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Inhibitory Effect of Yuzu Essential Oil on the Formation of *N*-Nitrosodimethylamine in Vegetables

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The inhibitory effect of yuzu (*Citrus junos* Tanaka) essential oil on the formation of *N*-nitrosodimethylamine (NDMA) in the presence of vegetables (31 species) or saliva was investigated by HPLC. Most vegetable extracts enhanced the formation of NDMA. However, the formation ratio of NDMA in vegetable extracts was decreased by yuzu oil in the range of 59 to 22%. In the presence of yuzu oil and saliva, its ratio ranged between 62 and 24%. These results indicated that yuzu oil inhibited the formation of NDMA even in vegetables and saliva. The contents of ascorbic acid, nitrate, and nitrite in the 31 vegetable species were 0.3−65 mg/100 g, 3−581 mg/100 g, and 10−750 μg/100 g, respectively. Ascorbic acid and nitrite had little effect on the inhibition or formation of NDMA at their intact levels. Nitrate accelerated the formation of NDMA, and the addition of saliva further enhanced it. The mechanism of inhibition of NDMA formation by α-terpinene was studied. It was assumed from the results of LC-MS that a new compound formed by the reaction of α-terpinene with nitrite would be a derivative of α-terpinene with dinitroso groups. The molecular weight of this compound was 194. It is suggested that terpene hydrocarbons in citrus essential oils would contribute to the decrease of NDMA formation.

KEYWORDS: ***N*-Nitrosodimethylamine; HPLC; inhibitory effect; yuzu essential oil; vegetable; ascorbic acid; nitrate; nitrite; α-terpinene; LC-MS**

INTRODUCTION

N-Nitroso compounds are known as strong carcinogens. Among them, the most examined are nitrosamines (*1*). Nitrosamines are formed by *N*-nitrosation of secondary amines with nitrite in an acidic condition. The precursors commonly occur in a wide variety of foods. Secondary amines also occur in meats, cured meat products, and smoked fish. Nitrite is often added to foods such as hams and sausages as an antimicrobial or coloring substance (*2*). Nitrate is a precursor of nitrite, and vegetable foods are a main source of nitrate (*3*). The content of nitrate in crops depends on the species, genetic and environmental factors, and cultivation conditions. In certain crops, for instance, the levels are as high as 1000 mg/kg or more. The level of nitrites in plants and water, on the other hand, is commonly very low (*4*). It is well-known that human saliva is a major source of nitrite (*3*). About 75% of the nitrate taken into the body is excreted in urine, and 25% is secreted in saliva. Twenty percent of the nitrate in saliva is readily reduced to nitrite by nitrate oxidoreductase (EC 1.6.6.1. NADH). WHO/FAO have suggested that the acceptable daily intakes (ADI) of nitrate and nitrite per kilogram of body weight are 3.7 and 0.6 mg, respectively (*5*). However, nitrate intake in Japan is ∼1.5 times the ADI. About 87% of the nitrate source is from vegetable foods (*6*), because the Japanese diet tends to be richer in vegetables than that of Europe, the United States, and other countries. It is reported that the mortality of Japanese by stomach cancer is higher than that of the Europeans because of a large intake of nitrates from foods (*7*). Nevertheless, the probability of the development of cancer is statistically very low because substances that inhibit the formation of nitroso compounds, such as ascorbic acid, tocopherols, and polyphenols, are taken simultaneously from ordinary food (*8*−*10*). It is reported that the extracts from persimmon (*Diospyros kaki* Thunb.), knotweed (*Polygonum longisetum*), and Japanese aucuba (*Aucuba japonica*) remarkably inhibited the mutagenicity of the *C*-nitro and *C*-nitroso compounds formed by the reaction of sorbic acid and sodium nitrite (*11*). The consumption of whole strawberry, kale juice, and garlic juice has been reported to be effective in the inhibition of *N*-nitrosodimethylamine (NDMA) (*12*). Terpene hydrocarbons such as limonene and carvone reduced the occurrence of cancer in mice due to activation of glutathione *S*-transferase (*13*). It is also reported that limonene, one of the major volatile components of citrus essentials oils, possessed anticarcinogenic activity against mammary, lung, stomach, and skin cancers (*14*, *15*). As reported before (*16*), the authors demonstrated that citrus essential oils and their components had an inhibitory effect on the formation of NDMA in a model

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Table 1. Vegetable Samples

tissue	common name	botanical name
leaves	cabbage	*Brassica oleracea* L. var. *capitata* L.
	celery	*Apium graveolens* L. var. *dulce* DC.
	Chinese cabbage	*Brassica campestris* L.
	komatsuna	*Brassica campestris* L.
	lettuce	*Lactuca sativa* L.
	mitsuba (Japanese horwort)	*Cryptotaenia japonica* Makino
	potherb mustard	*Brassica rapa* var. *lancinifolia*
	spinach	*Spinacia oleracea* L.
roots	carrot	*Daucus carota* L.
	carrot *(kintoki)*	*Daucus carota* L.
	garlic	*Allium sativum* L.
	ginger	*Zingiber officinale* Rosc.
	lotus (root)	*Nelumbo nucifera* Gaertn
	onion (white)	*Allium cepa* L.
seeds	bean sprouts	*Vigna radiata* Wilcz.
peas	peas	*Pisum sativum* L.
fruits	bitter gourd (balsam pear)	*Momordica charantia* L.
	cucumber	*Cucumis sativus* L.
	eggplant	*Solanum melongena* L.
	sweet pepper	*Capsicum annuum* L.
	tomato	*Lycopersicon esculentum* Mill
herbs	coriander	*Coriandrum sativum* L.
	dill	*Anethum graveolen* L.
	Italian parsley	*Petroselinum sativum* Hoffm.
	lemon balm	*Melissa officinalis* L.
	mint	*Mentha* sp.
	rocket	*Eruca vesicaria sativa* Mill
	salad parsley	*Petroselinum sativum* Hoffm.
	thyme	*Thymus vulgaris* L.
mushrooms	hen of the wood	*Grifola frondosa* (Dicks: Fr.) S.F. Gray
	shiitake	*Lentinus edodes* (Berk.) Sing.

