |
| n.i. | 1167 | | 25 | 40 | | sweet, fruity |
| (E)-Linalool oxid (furanoid) | 1172 | 1172 | 8 | 20 | flower | floral notes, fruity |
| Hexyl butanoate | 1178 | 1185 | 8 | 20 | apple peel | apple, musty |
| n.i. | 1270 | | 25 | 10 | | flower |
| Geranial (α-Citral) | 1278 | 1277 | 17 | 10 | lemon, mint | citrus, fruity |

$RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; $RI_{ref}$ – reference RI obtained from authentic standard compounds or databases (https://webbook.nist.gov/chemistry/ and http://www.flavornet.org); NIF – nasal impact frequency in %; lit – odor quality obtained from databases (http://www.thegoodscentscompany.com/); exp – odor description as collected in the experiments;* t - tentatively identified - coelution by 1-octen-3-ol and 1-octen-3-one, not clearly detected on this column;

The aroma profile of the peel from two picking dates (P1 and P4) of the apple variety Ilzer Rose based on GCO results are presented in Figure 41. During the ripening process, the apple fruit changes with respect to the volatile profile and also within the odor profile. The impact of the esters increased during ripening, and the lipid oxidation product 1-octen-3-one decreased. However, these findings did not fully explain the impact of the ripeness on the odor of the peel. To develop a full picture of the impact, the odor activity values need to be evaluated.

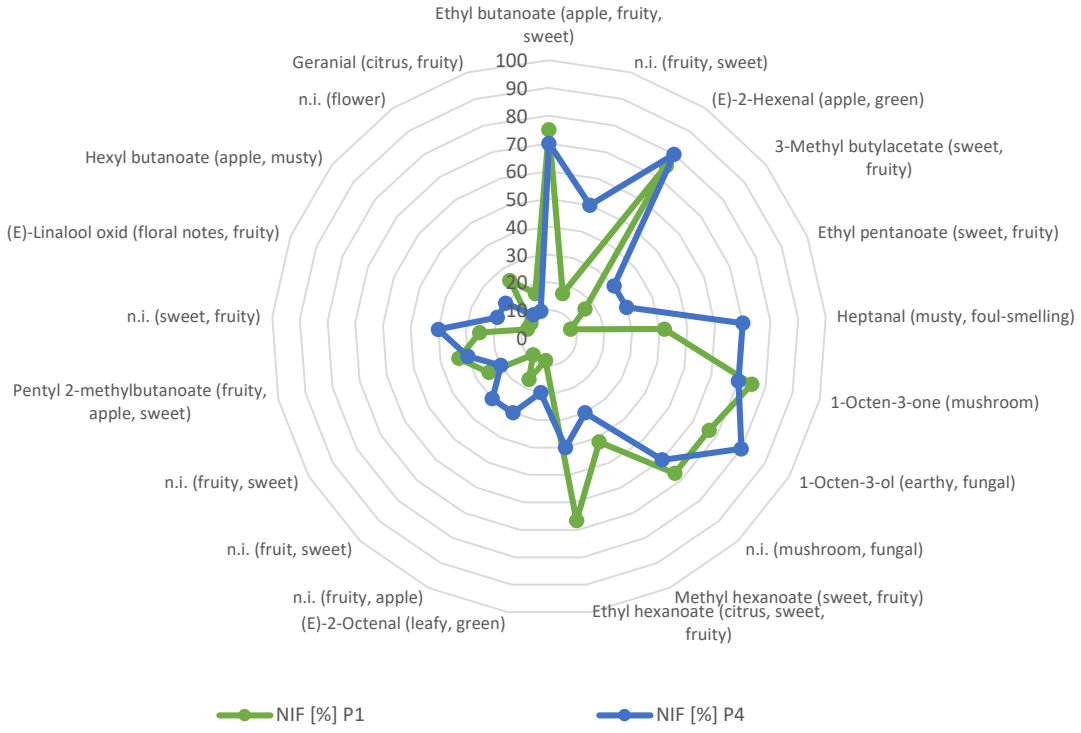


Figure 41: Aroma profile of the two Ilzer Rose peel samples of the first (P1) and fourth (P4) picking dates.

The odor thresholds of interesting (not documented by now) apple compounds were determined by the sensory expert panel. The chosen apple flavor compounds did not have a documented odor threshold in water and for the experiments was water the selected matrix. All experiments were performed in duplicate and the best-estimate threshold (BET) was calculated as the geometric mean value of the last missed concentration and the concentration of the next level. Table 17 lists the determined odor threshold values of the selected compounds together with each odor characterization.

Table 17: Odor thresholds of selected apple flavor compounds together with the characterizations of the odors, odor threshold values are given in terms of the BET value, n= 12 panelists, duplicate determination

| Aroma compound | Odor threshold [$\mu gL^{-1}$] in water | Odor characterization* |
|---|---|---|
| Hexyl 2-methylbutenoate | 4.2 | green |
| Butyl 3-methyl butanoate | 11 | fruity, apple, peach, pineapple |
| 2-Methylpropyl propanoate | 9.3 | fruity |
| Propyl hexanoate | 5.5 | berry, fruity, petrol, pineapple, roasted garlic |
| Ethyl 2-butenoate | 46 | fermented, musty, sweet |

| | | |
|---|---|---|
| 2-Methylpropyl octanoate | 50 | fruity, green, floral |
| 5-hexenyl acetate | 7.9 | quince |
| 2-Methylpropyl butanoate | 15 | fruity, sweet, candy, berry, cherry, apple |
| 3-Methylbutyl propanoate | 2.1 | fruity, sweet, banana, pineapple |
| Octyl 2-methyl butanoate | 5.1 | waxy, fruity, green, musty |

*Flavor & Extract Manufacturers Assoc. (https://www.femaflavor.org) and The Good Scent Company (http://www.thegoodscentscompany.com/search2.html)

In the next step, the odor activity values (OAVs) were calculated by dividing the concentrations of the individual substances from the GC-MS measurements (Table 15) by their odor thresholds (presented in Table 18).

As apple fruits mainly consist of water, odor thresholds determined in water were used. Based on OAV-concept, 35 substances should be odor-active (OAVs ≥ 1). According to the results of GC-O measurements, the OAV results must be interpreted with caution. However, there are odor-active compounds, such as (E)-hexenal, butyl 2-methylbutanoate, hexyl propanoate, hexyl 2-methylbutanoate, butyl 2-methylbutanoate, pentyl-2-methyl butanoate, propyl 2-methyl butanoate, hexyl butanoate, (E)-octenal, 2-methylbutyl acetate, pentyl acetate, and hexyl acetate, with a determined OAVs ≥ 1. These compounds have a fruity-apple-green-sweet description and are important for the odor of the Ilzer Rose. Nevertheless, the specific Ilzer Rose flavor with the floral notes is not explainable with these results. Due to the fact, that there are several compounds which are impossible to identify, further research should be undertaken to investigate the odor specific compounds for the apple variety Ilzer Rose.

Table 18: Calculated odor activity value (OAV) of volatile compounds of the apple peel with their odor thresholds in water

| | | OAV | | | |
|---|---|---|---|---|---|
| **Volatile Compounds** | **Odor Threshold[lit] (µg/kg)** | **2018 P1** | **2018 P2** | **2018 P3** | **2018 P4** |
| 1-Butanol | 500.0 | < 1 | < 1 | < 1 | < 1 |
| 1-Penten-3-ol | 400.0 | < 1 | < 1 | < 1 | < 1 |
| 2-Methyl-1-butanol | 300.0 | < 1 | < 1 | < 1 | < 1 |
| 1-Pentanol | 4000.0 | < 1 | < 1 | < 1 | < 1 |
| (Z)-2-Hexen-1-ol | 70.0 | 8 | 12 | 19 | 19 |
| 1-Hexanol | 2500.0 | < 1 | < 1 | < 1 | < 1 |
| Ethyl propanoate | 10.0 | < 1 | < 1 | < 1 | < 1 |
| Ethyl 2-methylbutanoate | 0.3 | < 1 | < 1 | < 1 | < 1 |
| Ethyl hexanoate | 1.0 | < 1 | < 1 | < 1 | < 1 |
| Butyl 2-methylbutanoate | 17.0 | 1 | 1 | 13 | 13 |
| Methyl butanoate | 76.0 | < 1 | < 1 | < 1 | < 1 |
| Methyl 2-methylbutanoate | 0.3 | < 1 | < 1 | < 1 | < 1 |
| Propyl butanoate | 124.0 | < 1 | < 1 | < 1 | < 1 |
| Butyl propanoate | 200.0 | < 1 | < 1 | < 1 | < 1 |
| Methyl hexanoate | 84.0 | < 1 | < 1 | < 1 | < 1 |
| Propyl 2-methylbutanoate | 0.2 | 74 | 124 | 314 | 314 |
| Butyl butanoate | 100.0 | 1 | 2 | 4 | 4 |
| Pentyl butanoate | 210.0 | < 1 | < 1 | < 1 | < 1 |
| n-Propyl hexanoate | 5.5* | 3 | 4 | 9 | 9 |
| Hexyl propanoate | 8.0 | 4 | 7 | 24 | 24 |
| 2-Methylbutyl 2-methylbutanoate | 75.0 | 1 | 1 | 4 | 4 |
| Methyl octanoate | 200.0 | < 1 | < 1 | < 1 | < 1 |
| Pentyl 2-methylbutanoate | 43.0 | 0 | 0 | 3 | 3 |
| Hexyl 2-methylpropanoate | 13.0 | 0 | 5 | 3 | 3 |
| Hexyl butanoate | 250.0 | 1 | 1 | 3 | 3 |
| Butyl hexanoate | 700.0 | < 1 | < 1 | < 1 | < 1 |
| Hexyl 2-methylbutanoate | 4.2* | 101 | 84 | 817 | 817 |
| 3-Methylbutyl hexanoate | 15.0 | 1 | 1 | 8 | 8 |
| Pentyl hexanoate | 200.0 | < 1 | < 1 | < 1 | < 1 |
| Butyl 2-methylbutanoate | 0.02 | 208 | 261 | 1674 | 1674 |
| Propyl octanoate | 8.0 | 2 | 1 | 2 | 2 |
| Hexyl hexanoate | 820.0 | 0 | 0 | 2 | 2 |
| Butyl acetate | 66.0 | 0 | 1 | 3 | 3 |
| 2-Methylbutyl acetate | 5.0 | 32 | 31 | 143 | 143 |
| Pentyl acetate | 5.0 | 6 | 12 | 34 | 34 |
| Hexyl acetate | 2.0 | 524 | 1032 | 1847 | 1847 |
| (Z)-2-Hexen-1-ol acetate | 7.0 | 268 | 463 | 745 | 745 |
| (E)-2-Butenal | 0.2 | 554 | 859 | 1654 | 1654 |
| n-Pentanal | 200.0 | 1 | 2 | 3 | 3 |
| (E)-2-Pentenal | 1500.0 | < 1 | < 1 | < 1 | < 1 |
| Hexanal | 5.0 | 309 | 282 | 718 | 718 |
| (E)-2-Hexenal | 17.0 | 162 | 231 | 457 | 457 |

| | | | | | |
|---|---|---|---|---|---|
| (E,E)-2,4-Hexadienal | 60.0 | 1 | 1 | 2 | 2 |
| (E)-2-Heptenal | 13.0 | 38 | 67 | 149 | 149 |
| (E)-2-Octenal | 3.0 | 4 | 7 | 17 | 17 |
| Nonanal | 1.0 | 4 | 7 | 15 | 15 |
| Decanal | 2.0 | 5 | 5 | 10 | 10 |
| 2(E)-Decenal | 0.4 | 28 | 71 | 182 | 182 |
| 2-Butanone | 50000.0 | < 1 | < 1 | < 1 | < 1 |
| 2-Pentanone | 70000.0 | < 1 | < 1 | < 1 | < 1 |
| 3-Pentanone | 1.5 | < 1 | < 1 | < 1 | < 1 |
| Acetoin (3-Hydroxy-2-butanone) | 800.0 | < 1 | < 1 | < 1 | < 1 |
| 1-Octen-3-one* | 0.005 | n.c. | n.c. | n.c. | n.c. |
| 6-Methyl-5-hepten-2-one | 68.0 | 6 | 9 | 20 | 20 |
| Limonene | 10.0 | 1 | 1 | 3 | 3 |
| Linalool oxide | 6 | 2 | 2 | 6 | 6 |
| Neral (cis-Citral) | 30.0 | < 1 | < 1 | < 1 | < 1 |
| Geranial (α-Citral) | 32.0 | < 1 | < 1 | < 1 | < 1 |
| Geranyl acetone | 60.0 | < 1 | < 1 | < 1 | < 1 |
| β-Farnesene | 87.0 | < 1 | < 1 | < 1 | < 1 |
| α- Humulene | 64.0 | < 1 | < 1 | < 1 | < 1 |
| α-Farnesene | 87.0 | 14 | 11 | 77 | 77 |

lit – odor thresholds obtained from Leffingwell & Associates (https://www.leffingwell.com/odor.htm); *determined from the expert panel of the Graz University of Technology; * not calculated - coelution by 1-octen-3-ol and 1-octen-3-one, not clearly detected on this column;

