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Sterol Composition and Distribution in Carnivorous Plants, *Sarracenia flava*, *Sarracenia purpurea,* and *Dionaea muscipula*

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ABSTRACT

Lipids extracted from carnivorous plants comprising 2 pitcher plants (*Sarracenia flava* and *purpurea*) and *Dionaea muscipula* and from grasses harvested in the same area, were saponified and the unsaponifiables (UNS) chromatographed to yield the sterols which were surveyed by GC and analyzed by GC/MS. β-Sitosterol was most prominent in the carnivorous plants, especially, in the roots of the *Sarracenia* plants and in *Dionaea* top portions, but stigmasterol ranged far lower and was undetected in several mixtures. As based on the 3 sterols, cholesterol comprised 4.5 and 5.7% of the grass UNS and was comparable to the levels for *S. flava* root and *D. muscipula* leaf products. However, the values ranged higher in UNS (up to 29% cholesterol) for most of the carnivorous plant mixtures screened. Possibly, nonphotosynthetic pathways involving insect prey and bacterial action, appear likely for the carnivorous plants investigated.

INTRODUCTION

The carnivorous plants gain nutrition by photosynthesis as well as by digestion of insect prey which they lure and trap by passive (pitfalls) or active ('steel' trap) modes. Although the absorption of nutrients from animals might have constituted one approach in areas with poor soil characteristics, relative to chemical makeup, it is currently felt that this mechanism may be of lower importance. Furthermore, many questions arise as to the digestion and assimilation of prey nutrients and the need for bacterial involvement in the process (Albert et al., 1992; Juniper et al., 1989). The problems and inconsistencies, notwithstanding, a study of the animal-based sterol, cholesterol, relative to deposition and the distribution in plants with active and passive modes of entrapment may contribute novel concepts in our knowledge of the intermediary metabolism.

Sterols, both free and esterified in *Nepenthes*, were shown to contain β-sitosterol, as the main component; 1% of either mixture comprised cholesterol (Wan et al.,1976). β-

Sitosterol was also prominent in the roots of *S. flava* (Miles and Kokpol, 1976) and in the roots and top portions of *S. purpurea* (Hooper and Chandler, 1984) and stigmasterol has been isolated from the pitchers at variable levels (Palmer et al., 1978; Hooper and Chandler, 1984). As based on the injection of tritiated precursor (5α-lanost-24-ene-3β, 9α-diol) directly into immature *Sarracenia purpurea* pitchers, the label could not be detected 10 days later in stigmasterol, indicative of a limited synthesis by the young plant (Palmer et al, 1978). Among other unsaponifiables (UNS), aliphatic hydrocarbon mixtures displaying wide variations in components have been reported for two *Drosera* species and *S. psittacina* (Sever et al., 1972; Miles et al., 1975) and compared with the ranges in fatty acids by the last group. The carnivorous plants present complex mixtures of carotenes, terpenes and other phytochemicals as reviewed by Juniper et al. (1989).

The current study was undertaken to determine the presence of cholesterol in UNS derived from *Dionaea muscipula* and the two pitcher plants, *S. flava* and *S. purpurea* and its relative distribution among the phytosterols. A sample of grasses harvested in the same area was included as a control. A preliminary report on the plant lipids has been presented earlier (Gershbein and Brown, 1991).

MATERIALS AND METHODS

Redistilled AR solvents were employed throughout and all glassware was degreased with 2:1 chloroform-methanol (v/v) and ethyl ether, the respective lipid - extracting media. Filtrates, after drying over anhydrous sodium sulfate, were concentrated under vacuum, the last portion of solvent being removed under nitrogen. All lipid samples were stored at 25°C under nitrogen. Sigma Chemical Co., St. Louis, MO was the source of the acetate standards.

Mature plants collected during August in the city limits of Wilmington, NC and comprising *Dionaea muscipula, Sarracenia flava, Sarracenia purpurea* and grasses indigenous to the area, were forwarded to the laboratory. The 'grasses' comprised a mixture of *Fimbristylis autumnalis* and *Panicum scoparium* in addition to broom sedge (*Andropogon virginicus*), bunch grass (*Muhlenbergia capillaris*), sweet bay (*Magnolia virginiana*), red bay (*Persea boronis*), bitter gallberry (*Ilex glabra*) and dog tongue (*Trilisa odoratissima*).The plants were cleaned of all debris, washed copiously with 0.90% NaCl and separated according to root, rhizome, leaf and tuber. The plant material was macerated and the lipid extracted with ethyl ether in a blender and after 72 h at 25°C, the contents were filtered and the residue extracted with ethyl ether. The filtrates were concentrated, and the residue taken up in ethyl ether, washed with several portions of water and dried. Removal of solvent yielded the lipids. A portion of the plant material from several batches was also submitted to Soxhlet extraction with chloroform-methanol and the lipids processed separately.

