PHYTOCHEMICAL COMPARISON OF *PUCCINELLIA ARCTICA* TO *POA PRATENSIS*, *PUCCINELLIA LANGEANA*, AND *PUCCINELLIA PHRYGANODES* FOR EVIDENCE OF CHEMICAL DEFENSE

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A

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By

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Abstract

Puccinellia arctica is a species of arctic grass that is unpalatable to Canada geese, *Branta canadensis*, and may be an effective and non-lethal means of controlling the growing populations of urban Canada geese that are problematic in many areas of North America. The secondary metabolite profile of *P. arctica* was compared to the metabolite profiles of three palatable grass species to determine the plausibility that *P. arctica* is chemically defended. The volatile and non-volatile secondary metabolite profiles of both *P. arctica* and the palatable grasses were the same. No alkaloids were detected in any of the grasses. Condensed tannin levels were similar in all of the grasses. Gallotannin levels were higher in the palatable grasses than in *P. arctica*. However, ellagitannin levels were higher in *P. arctica* than in the palatable grasses and may be responsible for its unpalatability to Canada geese.

ACKNOWLEDGMENTS

The natural products chemist T. A. Geissman once said that "the future...is to use the chemical information as the starting point for enquiry into questions that lie in the realms of biology." This statement eloquently summarizes what has become the central theme of this thesis. For me, it also now embodies what is one of the most fascinating, promising, and important uses for the science of chemistry.

I would like to start by thanking the members of my committee – Dr. Tom Clausen, Dr. Cathy Cahill, Dr. John Keller, and Dr. Richard Stolzberg for their unwavering guidance, encouragement, and belief in me. I am especially grateful to Dr. Tom Clausen for giving me the opportunity to turn what started out as a ziplock bag full of grass clippings into an interesting and rewarding thesis project.

I would also like to thank Dr. John Bryant for introducing me to the biological side of natural products chemistry. Together, both he and Dr. Clausen have opened my eyes to the intriguing world of chemical ecology/chemical defense and all of the important work that is being done by both chemists and biologists in this field.

I would like to thank Peter Scorup of Northern Native Seeds for providing me with both the plant material and basis for my project. His encyclopedic knowledge and photographs of *Puccinellia arctica* have also been most helpful.

I am eternally indebted to the true heart and soul of the chemistry department, Sheila Chapin and Marlys Schneider. Without their help, literally none of this work would have been possible.

I would also like to thank my fellow graduate students Maggie Isbell, Emily Reiter, Chance Riggins, Jane LeBlond, Lilly Allen-Dalley, Jeff Bickmeier, and Dennis Nicks for keeping me company and on my toes when in the lab. I would like to thank James Hetfield, Yarko Toivannen, Keeley Elgin, Angus Young, Leon Kennedy, and Lara Croft for helping me to relax and keep out of trouble when not in the lab.

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1.1. The Canada Geese Problem

Canada geese, *Branta canadensis*, are a relatively large member of the goose family. They typically weigh 8-13lbs and can have a wingspan of up to 5ft.[1] They have grayish brown bodies with black necks. Their black heads are adorned with white check patches.

Canada geese are the only geese in many localities, and these migratory birds appear throughout most of North America.[1] They breed over a vast, ecologically diverse area that covers both treeless and wooded country.[2] They typically nest near water such as lakes, ponds, larger streams, marshes, muskegs, and hummocky coastal plains.[2] Migratory Canada geese forage on plant leaves, grasses, berries, seeds, aquatic roots, and insects.[1]

Over the past half-century, populations of urban Canada geese have become established in many urban areas of North America.[3] Initially, the majority of landowners enjoyed seeing the geese on their property and often encouraged the geese to stay by providing food.[3] However, over time these urban geese populations have become problematic.

Grazing on lawns has become the principal food source for urban geese in urban areas.[3] This has created a problem when the geese damage turf by foraging on grass in backyards, golf courses, parks, landscaped areas, and playing fields.[4]

The feces the geese leave behind reduces the aesthetic and recreational value of the grazed areas.[5] A typical goose can defecate up to three pounds of feces in a day, which is more than a large dog.[6] The accumulated feces can negatively impact both water quality

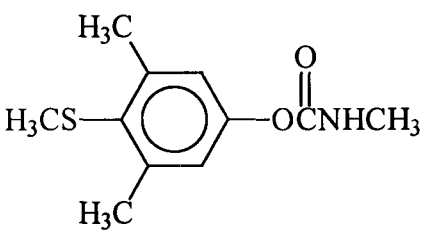
and public health.[4] In fact, public parks and beaches have been closed due to health concerns stemming from geese feces.[5] Buildup of feces in local waters can also lead to over-fertilization and cause algal blooms.[6]

Urban geese populations may also have a serious impact upon the safety of air travel. A United States Air Force AWACS (Airborne Warning and Control System) aircraft crashed while taking off from Elmendorf Air Force Base near Anchorage, AK in September of 1995. All twenty-four people on board were killed. The cause of the crash was attributed to collision with a flock of urban Canada geese.[6]

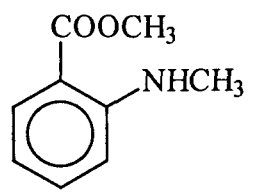
The incidence of nuisance problems associated with urban geese is pervasive and occurs throughout most of the eastern United States and in other parts of the U. S. and Canada.[5] In Alaska, Anchorage has a geese population of 5000 that is expected to grow to over 15,000 during the next decade.[6] Urban Canada geese thus pose a very real problem in many urban areas including Anchorage.

Unfortunately, the presently available solutions to the problems caused by Canada geese are not entirely satisfactory. Trapping and relocating geese is very labor intensive, and other geese usually move into the cleared area.[5] Issues of logistics, cost, and effectiveness often limit traditional management methods such as mechanical scare devices, traps, noisemakers, and pyrotechnic devices.[4] Hunting can ease geese problems by reducing geese populations and by making the geese less willing to occupy sites used by people.[3] However, local ordinances and limited hunter access frequently restrict hunting in urban areas.[3] Hunting may also be an unacceptable solution in some areas due to political reasons.

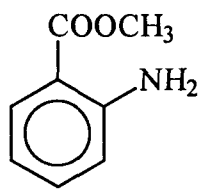
Aversive conditioning using a chemical repellant is another possible solution. Methiocarb can effectively repel nuisance geese from treated forage sites.[5] However, it may no longer be used in the United States as a geese repellant.[3] Dimethyl anthranilate and methyl anthranilate are also unpalatable and aversive to birds.[7,8,9] Nonetheless, improvements to their formulation and encapsulation processes need to be made to increase their effectiveness.[4] Methyl anthranilate has been used to repel Canada geese in Anchorage, AK. However, its effectiveness depends upon continued application throughout the growing season and is thus expensive.[10] Figure 1 displays the structures of methiocarb, dimethyl anthranilate, and methyl anthranilate.



methiocarb



dimethyl anthranilate



methyl anthranilate

Figure 1. Structures of three potential commercial antifeedants for geese.

Fescue grass infected with an alkaloid producing endophytic fungus has been observed to deter geese.[11] However, no proposal has been made to use endophytic fungus infections as a geese management tool.

Urban Canada geese select foraging sites that contain nearby water, an unobstructed line of sight, and palatable forage grass.[3] Landscape modifications such as draining bodies of water, planting shrubs and hedges to decrease line of sight, and converting grass forage areas to non-grass vegetation are viable solutions to nuisance geese problems.[3,12] However, these modifications require large scale changes to the urban lifestyle and may lead to concentration of the geese in areas that can not be readily converted, such as playing fields.[10]

Finally, manipulation of the grass that the geese forage on represents one possible long-term solution to the urban geese problem. If the grass can be made unpalatable to the geese, they will not forage on it and will move on to other areas with better food supplies.

1.2. Goals of this Thesis

Northern Native Seeds is located in Palmer, AK. The company has received Alaska Science and Technology Foundation (ASTF) funding to study the suitability of using arctic alkali grass, *Puccinellia arctica*, as a long-term, non-lethal geese deterrent. *P. arctica* is unpalatable to geese on the North Slope of Alaska. Using the ASTF funding, the company is investigating if *P. arctica*: is effective in deterring geese in an urban setting, can thrive and be propagated under urban mowing and fertilizing conditions, can

withstand foot traffic in high use areas, and is safe to plant in areas where children and pets will be present. If *P. arctica* is deemed suitable, it would provide a valuable urban geese management tool. The grass could be raised in Palmer and exported as seed to Anchorage and other areas of North America with urban geese problems. This would provide a possible long-term solution to the urban Canada geese problem.

Herbivores selectively browse plants based upon the plants' chemical and mechanical defenses, in addition to its nutritional value.[13] Thus, *P. arctica* may be unpalatable to geese due to a single factor or to several factors acting in conjunction. It may be tough and thus difficult for the geese to eat. The grass may be of low nutritional value to the geese, and they may be foraging on more nutritional species. The grass may also be chemically defended. Secondary metabolites produced by the grass may deter the geese from eating it. If *P. arctica* is chemically defended, and if these defensive chemicals are highly toxic, it might be unsuitable for planting in urban areas where children or pets could consume it.

The concept that secondary plant metabolites affect plant utilization by herbivores had its beginnings in 1970.[14] Over time, this theory has been endorsed and supported by experimental evidence.[15] Now, some secondary plant metabolites are often thought of as weapons in an ecological "arms race" waged between herbivores and plants.[16]

This thesis was instigated at the request of Northern Native Seeds in the summer of 1998. The purpose of this thesis was to perform a phytochemical comparison between *P. arctica* and three palatable grass species to determine the plausibility that *P. arctica* is chemically defended. Two of the other grasses, *Puccinellia langeana* and *Puccinellia*

phryganodes, are also found on the North Slope, but are very palatable to geese. The last grass species, *Poa pratensis*, is the palatable lawn grass that geese are currently feeding on in Anchorage.

The secondary metabolite profiles of the four grasses were compared against one another to look for possible defensive compounds present in *P. arctica* but not in the other species. Volatile compounds were analyzed by GC/MS. TLC was used to analyze non-volatile compounds. Alkaloids, secondary metabolites known to chemically defend fungus infected fescue grass from Canada geese,[11] were screened for using TLC and alkaloid spray reagents. TLC indicated the possible presence of tannins, secondary metabolites also known to repel birds,[17,18] and these were tested for using the Bate-Smith, acid butanol, rhodanine, and nitrosylation assays.

1.3. The *Puccinellia*

The *Puccinellia*, a small genus of slender grasses included in the family Gramineae, are named after the Italian botanist Professor Beneditto Puccinelli.[19] They usually occur in the saline soils of coastal and inland areas.[20] The *Puccinellia* are predominantly distributed from the middle latitudes to the high arctic in the Northern Hemisphere. However, some species do occur in the high latitudes of Australia, Africa, and South America.[20]

Some of the species in *Puccinellia* are comparatively long-lived, with a mean age of 35yr.[21] The mean age of their first reproduction may also be later than more temperate grass species.[21]

Apomixis, reproduction not dependent upon fertilization, is prevalent among the *Puccinellia* and they may be subdivided into almost endless subspecies.[19] In fact, chloroplast DNA restriction site variation data suggests that a significant number of the species in this genus are barely differentiated.[20]

1.3.1. *Puccinellia arctica*

P. arctica, also called *Puccinellia poacea*, *Glyceria arctica*, and arctic alkali grass, grows in low tundra and damp sand near coastal shores.[19] It is known to occur in the Alaska Yukon and the eastern and western Canada arctic.[19] It is a perennial that grows in tufts 10-55cm tall.[22] *P. arctica* has folded leaf blades and 3-6 green or purplish flowers.[22]

Five different *P. arctica* genotypes, 1, 2, 3, 4, and X, were studied in this analysis. They are cultivars that express different phenotypes, and are thus assumed to be different genotypes as well. The numbers 1, 2, 3, and 4 represent the order that the genotypes were planted at the Northern Native Seeds nursery in Palmer, AK. Genotype X is so named because it grows near X-pad in the Prudhoe Bay oil field. Figure 2 shows several of the genotypes in the Palmer, AK nursery. Most of the *P. arctica* genotypes look very similar when viewed separately on the North Slope, but their differences become apparent when seen growing together in a common nursery.[10]

All of the *P. arctica* genotypes used by Northern Native Seeds were originally collected on the North Slope. However, this may not be where the plants originated since the grass occurs throughout Canada and Alaska.[10] The five *P. arctica* genotypes are

Figure 2. *P. arctica* seed rows in the Palmer, AK nursery. At center are five rows of genotype 2, which is darker colored than genotype 1 (left) and genotype 3 (right). A single row of beach wild rye separates each of the genotypes. (Photograph used by permission of Peter Scorup of Northern Native Seeds.)

unpalatable to Canada geese to different degrees, with genotype 3 being the least palatable and genotype 4 being the most palatable.[23] They may all be chemically defended. Figure 3 shows grazing geese avoiding a *P. arctica* test plot in Anchorage, AK.

A Chemical Abstracts literature search showed no citations for *P. arctica*. The OCLC FirstSearch General Scientific Abstracts and OCLC FirstSearch Cambridge Scientific Databases also failed to yield any citations for *P. arctica*. There was no mention of *P. arctica* in the Science Citation Index or Cambridge Scientific Abstracts. It appears that little published work has been done on this remote arctic plant.

1.3.2. *Puccinellia langeana*

P. langeana, also known as *Puccinellia paupercula*, *Puccinellia alaskana*, *Atropis laeviuscula*, and dwarf alkali grass, thrives in the high tide area of silty coastal shores and the brackish margins of lagoons.[19] It has been found in the Alaska Yukon, eastern Siberia, the western and eastern Canada arctic, northeastern Europe, western Greenland, and northwestern Asia.[19] *P. langeana* is a perennial that grows in tufts 5-25cm tall.[22] It has folded leaf blades and 3-5 green to purplish flowers.[22] This plant is palatable to birds on the North Slope,[10] and was thus used as a negative control during analysis.

Three different *P. langeana* samples were analyzed. One was collected at Prudhoe Bay in 1998 at the same time as the *P. phryganodes* sample. The other two samples were collected from Prudhoe Bay in 1999 and represent two different genotypes. The D.S. #9

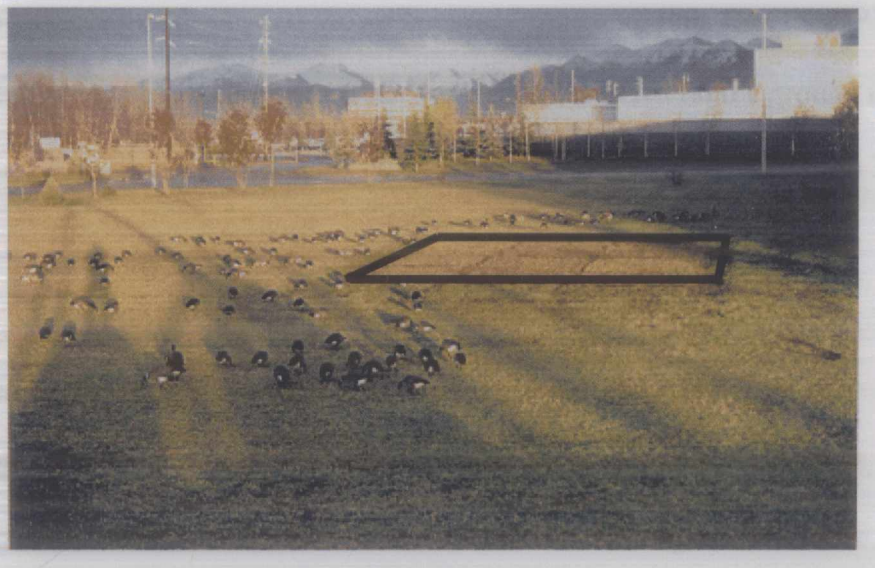


Figure 3. Grazing geese avoiding *P. arctica* test plots in Anchorage, AK. The *P. arctica* test plots are seen here as the outlined cluster of square sections near the center of the photograph. (Photograph used by permission of Peter Scorup of Northern Native Seeds.)

genotype tends to be upright and grows in more xeric conditions than the BPX plants. The BPX genotype tends to be prostrate with seeds heads flat on the ground until they are nearly ripe. Then the seed heads lift up and expose the seed to the wind at a 30-45° angle.[10]

1.3.3. *Puccinellia phryganodes*

P. phryganodes, also named *Glyceria vilfoidea* and creeping alkali grass, inhabits muddy tracts, brackish marshes, and the areas between the high and low tide lines of coastal shores.[19] It is circumpolar in distribution.[19] *P. phryganodes* is a perennial that grows in mats 5-15cm tall.[22] It is usually sterile, but when in bloom it has 3-5 purplish flowers.[22]

However, more recent work has shown that there is a large amount of variability, as determined by enzyme electrophoretic mobility's, both within and between populations of this grass.[24] This indicates that sexual reproduction may in fact play a larger role in the life cycle of *P. phryganodes* than previously believed. This plant is palatable to birds on the North Slope,[10] and was therefore used as a negative control during analysis.

1.4. *Poa pratensis*

Poa pratensis, better known as Kentucky bluegrass, grows along roadsides, riverbanks, muskegs, spits, and meadows practically anywhere in the sub-arctic and temperate regions of the Earth.[22] It is extremely tolerant of mowing and grazing, and is extensively used as a lawn and pasture grass.[25] *P. pratensis* is a perennial that can form

sods 25-100cm tall.[22] It has folded leaf blades and 2-6 green or purplish flowers.[22] It is one of the major species of lawn grass used in Anchorage, and is palatable to birds.[10] It was thus used as a negative control for analysis.

Chapter 2. Results and Discussion

2.1. Comparative GC/MS Analysis

The four plant species showed a high degree of similarity when volatile compounds in their extracts were analyzed by GC/MS. Figures 4 and 5 show typical gas chromatograms for the CH_2Cl_2 and MeOH extracts, respectively. Figure 6 shows the structures for compounds **I-VII** identified by GC/MS.

All of the CH_2Cl_2 extracts were similar and contained the same peaks. The two major peaks were identified as octadecanal **(I)** and 1-dotriacontanol **(II)**. There were no peaks found in the *P. arctica* samples that were not also found in the palatable plant samples.

Like the CH_2Cl_2 extracts, all of the MeOH extracts showed a high degree of similarity. Five compounds were identified in all of the MeOH extracts. These were 9,12,15-octadecatrienoic acid methyl ester **(III)**, 9,12-octadecadienoic acid methyl ester **(IV)**, hexadecanoic acid methyl ester **(V)**, (3β,24S)-stigmast-5-en-3-ol **(VI)**, and neophytadiene **(VIII)**. In addition to these, the *P. phryganodes* MeOH extract also contained (3β,24E)-stigmasta-5,24(28)-dien-3-ol **(VII)**. There were no peaks found in the *P. arctica* samples that were not also found in the palatable plant samples.

