Comparison of Tomato Phenolic and Ascorbate Fractions on the Inhibition of *N*-Nitroso Compound Formation

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Whole tomato juice has previously been shown to inhibit *N*-nitrosoamino acid formation *in vivo* to a greater extent than expected on the basis of its ascorbate content. The present work compares *in vitro* inhibition of nitrosation by ascorbate and phenolic fractions from tomatoes. Tomato extracts were separated into phenolic and ascorbate portions, and the phenolic portion was further fractionated. Fractions were tested for inhibition of *N*-nitrosomorpholine (NMOR) formation. Several phenolic fractions as well as the ascorbate fraction inhibited NMOR formation. Most of the inhibition from the phenolic fraction was due to *p*-coumaric and chlorogenic acids. The ascorbate fraction contained unidentified compounds that inhibited nitrosation. While both fractions substantially inhibited nitrosation, the ascorbate fraction was more inhibitory than the phenolic fraction.

INTRODUCTION

An inverse relationship exists between fresh fruit and vegetable consumption and certain cancer risks including gastric cancer (National Research Council, 1982). The underlying factors are unknown, but one contributing mechanism may be the inhibition of carcinogenic *N*-nitroso compound (NOC) formation within the body (Mirvish, 1983). Several studies have shown that NOC are formed endogenously (Mirvish, 1975; Ohshima and Bartsch, 1981; Ohshima et al., 1982). Precursors to these NOC include nitrate, nitrite, amines, and amides, all of which come from both dietary and endogenous sources (Leaf et al., 1989; Zeisel et al., 1989). NOC form at acidic and neutral pHs depending on the conditions and type of nitrosating agent available (Challis, 1981). The stomach is a prime location for NOC formation because of its acidic pH and availability of dietary precursors. However, NOC can also form in sites other than the stomach (Leaf et al., 1991).

Ascorbic acid and phenolics each inhibit the nitrosation of secondary amines *in vitro* (Mirvish et al., 1972) and *in vivo* (Ohshima and Bartsch, 1981; Leaf et al., 1987) by competing for nitrosating agent. Since epidemiology indicates that diets rich in fresh fruits and vegetables and not ascorbic acid *per se* lower cancer risk (National Research Council, 1982), other components in these foods could also inhibit the formation of carcinogenic NOC. Previous studies have shown that complex mixtures of plant foods inhibit NOC formation (Kurechi et al., 1980; Stich et al., 1983; Li and Liu, 1991). It has been suggested but not directly demonstrated that this is due to their ascorbate or phenolic content. We recently compared the ability of ascorbic acid alone to that of an equal amount of ascorbic acid taken with fruit and vegetable juices to inhibit the formation of *N*-nitrosoamino acids in humans on controlled diets. Tomato, green pepper, pineapple, strawberry, 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). This suggests that components in addition to ascorbic acid may be important in the *in vivo* inhibition of nitrosation.

The objective of this study was to compare the ability of the phenolic and ascorbate fractions of tomato juice to inhibit nitrosation and to identify the more inhibitory phenolic substances. To accomplish this, fresh tomato extract was fractionated and compared with ascorbic acid for ability to inhibit the *in vivo* formation of *N*-nitrosomorpholine.

MATERIALS AND METHODS

Reagents were obtained as follows: methylene chloride, acetonitrile, and distilled water, Burdick and Jackson Laboratories (Muskegon, MI); test combination 409677, Boehringer-Mannheim (Mannheim, Germany), used to determine the ascorbic acid content of juice and extracts; *N*-nitrosomorpholine (NMOR) and *N*-nitrosodipropylamine (NDPA), synthesized (Lijinsky et al., 1970); chlorogenic acid, *p*-coumaric acid, morpholine, Sigma Chemical Co. (St. Louis, MO). Ripe tomatoes were purchased locally. All remaining chemicals (nitrite, HCl, methanol, and sodium azide) were of ACS reagent or analytical grade and purchased from Mallinckrodt Inc. (Paris, KY) or Fisher Scientific (Fair Lawn, NJ). Ascorbic acid was of pharmaceutical grade and acquired from Hoffmann La-Roche (Nutley, NJ).

NOC are potent animal carcinogens and must be handled appropriately.

