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Quantification of Vanillin, Ethyl Vanillin and Methyl Vanillin Among Thai Rice Cultivars by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

**Jiraporn Burakorn*, Pran Pinthong, Yuttapoom Sampantharak,
Montakan Aimkaew and Kamonchanok Srithai**

*Department of Science Service, Ministry of Higher Education, Science, Research and Innovation,
Bangkok 10400, Thailand*

(*Corresponding author's e-mail: juntarama@yahoo.com)

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Abstract

A method was developed and validated for vanillin, ethyl vanillin, and methyl vanillin determination in Thai rice cultivars by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) under the selected reaction monitoring mode. In rice samples, the limit of detection was 13.3, 11.2 and 5.0 μg/kg for vanillin, ethyl vanillin, and methyl vanillin. A high correlation coefficient was obtained ($R^2 = 0.9999$, 0.9999 and 0.9998) within the linear range (0 - 300 μg/L) in the MS method. The recoveries ranging from 90.63 % ± 6.25 to 108.7 % ± 2.35 were obtained in different spiking concentrations. The average RSD% of intra-day for vanillin, ethyl vanillin, and methyl vanillin was 6.90, 5.13 and 2.16 %, and the inter-day were 6.72, 5.30 and 5.64 %, respectively. The developed method was applied to 24 commercial rice samples. Vanillin, were found in 24 samples ranging from 95.2 to 773.2 μg/kg. However, ethyl vanillin and methyl vanillin were not detected under LOD. The developed UPLC-MS/MS method for vanillin, ethyl vanillin and methyl vanillin determination include the merits of simple preparation steps and high sensitivity.

Keywords: Rice, Vanillin, Ethyl vanillin, Methyl vanillin, LC-MS/MS

Introduction

Rice is one of the most significant foods and high impact on economic of the world which 60 percentage of the world's population consume rice [1]. Thai cultivated rice in the main region and it considers the main food [2]. Thailand is one of the world's largest producers and exporters of rice, with the major rice growing areas located in the northeast region and in the lower north and central plains of the Chao Phraya River basin [24]. Rice samples can be removed the inedible outer hull, it includes the germ, bran and endosperm which called whole grain rice or brown rice base on the different pigmentations of the outer layer such as brown, black, purple, red etc. Brown rice and white rice have been associated with the phenomenon of polyphenols in its chemical composition. Phenolic acids are considered as an important group of the phenolic fraction of rice, including vanillic acid [1]. Vanillin is an aldehyde analogue which is also grouped within and relate to as phenolic acids [3]. Vanillin is a volatile compound and an important aroma in white and colored rice. The intensity of vanillin increases dramatically when rice is cooked [25].

Aroma intensities of rice are correlated with the mixture of aroma compounds it contains. 2-acetyl-1-pyrroline (2AP) has been described as a major aroma compound and as a characteristic compound in fragrant rice [4,5]. One hundred and forty volatile compounds were recognized of Thai local rice cultivars. Among the detected compounds, 18 volatile compounds, including vanillin contributed to the aroma intensities of both fragrant and non-fragrant rice [4]. The role of vanillin is a significant volatile compound in rice aroma and flavor besides 2AP [6]. The studies were reported that vanillin contribute to aroma intensities of rice [7-10]. Basmati, small-grained rice, cooked rice and in glutinous rice contain vanillin as an important flavor ingredient [11-13].

Analytical determination of the quantitative vanillin profile to define rice's nutritional and flavor qualities. The definition of the quantitative profile of vanillin in rice involves the separation, identification, and quantification of the analytes extracted in the previous steps. High performance liquid chromatography (HPLC) represents rice's most adopted analytical technique for analyzing vanillic acid and vanillic aldehyde or vanillin. Vanillic acid in white rice and brown rice were found at 0.02 and 0.06 mg/100 g, respectively using HPLC-DAD with C18 stationary phase and Trifluoroacetic acid in acetonitrile as mobile phase [14]. Commercial rice samples from Spain were found vanillin analyzed by HPLC-DAD [16]. Phenolic compounds in rice were identified [16]. This study adopted a normal-phase silica gel column to separate the ethyl acetate rice extract into sub-fractions. Then the sub-fractions were analyzed by NMR and ESI-MS spectroscopy. The results presented the identification of 10 compounds, including vanillic aldehyde. In addition, gas-chromatography (GC) can also be sometimes employed to investigate the quantitative profile of vanillin in rice.

