Volatile fingerprint of unroasted and roasted cocoa beans (Theobroma cacao L.) from different geographical origins

Volatile fingerprint of fermented and roasted cocoa beans (*Theobroma Cacao* L.) from different geographical origins

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ABSTRACT

The aroma characterization of 59 fermented and dried cocoa beans from 22 different geographical origin, covering the whole world cocoa production (America, Africa and Asia), was performed by HS/SPME/GC/MS analyses. Analysis of cocoa beans before and after roasting were performed to follow the aroma modification with the aim to achieve a volatile fingerprinting and a discrimination model based on beans origin.

A total of 62 volatiles was identified in fermented and dried beans, while 76 volatiles were identified in roasted cocoa beans. The compounds belong to several chemical groups including esters, alcohols, organic acids, aldehydes, ketones and pyrazines. Datasets were submitted to multivariate statistical analysis (Principal Component Analysis, PCA). Results allowed to discriminate not roasted cocoa beans based on their geographical origin; in particular, samples coming from African countries were separated from samples of Caribbean regions, whereas samples from Asia and Oceania were not discriminated. Principal component analysis, applied on the corresponding roasted samples showed that although the same roasting treatment has been applied to all the samples, the differences among the fermented samples are also maintained in the aromatic profile resulting after roasting confirming the discrimination model.

The volatile fingerprint showed interesting potential as authentication tools of raw and roasted cocoa beans, and from a technological point of view to give information in order to pilot the secondary processing steps of cocoa towards the final product designing.

Keywords

Fermented cocoa beans, roasted cocoa beans, flavour, fingerprint, geographical origin, PCA

INTRODUCTION

Cocoa authenticity is becoming an important issue due to the growing market of high quality cocoa of mono-origin. The geographic traceability of cocoa is important for both cocoa transforming industries and consumers. Food authenticity resides in food molecular composition and in the case of cocoa only recently studies have been focused on different classes of molecules trying to link them to cocoa geographic origin.

Cocoa of different geographical origins have different organoleptic characteristics and influence the final quality of chocolate (Cambrai et al., 2010). In particular, chocolate and cocoa aroma resides in large part in the corresponding volatile fraction, which is composed of a complex mixture of over 500 compounds and this complexity is demonstrated by the inability to reproduce in the laboratory cocoa typical flavor (Dimick, Hoskin, 2003). The cocoa aroma development is the result of many different technological processes applied to cocoa beans but it also depends on the cocoa variety and origin. Fermentation and roasting are the two crucial steps for aroma development during cocoa processing.

During fermentation numberless volatile compounds and flavor precursors were produced by microbial processes, which involves several biochemical reaction on carbohydrates, proteins and polyphenols already present in the beans. Several microorganisms have been reported as producers of volatile compounds during cocoa fermentation (Thompson, Miller, & Lopez, 2001). Alcohols, aldehydes and ketones have been reported as the major groups of compounds found in raw cocoa beans at the beginning of the fermentation (1 or 2 days). However, alcohols, esters and acids (acetic acid mainly) were developed in the middle of fermentation (3–5 days); finally acids, esters and alcohols were the most important groups of volatile compounds at the end fermentation (6–8 days) (Rodriguez-Campos, Escalona-Buendía, Orozco-Avila, Lugo-Cervantes, Jaramillo-Flores, 2011).

Carbohydrates are mainly present in the cocoa beans as simple sugars like sucrose, fructose and glucose; during fermentation, sucrose is almost completely hydrolyzed to fructose and glucose by the invertase enzyme.

During the fermentation of the beans the proteolysis, catalysed by aspartic endoprotease and carboxypeptidase, leds to the releasing of amino acids and oligopeptides. (Voigt & Biehl, 1995).
Although it is well known that relevant differences in enzyme activities exist between cocoa genotypes, simple and general relationships have not been yet established between genotype flavour and key enzyme activities in unfermented beans. Furthermore, how enzymatic processes are regulated, which substrates and products are related to desirable flavours, and which are the limiting factors for the enzymatic contribution to fermentation processes remain unclear.

During roasting process, flavor precursors generated during the fermentation step are transformed into flavor compounds through the Maillard reaction. Kirchhoff et al., (1989) observed a correlation between free amino acid accumulation and generation of specific aroma precursors.
The main aim of fermented beans roasting is to obtain two results: the elimination of undesirable compounds with low boiling point, such as acetic acid, and the formation of the typical flavor of roasted beans. The heat treatment actives reactions between reducing sugars and free amino acids or short-chain peptides, so leading to a significant reduction in the concentration of free amino acids and reducing sugars.
The compounds that originate during roasting are alcohols, ethers, furans, pyranes, thiazoles, acids, esters, imines, amines, ossazols and pyrroles. Nevertheless, the most important class of compounds are pyrazines and Strecker aldehydes. While pyrazines are not present in unfermented beans, fermented and dried beans contain low concentrations of them. During roasting, depending on time and temperature of treatment, the concentration of pyrazines further increases, giving rise to increasingly substituted pyrazines. Specific compounds recognized as cocoa/ nutty notes were: 2,3-dimethylpyrazine, trimethylpyrazine, tetramethylpyrazine, 3(or 2),5-dimethyl-2(or 3)-ethylpyrazine, 3,5(or 6)-diethyl-2-methylpyrazine. Three Strecker aldehydes present a strong chocolate flavor: 2-ethylpropanal, 2-methylbutanal, and 3-methylbutanal (Caligiani, Marseglia & Palla, 2015).

Flavor quality of chocolate usually depends on the cocoa beans source; beans from different origins can show distinct flavor characteristics such as acidic, hammy or smokey notes (Powell, 1983). Some authors have studied the influence of geographical origin of cocoa on the composition of the volatile fraction. Most studies concern the fermented, dried, and, most often, roasted beans (Hernandez & Rutledge, 1994; Muggler-Chavan & Reymond, 1967). The profile comparison and multivariate analyses allowed to distinguish beans (roasted or not) from various geographical origins (Cambrai et al., 2010).

Most of studies therefore are focused on a limited dataset, few are focused on fermented cocoa beans and rare are the works focused on fermented cocoa bean from a significant numbers of different countries representative of the world production, as instead considered in the present work.

SPME has been used to determine the volatile fingerprint of cocoa bean (Humston, Zhang, Brabeck, McShea, & Synovec, 2009). This technique has been reported to be cheap, solventless, fast and also with high reproducibility, low limits of detection and high sensitivity (Balasubramanian & Panigrahi, 2011; Ducki, Miralles-Garcia, Zumbé, Tornero, & Storey, 2008).

Cocoa aroma is generally studied after roasting, however, also fermented cocoa beans present a specific aroma that could be linked to the geographic origin and as a consequence to the spontaneous fermentation.

