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Quantification of Vanillin in Fruits of *Vanilla planifolia* by High-Resolution Liquid Chromatography

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Abstract: *Vanilla planifolia* is an orchid whose origin is the humid tropic of the south of Mexico, a cause of high quality and fine aroma; vanillin has a high added value in the worldwide market; therefore, this current investigation has been focused on the quantification of vanillin concentration which was collected on three different traditional agroecosystems located on the northeastern Sierra of the state of Puebla, commonly known as Totocapan Region in Mexico. For this, was performed an ethnobotanical exploration on every cultivar to collect *V. planifolia* crops. Then it ripened through a traditional process, and at the end, a sample was taken and carried out at High-Performance Liquid Chromatography (HPLC) protocol using methanol. The results showed significant differences in vanillin concentration and were dependent on the cut-off date. In December, we found concentrations of 9.73 g/s, on march 7.86 g/s, and April 14.26 g/s. According to these results, vanilla producers could choose the cut-off dates and negotiate a better price; besides this, *V. planifolia* has been protected by a denomination of origin. With this result, we can conclude that the crop yield period recommended by the Mexican Standard NMX-FF-074-1996 did not consider the agroecological environmental conditions of the north sierra mountains and northeastern mountains of the state of Puebla, where the present research was performed. The actual regulations correspond to the environmental conditions of the state of Veracruz, where the latitude varies from 10 to 2550 meters above sea level. Therefore, and according to the actual climate that affects the region, it is important to consider this to choose a vanilla crop yielding.

Keywords: agroecosystems; aromatic compound; genetic resource; traditional knowledge; vanillin.

1. Introduction

Vanilla is one of the most profitable crops in the tropical humid and represents a great opportunity for exportation by its quality. Due to the highest quality of vanillin synthesized by *Vanilla planifolia*, it is considered one of the most coveted fine chemicals in the international

food market [1]. *The vanilla* genus belongs to the Orchidaceae family, and it is composed of 110 species with a pantropical distribution (between 27 ° N-S latitude). In Mexico and Central America are reported 56 species of *Vanilla*, where only 15 species of the genus produce vanilla fragrant crops. However, the most worldwide cultivated vanilla species is *Vanilla planifolia* due to its ability to synthesize vanillin, a flavoring agent used in foods, beverages, and pharmaceuticals [2], therefore having great economic importance. Vanilla is a perennial, terrestrial, climbing, and epiphytic plant with flexible, cylindrical, simple or branched stems. Its surface appearance is bright green with alternate sheets and plant guides to achieving up to 50 length meters [3]. On other other hand, vanilla plants could have from 10 to 15 inflorescences, and every one is composed of over 10 individual's flowers; these have a pearl-wooden appearance or yellowish-white color who's some flower buds are opened every morning during the spring, but in the afternoon are closed; when the flowers are pollinated-naturally or artificially- gave rise a fleshy and elongated fruit known as silicuiforme capsule whose length come from 15 to 25 cm [4]. A vanilla planting needs around 3 or 4 years to begin to yield fruits and flowers, the investment is quite considerably by the farmers, especially if they do not have external funding, therefore the investment return is slow, without ruling the environmental risks that the crops are exposed, besides the volatility of the financial markets [5]. The agricultural or horticultural challenge of the *V. planifolia* production is the development of the appropriate techniques of cultivation to support the entire life cycle of this orchid according to its habitat preferences, such as epiphytic growth, mycorrhizal symbiosis, complex reproductive traits, and growth dynamics [6]. Vanilla intended for trade are obtained basically of the ripening and dehydrated fruits of *V. planifolia*, and in minor proportion, vanillin is extracted from *V. tahitensis* and *V. pompona*. Manual pollinization is one of the tasks to be accomplished as efficiently as possible because it depends on the poled yields in the cultivars. Due to the biological reproduction features, vanilla plants require a specific pollinator that is well adapted to the anatomy of the flower, therefore is difficult to achieve good yields when compared with natural pollination, therefore, is necessary the adequate handling of the flower to ensure high vanillin yields of the vanillin crops [7]. It is important to recall that the vanillin extracts are used to enhance and provide special flavor in a great variety of foods (ice creams, chocolate, fine bread, carbonated beverages, cakes, candies, gourmet food, among others), liquors, pharmaceutical products, perfume fragrances, and fine cosmetic products. On the other hand, it has been found that vanillin has highly desirable properties as an insecticide, fungicide, anticancer agent, appetite stimulant on babies and animals, masker of residual industrial wastes, among other potential uses [8]. According to the above description, the main purposes of the current investigation are: use High-Performance Liquid Chromatography (HPLC) to use a reliable technique for vanillin detection and quantify the amount of vanillin synthesized after the ripening of *V. planifolia* fruits which was collected from three different traditional agroecosystems, normally used in the northeastern Sierra of the state of Puebla, Mexico.