The lipids were saponified by refluxing with 20% NaOH in 95% ethanol (1.5 ml/g lipids) for 17 h, after which the mixture was cooled, diluted with water and extracted portionwise with ethyl ether. The ethereal layer was washed with water, the filtrate dried and concentrated, yielding the UNS. (The fatty acids or saponifiables in the initial alkaline solution, were processed following acidification, extraction of the acids with ether, washing with water portions and concentration of the dried filtrate).

UNS as a 1% solution in petroleum ether (b. 30-60°C) was fractionated over alumina (Alcoa F-20; 40 g/g UNS) and the column eluted with petroleum ether as such and containing 5% and 10% chloroform, 100% chloroform and finally 100% methanol. Each solvent was used at 25-30% of the initial volume. Fractions I-V resulted on removal of the respective fluids. Hydrocarbons occurred in Fractions I, II and III and the alcohols and sterols, in Fraction V. Some ketonic matter was present in Fraction III. Fraction V was acetylated by refluxing with acetic anhydride and pyridine for 45 min, cooled and n-hexane added. This solution was washed with portions of 5% HCl then water, until the aqueous layer was neutral. Drying of the hexane solution and concentration of the filtrate followed. The extraction of lipids and the fractionation procedures have been applied by this laboratory to mammalian samples with success (O'Neill and Gershbein, 1976; Gershbein et al., 1980).

GC and GC/MS Analysis

A Shimadzu GC-6AM instrument with DB-1 wide bore capillary column using an flame ionization detector was applied to the preliminary survey of components. The injector temperature was set at 250°C and that of the column was started at 130°C and maintained for 60 s and then increased to 280°C at the rate of 5°C/min.

For GC/MS, a Hewlett Packard 5890 gas chromatograph operated in the splitless mode was interfaced to a Hewlett Packard Mass Selective Detector. GC conditions: 90°C for 0 min, 5°C/min to 330°C; capillary column: J&W DB-5MS, 30 m, 0.25 mm i.d., 0.1 μm film thickness. The instrument was operated in the selected ion monitoring mode. Initially, ions with m/z = 179 were passed through the mass selective detector to detect the internal standard, phenanthrene. At 25 min into the run (after elution of the phenanthrene), the mass spectrometer was reset to monitor ions at m/z = 255 (common to the steroid derivatives studied), 368 (M^+- acetic acid for cholesteryl acetate), 394 (M^+- acetic acid for stigmasteryl acetate) and 396 (M^+- acetic acid for β-sitosteryl acetate) in order to detect important fragment ions from the extracted plant sterols. The instrument was set for low resolution and maximum sensitivity.

Samples initially screened by GC as such were combined with 0.5 ml of internal standard solution (916 μg/ml in heptane) and then diluted with this solvent to fill the autosampler vial to about 1.5 ml. The samples and appropriate standards were analyzed by GC/MS. The resulting peak areas (from mass spectrograms based on m/z values of 368, 394 and 396) were divided by the corresponding internal standard peak areas. The peak ratios from the standards were fit to a least squares line (non-zero intercept) which was used to calculate the amount of each analyte in the samples. Squalene in the hydrocarbon fractions was analyzed by GC (O'Neill and Gershbein, 1961).

RESULTS

Total lipids extracted from the cleaned plants employing solvent at 25°C or Soxhlet extraction and the distribution of saponifiables (fatty acids) and UNS on saponification are presented in Table 1. The percentages for the lipids and UNS are cumulative and include all respective plant portions as the leaf or pitcher, tuber, rhizome and root. Of the products obtained by chromatography over alumina, Fractions I, II and III, occurred in low

or trace amounts, the main one comprising alcohols + sterols (Fraction V) but with UNS recovery being lowest with *Dionaea muscipula*.

Hydrocarbons which occurred in Fractions I-III, ranged high in the sterol precursor, squalene. Saturated homologs were prominent in Fraction I, as illustrated for *S. flava* (Fig. 1). Chromatograms for Fraction V acetates derived from the plant samples and in which the peaks for cholesterol, stigmasterol and β-sitosterol are pinpointed, are shown in Fig. 2. Mammalian steroids as testosterone acetate could not be detected among the peaks of Fractions V.

