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Abstract: Cocoa (*Theobroma Cacao, L.*) represents an important market that gained relevance and became an esteemed commodity thanks to cocoa powder, chocolate, and other related products. This work has analyzed 59 cocoa powder samples from the European market. Three distinct subgroups were identified: organic or conventional, alkalized or not alkalized, and raw or roasted processing. The impact of the technological process was evaluated on the pH, color, and compositional traits, as content of biogenic amines and salsolinol. The phenolic fraction was also investigated trough both common and emerging methods. Results depict that the influence of the agronomical practices (organic/conventional) did not affect significatively ($p<0.05$) the composition of cocoa powders; similarly, the roasting process was not discriminative for the compounds traced. On the other hand, the alkalinization process greatly impacted on color, and pH, no matter of cocoa provenience, obtention, or other processes, also resulting reducing the phenolic fraction of treated samples. Principal components analysis confirmed that the alkali process acts on pH, color, and phenolic composition, but not on other bioactives molecules (BAs and salsolinol). All samples resulted safe while alkalized powders have a great reduction in beneficial biocompounds. A novel strategy could be to emphasize non-alkalized powders in the label, to meet the demand for more beneficial products.

Keywords: biogenic amines; polyphenols; salsolinol; organic processing; raw cocoa; alkalization treatment

1. Introduction

Cocoa powder is consumed and appreciated worldwide and, besides being used as the main ingredient in cocoa-based beverages, it is also widely exploited for formulating other products. When added to a recipe even in small amounts, cocoa powder has indeed a great effect on color and flavor as well as on functionality; cocoa is in fact one of the richest matrices in terms of bioactive compounds with a wide range of health properties, including antioxidant and anti-inflammatory activity [1]. Recently, [2] introduced the concept of the mood pyramid, which emphasizes the impact that bioactive molecules have on the mood and on cognitive aspects as a consequence of cocoa consumption.

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Cocoa has a complex composition and undergoes to intense processings. Its health benefits depend on the presence of multiple bioactive compounds, namely biogenic amines, polyphenols, methylxanthines, and to their interactions [3].

As far as the biogenic amines (BAs) are concerned, they are represented by polyamines, vasoactive amines (i.e., histamine, tyramine), and neurotransmitters that influence the mood (dopamine, serotonin, salsolinol). BAs can either naturally be present in cocoa or formed or be modified during technological processes via (i) microbial and/or oxidative decarboxylation of corresponding amino acid precursors, (ii) amination and transamination of ketones and aldehydes, (iii) thermal decarboxylation of amino acids in the presence of α-dicarbonyl compounds (Maillard reaction), (iv) lipid peroxidation [4–6].

Even though some of them at low levels may play important roles for plant protection as well as for human physiological functions, a high intake of vasoactive amines or an intake in presence of potentiating factors (drugs, alcohol, polyamines) are considered detrimental for human health and safety issues may arise [7,8]. Researchers investigate novel strategies to reduce BAs content in foods, at different stages of food processing [9–11], but in many cases it is difficult to avoid and/or contrast their occurrence, as in the case of fermented products. Furthermore, regulatory limits for BAs are still not considered by the current law (except for histamine levels in fish and fish products (Reg. CE 854/04 and Reg. CE 2073/05). For all these reasons, the knowledge of BAs content in cocoa and its derivatives, as well as in other foods, could be a useful tool for contributing to reduce consumers' exposure to these bioactive compounds.

In recent decades a great interest has been given in compounds belonging to the tetrahydroisoquinoline family, widespread in nature, that occurs naturally in plants, as well as in the brain of humans, primates and rodents [12]. Tetrahydroisoquinoline derivatives may be formed endogenously, as metabolites of BAs or their precursors, as well as be delivered exogenously for food intake. They can be divided into compounds with catechol- and non-catechol structure. Catechol derivatives include, among others, salsolinol (6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline), widely available in many beverages and foodstuffs [13], also employed as a marker for the postharvest senescence of fruit [14].

Salsolinol is a dopaminergic active compound which binds to the D2 receptor family, especially to the D3 receptor. It seems to be one of the main psychoactive compounds present in cocoa and chocolate and might be included in chocolate addiction [15].

Among bioactive positive components of cocoa, polyphenols emerge. In fresh unfermented cocoa beans polyphenols were detected for approximately 2% w/w [16] and approximately 6–8% on a dry weight basis [17]. Cocoa beans contain several classes of phenolic compounds among which, flavanols (28-39%), their oligomeric and polymeric derivatives (58-65%) (proanthocyanidins) and anthocyanins (4%) [18]. Traces of catechin esters, gallocatechin, epigallocatechin and epicatechin gallate can be also found [19].

Flavanols are the phenolic compounds most studied in cocoa, in particular the monomers (−)-epicatechin and (+)-catechin, and their polymers. Flavan-3-ols may group among them and constitute dimeric, oligomeric, or polymeric combinations of 10 or more units to constitute the proanthocyanidins, among which procyanidins (oligomers of epicatechin) are included. Recent reports indicate that cocoa beans and cocoa-derived products contain also considerable amounts of phenolic acids, stilbenes, and N-phenylpropenoyl-L-amino acids (NPAs) that are polyphenol/amino acid conjugates [17]. Substantial quantities of methylxanthines, another group of bioactive secondary metabolites derived from the purine base xanthine generated via repeated methylation, are also present. Together with polyphenols, methylxanthine compounds are responsible for the astringent and bitter sensation and, based on their pattern and concentration, can deeply affect the taste of cocoa-derived products.

In general, the composition of bioactive molecules in cocoa depends on different factors such as genotype, origin, difference between cultivars, different growth, and postharvest conditions [10]. In cocoa powder, the content and pattern of these compounds are deeply affected by cocoa transformation, a multi-step process that includes several unit operations such as fermentation,

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drying, roasting, and nib-grinding. The heat generated during this last step melts the cocoa butter and causes the formation of a liquid mass, also called cocoa liquor, which is then pressed to separate the cocoa butter from the solids to obtain the powder. Cocoa powder can be further submitted to an alkalizing treatment, also known as Dutch process. In this procedure, an alkali treatment is carried out on cocoa nibs before roasting, although the cocoa liquor itself, or even the powder, can also be directly treated [20]. The main aim of this operation is to modify flavor and color, even though other important technological consequences come along such as the increase of the pH and the improvement of the solubility of the powder in water [21]. Alkalization has been proven to strongly affect the content of phenolic compounds and their antioxidant capacity, generally causing a reduction, which is strictly related to the degree of alkalization [18] Despite the beneficial effects on solubility and sensory properties it could be observed a negative consequence on nutritional aspects in alkalinized cocoa powders, as oxidation, polymerization and degradation of polyphenols [22].

While the concentration of phenolic compounds in cocoa products and how processing can impact on such components has been the focus of intense research in the last two decades, very few works have been addressed to determine the content of BAs and polyphenols in cocoa powder which thus is worth to be investigated [1].

For this reason, this survey on 59 commercial samples of cocoa powder was to assess their real quality and safety associate to their intake. On selected samples, the following evaluations were carried out: color, pH, total polyphenolic content, antioxidant activity, concentration and pattern of biogenic amines. In addition, the research was aimed at knowing the effects of the possible processing conditions on the contents of bioactive compounds in the various subclasses of samples investigated.

2. Materials and Methods

2.1. Origin of the Samples

Fifty-nine commercial samples of cocoa powder were collected directly from European market; the origin of the samples was related to largest eight providers (85%), followed by those of other minor providers (15%), reflecting the distribution of international production on the market. Providers were identified with an alphabetical code (Table 1), at each sample has been assigned an identification numeric code; the geographical origin of raw cocoa beans has been reported in Table 1.

Table 1. Geographic origin of the samples sourced from Italian market.

