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Antioxidant Compounds in Traditional Indian Pickles May Prevent the Process-Induced Formation of Benzene

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ABSTRACT

Pickles in the Indian market contain ascorbic acid from the raw material used and benzoate as an added preservative that are involved in the formation of benzene in soft drinks. In this work, 24 market pickle samples were surveyed for benzene content, as well as its precursors and other constituents that influence its formation. The analysis showed that pickle samples were high in acid content (low pH) and showed significant amount of ascorbic acid, minerals (Cu and Fe), and benzoic acid present in them. Also, most samples exhibited high antioxidant activity that might be attributed to the ingredients used, such as fruits and spices. The solid-phase microextraction headspace gas chromatography–mass spectrometry method was developed in-house for benzene analysis. Eleven of 24 samples had benzene, with the highest concentration of 4.36 ± 0.82 μg of benzene per kg of pickle for a lime pickle that was also reported to have highest benzoic acid and considerably less hydroxyl radical (•OH) scavenging activity. However, benzene levels for all 11 samples were considerably below the World Health Organization regulatory limit of 10 μg/kg for benzene in mineral water. Studies on model systems revealed that the high antioxidant activity of Indian pickles may have had a strong inhibitory effect on benzene formation.

Benzene is placed in group 1 of the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, indicating it to be a proven carcinogen. Primarily, it causes acute myeloid leukemia in adults. Acute lymphocytic leukemia, chronic lymphocytic leukemia, multiple myeloma, and non-Hodgkin lymphoma are also linked with its exposures *(23)*. Automobile exhaust and active and passive smoking are mainly considered as principal routes for its exposure *(44)*. The exposure from food and water is relatively low, being below 1.5 to 2% of total exposure *(11)*. High levels of benzene metabolites, frequently reported among children and nonsmoking workers without occupational exposure, however, suggested that there may be significant unidentified sources of benzene *(24)*. Use of contaminated water during manufacture of foods, smoking in meat preparations *(47),* thermal treatment causing degradation of β-carotene, phenylalanine and terpenes *(27, 29),* food irradiation *(1, 8),* use of food-grade hexane for vegetable oil extraction *(32),* and artificial CO_2 used for carbonation in beers *(30, 31)* are some of the possible routes through which benzene might be either formed or enter into foodstuffs. However, the interaction of benzoic acid (BA) and ascorbic acid (AA) in the presence of metals, particularly Cu and Fe, and oxygen has been shown *(17)* to be the most likely route for benzene formation. Subsequently, numerous surveys were conducted by researchers in many countries to assess the risk, if any, posed by food products, such as juices and beverages, that were rich in AA and preserved by using sodium benzoate *(9, 10, 13, 28, 30, 36, 39)*. Traditional Indian pickles essentially belong to the "pickle in oil" category, and to meet the regulations of the Food Safety and Standards Authority of India, they must have a minimum drained weight of 60%, with all fruits and vegetables pieces being practically submerged in oil *(14)*. These pickles generally possess low pH, may or may not contain sugar, are rich in both AA and minerals, and are preserved by using sodium benzoate. All these features are favorable factors to produce benzene *(41)*. The presence of oil in pickles can retain more benzene, if formed in the pickles *(42)*.

Headspace gas chromatography–mass spectrometry (HS-GC/MS) is widely used for benzene quantification. Methods, such as gas chromatography-flame ionization detector and proton transfer reaction-mass spectrometry, are also reliable techniques but lack sensitivity at lower analyte concentrations. Solid-phase microextraction (SPME) is a relatively new sample extraction technique that integrates sampling, extraction, the concentration and introduction of a sample in a single stage, and which significantly minimizes the use of solvents *(16, 21, 45)*. Moreover, the requirement of a small amount of sample for SPME renders the technique highly suitable for the analysis of benzene in biological fluids *(16)*. There are many reports on benzene analysis wherein headspace SPME followed by GC/MS has been used for quantifying benzene *(12, 34)*.

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However, these reports are on samples other than food matrices. To the best of our knowledge, no work exists on SPME-HS-GC/MS analysis of benzene in foods.

The present work was, therefore, undertaken to survey pickles in the Indian market for the content of benzene by using SPME-HS-GC/MS analysis. The content of copper and iron that are known to catalyze the oxidative decarboxylation of BA to benzene, the salt content, and the antioxidant activities of the pickles that could mitigate the benzene formation were also estimated. A model system was also prepared to determine the role of individual components in benzene formation and to understand the rationale of the results obtained.

MATERIALS AND METHODS

Twenty-four pickles samples included in this survey were purchased from local supermarkets at various locations in India, such as Mumbai, Chandigarh, Hyderabad, and Perumbavoor. The key criterion for selecting a sample was the presence of sodium benzoate (E211) added as a preservative in the product. The pickles were made from fruits (lime, gooseberry, or mango) or vegetables (kenaf, garlic, or ginger) either singly or in blends and were of various brands and packaged in different packaging materials.