Chilled ripe tomatoes were homogenized in a Waring blender and filtered through cheesecloth into a beaker on ice. The extract was adjusted to pH 2.5 with 2 N HCl and centrifuged. The supernatant (referred to as "tomato extract") was separated into an ascorbate portion and a phenolic portion by the method of Seo and Morr (1984) using C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA). The Sep-Pak was preconditioned with 5 mL of MeOH, followed by 5 mL of distilled water and 5 mL of 0.01 N HCl. Ten milliliters of tomato extract was loaded on the Sep-Pak and the unretained eluent collected (referred to as the "ascorbate portion"). The column was then washed with 10 mL of 0.01 N HCl and the eluent discarded. The retained components were eluted with 75% MeOH. The MeOH extract was vacuum evaporated to dryness and resuspended in distilled water (referred to as the "phenolic portion").

HPLC was conducted using a Beckman Model 421A controller, Model 110B pumps, a Model 160 absorbance detector at 254 nM, a model 210A injector (100-μL sample loop), and a Hewlett-Packard Model 3390A integrator. Separations were made on a Brownlee Spheri-5 ODS 5-μm 220 × 4.6 mm i.d. column with a Brownlee RP-18 Newguard 7-μm 15 × 3.2 mm guard column. 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 1 mL/min.

NMOR and NDPA (5–7 μL) were analyzed with a Hewlett-Packard Model 5890 GC and a Model 543 thermal electron analyzer (TEA) detector. The GC was operated isothermally at 155 °C using a 3 m × 2.0 mm i.d. glass column packed with 10%

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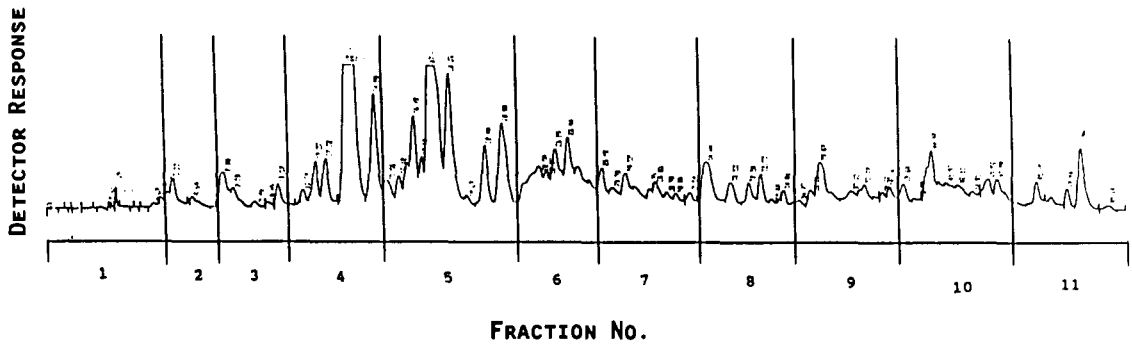

Figure 1. HPLC chromatogram of phenolic portion divided into 11 fractions. Retention times for each fraction are given in Table 1.

Carbowax 20M on 80–100 WHP Chormosorb: injection port, 180 °C; pyrolyzer, 550 °C; interface, 200 °C; cold trap, −160 °C. NMOR and NDPA were quantified by comparison to external standards (370 and 340 ng/mL, respectively, in MeOH).

The ability of extracts, fractions, or single compounds to inhibit nitrosation was determined by comparison to distilled water in a model system consisting of 1.0 mL of 0.6 M citrate buffer (pH 2.0), 1.5 mL of test fraction (or distilled water control), and 20 mM morpholine. Each fraction was vacuum evaporated (40 °C) to dryness and brought up to 1.5 mL with distilled water for the nitrosation assay. Sodium nitrite was added to make the system 0.26 mM, and the mixture was incubated at 37 °C for 30 min in a screw-capped tube. The reaction was stopped by adding sufficient sodium azide to make the solution 0.01 M (Eisenbrand et al., 1981). The NMOR was extracted with methylene chloride (1 × 1 mL) and quantified by GC-TEA. NDPA (340 ng in 1 mL of MeOH) was added immediately prior to extraction as an internal standard. Recovery averaged 92 ● 5%.