## 4.5. Apple peel of the variety Ilzer Rose as terpene factory

More than 300 volatile compounds have been reported in apple flavor, the important flavor compound groups are esters, alcohols, and aldehydes. In addition, a few terpenes, have been identified so far in apples (see section 4.4) (Rapparini, Baraldi, & Facini, 2001). However, for the flavor of apple mono- and sesquiterpenes have not been regarded to be high relevance so far. As mentioned in 2.2.4 the terpenes are generally formed either via the mevalonic (MVA) pathway (sesquiterpenes) of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (monoterpenes). One of the more significant findings to emerge from this study is that a huge amount of terpenes is first reported in the apple peel of the heritage apple variety Ilzer Rose. In this section, the identification and the impact of ripening on the terpenoids in the peel of Ilzer Rose apples were determined using comprehensive GC×GC-MS.

This highly sophisticated analytical method provides a high separation capacity as well as enormous sensitivity. It is the first time that volatile compounds of apple peel samples were reported by using Comprehensive GC×GC-MS. A measurement with this system results in very complex chromatograms with more than 300 different compounds of each investigated apple peel sample. The chromatograms of the four different ripening stages of the harvest 2018 can be seen in Figure 42. Quantification was based on the use of 2-octanol as the internal standard.

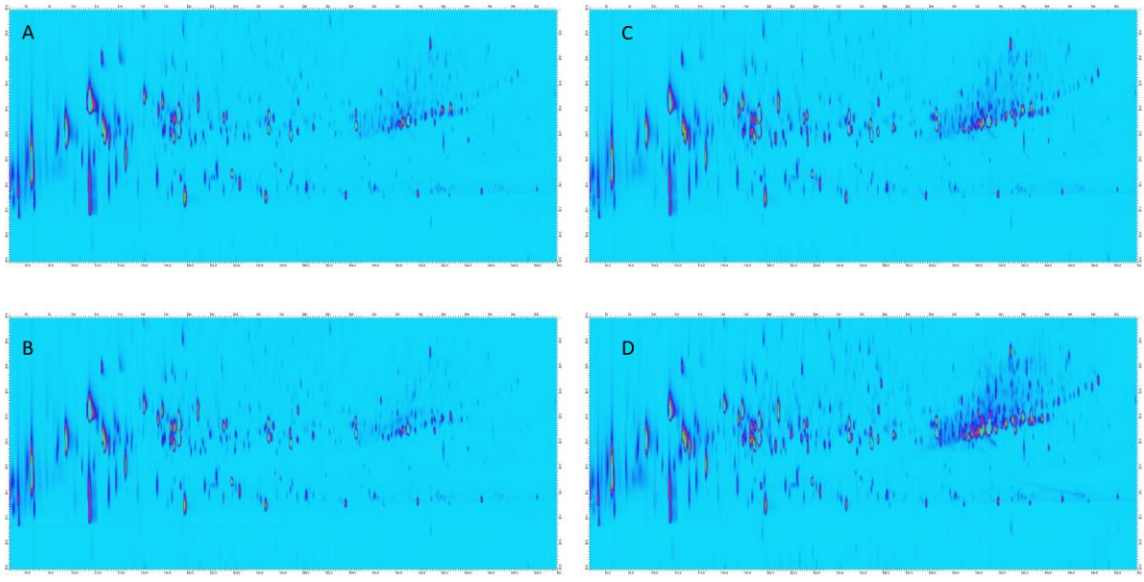


**Figure 42: Comprehensive GCxGC-MS chromatograms of the four different ripening stages of the peel of Ilzer Rose apples from the harvest 2018;** A = 2018 Krispel 1, B = 2018 Krispel 2, C = 2018 Krispel 3, D = 2018 Krispel 4; Retention times in the first dimension (x-axis) are given in minutes; retention times in the second dimension (y-axis) are given in seconds.

As seen in Figure 42, several volatile compounds are increased in the range of 34 and 46 minutes of retention time (first dimension) in the overripe apple peel samples (D). This segment is shown in more detail in Figure 43. The compounds such as (1) hexyl hexanoate, (2) 2-hexenyl hexanoate, (3) α-ylangene, (4) (E)-geranyl acetone, (5) 2-pentyl octanoate, (6) cis-caryophyllene, (7) cis-thujopsene, (8) cis-ß-farnesene, (9) cis-α-bisabolene, (10) (+)-valencene, (11) ß-guaiene, (12) α-

curcumene, (13) humulene, (14) cis-α-bergamotene, (15) α-farnesene, (16) caryophyllene oxide, (17) (E)-γ-bisabolene, (18) hexyl octanoate, (19) cis-sesquisabinene hydrate, (20) trans-longipinocarveol, (21) cadalene, (22) ß –atlantol were identified in this segment. The high amounts of α-farnesene, hexyl hexanoate, 2-hexenyl hexanoate, and α-bergamotene are not surprising, because it was reported before that terpenes, esters, and ketones increased during ripening. Monoterpenes such as limonene, citral, and linalool are formed by the methylerythritol phosphate (MEP) pathway and the MEP takes place in the plastid of the plant (Tholl, 2006). Monoterpenes are generally volatile and play an important role as components in the flavor industry (Dong, Jongedijk, Bouwmeester, & Van Der Krol, 2016). Sesquiterpenes such as β- caryophyllene, α-caryophyllene, curcurmene, bisabolene, cadalene are formed by the mevalonate pathway and this pathway takes place in the cytosol of the plant (Tholl, 2006). Terpenoids are associated with primary metabolism (such as carotenoid pigments) others are typical plant secondary metabolites (Aharoni et al., 2006). The production of terpenes is depending on the activity of terpene synthases, and enzymes that use geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate as substrate. (+)-Valencene is a well-known sesquiterpene which is mostly associated with citrus fruit, but the compound has been also detected in celery, mango, and olives (Sharon-Asa et al., 2003). Thus, it is not a surprise that this compound is also found in apple peels in this study. α-Caryophyllene (humulene) is the most characteristic terpene of hops and β- caryophyllene is the primary sesquiterpene contributing to the spiciness of black pepper (Hartsel, Eades, Hickory, & Makriyannis, 2016) and is also a common component of floral scent (Chen et al., 2003). The compound (+)-thujopsene is found in flowers and is a very important component of conifers. Linalool has been identified as the major floral terpene in other apple varieties (Rapparini et al., 2001), but in this study this compound is found in low concentrations only.

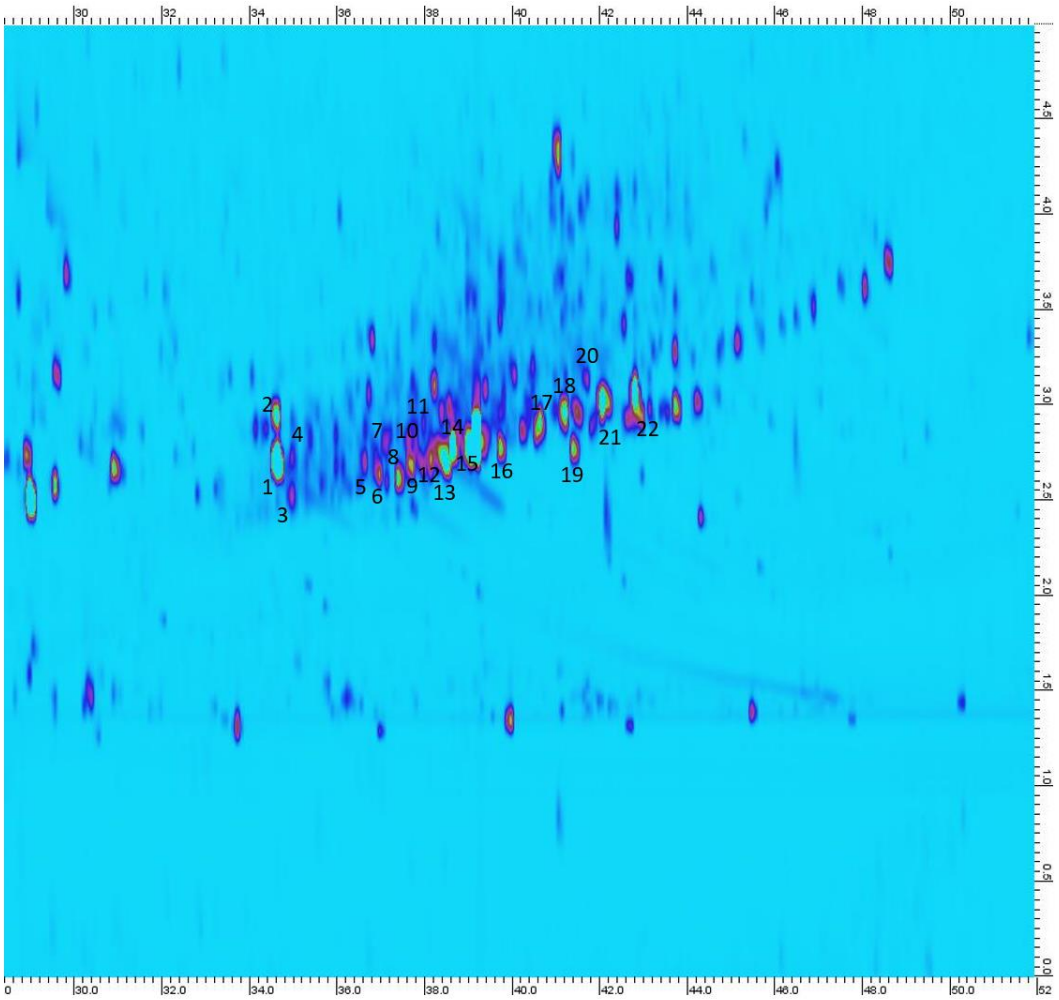


**Figure 43: Harvest 2018 - Segment of comprehensive GC×GC-MS chromatogram of (D) peel of overripe Ilzer Rose apple fruit;** Compound numbers: (1) hexyl hexanoate, (2) 2-hexenyl hexanoate, (3) α-ylangene, (4) (E)-geranyl acetone, (5) 2-pentyl octanoate, (6) cis-caryophyllene, (7) cis-thujopsene, (8) cis-ß-farnesene, (9) cis-α-bisabolene, (10) (+)-valencene, (11) ß-guaiene, (12) α-curcumene, (13) α -caryophyllene , (14) cis-α-bergamotene, (15) α-farnesene, (16) caryophyllene oxide, (17) (E)-γ-bisabolene, (18) hexyl octanoate, (19) cis-sesquisabinene hydrate, (20) trans- longipinocarveol, (21) cadalene, (22) ß -atlantol; Retention times in the first dimension (x-axis) are given in minutes; retention times in the second dimension (y-axis) are given in seconds.