Provider code (N of samples collected)	number code	Geographic origin
BC (N=15)	1; 5; 8; 12-14; 38-46	Ecuador; Sao Tomè; Dominican Republic; Peru
CA (N=1)	6	Colombia
CE (N=2)	4; 10	Ivory Coast
CG (N=8)	7; 16; 19; 21-25	Ivory Coast
CM (N=1)	15	Ivory Coast
DN (N=5)	11; 28-31	Ghana
IM (N=13)	17; 18; 20; 32-37; 47-48; 50-51	Ecuador; Peru
JR (N=3)	26-27; 53	Ecuador
ME (N=2)	58-59	Bali
PD (N=6)	9; 49; 54-57	Vietnam
UO (N=1)	3	Colombia
VV (N=1)	2	Bali
ZI (N=1)	52	Ivory Coast

In Table 3 information about the technological treatment of each investigated sample is shown; a total amount of 59 cocoa powder samples were analysed of which: 18 were commercially labelled as “organic”, 9 were claimed as “raw” (obtained from not roasted cocoa beans) while 29 samples were alkalized (as declared by providers).

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2.2. Colour Analysis

Color analysis on the cocoa samples was carried out by a Minolta Bench-top Colorimeter CR-5 CM-500 spectrophotometer (Konica Minolta, Tokyo, Japan). Before analysis, two calibrations were carried out, one with black standard and the other one with white standard. For each measurement, a single layer (4-5 mm) of cocoa powder was spread on a Petri dish. The analysis was repeated five times on each sample. The following color coordinates were determined: Lightness (L*), red/green (+/−) co- ordinate (a*), and (+/−) yellow/blue co-ordinate (b*), hue angle (h° = actang b*/a*). The a*/b* ratio was calculated to determine the red note of cocoa powder samples. The color difference [23], between the set of the not alkalized cocoa powder samples (control) and alkalized ones, was calculated as follows:

$$\Delta E_{NA} = (\Delta L^{*\,2} + \Delta a^{*2} + \Delta b^{*\,2})^{\,0.5}$$

2.3. Moisture and pH Determination

The pH of defatted cocoa powder was measured by diluting it in distilled water (1:1) after 1h of stop, by using an electrode probe connected to a pHmeter (Mettler Toledo, FE20, Ohio, USA).

Moisture content was determined according to AOAC official procedures [24]. In particular, 1 g of sample was dried in a forced-air drying oven at 105°C up to a constant weight.

2.4. Fat Content Determination

Cocoa powder samples were defatted by hexane washing , according to [25]. In brief, to 4 g of sample 25 mL of hexane were added; the mixture was then vortexed for 1 min and centrifuged (5000 g for 10 min) with an ALC4237R refrigerated centrifuge (ALC Intl., Cologno Monzese, Italy), each time discharging the supernatant. The defatting process was performed three times; eventually, to remove completely the hexane from the sample, the lipid-free cocoa powder were air-dried at room temperature. The total fat content was estimated by calculating the residual sample weight with respect to the starting weight.

2.5. Extraction of the Phenolic Fraction

All the reagents and solvents employed for assays are of analytical grade and were purchased from Sigma Aldrich (St Louis, MO, USA).

2.5.1. Polyphenols Conventional Liquid-Liquid Extraction

The defatted samples were further ground with mortar and pestle to reduce the powder size and allow a better contact of the extracting solvent with the sample. The sample extraction was carried out according to [25] with some modifications. One gram of defatted sample was added to 5 mL of 70:29.5:0.5 acetone/water/acetic acid; the mixture was vortexed for 1 minute, then sonicated in an ultrasonic bath at 20 °C for 10 minutes and finally centrifuged (10000g for 10 min). The supernatant was recovered and filtrated through cellulose filters. The extracted polyphenols were then stored in the freezer at -20°C until analyses.

2.5.2. Dimethylsulfoxide-Based Polyphenols Fast Extraction

0.1 g of cocoa powders, without any pretreatment, were solubilized in 1.5 mL of DMSO, vortexed for 1 min, and sonicated in an ultrasonic bath for 5 min at 20°C according to the strategy proposed by [26,27]with some modifications. The dispersion was centrifugated at 10000 g for 5 min, and the supernatant was recovered and stored at – 20°C in the dark.

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2.6. Phenolic Compounds Evaluation

2.6.1. Folin-Ciocalteau Assay

20 µL of a properly diluted cocoa sample, extracted in the conventional way according to section 2.6.2, was added to 20 µL of Folin-Ciocalteu reagent and orbitally stirred for 3 min with an orbital shaker (SSL1, Stuart equipment, Belfast, UK). Then, 400 µL of sodium carbonate (7.5% Na_2CO_3) and deionized water were added up to the final volume of 1000 µL. The solution was stirred at room temperature for 60 min, in the dark, and the total polyphenol content was determined at 760 nm; absorbance values were recorded using a JENWAY 6400 Spectrophotometer from Barloworld Scientific (Staffordshire, UK). Gallic acid was employed as a reference standard to calibrate the method.

2.6.2. AuNPs-Based Assay

A Gold nanoparticles (AuNPs) based colorimetric assay was performed according to [28] to evaluate the samples' reducing capacity. The assay was conducted in a 1.5 mL obscured microcentrifuge tubes; cocoa samples extracted according to section 2.6.3 were employed for the analysis which were diluted in DMSO prior analysis. 30 µL appropriate diluted cocoa extract was mixed with 210 µL of DMSO and stirred for 1 min at 300 rpm with the orbital shaker. Then, 25 µL of 2.0×10^{-2} mol L^{-1} $HAuCl_4$ and 235 µL of 1.0×10^{-2} mol L^{-1} PB pH 8.0 were added. Afterward, the solution was mixed for 1 min at 300 rpm with an orbital shaker and left to react for 5 min at 45°C in a water bath. Finally, the reaction was blocked at −20°C for 5 min, and the absorbance intensity, ascribed to the AuNPs formation, was recorded at 540 nm against the blank (reaction mix without sample). Gallic acid was employed as a reference standard to calibrate the method.

2.6.3. Electrochemical Measurement of Catechins

According to the method proposed by [29], the antioxidant activity on charge of flavan-3-ol structures has been evaluated using an electrochemical method. Thus, a screen-printed electrode (with a three-electrode configuration: working and counter electrode of graphite, and silver as pseudo-reference electrode, from EcoBioServices, Florence, Italy) modified with carbon black N220 from Cabot Corporation (Ravenna, Italy). and molybdenum disulfide from Sigma Aldrich (St Louis, MO, USA) according to [29] has been employed; from this point forward, the electrochemical sensor will be named nanostructured sensor (nSensor). The cocoa sample extracted according to section 2.3.6 was diluted in 0.1 mmol L^{-1} PB pH 7.0 to fit the dynamic range and 100 µL of samples was used to perform the analysis. Different Pulse Voltammetry (DPV), performed with a portable Palmsens 4 Potentiostat (Palm Instruments BV, Houten, Netherlands), was employed for the measurement, in a potential window from -0.15 to 0.35 V, with a pulse width of 50 ms, pulse amplitude of 20 mV, and a scan rate of 50 mV s^{-1}. Before the use and to regenerate the electrode, 5 consecutive cyclic voltammetry was conducted in 0.1 mmol L^{-1} PB pH 7 in 0.1 mol L^{-1} KCl, potential range from -0.30 V to +0.7 V (vs. pseudo-Ag/AgCl), at a scan rate of 0.5 V s^{-1}. Catechin was employed to calibrate the method, but the data was reported as gallic acid equivalents (GAeq) using an experimental correction factor, obtained by the ratio 'gallic acid calibration slope/catechin calibration slope'.

2.7. Biogenic Amines Determination

Defatted samples were subjected to BAs extraction, detection, identification and quantification by HPLC, optimizing the method described by [30]. All chemicals were of analytical reagent grade and supplied by Carlo Erba (Milan, Italy). Standards were obtained from Sigma (Bellefonte, USA).