A sample of *P. arctica*, collected from Prudhoe Bay in the summer of 1998 for a preliminary screening analysis prior to this thesis, was found to contain a small peak tentatively identified as dihydroactiniolide. This biologically active compound, first isolated from the leaves of *Actinidia polygama*[26], is found in many plant sources including tobacco[27] and tea.[28] It is a phytotoxin secreted by the aquatic plant spikerush

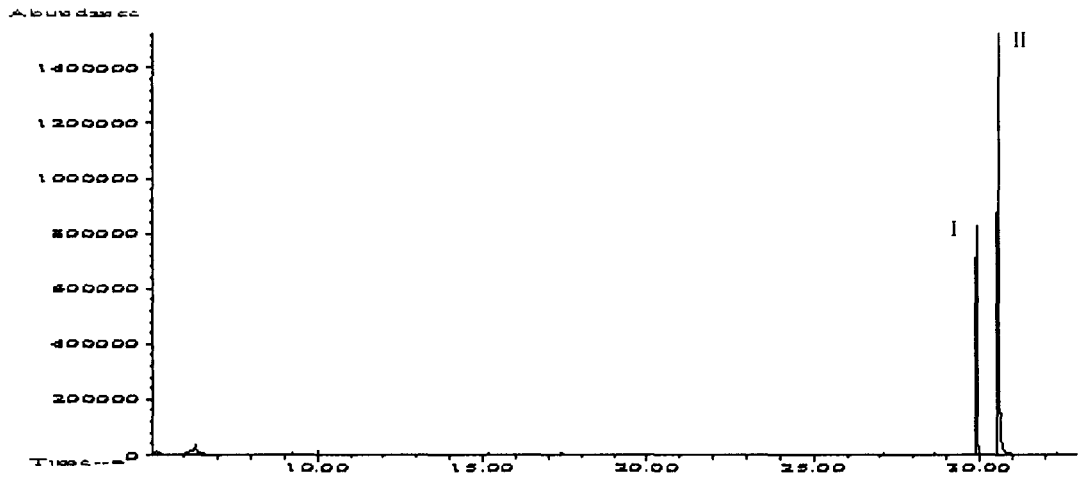


Figure 4. Typical CH_2Cl_2 extract GC chromatogram. Numbers correspond to compounds identified by MS as displayed in Figure 6. Time is in minutes.

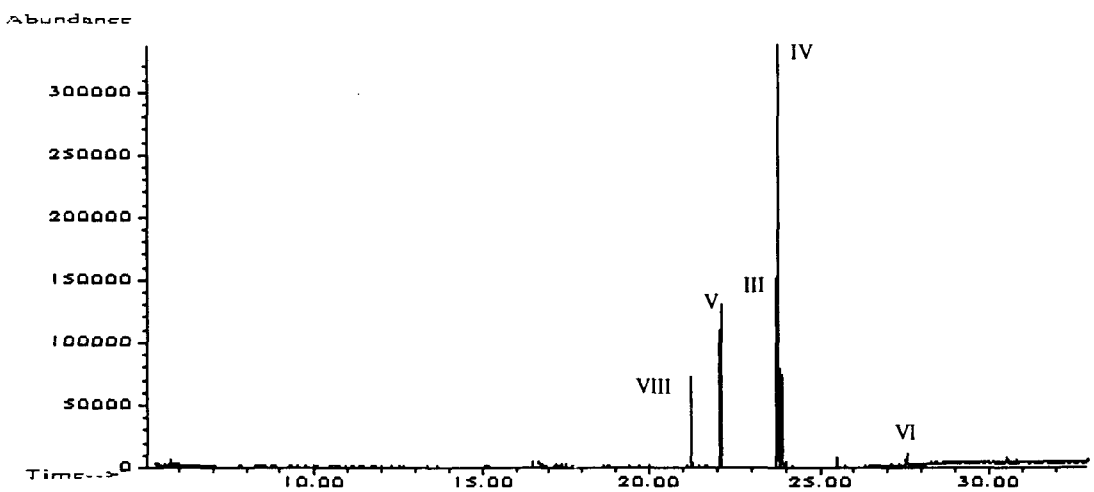
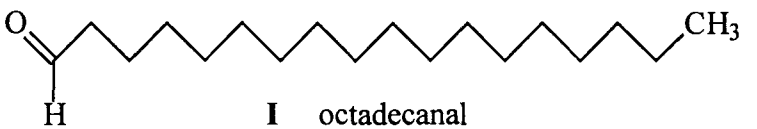
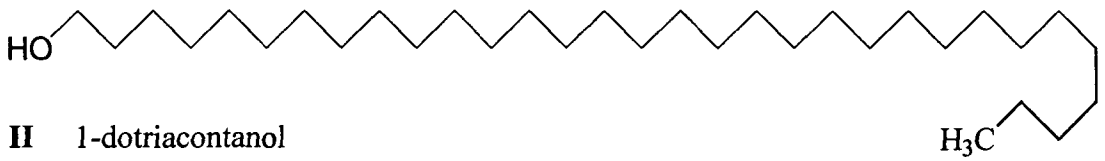


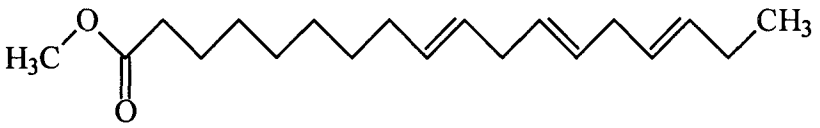
Figure 5. Typical MeOH extract GC chromatogram. Numbers correspond to compounds identified by MS as displayed in Figure 6. Time is in minutes.



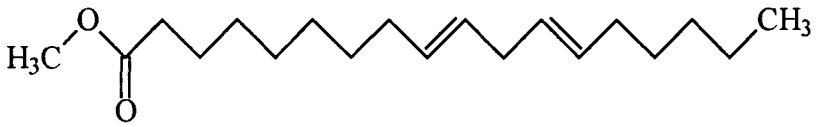
I octadecanal



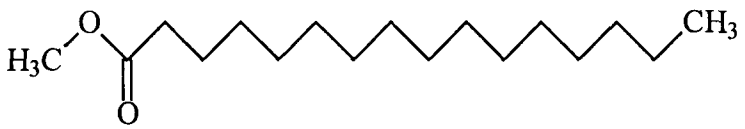
II 1-dotriacontanol



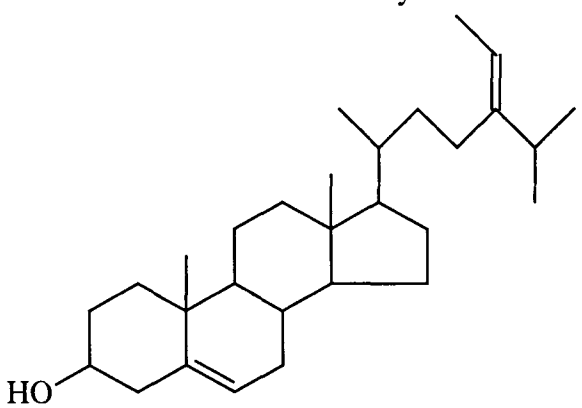
III 9,12,15-octadecatrienoic acid methyl ester

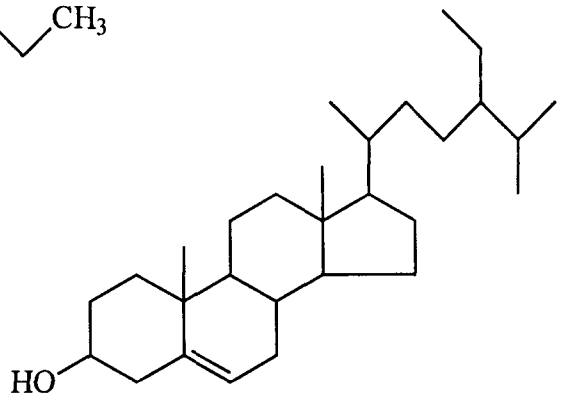


IV 9,12-octadecadienoic acid methyl ester



V hexadecanoic acid methyl ester





VI (3B,24S)-stigmast-5-en-3-ol

VII (3B,24E)-stigmasta-5,24(28)-dien-3-o

Figure 6. Structures of compounds **I-VII** identified by GC/MS. Numbers correspond to the peaks in Figures 4 and 5.

(*Eleocharis* spp.) that is capable of inhibiting both root length elongation and seed germination in other plants.[29] Additionally, it is a queen recognition pheromone of the red fire ant (*Solenopsis invicta*)[30] and a scent gland secretion of the red fox (*Vulpes vulpes*).[31] Figure 7 displays the structure of dihydroactiniolide.

Given its biologically active nature, it was initially thought that this compound might be playing a defensive role in *P. arctica.* However, none of the subsequent *P. arctica* samples analyzed for this thesis contained dihydroactiniolide. It now seems likely that this compound, if indeed present in the preliminary screening sample, was merely contamination from another source such as a fox.

Thus, comparative GC/MS analysis of both *P. arctica* and the palatable plants found no volatile compounds that could be responsible for chemical defense in *P. arctica.* All of the compound identifications were tentative. No authentic samples were available for comparison of retention times and mass spectral fragmentation patterns. Until authentic samples are run, or the compounds are isolated and identified by NMR/IR, the compound identifications will represent only possible structures. However, given the fragmentation process of mass spectroscopy it is likely that if the compound structural assignments are not correct then they will be similar to the actual compound structures.

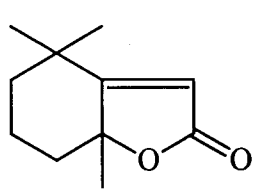


Figure 7. Structure of dihydroactiniolide.

2.2. Comparative Thin-Layer Chromatography

Four different solvent systems, $CHCl_3$, 5% (v/v) MeOH in $CHCl_3$, 10% (v/v) MeOH in $CHCl_3$, and 40% (v/v) MeOH in $CHCl_3$, were required to separate the TLC spots in the MeOH extracts. Three different solvent systems, $CHCl_3$, 5% (v/v) MeOH in $CHCl_3$, and 10% (v/v) MeOH in $CHCl_3$, were needed to separate the TLC spots in the CH_2Cl_2 extracts. The TLC plates were visualized with UV light and charring with 5% (v/v) H_2SO_4 in EtOH. The UV light visualizes any organic compounds capable of interacting with light at 254nm. The 5% (v/v) H_2SO_4 in EtOH reacts with all organic compounds present on the TLC plate. Both of these non-selective visualization methods are fairly sensitive and have a detection limit of around 10μg, depending upon compound.[32] An appropriate extract of the *P arctica* mature genotype 3 plant sample from May was spotted on all of the MeOH and CH_2Cl_2 extract TLC plates to serve as a standard.

The four plant species showed a high degree of similarity when non-volatile compounds in their extracts were analyzed by TLC. Figures 8 and 9 show typical TLCs for the MeOH and CH_2Cl_2 extracts, respectively. As seen in Figure 8, the MeOH extracts of *P. arctica* contained the same compounds as the MeOH extracts of the palatable plants with the exception of two spots. One spot had R_f values of 0.23 in 5% (v/v) MeOH in $CHCl_3$ and 0.56 in 10% (v/v) MeOH in $CHCl_3$. The other spot had a R_f of 0.33 in 10% (v/v) MeOH in $CHCl_3$. These purplish spots were present in all of the May *P. arctica* samples and yet not in the palatable *P. langeana* or *P. phryganodes* samples available at that time. It was initially thought that these could represent possible defensive compounds in *P. arctica.*

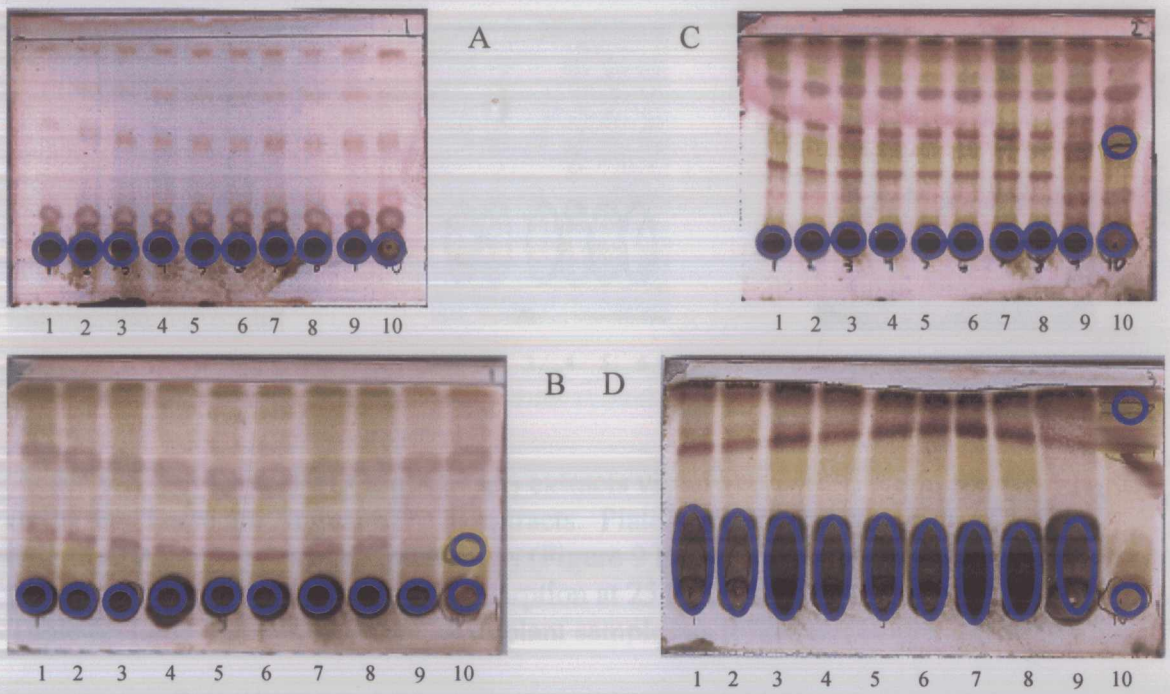


Figure 8 A-D. Typical TLCs of MeOH extracts visualized with UV and 5% (v/v) H_2SO_4 in EtOH. These are the May MeOH extracts. Plates were eluted with either $CHCl_3$ (Figure 8 A), 5% (v/v) MeOH in $CHCl_3$ (Figure 8 B), 10% (v/v) MeOH in $CHCl_3$ (Figure 8 C), or 40% (v/v) MeOH in $CHCl_3$ (Figure 8 D). Circles indicate UV absorption at 254nm. Plant samples are described in Table 6. Lane numbers correspond to plant samples as follows:

Lane 1 – arc/1young/may
Lane 2 – arc/1mature/may
Lane 3 – arc/2young/may
Lane 4 – arc/2mature/may
Lane 5 – arc/3young/may
Lane 6 – arc/3mature/may standard
Lane 7 – arc/4young/may
Lane 8 – arc/4mature/may
Lane 9 – lang/septem
Lane 10 – phryg

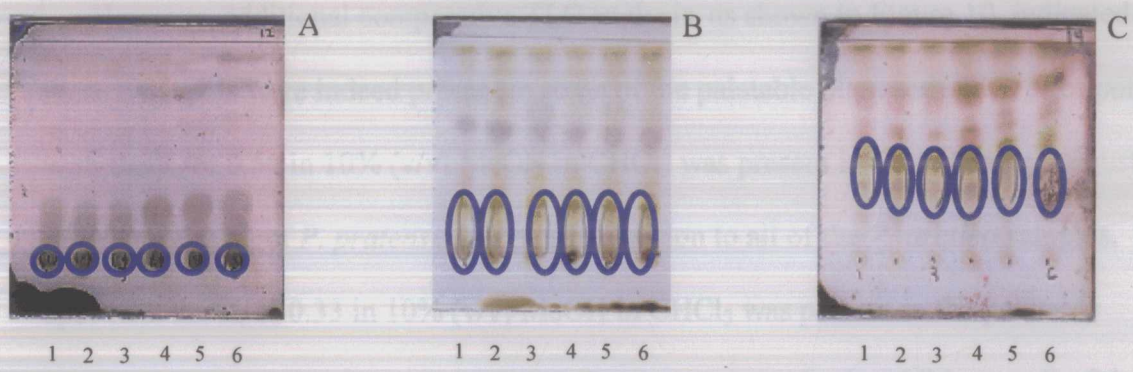


Figure 9 A-C. Typical TLCs of CH_2Cl_2 extracts visualized with UV and 5% (v/v) H_2SO_4 in EtOH. These are August CH_2Cl_2 extracts. Plates were eluted with either $CHCl_3$ (Figure 9 A), 5% (v/v) MeOH in $CHCl_3$ (Figure 9 B), or 10% (v/v) MeOH in $CHCl_3$ (Figure 9 C). Circles indicate UV absorption at 254nm. Plant samples are described in Table 6. Lane numbers correspond to plant samples as follows:

Lane 1 – arc/2prud/august
Lane 2 – arc/3prud/august
Lane 3 – arc/Xprud/august
Lane 4 – arc/3mature/may standard
Lane 5 – lang/DS9/august
Lane 6 – lang/BPX/august

However, additional comparative TLC analysis, as shown in Figure 10, indicated that these two spots were indeed present in some of the palatable plant samples. The spot with the R_f of 0.56 in 10% (v/v) MeOH in $CHCl_3$ was present in the palatable August *P. langeana* and all of *P. pratensis* samples in addition to all of the *P. arctica* samples. The spot with the R_f of 0.33 in 10% (v/v) MeOH in $CHCl_3$ was present in the palatable August *P. langeana* and August seedling *P. pratensis* samples in addition to all of the *P. arctica* samples. Therefore, these spots were ruled out as being possible defensive compounds.

As seen in Figures 8 and 10, the palatable *P. phryganodes* MeOH extract contained a compound not present in any of the other plant samples. This yellow spot was UV active and had R_f values of 0.21 in 5% (v/v) MeOH in $CHCl_3$, 0.50 in 10% (v/v) MeOH in $CHCl_3$, and 0.97 in 40% (v/v) MeOH in $CHCl_3$. As will be discussed in Section 2.3.3., this compound yielded a positive result when tested with the iodine-potassium iodide alkaloid spray reagent.

All of the MeOH extracts contained UV active compounds that were immobile on TLC except under extremely polar solvent conditions. It seems possible that these compounds are tannins, which are known to be rather immobile on TLC.[15]

As seen in Figure 9, all of the CH_2Cl_2 extracts contained the same compounds. All of the CH_2Cl_2 extracts contained UV active compounds that became mobile with 5% (v/v) MeOH in $CHCl_3$.

