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Novel Time- and Location-Independent Postharvest Treatment of Cocoa Beans: Investigations on the Aroma Formation during "Moist Incubation" of Unfermented and Dried Cocoa Nibs and Comparison to Traditional Fermentation

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Supporting Information

ABSTRACT: The aroma properties of cocoa nibs obtained by applying a novel postharvest treatment were investigated using methods of the molecular sensory science approach, i.e., solvent extraction and solvent-assisted flavor evaporation, aroma extract dilution analysis (AEDA), stable isotope dilution analysis, calculation of odor activity values (OAVs), and orthonasal sensory evaluation; those properties were then compared to the unfermented and dried raw material and a traditionally fermented sample of the same harvest. For the treatment, unfermented and dried cocoa nibs were, first, rehydrated with lactic acid and ethanol solution to adjust the pH value to 5.1 and, second, incubated under aerobic conditions for 72 h at 45 °C and subsequently dried. This treatment was used to induce enzymatic reactions within the cotyledon matrix, which also occur inside the bean during microbial fermentation of the surrounding fruit pulp. The results of the AEDA showed that many of the key aroma compounds found in fermented and dried cocoa increased during the incubation treatment. Especially some "fruity" esters were found with an equal or even higher flavor dilution (FD) factor in the incubated sample compared to the fermented sample, whereas the fermented sample showed high FD factors for "pungent, sour" and "sweaty" acids, such as acetic acid and 2- and 3-methylbutanoic acids. The quantitative data and calculated OAVs for the samples supported the findings of the AEDA, underlining the potential of this approach as a controllable and reproducible alternative postharvest treatment.

KEYWORDS: *cocoa postharvest treatment, cocoa incubation, cocoa fermentation, cocoa aroma formation, stable isotope dilution analysis (SIDA)*

INTRODUCTION

Postharvest treatment of fresh cocoa beans is a crucial step along the processing chain from the tree to a finished product, like chocolate. During fermentation and drying, the adhering pulp of the beans is removed, the germ is inactivated, and moisture in the beans is reduced to provide storability of raw cocoa. Moreover, it is a very important step for the formation of cocoa aroma and taste as a result of biochemical processes leading to the liberation of aroma precursors, formation of aroma compounds, and transformation of taste-active compounds, such as polyphenols.[1,2]

During standard postharvest treatment, cocoa pods are opened manually after harvest and the fresh cocoa beans with the adhering mucilaginous pulp are removed. The bean−pulp mass is then usually heaped up or filled in wooden fermentation boxes or baskets and subsequently covered.[3] These processes are usually not performed in a sterile environment, and non-sterile instruments and fermentation containers are used; therfore, the mass is inoculated with ubiquitous microbial flora, leading to a spontaneous fermentation.[1] In the initial phase of fermentation, yeasts are dominant and degrade the cocoa pulp surrounding the cocoa beans, metabolizing carbohydrates under anaerobic conditions, leading to the formation of ethanol and carbon dioxide.[4] As a result of pectinolytic activity of the involved yeasts, pulp liquefies and drains off.[5] After the yeasts, lactic acid bacteria dominate the fermentation and lactic acid is produced. During the subsequent aerobic phase of fermentation, supported by occasional mixing or turning of the mass, acetic acid bacteria metabolize ethanol to acetic acid, causing a rise in the temperature up to approximately 50 °C.[3] The pH value of the cocoa beans drops as a result of the uptake of lactic and acetic acids from initially approximately 6.4 down to 4.0−5.0.[4] This induces the inactivation of the embryo and initiates the degradation of cell walls and membranes, facilitating contact of endogenous enzymes with matrix ingredients, such as proteins, carbohydrates, and polyphenols, within the cytoplasm throughout the bean.[6,7] During this treatment, important aroma precursors, such as peptides, amino acids, and glucose and fructose are liberated.[8,9] Furthermore, polyphenols are oxidized and transformed, resulting in the browning of the bean and a reduction of bitterness and astringency.[10,11] Typically, after 5−7 days of fermentation, the beans are then spread for sun drying on the ground or trays, until the moisture content of the beans is below approximately 7%, which provides storability for transportation.[12] An alternative drying method is using artificial dryers with moderate temperatures up to 60 °C.[3] During the

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drying process, oxidation and polymerization of polyphenols continue and undesired volatile acids are reduced to some extent.[13] Already after drying, thermally induced compounds, such as Strecker aldehydes, furanones, and pyrazines, can be detected in low contents.[14,15]

Cocoa bean fermentation can be considered as a spontaneous process, which is not easy to control; natural fluctuations of raw material, unstandardized fermentation techniques, and natural variables, such as changing weather conditions, during postharvest processing have a great influence on the final quality of the dried product.[1,16] Therefore, quality fluctuations are often an issue in the chocolate industry.