In this paper, aroma characterization of 59 fermented and dried cocoa beans samples from 22 different geographical origins was performed by HS/SPME/GC/MS analyses. Sampling is representative of the average world production (America, Africa, Asia).

Analysis of cocoa beans before and after roasting were performed to follow the aroma modifications, with the aim to better understand the effect of the geographical origin on the intrinsic aroma of fermented cocoa and the related aroma potential after roasting. The goal of this work is to see how identical roasting condition results in different volatile fingerprinting profiles of cocoa beans from different geographic origins. Another goal was to check if volatile profile can be used to trace the

cocoa origin, both before and after roasting, in order to achieve elements useful to both cocoa transforming industries and consumers.

Materials and Methods

2.1 Chemicals

The compounds used as standards such as 3-Methylbutanal, 2,3-Butanedione, 2-Pentanol, 3-Methyl-1-butanol, Acetoin, 2,3-Dimethylpyrazine, Trimethylpyrazine, Tetramethylpyrazine, Benzaldehyde, 2,3-Butanediol, 1-Phenylethanol, 1H-Pyrrole-2-carboxaldehyde, toluene, n-alkanes (C8-C17) were all obtained from Sigma-Aldrich.

2.2 Samples

59 fermented and dried cocoa beans samples from 22 different geographical origins (Mexico, Cuba, Santo Domingo, Grenada, Sulawesi, Trinidad, Venezuela, Equador, Perù, Brazil, Sierra Leone, Ivory Coast, Ghana, Nigeria, São Tomé, Uganda, Tanzania, Madagascar, Malaysia, Java, Papua Guinea New, Flores) were investigated. Samples were provided by Barry Callebaut, Belgium. All fruits were harvested in 2012, and were fermented and dried in the countries of origin according to traditional procedures. The samples analysed were of Forastero variety, except one sample of Criollo variety coming from Mexico. The countries of origin and the numbers of cocoa beans samples collected were shown in Table 1.

The samples of fermented cocoa beans were analysed before and after roasting. Roasting of the beans was performed in a laboratory scale with a coffee-roaster (Probat BRZ 4, Emmerich, Germany). Cocoa beans were freshly roasted for each experiment, and roasting conditions were optimized by variation of roasting time and temperature. A temperature of 140 °C applied for 30 min was finally chosen to simulate the industrial process generally adopted by Barry Callebaut, Belgium.

For chemical analysis, the samples were frozen with liquid nitrogen, ground with a coffee mill to obtain a powder and immediately analysed.

2.3 Extraction of volatile compounds

The volatile compounds from cocoa beans sample were extracted using the head space solid phase microextraction (HS-SPME). The cocoa samples were analysed according to the method described by Rodriguez-Campos et al. (2011) with minor modifications.

A DVB/CAR/PDMS, 50/30 μm fiber was used. Both SPME manual holder and fibers were purchased from Supelco (Bellefonte, PA, USA).

Before use, the fiber was conditioned following the instructions of the manufacturer. The experimental conditions were as follows: 1.5 g of cocoa powder was placed in a 30-mL vial, added to 10 μL of a toluene solution 250 mg/kg in water (2.5 μg) as internal standard and sealed with Black Viton septa (Supelco, Bellefonte, PA, USA).

The SPME fibre was then exposed to the headspace of the sample. During the equilibration step (10 min) and the extraction step (20 min), the vial was maintained at 60 °C under magnetic stirring. After sampling, the extracted volatile compounds were thermally desorbed in the GC injector (splitless mode).

2.4. Separation and identification of volatile compounds

A Trace 1300 GC Thermo scientific (Thermo Fisher Scientific Inc.) system coupled to an ISQ mass spectrometer was used to perform the analyses. The capillary column used in the GC–MS was a SUPELCOWAX 10 capillary column (Supelco, 30 m × 0.25 mm, f.t. 0.25 μm). Helium at 15 psi was used as carrier gas with a flow rate of 1.0 ml min^{-1}.

Thermal desorption of the compounds from the fiber coating took place in the GC injector at 250 ◦C for 3 min. Analyses were performed at programmed temperature starting from 40 °C for 3 min, increasing of 5 °C per minute to 200 °C and maintaining this final temperature for 5 min.

The identification of compounds was based on three criteria: (1) by comparing the mass spectra with the NIST 14 of mass spectra (2) by comparing the retention index with literature data, and (3) whenever possible, the identification was confirmed by using pure standards of the components.

Retention indices were calculated using n-alkanes (C8-C17) as reference compounds according to Bianchi et al., (2007).

Semiquantitative analysis was carried out by comparing the peak area of each compound with that of the internal standard. Because of the non-availability of standards for all the detected substances, the response factor was considered as one for all the identified compounds.

Results and Discussion

In this research 59 samples of fermented cocoa beans were analyzed by HS-SPME GC-MS before and after roasting. A total of 76 aroma compounds were determined and grouped into different chemical classes (Tab 2, 3). The concentrations of each compound in each sample analysed are reported in supplementary materials**..**

	Fermented mg/kg	Roasted mg/kg	Fermented %	Roasted %
Aldehydes	1.62	7.98	7.46	15.62
Ketones	1.13	1.88	5.22	3.68
Esters	4.21	6.30	19.37	12.33
Alcohols	5.78	10.74	26.57	21.01
Phenols	0.08	0.21	0.36	0.42
Pyrazines	2.88	11.65	13.24	22.79
Hydrocarbons	0.12	0.18	0.57	0.35
Furanes and pyranes	0.00	0.77	0.00	1.50
Pyrroles	0.08	1.27	0.37	2.49
Terpenes	0.08	0.17	0.36	0.34
Acids	4.85	7.60	22.31	14.87
Others	0.91	2.35	4.18	4.60
Total sum	21.75	51.12		

Tab. 2 *Mean total sum of volatile compounds (mg/kg) determined on all cocoa samples before and after roasting divided by chemical classes*

