# Chemistry of Cocoa Bean Roasting

by

# Paweł Jerzy Andruszkiewicz

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Approved Dissertation Committee

**Prof. Dr. Nikolai Kuhnert**
Jacobs University Bremen

**Prof. Dr. Matthias S. Ullrich**
Jacobs University Bremen

**Dr. Maria F. Williams**
Barry Callebaut, Lebbeke-Wieze, Belgium

Date of Defense: 15$^{th}$ November 2019

Department of Life Sciences & Chemistry

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# Abstract

Chocolate production relies on its extremely complex raw material – cocoa beans. Their intrinsic nature externalizes as a unique chemical composition, which becomes even more prominent after every step of processing chain: fermentation, drying, and roasting. Over the years, numerous researchers pushed the boundaries of cocoa science and managed to achieve many key milestones regarding, among others, cocoa taste, aroma and their precursors, variability during processing and country of origin, or genetic background. However, some aspects, such as thermal processing, are less understood, and their impact is not yet well-established within the chocolate production context. At the same time, the cocoa industry has to continually meet market demands and deliver high-quality products regardless of supply chain capability. Therefore, this thesis aims to use mass spectrometric chemical profiling methods to deepen the scientific understanding of roasting of cocoa with an emphasis on its organoleptic properties.

A series of cyclic dipeptides (2,5-diketopiperazines or DKPs) responsible for the bitter taste of cocoa was studied. Along already known to the literature, new species were identified, and a kinetic model of their formation was established. Their relative concentrations were correlated with their putative peptide precursors in the raw material. Significant positive correlations indicated that short peptides in unroasted cocoa formed during fermentation are taste precursors for bitter 2,5-diketopiperazines. Additionally, it was shown that most DKPs were generated during the degradation of the single most abundant peptide precursor.

Furthermore, an investigation of Maillard reaction during cocoa processing, demonstrated a higher than suspected so far variety of Amadori compounds. These first stable reaction products of simple sugars with amino acids were proven to form from oligopeptides as well. Their generation and degradation trends were shown for the first time on cocoa beans processing series from Ghana. Fermentation, drying, and roasting changes of other components such as oligopeptides, sugars, aroma volatiles were illustrated as well.

Finally, an HPLC-MS based design of experiments model of cocoa roasting was established. Standard process parameters, such as time, temperature, the addition of water, acid and base, were investigated to demonstrate their influence over the chemical composition of the resulted product. Relative concentrations of procyanidin monomers, dimers, and trimers, an Amadori compound, and a peptide were considered as markers. For each, high-quality models were accomplished and validated, which displayed sound prediction accuracy. These proof-of-concept results show great promise in the optimization of cocoa roasting.

In memory of my grandfather

Jerzy Tyrała

*Wybaczyć sobie*
*to porzucić nadzieję*
*na lepszą przeszłość*

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# List of Abbreviations

| | |
|---|---|
| **ARPs** | Amadori rearrangement products |
| **CDPS** | cyclopeptide synthase |
| **DCM** | dichloromethane |
| **DKP** | diketopiperazine |
| **DoE** | design of experiments |
| **DoT** | dose over threshold |
| **DPPH** | diphenylpicrylhydrazyl |
| **EIC** | extracted ion chromatogram |
| **EI-MS** | electron ionization mass spectrometry |
| **ESI** | electrospray ionization |
| **FRAP** | ferric ion reducing antioxidant power |
| **GC** | gass chromatography |
| **HILIC** | hydrophilic interaction chromatography |
| **HMW** | high molecular weight |
| **HPLC** | high-performance liquid chromatography |
| **HPLC-MS** | high-performance liquid chromatography coupled to mass spectrometry |
| **IEX** | ion exchange |
| **LC** | liquid chromatography |
| **LMW** | low molecular weight |
| **m/z** | mass to charge ratio |
| **MeOH** | methanol |
| **MLR** | multiple linear regression |
| **MRPs** | Maillard reaction products |
| **MS** | mass spectrometry |
| **MS/MS** | tandem mass spectrometry |
| **MS$^n$** | tandem mass spectrometry |
| **NIST** | National Institute of Standards and Technology |
| **NRPS** | non-ribosomal peptide synthase |
| **OFAT** | one factor at a time |
| **PAC** | potent aroma compound |
| **PCA** | principal component analysis |
| **PLS-DA** | partial least squares - discriminant analysis |
| **PTFE** | polytetrafluoroethylene |
| **RCSs** | reactive carbon species |
| **RSD** | relative standard deviation |
| **RSM** | response surface methodology |
| **SIM** | selected ion monitoring |
| **TDU** | thermal desorption unit |
| **TOF** | time of flight |

# Chapter 1

# Introduction

Chocolate, as much as we love it today, originated from a place where it had much higher status. The use of cocoa is traced back to Honduras, at least 1100 BCE (Henderson, Joyce, Hall, Hurst, & McGovern, 2007). It is very difficult to assess the exact time and place from which cocoa spread to the rest of South America, but it is possible to track it in ceramics – cocoa is the only substance in the region that contains both theobromine and caffeine. Cocoa seeds (it is not known whether fermented) were roasted and created a base for a chocolate drink, to which water, chili powder, vanilla, and sometimes maize were added. It is assumed that everyone had access to cocoa, but elites had it more (Christopher, 2013). Since *Theobroma cacao* was labor-intensive, the ability to produce and being in possession of more quantities was impressive and was a sign of high social status. Notably, pots with traces of cocoa were found in the burial grounds of ancient nobility (Prufer & Hurst, 2007). It is known that cocoa seeds were used as a form of currency, but some accounts suggest that they were an important part of many rites of passage as well, such as birth, initiation, marriage (Prufer & Hurst, 2007).

Europeans had to wait until the early 16th century, when the Spanish conquest of Mexico took place, to have a taste of cocoa. At first, the Spanish did not appreciate cocoa, but it is speculated that through continued material dependence on Indians, it made them get used to dietary and domestic practices during the occupation (Norton, 2006). This way, the original chocolate drink found its way into Europe where its method of consumption started to change (S. T. Beckett, 2009). At first, it was sweetened, later on, people started to add milk to it, but the most important invention happened in 1828. Van Houten developed a manual press used to extract fat from cocoa beans leading to an opening of the first chocolate factory in 1847, which used recently discovered steam engines. Over the years, more inventions were made, which lead to the invention of the chocolate products we know today.

Nowadays, the chocolate-making process is still empirical, but there is more and more scientific research appearing on the quality of chocolate and how it can be influenced at every step of production, starting with the plant itself. This work tries to address the complex chemical transformations during the thermal processing of cocoa beans.

## 1.1 Chemistry of Cocoa Processing

Cocoa, which is produced from the seeds of a plant *Theobroma cacao*, is a very special commodity as it involves both fermentation and thermal treatment as its processing steps. This is a very important feature as degradation processes, first during fermentation, then continued at the time of roasting, give rise to thousands of compounds, in turn, influencing the quality and organoleptic properties of the final product – chocolate. However, without another aspect of its uniqueness is its composition – the seeds of *Theobroma cacao* plant contain special components which upon special processing, yield very special organoleptic properties.

Generally, *Theobroma cacao* is a very fragile plant of unique growing conditions and susceptible to change in the environment. It is cultivated around the Equator in West Africa,

South East Asia, and South America, with Ivory Coast being the largest producer (about 40% of world crop) (Fowler, 2008). Historically, there are three main cultivars of *Theobroma cacao* – Criollo, Forastero, and Trinitario, each with distinct characteristics. However, now, there are numerous genetic hybrids known to exist (Turnbull & Hadley, 2015). Criollo was the original cocoa variety from South and Central America (Motamayor et al., 2008). It has characteristic white cotyledons and a typically favorable flavor. It is also fragile and produces low yields. The Forastero variety underwent genetic changes through selective breeding, producing cocoa resistant to diseases, with a higher yield, and lesser flavor potential. Trinitario is known to be a hybrid between the two, with mixed characteristics. Additionally, there is a fourth type – Nacional, which originates from Ecuador. They are known to produce the so-called "Arriba" flavor. Each type has the potential to produce chocolate with different attributes, depending on further processing. The chemical and physical transformations start with the harvest of cocoa fruits from the trees.

## 1.1.1 Fermentation and Drying

*Fermentation*

Fermentation and drying are the first extrinsic steps with possible direct control over the product quality as their proper execution guarantees the formation of essential flavor precursors. After the harvest, cocoa beans with the pulp surrounding them are removed from the pod (the protective shell of the cocoa fruit) and are subsequently fermented for approximately five days (Fowler, 2008), although this depends on the beans themselves and their purpose. Some of the beans are produced to obtain only the cocoa butter; therefore, they are not fermented at all. The fermentation is usually performed in boxes or heaps (e.g., covered in banana leaves) and can be done in any quantities between 25 and 2500 kilograms of fresh cocoa beans. The uniformity of the process has to be ensured, especially in larger scales, by, e.g. good aeration, mixing, and draining of the liquefied pulp (so-called sweatings). The fermentation is done by employing microorganisms from the environment (spontaneous fermentation), but the addition of starter or other microbe cultures is also possible (controlled fermentation) (Schwan, Fleet, & Fleet, 2014). The general idea behind this process is that the polymeric constituents (e.g., carbohydrates and proteins) are trapped inside the compartmentalized structures of the bean, which can be destroyed during the fermentation. This homogenization process enables further degradation and the development of flavor precursors. This is supported by the observation that omitting fermentation yields cocoa without its characteristic organoleptic attributes (Rohan, 1964).

During the fermentation, the microorganisms feed on the pulp surrounding the cocoa beans since it is a good source of carbohydrates (it contains about 10-15%). Their activity and production of secondary metabolites are responsible for the death of the beans. At first, anaerobic yeasts propagate and convert the sugars into ethanol under anaerobic and slightly acidic conditions. After about two days of fermentation, the lactic acid bacteria become dominant and convert sugars and organic acids into lactic acid. At the end of the fermentation, when the aeration increases, acetic acid bacteria are the most abundant. In an exothermic reaction, they are converting ethanol into acetic acid, raising the temperature to 50°C or even more. This general trend of microbial activity is true for all the fermentation but can vary depending on the region.
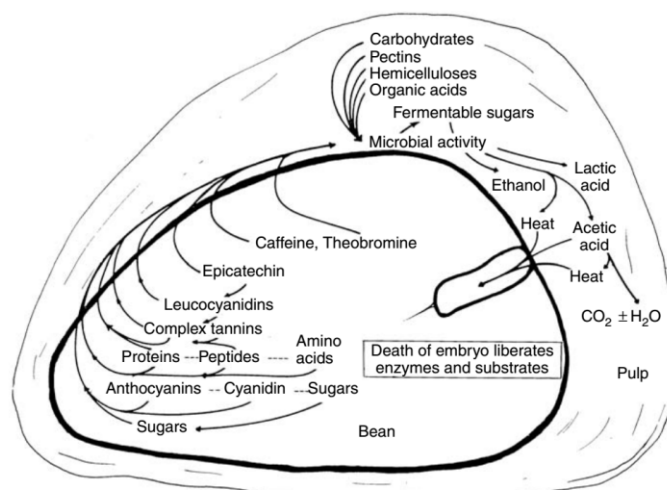
**Figure 1 Chemical changes during fermentation occurring inside and outside the cocoa bean. Reprinted with permission (Fowler, 2008).**

At the same time, at the beginning of the fermentation, the cocoa bean maintains its metabolic activity and tries to initiate the germination. There are two essential cell types within the bean cotyledons – the pigment cells containing methylxanthines (theobromine and caffeine) as well as various polyphenols, and the storage cells containing lipids and proteins. Within the first two days of fermentation, because of the microbial metabolites (acids and alcohol), the destruction of cell walls and membranes occurs, which leads to bean death. Various chemical compounds and enzymes, till now contained in different cells, mix and react with each other, forming flavor precursors (see **Figure 1** above). The enzymatic activity is affected by the acidity and temperature rise during the fermentation. Additionally, at this time, the polyphenols can interact with proteins, decreasing their solubility (Zak & Keeney, 1976).

There are different cocoa enzymes involved in the fermentation (Jürgen Voigt & Lieberei, 2014). Hydrolases, such as invertase, cleave off glycoside bonds. So far, their activity was not found to be connected to high flavor potential. More importantly, polyphenol oxidases catalyze the oxidation of polyphenolic compounds (such as abundant catechins) to quinones. These highly reactive carbonyl compounds can interact with proteins forming polymeric brown complexes, which are responsible for the browning of cocoa during the fermentation (Jürgen Voigt & Lieberei, 2014). There are also other minor enzymes participating in biochemical transformations of the beans, such as other oxidases or glutamate decarboxylase. However, the most important process at this stage is the enzymatic degradation of cocoa proteins, which was found to be the main source of cocoa flavor precursors (G. Ziegleder & Biehl, 1988; Jürgen Voigt & Lieberei, 2014), by the activity of the proteolytic enzymes – aspartic endoprotease and carboxypeptidase, as well as aminopeptidase. A series of investigations suggested that the combination of the first two enzymes at an optimal pH of 5.2 degraded the cocoa seed proteins releasing a mixture of hydrophilic peptides and hydrophobic amino acids (J. Voigt, Biehl, et al., 1994), which upon roasting in presence of reducing sugars and deodorized cocoa butter yielded cocoa-specific aroma (Jürgen Voigt & Lieberei, 2014). This was in line with the fact that the degradation of purified storage protein from unfermented beans with only endoproteases did not produce cocoa aroma precursors (J. Voigt, Heinrichs, Voigt, & Biehl, 1994). Most importantly, it was found that the microorganisms are not essential for the formation of those precursors, only the organic acids their produce create conditions needed for the

proteolytic activity of the endogenous cocoa enzymes (Ostovar & Keeney, 1973; Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; J. Voigt, Heinrichs, et al., 1994). This explains numerous successful attempts to mimic cocoa fermentation via artificial methods (Eyamo Evina, De Taeye, Niemenak, Youmbi, & Collin, 2016; John et al., 2016). The precursors formed during the fermentations have an essential role in the chemical transformations happening during the thermal treatment of cocoa.

*Drying*

After the fermentation, the cocoa beans are dried. The most common method is the sun-drying, where the beans are spread out during the day in layers on a horizontal surface. It takes a few days (about a week) to reduce the moisture content to about 7%, which is extremely important in preventing mold growth. Over-drying is possible, which makes the cocoa beans more fragile and harder to handle during transport. Additionally, other artificial drying methods were developed in case of unfavorable weather conditions. It is worth noting that during the drying (and fermentation) because of the slightly elevated temperature, some of the flavor-relevant chemical reactions can occur to a small extent. Most importantly, the Amadori compounds (first stable Maillard reaction intermediates) are present in dried cocoa beans (Meitinger, Hartmann, & Schieberle, 2014). After the drying process, the cocoa beans are ready to be shipped to a country where they can be stored and processed. At this point, they are considered raw material for chocolate and cocoa powder production.

## 1.1.2 The Cocoa Beans – Raw Material Composition

The fermented and dried cocoa bean is the essential raw material for chocolate and cocoa powder production; therefore, its chemical composition influences the outcomes at every step of the processing. As *Theobroma cacao* is cultivated in many countries around the Equator, in many varieties, and the processing not being uniform, it is known that the outcome in the form of chocolate can be quite distinct. A number of studies were devoted to the description of the chemical (Niemenak, Rohsius, Elwers, Omokolo Ndoumou, & Lieberei, 2006; Othman, Ismail, Abdul Ghani, & Adenan, 2007; Afoakwa, Quao, Takrama, Budu, & Saalia, 2013; Mayorga-Gross, Quirós-Guerrero, Fourny, & Vaillant, 2016; Sirbu, Grimbs, Corno, Ullrich, & Kuhnert, 2018) variation between cultivars and their effect on the organoleptic properties of the finished product (M Owusu, Petersen, & Heimdal, n.d.; S. Jinap, Dimick, & Hollender, 1995; Cambrai et al., 2010; Torres-Moreno, Tarrega, & Blanch, 2014). However, the general composition of the raw bean is as follows: lipids (~50%), proteins (~15%), cellulose (~13%), polyphenols (~6%), ash (~4%), starch (~3%), and others (~7%) (Bertazzo, Comai, Mangiarini, & Chen, 2013). Here we will discuss the main classes of compounds that constitute a fermented and dried cocoa bean.

*Lipids*

The lipid fraction is the most abundant class of compounds in cocoa (about 50-60% of the dry mass) (Bertazzo et al., 2013). However, it is not affecting the cocoa flavor directly; therefore, it is not of the most interest of researchers. Still, it is an essential part of chocolate production. It is a matrix for every chemical transformation, and it solvates aroma compounds forming during roasting very well. Most important properties industrially, such as melting point and hardness, are a result of the composition of the cocoa butter, which in turn is affected by the genetic variety of cocoa plants, the region of growth, and growing conditions (including farming practices), and processing. Most of the lipids content (about 98%) is known to be triacylglycerols, rest being other components, such as free fatty acids, mono- and diacylglycerols, glycolipids, phospholipids, and unsaponifiable matter. The

triacylglycerols are glycerol and fatty acid esters, and in cocoa, the most common fatty acids are palmitic acid, stearic acid, oleic acid, and linoleic acid. A large variety of these compounds are known to unfermented cocoa (Sirbu, Corno, Ullrich, & Kuhnert, 2018), and their composition in cocoa butter from different countries of origin affects its physical properties (Sirbu, Grimbs, et al., 2018). Despite being the least abundant amongst lipids, the polar lipids, especially phospholipids, are an important component affecting the physical properties of chocolate. However, their effect on chocolate melting behavior is typically enhanced by the addition of external phospholipids as emulsifiers, mainly soy or egg lecithin, during actual chocolate production (Afoakwa, Paterson, Fowler, & Vieira, 2008; Ribeiro et al., 2015; Kindlein, Elts, & Briesen, 2018).

*Carbohydrates*

Dried cocoa beans contain mono-, oligo-, and polysaccharides. The polysaccharides, which are the components of cell structures, consist of cellulose (about 12%), starch (3-7%), pectins, and hemicellulose (Bertazzo et al., 2013). Glucose and fructose are the most abundant free sugars in cocoa beans (Reineccius, Andersen, Kavanagh, & Keeney, 1972). As reducing sugars, they take an important part in chemical reactions occurring during the thermal treatment of cocoa. However, other low molecular weight carbohydrates as mannitol, *myo*-inositol, sucrose, melibiose, raffinose, and stachyose were reported (Megías-Pérez, Grimbs, D'Souza, Bernaert, & Kuhnert, 2018; Megías-Pérez, Ruiz-Matute, Corno, & Kuhnert, 2019)

*Proteins, peptides and Amino Acids*

Proteins, after the lipids, are the second most abundant component of unfermented cocoa beans. However, after the fermentation, all of the main cocoa storage proteins are degraded into smaller peptides, which along with sugars, are essential precursors of chocolate flavor (J Voigt, Wrann, Heinrichs, & Biehl, 1994; Scalone et al., 2019). Their content in the raw material is highly dependent on fermentation practices and unfermented bean protein profile while being independent of cocoa origin (Kumari et al., 2018; D'Souza et al., 2018). The amino acids are a small fraction of cocoa, present in amounts less than 1% of dry weight. From a chemical point of view, they are the main nucleophiles in cocoa, which could participate in numerous reactions during roasting (and prior).

*Polyphenols*

Cocoa is one of the polyphenol-rich foods, which can contain up to 18% of dried bean mass (Bravo, 2009). Polyphenolic compounds in cocoa are the bitter and astringent species that are associated with dietary health benefits (Borchers, Keen, Hannum, & Gershwin, 2000; Paoletti, 2012). Additionally, many polyphenols are colored compounds, and to them, cocoa owes its characteristic reddish-brown coloration (Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012). Polyphenols are plant's secondary metabolites, but surprisingly their quantity in the material is dependent not only on genotype and origin (Jonfia-Essien, West, Alderson, & Tucker, 2008; Elwers, Zambrano, Rohsius, & Lieberei, 2009), but harvesting practices and post-harvest processing as well (Afoakwa et al., 2013; Kothe, Zimmermann, & Galensa, 2013; Oracz, Zyzelewicz, & Nebesny, 2015). Furthermore, they can be used to discriminate cocoa beans based on their origin and fermentation status (D'Souza et al., 2017). There are three classes of polyphenols in cocoa: flavan-3-ols (37% of polyphenols), anthocyanins (about 4%), and proanthocyanidins (~58%) (Wollgast & Anklam, 2000). The main one is (-)-epicatechin, which constitutes up to 35%. There were polymers of catechins (proanthocyanidins) found in cocoa up to 10 units (D'Souza et al., 2017). They can interact with peptides and proteins (Siebert, Troukhanova, & Lynn, 1996); however, the mechanism

is unknown. Moreover, they can be oxidized by plant enzyme polyphenol oxidase. Additionally, they could be participating in Maillard reactions.

*Other Components*

Other notable components are present in cocoa that originate from the bean itself and the fermentation process. First, the methylxanthines, endogenous cocoa alkaloids are present in considerable amounts. These are caffeine and theobromine, which most importantly influence bitter taste, but have other bioactive properties as well (Tarka & Cornish, 1982; Shively & Tarka, 1984). Additionally, the microorganisms taking part in fermentation produce a variety of volatile compounds – organic acids, alcohols, and their esters. These are all aroma-active molecules and have a significant effect on the organoleptic properties of cocoa (Rodriguez-Campos et al., 2012) and can be influenced during further processing (Margaret Owusu, Petersen, & Heimdal, 2012).

Additionally, low amounts of compounds found mainly during roasting can be found in dried beans, most likely due to the slightly elevated temperature during fermentation and drying. These include volatile aldehydes and ketones (Ramli, Hassan, Said, Samsudin, & Idris, 2006), non-volatile bitter 2,5-diketopiperazines (cyclic dipeptides), as well as Amadori compounds (Meitinger et al., 2014), the first stable Maillard reaction intermediates.

## 1.1.3 Roasting

*Roasting Process*

Processing of the raw material (fermented and dried cocoa beans) focuses on the development of the two essential properties of chocolate – its flavor and texture. This means that both chemical and physical changes occur during the production. However, roasting is only one of the numerous steps taking place in the factory (Kamphuis, 2008). First, the cocoa beans have to be cleaned from any non-cocoa impurities acquired during post-harvest treatment and transport. This can be done via various methods such as sieving, counter-air flow techniques, magnets, fluidized bed techniques, etc., each focusing on the removal of different contaminants, like ferrous materials, high-density particles, and dust. This is followed by the removal of the shell, which can produce undesirable off-flavors and can cause problems during the grinding. Apart from the obvious quality reasons, there is a worldwide industry standard limiting the maximum cocoa shell content in a cocoa product (5% of dry defatted matter) (Codex Alimentarius, 2014). This is done by breaking the beans and separating resulted nibs from the shell via winnowing. This separation affects the nib yield, and it is important to produce as few small nib particles as possible (as they can be easily lost) to keep the process financially viable. At this point, the cocoa can be alkalized (optional), which is a process of heating the nibs in a water solution of certain alkali (typically potassium carbonate). This is done mainly during the production of cocoa powder, but in some cases, it can be used to introduce certain flavors to the chocolate. Although, the main reasons for alkalization are as follows: increase of dispersibility in water (important for cocoa powder and beverages), acidity decrease, and color modification (especially darkening) (Afoakwa, Paterson, Fowler, & Ryan, 2008). It also affects the lipid fraction of cocoa, especially its crystallization behavior (Marty-Terrade & Marangoni, 2012). The resulted alkalized nibs have to be dried before roasting. After the thermal treatment, the cocoa is ground producing a cocoa liquor (or cocoa mass), which is liquid while still warm, and solidifies while cooled. Then, it can be pressed out to yield cocoa powder and butter, or furtherly processed into the chocolate.
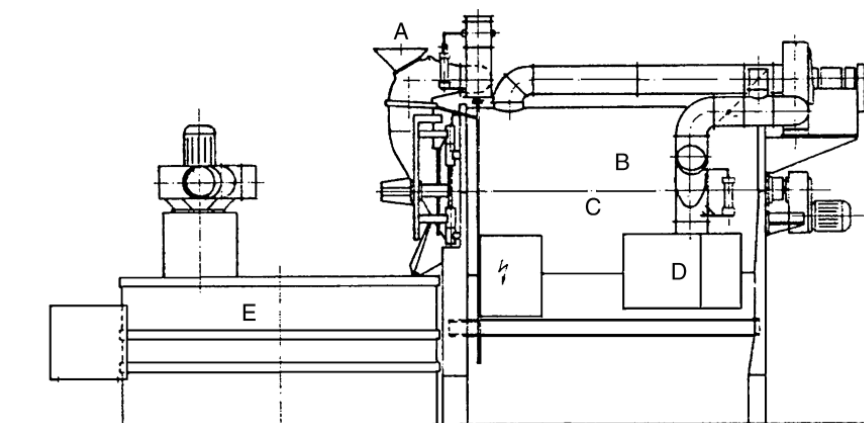
**Figure 2 Schematic of a batch drum roaster. Reprinted with permission (Kamphuis, 2008).** Barth Ludwigsburg GmbH & Co., Germany, 1998. A – funnel tube, B – reaction drum, C – perforated pipe for alkali solution, D – gas or oil heating, E – cooling pan.

The roasting methods vary (see *Industrial Roasting Equipment* subsection below) and is mostly done on either whole cocoa beans or cocoa nibs. The usage of the former was the original approach to cocoa processing. It is known for the preservation of unroasted beans' original flavors (heat transfer is slower for whole beans), and the deshelling is relatively easy, as the shell gets loose after the treatment. In contrast, the nib roasting is performed when performing alkalization. Batch drum roasters (see **Figure 2** above) are frequently used, to which the nibs are transferred, and then a specific roasting profile is applied. The heat (generated by e.g. gas burners) is transferred from the wall of the drum to the nib, and the drum tumbling prevents burning. At the beginning of the roasting, mandatory debacterization is carried out by the addition of water, which can affect the properties of cocoa as well (Krysiak, 2006). It is an essential procedure as post-harvesting practices pose a high risk of contamination with *Salmonella* and other vegetative bacteria (Burndred & Peace, 2017). High water activity and the presence of steam are important to the effective destruction of microorganisms. Typically, recipes feature a roasting profile, during which the temperature increase from the ambient temperature to a maximum roasting temperature over the course of roasting. The maximum roasting temperature can vary from 110 to 140 °C. The profile is not linear and depends on the desired outcome (low, medium, or high roast). After the roasting, the nibs have to be cooled down in a separate cooler. The same approach can be in a continuous process. Other roasting equipment can be employed as well, such as continuous vertical air roasters, shelf systems, or others (see the next subsection).

There are numerous studies on the effect of roasting on organoleptic properties of cocoa beans (Selamat Jinap & Dimick, 1991; S Jinap, Rosli, Russl, & Nordin, n.d.; Krysiak, 2006; Ramli et al., 2006; Arlorio et al., 2008; Baggenstoss, Poisson, Kaegi, Perren, & Escher, 2008; Oliviero, Capuano, Cämmerer, & Fogliano, 2009; Kothe et al., 2013; Margaret Owusu et al., 2012; Ioannone et al., 2015), but it is mainly an empirical process, which only a few insights are known so far on how to influence it. In general, the roasting decreases acidity and converts all precursors formed during the fermentation and drying into flavor-active compounds via Maillard reaction and similar processes (Afoakwa, Paterson, Fowler, & Ryan, 2008), as well as reduces the amount of some compounds, such as polyphenols (Żyżelewicz et al., 2016).

*Industrial Roasting Equipment*

Over the years, the chocolate industry was able to improve and innovate on the roasting process in general. Flavor development during the thermal treatment was better understood over time, which enabled its adjustment based on cocoa characteristics. This, in turn, impacted the design of novel roasting equipment, opening the possibility of use e.g., heating technologies such as infrared microwaves or superheated steam. Generally, the equipment used to roast depends on the type of raw material (whole beans, nibs, liquor) and the purpose of the use of the final product (Hii & Borém, 2020). The whole beans require simpler equipment and therefore is used by small- and medium-scale industries. Alternatively, large-scale industry uses beans to produce premium chocolate, as the shell facilitates the retention of aroma compounds (Kamphuis, 2008). Alternatively, nib roasting (most widely-used roasting method) provides an opportunity for other treatments before the roasting (i.a. acidification, alkalization), makes the roasting more uniform, and preserves butter content. Finally, some smaller businesses perform liquor roastings as they promote off-flavor vaporization, which decreases the required conching time (see **Table 1** below). Proper roasting equipment selection is needed for each of these methods. (Krysiak, 2006) showed that time, temperature, air flow rate, and velocity are essential roasting parameters, which influence temperature distribution, water evaporation, and in turn, heat and mass transfer. Ultimately, different equipment (such as convection oven or drum roaster) will yield different outcomes for bean, nib, and liquor roasting.

**Table 1 Comparison of roasting methods. Adapted from (Kleinert, 1994).**

| Characteristics | Whole Bean Roasting | Nib Roasting | Liquor Roasting |
|---|---|---|---|
| Flavor development | Favorable | Favorable | Favorable |
| Flavor profile modification (e.g. alkalization) | Poor | Favorable | Medium |
| Extract undesirable volatiles | Poor | Favorable | Favorable |
| Loss of fat to shell | Poor | Favorable | Favorable |
| Obtain microbial kill | Favorable | Favorable | Medium |
| Loosen shell | Favorable | * | * |
| Good grindability | Favorable | Favorable | Poor |

*Need preliminary thermal treatment

Small-scale chocolate producers can choose from a variety of simple beans and nibs roasting methods providing unique taste and flavor. Even now, some of the traditional chocolates are being produced using stainless steel frying pans or regular convection ovens (Hii & Borém, 2020). Alternatively, more modern approaches include microwave roasting (Yoshida & Kajimoto, 1994) and the use of superheated steam, which can reduce roasting duration and bitterness generation as well as the preservation of volatile compounds (Zzaman & Yang, 2014). However, the use of drum-type roasters remains as the most popular method of small-scale bean roasting (see the previous section and **Figure 2** above). They grant the most flexibility and uniformity and have a capacity of up to 50 kilograms per batch. The other aspect of small-scale processing is gourmet roasting. The availability of diverse sources of single-origin beans and customer demand for specialty chocolates imposed unique approaches to different raw materials. Gourmet roasters enable modification of crucial roasting parameters (such as temperature, time, airflow, steam injection, cooling time, fluidization) to generate optimal roasting outcomes for each type of a bean.

Scaling-up to industrial-scale roasters poses two main challenges – uniformity of roasting and effective microbial killing must be ensured with increasing scale (Hii & Borém, 2020). Debacterization is achieved by the inclusion of a water tank or steam generator system to the roaster. Furthermore, industrial roasting is often performed in a continuous fashion with an additional rapid cooling system prior to the exit from the roasting equipment (Beckett, 2009).

Continuous and batch drum roasters are frequently used by the cocoa industry to roast nibs (and alkalize them). One such example is the Sirocco roaster, which is still used to roast cocoa beans, nuts, and coffee (Minifie, 2012). Rotating drums are supplied with hot air causing turbulence in roasted beans and ensuring uniformity. This gentle heating system also reduces the risk of burning. After the roasting, the material is transferred automatically onto a perforated metal plate equipped with a cooling system. Generally, drum roasters were described above in the *Roasting Process* subsection. Other equipment used by the cocoa industry includes continuous vertical air roasters (shaft roaster), which are a subtype of shelf system roasters (Beckett, 2009), fluid bed roasters, or more specifically, roasters like Probat-Roaster and Buhler STR 2 Roaster (Minifie, 2012).

Shelf systems (Beckett, 2009) can be used to roast cocoa beans or nibs in a continuous fashion. The roaster is divided into top and bottom sections with multiple shelves. The raw material is supplied from the top onto the shelves. The unique ventilation system lets the hot air pass through and come into contact with cocoa. After the roasting, the shelves tilt and release thermally processed material onto lower ones where the cooling procedure takes place. A specific type of this roaster was manufactured by Lehmann (Lehmann Maschinenfabrik GmbH) (Kamphuis, 2008).

Another popular choice of the roasting method consists of systems using heating by convection in a current of hot air, which is used to roast nibs and whole cocoa beans (Minifie, 2012). One of the examples is a fluid bed roaster. In this setup, the raw material is supplied to the bottom of the fluid bed zone, where it is fluidized in a stream of hot air. This method allows each of the bean (or nib) to be in contact with air uniformly from all directions, ensuring homogenous roasting. The cooling process is also done by fluidization but in a stream of cold air. Probat-Roaster is a specific example of convection roasting. Cocoa beans are supplied from the top and fall down through three sections equipped with air heaters. The cold air is provided by an inlet at the bottom of the apparatus. It travels upward, heats up, and transmits the energy to the beans going in the opposite direction. The roasting process finishes when the beans encounter cold air, cool down, and reach the outlet. A similar approach is used in Buhler Roaster. The significant difference is that the roaster is not divided into separate heating sections. The raw material is traveling downward in a long tunnel, alongside which heaters are placed. Air circulates around them and roasts the beans. The roasted material falls down to a cooling section, through which cold air passes. After cooling down, the beans are collected through an outlet at the bottom of the apparatus.

### 1.1.4 Organoleptic Properties of Roasted Cocoa

In the end, the organoleptic properties of cocoa are shaped by its complex processing steps – most notably fermentation, drying, and roasting. In general, the fermentation is responsible for the degradation of polymers to flavor precursors, the drying already starts their chemical transformations, and the roasting develops them into aroma and taste components in a complex cascade of concurring reactions. Chemical identities of aroma and taste compounds present after these processes are well described in the literature

(Keeney, 1972; G. Ziegleder & Biehl, 1988; Bonvehí, 2005; Frauendorfer & Schieberle, 2006; Stark, Bareuther, & Hofmann, 2006; Aprotosoaie, Luca, & Miron, 2016). However, the complexity of cocoa flavor is astounding, and the behavior of all flavor-active components in the cocoa matrix and their interactions and formation are not well understood.

*Aroma*

There are about 600 odor-active compounds already described in cocoa (Aprotosoaie et al., 2016). They may originate from different steps of processing, but it is important that the volatiles already present in the raw material are negatively affected by increased roasting intensity, while new ones are generated at the same time. **Table 2** shows the most important aroma compounds present in the cocoa after roasting and processing into a powder. Mostly, they belong to following classes of molecules: alcohols, carboxylic acids, aldehydes, ketones, esters, and pyrazines. The amount of the possible compounds is overwhelming; however, they can be narrowed down to compounds or classes of compounds important to specific sensations (Aprotosoaie et al., 2016). As such, alcohols confer mostly fruity and floral aromas. They are formed mostly during fermentation as a result of microbial activity, but to some extent, thermal degradation of amino acids as well. Linalool and 2-phenylethanol are important representatives producing distinct floral aroma (S. Jinap, Rosli, Russly, & Nordin, 1998), and typically are present in higher quantities in Criollo cocoa beans typical for South America (Frauendorfer & Schieberle, 2008). A linalool/benzaldehyde ratio was proposed as a flavor index, with a score of 0.3 or more indicating fine-grade cocoas (Gottfried Ziegleder, 1990). The formation of carbonyl compounds such as aldehydes and ketones are mostly dependant on the Strecker degradation of amino acid residues during roasting. Although, they can be formed during fermentation and drying as well. Typical examples, such as 2- and 3-methylbutanals are responsible for malty and chocolate notes, whereas acetophenone is characteristic for its sweet and floral aromas (Rodriguez-Campos et al., 2012). Esters in cocoa are derived from alcohols and organic acids. Hence the fermentation is their main source. This is also why the ethyl- and methyl- esters, as well as acetates, dominate in fermented cocoa (Rodriguez-Campos et al., 2012). In general, they are responsible for fruity aromas. For example, flowery and honey 2-phenylethylacetate is an important compound for fine-flavor cocoas. On the other hand, amyl acetates are considered off-notes, and their formation should be avoided during the fermentation (Rodriguez-Campos et al., 2012). Pyrazines are the heteroaromatic compounds essential to roasted and nutty aromas of cocoa. Mostly, they form from α-aminoketones coming from the Strecker degradation.

There are about 80 pyrazines influencing cocoa flavor (Afoakwa, Paterson, Fowler, & Ryan, 2008), but only the tetramethylpyrazine contributes to 90% of total pyrazines (Rodriguez-Campos et al., 2012). However, there are pyrazines present with other combinations of substituents e.g., methyl-, ethyl-, propyl-, furyl-, vinyl-, methoxy-, and formation of a greater variety of them in model systems were found to be connected to the presence of peptide Amadori compounds (Zou, Liu, Song, & Liu, 2018). There are also furanones and pyrones formed during drying and roasting via degradation of simple sugars. Furaneol, hydroxymaltol, and cyclotene have a pleasant caramel aroma and are destroyed during alkalization (Rodriguez-Campos et al., 2012). Other compounds, such as some of the organic acids, and phenols (phenol, 2-methoxyphenol), produce unpleasant off-notes.

**Table 2 Important aroma compounds in the cocoa powder (Frauendorfer & Schieberle, 2006).**

| Odorant | Odor Quality | Concentration in Cocoa [µg/kg] | Odor Threshold [µg/kg] | Odor Activity Value |
|---|---|---|---|---|
| acetic acid | sour | 332000.00 | 124.00 | 2680.00 |
| 3-methylbutanal | malty | 25770.00 | 13.00 | 1980.00 |
| 3-methylbutanoic acid | sweaty | 8550.00 | 22.00 | 390.00 |
| phenylacetaldehyde | honey-like | 6600.00 | 22.00 | 300.00 |
| 2-methylbutanal | malty | 14314.00 | 140.00 | 102.00 |
| 3-hydroxy-4,5-dimethyl-2(5H)-furanone | spicy | 15.00 | 0.20 | 75.00 |
| 2-acetyl-1-pyrroline | popcorn-like | 5.90 | 0.10 | 59.00 |
| 4-hydroxy-2,5-dimethyl-3(2H)-furanone | caramel-like | 620.00 | 25.00 | 25.00 |
| 2-phenylacetic acid | honey-like | 7700.00 | 360.00 | 21.00 |
| 2,3-diethyl-5-methylpyrazine | earthy | 8.20 | 0.50 | 16.00 |
| methylpropanoic acid | rancid | 2800.00 | 190.00 | 15.00 |
| 2-ethyl-3,5-dimethylpyrazine | earthy | 31.00 | 2.20 | 14.00 |
| 2-methoxyphenol | spicy, smoky | 230.00 | 16.00 | 14.00 |
| 2-isobutyl-3-methoxypyrazine | bell pepper-like* | 0.85 | 0.80 | 12.00 |
| 2-methylbutanoic acid | sweaty | 1750.00 | 203.00 | 8.70 |
| 3-methylindole | fecal | 55.00 | 16.00 | 3.50 |
| 2-phenylethanol | flowery | 590.00 | 211.00 | 2.80 |
| dimethyl trisulfide | sulfury | 6.90 | 2.50 | 2.80 |
| butanoic acid | sweaty | 320.00 | 135.00 | 2.40 |
| linalool | flowery | 72.00 | 37.00 | 2.00 |
| 4-methylphenol | horse stable, phenolic | 124.00 | 68.00 | 1.80 |
| 2-phenylethyl acetate | flowery | 315.00 | 233.00 | 1.30 |
| 2-ethyl-3,6-dimethylpyrazine | earthy | 70.00 | 57.00 | 1.20 |
| 2-methyl-3-(methyldithio)furan | cooked meat-like | 0.47 | 0.40 | 1.20 |
| 2,3,5-trimethylpyrazine | earthy | 200.00 | 290.00 | <1 |
| δ-octalactone | coconut-like | 185.00 | 2490.00 | <1 |
| δ-octenolactone | coconut-like** | 151.00 | 4730.00 | <1 |
| δ-decenolactone | coconut-like | 82.00 | 590.00 | <1 |
| γ-decalactone | coconut-like | 60.00 | 320.00 | <1 |
| γ-nonalactone | coconut-like | 43.00 | 148.00 | <1 |
| δ-decalactone | coconut-like | 14.00 | 400.00 | <1 |
| 3-ethylphenol | phenolic | - | - | - |
| cis-isoeugenol | smoked | - | - | - |
| methylpropanal | malty | - | - | - |
| phenylmethanol | flowery | - | - | - |
| phenylmethyl acetate | fruity, flowery | - | - | - |
| vanillin | vanilla-like | - | - | - |
| γ-octenolactone | coconut-like | - | - | - |

Odor activity value is the concentration of the aroma volatile divided by its odor threshold

*(Pelosi, Baldaccini, & Pisanelli, 1982)

**(Frauendorfer & Schieberle, 2019)
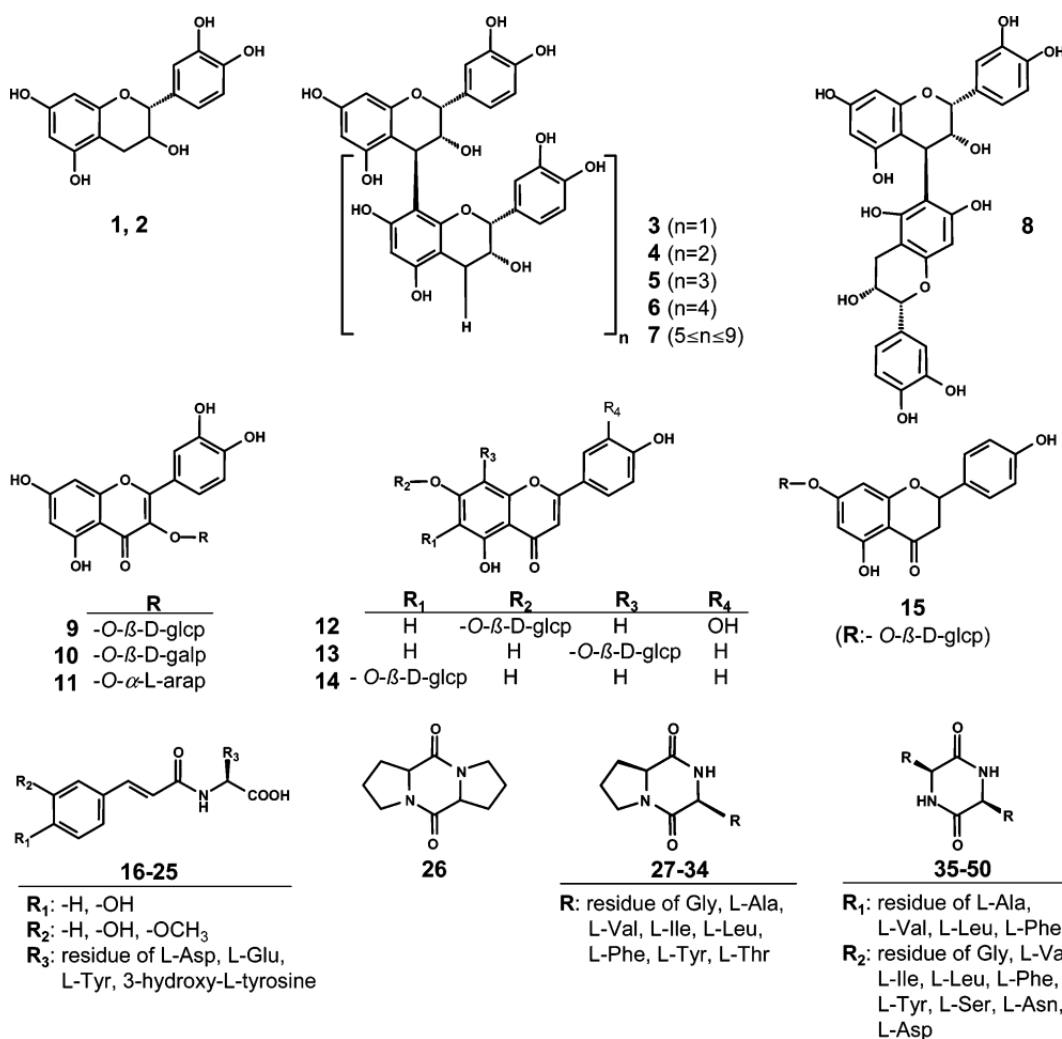
**Figure 3 Structures of compounds known to be essential to cocoa taste. Reprinted with permission (Stark et al., 2006).**

*Taste*

The cocoa taste was found to be much less complex. Extensive studies were performed in order to separate the non-volatile roasted cocoa fractions, resulting in insights on presence and activity of following taste compound classes: 2,5 diketopiperazines (Stark & Hofmann, 2005b), N-phenylpropenoyl-L-amino acids (Stark & Hofmann, 2005a), and polyphenols (Stark, Bareuther, & Hofmann, 2005). Their direct influence on taste was studied via taste-dilution analysis (Stark et al., 2006). In the end, a list of 34 compounds was established with following essential organoleptics of the roasted cocoa beans (see structures and numbering on **Figure 3** above): bitter theobromine and caffeine, seven bitter diketopiperazines (**28-31**, **36**, **37**, and **41**), seven puckering-astringent and bitter flavan-3-ols (**1-6**, and **8**), four astringent polyphenol glycosides (**9-12**), six-puckering astringent N-phenylpropenoyl amino acid amides (**17**, **18**, and **22-25**), the sour citric acid, acetic acid, succinic acid, malic acid, lactic acid, oxalic acid, γ-aminobutyric acid and β-aminoisobutyric acid. It is worth to note, that compounds in this list could be degraded during extensive roasting (or later during conching). This applies to volatile organic acids or polyphenols, but increased roasting intensity as well means the production of more e.g., diketopiperazines. Thus, there is a thin balance between flavor development and overroasting. However, as cocoa is a complex matrix, there are a lot of major cocoa components generally known to have taste excluded from this list, but also minor and

possible unknown compounds as well. For instance, there are 34 different diketopiperazines in cocoa found to date (Andruszkiewicz, D'Souza, Altun, Corno, & Kuhnert, 2019), most of them in small relative amounts below their tasting thresholds. Currently, there is no indication of whether they can interact with the taste receptors and enhance the taste of more abundant diketopiperazines or interact in completely another way with themselves or other compounds while in the oral cavity. This is also true for other compounds with even more variety of structures present in cocoa – polyphenols which are in general bitter and astringent (D'Souza et al., 2017), as well as short peptides known typically for being bitter (D'Souza et al., 2018; Kumari et al., 2018). Still, the interactions of all cocoa components at once with taste receptors warrants further investigations.