HS-solid phase microextraction (SPME)/GC-FID technique was used for quantitative determination -Acetyl-1-pyrroline (2AP) and other aroma volatiles in aroma rice [6]. In this particular situation, Indian rice types such as Basmati, Ambemohar, Kolam, Indrayani, and local abundantly contributed to the variation in 2AP, hexanal, nonanal, decanal, benzyl alcohol, vanillin, guaiacol, and indole.

Vanillic acid, an oxidized form of vanillin in rice cultivars were analyzed by HPLC-PAD and LC/Q-TOF (quadrupole-time of flight) MS; however, sample preparation was complicated. Rice flour samples were extracted twice with methanol, combined, and concentrated to dryness using a rotary evaporator [15]. Even though vanillin derivatives were analyzed by HPLC and Q-TOF, but the application of advanced liquid chromatography-tandem mass spectrometry (LC-MS/MS) to residue analyses has allowed a broad range of compounds to be determined, allowing the comprehensive evaluation of complex samples, such as food [17]. This experiment aimed to determine the vanillin, ethyl vanillin, and methyl vanillin of white rice in Thailand by UPLC-MS/MS because its highly accurate, specific, and sensitive analytical method applicable to quantitative analysis of very small amounts of substances in food raw materials and simple sample preparation. In this sense, LC-MS/MS with a triple quadrupole in multiple reaction monitoring (MRM) modes has become the most generally adopted technique for quantitating residues in food [18].

Materials and methods

Rice samples

The cultivar rice grown by farmers of Khon Kaen province, Thailand, was used to develop the method. Twenty-four rice samples in Thailand's fields during 2022 were purchased from supermarkets in Bangkok, Thailand, to validate the method and quantify vanillin and its derivatives.

Chemicals and reagents

Analytical grade standards of Acetonitrile (J.T. Baker, New Jersey, USA), Formic acid (EMD Millipore Corporation, St. Louis Missouri, USA) were used for peak identification and quantification. Standard vanillin, methyl vanillin, were purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany) and ethyl vanillin was purchased from LGC (LGC, London, UK) Dilutions of standards were made in acetonitrile: water (70:30) (Sigma-Aldrich, Steinheim, Germany).

UPLC-MS/MS analysis

Identification of Vanillin, Ethyl vanillin and Methyl vanillin were done using ultra-high performance liquid chromatography-tandem mass spectrometry (Waters Acquity UPLC H-Class system, Massachusetts, USA). Chromatographic separation was performed on a UPLC-TQD C-18 column (ACQUITY® UPLC BEH C18 column, Waters, USA) (2.1×50 mm. i.d., 1.7 μm particle size) at room temperature; the mobile phase, at a flow rate of 0.3 mL/min, comprised a mixture of 2 solvents: A, 0.1 % formic acid aqueous solution, and B, Acetonitrile. The eluted was continuously monitored by Triple quadrupoles detector (TQD) (Waters, Massachusetts, USA). UPLC-MS/MS was performed with electrospray ionization (ESI) operating at positive ion mode. The tandem spectrometer was operated in the MRM mode. The optimized parameters were as follows; ion capillary temperature at 350 °C, ion spray voltage at 1,200 V. The quantification of each analyte was acquired with transitions of the protonated ion at m/z 153.03 → 92.88 (dwell time 0.052 s, cone voltage 31 V, and collision energy 18 eV) for vanillin, 166.89 → 92.86 (dwell time 0.052 s, cone voltage 17 V, and collision energy 13 eV) for ethyl vanillin, 166.87 → 138.38 (dwell time 0.052 s, cone voltage 17 V, and collision energy 11 eV) for methyl vanillin, respectively. Data acquisition was carried out using the MassLynx 4.1 software (Waters Acquity UPLC H-Class system, Massachusetts, USA).

