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# Quantification of Flavor-Related Compounds in the Unburned Contents of Bidi and Clove Cigarettes

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Bidi cigarettes, small hand-rolled cigarettes produced primarily in India, are sold in the United States in a wide variety of candy-like flavors (e.g. dewberry, chocolate, clove) and are popular with adolescents. Many flavored bidis contain high concentrations of compounds such as eugenol, anethole, methyleugenol, pulegone, and estragole; several of these compounds have known toxic or carcinogenic properties. Clove cigarettes, or kreteks, are another highly flavored tobacco product with high levels of eugenol due to clove buds present in the tobacco filler. In this study, compounds in the burnable portion—the filler and wrapper material actually consumed during the smoking of bidis, kreteks, and U.S. cigarettes—were analyzed. Flavor-related compounds were solvent extracted from the burnable portion of each cigarette with methanol. An aliquot of the methanol extract was heated, and the sample headspace was sampled with a solid-phase microextraction fiber and introduced into a gas chromatograph−mass spectrometer for analysis in selected-ion monitoring mode. High levels of eugenol were detected in five clove-flavored bidi brands ranging from 78.6 to 7130 $\mu$g/cigarette ($\mu$g/cig), whereas diphenyl ether (128−3550 $\mu$g/cig) and methyl anthranilate (154−2360 $\mu$g/cig) were found in one grape-flavored bidi brand. A nontobacco herbal bidi brand contained the greatest variety of compounds, including anethole (489−665 $\mu$g/cig), eugenol (1670−2470 $\mu$g/cig), methyleugenol (27.7−36.6 $\mu$g/cig), safrole (32.4−34.4 $\mu$g/cig), myristicin (170−247 $\mu$g/cig), and elemicin (101−109 $\mu$g/cig). Filler from kreteks was found to contain high levels of eugenol, anethole, and coumarin. Flavored bidis and clove cigarettes contain a number of compounds that are present at levels far exceeding those reported in U.S. cigarette tobacco. Research is underway to determine the levels of these compounds delivered in smoke. It is not known what effect inhalation of these compounds has on smokers.

**KEYWORDS: Bidi cigarettes; flavors; alkenylbenzenes; solid-phase microextraction; gas chromatography; mass spectrometry**

## INTRODUCTION

Bidis originated in India around 1905 (*1*) as small, unflavored cigarettes (*2*). Present-day bidis consist of sun-dried, Oriental tobacco flakes hand-rolled (*3*) in leaf material from ebony trees in the family Ebenaceae Gürke, including *Diospyros ebenum*, *Diospyros melanoxylon*, *Diospyros ebenaster*, and *Diospyros ismailii* (*4*) (see traditional bidi, **Figure 1**), and usually bound together with a short length of thread (*5*). It is estimated that 1.2 trillion unflavored bidis are consumed annually in India (*6*). Bidis began gaining market share in the United States in the 1970s (*7*) and became more prevalent among young smokers during the late 1990s (*5*).

The popularity of bidis among American youth is partially attributed to their marketing in appealing candy-like flavors (dewberry, grape, chocolate, clove, etc.) and brightly colored packs that enhance visibility and product appeal. According to surveys, the most frequent reason youth give for smoking bidis is that they "taste better than cigarettes" (*5*). Flavor compounds present in bidis sold in the United States are likely added to improve taste or mask unpleasant odors (*8*). Presently, there are 599 additives that domestic cigarette manufacterer's report using in U.S. cigarettes, including individual flavor compounds, spices, and essential oils (*9*). Because pleasing taste is important to youth smokers (*5*), the use of bidi flavoring agents may play a role in enhancing product appeal with smokers, especially those who find the aroma or taste of conventional cigarette smoke unappealing (*10*).

Many flavored bidi brands contain high levels of selected flavor-related compounds (*11*). Some flavor-related constituents previously found in bidis such as methyleugenol (*12*) and

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**Figure 1.** Photograph of a 2R4F research cigarette, a traditional (tobacco-containing) bidi, and a large filtered herbal bidi. The entire contents of each cigarette type are shown to the right. Individual components separated from the herbal bidi filler include (a) brown bark-like material, (b) yellow fibrous material, (c) slender, striated seeds, and (d) yellow granules.

estragole (*13*) have been shown to be carcinogenic in laboratory animals. Another compound, pulegone, exhibits hepatotoxic and pneumotoxic activity in laboratory animals (*14*) and, in rat liver microsomes, is known to cause irreversible destruction of cytochrome $P_{450}$ enzymes (*15*). Moreover, eugenol, a spicy flavor compound present in clove cigarettes, can act as an anesthetic that numbs the throat, making smoke easier to inhale; hence, clove cigarettes have been referred to as trainer cigarettes (*16*). Exposure to eugenol in clove cigarette smoke can cause hemorrhagic pulmonary edema, hemoptysis, respiratory infection, and aspiration pneumonitis in some individuals (*16*). As with clove cigarettes, it is possible that bidi cigarettes containing certain flavorings that reduce pain perception, such as eugenol, could play a role in smoking initiation. In general, flavor compounds present in bidis generally impart a pleasant taste or aroma, whereas diphenyl ether, a compound that has been tentatively identified in bidis (*11*), is characterized as having a harsh metallic aroma (*17*) and is known to irritate mucus membranes and the upper respiratory tract (*18*), and prolonged occupational exposure has resulted in damage to the liver, kidney, spleen, or thyroid (*19*). It is not known what effect diphenyl ether has on the human body when repeatedly inhaled in cigarette smoke.

