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N-nitrosamine inhibition by strawberry, garlic, kale, and the effects of nitrite-scavenging and N-nitrosamine formation by functional compounds in strawberry and garlic

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Abstract

High nitrate and amine-rich food intake may result in an increased risk of endogenous formation of carcinogenic N-nitroso compounds (NOCs). We studied the effects of whole strawberries, garlic and kale on the formation of N-nitrosodimethylamine (NDMA) in a traditional Korean diet (amine and nitrate-rich) in simulated saliva and gastric conditions. The addition of whole strawberries, kale and garlic juices in the diet reduced NDMA formation by amine and nitrate. Strawberries and garlic juice were more effective than kale. To identify active compound, isolated fractions were prepared from strawberries or garlic juice samples by preparative liquid chromatography. Inhibition of NDMA formation and nitrite-scavenging ability of each fraction (strawberries: S1–S4; garlic juice: A1–A5) was tested. Fraction A4 inhibited NDMA formation by $50.5 \pm 5.4\%$ and increased the effect of nitrite-scavenging by $72.3 \pm 8.7\%$. Fractions A3 and A4 were identified as 1,2-benzendicarboxy acid (A3) and S-oxodially disulfide (A4) compounds by mass spectrophotometry and ^{13}C nuclear magnetic resonance.
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Keywords: N-nitrosodimethylamine; Nitrite; Strawberry; Garlic; Kale

1. Introduction

Human exposure to carcinogen N-nitrosamines (NAs) occurs through endogenous and exogenous sources such as foods and beverages (Chung, 1996, 2000). NAs have been related to an increased risk of gastric, esophageal, nasopharyngeal, and bladder cancer (Mirvish, 1995). N-nitrosodimethylamine (NDMA) is the most commonly known volatile NA in food samples (Yurchenko & Mölder, 2006). Dimethylamine (DMA) and nitrite are the immediate precursor of carcinogenic NDMA. Dimethylamine, diethylamine, trimethylamine, and trimethylamine oxide occurs naturally within fish and sea creatures such as squid, octopus and elasmobranches and the amines are the main precursors of NDMA (Forster & Goldstein, 1976; Lin & Chung, 1984; Lin, Lee, & Chang, 1983; Wang & Bolen, 1997). Vegetable and vegetable products are an important source of nitrate in an average Korean diet (Chung et al., 2003). Nitrate itself is relatively nontoxic, but approximately 5% of all ingested nitrate is converted to the more toxic nitrite in the oral cavity (Shapio, Hotchkiss, & Roe, 1991; Spiegelhalder, Eisenbrand, & Preussmann, 1976). Nitrite can react with secondary amines under the acidic conditions of the stomach to form NA.

There are many modulators of endogenous N-nitroso compounds (NOCs) formation. Chemical catalysts, such as thiocyanate, and inhibitors, such as ascorbic acid, α-

tocopherol, allyl sulfur compounds and phenolic compounds, influence the endogenous formation of NOCs (Bartsch, Ohshima, & Pignatelli, 1988; Chung, Lee, & Sung, 2002; Helser, Hotchkiss, & Roe, 1992; Kurechi, Kikugawa, & Fukuda, 1980; Mirvish, 1986; Shenoy & Choughuley, 1992; Wu, Wang, Li, & Han, 1993; Xu, Song, & Reed, 1993).

Fruits and vegetable are rich sources of antioxidant compounds such as ascorbic acid and phenolic compounds which scavenge free radicals and reactive oxygen species. Strawberry (*Fragaria ananassa*) is a very popular fruit and are reported to serve as one of our most important dietary sources of phenolic compounds (Kahkonen, Hopia, & Heinonen, 2001; Williner, Pirovani, & Guemes, 2003). Strawberry contains the highest amount of vitamin C (99 mg/100 g) among fruits (National Rural Living Science Institute, 1996). Kale (*Brassica oleracea* var. *acephala*) has a high level of vitamins and functional compounds and widely used in the juice industry. Kale extract was observed to protect against tumor formation (Yurtsever & Yardimci, 1999). Kale juice exhibited positive results, with characteristic color reactions representing protein, carbohydrate, and phenolic compounds (Lee, Park, & Rhee, 1997). Shenoy and Choughuley (1992) reported that onion and garlic (*Allium sativum* L.) juices were effective in reducing the chemical formation of NAs and suggested that allyl sulfur compounds in garlic juice might also act as nitrite scavengers.

