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Biogenic Amines as Quality Marker in Organic and Fair-Trade Cocoa-Based Products

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Abstract: In this study, the quantitative determination of eight biogenic amines (cadaverine, serotonin, histamine, spermidine, spermine, tyramine, putrescine and β-phenylethylamine) by an liquid chromatography method with evaporative light scattering detection was performed. The analysis of several samples of conventional, organic and fair trade cocoa-derivatives showed that organic and fair trade samples always contain much lower amine concentrations in comparison with their conventional counterparts, supporting the idea that biogenic amines can be regarded as cocoa quality markers. Irrespective of the kind of sample, results also showed that the most abundant amines were histamine, tyramine, spermidine, putrescine and spermine while β-phenylethylamine, cadaverine and serotonine have been found more rarely, all the amines never reaching dangerous amounts for consumer health. With the aim to confirm the experimental results, clustering analysis was performed on samples and instrumental results using principal component analysis.

Keywords: biogenic amines; cocoa-based products; organic products; fair trade; food safety; food quality

1. Introduction

Cocoa (*Theobroma cacao*) plantations are one of the most important forms of land use and are of enormous economic importance to developing countries in the humid tropics. Global production of cocoa is highly concentrated in West Africa (Ghana, Cote d'Ivoire, Cameroon and Nigeria) where more than 60% of total cocoa beans are produced, followed by South America (Caribbean, Brazil, Ecuador and Dominican Republic) and Asia (Indonesia, Malaysia and Papua New Guinea) [1].

The cocoa derivatives supply chain is very long, complex and includes many different actors. It begins with the cocoa farmers, who grow, harvest, extract, ferment, dry and pack the cocoa beans. Then, the cocoa beans from several farmers are collected and often mixed by local buyers, traders, local buying stations and exporters until they reach the chocolate manufacturing plant, mostly located in Europe and North America (Figure 1). It follows that the cocoa manufacturers often receive very heterogeneous batches of cocoa beans. It has been reported that several characteristics of cocoa derivatives strongly depend on the processes done at the very beginning of the supply chain and differences in the farming practices regarding growing, fermenting and drying the cocoa beans seem to be responsible for many of the qualitative characteristics of the cocoa beans [2,3]. When analyzing samples of cocoa beans from different countries it was found that several attributes were very different [4]. Differences were not only country-dependent, but also farmer-dependent, as significant differences can also be found in samples of cocoa beans from the same country [5]. These findings seem to confirm the theory that higher heterogeneity is present in samples from countries where cocoa farming is not the most important economical source, meaning that very different practices are used and an urgent need of standardization and traceability is present.

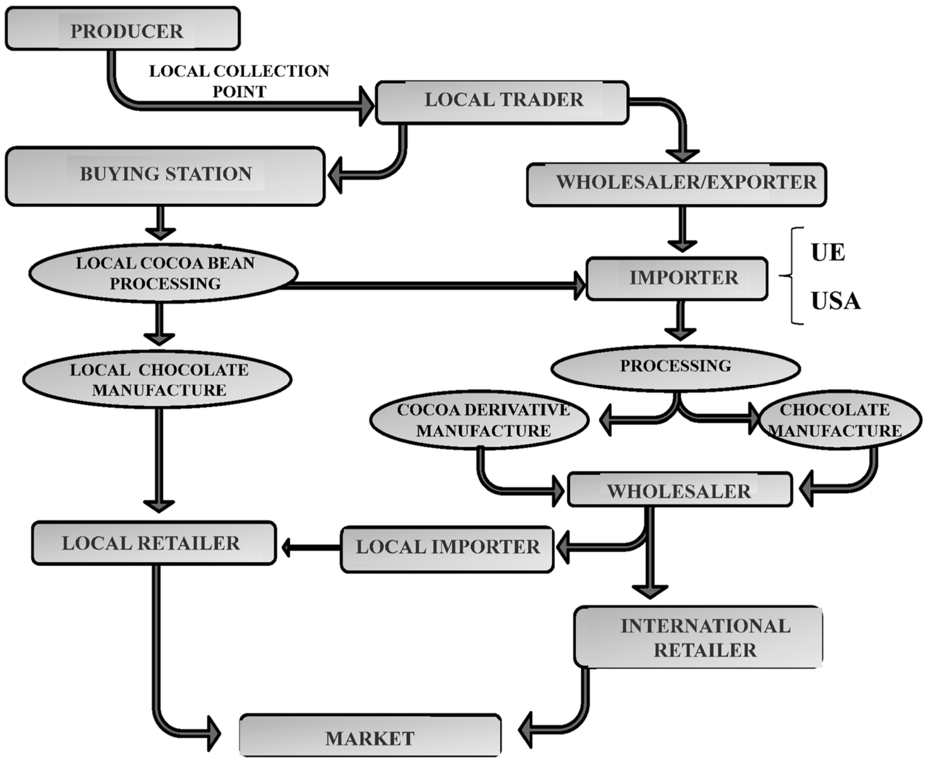

Figure 1. Supply chain of cocoa-based products from conventional agricultural.