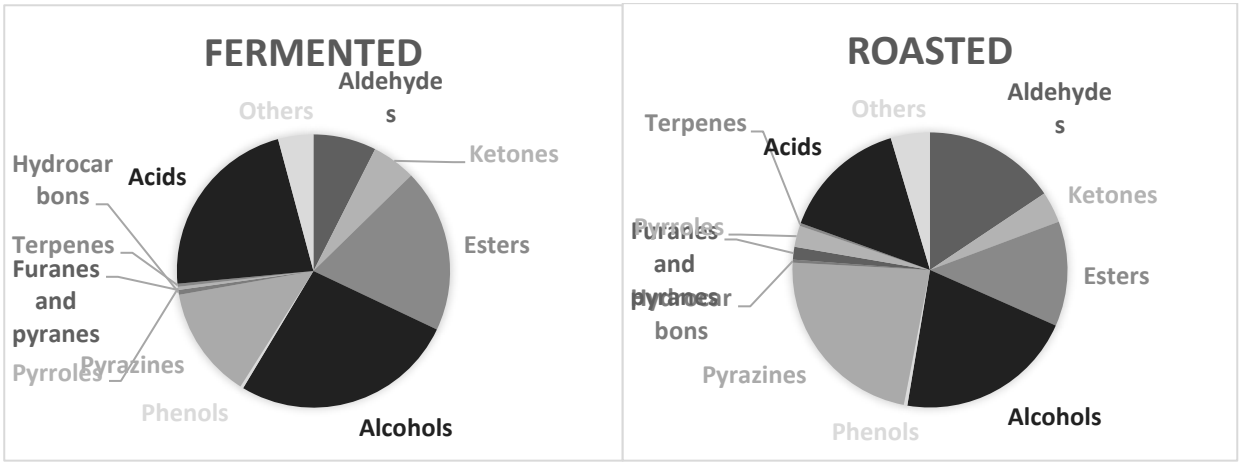

Figure 1: Mean distribution of the main classes of aroma compounds in b) fermented unroasted cocoa beans and c) roasted cocoa beans.

The total sum of volatiles in cocoa samples is highly variable, both in fermented and roasted beans, as demonstrated by data reported in tab 1. Roasting had a pronounced impact on the concentration of the volatiles, giving origin to total values of volatile about doubled respect to the fermented beans, from 21.75 to 51.12 mg/kg respectively (tab 2).

To better understand the cocoa aroma modification after roasting, figure 1a and 1b report, respectively, the mean distribution in classes of aroma compounds before and after roasting. In unroasted beans, the total relative concentrations of esters (4.21/19.37) and alcohols (5.78/26.57) were higher than the concentrations of aldehydes (1.62/7.46) and ketones (1.13/5.22), pyrazines (2.88/13.24) and other compounds (0.91/4.18), as shown in Figure 1. After roasting, the distribution of the different classes of compounds changes; pyrazines (11.65/22.79) and aldehydes (7.98/15.62) increased and new compounds were formed from the Maillard reactions, as pyrroles, furanes and pyranes.

Aroma compounds before roasting are mainly of fermentative origin, even if a slight effect of drying procedures has been also reported, so in principle their amount is not strictly linked to the development of aroma after roasting. However, a positive correlation was observed between total aroma before and after roasting (correlation coefficient: 0.76). This was tentatively explained by the fact that the most aromatic beans before roasting ex mexico correspond to the most fermented ones, which are richer of

amino acids of proteolytic origin and so they are also able to develop more aromatic compounds during roasting.

Composition of volatile fraction of fermented cocoa beans

The sum of each main classes of compounds for each sample is reported in Figure 2, showing a large variability in the aromatic compounds in fermented cocoa beans of different origin.

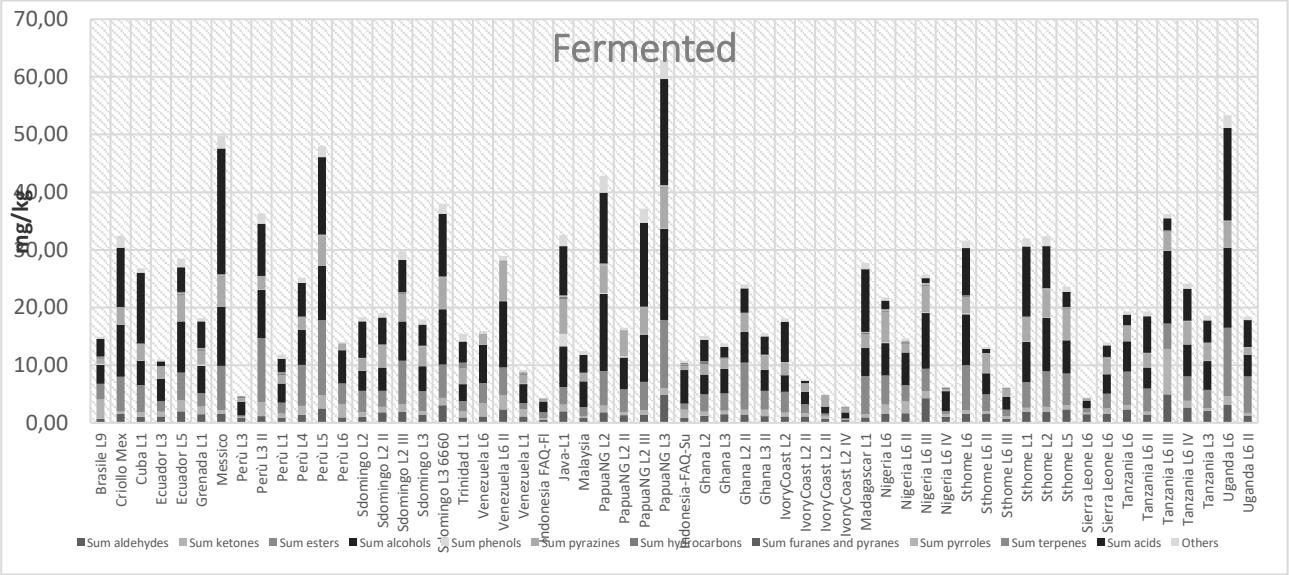

Figure 2: Total amounts of a) esters, b) aldehydes/ketones, c) pyrazines and d) alcohols in each cocoa sample of different geographical origin.

Esters. Esters of acetic acid are predominant. 3-methyl-1-butanol acetate, 2-phenylethyl acetate, methyl acetate, 1-butanol-3-methylbenzoate, and butyrolactone were the major esters found in the samples analyzed, as reported in Table 1 supplementary data. Ester concentration is quite variable in fermented cocoa beans of different origin, ranging from minimum value of 0.13 mg/kg for one sample from Ivory Coast, to 13.01 mg/kg for one sample from Perù.

The concentration of esters compounds depends on the duration of the fermentation process, in fact it decreases as fermentation time increases (Rodriguez-Campos et al., 2012).

To get a cocoa flavor of high quality is desirable the presence of 2-phenylethyl acetate and ethylphenyl acetate in high concentrations, as they are considered essential for the formation of the

typical cocoa aroma. The production of these esters can be a result of yeast metabolism during the fermentation process, which produces key cocoa aromas such as flowery and honey flavour notes (Aculey et al., 2010; Frauendorfer & Schieberle, 2008). Samples containing the higher amounts of 2-phenyletyl acetate (> 1mg/kg) are Criollo variety (considered a fine cocoa) and mainly American cocoa beans (Mexico, Perù, S.Domingo), Uganda, S.Thome and samples from Papua New Guinea, the last containing also the higher amount (0.39 mg/kg) of ethylphenyl acetate (see supplementary materials S1).

