Many minor chemical constituents of the food and forage legume *P. arvense* have not been studied in enough detail to yield information concerning their chemical or physical properties. This work shows that *P. arvense* seed certainly contain saponins which can be classified as triterpenoid in nature on the basis of the chromatographic similarities of their sapogenins to the soysapogenols. In addition to shedding light on their chemical and physical properties, this study of *P. arvense* saponins might also be used to show subtle chemotaxonomical differences between this legume and varieties of the same or different species.

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ISOLATION AND SPECTROMETRIC STUDY OF A COMPLEX PHENOLIC PRODUCT FROM *PISUM ARVENSE* SEED

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Abstract

The heptane-extractable hydrolysis fraction of *Pisum arvense* L. seed contains six products as shown by silica gel analytical thin-layer chromatography (TLC). Isolation of this fraction's main component by alumina column chromatography and silicia gel preparative TLC followed by infrared, nuclear magnetic resonance and mass spectrometry of the isolated component indicate it to be a trihydroxybenzene derivative of molecular formula $C_{26}H_{42}O_5$. A likely structure is derived and its plausibility discussed in detail by correlating the obtained spectral data.

Introduction

The saponins of certain food and forage legumes have recently come under scrutiny because of their antinutritional and antibiological properties (Applebaum *et al.,* 1969; Gestetner *et al.,* 1968; Shaney *et al.,* 1970). Among the legume saponins studied by this laboratory are those from the seed of *Pisum arvense* L. (Gaither and Brown). Since this species is an important food and money crop for many Southern United States farmers (Isbell, 1959), we are engaged in exploring the economic as well as medicinal applications that heretofore unstudied minor constitutuents of this legume may possess. This work describes the isolation and partial characterization of the major component contained in the heptane-extractable hydrolysis fraction of *P. arvense* seed (HHP). This fraction has previously been reported to be composed of biologically active compounds (Barker, 1970).

Experimental

Isolation of HHP. Pisum arvense L. (Field Pea, crowder variety) seed were purchased locally. These were washed in distilled water, dried at 80° for 24 hr, then macerated in a

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Waring Blendor before extraction for 48 hr with diethyl ether in a soxhlet apparatus to remove lipids and pigments. The ether-free meal was refluxed with 2 N hydrochloric acid (20 ml/gm) for 4 hr, suction filtered through Celite coated Whatman No. 1 filter paper, dried at 80° for 24 hr, and finally extracted for 24 hr in a Soxhlet extractor with n-heptane. The resulting yellow solution was decolorized with carbon. Desolventization yielded a white, waxy product in 6.9% yield (lipid-free basis).

Separation of HHP components. All thin-layer chromatography (TLC) samples were spotted or streaked as chloroform solutions. Analytical TLC of HHP on activiated (1 hr, 100°) 250 micron silica gel precoated glass plates (Mallinckrodt SilicAR TLC-7GF, Sargent Welch Co.) was carried out in cyclohexane—ethyl acetate (13:1, v/v). After evaporation of solvent, the plates were sprayed with 50% sulfuric acid and heated for 10 min at 110°. The color and migration of each spot was observed and recorded (Heftmann, 1966).

Column chromatography of HHP occurred over deactivated alcoa grade F-20 alumina (Walter *et al.*, 1955) (500 gm) using a Glenco Scientific 24 x 300 mm glass column fit with a 70 micron porous Teflon support disc. After applying the sample (2 gm) as a benzene solution, elution was continued with the same solvent (900 ml, cut 1) followed by 1% (750ml, cut 2; 1000 ml, cut 3) then 5% (1000 ml, cut 4) absolute methanol in benezene, and finally absolute methanol (2000 ml, cut 5). Each column fraction (50 ml) was monitored by analytical TLC, and appropriate fractions were combined into the cuts previously designated.

Preparative TLC of HHP column cut 3 was performed on activated (2 hr, 60°) 2 mm Silica Gel F-254 precoated glass plates purchased from Brinkmann Instruments, Inc. Each plate was loaded with 30 mg of material. The plates were continuously developed in cyclohexane—ethyl acetate (6:4, v/v) for 6 hr after which the mobile phase was evaporated. The plates were then sprayed on the outer 1 cm of each edge perpendicular to the sample streak with the 50% sulfuric acid reagent (Heftmann *et al.*, 1966) and heated for 20 min at 80°. Appropriate zones were scraped from the plates and extracted overnight with chloroform in a Soxhlet extractor.