We investigated the formation of carcinogenic NAs under simulated gastric conditions during incubation of amine-rich food and nitrate, and its possible inhibition by adding strawberry, garlic, or kale. In addition, we studied the effects of nitrite-scavenging and NDMA formation by functional compounds in strawberries and garlic. The objective of the study was to evaluate the effect of strawberry, garlic and kale juices on endogenous formation of NDMA in a simulated human digestive system and was to research functional compounds in strawberries and garlic.

2. Materials and methods

2.1. Materials

Strawberry, garlic, and kale were obtained from a local supermarket and were washed with tap water to eliminate external pesticides, soil, and other contaminants. The strawberries, garlic and kale were liquefied separately in a juicer (JMH-8800, HANIL, Korea). The resultant juices were used in treatment diets (TD1, TD2 and TD3).

The strawberry juice was adjusted to pH 2.5 with 3 N HCl and centrifuged. The methanol soluble portion of the supernatant was separated with C_{18} Sep-Pak cartridges (Water Associates, Milford, MA) using the method of Seo and Morr (1984).

The garlic was homogenized after the addition of distilled water. Homogenized garlic in a covered tube was incubated at 30 °C for 20 min and was centrifuged. Metha-

nol was added into the supernatant so that the final mass ratios were the supernatant:methanol = 1:1.5.

The methanol soluble portion in strawberries and the water and methanol soluble portion in garlic after centrifuging was evaporated under vacuum to dryness and dissolved in distilled water. The samples were used for the preparative high-performance liquid chromatography (Pre-HPLC) fraction.

2.2. Monitoring NDMA formation in an artificial digestion system

Simulated saliva was composed by calcium (1.6 mmol/L), chloride (15.5 mmol/L), phosphate, inorganic (1.6 mmol/L), potassium (14.1 mmol/L), sodium (17.4 mmol/L), ammonia (3.5 mmol/L), glucose (1.1 mmol/L), urea (1.5 mmol/L), α-amylase (100.0 units/mL) and lysozyme (670.0 units/L). The mixture of simulated saliva was adjusted to pH 6.7. Simulated gastric juice was composed of calcium (1.8 mmol/L), potassium (11.6 mmol/L), sodium (49.0 mmol/L), free HCl (57.5 mmol/L), total chloride (119.0 mmol/L) and pepsin (36.4 units/mL). The mixture of simulated gastric juice was adjusted to pH 2.0.

In the meal (CD1, TD1–TD3) listed in Table 1, about 20 g of food samples were minced into small pieces and accurately weighed and homogenized in a mixing blender to which 10 ml of simulated saliva was added. The mixture was then incubated at 37 °C for 5 min before adding 40 ml of simulated gastric juice. The volume ratio of saliva to gastric juice was 1:4 (10 ml and 40 ml, respectively), in con-

Table 1
Diet[a] composition of CD1[b], TD2[c], TD3[d] and TD4[e]

Food materials	(Weight, g)			
	CD1	TD1	TD2	TD3
Cooked rice	250	250	250	250
Egg soup	200	200	200	200
Braised squid (amine precursor)	50	50	50	50
Fried lotus root	48	48	48	48
Macaroni salad	65	65	65	65
Distilled water (NaNO₃ 548 mg)[f]	100	100	100	100
Drinking water	200	–	–	–
Whole strawberries	–	300	–	–
Garlic juice (75 g garlic juice in drinking water)	–	–	200	–
Kale juice	–	–	–	200

