Technische Universität München

Fakultät für Chemie

Molecular Background of Smoky Off-Flavors in Cocoa

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Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung einer

Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Michael Rychlik

Prüfer der Dissertation:
1. Priv.-Doz. Dr. Martin Steinhaus
2. Prof. Dr. Wilfried Schwab

Die Dissertation wurde am 13.04.2022 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 24.05.2022 angenommen.

Für meine Eltern Sabine und Wolfgang

&

meine Geschwister Katharina und Alexander

The experimental part of the present work was performed between April 2017 and April 2020 at the Leibniz Institute for Food Systems Biology at the Technical University of Munich (before September 15, 2017: German Research Centre for Food Chemistry), Freising, Germany, under the supervision of Priv.-Doz. Dr. rer. nat. habil. Martin Steinhaus.

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1   Summary

The odor-active compounds contributing to the highly appreciated aroma of cocoa as well as of cocoa-containing products such as chocolate have already been investigated in several studies. Sometimes, however, cocoa is tainted with undesirable off-flavors that already occur before roasting at the level of the fermented beans and may lead to consumer rejection if transferred to the final product. Among the known types of cocoa off-flavors, smoky off-notes are the most prevalent, but little has been known about the compounds that are causative for this off-flavor.

To clarify the molecular background of the smoky off-flavor, the volatiles from a sample of fermented cocoa with a pronounced smoky off-flavor and a reference sample with flawless aroma were isolated by solvent extraction and solvent-assisted flavor evaporation (SAFE). The parallel application of an aroma extract dilution analysis (AEDA) to both isolates revealed significantly higher flavor dilution (FD) factors for seven potential off-flavor compounds in the off-flavor sample. These compounds were 2-methoxyphenol (smoky, hammy), 3-methylphenol (smoky, phenolic), 4-methylphenol (horse stable, phenolic), 3-ethylphenol (smoky), 4-ethyl-phenol (smoky), 3-propylphenol (smoky, phenolic), and 4-propylphenol (phenolic). Screenings of further samples with smoky off-notes of varying intensity confirmed the selection of these seven potential off-flavor compounds. The quantitation of the compounds by GC-MS using stable isotopically substituted odorants as internal standards in several off-flavor samples and reference samples substantiated the screening results: consistently higher concentrations were obtained in the off-flavor samples than in the reference samples. To finally assess the contribution of the individual compounds to the smoky off-flavor in cocoa, odor activity values (OAVs) were calculated by dividing the concentrations by the odor threshold values of the potential off-flavor compounds in deodorized cocoa butter. In all off-flavor samples, 2-methoxy-phenol showed the highest OAV, followed by 4-methylphenol, 3-ethylphenol, 3-methylphenol, 4-ethylphenol, and 3-propylphenol. In the reference samples, only 2-methoxyphenol and 4-methylphenol showed OAVs >1. 4-Propylphenol did not exceed its odor threshold value in any of the samples analyzed and thus, was considered not relevant to the smoky off-flavor. For the other six compounds, maximum tolerable concentrations based on their odor threshold values were suggested.

After the importance of the six phenolic compounds 2-methoxyphenol, 4-methylphenol, 3-ethylphenol, 3-methylphenol, 4-ethylphenol, and 3-propylphenol to the smoky off-flavor had been shown, the second part of this work was focused on their behavior during further cocoa processing. Additionally included were the compounds (−)-geosmin (moldy, beetroot-like), 3-methyl-1*H*-indole (fecal, mothball-like), 1*H*-indole (fecal, mothball-like), and 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF; caramel-like, musty) which had been identified as potential off-flavor compounds in fermented cocoa samples with moldy-musty off-note. First, the impact of roasting was investigated. Two samples with smoky off-note, two samples with moldy-musty off-note, and a reference sample were roasted at three different temperatures and subse-quently processed into cocoa liquor. Secondly, the impact of further processing into chocolate mass and conching was analyzed on the basis of a spiked cocoa liquor. The concentrations of the off-flavor compounds in the cocoa liquor and the chocolate mass after conching were determined by GC-MS and compared to the concentrations before the processing. A

homogenous behavior was observed for the phenolic compounds causative for the smoky off-flavor. Concentrations increased slightly during roasting and processing into cocoa liquor but the changes were approximately balanced by losses during processing into chocolate mass and conching. Therefore, the previously suggested maximum tolerable concentrations in fermented cocoa beans did not need to be adjusted. The compounds potentially contributing to the moldy-musty off-flavor showed a less homogenous behavior. The concentrations of 3-methyl-1H-indole decreased during roasting and processing into cocoa liquor. Its limit could be raised at least when roasting was done at high temperatures. Concentrations of 1H-indole, however, increased significantly during roasting and processing into cocoa liquor. The resulting concentrations significantly exceeded the odor threshold value and the increase was not compensated by losses during processing into chocolate mass and conching. Therefore, the incoming goods inspection in the chocolate industry should include the analysis of a cocoa bean sample after test roasting to correctly evaluate the off-flavor potential of 3-methyl-1H-indole and 1H-indole.

2 Zusammenfassung

Die geruchsaktiven Verbindungen, die zum wertgebenden Aroma von Kakao und kakao-haltigen Produkten wie Schokolade beitragen, wurden bereits in verschiedenen Studien unter-sucht. Gelegentlich ist Kakao jedoch bereits vor der Röstung auf der Stufe der fermentierten Bohnen (im Deutschen meist als Rohkakao bezeichnet) mit unerwünschten Fehlaromanoten behaftet, die auf die Endprodukte übergehen können und dann zu einer Ablehnung durch den Verbraucher führen. Unter den bekannten Fehlaromen von Rohkakao ist die rauchige Note am weitesten verbreitet, die dafür verantwortlichen Verbindungen waren bisher jedoch wenig erforscht.

Um den molekularen Hintergrund des rauchigen Fehlaromas zu klären, wurden die flüchtigen Bestandteile einer Rohkakaoprobe mit ausgeprägter rauchiger Fehlnote und einer Referenzprobe mit einwandfreiem Aroma zunächst durch Lösungsmittelextraktion und Solvent-Assisted Flavor Evaporation (SAFE) isoliert. Die beiden Isolate wurden einer parallelen Aromaextraktverdünnungsanalyse (AEDA) unterzogen. Dabei wurden sieben Verbindungen als potenzielle rauchige Fehlaromaverbindungen in der Fehlaromaprobe identifiziert. Sie zeigten in der Fehlaromaprobe deutlich höhere Flavor-Dilution (FD) Faktoren als in der Referenzprobe. Diese Verbindungen waren 2-Methoxyphenol (rauchig, schinken-artig), 3-Methylphenol (rauchig, phenolisch), 4-Methylphenol (nach Pferdestall, phenolisch), 3-Ethylphenol (rauchig), 4-Ethylphenol (rauchig), 3-Propylphenol (rauchig, phenolisch) und 4-Propylphenol (phenolisch). Screenings weiterer Proben mit rauchigen Fehlaromanoten in unterschiedlichen Intensitäten bestätigten die Auswahl dieser sieben potenziellen Fehlaroma-verbindungen. Mit Hilfe von isotopensubstituierten Geruchsstoffen als interne Standards wurden die potenziellen Fehlaromaverbindungen mittels GC-MS quantifiziert. Die quanti-tativen Daten bestätigten die Screening-Ergebnisse. In den Fehlaromaproben wurden durchweg höhere Konzentrationen als in den Referenzproben nachgewiesen. Um schließlich den Beitrag der einzelnen Geruchsstoffe zum rauchigen Fehlaroma in Kakao zu bewerten, wurden Odor Activity Values (OAVs) bestimmt. Diese wurden jeweils aus der Konzentration der potenziellen Fehlaromaverbindung dividiert durch ihren Geruchsschwellenwert in desodorierter Kakaobutter berechnet. Insgesamt zeigte 2-Methoxyphenol die höchsten OAVs, gefolgt von 4-Methylphenol, 3-Ethylphenol, 3-Methylphenol, 4-Ethylphenol und 3-Propyl-phenol. In den Referenzproben wiesen nur 2-Methoxyphenol und 4-Methylphenol OAVs >1 auf. 4-Propylphenol überschritt seinen Geruchsschwellenwert in keiner der untersuchten Proben und wurde daher für das rauchige Fehlaroma als nicht relevant betrachtet. Für die anderen sechs Fehlaromaverbindungen wurden auf der Grundlage ihrer Geruchs-schwellenwerte maximal tolerierbare Konzentrationen in Rohkakao vorgeschlagen.

Nachdem die Bedeutung der sechs phenolischen Verbindungen 2-Methoxyphenol, 4-Methyl-phenol, 3-Ethylphenol, 3-Methylphenol, 4-Ethylphenol und 3-Propylphenol für das rauchige Fehlaroma geklärt war, sollte im zweiten Teil der Arbeit gezeigt werden, wie sich diese Fehlaromaverbindungen bei der weiteren Verarbeitung des Kakaos verhalten. Parallel dazu sollte auch geklärt werden, wie sich die Verbindungen (−)-Geosmin (schimmelig, nach rote Beete), 3-Methyl-1*H*-indol (fäkal, nach Mottenkugeln), 1*H*-Indol (fäkal, nach Mottenkugeln) und 4-Methoxy-2,5-dimethylfuran-3(2*H*)-on (MDMF; nach Karamell, muffig), die als potenzielle Fehlaromastoffe in Rohkakaoproben mit schimmelig-muffiger Fehlnote identifiziert worden

waren, bei der Verarbeitung verhalten. Zunächst wurde der Einfluss der Röstung untersucht. Zwei Proben mit rauchigem Fehlaroma, zwei Proben mit schimmelig-muffigem Fehlaroma und eine Referenzprobe wurden bei drei verschiedenen Temperaturen geröstet und zu Kakaomasse verarbeitet. In einem weiteren Versuch wurde der Einfluss der Weiterverarbeitung zu Schokoladenmasse und nachfolgendem Conchieren anhand einer aufdotierten Kakaomasse bestimmt. Die Konzentrationen der Fehlaromastoffe wurden jeweils nach der Verarbeitung zu Kakaomasse beziehungsweise nach dem Conchieren bestimmt und mit den Konzentrationen vor den jeweiligen Verarbeitungsschritten verglichen. Bei den für das rauchige Fehlaroma verantwortlichen phenolischen Verbindungen wurde ein homogenes Verhalten beobachtet. Die Konzentrationen stiegen durch das Rösten und die Verarbeitung zu Kakaomasse leicht an, die Zunahme wurde aber durch Verluste bei der Verarbeitung zu Schokoladenmasse und beim Conchieren in etwa wieder ausgeglichen. Die zunächst vorgeschlagenen maximal tolerierbaren Konzentrationen in Rohkakao mussten für die Verbindungen mit rauchigem Fehlaroma nicht angepasst werden. Die potenziell zu schimmelig-muffigen Fehlaromen beitragenden Verbindungen zeigten ein weniger homogenes Verhalten. Die Gehalte von 3-Methyl-1H-indol verringerten sich durch das Rösten und die Verarbeitung zu Kakaomasse. Sein Grenzwert könnte zumindest dann angehoben werden, wenn bei höheren Temperaturen geröstet wird. Die Konzentrationen von 1H-Indol hingegen stiegen während des Röstens und der Verarbeitung zu Kakaomasse erheblich an, was zu Konzentrationen deutlich über dem Geruchsschwellenwert führte. Der Anstieg wurde durch Verluste während der Verarbeitung zu Schokoladenmasse und beim Conchieren nicht ausgeglichen. Die Ergebnisse legten daher nahe, bei der Wareneingangskontrolle in der Schokoladenindustrie auch die Analyse einer Kakaobohnenprobe nach einer Teströstung einzubeziehen, um das Fehlaromapotenzial von 3-Methyl-1H-indol und 1H-Indol korrekt zu bewerten.

3 Abbreviations and Nomenclature

Abbreviations:

AEDA	Aroma extract dilution analysis
ASTM	American Society for Testing and Materials
AV	Acidic volatiles
3-AFC	3-Alternative forced choice
cAEDA	Comparative aroma extract dilution analysis
CI	Chemical ionization
CL	Cocoa liquor
DCM	Dichloromethane
EI	Electron ionization
FD	Flavor dilution
FFAP	Free fatty acid phase
FID	Flame ionization detector
GC	Gas chromatography
GC-O	Gas chromatography−olfactometry
GC-FID	Gas chromatography−flame ionization detector
GC × GC	Comprehensive two-dimensional gas chromatography
GC-MS	Gas chromatography−mass spectrometry
GC-HRMS	Gas chromatography−high resolution mass spectrometry
GC-GC-HRMS	Two-dimensional heart-cut gas chromatography−high resolution mass spectrometry
GC-GC-MS	Two-dimensional heart-cut gas chromatography−mass spectrometry
HS-SPME	Headspace solid phase microextraction
i.d.	Inner diameter
MCSS	Moving column stream switching
MOF	Moldy off-flavor
NBV	Neutral and basic volatiles
OAV	Odor activity value
OF	Off-flavor
OTV	Odor threshold value
OVF	Overfermented
PTV	Programmed temperature vaporizing

REF   Reference

RI   Retention index

SAFE   Solvent-assisted flavor evaporation

SIDA   Stable isotope dilution assay

SOF   Smoky off-flavor

TOF   Time-of-flight

Nomenclature:

γ-Decalactone	5-Hexyloxolan-2-one
Dimethyl trisulfide	Dimethyltrisulfane
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
Furaneol®	4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one
Geosmin	(4*S*,4a*S*,8a*R*)-4,8a-Dimethyloctahydronaphthalen-4a(2*H*)-ol
Geraniol	(2*E*)-3,7-Dimethylocta-2,6-dien-1-ol
MDMF	4-Methoxy-2,5-dimethylfuran-3(2*H*)-one
Methional	3-(Methylsulfanyl)propanal
γ-Nonalactone	5-Pentyloxolan-2-one
δ-Octalactone	6-Propyloxan-2-one
Sotolon	3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one
Vanillin	4-Hydroxy-3-methoxybenzaldehyde

4 Introduction

4.1 Molecular Sensory Science

4.1.1 The Olfactory System

Aroma is one of the foods' key properties as it strongly influences whether food is selected or rejected by the consumer. Surveys conducted in the recent years have consistently shown that the characteristic of "being tasty" is the main driver for the consumers' food selection. This overall sensory impression, which is created during food consumption, is determined by the sensory properties appearance, texture, consistency, taste and, most importantly, by the aroma.[1,2]

The aroma of food is typically caused by a mixture of odor-active compounds, which are volatile substances with a certain water and fat solubility, a sufficient vapor pressure, and a relatively low molecular weight of ~300 g/mol or less. Odor-active compounds can reach the olfactory epithelium either through the nose (orthonasally) or, during food consumption, through the throat (retronasally) as shown in Figure 1.[3,4]
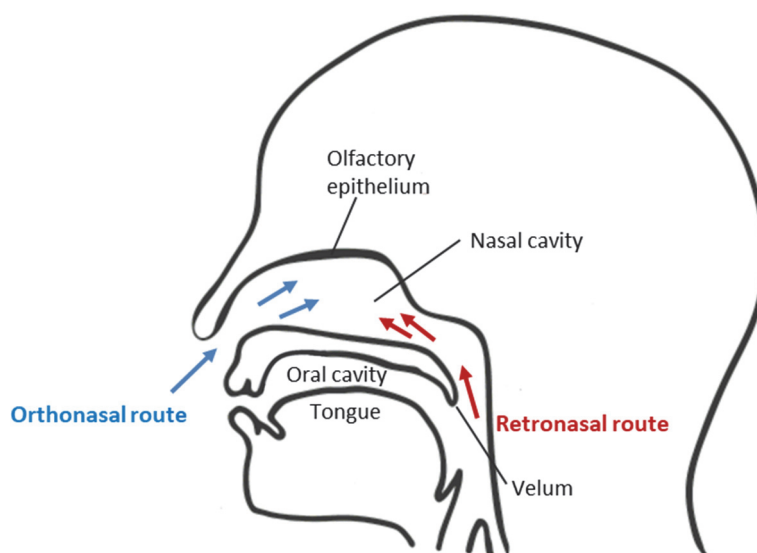
Figure 1: Orthonasal and retronasal odor perception

Once an odor-active compound has reached the olfactory epithelium, it can bind to one of the approximately 400 types of G-protein-coupled odorant receptors located in the membrane of the cilia of the olfactory neurons. A specific type of receptor can typically be activated by a number of odor-active compounds, just as a specific odor-active compound can typically bind to several types of receptors. In sum, this leads to activation patterns that are characteristic for each aroma impression.[4-10]

Odor-active compounds entering the olfactory system (Figure 2) are first dissolved in the olfactory mucosa, which contains specific odorant binding proteins. These proteins have a high affinity to the odorants and may transport the odorants to the odorant receptors. When an odorant binds to an odorant receptor (Figure 2, 1.), certain structural characteristics of the odorant activate the receptor by causing a conformational change of the receptor molecule.

This triggers a reaction cascade in the olfactory receptor cell, in which an influx of Na^+ and Ca^{2+} ions and an efflux of Cl^- ions is observed. The depolarization of the receptor cell membrane creates an action potential (2.) which is transmitted via the olfactory nerve beyond the ethmoid bone into the olfactory bulb (bulbus olfactorius). In the olfactory bulb, the axons of those odorant receptor cells that express the same type of receptor protein are bundled into glomeruli (3.). Each glomerulus activates one specific mitral cell, which relays a signal to regions of the limbic system and to the cerebral cortex by synaptic transmission (4.).[3-6,11]
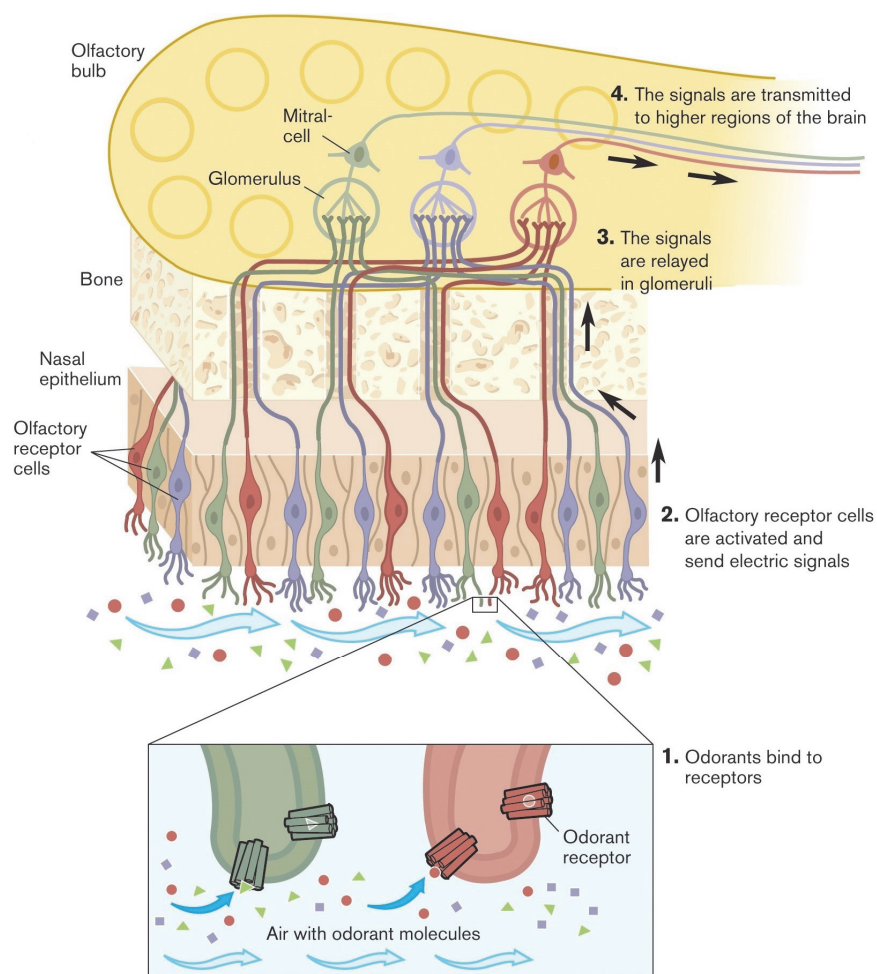
Figure 2: The olfactory system[12]

The physical and chemical characteristics of a compound, the ambient conditions, and the food matrix from which it is released all affect the amount of molecules that reach the human nose. However, to be odor-active, a compound must not only be sufficiently volatile to reach the human nose, but it must also be able to bind to and activate at least one of the approximately 400 types of odorant receptors.[3,4,6,9] Furthermore, it must be present at a concentration that exceeds its specific odor threshold value (OTV). OTVs can vary widely depending on the odorant and the food matrix from which the odorant is released. In water, for example, the vinegar-like smelling acetic acid shows an OTV of 5600 µg/kg,[13] whereas the bell pepper-like smelling 3-isobutyl-2-methoxypyrazine shows an OTV of 0.0062 µg/kg.[14]

Theoretically, all odorants present in concentrations above their respective OTV may contribute to the overall aroma of the food, but in fact only a small number of these odorants affect the overall sensory properties. These compounds are referred to as key odorants.[15] Key odorants that occur in various types of foods are named generalists, whereas individualists are unique to a certain type of food.[16,17] To identify the key odorants of a complex food, the sensomics concept is the method of choice.

4.1.2 Identification of Key Food Odorants

The procedure for the characterization and identification of key odorants according to Schieberle[18] and Grosch[19] was first summarized in 1995 and is nowadays also referred to as the Sensomics concept. It consists of the steps displayed in Figure 3.
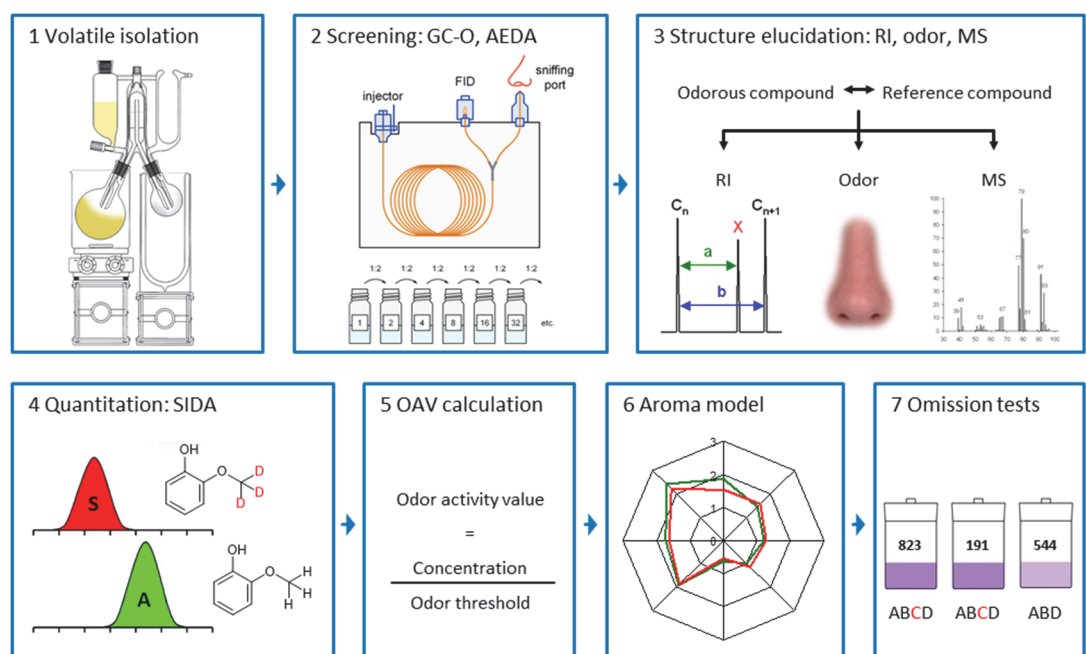
Figure 3: Identification of key odorants (illustration: Martin Steinhaus)

The identification of key odorants starts with the isolation of the volatiles. The isolation usually begins with an extraction by a non-polar organic solvent with a low boiling point such as diethyl ether or dichloromethane. This solvent extract still contains a large number of nonvolatiles, which need to be removed before further analysis. For this purpose, the solvent-assisted flavour evaporation (SAFE)[20] represents a gentle method that offers substantial advantages over other separation methods described in the literature. Low temperatures (≤40 °C), eva-poration of small sample extract portions under high vacuum, and subsequent recondensation of the volatile compounds using liquid nitrogen cooling minimize the risk of artifact formation and compound degradation.[15] In addition, compared to older approaches such as the "high vacuum transfer" (HVT), the recovery of high boiling odorants is increased and the duration is reduced.[20] The volatiles in the SAFE distillate can be fractionated by acid–base extraction into a fraction containing neutral and basic volatiles (NBV) and a fraction containing acidic

volatiles (AV). This may avoid interferences during the subsequent analysis.[18] The solvent phase obtained after SAFE or the phases after the optional fractionation, respectively, are concentrated under mild conditions using a Vigreux column and a Bemelmans microdistillation device.[21]

A defined volume of such a concentrate is subjected to gas chromatography-olfactometry (GC-O)[15] which is the essential step for the differentiation between odor-active volatiles and the multitude of odorless volatiles. The volatiles are separated on a GC capillary column. The end of the column is connected to a Y-shaped glass splitter which divides the column effluent into two equal parts. By using two deactivated fused silica capillaries of the same length, one part of the effluent is transferred to a flame ionization detector (FID) and the other part is transferred to a heated exit serving as "sniffing port" for the GC-O assessor (Figure 4). During a GC-O analysis, the assessor places the nose directly above the sniffing port while simultaneously a recorder or a computer system plots the FID signal. Whenever an odor is perceived, the assessor notes the odor quality as well as the associated retention time. A GC-O chromatogram is a combination of the data obtained by the GC-O assessor and the data obtained from the FID.
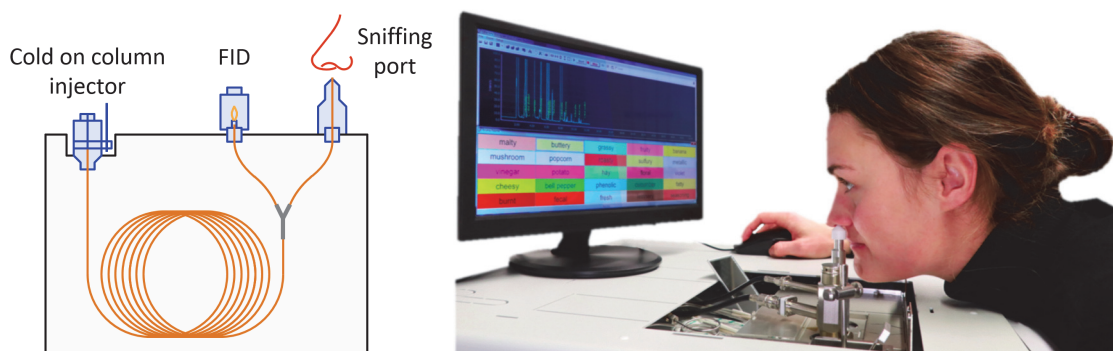
Figure 4: Schematic principle (left) and application (right) of GC-O (illustrations: Martin Steinhaus, Daniela Füllemann)

An aroma extract dilution analysis (AEDA)[22] is conducted to rank the odor-active compounds in the volatile isolate according to their odor potency. The volatile isolate is stepwise diluted, typically 1:2, with solvent to obtain dilutions of 1:2, 1:4, 1:8, 1:16, etc. Each diluted sample is subjected to GC-O analysis. The stepwise dilution and the GC-O analysis of each diluted sample is continued until no odor-active compound can be detected during GC-O analysis. Each odor-active compound is then assigned a flavor dilution (FD) factor representing the dilution factor of the highest diluted sample in which the compound was still perceivable during GC-O analysis.

A comparative AEDA (cAEDA)[18] is a valuable tool to compare two or more samples with respect to their odor-active compounds. For example, a cAEDA can be applied to the volatile extracts of a sample with an off-flavor and a reference sample without an off-flavor.[15] If an odorant has a significantly higher FD factor in the off-flavor sample compared to the reference sample and an odor quality that resembles the off-flavor, this odorant may contribute to the off-flavor (Figure 5).

