1. Introduction

Apples (*Malus domestica* Borkh.) are the most consumed fruit in Germany. As a locally grown fruit, they are also of great commercial importance for German fruit producers. Additionally, apple orchards are a defining feature of the landscape, particularly in the Lake Constance region and in the “Altes Land” near Hamburg. As the English proverb “an apple a day keeps the doctor away” suggests, various positive health benefits are attributed to apples and they are known as important sources of polyphenols and fiber in the Western diet. However, due to the structural homology of the apple allergen Mal d 1 to the main allergen in birch (*Betula verrucosa*) pollen, Bet v 1, approximately 2.5 million birch pollinosis patients in Germany suffer from a cross-allergic reaction against fresh apples.

Symptoms of an apple allergy are usually mild and restricted to the mouth and throat area, as the conformational immunoglobulin E (IgE) epitopes of Mal d 1 are labile and destroyed during gastric digestion due to the loss of the protein’s 3D-structure. Nevertheless, allergic consumers often avoid the consumption of fresh apples. Clinical studies and consumer surveys have revealed differences in the allergenic potential of the various apple varieties. Commercial breeds like Golden Delicious and Jonagold are reported as especially allergic, while traditional varieties, for example Gewürzluiken or Goldparmäne, are often better tolerated. In this context three hypotheses are discussed in the literature:

I) Apple varieties differ in their total Mal d 1 content.
II) Variances in the isoallergen profile among varieties occur and the isoallergens vary in their allergenic potential.
III) Interactions of the allergen with polyphenols modify the 3D-structure of Mal d 1, which might result in destruction or shielding of the IgE epitope and/or precipitation, thereby reducing the allergenicity.

A combination of the discussed theories is also possible. Therefore, the aim of this dissertation project was to improve the understanding of the impact factors affecting the tolerability of apples.

To test the merit of the first two hypotheses, a mass spectrometry-based bottom-up proteomic method for the isoallergen specific

Mal d 1 absolute quantification by isotope dilution analysis was developed (chapter 3.2.). This was necessary as the enzyme linked immunosorbent assays (ELISA) employed for Mal d 1 quantification, so far, are constrained by their inability to distinguish between different isoallergens. Isoallergens are allergens of the same species with a sequence homology of over 67% and a similar biological function. The use of a slightly modified Mal d 1.0108 isoform, expressed in *Escherichia coli* (chapter 3.1.), as an extraction standard enabled monitoring of the allergen's extractability from the apple samples and correction of the quantified Mal d 1 content accordingly.

The developed quantification method was used to characterize the isoallergen profile and to quantify the total Mal d 1 content of 20 different commercial and traditional varieties (chapter 3.3.). This is of interest, as differences among well tolerated and allergic varieties might indicate impact factors affecting variety specific allergenicity. Effects of storage (up to twelve weeks), production method and differences among individual apples were studied in further samples in detail (chapter 3.4.). As no clinical studies were conducted during this thesis, data about the allergenic potential of an apple variety was obtained from a consumer survey conducted by the BUND Lemgo. In this survey, consumers suffering from an apple allergy have been invited to report their tolerance level to different apple varieties online.

To conform to consumer demands of slow browning and sweet apples, commercial apple breeds often contain a reduced phenolic content compared to traditional varieties. Phenolics are the educts for the enzymatically catalyzed browning reaction observed when intact apple flesh is damaged. Therefore, a reduced phenolic content limits the browning of apples. However, polyphenols can form covalent bonds or interact non-covalently with proteins. This might lead to shielding or destruction of IgE epitopes or denaturation of the allergen, which might have a positive impact on the tolerance of an apple variety. To improve the understanding of the impact of polyphenols on the allergenicity, the phenolic profile and content of traditional and commercial apple varieties were determined (chapter 3.5. and 3.6.) and correlated with the Mal d 1 content and the reported allergenicity (chapter 3.6.).