A lot of previous research has focused on investigating the influence of different fermentation methods, such as heap and box fermentations, pre-conditioning of the beans in terms of partial removal of the pulp or pod-storage periods, the influence of the microbial flora on the cocoa bean, and the use of starter cultures, with the aim for a better process control.[17−20] Moreover, since the 1960s, *in vitro* trials with pH-adjusted solutions and controlled temperature profiles based on the traditional fermentation technique/process were conducted, to investigate enzymatic degradation of dried protein extracts from cocoa beans, with the aim to possibly understand the processes within the cocoa beans induced by fermentation more thoroughly.[21] Later such "fermentation-like incubation" or "artificial fermentation" trials were conducted with fresh whole beans, to mimic the crucial parameters of natural fermentation without proliferation of microorganisms aiming to study in depth the chemical and structural changes inside cocoa beans and to possibly gain a better process control during postharvest treatment.[22−24] This way, it could be shown that not the microorganisms themselves but their metabolism and metabolites create the conditions in the fermentation heap and beans, i.e., the rise in the temperature, the acidification of the tissue of heap and beans, and thereby inactivation of the embryo, which are all factors facilitating the most important biochemical transformations within the beans. These crucial steps lead to the diffusion and homogenization of soluble constituents throughout the bean, enabling the contact of enzymes and substrates until the beans are dried to their final moisture content of approximately 7%.[6,8] Further research demonstrated that a phase inversion within the bean takes place during fermentation, which leaves aqueous inclusions with dissolved constituents trapped inside a continuous fat phase.[25] On the contrary, when fresh beans are dried instantly after harvest, the seed also loses viability and the same phase inversion takes place but there is no prior release and homogenization of enzymes and substrate into the aqueous phase within the beans; therefore, potential reactants for aroma and aroma precursor formations stay separated, unless the beans would be further processed with moisture.[25]

The activity of the remaining enzymes in under- or unfermented cocoa was also the subject of a prior investigation.[26] *In vitro* incubation trials with defatted cocoa powders made from unfermented and dried cocoa showed sufficient remaining activity to reach equal aroma precursor concentrations compared to regular fermented cocoa.

In a more recent study investigating incubations of fresh cocoa beans in acetic acid solutions, it was reported for the first time that a prototype chocolate was made from the material after drying.[23] First results showed pleasant and typical chocolate taste with slight astringency, bitterness, and acidity. However, the sensory data were not published, and aroma formation during incubation of cocoa beans has not yet been analyzed on a molecular level.

Although incubation-like fermentation of fresh beans could be proven as an alternative postharvest treatment technique, it is not yet feasible to be successfully implemented on farm sites, because of the high economical costs for farmers, who would be required to put a sophisticated infrastructure in place.

To possibly overcome this problem, a novel approach for postharvest treatment has been set up. Unfermented and dried cocoa nibs, thus, storable and easy to transport, were used to test a time- and location-independent postharvest treatment by means of rehydration, adjusting the pH of the nibs, followed by incubation with subsequent artificial drying. The aim of this study was to investigate the effect of such an incubation treatment of unfermented and dried cocoa nibs on the resulting aroma constitution of the material after drying and to compare it (a) to the material before treatment and (b) with traditionally fermented and dried cocoa beans of the same harvest. For this reason, methodologies of the molecular sensory science approach, such as sensory analysis, screening for aroma compounds by gas chromatography−olfactometry (GC−O) in combination with aroma extract dilution analysis (AEDA), and quantitation by stable isotope dilution analysis (SIDA), were used,[27] with the aim to gain a better understanding on the aroma formation during incubation.

MATERIALS

Chemicals. For identification and determination of retention indices, the following chemicals were used (sources given in parentheses): acetic acid, 4-allyl-2-methoxyphenol, butanoic acid, 2,3-diethyl-5-methylpyrazine, dimethyl trisulfide, 2-ethyl-3,5-dimethylpyrazin, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 4-methylpentanoate, ethyl methylpropanoate, ethyl 3-phenylprop-2-enoate, ethyl 3-phenylpropionate, 3-ethylphenol, ethyl phenylacetate, 2-heptanol, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 2-isobutyl-3-methoxypyrazine, 2-isopropyl-3-methoxypyrazine, linalool, 2-methoxyphenol, 2- and 3-methylbutanals, 3-methyl-1-butanol, 2- and 3-methylbutanoic acids, 3-methylindole, 2-methyl-3(methyldithio)furane, methyl 3-phenylprop-2-enoate, methylpropanoic acid, (*E*,*E*)-2,4-nonadienal, (*E*,*Z*)-2,6-nonadienal, 1-octen-3-one, phenylacetaldehyde, phenylacetic acid, 2-phenylethanol, 2-phenylethyl acetate (Sigma-Aldrich, Buchs, Switzerland), 2-heptanol acetate (BOC Sciences, Shirley, NY, U.S.A.), and *trans*-4,5-epoxy-(*E*)-2-decenal (AromaLAB GmbH, Martinsried, Germany).

For quantitation, the following isotopically substituted standards were used (sources given in parentheses): 2-($^2H_3$)methylbutanal, ethyl 3-($^2H_3$)methyl-1-(2,2,3,4,4,4-$^2H_6$)butanoate, ($^2H_6$)dimethyl trisulfide, 2-($^2H_5$)ethyl-3,6-dimethylpyrazine, 3-($^2H_3$)methyl-(2,2,3,4,4,4-$^2H_6$)-butanoic acid, ethyl (2,3,4,5,6-$^2H_5$)phenylacetate, 2-($^2H_3$)-methoxyphenol, 2-(2,3,4,5,6-$^2H_5$)phenylethanol, 4-ethyl-(2,6-$^2H_2$)-phenol, 2-methyl-5-(^{13}C)methyl-4-hydroxy-2,3-dihydro-(5-^{13}C)furan-3-one (AromaLAB GmbH, Martinsried, Germany), and ($^{13}C_2$)acetic acid (Sigma-Aldrich, Buchs, Switzerland).

For the incubation trials, lactic acid (Sigma-Aldrich, Inc., Buchs, Switzerland) and ethanol (Alcosuisse AG, Bern, Switzerland) were used.