Standard L-ascorbic acid, EDTA (disodium-calcium salt), trichloroacetic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were obtained from Sigma Chemicals (Steinheim, Germany). Standards of 2-deoxy-D-ribose and mannitol were supplied by Aldrich Chemical Co. (Milwaukee, WI). Benzene (99.9%) and benzene-d_6 with an isotopic purity of 99.9% were both supplied by Supelco (Bellefonte, PA) and stored at 4°C. All solvents, including methanol, diethylether, hexane, petroleum ether, *m*-phosphoric acid, hydrogen peroxide, and 2,6-dichlorophenolindophenol were procured from SD Fine Chemicals Ltd. (Mumbai, India). Sodium chloride and sodium hydroxide were purchased from CHEMCO chemicals, Mumbai, India. Sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Qualigens (Mumbai, India). Inorganic salts, such as ferric chloride ($FeCl_3$), potassium chromate (K_2CrO_4), and potassium ferricyanide [$K_3Fe(CN)_6$], were from HiMedia (Mumbai, India). Acetic acid and thiobarbituric acid were supplied from SRL Chemicals (Mumbai, India) and The British Drug Houses Ltd. (Poole, UK), respectively. All the solvents were distilled before use, and all the reagents were of analytical grade, unless otherwise stated.

Pretreatment on pickle samples and storage. All pickle samples were stored at 4°C after purchase until analysis. Cold stored samples were approximately weighed and homogenized by using a shear mixer (Omni Mixer, Sorvall, Waterbury, CT) at speed 2 for 4 min to form a semisolid paste. Samples (15 g) were taken immediately for moisture analysis. The remaining homogenates were transferred to glass jars and stored at 4°C in the dark. These homogenized samples were used for all analysis. However, for analysis of copper and iron, pickle samples were crushed in mortar with pestle, so as to avoid contamination, and stored at 4°C in glass jars until use.

Analysis of pickle samples. Moisture analysis was done according to the protocol of the Indian Standards Institution *(22)* by using a hot air oven operating at 105 ± 2°C until constant weight was obtained. Total acidity was determined by the titrimetric method, and results were expressed in terms of acetic acid. The pH of the homogenized pickle was determined by using a pH meter. The content of sodium chloride or salt was determined according to Mohr's titration *(2)* after extracting it in warm water (~70°C), and the filtrate collected through Whatman filter 541 was used for salt determination. Fat analysis was carried out according to the standard protocol *(3)*. Semicontinuous extraction of the sample was carried out with petroleum ether by using Soxhlet assembly at a condensation rate of 5 to 6 drops of solvent per second for about 4 h. Contents in the flask were completely dried (100°C for 30 min) for quantification of fat.

AA was estimated by using the standard 2,6-dichlorophenolindophenol titrimetric method *(4)* and also by standard microfluorometric method *(5)*. Difference in AA content, as determined by the microfluorometric method (which measures both AA and dehydroascorbic acid) and the titrimetric method (which determines only AA), was used to calculate the percent degradation of AA. Prior to analysis, pickle extract containing AA was treated with distilled ether to completely remove any oil and color. Ether layer was discarded, and the treated extract was used thereafter for further analysis.

Analysis of BA was carried out by using the standard spectrophotometric protocol *(6)*. Estimation of copper and iron was done by an inductively coupled plasma atomic emission spectrometer (ARCOS, Kleve, Germany), with axial viewing of the emitted radiation. Briefly, a peristaltic pump was used to introduce the solutions into the inductively coupled plasma atomic emission spectrometer at a flow rate of 1.5 ml/min. The dissolved samples were introduced through the pump by using Tygon PVC tubing (Lima, OH). Operating parameters for the instrument included forward power at 1,400 W, coolant gas flow rate at 12.0 liters/min, auxiliary gas flow rate at 1.0 liter/min, and nebulizer gas flow rate at 0.8 liter/min. The elemental concentration was determined by using linear regression ($R^2 = 0.999$) for both elements, established in the range of 1.0 to 100 mg/kg for copper and 1.0 to 1,000 mg/kg for iron.

Analysis of antioxidant activity of the pickle samples. For determination of antioxidants, extraction of the pickle samples were performed by using the E-914 speed extractor (BUCHI Corp., Flawil, Switzerland), equipped with 40-ml stainless steel cells and 240-ml collection vials. The homogenized sample (2 g) was mixed with a minimum amount of diatomaceous earth to obtain a flowing powder that was packed in a cellulose thimble. Extraction conditions were an oven temperature of 50°C, pressure of 100 bar, heating up for 1 min, hold time of 2 min, and discharge of 2 min. The first two cycles were performed for washing samples by using hexane, followed by third cycle of extraction with distilled methanol. After every extraction, cells were first flushed with solvent for 2 min and then purged with N_2 (120 s). The extracts so obtained were dried under vacuum by using a rotary evaporator (BUCHI Corp.). The final volume was made to 5 ml with methanol (or distilled water, as required) and then passed through a 0.45-μm polyvinylidene fluoride filter (Simplepure). These filtrates were used for all the antioxidant assays.

DPPH assay (radical scavenging). The ability of the extracts to scavenge DPPH free radicals was determined by the method of Gyamfi et al. *(19),* with some modifications. A 100-μl aliquot of each diluted extract (10 μl of original extract made up to 1,000 μl with MeOH) was mixed with 1.0 ml of 0.1 mM DPPH radical in methanol. The controls contained all the reaction reagents, except the extract. After 30-min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. AA was used as a positive control. The values are presented as the

mean of triplicate analyses and expressed as milligrams of AA per kilogram of sample.