Thus, comparative TLC analysis of both *P. arctica* and the palatable plants found no non-volatile compounds that could be responsible for chemical defense in *P. arctica*.

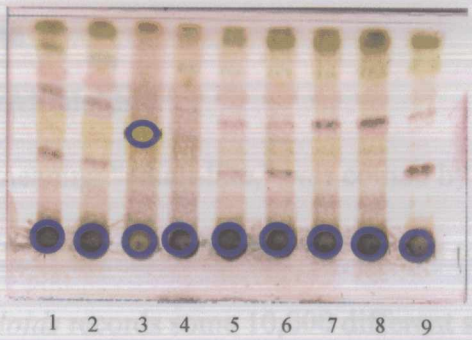


Figure 10. Comparative TLC of MeOH extracts visualized with UV and 5% (v/v) H_2SO_4 in EtOH. The plate was eluted with 10% (v/v) MeOH in $CHCl_3$. Circles indicate UV absorption at 254nm. Plant samples are described in Table 6. Lane numbers correspond to plant samples as follows:

Lane 1 – arc/3mature/may standard
Lane 2 – arc/3prud/august
Lane 3 – phryg
Lane 4 – lang/septem
Lane 5 – lang/BPX/august
Lane 6 – lang/DS9/august
Lane 7 – prat/nomow/july
Lane 8 – prat/mow/july
Lane 9 – prat/seed/august

2.3. Alkaloid Screening

2.3.1. Introduction to Alkaloids

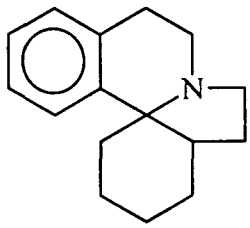
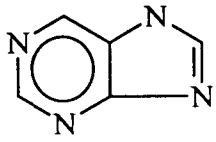
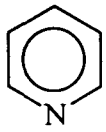
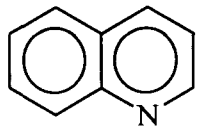
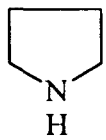
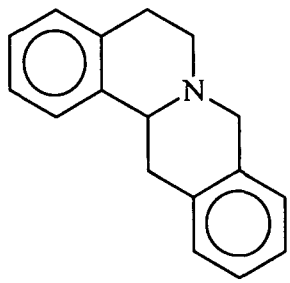
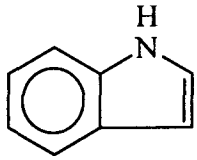
The term alkaloid, meaning "alkali-like," was coined by W. Meissner in 1819,[33] and refers to what is one of the largest and most varied groups of secondary metabolites.[34] The *Dictionary of Alkaloids* records some 10,000 different structures.[35] Because of the vast number of structurally distinct alkaloids, no single definition can possibly encompass them all. A good working definition of an alkaloid is a basic substance containing at least one nitrogen atom, usually as part of a cyclic system.[36] Another applicable definition of an alkaloid is a cyclic compound that contains nitrogen in a negative oxidation state and has a restricted distribution in living organisms.[37]

The nomenclature of alkaloids has not been systematized due to both historical reasons and to the vast number of individual compounds.[33] It is common practice to classify alkaloids based upon either chemical structure or the plant genera in which they occur.[33] Table 1 shows several of the many structural classes of alkaloids.

Amino acids are the most common precursors of alkaloids.[36] As a group, the alkaloids are extremely heterogeneous in structure. On one hand are the relatively simple structures like coniine, the major toxin in poison hemlock *Conium maculatum*.[38] On the other are pentacyclic structures like strychnine, a poison from *Strychnos nux-vomica* used in rodent baits.[38] Plant purine and pyrimidine bases, such as caffeine, are usually included in the alkaloids as well.[36]

Alkaloids are frequently optically active, typically colorless, and usually crystalline at room temperature (with nicotine being among the notable exceptions).[36] Alkaloids are

Table 1. Several classes of alkaloid structures.[39]

Class	Structure	Examples
Erythrinans		erythratine
Purines		caffeine theophylline
Pyridines		ricinine nicotine
Quinolines		dictamnine
Pyrrolidines		hygrine stachydrine nicotine
Protoberberines		berberine
Indoles		gramine physostigmine

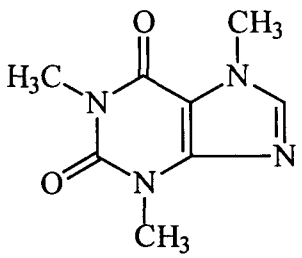
usually bitter and a simple, but not necessarily recommended, taste test can be done to detect alkaloids in fresh plant material. The alkaloid quinine, one of the bitterest known compounds, tastes bitter at a concentration of 1×10^{-5} M.[36] Figure 11 shows the structures of the alkaloids coniine, strychnine, caffeine, nicotine, and quinine.

Most of the alkaloids have been isolated from higher plants. In fact, 20% of the flowering plant species contain alkaloids.[34] The amount of any given alkaloid present in plant tissue can fluctuate dramatically based upon environmental conditions. In addition to the levels of nitrogen, phosphorus, and potassium, both the temperature and moisture content of the soil as well as the height above sea level can influence how much alkaloid is produced in any given plant.[40] There are even significant diurnal variations in alkaloid levels.[41]

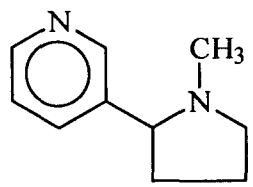
Alkaloids have also been isolated from many other organisms including algae, microbes, marine invertebrates, arthropods, and amphibians. Each alkaloid bearing species typically has a distinct pattern of alkaloid type and distribution.[34]

Alkaloids are frequently toxic and often have remarkable physiological activities.[36] In fact, physiological activity was once included in the classical definition of an alkaloid.[37] Alkaloids have been widely used as both medicinal drugs and poisons since ancient times.[30] Many alkaloids interfere with the action of chemical transmitters such as dopamine, serotonine, acetylcholine, and epinephrine.[34]

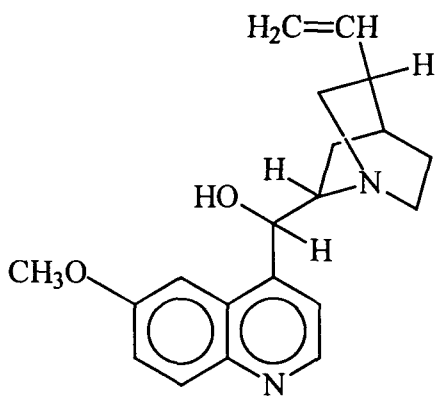
Alkaloids are known to play a role in the chemical defense of plants against herbivores.[40] Many alkaloids have been shown to deter insect herbivory.[42] They also provide protection against herbivory for many aquatic plants[43] This herbivore



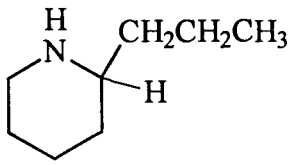
caffeine



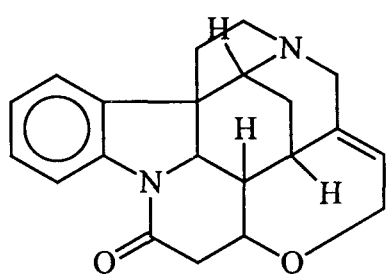
nicotine



quinine



coniine



strychnine

Figure 11. Structures of caffeine, nicotine, quinine, coniine, and strychnine.

deterrence may be due to both the toxic effects of alkaloids and to their bitterness.[34] Alkaloids may also play allelopathic[44] and antibiotic[34] defensive roles in plants.

Of possible importance in the analysis of *P. arctica* is the fact that several ergopeptine alkaloids, produced by a fungus growing subcutaneously in a species of fescue grass, have been shown to protect the grass from herbivory by Canada geese.[11] In this case, it appears that the deterrence is based more on the toxic effects of the alkaloids than on their bitter taste.[11] It has been hypothesized that grazing on the infected grass has played a part in the decline of some geese populations by putting them at a nutritional disadvantage.[11]

2.3.2. Alkaloid Spray Reagents

One of the preeminent methods for rapidly detecting a class of compounds in a crude mixture is by thin-layer chromatography using a spray reagent specific to that compound class to visualize the plate. This holds true for alkaloids in plant extracts. In fact, TLC has been applied to the analysis of alkaloids far more than any other chromatographic technique.[32]

There are many spray reagents that can colorimetrically indicate the presence of alkaloids. However, the thousands of known alkaloids have differing ring systems, nitrogen hybridizations, and chemical reactivities. Additionally, few spray reagents are specific only to alkaloids. Because of this, multiple spray reagents are often employed to corroborate that a positive result truly indicatives the presence of an alkaloid.

Dragendorff's reagent is the most widely used alkaloid spray reagent and was employed in this study.[39] The two other spray reagents used in this analysis were iodoplatinate and iodine-potassium iodide. It has been hypothesized that the minimum requirements for non-alkaloids to react with Dragendorff's reagent are either a conjugated carbonyl or lactone group, or else a hydroxyl group and an isolated double bond.[32]

Dragendorff's reagent reacts with tertiary and quaternary nitrogen atoms and has a sensitivity of between 0.01-1μg, depending on the alkaloid.[32] Alkaloids yield an intense orange color when visualized with this reagent. It will not react with primary or secondary nitrogen atoms, but will react with many non-alkaloid compounds including polyethylene glycols, chalcones, cholines, peptones, and proteins.[32] It is interesting to note that while Dragendorff's reagent reacts with proteins, it does not colorize amino acids.[32]

Iodoplatinate also reacts with tertiary and quaternary nitrogen atoms.[32] Its sensitivity is approximately the same as Dragendorff's reagent, 0.01-1μg depending on the alkaloid.[32] Iodoplatinate has several advantages over Dragendorff's reagent. Since it is non-destructive, alkaloids can be recovered after visualization.[32] Iodoplatinate reacts with different alkaloids to give different colors, ranging from brown to violet and blue.[32] This enables alkaloids with the same R_f values to be differentiated. Iodoplatinate will react with non-alkaloid compounds including biotin and phenothiazines.[32]

Iodine-potassium iodide reacts with alkaloids to give brownish orange spots on a yellow background. It is often used to detect purine class alkaloids as well as alkaloids isolated from the genera *Solanum* and *Lycopersicum*.[45]

2.3.3. Results

An initial assay detected a compound in the *P. arctica* MeOH extracts that tested positive with the Dragendorff's reagent. However, it was subsequently determined that this was a false positive caused by several contaminated TLC plates.

Figures 12 and 13 show typical TLCs of the MeOH and CH_2Cl_2 extracts visualized with UV and alkaloid spray reagents. As discussed in Section 2.2., the *P. phryganodes* MeOH extract contained a compound not found in any of the other plant species. This UV active compound, with an R_f of 0.50 in 10% (v/v) MeOH in $CHCl_3$, tested positive when treated with the iodine-potassium iodide spray reagent. However, it did not react with any of the other alkaloid spray reagents. Given that this compound only reacted with one spray reagent, and the fact that the plant is highly palatable, the identification of this compound as an alkaloid is questionable.

None of the other MeOH extracts yielded a positive result with any of the alkaloid spray reagents. None of the CH_2Cl_2 extracts tested positive for alkaloids either. It is thus concluded that none of the *P. arctica* samples contain alkaloids, and that alkaloids are not responsible for chemical defense in *P. arctica*. However, given the broad range of solubility and other properties of alkaloids, it is possible that a general screening procedure such as this one may have failed to detect alkaloids.[46]

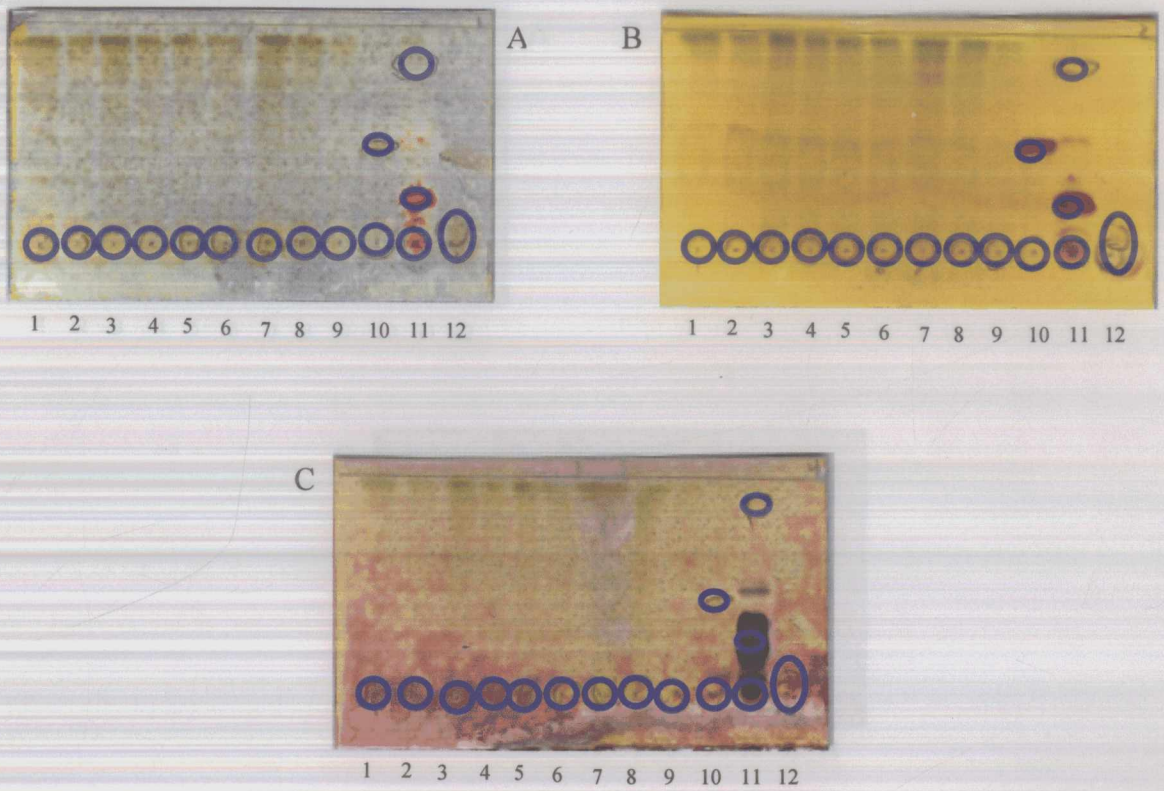


Figure 12 A-C. Typical TLCs of MeOH extracts visualized with UV and alkaloid spray reagents. These are the May MeOH extracts. Plates were eluted with 10% (v/v) MeOH in $CHCl_3$ and sprayed with either Dragendorff's reagent (Figure 12 A), iodine-potassium iodide (Figure 12 B), or iodoplatinate (Figure 12 C). Circles indicate UV absorption at 254nm. Plant samples are described in Table 6. Lane numbers correspond to plant samples as follows:

Lane 1 – arc/1young/may
Lane 2 – arc/1mature/may
Lane 3 – arc/2young/may
Lane 4 – arc/2mature/may
Lane 5 – arc/3young/may
Lane 6 – arc/3mature/may
Lane 7 – arc/4young/may
Lane 8 – arc/4mature/may
Lane 9 – lang/septem
Lane 10 – phryg
Lane 11 – tobacco MeOH extract positive control
Lane 12 – catechin negative control

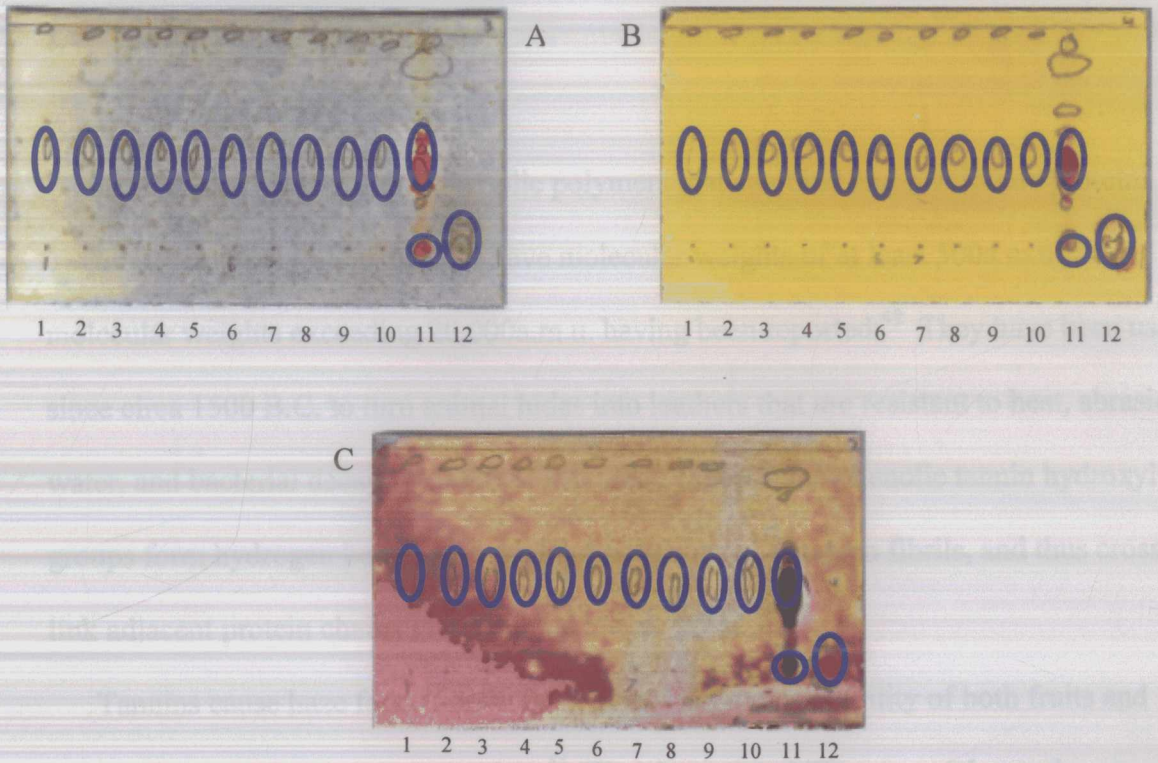


Figure 13 A-C. Typical TLCs of CH_2Cl_2 extracts visualized with UV and alkaloid spray reagents. These are the May CH_2Cl_2 extracts. Plates were eluted with 10% (v/v) MeOH in $CHCl_3$ and sprayed with either Dragendorff's reagent (Figure 13 A), iodine-potassium iodide (Figure 13 B), or iodoplatinate (Figure 13 C). Circles indicate UV absorption at 254nm. Plant samples are described in Table 6. Lane numbers correspond to plant samples as follows:

Lane 1 – arc/1young/may
Lane 2 – arc/1mature/may
Lane 3 – arc/2young/may
Lane 4 – arc/2mature/may
Lane 5 – arc/3young/may
Lane 6 – arc/3mature/may
Lane 7 – arc/4young/may
Lane 8 – arc/4mature/may
Lane 9 – lang/septem
Lane 10 – phryg
Lane 11 – tobacco CH_2Cl_2 extract positive control
Lane 12 – catechin negative control

2.4. Tannin Assays

2.4.1. Introduction to Tannins

Tannins are water-soluble phenolic polymers that can bind and precipitate proteins.[47] Tannins, being polymeric, usually have molecular weights of at least 500a.m.u., with molecular weights exceeding 20,000a.m.u. having been reported.[48] They have been used since circa 1500 B.C. to turn animal hides into leathers that are resistant to heat, abrasion, water, and bacterial decay.[49] During the tanning process, the phenolic tannin hydroxyl groups form hydrogen bonds with the peptide groups of collagen fibrils, and thus cross-link adjacent protein chains in the animal hides.[50]

Tannins cause haze formation in beer, and affect the palatability of both fruits and vegetables as well as wines and ciders.[51] They have the ability to complex and precipitate biomolecules such as proteins, polysaccharides, and alkaloids.[52] Tannins are present in the wood, fruits, bark, and leaves of most trees,[53] and are also found in many plants. They may make up as much as 20% of the dry mass of some woods, such as quebracho.[53]

Tannins are present in many of the foods, beverages, and stimulants that are used by humans and are now recognized as carcinogens if taken in large amounts over an extended period of time.[54] Much of the effectiveness of herbal folk medicines is due to tannins.[55]

The two major types of tannins, condensed and hydrolyzable, have traditionally been thought of as being chemically distinct. However, more recent research suggests that

many plants may in fact produce condensed tannins containing some hydrolyzable tannin moieties, and vice versa.[56]

Condensed tannins, also called proanthocyanidins, are the most widely distributed tannins in vascular plants.[50] They are found in almost all gymnosperms and ferns, and are widespread among the angiosperms.[46] They are flavanoid polymers with carbon-carbon bonds joining the individual flavanoid units. These carbon-carbon bonds are not susceptible to hydrolysis, but can be oxidatively cleaved under acidic conditions. The atoms in flavanoid ring systems are typically numbered according to the scheme shown in Figure 14.