2. Materials and Methods

The study was conducted in the Northeastern Sierra of the State of Puebla, which comprises two agroecosystems of the municipality of Olintla: Vicente Guerrero and Bibiano Hernández and one agroecosystem of the municipality of Huehuetla: Lipuntahuaca (Figure 1). According to CONEVAL [9] and INEGI [10], the municipality of Olintla has a total population of 11,641 inhabitants and 2,424 households and private dwellings in total. It is located between the coordinates 20º 02' 18" and 29º 10' 30" north latitude of the Tropic of Cancer and between

97º 36’ 54” and 97º 43’ 06” west longitude of the Greenwich meridian and in the case of the municipality of Huehuetla, INEGI [10], indicates that there is a total population of 18,803 inhabitants. There are 4,097 private dwellings in total.

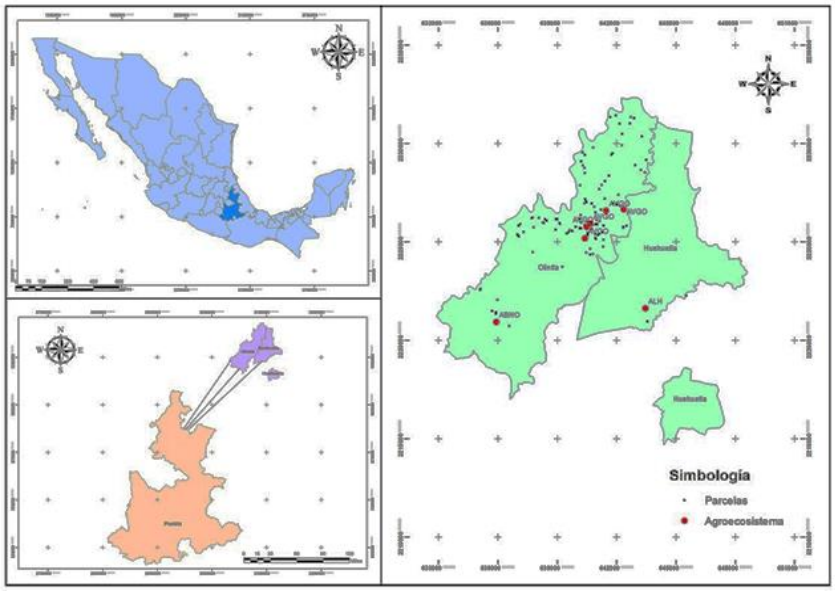


Figure 1. Location of the study area.

2.1. Biological material.

The sampling sites were geo-referenced, considering the particular characteristics and altitudes of the different areas with vanilla plantations. The following characteristics were considered for the selection of vanilla plants: a) free of pests and diseases, b) good shading and, c) in a reproductive state. Therefore, the selected plants were labeled for continuous observation and to detect the factors that intervene in the development and quality of their fruits. The first harvest carried out in Vicente Guerrero began on 10 December 2017, following the Mexican Standard [11]. The second harvest was carried out in Bibiano Hernández on 5 March 2018, both localities belonging to the municipality of Olintla, and, finally, the third harvest was carried out at the end of April in the locality of Lipuntahuaca, Huehuetla, Puebla (Table 1).