Shortly, 1.0 g of sample was added of 5.0 mL of 0.1 N HCl and stirred in vortex (1 min) and ultrasound (20 min). It was centrifuged at relative centrifugal force of 4472*xg* for 10 minutes (refrigerated centrifuge NEYA 16r, Mumbai, India) and the supernatant recovered. Then, 150 µL of saturated $NaHCO_3$ was added to 0.5 mL of the supernatant, adjusting the pH to 11.5 with 0.1 N NaOH. For derivatization, 2.0 mL of dansyl chloride/acetone (10 mg mL^{-1}) was added and incubated at 40°C for 1 h under agitation (195 stokes) (Dubnoff Bath-BSD/D, International PBI. Milano. Italy).

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To remove excess of dansyl chloride, 200 µL of 30% ammonia was added, allowed to stand for 30 min at room temperature, and diluted with 1950 µL of acetonitrile.

In a Spherisorb S30ODS Waters C18-2 column (3 µm, 150 mm x 4.6 mm ID), 10 µL of sample were injected with gradient elution, acetonitrile (solvent A) and water (solvent B) as follows: 0-1 min 35% B isocratic; 1-5 min. 35-20% B linear; 5-6 min, 20-10% linear B; 6-15 min, 10% B isocratic; 15-18 min, 35% linear B; 18-20 min, 35% B isocratic. Identification and quantification of cadaverine (CAD), dopamine (DOP), ethylamine (ETH), histamine (HIS), 2-phenylethylamine (PHE), putrescine (PUT), salsolinol (SAL), serotonin (SER), spermidine (SPD), spermine (SPM), and tyramine (TYR) was performed by comparing retention times and using the calibration curves ofpure standards, respectively. The results were reported as mg of BA kg^{-1} of defatted dry weight (of DFW).

2.8. Statistical Analyses

All determinations were done in triplicate, except where differently indicated.

The means and standard deviations for each numeric variable in the sample were computed. Differences in mean value between organic and non-organic, raw and non-raw, alkalized and non-alkalized cocoa samples were assessed through the t-test but when variable distribution was normal on the basis of the Shapiro-Wilk test, in other cases the Mann-Whitney (MW) test [31] was used. Since in some cases the sample size was limited (e.g., raw and organic samples) the Monte Carlo method with $1 \cdot 10^6$ simulation was applied to both Mann-Whitney and t-test in order to generate random replicate values that closely approximate the distribution of samples that are likely to be collected in a broader survey. All statistics were performed using XLSTAT 2021 software (Addinsoft, Paris, France).

In addition, a principal component analysis (PCA) [32] was carried out to obtain a representation of the numeric variables (excluding "total biogenic amines") in a space of reduced dimensionality. The dimensionality of such space, i.e., the number of principal components (PCs) to retain in the analysis, was defined through the elbow method. A score plot of the first 2 PCs was produced to visualize the distribution of the cocoa samples in the most meaningful bidimensional space. PCA was performed using R Statistical Software (v4.2.2) [33].

3. Results and Discussion

An extensive survey was carried out on 59 cocoa powder samples from the European market. Cocoa samples were segmented in distinct subgroups: organic/ conventional, according to the agronomic techniques used for their production, alkalized/not alkalized according to the alkalinization process used prior to roasting, and raw/not raw according to the roasting process adoption. In particular, the 'organic' mark and the 'raw' wording were present on the labels since they distinguish two types of cocoa powders 'commercial categories.

3.1. Colour Indices and pH

Colour parameters and pH, reported in Table 2, were used as technological indices of roasting process and alkalinization, respectively.

Table 2. Technological parameters of cocoa powder samples belonging to different categories.

	L*	**a***	**b***	**C**	**h***	**pH**
not organic (N. 41)	41.49±7.50	12.97±1.63	19.59±3.19	23.58±3.02	56.17±4.99	6.02±0.78
	(24.77-55.61)	(9.05-16.35)	(10.95-25.64)	(14.20-28.67)	(41.29-63.63)	(4.75-7.32)
organic (N. 18)	40.71±6.08	13.45±1.46	20.16±2.20	24.30±2.00	56.19±4.09	6.38±0.84
	(30.08-52.09)	(10.93-15.69)	(14.86-24.05)	(20.02-27.27)	(47.96-64.79)	(5.08-7.32)
p-value	0.675	0.320	0.499	0.363	0.985	0.121

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not raw (N. 50)	40.32±7.29	19.34±1.54	19.87±2.61	24.00±2.42	55.93±4.62	6.24±0.81
	(24.77-55.61)	(9.71-16.35)	(11.17-24.12)	(16.93-27.61)	(41.29-64.79)	(4.75-7.32)
raw (N. 9)	46.46±5.39	11.92±1.30	19.22±4.43	22.69±4.18	57.50±5.19	5.55±0.58
	(33.17-51.25)	(9.05-13.41)	(10.95-25.64)	(14.20-28.67)	(47.96-63.41)	(5.08-6.87)
p-value	0.019	0.012	0.716	0.238	0.246	0.009
not alkalized (N. 30)	46.52±5.52	12.18±1.42	20.51±2.92	23.89±2.95	59.11±3.39	5.39±0.28
	(32.86-55.61)	(9.05-14.82)	(10.95-25.64)	(14.20-28.67)	(50.46-64.79)	(4.75-5.95)
alkalized (N. 29)	35.80±4.44	14.10±1.06	19.00±2.76	23.70±2.58	53.14±3.87	6.90±0.28
	(24.77-42.23)	(11.86-16.35)	(11.17-22.35)	(16.93-27.53)	(41.29-57.93)	(6.01-7.32)
p-value	<0.001	<0.001	0.055	0.806	<0.001	<0.001

No differences in pH and colour between organic and conventional samples were observed since many factors, such as the fermentation process, alkalinization and roasting, could largely influence the cocoa and cocoa powder colour and pH [22,25,34]. The ranges of luminosity and hue angle values observed in this study are consistent with those previously reported by [22] but slightly wider (±10% approximately for both the indices).

As expected, alkalization increased the pH of cocoa powder (Table 2). According to the classification of alkalized cocoa powder [35], all investigated commercial samples resulted into the medium-alkalized range (pH 6.5-7.2) whereas none samples were assorted into the high-alkalized range (pH > 7.6). The pH of alkalized samples is within the range previously reported by [22]. The pH of the not alkalized samples resulted in the range of natural cocoa powder (pH 5.3-5.8).

Alkalized samples showed also lower luminosity (L*) and hue angle (h°) values, by indicating a higher browning extent. It is known that Maillard condensation is favoured in basic environment since the nucleophilic amino nitrogen group is not protonated [36] Moreover, recent studies reported about the relationship between the generation of polar and non-polar chromophores and the chemical arrangements of flavan-3-ols(+)catechin or epicatechin which occur in cocoa matter during alkalization process [37,38]. The colour difference between alkalized and not alkalized samples as calculated by the ΔE_{NA} index is of about 1.5, which is below the 2.3 threshold identified for the just noticeable difference by the human eye [39].

The raw samples showed lower L* values than roasted ones, with an average difference of about 6 points; previous studies indicated a reduction of about 3 points independent from the roasting temperature [34] but differences in prime material characteristics (chemical composition, pH, moisture content) could largely affect the extent of the browning reaction. The pH values of raw samples were lower than those of roasted ones because all the raw samples but one was not alkalized.

3.2. Fat and Moisture

The moisture content for each sample was measured and constant values were found (2% ± 0.07) and this was considered equal for all powders. The total fat content was estimated via gravimetry (section 2.4); no significant trends were observed for the different cocoa powders' classes, on the other hand, a heterogeneous fat content, ranging from 5.6% to 27.3%, was recorded (Table 3).

Table 3. Technological samples' features; fat and phenolic compounds evaluation; data are expressed as mean value (n= 3) and standard deviation.