Iron (III) to iron (II)–reducing activity (FRAP assay). The ability of the extracts to reduce iron (III) was assessed by the method of Oyaizu *(37)*. A 100-μl aliquot of each diluted extract (10 μl of original extract was made up to 1,000 μl) was mixed with 250 μl of phosphate buffer (0.2 M and pH 6.6) and 250 μl of 1% aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30-min incubation at 50°C, 250 μl of 10% trichloroacetic acid was added, and the mixture was centrifuged for 10 min. A 250-μl aliquot of the upper layer was mixed with 250 μl of water and 50 μl of 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700 nm. Iron (III)–reducing activity was determined as AA equivalents (milligrams of AA per kilogram of sample). The values are presented as the means of triplicate analyses.

Nonsite-specific hydroxyl radical–mediated 2-deoxy-D-ribose degradation ($^{\bullet}$OH scavenging potential). The ability of the extracts to inhibit nonsite-specific hydroxyl radical–mediated peroxidation was carried out according to Halliwell et al. *(20)*, with some modifications. The reaction mixture contained 100 μl of extract dissolved in water, 500 μl of 28.0 mM 2-deoxy-D-ribose in KH_2PO_4-NaOH buffer (0.1 M and pH 7.4), 200 μl of 20.0 mM $FeCl_3$ and 104 mM EDTA (1:1, vol/vol) solution, 100 μl of 20.0 mM H_2O_2, and 100 μl of 2.0 mM aqueous AA. The tubes were vortexed and incubated at 50°C for 30 min. Thereafter, 1 ml of 2.8% trichloroacetic acid and 1 ml of 1.0% thiobarbituric acid were added to each tube. The samples were vortexed again and heated in a water bath at 90°C for 30 min. The extent of oxidation was estimated from the absorbance of the solution at 532 nm. The antioxidant activities of the extracts were expressed as mannitol equivalents (grams of mannitol per kilogram of sample). The values are presented as the means of triplicate analyses.

Quantification of benzene. For sample preparation, a freshly homogenized pickle sample was accurately weighed (5 g), and a slurry was made with deionized water (10 ml; Milli-Q, Millipore Corp., Bangalore, India), having a minimum resistivity of 18.0 MΩ/cm. The slurry was centrifuged (18,514 × *g* for 15 min at 4°C), and the supernatant was decanted into a 40-ml SPME glass vial (Supelco) containing NaCl (5 g). The total volume was made to 15 ml with deionized water. Samples were spiked with benzene-d_6 to a final concentration of 0.075 μg/liter, and the vials were sealed completely till extraction was carried out. Conditions were preoptimized for benzene extraction by using headspace SPME. The sample was equilibrated at 40°C for 30 min. The low equilibration temperature ensures no artifactual benzene formation *(25)*. Extraction was carried out thereafter with the SPME fiber assembly 50/30 μm carboxen/divinylbenzene/polydimethylsiloxane (Supelco) for 15 min. The fiber was injected in the GC injector port at 270°C. Fiber was conditioned for 5 min at 270°C before every extraction.

GC/MS analysis. GC/MS analysis was carried out by using a Shimadzu QP-5050A series GC/MS (Kyoto, Japan) equipped with a GC-17A gas chromatograph and provided with a DB-5 (dimethyl polysiloxane, J&W Scientific, Folsom, CA) capillary column (length = 30 m, internal diameter = 0.25 mm, and film thickness = 0.25 μm). The operating conditions were as follows: column temperature started at 35°C with a 5-min hold time, and then increased from 35 to 60°C at the rate of 4°C/min, held at this temperature for 2 min, and programmed further to 280°C at the rate of 40°C/min, with a hold time at final temperature for 5 min; injector and interface temperatures maintained at 270 and 280°C, respectively; carrier gas (He), at a linear flow rate of 0.9 ml/min; ionization voltage, 70 eV; and electron multiplier voltage, 1 kV. The identification of benzene and its quantification was based on the retention time of the quantification ion (*m*/*z* 78 in selected ion monitoring mode), which was 2.83 ± 0.03 min and identification ions (*m*/*z* 77 and 52 in selected ion monitoring mode), being within the 0.5% margin of the relative retention time as determined in the standard sample. Ion ratios of *m*/*z* 77 to 78 and *m*/*z* 52 to 78 were used to confirm the identity of benzene. Similarly, benzene-d_6 was monitored by recording its identification ion with *m*/*z* value of 84.

Method validation. The linearity of the method was determined over the concentration range of 0.0 to 0.1 μg/liter of benzene and 0.075 μg/liter of benzene-d_6 spiked in the blank pickle sample. Five g of the pickle sample was spiked with 0.025 μg/liter of benzene and analyzed, and the percentage recovery was calculated. For the determination of precision, a sample containing 0.05 μg/liter was analyzed six times. The limit of detection (LOD) was calculated as three times the residual standard deviation of the *y* intercept divided by the slope of the standard curve. The limit of quantification (LOQ) was calculated as three times the LOD.

