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Identification and Quantitation of the Rice Aroma Compound, 2-Acetyl-1-pyrroline, in Bread Flowers (*Vallaris glabra* Ktze)

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The aroma impact compound, 2-acetyl-1-pyrroline (2AP), has been identified for the first time in headspace of fresh bread flowers (*Vallaris glabra* Ktze) in which volatile components were extracted by solid-phase microextraction (SPME) at room temperature prior to analysis by gas chromatography−mass spectrometry. A total of 50 volatiles were detected. Among these, 23 volatiles were identified, predominantly in a group of terpenes. More volatiles were found in the extract of fresh bread flowers obtained by continuous steam distillation and solvent extraction (SDS). Of the 40 volatiles identified, the additional components were mainly straight-chain saturated hydrocarbons. 2AP was found in the extracts obtained by both SPME (0.37%) and SDS (2.71% relative proportion). Quantitative analyses of 2AP in bread flowers and other plant materials were performed by solvent extraction employing acidic solutions and capillary GC with flame ionization detection. The highest concentration of 2AP was found in dried flowers of *V. glabra* at 26.1 mg/kg. By comparison with other plant sources, fresh leaves of *Pandanus amaryllifolius* Roxb contain 2AP at 10.3 mg/kg and Thai fragrant rice, Khao Dawk Mali 105, at 3.0 mg/kg.

KEYWORDS: 2-Acetyl-1-pyrroline; solid-phase microextraction; SPME; bread flowers; *Vallaris glabra*

INTRODUCTION

Vallaris glabra Ktze is a climbing type of fragrant plant with blade broadly elliptic leaves of 4−6 × 7−9 cm in size and inflorescence of long-stalked cuplike white flowers of 1−1.5 cm in diameter. The flowers have a common name, "bread flowers". The plant is well known to the natives of Thailand because of a pleasant smell continually liberated from its flowers during day and night. Natives of the nearby countries recognize this pleasant smell as pandan-like aroma. In the central part of Thailand, these flowers have a vernacular name, "dok khao mai", meaning "flowers that having scent of newly cooked fragrant rice", an odor familiar and very agreeable to those who consume Thai fragrant rice, mainly Khao Dawk Mali 105 (KDML 105). It is therefore of great interest to determine whether the compounds responsible for the scent of *V. glabra* share their identities with the aroma compounds of both pandan leaves and fragrant rice.

A five-membered N-heterocyclic ring compound, 2-acetyl-1-pyrroline (2AP), was identified for the first time as the important aroma component of cooked rice (*1*) and the volatile oil of freeze-dried pandan leaves (*Pandanus amaryllifolius* Roxb.) (*2*). Having the lowest odor threshold value, 0.1 nL/L of water, of all identified cooked rice volatiles (*3*), the compound has gained increasing interest among food aroma researchers. Apart from being identified and quantified in various rice varieties, both nonfragrant and fragrant rices (*4*−*10*), there have been a number of reports on identification of this key aroma compound in many types of processed and cooked food, e.g., canned sweet corn (*11*), toasted wheat bread (*12*), moderately roasted sesame (*13*), baguette crusts (*14*), cooked tail meat of freshwater crayfish (*Procambarus clarkii*) (*15*), boiled potatoes (*16*), roasted seeds of wild mango (*Irvingia gabonensis*) (*17*), enzyme-hydrolyzed oyster cooker effluent (*18*), Italian-type salami and Parma ham (*19*), cooked tail meat of the American lobster (*Homarus americanus*) (*20*), and heat-treated nonfat dry milks (*21*).

The aroma character of 2AP was also found to play a role in the odor of cooked blue crab (*Callinectes sapidus*) claw meat (*22*), maize flour extrudates (*23*), taro (*Colocasia esculenta* (L.) Schott) volatiles (*24*), and Iberian dry-cured ham (*25*). In addition, it was also traced in the extracts of boiled carp fillet (*Cyprinus carpio* L.) (*26*), two strains of mung bean in Bengal (*27*), and cooked acha (*Digitaria exilis* Stapf) (*28*).