The biplot (Figure 44) aids to visualize the correlations in the comprehensive GC×GC-MS data of the apple peel concentrations (60 volatile compounds are presented with the highest amounts) of volatile profiles each ripening stage of the apple variety Ilzer Rose. The biplot as results of the PCA analysis shows that the samples from fourth picking date (P4 = IL18 E4), located in quadrant IV, correlate with most of the terpenes which is in agreement with recently published results of studies on the ripening process (Cárdenas-Pérez et al., 2017, Giné-Bordonaba et al., 2019a). Samples of picking date three (P3 = IL 18 E3), located in quadrant III, resulted in the highest correlation level of a few esters such as hexyl 2-methyl butanoate, (E)-2-hexenyl hexanoate, 2-hexenyl propanoate, 2-methylbutyl 2-methyl butanoate, and pentyl 2-methyl butanoate. Volatile compounds that highly correlate with sample from the second picking date (P2 = IL 18 E2), located in quadrant II, are (*E*)-2-hexenal, hexanal, (*E*)-2-hexenyl butanoate, ethyl octanoate, (*E*)-hex-4-en-1-yl butanoate, (*Z*)-2-hexenyl acetate, hexyl acetate, and (*E,E*)-2,4-heptadienal. Samples from picking date 1 (P1 = IL 18 E1), located in quadrant I, are not correlated with any volatile compound.

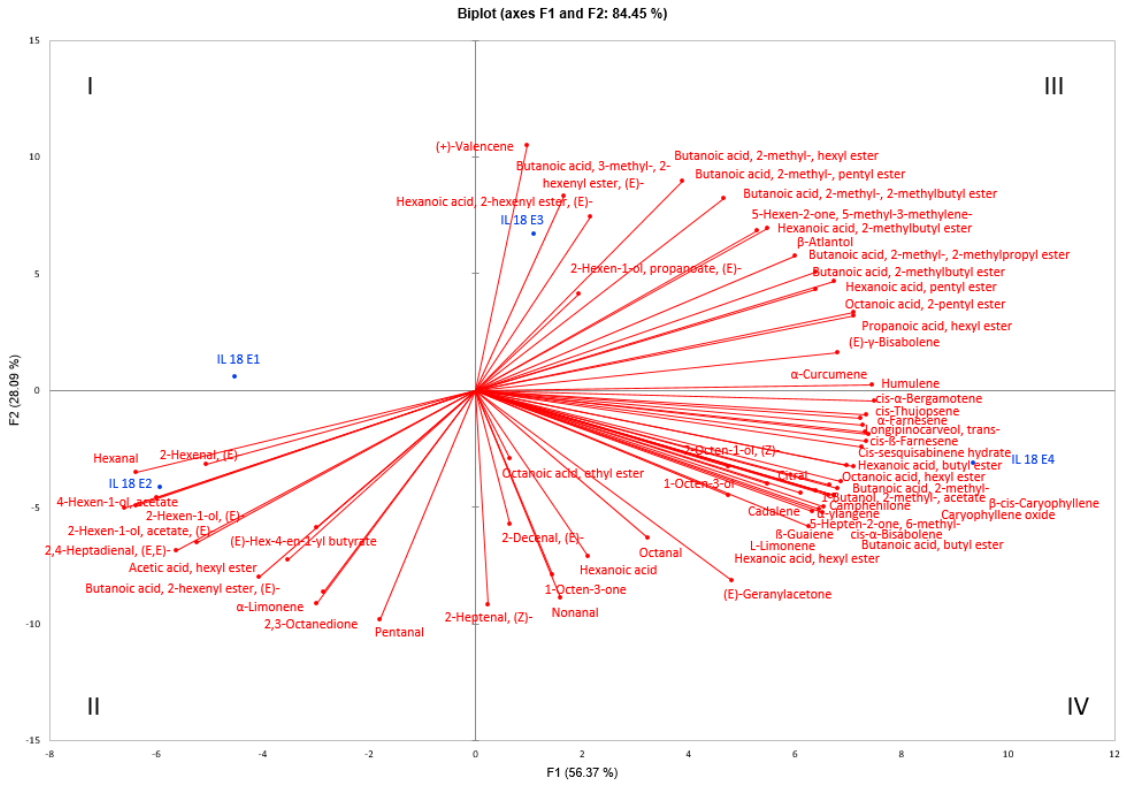


**Figure 44: Harvest 2018 –** Biplot scores and factor loadings obtained by PCA based on the comprehensive GC x GC-MS results from the volatile compounds in apple peel samples of the apple variety Ilzer Rose with the four different picking dates (E1, E2, E3, E4).

As shown in Table 19, the 60 volatile compounds with the highest concentration were presented of the four different ripening stages (harvest 2018). It is shown that mainly terpenes stood out for the differentiation between the four ripening stages since the levels of these compounds increased after each ripening stage. Picking date four resulted in the highest level of terpenes, such as α-farnesene, camphenilone, cis-α-bergamotene, α-caryophyllene, (E)-γ-bisabolene, cis-α-bisabolene, α-curcumene, cis-ß-farnesene, cis-sesquisabinene hydrate, cis-thujopsene, α-ylangene, α-limonene, (E)-geranyl acetone, ß-longipinene, trans-longipinocarveol, ß-guaiene, β-cis-caryophyllene, caryophyllene oxide, and cadalene. The majority of these terpenes could not be identified based on the current one-dimensional SPME-GC-MS method. To achieve a successful detection of these compounds, analysis using comprehensive GC×GC-MS was necessary.

**Table 19:** Volatile compounds identified by HS-SPME comprehensive GC×GC-MS analysis of the Ilzer Rose apple peel samples of four ripening stages; estimated quantities in $mgkg^{-1}$ +/i RSd (%) of the investigated apples calculated by comparison with internal standard (2-Octanol); TIC total ion chromatogram;

| Volatile Compounds | 2018 P1 | 2018 P2 | 2018 P3 | 2018 P4 | RI $[OV101/DB1]_{lit}$ | RI $[Rxi\text{-}1HT]_{exp}$ |
|---|---|---|---|---|---|---|
| α-Farnesene | 1.477 | 1.02 | 1.674 | 3.089 | 1518 | 1500 |
| (E)-2-Hexenal | 3.800 | 3.35 | 1.768 | 1.720 | 827 | 826 |
| (Z)-2-Hexen-1-ol acetate | 2.253 | 2.72 | 1.532 | 1.441 | 998 | 996 |
| Hexanal | 2.349 | 1.73 | 1.081 | 1.256 | 772 | 775 |
| Hexyl acetate | 1.431 | 1.79 | 1.066 | 1.159 | 1008 | 996 |
| Hexyl hexanoate | 0.521 | 0.63 | 0.543 | 1.025 | 1369 | 1371 |
| Hexyl 2-methylbutanoate | 0.709 | 0.59 | 1.046 | 0.817 | 1222 | 1223 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Butyl hexanoate | 0.478 | 0.513 | 0.54 | 0.745 | 1176 | 1176 |
| 6-Methyl-5-hepten-2-one | 0.367 | 0.37 | 0.35 | 0.698 | 965 | 964 |
| Camphenilone | 0.205 | 0.176 | 0.197 | 0.694 | 1036 | 1032 |
| (E)-2-Heptenal | 0.504 | 0.716 | 0.496 | 0.647 | 927 | 930 |
| (Z)-2-Hexen-1-ol | 1.075 | 0.984 | 0.548 | 0.609 | 863 | 853 |
| 2-Methylbutyl acetate | 0.430 | 0.284 | 0.294 | 0.579 | 863 | 864 |
| cis-α-Bergamotene | 0.278 | 0.174 | 0.309 | 0.573 | 1411 | 1483 |
| α- Humulene | 0.191 | 0.096 | 0.256 | 0.545 | 1465 | 1479 |
| (E)-2-Hexenyl butanoate | 0.440 | 0.630 | 0.427 | 0.463 | 1177 | 1176 |
| (E)-γ-Bisabolene[t] | n.d. | n.d. | 0.151 | 0.307 | 1528 | 1562 |
| Nonanal | 0.188 | 0.317 | 0.196 | 0.301 | 1079 | 1083 |
| 1-Octen-3-one | 0.180 | 0.329 | 0.214 | 0.301 | 956 | 957 |
| Pentanal | 0.362 | 0.350 | 0.156 | 0.299 | 674 | 675 |
| Octanal | 0.125 | 0.274 | 0.194 | 0.282 | 982 | 982 |
| (E)-2-Hexenyl hexanoate | 0.116 | 0.262 | 0.782 | 0.274 | 1379 | 1371 |
| Butyl butanoate | 0.132 | 0.101 | 0.112 | 0.271 | 969 | 980 |
| 5-Methyl-3-methylene-5-Hexen-2-one | 0.059 | 0.045 | 0.294 | 0.213 | 874 | 874 |
| Pentyl hexanoate | 0.081 | 0.034 | 0.169 | 0.212 | 1271 | 1272 |
| cis-α-Bisabolene | 0.086 | 0.029 | 0.031 | 0.210 | 1476 | 1457 |
| α-Curcumene | 0.061 | 0.047 | 0.112 | 0.203 | 1469 | 1469 |
| cis-ß-Farnesene | 0.057 | 0.052 | 0.090 | 0.200 | 1449 | 1448 |
| 2-Methylbutanoic acid | 0.047 | 0.032 | 0.044 | 0.183 | 839 | 847 |
| Hexyl octanoate | 0.061 | 0.090 | 0.100 | 0.180 | 1555 | 1567 |
| 2-Methylpropyl 2-methylbutanoate | 0.078 | 0.047 | 0.158 | 0.168 | 1015 (HP5) | 1028 |
| Pentyl octanoate | 0.060 | 0.046 | 0.138 | 0.163 | 1468 | 1436 |
| 2-Methylbutyl hexanoate | 0.067 | 0.060 | 0.177 | 0.157 | 1236 | 1238 |
| 4-Hexen-1-ol acetate | 0.334 | 0.527 | 0.201 | 0.157 | n.d. | 988 |
| Cis-sesquisabinene hydrate | 0.041 | 0.053 | 0.075 | 0.153 | 1559 (HP5) | 1569 |
| Hexanoic acid | 0.134 | 0.110 | 0.084 | 0.141 | 973 | 970 |
| (E)-2-Hexenyl 3-methylbutanoate | 0.069 | 0.131 | 0.272 | 0.139 | 1245 (HP5) | 1221 |
| (E)-2-Hexenyl propanoate | 0.087 | 0.128 | 0.151 | 0.121 | 1088 | 1089 |
| 1-Octen-3-ol | 0.055 | 0.100 | 0.088 | 0.117 | 959 | 966 |
| cis-Thujopsene | 0.038 | 0.025 | 0.051 | 0.115 | 1441 | 1441 |
| 2(E)-Decenal | 0.056 | 0.133 | 0.090 | 0.107 | 1236 | 1238 |
| α-ylangene | 0.034 | 0.027 | 0.027 | 0.099 | 1382 | 1380 |
| 2-Methylbutyl 2-methylbutanoate | 0.058 | 0.000 | 0.108 | 0.094 | 1090 | 1091 |
| β-Atlantol[t] | 0.058 | 0.030 | 0.080 | 0.086 | 1608 (DB5) | 1640 |
| Hexyl propanoate | 0.035 | 0.048 | 0.075 | 0.086 | 1088 | 1089 |
| Neral (cis-Citral) | 0.035 | 0.041 | 0.036 | 0.085 | 1240 | 1245 |
| L-Limonene | 0.044 | 0.043 | 0.040 | 0.075 | 1014 | 1017 |
| (E)-Geranyl acetone | 0.045 | 0.055 | 0.039 | 0.075 | 1431 | 1431 |
| 2-Methylbutyl butanoate | 0.054 | 0.029 | 0.065 | 0.074 | 1047 | 1043 |
| Ethyl octanoate | 0.000 | 0.115 | 0.077 | 0.073 | 1180 | 1182 |
| Pentyl 2-methylbutanoate | 0.051 | 0.034 | 0.097 | 0.072 | 1123 | 1124 |
| (+)-Valencene | 0.089 | 0.039 | 0.135 | 0.064 | 1465 | 1460 |
| trans-Longipinocarveol[t] | 0.000 | 0.000 | 0.017 | 0.060 | 1618 (HP-5MS) | 1603 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ß-Guaiene | 0.030 | 0.029 | 0.027 | 0.050 | 1478 | 1464 |
| (E)-4-Hexenyl butanoate[t] | 0.040 | 0.071 | 0.046 | 0.047 | 1197 (HP-5MS) | 1170 |
| α-Limonene | 0.054 | 0.054 | 0.032 | 0.044 | 1020 | 1021 |
| (Z)-2-Octen-1-ol | 0.000 | 0.031 | 0.027 | 0.042 | 1055 | 1053 |
| (E,E)-2,4-Heptadienal | 0.058 | 0.107 | 0.038 | 0.042 | 984 | 971 |
| ß-Longipinene[t] | 0.033 | 0.057 | 0.021 | 0.041 | 1402 (ZB5) | 1407 |
| cis-Caryophyllene[t] | 0.000 | 0.000 | 0.000 | 0.035 | 1434 (SPB-1) | 1441 |
| Caryophyllene oxide | 0.000 | 0.000 | 0.000 | 0.033 | 1534 | 1554 |
| 2,3-Octanedione | 0.028 | 0.031 | 0.000 | 0.021 | 959 | 963 |
| Cadalene | 0.000 | 0.000 | 0.000 | 0.020 | 1651 | 1634 |

t - tentative identification; $RI_{exp}$ – retention index as determined in the experiments; the RIs were experimentally determined using the standard method involving retention time (tR) of n-alkanes, which were injected under the same chromatographic conditions; $RI_{ref}$ – reference RI obtained from authentic standard compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002 or database (https://webbook.nist.gov/chemistry/ )

