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Control of undeclared flavoring of cocoa powders by the determination of vanillin and ethyl vanillin by HPLC

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ABSTRACT

A simple protocol for the extraction of vanillin and ethyl vanillin in cocoa powders, followed by analyte quantification using HPLC, has been proposed in this work. After optimizing both, extraction and separation conditions, both analytes were determined in less than 4 min, with relative standard deviation values lower than 2.05% in all cases. Detection limits within 0.04 and 0.06 mg L^{-1} were achieved. The applicability of the proposed method was evaluated by performing a recovery study, in which a cocoa sample was fortified with both flavors at different concentration levels. In all cases, the recovery values obtained were comprised between 97.5 and 103.1%. The developed method was also successfully applied to analyze 66 commercially available cocoa powders from different brands and markets finding that the 11% of powders contained vanillin and/or ethyl vanillin in concentration ranges comprised between 5.6 and 90.8, and 5.1 and 12.2 mg/100g for vanillin and ethyl vanillin, respectively. Finally, during the sensory evaluation of cocoa powders, it was revealed that samples containing vanillin show a very round aromatic profile that is very appreciated by the consumers. Thus, the developed method could be useful to control undeclared flavoring and to guarantee food quality and for protecting good manufacturers.

Keywords

Artificial flavouring
Cocoa quality control
Flavor enhancer addition

1. Introduction

Due to both, coloring and flavoring properties, cocoa powder is a key ingredient in the formulation of a myriad of food products such as chocolate and chocolate substitutes, confectionery, bakery and dairy products. Given the general tendency to restrict the use of artificial colorings in all these products, cocoa powders with high coloring capacity are gaining positions in the market. To obtain cocoa powders with high coloring capacity, cocoa nibs or cocoa cake are submitted to Dutching or alkalization. In this process the raw material is treated with an alkali, most commonly sodium carbonate dissolved in water, to transform the properties of the cocoa (Miller et al., 2008).

The purpose of the alkalization is manifold. On the one hand it reduces the acidity and astringency of the product, improving and intensifying the aromatic characteristics of cocoa powder. Reductions in astringency are effected by further polymerizations of flavonoids during alkali treatments (ADM Cocoa Manual, 2006). On the other hand, alkalization promotes a series of reactions between pigments and the alkali in the presence of heat, water and oxygen that promote a color development among light brown to red, and in some cases very dark colors (i.e. dark brown or even black). Finally, alkalization increases the dispersability of cocoa powder which improves the applicability of cocoa powders in different industries (e.g. dairy products) (Afoakwa, Paterson, Fowler, & Ryan, 2008). After alkalization, natural cocoa powder (pH of approximately 5.5), achieves values of pH of about 7 or 8 (Kostic, 1997).

Despite the benefits described, alkalization can lead to the appearance of defects in production. Cocoa nibs or cakes processed in non-optimal conditions can develop smoke-like profiles as well as flatter chocolate notes that decrease the quality of the products. Addition of flavor enhancers (e.g. vanilla extracts) to cocoa during the milling process could mask these undesired effects. Vanilla extract, a mixture of several hundred compounds in addition to vanillin, is one of the most widely used flavoring ingredients in foods and beverages. Although authentic vanilla extract is thought to provide the most desirable flavor characteristics, the supply of vanilla beans is limited and prices are high (Shen et al., 2014). Because of the high cost of authentic vanilla extracts, artificial vanilla flavorings are often used. These flavorings usually contain

synthetically produced vanillin and (or) ethyl vanillin. Ethyl vanillin is more expensive, but has much more flavoring strength than vanillin (Kumar, Sharma, & Mishra, 2012).

Despite some flavor enhancer (i.e. vanillin) addition is accepted by Codex Alimentarius (Codex Alimentarius, 2013), flavored cocoa cakes are not very well accepted in certain markets. As a consequence, non-declared flavor addition is a tempting practice to improve alkalized cocoa flavor. As a consequence, provide effective methods to control undeclared cocoa flavoring with vanillin and ethyl vanillin is necessary to guarantee food quality and for protecting good manufacturers.