Table 1. Cutting dates for each agroecosystem.

Agro-ecosystem	Sp	Altitude (masl)	Pollination	Harvest
AVGO	*V. planifolia*	600	april-17	december-17
ABHO	*V. planifolia*	745	april-17	march-18
ALH	*V. planifolia*	400	april-17	april-18

2.2. Processing of vanilla fruits.

For the present investigation, it was necessary to carry out the process of processing the green vanilla fruits; this process consisted of applying a drying technique under traditional management carried out by Mrs. Rosa de Jesús Acosta. The processing process consisted of the following steps: 1) Collection of harvested fruits: Plantations in good condition were selected, 2) Peeling of fruits: Detaching the fruits completely from the rachis or bunch, 3) Selection of fruits: Healthy fruits are selected by size, since their commercialisation (price) will strictly depend on their size, colour, aroma, texture and moisture content (gourmet; Primera, extra, colas and zacatillo) [11], 4) Vegetative death of fruits: It consisted of submerging the

fruits in hot water at 100% for 5 to 8 seconds and 5) Sunbathing and sweating process: It consisted of giving sunbaths to the fruits with a time of about two hours during the day, until reaching a temperature of 48-50ºC, they are immediately lifted from the sun and placed in sweating boxes, for this they were wrapped very well to avoid losing heat immediately, since otherwise it does not favour the enzymatic activity and synthesis of compounds that give the characteristic aroma to the vanilla (Figure 2). Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided before publication.

Figure 2. Traditional processing process of *Vanilla planifolia* fruits from the study area. (a) Collection of harvested fruits; (b) fruit peeling; (c) fruit selection; (d) sunning and sweating process; (e) processed fruits.

The main problem in this processing process is that it is closely dependent on climatic conditions, especially during the first phase when the fruits are most susceptible to microbiological contamination, and the general characteristics desired in this process are that they are whole, without openings, and full of seeds; that they have the characteristic aroma, a uniform dark brown color and no stains [12]. This process is very important, points out that the main axis to be considered for the conservation of vanilla is the strengthening and development of the Totonaca culture and its traditional production and processing systems. On the other hand, it also states that the method of both cultivation and processing is still purely artisanal, and despite the many studies already carried out, there is still a lack of research [13]. Díaz *et al*., (2018) point out that Papantla is one of the main vanilla processing centers in Mexico and around the world, as it is located in an area with the best climatic conditions required for the processing process; however, farmers have generated traditional knowledge about the best site for processing [14].

2.3. Sample preparation and vanillin extraction.

Three fruits were weighed for each agroecosystem, then ground with the aid of a hand mill, and the crushed fruits obtained from each sample were placed in a plastic bag. The 15 mL "Sarstedt" plastic tubes with screw caps were then weighed with the aid of a balance, weighing

0.150 mg. In each tube, the vanilla powder from the different agroecosystems was introduced; for Vicente Guerrero and Bibiano Hernández, 0.1506 mg was weighed, for the locality of Lipuntahuaca 0.1504 mg was weighed, and in the same tube, 3 mL of 80% methanol was added for each sample, the methanol was measured with a micropipette. Therefore, the tubes of each sample were shaken for 3 seconds. Then they were ultrasonically bathed with a "Branson" ultrasonic washing bath for 10 min, left to rest for 5 min, and then ultrasonically bathed again for 10 min. Subsequently, the vanillin standard was prepared, using 0.043 mg in a dilution of 0.5 mg/mL of methanol. Afterward, each sample was filtered with a 0.45 µm acrodisc; the syringe was introduced to add the vanillin dilution in the samples contained in each vial; it is worth mentioning that, when making an injection, the syringe must be disinfected with ethyl alcohol; this to prevent the samples from dissolving in each injection and, therefore, the samples were injected and introduced in each vial. Once the pure sample was obtained, quantitative analysis was performed to identify whether the samples contained vanillin. In the HPLC, 11 injections were performed; each injection lasted 28.5 minutes.