# Conclusions

# 5. Summary and conclusion

The aim of the present research was to examine a characterization of the flavor of heritage apple varieties from Styria. The investigated heritage apple varieties were Goldrenette, Schafsnase, Ilzer Rose, Kronprinz Rudolf, Herbstkalvil, Krummstiel, Golden Delicious, and these varieties were all grown and purchased in Styria. The second aim of this study was to investigate the formation of the volatile compounds in intact apple fruits, but also in the apple peel and the apple flesh. The apple variety Kronprinz Rudolf was also analyzed by differences depending on the growing conditions (plantation growing and meadow orchards) and the apple variety Ilzer Rose was determined on the impact of the ripening process.

For the flavor investigations, two different complementary approaches were used, the sensory evaluation and the analytical techniques. The complete sensory characterization of the intact apples and of sliced apples after enzyme inactivation was performed by an expert panel. The volatile and odor active compounds were identified by using gas chromatography-mass spectrometry (GC-MS) and gas chromatography – olfactometry (GC-O) after headspace solid-phase microextraction (HS-SPME). Besides, the analysis of the flavor was also performed by the use of comprehensive GC×GC-MS.

The flavor profiles of the heritage apple varieties were characterized by the important groups of alcohols (e.g. 2-butanol and 1-hexanol), aldehydes (e.g. (E)-2-hexenal and hexanal), esters (e.g. ethyl butanoate and methyl butanoate), and terpenes (e.g. α-farnesene and α-bergamotene). This study is the first comprehensive GC×GC-MS investigation of the apple peel of a heritage apple variety and the results of this analysis presents that the number of terpenes is very high in the apple peel. Terpenes, for example α -Caryophyllene, (E)-γ-Bisabolene, cis-α-Bisabolene, α-Curcumene, were reported the first time in apple fruits. These findings provide new insights for the flavor of apples and suggest an underestimated role for the terpenes in apple fruits. One of the more significant findings to emerge from this study is that the ripeness process in apples has a high impact on the formation of the secondary flavor compounds. These compounds were increased in the ripeness process. In combination with high concentrations of alcohol, this leads to a shift in the distribution of volatile compounds in the apple peel in favor of the terpenes and aldehydes. The impact of the different growing conditions was not significant on the flavor of the heritage apple varieties.

A limitation of this study is that the apple harvest years 2016 and 2017 were disastrous and that the investigations of the apple flavor were minimalized for these years. In spite of its limitations, the study offers valuable insights into the flavor of the heritage apple varieties from Styria. Further work is needed to fully understand the flavor formation pathways and the different impacts on them. The findings of this study have a number of important implications for future practice.

# Appendix

# 6. References


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# 7. List of Figures

# 8. List of Tables

# 9. Curriculum Vitae

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| 11/2015 – 11/2019: | University assistant and Ph.D. Student<br>at the Graz University of Technology (Austria),<br>Institute of Analytical Chemistry and Food Chemistry<br><br>Ph.D. thesis: 'Flavor analysis of heritage apple varieties from Styria' |
| 11/2013 – 12/2015: | Project Scientist at the Graz University of Technology (Austria),<br>Institute of Analytical Chemistry and Food Chemistry;<br>Project staff: organisation and coordination of consumer and expert sensory panels; research in the field of human sensory science and flavor analysis |

**Academic Education**

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|---|---|
| 02/2012 – 09/2013: | Master studies in Biotechnology at the Graz University of Technology (Austria)<br>Degree: Diplom-Ingenieurin (DI) and Master of Science (M.Sc.)<br><br>Master thesis: 'Analytische und sensorische Charakterisierung von alten<br>steirischen Streuobstapfelsorten' |
| 10/2005 – 01/2012: (Austria) | Bachelor studies in Molecular Biology at the University of Graz<br><br>Degree: Bachelor of Science (B.Sc.) |
| 10/2003 – 06/2005: | Human Medicine at the Medical University of Graz |
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**Honors and awards**

"Foodscapes Award" for best M.Sc. thesis (2014)

**Book Chapters in Reviewed Books**

I. Tauber, G. Innerhofer, E, Leitner, B. Siegmund, Characterisation of the Flavour of the Old Austrian Apple Variety 'Ilzer Rose', in B. Siegmund & E. Leitner (Eds.) Flavour Science – Proceedings of the 15th Weurman Flavour Research Symposium, Verlag der TU Graz, 2018, ISBN, 978-3-85125-593-5, pp. 135-148; doi: 103217/978-3-85125-593-5-29.

I. Ragger, U. Heil, G. Innerhofer, E. Leitner, B. Siegmund*, Flavour characterisation of old apple varieties as a contribution to preserve meadow orchards as typical cultural landscapes, in Taylor A.J., Mottram, D.S. (Eds.) Flavour Science, Context Products Ltd., UK, 2015, ISBN 978-1-899043-70-5, pp. 207-212.

**Oral Presentations**

Tauber, I.; Innerhofer, G.; Siegmund, B.; Flavour analysis of a heritage Austrian apple variety at different ripening stages – in: Book of Abstracts (2019), XX EuroFoodChem Conference; 17.-19.06.2019 in Porto, Portugal

Tauber, I.; Innerhofer, G.; Leitner, E.; Siegmund, B.; Aromastoffanalytik von alten steirischen Apfelsorten – in: Bericht 74. ALVA Jahrestagung: Weinbau und Klima; 27.-28.05. 2019 in Klosterneuburg, Austria

Tauber, I.; Innerhofer, G.: Siegmund, B.; Alte Steirische Apfelsorten – die Sorte macht das Aroma. – in: Österreichische Lebensmittelchemiker Tage 2018: Zusatzstoffe; 25.-27.04.2018 in Seggau, Austria

Tauber, I.; Innerhofer, G.: Siegmund, B.: „Fresh, juicy,Styrian" – The Flavor of the old apple variety 'Ilzer Rose' – in Book of Abstracts (2016), 12th ASAC Junganalytikerforum; 10.-11.06.2016 in Graz, Austria

**Poster Contributions**

Tauber, I.; Innerhofer, G.; Leitner, E.; Siegmund, B.; Apple skin of old apple variety as terpene factory - in: Book of Abstracts (2018), Bioflavour in Frankfurt/Main; 18.-21.09.2018 in Frankfurt/Main, Germany

Tauber, I.; Innerhofer, G.; Leitner, E.; Siegmund, B.; Apple Flavour Characterisation from Skin to Flesh – on the basis of the Old Apple Variety 'Ilzer Rose'- in: Book of Abstracts (2017), 15th Weurman Flavour Research Symposium; 18.-22.09.2017 in Graz, Austria

Tauber, I.; Innerhofer, G.; Siegmund, B.; Der Duft von alten Apfelsorten – in: Bericht ALVA Jahrestagung 2017: Zukunft Obstbau; S.403-405; 22.-23.05.2017 in Wesenhofen/Donau, Austria

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Tauber, I.; Heil, U.; Innerhofer, G.; Leitner, E.; Siegmund, B.: The Scent of Apples – A Non-Literary Investigation. – in: Book of Abstracts (2016), Seventh European Conference on Sensory and Consumer Research-Eurosense; 11.-14.09.2016 in Dijon, France

# 10. Appendix

## Characterisation of the flavour of the old Austrian apple variety 'Ilzer Rose'

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### Abstract

The old apple variety 'Ilzer Rose', coming from the region near the village Ilz (Austria), is an old variety that has been described since approximately 1900. The rather small, intense-red apples with white flesh have a very pleasant, intense fruity and rose-like flavour. The aim of this study was to characterize the flavour of the old apple variety 'Ilzer Rose' but also to identify differences in distribution of volatiles between the skin and the flesh of the apples. The use of comprehensive GC x GC-MS resulted in the detection of more than 600 volatile compounds and offers a completely new picture of the apple volatilome.

### Introduction

Styria is Austrian's apple cultivation hot spot. About 80% of the annual yield (corresponding to about 130.000 tons) is harvested in this region. The majority of apples – mainly new apple varieties as Golden Delicious, Gala or Idared – are cultivated in plantations. However, about 25% of the apples are grown in so-called meadow orchards. The traditional meadow orchards have been part of a specific type of landscape for hundreds of years and have hosted an enormous number of old apple varieties since then. Even though these varieties have been cultivated in this region for many decades, their flavour properties have not been characterised so far. Most varieties lack a molecular characterisation of flavour compounds.

In general, the flavour of apples is composed by several hundred different volatile compounds such as alcohols, aldehydes, esters, etc. The composition of the apple volatiles depends on variety, climate, maturity/ripening level and storage conditions [1]. Primary flavour compounds are formed via the enzymatic and biological processes in the intact fruit during growth, maturation and ripening, whereas secondary flavour compounds develop as results of tissue disruption. Apple flavour compounds are produced by several biosynthetic pathways, such as the β-oxidation of fatty acids, which is the primary biosynthetic pathway for ester formation. After cell disruption, the lipoxygenase (LOX) pathway is active and is mainly responsible for the formation of straight chain C6 and C9 aldehydes whereas amino acid degradation reactions lead for example to methyl branched aldehydes and alcohols. It is generally assumed that terpene biosynthesis plays a minor role for apple flavour. However, terpenes are formed via the mevalonic (MVA) pathway or the 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway. In general, compounds such as (*E*)-2-hexenal, hexanal, ethyl-2-methylbutanoate, ethyl butanoate and propyl butanoate are regarded to play a significant role for the apple flavour.

The formation of flavour compounds depends on the presence of precursor compounds and enzyme activities of the fruits, for processed fruits also on the conditions used during fruit processing. In this study we aimed to investigate primary flavour compounds in different parts of 'Ilzer Rose' apples. To reach this aim we applied 1-dimensional GC-MS as well as comprehensive GC x GC-MS for the identification of

*B. Siegmund & E. Leitner (Eds): Flavour Sci.*, 2018, Verlag der Technischen Universität Graz
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'Ilzer Rose' volatiles after enrichment by Headspace Solid Phase Microextraction (HS-SPME). The enormous capacity regarding separation as well as sensitivity of comprehensive GC x GC-MS allows deep insight into the flavour composition of this old apple variety. In addition, sensory methods were used to characterize the overall flavour properties.

## Experimental

*Apple samples*

Apples were harvested in 2016 from traditionally grown trees from meadow orchards in Styria. Apple skin was carefully separated from the flesh. To inactivate apple enzymes as far as possible, apple flesh and skin were prepared separately according to Aprea et al [2] prior to GC analysis.

