Volatile Sulfur Compounds in Fresh Orange and Grapefruit Juices: Identification, Quantitation, and Possible Importance to Juice Flavor

Philip E. Shaw,* James M. Ammons, and Robert S. Braman

A sensitive method for detecting volatile sulfur compounds was used to determine sulfur compounds in vapors above orange and grapefruit juices. Hydrogen sulfide and methyl sulfide were found in all samples; SO_2, COS, MeSH, and some higher alkyl sulfides were detected in some. Of all these, only hydrogen sulfide has been reported as a constituent of orange and grapefruit juices. Since hydrogen sulfide and methyl sulfide were found in the parts-per-billion concentration range, levels which are above their reported aroma thresholds in air, they probably contribute to the flavor of fresh orange and grapefruit juice.

The presence of hydrogen sulfide in freshly extracted orange juice has long been recognized, but its identification, quantitative determination, and contribution to fresh orange flavor have received little attention (Veldhuis, 1971). Unidentified volatile sulfides have been implicated as constituents of fresh orange juice (Nolte et al., 1942) and of aqueous orange essence (Wolford and Attaway, 1967). In the latter study, removal of the volatile sulfides, as complexes of mercuric chloride, caused noticeable change in the aroma of the essence. However, none of the sulfides were positively identified.

In some of the earliest studies on volatile constituents of orange and grapefruit juices, hydrogen sulfide was identified and the quantity present was estimated. Kirchner et al. (1950) distilled freshly prepared juices of California Valencia orange and Marsh seedless grapefruit, treated the distillates with a lead salt, and estimated that the concentrations of H_2S in the juices were 1.6 ppm for the orange and 0.9 ppm for the grapefruit. Kirchner and Miller (1957) detected a trace of hydrogen sulfide in fresh and freshly canned Valencia orange juice but none in a stored canned sample of the same juice. Kirchner et al. (1953) detected a trace of hydrogen sulfide in fresh and stored canned grapefruit juice but none in a freshly canned sample of that juice. In the 1953 and 1957 studies, the authors did not describe their method for positive identification of hydrogen sulfide, nor did they attempt to quantitatively determine it. No attempts were made in any of the above studies to assess the contribution of hydrogen sulfide to fresh citrus flavor or to any possible off-flavors in the juice samples. It is interesting that in no subsequent studies, even those conducted with more sophisticated equipment such as gas chromatography for identifying volatile constituents, has hydrogen sulfide or any other volatile sulfide been identified as a constituent of orange or grapefruit juices or oils (Shaw, 1977). However, Imagawa et al. (1974) did detect hydrogen sulfide and methyl sulfide in the headspace gases from concentrated juice of *Citrus unshiu* (Satsuma); also, they found the level of methyl sulfide much higher in headspace gases from concentrated juice than in those from fresh juice.

Taste and aroma threshold values for hydrogen sulfide and methyl sulfide reported by several workers have been summarized by Stahl (1973). Aroma threshold values in air range from 0.18 to 130 ppb for hydrogen sulfide and from 0.0094 to 20 ppb for methyl sulfide. Campbell et al. (1958) determined a taste threshold of 51 ppb hydrogen sulfide in water and 210 ppb in brewed coffee. They noted that levels above 210 ppb in brewed coffee were difficult to measure due to losses by volatilization. Guadagni et al. (1969) found a synergistic effect between the aromas of methyl sulfide and tomato juice in aqueous solution.

In the current study, levels of hydrogen sulfide in the headspace gases of orange and grapefruit juices were determined by a procedure that detects hydrogen sulfide as well as other volatile sulfur-containing compounds at levels lower than 1 ppb. Effects of added hydrogen sulfide on the composition of volatile sulfides in the headspace gases of fresh and processed orange juice were studied, and the results correlated with the composition of volatile sulfides in freshly squeezed juice.

EXPERIMENTAL SECTION

Samples of Marsh seedless grapefruit were obtained in May 1979 from the Subtropical Horticulture Research Station, Miami, FL. Samples of Valencia oranges were obtained in June 1979 either from the Science and Education Research Center, Lake Alfred, FL, or from a local market. California Navel oranges and frozen concentrated orange juice samples were purchased from a local market.