Aldehydes and ketones. A total of 13 compounds belonging to the class of aldehyde and ketones, were detected, ordered by the most abundant to the least: benzaldehyde, 2-nonanone, 2-heptanone, acetophenone, acetoin, 3-methylbutanal, 2,3-butanedione, phenylacetaldehyde, 2-methylbutanal, nonanal, 1H-pyrrole-2-carboxaldehyde and 2-phenyl-2-butenal. A high concentration of aldehydes and ketones is desired in cocoa beans as it helps to impart a fruity and flowery notes (Serra-Bonvehi 2005), total concentration in the samples analyzed ranging from 0.13 to 7.82 mg/kg.

The presence of 2-and 3-methylbutanal is appreciated because these aldehydes exhibit notes of malt and chocolate. The formation of these two aldehydes can be attributed to degradation of isoleucine and leucine by lactic acid bacteria during fermentation (Jinap et al., 1994, Frauedorfer & Shieberle, 2008). Their contents reaches values of 0.17 mg/Kg and 0.46 mg/Kg respectively in one sample from Tanzania, and values of 0.14 and 0.43 in one sample of Nigeria.

2-nonanone, acetophenone, acetoin and diacetyl were the major ketones identified. Diacetyl is a well-known secondary metabolite of yeasts and bacteria and imparts a buttery aroma to many foodstuffs, it ranging from minimum value of 0.01 mg/kg for Perù and Ivory Coast samples to 0.33 for one samples of Nigeria. Acetoin can be produced from pyruvate and butanediol during alcoholic fermentation (Pretorius, 2000) and has been reported as a precursor of tetramethylpyrazine (Hashim, Jinap, Muhammad & Ali, 1999). Samples containing the lowest levels of acetoin are costa avorio 0.01 mg/kg malesia 0.04 mg/kg sulawesi 0.03 mg/kg, samples containing the higher amount are venezuela 0.52 mg/kg perù 0.40 mg/kg s domingo 0.33 mg/kg.

Alcohol. The concentration of alcohols increases in the beans that undergo long fermentation processes, but it decreases during the drying process (Rodriguez-Campos et al., 2012). High alcohol contents are desirable to obtain cocoa products with flowery and candy notes (Aculey et al., 2010; Frauendorfer & Schieberle, 2008), total concentration founded in the samples analyzed ranging from 1.05 to 15.86 mg/kg. Several alcohols were detected in the samples under study: 2 phenylethanol, 2,3-butanediol, 2-heptanol, 3-methyl-1-butanol, 2-nonanol, 2-pentanol, 2-ethyl-1-hexanol, benzyl alcohol, phenol, 1-phenylethanol, 4-methyl phenol, 3-methyl phenol. Some identified alcohols are responsible for producing desirable flavour notes, i.e. 3-methyl-1-butanol, 2-heptanol and 2-phenylethanol (Ducki et al., 2008; Jinap, Wan-Rosli, Russly, & Nordin, 1998).

2,3-butanediol is normally considered as a fermentation product (Owusu et al., 2011), it ranging from a minimum value of 0.15 mg/Kg in one sample from sierra leone to 4.46 mg/Kg in a sample from papua.

The benzyl alcohol and 2-phenylethanol are known products of yeast metabolism (Delfini, Gaia, Bardi & Mariscalco, 1991; Nykanen, 1986) providing flowery flavor notes (Jinap et al., 1998) and increase with an increase of fermentation time (Crafack et al., 2014). Samples containing the higher amounts of 2-phenyethanol are Tanzania 0.24 3.03 papua 0.18 4.21 Nigeria 0.14 2.96, while peru 0.02 0.25 costa av 0.02 0.17 are the samples containing the lowest amounts (see supplementary materials S1).

Pyrazines. Pyrazines are considered the most important of the volatile components in cocoa beans. They originate in part by microbiological processes during fermentation but especially by Strecker degradation that accompanies the Maillard reaction during roasting. Pyrazines are substances with low molecular weight and high volatility and constitute the fundamental part of the cocoa aroma together with esters, alcohols, aldehydes and hydrocarbons.

Five pyrazynes, namely tetramethylpyrazine, trimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethyl-6-ethyl pyrazine and 2,3-dimethyl-5-ethyl pyrazine were present in all the fermented samples but in different proportions ranging from 0.21 to 7.08 mg/kg. A major quantitative difference involved primarily the dimethyl, trimethyl and tetramethyl pyrazine peaks.

Tetramethylpyrazine, the most abundant, reachs the highest values in American/Asiatic samples from Venezuela (6.02 mg/Kg), Papua New Guinea (5.04 mg/Kg) and Java (4.41 mg/Kg) while the lowest values in African samples from Sierra Leone 0.15 (mg/Kg) and Nigeria (0.30 mg/Kg). Tetramethylpyrazine is known to be a metabolic product of *Bacillus subtilis*, and its presence is an indication of *B. subtilis* activity during the fermentation of cocoa beans (Gill, MacLeod & Moreau, 1984). This compound is one of the important components of cocoa flavor that can be used as cocoa flavor enhancer (Rohan & Stewart 1965; Nebesny & Rutkowski 1998).

PCA

In order to obtain an easily interpretable quali-quantitative description of the eventual differences existent in the aroma profile of cocoa beans, a PCA was performed on all the bean samples of different geographic origins. PCA was performed on the matrix of 62 volatiles for 59 fermented cocoa samples, and the principal components were constructed with the correlation method. Four Components were extracted, accounting for 57 % of the variation in the volatile fingerprint. PC1, PC2 and PC4, accounting for 50.5 % of total variance explained, were plotted in a 3D score plot (Fig. 2a), which shows a clustering of cocoas according to their geographical macro areas of origin. Plotting the first two components only, no specific grouping was evidenced, so PC4 was also considered, because it appears the component mainly related to the origin of cocoa. PC3 was not included in the score plot because it contributes only to a large separation of a single sample from Java. Variables with high positive value on PC3, related to Java sample, correspond to phenol and methyl phenols (smoky note), which have unusual high values only in Java cocoa.