According to the suggestions made by most of the previous reports, 2AP was presumed to be a Maillard reaction product because of its occurrence after boiling or cooking of materials. The isolation methods used in the previous reports were mainly under thermal treatments such as continuous steam distillation and solvent extraction (SDS). However, a recent report by our group (*29*) on the quantification of 2AP in uncooked Thai fragrant rice, KDML 105, revealed its presence as a naturally occurring compound in solvent extracts at room temperature. In this present work, flowers and leaves of *V. glabra* were studied as another potential natural source of 2AP. Solid-phase microextraction (SPME), a mild extraction method, was em-

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ployed prior to analysis by gas chromatography−mass spectrometry (GC-MS) in order to characterize the headspace volatiles. Additionally, the concentration of 2AP in the flowers of *V. glabra* was determined in comparison to that in other known sources, *P. amaryllifolius* leaves and KDML 105 rice seeds. The quantitation was accomplished by solvent extraction of 2AP and subsequent analysis by capillary GC using a flame ionization detector (FID).

MATERIALS AND METHODS

Plant Materials. *V. glabra*, grown in the Department of Biology, Faculty of Science, Chiang Mai University, located in the northern part of Thailand, was used as plant material source. Its flowers and leaves were collected early in the morning on the day of experiment in March 2002. Both *P. amaryllifolius* leaves and KDML 105 rice were obtained from the Department of Agronomy, Faculty of Agriculture, Chiang Mai University. Fresh leaves of *P. amaryllifolius* were cut early in the morning on the day of experiment. The dried plant materials were obtained by air-drying at 30 °C until a constant weight was achieved. KDML 105 rice harvested in November 2001 was air-dried at 30 °C until the moisture content came down to 14.5%. The seeds were kept at −4 °C for 2 months, and then they were hulled by hand no more than 24 h before the experiment.

Chemicals. 2AP was synthesized as outlined by Buttery et al. (*2*), but it is known to contain many other products. Purification of the synthetic 2AP was accomplished by utilizing a gas chromatographic column packed with 3% dimethylpolysiloxane coated on 80/100 mesh solid supports. The principal peak for 2AP emerging from the GC detector outlet was collected in 3-mm-o.d. Pyrex tubes, sealed under vacuum, and stored at −20 °C before being subjected to examination by capillary GC-MS and infrared spectroscopy (IR). Standard solutions of 2AP were made by dissolving a known weight of the purified 2AP in a precisely measured volume of 0.1 M HCl. An exact weight of the internal standard, 2,4,6-trimethylpyridine (TMP), 99% purity (Aldrich Chemical Co., Milwaukee, WI), was dissolved in a precisely measured volume of 0.1 M HCl to give an internal standard solution with a 0.25 mg/L concentration of TMP.

Extraction of Volatiles. *Steam Distillation.* Fifty grams of fresh bread flowers without stalks and sepals was placed into a 500-mL two-neck round-bottom flask of a direct simple steam distillation apparatus containing 250 mL of distilled water. After heating, the temperature of the mixture was increased to and maintained at 98 °C. The vapor containing extracted volatiles from the flowers was then conveyed to a condenser and was condensed and accumulated at the bottom of a receiving flask placed in an ice−water bath until the distillate volume reached 100 mL. It was then transferred into a 500-mL separatory funnel and extracted twice, each time using 150 mL of dichloromethane. The organic layers were combined and concentrated to ∼50 mL using a rotary evaporator under reduced pressure at a temperature of 26 °C. After drying with anhydrous sodium sulfate, the extract was further concentrated to a volume of 1 mL. The concentrated extract was left uncovered at room temperature to allow the remaining solvent to evaporate more until the total volume decreased to 0.2 mL. It was then subjected to GC-MS analysis.

SPME. Ten fresh bread flowers without stalks and sepals, weighing approximately 1.2 g, were sealed in a 27-mL bottle fitted with a PTFE/silicone septum (Restek Corp., Bellefonte, PA) and an aluminum cap. The sample bottle was left at room temperature (27 °C) for 30 min. A SPME fiber (Supelco, Bellefonte, PA) of 1 cm in length, coated with poly(dimethylsiloxane) (PDMS) at 100 μm thickness, was mounted in the manual SPME holder (Supelco) and was preconditioned in a GC injection port set at 250 °C for 1 h. By insertion through the septum of the sample bottle, the fiber was then exposed to the sample headspace for 10 min prior to desorption of the volatiles into the splitless injection port of the GC-MS instrument.