The filler in conventional U.S. cigarettes (tobacco) and kreteks (tobacco and clove buds) is typically flavored prior to cigarette rolling (*20*, *21*). In a previous study, the levels of flavor-related additives were measured in bidi tobacco filler so that the levels could be directly compared to those in the filler from selected U.S. cigarettes (*11*). Although it is not known if bidis are flavored before or after assembly, a substantial amount of flavor-related material is present on both the leaf wrapper and tobacco filler (*11*). We subsequently analyzed flavor-related compounds in the entire portion of a cigarette consumed during smoking (hereafter, burnable portion), that is, from the cigarette tip to the burn termination point near the mouthpiece end. Use of the word "cigarette," as used here, refers collectively to any cigarette whether an American cigarette, kretek, or bidi. We analyzed the "burnable portion"—the consumed fraction of the tobacco column—of the cigarettes to determine the amount of flavor-related compounds available for transfer to mainstream smoke. The burnable portion in bidis includes the tobacco and leaf wrapper; in kreteks and U.S. cigarettes, the burnable portion consists of the tobacco filler plus the cigarette paper that would be consumed by smoking (**Figure 2**).

Research addressing inhalation exposure to flavor-related compounds in bidis is limited, in terms of both qualitative and quantitative assessment. We identified and measured the levels of flavor-related chemicals, including menthol, pulegone, methyl anthranilate, diphenyl ether, *cis*-ethyl-3-methyl-3-phenylglycidate (*cis*-EMPG), coumarin, elemicin, estragole, eugenol, methyleugenol, myristicin, *trans*-anethole (hereafter referred to as anethole), and safrole, in selected tobacco products. These 13 compounds were measured in 12 bidi brands (10 flavored and 2 unflavored) that were either of a traditional, smaller diameter construction or larger sized (**Figure 1**). Some traditional bidis contain a small cotton plug in the mouthpiece to act as a filter, whereas one bidi manufacturer uses a cellulose acetate-type filter fitted in the mouthpiece. In this study, we also analyzed three clove cigarette brands and three American cigarette brands. Several larger-sized bidis made by one manufacturer are available in tobacco-containing varieties (regular, clove, menthol, etc.) and one herbal bidi, which contains no tobacco (**Figure 1**).

## MATERIALS AND METHODS

**Sample Collection and Processing.** With the exception of Azad brand bidis and Gold Kishen bidis, all brands (bidis, kreteks, and U.S. cigarettes, bidis) were purchased between the spring of 2001 and summer of 2002 at convenience stores in the metropolitan Atlanta area. For each brand, three packs were purchased for analysis. We obtained all Azad bidi brands from the manufacturer's website (http://www.azadbidi.com) and Gold Kishen brand bidis from street vendors

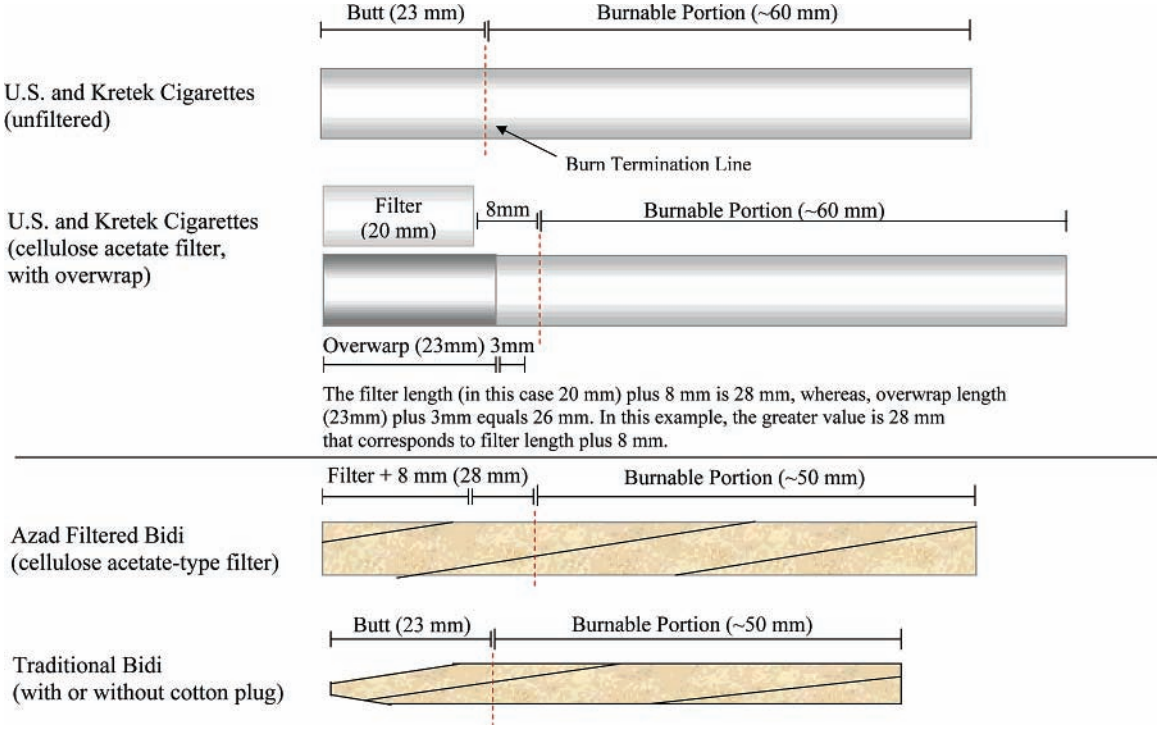


**Figure 2.** Diagram of burnable portion for U.S. (filtered and unfiltered) and bidi cigarettes (traditional and large filtered).

in India. The packs were placed in resealable plastic bags and placed in an ultralow (−70 °C) freezer for long-term storage. These samples do not represent all available brands. Prior to analysis, cigarettes were selected from the packs, individually labeled, sealed in test tubes, and stored temporarily in a laboratory freezer at −20 °C. Our analysis included five clove-flavored bidis (Kailas, Irie, Darshan, Shiv, and Azad brands), two menthol-flavored bidis (Azad and ShivSagar), one grape-flavored bidi (Darshan), one cinnamon-flavored bidi (ShivSagar), and two unflavored bidis (Gold Kishen and Azad natural). For comparison, we analyzed three clove cigarettes and three U.S. cigarette brands. For each brand, we measured flavor-related compounds in the burnable portion of a single cigarette from three different packs.