Sample preparation [19]

Rice was ground using ultra-centrifugal mill. Rice powder was weighted 1 ± 0.01 g in centrifuged tube and then added acetonitrile and water (70:30 by volume) 10 mL. The mixed solution was put in an incubator shaker at 300 rpm for 20 min and mixed well in a stirring mixer. The mixed solution was mixed again using an ultrasonic bath for 15 min. After that, the mixed solution was centrifuged at 3,000 rpm for

10 min. The supernatant (10 mL) was filtrated through a 0.22 µm nylon filter prior to injection. A 2 µl aliquot of the sample was injected into the UPLC system.

Calibration procedure

Vanillin standards were diluted with acetonitrile: water (70:30) for prepared 7 calibration levels at 0, 5, 10, 20, 50, 100 and 300 µg/L. All samples were injected into UPLC-MS/MS in triplicate. The limits of detection (LODs) and the limits of quantification (LOQs) of the samples were determined by the calibration curves with regression coefficient, slope, and intercept. The standard curves were used to calculate sample concentrations by interpolating analyst peak area data.

Recovery, precision, accuracy and limits of quantification

The blank rice samples were added with the vanillin standard to final concentration at 5, 10, 50 and 300 µg/kg for determined recoveries, inter-day and intra-day precision which the samples were analyzed under same conditions in 6 replicate determinations (each concentration in triplicate) [20,22,23]. The intra-day variability of the samples was analyzed within 1 day. The inter-day precision and accuracy were investigated for 3 days. The percentage coefficient of variation (CV) in the peak areas at each concentration was calculated to the precision. The known amount of vanillin standards added to each sample relative to the analyst concentrations expressed the accuracy. The lowest concentration of the analyst samples was calculated as 3 times the signal-to-noise ratio, which was clearly detected as LOD.
The accuracy was shown as the percentage recovery [20].

$$\% \text{ Recovery } (\%R') = \frac{\bar{x}' - \bar{x}}{x_{\text{spike}}} \times 100$$

Compare the difference between mean spiked value $\bar{x}'$ and mean value $\bar{x}$ with the added concentration x_{spike}. Calculate the relative spike recovery *R'* (%) at the various concentrations.

Validation of method using Thai rice samples

The optimized conditions were validated using 24 Thai rice samples for vanillin, methyl vanillin, and ethyl vanillin.

Statistical analysis

All analyses were performed in triplicates and reported as means ± standard deviation (SD). SPSS software (Version 24.0) was used to analyze data with a one-way analysis of variance (ANOVA) test. The significant differences among the 24 rice samples were analyzed by ANOVA ($p \leq 0.05$).

Results and discussion

UPLC method validation

The proposed method was validated for linearity, accuracy, precision, and limits of detection and quantification. Method validation was performed according to the guidelines for validating quantitative and qualitative test methods by the [20-23].

Linearity

The standard solution at 7 different concentrations: 0, 5, 10, 20, 50, 100 and 300 (µg/L), and the integrated peak areas were plotted calibration graphs based on linear regression analysis. **Figure 1** shows the correlation coefficients (R^2) obtained from the standard curves for vanillin (0.9999), methyl vanillin (0.9998), and ethyl vanillin (0.9999). There was an intense linear correlation between the concentrations of the compounds with the peak areas.

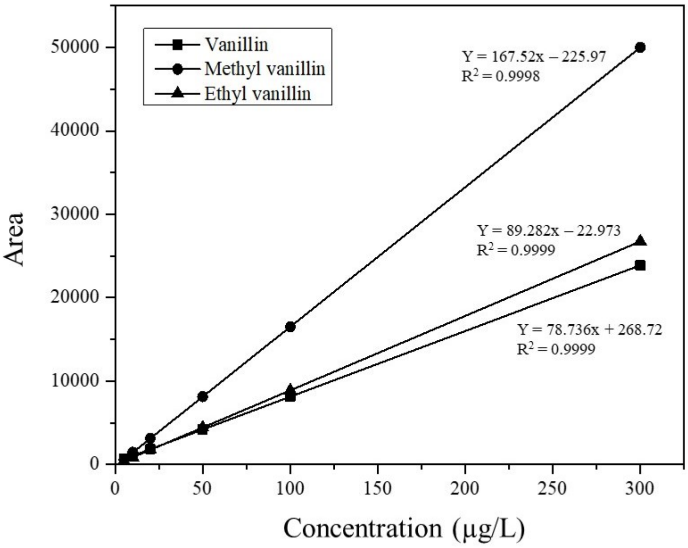

Figure 1 Standard curve of vanillin, ethyl vanillin and methyl vanillin.

