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The Role of Analytical Chemistry in Repellent Research

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ABSTRACT

Development of effective repellents requires in depth investigation of the interaction of animals with their chemical environment. This multidisciplinary field, chemical ecology, has received much attention in the area of plant/invertebrate herbivore interactions. At the Denver Wildlife Research Center (DWRC), we have benefited by the close collaboration of chemists and biologists when studying the interactions of vertebrates with natural products. Typically, this combines chemical assays that provide information on the characteristics of chemical cues with bioassays that generate information on the mechanisms that drive animal behavior. The chemistry role in this research can be either supportive or interactive. Two recent studies illustrate the integral role of chemistry in the study of plant/animal interactions.

KEY WORDS

Analytical chemistry, chemical ecology, plant/animal interactions, repellent

INTRODUCTION

Laboratory bioassays are staples of repellent research. The goal of these investigations is to elucidate the basis of chemically mediated animal behavior. Chemical assays are equally as important but are less frequently applied to repellent research. The analytical techniques available to today's chemists are invaluable tools in most research situations. In particular, we have gained useful information by applying chemical extraction, separation, and detection techniques to the investigation of plant/animal interactions.

The most basic role for analytical chemistry in repellent research, and the easiest to overlook, is the support role. A supportive role may consist of performing chemical assays on test compounds and formulations. Because many of thc compounds that have been reported to deter feeding are commercially available, it is common practice to obtain the technical material and run a series of bioassays employing established test paradigms. However, while not a frequent occurrence, the manufacturer's reported purity is not always correct. As one can imagine, huge errors in data interpretation can occur if a 50% pure test substance was assumed to be the 95% reported on the certificate of analysis. The results could be skewed by overestimating the test substance concentration. This problem can be exacerbated when minor impurities or undesired isomers present in the test substance have greater or different biological activities than the compound of interest. A notorious example of stereospecific bioactivity is the drug thalidomide (Glanz 1993). It is now thought that one enantiomer produced the desired pharmacological effect while the other caused severe birth defects.

This is a very important issue in the field of repellent research. An excellent example can be seen in the importance of stereochemistry and biological activity. Chemosensory responses have long been known to be stereospecific. A classic example often cited in text books is the odor differences between enantiomeric isomers of carvone. Friedman and Miller (1971) concluded that carvone enantiomers have distinctly different odors. For instance, the (-) isomer has a distinct spearmint odor, while the (+) isomer has the odor associated with caraway. Taste perception is also subject to stereospecificity. Generally, the L-amino acids are bitter while the D-amino acids are perceived as sweet (Kato et al. 1989). Thus, differences in chemosensory responses have obvious impacts on bioactivity in general and, logically, impacts on repellency. Analytical techniques are readily available which allow for identification and detection of enantiomers.

Incorrectly formulated products can lead to similarly disastrous results. Because of the complexities of some formulations, this is a potentially more frequent problem. For instance, encapsulation is subject to a number of critical formulating conditions (DeZarn 1995). Improper conditions can yield overformulated final products. Conversely, underformulated products could result from the loss of the test compound due to volatility, improper encapsulation processes, the use of impure technical material, etc. Loss of the active ingredient during shipping or storage is also common. Analyzing formulations shortly before they are applied to the test system can save a lot of time and money. One such example is the work of Avery et al. (1996). In this study, the formulations were found to be 40% of the target concentration. This knowledge made it much easier to explain the results of the field tests.

Beyond simple technical or formulation analyses, the application of analytical chemistry to repellent research can provide valuable information. We have successfully applied analytical chemistry techniques to the research of chemical cues in mammal foraging. The object of one such study was the development of a herbivore repellent to minimize forest damage initiated by mountain beaver (*Aplodontia rufa*). Mountain beaver inhibit reforestation efforts by clipping seedlings and girdling 15- to 20-year-old trees. Campbell and Evans reported in 1988 that mountain beaver had damaged in excess of 121,000 ha of highly productive forest land, resulting in estimated losses of millions of dollars. We investigated whether toxic cardiac glycosides mediate herbivore avoidance of digitalis (*Digitalis purpurea*) extracts by conducting a series of multiple-choice feeding trials. Samples of these extracts were also analyzed chromatographically.