				Total phenolic content (mg $_{GAeq}$ g^{-1})		
sample	**Label**	**alkalization**	**Fat (%)**	**Folin**	**AuNPs**	**nSensor**
1		raw	5.6 ± 0.9	141.0 ± 3.7	136.9 ± 7.1	57.1 ± 0.4
2	organic	raw	15.1 ± 1.3	72.0 ± 1.4	79.1 ± 0.5	33.0 ± 0.9
3	organic	raw	8.5 ± 0.7	60.8 ± 1.6	51.7 ± 0.2	21.6 ± 0.4

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4			alkalized	24.6 ± 2.1	36.2 ± 2.3	43.8 ± 0.1	18.3 ± 0.8
5	organic	raw		7.0 ± 0.6	51.8 ± 1.1	71.0 ± 0.8	29.6 ± 0.8
6	organic			8.5 ± 0.7	67.8 ± 1.0	52.0 ± 6.1	21.7 ± 2.0
7				9.3 ± 1.2	48.1 ± 0.2	70.1 ± 7.2	29.3 ± 0.6
8				9.1 ± 1.8	73.7 ± 1.2	39.5 ± 6.0	16.5 ± 2.2
9				9.1 ± 0.8	53.5 ± 0.7	72.9 ± 2.3	30.5 ± 1.8
10			alkalized	20.7 ± 1.8	35.7 ± 1.6	39.0 ± 2.1	16.3 ± 1.7
11				7.9 ± 0.7	31.1 ± 2.4	39.4 ± 1.5	16.5 ± 0.8
12				8.8 ± 0.7	24.9 ± 3.0	31.9 ± 0.6	13.4 ± 1.5
13	organic		alkalized	10.4 ± 0.9	15.8 ± 0.2	13.9 ± 0.1	5.9 ± 0.7
14	organic		alkalized	16.5 ± 1.4	14.2 ± 0.1	11.9 ± 0.2	5.1 ± 0.8
15				14.3 ± 1.2	20.3 ± 0.2	31.2 ± 0.9	13.1 ± 0.9
16				8.7 ± 0.7	25.3 ± 1.7	40.1 ± 1.1	16.8 ± 0.6
17	organic		alkalized	17.5 ± 1.5	20.1 ± 1.7	20.4 ± 1.0	8.6 ± 1.5
18			alkalized	10.2 ± 0.9	25.9 ± 1.0	18.7 ± 0.9	7.9 ± 1.0
19	organic		alkalized	8.1 ± 0.7	9.2 ± 0.2	8.9 ± 0.3	3.8 ± 0.4
20			alkalized	12.4 ± 1.1	22.3 ± 0.2	21.1 ± 0.4	8.9 ± 0.9
21			alkalized	8.7 ± 0.9	16.0 ± 0.1	10.5 ± 0.2	4.5 ± 0.9
22			alkalized	19.9 ± 1.8	14.0 ± 0.0	6.6 ± 0.1	2.8 ± 0.8
23			alkalized	20.5 ± 1.7	9.5 ± 0.0	8.6 ± 0.5	3.7 ± 0.4
24			alkalized	11.5 ± 1.0	8.8 ± 0.1	4.5 ± 0.2	2.0 ± 0.7
25				22.1 ± 1.9	33.3 ± 0.0	38.2 ± 5.2	16.0 ± 1.3
26	organic	raw		9.7 ± 0.8	43.3 ± 0.3	63.9 ± 1.7	26.7 ± 1.7
27	organic	raw		20.9 ± 1.8	32.8 ± 0.6	38.1 ± 0.9	15.9 ± 1.1
28				9.0 ± 0.8	37.6 ± 2.7	48.9 ± 2.2	20.5 ± 2.3
29				20.7 ± 1.8	27.3 ± 0.4	32.2 ± 0.3	13.5 ± 1.3
30			alkalized	7.9 ± 0.7	3.6 ± 0.4	13.5 ± 0.1	5.7± 0.8
31			alkalized	7.5 ± 0.6	16.9 ± 0.0	9.9± 0.4	4.2 ± 0.6
32				9.3 ± 0.8	43.4 ± 1.2	49.2 ± 0.1	20.6 ± 0.8
33				10.3 ± 0.9	44.2 ± 1.6	48.0 ± 1.1	20.1 ± 0.2
34			alkalized	6.4 ± 0.5	33.2 ± 0.0	12.4 ± 0.0	5.2 ± 0.1
35				17.9 ± 1.5	39.9 ± 0.9	38.3 ± 1.9	16.0 ± 0.5
36	organic		alkalized	9.6 ± 0.8	45.2 ± 2.1	59.4 ± 1.4	24.8± 1.6
37	organic		alkalized	15.9 ± 1.4	49.8 ± 2.0	45.7 ± 0.4	19.1 ± 1.2
38				9.5 ± 0.8	40.2 ± 1.9	50.2 ± 0.4	21.0 ± 1.8
39				20.7 ± 1.8	67.6 ± 0.5	49.7 ± 5.2	20.8 ± 0.7
40			alkalized	8.0 ± 0.9	21.1 ± 0.2	7.2 ± 0.3	3.1 ± 0.6
41			alkalized	19.3 ± 1.6	22.5 ± 0.2	16.4 ± 0.2	6.9 ± 0.1
42			alkalized	8.9 ± 0.8	17.1 ± 0.1	9.6 ± 0.3	4.1 ± 0.3
43			alkalized	8.2 ± 0.6	30.9 ± 2.0	14.8 ± 1.0	6.2 ± 0.9
44			alkalized	7.6 ± 0.6	15.3 ± 0.2	10.9 ± 0.1	4.6 ± 0.6
45	organic			9.3 ± 0.8	51.8 ± 1.5	55.5 ± 2.5	23.2 ± 0.2
46	organic		alkalized	7.3 ± 0.6	25.6 ± 1.9	35.5 ± 0.7	14.9 ± 0.5
47			alkalized	21.0 ± 1.8	26.0 ± 0.5	21.8 ± 1.8	9.2 ± 1.1
48	organic		alkalized	16.6 ± 1.4	35.0 ± 1.2	30.7 ± 0.4	12.9 ± 0.3
49				27.3 ± 2.3	56.8 ± 2.0	63.8 ± 0.4	26.7 ± 2.1
50	organic		alkalized	17.5 ± 1.7	26.6 ± 2.6	14.6 ± 0.4	6.2 ± 1.2
51	organic		alkalized	17.4 ± 1.4	65.4 ± 1.8	58.2 ± 0.2	24.3 ± 1.6
52			alkalized	18.8 ± 1.7	14.9 ± 0.2	8.8 ± 0.1	3.7 ± 0.3
53	organic	raw	alkalized	17.1 ± 1.3	25.2 ± 1.5	13.3 ± 0.6	5.6 ± 0.8
54				22.1 ± 1.9	46.2 ± 0.5	45.9 ± 0.3	19.2 ± 1.5
55				26.1 ± 2.2	64.3 ± 0.7	59.7 ± 0.3	24.9 ± 0.7
56				23.5 ± 2.0	37.0 ± 0.4	42.5 ± 2.4	17.8 ± 0.8
57				18.9 ± 1.6	28.7 ± 1.1	63.6 ± 1.6	26.6 ± 2.1
58		raw		16.2 ± 1.4	69.7 ± 0.1	71.5 ± 1.2	29.8 ± 2.1
59		raw		11.2 ± 1.0	50.4 ± 0.5	68.8 ± 2.0	28.7 ± 0.8

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3.3. Polyphenols Evaluation

The phenolic fraction of the cocoa samples set was characterized by using classical and emerging spectrophotometric methods i.e., the Folin Ciocalteu (Folin) and gold nanoparticles (AuNPs) based assays, respectively; moreover, the phenolic fraction has been also estimated using an electrochemical nanostructured sensor (nSensor) developed by [29] the nSensor allows the selective determination of total flavonoids including their polymers. For all methods, the detailed procedures are reported in section 2.