Preparation of model systems. All model systems were made in SPME vials. The system pH was maintained by using citrate buffer (pH = 3.2, 0.1 M, and $pK_{a1} = 3.14$), and each model contained NaCl (11.5%). These parameters for pH and salt concentration were the sample group average values obtained in preliminary analyzed data. The concentration of BA, AA, copper, iron, and antioxidants were varied. The total volume was adjusted to 15 ml. Nine different models (including one blank) were developed. Models were subjected to gamma irradiation (1 kGy) by using a ^{60}Co gamma irradiator (dose rate 4.1 kGy/h; GC 5000, BRIT, Mumbai, India) to facilitate formation of $^{\bullet}$OH that, subsequently, accelerates formation of benzene. Analysis for benzene quantification in the model system was then performed in triplicate by using SPME-HS-GC/MS, as described earlier.

Statistical analysis. Multivariate analysis was performed by the XLSTAT, version 2013.4.05 (Addinsoft Co., Paris, France), an add-in software to Microsoft Excel. The variance in the data set was studied by using principal component analysis. The loading plot was drawn by using principal components 1 and 2. Analysis of variance was performed to study the effect of different constituents on formation of benzene and multiple comparison of mean was carried out by Duncan's multiple range test. DSAASTAT, version 1.101, by Andrea Onofri (Perugia, Italy) was used for statistical analysis of data.

RESULTS AND DISCUSSION

Analysis of pickle samples. Moisture is an intrinsic component of any food product and has a significant effect on its shelf life *(48)*. Fruits used for pickle preparation contain more than 80% moisture and constitute more than 60% of the weight of the pickles, making pickles a high moisture food. The moisture content of all 24 pickle samples is given in Table 1. The moisture content ranged from 425 ± 4 for sample 24 to 701 ± 14 g/kg for sample 20. The major role of acids is to reduce the overall pH, which suppress bacterial growth. Acetic acid (E260) is added to

TABLE 1. *Analysis of pickle samples*[a]