The results of the behavioral and chromatographic analyses indicated that the presence of the cardiac glycosides in the extracts was not essential to illicit avoidance behavior.

In another study, chemical assays were employed to relate the chemical characteristics of a forage to the feeding behavior of black bears (*Ursus americanus*). Bears strip the bark from trees to feed on sapwood in the spring, presumably because the carbohydrates present in the sapwood are an excellent source of high energy food (Radwan 1969). Bear foraging is detrimental to the health and economic of value of timber stands, in addition to impeding reforestation efforts. Complete girdling due to foraging is lethal, while partial girdling leads to lower growth rates, insect infestation, and/or disease (Kanaskie et al. 1990). Complete losses can potentially occur in stands with tree densities near 200 trees/acre since an individual bear can peel the trunks of 50 to 70 trees per day (Schmidt and Gourley 1992). However, bitter tasting terpenoids, present in sapwood, may deter feeding (Harborne 1991). Chromatographic techniques were developed for the analysis of Douglas-fir (*Pseudotsuga menziesii*) sapwood for terpenoids and carbohydrates.

METHODS

Digitalis Extracts

Liquid-solid digitalis extracts (1.0 L) were prepared at room temperature with methanol (L–S:M), hexane (L–S:H), chloroform (L–S:C), or water (L–S:W). Liquid-liquid extracts were derived from 1.0 L hot water extracts. These were prepared by washing the aqueous digitalis extract with 1.2 L of hexane followed by 1.2 L chloroform. The hexane (L–L:H1) and chloroform (L–L:C2) phases, as well as the resultant filtrate (RF:A) were collected. This process was repeated with another hot water digitalis extract, but the order of the hexane and chloroform washes was reversed. Again, the chloroform (L–L:C1) and hexane (L–L:H2) phases were collected as was the resultant filtrate (RF:B). Emulsions formed during the extraction process were discarded.

All extracts were analyzed for digitoxin and gitoxin with a Hewlett Packard 1090M High Performance Liquid Chromatograph (HPLC) equipped with a diode array detector (Hewlett Packard Co., Palo Alto, CA). For more selective detection, we have also developed a liquid chromatographic technique that couples the separation capability of LC with selective pulsed amperometric detection (PAD) (Kelly et al. 1995). This electrochemical detector is specific for those compounds that are electroactive. In this case, the sugar moieties of the cardiac glycosides are easily detected by their oxidation on a gold electrode (Rocklin and Pohl 1983).

A series of multiple-choice tests were used to determine mountain beaver response to extracts derived from digitalis. First, mountain beaver were given a choice of seven treatments. Treatments consisted of apple cubes (1 cm^3) treated with the liquid-liquid extracts (L–L:H1, L–L:H2, L–L:C1, L–L:C2) and the resultant filtrates (RF:A, RF:B) along with a water control. Response of mountain beaver to a six-choice test (L–S:H, L–S:C, L–S:M, L–S:W, RF:B, and a water control) was assessed in the second test. The third behavioral assay was a four-choice test conducted with the solvents (methanol, hexane, chloroform, and a water control) used to prepare the extracts. Peeled apples were selected as the test food because they are readily ingested by mountain beaver.

Ten mountain beaver were presented trays containing 20 apple cubes. All cubes in a tray were treated with one of the extracts or a control (water). Water and feed were available ad libitum. The number of apple cubes remaining in each tray was determined at the end of each 24-hr period. The treatment period was 4 days. The data for each behavioral assay were assessed separately in single factor analyses of variance (ANOVA). Mountain beaver avoidance response was rated as: (high) greater than control; (low) similar to control; or (moderate) greater than control but less than extracts rated as high.