Condensed tannins are typically joined through either a 4,8 linkage, as shown in Figure 15, or a 4,6 linkage. The individual flavanoid monomers typically contain three asymmetric carbon atoms (carbon atoms 2, 3, and 4 in Figure 14), which gives rise to several possible stereoisomers.[16] Condensed tannin polymers are highly irregular, sometimes branch, and are often composed of several different flavanoid monomers.[56]

A single plant source may contain many different oligomeric condensed tannins.[57] The more highly polymerized condensed tannins are more abundant than the dimeric and trimeric oligomers, but are also less well characterized.[58] The condensed tannins are usually responsible for the effects ascribed to the presence of tannins in plants.[56]

Hydrolyzable tannins have a much more limited distribution than condensed tannins. They are typically found only in angiosperms.[50] Hydrolyzable tannins are formed around a polyol, typically D-glucose, with its hydroxyl groups esterified. As their name implies, they are joined by carboxylic ester linkages that are easily hydrolyzed by acids,

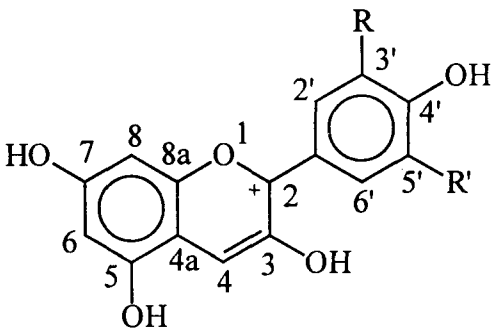


Figure 14. Position numbering in flavanoid ring systems. For pelargonidin R = R' = H. For cyanidin R = OH and R' = H. For delphinidin R = R' = OH.

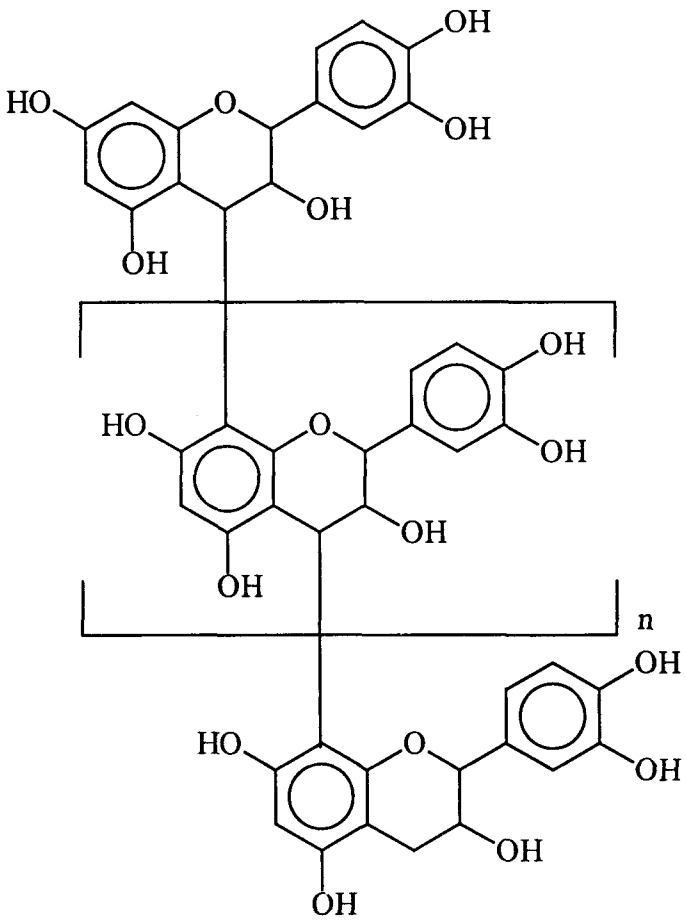


Figure 15. A procyanidin condensed tannin with a 4,8 linkage.

bases, and esterase enzymes. The polyol is usually esterified to either gallic acid or hexahydroxydiphenic acid, Figures 16 and 17 respectively. Polyols esterified to hexahydroxydiphenic acid are termed ellagitannins, with Figure 18 displaying a typical ellagitannin. If the polyol is esterified to gallic acid it is termed a gallotannin. As shown in Figure 19, most gallotannins contain additional galloyl groups esterified to the metahydroxyls of the inner galloyl groups in what are termed depside linkages.

Tannins were rarely discussed in an ecological sense before several papers published by Feeney between 1968 and 1970.[14,59,60] Then in 1976, separate papers by Feeney and Rhoades and Cates put forth the hypothesis that the presence of tannins in plants may be able to deter herbivory.[61,62]

Unfortunately, much of the now prolific literature concerning the ecological role of tannins is confused and conflicting.[63,64] Many authors citing the papers of Feeney and Rhoades and Cates transform their hypothesis into a fact.[64] Numerous papers have attempted to confirm the herbivore deterrent qualities of tannins by using correlations and generalizations.[64] Recent reviews have warned of the dangers and limitations of these practices.[64,65,66]

Technical issues have troubled much of the work done to investigate the ecological role of tannins. These include the use of unpurified tannins, differing tannin extraction procedures, modified natural herbivore diets, artificial herbivore diets, and correlation studies.[63,64,65] Often, the use of different experimental procedures can result in different conclusions being drawn from the study.[67]

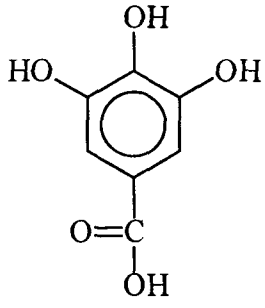


Figure 16. Gallic acid.

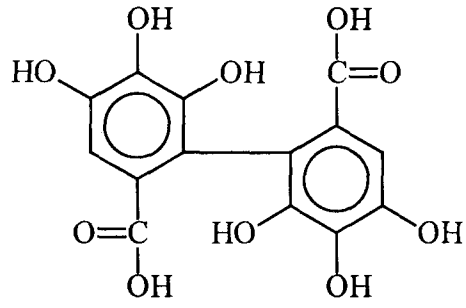


Figure 17. Hexahydroxydiphenic acid.

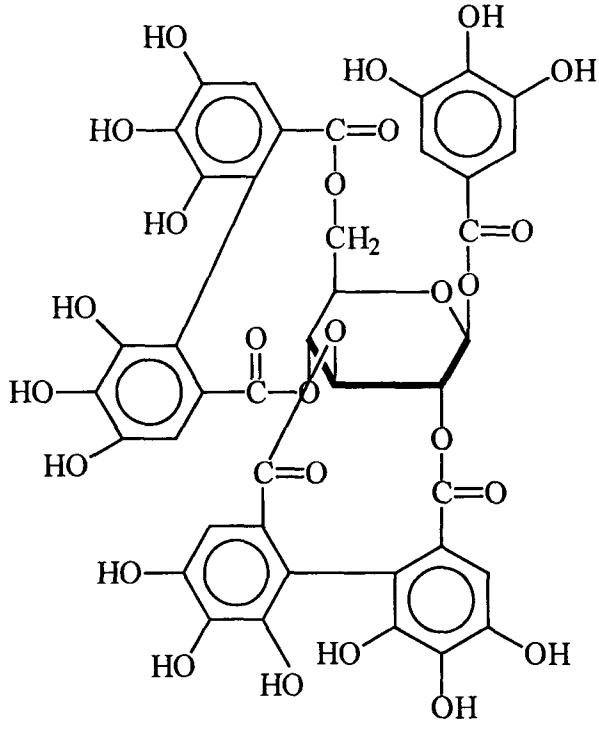


Figure 18. An ellagitannin.

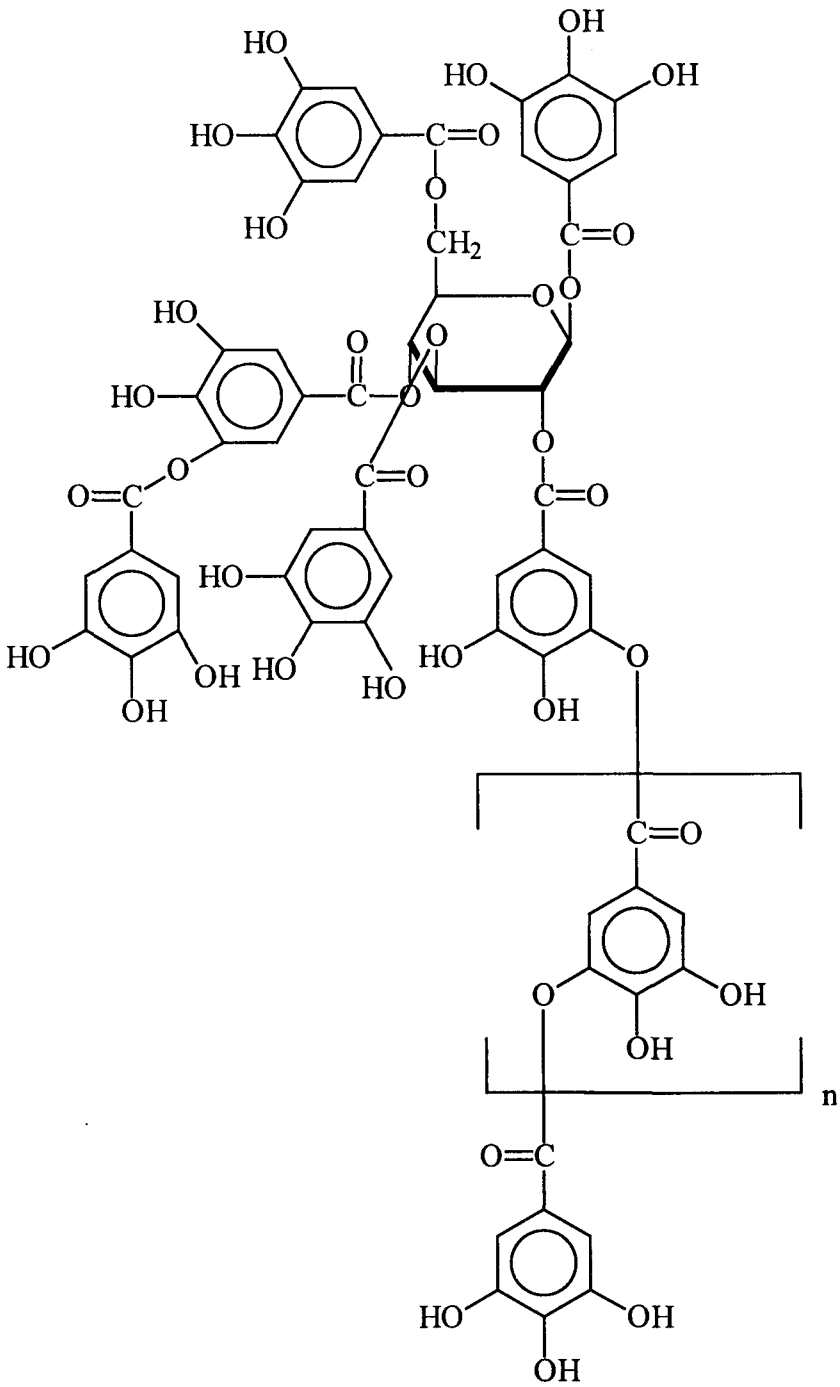


Figure 19. A gallotannin with additional galloyl groups esterified in depside linkages.

There is little consensus about the importance and mode of action of tannins in plant-herbivore interactions. Numerous studies indicate that tannins act as herbivore deterrents by complexing with dietary proteins and digestive enzymes in the gut, while other work shows that the systemic toxicity of tannins is mainly responsible for their role in chemical defense.[63] Some authors have found that the lower molecular weight polyphenols associated with tannins are actually the defensive chemicals.[51,64] It is often maintained that tannins coagulate mucoproteins and mucopolysaccharides in the oral cavity and cause astringency, a feeling of extreme dryness and roughness, that is unpleasant and thus deterrent to herbivores.[15,52] Several papers have implied that tannins have no affect on herbivores, or may even act as nutritional supplements.[63]

In light of these conflicting theories and findings, an argument has been made that specific compounds, and not merely general classes of secondary metabolites, provide for chemical defense in plants.[68,69] Zucker first hypothesized in 1983 that tannins of different structure and stereochemistry would precipitate proteins differently.[70]

There is a significant amount of variation in tannin structure, even between similar plant species.[63] Though both hydrolyzable and condensed tannins interact similarly with proteins,[71] the physiological effects of tannins are structure specific[70] and also depend on herbivore gut physiology.[63] This is because tannins vary immensely in their affinity for proteins, and proteins vary immensely in their tannin affinity.[72,73,74] As a result, tannins differ in their antiherbivore activity and herbivores differ in their sensitivity to tannins.[63] A tannin may thus have drastically different effects on different herbivores.[63]

Despite the complexity of the issue, tannins have been proven to defend plants against such varied herbivores as hares, goats, sheep, deer, *Colobus* monkeys, gorillas, giant tortoises, and insects.[50,75,76,77,78] Tannins may also protect plants against pathological microbes.[79] Of more pertinence to this analysis, tannins have been shown to protect sorghum grain from bird herbivory.[17,18]

Proline-rich proteins (PRPs) secreted in saliva are the principal means of mammalian herbivore defense against tannins.[52] They complex with and precipitate dietary tannins, thus providing protection from the tannins' anti-nutritional and toxic effects.[80] The tannin polyphenol rings bind with the PRPs via a hydrophobic stacking against the *pro-S* face of the proline residues.[52] The resulting feeling of astringency in the oral cavity may also serve to warn of the presence of tannin in browse. Herbivores may alternatively learn to avoid tannin rich plants over time by associating their taste with negative effects, rather than by instinctively avoiding them.[64,76,81]

The results of tannin assays have many inherent sources of inconsistency. A plants developmental stage and the environmental conditions it is grown in will both affect the amount of tannin it contains.[15,82] There is a large amount of variability between the tannin structures and levels found in different plant species, in different cultivars of the same species, and in different tissues of the same plant.[15] Many plant species contain numerous distinct types of tannin, frequently including both condensed and hydrolyzable.[15] Environmental, physiological, and seasonal changes may alter both the relative amounts of each tannin type and their extractability within any given plant.[15]

Extraction conditions, tannin structure, plant species, type of plant tissue, tissue maturity, tissue moisture content, and tissue preservation conditions can all affect tannin extractability.[15] Tannin extraction is almost always incomplete, and extracted levels may be small relative to unextracted tannin remaining in the plant tissue.[83] No single set of extraction conditions will work for all plant samples.[84]

Tissue damage can cause chemical reactions that reduce tannin extractability, and assayed tannin levels may be quite different from tannin levels actually present in plant tissue. Because of this, it is best to extract and analyze plant material immediately after collection.[83] Unfortunately, this is not always possible. When plant material can not be analyzed immediately after collection, it should be lypholized.[84]

In light of this, it should be recognized that differences in plant tannin levels may be due to differences in tannin extractability as well as to differences in actual tannin levels.[15] Additionally, since some tannins may be more biologically active than others, total tannin levels may be inappropriate predictions of palatability.

2.4.2. Bate-Smith Assay

As seen in Section 2.2., there were compounds present in the plant samples that required extremely polar solvents in order to become mobile on TLC. It seemed possible that these compounds were condensed tannins, which are known to be rather immobile on TLC.[15] The Bate-Smith assay was performed to confirm the presence of condensed tannins in the plant samples before the more quantitative, and also more expensive and labor intensive, tannin assays were performed. The Bate-Smith assay, or one of its

variants, was the standard method for determining condensed tannins until it was replaced by the acid butanol assay in 1986.[85]

The Bate-Smith assay was developed by Bate-Smith in 1954, and converts condensed tannins to the red colored compound cyanidin via the pathway shown in Figure 20. A condensed tannin dimer first undergoes acid-catalyzed cleavage of the carbon-carbon interflavanoid bond. The "upper" unit of each condensed tannin dimer yields a carbocation that can eventually form cyanidin. Since condensed tannin polymer chains are lengthened by the addition of further "upper" units,[86] the yield of cyanidin should increase with chain length.[85]

In the next step of the pathway, the carbocation goes on to form a flav-3-ene intermediate. This intermediate undergoes a final oxidation step, abstraction of a hydride ion from the C-2 carbon, to yield cyanidin.[85,87]

The Bate-Smith assay is qualitative, a red color indicates the presence of condensed tannins while an absence of red color indicates that condensed tannins are not present. Several authors have noted the drawbacks of this assay, which include low sensitivity, a lack of reproducibility in the yield of cyanidin, and the inability to relate cyanidin yield to condensed tannin concentrations.[85] As long as these drawbacks are kept in mind, the Bate-Smith assay remains useful as a simple, rapid, and inexpensive initial qualitative screening assay for condensed tannins that can be done in the field.