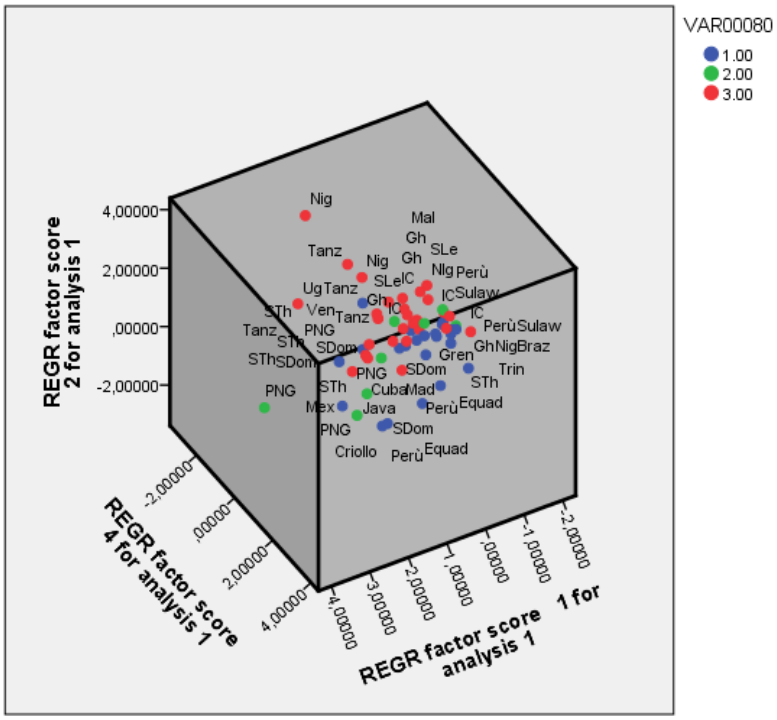

Figure 3: Principal component analysis: (a) 3d-score plot of all fermented and not-rosted cocoa bean samples on PC1, PC2 and PC4 and 2d-score plot on (b) PC1 vs PC4.

The score plot shows that samples of cocoa beans are divided into two principal groups, being the group corresponding to the African samples separated from the group of Caribben samples. The loading values of the variables associated to the first four principal components are reported in Table 3 supplementary material. The variables with positive values on PC4, that characterize the group of American beans are C7-compounds as, 2-heptanone, 2-heptanol acetate, 2-heptanol, while the variables with the higher negative values on PC4, related to the African beans, are mainly aldehydes and phenols as 2-methylbutanal, 3-methyl butanal, nonanal, phenol, methylphenol.

Composition of volatiles in roasted cocoa beans

The 59 samples of spontaneously fermented and dried cocoa beans were analyzed by HS-SPME GC-MS after roasting. Roasting conditions were the equivalent to industrial processes, 140°C for 30 minutes.

A total of 76 aroma compounds (Table 2) was detected. Roasting had a pronounced impact on the composition and relative concentration of the volatiles identified in cocoa beans showing a general increasing (Figure 2b, 4); in fact, 14 novel compounds were detected after roasting: 1,3-Propanediolo diacetate, 2-Methylpropanal, 2-Isopropyl-5-methyl-2-hexenal, 5-Methyl-2-phenyl-2-hexenal, 2-Ethyl pyrazine, 2,3-Dimethylpyrazine, 2-Ethyl-6-Methyl pyrazine, 3-Ethyl-2,5-Dimethyl pyrazine, n-Butylbenzene, 3-Furfuryl alcohol, 4-hydroxy-2,5-dimethyl 3(2H)furanone, 4H-pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl (dihydroxy-maltol), (1-ethyl-2-pyrrolidinyl)-methanol, Propanoic acid-2-metil.

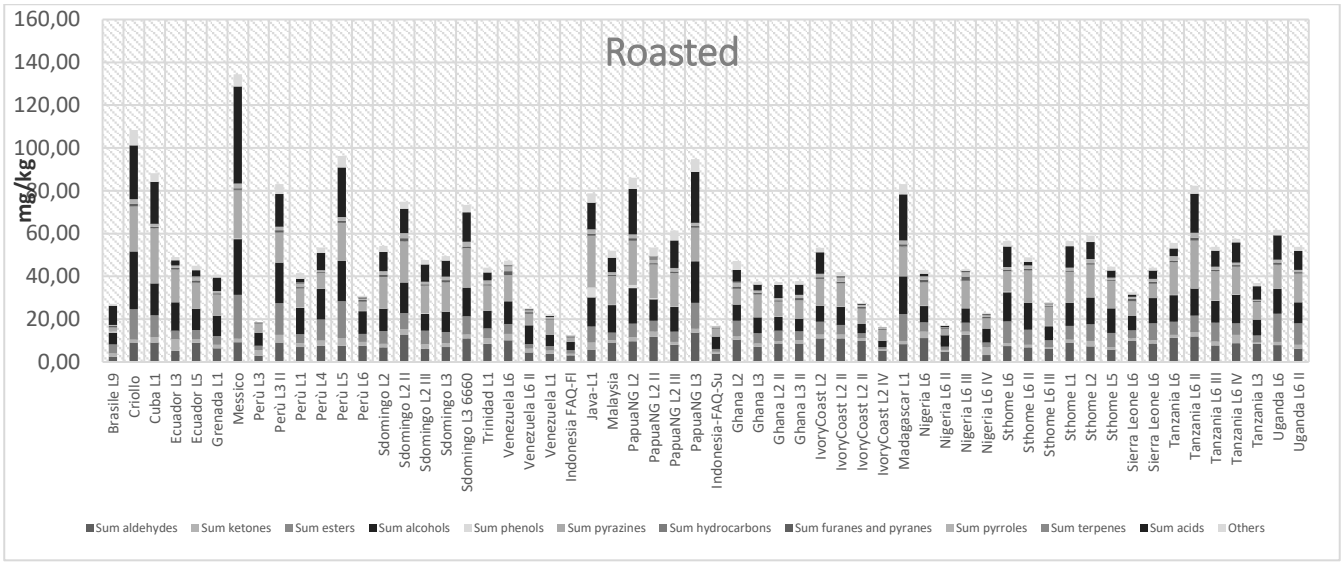

Fig 4

The global mean variation in cocoa specific aroma compounds before and after roasting is evidenced in Table 1, sub-divided in classes of compounds.

Whilst the total concentration of ketones, terpenes and terpene alcohols changed only slightly or remained at the same level, the concentration of aldehydes, alcohols and pyrazines increased remarkably upon roasting and were the classes that undergo major modification.

Figure 5:

Aldehydes. In the samples analysed total sum of aldehydes ranging from 2.49 to 13.75 mg/Kg. Pronounced increases of the Strecker aldehydes, 2-methylbutanal, 3-methylbutanal, benzaldeyde and benzenacetaldehyde were observed during roasting, whilst 2-methylpropanal, 2-isopropil-5-methyl-2-hexenal and 5-methyl-2-phenyl-2-hexenal, not present in the un-roasted beans, were identified in the roasted ones.

5-methyl-2-phenyl-2-hexenal is formed through aldol condensation of phenylacetaldehyde with acetaldehyde and 2-methylpropanal (Ziegleder, 2009), and is reported as being a key constituent of chocolate aroma (Bonvehí, 2005; Counet & Callemien, 2002; Owusu et al., 2011) which possesses a deep bitter, persistent cocoa note (Van Prang et al. 1968).