The allergic reaction is usually restricted to the oral cavity, therefore the release of polyphenols from apple flesh and peel is of interest, as

only phenolics released from the matrix can interact with the allergen. Hence, to determine the bioaccessibility of polyphenols during the oral phase, *in vitro* and *ex vivo* experiments with simulated saliva fluid and centrifuged and non-centrifuged saliva were conducted (chapter 3.5. and 3.6.). Additionally, the release of Mal d 1 during simulated oral digestion was determined (chapter 3.7.).

To get a better understanding of Mal d 1–polyphenol interactions, various analytical approaches were applied to characterize the interaction type and strength in model systems containing Mal d 1 and polyphenols (chapter 3.7.). Isothermal titration calorimetry was used to quantify the heat release during interactions, enabling conclusions about the strength and the driving force of interactions. Using untargeted mass spectrometry, possible covalent modifications by epicatechin at amino acid side chains were identified. However, this approach failed in apple samples.

The combined results about Mal d 1 and polyphenol profile and content, the release of Mal d 1 and polyphenols during the oral digestion phase, as well as the results of the interaction studies, permitted the identification of potential parameters affecting the variety specific allergenicity of apples.

2. Theoretical Background

2.1. The Apple – King of Fruits

2.1.1. Economic Importance and Historical Background of Apple Cultivation

Historical background. Apples belong to the botanical family of the Rosaceae (Brown, 2012). Other fruits of the same family are, for example, pear, peach and cherry. The history of apple consumption and domestication is long. The consumption of gathered crabapples during the Neolithic period is indicated by findings of often charred apples and seed coats at archeological sites (Cornille et al., 2019). The progenitor of our "modern" domesticated apples *(Malus domestica* Borkh.) is most likely *Malus sieversii* Roem. (Cornille et al., 2014). It is hypothesized that the origin of domestication and cultivation of these apple trees by humans was in the Tian Shan mountains in Central Asia around 10000 – 4000 BCE (Cornille et al., 2014). From there the knowledge of apple cultivation and cultivars were spread along the Silk Road from Asia to Europe during classical times (Cornille et al., 2019; Duan et al., 2017). During this spreading, hybridization with different crabapples (*Malus baccata* Borkh., *Malus orientalis* Ugilitz. and *Malus sylvestris* Mill.) occurred, leading to the nowadays consumed version of *Malus domestica* (Cornille et al., 2014). Grafting techniques were known to the ancient Greeks and Romans, the latter disseminated them throughout their empire (Cornille et al., 2014; Spengler, 2019). From Europe, settlers spread the knowledge of apple cultivation to the rest of the world (Cornille et al., 2019).

Economic importance. Today, apples are the most widely grown temperate fruit in the world and cultivation has expanded to subtropical and tropical climate zones (Brown, 2012). In 2021, approximately 93 million tonnes of apples were produced worldwide (FAO - Food and Agriculture Organization of the United Nations, 2023), making apples the third most produced fruit after bananas (125 million tonnes) and watermelons (102 million tonnes) (FAO - Food and Agriculture Organization of the United Nations, 2022). China is the largest producer, accounting for 49% of the global production, followed by Turkey and the USA (figure 2-1) (FAO - Food and Agriculture

Organization of the United Nations, 2023). In the EU, Poland, Italy, France and Germany are the four biggest apple producing countries. More than 60% of the produced apples are marketed as fresh fruit (Brown, 2012). In Germany, apples are the most consumed fruit with an estimated annual consumption of 24.4 kg/capita (2020/21) (BLE Referat 414 - Landwirtschafltiche Statistik, Berichte, 2022).

