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The biochemistry of cocoa flavor – A holistic analysis of its development along the processing chain

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Summary

The seeds of the cacao tree *Theobroma cacao* L. are the key ingredient of chocolate (CODEX ALIMENTARIUS, 1981). They have a unique and complex flavor profile. Up to 87 descriptors can be distinguished (JANUSZEWSKA et al., 2018). Beside the typical chocolate aroma, floral, fruity and nutty aroma notes may occur. Regarding taste attributes, typical cocoa bitterness and acidity are of key importance. A pleasant, mouth-filling astringency does complete the typical flavor characteristics. These attributes can be more or less intense, or even absent as in the case of floral, fruity and nutty notes, resulting in a great diversity in the flavor profile between countries, regions and even plantations (SUKHA et al., 2007). This diversity depends on a network of multiple interacting factors along the processing chain, from the genetic background of the cacao trees, seed physiology and climatic conditions over fermentation, drying and roasting up to chocolate ingredients such as sugar (ANDERSSON et al., 2006; AFOAKWA et al., 2008; MUÑOZ et al., 2019). The purpose of this review is to analyze the current knowledge about the impact of these factors and their interaction on the composition of taste- and aroma-active substances as well as respective precursors and how specific flavor profiles can be explained through them. Gaps in research as well as potential applications in cocoa processing and chocolate manufacturing are discussed.

Origin and distribution of the cacao tree and the genetic determination of the flavor potential

The cacao trees origin are the rainforests of the eastern Andean slopes of the Amazonian basin. In these areas, the greatest genetic diversity has been observed. From the center of origin a natural distribution along the rivers of the Amazonian basin took place (Fig. 1A) (BARTLEY, 2005; MOTAMAYOR et al., 2008). At trade level, cacao trees from this huge area are grouped as 'Forastero' (ROHSIUS, 2007). Scientifically, the group of Forastero is divided into eight genetic clusters (MOTAMAYOR et al., 2008). Approximately 1,500 years BC cacao was distributed by Olmec's to Central America where it gained increasing cultural and economic importance, especially in the civilizations of Maya and Aztecs. It was cultivated in plantations and apparently subjected to breeding (BARTLEY, 2005; ROHSIUS, 2007). This lead to a clear genetic distinction from cacao trees in the Amazonian basin. The cacao from these areas in Central America is named 'Criollo' at both, trade and scientific level and forms an independent ninth genetic cluster (MOTAMAYOR et al., 2008). The genetic diversity manifests also in the biochemical composition of the cacao seeds. Whilst Forastero seeds are mostly of dark violet color, Criollo seeds are usually of lighter color up to white, due to a lack in anthocyanin (Fig. 1A). Sensory attributes also are affected. Criollo is often described as mild and less astringent and bitter (ELWERS et al., 2009). Recently, traces of an even earlier cultivation and use of cacao located in South America at the Santa Ana-La Florida site in southeast Ecuador, dating back about 5,300 years BC, have been reported by ZARILLO et al. (2018).

A younger cultivation history might have the cacao nowadays grown in Ecuador. First records about commercial plantings are from the

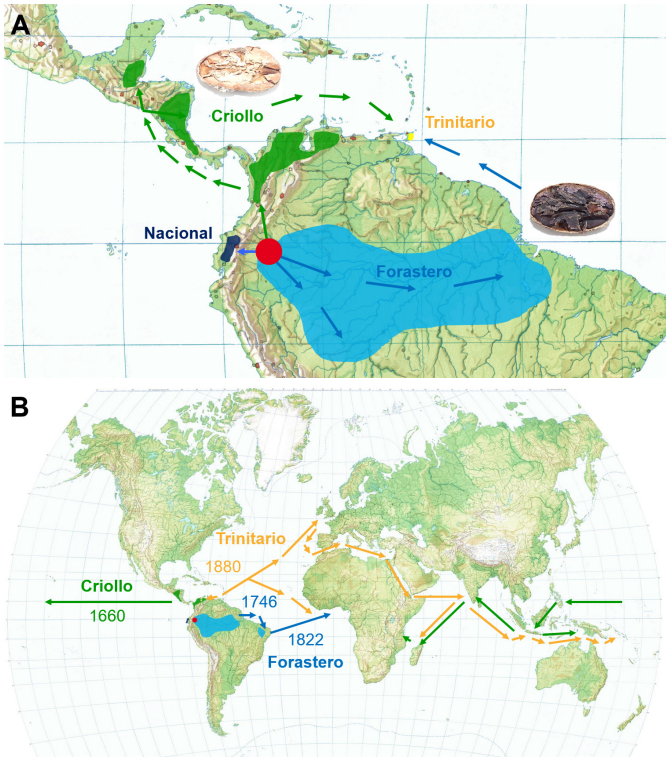

Fig. 1: Historical distribution of the cacao tree (*Theobroma cacao* L.) in the Americas (A) and Worldwide (B) modified from ROHSIUS (2007). Red dot: Center of Origin. (Map: http://www.reliefs.ch/blasse_weltkarte.jpg. Iimages of cocoa taken from ROHSIUS (2007)).

Europeans and date back 420 years (Fig. 1A). According to historical sources, the first plantations were located at the Guayas river shores and spread in its tributaries the rivers Daule and Babahoyo – upward rivers – probably the origin of the trade name 'Arriba' of Ecuadorean cocoa (LOOR et al., 2010). However, a previous distribution by the native population seems most likely (ROHSIUS, 2007; ZARILLO et al., 2018). Selections for distribution might have been made based on the aromatic, floral fruit pulp aroma (LIEBEREI, personal communication). As traditional cultivar, this cacao also forms a distinct tenth genetic cluster named 'Nacional' at scientific level (MOTAMAYOR et al., 2008), a term sometimes also used by the trade, i.e. 'Nacional' or 'Arriba Nacional'. Nacional is known to develop a strong floral aroma (LOOR et al., 2010).

Criollo was the first cocoa encountered by Europeans after the discovery of the American continent in 1492 A.D. When approximately 100 years later the cocoa consumption in Europe increased strongly, the cultivation of Criollo was extended to the Caribbean islands, including Trinidad (ROHSIUS, 2007). In 1670 A.D. these plantations were largely destroyed by a disease and new cacao trees from the populations in South America, discovered in the meanwhile, were introduced. In the rehabilitated plantations, now containing Criollo

and cacao from the genetic clusters in the Amazonian basin, crossings occurred. Recalling their origin Trinidad, these crossings are named Trinitario at scientific and at trade level (Fig. 1A) (Bartley, 2005; Rohsius, 2007). They can be assigned to different genetic clusters, depending on whether the Criollo or the Forastero background is dominant (Johnson et al., 2009; Gopaulchan et al., 2017). Thus, whilst at scientific level ten genetic clusters are distinguished, at trade level there are four groups: Forastero, Criollo, Nacional and Trinitario. From Criollo, Nacional and Trinitario trees so called fine or flavor cocoa is produced (International Cocoa Organization, 2019). It is characterized by the presence of ancillary flavor notes such as the floral aromas in case of Nacional seeds (Loor et al., 2010), fruity notes in case of Trinitario cocoa and nutty flavors, characteristic for example for some Criollo varieties (e.g. Sukha and Butler, 2005). Such aromas do not occur in most Forastero seeds. For them a strong basic chocolate aroma is typical (Sukha et al., 2007). Accordingly, the potential to develop fine flavor notes is genetically determined. Therefore, the genetic structure of the plantations is one essential criterion to gain fine flavor status. Two more criteria need to be fulfilled, appropriate post-harvest processing, i.e. fermentation, and the exportation of cocoa. Approximately 8% of the cocoa produced worldwide is fine or flavor cocoa, 92% are bulk cocoa (Quarterly Bulletin of Cocoa Statistics, 2019).

From 1660 A.D. on the Spanish distributed Criollo trees to Asia and East Africa via the Pacific route (Fig. 1B) (Bartley, 2005; Rohsius, 2007). Indeed, in several countries and regions, such as Madagascar and Java, Criollo descendants are still planted and they have fine flavor status (International Cocoa Organization, 2019). In 1746 A.D., the first plantations of Forastero, of genotypes belonging to the Amelonado cluster, were established in Bahia (Bartley, 2005; Rohsius, 2007; Motamayor et al., 2008). Indeed, Brazil nowadays produces bulk cocoa (International Cocoa Organization, 2019). From Bahia, starting in 1822 A.D., cocoa trees were distributed to West Africa (Fig. 1B). Descendants of these trees were later referred to as 'West African Amelonado' (Rohsius, 2007). Nowadays, more than 76% of the worldwide cocoa production is produced in West Africa (Quarterly Bulletin of Cocoa Statistics, 2019). Because of the genetic background of the trees, exclusively bulk coco is produced (International Cocoa Organization, 2019). 17.2% of the cocoa comes from South America, 6.3% from Asia and Oceania (Quarterly Bulletin of Cocoa Statistics, 2019).