Sapwood Preference

Forest stands of Douglas-fir in western Washington and Oregon were monitored for bear foraging activity. Recent bark peeling (less than 5 days old) was located in two sites, and all foraged trees in each stand were sampled. Sapwood was collected by removing two 40- × 10-cm patches of bark on opposite sides of the tree and scraping the sapwood (phloem tissue and xylem oleoresin located immediately below the cork cambium) into a plastic bag. The freezer bag and contents were immediately frozen and kept on dry ice until placed in a laboratory freezer at -24 °C. Samples were maintained frozen until homogenized with a mallet and divided. One portion was maintained frozen until analysis for terpenoids, while the other was lyophilized and analyzed for carbohydrates. The surface area of removed bark as well as DBH (diameter at breast height) and circumference at the base were determined for each tree. Basal surface area was calculated as the surface area of the tree from base to breast height (1.5 m). Sapwood density was determined as the mass of sapwood per 800 cm^2 sample area.

Terpenoids analyses were performed according to published procedures (Kimball et al. 1995). This method combines a simple ethyl acetate extraction with sensitive gas chromatographic (GC) analysis. High resolution capillary gas chromatography was combined with mass selective detection (MSD) which not only provided sensitive terpenoid detection but also excellent identification capabilities. Each terpenoid found in an extract was first identified by its mass spectrum and then verified by comparison to a reference standard. Carbohydrates were analyzed by extracting the lyophilized material with 50% aqueous ethanol and injecting the extracts onto an ion chromatograph equipped with a PAD. As opposed to the reversed-phase liquid chromatographic technique employed for the digitalis research, anion exchange chromatography was coupled with selective PAD detection.

To investigate the physical and chemical characteristics related to trees preferred or rejected by bears, trees sustaining damage greater than 3,000 cm^2 were considered to be preferred, while trees sustaining some damage but less than 300 cm^2 were considered to be sampled and rejected. Two sample t-tests were employed to detect differences in chemical and tree size characteristics between preferred and rejected trees. Differences between the sapwood concentrations of each individual compound, sum of all quantified terpenoids, sum of all quantified sugars, sum of sweet sugars (fructose, sucrose, and glucose), basal surface area, DBH, and sapwood density were investigated.

RESULTS AND DISCUSSION

Digitalis Extracts

Mountain beaver (n = 9) responses varied among treatments in the first behavioral assay ($P<0.0001$). One animal was not included in the analysis because it failed to ingest any treatment or control cubes throughout the assay. Mountain beaver took fewer ($P<0.05$) apple cubes treated with either of the resultant filtrates (RF:A, RF:B) or the initial phase L–L extracts (LL:H1, LL:C1) than they did control cubes or cubes treated with the second phase liquid-liquid extracts (LL:H2, LL:C2). None of the solvents used to prepare extracts were aversive (Table 1).

Table 1. Presence of Cardiac Glycosides and Relative Avoidance of Digitalis Extracts (After Nolte et al. 1995)

Extract	Digitoxin or Gitoxin Present?	Relative Avoidance
L-S:W	Yes	High
L-S:M	No	High
L-S:C	No	Low
L-S:H	No	Low
L-L:C1	Yes	High
L-L:H2	No	Low
RF:A	No	High
L-L:H1	Yes	High
L-L:C2	Yes	Moderate
RF:B	No	High
Water	No	Low
Methanol	No	Low
Chloroform	No	Low
Hexane	No	Low

All extracts were analyzed for digitoxin and gitoxin to determine if the cardiac glycosides were present (Table 1). Digitoxin and gitoxin were detected in the hot water liquid-solid extract prior to being subjected to the liquid-liquid extraction. HPLC analysis also indicated that digitoxin and gitoxin were present in both chloroform liquid-liquid extracts (L–L:C1 and L–L:C2) as well as the initial hexane liquid-liquid extract (L–L:H1). Cardiac glycosides were not present in the second hexane L–L extract (L–L:H2) or either of the resultant filtrates (RF:A and RF:B). Though the room temperature water liquid-solid extract contained digitoxin and gitoxin, neither of these cardiac glycosides were detected in the other liquid-solid extracts (L–S:M, L–S:H, L–S:C). The limits of detection for digitoxin and gitoxin were 0.67 and 0.48 μg/mL, respectively.

