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Characterization of the odor-active compounds responsible for atypical aroma notes in cocoa (*Theobroma cacao* L.)

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Contents

1 Summary

Cocoa is the key raw material in chocolate manufacturing. The quality of cocoa is crucial for the pleasant aroma of the final confectionary products. Ideally, cocoa shows a rich aroma with floral, sour, malty, earthy and fruity notes. However, chocolate manufacturing companies occasionally report the presence of off-flavors in fermented cocoa at the level of the incoming goods inspection. Among them moldy-musty and coconut-like notes are frequently observed. It is hard to establish the presence of off-flavors on the base of sensory data only, as it has mainly been done so far, because this type of assessment is very subjective. Therefore, there is a strong need for an objective method based on analytical data to affirm whether a cocoa batch is suitable or not for further manufacturing. To clarify the molecular background of the moldy-musty and the coconut-like notes, the volatiles were isolated from fermented cocoa samples tainted with off-flavors by solvent extraction and solvent-assisted flavor evaporation (SAFE). The odor-active compounds were determined by comparative aroma extract dilution analyses (cAEDA), using flawless cocoa samples as reference.

The first part of the investigation was focused on cocoa samples with a moldy-musty off-flavor. Application of cAEDA revealed (−)-geosmin, 4-methoxy-2,5-dimethylfuran-3(2*H*)-one, 1*H*-indole, and 3-methyl-1*H*-indole as potential off-flavor compounds. This compound selection was based on their odor quality and higher flavor dilution factors in the off-flavor cocoa than in the reference sample. Quantitation of the four compounds in nine off-flavor cocoa samples and calculation of odor activity values (OAVs; ratios of the concentrations to the odor threshold values) suggested crucial roles of (−)-geosmin and 3-methyl-1*H*-indole for the off-flavor. In the chocolate industry, their quantitation can be used to objectively assess the moldy-musty off-flavor at the level of the incoming goods inspection. Because both compounds are inhomogeneously distributed between testa and embryo, separate quantitation in the two parts of the seeds is required.

The second part of the investigation was focused on cocoa samples with a coconut-like note. Application of cAEDA revealed coconut-like smelling compounds δ-octalactone, δ-2-octenolactone, γ-nonalactone, γ-decalactone, δ-decalactone and δ-2-decenolactoneas as potential causative odorants. Quantitation of these six compounds and calculation of OAVs suggested δ-2-decenolactone as the crucial compound. Chiral analysis showed the presence of pure (*R*)-δ-2-decenolactone, commonly referred to as massoia lactone. Its key role for the coconut note was finally demonstrated in a spiking experiment: the addition of (*R*)-δ-2-decenolactone to the reference cocoa in an amount corresponding to the concentration difference between the two samples was able to provoke a coconut note in an intensity comparable to the one in the atypically smelling cocoa. To avoid an undesired coconut note caused by (*R*)-δ-2-decenolactone in the final products, the chocolate industry may consider its odor threshold value, that is 100 µg/kg, as a potential limit for the acceptance of fermented cocoa in the incoming goods inspection.

2 Abbreviations and nomenclature

Abbreviations:

AEDA	aroma extract dilution analysis
cAEDA	comparative aroma extract dilution analysis
3-AFC	3-alternative forced choice
ASTM	American Society for Testing and Materials
AV	acidic volatiles
CI	chemical ionization
EI	electron ionization
FD	flavor dilution
FFAP	free fatty acid phase
FID	flame ionization detector
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detector
GC-GC-MS	two-dimensional heart-cut gas chromatography-mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GC-O	gas chromatography-olfactometry
HRMS	high-resolution mass spectrometry
NBV	neutral and basic volatiles
NMR	nuclear magnetic resonance
OAV	odor activity value
OR	olfactory receptor
OTV	odor threshold value
RI	retention index
SAFE	solvent-assisted flavor evaporation
SIDA	stable isotope dilution assay

Nomenclature:

γ-decalactone	5-hexyloxolan-2-one
δ-decalactone	6-hexyloxolan-2-one
δ-2-decenolactone	(6*R*)-6-pentyl-5,6-dihydro-2*H*-pyran-2-one
(−)-geosmin	(4*S*,4a*S*,8a*R*)-4,8a-dimethyloctahydronaphthalen-4a(2*H*)-ol
HDMF	4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (Furaneol®)
linalool	3,7-dimethylocta-1,6-dien-3-ol
MDMF	4-methoxy-2,5-dimethylfuran-3(2*H*)-one
γ-nonalactone	5-pentyloxolan-2-one
δ-octalactone	6-propyloxan-2-one
δ-2-octenolactone	(6*R*)-6-propyl-5,6-dihydro-2*H*-pyran-2-one
vanillin	4-hydroxy-3-methoxybenzaldehyde

3 Introduction

3.1 Molecular sensory science

3.1.1 The physiology of odor perception

The sense of smell is of paramount importance for most species in the animal kingdom: for the orientation in the environment, for the search for food but also to detect warning signals, such as rotten foodstuff or smoke from a fire. Although the survival of humans does not crucially depend on their sense of smell, the perception of odors plays a crucial role in several aspects of life. Aroma, together with taste, texture and appearance, represents a significant quality parameter for food selection by consumers.

Odor is caused by volatile molecules that enter the nasal cavity and interact with specialized proteins called olfactory receptors (ORs). The existence of such receptors was first evidenced in 1991 by the Nobel laureates Axel and Buck who identified a novel gene family encoding ~ 390 ORs, thereby setting a milestone in the understanding of odor perception on the molecular level. The majority of the ORs are found on the cellular surfaces in an epithelium located at the posterior nasal roof. There, ~ 10 million olfactory receptor neurons are present in humans and for this reason, this area is also known as *regio olfactoria* (Figure 1).

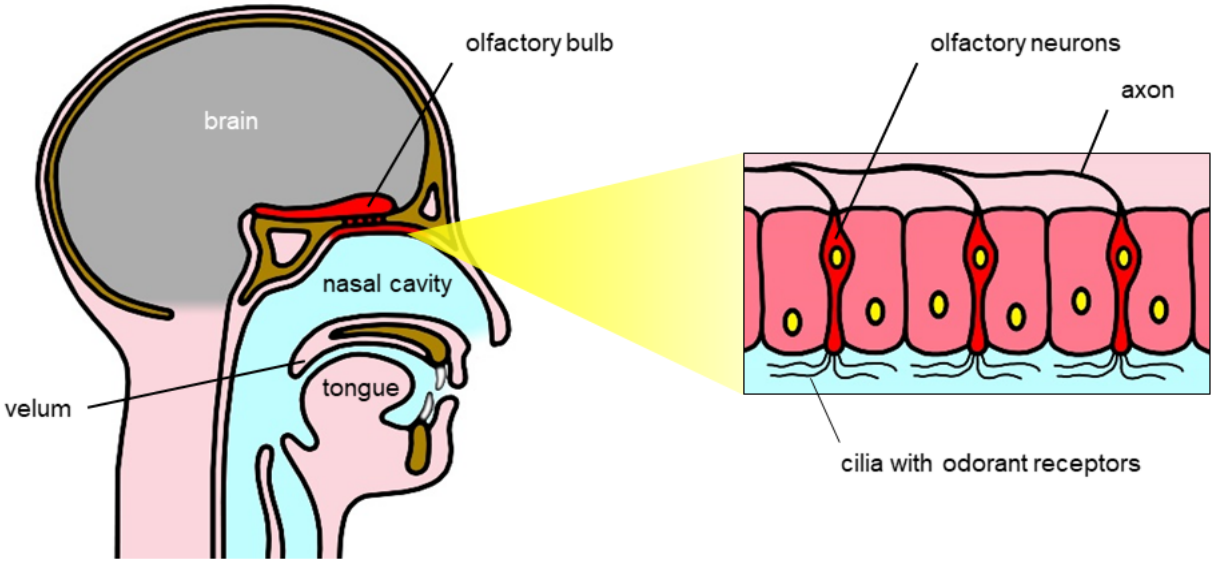

Figure 1: The human olfactory system. Illustration: Martin Steinhaus

The olfactory neuron is a bipolar nerve cell. On one side, its axon projects to the olfactory bulb of the brain. On the other extremity, it has a dendritic process which extends to the mucosal surface, where it gives rise to specialized hairy structures. These so-called cilia constitute an extensive, receptive surface for the interaction with odorants as in their membrane numerous olfactory receptors are located.[1]

On the molecular level, the binding of an odorant to an olfactory receptor is the first step of a complex cascade of events (Figure 2). The binding causes first a conformational change of the intracellular G protein to which the receptor is coupled. The activation of the G protein stimulates the cyclization of ATP by adenylyl cyclase, affording cyclic AMP (cAMP). This molecule acts as a second messenger, triggering the activation of ion channels. This allows Ca^{2+} ions along with Na^{+} ions to enter. High Ca^{2+} levels provoke the activation of Ca^{2+} dependent Cl^{-} channels, through which Cl^{-} ions leave the cell. The combination of these phenomena results in the depolarization of the cell membrane.[2]

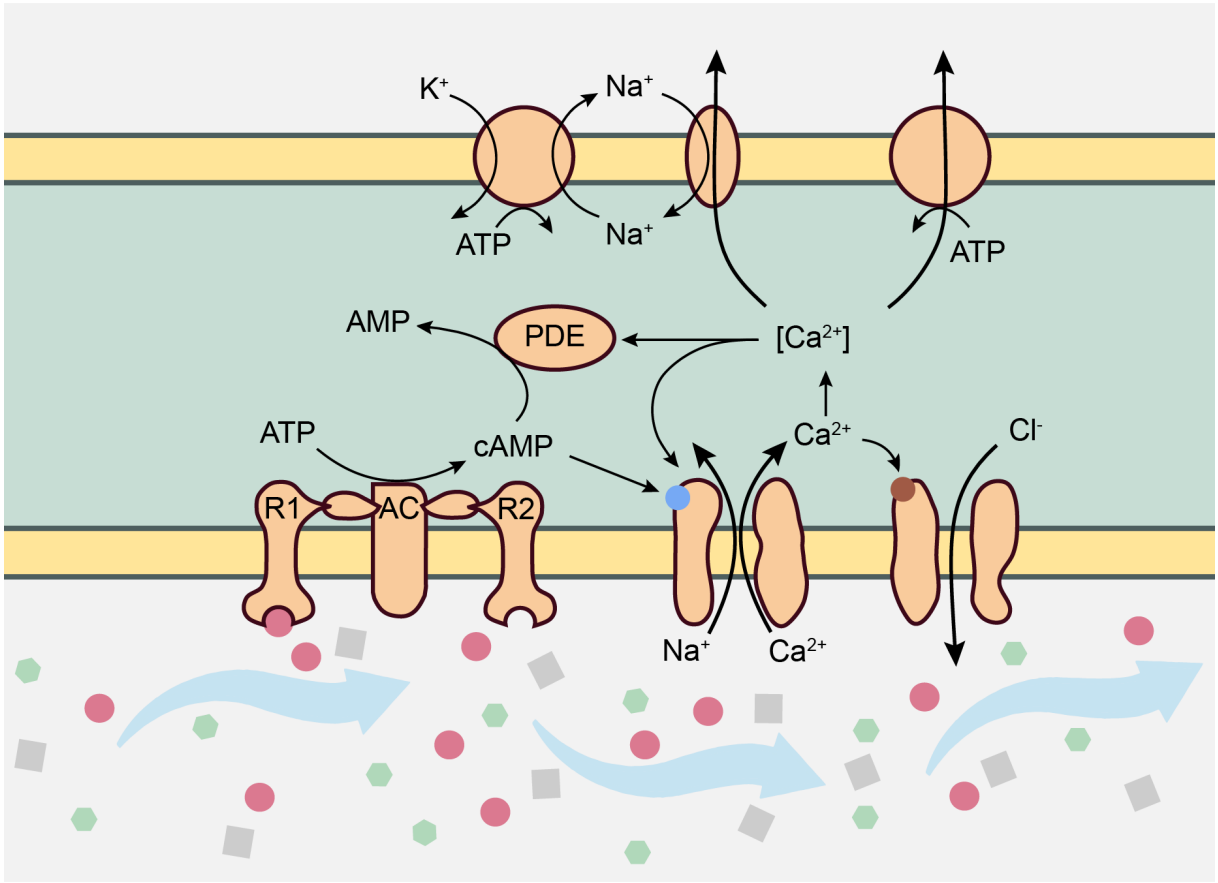

Figure 2: Signal transduction triggered by the binding of an odorant to an olfactory receptor located in the cilia membrane. Illustration inspired by Frings et al. and The Nobel Prize Foundation[3-4]

The depolarization proceeds as a neural impulse, which propagates via the axon of the olfactory neuron to the olfactory bulb (*bulbus olfactorius*) in the brain. In each cell, only one type of olfactory receptor is expressed. In the brain, axons of receptor cells

of the same receptor type gather into a bundle, a *glomerulus*. At this level, each odor results in a characteristic activation pattern. These activation patterns are transmitted via mitral cells to higher regions of the brain where the characteristic patterns are recognized and converted into the conscious experience of a specific odor.[4-5] The perception experience terminates because the odor-active compounds are released with the exhaled air, are removed by the draining mucus or undergo metabolic degradation.

Odor-active compounds have two ways to enter into contact with the olfactory epithelium: orthonasally and retronasally. In the orthonasal perception, odorants evaporate from the food and enter the nasal cavity through the nostrils along with the ambient air during inhalation. Together with the visual properties, the orthonasal odor properties provide the first sensory impression of the food before it is consumed. The retronasal odor perception takes place after the food has been introduced in the oral cavity, in particular during the swallowing process. Indeed, at this stage the odor-active compounds are transferred from the rear into the nasal cavity, causing an intense odor event. During mastication, normally no odor perception occurs yet, as the velum is closed.[5-6]

3.1.2 Characteristics of odor-active compounds

An important prerequisite for a substance to be odor-active is its volatility. This property is influenced by the molecular weight and the polarity. The polarity must be well balanced in order to allow the substance to initially dissolve in the olfactory mucus and to subsequently form hydrophobic interactions with the odorant receptors. Volatility represents an essential attribute for a compound to be odor-active but it is not sufficient. In fact, the compound has also to be present in the food matrix in adequate amounts to provoke a sufficient receptor response. The minimum concentration required for detection is commonly known as odor threshold value (OTV) and is substance-specific. A compound may only contribute to the overall aroma of a food if the odor threshold is reached or exceeded. The odor thresholds of odorants present in food cover a wide range of concentrations, actually over 10 orders of magnitude. For instance, (1*R*)-phenylethanethiol, a potent odor-active compound in curry leaves, possesses an odor threshold value in water of 0.00054 µg/kg,[7] while e.g., 2-methylpropanoic acid, a well-known odorant in cheese, can only be detected above a concentration of 60000 µg/kg.[8]

Nowadays it is still hardly possible to directly measure the absolute concentration of a volatile needed to interact at the human receptor level. For this reason, odor thresholds in a selected matrix are often employed as a useful tool to estimate the odor activity of a volatile compound.[9] The odor threshold value depends primarily on the volatility and the structure of the odorant. Subtle variations in the molecular structure can lead to clear differences in the perceived odor and in the odor threshold value. Pyrazines are a representative example: depending on the substitution, their odor is green, nutty, popcorn-like, musty, pea-like or coffee-like and their OTVs range from 0.000002 to 10

mg/L in water.[10-12] In addition to the functional groups, also the conformation of the odor-active molecule can be decisive for its odor quality and odor threshold value. For instance, vanillin smells like vanilla whereas isovanillin is almost odorless. (*S*)-(+)-Carvone smells like caraway, (*R*)-(−)-carvone reminds of the spearmint smell. Naturally occurring (−)-geosmin has an OTV 11 times lower than the synthetic (+)-isomer.[13] Even the odorant concentration in the food can play a role in the odor quality perception. At low concentrations, only receptors to which the odorant binds with high affinity are activated. At higher concentrations also receptors to which it binds with lower affinity are activated. This phenomenon can lead to a different odor quality perception depending on the odorant amount. For example, at low concentrations, β-ionone smells like violets, but at higher concentrations it smells like cedar wood.[5] Although several studies were undertaken to predict odor threshold values from the chemical structure, no successful approach is known so far.

3.1.3 Interpersonal variations in the odor perception

The intensity and the quality of the odor perception is not only influenced by the properties of the odor-active compound but also by the sniffer's individual sensitivity to that odor. Although every person has almost the same number of functional OR genes (*i.e.* the same number of different olfactory receptors), only monozygotic twins have an identical set of receptors. Every person possesses a unique combination of ORs, that is as characteristic as his fingerprint.[14]

As ~ 400 types of different ORs exist in humans, single nucleotide polymorphisms (SNP) in at least one receptor gene are rather common in the population. This can result in a non-functional receptor and a corresponding olfactory blindness to certain odorants, known as specific anosmia.[15-16] People with an anosmia for a compound cannot perceive it at a concentration at which it is clearly recognized by most other people.[17] For example, 36% of the human population is anosmic for 2-methylpropanal, which is a key odorant in malt.[18]

This diverse sensitivity is also involved in the preference or dislike of a food. Much effort is currently being made to deeper understand the role of genetic variants in the heterogeneous perception of foods' odor and taste.[19-21] An iconic case is cilantro, a herb popular in many cuisines around the world. Some people like it but many others claim that it has an unpleasant soapy aroma. This peculiar impression is largely attributed to several aldehydes present in the herb.[22] It was recently discovered that cilantro preference or dislike may correspond to genetic variants in the olfactory receptors. Indeed, in the cilantro preference group studied, a single nucleotide polymorphism significantly associated with soapy aroma perception was found. This polymorphism involves the genes that encode for OR6A2, an olfactory receptor with a high binding specificity for aldehydes that give cilantro its characteristic odor.[23]

Variations in the odor perception do not only stem from genetic causes. Indeed, some pathologic conditions, from a simple cold to more severe conditions such as

craniocerebral trauma or COVID-19 can provoke a permanent or temporary dysfunction of the sense of smell.[24-26]

3.2 The identification of key odorants

Often hundreds of volatiles have been identified in a single food (e.g. more than 800 in roasted coffee).[27] The majority of volatile food constituents do not interact with the olfaction system at the concentrations they are present in the food and therefore these volatiles are not odor-active. Odor-active compounds evoke at least a sensory signal on the primary perception level (receptor cell) and may be consciously perceived when tested individually, but not necessarily when present in a mixture with other odor-active compounds. The compounds that actually do have an influence on the overall olfactory properties of a complex food system are defined as key odorants. The process to unequivocally identify the key odorants in a food is part of the so-called molecular sensory science approach and comprises four steps: 1) isolation of the volatile compounds, 2) odorant screening by application of an aroma extract dilution analysis (AEDA), 3) calculation of odor activity values and 4) aroma reconstitution and omission experiments. In this approach, analytical methods are combined with sensory methods.[28-29]

3.2.1 Isolation of volatiles

The first step to isolate the volatiles from food is typically solvent extraction. As most odorants are lipophilic, they can be extracted from the food matrix by nonpolar organic solvents. Solvents with low boiling points are preferred as their subsequent removal for extract concentration is facilitated and the risk of compound degradation and artifact formation is minimized. Widely employed solvents are dichloromethane (b.p.: 40 °C) and diethyl ether (b.p.: 35 °C).

Extraction is followed by the solvent-assisted flavor evaporation (SAFE), a technique which aims at the separation of the volatiles from the nonvolatile compounds. Removal of nonvolatiles is essential because the analytical method of choice for the subsequent screening is gas chromatography (GC). During SAFE the volatile compounds evaporate at moderate temperatures under high vacuum and are subsequently recondensed with the aid of liquid nitrogen. This approach minimizes the degradation of odorants as well as the formation of artifacts. Due to these advantages, SAFE has become the mostly used approach for the isolation of volatiles from complex food matrices. The distillate obtained by SAFE contains both, odor-active and odorless volatile compounds.[30] In the next step, the distillate is concentrated using, for example, a Vigreux column and a Bemelmans microdistillation device.[31]

3.2.2 Odorant screening

The aroma concentrates are subjected to odorant screening by gas chromatography-olfactometry (GC-O). As in a standard GC setup, a fused silica capillary column is

placed in a thermostated oven (Figure 3). The sample is applied cold on-column in order to avoid thermal degradation in the injector block. Inside the oven, a controlled temperature program is applied. Volatile molecules are separated in the column according to their boiling points and their polarities. At the column end, the effluent is divided 1:1 by a Y-shaped glass splitter: one part of the effluent reaches the flame ionization detector (FID), which is connected to a recorder, while the other part reaches a heated exit called sniffing port.

During a GC-O run, a trained assessor places the nose closely above the sniffing port and evaluates the effluent odor by sniffing. By doing so, the nose is used as a second detector. Whenever an odor is perceived, the position as well as the respective odor quality are noted on the FID chromatogram plotted by the recorder.

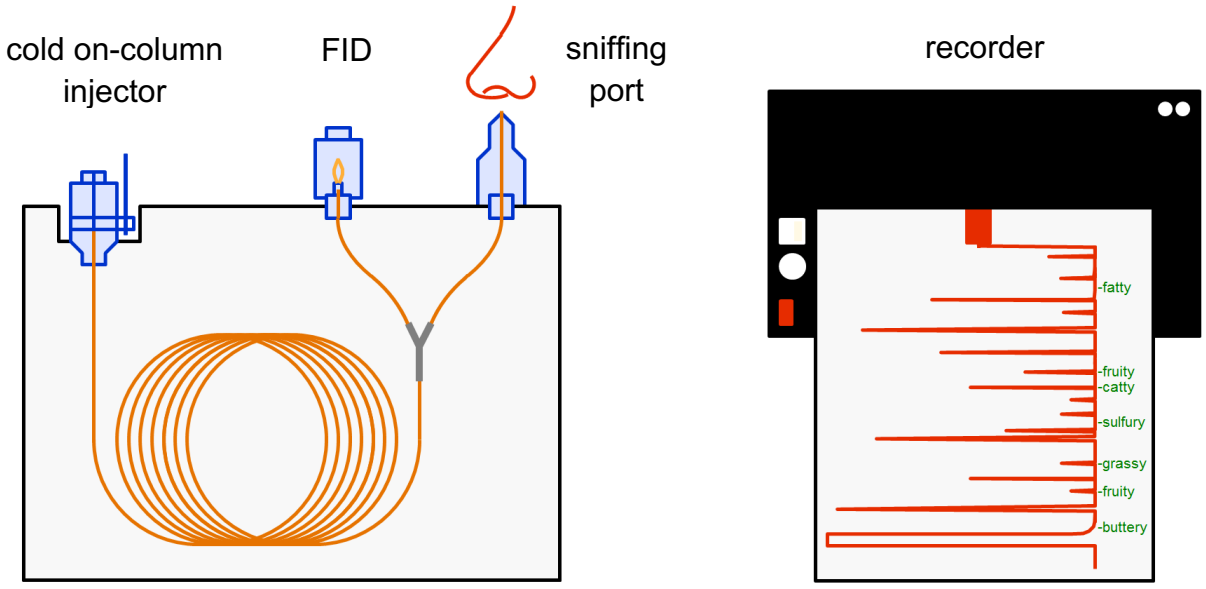

Figure 3: Scheme of a GC-O/FID system. Illustration: Martin Steinhaus

To distinguish the most potent odor-active compounds from the less potent, a ranking is performed by means of an aroma extraction dilution analysis (AEDA). The initial volatile isolate is stepwise diluted with solvent at a ratio of 1:2, thus obtaining dilutions of 1:2, 1:4, 1:8, 1:16, etc. Each diluted sample is evaluated by GC-O analysis. After several dilution steps, only the most potent odorants can be detected at the sniffing port. The procedure is continued until not a single odor is perceived in the GC effluent during the entire run. The dilution factor of the highest diluted sample in which the odorant was detected is defined as the flavor dilution (FD) factor of the odorant (Figure 4).[32] Due to specific anosmia, an assessor might be insensitive to certain odorants. For this reason, GC-O analyses must always be carried out by at least two assessors with complementary olfactory abilities, *i.e.*, a specific anosmia present in the one assessor must not be present in the other, and *vice versa*.[16] GC-O analysis is fundamental to prevent overlooking important odor-active trace compounds. Indeed,

they might not be detectable by an FID due to their low concentrations but clearly detectable by sniffing at the sniffing port.

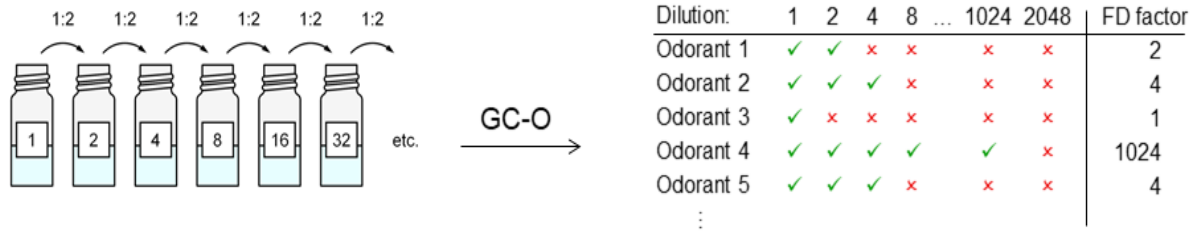

Figure 4: Aroma extract dilution analysis: stepwise extract dilution, GC-O and FD factor determination (illustration: Martin Steinhaus)

Structure elucidation of the compounds detected during screening is performed by the comparison with authentic reference compounds analyzed in appropriate dilution under the same conditions. The parameters taken into account for a first comparison are the retention indices (RIs) on two capillaries of different polarity and the odor quality as perceived at the sniffing port. Retention indices are calculated from the retention times of the odor-active regions and the retention times of adjacent *n*-alkanes by linear interpolation.[33] Subsequently, mass spectra are obtained by gas chromatography-mass spectrometry (GC-MS) in the electron (EI) and chemical ionization (CI) modes.[29]

To facilitate the structure assignments and avoid coelution during GC-MS analyses, a fractionation into neutral and basic volatiles (NBV) and acidic volatiles (AV) can be performed. The compounds in fraction NBV can be further fractionated according to their polarity by silica gel chromatography. Furthermore, thiols can be selectively isolated by mercurated agarose gel.[34] After fractionation, the odorants are localized in the individual fractions by GC-O before they are subjected to GC-MS analysis.

In case of chiral compounds, the assessment of the enantiomeric ratio is fundamental, since the odor as well as the OTV of the isomers can differ significantly.[35-37] If a reference compound for structure elucidation is not commercially available, it needs to be obtained by synthesis. The structure of the synthesized compound has to be confirmed by means of nuclear magnetic resonance (NMR) spectrometry.