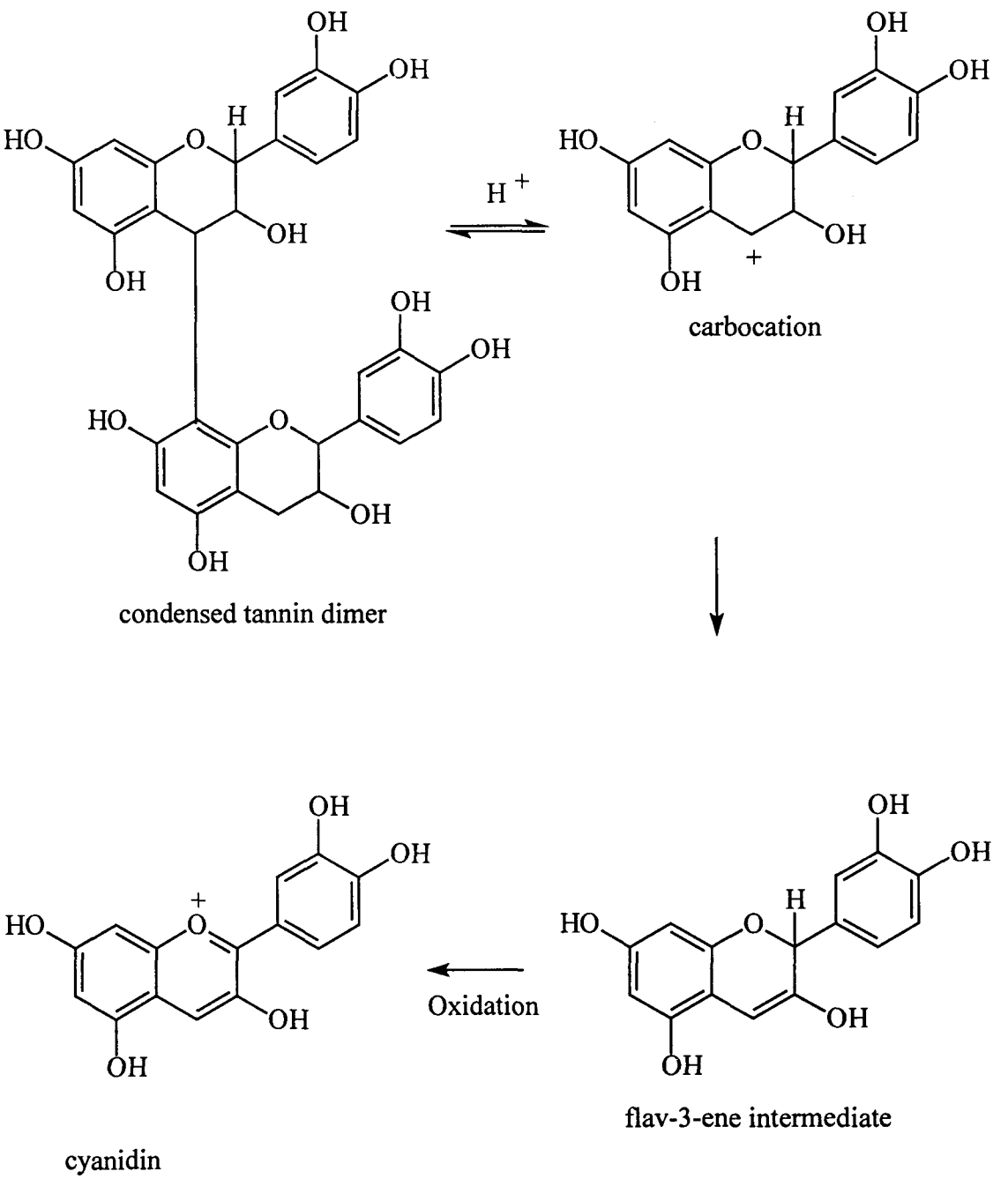


Figure 20. Pathway of cyanidin formation from condensed tannin dimer in the Bate-Smith assay.[85] A condensed tannin dimer undergoes acid-catalyzed cleavage to yield a carbocation. The carbocation then forms a flav-3-ene intermediate that undergoes an oxidation reaction to yield cyanidin.

Table 2 shows the results of the Bate-Smith assay. At first glance, there was no clear correlation between unpalatability and the presence of condensed tannin. Some of the *P. arctica* samples tested positive for condensed tannin while others did not. Additionally, some of the palatable plant samples contained condensed tannin.

However, a closer look indicates that there is a pattern to the results. Among *P. arctica*, the mown samples from July as well as the August seedling and Prudhoe Bay samples tested positive for condensed tannin. Both of the *P. langeana* samples from August also tested positive. These results correlate with the results of the acid butanol assay in Section 2.4.3. The samples that tested positive for condensed tannin in the Bate-Smith assay contained the highest amounts of condensed tannin as measured by the acid butanol assay.

A sample of *Acer saccharum*, sugar maple, was assayed to serve as a positive control. The members of the *Acer* genus have been examined exhaustively for tannins,[88] and *A. saccharum* is known to contain condensed tannin, gallotannin, and ellagitannin.[89,90,91] *A. saccharum* tested positive for condensed tannin with the Bate-Smith assay. On the basis of these initial results, the plant samples were quantitatively assayed for condensed and hydrolyzable tannins as discussed in Section 2.4.3., 2.4.4., and 2.4.5..

Table 2. Bate-Smith assay results. A + designates a red color indicative of cyanidin formation. A – designates an absence of red color and no cyanidin formation. Plant samples are described in Table 6.

Plant Sample	Result
arc/1mature/may	–
arc/2mature/may	–
arc/3mature/may	–
arc/4mature/may	–
arc/1young/may	–
arc/2young/may	–
arc/3young/may	–
arc/4young/may	–
arc/1mow/july	+
arc/2mow/july	+
arc/3mow/july	+
arc/4mow/july	+
arc/1nomow/july	–
arc/2nomow/july	–
arc/3nomow/july	–
arc/4nomow/july	–
arc/2prud/august	+
arc/3prud/august	+
arc/Xprud/august	+
arc/1seed/august	+
arc/2seed/august	+
arc/3seed/august	+
arc/4seed/august	+
lang/septem	–
lang/DS9/august	+
lang/BPX//august	+
phryg	–
prat/mow/july	–
prat/nomow/july	–
prat/seed/august	–
Acer saccharum	+

2.4.3. Acid Butanol Assay

The acid butanol assay is the easiest, most specific, and best method for quantifying condensed tannin levels.[15,92] It employs the same acid-catalyzed reaction pathway as the Bate-Smith assay to turn condensed tannins into cyanidin, which has a λ_{max} of 550nm under the reaction conditions. However, side reactions may form red-brown polymers that absorb near 450nm.[93] The yield of cyanidin, while dependant upon condensed tannin structure and storage conditions of the plant material, is generally correlated to condensed tannin level.[15]

The acid butanol assay uses a n-BuOH-HCl-Fe^{III} solvent system that allows for reproducible yields of cyanidin.[85] The cyanidin levels are quantified by measuring the absorbance at 550nm. The replacement of the aqueous Bate-Smith solvent with n-BuOH increases the cyanidin absorbance and decreases the absorbance of the red-brown polymer side products.[93] The addition of Fe^{III} can increase the yield of cyanidin by as much as 300%.[85] However, the temperature and reaction time need to be carefully controlled,[53] or else the absorption maxima will become ill defined.[94]

The acid butanol assay allows for the determination of relative condensed tannin content among multiple plant samples. The results are reported in terms of absorbance at 550nm. The relative condensed tannin content is sometimes calibrated against a condensed tannin standard to make an estimate of the absolute condensed tannin content of a sample, expressed in terms of a percentage of condensed tannin by weight. However, this practice requires the use of an appropriate standard.

It is improper to use condensed tannin isolated from one plant species as a standard for another plant species.[95] The structural variability between the different tannins would result in different responses to the tannin assay.

A tannin sample could be isolated from the plant of interest and used as a standard, as is recommended by one author.[95] However, in complex plant systems obtaining a pure tannin sample to use as a standard may prove to be impossible.[92] Large condensed tannins are difficult to extract, while the smaller condensed tannins are lost during chromatographic separations. Because of this, tannin isolations frequently yield condensed tannins that comprise a limited range of molecular weights.[64] Tannins may be oxidized or polymerized during isolation steps, which can cause changes in their structure.[64] Isolated tannins are not bound to proteins or polysaccharides, and thus may have lower biological activity than bound tannins.[64]

The only commercially available condensed tannin is isolated from the *Querbracho spp.*, and is thus not suitable as a standard for the *Puccinellia* or *Poa* samples. Additionally, the cyanidin yield from quebracho tannin is very low.[96] An unsuccessful attempt, using standard procedures[95,97,98], was made to extract and purify condensed tannin from *P. arctica* to use as a standard for the acid butanol assay. The isolated tannin contained an impurity that consistently caused the tannin to precipitate out of solution. As a result, it proved impossible to perform an analysis to confirm either identity or purity of the isolated tannin.

Cyanidin may also be used as a standard for the acid butanol assay.[92] However, the resulting values are well below the actual tannin content since the maximum reported

cyanidin yield from condensed tannin is 58% (average chain length 9.4 units).[94] Additionally, both condensed tannin linkage type, 4,6 versus 4,8, and hydroxylation pattern will affect cyanidin yield.[94]

Cyanidin may be obtained commercially,[99] but its price is prohibitive for most studies. As described in the Appendix, an unsuccessful attempt was made to synthesize cyanidin from quercetin. It proved to be impossible to isolate cyanidin in the purity necessary for use as a standard. In the end, the acid butanol assay was run without a standard and the relative amounts of condensed tannin in the plant samples were determined.

Figure 21 displays the calibration curve that was obtained when the acid butanol assay was performed on commercially available quebracho tannin. The equation for the regression line was $A_{550} = 1.0(\text{mg quebracho tannin}) + 0.0035$. The regression had an R^2 value of 0.98. Upon examination of the calibration curve, the acid butanol assay was determined to give a linear response up to an absorbance of 0.23. This covered the range of absorbances found in the plant samples.

Table 3 shows the results of the acid butanol assay. Replicate assays were performed on six plant samples. A t-test ($p=0.05$),[100,101] which included the replicates, showed that there was no statistical difference between the mean absorbance values of the *P. arctica* samples and palatable grass samples. *Acer saccharum*, known to contain condensed tannin as detailed in Section 2.4.2., was assayed as a positive control.

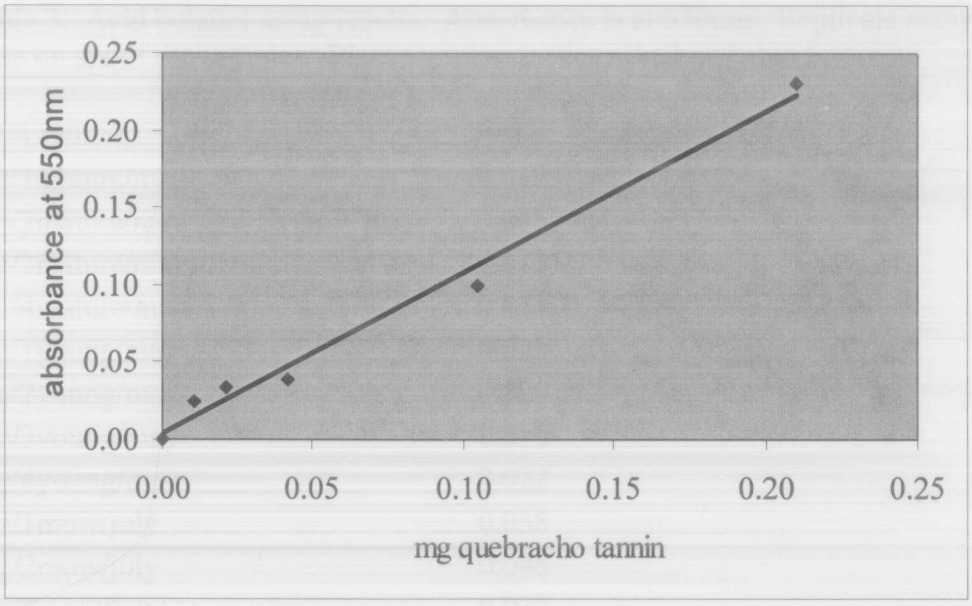


Figure 21. Quebracho tannin calibration curve.

Table 3. Acid butanol assay results. Absorbance is at 550nm. Replicate assays were done on six of the samples. Plant samples are described in Table 6.

Plant sample	A_{550}	Replicate A_{550}
arc/1mature/may	0.017	0.024
arc/2mature/may	0.015	
arc/3mature/may	0.013	
arc/4mature/may	0.011	
arc/1young/may	0.023	
arc/2young/may	0.020	
arc/3young/may	0.018	
arc/4young/may	0.022	
arc/1mow/july	0.038	
arc/2mow/july	0.046	
arc/3mow/july	0.037	
arc/4mow/july	0.033	
arc/1nomow/july	0.018	
arc/2nomow/july	0.021	
arc/3nomow/july	0.026	
arc/4nomow/july	0.021	0.015
arc/2prud/august	0.031	0.036
arc/3prud/august	0.034	
arc/Xprud/august	0.035	
arc/1seed/august	0.031	
arc/2seed/august	0.035	
arc/3seed/august	0.034	
arc/4seed/august	0.039	
lang/septem	0.020	
lang/DS9/august	0.031	
lang/BPX/august	0.033	0.029
phryg	0.018	
prat/mow/july/	0.013	0.008
prat/nomow/july	0.025	
prat/seed/august	0.017	0.023
Acer saccharum	0.19	

It was found to have an absorbance value considerably greater than the grass samples.

As discussed in Section 2.4.2., the acid butanol assay results correlated with the Bate-Smith assay results. The *P. arctica* mown samples from July as well as the August seedling and Prudhoe Bay samples tested positive for condensed tannin in the Bate-Smith assay. Both of the *P. langeana* samples from August also tested positive. The samples that tested positive for condensed tannin in the Bate-Smith assay contained the highest amounts of condensed tannin as measured by the acid butanol assay.

Figures 22 to 27 show the groupwise comparisons for the May mature, May young, July mown, July unmown, August Prudhoe, and August seedling samples respectively. Among the groupwise comparisons, the mean absorbance values of the *P. arctica* grass samples were statistically higher (t-test with p=0.05) than the palatable grass samples for the July mown and August seedling samples. The seedling results are interesting, since there is evidence that *P. arctica* seedlings are highly unpalatable and gradually become more palatable as they age.[10]

The mown and unmown grass samples are also interesting. It appears that *P. arctica* tannin levels increased in response to mowing. Mowing has been observed to increase condensed tannin levels in other grasses as well,[41] and these results can be explained using the carbon nutrient balance (CNB) hypothesis.[102]

The CNB hypothesis states that plant secondary metabolism is affected by changes in the amounts of both carbon and nutrients that are available. When plant growth is limited more by nutrient availability, perhaps due to low levels of nitrogen in the soil, the plant

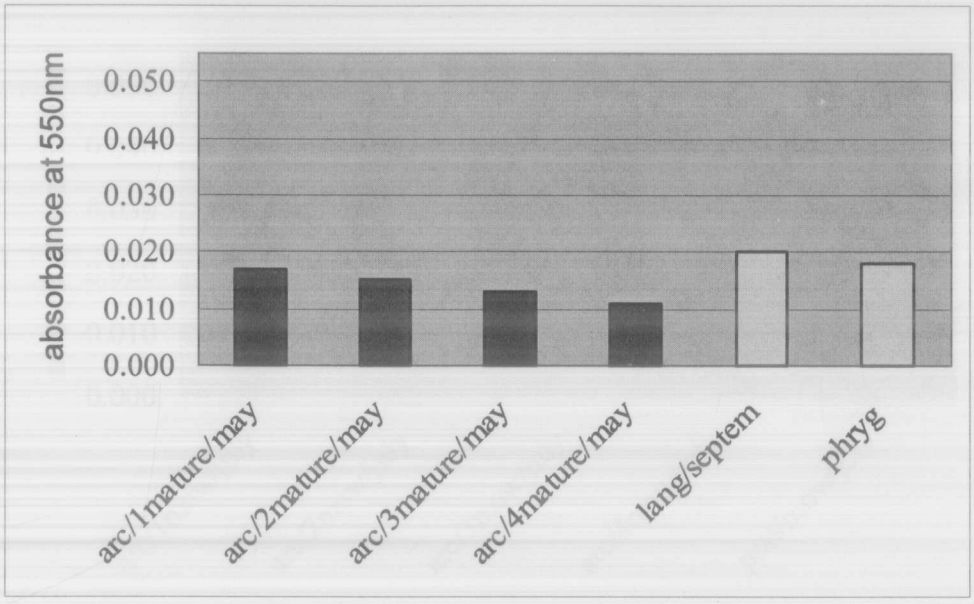


Figure 22. Acid butanol assay results for May mature samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

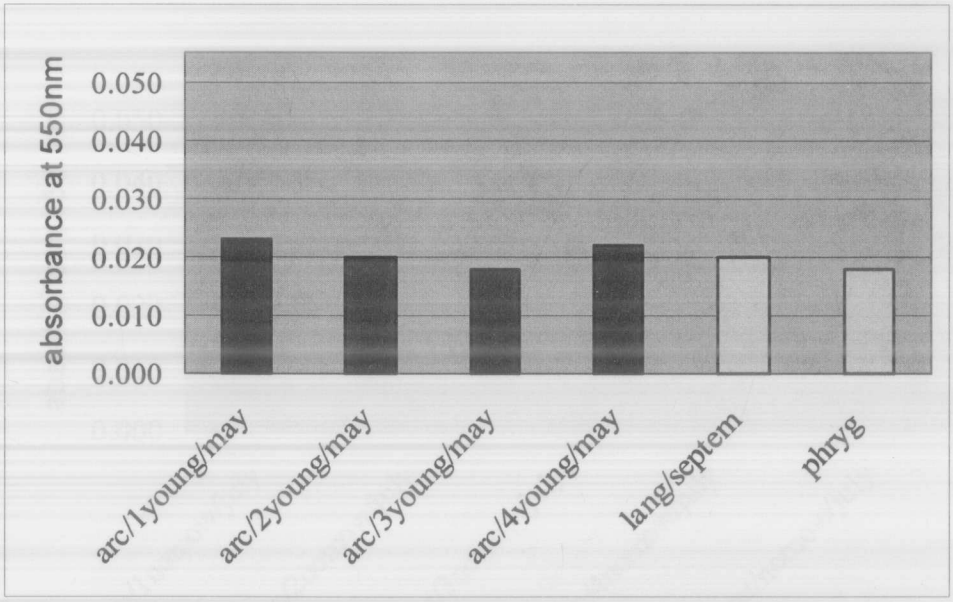


Figure 23. Acid butanol assay results for May young samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