Raw Materials. Cocoa of Trinitario variety was obtained from a cocoa supplier in Costa Rica during the harvest of 2016. The pods were harvested and opened within 24 h. The bean−pulp mass was removed and transferred to the first of four perforated wooden fermentation boxes arranged in tiers with a capacity of approximately 800 kg of bean−pulp mass. The mass was covered with banana leaves and fermented for 48 h before it was transferred to the next box in to aerate and mix the mass. This mixing step was repeated after approximately 72 and 96 h, respectively, before fermentation was stopped by drying after approximately 120 h. The beans were dried in a drying hall on tables

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Table 1. Odor-Active Compounds Identified in Aroma Distillates Isolated from Incubated and Dried Cocoa, Unfermented and Dried Cocoa, and Traditionally Fermented and Dried Cocoa during AEDA

			retention index			FD factor[a]	
number[b]	odorant[c]	odor quality[d]	FFAP	OV-1701	incubated cocoa	unfermented cocoa	fermented cocoa
1	2- and 3-methylbutanals[f]	malty		714	8[e]		
2	ethyl methylpropanoate[f]	fruity	954	810	64		64
3	ethyl 2-methylbutanoate[g]	fruity	1041	905	128	128	512
4	ethyl 3-methylbutanoate[g]	fruity	1059	908	64	16	64
5	ethyl 4-methylpentanoate[g]	fruity	1180		32	32[i]	4
6	3-methyl-1-butanol[g]	malty	1204		16	4	
7	2-heptanol acetate[g]	fruity	1255		32	16[i]	4
8	1-octen-3-one[h]	mushroom-like	1293	1072	32	4[i]	32
9	2-heptanol[g]	citric, sweet	1313		32	32[i]	128
10	dimethyl trisulfide[h]	cabbage-like	1360	967			128
11	unknown	earthy	1403			32	32
12	2-isopropyl-3-methoxypyrazine[h]	bell pepper-like	1418		32	32	32
13	acetic acid[g]	pungent, sour	1441		256	128	1024
14	2-ethyl-3,5-dimethylpyrazine[h]	earthy	1450	1152	32	8	128
15	2,3-diethyl-5-methylpyrazine[h]	earthy	1483	1228	4	16[i]	4
16	unknown	bell pepper-like	1498			4	4
17	2-isobutyl-3-methoxypyrazine[h]	bell pepper-like	1510	1241	128	64	256
18	linalool[a]	citrus-like, bergamot-like	1543	1195	16		16
19	methylpropanoic acid[g]	pungent, sweaty	1557				512
20	(*E*,*Z*)-2,6-nonadienal[g]	fatty, green	1572			4	4
21	butanoic acid[g]	rancid	1617		8	16	16
22	phenylacetaldehyde[h]	sweet, honey	1634	1193	32		16
23	2-methyl-3(methyldithio)furane[h]	meaty, nutty	1653	1271			64
24	2- and 3-methylbutanoic acids[h]	pungent, sweaty	1666		128	16	512
25	unknown	fruity, cinnamon-like	1676		128		
26	(*E*,*E*)-2,4-nonadienal[h]	fatty, green	1686	1348	32	4[i]	4
27	unknown	meaty, nutty	1712			8	256
28	ethyl phenylacetate[g]	flowery, fruity	1783	1364	32	4	64
29	2-phenylethyl acetate[g]	dried fruits-like, flowery	1802	1477	256	16	256
30	2-methoxyphenol[g]	smoky	1854	1226	16	4	1024
31	ethyl 3-phenylpropionate[h]	dried fruits-like, flowery	1873		64	16	
32	2-phenylethanol[g]	flowery	1896	1283	1024	256	1024
33	*trans*-4,5-epoxy-(*E*)-2-decenal[h]	metallic	1996		8	16	
34	unknown	sweet, fruity	2020		8		
35	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone[h]	caramel-like	2035	1240	64	16	64
36	methyl 3-phenylprop-2-enoate[h]	fruity, cinnamon-like	2068		4		16
37	ethyl 3-phenylprop-2-enoate[g]	fruity, cinnamon-like	2122		512	256	256
38	4-allyl-2-methoxyphenol[h]	smoky	2164		4	4[i]	4
39	3-ethylphenol[h]	phenolic, animalic	2188		256	128[i]	1024
40	3-hydroxy-4,5-dimethylfuran-2(5*H*)-one[h]	seasoning	2208	1347	128	8	64
41	unknown	flowery	2314		8		64
42	unknown	cardboard-like	2326		4	8	16
43	3-methylindole[h]	mothball-like	2490				4
44	phenylacetic acid[g]	beeswax-like	2576		4	4	64

[a]Flavor dilution factor determined by AEDA on capillary free fatty acid phase (FFAP). [b]Number of identified compound based on the retention index on FFAP. [c]Odorant name. [d]Odor quality perceived at the sniffing port. [e]Flavor dilution factor determined by AEDA on capillary OV-1701. [f]Identification based on the retention index and odor quality of the compound found in the literature.[14,15] [g]Identification by comparison of the odor quality at the sniffing port, mass spectrum (EI mode), and retention index on FFAP to the reference substance. [h]Identification by comparison of the odor quality at the sniffing port and retention index on FFAP to the reference substance. [i]Substances not yet reported in unfermented and dried cocoa.

for approximately 10 days under continuous mixing of the cocoa beans. For the unfermented material, the bean−pulp mass was directly dried with the same method. The dried fermented and unfermented beans were filled in jute bags and shipped to Switzerland. Until further use, the beans were stored at 12 °C.