Different methods have been usually proposed to determine vanillin and ethyl vanillin in vanilla extracts or food products. These methods include capillary electrophoresis (Ohasi, Omae, Hashida, Sowa, & Imai, 2007; Panossian, Mamikonyan, Torosyan, Gabrielyan, & Mkhitaryan, 2001), gas chromatography (Sostaric, Boyce, & Spickett, 2000; Perez-Silva et al., 2006), thin-layer chromatography (Poole, Daly, & Poole, 1993), liquid chromatography coupled to both UV (Jagerdeo, Passetti, & Dugar, 2000; Lavine, Hendayana, & Tetreault, 1994; Lavine, Corona, & Perera, 2012; Pyell, Pletsch-Viehman, & Ramus, 2002; Waliszewski, Pardio, & Ovando, 2007) and MS detectors (de Jager, Perfetti, & Diachenko, 2007; Rychlik, 2008), ultra-performance liquid chromatography working with monolithic columns (Sharma, Sharma, Sinha, Kumar, & Gupta, 2009), and even adsorptive stripping voltammetry (Yardım, Gülcan, & Şentürk, 2013). However, most of these methods are described to analyse very simple matrices (i.e. vanilla extracts, beverage alcohol products, among others). Only a few have determined these flavors in cocoa or chocolate samples (Richards, & Wailes, 2012; Risner, & Kiser, 2008); however, these methods employed longer extraction protocols than the one proposed in this manuscript.

The aim of this study was to develop a simple extraction protocol of vanillin and ethyl vanillin flavors from cocoa powder samples, followed by their quantification by HPLC. For this purpose, different extraction and separation conditions were optimized, and the optimal conditions were applied to the quantification of both flavors in cocoa powders. Next, a sensory evaluation of the samples will be also performed.

2. Materials and methods

2.1. Reagents and samples

Supragradient HPLC grade methanol (MeOH) (Scharlau, Barcelona, Spain), vanillin 99% and ethyl vanillin ≥ 98.5% (Sigma, Saint Louis, MO, USA), and deionized water (Aquinity deionizer, Membrapure GmbH, Berlin, Germany) were used.

A total of 66 cocoa powder samples were employed in this study. These samples were either purchased in the local market or kindly donated by Olam Food Ingredients Company. Samples were classified in three different categories according to the extractable pH range: natural cocoa powders (pH 5-6), light alkalized (pH 6-7.2), medium alkalized (pH 7.2-7.6) and strong alkalized powders (pH > 7.6) (Miller et al., 2008). According to this classification, from the 66 samples employed, 11 were natural cocoa, 12 light alkalized, 27 medium alkalized and 16 strong alkalized. For extractable pH determination, 10 g of cocoa powder were suspended in 90 mL of boiling distilled water and stirred. Mixture was allowed to cool down to 20-25ºC in a cold water bath (ADM Cocoa Manual, 2006). Then pH was measured in a digital pH-meter micropH 2001 (Crison Instruments, S.A., Barcelona, Spain).

2.2. Vanillin and ethyl vanillin extraction protocol

In order to extract vanillin and ethyl vanillin from the samples, 1 g of cocoa powder was weighted, and suspended in 10 mL of MeOH:H_2O (1:1, v/v). The mixture was kept under continuous shaking during 5 min, and then passed through a 0.45 µm pore-size nylon filter (Albet, Barcelona, Spain). The solution was immediately injected in the chromatograph or stored in a freezer.

2.3. Instrumentation and working conditions

A Hitachi LaChrom Elite liquid chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with an auto-sampler (model L-2200) and UV detector (model L-2400) was used. Separation was carried out with a Kromaphase 100 C18 analytical column (250 × 4.6 mm i.d., 5 µm) (Scharlab, Barcelona, Spain). Mobile phase was prepared by mixing MeOH and water. Elution started with a linear gradient from 50% to 100% MeOH for 2 min, followed by an isocratic elution with 100% MeOH for 2 more min. UV detection was performed at 231 nm. In all cases, 10 µL was injected, being the flow rate 1.2 mL min^{-1}.