## 1.2 The Maillard Reaction

Louis-Camille Maillard was a French biochemist living at the turn of the 19th century, whose scientific accomplishments influenced many branches of chemistry, medicine, and biology (Billaud & Adrian, 2003). His broad spectrum of work included: synthesis and crystallization of proteins, medicinal research on urine, analysis of trace metals, a theory concerning the natural formation of humic matters. Most notably, he laid the groundwork for the research on amino acid and sugar reaction, which was later named after him – the Maillard reaction. It was firstly reported in 1912 (Billaud & Adrian, 2003).
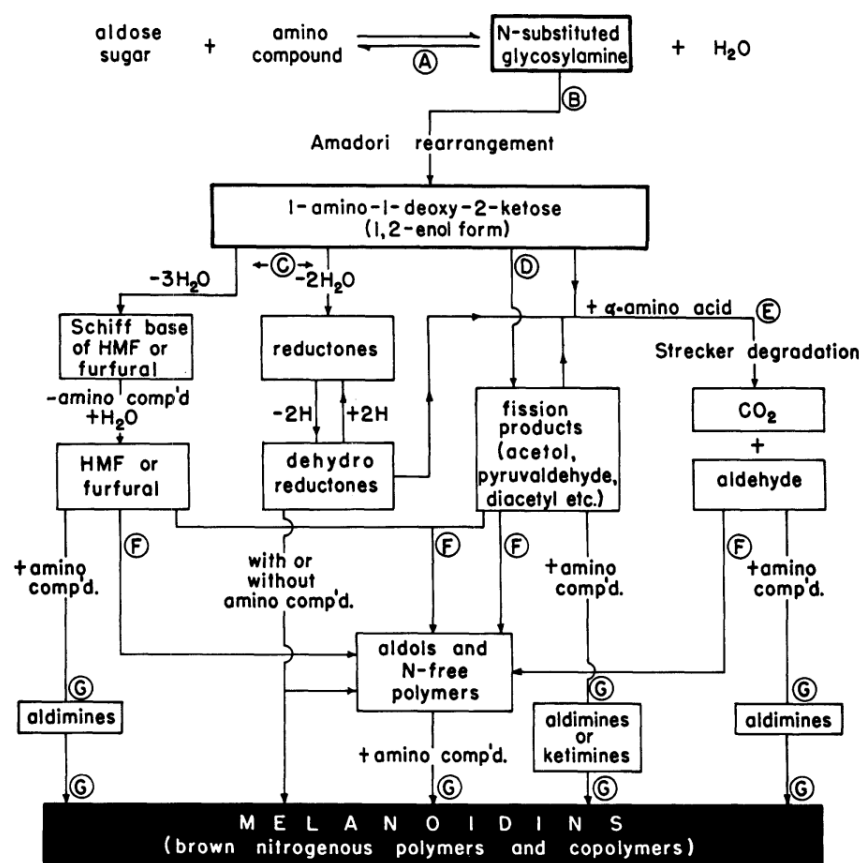


**Figure 4 Hodge's scheme of the Maillard reaction. Reprinted with permission (Hodge, 1953).**

It started with research on peptide synthesis (Billaud & Adrian, 2003). Maillard used glycerol as a "condensing agent" and succeeded in synthesizing several polypeptides. In pursuit of perfecting his method, he tried to use other polyalcohols instead – e.g., hexose

sugars. Surprisingly enough, he noticed browning of the reaction mixture, which started his more in-depth research in what later became the Maillard reaction (or non-enzymatic browning). Even though he was a pioneer, he had some observations that are still relevant today. He noticed the importance of carbonyl group of the sugar moieties, and that the nature of the reactants does not influence the browning reaction (any sugar, amino acid, peptide or protein can give browning products). Maillard compared his findings with the caramelization process, during which extensive dehydration occurs. However, he observed that his browning reaction could slowly proceed as low as 15 °C as long as it was heated before. This increased the applicability of the Maillard reaction to *in vivo* models. Moreover, he was able to assess the composition of late insoluble products (58.85% C, 4.92% H2 and 3.45% N), and observed $CO_2$ emission during the reaction, which he attributed solely to amino acid decomposition.

His findings were overlooked for some years (Billaud & Adrian, 2003), but around the Second World War period, scientists started to connect Maillard's studies non-enzymatic browning and food deterioration. The first summary of the Maillard reaction came from Hodge (Hodge, 1953). Based on growing research, he divided complicated cascade of non-enzymatic browning of amino acids and sugars into three stages (see **Figure 4**), and pointed out important groups of products and mechanisms of formation for each of them (adopted with changes from (Nursten, 2005)):

1. Initial Stage (colorless products)

    - Sugar and Amino Acid Condensation

    - Amadori Rearrangement

    - Free Radical Pathway (Namiki Pathway)

2. Intermediate Stage (yellow and UV absorbing products)

    - Sugar Dehydration (and Enolization)

    - Sugar Fragmentation

    - Amino Acid Degradation

3. Final Stage (highly colored melanoidins)

    - Aldol Condensation

    - Aldehyde-Amine Condensation

In short, the Maillard reaction begins with a simple condensation of sugars and amino compounds, which form Amadori rearrangement products (ARPs). These can degrade in different ways, with the help of sugar fission products, yielding small molecular weight compounds (which can be aroma compounds). In the end, they condense in various ways, forming complex nitrogenous polymers – melanoidins. Although the Maillard reaction has other significant consequences (e.g., in synthetic chemistry, biochemistry), it is most known to its contribution to changes in organoleptic properties during food processing.

Since Hodge, numerous books and research articles were written on the Maillard reaction (especially in the perspective of food chemistry), summarizing its most important elements (Ames, 1992; Nursten, 2005). Moreover, new aspects of the non-enzymatic browning, such as its pH dependence (Tressl, Nittka, Kersten, & Rewicki, 1995), came to our understanding. Most notably, free radical involvement into the basic-pH Maillard reaction was described (Hayashi & Namiki, 1986) and how it is different from acidic-pH melanoidin

formation (see **Figure 5**). The Maillard reaction knowledge expanded over the years, with the prospect of using this knowledge to influence the properties of thermally-processed foods (Ames, 1990; Parisi & Luo, 2018).
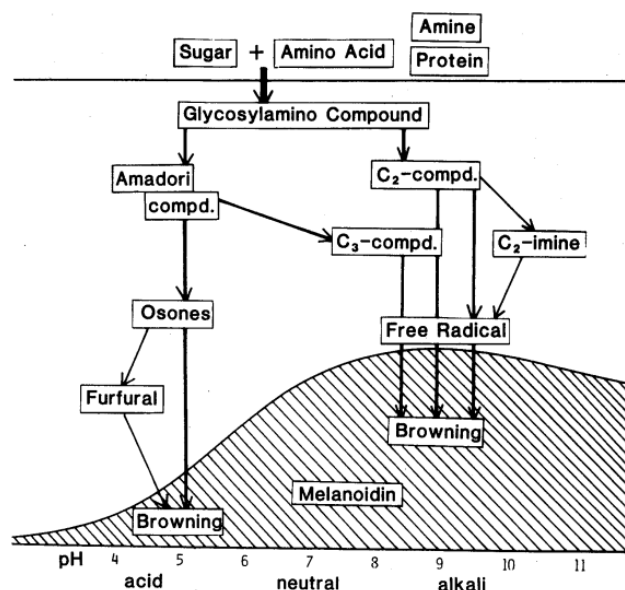


**Figure 5 Namiki's scheme of melanoidin formation and reaction pH-dependence. Reprinted with permission (Hayashi & Namiki, 1986).**

## 1.2.1 Initial Stage

*Initiatory Condensation and Amadori Rearrangement*

The first step (see **Figure 6**) is condensation of the carbonyl group (e.g., reducing sugar) and amino group (originated from an amino acid, protein, or amine). The product of this reaction is the N-substituted glycosylamino compound, which reversibly forms Schiff base derivatives. It begins with the nucleophilic attack of the unshared electron pair of an amino group onto the electrophilic carbon of the carbonyl group. This reaction usually needs an acid catalyst to protonate the carbonyl group and enhance its reactivity. The reaction rate is maximum at weakly acidic pH ranges (Namiki, 1988). Loss of one molecule of water leads to the formation of Schiff base, which immediately cyclizes to the N-substituted glycosylamine. It is a reversible step as hydrolysis of this compound can occur.

Glycosylamines are unstable and quickly form N-substituted 1-amino-1-deoxy-2-ketoses in so-called Amadori rearrangement, which, if happens specifically for aldose as a sugar (e.g., glucose), it forms an Amadori compound. It is catalyzed by weak acids, which protonate the Schiff base (Namiki, 1988). This step is considered irreversible, but there is some evidence to the contrary (Davidek, Clety, Aubin, & Blank, 2002). The Initial Stage of the Maillard reaction ends with the Amadori product serving as a base for further chemical transformations.

It is worth mentioning the Initial step of the Maillard reaction is favored by acidic conditions. It is contrary to the observed fact that browning reactions are favored by basic pH (Namiki, 1988). Furthermore, the reaction happens spontaneously, even at room temperature (Benzing-Purdie, Ripmeester, & Ratcliffe, 1985). In raisins, for example, the main production of Amadori rearrangement products occurs during the storage, not the processing (Sanz, del Castillo, Corzo, & Olano, 2001). This suggested similar behaviors of sugars and proteins can exist in physiological conditions, which in turn led to the discovery

of protein cross-linking mechanism in e.g., humans via N-glycosylation reaction pathways (Henning & Glomb, 2016). Similarly, this opened up possibilities for the identification of protein-specific Maillard reaction mechanisms in foods (Gerrard, 2002).
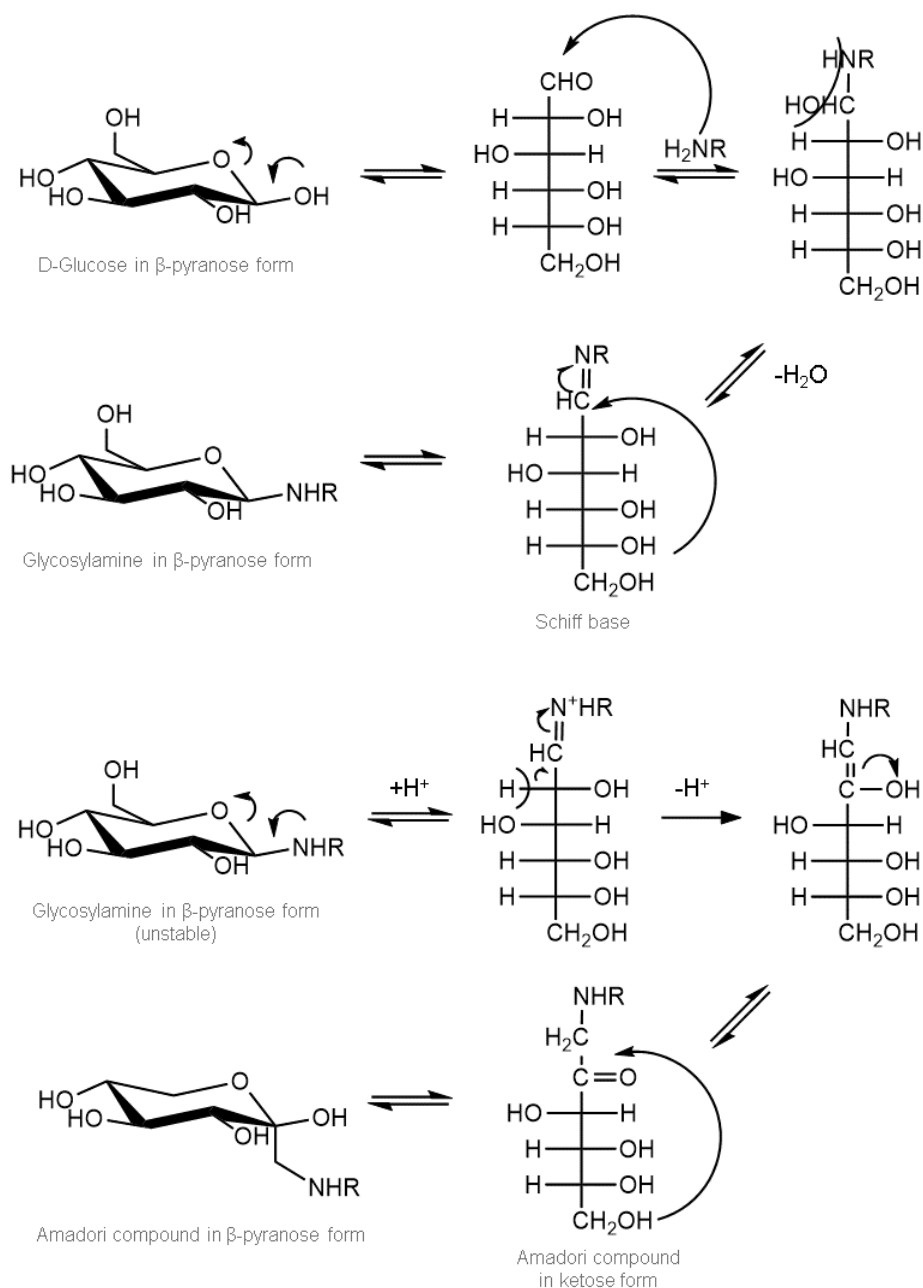


**Figure 6 Scheme of Initial Stage of Maillard reaction – Amadori rearrangement.**

Ketoses (such as fructose) undergo similar rearrangement, Heyns rearrangement, which leads to the formation of 2-amino-2-deoxyaldoses - Heyns compounds (Pilková, Pokorný, & Davídek, 1990). **Figure 7** illustrates the differences in the structure between Amadori and Heyns compounds and their substrates. In consequence, Heyns compounds are known to produce more variety of deoxyosones (Bruhns, Kaufmann, Koch, & Kroh, 2018), which in turn can undergo different chemical transformations.

It is worth noting that Amadori rearrangement is possible to occur for ammonia as a nucleophile as well (Kort, 1970), and respective pyrazines were observed as the products of such reaction (Shibamoto & Bernhard, 1977).
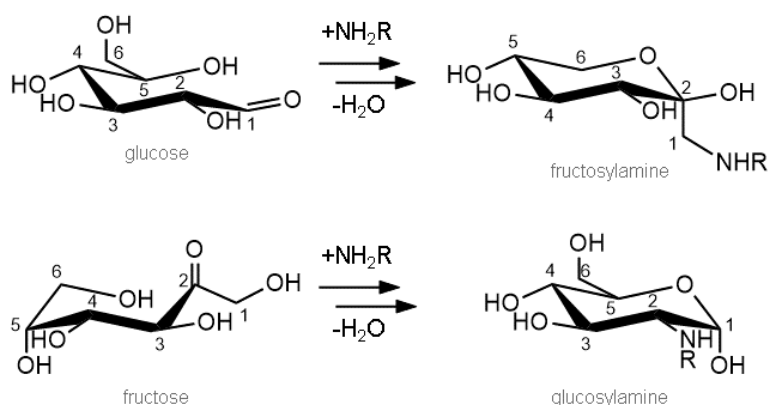


**Figure 7 Distinction of Amadori and Heyns rearrangements.**
Carbon atoms are numbered for easier comparison. The substrate in one rearrangement resembles the product in other, hence the nomenclature.

*Free Radical Pathway (Namiki Pathway)*

Before Namiki popularized the idea, general knowledge about radical involvement in the Maillard reaction was unknown. It was known, though, that during the Intermediate Stage of Maillard reaction, cleavage and fragmentation of sugars could occur with the involvement of retro-aldol reaction mechanisms (Bettina Cämmerer, Wedzicha, & Kroh, 1999). This happened during the main route of melanoidin formation in acidic conditions, which did not explain the browning process was happening in neutral and alkaline pH ranges. It was proved (Hayashi & Namiki, 1986) that sugar fragmentation can occur during the early stage of Maillard reaction even before actual Amadori rearrangement and is fueling the formation of radical species. This complemented previous observations and supported the idea of a radical pathway happening almost exclusively in basic conditions.

It is worth mentioning that it was observed methylglyoxal and glyoxal (important carbonyl reactive species), which were previously thought to be present only during the Intermediate Stage of Maillard reaction, form during Initial Stage as well (Hayashi & Namki, 1980). Their respective imines were isolated (Hayashi & Namiki, 1986), which are precursors of the pyrazinium radical structure described below (see **Figure 8**).

Radical pathway (Namiki pathway) involves glycosylamine or Amadori compound retroaldolization at the bonds between carbons $C_2$-$C_3$. Actual radical involvement begins after condensation of two identical fragments into symmetrical dihydropirazine when radical forms subsequently by one-electron oxidation. The presence of pyrazinium radical cation (see **Figure 8**) was confirmed by electron spin resonance spectroscopy (Hayashi, Ohta, & Namiki, 1977), mechanism of formation of which was later shown (Hayashi & Namiki, 1981). Subsequent studies described the formation of dihydroxydiethyldihydropyrazine, 2-hydroxy-1,4-diethyl-1,4-dihydropyrazine, and its colored oligomers via this pathway (Rizzi, 2003). It was shown on an example of coffee (T. Hofmann, Bors, & Stettmaier, 2002), that similar lysine-derived species, named CROSSPY, forms during roasting, can participate in peptide cross-linking, and is a browning precursor. It has to be stressed that protein cross-linking mostly happens on the lysine side-chain, as it has an amine group which can participate in pyrazinium ion

formation, but it's not exclusive to lysine – studies show the formation of similar species in peptides without lysine residue (Rizzi, 2003), suggesting that N-termini of amino acids also participate in this reaction.
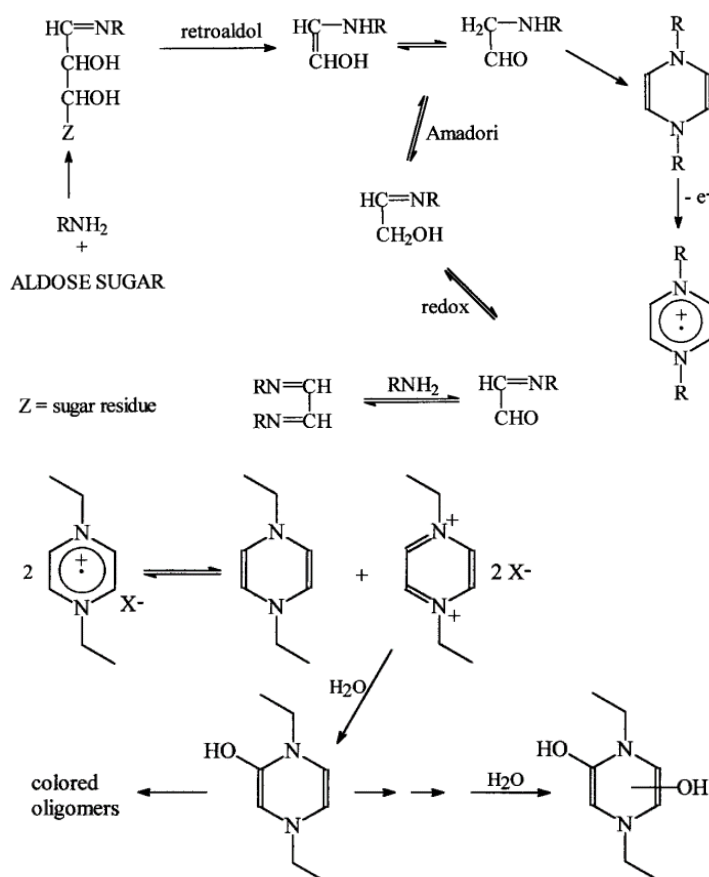


**Figure 8 Pyrazinium ion formation (upper) and its browning mechanism (lower part of the scheme). Reprinted with permission (Rizzi, 2003).**

The formation of radical species during the early stages of the Maillard reaction seems to be dependent on pH, sugar structure, and in a way, amino acid structure. A study investigating ionic and radical involvement in the early stage of the Maillard reaction (B. Cämmerer & Kroh, 1996) was performed using DPPH- and phenanthroline-based assays to quantify these phenomena. It was shown that in acidic pH, the radical involvement was small and increased over time, but in neutral or basic conditions, it was large and immediate (estimated to be at least 60% depending on the nature of reactants). The effect of different amino acids was shown on an example of glycine and phenylalanine, which affects the stability of radicals in a small way and was explained by better stabilization of radicals by $\pi$-electrons of phenylalanine side-chain. On the other hand, various sugars produce differences in radical participation but result in similar browning. This depends on the sugar moiety and its capability of producing retroaldol products. Another study (Georgescu, Georgescu, Leonte, & Florea, 1999) showed a smaller involvement of radicals in the case of disaccharides as the glycoside bond cleavage was the rate-limiting step.

Furthermore, autoxidation pathways of sugars and Amadori compounds can produce sugar fragments involving the creation of free radicals (Rizzi, 2003). General mechanism of sugar (including an $\alpha$-hydroxyketo group in its structure) autoxidation involves enolate oxidation by a metal ion, subsequent radical anion formation, and finally oxidation by molecular

oxygen into dicarbonyl compound. Besides forming smaller dicarbonyl compounds (glyoxal, etc.) autoxidation of glucose can lead to the formation of glucosone (not the deoxy- counterpart) (Liedke & Eichner, 2002). Experiments showed that glucose is autoxidized to glyoxal without preceding glucosone formation (Wells-Knecht, Zyzak, Litchfield, Thorpe, & Baynes, 1995). In a similar, heavy metal ion driven reaction, Amadori compounds were shown to degrade into corresponding glucosones (Liedke & Eichner, 2002), and the degradation is optimal in neutral pH (basic pH not included in the study). Similar reactions are typical to the Intermediate Stage of the Maillard reaction (Sugar Dehydration). In the absence of such ions, Amadori compounds could form products resulting from $C_2$-$C_3$ or $C_3$-$C_4$ enolate cleavage (Rizzi, 2003).

It is worth noting that the chemiluminescence phenomenon was observed during the early stages of the Maillard reaction and presumably originates from radical formation, such as singlet oxygen or peroxy compounds (Namiki et al., 1993).

## 1.2.2 Intermediate Stage

The Intermediate Stage of the Maillard reaction revolves around Amadori compounds and their reactivity – mainly in terms of degradation. In the original Hodge work (Hodge, 1953), the Intermediate Stage is described as reactions of Amadori rearrangement products, which produce first colored and UV-absorbing compounds. In general, it consists of sugar dehydration and enolization, its fragmentation (retroaldolization), and degradation of the amino acid itself (Strecker degradation). During this stage, low molar weight heterocyclic compounds are produced, which during the Final Stage of the Maillard reaction can polymerize yielding high molecular weight brown melanoidins. Intermediate Stage produces the main classes of aroma compounds as well (Strecker aldehydes, pyrazines, etc.).
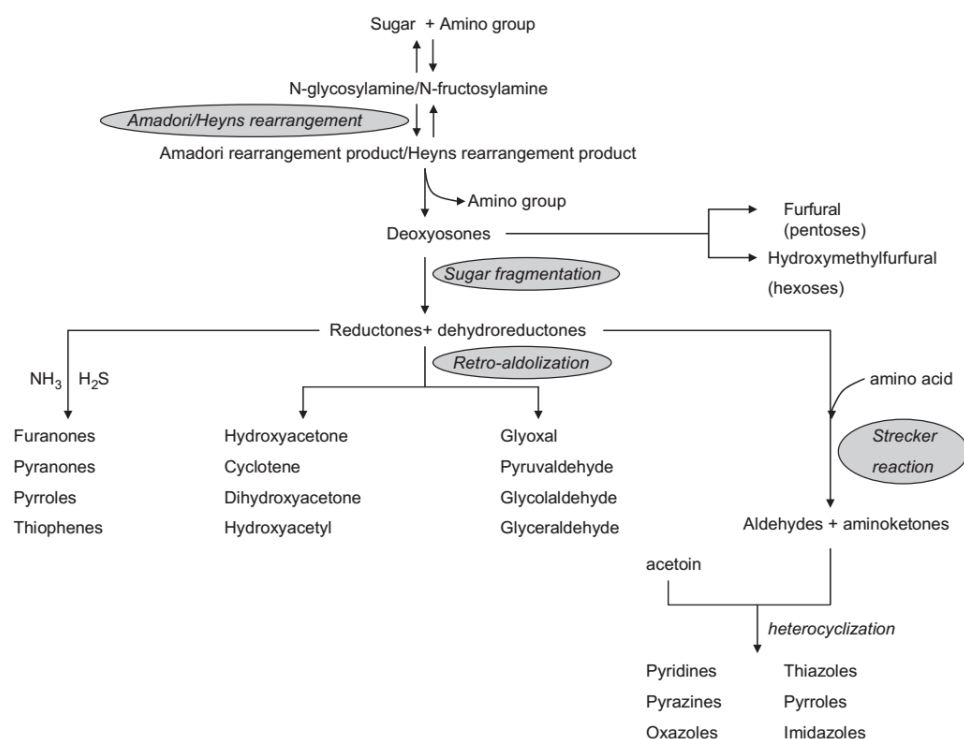


**Figure 9 Overview of aroma compounds as end products of Maillard reaction, which form mainly during the Intermediate Stage. Reprinted with permission (van Boekel, 2006).**

The main pathways occurring during the Intermediate Stage of Maillard are sugar dehydration and fragmentation and degradation of amino acid moieties. The first step is the Amadori compound enolization, which yields deoxyglucosones. Then, sugars, their respective osones, Amadori compounds, and their Schiff bases (reverse Amadori rearrangement) can dehydrate and cyclize with the formation of heterocycles. At the same time, all of the above can participate in retroaldolization and β-cleavage reactions, during which reactive carbonyl species (RCSs) and aminoketoses form. They, in turn, react via aldol condensation and cyclize yielding more variety of heterocycles. RCSs fuel Strecker degradation as well, which is the last type of reaction occurring during this stage, which produces Strecker aldehydes and more aminoketoses, which can dimerize into heterocyclics as well. An overview of important groups of compounds formed during the Intermediate Stage, which are mostly aroma compounds, is presented in **Figure 9**.

*Sugar Dehydration (and Enolization)*

The first attempts to organize Maillard reaction knowledge did not take into account the pH effect on different pathways of Amadori compound degradation. Later on (Tressl et al., 1995), it was concluded that initial dehydration of the Amadori compound is followed by 1,2- or 2,3- enolization (depending on pH, see **Figure 10**), and possible hydrolysis of formed Schiff base resulting in dicarbonyl and amino acid liberation. Each enolization reaction produces different variants of deoxyosones, which in the end, follow different reaction pathways.

Three main pathways of Amadori products degradation during the Intermediate Stage of Maillard reaction were established (Ames, 1992):

- 3-deoxyglucosone – 1,2-enolization in acidic conditions,

- 1-deoxyglucosone (1-deoxyhexosulose) – 2,3-enolization in basic conditions,

- 1-amino-1,4-dideoxyosone – similar to 2,3-enolization, but with retention of the amino acid moiety of the Amadori compound.

Additionally, the Amadori compound themselves can participate in fission and Strecker degradation reaction (later shown), which can be described as the fourth pathway of Amadori rearrangement products (ARPs) formation.

Another route, via the 4-deoxyosone route, was described (Ames, 1992), but it does not form from the Amadori compound (and it could not be isolated) but speculated to be formed based on the later products formed. On another note, Heyns compounds were proven to have more ways to rearrange and produce more variety of deoxyosones (Bruhns et al., 2018).

It was shown that 1,2-enolization of the Amadori compound occurs more easily when its nitrogen atom is nearly completely protonated, hence the pathway of 3-deoxyosone formation is favored in acidic media (Namiki, 1988). The 1,2-enaminol form is followed by the generation of 3-deoxyosone and loss of the amino acid group via hydrolysis and then furfural and hydroxymethylfurfural, which are known to be main products of this reaction both in model systems and food (Namiki, 1988). These important intermediates were shown to be produced in acidic degradation of carbohydrates and caramelization as well (Feather & Harris, 1973). It is worth mentioning that the furfural derivatives can participate in aldol condensation reactions and condensations with amino compounds (Hoydonckx, Van Rhijn, Van Rhijn, De Vos, & Jacobs, 2007), being a very prominent color precursor. Further products of this pathway could participate in protein cross-linking (Ames, 1992).

Finally, 3-deoxyosones can react with Amadori compounds yielding pyrrolealdehydes (Ames, 1992).

1-Deoxyosones are formed from Amadori products via 2,3-enolization, which is known to be favored in neutral and basic reaction conditions and participate in reductone production. It was confirmed by studies made on radio-labeled amino acids (Keyhani & Yaylayan, 1996) that 2,3-enaminol loses the amino acid group and forms 1-deoxyosone. Moreover, methylfuranone derivatives can be produced in this pathway via the hemiketal form of 1-deoxyosone; however, only in weakly basic conditions (Namiki, 1988). Similar but six-membered ring hemiketal form can yield pyranone derivatives in this way (Ames, 1992). The product structure of this pathway depends on the structure of Amadori product sugars (including disaccharides) and in general, participates in protein cross-linking. Another study showed that pyrrolinone-type reductones form from 1-deoxyosones and play an important role in melanoidin formation (Ames, 1992).
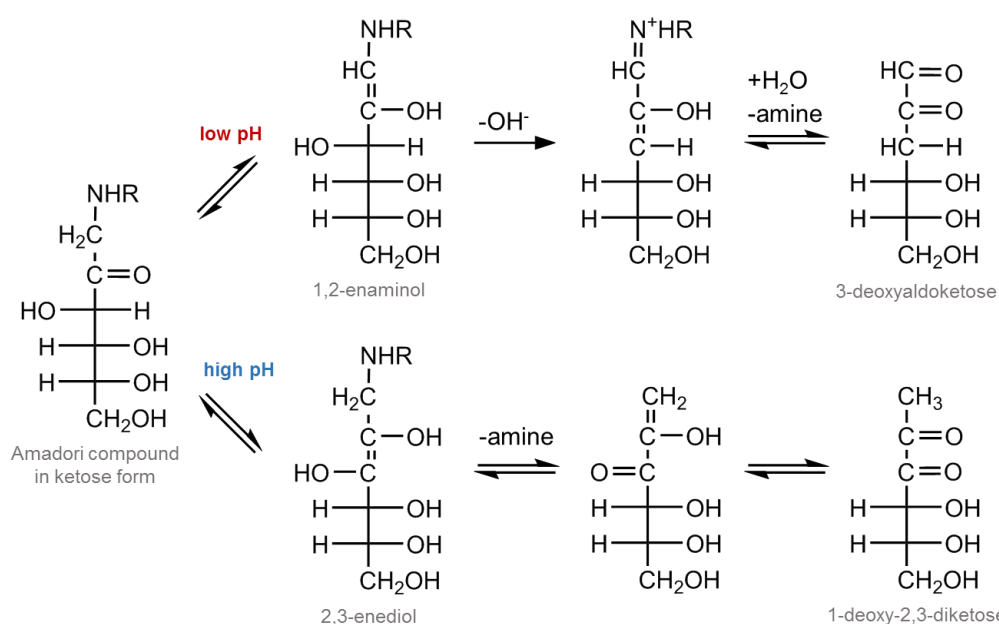


**Figure 10 Scheme of Amadori compound enolization, the first step of the Intermediate Stage of Maillard reaction.**

Another pathway of Amadori compound degradation involving deoxyosone formation was established but without the elimination of amino acid moiety. The products of this reaction, 1-amino-1,4-dideoxyosones, are formed in larger amounts from disaccharides. This pathway produces a variety of furan derivatives, including aminopyrroles, as well as aminoreductones, which as well can participate in protein cross-linking (Ames, 1992).

*Sugar Fragmentation*

Sugar fragmentation, besides Namiki Pathway, can occur in the Intermediate Stage of Maillard reaction and is mainly occurring but not exclusive to basic reaction conditions. The main contribution of this part of the reaction includes the formation of sugar fission products – dicarbonyls, α-hydroxycarbonyls, etc. – such as methylglyoxal, glyoxal, diacetyl, and others. The reaction mechanism involves retroaldolisation and is shown in **Figure 11**. This group of compounds was labeled as Reactive Carbonyl Species (RCSs) and in addition can come from direct sugar caramelization, Amadori product degradation and additional amination of Amadori products (Hayashi & Namiki, 1986), as well as in very small amounts as glucose degradation products (Thornalley, Langborg, & Minhas,

1999). These are highly reactive and important to both chromogenic and aromagenic pathways of the Maillard reaction (Yaylayan, 2003). Their browning capabilities were studied in reactions with lysine (in non-acidic conditions), with the order being glycolaldehyde > glyceraldehyde > dihydroxyacetone > methylglyoxal >> glyoxal (Namiki, 1988). A study showed that diacetyl reacted in acidic conditions with amino acids yielded oxazolines, pyrazines, and pyrroles (Namiki, 1988). There is evidence for polyphenol reactions with reactive carbonyl species, such as glyoxal and methylglyoxal, which prevent those compounds' involvement in the generation of volatiles (Y. Wang & Ho, 2012). RCSs are also forming in physiological conditions taking part in the formation of so-called advanced glycation endproducts (AGEs), which are linked to various chronic and age-related diseases (Henning & Glomb, 2016).
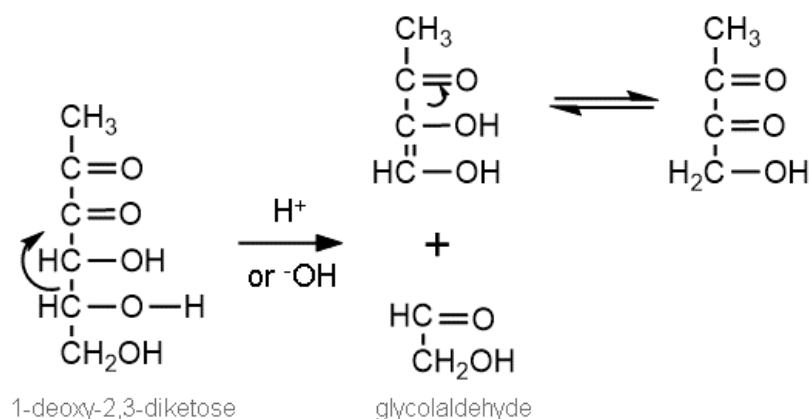


**Figure 11 An example of the retro-aldol reaction of the 1-deoxy-2,3-diketose with diacetyl derivative and glycolaldehyde as the end products.**

Another fission reaction can occur for a sugar moiety still being a part of Amadori product or 1-amino-1,4-dideoxyosone. These are one of the sources of aminoketoses in the reaction, which in turn can react with each other (symmetrically or not) yielding pyrazines, important aroma components of many heated foods.

*Amino Acid Degradation (Strecker Degradation)*

Strecker degradation, the main form of amino compound degradation during the Maillard reaction (Amadori compounds can participate by themselves (Yaylayan, 2003)), is fueled by the formation of dicarbonyl compounds, such as deoxyosones and sugar fission products. Additionally, other species occurring in food such as polyphenolics, lipids, lipid oxidation products, and terpenoids can be the source of the carbonyl groups (Rizzi, 1999). The main products of this reaction are aroma-active Strecker aldehydes and carbon dioxide (the major source of carbon dioxide during Maillard reaction). However, Strecker degradation is an important way of transferring amino groups from amino acid onto dicarbonyles, yielding aminoketoses, with structures depending on dicarbonyles, which they formed from. These, in turn, can dimerize cyclically (not necessarily in a symmetrical way), producing a group of important heteroaromatic aroma-active compounds – pyrazines and their derivatives. It was reported that peptides undergo analogous degradation with the formation of pyrazinones (pyrazine species with a carbonyl group as one of the ring substituents) (Izzo & Ho, 1992).

Amadori compounds, while reacting with amino acids, could form an imine adduct, which in turn degraded with the formation of a Strecker aldehyde (Rizzi, 1999). Moreover, it was

proved via model system experiments on an example of phenylalanine, that phenylacetaldehyde could be formed in oxidative degradation of respective Amadori compound by addition of copper (II) ions (Thomas Hofmann & Schieberle, 2000).

Altogether, Strecker degradation, along with Amadori rearrangement products of ammonia, are key reactions triggering the aromagenic pathway of the Maillard reaction. Chemical transformations of their products (e.g., aminoketones) are key to the formation of aroma volatiles such as pyrroles, oxazoles, pyrazines, imidazoles, pyrazinones, and pyridines.

### 1.2.3 Final Stage

The final stage of the Maillard reaction produces heterogeneous, nitrogen-containing brown polymers that can absorb up to 420 nm and are responsible for the color of many heat-treated foods. Their properties were closely studied (Ames, 1992; H.-Y. Wang, Qian, & Yao, 2011); however, due to the complexity of the process of their formation, their structure is relatively unknown and mostly hypothesized. It has been assumed that they are high molecular weight compounds (HMW), but their size can be controlled in model systems and vary in actual foods, as well as a low molecular fraction (LMW) has been reported (H.-Y. Wang et al., 2011). Additionally, it has been confirmed via gel permeation chromatography and electrofocusing (pI range between 3.5-4.85), that they are negatively charged at neutral pH (Kwak, Lee, Murata, & Homma, 2005). As a consequence of their nature, it is difficult to quantify melanoidins. However, their extinction coefficient was determined; hence, it is possible to estimate melanoidin concentrations using spectrophotometric methods (Brands, Wedzicha, & van Boekel, 2002; Bekedam, Schols, van Boekel, & Smit, 2006).

Preliminary studies on melanoidin structures on bread crust, coffee, tomato sauce, and model systems reported them to be mostly comprised of carbonyl compounds with furan, pyrrole, pyrazine, and pyridine rings (Adams, Borrelli, Fogliano, & De Kimpe, 2005). Additionally, there is an interesting relationship between pH and structure and color of low molecular weight melanoidin observed in model systems (although the experiment conditions varied not only in pH values). A blue pyrrolopyrrole dimer connected with a methine bridge was identified after the reaction in an aqueous alkaline solution of various combinations of xylose or glucose with either glycine or β-alanine (under the nitrogen). In the same studies and conditions, a red pyrrolopyrroleazepine pigment was found (Hayase et al., 2008). Both had polymerizing activities. Similar yellow compounds, acetyl pyridinone, and acetyl azepinone were detected in acidic xylose-glycine and glucose-glycine systems (Ames, Bailey, & Mann, 1999). Finally, in neutral conditions, the pentose and methylamine model system yielded an N-substituted pyrrole polymer chromophore, which condensates readily (Hayase, Usui, & Watanabe, 2006). The polymerizing activities of such low molecular weight compounds seem to play a crucial role in the formation of higher molecular weight polymers. As such, pH seems to dictate the structure of LMW compounds and time and temperature the molecular weight, typically resulting in the formation of the HMW melanoidins.

Even though we know some structures of the precursors to the HMW melanoidins, their mechanism of formation is still unclear. However, there have been three plausible theories proposed. First, the high molecular weight pigments can be formed from by polymerization of LMW Maillard reaction intermediates such as furans, pyrroles, pyrrolopyroles (like compounds mentioned above) (H.-Y. Wang et al., 2011). This could explain the existence of low molecular weight fraction of melanoidins (as unreacted precursors/monomers), and

the molecular weight dependence on heating time. Second, the LMW MRPs polymerization could occur by their cross-linking with reactive amino acid side chains, such as lysine, arginine, or cysteine (Thomas Hofmann, 1998b). A series of such brown-red pigments responsible for darker coloration was described both in model systems and in foods (Thomas Hofmann, 1998a; Lindenmeier, Faist, & Hofmann, 2002). Finally, the third hypothesis suggests that the degradation products of sugars form a backbone that is linked to multiple amino compounds, such as amino acids. This is supported by the findings that the melanoidins formed in glucose-only model systems exhibit similar adsorption isotherm to the model systems of glucose and amino acids (Ćosović, Vojvodić, Bošković, Plavšić, & Lee, 2010). Another model system study (mono-, di-, and oligomeric carbohydrates with amino acids) suggest, that intact glucose and its oligomers can be a part of this backbone (Bettina Cämmerer, Jalyschko, & Kroh, 2002), which also could react with amino compounds and form branched structures (Bettina Cämmerer, Jalyschkov, & Kroh, 2002). The existence of all these structures implies that the actual melanoidins could be a mixture of all three cases. Interestingly enough, the melanoidins in foods seem to be mostly high molecular weight compounds, such as in: coffee (Bekedam et al., 2006) (12-14 kDa), sweet wines (>12 kDa) (Rivero-Pérez, Pérez-Magariño, & González-San José, 2002), roasted malt (>60 k Da) (Faist, Lindenmeier, Geisler, Erbersdobler, & Hofmann, 2002), and roasted cocoa (>5 kDa) (Summa et al., 2008). The difference in molecular weights of melanoidins in foods shows different availability of Maillard precursors and treatment methods (or changes in extractability). In the case of cocoa, different melanoidins could form during the alkalization (blue dimer mentioned above), which could be a reason for its darker color.

Since Melanoidins are prevalent in heated foods, which also means in our diet, their potential biological effects have become of great interest. Melanoidins were shown to have antioxidant activity, antimicrobial activity, tumor growth-inhibiting properties, and modulating properties towards detoxification enzymes (Langner & Rzeski, 2014).

## 1.2.4 Overview of Other Aspects of the Maillard Reaction

Although the Maillard reaction in food is complex enough, there are many variable parameters as well as food constituents affecting its course. Here, a brief description of other complementing and competing processes is presented.

*Caramelization*

Thermal degradation of carbohydrates, termed caramelization, belongs to the category of non-enzymatic browning reactions (Fennema, 1996). It requires a temperature of at least 120 °C, which is much higher than for the Maillard reaction (Kroh, 1994). This process is facilitated by either acidic or basic pH, which was utilized by the food industry to produce deeply colored pigments. They were, in turn, used as coloring additives to foodstuffs and beverages (Sengar & Sharma, 2014). In addition, this reaction is able to produce specific flavor compounds (Pittet, Rittersbacher, & Muralidhara, 1970).

The caramelization process depends on the carbohydrates subjected to heating – mono-, oligo-, or polysaccharides (Kroh, 1994). However, the general mechanism reflects the chemical transformations of *Sugar Fragmentation* during the Maillard reaction (see **Section 1.2.2**), without the aspect of an amine as a nucleophile. In principle, the enolization of monosaccharides and their subsequent dehydration leads to the formation of various deoxyosones, which are the basis for heterocyclic compound production. Moreover, aldol condensation reactions can occur, as well as sugar fission via the retro-aldolization pathway.

Caramelization can produce low and high molecular weight compounds. The most prominent example of the former is the hydroxymethylfurfural (HMF), as well as hydroxyacetylfuran (HDF) and hydroxydimethylfuranone (HAF) which all form as a consequence of dehydration of respective deoxyosones. HMF can degrade further into other heterocyclic derivatives (Kroh, 1994). Although similar decomposition products can be generated from oligomeric sugars, they also undergo dehydration reaction without breakage of the bonds between the monomeric constituents. This process yields the formation of, e.g., difructose anhydrides, which were described in commercial caramels (Ratsimba, Fernández, Defaye, Nigay, & Voilley, 1999).

High molecular weight colored polymeric species can be created during caramelization. They are most likely derived from various furfurals via condensation reactions occurring with each other. However, these are poorly characterized. Additionally, unknown structures of species named caramelan, caramelen, and caramelin, were observed to form as a result of drastic dehydration of polymeric carbohydrates (Tomasik, Pałasiński, & Wiejak, 1989).

*Thermal Degradation of Proteins and Peptides*

Proteins and peptides are highly reactive molecules containing multiple moieties, which can react with each other or with the food matrix, undergoing complex transformations during heating (Bikaki, 2019). There are three elements in protein and peptide structure, which can be subjected to chemical modifications during heating – the N-terminus, the C-terminus, and the side chains of the amino acids connected to the backbone. Additionally, thermal decomposition can result in the breakage of the peptide chain (hydrolysis) as well as the cross-linking of different protein fragments (condensation).

Elevated temperature imposes favorable conditions for the release of volatile inorganic molecules, such as $CO_2$ (decarboxylation of either C-terminus or side-chain) (Bobrowski & Schöneich, 1996; Bikaki & Kuhnert, 2019). Deamidation reactions can occur as well for amide-containing side chains of glutamine and asparagine. The hydrolysis of amide bond occurring during heating causes a release of ammonia, which in turn could react in the Maillard reaction with the formation of simple Amadori compounds (III, Izzo, Zhang, & Ho, 1996). Additionally, dehydration induced by heating forces the peptides to form at least five-membered rings. An example of such reaction is a common thermal degradation product formed by spontaneous cyclization of two amino acids at the N-terminus of a peptide with an elimination of a cyclic dipeptide – 2,5-diketopiperazine. Diketopiperazines, as many amino acids and dipeptides, contribute to the bitter taste of foods (Belitz & Wieser, 1985; Ishibashi, Kouge, Shinoda, Kanehisa, & Okai, 1988). Their formation is promoted by increased temperatures and is relevant to foods processed in more intensive conditions (see **Chapter 2**). It was proposed that a similar cyclization reaction could take place with a carbonyl oxygen atom acting as a nucleophile, yielding a five-membered oxazole ring (Bikaki, 2019).

Since oxygen is an abundant component present during the thermal processing of food, it can frequently take part in their free radical-mediated oxidation (Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015). The reaction mechanism includes radical formation in the presence of oxygen, which in turn reacts with the peptides and proteins. In consequence, loss of sulfhydryl groups, the formation of protein carbonyls, the formation of cross-links, and the modification of aromatic amino acids are observed.