*Gas chromatographic analysis*

Aliquots of the homogenised samples (250 mg each for 1-dim GC-MS and 50 mg for comprehensive GC x GC-MS) were transferred into headspace vials, 2-octanol was used as internal standard (50 ng absolute). Four replicates of each sample were prepared and analysed. After enrichment of the volatiles by HS-SPME (30°C, 20 min, 50/30 µm DVB/CAR/PDMS fibre, 2 cm stable flex fibre) analyses were performed with 1-dimensional GC-MS (Agilent GC 7890, MS 5975c VL MSD, Santa Clara, CA, USA; HP5 30 m*0.25 mm*1 µm, EI (70eV)) and comprehensive GC x GC-MS (Shimadzu GC-2010 Plus coupled with Shimadzu GCMS-QP2010 Ultra, , Shimadzu Europa Gmbh; 1st dim.: ZB-5MS 30 m *0.25 mm*0.25 µm and 2nd dim.: BPX50 2.5 m *0.15 mm*0.15 µm, Zoex cryo modulator, 5s modulation frequency, Hot Jet 280°C, 350 msec pulse time; EI (70 eV)). Identification of the compounds was based on the comparison of the obtained mass spectra to those from MS libraries or authentic reference compounds as well as on retention indices (RI). Linear-temperature programmed RI were calculated using n-alkanes ($C_5$-$C_{26}$) and compared to data from authentic reference compounds and data from literature. For comprehensive GC x GC-MS retention indices were calculated for the 1st dimension.

*Sensory evaluation*

For sensory evaluation, the fruits were cut into cylinders and treated with an antioxidant solution according to Corollaro et al. [3] to avoid (i) browning of the apple pieces and (ii) excessive formation of secondary flavour compounds. Sensory evaluation was performed by 14 well-trained panellists under standardised conditions using quantitative descriptive analysis (QDA®). All panellists had vast experience in evaluating fruits and had undergone apple-specific training prior to this study. Data acquisition was performed by the use of Compusense Sensory Software (Compusense Inc., Guelph, Canada).

## Results and discussion

It was the aim of this study to characterize the flavour of the old apple variety 'Ilzer Rose', but also to investigate the distribution of the volatile compounds between the skin and the flesh of the apples.

Sensory evaluation was performed from standardised 'Ilzer Rose' apple pieces after inactivation of apple enzymes at the sample surface. Nine different odour/flavour attributes were chosen by the panel to describe the sensory characteristics of 'Ilzer Rose'.

Results from QDA® demonstrate the pronounced rose-like/floral and fruity properties of 'Ilzer Rose' apples (Figure 1).

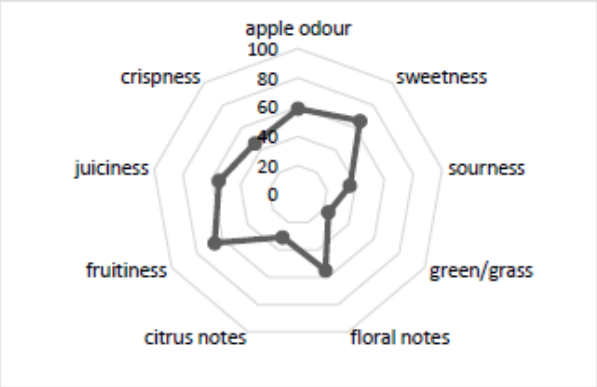


**Figure 1**: Results from QDA® of 'Ilzer Rose' after inactivation of fruit enzymes at the sample surface

A total of 82 volatile compounds was identified from the skin of the 'Ilzer Rose' by 1-dimensional GC-MS, in contrast to only 55 volatiles in the flesh alone. Significantly higher concentrations of most volatile compounds were found in the skin than in the flesh of the Ilzer Rose apples. Table 1 gives a comparison of the relative concentrations of selected volatiles in the skin and the flesh, respectively. Interestingly, not only the carotinoid cleavage product 6-methyl-5-hepten-2-one and the sesquiterpene α-farnesene – that had already been described in apple coating decades ago [5] – are significantly higher in concentration in the skin, but also esters like hexyl butanoate, hexyl 2-methyl butanoate and hexyl hexanoate (Table 1).

**Table 1**: Selected volatile compounds semi-quantified in the headspace of the apple skin and flesh samples by 1-dim GC-MS. Concentrations are expressed as relative concentrations to the internal standard 2-octanol

| Compound | *RI (HP5)* $_{exp}$ | *RI (HP5)* $_{lit}$ | *Skin (mg kg$^{-1}$)* | *Flesh (µg kg$^{-1}$)* |
|---|---|---|---|---|
| 6-Methyl-5-hepten-2-one | 986 | 987[a] | 1.6 | n.d. |
| Hexyl acetate | 1008 | 1014[b] | 6.6 | 7 |
| Hexyl butanoate | 1188 | 1193[a] | 3.5 | 3 |
| Hexyl-2-methyl butanoate | 1236 | 1236[c] | 2.5 | 3 |
| Hexylhexanoate | 1384 | 1386[c] | 5.0 | n.d. |
| α-Farnesene | 1516 | 1508[d] | 24.6 | 25 |

[a] RI obtained from authentic reference compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001–2002
[b] RI obtained from www.flavornet.org
[c] RI obtained from http://webbook.nist.gov/
[d] RI obtained from literature [4]

Chromatograms obtained from comprehensive GC x GC-MS analysis clearly demonstrate the differences between flesh and skin (Figure 2). More than 600 volatile compounds were (tentatively) identified in 'Ilzer Rose' apples, many of them seen in the apples for the first time. These results are in accordance with recently published data on the volatilome of strawberries – nearly 600 volatiles were described from strawberries after analysis by comprehensive GC x GC-MS [6]. The identified compounds include

well-known apple volatiles like esters, alcohols, aldehydes and ketones, but also a large number of mono- and sesquiterpenes. The presence of high numbers of terpenes predominantly in the skin of 'Ilzer Rose' is of special interest as, so far, terpenes have not been regarded to be important contributors to apple flavour. However, they might be the reason for the expressed floral/rose-like notes that are known from 'Ilzer Rose' apples.

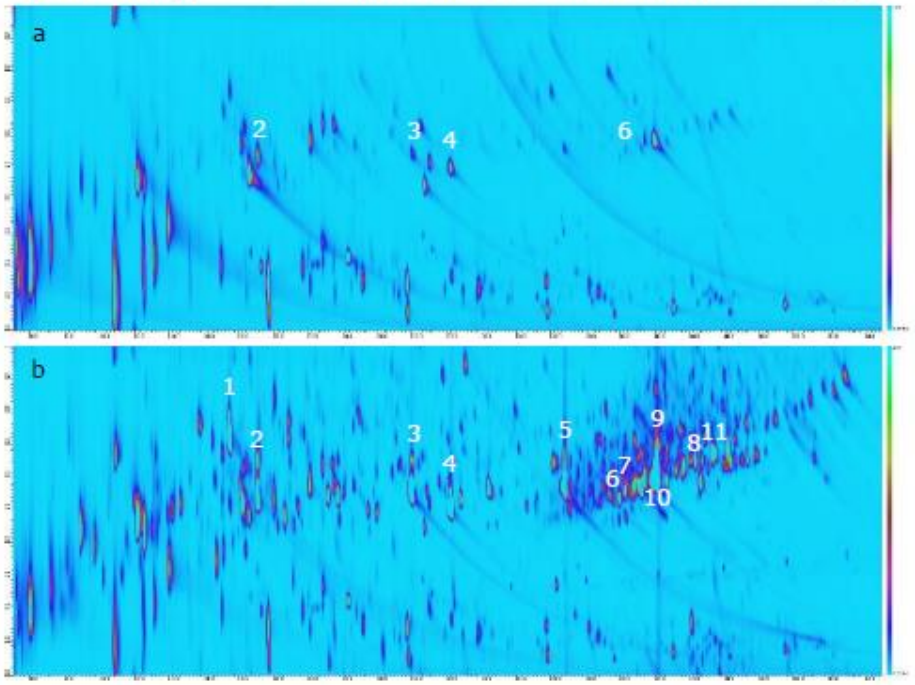


**Figure 2**: Chromatograms obtained from comprehensive GC x GC-MS; analysis of the (a) flesh and (b) skin of Ilzer Rose apple. Retention times in the first (x-axis) are given in minutes, retention times in the second dimension (y-axis) are given in seconds. (1) 6-methyl-5-hepten-2-one, (2) hexyl acetate, (3) hexyl butanoate, (4) hexyl-2-methylbutantoate, (5) hexyl hexanoate, (6) α-farnesene, (7) cis-β-farnesene', (8) cis-thujopsene', (9) β-longipinene', (10) β-vatirenene', (11) cis- α-santalol'; 'tentatively identified by probability-based matching of the obtained mass spectra with the mass spectra from the NIST library

The results obtained from this study demonstrate that the use of comprehensive GC x GC-MS offers a completely new insight into the apple volatilome. The preliminary results from this study serve as a basis for future investigations of volatiles in different parts of apples in general and of the floral, rose-like odour of 'Ilzer Rose' in particular.

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# Flavour characterisation of old apple varieties as a contribution to preserve meadow orchards as typical cultural landscapes

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[*] Iris Ragger and Ulrike Heil contributed equally to these investigations

The flavour of selected old apple varieties cultivated in meadow orchards were investigated in comparison to new apple varieties. Sensory evaluation and gas chromatographic analyses (GC-MS and GC-O) were performed of intact apples and juices. The old varieties *Kronprinz Rudolf* and *Bohnapfel* are characterized by a high number of volatile esters that are responsible for expressed fruitiness of the apples and products thereof. The outstanding amounts of ethyl 2-methylbutanoate are intrinsic for *Kronprinz Rudolf* and may count for red-berry, strawberry-like notes that have often been addressed as off-flavour. The comparison of juices from two vegetation periods with different climatic conditions showed that apples are highly susceptible to abiotic stress resulting in a decelerated ripening process as well as to reduced flavour formation.



## Introduction

Apple cultivation is of high agricultural as well as economic importance for the southern Austrian regions (Styria). In 2013, apples were cultivated on 6,200 hectares; 155,000 tons of apples were harvested which represents about 80% of the annual Austrian apple harvest. The bigger part thereof is cultivated in apple plantations. As elsewhere, the number of cultivated apple varieties is limited; the selection of varieties has mainly been based on growth and storage properties. Flavour has played a minor role. However, about 25% of the annual harvest is cultivated in extensive form on so-called meadow orchards. Recently, there have been strong intentions to preserve the meadow orchards as it represents an enormous pool for old apple varieties that are usually not cultivated in plantations.

From an economic point of view, the meadow orchard can only be preserved when economic benefits are obtained from this cultural landscape. Besides cultural and touristic aspects, the production of high quality foods showing distinct flavour characteristics is one way to contribute to the preservation of the meadow orchards. Single origin products (mainly apple juice and apple wine) of very high quality have been produced recently. Nevertheless, good knowledge of the flavour characteristics of the apple varieties is a prerequisite to emphasise the variety-specific flavour notes – the flavour properties of these old apple varieties have not been investigated before. Products of the very popular old apple variety *Kronprinz Rudolf* sometimes show red-berry, strawberry like sensory notes that are frequently perceived as an off-flavour. Therefore, the investigation of the intrinsic aroma characteristics of the most prominent apple varieties was one part of this study.



Part two of the present study was dedicated to the influence of climatic conditions on the flavour formation of apples. The results of abiotic stress (e.g. drought or high temperatures) are crop specific and, as a consequence, will influence the properties of plants and/or fruits in various ways [1]. High temperatures of up to 40 °C may induce various physiological and metabolic disorders in the fruit. Enzyme activity or functionality may be modulated or even lost, which may for example influence the ethylene production and, as a consequence, the ripening process. Finally, heat stress may also influence photosynthesis; increased leaf temperature may lead to closing of the stomata and, consequently, down-regulation of the $CO_2$ assimilation and carbohydrate formation. Little is known about the influence of high temperatures in combination with low amounts of precipitation on the flavour formation of apples.

Climatic conditions were completely different in 2012 and 2013. Whereas 2012 was an average year with respect to temperatures and amounts of precipitation, 2013 was an atypical year with a warm spring resulting in a short blooming period as well as rainless weeks in summer with temperatures of up to 40 °C, which is far above-average for this region. These climate differences in two consecutive years enabled us to investigate the susceptibility of apples to abiotic stress and the influence of heat stress on flavour formation in the fruit and the properties of the final product.

## Experimental

### *Materials*

Apples from the varieties *Kronprinz Rudolf (KR)*, *Bohnapfel (BA)*, *Maschanzker (MA)* as well as *Golden Delicious (GD)* and *Braeburn (BB)* were harvested when they were at their horticultural maturity. The apples were stored in a cooling device at 4 °C until they were further processed. Apple juices were produced from 50 kg apples per variety using a vertical pneumatic press. After fining, the raw juices were pasteurised in the bottle.

