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ANALYSIS OF COMPOSITION OF SUGAR MEALS OF WILD MOSQUITOES BY GAS CHROMATOGRAPHY

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ABSTRACT. Gas chromatography (GC) was successfully used for the first time to determine the components of natural sugar meals in individual mosquitoes and to determine whether carbohydrases are present in the crops of these insects. Crops of wild mosquitoes collected from a 2-ha cypress swamp north of Gainesville, FL, contained fructose, glucose, sucrose, maltose, turanose, melibiose, erlose, melezitose, raffinose, and a few unidentified carbohydrates. Time course studies with male and female *Aedes albopictus* showed rapid hydrolysis (>90%) of sucrose occurring within 2 h of ingestion, whereas melezitose remained relatively unchanged even 8 h after ingestion. The crop extraction/GC analysis technique is an improvement over the cold anthrone test traditionally used for sugar analysis. This procedure is a rapid one-step process used to determine natural sugar sources, hydrolysis, occurrence, and preferences for individual wild sugar-feeding Diptera.

KEY WORDS Mosquito, nectar feeding, sugar feeding, honeydew, gas chromatography, melezitose, quantitation

INTRODUCTION

Having adequate dietary sugar is a critically important aspect of the survival of mosquitoes and other dipterans that is often overlooked. Some species commonly have been observed feeding on natural sugar sources including floral nectars (Haeger 1955; Breeland and Pickard 1961, 1967; Sandholm and Price 1962; Grimstad and DeFoliart 1974; Magnarelli 1980, 1983; Vargo and Foster 1984; Yee et al. 1992; Yuval 1992; Foster 1995), extrafloral nectars (Haeger 1955, McCrae et al. 1976, Gadawski and Smith 1992, Taylor and Foster 1996), honeydew (Nielsen and Greve 1950, Haeger 1955), fruit (Joseph 1970), and other sources (Patterson et al. 1969, Worth 1975, Mogi and Miyagi 1989). Several aspects of mosquito sugar feeding have been reviewed by Yuval (1992) and Foster (1995).

Few attempts have been made to determine the exact sugar composition in dipteran crops using modern chromatography. Thin-layer chromatography has been used for pooled samples of tabanids by Magnarelli and Anderson (1977) and of mosquitoes by Magnarelli (1980). Hoppe (1983) and Burgin and Hunter (1997) used high-performance thin-layer chromatography for qualitative determination of sugars found in tabanids and black flies, respectively. High-performance liquid chromatography was successfully used by MacVicker et al. (1990) to examine the crops of 5 Italian sand fly species. Despite its common use in other systems, only 4 studies have used gas chromatography (GC) for investigating dipteran diets, including those of Schaefer and Miura (1972) for *Culex tarsalis* Coq., Moore et al. (1987) and Alexander (1988) for phlebotomine sand flies, and Chang et al. (1977) in tephritid fruit flies. All of these studies analyzed pooled crop samples.

As indirect evidence for "nectar" feeding, thousands of mosquitoes and other Diptera have been tested using the cold anthrone method, which determines the presence or absence of reducing sugars (e.g., fructose) found in almost all plant nectars. Many authors (Van Handel et al. 1972; Bidlingmayer and Hem 1973; Magnarelli 1978, 1979, 1980; Nasci and Edman 1984; Reisen et al. 1986; Andersson and Jaenson 1987; Edman et al. 1992; Smith and Kurtz 1994) have used the cold anthrone test developed by Van Handel (1972) to qualitatively establish the presence or absence of crop "fructose." According to Van Handel (1967), the cold anthrone reagent reacts with fructose, inulin, sucrose, melezitose, raffinose, and other sugars. These sugars all contain the fructose moiety and give positive or negative responses based on a color change. Unfortunately, the cold anthrone test does not provide any information about the origin or exact composition of sugars found in the dipteran crop and provides no quantitative information. Indeed, if no enzymatic breakdown of sugars occurs in the crop, determining if flies have preferences for certain families of plants or types of nectar (sucrose-rich for example) should be possible. Furthermore, we suspect that many of the reports of "nectar" feeding due to the presence of "fructose" in the crop may actually have been due to a positive anthrone reaction to melezitose (a common trisaccharide found in honeydew) or other reducing sugars not commonly found in floral nectaries.