Quantitative analysis demonstrated that the gitoxin concentration was similar in all extracts which contained cardiac glycosides. These concentrations ranged from 5.7 to 8.3 μg/mL. However, the gitoxin concentration in the room temperature water extract (L–S:W) was much less than the concentration observed in the other glycoside containing extracts. The gitoxin concentration in L–S:W was 3.8 μg/mL while the concentration ranged from 11.9 to 14.7 μg/mL in the other cardiac glycoside containing extracts.

Chemical analysis of all extracts subjected to bioassay revealed that the presence of digitoxin or gitoxin was not required to elicit an aversive response. High avoidance was observed in RF:A, RF:B, and L–S:W, all of which were void of cardiac glycosides. Furthermore, avoidance of the cardiac glycoside containing extracts was not correlated with the concentration of digitoxin or gitoxin. For example, S–L:W, which produced high relative avoidance, contained 5.7 and 3.8 μg/mL of digitoxin and gitoxin, respectively, while the digitoxin and gitoxin concentrations of the moderately avoided L–L:C2 were 5.4 and 11.9 μg/mL, respectively.

Though other cardiac glycosides have been identified in digitalis (Ikeda et al. 1995), digitoxin and gitoxin should be good indicators of the cardiac glycosides present in the digitalis extracts. Digitoxin and gitoxin are two of the three main secondary glycosides formed from the primary glycosides that are present in living, undamaged digitalis plants. Since the polarities and solubilities of all the primary and secondary glycosides are similar, we used the presence of digitoxin and gitoxin as "markers" for the presence of all possible cardiac glycosides.

The choice of extraction solvents also generated information concerning the compounds that are responsible for the aversive responses observed in bioassay. The four solvents chosen to prepare liquid-solid extracts ranged in polarity from water (very polar) to hexane (very nonpolar). Only extracts prepared with polar solvents (i.e., methanol and water) were shown to be aversive in bioassay. This is also true of the aversive liquid-liquid extracts because they were all derived from aqueous liquid-solid extracts. The nonpolar extracts, chloroform and hexane, did not invoke aversive responses. Therefore, because solutes are generally soluble in solvents of like polarity, the aversive cues present in digitalis extracts are likely polar and thus unlikely to be volatile.

Sapwood Preference

For Site 1, t-test results identified significant differences in sucrose concentrations between rejected and preferred trees (Figure 1). The results from Site 2 were more diverse. The concentrations of the terpenoids 3-carene, caryophyllene, citronellyl acetate, linalool, sabinene, terpinen–4–ol, thujene, α–terpinene, terpinolene, *p*–cymene, and γ–terpinene all showed