Alcohol. Total sum of alcohol ranging from 2.76 to 27.10 mg/Kg. The most abundant alcohols identified in the roasted beans were 2,3-butanediol, 2 phenilethanol and 2-heptanol, although they are normally considered as fermentation products.

2-heptanol is also important to cocoa flavor, which can be used as cocoa flavoring materials. This compound has been identified as volatile compounds of thermally degradable amino acids and has a strong green flavor and sweet aroma, which could contribute to the cocoa bean flavor.

Pyrazines. The group of pyrazines is one of the most important volatile compounds in roasted cocoa, in samples analysed in the present work total sum ranging from 1.78 to 25.38 mg/Kg. Serra-Bonvehí (2005) mentioned that pyrazines represented 40% of the aroma in roasted cocoa, in our samples they represent about 23% of the aroma in roasted beans, showing an increasing of the mean total sum of about 70% after roasting.

Besides the 2,6-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazines, 2,3-dimetil-5-etilpirazina and 2,3,5-trimetil-6-etilpirazina identified in the un-roasted beans, four new pyrazines were formed as a result of roasting: 2-ethyl pyrazine, 2,3 dimethylpyrazine, 2-ethyl-6-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine.

Important increases of 2,3-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazine were observed upon roasting with the latter being the most abundant pyrazine.

Tetramethylpyrazine is one of the important components of cocoa flavor that can be used as cocoa flavour enhancer (Rohan and Stewart 1965; Nebesny and Rutkowski 1998). From organoleptic descriptions, trimethyl- and tetramethylpyrazine possess a nutty, grassy and pungent persistent cocoa note (Van Praag et al. 1968). Samples containing the higher amounts of tetramethylpyrazine are Cuba (16.16 mg/Kg) Java (14.08 mg/Kg) and Tanzania (11.84 mg/Kg), while Flores (0.97 mg/Kg) Nigeria (0.99 mg/Kg) and Brazil (1.11 mg/Kg) are the samples containing the lowest amounts (see supplementary materials S1).

The second most represented pyrazine is 2,3,5-trimethylpyrazine, which accounted for a minimum of 0.37 mg/Kg (in sample Flores) to a maximum of 6.39 (in sample Mexico); its aroma contribution has been described as green and earthy, cocoa- and roasted nuts-like [16,21,17]

PCA

Data obtained were submitted to multivariate chemometric analysis (PCA).

PCA was performed on the matrix of 76 volatiles for 59 fermented cocoa samples, and the principal components were constructed with the correlation method. Two Components were extracted, accounting for 38 % of the variation in the volatile fingerprint.

PC2, PC3 and PC4, accounting for 51 % of total variance explained, were plotted in a 3D score plot (Fig. 2a), which shows a clustering of cocoa beans according to their origin. Plotting the first two components only, it was not possible to obtain a clear separation of cocoa beans origin, so PC4 was also considered to improve the differentiation of origin (Fig. 2c).

PC1 is not specifically linked to the geographical origin because essentially separates criollo (higher positive values on PC1) from the others. Criollo is a different variety known to be a fine flavor cocoa.

On the opposite side of PC1 samples from sulawesi are found that are generally poor fermented and with low aroma potential. Therefore PC1 is the component explainingmanily the system variability linked to the different ferementation level or different variety.

To gain insight in the observed spectral clustering, the PCA loadings were inspected (Fig. 3).

PC1 is the component mainly related to the differentiation based on fermentation level or different variety; it explains 27 % of the total variance in the samples, and the highest positive component loadings were Etanone-1-(1H-pirrol-2-il), Trimethylpyrazine, 1,3-Butanediol, Benzyl acetate; the signals 2,3-Dimethylheptane 2-Heptanone 2-Pentanol 2-Nonanone were those with higher negative loadings on PC1.

PC2 explains 38% of the variation and it is important for the separation of american and asiatic samples. PC2 is able to separate a small group of carribean cocoa beans and the highest positive loadings related to asiatic samples were the aldehydes Nonanal, 2-Isopropyl-5-methyl-2-hexenal, 2-Methylpropanal, 3-Methylbutanal and 2,6-Dimethyl pyrazine; the highest negative related to caribben samples loadings on PC2 were the esters 2-Phenylethyl acetate, Isobutyl acetate and 3-Methyl-1-butanol acetate.

PC3 (46%) contributes only to grouping a small group of Asiatic samples characterized by high negative value on PC3 correspond to phenol and methyl phenols (smoky note), variables with high positive value were 2 heptanone 2 heptanol acetato 2 nonanone.

PC4 explains 51% of the variance and is important for the differentiation between africa and america (seeFig. 2c). The highest positive loadings that characterize samples from africa were the alchools 2-pentanol, 1-phenilethanol, 3-Methyl-1-butanol and ketons 2,3-butanedione, acetophenone. The highest negative loading related to american beans were tetramethylpyrazine, phenol and methyl phenols (smoky note).