[a] Based on 2500 calories a day for male adults by 2000 Recommended Dietary Allowances (RDA) (Korea).
[b] Control diet, CD1 [diet including a high precursor of NDMA (amine and nitrate) + drinking water (200 ml)].
[c] Treatment diet, TD1 [diet including a high precursor of NDMA (amine and nitrate) + whole strawberries (300 g)].
[d] Treatment diet, TD2 [diet including a high precursor of NDMA (amine and nitrate) + garlic juice (200 ml; 75 g garlic juice in drinking water)].
[e] Treatment diet, TD3 [diet including a high precursor of NDMA (amine and nitrate) + kale juice (200 ml)].
[f] NaNO₃ 548 mg corresponding to 400 mg nitrate dissolved in 100 ml of distilled water.

formity with the approximately five-fold dilution of saliva in the stomach as estimated by Boyland and Walker (1974). The mixture was adjusted to pH 2.5 with 3 N HCl and was then incubated in 37 °C incubator for 1 h with gentle shaking. The sample was steam-distilled once by a modification of the method of Hotchkiss, Barbour, and Scanlan (1980). Samples were acidified to pH 1 with sulfuric acid containing sulfamate to prevent artificial nitrosamine formation. One milliliter of 1.50 ppm N-nitrosodi-n-butylamine (NDBA) was added as an internal standard. The sample was steam-distilled on a steam generator and 150 ml of the distillate was collected and then was transferred to a 250 ml flask to which 60 ml of dichloromethane (DCM) and 500 mg of sodium chloride were added. The distillate was extracted three times with 180 ml DCM. The pooled DCM extracts were dried over anhydrous sodium sulfate, concentrated to 3–5 ml in a Kuderna-Danish apparatus, and then blown down under nitrogen to a final volume of 1.0 ml. The concentrated sample was injected into a gas chromatography (GC, Hewlett-Packard model 5890A, Hewlett-Packard,

Avondale, PA)-thermal energy analyzer (TEA, Model 543, Thermo Electron Corp., Waltham, MA).

Conditions for GC–TEA were as follows: DB-5 fused silica capillary column; length 30 m, inside diameter 0.53 mm; flow rate, 5 ml/min; oven temperature programmed, 50–80 °C at 3 °C/min and 80–180 °C at 10 °C/min; injection port temperature, 180 °C; pyrolizer temperature, 550 °C; interface temperature, 200 °C; cold trap temperature, −160 °C; analyzer pressure, 1.9 Torr.