Incubation of Cocoa Material. To obtain the nibs for incubation trials, the beans were broken (Limprimita cocoa breaker, Capco/Castlebroom Engineering, Ltd., Ipswich, U.K.), deshelled (cocoa winnower large, Capco/Castlebroom Engineering, Ltd., Ipswich, U.K.), and classified by manually sieving through a 6 and 3 mm sieve, to make sure only nibs within this size range were used.

Eight portions of 150 g (±0.1 g) of unfermented and dried cocoa nibs were filled into polypropylene vacuum bags with the size of 20 × 30 cm (VC999 Verpackungssysteme AG, Herisau, Switzerland). To each

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bag, 80 g (±0.1 g) of 0.1 mol/L lactic acid solution containing 5% (v/v) ethanol was added, to adjust the pH value from 6.3 to 5.1 in the nibs and reach a final moisture content of 35% as determined in pre-trials. The addition of ethanol resulted in the suppression of the proliferation of microorganisms because it could be proofed in preliminary experiments (unpublished results). The bags were evacuated and sealed with a vacuum chamber machine (type K4, VC999 Verpackungssysteme AG, Herisau, Switzerland) to a pressure of <200 Pa and stored for 12 h at 4 °C to rehydrate the cocoa samples prior to incubation. Subsequently, the bags were opened and fumigated with oxygen to simulate the aerobic phase of traditional fermentation (type Biogon, PanGas AG, Dagmersellen, Switzerland) using the vacuum chamber machine (as described above) and sealed afterward, respectively. The incubations were performed in a laboratory incubator (B 5042, Heraeus GmbH, Hanau, Germany) at 45 °C for 72 h, mixing the nibs every 12 h by manual shaking of the bags. To stop the treatment, the sample bags were opened and the material of all bags was mixed before drying in aluminum trays with a layer thickness of a maximum of 1 cm under occasional turning in a laboratory oven with air circulation (VD23, Binder GmbH, Tuttlingen, Germany) at 40 °C for 24 h. Thereby, the final products reached a moisture content of <6%. The samples are hereafter referred to as "incubated", "unfermented", and "fermented" in the text. The incubation of the beans was performed under strict aerobic conditions as a result of the fact that sensory evaluations of cocoa materials obtained from incubation trials for 72 h under aerobic conditions showed more pleasant aroma qualities in contrast to the samples incubated under anaerobic conditions, as determined during pre-studies (unpublished data).

METHODS

Measurement of the pH Value. For the determination of the pH value, 5 ± 0.1 g of cocoa bean mass was frozen with liquid nitrogen and milled with a laboratory hammer mill (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Then, 45 mL of boiling deionized water was added. Before the pH value was measured (pH-meter type 827 pH lab, Metrohm AG, Herisau, Switzerland), the cocoa and deionized water slurry was cooled to 20 °C by stirring in a thermostated water bath.

Measurement of the Moisture Content. A total of 5 ± 0.1 g of sample was milled, as described above, and dried in a laboratory oven (VD23, Binder GmbH, Tuttlingen, Germany) at 103 ± 1 °C for 4−6 h using sea sand for homogeneous drying until weight equilibrium was reached.

Orthonasal Sensory Analysis. The sensory analysis was performed with a panel of six trained panelists. The panel was trained with the following reference substances dissolved in sunflower oil for the given aroma perception given in parentheses: 3-methyl 1-butanol (malty), ethyl 2-methylbutanoate (fruity), phenylacetaldehyde (floral), acetic acid (pungent), and 3-methylbutanoic acid (sweaty) (Sigma-Aldrich, Inc., Buchs, Switzerland).

For the evaluation of the cocoa material, 20 ± 1 g of sample was milled as described above and subsequently filled into a closed Erlenmeyer flask. For the orthonasal evaluation, the samples were randomized and the panel assessed the intensity of the selected aroma attributes with scores between 0 (not detectable) and 5 (strong). The results were calculated as means (n = 6).

Isolation of Volatiles for GC−O. Aroma-active compounds were isolated in the same manner as previously described.[28]

For the isolation of the volatile compounds, 20 g of cocoa material was frozen in liquid nitrogen, milled with a laboratory hammer mill (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany), and extracted with 200 mL of diethyl ether by vigorous stirring at room temperature for 12 h. Separation of the volatiles from the non-volatiles was performed using a solvent-assisted flavor evaporation (SAFE) distillation unit, and the extract was subsequently concentrated to a final volume of 300 μL as previously described.[28]

Quantitation of Selected Aroma Compounds. For the quantitation of relevant aroma compounds in the samples identified by GC−O, SIDA was applied. Sample preparation was performed in the same manner as previously described.[28,29] To detect compounds in high and low concentrations, 2 and 50 g of sample were extracted with 20 and 200 mL of diethyl ether, respectively, after isotopically substituted internal standards were added. To calculate the concentrations of the target compounds, a three-point calibration line was used. The calibration lines were generated by analysis of mixtures of analytes and isotopically substituted standards in three different ratios and plotting the area ratios of selected ions of standard and analytes against the ratio of the respective concentrations. The absolute amounts of the analytes in the samples were determined via the calibration line. The ions used for quantitation and the calibration lines can be found in Table S1 of the Supporting Information. All samples were analyzed in triplicates, and the results were calculated as means.

