Original paper

Characterization of Potent Odorants in Three Different Cultivars (Madagascar, Comoro and Tahiti) of Vanilla Bean by Aroma Extract Dilution Analysis (AEDA)

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The aroma concentrates of three different cultivars of vanilla bean were prepared by combining the solvent extraction and solvent assisted flavor evaporation (SAFE) techniques. Aroma extract dilution analysis (AEDA) of the volatile fraction revealed 36 odorants, including nine newly identified vanilla odorants, which were identified or tentatively identified from the 49 odor-active peaks with FD factors between 4¹ and 4⁹. Based on these results, it was proposed that the potent odorants, except for vanillin, were able to influence the characteristic flavor of vanilla beans from different growing regions.

Keywords: vanilla, flavor, aroma, cultivar, AEDA

Introduction

Vanilla beans are used as an important raw material for the flavoring of various kinds of foods and fragrances. The pods are harvested from the vines of the *Vanilla* genus in the Orchidaceae family. While about 110 species of vanilla are currently known, only two species (*V. planifolia* and *V. tahitensis*) are cultivated commercially. *V. planifolia* is cultivated in many places of the world, such as Madagascar and Indonesia. In particular, Bourbon vanilla, which comprises the Madagascar, Comoro, Réunion cultivars, is well known as a high quality product. Meanwhile, *V. tahitensis*, which was recently reported to be a hybrid of *V. planifolia* and *V. odorata* (Lubinsky *et al.*, 2008), is mainly cultivated in Tahiti and Papua New Guinea.

The most important characteristic of vanilla bean is its sweet attractive aroma, which has prompted numerous reports regarding its volatile compounds. More than 500 volatile compounds have been reported for vanilla (Klimes and Lampasky, 1976, Adedeji *et al.*, 1993, Toth *et al.*, 2011), with vanillin as the key aroma compound. Although there are some reports identifying the potent odorants of vanilla bean using the gas chromatography-olfactometry (GC-O) technique (Pérez-Silva *et al.*, 2006; Zhang

and Mueller, 2012; Brunschwig *et al.*, 2012; Takahashi *et al.*, 2013a,b), there are few studies (Zhang and Mueller, 2012; Brunschwig *et al.*, 2012) comparing the odorant profiles of different vanilla cultivars using GC-O techniques such as aroma extract dilution analysis (AEDA). Therefore, the characteristic odorants of individual vanilla cultivars remain unclear.

In the present paper, we characterized the potent odorants of three different vanilla cultivars (Madagascar, Comoro and Tahiti) using AEDA. Furthermore, the relationship between species and the characteristic aroma profile was also investigated, focusing on 3- or 4-methoxylated aromatic compounds, in *V. planifolia* and *V. tahitensis*.

Materials and Methods

Materials and Chemicals The Madagascar (16 – 20 cm, harvested in 2010), Comoro (14 – 17 cm, harvested in 2011) and Tahitian (14 – 20 cm, harvested in 2011) cultivars of vanilla bean were obtained from Kaneda Corporation (Tokyo, Japan).

The organic solvents (diethyl ether and pentane) used were of >99% purity (Kanto Chemical Co., Inc., Tokyo, Japan). **1, 2, 4, 5, 8, 9, 12, 13, 15, 19, 21, 22, 23, 25, 27, 28, 30, 31, 33, 35, 36, 38, 42,**

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44, 46, 48, 49, 50, 51, 52, 54 were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 3, 14, 20, 37, 45, 53 were purchased from Sigma Aldrich Japan Co., Ltd. (Tokyo, Japan). 6 (Czerny *et al.*, 1996), 11 (Czerny *et al.*, 1996), 16 (Guth and Grosch, 1990), 26 (Kumazawa *et al.*, 2006), 47 (Kaneko *et al.*, 2013) were synthesized according to the literature.

Isolation of Vanilla Volatiles The vanilla aroma concentrates were obtained by organic solvent extraction, SAFE distillation and a concentration method. After removing the vanilla pod, 1 g of vanilla bean was suspended in 1 mL of distilled water, to which 20 mL of a mixture of pentane/diethyl ether (1:1 v/v) was added, and then the aroma compounds were extracted in an ultrasonic bath for 60 min.