2.4. Sensory evaluation

Sensory evaluation of the 27 medium alkalized samples was conducted on dry powders (smell) and in water solution (flavor). Assessment was carried out following the general guidance of the International Organization for Standardization (ISO 6658, 2005). A selected and trained profiled panel of 10 assessors (6 females and 4 males) with previous experience in cocoa assessment performed the sensory evaluation of samples. The members of the panel were selected and trained according to the guidelines of the International Standard (ISO 8586, 2012).

For smell evaluation, samples were presented in white containers containing 50g of sample. For flavor evaluation, cocoa powders were mixed with hot water (4g in 100mL) and presented at 40 ºC to the panellists. Quantitative Descriptive Analysis (QDA) was the method selected in this study since this methodology provide a detailed description of the sensory characteristic of a product (Stone & Sidel, 2004). Sensory descriptors were basic cocoa notes, chocolate flavor, bitterness, acidity, astringency, alkalinity and vanilla (smell and flavor) notes. Panellists rated the intensity of the sensory descriptors for each sample, in triplicate, using unstructured scales of 10 cm, labelled on both ends with intensity terms, in the left (lower anchor) and in the right (upper anchor). A maximum of four samples were analysed by each panellist per session.

2.5. Statistical analysis

For the sensory analysis, a one-way ANOVA was performed for each sensory attribute evaluated in order to test if there were significant differences depending on the vanillin and ethyl vanillin addition. The least significance procedure (LSD) was used to test for the differences between averages at the 5% level of significance. The data were statistically processed using Statgraphics Centurion XVI (Manugistics Inc., Rockville, MD, USA). Correlation studies were carried out to establish the relationship between sensory attributes with the total amount of vanillin and ethyl vanillin by using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL, USA)).

3. Results and discussion

3.1. Optimization of vanillin and ethyl vanillin extraction

In order to extract vanillin and ethyl vanillin from the cocoa powder samples, both extraction solvent and extraction time were optimized. In all cases, the recovery percentages were calculated from the amount of vanillin and ethyl vanillin present in the starting sample and the amount of the analytes recovered after extraction. Three replicates were performed for each experiment.

For the optimization studies, a natural cocoa sample, which has not been flavored with vanillin and ethyl vanillin, was used. In each experiment, 1 g of cocoa was spiked with both analytes at a 0.1 wt%.

First, the extraction efficiency of three different solvents, such as H_2O, MeOH and MeOH:H_2O 1:1 (v/v), was tested. In all cases, the extraction was performed by adding 10 mL of each solvent to 1 g of cocoa sample containing 0.1 wt% of vanillin and ethyl vanillin, being this mixture kept under constant stirring during 10 min. As it can be observed in Fig. 1, only the MeOH:H_2O 1:1 (v/v) binary mixture provided recovery values of ca. 100%. Thus, this solvent was selected for further studies.

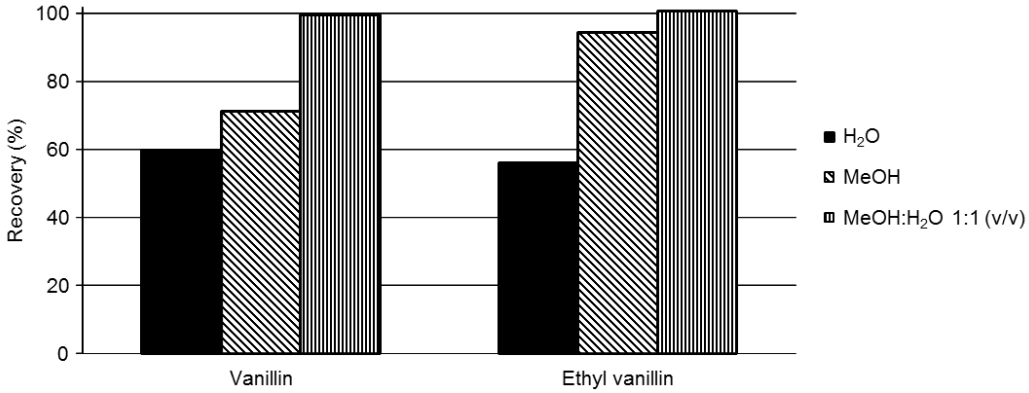

Fig. 1. Effect of solvent type on vanillin and ethyl vanillin recoveries from cocoa powder samples.

