Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 69 (2006) 882–887
Talanta
www.elsevier.com/locate/talanta

Focused microwaves-assisted extraction and simultaneous spectrophotometric determination of vanillin and *p*-hydroxybenzaldehyde from vanilla fragans

Arturo Longares-Patrón, M.P. Cañizares-Macías *

Department of Analytical Chemistry, Faculty of Chemistry, Universidad Nacional Autónoma de México (UNAM), 04510 México D.F., Mexico

Received 26 August 2005; received in revised form 9 November 2005; accepted 13 November 2005
Available online 13 December 2005

Abstract

A new method to quick extraction of vanillin and *p*-hydroxybenzaldheyde (PHB) of vanilla beans from vanilla fragans is proposed. Samples were irradiated with microwaves energy to accelerate the extraction process and photometric monitoring was performed at 348 and 329 nm (vanillin and PHB, respectively). The simultaneous determination of vanillin and PHB from extracts was performed using the Vierordt's method, which showed a precision, expressed as relative standard deviation, smaller 2.5% for both analytes. Conditions such as microwaves irradiation power, number of irradiation and non-irradiation cycles, irradiation time and ethanol concentration were optimized by means of multivariate screening that showed that irradiation power and number of irradiation cycles are the most significant condition in the vanilla extraction process. The focused microwave-assisted extraction (FMAE) was applied to commercial (dried vanilla beans from fresh green vanilla beans), lyophilised and dried (commercial vanilla dried at 135 °C in oven) vanilla beans samples. The results showed that the extraction of vanillin and PHB in the commercial vanilla samples were higher than in dried and lyophilised samples. With the proposed FMAE a decrease in the extraction time of 62 times and an increase in the vanillin and PHB concentrations between 40 and 50% with respect to the official Mexican extraction method, were obtained.

Keywords: Focused-microwave extraction; Vanillin and *p*-hydroxibenzaldehyde; Spectrophotometric determination

1. Introduction

Vanilla plant is an orchid of Mexican origin. Aztecs of Mexico cultivated Vanilla, which was carried to Europe by the Spaniards after 1521 and is now cultivated in a several tropical countries: Mexico, Madagascar, Tahiti and Indonesia are the major producers. Vanillin, in fact, appears in trace amounts in other plants, including commercial products such as tobacco. However, the beans of the Vanilla orchid still remain the only commercial source of natural vanillin [1].

Numerous procedures of nurturing, harvesting and drying to produce vanilla beans are needed. From pollination, which is hand made, it can take up to two years to obtain a great quality product: 4 kg of fresh beans are necessary to obtain 1 kg of dried commercial beans. The source and cost of the vanilla varies from expensive natural extracts from beans harvested from the vanilla orchid to relatively cheap synthetically produced vanillin. The natural extracts tend to be a complex mixture of 100–200 flavour components, many of which are present in trace amounts [2–4].

Nowadays, laboratories involved in vanilla analysis call for faster methods to analyze a large number of samples in a time as short as possible. Auxiliary energies such as microwaves can accelerate the extraction, thus shortening the analysis time. In fact, microwaves reduce substantially the required time for several process [5,6]. The use of microwaves in analysis includes the sample preparation for the analysis of flavours and off flavours in fish, cheese and olive oil [7] and sample digestion for elemental analysis [8], among others. So, microwaves–assisted extraction, which is known as more environmental-friendly process with economic advantages than the current extraction methods, has also been used for the extraction of biologically active compounds from different matrices [9–11]. The vanillin/*p*-hydroxybenzaldehyde ratio is a quality parameter of

* Corresponding author. Tel.: +52 55 56 22 37 88; fax: +52 55 56 22 37 23.
E-mail address: pilarm@servidor.unam.mx (M.P. Cañizares-Macías).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.11.030

the vanilla extract and many methods have been used to determinate both parameters: HPLC [12], capillary electrophoresis [13], micellar electrokinetic capillary chromatography [14], gas chromatography [15], etc. These methods are adequate when the aim is to determinate several components from vanilla beans but for the analysis of only two components are very expensive and longer. So, Ruíz et al. [16] have developed a spectrophotometric method for the determination of vanillin and *p*-hidroxibenzaldehyde using derivative spectroscopy based on the interference-free character derivative amplitudes measured from an isodifferential point because the absorptions spectra are overlapped.