In this context, over the last decade, in the cocoa sector, besides economical aspects, the importance of social and environmental issues increased considerably. As a consequence, certification has been placed at the center of an international debate inside the cocoa community, as it is one of the available tools in the market to ensure the application of principles for sustainable production of commodities [6]. There are different sustainability initiatives to certify cocoa, mostly related with the organic production. Cocoa, in fact, is a very suitable commodity for organic trade. It is consumed in large quantities, has structured trade channels, and is processed into a luxury item that has a high perceived value and few substitutes. The organic market only represents a very small share of the total cocoa market [1] as it is estimated at less than 1% and mainly concentrated in Latin American countries. However, the demand for organic cocoa products is growing at a very strong pace, as consumers are increasingly concerned about the safety of their food supply along with other environmental issues. Moreover, under the quality point of view, organic cocoa-based products are generally made with cocoa for fine flavor/single-source cocoa requiring the necessity of a flavor profile that is not only unique but can also stand alone in chocolate with 70% cocoa content. In this sense, there is general consensus among chocolate industry experts that the market for organic cocoa will be increasingly restricted to producers of high quality cocoa [7].

Other certification schemes in the cocoa sector are Rain Forest Alliance, Fairtrade and UTZ Certified which are mainly social standards but also require "good practices in cocoa production". Certification comprises a set of principles addressing social and economic concerns of farmers, farmer groups and communities including environmental requirements. Within their scope, the different certification schemes vary in their main focus or strategy for achieving a more sustainable cocoa production with some of them focusing on the creation of sustainable trade relations (e.g., Fairtrade) and others with a greater focus on increasing farmer productivity as a way to strengthen farmers (e.g., UTZ Certified). It can be said that overall they seek improvements in farmers' livelihoods, focus on developing good agricultural practices and on capacity building. In particular, production, processing and marketing activities can be made more efficient and higher-value, by reducing raw material costs (improving productivity or product quality), adapting the product to meet particular consumer preferences or increases the reliability of supply [8].

Generally speaking, cocoa derivatives quality assessment is a very complex issue to address, as it is influenced by a variety of factors to which the cocoa beans are subjected from the opening of the fruit until the end of industrial processing. It follows that the identification and determination of quality markers could be helpful in relation with the starting quality of the cocoa beans. In particular, quality traits (i.e., flavor) of cocoa-based products are strictly related with the chemical composition of the roasted seeds, which, in turn, is affected by the chemical composition of the raw materials and by the postharvest processing conditions [9]. Among cocoa compounds, free amino acids, oligopeptides, and reducing sugars formed during beans fermentation are generally considered important quality markers. These precursors are further converted in the typical cocoa flavor molecules via Maillard reactions and Strecker degradation during the roasting and conching processes [10]. However, it should be noted that the reactions to which these classes of compounds are subjected, are mostly omnidirectional thus leading not only to degradation but also to the formation of new substances. This is the case of the formation of biogenic amines (BAs), which are low molecular weight organic bases with high biological activity and causing undesirable physiological and toxicological effects if ingested at too high concentrations [11]. Although they are mainly formed in foods during the microbial decarboxylation of free amino acids, some studies clearly indicated that they can be also take origin during thermal processes of foods as a result of the oxidative decarboxylation of the corresponding amino acid precursor [12–15]. In particular, together with cocoa cultivar, parameters of roasting showed a significant effect on the concentrations of BAs in cocoa beans, increasing with temperature and humidity [14]. Strecker degradation seemed to be responsible for the formation of biogenic amines in the way of thermal decarboxylation of amino acids in the presence of α-dicarbonyl compounds [12,13] formed during the Maillard reaction or lipid peroxidation products [16,17].

As BAs have been widely exploited as important indicators of safety and quality in a variety of foods [11], in previous studies, we reported the quantitative determination of BAs in cocoa derivatives [18], also applying innovative analytical approaches [19]. Samples were mostly conventionally obtained, but some organic-fair trade products were also considered. As we pointed out remarkable differences between these classes of samples in terms of their amine profiles and distributions, the goal of the present work is the determination by liquid chromatography coupled with an evaporative light scattering detector (LC-ELSD) of BAs in a larger number of cocoa products to confirm our preliminary results. Furthermore, a statistical elaboration by Principal Component analysis has been performed as clustering tool to recognize possible data patterns and relationship between biogenic amines and cocoa products.

2. Materials and Methods

2.1. Samples

Data on samples of conventional, organic and fair trade cocoa-based products considered in this study derived partly from previous researches [18,19] and partly from new analyses. In particular, 16 new samples (9 conventional and 6 organic) have been selected, considering trade mark, origin and cocoa content in such a manner to accomplish a proper comparison with previously obtained data. All fair-trade samples considered in this study have been obtained using organic cocoa and ingredients.

2.2. Chemicals

BAs spermine (SPM, tetrahydrochloride), spermidine (SPD, trihydrochloride), putrescine (PUT, dihydrochloride), histamine (HIS, dihydrochloride), tyramine (TYR, hydrochloride), cadaverine (CAD, dihydrochloride), β-phenylethylamine (PHE, hydrochloride), serotonine (SER, hydrochloride), dansyl-chloride were purchased by Sigma-Aldrich (Milford, MA, USA), as well as perchloric acid (70%), ammonia (30%), trifluoroacetic acid (TFA) and LC solvents (acetonitrile and methanol LC grade). Ultrapure water was obtained from Milli-Q System (Millipore Corp., Milford, MA, USA). Filters (0.45 μm and 0.20 μm) were purchased by Sigma-Aldrich (Milford, MA, USA). SPE C_{18} cartridges (0.5 g) were obtained from Supelco Inc. (Bellefonte, PA, USA).