This aroma extract was spiked with an internal standard (5 ng 2-octanol), dried using an excess amount of anhydrous sodium sulfate, and then distilled by solvent assisted flavor evaporation (SAFE) (40°C, $<5.0 \times 10^{-3}$ Pa). The vanilla aroma concentrate was obtained by rotary evaporation concentration, followed by nitrogen steam evaporation to about 100 μ L. The resulting aroma concentrate was used as the sample for AEDA and GC-MS analyses.

Enrichment of Odorants for Identification For the identification experiments, the vanilla volatiles were isolated from a larger amount of vanilla (50 g) by combining the solvent extraction and the SAFE technique as described above. The aroma concentrate (approximately 100 μ L) was applied to a glass column (15°C, 20 cm $\times$ 0.7 cm i.d.) filled with 5 g of silica gel (Wakogel® C-200, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) in n-pentane. Elution was performed using the following solvents: n-pentane (30 mL, fraction A), n-pentane/diethyl ether (30 mL, 10 + 1, v/v, fraction B), n-pentane/diethyl ether (30 mL, 10 + 2, v/v, fraction C), n-pentane/diethyl ether (30 mL, 1+1, fraction D) and diethyl ether (30 mL, fraction E). The solution was concentrated to approximately 100 μ L as described above.

Gas Chromatography-Olfactometry (GC-O) An Agilent 6850 gas chromatograph equipped with a thermal conductivity detector (TCD) and DB-WAX (J&W Scientific, California, USA) fused silica capillary column (30 m, 0.25 mm i.d., 0.25 μ m film thickness) was used for GC-O analysis. One microliter of the sample was injected (splitless mode). The column temperature was programmed from 40 to 210°C at the rate of 5°C/min. The injector and detector temperatures were both 250°C. Helium was used as the carrier gas at the flow rate of 1 mL/min. A glass sniffing port was connected to the outlet of the TCD and heated to $>210^\circ\text{C}$ by a ribbon heater. Moist air was pumped into the sniffing port at 100 mL/min to quickly remove the odorant eluted from the TCD out of the sniffing port. The vanilla aroma concentrates were used for GC-O analysis. Determination of the odor qualities detected by sniffing was achieved by triplicate determinations for each sample.

Aroma Extract Dilution Analysis (AEDA) The original odor concentrate of the vanilla aroma was stepwise diluted with

dichloromethane to 4^n ($n = 1 - 9$), and 1 μ L of each fraction was analyzed by capillary GC on a DB-WAX column. The odorants were then detected by GC eluate sniffing (GC-O). The flavor dilution (FD) factors of the odorants were determined by AEDA. Before the FD factor measurement, three panelists repeatedly checked the retention time and odor quality of the odorants using each diluted sample (4^1 and 4^2), then the FD factor of the odorants was determined by detection of not less than two panelists at each dilution step.

Gas Chromatography–Mass Spectrometry (GC-MS) An Agilent 7890A gas chromatograph equipped Agilent 5975C inert XL series MSD and DB-WAX fused silica capillary columns (60 m, 0.25 mm i.d., 0.25 μ m film thickness) was used for identification and semi-quantification purposes. The column temperature was programmed from 80 or 40°C to 230°C at the rate of 3°C/min. The injector temperature was 250°C. Helium was used as the carrier gas at the flow rate of 1 mL/min. One microliter of the sample was injected, and the split ratio was 1:30 or splitless. Mass spectrometry was conducted at an ionization voltage of 70 eV (EI) and an ion source temperature of 150°C.

Identification and Semi-quantification of Aroma Compounds The identification of each key aroma compound was performed by comparing its Kovats GC retention index (RI) and mass spectrum to those of the authentic compounds by GC-MS, in addition to comparison of its RI and odor quality with those of the authentic compounds by GC-O. Semi-quantitative data of the respective key aroma compounds were calculated on the basis of the internal standard material using the estimated response factor of 1 by GC-MS.

Results and Discussion

Potent odorants of the three different vanilla cultivars (Madagascar, Comoro and Tahiti) Three different cultivars, belonging to *V. planifolia* (Madagascar, Comoro) and *V. tahitensis* (Tahiti), of commercial vanilla beans were used. The aroma of each vanilla bean had a different character, such as fatty and whisky-like for Madagascar, smoky for Comoro, and spicy and anise-like for Tahiti.