### *Sensory evaluation*

Sensory evaluation was performed in the sensory lab by a trained panel consisting of 14 well-trained panellists. Descriptive analysis as well as projective mapping was performed of the intact apples as well as of the juices. For the sensory evaluation of the intact apples, about 300 g of intact apples were transferred into glass jars with glass lids. For the evaluation, the lid was opened and the smell of the samples was investigated. After projective mapping of any sample type, multivariate analysis was performed by processing the x/y data of each panellist.

### *Analysis of the volatile compounds*

GC-MS analysis was performed after enrichment of the volatiles by headspace SPME. For all SPME measurements a 50/30 µm DVB/Carboxen/PDMS fibre (2 cm stable flex) was used. Juice samples (200 µL) were analysed by using a CTC sampling device (20 min at 40 °C). For the investigation of the intact samples, 300 g apples were put into a glass jar that was closed with a Teflon lid equipped with GC septa to be able to insert the SPME fibres. After equilibration (10 min at room temperature), SPME fibres were exposed into the headspace over the apples (10 min at room temperature) by using manual SPME sampling devices. For all GC-MS analyses, thermodesorption was performed in the GC-injection system at 270 °C. The initial GC oven temperature was -10 °C for better peak separation of the compounds with high volatility. Quantification of compounds of interest was performed by standard addition experiments. GC-O of the apple volatiles was



performed using FID detection in combination with the olfactory detection port (split ratio FID: ODP 1:1). GC-O was performed by trained panellists, using at least five panellists per sample at every dilution step.

## Results and discussion

### *Sensory properties and volatile compounds of intact apples and juices*

The first part of this study was dedicated to the investigation of the flavour characteristics of the apple varieties of interest by using sensory as well as gas chromatographic techniques. Headspace SPME GC-MS of the intact apples resulted in rather complex chromatograms, where the chromatograms were dominated by a large number of different fruit esters. The number of fruit esters determined for the different varieties correlated well with the intensity of the fruitiness that was addressed by the panellists. For *KR* and *BA* (both were described to show expressed fruitiness) about 50 different esters were identified in the chromatograms, whereas the other varieties, which lacked fruity descriptors, only showed about 30 different esters. Figure 1 gives a comparison of the amounts of selected esters. Interestingly, it is not only the number of identified esters, but also the 'ester patterns' that show significant differences between the investigated varieties. Ethyl butanoate, ethyl 2-methylbutanoate and ethyl hexanoate - esters that are known to be very fruity with low odour detection thresholds - could only be detected in the apple varieties *KR* and *BA*, whereas the other varieties were dominated by esters with longer chain lengths, lower volatility, and higher odour thresholds.

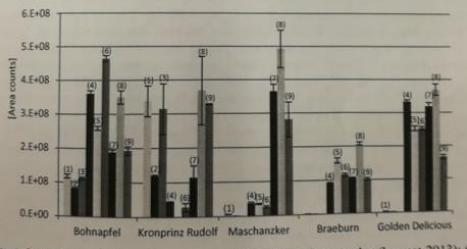


**Figure 1**. Amounts of selected esters detected from the intact apples (harvest 2013); (1) ethyl butanoate, (2) ethyl 2-methylbutanoate, (3) ethyl hexanoate, (4) butyl acetate, (5) 2-methylbutyl acetate, (6), hexyl acetate, (7) butyl butanoate, (8) hexyl butanoate, (9) hexyl hexanoate; amounts are given in terms of area counts; n=4

Proceeding from the intact apples to the single origin juices, sensory analysis showed that the characteristics that were described from the intact apples were well transferred into the respective juices (see Figure 2 for the results of projective mapping). Again the juices from *BA* and *KR* showed expressed fruitiness, whereas *MA*, *BB* and *GD* were significantly less fruity, with woody, spicy notes as well as notes that were reminscent of cooked apples. In addition, the perceived sweetness and acidity played an important role



for sensory differentiation. These findings also correlate well with analytical data for sugar and titratable acids (data not shown).

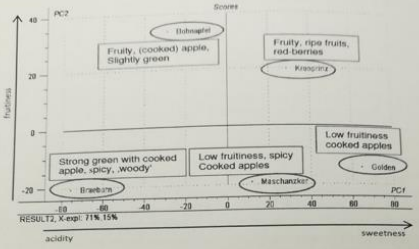


**Figure 2**. Projective mapping of the single origin apple juices (harvest 2013)

Quantification and subsequent calculation of the odour activity values (OAV) of the volatile compounds of interest (Table 1) confirmed the findings that were described before. β-Damascenone is the compound with the highest OAV in all juices and the importance of β-damascenone for the flavour of apple juice has been described before [2, 3]. Ethyl butanoate and ethyl 2-methylbutanoate only play an important role for the flavour of the varieties *BA* and *KR*. Results shown in Table 1 demonstrate, that ethyl 2-methylbutanoate, that has been described with very fruity, apple, red berry-strawberry like notes, plays an outstanding role in *KR*. These results explain that the strawberry-like notes, that have frequently been addressed as an off-flavour in *KR* products previously, are not an off-flavour, but intrinsic for this old apple variety. Additionally, the *KR*'s OAV for 2-hexenal (*E*) is significantly lower than those of the other investigated varieties, explaining why this variety is lacking green notes in the juice. Different LOX enzyme activity, or differences in lipid membrane composition, are likely to be the reasons for these findings.

The results of these investigations showed that the old apple varieties grown in Austrian meadow orchards possess a wide flavour diversity that cannot be found in the apple varieties that are usually grown in apple plantations. Further investigations will be performed with respect to intrinsic flavour properties of other old varieties that are native in meadow orchards.

### *Influence of the climatic conditions on the flavour formation*

Comparing the sensory properties of the juices obtained from the fruits of 2012 and 2013, we noticed that the variety characteristics were present in products from both years, but less pronounced in the extremely hot year 2013 than in the average year 2012. In contrast to what we expected from crop obtained from a hot year, juices from 2013 showed rather unbalanced flavour with low sweetness, high acidity, less fruitiness than in 2012 and, on the contrary, unripe and green notes that were perceived more intensely than in 2012. For

**Table 1.** Odour activity values for selected volatile compounds in the single origin juices from the harvest 2013. Quantification was performed by standard addition experiments; odour thresholds were extracted from literature (www.leffingwell.com)

| Compound/variety | BA | KR | MA | BB | GD |
|---|---|---|---|---|---|
| Ethyl butanoate | 16 | 142 | - | - | - |
| Ethyl 2-methylbutanoate | 524 | 7 480 | - | - | - |
| Butyl acetate | >1 | >1 | >1 | >1 | - |
| Hexyl acetate | 7 | - | 7 | 4 | 3 |
| 2-Hexenal (*E*) | 7 | 0,7 | 17 | 6 | 14 |
| 1-Hexanol | >1 | >1 | >1 | >1 | 8 |
| ß-Damascenone | 22 813 | 27 192 | 19 284 | 38 781 | >1 38 069 |

four of the investigated juices, the total soluble sugar content was significantly lower in 2013 than in 2012.

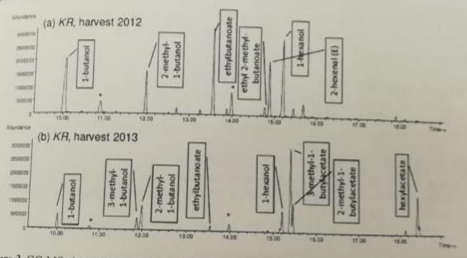


**Figure 3.** GC-MS chromatograms of *KP* juice from 2012 and 2013; compounds showing the highest concentration differences are marked, * SPME fibre degradation products

Results from GC-O showed that the total number of odour impressions was lower from the juices obtained from 2013 than from 2012. Closer examination of the volatile compounds (Figure 3; *KP* juice as a representative example), showed that esters like ethyl butanoate or ethyl 2-methylbutanoate as well as C6 aldehydes and aliphatic alcohols showed significantly higher concentrations in the juices from 2012 than from 2013. On the contrary, 3-methyl-1-butanol, 3-methylbutyl acetate as well as hexyl acetate were present in higher concentrations in the 2013 juices in comparison to the 2012 juices.

All these findings indicate that the 2013 ripening process was decelerated due to the extreme climatic conditions, producing fruits of lower maturity than in 2012. The lower concentrations of the aliphatic esters and alcohols are direct indicators thereof, as they are mainly formed via ß-oxidation in course of ripening process. A potential enzyme modulation due to heat stress could be another reason for these findings. Decreased amounts of C6 aldehydes, mainly products of the LOX pathway formed by cell disruption during apple processing, may be due to stress-induced changes in cell wall structures, substrate availability or enzyme modulation.



Volatile metabolites associated with the amino acid pathway seem to react in the adverse direction when climatic conditions are extreme. An increase in amino acid formation as a stress reaction of the plant has been reported before [4]. The high amounts of 3-methyl-1-butanol as degradation product of isoleucine as well as the corresponding acetate might be based on a stress-induced higher amount of the corresponding free amino acid. An increase in amino acids has been reported previously as potential metabolic change as a possible reaction of plants upon abiotic stress. Interesting to see is the increase in concentration of hexyl acetate in 2013. Fellner et al., 2000 [5] propose an interaction between the LOX pathway and the amino acid pathway, whereupon higher availability of leucine, isoleucine or valine in the presence of aminotransferase leads to increased concentration of acetyl-CoA in the system and, finally, to a higher amount of hexyl acetate in the fruit. In this context, the increased concentration of hexyl acetate might as well be seen as a result of stress induced higher contents of free amino acids in the apples.

The results obtained by the comparative investigation of apple products of two consecutive years with completely different climatic conditions gives reasons to believe that the apple is a crop that is highly susceptible to abiotic stress. Further investigations are needed to validate these findings and to be able to benefit from these findings for example with respect to apple breeding.

**Acknowledgement**

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# Decoding the flavour of 'Ilzer Rose' – an old apple variety from the south-east of Austria

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## Introduction

Fig. 1: Example of a traditional meadow orchard

Apple cultivation has a long tradition in southern parts of Austria (Styria). About 75% of the Austrian apples are cultivated and harvested within this region. In apple plantations, popular international apple varieties like Golden Delicious, Gala and Idared cover more than 60% of the fruits. On the contrary, about 25% of the Styrian apples are grown in so-called meadow orchards (Fig.1). One benefit of the traditional meadow orchards is the enormous number of apple varieties that have been domestic in this specific type of landscape for hundreds of years. Recent developments regarding for example apple price on the European market as well as the general consumers' trend to prefer regional products have put old apple varieties back on the map.

The old apple variety 'Ilzer Rose' is one of these varieties which have been described especially from this region near the village Ilz since about 1900. The rather small, intense-red apples with white flesh possess a very pleasant, intense fruity and slightly rose-like flavour which makes it interesting for producers of high quality apple juices, ciders and other products thereof.

## Materials and Methods

The formation of secondary flavour compounds is dependent on enzyme activity of the fruits, but also on the conditions used during fruit processing. To be able to focus on primary flavour compounds, apple enzymes were inactivated as far as possible by applying a procedure described in [1].

The volatiles of the flesh of the 'Ilzer Rose' were in the focus of the present study. For the flavour characterisation we used the following techniques:
Identification of the volatile and odour active compounds by using gas chromatography-mass spectrometry and gas chromatography-olfactometry after headspace SPME. In GC-olfactometry, the human noses of trained panellists were used as selective and sensitive detectors to identify the odour active compounds. Detection frequency was used to identify to most potent odourants of this apple variety.