Precision

Three different concentrations of vanillin, methyl vanillin, and ethyl vanillin (100, 500 and 3,000 µg/kg) were analyzed in 6 independent series in triplicate during the same day (Intra-day precision) and over 3 consecutive days (Inter-day precision) [20,22,23]. A measure of precision was the relative standard deviation (RSD), as shown in **Table 1**. The method for determining all of the analyzed compounds was a high degree of precision.

Limits of detection and quantification

The signal-to-noise ratio was adopted to estimate the limit of detection (LOD) and the limit of quantification (LOQ). The lowest concentration resulting in a peak area of 3 times and baseline noise, was the LOD. The term of the lowest concentration that provides a signal-to-noise ratio higher than 10 was LOQ. The limit of detection and quantification observed for vanillin, ethyl vanillin, and methyl vanillin are 44.2, 37.3 and 16.6 µg/kg respectively (**Table 1**). Sudtasarn *et al.*, (2022) reported that LOQ of vanillin, ethyl vanillin and methyl vanillin in rice were 40, 5 and 5 µg/kg respectively when using LC-MS/MS. However, the data was not showed the regression equation, linearity range, LOD, RSD%, Intra-day RSD% and Inter-day RSD% [27].

Accuracy

The accuracy of the method was evaluated as the recovery (R) [20,22,23]. The analyzed compound at known amounts of 100, 500 and 3,000 µg/kg were injected under identical conditions in 6 replicates determinations [26]. The R (%) value was calculated [20]. The proposed afforded a recovery of 90.63%±6.25 for vanillin based on ANOVA analysis by SPSS software (**Table 1**).

Table 1 Regression equations, linearity ranges, correlation coefficients, limits of detection (LOD), limits of quantification (LOQ), recoveries, and relative standard deviations (RSD) for vanillin, methyl vanillin, and ethyl vanillin determination.

Com pounds	Regression equation	Linearity range (µg/L)	Correla tion coeffi cient (R^2)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (Mean%±SD)	Intra-day RSD/% (*n* = 6)	Inter-day RSD/% (*n* = 6)
Vanillin	y = 78.736x + 268.72	0 - 300	0.9999	13.3	44.2	90.63 ± 6.25	6.90	6.72
Methyl vanillin	y = 167.52x - 225.97	0 - 300	0.9998	5.0	16.6	108.7 ± 2.35	2.16	5.64
Ethyl vanillin	y = 89.282x - 22.973	0 - 300	0.9999	11.2	37.3	102.7 ± 5.27	5.13	5.30

Determination of vanillin, methyl vanillin, and ethyl vanillin in Thailand rice samples

In order to evaluate whether the method is suitable for use as a routine analytical tool, vanillin, methyl vanillin, and ethyl vanillin were determined in 24 Thailand rice samples, and the results are given in **Table 2**. The analyses were performed in triplicate. An accurate, precise, and sensitive method was developed for the simultaneous detection, quantification, and confirmation of vanillin, ethyl vanillin, and methyl vanillin in rice using UPLC-MS/MS. The validation results have shown excellent recoveries R% (90.63 % ± 6.25 - 108.7 % ± 2.35) and precision (RSD%) of intra-day and inter-day (2.16 - 6.90 %) for all samples studied. The method was applied successfully for the analysis of 24 real samples. Twenty-four samples were found to be vanillin. However, ethyl vanillin and methyl vanillin were not found in all rice samples that closely relate to the results of Sudtasarn *et al.* (2022) which found vanillin in rice samples but not detected ethyl vanillin and methyl vanillin [27]. The vanillin in rice have been reported lower 40.00 - 378.90 µg/kg [27]. Sansenya and Wechakorn (2020) reported rice from Phetchabun province, Thailand found vanillin (Peak area 0.4 %) using GC-MS [25]. This research was found vanillin in rice 95.2 - 773.2 µg/kg. The identity of vanillin in Thai rice presents more aroma when cooked. However, further research should analyze more rice samples for covering a wide range of rice. In addition, vanillin was found in different milling fractions of rice bran from black rice (3.12 - 22.31 µg/g) [28]. Chu *et al.* (2020) reported vanillin content changed in Chinese wild rice at different germination stages (1.31 - 4.82 µg/g) [29]. The MRM chromatogram of vanillin, ethyl vanillin, and methyl vanillin in the standard solution and rice brand 9 sample was shown in **Figure 2**.