GC−O and Gas Chromatography−Mass Spectrometry (GC−MS). GC−O in combination with AEDA, identification, and SIDA with GC−MS of selected compounds was performed in the same manner, using the same equipment, as previously described.[28,29]

RESULTS AND DISCUSSION

Identification of Odor-Active Constituents in Fermented, Unfermented, and Incubated Samples. Table 1 shows the results of the performed AEDA. Overall, 44 compounds with a flavor dilution (FD) factor of >4 have been detected in the investigated cocoa materials: 39 compounds in the fermented−dried sample, 35 compounds in the incubated sample, and 32 compounds in the unfermented−dried sample, respectively. Thereby, 7 of the before mentioned aroma compounds with a FD factor of >4 could not be identified by the identification criteria mentioned in Table 1.

Many of the well-known key aroma compounds of fermented and dried cocoa, such as ethyl 2- and ethyl 3-methylbutanoates, acetic acid, 2-ethyl 3,5-dimethylpyrazine, 2,3-diethyl 5-methylpyrazine, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine, butanoic acid, 2- and 3-methylbutanoic acids, 2-phenylethyl acetate, 2-methoxyphenol, 2-phenylethanol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, ethyl phenylacetate, ethyl 3-phenylprop-2-enoate, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, and phenylacetic acid, could be detected in the unfermented, incubated, and traditionally fermented samples. This is in agreement with the literature, where all of these key odorants are already detectable during AEDA or have been quantitated in unfermented cocoa.[15,29]

The incubated sample showed the highest FD factors for 2-phenylethanol (FD of 1024) and ethyl 3-phenylprop-2-enoate (FD of 512). The unfermented material showed lower FD factors in comparison to the other samples, with the highest FD factor of 256 for the floral 2-phenylethanol and the fruity ethyl 3-phenylprop-2-enoate in comparison to the fermented and incubated samples. In the fermented sample, the odorants acetic acid, 2-methoxyphenol, 2-phenylethanol, and 3-ethylphenol could be detected with the highest FD factor of 1024. Overall, the fermented sample showed more odor-active compounds with high FD factors.

Esters with fruity odors, such as ethyl methylpropanoate, ethyl 3-methylbutanoate, ethyl 4-methylpentanoate, 2-heptanol acetate, 2-phenylethyl acetate, ethyl 3-phenylpropionate, and ethyl 3-phenylprop-2-enoate, were analyzed with an equal or even higher FD factor in the incubated sample compared to the other samples, suggesting a possible biochemical formation during incubation.

In contrast to that, the traditionally fermented sample showed higher FD factors in comparison to the unfermented and incubated samples, especially for the unpleasant smelling odorants acetic acid, methyl propanoic acid, butanoic acid,

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Table 2. Results of the Quantitation of the Odorants from Incubated and Dried Cocoa, Unfermented and Dried Cocoa, and Traditionally Fermented and Dried Cocoa

	content (μg/kg)					
	incubated cocoa		unfermented cocoa		fermented cocoa	
odorant[a]	mean	RSD[b] (%)	mean	RSD[b] (%)	mean	RSD[b] (%)
acids						
acetic acid	166000	5.1	377000	0.5	1050000	1.5
2-methylbutanoic acid	6470	1.1	2410	21.4	20100	2.5
3-methylbutanoic acid	3700	1.7	3270	35.5	72500	2.9
alcohols						
2-phenylethanol	1990	4.9	1970	1.5	1790	6.6
aldehydes						
3-methylbutanal	4480	4.7	139	33.1	622	9.4
2-methylbutanal	2010	3.5	60.7	10.2	705	1.3
esters						
ethyl phenylacetate	963	1.6	34.4	1.6	281	0.8
ethyl 3-methylbutanoate	120	6.9	<14		34.2	2.7
2-phenylethyl acetate	110	1.4	99.1	0.4	1300	1.2
ethyl 2-methylbutanoate	58.2	3.2	9.56	7.7	22.1	2.8
furanones						
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	0.6	8.3	4.79	33.3	26.6	11
phenols						
2-methoxyphenol	0.19	24.2	1.11	11.2	221	6.0
3-ethylphenol	<1.4		<1.4		7.66	8.7
pyrazines						
2-ethyl-3,5-dimethylpyrazine	1.86	6.6	3.93	5.3	39.5	3.1
2-ethyl-3,6-dimethylpyrazine	0.98	3.5	0.8	1.3	0.63	1.8
other odorants						
dimethyl trisulfide	<0.6		<0.6		2.5	15

[a]Odorant name. [b]Relative standard deviation was calculated from quantitative data obtained from three extractions of each sample.

and 2- and 3-methylbutanoic acids with FD factors of 1024, 512, 16, and 512, respectively. These acids are well-known as major compounds of fermented cocoa but have also been found in unfermented cocoa.[15]

Furthermore, methyl propanoic acid, dimethyl trisulfide and 2-methyl-3-(methyldithio)furan were detected exclusively in the traditionally fermented cocoa with high FD factors, indicating a possible formation during microbial fermentation and presumably a subsequent diffusion into the cocoa beans.[14,15]

Linalool has only been detected in the incubated and fermented samples (FD of 16). This is in accordance with a previous study, indicating that linalool may be released from glycosides within the bean during postharvest treatment.[29] On the other hand, the malty odorant 3-methyl 1-butanol could only be detected in the unfermented (FD of 4) and incubated (FD of 16) samples, suggesting a possible degradation during fermentation into the corresponding acid or transformation to the respective ester. Further malty smelling compounds, such as the Strecker aldehydes 2- and 3-methylbutanals, were only perceivable in the incubated sample during AEDA with a relatively low FD factor of 8.

