Comparison of the Aroma Composition and Sensory Properties of Dark Chocolates made with Moist Incubated and Fermented Cocoa Beans

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Abstract

In a previous investigation “moist incubation”, was described as a novel postharvest treatment for cocoa, and the aroma composition of the resulting cocoa nibs was compared to unfermented and fermented cocoa nibs. For this treatment unfermented and dried nibs are rehydrated with an aqueous solution containing lactic acid and ethanol to adjust the pH-value and are subsequently incubated at 45°C under aerobic conditions for 72 h before drying. The aim of the present study was to investigate the sensory properties and aroma composition of dark chocolates made of these materials after roasting. Therefore, gas-chromatography-olfactometry (GC-O) in combination with aroma extract dilution analysis (AEDA), quantitation with isotopically labelled standards, odor activity value (OAV) determination and sensory analysis were performed. The three different chocolates had distinct sensory and OAV profiles. The sensory profiles showed a higher intensity of fruity aroma notes and lower intensity of bitterness and astringency in the chocolate made with the moist incubated cocoa, while the chocolate made of fermented cocoa reached higher scores in the roasty aroma notes. Furthermore, higher OAVs were determined for the Strecker aldehydes in the chocolate made of the moist incubated cocoa, whereas higher OAVs for the pyrazines and the acids were detected in the chocolate made of fermented cocoa. In contrast, the chocolate produced with the unfermented cocoa showed low cocoa specific aroma notes and high levels of astringency and bitterness. The detected differences reveal interesting insights into the influence of different postharvest treatments on the resulting aroma composition in the final chocolate. Furthermore, the alternative postharvest treatment was demonstrated to result in chocolates with a pleasant sensory profile.

Keywords

Cocoa postharvest treatment; cocoa incubation; dark chocolate; cocoa aroma formation; sensory evaluation

Introduction

Cocoa is the main ingredient for chocolate, being one of the most favored sweets worldwide, beloved for its very distinct sensory properties. Before fresh cocoa beans can provide a desired aroma as well as the favored slight bitterness and astringency, biochemical transformation within the fresh beans` chemical composition is needed. During the traditional postharvest treatment, a spontaneous microbial degradation of the adhering fruit pulp surrounding the beans, leads to conditions inducing the desired biochemical changes in the beans.[1,2] The key factors can be summarized as the acidification of the beans` tissue, targeting a pH value of approximately 4.5-5.5, a temperature rise to approximately 45-50 °C, and the availability of oxygen.[2]

The reconstruction of the traditional fermentation process under controlled conditions in vitro without the influence of microorganisms was subject of many previous studies.[3–7] For this "fermentation-like incubation" beans were removed from the fresh cocoa pod, depulped and then incubated at controlled temperatures in pH-adjusted solutions. It was shown that the formation of aroma-relevant precursors as well as a directed transformation of polyphenols[5] could be achieved to the same extent as during traditional fermentations so a possible commercial use was discussed.[4] However, this process is restricted to the use of fresh beans, hence the process has to take place on or close to a farm site. Furthermore, rather high expenditures for infrastructure are needed. Therefore, an alternative approach, independent from time- and location referred to as "moist incubation" has been proposed.[8] In contrast to the fermentation-like incubation using beans freshly removed from the cocoa pod, for the moist-incubation unfermented and dried cocoa nibs are used, which are storable and may be transported to any production site. For the treatment unfermented and dried nibs are rehydrated with an aqueous solution containing lactic acid and ethanol to adjust the pH-value and are subsequently incubated at 45°C under aerobic conditions for 72 h before drying. During a first investigation,[8] the aroma formation before and after this treatment was investigated on a molecular level and compared to fermented cocoa. The results indicated

that aroma formation within the beans can be achieved independently of microbial degradation of the pulp, when applying the moist incubation treatment on unfermented and dried beans. However, the results showed differences in the abundancy of certain cocoa key odorants. Esters and Strecker aldehydes were found in equal or higher quantities in the moist incubated sample compared to the fermented sample. On the other hand, the fermented sample showed higher quantities in compounds such as acetic acid and 2- and 3-methylbutanoic acid. The material was investigated after applying the postharvest treatment including a drying step to directly compare the effect of the applied postharvest treatment without further processing like roasting. However, it remained unclear, if the detected differences are still detectable in the final product, the chocolate.

Therefore, the aim of the present study was to characterize the sensory properties and decode the aroma profiles on a molecular level of the same materials used in the previous study (moist incubated, fermented, and unfermented cocoa)[8] after processing them into model chocolates. Gas-chromatography-olfactometry (GC-O) in combination with aroma extract dilution analysis (AEDA), quantitation with isotopically labelled standards and sensory analysis[9] were performed to decode the aroma properties of the three model chocolates on molecular level and gain further insights on the influence of the different postharvest treatments on the generation of cocoa key odorants in the final products, the chocolates.

Material and Methods

Chemicals

For identification and determination of retention indices, the following chemicals were used: acetic acid, (*E,E*)-2,4-decadienal, 2,3-diethyl-5-methylpyrazine, dimethyl trisulfide, 2-ethyl-3,5-dimethylpyrazine, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl methylpropanoate, 2-ethyl-5-methylpyrazine, ethyl 3-phenylprop-2-enoate, ethyl 3-phenylpropanoate, 3-ethylphenol, ethyl phenylacetate, 3-hydroxy-4,5-dimethylfuran-2(*5H*)-one, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, 2-isobutyl-3-methoxypyrazine,

2-methoxyphenol, 2- and 3-methylbutanal, 2- and 3-methylbutanoic acid, 2-methyl-3(methyldithio)furane, methylpropanoic acid, (*E,E*)-2,4-nonadienal, phenylacetic acid, 2-phenylethanol, 2-phenylethyl acetate, and trimethylpyrazine were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland).