Sample no.	Moisture (g/kg)	Total acids (acetic acid, g/kg)	pH	Salt (g/kg)	Fat (g/kg)	AA (mg/kg)		AA, degraded (%) [(F−T)/F] × 100	Benzoic acid (mg/kg)	Metals (mg/kg)		Benzene (μg/kg pickle)
						Titrimetric method (T)	Microfluorometric method (F)			Cu	Fe	
1	620 ± 9	15.5 ± 0.2	3.33 ± 0.06	174.5 ± 0.3	145.9 ± 0.9	38 ± 0	491 ± 1	92 ± 0	222 ± 5	5.0 ± 0.1	47.6 ± 0.3	ND[b]
2	569 ± 12	22.2 ± 0.3	2.93 ± 0.06	174.8 ± 0.3	141.7 ± 2.6	69 ± 1	266 ± 1	74 ± 0	273 ± 5	13.5 ± 0.2	32.1 ± 0.2	ND
3	568 ± 7	13.8 ± 0.2	3.50 ± 0.00	151.2 ± 0.7	157.8 ± 3.5	34 ± 0	352 ± 2	90 ± 0	220 ± 5	4.5 ± 0.3	26.2 ± 0.4	ND
4	545 ± 13	12.1 ± 0.2	3.67 ± 0.06	150.1 ± 0.3	171.7 ± 2.8	16 ± 0	684 ± 6	97 ± 0	258 ± 7	2.1 ± 0.1	34.9 ± 0.6	<LOQ
5	503 ± 20	13.3 ± 0.2	2.80 ± 0.10	132.9 ± 0.3	120.4 ± 0.7	20 ± 0	661 ± 2	96 ± 0	220 ± 12	1.8 ± 0.0	30.9 ± 0.2	ND
6	536 ± 19	22.5 ± 0.1	3.07 ± 0.06	139.9 ± 0.7	159.2 ± 2.9	66 ± 0	408 ± 3	83 ± 0	296 ± 8	1.1 ± 0.0	36.9 ± 0.4	<LOQ
7	534 ± 12	9.4 ± 0.1	3.13 ± 0.06	123.8 ± 0.0	166.5 ± 2.6	32 ± 0	476 ± 6	93 ± 0	270 ± 2	1.2 ± 0.0	20.6 ± 0.7	0.24 ± 0.04
8	470 ± 18	20.7 ± 0.2	1.13 ± 0.06	54.8 ± 0.3	4.6 ± 0.6	12 ± 0	316 ± 29	96 ± 4	219 ± 6	1.1 ± 0.1	62.3 ± 0.3	ND
9	613 ± 7	24.7 ± 0.3	2.80 ± 0.00	113.6 ± 0.6	128.7 ± 1.7	69 ± 1	1,037 ± 1	93 ± 0	219 ± 9	2.4 ± 0.1	30.6 ± 0.5	ND
10	633 ± 11	15.0 ± 0.2	3.47 ± 0.06	129.4 ± 0.9	146.6 ± 3.1	34 ± 0	447 ± 20	92 ± 2	235 ± 4	3.7 ± 0.0	28.4 ± 0.2	<LOQ
11	535 ± 25	16.1 ± 0.0	3.40 ± 0.10	138.2 ± 0.7	217.8 ± 15.6	6 ± 0	734 ± 18	99 ± 1	287 ± 7	2.4 ± 0.2	146.9 ± 0.5	ND
12	594 ± 18	7.7 ± 0.1	4.03 ± 0.06	129.7 ± 0.9	157.6 ± 6.3	17 ± 0	546 ± 3	96 ± 0	287 ± 3	4.0 ± 0.2	27.2 ± 0.5	ND
13	545 ± 14	24.1 ± 0.1	2.80 ± 0.00	112.2 ± 0.3	124.9 ± 5.3	58 ± 0	597 ± 6	90 ± 0	294 ± 2	3.2 ± 0.1	32.0 ± 0.4	<LOQ
14	574 ± 18	20.9 ± 0.2	3.27 ± 0.06	149.1 ± 0.7	159.5 ± 1.4	60 ± 0	861 ± 5	93 ± 0	263 ± 4	2.4 ± 0.0	21.9 ± 0.5	ND
15	437 ± 6	24.3 ± 0.2	3.40 ± 0.00	102.9 ± 0.6	162.0 ± 5.1	16 ± 0	772 ± 1	97 ± 0	251 ± 8	1.7 ± 0.0	23.0 ± 0.5	0.25 ± 0.03
16	629 ± 15	15.4 ± 0.1	3.13 ± 0.06	87.7 ± 1.1	146.9 ± 0.6	63 ± 1	1,025 ± 16	93 ± 0	260 ± 11	5.2 ± 0.1	44.5 ± 0.4	ND
17	605 ± 24	20.2 ± 0.1	2.60 ± 0.10	118.2 ± 0.9	125.4 ± 4.8	66 ± 0	714 ± 12	90 ± 0	250 ± 6	6.9 ± 0.0	21.5 ± 0.3	ND
18	547 ± 13	7.7 ± 0.1	3.40 ± 0.10	70.9 ± 0.9	147.5 ± 4.9	41 ± 0	1,916 ± 2	97 ± 0	269 ± 10	2.7 ± 0.1	22.8 ± 0.4	ND
19	609 ± 14	8.6 ± 0.0	3.67 ± 0.06	79.0 ± 0.7	73.8 ± 2.8	28 ± 0	629 ± 5	95 ± 0	237 ± 16	1.8 ± 0.0	52.7 ± 0.4	ND
20	701 ± 14	12.3 ± 0.2	4.37 ± 0.06	18.7 ± 0	31.0 ± 1.4	45 ± 0	448 ± 0	89 ± 0	237 ± 0	0.8 ± 0.0	10.8 ± 0.3	0.16 ± 0.01
21	646± 13	15.5 ± 0.2	3.27 ± 0.06	84.5 ± 0.3	41.8 ± 0.6	69 ± 0	482 ± 2	85 ± 0	229 ± 1	6.8 ± 0.0	15.6 ± 0.3	0.25 ± 0.01
22	605 ± 13	19.0 ± 0.2	3.20 ± 0.00	121.3 ± 1.1	86.8 ± 4.0	74 ± 0	490 ± 1	84 ± 0	315 ± 11	4.9 ± 0.1	32.4 ± 0.5	4.36 ± 0.82
23	547 ± 15	15.1 ± 0.2	3.47 ± 0.06	89.3 ± 0.3	46.3 ± 0.7	34 ± 0	427 ± 1.	91 ± 0	222 ± 0	4.6 ± 0.2	31.0 ± 0.4	0.20 ± 0.01
24	425 ± 4	13.6 ± 0.0	3.63 ± 0.06	111.2 ± 0.7	93.7 ± 2.8	22 ± 0	683 ± 7	96 ± 0	274 ± 0	2.1 ± 0.1	28.2 ± 0.4	0.17 ± 0.01

[a] Values are mean ± standard deviation of three or more determinations.
[b] ND, not detected.

make up to the required acidity in commercial pickles. The calculated total acid content of all the pickle samples are given in Table 1. Total acid content ranged from a minimum of 7.7 ± 0.1 for sample 12, a garlic pickle, to the maximum value of 24.7 ± 0.3 g/kg for sample 9, a lime pickle. All the lime pickles (samples 2, 9, 13, 14, and 17) exhibited a total acid content of more than 20.0 g/kg. Likewise, samples 7 and 18 (mango), 12 (garlic), and 19 (ginger) had total acid of <10.0 g/kg. Similarly, the pH for most samples (16 out of 24) exhibited a range between 3.00 and 4.00 (Table 1). All samples having a pH below 3.00 were lime pickle samples, except sample 8, which presented the lowest value of 1.13 ± 0.06, and contained tamarind as an ingredient.