From 1880 A.D. on, Trinitario varieties were distributed from Trinidad to South America, Asia and Africa (Fig. 1B) (Bartley, 2005; Rohsius, 2007). Indeed, countries with Trinitario plantations, such as Trinidad itself or Vietnam have fine flavor status. Beside the genetic structure of the plantations, they also do fulfill the criteria of appropriate fermentation and exportation of cocoa, like all the countries mentioned. A full list of the countries producing and exporting fine or flavor cocoa can be found in the annex c of the international cocoa agreement (International Cocoa Organization, 2019).

Because of this historical distribution and the genetic structure of plantings it is connected with, as well as traditional processing protocols analyzed in the following paragraphs, countries are associated with specific flavor profiles. In the last decades, changes in the genetic composition of plantations have been observed worldwide (Meinhardt, 2019). These changes are due to new selections and breeding approaches, the introduction of genetic material from other countries and farmer selections. Consequently, typical traditional flavor profiles are changing. For studies about the development of specific flavor profiles, it is therefore important to analyze the genetic background of cocoa samples. Meinhardt (2019) has recently proposed a methodology based on Single Nucleotide Polymorphisms (SNPs), allowing the verification of the genetic background of single fermented and dried cocoa seeds. Based on this method, one hundred seeds per cocoa sample can easily be analyzed, providing detailed insight into the genetic background. The great genetic diversity, described by Motamayor et al. (2008), further underlines the necessity of a more detailed understanding of the impact of the genetic clusters and the genotypes on flavor and how specific flavor attributes are obtained.

Cocoa seed and fruit pulp morphology

Cocoa fruits are of various shape and color and contain up to 50 seeds. Each seed is surrounded by an aqueous, sweet-sour tasting and sometimes aromatic fruit pulp, which derives from the fruits endocarp (Fig. 2) (Lieberei and Reisdorff, 2007). The aroma of the fruit pulp that can be of floral or fruity character has been linked to the fine flavor attributes of the seeds. It is supposed that the respective aroma-active substances migrate into the seeds during fermentation (e.g. Eskes et al., 2018). The fruit pulp stays attached to a hardened but flexible seed shell, protecting the embryo from mechanical damage (Lieberei and Reisdorff, 2007). It contributes to between 10 and 14% of the seeds dry weight. The embryo consists of two large storage cotyledons, comprising 86-90% of the dry weight (Muñoz et al., 2019), a small hypocotyl and an even smaller radicle (Fig. 2). Cocoa seeds are recalcitrant and contain about 27 to 40% of water upon ripeness (Stoll, 2010).

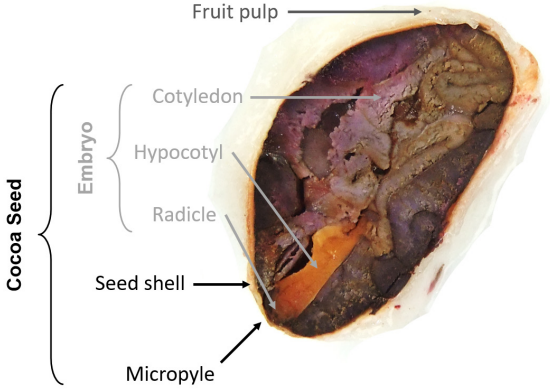

Fig. 2: Cocoa seed morphology

Fermentation, drying and their impact on the flavor profile

Fresh cocoa seeds have a very low storage stability, because of their high water content and the aqueous fruit pulp. They are characterized by an unpleasant bitter taste and a mouth-drying astringency, except for some Criollo genotypes. Fresh cocoa seeds do not develop any chocolate aroma and no typical bitterness during roasting, because they do not contain the necessary precursors (Ziegleder and Biehl, 1988; Stark and Hofmann, 2005). These attributes drastically change during primary post-harvest processing, consisting of fermentation and drying.

For fermentation, the seeds with the attached fruit pulp are removed from the fruits and transferred into wooden boxes or heaped on banana leaves. Sizes do vary and the boxes may contain from 200 kg of seeds up to several tons. During fruit opening, a spontaneous inoculation of the fruit pulp with microorganisms from the environment takes place. Further inoculation may come from the boxes, the surface of the banana leaves or from fruit flies (Schwan and Wheals, 2004; Weckx and De Vuyst, 2016). Formulations of starter cultures have been suggested (e.g. Lefeber et al., 2012), but are not frequently applied so far. The microorganisms find in the fruit pulp an ideal growth media. Their activity results in a degradation of the fruit pulp, heating of the fermentation mass and acidification of the seed tissue (Schwan and Wheals, 2004; Weckx and De Vuyst, 2016).

Under these conditions, the chocolate aroma precursor formation from seed storage components initiates and the precursors of typical cocoa bitterness are produced. Substances responsible for unpleasant bitterness and astringency are oxidized (ZIEGLEDER and BIEHL, 1988; STARK et al., 2006; ELWERS et al., 2009; VOIGT et al., 2016). The use of starter cultures can reliably improve the fermentation, potentially leading to higher consistency within this important processing step and consequently also with regard to flavor quality (LEFEBER et al., 2012).

The oxidation processes and the aroma precursor formation proceed during the drying process, following upon completion of fermentation (ZIEGLEDER, 2016). The residual moisture content is reduced to approximately 7%. The cocoa seeds become storage stable for several years, are low in unpleasant bitterness and astringency and develop chocolate aroma and typical cocoa bitterness during roasting.

Fermentation time may vary from as few as two days up to seven days. With increasing fermentation time unpleasant bitterness and astringency are more and more reduced. Increasing amounts of chocolate flavor precursors are produced (ROHSIUS, 2007; KUMARI et al., 2016), resulting in increased chocolate flavor intensity after roasting. However, fine flavor aroma, such as floral notes, seem to be reduced in their intensity with increasing fermentation time and can be completely lost (ESKES et al., 2018). Indeed, cocoa with fine flavor potential is usually fermented for less time than bulk cocoa, to obtain an optimal flavor profile (ROHSIUS, 2007). Such traditional local fermentation protocols thus are essential for the origin-specific flavor profiles. In other words, the flavor potential is genetically determined, but the flavor profile is defined during fermentation and drying. Hereby, fermentation protocols include the opportunity to diversify flavor. Cocoas with distinct flavor profiles, for example with light chocolate aroma and pronounced floral notes, or with intense chocolate aroma and light or no floral notes, can be produced from the same batch of fresh beans using adapted protocols. However, the potential to develop floral aroma or other fine flavor notes remains genetically determined. Beside the duration, also the turning interval has great impact onto the flavor profile (CAOBISCO/ECA/FCC Cocoa Beans Manual, 2015).

The concept of terroir in cocoa

Terroir is a highly important concept in viticulture (VAN LEEUWEN and SEGUINE, 2006). In cocoa, where terroir effects similar to wines are implied in many origin specific cocoas and chocolates, the impact has not yet been systematically tested (SUKHA et al., 2017). In general, Terroir can be defined as an interactive ecosystem, in a given place, including climate, soil and the crop plant (SEGUIN, 1988; VAN LEEUWEN and SEGUINE, 2006). Human factors, such as history, socioeconomics, as well as crop-cultivation and processing techniques, are also part of terroir. Due to the many factors being involved and because these factors are interacting, Terroir is difficult to study (SEGUIN, 1986; VAN LEEUWEN and SEGUINE, 2006).

For cocoa, especially the importance of traditional origin-specific fermentation protocols for the flavor profile is well known and has been described in the above paragraph. In addition to this, SUKHA et al. (2017) report impact of the growing environment on the intensity of the astringency, the bitter taste and the fruity aroma. The processing location may apparently affect the intensity of fruity, floral and nutty aromas and of the acidic taste. It remains however elusive, which components of the processing location and which environmental factors affect the flavor development. Additional insides to this are provided by HEGMANN (2015), who shows that higher amounts of rainfall may potentially result in increased intensity of floral notes. Findings of ZUG et al. (submitted) indicate that there might be an impact of the soil pH on components contributing to astringency and bitterness. However, as pointed out by SUKHA et al. (2017), systematical studies are needed to better understand Terroir effects in cocoa.

Quality analysis by means of the cut test

Fermentation and subsequent drying go along with a change in color of the cotyledons. Fresh seeds can be violet, slight-pink, rosé or white, depending on the genetic background (ROHSIUS, 2007). When dried unfermented, violet seeds are of light-violet color and waxy consistence (Fig. 3). They are called 'slaty'. Upon acidification, the color turns into intense dark violet (Fig. 3). These seeds are still under-fermented and called 'violet'. With ongoing fermentation, the color starts turning brownish, often from the center of the seeds. Such seeds are partially fermented. Only upon completion of the fermentation process, their cotyledon color turns fully brownish (Fig. 3). Consequently, the respective seeds are called 'brown' (ROHSIUS, 2007). Thus, cotyledon color allows an assumption about the flavor attributes of a batch of cocoa. When a batch contains significant amounts of slaty seeds, it will usually be characterized by unpleasant bitterness and astringency and a lack of chocolate flavor after roasting. With increasing amounts of partially and fully fermented seeds and a residual quantity of violets, light chocolate aroma will become characteristic. Fine aroma attributes are preserved. When most of the seeds are fully fermented, the chocolate flavor will be well pronounced, but fine aroma might be lost.