GC-MS. A gas chromatograph−mass spectrometer (Agilent 6890 and HP 5973 mass-selective detector, Agilent Technologies, Palo Alto, CA) equipped with a fused silica capillary column, HP-1MS, with dimethylpolysiloxane as nonpolar stationary phase (30 m × 0.25 mm i.d. × 0.25 μm) (Agilent Technologies) was utilized for analysis of volatiles obtained from distillation and SPME of bread flowers. For the steam distillation extract, the sample was injected with a split ratio of 20:1. The injection port temperature was 250 °C. The column temperature program started at 35 °C upon injection. The temperature was increased at a rate of 2 °C/min to 100 °C, and then at a rate of 5 °C/min to 230 °C, and held there for 2 min. When volatiles adsorbed on the SPME fiber were to be analyzed, the injection port was set to splitless mode. The initial column temperature was set at 35 °C. It was first ramped at a rate of 2 °C/min to 50 °C, then at a rate of 3 °C/min to 100 °C, and finally at a rate of 5 °C/min to 180 °C. Purified helium gas at a flow rate of 1 mL/min was used as the GC carrier gas. The mass spectrometer was operated in the electron impact (EI) mode with an electron energy of 70 eV; ion source temperature, 230 °C; quadrupole temperature, 150 °C; mass range *m*/*z* 29−550; scan rate, 0.68 s/scan; EM voltage, 1423 V. The GC-MS transfer line was set to 280 °C.

GC-MS Data Analysis. Identification of volatile components in both steam distillate extract and SPME was performed by matching their mass spectra with reference spectra in the Wiley 275 Mass Spectral Library (Revision C.00.00) and the NIST 98 Mass Spectral Library (Revision D.01.00/1.6d), both purchased from Agilent Technologies. In addition, published Kováts indices (*30*−*32*) and retention times of known standards, for some available compounds, were used to aid structural confirmation. Quantitative analysis of each volatile component in percent was performed by peak area normalization measurements.

Quantitative Analysis. *Solvent Extraction.* Five (5.00)-gram samples of fresh bread flowers without stalks and sepals, finely chopped fresh leaves, and ground rice seeds were weighed. One (1.00)-gram samples of dried bread flowers and finely chopped fresh pandan leaves were weighed. Four replications were carried out. The samples were then added to 125-mL flasks containing 50 mL of 0.25 mg/L TMP internal standard solution. The mixture was stirred for 30 min before filtration. Thirty milliliters of the filtrate was transferred to a 125-mL pear-shaped separatory funnel. This was followed by the addition of approximately 1.2 mL of 5.0 M NaOH to make the solution slightly basic, and then 50 mL of dichloromethane was immediately added as an organic solvent. The extraction was done twice, and 90.0-mL portions of the combined dichloromethane solution were used. After being dried with anhydrous sodium sulfate, the extract was concentrated to 1 mL using a rotary evaporator under reduced pressure and a temperature of 26 °C. The concentrated extract was transferred to a V-shaped vial and left open to the air at room temperature until its volume decreased to 0.1 mL before it was subjected to quantitative analysis by capillary GC with a FID. The whole experimental process was repeated for the extraction and analysis of standard 2AP of known amounts in a dilution series in order to obtain a standard calibration curve.

GC Conditions. GC analyses were performed on a Agilent 6890 GC equipped with a Agilent 7683 injector and a FID (Agilent Technologies). A fused silica capillary column HP-5MS, biphenyldimethylpolysiloxane, with dimension 30 m × 0.25 mm i.d. and 0.25 μm film thickness (Agilent Technologies) was programmed starting at 45 °C. The temperature was ramped to 120 °C at a rate of 5 °C/min, resulting in an overall separation time of 15 min. The GC injector was in a split mode with a split ratio of 10:1. The injector temperature was set at 250 °C. Purified helium gas at a flow rate of 1 mL/min was used as the GC carrier gas.