solution. In particular, the essential oil of yuzu (*Citrus junos* Tanaka), which is the most popular sour citrus fruit used for cuisine, dressing, and cosmetics in Japan, exhibits a high inhibitory effect on the formation of NDMA. However, the inhibitory effect of citrus essential oils in vegetable foods in the presence of saliva has not been studied. The mechanism of inhibition of NDMA formation has also been unknown. This study aims to investigate the inhibitory effect of vegetable foods and citrus essential oils in the presence of saliva and the mechanism of the inhibitory effect of terpene hydrocarbons in citrus essential oils on the formation of NDMA.

MATERIALS AND METHODS

Reagents. Dimethylamine, sodium nitrite, polyoxyethylenesorbitan monolaurate (Tween 20), and α-terpinene were purchased from Sigma Chemical Co. (St. Louis, MO). NDMA standard, acetic acid, and limonene, which were extra pure reagents, and L-ascorbic acid, metaphosphoric acid, zinc sulfate, potassium nitrate, phosphoric acid, sulfamic acid, and silver sulfate of special grade, and chloroform for HPLC were obtained from Wako Pure Chemical Industries (Osaka, Japan). Ethanol, sodium hydroxide, and sulfuric acid of special grade, and methanol for HPLC were from Nacalai Tesque (Kyoto, Japan). 2,6-Xylenol, 4,6-dinitro-*o*-cresol, myrcene, and terpinolene were provided by Tokyo Kasei Kogyo (Tokyo, Japan).

Yuzu Essential Oil Preparation. Yuzu (*C. junos* Tanaka) fruit was obtained from the Kochi Fruit Tree Experimental Station in November 2001. Cold-pressed peel essential oil of yuzu was prepared according to the method described previously (*17*).

Preparation of Vegetable Samples for NDMA Assay. Thirty-one kinds of vegetable samples used in this study, including leaves, roots, seeds, fruits, herbs, and mushrooms, were purchased at the market as shown in **Table 1**. Each sample (100 g) was treated with a JC-V 60 juicer (Toshiba Co., Tokyo, Japan) for 1 min. The juice was centrifuged at 9000*g* for 10 min at 5 °C. The supernatant (25 mL) was adjusted to pH 3.6 with an aqueous solution of 10% acetic acid and then diluted to 100 mL with Milli-Q water. The dilute was filtered through no. 5C filter paper (Advantec, Toyo Roshi, Tokyo, Japan) and then through a 0.2 μm membrane filter (Millipore, Tokyo, Japan). These vegetable extracts were used for the NDMA assay.

Sampling of Saliva. The saliva from three donors in our laboratory, who were in good health and nonsmokers, was sampled, mixed together, and immediately kept in ice until it was used in the experiment.

Assay for NDMA. The assay for NDMA was carried out by modifying the method used by Sawamura et al. (*16*).

Each 0.5 mL of 25 mM dimethylamine and 25 mM sodium nitrite, the pH values of which were preliminarily adjusted to 3.6 with an aqueous solution of 10% acetic acid, and 0.1 mL of 6.55% (w/v) Tween 20 were mixed with 0.01 mL of yuzu oil and 0.2 mL of vegetable extract in a brown vial (4 mL). The vial was closed with a tightly screwed cap with an inner seal made of Teflon. Milli-Q water was used in the control instead of yuzu oil and vegetable extract. The total volume was 1.31 mL.

In the case of the saliva experiment, each 0.4 mL of 31.25 mM dimethylamine and 31.25 mM sodium nitrite, which were preliminarily adjusted to pH 3.5 with an aqueous solution of 10% acetic acid, and 0.1 mL of 6.55% (w/v) Tween 20 were mixed with 0.01 mL of yuzu oil, 0.2 mL of vegetable extract, and 0.2 mL of saliva in a brown vial (4 mL). The vial was closed with a tightly screwed cap with an inner seal made of Teflon. The control contained Milli-Q water instead of yuzu oil and vegetable extract. The total volume was 1.31 mL.

The reaction mixture described above was incubated at 37 °C for 24 h with shaking at 50 strokes/min. After a 24 h, the reaction mixture with saliva was centrifuged at 10000*g* for 10 min. Then, all sample solutions were filtered through a 0.2 μm membrane filter prior to injection into a high-performance liquid chromatograph (HPLC). The measurement was performed in triplicates.

HPLC Operating Conditions for NDMA Assay. The measurement of NDMA was performed by HPLC. The apparatus was composed of a Jasco PU-1580-53 pump, a Jasco DG-1580-53 degasser, a UV 8000 detector (Toyosoda, Tokyo, Japan), a Rheodyne model 7125 injector with a 20 μL loop, a column bath U-620 (Sugai, Tokyo, Japan), and a D-2000 chromatointegrator (Hitachi, Tokyo, Japan). Absorbance was monitored at 220 nm. A reverse-phase column, CAPCELL PAK C18 MG column (4.6 mm i.d. × 250 mm, Shiseido, Japan), was used. The column temperature was kept at 37 °C, and 5% methanol was used as the mobile phase at 1.0 mL/min.

Determination of Ascorbic Acid. The contents of ascorbic acid in the 31 vegetable samples were determined according to the method described by Sawamura et al. (*18*). Vegetable samples were treated with a JC-V 60 juicer (Toshiba). The juice prepared was centrifuged at 9000*g* for 10 min at 5 °C. The supernatant (5 mL) was mixed with ethanol (10 mL) and 8% (w/v) metaphosphoric acid (5 mL). The mixture was filtered through a 0.2 μm membrane filter (Millipore), and 10 μL of the filtrate was injected into the HPLC.

