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VOLATILES OF EXOGENOUS ORIGIN FROM THE HUMAN ORAL CAVITY

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SUMMARY

The volatiles found in the headspace above male and female saliva were examined by combined gas chromatography—mass spectrometry. This has led to the identification of a number of constituents of exogenous origin. The most likely source of these products are atmospheric and water pollutants as well as food stuffs and cosmetic products. Volatiles from saliva represent a potential medium for the detection of reproductive states as well as

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local and systemic diseases. Consequently, knowledge of compounds not arising from the body's metabolic process is important to prevent their identification as anomalous metabolites.

INTRODUCTION

Volatile compounds present in breath halitus emitted through the oral cavity provide significant information pertaining to three areas: (a) clinical detection and diagnosis of oral and systemic disorders; (b) the relationship between odor production to menstrual hormonal variations; and (c) the potential for human odor communication. Compounds in expired air have already received attention as a diagnostically important media for measuring pathological changes involving periodontal tissue destruction [1—3]. In addition, characteristic volatiles are reported to occur in the expired air of patients afflicted with various systemic disorders including cirrhosis of the liver [4, 5], uremia [6], diabetes mellitus [7] and large bowel cancer [8].

Anecdotal information as well as scientific reports have evoked considerable speculation concerning the possibility of human chemical communication [9—11]. Previous investigations pertaining to this possibility have conjectured that the vagina and/or the axillae are the odor producing areas of significance [12—16].

Recently, it has been reported from this laboratory that monthly changes in circulating sex steroids found in ovulating females influence the levels of volatile sulfur compounds of mouth air [17]. Subsequently, we have undertaken an investigation of volatiles in the headspace above incubated saliva from cycling females to determine the patterns of other compounds that may also be indicative of ovulation. The initial results of this study identified a number of volatile components, some of which appear to be artifacts ascribed to environmental contaminants, deodorant products and diet. To eliminate misinterpretation, it is important to recognize these exogenous materials in biological samples.

EXPERIMENTAL

Subjects

Volunteers were drawn from among the employees of the Monell Chemical Senses Center as well as the patient populations of the Veterans Administration Hospital and Out-Patient Clinic, the School of Dental Medicine, University of Pennsylvania and the Family Planning Service, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. The patient population consisted of male and female subjects (ages 20—40 years) with good general health.

All female subjects received a complete physical and pelvic examination. They were required to maintain an accurate basal body temperature chart during complete cycles prior to and during sample collection.

The intake of foods which could possibly introduce exogenous sources of compounds (i.e., members of the Alliceae family — garlic, onion, etc., or Cruciferae family — broccoli, cabbage, etc.) were recorded on a daily basis. In

addition, the usage of any medication whether prescription or over the counter, as well as tobacco consumption (frequency and type) were recorded. Male subjects were also requested to record foods, medication and tobacco consumption in the same manner as the female subjects.

Each subject also received a thorough oral exam to determine the health status of oral soft tissues. The oral health status was based on the determination of four universally accepted and highly reproducible indices [18]. Only subjects displaying low index ratings (i.e., good oral health) were allowed to participate in the study.

Salivary collection

Each subject donated a maximum of 10 ml of gum base (polyvinyl acetate, Life Savers Inc.) stimulated saliva within a 10-min interval. Subjects were instructed to abstain each morning prior to sample collection from food, liquid and desist from smoking and exercising oral hygiene. Collected samples were stored at −10° C until instrumental analysis was conducted.

Collection of volatiles

For each analysis, a 5-ml aliquot of saliva was placed into an individual 25-ml, 2-neck, round bottom flask, adapted with PTFE joints and equipped with a nitrogen line and collection tube. Diethyl phthalate was employed as the internal standard and added to each saliva sample prior to the onset of collection. Volatile organic compounds were collected and concentrated from the headspace of saliva, maintained at 37° C, using Tenax (Applied Science Labs., State College, PA, U.S.A.) polymer traps (150 mm × 1.5 mm I.D.). Collection times employed for each sample included two back-to-back collections of 1.5 h followed by one of 21 h. The remaining 5 ml of saliva were frozen at −60° C until needed for duplicate gas chromatography and gas chromatography—mass spectrometry (GC—MS) analyses [3].

Using the above procedure, the salivary volatiles were continuously collected during the indicated periods of incubation. Preliminary work with recovery of various standard mixtures of volatile organic compounds (acetic, propionic, isovaleric, butyric and heptanoic acids; CH_3SSCH_3, pyridine, picolines, benzaldehyde, 3-hydroxy-2-butanone, furfural alcohol, phenol, *p*-cresol, decanol, tetradecanol, indole, diphenylamine) from physiologic saline solutions shows that collection efficiency increases with time and decreases with compound polarity.