2.4. Quantification of vanillin by HPLC.

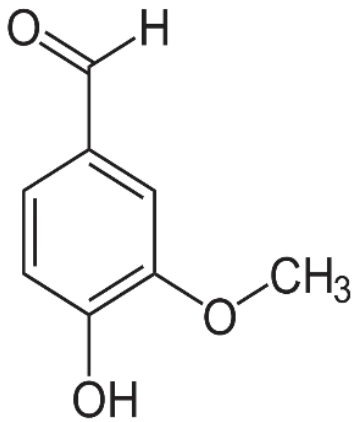
The extracts were analyzed by High-Performance Liquid Chromatography. Agilent technology chemstation family software products HPLC instrument was used. Version B.02.01. March 2006, with UV detector. A nucleosil 100 A 125 x 4.0 mm i.d., 5 µm, nucleosil type column at an injection volume of 10 µL with a flow rate of 1.0 mL min-1 was used as equipment conditions. The mobile phase corresponded at 0.10 min to A and B percentages of 85 and 15% respectively; at 20 min to A and B percentages of 65 and 35% respectively and finally at 23 min to A and B percentages of 65 and 35% respectively.

3. Results and Discussion

3.1. Production system in the study area.

The different sampling sites were identified and geo-referenced in most vanilla producers located in the study area. *V. planifolia* species were found from 322 to 990 m above sea level.


a



b

Figure 3. Fruit sample and vanillin content in *Vanilla planifolia*. (a) Aromatic compound "vanillin"; (b) vanillin molecule (molar mass: 152.14 g/mol; $C_8H_8O_3$).

This wide altitudinal range leads to a significant change in temperature and the growth and development of some other plants and micro-organisms, which probably also affects the

soil type. These factors affect the growth, development, and therefore the vanillin content, and therefore, different periods of physiological maturity of the fruits. If so, different harvesting dates would have to be established - for the lower or warmer parts and the higher or colder parts - otherwise, we would be selling unripe fruits in the case of the higher areas, and on the other hand with low levels of concentration of aromatic compounds, as shown in Figure 3.

Table 2 shows the characterization of agroecosystems with *V. planifolia* in the study area.

Table 2. Characterization of agroecosystems with *V. planifolia* in the study area.

Characteristic	**ALH⊄**	**AVGOξ**	**ABHOΨ**
Altitude	350-550	400-600	600-800
Climate	Semi-warm humid with rainfall all year round (100%)	Semi-warm humid with rainfall all year round (100%)	Semi-warm humid with rainfall all year round (100%)
Production system and type of stakes	On acahual plantations with tutors such as pinion	On acahual plantations with stakes such as pichoco, pinion, capulín, cocuite, pichoco.	On acahual plantations with stakes such as pichoco, pinion, capulín, cocuite, pichoco.
Harvest date	April	December	March
Type of processing	Traditional	Traditional	Traditional
Soil type	Leptosol (98%)	Leptosol (99%)	Leptosol (99%)

Source: ⊄Agro-ecosystem Lipuntahuaca, Hueuetla; ξAgro-ecosystem Vicente Guerrero, Olintla; ΨAgro-ecosystem Bibiano Hernández, Olintla [14, 18].

The climate of the two localities of the municipality of Olintla and the locality of Lipuntahuaca, Huehuetla, are very similar in terms of temperature and precipitation, the same is true for the type of soil existing in both municipalities, in which the Leptosol soil predominates, characterized by being thin or extremely gravelly soils, on continuous rock, this type of soil is very common in mountainous regions [14,15]. It was found that in the study area (Olintla, Caxhuacan, Huehuetla, and Tuzamapan Puebla), vanilla has been produced for several years, this product has been sold green to buyers in the municipalities of Zozocolco de Guerrero, Veracruz, who resell it to processors in Papantla; hence it is extremely important to know the production process. Otherwise, we will be selling green vanilla, and buyers from other countries such as Germany, France, England, and the United States, who are the main buyers, could generalize that our vanilla is of low quality.

Figure 4. *Vanilla planifolia* fruits found in the community of Vicente Guerrero, Olintla.