HPLC Conditions for Determination of Ascorbic Acid. The determination of ascorbic acid was carried out by HPLC (*18*). A Jasco HPLC, composed of an 880-PU pump and an 875-UV detector set at 243 nm with a Rheodyne model 7125 10 μL loop injector, was used. Ascorbic acid was separated with a Wakosil 5C 18 column (4.6 mm i.d. × 150 mm, Wako Pure Chemical Industries) at 40 °C. The mobile phase was 0.2% (w/v) metaphosphoric acid running at a flow rate of 1.0 mL/min. Peak areas were calculated with a D-2500 chromatointegrator (Hitachi).

Determination of Nitrite and Nitrate. The contents of nitrite and nitrate in the 31 vegetable samples were determined by HPLC according to the method of Kunugi et al. (*19*). The vegetable extracts were prepared as follows: each vegetable sample (10 g) was homogenized in hot water at 80 °C using a JC-V 60 blender (Toshiba). The homogenate was then transferred to a 200 mL volumetric flask. To each were added 10 mL of 0.5 M sodium hydroxide and 12% (w/v) zinc sulfate. The mixture was incubated at 80 °C for 20 min. Then, it was cooled by running water and diluted to 200 mL. This solution was filtered through no. 5C filter paper. The vegetable extracts obtained were used for the determination of nitrite and nitrate.

Nitrite. Silver sulfate (0.1 g) was added to a test tube with a cap, to which a mixture of 4 mL of sulfuric acid (1+3) and 5 mL of vegetable

extracts had been added previously. The solution was mixed thoroughly and then treated with 1 mL of 2,6-xylenol solution. After incubation at 40 °C for 30 min, exactly 1 mL of 4,6-dinitro-*o*-cresol solution (internal standard) and 4 mL of chloroform were added to the reaction mixture. Ten microliters of the extract in chloroform was analyzed by HPLC.

Nitrate. Sulfamic acid (0.1 g) was added to a mixture of 8 mL of $H_2SO_4-H_3PO_4$ (3:1) and 1 mL of vegetable extract in a test tube, which was closed with a cap. After it was allowed to stand for 3 min, silver sulfate (0.1 g) and 1 mL of 2,6-xylenol solution were added. Upon incubation at 40 °C for 5 min, exactly 1 mL of 4,6-dinitro-*o*-cresol solution (internal standard) and 4 mL of chloroform were added. Ten microliters of the extract in chloroform was analyzed by HPLC.

HPLC Conditions for the Determination of Nitrite and Nitrate. HPLC operation conditions were as follows: pump, Jasco 880-PU; detector, Jasco 875-UV; column, Cosmosil 5SL (4.6 mm i.d. × 250 mm, Nacalai Tesque, Kyoto, Japan); eluent, chloroform/methanol/acetic acid (98:1:1); flow rate, 1.0 mL/min; column temperature, room temperature (20−25 °C); wavelength, 310 nm; injection volume, 10 μL.

Addition of Ascorbic Acid, Nitrite, and Nitrate to the Model Reaction Mixture for NDMA Formation. Several concentrations of ascorbic acid, nitrite, and nitrate solutions equivalent to those in the vegetable extracts used were prepared. Half a milliliter of 25 mM sodium nitrite (pH 3.6), 0.5 mL of 25 mM dimethylamine (pH 3.6), and 0.1 mL of Tween 20 (6.55%) were mixed with 0.2 mL of each authentic compound (ascorbic acid, nitrite, or nitrate) and 0.01 mL of Milli-Q water in a brown vial (4 mL). The total volume was 1.31 mL. The control contained 0.2 mL of Milli-Q water instead of the authentic compound. Incubation and other procedures for NDMA assay were the same as described in the experiment on the effect of vegetables on the formation of NDMA.

Addition of Saliva to the Model Reaction Mixture in the Presence of Nitrate. Sodium nitrite (0.4 mL, 31.25 mM) (pH 3.5), 0.4 mL of 31.25 mM dimethylamine (pH 3.5), and 0.1 mL of Tween 20 (6.55%) were mixed with 0.2 mL of saliva, 0.2 mL of nitrate, and 0.01 mL of Milli-Q water. The total volume was 1.31 mL. Milli-Q water (0.2 mL) instead of nitrate was used in the control. The procedure for NDMA assay was the same as described for the experiment on the effect of vegetables on the formation of NDMA in the presence of saliva.

Addition of Terpene Hydrocarbon (0.01 mL) to Model Reaction Mixture for NDMA Formation. Half a milliliter of 25 mM sodium nitrite (pH 3.6), 0.5 mL of 25 mM dimethylamine (pH 3.6), and 0.1 mL of Tween 20 (6.55%) were mixed with 0.01 mL of a terpene hydrocarbon (limonene, myrcene, α-terpinene, or terpinolene) and 0.2 mL of Milli-Q water in a brown vial (4 mL). The total volume was 1.31 mL. Incubation and other procedures for NDMA assay were the same as described above.

Addition of α-Terpinene (0.1 mL) to Model Reaction Mixture for NDMA Formation. Half a milliliter of 25 mM sodium nitrite (pH 3.6), 0.5 mL of 25 mM dimethylamine (pH 3.6), and 0.1 mL of Tween 20 (6.55%) were mixed with 0.1 mL of α-terpinene and 0.11 mL of Milli-Q water in a brown vial (4 mL). The final volume was 1.31 mL. Incubation and other procedures for NDMA assay were the same as described above.

Preparation of Standard Terpene Compounds. Each 0.01 mL of terpene compound including limonene, myrcene, α-terpinene, and terpinolene was mixed with 0.1 mL of Tween 20 (6.55%) and 1.2 mL of Milli-Q water.