An assumption about the genetic background also is possible, as slight-pink, rosé and white seeds maintain their lighter appearance during fermentation and drying, turning to light brown, beige or remain of almost white color (ROHSIUS, 2007). Forastero seeds are usually dark violet, turning dark brown during fermentation and drying. In contrast, pure Criollo seeds stay almost white (Fig. 1A). Trinitario seeds can display a mixture of colors, from dark brown, over light brown to beige and almost white. The ratios may largely vary. However, exceptions do occur. Catongo for example, a Forastero cacao, has white seeds too (ROHSIUS, 2007). The evaluation of cotyledon color, the Cut Test, is a widely used industry standard (CAOBISCO/ECA/FCC Cocoa Beans Manual, 2015).

Fig. 3: Color change of Cocoa seeds during fermentation and drying. (images of cocoa taken from ROHSIUS (2007)).

Fresh cocoa bean ingredients and their impact on flavor

Cocoa beans are characterized by a unique composition of different nutrients. Beside fat, which contributes to about 50% of the dry weight, the seeds contain 12% protein and 7% carbohydrates. Moreover, they are rich in phenolic substances, about 15% of the dry weight. Alkaloids contribute to about 4% of the dry weight (e.g. KADOW et al., 2015). Both groups, phenolic substances and alkaloids, are of key importance for the flavor profile of fresh cocoa seeds.

Amongst the phenolic substances are epicatechin, the predominant one in quantity, and catechin. Both are often linked to unpleasant bitterness and astringency (e.g. ELWERS et al., 2009), especially in unfermented and under-fermented seeds. The flavor attributes of epicatechin and catechin are described as both, bitter and puckering astringent (STARK et al., 2006). ELWERS et al. (2009) observed no significant variation between seeds with different genetic background regarding the epicatechin concentration. However, the concentration varies from 14 - 21 $g \cdot kg^{-1}$ of cocoa nibs. Broader variation was found

for catechin. Whilst some genoytpes do not contain any catechin, in others up to 1.1 $g \cdot kg^{-1}$ were measured (Elwers et al., 2009). To valuate the contribution of a substance to a sensory attribute, its threshold concentration (TC) for human perception needs to be considered (e.g. Stark et al., 2006). Only if the concentration is above threshold, a contribution to the taste profile is likely and the substance can be regarded as probably taste-active. The TC of the bitter taste of epicatechin and catechin in cocoa butter is 2.1 $g \cdot kg^{-1}$ and 0.9 $g \cdot kg^{-1}$, respectively. Dividing the concentration measured in the seeds by the TC, the dose over threshold factor (DoT) is calculated, i.e. the factor by that the concentration exceeds the specific TC. A DoT factor greater than one makes it likely, that the respective substance contributes to the taste profile (Stark et al., 2006). The DoT factor for bitter taste of epicatechin varies from 6.5 to 10.3, whilst for catechin a maximum DoT of 1.2 results, based on the concentrations reported by Elwers et al. (2009). Thus, in fresh beans epicatechin contributes most likely to the unpleasant bitter taste. Catechin might contribute as well. The DoT factors for the puckering astringency caused by epicatchin vary from 9.5 to 15.1 between samples. For catechin a DoT of 0.9 is not exceeded. Thus, epicatechin contributes most likely to the unpleasant astringency of unfermented and under-fermented seeds. Catechin apparently does not.

Alkaloids, in particular theobromine, also contribute to the bitter taste of fresh cocoa seeds. The concentration in fermented and dried seeds varies from 9.1 to 17.6 $g \cdot kg^{-1}$ (Rohsius, 2007), depending on the genetic background. The respective DoT factor range is 15.3 to 29.5. During fermentation and drying the theobromine content slightly decreases to approximately 81% of the initial amount (Peláez et al., 2016). Thus, it can be assumed that the DoTs in unfermented seeds are slightly higher, 19 to 37 approximately. Caffeine occurs in lower concentrations, 0.5 to 4.2 $g \cdot kg^{-1}$ in fermented dried seeds (Rohsius, 2007). DoT factors are between 0.4 and 3.1. However, caffeine seems to decrease to approximately 50% of the initial amount during fermentation and drying (Muñoz et al., 2019). Accordingly, DoT factors in fresh seeds might be as high as 0.8 to 6.2. Nevertheless, it apparently depends on the genotype, if caffeine contributes to the bitter taste of fresh seeds or not.

For puckering astringency, also conjugates of cinnamic acid and amino acids are of importance. Caffeic acid aspartate for example occurs in concentrations of up to 3.3 $g \cdot kg^{-1}$ (Elwers et al., 2009). Having a low TC, the DoT factor of caffeic acid aspartate can be as high as 59 in fresh seeds, indicating its importance for puckering astringency. However, some genotypes do only contain marginal amounts or even no caffeic acid aspartate (Elwers et al., 2009).

Fresh fruit pulp ingredients and their impact on flavor

Solid in under-ripe fruits, the fruit pulp turns mucilageous in fully ripe fruits, because of the fruit own pectinolytic activity. The fruit pulp of ripe fruits is aqueous, containing about 83% of water (Pettipher, 1986). It is also rich in carbohydrates. A sucrose concentration of 13 up to 43 $g \cdot kg^{-1}$ is typical (Pettipher, 1986), explaining the sweet taste. The TC for sucrose is 4.3 $g \cdot kg^{-1}$, in water (Stark et al., 2006). The DoT range is 3 to 10, accordingly. The sweetness is contrasted by a pleasant acidity, caused by the approximately 2% of organic acids that the fruit pulp contains. The greatest part of it is citric acid. 3 to 13 $g \cdot kg^{-1}$ are typical. The TC of citric acid is 0.5 $g \cdot kg^{-1}$ in water (Stark et al., 2006). The respective DoT range is 6 to 26.

Amongst these major components, the fruit pulp contains variable amounts of aromatic substances: terpenes, alcohols and esters. Fruit pulp of fine or flavor cocoas seems to contain much higher amounts than fruit pulp of bulk cocoas does (Kadow et al., 2013). In line with this, the aroma intensity for example for floral notes of such genotypes, apparently caused by linalool and β-ocimene, is rated as high as nine and five on a scale from zero to ten in Solid Phase Micro-Extraction GC-olfactometry analysis. No floral aroma was observed in the bulk cocoa fruit pulp sample (Kadow et al., unpublished data). Kadow et al. based their study i.e. also the genotype selection on previous work by Eskes et al. (2007). The authors observed as well floral and fruity aroma only in the fruit pulp of the fine flavor genotypes they analyzed. These aroma notes were absent in bulk cocoa fruit pulp (Eskes et al., 2007). Linalool has often been linked to fine flavor quality and the floral notes of Ecuadorean Arriba (Ziegleder, 1990). Besides linalool, also 2-phenylethanol may contribute to floral aroma of fresh fruit pulp. Depending on the genotype, Chetschik et al. (2018) report concentrations of 250 $\mu g \cdot kg^{-1}$ of 2-phenylethanol and 14 $\mu g \cdot kg^{-1}$ of linalool. Like for taste-active substances, aromatic substances need to be above TC to contribute to the overall aroma impression. The TCs for 2-phenylethanol and linalool are 211 and 37 $\mu g \cdot kg^{-1}$, respectively. Apparently, only 2-phenylethanol contributes to the floral aroma of this specific fruit pulp, at least in its fresh status. 3-methylbutyl-acetate found in concentrations of 5 $\mu g \cdot kg^{-1}$ may provide a fruity character instead (Chetschik et al., 2018). The TC for 3-methylbutyl-acetate can be as low as 0.75 $\mu g \cdot kg^{-1}$.

Beside the genetic impact, the linalool concentration in the fruit pulp can be influenced by the degree of fruit ripeness and by climatic conditions. Hegmann (2015) report 10-fold increased linalool amounts during the rainy season. The findings add further information and evidence to the concept of Terroir in cocoa.

Biochemical changes during fermentation and their impact on flavor

Changes of Fruit Pulp Ingredients

The anaerobic phase

Beside sucrose, the fruit pulp contains significant amounts of fructose and glucose (Pettipher, 1986). Together with the acidic pH of about 3.6 ideal growth conditions for yeasts do result. Amongst other yeasts and in most of the fermentations worldwide, *Saccharomyces cerevisiae* establishes in the fruit pulp at the beginning of the fermentation process (Fig. 4). *Pichia* species such as *Pichia kluyveri* and *Candida* species have also often been found (Schwan and Wheals, 2004; De Vuyst and Weckx, 2016). Tight packaging of the cocoa seeds in the fermentation boxes and in the heaps together with the metabolic activity of the yeasts result in anaerobic conditions. Consequently, the yeasts do degrade the pulp sugars to alcohol, mainly ethanol (Fig. 4). In addition, they secret pectinase, resulting in further liquefaction of the fruit pulp (Schwan and Wheals, 2004; De Vuyst and Weckx, 2016). *Pichia* and *Candida* species do additionally metabolize the citric acid, causing an increase of the pH value of the fruit pulp (Schwan and Wheals, 2004). Moreover, *P. kluyveri* produces elevated amounts of esters with fruity aroma (Fig. 4). It is therefore used as starter culture (Crafack et al., 2013). Also *Saccharomyces cerevisiae*, strain H5S5K23, has been suggested for starter culture formulations, as it contributes to enhanced and consistent ethanol production (Lefeber et al., 2012). The increasing pH value, resulting from the citric acid degradation, allows the growth of bacteria. Lactic acid bacteria (LAB) do establish in the fermentation mass, shortly after the yeasts, amongst them many *Lactobacillus* species such as *L. plantarum* and *L. fermentum*. The great majority of LAB isolated from cocoa fermentations utilize glucose via the Embden-Meyerhof pathway yielding more than 85% lactic acid (Fig. 4). However, some species utilize glucose via the hexose monophosphate pathway producing 50% lactic acid, and in addition ethanol, acetic acid, glycerol and mannitol (Schwan and Wheals, 2004). At which time point in the fermentation LAB can establish could depend on the citric acid concentration. If the concentration is rather low, as observed for example in Malaysian cocoa, LAB could establish earlier, resulting in a LAB dominated fermentation. This may have impact on the fla-