Finally, multiple cross-linking reactions between peptides and proteins can occur with the involvement of the reactive side chains of amino acids (Gerrard, 2002). Such cross-links can occur via many mechanisms and affect the functional and nutritional properties of food.

*Peptide-specific Maillard Reaction*

Although there is much emphasis put on amino acids as objects of studying Maillard reaction, some research showed that short peptides could produce unique MR products, such as pyrazinone as well as some pyrazines in larger quantities (Izzo & Ho, 1992; Van Lancker, Adams, & De Kimpe, 2010). Recent investigations have shown that short peptides form Amadori compounds in model systems, which in turn generate a greater variety of pyrazines than amino acid model systems (Scalone, Cucu, De Kimpe, & De Meulenaer, 2015; Zou et al., 2018). Peptides were shown to produce more substituted pyrazines with the N-terminal amino acid being a decisive factor in the type of the pyrazine created (Van Lancker, Adams, & De Kimpe, 2012). Interestingly enough, in a peptide model system heated above 120 °C peptide cross-links were produced more readily in comparison to lower temperatures. At the same time, the amount of short bitter peptides decreased (Lan et al., 2010). Additionally, high molecular weight peptide MR systems (molecules between 1000 and 5000 Da) yielded better taste after thermal treatment (Ogasawara, Katsumata, & Egi, 2006).
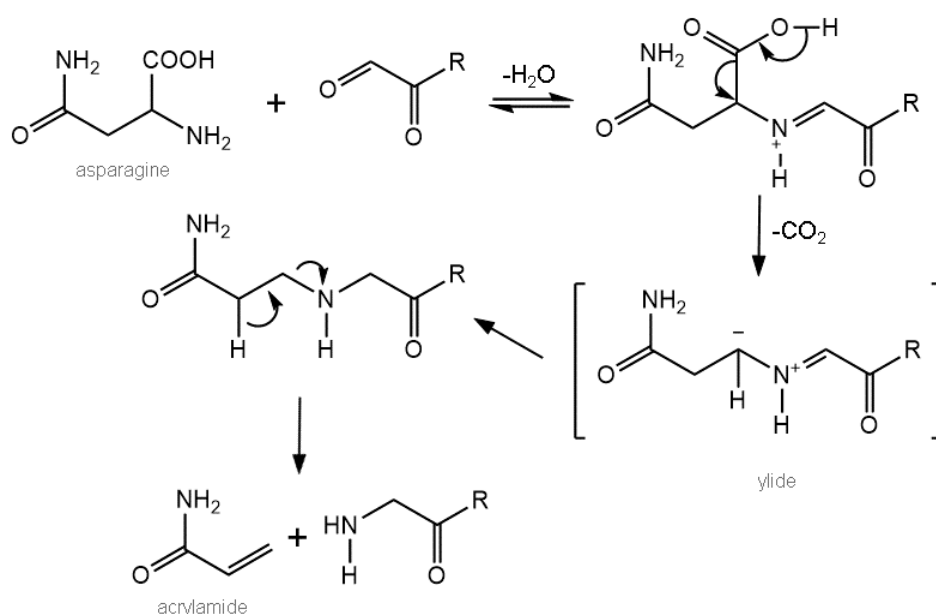


**Figure 12 Formation of acrylamide from asparagine via the Strecker degradation pathway.**

However, the Maillard reaction can impose a risk to food safety, as acrylamide formation was mechanistically connected with the degradation of asparagine and other amino acids (Friedman & Mottram, 2005). The reaction required a carbohydrate reactant to occur (see **Figure 12**). Recently, investigations revealed that the formation of acrylamide was possible for peptides as well. Peptides containing tryptophan, methionine, aspartic acid, phenylalanine, alanine, and glutamic acid without any dicarbonyl source present were able to produce acrylamide in a heated model system (Casado, Montaño, Spitzner, & Carle, 2013). In addition, browning and acrylamide formation followed almost the same kinetic pattern during baking (Gökmen, Açar, Arribas-Lorenzo, & Morales, 2008).

Ultimately, peptide and protein Maillard reaction model systems have shown significant differences in compounds produced in comparison to their amino acid counterparts. This opens up more possibilities in flavor chemistry of food and warrants further research.

*Maillard Reaction under Physiological Conditions and Advanced Glycation Endproducts (AGEs)*

At first, the Maillard reaction was associated only with the thermal treatment of food and its ensuing organoleptic properties. However, further studies have shown Amadori rearrangement products forming in mild conditions, which resulted in the investigations of analogous reactions under physiological conditions. It was an interesting development as so far, O-glycosylations of proteins were typically considered as the mainly occuring glycosylations in biochemical studies *in vivo*. Additionally, in this case, such reactions can be affected by enzymatic activity as well. The products of the Maillard reaction under physiological conditions were termed advanced glycosylation end products (AGEs).

In principle, the reaction mechanism is similar to its food counterpart (Henning & Glomb, 2016). It starts with the Amadori arrangement and the formation of glucosones. Their subsequent fragmentation reactions yield reactive carbonyl species (RCSs), such as glyoxal or diacetyl, which are essential precursors for AGEs. However, the *Sugar Fragmentation* occurs differently (see **Section 1.2.2**), as the mild conditions are inadequate for retroaldolization and other sugar fission reactions. The main pathway *in vivo* is the amine-induced or hydrolytic cleavage of the β-dicarbonyl compounds formed after the isomerization (by enolization) of respective glucosones (Henning & Glomb, 2016). In addition to simple sugars, ascorbic acid can react in a similar way yielding 2,3-diketogulonic acid as a product. These RCSs can undergo further chemical transformations and form cross-links between side chains of basic amino acids, such as lysine and arginine, in a similar way to those presented in **Figure 8**. For example, two glyoxal molecules can react and cross-link two separate lysine side-chains and form a structure that is shown in **Figure 13** below (Horvat & Jakas, 2004).

The risk of the formation of such species is that the AGEs are associated with many chronic and age-related diseases such as diabetes, uremia, atherosclerosis, cataractogenesis, and Alzheimer's disease (Henning & Glomb, 2016). For example, they can advance the age-associated structural and physiological changes in the cardiovascular system (Zieman & Kass, 2004). Additionally, AGE formation in vivo is promoted by a general increase in oxidative stress associated with many chronic diseases (Henning & Glomb, 2016), which can create a positive feedback loop. This can happen in the case of e.g., diabetes (Sato et al., 2006). Furthermore, AGEs interact with a receptor – RAGE, which is a multiligand receptor propagating dysfunction in cells and leading to many inflammatory disorders. It is expressed at low levels in healthy tissues but becomes upregulated at sites where its ligands accumulate (Chavakis, Bierhaus, & Nawroth, 2004). Even though the MRPs form in vivo and food differently, because of the binding to the RAGE receptor, digestion and absorption of dietary AGEs can pose health risks (Henle, 2007)

Additionally, similar to AGEs, lipid-derived intermediates can also take part in derivatization and cross-linking of peptides and are called advanced lipoxidation end products (ALEs) (Miyata, Kurokawa, & Strihou, 2000; Thorpe & Baynes, 2003).

pentosidine          pyrraline imine
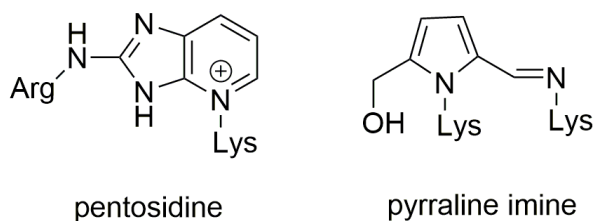
**Figure 13 Structures of the advanced glycosylation end products (AGEs), which participate in the cross-linking of peptides and proteins.**

*Maillard Reaction in Cocoa*

Mechanistic knowledge of Maillard reaction in cocoa and food in general is mainly acquired by reactions carried out in the model systems. Hence it is only an approximation of what happens in the actual food during thermal processing. Some reactions are executed in a specific medium to estimate the matrix behavior, such as cocoa butter roasting model (Arnoldi, Arnoldi, Baldi, & Griffini, 1988). This does not change that although we have the knowledge of most important flavor components of roasted cocoa (see **Section 1.1**) (Hashim, 2000; Aprotosoaie et al., 2016), there is still much to discover in the field of cocoa processing chemistry. Especially, when FT-ICR studies have shown 10,000 signals for unroasted fermented cocoa (Milev, Patras, Dittmar, Vrancken, & Kuhnert, 2014), and only a few Amadori precursors were observed in cocoa (Meitinger et al., 2014). Additionally, the number of peptides after fermentation is unproportionally higher than the reported amount of reducing sugars for cocoa, which is unusual amongst foods undergoing intensive non-enzymatic browning.

Another unique aspect of the Maillard reaction in cocoa is the high abundance of polyphenolic compounds (Oracz et al., 2015). It is generally known that the Maillard reaction benefits from oxidative conditions, which could be inhibited by the antioxidant-rich fraction of cocoa polyphenols (D'Souza et al., 2017). It was shown that higher polyphenol content decreased the amount of pyrazines formed during roasting (Misnawi, Jinap, Jamilah, & Nazamid, 2004). However, the antioxidant capacity is as well lowered by the roasting (Summa et al., 2006; Oracz & Nebesny, 2019). There is also a suspicion that polyphenols could decrease susceptibility of cocoa peptides to the Maillard reaction-related cross-linking (Jumnongpon et al., 2012).

Hence, the investigations contained in this thesis are aimed at studying the organoleptic Maillard-related components of roasted cocoa, directly in the matrix itself.

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# Aims and Objectives

Roasting of cocoa beans is one of the most important steps in chocolate production. Over its course, many chemical transformations occur, which drastically change the organoleptic properties of the raw material. Within the cocoa industry, it has been an empirically driven process, and the science of thermally processed food still does not provide enough insight into the roasting chemistry. Another layer is added to this challenge when one considers that the dried cocoa beans as the raw material are chemically complex and highly diverse. Their variability is expressed at every stage of the processing chain and is mainly manifested as origin and variety of raw material, degree of fermentation, and different drying practices. COMETA (COcoa METAbolomics) project, a collaboration between Barry Callebaut AG and Jacobs University Bremen, was founded to address above-mentioned challenges and to provide a link between the cocoa quality, and its origin as well as post-harvest practices. However, the knowledge of raw material has to be met with an even deeper understanding of its processing. Therefore, this thesis work utilized already existed HPLC-MS methodology and the knowledge generated by COMETA project to provide industrially relevant insights to the roasting and its influence on organoleptic properties. Consequently, within this Ph.D., the general aims were as following:

- to identify important components and markers of roasted cocoa;
- relate them to their precursors and degradation products within a wider context of chocolate production;
- show their variability and relevance to material coming from different countries of origin;
- look for correlations between different components before and after processing;
- while focusing on organoleptic and quality markers, including Maillard reaction products;
- additionally, construction of a Design of Experiments (DoE) cocoa roasting model;
- all within the aims of the COMETA project, which should complement other member's work.

# Chapter 2

# Thermally-induced Formation of Taste-active 2,5-Diketopiperazines from Short-chain Peptide Precursors in Cocoa

Paweł J. Andruszkiewicz, Roy N. D'Souza, Irem Altun, Marcello Corno, and Nikolai Kuhnert

## Abstract

2,5-diketopiperazines (DKPs) are cyclic dipeptides responsible for the specific bitter taste of cocoa formed during roasting. The 2,5-diketopiperazine and peptide composition of four different cocoa bean samples from different origins were studied using LC-MS techniques. 34 diketopiperazines were identified, of which 10 are newly reported in cocoa. Their formation was followed during two different roasting time-series using a zero-order and an alternative Prout-Tompkins solid-state kinetic models. The activation energies of diketopiperazine formation showed a distribution close to normal with individual values depending on the nature of the substituents. The relative concentrations of the DKPs were correlated with their putative peptide precursors in unroasted cocoa bean samples. The results showed a significant positive correlation, indicating that oligopeptides formed in cocoa bean fermentation are taste precursors for bitter-tasting diketopiperazines. Unexpectedly, for most diketopiperazines, a single major peptide precursor could be suggested.

## 2.1 Introduction

2,5-Diketopiperazines (DKPs) are cyclic dipeptides formed from two amino acids. These molecules are found in nature as well as in processed food and have gained attention along with their derivatives due to their bioactivity. For example, cyclo(His-Pro) is endogenous to some mammals, where it is present in various body fluids and has multiple biological effects, e.g., secretion control of thyrotropin and growth hormone (Prasad, 1995). In a related study, the spontaneous non-enzymatic cyclization of the recombinant human growth hormone led to a DKP release (Battersby, Hancock, Canova-Davis, Oeswein, & O'Connor, 1994). In the plant kingdom, the tryptophan-based DKP alkaloid, Cristatin A, which was found to be produced by *Lepidagathis cristata*, has been reported to be the active ingredient in its plant extract and has been used for the treatment of skin infections and fever in traditional Ayurvedic medicine (Ravikanth, 2001). Various microbes produce DKPs and their analogs, either intentionally or as by-products, via cyclopeptide synthase (CDPS) or non-ribosomal peptide synthetase (NRPS) pathways (Belin et al., 2012), where

they have been suggested to act as metal ion chelators. It has also been reported that lactic acid bacteria can inhibit the growth of fungi by the synthesis of antifungal cyclic dipeptides (Magnusson, Ström, Roos, Sjögren, & Schnörer, 2003). On the other hand, for some pathogenic fungi and bacteria, metabolites with a DKP-scaffold play an important role in their virulence. Therefore, the synthetic pathways (NRPS-dependent and CDPS-dependent) could be potential drug targets for antimicrobial agents (Gu, He, Yan, & Zhang, 2013). The different properties of DKPs attracted medicinal chemists to develop new methods for their synthesis and subsequently base new lead structures on them (Borthwick, 2012).

DKPs are common constituents in many food products (Borthwick & Da Costa, 2017), where they impart a bitter taste, even more, bitter than their acyclic counterparts (Ishibashi, Kouge, Shinoda, Kanehisa, & Okai, 1988). DKPs have been found in beer (Gautschi et al., 1997), roasted coffee (Ginz & Engelhardt, 2000, 2001), chicken essence (Chen, Liou, & Chen, 2004), wheat sourdough and bread (Ryan, Dal Bello, Arendt, & Koehler, 2009), and even wine (Stamatelopoulou et al., 2018). In food, DKPs are degradation products of thermally processed peptides or proteins, which cyclize at their N-termini preferably in either acidic or basic conditions (Anteunis, 2010), and where they can also undergo epimerization at either of the two stereocentres (Eguchi & Kakuta, 1974; Steinberg & Bada, 1981). A kinetic study following DKP-formation from protected amino acid precursors has also been previously reported (Goolcharran & Borchardt, 1998).

Cocoa bean processing involves both microbial fermentation as well as subsequent thermal treatment. During bean fermentation, polymeric cocoa constituents, such as proteins, degrade upon microbial action and enzymatic activity of the bean itself. At the time of thermal processing, the oligopeptides created during fermentation can participate in various reactions, most notably the Maillard reaction (van Boekel, 2006), which is an essential contributor to cocoa flavor and color. However, it has been known that bitterness in cocoa is partially attributed to diketopiperazines that act synergistically with theobromine (Pickenhagen et al., 1975), which is also found in significant amounts in cocoa. A study on DKPs in cocoa and their taste attributes showed quantitative data and descriptions of their taste thresholds by a taste panel (Stark & Hofmann, 2005). Furthermore, taste recombinant experiments revealed DKPs as one of the classes of compounds that are essential contributors to the characteristic bitter taste of roasted cocoa along with theobromine, caffeine, and flavan-3-ols (Stark, Bareuther, & Hofmann, 2006). In this contribution, we present a study on previously reported and unreported DKPs in cocoa, their kinetics of formation, and probable precursor oligopeptides.

## 2.2 Material and Methods

### 2.2.1 Chemicals and Reagents

Petroleum ether, dichloromethane, acetone, HPLC-grade isopropanol, HPLC-grade acetonitrile, and HPLC-grade methanol were obtained from Carl Roth (Germany). Acetic acid, formic acid, hesperetin, and sodium hydroxide were obtained from Sigma-Aldrich (Germany). Milli-Q water (18.2 M$\Omega$•cm at 25 °C) was used throughout all experiments.

## 2.2.2 Sample Collection and Preparation

4 different fermented, dry cocoa bean samples from Ivory Coast (2 separate samples), Ecuador, and Indonesia were obtained by Barry Callebaut AG. More detailed information about sample origin and raw material processing is shown in **Table 1**.

At least 30 g of cocoa beans from a sample was first de-shelled manually using a scalpel and then ground to a fine powder using a knife mill (Retsch Grindomix GM200, Germany) at 10,000 rpm. The samples were stored at 4 °C both prior and after grinding.

**Table 1 Information on origin and raw material processing of cocoa beans used in this study.**

| Name | Country of Origin | Nearest Town/Village | Hybrid | Harvest Time | Fermentation Type | Fermentation Time [days] | Drying Type | Drying Time [days] |
|------|-------------------|----------------------|--------|--------------|-------------------|--------------------------|-------------|--------------------|
| VA09 | Ivory Coast | Daloa /Guessabo | G11UPA402XT413 | April 2014 | Spontaneous | 7 | Sun | n/a |
| VB01 | Ivory Coast | Daloa /Guessabo | G3UPA409XC1 | October 2014 | Spontaneous | 7 | Sun | 10 |
| IA03 | Indonesia | Sukabumi, Jakarta | TSH 858 | June 2014 | Spontaneous | 6 | Sun | 7 |
| EB02 | Ecuador | Quevedo | CCN 51 | October 2014 | Spontaneous | 6 | Sun | 5 |

## 2.2.3 Model Roasting

Fermented dry cocoa powder from Ivory Coast (VA09) was roasted in two time series at varying temperatures (120 °C and 150 °C). For each of the roasting series, seven 12 g samples were weighed out on aluminum weighing boats and put into a forced draft oven. A sample was collected from the oven every 10 minutes during the course of 70 minutes. Liquid nitrogen was used to quench ongoing chemical reactions immediately after collection. Three additional cocoa powders from Ecuador (EB02), Indonesia (IA03), and Ivory Coast (VB01) were treated for 70 minutes at 150 °C.

The roasted powder was defatted using petroleum ether in an automated Soxhlet extraction apparatus (Büchi B-811, Germany) for 8 hours. The defatted powder was dried under vacuum and stored at 4 °C until further use.

## 2.2.4 Peptide and Diketopiperazine Extraction

Peptide extraction was carried out following a previously established protocol (D'Souza et al., 2018). 5 mL of acidified methanolic solution (MeOH:$H_2O$:$CH_3COOH$::70:28:2) and 50 mg of defatted power were mixed and sonicated in an ultrasonication bath for 10 mins, then stirred for a further 30 mins, and finally syringe-filtered through a PTFE membrane filter (0.45 micron). The methanolic extract was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and used directly after for HPLC-MS experiments.

Diketopiperazine extraction was performed using a simplified version of an already published method (Stark & Hofmann, 2005). 6 g of defatted cocoa powder was sonicated with 100 mL of 70% acetone in the water at room temperature for 15 minutes, followed by 15 minutes of stirring. The extract was filtered with a filter paper, and acetone was evaporated with a rotary evaporator (40 °C). The remaining water phase was extracted with 3 × 50 mL of DCM and dried under reduced pressure (40 °C). Samples were stored at -25 °C. Before the HPLC-MS measurements, samples were suspended in methanol to a concentration of 0.75 mg/mL.

## 2.2.5 HPLC-TOF-MS Measurements

HPLC separation conditions were adopted from a previously established method (D'Souza et al., 2018), and in the case of both peptide and DKP analysis, an Agilent 1260 HPLC system equipped with a Poroshell 120 EC-C18 column (RRHD, 2.1 x 100 mm, 2.7 µm particle size) was used. Milli-Q water and acetonitrile, both with the addition of 0.05% of formic acid, were used as a binary solvent system (Solvent A and Solvent B respectively), with sample injection volume being 2 µL. The gradient, at a constant flow rate of 0.4 mL/min and column temperature of 40 °C, used for peptide analysis was (t (min), %B): (0, 8); (1, 8); (2.5, 12); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95). The gradient employed for diketopiperazine measurements was (t (min), %B): (0, 5); (1, 5); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95). The described HPLC system was coupled to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with an electrospray ionization source (nebulizer pressure of 1.8 bars, the dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). MS data for both peptide and DKP analyses were acquired in positive ion mode (including selected MS/MS experiments) and reported NIST monoisotopic atomic masses of the elements (Coursey, Schwab, Tsai, & Dragoset, 2015) were used to calculate monoisotopic molecular masses of detected compounds. 0.1 M sodium formate solution was used to calibrate the TOF analyzer before each sample measurement. The UV signal of theobromine at 280 nm was used as an internal standard for the relative quantification of diketopiperazines in all cases due to its robust temperature stability (Wesołowski & Szynkaruk, 2001; Wesolowski & Szynkaruk, 2008; Kamel, 2015). The relative concentration of each DKP was calculated as a ratio of respective MS peak area to the UV peak area of theobromine at 280 nm.

## 2.2.6 Statistical Analysis

Peptide extracts of raw VA09 sample were injected four times to ensure reproducibility. Data for peptides shown in **Figure 5** showed RSDs ranging from 4.6-7.6 % (see **Supplementary Information**).

For DKP quantification, analytical replicates were determined. Eight extracts were injected per duplicate and one per quadruplicate. The majority of DKP signals showed RSDs ranging from 0.4-3.6 %, with most intensive peaks having less variation (2% and less, see **Supplementary Information**).

## 2.2.7 Reaction Kinetics and Thermodynamics

In this paper, we describe the kinetics of DKP-formation using zero-order and solid-state reaction models. The relation between product concentration and time for zero-order reaction is shown in **Eq. 1**, where $P$ is the product concentration (the relative amount of DKPs), $A_0$ is the initial substrate concentration, $k$ is the rate constant, and $t$ is the reaction time.

$$P = A_0 + k \times t \tag{1}$$

The relation between product concentration and time for Prout-Tompkins solid-state reaction is shown in **Eq. 2**, where $\alpha$ is the fractional extent of conversion, $A_0$ is the initial substrate concentration, $k$ is the rate constant, and $t$ is the reaction time.

$$ln \frac{\alpha}{(1-\alpha)} = A_0 + k \times t \tag{2}$$

The $\alpha$ parameter is used to describe the proceeding reaction (e.g., weight loss), as it is not practical to assess the substrate concentrations in our system. In this case, it was treated as a fitting parameter. The rate constants obtained for each DKP using **Eq. 1** and **Eq. 2** (for each model) at two different temperatures were used to calculate activation energy ($E_a$) of the respective DKP using the standard Arrhenius equation, as shown in **Eq. 3**, where $E_a$ is activation energy, $R$ is the universal gas constant, and $k_1$ and $k_2$ are the reaction rate constants at temperatures $T_1$ and $T_2$, respectively.

$$E_a = \frac{R \cdot ln\left(\frac{k_2}{k_1}\right)}{\frac{1}{T_1} - \frac{1}{T_2}} \tag{3}$$

## 2.3 Results and Discussion

We present a comprehensive study on previously reported and unreported cocoa DKPs, their kinetics of formation, and their correlation to putative peptide precursors. The identification, structure elucidation, and kinetic studies were performed on the same set of Ivorian cocoa bean samples (VA09), roasted within a period of 70 min (sampled at 10 min intervals) at two different roasting temperatures (120 °C and 150 °C). The collected data was compared to three additional samples from other cocoa origins (Ivorian VB01, Indonesian IA03, and Ecuadorean EB02) to ensure the generality of the observations made.
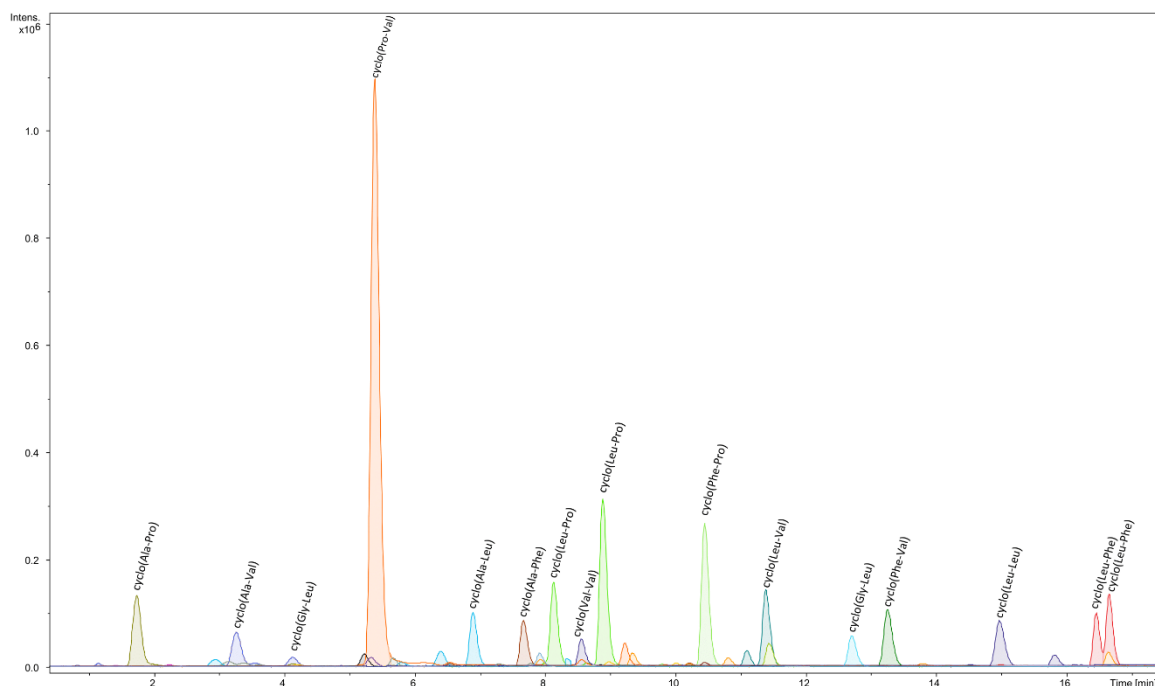


**Figure 1 Positive ion mode HPLC-MS extracted ion chromatograms of the most intensive 2,5-diketopiperazine peaks formed after roasting of Ivorian cocoa beans (VA09) at 150 °C for 70 minutes.**

### 2.3.1. Identification of Cocoa Diketopiperazines

The HPLC-MS-MS identification was based on MS data obtained from diketopiperazine extracts (dichloromethane fraction) of cocoa from the Ivory Coast roasted at 150 °C for 70 minutes (VA09). The HPLC-MS detection of all identified DKPs was confirmed on all other samples. The UV peak area of theobromine at 280 nm was used as an internal standard since it was found stable and invariant under all our experimental conditions with an RSD < 2 % (Wesołowski & Szynkaruk, 2001; Wesolowski & Szynkaruk, 2008; Kamel, 2015). All quantitative MS data are reported relative to the internal standard. A summary of the most abundant DKPs, as a collection of extracted ion chromatograms, is shown in **Figure 1**. While we were able to observe several isomeric peaks of some of the cyclic dipeptides, the DKPs in this paper lack absolute stereochemical assignment. According to the literature, we assign the major diastereoisomer as *cis*-isomers (Stark & Hofmann, 2005). Additionally, isoleucine- and leucine-containing DKPs could not be differentiated. Structures of all identified 2,5-diketopiperazines are shown in **Figure 2** and will be referenced by the numbers established there.
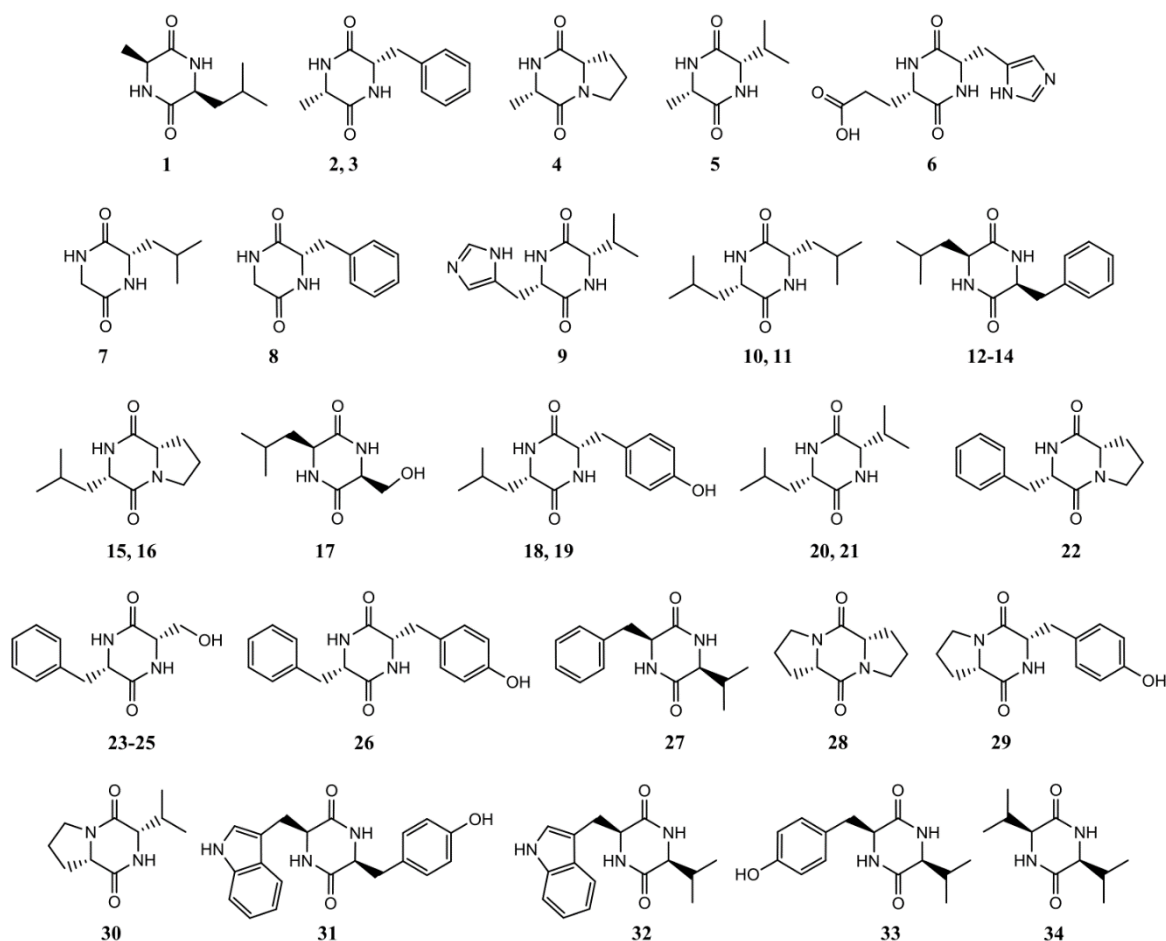


**Figure 2 Structures of all identified 2,5-diketopiperazines (all amino acids shown as L-stereoisomers, leucine and isoleucine were non differentiable).**

The MS$^n$ studies of different DKPs from various sources by ESI-MS and EI-MS were previously reported in the literature (Szafranek, Palacz, & Grzonka, 1976; Furtado et al., 2007; Guo, Cao, Zong, Liao, & Zhao, 2009), and their fragmentation patterns are

systematic and well understood. This fact facilitated the identification of DKPs based on the construction of neutral loss chromatograms, and all MS$^n$ extracted ion chromatograms using reported fragment ions and neutral losses. We show a scheme representing all possible product-ions generated during the fragmentation of diketopiperazines in **Figure 3**.

The most common fragmentation pathways reported result from the loss of CO ($l$ ion), loss of CO and NH ($k$ ion), loss of any amino acid residue (either $c$, $c'$, $c''$ or $d$, $d'$, $d''$ ions), and loss of one of the amino acid side chains ($a1$ or $a2$ ions). On the other hand, it was reported that the DKPs can be solely identified based on $m/z$ value of its pseudomolecular ion as long as it is accompanied by the presence of iminium ions ($e1$ and $e2$ ions) of their monomeric amino acids (Chen et al., 2004; Stark & Hofmann, 2005). Iminium ions are produced preferentially should the DKP contain aromatic amino acid moieties.

$$D = M_{AA1} + M_{AA2} - 2H_2O + H$$



$a1 = D - (M_{AA1} - NH_2 - COOH - CH)$
$a2 = D - (M_{AA2} - NH_2 - COOH - CH)$
$b1 = M_{AA1} - NH_2 - COOH - CH + H$
$b2 = M_{AA2} - NH_2 - COOH - CH + H$

$c = c' = c'' = M_{AA1} - H2O + H$
$d = d' = d'' = M_{AA2} - H2O + H$
$e1 = M_{AA1} - COOH - H + H$
$e2 = M_{AA2} - COOH - H + H$
$f1 = D - (M_{AA1} - COOH - H)$

$g1 = D - (M_{AA1} - NH_2 - COOH)$
$g2 = D - (M_{AA2} - NH_2 - COOH)$
$h1 = M_{AA1} - NH_2 - COOH + H$
$h2 = M_{AA2} - NH_2 - COOH + H$
$i1 = M_{AA1} - NH_2 - OH + H$
$i2 = M_{AA2} - NH_2 - OH + H$
$j1 = D - (M_{AA1} - NH_2 - OH)$
$j2 = D - (M_{AA2} - NH_2 - OH)$

$k = D - CO - NH$
$l = D - CO$
$m = D - NH3$
$n = M_{AA1} + M_{AA2} - 2NH_2 - 2COOH + N$
$o = D - b1 - b2 - 2H$
$p = D - b1 - b2 - 3H$
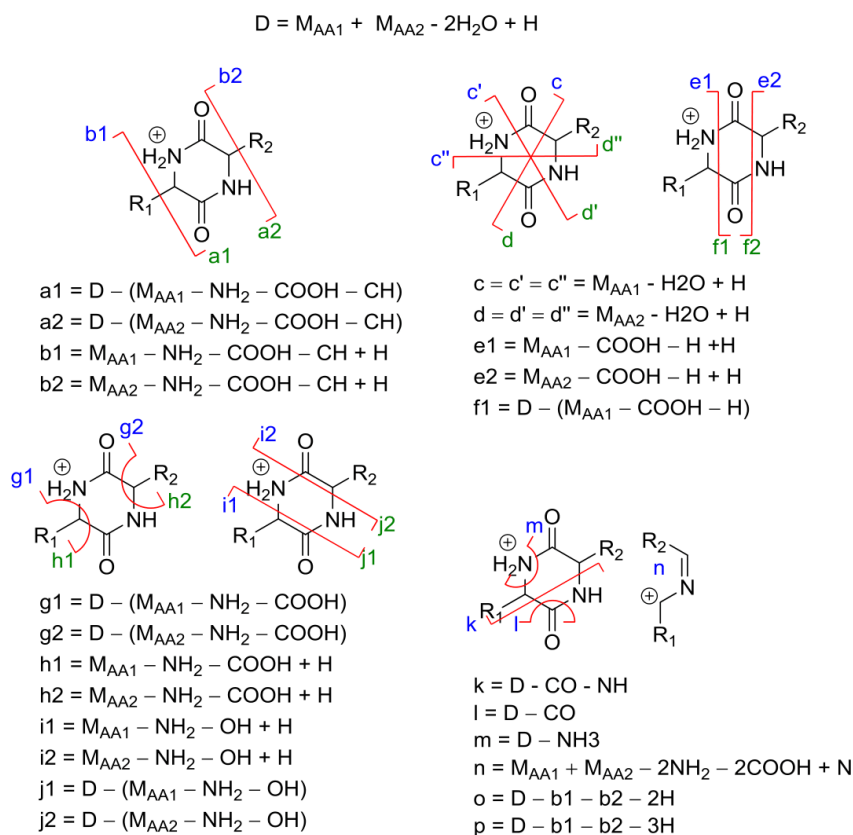
**Figure 3 Scheme of all fragmentations possible for 2,5-diketopiperazines.**

**Figure 4a)** shows the annotated fragmentation pattern of cyclo(Pro-Val) (**30**, $m/z$ 197.1278), the most abundant DKP in cocoa (Stark & Hofmann, 2005). Its most intense fragment ion at $m/z$ 154.0732 ($a1$ ion) is formed due to the loss of its valine side chain. The $n$ ion ($m/z$ 124.1122) is formed via CO loss, followed by the creation of a five-membered ring, which undergoes further rearrangements. The $c$ ion ($m/z$ 98.0601) is formed by the loss of the entire valine residue. **Figure 4b)** shows the annotated fragmentation pattern of cyclo(Phe-Tyr) (**26**, $m/z$ 311.1382). The two most important fragment peaks, $m/z$ 136.0757 ($e2$ ion) and 120.0808 ($e1$ ion), are the iminium ions of tyrosine and phenylalanine residues, respectively. The $p$ ($m/z$ 114.0425) and $o$ ($m/z$ 113.0348) ions form by the loss of both

**Table 2 LC-MS$^2$ identification of 2,5-diketopiperazines in extract of roasted Ivorian cocoa powder (VA09 sample roasted in 150°C for 70 minutes).**

| No. | Name | RT | m/z (exp) | m/z (theo) | Δ$_{ppm}$ | Ion Fragments (Intensity:Matched fragment) |
|---|---|---|---|---|---|---|
| 1 | cyclo(Ala-Leu) | 6.8 | 185.1281 | 185.1285 | 1.9 | 131.9745 (100); 114.0915 (71:d); 108.9575 (68); 136.952 (67); 112.1119 (45:n); 125.9646 (37); 113.9657 (32); 116.9736 (30); 113.0344 (30:o); 154.9637 (27); 146.9609 (19); 165.0801 (15); 123.9512 (15); 140.108 (14); 118.9668 (13); 128.9443 (11); 187.1191 (10); 139.1343 (10); 86.0942 (10:e2) |
| 2 | cyclo(Ala-Phe) | 7.6 | 219.1122 | 219.1128 | 2.8 | 120.0804 (100:e2); 128.0607 (23:a2); 146.0965 (17:n); 113.0337 (15:o); 103.0567 (11) |
| 3 | cyclo(Ala-Phe) | 10.2 | 219.1128 | 219.1128 | 0 | 173.1077 (100); 159.0925 (67); 145.079 (50); 121.0766 (49); 201.1014 (47); 117.0586 (34); 132.0762 (32); 158.082 (31); 130.0676 (20); 171.0912 (19); 185.0719 (16); 162.0798 (14); 175.0887 (12); 160.0736 (12); 148.9798 (12); 148.0736 (12:d); 139.0879 (12); 188.0974 (11); 120.0801 (11:e2); 157.0756 (10) |
| 4 | cyclo(Ala-Pro) | 1.7 | 169.0970 | 169.0972 | 0.9 | 169.097 (100); 98.0611 (99:d); 141.1014 (91:l); 154.0753 (62:a1); 124.0735 (62); 118.0527 (57); 96.0813 (43:n); 131.0588 (41); 137.0692 (39); 107.062 (32); 157.9887 (30); 94.0545 (23); 137.984 (22); 125.9601 (22); 152.9693 (16); 121.964 (16); 139.0879 (15); 109.0505 (15); 81.0701 (15) |
| 5 | cyclo(Ala-Val) | 3.2 | 171.1122 | 171.1128 | 3.5 | 125.9614 (100); 172.0749 (81); 144.0782 (42); 154.0589 (34); 113.0344 (32:o); 142.0691 (30); 128.0592 (24:a2); 148.9761 (23); 144.9357 (23); 123.0561 (19); 170.0614 (18); 131.9731 (17); 121.9643 (17); 127.0527 (16); 118.9414 (15); 135.9527 (14); 115.0571 (14); 123.9672 (13); 107.9538 (13) |
| 6 | cyclo(Glu-His) | 10.4 | 267.1100 | 267.1088 | 4.6 | 136.0588 (100); 181.0777 (82); 138.0902 (80); 121.0655 (64); 194.0848 (45:a1); 130.0674 (40); 145.0627 (39:j2); 124.0704 (39); 154.0652 (38); 152.0662 (38); 149.1132 (29); 148.0752 (29); 185.084 (26); 251.1168 (23); 210.1151 (23); 164.1014 (22); 110.0597 (22); 120.0803 (21); 176.0645 (20); 210.0852 (19); 136.1019 (19); 119.0621 (18); 232.1043 (17); 222.0877 (17); 179.1536 (17); 179.1071 (17); 107.0459 (15); 194.1164 (14); 134.0911 (14); 172.1017 (13) |
| 7 | cyclo(Gly-Leu) | 4.1 | 171.1128 | 171.1128 | 0 | 125.961 (100); 144.9363 (37); 148.9768 (27); 131.062 (17); 173.1061 (13); 136.9512 (13); 114.092 (12:d); 126.9235 (11) |
| 8 | cyclo(Gly-Phe) | 5.1 | 205.0968 | 205.0972 | 1.7 | 120.0806 (100:e2); 114.0438 (28:a2); 114.0438 (28:p); 153.9598 (18); 132.0801 (16:n); 125.9605 (16); 108.9579 (15); 103.0542 (14); 136.9533 (13); 130.065 (13); 123.946 (13); 131.9735 (11); 131.0489 (11); 118.9433 (10) |
| 9 | cyclo(His-Val) | 12.2 | 237.1330 | 237.1346 | 6.8 | 194.129 (100:k); 194.0801 (65:a2); 136.0512 (51); 146.9619 (43); 180.0765 (34); 151.0774 (33); 122.0825 (27); 167.1177 (22); 164.0913 (22); 135.0672 (22); 166.135 (20); 125.0703 (20); 203.9412 (19); 237.1381 (18); 135.0927 (17); 164.0588 (16); 149.085 (16); 173.9829 (15); 179.0551 (14); 179.1056 (13); 149.1043 (13); 219.9365 (12); 125.9642 (12); 123.9424 (12); 145.0725 (10); 131.9768 (10); 110.0976 (10) |
| 10 | cyclo(Leu-Leu) | 14.4 | 227.1753 | 227.1754 | 0.5 | 153.9591 (100); 131.9745 (65); 136.9527 (51); 125.964 (49); 123.9456 (45); 146.9617 (44); 154.1579 (32:n); 181.9512 (30); 108.9577 (29); 116.9703 (28); 181.0883 (25); 168.0811 (22); 154.0643 (19); 117.9362 (17); 159.97 (14); 210.128 (13); 203.9342 (13); 141.9571 (13); 134.962 (13); 122.0496 (13); 115.0564 (13); 150.9553 (12); 227.162 (11); 172.095 (11); 148.9636 (11); 113.0364 (11:o) |
| 11 | cyclo(Leu-Leu) | 14.9 | 227.1748 | 227.1754 | 2.7 | 154.1593 (100:n); 114.0905 (52:c); 114.0905 (52:d); 113.0366 (44:o); 153.959 (34); 86.0968 (30:e1); 86.0968 (30:e2); 146.9631 (20); 125.9628 (18); 112.1111 (18); 170.1032 (15:a1); 170.1032 (15:a2); 126.0916 (15); 107.9533 (15); 168.081 (14); 131.9743 (12); 135.9512 (11) |
| 12 | cyclo(Leu-Phe) | 15.0 | 261.1590 | 261.1598 | 2.9 | 120.08 (100:e2); 113.0353 (26:o); 105.0692 (13:h2); 188.1466 (11:n); 188.0985 (11); 150.1177 (11) |