Reference compounds

The employed 99% pure internal standard diethyl phthalate was supplied through the courtesy of International Flavors and Fragrances (Union Beach, NJ, U.S.A.). Reagent grade allyl isothiocyanate was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Nanograde methylene chloride was employed as the solvent in dilution studies with the other reference materials.

GC—MS of incubated saliva headspace

GC—MS was used to study the mixture of organic compounds in the headspace above incubated saliva.

While the first 1.5-h and 21-h samples were generally used for GC—MS analyses; the second 1.5-h sample was employed for calculation of chromatographic peak areas.

The GC—MS system consisted of a Perkin-Elmer 990 gas chromatograph interfaced to a Hitachi RMU-6L mass spectrometer via a Watson-Biemann separator [19]. The separator was set at 270°C, the ionization chamber at 175°C and the ionization voltage was maintained at 70 eV. Mass spectra were scanned from *m/z* 12 to 400 in 6 sec. Identification of all compounds was confirmed by comparison of their mass spectra and chromatographic retention times with those from commercially available samples. Relative GC retention times were obtained by comparison of headspace components in relation to authentic samples with a series of C_2—C_{18} fatty acid ethyl esters to obtain their "ethyl ester index" [20].

Organic materials collected on the Tenax tubes were desorbed from the polymer by rapidly heating the tube to 240°C and maintaining that temperature for 15 min. The organics were transferred and condensed onto the front 15 cm of a chromatographic column which was cooled with dry ice. After desorption was complete, the dry ice was removed, the Tenax tube was removed from the injection port, the carrier flow was resumed through the column and the oven of the chromatograph was brought to its starting temperature of 70°C. The separation was effected on a 3.3 m × 2 mm I.D. Carbowax 20M glass column. Analysis conditions were as follows: injection port 260°C, flame ionization detector 260°C, and a helium carrier gas flow-rate of 40 ml/min. The temperature program employed was initially held at 70°C for 4 min, increased from 70°C to 230°C at 4°/min and the final temperature of 230°C was held for 12 min. The same amplifier sensitivity of $2 \cdot 10^{-10}$ A full scale was employed for each analysis. Peak areas were calculated by triangulation (height × width at half height).

RESULTS

Volatiles found in the salivary headspace of saliva collected from 17 subjects (3 females and 14 males) with essentially normal oral health are shown in Table I. Those compounds which appear to be exogenous are distinguished by an asterisk.

The GC profiles shown in the figures and the compounds listed in Table I are representative of the salivary volatiles seen for males and females with good to moderate oral health. As reported previously, in both males and females, oral degenerative diseases such as periodontitis, yield qualitative and quantitative changes in the salivary volatiles [3]. In addition, preliminary data from the salivary volatiles of the three females discussed also here indicate that quantitative changes occur in these volatiles during the menstrual cycle; however, daily examination of salivary volatiles from males over comparable 30-day intervals has not yet been completed.

Perhaps the most interesting of the compounds in Table I is allyl isothiocyanate which appeared in 5 of 50 saliva samples obtained from one female across two menstrual cycles. Fig. 1 is a typical GC profile of the headspace volatiles from this subject. The average amount of allyl isothiocyanate isolated

TABLE I

COMPOUNDS FOUND IN THE SALIVARY HEADSPACE

All the compounds listed except for heptadecane and an unknown sesquiterpene were also confirmed by their relative chromatographic retention times.

Compounds	Fig. 1	Fig. 2	Identified by mass spectrum
Acetone	A	A	yes
Ethyl alcohol	B	B	yes
Benzene	C	C	yes
Tetrachloroethylene*	D	D	yes**
Toluene*	E	—	yes
C_2—C_4 alkylbenzenes*	—	—	yes**
Dimethyldisulfide	—	F	yes
Styrene*	—	G	yes**
Acetoin	—	H	yes
Limonene	I	—	yes
Allyl isothiocyanate*	J	—	yes
Dimethyltrisulfide	—	K	yes
2-Ethyl-1-hexanol*	L	L	yes
Benzaldehyde	M	M	yes
β-Bourbonene	N	—	yes
β-Caryophyllene	O	—	yes
Heptadecane	P	P	yes**
Unknown sesquiterpene	Q	—	yes**
Naphthalene*	R	—	yes**
Isopropyl dodecanoate*	—	S	yes
Benzyl alcohol	T	T	yes
Phenylethanol	U	—	yes
Butylated hydroxytoluene (BHT)*	V	—	yes**
Dodecanol	W	W	yes
Phenol	X	X	yes
Isopropyl myristate*	—	Y	yes
p-Cresol	Z	Z	yes
Tetradecanol	a	—	yes
Isopropyl palmitate*	—	b	yes
Indole	c	c	yes
Skatole	—	d	yes
Diphenylamine	e	e	yes