3.2.3 Odor activity value calculation

To approximate the contribution of the odorants to the overall aroma, it is helpful to calculate their odor activity values (OAVs). The OAV is defined as the ratio of the odorant concentration to the odor threshold value. When the OAV is greater than 1, the compound may be relevant for the overall food aroma.

$$OAV = \frac{Conc.}{OTV}$$

OAV: odor activity value

Conc.: odorant concentration (µg/kg)

OTV: odor threshold value in an appropriate matrix (µg/kg)

The gold standard for odorant quantitation is the application of stable isotope dilution assays (SIDA): a ^{2}H or ^{13}C substituted analogue of the analyte is added to the sample prior to the workup as an internal standard.[38] The advantage of this approach is that any loss of the analyte is fully compensated, because the standard, which has virtually the same chemical and physical properties as the analyte, is behaving in an identical way under the workup conditions.

By means of GC-MS, the peak areas of the selected quantifier ions for the analyte and the internal standard are recorded. Odorant concentrations in the food sample can then be calculated from the analyte peak area, the internal standard peak area, the amount of food used for the workup and the amount of standard added, by employing a calibration line equation, obtained by linear regression after analysis of analyte/standards mixtures in different concentration ratios. Due to the rapid development of GC-MS techniques within the last decades, it is become possible to determine the concentration of volatiles even when present in trace amounts.[38]

In order to assess the impact of the individual compounds for the overall food aroma, their concentrations are determined. To determine the odor threshold value of an odorant, a matrix with similar properties of those of the food of interest has to be selected. In fact, the matrix' structural properties influence the odorants' release into the air, resulting in a specific odor threshold value in each matrix. Typical matrices employed to simulate foods and beverages for these experiments are oil, water, starch or ethanol-water mixtures.[9, 39] Odor threshold values are determined according to the American Society for Testing and Materials (ASTM) procedure for the determination of odor and taste thresholds by a forced-choice ascending concentration series method of limits.[40] The matrix is spiked with the test substance at different decreasing concentrations. The spiked samples are filled into vessels and presented, together with unspiked samples, to a sensory panel consisting of 15–20 trained assessors. The panel is asked to evaluate the samples by sniffing and identify the differing one. From the pattern of correct/incorrect responses, an individual threshold value is derived for each assessor. The odor threshold value of the panel is then calculated as the geometric mean of the individual thresholds as detailed by Czerny et al.[9]

3.2.4 Recombination and omission experiments

Although OAVs are a valuable tool to approximate the contribution of individual odor-active compounds to the overall aroma of a food, they do not take into account the interactions associated with the perception of odorants in a mixture. To clarify to which

extent the single odorants are crucial for the overall olfactory profile, omission experiments are applied to an aroma reconstitution model.

The aroma reconstitution, also referred to as aroma recombination or aroma re-engineering, consists of a simulation test: a model matrix is spiked with odorants with OAVs ≥ 1, generating an “aroma model” or “aroma reconstitute”. The ideal model matrix mimics the situation in the original food and needs to possess a similar water and lipid content as well as the same pH. If required, also the concentration of further major components, which might have an impact on the odorant release, should be reproduced in the matrix. Such components may include sugars, starch, minerals etc. The model is subsequently compared with the original food and the aroma similarity is evaluated by a trained sensory panel. This experiment is considered successful when a good match between the food model and the original food is achieved, meaning that all the key odorants were successfully identified and quantitated in the previous steps and, in particular, that no odor-active compound has been overlooked during screening. On the contrary, if the model significantly differs from the original food, the selection of the compounds to be quantitated needs to be broadened.[16, 29]

After successful aroma reconstitution, the final step of the approach for the identification of the key odorants consists of omission experiments. In each experiment, a single odor-active compound is omitted from the aroma reconstitution model. The incomplete model is then compared with the complete reconstitution model in a 3-alternative forced choice (3-AFC) test by the sensory panel. If a significant difference is obtained, the relevance of the omitted compound for the overall aroma of the complete reconstitution model is confirmed. The *p*-value is typically used as numeric approximation of its importance.[34, 41]

3.3 Cocoa and chocolate

Cocoa is derived from the seeds of the cocoa tree (*Theobroma cacao* L.) and represents the key ingredient in chocolate manufacturing. Chocolate bars are made of dark chocolate, milk chocolate or white chocolate, often in combination with nuts, raisins, or crisped rice or with soft fillings made of marzipan, coconut, caramel or yoghurt. Chocolate is also an ingredient in a broad range of confectionary products such as bars, cookies, cakes, and puddings. On certain Western holidays, including Christmas, Easter, Valentine's day and Hanukkah, it is traditional to make gifts made out of chocolate molded into different shapes e.g. eggs, hearts, and coins.

The world cocoa production in the 2018-2019 crop exceeded 4 million tons.[42] The growing consumption and popularity of cocoa products is mainly to attribute to their unique sensory properties. In addition, in the last decades several health benefits connected to cocoa consumption have been highlighted.[43] The niche market of fine or flavor cocoa is experiencing the highest percentage of growth compared to other cocoa segments. This increasing demand is driven by consumers searching for healthier chocolate, chocolate of single origin or chocolate with peculiar organoleptic properties such as floral, fruity, caramel and nutty aroma notes.

3.3.1 The cocoa tree

The cocoa tree belongs to the Malvaceae family, is evergreen, 4–8 m tall and native to the tropical regions of the Americas. During the 16th century, cocoa reached Europe, where chocolate beverages quickly became an appreciated commodity among the nobility. Linneus gave to the plant the scientific name *Theobroma cacao* in 1753. Theobroma means food of the gods in Latin and cacao derives from the Aztec word xocolatl, from xococ (bitter) and atl (water).[44-45]

The natural habitat of the cocoa plant is the tropical evergreen forest. As the tree is sensitive to direct sunlight and wind, it is frequently cultivated with other shade-giving trees. Other climatic factors, such as temperature and rainfall also influence the tree development. High humidity plays a key role and has to be around 70% during the day and around 100% during the night.[46] This optimum type of climate is found in regions within a latitude of 10° from the equator. In this geographical area the world leaders in cocoa production, such as Ivory Coast, Ghana, Ecuador, Dominican Republic and Indonesia are located.[47] Despite the fact that cocoa is native to tropical America, West African countries, particularly Ivory Coast and Ghana, are nowadays the most important cocoa producers, accounting for ~ 60% of the world cocoa supply, followed by Central and South America with 14% and Southeast Asia with 16%.[48] The majority of the production is done in small or medium-sized farms; only 30% of the cocoa production originates from high-end farming.[49]

The fruit grows directly from the trunk and from thicker branches and is commonly named cocoa pod. It is an egg-shaped berry with a yellow to brown color at maturity (Figure 5).

Figure 5: Different stages of the cocoa pod ripening. Photo: Martin Steinhaus

Each pod contains 30 to 50 seeds which are surrounded by a white mucilaginous pulp with a sweet-sour taste (Figure 6).[50] Fresh seeds are characterized by an unpleasantly strong astringency and bitterness.

Figure 6: Opened cocoa pod. Photo: Martin Steinhaus

Depending on the genetic background, fresh seeds can be violet, light pink or white.[51] The seed consists of an inner part surrounded by a shell, botanically referred to as the embryo and the testa, respectively (Figure 7).[52] The embryo is formed from two large storage cotyledons, representing ~ 85% of the seed's dry weight. The testa protects the embryo from mechanical damage and represents ~ 15% of the dry weight.[53]

Figure 7: Fermented cocoa seed in which the two main parts (testa and embryo) are clearly distinguishable. Photo: Caterina Porcelli and Martin Steinhaus

3.3.2 Cocoa varieties

There are four main cultivar groups of cocoa used to manufacture cocoa products: Criollo, Nacional, Forastero and Trinitario. They are distinguished by the geographic origin, the morphological features of the fruit and the aroma characteristics of the cocoa seeds.[50, 54]

Criollo, the variety used by the Mayas, is rare and highly prized due to its high sensitivity to climate fluctuations and to diseases and the consequently rather small yield. It is less bitter and less astringent than other varieties and shows more floral aroma notes.[50] Only 5–10% of the global chocolate production is made with this cocoa variety. On most cocoa plantations, Criollo cocoa is rarely grown alone but is typically cultivated along with Forastero cocoa. The latter is widely cultivated and the plant is more robust and provides higher yields. The Forastero variety is classified as bulk or ordinary cocoa grade. It shows less fine chocolate notes in the aroma when compared to the Criollo type and a stronger basic cocoa note. Due to their low price, Forastero seeds are employed for 80% of the world chocolate production. A further cultivar is Trinitario cocoa, generated by hybridization of Criollo and Forastero varieties and combining the fine flavor of the first and the resistance of the second. It has strong basic chocolate aroma characters and some wine-like flavor and is employed in ~ 10–15% of chocolate production. The Nacional variety grows only in Ecuador and is characterized by floral and green aroma notes. Criollo, Trinitario and Nacional types are classified as fine or flavor cocoas due to their peculiar aroma and are mainly used to manufacture dark specialty chocolate.[44, 54-55]

As the different cocoa cultivar groups significantly differ with respect to the development of sour, bitter, astringent, nutty and smoky-hammy notes, chocolate produced with a single bean variety can differ considerably in flavor from chocolate obtained from bean blends.[56]

3.3.3 Cocoa processing and chocolate manufacturing

Cocoa undergoes several processing steps before it is consumed as chocolate product. Numerous parameters in these processes can impart an enormous variability to flavor and overall quality.

The processing of cocoa starts with the harvest of the pods (Figure 8), which is usually carried out manually using machetes. The pods are opened and both, the fresh seeds and the surrounding mucilaginous pulp, are transferred into wood boxes or are heaped on palm or banana leaves. This has to be done within 24 hours to avoid pre-fermentation and seed germination. The fermentation time span ranges from 2 to 10 days and is variety specific. For Criollo 2 or 3 days are sufficient to reach optimum fermentation, while Forastero requires between 5 and 8 days. Beside the duration, also the mixing intervals can influence the final flavor profile.[48, 50]

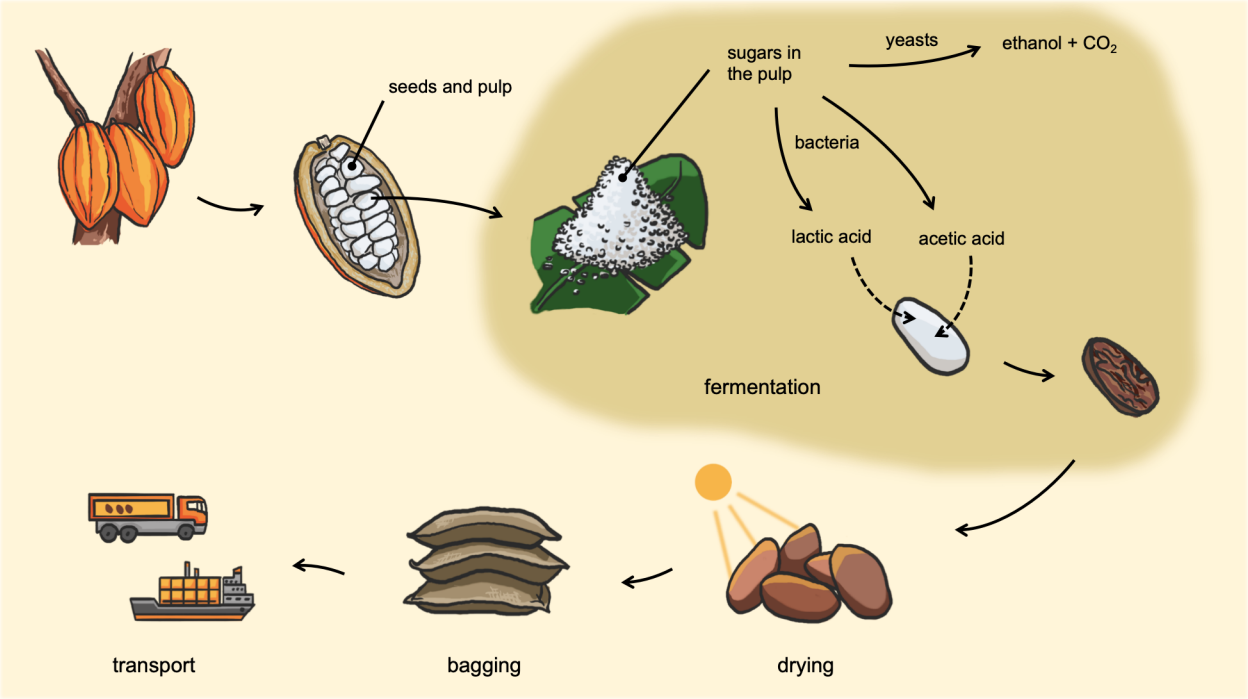

Figure 8: Cocoa processing steps carried out in the country of origin. Illustration: Caterina Porcelli

During fermentation, the cocoa beans undergo complex processes that alter their original chemical and physical properties and lead to the death of the seeds. The fresh and moist cocoa seeds are colonized by microorganisms present in the surrounding environment, e.g. in the air, in the fermentation boxes, on the surface of the leaves used as basis for the heaps or on fruit flies. Overall, the microorganisms' activities determine the degradation of the fruit pulp, the temperature increase of the fermentation mass and the acidification of the seed tissue.[50, 57]

At the begin of the fermentation process, the high amount of sugars (sucrose, glucose, and fructose) present in the fruit pulp together with its acidity (pH < 4, mainly originating from citric acid) offer an optimum medium for yeasts to grow. During the first 24–36 hours, yeasts convert the sugars to ethanol and carbon dioxide under anaerobic conditions. Lactic acid bacteria (LAB) produce lactic acid (lactic fermentation). At this stage most seeds are still alive.

Due to the secretion of pectinase, the pulp is liquefied and drains off.[50]. As a result, the conditions become aerobic. Oxygen-mediated reactions start, one of the most important being the conversion of soluble polyphenols into insoluble polymers catalyzed by polyphenol oxidases, which results in reduced bitterness, reduced astringency and browning.[12, 58] Lactic acid bacteria are replaced by acetic acid bacteria (AAB), which oxidize ethanol to acetic acid (acetic fermentation). This process is exothermic and leads to heating of the fermenting mass up to 48 °C. The temperature increase in combination with the pH drop caused by the acetic acid uptake provokes the death of the embryo.[59]

The enzymatic reactions occurring during cocoa fermentation generate essential flavor precursors such as peptides and free amino acids. Their concentrations at the end of the fermentation process are crucial for the optimal development of typical odorants during subsequent roasting. Indeed, a considerable number of investigations have demonstrated that in case the fermentation step is skipped, too short or carried out in an inappropriate manner, the beans develop only weak cocoa aroma when roasted.[12, 54, 60]

Even though the fermentation stage is considered the most crucial in the transformation process from seed to chocolate, it is often carried out by the farmers or small enterprises in an empirical way, frequently without a meticulous control of the processing conditions. This can result in cocoa batches with heterogeneous quality and also in the occasional development of off-flavors. A typical issue is overfermentation which means fermentation beyond the optimum time. In that case, aerobic bacteria can lead to an increase of the pH value, to blackening of the beans and to unwanted flavors.[59]

After fermentation, the seeds have a moisture content of ~ 60%. This value is reduced to ~ 7% in the subsequent drying step. The reduction of the moisture content is essential to avoid mold growth and overfermentation during storage.[54] In most cocoa growing countries, the relatively low rainfall during the harvest season allows sun drying which normally takes 7 days. The seeds are distributed on mats or on wooden floors, which are in some cases sheltered from rainfalls. Sun drying is preferable as it proceeds slowly, resulting in a more effective removal of acetic acid.[58] If the climatic conditions are inadequate for sun drying, the seeds are dried artificially. In this approach, wood fires or oil burners heat the air, which then passes through the cocoa seeds. The direct contact between the seeds and the smoke has to be prevented, as cocoa easily adsorbs volatile phenols from smoke, resulting in a smoky-hammy off-flavor.[61] Too rapid drying or the exposure to excessive heat may result in insufficient removal of volatile acids such as acetic acid. On the other hand, incomplete drying or rain soaking can lead to microbial growth and the development of a moldy-musty off-flavor.[48] The oxidative processes started during fermentation continue during drying.[52] Furthermore, Maillard reaction may already occur at this stage.

After drying, the cocoa seeds can be stored up to 12 months in silos or jute bags before they are shipped from the country of origin to the chocolate manufacturing companies. There, the seeds are cleaned to remove stones, twigs, and other debris. Afterwards, the seeds are roasted (Figure 9). Roasting is another processing step crucial for the development of the characteristic chocolate aroma. Moreover, roasting further reduces acetic acid and decreases the moisture content to 1–2%.[48]

Cocoa is normally roasted at temperatures between 120 and 140 °C depending on the cocoa variety.[62] The temperature of choice depends also on the subsequent use: a high degree of roasting is required for the production of cocoa powder whereas a low degree of roasting is desired for the production of cocoa butter. Overall, the temperatures are much lower than those applied for the roasting of nuts and coffee. Cocoa overroasting (at temperatures higher than 140 °C) causes the development of

a burnt, bitter and coffee-like off-flavor.[12] Cocoa can be roasted as whole seeds, as nibs or as liquid cocoa liquor. The roasting of smaller particles has the advantages of a better controlled and homogeneous roasting level and of a more efficient removal of acetic acid. Furthermore, a shorter roasting time can be applied when the particle size is reduced: for whole seeds ~ 30 min are needed, for nibs 12 min and for liquor only 2 min.[52] The thermal treatment leads to an intense Maillard reaction. Carbonyl groups of reducing sugars react with the amino groups of free amino acids and short-chain peptides. Compounds formed during roasting include alcohols, ethers, furans, thiazoles, pyrones, acids, esters, imines, amines, oxazoles, and pyrroles.[62]

An optional alkalization treatment, also known as “Dutching” is sometimes included in the roasting process but can also be performed prior to roasting. It consists of the addition of alkalizing agents such as potassium carbonate, sodium carbonate or sodium hydroxide and has the aim to partially neutralize the acids in the cocoa. This treatment is performed when the production of cocoa powder is intended. Alkalization increases the dispersability of cocoa powder in beverages and contributes to a darker color.[58, 63]

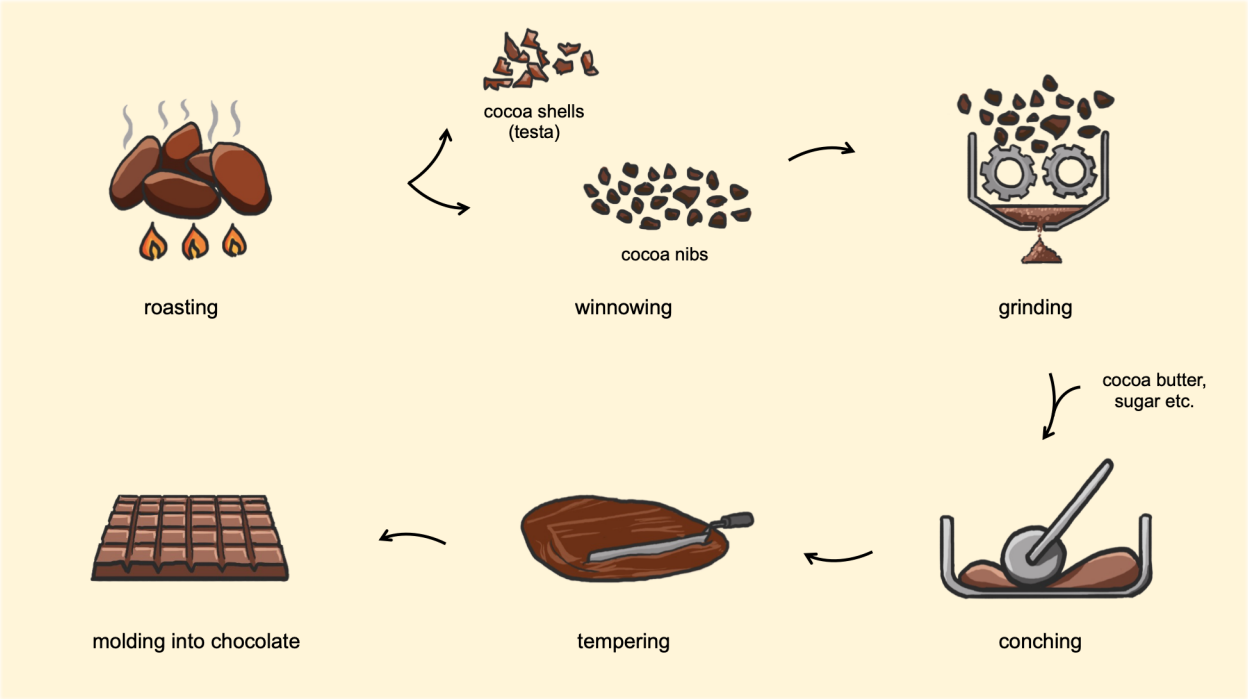

Figure 9: Chocolate production. Illustration: Caterina Porcelli

The roasted cocoa is ground and thereby the cocoa butter melts resulting in cocoa liquor. By pressing, a part of the cocoa butter can be separated. The pressing residue can be used to make cocoa powder, also referred to as cocoa solids. However, in most cases cocoa liquor is directly processed into chocolate mass by the addition of sugar, cocoa butter and, depending on the desired chocolate product, other ingredients such as milk powder and vanillin.

The penultimate step of cocoa processing is the conching, a multiday mixing and heating treatment. Chocolate mass is stirred and ground at temperatures above 40 °C in containers by metal cylinders, which make constant back and forth movements. Conching aims at creating a smoother texture by reducing the size of the cocoa and sugar particles to a level below that the tongue can detect (~ 20 µm). In addition, it improves the flavor by further reducing the amount of acetic acid. Dark chocolate is typically treated at temperatures between 70 and 80 °C. High-quality chocolate undergoes conching for about 72 hours, whereas bulk grade chocolate is conched up to 6 hours. After the treatment is completed, the chocolate mass is stored in tanks heated at 45–50 °C until final processing.

To induce chocolate hardening, a temperature decrease of the mass is required. The triglycerides of the cocoa butter start to form crystals. Once a sufficient number of crystals have developed, chocolate can be poured into molds and further cooled. During cooling the crystals increase in number and size very quickly until the chocolate is thoroughly solidified.

Cocoa butter can arrange in 6 different crystal forms (polymorphous crystallization), each characterized by different properties such as melting point and breaking behavior (Table 1). The goal of chocolate makers is to exclusively obtain crystals of form V when chocolate starts to cool down. Indeed, consistently small cocoa butter crystals result in the uniform sheen and hard snap of properly processed chocolate. On the other hand, uncontrolled crystallization of cocoa butter results in crystals of variable dimensions, in some cases big enough to be very clearly visible. This causes the surface of the chocolate to appear greyish and matt and the chocolate to crumble rather than to snap when broken.

Table 1: Different crystal forms of cocoa butter and their respective properties[64]

Crystal form	Melting temperature	Properties
I	17 °C	Soft, crumbles, too prone to melting
II	21 °C	Soft, crumbles, too prone to melting
III	26 °C	Firm, poor snap, too prone to melting
IV	28 °C	Firm, good snap, too prone to melting
V	34 °C	Glossy, firm, optimum snap, melts near body temperature (37 °C)
VI	36 °C	Hard, requires several days to form

To ensure that a sufficient amount of crystals of form V are present in the cocoa butter before molding, conched chocolate undergoes a final processing step known as tempering, which aims at the proper pre-crystallization of the cocoa butter. This process does not only guarantee the desired appearance and snap of the finished product, but it also causes the chocolate to contract during cooling, making it easier to unmould. Tempering is achieved by spreading the chocolate on a metal or marble worktop forming a thin layer. The chocolate is kept under constant stirring carefully monitoring the temperature throughout the process. This treatment can be replaced by “seeding” the liquid chocolate with pre-crystallized chocolate. In both cases, the final result is a stable crystalline structure with the desired properties. Sometimes, tempering is performed prior to conching to shorten the conching times by about 30%.[12]

Chocolate is extremely sensitive to temperature, light exposure and humidity. Ideal storage conditions correspond to temperatures between 15 and 17 °C and a relative humidity of less than 50%. Being highly prone to absorb other aromas, it is usually wrapped in aluminum foil and stored away from other foods.

3.3.4 Cocoa volatiles

Despite the fact that taste- and odor-active compounds represent a very low percentage of the overall composition, they play a crucial role for the quality of cocoa and of the products made thereof. This has stimulated research on cocoa aroma since 1912, when Bainbridge and Davies performed the pioneering studies which led to the identification of linalool, acetic acid and several esters in a distillate prepared from two tons of roasted cocoa beans.[65] In the following decades, comprehensive investigations have aimed at disclosing the complex aroma of cocoa, leading to the identification of hundreds of volatile compounds in different types of roasted cocoa.[66-67]

Nowadays, ~ 600 odorants are known.[12] Several alkyl-substituted pyrazines, such as tetramethylpyrazine, as well as aldehydes, such as phenylacetaldehyde, 2-methylbutanal and 3-methylbutanal were quite early suggested as important contributors to cocoa aroma.[68-69] In 2006, Frauendorfer et al. carried out the first systematic study on the key aroma compounds in roasted cocoa by using molecular sensory science approaches.[63]

The aroma character of roasted cocoa is influenced by the genotype, the farming practices, the environmental conditions but, above all, by the complex biochemical and chemical reactions occurring during the postharvest processing and roasting.[54, 63]

3.3.5 Cocoa off-flavors

Ideally, fermented cocoa shows a rich aroma with floral, sour, malty, earthy and fruity notes.[54] During further processing additional odor notes develop and the overall aroma intensifies.[62, 70] Occasionally, however, fermented cocoa beans are tainted with off-flavors. Among these, moldy-musty, smoky, coconut-like, cheesy, fecal, and mushroom-like odor notes are most prevalent.[61, 71-72] When these undesirable notes are pronounced and survive the manufacturing process, they can severely affect the sensory quality of the final chocolate products. Consumers used to a specific product flavor tend to be very susceptible even to minor variations. In the worst case, cocoa off-flavors may lead to complaints, expensive product recalls and severe damage to the brand reputation.[73]

Whereas the compounds responsible for the pleasant cocoa aroma and their development during processing have been studied in detail,[12, 63, 65-66] there was only little information available in the scientific literature on the identity of compounds causing flavor defects.

In 2016 Aprotosaie et al. published an extensive study on the flavor of cocoa. However, the occurrence of off-flavors was only briefly mentioned.[54] The only investigation dealing specifically with cocoa off-flavors dates back to 1992, when Ney reported the occurrence of smoky, acidic, moldy, hammy, bitter and astringent off-notes in cocoa.[71] Among them, the moldy off-flavor is described as particularly detrimental. However, no investigation on the causative compounds was carried out.