Next, using the same experimental conditions indicated above, and 10 mL of MeOH:H_2O 1:1 (v/v), different extraction times (1, 5, 10, 15, and 20 min) were tried. In all cases, recovery values were ca. 100% (data not shown); thus, a time of 5 min was selected.

3.2. Optimization of the chromatographic conditions

Vanillin and ethyl vanillin separation was studied using the 0.1 wt% spiked cocoa sample in order to obtain not only an optimal resolution of both analytes, but also separation from other matrix peaks that absorb at the maximum vanillin wavelength (231 nm). As previously reported by Waliszewski et al. (2007), the available HPLC methods for vanillin quantification in literature used MeOH and H_2O as mobile phase. Thus, different proportions of these solvents with both, gradient and isocratic elution, were firstly tried using a flow rate of 1 mL min^{-1} and an injection volume of 10 µL. The best results in terms of resolution and analysis time were obtained using a linear gradient from 50% to 100% MeOH for 2 min, followed by an isocratic elution with 100% MeOH for 2 more min. The HPLC system was next re-equilibrated with the initial composition for 5 min prior to the next injection.

Next, the effect of the mobile phase flow rate was tested in the 0.5–1.5 mL min^{-1} range. As expected, both retention time and peak width decreased as the flow rate increased for both analytes. However, a matrix peak and vanillin peak were not baseline resolved at flow rates higher than 1.2 mL min^{-1}. Thus, this rate was chosen for further work.

Finally, the effect of the injection volume was investigated over the range from 5 to 20 µL. In general, the peak area of both analytes increased with increasing injection volume. Thus, a volume of injection of 10 µL was selected as a compromise between sensitivity and peak resolution. A chromatogram showing the separation of vanillin and ethyl vanillin in the 0.1 wt% spiked cocoa sample under the selected conditions is shown in Fig. 2. Efficient, reproducible, and sensitive separation and detection of both analytes was obtained in less than 4 min, which allows a quick and reliable detection and quantification of cocoa sample flavoring with both vanillin and ethyl vanillin flavors.

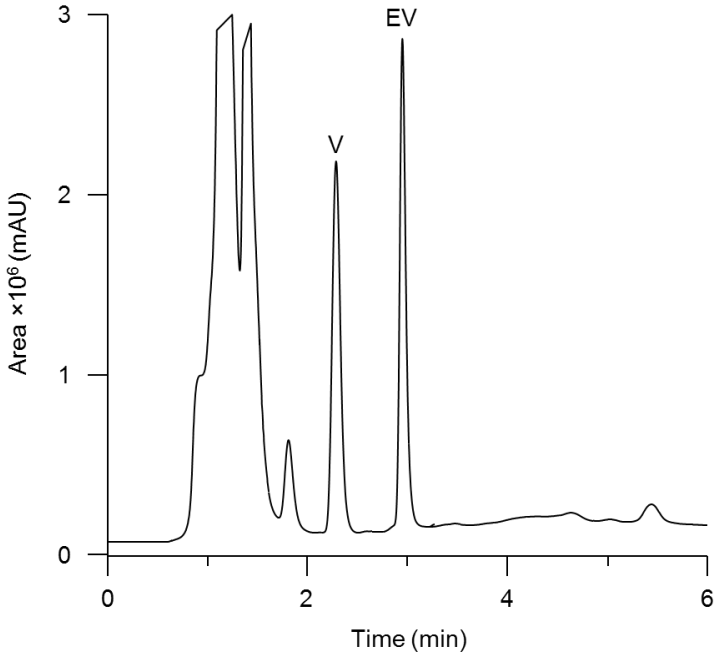

Fig. 2. Chromatogram showing the separation of vanillin and ethyl vanillin in a 0.1 wt% spiked cocoa sample. Chromatographic conditions: linear gradient from 50% to 100% MeOH for 2 min, followed by an isocratic elution with 100% MeOH for 2 min more min; UV detection, 231 nm; injection volume, 10 µL; flow rate, 1.2 mL min^{-1}. Peak identification: V, vanillin; EV, ethyl vanillin.