2.3. Amine Standard Solutions and Calibration

The LC-ELSD chromatographic parameters, as well as the analytical performances of the method, have been already described in detail [19]. Briefly, the mobile phase were composed by (A) acetonitrile/water 20/80 (*v*/*v*) mixture containing trifluoroacetic acid (0.05%, *v*/*v*) and (B) acetonitrile/water 20/80 containing trifluoroacetic acid (0.35%, *v*/*v*). The chromatographic separation was carried out using a linear binary gradient according to the following scheme: elution program began with A:B 100:0 (*v*/*v*) reaching after 10 min A:B 70:30 (*v*/*v*) and further 8 min to reach A:B 40:60 (*v*/*v*). Then 2 min of isocratic elution were carried out and further 5 min to reach A:B 0:100 (*v*/*v*). Finally, 10 min were necessary to restore again the starting conditions (A:B 100:0, *v*/*v*). Flow was kept constant at 0.7 $mL{\cdot}min^{-1}$, for a total analysis time of 35 min and a time interval of 10 min between two successive injections was applied. The flow of nebulizer gas (N_2) was maintained at 3.3 bar and the drift tube temperature was set at 40 °C.

The identification of the amines was performed by comparing the retention times of peaks in the samples with those of standard solutions and by addition of the suspected amine to the samples. A calibration plot, reporting the peak area against standard concentration, was constructed for twelve concentration levels and six independent replicates for each concentration level were performed. Quantitative determination was accomplished by direct interpolation in the standard curves for each amine. In order to reassure repeatability, standards were run throughout the day and also between days.

2.4. BA Extraction and Purification

Once packaging was open, to avoid sample alteration the extraction of BAs from each kind of sample was accomplished during the same day, following the procedure of Pastore et al. (2005) [14] with some modifications. All the extracts were then stored at 4 °C. A total of 25 mL of perchloric acid 0.6 M were added to about 5 g of sample (or sample spiked with a BAs standard mix), in a 50 mL test tube. The mixture was homogenized (vortex at 40 Hz for 5 min), centrifuged (10,000 *g* for 15 min), filtered (syringe filter 0.45 μm), collected in a plastic vial and purified by SPE on a C_{18} sorbent (conditioning: 2.0 mL of H_2O and 2.0 mL (two times) of CH_3OH; loading: 5.0 mL of the basified sample; washing: 2.0 mL of NH_4OH at pH 11.0; eluting: 2.0 mL (two times) of CH_3OH). The eluting solution was dried with nitrogen gas and the residue was re-dissolved in a plastic test tube with 1.3 mL of perchloric acid 0.6 M.

Recovery experiments were performed by spiking—before the extraction procedure—samples 1, 22 and 48 with different aliquots of a BAs standard mix to evaluate the method performances at three different levels of concentration. Each experiment has been replicated six times and recovery values were always higher than 90% with standard deviation lower than 3% [18,19].

2.5. Equipment

LC analysis were performed with a Jasco PU-2080 instrument equipped with a Rheodyne 7725 injector with a 20 mL sample loop and a gradient pump (PU-2089 plus, Jasco Inc., Easton, MD, USA). The system was interfaced with an ELS detector (1200 Series, Agilent Tech., Lexington, MA, USA). Data were collected and analyzed with an integrator Jasco-Borwin1. APrimsep 200 column (SIELC Technologies, Prospect Heights, IL, USA) with Primsep 200 Guard Kit (10 mm × 4.6 mm I.D., 5 mm) was applied. A micro-processor pH meter (Hanna Instruments, Eboli (SA), Italy), equipped with a combined glass–calomel electrode, was employed for pH measurements. A centrifuge (Thermo Scientific, Milan, Italy) was used for the pre-treatment of the samples.

2.6. Data Handling

Principal component analysis was applied to describe data patterns and to highlight differences of BE distribution in different cocoa derivatives. PCA aims to extract the important information from the data table and to express this information by projection of the samples and variables on a set of new orthogonal variables called principal components, PCs. The Unscrambler X software version 10.3 from CAMO (Computer Aided Modelling, Trondheim, Norway) was used for the multivariate data elaboration.

3. Results and Discussion

In Table 1, the concentrations of BAs found in 48 cocoa-based products are reported. As can be seen, HIS, TYR, SPD, PUT and SPE were generally found, while PHE, CAD and SER have been found only rarely. Quantities of total BAs ranged from 5.7 $\mu g \cdot g^{-1}$ in sample 4 to 79.0 $\mu g \cdot g^{-1}$ in sample 47, with wide variations depending on the sample. The recorded data are of the same order of magnitude of those reported in previous studies concerning cocoa beans and chocolate from conventional agricultural which showed a total BAs concentration from a few $\mu g \cdot g^{-1}$ and never exceeding 35 $\mu g \cdot g^{-1}$ [20,21]. The same order of magnitude of total BAs concentrations was recorded by Baranowska and Plonka (2015) proposing an analytical method to simultaneously determine BAs and other analytes in cocoa products by LC-DAD and LC-FL [22]. Other studies, for the same kind of samples, in contrast with our results never contained levels of BAs higher than 0.5 $\mu g \cdot g^{-1}$ [13]. This discrepancy can be explained considering that BAs levels and distributions are influenced by many parameters regarding either the hygienic conditions of the raw materials or the production process, as well as the preservation techniques and the packaging of the product making a direct overlapping of the data arising from different studies generally difficult to accomplish.