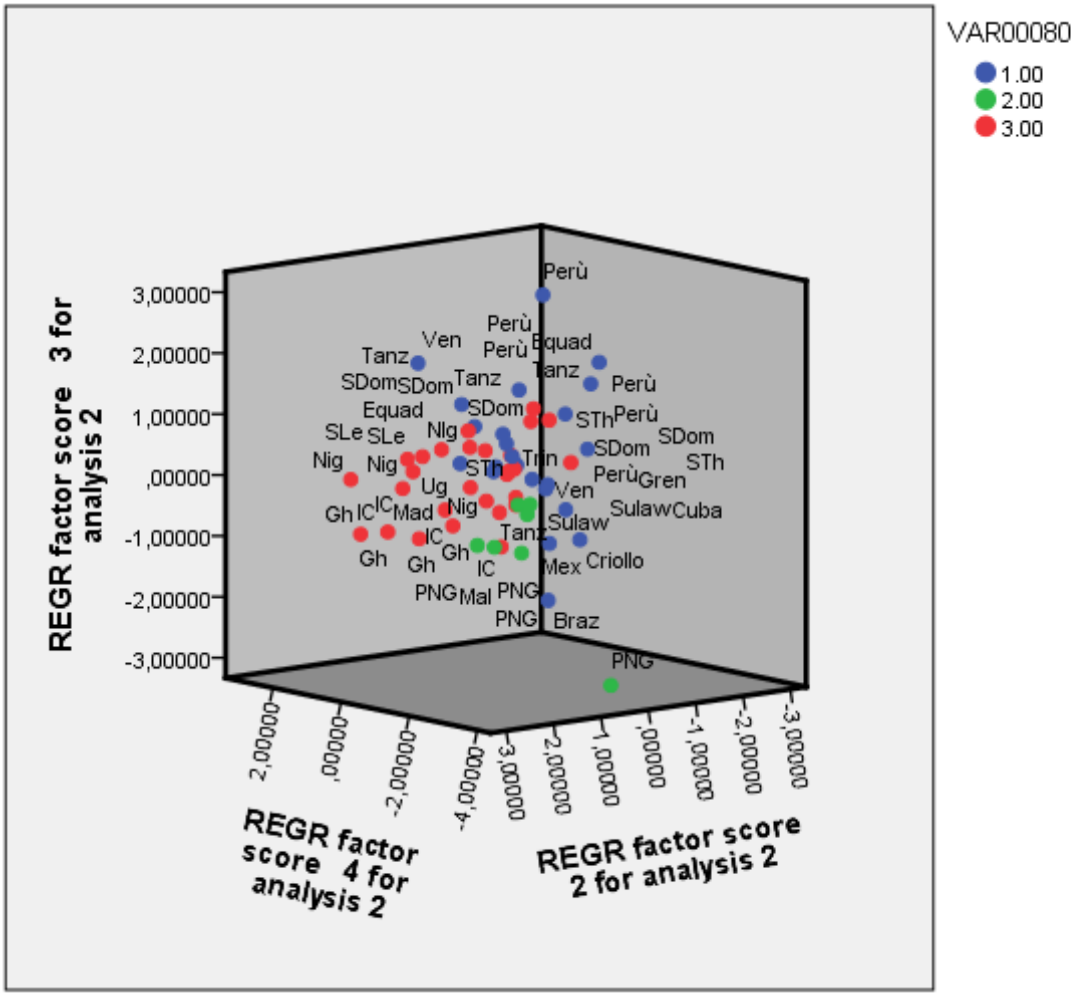

Figure 5: Principal component analysis: 3d-score plot of all fermented and not-rosted cocoa bean samples on PC2, PC3, PC4

Results showed that although the same roasting treatment has been applied to all the samples, the differences among the fermented samples are also manteined in the aromatic profile resulting after roasting. As reported for not roasted cocoa beans, the volatile fingerprint showed interesting potential

for authentication of both raw and roasted cocoa beans suggesting that the native aromatic profile of fermented beans continues to differentiate cocoa showing a pronunced impact on the characteristics of the final product.

Conclusions

Only few studies are present in literature about the analytical methods to determine the metabolic differences of cocoa composition related to cocoa beans origin. Most of studies are focused on a limited dataset, few are focused on raw cocoa beans and rare are the works focused on raw cocoa bean from a significant numbers of different countries. One of the main strength point of the samples considered in this study is that they are representative of the entire world cocoa production.

HS SPME GC MS analysis of cocoa aroma combined with chemometric tools has been revealed as a promising application for the authentication of high quality cocoa, both in raw and roasted form. A total of 62 volatiles were identified in raw beans while 76 were those identified in roasted cocoa beans. The compounds belong to the following chemical groups: esters, alcohols, organic acids, aldehydes and ketones, pyrazines and other compounds.

Data obtained were submitted to multivariate chemometric analysis (PCA). Results showed that cocoa volatilome combined with chemometrics are able to discriminate cocoa samples from macroareas as Africa and America. Samples from Asia/Oceania are instead not discriminated. The same statistical analysis applied on the corresponding roasted samples showed that although the same roasting treatment has been applied to all the samples, the differences among the fermented samples are also manteined in the aromatic profile resulting after roasting.

As reported for fermented cocoa beans, the volatile fingerprint showed interesting potential also for authentication of roasted cocoa beans, and this could be an advantage from a commercial point of view since it is important to determine the quality of chocolate and the veracity of labeling after technological processing to trace the cocoa production site’s country.

466 The latter important practical application could be the improvement of chemical composition
467 knowledge on cocoa raw material to give information to pilot the secondary processing steps of cocoa
468 towards the desired final product characteristics.
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Table 1 Geographical origin of cocoa samples together with the number of samples for single country.

Contienent	Country	N° of samples
América	Mexico (var. Criollo)	1
	Mexico	1
	Cuba	1
	Dominican Republic	5
	Grenada	1
	Trinidad	1
	Venezuela	4
	Ecuador	2
	Brazil	1
	Perú	6
África	Sierra Leone	2
	Ivory Coast	4
	Ghana	4
	Nigeria	4
	S˜ao Tom´e	6
	Uganda	2
	Tanzania	5
	Madagascar	1
Asia	Java	1
	Malaysia	1
	Flores	1
	Sulawesi	1
	Papua New Guinea	4

Table 3:

concentration (mg/kg) of cocoa aroma compounds before and after cocoa beans roasting.

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N°	Compound name	R.T.	RI	Odour Description[a]	Identification[b]	Anova test	Mean in fermented beans	Std dev	Mean in roasted beans	Std dev
	Esters									
1	Methyl acetate	2,68	832		MS, IK		0,217		0,208	
2	Ethyl acetate	3,35	893	Fruity, pineapple	MS, IK		0,105		0,194	
3	Isobutyl acetate	5,81	1019	Fruity	MS, IK		0,094		0,116	
4	Butyl acetate	7,35	1077		MS, IK		0,310		0,357	
5	3-Methyl-1-butanol acetate	8,73	1128	Banana	MS, IK		2,000		2,277	
6	2-Heptanol acetate	12,77	1269		MS	<0.05	0,294		0,694	
7	Ethyl octanoate	17,48	1442	Fruity, flowery	MS, IK	<0.05	0,187		0,312	
8	2-Methyl-propanoic acid ethyl ester	22,24	1632		MS	<0.05	0,035		0,057	
9	Butyrolactone	22,49	1642	Bready	MS, IK	<0.05	0,082		0,211	
10	Ethyl decanoate	22,56	1645	Fruity, floral, pear, grape	MS, IK	<0.05	0,035		0,064	
11	1,3-Propanediolo diacetato*	23,04	1666		MS	<0.05	0,000		0,080	
12	Benzoic acid ethyl ester	23,32	1678		MS	<0.05	0,063		0,122	
13	Benzyl acetate	24,78	1741	Flowery, rose, jasmine	MS, IK	<0.05	0,047		0,101	
14	Benzene acetic acid methyl ester	25,47	1772		MS	<0.05	0,020		0,045	
15	Ethylphenyl acetate	26,03	1797	Flowery, rose, fruity, sweet	MS, IK	<0.05	0,087		0,186	
16	2-Phenylethyl acetate	26,69	1827	Honey, flowery	MS, IK	<0.05	0,550		1,078	
17	Ethyl laurate	27,06	1845	leaf, fruity, floral	MS, IK	<0.05	0,000		0,003	
18	1-Butanol-3-methylbenzoate	27,18	1850		MS		0,086		0,194	
	Aldehydes									
19	2-Methylpropanal*	2,55	820	Malty, chocolate	MS, IK	<0.05	0,000		0,259	
20	2-Methylbutanal	3,75	917	Malty, chocolate, cocoa	MS, IK	<0.05	0,037		0,500	
21	3-Methylbutanal	3,82	920	Malty, chocolate, cocoa	MS, IK, STD	<0.05	0,115		2,929	
22	2-Isopropyl-5-methyl-2-hexenal*	15,58	1370		MS	<0.05	0,000		0,953	
23	Nonanal	16,41	1401	Tallowy, soapy-fruity	MS, IK	<0.05	0,016		0,041	
24	Benzaldehyde	19,85	1534	Bitter almond, grass, fruity	MS, IK, STD	<0.05	1,369		2,234	
25	Phenylacetaldehyde	22,8	1656	Green, honey, flowery	MS, IK	<0.05	0,068		0,283	
26	2-Phenyl-2-butenal	29,19	1946	Flowery, cocoa, roasted, rum	MS, IK	<0.05	0,016		0,408	
27	5-Methyl-2-phenyl-2-hexenal*	32,06	2086	Sweet, roasted cocoa	MS, IK	<0.05	0,000		0,376	
	Ketones									
28	2,3-Butanedione	5,05	985	Buttery	MS, IK, STD		0,087		0,077	
29	2-Heptanone	10,43	1187	Fruity, green, flowery	MS, IK		0,296		0,277	