For quantitation, the following isotopically substituted standards were used: 2-($^2H_3$)methylbutanal, 3-($^2H_3$)methyl(3,4,4,4-$^2H_4$)butanal, ethyl 3-($^2H_3$)methyl(2,2,3,4,4,4-$^2H_6$)butanoate, ($^2H_6$)dimethyl trisulfide, 2-($^3H_2$)methyl-3,5-dimethylpyrazine, 2-($^2H_5$)ethyl-3,6-dimethylpyrazine, 2-(1,1-$^2H_2$)ethyl-3(1,1-$^2H_2$)ethyl-5-($^2H_3$)methylpyrazine, 2-methyl-3-(($^2H_3$)methyldithio)furane, 3-($^2H_3$)methyl-(2,2,3,4,4,4-$^2H_6$)butanoic acid, 2-($^2H_5$)phenylethyl acetate, ($^2H_5$)ethyl-3-phenylpropanoate, 2-($^2H_5$)phenylethanol, 4-hydroxy-2-methyl-5-(^{13}C)methyl(5-^{13}C)furan-3(*2H*)-one, ethyl-3-($^2H_5$)phenyl(2,3-$^2H_2$)prop-2-enoate, phenyl($^{13}C_2$)acetic acid (AromaLAB GmbH, Martinsried, Germany), ($^{13}C_2$)acetic acid (Merck KGaA, Darmstadt, Germany).

Raw Materials

The moist incubated and dried cocoa, the unfermented and dried, as well as the fermented and dried cocoa material as obtained during the previous study[8] were used to prepare the prototype chocolates. Cocoa of the cultivar Trinitario was harvested on a farm in Costa Rica and a batch of approximately 800 kg was filled in a wooden fermentation box and covered with banana leaves to start the fermentation. Mixing and aeration by transferring the mass to the next box was firstly performed after 48 h and was repeated every 24 h until a total fermentation time of approximately 120 h was reached. The beans were then spread on trays in a drying hall and dried under occasional mixing for approximately 10 days. To obtain unfermented material, one part of the fresh beans was directly spread on wooden drying trays to suppress fermentation and dried on trays in the same way as fermented cocoa beans. Samples of fermented and unfermented beans were shipped to Switzerland and stored at 12 °C until they were broken and deshelled to obtain unfermented and fermented cocoa nibs. For the moist incubated material eight

97 portions of 150 g (± 0.1 g) unfermented nibs were rehydrated under vacuum in a sealed bag for 12 h at 4
98 °C with 80 g (± 0.1 g) of aqueous solution containing lactic acid (0.1 mol/L) and ethanol (5 % v/v) to
99 reach a pH value in the cocoa solids of 5.1 and a final moisture content of 35 %. The bags were then
100 opened, fumigated with oxygen, sealed and then incubated at 45 °C for 72 h in a laboratory incubator
101 under occasional mixing by turning the bags every 12 h. After incubation the material was mixed and
102 dried on trays using a laboratory oven at 40 °C for 24 h in a laboratory oven with air circulation under
103 occasional turning until a final moisture content < 6 % was reached.
104 For the formulation of the chocolate prototypes commercially available deodorized cocoa butter (Carma,
105 Barry Callebaut AG, Zurich, Switzerland), white crystal sugar (Schweizer Zucker AG, Frauenfeld,
106 Switzerland), and sunflower-lecithin (Bunge Ltd., Chesterfield, USA) were used.
107 **Preparation of Prototype Chocolates**
108 Sample materials were frozen with liquid nitrogen (PanGas AG, Dagmersellen, Switzerland) and ground
109 with a kitchen blender (Thermomix®, Vorwerk AG, Dierikon, Switzerland) to a particle size < 2 mm. To
110 provide a reproducible, homogenous, quick roasting, and subsequent quick cooling of the cocoa material,
111 an adapted roasting method based on a thin layer roasting technique developed by Mohr (1970)[10] was
112 performed: approximately 50 g of finely ground cocoa powder was evenly distributed with a maximum
113 layer thickness of 3 mm on one half of a 30 cm × 60 cm sheet of aluminum foil and covered with the
114 other half after folding in the middle. The edges were folded to keep the cocoa powder in place. These
115 envelopes were then roasted for 10 min in an oven (type H 5081-60 BP, Miele AG, Spreitenbach,
116 Switzerland) between two preheated (125 °C ± 2 °C) tailor-made solid aluminum plates (35 cm × 30 cm
117 × 1.5 cm) with a thermocouple (type T, EBI 40 TC-01, Xylem Analytics Germany Sales GmbH & Co.
118 KG, Ingolstadt, Germany) attached measuring the temperature of the plate at the point of contact with
119 the envelope. Pre-trials showed that the temperature of the cocoa powder inside the envelope reached the
120 temperature of the aluminum plates within 60 s. A roasting time of 10 minutes was defined in pre-trials,
121 in which this process time resulted in the most balanced aroma profile of the material. After the roasting,

the envelopes were removed and placed flat on a steel surface to quickly cool down to room temperature. The roasted cocoa material was hereafter mixed with crystal sugar and refined two times with a 3-roll refiner (Type SDY 200, Bühler AG, Uzwil, Switzerland) to reach a particle size below 25 µm. This premix was manually homogenized with cocoa butter and lecithin to prepare a 70 % prototype chocolate, containing 50 % cocoa mass, 20 % cocoa butter, 29.5 % sugar, and 0.5 % lecithin. The chocolate masses were not conched to avoid further changes in the aroma constitution after roasting. Pre-trials showed that good textural properties can be achieved by this preparation technique. The chocolates were then manually pre-crystallized and filled in chocolate bar molds. After complete crystallization, the bars were wrapped in aluminum foil, vacuum packed, and frozen at -20 °C until use for analysis. The chocolate samples are hereafter referred to as "incubated chocolate", "fermented chocolate", and "unfermented chocolate".

Methods

Sensory Analysis

The sensory evaluation of the obtained chocolates was carried out in the form of profiling with a trained panel (n=8), referring to the *Quantitative Descriptive Analysis* (QDA®) method, and according to the ISO 13299:2016 standard. Altogether, 10 attributes in the three main categories aroma, taste, and texture were defined (Table S1). For profiling of the chocolate samples, the intensity of the chosen attributes was rated on a continuous line scale from "0 = not perceivable" to "10 = very intense". These evaluations were done in the sensory lab of the Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland. The samples were blinded by labeling with random three-digit codes. The experimental design was set up according to a *Randomized Complete Block Design* (RCBD), meaning that each sample was randomly assigned to each panelist and each panelist was then evaluating all three samples in one single session. Panelists were invited for an additional session to do a second evaluation of all three test samples. The presentation of the samples was carried out one by one, following a sequential-monadic presentation order. For neutralization between the samples water and saltless crackers were used. The

data was analyzed using the statistical software XLSTAT 2018 (Addinsoft, New York, USA), carrying out a two-way analysis of variance (ANOVA) and a post-hoc test (Fisher`s L.S.D.) to determine significant differences between the samples. The results of the evaluation and the corresponding standard deviations can be found in Table S2.