Recently, the application of the molecular sensory science approach lead to the unequivocal identification of 2-methoxyphenol, 3- and 4-ethylphenol, 3- and 4-methylphenol and 3-propylphenol as the crucial odorants for the smoky off-flavor in cocoa,[61] but no such investigation has been carried out on the frequently reported moldy musty off-flavor and the atypical coconut note so far.

Moldy-musty off-flavors in cocoa are often found in combination with mold growth. This is often associated with insufficient mixing during fermentation, overfermentation, slow or insufficient drying, or storage and transportation under high humidity. In some cases, mold is very clearly visible on the surface of the seeds (Figure 10). However, in many other cases it is at the first stages of its development and can be detected only by performing the so-called cut-test, a widely applied quality control method in the cocoa industry.[74]

Figure 10: Fermented cocoa with very well-visible mold growth on the seeds' surface. Photo: Caterina Porcelli

Another atypical odor has recently been detected at the incoming goods inspection level in the cocoa industry, namely an odor described as coconut-like. This note is clearly not as aversive as the smoky and moldy-musty off-flavors and such cocoa might even be considered suitable to manufacture specialty chocolate. Nevertheless, a pronounced atypical coconut-like note unpredictably occurring in the final chocolate products is clearly undesired.

4 Objectives

The quality of cocoa is crucial for the pleasant aroma of chocolate products. Before further processing in the chocolate industry, fermented cocoa undergoes a critical evaluation by a sensory panel at the incoming goods inspection level to detect batches tainted with off-flavors.[75] The assessment of flavor defects by a sensory panel, however, is prone to false negative results, e.g. considering the fact that their perception is very subjective and specific anosmias are quite frequent in the population.[16] Therefore, there is a strong demand for more objective methods such as the targeted quantitation of the compounds causative for the individual off-flavors.

Although the presence of off-flavors in cocoa is a known phenomenon, still little data is available in the scientific literature on the causative compounds. The primary aims of the present research project were to characterize the odor-active compounds causing a moldy-musty and a coconut-like odor in cocoa by (i) screening the volatiles isolated from tainted cocoa for potential off-flavor compounds by aroma extract dilution analysis (AEDA) and (ii) substantiating the role of the individual compounds by quantitation and calculation of odor activity values (OAVs).

5 Results and discussion

The present thesis is a publication-based dissertation. Data was summarized in three articles published in international peer reviewed scientific journals. For each publication, a copy of the original, a summary including the individual contributions of the authors, as well as the reprint permission of the publisher can be found in the appendix.

5.1 Molecular background of a moldy-musty off-flavor in cocoa

5.1.1 Odorant screening

For the characterization of the compounds responsible for the moldy-musty off-flavor in cocoa, a sample of fermented cocoa seeds (M1) was selected for its pronounced moldy-musty off-flavor and employed in the screening for odor-active compounds. A sample of fermented cocoa seeds with a pleasant aroma without off-notes (REF) was selected as reference and analyzed in parallel.[76]

To isolate the volatile compounds, cocoa seeds were homogenized and the powder obtained was mixed with organic solvent. Nonvolatiles were removed by SAFE. The SAFE distillates were concentrated and subjected to comparative analysis by AEDA. Results revealed 57 odorants exhibiting an FD factor of 8 or higher in at least one of the two samples. Structure assignment of the odor-active compounds was achieved by comparison of the retention indices on two columns of different polarity (FFAP and DB-5), the odor properties as perceived at the sniffing port of the GC-O system and the mass spectra obtained by GC-MS (in EI and CI mode) with data from authentic reference compounds analyzed under the same conditions. The aroma concentrates were first separated into a fraction containing the neutral and basic volatiles (NBV) and a fraction containing the acidic volatiles (AV) by acid-base extraction. To facilitate the structure assignements of the odorants detected by GC-O and avoid coelution during GC-MS analyses, the NBV fraction was further fractionated into five sub-fractions of different polarity by using silica gel chromatography. By application of this approach each odor-active region could be unequivocally assigned to the causative compound.

In both the samples, several well-known cocoa odorants, previously reported in numerous studies, were detected. Among these were honey-like, floral-smelling 2-phenylethanol, floral, citrusy-smelling linalool, rancid, sweaty-smelling 2-methyl-propanoic acid and fruity and blueberry-like smelling ethyl 3-methylbutanoate.[54, 77-78]

The compounds potentially contributing to the moldy-musty off-flavor were identified by their odor qualities in combination with an FD factor being higher in the off-flavor sample than in the reference sample. The criteria were met by 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF), geosmin, 1*H*-indole and 3-methyl-1*H*-indole (Table 2).

Table 2: Potential off-flavor compounds identified among the volatiles isolated from fermented cocoa seeds with a pronounced moldy-musty off-flavor (M1) in comparison to a reference cocoa sample (REF)

odorant[a]	odor	RI[b]		FD factor[c]	
		FFAP	DB-5	M1	REF
MDMF[d]	caramel, musty	1577	1055	256	<1
geosmin	moldy, beetroot	1816	1413	2048	<1
1*H*-indole	fecal, mothball	2452	1294	32	4
3-methyl-1*H*-indole	fecal, mothball	2481	1390	16	2

[a]Structure assignments were based on retention indices (FFAP, DB-5), mass spectrum and odor quality; data was compared to those of authentic reference compounds analyzed under the same conditions. [b]Retention index; calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [c]Flavor dilution factor. [d]4-methoxy-2,5-dimethylfuran-3(2*H*)-one.

Among the four potential off-flavor compounds, moldy, beetroot-like smelling geosmin showed the highest FD factor, namely 2048, in the moldy-musty cocoa sample (M1), but was not detected in the reference sample (REF). This compound is a well known product of microbial metabolism for which very low odor detection threshold values in the range of 1−20 ng/kg had been reported.[79] Geosmin-associated off-flavors have been reported in fish, cereals, drinking water and wine but so far not in cocoa.[80-83] It is yet unclear whether geosmin formation in cocoa is caused by molds or rather by bacteria such as *Streptomyces* species.

Another potential off-flavor compound revealed by the comparative AEDA was caramel-like and musty-smelling 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF). In the moldy cocoa sample, its FD factor was 256, whereas, like geosmin, it was not detected in the reference sample. The occurrence of MDMF has been shown in different kinds of fruit such as mango, guava, kiwi, and strawberry.[11, 84] The biosynthesis of MDMF starts from fructose 1,6-bisphosphate and proceeds via the formation of 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF). The latter is then converted into MDMF by an S-adenosyl-L-methionine-dependent O-methyl-transferase.[85] Although the odor of MDMF at lower concentrations is pleasant and described as sweet and caramel-like, the odorant exhibits an additional rather unpleasant musty note at higher concentrations.

Comparative AEDA also led to the identification of 1*H*-indole and 3-methyl-1*H*-indole. Their odor quality, described as fecal and mothball-like was not directly linked to the overall-dominating moldy and musty off-flavor but nevertheless considered highly unpleasant. Both compounds were also detected in the reference cocoa sample, nevertheless their FD factors were higher in the off-flavor cocoa sample (32 and 16 vs. 4 and 2). 1*H*-Indole and 3-methyl-1*H*-indole have been reported as products of the decomposition of the amino acid L-tryptophan mediated by bacteria.[86]

5.1.2 Enantiomeric distribution of geosmin

Geosmin is a chiral molecule with three stereogenic centers. Its identification in the cocoa sample with moldy-musty off-flavor was accomplished using a commercially available racemic mixture of (+)- and (−)-geosmin. Nevertheless, it has been demonstrated that the two enantiomers significantly differ in their odor potency. The OTV of the (−)-isomer is ~ 10 times lower than that of the (+)-isomer.[13, 80, 87]

However, no investigation on the enantiomeric distribution in cocoa was reported in the literature so far. Due to the very high FD factor in the moldy sample, a deeper study on this compound was performed, with the aim to determine its enantiomeric distribution in the moldy-musty smelling cocoa sample. Aroma concentrates obtained from cocoa were analyzed by two-dimensional GC with heart-cutting using a β-cyclodextrin-modified stationary phase in the second dimension and a high resolution mass spectrometer as the detector (Figure 11A). A solution of the racemic mixture was analyzed under the same conditions (Figure 11B).

The chromatogram showed that exclusively one of the two enantiomers of geosmin was present in the cocoa sample. Considering the elution order of the enantiomers on the chiral stationary phase employed, which had been published previously,[88] the moldy smelling odorant detected in the off-flavor cocoa sample was unequivocally identified as (−)-geosmin. The presence of this isomer has also been reported in fish, drinking water and wine whereas no natural occurrence of the (+)-isomer has been demonstrated so far.[13, 89]

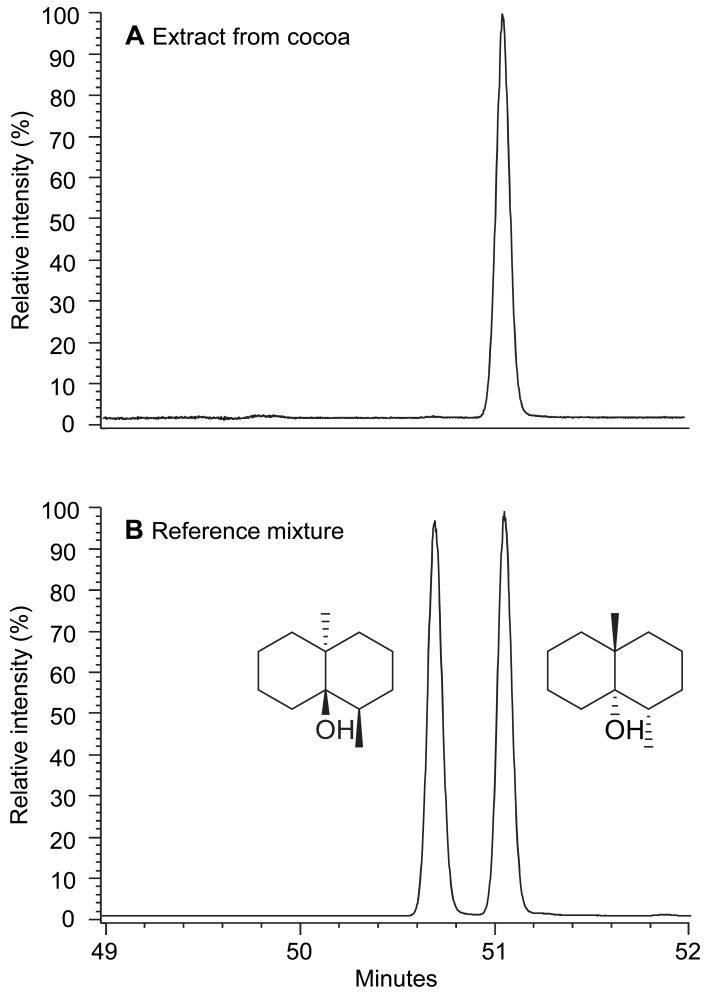

Figure 11: Analysis of an extract obtained from the pronounced moldy cocoa sample (A) in comparison to a racemic reference mixture of geosmin (B) by GC-GC-HRMS with a β-cyclodextrin-modified stationary phase in the second dimension

5.1.3 Quantitation and calculation of OAVs

To substantiate the role of the four off-flavor compounds identified in the screening experiments for the moldy-musty off-flavor, their concentrations were determined by GC-MS analysis and their odor activity values were calculated in a total of ten cocoa samples. The samples included the off-flavor cocoa sample and the reference sample previously used for odorant screening. In addition, six cocoa seed samples and two cocoa liquor samples showing a more or less pronounced moldy-musty off-flavor were included in the quantitation experiments (M2−M9).

Quantitation was accomplished by using SIDA in combination with heart-cut GC-GC-MS analysis. Cocoa samples were homogenized and mixed with organic solvent. To compensate for losses during the workup, stable isotopologues of the target compounds were added as internal standards. Stable isotopically substituted odorants

4-($^2H_3$)methoxy-2,5-dimethylfuran-3(2*H*)-one and 3-($^2H_3$)methyl(2,4,5,6,7-$^2H_5$)-1*H*-indole were prepared according to procedures described in the literature.[90-91] (2,3,4,5,6,7-$^2H_6$)-1*H*-indole was prepared from the isotopically unmodified compound using the same procedure published for the synthesis of 3-($^2H_3$)methyl(2,4,5,6,7-$^2H_5$)-1*H*-indole, which was based on a forced H/D exchange.[91]

For ($^2H_3$)geosmin, a novel synthetic eight-step procedure was developed as depicted in Figure 12.[92] Inexpensive racemic 2-methylcyclohexan-1-one (**1**) was reacted with an auxiliary chiral amine using previously published procedures.[93-94] After Michael addition of the resulting imine to pent-1-en-3-one, the amine was removed by hydrolysis with acetic acid followed by an intramolecular aldol condensation to afford the bicyclic intermediate (4a*R*)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one (**3**). The double bond in **3** was epoxidized using mCPBA. The epoxide was then subjected to reduction with $LiAlD_4$, which lead to the incorporation of two deuterium atoms, resulting in diol **4**. Selective tosylation of the diol, followed by a second reduction step with $LiAlD_4$, finally afforded trideuterated (–)-geosmin [(4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$)octahydronaphthalen-4a(2*H*)-ol)] (**5**). The final product was purified by flash chromatography using a diol stationary phase. The incorporation of three deuterium atoms was confirmed by GC-MS. Further investigation on the structure was performed by one- and two-dimensional NMR experiments. Overall, the synthetic approach developed afforded ($^2H_3$)geosmin with 24% yield and 91% enantiomeric purity.

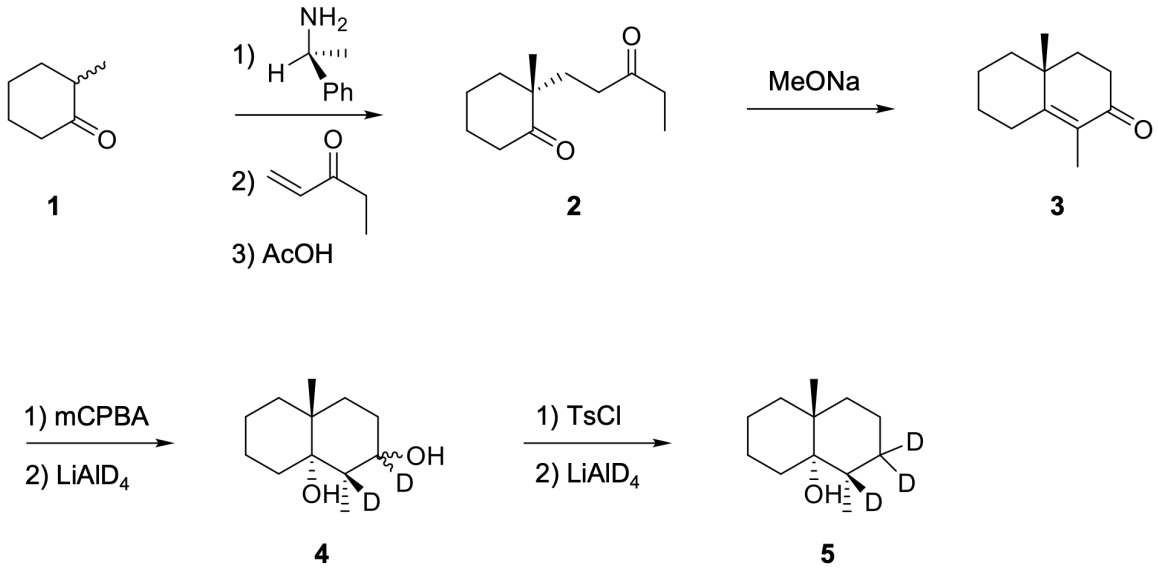

Figure 12: Synthetic approach affording ($^2H_3$)geosmin [(4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$)octahydronaphthalen-4a(2*H*)-ol][92]

After addition of the stable isotopologues of the target compounds, nonvolatiles were removed by SAFE. The SAFE distillates were concentrated and the concentrates were analyzed by heart-cut GC-GC-MS analysis in CI mode.

To assess the impact of the individual compounds for the moldy-musty off-flavor in cocoa, OAVs were calculated by dividing the concentrations by the respective OTVs. OTVs were determined in deodorized cocoa butter by using the standard practices of the ASTM.[40] The results of the OTV and OAV calculations are shown in Table 3. The OTVs determined for 3-methyl-1*H*-indole and (–)-geosmin were 1.1 µg/kg and 1.6 µg/kg, respectively. Both were clearly lower than the OTVs of 1*H*-indole and MDMF, which were 51 µg/kg and 350 µg/kg, respectively.

The OAVs calculated for the four potential off-flavor compounds in the reference sample were all < 1, meaning that their concentrations were not exceeding the respective OTVs. In the case of 1*H*-indole, the OAVs were < 1 also in off-flavor samples, suggesting a minor importance of this compound for the overall moldy-musty off-flavor in cocoa. MDMF showed an OAV > 1 only in M1, the cocoa sample with the most pronounced moldy-musty odor. Considering the facts that the OAV of MDMF in all the other off-flavor cocoa samples was consistently < 1 and, even in M1, with an OAV of 1.3, its concentration was only slightly exceeding its threshold, MDMF was also considered to play a rather minor role for the moldy-musty off-flavor in cocoa. Interestingly, (–)-geosmin showed OAVs > 1 only in four of the nine off-flavor cocoa samples investigated whereas in all off-flavor cocoa samples 3-methyl-1*H*-indole showed OAVs >. Furthermore, OAVs of 3-methyl-1*H*-indole were significantly higher than OAVs of (–)-geosmin in eight of the nine off-flavor cocoa samples.

Table 3: Odor activity values (OAVs) of off-flavor compounds in nine cocoa samples with moldy-musty off-flavor (M1−M9) and in a reference cocoa sample without off-flavor

odorant	OTV[a] (µg/kg)	OAV[b]									
		M1[c]	M2	M3	M4	M5	M6	M7	M8	M9	REF[c]
MDMF	350	1.3	<1	<1	<1	<1	<1	<1	<1	<1	<1
(–)-geosmin	1.6	7.2	<1	2.2	2.2	2.7	<1	<1	<1	<1	<1
1*H*-indole	51	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
3-methyl-1*H*-indole	1.1	29	12	11	1.9	20	11	60	74	26	<1

[a]Odor threshold value in deodorized cocoa butter. [b]Odor activity values were calculated as ratio of the concentration in the cocoa sample to the OTV. [c]Samples M1 and REF were the samples previously used for odorant screening by AEDA (cf. Table 2).

Nevertheless, these data could not provide an explanation for the overall olfactory profile of the off-flavor cocoa samples, which rather resembled the moldy, beetroot-like odor of (–)-geosmin than the fecal, mothball-like odor of 3-methyl-1*H*-indole. This led to the hypothesis that the two odorants might not be homogeneously distributed within the cocoa seeds.

Indeed, (−)-geosmin, being mainly produced by aerobic microorganisms, might be present at higher concentrations on the seeds' surface. By contrast, 3-methyl-1*H*-indole as product of anaerobic species might be depleted on the surface.[86, 95]

5.1.4 Distribution of (−)-geosmin and 3-methyl-1*H*-indole in the seeds

To gain a deeper insight into the distribution of (–)-geosmin and 3-methyl-1*H*-indole in the cocoa seeds, the two compounds were quantitated after separation of the seed shell (testa) from the inner part (embryo). This separation was achieved by hand with the aid of a kitchen knife and was performed for two different off-flavor samples. Results of the investigation are shown in Table 4.

In sample M5, in which (–)-geosmin showed an overall OAV of 2.7 (cf. Table 3), an OAV of 5.6 was determined in the testa, whereas in the embryo the OAV was only 1.8. Similarly, in sample M6, in which (–)-geosmin had shown an overall OAV < 1 in the previous quantitation experiment (cf. Table 3), the OAV was higher in the testa (1.8) but considerably lower in the embryo (0.2).

3-Methyl-1*H*-indole showed the opposite behavior. In sample M5 its OAV in the testa was only 6.1, whereas in the embryo an OAV of 16.6 was calculated. In sample M6, the OAVs in testa and embryo were 3.3 and 7.8, respectively, meaning that the concentration was consistently lower in the testa than in the embryo.

Table 4: Distribution of (–)-geosmin and 3-methyl-1*H*-indole between testa and embryo in two cocoa samples with moldy-musty off-flavor (M5−M6)

odorant	sample, part	OAV[a]	distribution (%)[b]
(–)-geosmin	M5, testa	5.6	42
	M5, embryo	1.8	58
	M6, testa	1.8	65
	M6, embryo	0.2	35
3-methyl-1*H*-indole	M5, testa	6.1	8
	M5, embryo	16.6	92
	M6, testa	3.3	8
	M6, embryo	7.8	92

[a]OAVs were calculated as ratio of the concentration in the cocoa bean part to the OTV of 1.6 µg/kg for (–)-geosmin and 1.1 µg/kg for 3-methyl-1*H*-indole. [b]Calculated from the concentrations in the cocoa bean parts and the mass percentages of testa to embryo, which were 19% to 81% in sample M5 and 17% to 83% in sample M6, respectively.

In conclusion, the higher concentration of (–)-geosmin on the surface of the cocoa seeds in combination with the lower concentration of 3-methyl-1*H*-indole explained the overall dominating moldy-musty odor note perceived in the off-flavor cocoa samples. A further consequence of the inhomogeneous distribution whithin the cocoa seeds that need to be considered is that the testa is removed during further cocoa processing. Indeed, although the testa constitutes less than 20% of the seed weight, it could be shown that it contained the major portion of (–)-geosmin, namely 42 and 65% of the total amount determined in the two samples investigated in our study (cf. Table 4, column “distribution”). The testa removal plays a less crucial role for 3-methyl-1*H*-indole, because with 8% only a minor percentage of this compound is located in the testa.

In summary, the results of the current investigation suggest that the analytical monitoring of fermented cocoa at the incoming goods inspection level in the chocolate industry should include the quantitation of (–)-geosmin and 3-methyl-1*H*-indole to objectively detect moldy-musty off-flavors. Since it was shown that both compounds are inhomogeneously distributed between the testa and the embryo, separate quantitation in the two parts of the seeds is recommended. Sensory threshold values of 1.6 and 1.1 µg/kg are suggested as maximum tolerable concentrations for (–)-geosmin and 3-methyl-1*H*-indole, respectively.

5.2 Molecular background of an atypical coconut-like odor in cocoa

5.2.1 Odorant screening

To elucidate the compounds responsible for an atypical coconut odor in cocoa, a sample of fermented cocoa (C1) was selected for its pronounced coconut note and used for a screening for odor-active compounds. A sample of fermented cocoa with a typical aroma profile without any off-note (REF) was analyzed in parallel and used as reference.[96]

To isolate the volatile compounds, cocoa seeds were homogenized and the powder obtained was mixed with organic solvent. Nonvolatiles were removed by SAFE. The SAFE distillates were concentrated and subjected to a comparative analysis by AEDA. Results revealed 48 odorants exhibiting an FD factor of 4 or higher in at least one of the two samples. Structure assignment was performed as detailed for the moldy-musty off-flavor (cf. section 5.1.1). This resulted in the unequivocal assignment of 46 odor-active regions to the respective causative compounds.

Among the compounds identified, six showed a coconut odor. All six coconut-like smelling compounds were γ- or δ-lactones, namely δ-octalactone, γ-nonalactone, δ-2-octenolactone, γ-decalactone, δ-decalactone and δ-2-decenolactone (Table 5). δ-2-Decenolactone showed the highest FD factor in the cocoa sample C1 with the atypically pronounced coconut-like odor, namely 2048. With 256, the FD factor of δ-2-decenolactone in the reference sample was clearly lower. High FD factors in sample C1 were additionally obtained for γ-nonalactone (1024), γ-decalactone (1024), and δ-octalactone (512). For these three compounds, however, the FD factors indicated no difference between sample C1 and the reference sample. Low FD factors of δ-2-octenolactone and δ-decalactone suggested only a minor role of these compounds for the atypical odor in C1.

Lactones are biosynthesized from fatty acids. After introduction of a hydroxy group, the chain length is reduced by β-oxidation. The shortened hydroxy carboxylic acids undergo cyclization under acidic conditions, finally affording the lactones.[97] Some γ- and δ-lactones have been previously reported as major contributors to the aroma of different kinds of fruits such as apricots and peaches,[98-101] milk products,[84, 102] wines and spirits.[103] The six coconut-like smelling lactones identified in the current study have been reported in cocoa before.[61, 63, 76-77] Nevertheless, none of them has been reported as responsible for an atypical aroma note in fermented cocoa so far. Considering the fact that their coconut-like odor quality exactly matched the atypical note detected in cocoa sample C1, the six odorants were suggested to play a crucial role, whereas the substantial contribution of other compounds was assumed rather unlikely.

Table 5: Compounds with coconut-like odor identified among the volatiles isolated from fermented cocoa seeds with an atypically pronounced coconut odor (C1) and a reference cocoa sample (REF)

odorant[a]	odor	RI[b]		FD factor[c]	
		FFAP	DB-5	C1	REF
δ-octalactone	coconut	1984	1250	512	512
γ-nonalactone	coconut	2029	1393	1024	1024
δ-2-octenolactone	coconut, creamy	2047	1264	16	16
γ-decalactone	coconut, peach	2140	1466	1024	1024
δ-decalactone	coconut	2210	1494	32	1
δ-2-decenolactone	coconut	2255	1475	2048	256

[a]Structure assignments were based on retention indices (FFAP, DB-5), mass spectrum and odor quality; data was compared to those of authentic reference compounds analyzed under the same conditions. [b]Retention index; calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [c]Flavor dilution factor.

5.2.2 Quantitation and calculation of OAVs

The results obtained by screening were substantiated by quantitation of the six coconut-smelling compounds identified as potential causative compounds and by calculation of odor activity values. The investigation was extended to two additional cocoa samples (C2 and C3) which also showed an atypical, though less pronounced coconut note than sample C1.

Quantitation was accomplished by heart-cut GC-GC-MS analysis. Cocoa samples were homogenized and mixed with organic solvent. To compensate for losses during the workup, stable isotopically substituted were added prior to the workup as internal standards.

[6-(2,3-$^2H_2$)Propyloxan-2-one],[104] [5-(1,2-$^2H_2$)pentyloxolan-2-one], [5-(1,2-$^2H_2$)hexyl oxolan-2-one][105] and [6-pentyl(3,3,4,4-$^2H_4$)oxan-2-one][102] were employed for the quantitation of δ-octalactone, γ-nonalactone, γ-decalactone and δ-decalactone, respectively. Isotopologues were not available for δ-2-octenolactone and δ-2-decenolactone. Instead, the deuterated isotopologues of the corresponding saturated lactones were used as internal standards. After addition of the standards, nonvolatiles were removed by SAFE. The SAFE distillates were concentrated and the concentrates were analyzed by heart-cut GC-GC-MS analysis in the CI mode.