RESULTS AND DISCUSSION

The contribution of 2AP to the aroma of flowers has not been investigated previously, as most of the past studies were directed toward identification of this compound in foodstuffs. Because *V. glabra* flowers possess a strong characteristic odor similar to that of pandan leaves and fragrant rices, the flowers were thought to contain a certain amount of 2AP. SPME was the method of choice in the present work for qualitative analysis of 2AP in the sample headspace due to its simplicity and the fact that additional chemical treatment was not required, thus reducing unwanted chemical reactions. The technique had

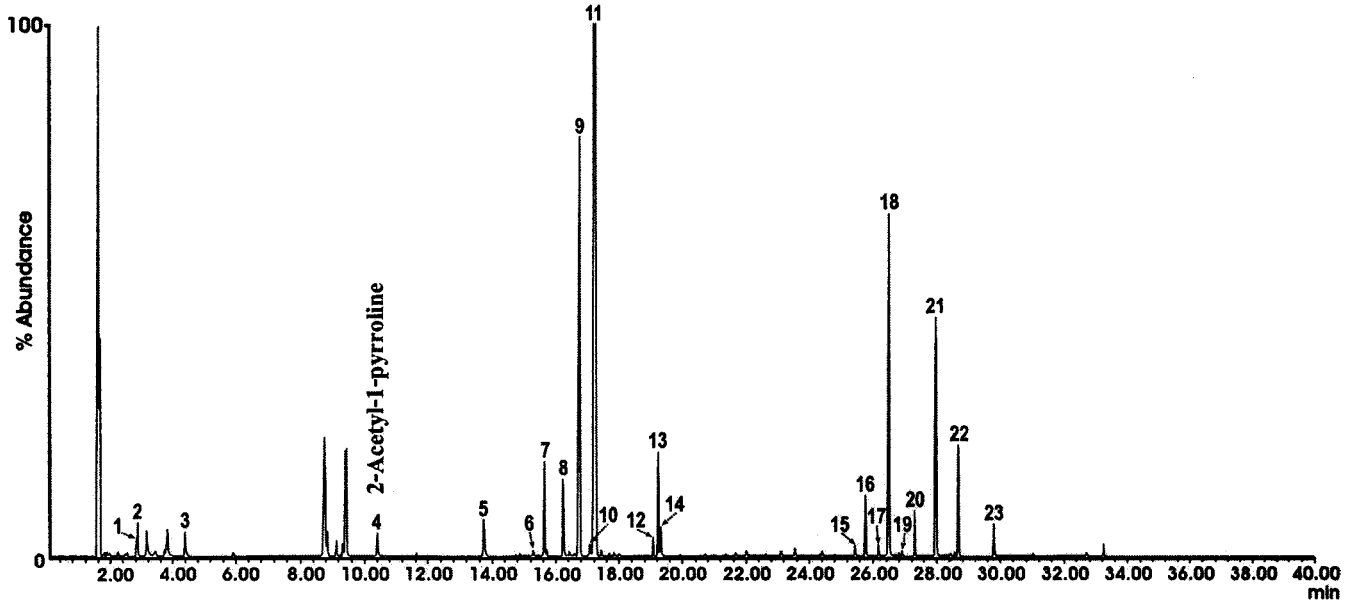

Figure 1. GC-MS total ion chromatogram of fresh bread flower volatiles extracted from the sample headspace by SPME using a PDMS fiber. The numbers refer the compounds listed in **Table 1**.