The heterocyclic odorants 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone and 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one were analyzed with higher FD factors in the fermented and incubated samples. This is in accordance with another study, where both odorants were detectable in unfermented cocoa and increased during fermentation.[15]

Quantitation of Selected Aroma Compounds in Fermented, Unfermented, and Incubated Cocoa Samples. The results of the quantitation of selected aroma compounds in the incubated and dried, unfermented and dried, and fermented and dried cocoa are shown in Table 2.

Among the acids, acetic acid was the most abundant compound in all of the samples, followed by 2- and 3-methylbutanoic acids and methylpropanoic acid. The incubated sample showed the lowest concentration of acetic acid (166 000 μg/kg), followed by the unfermented sample (377 000 μg/kg) and the fermented sample (1 050 000 μg/kg). The values for the unfermented and fermented samples are well in alignment with the values found in the literature.[14,15,30] The incubated sample showed lower acetic acid concentrations, suggesting possible losses during incubation and drying, and clearly shows that acetic acid is not formed during the moist incubation as opposed to the traditional fermentation. Furthermore, 2-methylbutanoic acid was found in higher levels in the fermented sample (20 100 μg/kg) compared to the incubated and unfermented samples (6470 and 2410 μg/kg). 3-Methylbutanoic acid was found with 20-fold higher levels in the fermented sample (72 500 μg/kg) compared to the incubated (3700 μg/kg) and unfermented (3270 μg/kg) material. These acids are known to be formed during the aerobic phase of the fermentation by microbial conversion of the precursors leucine and isoleucine.[31,32] Moreover, they may also be formed enzymatically inside the bean during regular plant metabolism, although to a much lesser extent in comparison to the microbial formation. This way, their presence in the dried and unfermented cocoa seems reasonable and in alignment with the findings of previous investigations.[15,33] Furthermore, they have also been reported to be generated during the Strecker reaction in the presence of oxygen.[34] Even though the drying temperature after incubation

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and during drying of the unfermented sample was below 40 °C, it is also possible that these acids derive from the Strecker degradation of amino acids during the drying process. High contents of short-chained carboxylic acids are undesired in the final product, because their sensory perception is described as mostly pungent, rancid, cheesy, or vomit-like.[15,35] Therefore, some of the downstream processes during chocolate manufacture, such as roasting and conching, aim at reducing these compounds.[36] Using the incubation as a postharvest treatment may therefore minimize processing times and costs, because these later downstream processes may not be needed.

For the quantitated alcohols, 2-phenylethanol was found in comparable concentrations (1990 μg/kg) in both the incubated and dried as well as the unfermented and dried material (1970 μg/kg). The content in the fermented and dried sample was a little lower in comparison (1790 μg/kg). In a previous study, it was shown that 2-phenylethanol is already present in small concentrations in unfermented and fresh cocoa beans and increases drastically during fermentation.[29] The occurrence of 2-phenylethanol in fermented cocoa has mainly been linked to the metabolism of yeasts via the Ehrlich pathway.[37] The presence of this compound in similar concentrations in the incubated, unfermented, and fermented samples however shows that an enzymatic formation or release from glucosides is also possible.[33,38,39] The lower amount of 2-phenylethanol in the traditionally fermented sample obtained in this investigation compared to the unfermented and incubated material might suggest that 2-phenylethanol is converted by microorganisms into further well-known cocoa aroma compounds, such as phenylacetic acid or 2-phenylethyl acetate.[40] The results of the quantitation of 2-phenylethyl acetate also support this assumption. This compound was the only ester that was found with a much higher concentration in the traditionally fermented sample (1300 μg/kg) compared to the incubated sample (110 μg/kg) and the unfermented sample (99.1 μg/kg). The incubated and dried sample contained higher levels of the esters ethyl 2- and ethyl 3-methylbutanoates (58.2 and 120 μg/kg) compared to the unfermented and dried (9.56 and <14 μg/kg) and fermented and dried (22.1 and 34.2 μg/kg) samples. Furthermore, a much higher level was found for ethyl phenylacetate (963 μg/kg) in the incubated sample than in the unfermented (34.4 μg/kg) and traditionally fermented (281 μg/kg) samples. The higher levels of esters in the incubated sample may be linked to the addition of 5% ethanol to the incubation medium, which may have resulted in a promoted formation of these esters via cocoa enzymes during incubation.

The Strecker aldehydes 2- and 3-methylbutanals were found with the highest concentrations in the incubated and dried sample (2010 and 4480 μg/kg), followed by the traditionally fermented sample (705 and 622 μg/kg) and unfermented and dried sample (60.7 and 139 μg/kg). The values given in the literature for 2- and 3-methylbutanals in fermented cocoa range between 1100 and 3900 μg/kg.[14,15] The values obtained in this study are well in line with those found in the literature; however, the levels of the traditionally fermented sample were analyzed in somewhat lower quantities. These aldehydes can derive from their corresponding amino acids by thermal formation but are also intermediates of the amino acid metabolism within plant cells.[38,40] The results may indicate a promoted release of the corresponding precursors, like amino acids, during incubation in comparison to the traditional fermentation and a subsequent conversion of those during the drying step. Furthermore, the addition of water to dry-processed foods is also known to promote the release of Strecker aldehydes from precursors,[41] whereby oxazolines are assumed as potential precursors.[42] However, oxazolines could not be proven until now in dried foods. In a previous investigation, the quantitated levels of Strecker aldehydes in chocolates made from fermented and dried nibs applying a novel technological process using water without a traditional roasting step were comparable to the levels found in traditionally manufactured chocolate,[28] indicating that there must be precursors releasing Stecker aldehydes upon the reaction with water. Therefore, the high contents of the Strecker aldehydes in the incubated sample in comparison to the other two samples might be explained by the fact that those compounds are released from yet unknown precursors upon the contact of water during the moist incubation.