Odorant	FD factor reference sample	FD factor off-flavor sample
Odorant 1	<1	2048
Odorant 2	1024	512
Odorant 3	512	512
Odorant 4	<1	256
Odorant 5	256	128
⋮		

🟩 Characteristic food odorant
🟥 Potential off-flavor compound
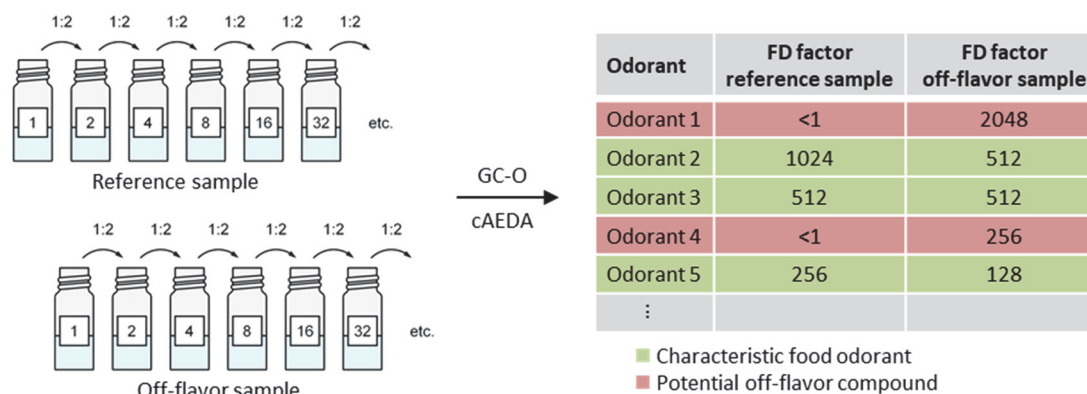
Figure 5: Basic principle of a cAEDA and theoretical result (illustration: Martin Steinhaus, Daniela Füllemann)

The structure assignment[15] of odor-active compounds perceived during GC-O is performed by comparing specific parameters with the data of authentic reference substances analyzed in appropriate dilutions on the same instruments. These parameters include the odor quality perceived during GC-O analysis, the retention indices (RIs) on two GC columns of different polarity, and the mass spectra obtained by gas chromatography-mass spectrometry (GC-MS), both in chemical ionization (CI) and in electron ionization (EI) mode.

Even though AEDA and cAEDA are valuable screening methods for odor-active compounds, they cannot finally clarify the contribution of the identified odorants to the overall aroma of the food sample. One reason for this is that during GC-O analysis, all odorants are fully evaporated regardless of their volatility. In addition, matrix effects are not considered and there is no compensation for losses during the workup. Therefore, the GC-O results need to be further substantiated. This is typically achieved by the quantitation of the odorants, for which the stable isotope dilution analysis (SIDA)[23] is considered the gold standard.[15,18] If AEDA or cAEDA have been conducted previously, SIDA may first be applied to the odorants with the highest FD factors (AEDA) or to the odorants that account for the greatest difference between the compared samples (cAEDA).

In SIDA, deuterium- or ^{13}C-substituted analogues[24] of the target compounds are used as internal standards and are added to the sample at the beginning of the workup (Figure 6). Due to the almost identical chemical and physical properties of a target analyte and its isotopically substituted analogue, losses during a mild workup are considered compensated. Any loss of the target analyte during workup coincides with a corresponding loss of its isotopically substituted analogue, so that the concentration ratio of both compounds remains constant. However, it is absolutely crucial to homogenize the mixture of sample and internal standard until equilibrium is reached before the start of the workup procedure. The time required for equilibration is depending on the food matrix.[25] The concentration ratio is then determined by GC-MS analysis, preferably in CI mode to obtain an intense signal for the molecular ion.[15] The concentration of the target analyte in the food sample is finally calculated using the area ratio of the target analyte to the internal standard, the amount of added internal standard, and the amount of sample used for the workup by employing a calibration line equation determined from the analysis of target analyte/internal standard mixtures in different concentration ratios.
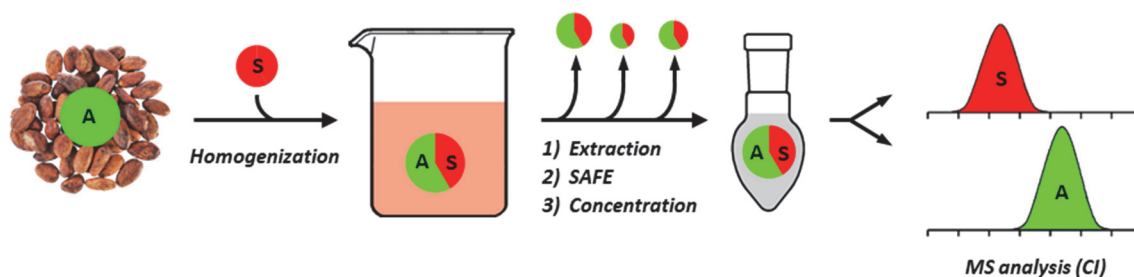
Figure 6: Application of SIDA (illustration: Martin Steinhaus)

Once the exact concentrations of the target analytes have been determined, the next step in identifying the key odorants is to calculate odor activity values (OAVs) in order to assess the odor potency of the individual compounds.[26,27] The OAV is defined as the ratio of the exact concentration of an odorant to its OTV. The OTV can be determined according to the American Society for Testing and Materials (ASTM) standard practice for determination of odor and taste thresholds by a forced-choice ascending concentration series method of limits.[28] To obtain meaningful OAVs, the matrix in which the OTV is determined should be similar to the original food matrix.

It can be assumed that odorants with an OAV <1 do not contribute to the overall aroma of the food. Odorants with an OAV ≥1 may contribute to the overall aroma of the food; however, this does not necessarily have to be the case. Nevertheless, OAVs give a better estimation of the major odorants in the food sample than FD factors, as OAVs are based on exact concentrations, consider matrix interactions, and include each compounds' individual volatility.

To verify that all important odorants have been considered in the previous steps, an aroma reconstitution experiment is performed. For this purpose, odorants with an OAV ≥1 are added to a model matrix in the concentrations determined in the food sample. The model matrix should mimic the food sample by having at least the same content of water, lipids, sugars, and the same pH-value. In a quantitative olfactory profile analysis, a trained sensory panel evaluates the aroma reconstitution model in comparison to the original food sample. The aroma reconstitution is considered successful if the aroma profiles of the reconstitution model and the food sample are in a good agreement.[18] In case the subject of a study was the determination of odorants causative for an off-flavor, a reconstitution experiment might be done by adding the potential off-flavor compounds in their determined concentrations to an appropriate reference material without the off-flavor.[15]

After a successful aroma reconstitution, omission tests are the final step of the sensomics concept for the identification of the key odorants in a food.[19] An aroma reconstitution model is prepared with omission of a single odorant. This incomplete aroma reconstitution model is then compared to the complete aroma reconstitution model in a 3-alternative forced choice (3-AFC) test. If this test reveals a significant difference between the two aroma reconstitution models, the missing odorant plays a substantial role in the overall aroma of the sample and can thus be considered a key odorant.

4.2 Cocoa and Chocolate

4.2.1 The Cocoa Tree

The cacao tree or cocoa tree, *Theobroma cacao* L., belongs to the Mallow family (Malvaceae).[29] There are 22 species assigned to the genus *Theobroma*, all of them native to the tropical rainforests of Central and South America. The cocoa tree is the only *Theobroma* species that is widely cultivated. The cocoa tree is an evergreen tree of up to 15–20 m height. It grows in the dense shade of other trees in its natural habitat, the lower story of the tropical rainforest. In cultivation, the cocoa tree is usually planted mixed with other food crops such as plantains, which provide shade that is especially important for young cocoa trees. The growth height is limited to 4–8 m to facilitate harvesting. Cocoa requires high annual rainfall, preferably 1500–2500 mm, which should be evenly distributed throughout the year. Additionally, it prefers high humidity, typically 70–80% during the day and up to 100% at night. Temperature requirements range from 18–32 °C. Cocoa grows in different soils, but prefers a good drainage and a neutral to slightly acidic pH (5.0–7.5).[30-32]

Under good growing conditions, the cocoa tree may start to flower at an age of 2–3 years.[31] The flowers are formed directly on the trunk or on older branches (cauliflory) and are relatively small, about 15 mm in diameter (Figure 7).[30] After developing for approximately 30 days, the flowers start opening during the afternoon and are fully open the next morning. Then, anthers release their pollens. The flowers have to be pollinated and fertilized on this day; otherwise, they fall off the following day. A healthy cocoa tree produces between 20000 and 100000 flowers each year, however, only 1–5% of these are pollinated by various small insects.[32]

Figure 7: Left: cauliflory; right: cocoa flowers (pictures: Daniela Füllemann)

After fertilization, the ovary develops over various growth stages into the fruit, the cocoa pod. Depending on the type of cocoa, a ripe cocoa pod can vary strongly in its appearance. It may have different shades of red, orange or yellow, have different surface textures, and be either round or more elongated-oval in shape. It is 10–35 cm long and weighs 200–1000 g.[30-32] Inside the cocoa pod, there are usually 30–50 cocoa seeds, commonly referred to as beans, attached

to a central placenta and embedded into an edible endocarp, also called pulp (Figure 8).[30,32,33] During fermentation, the odorants present in the pulp might serve as a reservoir for valuable cocoa odorants.[34] A cocoa bean consists of the embryo and the seed shell (also known as seed coat or testa). The embryo itself consists of two large storage cotyledons, which account for 86–90% of the dry weight of the bean, as well as a small hypocotyl and a small radicle.[33,35] The cotyledons consist mainly of two types of cells, namely storage cells containing fat and proteins, and pigment cells containing polyphenols and alkaloids.[31]

Figure 8: Opened, fresh cocoa pod with seeds embedded into the pulp (picture: Franziska Krause)

In summary, the dry weight of fresh cocoa seeds consists of ~50% fat, ~12% protein, and ~7% carbohydrates. In addition, cocoa seeds are rich in phenolic substances and alkaloids, which contribute about 15% and 4%, respectively, to the dry weight.[33]

4.2.2 Cocoa Main Types

In 1944, three main types, groups, or varieties were proposed for the cocoa tree, namely Criollo, Forastero and Trinitario.[36]

Criollo cocoa beans are white, ivory or very pale purple in color. They have a highly regarded mild, nutty cocoa flavor and are thus considered as "fine flavor" cocoa. However, the trees produce only low yields and are susceptible to diseases and parasites. Once probably domesticated by the Maya, the Criollo is nowadays rarely found and only survived on some old plantations in Central and South America, Madagascar, Samoa, and Sri Lanka.[30,31]

The Forastero type originated in the Amazon basin and is nowadays widely distributed in Brazil and West Africa.[32] Due to their melon-shaped pods, the main subtype of Forastero is called Amelonado. It is extensively cultivated in West Africa. Generally, Forastero is a more vigorous type that produces higher yields of dark purple colored beans. They develop a strong basic cocoa flavor and are therefore classified as "bulk" or basic flavor cocoa.[32,37]

The Trinitario is considered as a hybrid of Criollo and Forastero cocoa and probably originated in the 18th century in Trinidad.[36] Due to hybridization, some Trinitario varieties produce beans with fine flavor characteristics reminiscent, for example, of dried fruits or molasses, while being more resistant to diseases.[31,32]

Besides these three main types, another type called Nacional is also often mentioned, which is only grown in Ecuador. Due to its high susceptibility to diseases, especially to witches broom, the pure Nacional almost disappeared. However, there is a further type only cultivated in Ecuador that is attributed to the Nacional type. It produces a fine flavor cocoa with floral and spicy notes known as "Arriba" flavor.[30,38]

In a recent study by Motamayor et al.[39], the classification of cocoa types was based on a genetic study of authentic samples. Results revealed a differentiation of *Theobroma cocoa* L. into ten major groups instead of the traditional classification into three main types. The ten major groups included Criollo, Amelonado, and Nacional, but not Trinitario and Forastero.

4.2.3 Cocoa Cultivation

Cocoa is cultivated commercially in regions between 20° north and 20° south of the equator, where conditions such as rainfall and soil type meet the cocoa-specific requirements.[31] The three main cultivation areas and their percentage of global annual production in the crop year 2018/19 were as follows: West Africa ~76%, South America ~18%, Asia and Oceania ~6%. The seven largest cocoa producing countries in 2018/19 were Côte d'Ivoire, Ghana, Ecuador, Cameroon, Nigeria, Indonesia, and Brazil, which together accounted for ~88% of the global annual production (Figure 9). Côte d'Ivoire and Ghana alone supplied 45% and 17% of the global annual production, respectively. In total, the global production of fermented and dried cocoa beans in the crop year 2018/19 amounted to more than 4.7 million tons.[31,40,41]

■ The two largest cocoa producing countries   ■ Belonging to the seven largest cocoa producing countries
■ Other cocoa producing countries
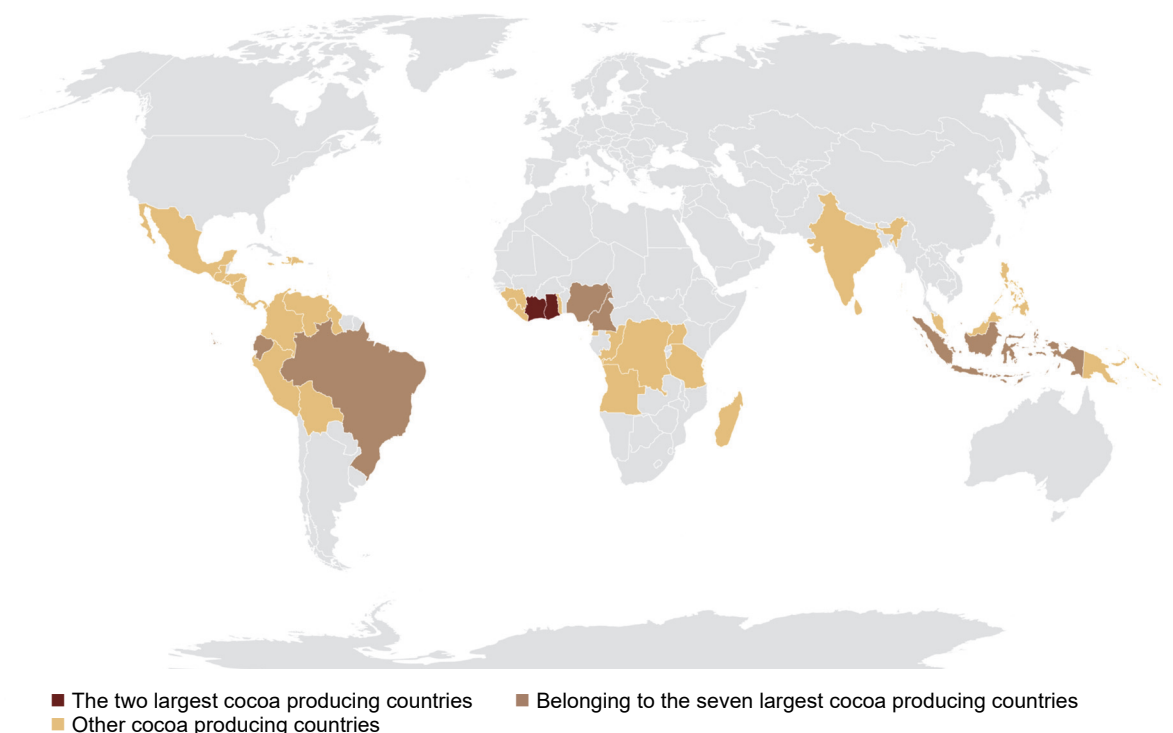
Figure 9: Cocoa producing countries in the crop year 2018/19[42,43]

Yields can vary considerably at different plantations, influenced by various factors such as the type of cocoa planted, environmental factors (climate, soil), cultivation system (tree density, shade level), maintenance practice (fertilization, irrigation, pruning), age of trees, and disease-related losses. There are plantations that yield more than 2500 kg/ha of fermented and dried cocoa beans.[31] However, most of the world's cocoa (up to 90%) is grown by smallholders in mixed cropping systems. In West Africa, where about three-quarters of the world's annual crop is grown, farm sizes of less than 2 ha are common and yields typically range from 250–600 kg/ha.[31,44]

Cocoa is susceptible to a number of diseases and pests that result in a loss of approximately 30% of the primary annual crop.[31] A disease caused by a viral infection is the swollen shoot disease, which is spread by small insects and results in a significant yield reduction in West African cocoa plantations.[31,32] The worldwide distributed black pod disease is caused by the *Phytophtora* spp. fungi and leads to rotting of cocoa pods and beans.[32] Other fungal diseases include witches' broom, caused by *Moniliophthora perniciosa* and frosty pod rot, caused by *Moniliophthora roreri*, which are both common in Central and South America.[31] In addition, cocoa trees can be infested by insects such as capsids, mirids, and the cocoa pod borer.[31,32] Diseases and pests can be controlled by using appropriate planting material, removing infested material and, if sufficiently economical, by applying fungicides or insecticides.[31]

Most of the cocoa cultivated worldwide is classified as bulk cocoa, which has a strong but simple cocoa flavor and is mainly used to make milk and dark chocolate, cocoa butter, cocoa liquor, and cocoa powder.[31] Generally, bulk cocoa is predominantly produced from high-yielding and disease-resistant Forastero trees grown in West Africa or Brazil.[31] Fine flavor cocoa, on the other hand, is produced mainly in Latin America from Criollo, Trinitario, and Nacional trees, and accounts currently for an estimated 12% of the total global cocoa bean exports.[45] There is no generally accepted criterion for fine flavor cocoa beans, except that they should come from trees of these three types and show fruity, floral, herbal, woody, and nutty odor qualities in addition to a typical cocoa aroma.[45] In the literature, the fruity and floral characteristics of fine flavor cocoas such as Criollo and Nacional were partly attributed to high concentrations of citrusy, floral smelling linalool.[46-48] The formation of odor-active compounds, however, is not only dependent on factors like genotype and growing conditions of the cocoa trees, but also on the type of cocoa processing. Processing steps such as fermentation, drying, and roasting significantly influence the aroma of cocoa beans.

4.2.4 Cocoa Harvest, Fermentation and Drying

Cocoa pods are harvested almost throughout the year, with a main harvesting season and additional secondary or intermediate harvesting seasons.[31] Ripe cocoa pods are indicated by a change in color, typically from dark red or green to yellow or orange.[31] To remove the ripe pods from the tree, knives or special wooden tools with long handles are used.[32] The pods may be stored in heaps for a few days before being opened by using knives, machetes, or by cracking the pods with a wooden tool. When using knives, care must be taken not to damage the cocoa beans inside. The beans are collected by hand while the placenta is removed.[31,32] The beans including the adhering pulp undergo fermentation, which is an essential step for the direct formation of some odor-active compounds and, above all, for the formation of important

odorant precursors. It has been shown that these precursors are essential for the formation of major chocolate odorants during the subsequent roasting process.[49-52]

Fermentation of cocoa beans is a spontaneous, complex process with a wide variety of methods used around the world. There are, however, two main fermentation methods, namely heap fermentation, typical of West African countries, and box fermentation, common in most Central and South American countries and in Asia.[31,32,53,54] In heap fermentation, cocoa beans are collected in heaps covered with banana leaves. Heap sizes can range from 20 to 5000 kg.[31,54] In box fermentation, mostly wooden boxes are used that can hold between 25 and 2000 kg of cocoa beans (Figure 10).[31,54] In box fermentation, the fermenting mass is regularly turned, typically every 24–48 h. The cocoa beans fermented in heaps are not mixed at all or only once after 48 h.[54] Fermentation time depends primarily on the type of cocoa. Fine flavor types such as Criollo are usually fermented for a short time (1–3 days) and bulk cocoa types such as Forastero are fermented for a longer time (5–10 days).[32,38]

Figure 10: Box fermentation in Nicaragua (photo: Franziska Krause)

Fermentation includes anaerobic and aerobic processes. Yeasts convert sugars from the pulp to ethanol.[55] Due to the rising pH caused by the degradation of citric acid in the pulp and due to the increase in aeration, yeast activity decreases and lactic acid bacteria gain impor-tance.[37,55] They convert sugars and some organic acids into lactic acid.[55] Subsequently, acetic acid bacteria predominate and oxidize ethanol in an exothermic reaction to acetic acid. This is mainly responsible for a final temperature of the fermenting mass of up to 45–50 °C.[31,54,55] The progress of the process is also noticeable in the odor of the material. It changes from an alcoholic odor to a strongly acetic odor.[54] The diffusion of acids into the cotyledons,[55] the rising temperature, and the low pH lead to the inhibition of germination. Cell structures break down and increase cell permeability.[54] Consequently, this allows fat, proteins, polyphenols, and alkaloids to react in predominantly enzymatic reactions.[31] The total amount of polyphenols decreases during fermentation,[56] resulting in reduced bitterness and astringency and a change in the color of the fermented beans.[31,38,57] The amount of methylxanthines such as theobromine

and caffeine is also reduced.[31] Enzymatic hydrolysis of storage proteins leads to oligopeptides and amino acids. Both are, together with reducing sugars, important reactants in the formation of flavor precursors.[31]

The duration of fermentation and the final pH are crucial for the formation of flavor precursors. The final pH is influenced by the diffusion rate of organic acids into the cotyledons. An optimal final pH value (5.5–5.0)[57] after fermentation leads to strong cocoa-specific flavors after roasting. In contrast, lower pH values (4.5–4.0)[57] lead to weak cocoa-specific flavors. Higher pH values (pH >5.8)[57] as well as overfermentation favor the formation of off-flavor notes.[31,32,55]

After fermentation is complete, the next step in cocoa processing is drying. The drying process depends mainly on the weather. Thus, in regions with dry and sunny weather, natural or sun drying is applied more frequently. In contrast, in regions with higher humidity or fewer hours of sunlight, artificial drying is important.[31,54] In natural drying, cocoa beans are spread out in thin layers in the sun on mats, sheets, tables, trays, or terraces during the day (Figure 11).[31,54] To achieve uniform drying results, they are turned regularly, protected from rain and covered during the night. If there is insufficient sunlight or if the cocoa harvest season overlaps with the rainy season, artificial drying methods using conduction driers, convection driers, or mechanical driers are required.[54] Complete drying is important to proceed with reactions started during fermentation, such as the oxidation of polyphenols. These oxidation reactions are important for the formation of flavor precursors, the development of brown color, as well as the reduction of bitterness, astringency, and acidity.[32,37,38,54] In addition, it is essential to reduce the water content of the fermented beans to a final value between 4–8% to avoid overfermentation, inhibit mold growth, and improve the shelf life of beans during shipping and storage.[31,32,37,38,54]

Figure 11: Drying of cocoa beans on tables in Nicaragua (photo: Franziska Krause)

From the growing countries, fermented and dried cocoa beans are shipped worldwide, as further cocoa processing often takes place outside the growing countries. Europe, for example, accounts currently for more than one-third of the global processing.[58] Cocoa beans are transported in jute bags, in bulk in shipping containers, or as mega-bulk in the hold of a ship.[31,59]

During shipping and storage, care must be taken to ensure that the beans do not come into contact with moisture or odorous materials. Cocoa is highly susceptible to absorb extraneous odors.[31]

First quality assessments of fermented cocoa include weighing of the beans and sensory testing. Additionally, the cocoa is checked for visible mold on the outside of the beans and for insect damage. The so-called cut-test is performed to determine the level of fermentation. Underfermented beans are identified by their greyish, "slaty" color. Additionally, mold inside the beans can be detected.[31,60]

4.2.5 Composition of Cocoa Beans

The major ingredients of the fermented and dried cocoa nibs, the seed shell, and the embryo are listed in Table 1.

Table 1: Composition of the cocoa nibs, the seed shell, and the embryo (adapted from Belitz et al.[61])

Ingredient	Cocoa nibs (%)	Seed shell (%)	Embryo[a] (%)
Water	5.0	4.5	8.5
Fat	54.0	1.5	3.5
Crude protein	11.5	10.9	25.1
Starch	6.0		
Polyphenols	6.0		
Cellulose	9.0	26.5	4.3
Carboxylic acids	1.5		
Ash	2.6	8.0	6.3
Theobromine	1.2	1.4	
Caffeine	0.2		

[a]Embryo without the cotyledons.

The main fatty acids in cocoa fat (cocoa butter) are palmitic acid (C16:0; ~25%), stearic acid (C18:0; ~37%), and oleic acid (C18:1; ~34%).[61] Among the polyphenols, catechins (~37%), anthocyanins (~4%), and proanthocyanidins (~58%) are the most abundant.[61-63] Citric acid, acetic acid, malic acid, lactic acid, and succinic acid contribute significantly to the total amount of organic acids.[31,61]

4.2.6 Cocoa Bean Processing and Chocolate Production

The further processing of fermented and dried cocoa beans is illustrated in Figure 12. Fermented and dried cocoa beans are first cleaned to remove non-cocoa components such as dust, stones, wood, glass, and pieces of metal that can damage subsequent processing machinery. Cleaning is usually achieved by sieves, magnets, and airflow techniques.[64]

Subsequent steps include the removal of the cocoa shell, breaking, winnowing, and roasting. The order of the steps depends on the applied type of roasting. Removal of the shell is

important because shells do not contribute positively to the cocoa flavor. Due to their rather hard and fibrous texture, shells may hamper further processing.[64] In addition, undesirable contaminants such as toxins and heavy metals may be accumulated in the cocoa shell.[65,66] Heat impact, for example during roasting, facilitates the shell removal.[64] Breaking of the beans is followed by winnowing, which involves sorting the nibs by particle size using sieves and air classifiers.[64,67] In addition, winnowing ensures that the nibs are virtually free from shells and foreign material.[67]
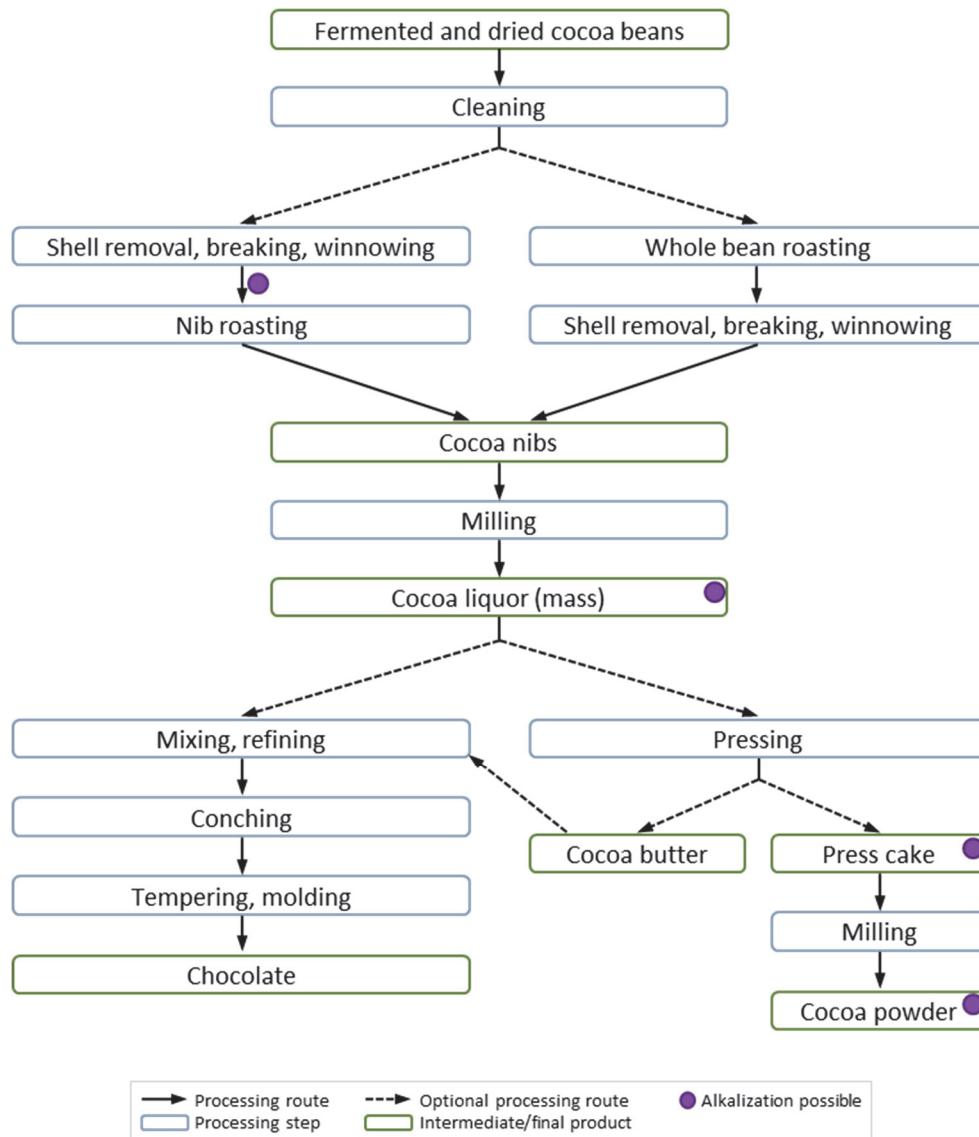
Figure 12: Simplified illustration of cocoa bean processing

Roasting is an extremely important step in cocoa processing, as it leads to the formation of valuable odor-active compounds from their precursors. Additionally, it reduces unwanted volatile acids, water, and microorganisms.[67] In the cocoa industry, roasting temperatures in the range of 110–140 °C are most commonly applied.[47,64,68]