The volatile concentrates of the vanilla beans were prepared by combining the solvent extraction and SAFE techniques. The extraction solvent was optimized by comparing and assessing the aroma between the vanilla beans themselves and their extracts. Although the concentration process could result in the loss of some of the more volatile organic compounds, the volatile concentrates obtained using this sampling operation (without excessive heating) reproduced well the characteristic aromas of the vanilla beans. Thus, this was regarded as the preferred method to obtain analytical samples for comparing individual potent odorants using the GC-O technique.

The AEDA technique was applied to the volatile concentrates prepared from the three different vanilla bean cultivars

(Madagascar, Comoro and Tahiti), and 49 odor-active peaks with FD factors between 4^1 and 4^9 were determined, thus confirming that the aroma of the vanilla beans consisted of many potent odorants (Table 1.). However, since many of the peaks of the unknown odorants on the gas chromatogram overlapped with other compounds (co-eluted), it was difficult to quantitate and qualitate these odorants at low levels. Therefore, to identify these low amounts of unknown odorants, highly concentrated volatile fractions were prepared from 50 g of Madagascar vanilla bean. The obtained volatile concentrate was fractionated into five fractions (from A to E) by silica gel column chromatography, so that these additional small amounts of odorants could be identified or tentatively identified by GC-MS and GC-O analyses. Finally, in these volatile concentrates, 36 odorants could be identified or tentatively identified from 49 odor-active peaks by GC-MS and GC-O. Besides these potent odorants, which included nine newly identified odorants (**3**, **4**, **6**, **8**, **11**, **15**, **26**, **38**, **47**), there were some important and unidentified remaining odorants in the vanilla aroma.

Because the odor thresholds of the newly identified compounds were extremely low, these odorants in the vanilla can be assumed to be too low in concentration and thus difficult to identify. Pyrazines (**3**, **6**, **11**) and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (**38**) are known to be produced from sugars and amino acids through multistep reactions (Koehler *et al.*, 1969; Huang *et al.*, 1994; Blank *et al.*, 1996). Unsaturated aldehydes (**15**, **26**) are reported to be generated from unsaturated fatty acids, such as linolenic acid, by autoxidation and enzymatic oxidation (Belitz *et al.*, 2004). Additionally, it is well known that methoxypyrazines (**4**, **8**) are produced by enzymatic reaction (Dunlevy *et al.*, 2010). Therefore, the contents of these compounds are assumed to be dependent on their precursor contents and the manufacturing conditions of the vanilla bean.

As a result of the AEDA, vanillin (**46**) was obviously the most important odorant for each cultivar, which is consistent with previous reports. The odorant composition of cultivars of the same species (Madagascar and Comoro) was similar; various phenyl compounds (guaiacol (**22**), p-cresol (**30**), ethyl cinnamate (**33**) and eugenol (**36**)), which are derivatives of phenylpropanoids, showed high FD values in both vanillas. In contrast, certain compounds were characteristic for individual cultivars. For example, 2-alkenals and 2,4-dienals (**9**, **15**, **19**) in Madagascar vanilla, reported as lipid oxidation products formed during the curing process (Dunphy and Bala, 2014), were found to be higher than in Comoro vanilla. Meanwhile, guaiacol (**22**) showed the highest FD value, especially in Comoro vanilla, and was assumed to be responsible for the characteristic smoky note.

In food, guaiacol is reported to be produced by microorganisms or thermal degradation (Huang *et al.*, 1993; Witthuhn *et al.*, 2012; Belitz *et al.*, 2004). The curing of vanilla beans generally includes fermentation and sun drying processes. Therefore, the higher guaiacol content of Comoro vanilla compared to Madagascar

vanilla is assumed to be due to differences in curing conditions, such as the bacterial flora or drying conditions, between Madagascar and Comoro. In addition, plant maturity affects the lipoxygenase activity, which is involved in the production of 2-alkenals and 2,4-dienals (Belitz *et al.*, 2004). Thus, it was assumed that differences in cultivation conditions between Madagascar and Comoro affects the aroma profile of vanillas. However, we compared only one sample of each cultivar in this study. In order to confirm the accuracy of these determinations, a larger study that compares various kinds of vanilla products is needed.

On the other hand, the Tahitian vanilla differed in odorant composition from *V. planifolia*. It had fewer odorants than *V. planifolia*, and four odorants (**27**, **35**, **42**, **49**) showed characteristically high FDs. Surprisingly, these four odorants had the 4-methoxy aromatic structure, which is assumed to contribute the spicy and anise note.