BAs present at higher concentrations in all samples were generally HIS (1.9–47.2 $\mu g \cdot g^{-1}$), TYR (1.3–31.7 $\mu g \cdot g^{-1}$) and PUT (not detectable (nd)–32.7 $\mu g \cdot g^{-1}$) followed by SPD (nd–10.2 $\mu g \cdot g^{-1}$) and SPM (nd–9.3 $\mu g \cdot g^{-1}$), which were present in most of the samples at lower concentrations. SER was present only in samples 24, 25, 39 and 40 due to the presence of coffee as ingredient, and never exceeding 5.2 $\mu g \cdot g^{-1}$ [23]. Finally, CAD and PHE were detected only in few conventional samples (1–3, 42, and 43 and 1–3, 8 and 13, respectively) at very low concentrations (nd–6.1 $\mu g \cdot g^{-1}$ and nd–3.2 $\mu g \cdot g^{-1}$, respectively). These findings confirm previous results [18], indicating that these amines could be regarded as possible discriminating parameters among conventional and organic/fair trade cocoa products.

As can be seen in Table 1, organic and fair-trade samples usually contain much lower amounts of BAs in comparison with their conventional counterparts, for all product categories (i.e., cocoa powder, chocolate, confectionaries or powders to prepare cocoa-based desserts). As already underlined, the root causes of cocoa quality anomalies can be firstly related with poor farm management, infestation and other diseases. To this regard, primary forest is considered the ideal environment for cocoa production; however, in particular for conventionally produced cocoa, it is often cut through the practice of slash and burn. Since forest land is no longer available, new cocoa plantations must be established on agricultural land. In some cases, cocoa is grown in monocultures under largely unshaded conditions although it is well known that cacao plants actually do much better in shade. Such a production system enhances loss of soil fertility and biodiversity finally leading to poor growth and premature aging of the cocoa trees. Many processing parameters can also have dramatic effects on cocoa quality such as: poor handling, bad fermentation, inadequate drying and hence high moisture content, capable of making the product vulnerable to mold and bacterial growth, poor and long-time storage, leading to fat degradation and pest infestation. All these aspects strongly support the increase of BAs concentrations.

Table 1. Main characteristics of the samples and biogenic amines content in cocoa-based samples. Data represent mean ± RSD (n = 3), $p < 0.05$.