Salt enhances the overall flavor and suppresses bitterness *(26),* thereby improving the palatability of foods. Reduction of salt content in foods such as pickles and meat products might lead to undesirable changes in terms of flavors and might favor microbial spoilage *(18)*. For this reason, salt is added up to a minimum of 12% in pickled fruits and vegetables *(14)*. Table 1 shows the salt content in the pickles analyzed in this study. It ranged between 18.7 ± 0 for sample 20 to 174.8 ± 0.3 g/kg for sample 2. However, the salt content in most cases was between 100 and 150 g/kg. Fat addition enriches food in terms of calories and essential fatty acids, and it also influences texture. Analysis of the pickles for fat content showed them to contain around 150 g fat/kg sample (Table 1).

Fruits, such as lemon (420 mg/kg) and raw mango (210 mg/kg), are rich sources of vitamin C *(40)*. However, the blanching of fruits and vegetables causes high losses of AA *(35)*. Results obtained from this method clearly suggested all pickle samples to have fairly high AA content initially (Table 1). It is apparent that processing, such as blanching and subsequent storage, resulted in significant loss (more than 90% in most cases) of AA. At the same time, there is a probability that some amount of AA might have participated in oxidative decarboxylation of BA to form benzene and, ultimately, was converted to dehydroascorbic acid.

BA (E210) exhibits excellent bacteriostatic and fungistatic effect in the pH range of 1.5 to 4.0 *(46)*. Its sodium salt (E211) is generally used and has better antibacterial activity and suitability for food matrices. BA is permitted as a preservative up to 250 mg/kg in pickles by the Food Safety and Standards Authority of India *(15)*. The BA content in pickles analyzed in this study ranged from 219 ± 9 to 315 ± 11 mg/kg, with the majority of the samples having concentration around 250 mg/kg. Thirteen of the total 24 pickle samples were found to contain more than the prescribed limit of 250 mg/kg BA.

Because copper and iron act as catalysts in decarboxylation of BA, they were analyzed in all the procured pickle samples (Table 1). Copper was found to be in the range of 0.8 ± 0.0 to 13.5 ± 0.2 mg/kg. Iron was present comparatively in greater amount ranging from 10.8 ± 0.3 to 146.9 ± 0.5 mg/kg. Sample 11 containing the highest value of 146.9 mg/kg iron had a leafy vegetable *gongura* (*Hibiscus cannabinus* L.), which is widely grown and consumed in Andhra Pradesh, India.

TABLE 2. *Analysis of antioxidants in pickles*[a]

Sample no.	DPPH (mg of AA/kg pickle)	FRAP (mg of AA/kg pickle)	•OH radical scavenging (g mannitol/kg pickle)
1	1,158 ± 40	1,832 ± 4	346 ± 0
2	375 ± 2	952 ± 7	596 ± 1
3	880 ± 2	1,377 ± 4	107 ± 0
4	364 ± 0	1,012 ± 6	488 ± 0
5	465 ± 0	1423 ± 9	210 ± 0
6	12,828 ± 130	12,832 ± 62	526 ± 0
7	1,543 ± 43	2,175 ± 1	188 ± 0
8	383 ± 3	1,387 ± 2	168 ± 0
9	595 ± 5	1,179 ± 24	355 ± 1
10	1,122 ± 9	1,330 ± 6	138 ± 0
11	1,031 ± 28	1,844 ± 1	89. ± 0
12	450 ± 2	1,354 ± 7	274 ± 0
13	724 ± 2	964 ± 1	796 ± 3
14	541 ± 1	1,095 ± 7	598 ± 2
15	843 ± 5	1,955 ± 1	544 ± 2
16	12,650 ± 109	13,164 ± 71	485 ± 3
17	779 ± 5	1,359 ± 11	668 ± 3
18	4,481 ± 25	5,241 ± 31	168 ± 0
19	1,633 ± 7	2,136 ± 9	394 ± 0
20	416 ± 0	1,171 ± 4	511 ± 0
21	12,454 ± 86	12,934 ± 76	330 ± 0
22	713 ± 4	1,170 ± 8	107 ± 0
23	4,363 ± 18	4,850 ± 42	467 ± 1
24	1,213 ± 18	1,930 ± 0	36. ± 0

[a] Values are mean ± standard deviation of three or more determinations.

Antioxidant activity. Analysis of the antioxidant activities of the pickle samples is given in Table 2. A wide range for both DPPH scavenging activities (from 364 ± 0 to 12,828 ± 130 mg of AA per kg of sample) and FRAP (from 952 ± 7 to 13,164 ± 71 mg of AA/kg sample) was observed among the pickle samples. Three samples, namely, 6, 16, and 21, exhibited a value >12,000 mg of AA per kg of sample, all of which were gooseberry pickles. This indicates high antioxidant activities associated with gooseberries *(33, 38)*. Samples 18 and 23 had similar DPPH scavenging potential, both of which were mango pickles. Similarly, •OH scavenging activities were between 36 ± 0 to 796 ± 3 g of mannitol per kg of pickle (Table 2). All the lime pickle samples (2, 13, 14, and 17) had greater values (>500 g of mannitol per kg of sample) for •OH scavenging. The •OH scavenging activities for pickles made of chillies (sample 4), gooseberry (sampled 6 and 16), and ginger (sample 15) were also considerably higher.