| 13 | cyclo(Leu-Phe) | 16.3 | 261.1585 | 261.1598 | 4.8 | 120.0805 (100:e2); 113.0359 (17:o); 132.0811 (11); 188.1436 (10:n) |
|---|---|---|---|---|---|---|
| 14 | cyclo(Leu-Phe) | 16.6 | 261.1590 | 261.1598 | 2.9 | 120.0806 (100:e2); 113.0356 (20:o); 188.1426 (17:n) |
| 15 | cyclo(Leu-Pro) | 8.0 | 211.1437 | 211.1441 | 1.9 | 154.073 (100:a1); 138.1266 (45:n); 126.0815 (26); 136.1024 (23); 152.0659 (19); 121.0785 (15); 112.035 (15); 137.0695 (12); 86.0973 (12:e1); 210.1362 (10); 114.0916 (10:c) |
| 16 | cyclo(Leu-Pro) | 8.8 | 211.1439 | 211.1441 | 1.0 | 138.1276 (100:n); 154.0739 (88:a1); 114.0911 (45:c); 211.1424 (26); 98.0611 (24:d); 153.0663 (16); 183.1497 (11:l); 127.0864 (10) |
| 17 | cyclo(Leu-Ser) | 2.2 | 201.1223 | 201.1234 | 5.3 | 125.9613 (100); 146.9607 (77); 153.9556 (53); 123.9447 (49); 108.958 (48); 131.9737 (27); 149.9832 (16); 140.952 (15); 135.9539 (15); 132.9569 (15); 86.0958 (12:e1); 113.963 (11) |
| 18 | cyclo(Leu-Tyr) | 7.9 | 277.1539 | 277.1547 | 2.8 | 136.0753 (100:e2); 107.0487 (62); 119.048 (24); 121.0644 (11:h2) |
| 19 | cyclo(Leu-Tyr) | 9.3 | 277.1543 | 277.1547 | 1.3 | 136.0755 (100:e2); 107.0497 (63); 119.0503 (30); 121.0616 (18:h2); 204.1375 (17:n); 114.0436 (14:p); 120.0811 (13) |
| 20 | cyclo(Leu-Val) | 11.0 | 213.1593 | 213.1598 | 2.1 | 140.1426 (100:n); 113.0376 (45:o); 123.0681 (32); 86.0968 (26:e1); 167.0746 (23); 130.0676 (23); 143.0708 (20); 149.0767 (16); 144.9349 (13); 171.0946 (12); 157.0818 (12); 185.107 (11); 185.0744 (11); 172.0746 (11); 166.9443 (11); 114.0902 (11:c); 196.0877 (10) |
| 21 | cyclo(Leu-Val) | 11.3 | 213.1593 | 213.1598 | 2.1 | 140.1432 (100:n); 113.0364 (45:o); 114.0896 (29:c); 168.1364 (11); 130.0657 (10) |
| 22 | cyclo(Phe-Pro) | 10.4 | 245.1275 | 245.1285 | 3.9 | 154.0732 (100:a1); 120.0804 (89:e1); 153.065 (15); 172.1112 (12:n) |
| 23 | cyclo(Phe-Ser) | 4.2 | 235.1066 | 235.1077 | 4.8 | 120.0801 (100:e1); 113.037 (33:o); 203.9375 (20); 146.064 (13); 118.066 (13); 103.054 (11) |
| 24 | cyclo(Phe-Ser) | 8.9 | 235.1070 | 235.1077 | 3.1 | 135.0935 (100); 147.0917 (89); 163.0863 (23:j2); 120.0767 (21); 133.0769 (18); 122.0839 (15); 177.0683 (12); 145.0787 (12) |
| 25 | cyclo(Phe-Ser) | 10.0 | 235.1064 | 235.1077 | 5.6 | 160.0651 (100); 132.0686 (34); 173.0705 (32); 184.063 (28); 170.0831 (20); 159.0553 (18); 156.0658 (18); 131.0607 (17); 120.0794 (16:e1); 199.086 (13); 147.0627 (13); 145.0769 (13); 118.0631 (12); 197.0703 (11); 155.0589 (10) |
| 26 | cyclo(Phe-Tyr) | 11.3 | 311.1382 | 311.1390 | 2.6 | 136.0755 (100:e2); 120.0808 (98:e1); 107.0492 (77); 114.043 (32:p); 119.0501 (28); 146.0614 (20); 113.0348 (11:o); 238.1217 (10:n); 130.0656 (10) |
| 27 | cyclo(Phe-Val) | 13.1 | 247.1433 | 247.1441 | 3.3 | 120.0807 (100:e1); 174.1272 (32:n); 113.0342 (23:o); 129.0672 (11) |
| 28 | cyclo(Pro-Pro) | 2.9 | 195.1121 | 195.1128 | 3.6 | 138.0662 (100); 110.0709 (33); 123.0427 (22); 135.0429 (11); 98.0598 (10:c); 98.0598 (10:d) |
| 29 | cyclo(Pro-Tyr) | 5.3 | 261.1231 | 261.1234 | 1.0 | 107.049 (100); 136.0754 (83:e2); 154.0746 (63:a2); 119.0494 (26); 113.0712 (13:j2) |
| 30 | cyclo(Pro-Val) | 5.3 | 197.1279 | 197.1285 | 2.8 | 154.0735 (100:a2); 124.1117 (34:n); 138.0658 (21); 153.0645 (14); 98.0597 (12:c) |
| 31 | cyclo(Trp-Tyr) | 11.2 | 350.1508 | 350.1499 | 2.5 | 130.0652 (100:b3); 190.1232 (17) |
| 32 | cyclo(Trp-Val) | 12.6 | 286.1545 | 286.1550 | 1.8 | 130.0651 (100:b3) |
| 33 | cyclo(Tyr-Val) | 7.9 | 263.1384 | 263.1390 | 2.4 | 107.049 (100:b3); 136.0758 (89:e1); 190.1222 (26:n); 119.0487 (26); 133.0608 (21); 114.043 (16:p); 177.1006 (15); 135.0665 (15); 121.0636 (12:h1); 118.0692 (11) |
| 34 | cyclo(Val-Val) | 8.5 | 199.1436 | 199.1441 | 2.5 | 126.1267 (100:n); 113.0346 (39:o); 140.0571 (33); 111.0569 (27); 125.9623 (25); 109.0887 (15); 146.9616 (13); 149.9835 (11); 123.0603 (11); 100.0721 (11) |

amino acid side chains as well as protons. Again, the *n* ion (*m/z* 238.1226) is formed in a rearrangement involving a five-membered ring. All high-resolution tandem MS data for other diketopiperazines are summarized in **Table 2**.
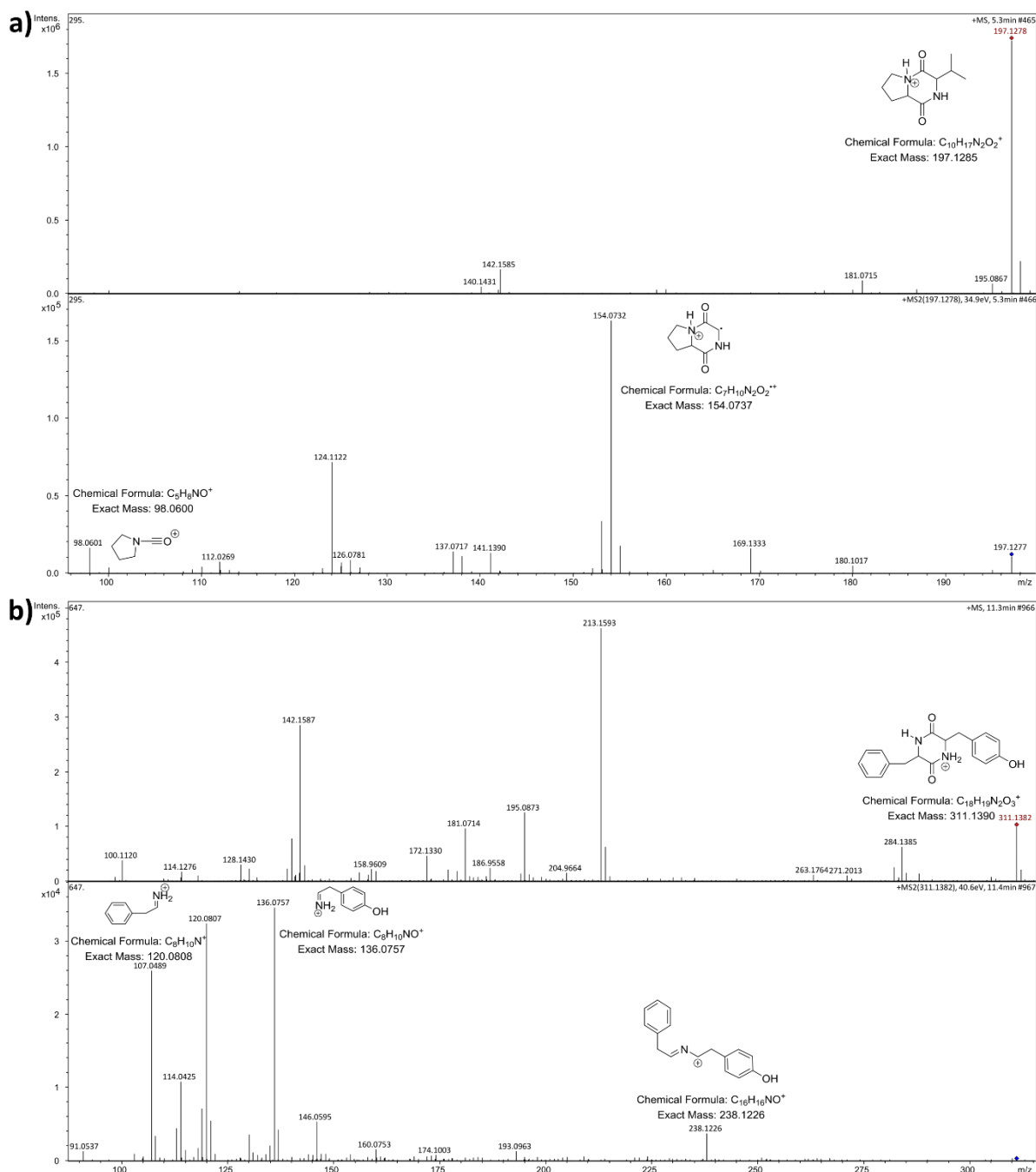


**Figure 4 MS² fragmentation of a) cyclo(Pro-Val), and b) cyclo(Phe-Tyr), with major fragments annotated.**

The identification of DKPs in this paper was carried out by comparison of experimental *m/z* values with those calculated for all 180 theoretically possible DKPs, as well as by matching their corresponding fragment ions to those shown in **Figure 3**. Out of 34 DKPs, 20 exhibited characteristic iminium ions of either amino acid in their fragmentation spectra, whereas the rest of the DKPs shown were identified based on other specific ions. We have confidently identified DKPs which correspond to (structure numbers correspond to those in **Figure 2** and **Tables 2**

and **3**): cyclo(Ala-Leu) (**1**), cyclo(Ala-Phe) (**2**, **3**), cyclo(Ala-Pro) (**4**), cyclo(Ala-Val) (**5**), cyclo(Gly-Leu) (**7**), cyclo(Gly-Phe) (**8**), cyclo(Leu-Phe) (**12-14**), cyclo(Leu-Pro) (**15**, **16**), cyclo(Leu-Val) (**20**, **21**), cyclo(Phe-Pro) (**22**), cyclo(Phe-Ser) (**23-25**), cyclo(Phe-Val) (**27**), cyclo(Pro-Pro) (**28**), cyclo(Pro-Tyr) (**29**), cyclo(Pro-Val) (**30**), cyclo(Tyr-Val) (**33**), cyclo(Val-Val) (**34**), all of which were previously reported in cocoa (Stark & Hofmann, 2005). In addition, we have discovered the presence of cyclo(Glu-His) (**6**), cyclo(His-Val) (**9**), cyclo(Leu-Leu) (**10**, **11**), cyclo(Leu-Ser) (**17**), cyclo(Leu-Tyr) (**18**, **19**), cyclo(Phe-Tyr) (**26**), cyclo(Trp-Tyr) (**31**), cyclo(Trp-Val) (**32**), which to our best knowledge were not yet reported in cocoa. Seven out of herein reported cocoa DKPs were studied (along with other cocoa constituents) by a taste-recombinant approach and confirmed to be essential taste components specific to roasted cocoa (Stark et al., 2006). This were (stereospecific): cyclo(L-Pro-L-Ala), cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Ile), cyclo(L-Pro-L-Leu), cyclo(L-Ala-L-Ile), cyclo(L-Ala-L-Leu), cyclo(L-Val-L-Leu). Additional DKPs detected here for the first time in cocoa were previously reported in roasted malt, aged sake, beer, chicken essence, roasted coffee, comte cheese, hydrolyzed vegetable protein (Borthwick & Da Costa, 2017). Most importantly, the novel cocoa DKPs, cyclo(L-Leu-L-Leu) and cyclo(L-Ile-L-Ile), were reported to appear in chicken essence (Chen et al., 2004) and to have a bitter taste (Li, Tang, Duan, Gao, & Xue, 2013; Ishibashi et al., 1988). Cyclo(L-Leu-L-Leu) also was detected in *Bacillus subtilis* (Lu et al., 2009) as an intermediate for the ion-chelator, pulcherrimin (Cryle, Bell, & Schlichting, 2010). The following DKPs were to our best knowledge not yet reported in the literature: cyclo(Glu-His) (**6**), cyclo(His-Val) (**9**), cyclo(Leu-Ser) (**17**), cyclo(Leu-Tyr) (**18**, **19**), cyclo(Phe-Tyr) (**26**), cyclo(Trp-Tyr) (**31**), and cyclo(Trp-Val) (**32**).

Interestingly, we have also discovered the presence of GABA-containing cyclic dipeptides, cyclo(His-GABA), and cyclo(Pro-GABA), which cannot be classified as DKPs. Owing to the neurotransmitter properties of GABA (Fields, Heinricher, & Mason, 1991; Chebib & Johnston, 1999), these cyclic dipeptides could be potential bioactive lead compounds with unexpected properties, which warrant further investigation.

### 2.3.2 Putative Precursor Degradation

DKPs form from peptide by cyclization of the N-terminal primary amine onto the second from last amide moiety. We studied the degradation of peptides that matched the sequence of DKPs identified in our samples as those could be potentially the precursors for given diketopiperazines. We tracked almost 100 peptides along with the roasting (see **Supplementary Information**) with the N-terminal sequence matching the N-terminal sequence of DKPs we report. In a great majority of cases, we observed a loss of intensity of peptide peaks over the roasting time. This trend is shown in **Figure 5**, where an example of some of the most intensive peptide peaks are depicted. It confirms the anticipated behavior of peptides during cocoa roasting. Despite the general degradation trend, we cannot assume that all peptides contribute to the DKP formation in the same way, as cocoa is a complex food matrix with a plethora of possible reactants. Hence peptides can react in other competing pathways as well. This was confirmed by our data and suggested to use DKP quantities rather than peptide quantities in a kinetic model. Therefore, we decided to use DKP (product) data to establish a kinetic model.

It has to be underlined that in the majority of the cases, one N-terminal peptide sequence was represented by one most-intensive peptide, and only a small fraction of intensities belonged to other peptide sequences. This could suggest that the formation of the DKPs depends mainly on the most intensive peptides rather than on the collective contribution of many. However, the degradation trend of particular peptides could not be fit into a kinetic model (see trends in

**Figure 5**). Nevertheless, the control over DKP formation could be accomplished, in principle, by the optimization of peptidase activity in cocoa fermentation.
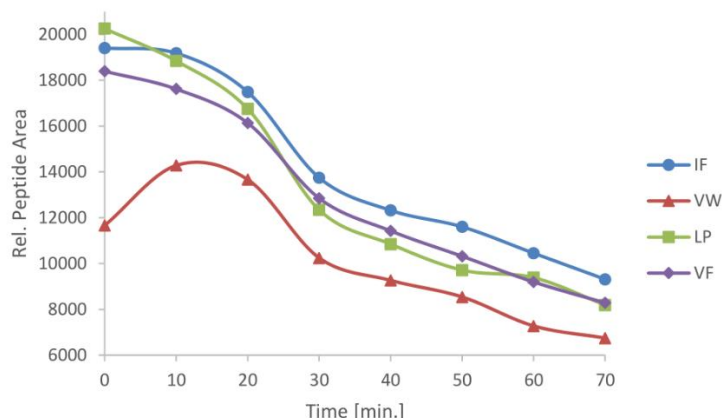


**Figure 5 Degradation of selected putative dipeptide precursors across 150 °C roasting series of Ivorian cocoa beans (VA09).**

### 2.3.3 Kinetics of Cocoa 2,5-Diketopiperazines Formation

In a roasting series, bulk cocoa bean powder samples were roasted at two commonly employed roasting temperatures (120 °C and 150 °C), where samples were collected at 10-minute time intervals until the end of roasting after 70 minutes.

The experimental data revealed a rather complex behavior, and we employed two kinetic models, zero-order (linear) and solid-state (sigmoidal), to characterize and compare the kinetics of 2,5-diketopiperazines formation. The linear model assumes a typical solid-state reaction and allows direct extraction of activation parameters for the purpose of comparison. The second model better accounts for the experimentally observed shape. The reaction, in this case, is considered quasi-autocatalytic since the formation of a DKP from a peptide yields a further structurally different DKP available as a subsequent reactant. This behavior is seen in the early stages of some reactions in solid-state and can be described by the Prout-Tompkins equation (**Eq. 2**). Furthermore, we calculate the kinetic parameters using changes of the product (relative DKP quantities) because using substrates in such a complex matrix was not feasible. All the calculated parameters are shown in **Table 3**. Relative DKP concentrations quantified from MS data at regular intervals enabled the determination of reaction rate constants for the linear (zero-order reaction) model. Representative curves for **cyclo**(Pro-Val) (**30**) and cyclo(Phe-Tyr) (**26**) are shown in **Figure 6A**-**D**). The curves were fitted only to the first five data points in case of 120°C roasting, and four data points for 150°C roasting, which correspond to the linear range of the concentration-time curves. Using Arrhenius theory (**Eq. 3**), the reaction rate constants at 120 °C and 150 °C were employed to estimate reaction activation energies for each DKP, which range from about 45 to 190 kJ/mol. The histogram of activation energies of DKP formation is shown in **Figure 7a**). The histogram is slightly skewed towards lower activation energies; however, the DKPs in this study have on average energy activation of 85 kJ/mol, and the distribution is close to the normal distribution. Furthermore, it suggests that all the DKPs might form by a similar reaction mechanism. A number of studies were reported on semi-empirical studies of energy activation of peptide bond formation (Oie, Loew, Burt, Binkley, & MacElroy, 1982; Oie, Loew, Burt, & Macelroy, 1983), where ammonia-glycine and ammonia-formic acid model reactions revealed that it should equal to approximately 160 kJ/mol.

**Table 3 Calculated kinetic parameters of 2,5-diketopiperazines formation during cocoa roasting (VA09 sample roasted in 120 °C and 150 °C, both for 70 minutes).**

| No. | Name | Zero Order Kinetic Model | | | Reduced Prout-Tompkins Kinetic Model | | |
|---|---|---|---|---|---|---|---|
| | | $k_{120\,°C}$ [min$^{-1}$] | $k_{150\,°C}$ [min$^{-1}$] | $E_a$ [kJ × mol$^{-1}$ × K$^{-1}$] | $k_{120\,°C}$ [min$^{-1}$] | $k_{150\,°C}$ [min$^{-1}$] | $E_a$ [kJ × mol$^{-1}$ × K$^{-1}$] |
| 1 | cyclo(Ala-Leu) | 0.98390 | 6.24850 | 85.16909 | 0.03179 | 0.09500 | 50.43610 |
| 2 | cyclo(Ala-Phe) | 0.88963 | 5.05801 | 80.07094 | 0.02071 | 0.06472 | 52.49499 |
| 3 | cyclo(Ala-Phe) | 0.02119 | 1.29035 | 189.31860 | 0.12605 | 0.53417 | 66.53010 |
| 4 | cyclo(Ala-Pro) | 4.36769 | 16.70096 | 61.79446 | 0.03115 | 0.10668 | 56.72287 |
| 5 | cyclo(Ala-Val) | 1.08700 | 5.81653 | 77.27717 | 0.02740 | 0.07227 | 44.68912 |
| 6 | cyclo(Glu-His) | 0.21214 | 0.68126 | 53.75348 | 0.02220 | 0.07496 | 56.06466 |
| 7 | cyclo(Gly-Leu) | 0.20606 | 1.05363 | 75.18188 | 0.05135 | 0.14617 | 48.19992 |
| 8 | cyclo(Gly-Phe) | 0.25495 | 1.34302 | 76.55488 | 0.02924 | 0.07732 | 44.80252 |
| 9 | cyclo(His-Val) | 0.00611 | 0.07839 | 117.54067 | 0.01235 | 0.06992 | 79.87689 |
| 10 | cyclo(Leu-Leu) | 0.01314 | 0.21620 | 129.03490 | 0.03124 | 0.10395 | 55.38756 |
| 11 | cyclo(Leu-Leu) | 1.15981 | 6.58134 | 79.98162 | 0.02421 | 0.07273 | 50.67154 |
| 12 | cyclo(Leu-Phe) | - | - | - | - | - | - |
| 13 | cyclo(Leu-Phe) | 1.13835 | 4.92093 | 67.44714 | 0.01542 | 0.04095 | 45.01394 |
| 14 | cyclo(Leu-Phe) | 1.31742 | 7.39818 | 79.50159 | 0.02150 | 0.06468 | 50.75091 |
| 15 | cyclo(Leu-Pro) | 3.44189 | 16.51731 | 72.26027 | 0.03600 | 0.08479 | 39.47232 |
| 16 | cyclo(Leu-Pro) | 7.26174 | 32.23643 | 68.67068 | 0.03152 | 0.07257 | 38.42336 |
| 17 | cyclo(Leu-Ser) | 0.03655 | 0.17673 | 72.60619 | 0.02904 | 0.08883 | 51.51722 |
| 18 | cyclo(Leu-Tyr) | 0.11789 | 0.41407 | 57.88109 | 0.01190 | 0.02504 | 34.27772 |
| 19 | cyclo(Leu-Tyr) | 0.19136 | 0.98588 | 75.53091 | 0.02046 | 0.06915 | 56.10921 |
| 20 | cyclo(Leu-Val) | 0.18292 | 1.31640 | 90.93000 | 0.03551 | 0.11828 | 55.44040 |
| 21 | cyclo(Leu-Val) | 1.50380 | 9.23597 | 83.62756 | 0.02861 | 0.08633 | 50.87650 |
| 22 | cyclo(Phe-Pro) | 5.72020 | 24.46748 | 66.95965 | 0.02446 | 0.05803 | 39.79447 |
| 23 | cyclo(Phe-Ser) | 0.05950 | 0.18059 | 51.15006 | 0.01781 | 0.04202 | 39.54202 |
| 24 | cyclo(Phe-Ser) | 0.07271 | 1.47297 | 138.61089 | 0.11485 | 0.39241 | 56.60863 |
| 25 | cyclo(Phe-Ser) | 0.01378 | 0.43003 | 158.51122 | 0.08356 | 0.55649 | 87.35983 |
| 26 | cyclo(Phe-Tyr) | 0.47965 | 1.27444 | 45.02284 | 0.01152 | 0.02297 | 31.81363 |

| 27 | cyclo(Phe-Val) | 1.18302 | 7.08895 | 82.49205 | 0.02329 | 0.07384 | 53.16801 |
| 28 | cyclo(Pro-Pro) | 0.31688 | 1.60113 | 74.63503 | 0.56121 | 2.18580 | 62.64304 |
| 29 | cyclo(Pro-Tyr) | 0.34017 | 1.60954 | 71.60888 | 0.02193 | 0.06158 | 47.56048 |
| 30 | cyclo(Pro-Val) | 30.14430 | 128.39839 | 66.76620 | 0.04360 | 0.11755 | 45.69523 |
| 31 | cyclo(Trp-Tyr) | 0.03975 | 0.02230 | -26.64043 | 0.01292 | 0.00846 | -19.52315 |
| 32 | cyclo(Trp-Val) | 0.53591 | 3.62119 | 88.02690 | 0.02553 | 0.08365 | 54.67402 |
| 33 | cyclo(Tyr-Val) | 0.19009 | 0.97400 | 75.28014 | 0.02268 | 0.07946 | 57.77211 |
| 34 | cyclo(Val-Val) | 0.26379 | 2.13656 | 96.37555 | 0.04062 | 0.11516 | 48.00711 |

The intramolecular nature of our reaction systems justifies lower activation energies in accordance with kinetic theory. Additionally, proline-based DKPs, which should be easier formed, had decreased activation energies when compared to the average. For DKP formation, a peptide needs to change conformation from a *trans*-amide geometry to a *cis*-amide geometry, which is favored for proline as a secondary amide. Furthermore, the highest intensity of cyclo(Pro-Val) (**30**) could be furthermore explained by the steric buttressing effect of valine side-chain, which makes the cyclization reaction easier.

However, the DKPs seem to stagnate or decrease in concentration later during the roasting. This cannot be explained with a linear model, and we believe it is associated with the DKPs following more complex formation and degradation reaction kinetics with unknown mechanisms. We fitted the data with a solid-state model, which is presented in **Figure 6a-d)** (only acceleratory period of the reaction) and **6a'-d')** (all data points). This model yielded a satisfactory fit to the sigmoidal shape of the concentration-time curve. During the last points of the kinetic curve, a decrease in concentration is observed, which we assume is caused either by epimerization, which is common while heating diketopiperazines in non-neutral pH (Eguchi & Kakuta, 1974; Steinberg & Bada, 1981), or by further degradation of the DKPs. Evidence for epimerization is available in our data since multiple isomers are observed, and the minor isomer increases at later time points (see **Supplementary Information**). It is apparent for cyclo(Phe-Ser), as one isomer after 30 minutes of heating at 150 °C increases in concentration (**23**), one decreases (**24**), and one stays at the same level (**25**). The histogram of activation energy for reduced Prout-Tompkins solid-state models is shown in **Figure 7b)**. While the full model (all data points) describes the sigmoidal trend well, the reduced model (acceleratory stage) yields more probable kinetic parameters (**Table 3**). The activation energy spans from 31 to 87 kJ/mol, with an average of 51 kJ/mol. The proline derivatives fit well within this model with activation energy below the average.

We believe that these kinetic data are useful in tailoring the DKP content in industrial cocoa roasting and, possibly, controlling cocoa bitterness. Previous findings show the dose-over-threshold (DoT) factors for seven key cocoa DKPs (**1**, **4**, **15**, **16**, **20,** or **21**, **30**) for bitterness as being close to unity (Stark & Hofmann, 2005). This means that slight increases or decreases in the concentration of these DKPs could influence cocoa bitterness and, in turn, product quality. On the other hand, mentioned cyclic dipeptides were shown to be essential to characteristic roasted cocoa taste, and lowering their concentrations could lead to loss of this taste attribute.

It is also worth mentioning that some of the DKPs appear to be already present in small amounts in dry, non-thermally processed samples (see **Figure 6**). This may be either caused by microbial activity or by prolonged elevated temperature during fermentation and subsequent loss of water during the drying process. Microorganisms have biosynthetic pathways, during which DKPs can be produced or incorporated into much more complex structures (Belin et al., 2012; Gu et al., 2013). Alternatively, they could be formed during fermentation by thermal pathways since microbial fermentation is accompanied with temperature increases in the fermentation heap caused by microbial metabolism up to 50 °C (Camu et al., 2007; Papalexandratou, Vrancken, De Bruyne, Vandamme, & De Vuyst, 2011).
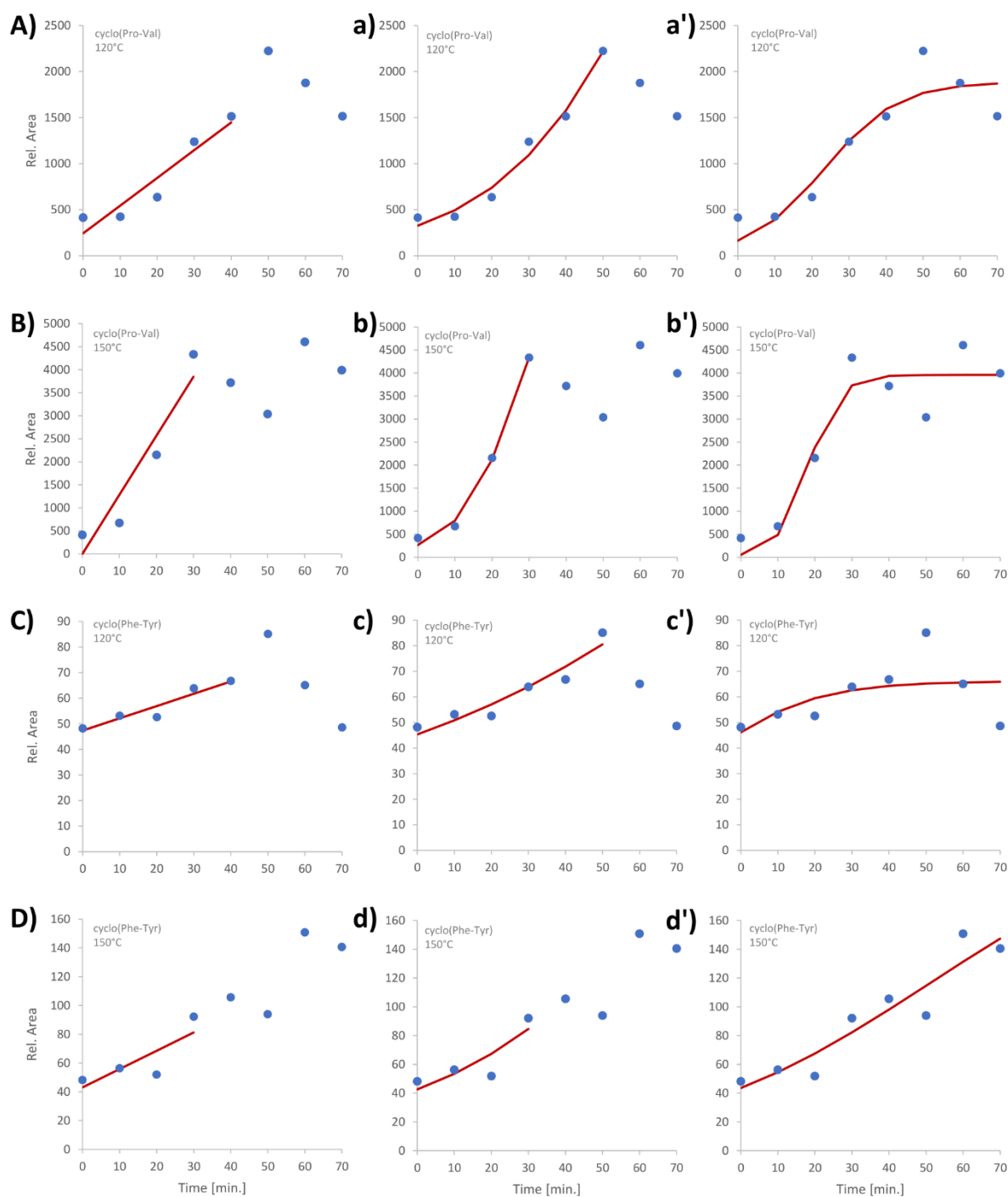
**Figure 6 Cyclo(Pro-Val) (A, B, a, b, a', b') and cyclo(Phe-Tyr) (C, D, c, d, c', d') kinetic data for A-D) zero-order, a-d) reduced Prout-Tompkins, and a'-d') full Prout-Tompkins model.** The temperatures are 120°C for A, a, a', C, c, c', and 150°C for B, b, b', D, d, d' respectively. Kinetic data derived from VA09 (120 and 150 °C) roastings.
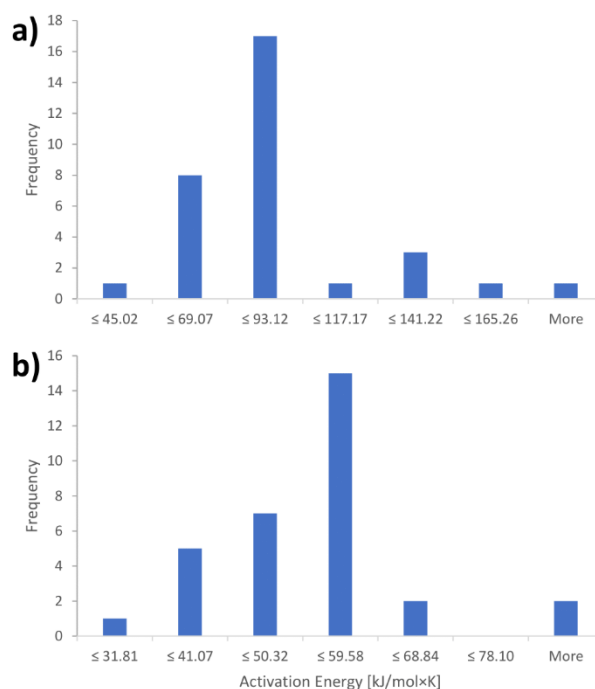
**Figure 7 Histogram of apparent activation energies for a) zero-order, and b) reduced Prout-Tompkins model.**

### 2.3.4 Correlation of DKPs Formation and Peptide Degradation

DKPs form relatively easily from heated non-protected dipeptides in non-neutral conditions (Anteunis, 2010). Synthesis can be performed from variously substituted amino acids (Borthwick, 2012), but furnishes poor yields when using unprotected amino acids. In cocoa, DKPs presumably originate from small peptides by intramolecular cyclization at their N-termini. An attempt is made to correlate DKPs (cocoa roasted at 150°C for 70 minutes) with peptides present in dry cocoa beans and which have the same DKP sequence on their N-termini. Peptide analysis was conducted according to a previous study on the peptidome of fermented cocoa (D'Souza et al., 2018). Here, 800 different short peptides produced in cocoa fermentation by endogenous exo- and endopeptidases were identified. The relative LC-MS peak areas of all peptide precursors of DKPs containing the two amino acids of the respective DKP at their N-termini were summed up in order to correlate them with similarly treated DKP data (all isomers summed). The scatter plots with $R^2$, Pearson, and Spearman coefficients are presented in **Figure 8**. The correlation for all DKPs and peptides is not satisfactory (see **Supplementary Information**) but improves greatly when proline DKPs are treated separately. Proline-containing DKPs have a much steeper increase in concentration, while the relative peptide concentration range for other DKPs is the same. All the origins maintain the same correlation trend.

This correlation exercise allows the definition of tentative DKP precursor peptides. As mentioned earlier, the majority of DKPs can be assigned to a single peptide precursor. For example, cyclo(Phe-Val) (**27**) is derived mainly from dipeptide with a sequence of VF or FV. Only in two cases, multiple peptide precursors contribute in the same extent to the formation of single DKP – cyclo(Ala-Phe) (**2**, **3**) originates from FA or AF, FAF, FAR, FAW, and cyclo(Gly-Leu) (**7**) from GI, GIND, GINDY, GINDYR. This observation is rather surprising since theoretically, the so far identified 800 peptides in cocoa formed by

fermentation should give rise to, on average, four to five DKP precursor peptides. Additionally, it is worth mentioning that not all peptides form detectable amounts of DKPs.
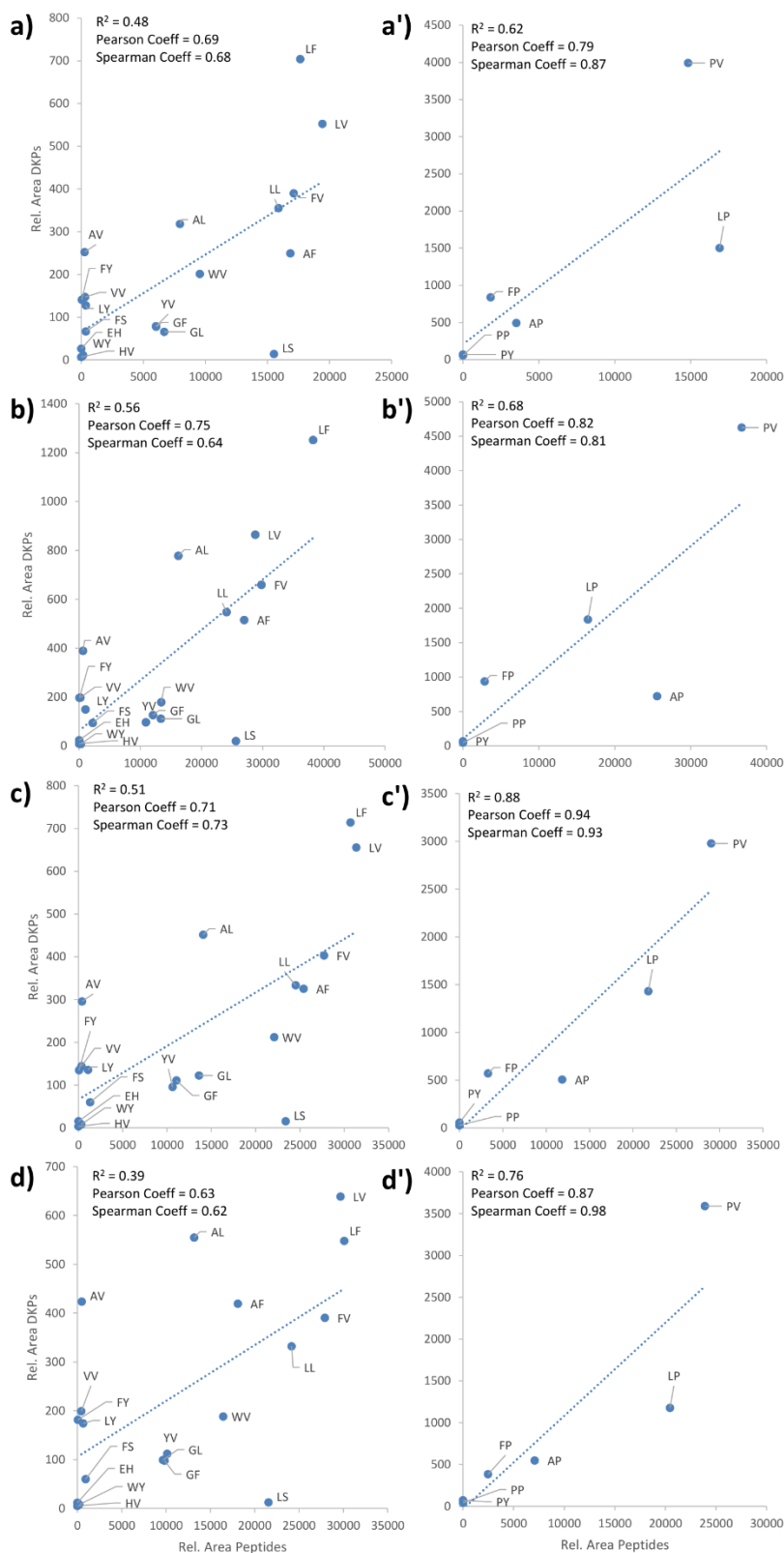


**Figure 8 Correlation of DKP content with putative peptide precursors for samples originated from: a) Ivory Coast (VA09 150 °C roasting), b) Ecuador (EB02 150 °C roasting), c) Indonesia (IA03 150 °C roasting), and d) Ivory Coast (VB01 150 °C roasting).**

Graphs annotated without prime symbol present data for every DKP besides proline-containing DKPs, and with a letter and a prime symbol only proline-containing DKPs.

## 2.4 Conclusions

In conclusion, we have identified a series of new, unreported DKPs formed in cocoa roasting. DKP formation was followed over time under different roasting conditions. The DKPs formation was fitted to zero-order Arrhenius kinetics yielding activation energies, while a better fit was shown to be obtained by a Prout-Tompkins solid-state kinetic model. Concomitantly, peptide degradation in cocoa roasting was monitored. The peptide composition of the unroasted bean correlates well with roasted bean's DKPs composition. This means the peptide compositions could be used as a predictive tool to assess the bitterness of the product after the roasting. Tentative DKP precursor peptides were identified with most DKPs originating from a single peptide precursor.

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## Conflict of interest statement

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# Chapter 3

# Novel Amadori and Heyns Compounds Derived from Short Peptides Found in Dried Cocoa Beans

Paweł J. Andruszkiewicz, Roy N. D'Souza, Marcello Corno, and Nikolai Kuhnert

## Abstract

Chemical transformations of Amadori compounds are responsible for the formation of aroma volatiles at the end of the Maillard reaction cascade, which in turn contributes to unique organoleptic characteristics of chocolate. A large amount of short peptides reported in fermented cocoa suggests the existence of a much larger variety of these flavor precursors than previously suspected. An HPLC-MS-MS study was performed on dried Malaysian cocoa beans to identify novel Amadori and Heyns compounds. In total, 34 species were found, including 26 previously unknown derived from di- and tripeptides. We illustrate how the structures were elucidated via tandem MS experiments, as well as present a comparative study on their relative quantities in samples coming from 11 countries of origin. There were significant differences between them, and discrimination was possible by principal component analysis based on Amadori content alone. However, the PCA separation could be a result of various post-harvest practices exerted among said countries.

## 3.1 Introduction

Chocolate production, from the bean to the factory, involves many processes, which involve slight to drastic elevations of the temperature. During cocoa beans fermentation, the temperature reaches approximately 50°C (Lagunes Gálvez, Loiseau, Paredes, Barel, & Guiraud, 2007; Camu et al., 2008). Although it is difficult to establish it for traditional drying methods such as sun-drying, artificial drying is performed at roughly 60°C (Rodriguez-Campos et al., 2012). Roasting takes place under the roughest conditions, with the final temperature reaching between 110 and 140°C (Beckett, 2009). Increasing the intensity of thermal treatment lets the cocoa develop its extraordinary character. Paired with its unique composition, it enables many intricate chemical reactions to occur. Most prominently, the Maillard reaction, which starts with transformations arising from the reaction of amino acids or peptides and sugars. They can start even at room temperature; however, it becomes dominant in temperatures above 100°C (Ruan, Wang, & Cheng, 2018). It has an enormous effect on the organoleptic properties of foods as it produces many aroma volatiles as well as colored polymers (Yaylayan, 2003). Hence, it is important to the food industry as it influences the product quality of heated foods (Ames, 1990, 1998). The first major stable compounds of the Maillard reaction are Amadori and Heyns compounds, condensation, and rearrangement products of amino acids with either glucose or fructose,

respectively. They are of most importance as their thermal degradation directly leads to the formation of aroma compounds and melanoidins (brown pigments). They are very well studied in model systems (Hofmann & Schieberle, 2000; Davidek, Clety, Aubin, & Blank, 2002) as well as they were detected in some foods (Meitinger, Hartmann, & Schieberle, 2014; Yuan, Sun, Chen, & Wang, 2016, 2017). Additionally, they have gained much attention after the discovery of their formation in physiological conditions and their involvement in the generation of advanced glycosylation end products (AGEs) (Singh, Barden, Mori, & Beilin, 2001; Horvat & Jakas, 2004). More importantly, Amadori compounds of dipeptides were found in model systems. It was shown that their degradation produces more variety of pyrazines than their amino acid counterparts(Zou, Liu, Song, & Liu, 2018). Hence, the importance of studying them in an actual food matrix arises. So far, there were only a few single amino acids Amadori compounds identified in cocoa (Meitinger et al., 2014). However, taking into account many short peptides found across cocoa fermentation (D'Souza et al., 2018) and the mild conditions needed to generate Amadori compounds, more variety should exist in the raw material for chocolate production. Thus, we present an HPLC-MS-MS study on Malaysian cocoa beans, which aimed to identify more novel Amadori compounds in dried cocoa beans. Additionally, for the first time, we present a comparative analysis of relative quantities of Amadori compounds in different cocoa samples. This research can help with differentiation as well as establishing quality standards for dried cocoa beans.

## 3.2 Material and Methods

### 3.2.1 Chemicals and Reagents

Acetone, dichloromethane, HPLC-grade acetonitrile, HPLC-grade isopropanol, HPLC-grade methanol, and petroleum ether were supplied by Carl Roth (Germany). Acetic acid, formic acid, hesperetin, and sodium hydroxide were supplied by Sigma-Aldrich (Germany). Milli-Q water (18.2 MΩ•cm at 25 °C) was used in all the experiments performed.

### 3.2.2 Sample Collection and Preparation

A sample of fermented and dried cocoa from Malaysia (internally labeled as MC01) was supplied by Barry Callebaut AG. This sample was used for the tandem MS identification of novel Amadori compounds. It was taken from a farm near Kuala Lipis, Malaysia, and belonged to the PBC 123 clone. It was harvested as a part of the main crop. The cocoa beans were fermented for 144 hours and then dried for 96 hours. After the shipment, the sample was stored at 4 °C before any processing.

There were thirteen additional samples obtained by Barry Callebaut AG in order to perform relative quantification of Amadori compounds and discriminant analysis. They came from: Brazil (BC03), Cameroon (PC20), Dominican Republic (E2), two from Ghana (PD04 and PD08), Indonesia (IA03), two from Ivory Coast (PD07 and PD11), Java (PC23), Madagascar (PC21), Papua New Guinea (PC18), Nigeria (NE01), and San Thome (PC19). The Brazilian sample (BC03, CCN-51 hybrid) was harvested on an estate (GPS 14° 02' 53"S, 039 °23' 02"W), and within 12 hours, the fermentation was started. The beans were fermented for 6 days and directly after sun-dried. It was noted that the crop yield and quality was poor because of a very dry period at that time. The sample from Indonesia (IA03, TSH 858 hybrid) was obtained from a farm near Sukabumi, Jakarta. The beans were fermented

12 hours within harvest, which lasted for 6 days, followed by 7 days of sun-drying. The post-harvest treatment of the remaining samples is unknown.

Approximately 30 g of cocoa beans from each sample were de-shelled and ground to a fine powder using a knife mill (Retsch Grindomix GM200, Germany) at 10,000 rpm. Obtained ground powder was stored at 4 °C until further processing. The powder was defatted for 8 hours using petroleum ether as an extraction solvent in an automated Soxhlet extraction apparatus (Büchi B-811, Germany). The defatted powder was dried under vacuum and stored at 4 °C until further use.

### 3.2.3 Extraction

Extraction of the sample for Amadori compound identification as well as relative quantification of both Amadori compounds and oligopeptides was performed according to a previously established protocol (D'Souza et al., 2018). 5 mL of extraction buffer (MeOH:$H_2O$:$CH_3COOH$::70:28:2) was added to 50 mg of defatted cocoa powder, mixed and sonicated in an ultrasonic bath for 10 minutes, then stirred for 30 minutes, and finally filtered through a PTFE syringe membrane filter (0.45 micron). The obtained extract was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and directly used for HPLC-MS experiments.

### 3.2.4 HPLC-TOF-MS-MS Measurements

HPLC separation conditions for every experiment were adapted from a previously established method (D'Souza et al., 2018). Agilent 1260 HPLC system equipped with a Poroshell 120 EC-C18 column (RRHD, 2.1 x 100 mm, 2.7 µm particle size) was used. Milli-Q water and acetonitrile with the addition of 0.05% of formic acid were used as Solvent A and Solvent B, respectively. The sample injection volume employed was 2 µL. The flow rate was constant at 0.4 mL/min, and the column oven temperature was set up to 40 °C. The chromatographic gradient used for the analysis was as follows: (t (min), %B): (0, 8); (1, 8); (2.5, 12); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95). The above-mentioned HPLC system was coupled to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with an electrospray ionization source (nebulizer pressure of 1.8 bars, dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). The data were acquired in positive ion mode and reported NIST monoisotopic atomic masses of the elements (Coursey, Schwab, Tsai, & Dragoset, 2015) were used to calculate monoisotopic molecular masses of detected compounds. 0.1 M sodium formate solution was used to calibrate the TOF analyzer before each sample measurement. During the MS-MS measurements, the collision energy was set to change dynamically and was proportional to the mass of the fragmented molecule. The MS relative quantification of Amadori compounds was performed using Bruker QuantAnalysis software, whereas the peptides using a previously established protocol (D'Souza et al., 2018). In both cases, all samples were normalized using a sum of all detected MS peaks.