Sample #	Sample Type	% Cocoa	Cocoa Origin	Cultivation	Biogenic Amines (μg·g^{-1})								
					PHE	*PUT*	*CAD*	*HIS*	*TYR*	*SPD*	*SER*	*SPM*	*Total*
1	Cocoa powder	80	NR	Conventional	2.0 ± 0.1	9.1 ± 0.3	5.3 ± 0.3	23.8 ± 0.6	13.1 ± 0.4	9.7 ± 0.3	-	9.3 ± 0.3	**72.3 ± 0.5**
2	Cocoa powder	80	NR	Conventional	1.8 ± 0.1	7.1 ± 0.4	3.3 ± 0.2	20.1 ± 0.4	6.4 ± 0.3	10.2 ± 0.2	-	-	**48.9 ± 0.6**
3	Cocoa powder	80	NR	Conventional	3.2 ± 0.2	10.1 ± 0.2	6.1 ± 0.3	15.8 ± 0.4	9.7 ± 0.2	5.9 ± 0.3	-	1.3 ± 0.1	**50.8 ± 0.5**
4	Cocoa powder	80	Dominican Republic	Organic-Fair Trade	-	1.0 ± 0.1	-	2.4 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	-	-	**5.7 ± 0.2**
5	Cocoa powder	80	Cameroon	Organic-Fair trade	-	-	-	2.9 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	-	0.6 ± 0.1	**7.0 ± 0.2**
6	Cocoa powder	80	Panama	Organic-Fair trade	-	0.8 ± 0.1	-	2.2 ± 0.1	1.6 ± 0.1-	1.4 ± 0.1	-	0.9 ± 0.1	**6.9 ± 0.2**
7	Dark Chocolate	60	NR	Conventional	-	4.7 ± 0.2	-	20.0 ± 0.5	8.1 ± 0.2	8.2 ± 0.3	-	5.3 ± 0.2	**46.3 ± 0.4**
8	Dark Chocolate	70	NR	Conventional	1.0 ± 0.1	5.4 ± 0.3	-	35.0 ± 0.4	6.7 ± 0.3	9.8 ± 0.2	-	7.1 ± 0.3	**65.0 ± 0.3**
9	Dark Chocolate	70	NR	Conventional	-	6.1 ± 0.4	-	30.3 ± 0.5	9.4 ± 0.4	8.2 ± 0.3	-	6.8 ± 0.3	**60.8 ± 0.4**
10	Dark Chocolate	70	Ecuador	Organic-Fair Trade	-	4.0 ± 0.2	-	20.0 ± 0.4	12.0 ± 0.3	7.3 ± 0.2	-	4.9 ± 0.1	**48.2 ± 0.3**
11	Dark Chocolate	70	Dominican Republic	Organic-Fair Trade	-	1.2 ± 0.1	-	3.4 ± 0.2	2.1 ± 0.2	1.8 ± 0.2	-	1.9 ± 0.1	**10.4 ± 0.3**
12	Dark Chocolate	70	Ecuador	Organic-Fair trade	-	1.9 ± 0.2	-	5.8 ± 0.3	2.1 ± 0.1	2.1 ± 0.1	-	2.8 ± 0.2	**14.7 ± 0.2**
13	Dark Chocolate orange flavour	49	NR	Conventional	1.1 ± 0.1	2.3 ± 0.1	-	10.5 ± 0.3	7.0 ± 0.2	5.0 ± 0.2	-	4.3 ± 0.2	**30.2 ± 0.2**
14	Dark Chocolate orange flavour	55	Cameroon	Organic	-	0.9 ± 0.1	-	3.8 ± 0.1	2.2 ± 0.3	3.2 ± 0.2	-	5.0 ± 0.1	**15.1 ± 0.2**
15	Dark Chocolate with cocoa beans	73	Dominican Republic	Organic-Fair Trade	-	0.9 ± 0.1	-	1.9 ± 0.1	2.3 ± 0.2	1.0 ± 0.1	-	1.6 ± 0.1	**7.7 ± 0.2**
16	Extra Dark Chocolate	75	NR	Conventional	-	7.9 ± 0.1	-	19.5 ± 0.3	12.4 ± 0.3	9.0 ± 0.2	-	8.8 ± 0.1	**57.6 ± 0.3**
17	Extra Dark Chocolate	75	Panama	Organic-Fair trade	-	1.2 ± 0.1	-	2.9 ± 0.2	1.6 ± 0.1	1.1 ± 0.1	-	1.9 ± 0.1	**8.6 ± 0.1**
18	Milk Chocolate	32	NR	Conventional	-	2.1 ± 0.1	-	37.1 ± 0.5	4.7 ± 0.1	3.5 ± 0.2	-	-	**47.4 ± 0.3**
19	Milk Chocolate	32	NR	Conventional	-	3.1 ± 0.1	-	45.4 ± 0.4	14.1 ± 0.2	6.1 ± 0.3	-	-	**68.7 ± 0.6**
20	Milk Chocolate	33	NR	Conventional	-	3.9 ± 0.2	-	27.9 ± 0.6	19.6 ± 0.3	4.7 ± 0.3	-	-	**56.1 ± 0.4**
21	Milk Chocolate	35	Ghana	Organic	-	1.6 ± 0.1	-	7.3 ± 0.2	2.1 ± 0.1	1.4 ± 0.2	-	-	**12.4 ± 0.3**
22	Milk Chocolate	34	Dominican Republic	Organic-Fair Trade	-	0.9 ± 0.1	-	12.0 ± 0.2	1.8 ± 0.1	1.0 ± 0.1	-	-	**15.7 ± 0.2**
23	Milk Chocolate	39	Ecuador	Organic-Fair trade	-	0.8 ± 0.1	-	10.0 ± 0.2	2.0 ± 0.1	1.3 ± 0.1	-	-	**14.1 ± 0.2**