Elemental Analysis. Nitrogen, halogen, and sulfur were assayed according to Shriner, Fuson, and Curtin; (1964).

Spectrometry. Infrared (IR) spectra were obtained on a Beckman IR-20 spectrophotometer using neat samples in a Beckman Mini-cell which had the silver chloride windows turned back to back. Nuclear magnetic resonance (NMR) spectra were taken on a Varian T-60 NMR spectrometer using 20% solutions in deuterated chloroform with 3% tetramethylsilane added as internal reference. A Bell and Howell 21-491 mass spectrometer yielded mass spectra (MS).

Results and Discussion

The residue isolated by the elaborated procedure proved heterogeneous since analytical TLC showed it to be composed of at least six fractions (Figure 1). These data strongly suggest that component D predominates over the others. Analytical TLC of HHP samples as small as 6 microgm rendered no component visible except D, further indicating that this fraction is the main component. Preparative TLC of HHP in cyclohexane—ethyl acetate (13:1, v/v) or any other solvent system tried did not result in adequate separation for isolation of any of the components in pure form; therefore, HHP was preliminarily fractionated over a column of deactivated alumina (Walter *et al.*, 1955). Column cut 3 (429 mg) was found to be composed primarily of component D and a small quantity of component C. It was subjected to preparative TLC, and 185 mg of a colorless, viscous oil was obtained which when analyzed by analytical TLC had identical Rf and color reaction with the crude HHP component D. The isolated fraction also proved homogenous to analytical TLC in cyclohexane—ethyl acetate (6:4, v/v, Rf approx. .42), benezene—ethyl acetate (3:1, v/v, Rf approx. .59), and n-hexane—acetone (5:1, v/v, Rf approx. .82).

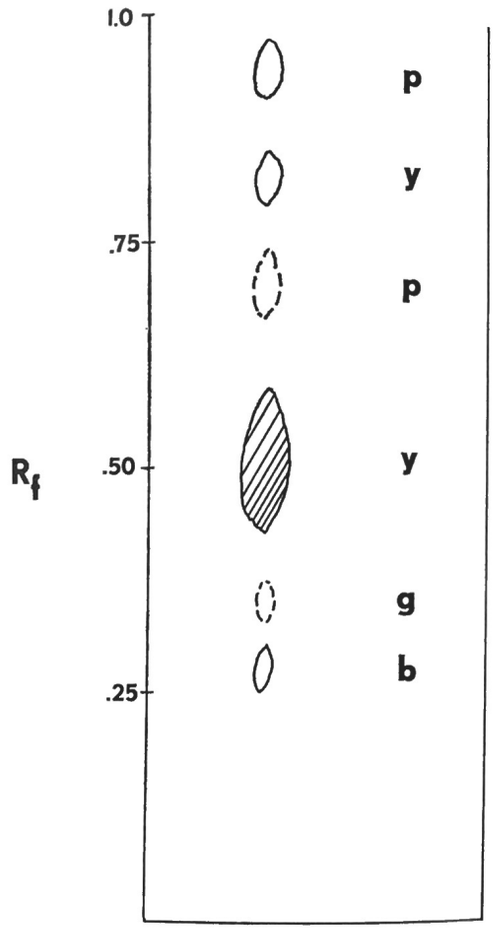


Figure 1. Analytical thin-layer chromatography of heptane-extractable hydrolysis fraction of *Pisum arvense* seeds on activated 250 micron silica gel plates using cyclohexane—ethyl acetate (13:1, v/v) as developing solvent. Colors obtained with the 50% sulfuric acid visualization reagent abbreviated as b, brown; g, green; p, purple; and y, yellow. Fraction designations in order of decreasing Rf are A, B, C, D, E, and F, respectively.

The IR spectrum of component D showed strong aliphatic (2870, 2830, and 1381 cm^{-1}) and aromatic (1600, 1580, and 1462 cm^{-1}) absorptions. Carbonyl stretch at 1732 cm^{-1}, C—O—C vibrations at 1280 cm^{-1}, and a weak absorption at 648 cm^{-1} indicated the component to be an acetate ester (Conley, 1966).

Aromatic protons (1H) were evidenced in the NMR spectrum by a complex multiplet at *tau* 2.25 parts per million (PPM). Protons (1H) within the vicinity of electronegative groups also appeared in a broad triplet at *tau* 5.87 PPM while aliphatic protons (12H) were seen in broad absorptions between *tau* 8.35 and *tau* 9.20 PPM. These observations support the IR data concerning the aromatic and alphatic character of the molecule.