Sample Preparation and Isolation of Volatiles for GC-O Analysis and Quantitation

Aroma compounds were isolated in the same manner as previously described.[8,11] To prepare an extract for the GC-O analysis and identification of aroma compounds 20 g of chocolate were cut into fine pieces with a kitchen knife and extracted with 200 ml diethyl ether by stirring at room temperature for 12 h. For the quantitation of compounds in high and low concentrations samples of 2 g and 50 g were extracted with 20 mL and 200 mL respectively in the same manner, after isotopically labelled standards of the target compounds were added. Separation of the volatiles from the non-volatiles for the GC-O extract as well as the extracts used for quantitation was performed using a SAFE distillation unit and the extract was subsequently concentrated to a final volume of 300 µL.

Identification of Aroma Compounds Using Gas Chromatography-Olfactometry (GC-O) and Gas Chromatography-Mass Spectrometry (GC-MS)

GC-O in combination with AEDA and identification with GC-MS of selected compounds was performed in the same manner, using the same equipment, as previously described.[8,11,12]

Quantitation of Selected Aroma Compounds

The quantitation of selected compounds was done in the same manner, using the same equipment, as previously described.[8] The concentration of target compounds was calculated using a five-point calibration line. To obtain the calibration lines, mixtures of analytes and isotopically substituted standards in five different ratios (1:5, 1:2; 1:1, 2:1; 5:1) were analyzed. The peak area ratios of selected ions of standard and analytes were plotted against the ratios of the respective concentrations. Quantitation of analytes in the samples were determined with the calibration line using linear regression. The ions used for quantitation, and the calibration lines can be found in the supporting information Table S3. All

samples were analyzed in triplicates (unless stated differently in the results table) and the results were calculated as mean values.

Results and Discussion

Sensory Profiles of the Dark Chocolates Made with Incubated, Fermented, and Unfermented Chocolates

The sensory scores in the defined attributes and the illustrated sensory profiles are shown in Figure 1. While the incubated and the fermented chocolates were described having a differing, but typical dark chocolate flavor profile with the pleasant attributes of dark chocolate such as malty, roasty, fruity, and flowery aroma notes, as well as slight bitterness and low astringency, the unfermented chocolate was not perceived as typical. The data showed a significant difference (α=0.05) in many of the attributes between the three samples (Table S1). The incubated chocolate showed a somewhat higher intensity score among flowery, fruity, malty, and caramel-like aroma notes. On the other hand, roasty aroma notes were rated higher in the fermented chocolate. The unfermented chocolate was rated with an overall low aroma intensity with the highest score for the attribute green. Furthermore, the samples showed differences in the perception of the taste attributes. The incubated chocolate was perceived sweeter than the other chocolates. Additionally, the bitterness and astringency were perceived in a lower intensity compared to the other samples. Among the three samples, the unfermented chocolate reached the highest scores for both attributes, suggesting the desired transformation of polyphenols, usually induced by the fermentation, and drying, was suppressed compared to the moist incubation and fermentation.

Identification of Odor-Active Constituents in the Incubated, Fermented, and Unfermented Chocolates

Table 1 shows the results of the performed AEDA. Overall, 29 compounds with a flavor dilution factor (FD factor) >4 have been detected in the investigated chocolates: 26 compounds in the incubated chocolate sample, 25 compounds in the fermented chocolate, and 15 compounds in the unfermented

196 chocolate, respectively. One aroma compound with a FD factor >4 could not be identified by the
197 identification criteria mentioned in Table 1.

198 Many well-known aroma compounds which have also been previously found in roasted cocoa and dark
199 chocolates such as 2- and 3- methylbutanal, 2-ethyl-3,6-dimethylpyrazine, acetic acid, 2-isobutyl-3-
200 methoxypyrazine, 2-methyl-3(methyldithio)furane, 2- and 3-methylbutanoic acid, 2-phenylethyl acetate,
201 ethyl 3-phenylpropionate, 2-phenylethanol, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, ethyl 3-phenyl-
202 prop-2-enoate, 3-hydroxy-4,5-dimethylfuran-2(*5H*)-one, and phenylacetic acid[11,13] were detected in all
203 samples analyzed during this investigation. Additionally, an unknown compound with a meaty and nutty
204 odor was detected with high FD factor of 256 in the incubated chocolate and a FD factor of 128 in the
205 fermented chocolate This unknown compound was also detected with a somewhat lower intensity in the
206 unfermented chocolate with a FD factor of 16.

207 Overall, the fermented and incubated chocolates showed a comparable number of typical dark chocolate
208 and cocoa key aroma compounds, with comparable FD factors. As expected, fewer compounds with
209 generally lower FD factors were detected in the unfermented chocolate, indicating a higher concentration
210 of aroma precursors present in the material after fermentation or moist incubation.

211 The highest FD factor of 1024 was found for the caramel-like 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone
212 in both the fermented and the incubated chocolates. This fact fits well with previous findings, where 4-
213 hydroxy-2,5-dimethyl-3(*2H*)-furanone was identified as one of the key odorants in the cocoa mass after
214 roasting and also in chocolate.[11,14–16]

215 A major difference in the AEDA results can be seen for the earthy, nutty, and roasty smelling pyrazines.
216 2,3,5-Trimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, 2- ethyl 3,5-dimethylpyrazine, and 2,3-diethyl
217 5-methylpyrazine were detected with higher FD factors in the fermented chocolate compared to the
218 incubated chocolate, whereas only 2,3,5-trimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine were
219 detected with a low FD factor of 4. In the past, many different pyrazines have been identified in the
220 aroma of roasted cocoa and chocolate and they are generally regarded as important contributors to the

aroma of chocolate.[17–19] In contrast to that, a recent study where roasted Forastero beans were analyzed, it was concluded that the pyrazines are of minor importance for the cocoa aroma.[14]

Furthermore, another difference between the aroma composition of the three chocolates of the present study was detected among the organic acids, acetic acid, 2- and 3-methylbutanaoic acid, and methylpropanoic acid. These components showed higher FD factors in the fermented chocolate compared to the incubated and the unfermented chocolate. Even though they are usually referred to unpleasant odor descriptions such as pungent, sour, vinegar-like, sweaty, or rancid, in many studies these acids reach the highest FD factors and consequently reach very high odor activity values in cocoa and chocolate.[13–16,20] The fact that many traditional chocolate making processing steps, like drying, roasting, thin layer treatment of cocoa liquor, and conching aim at reducing these compounds,[19,21,22] underlines that these compounds, especially when present in high concentrations, are rather undesirable.

Furthermore, the important malty Strecker aldehydes 2- and 3-methylbutanal were detected with comparable FD factors in the incubated chocolate and in the fermented chocolate, respectively. These compounds can be directly linked to the presence of their parent amino acids leucine and isoleucine in the raw material. These amino acids are known to be important precursors released during fermentation within the bean.[2,22] Only minor differences were detected in terms of FD factors between the fermented and incubated chocolates for the fruity and flowery esters. While ethyl methylpropanoate, ethyl 2-methylbutanoate, and ethyl 3-methylbutanoate, ethyl phenylacetate, and 2-phenylethyl acetate reached rather low FD factors in the incubated and fermented chocolates, ethyl 3- phenylpropionate and ethyl 3-phenylprop-2-enoate showed high FD factors. Furthermore, the flowery smelling alcohol 2-phenyl-ethanol was detected in all three chocolates with a relatively high FD factor of 256 for the fermented and incubated chocolate and FD 128 for the unfermented chocolate sample, respectively. Previous studies showed that this odorant is already present in unfermented cocoa and concentrations do not change significantly during roasting.[14–16]

Other well-known odorants of cocoa and chocolate such as the cabbage-like dimethyl trisulfide and the cooked meat-like 2-methyl-3-(methyldithio)furane showed slightly higher FD factors in the incubated chocolate compared to the fermented chocolate. In the unfermented chocolate dimethyl trisulfide could not be detected and 2-methyl-3-(methyldithio)furane showed a lower FD factor in comparison to the incubated and fermented chocolates. Other studies showed that these odorants are present in cocoa after fermentation and increase during roasting.[14–16] In a recent study, where two commercially available dark chocolates with 90 % and 99 % cocoa content were investigated, dimethyl trisulfide reached the highest FD factors during GC-O analysis and even showed the highest OAV during quantitation of both samples, underlining the importance of these compounds for the cocoa aroma.[13]

Furthermore, the fatty and green components (*E,E*)-2,4-nonadienal and (*E,E*)-2,4-decadienal were detected in the incubated chocolate with FD factors of 4 and 64 and in the fermented chocolate with slightly higher FD factors of 64 and 128, respectively. These compounds have been identified in chocolate and cocoa and are known to be thermally induced lipid oxidation products.[23,24] However, both compounds were not detected in the unfermented chocolate.

Another main difference in the GC-O profiles of the samples was found for the phenolic and animalic smelling 3-ethylphenol and the smoky smelling 2-methoxyphenol. 3-Ethylphenol was exclusively detected in the unfermented chocolate with a FD factor of 16. 2-Methoxyphenol was detected with a high FD factor of 256 in the fermented chocolate. On the other hand, the incubated chocolate showed a low FD factor of 4 and this odorant was not detectable in the unfermented chocolate. The same trend for this compound was observed in the corresponding unroasted raw materials, suggesting that its formation is strongly linked to microbial activity during fermentation.[8]

Quantitation of Selected Aroma Compounds in the Investigated Chocolate Samples

The results of the quantitation of the selected aroma compounds in the incubated, the fermented and the unfermented chocolates are shown in Table 2. Among the quantitated volatiles, acetic acid was the most abundant compound in all the samples. The lowest concentration was measured in the incubated

chocolate (23.4 mg/kg), followed by the unfermented chocolate (40.0 mg/kg). The fact that less acetic acid was found in the incubated chocolate is in accordance with the values measured in the raw material before roasting. This way, a loss of acetic acid during the drying step of this treatment procedure can be suggested.[8] The higher value for the fermented chocolate (55.7 mg/kg) is in accordance with another study, where quantities of 53.7-87.7 mg/kg were found in commercially available dark chocolates with cocoa contents ranging from 70-85 %.[11] Furthermore, higher concentrations of 2- and 3-methylbutanoic acid were also measured in the fermented chocolate (1,760 µg/kg and 3,450 µg/kg) compared to the incubated chocolate (418 µg/kg and 1,330 µg/kg). In other studies, concentrations ranged from 391-1,670 µg/kg for 2-methylbutanoic acid and 438-3,320 µg/kg for 3-methylbutanoic acid were measured, showing the values obtained in the present study can be compared to the ones of commercially available chocolates.[11,13] For the unfermented chocolate, far lower concentrations for these compounds were observed (135 µg/kg and 195 µg/kg). 2- and 3-Methylbutanoic acid are known to increase during traditional fermentation, but they can also be generated during Strecker degradation from their corresponding parent amino acids leucine and isoleucine during thermal treatment.[1,21,25] A slight increase after roasting of cocoa beans was detectable in different studies.[14–16] Therefore, the high concentrations in the fermented chocolate of the present study may derive from both processing steps – the fermentation and the subsequent roasting –, while the moderate content of the incubated chocolate might be linked to the formation of these compounds during the roasting process.

Interesting results were also found for the malty compounds 2- and 3-methylbutanal. These odorants showed higher concentrations in the incubated chocolate (274 µg/kg and 916 µg/kg) compared to the fermented chocolate (104 µg/kg and 587 µg/kg) and the unfermented chocolate (53.1 µg/kg and 208 µg/kg). The same trend was found when the raw material was analyzed before roasting and preparation of chocolate. These important compounds are known to be formed by Strecker degradation from their parent α-amino acids leucine and isoleucine.[24] Previous studies showed, that Strecker aldehydes can be released upon contact with water in dry foods[26,27] and also from fermented and dried, unroasted cocoa

beans after treatment with water.[28] The results of the present study suggest that the combined effect of the moist treatment as well as the formation of aldehydes during drying and subsequent roasting could lead to overall higher amounts in the incubated chocolate in comparison to the fermented chocolate. Furthermore, the pyrazine concentrations of the incubated chocolate for 2-ethyl-3,5-dimethylpyrazine (7.91 µg/kg), 2-ethyl-3,6-dimethylpyrazine (26.0 µg/kg), 2,3,5-trimethylpyrazine (7.11 µg/kg), and 2,3-diethyl-5-methylpyrazine (0.23 µg/kg) were comparable to the concentrations found in the unfermented chocolate (8.88 µg/kg, 15.7 µg/kg, 6.34 µg/kg, and 0.13 µg/kg), while the concentrations in the fermented chocolate showed 5- to 20-fold higher values (111 µg/kg, 120 µg/kg, 136 µg/kg, 1.84 µg/kg). Pyrazines are known to be formed from α-aminoketones during Strecker degradation in the Maillard reaction[29], but it was shown by Scalone et al. (2015) that they may also derive from oligopeptides.[30] Furthermore, it was shown that the use of oligopeptides as precursors promotes pyrazine formation compared to the use of free amino acids in model systems.[30,31] Short peptides have gained increasing attention as being significantly responsible precursors for cocoa aroma formation. Recently, 34 Amadori and Heyns compounds deriving from di- and tripeptides in fermented and dried cocoa have been identified for the first time.[32] Unfortunately, the authors did not investigate the volatile profiles deriving from these Maillard reaction intermediates. However, their presence in fermented and dried cocoa and the findings that oligopeptides are known to promote the formation of pyrazines suggests that these precursors may have been formed to a larger extent during microbial fermentation compared to the moist incubation. Furthermore, the results in a study from Zou et al. (2018) showed a promoted formation of Strecker aldehydes when free amino acids were used as Maillard reaction precursors as compared to oligopeptides.[31] The higher quantities of pyrazines found in the fermented sample and the higher quantities of Strecker aldehydes in the incubated sample could therefore indicate that the formation of peptides was promoted during fermentation, while higher quantities of free amino acids were generated after the moist incubation treatment. However, this has to be proven by the respective precursor measurements in the different materials. A possible reason for the different precursor formation may be

due to differences in the pH value reached in the cotyledon during the postharvest treatment. It is known that lower pH values reached during fermentation promote the formation of oligopeptides, while higher pH values promote the formation of amino acids, especially the important precursors leucine, isoleucine, valine and phenylalanine.[33]

The floral smelling 2-phenylethanol was quantitated in comparable amounts in the incubated (1,880 µg/kg) and the unfermented chocolate (1,830 µg/kg), while lower amounts were found in the fermented chocolate (1,530 µg/kg). This is in accordance with values obtained during a previous study, where lower amounts were measured in the fermented cocoa beans.[8] 2-Phenylethanol can be converted by microorganisms to form phenylacetic acid and 2-phenylethyl acetate during fermentation.[22] This is also in line with the higher concentrations for the beeswax-like phenylacetic acid and 2-phenylethyl acetate in the fermented sample compared to the incubated and the unfermented chocolates. Another ester which was found with a slightly higher concentration in the fermented chocolate is ethyl 3-phenylprop-2-enoate (64.0 µg/kg) compared to the incubated (48.3 µg/kg) and the unfermented chocolate (33.6 µg/kg). On the other hand, the esters ethyl 3-methylbutanoate and ethyl 3-phenylpropanoate were measured in higher concentrations in the incubated chocolate with 3.94 µg/kg and 10.0 µg/kg compared to 1.88 µg/kg and 3.46 µg/kg in the fermented and 0.56 µg/kg and 3.93 µg/kg in the unfermented chocolate, suggesting that these esters may have been formed to a larger extent enzymatically in the cocoa bean material during the moist incubation.

Another important odorant is the caramel-like 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone. It reached the highest FD factors in the AEDA with a comparable concentration in the incubated chocolate (504 µg/kg) compared to the fermented chocolate (548 µg/kg), while the unfermented chocolate showed a much lower concentration (72.7 µg/kg). These results are in line with concentrations given in the literature.[11,14–16]

In addition to that, two sulfur containing odorants were quantitated: the cabbage-like dimethyl trisulfide and the meaty and nutty 2-methyl-3(methyldithio)furane. Both odorants have been identified in fermented cocoa beans and an increase of their concentrations was shown during roasting.[14,15] In the

incubated chocolate and the fermented chocolate of the present study, concentrations of dimethyl trisulfide were comparable, reaching 6.31 µg/kg and 4.45 µg/kg, respectively, being in line with values given in literature.[11,13–15] On the other hand, the concentration of this odorant was drastically lower in the unfermented chocolate with 0.69 µg/kg, suggesting that the corresponding precursors were formed during the moist incubation treatment and the fermentation in comparable intensities, while the necessary precursors were missing in the unfermented chocolate.

Comparison of the Calculated *Odor Activity Values*

The calculated OAVs of the odorants in the investigated chocolates are shown in Table 3. Eleven compounds with an OAV> 1 were detected in the incubated chocolate, twelve in the fermented and eight in the unfermented chocolate. Overall, the incubated and fermented chocolates reached higher values compared to the unfermented chocolate. The values determined for the incubated chocolate were somehow lower in comparison to the OAVs of the fermented chocolate. A major difference in the OAV profile of these two chocolates is the 13-fold higher OAV for 2-ethyl-3,5-methylpyrazine in the fermented chocolate, and the somewhat higher OAV determined for the Strecker aldehydes 2- and 3-methylbutanal in the incubated chocolate.