Principal component analysis was used to visualize the effect of different variables on benzene formation. First two principal components (F1 and F2) explained 26.13 and 20.78% of data variance, respectively. From the loading plot (Fig. 1), it was observed that the formation of benzene is positively correlated with BA ($R^2 = 0.439$), AA ($R^2 = 0.308$), Cu^{2+} ($R^2 = 0.309$), and pH ($R^2 = 0.017$), whereas negatively correlated with presence of fat ($R^2 = -0.182$) and

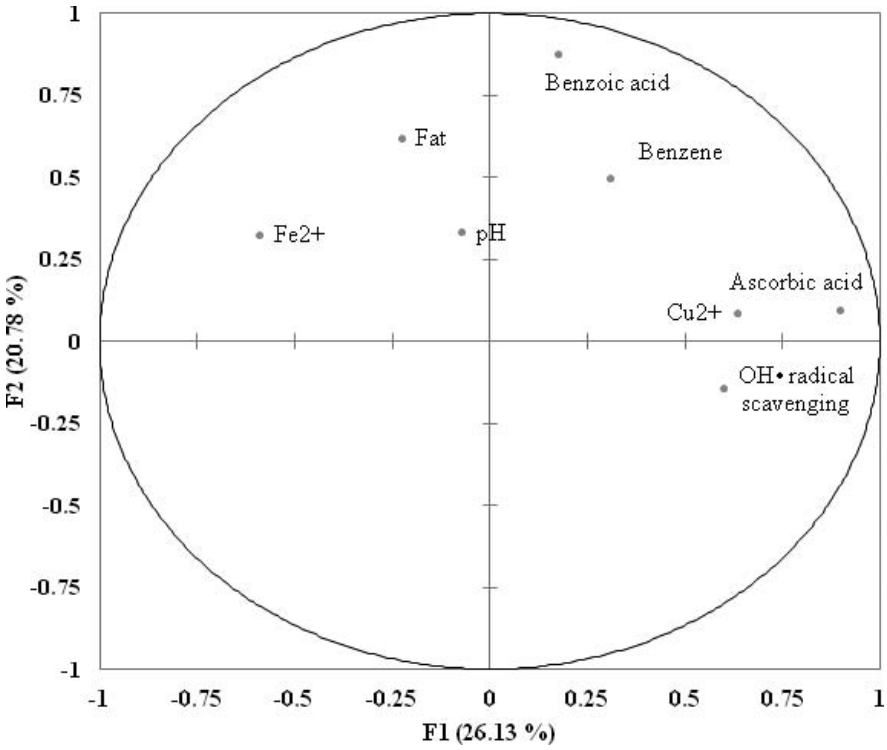

FIGURE 1. *Principal component analysis for studying the effect of various variables on formation of benzene in pickles.*

antioxidants (R^2= –0.26). These observations are in coherence with literature findings *(42, 43)*.

Benzene quantification by using headspace SPME-GC/MS. The method was validated in-house for specificity, linearity ($R^2 = 0.9695$), precision (coefficient of variation $\leq$ 12.37%), and accuracy (percent recovery = 79.39%). Homemade lime pickle containing no benzoate was used for this purpose. The LOD and LOQ were found to be 0.01 and 0.03 μg/kg of pickle sample, respectively. These values of detection limits are far below the maximum limit for benzene suggested by the European Union (1 μg/kg), U.S. Food and Drug Administraion (FDA; 5 μg/kg), and World Health Organization (10 μg/kg). This low order detection limit for benzene has been reported earlier for the SPME-HS-GC/MS technique but for nonfood samples *(16, 34)*.

The benzene content was quantified in the pickle samples by using an internal standard and is represented in Table 1. Note that 13 samples did not show any benzene, while 4 samples contained benzene at levels which were below the LOQ for this method. The remaining seven pickle samples were positive for benzene when it ranged from 0.16 ± 0.01 μg/kg (sample 20) to as high as 4.36 ± 0.82 μg/kg (sample 22). Note that the latter value of benzene content in the pickles is below the FDA and World Health Organization limits of 5 and 10 μg/kg in mineral water, respectively.

Note that samples 21 to 24 contained relatively high amount of benzene as compared with other samples. These were all purchased from Perumbavoor, Kerala, India. Furthermore, sample 22 in which the highest content of benzene was found also showed the highest content of BA (315 ± 11 mg/kg) and AA (74 ± 0 mg/kg) among all the screened samples. Also, •OH radical scavenging activity for this sample (107 ± 0 mg mannitol per g of pickle) was relatively lower than most of the other samples. This is in accordance with Vinci et al. *(43)* who reported that the higher the content of BA and AA in a food system, the higher the benzene formation. Benzene was detected in samples 7 (0.24 ± 0.04 μg of benzene per kg of pickle) and 22 (4.36 ± 0.82 μg of benzene per kg of pickle), which were made of lime, while samples 15 (0.25 ± 0.03 μg of benzene per kg of pickle) and 24 (0.17 ± 0.01 μg of benzene per kg of pickle) were ginger pickles. Additionally, six of seven samples containing benzene were products that were packed in a flexible polymeric pouch. Low pH and the absence of sugar are some of the factors that further promote benzene formation from BA *(7, 43)*.