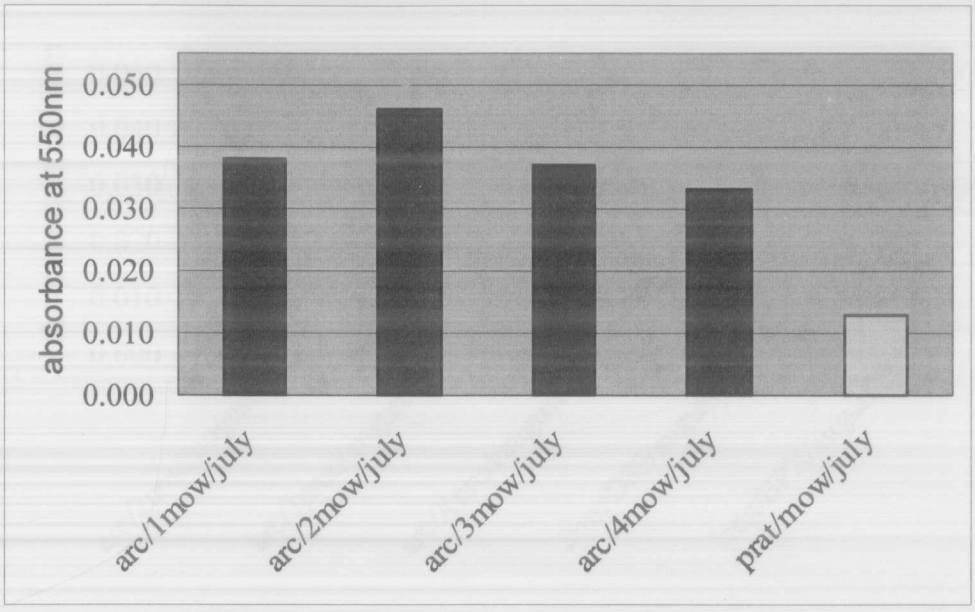


Figure 24. Acid butanol assay results for July mown samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

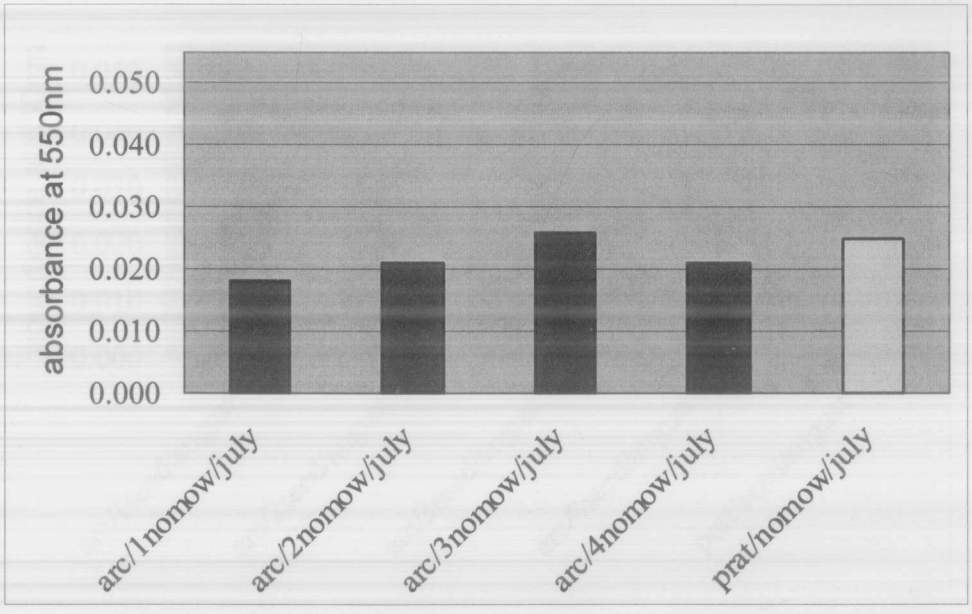


Figure 25. Acid butanol assay results for July unmown samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

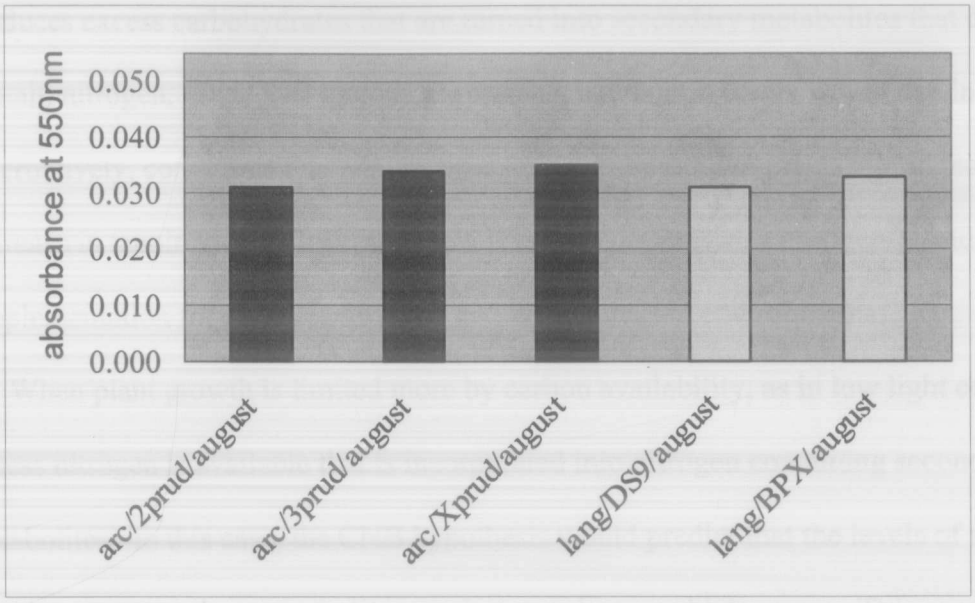


Figure 26. Acid butanol assay results for August Prudhoe samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

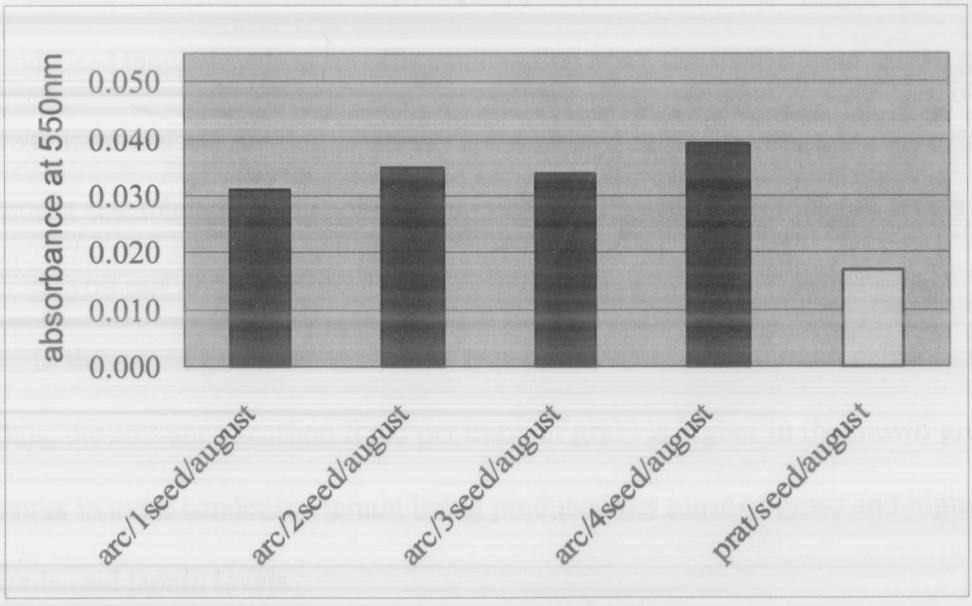


Figure 27. Acid butanol assay results for August seedling samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

produces excess carbohydrates that are turned into secondary metabolites that do not contain nitrogen. The CNB hypothesis predicts that tannin levels would rise in this case. Alternatively, conditions that alleviate the nutrient stress upon plants would result in a decrease in tannin levels. Indeed, tannin levels do decline with nitrogen[103] and water[104] supplementation for plants in nitrogen and water stressed environments.

When plant growth is limited more by carbon availability, as in low light conditions, excess nitrogen is available that is incorporated into nitrogen containing secondary metabolites. In this case, the CNB hypothesis would predict that the levels of non-nitrogenous secondary metabolites, such as tannins, would decrease. This aspect of the CNB hypothesis has also been supported by experimental evidence in that tannin levels decline with decreased sunlight.[105]

An argument can be made that explains the observed increases in mown grass condensed tannin levels as an effect of shading upon the CNB. In unmown grass, the lower leaves of the grass are blocked from the sun by grass that is growing above them. Thus, a sizeable percentage of the unmown grass leaves have lowered levels of photosynthesis and therefore fix less carbon.

In the mown grass, the leaves are exposed to more sunlight and can fix more carbon. Thus, the amount of carbon fixed per mass of grass is higher in the mown grass. This results in more condensed tannin being produced per mass of grass and higher assayed condensed tannin levels.

In all of the other groupwise comparisons, there was no statistical difference (t-test with p=0.05) between the mean absorbance values of *P. arctica* and the palatable grass

samples. It should also be noted that all of the plant samples, both *P. arctica* and palatable, had absorbance values at least a factor of 4 lower than *A saccharum*. If condensed tannin levels are indeed higher in *P. arctica* seedlings and mown plants than in the palatable plants, they may not be high enough to be ecologically significant. Additionally, the number of samples used in the seedling and mown comparisons was very small. An error in the measurement of the *P. pratensis* mown sample, for instance, could drastically affect the results.

In conclusion, there was no statistical difference between the mean absorbance values of *P. arctica* and the palatable grass samples. Because of this, it is concluded that condensed tannins are not responsible for chemical defense in *P. arctica*. There was some evidence that seedling and mown *P. arctica* samples may have had elevated condensed tannin levels. However, more work needs to be done to determine if this is indeed so and if the effect is ecologically significant.

2.4.4. Rhodanine Assay

Bate-Smith devised a technique in 1977 for measuring gallotannin levels using potassium iodate.[90] However, the iodate used in this assay also reacts with ellagitannins and other plant phenolics.[106] Precipitates can form in the reaction and interfere with absorbance measurements.[90] Reaction time and temperature must be carefully controlled, and it is difficult to obtain reproducible results.[106]

In 1973, Thies and Fischer developed a method to measure gallic acid levels in commercially available gallotannin.[107] However, this method is also subject to

precipitate formation and unreproducible results.[106] The rhodanine assay is an improvement of the method originally devised by Thies and Fischer, and is unaffected by the presence of other plant phenolics such as ellagic acid, ellagitannin, and condensed tannin.[106] It is the recommended method for measuring gallotannins.[15]

In this assay, gallotannins are first hydrolyzed into gallic acid. Rhodanine then reacts with the adjacent hydroxyl groups of gallic acid to yield a red colored complex with a λ_{max} of 520nm.[106] Careful control of pH, order of reagent addition, and reaction time is necessary for reliable results.[106]

Like the acid butanol and nitrosylation assays, the rhodanine assay enables the relative determination of tannin content among plant samples. Gallotannin content is reported in terms of the percentage of esterified gallic acid by weight. This assay does not provide an absolute measure of the tannin content in a sample. The number of gallic acid residues in a given amount of gallotannin is variable.[108] Some ellagitannins may contain galloyl moieties as well.[108] Since gallic acid esters in plants comprise simple mono- and digalloylesters in addition to gallotannin,[109] gallotannin content may be overestimated with the rhodanine assay.[106] However, other colorimetric methods have the same drawback.[51,90]

Figure 28 displays the calibration curve that was obtained when the rhodanine assay was performed on commercially available gallic acid. The equation for the regression line was $A_{550} = 4.6(\text{mg gallic acid}) + 0.022$. The regression had an R^2 value of 0.99. Upon examination of the calibration curve, the rhodanine assay was determined to

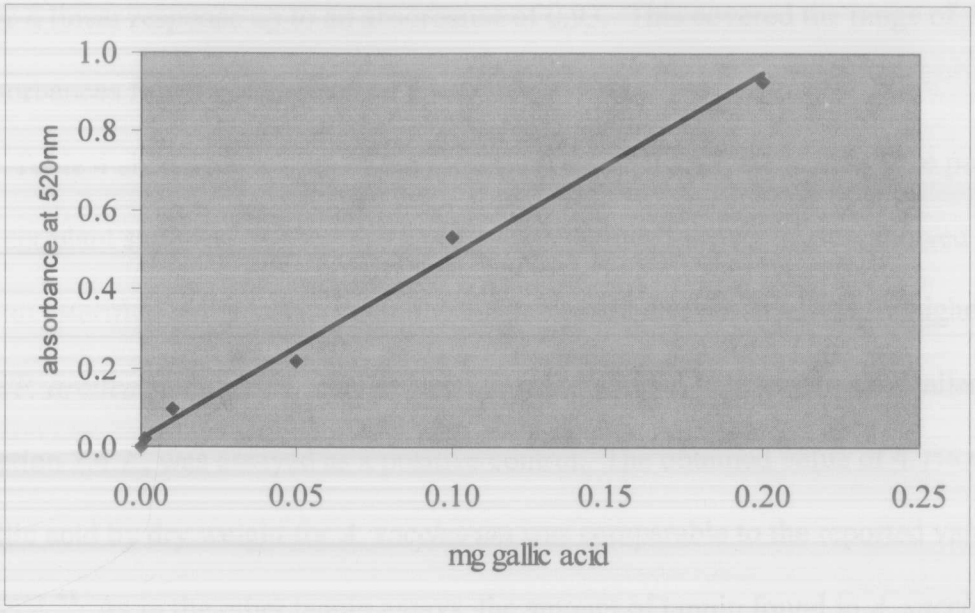


Figure 28. Gallic acid calibration curve.

give a linear response up to an absorbance of 0.93. This covered the range of absorbances found in the plant samples.

Table 4 shows the results of the rhodanine assay. Replicate assays were performed on six plant samples. A t-test (p=0.05), which included the replicates, showed that the mean esterified gallic acid level in the palatable samples was statistically higher than in the *P. arctica* samples. *A. saccharum*, known to contain gallotannin as detailed in Section 2.4.2., was assayed as a positive control. The obtained value of 4.7% esterified gallic acid by dry weight for *A. saccharum* was comparable to the reported value of 3.35%.[88] As in the other tannin assays, the amount of tannin found in *A. saccharum* was considerably greater than the amount of tannin in the grass samples.

Figures 29 to 34 show the groupwise comparisons for the May mature, May young, July mown, July unmown, August Prudhoe, and August seedling samples respectively. Among the groupwise comparisons, the mean ellagic acid level in the *P. arctica* grass samples was not statistically different (t-test with p=0.05) than the mean of the palatable grass samples for the August Prudhoe samples. For all other comparisons, the mean ellagic acid levels in the palatable samples were statistically greater (t-test with p=0.05) than the mean of the *P. arctica* grass samples. If gallotannins were playing a defensive role in *P arctica*, then their levels should be higher in the *P. arctica* grass samples.

Table 4. Rhodanine assay results. Replicate assays were done on six on the samples. Plant samples are described in Table 6.