Gas chromatography is an excellent and powerful tool for identifying and quantifying the exact dietary sugar preferences, occurrence, and composition of sugar meals in wild flies. Processing crop contents for GC is only slightly more labor intensive than the methods used for the cold anthrone

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test. A sample of <1 μl is all that is required. The technique is relatively rapid, employing silylation of crude crop contents and subsequent GC analysis. We describe here a method that allows researchers to determine the individual sugar meal components for mosquitoes and other flies, or the apparent presence or absence of carbohydrases in the crops of mosquitoes and other flies by monitoring the decline in parent sugar concentrations, and the increase in the metabolites over time.

MATERIALS AND METHODS

Specimen preparation: Representative samples of wild mosquitoes were vacuumed from resting sites shortly after sunrise in a ca. 1-ha cypress swamp surrounded by pine flat woods located north of Gainesville, FL. Mosquitoes were kept alive, chilled, and processed within 4 h of capture. Specimens were sacrificed by laterally inserting a #0 or smaller insect pin just above the mesothoracic spiracle. Legs and wings were removed using fine forceps. The crop (ventral diverticulum) was exposed by grasping the 3rd or 4th abdominal segment and pulling back slowly so that the crop would emerge between the abdominal segments. For crops containing liquid, <1 μl of fluid was sampled using a fine-tipped 10-μl capillary tube made by heating and pulling glass tubing. The capillary tube contents were transferred to a 200-μl glass tube inserted into a 3-ml glass GC sampling vial (National Scientific Company, Lawrenceville, GA) and secured with a Teflon-lined cap. A separate vial was used for each specimen.

Sample preparation for GC analysis: Sugars are highly polar compounds and their analysis by GC requires silylation to derivitize the polar carboxyl and hydroxyl groups. The derivitizing agent Tri-Sil Z® (Pierce Chemical Company, Rockford IL), composed of TMSI (*N*-tri-methyl-silyl imidazole), in dry pyridine was used to process crop contents. Tri-Sil Z (100 μl) was added to each vial containing one crop extract and each vial was vortexed, heated at 60–70°C for 15 min and frozen until analysis. Using 1 μl out of the 100-μl Tri-Sil Z preparation, GC was performed using an Hewlett Packard 6890 instrument with an on-column auto injector, flame ionization detector, and equipped with a DB-5 fused silica capillary column (30 m × 0.25 μm, J & W Co., Folsum, CA). The column was heated from 60 to 300°C at a ramp of 20°C/min for 20 min. Pyridine and acetonitrile were used as solvents to clean the syringe between samples. The following sugars (Aldrich Chemicals, Milwaukee, WI) were made up as 0.1% standards in distilled water: D(−)fructose, D-glucose, sucrose, maltose, D(+) melezitose, L-arabinose, L-rhamnose, D(+)melibiose, D(+)raffinose, turanose (a hydrolysis product of melezitose), and trehalose. Trehalose is a sugar present in insect hemolymph (Friedman 1985); the other sugars have all been found associated directly or indirectly with plants (Percival 1961, Van Handel et al. 1972). Melezitose and turanose are known to be associated with honeydew (Auclair 1963). The data and resulting chromatograms and integrations were recorded and processed using a PE Nelson 900 Series (970A) interface with Turbochrome® software (Ver 4.1, 1995, Perkin-Elmer Corp., Cupertino, CA).

Crop sucrose and melezitose hydrolysis: Thirty to 50 laboratory-reared *Aedes albopictus* (Skuse) (July 1995, U.S. Department of Agriculture, Gainesville, FL) pupae were placed in clean 35-mm film canister lids and allowed to emerge in new 200-ml urine cups with fitted fine mesh screen lids. A total of 25 cups with pupae was assembled. The adult mosquitoes were deprived of sugar and water for 2 days and kept in a rearing chamber at 28°C, 80% relative humidity and 14 h light: 10 h dark. The mosquitoes were then provided unlimited access to 10% standard solutions of sucrose and melezitose for 2 h. Blue food coloring was added to the sugar solutions to aid detection in the crop. Mosquitoes were fed by placing 10 drops of the standard sugar solutions atop the screens of each cup. Mosquitoes from each cup were sacrificed until several samples of males and females were found to have contents in their crops. Individual mosquito samples were taken at 2, 4, 8, and 20 h after ingesting one of the sugar solutions. A sample of the dyed standard sugar solutions was used as a control (time 0). Mosquitoes were otherwise processed and analyzed as discussed above.