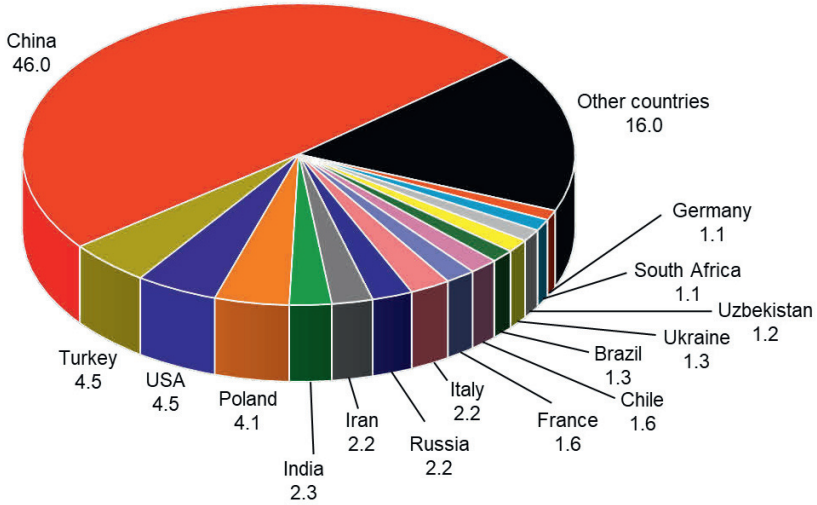

Figure 2-1: *The 15 most important apple producing countries worldwide. The yield of commercial apple producers in the harvest year 2021 are stated in million tonnes (FAO - Food and Agriculture Organization of the United Nations, 2023).*

2.1.2. Apple Production and Storage

Apple production. Even through it is estimated that over 10000 – 20000 apple varieties have been breed so far and still new varieties are generated in breeding programs, only around 12 varieties are of importance on the European market (Bannier, 2011; Bundesanstalt für Landwirtschaft und Ernährung Deutschland, 2021; Szot et al., 2022). The most common dessert apple varieties of Germany are Elstar, Granny Smith, Golden Delicious, Gala, Jonagold/-prince/-red, Braeburn and club varieties like Kanzi® or Pink Lady®. Approximately 80% of the grown apples in Germany are apples from the varieties Elstar, different mutations of the Jona-group, Braeburn and Gala (Statistisches Bundesamt (Destatis) Germany, 2023).

Most of the varieties in modern commercial apple breeding are scions of six ancestor varieties, namely Golden Delicious, Cox Orange, Jonathan, McIntosh, Red Delicious and James Grieve (Bannier, 2011). As nowadays scions of these six varieties are used in the breeding programs, incest like relationships are common among modern breeds. This is especially pronounced in scions of the Golden Delicious variety, as it has been involved in over 50% of all new varieties since 1920 (Bannier, 2011). The high rate of inbreeding is problematic due to the reduction in genetic diversity.

In marketing and the literature about apple allergy the terms “old” and “new” varieties are often employed and it is insinuated that the “old” varieties, usually not of commercial importance and often grown in traditional orchard meadows (Streuobstwiesen), are better tolerated by allergic patients (BUND-Ortsgruppe Lemgo, 2023a; Vegro et al., 2016). However, the denomination of “old” and “new” is difficult, as the origin dates of so called “new” breeds are often as historic as the varieties termed as “old” (table 2-1). Therefore, in this thesis the terms “commercially important” and “traditional” varieties are used.

Table 2-1: *Year of first description of selected commercial and traditional apple varieties according to Baric et al., 2020.*

breeds of commercial importance	**reported year of first description**	**traditional varieties**	**reported year of first description**
Granny Smith	1868	**Goldparmäne**	before 1700
Golden Delicious	ca. 1890	**Bohnapfel**	after 1750
Gala	1934	**Altländer Pfannkuchenapfel**	before 1840
Jonagold	1943	**Gewürzluiken**	1885
Elstar	1955	**Brettacher**	ca. 1900
Braeburn	1989	**Ingrid Marie**	1910

Apples are either produced by conventional, integrated pest management/integrated production (IP) or organic production (ORG) methods. These methods differ by the allowed pesticide formulations. Independent of the production method applied, pest control and disease prevention is necessary for commercial fruit production (Simon et al., 2011). In organic farming, only substances that are registered in the positive list in the EU Commission Implementing Regulation (EU) 2021/1165 (European Parliament and of the Council of the European Union, 2018) may be used. In general, synthetic-chemical pesticides and fertilizers are forbidden (European Commission, 2021; Federal Ministry of Food and Agriculture Germany, 2022), while in conventional production and IP these formulations can be utilized. Production according to the ORG or IP guidelines is compulsory in the EU, as conventional farming is no longer allowed (Damos et al., 2015; European Parliament and of the Council of the European Union, 2009). IP is a combination of ORG and conventional production methods aiming to minimize the use of pesticides. In IP, chemically synthesized pesticides should only be employed as the last resort, if other pest management tools, e.g., biological or physical methods are not available or fail (Damos et al., 2015).