A extraction method assisted by microwaves energy to obtain a natural vanilla extract from vanilla beans for the determination of *p*-hydroxybenzaldehyde and vanillin was developed. The method was compared with the official Mexican method [17]. For both extraction method the determination of the analytes by absorption spectrophotometry using the Vierordt's method [18] was carried out. This method is based in the absorbencies measurement at maximum wavelength for each analyte and their quantification using a two equations system.

2. Experimental

2.1. Instrumentation

A Cary 3 UV–vis spectrophotometer (Sydney, Australia) equipped with a 1 cm quartz cell and a software to measure the hydrolysis of vanillin and PHB were used. A Microdigest 301 focused-microwave device of 300 W from Prolabo, France, as energy source to accelerate the extraction of vanillin and PHB from vanilla beans was used. An Explorer Ohaus balance with precision of 0.1 mg was also used. A LABCONCO Freeze Dryer 4.5 and a Precision Scientific Vacuum Pump with 1/2 HP for the samples lyophilization were used.

2.2. Reagents and solutions

Vanillin and *p*-hydroxybenzaldehyde were obtained from Sigma and stock solutions (100 μg/ml) were prepared by dissolving 0.01 g in 5 ml of absolute ethanol (Baker) and diluted with distilled water to a volume of 100 ml. Stock solutions were stored at 4 °C and used for a period of 1 week.

Standard solutions were obtained by taking suitable volumes of the stock solution, expected to contain between 0.2 and 20 μg/ml of vanillin or PHB or their binary mixture, were transferred into 100 ml volumetric flasks. Approximately 80 ml of water and 2 ml 0.1N NaOH (Baker) were added, mixing and diluting with water to the mark. Several mixtures of both compounds in that concentrations range were prepared.

2.3. Vanilla samples

Chopped commercial vanilla (vanilla fragans) beans (2 mm × 2 mm, approximately) from the region of Papantla, Veracruz, Mexico, to obtain vanilla extracts were used.

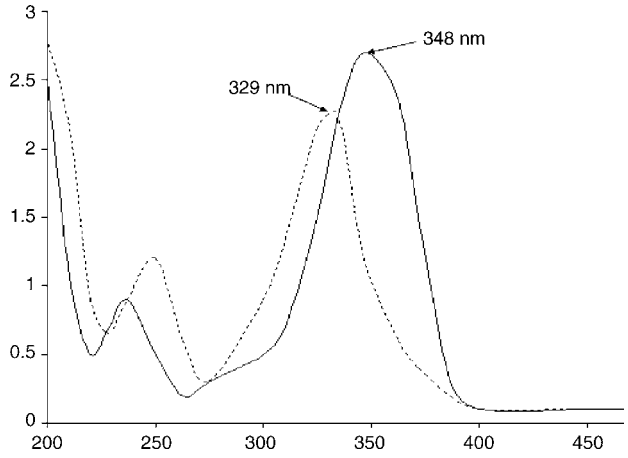

Fig. 1. Absorptions spectra of vanillin: 348 nm (—) and PHB, 329 nm (- - -).

2.4. Procedure

2.4.1. Spectrophotometric determination of vanillin and PHB

The vanillin and PHB were monitored in accordance with the maximum wavelengths for each compound, 348 and 329 nm, respectively. As the absorptions spectra of these compounds are overlapped, as is shown in Fig. 1, the Vierordt's method was evaluated for the quantification of vanillin and PHB from vanilla beans.

2.4.2. Vierordt's method

For the two studied compounds four calibration graphs were obtained. These graphs were done for each compound at 348 and 329 nm by varying the concentration of the analytes separately. The slope for each standard curve represents the molar absorptivity of the study compound at the fixed measurement wavelength. The vanillin and *p*-hydroxybenzaldehyde concentrations from vanilla beans by applying the additive principle yields two simultaneous equations, which were solved algebraically by substitution method.