RESULTS AND DISCUSSION

Our preliminary analyses showed a wide variation in the types of sugars present in wild mosquitoes. Our chromatographic analysis of combined standard sugars indicated that these common plant mono-, di-, and trisaccharides have unique retention times (Fig. 1a). Some of the sugars, such as fructose, glucose, and others, are anomeric molecules and display one peak for each form. Qualitative analysis of crude crop contents of wild *Culiseta melanura* (Coquillett) (Figs. 1b, 1c) and *Anopheles quadrimaculatus* s.l. Say (Figs. 1d, 1e), indicated that most of the peaks are identifiable by GC. Figures 1b–1e show that these female mosquitoes had recently fed on sugar sources containing fructose, glucose, sucrose, turnanose, melibiose, melezitose, raffinose, and a few unknowns. Later GC runs identified the unknown trisaccharide as erlose (glucosucrose). Honeydew has been found to contain oligosaccharides (e.g., melezitose, erlose, turanose, and trehalulose) that are largely unique to homopteran exudates and are uncommon in plant nectars (Hudson 1946, Auclair 1963, Lombard et al. 1987, Bates et al. 1990, Byrne and Miller 1990, Yee et al. 1996). The presence of a large sucrose peak in *Cs. melanura* (Fig. 1c) suggests that either salivary sucrase is not present in this species or, more likely,