*Probable exogenous constituent.
**These compounds compared with literature mass spectrum only, other compounds compared with authentic sample mass spectrum, as well.

from the saliva samples was 124 ± 24 (S.E.M.) ng with a range of 70—200 ng in 10.8 l of headspace. The mass spectra of both authentic allyl isothiocyanate and the compound recovered from a saliva sample are shown in Fig. 2. The probable origin of this component is the gourmet mustard consumed by this subject. Allyl isothiocyanate is a known constituent of mustard seeds [21].

Fig. 3 is a GC pattern from a male subject in whose salivary headspace sample isopropyl esters of C_{14} and C_{16} fatty acids were identified. These esters are known components of deodorant and cosmetic preparations [22], and have

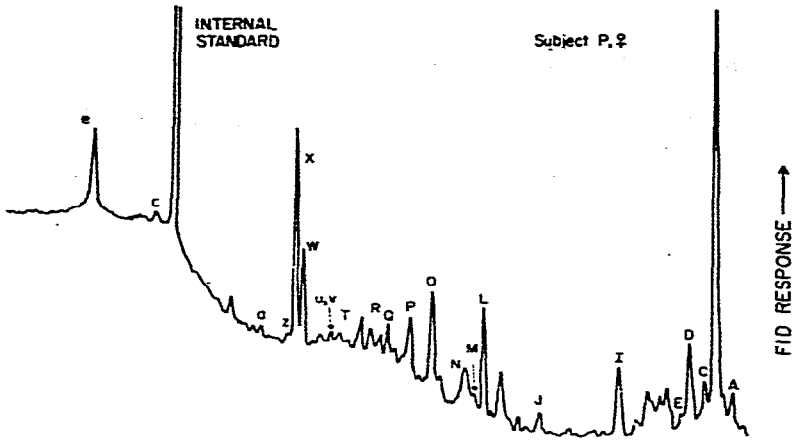

Fig. 1. Gas chromatogram of volatiles collected from the headspace above incubated whole saliva (90 min) of female subject P. This saliva sample was taken on day 15 of the subject's menstrual cycle. Volatiles were collected on Tenax and chromatographed on a 3.3-m 10% Carbowax 20M column with diethyl phthalate as the internal standard. See Table I for peak identification.

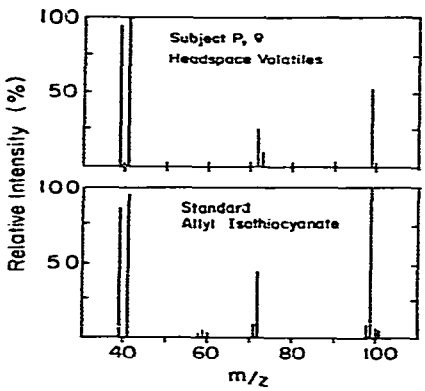

Fig. 2. Mass spectra of allyl isothiocyanate from the salivary volatiles of subject P (top) and reagent grade allyl isothiocyanate (bottom). Mass spectra of both were obtained as the compound eluted into the ion source from a 3.3-m 10% Carbowax 20M column.

been identified in other body fluids [16]. The subject revealed that the saliva sample was collected during the time he was applying a spray deodorant. Consequently, this sample was contaminated from the residual spray.

The aromatic hydrocarbons have been identified by other investigators as man-made emissions in urban air [23—26]. They may also arise, in part, from the Tenax tube traps, since toluene, styrene and alkylbenzenes have been occasionally observed in our blanks.

Butylated hydroxytoluene has been previously reported in saliva [27] and other body fluids [28]. It is an antioxidant used in a variety of foods. This compound has been sporadically observed in our samples.