For analysis, cigarettes were cut at the burn termination line—the smoking termination point under standardized machine smoking conditions. In small, traditional-style filtered and unfiltered bidis, this point is 23 mm from the mouthpiece end. In larger cigarettes with cellulose acetate-type filters, including Azad bidis, kreteks, and U.S. cigarettes, the burn termination line is the filter length plus 8 mm or the filter overwrap length plus 3 mm, whichever is greater (**Figure 2**).

**Materials.** We obtained menthol, methyl anthranilate, *cis*-ethyl 3-methyl-3-phenylglycidate, and diphenyl ether from Aldrich Flavors and Fragrances (Milwaukee, WI); methanol was purchased from Tedia (Fairfield, OH). Other chemicals were obtained as described earlier (*19*). The manufacturers provided the lot purity of each chemical, which we used to calculate the amount of flavor-related compounds added to the standard solutions; the purity of elemicin is 95%.

Wide-mouth 60-mL amber glass bottles fitted with custom-made pierceable Teflon septum caps (Lab Depot, Alpharetta, GA), and 50-$\mu$L syringes were purchased from SGE Incorporated (Austin, TX). Precleaned 10-mL solid-phase microextraction (SPME) vials and steel crimp seals with preassembled Teflon-coated silicon septa were assembled in an off-site ultraclean room (Microliter Analytical Inc., Suwanee, GA). For SPME, 75-$\mu$m Carbowax/divinylbenzene StableFlex (CW-DVB) fibers were obtained from Supelco (Bellefonte, PA). Fibers were conditioned at 250 °C for 30 min prior to initial use. The contents of 60-mL bottles used for solvent extraction were agitated on an orbital shaker (Lab-Line, Barnstead International, Dubuque, IA). All chemical standards were weighed on an analytical balance (Sartorius AG, Göttingen, Germany) to an accuracy of 0.01 mg. Tobacco samples were weighed to an accuracy of ±0.2 mg on a top-loading balance (Ohaus Corp., Pine Brook, NJ).

Flavor-related analytes were quantified using 3′,4′-(methylenedioxy)-acetophenone (MDA) as an internal reference compound. An internal reference solution was prepared by adding approximately 50 mg of MDA solid to a newly opened 4-L bottle of methanol and shaking it vigorously to yield a final concentration of 12.5 $\mu$g/mL of MDA in the extraction solution.

The 4-L bottle was then fitted with a Dispensette III pipettor (BrandTech Scientific Inc., Essex, CT) to dispense 40-mL aliquots of MDA extraction solution into the 60-mL bottles. The MDA-containing extraction solution was used for all blanks, standards, quality controls (QCs), and cigarette samples. Preparation of all samples in an analytical batch was done with the same batch of extraction solution. A new calibration curve was prepared and analyzed each time a new bottle of MDA-containing extraction solution was prepared.

Stock solutions containing the 13 flavor-related compounds were prepared by dilution of neat standards in methanol in a 50-mL volumetric flask. The resulting solutions were sealed in 10-mL glass ampules, wrapped in foil, and stored in darkness at −20 °C to prevent photodegradation. Aliquots from 10 calibration solutions of various concentrations were transferred into amber vials with Teflon-lined caps for short-term storage. New calibration solutions were prepared about every 90 days.

We analyzed a blank tobacco sample consisting of tobacco filler taken from 1R3F Kentucky Research Cigarettes (University of Kentucky, Lexington, KY) with each sample batch. The residual analyte levels of the blank tobacco were below the limit of detection.

**Overview of Analytical Methodology.** In this study, we analyzed compounds in the burnable portion, which is the part of the filler and wrapper material actually consumed during smoking. Flavor-related compounds in the burnable portion of each cigarette were extracted with methanol; an aliquot of the methanol extract was transferred into a vial. Subsequently, the vial was heated and the sample headspace sampled with a SPME fiber. Compounds trapped on the SPME fiber were introduced into a gas chromatograph−mass spectrometer (GC-MS) for analysis in selected-ion monitoring (SIM) mode. We utilized SPME of the methanol extract instead of direct injection to reduce chromatographic interferences and eliminate excessive soiling of the inlet liner.

**Table 1.** Approximate Retention Times, Concentration Ranges, and Quantitation and Confirmation Ion Parameters for the Flavor Compounds Investigated in This Study

| analyte | RT[a] (min) | concn range ($\mu$g) | quant ion (amu);[b] SIM dwell time (ms) | conf ion (amu);[c] SIM dwell time (ms) |
|---|---|---|---|---|
| menthol | 6.4 | 32.5–27100 | 123; 10 | 138; 10 |
| estragole | 6.8 | 0.472–393 | 148; 25 | 147; 25 |
| pulegone | 7.6 | 0.674–562 | 109; 50 | 137; 50 |
| *trans*-anethole | 8.7 | 20.8–17350 | 148; 50 | 133; 50 |
| safrole | 8.9 | 0.436–363 | 162; 50 | 135; 25 |
| methyl anthranilate | 10.3 | 27.0–22500 | 151; 50 | 120; 50 |
| eugenol | 10.6 | 21.4–17900 | 149; 10 | 137; 75 |
| methyleugenol | 12.1 | 0.362–302 | 178; 25 | 163; 100 |
| diphenyl ether | 12.3 | 11.0–9140 | 170; 50 | 141; 50 |
| coumarin | 13.0 | 4.94–4120 | 146; 40 | 118; 25 |
| *cis*-EMPG[d] | 13.2 | 12.2–10190 | 132; 25 | 104; 25 |
| MDA (IR)[e] | 13.4 | 500 | 149; 10 | 121; 10[f] |
| myristicin | 16.0 | 2.07–1720 | 192; 10 | 165; 125 |
| *cis*-elemicin | 16.7 | 0.280–233 | 208; 25 | 193; 50 |

[a] RT, retention time (approximate). [b] Quantitation ion. [c] Confirmation ion. [d] *cis*-EMPG, *cis*-ethyl methylphenylglycidate. [e] 3′,4′-(Methylenedioxy)acetophenone (MDA), internal reference compound used for quantification. [f] Ion monitored for peak identification.