3.3. Analytical performance of the HPLC method and application to the determination of vanillin and ethyl vanillin in cocoa powder samples

Precision was determined by studying the intra- and interday repeatabilities of retention times and peak areas obtained by injecting the same 5 mg L^{-1} solution containing vanillin and ethyl vanillin ten times per day during 3 days (see Table 1). In all cases, the relative standard deviation (RSD) values were lower than 0.25 and 2.05 for retention times and peak areas, respectively.

Table 1. Analytical figures of merit for the determination of vanillin and ethyl vanillin in cocoa powder samples.

Analyte	Intra-day repeatability[a], RSD (%), n= 10		Inter-day repeatability[a], RSD (%), n = 3 days		Linear range (mg L^{-1})	Sensitivity[b]	r^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
	Peak area ratio	t_r	Peak area ratio	t_r					
Vanillin	1.15	0.10	1.58	0.12	0.5 – 25 50 – 200	145330 183554	0.9998 0.9996	0.06	0.21
Ethyl vanillin	1.88	0.22	2.05	0.25	0.5 – 25 50 – 200	138957 148039	0.9998 0.9999	0.04	0.14

[a] Obtained from ten injections of the same standard solution in one day and along three consecutive days.
[b] Obtained from the slope of calibration curve constructed using peak area vs analyte concentration (mg L^{-1}).
RSD relative standard deviation; t_r: retention time; r^2: determination coefficient; LOD: limit of detection; LOQ: limit of quantification.

External calibration curves of peak areas were constructed by injecting six standard solutions in the linear ranges indicated in Table 1. As it can be observed r^2 > 0.9996 were obtained. The sensitivity of each analyte obtained from the calibration curve constructed using peak areas was also given in Table 1. The limits of detection (LOD) of each analyte were calculated by multiplying by 3 the standard deviation of the peak area divided by the slope of the calibration curve (ICH guidelines). The standard deviation values for each analyte were obtained by injecting ten times aliquots of a solution containing known low concentrations of analyte that fulfill the signal-to-noise

ratio of 3. LOQ was obtained by multiplying by 3.3 the LOD values. As observed in Table 1, LODs and LOQs were 0.06 and 0.21 and 0.04 and 0.14 mg L^{-1} for vanillin and ethyl vanillin, respectively. These values were lower than others previously published in literature (Jagerdeo et al., 2000; Ohashi et al., 2007; Waliszewski et al., 2007). Additionally, standard addition calibration curves were obtained by adding to the samples at least four solutions with increasing concentrations, taking into account the linearity ranges given in Table 1. All curves were linear with $r^2 > 0.9995$, and in all cases the slope of calibration curve did not differ significantly from that obtained with the external calibration method. From these results, it can be concluded that no matrix effect was observed in the determination of these analytes in the cocoa samples analyzed. Thus, external calibration curves were employed for analyte quantification in all analyzed samples.

Next, the applicability of the method was evaluated by performing a recovery study. For this purpose, a cocoa sample in which either vanillin or ethyl vanillin were not originally present, was fortified with both flavors at 6 different concentration levels (see Table 2). As it is shown in this table, recovery values, which were estimated from measured versus added amounts, ranged between 97.9% and 103.1% and between 97.5 and 102.8 % for vanillin and ethyl vanillin, respectively. Thus, the results obtained demonstrated the applicability of the proposed methodology for the accurate determination of both flavors.

