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On-line dilution and detection of vainillin in vanilla extracts obtained by ultrasound

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Abstract

A continuous flow method coupling the dilution of extracts, obtained by application of ultrasound, and the on-line detection of vanillin is proposed. The flow method allowed the quantification of vanillin in a range between 200 mg l^{-1} and 2000 mg l^{-1} with a repeatability and reproducibility of 3.79% and 3.03%, respectively, for a standard of 1200 mg l^{-1}. The extraction conditions such as irradiation power, irradiation time, non-irradiation time and number of cycles are some of the most significant conditions in the vanilla extraction by ultrasound assisted extraction (USAE). The obtained results were compared with other conventional extraction methods: Soxhlet and maceration in accordance with the Mexican official method. The results showed that with the application of the USAE the extraction efficiency was increased between 19% and 72% in comparison with Soxhlet and maceration, respectively, besides, the extraction time decreased between 83% and 98%.

Keywords: Continuous-flow system; Dilution/detection on-line; Ultrasound irradiation; Vanillin; Extraction

1. Introduction

Vanilla is one the World's most popular flavor. Its fruity, floral fragrance combined with a deep, aromatic body makes it unique and universally favored. Numerous procedures of nurturing, harvesting and drying to produce vanilla beans are needed. As, by means of pollination, which is hand made, two years are needed to obtain a great quality product, and only 1 kg of dried commercial beans is obtained from 4 kg of fresh beans (Ramachandra Rao & Ravishankar, 2000), all these factors cause an increased vanilla cost. For this reason the artificial vanilla flavors have been used extensively, but the organoleptic and chemistry characteristics are very low quality. According to recent researches, over 250 flavor compounds have been found in natural extracts of vanilla causing them have better quality than artificial extracts. Quantitatively, vanillin is the major compound present in the beans although other phenolic compounds such as *p*-hydroxybenzaldehyde (PHB) and vanillic acid, among others, give to the beans special characteristics. Generally, the rate vanillin/PHB is found between 10 and 15, and for other compounds this rate is higher than 100 (Boyce, Haddad, & Sostaric, 2003; Hartman et al., 1992; Li, Jiang, Mao, & Shen, 1998; Longares-Patrón & Cañizares-Macías, 2006). Therefore, the determination of vanillin is one of the most important parameters in natural extracts; for its determination the standard method is based in the hydrolysis of vanillin and the measurement of its absorbance at 348 nm (AOAC, 1995, chap. 36) Other methods such as high performance liquid chromatography with ultra-violet detection (Anklam, Gagliore, & Muller, 1997; Boyce et al., 2003; Negishi & Ozawa, 1996); gas chromatographic separation with flame ioniza-

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tion, mass spectrometric detection (Pérez-Silva et al., 2006; Sostaric, Boyce, & Spickett, 2000) or derivatization followed by spectrometric determinations (Ni, Zhang, & Kokot, 2005); direct thermal desorption-gas chromatography using flame ionization or mass spectrometric detection (Hartman et al., 1992); and by electrochemical sensors (Bettazzi, Palchetti, Sisalli, & Mascini, 2006) have also been used to analyze vanilla. All these methodologies involve a time consuming extraction step combined with some type of separation, laborious isolation and expensive equipment.

The isolation of the target analytes from a solid matrix is one of the most critical analytical steps and the problems arise such as the possibility of analyte loss or contamination during sample preparation, a long required time, and large solvent consumption (Luque-García & Luque de Castro, 2004). The conventional Soxhlet extraction used to extract analytes from solid samples is a well-established model and it is used to evaluate new extraction methods. Although this extraction method is straightforward and cheap it is very slow and tedious (García-Ayuso & Luque de Castro, 1999).

In the last decade, there has been an increasing demand for new extraction techniques enabling automation, shortening extraction times and reduction of organic solvents consumption (Sterbová, Matejícek, Vlcek, & Kubán, 2004; Zuo et al., 2004). Advances in preparation of solid samples have brought a great number of new techniques such as focused microwave-assisted energy (Luque-García & Luque de Castro, 2003; Luque-García, Velasco, Dobarganes, & Luque de Castro, 2002; Stashenko, Jaramillo, & Martínez, 2004) and ultrasonic irradiation (Romdhane & Gourdon, 2002; Toma, Vinatoru, Paniwnyk, & Mason, 2001; Vinatoru, 2001; Zuo et al., 2004); which accelerate the different steps of the analytical process in comparison with other methods where these energies are not used. Therefore, the ultrasound-assisted methods have been used for food analysis improving their results, for example, as a means of accelerating the oxidation process in virgin olive oil with the aim to determining the oxidative stability in shorter time (Cañizares-Macías, García-Mesa, & Luque de Castro, 2004) or when extracting aroma compounds in aged samples of brandy (Caldeira, Pereira, Clímaco, Belchior, & Bruno de Sousa, 2004) or white wine (Hernanz-Vila, Heredia-Mira, Beltrán-Lucena, & Fernández-Racamales, 1999).

Some authors have used ultrasound to extract vanillin from vanilla beans or vanilla extracts. In the first case the authors use an ultrasound bath and the obtained extraction efficiency is similar to that obtained when the conventional extraction (maceration) is used (Sharma et al., 2006). In other research, the authors use biphasic sonoelectroanalysis to extract vanillin from natural vanilla essence using an ultrasonic probe and ethyl acetoacetate as electrochemical, extractant of vanillin and sonoelectrochemical solvent (Hardcastle, Paterson, & Compton, 2001).

A fast method to extract vanillin from vanilla beans by using an ultrasound probe in order to accelerate the extraction process is proposed here. The sound waves were applied directly into the extractant and the extracted vanillin was monitored spectrophotometrically at 347 nm using a continuous flow manifold where the dilution of the extract and the detection of vanillin were coupled on-line. The results were compared with those obtained by Soxhlet and maceration [SECOFI, Mexican official method (1996)].

2. Materials and methods

2.1. Instrumentation

A Cary 3 UV–Vis spectrophotometer (Varian, Sydney, Australia) equipped with 18 μl quartz cell and a software to measure the hydrolysis of vanillin were used. A Gilson Minipuls-3 (Villiers-le Bel, France) 8-channel peristaltic pump, 4 Rheodyne 5041 (Rohnert Park, CA, USA) low pressure injection valves and PTFE tubing of 0.5 mm internal diameter were used to built the hydrodynamic manifold. A Branson 450 digital sonifier (20 kHz, 400 W) equipped with a cylindrical titanium alloy microprobe (13 mm diameter) was used as ultrasound irradiation to extract vanillin from vanilla beans. An Explorer Ohaus balance with a precision of 0.1 mg was used to weigh the samples.

2.2. Reagents and solutions

Vanillin was obtained from Sigma (Mexico) and the stock solutions (5000 mg/l) were prepared by dissolving 0.5 g in 10 ml of ethanol (J.T. Baker, Mexico) and diluted with distilled water to a volume of 100 ml. Stock solutions were stored at 4 °C, so it was possible to use them for one week.

Standard solutions were prepared by taking suitable volumes of the stock solution, expected to contain between 200 mg l^{-1} and 2000 mg l^{-1} of vanillin.

A 0.01 N NaOH solution was prepared by dissolving 0.4 g of NaOH (J.T. Baker, Mexico) with 1000 ml of distilled water.

A 10 mg l^{-1} bromocresol green solution from Merck (Mexico) was prepared by dissolving 0.01 g of dye with 1000 ml of a solution of borax 10^{-2} mol l^{-1} (J.T. Baker, Mexico), which was stored at room temperature.

2.3. Vainilla samples

Extra commercial vanilla (*Vanilla fragans*) −15 cm long and 1 cm wide-beans from the region of Papantla, Veracruz, Mexico, were handly chopped (<3 mm) and approximately 1.0 g was used to obtain vanilla extracts. This value was chosen because this amount is enough to obtain an adequate vanillin concentration that can be quantificated with the proposed flow system.

2.4. Procedure

2.4.1. Flow injection manifold

Fig. 1 shows the generic flow injection manifold used for diluting and detecting vanillin. The automatic dilution loop is the key part of the manifold. It provides automatic mixing, diluting and homogenization of sample or standard prior to injection into the flow injection analysis (FIA) system. The dilution loop is formed by an open-close system (Cañizares-Macías, Hernández-Garciadiego, & Gómez-Ruíz, 2001) that is achieved with the injection valves V_1 and V_2. According to Fig. 2a the dilution solution (distilled water) is aspired into dilution loop by means of the valve V_2. When the loop is completely filled, the valve V_2 is closed and the valve V_1 is changed of position and then 117 µl of a standard/extract solution are introduced in the loop (Fig. 2b). Later, the valve V_1 is switched and the system is closed to mix, homogenize and dilute the standard/sample with the diluent (Fig. 2c). After suitable dilution time (4 min), the valve V_2 is switched (Fig. 2d) and the loop of the valve V_3 is filled with the diluted standard/sample. Therefore, a volume of 100 µl from V_3 is injected into a carrier of 0.01 N NaOH, which carries the sample to the reactor where the vanillin is hydrolyzed. Finally, the hydrolyzed plug passes through a flow cell located in the UV–Vis spectrophotometer, where the product is measured at 347 nm (reaction module).

2.4.2. Extraction from vanilla beans

Three extraction procedures to extract vanillin from vanilla beans were evaluated:

1. *Mexican official method* (MOM): This method was carried out in accordance with the Mexican official norm (SECOFI NMX-FF-074-1996-SCFI). The vanilla beans were poured in a glass container covered with 2 ml of ethanol and 1 ml of water and the solution was macerated for 12 h. Then, two milliliters of ethanol were added to the solution 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. *Conventional Soxhlet extraction* (CSE): The vanilla beans were placed into a filter paper cartridge (10 cm × 10 cm). The overall Soxhlet glassware was fitted to a distillation flask containing 100 ml of ethanol and 2–3

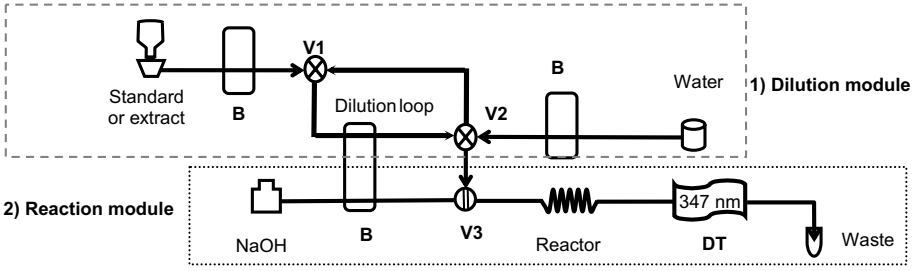

Fig. 1. General flow injection manifold used combining the dilution of the extracts with FIA determination of vanillin. B: peristaltic pump, V: injection valve, DT: detector.

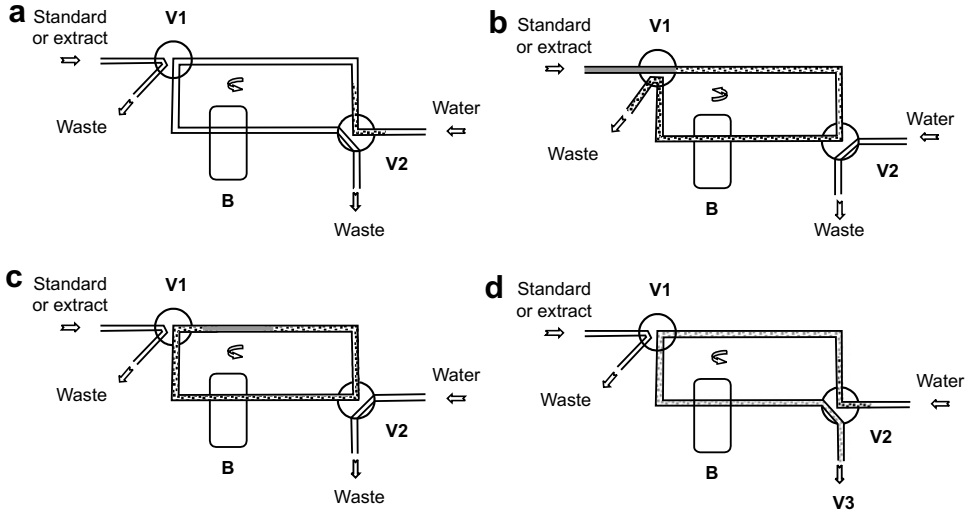

Fig. 2. Schematic diagrams for the implementation of an automatic dilution loop: (a) introduction of the dilute; (b) sampling position (standard or extract); (c) homogenization of the standard or extract; (d) injection of diluted standard/extract into the reaction module (see text for details).

boiling regulators glass. After 8 h extraction, the extract was left to cool down until it reached room temperature in order to be analyzed.

3. *Ultrasound-assisted extraction* (USAE): The vanilla beans were poured in a glass beaker covered with 50 ml of ethanol. The sonicator probe was placed into the flask at 2.5 cm from the surface of solvent and at 1.5 cm high from the bottom of sample for direct interaction. 40 cycles of 59.9 s ultrasound irradiation (200 W) were applied to the sample with a delay time between them of 59.9 s. The obtained extract was cloudy so that it was necessary to leave it to stand in order to obtain a translucent amber solution to be analyzed.

3. Results and discussion

3.1. Optimization of the flow injection manifold

Before the optimization of the dilution loop it was necessary the construction of a calibration curve of vanillin with the objective of knowing the linear range. For this test only the module reaction was used. To optimize the reaction module, the hydrodynamic and chemical parameters were studied. The optimum values were: flow-rate, 1.0 ml min^{-1}; injection volume, 100 μl; NaOH concentration, 0.01 N and reactor length, 100 cm. To select the optimum values the absorbance signal was measured. Therefore, different injection volumes were studied and the results showed that volumes higher than 100 μl caused similar signals. On the other hand, the 0.01 N NaOH concentration was enough to hydrolyze the vanillin, concentrations higher than 0.01 N caused double peaks. With flow-rates higher than 1 ml min^{-1} the absorbance signal was lower and with reactor lengths higher than 100 cm the signal was similar but the analysis time was longer. The found linear range was of 1 mg l^{-1} to 30 mg l^{-1}. Therefore, if the extracts contain until 3000 mg l^{-1} (Boyce et al., 2003), the dilution should be approximately 100.

The performance of the automatic dilution loop was assessed in order to ensure the best precision. For this purpose, the system was optimized and tested by using a stock solution (bromocresol green), which acted as sample/standard, and sodium tetraborate buffer, which functioned as diluent.

The studied parameters to optimize the dilution were: dilution loop volume, sample or standard aspiration time, flow-rate and dilution time.

Dilution loop volume and aspiration time: to select the optimum values of these parameters, the dilution factor (D_{ef}) was calculated from the rate between the absorbance value of the bromocresol green solution without passing through the dilution loop but injected in the reaction module, and the obtained absorbance value after the dilution system. Different dilution loop volumes were evaluated with the aim to obtain the best dilution in accordance with the aspiration time and the D_{ef}. A flow-rate of 1 ml min^{-1} was selected as optimum because it is the same used in the reaction module. Besides, flow-rate higher caused bubbles in the dilution loop when the standards/extracts were analyzed because they were prepared in an alcoholic solution. On the other hand, flow-rates lower caused too long homogenization time. The sample volume, which is related with the aspiration time, was studied in a range between 60 μl and 600 μl (from 3 to 36 s of aspiration time) in accordance with the concentrations of vanillin in real samples.

In these conditions, the optimum values were 7 s of aspiration time (117 μl) and 1.2 ml for the dilution loop. The obtained D_{ef} was of 79, which allowed the suitable dilution of the vanillin from extract samples for the quantification of vanillin. With higher volumes of sample the dilution was lower and the measured signal was higher to the maximum absorbance value of the calibration curve. On the other hand, at lower volumes the dilution was very high and some solutions were found out of the linear range.