2.4.3. AOAC method

The official method to determine vanillin is based in the hydrolysis of vanillin with NaOH and their measurement at 348 nm only. At this wavelength the PHB is interference and its value is not removed, so both analytes are measured. To compare the obtained results by the Vierordt's method the extracts were also measured using the AOAC method.

2.4.4. Vanilla beans samples treatment

The vanilla extracts were obtained using vanilla beans, which were treated in different ways: (a) *Dried vanilla beans*: stock commercial beans were dried following the next procedure: the oven was regulated at 135 ± 2 °C; 10 g of vanilla beans were weighted into each dish and shaken until contents were evenly distributed with covers removed, dishes were placed and covered in the oven as quickly as possible and the samples were dried for 2 h. The covers of the dishes were placed and transferred to the desiccator to cool. The sample was weighted and calcu-

Table 1
Statistical analysis of calibration curves for the determination of vanillin and PHB at 348 and 329 nm by Vierordt's method

Compound	Wavelength	Linear range ($\mu g\,ml^{-1}$)	Regression coefficient	R.S.D. (%)	Slope
Vanillin	348	0.75–13.2	0.9998	0.58	0.1655
	329	0.3–13.2	0.9999	0.49	0.1086
PHB	348	0.3–13.4	0.9998	0.70	0.1024
	329	0.3–6.7	0.9998	0.44	0.2216

lated the loss in weigh as water. (b) *Lyophilized vanilla beans*: 10 g of vanilla beans were placed in a lyophilization tube; liquid nitrogen was added and the tube placed in the freeze dryer for a period of 4 h at pressure of 5 μmHg. (c) *Commercial beans*: these beans were not treated and were analyzed directly using the different extraction methods.

2.5. Extraction from vanilla beans

Two kinds of extraction were evaluated to extract vanillin and PHB from vanilla beans:

(1) *Mexican official method*: This method was carried out in accordance with the Mexican Official Norm (NMX-FF-074-1996-SCFI). One gram of vanilla beans was poured in a 10 ml volumetric flask covered with 2 ml of ethanol and 1 ml of water. The solution was macerated for 12 h. Two milliliters of ethanol were added mixing well all the content. Maceration continued for 3 days. The solution was drained funnel dry, packing solids firmly and percolating slowly with a 50% ethanol solution until reaching a final volume of 10 ml.

(2) *Proposed focused microwave procedure*: One gram of vanilla beans was poured into a 10-cm test tube, which was placed into a water bath. Twenty-five milliliters of a 70% (v/v) ethanol–water solution were added to the sample. A refrigerant was adapted to the test tube to avoid loss of the extractant and a 150 W microwaves irradiation power was applied to the sample. Monitoring of vanillin and PHB was carried out after application of twenty cycles of 1 min irradiation each one with a delay time between them of 3 min.

2.5.1. Treatment of the Vanilla fragans extracts to measure vanillin and PHB

One milliliter of vanilla extract was poured into 100 ml volumetric flask. 80 ml of water and 2 ml 0.1N NaOH were added, mixing and diluting to the mark with distilled water. The extracts were monitored at 348 and 329 nm.

3. Results and discussion

3.1. Determination of molar absortivities of vanillin and PHB at 329 and 348 nm

The Vierordt's method allows to calculate the concentration of two analytes when their absorption spectras exhibit overlapping of the spectral bands. Fig. 1 shows the absorption spectras of vanillin and PHB. At 348 and 329 nm is showed that the spectral bands are overlapped.

To calculate molar absorptivities, standards of each compound were prepared in a range of 0.2–20 $\mu g\,ml^{-1}$ and they were monitored at 329 and 348 nm and thus the calibration graphs were constructed where the slope of each graph is equivalent to molar absorptivity for each studied wavelength. The linear ranges for each compound at studied wavelengths are shown in Table 1.

In accordance with the obtained graphs the equations system used for the quantification of vanillin and PBH in vanilla samples was the next:

$$A_{348} = 0.1024C_{PHB} + 0.1655C_V \quad (1)$$

$$A_{329} = 0.2216C_{PHB} + 0.1086C_V \quad (2)$$

where A_{348} and A_{329} are measured absorbances at 348 and 329 nm. C_{PHB} and C_V are PHB and vanillin concentrations, respectively.