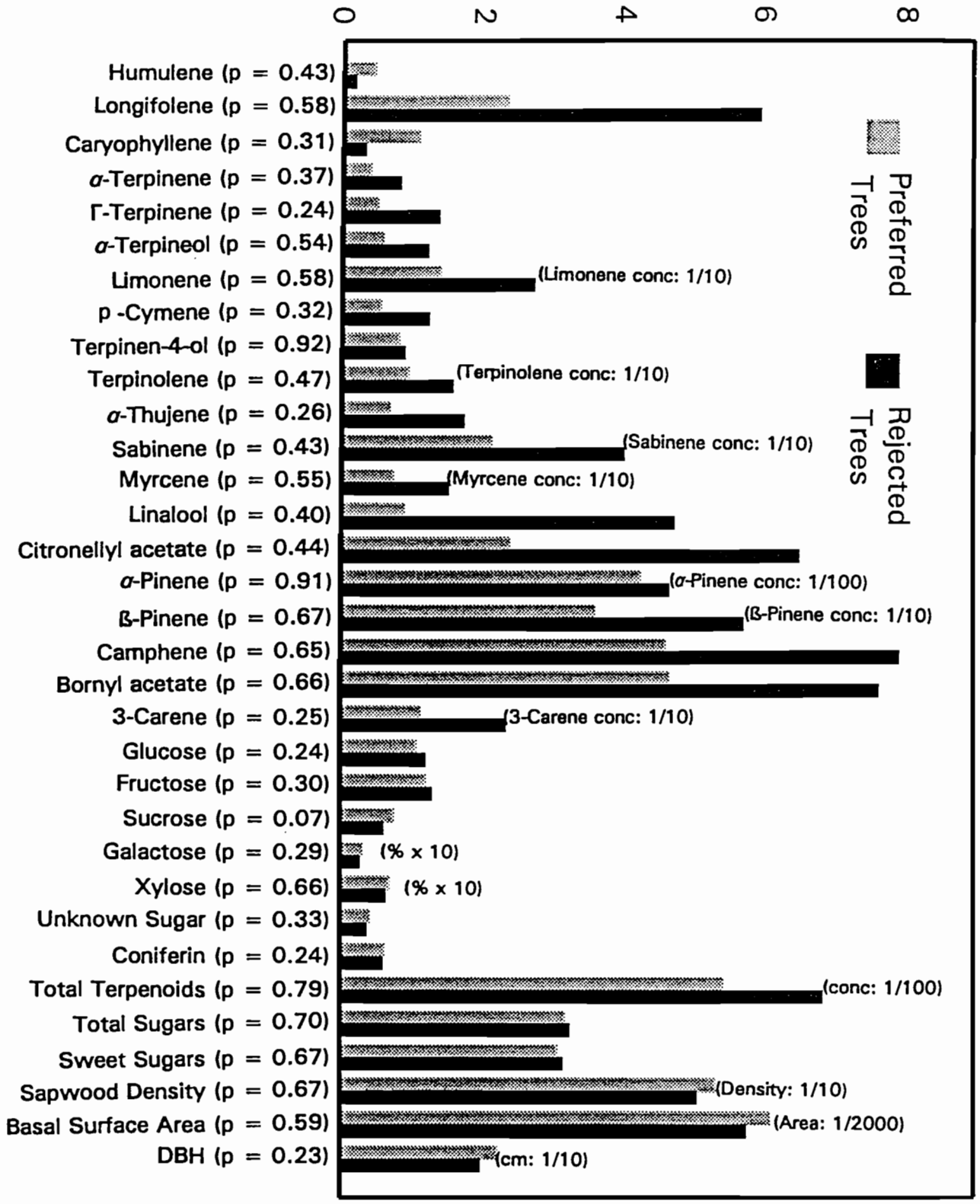

FIGURE 1. T-test results from Site 1. The corresponding units, except where noted, are: terpenoids (μg/g), sugars (%), and tree measurements (cm).

differences between the damage extremes as did the sum of terpenoids, sugars, and sweet sugars (Figure 2).