2.3. Inhibition of NDMA formation and nitrite-scavenging ability by Prep-HPLC fractions

The water and methanol soluble portion in garlic were separated by Prep-HPLC (Water Model 201) using a Shimadzu-Prep-ODS column (7.8 mm × 250 mm), a model LKB VWM absorbance detector at 254 nm and a model LKB 2221 integrator. The mobile phase was methanol (60%) and water containing 0.1% formic acid (40%) at a flow rate of 5 ml/min.
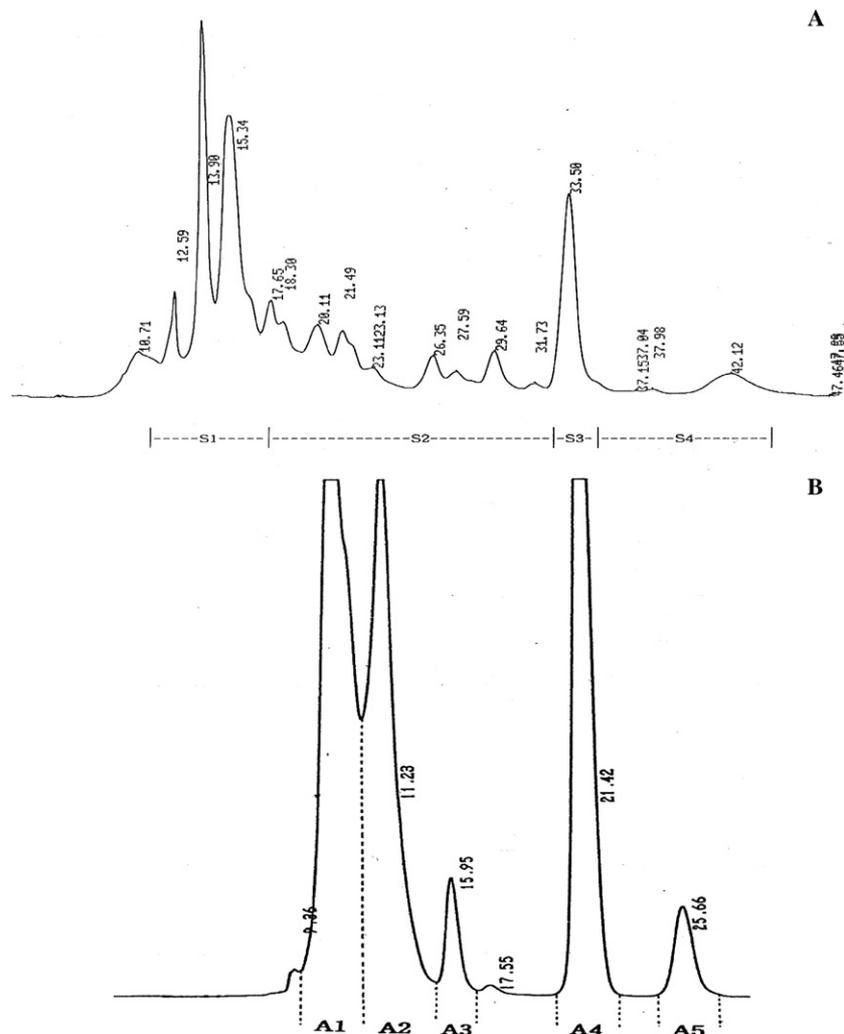

Fig. 1. Preparative-HPLC chromatogram of methanol soluble portion obtained strawberries (A) and water and methanol soluble portion obtained garlic (B).

The methanol soluble portion in strawberries were used for fraction by Prep-HPLC (Pharmacia LKB LCC 2252) using a Shimadzu-Prep-ODS column (7.8 × 250 mm), a UV detector (LKB VWM) operating at 245 nm, and a model LKB 2221 integrator. The gradient was solvent A (2% acetic acid in water) and solvent B (40% acetonitrile in water) from 10% B for 1 min to 80% B in 50 min at a flow rate of 5 ml/min.

The Prep-LC fractions (Fig. 1A: S1–S4; Fig. 1B: A1–A5) were taken from a water and methanol soluble portion of 1.8 kg of garlic or a methanol soluble portion of 1.8 kg of strawberries were vacuum evaporated to dryness and brought up to 36 ml (garlic) or 6 ml (strawberries) with distilled water for the assays about inhibition of NDMA formation and nitrite-scavenging ability. NDMA formation was monitored in a 0.2 M citrate buffer (pH 2.5) containing DMA (200 mM) and sodium nitrite (100 mM). When testing the inhibition of the portion, two levels (strawberries: 0 and 1.0 ml) or four levels of the fractions (garlic: 0, 0.5, 2.0 and 3.0 ml) were added into the reacting mixture. The NDMA-generating solution was incubated for 1 h in a shaker incubator maintained at 37 °C. The reaction was stopped by adding ammonium sulfamate (500 mg), and the resulting NDMA was extracted with DCM (1 ml × 1). The extracts were analyzed for NDMA concentration by GC–TEA.

Nitrite-scavenging ability of each fraction (S1–S4, A1–A5) was carried out by the method of Kang, Park, and Lee (1996) and determined by measuring the residuary nitrite capacity at absorbance 520 nm.