There are three different types of roasting, namely whole bean roasting, nib roasting, and cocoa liquor (mass) roasting.[67] Whole bean roasting is the traditional approach in the

production of cocoa liquor, because the shell may preserve valuable odorants. Thus, whole bean roasting is essential in the production of cocoa liquors with special flavor notes.[64] In case of whole bean roasting, shell removal, breaking, and winnowing follow roasting.[64,67] Nib roasting is mainly used in the production of cocoa powder. In nib and liquor roasting, additional heating is often required to remove the shell before roasting. This may result in a poor separation into nibs and shells.[67]

Alkalization is an optional step in cocoa processing. It is predominantly applied to nibs, but also to cocoa liquor, cocoa press cake, and cocoa powder.[64,67] The cocoa products are mixed with an aqueous alkaline solution and the mixture is heated for a certain time, sometimes under pressure, until the color changes. After that, the product is dried again.[64] Alkalization raises the pH from 5.2–5.6 to 6.0–7.6 and above.[67,69] In addition, alkalization modifies the flavor and increases solubility.[69] For the production of cocoa powder, alkalization of cocoa nibs prior to roasting is widely applied.[64]

Grinding of cocoa nibs is a multistage process that results in an important intermediate product, the cocoa liquor, also referred to as cocoa mass.[64] During grinding, the cocoa butter is released from the cells and simultaneously, the mass is heated by friction. This leads to the formation of the viscous cocoa liquor.[67] Grinding is often followed by heat treatment to reduce the microbial load.[64,67]

Pressing cocoa liquor using hydraulic presses results in the separation into cocoa press cake and cocoa butter. The fat content of cocoa liquor, which is about 47–56%[64], is reduced to 10–24%[67] in the press cake. Cocoa butter has a light yellow color and a slight cocoa aroma. For the production of some cocoa products, deodorized cocoa butter is used, from which the cocoa aroma has been partially or completely removed.[64] Cocoa butter can crystallize in six different polymorphic forms (I–VI), which have melting points ranging from 16 °C to 36 °C.[67,70] However, only form V is desirable in chocolate products due to its stability, gloss, and specific melting characteristics.[70]

Milling of the cocoa press cake results in cocoa powder. During this process, tempering is important to allow the fat in the powder to crystallize in a stable form and to prevent both color changes and the formation of lumps. Cocoa powder with different fat contents can be blended to obtain a specific final fat content.[64,67]

To make chocolate, cocoa liquor, cocoa butter, sugar, and, if applicable, milk powder are mixed and then refined.[67] Refining aims at particle size reduction and homogenous particle size distribution, both of which affect the rheological and sensory properties.[67,71] It is achieved by a combination of thermostatically regulated two- and five roll refiners.[67] Conching is an essential process in chocolate manufacturing. It significantly improves texture and flavor of the chocolate.[67] Additionally, moisture is reduced. The conching process can be described as slow agitation of the chocolate mass at temperatures of ~50–80 °C over a period of several hours up to three days.[47,67,72] To further reduce the viscosity, emulsifiers such as lecithin may be added during the conching.[67] Proper tempering prior to molding is essential for the crystallization of the cocoa butter in polymorphic form V. Thus, it vitally influences the quality of the final chocolate product.[67]

4.3 Odor-Active Compounds in Cocoa

4.3.1 Molecular Background of the Characteristic Cocoa Aroma

Various compounds derived from cocoa contribute significantly to the highly appreciated aroma of cocoa products such as chocolate. The processing of the cocoa beans, especially the fermentation, drying, and roasting, are of great importance for the formation of valuable odor-active compounds. Furthermore, with correct processing, undesirable odor-active compounds can be reduced.[47,49]

The characteristic aroma of fermented and dried cocoa beans is predominantly described as acidic, but in addition, earthy, malty, caramel-like, honey-like, fruity, floral and sweaty odor notes are also clearly perceptible.[73,74] Compounds associated with the typical odor of fermented cocoa beans are according to the literature: acetic acid (vinegar-like), 2,3-diethyl-5-methylpyrazine (earthy), 2-/3-methylbutanal (sweet-malty), 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (caramel-like), phenylacetaldehyde (honey-like), ethyl 2-methylbutanoate and ethyl 2-methylpropanoate (fruity), 2-phenylethanol (floral), 2-methylpropanoic acid (cheesy, rancid), and 2- and 3-methylbutanoic acids (sweaty, rancid).[32,38,47,73-76]

Fermentation is essential for the formation of precursors from which important odorants are formed during roasting.[51,77] In fact, it has been shown that unfermented cocoa beans do not develop a cocoa-typical aroma during roasting.[49] The formation of odorants from precursors during roasting is associated with the Maillard reaction.[78] In an early phase of the Maillard reaction, reducing sugars react with amines, amino acids, and peptides, especially at elevated temperatures.[61,78] Thus, it is important that sucrose is converted to reducing sugars and proteins are hydrolyzed to free amino acids and peptides during cocoa fermentation.[31,79] A reducing aldose reacts with a free amino acid or a peptide to a Schiff base. After rearrangements, an important intermediate of the Maillard reaction, a 1-amino-1-deoxy-ketose or Amadori compound is formed.[61,78] In case of a ketose, the resulting product is an 2-amino-2-deoxy-aldose or Heyns compound.[61,78] Amadori compounds such as Fru-Phe and Fru-Leu have been detected in fermented and dried cocoa beans. It was shown that heating of these compounds yields chocolate-like odor notes.[80,81] Amadori compounds also undergo further reactions leading to the formation of α-dicarbonyls (deoxyosones). In the Strecker degradation, such an α-dicarbonyl compound reacts with an amino acid to form an imine. This intermediate is then decarboxylated and CO_2, an α-aminoketone, and an aldehyde, the Strecker aldehyde, are released.[61,78] It is important to mention that beyond these examples, the Maillard reaction comprises a multitude of possible reactions that depend on the availability of amino acids, sugars, and other precursors, as well as the temperature and pH value.[61]

Strecker aldehydes include important odorants such as phenylacetaldehyde (from L-phenylalanine), 3-methylbutanal (from L-leucine), and 3-(methylsulfanyl)propanal (methional, from L-methionine).[38,50,82] The formation of Strecker aldehydes has been confirmed in model reactions even under mild conditions.[83] Besides the Strecker aldehydes, the corresponding acids such as phenylacetic acid and 3-methylbutanoic acid are formed.[82] According to the literature, the formation of the acids significantly depends on the availability of oxygen, the reaction of the α-dicarbonyl with the amino acid, and the pH.[82] However, it was shown that the acids are not formed extensively by the direct oxidation of the aldehyde.[82]

Further reactions may include the partly esterification of alcohols with acetic acid, leading to acetates.[47] Acids such as 2- and 3-methylbutanoic acid may be esterified with ethanol, resulting in ethyl esters.[47]

The aroma of roasted cocoa beans differs clearly from that of fermented beans. It is described as less acidic, while malty, caramel-like, earthy, honey-like, and roasty attributes predominate.[73,74] In roasted cocoa, an important odorant class are alkylated pyrazines.[38,47,84] They have been reported to account for nearly one fifth of the total volatiles.[84] The type of substitution affects both the odor threshold values, which can vary by several orders of magnitude, and the odor qualities. The odor qualities of the alkylated pyrazines have been described as earthy, pea-like, sweet, roasty, nutty, and bell pepper-like, for example.[85] It has been postulated that during cocoa roasting, a dimerization of α-aminoketones leads to dihydropyrazines and subsequent oxidation results in the final pyrazines.[86]

Aldehydes and pyrazines have been mentioned in the literature as the major substance classes for the aroma of roasted cocoa.[47] Further important substance classes include esters, acids, hydrocarbons, terpenoids, amines, alcohols, ketones, furans, sulfur compounds, oxazoles, phenols, pyrroles, pyridines, and lactones.[37,47,84,87,88]

Terpenoids such as linalool might be relevant for distinguishing fine flavor cocoa from bulk cocoa, since clearly higher concentrations of linalool were found in fine flavor cocoa varieties than in bulk cocoa varieties. Therefore, the linalool content or the linalool/benzaldehyde ratio have been suggested as an indicator for fine flavor cocoa varieties.[46]

During further processing into chocolate, in particular the conching step affects the flavor, as the contents of volatile acids and short-chain fatty acids are significantly reduced.[47] However, conching also leads to some reduction in desired odor-actives compounds, while the concentrations of some compounds such as the caramel-like smelling 4-hydroxy-2,5-dimethylfuran-3(2H)-one (Furaneol®) may be increased.[88,89] In the final chocolate product, compounds from further ingredients such as milk powder may additionally contribute to the overall chocolate flavor.[88]

It has been shown that the addition of water to dry processed foods such as cocoa or chocolate leads to the formation of Strecker aldehydes. In detail, after the addition of water, the concentrations of 2-methylbutanal, 3-methylbutanal, and phenylacetaldehyde in roasted cocoa beans increased 10–47-fold.[52] Recently published studies addressed the targeted use of water addition in chocolate production by the use of a novel processing technology. Results revealed that water addition leads to higher amounts of Strecker aldehydes and an increase in other characteristic key odorants, while volatile acid contents were reduced.[90,91] It is assumed that upon contact with saliva during chewing, the same formation reactions lead to the release of further Strecker aldehydes. This would result in a more intense perception of these compounds when chocolate is consumed.[52]

4.3.2 Molecular Background of Cocoa Off-Flavors

The occasional presence of off-flavors in fermented cocoa is well documented in the literature and is reflected in specific quality requirements of the cocoa processing industry. If cocoa with off-flavors is further processed, the off-note may be transferred to the final product, which can lead to consumer complaints. Typical cocoa off-notes are described as fecal, mushroom-like, moldy-musty, hammy, and smoky, with the latter being the most commonly reported off-flavor.[32,47,55,92-95]

The occurrence of moldy off-flavors, which are also referred to as moldy-musty off-flavors, may be caused by mold growth either inside or on the surface of cocoa beans. Mold growth on cocoa beans has been associated with insufficient drying or storage at elevated moisture levels.[31,32] Only 3% of moldy beans can cause moldy-musty off-flavors in chocolate.[31,92,93] In the literature, methyl ketones such as 2-pentanone, 2-heptanone, and 2-nonanone were considered responsible for these off-flavors.[93,96] However, their odor qualities are described as fruity, soapy, fatty, and musty,[97] and therefore, these compounds cannot explain the moldy off-flavor in cocoa beans. In a recent study by Porcelli et al.[98], the moldy, beetroot-like smelling (–)-geosmin, which is a well-known off-flavor compound in wine, drinking water, cereals, and fish,[99-102] was identified for the first time in fermented cocoa samples with moldy-musty off-flavors. Additionally, the fecal, mothball-like smelling 3-methyl-1*H*-indole showed high OAVs. Thus, both odorants were suggested to contribute to the moldy-musty off-flavor.[98]

The hammy off-flavor is often associated with the overfermentation of cocoa beans.[32,37,38,55,93] In the literature though, it is also occasionally linked to the smoky off-flavor and then referred to as smoky-hammy off-flavor.[31,103] Moreover, it is reported that the distinction between hammy and smoky off-flavors is often not easy in practice.[47] Ney et al.[93] differentiated between hammy off-flavors on the one hand and smoky off-flavors on the other hand. They suggested short-chain carboxylic acids such as propanoic acid, butanoic acid, pentanoic acid, and 3-methyl-butanoic acid as causative for the hammy off-flavor. According to Ney et al., the hammy off-flavor may be prevented by better control of the fermentation, especially by avoiding over-fermentation.[93] Schwan and Wheals[55] also linked the hammy off-flavor to the overfermentation of cocoa beans and higher contents of short-chain fatty acids produced by microorganisms. However, the odor qualities of short-chain carboxylic acids are described as sweaty and cheesy, but not hammy.[97] Thus, it is questionable whether these compounds actually contribute to the hammy off-flavor.

The smoky off-flavor typically occurs after inappropriate artificial drying using wood fires if the cocoa beans come into contact with the smoke.[47,55,76,93,95,103-105] However, smoky off-notes may also be caused by overfermentation.[76] This seems plausible, since carboxylic acids from plants can be converted into volatile smoky smelling phenols by microbial enzymes. For example, 2-methoxyphenol and 4-ethyl-2-methoxyphenol can be formed from ferulic acid.[106-109] In addition, both smoke contamination[110] and microbial formation[111-113] of volatile phenols are known to cause smoky off-notes in wine. In cocoa, however, the responsible compounds have not yet unequivocally been identified.

Lehrian et al.[103] associated smoky/hammy off-flavors in fermented cocoa with high contents of phenolic compounds and wood smoke contamination during cocoa drying. The phenols were

quantitated by a photometric sum method; however, neither the exact structures were identified nor their contribution to the overall aroma was elucidated.[103]

Ney et al.[93] also suggested phenolic compounds, particularly 2,6-dimethoxyphenol, as causative for the smoky off-flavor. Phenolic compounds might be absorbed by the cocoa beans when dried over or next to open fire. However, Ney's conclusion is questionable, because the OTV of 2,6-dimethoxyphenol is relatively high (29 µg/kg)[97] compared to other smoky smelling phenolic compounds such as 2-methoxyphenol (0.84 µg/kg)[97] and 4-vinyl-2-methoxyphenol (0.15 µg/kg)[97], which are both known to contribute to the smoky notes of alcoholic beverages matured in toasted wood barrels.[114,115]

Serra Bonvehí and Ventura Coll[104] analyzed cocoa powder samples that were correctly processed and samples that had either little contact or strong direct contact with wood smoke. Samples were compared based on the concentrations of nine selected phenolic compounds. Concentration data was acquired by GC-FID and evaluated by multivariate statistical analysis. The data analysis revealed five phenolic compounds showing significant differences in the smoked samples. For these compounds, maximum tolerable concentrations in cocoa powder were suggested: phenol (2 mg/kg), 3-methylphenol (0.9 mg/kg), 2,3-dimethylphenol (0.55 mg/kg), 3-ethylphenol (0.90 mg/kg), and 4-ethylphenol (0.70 mg/kg). A maximum value of 9.6 mg/kg was proposed for the total phenol content. However, the suggested maximum tolerable concentrations were solely based on statistical analysis and not verified by sensory tests.

Rodriguez-Campos et al.[76] evaluated the effect of fermentation time and drying temperature on volatile compounds in cocoa by HS-SPME-GC-MS. Smoky off-flavor notes were attributed to undesirable phenolic compounds from smoke contamination. 2-Methoxyphenol was observed only at the end of the fermentation period after six and eight days, with concentrations ranging from ~0.5–1.0 mg/kg. Somewhat higher concentrations (~1.5 mg/kg) were found after artificial drying.

Perotti et al.[105] compared smoky with non-smoky cocoa bean and cocoa liquor samples by HS-SPME-GC × GC-TOF-MS analysis. Statistical data evaluation of the cocoa volatiles revealed ten marker compounds correlated with the smoky off-flavor. However, their actual contribution to the smoky off-flavor was not investigated by sensory tests. The six marker compounds naphthalene, 2-methoxyphenol, 2-methoxy-4-methylphenol, phenol, 4-ethyl-2-methoxy-phenol, and 4-methylphenol were quantitated by HS-SPME-GC-MS. Results revealed 7–125-fold higher concentrations in smoky samples than in non-smoky samples. Based on the concentration data, the authors suggested an operative limit for accepting incoming cocoa bean samples of 10 µg/kg for each of the selected smoky marker compounds and neglected the fact that the odor thresholds of the compounds differ widely.[97] Moreover, this general operative limit is up to 200 times lower than the limits suggested by Serra Bonvehí and Ventura Coll.

5   Objectives

As detailed in the introduction, the seeds of the cocoa tree, *Theobroma cacao* L., are key contributors to the valuable aroma of chocolate products. However, fermented cocoa is occasionally affected by off-flavors that may lead to consumer rejection if transferred to the final products. Among the off-flavors in cocoa, the smoky off-note is the most prevalent. The detection of off-flavors is an important part of the incoming goods inspection of the cocoa processing industry. Currently, this is mainly done by rather subjective sensorial evaluations, while objective approaches based on the concentrations of the causative compounds are lacking. To date, only a few studies focused on the identification of the compounds causing smoky off-flavors in cocoa. Furthermore, the limits suggested for these compounds vary widely.

The aims in the first part of the present study were (1) to identify the compounds potentially contributing to the smoky off-flavor in cocoa by GC-O in combination with aroma extract dilution analyses, (2) to substantiate the screening results by quantitation and calculation of odor activity values, and (3) to suggest maximum tolerable concentrations applicable at the incoming goods inspection of the cocoa processing industry. The aim of the second part of the study was to investigate the concentration changes of the compounds contributing to the smoky off-flavor and the compounds contributing to the moldy-musty off-flavor in fermented cocoa during further processing towards chocolate.

6 Results and Discussion

The present thesis is a publication-based dissertation. The data was summarized in two articles published in international peer-reviewed scientific journals. Copies of the two publications, the summaries with the individual contributions of the authors, and the reprint permissions of the publishers are included in the appendix.

6.1 Screening for Potential Off-Flavor Compounds

An initial odorant screening was applied to a sample of fermented cocoa with a pronounced smoky off-flavor and a reference sample with a flawless aroma and no off-flavor. Both samples were obtained from German chocolate manufacturers. Before analysis, the samples were stored in vacuum-sealed bags at +4 °C.

The cocoa bean samples were cooled with liquid nitrogen and ground into a fine powder. The powders were mixed with an organic solvent and the mixture was stirred at room temperature. Nonvolatiles were removed by SAFE. The SAFE distillates were concentrated using a Vigreux column and a Bemelmans microdistillation device. The concentrates were subjected to cAEDA. Results revealed 46 odor-active regions with FD factors ≥8 in at least one of the two samples. Preliminary structure assignments were achieved by comparing RIs and odor qualities perceived at the sniffing port during cAEDA with the corresponding data in databases and the literature.[97,116] The preliminary assignments were verified by the analysis of the respective reference compounds in appropriate dilutions. Two GC systems of different polarity (DB-FFAP and DB-5) were employed. Final structure identification was achieved by GC-MS. Prior to GC-MS analysis, the SAFE distillates were further fractionated to avoid coelutions. First, the distillates were fractionated into a fraction containing the neutral and basic volatiles (NBV) and a fraction containing the acidic volatiles (AV). The fraction of NBV was further fractionated into five subfractions of different polarity by silica gel liquid chromatography. For the final structure confirmation, the mass spectra of the cocoa volatiles in CI and EI mode were compared with the mass spectra of the authentic reference compounds analyzed under the same conditions. Finally, the structures of 42 of the 46 odorants in the off-flavor sample and the reference sample could be unambiguously assigned.[117] The odorants with FD factors ≥16 in the off-flavor sample are listed with decreasing FD factors in the off-flavor sample in Table 2.

In the off-flavor sample, the highest FD factor of 4096 was determined for smoky smelling 4-ethylphenol (**41**). In the reference sample, however, it was not detected (FD <1). Remarkably high FD factors of 2048 in the off-flavor sample were obtained for smoky, hammy smelling 2-methoxyphenol (**30**) and for smoky, phenolic smelling 3-/4-propylphenol (**44**). Among these compounds, only 2-methoxyphenol (**30**) was detected in the reference sample (FD 512), which was, unlike the previously mentioned phenols, a known odorant in fermented cocoa.[73-75,90] Because of the higher FD factors in the off-flavor sample and the smoky, hammy, and phenolic odor qualities, compounds **41**, **30**, and **44** were considered potential contributors to the smoky off-flavor.[117,118]

Table 2: Odor-active compounds in the SAFE distillates obtained from a fermented cocoa sample with a pronounced smoky off-flavor (OF) and a reference sample without off-flavor (REF): odorants with FD factors ≥16 in the sample OF are listed with decreasing FD factors

No.[a]	Odorant[b]	Odor[c]	RI[d]		FD factor[e]	
			FFAP	DB-5	OF	REF
41	**4-Ethylphenol**	**smoky**	2190	1166	**4096**	**<1**
30	**2-Methoxyphenol**	**smoky, hammy**	1861	1091	**2048**	**512**
44	**3-/4-Propylphenol**	**smoky, phenolic**	2269	1259	**2048**	**<1**
32	2-Phenylethan-1-ol	floral	1913	1117	1024	4096
40	γ-Decalactone	peach	2150	1451	512	2048
6	Ethyl 3-methylbutanoate	fruity	1062	855	512	512
26	2-Methyl-3-(methylsulfanyl)furan[f]	meat	1665	1177	512	512
21	3-Isobutyl-2-methoxypyrazine	bell pepper	1519	1183	512	256
4	Ethyl butanoate	fruity	1028	802	512	16
28	2-Phenylethyl acetate	honey, floral	1811	1261	256	1024
31	Ethyl 3-phenylpropanoate	floral, cinnamon	1884	1351	256	512
37	4-Hydroxy-2,5-dimethylfuran-3(2H)-one[f]	caramel	2033	1075	256	256
34	trans-4,5-Epoxy-(2E)-dec-2-enal[f]	metallic	2005	1381	256	4
36	γ-Nonalactone	coconut	2029	1367	256	4
39	**3-Methylphenol**	**smoky, phenolic**	2088	1075	**256**	**<1**
1	Ethyl 2-methylpropanoate	fruity	975	759	128	128
25	2-/3-Methylbutanoic acid	sweaty	1661	868	64	512
2	Butane-2,3-dione[f]	buttery	985	<700	64	128
13	Dimethyl trisulfide	sulfury, cabbage	1373	971	64	64
24	Phenylacetaldehyde	floral, honey	1650	1045	64	64
17	3-Isopropyl-2-methoxypyrazine	earthy	1427	1095	64	32
11	3-Hydroxybutan-2-one	buttery, nutty	1286	715	64	4
38	**4-Methylphenol**	**horse stable, phenolic**	2085	1073	**64**	**<1**
10	Unknown	fruity	1257	-	32	32
43	3-Hydroxy-4,5-dimethylfuran-2(5H)-one[f]	seasoning	2203	1108	16	256
20	2,3-Diethyl-5-methylpyrazine	earthy	1488	1158	16	32
9	2-/3-Methylbutan-1-ol	malty	1202	735	16	8
42	**3-Ethylphenol**	**smoky**	2200	1168	**16**	**<1**

[a]Numbers according to Table 1 in Publication 1.[117] [b]Odorants showing an FD factor of ≥16 in the off-flavor sample. Structure assignments were based on the comparison of the retention indices on two columns of different polarity (DB-FFAP, DB-5), the odor quality perceived at the sniffing port during GC-O, and the mass spectra obtained by GC-MS analysis (CI and EI) with data of authentic reference substances analyzed under the same conditions. [c]Odor quality as perceived at the sniffing port during GC-O. [d]Retention index; calculated from the retention time of the odorant and the retention times of adjacent n-alkanes by linear interpolation. [e]Flavor dilution factor; dilution factor of the highest diluted sample obtained from concentrated fractions NBV or AV by serial dilution in which the odorant was detected during GC-O analysis by any of three panelists. [f]A clear mass spectrum could not be obtained in the cocoa volatile concentrates; identification was based on the remaining criteria detailed in footnote b.

In the reference sample, the three highest FD factors of 4096, 2048, and 1024 were obtained for floral smelling 2-phenylethan-1-ol (**32**), peach-like smelling γ-decalactone (**40**), and honey, floral smelling 2-phenylethyl acetate (**28**), all of which are known to contribute to the pleasant aroma of fermented cocoa.[73-75,90] These compounds were also identified in the off-flavor sample, but with slightly lower FD factors, namely 1024, 512, and 256.[117] In general, the

odorants detected in the reference sample agreed well with the major odorants in fermented cocoa reported in the literature.[73-75,90]

Due to their conspicuous odor qualities and their significantly higher FD factors in the off-flavor sample than in the reference sample, further odorants were additionally considered as potential off-flavor compounds. These were smoky, phenolic smelling 3-methylphenol (**39**), horse stable-like, phenolic smelling 4-methylphenol (**38**), and smoky smelling 3-ethylphenol (**42**), for which FD factors of 256, 64, and 16, respectively, were determined in the off-flavor sample. In the reference sample, however, these phenols could not be detected (FD <1).[117,118]

In summary, the compounds 4-ethylphenol (**41**), 2-methoxyphenol (**30**), 3-/4-propylphenol (**44**), 3-methylphenol (**39**), 4-methylphenol (**38**), and 3-ethylphenol (**42**) were identified as potentially causative for the smoky off-flavor.[117] Their structures as well as their FD factors in the off-flavor sample and the reference sample are shown in Figure 13.

4-Ethylphenol
(FD 4096 / <1)

2-Methoxyphenol
(FD 2048 / 512)

3-Propylphenol and 4-Propylphenol
(FD 2048 / <1)

3-Methylphenol
(FD 256 / <1)

4-Methylphenol
(FD 64 / <1)

3-Ethylphenol
(FD 16 / <1)
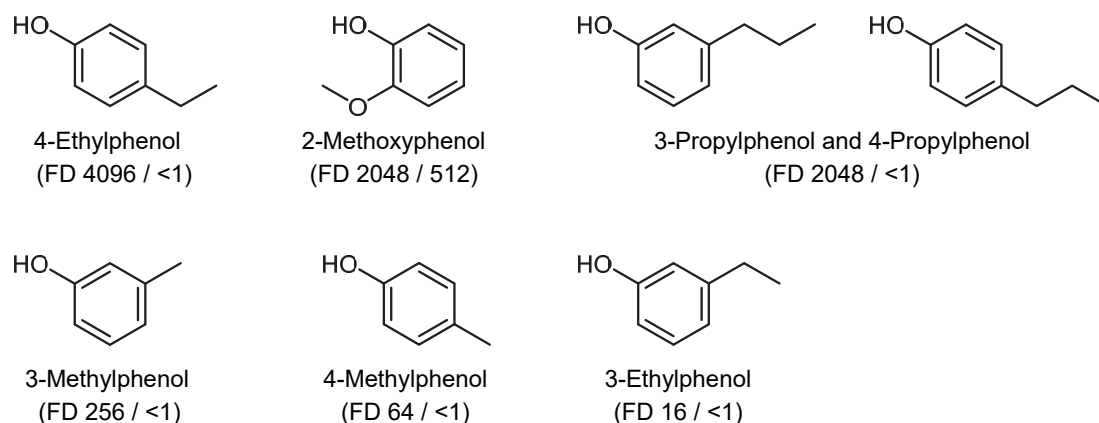
Figure 13: Potential off-flavor compounds and their FD factors in the samples OF / REF

The isomers 3- and 4-propylphenol were not separated on the DB-FFAP column used for AEDA. A coelution of both isomers was also observed when using a non-polar DB-5 column. To clarify whether both isomers or only one of the two is present in the cocoa sample, isomer separation, however, was required. This could be achieved by using a heart-cut GC-GC-HRMS system equipped with a polar FFAP column in the first dimension and a chiral BGB-176 column with a β-cyclodextrin-based stationary phase in the second dimension. Chiral GC columns are typically used for the separation of enantiomers, but often show excellent separation of positional isomers as well.[15] By means of this GC-enantioGC-MS approach, the fraction of AV was analyzed in parallel to the reference compounds. Results revealed the presence of both isomers, 4-propylphenol (**44a**) and 3-propylphenol (**44b**), in the cocoa off-flavor sample.[117]

The odorant screening of fermented cocoa samples was extended to further six samples with smoky off-flavors and a further reference sample (REF2). All samples were obtained from German chocolate manufacturers and originated from various cocoa producing countries. A trained sensory panel consisting of seven panelists ranked the seven off-flavor samples in order of decreasing intensity of the smoky off-note from extremely intense (OF1) via intense (OF2–OF6) to moderate (OF7). In addition, a sample with an off-note typical for overfermented cocoa beans (OVF) was included in the screening, because overfermentation had often been

associated with the development of hammy off-flavors.[32,37,38,55,93] Detailed fermentation parameters of the sample, however, were not known. Both reference samples (REF1, REF2) were without off-flavor; however, REF1 showed a very pleasant and intense cocoa aroma, whereas the aroma of REF2 was rather weak.

A quick GC-O screening was applied to the seven additional samples by comparing the intensities of the potential off-flavor compounds without dilution (Table 3). The results revealed the presence of the seven previously identified compounds and the absence of further compounds potentially causative for the smoky off-flavor. The data showed clear differences between the off-flavor samples and the reference samples. In OF1–OF7, all potential off-flavor compounds were at least weakly perceivable. In contrast, in REF1 and REF2, only 2-methoxyphenol (**30**) was perceived with a moderate intensity. The sample with the most intense smoky off-flavor, OF1, also showed the highest overall intensities. The sample OVF was rather similar to the reference samples than to the off-flavor samples. Smoky, hammy smelling 2-methoxyphenol (**30**) was perceived with a high intensity. In contrast to REF1 and REF2, 3-ethylphenol (**42**) was also perceived, but only weakly.