This equation system was solved algebraically by substitution of the Eq. (1) in Eq. (2) of C_V or C_{PHB}. The precision, expressed as relative standard deviation, was evaluated for both analytes to the studied wavelengths with excellent results (less than 1%). These results are also shown in Table 1.

3.2. Analysis of vanillin and PHB mixtures using Vierordt's method

Working mixtures were prepared by dissolving the appropriate amounts of the vanillin and PHB standards. The different concentrations were selected considering the real ratio between both analytes in the vanilla beans (10:1) as well as higher and smaller ratios. The concentrations were calculated using the Eqs. (1) and (2). The mean recovery percentage expressed as percentage of the contents resulting from average of three determinations for each synthetic mixtures are shown in Table 2. The obtained recoveries are up to 96% when the vanillin/PHB ratio is lower than 20:1 but when the vanillin/PHB ratio is 20:1 the error in the PHB concentration value is up to 50%, although this ratio is not normal in vanilla beans. So, to determine vanillin and PHB from natural vanilla extracts (obtained from focused-microwave energy and official method) was decided to use the Vierordt's method because generally the ratio between vanillin and PHB is 10:1 and the selected method has an excellent recovery for both analytes to this ratio.

Table 2
Resolution of synthetic mixtures vanillin and PHB by using the Vierordt's method

Ratio (vanillin/PHB)	Theoretical ($\mu g\,ml^{-1}$)		Vierordt's method			
	Vanillin	PHB	Vanillin		PHB	
			Found ($\mu g\,ml^{-1}$)	Recovery (%)	Found ($\mu g\,ml^{-1}$)	Recovery (%)
2/1	1.99	1.1	2.04	102.51 ± 0.65	1.06	97.36 ± 0.58
5/1	5.97	1.1	6.10	102.17 ± 0.43	1.07	97.27 ± 0.66
10/1	9.95	0.96	9.89	99.40 ± 0.65	0.93	96.87 ± 0.61
15/1	10.0	0.66	9.84	98.40 ± 0.50	0.69	104.50 ± 0.48
20/1	9.95	0.55	10.08	101.31 ± 0.42	0.27	49.09 ± 0.50

3.3. Optimization of the focused microwave- assisted extraction (FMAE)

3.3.1. Multivariate optimization

Commercial vanilla beans were used to optimize the variables affecting the process of extraction by focused-microwave. The studied variables were: microwave irradiation power, non-irradiation time, ethanol concentration and microwave irradiation time, which is directly connected with the number of cycles. For the optimization of the microwaves parameters was only measured the vanillin concentration at 348 nm to simplify the calculation.

A screening study of the behaviour of the main variables affecting the extraction efficiency was performed by means of the experimental design methodology [Statgraphics Plus for Windows v 2.1, Rockville, MD, 1992]. A central design based on a two-level-full-factorial design was selected, on the basis of the low number of variable to be studied. A full factorial design was built for the screening study to determine the behaviour of the main variables which affect the microwaves-assisted acceleration of extraction power. Table 3 shows the upper and lower values given to each factor. Such values were selected from the available data and experience gathered in the preliminary experiments. The irradiation time was established at 1 min/cycle because higher time of continuous irradiation caused overheating in the ethanol solution and so ethanol and water spray as result. Thus the screening study was carried out considering 1 min of irradiation time per cycle, so that the studied parameter was number of cycles instead of irradiation time.

Analysis of variance (ANOVA) and the estimated effects on the extraction were performed on the design to assess the significance of the model. The conclusion was that the number of cycles (total irradiation time) and irradiation power were the most influential factors on the extraction efficiency. The ethanol concentration and non-irradiation time were not statistically significant factors under the tested ranges. However, the vanillin concentrations improved when the value of the ethanol concentration was highest and the non-irradiation time was the lowest. Thus, for subsequent experiments values of 70% ethanol and 3 min non-irradiation time were used. The number of cycles and irradiation power had a positive effect, so higher values should also be tested. The increase in the irradiation power (upper at 150 W) caused water spray from water bath and a higher number of cycles (upper at 20 cycles) did not increase the extraction of vanillin from vanilla beans significantly. So the optimal values for these two parameters were: irradiation power; 150 W and number of cycles, 20 of one min. With these conditions the total extraction time was of 70 min.