The highest OAV in the incubated chocolate were observed for dimethyl trisulfide (210), 3-methylbutanoic acid (120), and phenylacetic acid (114), followed by acetic acid (61), 3-methylbutanal (61), and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (19). On the other hand, the fermented chocolate showed the highest values for 3-methylbutanoic acid (313), phenylacetic acid (162), and dimethyl trisulfide (148), followed by acetic acid (145) and 2-ethyl-3,5-dimethylpyrazine (65). The highest OAV in the unfermented chocolate was reached by acetic acid (104), followed by phenylacetic acid (18), and 3-methylbutanal (14).

The different OAVs for all odorants of the three chocolates are reflected in the differences found for their sensory profiles. The high OAVs of pyrazines in the fermented sample might be linked to the intense

perception of the attribute roasty, and the lower scores in the fruity and flowery perception in comparison to the incubated sample during sensory evaluation. However, the fact that the incubated and the fermented chocolate both showed typical dark chocolate aroma properties despite the low amounts of pyrazines in the incubated sample confirms the findings of Frauendorfer et al. (2019) that pyrazines are of negligible importance for the cocoa aroma.[14]Furthermore, a twofold higher OAV for the fruity ester ethyl 3-methylbutanoate was determined for the incubated chocolate (4) in comparison to the fermented chocolate (2). Even though these OAVs were both comparably low, it is possible that fruity and flowery notes were suppressed by the stronger roasty aroma notes in the fermented chocolate, while the low concentrations of pyrazines support the flowery and fruity aroma perception of the incubated chocolate. A major difference in the aroma composition has been discovered among the pyrazines, which were measured in much higher concentrations in the fermented chocolate compared to the incubated and unfermented chocolate. This may be due to a promoted formation of oligopeptides during fermentation, while the higher amounts of Strecker aldehydes in the incubated chocolate suggests a promoted formation of free amino acids.

The aroma of all chocolates was perceived as different from each other, which is not surprising, because of the given differences of the postharvest treatments used in comparison. Overall, the fermented chocolate as well as the incubated chocolate showed a typical aroma and taste profile. In comparison, the unfermented chocolate did not elicit the pleasant attributes of dark chocolate, such as malty, roasty, fruity, and flowery aroma notes and was mostly perceived as green. Thereby, the green odor impression might be a result of the low abundancies of 2- and 3-methylbutanal, the pyrazines, dimethyl trisulfide and 4-hydroxy-2,5-dimethyl-3(2H)-furanone and dimethyl trisulfide and on the other hand the relatively high odor activity value of acetic acid and and also the presence of 2-isobutyl-3-methoxypyrazine, which concentration and OAV was not determined due to its relatively low FD-factor. Furthermore, a high adstringency and bitterness could be perceived in the unfermented chocolate. In this study, it was observed that reduction of astringency and bitterness as reached during fermentation and drying, can also

be achieved by applying the moist incubation treatment. This might be linked to the extant polyphenol oxidase activity of the incubated cocoa tissue in addition to the use of an oxygen atmosphere during the incubation, as well as the contact of the incubated cocoa powder during drying. The present study showed that the moist incubation treatment of unfermented and dried nibs provides an intermediate product, which can be used to produce a chocolate with a pleasant aroma and taste. Thus, the proposed technique has the potential to serve as an alternative reproducible time- and location-independent postharvest treatment, which can be easily controlled. Besides, this study gives interesting insights about the formation of typical cocoa aroma compounds, especially the Strecker aldehydes and pyrazines, which were formed to different extents, depending on the applied postharvest treatment. To understand the underlying mechanisms leading to the obtained aroma and taste profile, more research regarding the non-volatile components is necessary.

Abbreviations Used

AEDA, Aroma Extract Dilution Analysis; FD, Flavor Dilution; FFAP, Free Fatty Acid Phase; GC-MS, Gaschromatography-Mass Spectrometry; GC-O, Gaschromatography-Olfactometry; OAV, Odor Activity Value; SAFE, Solvent Assisted Flavor Evaporation; ZHAW, Zurich University of Applied Sciences

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Conflict of Interest

The authors declare no competing financial interest.

Supporting Information Description

Table S1. Attributes, Definitions and References for the Evaluation of the Incubated, Unfermented and Fermented Chocolates

Table S2. Means Scores of Attributes, p-values from ANOVA and Results from Post-Hoc Test (Fisher`s L.S.D.) from the Sensory Evaluation of the Incubated, Unfermented and Fermented Chocolates

Table S3. Cocoa Odorants, Standards, Selected Ions (m/z) of Analytes, Standards and Calibration Lines Used For Quantitation

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Figure Captions

Figure 1. Sensory Profiles of the Incubated (IC), Unfermented (UC), and Fermented Chocolate (FC)

Table 1 Flavor Dilution Factors of Compounds Determined in Aroma Distillates Isolated from Incubated Chocolate (IC), Unfermented Chocolate (UC) and Fermented Chocolate (FC) during AEDA

			retention index on		FD factor[d]		
no.[a]	odorant[b]	odor quality[c]	FFAP	OV-1701	IC	UC	FC
1	2- and 3-methylbutanal[e,f]	malty	927	710	256	16	128
2	ethyl methylpropanoate[g]	fruity	950	818	8	<4	<4
3	ethyl 2-methylbutanoate[g]	fruity	1019	907	8	<4	16
4	ethyl 3-methylbutanoate[g]	fruity	1042	910	8	<4	<4
5	dimethyl trisulfide[g]	cabbage-like	1358	1030	64	<4	16
6	2-ethyl 5-methylpyrazine[g]	earthy	1376	n.d.	4	<4	<4
7	trimethylpyrazine[g]	earthy	1391	1080	4	<4	64
8	2-isopropyl-3-methoxypyrazine[g]	bell pepper-like	1414	1139	4	<4	16
9	2-ethyl-3,6-dimethylpyrazine[g]	earthy	1430	1153	4	4	256
10	acetic acid[h]	pungent	1439	n.d.	64	16	256
11	2-ethyl-3,5-dimethylpyrazine[g]	earthy	1446	1160	<4	<4	16
12	2,3-diethyl-5-methylpyrazine[g]	earthy	1475	1220	<4	<4	64
13	2-isobutyl-3-methoxypyrazine[g]	bell pepper-like	1505	1239	4	16	16
14	methylpropanoic acid[h]	pungent, sweaty	1551	n.d.	4	<4	16
15	2-methyl-3(methyldithio)furane[g]	meaty, nutty	1649	1265	128	16	64
16	2- and 3-methylbutanoic acid[h]	pungent, sweaty	1653	n.d.	128	16	128
17	*(E,E)*-2,4-nonadienal[g]	fatty, green	1686	1350	8	<4	64
18	unknown	meaty, nutty	1714	n.d.	256	16	128
19	ethyl phenylacetate[h]	flowery, fruity	1769	1360	4	<4	16
20	(*E,E*)-2,4-decadienal[h]	fatty, green	1795	n.d.	64	<4	128