Table 3: Odor intensities of potential off-flavor compounds perceived during GC-O of different fermented cocoa samples

Sample	Sample description	Odor intensities of potential off-flavor compounds[a]					
		2-Methoxy-phenol	4-Methyl-phenol	3-Methyl-phenol	4-Ethyl-phenol	3-Ethyl-phenol	3-/4-Propyl-phenol
OF1	extremely intense smoky OF	+++	+++	+++	+++	++	+++
OF2	intense smoky OF	+++	+++	++	+++	+	++
OF3	intense smoky OF	+++	++	++	+++	++	+++
OF4[b]	intense smoky OF	+++	++	++	+++	+	+++
OF5	intense smoky OF	+++	++	++	+++	++	++
OF6	intense smoky OF	+++	+	++	+++	+	++
OF7	moderate smoky OF	+++	++	+	+++	+	++
OVF	overfermented odor	+++	−	−	−	+	−
REF1[b]	typical, pleasant odor	++	−	−	−	−	−
REF2	typical, weak odor	++	−	−	−	−	−

[a]Intensities of potential off-flavor compounds: zero (−), weak (+), moderate (++), or strong (+++) intensity. [b]Samples OF4 and REF1 were the samples previously used for the odorant screening by AEDA.

In addition to the fermented cocoa samples, cocoa liquor samples were investigated. Cocoa liquor is an important intermediate product in chocolate making. For its production, cocoa beans are typically roasted and then ground. Five cocoa liquor samples with slight smoky off-flavors (CL1–CL5) and one cocoa liquor sample with a typical odor and no off-flavor (REF3) were screened by GC-O. Due to the slight smoky off-notes of CL1–CL5, a ranking of the samples was not conducted. Nevertheless, the intensities of the potential off-flavor compounds (Table 4) clearly distinguished CL1–CL5 from REF3. In REF3, only 2-methoxyphenol (**30**) and 4-ethylphenol (**41**) were weakly perceived but none of the other potential off-flavor compounds. In CL1–CL5, 2-methoxyphenol (**30**) showed either moderate or strong intensities and 4-methyl-phenol (**38**), 3-methylphenol (**39**), 4-ethylphenol (**41**), and 3-ethylphenol (**42**) were either not detectable, or perceived with weak intensity (except for a moderate intensity of 4-ethylphenol in CL4). 3- and 4-propylphenol (**44**) were not detected in any of the samples. An important result of this screening was that no additional compound with smoky, hammy, and/or phenolic odor quality was detected in the cocoa liquor samples. In total, the intensities in the cocoa liquor samples were lower than in the fermented cocoa samples with smoky off-flavors (OF1–OF7), which was in accordance with the overall sensory impression.

Table 4: Odor intensities of potential off-flavor compounds perceived during GC-O of different cocoa liquor samples

Sample	Sample description	Odor intensities of potential off-flavor compounds[a]					
		2-Methoxy-phenol	4-Methyl-phenol	3-Methyl-phenol	4-Ethyl-phenol	3-Ethyl-phenol	3-/4-Propyl-phenol
CL1	slight smoky OF	+++	+	−	−	−	−
CL2	slight smoky OF	+++	−	−	−	+	−
CL3	slight smoky OF	++	+	+	−	+	−
CL4	slight smoky OF	+++	+	+	++	−	−
CL5	slight smoky OF	++	+	+	−	+	−
REF3	typical odor	+	−	−	+	−	−

[a]Intensities of potential off-flavor compounds: zero (−), weak (+), moderate (++), or strong (+++) intensity.

In summary, the application of cAEDA to a sample of fermented cocoa with pronounced smoky off-flavor and to a reference sample with flawless aroma and no off-flavor revealed seven phenolic compounds with smoky, hammy, and/or phenolic odor qualities that potentially contributed to the smoky off-flavor. These compounds were 2-methoxyphenol (**30**), 4-methyl-phenol (**38**), 3-methylphenol (**39**), 4-ethylphenol (**41**), 3-ethylphenol (**42**), 4-propylphenol (**44a**), and 3-propylphenol (**44b**).[117] The GC-O screening of further six fermented cocoa samples with smoky off-flavors in different intensities, five cocoa liquor samples with slight smoky off-flavors, and two additional reference samples without off-flavor confirmed the selection of the seven potential off-flavor compounds.

6.2 Quantitation of Potential Off-Flavor Compounds

To clarify the individual roles of the seven potential off-flavor compounds for the smoky off-flavor, 2-methoxyphenol, 4-methylphenol, 3-methylphenol, 4-ethylphenol, 3-ethylphenol, 4-propylphenol, and 3-propylphenol were quantitated in the fermented cocoa off-flavor samples (OF1–OF7) and in the fermented cocoa reference samples (REF1, REF2).[117] In addition, the fermented cocoa sample with an off-note typical for overfermented cocoa beans (OVF), the five cocoa liquor samples with slight smoky off-flavors (CL1–CL5), and the cocoa liquor reference sample without off-flavor (REF3) were included in the quantitation experiments.

The samples were cooled with liquid nitrogen and ground into a fine powder. The powders were mixed with organic solvent. To the mixture, isotopically substituted odorants were added as internal standards to compensate for losses during the workup. After extraction, insoluble material was removed by filtration. The volatiles were separated from the nonvolatiles by SAFE. The volatile isolates were concentrated and the volatile concentrates were subjected to heart-cut GC-GC-MS(CI) or to heart-cut GC-GC-HRMS(CI). The odorant concentrations were calculated from the area counts of the analyte peak, the area counts of the internal standard peak, the amount of internal standard added, and the amount of sample material used for the workup by employing a calibration line obtained from the analysis of analyte/standard mixtures in at least five different concentration ratios.[117]

For the quantitation of 4-ethylphenol, 3-ethylphenol, 4-propylphenol, and 3-propylphenol, the isotopically substituted odorants 4-(1,1-^{2}H$_2$)ethylphenol and 4-(1,1-^{2}H$_2$)propylphenol were synthesized from the corresponding ketones. In detail, the educts were 1-(4-hydroxyphenyl)ethan-1-one and 1-(4-hydroxyphenyl)propan-1-one, respectively.[117] For the modified Clemmensen reduction (Figure 14), zinc powder was first activated with hydrochloric acid and then dried. Preliminary experiments revealed that the use of zinc amalgam was not necessary for the formation of the desired products. Under an argon atmosphere, deuterium oxide, deuterium chloride solution in D$_2$O, and the educts dissolved in anhydrous tetrahydrofuran were added to the activated zinc powder and the mixture was stirred at room temperature. The reaction products were extracted with dichloromethane and their structures were confirmed by the retention indices on two columns of different polarity, the odor qualities perceived during GC-O, and the GC-MS spectra in EI and CI mode.
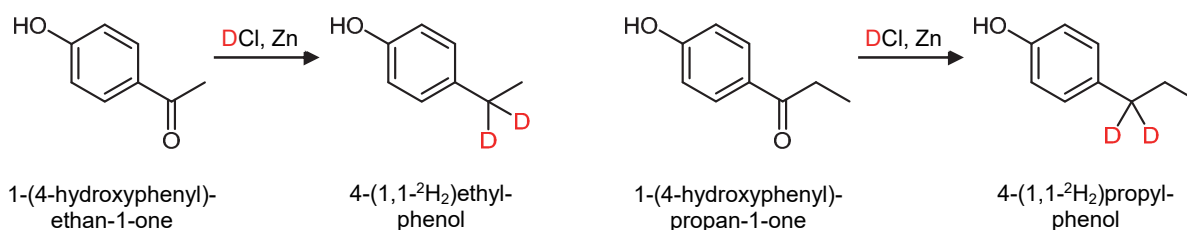

Figure 14: Syntheses of 4-(1,1-^{2}H$_2$)ethylphenol (left) and 4-(1,1-^{2}H$_2$)propylphenol (right)

In the fermented cocoa samples (Table 5), the odorant quantitation revealed concentrations ranging from 0.202 µg/kg (4-propylphenol in OVF) to 815 µg/kg (2-methoxyphenol in OF1). In samples OF1–OF7, concentrations of all off-flavor compounds were consistently higher than in REF1 and REF2. An exception was a low concentration of 4-propylphenol in OF7.[117] In sample OVF, concentrations of 3-methylphenol, 3-ethylphenol and 4-propylphenol were even lower than in the reference samples. For 4-methylphenol and 3-propylphenol, the concentrations were almost identical to the concentrations in REF2. However, the concentration of smoky, hammy smelling 2-methoxyphenol was approximately 5-fold higher in sample OVF than in REF1 and REF2. In general, in all fermented cocoa samples, the highest concentrations were obtained for 2-methoxyphenol.

In the cocoa liquor samples (Table 6), odorant concentrations ranged from 0.204 µg/kg (3-propylphenol in REF3) to 354 µg/kg (2-methoxyphenol in CL2). The highest concentrations in the cocoa liquor samples were clearly lower than the highest concentrations in the fermented cocoa samples. This is in accordance with the sensorial sample description, as CL1–CL5 showed a slight smoky off-flavor and OF1–OF7 a moderate, intense, or extremely intense smoky off-flavor. In CL1 and CL2, the concentrations of 4-methylphenol, 3-methylphenol, and 4-ethylphenol were lower than in REF3. In CL1, this was additionally the case for 3-ethylphenol. In contrast, the concentration of 2-methoxyphenol was in CL1 ~4.5-fold and in CL2 ~5.5-fold higher than in REF3. Among all investigated cocoa liquor samples, the second highest concentration of the smoky smelling 4-ethylphenol was obtained in REF3. In detail, concentrations of 4-ethylphenol in CL1–CL3 and CL5 were below the value of 31.0 µg/kg in REF3. However, total concentrations of all potential off-flavor compounds were in REF3 clearly lower than in CL1–CL5.

The total concentrations are additionally displayed in a stacked bar diagram (Figure 15). The fermented cocoa sample OF1 with an extremely intense off-flavor was the only sample with a total concentration of >2000 µg/kg. The samples with intense smoky off-flavors, OF2–OF6, showed total concentrations ranging between ~1200 µg/kg and ~2000 µg/kg. In contrast, the total concentrations in REF1–REF3 were below ~125 µg/kg. Samples OF7 and OVF showed both a total concentration of ~375 µg/kg. In sample OF7, 2-methoxyphenol, 4-methylphenol, 3-methylphenol, and 4-ethylphenol mainly contributed to the sum. In contrast, in sample OVF, the total concentration was mainly derived from the concentration of 2-methoxyphenol. This might suggest a crucial role of 2-methoxyphenol for the off-note associated with overfermented cocoa. Among the cocoa liquor samples, total concentrations in CL1–CL5 ranged between ~175 µg/kg and ~380 µg/kg, whereas in REF3, the total concentration was below 125 µg/kg. In CL3–CL5, total concentrations consisted mainly of the concentrations of 2-methoxyphenol, 4-methylphenol, 3-methylphenol, and 4-ethylphenol. In contrast, the total concentrations of ~300 µg/kg in CL1 and ~380 µg/kg in CL2 were mainly due to the concentrations of 2-methoxyphenol. Total concentrations obtained for CL1 and CL2 were comparable with the concentrations in the fermented cocoa sample OVF.

Results and Discussion

Table 5: Concentrations of potential off-flavor compounds in different samples of fermented cocoa

Sample	Sample description	concentration (µg/kg)[a]						
		2-methoxyphenol	4-methylphenol	3-methylphenol	4-ethylphenol	3-ethylphenol	4-propylphenol	3-propylphenol
OF1	extremely intense smoky OF	815	527	635	275	143	13.5	21.3
OF2	intense smoky OF	431	196	430	352	103	9.25	19.4
OF3	intense smoky OF	552	291	443	233	83.2	6.43	15.8
OF4	intense smoky OF	354	171	346	205	71.9	9.93	17.5
OF5	intense smoky OF	632	352	435	447	80.9	11.9	17.8
OF6	intense smoky OF	610	209	388	147	63.4	3.35	9.80
OF7	moderate smoky OF	120	56.9	80.1	95.4	12.0	0.573	2.47
OVF	overfermented OF	345	5.15	0.963	6.78	0.299	0.202	0.204
REF1	typical, pleasant odor	71.0	19.1	5.82	11.9	0.379	0.893	0.255
REF2	typical, weak odor	65.0	5.15	1.06	2.49	1.63	0.328	0.210

[a]Mean of duplicates or triplicates; individual concentrations and standard deviations are available in the Supporting Information file of Publication 1.[117]

Table 6: Concentrations of potential off-flavor compounds in different samples of cocoa liquor

Sample	Sample description	concentration (µg/kg)[a]						
		2-methoxyphenol	4-methylphenol	3-methylphenol	4-ethylphenol	3-ethylphenol	4-propylphenol	3-propylphenol
CL1	slight smoky OF	279	8.44	0.794	5.83	0.247	1.51	1.11
CL2	slight smoky OF	354	9.62	2.41	14.2	4.06	2.31	1.20
CL3	slight smoky OF	111	26.9	18.6	14.6	3.56	0.206	0.269
CL4	slight smoky OF	166	60.3	39.8	40.7	0.473	1.10	0.478
CL5	slight smoky OF	112	37.2	29.2	18.5	5.03	2.32	1.35
REF3	typical odor	63.1	14.3	4.35	31.0	0.451	0.208	0.204

[a]Mean of duplicates or triplicates, standard deviations were <25%.
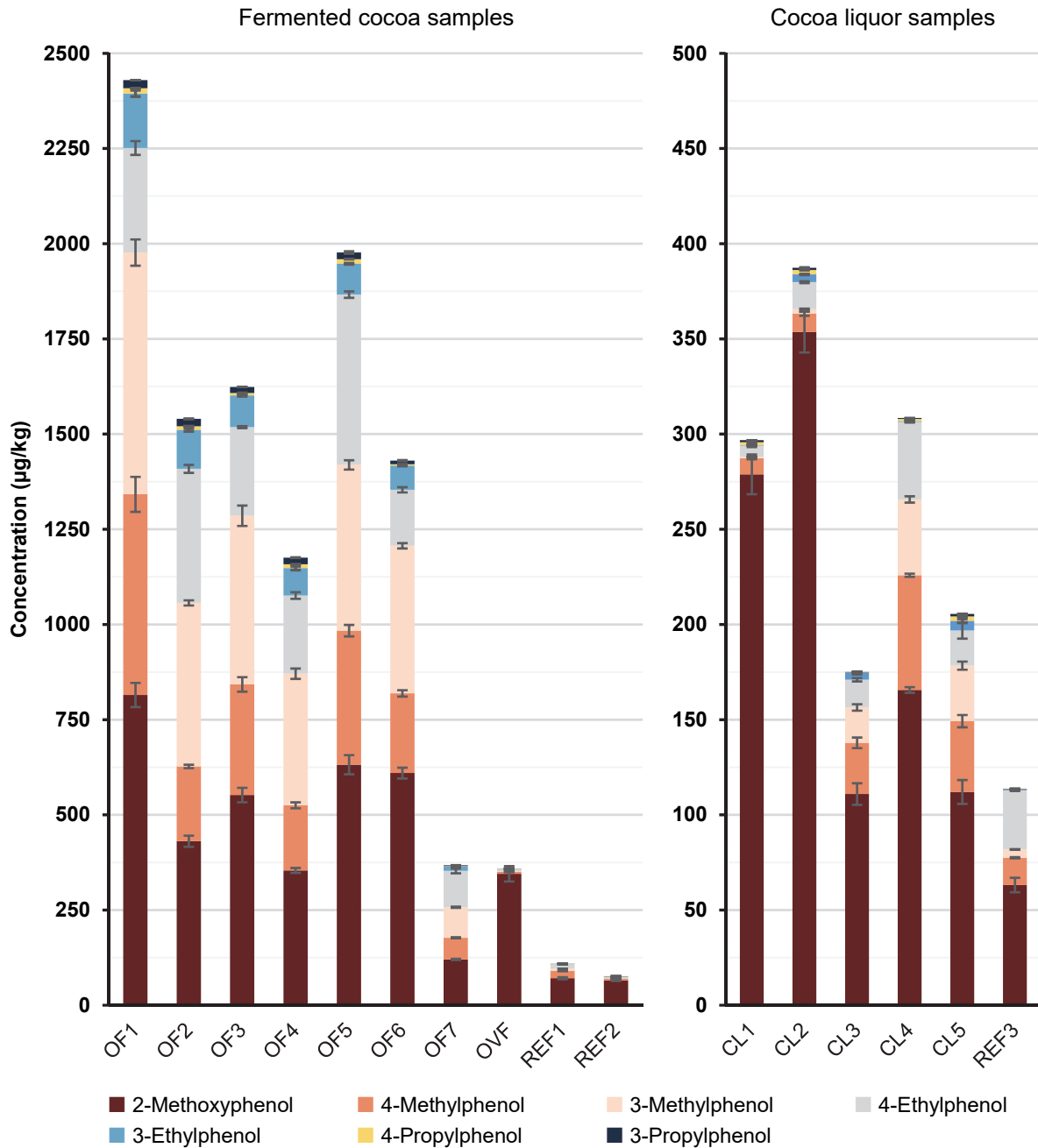
Figure 15: Concentrations of potential smoky off-flavor compounds in fermented cocoa samples (left) and cocoa liquor samples (right)

Among the potential smoky off-flavor compounds identified in the current study, 2-methoxyphenol, 4-methylphenol, 3-methylphenol, 4-ethylphenol, and 3-ethylphenol have been reported in connection with the smoky off-flavor in cocoa before.[76,104,105] Rodriguez-Campos et al.[76] reported 2-methoxyphenol concentrations of ~400 µg/kg to ~1200 µg/kg in overfermented cocoa. The highest total concentration of ~1500 µg/kg was obtained in a wood-fire dried sample. Serra Bonvehí and Ventura Coll[104] investigated the concentrations of 2-methoxyphenol, 4-methylphenol, 3-methylphenol, 4-ethylphenol, and 3-ethylphenol in uncontaminated and wood smoke contaminated cocoa samples. However, the concentrations obtained for uncontaminated cocoa of 1750−3220 µg/kg for 2-methoxyphenol, 780−1370 µg/kg for 4-methylphenol, 330−770 µg/kg for 3-methylphenol, 370−610 µg/kg for 4-ethylphenol, and

280−710 µg/kg for 3-ethylphenol already exceeded the concentrations we found in OF1–OF7. The concentrations reported by Serra Bonvehí and Ventura Coll for the wood smoke contaminated samples were even higher. Perotti et al.[105] suggested 2-methoxyphenol and 4-methylphenol as marker compounds for smoky off-flavors in cocoa. For 2-methoxyphenol, they reported an average concentration of 8.2 µg/kg in cocoa without off-flavor and 68.7 µg/kg in cocoa with smoky off-flavor. Thus, the concentrations in cocoa with smoky off-flavors reported by them was both in the range of our reference samples (65 µg/kg and 71 µg/kg) as well as of flawless cocoa samples previously reported in the literature (61 µg/kg and 110 µg/kg).[73,74] For 4-methylphenol, they reported average concentrations of 143.0 µg/kg in cocoa with a smoky off-flavor, whereas we found 56.9−527 µg/kg in samples OF1−OF7.

6.3 Determination of Odor Threshold Values

For the calculation of OAVs, the concentrations need to be divided by the OTVs of the compounds. The OTVs of the potential smoky off-flavor compounds were determined in deodorized cocoa butter. According to the American Society for Testing and Materials (ASTM) standard practice for determination of odor and taste thresholds by a forced-choice ascending series method of limits, OTVs were determined from a series of 3-AFC tests with ascending concentrations of the potential off-flavor compounds.[28] In detail, exact portions of melted deodorized cocoa butter were placed into PTFE vessels with lids. To prepare the test samples, an exact amount of the odorant dissolved in ethanol was added. Between two consecutive 3-AFC tests, odorant concentrations increased 3-fold. To the blank samples, only ethanol was added. Samples were carefully homogenized after spiking. Tests were performed at 32 °C sample temperature and a room temperature of 22±2 °C by a sensory panel consisting of 12−23 trained panelists.[117] The panelists first performed the 3-AFC test with the lowest concentration and proceeded towards the tests with the higher concentrations. For each 3-AFC test in a series, the panelists indicated the sample that was different from the other two. The odor detection threshold of each panelist was estimated as the geometric mean of the highest odorant concentration that resulted in an incorrect answer and the next higher concentration that resulted in a correct answer. Finally, the OTV of an odorant was calculated as the geometric mean of the odor detection thresholds of all panelists. In general, the odor detection thresholds of the individual panelists often vary significantly. This also applied to the detection thresholds of the seven compounds potentially contributing to the smoky off-flavor (Figure 16).
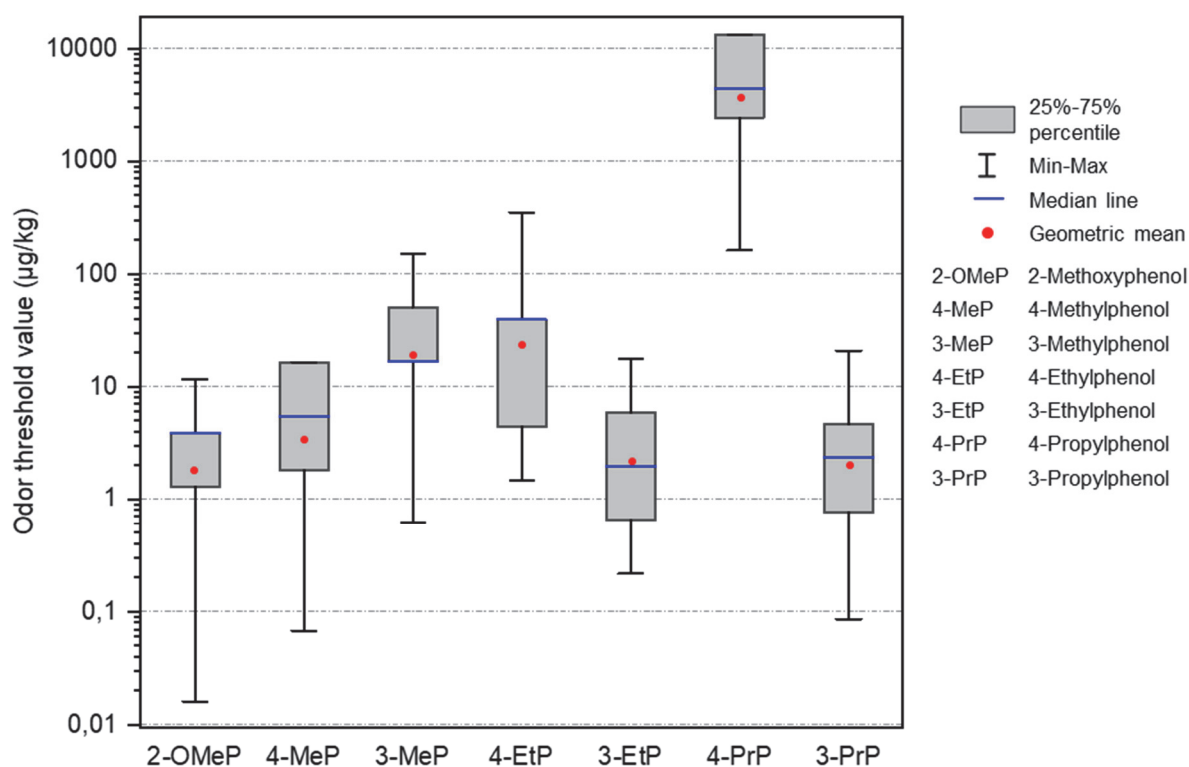
Figure 16: Individual detection thresholds of the potential off-flavor compounds and their variations

The boxplots displayed in Figure 16 indicate the minima, the maxima, the 25%−75% percentiles, the medians of the individual odor detection thresholds as well as the geometric means defined as the OTVs of the compounds. For each potential off-flavor compound, the odor detection thresholds of the individual panelists varied at least by two orders of magnitude. The lowest individual detection thresholds were determined for 2-methoxyphenol (~0.015 µg/kg), followed by 4-methylphenol (~0.06 µg/kg), and 3-propylphenol (~0.09 µg/kg). In contrast, the lowest individual detection threshold of 4-propylphenol was ~150 µg/kg. 4-Propylphenol clearly differed in its odor activity from the other potential smoky off-flavor compounds.

In summary, the OTVs were 1.8 µg/kg for 2-methoxyphenol, 3.3 µg/kg for 4-methylphenol, 19 µg/kg for 3-methylphenol, 23 µg/kg for 4-ethylphenol, 2.2 µg/kg for 3-ethylphenol, 3700 µg/kg for 4-propylphenol, and 2.0 µg/kg for 3-propylphenol.[117] However, given the large variation in the individual odor detection thresholds, there might be consumers that are still able to perceive compounds even though the OAV is <1. This has to be carefully considered when limits for the incoming goods inspection in the chocolate industry are discussed.

6.4 Calculation of Odor Activity Values

To assess the contribution of the individual potential off-flavor compounds to the smoky off-flavor, OAVs were calculated by dividing the concentrations by the OTVs of the compounds in deodorized cocoa butter. The concentration of 4-propylphenol was below its OTV in all samples. Thus, with an OAV <1, 4-propylphenol was considered not relevant for the smoky off-flavor in the cocoa samples. In contrast, OAVs >1 were determined for all other potential off-flavor compounds in the fermented cocoa samples with a smoky off-flavor (OF1−OF7) (Table 7). Among these samples, the highest OAVs were determined for 2-methoxyphenol (67−450), followed by 4-methylphenol (17−160), 3-ethylphenol (5.5−65), 3-methylphenol (4.2−33), 4-ethylphenol (4.1−19), and 3-propylphenol (1.2−11) Thus, the OAV calculations eventually confirmed their role as off-flavor compounds.[117] In the sample with the overfermented off-note (OVF), OAVs >1 were determined only for 2-methoxyphenol (190) and 4-methylphenol (1.6). For both compounds, OAVs >1 were also observed in the reference samples REF1 and REF2: OAVs were 39 and 36 for 2-methoxyphenol, and 5.8 and 1.6 for 4-methylphenol. Thus, the OAVs of 4-methylphenol in the reference samples and in sample OVF were comparable. However, for 2-methoxyphenol, the OAV in sample OVF was almost 5-fold higher than the OAVs in the reference samples. Therefore, the data suggested an essential role of 2-methoxyphenol to both the smoky and the overfermented off-note in cocoa.

In the cocoa liquor samples (Table 8) with slight smoky off-flavor (CL1−CL5), the highest OAVs were determined for 2-methoxyphenol (62−200) and 4-methylphenol (2.6−18). OAVs of 3-methylphenol, 4-ethylphenol, and 3-ethylphenol were clearly lower. In detail, OAVs of these three compounds were occasionally >1, but in no case beyond 2.3. The concentrations of 3-propylphenol exceeded its odor threshold value in none of the cocoa liquor samples. In CL1, OAVs >1 were determined only for 2-methoxyphenol and 4-methylphenol. Thus, the other off-flavor compounds were considered negligible in this sample. With an OAV of 160, 2-methoxyphenol was most important for the off-note of CL1. In CL2, the highest OAV of 200 was determined for 2-methoxyphenol. Additionally, OAVs >1 were determined for 4-methylphenol (2.9) and 3-ethylphenol (1.8). In samples CL3−CL5, OAVs of 2-methoxyphenol with 62−92 were lower than in CL1 and CL2. However, with OAVs of 8.2−18 in CL3−CL5, 4-methylphenol probably affected the slight smoky off-flavor of these samples somewhat more than in CL1 and CL2. The OAVs in the cocoa liquor reference sample (REF3) were 35 for 2-methoxyphenol and 4.3 for 4-methylphenol. Both were in accordance with the OAVs of the fermented cocoa reference samples (REF1, REF2). Additionally, in REF3 a rather low OAV of 1.3 was determined for 4-ethylphenol. In total, the data suggested that especially 2-methoxyphenol and 4-methylphenol played a significant role for the slight smoky off-flavor of the cocoa liquor samples. This was in agreement with the data obtained for the fermented cocoa samples, in which both compounds showed the highest overall OAVs.

The OAVs of the off-flavor compounds in all samples are displayed in Figure 17. In agreement with the sensory ranking of samples OF1−OF7, samples OF1 and OF7 were scored with the highest and the lowest intensity of the smoky off-flavor, respectively. However, within OF2−OF6, the total OAVs did not fully reflect the sensory scoring. This could be explained by the fact that the intensity of the perceived smoky off-flavor not only depends on the total concentrations of the off-flavor compounds, but also on the concentrations of additional odorants present in the sample, which may mask the off-flavor to some extent.[117]

Results and Discussion

Table 7: Odor Activity Values (OAVs) of potential off-flavor compounds in different samples of fermented cocoa

Sample	Sample description	Odor Activity Value (OAV)[a]						
		2-methoxyphenol	4-methylphenol	3-methylphenol	4-ethylphenol	3-ethylphenol	4-propylphenol	3-propylphenol
OF1	extremely intense smoky OF	450	160	33	12	65	<1	11
OF2	intense smoky OF	240	60	23	15	47	<1	10
OF3	intense smoky OF	310	88	23	10	38	<1	7.9
OF4	intense smoky OF	200	52	18	8.9	33	<1	8.8
OF5	intense smoky OF	350	110	23	19	37	<1	8.9
OF6	intense smoky OF	340	63	20	6.4	29	<1	4.9
OF7	moderate smoky OF	67	17	4.2	4.1	5.5	<1	1.2
OVF	overfermented OF	190	1.6	<1	<1	<1	<1	<1
REF1	typical, pleasant odor	39	5.8	<1	<1	<1	<1	<1
REF2	typical, weak odor	36	1.6	<1	<1	<1	<1	<1

[a]OAVs were calculated as concentration divided by the odor threshold value in deodorized cocoa butter.