Among the furanones, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone was found with the highest concentration in the fermented sample (26.6 μg/kg), followed by the unfermented (4.79 μg/kg) and incubated (0.6 μg/kg) samples. The values for the fermented and unfermented samples are in accordance with values given in the literature, whereby this compound could not be detected in unfermented cocoa and reached levels to approximately 35 μg/kg after fermentation and drying.[14,15] 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone is a known sugar degradation product, which is mostly supposed to be thermally generated; therefore, a formation during drying from carbohydrates seems possible to a small extent.[15] However, in comparison, the higher amounts obtained in the fermented sample might indicate a promoted microbial formation of the required precursors during fermentation and the formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone during drying.

2-Methoxyphenol was found in more than 200-fold higher concentrations in the fermented sample (221 μg/kg) compared to the unfermented (1.11 μg/kg) and incubated (0.19 μg/kg) samples, which is in accordance with concentrations given in the literature for unfermented and fermented samples.[14,15,29] This clearly indicates a fermentative formation with subsequent diffusion into the bean. Interestingly, the contents in the incubated and unfermented samples are very low, even though 2-methoxyphenol is also assumed to be formed enzymatically from ferulic acid inside the bean during fermentation and may also be formed thermally during drying.[15] The obtained results indicate that the availability of the presumed precursor ferulic acid, which may derive from lignin or glycosides, is much higher during fermentation. Thus, a microbial-induced release from the testa or pulp containing lignin or the corresponding glycosides seems reasonable.[43] An interesting fact is that the testa was removed prior to incubation, which also supports the assumption that the high content in the fermented sample mostly derives from outside the cotyledon. 3-Ethylphenol could only be quantified in the fermented sample (7.66 μg/kg), even though it was also detectable during AEDA in the other samples.

2-Ethyl-3,6-dimethylpyrazine showed little differences between the different samples, with the highest value obtained in the incubated sample. However, contents found in the literature are about 15-fold higher, suggesting variation between the different types of cocoa used.[14] On the other hand, the values obtained for 2-ethyl-3,5-dimethylpyrazine showed greater variation between the different treatments in the investigated materials. The highest value was measured for the fermented sample (39.5 μg/kg), which is well in line with values found in the literature.[14,15]

Dimethyl trisulfide could not be detected in the incubated and unfermented samples but could be quantified in the fermented

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Table 3. OAVs Calculated For Incubated and Dried Cocoa, Unfermented and Dried Cocoa, and Traditionally Fermented and Dried Cocoa

odorant[a]	odor threshold[b] (μg/kg)	OAV		
		incubated cocoa	unfermented cocoa	fermented cocoa
acetic acid	124[c]	1340	3040	8440
3-methylbutanal	13[c]	340	11	48
ethyl 2-methylbutanoate	0.26[c]	220	37	85
ethyl 3-methylbutanoate	0.62[c]	190	<1	55
3-methylbutanoic acid	22[c]	170	150	3300
2-methylbutanoic acid	203[d]	32	12	99
2-methylbutanal	140[c]	14	<1	5
2-phenylethanol	211[c]	9	9	8
ethyl phenylacetate	300[e]	3	<1	<1
2-ethyl-3,5-dimethylpyrazine	2.2[c]	<1	2	18
2-methoxyphenol	16[c]	<1	<1	14
2-phenylethyl acetate	233[d]	<1	<1	6
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	25[f]	<1	<1	1
dimethyl trisulfide	2.5[c]	<1	<1	1

[a]Odorant name. [b]Orthonasal threshold value determined in oil. [c]Orthonasal threshold value determined in oil according to ref 46. [d]Orthonasal threshold value determined in oil according to ref 47. [e]Orthonasal threshold value determined in oil according to ref 48. [f]Odor activity value calculated as the ratio of the amount in the sample to the threshold value determined in oil.

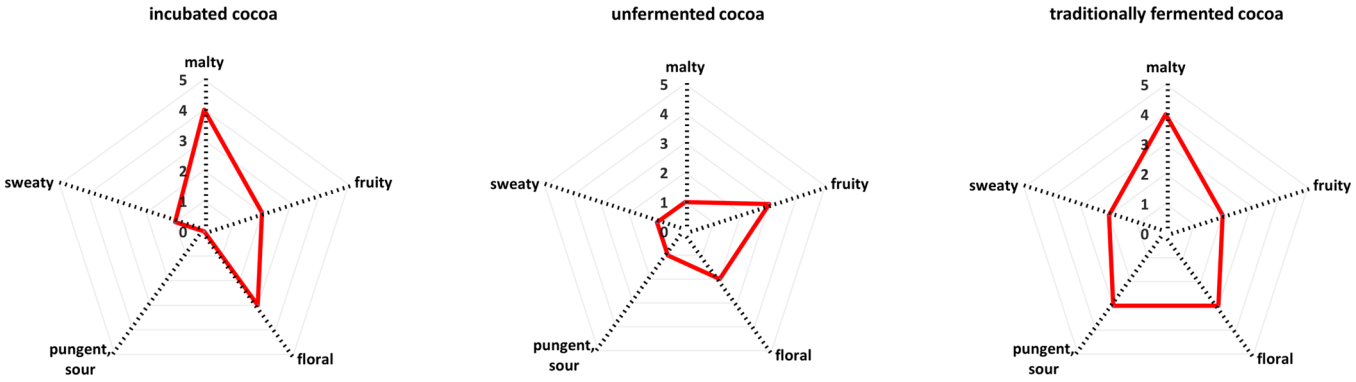

Figure 1. Orthonasal sensory profiles of the incubated and dried, unfermented and dried, and fermented and dried cocoa material.