Table 1 shows the MS fragmentation pattern of the component under study. The molecular ion is seen at m/e 434. Correlation of this ion, NMR data, and negative sodium fusion assay to a list of possible formulae suggests that $C_{26}H_{42}O_5$ is the molecular formula for component D. The relative non-abundance of the molecular ion makes one suspect that component D is not composed of several fused aliphatic rings since cyclization induces a great degree of symmetry to a molecule implying several tertiary and quarternary carbon atoms to be present, and in the case of the molecular ion, a carbonium ion of high stability was not formed.

TABLE 1

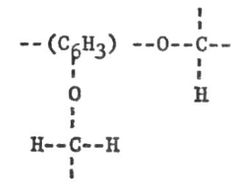
Mass Spectrum Fragmentation pattern of Isolated Component.

m/e	R. I.[a] (%)	m/e	R. I.[a] (%)	m/e	R. I.[a] (%)	m/e	R. I.[a] (%)
26	3.10	52	1.11	83	8.30	151	9.42
27	7.87	53	2.07	84	6.01	152	1.89
28	2.50	54	1.51	85	1.80	169	31.12
29	23.00	55	18.03	93	2.65	170	2.94
30	1.03	56	8.70	98	1.74	180	1.07
31	1.40	57	37.31	104	11.20	193	1.00
37	1.51	58	3.37	105	4.35	263	1.24
38	1.18	65	5.80	112	18.34	280	13.68
39	4.84	67	2.83	113	19.90	281	2.52
40	1.03	69	7.88	114	2.49	282	1.03
41	24.90	70	23.00	121	2.86	391	13.68
42	4.18	71	24.25	122	1.51	392	3.73
43	29.00	72	2.61	123	1.04	393	2.69
44	1.20	75	2.90	132	3.36		
50	2.34	76	6.84	134	1.07		
51	1.24	77	4.56	150	100.00	434	0.13

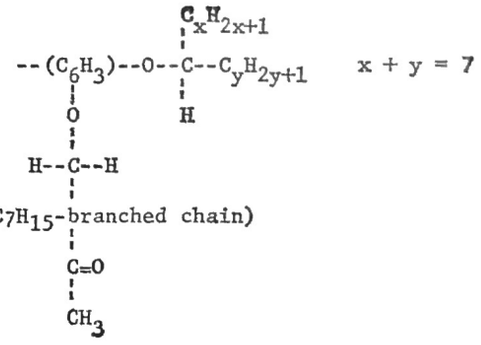
[a]R. I. = Relative Intensity. Except for the parent peak, those peaks under 1% are not reported.

Therefore, the initial cleavage of 43 mass units probably was not due to the $C_3H_7^+$ ion commonly seen in MS studies of cyclic compounds (Reed, 1963). Scrutiny of the P _ 42 peak shows the ^{13}C contribution to allow for at least 24 carbons; consequently, the P _ 43 fragment was probably formed by the loss of a CH_3CO^+ ion from a branch point on the molecular ion. Again the large degree of aliphatic character of the molecule is indicated by the loss of C_8-111 and 112 hydrocarbon units (McLafferty, 1963) from the m/e 391 and m/e 280 fragments, respectively, and by the series of intense peaks found at m/e 71, 69; 57, 55; 43, 41; and 29, 27 (Reed, 1963). Loss of 18 mass units from the m/e 168 fragment is probably due to the ubiquitous water molecule (Beynon, 1960) and results in the base peak at m/e 150 while the aromaticity previously suspected is once again butressed by the characteristic benzene ring fragments at m/e 75, 76, and 77 (Reed, 1963).

In the NMR spectrum the integration ratio of 1:1:12 and the deshielded protons at *tau* 5.87 PPM can best concur with the MS data above if a trisubstituted benzene ring (C_6H_3) is present along with three hydrogens adjacent to phenoxyl groups:



Since two C_8-hydrocarbon groups have been indicated, these could form the alkyl portions of the resulting diether with the CH_3CO^+ ion most likely occurring at a branch point on one of these hydrocarbon chains:



The addition of acetate indicated by the IR spectrum completes the molecule and furnishes requirements which satisfy the molecular formular, IR, and NMR spectral data: