Improved Extraction of Vanillin 4-Hydroxy-3-methoxybenzaldehyde from Cured Vanilla Beans Using Ultrasound-Assisted Extraction: A Comparison of Ultrasound-Assisted and Hot Water Bath Extraction

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ABSTRACT

This work was carried out to improve the extraction of vanillin from cured vanilla beans through optimizing operating conditions namely ethanol concentration, time, temperature, bean size and ratio of vanilla/solvent using ultrasonic extraction. Hot water bath and ultrasound bath extractions were also compared. Results showed that the optimization of vanillin extraction by ultrasonic horn with 40% ethanol for 1h at 30° C was comparative to water bath extraction at 56° C for 15h. However, optimization for vanillin extraction in hot water bath took a long time and was also at higher ethanol concentration (50%) i.e. 600ppm for hot water bath extraction, and 400ppm ultrasonic bath. Ultrasonic horn required only an hour to release similar vanillin concentration 560ppm. It can therefore be concluded that to extract vanillin from cured vanilla beans, ultrasonic application at 40% ethanol concentration for 1h at 30° C and size of bean <25mm are recommended.

Key Words: Vanilla; Ultrasonic assisted extraction (USAE); USAE horn, USAE bath, Ethanol

Vanilla 4-Hidroksi-3-metoksibenzaldehit’in Vanilya Tanesinden Ultrason Destekli Ekstraksiyon Yöntemyle Gelişirilmiş Ekstraksiyonu: Ultrason Destekli ve Sicak Su Banyosu Ekstraksiyonunun Karşılaştirılması

ÖZET

Bu çalışma vanilya tanesinden vanilin ekstraksiyonu sırasında etanol konsantrasyonu, zaman, sıcaklık, tane boyutu ve vanilya tane boyutu ile miktar/cözümcü oranı, ultrasonik uygulama gibi çalışma koşullarının optimizasyonu amacıyla yapılmıştır. Sicak su banyosu ile ultrason banyosunda ekstraksiyon da karşılaştırılmıştır. Sonuçlar 30°C’dede %40 etanol içinde 1 saat ultrasonik uygulamanın 56°C’dede su banyosundaki 15 saat ekstraksiyonı kyaslanabilir olduğunu göstermiştir. Ancak, sicak su banyosunda vanilin ekstraksiyonu uzun bir zaman almış olup, sicak su banyolu ekstraksiyon için daha yüksek etanol konsantrasyonu (%50) (sicak su banyolu ekstraksiyonda 600ppm ve ultrasonik su banyolu ekstraksiyonda 400ppm) gerekli olmuştur. Ultrasonik uygulamada benzer vanilin konsantrasyonuna (560ppm) ulaşmak için gerekli zaman sadece bir saat olmuştur. Sonuç olarak, vanilya tanesinden vanilin ekstraksiyonu için 30°C’de %40 etanol konsantrasyonunda bir saatlik ultrasonik uygulama ve <25mm tane boyutu tavsiye edilmektedir.

Anahtar Kelimeler: Vanilya, Ultrasonik destekli ekstraksiyon, Ultrasonikasyon, Etanol
INTRODUCTION

Vanilla extract is widely used in the food and the confectionery industry. The main component of the vanilla aroma is vanillin (C9H8O3 or 4-hydroxy-3-methoxybenzaldehyde) [1]. Recent reports have shown that vanillin can act as an antioxidant improving the keeping quality of precooked dried cereal flakes and afforded significant protection against protein oxidation and lipid peroxidation in rat liver. Vanillin exhibits strong antimicrobial properties with activity demonstrated against a number of yeast and mould strains in laboratory media, fruit-based agar systems, fruit purees and fruit juices. Vanillin is primarily a membrane active compound, resulting in the dissipation of ion gradients and the inhibition of respiration, the extent to which is species-specific. These effects initially do not halt the production of ATP [2]. The name vanilla is derived from its flavor character and flavoring strength from one of its most important components, vanillin. Vanillin content attributes of almost one-third of the flavor strength of vanilla products [3]. In international commerce most of vanilla is derived from *Vanilla planifolia* Andrews (synonym *Vanilla fragrans* Ames). Bourbon vanilla, cultivated in Madagascar and other islands in the Indian Ocean, represents approximately 75% of the world production. Seventy-eight compounds were identified as odor active compounds in the vanilla bean extracts with 10 confirmed with authentic references. Vanillin or 4-hydroxy-3-methoxybenzaldehyde is the most abundant constituent followed by guaiacol [4]. Soft independent modeling of class analogy (SIMCA) discriminating power showed that the most important compounds responsible for the differentiation between samples of vanilla by selected ion flow tube mass spectrometry (SIFT-MS) were vanillin, anise alcohol, 4-methylguaiacol, p-hydroxy benzaldehyde/trimethyl pyrazine, p-cresol/anisole, guaiacol, isovaleric acid, and acetic acid. ATR-IR spectroscopy analysis showed that the classification of samples was related to major bands at 1523, 1573, 1516, 1292, 1774, 1670, 1608, and 1431 cm\(^{-1}\), associated with vanillin and vanillin derivatives [5]. Food and Drug Administration (FDA) published in its publication section 169.175 that the vanilla extract is the constituent followed by guaiacol [4]. Soft independent modeling of class analogy (SIMCA) discriminating power showed that the most important compounds responsible for the differentiation between samples of vanilla by selected ion flow tube mass spectrometry (SIFT-MS) were vanillin, anise alcohol, 4-methylguaiacol, p-hydroxy benzaldehyde/trimethyl pyrazine, p-cresol/anisole, guaiacol, isovaleric acid, and acetic acid. ATR-IR spectroscopy analysis showed that the classification of samples was related to major bands at 1523, 1573, 1516, 1292, 1774, 1670, 1608, and 1431 cm\(^{-1}\), associated with vanillin and vanillin derivatives [5]. Food and Drug Administration (FDA) published in its publication section 169.175 that the vanilla extract is the solution in aqueous ethyl alcohol of the sapid and odorous principles extractable from vanilla beans. In vanilla extract the content of ethyl alcohol is not less than 35 percent by volume and the content of vanilla constituent is not less than one unit per gallon [6]. In addition, Daphna Havkin-Frenkel et al [7] confirmed in its publication that the vanillin is the most abundant component of vanilla extract. However, the selection of the method to isolate the active component with best yield from natural sources is mainly important and dependent on the nature of compounds, raw material to be processed, and extraction method. Extraction of biologically active components from plant also is one of the more sustainable approaches that may be employed [8]. Extraction of vanillin from vanilla pods is well explored using a variety of solvents ranging from hexane, ethanol, methanol, acetonitrile, acetone, chloroform and ethanol/water [7, 8] under conventional methods or maceration. Similarly, methods are available on extraction of vanillin including solid-phase micro-

In recent years, an increasing of demand for new extraction techniques enabling automation, shortening extraction times and reduction of organic solvent consumption has seen. Among these more efficient extraction techniques are ultrasonic extraction (USE), microwave extraction (MAE), supercritical fluid extraction and accelerated solvent extraction [13]. The advantages of using ultrasound for food processing, includes: more effective mixing and micro-mixing, faster energy and mass transfer, reduced thermal and concentration gradients, reduced temperature, selective extraction, reduced equipment size, faster response to process extraction control, faster start-up, increased production, and elimination of process steps [14]. The high intensity ultrasonicication can accelerate heat and mass transport in a variety of food process operations and has been successfully used to improve drying, mixing, homogenization and extraction. Ultrasonication is the application of high-intensity, high-frequency sound waves and their interaction with materials [15, 16]. The current study was therefore carried out to optimize extraction of vanillin from the cured vanilla beans using ultrasonic assisted extraction (USAE) by optimizing different operating extraction parameters namely solvent concentration, temperature, time of extraction, size of cut bean and ratio of solvent-vanilla. Furthermore the comparison of USAE horn method with water bath extraction was also investigated.

MATERIALS and METHODS

Materials

The cured vanilla beans were imported from a local farmer (Ambanja, Antsiranana, Madagascar). Vanillin standard (V1104-2) was purchased from Sigma–Aldrich (Shanghai, China). Ethanol and NaOH were obtained from Sinopharm Chemical reagent Co. (Shanghai, China). All other chemicals and solvents used were of analytical grade.

Ultrasonic Extraction

Ultrasonic bath extraction was performed by using a Kun Shan KQ-5200 ultrasound cleaner (max. power, 200W, 40 kHz; Kun Shan Ultrasound Instrument Co., Kunshan China). The USAE generators probe, (JYD-900L, Shanghai Zhixin Co., Ltd., China), which can deliver a maximum power of 100W at 25 kHz, equipped with a thermometer to measure the reaction temperature, was also used. The experimental ultrasound apparatus used in this work has been described in detail in previous work of Bashari et al [17]. Ultrasonic horn was operated in pulsed mode (5 s on followed by 5 s off), operated at maximum supplied power 34 W [8] and frequency 20 kHz (this frequency was found to be effective for maximum extraction of plant contents) [18]. A circulating water bath (DC2006, Shanghai Hengping...
Apparatus Factory, Shanghai, China) with an accuracy of 0.1 K was adopted to keep the reaction temperature at a constant. During the extraction operation, 1.5 g of cured vanilla bean (cut into small pieces and milled using mortar) and was mixed with 100mL of solvent (ethanol-water).

Extraction of Vanillin by Hot Water Bath Shaking

Maceration was performed at 56°C using shaking machine for 15h according to the method subscribed by Ranadive [3]. Cut vanilla bean (1.5 g) was putted with the corresponding solvent (100mL, 35% to 90% of ethanol in water) for 15 h. The extracts were collected for further analysis.

Vanilla Analysis

The cured vanilla beans (1.5 g) were cut into small pieces and milled then putted into the 100 mL volumetric flask. 100 mL of solvent was added according to different concentration (35, 40, 50, 60, 70, 80 and 90%). The pre-leaching for 30 minutes was used to improve the extraction method [8].

To prepare the standard solution, 0.05 g vanillin was dissolved in solution containing 3 mL ethyl alcohol and 50 mL of distilled water. Then, 3 mL vanillin solution was added into a 1 liter flask + 2mL of 0.1N NaOH solution and brought the volume to 1 liter with distilled water. The blank was prepared by adding 2mL 0.1N NaOH + 98 mL distilled water.

NaOH was used to increase the pH of vanilla extract for UV absorbance. Native vanillin is acidic (pH of a 5% solution of vanillin in water is 4.3). The phenol group of vanillin has a pKₐ value of 7.38. With increasing pH the molecule will lose a proton, and become negatively charged and more soluble in water [19].

The absorbance of 1ppm vanillin was calculated as:

$$A_{348} - (0.29A_{270} + 0.71A_{380})/3$$

where, $A_{348}$ is the peak of absorption of vanillin. The background absorption from polyphenolic and other interfering compounds in the sample is corrected by subtracting a percentage of the absorbance at 270 and 380nm.

The standard vanillin factor was calculated as:

$Standard Vanillin Factor (SVF) = Absorbance (corrected)$

The yield of vanillin in the vanilla extract was calculated as:

$Vanillin Content = \frac{A_{348} (multiplier from the table 1)}{Standart vanillin factor (SVF)}$

**Table 1. Sample preparation for vanillin determination [3]**

<table>
<thead>
<tr>
<th>Vanilla product</th>
<th>To make solution A</th>
<th>To make solution B</th>
<th>0.1 NaOH</th>
<th>Multiplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 fold Extract</td>
<td>5 mL</td>
<td>2 mL</td>
<td>1 mL</td>
<td>1</td>
</tr>
</tbody>
</table>

Statistical Analysis

All treatments and analysis were conducted in triplicate and the results were expressed as means ± SD. The data were evaluated by statistical analysis of variance (ANOVA) and Duncan’s multiple range test (MRT) using SPSS software version 19.0 to compare the effects of various factors under ultrasonic treatment. The standard deviation of the replicate values is shown as error bars in the values depicted on Y axis (P < 0.05). The analysis data to get the graphic was done using Origin pro8.Ink software (Origin Lab Corporation, Massachusetts, USA).

RESULTS and DISCUSSIONS

Comparison between the Extraction Methods

For extraction of natural component, several experiments have been conducted to determine the optimal conditions to maximize the amount of active contents extracted and to improve the quality of extraction. Classical extraction technologies were based on the use of an appropriate solvent to remove lipophilic compounds from the interior of plant tissues. The choice of a suitable solvent in combination with sufficient mechanical agitation influences mass transport processes and subsequently efficiency of the extraction and affects the quality of released product [11]. In this study, the amount of vanillin released using three extraction methods: USAE horn, USAE bath and hot water bath for 15h was analyzed.

Figure 1 shows the amount of vanillin released using USAE horn and USAE bath methods compared with that observed using hot water bath extraction method for 15h at the same different solvent concentrations. The result showed that the amounts of vanillin released using hot water bath and USAE horn were higher than that released using USAE bath method. Therefore, the use...
of USAE horn resulted in intensification of the extraction operation of vanillin as can be shown in Figure 1. On the other hand, hot water extraction method operated at 56°C and 50% ethanol (the ethanol concentration in which the higher vanillin content is released) yielded around 650 ppm in 15 h, whereas USAE extraction method required only 1 h for release of around 560 ppm vanillin concentration at more low proportions of ethanol (40%) and under ambient operating temperature. In addition, USAE horn released around 560 ppm, however, only 400 ppm for USAE bath and as can be seen in Figure 1. The use of USAE bath method required higher ethanol concentration to get the high concentration of vanillin yielded (80%). Thus, for extracting the vanillin from cured vanilla bean, as a general rule, for the same element and matrix, an ultrasonic bath required higher concentrations of solvent than ultrasonic horn to obtain comparable extraction efficiencies [20]. This result also showed that, the ultrasonic horn system was a powerful extraction method because the ultrasonic intensity was delivered on a small surface [18]. Also, it can be established that used of ultrasound horn could reduce the requirement of ethanol solvent extraction and less time was consumed for similar/or higher levels of vanillin extracted, compared to two another extraction methods. Therefore, the ultrasonic horn was potential technology, faster and more complete than maceration or USAE bath.

![Figure 1. Comparison of three methods of extraction: USAE horn & USAE bath (for 1h) at room temperature and hot water bath shaking for 15 h at 56°C (1.5 g of cut and milled vanilla bean with different concentration of ethanol for 15h extraction for water bath shaking and 1h for USAE extraction at room temperature)](image)

According to the literature, the vanillin is soluble in ethanol (50 mg/mL), yielding a clear, colorless solution. It is also soluble in water (10 mg/mL) and glycerol (50 mg/mL) at room temperature [21]. Thus the Figure 2 shows the extraction of vanillin from cured vanilla beans was influenced by the ethanol concentration in water [8]. As can be seen in Figure 2, the higher vanillin content was observed when 40% ethanol concentration was used at room temperature. The above observation may explain due to the fact that, in aqueous solutions containing low percentages of alcohol or other organic solvents, the structure of vanillin clusters is preserved, because the hydrophobicity of organic solvents enhances aromatic stacking. However, when vanillin is exposed to mostly an aqueous microenvironment, there might be a diminution in π-π stacking of aromatic rings (interactions). This phenomenon, stemming from the hydrophobicity of aromatic compounds contributes to the tendency of vanillin to form aggregates [22]. Moreover, water molecules enveloping vanillin crystals may form hydrogen bonds that link neighboring vanillin molecules in a cluster. Hence, an aqueous environment might enhance the solubility of vanillin and, in consequence, break up the multi-molecular structure of vanillin aggregates. Also, reinforcement of water structuring by the addition of small amounts of alcohol decreases the solubility of vanillin, whereas disruption of water structure by higher alcohol concentrations greatly enhances solvent–solute interfacing and increased solubility of vanillin. Thus, in this study, the using of ultrasonic horn has disrupted vanilla cell walls thereby facilitated the release of extractable compounds i.e. vanillin, enhanced mass transport of solvent from the continuous phase into vanilla cells, and less consuming of solvent (40%). It was reported that, disruption of water structure by some alcohol concentrations greatly enhances solvent–solute interfacing and increased solubility of vanillin [22]. Thus it was appeared that an optimum ratio of alcohol to water might results in maximum amount of vanillin released. With this observation, effect of mixture of water–ethanol on the extent of extraction has been investigated and it was indeed demonstrated that the extraction of vanillin from cured vanilla beans was greatly influenced by the ethanol concentration in water and extraction method used.

![Figure 2. Optimum concentration of ethanol: water ratio for USAE horn extraction (1.5 g of cut and milled vanilla bean with different concentration of ethanol for 1h extraction at room temperature)](image)

Optimization of Ethanol Concentration

For this study we could summarize that 40% (v/v) ethanol/water solution resulted in maximum extraction of vanillin from cured vanilla beans, beyond this concentration, any further increase in the ethanol concentration was found to be detrimental for the extraction. This observation could be attributed to higher polarity of solvents.
ultrasound horn reduced the requirement of solvent ethanol and less time consuming. Quantitatively speaking, use of 90% ethanol concentration for same duration 1h released only about 370ppm of vanillin whereas 40% ethanol concentration resulted in 600ppm vanillin concentration under same condition. Therefore, for the all next optimization studies, (i.e. for temperature, time, size of bean, and sample-solvent ratio) the ethanol concentration was kept at 40% of ethanol in water and the vanilla bean of 1.5g.

**Optimal Temperature for USAE Horn Extraction**

The effect of temperature on the release of vanillin extracted by USAE horn technique using same concentration of solvent (40%) and same quantity of vanilla bean is shown in Figure 3. The temperature, time and particle size were the main factors affected the ultrasonic extraction [23]. For the extraction, the increase of temperature increased also the solubility of vanillin in water or in ethanol/water [19]. Thus, using USAE horn; it was found that, the optimum temperature for vanilla extraction was 30±2°C. At this temperature the vanillin released was around 683 ppm; however, as it can be seen in Figure 3, below 30°C the concentration of vanillin released was lower. In addition beyond this temperature, any further increase was found to be detrimental for the extraction process (the extent of vanillin extraction was about 605 ppm at 40±2°C and 674 ppm at 50±2°C). Thereby, the use of ultrasonic horn had a beneficial effect on the temperature factors i.e. use less energy consuming and had more advantage for extraction of heat sensible components.

![Figure 3. Effect of temperature on vanilla extraction using USAE horn method (1.5 g of cut and milled vanilla bean, with 40% ethanol concentration for 1h extraction at different temperature)](image)

**The Effect of Time on Extraction**

Ultrasound has the potential to be used in extraction processes to improve efficiency and to reduce processing time [11]. The result of this study has also demonstrated that the use of ultrasonic had a large benefit to decrease the extraction time of vanillin. The vanillin concentration released for 1 h was around 510 ppm (Figure 4) and no further change has been shown after this duration until 3h extraction time (around 550 ppm). Similar result for using ultrasound horn has been reported in the literature for extraction of vanillin using similar method USAE by Jadhav et al. [8]. Thus, it appeared that application of ultrasonic allowed vanillin to dissolve in the solvent thereby boosting yield with shorter time by disrupting the vanilla cell wall. Due to cavitation, the cracks were developed in the vanilla cell wall which increased permeability of tissues facilitating the entry of the solvent into the inner part of the material as well as washing out of the extracts. Also the turbulence and acoustic streaming of ultrasonic could significantly increase the solid liquid mass transfer coefficients due to micro scale effects in the system. The mechanical effects of ultrasound could also increase the contact surface area between solid and liquid phases due to possibility of size reduction in the solid matrix. The enhanced rates of mass transfer would mean that enhanced rate of the solvent being brought to the solid surface and also the transfer of the soluble constituents into the solvents was enhanced [24].

![Figure 4. Effect of time on vanilla extraction using USAE horn (1.5g of cut and milled vanilla bean, with 40% ethanol concentration for 1h extraction at room temperature for different extraction times)](image)

**The Effect of the Size of Cured Vanilla Bean on Extraction**

The size of cut beans affected analytical performance in the ultrasonic extraction solid liquid extraction of vanillin (Figure 5). If the samples were not homogenously distributed in the matrix i.e. the size of cut bean was large, grinding samples to a small size increased the homogeneity of the suspension, thereby increased the concentration of vanillin obtained [20]. The correlation of size of bean on vanillin recovery was quite significant with $R^2=0.9976$. When the size of cut bean was large (1cm) the vanillin concentration recovered was around 260ppm; on the other side, for small size cut and milled bean, the obtained vanillin was around 770ppm, and was almost 3 times of vanillin yielded for 1cm cut bean. Therefore use of ultrasonic horn to extract the vanillin from the vanilla bean was strongly correlated with the size of the cut bean ($R^2=0.9976$). This can be explained that there was a direct relation between total area of matrix and analyte extraction. This result was in good agreement with that observed by Walter et al. [23] for analyzing the factors affecting ultrasonic extraction.
The effect of different quantity of bean/solvent ratio 1g: 100mL to 10g: 100mL (w/v) at room temperature was also examined. This result showed (Figure 6) that the ultrasonic horn extraction was a convenient technique for the extraction of vanillin using liquid solvents and was proven for a fast and complete extraction process. This can be explained because of the surface area between the solid and liquid phase was significantly larger due to the cell disruption and particle dispersion [25]. The investigation (Figure 6) confirmed that the extraction yield increased with increasing of solvent/material ratio with $R^2=0.9774$. It can be explained the high power of ultrasonic forces provided the necessary energy for the complete extraction, therefore less solvents was needed and also able to extract high amounts of active compound (vanillin) within a short processing time.

**Summarization of the Optimal Parameters for Vanillin Extraction using USAE Horn Method**

The reproducibility of the measurements was assessed for vanillin extractions in triplicate at different parameters, which are summarized in Table 2. The reproducibility of these results was good, at between 2 and 15% relative error. This large range of degree error was because of the non-homogenous of vanilla seeds (in which the vanillin active compounds found) in the vanilla bean.

![Vanillin concentration vs. Size of cut bean](image1)

**Figure 5.** The effects of the size of the cut vanilla bean on the extraction of vanillin from vanilla bean using ultrasonic assisted horn (Different size of cut vanilla bean (1.5g) with 40% ethanol concentration for 1h extraction at room temperature)

![Vanillin concentration vs. Cured vanilla beans](image2)

**Figure 6.** The effects of the bean quantity-to-solvent ratio on the vanillin extracted using USAE horn method (Different amounts of cut and milled vanilla bean, with 40% ethanol concentration for 1h extraction at room temperature)

**CONCLUSION**

The study findings have revealed that the use of various extraction methods coupled with identifying the ideal combinations for different operating extraction parameters such as temperature, extraction time, solvent concentration and size of bean can significantly optimize vanillin extraction from cured vanilla beans. From the three extraction methods used, it was found out that vanillin extraction was effectively optimized in USAE horn method at 40% ethanol concentration for 1 hour and at ambient temperature of 30°C while for water bath extraction method, it was at 50% ethanol concentration for 15 hours and at 56°C. It can therefore be concluded that the use of USAE horn method at 40% ethanol concentration for 1 hour at ambient temperature of 30°C was the most effective extraction method in effectively optimizing vanillin extraction from cured vanilla beans. It was also observed that the size of the cut vanilla bean had the strong effect on the vanillin extracted using USAE horn, and the amount of vanillin yielded increased proportionally with the amount of vanilla bean in the mixture.

**Table 2. Summarization of optimal condition for USAE horn method**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Optimum condition</th>
<th>Average yield and ±standard deviation(ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (%)</td>
<td>40</td>
<td>574.9± 5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>30±2</td>
<td>683.9± 6</td>
</tr>
<tr>
<td>Time (h)</td>
<td>1</td>
<td>471.2± 9</td>
</tr>
<tr>
<td>Size of cut bean (mm)</td>
<td>2.5</td>
<td>770.8± 5</td>
</tr>
<tr>
<td>Sample-solvent ratio</td>
<td>Proportional</td>
<td>418.8(1g) to 3383.5(10g)</td>
</tr>
</tbody>
</table>

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**REFERENCES**