(table continues

Table 1 Flavor Dilution Factors of Compounds Determined in Aroma Distillates Isolated from the Incubated Chocolate (IC), Unfermented Chocolate (UC) and Fermented Chocolate (FC) (continued)

			retention index on		FD factor[d]		
no.[a]	odorant[b]	odor quality[c]	FFAP	OV-1701	IC	UC	FC
21	2-phenylethyl acetate[h]	dried fruits-like, flowery	1799	1477	4	4	16
22	2-methoxyphenol[g]	smoky	1849	1226	4	<4	256
23	ethyl 3-phenylpropionate[g]	dried fruits-like, flowery	1867	n.d.	256	128	128
24	2-phenylethanol[h]	flowery	1898	1283	256	128	256
25	4-hydroxy-2,5-dimethyl-3*(2H)*-furanone[h]	caramel-like	2025	1240	1024	128	1024
26	ethyl 3-phenylprop-2-enoate[g]	fruity, cinnamon-like	2118	n.d.	128	128	128
27	3-ethylphenol[g]	phenolic, animalic	2170	n.d.	<4	16	<4
28	3-hydroxy-4,5-dimethylfuran-2*(5H)*-one[g]	seasoning	2190	1347	16	16	16
29	phenylacetic acid[g]	beeswax-like	2546	n.d.	32	16	64

a) number of identified compound based on retention index on capillary column FFAP, b) odorant name, c) odor quality perceived at sniffing port, d) flavor dilution factor determined by AEDA on capillary FFAP, e) flavor dilution factor determined by AEDA on capillary OV-1701, f) identification based on retention index and odor quality of compound found in literature[34], g) identification by comparison of odor quality at sniffing port and retention index on FFAP with reference substance , h) identification by comparison of odor quality at sniffing port, mass spectrum and retention index on FFAP with reference substance

Table 2 Results of the Quantitation of the Odorants in the Incubated Chocolate (IC), Unfermented Chocolate (UC) and Fermented Chocolate (FC)

Sample	IC		UC		FC	
odorant[a]	content (µg/kg)					
	mean	rel. SD[b] (%)	mean	rel. SD[b] (%)	mean	rel. SD[b] (%)
acetic acid	23400	3.3	40000	2.5	55700	2.5
2-methylbutanoic acid	418	1.4	134	4.4[c]	1760	3.0
3-methylbutanoic acid	1330	1.8	189	1.0[c]	3450	3.2
phenylacetic acid	2950	3.4	724	12.5	4210	1.7
2-phenylethanol	1880	0.5	1830	0.5	1530	0.3
2-methylbutanal	274	1.2	53.1	2.2[c]	104	0.0
3-methylbutanal	916	3.0	208	10.9	587	8.0
ethyl 3-methylbutanoate	3.94	2.8	0.56	13.6[c]	1.88	2.4
2-phenylethyl acetate	36.6	3.1	42.5	0.3[c]	257	1.1
ethyl-3-phenylprop-2-enoate	48.3	0.4	33.6	4.4	64.0	4.1
ethyl-3-phenylpropanoate	10.0	1.2	3.93	1.3	3.46	2.4
4-hydroxy-2,5-dimethyl-3*(2H)*-furanone	504	1.9	72.7	1.1	548	4.1
2-ethyl-3,5-dimethylpyrazine	7.91	1.5	8.88	0.8	111	0.7
2-ethyl-3,6-dimethylpyrazine	26.0	0.3	15.7	1.0	120	0.8
2,3,5-trimethypyrazine	7.11	4.7	6.34	0.5	136	0.7
2,3-diethyl-5-methylpyrazine	0.23	8.9	0.13	3.2	1.84	16.0
dimethyl trisulfide	6.31	2.2	0.69	11.0	4.45	2.5
2-methyl-3-(methyldithio)-furane	0.29	4.1	0.24	6.7	0.21	8.8

a) odorant name, b) relative standard deviation was calculated from quantitative data obtained from three extractions of each sample c) relative standard deviation was calculated from quantitative data obtained from two extractions of sample

Table 3 Odor Activity Values Calculated for the Incubated Chocolate (IC), Unfermented Chocolate (UC) and Fermented Chocolate (FC)

		IC	UC	FC
odorant[a]	odor treshold[b] [µg/kg]		OAV[c]	
dimethyl trisulfide	0.03	210	<1	148
3-methylbutanoic acid	11	121	17	314
phenylacetic acid	26	113	28	162
acetic acid	350[d]	67	114	159
3-methylbutanal	15	61	14	39
4-hydroxy-2,5-dimethyl-3(2H)-furanone	27	19	3	20
2-methylbutanal	34	8	<1	5
2-phenylethanol	490	4	4	3
2-ethyl-3,5-dimethylpyrazine	1.7	5	4	65
ethyl 3-methylbutanoate	0.98	4	1	2
2-methylbutanoic acid	110	4	<1	16
2-ethyl-3,6-dimethylpyrazine	76	<1	<1	2

a) odorant name, b) orthonasal threshold value determined in oil according to reference[34], c) odor activity value calculated as ratio of amount in sample to threshold value determined in oil, d) orthonasal threshold value determined in oil according to reference[35]