To assess the impact of the individual compounds for the atypical coconut note in cocoa, OAVs were calculated as ratio of the concentrations in the four samples to the OTVs of the individual compounds. OTVs were determined in low odor sunflower oil by using the standard practices of the American Society for Testing and Materials (ASTM).[40] The results of the OTV and OAV calculations are shown in Table 6.

Table 6: Odor activity values (OAVs) of six lactones in three cocoa samples with an atypically pronounced coconut odor (C1−C3) and in a reference cocoa sample (REF)

odorant	OTV[a] (µg/kg)	OAV[b]			
		C1[e]	C2	C3	REF[e]
δ-octalactone	1600[c]	<1	<1	<1	<1
γ-nonalactone	1300[c]	<1	<1	<1	<1
δ-2-octenolactone	4700[c]	<1	<1	<1	<1
γ-decalactone	4800[c]	<1	<1	<1	<1
δ-decalactone	4300[c]	<1	<1	<1	<1
δ-2-decenolactone	120[d]	13	4.7	1.7	<1

[a]Odor threshold value in low odor sunflower oil. [b]Odor activity values were calculated as ratio of the concentration in the cocoa sample to the OTV. [c]OTV of a racemic mixture. [d]OTV of a commercial sample with unknown enantiomeric ratio. [e]Samples C1 and REF were the samples previously used for odorant screening by AEDA (cf. Table 5).

However, the OAVs calculated included an approximation due to the chirality of the lactones. The OTVs of δ-octalactone, γ-nonalactone, δ-2-octenolactone, γ-decalactone and δ-decalactone were determined using racemic mixtures, whereas the enantiomeric distribution in the cocoa samples was unknown. Furthermore, the OTV of δ-2-decenolactone was determined using a commercial mixture whose enantiomeric composition was also unknown. For δ-octalactone, γ-nonalactone, δ-2-octenolactone, γ-decalactone and δ-decalactone OAVs < 1 were determined in all four samples, meaning that they were not exceeding the OTVs of the racemates. In contrast, δ-2-decenolactone showed approximated OAVs of 13 in sample C1, 4.7 in sample C2 and 1.7 in sample C3, whereas in the reference sample, the OAV was < 1. These results suggested that δ-2-decenolactone was mainly responsible for the atypical coconut-like odor of samples C1, C2, and C3.

δ-2-Decenolactone was first reported in 1937 by Abe who identified it as the major odor-active compound in the bark of the massoia tree (*Cryptocaria massoia*), a tropical tree growing wild in the rain forests of New Guinea. Massoia bark oil was widely used in the past as coconut flavoring before cheaper synthetic flavorings became available.[106] In massoia bark, exclusively the (*R*)-isomer of δ-2-decenolactone is present.[107-108] Therefore, (*R*)-δ-2-decenolactone is also often referred to as massoia lactone.

5.2.3 Chiral analysis of δ-2-decenolactone

To gain a deeper understanding of the role of δ-2-decenolactone for the atypical note in the coconut-like smelling cocoa sample C1, a spiking experiment was considered the method of choice: δ-2-decenolactone would be added to the reference cocoa sample REF to reach the same concentration as previously determined in C1 and the mixture would be sensorially evaluated.

To do so, the enantiomeric distribution of the δ-2-decenolactone in the spiking solution as well as in the cocoa sample has to be taken into account. Indeed, it has been demonstrated that enantiomers often significantly differ in their odor threshold values and even in their odor qualities. This is true, for example, for a homologous series of saturated γ- and δ-lactones.[98, 103] In most cases, the (*R*)-enantiomers showed a higher odor potency than the (*S*)-enantiomers. For δ-2-decenolactone, however, no such data was available in the literature.

To investigate the enantiomeric distribution of the commercial δ-2-decenolactone, a reference solution was analyzed by enantioGC using a β-cyclodextrin-modified stationary phase and by following the same principle as described in Section 5.1.2 for geosmin. The experiment revealed an enantiomeric ratio of 17/83. Analysis of a natural massoia bark oil enabled to assign the elution order as S before R on the column employed. Thus, it could be concluded that the commercially obtained reference compound consisted of 83% (*R*)-enantiomer and 17% (*S*)-enantiomer.

GC-O showed that both enantiomers possess a coconut-like odor. The method of Ullrich and Grosch[108] was applied to evaluate their individual odor potency: the odor threshold values in air of both δ-2-decenolactone enantiomers were determined by AEDA. Results (Table 7) showed that also in δ-2-decenolactone, the (*R*)-enantiomer represented the more potent odorant. With 1.6 ng/L, its odor threshold value was ~ 30 times lower than that of the (*S*)-enantiomer.

Table 7: Odor threshold values (OTVs) of (*R*)- and (*S*)-δ-2-decenolactone in air

Odorant	Odor quality	OTV_{air} (ng/L)
(*R*)-δ-2-Decenolactone	Coconut	1.6
(*S*)-δ-2-Decenolactone	Coconut	52

Afterwards, the enantiomeric distribution of δ-2-decenolactone in cocoa was investigated: the volatile isolates were analyzed by two-dimensional gas chromatography with heart-cutting using the chiral column in the second dimension and a high resolution mass spectrometer as the detector. Results showed that the δ-2-decenolactone in cocoa was pure (*R*)-enantiomer. In addition to massoia bark and fermented cocoa, enantiopure (*R*)-δ-2-decenolactone has also been reported in Merlot and Cabernet Sauvignon musts and wines, where it contributes to dried fruit aroma notes.[107]

Knowing the enantiomeric purity of δ-2-decenolactone in fermented cocoa and the difference in the odor potency of the two enantiomers, a better approximation of its relevance for the atypical coconut-like odor was possible. From the OTVs of the individual enantiomers in air (cf. Table 7) and the OTV of the reference mixture with 83% (*R*)- and 17% (*S*)-enantiomer in oil (120 µg/kg; cf. Table 6), the OTVs of the individual enantiomers in oil were approximated. The calculations resulted in OTVs in oil of 3300 µg/kg for the (*S*)-δ-2-decenolactone and 100 µg/kg for the (*R*)-δ-2-

decenolactone. Using the latter for the OAV calculations of (*R*)-δ-2-decenolactone in the cocoa samples resulted in values of 16 in C1, 5.6 in C2, and 2.1 in C3, whereas in the reference sample REF the OAV was below 1 (Table 8). In summary, these data substantiated the hypothesis that (*R*)-δ-2-decenolactone was the responsible compound for the atypical coconut-like odor in the fermented cocoa samples. Final evidence was eventually provided for sample C1 by means of a spiking experiment.

Table 8: Odor activity values (OAVs) of (*R*)-δ-2-decenolactone in three samples of fermented cocoa with an atypical coconut-like odor (C1, C2, C3) and in the reference cocoa sample without a coconut-like odor note (REF)

Odorant	OTV_{oil}[a] (µg/kg)	OAV[b]			
		C1	C2	C3	REF
(*R*)-δ-2-Decenolactone	100	16	5.6	2.1	0.84

[a]Odor threshold value in oil; approximated from the odor threshold value in air (cf. Table 4) and the odor threshold value of the 83/17 (*R*)/(*S*)-mixture in oil as 0.83 × 120 µg/kg + (1.6/51.5) × 0.17 × 120 µg/kg
[b]Odor activity value; calculated as ratio of the concentration in the cocoa sample (cf. Table 2) to the OTV in oil

5.2.4 Odorant spiking

The key role of (*R*)-δ-2-decenolactone for the coconut note was finally demonstrated in a spiking experiment consisting in the addition of the compound to the reference cocoa in an amount corresponding to the concentration difference between this sample and C1, in order to check whether this amount was able to provoke a coconut note in an intensity comparable to the one in the atypically smelling cocoa.

A sample of the reference cocoa REF was spiked with 1800 µg/kg of the commercial δ-2-decenolactone, corresponding to 1490 µg/kg (*R*)-δ-2-decenolactone and thus the concentration difference between the reference sample REF and the sample with the atypically pronounced coconut note C1 (cf. Table 8). In this experiment, the odor contribution of the (*S*)-isomer was considered negligible; approximated from its abundance in the commercial reference solution (17%) and its relative odor potency (1.6/51.5; cf. Table 7), this contribution was only 0.5%, whereas 99.5% of the odor could be attributed to the (*R*)-isomer.

The spiked sample was orthonasally compared to the reference cocoa without addition of (*R*)-δ-2-decenolactone and to the cocoa sample C1 with the atypical coconut-like odor note. A panel of 19 trained assessors evaluated the three samples and assigned scores ranging from 0 to 3 with 0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong to eight predefined odor descriptors previously collected by free-choice profiling. For each descriptor an odor reference was provided consisting of an aqueous odorant solution in a concentration ~ 100 times above the orthonasal odor threshold value. The eight descriptors and the corresponding reference odorants were “coconut-like” (γ-nonalactone), “vanilla-like” (vanillin), “honey-like” (phenylacetaldehyde), “banana-like” (3-methylbutyl acetate), “fruity” (ethyl 2-methylbutanoate), “vinegar-like”

(acetic acid), “earthy” (2,3,5-trimethylpyrazine), and “malty” (3-methylbutanal). The scores of the individual assessors were averaged by calculating the arithmetic mean.

Application of a quantitative olfactory profile analysis (Figure 13) showed that the spiking with the (*R*)-δ-2-decenolactone was able to provoke the atypically pronounced coconut odor. The rating of the coconut note in the spiked sample (Figure 13a) was clearly higher than that in the reference cocoa without any addition of (*R*)-δ-2-decenolactone (Figure 13b) and in the same range as the rating in sample C1 (Figure 13c).

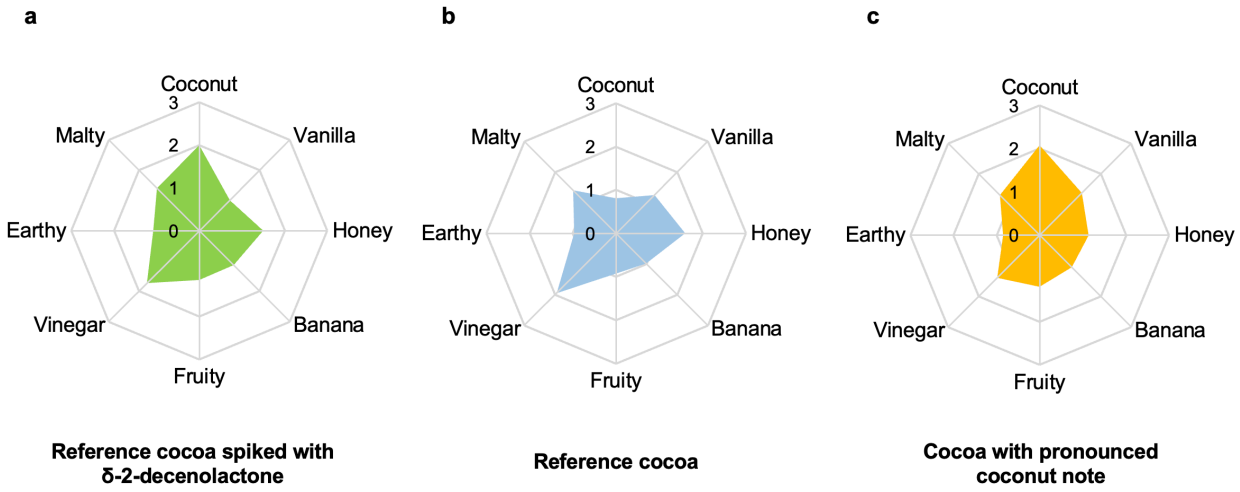

Figure 13: Olfactory profiles of the reference cocoa sample spiked with δ-2-decenolactone (**a**), the reference sample without addition (**b**) and the sample C1 with the atypically pronounced coconut note (**c**). Assessors rated the intensity of each descriptor on a scale from 0 to 3 with 0 = not detectable, 1 = weak, 2 = moderate and 3 = strong

With the spiking experiment, the crucial role of (*R*)-δ-2-decenolactone for the atypically pronounced coconout-like aroma note of sample C1 could be confirmed. The chocolate industry may consider its odor threshold value, that is 100 µg/kg, as a potential limit for the acceptance of fermented cocoa at the level of the incoming goods inspection to avoid an undesired coconut note caused by (*R*)-δ-2-decenolactone in the final products. This value can allow for a more objective decision-making on acceptance or rejection of cocoa batches than sensory testing can provide.

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7 Appendix

7.1 Publication 1

7.1.1 Bibliographic data

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RESEARCH ARTICLE

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Enantioselective synthesis of tri-deuterated (–)-geosmin to be used as internal standard in quantitation assays

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For the accurate and sensitive quantitation of the off-flavor compound geosmin, particularly in complex matrices, a stable isotopologue as internal standard is highly advantageous. In this work, we present a versatile synthetic strategy leading from (4a*R*)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one to tri-deuterated (–)-geosmin ((4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$) octahydronaphthalen-4a(2*H*)-ol). The starting material was readily accessible from inexpensive 2-methylcyclohexan-1-one using previously published procedures.

KEYWORDS

(–)-($^2H_3$)geosmin, (–)-geosmin, (4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$)octahydronaphthalen-4a(2*H*)-ol, (4*S*,4a*S*,8a*R*)-4,8a-dimethyloctahydronaphthalen-4a(2*H*)-ol, deuteration, internal standard, musty and earthy off-flavor

1 | INTRODUCTION

(–)-Geosmin (Figure 1) is a highly odorous molecule with a characteristic musty and earthy smell and a low odor detection threshold value in the range of 1–20 ng/kg.[1,2] Its name is derived from the ancient Greek words "geo" meaning earth and "osme" meaning odor.

In nature, geosmin is produced as secondary metabolite by several types of microorganisms, including actinomycetes, cyanobacteria, myxobacteria, and fungi.[3–5] The biosynthesis involves a Mg^{2+}-dependent sesquiterpene synthase, which converts farnesyl diphosphate (FPP) to a mixture of sesquiterpenoids including geosmin.[3] The compound can cause a musty and earthy off-flavor in foods and beverages such as drinking water, wine, fish, and cereals.[6–8] In the worst case, the off-flavor may lead to consumers' rejection and significant economic loss. Recently, we identified geosmin in fermented cocoa and demonstrated that it may be transferred in odor-active amounts to chocolate. To avoid this, an accurate and sensitive method for its detection and quantitation in fermented cocoa is essential. In gas chromatography–mass spectrometry (GC–MS) and in liquid chromatography–mass spectrometry (LC-MS), the use of a stable isotopically substituted analog of the target compound as internal standard is currently considered the best approach.[9,10] Although racemic deuterated geosmin is available from chemical companies, it is highly expensive (~20 000 € for 150 mg). Therefore, we attempted to find a convenient synthetic route to deuterated geosmin as an alternative to the commercial product.

Most strategies reported in the literature on the synthesis of isotopically unmodified geosmin proceeded through 1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one.[11–13] This dimethyloctalone intermediate was obtained from 2-methylcyclohexan-1-one via a Robinson annulation with pent-1-en-3-one, the latter generated in situ from 1-chloropentan-3-one by an acid-catalyzed dehydrochlorination as detailed by Zoretic et al. in 1975.[14] This approach was also adopted in a paper on the synthesis of deuterated geosmin published in 1991, which to our knowledge is the only one reporting the preparation of an isotopically modified geosmin so far.[15] In this work, the deuteration was accomplished by reacting cyclohexene oxide with ($^2H_3$)methylmagnesium iodide followed by oxidation of the obtained 2-($^2H_3$)methylcyclohexan-1-ol to 2-($^2H_3$)methylcyclohexan-1-one. The above mentioned

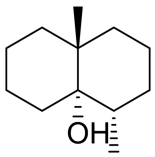
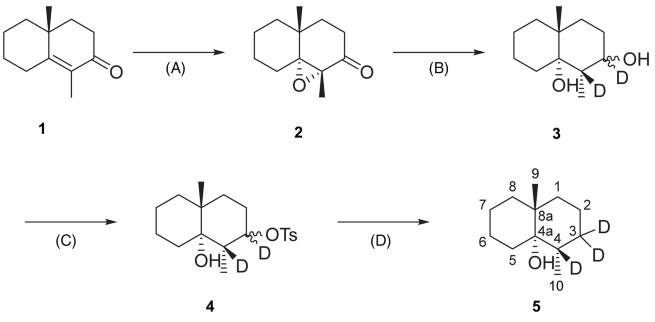


FIGURE 1 Structure of (–)-geosmin

Robinson annulation then yielded the trideuterated dimethyloctalone. However, this approach would lead to a racemic product as well as impurities due to the lack of stereo- and regiocontrol. Therefore, we decided to synthesize deuterated geosmin from the isotopically unmodified dimethyloctalone obtained in a stereoselective approach previously reported by Revial et al. They used the auxiliary chiral amine (1*S*)-1-phenylethan-1-amine to convert commercially available *rac*-2-methylcyclohexan-1-one via (2*R*)-2-methyl-2-(3-oxopentyl)cyclohexan-1-one to the enantiopure dimethyloctalone (4a*R*)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one.[12,16,17] Our aim was to use the latter compound and convert it to deuterated (–)-geosmin.

2 | RESULTS AND DISCUSSION

Enantiopure (4a*R*)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one **1** was converted to trideuterated geosmin by a sequence of four synthetic steps as depicted in Scheme 1. The first step was the epoxidation of the double bond in **1**. Based on the work of Gosselin et al.,[13] *m*CPBA was chosen as oxidizing agent. For the synthesis of isotopically unmodified geosmin, they compared the suitability of *m*-chloroperbenzoic acid and hydrogen peroxide for the epoxidation of **1**. The use of the peroxy acid afforded a 96:4 mixture of the α- and β-epoxyketones and an overall yield of 80% of the α-epimer, whereas such a high stereoselectivity could not be achieved with hydrogen peroxide. Gosselin et al.[13] concluded that the steric hindrance induced by the angular methyl group over the β-face in **1** accounts for the preferential attack of the bulky aromatic peroxy acid molecule on the α-face. We adopted the approach of Gosselin et al. for the epoxidation of **1** but applied $NaHCO_3$ as an additional base because preliminary experiments had revealed that this slightly increased the yield (data not shown). This was to be expected as the acidity of *m*-chloroperbenzoic acid can lead to side products.[18] Protonation of the double bond in the educt could lead to the formation of an alcohol whereas protonation of the epoxide would lead to the formation of a diol. The epoxidation step proceeded with a yield of 78%. The epoxide was then subjected to reduction with $LiAlD_4$, which led to the incorporation of two deuterium atoms and finally resulted in diol **3**.



SCHEME 1 Synthesis of the target molecule (4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$)octahydronaphthalen-4a(2*H*)-ol [($^2H_3$)geosmin]. Reagents and conditions: (a) *m*CPBA, $NaHCO_3$ in CH_2Cl_2, 0°C for 2 h, then RT for 24 h; (b) $LiAlD_4$, THF reflux, 2 h; (c) TsCl, Py in $CHCl_3$, 10°C for 72 h; (d) $LiAlD_4$, $CHCl_3$ reflux, 4 hours. Steps (c) and (d) were performed one pot

By selective tosylation, the secondary hydroxy group of **3** was converted into a good leaving group, affording **4**. Without isolation of **4**, a second reduction step with $LiAlD_4$ replaced the tosyl group by deuterium, finally leading to the trideuterated target molecule (4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$)octahydronaphthalen-4a(2*H*)-ol **5**, that is, ($^2H_3$)geosmin. The compound was purified by flash chromatography. The overall yield from **1** was 24%. The enantiomeric distribution of ($^2H_3$)geosmin was determined by GC–MS using a β-cyclodextrin-based chiral column. The elution order was taken from a previous report on the enantioseparation of geosmin in wine.[19] Results indicated an enantiomeric purity of 91%, which confirmed the proposed enantioselectivity of the synthetic approach.

The incorporation of three deuterium atoms was confirmed by GC–MS. The EI mass spectrum of ($^2H_3$)geosmin (Figure 2A) showed a molecular ion of *m*/*z* 185, whereas the spectrum of the isotopically unmodified geosmin showed a molecular ion of *m*/*z* 182 (Figure 2B). No signals of *m*/*z* 182, 183, and 184 were present in the spectrum of the synthesized molecule, showing that no undeuterated, monodeuterated, and dideuterated geosmin isotopologues were present. Thus, the approach resulted in a uniformly trideuterated product. Further evidence was achieved by NMR. ^1H and ^{13}C NMR spectra allowed to unambiguously assign the positions of the three deuterium atoms. The singlet obtained in the ^1H NMR spectrum confirmed the presence of the deuterium atom at C4. Moreover, the multiplicity of the signals obtained in the ^{13}C NMR spectrum for carbons C3 and C4 indicated the coupling with two and one deuterium atoms, respectively.

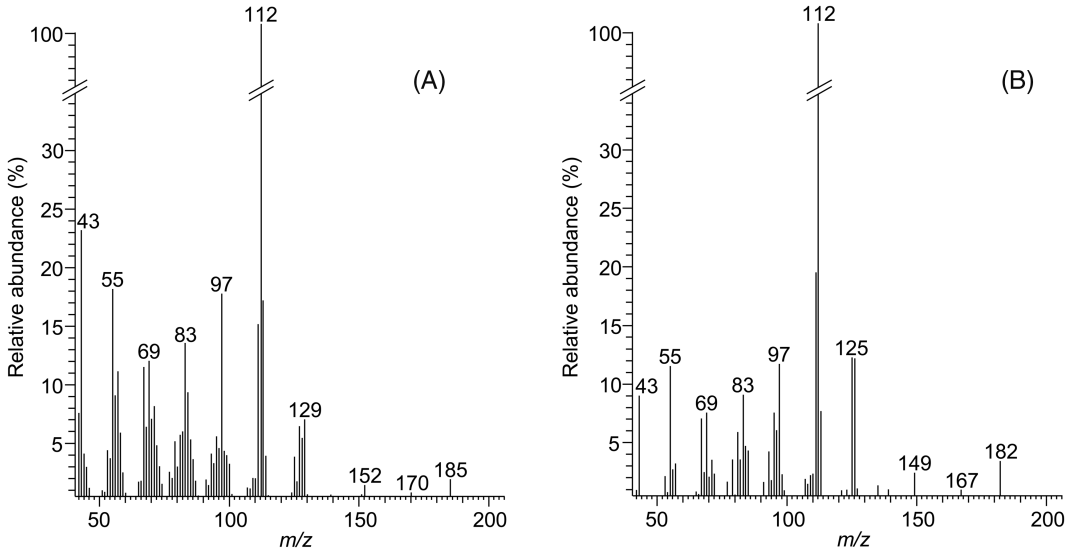


FIGURE 2 Mass spectrum of the synthesized ($^2H_3$)geosmin (A) in comparison to the mass spectrum of the isotopically unmodified compound (B)

3 | CONCLUSIONS

By using $LiAlD_4$ for the incorporation of deuterium atoms, we developed a quick and convenient approach for the preparation of uniformly trideuterated geosmin from the dimethyloctalone (4a*R*)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one with 24% yield and 91% enantiomeric purity. The synthesis proceeded in four steps among which two were performed one-pot. In combination with the stereoselective preparation of the dimethyloctalone from inexpensive racemic 2-methylcyclohexanone according to Revial et al., we achieved an overall yield of 16%. ($^2H_3$)Geosmin can be used as an internal standard for the sensitive quantitation of geosmin in complex matrices by GC–MS or LC–MS.

4 | EXPERIMENTAL

4.1 | Chemicals and materials

The chemicals used were obtained from commercial sources: *m*-chloroperbenzoic acid (77%), *p*-toluenesulfonyl chloride, pyridine, sodium sulfate, and lithium aluminum deuteride were purchased from Merck (Darmstadt, Germany); sodium bicarbonate from Alfa Aesar (Karlsruhe, Germany); tetrahydrofuran from Santa Cruz Biotechnology (Heidelberg, Germany). Diethyl ether and dichloromethane were purchased in technical grade from Fisher Scientific (Loughborough, UK) and VWR (Darmstadt, Germany), respectively, and they were freshly distilled before use. Hexane, tetrahydrofuran, and chloroform were purchased in technical grade and stored over molecular sieves (4 Å). Chloroform was filtered through alumina before use to eliminate traces of ethanol present as stabilizer. Silica gel 60 (particle size: 0.035–0.070 mm) and LiChroprep® DIOL (particle size: 0.040–0.063 mm) used for purification as well as precoated silica gel thin-layer chromatography (TLC) plates (layer thickness 750 µm, no fluorescence indicator) used for reaction monitoring were purchased from Merck. Hexane and diethyl ether mixtures in different proportions were used as mobile phase. Cerium ammonium molybdate or potassium permanganate solutions were employed in TLC as stains for substance detection, followed by heat treatment (200°C).

4.2 | Gas chromatography–mass spectrometry

EI mass spectra were recorded using a GC–MS system consisting of a Trace GC Ultra gas chromatograph coupled to a single quadrupole ISQ mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany). Compounds were dissolved in dichloromethane at a concentration of ~20 µg/mL. An aliquot (1 µL) was introduced by an autosampler GC PAL, PAL Firmware 2.5.2 (Chromtech, Bad Camberg, Germany), into a PTV injector (Thermo Fisher Scientific) at 40°C. The injector temperature was raised at 12°C/s to 60°C (held for 0.5 min) and then by 10°C/s to 240°C (held for 1 min). The carrier gas was helium at a flow rate of 2 mL/min. The splitflow

was 24 mL/min. The column was a DB-1701 coated fused silica capillary, 30 m × 0.25 mm i.d., 0.25-μm film thickness (Agilent, Waldbronn, Germany). The initial oven temperature was 40°C. After 2 min, it was raised at 6°C/min to 230°C (held for 5 min). Mass spectra were acquired at an ionization energy of 70 eV and a scan range of 40–300 *m/z*. The mass spectra were evaluated using Xcalibur 2.0 software (Thermo Fisher Scientific). Chemical ionization (CI) mass spectra were recorded using an enantioGC–MS system consisting of a Trace 1310 gas chromatograph coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific). Compounds were dissolved in dichloromethane at a concentration of ~10 μg/mL. An aliquot (1 μL) was introduced by a TRI Plus RSH autosampler (Thermo Fisher Scientific) into a PTV injector (Thermo Fisher Scientific) used in on-column mode. The carrier gas was helium at a flow rate of 1 mL/min. The column was a BGB-176 coated fused silica capillary, 30 m × 0.25 mm i.d., 0.25-μm film thickness (BGB Analytik, Rheinfelden, Germany). The initial oven temperature was 40°C. After 2 min, it was raised at 2°C/min to 200°C, and the final temperature was held for 5 min. Mass spectra were acquired with targeted SIM mode at an ionization energy of 70 eV and a scan range of 50–500 *m/z*. The reagent gas was isobutane. The mass spectra were evaluated using Xcalibur 2.0 software (Thermo Fisher Scientific).