### 3.2.5 Discriminant Analysis

The discriminant analysis of the mass spectrometric data was performed using Orange software (Demsar et al., 2013). InfoGain method was used to rank all the features, and subsequently, the top 14 most discriminant were chosen for the principal component analysis (PCA).
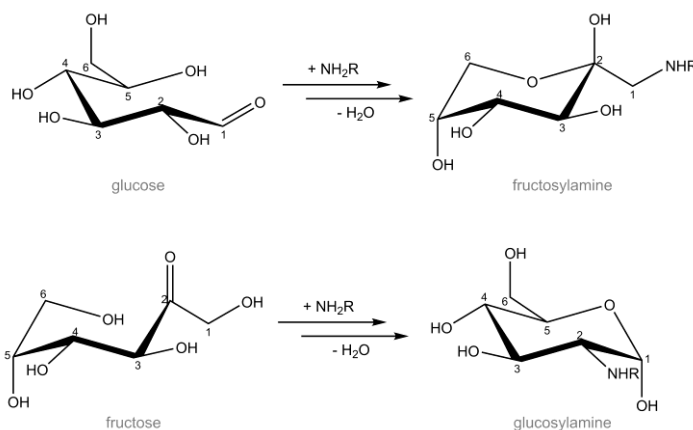


**Figure 1 Difference between Amadori (fructosylamine) and Heyns compounds (glucosylamine), which originate from glucose and fructose, respectively.**

## 3.3 Results and Discussion

The identification of all of the early Maillard reaction products was performed on a methanolic extract of a fermented and dried Malaysian cocoa beans sample. This country of origin was chosen as it is a common knowledge that Malaysian beans tend to have, on average lower protein content than others. Therefore, the results of the identification can be easily extrapolated to other bean origins. Recent studies have shown a large amount (~800) of previously unknown peptides present in coca (D'Souza et al., 2018). We were able to generate a mass list of hypothetical Amadori compounds derived from those peptides, which we compared with obtained MS data. Examination of tandem mass spectra revealed thea presence of thirty-two short-peptide Amadori compounds, surprisingly including Heyns compounds as well (the difference between them is shown in Figure 1). In cocoa, Tthe amounts of fructose and glucose (0.0-103.4 and 4.3-175.0 mg/100g of dry mass respectively) is considerably low (Megías-Pérez, Grimbs, D'Souza, Bernaert, & Kuhnert, 2018) in comparison to amino acid content reported for fermented beans in the literature (Rohsius, Matissek, & Lieberei, 2006), which is between 500 to 2520 mg/100g of dry mass. Moreover, approximate Amadori content amounting to 80 mg/100g of unroasted beans (Meitinger et al., 2014) makes their discovered diversity even more surprising (Megías-Pérez et al., 2018).

### 3.3.1 Identification of Novel Amadori Compounds

The HPLC-ESI-MS-MS experiments revealed the presence of thirty-two different short-peptide Amadori and Heyns compounds: six derived from amino acids and already known in cocoa (Meitinger et al., 2014), twenty-three dipeptide-containing and three tripeptide-containing compounds which are to our knowledge novel in the food literature. The summary of all the identified structures is shown in **Table 1**. They novel compounds elucidated in this paper are as follows: Fru-VP (**1**), Fru-FG (**2**), Fru-IA (**3**), Glc-AY or YA or FS or SF (**4**), Fru-FS (**5**), Fru-L(I)G (**6**), Fru-VA (**7**), Fru-L(I)P (**8**), Fru-L(I)V (**9**), Fru-

L(I)V or VL(I) (**10**), Glc-WVT (**11**), Fru-TVW (**12**), Glc-TVW (**13**), Fru-IT (**14**), Fru-L(I)L(I) (**15**), Fru-L(I)E (**16**), Fru-FT (**17**), Fru-IF (**18**, **19**), Fru-FL(I) (**20**, **21**), Glc-VY (**22**), Fru-FE (**23**), Fru-MF (**24**), Fru-VS (**25**), Fru-L(I)S or VT (**26**), Fru-V (**27**). Structures previously described in the literature, which we have detected are:, Fru-L(I) (**28**), Fru-M (**29**), Fru-F (**30**), Fru-Y (**31**, **32**). An extracted ion chromatogram of chosen examples of compounds is shown in **Figure 2**. After the N-glycosylation of peptides with glucose, the sugar structure resembles fructose (it is opposite for fructose reactions), hence Fru- (fructosyl-) and Glc- (glycosyl-) nomenclature. In some cases, the spectra recorded were ambiguous to an extent, and the absolute assignment of the compounds was not possible. Firstly, in the absence of either Amadori- or Heyns-specific fragmentation peaks (**6**, **10**, **12**, **18**, **19**), we assumed that the compound investigated was an Amadori compound as those should be much more abundant in cocoa. Secondly, in some cases, the sequence of the peptide chain was not clear, and we annotated it accordingly. Additionally, the stereochemistry of the amino acids involved could not be determined, and we assume that all of them are L-isomers. The spectra of all of the compounds are included in the **Supplementary Information**. Here, we will discuss the general behavior of Amadori and Heyns compounds during tandem MS fragmentation as well as apply our approach to three particular examples.
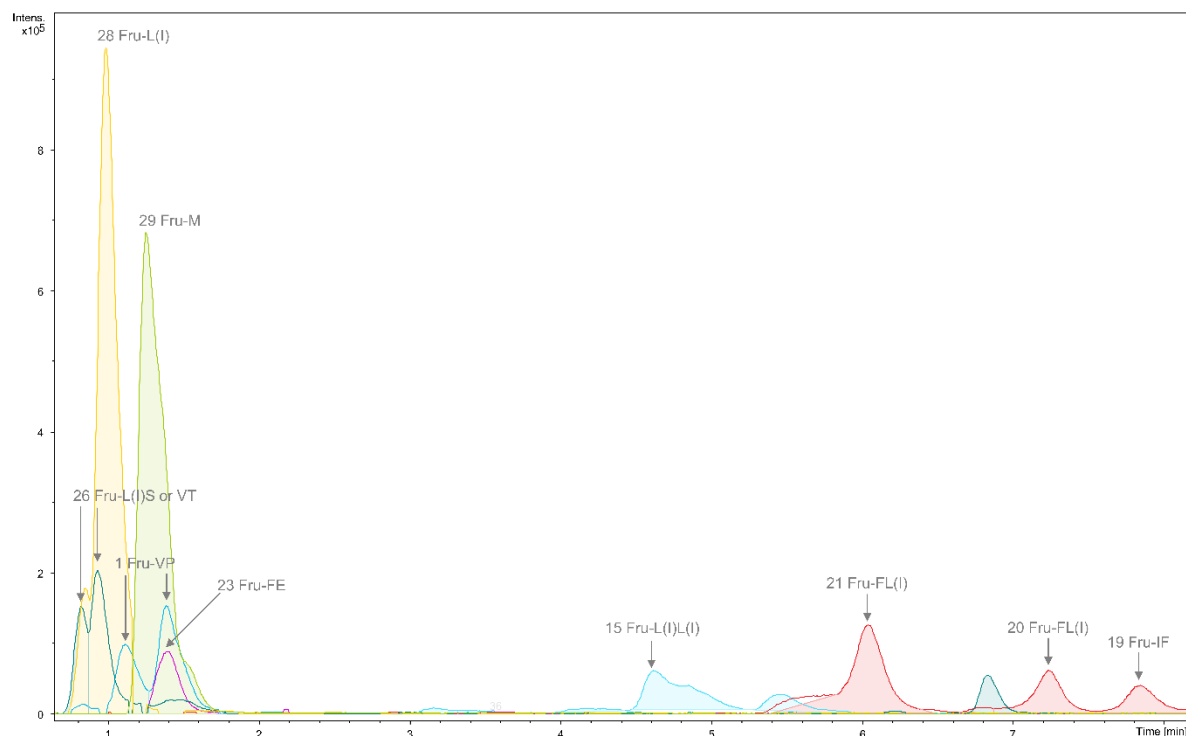


**Figure 2 Extracted ion chromatogram showing some of the identified Amadori compounds.**

The tandem MS fragmentation patterns of single amino acid Amadori compounds are known and well understood based on both analytical standards and Maillard reaction model systems (Hau, Devaud, & Blank, 2004; Davidek, Kraehenbuehl, Devaud, Robert, & Blank, 2005; Wang, Lu, Liu, & He, 2008). The distinction between Amadori and Heyns compounds using their specific fragmentation patterns is possible and described in the literature as well (Yuan et al., 2016, 2017). The detection and identification of those species was achieved in number of foods including cocoa, tomatoes, varieties of peppers, asparagus, cauliflowers, carrots, celery, coffee, barley, wheat, garlic, raisins, and jujubes (Eichner, Reutter, & Wittmann, 1993; Meitinger et al., 2014; Yuan et al., 2016, 2017).

Moreover, the presence of short peptide-derived Amadori compounds was confirmed in Maillard reaction model systems by mass spectrometry techniques (Mennella, Visciano, Napolitano, Castillo, & Fogliano, 2006; Zou et al., 2018).

Generally, the N-glycosylated amino acids fragment producing ions with characteristic multiple neutral losses of $H_2O$ (up to three) along with consecutive losses of $CO_2$ and $H_2CO$ as the most abundant peaks. The remaining peaks come from the fragmentation of the amino acid itself, most notably the peak of protonated amino acid $[AA+H]^+$. The distinction between Amadori and Heyns compounds is possible based on unique neutral losses of 96 and 150, which is $[M+H-2H_2O-CH_3OH-CO]^+$ ion specific for Heyns and $[AA-H+CH_2]^+$ ion specific for Amadori compounds respectively. Another paper describes fragmentation patterns of more complex glycosylated peptides: Amadori compounds of tri- and tetrapeptides, glycopeptide esters, a glycosylamine, and a cyclic Amadori compound (Jeri?, Versluis, Horvat, & Heck, 2002). The data reported show each of the compound class fragments similarly, differing only in abundance of specific ions, especially the multiple neutral losses of $H_2O$ as well as losses of $CO_2$ and $H_2CO$.

**Figure 3** shows spectra we obtained for following N-glycosylated peptides: a) (**27**) Fru-V, b) (**14**) Fru-IT, and c) (**13**) Glc-TVW. Only peaks with intensity larger than 1% were considered. Fru-V (**27**, *m/z* 280), similarly to other single amino acid Amadori compounds in this investigation, was already studied via mass spectrometry techniques, and its fragmentation pattern is previously known (Hau et al., 2004; Davidek et al., 2005; Wang et al., 2008; Yuan et al., 2016). Furthermore, it is amongst seven Amadori compounds known to cocoa (Meitinger et al., 2014). The most abundant peaks in its fragmentation come from a typical pattern of sugar dehydration and subsequent loss of carbon dioxide or formaldehyde. The parent ion (*m/z* 280) loses an $H_2O$ molecule producing the first fragmentation peak at *m/z* 262, followed by consecutive loss of $CO_2$ resulting in the *m/z* 234 peak. Similarly, the loss of two water molecules leads to the formation of *m/z* 244 ion, which after the decarboxylation, yields the most abundant daughter ion $[M+H-2H_2O-CO]^+$ at *m/z* 216. This compound does not produce a stable $[M+H-3H2O]^+$ ion, instead forming the cyclic $[M+H-3H_2O-CO]^+$ and $[M+H-3H_2O-H_2CO]^+$ ions directly, at *m/z* 198 and 196 respectively, which are much less abundant overall. The amino acid component can be cleaved off from the sugar as well, yielding a protonated peptide peak (*m/z* 118), which in turn helps the structure elucidation. Lastly, the bond between $C_1$ and $C_2$ carbon of the carbohydrate can break yielding the $[M-H+CH_2]^+$ ion (*m/z* 130), which confirms that the structure belongs to an amino acid and an aldohexose that underwent condensation and furtherly rearranged into the Amadori compound. Strangely enough, the spectrum contains a much smaller peak specific for Heyns compounds $[M+H-2H_2O-CH_3OH-CO]^+$ at *m/z* 156, which could be a result of the poor chromatographic resolution. The above-mentioned peaks derived from amino acid are mostly much less abundant than those resulting from the dehydration of the hexose. The fragmentation pattern matches a previously proposed fragmentation mechanism of N-glycosylated amino acids (Wang et al., 2008).

**Table 1 Amadori and Heyns compounds identified in the study.**

Fru is an abbreviation for fructosyl (Amadori compound), Glc is an abbreviation for glycosyl (Heyns compound). Amino acid names are depicted by their standard single-letter codes. L(I) indicates that the distinction between leucine and isoleucine was not possible

| ID | Compound Identity | RT | Exp. m/z | Calc. m/z | Error [ppm] | Mol. Formula | Intensity | Ion Fragments |
|---|---|---|---|---|---|---|---|---|
| 1 | Fru-VP | 1.1 | 377.1923 | 377.1918 | 1.23 | $C_{16}H_{28}N_2O_8$ | 285782 | 216.1242(100); 341.171(90); 293.1508(76); 359.1811(22); 215.1401(17); 234.1348(12); 116.0718(11); 323.1618 (9); 198.1137(7); 242.1042(6); 227.1424(3); 150.0938(4); 260.1168(2); 128.0728(2) |
| 2 | Fru-FG | 1.3 | 385.1606 | 385.1605 | 0.25 | $C_{17}H_{24}N_2O_8$ | 177288 | 301.1188(100); 349.138(44); 264.1239(39); 120.0815(31); 331.1282(24); 223.1065(17); 246.1125(16); 313.1165(12); 198.0915(11); 283.1065(7); 367.1477(6); 154.0532(5); 235.0992(2); 289.1188 (1) |
| 3 | Fru-IA | 0.9 | 365.1919 | 365.1918 | 0.07 | $C_{15}H_{28}N_2O_8$ | 518548 | 281.1508(100); 230.1396(51); 329.1721(49); 86.0978(20); 311.1598(20); 212.128(17); 203.1402(10); 347.1825(9); 164.1083(8); 168.0653(7); 168.0653(7); 293.1525 (5); 248.1484(3); 215.1389(3); 263.1394(3) |
| 4 | Glc-AY or YA or FS or SF* | 1.3 | 415.1713 | 415.1711 | 0.40 | $C_{18}H_{26}N_2O_9$ | 55928 | 331.1294(100); 361.139(25); 379.1522(17); 166.0855(14); 235.1066(14); 253.1187(12); 207.1124(10); 120.0814(9); 343. 1279(9); 315.1322(8); 244.0982(8); 292.1207(7); 313.1161(7); 397.1602(4); 285.1216(4); 319.1261(3); 265.1177(1) |
| 5 | Fru-FS | 0.9 | 415.1712 | 415.1711 | 0.13 | $C_{18}H_{26}N_2O_9$ | 114360 | 331.1296(100); 379.15(43); 264.1224(25); 120.0812(15); 361.1402(15); 246.1132(10); 253.1178(8); 198.0934(8); 232.0813(7); 343.1319(6); 285.1240(6); 397.1593(5); 265.1239(5); 136.0773(5); 313.1175(4); 292.1218(2) |
| 6 | Fru-L(I)G** | 0.9 | 351.1760 | 351.1762 | -0.41 | $C_{14}H_{26}N_2O_8$ | 339452 | 267.1356(100); 230.1394(38); 315.1562(36); 297.1448(23); 86.098(22); 212.1295(14); 164.1087(8); 333.1672(7); 189.1250(7); 154.0448(6); 279.1338(5); 249.1229(4); 220.0839(4); 202.0736(4) |
| 7 | Fru-VA | 0.7 | 351.1768 | 351.1762 | 1.61 | $C_{14}H_{26}N_2O_8$ | 322514 | 267.1356(100); 216.1235 (44); 230.1394(38); 315.1562(36); 297.1448(23); 86.098(22); 212.1295(14); 150.0931 (14); 164.1087(8); 198.1139(8); 333.1672(7); 189.1250(7); 279.1338(5); 168.0657 (5); 129.1031(5); 249.1229(4); 220.0839(4); 202.0736(4); 234.1004 (3); 201.1245(2) |
| 8 | Fru-L(I)P | 2.1 | 391.2066 | 391.2075 | -2.26 | $C_{17}H_{30}N_2O_8$ | 92296 | 309.1814(100); 230.139(58); 307.1658(57); 355.1883(53); 357.2017(33); 216.1234(28); 339.1901(24); 212.1241(14); 150.0917(13); 225.1611(12); 116.0704(11); 373.1955(11); 248.1509(11); 229.1565(10); 337.1784(7); 258.1364(7); 86.0981(7); 276.1435(6); 164.1073(6); 132.1032(6); 276.1435 (5); 198.1141(4); 291.1712(3); 241.1559(3) |
| 9 | Fru-L(I)V | 1.3 | 393.2229 | 393.2231 | -0.55 | $C_{17}H_{32}N_2O_8$ | 95486 | 309.1819(100); 230.1383(69); 357.2024(49); 86.0977(26); 339.1913(24); 355.1852(21); 375.2125(13); 231.1694(13); 196.1299(12); 164.1081(12); 212.1281(11); 129.1042(9); 263.1725(6); 248.1532 (6); 226.1087(6); 132.1036(6); 243.1719(2) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | Fru-L(I)V or VL(I)** | 2.0 | 393.2237 | 393.2231 | 1.54 | $C_{17}H_{32}N_2O_8$ | 200348 | 309.1812(100); 357.2036(36); 216.1223(31); 230.1396(30); 339.1908(23); 355.1868(19); 307.1673(18); 231.1695(10); 210.1136(9); 198.1140(9); 375.2151(7); 150.0935(6); 321.1767(4); 291.1750(4); 263.1801(4); 164.1066(3); 86.0968(3) |
| 11 | Glc-WVT* | 4.3 | 567.2647 | 567.2661 | 2.47 | $C_{20}H_{28}N_4O_5$ | 26832 | 283.0826(100); 258.1153(96); 240.1019(89); 217.0505(81); 513.2342(55); 384.1935(49); 257.1143(46); 321.1462(39); 366.18(37); 303.13(36); 388.1881(29); 267.117(29); 564.2985(26); 402.1727(25); 483.2169(25); 336.1712(25); 531.2508(23); 286.1599(22); 495.2273(21); 258.1587(20); 549.2571(18); 412.2026(16); 461.1362(10); 102.0561(9); 188.0704(8); 430.1996(8); 156.076(8); 120.0697(8); 306.1531(7); 405.2088(7); 471.2283(2) |
| 12 | Fru-TVW** | 5.9 | 567.2658 | 567.2661 | 0.53 | $C_{20}H_{28}N_4O_5$ | 28330 | 281.1496(100); 317.1723(98); 483.2216(90); 302.0995(67); 279.1356(66); 304.1649(65); 265.1201(65); 299.163(64); 513.2355(55); 309.1476(49); 251.1387(46); 373.1733(36); 469.2047(34); 355.16(32); 205.1007(31); 237.1232(31); 345.1648(28); 327.1567(27); 305.1695(24); 173.1276(16); 201.1222(15); 188.0715(14); 531.246(12); 549.251(12); 159.0892(7); 102.0576(7); 405.2048(1) |
| 13 | Glc-TVW* | 5.8 | 567.2648 | 567.2661 | 2.29 | $C_{20}H_{28}N_4O_5$ | 24936 | 304.1678(100); 299.1614(99); 483.2264(85); 265.1193(85); 279.1357(79); 281.1514(79); 251.1376(66); 317.1708(54); 309.1462(53); 205.0991(42); 469.2109(41); 237.1267(31); 327.1538(30); 513.2293(27); 136.0746(26); 305.171(25); 345.1654(24); 173.1305(15); 219.1177(15); 107.0516(15); 363.1836(13); 549.2705(11); 451.1959(10); 531.2362(9); 409.1762(9); 102.0569(8); 471.2253(6); 405.2123(5) |
| 14 | Fru-IT | 0.8 | 395.2024 | 395.2024 | 0.00 | $C_{16}H_{31}N_2O_9$ | 189550 | 311.1595(100); 359.1819(50); 86.0976(49); 230.1393(46); 341.1708(17); 233.15(10); 136.0758(9); 212.1311(7); 132.1047(6); 323.1643(6); 198.0776(6); 173.1296(6); 248.1505(5); 164.1069(5); 293.1476(5); 276.1469(4); 377.1927(4); 96.0445(3); 147.0792(2); 264.1149(2); 245.1533(1) |
| 15 | Fru-L(I)L(I) | 3.9 | 407.2393 | 407.2388 | -1.23 | $C_{18}H_{35}N_2O_8$ | 103464 | 323.1973(100); 230.1388(41); 371.2179(38); 353.2073(24); 127.0399(23); 86.0978(19); 145.0508(18); 103.0398(15); 210.1128(11); 389.23(11); 303.0877(11); 164.1078(10); 196.136(9); 258.1344(9); 212.1289(8); 245.1863(8); 248.1493(7); 97.0291(7); 276.1433(5); 120.0824(4); 257.1863(3) |
| 16 | Fru-L(I)E | 0.8 | 423.1978 | 423.1973 | -1.18 | $C_{17}H_{31}N_2O_{10}$ | 420024 | 339.1561(100); 387.1771(49); 230.1392(31); 369.1655(23); 261.1451(14); 86.0982(14); 212.1286(9); 405.1878(8); 274.0909(7); 248.1473(6); 196.1326(6); 321.1442(5); 164.1065(4); 273.1411(3); 148.0619(3); 185.1306(2) |
| 17 | Fru-FT | 1.0 | 429.1868 | 429.1868 | 0.00 | $C_{19}H_{29}N_2O_9$ | 101052 | 345.1445(100); 393.1679(32); 427.2574(29); 264.1244(19); 327.1322(19); 120.0826(16); 375.1562(15); 246.1079(13); 267.1355(13); 217.1554(11); |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | 411.1759(10); 228.1024(7); 198.0765(7); 86.0964(5); 129.1029(5); 103.0404(4); 292.0312(4); 279.1412(3) |
| 18 | Fru-IF** | 5.0 | 441.2235 | 441.2231 | 0.74 | $C_{21}H_{32}N_2O_8$ | 79852 | 357.1811(100); 405.2012(36); 230.1395(32); 387.1892(19); 279.1714(9); 244.0987(8); 86.0972(8); 212.13(8); 423.2144(8); 164.1084(6); 292.1182(6); 311.176(5); 196.1352(5); 120.0826(4); 369.1843(3); 136.0777(2); 194.1207(2) |
| 19 | Fru-IF** | 6.1 | 441.2214 | 441.2231 | -3.93 | $C_{21}H_{32}N_2O_8$ | 60232 | 357.1809(100); 405.2013(28); 230.1385(23); 387.1923(18); 244.0955(12); 279.1703(10); 439.1565(8); 212.1297(7); 86.0971(7); 164.1068(7); 196.1348(6); 292.1175(6); 423.2108(6); 311.1724(4); 220.0978(4); 194.1176(3); 127.0395(3); 136.0770 (2) |
| 20 | Fru-FL(I) | 6.7 | 441.2224 | 441.2231 | -1.66 | $C_{21}H_{32}N_2O_8$ | 68270 | 357.1813(100); 405.2022(43); 387.1933(27); 120.0814(27); 264.1245(25); 279.1717(15); 246.1129(10); 210.1119(10); 230.1184(8); 423.2119(8); 311.176(6); 198.0902(5); 369.1827(5); 291.1709(3); 86.0971(2) |
| 21 | Fru-FL(I) | 4.7 | 441.2241 | 441.2231 | 2.14 | $C_{21}H_{32}N_2O_8$ | 40928 | 357.1799(100); 405.202(53); 264.1232(33); 120.0817(27); 387.1922(23); 279.1677(17); 246.1115(15); 230.1157(12); 311.1782(9); 423.2117(8); 210.1145(7); 369.1782(7); 291.1701(5); 198.0916(5); 86.098(4); 136.0762(3); 162.0936(3); 323.1751(3); 177.0578(3); 345.1789(2) |
| 22 | Glc-VY* | 1.1 | 443.2021 | 443.2024 | -0.66 | $C_{20}H_{30}N_2O_9$ | 55624 | 359.1601(100); 407.181(58); 389.1704(26); 216.1236(21); 280.1188(19); 281.1511(15); 136.075(11); 120.0819(11); 425.1889(10); 264.1229(8); 260.0907(8); 86.0968(6); 308.1144(6); 246.1138(5); 150.0909(5); 185.1669(5); 198.1142(5); 326.1211(4); 166.0874(4); 313.1521(4); 442.2366(4); 341.1482(3); 293.1507(2) |
| 23 | Fru-FE | 1.1 | 457.1814 | 457.1817 | 0.66 | $C_{20}H_{29}N_2O_{10}$ | 193494 | 373.1397(100); 421.1615(50); 295.1275(23); 264.1211(21); 403.15(19); 120.0812(18); 439.1703(13); 246.1121(11); 226.0729(8); 274.0939(6); 198.0937(6); 148.0595(6); 228.1021(5); 310.1309(4); 355.1246(3); 327.1314(2); 307.1293(1) |
| 24 | Fru-MF | 4.5 | 459.1797 | 459.1796 | -0.22 | $C_{20}H_{31}N_2O_8S$ | 74878 | 375.1387(100); 423.1572(53); 248.0966(41); 292.1189(32); 405.1501(29); 212.0756(28); 104.0536(23); 244.0987(22); 297.1283(20); 230.0861(17); 441.17(14); 357.1358(12); 182.0629(11); 232.0975(10); 387.1351(7); 166.0851(7); 339.138(7); 274.1113(7); 307.1107(7); 129.1041(6); 200.0938(5); 367.1521(4); 327.1306(3); 258.0761(3); 309.1229(2) |
| 25 | Fru-VS | 0.7 | 367.1709 | 367.1711 | -0.69 | $C_{14}H_{26}N_2O_9$ | 206724 | 283.1299(100); 331.1506(33); 216.1248(32); 313.1405(19); 365.1058(11); 84.0821(10); 349.1589(10); 230.1381(9); 116.0706(9); 184.0605(9); 203.0541(8); 156.0738(6); 129.1023(6); 253.1293(6); 265.1202(6); 205.1199(4); 97.0293(4); 175.1123(4); 150.0941(3) |
| 26 | Fru-L(I)S or VT | 0.7 | 381.1879 | 381.1868 | 3.03 | $C_{15}H_{28}N_2O_9$ | 595386 | 297.1458(100); 345.167(47); 216.1249(25); 327.1559(22); 230.1405(17); 363.1765(10); 219.1351(9); 212.1299(8); 86.0974(8); 150.0941(7); 136.0774(6); 198.0769(6); 231.1417(6); 309.1461(6); 279.1372(6); |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 258.1393(4); 120.0665(4); 232.0821 (3); 164.1092(3); 182.1179(3); 251.1064 (2) |
| 27 | Fru-V | 0.7 | 280.1398 | 280.1391 | 2.70 | $C_{11}H_{21}NO_7$ | 1054608 | 216.1245(100); 244.1185(60); 262.1296(46); 130.0872(28); 198.1133(21); 118.0875(18); 84.0823(12); 196.0976(10); 234.1343(10); 161.0694(9); 102.0922(9); 150.0925(8); 114.092(7); 154.0877(3); 110.0718(3); 180.1025(3); 156.1015(2); 226.107(2) |
| 28 | Fru-L(I) | 0.7 | 294.1556 | 294.1547 | 3.11 | $C_{12}H_{23}NO_7$ | 831328 | 230.1398(100); 258.1349(55); 276.1445(38); 212.1288(21); 86.0977(18); 132.1027(13); 210.1135(12); 161.0683(11); 144.1031(11); 120.082(8); 97.0294(7); 248.1525(7); 220.084(4); 194.0473(2) |
| 29 | Fru-M | 0.7 | 312.1114 | 312.1111 | -0.96 | $C_{11}H_{22}NO_7S$ | 138344 | 276.0911(100); 230.0855(44); 84.0812(33); 248.0965(32); 133.0329(31); 88.04(25); 294.101(23); 228.0675(22); 104.0535(21); 150.058(18); 127.04(17); 118.0875(15); 212.0735(14); 258.0801(10); 220.0835(9); 97.0305(8); 200.0917(8); 143.0621(7); 161.0657(6); 111.0448(4); 192.0741(3); 266.1073(3); 181.0937(2); 162.0585(2) |
| 30 | Fru-F | 1.0 | 328.1396 | 328.1391 | 2.39 | $C_{15}H_{21}NO_7$ | 2873394 | 264.1243(100); 292.1191(88); 120.082(72); 310.1295(45); 132.0823(44); 244.0981(40); 166.0872(37); 246.1138(26); 178.0874(21); 161.0691(16); 127.0396(15); 143.0589(11); 198.0921(11); 97.0297(10); 228.1029(6); 282.1357(5); 112.0404(4); 204.1021(3); 232.0972(2); 274.108(2) |
| 31 | Fru-Y*** | 0.7 | 344.1345 | 344.1340 | -1.58 | $C_{15}H_{22}NO_8$ | 306860 | 280.1187(100); 308.1133(75); 326.1232(62); 136.0759(27); 260.0924(20); 165.0566(17); 86.0971(15); 182.0821(13); 147.0775(13); 194.0822(13); 262.108(12); 130.0512(10); 234.1128(9); 97.0302(9); 107.0497(8); 248.0914(8); 217.0809(7); 298.131(6); 202.0757(5); 175.1174(5); 156.0672(4); 288.1098(4); 220.0968(3) |
| 32 | Fru-Y | 0.8 | 344.1346 | 344.1340 | -1.74 | $C_{15}H_{22}NO_8$ | 727500 | 280.1198(100); 308.1141(83); 326.1248(47); 165.0559(23); 136.0769(22); 260.0922(20); 148.0764(18); 262.1088(18); 86.0972(15); 194.0823(11); 182.0824(9); 107.0499(8); 123.0453(8); 97.0286(8); 244.1278(7); 214.0871(6); 298.1267(4); 234.1123(4); 202.0707(4); 224.0896(3) |

*Heyns-specific peaks are more intensive than Amadori-specific peaks

**There are no either Amadori or Heyns-specific peaks

***Splitting peak with different Amadori and Heyns-specific peaks than its counterpart (compound 32)

Remaining structures have clear Amadori-specific fragmentation peaks, and the Heyns-specific peaks are less abundant or non-existent

**a) (27) Fru-V**

+MS, 0.7min #151

280 [M+H]⁺ 280.1398

266.1244
246.1456
262.1296
276.1465 2+
219.1347
234.0991
205.1187
215.1393
182.0819 187.1082
175.1175
163.0604
148.0613
133.0611
118.0871
86.0976

+MS2(280.1398), 26.1eV, 0.7min #152

216 [M+H-2H₂O-CO]⁺ 216.1245

156 [M+H-2H₂O-CH₃OH-CO]⁺

234 [M+H-H₂O-CO]⁺ 234.1343

244 [M+H-2H₂O]⁺ 244.1185

262 [M+H-H₂O]⁺ 262.1296

196 [M+H-3H₂O-H₂CO]⁺

198 [M+H-3H₂O-CO]⁺ 198.1133

118 [AA+H]⁺ 118.0875

130 [AA-H+CH₂]⁺ 130.0872

84.0823
102.0922
110.0718
114.0920
150.0925
154.0877
161.0694
180.1025

**b) (14) Fru-IT**

+MS, 0.8min #193

294.1555

276.1455 2+

395 [M+H]⁺

381.1886 2+
344.1346
351.1762
361.1729
329.1826
312.1118
261.1453
247.1294
233.1501
219.1344
182.0818
152.0577
132.1027
86.0974
193.0350
395.2024

+MS2(395.2024), 29.4eV, 0.8min #212

293 Sug-** b2-3H₂O-H₂CO
311 [M+H-3H₂O-H₂CO]⁺ 311.1595

198 Sug-L(I)T y1-3H₂O-H₂CO

276 Sug-TL(I) y1-H₂O

323 Sug-** b2-3H₂O

164 Sug-L(I)T a1-3H₂O-H₂CO

136 Sug-IT d-3H₂O-H₂CO

341 [M+H-3H₂O]⁺ 341.1708

245 [**-H+CH₂]⁺

233 [**+H]⁺

359 [M+H-2H₂O]⁺ 359.1819

86 L(I)T a1 86.0976

230 Sug-L(I)T a1-H₂O 230.1393

212 Sug-L(I)T a1-2H₂O

248 Sug-L(I)T a1 248.1505

377 [M+H-H₂O]⁺ 377.1927

132 TL(I) y1 136.0758

96.0445
147.0792
173.1296
198.0776
264.1149
276.1469
293.1476
323.1643

**c) (13) Glc-TVW**

+MS, 5.8min #1260

380.1818

277.1190

299 Sug-**W b2-2H₂O-CO

304 T** y2

305 **V c2

567 [M+H]⁺ 567.2648

215.1032
166.0868
195.0882
291.0860
316.2240
360.1447
447.2249
480.2339
542.2321

+MS2(567.2648), 33.7eV, 5.9min #1267

281 Sug-**W b2-3H₂O-CO
304.1678

279 Sug-**W b2-3H₂O-H₂CO
265 Sug-V** d 265.1193

309 Sug-**W b2-3H₂O
317 Sug-**W a2-H₂O

471 [M+H-2H2O-CH3OH-CO]⁺

483 [M+H-3H₂O-H₂CO]⁺ 483.2264

279.1357

251 Sug-**W a2-3H₂O-H₂CO 251.1376

327 Sug-**W b2-2H₂O

513 [M+H-3H₂O]⁺ 513.2293

531 [M+H-2H₂O]⁺

345 Sug-**W b2-H₂O

317.1708

205 **W y1 205.0991

549 [M+H-H₂O]⁺

102 T** b1

136.0746

173 **W a2 173.1305

327.1538

363 Sug-W** y2-H₂O 345.1654

405 [**+H]⁺

549.2705
567.2618

107.0516
219.1177
237.1267
363.1836
409.1762
451.1959
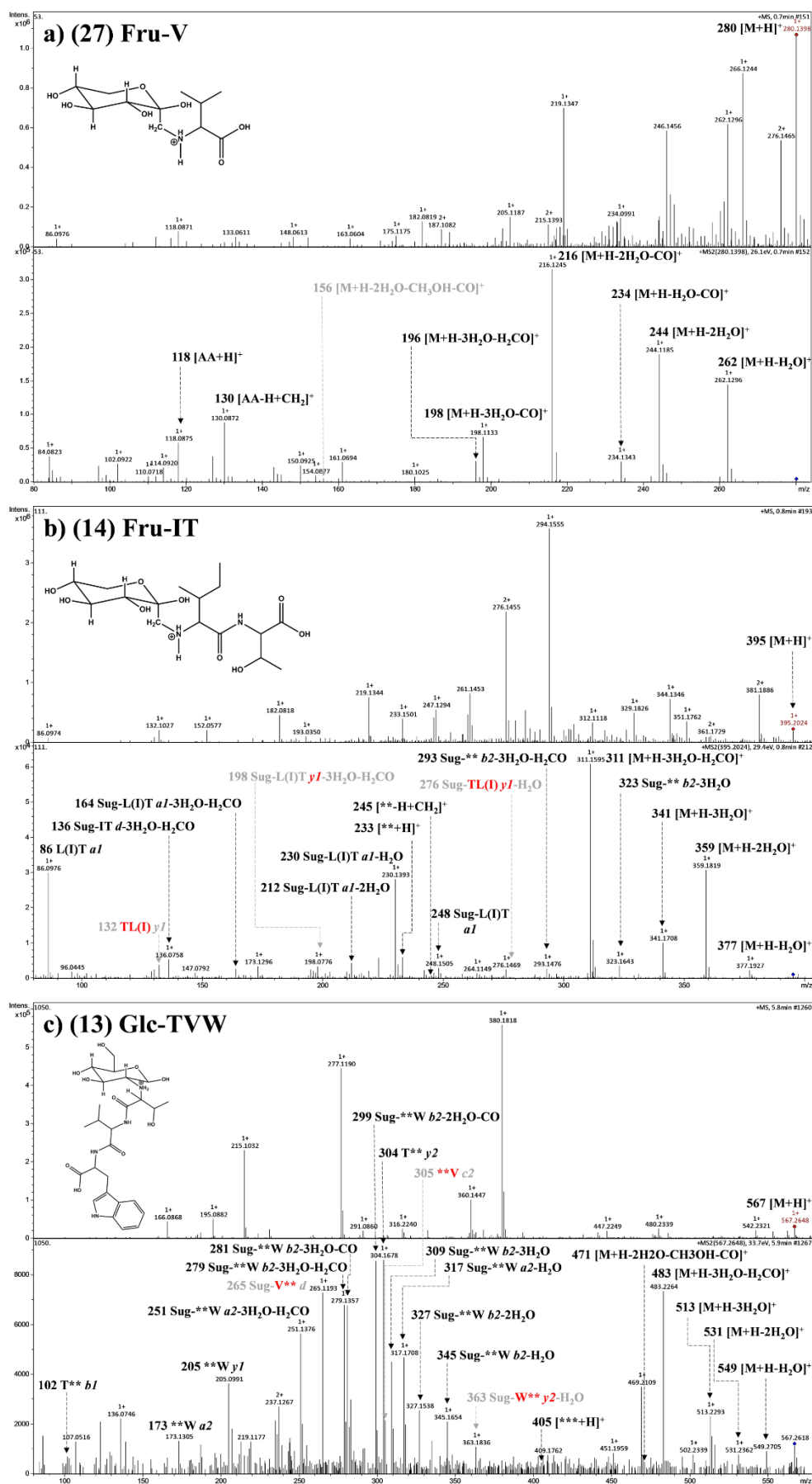469.2109
502.2339
513.2293
531.2362

93

**Figure 3 (previous page) An example of HPLC-ESI-MS-MS fragmentation spectra of N-glycosylated peptides for a) (27) Fru-V, b) (14) Fru-IT, and c) (13) Glc-TVW, Fru- being fructosyl (Amadori compound), and Glc- glycosyl- (Heyns compound) species respectively.** All important ion fragments (>1% relative intensity) are annotated. "AA" and "*" indicate any single amino acids that are part of the individual structures. Letters and numbers in italics (e.g., a1) are the notations based on the standard peptide fragmentation nomenclature. Grayed-out text indicates fragments specific to other isomers of the elucidated structure, while the red highlights point out how those peaks contradict our assignment. L(I) annotation implies that the fragment is the same for both leucine and isoleucine.

Additional amino acid causes the fragmentation pattern to be more complex. Still, the most abundant peaks for Fru-IT (**Figure 3b)**, $m/z$ 395) are the ones originating from the dehydration of the hexose, but the ratio between individual peaks changes. Formation of the $[M+H-3H_2O-H_2CO]^+$ ion at $m/z$ 311 is favored, and the decarboxylation does not occur for the $[M+H-H_2O]^+$ ($m/z$ 377), $[M+H-2H_2O]^+$ ($m/z$ 359), and $[M+H-3H_2O]^+$ ($m/z$ 341) ions. Additionally, another dehydration can take place on the C-terminus of the peptide chain ($m/z$ 323). On the other hand, Amadori-specific ($m/z$ 245) and protonated peptide ($m/z$ 233) peaks are not abundant, but other patterns arise as the fragmentation of both hexose and peptide components of the structure can break down at the same time. Most notably, a series of *a1* peptide fragments still attached to a hexose are present, alongside their sugar dehydration and decarboxylation products. Among them, the Sug-L(I)T *a1*-$H_2O$ peak at $m/z$ 230 was the most abundant (46% relative intensity). The specific *d* fragment ion at $m/z$ 136 enabled us to confirm the presence of isoleucine moiety in the dipeptide. Besides other small peaks, three daughter ions contradicting our structure assignment were present (**Figure 3b)**, grayed out). Especially, the $m/z$ 276 and 198 peaks containing *y1* peptide fragments attached to hexoses, which could not be formed without some unlikely rearrangement.

Unsurprisingly, the N-glycosylated tripeptide Glc-TVW (**Figure 3c)**, $m/z$ 567), exhibit the most complex fragmentation pattern. The sugar component degradation behavior is similar to the one displayed by the previous dipeptide, but the relative intensity of the peaks decreased with respect to the rest of the spectra. The $[M+H-3H_2O-H_2CO]^+$ ion at $m/z$ 483 is not the most abundant fragment. Heyns-specific peak, as well as the protonated peptide, is smaller, but present ($m/z$ 471 and 405 respectively). The most abundant peak in the spectra is a *y2* peptide fragment of either TVW or TWV at $m/z$ 304, followed by the Sug-**W *b2*-$2H_2O$-CO at $m/z$ 299. Most of the remaining daughter ions originate from the *b2* fragment ion and fragmentation of the protonated peptide. Additionally, there are also three peaks not conforming to our expectations, similarly to the previously described dipeptide.

The collision energy depended on the mass of individual parent ion, but still, all the compounds in this study were fragmented in similar conditions (energy between 26 and 34 eV). Although only the twenty-three-dipeptide group is large enough to derive some general pattern of behavior. Still, we have identified six N-glycosylated amino acids known in cocoa, twenty-three N-glycosylated dipeptides, and three N-glycosylated tripeptides that were previously unreported. Twenty-three of them are undoubtedly Amadori compounds (**1-3**, **5**, **7-9**, **14-17**, **20**, **21**, **23-32**), four Heyns compounds (**4**, **11**, **13**, **22**), and five lack distinguishing fragments but were assigned as Amadori compounds (**6**, **10**, **12**, **18**, **19**). Recent literature does not indicate that the analytical standards of Amadori compounds can fragment producing Heyns-specific peaks. In total, nineteen were ambiguously assigned because of unclear peptide sequence or lack of Amadori- or Heyns-specific peaks (**see Table 1**).

Each type of N-glycosylated species in this study is clearly distinguishable from each other (see **Figure 3** and **Supplementary Information**). The single amino acids containing compounds have the most simple fragmentation spectra among all and exhibit a particular pattern of the hexose-specific dehydration and decarboxylation. In most of them (the only exception is Fru-M, **29**), the most abundant peak is the $[M+H-2H_2O-CO]^+$ ion. The amino acid part-specific peaks are not abundant and are limited only to the protonated amino acid peak $[AA+H]^+$. In the case of dipeptide Amadori and Heyns compound, the spectra change slightly. Still, the peptide degradation occurs in a smaller degree, and the main peaks produced belong to the hexose degradation. However, the ratio between the hexose peaks changes, with the most abundant one being the $[M+H-3H_2O-H_2CO]^+$ for most cases, except the proline-containing compounds. Fru-VP (**1**) and Fru-L(I)P (**8**) display the Sug-VP $a1$-$H_2O$ and the $[M+H-3H_2O-CO]^+$ as the most abundant peaks, respectively. As the length of the amino acid chain increase by one, the variety of the peptide-specific fragments increases. This is true for the tripeptides, as well. In their case, the hexose degradation pattern is very similar, but the most abundant peaks are the ones that combine both hexose and peptide fragmentations. The complexity of spectra dramatically increases.

Additionally, we have identified some irregularities in the behavior of most of the presented compounds during HPLC-ESI-MS-MS fragmentation. First of all, the differentiation between Amadori and Heyns compounds was ambiguous in some cases. Only nineteen from thirty-two compounds had obvious fragments of either Amadori or Heyns compounds. Surprisingly, seven had both of them (**2**, **4**, **21**, **25**, **27**, **30**, **31**), and six none of them (**6**, **10**, **12**, **15**, **18**, **19**). This behavior is not clear, and literature does not indicate that it occurs for MS-MS fragmentation of analytical standards. Secondly, most di- and tripeptide-containing structures produced fragments that would indicate a different sequence of amino acids in the peptide chain that suggested by other daughter ions (e.g., **Figure 3b**), $m/z$ 132). However, those peaks appear infrequently; therefore, they did not affect our assignment. Their appearance in the spectra would have to be associated with either poor chromatographic resolution of different isomers, or an unlikely rearrangement. Finally, in some cases of di- and tripeptide-containing structures, an ion comprised of $y$ peptide fragment and hexose fragment is produced (e.g., **Figure 3b**), $m/z$ 276, and 198). This is highly unexpected, as the formation of $y$ ions would mean that the peptide part of the compound broke off the hexose part. There is no clear explanation for this behavior.
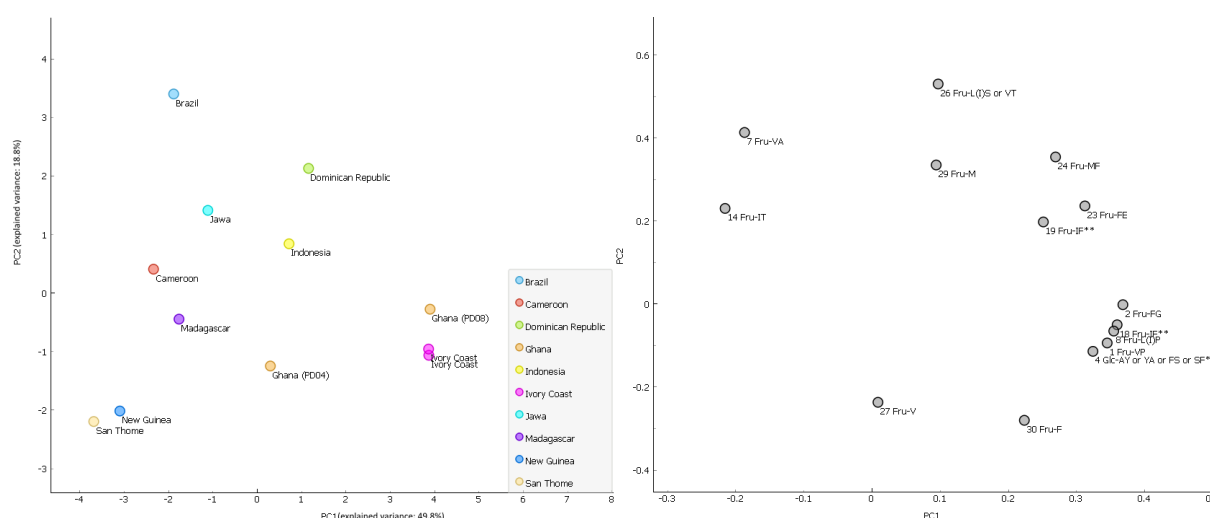


**Figure 4 Principal component analysis of dry cocoa beans from different origins based on their Amadori and Heyns compounds content (explained variance: PC1 49.8%; PC2 18.8%).**

### 3.3.2 Discriminant Analysis of Different Cocoa Samples Based on Amadori Compound Content

Employing both reported previously and newly identified Amadori and Heyns compounds, discriminant analysis was performed, which successfully distinguished the samples based on their geographical origin (see PCA in **Figure 4**). At first, all the chemical species were included, however, using only the top 14 most important features (ranked by InfoGain method of Orange software) improved the separation. The principal component analysis distinguishes African countries very well, even among themselves. The Brazilian sample stood out as well, most likely because of bad weather conditions during the year of the harvest. The rest of the samples separate as well, yet it is difficult to speculate why without including more samples from each of the regions. Most notably, the separation based on origins is doubtlessly affected by a variety of the cocoa hybrid as well as post-harvest practices (such as drying and fermentation), and those should be taken into account in further studies.