Folin assay returns information concerning the total phenolic content and relies on the ability of the phenolic compounds to quantitatively reduce the Folin reagent, whereas the AuNPs-based assay evaluates the total phenolic content (TPC) coupled to the phenolic compounds' intrinsic antioxidant capacity. Indeed, the AuNPs assay principle relies on the PCs' ability to reduce the gold cations Au(III) in the metal form Au (0) and stabilize them in the form of colloidal nanoparticles (AuNPs) [28]; the latters give rise to the optical response. In this case, the phenolic structure plays a key role in the AuNPs formation, due to the resulting antioxidant capacity and stabilizing ability; for these reasons, the method returns information on the TPC intrinsic reactivity [40]. Despite the different principles, Folin and AuNPs assays return similar data, a slight overestimation for the Folin assay was observed since Folin is not selective, the overestimation could be attributed to other reducing species [41]; on the other hand, AuNPs assay is more influenced by the phenolic compounds intrinsic antioxidant capacity. Nevertheless, the high correlation between the two methods has been confirmed by the high Pearson coefficient (R= 0.95) and the correlation equation's slope close to 1 (y=1.0647x-4.3022) indicating the numerical match of the data. The strong correlation observed is remarkable due to the different PCs extraction procedures performed before the assay, i.e., a conventional extraction for the Folin (see section 2.5.1.) and a direct phenolic compounds solubilization form cocoa powder, by using DMSO, for the AuNPs-based assay (see section 2.5.2.); this data are confirming that the DMSO-based free-extraction approach is a promising strategy to straightforwardly and rapidly determine PCs in fat-rich samples.

Overall, the same TPC trend was pointed out by the two methods; however, the alkalinized cocoa powders reported a lower TPC amount concerning non-alkalinized samples, whereas higher TPC was observed for raw cocoa powder (Table 3). No significant differences were highlighted between organic and non-organic samples.

On the other hand, the nSensor-based method allows to estimate selectively the flavonoids and their polymers. The method relies on the flavonoids' ability to donate electrons at the sensor working electrode surface under an applied potential which promotes the selective oxidation of these structures [29]. Even in this case, the sensor allows evaluating the electron donor ability of flavonoids; the latter is intrinsically influenced by their antioxidant capacity. As expected, the nSensor's data are lower than the two spectrophotometric assays, about half compared to the AuNPs, and mainly one-third compared to the Folin, due to the method selectivity (Table 3). Despite this, the flavonoid content is consistent with the TPC trends observed for Folin and AuNPs. As observed by the data, a higher correlation with the AuNPs test was found (R=0.97) than the Folin method (R=0.95) confirming that the nSensor, in addition to selectivity, returns information more related to the antioxidant capacity of the studied phenolic structures.

The TPC values of the samples covered a quite broad interval of contents (Table 3), ranging from 3.63 to 140.89 mg GAE g^{-1}, giving an indication of the wide variability in the total content of phenolic compounds in cocoa powders, which, in turn, is related to the variability of the raw materials as well as of the processing conditions adopted. Such a wide range comprise values reported in other studies [22,23,35,42].

For this reason, as carried out for colour pamateres and pH values (Table 2), also samples for total phenolic content values, as evaluated by the Folin and the nSensor-based methods, were segmented according to technology of production (organic vs conventional) and processing (alkalinization and roasting) and the results reported in Table 4; in order to evaluate the effect of technology of production and processing on phenolic content, the latter was calculated on defatted dry basis.

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A significant impact on both Folin and nSensor values was observed as a consequence of the roasting and alkalization processes while no significant impact was found as a consequence of different agronomic practices.

Table 4. Phenolic compounds (mean ± standard deviation; min; and max value) of cocoa powder samples belonging to different categories.

	Phenolic compounds (mg $_{GAEeq}$ g^{-1}_{DDW})	
	TPC	**nSensor**
conventional (N. 41)	42.18±27.45	18.13±12.57
	(3.94-149.2)	(2.21-60.47)
organic (N. 18)	45.39±22.27	19.21±10.51
	(9.97-84.71)	(4.11-38.86)
p-value	*0.467*	*0.477*
not raw (N. 50)	38.61±21.19	16.20±9.91
	(3.94-86.99)	(2.21-36.69)
raw (N. 9)	68.44±35.19	31.03±14.64
	(30.40-149.2)	(6.77-60.47)
p-value	*0.003*	*0.003*
not alkalized (N. 30)	57.48±25.38	26.57±9.32
	(23.67-149.2)	(14.65-60.47)
alkalized (N. 29)	28.35±16.38	10.07±7.73
	(3.94-79.23)	(2.21-29.44)
p-value	*<0.001*	*<0.001*

Specifically, the raw and not alkalized cocoa powders were characterized by higher contents of total polyphenols and flavanols (nSensor) than roasted and alkalized ones; as both roasting and alkalization processes are indeed reported to significantly impair phenolic compounds and their bioactivity [43].

According to literature, during roasting, a TPC decrease by 28% up to more than 50% could be found depending on the processing temperature adopted [44] and it was related to the oxidation of flavanols and proanthocyanidins. On average, also in the samples under study, the reduction of both TPC and nSensor values in the roasted samples accounted for about a 50% when compared with the raw cocoa powders.

The chemical oxidation of polyphenols, which takes place during roasting, induces polymerization reactions [44] which are responsible for browning [45]. The energy of activation of polyphenols oxidation (between 60 and 80 kJ mol^{-1}) is lower than that of melanoidin formation (132 kJ mol^{-1}), thus the browning observed during roasting is not solely dependent on Maillard reaction occurrence but also to polyphenols oxidation with a consequencial effect on colour parameters (Table 2).

As far as alkalization is concerned, it has been proven to strongly affect the content of phenolic compounds, generally causing a reduction, which is strictly related to the degree of alkalization when all other conditions are equal. In fact, losses in the content of total polyphenols of around 27%, 54% and 63% are reported for light, medium and heavy alkalinized cocoa powders, respectively [35]. In the samples under investigation in the present study, the reduction of both TPC and flavanols (nSensor) values in the alkalized samples accounted, on average, for about 50% and 60%, respectively, when compared with the not alkalized cocoa powders. Similar percentages of reduction (45.5%) in

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the content of total polyphenols were reported also in a work by [46]in which 11 cocoa powder samples (6 alkalinized and 5 not alkalinized) were considered.

3.3. Biogenic Amines

As known, biogenic amines (BAs) are ubiquitous compounds highly found in fermented products. Cocoa, and its derivatives, may contain variable quantities of BAs in respect to several characteristics. Mainly. technological processes are responsible for their accumulation in foods. but even agricultural practices. geographical origins, and species varieties have a direct influence on the final content [7]

A lot of studies have seen the evolution of BAs in cocoa seeds during fermentation and roasting [10,47,48].

The present research shows that most of cocoa samples sourced from Italian market contain very low levels of biogenic amines. As shown in Table 5, median resulted 0.00 mg kg^{-1} DDW, for all each biogenic amines, demonstrating that their occurrence is quite random and would appear to be unrelated to a specific variable processing or chemical characteristics.

Table 5. Results of biogenic amines analysis in cocoa powder sourced from Italian market (N=59).

	ETH	DOP	SER	HIS	SPD	SAL	SPM	total BAs
N of positive (%)	9 (15%)	3 (5%)	13 (22%)	11 (19%)	5 (8%)	4 (7%)	3 (5%)	18 (31%)
BAs (mg kg^{-1} DDW)								
Median	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
median (of positive *samples)*	22.89	88.32	82.13	69.70	23.88	0.22	25.35	154.08
Mean	6.11	5.80	18.53	11.40	3.53	0.06	1.46	46.89
dev st	20.23	27.23	38.97	28.21	15.92	0.31	6.84	95.07
Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Maximum	91.54	176.95	181.81	139.28	105.96	1.86	43.66	480.87

BAs were detected in 31% of the samples, with a median value (relative to positive BAs samples) of 154.08 mg Kg^{-1}DDW, in a range of 0.00-480.87 mg Kg^{-1}DDW (Table 3).