4.3 | NMR spectroscopy

One-dimensional and 2-D NMR data (^{1}H, ^{13}C, gs-COSY, gs-HSQC, and gs-HMBC) were acquired with an Avance III 400 MHz system (Bruker, Rheinstetten, Germany) equipped with a *Z*-gradient 5-mm multinuclear observe probe ($BBFO_{plus}$) at 298 K. The compounds were dissolved in $CDCl_3$ containing 0.03% (v/v) TMS (Eurisotop, Saint-Aubin, France). All spectra were referenced to TMS (0.0 ppm). For data processing and analysis, Topspin 3.2 (Bruker) and MestReNova 12.0.4 (Mestrelab Research, Santiago de Compostela, Spain) were used.

4.4 | (1a*R*,4a*R*,8a*R*)-1a,4a-dimethylhexahydro-1a*H*-naphtho[1,8a-*b*]oxiren-2(3*H*)-one (2)

Compound **1** (400 mg, 2.25 mmol) was dissolved in dichloromethane (30 mL), and sodium hydrogen carbonate (378 mg, 4.50 mmol) was added. The mixture was cooled on ice, and *m*-chloroperbenzoic acid (581 mg, 3.37 mmol) was added under argon over a period of 10 min. Quickly, a white precipitate was formed. After 2 h, the suspension was brought to room temperature and left under magnetic stirring for additional 24 h. The suspension was washed with a mixture of a saturated aqueous sodium thiosulfate solution and a saturated aqueous sodium carbonate solution (1 + 1, v + v; 2 × 50 mL), followed by brine (50 mL) and finally dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel to afford 342 mg of **2** (78% yield). TLC: R_f 0.48 (hexane/diethyl ether, 4 + 1, v + v). MS (EI): *m/z* (%): 109 (100), 67 (41), 133 (30), 81 (26), 43 (25), 176 (18), 151 (18), 137 (15), 55 (13), 110 (11). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, gs-COSY): δ 2.43 (ddd, ^{2}J = 19.2 Hz, ^{3}J = 8.2, 1.9 Hz, 1H, H-3α), 2.31 (ddd, ^{2}J = 19.2 Hz, ^{3}J = 11.4, 7.6 Hz, 1H, H-3β), 2.04 (m, 1H, H-4α), 1.97 (m, 1H, H-7α), 1.83 (m, 1H, H-8α), 1.65 (m, 1H, H-6α), 1.58 (m, 1H, H-6β), 1.56 (m, 2H, H-5), 1.55 (m, 1H, H-8β), 1.50 (m, 1H, H-7β), 1.38 (s, 3H, H-9), 1.22 (m, 1H, H-4β), 1.05 (s, 3H, H-10). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, gs-HSQC, gs-HMBC): δ 208.2 (C-2), 72.0 (C-8a), 65.5 (C-1a), 38.3 (C-5), 34.4 (C-4a), 33.5 (C-3), 32.1 (C-4), 26.4 (C-7), 24.1 (C-8), 21.0 (C-6), 20.7 (C-10), 11.3 (C-9).

4.5 | (1*S*,4a*R*,8a*S*)-1,4a-dimethyl(1,2-$^{2}H_2$)octahydronaphthalene-2,8a(1*H*)-diol (3)

Under an argon atmosphere, lithium aluminum deuteride (185 mg, 4.41 mmol) was suspended in dry THF (15 mL), and the flask was heated to gentle reflux. Epoxide **2** (342 mg, 1.76 mmol) was dissolved in dry THF (5 mL) and added dropwise. After 2 h, the flask was cooled on ice, and a saturated aqueous solution of sodium sulfate (5 mL) was slowly added. Hydrochloric acid (1%; 1 mL) was added, and the mixture was stirred. The aqueous layer was separated and extracted with diethyl ether (2 × 20 mL). The organic phase and the diethyl ether extracts were combined, washed with brine (2 × 20 mL), and dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure, and the crude product was purified by flash chromatography on silica gel to afford 198 mg of **3** (56% yield). TLC: R_f 0.28 (hexane/diethyl ether, 2 + 3, v + v). MS (EI): *m/z* (%): 112 (100), 125 (71), 126 (42), 97 (38), 182 (31), 200 (19), 111 (17), 43 (14), 55 (13), 113 (13). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, gs-COSY): δ 1.85 (ddd, ^{2}J = 12.2 Hz, ^{3}J = 3.4, 2.4 Hz, 1H, H-3α), 1.74 (dd, ^{2}J = 13.6 Hz, ^{3}J = 4.2, 1H, H-4α), 1.63-1.53 (m, 6H, H-3β, H-5α, H-6α, H-7, H-8α), 1.44 (m, 1H, H-6β), 1.42 (m, 1H, H-8β), 1.09 (m, 1H, H-5β), 1.07 (m, 1H, H-4β), 1.05 (s, 3H, H-10), 0.97 (s, 3H, H-9). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, gs-HSQC, gs-HMBC): δ 75.6 (C-8a), 72.1

(t, $^2J_{D\text{-}C}$ = 22.2 Hz, C-2), 41.9 (t, $^2J_{D\text{-}C}$ = 19.0 Hz, C-1), 36.9 (C-4a), 35.1 (C-5), 33.7 (C-4), 30.5 (C-3), 30.2 (C-8), 20.7 (C-7), 20.4 (C-10), 20.2 (C-6), 9.8 (C-9).

4.6 | (4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$) octahydronaphthalen-4a(2*H*)-ol (5)

Diol **3** (198 mg, 0.99 mmol) was dissolved in chloroform (40 mL). Under an argon atmosphere, pyridine (800 µl, 9.90 mmol) and subsequently *p*-toluenesulfonyl chloride (1.91 g, 10.0 mmol) were added slowly under stirring. The mixture was kept for 72 h at 10°C. A suspension of lithium aluminum deuteride (83.4 mg, 1.99 mmol) in dry THF (10 mL) was added dropwise, and the reaction mixture was heated at reflux. After 4 h, the mixture was cooled down to room temperature. Diethyl ether (20 mL) was added, followed by water (5 mL), and subsequently aqueous hydrochloric acid (1%; 20 mL). The organic phase was separated and washed with an aqueous sodium hydrogen carbonate solution (5%; 20 mL) and brine (20 mL). After drying over anhydrous sodium sulfate and filtration, the solvents were removed under reduced pressure, and the crude product was purified by flash chromatography on a diol phase to give 98.5 mg of **5** (55% yield) with an enantiomeric purity of 91% (enantioGC-MS, BGB-176 column). TLC: R_f 0.41 (hexane/diethyl ether, 2 + 3, v + v). MS (EI): *m/z* (%): 112 (100), 43 (23), 97 (19), 113 (18), 55 (18), 111 (16), 41 (15), 83 (14), 67 (12), 69 (12). The full MS (EI) is depicted in Figure 2. MS (CI): calculated mass for $C_{12}H_{19}D_3O$: 185.3214; found 185.3218. ^1H NMR (400 MHz, $CDCl_3$, 298 K, gs-COSY): δ 1.67–1.54 (m, 7H, H-1α, H-2α, H-5α, H-6α, H-7, H-8α), 1.50–1.35 (m, 3H, H-2β, H-5β, H-6β), 1.03 (s, 3H, H-10), 0.99–0.96 (m, 2H, H-1β, H-8β), 0.78 (s, 3H, H-9). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, gs-HSQC, gs-HMBC): δ 74.5 (C-4a), 37.3 (C-8a), 35.7 (C-8), 35.1 (C-1), 34.3 (t, $^2J_{D\text{-}C}$ = 22.3 Hz, C-4), 30.5 (quint, $^2J_{D\text{-}C}$ = 19.4 Hz, C-3), 29.9 (C-5), 21.4 (C-7), 20.8 (C-2/6), 20.7 (C-2/6), 20.3 (C-10), 14.9 (C-9)

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Enantioselective synthesis of tri-deuterated (–)-geosmin to be used as internal standard in quantitation assays

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7.1.4 Summary and individual contributions

(–)-Geosmin (from the ancient Greek words "geo" = earth and "osme" = odor) is a highly odorous molecule with a moldy and earthy smell, which reminds of beetroots. It is perceivable at very low concentrations, indicated by a sensory detection threshold in the range of 1–20 ng/kg. (–)-Geosmin is known to cause an unpleasant odor in foods and beverages such as drinking water, wine, fish and cereals. Recently we detected (–)-geosmin also in fermented cocoa and demonstrated that it can be transferred in odor- active amounts into the final chocolate products and lead to rejection by the consumers and significant economic loss.

To reliably detect and quantitate odor-active compounds present at trace levels in complex matrices, a sensitive and selective method is essential. In gas chromatography-mass spectrometry (GC-MS) and in liquid chromatography-mass spectrometry (LC-MS), the use of a stable isotopically substituted analogue of the target compound as internal standard is currently considered the best approach. Although racemic deuterated geosmin is available from chemical companies, it is highly expensive (~ 20.000 € for 150 mg). Therefore, we aimed at finding a convenient synthetic route to deuterated (–)-geosmin as an alternative to the commercial product.

A versatile synthetic strategy leading to tri-deuterated (–)-geosmin ((4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^{2}H_3$)octahydronaphthalen-4a(2*H*)-ol) was developed. Inexpensive racemic 2-methylcyclohexan-1-one was reacted with an auxiliary chiral amine using previously published procedures. After Michael addition of the resulting imine to pent-1-en-3-one, the amine was removed by hydrolysis with acetic acid followed by an intramolecular aldol condensation to afford the bicyclic intermediate (4a*R*)-1,4adimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one. After epoxidation, two deuterium atoms were incorporated by reduction with lithium aluminum deuteride. A second reduction step employed after selective tosylation of the resulting diol finally afforded trideuterated (–)-geosmin. The product was purified by flash chromatography using a diol stationary phase and its structure was confirmed by GC-MS as well as by one- and two-dimensional NMR experiments. By application of this novel eight step procedure, trideuterated (–)-geosmin was obtained with 91% enantiomeric purity.

Caterina Porcelli evaluated the existing literature and designed the study. Caterina performed the synthesis and purification steps and prepared the manuscript. Johanna Kreissl provided support in NMR and GC-MS data evaluation. Martin Steinhaus conceived and directed the study, supervised Caterina’s work and revised the manuscript.

7.2 Publication 2

7.2.1 Bibliographic data

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7.2.2 Publication reprint

For a reprint of publication 2, please turn to the next page.

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Molecular Background of a Moldy-Musty Off-Flavor in Cocoa

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ABSTRACT: The parallel application of aroma extract dilution analysis to the volatiles isolated from a sample of fermented cocoa seeds with a pronounced moldy-musty off-flavor and to the volatiles isolated from a flawless reference sample revealed (−)-geosmin, 4-methoxy-2,5-dimethylfuran-3(2*H*)-one, 1*H*-indole, and 3-methyl-1*H*-indole as potential off-flavor compounds on the basis of their odor quality and higher flavor dilution factors in off-flavor cocoa than in the reference sample. Quantitation of the four compounds in nine off-flavor cocoa samples and calculation of odor activity values (ratio of the concentrations to the odor threshold values) suggested the crucial roles of (−)-geosmin and 3-methyl-1*H*-indole for the off-flavor. In the chocolate industry, their quantitation can be used to objectively assess the off-flavor at the level of incoming goods inspection. Because both compounds are inhomogeneously distributed between the testa and the embryo, separate quantitation in the two parts of the seeds is required.

KEYWORDS: *cocoa (Theobroma cacao L.), moldy-musty off-flavor, aroma extract dilution analysis (AEDA), (−)-geosmin, 3-methyl-1H-indole, stable isotopically substituted odorants*

INTRODUCTION

Cocoa is a major ingredient in chocolate products and mainly responsible for their pleasant aroma. For the development of a characteristic aroma, the fermentation applied to the seeds of the cocoa tree (*Theobroma cacao* L.) as well as the subsequent roasting of the fermented and dried seeds is crucial.[1−3] The fermentation step is carried out directly after harvest in the country of cocoa origin. The fermented cocoa is then typically shipped internationally to chocolate companies, where chocolate production starts with the roasting step.

During the incoming goods inspection, the fermented cocoa undergoes a testing by a sensory panel to detect batches tainted with off-flavors.[4] Occasionally occurring off-flavors include moldy-musty, smoky, cheesy, fecal, and mushroom-like odor notes.[5−7] The undesirable odors can survive the manufacturing process. When they are still perceptible in the final chocolate products, they may lead to consumer complaints, expensive product recalls, and severe damage to the brand reputation.[8,9] The assessment of flavor defects by a sensory panel, however, is prone to false negative results, for example, considering the fact that specific anosmias are quite frequent in the population.[10] Therefore, there is a strong demand in the cocoa industry for more objective methods such as the targeted quantitation of the compounds being responsible for the individual off-flavors. However, despite the fact that numerous studies on the key odorants in cocoa have been conducted,[11−14] there is currently only little information available in the scientific literature on the identity of compounds causing flavor defects.[5,6] Recently, a molecular sensory science approach led to the unequivocal identification of 2-methoxyphenol, 3- and 4-ethylphenol, 3- and 4-methylphenol, and 3-propylphenol as the crucial odorants for the smoky off-flavor in cocoa,[7] but no such investigation has been carried out on the frequently reported moldy-musty off-flavor so far.

Thus, the aim of the present study was to clarify the odor-active compounds causing the moldy-musty off-flavor in cocoa by (i) screening the volatiles isolated from tainted cocoa for potential off-flavor compounds by aroma extract dilution analysis (AEDA)[15] and (ii) substantiating the role of these compounds by quantitation and calculation of odor activity values (OAVs) as ratios of the concentrations to the odor threshold values (OTVs).[10,16]

MATERIALS AND METHODS

Cocoa Materials. A total of ten cocoa samples were obtained from German chocolate manufacturers. Among them, nine (M1−M9) were tainted with a moldy-musty off-flavor, whereas one was flawless (REF) and was used as a reference. Samples M1−M7 and REF were whole cocoa seeds, while M8−M9 consisted of cocoa liquor. Cocoa seeds have been harvested, fermented, and dried by local farmers in their countries of origin. Cocoa liquors have been produced from fermented and roasted cocoa seeds. All the samples were stored at 5 °C until analysis. The separation of the testa and embryo was achieved by hand with the aid of a kitchen knife. Deodorized cocoa butter was obtained from Cargill (Berlin, Germany).

Chemicals. Reference odorants **1**−**6**, **8**−**10**, **13**, **15**−**25**, **27**−**40**, **43**−**50**, and **52**−**57** were purchased from Merck (Darmstadt, Germany). Odorants **12**, **26**, and **51** were obtained from Acros Organics (Schwerte, Germany). Odorants **7** and **41** were purchased from Alfa Aesar (Karlsruhe, Germany). Odorants **11**,[17] **14**,[18] and **42**[19] were synthesized according to literature procedures. Stable isotopically substituted odorants ($^2H_3$)-**24**, 4-($^2H_3$)methoxy-2,5-dimethylfuran-3(2*H*)-one and ($^2H_8$)-**55**, 3-($^2H_3$)methyl(2,4,5,6,7-$^2H_5$)-1*H*-indole

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were prepared according to published procedures.[20,21] ($^2H_6$)-**54**, (2,3,4,5,6,7-$^2H_6$)-1*H*-indole, was synthesized from the isotopically unmodified compound using the approach published for the synthesis of ($^2H_8$)-**55**, which was based on a forced H/D exchange.[21] ($^2H_3$)-**35**, (4*S*,4a*S*,8a*R*)-4,8a-dimethyl(3,3,4-$^2H_3$)octahydronaphthalen-4a(2*H*)-ol), was synthesized by the 8-step procedure detailed in Figure 1. In

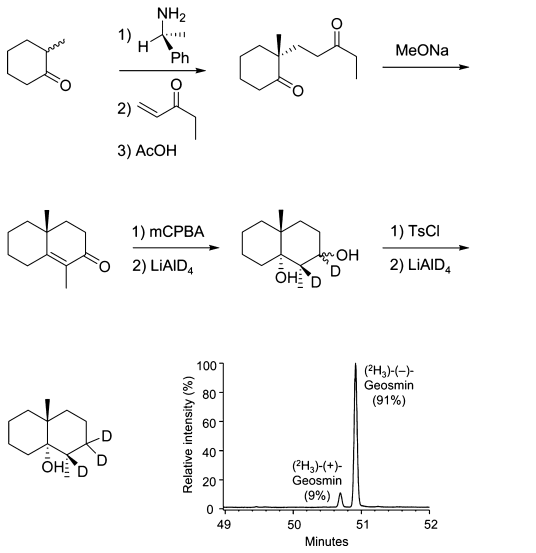

Figure 1. Synthetic approach to trideuterated geosmin and GC separation of the products indicating the enantiomeric purity.[24]

brief, inexpensive racemic 2-methylcyclohexan-1-one was reacted with an auxiliary chiral amine as suggested by Revial et al.[22,23] After Michael addition of the resulting imine to pent-1-en-3-one, the amine was removed by hydrolysis with acetic acid followed by an intramolecular aldol condensation to afford bicyclic (4a*R*)-1,4a-dimethyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one. After epoxidation, two deuterium atoms were incorporated by reduction with lithium aluminum deuteride. A second reduction step employed after selective tosylation of the resulting diol finally afforded trideuterated geosmin with 91% enantiomeric purity.[24] Miscellaneous chemicals are detailed in the Supporting Information.

Gas Chromatography. GC-olfactometry (GC-O) analyses were performed by using a system equipped with a cold on-column injector, a custom-made sniffing-port,[25] a flame ionization detector (FID), and either a DB-FFAP or a DB-5 column. GC−MS analyses were performed by using a one-dimensional GC−MS system with a sector mass spectrometer, a two-dimensional heart-cut GC−GC−MS system with a Paul ion trap mass spectrometer, or a two-dimensional heart-cut GC−GC−HRMS system with an Orbitrap mass spectrometer. Further details on the GC systems are available in the Supporting Information.

Aroma Extract Dilution Analysis. Fermented cocoa seeds and cocoa liquor were immersed in liquid nitrogen. The cooled seeds were coarsely granulated with a laboratory knife mill Grindomix GM 200 (Retsch, Haan, Germany) at 3800 rpm (2 × 15 s) and then further ground into a fine powder with a 6875 Freezer Mill (SPEX SamplePrep, Stanmore, UK). Dichloromethane (100 mL) was added to the powder (50 g), followed by magnetic stirring at room temperature (16 h). The mixture was filtered, and the residue was stirred (1 h) with a second portion of dichloromethane (100 mL). The combined filtrates were dried over anhydrous sodium sulfate. Nonvolatiles were removed by solvent-assisted flavor evaporation (SAFE) at 40 °C.[26] The SAFE distillate was separated into a neutral and basic volatile (NBV) fraction and an acidic volatile (AV) fraction by shaking with an aqueous sodium carbonate solution (cf. Supporting Information).

The fractions NBV and AV were analyzed by GC-O using the DB-FFAP column. The analyses were performed by three trained and experienced assessors (2 females, 1 male, and aged 25−48). Each assessor repeated the GC-O analyses until the results were reproducible. Then, fractions NBV and AV were stepwise diluted with dichloromethane at a ratio of 1:2, and the diluted samples were also subjected to GC-O analysis. A flavor dilution (FD) factor was assigned to each odorant which corresponded to the dilution factor of the highest diluted sample in which the odorant was detected during GC-O by any of the three assessors.[10,15]

To facilitate the structure assignments of the odorants detected by GC-O and AEDA and avoid coelutions during GC−MS analyses, the fraction NBV was subfractionated into five fractions by liquid chromatography (cf. Supporting Information).

Odorant Quantitation. To quantitate odorants **24**, **35**, **54**, and **55**, dichloromethane (50−100 mL) was added to cocoa seeds powder, cocoa testa powder, cocoa embryo powder, or cocoa liquor powder (15−50 g). Isotopologues of the target odorants (~0.05−0.25 μg) dissolved in dichloromethane (50−200 μL) were added as internal standards. After magnetic stirring at room temperature (16 h), the mixture was filtered, and the residue was stirred (1 h) with a second portion of dichloromethane (50−100 mL). The combined filtrates were dried over anhydrous sodium sulfate. Nonvolatiles were removed by SAFE at 40 °C, and the SAFE distillate was concentrated (200 μL). The concentrates were analyzed with the heart-cut GC−GC−MS system (**24**, **54**, and **55**) or the heart-cut GC−GC−HRMS system (**35**). Details on the heart-cut approach are provided in the Supporting Information.

Peak areas corresponding to the analyte and internal standard were obtained from the extracted ion chromatograms using characteristic quantifier ions. Odorant concentrations in the cocoa samples were calculated from the area counts of the analyte peak, the area counts of the standard peak, the amount of cocoa used for the workup, and the amount of standard added, by employing a calibration line equation. The calibration line equation was obtained by linear regression after analysis of analyte/standard mixtures in different concentration ratios. Individual quantifier ions and calibration line equations are available in the Supporting Information.

Odor Threshold Values. OTVs were determined orthonasally in liquid deodorized cocoa butter by using the standard practices of the American Society for Testing and Materials (ASTM).[27] Spiked samples were prepared by adding the test substance in ethanolic solution (10−20 μL) to 10 g of cocoa butter. The same volume of pure ethanol was added to the reference samples. Between two consecutive 3-AFC tests, odorant concentrations differed by a factor of 3. Samples were presented to the assessors in cylindrical PTFE vessels (5.7 cm height × 3.5 cm i.d.) with lids. The sample temperature was 32 °C. The tests were carried out by 15−20 trained assessors in separate booths of a special room exclusively dedicated to sensory evaluations. The room temperature was 22 ± 2 °C.

RESULTS AND DISCUSSION

Screening for Off-Flavor Compounds. Parallel application of an AEDA to the volatiles isolated from a sample of fermented cocoa seeds with a pronounced moldy off-flavor and to the volatiles isolated from a reference sample of fermented cocoa seeds without any off-flavor revealed a total of 57 odorants exhibiting an FD factor of ≥8 in at least one of the two samples (Table 1). By comparing the retention indices (RIs) on FFAP and DB-5 columns, the mass spectra as obtained by GC−MS, and the odor properties as perceived at the sniffing port with data obtained from authentic reference compounds, all 57 odorants could be unequivocally identified. In both the samples, the highest FD factors (1024−2048) were determined for honey, floral-smelling 2-phenylethanol (**39**), floral, citrusy-smelling linalool (**22**), rancid, sweaty-smelling 2-methylpropanoic acid (**23**), and fruity and blueberry-like smelling ethyl 3-methylbutanoate (**4**). These compounds are

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Table 1. Odor-Active Compounds Identified among the Volatiles Isolated from Fermented Cocoa Seeds with a Pronounced Moldy-Musty Off-Flavor (M1) in Comparison with the Reference Sample without Off-Flavor (REF)

			RI[b]		FD factor[c]	
no.	odorant[a]	odor	FFAP	DB-5	M1	REF
1	ethyl 2-methylpropanoate	fruity	949	765	4	8
2	butane-2,3-dione	buttery	980	593	16	16
3	ethyl 2-methylbutanoate	fruity	1045	848	16	32
4	ethyl 3-methylbutanoate	fruity, blueberry-like	1058	852	512	1024
5	3-methylbutyl acetate	fruity, banana	1119	880	1	8
6	(4*Z*)-hept-4-enal	fish oil	1239	901	8	8
7	3-hydroxybutan-2-one	buttery	1281	800	32	64
8	oct-1-en-3-one	mushroom	1294	979	64	128
9	heptan-2-ol	coconut, dill	1304	903	4	8
10	(2*E*)-hept-2-enal	green, fatty	1311	957	8	16
11	2-acetyl-1-pyrroline	popcorn	1322	922	32	64
12	dimethyl trisulfide	sulfury, cabbage	1367	968	256	512
13	2,3,5-trimethylpyrazine	earthy	1378	1003	256	128
14	2-propanoyl-1-pyrroline	popcorn	1399	1024	4	32
15	oct-1-en-3-ol	mushroom	1411	982	32	16
16	3-isopropyl-2-methoxypyrazine	earthy, pea	1424	1094	16	8
17	acetic acid	vinegar	1447	612	64	16
18	3-(methylsulfanyl)propanal	cooked potato	1451	905	512	128
19	2,3-diethyl-5-methylpyrazine	earthy	1487	1158	64	4
20	3-isobutyl-2-methoxypyrazine	bell pepper	1514	1184	128	128
21	methyl 3-(methylsulfanyl)propanoate	earthy, cabbage	1520	1034	16	8
22	linalool	citrusy, floral	1534	1102	256	1024
23	2-methylpropanoic acid	cheesy, sweaty	1559	789	512	1024
24	MDMF[d]	caramel, musty	1577	1055	256	<1
25	butanoic acid	cheesy, sweaty	1630	821	512	512
26	phenylacetaldehyde	floral, honey	1640	1045	256	512
27	acetophenone	bitter almond, rubber	1640	1064	8	1
28	3-methylbutanoic acid	cheesy, sweaty	1660	871	64	128
29	2-acetylthiazole	popcorn	1660	1017	1	32
30	2-methylbutanoic acid	sweaty	1669	878	64	128
31	geranial	citrusy	1710	1269	32	64
32	citronellol	rose	1780	1230	16	32
33	(2*E*,4*E*)-deca-2,4-dienal	fatty, fried	1800	1317	8	4
34	2-phenylethyl acetate	honey, floral	1808	1256	64	128
35	(−)-geosmin	moldy, beetroot	1816	1413	2048	<1
36	geraniol	rose, citrusy	1837	1256	16	32
37	2-methoxyphenol	smoky	1855	1090	64	32
38	ethyl 3-phenylpropanoate	fruity, cinnamon	1874	1418	16	8
39	2-phenylethanol	floral, honey	1903	1116	128	2048
40	maltol	caramel	1969	1110	32	32
41	δ-octalactone	coconut	1969	1284	16	32
42	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	metallic	1997	1382	512	512
43	γ-nonalactone	coconut	2031	1360	16	32
44	HDMF[e]	caramel	2049	1071	1	512
45	4-methylphenol	fecal, horse stable	2077	1077	64	16
46	3-methylphenol	smoky, phenolic	2089	1083	16	8
47	δ-decalactone	coconut	2194	1494	2	8
48	sotolon	seasoning	2200	1097	1	512
49	2-methoxy-5-vinylphenol	smoky, clove	2219	1331	8	4
50	2,6-dimethoxyphenol	smoky, clove	2281	1346	16	8
51	cinnamyl alcohol	floral	2297	1335	1	16
52	δ-dodecalactone	peach, coconut	2384	1680	4	8
53	γ-(6*Z*)-dodec-6-enolactone	peach	2403	1659	8	8
54	1*H*-indole	fecal, mothball	2452	1294	32	4
55	3-methyl-1*H*-indole	fecal, mothball	2481	1390	16	2
56	phenylacetic acid	honey	2563	1261	32	128
57	vanillin	vanilla	2583	1408	8	256

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Table 1. continued

[a]Odorants exhibiting an FD factor of ≥8 in at least one of the two cocoa samples. Structure assignments were based on RIs (FFAP, DB-5), mass spectrum, and odor quality; these data were compared with those of authentic reference compounds analyzed under the same conditions. [b]Retention index; calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [c]FD factor. [d]4-Methoxy-2,5-dimethylfuran-3(2*H*)-one. [e]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one.

well-known cocoa odorants and have been reported in numerous studies before.[2,28−30] Potential off-flavor compounds were identified in Table 1 by a corresponding odor description and by a clearly higher FD factor in the off-flavor cocoa sample than in the reference sample. This applied to compounds **24**, **35**, **54**, and **55**.