Table 8: Odor Activity Values (OAVs) of potential off-flavor compounds in different samples of cocoa liquor

Sample	Sample description	Odor Activity Value (OAV)[a]						
		2-methoxyphenol	4-methylphenol	3-methylphenol	4-ethylphenol	3-ethylphenol	4-propylphenol	3-propylphenol
CL1	slight smoky OF	160	2.6	<1	<1	<1	<1	<1
CL2	slight smoky OF	200	2.9	<1	<1	1.8	<1	<1
CL3	slight smoky OF	62	8.2	1.0	<1	1.6	<1	<1
CL4	slight smoky OF	92	18	2.1	1.8	<1	<1	<1
CL5	slight smoky OF	62	11	1.5	<1	2.3	<1	<1
REF3	typical odor	35	4.3	<1	1.3	<1	<1	<1

[a]OAVs were calculated as concentration divided by the odor threshold value in deodorized cocoa butter.

The comparison of the fermented cocoa sample OVF and the cocoa liquor samples CL1 and CL2 revealed similar OAVs of 190, 160, and 200 for smoky, hammy smelling 2-methoxyphenol. The compounds 4-methylphenol, 3-methylphenol, 4-ethylphenol, 3-ethylphenol, and 3-propylphenol, however, showed significantly lower OAVs than in all other analyzed off-flavor samples. Thus, it might be assumed that processing of a sample such as OVF could result in a cocoa liquor such as CL1 or CL2, provided that there is no major loss or extensive formation of off-flavor compounds during roasting and processing into cocoa liquor. However, little was yet known about the influence of processing on the concentrations of cocoa off-flavor compounds. Thus, the aim of the second part of this study (cf. section 6.6 and 6.7) was to further investigate the behavior of major cocoa off-flavor compounds during further processing.
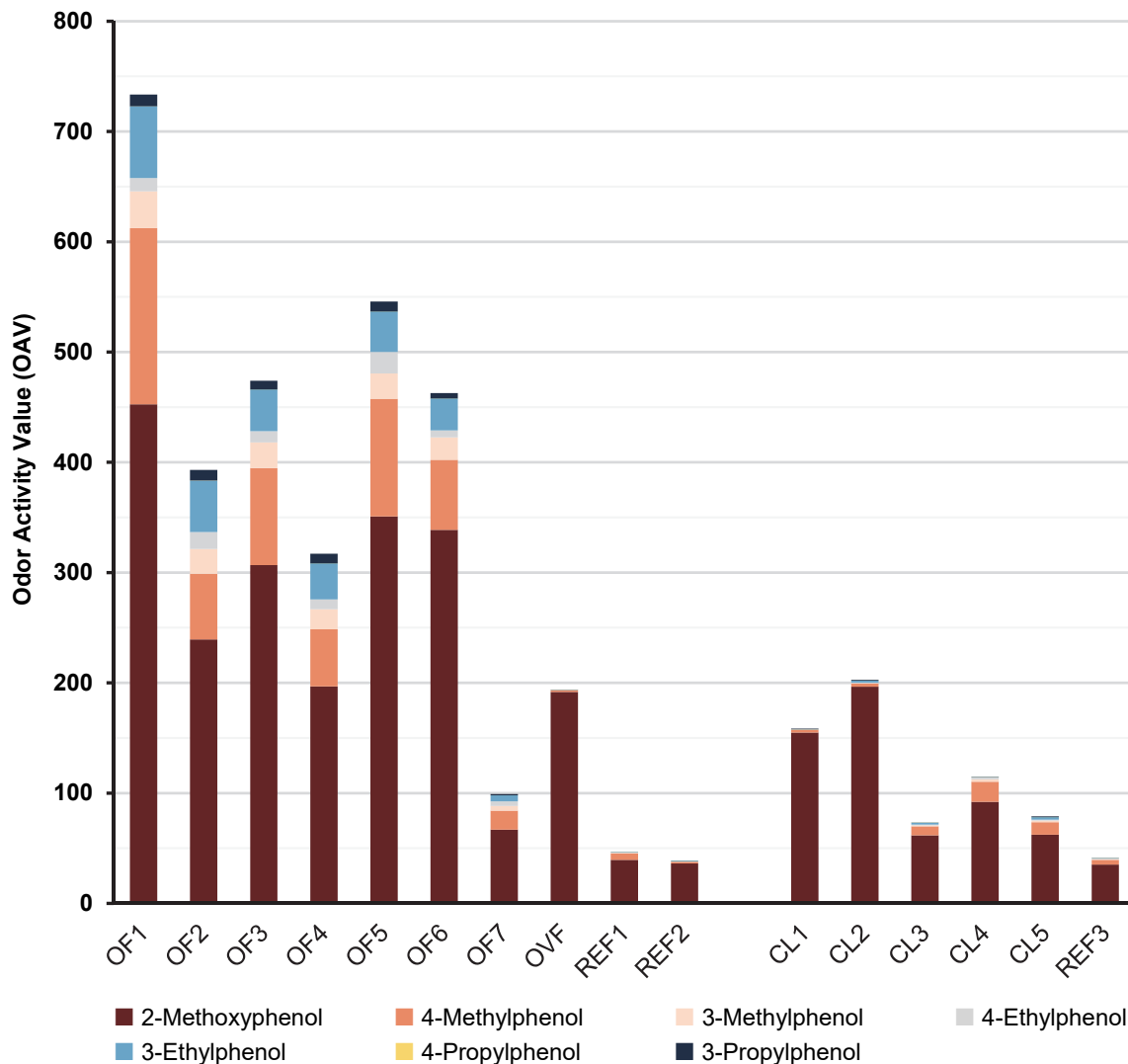

Figure 17: OAVs of the off-flavor compounds in fermented cocoa samples and cocoa liquor samples

6.5 Maximum Tolerable Concentrations

For the practical application of the results obtained so far, maximum tolerable concentrations of the potential off-flavor compounds in fermented cocoa were suggested. Such limits could be applied during the incoming goods inspection in the cocoa processing industry. Due to the wide range of odor threshold values in deodorized cocoa butter (1.8−3700 µg/kg), individual values were required. The results of the current study revealed OAVs <1 in the fermented cocoa reference samples (REF1, REF2) for 3-methylphenol, 4-ethylphenol, 3-ethylphenol, and 3-propylphenol. Thus, we suggested to derive their maximum tolerable concentrations from the rounded threshold values in deodorized cocoa butter. For the compounds with OAVs >1 in the fermented cocoa reference samples, namely 2-methoxyphenol and 4-methylphenol, the rounded maximum concentrations in the fermented cocoa reference samples were suggested as maximum tolerable concentrations. The concentrations in the reference samples were 71.0 µg/kg and 65.0 µg/kg for 2-methoxyphenol and 19.1 µg/kg and 5.15 µg/kg for 4-methyl-phenol (cf. Table 5). Thus, 70 µg/kg and 20 µg/kg were suggested as maximum tolerable concentrations for the incoming goods inspection in the cocoa processing industry.[117]

In summary, the suggested maximum tolerable concentrations were 70 µg/kg for 2-methoxy-phenol, 20 µg/kg for 4-methylphenol, 20 µg/kg for 3-methylphenol, 20 µg/kg for 4-ethylphenol, 2 µg/kg for 3-ethylphenol, and 2 µg/kg for 3-propylphenol. Due to its high odor threshold value, 4-propylphenol does not have to be considered.[117]

In the literature, a limit of 10 µg/kg was suggested for 2-methoxyphenol and 4-methylphenol.[105] However, with respect to the concentrations obtained in the reference samples, this low limit was considered not reasonable. In addition, clearly higher limits of 900 µg/kg for 3-methyl-phenol, 900 µg/kg for 3-ethylphenol, and 700 µg/kg for 4-ethylphenol were also previously suggested.[104] However, considering the odor threshold values in deodorized cocoa butter, the concentrations suggested as limits would already cause an off-flavor.[117]

The concentration data obtained for the cocoa liquor samples (cf. Table 6) supported the suggested maximum tolerable concentrations. In the cocoa liquor reference sample (REF3), the concentrations were below the suggested maximum tolerable concentrations (with the only exception of 4-ethylphenol slightly exceeding its suggested maximum tolerable concentration). In contrast, in the cocoa liquor samples with slight smoky off-flavors (CL1−CL5), in particular the concentrations of 2-methoxyphenol and 4-methylphenol were exceeding the maximum tolerable concentrations of 70 µg/kg and 20 µg/kg, respectively.

However, if further processing of fermented cocoa, e.g. roasting or conching, leads to a significant increase or decrease in the concentrations of the off-flavor compounds, adjustments of the suggested maximum tolerable concentrations might be necessary. Thus, the behavior of the off-flavor compounds during further processing was tested in the following part of this work.

6.6 Impact of Roasting and Processing into Cocoa Liquor on the Concentrations of Off-Flavor Compounds

Two fermented cocoa samples with smoky off-flavors (SOF1, SOF2) and a reference sample with a characteristic aroma and without off-notes (REF) were roasted and processed into cocoa liquor. The previously identified six smoky off-flavor compounds were quantitated in the cocoa liquors and the concentrations were compared to the data before roasting (cf. Table 5).[117] Additionally, two samples with a moldy-musty off-flavor (MOF1, MOF2) were likewise roasted and processed into cocoa liquor. In these samples, as well as in sample REF, (−)-geosmin (moldy, beetroot-like), 3-methyl-1*H*-indole (fecal, mothball-like), 1*H*-indole (fecal, mothball-like), and 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF; caramel-like, musty), identified as potentially causative for the moldy-musty off-flavor,[98] were quantitated before and after roasting and processing into cocoa liquor.

All samples were roasted individually in a pre-heated convection oven for 25 min according to the protocol "Elements of harmonized international standards for cocoa quality and flavour assessment" of the Cocoa of Excellence (CoEx) Programme.[119] Three different roasting temperatures were applied, namely 110, 125, and 140 °C. After roasting, the shells were removed manually and the cocoa beans were processed into cocoa liquor using a mortar grinder. Prior to the sample workup, cocoa liquor samples were milled into a fine powder using a cryogenic mill. Internal standard addition, solvent extraction, isolation of volatiles by SAFE, concentration, analysis by heart-cut GC-GC-MS(CI) or heart-cut GC-GC-HRMS(CI), and calculation of the concentrations in the samples were performed according to the procedure previously described (cf. section 6.2).[120]

The concentrations and the OAVs of the six smoky off-flavor compounds in the samples with smoky off-flavors (SOF1, SOF2) and the reference sample (REF) before and after roasting and processing into cocoa liquor are displayed in Table 9. OAVs were calculated with the OTVs in deodorized cocoa butter (cf. section 6.3).[117]

Results indicated no general decrease of the smoky off-flavor compounds due to roasting and processing into cocoa liquor in samples SOF1 and SOF2. The only clear decrease was observed for 3-propylphenol in SOF2. From a concentration of 17.5 µg/kg corresponding to an OAV of 8.8 in the fermented cocoa, 3-propylphenol finally decreased by a factor of ~3 to 5.56 µg/kg and an OAV of 2.8 after roasting at 140 °C and processing into cocoa liquor. However, such a behavior was not observed in SOF1. In detail, with concentrations ranging from 21.3 to 28.4 µg/kg (OAVs of 11−14), only minor changes were observed in SOF1.

The off-flavor compounds 2-methoxyphenol, 4-methylphenol, 4-ethylphenol, and 3-ethylphenol showed a tendency towards a slight increase in SOF1 and SOF2. However, the increase was never greater than 1.4-fold and notably, the order of the off-flavor compounds with respect to the OAVs was not changed: the highest OAVs before as well as after roasting and processing into cocoa liquor were obtained for 2-methoxyphenol, followed by 4-methylphenol, 3-ethylphenol, and 3-methylphenol.[120]

In the reference sample (REF), OAVs >1 before roasting were only observed for 2-methoxyphenol and 4-methylphenol. After roasting and processing into cocoa liquor, however, the concentrations of 4-ethylphenol increased up to 5.4-fold and the concentrations of 3-ethylphenol

increased up to 15-fold. Thus, the concentrations of 4-ethylphenol and 3-ethylphenol were slightly exceeding their OTVs in the cocoa liquors.[120] In the reference sample, the concentrations of 2-methoxyphenol and 4-methylphenol rather decreased during roasting and processing into cocoa liquor.[120] In contrast, an increase of both compounds after roasting of two cocoa samples without off-flavors were reported in another study.[73,74] This difference might be due to the different roasting conditions applied.

The concentrations and the OAVs of the four potential off-flavor compounds in the samples with moldy-musty off-flavors (MOF1, MOF2) and the reference sample (REF) before and after roasting and processing into cocoa liquor are displayed in Table 10. The OTVs in deodorized cocoa butter used for the OAV calculations were 1.6 µg/kg for (−)-geosmin, 1.1 µg/kg for 3-methyl-1*H*-indole, 51 µg/kg for 1*H*-indole, and 350 µg/kg for MDMF.[98]

The moldy, beetroot-like smelling (−)-geosmin was present in odor-active amounts only in sample MOF1. Roasting at 125 and 140 °C almost doubled its concentration, whereas roasting at 140 °C reduced its concentration. These results were rather difficult to explain as (−)-geosmin is not known to be formed thermally.[99,102,121] Additionally, (−)-geosmin was recently found to be enriched in the cocoa shells,[98] which, however, had been removed after roasting and before processing into cocoa liquor. Therefore, in the roasted samples rather a lower concentration than in the unroasted sample was expected. Based on the single dataset obtained from MOF1, a clear conclusion was not possible.

The fecal, mothball-like smelling 3-methyl-1*H*-indole was significantly reduced during roasting at higher temperatures and processing into cocoa liquor. After roasting at 125 and 140 °C, OAVs decreased in MOF1 from 1.8 to <1 and in MOF2 from 60 to 1.5, respectively. Thus, the limit of 3-methyl-1*H*-indole might be raised when roasting is conducted at higher temperatures.

The fecal, mothball-like smelling 1*H*-indole also showed considerable changes after roasting and processing into cocoa liquor. Whereas the concentrations were below the OTV in the fermented cocoa, they increased continuously with rising roasting temperatures. This resulted in OAVs between 1.7 and 8.0 in the samples roasted at 140 °C. Additionally, in the sample REF after roasting at 125 and 140 °C, 1*H*-indole was the only of the four off-flavor compounds that exceeded its OTV. Thus, it might be advisable to monitor the concentrations of 1*H*-indole rather after test roasting and not in the fermented but unroasted cocoa.

The concentrations of caramel-like, musty smelling MDMF significantly decreased in MOF2 and increased in MOF1 and REF. Higher concentrations after conching were also reported for the structurally similar Furaneol®.[89] Accordingly, the increasing concentrations of MDMF in MOF1 and REF could be explained by a formation from precursors present in the cocoa beans.[122] The instability of Furaneol® could possibly also apply to MDMF and thus explain the decreasing concentrations in MOF2.[123-125] However, the concentrations stayed below the relatively high OTV and thus, there seems to be no need for monitoring MDMF.

Results and Discussion

Table 9: Changes in the concentrations and in the odor activity values (OAVs) of crucial off-flavor compounds in two samples with smoky off-flavor (SOF1, SOF2) and a reference sample without off-flavor (REF) caused by roasting at 110, 125, and 140 °C, and processing into cocoa liquor

Sample		2-Methoxyphenol		4-Methylphenol		3-Methylphenol		4-Ethylphenol		3-Ethylphenol		3-Propylphenol	
		Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV
SOF1	Before[a]	815	450	527	160	635	33	275	12	143	65	21.3	11
	110°C	871	480	476	140	575	30	320	14	179	81	28.4	14
	125°C	886	490	450	140	517	27	308	13	158	72	21.9	11
	140°C	1050	580	519	160	611	32	368	16	191	87	28.1	14
SOF2	Before[a]	354	200	171	52	346	18	205	8.9	71.9	33	17.5	8.8
	110°C	393	220	157	48	312	16	236	10	96.7	44	14.3	7.1
	125°C	488	270	176	53	329	17	209	9.1	79.8	36	6.93	3.5
	140°C	445	250	170	51	302	16	249	11	84.2	38	5.56	2.8
REF	Before[a]	71.0	39	19.1	5.8	5.82	<1	11.9	<1	0.379	<1	0.255	<1
	110°C	57.7	32	16.0	4.9	4.97	<1	35.1	1.5	2.17	1.0	0.264	<1
	125°C	67.5	38	16.6	5.0	4.39	<1	45.7	2.0	5.98	2.7	0.347	<1
	140°C	65.9	37	21.5	6.5	6.45	<1	64.5	2.8	3.21	1.5	0.292	<1

[a]Data taken from Publication 1.[117] [b]Mean of duplicate or triplicate workups; individual concentrations and standard deviations of the samples roasted at 110, 125, and 140 °C are available in the Supplementary Information file of Publication 2.[120]

Results and Discussion

Table 10: Changes in the concentrations and in the odor activity values (OAVs) of crucial off-flavor compounds in two samples with moldy-musty off-flavor (MOF1, MOF2) and a reference sample without off-flavor (REF) caused by roasting at 110, 125, and 140 °C, and processing into cocoa liquor

Sample		(−)-Geosmin		3-Methyl-1H-indole		1H-Indole		MDMF	
		Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV	Conc. (µg/kg)[b]	OAV
MOF1	Before[a]	3.54	2.2	2.07	1.8	8.10	<1	14.9	<1
	110°C	6.26	3.9	2.39	2.2	24.7	<1	26.4	<1
	125°C	6.19	3.9	<1.0	<1	67.5	1.3	38.2	<1
	140°C	2.43	1.5	<1.0	<1	84.2	1.7	38.4	<1
MOF2	Before[a]	<0.2	<1	66.4	60	5.46	<1	226	<1
	110°C	<0.2	<1	8.59	7.8	102	2.0	3.64	<1
	125°C	<0.2	<1	1.65	1.5	248	4.9	3.68	<1
	140°C	<0.2	<1	2.47	2.2	406	8.0	2.52	<1
REF	Before[a]	<0.2	<1	<1.0	<1	<1.0	<1	<1.0	<1
	110°C	<0.2	<1	<1.0	<1	47.0	<1	1.32	<1
	125°C	<0.2	<1	<1.0	<1	82.7	1.6	1.45	<1
	140°C	<0.2	<1	<1.0	<1	96.2	1.9	1.62	<1

[a]Data taken from Porcelli, C.; Neiens, S. D.; Steinhaus, M. Molecular background of a moldy-musty off-flavor in cocoa. *J. Agric. Food Chem.* **2021**, *69*, 4501–4508.[98] [b]Mean of duplicate or triplicate workups; individual concentrations and standard deviations of the samples roasted at 110, 125, and 140 °C are available in the Supplementary Information file of Publication 2.[120]

6.7 Impact of Processing into Chocolate Mass and Conching on the Concentrations of Off-Flavor Compounds

Two samples of a cocoa liquor with a characteristic aroma and no off-flavor were spiked with the ten previously identified odorants. One spiked cocoa liquor sample was processed into a dark chocolate mass and the other sample was processed into a milk chocolate mass.

Spiking was achieved by adding exact amounts of the off-flavor compounds dissolved in ethanol to portions of molten cocoa liquor. The final concentrations in the spiked cocoa liquor resulted from the spiked amounts plus the amounts naturally present in the cocoa liquor. Spiked amounts were derived from the concentrations previously determined in the cocoa off-flavor samples.[98,117] The final concentrations ranged between ~5.1 µg/kg for (−)-geosmin and ~900 µg/kg for 2-methoxyphenol. The portions of spiked cocoa liquor were used to make the dark chocolate and the milk chocolate mass. Both chocolate masses were conched at a temperature of 80 °C for 12 h and subsequently moulded into chocolate. Quantitation of the off-flavor compounds in the chocolate was conducted as previously described for the cocoa liquor samples.[120]

The smoky off-flavor compounds revealed recoveries of 37−70% in the dark chocolate mass and 41−74% in the milk chocolate mass. For these compounds, no significant differences between dark and milk chocolate mass were obtained. The recoveries of 4-methylphenol, 4-ethylphenol, and 3-ethylphenol ranged from 66 to 74% in both chocolate masses. Somewhat lower recoveries between 51 and 63% were obtained for 3-methylphenol and 3-propylphenol. The lowest recoveries of 37% in the dark chocolate mass and 41% in the milk chocolate mass were obtained for 2-methoxyphenol. In contrast, an increase of 2-methoxyphenol was reported after conching of two different chocolate samples in another study.[89] However, these experiments were conducted at much lower concentration levels, which probably contributed to the different results. In summary, the concentrations of the smoky off-flavor compounds consistently decreased during processing into chocolate mass and conching, which compensated for the minor increases during roasting and processing into cocoa liquor (cf. section 6.6). Thus, there was no need to adjust the maximum tolerable concentrations previously suggested (cf. section 6.5).[120]

Quantitation of the off-flavor compounds potentially contributing to the moldy-musty off-flavors revealed recoveries between 79 and 82% for (−)-geosmin and 3-methyl-1*H*-indole in both chocolate masses. 1*H*-Indole showed higher recoveries of 101% in the dark chocolate mass and 93% in the milk chocolate mass. The recoveries of MDMF with 9% and 11% were low. In summary, the maximum tolerable concentrations of 3-methyl-1*H*-indole in fermented cocoa might thus be raised at least when roasting is conducted at higher temperatures. Due to the clear increase during roasting that was not compensated by losses during processing into chocolate mass and conching, the incoming goods inspection should include test roasting before the analysis of 1*H*-indole. MDMF does not have to be considered due to its high OTV and its significant decrease during processing into chocolate mass and conching.[120]

6.8 Conclusion

In summary, this study revealed 2-methoxyphenol, 4-methylphenol, 3-methylphenol, 4-ethyl-phenol, 3-ethylphenol, and 3-propylphenol as causative for the smoky off-flavor in cocoa. Based on their OTVs and their higher concentrations in the off-flavor samples than in the reference samples, maximum tolerable concentrations for these compounds were suggested as shown in Figure 18 together with the maximum tolerable concentrations of three major off-flavor compounds identified in cocoa with moldy-musty off-flavor.

For the application of the maximum tolerable concentrations in the incoming goods inspection of the chocolate industry, the high variance of the individual odor detection thresholds and the changes of the odorant concentrations during processing need to be considered. The changes during processing were of little relevance for the compounds identified in the smoky off-flavor samples, because minor increases during roasting and processing into cocoa liquor were compensated by losses during processing into chocolate mass and conching. In contrast, 3-methyl-1H-indole contributing to the moldy-musty off-flavor substantially decreased after roasting at higher temperatures and processing into cocoa liquor, whereas 1H-indole signifi-cantly increased. Thus, in the incoming goods inspection of the cocoa industry, it is suggested to apply test roasting using the company-specific roasting parameters to fully evaluate the off-flavor potential of 3-methyl-1H-indole and 1H-indole. Finally, based on the results of this study, a decision can then be made whether to use or to reject the cocoa beans.

3-Ethylphenol
(2 µg/kg)

3-Propylphenol
(2 µg/kg)

4-Methylphenol
(20 µg/kg)

3-Methylphenol
(20 µg/kg)

4-Ethylphenol
(20 µg/kg)

2-Methoxyphenol
(70 µg/kg)

3-Methyl-1H-indole
(1.1 µg/kg)

(−)-Geosmin
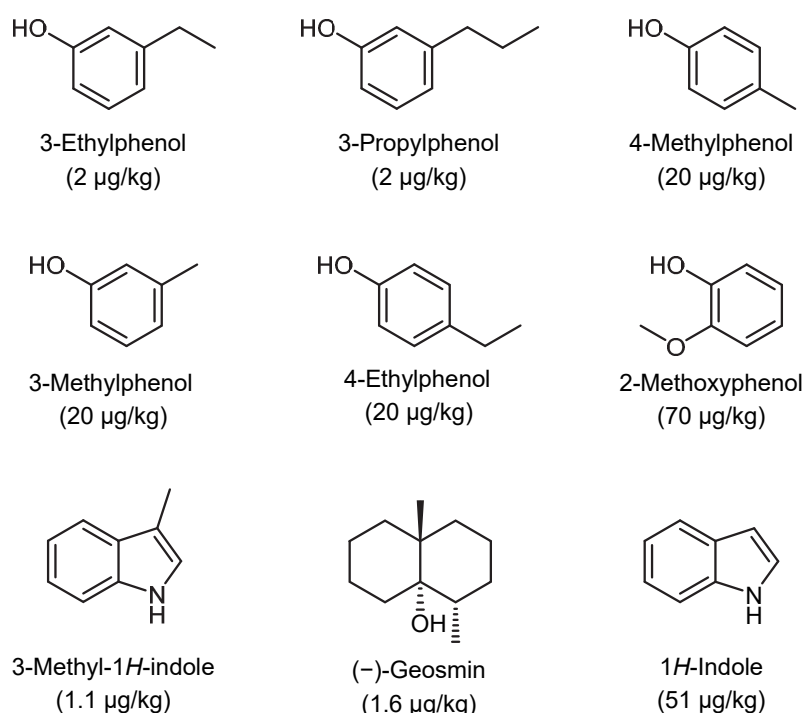(1.6 µg/kg)

1H-Indole
(51 µg/kg)

Figure 18: Suggested maximum tolerable concentrations applicable in the incoming goods inspection in the cocoa industry

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8 Appendix

8.1 Publication 1: Characterization of Odorants Causing Smoky Off-Flavors in Cocoa

8.1.1 Bibliographic Data

Title:	Characterization of Odorants Causing Smoky Off-Flavors in Cocoa
Authors:	Daniela Füllemann, Martin Steinhaus
Journal:	Journal of Agricultural and Food Chemistry
Publisher:	American Chemical Society
Publication date:	September 1, 2020 (online)
Issue date:	September 30, 2020
Volume:	68
Issue:	39
Pages:	10833–10841
DOI:	https://doi.org/10.1021/acs.jafc.0c04633
Hyperlink:	https://pubs.acs.org/doi/abs/10.1021/acs.jafc.0c04633

8.1.2 Publication Reprint

For a reprint of publication 1, please turn to the next page.

Article

Characterization of Odorants Causing Smoky Off-Flavors in Cocoa

Daniela Füllemann and Martin Steinhaus*

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ABSTRACT: Application of an aroma extract dilution analysis in parallel applied to the volatiles isolated from a sample of fermented cocoa with a pronounced smoky off-flavor and a reference sample with flawless aroma revealed seven potential off-flavor compounds with smoky/phenolic odor qualities and higher flavor dilution factors in the off-flavor sample than in the reference sample. These compounds were 2-methoxyphenol, 3- and 4-methylphenol, 3- and 4-ethylphenol, and 3- and 4-propylphenol. Their quantitation in seven off-flavor samples and two reference samples without off-flavor showed that 4-propylphenol did not exceed its odor threshold value. From the concentrations obtained for the other six compounds and their odor threshold values in deodorized cocoa butter, maximum tolerable concentrations were derived for the incoming goods inspection in the chocolate industry. The suggested limits are 2 μg/kg for 3-ethylphenol and 3-propylphenol, 20 μg/kg for 4-methylphenol, 3-methylphenol, and 4-ethylphenol, and 70 μg/kg for 2-methoxyphenol.

KEYWORDS: *Theobroma cacao, aroma extract dilution analysis, 2-methoxyphenol, 4-methylphenol, 3-ethylphenol, 3-propylphenol, stable isotopically substituted odorants*

INTRODUCTION

The seeds of the cocoa tree *Theobroma cacao* L., referred to as cocoa beans, are the key raw material for the production of chocolate. After harvest, the fruits are opened and both the seeds and the pulp are fermented in heaps or boxes for two to 10 days.[1] During the fermentation, the pulp liquefies and runs off. The seeds are dried and traded as "fermented cocoa" at an annual amount of more than 4.5 million metric tons.[2] To process the fermented cocoa seeds to chocolate, the whole beans or the beans crushed into cocoa nibs are roasted and milled to obtain cocoa liquor. By pressing, a part of the cocoa butter can be separated. The pressing residue can be used to make cocoa powder. Cocoa liquor is processed into chocolate mass by the addition of sugar, cocoa butter, and if applicable, other ingredients such as milk powder. The mixture undergoes further milling and finally a conching process that leads to a homogeneous final product.