2.4. Confirmation by MS and ^{13}C NMR

Fractions A3 and A4 were completely dried and added to 5 ml of 10% H_2SO_4 in methanol. After 5 h of heating, the reactant was cooled, removed from the methanol, a small amount of distilled water was added, and then extracted with ether and ethylacetate. The extract was dried with sodium sulfate, condensed, and separated using thin layer chromatography (TLC, cellulose plate, ethylacetate:acetone 5:1). The sample separated using TLC was injected into a JM-700 mass spectrometer (Jeol, Japan). ^{13}C nuclear magnetic resonance (NMR, Bruker DRX 500 MHz, Germany) spectra were obtained using a Unity Plus 500 spectrometer (Bruker, Germany).

2.5. Statistical analyses

Each experiment was performed a minimum of three times. Statistical analyses were performed using unpaired Student's t-test. Data are reported as the mean ± SD. Statistical significance level was set to $P < 0.05$.

3. Results and discussion

We studied the amount of NDMA formation after the incubation of nitrate (400 mg/day) in combination with an amine-rich diet with additional drinking water (see Table 1 for diet composition, CD1), and we studied the decreasing of the NDMA amount by addition of whole strawberries, garlic juice or kale juice instead of water in CD1 diet. The NDMA amount in diets containing water (CD1), whole strawberries (see Table 1 for diet composition, TD1), garlic juice (see Table 1 for diet composition, TD2) or kale juice (see Table 1 for diet composition, TD3) with an amine-rich diet was 3.0 ± 0.3, 1.3 ± 0.1, 1.2 ± 0.1 and 1.7 ± 0.1 μg/kg, respectively (Table 2). This result showed that the NDMA amount in the TD1, TD2 and TD3 diets, whole strawberries, garlic juice or kale juice respectively, was significantly decreased compared to the CD1 diet which included only water ($P < 0.05$).

The present study was designed to investigate the inhibition of NDMA of whole strawberries, garlic or kale in simulated saliva and gastric juice as model system of human consumption. These results suggest that in the model system, whole strawberries, garlic juice and kale juice was showed by deceasing of the carcinogenic NDMA formation in this model system. In the model system of human consumption, we used whole strawberries, garlic and kale as a source of dietary ascorbic acid, allyl sulfur compounds and phenolic compounds. Our study was shown to inhibit the formation of NDMA in vitro by the extract of fruits and vegetables and we suggested that the inhibition might be mainly due to ascorbic acid, phenolic compounds and allyl sulfur compounds in strawberry, kale and garlic. This is in accordance with other studies that ascorbic acid and phenolic compounds each inhibit the nitrosation of secondary amines in vitro (Mirvish, Wallcave, Eagen, & Shubik, 1972) and in vivo (Chung et al., 2002; Leaf, Vecchio, Roe, & Hotchkiss, 1987; Ohshima & Bartsch, 1981) by competing for the nitrosating agent.

Fruits and vegetables contain multiple compounds such as ascorbate, sulfur compounds, phenolic compounds, fiber, folic acid, carotenoids, flavonoids, vitamins, and many other agents with possible anticancer effects but

Table 2
The contents of NDMA in experimental diets under simulated gastric digestion

Diets groups	NDMA (μg/kg)	Recovery of NDBA (%)
CD1	3.0 ± 0.3	90.5 ± 2.2
TD1	1.3 ± 0.1[a]	92.5 ± 3.0
TD2	1.2 ± 0.1[a]	90.0 ± 2.7
TD3	1.7 ± 0.1[a]	94.5 ± 3.5

Reaction condition were as follows; CD1: amine-rich diet (Table 1) + nitrate (400 mg/day) + drinking water (200 ml) + simulated saliva (200 ml) and gastric juice (800 ml), TD1: amine-rich diet (Table 1) + strawberries (300 g) + simulated saliva (200 ml) and gastric juice (800 mL), TD2: amine-rich diet (Table 1) + nitrate (400 mg/day) + garlic juice (200 g; 75 g garlic juice in drinking water) + simulated saliva (200 ml) and gastric juice (800 ml), TD3: amine-rich diet (Table 1) + nitrate (400 mg/day) + kale juice (200 ml) + simulated saliva (200 ml) and gastric juice (800 ml). The incubation was performed at 37 °C for 1 h.
[a] Significantly different from control. The level accepted as statistically significant difference was $P < 0.05$. Data are expressed as the mean values ± standard deviation ($n = 3$).

mechanisms responsible for their anticancer properties are not fully understood. Tomatoes, green peppers, pineapples, strawberries, and carrot juice all had a greater ability to inhibit endogenous nitrosation in vivo than an equal amount of ascorbic acid taken with water alone (Helser et al., 1992). Therefore, it seems possible that also phenolic compounds in strawberries might inhibit nitrosation.