Plant Sample	Esterified Gallic Acid % Dry Weight	Replicate
arc/1mature/may	0.12	0.14
arc/2mature/may	0.13	
arc/3mature/may	0.13	
arc/4mature/may	0.15	
arc/1young/may	0.15	
arc/2young/may	0.13	
arc/3young/may	0.13	
arc/4young/may	0.12	
arc/1mow/july	0.13	
arc/2mow/july	0.090	
arc/3mow/july	0.068	
arc/4mow/july	0.080	
arc/1nomow/july	0.13	
arc/2nomow/july	0.14	
arc/3nomow/july	0.11	
arc/4nomow/july	0.052	0.037
arc/2prud/august	0.026	0.042
arc/3prud/august	0.090	
arc/Xprud/august	0.12	
arc/1seed/august	0.030	
arc/2seed/august	0.037	
arc/3seed/august	0.026	
arc/4seed/august	0.065	
lang/septem	0.31	
lang/DS9/august	0.029	
lang/BPX/august	0.12	0.10
phryg	0.27	
prat/mow/july/	0.27	0.31
prat/nomow/july	0.24	
prat/seed/august	0.25	0.22
Acer saccharum	4.7	

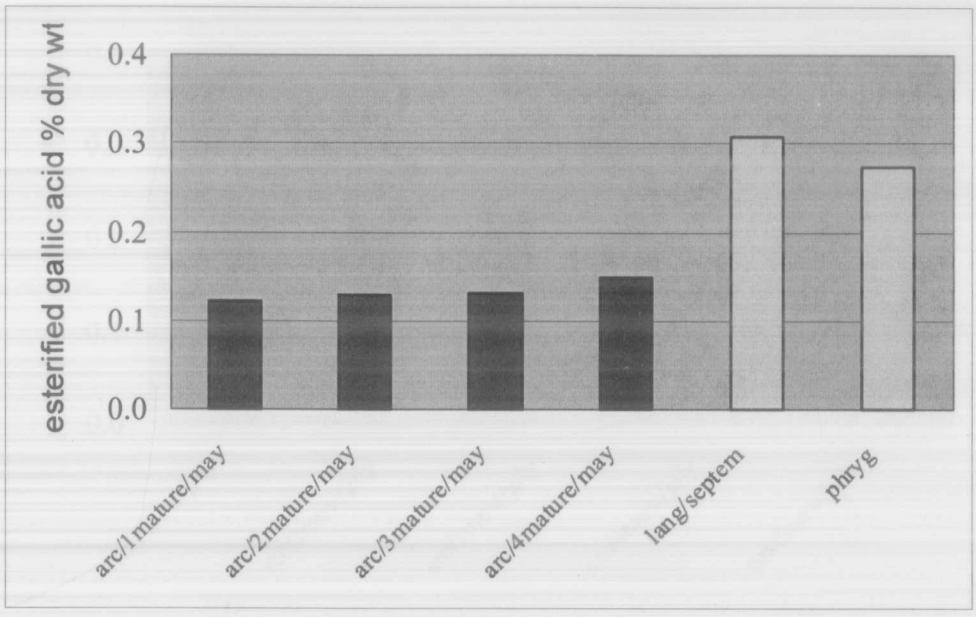


Figure 29. Rhodanine assay results for May mature samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

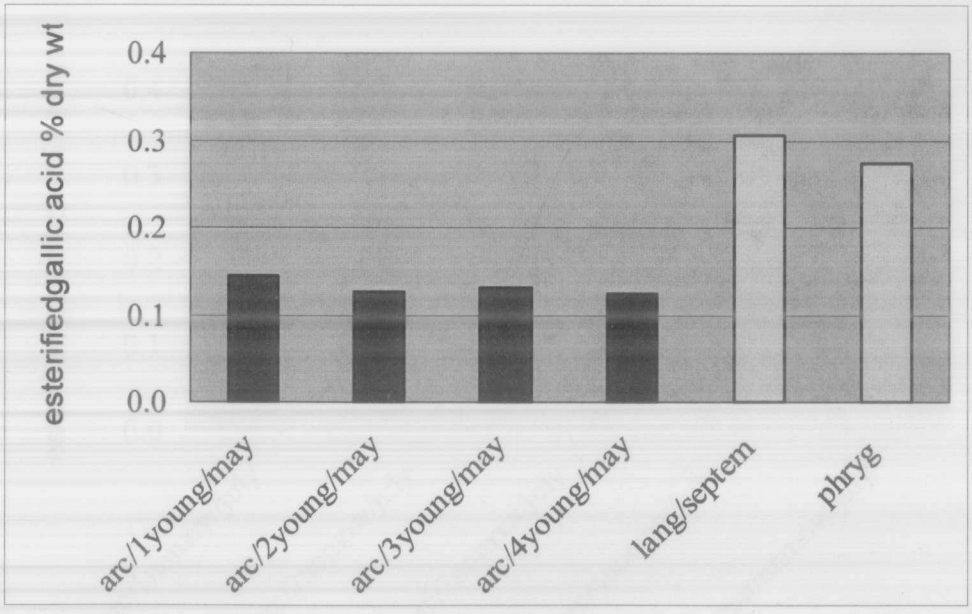


Figure 30. Rhodanine assay results for May young samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

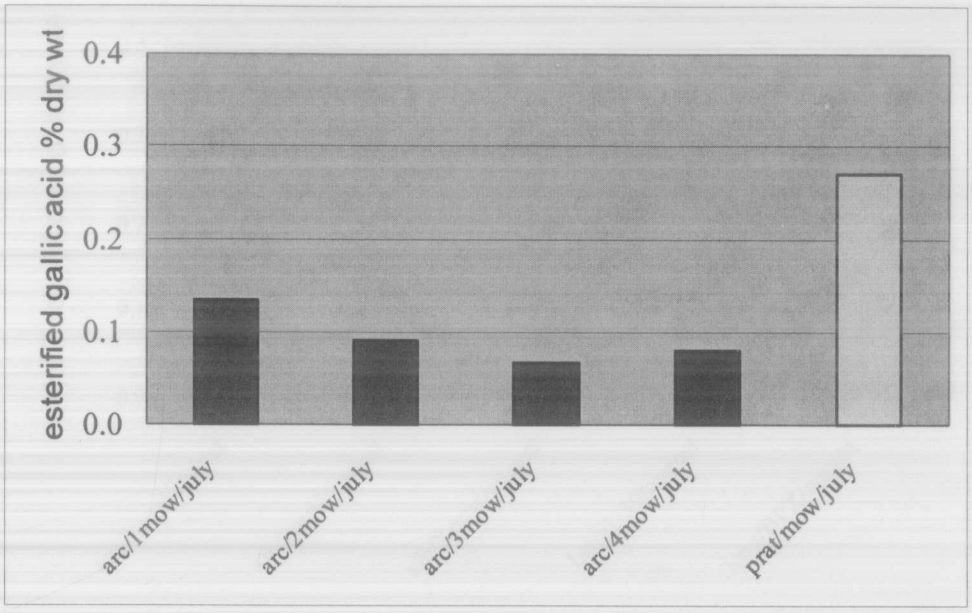


Figure 31. Rhodanine assay results for July mown samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

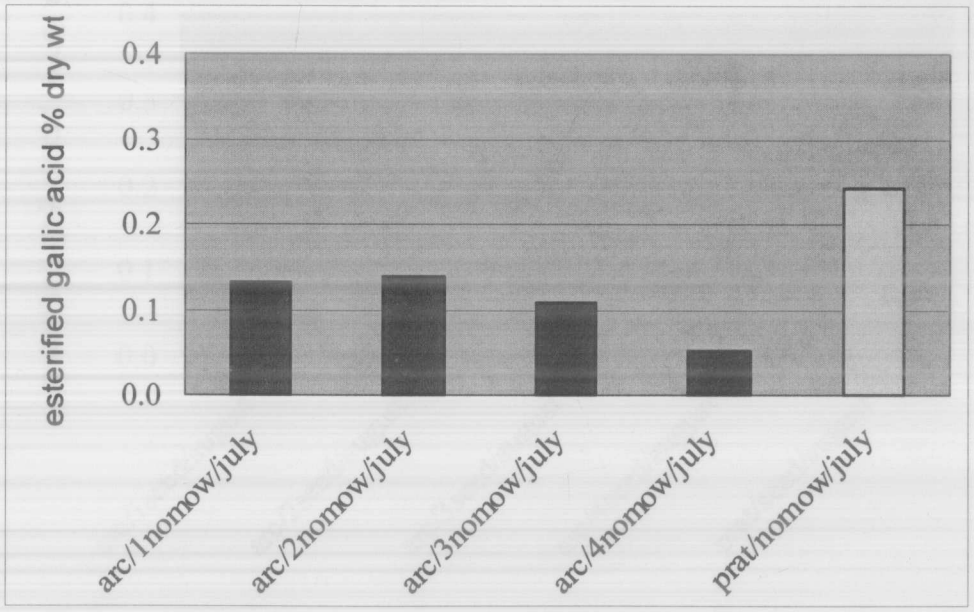


Figure 32. Rhodanine assay results for July unmown samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

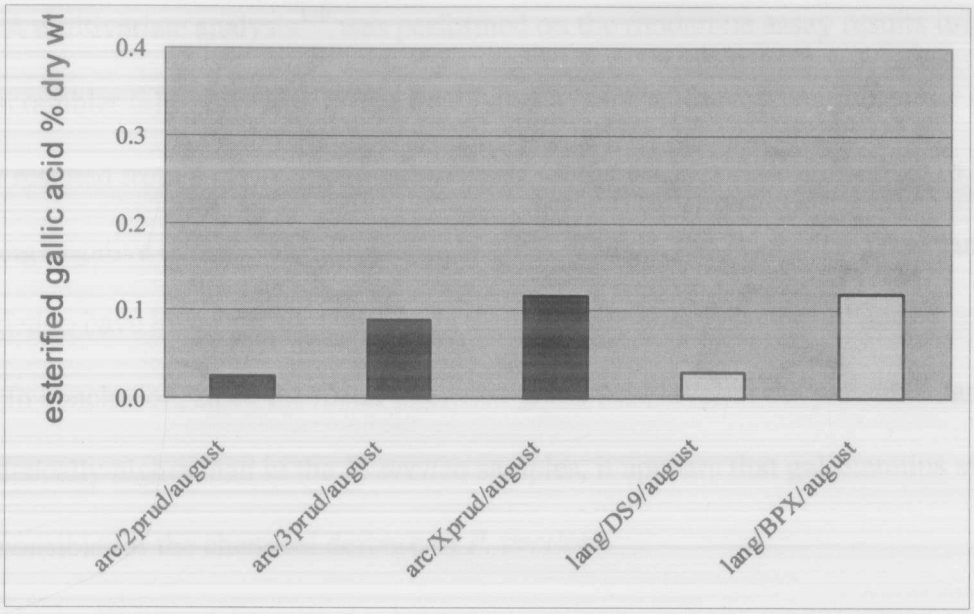


Figure 33. Rhodanine assay results for August Prudhoe samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

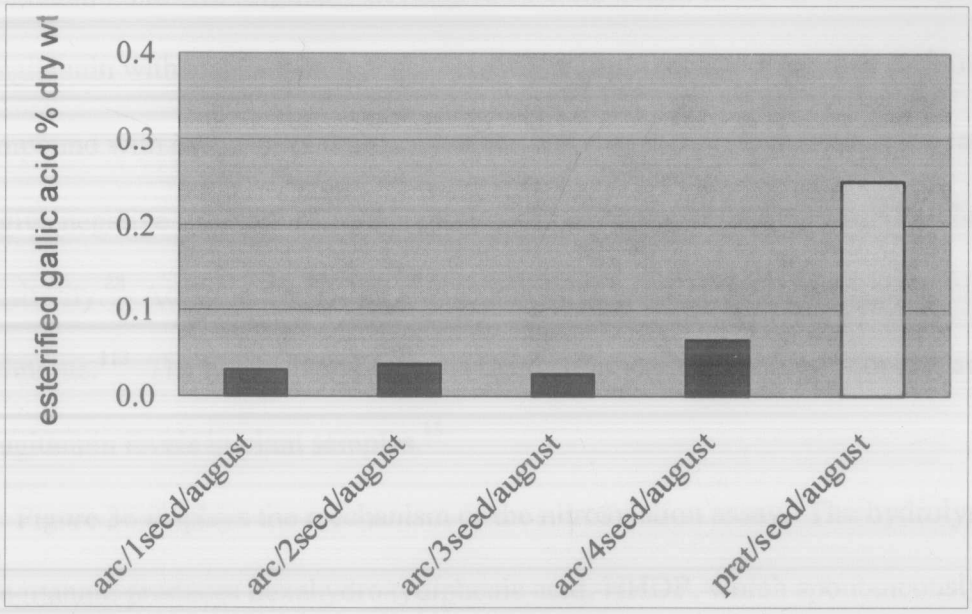


Figure 34. Rhodanine assay results for August seedling samples. ■ Indicates *P. arctica* samples. ▭ Indicates palatable samples. Plant samples are described in Table 6.

A multivariate analysis[110] was performed on the rhodanine assay results using the Unscrambler 6.11 software.[111] Figure 35 displays the x loading weights and y loadings that resulted from a pls1 regression analysis. Gallic acid levels were shown to have a strong negative correlation with unpalatability. That is, the *P. arctica* grass samples tended to have lower gallotannin levels than the palatable grass samples.

In conclusion, since the mean esterified gallic acid level in the palatable samples was statistically higher than in the *P. arctica* samples, it appears that gallotannins are not responsible for the chemical defense of *P. arctica*.

2.4.5. Nitrosylation Assay

The nitrosylation assay is an improvement of a quantitative technique to measure ellagitannin that was originally developed by Bate-Smith in 1972.[112] Bate-Smith reacted ellagitannin with an acidic solution of $NaNO_2$ in the absence of oxygen to form a blue compound with a λ_{max} of 600nm. However, the Bate-Smith technique has been reported to give negative results for some plants known to contain ellagitannin,[51] and has a low sensitivity.[88] Additionally, gallic acid is known to interfere with the Bate-Smith technique.[113] The nitrosylation assay is the recommended technique for measuring ellagitannin levels in plant samples.[15]

Figure 36 displays the mechanism of the nitrosylation assay. The hydrolysis of ellagitannin produces hexahydroxydiphenic acid, HHDP, which spontaneously lactonizes to form ellagic acid. Ellagic acid has two equivalent unsubstituted carbons that are

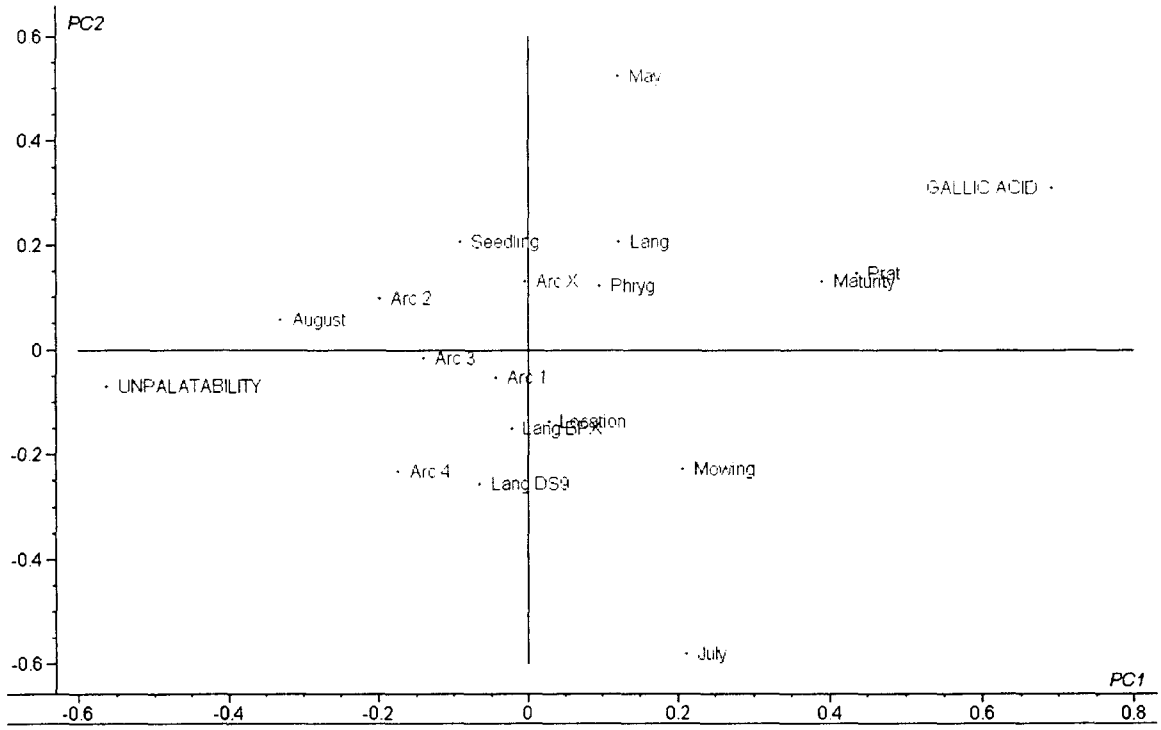


Figure 35. X loading weights and y loadings for the rhodanine assay. This plot shows the results of a multivariate analysis done on the rhodanine assay results using The Unscrambler 6.11 software. The x axis is the first principal component, and the y axis is the second principal component. Note how gallic acid and unpalatability are on opposite sides of the x axis. This indicates a strong negative correlation. The explained variance for the model is X-explained 16%, 8% and Y-explained 72%, 9%.

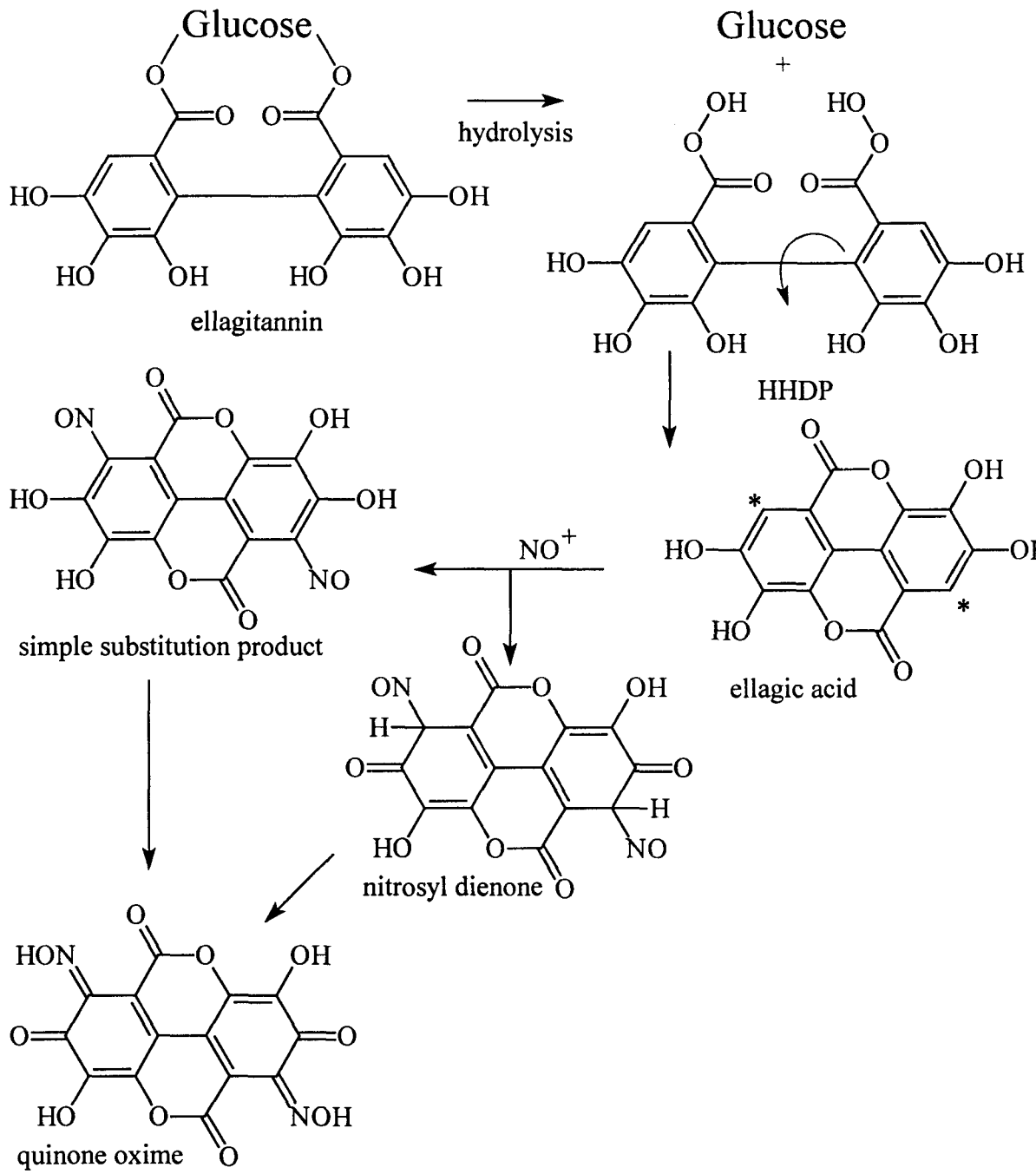


Figure 36. Mechanism of quinone oxime formation from ellagitannin in the nitrosylation assay.[88] An ellagitannin undergoes hydrolysis to yield HHDP, which spontaneously forms ellagic acid. The unsubstituted carbons of ellagic acid, indicated by asterisks, are susceptible to electrophilic attack by NO^+. The two possible products, a simple substitution product and a nitrosyl dienone, both decay to a quinone oxime.

susceptible to electrophilic attack by NO^+. This gives rise to two different possible nitrosylation products through different mechanisms. It is unclear which mechanism predominates in this reaction.