24	Milk Chocolate with coffee cream	15	NR	Conventional	-	2.3 ± 0.1	-	11.9 ± 0.2	6.9 ± 0.1	1.1 ± 0.1	5.2 ± 0.1	3.1 ± 0.2	**30.5 ± 0.2**
25	Milk Chocolate with coffee cream	15	Bolivia	Organic	-	1.0 ± 0.1	-	7.9 ± 0.1	4.1 ± 0.2	1.2 ± 0.1	3.0 ± 0.2	2.0 ± 0.1	**19.2 ± 0.3**
26	White Chocolate	NR	NR	Conventional	-	-	-	38.1 ± 0.5	10.6 ± 0.2	6.2 ± 0.1	-	3.5 ± 0.1	**58.4 ± 0.4**
27	White Chocolate	39	NR	Conventional	-	-	-	35.8 ± 0.5	17.1 ± 0.3	6.1 ± 0.2	-	-	**59.0 ± 0.6**
28	White Chocolate	39	NR	Conventional	-	-	-	47.2 ± 0.5	20.6 ± 0.4	4.7 ± 0.3	-	2.8 ± 0.2	**75.3 ± 0.4**
29	White Chocolate	39	Cameroon	Organic	-	-	-	9.5 ± 0.3	4.1 ± 0.2	2.8 ± 0.2	-	1.1 ± 0.1	**16.4 ± 0.3**
30	White Chocolate	39	Dominican Republic	Organic-Fair trade	-	-		12.0 ± 0.3	3.3 ± 0.1	2.4 ± 0.1	-	-	**17.7 ± 0.2**
31	White Chocolate	39	Ecuador	Organic-Fair trade	-	-		12.4 ± 0.3	3.0 ± 0.1	2.0 ± 0.1	-	1.2 ± 0.1	**18.6 ± 0.2**
32	Milk chocolate egg	15	NR	Conventional	-	13.1 ± 0.2	-	23.1 ± 0.3	28.9 ± 0.4	-	-	-	**65.1 ± 0.3**
33	Cocoa and hazelnut spread	NR	NR	Conventional	-	15.2 ± 0.2	-	21.9 ± 0.4	31.2 ± 0.5	2.9 ± 0.1	-	6.1 ± 0.2	**77.3 ± 0.4**
34	Dark cocoa spread	NR	NR	Conventional	-	10.9 ± 0.2	-	16.3 ± 0.3	24.6 ± 0.3	4.0 ± 0.1	-	5.6 ± 0.2	**61.4 ± 0.3**
35	Dark cocoa spread	25	Ghana	Organic	-	4.6 ± 0.2	-	8.1 ± 0.2	11.2 ± 0.2	2.2 ± 0.1	-	3.8 ± 0.2	**29.9 ± 0.2**
36	Soluble chocolate powder	NR	NR	Conventional	-	9.6 ± 0.2	-	8.7 ± 0.2	7.1 ± 0.2	4.4 ± 0.2	-	-	**29.8 ± 0.2**
37	Soluble chocolate powder	20	Cameroon	Organic	-	6.9 ± 0.2	-	3.5 ± 0.1	5.4 ± 0.2	2.1 ± 0.1	-	-	**17.9 ± 0.3**
38	Gianduja Chocolate	32	NR	Conventional	-	18.1 ± 0.3	-	28.4 ± 0.2	16.2 ± 0.2	6.2 ± 0.2	-	4.1 ± 0.1	**73.0 ± 0.4**
39	Coffee confectionary	32	Bolivia	Organic-Fair Trade	-	0.9 ± 0.1	-	4.9 ± 0.1	4.3 ± 0.2	1.3 ± 0.1	2.8 ± 0.2	1.1 ± 0.1	**15.3 ± 0.2**
40	Coffee confectionary	30	Dominican Republic	Organic-Fair trade		0.9 ± 0.1		5.0 ± 0.1	4.3 ± 0.2	1.3 ± 0.1	2.9 ± 0.1	1.0 ± 0.1	**15.4 ± 0.2**
41	Giandujotto confectionary	21	NR	Conventional	-	32.7 ± 0.4	-	10.5 ± 0.2	11.3 ± 0.2	6.1 ± 0.2	-	-	**60.6 ± 0.3**
42	Cocoa glaze	15	NR	Conventional	-	7.6 ± 0.2	4.3 ± 0.1	19.7 ± 0.2	21.1 ± 0.4	8.6 ± 0.2	-	6.7 ± 0.3	**68.0 ± 0.3**
43	Chocolate syrup	20	NR	Conventional	-	11.2 ± 0.2	5.1 ± 0.2	17.3 ± 0.2	29.9 ± 0.5	7.9 ± 0.2	-	6.4 ± 0.2	**77.8 ± 0.4**
44	Chocolate syrup	20	Ecuador	Organic	-	5.6 ± 0.2	-	7.9 ± 0.2	12.1 ± 0.2	5.0 ± 0.1	-	4.1 ± 0.2	**34.7 ± 0.3**
45	Powder to prepare cocoa drink	25	Dominican Republic	Organic-Fair trade	-	0.9 ± 0.1	-	9.8 ± 0.2	7.9 ± 0.2	-	-	1.7 ± 0.1	**20.3 ± 0.2**
46	Powder to prepare cocoa mousse	NR	NR	Conventional	-	13.3 ± 0.2	-	25.1 ± 0.4	19.7 ± 0.2	7.4 ± 0.2	-	6.0 ± 0.2	**71.5 ± 0.3**
47	Powder to prepare cocoa pudding	NR	NR	Conventional	-	10.1 ± 0.2	-	23.4 ± 0.4	31.7 ± 0.5	4.9 ± 0.2	-	8.9 ± 0.1	**79.0 ± 0.4**
48	Powder to prepare cocoa pudding	19	Dominican Republic	Organic-Fair trade	-	3.7 ± 0.2	-	15.0 ± 0.3	12.9 ± 0.3	3.6 ± 0.3	-	2.9 ± 0.2	**38.1 ± 0.3**