In this regard, it is known by the processors in the Papantla area that a large part of the vanilla production they process comes from the mountainous area of Puebla (Díaz *et al*., 2017) [14]. However, it was found that in the Sierra Norte and Northeast of the state of Puebla, fruits of good size or gourmet quality, as referred to in the NORMA (NOM-182-SCFI-2011) [16] are produced, since it is very common to find fruits ranging from 12 cm to 25 cm in length, although fruits of 25 to 28 cm have been found. Generally, the fruits found in the study area are larger in diameter, reaching an average of 20 cm in length, so they do not have any problem being considered gourmet quality fruits (Figure 4).

During the research, it was found that most of the producers have traditional production systems, that is, under acahuales with secondary vegetation in which they use different species of stakes, including the following: piñón (*Jatropha curcas*), capulín (*Prunus salicifolia*), cocuite (*Gliricidia sepium*), gasparito (*Erythrina americana*), naranjo (*Citrus sinensis* L.), and pichoco (*Eritrina* sp.), among others.

3.2. Fruit processing.

On 29 December 2017, the processing process began for the first fruits obtained from the locality of Vicente Guerrero, Municipality of Olintla, Guerrero Puebla, under traditional management carried out by Mrs. Rosa de Jesús Acosta. In summary, the process of processing consisted of the following steps: a) Collection of harvested fruits, b) Peeling of fruits: Detach the fruits completely from the rachis or bunch, c) Selection of fruits: Healthy fruits are selected by size since their commercialization (price) [11] will depend strictly on their size, color, aroma, texture and moisture content (gourmet; first, extra, tails and zacatillo), d) Vegetative death of fruits: It consisted of submerging the fruits in hot water at 100% for 5 seconds and e) Sunbathing and sweating process: it consisted of giving sunbaths to the fruits for about two hours during the day until reaching a temperature of 48 to 50°C, they are immediately lifted from the sun and placed in sweating boxes, for this they were wrapped very well to avoid losing heat immediately; otherwise it does not favor the enzymatic activity and synthesis of compounds that give the characteristic aroma of vanilla.

The main problem in this processing process was found to be that it is closely dependent on climatic conditions, especially during the first phase when the fruits are most susceptible to microbiological contamination. The general characteristics desired in this process are that they are whole, without openings and full of seeds; that they are shiny, thick, and flexible; and that they have the characteristic aroma [17,18], a uniform dark brown color and no stains as reported by Ms Rosa Acosta, a traditional vanilla processor from the Community of Potingo, Nautla, Veracruz [12]. Finally, it is worth highlighting what Salazar (2011) points out, in which he mentions that one of the main axes to be considered for the conservation of vanilla is the strengthening and development of vanilla culture in Totonacapan lies in the study and strengthening of traditional production and processing systems [13,19-21]. However, it states that both cultivation and processing methods are still artisanal [12].

3.3. Calibration curve with the vanillin standard.

To analyze the amount of vanillin in each of the fruit samples in the present investigation, it was necessary to prepare a calibration curve. For this purpose, a vanillin standard (vanillin, $C_8H_8O_3$; 152.15 g/mol) from PhytoLab was used. For this purpose, the following dilutions were used: 1-8 mg, obtained from a 0.5 mg/mL methanol dilution and

injected into 10 mL. The concentration of vanillin was quantified using Agilent Technologies Chemstation Family HPLC equipment with the help of Products Software, version B.02.01. March 2006. The data obtained for the vanillin calibration curve are shown in Table 3 and Figure 5.

Table 3. Level, quantity, and area of vanilla.

Lv 1[1]	Quantity (mg)	Area
1	1	351.21
2	2	678.26
3	4	1287.81
4	8	2641.81

*Level or number of repetitions and quantity or concentration.

The calibration curve used to determine the amount of vanillin present in each of the vanilla samples from three agroecosystems with different altitudes and, above all, different harvesting dates are shown below.