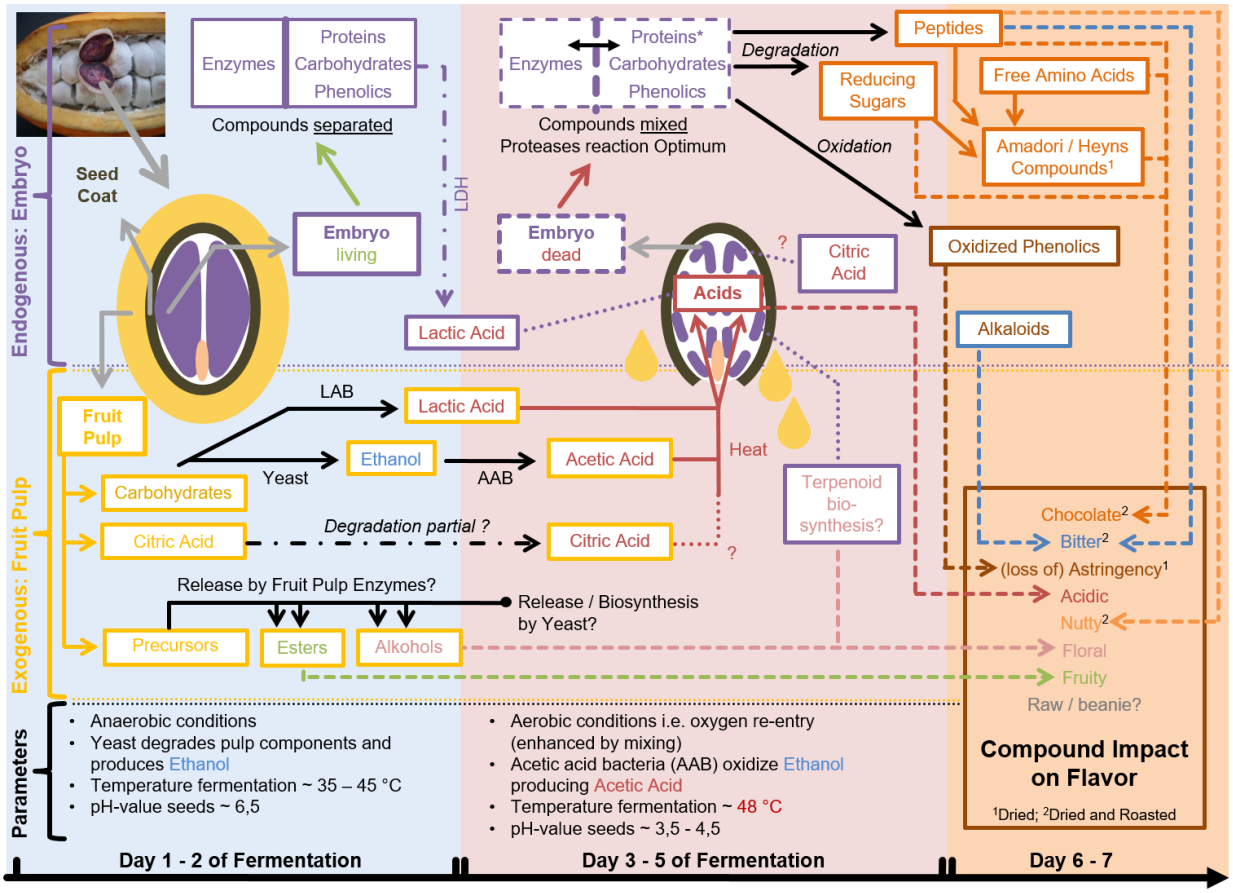

Fig. 4: Physicochemical networks during standard cocoa fermentation. Based on the discussions with Reinhard Lieberei, Christina Rohsius and Silke Elwers.

vor profile, as lactic acid has physico-chemical properties that differ from those of acetic acid and is hardly removed from the seeds during further processing. In their study, Ho et al. (2015) conclude that LAB may not be required for successful fermentation of cocoa seeds. However, Lefeber et al. (2012) report that a full starter culture, comprising beside *S. cerevisiae* H5S5K23 also *L. fermentum* 222 and *A. pasteurianus* 386B have been suggested for starter culture formulations as this allows a consistent production of high-quality fermented dry cocoa beans (Lefeber et al., 2012)

The aerobic phase

With its increasing liquefaction, the fruit pulp starts draining from the fermentation mass. Oxygen does re-enter. At this point in time, the fermentation mass is often turned, to allow equal oxygen distribution. With oxygen available, acetic acid bacteria (AAB), such as *Acetobacter aceti* and *Gluconobacter oxydans*, start dominating in the fermentation mass. In their review, Schwan and Wheals (2004) provide lists of yeast, LAB and AAB species detected in cocoa fermentations. AAB oxidize the ethanol, previously produced by yeast, to acetic acid (Fig. 4). Concentrations of a maximum of 6 $g \cdot L^{-1}$ of fruit pulp have been observed. The oxidation goes along with a heating of the fermentation mass and temperatures of 48 °C are usually reached (Schwan and Wheals, 2004). The acetic acid starts migrating into the seed tissue, where 1.5 to 12 $g \cdot kg^{-1}$ are found in fermented and dried seeds (Rohsius et al., 2010). At the beginning, this migration seems to take place mainly via the micropyle (Fig. 2; Fig. 4). The seed shell at this point is impermeable for acetic acid, which is accumulating in a sclerenchymatic cell layer with thick cell walls (Andersson et al., 2006). Thus, the velocity of the seed tissue acidification seems to depend on how fast the micropyle opens, a seed physiological variable, and on how fast the sclerenchymatic cell layer is saturated with acetic acid. If genetic variability exists with regard to this cell layer, and thus regarding the velocity of the acetic acid migration, has not yet been investigated.

Fine flavor substances

The aromatic alcohols, terpenes and esters, which the fruit pulp contains depending on the genotype, also undergo changes during the fermentation process. 2-phenylethanol increases 480fold, reaching a concentration of 120$mg \cdot kg^{-1}$ (Chetschik et al., 2018). Like for tastants the DoT, an odor activity value (OAV), i.e. the 2-phenylethanol concentration divided by its TC, can be calculated for aroma substances (Frauendorfer and Schieberle, 2008). The OAV of 2-phenylethanol increases from 1.2 in the fresh fruit pulp to 569 during fermentation. Accordingly, 2-phenylethanol now strongly contributes to the floral aroma of the fruit pulp. Fruit pulp aroma attributes are often used as indicator for fine flavor quality (Eskes et al., 2018). However, the findings of Chetschik et al. (2018) indicate that at least some floral aromas might be rather weak in ripe fruits and do strongly increase only during fermentation. In the seeds, the concentration of 2-phenylethanol increases from 42 $\mu g \cdot kg^{-1}$ to 16 $mg \cdot kg^{-1}$(Chetschik et al., 2018). Thus, the concentration in the fruit pulp is higher than in the seed tissue at any time during fermentation and might be the driving force of a migration into the cotyledons as suggested by Eskes et al. (2007; 2012; 2018). The authors found that when floral aroma notes are present in the fresh fruit pulp, these notes can be detected by means of sensory evaluation in the seeds after fermentation and drying (Eskes et al., 2007). When adding aromatic Annona fruit pulp to bulk cocoa fermentations, the Annona aroma traits were found in the dried seeds (Eskes et al.,

2012). Indeed, when subjecting fresh cocoa seeds to an air stream containing linalool or β-ocimene, the substances could both be detected in the seed tissue afterwards (KADOW et al., unpublished data). The OAV for 2-phenylethanol in the seed tissue increases from 0.2 and thus below threshold to 76. Accordingly, 2-phenylethanol confers most likely floral aroma to the seeds. A substantial decrease with increasing duration of the fermentation, especially in the seeds, has not been observed (CHETSCHIK et al., 2018). Thus, a loss of fine flavor attributes during prolonged fermentation is not necessarily caused by a loss of the respective fine aroma substances. The linalool concentration increases 12-fold in the fruit pulp during fermentation, reaching a concentration of 170 $\mu g \cdot kg^{-1}$ (CHETSCHIK et al., 2018), corresponding to an increase of the OAV from below threshold to 4.6. However, fresh cotyledons already contain 114 $\mu g \cdot kg^{-1}$. After five days of fermentation a concentration of 1130 $\mu g \cdot kg^{-1}$ is reached. Thus, the concentration is lower in the fruit pulp than in the cotyledons at any time of the fermentation process. A gradient, explaining a migration into the seeds, is not present in the case of linalool. It seems to be produced in both tissues and to a greater extent in the cotyledons (CHETSCHIK et al. 2018), where with an OAV of 31 linalool most likely contributes besides 2-phenylethanol to the floral aroma. Accordingly, fine flavor components may be fruit pulp derived, as in the case of 2-phenylethanol, or seed tissue derived as for linalool (Fig. 4). However, it needs to be considered that the fruit pulp largely consist of water. Linalool has a low solubility in water. The cotyledons instead contain high amounts of fat, with presumably better linalool solubility. Thus, a simple comparison of concentration gradients based on the weight of the tissues, may not adequately reflect the actual linalool migration gradient.