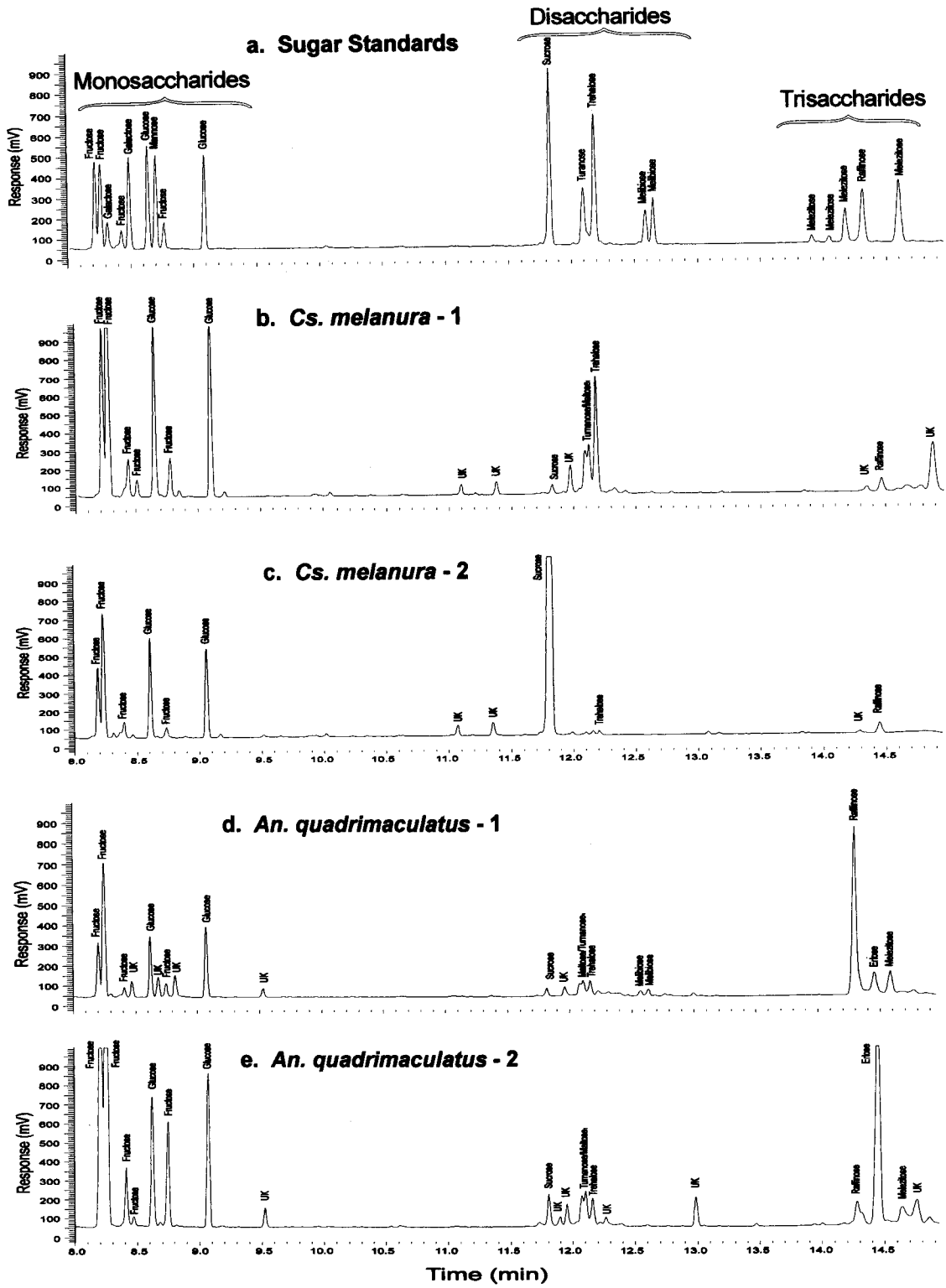

Fig. 1. (a) Chromatogram of combined standards (ca. 0.1%) for common sugars associated with plants. (b, c) Representative chromatograms of crude crop extracts of wild-caught *Culiseta melanura*. (d, e) Representative chromatograms of crude crop extracts of wild-caught *Anopheles quadrimaculatus*. Multiple peaks of the same sugar are anomeric forms.

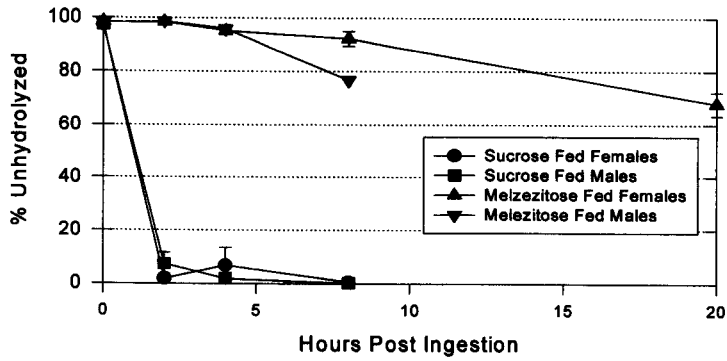

Fig. 2. Timed feeding trials for male and female *Aedes albopictus* showing the hydrolysis of melezitose and sucrose occurring in the crop (n = 2–5).

was not secreted at the time of feeding. Another possibility is that the sugar source contains carbohydrase-inhibiting compounds that prevent the immediate hydrolysis of sucrose into glucose and fructose.

Much of the evidence for mosquito nectar feeding has come from direct field observations rather than through specific chemical qualitative crop content analysis. West and Jenkins (1951), Sandholm and Price (1962), Gadawski and Smith (1992), Breeland and Pickard (1961, 1967), and Magnarelli and Anderson (1977) observed some mosquito species feeding at sugar sources during the day. Others observed them feeding on sugar sources at night, dawn, or dusk (Grimstad and DeFoliart 1974, Magnarelli 1983, Vargo and Foster 1984, Andersson and Jaenson 1987, Bowen 1992, Yee et al. 1992). Many mosquito species have been found to contain "fructose" in their crops (Bidlingmayer and Hem 1973), yet some of these species have not been observed sugar feeding.

Sucrose- and melezitose-fed mosquitoes

Extensive work has been done characterizing protein acquisition and the enzymatic processes of protein (blood meal) digestion in the mosquito midgut. However, relatively little has been done to assess the relative importance of sugar acquisition and its subsequent digestion. Gas chromatography analysis provides a rapid method of indirectly determining the presence or absence of carbohydrases in the crop (from saliva shunted to the crop).