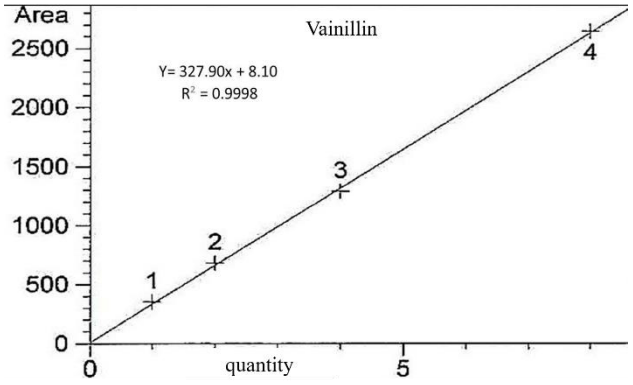


Figure 5. Calibration curve for the quantification of vanillin in each of the *Vanilla planifolia* samples.

3.4. Quantification of vanillin in the AVGO agroecosystem.

Once the HPLC equipment had been calibrated, the vanillin content present in each of the samples of vanilla fruits from the three agroecosystems described in Table 5 was quantified. To do this, each of the vials previously labeled with its identification data was placed in the tray of the HPLC equipment.

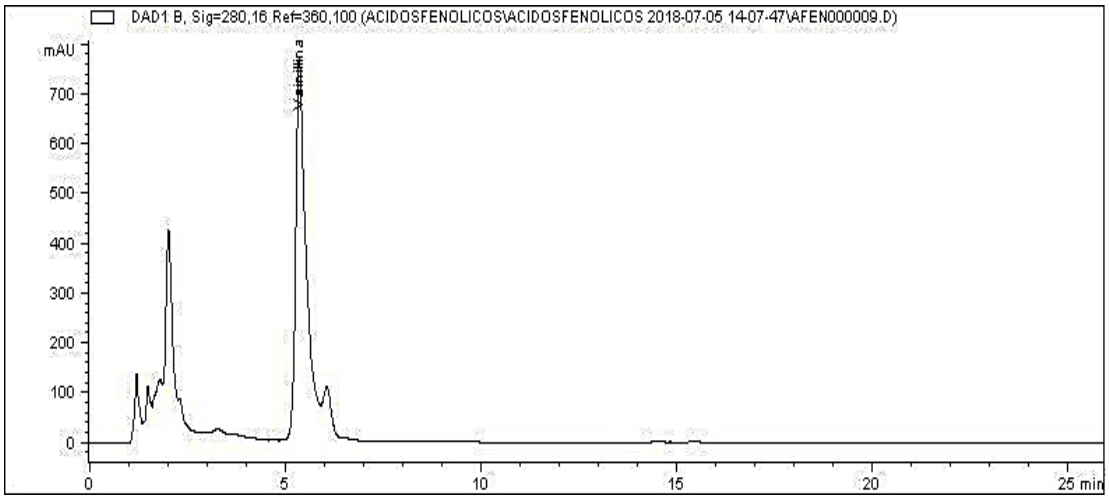


Figure 6. Chromatogram of quantification of vanillin content in fruits of the agroecosystem (AVGO) located in the locality of Vicente Guerrero, Olintla, Puebla.

The vanillin content of the vanilla samples from the different agroecosystems was then quantified by HPLC, where significant differences were found concerning the vanillin content in each of the samples. Among the variables responsible for these differences are the harvest date, which determines the degree of physiological maturity of the fruits, the altitude above sea level of each agroecosystem, and, therefore, the *V. planifolia* fruit samples extracted from these agroecosystems. Once the samples were run on the HPLC, the vanillin content of each of the samples was quantified and plotted, in which certain differences in vanillin concentration and retention time can be observed at the time of appearance of the graph denoting the presence of vanillin in each of the samples. The results show concentrations of 9.73 grams per dry weight of vanillin, with a retention time of 5.35 minutes, in fruits located at 600 m above sea level on average, and with a period of 9 months (Apr-2017-Dec-2017) of growth and development of the fruits, i.e., after the flowers have been pollinated naturally or manually by the producers (Figure 6).