For more detailed study, the methanol-soluble fraction of the strawberry juice contained several compounds that were separated by Prep-LC into four fractions (Fig. 1A), S1, S2, S3, and S4, and inhibited NDMA formation by 60.9 ± 4.2, 64.1 ± 3.8, 67.4 ± 5.5, $65.4 \pm 7.3\%$, respectively (Table 3). Fraction S1 showed the greatest nitrite scavenging activity ($68.3 \pm 3.9\%$). The water and methanol soluble portion of the garlic contained several compounds that were separated by Prep-LC into 5 fractions (Fig. 1B).

The A1, A2, A3, and A4 fractions inhibited NDMA formation from 10.5% to 78.9% in a dose-dependent manner (Table 4). The A1 fraction (0.5, 2.0, and 3.0 ml) dose-dependently decreased NDMA formation to 15.8, 55.3 and 71.1%, respectively. Inhibition of NDMA formation by addition of the A2 fraction (0.5, 2.0, and 3.0 ml) increased from 10.5% to 78.9% and inhibition of NDMA formation in the presence of 0.5, 2.0, and 3.0 ml A4, respectively, increased from 23.7% to 50.5%.

The 3.0 ml fractions of A1, A2, A3 and A4 inhibited NDMA formation by 71.1 ± 6.5, 78.9 ± 6.1, 26.8 ± 2.5 and $50.5 \pm 5.4\%$, respectively, while fraction A3 (0.5, 2.0 ml) and A5 (3.0 ml) catalyzed NDMA formation by $18.4 \pm 2.5\%$, $9.7 \pm 1.5\%$, and $10.5 \pm 1.2\%$, respectively. These data indicated that fraction 2 accounted for the greatest inhibition potential. The NDMA formation was inhibited in a dose-dependent manner after exposure to the fractions A1, A2, A3 and A4, respectively.

The nitrite-scavenging ability of fractions A1, A2 and A4 were higher than fractions A3 and A5 (Table 4). Three milliliters fractions A1, A2 and A4 affected nitrite-scavenging ability by 89.3 ± 11.3, 91.2 ± 6.7 and $72.3 \pm 8.7\%$, respectively and the nitrite-scavenging ability of fractions A1–A5 were affected in a dose-dependent manner.

Strawberries contain phenolic compounds such as flavonols, anthocyanidins, hydroxycinnamic acid, ellagic acid

Table 3
Inhibition of NDMA formation and nitrite-scavenging ability by fraction S1–S4 taken from the methanol soluble portion of strawberries 1.8 kg after separation by Prep-HPLC

Fraction number	Added amount (ml)	Nitrite-scavenging (%)	Inhibition of NDMA formation (%)[a]
S1[b]	1.0	68.3 ± 3.9	60.9 ± 4.2
S2	1.0	37.2 ± 2.4	64.1 ± 3.8
S3	1.0	55.0 ± 5.1	67.4 ± 5.5
S4	1.0	16.0 ± 2.1	65.4 ± 7.3

[a] Compared with distilled water control, which is 0% inhibition.
[b] The fraction S1–S4 taken from the methanol soluble portion of strawberries 1.8 kg concentrated until 6 ml. Data are expressed as the mean values ± standard deviation ($n = 3$).