The criteria were best met by moldy, beetroot-like smelling compound **35**, which showed the highest FD factor, namely 2048, among all compounds in the off-flavor cocoa sample, but was not detected in the reference sample. Compound **35** was identified as geosmin, a well-known product of microbial metabolism for which very low OTVs in the range of 1−20 ng/kg had been reported.[31] Geosmin-associated off-flavors have been reported in cereals, drinking water, fish, and wine but so far not in cocoa.[32−35] Moldy-musty off-flavors in cocoa are often associated with mold growth, either directly visible on the bean surface or by performing the so-called cut-test, a widely applied quality control approach in the cocoa industry.[36] Mold growth is favored by insufficient mixing of cocoa batches during fermentation, by overfermentation, by slow or insufficient drying, and by storage in a humid environment. However, it is yet unclear whether molds or rather bacteria such as *Streptomyces* species account for geosmin formation in cocoa.[5,37]

Another potential off-flavor compound revealed by the comparative AEDA was caramel-like and musty-smelling 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF, **24**). Like geosmin, it was not detected in the reference sample. In the off-flavor cocoa sample, its FD factor was 256. MDMF is an odor-active compound widely found in different kinds of fruit such as guava, kiwi, mango, and strawberry.[38,39] It is enzymatically formed from fructose 1,6-bisphosphate together with 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF, **44**). Conversion of HDMF to MDMF is biochemically achieved by an S-adenosyl-L-methionine-dependent O-methyltransferase.[40] Although its odor at lower concentrations is pleasant and described as sweet and caramel-like, we found that at higher concentrations, it exhibits an additional rather unpleasant musty note.

The other two potential off-flavor compounds identified by the comparative AEDA were fecal and mothball-like-smelling compounds 1*H*-indole (**54**) and 3-methyl-1*H*-indole (**55**). Their odor quality was not directly linked to the moldy and musty off-flavor but nevertheless considered highly unpleasant. Both compounds were also detected in the reference sample, but their FD factors were higher in the off-flavor cocoa sample (32 and 16 vs 4 and 2). 1*H*-Indole and 3-methyl-1*H*-indole have been reported as products of the bacterial decomposition of the amino acid L-tryptophan.[41]

Enantiomeric Distribution of Geosmin. Geosmin is a chiral molecule including three stereogenic centers. Its identification in the off-flavor cocoa sample used in the AEDA was based on a commercially available racemic mixture of (+)- and (−)-geosmin. However, it has been shown that the two isomers clearly differ in their odor potency.[32,42,43] The OTV of the (−)-isomer is 11 times lower than that of the (+)-isomer.[42] Therefore, we attempted to determine the enantiomeric distribution of geosmin in the off-flavor cocoa sample. The extract obtained from cocoa was analyzed by two-dimensional GC with heart-cutting using a β-cyclodextrin-modified stationary phase in the second dimension and a high resolution mass spectrometer as the detector (Figure 2A). A

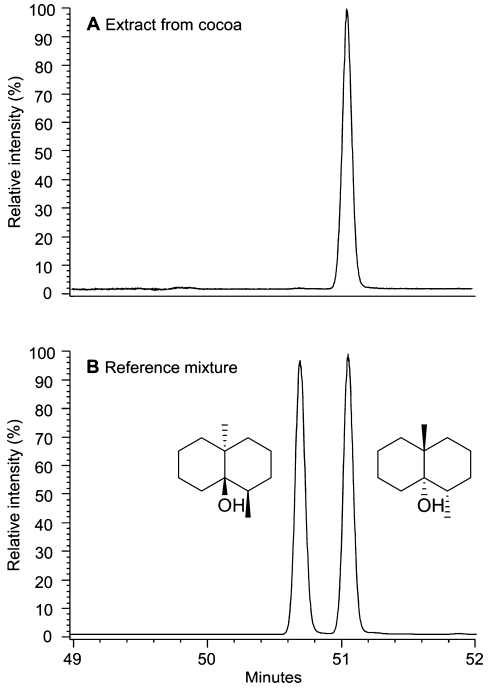

Figure 2. Analysis of an extract obtained from the off-flavor cocoa sample M1 (A) in comparison to a racemic reference mixture of geosmin (B) by GC−GC−HRMS with a β-cyclodextrin-modified stationary phase in the second dimension.

solution of the racemic reference was analyzed under the same conditions (Figure 2B). The result clearly showed that there was only one of the two enantiomers present in the cocoa sample. Considering the elution order of the two enantiomers on the stationary phase employed, which had been published previously,[44] the off-flavor compound in the cocoa sample was unequivocally identified as (−)-geosmin. The presence of (−)-geosmin has also been reported from drinking water, fish,[42] and wine,[45] whereas to the best of our knowledge, no natural occurrence of (+)-geosmin has been shown so far.

Quantitation of Off-Flavor Compounds. To substantiate the role of the identified off-flavor compounds for the moldy-musty off-flavor, their concentrations in ten cocoa samples were determined by GC−MS. The samples included the off-flavor cocoa sample and the reference sample previously used for odorant screening plus six additional cocoa seed samples and two cocoa liquor samples, all of which showed a more or less pronounced moldy-musty off-flavor. To

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Table 2. Concentrations of Off-Flavor Compounds in Nine Fermented Cocoa Samples with Moldy-Musty Off-Flavor (M1−M9) and a Reference Sample without Off-Flavor (REF)

	concentration (μg/kg)[a]									
odorant	M1[b,d]	M2[b]	M3[b]	M4[b]	M5[b]	M6[b]	M7[b]	M8[c]	M9[c]	REF[b,d]
MDMF	442	9.17	47.4	14.9	41.8	3.08	226	29.3	18.2	<1
(−)-geosmin	11.5	<0.2	3.48	3.54	4.28	0.922	<0.2	<0.2	<0.2	<0.2
1*H*-indole	9.14	5.28	15.0	8.10	2.82	9.90	5.46	<1	<1	<1
3-methyl-1*H*-indole	32.1	13.0	12.5	2.07	22.5	12.0	66.4	81.5	29.1	<1

[a]Mean of duplicates or triplicates; details are provided in the Supporting Information. [b]Cocoa seed samples. [c]Cocoa liquor samples. [d]Samples M1 and REF were the samples previously used for odorant screening by AEDA (cf. Table 1).

Table 3. OAVs of Off-Flavor Compounds in Nine Cocoa Samples with Moldy-Musty Off-Flavor (M1−M9) and a Reference Sample without Off-Flavor (REF)

		OAV[b]									
odorant	OTV[a] (μg/kg)	M1[c]	M2	M3	M4	M5	M6	M7	M8	M9	REF[c]
MDMF	350	1.3	<1	<1	<1	<1	<1	<1	<1	<1	<1
(−)-geosmin	1.6	7.2	<1	2.2	2.2	2.7	<1	<1	<1	<1	<1
1*H*-indole	51	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
3-methyl-1*H*-indole	1.1	29	12	11	1.9	20	11	60	74	26	<1

[a]OTV in deodorized cocoa butter. [b]OAVs were calculated as a ratio of the concentration in the cocoa sample to the OTV. [c]cf. Table 2, footnote d.

compensate for workup losses, stable isotopologues of the target compounds were used as internal standards.

The quantitations revealed concentrations of the off-flavor compounds which, in most cases, were clearly higher in the off-flavor cocoa samples than in the reference sample (Table 2). For caramel-like, musty-smelling MDMF, the concentration in the reference sample was below 1 μg/kg, whereas the concentrations in the off-flavor cocoa samples covered a broad range from 3.08 to 442 μg/kg. The highest concentration was obtained in the off-flavor cocoa sample M1, which was used for the AEDA screening. The same was true for moldy, beetroot-smelling (−)-geosmin. In M1, the (−)-geosmin concentration was 11.5 μg/kg, which was clearly higher than the concentration in the reference sample (<0.2 μg/kg). The off-flavor cocoa samples M2, M7, M8, and M9 showed the same low (−)-geosmin concentrations as the reference sample, whereas the concentrations in the other off-flavor cocoa samples were in the range from 0.922 to 4.28 μg/kg. Despite the fact that fecal and mothball-like-smelling odorants 1*H*-indole and 3-methyl-1*H*-indole both are derivatives of tryptophane, their concentrations in the cocoa samples were not correlated. The 1*H*-indole concentration in the reference sample was below 1 μg/kg. In the off-flavor cocoa samples, the concentration ranged from <1 to 15 μg/kg with the highest concentration found in sample M3. 3-Methyl-1*H*-indole could not be detected in the reference sample (<1 μg/kg), whereas in the off-flavor cocoa samples, its concentration ranged between 2.07 μg/kg in sample M4 and 81.5 μg/kg in sample M8. Interestingly, sample M8 showed a comparatively low concentration of 1*H*-indole. In summary, the data on 1*H*-indole and 3-methyl-1*H*-indole concentrations suggested that both compounds are rather independently formed, possibly even by different species of bacteria. Independent formation of 1*H*-indole and 3-methyl-1*H*-indole has previously been demonstrated for intestinal bacteria.[41]

Calculation of OAVs. To assess the impact of the individual compounds for the moldy-musty off-flavor in cocoa, OAVs were calculated as the ratio of the concentrations to the OTVs of the individual compounds. OTVs were determined in deodorized cocoa butter. The results of the OTV and OAV calculations are depicted in Table 3. With 1.1 μg/kg and 1.6 μg/kg, the OTVs of 3-methyl-1*H*-indole and (−)-geosmin were clearly lower than the OTVs of 1*H*-indole and MDMF, which were 51 and 350 μg/kg, respectively. It is noteworthy that specific anosmia to (−)-geosmin is well-known among humans, but its OTV was determined with a panel exclusively consisting of normosmic candidates.

The OAVs calculated for the four potential off-flavor compounds in the reference sample were all <1. For 1*H*-indole, also the OAVs in all the off-flavor cocoa samples were <1, which suggested a minor importance of these compounds to moldy-musty off-flavors in cocoa. MDMF showed an OAV >1 only in sample M1, which had been used in the screening experiment. Given the facts that the OAV of MDMF in the other off-flavor cocoa samples was consistently <1 and, even in M1, with an OAV of 1.3, its concentration was only slightly exceeding its threshold, MDMF was also considered to be of minor importance for the moldy-musty off-flavors in cocoa. Interestingly, only four of the nine off-flavor cocoa samples showed OAVs >1 for (−)-geosmin, whereas all off-flavor cocoa samples showed OAVs >1 for 3-methyl-1*H*-indole. Furthermore, OAVs of 3-methyl-1*H*-indole were higher than OAVs of (−)-geosmin in eight of the nine off-flavor cocoa samples. These data, however, did not explain why the overall olfactory profile of the off-flavor cocoa samples rather resembled the moldy, beetroot-like odor of (−)-geosmin than the fecal, mothball-like odor of 3-methyl-1*H*-indole. This prompted us to consider that the two compounds might not be homogeneously distributed in the cocoa seeds. (−)-Geosmin is mainly produced by aerobic microorganisms and therefore might be enriched on the surface of the seeds, whereas 3-methyl-1*H*-indole has been reported as a product of anaerobic species and therefore might be depleted on the surface.[41,46]

Distribution of (−)-Geosmin and 3-Methyl-1*H*-indole in Cocoa Seeds. To test our hypothesis on the inhomogeneous distribution of (−)-geosmin and 3-methyl-1*H*-indole in cocoa seeds, we quantitated the compounds in the seed shell (testa) and the inner part (embryo) separately. The experiments were carried out with two different off-flavor samples. Results (Table 4) confirmed our hypothesis.

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Table 4. Distribution of (−)-Geosmin and 3-Methyl-1*H*-indole between the Testa and Embryo in Two Cocoa Samples with Moldy-Musty Off-Flavor

odorant	sample, part	conc. (μg/kg)[a]	OAV[b]	distribution (%)[c]
(−)-geosmin	M5, testa	9.00	5.6	42
	M5, embryo	2.95	1.8	58
	M6, testa	2.89	1.8	65
	M6, embryo	0.322	0.2	35
3-methyl-1*H*-indole	M5, testa	6.73	6.1	8
	M5, embryo	18.3	16.6	92
	M6, testa	3.58	3.3	8
	M6, embryo	8.59	7.8	92

[a]Mean of triplicates; details are provided in the Supporting Information. [b]OAVs calculated as a ratio of the concentration in the cocoa bean part to the OTV of 1.6 μg/kg for (−)-geosmin and 1.1 μg/kg for 3-methyl-1*H*-indole (cf. Table 3). [c]Calculated from the concentrations in the cocoa bean parts and the mass ratios of the testa to embryo, which were 19:81% in sample M5 and 17:83% in sample M6, respectively.

In sample M5 with a high overall (−)-geosmin concentration, 9.00 μg/kg of the compound was determined in the testa, whereas in the embryo, the concentration was only 2.95 μg/kg. In sample M6, which had showed an overall (−)-geosmin concentration in the previous experiment that was below its OTV (cf. Tables 2 and 3), the concentration in the testa was exceeding the OTV. In the testa, a concentration of 2.89 μg/kg equivalent to an OAV of 1.8 was found, whereas the concentration in the embryo was only 0.322 μg/kg equivalent to an OAV of only 0.2. By contrast, 3-methyl-1*H*-indole showed the opposite behavior. In sample M5, its concentration in the testa was 6.72 μg/kg, whereas in the embryo, 18.3 μg/kg was determined. In sample M6, the concentrations in the testa and embryo were 3.58 and 8.59 μg/kg, respectively. Thus, the 3-methyl-1*H*-indole concentration was consistently lower in the testa than in the embryo.

In summary, the higher concentration of (−)-geosmin on the surface of the cocoa seeds in combination with the lower concentration of 3-methyl-1*H*-indole might explain the overall dominating moldy-musty odor note perceived in the off-flavor cocoa samples. Another consequence of the inhomogeneous distribution in the cocoa seeds is that a major part of (−)-geosmin is removed with the testa during further cocoa processing, although the testa constitutes less than 20% of the seed weight (cf. Table 4, column "distribution"). After classical bean roasting, the seeds are broken. The testa fragments are separated from the pieces of the cotyledons, the so-called cocoa nibs, by winnowing. While the cocoa nib fraction is the basis for chocolate manufacturing, the shell fraction is typically used as an agricultural fertilizer or as fuel. In the two samples analyzed in our study, 42 and 65% of the total (−)-geosmin were located in the testa. However, it is yet unclear, whether thermal diffusion during roasting could lead to a substantial transfer of (−)-geosmin from the testa to the embryo resulting in an increased concentration in the final product. If so, nib roasting could be the better alternative because in this process, the testae are removed before the thermal treatment. Furthermore, the winnowing process is not 100% effective; thus, a minor amount of testa fragments remains in the nib fraction. These aspects are of minor importance for 3-methyl-1*H*-indole because with 8%, only a minor percentage of this compound is located in the testa.

In conclusion, the results of our study suggest that the analytical monitoring of fermented cocoa at the incoming goods inspection in the cocoa industry should include the quantitation of (−)-geosmin and 3-methyl-1*H*-indole to objectively detect moldy-musty off-flavors. We recommend the use of the sensory threshold values of 1.6 and of 1.1 μg/kg as the maximum tolerable concentrations for (−)-geosmin and 3-methyl-1*H*-indole, respectively. To evaluate the risk of an off-flavor in the final product, separate analysis of (−)-geosmin in the testa and embryo is essential. In particular, this allows to correctly assess the impact of residual testa fragments in the nib fraction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00564.

Experimental data on miscellaneous chemicals, GC systems, fractionation of cocoa volatiles, and heart-cutting; quantifier ions and calibration line data used in quantitation; and individual concentration data used for mean calculations and standard deviations (PDF)

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Notes

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■ ABBREVIATIONS

AEDA, aroma extract dilution analysis; 3-AFC, three-alternative forced choice; AV, acidic volatiles; FD factor, flavor dilution factor; FFAP, free fatty acid phase; FID, flame ionization detector; GC, gas chromatography; GC-O, gas chromatography-olfactometry; HRMS, high-resolution mass spectrometry; i.d., inner diameter; MS, mass spectrometry;

NBV, neutral and basic volatiles; OAV, odor activity value; RI, retention index; SAFE, solvent-assisted flavor evaporation

NOMENCLATURE

2-acetyl-1-pyrroline, 1-(3,4-dihydro-2*H*-pyrrol-5-yl)ethan-1-one; cinnamyl alcohol, (2*E*)-3-phenylprop-2-en-1-ol; citronellol, 3,7-dimethyloct-6-en-1-ol; δ-decalactone, 5-hexyloxolan-2-one; γ-(6*Z*)-dodec-6-enolactone, 5-[(2*Z*)-oct-2-en-1-yl]-oxolan-2-one; *trans*-4,5-epoxy-(2*E*)-dec-2-enal, (2*E*)-3-[(2*R*,3*R*)/(2*S*,3*S*)-3-pentyloxiran-2-yl]prop-2-enal; (−)-geosmin, (4*S*,4a*S*,8a*R*)-4,8a-dimethyloctahydronaphthalen-4a-(2*H*)-ol; geranial, (2*E*)-3,7-dimethylocta-2,6-dien-1-al; geraniol, (2*E*)-3,7-dimethylocta-2,6-dien-1-ol; HDMF, 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one, also known as furaneol; linalool, 3,7-dimethylocta-1,6-dien-3-ol; maltol, 3-hydroxy-2-methyl-4*H*-pyran-4-one; MDMF, 4-methoxy-2,5-dimethylfuran-3(2*H*)-one; methional, 3-(methylsulfanyl)propanal; δ-octalactone, 6-propyloxan-2-one; γ-nonalactone, 5-pentyloxolan-2-one; sotolon, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one; vanillin, 4-hydroxy-3-methoxybenzaldehyde

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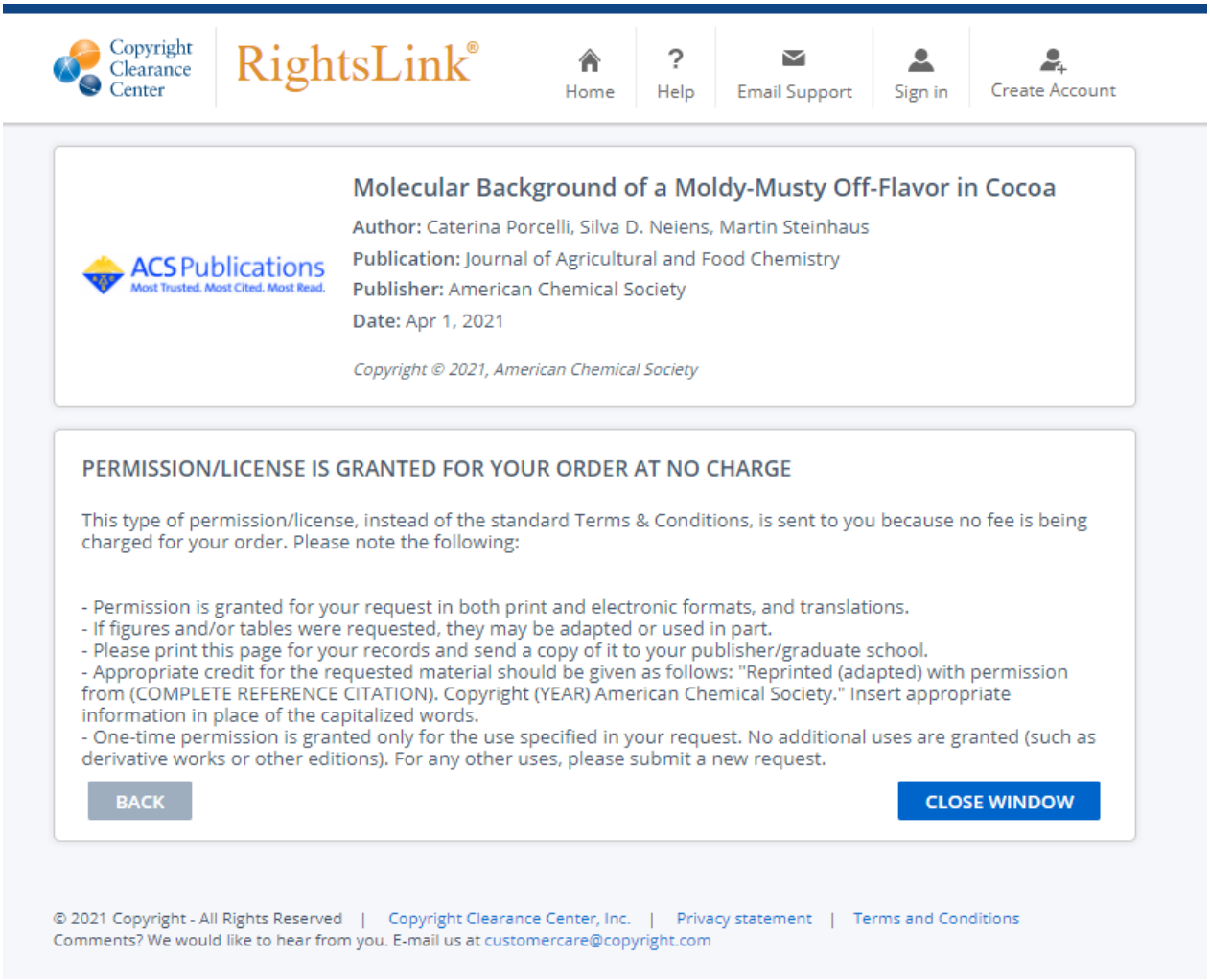

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Molecular Background of a Moldy-Musty Off-Flavor in Cocoa

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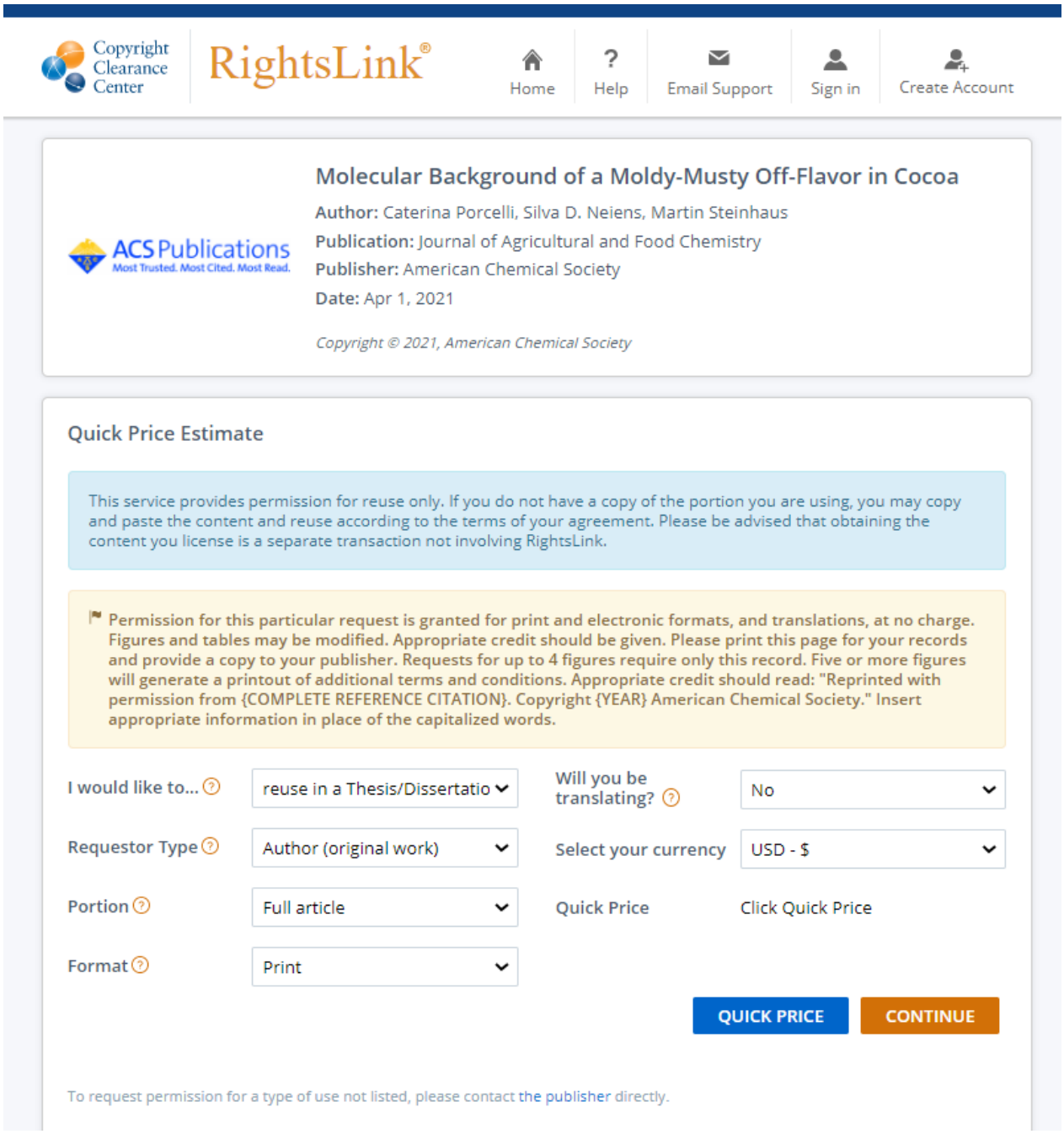

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7.2.4 Summary and individual contributions

The characteristic aroma of fermented cocoa can occasionally be tainted by the presence of off-flavors, among which a moldy-musty one is frequently reported. This unpleasant odor may survive the manufacturing process and be transferred into the final product, leading to consumer complaints, product recalls and damage to the brand reputation.