The structures of individual phenolic acids were determined by comparison of retention times by both gas chromatography and HPLC to those of authentic compounds after hydrolysis and methylation. Phenolic esters which were collected from the HPLC column were hydrolyzed to free acids using the method of Seo and Morr (1984) (evaporation to dryness, addition of 2 N NaOH, incubation at 23 °C for 6 h). Products were methylated with ethereal diazomethane and chromatographed and coninjected with standards using both HPLC and GC-FID. GC-FID conditions: column, 30 m × 0.53 mm i.d. Restek Rtx-50; He flow, 2 mL/min; column temperature, 70–240 °C at 8 °C/min; injector, 200 °C; detector, 250 °C. HPLC conditions for rechromatography of hydrolyzed products were the same as given above for original extracts. Order and time of elution for HPLC were similar to those reported in the literature (Winter and Herrmann, 1986).

RESULTS AND DISCUSSION

The C_{18} SPE procedure separates aqueous plant extracts into ascorbate and phenolic portions (Seo and Morr, 1984). Each of the two portions of tomato extract inhibited nitrosation, and each contained several compounds that were separated by HPLC. Phenolic acids such as caffeic acid (Kuenzig et al., 1984) and its quinic acid ester chlorogenic acid (Pignatelli et al., 1982) have been shown to inhibit N-nitrosation when tested as single compounds. The phenolic portion from 30 mL of tomato extract was separated by HPLC into 11 fractions, each containing several components (Figure 1). Several of these fractions inhibited NMOR formation (Table 1). Fraction 5 inhibited NMOR formation by 72%, while the whole phenolic portion inhibited NMOR formation by 85%, indicating that fraction 5 accounted for most of the inhibition in this fraction.

Fraction 5 was further separated by HPLC into seven subfractions (subfractions 5a–g; 14.5–24.0-min retention time; Figure 2), several of which inhibited NMOR formation (Table 2). Comparison of the retention times and cochromatography of unknown peaks with authentic compounds indicated that subfraction 5d contained chlorogenic acid (retention time = 18.36 min; Figure 2). However, this subfraction accounted for a smaller portion of the inhibition than subfractions 5c,e–g (Table 2).

Table 1. Inhibition of NMOR Formation by the Components from the Phenolic Portion of 30 mL of Tomato Juice before and after Separation by HPLC

HPLC separation	retention time (min)	% inhibition[a]
before separation		
phenolic portion		85
after separation		
fraction 1	0.0–5.0	0
fraction 2	5.0–7.8	0
fraction 3	7.8–11.3	0
fraction 4	11.3–15.4	3
fraction 5	15.4–21.1	72
fraction 6	21.1–25.0	28
fraction 7	25.0–30.0	15
fraction 8	30.0–35.0	14
fraction 9	35.0–39.2	7
fraction 10	39.2–44.5	28
fraction 11	44.5–49.5	31

[a] Compared to distilled water control, which is 0% inhibition.

The most inhibitory subfraction (5c) was hydrolyzed by the alkaline method of Seo and Morr (1984) to identify any phenolic acids present as glucosides or esters. Chromatography of the hydrolysate of subfraction 5c by both GC-FID and HPLC indicated the presence of free *p*-coumaric acid. While the glucoside of *p*-coumaric acid would not be expected to be inhibitory, analysis of the products after reaction under the acidic conditions of the nitrosation assay showed that free *p*-coumaric was liberated. This explains the inhibitory effect of the glucoside/ester. Because our nitrosation assay was similar in pH (2.0), time (30 min), and temperature (37 °C) to conditions found in the stomach, it is likely that glucosides of *p*-coumaric acid as well as glucosides of other phenolics would be hydrolyzed to some degree in the acidic environment of the stomach and would similarly inhibit NOC formation *in vivo*. Esters or glucosides of phenolic acids (e.g., caffeic, ferulic, and *p*-coumaric acids) are known to be present in tomato extract without regard to variety in concentrations of 2–71 mg/kg of fresh weight (Winter and Herrmann, 1986).