HPLC Conditions for NDMA Assay in the Presence of Terpene Hydrocarbons. The measurement of NDMA was carried out by HPLC. A Shimadzu HPLC composed of two LC-10 AD vp pumps, an SPD-M 10A vp detector, an SCL-10A vp system controller, and a DGU-14A degasser was used. A CAPCELL PAK C18 MG column (4.6 mm i.d. × 250 mm, Shiseido) was used. The column was maintained at room temperature. Methanol (5%) was used as mobile phase at 1.0 mL/min.

LC-MS Conditions. A Shimadzu LC-10 AD HPLC coupled with an LCMS-2010 mass spectrometer was used for LC-MS. A Shimadzu HPLC composed of two LC-10 AD vp pumps, an SPD-M 10 A vp detector, an SCL-10A vp system controller with an SIL-HT autosampler, a CTO-10AC vp column oven, and a DGU-14A degasser was used. A Cosmosil 5C18 column (3.0 mm i.d. × 150 mm, Nacalai Tesque) was used. The column temperature was kept at 37 °C. An aqueous solution of 5% methanol was used as mobile phase at 0.8 mL/min. The analytical conditions for LC-MS 2010 were as follows: nitrogen gas flow rate, 1.5 L/min; CDL temperature, 250 °C; block temperature, 200 °C; detector voltage, 1.5 kV; mode, ESI; probe voltage, 4.5 kV; scan, *m*/*z* 40−1500.

RESULTS AND DISCUSSION

Effects of Vegetable Extracts on NDMA Formation with or without Yuzu Oil. The effects of vegetable extracts in a given model on the formation of NDMA with or without yuzu oil were investigated. The results are shown in **Table 2**.

The inhibition ratio was calculated using the following equation:

$$\text{inhibition ratio (\%)} = 100\% - (\%\ \text{of NDMA formation})$$

Most vegetable extracts used in the present study little affected the inhibition of NDMA formation. Celery, Chinese cabbage, and salad parsley slightly inhibited the formation of NDMA to the extents of 1, 4, and 4%, respectively. There was no significant inhibitory effect of NDMA in cabbage and carrot (kintoki) ($P < 0.05$). The other 26 kinds of vegetable extracts even accelerated the formation of NDMA. The vegetables that presented the formation ratio of NDMA from 101 to 110% were mitsuba, potherb mustard, carrot, bean sprouts, bitter gourd, cucumber, sweet pepper, Italian parsley, and mint. The vegetables with effects ranging from 110 to 120% of NDMA formation ratio were as follows: komatsuna, lettuce, spinach, ginger, onion (white), peas, eggplant, tomato, lemon balm, thyme, and shiitake mushroom. The vegetables with effects increasing the ratio of NDMA from 120 to 140% were garlic, lotus (root), coriander, dill, rocket, and hen of the wood mushroom. Achiwa et al. (*20*) reported that vegetable juices inhibited the formation of NDMA in the range between 0 and 50%. In this study, among the 21 kinds of vegetable extracts, celery and Chinese cabbage presented the ratio of NDMA formation of <100%, and the other vegetable extracts exceeded 100%. Most herbs have been used as medicinal plants for thousands of years, primarily as a remedy or antiseptic for digestive disorders. There have been few studies about the effect of herbs on the formation of NDMA. In this study, the authors then examined eight species of herbs, including coriander, dill, Italian parsley, lemon balm, mint, rocket, salad parsley, and thyme. As shown in **Table 2**, only salad parsley slightly inhibited the formation of NDMA. The other herb samples, however, increased the formation of NDMA (104−136%). Two kinds of mushroom samples, hen of the wood and shiitake mushroom, also formed high amounts of NDMA (117 and 133%, respectively), as shown in **Table 2**. Neither of the two mushrooms had an inhibitory effect on the formation of NDMA.

The addition of yuzu oil to the reaction mixture containing the vegetable extracts showed high inhibitory effects on the formation of NDMA. The extent of inhibition in the presence of yuzu oil was between 78 and 41%. The vegetables having an inhibitory effect of >70% were celery, komatsuna, mitsuba, potherb mustard, carrot, peas, cucumber, and sweet pepper. The vegetables that presented the inhibition ratio of 70−60% were cabbage, Chinese cabbage, lettuce, spinach, carrot (kintoki), garlic, lotus (root), bitter gourd, eggplant, and salad parsley. Ginger, bean sprouts, onion (white), tomato, dill, Italian parsley, mint, hen of the wood mushroom, and shiitake mushroom had inhibitory effects ranging from 50 to 60%. Coriander, lemon balm, rocket, and thyme showed inhibition ratios of >40%. From these results, each of the vegetable samples presented the

Table 2. Contents of Nitrate, Nitrite, and Ascorbic Acid in Vegetable Samples and Formation of NDMA in Vegetable Extracts with or without Yuzu Oil and Saliva