Dilution time: to determine the time in which the sample/standard was homogenized, 117 μl of a solution of vanillin dissolved in ethanol were introduced into the dilution loop and were injected into the flow injection system (reaction module) at different times. The minimum time required for total homogenization, measured through absorbance, was 4.0 min with a flow-rate of 1 ml min^{-1}.

Under these conditions the calibration curve was constructed. The linear range was from 200 mg l^{-1} to 2000 mg l^{-1}, with the following lineal equation: $A = (0.00108 \pm 0.000038)[\text{vanillin}] + (0.1072 \pm 0.04695)$, where A is absorbance. The detection limit was 66.5 mg l^{-1}, the quantification limit 221.5 mg l^{-1} and the regression coefficient, $r = 0.9995$.

3.2. Flow injection manifold evaluation

To evaluate the precision of the method, within-laboratory reproducibility and repeatability were evaluated in a single experimental set-up with low and high levels of vanillin standard duplicates. Two triplicate measurements of each concentration were carried out in 7 days. The obtained results are listed in Table 1. To calculate the repeatability and within-laboratory reproducibility an ANOVA test was carried out. The obtained results, expressed as relative standard deviation (RSD), were: for a vanillin standard at 300 mg l^{-1}, 9.06% for repeatability

Table 1
Test results of the determination of within-laboratory reproducibility and repeatability for the proposed flow continuous method

Day	Vanillin measured (300 mg l^{-1})		Vanillin measured (1200 mg l^{-1})	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	300.86	286.31	1211.66	1211.85
2	360.43	359.63	1154.73	1227.71
3	329.79	329.79	1123.76	1131.54
4	364.95	323.88	1241.21	1241.21
5	351.00	352.39	1225.42	1112.02
6	326.28	333.15	1194.99	1202.45
7	324.91	326.74	1223.76	1220.16

and 9.14% for reproducibility; when the vanillin standard was of 1200 mg l^{-1} were 3.03% and 3.79%, respectively.

These results show that the proposed flow injection manifold is adequate to determine vanillin with an excellent precision and with the significant advantage to avoiding a previous treatment of the extracts before the detection, as the dilution of the sample on-line is made possible.

3.3. Extraction processes optimization

The vanilla extracts were obtained using different methods of extraction and the results were compared among them. The MOM was carried out in accordance with the reported by the Mexican official norm (1996). CSE and USAE were studied and optimized with the aim of extracting the maximum amount of vanillin. Two ethanol solutions at different concentrations were used as extractants when USAE was used, so the conditions of extraction could be compared with the CSE (using a 100% ethanol solution), and with the MOM (using a 70% ethanol solution).

3.3.1. Conventional Soxhlet extraction (CSE)

This method was used because it is one the most common when extractions from solid samples were carried out. The studied parameter was extraction time: 2 h, 4 h, 6 h, 8 h and 10 h. For each extraction time, three extractions were done and each one was measured using the proposed flow manifold. For longer times than 8 h the extraction time was too long and the concentration of vanillin was practically the same, therefore this time was selected as optimum.

3.3.2. Ultrasound-assisted extraction (USAE)

The height of the probe was fixed at 15 mm from bottom of the flask because this way the irradiation was direct and the bubbles or turbulence were avoided. A volume of 50 ml of ethanol was selected to obtain an extract into the range of vanillin quantification when 1.0 g of vanilla beans was used.

To optimize the extraction process by ultrasound irradiation, a screening study of the behavior 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.

This study involved the ultrasound device variables, namely: power between 80 W and 200 W, irradiation time between 10 s and 59.9 s, non-irradiation time between 0 s and 59.9 s (sonicator maximum value) and irradiation and non-irradiation cycles between 20 and 40. The monitoring of the concentration of vanillin was carried out using the flow manifold described in Section 2.4.1. The experimental matrix and the obtained results are shown in Table 2.