To determine the relative inhibitory ability of each tomato extract component, an amount of ascorbate, *p*-coumaric acid, or chlorogenic acid equivalent to that contained in 0.5, 1.0, and 5.0 mL of tomato extract, the combined ascorbate portion, and the combined phenolic portion were compared in nitrosation assays using equal volumes. Inhibition by an amount of aqueous ascorbic acid equivalent to that contained in the ascorbate portion was also tested. This ascorbic acid concentration (85 mg/L) was the same as that in the whole tomato extract and ascorbate fractions. No ascorbic acid was present in the

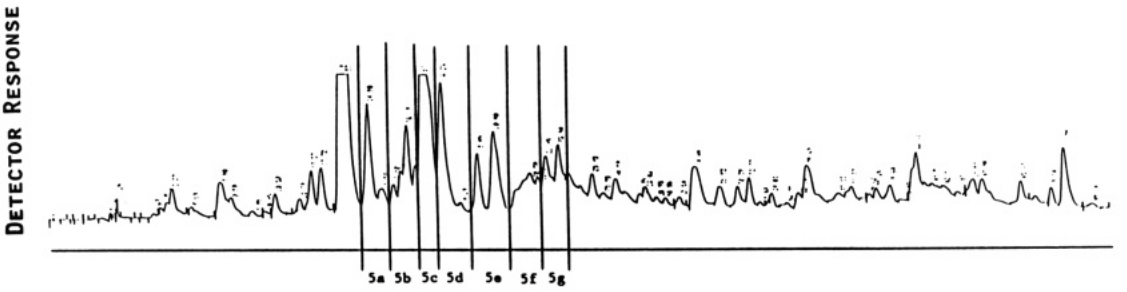

Figure 2. HPLC chromatogram of phenolic portion with fraction 5 (Figure 1) divided into seven subfractions. Retention times for each fraction are given in Table 2.

Table 2. Inhibition of NMOR Formation by Subfractions of Fraction 5 (Figure 1; Table 1) Taken from the Phenolic Portion of 30 mL of Tomato Juice

fraction	retention time (min)	% inhibition[a]
subfraction 5a	14.5–15.9	0
subfraction 5b	15.9–17.3	0
subfraction 5c	17.3–17.8	45
subfraction 5d	17.8–19.4	19
subfraction 5e	19.4–21.1	27
subfraction 5f	21.1–23.0	21
subfraction 5g	23.0–24.0	22

[a] Compared to distilled water control, which is 0% inhibition.

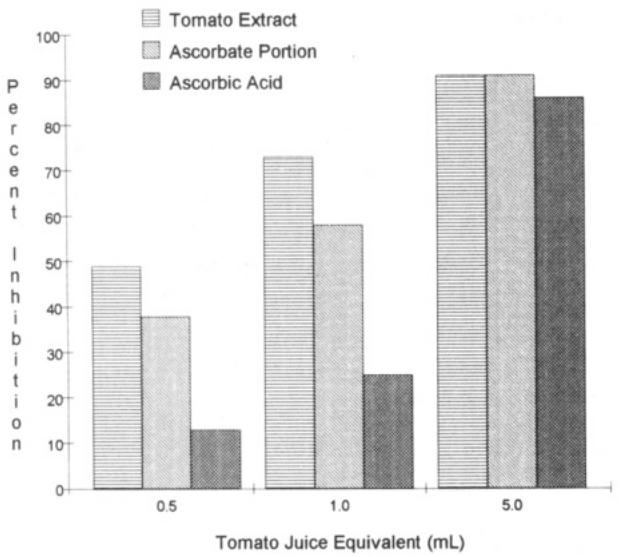

Figure 3. Percent inhibition of NMOR formation by different amounts of tomato extract derived from the same batch of juice and the same concentration of ascorbic acid compared to water. Ascorbate portion refers to the combined ascorbate portion isolated by C_{18} Sep-Pak.

phenolic portion. Chlorogenic (2.7 mg/100 mL) and *p*-coumaric acids (1.9 mg/100 mL) were similarly tested. The concentration of these phenolic acids was based on the average concentrations in the ripe tomato (Winter and Herrmann, 1986).