Apple storage. Apples from domestic production are available to consumers in high-quality all year long (Streif et al., 2010). This is possible due to optimized storage conditions, keeping the fruit fresh and crisp. However, unsuitable storage parameters, e.g., too low temperatures or too low oxygen partial pressure, can negatively affect the fruit quality (Streif et al., 2010; Wright et al., 2015). In general, only intact and healthy fruit can be stored for longer periods of time.

Ripening and ultimately senescence is a highly regulated process in apples, which is genetically controlled and characterized by a loss of water, formation of aroma components, metabolization of starch to sugars and reddening of the fruit due to chlorophyll degradation (figure 2-2) (Kvikliene et al., 2006; Prasanna et al., 2007; Streif et al., 2010). The fruit also softens, as the cell walls are degraded by enzymes and the cells lose their cohesion (Brummell and Harpster, 2001; Gwanpua et al., 2014; Wei et al., 2010).

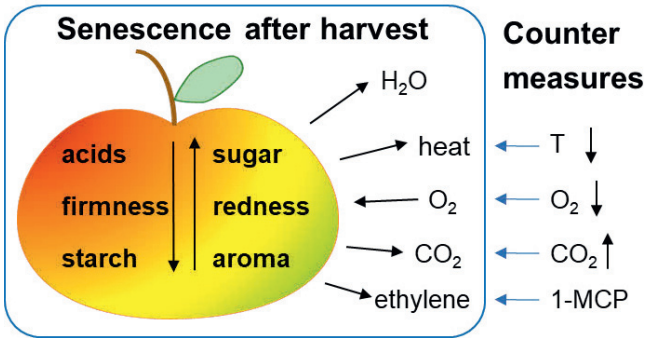

Figure 2-2: *Changes in apples during ripening and senescence and possible countermeasures taken during storage to slow down ripening. Graphic adopted from the master thesis of L. Straub, 2022.*

Many metabolic processes related to ripening are initiated and controlled by the gaseous phytohormone ethylene, which is also produced by the apple itself (Brummell and Harpster, 2001; Gwanpua et al., 2014; Prasanna et al., 2007). Additionally, exogenous ethylene can induce ripening in other apples.

Different storage methods and parameters are applied for apple storage depending on variety, region of production and technical facilities available (Prange and Wright, 2023). The methods all aim to slow down ripening by reducing respiration (Streif et al., 2010). The most basic method of storage is keeping the apples at low temperatures (-0.5°C – 4°C) and in darkness, to slow down the metabolism. Additionally, the low temperature can increase the humidity in the storage unit. A higher humidity lowers the water vapor deficit, which reduces the loss of water (Prange and Wright, 2023). More elaborate storage methods control the composition of the air in the storage units (Streif et al., 2010; Wright et al., 2015). By reducing the oxygen and increasing the carbon dioxide levels in addition to cold storage, respiration of an apple can be further slowed. The formation of ethylene is reduced as well by a reduced oxygen content (Gwanpua et al., 2014; Wright et al., 2015). As ethylene is important for the formation of some volatile aroma compounds, storage methods reducing ethylene formation might negatively affect fruit aroma, especially if the fruit are marketed directly after the removal from storage units without a post-harvest period at room temperature (Ferenczi et al., 2006).