In order to elucidate the molecular background of this off-flavor, the fermented cocoa seeds of a moldy-musty smelling sample were milled and treated with solvent. Nonvolatiles were removed from the extract by SAFE. The SAFE distillate was concentrated and screened for off-flavor compounds by AEDA. A flawless cocoa sample was analyzed in parallel and used as a reference.

Comparison of the two samples revealed 57 odorants showing an FD factor of ≥ 8 in at least one of the two samples. Among these, (−)-geosmin, 4-methoxy-2,5-dimethylfuran3(2*H*)-one, 1*H*-indole and 3-methyl-1*H*-indole were highlighted as potential off-flavor compounds. This selection was based on the odor quality and higher flavor dilution factors of the compounds in the off-flavor cocoa than in the reference sample. To substantiate the role of the four compounds for the off-flavor, their quantitation was performed in nine moldy-musty cocoa samples. Their odor threshold values (OTVs) were determined by sensory experiments and their odor activity values (OAVs) were calculated. The results obtained suggested crucial roles of (−)-geosmin and 3-methyl-1*H*-indole for the undesired odor. Furthermore, it could be demonstrated by separate quantitation of the two compounds in the seed shell (testa) and in the inner part (embryo) that they are inhomogeneously distributed. Indeed, higher concentrations of (−)-geosmin were found in the seed shell, whereas the concentration of 3-methyl-1*H*-indole was higher in the embryo. This might explain the overall dominating moldy-musty odor note perceived in the cocoa samples tainted with the off-flavor.

Caterina Porcelli designed and performed the experiments including volatile isolations, GC-O screenings, structure assignments, syntheses, quantitations and sensory experiments. Caterina evaluated the resulting data and prepared the manuscript. Martin Steinhaus conceived and directed the study, supervised Caterina`s work and revised the manuscript. Martin additionally participated in the sensory tests, including the GC-O analyses.

7.3 Publication 3

7.3.1 Bibliographic data

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7.3.2 Publication reprint

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ORIGINAL PAPER

Molecular characterisation of an atypical coconut-like odour in cocoa

Caterina Porcelli[1] · **Martin Steinhaus**[1]

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Abstract

Parallel application of an aroma extract dilution analysis (AEDA) to the volatiles isolated from a sample of fermented cocoa with an atypically pronounced coconut note and to the volatiles isolated from a reference cocoa sample revealed coconut-like smelling compounds *δ*-octalactone, *δ*-2-octenolactone, *γ*-nonalactone, *γ*-decalactone, *δ*-decalactone, and *δ*-2-decenolactone as potential causative odorants. Quantitation of these six compounds and calculation of odour activity values as ratios of the concentrations to the odour threshold values suggested *δ*-2-decenolactone as the crucial compound. Chiral analysis showed the presence of pure (*R*)-*δ*-2-decenolactone, commonly referred to as massoia lactone. Its key role for the coconut note was finally demonstrated in a spiking experiment: the addition of (*R*)-*δ*-2-decenolactone to the reference cocoa in an amount corresponding to the concentration difference between the two samples was able to provoke a coconut note in an intensity comparable to the one in the atypically smelling cocoa. To avoid an undesired coconut note caused by (*R*)-*δ*-2-decenolactone in the final products, the chocolate industry may consider its odour threshold value, that is 100 µg/kg, as a potential limit for the acceptance of fermented cocoa in the incoming goods inspection.

Keywords Cocoa (*Theobroma cacao* L.) · Coconut aroma · Aroma extract dilution analysis (AEDA) · (*R*)-*δ*-2-Decenolactone · Massoia lactone · (6*R*)-5,6-Dihydro-6-pentyl-2*H*-pyran-2-one

Abbreviations

AEDA	Aroma extract dilution analysis
AV	Acidic volatiles
FD	Flavour dilution
GC–O	Gas chromatography–olfactometry
HDMF	4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one
NBV	Neutral and basic volatiles
OAV	Odour activity value
OTV	Odour threshold value
RI	Retention index
SAFE	Solvent-assisted flavour evaporation

Nomenclature

2-Acetyl-1-pyrroline	1-(3,4-Dihydro-2*H*-pyrrol-5-yl) ethan-1-one
Ethyl cinnamate	Ethyl (2*E*)-3-phenylprop-2-enoate
Geranial	(2*E*)-3,7-Dimethylocta-2,6-dien-1-al
Geraniol	(2*E*)-3,7-Dimethylocta-2,6-dien-1-ol
Linalool	3,7-Dimethylocta-1,6-dien-3-ol
Maltol	3-Hydroxy-2-methyl-4*H*-pyran-4-one
Sotolon	3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one
Vanillin	4-Hydroxy-3-methoxybenzaldehyde

Introduction

Cocoa is the key raw material in chocolate manufacturing and its quality is crucial for the pleasant aroma of chocolate products. Cocoa is derived from the seeds of the cocoa tree (*Theobroma cacao* L.) which is grown throughout the tropics, particularly in Ivory Coast, Ghana, Nigeria, Cambodia, Indonesia, Ecuador, Peru, and Brazil. After harvest, the cocoa fruits, also known as the cocoa pods, are opened and the seeds, 30–50 per pod, are collected together with the surrounding white mucilaginous pulp. In a fermentation step of 2–10 days, the pulp is removed and the seeds, now referred to as the cocoa beans [1], are dried. Annually, more

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than 4 million metric tons of fermented and dried cocoa are produced [2]. The cocoa is then shipped internationally to confectionery companies. Chocolate production starts with roasting of the cocoa. The roasted material is ground into cocoa liquor to which further ingredients such as sugar, cocoa butter, and milk powder are added before chocolate is finally obtained by conching, tempering, and moulding.

At the incoming goods inspection in the chocolate industry, the fermented cocoa undergoes a critical sensory evaluation. Ideally, the cocoa shows a rich aroma with sour, malty, floral, and fruity notes [3]. During roasting, further pleasant odour notes develop and the overall aroma intensifies [4, 5]. Occasionally, however, the fermented cocoa beans are tainted with off-flavours among which smoky and mouldy-musty notes are most prevalent [6, 7]. These off-notes can persist during further processing and thus decrease the marketability of the final confectionery products. Whereas the compounds responsible for the pleasant cocoa aroma and their development during processing have been studied in detail [8–11], little has been known on the molecular background of cocoa off-flavours before we started to work on the subject recently. We screened fermented cocoa samples with smoky off-notes as well as samples with mouldy-musty off-notes for potential off-flavour compounds by gas chromatography–olfactometry (GC–O) in combination with aroma extract dilution analyses (AEDA) and substantiated the results by quantitation and calculation of odour activity values (OAVs) [12, 13]. Moreover, we studied the behaviour of the off-flavour compounds during chocolate manufacturing [14]. Finally, we suggested maximum tolerable concentrations for the individual compounds in fermented cocoa which are applicable at the incoming goods inspection level in the chocolate industry and allow for a more objective decision-making on acceptance or rejection of cocoa batches than sensory testing can provide.

Although smoky und mouldy-musty off-flavours occur most frequently in fermented cocoa, another atypical odour has recently become a problem for the quality control departments in the chocolate industry, namely an odour described as coconut-like. The coconut note is clearly not as aversive as the smoky and mouldy-musty off-flavours and such cocoa might even be suitable to make specialty chocolate. However, a strong coconut-like note just occasionally and unpredictably occurring in a mainstream chocolate product is clearly undesired. Customers used to a specific product flavour tend to be very susceptible even to minor variations.

Unequivocal information on the molecular background of such an atypically pronounced coconut-like note in cocoa was not available in the scientific literature. Therefore, the aim of our investigation was to identify the crucial odour-active compound(s) in a sample of fermented cocoa with a pronounced coconut-like odour by applying GC–O and AEDA followed by quantitation and calculation of OAVs. A reference sample with a typical aroma profile was analysed in parallel.

Materials and methods

Cocoa and massoia bark oil samples

Four cocoa samples were provided by chocolate manufacturers. Sample C1 showed the most pronounced coconut note. Samples C2 and C3 also showed the atypical coconut note, but less pronounced. The fourth sample (REF) served as a reference with a typical aroma profile. Sample C1 consisted of cocoa nibs, samples C2 and C3 were cocoa liquors, and REF consisted of whole fermented cocoa seeds. All the samples were stored at 5 °C before analysis. Massoia bark oil was purchased from Maienfelser Naturkosmetik Manufaktur (Wüstenrot, Germany).

Chemicals

Reference odorants **1**–**6**, **8**, **11**–**21**, **23**–**33**, **36**–**48**, and (2*E*)-dec-2-enal were purchased from Merck (Darmstadt, Germany). Odorants **7** and **34** were obtained from Alfa Aesar (Karlsruhe, Germany). Odorants **10** and **22** were purchased from Acros Organics (Schwerte, Germany). Odorants **9** and **35** were synthetized according to literature procedures [15, 16].

The stable isotopically substituted odorants were prepared by approaches described in the literature: ($^2H_2$)-**34**, [6-(2,3-$^2H_2$)propyloxan-2-one] [17]; ($^2H_2$)-**36** [5-(1,2-$^2H_2$)pentyloxolan-2-one], ($^2H_2$)-**42** [5-(1,2-$^2H_2$)hexyloxolan-2-one] [18]; and ($^2H_{2\text{-}4}$)-**44** [6-pentyl(3,3,4,4-$^2H_4$)oxan-2-one] [19].

Dichloromethane, diethyl ether, and *n*-pentane were purchased from CLN (Freising, Germany). Before use, they were freshly distilled through a column (120 × 5 cm) packed with Raschig rings. Ethanol LiChrosolv® and hexane LiChrosolv® were obtained from Merck. Silica gel 60 (0.040–0.63 mm) was purchased from VWR (Darmstadt, Germany) and purified as detailed previously [20]. Low odour sunflower oil, brand Thomy, was from Nestlé (Neuss, Germany).

AEDA

Fermented cocoa seeds and cocoa liquor were flash frozen with liquid nitrogen and coarsely crushed using a laboratory mill Grindomix GM 200 (Retsch, Haan, Germany) at 3800 rpm (2 × 15 s). The material was ground into a fine powder by using a 6875 Freezer Mill (SPEX SamplePrep, Stanmore, UK). The powder (50 g) was stirred with dichloromethane (100 mL) at room temperature for 16 h. The mixture was filtered through a folded paper filter and the

residue was stirred with a second portion of dichloromethane (100 mL) for 1 h. After filtration, the combined extracts were dried over anhydrous sodium sulphate and nonvolatiles were removed by solvent-assisted flavour evaporation (SAFE) at 40 °C [21]. The distillate was shaken with an aqueous solution of sodium hydrogen carbonate (0.5 mol/L; 2 × 100 mL). The aqueous extracts were combined. The organic phase was washed with brine (50 mL), dried over anhydrous sodium sulphate and concentrated (1 mL), first using a Vigreux column (50 × 1 cm) and subsequently a Bemelmans microdistillation device to afford the neutral and basic volatiles (NBV) fraction [22]. The aqueous phase was washed with dichloromethane (50 mL), acidified (pH 2) with hydrochloric acid (5 mol/L), and the acidic volatiles were re-extracted with dichloromethane (3 × 50 mL). The combined organic phases were washed with brine (50 mL), dried over anhydrous sodium sulphate, and concentrated (1 mL) to afford the acidic volatiles (AV) fraction.

The fractions NBV and AV were analysed using a GC–O/FID system (cf. Supplementary Information file) and an FFAP column. Three experienced assessors (2 females, 1 male, aged 27–50) carried out the analyses. GC–O runs were repeated until the individual results were reproducible. Afterwards, fractions NBV and AV were stepwise diluted 1:2 with dichloromethane to obtain dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, and 1:8192 of the initial solution and the diluted samples were also subjected to GC–O analysis by the same three experienced assessors. Finally, each odorant was assigned a flavour dilution (FD) factor defined as the dilution factor of the highest diluted sample in which the odorant was detected during GC–O analysis by any of the three assessors [23, 24].

To clarify the structures of the odorants, at first the retention indices (RIs) and odour qualities were compared with data from the Leibniz-LSB@TUM odorant database [25]. In the second step, reference samples of the proposed odorants were analysed by GC–O in parallel to the cocoa volatile isolates by using two capillary columns of different polarity (FFAP and DB-5). Final structure confirmation was achieved by GC–MS. To avoid coelution problems, fraction NBV was further fractionated into five subfractions by liquid chromatography before GC–MS analysis. For this purpose, hexane (1 mL) was added to fraction NBV (1 mL) and the mixture was re-concentrated to a volume of 1 mL. The concentrate was applied onto a slurry of purified silica gel (8 g) in pentane within a water-cooled (12 °C) glass column (1 cm i.d.). Elution was carried out with pentane/diethyl ether mixtures of 100 + 0, 90 + 10, 70 + 30, 50 + 50, and 0 + 100 (v + v; 50 mL each). The eluate was collected in five portions of 50 mL and each portion was dried over anhydrous sodium sulphate, filtered, and concentrated to 1 mL. The odorants previously detected in fraction NBV were localized in the individual subfractions by GC–O. Then the fractions were analysed in parallel to reference compound solutions with a GC–HRMS system (cf. Supplementary Information file) run in EI or CI mode using an FFAP or a DB-5 column.

Odorant quantitation

Samples were powdered as detailed above. Dichloromethane (50 mL) was added to the powder (5 g) and stable isotopically substituted odorants (~ 5 ng–2 µg) in dichloromethane (10 µL–2 mL) were added as internal standards. After magnetic stirring at room temperature for 16 h, the mixture was filtered through a folded paper filter. The residue was stirred (1 h) with a second portion of dichloromethane (50 mL). After filtration, the combined extracts were dried over anhydrous sodium sulphate. Nonvolatiles were removed by SAFE at 40 °C. The distillate was dried over anhydrous sodium sulphate and concentrated (100 µL).

The concentrates were analysed using a heart-cut GC–GC–HRMS system (cf. Supplementary Information file). First, the retention times of the target compounds and the internal standards in the first and second dimensions were determined by analysis of reference mixtures. During analysis of the cocoa volatiles, a heart-cut (1–2 min) of the eluate of the first column containing the respective target compound and the internal standard was transferred to the second column. Transferred substances were refocused in a cold trap. Finally, cooling of the trap was turned off, and the second oven and the mass spectrometer were started. Peak areas corresponding to the analyte and internal standard were obtained from extracted ion chromatograms using characteristic quantifier ions. The concentration of each target compound in the cocoa samples was then calculated from the area counts of the analyte peak, the area counts of the standard peak, the amount of cocoa sample, and the amount of standard added, by employing a calibration line equation previously obtained from the analysis of analyte/standard mixtures in different concentration ratios followed by linear regression. Individual quantifier ions and calibration line equations are available in the Supplementary Information file, Table S1.

Odour threshold values

The odour threshold values of compounds **34**, **36**, **42**, **44** and **45** in low odour sunflower oil were determined orthonasally by a series of three alternative forced choice tests according to the standard practice of the American Society for Testing and Materials [26]. Test samples (10 g) consisted of sunflower oil which had been spiked with the odorant, blank samples consisted of pure sunflower oil. Between two consecutive three alternative forced choice tests, odorant concentrations increased threefold. Samples (10 g) were

presented in cylindrical plastic vessels (5.2 cm height, 3.5 cm i.d.) with lids. Tests were performed at a temperature of 22 ± 2 °C in a special room exclusively dedicated to sensory evaluations. The panel consisted of 15–20 trained assessors.

The odour threshold values of the individual δ-2-decenolactone enantiomers in air were determined by AEDA using the GC–O/FID system (cf. Supplementary Information file) with the chiral MEGA-DEX DAC-Beta column and the approach detailed in [27]. The internal standard was (2*E*)-dec-2-enal with an odour threshold value of 2.7 ng/L [28].

Isolation of massoia bark oil volatiles

Massoia bark oil (2 mL) was diluted with dichloromethane (50 mL) and the mixture was subjected to SAFE. The distillate was dried over anhydrous sodium sulphate, concentrated (1 mL) using a Vigreux column (50 × 1 cm), and stored at − 20 °C before analysis.

Quantitative olfactory profiles

Powdered reference cocoa (5 g) was spiked with δ-2-decenolactone (9 µg of the commercial mixture) in ethanol (150 µL). This sample was subjected to a quantitative olfactory profile analysis together with a sample of the powdered reference cocoa without addition of δ-2-decenolactone and a sample of powdered cocoa C1 with the pronounced coconut-like note. To the latter two samples, 150 µL of pure ethanol were added. Samples (5 g) were presented in cylindrical PTFE vessels (5.7 cm height, 3.5 cm i.d.) with lids. Tests were performed in the special room detailed above. A panel of 19 trained assessors evaluated the three samples orthonasally and assigned scores ranging from 0 to 3 with 0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong to eight pre-defined odour descriptors previously collected by free-choice profiling. For each descriptor an odour reference was provided consisting of an aqueous odorant solution in a concentration ~ 100 times above the orthonasal odour threshold value. The eight descriptors and the corresponding reference odorants were "coconut-like" (γ-nonalactone), "vanilla-like" (vanillin), "honey" (phenylacetaldehyde), "banana-like" (3-methylbutyl acetate), "fruity" (ethyl 2-methylbutanoate), "vinegar-like" (acetic acid), "earthy" (2,3,5-trimethylpyrazine), and "malty" (3-methylbutanal). The scores of the individual assessors were averaged by calculating the arithmetic mean.

Results and discussion

Odorant screening

Parallel application of an AEDA to the volatiles isolated from a cocoa sample with an atypically pronounced coconut note (C1) and to the volatiles isolated from a reference cocoa (REF) revealed a total of 48 odorants with an FD factor of 4 or higher in at least one of the two samples, 46 of which were unequivocally identified (Table 1).

In both samples, the highest FD factors (2048–4096) were determined for fruity smelling compounds ethyl butanoate (**3**) and ethyl 2-methylbutanoate (**4**), bell pepper-like smelling 3-isopropyl-2-methoxypyrazine (**14**), cheesy smelling 2-/3-methylbutanoic acid (**23**), floral, citrusy smelling geranial (**25**), rosy smelling 2-phenylethyl acetate (**28**), cinnamon-like smelling ethyl 3-phenylpropanoate (**31**), honey-like, rosy smelling 2-phenylethan-1-ol (**32**), metallic smelling *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**35**), caramel-like smelling 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF; Furaneol®) (**38**), and soup seasoning-like smelling sotolon (**43**). These compounds are all well-known cocoa odorants and have been reported in numerous studies before [3, 9, 29–31].

Six of the 48 odorants depicted in Table 1 showed a coconut odour. All six compounds were γ- or δ-lactones, namely δ-octalactone (**34**), γ-nonalactone (**36**), δ-2-octenolactone (**37**), γ-decalactone (**42**), δ-decalactone (**44**) and δ-2-decenolactone (**45**). Their structures are depicted in Fig. 1. Given the fact that their coconut-like odour quality exactly matched the atypical note detected in cocoa sample C1, the six lactones were identified as potential causative compounds and a substantial contribution of other compounds was considered unlikely. In general, γ-lactones and δ-lactones are important contributors to the aroma of different kinds of fruits such as apricots and peaches [32–35], wines and spirits [36], and milk products [19, 37]. Their biosyntheses start from fatty acids. After introduction of a hydroxy group, the chain length is reduced by β-oxidation. Under acidic conditions, the shortened hydroxy carboxylic acids finally undergo cyclization to form the lactones [38]. The six coconut-like smelling lactones identified in the current study have been found in cocoa before [9, 12, 13, 29]; however, none of them has been reported as causative for an atypical aroma note in fermented cocoa so far. Among these six lactones, δ-2-decenolactone (**45**) showed the highest FD factor in the cocoa sample C1 with the atypically pronounced coconut-like odour, namely 2048. In this sample, higher FD factors were only obtained for 2 of the 48 odorants, namely for 2-phenylethyl acetate and sotolon (both 4096). Moreover, with 256 the FD factor of δ-2-decenolactone in the reference sample was clearly lower. High FD factors in sample

Table 1 Odorants in the volatile isolates obtained from the cocoa with the atypically pronounced coconut odour (C1) and from the reference cocoa (REF)

No	Odorant[a]	Odour quality[b]	RI[c]		FD factor[d]	
			FFAP	DB-5	C1	REF
1	Ethyl 2-methylpropanoate	Fruity	< 1000	765	16	4
2	Butane-2,3-dione	Butter	< 1000	< 700	16	8
3	Ethyl butanoate	Fruity	1031	804	256	2048
4	Ethyl 2-methylbutanoate	Fruity	1060	849	512	2048
5	Ethyl 3-methylbutanoate	Fruity, blueberry	1077	852	32	128
6	3-Methylbutyl acetate	Fruity, banana	1130	878	64	64
7	3-Hydroxybutan-2-one	Butter	1262	800	64	32
8	1-Octen-3-one	Mushroom	1298	979	16	8
9	2-Acetyl-1-pyrroline[e]	Popcorn	1332	922	4	4
10	Dimethyl trisulfide	Cabbage	1370	967	256	8
11	2,3,5-Trimethylpyrazine	Earthy	1383	1002	64	4
12	Unknown	Rose, citrus	1411		4	< 1
13	Ethyl cyclohexanecarboxylate	Fruity	1414	1131	< 1	256
14	3-Isopropyl-2-methoxypyrazine	Bell pepper	1430	1095	2048	256
15	Acetic acid	Vinegar, pungent	1436	< 700	64	32
16	3-(Methylsulfanyl)propanal[e]	Cooked potato	1454	905	1024	512
17	3-Isobutyl-2-methoxypyrazine	Bell pepper	1518	1184	256	128
18	(2*E*)-Non-2-enal	Green, fatty	1532	1160	64	64
19	Linalool	Citrus, bergamot	1545	1102	32	64
20	2-Methylpropanoic acid	Sweaty, cheese	1553	789	64	64
21	Butanoic acid	Sweaty, cheese	1618	821	128	512
22	Phenylacetaldehyde	Honey	1643	1046	256	128
23	2-/3-Methylbutanoic acid	Cheese	1655	874	2048	2048
24	2-Acetylthiazole	Popcorn	1667	1038	256	16
25	Geranial	Floral, citrus	1711	1269	2048	2048
26	(2*E*,4*E*)-Deca-2,4-dienal	Fatty, deep-fried	1780	1317	16	8
27	Ethyl phenylacetate	Honey	1795	1246	32	256
28	2-Phenylethyl acetate	Rose	1822	1256	4096	512
29	Geraniol	Citrus, rose	1843	1256	256	256
30	2-Methoxyphenol	Smoky	1858	1090	1024	512
31	Ethyl 3-phenylpropanoate	Cinnamon	1881	1418	1024	2048
32	2-Phenylethan-1-ol	Honey, rose	1919	1116	2048	2048
33	Maltol	Caramel	1961	1110	512	64
34	*δ*-Octalactone	Coconut	1984	1250	512	512
35	*trans*-4,5-Epoxy-(2*E*)-dec-2-enal	Metallic	2012	1382	1024	2048
36	*γ*-Nonalactone	Coconut	2029	1393	1024	1024
37	*δ*-2-Octenolactone	Coconut, creamy	2047	1264	16	16
38	HDMF[e]	Caramel	2028	1071	2048	128
39	Unknown	Burnt	2050		256	256
40	4-Methylphenol	Faecal, horse stable	2072	1078	32	32
41	Ethyl cinnamate	Sweet, cinnamon	2106	1469	16	16
42	*γ*-Decalactone	Coconut, peach	2140	1466	1024	1024
43	Sotolon[e]	Soup seasoning	2200	1107	4096	1024
44	*δ*-Decalactone	Coconut	2210	1494	32	1
45	*δ*-2-Decenolactone	Coconut	2255	1475	2048	256
46	3-Methyl-1*H*-indole	Faecal, mothball	2513	1390	8	4
47	Phenylacetic acid	Honey, beeswax	2547	1261	32	16
48	Vanillin	Vanilla	2573	1408	512	256

[a]Each odorant was identified by comparing its retention indices on two GC capillaries of different polarity (FFAP, DB-5), its mass spectrum obtained by GC–MS, as well as its odour quality as perceived at the sniff-

Table 1 (continued)

ing port during GC–O with data obtained from authentic reference compounds analysed under the same conditions

[b]Odour quality as perceived during GC–O analysis at the sniffing port

[c]Retention index; calculated from the retention time of the odorant and the retention times of adjacent *n*-alkanes by linear interpolation

[d]Flavour dilution factor; dilution factor of the highest diluted sample prepared from the concentrated SAFE distillate in which the odorant was detected during GC–O by any of three assessors

[e]A clear mass spectrum could not be obtained in the cocoa volatile isolates; identification was based on the remaining criteria detailed in footnote a

C1 were additionally obtained for γ-nonalactone (1024), γ-decalactone (1024), and δ-octalactone (512). For these 3 compounds, however, the FD factors indicated no difference between sample C1 and the reference sample. Low FD factors of δ-2-octenolactone (**37**) and δ-decalactone (**44**) suggested only a minor role of these compounds for the atypical odour in C1.

In summary, the odorant screening resulted in six compounds that potentially contributed to the atypical odour of the coconut-like smelling cocoa C1, but their individual roles needed further clarification. Therefore, the next steps in our study were to quantitate the six lactones and compare the concentrations with the odour threshold values of the individual compounds by calculating odour activity values.

Quantitation and calculation of odour activity values

The concentrations of the six coconut-like smelling lactones were determined by GC–MS analysis of volatile isolates obtained by solvent extraction and SAFE. To compensate for losses during sample workup, stable isotopically substituted odorants (cf. Supplementary Information file, Table S1) were added prior to the workup as internal standards. For compounds **34**, **36**, **42**, and **44**, deuterated isotopologues of the target compounds were employed. Isotopologues were not available for compounds **37** and **45**. Instead, the deuterated isotopologues of the corresponding saturated lactones **34** and **44** were used as internal standards. The results obtained for samples C1 and REF previously used for the odorant screening are shown in Table 2 together with the data of two additional samples (C2, C3) having an atypical, though less pronounced coconut note than sample C1. The concentrations of compounds **34**, **36**, **37**, **42**, and **44** were hardly suitable to explain the sensory difference between the samples. Although, for example, compounds **36** and **44** showed clearly higher concentrations in the samples with the atypical note than in the reference sample, the highest concentrations were obtained in sample C2, and not in C1 which showed the most intense coconut note. In contrast, the concentration of δ-2-decenolactone (**45**) was well in line with the sensory rating, thus being highest in sample C1 and lowest in the reference sample. This would be in line with a major role of δ-2-decenolactone for the coconut odour.