Table 2 Quantification of vanillin, methyl vanillin, and ethyl vanillin from Thailand rice samples.

No.	Types/brands	Vanillin (µg/kg) (mean±SD)	Methyl vanillin (µg/kg) (mean±SD)	Ethyl vanillin (µg/kg) (mean±SD)	Uncertainty (µg/kg)
1	Thai Jasmine Rice Brand Hong Thong	382.0 ± 20.7^{g}	nd		± 12.7
2	Thai Jasmine Rice Brand TraChat	325.7 ± 13.3^{i}	nd		± 10.7
3	Thai Jasmine Rice Brand Mah Boon Krong	317.3 ± 14.5^{i}	nd		± 10.5
4	Thai Jasmine Rice Brand Tra Chat Thong	352.7 ± 10.9^{h}	nd		± 11.5
5	Thai Jasmine Rice Brand Silver Pin	508.9 ± 22.7^{e}	nd		± 16.8
6	Thai Jasmine Rice Brand Sandee	522.2 ± 25.9^{de}	nd		± 17.3
7	Thai Jasmine Rice Brand Hong Thong	276.7 ± 26.1^{j}	nd		± 9.76
8	Thai Jasmine Rice Brand Tra Chat Life	539.0 ± 10.0^{d}	nd		± 17.4
9	White Rice	773.2 ± 27.2^{a}	nd		± 25.3
10	Thai Jasmine Rice	286.6 ± 05.3^{j}	nd		± 9.28
11	Thai Jasmine Rice Brand Takhli	271.5 ± 16.1^{j}	nd		± 9.08
12	Sao Hai Rice Brand 1	355.8 ± 12.7^{gh}	nd		± 11.6
13	Sao Hai Rice Brand 2	654.9 ± 07.3^{c}	nd		± 21.2
14	Khao Tah Haeng Rice	164.4 ± 13.5^{l}	nd		± 5.68
15	Thai Yellow Rice	720.3 ± 17.5^{b}	nd		± 23.4
16	Thai Pathumthani fragrant Rice	413.6 ± 05.2^{f}	nd		± 13.4
17	Thai Suphanburi fragrant Rice	369.5 ± 23.2^{gh}	nd		± 12.4
18	Tubtim Chumphae Rice Brand 1	411.5 ± 15.2^{f}	nd		± 13.5
19	Tubtim Chumphae Rice Brand 2	149.0 ± 12.7^{l}	nd		± 5.17

No.	**Types/brands**	**Vanillin (µg/kg) (mean±SD)**	**Methyl vanillin (µg/kg) (mean±SD)**	**Ethyl vanillin (µg/kg) (mean±SD)**	**Uncertainty (µg/kg)**
20	Sung Yod Rice	95.2 ± 14.4^{m}	nd		± 3.80
21	Rice Berry Brand 1	231.5 ± 04.9^{j}	nd		± 7.50
22	Rice Berry Brand 2	210.0 ± 13.7^{k}	nd		± 7.07
23	Glutinous Rice Brand 1	383.7 ± 8.1^{g}	nd		± 12.4
24	Glutinous Rice Brand 2	158.5 ± 8.4^{l}	nd		± 5.26
	Max	773.2	nd		
	Min	95.2	nd		
	Mean	369.7	nd		
	SD	177.9	nd		