The ability to trigger migraine episodes after intake of some foods, particularly cocoa products, may be due to BAs such as tyramine or phenylethylamine [49]; in our study, nor TIR neither PHE were found in cocoa powder. Moreover, CAD and PUT resulted also not detectable in investigated samples.

Most of samples were found to contain SER and HIS (22% and 19% positive to them, respectively). Any case, it is important highlight that the highest HIS level (139.28 mg Kg^{-1}DDW) was found in sample derived from conventionally grown cocoa, roasted cocoa bean, and processed by alkalization (sample named 34, see table S1). High levels of HIS may lead to hypotension, nausea, migraine, abdominal pain, and heart problems [50]. No observed adverse effect level (NOAEL) was observed after exposure to 50 mg histamine per person per meal for healthy individuals; these which would be hard to reach, even by eating 2,5 g of cocoa powder, which is the recommended dose by [51]. Moreover, the adverse role of HIS (and TYR) on the human health is enhanced in sensitive individuals (with histamine intolerance) and potentiated in association with intake of alcohol and some drugs having anti-depressant and anti-hypertensive effects [52]. It is important also to highlight that amines could be increased after gastrointestinal digestion, according to a recent study of [53]. These authors tested bioaccessibility of biogenic amines in cocoa dark chocolate through in vitro simulation of oral, gastric and intestinal digestions. High bioaccessibility with slight influence of digestive enzymes was found for all amines. In vitro digestion showed that pepsin increased accessibility of polyamines, while pancreatin positively acts on HIS and CAD accessibility.

Cocoa powder samples resulted rich of SER and DOP at very high levels, confirming literature data [5,6]. Dopamine and serotonin are neurotransmitter that play an important role in the brain's reward system. DOP is involved in motivation. reinforcement of behaviors, and pleasure; SER is a

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calming neurotransmitter. In the brain of the animal organisms**, endogenous dopamine and serotonin levels** are increased by many different types of drugs, including dark chocolate. Most literature argues that dopamine or serotonin syntesis is an essential way in which an organism can activate a positive behaviour [54,55]: SER synthesis has been correlated to the occurrence of its precursors (eg. tryptophan), phenylethylamine is a precursor of DOP. McCutcheon [56] put in evidence that dopamine is sensitive to nutritional value of certain foods [56]. Flavonoids, contained in dark chocolate, seems also to stimulate dopamine syntesis in brains [57]. The study was conducted on rats, and more research is needed to confirm the findings in humans.

In the light of all these speculations, the role of exogenous DOP and SER should be better understood, in order to correctly highlight the role that food rich in these compounds can have, and whether their intake really has a positive effect on neurologically, mood and behaviorally aspect.

Literature reports that cocoa and chocolate contain the tetrahydroisoquinoline alkaloid salsolinol up to a concentration of 25 mg/g; taking the detected concentration and the pharmacological properties into account, salsolinol seems to be one of the main psychoactive compounds present in cocoa and chocolate and might be included in chocolate addiction [15]. To our best knowledge, this is the first study reporting analyses of salsolinol content in cocoa powder. In the investigated samples, SAL was detected only in few samples (7%) and, as it is possible to see in Table 5, its maximum content resulted less than 2 mg kg^{-1} DDW, in not organic, not raw and not alkalized sample (N° 33, Table S1). In any case, the presence of salsolinol in food can be interpreted as not healthy, because of it could act as a neurotoxin which kills dopaminergic neurons [58]. High concentrations of salsolinol were detected in urine of Parkinson's patients, thus it has been speculated it contribute to pathophysiology of Parkinson's disease and chronic alcoholism. It has demonstrated that when about 50-60% of dopamine-producing nerve cells are lost, symptoms of Parkinson's disease begin to manifest. In animal studies, chronic administration of salsolinol induced parkinsonian-like symptoms. Moreover, little is known about its effects on the gut-brain axis activation (Kurnik et al., 2016). Other authors hypotized also a possible neuroprotective property of this chemical compound [12] . Moreover, Wen et al. [59] reported that salsolinol reduces doxorubicin-induced chronic heart failure, reduces serum myocardial injury marker levels, decreases tissue damage to the heart, and increases the relative mRNA expression levels of key enzymes downstream of the TCA cycle to increase cardiac energy metabolism. However, it should better clarify if exogenous salsolinol would have the same effects of one synthetized physiologically in animal organisms.

Polyamines as SPM and SPD were also detected, but only in a few samples, and with maximum concentration of about 44 mg kg^{-1} DDW and 106 mg kg^{-1} DDW, respectively. Spermidine and spermine are naturally present in food; in particular, SPD is most abundant in plant-based products, whilst SPM is generally higher in animal-derived foods [60]. Polyamines intake have important implications in human health, mainly for the intestinal and immune systems. Due to their antioxidant and anti-inflammatory effect, they are also important in the prevention of chronic diseases such as cardiovascular diseases. There are no recommendations for polyamine daily intake, however, dietary source becomes of greater importance in an aging population, because of *de novo* synthesis of polyamines tends to decrease with age [61]

In Table 6 are shown data for BAs of all investigated samples, collected for three subclasses. In general, observing these results, it is evident a significant positive effect on the restraint of biogenic amines in cocoa powder from poorly processed raw matter (organic grow cocoa, not roasted cocoa beans).

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Table 6. Biogenic amines (mg kg^{-1} DDW) levels; reported as mean; standard deviation and range (min-max); in the subclasses.

	ETH	DOP	SER	HIS	SPD	SAL	SPM	total BAs
conventional (N. 41)	7.15±22.05 (0.00-91.54)	8.34±32.46 (0.00-176.95)	23.54±43.42 (0.00-181.81)	15.75±32.76 (0.00-139.28)	5.08±18.96 (0.00-105.96)	0.09±0.37 (0.00-1.86)	2.10±8.15 (0.00-43.66)	62.04±107.95 (0.00-480.87)
organic (N. 18)	3.76±15.61 (0.00-66.28)	0.00±0.00 (0.00-0.00)	7.14±23.40 (0.00-95.62)	1.49±6.33 (0.00-26.84)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	12.39±39.99 (0.00-161.90)
p-value	0.464	0.455	0.573	0.158	0.199	0.107	0.174	0.832
not raw (N. 50)	7.21±21.82 (0.00-91.54)	6.84±29.51 (0.00-176.95)	21.87±41.51 (0.00-181.81)	13.45±30.23 (0.00-139.28)	4.16±17.24 (0.00-105.96)	0.07±0.34 (0.00-1.86)	1.72±7.41 (0.00-43.66)	55.33±101.10 (0.00-480.87)
raw (N. 9)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)	0.00±0.00 (0.00-0.00)
p-value	0.246	0.152	0.372	0.174	0.461	0.838	0.178	0.159
not alkalized (N. 30)	3.82±13.39 (0.00-69.61)	8.84±35.61 (0.00-176.95)	19.93±44.54 (0.00-181.81)	15.70±29.40 (0.00-86.25)	4.42±19.67 (0.00-105.96)	0.12±0.43 (0.00-1.86)	2.87±9.46 (0.00-43.66)	55.71±108.08 (0.00-480.87)
alkalized (N. 29)	8.48±25.50 (0.00-91.54)	2.65±14.25 (0.00-76.73)	17.08±32.96 (0.00-107.60)	6.95±26.71 (0.00-139.28)	2.61±11.08 (0.00-57.48)	0.00±0.02 (0.00-0.08)	0.00±0.00 (0.00-0.02)	37.77±80.33 (0.00-296.41)
p-value	0.399	0.781	0.425	0.161	0.686	0.171	0.175	0.380

Cocoa powder from conventional process (not organic) resulted at higher levels of HIS and other BAs, so their sum was also significant higher respect to organic samples. These results are agreed with literature. Some authors highlighted BAs as quality descriptors in cocoa products [47] and useful even to differentiate between conventionally and organically grown cocoa [62,63] They identified cadaverine, serotonin, histamine, spermidine, spermine, tyramine, putrescine, and β-phenylethylamine, showing that the organic samples contain lower concentrations of all these amines.