2-phenylethanol, which is clearly fruit pulp derived, can be synthesized through the Ehrlich pathway. Interestingly, this pathway depends on *S. cerevisiae*, present in cocoa fermentations worldwide. Phenylalanine is metabolized first into phenylpyruvate, next into phenylacetaldehyde and finally into 2-phenylethanol (CHETSCHIK et al., 2018). However, the phenylalanine content of the fruit pulp seems to be rather low. 1.3 $mg \cdot kg^{-1}$ have been found by PETTIPHER (1986). This quantity is not sufficient to explain the 120 $mg \cdot kg^{-1}$ of 2-phenylethanol, reported by CHETSCHIK et al. (2018). Thus, a 2-phenylethanol release from glycosylated precursors seems more likely (Fig. 4). However, it needs to be considered that PETTIPHER (1986) did his measurements on bulk cocoa fruit pulp. It cannot be excluded, that fruit pulp of fine flavor cocoas has higher phenylalanine concentrations. Another possibility is that phenylalanine is released from proteins, especially from storage proteins in the seeds, during fermentation. Nevertheless, when the free amino acid release from storage proteins initiates, the yeast populations in the fruit pulp already decreased. In addition, if storage protein derived phenylalanine would be the source of 2-phenylethanol formation, high concentrations should be found in any cocoa, not only in fine flavor cocoa.

Changes of Cotyledon Ingredients

Astringent and bitter-tasting substances

The acidification caused by the migration of acidic acid, together with the increasing temperature, result in cotyledon tissue death and thus in cell disintegration (SCHWAN and WHEALS, 2004). An aqueous and a fatty reaction space do result (e.g. ZIEGLEDER, 2014). Substrates and enzymes, previously stored in separate cell compartments get mixed. Such as the phenolic substances and polyphenol oxidase (PPO) in the aqueous reaction space (Fig. 4). Upon oxygen availability and a sufficiently high pH, the pH optimum of cocoa PPO is 6.4, PPO oxidizes catechin and epicatechin into the respective chinones. The chinones presumably react with proteins, carbohydrates, cell wall constituents and other cell substances, forming large insoluble polymers (AFOAKWA et al., 2008; ELWERS, 2008). However, the detailed reaction pathways that PPO activity leads to, still need further investigation. As consequence of the polymerization, the concentration of soluble catechin and epicatchin in the cotyledons decreases. ELWERS et al. (2009) report residual amounts of 8% for epicatechin and 1% for catechin. Only soluble epicatechin and catechin seem to contribute to unpleasant bitterness and astringency. Accordingly, the respective DoT factors for bitterness and astringency decrease (Fig. 4). Caffeic acid aspartate seems to be less affected. 33% of the initial amount remain soluble (ELWERS et al., 2009). However, many analyses on this decrease during fermentation, such as the ones of ELWERS et al. (2009), have been carried out on dried seeds. Thus, the impact of fermentation and the impact of drying cannot be fully distinguished. Future studies should consider to shock-freeze sample aliquots immediately in liquid nitrogen, to compare their biochemical composition to the one after drying.

Flavor precursor formation

Moreover, during fermentation a seed storage protein degradation by seed own enzymes takes place (VOIGT and BIEHL, 1995). It already starts during the first 24 hours of fermentation (KUMARI et al., 2016). These early degradation processes might be due to initiating germination of the seed. Cocoa seeds are recalcitrant and germination seems to be triggered by oxygen availability, thus quickly initiating after fruit opening (LIEBEREI, personal communication). Upon acidification and heating of the seeds, protein degradation greatly accelerates, as the involved proteases have a low pH and a high temperature optimum (HANSEN et al., 1998). The degradation consists in a two-step reaction. First, endoproteases cut the storage proteins into larger peptides of approximately 16 amino acids length on the first day of fermentation. Secondly, exoproteases cleave single amino acids (AA) from theses peptides (Fig. 4), resulting in increasing amounts of free AA and peptides of 13 to 14 AA length after 2 to 3 days of fermentation, 6 to 12 AA after 4 to 5 days of fermentation and finally 2 to 3 AA after 6 to 7 days of fermentation. In this way, from the vicilin-like globulin alone, contributing to 23% of the storage protein, about 560 different peptides can be formed (KUMARI et al., 2016).

The carbohydrates as well do undergo modification processes. In particular, sucrose is cleaved into fructose and glucose by invertase (HANSEN et al., 1998). Together with the free AA, these reducing sugars are precursors for the chocolate aroma formation during roasting (AFOAKWA et al., 2008). In addition, some of the peptides, such as arginine-asparagin-asparagin-proline-tyrosine-tyrosine-phenylalanine-proline-lysine, seem to contribute to chocolate aroma formation too (VOIGT et al., 2016). Di- and tri-peptides, such as valine-proline, are bitter-taste precursors (Fig. 3). They yield bitter tastants during the roasting process (STARK and HOFMANN, 2005).

Peptides are apparently also the precursors of nutty aroma notes (Fig. 4) (VOIGT et al., 2018). The formation of the specific peptides is favored by pH values above five. When the pH value is lower, ideally at about 4.8, increased amounts of shorter peptides and free AA seem to be produced (VOIGT et al., 2018), i.e. bitter taste and chocolate aroma precursors.

Fine Flavor Substance Biosynthesis

With regard to fine flavor substances, DE WEVER (2020) recently showed that in fine flavor cocoa the expression of genes from the secondary metabolites pathway including the terpenoid pathway is strongly upregulated during fermentation. This might explain the tenfold increase of the linalool concentration in the cotyledon tissue reported by CHETSCHIK et al. (2018). In bulk cocoa, no such upregulation of the respective genes was found (DE WEVER, 2020). The findings are further evidence that fine flavor substances are not always fruit pulp derived, but can be produced also in the cotyledons, depending on the type of substance.

Biochemical changes of cotyledon ingredients during drying and their impact on flavor

Aroma precursor formation

During the drying process, the free AA and the reducing sugars undergo further modifications. They react with each other, forming Amadori compounds, such as fructosyl-leucine and fructosyl-phenylalanine. Amadori compounds also are important precursors of chocolate aroma development (Fig. 4). ZIEGLEDER (2016) has studied their formation during the drying process in detail. Recently, ANDRUSZKIEWICZ et al. (2020) found that also di- and tri-peptides do react with the reducing sugars yielding the respective Amadori and Heyns compounds. Amongst them also fructosyl-valin-prolin. Presumably, these compounds do also function as aroma precursors (ANDRUSZKIEWICZ et al., 2020) suggesting a trade-off between di-peptide-based bitter taste and chocolate aroma. Depending on the drying protocol, more or less di-peptides could react to Amadori and Heyns compounds, hereby changing the ratios of bitter taste and chocolate aroma precursors. However, it still needs to be investigated if the di- and tri-peptide based Amadori and Heyns compounds are really formed during the drying process or already during fermentation.

Astringent and bitter-tasting substances

The oxidation of phenolic substances apparently proceeds, as long as a sufficient amount of free water is available. Consequently, unpleasant bitterness and astringency are further reduced. ELWERS et al. (2009) report epicatechin concentrations of 1.1 to 1.7 $g \cdot kg^{-1}$ in fermented dried seeds, corresponding to DoT factor ranges of 0.5 to 0.8 for bitter taste and 0.8 to 1.2 for puckering astringency. Catechin concentrations were as low as 11 $mg \cdot kg^{-1}$. The DoT factor for bitterness as well as for puckering astringency is 0.01. Thus, in the studied samples epicatechin and catechin do not contribute to the bitter taste of fermented dried seeds. Only epicatechin contributes marginally to the puckering astringency. However, TRAN et al. (2015) report residual epicatechin and catechin concentrations of up to 4 $g \cdot kg^{-1}$ and 0.25 $g \cdot kg^{-1}$, respectively. In such samples, epicatechin may contribute to both, bitterness and puckering astringency. The respective DoT factors are 1.9 and 2.8. The catechin concentration is still below threshold. The greater amounts found by TRAN et al. (2015) might be a result of the genetic background or, more likely, caused by a lower degree of fermentation. Residual amounts of cinnamic acid aspartate can be as high as 1.1 $g \cdot kg^{-1}$ (ELWERS et al., 2009). The respective DoT for puckering astringency is 19. Thus, cinnamic acid aspartate remains an important contributor to puckering astringency after fermentation and drying. Interestingly, it was found in higher concentrations in Criollo seeds, described as low in astringency, than in other cocoas (ELWERS et al., 2009).