Table 2. Recovery results of the determination of vanillin and ethyl vanillin in cocoa samples spiked at different levels.

Analyte	Spiked level (wt%)	Recovery (%)[a]
Vanillin	0.1	100.0 ± 1.1
	0.05	99.7 ± 1.3
	0.01	98.6 ± 2.0
	0.005	103.1 ± 0.9
	0.0025	97.9 ± 1.6
	0.001	100.4 ± 1.9
Ethyl vanillin	0.1	99.8 ± 1.6
	0.05	101.1 ± 1.6
	0.01	97.5 ± 1.3
	0.005	101.2 ± 1.4
	0.0025	102.8 ± 1.2
	0.001	99.8 ± 1.9

[a] Mean ± RSD, *n*=3.

Finally, all the cocoa powders were subjected to HPLC analysis. Among the 66 samples analyzed, vanillin and ethyl vanillin were only detected in 3 samples, while 4 samples contained only vanillin. Representative chromatograms of a sample containing both flavors (A), a sample containing only vanillin (B), and a sample that do not contained any flavor (C) are depicted in Fig. 3. The contents of vanillin and ethyl vanillin found in the samples are included in Table 3.

Table 3. Content of vanillin and ethyl vanillin found in cocoa powder samples expressed as mg of flavor per 100g of cocoa powder.

Sample	Vanillin (mg / 100g)	Ethyl vanillin (mg / 100g)
1	90.8 ± 1.0	---
2	24.9 ± 1.8	---
3	9.1 ± 1.4	5.1 ± 2.0
4	12.0 ± 1.0	7.0 ± 1.0
5	10.0 ± 1.1	12.2 ± 1.3
6	5.6 ± 0.7	---
7	30.5 ± 1.4	---

[a] Mean ± RSD, *n*=3.

As observed, in samples containing both vanillin and ethyl vanillin, the global content of flavoring molecules was lower than those containing exclusively vanillin, except for one of the samples, in which the content of vanillin was very low. It suggests that when presented together, both molecules have a synergistic effect in flavor enhancing.

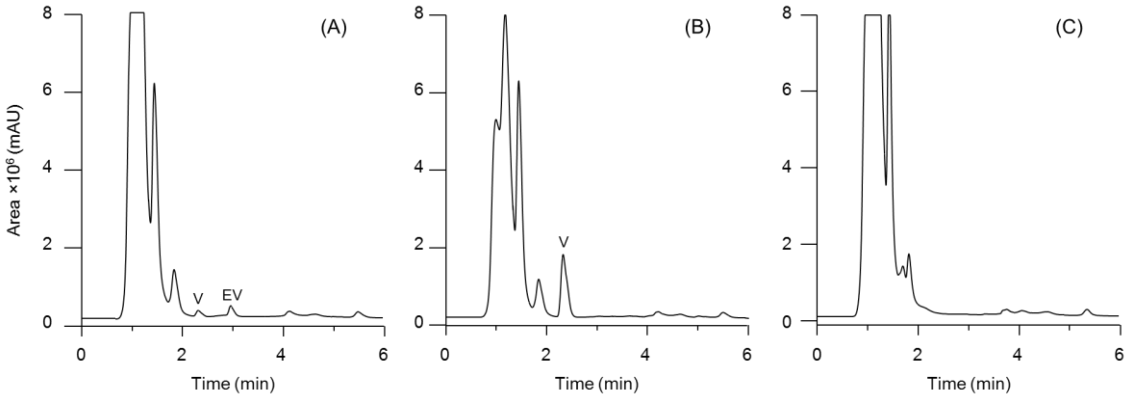

Fig. 3. Representative chromatograms of a cocoa sample containing vanillin and ethyl vanillin (A), a sample containing only vanillin (B), and a sample that do not contained any flavor (C). Chromatographic conditions and peak identification as in Fig. 2.