The results of the AEDA indicated that a variety of trace and low-odor threshold odorants play an important role in the characteristic aroma of individual vanilla cultivars. It is assumed that differences in the aroma profile were derived from differences in the species, mainly in the curing process and growth conditions. Further clarification of the characteristics of each vanilla cultivar could be achieved by a more comprehensive investigation and comparison of the cultivars.

The 3- or 4-methoxylated aromatic compounds responsible for species differences in vanilla aroma Based on the AEDA results, some compounds with the methoxybenzene (anisole) skeleton (**25**, **27**, **35**, **36**, **37**, **42**, **46**, **47**, **49**) were assumed to be responsible for the vanilla aroma. Table 2 shows the concentrations of anisole compounds (Figure 1.) in the three different cultivars. *V. planifolia* had a high content of 3-methoxylated compounds, such as vanillin and eugenol, while *V. tahitensis* contained abundant 4-methoxylated compounds, such as anisyl alcohol, anisaldehyde and isovanillin. This result suggests that *V. planifolia* and *V. tahitensis* have different biosynthesis routes for these aromatic compounds.

Vanillin is generally thought to be biosynthesized via a multi-step conversion from phenylalanine (Podstolski, 2011; Negishi *et al.*, 2009). The 3-methoxylation of vanillin was achieved by 3-orthomethyltransferase (3-OMT). 3-OMT is thought to be activated in *V. planifolia* to a greater extent than in *V. tahitensis*. In addition, enzymes that methylate the phenol group in the 4-position (4-OMT) are thought to be present or highly active in *V. tahitensis*.

Although many kinds of 4-OMTs have been reported in plants (Koeduka, 2010), there has been little discussion of 4-OMT in vanilla. Besides, the biosynthesis route of aroma compounds in *V. tahitensis* has not yet been reported. In the future, investigation of vanilla enzymes related to the methoxylation of the phenol group will be important for further elucidation of the aroma profile of various cultivars of vanilla.

Table 1. Odorants (FD ≥ 4) of the vanilla beans in three cultivars (Madagascar, Comoro and Tahiti)

No.	RI ^a	Compound ^b	Odor quality	FD factor ^c		
				Madagascar	Comoro	Tahiti
1	930	2- and 3-methylbutanal ^d	stimulus, malty	4	4	4
2	1296	1-octen-3-one	mashroom-like	16	4	nd ^e
3	1388	2-ethyl-6-methylpyrazine ^{df}	nutty, fruity	4	4	nd ^e
4	1428	2-isopropyl-3-methoxypyrazine ^{df}	peacy	16	4	4
5	1439	acetic acid	sour	4	16	4
6	1462	2-ethyl-3,5-dimethylpyrazine ^{df}	nutty	16	4	4
7	1502	NI	putrid	4	16	4
8	1521	2-isobutyl-3-methoxypyrazine ^{df}	earthy, musty	64	64	16
9	1530	(<i>E</i>)-2-nonenal ^d	sweet, fatty	64	4	4
10	1541	NI	lemon-like	nd ^e	nd ^e	4
11	1555	2-ethenyl-3,5-dimethylpyrazine ^{df}	nutty	16	4	4
12	1622	butanoic acid	sweaty, rancid	4	4	nd ^e
13	1660	3-methylbutanoic acid	sweaty, rancid	64	64	16
14	1665	NI	meaty, vitamine-like	16	16	nd ^e
15	1696	(<i>E,E</i>)-2,4-nonadienal ^f	sweet, fatty	256	16	nd ^e
16	1716	3-methyl-2,4-nonanedione ^d	green	4	16	4
17	1723	NI	sweet, lactone-like	16	nd ^e	nd ^e
18	1761	NI	sweet	4	nd ^e	nd ^e
19	1805	(<i>E,E</i>)-2,4-decadienal	sweet, fatty	64	16	nd ^e
20	1817	β -damascenone	sweet	64	64	nd ^e
21	1837	hexanoic acid	sour, green	nd ^e	4	4
22	1851	guaiacol	burnt	1024	65536	256
23	1913	4-octanolide	sweet, juicy, lactone-like	4	4	nd ^e
24	1938	NI	sweet, juicy	nd ^e	4	nd ^e
25	1948	2-methoxy-4-methylphenol	burnt, spicy	16	64	nd ^e
26	1996	<i>trans</i> -4,5-epoxy-(<i>E</i>)-2-decenal ^{df}	sweet, juicy	16	4	4
27	2020	anisaldehyde	spicy, sweet, cherry	64	64	4096
28	2023	4-nonanolide	sweet	64	64	nd ^e
29	2052	NI	metallic	nd ^e	4	nd ^e
30	2073	<i>p</i> -cresol	phenolic	256	256	16
31	2070	methyl cinnamate	fruity, strawberry-like	4	16	nd ^e
32	2102	NI	burnt, spicy	4	nd ^e	nd ^e
33	2124	ethyl cinnamate	fruity, strawberry-like	256	1024	64
34	2141	NI	lactone	nd ^e	4	nd ^e
35	2146	anisyl acetate	sweet, anise-like	4	4	64
36	2157	eugenol	spicy	1024	1024	16
37	2185	2-methoxy-4-vinylphenol	spicy	4	nd ^e	nd ^e
38	2190	3-hydroxy-4,5-dimethyl-2(5 <i>H</i>)-furanone ^f	caramel-like	256	64	64
39	2236	NI	juicy, sweet, butter-like	4	4	nd ^e
40	2247	NI	powderly	nd ^e	4	nd ^e
41	2259	NI	spicy	nd ^e	4	nd ^e
42	2274	anisyl alcohol	spicy, anise-like, cherry	16	16	1024
43	2304	NI	meaty	4	nd ^e	nd ^e
44	2446	coumarin	sweet	4	4	16
45	2544	phenylacetic acid ^d	sweet	4	4	nd ^e
46	2551	vanillin	vanilla-like	262144	262144	16384
47	2561	4-vinyl-2,6-dimethoxyphenol ^{df}	spicy, smoky	64	16	16
48	2613	3-phenylpropionic acid ^d	animal-like	4	16	16
49	2686	isovanillin	spicy, smoky	nd ^e	nd ^e	256