Table 2
Experimental design and results of the surface response methodology for USAE

Test	Cycles	Irradiation (s)	Non-irradiation (s)	Power (W)	Response (mg l^{-1})
A	20	59.9	59.9	80	258.76
B	5	59.9	0.1	80	151.11
C	20	10	59.9	200	332.79
D	5	10	0.1	200	76.09
E	20	10	0.1	80	66.24
F	5	59.9	0.1	200	67.56
G	12.5	34.95	30	140	110.26
H	20	10	0.1	200	241.38
I	5	10	0.1	80	34.16
J	20	10	59.9	80	94.49
K	5	10	59.9	200	68.52
L	20	59.9	59.9	200	208.63
M	20	59.9	0.1	200	159.64
N	5	10	59.9	80	23.04
O	5	59.9	59.9	200	103.24
P	5	59.9	59.9	80	65.99
Q	20	59.9	0.1	80	49.88

Analysis of variance (ANOVA) and the estimated effects on the extraction were performed on the design to assess the significance model with the summary of the model statistic given in Table 3.

ANOVA showed that the most influential factors on the extraction efficiency were the number of cycles and the power, because the *P*-value was less to 0.05 to a test of 95% of confidence, while the irradiation and non-irradiation times were not statistically significant factors. On the other hand, the estimated effects on the extraction into the model show a direct relation between: (a) cycles and non-irradiation time; (b) cycles and power and (c) irradiation time and power; as well as in inverse proportion between: (a) cycles and irradiation time and (b) non-irradiation time and power. Therefore, the results showed that the best responses happened when the power and the number of cycles were higher although the latter increased the analysis time. With these results other tests

Table 3
Analysis of variance (ANOVA) for the optimization of the USAE method

Source	SS[a]	DF[b]	MS[c]	*F*-ratio	*P*-value
Cycles (A)	42240.5	1	42240.5	15.83	0.0073
Irradiation (B)	1025.6	1	1025.6	0.38	0.5581
Non-irradiation (C)	5983.02	1	5983.02	2.24	0.1849
Power (D)	16523.8	1	16523.8	6.19	0.0473
AB	3723.44	1	3723.44	1.40	0.2822
AC	12413.3	1	12413.3	4.65	0.0744
AD	11661.8	1	11661.8	4.37	0.0815
BC	721.728	1	721.728	0.27	0.6216
BD	14854.7	1	14854.7	5.57	0.0563
CD	47.679	1	47.679	0.02	0.8980
Pure error	16011.2	6	2668.54		
Total (corr.)	125207.0	16			

[a] Sum of squares.
[b] Degrees of freedom.
[c] Mean of squares.

were carried out and optimum values for non-irradiation and irradiation (59.9 s in both cases) were selected, as shorter intervals caused loss of extract and probe overheat; the same happened if the power was higher than 200 W. As a consequence, when 59.9 s of non-irradiation and irradiation and 200 W of power were established and by changing the numbers of cycles to 40 and 50, vanilla extracts were obtained. The results showed that after 40 cycles the concentration of vanillin was practically the same but the analysis time increased a 20%, therefore the selected value was 40 cycles as a compromise between the concentration of vanillin and the analysis time. Therefore, the optimum conditions for the extraction of vanillin from vanilla using USAE were: power, 200 W; irradiation time, 59.9 s; non-irradiation time, 59.9 s and number of cycles, 40. Under these conditions the analysis time was of 80 min.

3.4. Influence of USAE on vanillin

To evaluate ultrasound extraction procedure, a 1200 mg l^{-1} alcoholic solution of vanillin was prepared. Three aliquots were separately submitted to the USAE extractions and analyzed by the proposed flow manifold. The results showed excellent recoveries of $99.9 \pm 0.62\%$. In addition to this test, the possible degradation or molecular decomposition of vanillin was studied by means of its absorption spectra before the ultrasound irradiation and after it. The obtained spectra were the same, therefore, with the results of both tests it is possible to conclude that vanillin molecule is not affected by the application of ultrasound.