	contents[a]			NDMA formation[b] (%)			
				absence of saliva[c]		presence of saliva[d]	
sample	nitrate (mg/100 g)	nitrite (μg/100 g)	ascorbic acid (mg/100 g)	juice alone	yuzu oil added	juice alone	yuzu oil added
cabbage	158 ± 1.7	230 ± 1.0	34 ± 1.7	100 ± 1.5	34 ± 0.4	103 ± 1.4	28 ± 0.4
celery	210 ± 2.0	100 ± 1.7	1 ± 0.2	99 ± 2.2	26 ± 0.5	96 ± 0.7	27 ± 1.1
Chinese cabbage	159 ± 2.6	630 ± 2.4	11 ± 1.0	96 ± 2.6	35 ± 1.3	107 ± 2.3	32 ± 0.9
komatsuna	484 ± 1.7	750 ± 2.6	29 ± 1.7	117 ± 2.2	26 ± 1.9	101 ± 2.7	36 ± 2.2
lettuce	81 ± 2.0	360 ± 1.7	2 ± 0.2	115 ± 1.3	37 ± 0.4	92 ± 2.1	24 ± 0.3
mitsuba	560 ± 1.8	230 ± 1.8	2 ± 0.3	105 ± 1.7	25 ± 0.2	91 ± 0.8	29 ± 0.4
potherb mustard	535 ± 1.6	650 ± 2.0	18 ± 1.4	106 ± 1.0	28 ± 0.7	100 ± 2.6	31 ± 1.1
spinach	123 ± 1.1	710 ± 2.5	65 ± 1.2	114 ± 2.7	37 ± 1.1	99 ± 1.5	28 ± 1.1
carrot	9 ± 0.8	70 ± 1.1	6 ± 0.5	110 ± 2.6	22 ± 1.6	115 ± 1.0	25 ± 0.1
carrot (kintoki)	14 ± 0.8	180 ± 1.9	10 ± 0.9	101 ± 1.7	34 ± 0.8	94 ± 0.5	29 ± 0.9
garlic	3 ± 0.3	10 ± 0.9	5 ± 0.4	124 ± 0.7	35 ± 0.5	114 ± 2.4	27 ± 1.5
ginger	10 ± 0.4	20 ± 0.9	0.4 ± 0.03	113 ± 2.3	41 ± 1.6	114 ± 1.2	35 ± 1.9
lotus (root)	7 ± 0.3	10 ± 0.2	30 ± 1.7	138 ± 1.5	39 ± 1.2	124 ± 1.7	44 ± 1.1
onion (white)	6 ± 0.2	10 ± 0.4	6 ± 0.2	115 ± 2.2	46 ± 0.7	99 ± 1.4	31 ± 0.2
bean sprouts	5 ± 0.3	30 ± 1.0	3 ± 0.2	110 ± 1.8	41 ± 0.7	91 ± 1.0	26 ± 0.8
peas	15 ± 0.7	210 ± 2.4	25 ± 0.7	112 ± 0.8	26 ± 0.2	92 ± 2.6	24 ± 0.7
bitter gourd	84 ± 1.0	250 ± 1.6	48 ± 0.7	109 ± 2.0	36 ± 0.4	119 ± 1.4	33 ± 0.9
cucumber	66 ± 0.8	150 ± 1.2	5 ± 0.2	102 ± 1.1	28 ± 0.8	84 ± 2.2	25 ± 0.1
eggplant	8 ± 0.2	130 ± 2.2	4 ± 0.3	115 ± 1.2	34 ± 0.2	104 ± 1.0	32 ± 1.0
sweet pepper	42 ± 0.9	90 ± 1.7	49 ± 1.1	102 ± 1.4	27 ± 0.9	110 ± 1.8	36 ± 1.9
tomato	50 ± 1.5	40 ± 1.1	5 ± 0.1	115 ± 0.8	45 ± 0.6	113 ± 1.3	39 ± 1.4
coriander	457 ± 1.1	110 ± 1.7	0.4 ± 0.01	136 ± 0.9	56 ± 1.1	121 ± 1.0	46 ± 0.8
dill	11 ± 0.8	20 ± 1.1	0.3 ± 0.01	129 ± 1.2	48 ± 1.9	109 ± 0.6	50 ± 0.5
Italian parsley	404 ± 1.1	10 ± 0.9	0.4 ± 0.01	106 ± 2.4	49 ± 1.2	113 ± 1.9	44 ± 1.4
lemon balm	336 ± 1.1	490 ± 2.5	3 ± 0.2	115 ± 2.0	58 ± 1.0	103 ± 1.4	41 ± 1.0
mint	581 ± 2.5	50 ± 1.0	0.4 ± 0.01	104 ± 1.3	43 ± 0.5	108 ± 1.0	45 ± 0.7
rocket	471 ± 2.2	50 ± 1.7	23 ± 0.9	136 ± 1.7	51 ± 1.0	94 ± 1.7	32 ± 0.4
salad parsley	460 ± 1.7	150 ± 1.6	1 ± 0.04	96 ± 1.6	35 ± 0.3	97 ± 0.5	37 ± 0.8
thyme	20 ± 1.5	160 ± 1.6	0.5 ± 0.04	120 ± 1.1	59 ± 0.7	145 ± 0.5	62 ± 0.4
hen of the wood	8 ± 0.3	10 ± 0.6	3 ± 0.2	133 ± 1.7	43 ± 0.6	145 ± 1.0	42 ± 0.3
shiitake mushroom	10 ± 0.5	10 ± 0.3	7 ± 0.2	117 ± 1.3	50 ± 0.6	109 ± 0.7	57 ± 0.3

[a,b] Values are mean ± standard deviation of triplicates/treament. [c] Compared with Milli-Q water control 1, which is 100% NDMA formation: control 1, a mixture of 0.5 mL of dimethylamine (25 mM), 0.5 mL of sodium nitrite (25 mM), 0.1 mL of Tween 20 (6.55%) and 0.21 mL of Milli-Q water. The total volume came to 1.31 mL. [d] Compared with Milli-Q water control 2, which is 100% NDMA formation: control 2, a mixture of 0.4 mL of dimethylamine (31.25 mM), 0.4 mL of sodium nitrite (31.25 mM), 0.1 mL of Tween 20 (6.55%), 0.2 mL of saliva, and 0.21 mL of Milli-Q water. The total volume was 1.31 mL.

inhibitory effect remarkably when yuzu oil was added. As shown in **Table 2**, among 31 kinds of vegetable samples, for instance, lotus (root), having a high formation ratio of NDMA of as much as 138%, presented a considerable inhibition by the addition of yuzu oil. The formation ratio of NDMA by lotus with yuzu oil was 39%, which was only about one-third of the NDMA formation ratio without yuzu oil. The previous study indicated that yuzu oil presented a higher inhibition ratio of >70% (*16*). In this study, although the inhibitory effect on NDMA formation was not found in vegetable samples alone, the mixture of yuzu oil and vegetable samples highly inhibited the NDMA formation.