3.3.2. Application of focused microwaves-assisted extraction in vanilla beans

A comparative study was performed between the proposed extraction method and Mexican Official method using different samples of vanilla beans: dried, lyophilized and commercial. All samples were extracted for both methods and the vanillin and PHB concentrations were calculated using the Vierordt's method. The extractions were done in triplicate for each kind of sample. In the same way these samples were analyzed by the AOAC method for the determination of vanillin [19] to probe the advantages of using the Vierordt's method. In AOAC method the measurement is carried out at 348 nm only, so the PHB is a positive interference and is not possible to quantificate it. The mean results are summarized in Table 4 and they show that the obtained values for vanillin by the AOAC method are higher than those obtained using the Vierordt's method, it is because the PHB increases the absorbance value at 348 nm.

To compare the obtained results between the AOAC method and the Vierordt's method a *t*-test analysis was carried out. H_o is

Table 3
Experimental values tested for the optimisation of the FMAE process

	Irradiation power (W)	Number of cycles[a]	Non-irradiation time (min)	Ethanol concentration (%)
Screening design				
Upper value	150	20	6	70
Lowest value	60	5	3	40
Optimal values	150	20	3	70

[a] The irradiation time was 1 min/cycle.

Table 4
Obtained concentration of vanillin and PHB in vanilla beans extracts obtained using the proposed FMAE and the Mexican official method extraction

Extraction method[a]	Vanilla sample	Vanillin (mg g^{-1} vanilla beans)		PHB (mg g^{-1} vanilla beans)
		A	B	
FMAE	Dried[b]	18.65 ± 0.45	21.36 ± 0.42	2.68 ± 0.067
	Lyophilized	22.11 ± 0.71	25.36 ± 0.63	2.81 ± 0.073
	Commercial	23.06 ± 0.46	25.96 ± 0.60	3.09 ± 0.071
Official method	Dried[b]	7.03 ± 0.25	8.10 ± 0.16	1.02 ± 0.032
	Lyophilized	10.99 ± 0.26	12.00 ± 0.36	1.25 ± 0.041
	Commercial	11.48 ± 0.31	12.82 ± 0.32	1.28 ± 0.038

[a] Test in triplicate.
[b] Commercial beans dried at 135 °C; A = Vierordt's method; B = AOAC method.

the obtained vanillin concentration by AOAC method is the same that the obtained by the sum of the concentrations of vanillin and PHB using the Vierordt's method, so $\mu_d = 0$. The t-statistic calculated was: $t = d(n)^{1/2}/s_d$, where d and s_d are the media and standard deviation respectively of d, which is the difference between the values of each couple, and $n = 6$. The number of liberty grades of t is $n - 1$.

The obtained $t_{experimental}$ was 0.254 and the $t_{theoretical}$ is 2.57 for a $P = 0.05$, so it is possible to conclude that the two methods are similar. Therefore the PHB interference has been effectively avoided by the application of the Vierordt's method and has been possible to quantificate both analytes.

The results also show that from dried vanilla, the extraction of vanillin and PHB is considerably smaller than from lyophilized and commercial vanilla, possibly because the temperature of dried caused the elimination of volatile compounds as vanillin and PHB. In this case, the loss of water was 16.8% with a precision, expressed as relative standard deviation, of 5.3%. The difference between the lyophilized and commercial vanilla is not significantly but the values are higher for the commercial one.

Table 4 also shows the excellent obtained results with the proposed method extraction (FMAE): an increase of 50 and 41% in the vanillin and PHB concentrations, respectively, with regard to official method were obtained. On the other hand, using the focused microwave energy the extraction time is only of 70 min and with the official method delays for 3 days.

3.3.3. Recovery assays

To evaluate the FMAE extraction procedure, a simulated natural vanilla mixed vanillin and PHB in a ratio 10/1, yellow-brown vegetal color (to obtain a similar color to the natural vanilla color), vanillic acid and *p*-hydroxybenzoic acid with concentration 10 times less at PHB concentration was prepared. Three aliquots were separately submitted to the FMAE extraction and analyzed by Vierordt's method. The results showed that a recovery percentage of 98.4 ± 3.2% for vanillin and of 97.5 ± 3.6% of PHB were obtained.