**Figure 5** shows bar charts of chosen Amadori compounds and their putative peptide precursors for some of the samples presented in the study. There seems to be a slight connection between the Amadori compound content and their corresponding peptides; however, this result furtherly enforces the earlier point, that the important factors shaping the Amadori content profile of cocoa are the hybrid type and the fermentation and drying. Unfortunately, our data lack this basic information for most of the samples. Nevertheless, there are significant differences in Amadori and Heyns compounds content between the presented samples. presented, Additionally, considering the well-known fundamental involvement of these compounds in aroma and color formation in heated foods. and Amadori  novel compounds described herein should be considered as important quality markers of cocoa quality and origin. in the future. However, direct links between aroma quality and presented compounds have yet to be established.

## 3.4 Conclusions

In our study, we have identified thirty-two N-glycosylated short-peptides (Amadori and Heyns compounds), of which twenty-six are novel to cocoa. We have demonstrated their general ESI-MS-MS fragmentation patterns, which agree with previous findings in the literature. Additionally, we have described some unusual daughter ions that occur in many of the presented compounds, which should be confirmed by experiments on authentic analytical standards. Our data shows that there is more variety in Amadori compounds in cocoa (and in food) than it was reported so far. We believe that further studies on these compounds can shed some light on chemical transformations occurring during cocoa processing. Moreover, we have relatively quantified all presented species in thirteen samples of cocoa originating from different countries. The principal component analysis has shown that these samples have different Amadori compound profiles, and it is possible to distinguish them on this basis alone, which suggests they could be an important quality marker. Nevertheless, further research is necessary as it could be a result of different fermentation and drying practices as well as a variety of cocoa. Still, we believe this research provides groundwork for the identification of further Amadori and Heyns compounds, as well as linking them to the quality and origin of different cocoa beans.
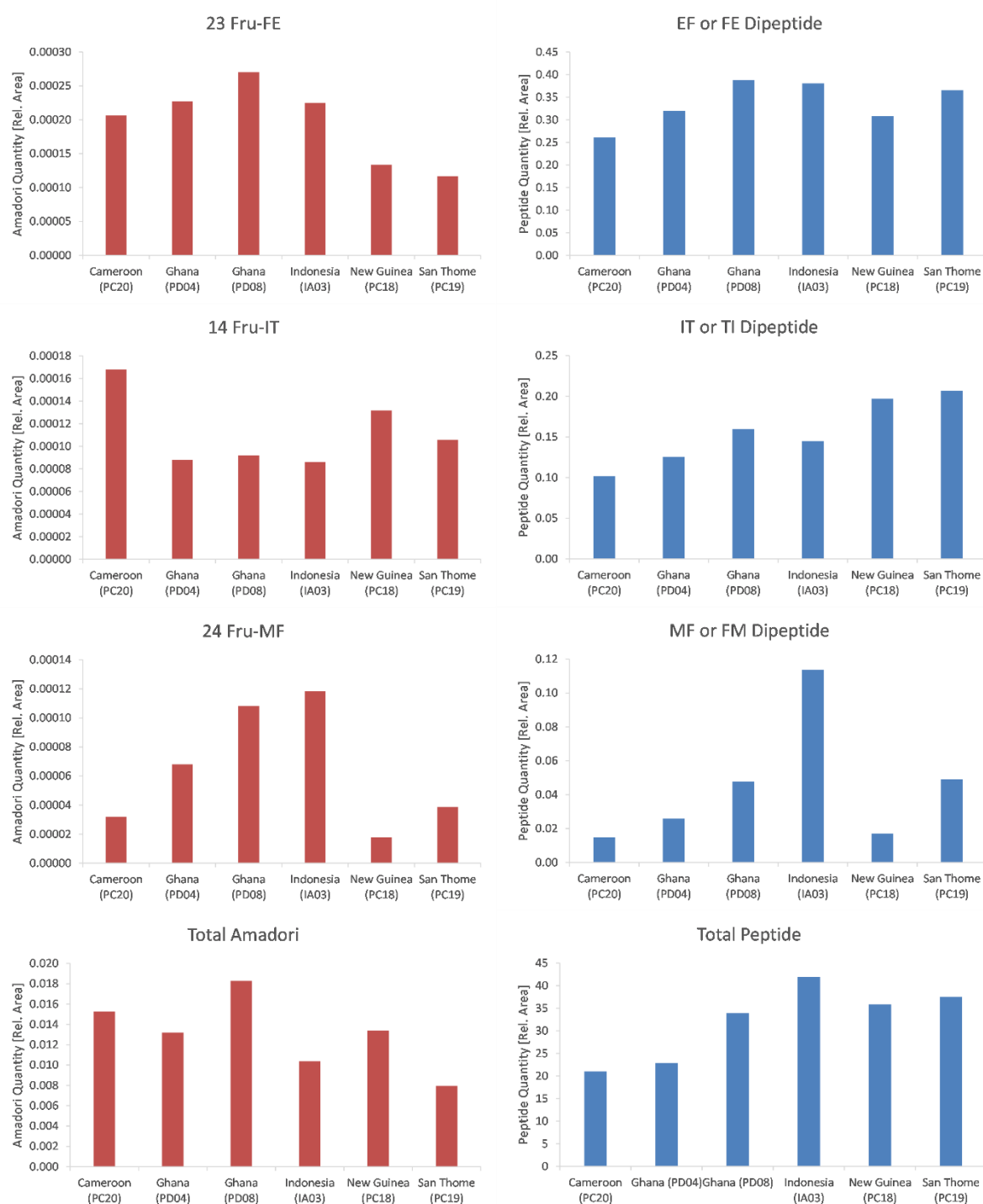
**Figure 5 Relative quantities of some of the Amadori compounds (left) and their putative peptide precursors (right) for chosen countries of origin.**

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## Conflict of interest statement

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# Chapter 4

# Chemical Profiling of Ghanaian Cocoa Bean Processing Series Using Mass Spectrometric Detection of Volatiles and Maillard Precursors

Paweł J. Andruszkiewicz, Roberto Megías-Pérez, Britta Behrends, Isabelle Leuven, Marcello Corno, and Nikolai Kuhnert

*Note:* The **Supplementary Information** for this chapter is available in **Appendix A**.

## Abstract

Chocolate quality highly relies on the quality of raw material. Cocoa beans are very complex chemically, with high variation across hybrid and origin. This requires a deep understanding and a unique approach to each raw material. Here, we present a mass spectrometric study of the chemical profile of Ghanaian cocoa beans across fermentation, drying, and roasting, with emphasis put on chemical compounds related to Maillard reaction: peptides, carbohydrates, Amadori compounds (including recently reported dipeptide species), pyrazines and other volatiles. We show their general trends as well as correlations between them, which can be helpful in quality control of Ghanaian cocoa beans.

## 4.1 Introduction

Cocoa beans form the essential resource in chocolate production; they have been intensely studied, but due to their chemical complexity only characterized in parts. Key milestones in cocoa research include the identification and elucidation of important taste-active non-volatiles (Stark, Bareuther, & Hofmann, 2006), aroma volatiles (Frauendorfer & Schieberle, 2006), as well as composition of important flavor precursors such as sugars and peptides and their variations based on different cocoa origins (Caligiani, Marseglia, Prandi, Palla, & Sforza, 2016; Kumari et al., 2018; Megías-Pérez, Grimbs, D'Souza, Bernaert, & Kuhnert, 2018). Their general behavior along the most critical processing steps – fermentation (Hashim, Selamat, Syed Muhammad, & Ali, 1998; Kumari et al., 2016; D'Souza et al., 2018), drying (Rodriguez-Campos et al., 2012), and roasting (de Brito et al., 2001). However, the behavior of the same cocoa material during fermentation, drying, roasting, and chocolate processing is less reported. Of course, the accumulated knowledge expands, and in the future, it should be possible to predict accurately how a given hybrid from a given country of origin will behave during particular processing practices, allowing to tailor cocoa quality parameters based on scientific models. Currently, it has been done in some extant for beans originating from Malaysia, Mexico, and Brazil (Hashim et al., 1998; de Brito et al., 2001; Rodriguez-Campos et al., 2012; Moreira, Vilela, Santos, Lima, & Schwan, 2018). However, it is crucial to continue to report chemical profiles of cocoa

processing, especially for the largest cocoa producers, as they supply most of the cocoa to the world.

Ghana is the second-largest supplier of cocoa beans in the world (Mattyasovszky, 2018). They have a specific fermentation routine, which involves pod storage for up to 5 days before fermentation (Afoakwa, Quao, Takrama, Budu, & Saalia, 2013), and then fermentation is done mostly in heaps (Daniel et al., 2009). Moreover, it is believed that cocoa beans from Ghana are generally well fermented (N. Camu et al., 2007). There were several studies on quality of cocoa beans from Ghana (Aculey et al., 2010; Afoakwa et al., 2013; Teye, Huang, Dai, & Chen, 2013). Other countries of origin as well (Ramli, Hassan, Said, Samsudin, & Idris, 2006; Milev, Patras, Dittmar, Vrancken, & Kuhnert, 2014; Caligiani et al., 2016; Grillo et al., 2019). However, only few studies considered impact of processing in general for cocoa beans (Hashim et al., 1998; de Brito et al., 2001; Rodriguez-Campos et al., 2012; Moreira et al., 2018). Here, we present a first report covering chemical profiling of multiple processing steps (raw, fermentation series, dried, and cocoa liquor) of beans from Ghana. Additionally, it is the first time both novel dipeptide (see **Chapter 3**) and known to date Amadori compounds from cocoa (Meitinger, Hartmann, & Schieberle, 2014)s are put into a frame of processing series. We believe it is important to report such studies, as covering as many hybrids and origins of cocoa as possible can not only give us information on how to ferment and dry the beans as well as what to expect after thermal processing of beans with specific precursor content.

## 4.2 Material and Methods

### 4.2.1 Chemicals and Reagents

Acetone, HPLC-grade acetonitrile, HPLC-grade isopropanol, HPLC-grade methanol, and dichloromethane were supplied by Carl Roth (Germany). Acetic acid, formic acid, hesperetin, and sodium hydroxide were supplied by Sigma-Aldrich (Germany). Milli-Q water (18.2 M$\Omega$•cm at 25 °C) was used during all the experimental work.

### 4.2.2 Sample Preparation

Fermented cocoa beans, as well as dried and roasted material, came from Eastern Region of Ghana and were supplied by Barry Callebaut AG. The cocoa pods were stored for four days prior to their manual opening and spontaneous fermentation in heaps. A kilogram of material was sampled every 24 hours of fermentation for a total of seven samples, including unfermented beans. Afterward, they were dried under the sun and shipped. Additionally, the dried fermented beans were roasted and processed into cocoa liquor by Barry Callebaut AG.

All cocoa material was ground with a Knife Mill GrindoMix GM 200 (Retsch, Germany). Samples were stored at 4 °C before further use. For the HPLC-MS analysis of peptides, Amadori compounds, and carbohydrates, about 6 g of the ground powders of each sample were defatted with dichloromethane in an automated Soxhlet extraction apparatus (Büchi B-811, Germany) for 18 hours. The defatted powder was dried under vacuum and stored at 4 °C before further experimental work.

### 4.2.3 Peptide and Amadori Compound HPLC-TOF-ESI-MS Measurements

The extraction of the defatted cocoa powder was carried out according to a previously established protocol (D'Souza et al., 2018). 50 mg of defatted cocoa powder was extracted with an acidified methanolic solution (MeOH:H2O:CH3COOH::70:28:2) by sonication in an ultrasonic bath for 10 minutes and subsequent stirring for 30 minutes. The obtained extract was filtered through a PTFE syringe membrane filter (0.45 micron). Afterward, it was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and immediately used for HPLC-MS experiments.

The detection of peptides via HPLC-MS was adopted from a previously published method (D'Souza et al., 2018), and the detection of Amadori compounds was possible using the same experimental setup (see **Chapter 3**). Poroshell 120 EC-C18 column (RRHD, 2.1 × 100 mm, 2.7 µm particle size) connected to an Agilent 1260 HPLC system was used. Milli-Q water and acetonitrile with the addition of 0.05% of formic acid were used as Solvent A and Solvent B, respectively. The sample injection volume was set to 2 µL, flow rate to 0.4 mL/min (constant), and the column oven temperature to 40 °C. The chromatographic gradient used for the both analyses was as following (t (min), %B): (0, 8); (1, 8); (2.5, 12); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95). The HPLC system was coupled to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (nebulizer pressure of 1.8 bars, the dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). The data were acquired in both positive ion mode and calibrated in HPC mode. Reported NIST monoisotopic atomic masses of the elements (Coursey, Schwab, Tsai, & Dragoset, 2015) were used to calculate monoisotopic molecular masses of detected compounds. To calibrate the TOF analyzer before each sample measurement, 0.1 M sodium formate solution was used. The sum of all measured peaks for each sample was used to calculate the reported relative amounts of compounds. Additionally, normalization by the moisture content of the samples was employed. The moisture content of the dry bean sample was used to normalize the moisture content of the liquor sample as the latter value was unknown. For both peptides and Amadori compounds, two samples were subjected to a series of intra- and interday repetitions (see Supplementary Information).

### 4.2.4 Carbohydrate HILIC-ESI-TOF-MS Measurements

For the extraction of low molecular weight carbohydrates from cocoa, defatted cocoa powder (150 mg) was subjected to two cycles of solid-liquid extraction (SLE) using ethanol 80% as extracting solvent, following the procedure described by (Megías-Pérez, Gamboa-Santos, Soria, Villamiel, & Montilla, 2014). All samples were prepared in duplicate. 1 mL of the sample was filtered through a CHROMAFIL Xtra PTFE-45/25 filter (Macherey-Nagel, (Macherey Nagel, Düren, Germany) and 10 µL of an internal standard solution of 1 mg mL-1 of Asp-Phe methyl ester were added.

Chromatographic analysis was performed using an Agilent 1100 Series HPLC (Agilent Technologies, Karlsruhe, Germany), according to a previous study (Megías-Pérez, Ruiz-Matute, Corno, & Kuhnert, 2019). A BEH X-Bridge column was used, with a trifunctionally-bonded amide phase (Waters Company, USA) and the following characteristics: 150 mm × 3.0 mm; 3.5 µm particle size and 135 Å pore size. Water and acetonitrile with 0.05% ammonium hydroxide were used as solvent A and B, respectively.

An injection volume of 3 µL and a flow rate of 0.4 mL min-1 were used. The HPLC instrument was coupled to a mass spectrometer described below.

The quantitative analysis of carbohydrates was performed using a microTOF mass spectrometer equipped with an ESI source (HCT Ultra, Bruker Daltonics, Bremen, Germany) operating in the positive mode in the range of 50–1200 m/z, according to a previously reported method (Megías-Pérez et al., 2018). 0.1 M sodium formate was used as a calibrant, with a solution injected through a six-port valve before each of the chromatographic runs. The electrospray source parameters were adjusted as follows: spray voltage, 4.5 kV, drying gas (N2, 99.5% purity); temperature = 220 °C; drying gas flow, 12 L/min; nebulizer (N2, 99.5% purity) pressure, 1.6 bar. Data acquisition was performed using HyStar 3.2 software. The specifics of quantification and all analytical parameters are described in detail in a previous study (Megías-Pérez et al., 2018).

### 4.2.5 GC-MS Analysis

For fermented as well as dried cocoa beans, 5 g of grounded and homogenized sample was weighed out in a 20 ml vial. The sample was spiked with 10 µl of a solution of a mix of internal standards (4-heptanone (1,108 µg/µl); 2,3-dimethoxytoluene (0,4249 µg/µl); 13C1-Acetic acid (42,48 µg/µl); 2-methylpentanoic acid (4,206 µg/µl)). After well stirring, a PDMS- coated Twister (Gerstel, Germany) was placed in the headspace of the 20 ml vial filled with grounded cocoa bean sample and closed with a magnetic cap. The vial was stirred for 60 min at a temperature of 70 °C in a heated agitator of MPS 2 (Gerstel, Germany). The sampled Twister was transferred to an empty TDU tube and closed with a transport adapter (TDU, Gerstel, Germany).

All cocoa liquor samples were liquefied at 50 °C. 50 g of melted and homogenized sample was weight in a 400 ml recipient. The sample was spiked with 20 µl of a solution of a mix of internal standards (4-heptanone (1,108 µg/µl); 2,3-dimethoxytoluene (0,4249 µg/µl); 13C1-Acetic acid (42,48 µg/µl); 2-methylpentanoic acid (4,206 µg/µl)). After the stirring, a microvial was filled with 100 µg of cocoa liquor sample and transferred to an empty TDU tube and closed with a transport adapter (TDU, Gerstel, Germany).

A gas chromatograph (GC 7890A Agilent Technologies, Palo Alto, CA) in combination with a mass selective detector (MSD 5975C Agilent Technologies, Palo Alto, CA) was used for analyzing the aroma compounds of the cocoa bean and the cocoa liquor samples. The GC-MS instrument was equipped with a MultiPurpose Sampler (MPS 2, Gerstel, Germany), a thermal desorption unit (TDU, Gerstel Germany), and a PTV inlet (CIS4, Gerstel, Germany). The aroma compounds of cocoa bean samples were desorbed from the sampled Twister by TDU desorption during which the PTV inlet and the TDU were in splitless mode. The TDU temperature started at 70 °C and increased to 250 °C at 60 °C/min (held for 5 min). The desorbed aroma compounds were trapped at -10 °C in the PTV inlet. After cryofocusing, the PTV inlet was heated to 280 °C at a rate of 12 °C/min (held for 20 min). The aroma compounds of cocoa liquor samples were desorbed by TDU desorption during which the PTV inlet was in solvent vent mode, and TDU was in splitless mode. The TDU temperature was set at 70 °C for 20 min. The desorbed aroma compounds were trapped at -10 °C in the PTV inlet. After cryofocusing, the PTV inlet was heated to 280 °C at a rate of 12 °C/min (held for 20 min).

The aroma compounds were separated by using a fused silica capillary column (HP-FFAP 50 m with 0.32 mm internal diameter and 0.50 µm film thickness, Agilent Technologies) preceding by a fused silica precolumn (2 m with 0.53 mm internal diameter). The GC oven temperature was set at 35 °C (held for 5 min) and increased to 240 °C at 5 °C/min (held for

4 min). Helium was used as a carrier gas with a flow rate of 2 ml/min. The MSD was operated at 70 eV in electron ionization (EI) mode. The transfer line was set at 230 °C. In order to improve aroma compound identification, the fragment ions were monitored for each potent aroma compound (PAC) by selected ion monitoring (SIM). The aroma compounds of the cocoa bean and the cocoa liquor samples were identified by comparing the mass spectrum of the aroma compound of the sample with the mass spectrum of a mass spectra database Wiley 275L. Semi-quantification of the aroma compounds was performed by comparing the peak area of the aroma compound to the peak area of the selected internal standard. The concentration of the aroma compounds was expressed in µg/kg or ppb.

## 4.3 Results and Discussion

Our study involved chemical profiling of cocoa beans from the second-largest producer in the world, Ghana. It is known for its distinct fermentation practices, which lead to high-quality well-fermented beans. The profiling involved mapping the most important Maillard precursors and volatiles across the most important industrial processing steps – fermentation, drying, and roasting. Here we present the data for peptides, carbohydrates, and volatiles, including pyrazines. Additionally, it is the first time that recently reported dipeptide Amadori compounds in addition to well-known cocoa Amadori compounds, which were tracked along processing series of cocoa beans. An attempt was made to correlate putative aroma precursors with actual aroma volatiles relative quantities. This dataset can be used as a basis for quality assessment of origin cocoa beans.

### 4.3.1 Peptides

Amino acids and peptides, as compounds playing a major role in cocoa processing, already have been shown to be viable as biomarkers differentiating cocoas based on their origin and processing conditions (Rohsius, Matissek, & Lieberei, 2006; Kumari et al., 2018). Small peptides are produced in the course of fermentation from main cocoa storage proteins such as vicilin and albumin by exo- and endo-peptidases released following a microbial attack on the bean. Gradually proteins are degraded, forming longer peptides early in the fermentation that are successively shortened to provide mainly di- and tri-peptides at the end of the fermentation, with around 800 of these small peptides already unambiguously identified by tandem mass spectrometry recently (D'Souza et al., 2018). In our study, peptide and amino acid quantities were obtained by HPLC-MS relative quantification established using methodology recently introduced (D'Souza et al., 2018). In the case of the latter, our detection methods enabled us only to identify arginine, phenylalanine, and tyrosine. The hydrophobic amino acids phenylalanine and tyrosine are known to be present in Ghanaian cocoa beans in a significant amount (Pätzold & Brückner, 2006; Rohsius et al., 2006), whereas arginine, as a basic amino acid, is easily ionizable, which explains their appearance in our chromatograms. They are detected in comparable amounts to oligopeptides (see **Table 1** in **Supplementary Information**) and have unusual formation pattern along the fermentation. However, they reach maximum concentration after 144 hours of fermentation as expected, then degrade upon drying and increase after roasting (possibly due to Amadori compound degradation or thermally induced peptide hydrolysis). The amino acid composition of different origins was already reported (Rohsius et al., 2006), with Ghana containing a significant amount (between ~14-16 mg/g fat free dry matter, with other origins ranging from 4 to 26), which can point towards greater than average protein content of unfermented bean or most possibly good fermentation practices. Among others, Ghanaian dried cocoa was shown to contain more amino acids compared to its fermented

counterpart (~30% increase), following a drastic drop in concentrations after roasting (Pätzold & Brückner, 2006). In our study, we observe an opposite trend, with a decrease after the drying and increase after roasting; however, in our study, we take into account the moisture content of the samples, which changes drastically after drying. Additionally, a study of Malaysian cocoa beans fermentation showed an increasing trend of total amino acid concentration, which reached a plateau after four days of fermentation finishing with ~13 mg per kg of free amino acids in the sample after six days (Hashim et al., 1998). However, a study on Brazilian cocoa fermentation showed different degradation and formation trends for different amino acids after drying and roasting (de Brito et al., 2001).
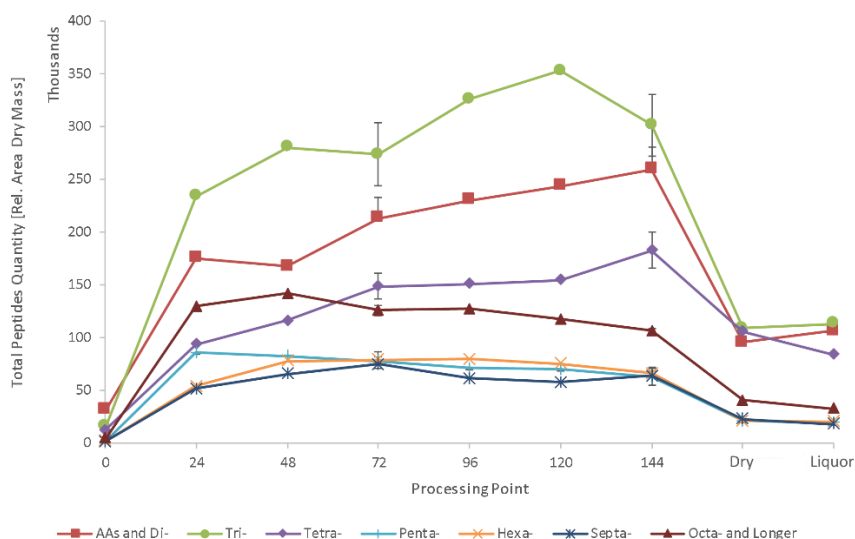


**Figure 1 Relative concentrations of oligopeptides in Ghanaian cocoa beans.**

In our study we have identified over five hundred unique peptides, which is less compared to a previous study on Ivorian cocoa (D'Souza et al., 2018), however it was reported that generally Ivorian total peptide content is lower than the Ghanaian (Marseglia et al., 2014; Caligiani et al., 2016). However, this could be explained by a probable difference in the diversity of peptides for both countries of origin.

In general, we characterize peptide profiles from MS data in two ways, firstly by summation of all peak areas over a certain class of peptides and secondly by a count of the number of all peptides detected in the LC-MS chromatograms. **Figure 1** demonstrates a general trend using the sum of peak areas for peptides according to the amino acid length of all of them across the processing steps of the Ghanaian cocoa beans. The trend shows a quick increase after 24 hours, which can be explained by proteolytic activity that generally is at its peak between second and third day of the fermentation (Hashim et al., 1998) and is caused by the bean death and the release of its proteolytic enzymes (Voigt & Lieberei, 2014). The upward trend immediately slows down and reaches its maximum between 120 and 144 hours of fermentation. At the end of fermentation, the most abundant class of peptides are tripeptides. The concentration of peptides decreases rapidly upon drying and roasting as expected as they are the major participants of the Maillard reaction. Previous studies on Ivorian beans have shown a similar trend. However, some of the smaller peptides started to decrease in quantity after about 96 hours, which is not observed here (D'Souza et al., 2018). Furthermore, the peptide formation trend mirrors the protein degradation trend shown by (Kumari et al., 2016). Additionally, shorter peptides (amino acids, di-, tri-, and tetrapeptides) show a marked increase between 72 and 144 hours fermentation, whereas

longer ones degrade simultaneously, which suggests that protein and peptide degradation is the most crucial process affecting peptide concentration during cocoa fermentation. The trend closely resembled one shown for Ecuador bean fermentation for non-protein nitrogen content (Lerceteau, Rogers, Petiard, & Crouzillat, 1999). As well, the trend after drying and roasting is similar for Brazilian beans (shown as amino-terminal groups) (de Brito et al., 2001). The data indicates good quality of this fermentation series, which is in accordance with general knowledge (N. Camu et al., 2007) and supported by the fact that the pod storage before fermentation slightly increases the activity of both aminopeptidase and carboxypeptidase (Voigt & Lieberei, 2014).

## 4.3.2 Carbohydrates

Carbohydrates are also a class of compounds with predictive capabilities for cocoa origin and processing (Megías-Pérez et al., 2018, 2019). The importance of carbohydrates is given by their participation as the second reaction partner in Maillard reactions. Absolute quantities of 35 dominant low molecular carbohydrates in wet and fermented cocoa beans have been reported recently and surprisingly revealed a rather low level in comparison to other foods characterized by intense Maillard browning. **Table 2** shows values for fructose, glucose (and galactose), inositols, stachyose, melibiose, 1-kestose, and raffinose for unfermented, fermented, and dried cocoa beans. **Figure 2** illustrates their changing concentrations across the whole processing series. An in-depth study of carbohydrates changes during the processing of Ghana beans was not reported yet. However, the values for unfermented and fermented dried beans follows closely the same trend as other origins (Ivory Coast, Malaysia, Indonesia, Brazil, Tanzania, and Ecuador) (Megías-Pérez et al., 2018). Sucrose degrades rapidly across the fermentation series, which results in an initial increase of reducing sugars (T. A. Rohan, 1967; Terence A. Rohan, 1964; Hashim et al., 1998). At the same time fructose degrades slowly and spikes up in concentrations in the dried sample. Differences in glucose and fructose trends, despite the greater initial concentration of the former one, can be either preferential metabolism of glucose or its polymerization during sucrose hydrolysis (Reineccius, Andersen, Kavanagh, & Keeney, 1972). The rest of the measured carbohydrates after the initial spike retains a relatively stable concentration (slight degradation trend, glucose is especially stable). Additionally, the sugar content can be affected by degradation of glycosylate polyphenols during the fermentation (Pettipher, 1986; Wollgast & Anklam, 2000) as well as the release of glycones of various compounds by cocoa glycosidases (their activities seem not to decrease during the fermentation) (Voigt & Lieberei, 2014). In general, Ghanaian beans seem to be on the higher end of carbohydrate content distribution comparing to other origins. Similar trends were reported separately for Malaysian (Hashim et al., 1998) and Brazilian beans,

**Table 1 Absolute quantities of carbohydrates as measured by HILIC-HPLC-ESI-MS.**

| Sugar | Unfermented Beans | Fermented 24h | Fermented 48h | Fermented 72h | Fermented 96h | Fermented 120h | Fermented 144h | Dried Fermented Beans | Cocoa Liquor* |
|---|---|---|---|---|---|---|---|---|---|
| Fructose | 5.25 | 9.20 | 7.06 | 9.61 | 7.16 | 6.73 | 4.91 | 13.44 | n/a |
| Glucose and Galactose | 7.65 | 13.23 | 9.42 | 10.49 | 8.44 | 7.69 | 6.59 | 7.17 | n/a |
| Sucrose | 10.56 | 12.80 | 5.03 | 0.75 | 1.37 | 0.65 | 0.10 | 1.88 | n/a |
| Myo-inositol | 0.81 | 1.04 | 1.42 | 1.34 | 1.12 | 1.13 | 1.13 | 2.09 | n/a |
| Scyllo-inositol | 0.04 | 0.10 | 0.10 | 0.13 | 0.08 | 0.08 | 0.06 | 0.28 | n/a |
| Stachyose | 2.77 | 5.60 | 3.40 | 3.40 | 1.66 | 1.21 | 0.85 | 1.81 | n/a |
| Melibiose | 0.04 | 0.09 | 0.16 | 0.18 | 0.08 | 0.06 | 0.04 | 0.05 | n/a |
| 1-kestose | 0.06 | 0.12 | 0.18 | 0.09 | 0.03 | 0.00 | 0.00 | 0.00 | n/a |
| Raffinose | 0.0007 | 0.0011 | 0.0011 | 0.0005 | 0.0002 | 0.0001 | 0.0001 | 0.0002 | n/a |
| Total | 27.17 | 42.17 | 26.78 | 25.99 | 19.95 | 17.55 | 13.68 | 26.72 | n/a |

All quantities displayed in mg/g of the dry matter

*Liquor data obtained had poor recovery (less than 50%) and data are omitted

which also show rapid degradation trends after the roasting (de Brito et al., 2001). The same degradation trends were shown on Mexican beans, this time with both fermentation (nine timepoints) and drying series (five timepoints) (Rodriguez-Campos et al., 2012). Here, we could not obtain reliable values after the roasting for Ghanaian beans, but the literature reports an expected sudden decrease of total carbohydrate content for Ghana after roasting (Redgwell, Trovato, & Curti, 2003). From the data, it must be concluded that carbohydrates are the rate-limiting factor in cocoa Maillard chemistry due to their lower concentration if compared to amino acids and peptides.
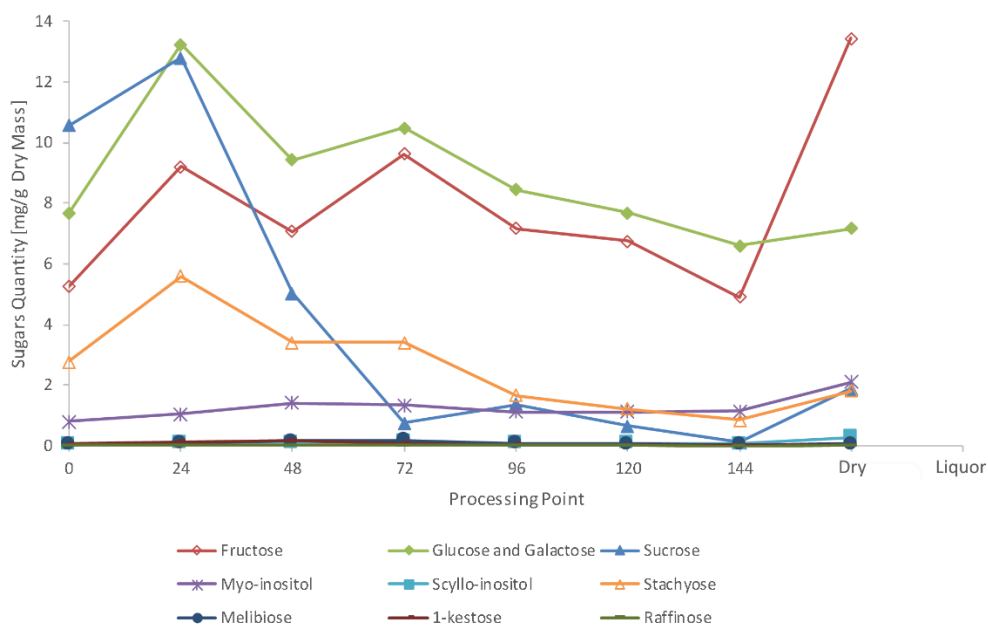


**Figure 2 Quantification of major carbohydrates in Ghanaian cocoa beans.**

### 4.3.3 Amadori Compounds

Amadori and Heyns compounds formed from amino acids and glucose or fructose respectively were located in the LC-MS chromatograms in positive ion mode based on their high-resolution masses and characteristic tandem mass spectra. Amadori and Heyns products could be, for most cases be distinguished based on their characteristic fragmentation and were assigned this way (see **Chapter 3**). Generally, the N-glycosylamines are known for their multiple neutral losses of $H_2O$, $CO_2$, and $H_2CO$ as the most abundant peaks. Additionally, breakage of the bond between sugar carbon and amine nitrogen is commonly producing a protonated amino acid or peptide peak. The distinction between Amadori and Heyns compounds is made based on neutral losses of 96 and 150, which are [M+H-2H2O-CH3OH-CO]$^+$ ion specific for Heyns compounds and [AA-H+CH2]$^+$ ion specific for Amadori compounds. Relative quantification was carried out from extracted ion chromatograms (EICs) created with a mass error of +/- 0.001 Da.

Here, we are the first ever to show a change of a comprehensive series of Amadori compounds (Meitinger et al., 2014), as well as recently reported (see **Chapter 3**) of novel Amadori compounds formed from dipeptides. Relative concentrations are changing across fermentation series, as well as dried and roasted beans from the same origin (Ghana) and batch. It is also an exclusive report on Amadori compound change in any food processing series. Reports on Amadori compounds formed from peptides have been mostly absent from the literature; however, as demonstrated here in cocoa, they have a special significance due to the dominance of short peptides in the fermentation process. Selected Amadori compounds, identified here, were already reported in foods including cocoa (Meitinger et al., 2014), other foods (Eichner, Reutter, & Wittmann, 1993; Sanz, del Castillo, Corzo, & Olano, 2001; Yuan, Sun, Chen, & Wang, 2016), and model systems (Martins, Marcelis, & van Boekel, 2003; Martins & Van Boekel, 2003; Yaylayan, 2003). **Table 2** displays relative quantities of selected Amadori compounds during the whole processing series. The single amino acid Amadori compounds follow an expected trend of very slow increase in concentration till the end of fermentation (see **Figure 3**) as the mild temperature increase resulted from microbial activity can lead to the formation of small quantities of these compounds. The temperature inside the heap in a Ghanaian fermentation was reported to increase slowly to about 45 °C, which was achieved after three days of fermentation (N. Camu et al., 2007) and stayed almost constant afterward. However, while drying the temperature is higher, therefore the greatest concentration of amino acid Amadori compounds is observed after drying. Understandingly, after roasting, their amounts drop significantly, forming MR-related products, almost to the level of the non-fermented bean. Interestingly enough, the dipeptide species reported recently (see **Chapter 3**) follow almost the same trend, but with slightly smaller quantities overall. Taking into the account possibility of the existence of still unidentified species, the dipeptide Amadori compounds must have at least the same influence on Maillard reaction progress as the single amino acid Amadori compounds. This is consistent with the literature, as recently it has been found that the dipeptide Amadori compounds increase the diversity of pyrazines found in Maillard reaction model system (Zou, Liu, Song, & Liu, 2018), which suggests a more important mechanistic influence of dipeptide Amadori compounds on pyrazine formation than just being a source of dicarbonyl reactants. However, more studies need to be performed.

**Table 2 Relative quantities of Amadori compounds as measured by HPLC-MS.**

| ID | Amadori Compound | Unfermented Beans | Fermented 24h | Fermented 48h | Fermented 72h**** | Fermented 96h | Fermented 120h | Fermented 144h**** | Dried Fermented Beans | Cocoa Liquor |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Fru-VP | 0.0000 | 2.9967 | 6.0734 | 5.6074 | 8.5931 | 8.2607 | 8.8924 | 18.7913 | 2.2573 |
| 2 | Fru-FG | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 1.0854 | 0.1011 |
| 3 | Fru-IA | 1.1324 | 1.3990 | 2.5915 | 1.7074 | 2.6056 | 2.6115 | 2.3293 | 4.7797 | 0.7140 |
| 4 | Glc-AY or YA or FS or SF* | 0.0000 | 0.0000 | 0.4901 | 0.5216 | 0.8908 | 0.9716 | 0.8553 | 2.2493 | 0.4901 |
| 5 | Fru-FS | 0.0000 | 0.0000 | 0.0000 | 0.1605 | 0.4863 | 0.6826 | 0.5556 | 1.6399 | 0.4653 |
| 6 | Fru-L(I)G** | 0.0000 | 0.5054 | 0.5006 | 0.6875 | 1.6598 | 1.7919 | 1.4266 | 3.2307 | 0.4141 |
| 7 | Fru-VA | 0.0000 | 0.1839 | 0.2790 | 0.2724 | 0.4848 | 0.3799 | 0.3775 | 0.4851 | 0.1412 |
| 8 | Fru-L(I)P | 0.0000 | 0.0000 | 0.0000 | 1.1416 | 0.0000 | 0.0000 | 0.0000 | 7.1369 | 0.0000 |
| 9 | Fru-L(I)V | 0.0000 | 0.0000 | 0.0000 | 0.5155 | 0.0000 | 0.0000 | 1.2401 | 0.8092 | 0.7660 |
| 10 | Fru-L(I)V or VL(I)** | 0.0000 | 0.0000 | 0.0000 | 0.6402 | 0.0000 | 0.0000 | 1.7862 | 6.7936 | 0.5201 |
| 11 | Glc-WVT* | 0.0000 | 0.0000 | 0.0000 | 0.3002 | 0.0000 | 0.0000 | 0.7830 | 0.9846 | 0.2349 |
| 12, 13 | Fru-TVW**, Glc-TVW* | 0.0000 | 0.0000 | 0.0000 | 0.0022 | 0.0663 | 0.0249 | 0.0000 | 0.7083 | 0.2749 |
| 14 | Fru-IT | 0.0000 | 0.6390 | 0.8046 | 0.8381 | 1.2905 | 1.2804 | 1.3619 | 1.5380 | 0.5688 |
| 15 | Fru-L(I)L(I) | 0.0000 | 0.0000 | 0.0000 | 0.5714 | 0.3456 | 1.0569 | 1.7273 | 4.7724 | 0.5811 |
| 16 | Fru-L(I)E | 0.8058 | 0.0000 | 1.0837 | 1.2236 | 2.7941 | 2.2510 | 3.7507 | 5.4820 | 1.3504 |
| 17 | Fru-FT | 0.0000 | 0.0000 | 0.2326 | 0.2877 | 0.3684 | 0.6875 | 0.6152 | 1.2011 | 0.2161 |
| 18 | Fru-IF** | 0.0000 | 0.0000 | 0.0000 | 0.6436 | 1.3274 | 1.1566 | 1.9969 | 5.4594 | 0.7255 |
| 19 | Fru-IF** | 0.0000 | 0.0000 | 0.0000 | 0.4167 | 0.9188 | 0.7795 | 0.9041 | 2.3172 | 0.2095 |
| 20 | Fru-FL(I) | 0.0000 | 0.0000 | 0.0000 | 0.1739 | 0.0000 | 0.5334 | 0.5963 | 1.6272 | 0.2902 |
| 21 | Fru-FL(I) | 0.0000 | 0.0000 | 0.0000 | 0.0918 | 0.0000 | 0.1085 | 0.2835 | 0.7158 | 0.1927 |
| 22 | Glc-VY* | 0.0000 | 0.5753 | 1.5440 | 1.3327 | 2.4457 | 2.2436 | 2.4692 | 4.7070 | 0.9425 |
| 23 | Fru-FE | 0.0000 | 0.5151 | 1.3197 | 1.0064 | 1.5410 | 1.8523 | 1.8052 | 3.4673 | 0.6770 |
| 24 | Fru-MF | 0.0000 | 0.0000 | 0.0000 | 0.4136 | 0.7214 | 0.7213 | 0.7676 | 1.5688 | 0.1780 |
| 25 | Fru-VS | 0.0000 | 0.0000 | 0.0000 | 0.3962 | 0.8335 | 1.1142 | 0.8634 | 1.9376 | 0.5050 |
| 26 | Fru-L(I)S or VT | 0.0000 | 1.3087 | 4.1946 | 1.6550 | 0.0000 | 0.0000 | 7.0844 | 8.5718 | 0.0000 |
| 27 | Fru-V | 2.3494 | 1.5105 | 1.8862 | 1.0740 | 2.0838 | 2.6678 | 1.7747 | 8.8851 | 1.4670 |

| 28 | Fru-L(I) | 11.1992 | 5.5313 | 7.5190 | 4.0478 | 10.0974 | 9.5875 | 7.2556 | 55.0650 | 10.2914 |
| 29 | Fru-M | 0.0000 | 0.4242 | 0.9151 | 0.3726 | 1.1126 | 1.1830 | 0.8204 | 2.4754 | 0.2428 |
| 30 | Fru-F | 7.2356 | 5.8898 | 9.0470 | 5.7792 | 13.0415 | 12.6000 | 10.2260 | 47.7223 | 7.9366 |
| 31, 32 | Fru-Y***, Fru-Y | 1.7369 | 0.5970 | 1.0275 | 0.6766 | 1.1245 | 1.2073 | 1.1895 | 2.6352 | 0.9652 |
| - | Total | 24.4594 | 22.0759 | 39.5084 | 32.5576 | 54.8327 | 55.7540 | 63.7379 | 208.8428 | 33.7186 |

All numbers represent relative MS peak areas (relative to the sum of all peaks in the sample) additionally normalized by the moisture content

*Heyns-specific peaks are more intensive than Amadori-specific peaks

**There are no either Amadori or Heyns-specific peaks

***Splitting peak with different Amadori and Heyns-specific peaks than its counterpart (compound 32)

Rest have clear Amadori-specific fragmentation peaks that are the only peaks or are bigger than Heyns

****Average of one inter- and three intra-day measurements

ID of the compounds are identical to those in the previous chapter, and some of the isomer relative quantities were summed

Moreover, **Figure 3** shows a general trend of Amadori compounds' precursors during the fermentation. There seems to be no connection between concentrations of particular compounds, which would imply all the degradation and formation reactions occurring at the same time to some extent during the fermentation process.
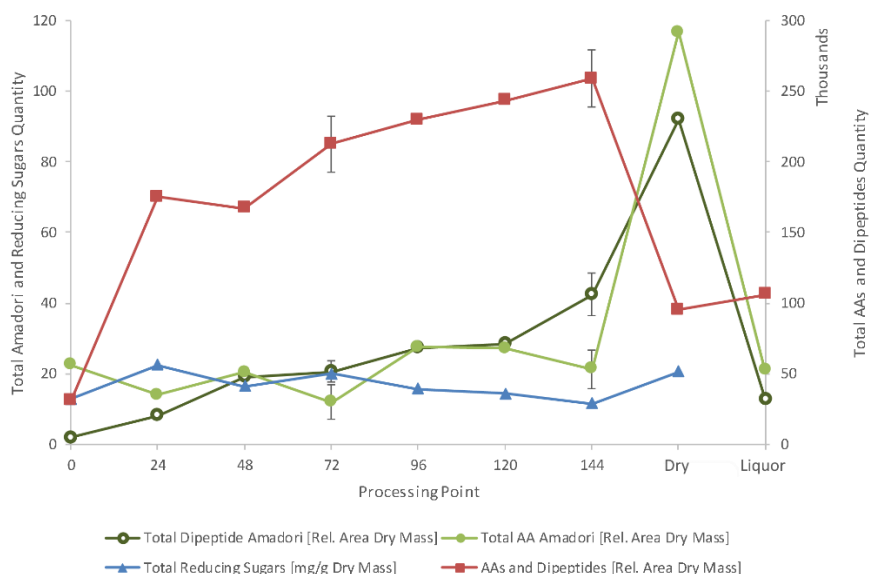


**Figure 3 Amadori compound formation during the processing of Ghanaian beans.**

It is important to note that our data show the formation of Amadori compounds during fermentation, in which due to microbial metabolism temperatures in the fermentation heap increase to 45°-50°C (Lagunes Gálvez, Loiseau, Paredes, Barel, & Guiraud, 2007; Nicholas Camu et al., 2008). At later stages of cocoa processing, Amadori degradation competes with its formation.