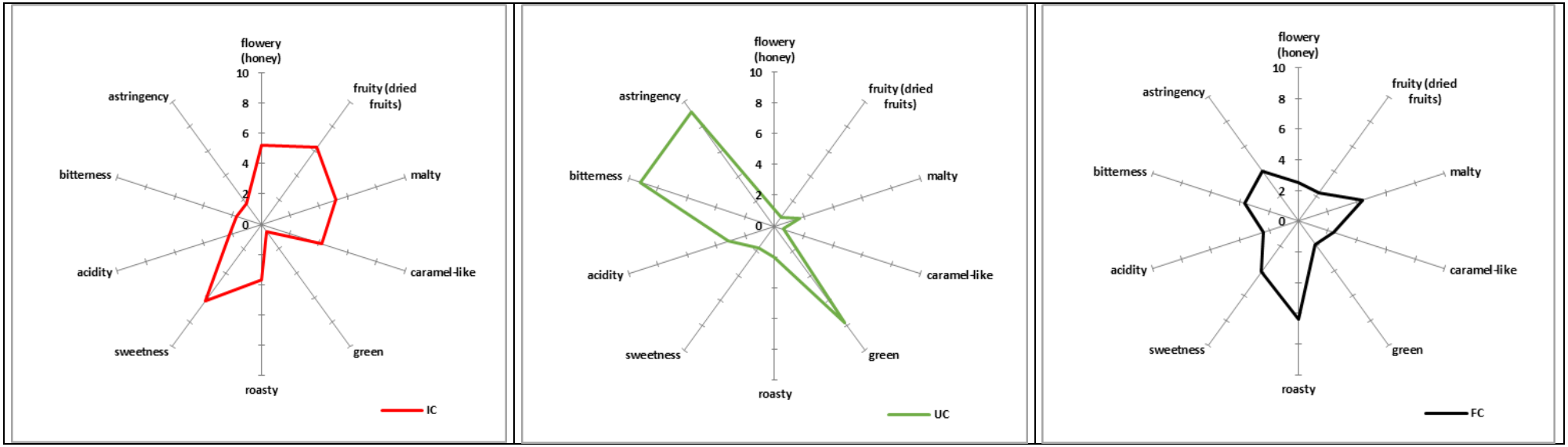
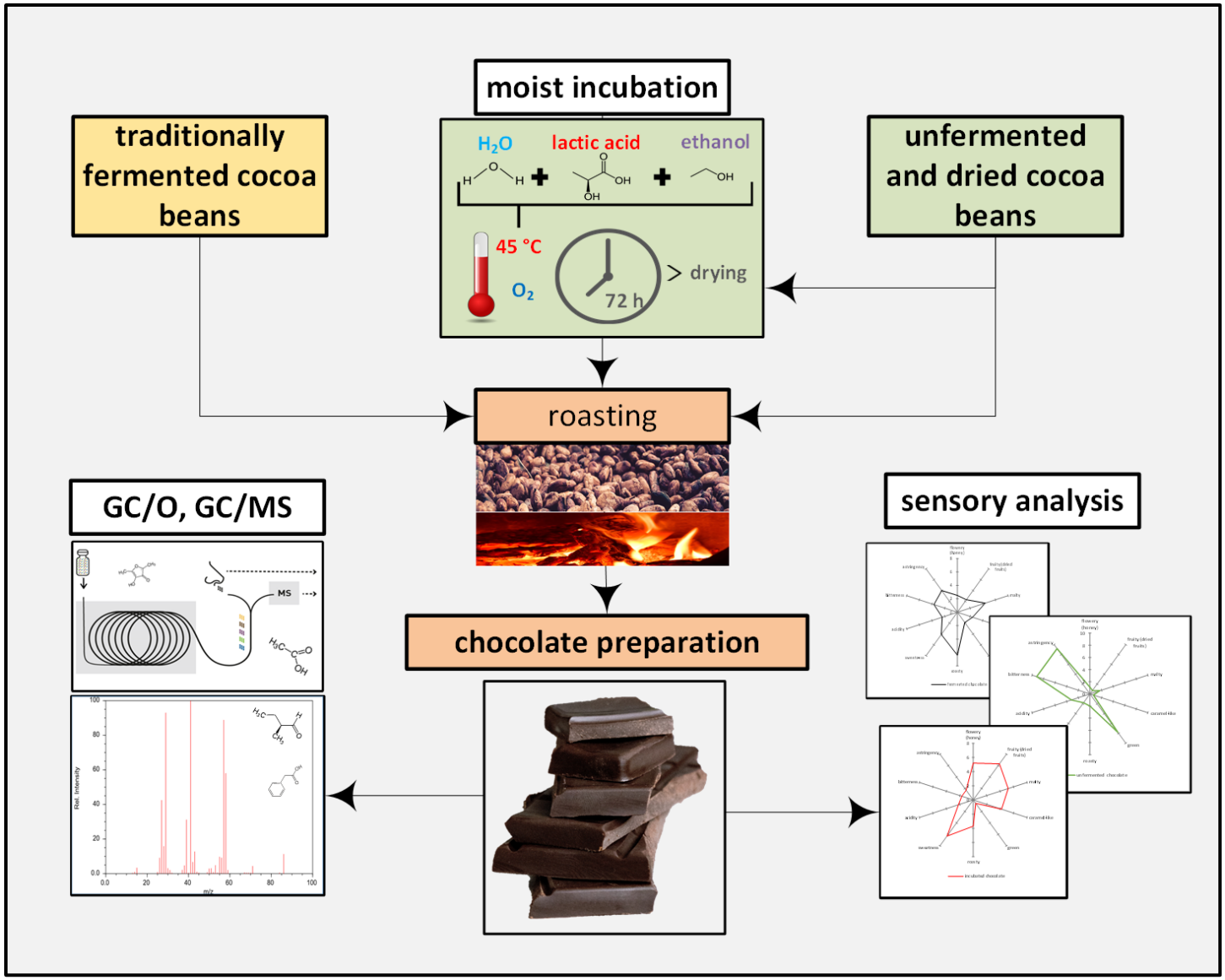


Figure 1 Sensory Profiles of the Incubated Chocolate (IC), Unfermented Chocolate (UC) and Fermented Chocolate (FC)

TOC:


moist incubation
traditionally fermented cocoa beans
unfermented and dried cocoa beans
H2O
lactic acid
ethanol
45 °C
O2
72 h
drying
roasting
GC/O, GC/MS
MS
sensory analysis
chocolate preparation