Preparation of Samples for Headspace Analyses. The fresh juice sample to be analyzed was prepared, hand reamed from the fruit, and 800 mL was filtered with a coarse kitchen strainer into a 10-L glass bottle. The bottle was then capped with a cork stopper through which an 8-mm i.d. glass tube had been inserted to a depth of 10 cm below the bottom of the cork. A rubber septum was attached on the upper end of the glass tube for withdrawal of headspace gas samples. The frozen concentrated orange juice sample was reconstituted to single-strength with tap water, and 750 mL of this sample was placed in the 10-L glass bottle. The brix and acid values for the samples were as follows: for Valencia orange juice, brix 9.8°, acid 0.70 g/100 mL, brix/acid ratio 10.0; Navel orange juice, brix 9.53°, acid 1.08 g/100 mL, brix/acid ratio 10.4; Marsh seedless grapefruit juice, brix 10.2°, acid 1.04 g/100 mL, brix/acid ratio 11.1; for reconstituted frozen concentrated orange juice, brix 12.09°, acid 0.81 g/100 mL, brix/acid ratio 14.93.

Preparation of Blanks. Blanks for monitoring retention or decomposition of H_2S in the headspace sampling system, which included the 10-L bottle with or without sample and preconcentration trap, were prepared as follows: (a) A standard 1-ppm gas mix of H_2S in air was prepared by injecting 10.0 μL of H_2S gas (Matheson) into a 10-L bottle. A calibration curve was prepared, showing the recorder responses for 0.25–5.0-mL samples of this mix (0.25–5.0 nL of H_2S) injected directly into the gas chro-

U.S. Citrus and Subtropical Products Laboratory, Southern Region, U.S. Department of Agriculture, Agricultural Research, Science and Education Administration, Winter Haven, Florida 33880 (P.E.S.), and the Department of Chemistry, University of South Florida, Tampa, Florida 33620 (J.M.A., R.S.B.).

Table I. Determination of Volatile Sulfides in Headspace Gases of Orange and Grapefruit Juices[a]

juice sample and time after preparation	concn present,[b] ppb in headspace gases					
	H_2S	Me_2S	MeSH	SO_2	COS	other S
Marsh grapefruit, 10 min	1.9	0.5	N	+	+	N
California Navel orange A						
42 min	11	3.5	N	+	+	N
60 min	18	3.3	+	+	+	N
California Navel orange B						
5 min	26	0.5	N	N	N	N
28 min	22	+	N	N	N	N
spiked 60 min[c]	23	12	+	+	+	+
Valencia orange A, 45 min	2.0	N	N	N	N	N
Valencia orange B, 10 min	1.9	0.5	N	+	+	N
reconst frozen concentrated orange juice						
7 min	2.6	N	N	N	N	N
spiked 8 min[d]	10	11	+	+	+	+
distilled water blank						
1 min	N	N	N	N	N	N
spiked 10 min[e]	+	+	+	+	+	+

[a] Sample size was 3.4 L for Valencia orange B and 0.57 L for all other samples. A and B samples were different juices made from the same batch of fruit. [b] N = not detected; + = present but not quantitated (not major fraction). [c] Spiked with 10 μL of H_2S in headspace gases at 53 min, then let stand 1 h. [d] Spiked with 10 μL of H_2S in headspace gases at 60 min, then let stand 8 min. [e] Spiked with 10 μL of H_2S in headspace gases initially, then let stand 10 min.

matograph, and a linear log–log relationship was obtained ($\ln y = 1.859 \ln x + 0.176$). Reproducibility was determined from the analysis of five 10-mL samples, each containing 10 nL of H_2S. The coefficient of variation was about 3%. (b) Distilled water (800 mL) was added to the 10-L glass bottle, and a 0.57-L trap sample (see below) was withdrawn in 1 min and analyzed. Then, 10 μL of H_2S was added to the headspace gases, and 10 min later a 0.57-L trap sample was withdrawn and analyzed. Results on these two blanks are listed in Table I.

Analytical Procedures. Volatile sulfides were quantitatively analyzed as described by Braman et al. (1978) with a gas chromatograph equipped with a flame photometric detector. Non-sulfur-containing compounds are not detected by this procedure. The samples for gas analyses were either 10-mL portions of headspace gases withdrawn through the septum with a gas-tight syringe or preconcentrated trap samples. The 10-mL headspace samples were withdrawn within 1 min after juice extraction.