**Solvent Extraction of Analytes from Matrix.** We added a 40-mL aliquot of MDA-containing extraction solution to 0.40 g of tobacco from the burnable portion of each cigarette. This was agitated on an orbital shaker at 160 rpm for 4 h at ambient temperature. Following extraction we removed a 25-$\mu$L aliquot of the extraction solution with a clean 50-$\mu$L syringe and injected it through the septum of a clean, sealed SPME vial. This sample preparation procedure was used for all blanks, calibration runs, QC materials, and unknown samples.

**Calibration Curve Preparation.** To prepare calibration curves, we spiked 200-$\mu$L aliquots of the appropriate standard solution onto a 0.40-g portion of blank tobacco. The standards were equilibrated with the tobacco for at least 10 min prior to extraction with the MDA solution. The calibration range for each analyte spanned $\geq$3 orders of magnitude (**Table 1**). Calibration linearity was excellent with correlation coefficients exceeding 0.980 for all analytes.

**Quality Control Samples.** QC samples were prepared and analyzed daily with each sample set to ensure reproducibility and long-term analytical stability of the reported results. The QC spiking solution was prepared by diluting 510 $\mu$L of the stock solution in 50 mL of methanol. To prepare a QC sample, a 200-$\mu$L aliquot of QC spiking solution was added to a 0.40-g portion of tobacco in an extraction bottle. Following a 10-min period to allow the analytes to interact with the sample matrix, a 40-mL aliquot of extraction solution was added to the QC sample. A 25-$\mu$L aliquot of the QC sample was transferred to a SPME vial for analysis. In total, 60 QC samples were analyzed over a 3-week period prior to the evaluation of unknown samples to characterize analyte QC limits (mean and 95th and 99th confidence intervals) in the tobacco matrix.

**Analysis of Unknown Samples.** Immediately prior to analysis, cigarette samples were removed from the freezer, equilibrated to room temperature, and then cut with a disposable scalpel along the burn termination point. The burnable portion was inserted into an extraction bottle, a 40-mL aliquot of extraction solution was added, and the bottle was immediately capped and agitated for 4 h at 160 rpm at ambient temperature. The extraction time (4 h), determined by a time-course extraction study, was sufficient for extracting the flavor-related compounds from the tobacco, leaf wrapper material, and clove buds present in clove cigarettes.

**GC-SIM/MS Parameters.** In this study, target analytes were separated with a DB-5MS column (30 m; 0.25 mm; 0.25 $\mu$m film; J&W Scientific, Folsom, CA) fitted in an Agilent 6890 gas chromatograph (GC) and coupled to an Agilent 5973 mass selective detector (MS). The GC-MS is a product of Agilent Technologies Inc. (Avondale, PA). The GC inlet, maintained at 230 °C in splitless mode, was fitted with a 0.75-mm i.d. injection sleeve (Supelco, Bellefonte, Pa). Research grade helium (99.9999%) (Airgas Inc., Jacksonville, FL), with a flow rate of 1.1 mL/min, was used as the column carrier gas; purge flow to split vent was opened at 0.75 min. The following GC ramp program was used: hold at 55 °C for 1 min; ramp at 40 °C/min to 115 °C; ramp at 2 °C/min to 128 °C; ramp at 1.5 °C/min to 132 °C; ramp at 5 °C/min to 155 °C; ramp at 40 °C/min to 270 °C. The total GC run time was 19.15 min. The transfer line was maintained at 280 °C. Mass spectral analysis was performed in SIM mode; SIM parameters, including ions and dwell times, are listed in **Table 1**.

A CTC CombiPAL autosampler (Leap Technologies, Carrboro, NC) was mounted atop the GC-MS system. Upon initiation, the CTC autosampler transported a vial from the sample tray to a heater block, where the vial was preheated at 95 °C for 2.0 min. Following preheating, the vial headspace was extracted for 30 s with a preconditioned 75-$\mu$m Carbowax/divinylbenzene StableFlex fiber. Analytes retained by the SPME fiber were subsequently desorbed in the GC inlet (230 °C), separated by GC, and detected by SIM/MS.

**Data Handling and Statistics.** Instrument files were transported into Xcalibur, version 1.3 (Thermo Electron, San Jose, CA), for integration. We manually integrated analyte peaks that were not found or improperly autointegrated. Xcalibur data were subsequently ported into a custom database in Microsoft Access 2000 (Microsoft Corp., Redmond, CA) for calculations. All statistical analyses were performed with SAS, version 8.2 (SAS Institute Inc., Cary, NC).

**Table 2.** Limits of Detection (LOD), Spiked Amounts, Recovery, and Relative Standard Deviation (RSD) Values Obtained by Spiking Tobacco with 200 $\mu$L of Flavor Spiking Solution onto Tobacco Samples ($n$ = 5) at Levels Similar to Those Found in Bidi Samples

| analyte | LOD ($\mu$g) | spiked amount ($\mu$g) | recovery (%) | RSD (%) |
|---|---|---|---|---|
| *trans*-anethole[a] | 20.8 | 1250 | 127 | 11.6 |
| coumarin | 9.9 | 296 | 103 | 4.3 |
| diphenyl ether | 10.9 | 658 | 120 | 9.3 |
| elemicin | 1.2 | 29.8 | 96.5 | 7.5 |
| *cis*-ethyl methylphenylglycidate | 24.5 | 734 | 77.7 | 9.5 |
| estragole[a] | 2.0 | 5.5 | 115 | 11.6 |
| eugenol | 42.9 | 2290 | 119 | 13.3 |
| menthol[a] | 32.5 | 1950 | 107 | 15.7 |
| methyl anthranilate | 27.0 | 1620 | 90.3 | 8.0 |
| methyleugenol | 6.3 | 21.7 | 74.1 | 3.4 |
| myristicin | 4.1 | 124 | 72.9 | 3.0 |
| pulegone[a] | 5.7 | 40.5 | 104 | 10.8 |
| safrole[a] | 1.8 | 26.2 | 127 | 13.3 |

[a] Only four sample results were available for these calculations.