sample with 2.5 μg/kg, which is in line with values obtained in the literature.[14] In addition to that, this compound was only detectable in the fermented sample during AEDA.

Volatile sulfurous compounds, such as dimethyl trisulfide, can derive from enzymatic degradation of sulfur-containing amino acids, such as methionine.[44] Methionine can be found in cocoa beans, meaning that an enzymatic formation within the bean is possible. The exclusive presence of dimethyl trisulfide in the traditionally fermented sample might be related to the lysis of yeast cells, resulting in the release of sulfurous amino acids as precursors for the dimethyl trisulfide formation, as recently demonstrated for wines with elevated amounts of this odorant.[45]

Odor Activity Values (OAVs) and Orthonasal Sensory Profiles. The calculated OAVs are shown in Table 3. The highest values for all samples were calculated for acetic acid, with the fermented sample reaching an OAV of 8440, the unfermented sample reaching an OAV of 3040, and the incubated sample reaching an OAV of 1340, respectively. The sweaty 2- and 3-methylbutanoic acids were also among the most odor-active compounds in both the incubated and unfermented samples, respectively, but values reached in the fermented and dried sample were up to 20 times higher. This can also be seen in the aroma profiles given in Figure 1. The traditionally fermented sample reached higher scores for the attributes "pungent" and "sweaty" compared to the incubated and unfermented samples. Furthermore, calculated OAVs for 2- and 3-methylbutanals in the incubated sample were 3−7-fold higher compared to the fermented sample. "Malty" notes were evaluated with equal intensity in the incubated and fermented samples by the sensory panel, but low intensities were perceived in the unfermented cocoa. Moreover, about 3-fold higher OAVs for the "fruity" esters ethyl 2- and ethyl 3-methylbutanoates were calculated for the incubated sample compared to the fermented sample. In addition to that, the fruity ethyl phenylacetate showed aroma activity only in the incubated sample, whereas 2-phenylethyl acetate reached an OAV of >1 only in the fermented sample. Although the incubated sample showed overall the highest OAV for the fruity smelling esters in comparison to the other samples, the unfermented sample was rated with the highest score for "fruity" during sensory analysis. "Floral" 2-phenylethanol reached comparable OAVs in all samples, reaching 9-fold the odor threshold level, although it was rated a little bit higher by the panel in the incubated and fermented material.

Furthermore, the fermented sample showed aroma activity values of >1 for the odorants 2-ethyl-3,5-dimethylpyrazine, 2-methoxyphenol, 2-phenylethyl acetate, dimethyl trisulfide, and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, whereas these com-

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pounds showed no aroma activity in the incubated and unfermented samples.

The study showed that the used incubation treatment of unfermented and dried cocoa facilitates contact of enzymes and substrates within the cotyledon tissue. The incubation clearly promoted the formation of many well-known key aroma compounds of cocoa beans, supposedly via enzymatic formation within the beans. On one hand, higher quantities of "malty" and "fruity" odorants were measured in comparison to the fermented sample, indicating an enzymatic formation of these compounds, presumably supported by aerobic conditions, the presence of ethanol during incubation, and a higher drying temperature. On the other hand, compounds linked to negative attributes as "pungent, sour" and "sweaty", i.e., acetic acid and 2- and 3-methylbutanoic acids, have been quantitated with much lower concentrations in the incubated sample compared to the fermented sample, suggesting that "sweaty" and "pungent, sour" aroma compounds are preferably formed by microbial activity. However, these compounds were repeatedly reported to be among the key aroma compounds in cocoa, even though the positive contributions of these odorants on the overall perception of cocoa aroma has not yet been thoroughly investigated. The findings show that this novel postharvest treatment has the potential to serve as a controllable and reproducible postharvest treatment, yielding cocoa material with less microbial-originated aroma compounds, such as volatile acids. This could not only be of impact for traditional chocolate manufacture, where many of the processing steps, like roasting or conching, aim at reducing these compounds, but it is also of importance for new technologies, which process cocoa nibs without roasting.[32]

To fully understand the consequences of the different aroma compositions within the beans derived from raw materials and estimate the true potential of the moist incubation of unfermented and dried cocoa, future trials should include a roasting step and the measurement of the aroma-active compounds of the final product, chocolate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b06119.

Cocoa odorants, standards, selected ions (m/z) of analytes and standards, and calibration lines used for quantitation (Table S1) (PDF)

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Notes

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■ ABBREVIATIONS USED

AEDA, aroma extract dilution analysis; FD, flavor dilution; FFAP, free fatty acid phase; GC−MS, gas chromatography−mass spectrometry; GC−O, gas chromatography−olfactometry; OAV, odor activity value; SAFE, solvent-assisted flavor evaporation; SIDA, stable isotope dilution analysis; ZHAW, Zurich University of Applied Sciences

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