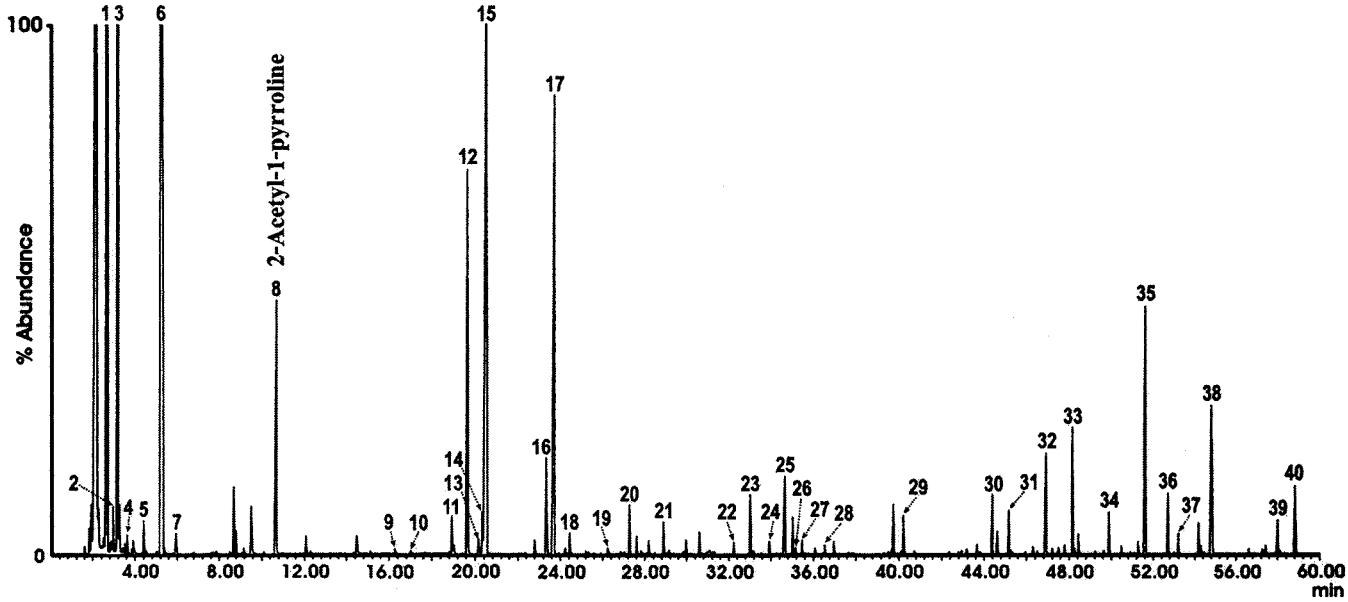

Figure 2. GC-MS total ion chromatogram of fresh bread flower volatiles extracted by SDS. The numbers refer the compounds listed in **Table 1**.

successfully been applied for screening of 2AP in the headspace of milled and brown Jasmine rice samples (*33*). It was noted that, because of the relatively low recovery of 2AP (<0.3%), heating of the rice samples to produce sufficient amounts of 2AP was performed at 80 °C.

In our study, to assess the natural occurrence of 2AP in the plant materials, the flowers were kept at room temperature (27 °C). The optimum extraction time was 10 min. Separation of more than 50 volatile components resulted, and a representative GC-MS total ion chromatogram (TIC) is shown in **Figure 1** for SPME. Among these, 23 volatiles were identified, constituting 91.57% of the total volatiles (**Table 1**). The majority of volatiles identified were in a group of monoterpenes and sesquiterpenes. The acyclic monoterpene alcohol, linalool, was found to be a principal constituent, accounting for 62.24% of the amount of all identified terpenes. The rest were present as minor constituents, identified as follows: monoterpenes *trans*-linalool oxide (7.21%), *cis*-linalool oxide (1.23%), epoxylinalol (1.66% and 0.26%), *trans*-β-ocimene (1.46%), β-myrcene (0.54%), and *cis*-β-ocimene (0.05%); sesquiterpenes *trans*-β-caryophyllene (5.99%), germacrene-D (4.03%), β-elemene (1.02%), α-humulene (0.77%), α-copaene (0.14%), and *trans*-β-bergamotene (0.07)%. 2AP was unambiguously identified (peak no. 4) with 0.38% relative concentration. Being a character impact compound with a relatively low odor threshold value, 0.1 nL/L of water (*3*), the contribution of 2AP to the aroma quality of *V. glabra* flowers is considered to be significant.