**(i) Sample preparation**
- Inactivation of genuine enzymes
- HS-SPME (2 cm 50/30 µm DVB/Carboxen/PDMS)

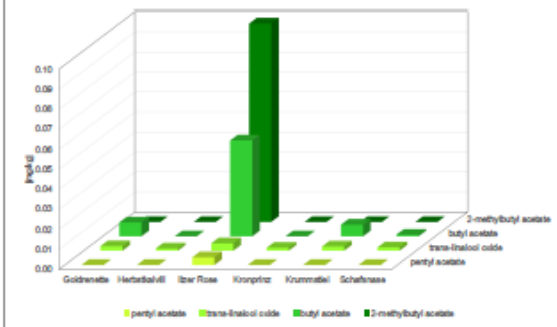
**(ii) Analysis of the volatiles**
(after Headspace Solid Phase Microextraction)
- Gas chromatography-mass spectrometry
- Gas chromatography-olfactometry (Detection Frequency)

## Results

Descriptive analysis of the enzyme-inactivated sliced 'Ilzer Rose' apples showed a distinct flowery/floral flavour with pronounced crispiness and fruitiness. GC-MS analysis of the volatile compounds revealed significantly higher concentrations of four volatiles compounds (butyl acetate, 2-methylbutyl acetate, trans linalool oxide and pentyl acetate), which are only present in very low amounts in other old apple varieties (Fig.2).
Results from GC-O (Fig. 3) demonstrate a certain contribution of these compounds to the 'Ilzer Rose' flavour, but do not explain the distinct flowery/floral odour. Further investigations are necessary to identify the volatile compounds that contribute to the typical 'Ilzer Rose' flavour.

**However, these results serve as a good basis to disclose the secret of 'Ilzer Rose' flavour!**

### GC-MS Analysis




estimated quantities in ng/kg of old apples calculated by comparison with IS (2-Octanol)

Fig. 2: Concentrations of the four compounds of interest in „Ilzer Rose' comperison to other old apple varietes

### GC-Olfactometry

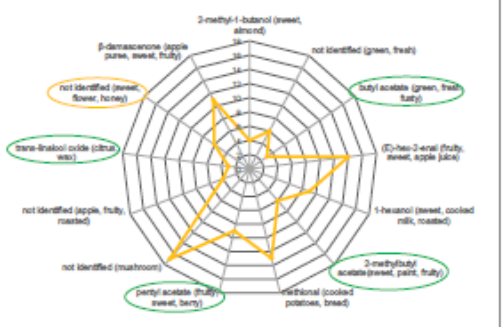



Fig. 3: Odour active compounds in 'Ilzer Rose' identified after GC-O (Detection Frequency)

**Reference:**
1. M.L. Corollaro, I. Endrizzi, A. Bertolini et al. (2013) Postharvest Biology and Technology, 77, 111-120.

# The Scent of Apples – A Non-Literary Investigation

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## Introduction

Styria is Austrian's apple cultivation hot spot. About 80% of the annual harvest (corresponding to about 130.000 tons) are harvested in this region. The majority of the apples – mainly new apple varieties as Golden Delicious, Gala, Idared or Braeburn - are cultivated in plantations.

Fig. 1: Example of a traditional meadow orchard

However, about 25% of the apples are grown in so-called meadow orchards (Fig. 1). These meadow orchards represent an enormous pool for apple varieties – mainly **old apple varieties** that have been traditionally grown there. In many cases these varieties show completely different sensory properties than fruits from new varieties.

*'The Scent of Apples'* is not only the title of a short story by Bienvenido N. Santos, but also the issue of our recent investigations concerning the perceived odour when sniffing intact ripe apples. Therefore, 15 different apple varieties were analyzed with respect to the volatiles emitted by the intact fruit. In this study, we aimed to answer the following to questions:

- **Is it possible to differentiate apple varieties by sniffing the intact apples only?**
- **By means of GC analysis – is it possible to interpret the differences by investigating the primary flavour compounds?**

## Materials and Methods

For the characterisation of the scent of the intact apples we used two different techniques that deliver complementary information regarding the primary flavour compounds of the investigated apple varieties.

**Apple varieties**

- 8 old varieties (***Bohnapfel***, *Boskop*, *Cox Orange*, *Ilzer Rose*, ***Kronprinz Rudolf***, *Leder Renette*, ***Maschanzker***, *Schafsnase*)
- 7 new varieties (***Braeburn***, *Elstar*, *Gala*, *Pinova*, ***Golden Delicious***, *Rubinette*, *Topaz*)

**Sensory evaluation**

- Trained panellists
- Intact apples in glass jars with lids
- Evaluation of the smell after opening the lid
- Descriptive analysis
- Projective mapping

**Analysis of the volatiles**

- Intact apples in glass jars
- Enrichment of the volatiles after equilibration
- Headspace-SPME of the volatiles
- GC-MS analysis

## Results

**Sensory evaluation** showed that it is possible to differentiate between the 15 apple varieties of interest according to the smell of the intact fruits (results not shown). However, there was no clear separation between old and new varieties. Figure 2 and 3 show the characteristics of three old and two new selected apple varieties that were of special interest for this study. The two old varieties *Bohnapfel* and *Kronprinz Rudolf* were separated from the other varieties of interest mainly due to their expressed fruitiness. Three esters with expressed fruitiness and low odour thresholds (i.e. **ethyl butanoate, ethyl-2-methyl butanoate, ethyl hexanoate**) can only be found in relevant amounts within the primary flavour compounds of *Bohnapfel* and *Kronprinz Rudolf*, explaining the expressed fruitiness of these varieties.

**Sensory Analysis**

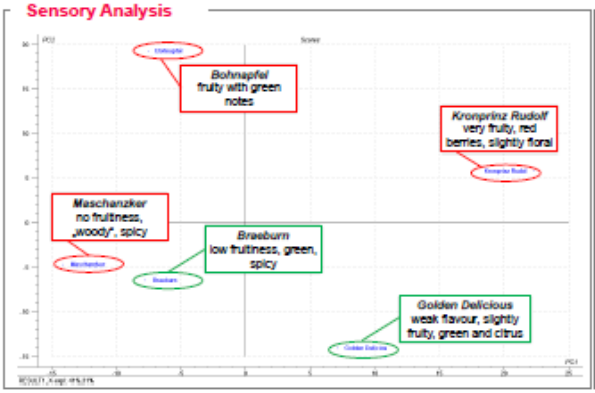


Fig. 2: Projective Mapping: Classification according to similarities/differences in odour from 5 varieties of interest red :old varieties, green: new varieties

**GC-MS Analysis**

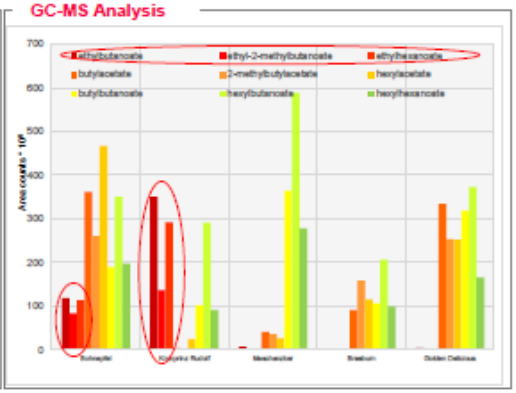


Fig. 3: Comparison of ester patterns of the five apple varieties of special interest

**Thanks to....** the members of the sensory test panel for evaluating the apple samples.

# Der Duft von alten Apfelsorten

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## Einleitung

Im Zuge der aktuellen Diskussionen um Sortenvielfalt steht das Aroma von unversehrten alten Streuobst-Apfelsorten im Mittelpunkt dieser Studie. Wir gingen der Fragestellung nach, ob sich Sortencharakteristika von Äpfeln schon im Geruch bzw. in den flüchtigen Verbindungen, die vom unversehrten Apfel emittiert werden, manifestieren. In Teilen der Steiermark stellt die Kulturlandschaft Streuobstwiese eine erhaltenswerte Kulturform dar. Die Entwicklung und Produktion von für diese Regionen typischen Produkten liefern einen wesentlichen Beitrag dazu, diese einzigartige Kulturlandschaft zu erhalten. Traditionellerweise werden dort vor allem so genannte alte Apfelsorten (z.B.: Maschanzker, Kronprinz Rudolf oder Bohnapfel) in extensiver Form angebaut.

**Abb. 1: Beispiele für alte Apfelsorten** (von links nach rechts): Bohnapfel, Kronprinz Rudolf und Maschanzker

## Material und Methoden

Das typische Apfelaroma wird geprägt durch verschiedene Alkohole, Ester und Aldehyde. Die Verbindungen (*E*)-2-Hexenal, Hexanal, Ethyl-2-methylbutanoat, Ethylbutanoat und Propylbutanoat spielen eine wichtige Rolle. Doch gibt es von Sorte zu Sorte Unterschiede im Aromaprofil und um die typischen Aromastoffe einer alten Apfelsorte zu identifizieren, wurden Techniken der instrumentell analytischen Aromastoffanalytik kombiniert mit sensorischen Analysen eingesetzt. Die primären Aromastoffe entstehen durch die enzymatisch kontrollierten Stoffwechselvorgänge und sind wichtig für das Aroma der unversehrten Äpfel.

**Apfelsorten**

- Maschanzker (alte Sorte)
- Bohnapfel (alte Sorte)
- Kronprinz Rudolf (alte Sorte)
- Braeburn (neue Sorte)
- Golden Delicious (neue Sorte)

**Sensorische Analysen**

- geschultes Sensorikpanel
- unversehrte Äpfel über den Rexgläsern abriechen
- Deskriptive Beurteilung
- Projective Mapping

**Analyse der flüchtigen Verbindungen**

- unversehrte Äpfel in Rexgläsern
- Anreicherung der flüchtigen Verbindungen aus dem Dampfraum über der Frucht mittels SPME
- GC- MS Analysen

## Ergebnisse

Die Ergebnisse dieser Untersuchungen geben einen interessanten Einblick in die Sortenvielfalt der in der Steiermark angebauten Apfelsorten. Die Überlagerungen aller fünf Aromaprofile der GC-MS Analysen zeigen, dass es signifikante Unterschiede bei der Zusammensetzung der Aromastoffe gibt. Ausgewählte Verbindungen (1-Butanol, 2-Methyl-1-butanol, Butylacetat, Butylbutanoat, Ethylhexanoat), die in allen fünf Aromaprofilen in unterschiedlich hohen Konzentrationen vorkommen sind in Abb.2 dargestellt. Nicht alle diese Verbindungen sind geruchsaktiv und haben Auswirkungen auf die Aromaausprägung des Apfelaromas, jedoch stellen wahrscheinlich einige dieser Aromastoffe aromarelevante Verbindungen dar. In Kombination mit der sensorischen Analyse kann man deutliche sortenabhängige Unterschiede erkennen (Abb.3), so zeigt die alte Apfelsorte Kronprinz Rudolf ein sehr fruchtig-beeriges Aroma, dass auch die hohe Konzentration an fruchtigen Esterverbindungen der GC-MS Messungen bestätigt.

**GC-MS Analyse**

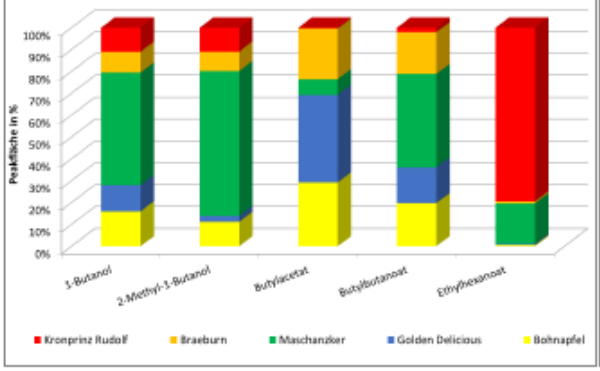


**Abb. 2:** Vergleich von fünf ausgewählten Verbindungen, die in allen untersuchten Apfelsorten vorkommen, mit deren Flächen

**Sensorische Analyse**

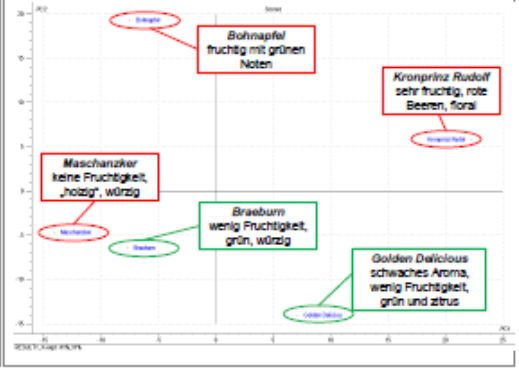


**Abb. 3: Projective Mapping:** Anordnung aufgrund von Ähnlichkeiten/Unterschiede im Aroma der 5 verschiedenen Apfelsorten; rot: alte Apfelsorten; grün: neue Apfelsorten

**Danksagung:** Besonderer Dank geht an die Mitglieder des Sensorikpanels des ACFC (Technische Universität Graz) für die sensorische Beurteilung der Proben.