To assure that did not exist interferences by matrix effect, the FMAE extraction method was carried out adding known amounts of vanillin and PHB to the vanilla sample at the beginning of each extraction. Approximately a 10 and 20% of vanillin and PHB were added taking as a basis the concentration determined in the vanilla beans with Vierordt's method. The results shown recoveries between 96.2 and 100.4% for vanillin and between 95.4 and 101.6% for PHB.

4. Conclusions

The obtained results prove that the application of focused-microwave energy to vanilla beans is appropriated for accelerating the extraction of vanillin and PHB from vanilla beans. The necessary time to obtain the vanilla extracts for the analysis of vanillin is substantially shorter (62 times) than the required by Mexican official method currently used in most laboratories for this analysis. In addition, the Vierordt's method allows the spectrophotometric determination of vanillin and PHB simultaneously from vanilla extracts with an excellent precision. With the proposed FMAE is possible to obtain a vanilla extract in shorter time to analyze vanillin and PHB, since these extracts had higher vanillin and PHB concentrations than that obtained by official method so the quality of the obtained extracts from FMAE is better. The results show that the proposed focused microwaves-assisted extraction method can be an excellent alternative to obtain a natural vanilla extract from vanilla beans in a shorter time than actually.

Acknowledgments

The Faculty of Chemistry of Autonomous National University of Mexico and the "Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica, PAPIIT" (grant no. IN219401) of the Dirección General de Asuntos del Personal Académico are gratefully acknowledged for financial support.

References

[1] H.P.S. Makkar, K.J. Beeker, Agric. Food Chem. 42 (1994) 731.
[2] A.S. Renadive, in: G. Charalambous (Ed.), Spices, Herbs and Edible Fungi, Elsevier Science, London, 1994, pp. 14–28.
[3] T.G. Hartman, K. Karmas, J. Chen, A. Shevade, M. Deagro, H.-I. Hwang, Am. Chem. Soc. Ser. 506 (1992) 61–76.
[4] N.J. Walton, M.J. Mayer, A. Narbad, Phytochemistry 63 (2003) 505.
[5] M. Erisson, A. Colmsjo, J. Chromatogr. A 877 (2000) 141.
[6] J.L. Luque García, M.D. Luque de Castro, Trends Anal. Chem. 22 (2003) 90.
[7] J.L. Luque García, J. Velasco, M.D. Luque de Castro, Food Chem. 76 (2002) 241.
[8] K.J. Lamble, S.J. Hill, Analyst 123 (1998) 103R.

[9] K. Ganzler, A. Salgó, K. Valkí, J. Chromatogr. 371 (1986) 299.
[10] C.S. Eskilsson, E. Björklund, J. Chromatogr. A 902 (2000) 227.
[11] X. Pan, G. Niu, H. Liu, Biochem. Eng. J. 12 (2002) 71.
[12] A.S. Renadive, J. Agric. Food Chem. 40 (1992) 1922.
[13] U. Butehorn, J.U. Pyell, Chromatogr. A 736 (1996) 321.
[14] M.C. Boyce, P.R. Haddad, T. Sostaric, Anal. Chim. Acta 485 (2003) 179.
[15] T. Sostaric, M.C. Boyce, E.E.J. Spickett, Agric. Food Chem. 48 (2000) 5802.
[16] C.C. Ruíz, A.H. Bayona, F.J. García Sánchez, Agric. Food Chem. 38 (1990) 178.
[17] Norma Mexicana, NMX-FF-074-1996, Non industrialized products for human consumption – spices and condiment whole and fought – vanilla (Vanilla fragans Salisbury Ames or Vanilla planifolia Andrews) Specifications (1996) Secretaria de Comercio y Fomento Industrial.
[18] S. Altinoz, S. Toptan, J. Food Comp. Anal. 16 (2003) 517.
[19] AOAC Official Methods of Analysis, Chapter 36, 1995.