Forty-five samples of sucrose- and melezitose-

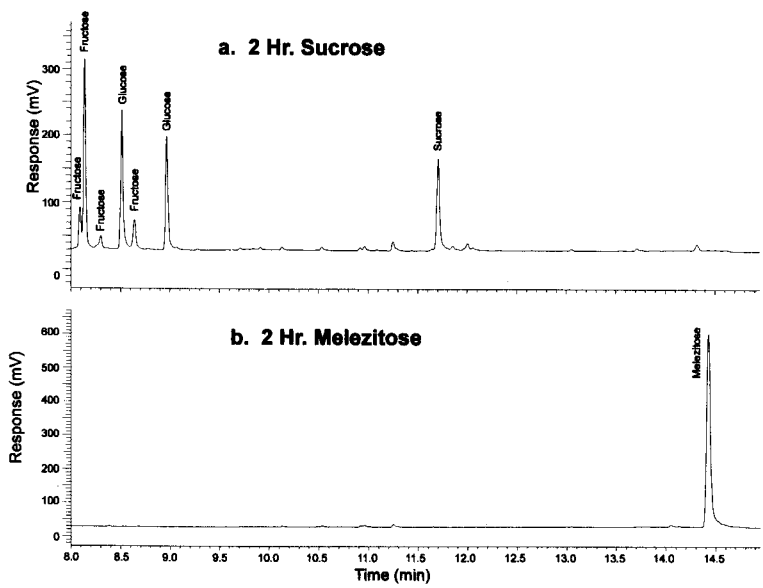

Fig. 3. (a) Representative chromatogram showing hydrolysis of sucrose into fructose and glucose 2 h after ingestion in *Aedes albopictus* Multiple peaks of the same sugar are anomeric forms. (b) Representative chromatogram showing hydrolysis of melezitose 2 h after ingestion in *Aedes albopictus*.

fed *Ae. albopictus* were processed. Figure 2 shows the time course of crop sucrose and melezitose, respectively, for males and females following ingestion. Almost complete hydrolysis (>90%) of sucrose occurred within 2 h of ingestion, whereas melezitose remained relatively unchanged even 8 h after ingestion. No significant difference was found between the response of the males and females fed sucrose or melezitose (P = 0.61 and 0.65, respectively; analysis of variance, SAS institute, Cary, NC). In general, the crops were largely empty 16 h after ingestion and the number of unidentified peaks increased after 4 h in the crop. Figure 3a shows a representative chromatogram of crude crop sucrose extract 2 h after ingestion. This chromatogram shows sucrose broken down into the anomers of fructose and glucose. Conversely, melezitose from *Ae. albopictus* remained relatively unaltered 2 h after ingestion (Fig. 3b) and was found to remain largely unhydrolyzed even 20 h after ingestion.

Crop and salivary enzymatic activity undoubtedly varies by species. Depending on the species and type of sugar, GC analysis can reveal important primary sugar meal preferences. Only a few hematophagous Diptera have been reported to contain salivary carbohydrases (Gooding 1975), which are presumably shunted to the crop upon ingestion of sugar meals. Marinotti and James (1990) and Marinotti et al. (1990) showed that *Aedes aegypti* L. possessed an α-glucosidase in the saliva that works to break down some sugars in the crop. Of the sugars tested, they found that sucrose was broken down the fastest, followed by maltotriose, maltopentaose, and maltose. Melezitose, trehalose, raffinose, starch, and other carbohydrates were only minimally affected. Indirect evidence for salivary or crop enzymes also exists. Schaefer and Miura (1972) found carbohydrases in the saliva of *C. tarsalis,* including invertase, maltase, melezitase, amylase, and lactase. Crops of sucrose-fed sand flies were analyzed with GC and found to contain the hydrolysis products fructose and glucose (Moore et al. 1987, Alexander 1988). Likewise, in preliminary GC runs, no evidence was found of hydrolysis products in melezitose-fed flies (Alexander 1988). At least for some species, it is possible to use GC to determine the proportions and sugars present in the crops of wild mosquitoes and the relative importance that melezitose or other unhydrolyzed sugars play in the diets of medically important Diptera.

In most cases, sugar-fed female mosquitoes should be able to enhance their survival, host-finding ability, and ultimately improve their chances of vectoring pathogens. A few studies have shown that female sugar-fed mosquitoes of 3 species have lower host avidity than starved or water-fed females (Foster and Eischen 1987, Xue and Barnard, unpublished data). However, other authors found that females with access to sugar sources are more persistent in their attempts at bloodfeeding than are sugar- and water-starved females (Walker and Edman 1985). Likewise, Kelly and Edman (1996) found higher parasite transmission rates in sugar-fed *Ae. aegypti.*

This paper describes a technique that allows accurate identification and quantification of sugars present in individual mosquito meals. The equipment used to perform the described analyses costs from $15,000 to $30,000 depending upon the manufacturer. Unlike other studies that typically use pooled samples, our GC technique, when applied to sufficient sample sizes, can provide valuable insight into the occurrence, composition, and importance of certain life-sustaining sugar sources for wild mosquito populations. From this information, attractants or other methods could be developed to help control or improve sampling of mosquito populations.

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