A major factor for the popularity of chocolate is its pleasant aroma, which is mainly derived from the cocoa seeds. Whereas fresh cocoa seeds exhibit little aroma, the aroma develops during fermentation and roasting.[3] Fermentation is crucial for the direct formation of some odor-active compounds but, more importantly, for the formation of thermolabile precursors that are converted to major chocolate odorants during the subsequent roasting process.[4−6]

The cocoa-derived compounds contributing to the pleasant aroma of chocolate have been studied in detail and include, e.g., honey-like smelling phenylacetaldehyde, sweet-malty smelling 2- and 3-methylbutanal, fruity smelling ethyl 2-methylbutanoate and ethyl 2-methylpropanoate, earthy smelling 2,3-diethyl-5-methylpyrazine, vinegar-like smelling acetic acid, and caramel-like smelling 4-hydroxy-2,5-dimethylfuran-3(2H)-one.[7−11] Sometimes, however, fermented cocoa is tainted with off-flavors that may be transferred to the final product and lead to

consumers rejecting the chocolate. Such off-flavors in fermented cocoa include smoky off-flavors, moldy off-flavors, fecal off-flavors, and mushroom-like off-flavors.[12−15] The most prevalently reported off-flavor in fermented cocoa, however, is the smoky off-flavor. Smoky odor notes are typically observed after smoke contact during inappropriate artificial drying by using wood fires[12,16−19] but may also be caused by overfermentation.[19]

Lehrian et al. showed that wood smoke contamination during cocoa drying led to smoky/hammy off-flavors associated with high contents of phenolic compounds.[16] These were quantitated as sum by photometry but no efforts were made to identify individual compounds and elucidate their odor contribution. Ney differentiated between smoky off-flavors, on the one hand, and hammy off-flavors, on the other hand.[12] He suggested 2,6-dimethoxyphenol as the crucial compound for the smoky type of off-flavor and short-chain carboxylic acids like propanoic, butanoic, pentanoic, and 3-methylbutanoic acid as causative for the hammy off-flavor. However, among smoky smelling phenolic compounds, 2,6-dimethoxyphenol shows a comparatively high odor threshold (29 μg/kg).[20] For example, the odor threshold value of 2-methoxyphenol, the most prevalent smoky smelling compound in food, is significantly lower (0.84 μg/kg).[20] Furthermore, the short-chain carboxylic acids do not show a hammy odor, but sweaty and cheesy notes,[20] thus making Ney's suggestions highly questionable. Serra Bonvehí and Ventura Coll compared correctly processed cocoa samples to
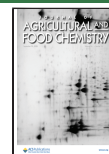
samples that were poorly smoked and samples that were strongly contaminated with direct wood smoke on the basis of the concentrations of nine selected phenolic compounds.[17] Multivariate statistical analysis revealed five compounds with significant differences, for which maximum tolerable concentrations were suggested: phenol (2 mg/kg), 3-methylphenol (0.9 mg/kg), 2,3-dimethylphenol (0.55 mg/kg), 3-ethylphenol (0.90 mg/kg), and 4-ethylphenol (0.70 mg/kg). However, the suggested maximum tolerable concentrations were not verified by sensory tests with individual compounds. A study by Rodriguez-Campos et al. attributed smoky off-flavors in cocoa caused by overfermentation to 2-methoxyphenol, which showed a clear increase with extended fermentation time.[19] Perotti et al. compared cocoa samples with and without smoky off-flavor by GC × GC-TOF-MS analysis.[18] Statistical data evaluation revealed naphthalene, 2-methoxyphenol, 2-methoxy-4-methylphenol, phenol, 4-ethyl-2-methoxyphenol, and 4-methylphenol as marker compounds associated with the smoky note. Whether these compounds actually contributed to the off-flavor was not investigated. On the basis of concentration data acquired by HS-SPME-GC-MS, Perotti et al. suggested an operative limit at the incoming goods inspection of 10 μg/kg for each of the compounds,[18] despite the fact that their odor threshold values differ widely.[20]

Summarizing the literature overview, the importance of phenolic compounds for the smoky off-flavor in fermented cocoa is unquestionable. However, it is still unclear to what extent the individual phenols contribute to the off-flavor. Furthermore, data show a clear controversy on the maximum concentration levels at which the individual compounds can be considered tolerable. For example, the limits suggested by Serra Bonvehí and Ventura Coll[17] are 55−200 times higher than the general operative limit recommended by Perotti et al.[18]

Thus, the aims of the present study were (1) to reinvestigate the odor-active compounds potentially contributing to smoky notes by using gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA) in parallel applied to a sample of fermented cocoa with a pronounced off-flavor and to a sample with flawless aroma characteristics, (2) to substantiate the role of the individual odorants by quantitation and calculation of odor activity values (OAVs), and finally (3) to suggest maximum tolerable concentrations for the incoming goods inspection in the chocolate industry on the basis of sensory threshold values.

■ MATERIALS AND METHODS

Samples. Fermented cocoa beans with (7 samples) and without (2 samples) smoky off-flavor were provided by German chocolate manufacturers. Using an expert panel of seven trained panelists, off-flavor samples were numbered according to the intensity of the smoky note from OF1 (extremely intense) to OF7 (moderate). Among the reference samples without off-flavor, REF1 was characterized by an intense and very pleasant cocoa aroma, whereas the aroma of sample REF2 was rather weak.

Reference Odorants. 1−2, 4−7, 9, 12−13, 15, 17−32, and 36−46 were obtained from Merck (Darmstadt, Germany) and 11, 16, and 33 were purchased from Alfa Aesar (Karlsruhe, Germany). Compounds 14[21] and 34[22] were synthesized as detailed in the literature.

Stable Isotopically Substituted Odorants. ($^2H_7$)-38 was purchased from Merck. ($^2H_3$)-30 was synthesized according to the literature.[23] ($^2H_2$)-41 and ($^2H_2$)-44a were synthesized as detailed below.

Miscellaneous Chemicals and Materials. Dichloromethane and diethyl ether were obtained from CLN (Freising, Germany). Deuterium chloride solution (35% in deuterium oxide), methyl

octanoate, and tetrahydrofuran were purchased from Merck. Deuterium oxide (99.96% deuterium), 1-(4-hydroxyphenyl)ethan-1-one, 1-(4-hydroxyphenyl)propan-1-one, pentane, and silica gel (0.040−0.63 mm) were purchased from VWR (Darmstadt, Germany). Before use, silica gel was washed with hydrochloric acid as detailed previously[24] and dichloromethane, diethyl ether, and pentane were freshly distilled through a column (120 × 5 cm) filled with Raschig rings. Deodorized cocoa butter was obtained from Cargill (Berlin, Germany).

Syntheses. *4-(1,2-$^2H_2$)Ethylphenol.* Zinc powder (6 g) was stirred (5 min) with a mixture of concentrated hydrochloric acid (0.3 mL) and deionized water (7.5 mL). The activated zinc powder was collected with a Büchner funnel, washed with deionized water (50 mL), and dried in a stream of nitrogen. The following reagents were added to the activated zinc powder under an argon atmosphere and continuous stirring at room temperature: deuterium oxide (4 mL), deuterium chloride solution (9 mL), and 1-(4-hydroxyphenyl)ethan-1-one (1.91 g, 14 mmol) dissolved in anhydrous tetrahydrofuran (5 mL). The reaction mixture was further stirred for 24 h. Three more portions of deuterium chloride solution (3 mL) were added after 4, 8, and 18 h. The reaction products were extracted by shaking the reaction mixture with aqueous sodium carbonate solution (0.5 mol/L, 3 portions, 100 mL total volume). The combined aqueous extracts were washed with dichloromethane (50 mL) and then acidified (pH 2) with hydrochloric acid (5 mol/L). The reaction product was re-extracted with dichloromethane (3 portions, 100 mL total volume), the combined organic phases were dried over anhydrous sodium sulfate, filtered, and the filtrate was made up to a final volume of 100 mL with dichloromethane. GC-FID (internal standard methyl octanoate; response factor 0.8540) revealed 389 mg 4-(1,2-$^2H_2$)ethylphenol corresponding to 22% yield and 93% purity. GC-MS confirmed the incorporation of two deuterium atoms but with some impurity of the trideuterated product. MS (EI): *m/z* (%) 109 (100), 124 (39), 110 (25), 125 (15), 108 (13), 78 (9), 79 (9). MS (CI): *m/z* (%) 125 (100), 126 (45), 127 (9), 124 (4).

4-(1,2-$^2H_2$)Propylphenol. The synthesis was performed according to the approach detailed above for 4-(1,2-$^2H_2$)ethylphenol using 1-(4-hydroxyphenyl)propan-1-one (2.10 g, 14 mmol) as an educt. GC-FID (internal standard methyl octanoate; response factor 0.8831) revealed 1143 mg 4-(1,2-$^2H_2$)propylphenol corresponding to 59% yield and 96% purity. GC-MS confirmed the incorporation of two deuterium atoms. MS (EI): *m/z* (%) 109 (100), 138 (33), 108 (19), 110 (11), 79 (7), 78 (7), 137 (5). MS (CI): *m/z* (%) 139 (100), 138 (15), 140 (12), 133 (8), 89 (7).

GC-O/FID. A gas chromatograph 8000 Series (Fisons Instruments, Mainz, Germany) was equipped with a cold on-column injector, a flame ionization detector (FID), and a custom-made sniffing port.[25] The column was either a DB-FFAP or a DB-5, both 30 m × 0.25 mm i.d., 0.25 μm film thickness (Agilent Technologies, Waldbronn, Germany). The carrier gas was helium at a constant flow of 1.1 mL/min. The column ended in a Y-shaped glass splitter conveying the effluent via two deactivated fused silica capillaries (50 cm × 0.25 mm i.d.) simultaneously to the FID (250 °C base temperature) and the sniffing port (230 °C base temperature). Injection volume was 1 μL. The oven temperature was 40 °C for 2 min then increased by 6 °C/min to 230 °C (DB-FFAP) and 240 °C (DB-5). The final values were held for 5 min. The FID chromatograms were plotted by a recorder. A trained assessor who placed the nose directly above the sniffing port carried out the GC-O. The assessor marked each odor-active region in the chromatogram and noted the odor. Retention indices (RI) were calculated by linear interpolation from the retention times of the odor-active regions and the retention times of adjacent *n*-alkanes.[26]

Gas Chromatography−Mass Spectrometry (GC-MS). A HP 5890 gas chromatograph (Hewlett-Packard, Heilbronn, Germany) was connected to a MAT 95 sector field mass spectrometer (Finnigan, Bremen, Germany). The system was equipped with a cold on-column injector. The carrier gas was helium at a constant flow of 1.9 mL/min. The installed columns and applied oven programs equaled those used in the GC-O/FID approach. The injection volume was 0.5 μL. Mass spectra in the electron ionization (EI) mode were obtained at 70 eV

with a scan range of m/z 30−300. Mass spectra in the chemical ionization (CI) mode were generated at 150 eV with isobutane as reagent gas and a scan range of m/z 85−350. Data were evaluated using the Xcalibur software (Thermo Fisher Scientific, Dreieich, Germany).

Heart-Cut GC-GC-MS. A Trace Gas Chromatograph Ultra (Thermo Fisher Scientific) was equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a cold on-column injector. The carrier gas was helium at a constant pressure of 110 kPa. The injection volume was 2 μL. The oven temperature was 40 °C for 2 min, then increased by 6−40 °C/min to 230−240 °C. The final values were held for 5 min. The column was either a DB-FFAP or a DB-5, both 30 m × 0.32 mm i.d. and 0.25 μm film thickness (Agilent Technologies). The column end was connected to a moving column stream switching (MCSS) system (Thermo Fisher Scientific). The MCSS system transferred the column effluent time-programmed via deactivated fused silica capillaries (0.32 mm i.d.) either simultaneously to a custom-made sniffing port (230 °C base temperature) and an FID (250 °C base temperature) or to a liquid nitrogen-cooled trap inside the oven of a second gas chromatograph (250 °C transfer line temperature). The second gas chromatograph was a CP 3800 (Varian, Darmstadt, Germany) equipped with either a DB-1701 or a DB-FFAP column, both 30 m × 0.25 mm i.d. and 0.25 μm film thickness (Agilent Technologies). The oven temperature was 40 °C for 2 min and then increased by 6−20 °C/min to 230 °C. The final value was held for 5 min. The end of the second GC column was connected to a Saturn 2200 mass spectrometer (Varian), which was operated in the CI mode with methanol as the reagent gas and a scan range of m/z 60−250. Data were evaluated using the MS workstation software (Varian).

Heart-Cut GC-GC-HRMS. A Trace 1310 gas chromatrograph (Thermo Fisher Scientific) was equipped with a TriPlus RSH autosampler (Thermo Fisher Scientific) and a programmed temperature vaporizing (PTV) injector. The carrier gas was helium at a constant flow of 1 mL/min. The injection volume was 1 μL. The oven temperature was 40 °C for 2 min, then increased by 6 °C/min to 240 °C. The final value was held for 10 min. The column was a DB-FFAP, 30 m × 0.32 mm i.d., and 0.25 μm film thickness (Agilent Technologies). The column end was connected to a Deans Switch (Thermo Fisher Scientific). The switch transferred the column effluent time-programmed via deactivated fused silica capillaries (0.32 mm i.d.) either simultaneously to a custom-made sniffing port (230 °C base temperature) and an FID (250 °C base temperature) or to a liquid nitrogen-cooled trap (250 °C transfer line temperature). The trap was connected to the second Trace 1310 gas chromatograph (Thermo Fisher Scientific), which was equipped with a BGB-176 column, 30 m × 0.25 mm i.d., and 0.25 μm film thickness (BGB Analytik, Rheinfelden, Germany). The oven temperature was 40 °C for 2 min, then increased by 2 °C/min to 200 °C. The final value was held for 5 min. The end of the second GC column was connected to a Q Exactive GC Orbitrap mass spectrometer (Thermo Fisher Scientific) operated in CI and high-resolution mode with isobutane as reagent gas. Data were evaluated using the Xcalibur software (Thermo Fisher Scientific).

Isolation of Cocoa Volatiles. Fermented cocoa beans were frozen with liquid nitrogen and ground into a coarse granulate by using a laboratory mill (Retsch, Haan, Germany) at 3800 rpm (3 × 5 s). The granulate was further ground into a fine powder by using a 6875 Freezer Mill (SPEX SamplePrep, Stanmore, UK). Dichloromethane (150 mL) was added to cocoa powder (50 g), and the mixture was vigorously stirred with a magnetic stirrer at room temperature for 16 h. The mixture was filtered through a folded paper filter, and the residue was further extracted with dichloromethane (150 mL) for 1 h. After filtration, the organic phases were combined, dried over anhydrous sodium sulfate, and the nonvolatiles were removed by SAFE at 40 °C.[27] The distillates, when tested on a fragrance test strip after evaporation of the solvent, fully represented the typical aroma of the samples. Particularly, the smoky note in the off-flavor sample distillates was clearly perceivable. The volatiles were fractionated by acid−base extraction. In a separating funnel, the SAFE distillate was shaken with three portions of an aqueous sodium carbonate solution (0.5 mol/L, 200 mL total volume). The aqueous extracts containing the acidic volatiles were combined. The organic phase containing the neutral and

basic volatiles was washed with saturated sodium chloride solution (50 mL), dried over sodium sulfate, and concentrated (1 mL) by using a Vigreux column (50 × 1 cm) and a Bemelmans microdistillation device (fraction NBV).[28] The aqueous phase containing the acidic volatiles was washed with dichloromethane (50 mL), acidified (pH 2) with hydrochloric acid (5 mol/L), and re-extracted with three portions of dichloromethane (150 mL total volume). The combined organic phases were washed with saturated sodium chloride solution (50 mL), dried over anhydrous sodium sulfate, and concentrated to 1 mL (fraction AV).

AEDA. The fractions NBV and AV were analyzed by GC-O using the FFAP column. Three trained and experienced assessors (2 female, 1 male, aged 26−48) carried out the analyses. GC-O runs were repeated until the results were reproducible. Then, fractions NBV and AV were stepwise diluted 1:2 with dichloromethane to obtain 1:2, 1:4, 1:8, 1:16, ..., and 1:8192 dilutions of the initial solutions and each diluted sample was analyzed by GC-O. 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 perceived by any of the three panelists.

Silica Gel Liquid Chromatography. Hexane (1 mL) was added to the fraction NBV, and the mixture was reconcentrated to 1 mL by microdistillation.[28] The concentrate was applied onto a slurry of purified silica gel (8 g) and pentane in a water-cooled (12 °C) glass column (1 cm i.d.). Elution was performed with pentane (50 mL), pentane/diethyl ether mixtures of 90 + 10, 70 + 30, 50 + 50 ($v + v$; 50 mL each), and diethyl ether (50 mL). The eluate was collected in five portions of 50 mL and each portion was concentrated to a final volume of 0.5 mL (subfractions NBV1−NBV5). The odorants previously detected in fraction NBV were localized in the individual subfractions by GC-O before fractions NBV1−NBV5 were subjected to GC-MS analysis.

Odorant Quantitation. For the quantitation of odorants **30**, **38**, **39**, **41**, **42**, **44a**, and **44b**, dichloromethane (25−100 mL) was added to fermented cocoa bean powder (1−10 g). The mixture was spiked with stable isotopically substituted odorants as internal standards (0.1−0.4 μg in < 1 mL dichloromethane). After magnetic stirring (14−16 h) at room temperature, the mixture was filtered and the filtrate was dried over anhydrous sodium sulfate. Nonvolatiles were removed by SAFE. The distillate was dried over anhydrous sodium sulfate and concentrated to a final volume of 200 μL. Aliquots of the concentrates were analyzed using the heart-cut GC-GC-MS system (**30**, **38**, **39**, **41**, and **42**) or the heart-cut GC-GC-HRMS system (**44a** and **44b**). Peak areas corresponding to the analytes and the internal standards were obtained from the extracted ion chromatograms of characteristic quantifier ions. From the area counts of the analyte peak, the area counts of the standard peak, the amount of standard added, and the amount of starting material, the concentration of each odorant in the cocoa samples was calculated by employing a calibration line equation. The calibration line equation was obtained from the analysis of analyte/standard mixtures in five different concentration ratios (5:1, 2:1, 1:1, 1:2, and 1:5). Individual calibration line equations and quantifier ions are available in the Supporting Information.

Determination of Odor Threshold Values. Orthonasal odor threshold values were determined in deodorized cocoa butter according to the American Society for Testing and Materials (ASTM) standard practice for determination of odor and taste thresholds by a forced-choice ascending concentration series method of limits.[29] Odorant concentrations were increased 3-fold between two consecutive three-alternative forced-choice (3-AFC) tests in the series. Individual test samples were prepared by placing portions (10 g) of the cocoa butter, previously melted at 38 °C, in cylindrical polytetrafluoroethylene vessels (height 5.7 cm, i.d. 3.5 cm) with lids and adding the odorant (31 pg−2 mg) dissolved in ethanol (10−20 μL). Reference samples were prepared by adding the same amount of pure ethanol. Tests were performed at a 32 °C sample temperature and 22 ± 2 °C room temperature in a specially designed sensory room with separated booths. The sensory panel consisted of 12−23 trained panelists.

Table 1. Odorants in the SAFE Distillates Obtained from Fermented Cocoa Beans: Reference Sample without Off-Flavor (REF) vs Off-Flavor Sample with Pronounced Smoky Odor (OF)

no.	odorant[a]	odor[b]	RI[c] FFAP	RI[c] DB-5	FD factor[d] REF	FD factor[d] OF
1	ethyl 2-methylpropanoate	fruity	975	759	128	128
2	butane-2,3-dione[e]	buttery	985	<700	128	64
3	unknown	fruity	1017	773	16	4
4	ethyl butanoate	fruity	1028	802	16	512
5	ethyl 2-methylbutanoate	fruity	1053	850	256	<1
6	ethyl 3-methylbutanoate	fruity	1062	855	512	512
7	3-methylbutyl acetate	fruity, banana	1119	876	16	8
8	unknown	fruity	1182	-	32	2
9	2-/3-methylbutan-1-ol	malty	1202	735	8	16
10	unknown	fruity	1257	-	32	32
11	3-hydroxybutan-2-one	buttery, nutty	1286	715	4	64
12	oct-1-en-3-one[e]	mushroom	1297	979	4	8
13	dimethyl trisulfide	sulfury, cabbage	1373	971	64	64
14	(3E,5Z)-undeca-1,3,5-triene[e]	fresh, pineapple	1387	1174	32	2
15	2,3,5-trimethylpyrazine	nutty, caramel	1402	1002	8	8
16	ethyl cyclohexanecarboxylate[e]	fruity, fresh	1414	1136	128	8
17	3-isopropyl-2-methoxypyrazine	earthy	1427	1095	32	64
18	acetic acid	vinegar, pungent	1450	<700	16	<1
19	3-(methylsulfanyl)propanal[e]	cooked potato	1454	905	32	8
20	2,3-diethyl-5-methylpyrazine	earthy	1488	1158	32	16
21	3-isobutyl-2-methoxypyrazine	bell pepper	1519	1183	256	512
22	2-methylpropanoic acid	cheesy	1564	786	16	<1
23	butanoic acid	cheesy	1625	814	32	8
24	phenylacetaldehyde	floral, honey	1650	1045	64	64
25	2-/3-methylbutanoic acid	sweaty	1661	868	512	64
26	2-methyl-3-(methylsulfanyl)furan[e]	meat	1665	1177	512	512
27	(2E,4E)-nona-2,4-dienal[e]	green, fatty	1705	1216	16	8
28	2-phenylethyl acetate	honey, floral	1811	1261	1024	256
29	geraniol	citrus, floral	1845	1259	8	<1
30	2-methoxyphenol	smoky, hammy	1861	1091	512	2048
31	ethyl 3-phenylpropanoate	floral, cinnamon	1884	1351	512	256
32	2-phenylethan-1-ol	floral	1913	1117	4096	1024
33	δ-octalactone	cinnamon	1970	1288	8	<1
34	trans-4,5-epoxy-(2E)-dec-2-enal[e]	metallic	2005	1381	4	256
35	unknown	cinnamon	2017	-	256	8
36	γ-nonalactone	coconut	2029	1367	4	256
37	4-hydroxy-2,5-dimethylfuran-3(2H)-one[e]	caramel	2033	1075	256	256
38	4-methylphenol	horse stable, phenolic	2085	1073	<1	64
39	3-methylphenol	smoky, phenolic	2088	1075	<1	256
40	γ-decalactone	peach	2150	1451	2048	512
41	4-ethylphenol	smoky	2190	1166	<1	4096
42	3-ethylphenol	smoky	2200	1168	<1	16
43	3-hydroxy-4,5-dimethylfuran-3(5H)-one[e]	seasoning	2203	1108	256	16
44	3-/4-propylphenol	smoky, phenolic	2269	1259	<1	2048
45	phenylacetic acid	honey, beeswax	2555	1258	128	4
46	vanillin	vanilla	2596	1407	256	8

[a]Odorants showing an FD factor of ≥8 in either of the two samples; structure assignments were based on the comparison of the retention indices on two columns of different polarity (DB-FFAP, DB-5), the odor quality perceived at the sniffing port during GC-O, and the mass spectra obtained by GC-MS analysis (CI and EI) with data of authentic reference substances analyzed under the same conditions. [b]Odor quality as perceived at the sniffing port during GC-O. [c]Retention index: calculated from the retention time of the odorant and the retention times of adjacent n-alkanes by linear interpolation. [d]Flavor dilution factor: dilution factor of the highest diluted sample obtained from concentrated fractions NBV or AV by serial dilution in which the odorant was detected during GC-O analysis by any of three panelists. [e]A clear mass spectrum could not be obtained in the cocoa volatile concentrates; identification was based on the remaining criteria detailed in footnote a.

■ RESULTS AND DISCUSSION

Screening for Potential Off-Flavor Compounds. The volatiles in parallel isolated from a sample of fermented cocoa with a pronounced smoky off-flavor and a sample of fermented cocoa with a characteristic, intense, and very pleasant aroma without any off-notes were separated into a fraction of acidic volatiles and a fraction of neutral and basic volatiles and then subjected to a comparative AEDA. Results revealed 46 odorous

regions in the chromatogram with FD factors ≥8 in at least one of the two cocoa samples (Table 1). Preliminary structure assignments were achieved by comparing the retention indices and the odor qualities with data from the literature and databases.[20,30] The results were verified by parallel GC-O analysis of authentic reference compounds and the cocoa volatile concentrates using two columns of different polarity (DB-FFAP and DB-5). Final structure confirmation was achieved by GC-MS. To avoid coelutions, the fractions of neutral and basic volatiles were further fractionated by silica gel liquid chromatography. GC-MS analyses of the fractions were performed in parallel to GC-MS analyses of reference compounds in the EI and CI mode by using the DB-FFAP as well as the DB-5 column.

With this approach, the structures of 42 out of 46 odorants were unequivocally assigned (1−2, 4−7, 9, 11−34, and 36−46). The compounds detected in the reference sample agreed well with the major odorants reported in previous studies on fermented cocoa.[9,10] The compounds potentially contributing to the smoky off-flavor were identified by smoky, hammy, and/or phenolic odor qualities plus an FD factor being clearly higher in the off-flavor sample than in the reference sample. This was the case for 2-methoxyphenol (30), 4-methylphenol (38), 3-methylphenol (39), 4-ethylphenol (41), 3-ethylphenol (42), as well as for 3-/4-propylphenol (44). Among these potential off-flavor compounds, smoky smelling 4-ethylphenol (41) showed the highest FD factor in the off-flavor sample, namely 4096. An FD factor of 2048 was assigned to smoky and hammy smelling 2-methoxyphenol (30) and phenolic smelling 3-/4-propylphenol (44). 3-Propylphenol (smoky, phenolic) and 4-propylphenol (phenolic) were not separated on the column used for AEDA (FFAP), thus resulting in a combined FD factor. Separation of the two propylphenol isomers was also unsuccessful on the DB-5 column but could be achieved by using a chiral BGB-176 column with a β-cyclodextrin-based stationary phase. Such chiral GC phases can not only be used for the differentiation of enantiomers but also often show superior separation of positional isomers.[31] To clarify whether the off-flavor cocoa sample contained 3-propylphenol, 4-propylphenol, or both, the fraction of acidic volatiles was analyzed in parallel to the reference compounds by GC-GC-HRMS using an FFAP column in the first dimension and the BGB-176 column in the second dimension. Results revealed the presence of both, 3-propylphenol (44b) and 4-propylphenol (44a) (Figure 1). For the other three potential off-flavor compounds, clearly lower FD factors were determined, namely 256 for smoky, phenolic smelling 3-methylphenol (39), 64 for horse stable-like and phenolic smelling 4-methylphenol (38), and 16 for smoky smelling 3-ethylphenol (42). Only one of the seven potential off-flavor compounds, namely 2-methoxyphenol (30), was also detected in the reference sample. Its FD factor in the reference sample, however, was lower than its FD factor in the off-flavor sample.

2-Methoxyphenol (30) has frequently been reported as cocoa odorant. It was found in odor-active amounts in flawless Criollo and Forastero cocoa beans before and after roasting.[9,10] 2-Methoxyphenol was also associated with overfermentation[19] and artificial wood-fire drying[19] and was suggested as a marker compound for smoky off-flavors in cocoa beans and liquors.[18] The latter also applies to 4-methylphenol (38).[18] 3-Methylphenol (39), 4-ethylphenol (41), and 3-ethylphenol (42) occurred in cocoa samples contaminated by wood smoke.[17]
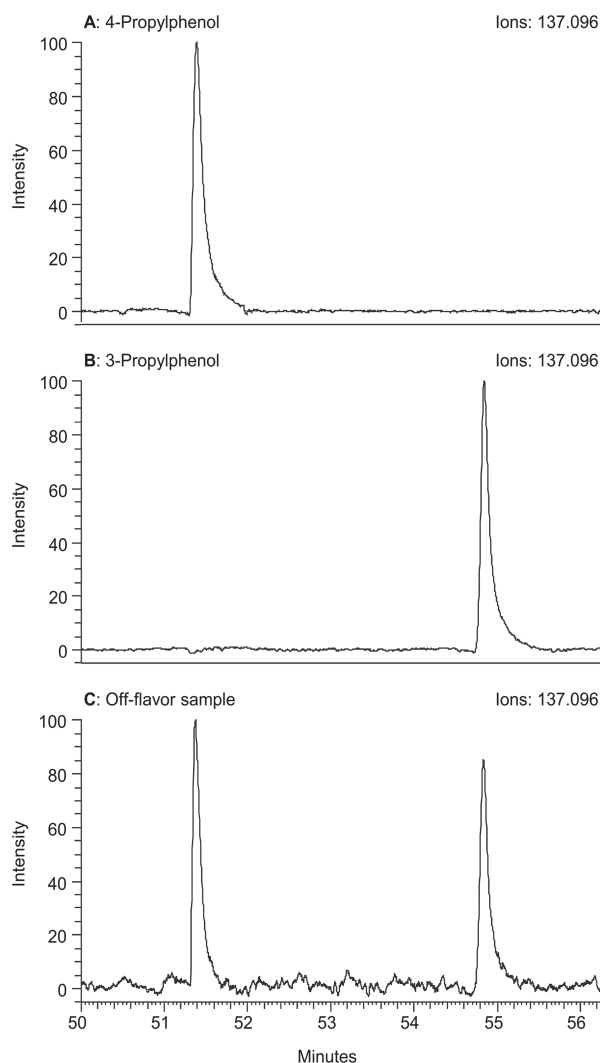

Figure 1. GC-GC-HRMS separation of 4-propylphenol and 3-propylphenol with a β-cyclodextrin modified stationary phase in the second dimension. Reference solutions of 4-propylphenol (A) and 3-propylphenol (B) compared to an extract obtained from a cocoa off-flavor sample (C).