A relatively new method for ripening control of apples is the application of the ethylene receptor blocker 1-methylcylcopropen (1-MCP) at the beginning of storage (Blankenship and Dole, 2003; Fan et al., 1999). This chemical is structurally similar to ethylene and blocks the ethylene receptors in apples, preventing ethylene induced ripening processes like fruit softening and ethylene synthesis (Blankenship and Dole, 2003; Storch et al., 2015). However, 1-MCP application is not allowed in organically produced fruits (European Commission, 2021). Additionally, not all apple varieties are suitable to be treated with 1-MCP (Streif et al., 2010).

2.2. Health-Related Constituents in Apples

Composition and health benefits accorded to a regular apple consumption. Apples consist of approximately 85% water, 14% carbohydrates, 0.3% protein, 0.3% fat and minor compounds such as minerals, vitamins, organic acids, carotenoids and phenolic compounds (Bondonno et al., 2017; Jensen et al., 2009). The carbohydrates entail sugars, mainly fructose and insoluble and soluble fiber (3%) (Jensen et al., 2009). Soluble fiber is predominantly pectin, while the insoluble fiber (> 50% of total fiber content) is mostly cellulose and hemicellulose (Bondonno et al., 2017).

Epidemiological studies indicate a positive health effect of a diet rich in fruits and vegetables (Boyer and Liu, 2004; Starowicz et al., 2020; van Duyn and Pivonka, 2000). To a regular apple consumption a reduced risk of cardiovascular disease, diabetes type II, several cancer types, osteoporosis and asthma is ascribed (Bondonno et al., 2017; Boyer and Liu, 2004; Ferretti et al., 2014; Hyson, 2011; Starowicz et al., 2020). These positive effects are mainly attributed to the polyphenols (PP), especially flavonoids, and fibers present in the fruit (see chapter 2.2.2).

2.2.1. Apple Allergy – Prevalence and Allergens

2.2.1.1. Food Allergy and its Diagnosis

Immunoglobulin E (IgE)-mediated food hypersensitivities. Besides the positive health benefits attributed to apples, they can also cause allergic reactions (Fernández-Rivas et al., 2006; Wagner et al., 2016). A food allergy is defined as an adverse, reproducible and specific health effect of the immune system, triggered by an exposure

to a given food (Boyce et al., 2010). The majority of food allergies are caused by IgE-mediated reactions, also known as immediate type hypersensitivities or type I reactions (Kumar et al., 2012). The onset of symptoms of IgE-mediated allergic reactions is fast, usually within minutes to hours after the exposure to the offending food. The immunochemical mechanism is complex and occurs in two steps, which are described in a simplified manner in the following paragraphs and figure 2-3.

The first step of an IgE-mediated food allergy, is the sensitization by the allergen (antigen), which is usually a protein or glycoprotein (Anvari et al., 2019; Bonilla and Oettgen, 2010; Bröker et al., 2019; Kumar et al., 2012; Valenta et al., 2015; van Ree and Aalberse, 2016). During primary sensitization the immune system is exposed to the allergen for the first time and recognizes the protein as foreign and pathogen. Antigen presenting cells (APC), especially dendritic cells, capture the protein and degrade it into peptide fragments, which are presented on the cell surface. In the next step, naïve T-cells with a T-cell receptor that can bind to the presented peptide on the APCs, are activated and differentiate to type 2 helper-T-cells (T_2H).

Every naïve B-cell expresses an antibody (IgM) with a specific binding region as receptors on its surface. If the receptors bind the antigens of the allergens and two vicinal receptors are cross-linked, the B-cell is activated. The B-cell takes up the antigens and presents peptide fragments on its surface. If a T_2H-cell binds an activated B-cell, it secretes cytokines (interleukin (IL)-4 and IL-13), which induce differentiation of the B-cell to a plasma cell and a class switch of the produced antibodies from IgM to IgE. Afterward, the plasma cell produces IgE antibodies with the same specificity as the B-cell receptors (IgM). The secreted IgE then bind to FcεRI receptors on the surface of effector cells, like mast cells or basophils. In general, IgE binding affinity is not directed towards the entire allergen, but rather towards a specific part, namely the epitope. During the sensitization stage, no or only mild allergic symptoms occur.