In all cocoa powder samples (N, 9) from raw cocoa bean BAs resulted not detectable, whilst the higher amounts for each single amine were detected in samples from roasted beans.

Histamine found in samples could result from thermal decarboxylation of histidine during roasting of cocoa bean [64]. Delgado-Ospina [5] have found the direct influence of different roasting temperatures on the increase of BAs especially on histamine and some polyamines, while a decrease

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on serotonin in dried roasted seeds. As pointed out from the authors, values were not hazardous for human consumption.

As showed in Table 6, alkalization process generally does not affect the occurrence of BAs, except for SAL and SPD, which were found only in not alkalized samples, (see also Table S1). It is speculable that results are probably correlate to the cocoa origin very varied for some big trade farm.

3.4. Principal Component Analysis

Principal component analysis was carried out to obtain a representation of the numeric variables in a space of reduced dimensionality. The dimensionality of such space was chosen based on the portion of variance explained by each component. Based on the graphical analysis of the scree plot, the four components chosen explained 31.2% (PC1), 18.8% (PC2), 14.7% (PC3) and 9% (PC4) of variance, respectively. By the observation of the trend of square cosine (Cos2), it was possible to deduce that all variables contribute for each component (quality of representation).

Figure 1 represents a heat map showing the loadings of each variable for PC1, PC2, PC3 and PC4. The PCA was performed to highlight how factors or variables can discriminate cocoa powder samples obtained with different technological processes. Figure 2 shows graphically the loadings on PC1 and PC2.

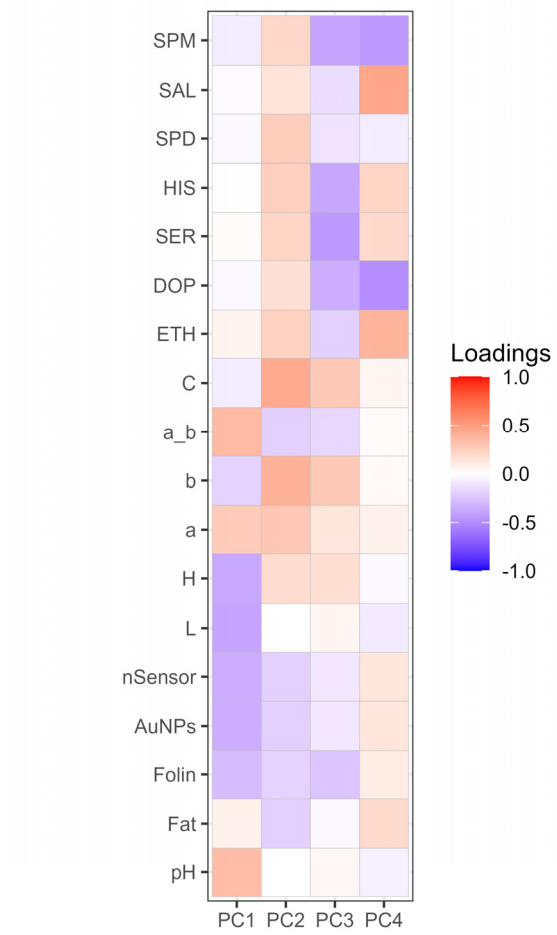

Figure 1. Heatmap diagram displaying the loadings of variables for principal components PC1, PC2, PC3 and PC4. Difference intensities are shown in the far right-hand side: deep blue and red colorations represent extremes of low and high intensity, respectively.

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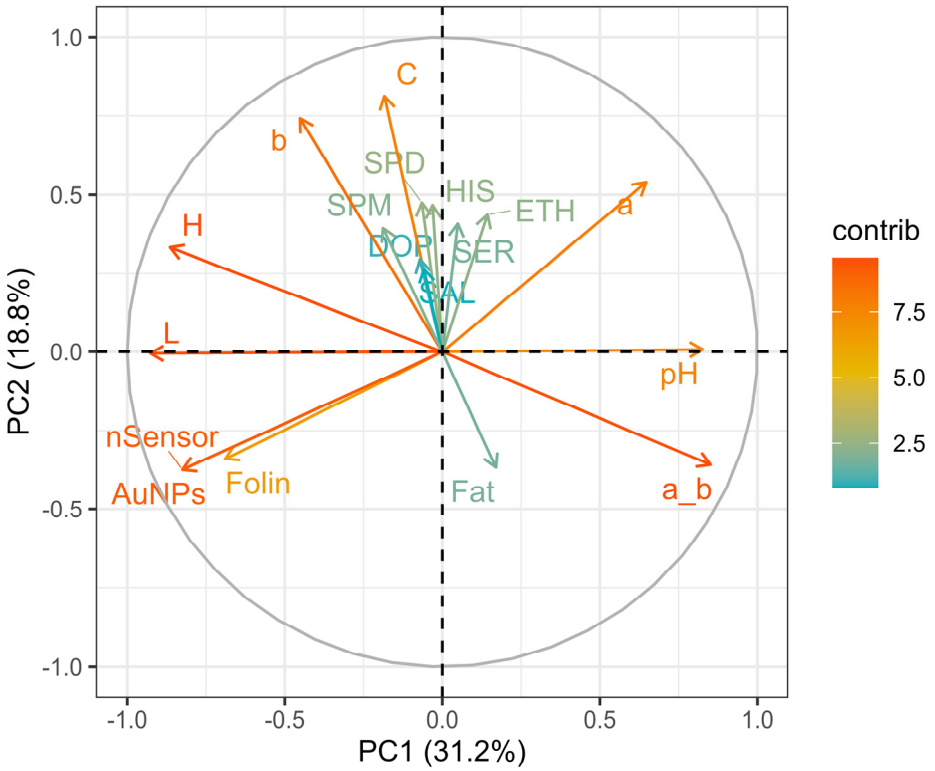

Figure 2. Loadings plot of variables for the first two components. Difference contribution are shown in the far right-hand side: deep green and orange colorations represent extremes of low and high contribution, respectively.

From Figure 1 it is visible how for PC1 and PC2 the different loadings impact on the discrimination of the samples. PC1 puts on the two extremes pH and L; this separation reflects somehow the nature of cocoa powders, informing about the effect of the alkalinization process. This, among all the changes, alters the pH and the color. As visible, the pH variable influences the ratio a*/b* that has a similar positive contribution (Figure 1) and a spatial localization in the PCA plot (Figure 2) pH (0.34) and red index a*/b* (0.36) showing a positive effect. Oppositely, L, (lightness) has a negative contribution (Figure 1) being localized in the opposite region of the PCA plot (Figure 2) (L) (-0.39). Of course, as for the pH's influence on a*_b* ratio, L correlates with H (hue angle) (-0.36); this property describes the color position on a color wheel expressed in degrees °, or by its main wavelength. Thus, even if the color itself does not change, L* differences increments or diminishes the color intensity, making it possible to distinguish among lighter or darker shades. Alkalized products tend to have higher pH's values and darker shades of brown. This is in line with the general market demand for cocoa and chocolate products, despite the recent introduction of ruby chocolate and related products [65]. Polyphenols, evaluated by nSensor, AuNPs assay and Folin assay, are all giving a negative contribution to PC1 (Figure 1) the spatial positioning on the PCA attests that these three variables have a great contribution highly discriminating the samples distributed around these variables Folin (-0.29) and AuNPs-based assay (-0.34). Once again, this technological treatment impacts on the final content of flavanols and other phenolic species measured with different indexes. As attested, alkalization reduces bitterness by limiting the presence of polyphenols and increasing the rate of Maillard reactions of conjugation among sugars and amino acids [18]. Concerning PC2,

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this component was mainly influenced by colorimetric parameters. The second component was associated with increasing of C*, b* and a*. PC2 also receives a positive contribution from all the biogenic amines and salsolinol as visible from Figure 1. The PCA anyway better explains how their role is limited. The less discriminative power of BAs and salsolinol of this PCA informs that these compounds are not influenced by the alkali process as happens for colorimetric indexes. From these analyses it must be stated that the technological process, namely alkalization, changes many features of cocoa powders acting also on compositive traits (pH, color, and phenolic composition) but with a limited extension on the evolution of some constitutive compounds (BAs and salsolinol) more dependent on the obtention of the raw matter and other previous technological process such as fermentation and roasting.