Theobromine and caffeine do not undergo modifications during fermentation and drying. However, leaching during fermentation results in a decrease especially of the caffeine concentration (MUÑOZ et al., 2019). Nevertheless, theobromine still significantly contributes to the bitter taste of fermented dried seeds (Fig. 4). The contribution of caffeine is genotype dependent, as the concentration can be below TC.

Acidic tastants

Moreover, during drying, acetic acid evaporates, resulting in reduced intensity of the acidic taste. The concentrations found by ROHSIUS et al., (2010) correspond to a DoT factor range of 12 to 100. Drying technicians often report that quick drying, e.g. in 3 to 4 days, results in higher residual acetic acid concentrations in the seeds. These observations are supported by the findings of JINAP and THIEN (1994) and ZIEGLEDER (2016). Higher acididty is thought to cause not only a more acidic taste, but also more intense unpleasant bitterness and astringency. The observations might be explained with the pH optimum of the PPO. Only after evaporation of larger acetic acid quantities, the oxidation rate would be sufficiently high. However, it could well be that sufficient oxidation is mainly a question of time.

Fine flavor and ancillary aromas

Intermediate duration of drying, i.e. five days, is thought to favor the preservation of fruity aroma notes. Extending it to about seven days, results in a dominant chocolate aroma instead. Finally, with very slow drying regimes, i.e. about ten to fourteen days, tobacco- and leather-like aromas seem to occur (e.g. Ingemann Fine Cocoa). More research is needed to elucidate the aroma-active substances and the biochemical processes occurring during drying.

Quality analysis through quantification of flavor-related ingredients

The knowledge about the substances contributing directly, or indirectly as precursors, to cocoa flavor, can be used for quality analysis. Within the Cocoa Atlas for example, 143 cocoa samples from 32 origins were analyzed with regard to their free AA content, the free sugar concentration, the polyphenol amount as a simple sum parameter and other valuable ingredients (ROHSIUS et al., 2010). Samples from Ecuador were found to contain between 5.7 and 12.6 $mg \cdot g^{-1}$ fat free dry matter (ffdm) of free AA. In the Ghana samples 13.8 to 17.3 $mg \cdot g^{-1}$ ffdm were found. Unfermented cocoa contains up to 4 $mg \cdot g^{-1}$ ffdm, well fermented seeds instead 8.9 to 14.9 $mg \cdot g^{-1}$ ffdm of free AA. Thus, whilst the Ghana samples are all well fermented, some of the Ecuador samples are almost not fermented. The analytical picture becomes more detailed when looking additionally at the reducing sugars. The Ghana samples contain between 7.4 and 11.1 $mg \cdot g^{-1}$ ffdm of glucose and fructose, the Ecuador samples 3.3 to 7.9 $mg \cdot g^{-1}$ ffdm. The reason for the low amount in some of the Ecuador samples is the incomplete cleavage of sucrose, resulting from a low degree of fermentation. Whilst the residual sucrose amounts exceed 23 $mg \cdot g^{-1}$ ffdm in some of the Ecuador samples, the highest amount found in the Ghana samples was 1.1 $mg \cdot g^{-1}$ ffdm. In several of the Ghana samples sucrose was not detectable. Finally, the total phenolics amount can be considered. The Ghana samples contained 80 to 96 $mg \cdot g^{-1}$ ffdm. With 65 $mg \cdot g^{-1}$ ffdm, in some of the Ecuador samples the total phenolics concentration was lower. However, the ones with low free AA concentration contained as much as 140 $mg \cdot g^{-1}$ ffdm total phenolics (ROHSIUS et al., 2010). Such high concentrations are linked with an intensity of three to four, on a scale with a maximum of five, for unpleasant bitterness and astringency (KADOW, unpublished data). Accordingly, the mentioned cocoa seed ingredients can be used as biochemical quality parameters. However, the work-up procedures and measurements are rather time intensive. Near infrared spectroscopy (NIRS) has proofed to be a suitable tool to predict the concentration of many biochemical quality parameters within seconds and in a single measurement (KRÄEHMER et al., 2015). The velocity of NIRS allows its use in quality testing upon the arrival of batches of cocoa, as well as in-process applications.

Biochemical changes in the cotyledons during Roasting and their Impact on Flavor

Aroma-active substances

Strecker aldehydes

During roasting, numerous volatiles are formed from the previous obtained aroma precursors, amongst them Strecker aldehydes. 3-methylbutanal for example, having malty aroma attributes, increases 64fold during roasting. Phenylacetaldehyde, having honey-like aroma, does also increase 64-fold. The precursors are glucose

and fructose forming diketones in a first reaction, and Leucine and Phenylalanine, respectively. The strong increase of both substances during roasting is a valuable indicator for their importance to the aroma profile (FRAUENDORFER and SCHIEBERLE, 2008). However, more important is the OAV, the factor that the substance concentrations are above the respective threshold for human perception. 3-methylbutanal has an OAV of 2610, phenylacetaldehyde an OAV of 250. Both substances indeed are amongst the 25 substances indispensable for typical chocolate flavor (FRAUENDORFER and SCHIEBERLE, 2006). However, when combining glucose and leucine in roasting models, the formation of 3-methylbutanal is marginal. 25-times more of 3-methylbutanal are formed, when the respective Amadaori compound fructosyl-leucine is used as precursor. This demonstrates, that Amadori compounds, and not free AA and reducing sugars, are the key precursors of chocolate aroma formation (ZIEGLEDER, 2016). It might also explain the above-described observation that prolonged drying results in dominant chocolate aroma and diminished fruity notes. The formation of Amadori compounds mainly takes place when the residual moisture is below 20% (ZIEGLEDER, 2016). Prolonged drying might result in a prolongation of this phase and in increased Amadori compound formation. Consequently, the chocolate aroma intensity would increase, dominating the aroma profile.
In addition to the aroma-active Strecker aldehydes, GRANVOGL et al., (2012) report the detection of oxazolines in dark chocolates, amongst them 2-isobutyl-5-methyl-3-oxazoline. The substance is rapidly hydrolyzed upon contact with water, yielding 3-methylbutanal. Thus, an important part of the chocolate aroma substances might be released only during chocolate consumption i.e. upon the contact with saliva (GRANVOGL et al., 2012). It remains to be determined, during which processing step the oxazolines are formed in cocoa.

Pyrazines
Pyrazines do also strongly increase during roasting. 2-ethyl-3,5-dimethylpyrazine for example does as well increase 64-fold, corresponding to an OAV of 14. It has a potato chip-like aroma character (FRAUENDORFER and SCHIEBERLE, 2008). 2-ethyl-3,5-dimethylpyrazine can apparently be released from peptide precursors such as arginine-asparagin-asparagin-proline-tyrosine-tyrosine-phenylalanine-proline-lysine, identified by VOIGT et al. (2016). However, further research is needed to fully understand the role of peptides in chocolate aroma formation. It might well be that the glucosyl- and fructosyl-di- and tri-peptides first found in cocoa beans by ANDRUSZKIEWICZ et al., (2020) are the more important aroma precursors, as in the case of the free AA-derived Amadori compounds.

Furans
Furans as well are of key importance for chocolate aroma. 4-hydroxy-2,5-dimethyl-3(2H)-furanon for example increases 16-fold during the roasting process and has an OAV of 25. Its aroma character is described as caramel-like (FRAUENDORFER and SCHIEBERLE, 2008). It can be formed from the precursor mixture glucose and phenylalanine. However, again the formation is more efficient, i.e. 21-fold, when the respective Amadori compound fructosyl-phenylalanine is used, underlining once more the importance of Amadori compounds as chocolate aroma precursors (ZIEGLEDER, 2016). Their formation mainly takes place during the drying process, when the residual humidity is between 7 and 20%. The optimum temperature for increased Amadori compound formation is about 60 °C (ZIEGLEDER, 2016). Consequently and as mentioned previously, the drying process needs great attention to optimal develop the flavor profile of cocoa.

The odor object chocolate aroma
Some substances do not show any increase during roasting, but maintain their high OAV. This is the case for instance for 3-methyl-butanoic acid, having an OAV of 390 before and after roasting. 3-methylbutanoic acid can be produced from free AA by yeast and bacteria (COBAN and DEMIRCI, 2017). It has, as a single substance, a rancid aroma character (FRAUENDORFER and SCHIEBERLE, 2008). Thus, it might be regarded as an off-flavor, present in the samples analyzed, but this is apparently not the case. FRAUENDORFER and SCHIEBERLE (2006) demonstrate that 3-methyl-butanoic acid, together with the Strecker aldehydes, pyrazines, furans and other aroma-active substances, forms a new odor object (HOFMANN et al., 2014), divers from the aroma attributes of the single substances: typical chocolate aroma. In total, 24 substances are needed to reproduce the typical aroma of roasted cocoa nibs and powder. FRAUNDORFER and SCHIEBERLE (2006) provide a list with all 24 substances in their publication.

Fine flavor substances
For fine flavor cocoas, usually milder roasting conditions i.e. lower roasting temperatures are applied to preserve the fine aroma notes (CAOBISCO/ECA/FCC Cocoa Beans Manual, 2015). As discussed, linalool and 2-phenylethanol are key substances for floral fine aroma. In unroasted Criollo seeds FRAUENDORFER and SCHIEBERLE (2008) report OAVs of 29 and 76 for linalool and 2-phenylethanol, respectively. Upon roasting of the seeds at 95 °C and for 14 minutes, the linalool concentration remained stable. The 2-phenylethanol concentration increased from 16 $mg \cdot kg^{-1}$ to 34.3 $mg \cdot kg^{-1}$, corresponding to an OAV of 163 in the roasted seeds (FRAUENDORFER and SCHIEBERLE, 2008). Thus, fine aroma substance concentration does not necessarily decrease during roasting. That fine aroma attributes may nevertheless be reduced in their intensity or lost during roasting could be due to the formation of a new odor object (HOFMANN et al., 2014) with increasing concentration of Strecker aldehydes, pyrazines and furans at higher roasting temperatures. Accordingly, the odor object would change from mild chocolate aroma with clearly perceivable floral notes to intense chocolate aroma. Additional studies with multiple roasting regimes would be of interest to clarify this point. For such studies, it is not only of great importance to consider exact quantities and thresholds of the aroma-active substances, but also the respective ratios (HOFMANN et al., 2014). Which substances are responsible for fruity and nutty aroma notes still needs to be determined.
Roasted bulk cocoa seeds do as well contain linalool and 2-phenylethanol. However, the concentrations are marginal in contrast to fine flavor seeds. FRAUENDORFER and SCHIEBERLE (2006) report a linalool concentration of 70 $\mu g \cdot kg^{-1}$ and a 2-phenylethanol concentration of 0.6 $mg \cdot kg^{-1}$. The OAVs are as low as 2 and 3 for linalool and 2-phenylethanol, respectively. Thus, fine aroma substances do not exclusively occur in fine flavor cocoas, but in bulk cocoa the concentrations are too low for a significant contribution to the aroma profile.

Taste-active and astringent substances
Peptide-derived bitter tastants
The taste-active substance profile also undergoes major changes during the roasting process. Di- and tri-peptides such as valine-proline undergo cyclization, yielding bitter-tasting diketopiperazines, cyclo(L-proline-L-valine) when valine-proline is the precursor. The amount of cyclo(L-proline-L-valine) formed during roasting increases with increasing fermentation time, as more and more valine-proline is produced. How much of the peptide precursors react to diketopiperazines also depends on the roasting conditions. ANDRUSZKIEWICZ et al. (2019) report a two-fold increase of cyclo(L-proline-L-valine) at elevated roasting temperatures in comparison to lower temperature roasting regimes. In fully fermented and roasted seeds a cyclo(L-proline-L-valine) concentration of 1.7 $g \cdot kg^{-1}$ was found, corresponding to a DoT factor of 2 in cocoa butter. In total, up to 34 diketopiperazines have been detected in cocoa (ANDRUSZKIEWICZ et al., 2019). An additive effect is assumed regarding their bitter taste, making them key bitter tastants of roasted cocoa (STARK

and HOFMANN, 2005; STARK et al., 2006). Variation might also occur, depending on the storage protein quantity. KUMARI et al. (2018) report quantities ranging from 2 to 13% of protein, in cocoa seeds from different origin. For example about 3.5% in cocoa seeds from Indonesia and up to 12% in samples from Ivory Coast. This should have impact on the flavor precursor quantity, which can be obtained during fermentation. Indeed, ROHSIUS (2007) finds free AA amounts of about 7 $mg \cdot g^{-1}$ ffdm in some Indonesian samples, whilst those from Ivory Coast contain 12 to 18 $mg \cdot g^{-1}$ ffdm. However, the author also reports free AA amounts of up to 25 $mg \cdot g^{-1}$ ffdm for some of the samples from Indonesia (ROHSIUS, 2007). This underlines the importance of considering the multiple factors that influence the biochemical changes and thus the flavor development in cocoa along the complex processing chain.

Alkaloid bitter tastants

Theobromine is more or less process stable and still contributes to the bitter taste after fermentation, drying and roasting. A concentration corresponding to a DoT factor of 19 in cocoa butter has been reported. In contrast, the caffeine concentration was below threshold (STARK et al., 2006). However, as pointed out previously, depending on the genotype, the caffeine concentration can also be above threshold.

In addition to this, ZHANG et al. (2014) report the formation of catechin-C-spiro-glycosides during Maillard reactions. One of these spirocyclic catechin Maillard products significantly suppresses the bitterness of caffeine. The compound was identified in cocoa and increases 3-fold during roasting (ZHANG et al., 2014). Thus, cocoa bitterness might be further modulated through partial suppression of the alkaloid bitter taste.

Bitter and astringent substances

The epicatechin concentration further decreases during roasting, due to degradation and epimerization into catechin (URBAŃSKA et al., 2019). STARK et al. (2006) find an epicatechin concentration of 2.5 $g \cdot kg^{-1}$ in roasted cocoa nibs. This corresponds to a DoT factor of 1.2 for its bitter taste and 1.8 for astringency. With 0.69 $g \cdot kg^{-1}$ the catechin concentration is higher than in the fermented and dried bean samples analyzed by ELWERS et al. (2009) and TRAN et al. (2015). This might simply depend on a variation between samples. However, the catechin concentration might also increase during roasting, due to epimerization of epicatechin (URBAŃSKA et al., 2019). Anyhow, the concentration is still below threshold of human perception for bitterness as well as astringency.

In summary, the bitter taste changes completely during fermentation, drying and roasting, from a phenolic substances and alkaloid based one, to diketopiperazine and alkaloid dominated bitterness. Thus, presumably diketopiperazines and alkaloids together are responsible for the typical bitter taste of cocoa and chocolate (STARK et al., 2006).

Purely astringent substances

Caffeic acid aspartate also seems to decrease further during roasting. STARK et al. (2006) report a concentration of 0.48 $g \cdot kg^{-1}$, corresponding to DoT factor of 8.5 for its puckering astringency. Another N-phenylpropenoyl amino acid strongly contributing to puckering astringency is N-caffeoyl-L-dopa. A concentration of 0.31 $mg \cdot kg^{-1}$ was found in roasted seeds (STARK et al., 2006). This corresponds to a DoT factor of 15.

Another group of substances with astringent character is newly formed. During roasting, epicatechin and catechin react in non-enzymatic reactions with glucose, yielding phenol glycoconjugates, such as catechin-6-C,8-C-β-D-diglucopyranoside and epicatechin-8-C-β-D-glucopyranoside. They are described as velvety, smoothly astringent (STARK and HOFMANN, 2006) and may contribute to a pleasant mouth-filling sensation similar as in red wines. Epicatechin-8-C-β-D-glucopyranoside has a DoT of 5.5, catechin-6-C,8-C-β-D-diglucopyranoside a DoT of 9.5 in roasted cocoa (EP 1 856 988 A1). In addition, flavan-3-ol-C-glycosides seem to modulate bitterness. When added to cocoa beverages, the bitterness of the beverages is describe as milder, softer and more pleasant. When added to theobromine solutions, the bitterness of the solution strongly decreases (STARK and HOFMANN, 2006), similar to the observations made by ZHANG et al. (2014) regarding catechin-C-spiro-glycosides. Increased concentrations of flavan-3-ol-C-glycosides were found in alkalized samples, with the respective DoTs being 13.5 for catechin-6-C,8-C-β-D-diglucopyranoside and 6.5 for epicatechin-8-C-β-D-glucopyranoside (EP 1 856 988 A1). This might explain the taste attributes of alkalized cocoa, which are often described as milder and low in bitterness and astringency.

Acidic substances

Finally, the acidic taste strongly depends on the acetic acid formed during fermentation and on citric acid, with DoT factors of 9 and 20, respectively. Citric acid, already present in the fresh fruit pulp, might migrate into the seeds during early stages of fermentation. It could also be produced seed internally e.g. through the citric acid cycle (KUMARI, 2018). In addition, succinic acid and malic acid slightly contribute to the overall acidity. A DoT of 1.9 is reported for succinic acid. The concentration of malic acid is at threshold and the DoT is consequently as low as 1 (STARK et al., 2006). Both acids can be derived through the citric acid cycle as well (KUMARI, 2018). In the sample analyzed by STARK et al. (2006), lactic acid did not contribute to the acidic taste. Its concentration was below threshold. However, the lactic acid concentration in fermented dried seeds may strongly vary (ROHSIUS, 2007; TRAN et al., 2015), presumably depending on the fermentation process. However, during the anaerobic phase of fermentation also seed internal lactic acid formation seems to occur (KADOW et al., unpublished data). Considering the maximum lactic acid amounts found by ROHSIUS (2007) a DoT of 10 could be reached for lactic acid.

In total, 34 taste-active substances are needed to reproduce the typical taste-profile of roasted cocoa nibs. A list with all 34 substances can be found in the publication of STARK et al. (2006).

Taste-saturation effects

To fully understand the importance of each of the flavor-active substances, saturation effects in human perception need to be considered. Saturation for N-caffeoyl-L-dopa is reached at a concentration of 0.6 $g \cdot kg^{-1}$, corresponding to a DoT factor of 64 in aqueous solution. This means that even if the concentration further increases, the intensity of the astringency perceived does not. The reported N-caffeoyl-L-dopa amount of 0.3 $g \cdot kg^{-1}$ (STARK et al., 2006) is below saturation and higher amounts would result in increased astringency. In contrast, for γ-aminobutyric acid, causing as well astringency, saturation is reached at a concentration of 8.2 $mg \cdot kg^{-1}$, corresponding to a DoT factor of 4. The γ-aminobutyric acid amount detected in roasted cocoa was as high as 520 $mg \cdot kg^{-1}$. Taking into account only the DoT of 251 would result in an overestimation of the contribution of γ-aminobutyric acid to the astringency (STARK et al., 2006). Also variations in the γ-aminobutyric acid concentration, from 250 to 1500 $mg \cdot kg^{-1}$, as reported by STOLL (2010), i.e. a DoT factor range of 121 to 727, need to be considered under the aspect of saturation. The variations observed are not sensorial perceived. In addition, the intensity of the astringency perceived is 2 on scale with a maximum of 5 for γ-aminobutyric when saturation is reached. In contrast, the maximum intensity for N-caffeoyl-L-dopa is 5, pointing out again the importance of this substance for puckering astringency (STARK et al., 2006).

Saturation effects do also occur regarding the bitter tastants. For cyclo(L-pro-L-val) saturation occurs at a DoT of more than 64. At

such high concentrations, the bitter intensity of cyclo(L-pro-L-val) is 5 on a scale with a maximum of 5. This underlines the bitter potential and the importance of diketopiprazines as bitter tastants in cocoa (STARK and HOFMANN, 2005).

Impact of the matrix on the perception of taste-active substances

The impact of aqueous and fatty matrices

When determining taste thresholds or training panelists, it is of vital importance to consider the impact of the matrix used to dissolve the taste-active substances. Basic trainings on bitter taste for example are often carried out in aqueous solution (e.g. CAOBISCO/ECA/FCC Cocoa Beans Manual, 2015). In water, caffeine for example has a bitter taste TC of 750 $\mu mol \cdot kg^{-1}$. In cocoa butter, the taste threshold increases nine-fold to a TC of 6.900 $\mu mol \cdot kg^{-1}$ (HOFMANN, 2015). The bitter taste TC of epicatechin as well increases nine-fold. Similarly, the TC for its astringent character increases six-fold. Amongst the astringent substances also quercetin-3-O-β-D-glucopyranoside has been detected in cocoa (STARK et al., 2006). It has a very low TC in water, of only 0.65 $\mu mol \cdot kg^{-1}$. Based on this TC, the quantities found in cocoa correspond to a DoT of 156 (STARK et al., 2006), suggesting a strong contribution of the substance to cocoa astringency. However, in cocoa butter the TC increases 369-fold (HOFMANN, 2015). Consequently, the quantities found in cocoa only correspond to a DoT 0.4. Accordingly, quercetin-3-O-β-D-glucopyranoside most likely does not contribute to cocoa astringency. Considering only the TC in water, the importance of quercetin-3-O-β-D-glucopyranoside for cocoa astringency would have been strongly overestimated. The example further underlines how essential the consideration of matrix effects is.

The impact of sugar addition

Such above described matrix effects also occur in the presence of sugar. Therefore, during chocolate manufacturing, the impact of the single substances on the flavor profile changes once again. The addition of sugar results in an increase of the TC for bitter taste of catechin and epicatechin. Consequently, the DoT factors decrease from 0.7 to 0.3 for catechin and from 1.2 to 0.6 for epicatechin, based on the concentrations found by STARK et al. (2006). Thus, in chocolate both substances do not contribute to the bitter taste. In contrast, the TC for the bitter taste of cyclo(L-pro-L-val) decreases, resulting in an increment of the DoT from 2 to 27. The concentration is still far below saturation of human perception. These findings further underline the importance of cyclo(L-pro-L-val) for the typical bitter taste of chocolates (STARK et al., 2006). The TC of theobromine increases slightly. The DoT factor for its bitter taste decreases from 19 to 17. Nevertheless, also after sugar addition, theobromine remains an important contributor to the bitter taste. The TC of caffeine is as well affected. The DoT factor range for caffeine after sugar addition is 0.3 to 2.5 based on the concentrations observed by ROHSIUS (2010).
Astringency is as well affected by the addition of sugar. The TC for puckering astringency of catechin and epicatechin increases and the DoTs decrease from 0.5 to 0.3 for catechin and from 1.8 to 1.7 for epicatechin, according to the concentrations found by STARK et al. (2006). Thus, a marginal contribution of epicatechin to puckering astringency in the final chocolate product is possible. Of greater importance remains N-caffeoyl-L-dopa. Its TC increases as well, but the DoT is still as high as 4.5 after sugar addition (STARK et al., 2006). About the impact of sugar addition onto the TC for human perception of the flavan-3-ol-C-glycosides, no data have been published so far.

Changes of the aroma-active substance ratios during conching

During the dry-phase of conching residual moisture and volatile acids, especially acetic acid, are effectively reduced in their concentration in the chocolate mass (e.g. BECKETT, 2008). Beside acetic acid, other aroma-active substances are also affected and their concentration changes during conching. Strecker aldehydes apparently show a general decline, with 3-methylbutanal decreasing by 28-36% (COUNET et al., 2002). In contrast to this, several pyrazines and furans apparently increase in their concentration. 2-ethyl-3,5-dimethylpyrazine only slightly, with a 1.4-fold increase. 4-hydroxy-2,5-dimethyl-3(2H)-furanon more substantial. 1.7 to 3.4-fold higher concentrations were found after conching (COUNET et al., 2002). Thus, not only the concentration of key aroma-active substances changes, but also the ratios between them. DANZL and ZARDIN (2014) describe a slight loss in chocolate aroma intensity during conching. However, the chocolate mass also becomes more harmonious in its flavor profile (DANZL and ZARDIN, 2014).

Quality analysis by means of aroma- and taste-active substances

The detailed knowledge about the substances responsible for a specific flavor attribute can be used in the training and formation of sensory panels. So far, in the first phase, trainings are often based on the recognition of the basic tastes, i.e. bitter, sweet, acidic, salty and umami, in aqueous solution. In a second phase, fine aroma attributes such as floral are trained, using for instance orange blossom water. In the third and final phase, panelists are exposed to a wide variety of origin chocolates to build a mental library of associations, linked to key chocolate flavor descriptors (CAOBISCO/ECA/FCC Cocoa Beans Manual, 2015). Making use of the reviewed knowledge, the cocoa specific tastants and aroma-active substances can be used to recognize in a first training phase single attributes of interest, for example puckering astringent, bitter or floral. The substances can be mixed into cocoa butter, instead of water, to best match the matrix of cocoa liquors and chocolates. In a second phase, different intensity levels can be trained, still on single attribute basis. This also allows the introduction and learning of optimum intensities for specific attributes. In a third phase, attributes can then be combined, until full taste or aroma profiles are used. In a fourth and final phase, the panelists evaluate liquors and chocolates. These liquors and chocolates should be analyzed with regard to their flavor-active substance composition, to guarantee suitability as standardized training material.
Analysis of the taste- and aroma-active substances and their precursors can also be used for quality control purposes. It can be applied in the testing of the raw material, i.e. the fermented, dried seeds, and for the identification of new suitable origins, matching a required flavor profile. Additional testing can be implemented along further critical control points of the processing chain, for example after roasting and grinding and after conching. In this way, a holistic monitoring of flavor quality can be established. However, such an approach requires high-throughput analytics.

Outlook on future research approaches

Most of the studies about flavor quality and its development have been carried out with a limited number of samples, given the complex analytical work behind them. Often, only a small part of the processing chain is considered, e.g. fermentation or the roasting process. New studies should possibly be carried out with greater sample number and cover all influence factors i.e. critical control points (CCP) of the processing chain. Key CCP are, based on the reviewed literature:

- the genetic background of the seeds
- fruit pulp aroma- and taste-active substances as well as precursors
- the microbiome during fermentation
- fermentation parameters e.g. temperature, pH, duration and turning interval
- seed aroma- and taste-active substances as well as precursors during fermentation

· the drying regime e.g. temperature, residual moisture and duration
· seed aroma- and taste-active substances as well as precursors during drying
· aroma- and taste-active substances after roasting
· the impact of the other chocolate ingredients e.g. sugar

In addition, factors such as the growing environment of the cacao e.g. climate and soil, fruit ripeness and characteristics of the processing location e.g. type of fermentation, box size and material should be monitored.

High throughput analytics are needed to process the samples. For the genetic background of the cocoa beans and the taste-active substances, such tools are available (Kauz et al., 2018; Meinhardt, 2019). For aroma-active substances, Hyper-Fast-GC has the potential to become a key tool. A metagenomics-based approach to monitor cocoa fermentation microbial communities has recently been published (Agyirifo et al., 2019).

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Personally, I do miss Reinhard as a colleague and as a friend.

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