3.5. Recovery assays

To assure that there were not interferences by matrix effect, the USAE method was carried out by adding determined amounts of vanillin (600 mg l^{-1} and 1600 mg l^{-1}) to the obtained extracts, which were measured with the proposed flow manifold. The results showed recoveries between 97.4% and 103.6% for 600 mg l^{-1} and between 97.7% and 102% for 1600 mg l^{-1}, assured that the measurements of vanillin were adequated.

3.6. Comparison between USAE, CSE and MOM

Under the optimum extraction conditions for each method, different extractions from vanilla in triplicate for the evaluation of the methods were carried out. Table 4 shows the obtained results, where the maximum concentration of vanillin by USAE is observed when the extractant is ethanol at 100%. On the other hand, when the extraction is carried out by using a 70% ethanol solution and USAE the vanillin concentration is lower. This result is interesting because the natural extracts are obtained with solutions of different rates ethanol–water, besides, the official method to quantificate vanillin uses a rate 70:30, therefore it is possible to assure the advantage of the application of energy as ultrasound in relation to the maceration used in the MOM.

Table 4
Concentration of vanillin in vanilla beans using different procedures of extraction

Concentration of vanillin (mg g^{-1})	Soxhlet	Maceration	Ultrasound	
			Extractant concentration (ethanol)	
			70%	100%
	21.03 ± 1.23	13.01 ± 0.34	22.47 ± 1.36	25.92 ± 0.78

The data show that the vanilla concentration in the extract yield by USAE is the best of all methods. When the Soxhlet method is used the extraction time is 83% larger and the vanillin concentration is lower (19% less in comparison with USAE when the extractant is 100% ethanol, and 6.4% less when the extractant is 70% ethanol). With regard to the MOM, the vanillin concentration in the extracts using USAE is 72% better and a severe reduction of the extraction time is obtained (98%). Therefore, the results show that USAE procedure not only increase efficiency but also decrease the extraction time in comparison with MOM and Soxhlet, which are the commonly used methods.

3.7. Significance test between USEA–CSE

To assure that USAE improve the vanillin extraction efficiency a *T*-test analysis between USAE and CSE was carried out. *F*-test for comparison of standard deviations between USAE (by using pure ethanol) and CSM was carried out and from the obtained result the best method for the *t*-test analysis was selected. The *F*-statistics calculated was: s_1^2/s_2^2, where s_1 and s_2 are the standard deviations of the obtained results for each studied method. Therefore, between USAE ($s_2 = 0.78$) and CSE ($s_1 = 1.23$), $F = 2.5$, for two degrees of freedom. The critical value is $F_2 = 39$ ($P = 0.05$; two tailed test). The results show that the standard deviations are not significant between methods. Therefore, the null hypothesis adopted is that the means of the results given by the two methods under study are equal. Therefore, the comparison of experimental means was carried out using the following equations:

$$|t| = \frac{x_1 - x_2}{s\sqrt{\left(\frac{1}{n_1}\right) + \left(\frac{1}{n_2}\right)}},$$

where s is calculated from:

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)}$$

and $|t|$ has $n_1 + n_2 - 2$ degrees of freedom and x_1 and x_2 are the means of the evaluated methods.

There are four degrees of freedom, so the critical value $t_4 = 2.78$ ($P = 0.05$). The obtained $|t|$ value was equal at

5.8. Therefore, it is possible to conclude that as the value of $|t|$ is higher than critical values, the difference between methods is significant at the 5% level, rejecting the null hypothesis. With this result it is conclude that the concentration obtained by using the CSE method is significantly lower.

4. Conclusions

The proposed flow injection manifold allows to determinate vanillin from vanilla beans extracts with an excellent precision and with the great advantage to avoid a previous treatment of the extracts before the detection, because it is possible the dilution of the sample on-line. The results also showed that USAE procedure increases drastically the efficiency and decreases the extraction time in comparison with MOM and Soxhlet methods, which are the commonly used methods. Therefore, the proposed USAE method can be an excellent alternative to obtain natural vanilla extract from vanilla beans in a shorter time than the current methods and possibly with best quality.

On the other hand, the use of a flow manifold allows the determination of vanillin from ultrasound extract in shorter time and with the possibility to automatize all the process.

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