### 4.3.4 Aroma Volatiles Including Pyrazines

Amadori compounds described above are the first stable precursors formed in the Maillard reaction, and their chemical transformations during the thermal treatment lead to the formation of colored compounds termed melanoidines as well as many aroma volatiles. Next to Maillard reaction derived aroma volatiles cocoa aroma is defined by further volatiles originating from either plant or microbial metabolism. We have employed headspace GC-MS analysis to profile major volatile compounds over the course of Ghana beans processing (see **Table 3**). The analysis was based on 50 selected key cocoa aroma volatiles, for which authentic standards were available. As expected, the total volatiles trend shows a steady increase over all of the steps of processing and then decreases after drying and roasting (see **Figure 4**). The most significant decrease in total volatiles after drying and roasting (not shown on the graphs) is caused by a considerable loss of acetic acid during the thermal treatment. Thirty-one main volatile compounds responsible for cocoa aroma have been already described in case of unroasted and roasted cocoa beans from Grenada, nineteen of which we report (Frauendorfer & Schieberle, 2008). However, the comparative effect of roasting cannot be determined, as the authors employed extreme under-roasting to preserve the volatiles (95 °C, 14 min.). The only similarity was the increasing pyrazine trend after the roasting. Surprisingly, the amount of ethyl 2-methylbutanoate (fruity note), linalool and phenylacetaldehyde (floral), compounds which are more prominent in high-quality origin

**Table 3 Semi-quantification of volatiles by GC-MS.**

| Aroma Volatile | Aroma | Unfermented Beans | Fermented 24h | Fermented 48h | Fermented 72h | Fermented 96h | Fermented 120h | Fermented 144h | Dried Fermented Beans | Cocoa Liquor* |
|---|---|---|---|---|---|---|---|---|---|---|
| 2-methylbutanal | Cocoa | 1918.76 | 3225.41 | 4611.50 | 5345.85 | 4615.05 | 7550.19 | 3895.59 | 2496.47 | 538.52 |
| 3-methylbutanal | Cocoa | 3303.05 | 6911.95 | 10206.33 | 11580.67 | 9607.87 | 14720.30 | 7062.62 | 6052.46 | 1823.95 |
| 5-methyl-2-phenyl-2-hexenal | Cocoa | 1.22 | 5.24 | 5.46 | 12.99 | 11.24 | 4.52 | 5.52 | 5.72 | 207.00 |
| Trimethylpyrazine | Roasted | 1.98 | 2.49 | 4.26 | 12.42 | 14.97 | 10.48 | 33.64 | 684.55 | 2094.91 |
| 2-ethyl-3,6-dimethylpyrazine | Roasted | 0.43 | 0.56 | 0.53 | 1.07 | 1.83 | 0.51 | 1.59 | 6.26 | 76.90 |
| 2-ethyl-3,5-dimethylpyrazine | Roasted | 0.89 | 0.99 | 0.81 | 1.89 | 1.80 | 0.81 | 2.52 | 53.01 | 224.40 |
| Acetic acid | Acidic | 4674664.63 | 3903938.45 | 5048983.48 | 5680371.84 | 5231029.99 | 5575164.33 | 6460540.22 | 3829186.14 | 586230.75 |
| Ethyl 2-methylpropanoate | Fruity | 3.50 | 10.46 | 12.53 | 18.00 | 12.90 | 5.44 | 1.62 | 6.51 | 1.20 |
| 2,3-butanedione | Fruity | 94.95 | 1113.65 | 1046.94 | 1411.32 | 1882.27 | 1072.35 | 669.57 | 2281.95 | 770.44 |
| Methyl 3-methylbutanoate | Fruity | 2.85 | 8.05 | 11.31 | 29.02 | 60.50 | 60.42 | 59.85 | 31.43 | 4.81 |
| Ethyl 2-methylbutanoate | Fruity | 10.39 | 40.08 | 56.43 | 48.76 | 42.42 | 31.01 | 16.67 | 65.97 | 17.33 |
| Ethyl 3-methylbutanoate | Fruity | 26.99 | 91.93 | 101.71 | 74.08 | 60.05 | 46.31 | 25.32 | 86.64 | 24.52 |
| γ-nonalactone | Fruity | 7.92 | 33.57 | 18.24 | 25.92 | 81.93 | 45.51 | 24.81 | 106.84 | 13.13 |
| 2-octen-d-lactone | Fruity | 0.00 | 2.30 | 2.70 | 4.41 | 9.86 | 3.66 | 6.34 | 9.06 | 30.77 |
| Massoya lactone | Fruity | 0.00 | 2.76 | 1.33 | 1.20 | 8.85 | 3.44 | 0.82 | 1.55 | 17.55 |
| Furaneol | Fruity | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 39.63 | 260.76 |
| Ethyl-3-phenylpropanoate | Fruity | 0.72 | 2.94 | 3.94 | 4.01 | 3.59 | 2.68 | 8.47 | 21.44 | 46.79 |
| Linalool | Floral | 461.73 | 201.88 | 278.67 | 340.86 | 253.42 | 176.32 | 481.14 | 277.27 | 363.01 |
| Phenylacetaldehyde | Floral | 266.26 | 775.80 | 1082.97 | 1922.31 | 1403.68 | 721.58 | 2002.50 | 132.94 | 87.60 |
| Ethyl phenylacetate | Floral | 10.43 | 12.22 | 20.62 | 13.80 | 8.30 | 3.95 | 8.95 | 31.05 | 62.32 |
| 2-phenylethyl acetate | Floral | 98.00 | 90.43 | 165.59 | 199.06 | 195.03 | 229.47 | 432.97 | 427.55 | 2127.37 |
| β-damascenone | Floral | 0.00 | 0.04 | 0.05 | 0.11 | 0.09 | 0.06 | 0.19 | 0.10 | 0.47 |
| 2-phenylethanol | Floral | 240.88 | 156.99 | 254.35 | 400.05 | 434.14 | 594.61 | 2144.72 | 1419.99 | 2223.63 |

| Compound | Descriptor | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Ethylcinnamate | Cinnamon-like, spicy | 0.00 | 1.15 | 1.82 | 3.95 | 1.59 | 0.81 | 4.53 | 2.95 | 15.43 |
| Vanillin | Vanilla-like | 0.00 | 1.19 | 1.76 | 4.71 | 7.29 | 3.40 | 7.02 | 30.48 | 51.27 |
| Dimethyl disulfide | Cabbage, meaty | 9.37 | 19.27 | 33.88 | 29.46 | 21.06 | 35.66 | 40.47 | 65.92 | 115.78 |
| Dimethyltrisulfide | Cabbage, meaty | 10.88 | 16.21 | 40.77 | 23.48 | 13.75 | 46.34 | 54.06 | 18.67 | 16.32 |
| 2-methyl-3-(methyldithio)-furan | Cabbage, meaty | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.56 |
| 2-methoxyphenol | Smoky, hammy | 2.64 | 4.31 | 6.80 | 31.44 | 42.38 | 23.31 | 141.49 | 142.90 | 290.43 |
| 4-methyl-2-methoxyphenol | Smoky, hammy | 0.00 | 0.56 | 0.66 | 1.86 | 1.55 | 1.24 | 18.41 | 4.65 | 16.77 |
| 3-methylphenol | Smoky, hammy | 3.48 | 4.84 | 2.62 | 5.05 | 5.65 | 2.45 | 4.59 | 13.65 | n/a |
| 4-methylphenol | Smoky, hammy | 0.00 | 3.06 | 1.71 | 3.45 | 4.16 | 1.59 | 3.04 | 5.47 | 19.87 |
| 4-ethylphenol | Smoky, hammy | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 2,6-dimethoxyphenol | Smoky, hammy | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3-isopropyl-2-methoxypyrazine | Musty, earthy | 0.56 | 0.50 | 0.67 | 0.83 | 0.67 | 0.30 | 0.79 | 0.47 | 0.61 |
| 3-isobutyl-2-methoxypyrazine | Musty, earthy | 1.13 | 0.84 | 1.45 | 2.18 | 1.38 | 0.64 | 2.04 | 1.68 | 3.43 |
| E-2-nonenal | Paper, cardboard-like | 7.78 | 32.82 | 18.03 | 15.23 | 60.09 | 79.98 | 16.01 | 106.88 | 81.79 |
| 2-methylpropanoic acid | Sweaty, cheesy | 155.42 | 484.98 | 860.04 | 5282.24 | 8682.40 | 5373.52 | 16849.37 | 12961.37 | 15261.82 |
| 3-methylbutanoic acid | Sweaty, cheesy | 232.32 | 903.38 | 1583.66 | 5208.34 | 8738.96 | 5950.07 | 21162.44 | 12887.98 | 13128.66 |
| 2-methylbutanoic acid | Sweaty, cheesy | 100.64 | 347.46 | 789.22 | 4027.68 | 6186.53 | 5092.96 | 19713.21 | 11611.34 | 12043.87 |
| Butanoic acid | Sweaty, cheesy | 162.44 | 172.30 | 139.42 | 252.15 | 408.20 | 229.08 | 336.38 | 744.07 | 848.82 |
| Skatol | Animal, fecal-like | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Total (without acetic acid) | - | 7137.59 | 14682.62 | 21368.78 | 36339.85 | 42885.48 | 42124.97 | 75239.30 | 52836.88 | 52912.80 |

All quantities displayed in µg/kg of the dry matter

*Moisture content not available for liquor sample, same moisture content as dry bean assumed for calculations

samples (such as from South America), was higher in Ghanaian beans. A study on four different hybrids from Brazil across processing series (unfermented, fermented, and chocolate samples) shown significant relative differences in acids, alcohols, aldehydes, ketones, and esters (Moreira et al., 2018). However, the variation decreased after processing into chocolate. Another study focusing on Mexican beans and drying practices showed that significant changes in volatiles, especially pyrazines, can be achieved by tuning the drying temperature (Rodriguez-Campos et al., 2012). Additionally, such important aroma volatiles as acetic acid, 3-methylbutanal (cocoa note), ethyl acetate (fruity), 2-phenylethyl acetate, and 2-phenylethanol (floral) responded differently to various drying conditions and could be optimized.
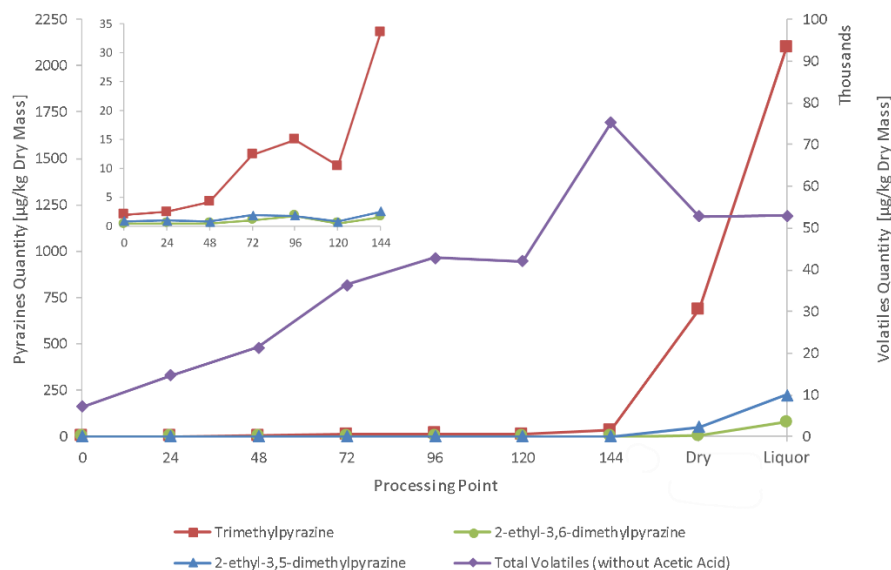


**Figure 4 Total volatiles (without acetic acid) and pyrazine trends over the course of Ghanaian beans processing.**

**Figure 5** shows the general trends of different classes of volatile compounds based on their aroma. Cocoa notes reported here are exclusively aldehydes, which form mainly by Strecker degradation of amino acids and can form slowly during fermentation (Aprotosoaie, Luca, & Miron, 2016). They are essential substrates for the formation of heterocyclic compounds, which can explain their fast decline after drying and roasting. Fruity and floral compounds mainly consist of esters, lactones, and alcohols. Esters are an important class of compounds that arise from amino acids (Aprotosoaie et al., 2016). Alcohols are a result of microbial activity, but can also form via thermal degradation of amino acids. In general, higher temperatures can decrease concentrations of all these compounds. Despite this, the floral compounds increase after the roasting because of the cumulative effect of 2-phenylethylacetate and 2-phenylethylethanol.
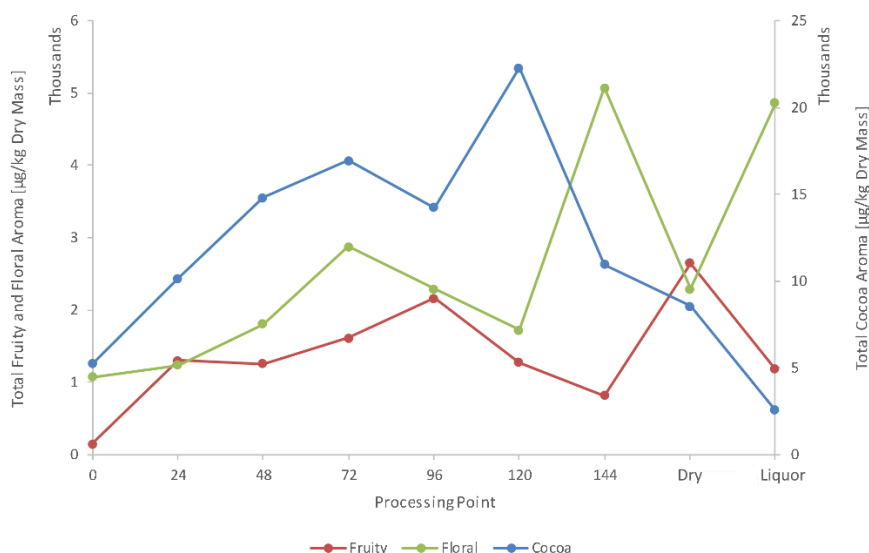
**Figure 5 General trends of volatiles present in Ghanaian cocoa beans with different aroma characteristics.**

**Figure 4** shows the formation trends of three key pyrazines: trimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine. Pyrazines are well-known desirable aroma compounds associated with thermal processing that are important products of Maillard reaction. However, they can form during fermentation as a result of microbial activity as well, and were found in vegetables most likely because of symbiotic relationships of microorganisms living inside root tissues of various plants (Rizzi, 1988). This could explain their presence already in unfermented cocoa beans (see **Table 3** and **Figure 4**). Additionally, the substitution pattern of pyrazines in microbial synthesis is affected by the type of amino acid used, and fructose yields more pyrazines than glucose (Ito, Sugawara, Miyanohara, Sakurai, & Odagiri, 1989; Yamaguchi et al., 1993). Some bacteria can use pyrazine as a sole energy and carbon source (Müller & Rappert, 2010). Still, only negligible amounts of pyrazines are formed during the fermentation, and almost all of them are formed during both drying and roasting, which is highlighted by the smaller graph in **Figure 4**. To our current knowledge, only one study was published considering pyrazine concentration changes during roasting of Ghanaian cocoa beans (Reineccius, Keeney, & Weissberger, 1972). The levels of trimethylpyrazine, the main pyrazine in our study, was a factor of 10 less than the value we report (between 110 and 170 µg/kg in three lots). The exact method of roasting is not described, and it is not known if our industrially processed samples can be compared. Fermentation status was not reported as well, and it was shown that both presence of dipeptide Amadori compounds (Zou et al., 2018) and quantity of dipeptides (Scalone, Cucu, De Kimpe, & De Meulenaer, 2015) in model systems influence pyrazine diversity and quantity, respectively. The investigation presents numbers in the same range for roasted cocoas from Brazil, Samoa, Ecuador, Mexico, and the Dominican Republic. On the other hand, another study on Malaysian cocoa reports that trimethylpyrazine reaches almost the same concentration as we report after four days of fermentation (Hashim et al., 1998). It is open to speculation whether depending on origin and its microbial population, it is possible to yield pyrazine quantities during fermentation over a factor of 50 higher, and it might be a result of other factors.

### 4.3.5 Correlation Analysis

A correlation matrix was prepared using a Spearman rank correlation for selected important components of this study (see **Table 4**) to confirm our assumptions regarding Amadori compounds. An analogous approach was applied to employ Pearson correlation (see **Supplementary Information**). Both methods demonstrate similar results; however, Spearman correlation coefficients are greater as it is a rank correlation used to show non-linear relationships between variables.

Although both single amino acid and oligopeptide Amadori compounds negatively correlate well with their sugar and peptide precursors, surprisingly the former correlate better with fructose and glucose, and the latter with sucrose. The reason for this disparity is unknown. Additionally, oligopeptide Amadori compounds (and to a lesser extent their precursors) display an exceptional positive correlation with aroma volatiles such as pyrazines (roasted notes represented in **Table 4**) and Strecker aldehydes (cocoa notes). This is consistent with the literature, as it was recently shown that Maillard model systems containing dipeptides and sugars produce more variety of pyrazines than their single amino acid equivalents (Zou et al., 2018). These results suggest some involvement of these species in the formation of Strecker aldehydes as well. Additionally, dipeptide Amadori compounds and peptides correlate positively with floral aroma volatiles across fermentation, which are mainly 2-phenylethanol and 2-phenylethyl acetate. These compounds are related to the microbial production of alcohols and subsequent esterification by acetic acid present in the fermenting heap. Furthermore, monosaccharides seem to be good indicators for the floral aroma potential. All these compounds appear to be promising biomarkers for the most critical aroma components of fermented cocoa beans.

**Table 4 Correlation matrix performed using Spearman rank correlation.**

| | Fructose | Glucose and Galactose | Sucrose | Σ Carbohydrates | Σ Peptides | Σ AA Amadori | Σ Dipeptide Amadori | Σ Amadori | Σ Roasted | Σ Cocoa | Σ Fruity | Σ Floral | Σ Volatiles (w/o Acetic Acid) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fructose | 1.00 | | | | | | | | | | | | |
| Glucose and Galactose | 0.93 | 1.00 | | | | | | | | | | | |
| Sucrose | 0.36 | 0.54 | 1.00 | | | | | | | | | | |
| Σ Carbohydrates | 0.43 | 0.61 | 0.96 | 1.00 | | | | | | | | | |
| Σ Peptides | -0.21 | -0.36 | -0.89 | -0.93 | 1.00 | | | | | | | | |
| Σ AA Amadori | -0.50 | -0.61 | -0.21 | -0.43 | 0.39 | 1.00 | | | | | | | |
| Σ Dipeptide Amadori | -0.29 | -0.43 | -0.93 | -0.96 | 0.96 | 0.32 | 1.00 | | | | | | |
| Σ Amadori | -0.54 | -0.64 | -0.89 | -0.96 | 0.89 | 0.50 | 0.93 | 1.00 | | | | | |
| Σ Roasted | -0.07 | -0.29 | -0.82 | -0.86 | 0.79 | 0.18 | 0.89 | 0.79 | 1.00 | | | | |
| Σ Cocoa | 0.29 | 0.18 | -0.57 | -0.50 | 0.68 | 0.00 | 0.54 | 0.46 | 0.39 | 1.00 | | | |
| Σ Fruity | 0.82 | 0.64 | 0.00 | -0.04 | 0.25 | -0.04 | 0.18 | -0.07 | 0.36 | 0.43 | 1.00 | | |
| Σ Floral | 0.04 | -0.14 | -0.75 | -0.71 | 0.61 | -0.14 | 0.75 | 0.64 | 0.93 | 0.39 | 0.29 | 1.00 | |
| Σ Volatiles (w/o Acetic Acid) | -0.21 | -0.39 | -0.86 | -0.93 | 0.89 | 0.36 | 0.96 | 0.89 | 0.96 | 0.43 | 0.29 | 0.82 | 1.00 |

## 4.4 Conclusions

Here, for the first time, we report a chemical profile of Ghanaian cocoa beans processing series for the same material with respect to important Maillard reaction-related compounds, which include peptides, sugars, and aroma volatiles. As well, for the first time, we show the change of single amino acids and dipeptides to form Amadori compounds across processing series. Most importantly, we show that the formation of aroma precursors is based on the Maillard reaction between peptides and carbohydrates occurring during elevated temperatures in the process of fermentation. The concentration of actual aroma volatiles, such as pyrazines and Strecker aldehydes, correlates to the relative amount of Amadori products as well as their precursors formed in fermentation. Surprisingly, novel oligopeptide Amadori compounds are correlated better with their precursors and important aroma volatiles. Hence, profiles of such compounds could be useful in the assessment of the quality of processing. However, expansions for other origins is necessary.

## Conflict of interest statement

The authors of this research paper receive research funding from Barry Callebaut. The terms of this arrangement have been reviewed and approved by the Jacobs University Bremen in accordance with its policy on objectivity in research.

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# Chapter 5

# HPLC-MS-based Design of Experiments Approach on Cocoa Roasting

Paweł J. Andruszkiewicz, Marcello Corno, and Nikolai Kuhnert

*Note:* Contents of this chapter are in the publication process at the time of publication of this thesis, and will be available online in the future. The **Supplementary Information** for this chapter is available in **Appendix B**.

## Abstract

Modern statistical methods, such as the design of experiments and response surface methodology, are widely used to describe changes in multiparameter processes during the processing of food in both science and technology contexts. However, these approaches are described to a lesser degree in the case of cocoa roasting than other foods and processes. Our study aimed to use the design of experiments to establish a model of cocoa roasting. We have used HPLC-MS techniques to link standard process parameters with chemical compounds changing in concentration during cocoa roasting. Influence of time, temperature, the addition of water, acid, and base, on relative concentrations of procyanidin monomers, dimers, and trimers, an Amadori compounds, and a peptide, was shown. High-quality models for each compound were established and validated, displaying good prediction accuracy.

## 5.1 Introduction

Roasting of cocoa beans and thermal processing of food, in general, have a much longer history must be considered mostly an empirically driven process. It is a common knowledge that applying different roasting conditions results in varying outcomes in terms of organoleptic properties, and these conditions were frequently optimized via simple methods such as "one-factor-at-a-time" (OFAT). Even though this method is still used to some degree, nowadays modern statistical techniques, such as the design of experiments (DoE) and response surface methodology (RSM) are becoming dominant in other fields of science and engineering. Both of these combined can provide enormous help with minimizing the number of experiments to be performed and maximizing the information output at the same time. Moreover, they are more and more used to describe processes both in food science and technology (Granato & de Araújo Calado, 2013). The benefits of these approaches were quickly recognized by, e.g. synthetic chemists, who no longer had to waste time optimizing their reaction yields by the OFAT approach (Leardi, 2009). The RSM proved to be very useful in analytical chemistry (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008), even in case of development and optimization of mass spectrometric methods (Hecht, Oberg, & Muddiman, 2016). It could also be applied to optimize extraction conditions in metabolomic studies (Gullberg, Jonsson, Nordström, Sjöström, & Moritz, 2004). Metabolomics can profit greatly from the design of experiments approach

as they often involve work on big data sets with many variables involved (Jacyna, Kordalewska, & Markuszewski, 2019), such as LC-MS based metabolomics (Eliasson etal., 2012). The same reasons make the response surface methodology a valuable tool to improve the industrial food processing (Yolmeh & Jafari, 2017). For example, it has been used during the optimization of roasting of various nuts to enhance their organoleptic properties (Özdemir & Devres, 2000; Kahyaoglu, 2008). On the other hand, the design of the experiments approach proved useful in the reduction of acrylamide formation, e.g. in bread, fries, and model systems (Bråthen & Knutsen, 2005; Mestdagh et al., 2008).

In this study, we focused on HPLC-MS assisted DoE approach to cocoa roasting. We explored how commonly used industrial roasting conditions influence the changes in the chemical composition of cocoa. So far, statistical approaches were not applied to cocoa roasting to the same degree as in the case of other foods and processes. However, among others, response surface methodology was used to explore impact of roasting conditions on sensory properties of chocolate (Rocha, Santana, Soares, & Bispo, 2017) and to optimize processing conditions towards maximum antioxidant activity and polyphenol content (Zzaman, Bhat, & Yang, 2014; Gültekin-Özgüven, Berktaş, & Özçelik, 2016). The literature on other cocoa processing steps optimization and modeling seems to be richer, especially in the case of the fermentation (Moreno-Zambrano, Grimbs, Ullrich, & Hütt, 2018; John et al., 2019). We believe that combining these approaches could not only help to optimize process parameters but as well shed some light on chemical transformations mechanisms during chocolate production.

## 5.2 Material and Methods

### 5.2.1 Chemicals and Reagents

Acetone, HPLC-grade acetonitrile, HPLC-grade isopropanol, HPLC-grade methanol, and dichloromethane were acquired from Carl Roth (Germany). Acetic acid, citric acid, formic acid, hesperetin, potassium carbonate, and sodium hydroxide were acquired from Sigma-Aldrich (Germany). Milli-Q water (18.2 MΩ•cm at 25 °C) was used during all the experimental work.

### 5.2.2 Nib Preparation

A 25 kg bag of fermented and dried cocoa beans from the Ivory Coast was supplied by Barry Callebaut AG. There was no additional contextual information provided for this sample.

A batch of approximately 200 g of the cocoa beans was crushed using a small-scale cocoa bean crusher. A produced mixture of crushed beans (cocoa nibs) and shells was separated using a small-scale winnower. The winnowing process was repeated until the nibs were completely separated. The whole process was repeated until approximately 3.5 kg of cocoa nibs were obtained, which were kept in the fridge (4 °C) until further processing.

### 5.2.3 Experimental Design and Validation

We have recently demonstrated the success of the DoE approach to cocoa fermentation (John et al., 2020) and decided to apply a similar methodology to cocoa bean roasting. The whole process of design of experiments approach was done utilizing the MODDE Pro 11 software (MKS Umetrics AB, Sweden). The roasting model was designed to include

temperature, time, humidity (water addition), and additives modifying pH (citric acid and potassium carbonate addition) as quantitative factors (see **Table 1** for respective factors and their ranges). The additive factor combined both acid and base additives into one scale, with negatives values being acid concentration, positive being base concentration, and "0" being roasting without additives. Six constraints were defined for the model (see **Figure 1**). In principle the model applies to all cocoa constituents detectable by mass spectrometry(Milev, Patras, Dittmar, Vrancken, & Kuhnert, 2014), however, only five representative responses were chosen to be studied here, each being a concentration (expressed as relative areas) of a compound measured by HPLC-MS: epicatechin, procyanidin B-dimer, procyanidin B-trimer, an Amadori compound Fru-Phe, and a peptide VP. The first three compounds were reported as sums of concentrations of all their isomers. The model for each response employed multiple linear regression (MLR) and was orthogonally scaled. Initially, twenty-three model terms were included as a part of each model. The optimization (RSM) was directly selected as the objective of the design. The design space was explored using D-optimal design, and a model with the highest G-efficiency (parameter representing the variance of predicted values) (*MODDE 11 User Guide*, 2015) was selected from the ones generated by the software (see **Figure 2**).

After performing sixty experiments designated by the software (as described in **Section 5.2.4**.), the models for each of the responses were refined by transforming the models and removing unnecessary model terms, in order to improve their fitting (measured by R2, Q2, validity, and reproducibility parameters, see **Figure 3**) (*MODDE 11 User Guide*, 2015).

The refined models were validated using the optimizer function of the software. One objective per response was chosen (see **Table 2**), and the software generated the factor values to achieve a given objective, including the respective 95% confidence intervals. The roasting experiments were performed in the same manner as the rest of the experiments (as described below, **Section 2.4**.).

### 5.2.4 Model Roasting

The cocoa nibs were subjected to 60 model roasting experiments generated by the MODDE 11 Pro software according to the design. Approximately 50 g of cocoa nibs were placed on a metal tray with a large surface area to ensure uniform heat transfer and easier moisture evaporation during the roasting. Depending on the experiment, water, or water and an additive could be added to the tray and mixed thoroughly until homogenized. Additives (citric acid or potassium carbonate) were dissolved completely in water beforehand. The samples were roasted in a laboratory forced draft oven for a time and at a temperature specified by the software. Afterward, they were removed from the oven and left to cool down in a desiccator at room temperature. Roasted nibs were stored in a fridge at 4 °C until further use.

### 5.2.5 Defatting and Extraction

For each of the 60 samples, approximately 30 g of roasted cocoa nibs were ground to a fine powder at 10,000 rpm using a knife mill (Retsch Grindomix GM200, Germany). It was stored in a fridge at 4 °C before further use. About 6 g of the ground powder was defatted with dichloromethane in an automated Soxhlet extraction apparatus (Büchi B-811, Germany) for 18 hours. The defatted powder was dried under vacuum and stored at 4 °C before further experimental work.

The extraction of the defatted cocoa powder was carried out according to a previously established protocol (D'Souza et al., 2018). 50 mg of defatted cocoa powder was extracted with an acidified methanolic solution (MeOH:H2O:CH3COOH::70:28:2) by sonication in an ultrasonic bath for 10 minutes and subsequent stirring for 30 minutes. The obtained extract was filtered through a PTFE syringe membrane filter (0.45 micron). Afterward, it was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and immediately used for HPLC-MS experiments.

### 5.2.6 HPLC-TOF-MS-MS Measurements

HPLC conditions were adopted from a previously published method (D'Souza et al., 2018). Agilent 1260 HPLC system equipped with a ZORBAX Eclipse Plus C-18 column was used. Milli-Q water and acetonitrile with the addition of 0.05% of formic acid were used as Solvent A and Solvent B, respectively. The sample injection volume was set to 2 μL, constant flow rate to 0.4 mL/min, and the column oven temperature to 40 °C. The chromatographic gradient used for the analysis was the following: (t (min), %B): (0, 8); (1, 5); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95). The HPLC system was coupled to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (nebulizer pressure of 1.8 bars, the dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). The data were acquired in both positive and negative ion mode and calibrated in HPC mode. Reported NIST monoisotopic atomic masses of the elements (Coursey, Schwab, Tsai, & Dragoset, 2015) were used to calculate monoisotopic molecular masses of detected compounds. 0.1 M sodium formate solution was used to calibrate the TOF analyzer before each sample measurement. The sum of all measured peaks for each sample was used to calculate the reported relative amounts of compounds. The relative concentration of each compound was calculated as a ratio of respective MS peak area to the sum of all peaks within the sample. In the case of epicatechin specifically, the relative amount is the sum of all the isomers with the same mass. The rest of the reported compounds are only single isomers.

## 5.3 Results and Discussion

The roasting of cocoa beans for chocolate production was always an empirical process, with parameters optimized by trial and error over the centuries. Scientific knowledge on roasting, its products, and the influence of process parameters on cocoa quality is progressing; however, the literature focusing on the prediction of quantitative values is scarce (Zzaman et al., 2014; Rocha et al., 2017). It is surprising as the literature on the design of experiments on food and its processing is much more abundant and considered important to the industry (Granato & Ares, 2014).

Here we present a design of experiments approach applied to cocoa roasting, which in principle can be used both to optimize roasting conditions, as well as for mechanistic investigations of the roasting chemistry itself. Additionally, such studies could be valuable as a part of a larger predictive model, for example when coupled with similar approaches to cocoa fermentation (John et al., 2019).

The design of experiments process can be divided into three phases (design, analysis, and prediction) and will be described here as such.

**Table 1 Factors took into the account in the cocoa roasting design of experiments approach.**

| Name | Abbr. | Units | Type | Settings | Transform | Precision |
|------|-------|-------|------|----------|-----------|-----------|
| Temperature | Temp | °C | Quantitative | 90 to 180 | None | 1 |
| Time | t | min. | Quantitative | 20 to 180 | None | 1 |
| Humidity | Hum | ml | Quantitative | 0 to 50 | None | 1.25 |
| Additive | Add | % of cocoa weight | Quantitative | -5 to 5 | None | 0.25 |

### 5.3.1 Design Phase

Typically, to model any process, in-depth knowledge of any parameters that can influence it is required. In order to do that, a separate screening design with a wide range of factors is built (*MODDE 11 User Guide*, 2015). However, as we mentioned, cocoa roasting is a long-established process, with its main parameters already known (Beckett, 2009). Therefore, we proceeded directly with an RSM (response surface methodology) optimization. **Table 1** shows factors taken into the account in case of our roasting model, which were: time, temperature, humidity (addition of water), and additives (addition of either citric acid or potassium carbonate).
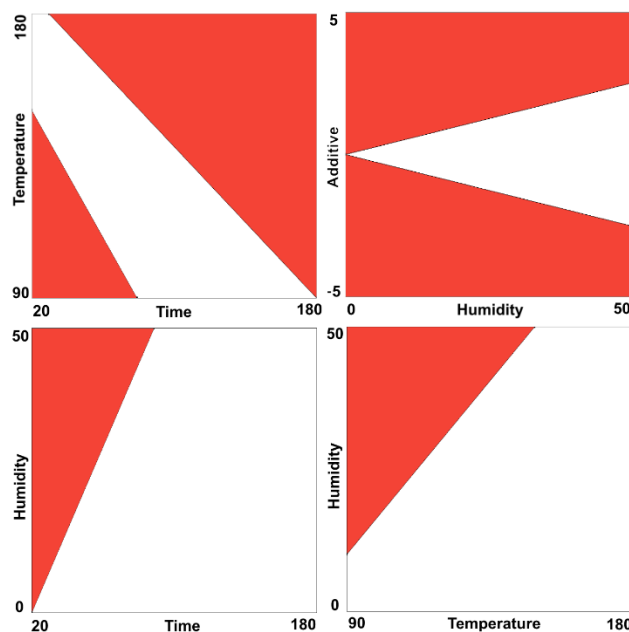


**Figure 1. Graphical representation of constraints applied to the design.**
Red area illustrates values excluded from the design space (white area).

These, except the acid addition, are the basic parameters commonly altered to produce different outcomes during the industrial roasting. Parameters used here include extreme under- und over-roasting boundary values in industrial roasting, which typically operates around 120 °C for 60-80 minutes. Additionally, there was a set of constraints imposed on the model to avoid any technical difficulties during the roasting (see **Figure 1**). The additive vs. humidity, to avoid the addition of either acid or base without addition of any water, and other humidity constraints promoted the water to be fully evaporated before the ending of the roasting (as in an industrial process). For this model, five responses were chosen to be studied by HPLC-MS: epicatechin (and its isomers), procyanidin B-dimer, procyanidin B-trimer, an Amadori compound Fru-Phe (fructosylphenylalanine), and a peptide VP. Each of them represents a class of compounds commonly known to participate in chemical
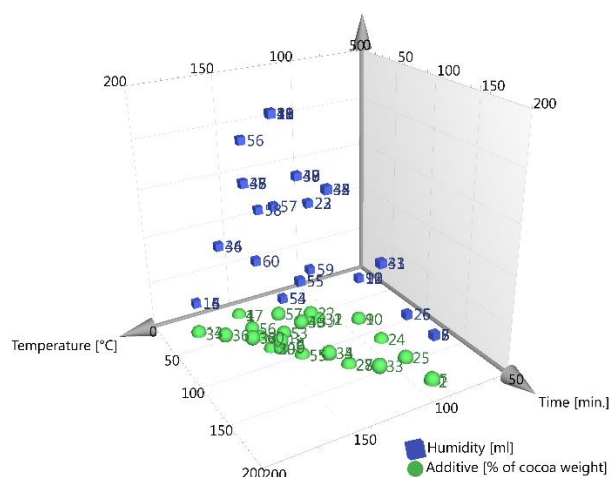
**Figure 2 Graphical representation of the design space.**

transformations during the cocoa roasting. The model for each response was based on multiple linear regression (MLR) and was orthogonally scaled. To maximize the predictive capabilities of the model, we added the maximum amount of model terms up to order of three, including second-order interactions between the factors to the model terms (coefficients), twenty-three in total. This included first-, second-, and third-order terms for each of the factors (number of the order indicates the number of factors taken into account in the coefficient, e.g., time*time*time is a third-order term). After all the parameters of the design were established, a list of possible models was generated using the D-optimal method. From the available designs, the one with the highest G-efficiency (quality parameter) was selected. A graph of all the experiments generated within the chosen design, considering all the factors, constraints, and design parameters, is shown in **Figure 2**. All the sixty roasting experiments were performed, and the results were investigated in the analysis phase.
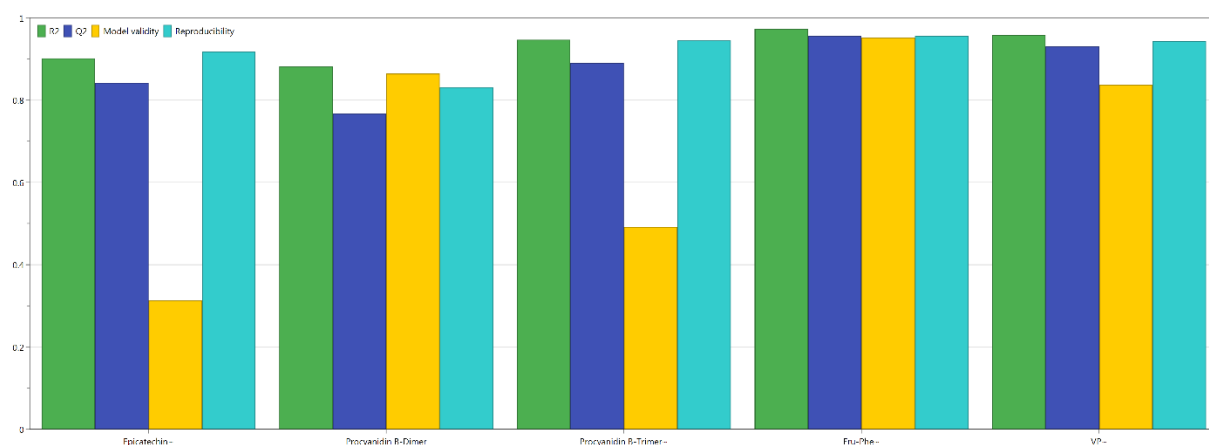


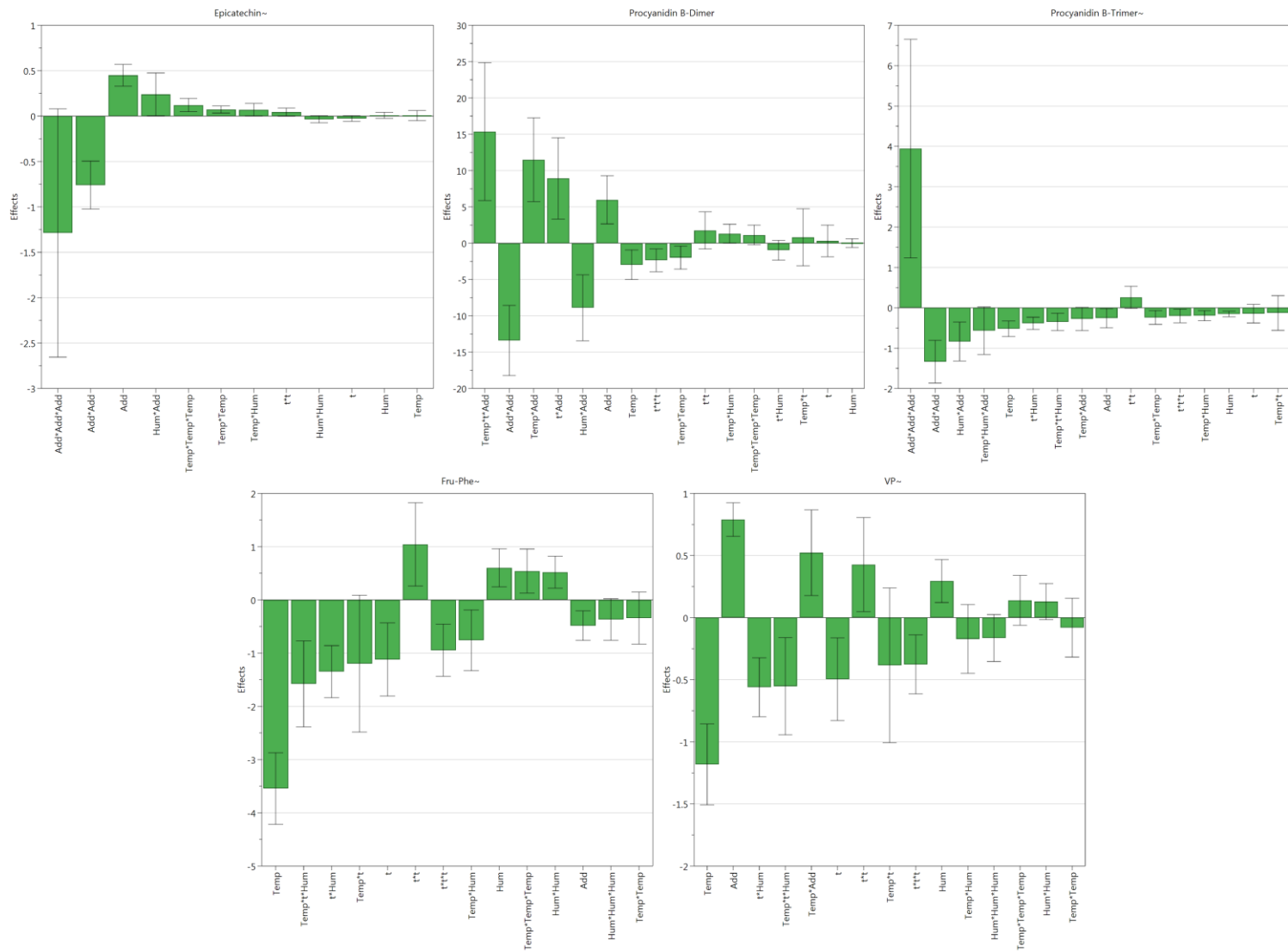**Figure 3 Model statistics obtained for the responses.**
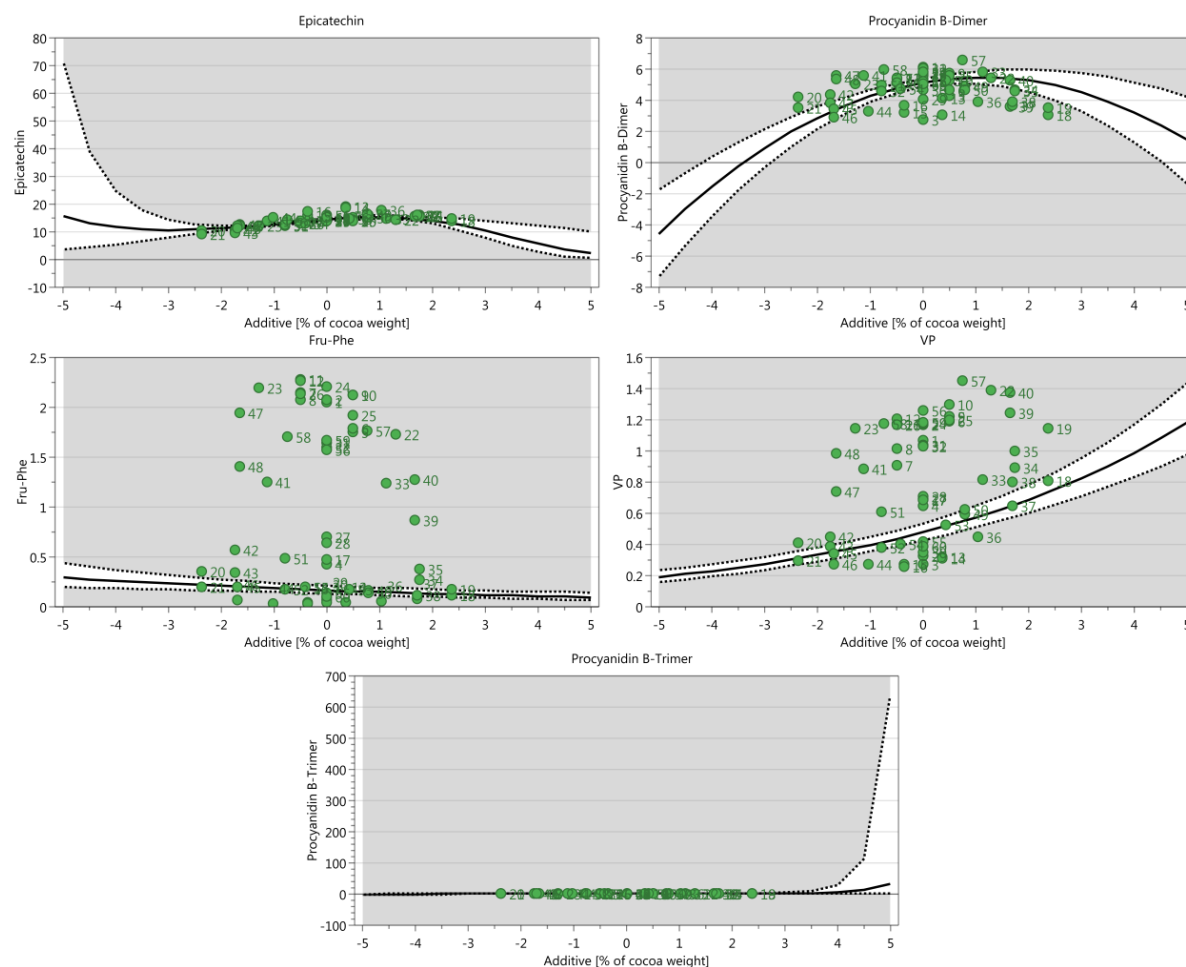
**Figure 4 The coefficients plot.**

**Figure 5 Factor effects plot for additives response.**

### 5.3.2 Analysis Phase

The HPLC-MS measurements provide the input for the responses, based on which the actual mathematical model can be constructed and refined. The summary of the fit graph (see **Figure 3**, values after refinement of the model) shows model quality for each response represented as four parameters: R2, Q2, model validity, and reproducibility (*MODDE 11 User Guide*, 2015). R2 describes the model fit, and the values of less than 0.5 suggest low significance. Q2 illustrates the predictive quality of the model, which as well should be above the 0.5 value. The model validity is an estimate of general model problems, like outliers or transformation problems, and each model should score at least 0.2 in model validity. The model reproducibility above 0.5 suggests reproducible results. Models for each of the responses show high values for every statistical quality parameter, which in turn suggests a high quality of each of the models. To achieve these results, logarithmic transformation was needed for some of the models. Additionally, to improve the quality of the models, some of the previously chosen model terms had to be removed. **Figure 4** shows all the model terms (coefficients) that are present in the refined model. Significant model terms (error bars of the respective term could not cross the zero on the coefficient plot) were kept, as well as the ones which changed the model quality in a major way upon deletion.