Based on Figure 5, we can observe that the fruits contain more vanillin than the fruits from the Papantla area, as shown by Salazar (2011), who quantified *V. planifolia* fruits from the community of Pipila, Papantla Veracruz, with a vanillin concentration of 18657. 20 ± 638.66 ppm, while for *V. planifolia* fruits from the community Vista Hermosa in the municipality of Jalpan Puebla, a value of 17264.20 ± 504.29 ppm of vanillin was quantified [13]. It was also found a concentration of 19118 µg/g of vanillin in *V. planifolia* fruits from the Papaloapan region. Based on the present research and the results of the aforementioned investigations, we can affirm that the vanilla fruits from the AVGO agroecosystem are of good quality in terms of size and vanillin content, which exceeds the levels indicated by the Mexican Official Norm [11], which classifies the fruits into three categories: extra, first and second, as shown in Table 4.

Table 4. Classification of vanilla based on the Official Mexican Standard [11].

Quality	Moisture content (%)	Vanillin content (%)	Sensory characteristics
Extra	25-27	≥2.5	Supple and shiny, dark brown or blackish colour, clean and delicate sweet aroma.
First	19-24.9	2-2.49	More or less flexible and shiny, dark brown or brown with reddish filaments, sweet aroma.
Second	19-18.9	1.3-1.99	Slightly flexible and shiny, light brown colour with small reddish cracks, mild aroma.

3.5. Quantification of vanillin in the agroecosystem ABHO.

On the other hand, the quantification of vanillin content in the agroecosystem (ABHO) located in Bibiano Hernández, Olintla, Puebla, Puebla, was carried out. In this sample of fruits, 7.86 grams per dry weight of vanillin was found, with a retention time of 4.94 minutes, in fruits located at 745 meters above sea level on average, with a period of 11 months (Apr-2017-Mar-2018) of growth and development of the fruits, after having been pollinated naturally and some manually by farmers. It was found that despite being evaluated one month later (higher physiological maturity), the vanillin content is lower than that of 9 months, so possibly, in this case, the altitude plays an important role (745 masl on average) in the formation of vanillin, since there is a difference of 1.97 grams per dry weight of vanillin (Figure 7).

In the ABHO, the temperature is at least two degrees lower than in the Vicente Guerrero and Lipuntahuaca agroecosystems, which surely influences the vanillin content to some extent fruits.

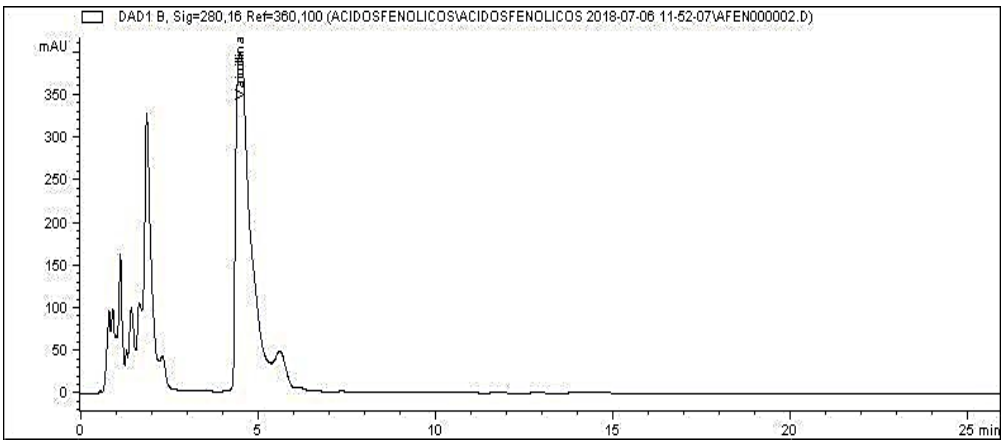


Figure 7. Chromatogram of quantification of vanillin content in the agroecosystem (ABHO) located in the locality of Bibiano Hernández, Olintla, Puebla.