Comparison of the ascorbate and phenolic portions (Figures 3 and 4) indicated that both inhibited nitrosation, but the ascorbate fraction gave greater inhibition. The amount of the ascorbate fraction contained in 0.5 mL of juice (containing 45 μg of ascorbate) inhibited morpholine nitrosation by 40%. The same amount of phenolic fraction gave 20% inhibition, suggesting that in whole juice the ascorbate fraction contributes more to inhibition than the phenolic fraction. Inhibition by the ascorbate fraction could not be totally explained by the ascorbate content. Inhibition in the ascorbate portion (40%) was greater than

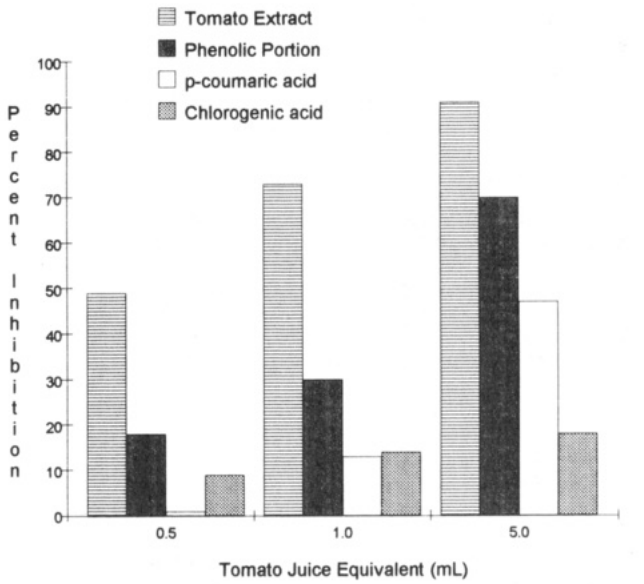

Figure 4. Percent inhibition of NMOR formation by different amounts of tomato extract and the same amount of *p*-coumaric acid or chlorogenic acid compared to water. All juices and extracts were from the same batch as those reported in Figure 3. Phenolic portion refers to the combined phenolic portion isolated by C_{18} Sep-Pak.

that shown by an equivalent amount of ascorbate in water (10%), indicating that other compounds with inhibitory activity were present in this fraction (Figure 3). Whole juice gave approximately 50% inhibition at the same concentration (i.e., 0.5 mL). With higher amounts of inhibitory compounds (e.g., the amount contained in 1 mL), ascorbic acid inhibited the formation of NMOR by 25%, while the whole ascorbate portion inhibited the formation of NMOR by 60% (Figure 3). With yet higher amounts of extract, (i.e., 5.0 mL), the contribution of any additional components in the ascorbate portion was overwhelmed by the nearly complete inhibition of nitrosation by ascorbic acid (Figure 3). A degradation product of ascorbic acid, 3-hydroxy-2-pyranone (Tatum et al., 1969), has been identified as an inhibitory compound in Chinese wild plum extract by Normington et al. (1986).

The inhibition observed in the phenolic portion was due to components other than ascorbic acid, since this portion was devoid of ascorbate. Chlorogenic acid and *p*-coumaric acid together accounted for most of the inhibition contained in the phenolic portion (Figure 4). These data indicate that chlorogenic and *p*-coumaric acids contribute significantly to inhibition by the phenolic portion.

The inhibition of nitrosation observed in the phenolic portion supports previous investigations done with single

phenolic compounds showing that they are effective inhibitors of nitrosation (Pignatelli et al., 1983; Stich et al., 1983; Kurechi et al., 1980). *p*-Coumaric acid was the most inhibitory phenolic found in tomato extract. Both free phenolic acids as well as their glucosides are capable of inhibiting nitrosation after the latter are hydrolyzed under conditions similar to those in the stomach.

Other fruits and vegetables that have been shown to inhibit nitrosation *in vivo* (Helser et al., 1992) contain phenolics similar to those in tomato, and comparable results from fractionation of these foods would be expected. These data show that ascorbate accounts for only a portion of the inhibition of nitrosation seen with whole tomato juice and support our *in vivo* observation (Helser et al., 1992) that whole juices provide more inhibition than an equivalent amount of ascorbic acid. Although the consumption of foods containing ascorbic acid has been well correlated with a decreased risk of gastric cancer, these data show that ascorbic acid is not the only component of tomatoes which inhibits nitrosation.

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