Table 4
Inhibition of NDMA formation and nitrite-scavenging ability by fraction A1–A5 taken from the water and methanol soluble portion of garlic 1.8 kg after separation by Prep-LC

Fraction number	Added amount (ml)	Nitrite-scavenging (%)	Inhibition of NDMA formation (%)[a]
A1[b]	0.5	27.3 ± 3.6	15.8 ± 0.4
	2.0	87.0 ± 10.1	55.3 ± 5.6
	3.0	89.3 ± 11.3	71.1 ± 6.5
A2	0.5	57.2 ± 4.5	10.5 ± 0.4
	2.0	85.2 ± 5.6	39.5 ± 1.0
	3.0	91.2 ± 6.7	78.9 ± 6.1
A3	0.5	30.0 ± 2.1	-18.4 ± 2.5
	2.0	48.5 ± 6.5	-9.7 ± 1.5
	3.0	55.6 ± 5.6	26.8 ± 2.5
A4	0.5	52.0 ± 6.7	23.7 ± 3.4
	2.0	64.6 ± 7.8	28.9 ± 3.2
	3.0	72.3 ± 8.7	50.5 ± 5.4
A5	0.5	2.4 ± 0.1	21.1 ± 3.1
	2.0	10.4 ± 0.2	2.6 ± 0.3
	3.0	13.7 ± 1.1	-10.5 ± 1.2

[a] Compared with distilled water control, which is 0% inhibition.
[b] The fraction A1–A5 taken from the water and methanol soluble portion of garlic 1.8 kg concentrated until 36 ml. Data are expressed as the mean values ± standard deviation ($n = 3$).

and ellagitannin (Seeram, Lee, Scheuller, & Heber, 2006) and have antioxidant, anticancer, anti-atherosclerotic and anti-neurodegenerative properties (Chung et al., 2002; reviewed in Hannum, 2004). All fractions (S1–S4) of the methanol soluble portions, with the exception of the ascorbic acid portion, in strawberry juice inhibited NDMA formation. Our data provides useful information about carcinogenic NDMA inhibition properties due to phenolic compounds in strawberries.

The sulfur compounds in *Allium* vegetables, such as ajoene, dially sulfides and S-allycysteine, have cancer preventive activity in chemically induced animal cancer models (Kim & Chun, 1999). *Allium* vegetables, including garlic and onions, were particularly effective in reducing the chemical formation of *N*-nitrosomorpholine (NMOR) (Dion, Agler, & Milner, 1997). In our study, garlic juice and Prep-LC fraction of water and methanol soluble portion of garlic inhibited NDMA formation in vitro, perhaps via sulfur compounds.