## RESULTS AND DISCUSSION

**Study Background.** In a previous study, we measured flavor-related additives in bidi tobacco for direct comparison with levels in U.S. cigarette tobacco. Flavor-related chemicals are present at high levels in both the leaf wrapper and tobacco throughout the entire bidi (*11*). We therefore measured the flavor-related additives present in the entire bidi (leaf wrapper and tobacco) actually consumed during smoking. This burnable portion (**Figure 2**) contains most of the flavor-related compounds available for transfer to the smoke.

**Determination of Limit of Detection (LOD), Recovery, and Reproducibility.** We calculated the analyte LODs as 3 times the standard deviation at zero concentration (**Table 2**) (*22*). We determined recoveries by quantitative analysis after spiking 200-$\mu$L aliquots of the standard solution onto five tobacco samples having levels before spiking that were below the LOD. We calculated recovery as the percent difference between the blank and analyte levels spiked onto the tobacco and reproducibility as the relative standard deviation of these values. Recoveries

**Table 3.** Amount of Flavor-Related Compounds Detected in the Burnable Portion of 10 Flavored Bidi Cigarette and 3 U.S. Cigarette Brands

| | analyte range, burnable portion[a] (μg/cig) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | anethole | eugenol | methyl-eugenol | safrole | myristicin | elemicin | menthol | methyl anthranilate | diphenyl ether |
| LOD (μg) | 20.8 | 42.9 | 6.3 | 1.8 | 4.1 | 1.2 | 32.5 | 27.0 | 10.9 |
| bidis | | | | | | | | | |
| Kailas clove[b] | 293–647 | 148–560 | ND[c] | ND | ND–18.2 | ND–7.35 | 186–403 | ND | ND |
| Shiv clove | ND | 984–2790 | ND | ND | 4.36–26.0 | ND–8.24 | 35.3–80.8 | ND | ND |
| Irie clove | 40.3– 94.7 | 1230–3230 | ND | ND | 6.13–10.3 | 1.76–2.68 | 68.8–101 | ND | ND |
| Darshan clove | 73.1–217 | 2390–4000 | ND | ND | ND | ND | 50.7–95.3 | ND | ND |
| Azad clove | ND–43.3 | 6210–7130 | ND–7.52 | ND | 4.98–34.7 | 1.47–12.8 | 58.3–260 | ND | ND |
| Azad herbal[d] | 489–665 | 1670–2470 | 27.7–36.6 | 32.4–34.4 (2) | 170–247 | 101–109 | 758–1380 | ND | ND |
| Darshan grape[e] | ND–88.0 | ND | ND | ND | ND | ND | 67.0–471 | 154–2360 | 200–3550 |
| Shiv cinnamon | ND | ND–89.0 | ND | ND | ND | ND | ND–59.8 | ND | ND |
| Shiv menthol[f] | ND | ND | ND | ND | ND | ND | 129–305 | ND | ND |
| Azad menthol | ND | ND | ND | ND | ND | ND | 930–2770 | ND | ND |
| U.S. cigarettes | | | | | | | | | |
| filter menthol | ND | ND | ND | ND | ND | ND | 3580–6950 | ND | ND |
| filtered | ND | ND | ND | ND | ND–7.62 | ND | ND–95.0 | ND | ND |
| nonfiltered | ND | ND | ND | ND | ND | ND | ND–72.3 | ND | ND |

[a] All data ranges contained three or more results unless noted otherwise in parentheses. [b] Kailas clove also contains pulegone (ND–6.59; $n = 2$). [c] ND, at or below the analyte LOD value. [d] Azad herbal also contains estragole (19.0–26.5 μg/cig). [e] Darshan grape also contains *cis*-EMPG (ND–122 μg/cig); values in one pack were much less than in the other two. [f] Shiv menthol also contains pulegone (ND–7.09).

**Table 4.** Retention Times, Calculated Mass, Measured Mass, and Mass Accuracy for Several Flavor Chemicals in the Unburned Contents of Azad Herbal (Tobaccoless) and Darshan Grape Bidis Analyzed by High-Resolution Time-of-Flight Mass Spectrometry

| flavor compound | brand | retention time (min) | molecular formula | calcd mass (amu) | measd mass (amu) | mass accuracy | |
|---|---|---|---|---|---|---|---|
| | | | | | | mDa | ppm |
| estragole | Azad herbal | 4.55 | $C_{10}H_{12}O$ | 148.0888 | 148.0889 | 0.1 | 0.8 |
| anethole | Azad herbal | 6.37 | $C_{10}H_{12}O$ | 148.0888 | 148.0890 | 0.1 | 1.0 |
| safrole | Azad herbal | 6.47 | $C_{10}H_{10}O_2$ | 162.0681 | 162.0681 | 0.1 | 0.4 |
| eugenol | Azad herbal | 7.95 | $C_{10}H_{12}O_2$ | 164.0837 | 164.0833 | –0.4 | –2.4 |
| methyleugenol | Azad herbal | 9.22 | $C_{11}H_{14}O_2$ | 178.0994 | 178.0994 | 0.0 | –0.1 |
| myristicin | Azad herbal | 12.94 | $C_{11}H_{12}O_3$ | 192.0786 | 192.0791 | 0.5 | 2.5 |
| elemicin | Azad herbal | 13.78 | $C_{12}H_{16}O_3$ | 208.1099 | 208.1095 | –0.4 | –2.2 |
| methyl anthranilate | Darshan grape | 13.40 | $C_8H_9NO_2$ | 151.0634 | 151.0633 | 0.1 | 0.5 |
| diphenyl ether | Darshan grape | 14.57 | $C_{12}H_{10}O$ | 170.0732 | 170.0728 | –0.3 | –2.0 |