The presence of 4-propylphenol (44a) and 3-propylphenol (44b) has not been reported in cocoa before.

The comparative screening for potential off-flavor compounds was extended to six additional fermented cocoa samples with a smoky off-flavor (data not shown). For all six samples, the results revealed the same set of potential off-flavor compounds (30, 38, 39, 41, 42, 44a, and 44b) as detected in the previous sample. In particular, no additional compound with smoky, hammy, and/or phenolic odor quality was detected.

Concentrations of Potential Off-Flavor Compounds. To substantiate the screening results, the potential off-flavor compounds were quantitated in the seven off-flavor samples (OF1−OF7) and in the reference sample (REF1) used in the screening experiments. The off-flavor samples were numbered according to the intensity of the smoky off-note, with OF1 showing the highest and OF7 the lowest intensity. A second sample without smoky off-flavor (REF2) was additionally included in the quantitations. The aroma of this second reference sample was weaker than the aroma of REF1 but did

Table 2. Concentrations of Potential Off-Flavor Compounds in Seven Fermented Cocoa Samples with Smoky Off-Flavor (OF1–OF7) and Two Fermented Cocoa Samples without Off-Flavor (REF1, REF2)

		concentration (µg/kg)[a]								
odorant	odor	OF1[b]	OF2[b]	OF3[b]	OF4[b,c]	OF5[b]	OF6[b]	OF7[b]	REF1[c]	REF2
2-methoxyphenol	smoky, hammy	815	431	552	354	632	610	120	71.0	65.0
4-methylphenol	horse stable, phenolic	527	196	291	171	352	209	56.9	19.1	5.15
3-methylphenol	smoky, phenolic	635	430	443	346	435	388	80.1	5.82	1.06
4-ethylphenol	smoky	275	352	233	205	447	147	95.4	11.9	2.49
3-ethylphenol	smoky	143	103	83.2	71.9	80.9	63.4	12.0	0.379	1.63
4-propylphenol	phenolic	13.5	9.25	6.43	9.93	11.9	3.35	0.573	0.893	0.328
3-propylphenol	smoky, phenolic	21.3	19.4	15.8	17.5	17.8	9.80	2.47	0.255	0.210

[a]Mean of duplicates or triplicates; standard deviations were <20%; individual values and standard deviations are provided in the Supporting Information. [b]Off-flavor samples are listed in order of decreasing off-flavor intensity from extremely intense (OF1) via intense (OF2–OF6) to moderate (OF7). [c]Samples OF4 and REF1 were the samples previously used for odorant screening by AEDA (cf. Table 1).

Table 3. Odor Activity Values (OAVs) of Potential Off-Flavor Compounds in Seven Fermented Cocoa Samples with Smoky Off-Flavor (OF1–OF7) and Two Fermented Cocoa Samples without Off-Flavor (REF1, REF2)

		odor activity value (OAV)[b]								
odorant	odor threshold value[a]	OF1[c]	OF2[c]	OF3[c]	OF4[c,d]	OF5[c]	OF6[c]	OF7[c]	REF1[d]	REF2
2-methoxyphenol	1.8	450	240	310	200	350	340	67	39	36
4-methylphenol	3.3	160	60	88	52	110	63	17	5.8	1.6
3-methylphenol	19	33	23	23	18	23	20	4.2	<1	<1
4-ethylphenol	23	12	15	10	8.9	19	6.4	4.1	<1	<1
3-ethylphenol	2.2	65	47	38	33	37	29	5.5	<1	<1
4-propylphenol	3700	<1	<1	<1	<1	<1	<1	<1	<1	<1
3-propylphenol	2.0	11	10	7.9	8.8	8.9	4.9	1.2	<1	<1

[a]Odor threshold value determined in deodorized cocoa butter. [b]Odor activity values were calculated as concentration/odor threshold value. [c,d]Cf. Table 2, footnotes b and c.

not show any off-note either. To compensate for losses during the workup, stable isotopically substituted odorants were used as internal standards.

The results (Table 2) revealed odorant concentrations ranging from 0.210 µg/kg (3-propylphenol in REF2) to 815 µg/kg (2-methoxyphenol in OF1). In the seven off-flavor samples, the concentrations of all potential off-flavor compounds were consistently higher than in the two reference samples (with the only exception of a low 4-propylphenol concentration in OF7).

Serra Bonvehí and Ventura Coll[17] reported clearly higher concentrations of major phenolic compounds. In uncontaminated cocoa, concentrations were 1750–3220 µg/kg for 2-methoxyphenol, 780–1370 µg/kg for 4-methylphenol, 330–770 µg/kg for 3-methylphenol, 370–610 µg/kg for 4-ethylphenol, and 280–710 µg/kg for 3-ethylphenol. In wood smoke contaminated cocoa, the concentrations were 3190–7710 µg/kg for 2-methoxyphenol, 1170–3070 µg/kg for 4-methylphenol, 870–2370 µg/kg for 3-methylphenol, 570–3170 µg/kg for 4-ethylphenol, and 710–4070 µg/kg for 3-ethylphenol. The high concentrations in the wood smoke contaminated cocoa samples can be ascribed to the artificial smoking process applied. However, it is noticeable that the concentrations of 2-methoxyphenol, 4-methylphenol, and 3-ethylphenol reported by Serra Bonvehí and Ventura Coll in the uncontaminated samples already exceeded the concentrations we found in the off-flavor samples. Artifact formation during the steam-distillation process applied by Serra Bonvehí and Ventura Coll for cocoa sample workup or coeluted compounds falsifying the GC-FID quantitations might account for that.

Rodriguez-Campos et al.[19] reported 2-methoxyphenol concentrations in fermented cocoa of 0 to ~1500 µg/kg. No

2-methoxyphenol could be detected in properly fermented and dried samples, whereas overfermentation led to concentrations of ~400 to ~1200 µg/kg. The highest concentration of ~1500 µg/kg was detected in a wood-fire dried sample.

Perotti et al.[18] focused on 2-methoxyphenol and 4-methylphenol, which they suggested as crucial marker compounds for a smoky off-flavor in cocoa. In cocoa without smoky off-flavor, they reported an average concentration of 8.2 µg/kg for 2-methoxyphenol, whereas we found 65 and 71 µg/kg. Our values were in the same range as the previously reported concentrations of 2-methoxyphenol in flawless Criollo and Forastero cocoa samples (110 µg/kg and 61 µg/kg, respectively).[9,10] For 4-methylphenol, Perotti et al. reported concentrations below their limit of quantitation in cocoa without a smoky off-flavor. In cocoa with a smoky off-flavor, average concentrations were 68.7 µg/kg for 2-methoxyphenol and 143.0 µg/kg for 4-methylphenol, whereas we found 120–815 µg/kg for 2-methoxyphenol and 56.9–527 µg/kg for 4-methylphenol.

OAVs of Potential Off-Flavor Compounds. To assess the importance of the individual off-flavor compounds for the smoky off-flavor in cocoa, OAVs were calculated by dividing the concentrations by the odor threshold values of the compounds. Odor threshold values were determined in deodorized cocoa butter from a series of 3-AFC tests with forced-choice and ascending concentrations of the potential off-flavor compounds according to the ASTM method.[29] Results (Table 3) revealed odor threshold values between 1.8 µg/kg and 3700 µg/kg. Low threshold values were obtained for odorants 2-methoxyphenol (1.8 µg/kg), 3-propylphenol (2.0 µg/kg), 3-ethylphenol (2.2 µg/kg), and 4-methylphenol (3.3 µg/kg). Somewhat higher threshold values were obtained for 3-methylphenol (19 µg/kg)

and 4-ethylphenol (23 μg/kg). The highest threshold value was determined for 4-propylphenol (3700 μg/kg). Thus, 4-propylphenol is clearly less odor-active than its isomer 3-propylphenol.

Calculation of OAVs revealed values >1 for all potential off-flavor compounds in the off-flavor samples with the exception of 4-propylphenol. The concentration of 4-propylphenol was below its odor threshold value in all samples analyzed. Thus, 4-propylphenol is not relevant for the smoky off-flavor in cocoa. For all other odorants analyzed, OAVs finally confirmed their role as off-flavor compounds. In all off-flavor samples (OF1−OF7), 2-methoxyphenol showed the highest OAV (67−450), followed by 4-methylphenol (OAVs 17−160), 3-ethylphenol (OAVs 5.5−65), 3-methylphenol (OAVs 4.2−33), 4-ethylphenol (OAVs 4.1−19), and 3-propylphenol (OAVs 1.2−11).

The intensity of the smoky off-flavor decreased going from OF1 to OF7. In line with the sensory scoring, OF1 showed the highest and OF7 the lowest concentrations of the off-flavor compounds. However, within off-flavor samples OF2 to OF6, the off-flavor compound concentrations did not fully mirror the sensory scoring, indicating that the intensity of the off-flavor does not only depend on the concentrations of the off-flavor compounds but also on the concentrations of genuine cocoa odorants that are able to mask the off-flavor to a certain extent.

In the reference samples without smoky off-flavor (REF1 and REF2), only 2-methoxyphenol and 4-methylphenol showed OAVs > 1. Specifically, OAVs were 39 and 36 for 2-methoxyphenol and 5.8 and 1.6 for 4-methylphenol. Thus, in both cases, OAVs were clearly lower than in the off-flavor samples.

Maximum Tolerable Concentrations of Smoky Off-Flavor Compounds in Fermented Cocoa. Given the diversity of sensory thresholds obtained for the potential off-flavor compounds (1.8−3700 μg/kg; cf. Table 3), a general limit applicable at the incoming goods inspection level, as suggested by Perotti et al., is not reasonable. Instead, for the compounds 3-methylphenol, 4-ethylphenol, 3-ethylphenol, and 3-propylphenol, which showed OAVs < 1 in the reference samples, the limits should rather be derived from the odor threshold values. 4-Propylphenol does not have to be considered because its concentration was below its odor threshold value, even in the off-flavor samples. Thus, we propose maximum tolerable concentrations based on the rounded threshold values of 2 μg/kg for 3-ethylphenol and 3-propylphenol and 20 μg/kg for 3-methylphenol and 4-ethylphenol. These values are clearly lower than the limits suggested by Serra Bonvehí and Ventura Coll, which are 900 μg/kg for 3-methylphenol, 900 μg/kg for 3-ethylphenol, and 700 μg/kg for 4-ethylphenol.[17] In accordance with our odor threshold data (cf. Table 3), they could already cause a smoky off-flavor. To derive limits for 2-methoxyphenol and 4-methylphenol, it has to be taken into account that they appeared in amounts exceeding their odor threshold values even in the reference samples, although their concentrations were below those in the off-flavor samples (cf. Table 2). As no smoky off-flavor was perceived in the REF1 and REF2 samples, the off-flavor was obviously masked by genuine cocoa odorants.[9,10] This suggests using the concentrations determined in REF1 (71.0 μg/kg and 19.1 μg/kg), which were higher than those in REF2 (65.0 μg/kg and 5.15 μg/kg), as a basis for the proposed maximum tolerable concentrations. Consequently, we suggest rounded values of 70 μg/kg for 2-methoxyphenol and 20 μg/kg for 4-methylphenol as maximum tolerable concentrations. However, it must be noted that the perception of the off-flavor

depends not only on the concentrations of the off-flavor compounds but also on the concentrations of genuine cocoa odorants contributing to the pleasant aroma of cocoa that are able to mask the off-flavor. Thus, the proposed limits for 2-methoxyphenol and 4-methylphenol need to be verified by an analysis of an extended number of cocoa samples.

In summary, the combination of odorant screening by AEDA, the targeted quantitation of crucial compounds, and the calculation of OAVs, all applied to samples with and without off-flavor, allowed to identify six compounds contributing to smoky off-notes in fermented cocoa and to suggest maximum tolerable concentrations applicable at the incoming goods inspection level in the chocolate industry. The structures of the compounds and the suggested maximum tolerable concentrations are depicted in Figure 2.
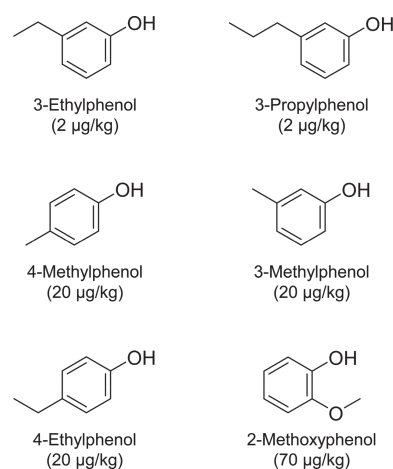

Figure 2. Smoky off-flavor compounds and their suggested maximum tolerable concentrations applicable at the incoming goods inspection level in the chocolate industry.

■ ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c04633.

> Quantifier ions and calibration line data used in the quantitations and individual concentration data used for mean calculations and standard deviations (PDF)

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https://pubs.acs.org/10.1021/acs.jafc.0c04633

■ **ABBREVIATIONS USED**

AEDA, aroma extract dilution analysis; 3-AFC, three-alternative forced-choice; AV, acidic volatiles; CI, chemical ionization; EI, electron ionization; FD factor, flavor dilution factor; FFAP, free fatty acid phase; FID, flame ionization detector; GC, gas chromatography; GC × GC, comprehensive two-dimensional gas chromatography; GC-O, gas chromatography-olfactometry; HRMS, high resolution mass spectrometry; i.d., inner diameter; HS-SPME, headspace solid phase microextraction; MCSS system, moving column stream switching system; MS, mass spectrometry; NBV, neutral and basic volatiles; OAV, odor activity value; PTV injector, programmed temperature vaporizing injector; RI, retention index; SAFE, solvent-assisted flavor evaporation; TOF, time-of-flight

Nomenclature

dimethyl trisulfide, dimethyltrisulfane; *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; geraniol, (2*E*)-3,7-dimethylocta-2,6-dien-1-ol; δ-octa-lactone, 6-propyloxan-2-one; γ-nonalactone, 5-pentyloxolan-2-one; γ-decalactone, 5-hexyloxolan-2-one; vanillin, 4-hydroxy-3-methoxybenzaldehyde

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8.1.3 Summary and Individual Contributions

The seeds of the cocoa tree *Theobroma cacao* L., commonly referred to as cocoa beans, are the key raw material for the production of chocolate. Cocoa beans typically show pleasant aroma characteristics that contribute positively to the aroma of the final products. However, fermented cocoa beans are sometimes tainted with undesirable off-flavors including smoky, moldy, fecal, and mushroom-like off-notes. If such off-flavors are transferred to the final products, they may lead to consumers rejecting these products. Among the known off-flavors, the smoky off-flavor is the most prevalent. Thus, the aim of this study was to identify the compounds causative for the smoky off-flavor in fermented cocoa beans.

The volatiles isolated from a sample of fermented cocoa with a pronounced smoky off-flavor and a reference sample with flawless aroma were subjected to cAEDA. In the reference sample, the detected compounds agreed well with the major odorants previously reported in fermented cocoa. In the off-flavor sample, seven conspicuous compounds were identified by smoky, hammy, and/or phenolic odor qualities. Additionally, their FD factors were clearly higher in the off-flavor sample than in the reference sample. Moreover, six out of the seven compounds were not even detected by GC-O in the reference sample. Thus, 2-methoxyphenol, 4-methylphenol, 3-methylphenol, 4-ethylphenol, 3-ethylphenol, and 3-/4-propylphenol were identified as potential off-flavor compounds in smoky off-flavor samples. The chromatographic separation of 3- and 4-propylphenol was finally achieved using a chiral BGB-176 column with a β-cyclodextrin-based stationary phase in the second dimension of a heart-cut GC-GC-HRMS system. The selection of these seven potential off-flavor compounds was confirmed by the screening of further samples with smoky off-notes in different intensities.

To further substantiate the screening results, the seven potential off-flavor compounds were quantitated in seven samples with smoky off-flavors in different intensities and two reference samples using stable isotopically substituted odorants as internal standards. Results revealed concentrations ranging from 0.210 µg/kg for 3-propylphenol in a reference sample to 815 µg/kg for 2-methoxyphenol in the off-flavor sample with the most intense smoky off-note. To assess the individual contribution of each compound to the smoky off-flavor, odor activity values (OAVs) were calculated using the odor threshold values (OTVs) of the potential off-flavor compounds in deodorized cocoa butter. Highest OAVs were obtained for 2-methoxyphenol (67−450), followed by 4-methylphenol (17−160), 3-ethylphenol (5.5−65), 3-methylphenol (4.2−33), 4-ethylphenol (4.1−19), and 3-propylphenol (1.2−11). Due to its high OTV, 4-propylphenol was not considered relevant for the smoky off-flavor. On the basis of the OTVs, maximum tolerable concentrations applicable at the incoming goods inspection of the chocolate industry were suggested, which amounted to 2 µg/kg for 3-ethylphenol and 3-propylphenol, 20 µg/kg for 3-methylphenol, 4-methylphenol and 4-ethylphenol, and 70 µg/kg for 2-methoxyphenol.

Daniela Füllemann designed and conducted the experiments including the volatile isolations, the GC-O screenings, the structure assignments, the syntheses of two isotopically substituted odorants, the quantitations, the determinations of OTVs in deodorized cocoa butter, and the calculation of OAVs. Daniela evaluated the data and prepared the manuscript. Martin Steinhaus conceived and directed the study, supervised Daniela's work, and revised the manuscript. Additionally, he participated in the sensory tests and in the GC-O analyses.

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Publication: Journal of Agricultural and Food Chemistry
Publisher: American Chemical Society
Date: Sep 1, 2020

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Date: Sep 1, 2020

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8.2 Publication 2: Impact of processing on important cocoa off-flavour compounds

8.2.1 Bibliographic Data

Title:	Impact of processing on important cocoa off-flavour compounds
Authors:	Daniela Füllemann, Silva D. Neiens, Martin Steinhaus
Journal	European Food Research and Technology
Publisher:	Springer Nature
Publication date:	October 8, 2021 (online)
Issue date:	January 2022
Volume:	248
Issue:	1
Pages:	197–205
DOI:	https://doi.org/10.1007/s00217-021-03873-0
Hyperlink:	https://link.springer.com/article/10.1007/s00217-021-03873-0

8.2.2 Publication Reprint

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

ORIGINAL PAPER

Impact of processing on important cocoa off-flavour compounds

Daniela Füllemann[1] · Silva D. Neiens[1] · Martin Steinhaus[1]

Abstract

The compounds responsible for smoky and mouldy–musty off-flavours in fermented cocoa have recently been elucidated; however, their behaviour during further processing into chocolate was still unclear. The compounds 2-methoxyphenol, 3-methylphenol, 4-methylphenol, 3-ethylphenol, 4-ethylphenol, and 3-propylphenol known to contribute to smoky off-flavours showed a tendency towards a minor increase during roasting and processing into cocoa liquor. This increase amounted to 1.4-fold at the most, however, was clearly compensated by losses of 30–63% during further processing into chocolate mass and conching. Among the off-flavour compounds identified in mouldy–musty smelling cocoa, faecal, mothball-like 3-methyl-1H-indole showed a clear decrease during roasting and processing into cocoa liquor, at least at rather high roasting temperatures, and a further decrease during processing into chocolate mass and conching. In contrast, faecal, mothball-like 1H-indole substantially increased during roasting and processing into cocoa liquor, namely from concentrations below its odour threshold value to concentrations up to 8 times beyond its odour threshold value. During processing into chocolate mass and conching, 1H-indole remained virtually unchanged. The data suggested that the monitoring of off-flavour compounds at the incoming goods inspection in the chocolate industry should not be limited to the fermented beans as such but additionally include the analysis of a bean sample after test roasting to correctly assess the off-flavour potential of 3-methyl-1H-indole and 1H-indole.

Keywords Cocoa · *Theobroma cacao* · Smoky off-flavour · Mouldy–musty off-flavour · Roasting · Conching

Abbreviations

GC–MS	Gas chromatography–mass spectrometry
Geosmin	(4S,4AS,8aR)-4,8a-Dimethyloctahydronaphthalen-4a(2H)-ol
MDMF	4-Methoxy-2,5-dimethylfuran-3(2H)-one
OAV	Odour activity value
SAFE	Solvent-assisted flavour evaporation

Introduction

To make chocolate, the fermented seeds of the cocoa tree (*Theobroma cacao* L.) are roasted and then ground into cocoa liquor. After addition of further ingredients such as sugar, cocoa butter, and milk powder, the mixture is ground to yield chocolate mass. Further homogenisation

and refining is achieved by conching, a process that lasts between hours and days, before the mass is finally moulded into chocolate [1, 2].

Fermented cocoa is occasionally tainted with off-flavours. Such batches must be sorted out during incoming goods inspection in the chocolate industry to avoid quality issues with the final product. In two recent studies, we identified the crucial odorants responsible for the smoky off-flavour and the crucial odorants responsible for the mouldy–musty off-flavour in fermented cocoa. We showed that the smoky off-note is caused by 2-methoxyphenol, 3-methylphenol, 4-methylphenol, 3-ethylphenol, 4-ethylphenol, and 3-propylphenol [3], whereas (−)-geosmin, 3-methyl-1H-indole, 1H-indole, and 4-methoxy-2,5-dimethylfuran-3(2H)-one (MDMF) are potential off-flavour compounds in mouldy–musty smelling cocoa samples [4]. Predominantly based on the odour threshold values in deodorized cocoa butter, maximum tolerable concentrations in fermented cocoa were suggested. These limits amounted to 1.1 µg/kg for 3-methyl-1H-indole, 1.6 µg/kg for (−)-geosmin, 2 µg/kg for 3-ethylphenol and 3-propylphenol, 20 µg/kg for 3-methylphenol, 4-methylphenol, and 4-ethylphenol,

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and 70 μg/kg for 2-methoxyphenol [3, 4]. The decision on acceptance or rejection can thus be made on a more objective basis than with sensory testing only. However, it was so far largely unclear, how further processing influences the concentrations of the off-flavour compounds. Given the elevated temperatures, a substantial impact could be expected from roasting and conching. Cocoa bean roasting is typically done at 120–140 °C for 20–30 min [2, 5, 6], whereas during conching, temperatures of ~ 50–80 °C are applied for up to 3 days [1, 2, 7].

Several studies have addressed the changes in odorant concentrations during cocoa processing [6, 8–15]. It was shown that the high temperatures during roasting converted thermolabile precursors formed during fermentation [16, 17] into important odour-active compounds such as Strecker aldehydes, pyrazines, and furanones [8–10, 18]. At the same time, the concentrations of some undesired odorants such as acetic acid were reduced [6, 11]. Conching not only improved the rheological properties of the chocolate but also affected chocolate flavour [14, 19]. Specifically, the concentrations of some undesired volatile acids decreased [14], whereas for example caramel-like smelling 4-hydroxy-2,5-dimethylfuran-3(2H)-one (HDMF; Furaneol®) increased during conching [14, 15]. So far, no study on the effect of cocoa processing focused on off-flavour compounds. Nonetheless, Frauendorfer et al. reported higher concentrations of 2-methoxyphenol and 4-methylphenol after roasting of Criollo and Forastero cocoa beans [9, 10]. Moreover, Counet et al. who quantitated 44 volatiles in dark chocolate before and after conching found that 2-methoxyphenol and 1H-indole increased [14]. In contrast, Beckett reported a reduction in the amount of phenols during conching; however, no data on individual compounds was provided [20].

To obtain a better view on the behaviour of the compounds contributing to smoky and mouldy–musty off-flavours during chocolate manufacturing, the aim of the current study was to determine their concentration changes induced by 1) cocoa roasting and processing into cocoa liquor and by 2) further processing into chocolate mass and conching. If substantial differences were to occur, the previously suggested maximum tolerable concentrations in fermented cocoa might need to be adjusted.

Materials and methods

Cocoa

The samples of fermented cocoa beans were obtained from German chocolate manufacturers. The samples SOF1 and SOF2 showed smoky off-flavours, the samples MOF1 and MOF2 were tainted with mouldy–musty off-flavours, and the reference sample REF exhibited a characteristic aroma without off-notes. All samples were stored at + 4 °C.

Chemicals

The reference odorants 2-methoxyphenol, 4-methylphenol, 3-methylphenol, 4-ethylphenol, 3-ethylphenol, 3-propylphenol, MDMF, 3-methyl-1H-indole, 1H-indole, and geosmin as well as 4-(^{2}H$_3$)methyl(^{2}H$_4$)phenol were purchased from Merck (Darmstadt, Germany). The following isotopically substituted odorants were synthesised as detailed in the literature: 2-[(^{2}H$_3$)methyloxy]phenol [21], 4-(1,1-^{2}H$_2$)ethylphenol [3], 4-(1,1-^{2}H$_2$)propylphenol [3], 2,5-dimethyl-4-[(^{2}H$_3$)methyloxy]furan-3(2H)-one [22], 3-(^{2}H$_3$)methyl(2,4,5,6,7-^{2}H$_5$)-1H-indole [23], and ((4S,4aS,8aR)-4,8a-dimethyl(3,3,4-^{2}H$_3$)octahydronaphthalen-4a(2H)-ol) [24]. (2,3,4,5,6,7-^{2}H$_6$)-1H-Indole was synthesised from the isotopically unmodified compound 1H-indole according to the approach published for the synthesis of 3-(^{2}H$_3$)methyl(2,4,5,6,7-^{2}H$_5$)-1H-indole [23]. Dichloromethane was freshly distilled through a column (120 cm × 5 cm) packed with Raschig rings before use. Ethanol (99.9%) was purchased from Honeywell (Seelze, Germany).

Roasting and processing into cocoa liquor

The fermented cocoa beans were roasted according to the protocol "Elements of harmonized international standards for cocoa quality and flavour assessment" of the Cocoa of Excellence (CoEx) Programme [25]. In brief, the fermented cocoa beans were placed in a single layer on a stainless steel mesh tray and put in a pre-heated convection oven FP53 (Binder, Tuttlingen, Germany) for 25 min. The roasting temperatures were 110, 125, and 140 °C. After roasting, the cocoa beans were allowed to cool down to room temperature before the shells were removed manually. The cocoa was processed into cocoa liquor using a preheated (45 °C) RM 200 mortar grinder (Retsch, Haan, Germany) equipped with a porcelain pestle and a porcelain mortar. The cocoa liquors were stored at + 4 °C until analysis.

Processing into chocolate mass and conching

Solutions of the off-flavour compounds (0.2–9 mg in 200 μl ethanol) were premixed with molten cocoa liquor (500 g). In the pilot plant of a German chocolate manufacturer, the spiked cocoa liquors were included into the recipes used for making a dark chocolate mass (10 kg; > 50% cocoa liquor; further ingredients: cocoa butter, sugar) and a milk chocolate mass (10 kg; < 10% cocoa liquor; further ingredients: cocoa butter, milk powder, sugar) and conched at a temperature of 80 °C. After 12 h, the mass was moulded into chocolate.

Odorant quantitation

Samples of the cocoa liquors and chocolates were immersed in liquid nitrogen, crushed using a GM 200 laboratory mill (Retsch) and further ground into a fine powder using a 6875 Freezer Mill (Spex SamplePrep, Stanmore, UK). To portions (1−50 g) of the powder, dichloromethane (25−100 mL) was added and the mixture was spiked with the isotopically substituted odorants (0.04–8 μg) in dichloromethane (40–400 μL) as internal standards. After magnetic stirring at room temperature for ~ 15 h, the mixture was filtered through a folded paper filter. The filtrate was dried over anhydrous sodium sulphate. Non-volatiles were removed by SAFE at 40 °C [26]. The distillate was concentrated using a Vigreux column (50 × 1 cm) and a Bemelmans microdistillation device [27] to a final volume of 200 μL. Portions of the concentrates (1–2 μL) were analysed by GC–MS. Quantitation of 2-methoxyphenol, 3- and 4-methylphenol, 3- and 4-ethylphenol, 3-methyl-1*H*-indole, 1*H*-indole, and MDMF was accomplished using a heart-cut GC–GC–MS system. For the quantitation of 3-propylphenol and (−)-geosmin, a heart-cut GC–GC–HRMS system was employed. Odorant concentrations were finally calculated from the peak area counts of the analyte peak and the internal standard peak in the extracted ion chromatograms of characteristic quantifier ions, the amount of sample used, and the amount of standard added, by employing a calibration line equation obtained by linear regression after the analysis of analyte/standard mixtures in at least five different concentration ratios, covering a range of at least 1:5 to 5:1. Details on the GC–MS systems, the quantifier ions, and the calibrations are provided in the Supplementary Information file.