All the samples containing vanillin or ethyl vanillin belonged to the group of medium alkalized cocoas. Generally, medium alkalized cocoas are rich in flavor where cocoa and chocolate notes are present, and where alkaline notes are not really intense. However, processes conducted in not optimal conditions can alter the sensory profile by flattering cocoa/chocolate notes and by enhancing alkaline ones.

Moreover, from these 7 samples, only 1 has declared the addition of vanillin in product label. It demonstrates that undeclared flavoring of cocoa powders is a fraudulent practice in the cocoa industry that should be controlled.

3.4. Sensory evaluation

To evaluate the effect of the vanillin and ethyl vanillin presence on sensory perception of cocoa samples, cocoa samples classified as medium alkalized powders were sensory evaluated by a trained panel. This group of samples was selected since all the samples containing vanillin and/or ethyl vanillin corresponded to this category.

Generally, samples showed the typical flavor pattern of medium alkalized powders: reduced natural acidity and bitterness of non-alkalised cocoas and the appearance of alkaline notes (Rodríguez, Pérez, & Guzmán, 2009). Despite this common pattern, significant differences ($p<0.001$) were detected between non-flavored and flavored samples regards to chocolate, bitterness, alkalinity and vanilla attributes (Table 4). Cocoa samples where vanillin and/or ethyl vanillin were detected received a higher rate in vanilla and chocolate flavor descriptors, both related with sweet and pleasant perception (Serra-Bonvehí, 2005). In contrast, these samples showed lower intensity in descriptors related to bitterness and alkalinity. No significant differences ($p>0.5$) were found in cocoa, acidity and astringency descriptors. These results state that vanillin/ethyl vanillin have the capability to round out the flavor profile of alkaline cocoas by enhancing pleasant notes and masking bitter and alkaline notes, resulting in an increased consumer's acceptance (Hoskin, 1994).

Table 4. Sensory scores[a] based on QDA results (Mean ± SD) of non-flavored and flavored samples.

	Cocoa	**Chocolate**	**Bitterness**	**Acidity**	**Astringency**	**Alkalinity**	**Vanilla (smell)**	**Vanilla (flavor)**
Non-flavored	5,5±1,2[a]	5,6±1,9[a]	7,1±0,8[a]	1,7±1,4[a]	6,1±1,4[a]	7,7±1,2[a]	3,8±2,5[a]	0,5±0,2[a]
Flavored	5,5±1,1[a]	8,0±1,4[b]	5,3±1,4[b]	2,2±1,5[a]	5,4±1,6[a]	5,6±1,1[b]	8,5±1,1[b]	6,3±4,1[b]

[a] Sensory scores quantified using unstructured scales of 10 cm (see section 2.4).
Means with different superscript letters for the same column are significantly different ($p < 0.001$).

To establish relationships between sensory attributes and the total amount of vanillin and ethyl vanillin, a correlation study was performed. In this case, only vanilla (smell and flavor) and chocolate attributes seemed to be correlated with vanillin and ethyl vanillin content, being the obtained Pearson correlation coefficients 0.776, 0.929 and 0.883, respectively.

4. Conclusions

In this work, a fast and simple method for the extraction and HPLC determination of vanillin and ethyl vanillin from cocoa powders with detection limits within 0.04 and 0.06 mg L^{-1}, respectively, was reported. Recovery values ranged between 97.5 and 103.1%, in all cases. Analysis of commercially available cocoa powder samples using the developed method revealed that 7 out of 66 samples contained vanillin and/or ethyl vanillin. Of those, only one brand declared vanillin content in the label. Sensory evaluation revealed that samples containing vanillin and or ethyl vanillin were significantly higher in pleasant aromatic notes (vanilla and chocolate) and lower in bitterness and alkalinity. This alkalization-masking effect of vanillin/ethyl vanillin addition could justify the declared use of flavoring agents. However, the fact that only one of samples containing vanillin/ethyl vanillin in its composition declared the sample aromatization states that aromatized cocoa might not be an accepted practice by consumers. In this scenario, the developed method is susceptible to be incorporated as routine method in the industry and control laboratories to verify the possible addition of undeclared flavorings to cocoa powders.

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