**Table 5.** Amount of Flavor-Related Compounds Detected in the Burnable Portions of Three Clove Cigarette Brands

| | analyte range, burnable portion[a] (μg/cig) | | | | | |
|---|---|---|---|---|---|---|
| | anethole | eugenol | estragole | myristicin | menthol | coumarin |
| LOD (μg) | 20.8 | 42.9 | 2.0 | 4.1 | 32.5 | 9.9 |
| Djarum Super 16[b] | 39.2–261 | 15400–34300 | ND[c] | 6.21–15.7 (2) | 130–259 | 60.5–146 |
| Gudang Garam Kings | 284–384 | 47500–78900 | 12.5–23.9 | ND | 271–558 | 180–260 |
| Wismalak Gelora Djaja | ND –23.1(2) | 45200–51300 | ND | ND | 314–376 | 172–383 |

[a] All data ranges contained three or more results. Kretek A and C are filtered, whereas Kretek B is unfiltered. [b] Djarum Super also contained elemicin (2.0–7.1 μg/cig; LOD = 1.2 μg). [c] ND, at or below the analyte LOD value.

for the compounds ranged from 72.9 to 127% (**Table 2**). Relative standard deviations (RSD) were <15%, except for menthol (15.7%) (**Table 2**). Pack-to-pack variation in actual samples may be significant in some brands and probably accounts for a portion of the variation seen (**Tables 3** and **5**). Chromatograms of the burnable portion from bidis showed a large number of symmetrical, unsaturated peaks (**Figure 3**).

**Flavor-Related Compounds in Bidis and U.S. Cigarettes.** Of the two unflavored bidi brands, Azad regular contained only menthol [51.0−192 μg/cigarette (μg/cig)] and Gold Kishen contained no analytes above the LOD; these two brands are not discussed further. Menthol was present in all 10 of the flavored bidi brands including nonmentholated brands. Menthol ranged from nondetectable to 2770 μg/cig. Four of the nonmentholated brands contained menthol at levels exceeding the two mentholated bidi brands. Menthol in the U.S. mentholated cigarettes exceeded the highest level in the bidis. Myristicin was the only other compound found at detectable levels in U.S. cigarettes.

Eugenol, the main flavor-related compound in cloves [*Syzygium aromaticum* (Linnaeus) Merrill & Perry] (*23*), was present in all five of the clove-flavored bidi brands (**Table 3**), at levels ranging from 148 to 7130 μg/cig. Among the bidi brands, the Azad clove bidi brand had the highest amount of eugenol. Eugenol levels in clove-flavored bidis varied significantly both between brands and between cigarettes of the same brand. For the Azad clove brand, the highest value for an individual cigarette was 14% greater that the mean value for that brand, whereas for the Shiv clove brand, the maximum level was 45% greater than the mean level. In addition to eugenol, most of the clove-flavored bidis contained anethole, myristicin, and elemicin, suggesting common flavoring schemes. Except