3.6. Quantification of vanillin in the ALH agroecosystem.

Finally, the quantification of vanillin content in the agroecosystem (ALH) located in the community of Lipuntahuaca, Huehuetla, Puebla, was carried out, in which 14.26 grams per dry weight of vanillin was found (Table 5), with a retention time of 5. 32 minutes, in fruits belonging to an agroecosystem located at 400 m above sea level on average and with a period of 12 months (Apr-2017-Apr-2018) of growth and development of the fruits after being pollinated under a traditional production system with tutors of the region such as piñon, capulin and other shrubs typical of the agroecosystem (Figure 8). In this agroecosystem, the vanilla cuttings are at least 6 years old since they were planted by the farmers, which means they have been in production for 3 years. It should be noted that they do not use insecticides or chemical fertilizers for their vanilla plants, as they use the leaves and trunks as a source of natural fertilizer, and the stones are used as physical barriers to retain the soil and organic matter.

Table 5. Vanillin content in *Vanilla planifolia* fruits from three agroecosystems of the Northeastern Sierra of Puebla.

Agro-ecosystem	Retention time	g/ps	Ppm
AVGO	5.35 min	9.73	9730
ABHO	4.94 min	7.86	7860
ALH	5.32 min	14.26	14260

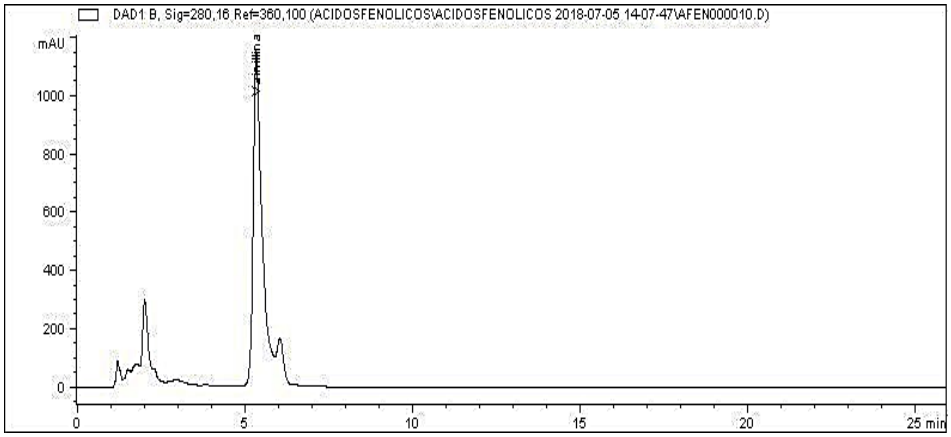


Figure 8. Chromatogram of quantification of vanillin content in the agroecosystem of Lipuntahuaca, Huehuetla, Puebla.

On the other hand, according to the study by Soto (2016), in which the aromatic profiles of six chemotypes of *Vanilla planifolia* from the regions of Puebla and Veracruz were analyzed,

the quantification of vanillin (4-hydroxy-3-methoxy-benzaldehyde) was 7050 mg/kg. Therefore only the main aromatic compounds were identified) [22].

4. Conclusions

The presence of vanillin was identified in the three agroecosystems of traditional vanilla (*V. planifolia*) production, in which differences were found in the vanillin content according to the variables evaluated (altitude and cutting date), as these are some of the most important environmental factors in the management of vanilla production systems. It was found that the cutting date is a very important factor since, after 9 months after the flowers have been pollinated, the vanillin content is higher. While altitude is an element that should be studied in more detail, it is recommended to evaluate temperatures during the production cycle, the distribution of precipitation, luminosity, cloud cover, and soil types. With the data from the present research, the producers in the study area now have more elements to decide to harvest based on the data obtained and the traditional knowledge they have about their production systems or to follow the Mexican Official Norm, which indicates that vanilla cutting should begin on 10 December, decide whether to wait longer to ensure that their fruits reach the necessary physiological maturity and therefore a higher vanillin content, which they could negotiate at a fairer price. Otherwise, they will not only sell their products at a low price but also put the quality of Mexican vanilla at risk and cast doubt on it.

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Conflicts of Interest

The authors declare no conflict of interest.

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