Preconcentrated trap samples were analyzed because the concentrations of volatile sulfur compounds in headspace samples were low. By this method of analysis (Braman and Ammons, 1978), 0.57 L of headspace was withdrawn through a gold foil trap in 30 s. The gold adsorbed the volatile sulfur compounds, and hydrogen sulfide and methyl mercaptan were derivatized in the trap with ethyl iodide to diethyl sulfide and methyl ethyl sulfide, respectively. By a stream of nitrogen the alkyl sulfides and other sulfur compounds were eluted from the trap onto the GC column for separation and detection. A typical GC separation is shown in Figure 1. Standard curves of gas chromatographic response vs. concentration were prepared for hydrogen sulfide and methyl sulfide with the diffusion tube standards of O'Keefe and Ortman (1966). By this procedure, hydrogen sulfide and methyl sulfide concentrations as low as 1 part per trillion can be quantified.

RESULTS AND DISCUSSION

Application of a new sensitive procedure for determining volatile sulfur compounds to the analysis of headspace gases of fresh and processed orange and grapefruit juices has afforded the results in Table I. Attempts to measure hydrogen sulfide levels within 1 min after juice extraction by direct injection of 10 mL of headspace gases onto the chromatographic column failed to show the presence of H_2S. Since this indicated that the level may have been

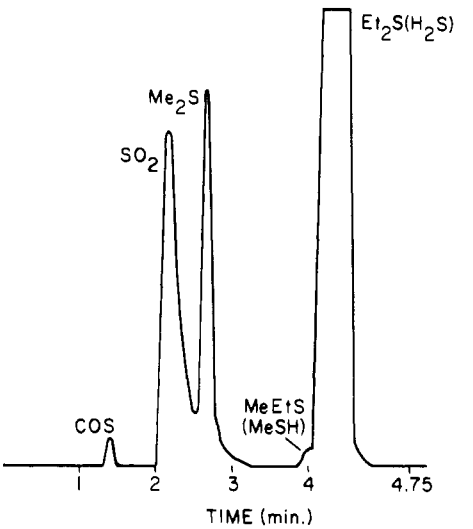

Figure 1. Typical separation of volatile sulfur compounds after preconcentration of headspace gases and derivatization (Navel orange juice sample A).

below the limit of detection for direct injection (25 ppm), a preconcentrated sample was then analyzed. This procedure of analyzing 10 mL of headspace and then a preconcentrated sample was followed for all the juices except the California Navel orange juice B. This sample was not analyzed by the direct injection method first because we wanted to obtain a preconcentrated sample at the earliest possible time.

Results for the preconcentrated samples are summarized in Table I. Sulfur dioxide and carbonyl sulfide were detected in the headspace of Marsh grapefruit juice by their gas chromatographic retention times, but no attempt was made to quantitate them. No methanethiol or higher molecular weight alkyl sulfides were detected in that sample.

The concentrations of sulfides in the headspace of two different samples of fresh California Navel orange juice from the same batch of fruit were monitored. In the headspace of sample A during the interval between 42 and 60 min after extraction, hydrogen sulfide increased and methanethiol increased to a detectable level (Table I). Of the headspace samples analyzed, the one for sample B after 5 min had the highest level of hydrogen sulfide (26 ppb).

Levels of both hydrogen sulfide and methyl sulfide were lower after 28 min than after 5 min. At neither of those sampling times were any of the other volatile sulfur-containing compounds detected. After 53 min, 10 μL of hydrogen sulfide gas was added to the headspace gases. One hour later, the hydrogen sulfide was at about the same concentration as it was at the 5-min sampling, but methyl sulfide was greatly increased and the other volatile sulfur compounds were all detected.

The headspace of fresh Florida Valencia orange juice A (Table I) was analyzed after 45 min and contained only hydrogen sulfide. A second larger sample of headspace (3.4 L) over another juice sample was therefore analyzed, and this 10-min sample contained about the same level of H_2S as the 45-min sample but also a quantifiable amount of methyl sulfide and detectable amounts of sulfur dioxide and carbonyl sulfide.

A relatively low level of volatile sulfides in the headspace of single-strength orange juice prepared from frozen concentrate would be expected because of expected losses of volatiles during preparation of the concentrate, and only hydrogen sulfide was detected in the 7-min sample (Table I). However, 8 min after the headspace gases had been spiked with 10 μL of hydrogen sulfide gas, the headspace contained 10 ppb H_2S, 11 ppb methyl sulfide, and detectable amounts of other volatile sulfur compounds.