Figure 3 completes the information given from the heat map and the PCA. Graphs in Figure 3 show a score plot for PC1 and PC2, and for PC1 and PC4, respectively. As visible from the first score plot, there is a strong separation between alkalized and non-alkalized samples highly spread on PC1. By overlapping the variables distribution (Figure 2), is clear that pH, and a*_b* ratio are discriminant for alkalized samples (right side of the plots), while L and H, b*, and C* for non-alkalized samples (these have more vivid colors and are lighter). Those samples, mainly blue circles, at the very far right side of the plot, may be powders on which the extension of the dutching process, was higher, or conducted with different reagents (i.e., $NaHCO_3$, K_2CO_3, KOH) and more intense conditions (temperature and pressure). Obtention of cocoa, thus their organic or conventional farming, and the rawness, do not have a great impact on the differentiation of samples. Anyway, in the first plot all the organic samples, and most raw beans obtained powders, no matter of the technological processes given, may be grouped. The second score plot illustrates again the marked difference between non-alkalized and alkalized samples, spread on the horizontal direction (PC1). Other traits (organicity, and rawness) are not potent enough to distinguish samples.

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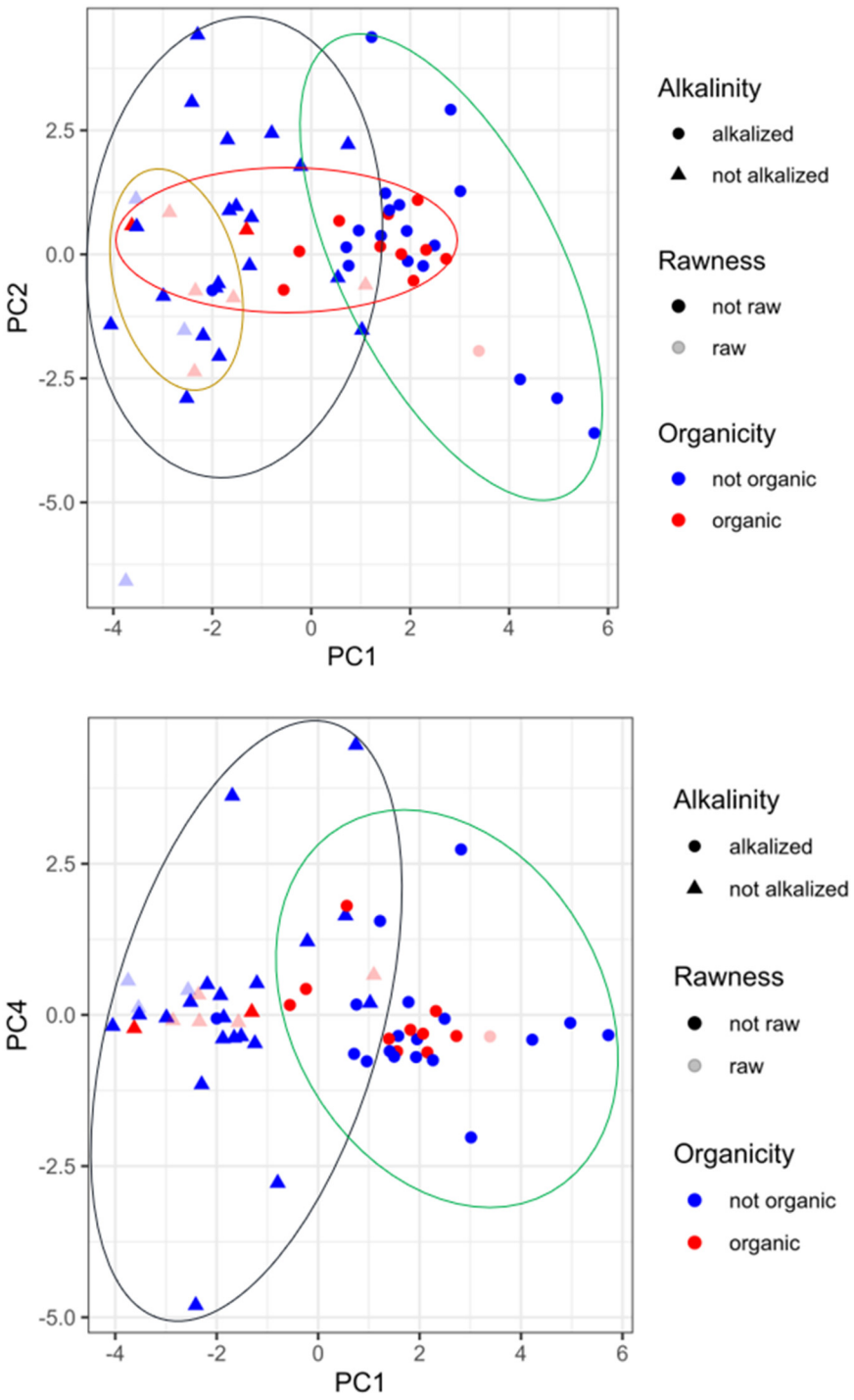

Figure 3. Score plot obtained by Principal Component analysis of pH, colorimetric parameters, biogenic amines, and polyphenols content in cocoa powder samples at different technological treatments. Legend: circle, alkalized cocoa powder; triangle, not alkalized samples**;** blue circle, not organic cocoa powder; red circle, organic samples; light blue symbol, cocoa powder from roasted beans; light red symbol, cocoa samples from raw beans.

5. Conclusions

This survey offers a vast insight into the qualitative state of marketed cocoa powders. Several compounds were followed, and multiple differences about technological processes and agronomical practices for cocoa production were considered. For the samples here described, the technological

process of alkalinization (or Dutch process), caused the main changes highly separating the treated form the untreated powders.

As known, cocoa is alkalized to improve cocoa powders solubility while reducing bitterness, acidity, and giving the tipycal color and aroma of commercial powders. In fact, for long time these characteristics were driving consumers choices for this product. Alkalinization on one hand had a tremendous impact on the phenolic fraction, reducing its presence, on the other hand, did not affect the presence and/or evolution of some compounds as biogenic amines and salsolinol.

Even if all samples may be considered safe (in respect to the content of the latter compounds) we registered positive samples to histamine known for its vasoactive effect and toxicity. The concern relies on the fact that available cocoa powders are often a blend of different producers possibly mixing high biogenic amines and salsolinol positive powders. This may pose a safety issue in consideration that the toxicity of a single biogenic amine may be enhanced by the activity of others especially on those subjects with specific sensitivity or who are overloading their detoxyfing systems. For what concerns the phenolic fraction, this paper offers the direct comparison of different methods from the most common as the Folin-Ciocalteau assay, a gold nanoparticles based assays, and an electrochemical nanostructured sensor.

In extreme conclusion, it can be affirmed that this work may be a starting point for deepening the topic about quality evaluation of cocoa powders and related product, also presenting innovative methods and uncovering low indagated active compounds as salsolinol, on which more research is needed.

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