*NI, not identified.

^aRI, retention index calculated on DB-WAX column using C5-C32 n-alkanes.^bThe compound was identified by comparing its RI and mass spectrum with the authentic compound by GC-MS in comparison of its RI and odor quality with the authentic compound by GC-O.^cFD factor, flavor dilution factor.^dThe compound was tentatively identified by GC-O analysis by comparison to the authentic compound, but no unequivocal mass spectrum was available by GC-MS.^end, not detected^fThe compound was newly identified in the vanilla beans.

Table 2. The concentrations of the 3 or 4-position methoxylated compounds in the vanilla beans

	No.	Compounds	Concentration ($\mu\text{g/g}$) ^a		
			<i>V. planifolia</i>		<i>V. tahitensis</i>
			Madagascar	Comoro	Tahiti
3-position^b methoxylated compounds	25	2-methoxy-4-methylphenol	2	6	0.5
	36	eugenol	0.6	0.7	nd
	37	2-methoxy-4-vinylphenol	nd	1	0.1
	46	vanillin	6201	8053	1501
	50	acetovanillone	4	5	1
	51	vanillyl alcohol	4	nd	1
4-position^b methoxylated compounds	27	anisaldehyde	0.3	0.3	19
	35	anisyl acetate	nd	0.3	14
	42	anisyl alcohol	8	6	1175
	49	isovanillin	nd	nd	34
	52	methyl anisate	nd	0.5	3
	53	anisyl formate	nd	nd	0.9
	54	anisic acid	nd	nd	238

nd, not detected

^a The concentration was determined by the ratio of the percentage peak area of each compound to the internal standard (2-octanol)^b The number of positions was counted regarding any carbonic group (CH_3 , $\text{CH}=\text{CHCH}_2$, CHO , COCH_3 , CH_2OH , COOCH_3 , COOH , CH_2OCHO , CH_2OAc) as the 1-position. **Fig. 1.** The structure of the 3 or 4-position methoxylated compounds in the vanilla beans

On the basis of these results, to better clarify the characteristic vanilla aroma of each cultivar, it is necessary to take into account not only vanillin, but also trace odorants such as those newly identified in this study. However, these results were obtained from only one product sample of each cultivar. To improve the accuracy of the flavor profile of each vanilla cultivar, it is important to understand the potent odorants of various vanilla products. Therefore, a more detailed study of the various kinds of vanilla products, such as from different raw materials and manufacturers, and their manufacturing conditions is needed.

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