A comparative study was carried out with the volatile constituents of fresh bread flowers obtained by steam distillation and solvent extraction (SDS). Forty volatiles were identified out of at least 80 components shown in the TIC in **Figure 2**. 2AP was also found in the SDS sample in higher concentration (2.71%). In total, only 13 volatiles were identified in both SPME and SDS samples, which were mainly the above mono- and sesquiterpenes. Linalool, the main terpenic constituent of the SPME sample, was also presented in the SDS sample. Some acyclic monoterpenes, hydrocarbons β-myrcene, *cis*-β-ocimene, and *trans*-β-ocimene, were not present in the TIC of the SDS

Table 1. The Identified Volatile Components of Fresh Bread Flowers Extracted by SPME and SDS

compound[a]	KI[b]	peak no.[c] SPME	peak no.[c] DIST	% RA[d] SPME	% RA[d] DIST
ethyl acetate[1,2,3]	589		1		16.02
3-methyl-1,3-pentadiene[1]	606	1		0.24	
4-methyl-1,3-pentadiene[1]	609	2		0.37	
3-methylbutanal[1,3]	615		2		0.22
benzene[1,3]	629		3		5.56
3-hydroxy-2-butanone (acetoin)[1]	661		4		0.12
3-methyl-1-butanol[1,3]	744	3	5	0.58	2.04
methylbenzene[1,3]	776		6		31.42
hexanal[1]	919		7		0.16
2-acetyl-1-pyrroline[1,3]	930	4	8	0.38	2.71
β-myrcene[1,2,3]	992	5		0.54	
cis-β-ocimene[1,2]	1022	6		0.05	
trans-β-ocimene[1,2]	1030	7		1.46	
benzyl alcohol[1]	1033		9		0.06
limonene[1,2,3]	1039		10		0.04
cis-linalool oxide[1,2]	1071	8	11	1.23	0.42
trans-linalool oxide[1,2]	1082	9	12	7.21	3.87
benzeneethanol[1]	1091	10	13	0.14	0.20
nonanal[1]	1094		14		0.46
linalool[1,2,3]	1095	11	15	62.24	10.32
epoxylinalol[1]	1131	12	16	0.26	1.19
epoxylinalol[1]	1136	13	17	1.66	6.72
naphthalene[1]	1151	14		0.47	
α-terpineol[1,2]	1208		18		0.23
nerol[1,2]	1239		19		0.05
geraniol[1,2]	1262		20		0.48
4-vinyl-2-methoxyphenol[1]	1295		21		0.32
α-copaene[1,2]	1361	15		0.14	
β-elemene[1,2]	1399	16	22	1.02	0.10
tetradecane[1]		17		0.16	
trans-β-caryophyllene[1,2,3]	1445	18	23	5.99	0.54
trans-β-bergamotene[1]	1457	19		0.07	
α-humulene[1,2,3]	1469	20	24	0.77	0.11
germacrene-D[1,2]	1479	21	25	4.03	0.70
2,4-bis(1,1-dimethylethyl)-phenol[1]	1492		26		0.06
pentadecane[1,3]		22	27	1.80	0.12
γ-elemene[1]			28		0.07
ethyl phthalate[1]		23		0.76	
heptadecane[1,3]			29		0.33
nonadecane[1,3]			30		0.52
hexadecanoic acid[1,3]			31		0.43
(*Z*,*Z*)-3,6-*cis*-9,10-epoxy-nonadecadiene[1]			32		0.90
heneicosane[1]			33		1.10
docosane[1]			34		0.35
tricosane[1]			35		2.14
hexanedioic acid, dioctyl ester[1]			36		0.54
tetracosane[1]			37		0.19
pentacosane[1]			38		1.67
hexacosane[1]			39		0.07
heptacosane[1]			40		0.98
total				91.57	93.53

[a] Identification: 1, mass spectrum (tentative); 2, Kováts indices; and 3, standard compound. [b] Kováts indices using a nonpolar dimethyl polysiloxane column. [c] Numbers correspond to those labeled on the total ion chromatogram obtained by GC-MS of each extraction method. [d] Relative areas presented in percentage of the total peak areas.

sample, which is commonly known to result from their relative instabilities. Some monoterpene alcohols, found only in the TIC of the SDS sample as minor constituents (nerol, geraniol, and α-terpineol), are isomerization products of linalool developed during the steam distillation process.