PHE, β-phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine; SER, serotonine; SPM, spermine. NR = Not Reported.

On the contrary, organic farming requires rigorous application of prescribed standards with strict credible certification and inspection regimes [24]. Organic production of cocoa poses requirements such as: implementation of organic approaches to soil fertility, pest and disease management not allowing the use of synthetic pesticides and fertilizers as well as other unnatural postharvest treatments for cocoa beans, packaging materials and stores; proper separation of organic cocoa and conventional cocoa during production and postharvest handling; implementing a good traceability system, based on clear labeling and record keeping in order to minimize contamination (Figure 2). Moreover, organic cocoa trees are typically shade grown, meaning that they grow under the canopy of other rainforest plants rather than in deforested swaths, producing cacao plants much more resistant to disease. Thus, it could be hypothesized that the organic farming rules coupled with the strict quality control of the single-source cocoa beans can limit the accumulation of BAs in organic and fair-trade cocoa derivatives.

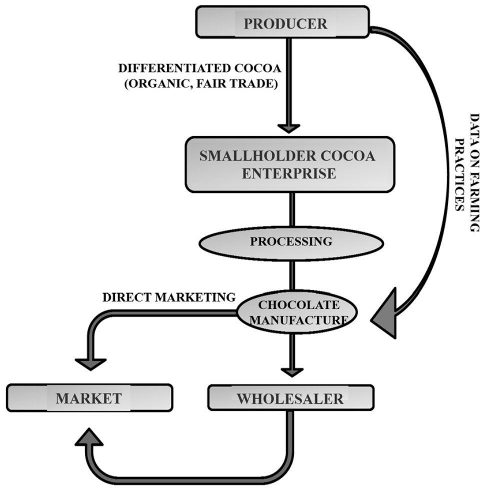

Figure 2. Supply chain of cocoa-based products from organic and fair trade agricultural.

The data reported in Table 1 seem to show a general trend indicating that a more complex production process, with the employment of a larger number of ingredients, generates a higher amine content, especially for conventional products. This can be explained considering that any additional step in the production process can add new sources of contamination. At the same time, the applied temperature during the each phase can promote the thermogenic BAs formation as well. It follows that, inside each class of samples (i.e., conventional and organic/fair trade), the BAs content increased passing from cocoa to chocolate and confectionary, reaching the highest values for powders used to prepare cocoa drink with the exception of sample 36, in which the low amount of BAs found, is probably in relation with the industrial process involved in the soluble cocoa technology where the use of water/vapor produces the loss of water-soluble BAs.

A further increase of BAs can be also related with other ingredients containing amines such as hazelnut (up to 25% in samples 33–35), milk powder (samples 45–48), corn syrup (samples 33–35, 43 and 44), or coffee (samples 24, 25, 39 and 40), although this effect seems to be less important for fair trade/organic products where not only cocoa beans but also most of the other ingredients and additives are certified as organic. It has been found in hazelnut up to about 35 $mg \cdot kg^{-1}$ of BAs, mostly SPD and SPM [20]. In the case of milk powder, the severe heat treatment applied during processing can strongly support the thermogenic formation of BAs. Moreover, in the case of product defects, tryptophan degradation and lipids peroxidation products can be formed as well also increasing the concentrations of BAs [13,16,17,25]. Corn syrup has been reported to contribute to BAs during malting and brewing [26]. In coffee samples PUT is generally the most abundant amine as well as other natural polyamines SPD and SPM present in coffee tissues; at lower concentrations SER have been also detected as well as agmatine [23,27].

Analytical data reported in table 1 have been arranged in a data matrix form and subdued to multivariate elaboration. Matrix rows represented the objects, in our case cocoa samples, and columns were the description of different composition in terms in BA content. A clustering study was performed by running the PCA algorithm. Before regression, the data were mean centered by dividing each row of data matrix by its average. PCA performance and selection of optimum number of principal component was evaluated by internal full cross validation procedure. PCA modeling gave 89% of explained variance by considering the first four PCs. The samples grouping was evident in scores plot (Figure 3a) and distinguish the two clusters, containing, respectively, sample from conventional or organic cultivations, was allowed. The discrimination was more evident if a single cocoa type product was observed; in all cocoa production types, the distance between conventional and organic cultivations in PCs space is clear. Loadings plot (Figure 3b) shows how the variables permit the distinction of the different cultivation process. Convectional cultivation samples had higher concentration level for all BA. In particular, cocoa powder samples from conventional cultivation (CP_C) had highest value for SPD, CAD and PHE amines [28,29].