DISCUSSION

It will be noted (Table 1) that the total lipids extracted from the carnivorous plants and grass mixture, from the same vicinity, occurred at 0.7-1.8% and of which UNS made up 7-10% of the lipids except for a higher level in *S. purpurea*. With the exception of a lower recovery for *D. muscipula*, UNS of the plant types consisted of 72-85% alcohols and sterols as based on column chromatography; the C-nos. ranged from C_{12} to C_{47} with more peaks in the grass mixture. The hydrocarbon-containing fractions eluted earlier with petroleum ether media, were high in squalene and displayed C-nos. of up to C_{46} (carnivorous plants) and to C_{38} (grasses). Considering the complexity of mixtures and of compounds reported for carnivorous plant types, the occurrence of many components in Fraction IV, and certainly, of those in Fraction V, would be expected. However, an exhaustive analysis of such agents is deferred presently, attention being directed to the sterols with emphasis on cholesterol.

In the evaluation of cholesterol levels in the various plant specimens (Table 2), sections of plants were sampled and the individual UNS isolated and chromatographed. Stigmasterol was quite low in the pitcher series as to be undetected in two of the *S. purpurea* root and pitcher in addition to the tuber of *D. muscipula*. β-Sitosterol ranged higher in the rhizome and roots of the *Sarracenia* plants as well as in *D. muscipula* top portions. Cholesterol occurred in each of the samples, including the grasses (0.67 and 0.79 mg/g UNS). Based on its percentage in the mixture with the two phytosterols, the cholesterol levels were low (4.0 and 5.7%) in grasses as *S. flava* root (4.5%) and *D. muscipula* leaf (4.2%), but definitely higher in the remaining six carnivorous plant samples (11-29%).

It should be pointed out that in the mixture with stigmasterol and β-sitosterol, the grasses presented a higher cholesterol level as compared to the findings of Wan et al. (1972), who deduced a value of 1% of the total from *Nepenthes*. Currently, a problem in the absorption of stigmasterol as mentioned in relation to tritiated precursors (Palmer *et al.*, 1978), is reflected in the findings. One would surmise that an elevation in cholesterol level in contrast to the grasses might indicate importance of the prey utilized by the carnivorous plants. The prominent prey of plants such as *S. flava* is diptera and such arthropods would contribute to cholesterol in Fraction V of the UNS portion. At least, the current pilot indicates that as based on cholesterol, the use of the carnivorous plants as harvested in late August in this American locale, may lead to a tool in following intermediary metabolism of this sterol. A possibility also exists that aside from prey,

cholesterol might arise from endogenous *de novo* biosynthesis so that labeling studies are definitely in order.

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Table 1. Total lipids of plant samples and pertinent fractions.

Sample, Mode of Extraction	*S. flava*, 25°C	*S. purpurea*, 25°C	*D. muscipula*, Soxhlet	Grasses, 25°C	Grasses, Soxhlet
Wt. cleaned plant, g	101.5	55.6	58.1	93.5	147.7
Wt. lipids, g	1.83	0.80	0.41	1.34	1.61
Lipids, %	1.82	1.44	0.71	1.43	1.09
Wt. saponifiables, mg (fatty acids)	238	150	76	435	93
Wt. UNS, mg	163	130	31	140	38
Wt. UNS, mg chromatographed over alumina	153	113	25	74	25
Fraction recovery, %					
I	Tr	2.7	Tr	4.0	Tr
II	Tr	0.9	Tr	Tr	Tr
III	Tr	Tr	Tr	Tr	Tr
IV	2.0	1.8	4.0	10.8	8.0
V	85.0	84.1	56.0	75.7	72.0

Tr, trace.

Table 2. Analysis of cholesterol and phytosterols in plant UNS as based on GC/MS of Fraction V Acetates.

	Cholesterol	Stigmasterol mg/g UNS	β-Sitosterol	Cholesterol in mixture, %
Sarracenia flava				
Rhizome	4.9	0.30	11.5	29.3
Root	2.2	2.2	44.4	4.5
Leaf	1.2	0.70	9.2	10.8
Sarracenia purpurea				
Rhizome	4.8	1.9	47.6	8.8
Root	5.0	ND	40.0	11.1
Leaf	1.9	ND	6.3	23.2
Dionaea muscipula				
Tuber (Soxhlet)	1.0	ND	3.3	23.3
Leaf (Soxhlet)	2.2	5.6	44.4	4.2
Grass Mixture				
Leaf	0.67	2.7	13.3	4.0
Leaf (Soxhlet)	0.79	5.3	7.9	5.7

Unless otherwise stated, the initial lipids were extracted at 25°C.
ND, not detected.

Figure 1. Chromatogram of Fraction I from plant UNS of *S. flava* chromatographed over alumina. A, Tricosane; B, Pentacosane; C, Squalene.

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Figure 2. Tracings of plant alcohols + sterols (Fraction V from UNS chromatography over alumina). Acetates of D, 5-Tetradecen-1-ol; E, 1-Hexadecanol; F, 1-Docosanol; G, Cholesterol; H, Stigmasterol; I, β-Sitosterol.

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