Results and discussion

Impact of roasting and processing into cocoa liquor on cocoa off-flavour compounds

Two samples of fermented cocoa beans with a smoky off-flavour, two samples of fermented cocoa beans with a mouldy–musty off-flavour, and a reference sample with a characteristic aroma and no off-flavour were roasted and then processed into cocoa liquor. Each of the five cocoa samples was roasted at three different temperatures, namely 110, 125, and 140 °C. Although roasting temperatures as low as 90 °C and as high as 170 °C have also been reported in the literature [1, 13, 28, 29], the range of 110–140 °C is the one most relevant in the chocolate industry [2, 6].

In the cocoa liquors obtained from the cocoa samples with a smoky off-flavour (SOF1, SOF2), the six phenols previously identified as causative for the off-note [3] were quantitated. In detail, the compounds and their odour qualities were 2-methoxyphenol (smoky, hammy), 3-methylphenol (smoky, phenolic), 4-methylphenol (horse stable-like, phenolic), 3-ethylphenol (smoky), 4-ethylphenol (smoky), and 3-propylphenol (smoky, phenolic). Quantitation was accomplished by GC–MS after solvent extraction and SAFE using stable isotopically substituted odorants as internal standards. The reference sample without off-flavour (REF) was analysed in parallel. The data obtained were compared to the concentrations before processing (Table 1). Moreover, the concentrations were divided by the odour threshold values of the compounds in deodorized cocoa butter to obtain odour activity values (OAVs) and thus to simultaneously assess the impact of processing and the odour relevance of the compounds (Fig. 1). The odour threshold values applied were 1.8 μg/kg for 2-methoxyphenol, 19 μg/kg for 3-methylphenol, 3.3 μg/kg for 4-methylphenol, 2.2 μg/kg for

Table 1 Changes in the concentrations (μg/kg) of crucial off-flavour compounds in two cocoa samples with a smoky off-flavour (SOF1, SOF2) and a reference sample without off-flavour caused by roasting at 110, 125, and 140 °C and processing into cocoa liquor

Odorant	SOF1				SOF2				Reference sample			
	Before[a]	110 °C[b]	125 °C[b]	140 °C[b]	Before[a]	110 °C[b]	125 °C[b]	140 °C[b]	Before[a]	110 °C[b]	125 °C[b]	140 °C[b]
2-Methoxyphenol	815	871	886	1050	354	393	488	445	71.0	57.7	67.5	65.9
3-Methylphenol	635	575	517	611	346	312	329	302	5.82	4.97	4.39	6.45
4-Methylphenol	527	476	450	519	171	157	176	170	19.1	16.0	16.6	21.5
3-Ethylphenol	143	179	158	191	71.9	96.7	79.8	84.2	0.379	2.17	5.98	3.21
4-Ethylphenol	275	320	308	368	205	236	209	249	11.9	35.1	45.7	64.5
3-Propylphenol	21.3	28.4	21.9	28.1	17.5	14.3	6.93	5.56	0.255	0.264	0.347	0.292

[a]Data taken from[3]

[b]Mean of duplicate or triplicate workups; individual concentrations and standard deviations are available in the Supplementary Information file, Tables S2–S4
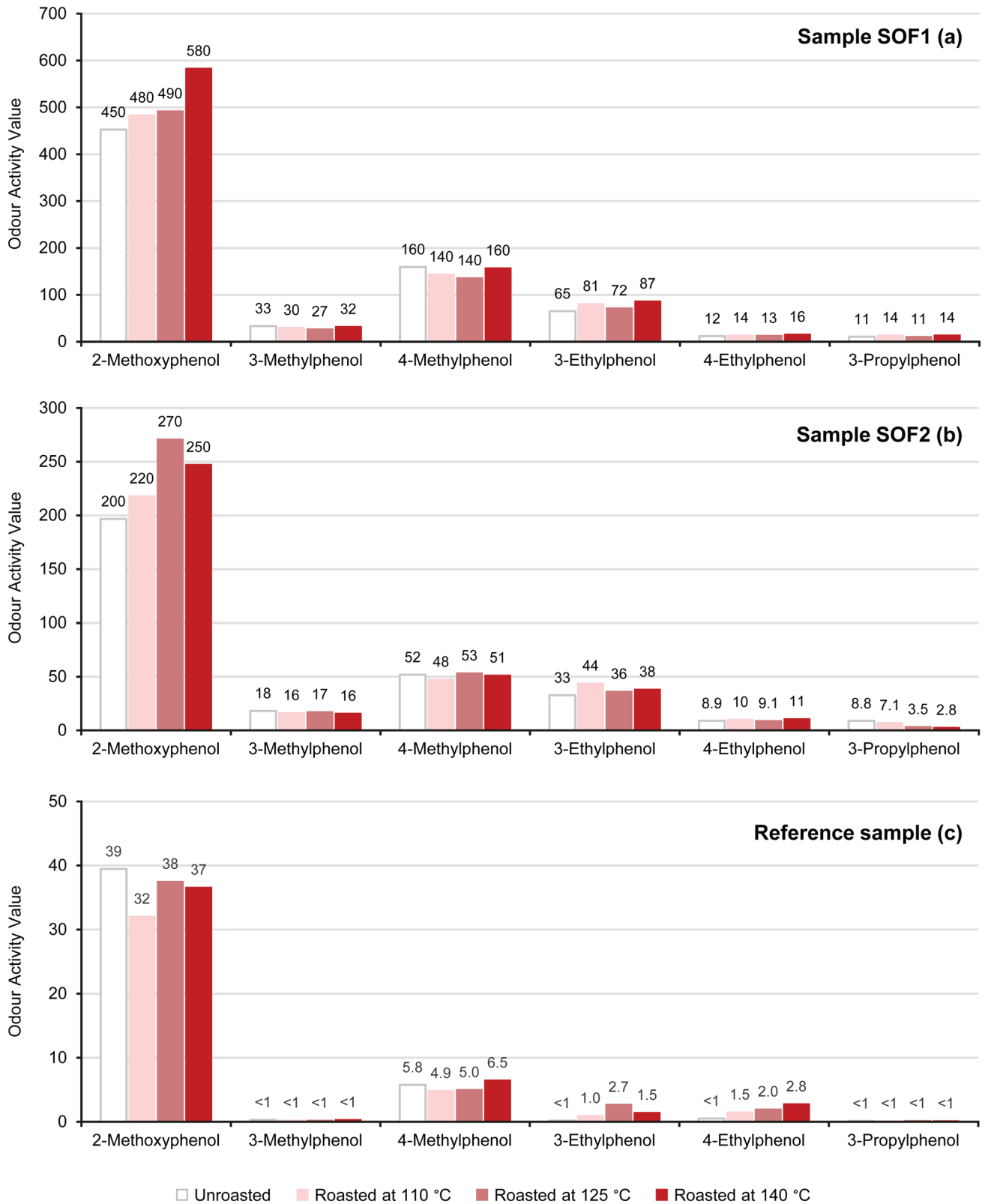
Fig. 1 Changes in the odour activity values of crucial off-flavour compounds in two cocoa samples with a smoky off-flavour (SOF1, SOF2) and a reference sample without off-flavour caused by roasting at 110, 125, and 140 °C and processing into cocoa liquor

3-ethylphenol, 23 µg/kg for 4-ethylphenol, and 2.0 µg/kg for 3-propylphenol [3].

Results indicated only minor changes of the compounds during roasting and processing into cocoa liquor. In particular, there was no general decrease of off-flavour compounds that could have been expected if—despite the rather high boiling points of the compounds of ~202−230 °C—evaporation during roasting had played a substantial role. In this case, an adjustment of the maximum tolerable concentrations in fermented cocoa during incoming goods inspection in the chocolate industry could have been considered. A clear decrease that reflected the roasting temperatures was only observed for a single compound in a single sample, namely 3-propylphenol in sample SOF2. The compound decreased during roasting and processing into cocoa liquor from 17.5 to 14.3 µg/kg at 110 °C, 6.93 µg/kg at 125 °C, and 5.56 µg/kg at 140 °C (Table 1) which corresponded to OAVs of 8.8 before roasting and 7.1, 3.5, and 2.8 after roasting and processing into cocoa liquor (Fig. 1). However, no such behaviour was observed for 3-propylphenol in sample SOF1. By contrast, some of the off-flavour compounds in the off-flavour samples rather showed a tendency towards a slight increase. This increase was clearly beyond the concentration effect associated with the loss of water during roasting. Given a typical water content of 4–8% in the unroasted cocoa and a final water content of ~2% in the roasted cocoa [1, 30, 31], such an increase would be clearly below the 1.1-fold. On the other hand, the increase was also never greater than 1.4-fold and, in particular, did not change the order of the off-flavour compounds in terms of OAVs: before and after roasting and processing into cocoa liquor, 2-methoxyphenol showed the highest OAVs among the six compounds in the two off-flavour samples followed by 4-methylphenol and 3-ethylphenol. In the reference sample without off-flavour, the levels of all off-flavour compounds were clearly lower, before roasting as well as after roasting and processing into cocoa liquor. 2-Methoxyphenol and 4-methylphenol were the only compounds exceeding their odour threshold levels before roasting. During roasting and

processing into cocoa liquor, both did not show substantial changes; however, the concentrations of 3-ethylphenol and 4-ethylphenol increased up to 15-fold for 3-ethylphenol and up to 5.4-fold for 4-ethylphenol, resulting in concentrations slightly beyond their threshold values, whereas 3-methylphenol concentrations and 3-propylphenol concentrations stayed below the threshold value.

Different from our results, Frauendorfer and Schieberle reported a clear increase of 2-methoxyphenol and 4-methylphenol during roasting of cocoa beans without an off-flavour [9, 10], although conditions with 95 °C roasting temperature and 14 min roasting time were rather mild. For 2-methoxyphenol, they found an increase from 110 µg/kg (Criollo beans) and 61 µg/kg (Forastero beans) to 230 and 100 µg/kg, respectively, whereas in our reference sample, the concentration even slightly decreased from 71 to 57.7–67.5 µg/kg, depending on the roasting temperature. For 4-methylphenol, Frauendorfer and Schieberle observed an increase from 5.3 (Criollo) and 4.6 (Forastero) to 9.9 and 7.6 µg/kg, respectively. In our reference sample, the concentration before roasting was 19.1 µg/kg and after roasting and processing into cocoa liquor it ranged from 16.0 to 21.5 µg/kg.

In the cocoa liquors obtained from the cocoa samples with a mouldy–musty off-flavour (MOF1, MOF2), (−)-geosmin, 3-methyl-1*H*-indole, 1*H*-indole, and 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF) were quantitated. This selection was based on our previous study [4]. Again, the reference sample without off-flavour (REF) was analysed in parallel. Results are depicted in Table 2 together with the concentrations before processing. Figure 2 shows the OAVs calculated from the concentrations and the odour threshold values of the compounds in deodorized cocoa butter. The odour threshold values used for these calculations were 1.6 µg/kg for (−)-geosmin, 1.1 µg/kg for 3-methyl-1*H*-indole, 51 µg/kg for 1*H*-indole, and 350 µg/kg for MDMF [4].

(−)-Geosmin, the compound with the most pronounced mouldy smell, was present in odour-active amounts only in sample MOF1. Its concentration in the samples roasted at 110 and 125 °C was almost twice the concentration in

Table 2 Changes in the concentrations (µg/kg) of crucial off-flavour compounds in two cocoa samples with a mouldy–musty off-flavour (MOF1, MOF2) and a reference sample without off-flavour caused by roasting at 110, 125, and 140 °C and processing into cocoa liquor

Odorant	MOF1				MOF2				Reference sample			
	Before[a]	110 °C[b]	125 °C[b]	140 °C[b]	Before[a]	110 °C[b]	125 °C[b]	140 °C[b]	Before[a]	110 °C[b]	125 °C[b]	140 °C[b]
(−)-Geosmin	3.54	6.26	6.19	2.43	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
3-Methyl-1*H*-indole	2.07	2.39	<1.0	<1.0	66.4	8.59	1.65	2.47	<1.0	<1.0	<1.0	<1.0
1*H*-Indole	8.10	24.7	67.5	84.2	5.46	102	248	406	<1.0	47.0	82.7	96.2
MDMF	14.9	26.4	38.2	38.4	226	3.64	3.68	2.52	<1.0	1.32	1.45	1.62

[a]Data taken from[4]

[b]Mean of duplicate or triplicate workups; individual concentrations and standard deviations are available in the Supplementary Information file, Tables S5–S7
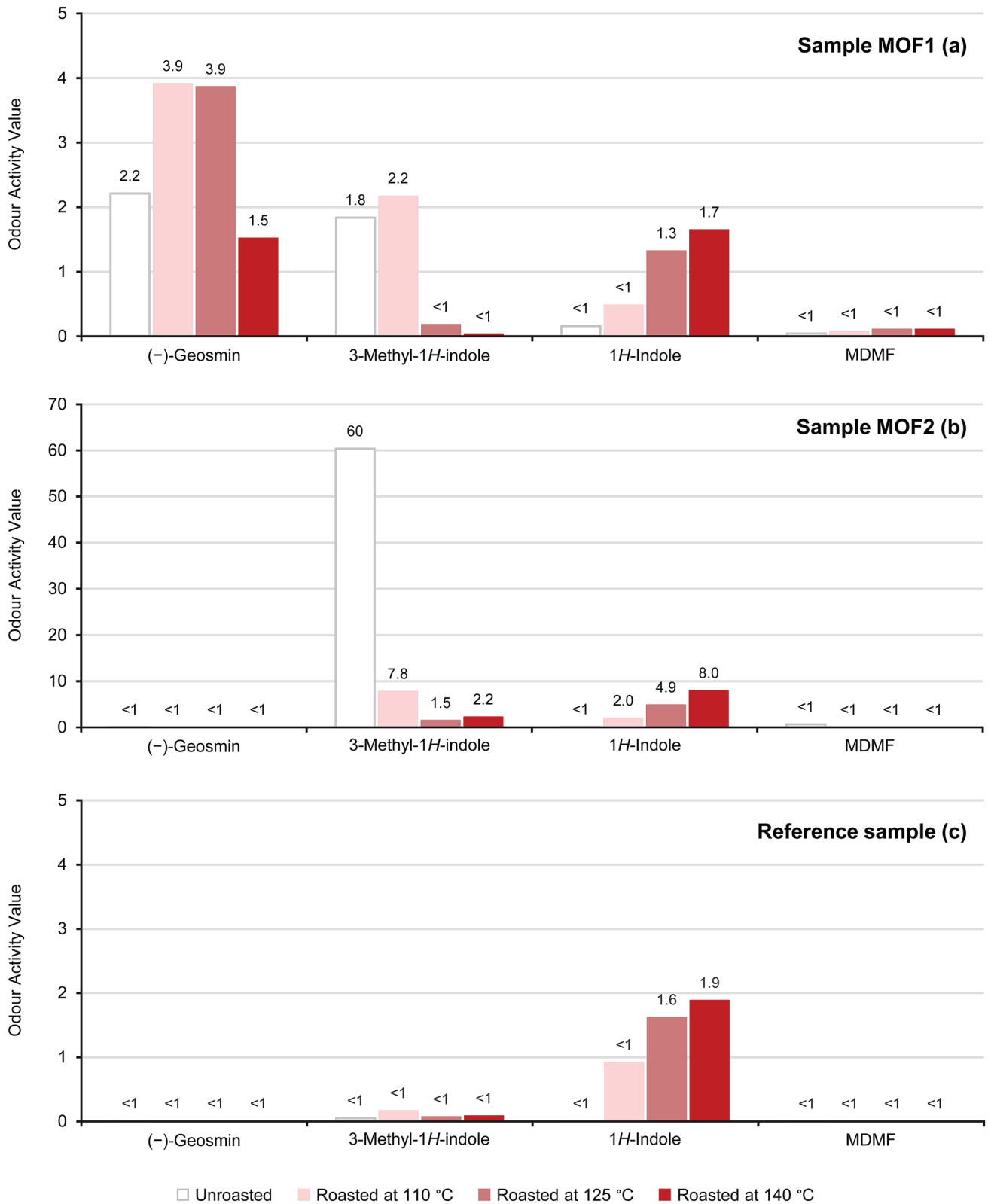
Fig. 2 Changes in the odour activity values of crucial off-flavour compounds in two cocoa samples with a mouldy–musty off-flavour (MOF1, MOF2) and a reference sample without off-flavour caused by roasting at 110, 125, and 140 °C and processing into cocoa liquor

the unprocessed sample. This is hard to explain, given that the compound is a well-known product of microbiological metabolism but not known to be formed thermally [32–34]. Furthermore, (−)-geosmin has been found enriched in the cocoa seed shells [4]. The shells, however, had been removed during processing of the roasted cocoa samples to cocoa liquor. Thus, rather a lower concentration was to be expected in the roasted samples. This, however, was only the case for the sample roasted at 140 °C, in which not more than 2/3 of the initial (−)-geosmin was recovered.

Faecal, mothball-like smelling 3-methyl-1H-indole was the most odour-active compound in the unprocessed off-flavour sample MOF2 and the second most odour-active compound in the unprocessed off-flavour sample MOF1. In both off-flavour samples, its concentration clearly decreased during roasting and processing into cocoa liquor, at least when higher roasting temperatures were applied. After roasting at 125 and 140 °C and processing into cocoa liquor, its OAV in sample MOF1 had decreased from 1.8 to clearly below 1 and in sample MOF2, the decrease was from 60 to 1.5 and 2.2, respectively.

The most conclusive change during roasting and processing into cocoa liquor was observed for faecal, mothball-like smelling 1H-indole. In all three cocoa samples, the concentration in the unprocessed cocoa was below the odour threshold value of 51 μg/kg. In the processed samples, the amounts increased continuously with increasing roasting temperature leading to OAVs between 1.7 and 8.0 in the samples roasted at 140 °C. In the three 140 °C samples, 1H-indole was the most odour-active among the four off-flavour compounds. Moreover, in the reference sample 1H-indole was the only compound of the four that exceeded the odour threshold, namely in the samples roasted at 125 and 140 °C.

Caramel-like and musty smelling MDMF showed a heterogeneous behaviour during roasting. Whereas in sample MOF1 and in the reference sample its concentration increased with the roasting temperature, a decrease was observed in sample MOF2. However, in none of the samples any concentration ever exceeded the odour threshold value of the compound.

Impact of processing into chocolate mass and conching on cocoa off-flavour compounds

A cocoa liquor with a characteristic aroma and no off-flavour was spiked with the compounds previously identified as crucial for smoky and mouldy–musty off-flavours. The final concentrations resulting from the amounts naturally present in the cocoa liquor plus the spiked amounts were in the ranges previously determined in cocoa samples with the respective off-flavours [3, 4]. Portions of the spiked cocoa liquor were processed into a dark chocolate mass and into a milk chocolate mass, respectively. After conching at 80 °C for 12 h, the off-flavour compounds were quantitated and the concentrations obtained were compared to the concentrations in the cocoa liquor before processing.

The results (Table 3) revealed recoveries between 9 and 101%. For none of the compounds, a substantial difference between the dark chocolate mass and the milk chocolate mass was observed. The six phenolic compounds crucial for the smoky off-flavours showed recoveries of 37–70% in the dark chocolate mass and recoveries of 41–74% in the milk chocolate mass. The lowest recoveries were

Table 3 Changes in the concentrations of crucial off-flavour compounds caused by processing spiked cocoa liquor into chocolate mass and conching

Odorant	Dark chocolate mass			Milk chocolate mass		
	Before (μg/kg)[a]	After (μg/kg)[b]	Recovery (%)	Before (μg/kg)[a]	After (μg/kg)[b]	Recovery (%)
2-Methoxyphenol	926	346	37	897	369	41
3-Methylphenol	362	202	56	360	226	63
4-Methylphenol	576	381	66	570	406	71
3-Ethylphenol	174	121	70	174	128	74
4-Ethylphenol	339	235	69	325	239	74
3-Propylphenol	21.1	10.7	51	21.0	11.5	55
(−)-Geosmin	5.10	4.02	79	5.10	4.16	82
3-Methyl-1H-indole	106	83.9	79	106	84.4	80
1H-Indole	83.5	84.3	101	28.4	26.4	93
MDMF	519	47.8	9	519	57.5	11

[a]Sum of the amount naturally present in the cocoa liquor and the spiked amount; details are available in the Supplementary Information file, Table S8

[b]Mean of triplicate workups; individual concentrations and standard deviations are available in the Supplementary Information file, Tables S9 and S10

obtained for 2-methoxyphenol. Interestingly, Counet et al. [14] reported higher concentrations for 2-methoxyphenol after conching. In two different chocolate samples, the 2-methoxyphenol concentrations increased by 50 and 13%, respectively. However, these experiments had been performed at much lower concentration levels (66 and 75 μg/ kg) than our experiments (926 and 897 μg/kg), which probably shifted the balance between losses and formation from precursors.

Among the four compounds potentially contributing to mouldy–musty off-flavours, (−)-geosmin and 3-methyl-1H-indole showed recoveries around 80%. The recoveries for 1H-indole were higher, namely 101% in the dark chocolate mass and 93% in the milk chocolate mass. Possibly, losses of 1H-indole during conching were in parts compensated by its formation from thermolabile precursors. The rather high recoveries of 1H-indole during conching were thus in line with its clear increase during roasting. MDMF behaved differently from all other compounds as its recovery with 9% and 11% was quite low.

Conclusions

Our data suggests that for the off-flavour compounds causing smoky off-flavours, namely 2-methoxyphenol, 3-methylphenol, 4-methylphenol, 3-ethylphenol, 4-ethylphenol, and 3-propylphenol, minor increases during roasting and processing into cocoa liquor are compensated by losses during processing into chocolate mass and conching. For these compounds, no adjustment of the previously suggested maximum tolerable concentrations on the level of the fermented cocoa is, therefore, necessary. The compounds potentially contributing to mouldy–musty off-flavours showed a less homogeneous behaviour. For (−)-geosmin, a clear statement is not possible, because only a single dataset was available to assess the changes during roasting and processing into cocoa liquor. Given the decomposition of 3-methyl-1H-indole during roasting and processing into cocoa liquor, its limit might be raised, at least when higher roasting temperatures are applied. By contrast, 1H-indole showed a clear increase during roasting and processing into cocoa liquor that was not compensated by processing into chocolate mass and conching. In the incoming goods inspection in the chocolate industry, monitoring of 1H-indole should therefore include roasting of the beans before analysis. MDMF needs not to be included in the monitoring. Its concentration in the fermented beans is typically below its relatively high odour threshold value and is further reduced during processing.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-021-03873-0.

Declarations

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8.2.3 Summary and Individual Contributions

Previous investigations on the odorants contributing to the smoky off-flavor and to the moldy-musty off-flavor in fermented cocoa beans had revealed 2-methoxyphenol, 3-methylphenol, 4-methylphenol, 3-ethylphenol, 4-ethylphenol, and 3-propylphenol as causative for the smoky off-flavor, and (−)-geosmin, 3-methyl-1*H*-indole, 1*H*-indole, and 4-methoxy-2,5-dimethylfuran-3(2*H*)-one (MDMF) as potential off-flavor compounds contributing to the moldy-musty off-flavor. To get a deeper insight into the behavior of these compounds during chocolate production, their concentration changes induced by (1) cocoa roasting at 110, 125, and 140 °C and processing into cocoa liquor and by (2) further processing into chocolate mass and conching were determined in the current study.

After roasting and processing into cocoa liquor, the quantitation of the off-flavor compounds in the smoky off-flavor samples revealed only minor concentration changes. In particular, there was no general decrease of the off-flavor compounds. An increase of 1.4-fold was determined for 2-methoxyphenol. In the reference sample, only 2-methoxyphenol and 4-methylphenol exceeded their odor threshold values in the fermented cocoa beans. After roasting and processing into cocoa liquor, also the concentrations of 3-ethylphenol and 4-ethylphenol slightly exceeded their odor threshold values. Quantitation of the compounds potentially contributing to the moldy-musty off-flavor revealed decreasing concentrations for 3-methyl-1*H*-indole at higher roasting temperatures. 1*H*-Indole substantially increased during roasting and processing into cocoa liquor resulting in concentrations up to 8 times beyond its odor threshold value. Due to the limited dataset, no clear statement was possible for (−)-geosmin. MDMF exceeded in none of the samples its comparatively high odor threshold value.

Conching experiments were conducted by spiking a cocoa liquor with the off-flavor compounds in the concentrations previously obtained in samples with smoky and moldy-musty off-flavor. The spiked cocoa liquor was used for the production of a dark chocolate mass and a milk chocolate mass. Results revealed for the smoky off-flavor compounds recoveries between 37 and 74%. For the off-flavor compounds detected in the moldy-musty cocoa samples, a less homogenous behavior was observed. Recoveries were 79–82% for (−)-geosmin and 3-methyl-1*H*-indole, 93–101% for 1*H*-indole, and only 9–11% for MDMF.

For the smoky off-flavor compounds, the minor increases during roasting and processing into cocoa liquor were balanced by the decreases during processing into chocolate mass and conching. Thus, adjustments of the previously suggested maximum tolerable concentrations were not necessary. For 3-methyl-1*H*-indole, the limit might be raised at least when roasting is conducted at higher temperatures. To correctly assess the off-flavor potential of 1*H*-indole, test roasting was suggested, because this compound substantially increased during roasting.

Daniela Füllemann and Silva D. Neiens designed and conducted the roasting and processing experiments and the spiking of the cocoa liquor for the conching experiments. Daniela conducted the volatile isolations, the quantitations, the calculation of OAVs, and the evaluation of the data of the smoky off-flavor compounds. Together with Silva, Daniela conducted the volatile isolations, the quantitations, the calculation of OAVs, and the evaluation of the data related to the moldy-musty off-flavor compounds. Daniela prepared the manuscript. Martin Steinhaus conceived and directed the study, supervised Daniela's and Silva's work, and revised the manuscript.

8.2.4 Reprint Permission

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8.3 List of Publications, Talks, and Poster Presentations

Publications

Publications in peer-reviewed journals:

Füllemann, D.; Steinhaus, M. Characterization of odorants causing smoky off-flavors in cocoa. *J. Agric. Food Chem*. **2020**, *68*, 39, 10833–10841. https://doi.org/10.1021/acs.jafc.0c04633

Füllemann, D.; Neiens, S.D.; Steinhaus, M. Impact of processing on important cocoa off-flavour compounds. *Eur. Food Res. Technol*. **2022**, *248*, 1, 197–205. https://doi.org/10.1007/s00217-021-03873-0

Miscellaneous journal contributions:

Füllemann, D.; Steinhaus, M. Klärung der molekularen Ursachen für schinkig-rauchige Fehlaromanoten in Kakao. *Lebensmittelchemie* **2020**, *74*, S1-003. https://doi.org/10.1002/lemi.202051003

Talks

Oral presentations at international scientific meetings:

Füllemann, D.; Steinhaus, M. Elucidation of the molecular background of smoky and hammy off-flavors in cocoa. American Chemical Society, 258th National Meeting, Agricultural and Food Chemistry Division, Symposium: Agnes Rimando Memorial International Student Symposium. San Diego, CA, USA, August 26, 2019.

Füllemann, D.; Steinhaus, M. Which compounds are responsible for smoky off-flavors in cocoa? First Shared Flavor-Seminar between Chulalongkorn University Bangkok (Chula), Leibniz Institute for Food Systems Biology at the Technical University of Munich (Leibniz-LSB@TUM) and the Chair of Food Chemistry and Molecular Sensory Science at the Technical University of Munich (TUM). Online, December 14, 2020.

Oral presentations at miscellaneous national meetings:

Füllemann, D.; Steinhaus, M. Elucidation of the molecular background of smoky and hammy off-flavors in cocoa. Forschungsseminar der Lebensmittelchemie; organized by the Chair of Food Chemistry and Molecular Sensory Science, Chair of Analytical Food Chemistry, Associate Professorship of Biotechnology of Natural Products, and Leibniz Institute for Food Systems Biology at the Technical University of Munich; promoted by the Graduate Center Weihenstephan. Freising, Germany, October 28, 2019.

Füllemann, D.; Steinhaus, M. Klärung der molekularen Ursachen für schinkig-rauchige Fehlaromanoten in Kakao. 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, 71th Bavarian Regional Meeting). Würzburg, Germany, March 10, 2020.

<u>Poster Presentations</u>

Füllemann, D.; Porcelli, C.; Steinhaus, M. Odour-active compounds in fermented cocoa showing hammy and mouldy off-flavours. 7. Runder Tisch Kakao. Hamburg, Germany, June 22–23, 2017.

Füllemann, D.; Steinhaus, M. Analysis of odorants causing smoky off-flavors in cocoa. Choco Tec 2018. Cologne, Germany, December, 3–5, 2018.

Füllemann, D.; Steinhaus, M. Elucidation of the molecular background of smoky off-flavors in cocoa. 12th Wartburg Symposium on Flavor Chemistry & Biology. Eisenach, Germany, May 21–24, 2019.