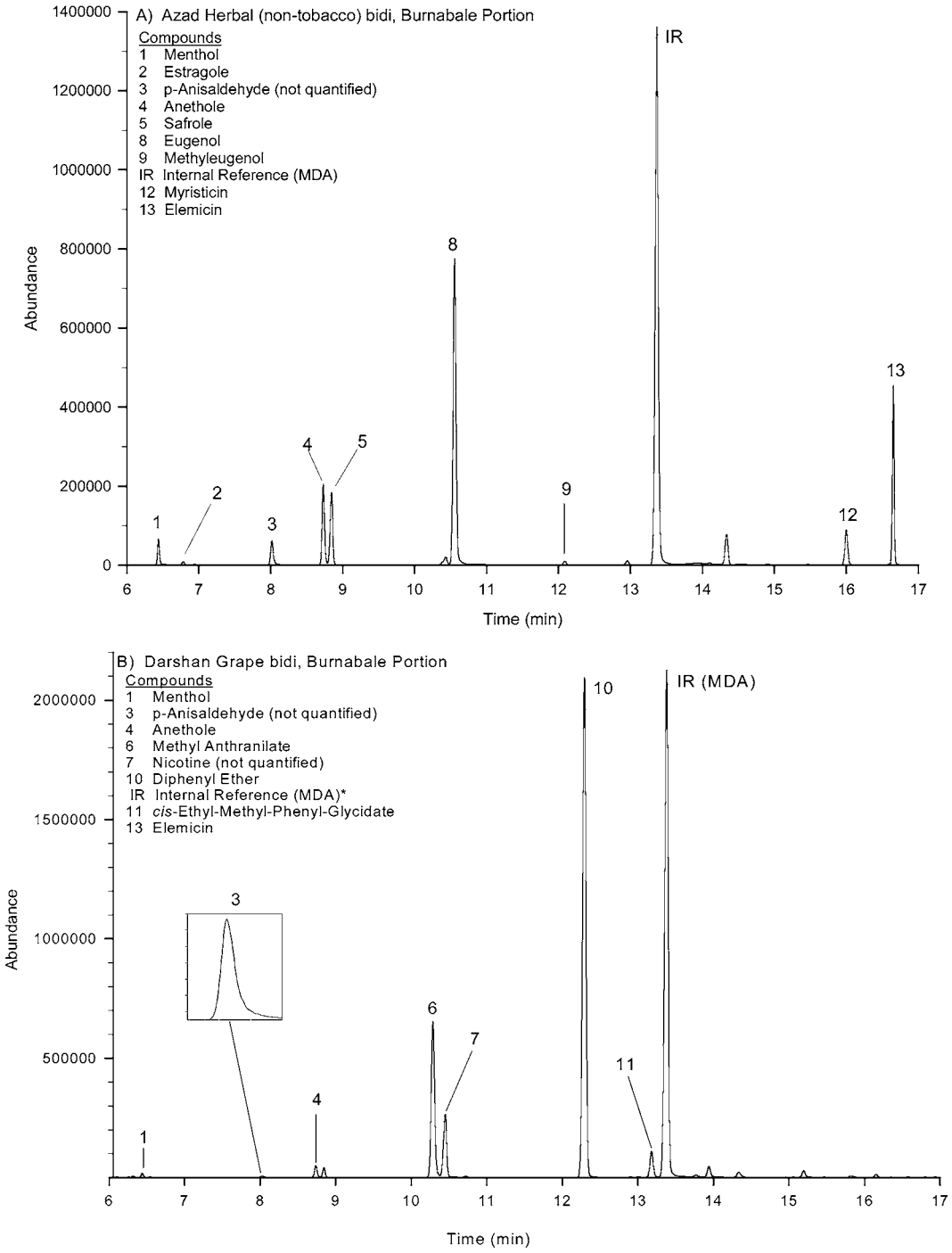


**Figure 3.** Selected-ion monitoring trace of the burnable portion of (**A**) Azad herbal and (**B**) Darshan grape bidis are shown below. The internal reference (IR) compound used for quantification in this study was 3′,4′-(methylenedioxy)acetophenone (MDA). One compound, *p*-anisaldehyde, was positively identified in both brands; however, it was not quantified. Nicotine was absent in the Azad herbal bidi but was identified (not quantified) in the Darshan grape bidi.

for the herbal bidi that contained many target analytes, these compounds (eugenol, anethole, myristicin, and elemicin) were rarely found in any brand except the clove-flavored bidis. Although the herbal bidi contained no tobacco, it contained high levels of several flavor-related compounds also found in tobacco-containing bidis. Among the bidi brands, Azad herbal bidis contained the highest levels of anethole, methyleugenol, myristicin, elemicin, estragole, and safrole.

The grape-flavored bidi contained several compounds not present in the other products tested in the study. Some grape bidis contained milligram per cigarette levels of diphenyl ether and methyl anthranilate, whereas *cis*-EMPG was present at much lower levels. This was the only grape-flavored bidi tested; therefore, we do not know if other grape-flavored bidi brands also contain these compounds. Cinnamon- and menthol-flavored bidis contained a limited number of the analytes we selected. They all contained menthol, but we detected only a few other flavor-related compounds (eugenol and pulegone) (**Table 3**). In contrast, clove, herbal, and grape bidis all contained a wide array of flavor-related compounds.

**High-Resolution Mass Analysis of Flavor-Related Analytes in Bidis.** In this study, we also utilized high-resolution mass spectrometry (GC-HRMS) to confirm the presence of compounds such as diphenyl ether, methyleugenol, and safrole in

**Table 6.** IUPAC and Common Names and CAS Registry Numbers of the Investigated Compounds

| IUPAC name | common name | CAS Registry No. |
|---|---|---|
| 1-methoxy-4-(2-propenyl)benzene | estragole | 140-67-0 |
| 5-methyl-2-(1-methylethylidene)cyclohexanone | pulegone | 15932-80-6 |
| 1-methoxy-4-(1-propenyl)-benzene | *trans*-anethole | 104-46-1 |
| 5-(2-propenyl)-1,3-benzodioxole | safrole | 94-59-7 |
| 1-hydroxy-2-methoxy-allylbenzene | eugenol | 97-53-0 |
| 1,2-dimethoxy-4-allylbenzene | methyleugenol | 93-15-2 |
| 2*H*-1-benzopyran-2-one | coumarin | 91-64-5 |
| 4-methoxy-6-(2-propenyl)-1,3-benzodioxole | myristicin | 607-91-0 |
| 3,4,5-trimethoxyallylbenzene | elemicin | 487-11-6 |
| diphenyl ether | diphenyl ether | 101-84-8 |
| (1$\alpha$,2$\beta$,5$\alpha$)-($\pm$)-5-methyl-2-(1-methylethyl)cyclohexanol | menthol | 2216-51-5 |
| 2-aminobenzoic acid methyl ester | methyl anthranilate | 134-20-3 |
| 3′,4′-(methylenedioxy)acetophenone | MDA | 3162-29-6 |
| 2,4,6-tris(trifluormethyl)-1,3,5-triazine | metri | 368-66-1 |
| *cis*-3-methyl-3-phenyloxirane carboxylic acid ethyl ester | *cis*-ethyl methylphenylglycidate | 19464-95-0 |

bidis. We analyzed the entire contents of Azad herbal and Darshan grape bidis with an Agilent 6890 GC (Palo Alto, CA) coupled with a GCT high-resolution time-of-flight mass spectrometer (Waters Corp., Milford, MA). The same SPME and GC parameters (GC ramp, inlet temp, etc.) employed in the quantification protocol with GC-SIM/MS were also used for the HRMS measurements. All mass measurements were recorded with a mass resolving power exceeding 7000 (full width at half-height) and a mass accuracy of $\pm$0.0005 amu. Mass spectral calibration and real-time mass adjustments were performed using a lock mass (284.9949 amu) from 2,4,6-tris-(trifluoromethyl)-1,3,5-triazine (CAS Registry No. 368-66-1) (see **Table 6** for CAS Registry Numbers of all investigated compounds) continuously introduced into the MS source. We further confirmed the identity of these compounds by mass spectral matching with a library search of full-scan spectra (R. P. Adams Mass Spectral Library; Allured Publishing, Carol Stream, IL). Using elemental composition software, the predicted molecular formula, exact mass measurements, and mass accuracy of target analytes were determined (**Table 4**). Using GC-HRMS, we confirmed the presence of estragole, anethole, safrole, eugenol, methyleugenol, myristicin, and elemicin in Azad herbal bidis and of diphenyl ether and methyl anthranilate in Darshan grape bidis.

**Quantification of Flavor-Related Analytes in Kretek Filler.** The three kretek brands analyzed contained high concentrations of eugenol, which ranged from 15400−78900 $\mu$g/cig. Eugenol levels in kreteks were 10-fold greater than found in the five clove-flavored bidi brands. We also detected compounds such as anethole, estragole, menthol, and coumarin in kreteks (**Table 5**). Myristicin and elemicin were present together in a single kretek brand. In addition, coumarin levels in kreteks (61−383 $\mu$g/cig) were higher than in bidis previously studied (*11*).