Surprisingly, despite pickles being rich in AA and minerals and being preserved with benzoates, they showed very low or no benzene formation. This was thought to be attributed to the presence of antioxidants that are present in ingredients used in the preparation of pickles. Also, spices are known to be powerful antioxidants. Indian pickles are exclusively prepared with the addition of various spices, resulting in high antioxidant activity in pickles. Results for antioxidant assays validated the fact that Indian pickles did possess high •OH radical scavenging capacities (Table 2), which may have mitigated the formation of benzene. This hypothesis was further confirmed by conducting a study on model systems.

Model systems. The results for quantification of benzene in model systems are presented in Table 3. Note that no benzene was detected in the blank model that contained citrate buffer and sodium chloride. However when BA was added at 315 mg/liter, benzene formation was observed at levels around 0.15 μg/liter. There might be significant benzene formation from BA at lower pH values,

TABLE 3. *Benzene quantification in model systems*[a]

Model no.	Model[b]	Concn of BA (mg/liter)	Concn of AA (mg/liter)	Concn of metals (mg/liter)[c]		Concn of AO (mg AA/liter)	Benzene (μg/liter)[d]
				$CuSO_4$	$FeSO_4$		
1	Blank	—	—	—	—	—	ND
2	BA (maximum)	315	—	—	—	—	0.15 ± 0.02 C
3	AA (maximum)	—	74	—	—	—	ND
4	AA + BA (minimum)	219	6	—	—	—	0.23 ± 0.05 B
5	AA + BA (maximum)	315	74	—	—	—	0.77 ± 0.19 A
6	AA + BA + metals (minimum)	219	6	3	29	—	0.22 ± 0.01 B
7	AA + BA + metals (max)	315	74	34	169	—	0.23 ± 0.00 B
8	AA + BA + metals + AO (minimum)	219	6	3	29	375	0.07 ± 0.00 D
9	AA + BA + metals + AO (maximum)	315	74	34	169	12,828	ND

[a] —, not applicable; ND, not detected; AO, antioxidant.
[b] Minimum and maximum are referred to as the least and the highest concentrations of mentioned parameters present in a particular model and are adapted from Tables 1 and 2.
[c] Minimum and maximum concentrations for both Cu and Fe are obtained from Table 1 and were converted to the corresponding minimum and maximum concentrations of $CuSO_4$ and $FeSO_4$, respectively.
[d] Values are mean ± SD of three or more determinations. Different letters indicate significant difference ($P \leq 0.05$) on benzene formation due to different treatments.

even if AA is absent. This is also supported by U.S. Department of Health and Human Services *(41),* which suggested that citric and erythorbic acid produce $^{\bullet}OH$ in the same way as AA. Also, no benzene was detected in the model containing only AA (74 mg/liter).

Further, the model system containing both AA and BA showed a very high content of benzene formation, which reached 0.77 μg/liter of benzene in model solution 5, where the maximum concentration of both AA and BA (as obtained from pickle analysis) was incorporated. This proved that the role of AA in the whole reaction mechanism was important, as it enabled more $^{\bullet}OH$ formation that consequently accelerated benzene formation. The outcome for model solutions 6 and 7 in which metals were also present in addition to AA and BA were rather contradictory when the total benzene formed was quantitatively less than in model solutions 4 and 5. It was observed that there was no statistically significant difference ($P \leq 0.05$) in the amount of benzene formed during the absence or presence of metal ions (model solutions 4 and 6). Thus, no significant effect of metal ion on formation of benzene was seen. Similar results were observed while analyzing pickle samples as evaluated by principal component analysis analysis. It is possible that irradiation might have induced other reactions that are responsible for making copper and iron unavailable to further contribute to benzene production. However, no further attempt was made to prove this inference. Finally, in model solutions 8 and 9 (Table 3), AA was added in excess to act as an antioxidant. In model solution 8, where relatively lower amount of AA (375 mg/kg) was added, benzene production was still witnessed to be around 0.07 μg/liter. This reestablished the findings in literature that lower levels of AA do drive decarboxylation reaction of BA forward. At the same time, it was imperatively noticed that the quantity of benzene formed was still lower than that in model solutions 4 and 6 (0.23 ± 0.05 and 0.22 ± 0.01 μg/liter, respectively). In model solution 9, the AA concentration was as high as 12,828 mg/kg, and complete inhibition of benzene formation was seen. Conclusively, it can be said that concentration of AA in model solution 8 was too low for it to behave as an ideal prooxidant, while that in model solution 9 was too high to act as an ideal antioxidant.

Overall, it can be concluded that preservation of pickles prepared from fruits and vegetables that are rich in AA by using sodium benzoate could predispose it to benzene formation, especially if there are no antioxidant-rich ingredients in the formulation. These concerns could be addressed by avoiding the use of sodium benzoate in the pickles as a preservative and using alternative methods of preservation, such as by irradiation.

In conclusion, analysis of 24 Indian pickles samples, which were rich in both AA and BA and also had significant quantities of prooxidant copper and iron, showed very low or no benzene content in the same. This was attributed to the very high antioxidant activity that is associated with Indian pickles and confirmed from studies on model systems. Thus, we conclude that consumption of Indian pickles is unlikely to pose any health risk associated with benzene.

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