Apart from terpenic constituents, other functionalities were present mainly in the TIC of the SDS sample, e.g., ethyl acetate, 3-hydroxy-2-butanone (acetoin), methylbenzene, and a significant number of straight-chain saturated hydrocarbons. These hydrocarbons occur abundantly, are generally found in plant materials, and may be utilized as waxy coatings on flowers. However, they are known to make limited contributions to the scent of the flowers. Ethyl acetate, the most common ester in fruits, and acetoin both occur widely in plants having fruity and buttery odors, respectively (*34*).

Furthermore, a volatile component tentatively identified as an isomer of 2AP in rice extract (*35*) was also found in both SPME and SDS sample. This component eluted from the chromatographic column just after 2AP. It was present in the TIC of SPME and SDS sample at retention times of 11.63 and 12.09 min, respectively, as the temperature programs for GC separation in both samples were different. Its mass spectrum possessed the same molecular ion as 2AP at *m*/*e* 111(35), and other major ions included 83(35), 55(100), 42(85), 41(31), and 39(17). In comparison with the six-membered ring analogue of 2AP, of which its enamino tautomer always occurs in observable amounts (*36*), this tentatively assigned isomer of 2AP would likely be an enamino tautomer of 2AP. However, its complete identification and role in aroma of any foodstuffs or plant materials have not yet been confirmed.

Table 2. Quantitative Results Obtained by Capillary GC for the Aroma Compound, 2AP, in Some Plant Materials

sample of plant materials	concn of 2AP (mg/kg)
V. glabra flowers, fresh	3.36[a]
V. glabra flowers, dry	26.12
V. glabra leaves, fresh	0.53[a]
P. amaryllifolius leaves, fresh	10.26[a]
KDML 105 brown rice seeds	3.00

[a] Based on wet weight of the plant materials.

Quantification of 2AP in bread flowers and leaves, pandan leaves, and fragrant rice was carried out using the method previously reported by our group (*29*). This method employed solvent extraction of 2AP by acidic solution (0.1 M HCl) and re-extraction into organic solvent prior to analysis with GC-FID. The whole extraction process was performed at room temperature to minimize unwanted reactions of organic components, which may result in degradation or formation of compounds, especially the target 2AP. The internal standard method using TMP was employed. Determination of 2AP in the plant extracts was performed by means of a standard calibration curve plotted as a correlation of the peak area ratios between the standard (2AP) and the internal standard (TMP), and concentrations of the standard 2AP in the dilution series, ranging between 0.62 and 5.00 mg/L. A linear calibration curve resulted, with a regression coefficient of 0.9997. Because of the basic nature of 2AP, when it is subjected to extraction by acidic solvent, it is assumed that almost all of the 2AP is extracted from the sample. Performing the extraction process a second time allowed recovery of the total amount of 2AP from each sample. It was found that further extraction of the sample that had been extracted twice gave an undetectable signal of 2AP. Accordingly, the plant extracts were presumed to contain virtually all of the 2AP.

The results of quantitation studies by capillary GC are listed in **Table 2**. *V. glabra* flowers were found to contain the highest amount of 2AP of all foodstuffs and natural sources that have ever been reported. It is also interesting that the *V. glabra* leaves were found to contain an amount of 2AP close to that of some milled fragrant rice seeds reported elsewhere (*4*, *5*, *29*, *35*, *37*,

38). The 2AP concentration in the KDML 105 brown rice seeds used in this experiment is much higher than those reported by our group using the same method of quantitative analysis (*29*). The variation of concentrations of this key aroma compound within the same rice variety is quite significant and may be due to various factors, such as harvest site, postharvest treatment, method of storage, storage time, and degree of milling.

Other researchers have attributed the presence of 2AP to cooking or heat-processing steps. The present work shows definitively that 2AP is present in the raw plant materials, and it will be important to examine the enzymatic reactions in its biosynthesis to fully understand the implications. Also, the biological formation of 2AP in *V. glabra* flowers in a very high concentration suggests the possibility of utilizing these flowers as another natural source for a food-flavoring additive.

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