Effects of Vegetable Extracts with Yuzu Oil and Saliva on the Formation of NDMA. The effects of vegetable extracts with saliva on the NDMA formation are shown in **Table 2**. Among the 31 kinds of vegetable extracts, cucumber showed a strong inhibitory effect of NDMA formation (as much as 16%). Celery, lettuce, mitsuba, spinach, carrots (kintoki), onion (white), bean sprouts, peas, rocket, and salad parsley presented low inhibitory effects of NDMA (<10%). The other vegetable extracts enhanced the formation of NDMA to some extent. The vegetables that presented the formation ratio of NDMA from 101 to 120% were as follows: cabbage, Chinese cabbage, komatsuna, carrot, garlic, ginger, bitter gourd, eggplant, sweet pepper, tomato, dill, Italian parsley, lemon balm, mint, and shiitake mushroom. Lotus (root), coriander, thyme, and hen of the wood mushroom increased the formation of NDMA in the range of 120−145%. However, each vegetable extract effectively inhibited the formation of NDMA in the presence of yuzu oil. The inhibition ratio of NDMA formation in vegetable extracts and yuzu oil was from 38 to 76%. The vegetables presenting inhibition ratios of the NDMA formation of >70% were cabbage, celery, lettuce, mitsuba, spinach, carrot, carrot (kintoki), garlic, bean sprouts, peas, and cucumber, and the vegetables presenting inhibition ratios between 60 and 70% were Chinese cabbage, komatsuna, potherb mustard, ginger, onion (white), bitter gourd, eggplant, sweet pepper, tomato, rocket, and salad parsley. Thyme showed a low inhibition ratio of 38% even in the presence of yuzu oil. However, the formation ratio of NDMA was as high as 145% in the absence of yuzu oil (**Table 2**). These results suggest that all of the vegetable extracts used in this study had an inhibitory effect against the formation of NDMA when yuzu oil was added. Yuzu oil, in other words, does not lose its inhibitory function even in the presence of saliva.

In **Table 2**, the information about the inhibitory effect of yuzu oil in vegetable extracts on NDMA formation in the absence or presence of saliva can be independently obtained. The data for NDMA formation ratio in the absence of saliva could not be directly compared with those in the presence of saliva, because the basic peak areas were different between control 1 and control 2. To elucidate the NDMA formation in vegetable extracts in the presence of saliva, the effect of saliva on NDMA formation in vegetable extracts was investigated. The amount of NDMA formed in a mixture of 0.5 mL of dimethylamine

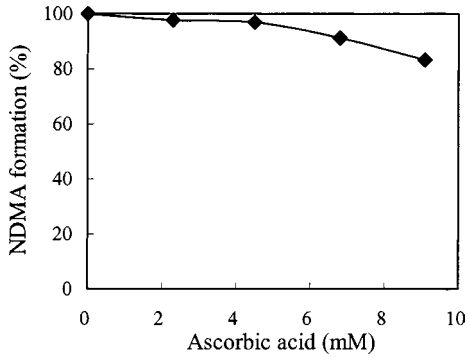

Figure 1. Effect of ascorbic acid on NDMA formation.

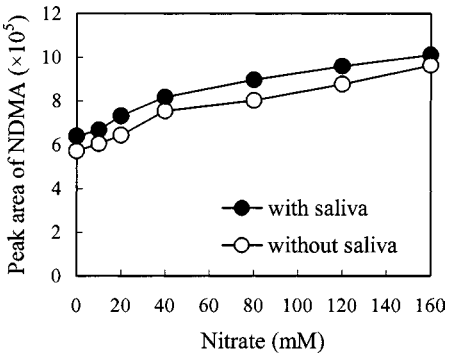

Figure 2. Effect of saliva on NDMA formation in nitrate.

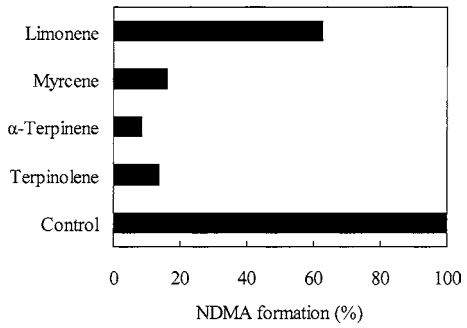

Figure 3. Effect of terpene hydrocarbons on NDMA formation.

(25 mM), 0.5 mL of sodium nitrite (25 mM), 0.1 mL of Tween 20 (6.55%), 0.2 mL of each vegetable extract, and 0.01 mL of Milli-Q water was used as control, where the reaction mixture was a little modified from the specification in the footnote of **Table 2**. The behavior of NDMA formed in a mixture of 0.4 mL of dimethylamine (31.25 mM), 0.4 mL of sodium nitrite (31.25 mM), 0.1 mL of Tween 20 (6.55%), 0.2 mL of each vegetable extract, 0.2 mL of saliva, and 0.01 mL of Milli-Q water was examined. It was demonstrated that the formation ratio of NDMA in each vegetable extract was in the range of 106−148% compared to control as long as saliva was present. The percentage was figured out as follows: the peak area of juice with saliva was divided by that of juice without saliva (the data are not shown here). In the case of eggplant, for example, the estimate was 106%, whereas in the case of hen of the wood mushroom, it was 148%. These estimations suggest that saliva can promote the formation of NDMA in vegetable extracts.