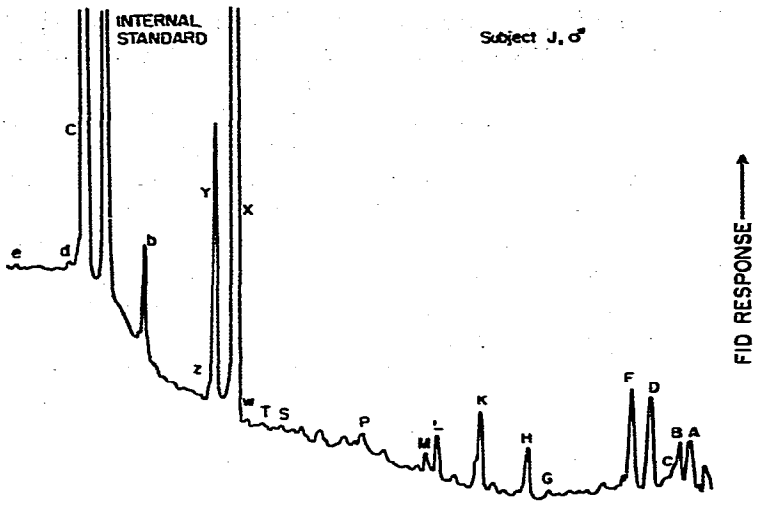

Fig. 3. Gas chromatogram of volatiles collected from the headspace above incubated whole saliva (90 min) of male subject J. Volatiles were collected on Tenax and chromatographed on a 3.3-m 10% Carbowax 20M column with diethyl phthalate as the internal standard. See Table I for peak identification.

The presence of tetrachloroethylene in Philadephia drinking water has been demonstrated by the EPA as well as our own analyses. This compound is also commonly found in urban air samples [23]. In addition, 2-ethyl-1-hexanol, a plasticizer, has been reported in body fluids [16].

DISCUSSION

The volatiles emitted by humans are gaining in importance as potential diagnostic aids [29]. However, a large number of exogenous organic compounds gain access to the body each day, some of which are stored by and only slowly expelled in secretions and excretions. Consequently, impurities make up some of the volatiles that are profiled by current headspace and GC—MS techniques.

Previous studies of volatiles produced within the oral cavity have employed small aliquots of mouth air for analysis [2, 30, 31]. These studies sought the nature of oral metabolites as well as those responsible for oral malodors. In the latter case, volatile sulfur compounds are involved in oral malodor and a gas chromatograph specifically equipped with a flame photometric detector is the method of choice for isolating and detecting the low levels of these compounds [1, 2]. In order to exploit the full potential of saliva as a diagnostic medium in the area of detection of reproductive and pathologic states, it is important to investigate a wide array of organic constituents present in healthy individuals. In our research, it has been found that part of the normal profile consists of organic constituents from diverse sources, e.g., urban air, drinking water, diet and cosmetic preparations.

Pollutants from man-made emissions in urban air, as well as chlorinated organic constituents found in municipal drinking water, have received considerable attention in the popular press because of their potential

detrimental health effects. The organic compounds identified in this study (i.e., aromatic hydrocarbons, tetrachloroethylene, etc.) have also been found in volatiles from plasma and axillary secretions [32–34, 16].

The role of diet as it affects the volatiles being profiled using GC–MS techniques has recently been reviewed [29]. Notable for the odors that they impart to the body, breath and urine are members of Alliceae (i.e., garlic, onions, etc.) and Cruciferae (cauliflower, cabbage, broccoli, etc.). Mustard is a member of the Cruciferae family and it appears that the occurrence of allyl isothiocyanate in our sample was directly related to the subject's preference for gourmet mustard*. In addition, this compound has been seen only on one other occasion in hundreds of other saliva samples analyzed in our laboratories.

Another constituent which appears clearly dietary in origin is butylated hydroxytoluene (BHT). Approximately 0.1 mg/kg body weight of phenolic antioxidants are consumed daily by man. Moreover, BHT can be metabolized by the human body and has been shown to inhibit the activation of certain carcinogens [35, 36].

The presence of the isopropyl esters was related, in our study, to the chance spraying of deodorant in the presence of the saliva being collected. However, in another study involving the analysis of secretions from the axillae, these compounds were present even after 16 days of not using any scented soaps or deodorant [16]. Since we have not seen these esters in detectable quantities in headspace of any other samples, they may not be absorbed into plasma, and hence into saliva, in any appreciable quantity. That saliva does reflect plasma levels of certain organic constituents has recently been shown [27]. In addition, a number of systemic diseases are manifested directly in the oral cavity by increased exfoliation of mucosal cells, ulceration and alterations of bacterial populations [37, 38]. Consequently, ongoing studies may show saliva to be an ideal sample which can be collected in a non-invasive manner and provide insight into body function.

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*This particular brand of mustard consumed contains a large number of mustard seeds.

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