30	Acetoin	13,43	1292	Buttery, cream	MS, IK. STD	<0.05	0,128	0,427
31	2-Nonanone	16,27	1396	Flowery, fatty	MS, IK		0,391	0,611
32	Acetophenone	22,96	1662	Floral, swet	MS, IK	<0.05	0,233	0,482
33	1H-inden-1-one-2,3-dihydro	30,87	2028		MS	<0.05	0,000	0,005
	Alcohols							
34	2-Pentanol	8,58	1122	Green	MS, IK, STD		0,162	0,198
35	3-Methyl-1-butanol	11,09	1210	Malty, chocolate	MS, IK, STD		0,477	0,568
36	2-Heptanol	14,31	1324	Citrusy, sweet	MS, IK	<0.05	0,527	0,904
37	2-Ethyil-1-hexanol	18,89	1496		MS, IK	<0.05	0,148	0,279
38	2-Nonanol	19,59	1524		MS, IK		0,227	0,290
39	2,3-Butanediol	20,16	1547	Sweet, flowery	MS, IK, STD	<0.05	1,334	2,536
40	1,3-Butanediol	21,07	1583		MS	<0.05	1,389	2,541
41	1-Phenylethanol	26,59	1823	Honey, floral	MS, IK, STD		0,049	0,108
42	Benzyl alcohol	27,98	1887	Sweet, fruity	MS, IK		0,074	0,157
43	2 Phenylethanol	28,71	1922	Floral	MS, IK	<0.05	1,393	3,158
	Phenols							
44	2-methylphenol	30,62	2016	Smoky	MS, IK		0,000	0,002
45	Phenol	30,71	2020	Smoky	MS		0,068	0,151
46	4-Methyl phenol	32,21	2093	Horse stable, phenolic	MS, IK	<0.05	0,004	0,040
47	3-Methyl phenol	32,36	2100	Smoky	MS, IK		0,004	0,020
	Pyrazines							
48	2,6-Dimethyl pyrazine	14,5	1331	Nutty, coffee, green	MS, IK	<0.05	0,114	0,192
49	2-Ethyl pyrazine*	14,68	1338	Peanut-butter, musty, nutty	MS, IK	<0.05	0,000	0,027
50	2,3-Dimethylpyrazine*	15	1349	Caramel, cocoa, sweet, baked	MS, IK, STD	<0.05	0,000	0,463
51	2-Ethyl-6-Methyl pyrazine*	16,08	1389		MS, IK	<0.05	0,000	0,129
52	Trimethylpyrazine	16,57	1407	Earthy, cocoa, fried potato, roasted	MS, IK, STD	<0.05	0,389	2,390
53	3-Ethyl-2,5-Dimethyl pyrazine*	17,71	1451		MS	<0.05	0,000	0,479
54	2,3-Dimethyl-5-ethyl pyrazine	18,11	1466	Cocoa, chocolate	MS, IK	<0.05	0,003	1,637
55	Tetramethyl pyrazine	18,49	1480	Chocolate, cocoa, roasted	MS, IK, STD	<0.05	2,336	5,983
56	2,3,5-Trimethyl-6-ethyl pyrazine	19,43	1517	Candy, sweet	MS, IK	<0.05	0,036	0,349
	Hydrocarbons							
57	2,3-Dimethylheptane	2,47	813		MS		0,046	0,021
58	1-Ethyl-2-methylbenzene	11,69	1231		MS	<0.05	0,001	0,005

59	Styrene	12,6	1263		MS, IK		0,065	0,060
60	n-Butylbenzene*	20,94	1578		MS	<0.05	0,000	0,069
61	Naphtalene	25,04	1753		MS, IK	<0.05	0,011	0,025
	Furanes/pyranes							
62	3-Furfuryl alchol*	23,14	1670	Bready	MS, IK	<0.05	0,000	0,367
63	4-hydroxy-2,5-dimethyl 3(2H)furanone*	31,23	2045	Caramel-like-STRAWBERRY	MS, IK		0,000	0,101
64	4H-pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl (dihydroxymaltol)*	35,84	2270	caramel?	MS	<0.05	0,000	0,301
	Pyrroles							
65	(1-ethyl-2-pyrrolidinyl)-metanol*	14,79	1342		MS	<0.05	0,000	0,441
66	Etanone-1-(1H-pirrol-2-il)	29,96	1983		MS		0,064	0,752
67	1H-Pyrrole-2-carboxaldehyde (2-acetyl-1H-pyrrole)	31,09	2039	Chocolate, hazelnut	MS, STD		0,016	0,081
	Terpenes							
68	β-myrcene	9,88	1168	Herbaceous, metallic	MS, IK		0,068	0,138
69	Linalool	20,54	1562	Rose, flowery	MS, IK	<0.05	0,011	0,035
	Acids							
70	Acetic acid	17,97	1461	Sour, vinegra	MS, IK		4,852	7,042
71	Propanoic acid-2-metil*	21,21	1589		MS		0,000	0,558
	Others							
72	unknown1	11,9	1240				0,063	0,000
73	unknown2	11,95	1240				0,079	0,354
74	unknown3*	14,89	1345		MS		0,000	0,363
75	unknown4	17,66	1449		MS		0,048	0,039
76	unknown5	20,76	1571				0,718	1,596

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587 *Table 4: Loadings values of the variable for the first four principal components*

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