In this study, it was intact saliva that was added into the reaction mixture for NDMA formation. To examine the effect of bacteria in saliva on the NDMA formation, the saliva samples were filtered through a 0.2 μm membrane filter (Millipore) for removal of bacteria before being added into the reaction mixture. The results indicated that the formation of NDMA increased in the presence of intact saliva samples to the extent of 1.4 times that of the filtered saliva samples. Therefore, it is suggested that the increase in the NDMA formation in the vegetable samples with saliva is caused by the reduction of nitrate by bacteria in saliva.

Effect of Ascorbic Acid on the Formation of NDMA. The contents of ascorbic acid in the 31 vegetable samples ranged from 0.3 to 65.0 mg/100 g, as shown in **Table 2**. Particularly, the contents of ascorbic acid in the herb samples such as Italian parsley, coriander, mint, thyme, and dill were <1 mg/100 g. The effect of ascorbic acid on NDMA formation is shown in **Figure 1**. The inhibition of NDMA formation depended on the concentration of ascorbic acid. These results are consistent with the reports that ascorbic acid can inhibit the NDMA formation (*8*−*10*). However, the amount of ascorbic acid equivalent to that in vegetables little affected the inhibition of NDMA formation. Spinach contained the highest amount of ascorbic acid (65 mg/100 g) among the vegetable samples, but this level effected only a 10% inhibition of NDMA formation.

Effect of Nitrite on the Formation of NDMA. The nitrite contents of the 31 vegetables were between 10 and 750 μg/100 g, as shown in **Table 2**. It is known that the rate of formation of nitrosamines is proportional to the square of nitrite concentration (*1*). Although the amount of nitrite equivalent to that analyzed in the vegetables was added to the reaction mixture, there was little increase in NDMA. The results indicated that a small amount of nitrite (10−750 μg/100 g) in vegetables did not affect the formation of NDMA. It is considered that nitrite in vegetables is not a main factor that conducts the formation of NDMA. It is reported that a minor part of nitrite source results from nitrite-containing foods such as preserved meat and certain vegetables, but such a level is generally very low (*21*). The main source of nitrite results from the reduction of nitrate into nitrite by oral bacteria (*22*, *23*).

Effect of Nitrate on the Formation of NDMA. The contents of nitrate in the 31 vegetables were between 3 and 581 mg/100 g, as shown in **Table 2**. The leafy vegetables and most herbs such as cabbage, celery, Chinese cabbage, komatsuna, mitsuba, potherb mustard, spinach, coriander, Italian parsley, lemon balm, mint, rocket, and salad parsley had a high level of nitrate (>100 mg/100 g). The effect of nitrate on NDMA formation is shown in **Figure 2** by open circle mark. The formation of NDMA increased with a small increase in the concentration of nitrate.

Effect of Saliva on the Formation of NDMA. The effect of saliva on the NDMA formation in nitrate was examined, as shown in **Figure 2**. NDMA was increasingly formed in the presence of saliva more than in its absence.

The authors showed that saliva enhanced the formation of NDMA in vegetable extracts as described above. Therefore, the increased formation of NDMA with saliva may be caused by the reduction of nitrate from vegetables by oral bacteria. This is additional evidence that saliva is a major source of nitrite for human.

Inhibitory Effect of Authentic Terpene Hydrocarbons in Citrus Essential Oils on the Formation of NDMA. The effects of common terpene hydrocarbons such as terpinolene, α-terpinene, myrcene, and limonene were examined on the NDMA formation. The effect of these four compounds on the NDMA formation is illustrated in **Figure 3**. Myrcene, α-terpinene, and terpinolene had an inhibitory effect of >80%. Limonene showed a low inhibitory effect, as much as 38%. These results were similar to those of previous studies (*16*).

To get knowledge on the inhibition mechanism of NDMA formation, α-terpinene, one of the terpene compounds, was