Value with the different letter in each rice type for vanillin, ethyl vanillin and methyl vanillin are significantly different at $p \leq 0.05$; *nd* not detected

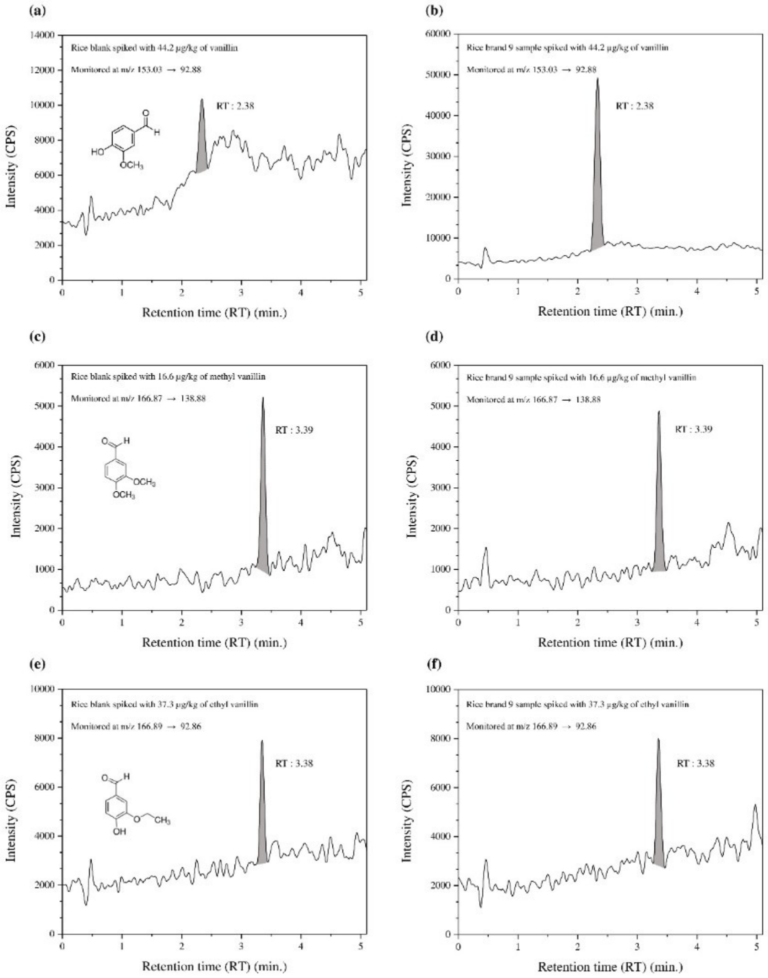

Figure 2 Chromatogram of rice samples spiked with vanillin, methyl vanillin, and ethyl vanillin at the LOQ levels; (a) rice blank sample spiked with vanillin at 44.2 µg/kg (b) rice brand 9 sample spiked with vanillin at 44.2 µg/kg (c) rice blank sample spiked with methyl vanillin at 16.6 µg/kg (d) rice brand 9 sample spiked with methyl vanillin at 16.6 µg/kg (e) rice blank sample spiked with ethyl vanillin at 37.3 µg/kg, and (f) rice brand 9 sample spiked with ethyl vanillin at 37.3 µg/kg.

Conclusions

This research developed and validated methods for determining vanillin, ethyl vanillin, and methyl vanillin in Thai rice cultivars by UPLC-MS/MS. The methods were high accuracy and precision and the detection limit was a high correlation coefficient ($R^2 = 0.9999$, 0.9999 and 0.9998) within the linear range (0 - 300 μg/L) in the MS method. The recoveries ranging was obtained from 90.63 % ± 6.25 to 108.7 % ± 2.35 in different spiking concentrations. The average RSD% of intra-day for vanillin and its derivatives was from 2.16 to 6.90 %, and the inter-day was 5.30 - 6.72 %. The developed methods were applied to 24 commercial rice samples in which vanillin was found in 24 samples but not detected ethyl vanillin and methyl vanillin.

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