The established model enabled us to study both single-factor influences on the response, as well as the interactions between them. **Figure 5** shows the effects of additives on all the

responses. The factor effects plots for temperature, time, and humidity can be seen in the **Supplementary Information**. The factor effects plot for time shows expected degradation trends for VP, Fru-Phe, procyanidin B-dimer, and B-trimer, with the most rapid degradations for the first two compounds, as they are reactive and participate in the Maillard reaction. While dimers and trimers degrade throughout roasting, epicatechin (and its isomers, e.g., catechin) is relatively stable. Unlike the rest of the compounds, epicatechin has a relative concentration uptrend when it comes to increasing temperature. This may be the result of the degradation of the procyanidin polymers. The humidity seems to have a minor effect on all the classes of compounds reported here. It is surprising, as higher water activity is reported to have an enhancing effect for various browning reactions (including Maillard reaction) (Eichnerl & Karel, 1987; Pereyra Gonzales, Naranjo, Leiva, & Malec, 2010). The most impactful of the factors appear to be additives, and it has the most diverse influence over all the responses. The Amadori compound (Fru-Phe) level is unaltered by either acid or base, whereas the epicatechin and its trimer seem to be affected only in most extreme conditions, as predicted by the model. The peptide (VP) degrades to a lesser extent basic conditions, and the procyanidin B-dimer is stable only in neutral pH. This also is not consistent with the literature as compounds participating in the Maillard reaction should degrade faster with increasing pH (Cerrutti, Resnik, Seldes, & Fontan, 1985; Ajandouz & Puigserver, 1999; Martins & Van Boekel, 2003). However, the reports regarded model systems, avoiding any matrix effects specific to cocoa. Interestingly enough, the behavior of epicatechin and procyanidin B-dimer matches the one described in the literature, as catechins are stable in acidic pH and degrade readily in basic conditions, and the dimers are only stable in neutral pH (Zhu et al., 2002; Kırca, Özkan, & Cemeroğlu, 2007).
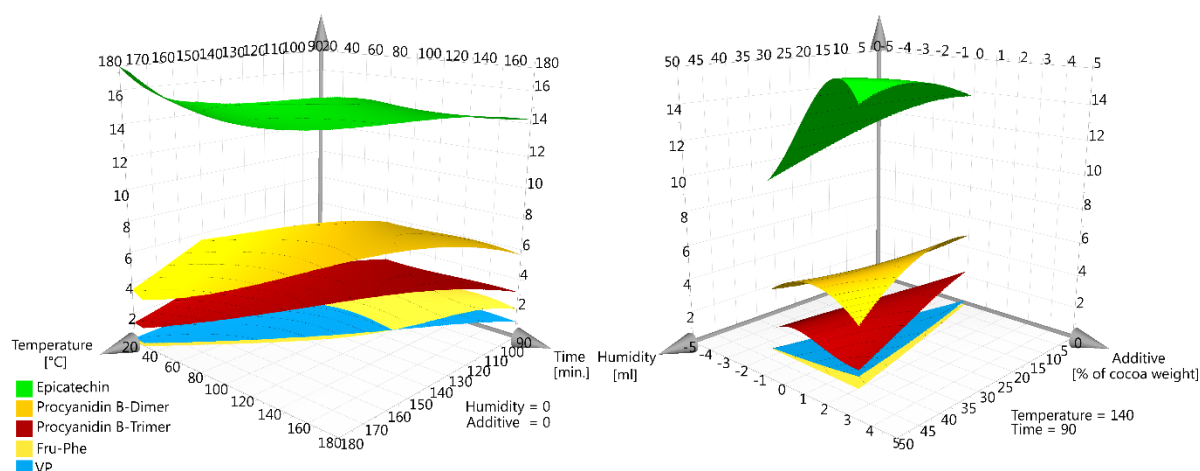


**Figure 6 Relationship between factors and responses represented by response surfaces.** Temperature and time dependence is displayed on the left (humidity and additives constant), and humidity and additive on the right (temperature and time kept constant).

The cumulative effects of each of the factors on the responses can be seen in the response surface plot shown in **Figure 6**. As stated before, temperature and time factors have an expected influence on most of the responses, as anticipated in a thermal degradation process. On the other hand, the figure shows the most variability of each of the classes of compounds under different pH and humidity conditions. This is in line with the literature and industrial knowledge as these are the most common parameters used to control the course of browning reactions (Martins & Van Boekel, 2003; Pereyra Gonzales et al., 2010; Beckett, 2009).

**Table 2 Validation experiments generated by the optimizer feature.**
This table uses abbreviations for factors earlier shown in **Table 1**. Displayed predicted compound concentrations are relative MS peak areas. Exp is the experiment number.

| Exp | Objective | T | t | Hum | Add | Epicatechin | Procyanidin B-Dimer | Procyanidin B-Trimer | Fru-Phe | VP |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Max VP | 114 | 56 | 26.06 | 0.98 | 15.83 | 5.69 | 3.07 | 2.22 | 1.52 |
| 2 | Max B-Dimer | 121 | 49 | 9.78 | 0.49 | 15.37 | 6.24 | 3.17 | 1.02 | 1.00 |
| 3 | Min Epicatechin | 146 | 87 | 47.15 | 2.31 | 9.82 | 3.80 | 1.48 | 0.27 | 0.35 |
| 4 | Min B-Trimer | 146 | 86 | 47.43 | 2.29 | 14.96 | 3.22 | 1.01 | 0.16 | 0.91 |
| 5 | Min Fru-Phe | 173 | 41 | 1.34 | 0.03 | 15.87 | 3.24 | 1.41 | 0.03 | 0.24 |

### 5.3.3 Prediction Phase and Model Validation

The refined model was tested for prediction accuracy. Five experiments with distinctive objectives were planned (see **Table 2**). The objectives were to maximize or minimize concentrations (represented by MS relative areas) of five of the chosen compounds. The software then generated a list of experiments to be performed. **Figure 7** shows their results. Each optimization result for a single response was supplied with a set of predictions for the rest of the responses to explore the robustness of the model. The actual data for epicatechin, dimer, and trimer for experiment 1 is not shown because of poor MS calibration for this experiment. Aside from the comparison of predicted (with 95% confidence interval) and observed values, the figure shows the relative concentrations of respective compounds in the raw material represented by a line.

For most of the compounds and experiments, the established model estimated the values of relative concentrations very well. The one major outlier was the peptide (VP), which behaved quite differently than predicted during roasting. This could be because peptides are probably the most reactive compounds in the cocoa beans, and there are many competing reactions in which they can participate. The polyphenols have good predictability, and as well there is an option of pushing their balance toward either their conservation or degradation. There seems to be a balance between catechins and their oligomer counterparts, which would have to be explored in an expanded model, possibly including higher oligomers. In the case of Fru-Phe, there is a possibility of decreasing its degradation with high accuracy. However, as its degradation is essential in the formation of aroma volatiles, this could most likely only decrease the quality of roasted cocoa. However, the validation of the model shows that most of the studied compounds' behavior is predicted very well. This approach, when expanded to different compounds classes, could provide useful insights on chemical transformations and their mechanisms occurring during roasting of cocoa.

## 5.4 Conclusions

In our study, we have applied common knowledge of cocoa processing to construct a design of experiments model of the roasting. We have illustrated it on an example of five well-known constituents of fermented and dried cocoa beans. Our results showed very good prediction and some unexpected behaviors of some of the compounds in comparison to established model systems knowledge. Nevertheless, we regard these initial results as very promising, especially for mechanistic studies of cocoa chemistry. Further expansions of this method could lead to optimization of cocoa processing, especially when coupled to similar, more systematized approaches of mathematical modeling of cocoa fermentation.
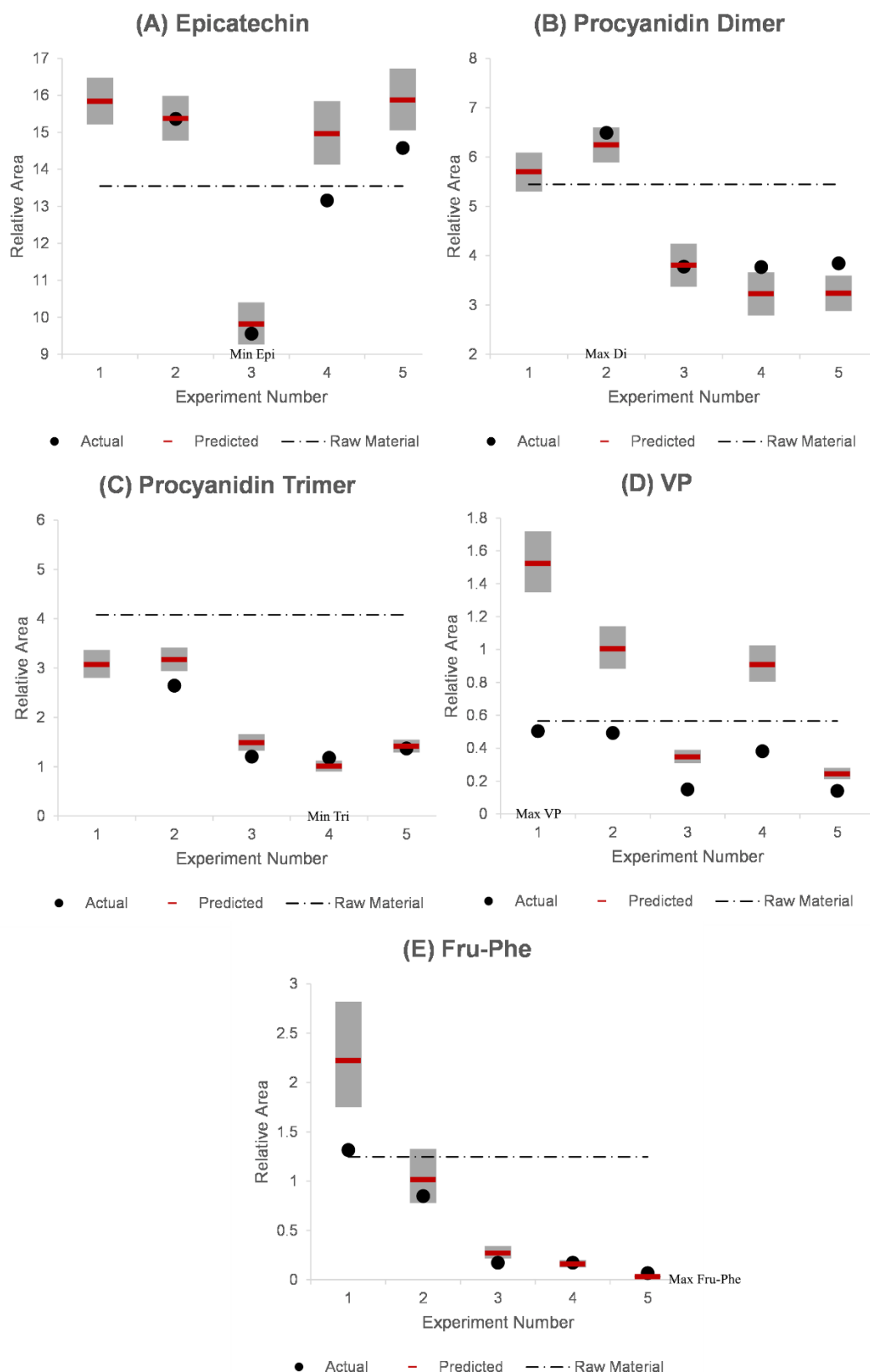
**Figure 7 Results of validation experiments.**
The graph shows actual relative concentrations of all five responses against the predicted values (including 95% confidence intervals in gray) and the relative concentrations in the raw material. Each graph has annotation with an objective and a compound name by the respective experiment number.

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## Conflict of interest statement

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# Chapter 6

# Natural Alkalization of Cocoa Nibs by Ion Exchange Chromatography

Paweł J. Andruszkiewicz, Roy N. D'Souza, Marcello Corno, and Nikolai Kuhnert

## Abstract

The alkalization process is a historical method of intrusive cocoa processing used in order to decrease acidity and bitterness, adjust the color, and increase dispersibility in water. As an alternative, we have developed a method of "natural" alkalization using ion-exchange chromatography without using any additives. On a laboratory scale, we were able to increase the pH of cocoa nibs above 9. This promising method should be optimized on an industrial scale with suitable equipment.

## 6.1 Experimental Setup

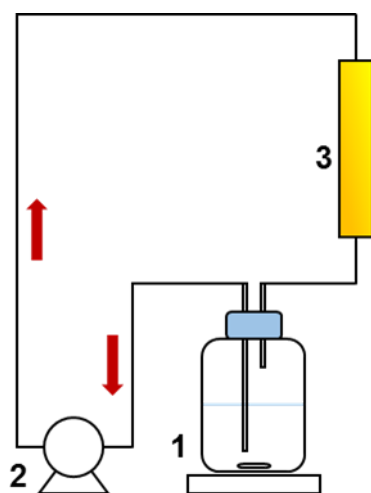| Ingredients | Cocoa nibs, liquid chromatography column with anion-exchange resin |
|---|---|
| Techniques | Ion exchange chromatography |
| Goal | Alkalization of cocoa nibs without additives |



**Fig. 1** Schematic of the experimental setup: 1 – Container with magnetic stirrer, water and cocoa nibs, 2- peristaltic pump, 3 – anion-exchange column. Direction of the flow is marked with red arrows.

The experimental setup consisted of container for cocoa nibs, peristaltic pump, and column packed with a strong anion-exchange resin ($^-$OH form), all connected with tubing to form a closed system (see Fig. 1). Before the experiment, the column was equilibrated with 1M NaOH, following washing with water till neutral pH. Cocoa nibs were put inside a paper filter and immersed in water inside the container. The paper filter, as well as three other filters present in the system, ensured that smaller particles would not block the small diameter tubing. Water in the container was constantly stirred to fasten pH equilibrium. The pump transported the solvent to the column and then back into the container. Measurements of pH were executed directly before and after the experiment. Additionally, another experiment was performed. Cocoa nibs were immersed in water, and an excessive amount of equilibrated anion-exchange resin was added to them. They were stirred for sixty hours, along with a control sample not
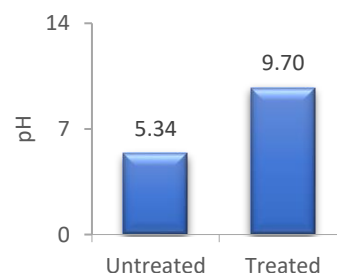


**Fig. 2** Changes in pH of cocoa nibs before and after ion exchange alkalization.

containing the resin. The resin was separated from the nibs by sieving. The sample pH was measured before and after the treatment, and both samples were roasted directly afterward.

## 6.2 Results

Despite the technical difficulties (blocked filters decreased the flow rate), a 30-minute long experiment was sufficient to increase the pH of cocoa nibs from 5.34 to 9.70 (Fig. 2). The difficulties were associated with too small sizes of the column filters, which should be relevant only at laboratory scale. After the sixty hour-long second experiment, the pH of the nibs with added resin was 9.97. The control sample had a stable pH changing slightly from 5.38 to 5.48 during that time. The results of the roasting of both samples are



**Fig. 3** Roasting results after the treatment during the second experiment.

visible in Fig. 3. The gloss coming from the treated sample is a result of lipids seated out during roasting.

## 6.3 Conclusions

Both experiments show that the pH modulation of cocoa nibs is possible using ion-exchange chromatography, avoiding the addition of external basic reagents, and achieving positive results (darkening) after the roasting. The product of this treatment could be roasted and industrially processed afterward, yielding cocoa with potentially the same attributes as alkalized powder. The final pH could be modified by changing parameters of the setup – amount and type of resin, as well as the amount of water. However, industrial optimization is needed.

## Acknowledgments

# General Discussion and Outlook

The objectives of this thesis were successfully met. The main parts of this work, HPLC-MS investigations on important flavor-related species, 2,5-diketopiperazines and Amadori compounds, were accomplished. Both classes, which are highly relevant to chemical transformations of cocoa (affecting its flavor), were carefully studied in terms of their formation in raw and roasted material, their precursors, and variability in different countries of origin. This work has shown the importance of compounds described above as quality markers across various steps of chocolate production. Furthermore, using those compounds as an example, it showed that modeling those compounds' behavior is achievable and useful for possible optimization of industrial processes as well as for the purpose of scientific investigation of cocoa roasting. Findings presented in this thesis complement similar studies performed on cocoa fermentation and contribute to a better understanding of cocoa processing and flavor development.

## Identification and Behavior of Novel Flavor-related Cocoa Constituents

### 2,5-Diketopiperazines and Their Precursors

Generally, the 2,5-diketopiperazines are known to be present in food and beverages, in which they contribute to flavor as well as show biological activity (Borthwick & Da Costa, 2017). Mass spectrometry was demonstrated to be a useful tool in researching them (Furtado et al., 2007; Guo et al., 2009). Most importantly, they were well studied in cocoa and shown to be essential to its taste (Stark & Hofmann, 2005; Stark et al., 2006). It was shown that among 25 2,5-diketopiperazines, seven particular ones together were at least needed to invoke a cocoa-like sensation in human receptors. Research done within this thesis expands our knowledge of DKPs to 34 chemical compounds, with the data suggesting more possible species outside the limit of quantification and detection. This poses a question whether more variety of less abundant 2,5-diketopiperatines affects the cocoa taste. Considering that the human bitter receptors are highly unspecific and could react to cumulative contribution of several types of compounds (Behrens & Meyerhof, 2006; Meyerhof et al., 2010, 2011), even less amounts of many types of DKPs could stimulate the taste receptors. To investigate this, a series of fractionation and taste dilution experiments similar to previous work (Stark et al., 2006) should be performed. The results of such experiments could increase our predictive capabilities and understanding of bitterness perception.

Most importantly, almost 100 peptides were identified as putative precursors. Such an important finding could be used to control populations of DKPs on fermentation level e.g., by employing specific peptidases to increase or decrease precursor availability. Subsequently, kinetic studies on cocoa roasting show complex behavior of both 2,5-diketopiperazines and peptide precursors, most likely because of multiple peptide reactions (not only yielding DKPs) that can occur at the same time within the matrix. Additionally, it is pointed out that undesirable farming practices can affect microbial populations during fermentation (Pacheco-Montealegre et al., 2020), which in turn could explain significant differences between peptide diversity in different origins of cocoa during and after fermentation (Kumari et al., 2018). Moreover, a recent study on fermentation proves that peptides are highly reactive and can undergo chemical transformations with different cocoa constituents at the same time (Kuhnert et al., 2020). Both facts can contribute to peptide variability in the raw material, and their subsequent complex behavior during the roasting. Still, this thesis shows that a positive linear correlation between DKPs and

their peptide precursors exists and yields impressive predictive capabilities. This direct connection proves that better control over processing in terms of bitterness can be achieved when applied to the most bitter 2,5-diketopiperazines. The results are especially promising as the same observations were made for beans from different countries of origin. These findings highlight that the most important contributor to DKP formation is precursor availability, which is greater for properly fermented cocoa beans. Data presented in this work contributes to the future attempts to the modeling of chemical reactions during roasting of cocoa and the prediction of their outcomes.

*Amadori Compounds and Their Relation to Flavor*

Non-enzymatic browning, or the Maillard reaction, is by itself very well studied, with researchers focusing on the end products of this complex reaction – aroma and colored compounds. At the same time, their essential precursors, Amadori and Heyns compounds, received less attention. Still, their degradation was directly linked to the formation of important flavor components of many foods (Martins et al., 2000). Primarily, they were studied in both food and model systems as chemical compounds consisting of single amino acid and a monosaccharide. However, the existence of dipeptide species was implied in the literature by model Maillard reaction studies (Zou et al., 2018). They revealed as well that those dipeptide species are affecting pyrazines (important aroma compounds) formation during heat treatment. Until recently, only seven Amadori compounds were reported to be present in cocoa. The latest FT-ICR-MS study on cocoa suggests that many more possible Amadori compounds may form during fermentation (Kuhnert et al., 2020).

Here, a series of dipeptide Amadori (and Heyns) compounds was reported for the first time. The presence of a greater variety of Amadori compounds in cocoa suggests the occurrence of more complex Maillard-related reactions during its roasting. In turn, even more variety of flavor products may possibly exist. Additionally, PCA separation of countries of origin based on Amadori compound content was possible, with various countries having different Amadori compound profiles. The previous study on peptidome (Kumari et al., 2018) suggests it could be due to processing differences during fermentation, not the origin itself. This is due to the fact that proper fermentation increases the amount and variety of peptide precursors available in the cocoa bean (D'Souza et al., 2018). Within this work, for the first time, a trend of Amadori compounds across processing series (fermentation, drying, and roasting of Ghanaian cocoa) was closely studied along with their precursor sugars and peptides as well as their volatile degradation products. The formation of those essential flavor precursors can be clearly a sign of adequately executed fermentation and drying, and they can be used as quality markers for dried cocoa beans. As a whole, these findings point attention towards particular chemical components that can be used as markers for well-processed and high flavor-potential raw material. Furthermore, the identification of all Amadori compounds in cocoa in the future could help us understand how fermentation practices and origin influence the precursor composition of raw cocoa beans as a whole. Most importantly, it would allow us to link the precursor composition to the flavor profile of the roasted product.

*Design of Experiments Modelling Approach*

The design of experiments proved to be a meaningful approach to investigation and optimization of various processes, including in the field of food science and technology (Granato & de Araújo Calado, 2013; Yolmeh & Jafari, 2017). It is very well described in the literature, and as mass spectrometry is a popular high-throughput method used in metabolomics, it was also used together with DoE (Eliasson et al., 2012; Rhoades & Weljie, 2016). Furthermore, it is not new to cocoa science, and a recent study describes the most important factors influencing cocoa fermentation revealed by the design of experiments approaches (John

et al., 2020). All in all, more and more modeling and computational studies on cocoa emerge in order to explain and predict its complex processing behavior (Moreno-Zambrano et al., 2018; Megias-Perez et al., 2020).

The motivations for this part of the research are provided from both scientific and industrial perspective. The results of the DoE approach to cocoa roasting can provide mechanistic insights into chemical transformations of cocoa constituents, which, in turn, can be translated into practical solutions. Based on common knowledge of cocoa roasting, a design of experiments approach was established using HPLC-MS data. Validation of results for five compounds well-known to the literature showed the high quality of the models, each displaying satisfying prediction accuracy. Despite some unexpected behaviors, the approach shows great promise in studying the mechanistic chemistry of cocoa roasting. The initial results proved the optimization of thermal processing in terms of specific compounds to be possible. In general, the extension of this approach could provide significant insights into the optimization of cocoa roasting. More specifically, the compounds selected for this study belong to, among others, classes of compounds mentioned in the previous two subsections of the discussion, namely 2,5-diketopiperazines, Amadori compounds, and peptides. From a mechanistic perspective, the most important connection between them is that peptides are precursors for both Amadori compounds (form during fermentation and drying) and DKPs (form during roasting). Apart from this, peptides can undergo many other chemical transformations, both alone and with other compounds. This means that further studies on this topic will allow us to establish the reactivity of peptides and quantify their contributions to specific classes of compounds. In this particular example, to the formation of Amadori compounds and 2,5-diketopiperazines, which both contribute to the flavor of cocoa. More importantly, modeling of roasting, as shown here, coupled with similar approaches to other processing steps (John et al., 2020), may give us the whole picture of chemical transformations for the entire chocolate production. This way, we will be able to show the exact chemical mechanisms governing over cocoa processing as well as predict and optimize them.

## Industrial Recommendations

This thesis addressed only a small selected fraction of existing open questions in the science of cocoa roasting. Even then, for each of the projects, more challenges essential from the industrial and scientific points of view were generated along the way. The impact of the most abundant 2,5-diketopiperazines on the bitterness of cocoa is already well studied. However, a great diversity of less abundant DKPs presented here poses a question of whether they have any effect in this regard. Human bitterness receptors are highly unspecific, and there is a possibility that even diketopiperazines below their taste thresholds could have a cumulative impact on bitterness perception. Therefore, from both scientific and industrial perspectives, it is necessary to perform an investigation on the taste of this compound class as a whole. Combined with our precursor correlation, a result of such a study would provide enough insight to propose a model of DKP-related cocoa bitterness. Additionally, these compounds should be thoroughly searched for in other cocoa beans from different origins, to set quality standards among cocoa-producing countries. Most importantly, the optimization of roasting conditions minimizing DKP content should be performed to reduce chocolate bitterness. Another approach targeting the fermentation would consist of carrying out the process in such a way to decrease the formation of specific peptide precursors. It could be done by using specifically engineered enzymes or microorganisms.

Amadori compounds are essential to flavor formation through the Maillard reaction. They have been studied in the model systems very well, but their complex behavior in food matrices is the

cause of their poor understanding. There is much to be achieved scientifically in studying mechanisms of their chemical transformations. However, the most important influence of the presented research on this topic is the implications for the cocoa industry. The data shown here clearly points out the differences in amounts of Amadori compounds in different varieties, origins, and qualities of cocoa beans. Their presence should be meticulously inspected at the point of both processing and sourcing. They should become an important part of the quality assessment of raw material for chocolate production. As peptides are important to Amadori compound formation, selecting cocoa beans, which were well-fermented, should increase their amount and their contribution to aroma formation. Furthermore, advancements in cocoa research in the future could point out why the differences in Amadori compound quantities exist between different varieties. This, in turn, could allow us to maximize their yield. Another question is - can we predict the flavor of cocoa based on quantities and chemical structures of Amadori compounds? The answer to this question could help the farmers set standards of fermentation to yield better-flavored cocoa and after roasting, better chocolate.

Finally, the proof-of-concept of the design of experiments approach on cocoa roasting demonstrated to be a promising tool in predicting the thermal treatment output in terms of chemical composition. It was applied to a small number of selected compounds. However, the extension of this method to other components (as well as other cocoa varieties) can help the optimization of roasting in terms of flavor generation. In addition, as the experiments were performed on a laboratory scale, upscaling to the industrial scale is required. For industrial purposes, a combination of previously shown results and the DoE approach could prove extremely helpful in the optimization of roasting towards achieving high-quality chocolate. Notably, when considering the results of similar experiments done on the fermentation process, which was done recently within the COMETA project. The scientific use of this model could also be useful in mechanistic studies of chemical transformations of different compound classes occurring during cocoa roasting, which again could help us improve the organoleptic properties of produced chocolate.

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# Appendix A

**Supplementary Information**

**Chemical Profiling of Ghanaian Cocoa Bean Processing Series with Emphasis on Maillard-related Compounds**

Paweł J. Andruszkiewicz, Roberto Megías-Pérez, Britta Behrends, Isabelle Leuven, and Nikolai Kuhnert

## 3. Reproducibility Data for Amadori Compounds

| ID**** | Ferm. 72h (Inter)* | Ferm. 72h (Intra)* | Ferm. 72h (Intra)* | Ferm. 72h (Intra)* | Ferm. 72h (Average) | Ferm. 72h (St. Dev.) | Ferm. 144h (Inter)* | Ferm. 144h (Intra)* | Ferm. 144h (Intra)* | Ferm. 144h (Intra)* | Ferm. 144h (Average) | Ferm. 144h (St. Dev.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.6008 | 5.8883 | 5.4957 | 5.4449 | 5.6074 | 0.1982 | 8.4522 | 9.2340 | 8.8608 | 9.0226 | 8.8924 | 0.3309 |
| 2 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 3 | 2.1119 | 1.5849 | 1.5628 | 1.5701 | 1.7074 | 0.2698 | 2.5738 | 2.3571 | 2.3910 | 1.9952 | 2.3293 | 0.2422 |
| 4 | 0.4901 | 0.5444 | 0.5432 | 0.5086 | 0.5216 | 0.0268 | 0.8360 | 0.9518 | 0.7863 | 0.8471 | 0.8553 | 0.0695 |
| 5 | 0.0000 | 0.2095 | 0.2274 | 0.2049 | 0.1605 | 0.1074 | 0.7365 | 0.4519 | 0.5106 | 0.5236 | 0.5556 | 0.1245 |
| 6 | 0.9130 | 0.5956 | 0.6269 | 0.6146 | 0.6875 | 0.1509 | 2.1691 | 1.1563 | 1.2142 | 1.1667 | 1.4266 | 0.4957 |
| 7 | 0.2904 | 0.2622 | 0.2645 | 0.2724 | 0.2724 | 0.0128 | 0.2305 | 0.4080 | 0.4317 | 0.4399 | 0.3775 | 0.0989 |
| 8 | 0.0000 | 1.5373 | 1.5298 | 1.4994 | 1.1416 | 0.7613 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 9 | 0.0000 | 0.6807 | 0.7392 | 0.6423 | 0.5155 | 0.3460 | 0.7034 | 1.3043 | 1.5394 | 1.4134 | 1.2401 | 0.3705 |
| 10 | 0.0000 | 0.8133 | 0.8791 | 0.8683 | 0.6402 | 0.4277 | 2.2159 | 2.1238 | 1.4884 | 1.3167 | 1.7862 | 0.4501 |
| 11 | 0.0000 | 0.4092 | 0.3868 | 0.4048 | 0.3002 | 0.2004 | 0.0000 | 1.0166 | 1.1114 | 1.0039 | 0.7830 | 0.5242 |
| 12, 13 | 0.0000 | 0.0000 | 0.0000 | 0.0089 | 0.0022 | 0.0045 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 14 | 0.9031 | 0.7913 | 0.8739 | 0.7842 | 0.8381 | 0.0594 | 1.6237 | 1.1384 | 1.4627 | 1.2226 | 1.3619 | 0.2222 |
| 15 | 0.3866 | 0.5503 | 0.6614 | 0.6872 | 0.5714 | 0.1368 | 1.4671 | 1.8240 | 1.8462 | 1.7717 | 1.7273 | 0.1762 |
| 16 | 0.7343 | 1.4139 | 1.3067 | 1.4395 | 1.2236 | 0.3312 | 2.7284 | 4.1266 | 4.0329 | 4.1148 | 3.7507 | 0.6828 |
| 17 | 0.2441 | 0.3079 | 0.3092 | 0.2895 | 0.2877 | 0.0304 | 0.5503 | 0.6436 | 0.6392 | 0.6278 | 0.6152 | 0.0438 |
| 18 | 0.0000 | 0.8429 | 0.8754 | 0.8560 | 0.6436 | 0.4293 | 2.3091 | 1.9067 | 1.9552 | 1.8165 | 1.9969 | 0.2160 |
| 19 | 0.0000 | 0.5419 | 0.5622 | 0.5628 | 0.4167 | 0.2780 | 1.0144 | 1.0469 | 0.7869 | 0.7681 | 0.9041 | 0.1470 |
| 20 | 0.0000 | 0.2311 | 0.2456 | 0.2191 | 0.1739 | 0.1165 | 0.6652 | 0.6228 | 0.5476 | 0.5496 | 0.5963 | 0.0577 |
| 21 | 0.0000 | 0.1251 | 0.1216 | 0.1207 | 0.0918 | 0.0613 | 0.3167 | 0.2824 | 0.3030 | 0.2320 | 0.2835 | 0.0371 |
| 22 | 1.3273 | 1.3300 | 1.4002 | 1.2731 | 1.3327 | 0.0521 | 1.9196 | 2.7508 | 2.6142 | 2.5923 | 2.4692 | 0.3731 |
| 23 | 0.8602 | 1.0464 | 1.1297 | 0.9895 | 1.0064 | 0.1133 | 1.4569 | 1.9511 | 1.9540 | 1.8588 | 1.8052 | 0.2364 |
| 24 | 0.5148 | 0.3937 | 0.3778 | 0.3680 | 0.4136 | 0.0683 | 0.7881 | 0.7683 | 0.8165 | 0.6975 | 0.7676 | 0.0508 |
| 25 | 0.0000 | 0.5239 | 0.5808 | 0.4799 | 0.3962 | 0.2673 | 0.7322 | 0.9590 | 0.9989 | 0.7636 | 0.8634 | 0.1350 |
| 26 | 1.6435 | 1.7284 | 1.7045 | 1.5435 | 1.6550 | 0.0825 | 0.0000 | 9.5637 | 9.2995 | 9.4744 | 7.0844 | 4.7242 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 27 | 1.6933 | 0.8724 | 0.8869 | 0.8434 | 1.0740 | 0.4133 | 2.3052 | 1.6197 | 1.5534 | 1.6204 | 1.7747 | 0.3551 |
| 28 | 7.3574 | 2.9422 | 3.0475 | 2.8443 | 4.0478 | 2.2079 | 10.7863 | 6.3495 | 5.8664 | 6.0201 | 7.2556 | 2.3624 |
| 29 | 0.6462 | 0.3418 | 0.2922 | 0.2103 | 0.3726 | 0.1902 | 1.2881 | 0.8090 | 0.7006 | 0.4837 | 0.8204 | 0.3399 |
| 30 | 8.6173 | 4.8508 | 4.9087 | 4.7401 | 5.7792 | 1.8933 | 14.1164 | 9.2301 | 8.8128 | 8.7448 | 10.2260 | 2.6024 |
| 31, 32 | 0.9073 | 0.6488 | 0.5813 | 0.5688 | 0.6766 | 0.1578 | 1.0970 | 1.1993 | 1.2045 | 1.2574 | 1.1895 | 0.0670 |
| - | 35.2416 | 32.0085 | 32.1213 | 30.8590 | 32.5576 | 1.8780 | 63.0822 | 65.7958 | 63.7284 | 62.3453 | 63.7379 | 1.4837 |

All numbers represent relative MS peak areas (relative to the sum of all peaks in the sample) additionally normalized by the moisture content

Ferm. = Fermented; St. Dev. = Standard Deviation

*Annotations in brackets designate inter- and intra-day repetitions

*Heyns peaks are bigger than Amadori peaks or Heyns are present and Amadori not

**There are no either Amadori or Heyns peaks

***Special case: Fru-Y has bigger Amadori peaks, but also has multiple Heyns peaks, unlike the other Fru-Y peak (splitting)

Rest have clear Amadori-specific fragmentation peaks that are the only peaks or are bigger than Heyns

****ID of compounds from my previous paper, two of the compounds' isomers were summed

## 4. Correlation Matrix (Pearson)

| | Fructose | Glucose and Galactose | Sucrose | Σ Carbohydrates | Σ Peptides | Σ AA Amadori | Σ Dipeptide Amadori | Σ Amadori | Σ Roasted | Σ Cocoa | Σ Fruity | Σ Floral | Σ Volatiles (w/o Acetic Acid) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fructose | 1.00 | | | | | | | | | | | | |
| Glucose and Galactose | 0.87 | 1.00 | | | | | | | | | | | |
| Sucrose | 0.14 | 0.56 | 1.00 | | | | | | | | | | |
| Σ Carbohydrates | 0.62 | 0.90 | 0.85 | 1.00 | | | | | | | | | |
| Σ Peptides | 0.33 | 0.07 | -0.70 | -0.34 | 1.00 | | | | | | | | |
| Σ AA Amadori | -0.67 | -0.72 | -0.33 | -0.63 | 0.02 | 1.00 | | | | | | | |
| Σ Dipeptide Amadori | -0.29 | -0.53 | -0.86 | -0.80 | 0.77 | 0.33 | 1.00 | | | | | | |
| Σ Amadori | -0.48 | -0.69 | -0.83 | -0.88 | 0.64 | 0.64 | 0.94 | 1.00 | | | | | |
| Σ Roasted | -0.37 | -0.53 | -0.71 | -0.72 | 0.51 | 0.16 | 0.89 | 0.79 | 1.00 | | | | |
| Σ Cocoa | 0.33 | -0.04 | -0.68 | -0.37 | 0.75 | 0.20 | 0.48 | 0.47 | 0.10 | 1.00 | | | |
| Σ Fruity | 0.61 | 0.33 | -0.44 | -0.03 | 0.75 | 0.02 | 0.33 | 0.28 | 0.12 | 0.59 | 1.00 | | |
| Σ Floral | -0.30 | -0.44 | -0.67 | -0.64 | 0.50 | -0.04 | 0.84 | 0.67 | 0.97 | 0.07 | 0.05 | 1.00 | |
| Σ Volatiles (w/o Acetic Acid) | -0.32 | -0.53 | -0.81 | -0.78 | 0.67 | 0.25 | 0.97 | 0.88 | 0.97 | 0.34 | 0.23 | 0.91 | 1.00 |

# Appendix B

**Supplementary Information**

**HPLC-MS-based Design of Experiments Approach on Cocoa Roasting**

Paweł J. Andruszkiewicz, Marcello Corno, and Nikolai Kuhnert
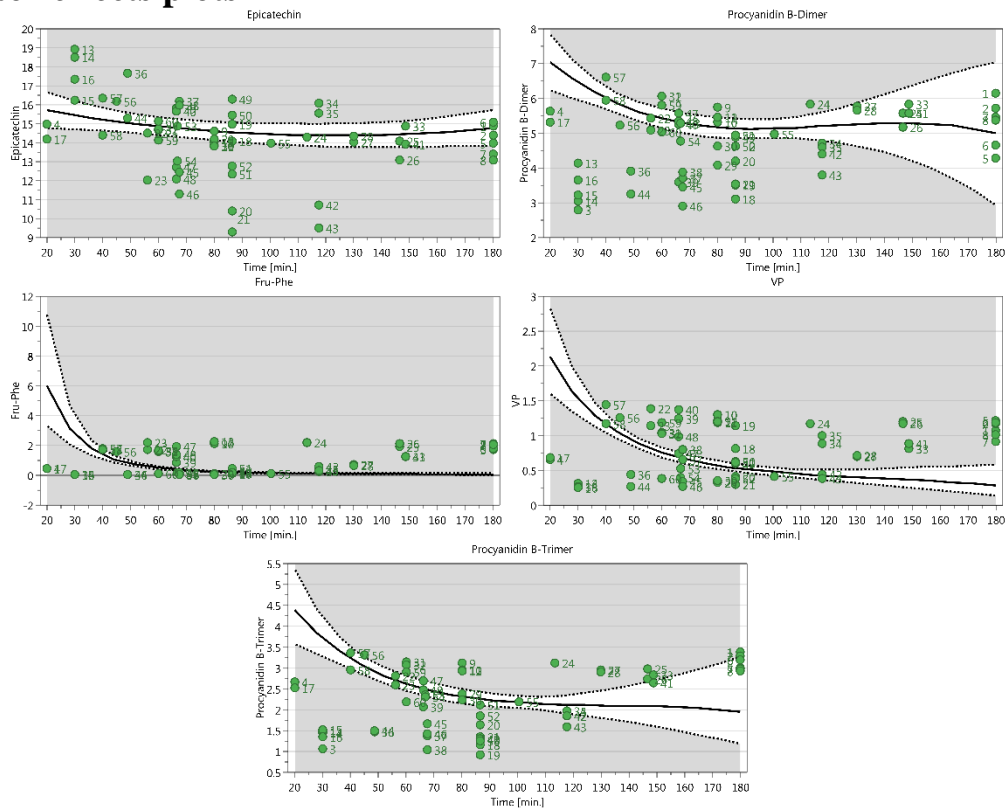
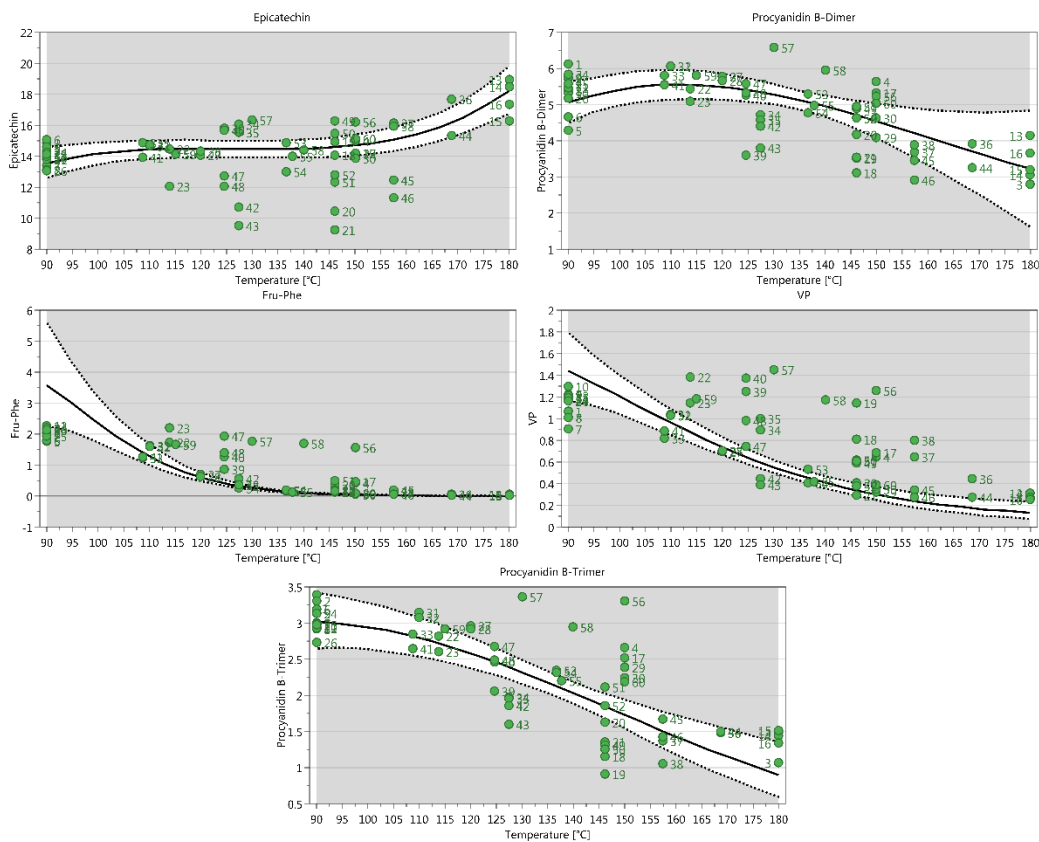# 1. Factor effects plots
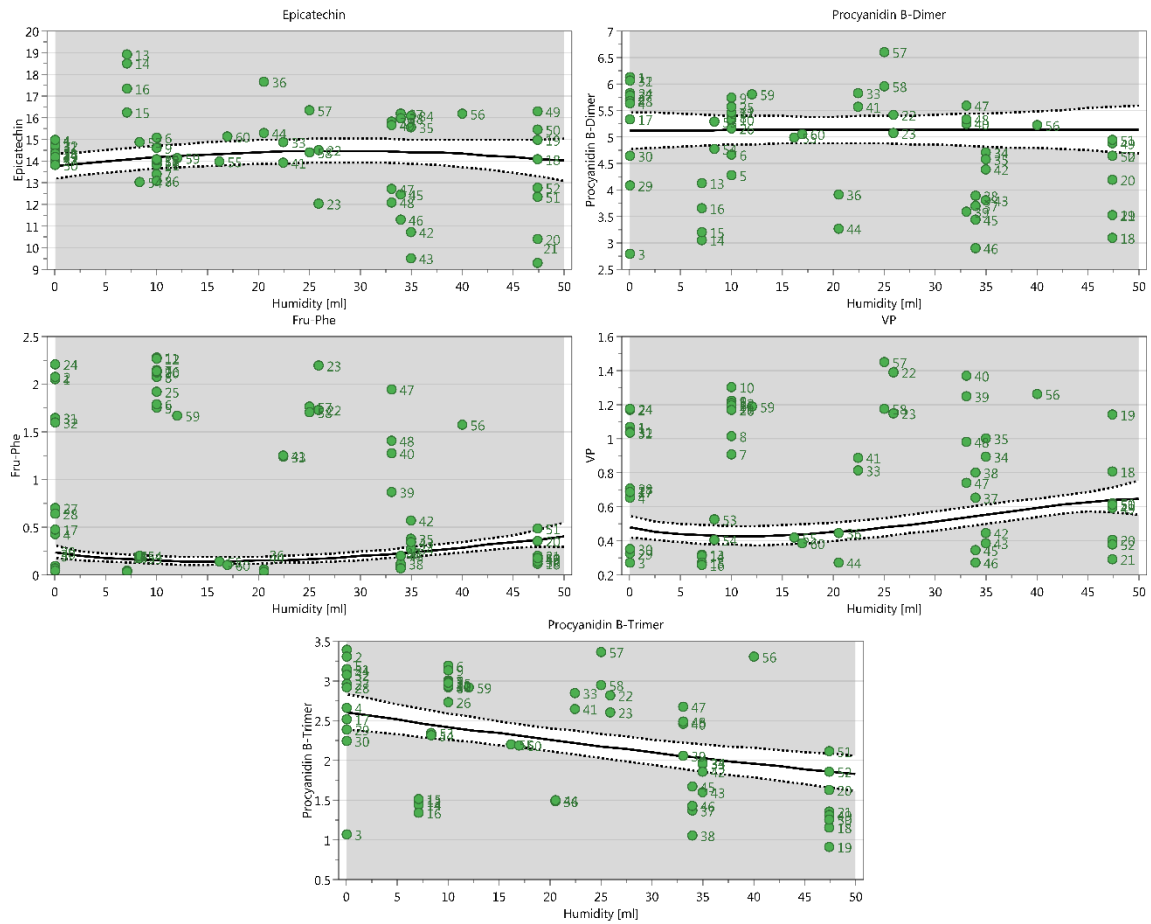


**Figure 1 Factor effects for time.**



**Figure 2 Factor effects for temeperature.**

**Figure 3 Factor effects for humidity.**