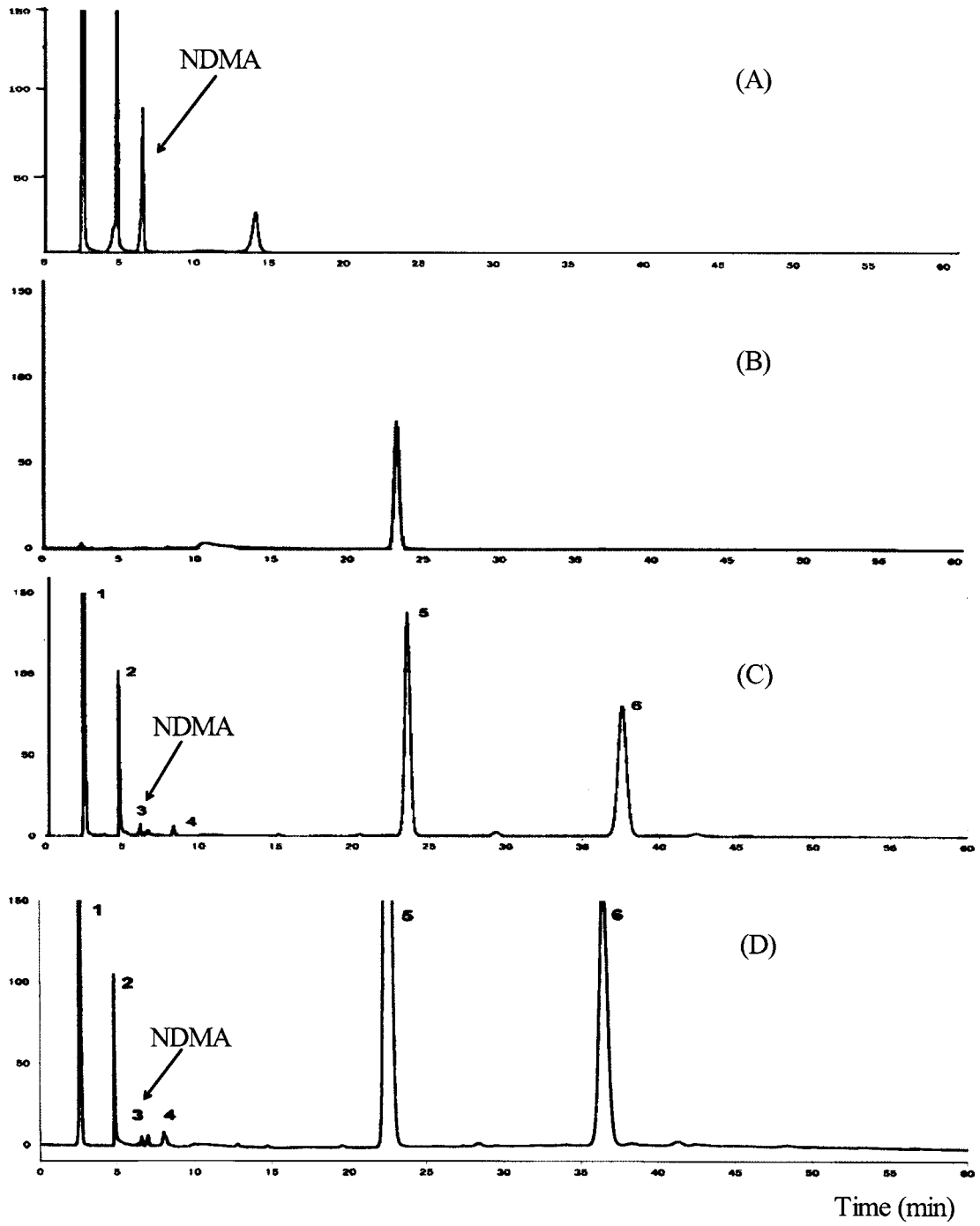

Figure 4. Chromatograms for NDMA formation with or without α-terpinene: (**A**) control for NDMA formation; (**B**) α-terpinene alone; (**C**) α-terpinene (0.01 mL) for NDMA formation; (**D**) α-terpinene (0.1 mL) for NDMA formation. Peaks: 1, nitrite; 2, acetic acid.

further investigated. The result is shown in **Figure 4**. The addition of α-terpinene to the reaction media developed one new compound (peak 6 in **Figure 4C**), the retention time of which was 37.6 min. It is considered possible that this compound could be an α-terpinene derivative which substitutes for NDMA. The behavior of formation of the α-terpinene derivative was further examined after 0.1 mL of α-terpinene was added to a model solution for NDMA formation. The result is shown in **Figure 4D**. The formation of the α-terpinene derivative (peak 6 in **Figure 4D**) considerably increased as compared to that described in **Figure 4C**. These results indicated that the α-terpinene derivative was not only formed by the reaction between α-terpinene and nitrite but also increased with an increase in the concentration of α-terpinene in the model solution for NDMA formation. It is considered that the decrease of NDMA formation in the presence of terpene hydrocarbons is due to the formation of terpene derivative by the reaction of terpene hydrocarbons with nitrite. Peak 6 was repeatedly isolated by HPLC. The isolated substance was concentrated under vacuum with a stream of nitrogen. This unknown sample was analyzed by LC-MS. The MS spectrum of α-terpinene derivative is shown in **Figure 5**. The peak of *m*/*z* 217 is most likely to be the relating compound of the α-terpinene derivative. When we assume the *m*/*z* 217 compound is bound with sodium, the molecular weight of the α-terpinene derivative would be 194. It is considered that the compound consisting of two or three double bonds such as α-terpinene, terpinolene, and myrcene would compete with dimethylamine to react with nitrite (*16*).

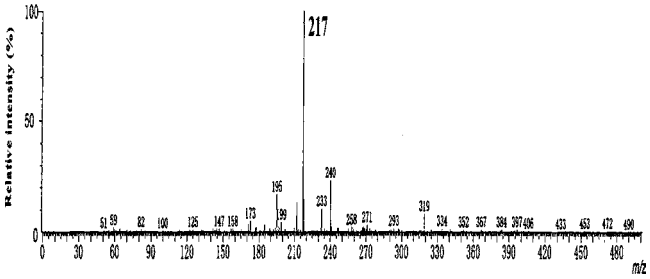

Figure 5. MS spectrum of α-terpinene derivative.

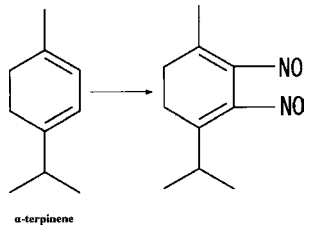

Figure 6. Postulated chemical structure of α-terpinene derivative.

α-Terpinene (0.01 mL) added to 1.31 mL of the model solution amounts to 63 μmol, and 0.5 mL of nitrite added to 1.31 mL of the model solution amounts to 12.5 μmol. Therefore, it is supposed that the reaction of nitrite with α-terpinene would make a compound with the nitroso group in the model solution for NDMA formation. Accordingly, the chemical structure of the α-terpinene derivative is postulated in **Figure 6**. It is a monocyclic and unsaturated compound with two nitroso groups.

In conclusion, NDMA formed by the reaction of nitrites with dimethylamine under acidic condition has attracted attention because of the potential toxicity. The present study focused on the effects of vegetables and yuzu essential oils on the formation of NDMA in the absence or presence of saliva. The mechanism of inhibition by several terpene compounds in citrus essential oil was also investigated.

The results of this study indicated that yuzu oil still possessed inhibitory activity on the formation of NDMA in a given reaction medium even in the presence of vegetables and saliva. It was also demonstrated that the decrease of NDMA formation in the presence of α-terpinene is due to the formation of α-terpinene derivative by the reaction of α-terpinene with nitrite. It is suggested that terpene compounds of citrus essential oils would contribute to the inhibition of NDMA formation.

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