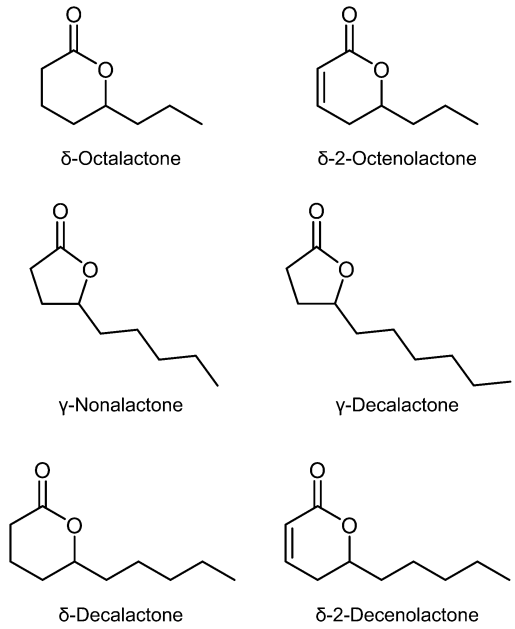


Fig. 1 Coconut-like smelling compounds identified in the cocoa samples

The picture became clearer, when the odour threshold values of the compounds were taken into account. For this

Table 2 Concentrations of lactones in three samples of fermented cocoa with an atypically pronounced coconut odour (C1, C2, C3) and in the reference cocoa sample (REF)

No	Odorant	Concentration (µg/kg)[a]			
		C1	C2	C3	REF
34	δ-Octalactone	0.195	0.744	0.355	0.422
36	γ-Nonalactone	152	191	162	125
37	δ-2-Octenolactone	0.319	1.59	0.900	0.706
42	γ-Decalactone	51.3	413	52.3	109
44	δ-Decalactone	76.0	461	92.3	6.98
45	δ-2-Decenolactone	1580	570	210	86.1

[a]Mean of duplicates or triplicates; individual values and standard deviations are provided in the Supplementary Information file, Table S2

Table 3 Odour activity values (OAVs) of lactones in three samples of fermented cocoa with an atypically pronounced coconut odour (C1, C2, C3) and in the reference cocoa sample (REF)

No	Odorant	OTV_{oil}[a] (µg/kg)	OAV[b]			
			C1	C2	C3	REF
34	δ-Octalactone	1600[c]	0.00	0.00	0.00	0.00
36	γ-Nonalactone	1300[c]	0.12	0.15	0.12	0.10
37	δ-2-Octenolactone	4700[c,d]	0.00	0.00	0.00	0.00
42	γ-Decalactone	4800[c]	0.01	0.09	0.01	0.02
44	δ-Decalactone	4300[c]	0.02	0.11	0.02	0.00
45	δ-2-Decenolactone	120[e]	13	4.7	1.7	0.71

[a]Orthonasal odour threshold value in low odour sunflower oil

[b]Odour activity value; calculated as ratio of the concentration in the cocoa sample to the OTV

[c]OTV of a racemic mixture

[d]Data from literature [9]

[e]OTV of a commercial sample with unknown enantiomeric ratio

purpose, the concentrations (cf. Table 2) were divided by the respective odour threshold values previously determined with low odour sunflower oil as matrix to obtain the odour activity values of the compounds in the four samples. The odour activity values thus represented the factors by which the concentrations in the cocoa samples exceeded the respective odour threshold values. The results, however, included an approximation due to the chirality of the compounds. The odour threshold values of compounds **34**, **36**, **37**, **42**, and **44** were determined with racemic mixtures, whereas the enantiomeric distribution in the cocoa samples was unknown. Moreover, the odour threshold value of δ-2-decenolactone (**45**) was determined with a commercial mixture, the enantiomeric composition of which was also unknown. Nevertheless, the result was very clear, because the concentrations of compounds **34**, **36**, **37**, **42**, and **44** were by far below the respective odour threshold values of the racemates in all four samples corresponding to odour activity values between 0.00 and 0.15 (Table 3). In contrast, δ-2-decenolactone (**45**) showed approximated OAVs of 13 in sample C1, 4.7 in sample C2, and 1.7 in sample C3. In the reference sample, the concentration of δ-2-decenolactone was below the OTV resulting in an OAV of 0.71. The low OAVs of compounds **34**, **36**, and **42** were somewhat surprising, given their rather high FD factors in the AEDA (cf. Table 1). This exemplifies the importance of avoiding overinterpretation of AEDA data. Instead, AEDA results should always be substantiated by quantitation and calculation of OAVs [24]. In summary, the OAV calculations suggested that δ-2-decenolactone (**45**) was mainly responsible for the atypical odour of samples C1, C2, and C3.

δ-2-Decenolactone was first reported in 1937 by Abe who identified it as the primary odour-active compound in the bark of the massoia tree (*Cryptocaria massoia*), a tropical tree growing wild in the rain forests of New Guinea. Before cheaper synthetic flavourings became available, massoia bark oil was widely used as coconut flavouring [39, 40]. In massoia bark, only the (*R*)-isomer of δ-2-decenolactone is present [41, 42]. (*R*)-δ-2-decenolactone is therefore also often referred to as massoia lactone.

Chiral analysis of δ-2-decenolactone

To confirm the crucial role of δ-2-decenolactone for the atypical note in the coconut-like smelling cocoa sample C1, a spiking experiment was considered the method of choice: δ-2-decenolactone would be added to the reference sample to reach the same concentration as determined in C1 and the mixture would be sensorially evaluated. This experiment, however, needed to consider the enantiomeric distribution of the δ-2-decenolactone in the cocoa beans and in the spiking solution, because enantiomers often widely differ in their odour threshold values and sometimes even in their odour qualities. This has, for example, clearly been demonstrated for a homologous series of saturated γ- and δ-lactones [32, 36]. In most cases, the (*R*)-enantiomers showed a higher odour potency than the (*S*)-enantiomers. For δ-2-decenolactone, however, no such data was available. Therefore, we subjected the δ-2-decenolactone reference sample to enantioGC analysis using a chiral β-cyclodextrin-based GC column. The experiment revealed an enantiomeric ratio of 17/83. Analysis of a natural massoia bark oil allowed to assign the elution order as S before R. Thus, our commercially obtained reference compound consisted of 83% (*R*)-δ-2-decenolactone and 17% (*S*)-δ-2-decenolactone. GC–O showed that both enantiomers have a coconut-like odour; however, they clearly differed in their odour potency. Using the method of Ullrich and Grosch [27], the odour threshold values in air of both δ-2-decenolactone enantiomers were determined by AEDA. Results (Table 4) showed that also in δ-2-decenolactone, the (*R*)-enantiomer represented the more potent odorant. With 1.6 ng/L, its odour threshold value was ~ 30 times lower than that of the (*S*)-enantiomer.

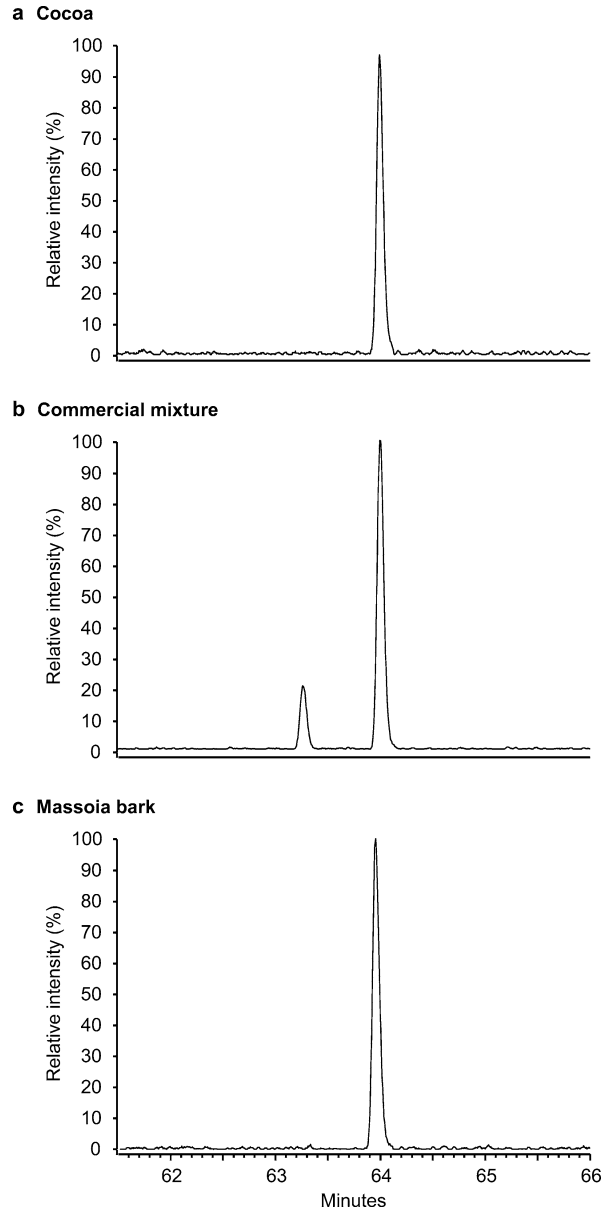


Fig. 2 Heart-cut GC–enantioGC–HRMS analysis of the volatile isolate obtained from the cocoa sample C1 with the atypically pronounced coconut note (**a**) in comparison to the commercial mixture of *δ*-2-decenolactone with 17% (*S*)- and 83% (*R*)-enantiomer (**b**) and the volatile isolate obtained from massoia bark oil (**c**)

As a next step, we determined the enantiomeric ratios of *δ*-2-decenolactone in the cocoa samples. For that purpose, the volatile isolates were subjected to two-dimensional gas chromatography with heart-cutting using the chiral column in the second dimension and a high resolution mass spectrometer as the detector. Results showed that the *δ*-2-decenolactone in cocoa was pure (*R*)-enantiomer. Figure 2 depicts the relevant chromatogram sections after injection

Table 4 Odour threshold values (OTVs) of (*R*)- and (*S*)-*δ*-2-decenolactone in air

No	Odorant	Odour quality	OTV_{air} (ng/L)
45a	(*R*)-*δ*-2-Decenolactone	Coconut	1.6
45b	(*S*)-*δ*-2-Decenolactone	Coconut	52

Table 5 Odour activity values (OAVs) of (*R*)-*δ*-2-decenolactone in three samples of fermented cocoa with an atypical coconut-like odour (C1, C2, C3) and in the reference cocoa sample without a coconut-like odour note (REF)

Odorant	OTV_{oil}[a] (µg/kg)	OAV[b]			
		C1	C2	C3	REF
(*R*)-*δ*-2-decenolactone	100	16	5.6	2.1	0.84

[a]Odour threshold value in oil; approximated from the odour threshold value in air (cf. Table 4) and the odour threshold value of the 83/17 (*R*)/(*S*)-mixture in oil as 0.83×120 µg/kg + $(1.6/51.5) \times 0.17 \times 120$ µg/kg

[b]Odour activity value; calculated as ratio of the concentration in the cocoa sample (cf. Table 2) to the OTV in oil

of the volatile isolate obtained from sample C1 with the atypically pronounced coconut note (Fig. 2a), the reference mixture with 83% (*R*)- and 17% (*S*)-enantiomer (Fig. 2b), and the volatile isolate obtained from massoia bark oil (Fig. 2c). In addition to massoia bark and fermented cocoa, enantiopure (*R*)-*δ*-2-decenolactone has also been reported in Merlot and Cabernet Sauvignon musts and wines, where it contributes to dried fruit aroma notes [40].

With the knowledge of the enantiomeric purity of *δ*-2-decenolactone in fermented cocoa and the knowledge on the difference in the odour potency of the two enantiomers, a better approximation of its relevance for the atypical coconut-like odour was possible. From the odour threshold values of the individual enantiomers in air (cf. Table 4) and the odour threshold value of the reference mixture with 83% (*R*)- and 17% (*S*)-enantiomer in oil (120 µg/kg; cf. Table 3), the odour threshold values of the individual enantiomers in oil were approximated. The calculations resulted in odour threshold values in oil of 3300 µg/kg for the (*S*)-*δ*-2-decenolactone and 100 µg/kg for the (*R*)-*δ*-2-decenolactone. Using the latter for the OAV calculations of (*R*)-*δ*-2-decenolactone in the cocoa samples resulted in values of 16 in C1, 5.6 in C2, and 2.1 in C3, whereas in the reference sample REF the OAV was below 1 (Table 5). In summary, these data supported the hypothesis that (*R*)-*δ*-2-decenolactone was the compound being responsible for the atypical coconut-like odour in the fermented cocoa samples.

Final evidence was eventually provided for sample C1 by a spiking experiment.

Odorant spiking

A sample of the reference cocoa REF was spiked with 1800 µg/kg of the commercially obtained *δ*-2-decenolactone which corresponded to 1490 µg/kg (*R*)-*δ*-2-decenolactone and thus the concentration difference between the reference sample REF and the sample with the atypically pronounced coconut note C1 (cf. Table 5). In this experiment, the odour contribution of the (*S*)-*δ*-2-decenolactone included in the commercially obtained *δ*-2-decenolactone was considered negligible; approximated from its percentage (17%) and its relative odour potency (1.6/51.5; cf. Table 4), this contribution was only 0.5%, whereas 99.5% of the odour could be attributed to the (*R*)-isomer.

The spiked sample was orthonasally compared to the reference cocoa without addition of (*R*)-*δ*-2-decenolactone and to the cocoa sample C1 with the atypical coconut-like odour note in a quantitative olfactory profile analysis. The result (Fig. 3) clearly showed that the spiking with the (*R*)-*δ*-2-decenolactone was able to provoke the atypically pronounced coconut odour. The rating of the coconut note in the spiked sample (Fig. 3a) was clearly higher than that in the reference cocoa without addition of (*R*)-*δ*-2-decenolactone (Fig. 3b) and in the same range as the rating in sample C1 (Fig. 3c).

Conclusion

The combination of a comparative odorant screening by AEDA, the targeted quantitation of potentially relevant compounds identified by their specific odour, and the calculation of odour activity values suggested *δ*-2-decenolactone as the compound causative for an atypically pronounced coconut odour in a sample of fermented cocoa. Chiral analysis indicated the presence of pure (*R*)-*δ*-2-decenolactone (Fig. 4). A spiking experiment finally confirmed the crucial role of (*R*)-*δ*-2-decenolactone for the coconut-like aroma note in this sample. In accordance with the concentrations determined in the three cocoa samples with an atypical coconut note and the reference cocoa without pronounced coconut note (cf. Table 5), the chocolate industry may consider 100 µg/kg, that is the odour threshold value of (*R*)-*δ*-2-decenolactone, as a provisional limit for the acceptance of fermented cocoa in the incoming goods inspection.

Further studies on the topic are required to achieve a more comprehensive understanding of the molecular basis of atypical coconut notes in cocoa. These should include a larger number of samples and clarify in particular whether in other cocoa samples further lactones may contribute to

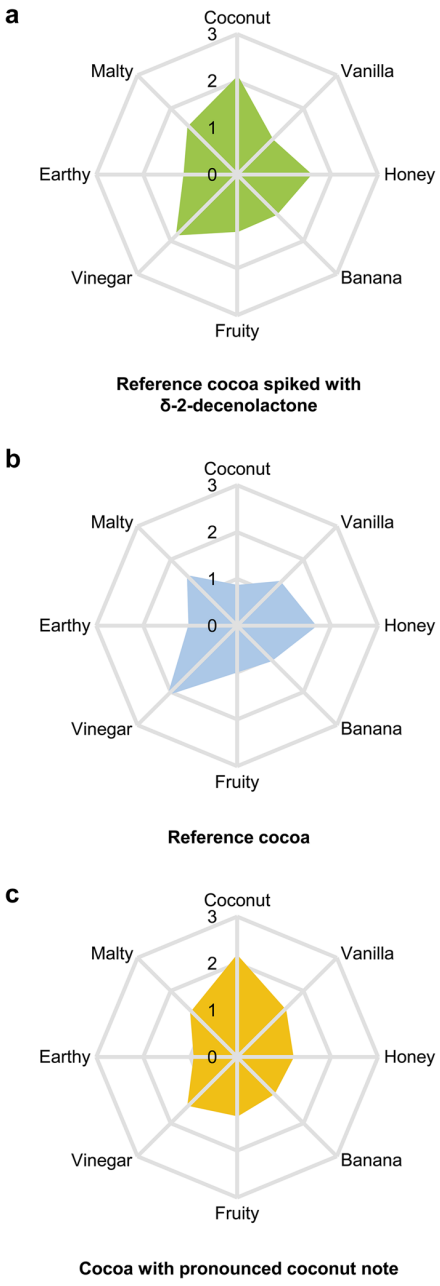
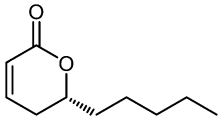


Fig. 3 Olfactory profiles of the reference cocoa sample spiked with *δ*-2-decenolactone (**a**), the reference sample without addition (**b**), and the sample C1 with the atypically pronounced coconut note (**c**). Assessors rated the intensity of each descriptor on a scale from 0 to 3 with 0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong (details are available in the Supplementary Information file, Table S3)

Fig. 4 (*R*)-*δ*-2-Decenolactone



the atypical coconut-like odour. In the absence of high concentrations of (*R*)-*δ*-2-decenolactone, even subthreshold concentrations of other lactones might lead to a perceivable coconut note as recently hypothesized in a study on milk chocolate [43]. An aspect that could also be considered in future investigations is the influence of water presence on the concentrations of lactones in cocoa [44].

Another open question is the source of the (*R*)-*δ*-2-decenolactone and other coconut-like smelling lactones in fermented cocoa. One possibility is their synthesis by microorganisms. For example, biosynthesis of (*R*)-*δ*-2-decenolactone was reported in *Fusarium solani* [45] and (*R*)-*δ*-2-octenolactone was found in *Lasiodiplodia theobromae* [46]. The use of infested pods might be crucial for the development of atypically strong coconut notes in fermented cocoa. This could also explain their rather occasional occurrence.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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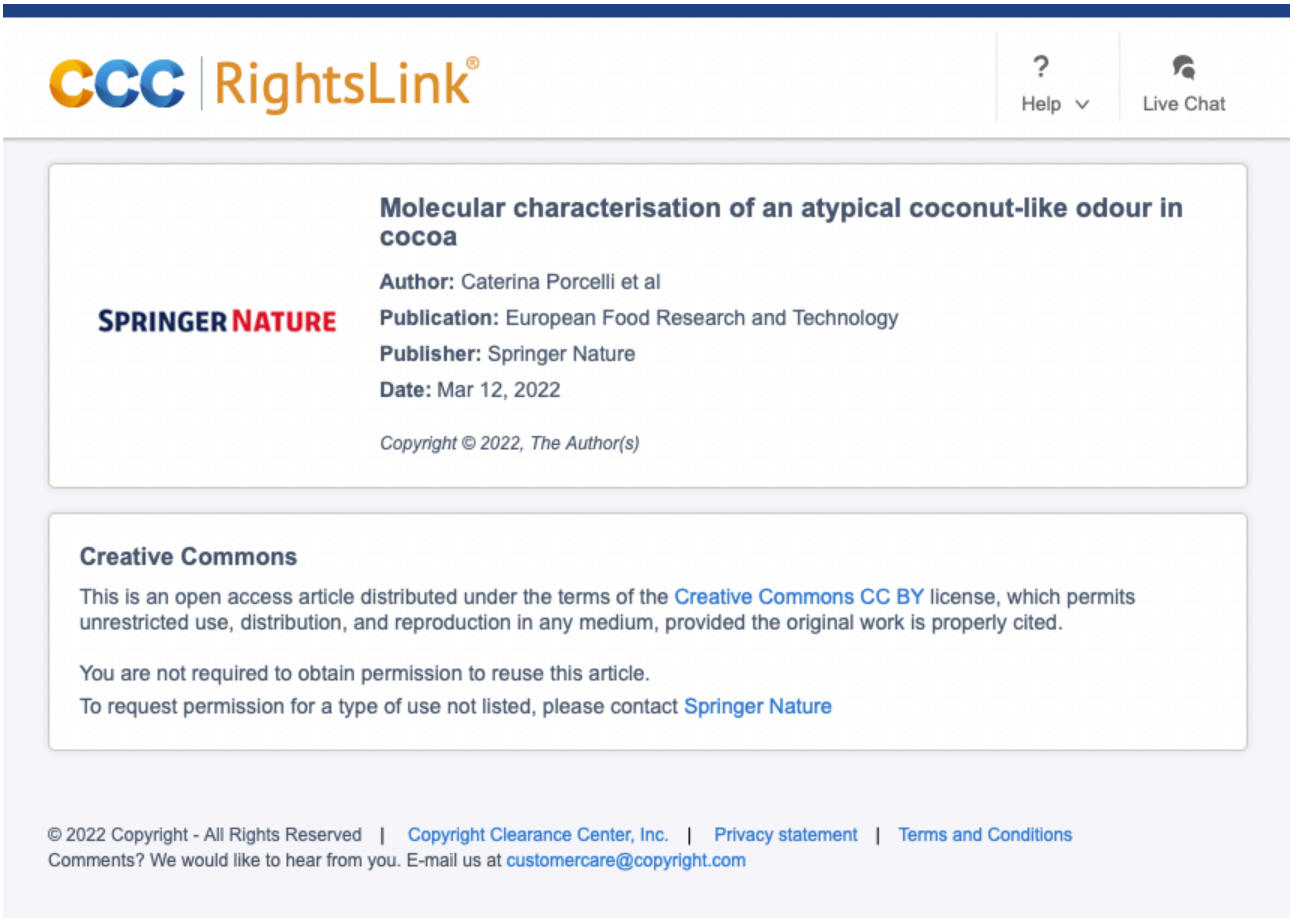

7.3.4 Summary and individual contributions

When sourcing fermented cocoa from the international market, the chocolate industry is occasionally confronted with batches tainted with atypical odors, such as a note described as coconut-like. This note is clearly not as aversive as the smoky and moldy-musty off-flavors and coconut-smelling fermented cocoa might even be suitable to manufacture specialty chocolate. However, such a note can lead to complaints from the customers, who tend to expect a reproducible flavor from a specific product and are rather susceptible even to minor variations.

In order to identify the causative compounds for this note, the fermented cocoa seeds of a coconut-like smelling sample were milled and treated with solvent. Nonvolatiles were removed from the extract by SAFE. The SAFE distillate was concentrated and screened for off-flavor compounds by aroma extract dilution analysis (AEDA). A reference sample with a typical aroma profile was analyzed in parallel.

The approach revealed 48 odorants with an FD factor of ≥ 4 in at least one of the two samples. Among these compounds, six showed a coconut-like odor, namely δ-octalactone, δ-2-octenolactone, γ-nonalactone, γ-decalactone, δ-decalactone and δ-2-decenolactone and were highlighted as potential causative odorants. To substantiate their role for the atypical coconut-like note, their quantitation was performed and their odor activity values (OAVs) were calculated as ratios of the concentrations to the odor threshold values (OTVs). The results obtained suggested δ-2-decenolactone as the crucial compound.

By chiral analysis, the presence of pure (*R*)-δ-2-decenolactone, commonly referred to as massoia lactone, was shown. Its key role for the coconut note could finally be demonstrated in a spiking experiment: the addition of (*R*)-δ-2-decenolactone to the reference cocoa in an amount corresponding to the concentration difference between the two samples was able to provoke a coconut note in an intensity comparable to the one in the atypically smelling cocoa. To avoid an undesired coconut note caused by (*R*)-δ-2-decenolactone in the final products, the chocolate industry may consider its odor threshold value, that is 100 µg/kg, as a potential limit for the acceptance of fermented cocoa in the incoming goods inspection.

Caterina Porcelli designed and performed the experiments including volatile isolations, GC-O screenings, structure assignments, syntheses, quantitations and sensory experiments. Caterina evaluated the resulting data and prepared the manuscript. Martin Steinhaus conceived and directed the study, supervised Caterina`s work and revised the manuscript. Martin additionally participated in the sensory tests, including the GC-O analyses.

7.4 List of publication, talks and poster presentations

Publications

Publications in peer reviewed journals:

Porcelli, C.; Kreissl, J.; Steinhaus M. Enantioselective synthesis of tri-deuterated (–)-geosmin to be used as internal standard in quantitation assays. *J Labelled Compd Radiopharm*. **2020**, *63*, 11, 476–481. DOI: 10.1002/jlcr.3874

Porcelli, C.; Neiens S. D.; Steinhaus M. Molecular background of a moldy-musty off-flavor in cocoa. *J Agric Food Chem* **2021**, *69*, 15, 4501–4508. DOI: 10.1021/acs.jafc.1c00564

Porcelli, C.; Steinhaus M. Molecular characterization of an atypical coconut-like odour in cocoa. *Eur Food Res Technol* **2022**, *248*, 1513–1523. DOI: 10.1007/s00217-022-03981-5

Miscellaneous journal contributions:

Porcelli, C.; Steinhaus M. Synthese von deuteriertem Geosmin für den Einsatz als interner Standard in Quantifizierungsassays. *Lebensmittelchemie* **2020**, *74*, 9. DOI: 10.1002/lemi.202051009

Talks

Oral presentations at scientific meetings:

Porcelli, C.; Steinhaus M. Enantioselective synthesis of tri-deuterated (–)-geosmin to be used as internal standard in quantitation assays. Lebensmittelchemische Gesellschaft (LChG), Fachgruppe in der Gesellschaft Deutscher Chemiker (GDCh), 71. Arbeitstagung des Regionalverbands Bayern (German Society of Food Chemistry, a division of the German Chemical Society, 71st Bavarian Regional Meeting). Würzburg, Germany, March 10, 2020.

Porcelli, C.; Steinhaus M. Elucidation of the molecular background of moldy-earthy off-flavors in cocoa. Forschungsseminar der Lebensmittechemie. Freising, Germany, October 10, 2020.

Poster presentations

Füllemann, D.; Porcelli, C.; Steinhaus M. Odour-active compounds in fermented cocoa showing hammy and mouldy off-flavours. Runder Tisch Kakao. Hamburg, Germany, June 22–23, 2017.

Porcelli, C.; Steinhaus M. Synthesis of stable isotopically substituted geosmin to be used as internal standard in the sensitive quantitation of geosmin in cocoa. Choco Tec. Cologne, Germany, December 3–5, 2018.