# Apple Flavour Characterisation from Skin to Flesh – On Basis of the Old Apple Variety 'Ilzer Rose'

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## Introduction

Fig. 1: An halved ‚Ilzer Rose' apple: intense-red apple with white flesh

Apple cultivation has a long tradition in Austria, especially in Styria. About 25% of Styrian apples are grown in so-called meadow orchards. The traditional meadow orchards have been a specific type of landscape for hundreds of years and accommodate an enormous number of old apple varieties. Even though these varieties have been cultivated in this region for many decades, the flavour properties are not described. For most varieties, a molecular characterisation of the flavour compounds is lacking.

The old apple variety 'Ilzer Rose' is one of these varieties which have been described especially from this region near the village Ilz since about 1900. The rather small, intense-red apples with white flesh have a very pleasant, intense fruity and slightly rose-like flavour which makes it interesting for producers of high quality apple juices, ciders and other products thereof.

## Materials and Methods

The formation of flavour compounds is dependent on enzyme activities of the fruits, but also on the conditions used during fruit processing. To be able to focus on primary flavour compounds, apple enzymes were inactivated as far as possible by applying a procedure described in [1].

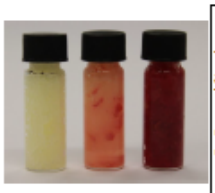


Fig. 2: The enzyme-inactivated ‚Ilzer Rose' apple: flesh only , flesh and skin, skin only (left to right)

### Sensory Methods

The inactivated apple samples were evaluated by an expert panel in white cups.
The following methods were used:

- Descriptive Analysis
- QDA Quantitative Descriptive Analysis®

### GC-Methods

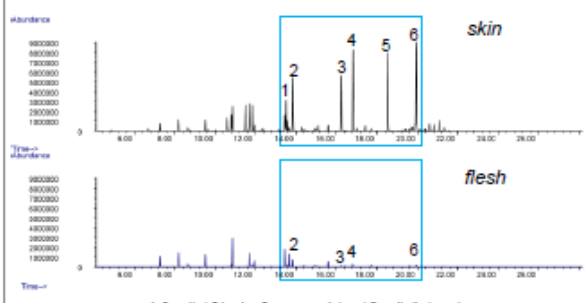
Gaschromatographic analysis were performed by using the following techniques:

- Inactivation of genuine enzymes
- Headspace Solid Phase Microextraction (2 cm 50/30 µm DVB/Carboxen/PDMS)
- 1-dim. Gas chromatography-mass spectrometry GC-MS on HP5
- Comprehensive GC x GC-MS
  1st dim.: 30 m ZB-5MS 0.25mm*0.25µm
  2nd dim.: 2.5 m BPX50 0.15mm*0.15µm

## Results

Descriptive analysis of the enzyme-inactivated sliced 'Ilzer Rose' apples showed a distinct flowery/floral flavour with pronounced crispiness and fruitiness. GC-MS analysis of the volatile compounds revealed significantly higher concentrations of a few volatile compounds in the skin compared to the flesh of the apple (Fig.3). Results from Comprehensive GC x GC-MS (Fig.4) show the differences more pronounced between the volatiles of the flesh and the skin. The amount of the sesquiterpenes and of 5 compounds (6-methyl-5-hepten-2-one, hexyl acetate, hexyl butanoate, hexyl-2-methyl butanoate and hexyl hexanoate) is many times higher in the skin than in the flesh. These results are well reflected in the sensory impression – it is the skin of Ilzer Rose showing the typical flowery/floral flavour and not the flesh alone.

### GC-MS Analysis




1: 6-methyl-5-hepten-2-one
2: hexyl acetate
3: hexyl butanoate
4: hexyl-2-methylbutanoate
5: hexyl hexanoate
6: α-farnesene

Fig. 3: 1 dim. GC-MS Analysis: Significantly higher concentrations of selected volatile compounds can be found in the skin sample in comparison to the flesh of the Ilzer Rose apple.

### Comprehensive GCxGC-MS Analysis

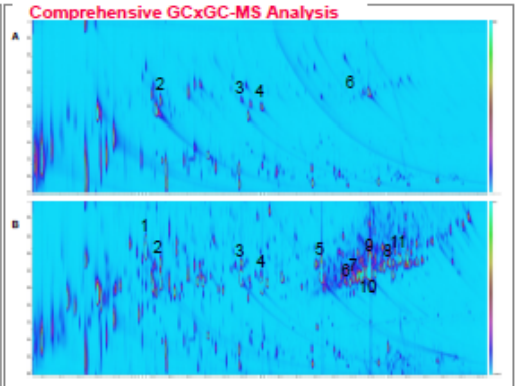



Fig. 4: Comprehensive GCxGC-MS Analysis: volatile compounds from Ilzer Rose apple, A: flesh sample; B: skin sample; (1) 6-methyl-5-hepten-2-one, (2) hexyl acetate, (3) hexyl butanoate, (4) hexyl-2-methylbutanoate, (5) hexyl hexanoate, (6) α-farnesene, (7) cis-β-farnesene, (8) cis-thujopsene, (9) β-longipinene, (10) β-Vetirenene, (11) cis- α-Santalol

Reference:
1. M.L. Corollaro, I. Endrizzi, A. Bertolini et al. (2013) Postharvest Biology and Technology, 77, 111-120.

# Apple skin of old apple varieties as terpene factory

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## Introduction

In Austria, the majority of apples is cultivated in plantations, however, 25% apples are grown in so-called meadow orchards. In this specific type of landscape an enormous number of old apple varieties have been grown for many decades. The flavour compounds of these old apple varieties have not been fully characterized so far, but the knowledge is important to bring them back on the map. More than 300 volatile compounds have been reported in apple flavour, the important flavour compound groups are esters, alcohols, and aldehydes. In addition, a few terpenes have been identified so far in apples [1]. However, for the flavour of apples mono- and sesquiterpenes have not been regarded to be of high relevance so far. Only recently, we discussed the presence of a large number of mono- and sesquiterpenes in the flesh and especially in the skin of an old domestic apple variety [2]. In general, terpenes are formed either via the mevalonic (MVA) pathway (sesquiterpenes) or the 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway (monoterpenes) (Fig. 1).

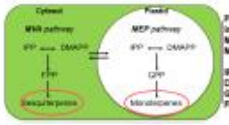

Fig. 1: Terpene formation pathways in the intact cell: MVA pathway: mevalonic acid pathway; MEP pathway: 2-C-methyl-D-erythritol-4-phosphate pathway; IPP: isopentyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: geranyldiphosphate; FPP: farnesyldiphosphate; adapted from [3]

Thus, it was the aim of this study to continue the work on the identification of the terpenoids in the skin of old apple varieties using highly sophisticated analytical methods including comprehensive GC x GC-MS providing high separation capacity as well as enormous sensitivity.

## Material and Methods

The investigated apple varieties were selected consciously for this study based on their sensory properties. With the exception of Golden Delicious, the apples were harvested from Styrian meadow orchards in autumn 2016.

- Ilzer Rose apple (specific rose apple variety)
- Kronprinz Rudolf apple (pronounced red berry-like fruitiness)
- Krummstiel apple (little fruitiness, tannic apple variety)
- Golden Delicious apple (new apple variety, sweet, fruity)

The apple skin was carefully separated from the flesh and the enzymes were inactivated as far as possible by adding a mixture of $CaCl_2$, NaCl and ascorbic acid [4] prior to gaschromatographic analysis. Aliquots of the homogenised samples (50 mg) were spiked with an internal standard (50 ng absolute of 2-octanol).

Gaschromatographic analysis were performed by using the following techniques:

- Headspace Solid Phase Microextraction (2 cm 50/30 µm DVB/Carboxen/PDMS)
- Comprehensive GC x GC-MS
  1st dim.: 30 m ZB-5MS 0.25 mm*0.25 µm
  2nd dim.: 2.5 m BPX50 0.15 mm*0.15 µm
  Zoex Cryo modulator (5 s mod. frequency)
  Hot Jet 280°C, 350 msec pulse time
  EI (70 eV)
- Identification was based on mass spectra and retention indices in the 1st dimension

## Results

The use of comprehensive GC x GC-MS unveiled more than 600 volatile compounds in the investigated apple varieties. The identified compounds include the well-known apple volatiles likes esters, alcohols, aldehydes and ketones. In addition, due to the high sensitivitiy of comprehensive GC x GC-MS, a large number of mono- and sesquiterpenes could be identified in apple skin. 10 terpenes with the highest relative amounts are highlighted in Fig. 2. Terpenes were identified in the skin of all investigated apple varieties, even though they do not seem to influence the sensory properties of varieties like Krummstiel. However, Ilzer Rose apple skin contains the highest relative amounts of volatile mono- and sesquiterpenes which might be the reason for the pronounced floral and rose-like flavour of the Ilzer Rose apples.

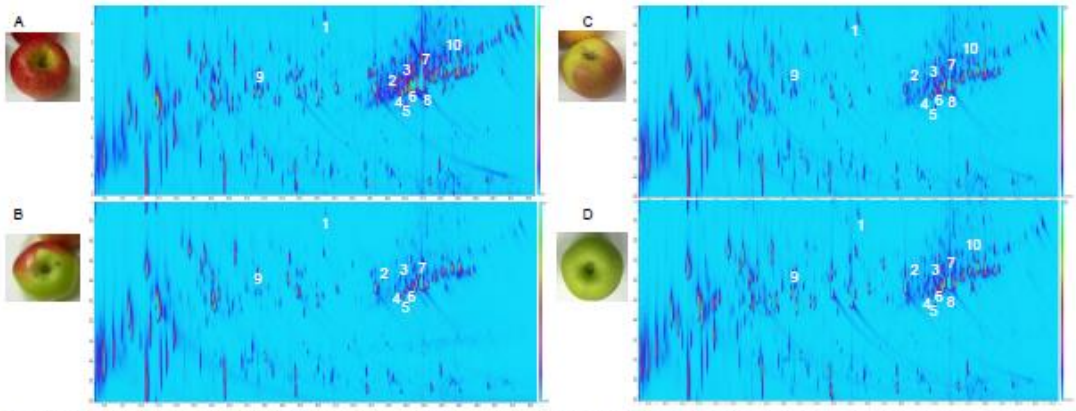


Fig. 2: Chromatograms obtained from comprehensive GC x GC-MS analysis: analysis of the skin of A: Ilzer Rose; B: Kronprinz Rudolf; C: Krummstiel; D: Golden Delicious apples. Retention times in the first (x-axis) are given in minutes, retention times in the second dimension (y-axis) are given in seconds. (1) citral (2) α-bulnesene; (3) β-guaiene; (4) α-bisabolene; (5) δ-β-farnesene, (6) α-farnesene, (7) β-longipinene; (8) β-vetinenene; (9) linalool; (10) α-ylangene; *tentatively identified by probability-based matching of the obtained mass spectra with the mass spectra from the NIST library

[1] Rapparini F, Baraldi R, Facini O., 2001 Seasonal variation of mono-terpene emission from Malus domestica and Prunus avium. In: Phytochemistry. 57:991-7.
[2] Tauber I., Innerhofer G., Leitner R., Siegmund B., 2016. Characterisation of the flavour of the old Austrian Apple variety 'Ilzer Rose'. In: B. Siegmund & E. Leitner (eds.) Flavour Science - Proceedings of the XV Weurman Flavour Research Symposium, Verlag der TU Graz, Austria, accepted for publication.
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[4] Aprea, E., Gika, H., Carlin, S., Theodoridis, G., Vrhovsek, U. and Mattivi, F., 2011. Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry. In: Journal of Chromatography A., pp. 4517-4524.

„Wenn es einen Glauben gibt, der Berge versetzen kann, so ist es der Glaube an die eigene Kraft.“

Marie von Ebner-Eschenbach