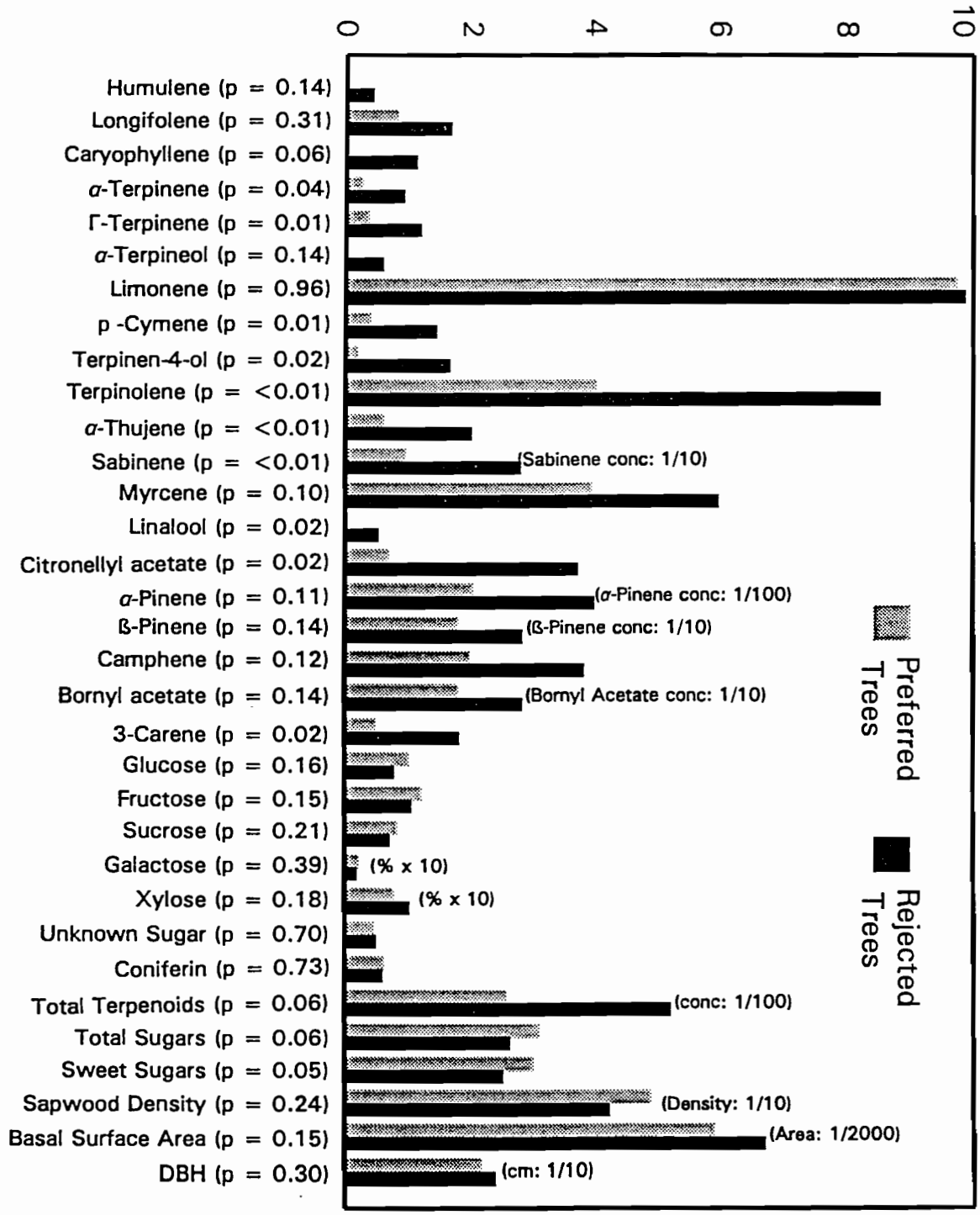

FIGURE 2. T-test results from Site 2. The corresponding units, except where noted, are: terpenoids (μg/g), sugars (%), and tree measurements (cm).

Again, chromatographic analyses generated important information relative to the investigation of the compounds related to feeding behavior. Significant differences were found between preferred and rejected trees for many of the chemical variables. Terpenoid concentrations were higher in the sapwood of rejected trees. These terpenoids are considered mammalian antifeedants because of their bitter taste (Harborne 1991). While bitter tastes are ineffective antifeedants for strict herbivores (Nolte et al. 1994), they would be expected to influence omnivore feeding behavior (Jacobs et al. 1978).

Conversely, the concentration of total sugars was higher in the sapwood of preferred trees. Carbohydrates, which may serve as positive cues that signal the nutritive value of the sapwood, supply energy at lower costs than proteins or fats (Hodge and Osman 1976). Similar to humans, omnivores are thought to perceive sucrose as "sweet" (Smith and Wilson 1989). Therefore, the sweet taste may result in a preference for sapwood with high concentrations of carbohydrates. These results suggest that low terpenoid and/or high carbohydrate levels are associated with sapwood preference, whereas high terpenoid and/or low carbohydrate levels are associated with sapwood rejection.

Both of these examples have demonstrated the importance of analytical chromatography to the investigation of chemicals which are related to mammal foraging behavior. Combining analytical chemistry with bioassays is an invaluable tool in the identification of biologically active compounds from plant materials.

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