Except for hydrogen sulfide, the volatile sulfur compounds found in this study have not been reported as natural constituents of orange or grapefruit. Alkyl thiols would have been detected (after derivatization with ethyl iodide) as their ethyl alkyl derivatives by gas chromatographic retention times (Braman and Ammons, 1978). Thus, diethyl sulfide and ethyl methyl sulfide, if present in the juice samples, would have been eluted with derivatized hydrogen sulfide and methanethiol, respectively, and would not have been detected separately.

A preconcentrated trap sample of headspace gases above one juice sample was chromatographed for volatile sulfides without added ethyl iodide so that ethyl sulfide and ethyl methyl sulfide would be detected if present in the juice; neither of those sulfides was present at detectable levels. The higher molecular weight alkane sulfides referred to in the last column of Table I consisted of three compounds whose retention times were in the range expected for ethyl propyl sulfide and ethyl butyl sulfide, but they remain unidentified.

We used several blanks to examine the stability of hydrogen sulfide in the headspace sampling system and to determine whether the hydrogen sulfide standard was contaminated by other volatile sulfides. A blank consisting of the hydrogen sulfide (from a lecture bottle) used to determine a calibration standard curve contained no other volatile sulfur compounds. Also, a blank consisting of a preconcentrated trap sample equivalent to 0.57 L of headspace gases over 800 mL of distilled water and sampled after 1 min contained no volatile sulfur compounds. However, when the headspace gases above 800 mL of distilled water were spiked with 10 μL of H_2S, and a preconcentrated trap sample was analyzed after 10 min, the other volatile sulfur compounds were detected in addition to H_2S (Table I). Injection of 10 μL of H_2S directly onto the gold trap in the presence of ethyl iodide caused the formation of ethyl sulfide as the only detectable volatile sulfur compound. These results suggest that hydrogen sulfide reacted with other components in water or in juice to produce all of the other volatile sulfides found in the headspaces of the juices.

Some possible sources for error were studied. To determine a calibration curve, we prepared an H_2S standard in the same 10-L bottle that was used for the juice experiments. In this way, we compensated for any losses due to sorption of H_2S by the sampling system. The COS peak in Figure 1 is very close to that for H_2S, but no H_2S was eluted with COS because underivatized H_2S and MeSH are not eluted from the gold trap under the conditions used.

It seems likely that hydrogen sulfide was one source of the other sulfur compounds found in this study. Under the acidic conditions of the juice samples, most of the hydrogen sulfide in the sample should be found in the headspace gases (Broderius and Smith, 1977), where it could form the other sulfur compounds we found. The rapid formation of SO_2 and COS from H_2S could easily be rationalized since H_2S oxidizes to SO_2 and may equilibrate with CO_2 to afford COS and H_2O. Results from the samples of orange juice and distilled water spiked with 10 μL of H_2S indicate that a complex series of reactions may have occurred to form the alkyl sulfur compounds from H_2S. Spiking increased the level of H_2S only slightly but resulted in detection of other volatile sulfur compounds. The fact that H_2S-spiked distilled water and reconstituted frozen concentrated orange juice produced the same mixture of volatile sulfides that fresh juice produced suggests that the sulfur compounds other than H_2S were formed nonenzymatically in reactions probably catalyzed by trace contaminants in the distilled water and aqueous mixtures.

Levels of hydrogen sulfide and methyl sulfide measured in this study are within the range of aroma threshold values reported for these compounds in air (H_2S, 0.18–130 ppb, and methyl sulfide, 0.0094–20 ppb). With the acting of possible synergistic effects (Guadagni et al., 1969), these two volatile, potent flavor compounds could well exert a marked effect on the flavor of fresh citrus juices. Initial attempts to evaluate the contribution of these volatile sulfides to the aroma of fresh orange juice were hampered by the transient nature of the aroma of freshly squeezed, unpasteurized orange juice and no definitive results were obtained. Work is in progress to properly evaluate the contribution of the volatile sulfides to fresh juice flavor.

CONCLUSION

Measureable levels of hydrogen sulfide, methyl sulfide, and other volatile sulfur compounds were present in fresh orange and grapefruit juices, and they persisted in the headspace gases for at least an hour after extraction. This study suggests that hydrogen sulfide reacts with other juice components to produce a complex mixture of volatile sulfur compounds.