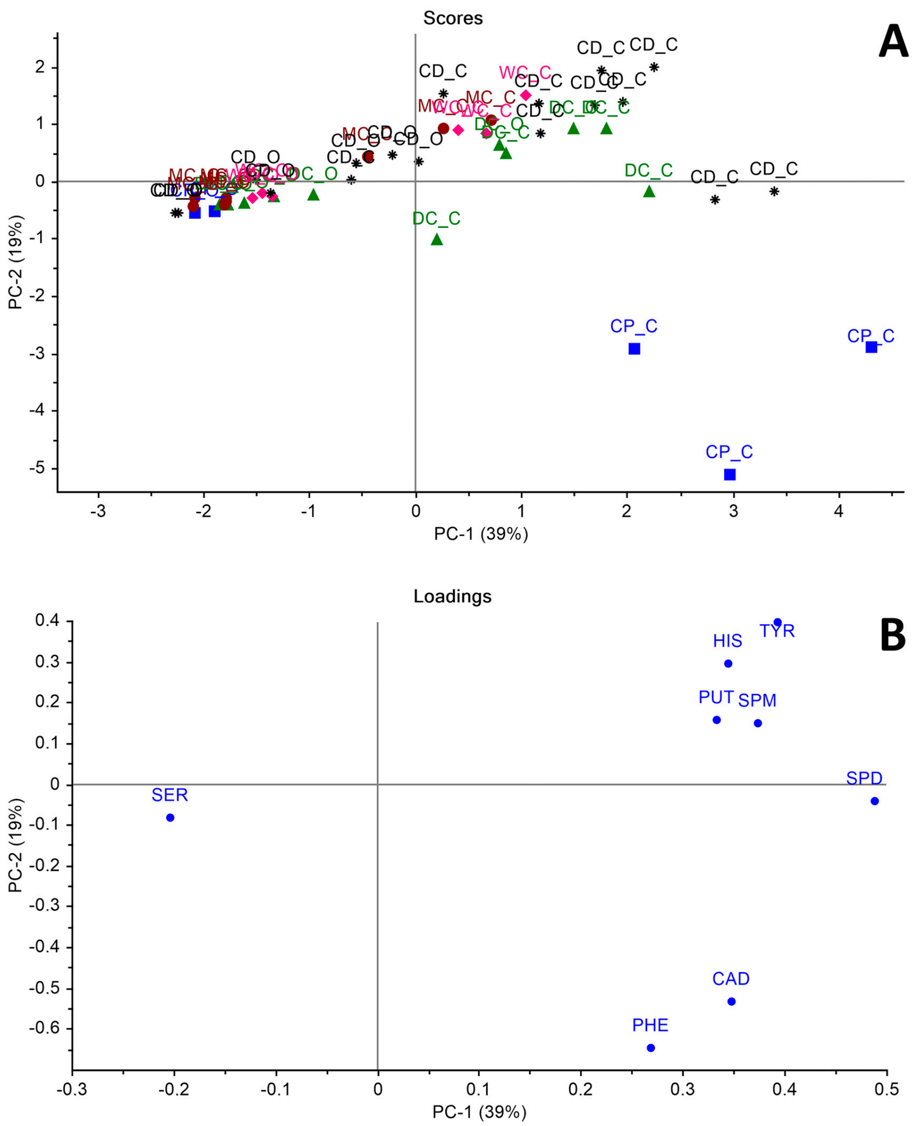

Figure 3. (**A**) Scores; and (**B**) Loading. Plots from principal components data analysis: cocoa powder samples (CP_C for conventional and CP_O for organics cultivations); dark chocolate samples (DC_C and DC_O); milk chocolate samples (MC_C and MC_O); white chocolate samples (WC_C and WC_O); and cocoa derivatives samples (CD_C and CD_O).

It has been reported that an intake higher than 40 mg biogenic amines per meal can be considered potentially toxic [15]. However, not all amines are equally toxic; consequently, HIS, TYR and PHE are of concern. For fermented foods 50 ppm–100 ppm, 100 ppm–800 ppm and 30 ppm for HIS, TYR and PHE, respectively, or a total of 100 ppm–200 ppm could be regarded as acceptable. According to Parente et al. (2001), HIS intake range within 8 mg–40 mg, 40 mg–100 mg and higher than 100 mg may cause slight, intermediate and intensive poisoning, respectively [30]. However, the European Commission Regulation (EC) No 1441/2007 poses only for histamine in fishery products, limits between a minimum level of 200 $mg \cdot kg^{-1}$ and a maximum level of 400 $mg \cdot kg^{-1}$. As can be noted from reported data, none of the analyzed samples represents a possible risk for consumer health, considering the proposed limits. Nevertheless, it should be kept in mind that chocolate is the most commonly craved food, thus leading to possible abnormal dietary intake; in this sense, organic cocoa-based products should be preferred as they contain much lower BAs concentrations in comparison with their conventional counterparts.

4. Conclusions

In recent years, cacao markets have become increasingly diversified in response to structural changes in consumer demand, generally related to consumers' concerns over health, environmental conservation and the social-economic welfare of small producers. In this sense, throughout the 1990s many initiatives were taken in the context of promoting more sustainable production processes (mainly organic) and socially-responsible trade practices (Fair Trade) within the cocoa sector. In addition to the conventional, mass market segment, market segments have emerged related to fine flavor and single source, organic, and fair trade, representing the fastest growing segments of the cacao industry. However, despite the important contribution of the cocoa sector to the national economies, the problems are still numerous, in particular for conventional farming. This sector, in fact, is characterized by smallholder production and low or inadequate investments in inputs as well as the lack of appropriate farmer training and good agricultural practices negatively influence crop quality. It follows that deliveries are frequently characterized by a great heterogeneity with regard to their quality attributes and a reliable quality assessment is of great importance for both, producers and purchasers. Although the quality assessment of cocoa and chocolate is generally related with their sensory characteristics, it is possible to consider for the same purpose some chemical compounds produced during processing and/or microbial deterioration of raw materials and derivatives. In this regard, we investigated the possible exploitation of BAs as quality index of organic and fair trade cocoa products. The determination of eight BAs in 48 samples by LC-ELSD showed that, irrespective of the kind of sample (i.e., cocoa powder, chocolate, confectionaries and powders), the BAs concentrations found in organic and fair trade products were always much lower than those found in the corresponding conventional products. These results were confirmed in multivariate data analysis by PCA thus demonstrating that BAs can represent a discriminating class of compounds between the different classes of products. Moreover, the presence of PHE and CAD only in conventional derivatives surely deserves a deeper investigation in order to further highlight significant differences between conventional and organic cocoa and cocoa derivatives.

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