4. Confirmation by MS and ^{13}C NMR

The substances in the fractions A3 and A4 were characterized by mass spectra (Fig. 2A and B) and ^{13}C NMR (Fig. 2C). Its' spectra was in accordance with the published library spectra and literature (Block & Ahmad, 1984; Block, Ahmad, Catalfamo, Jain, & Apitz-Casto, 1986) of allicin in garlic. Fractions A3 and A4 were identified as 1,2-benzendicarboxy acid compound (Fig. 1B: A3) by MS (Fig. 2A) and S-oxodially disulfide compound (Fig. 1B: A4) by MS (Fig. 2B) and ^{13}C-NMR (Fig. 2C).
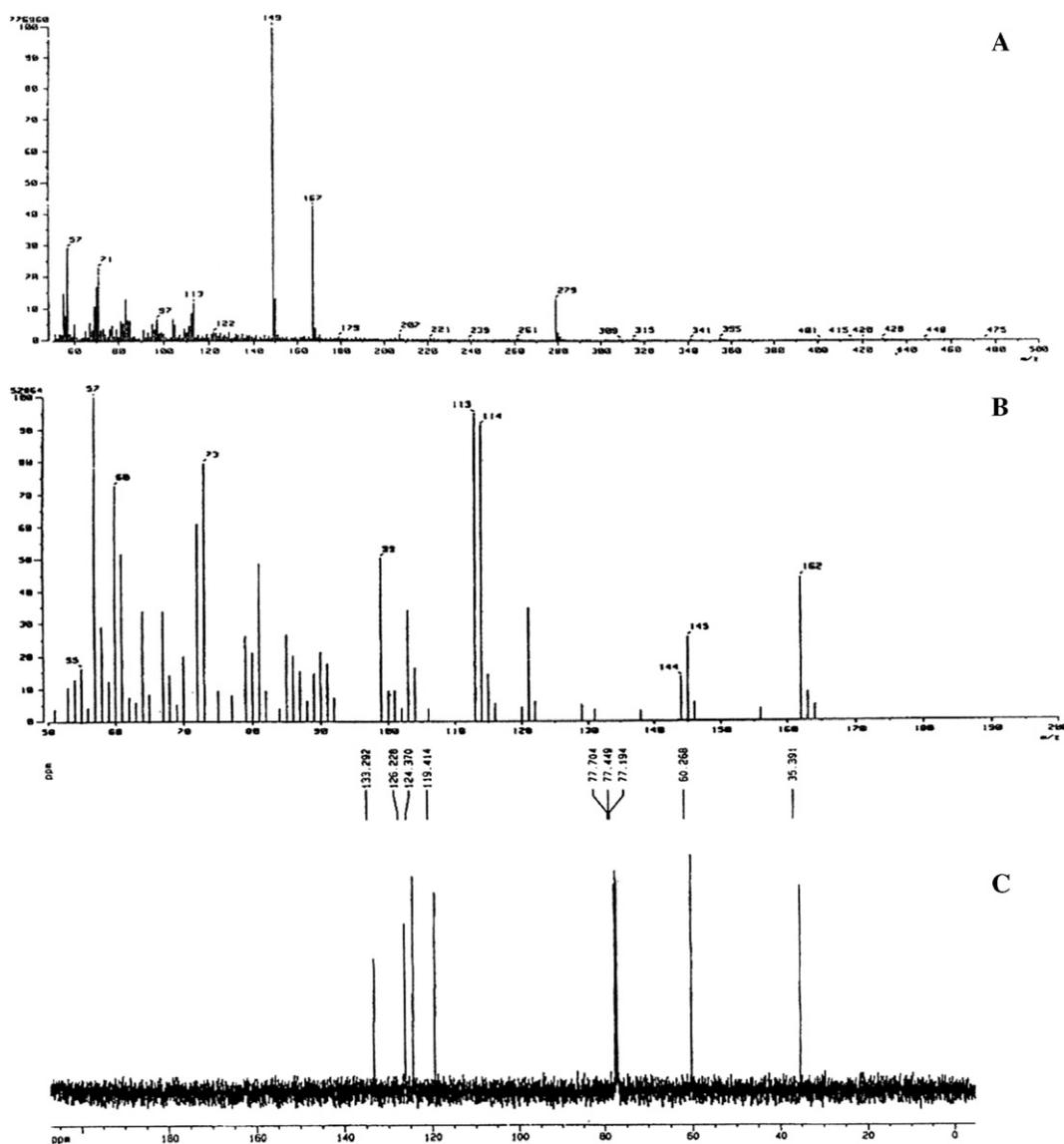
Fig. 2. Mass spectrum of fraction A3 (A) and A4 (B) and ^{13}C NMR (C) spectrum of fraction A4 separated from water and methanol soluble portion of garlic.

We failed in our attempt to confirm the spectra of fractions A1 and A2 due to complex structures including sugars. Sugars were not detected by NMR analysis because they are not soluble in CDCl$_3$.

In conclusion, consumption of a diet rich in nitrate and amine implied the risk of formation of carcinogenic nitrosamines, but we have shown that addition of whole strawberry, garlic or kale juice in combination with an amine-rich diet containing nitrosatable precursors with nitrate (400 mg/day) can significantly decrease NDMA concentration in a simulated human consumption system. This system represents a useful model for carcinogenic NDMA inhibition study in vitro before possible experimentation on animals or humans, and our data is important for the Korean people as phenolic compounds and sulfur compounds could be beneficial food additives. Thus fruits and vegetables contain multiple compounds that might exert protective effects against diseases such as cancer.

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