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Mutagenicity of 2-Alkyl-*N*-nitrosothiazolidines

Jun Sekizawa and Takayuki Shibamoto*

N-Nitrosothiazolidine and its 2-alkyl derivatives, which formed in the browning reaction with nitrite, were tested for mutagenicity by using the Ames *Salmonella*/microsome mutagenicity assay. Some *N*-nitrosothiazolidines showed positive mutagenic responses toward *Salmonella typhimurium* TA 100, and the order of mutagenic potency relative to their 2-alkyl substituents was as follows: unsubstituted > isopropyl > propyl > ethyl > butyl > isobutyl > methyl. The metabolic activation system (S-9 mix) was not required to detect mutagenicity. In fact, the addition of S-9 mix strongly suppressed the mutagenic activity of *N*-nitrosothiazolidines. The factors which caused deactivation of the mutagens in S-9 mix were also investigated.

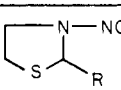
Tremendous numbers of chemicals are produced when foods are cooked. These chemicals include many nitrogen- and sulfur-containing compounds such as pyrazines, thiophenes, and thiazoles (dehydrogenated products of thiazolidines) (Wilson et al., 1973; Persson and von Sydow, 1973; Mussinan and Walradt, 1974). Extensive research in flavor chemistry has led to the isolation and identification of hundreds of these chemicals. Recently, increasing attention has been paid to the potential mutagenicity and carcinogenicity of these chemicals (Kosuge et al., 1978; Yamamoto et al., 1978; Yoshida, 1979).

Sakaguchi and Shibamoto (1978) observed the formation of thiazolidine and its alkyl derivatives by heating an aqueous cysteamine and acetaldehyde mixture in the course of their study of a nonenzymatic browning model system. Thiazolidines have a strong roasted flavor and can be readily nitrosated with sodium nitrite. The resultant nitroso derivatives showed some mutagenicity on *S. typhimurium* (Mihara and Shibamoto, 1979). In the present study, the effect of the alkyl side chain substituents of *N*-nitrosothiazolidines on their mutagenicities was investigated. The effect of the metabolic activation system (S-9 mix) and the mechanism of suppression of the mutagenicity of *N*-nitrosothiazolidines by S-9 mix were also discussed.

EXPERIMENTAL SECTION

Materials. Thiazolidine and its 2-alkyl derivatives were prepared from cysteamine and the corresponding fatty aldehydes (Nakarai Chemicals Ltd., Kyoto) as described by Ratner and Clarke (1973). Nitrosation of thiazolidines was performed as described by Ray (1978). The *N*-nitrosothiazolidines tested for mutagenicity are shown in Table I. Dimethyl sulfoxide (Me_2SO), polychlorinated biphenyl (PCB), and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NMNG) were purchased from Wako Pure Chemical (Osaka). 5,6-Benzoflavone (BF), benzo[*a*]pyrene (BP), and phenobarbital (PB) were obtained from Aldrich Chemical (Milwaukee, Wis.), Sigma Chemical (St. Louis, Mo.), and Yamazen Chemical (Osaka), respectively. 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) was donated by Dr. T. Matsushima (Tokyo University). All other chemicals were also obtained from reliable sources.

Table I. Chemicals Tested for Mutagenicity

	abbrev	R	mol wt
N-nitrosothiazolidine	NT		118
2-methyl-*N*-nitroso-thiazolidine	MNT	CH_3	132
2-ethyl-*N*-nitroso-thiazolidine	ENT	CH_3CH_2	146
2-propyl-*N*-nitroso-thiazolidine	PNT	$CH_3CH_2CH_2$	160
2-isopropyl-*N*-nitroso-thiazolidine	IPNT	$(CH_3)_2>CH$	160
2-butyl-*N*-nitroso-thiazolidine	BNT	$CH_3CH_2CH_2CH_2$	174
2-isobutyl-*N*-nitroso-thiazolidine	IBNT	$(CH_3)_2>CHCH_2$	174

Metabolic Activation System. Rat liver homogenate (S-9) was obtained from male Sprague-Dawley rats (200–210 g) which were injected either with PB and BF or with PCB, or from rats which were fed PB in their drinking water (Ames et al., 1975). Fifty microliters (per plate) of S-9 mix prepared from BP plus BF-induced rats was used in most cases. The amount of S-9 mix per plate (50 μL/plate or 100 μL/plate) did not lead to significant differences in the results.

Mutagenicity Test. Mutagenicity tests were conducted

Department of Environmental Toxicology, University of California, Davis, California 95616 (T.S.), and Ogawa & Co. Ltd., 1-12-3 Niitaka Yodogawa-Ku, Osaka, Japan (J.S.).

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