**Summary.** In this study, several flavor-related compounds were found in selected brands at very high levels. Five clove-flavored bidi brands contained eugenol at levels ranging from 148 to 7130 $\mu$g/cig, an almost 50-fold range from the lowest to highest values. The five clove-flavored bidi brands are produced by different manufacturers, which accounts for some of the observed variation; significant variability also occurred within a single pack and between packs of the same brand. An example of this variability is Shiv clove brand (**Table 3**), which varied from 984 to 4070 $\mu$g/cig of eugenol. Darshan grape was the only bidi brand containing a very wide range of diphenyl ether (128−1890 $\mu$g/cig) and methyl anthranilate (22.9−1290 $\mu$g/cig); two packs had similarly high levels, whereas a single pack had lower values. Lower values may be due to nonuniform flavor application and/or volatile losses due to product aging. Diphenyl ether and methyl anthranilate are likely associated with the grape flavorings used; however, without analyzing other grape-flavored bidi brands, we cannot confirm this association. Kreteks contained higher levels of eugenol, anethole, and coumarin (**Table 5**) than previously found in U.S. cigarettes (**Table 3**).

The Azad herbal bidi contained the greatest variety of compounds of the bidi brands tested. As mentioned earlier, the herbal components (i.e., powders, seeds, etc.) of the Azad herbal bidi were separated (**Figure 1**), methanol rinsed, and analyzed. Three compounds, safrole, myristicin, and elemicin, which were found in components of Azad herbal bidis (**Figure 1**; components a, b, and d), are fairly unique constituents of flavorings, including nutmeg and mace, derived from *Myristica fragrans* Houtton (*23*) (**Table 3**; **Figure 3**). The presence of these compounds in Azad herbal bidis strongly suggests that materials derived from nutmeg or mace may be a component of the herbal bidi filler. Myristicin and elemicin showed consistent levels in many of the tobacco-containing bidis tested, whereas safrole, a lower concentration constituent, was found only occasionally. Aside from bidis, safrole, myristicin, and elemicin occur together in cigarette tobaccos (*24*), and myristicin and elemicin were present in mainstream smoke from the same cigarettes; safrole was below the LOD in smoke (*25*).

Azad herbal bidi filler also contained long, striated seeds, which are shown in **Figure 1** (component c). Solvent-rinsed seeds, separated form the filler, were analyzed by GC-HRMS (>5 ppm mass accuracy) and found to contain three major compounds, estragole, *p*-anisaldehyde, and anethole. On the basis of the physical appearance of the seeds (*26*) and their corresponding chemical composition (*23*), they may be seeds from *Pimpinella anisum* L. (Anis). The majority of flavor chemicals present in Azad herbal bidis are thought to come from chemicals volatilized from botanical constituents (powders, spices, seeds, etc.) present in the filler.

Conversely, flavor chemicals present in most tobacco-containing bidis are likely introduced by adding a solution of flavoring materials to the tobacco prior to rolling or possibly by applying (spraying, dipping, etc.) the solution onto the completed bidi. Like flavorings added to domestic cigarettes (*9*) or kreteks (*21*), the bidi flavoring likely contains essential oils, extracts, and individual flavor chemicals. Variation in the composition and amount of exogenous flavoring added to bidis coupled with volatile losses that occur during production and storage may contribute to the variation between packs and, to a lesser degree, within an individual pack. The wide variation of diphenyl ether (128−3550 $\mu$g/cig) and methyl anthranilate (154−2360 $\mu$g/cig) in Darshan grape bidis (**Table 3**) is a good

example of between-pack variation; one pack had values much lower than the other two packs. Interestingly, the variability of flavor-related compounds in Azad herbal bidis was generally much smaller than that of tobacco-containing bidis, suggesting that the chemical content of herbal filler components is perhaps more consistent than the levels in tobacco-containing bidis, which are likely augmented with exogenous flavoring.

For flavored bidis, the typical tobacco odor is usually masked by a pleasant aroma (grape, cinnamon, clove, etc.). Clove-flavored bidis contain eugenol, a spicy flavor compound present in clove cigarettes, which can act as an anesthetic that numbs the throat, making smoke easier to inhale (*16*). As with clove cigarettes, it is also possible that bidi flavorings such as eugenol may diminish discomfort associated with initiation to the smoking of bidis. Bidis also deliver smoke levels of some toxic compounds (nitrosamines, polycyclic aromatic hydrocarbons, phenols, etc.) at levels higher than found in smoke from some conventional cigarettes (*2*, *27*). Moreover, bidi smokers have a greater incidence of various cancers (lung, liver, stomach, etc.) than cigarette smokers (*28*, *29*).

We analyzed a limited number of packs of bidis and kreteks (two or three of each brand). This small sample size restricts our statistical power to generalize the levels expected for a larger sampling of these products. However, we believe the data from even this relatively small sample set are an important indicator that bidi and kretek smokers are exposed to high levels of potentially harmful substances.

In this study, high levels of flavor-related compounds were measured in the burnable portion, which includes filler and wrapper material, of bidis and kreteks. The levels of smoke constituents transferred into bidi smoke have been measured and will be published elsewhere; estimates of potential exposure will be discussed in that work. It our estimation, compounds, such as diphenyl ether and eugenol, that are known to be hazardous to humans when inhaled in high concentrations pose significant health concerns, and usage of such compounds in smoking products, particularly at high levels, should be discouraged until detailed toxicity information is available.

## ABBREVIATIONS USED

GC, gas chromatograph or gas chromatography; HR-TOF-MS, high-resolution time-of-flight mass spectrometry; LOD, limit of detection; MS, mass spectrometer or mass spectrometry; SIM, selected-ion monitoring; SPME, solid phase microextraction.

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