The first possible mechanism is a simple substitution of H^+ by NO^+ at the electron dense unsubstituted carbons.[88] This yields the simple substitution product, which is a nitrosyl compound. The second possible mechanism, which differs from the first mechanism only by a keto-enol tautomerization, involves electrophilic attack and loss of H^+ from the ortho phenol group to form a nitrosyl dienone. The second mechanism is similar to the mechanism proposed for the nitrosylation of other phenolics,[114] The hydrolysis and subsequent nitrosylation of ellagitannin thus yields a red compound, either the nitrosyl compound or the nitrosyl dienone, with a λ_{max} of 538nm in pyridine.

The two possible nitrosylation products are short lived, and soon decay to form a yellow colored quinone oxime. Both an initial and a final absorbance measurement are recorded at 538nm in the nitrosylation assay. The difference between the two measurements represents the amount of red colored nitrosylation product that is formed, and is thus proportional to the ellagic acid concentration.[114] Since the nitrosylation product is short-lived, control of time and temperature is important.[114]

Like the acid butanol and rhodanine assays, the nitrosylation assay enables the relative determination of tannin content among plant samples. Ellagitannin content is reported in terms of the percentage of ellagic acid by dry weight. This assay does not provide an absolute measure of the tannin content in a sample. The nitrosylation assay is

not affected by common plant phenolics such as flavanoids, gallic acid, phenylpropanoids, gallotannins, or condensed tannins.[88]

Figure 37 displays the calibration curve that was obtained when the nitrosylation assay was performed on commercially available ellagic acid. The equation for the regression line was $A_{550} = 22(\text{mg ellagic acid}) + 0.0016$. The regression had an R^2 value of 0.98. Upon examination of the calibration curve, the nitrosylation assay was determined to give a linear response up to an absorbance of 1.0. This covered the range of absorbances found in the plant samples.

Table 5 shows the results of the nitrosylation assay. Replicate assays were performed on six plant samples. A t-test (p=0.05), which included the replicates, showed that the mean ellagic acid level of the *P. arctica* samples was statistically higher than the mean of the palatable samples. *A. saccharum*, known to contain ellagitannin as detailed in Section 2.4.2., was assayed as a positive control. The obtained value of 5.4% ellagic acid by dry weight for *A. saccharum* was comparable to the reported value of 4.62%.[88] As in the other tannin assays, the amount of tannin found in *A. saccharum* was considerably greater than the amount of tannin in the grass samples.

Figures 38 to 43 show the groupwise comparisons for the May mature, May young, July mown, July unmown, August Prudhoe, and August seedling samples respectively. The mean ellagic acid levels in the *P. arctica* grass samples were statistically higher (t-test with p=0.05) than the palatable grass samples for all of the comparisons.

A multivariate analysis was performed on the nitrosylation assay results using the Unscrambler 6.11 software. Figure 44 displays the x loading weights and y loadings

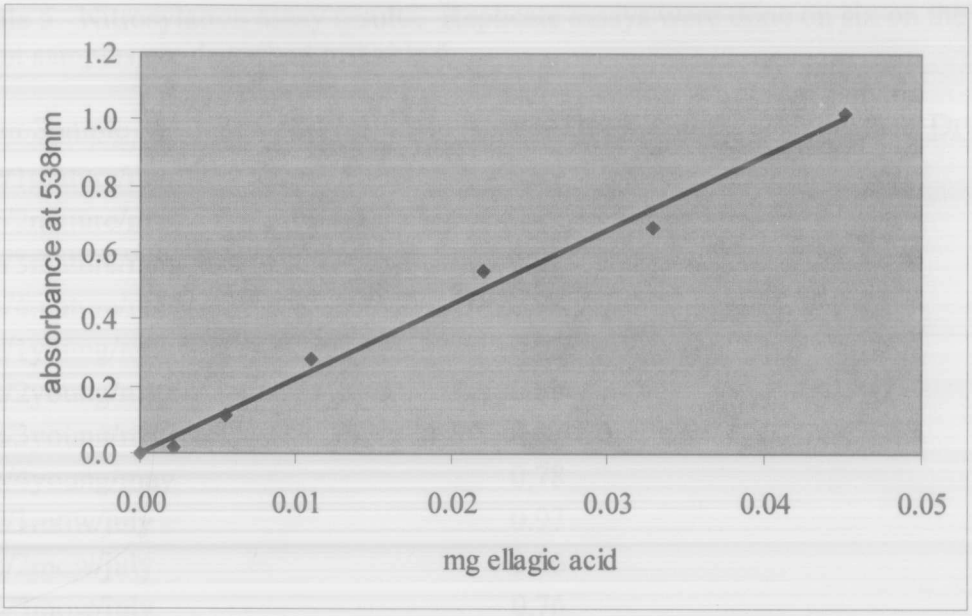


Figure 37. Ellagic acid calibration curve.

Table 5. Nitrosylation assay results. Replicate assays were done on six on the samples. Plant samples are described in Table 6.

Plant Sample	Ellagic Acid % Dry Weight	Replicate % Dry Weight
arc/1mature/may	0.99	1.0
arc/2mature/may	0.72	
arc/3mature/may	0.92	
arc/4mature/may	0.98	
arc/1young/may	0.83	
arc/2young/may	0.80	
arc/3young/may	0.87	
arc/4young/may	0.78	
arc/1mow/july	0.97	
arc/2mow/july	0.66	
arc/3mow/july	0.76	
arc/4mow/july	0.73	
arc/1nomow/july	0.97	
arc/2nomow/july	0.64	
arc/3nomow/july	0.58	
arc/4nomow/july	0.73	0.69
arc/2prud/august	0.78	0.77
arc/3prud/august	0.61	
arc/Xprud/august	0.59	
arc/1seed/august	0.99	
arc/2seed/august	0.99	
arc/3seed/august	0.97	
arc/4seed/august	0.94	
lang/septem	0.42	
lang/DS9/august	0.33	
lang/BPX/august	0.47	0.48
phryg	0.36	
prat/mow/july/	0.34	0.32
prat/nomow/july	0.34	
prat/seed/august	0.50	0.51
Acer saccharum	5.4	

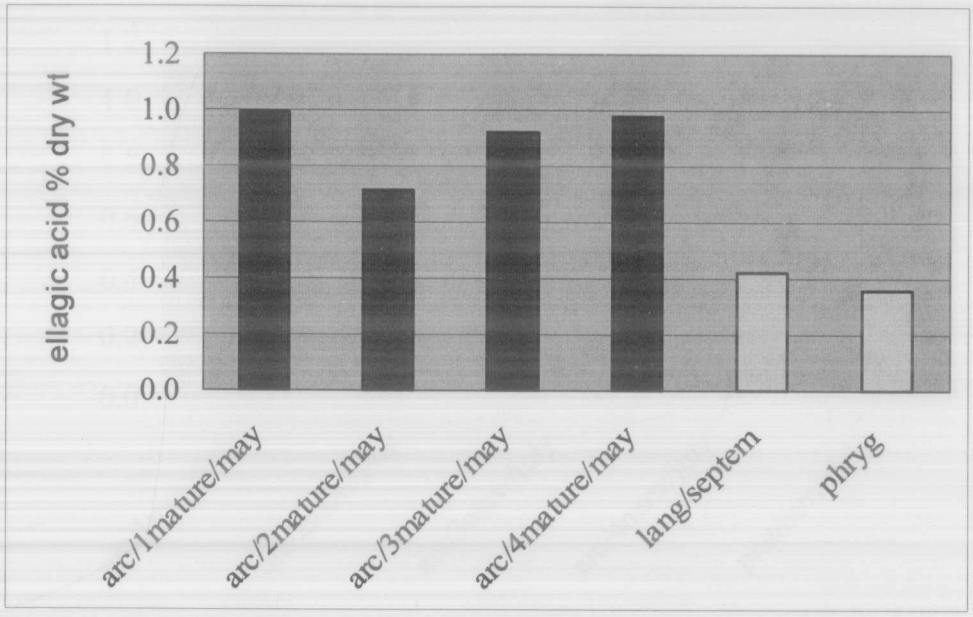


Figure 38. Nitrosylation assay results for May mature samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

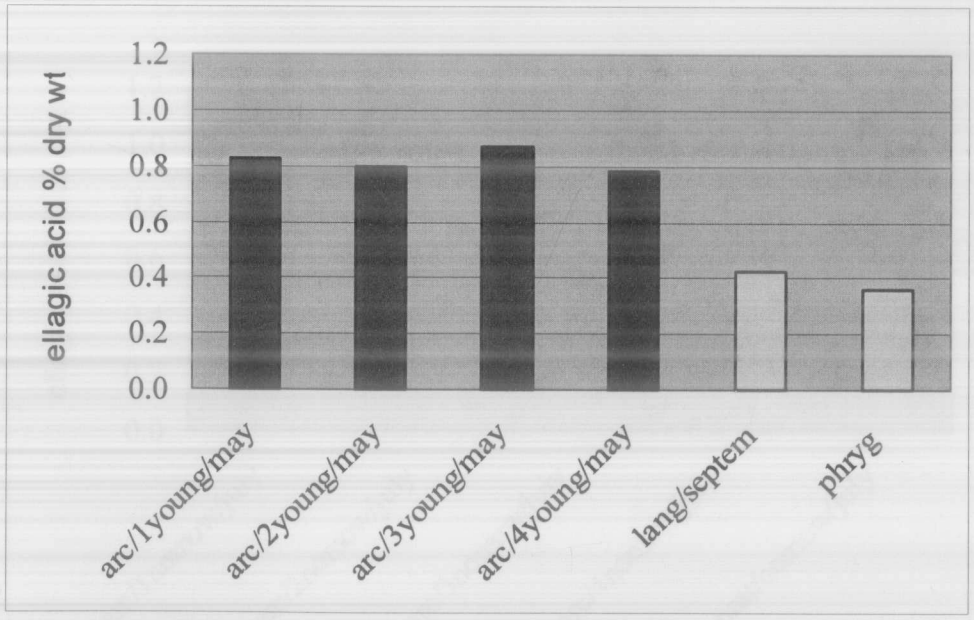


Figure 39. Nitrosylation assay results for May young samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

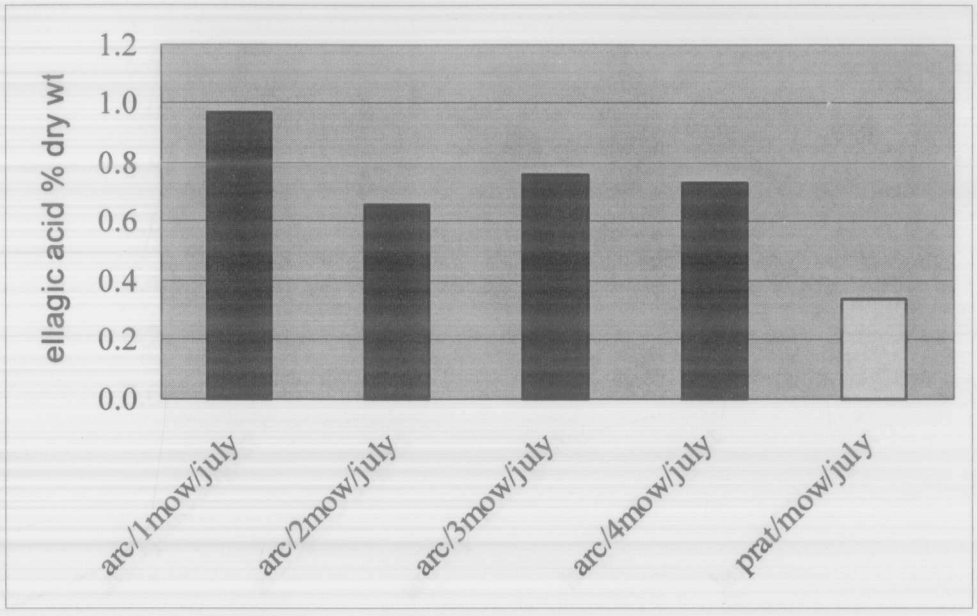


Figure 40. Nitrosylation assay results for July mown samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

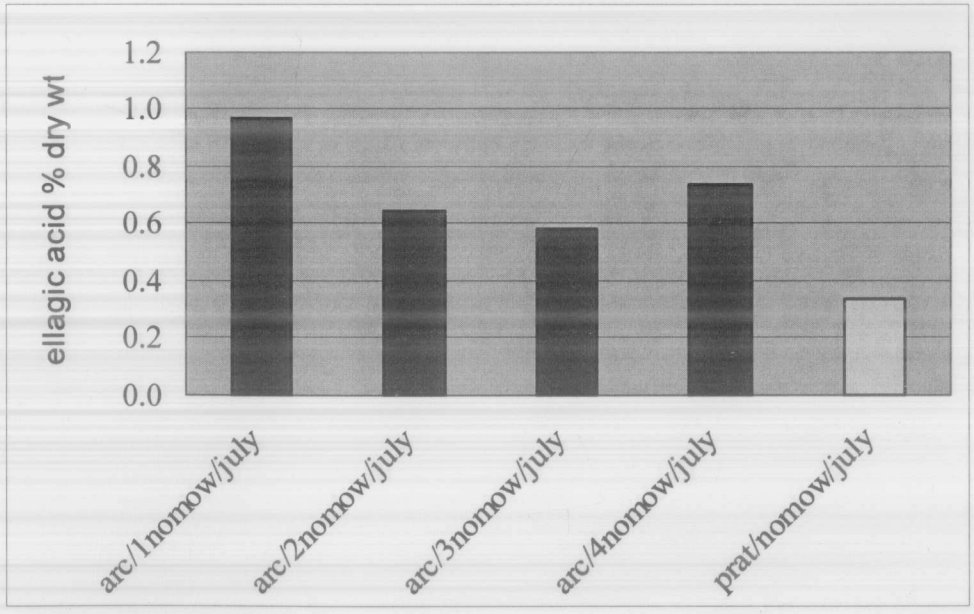


Figure 41. Nitrosylation assay results for July unmown samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

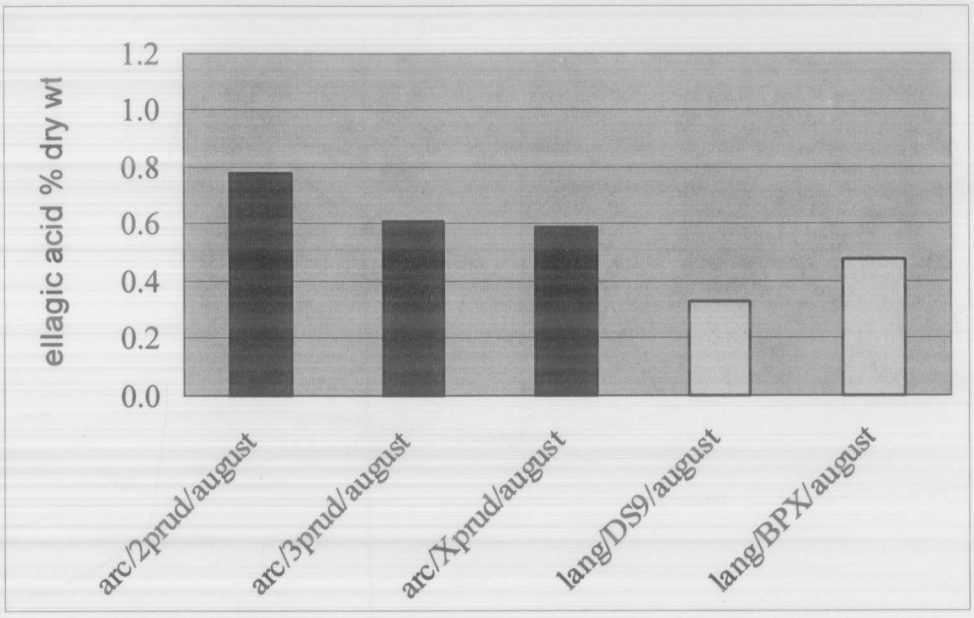


Figure 42. Nitrosylation assay results for August Prudhoe samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

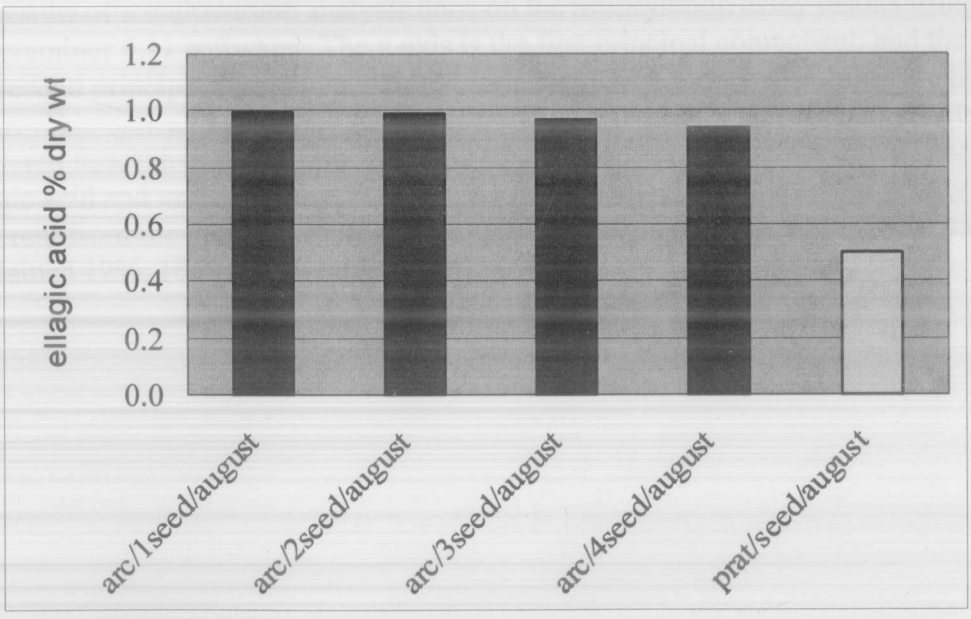


Figure 43. Nitrosylation assay results for August seedling samples. ■ Indicates *P. arctica* samples. □ Indicates palatable samples. Plant samples are described in Table 6.

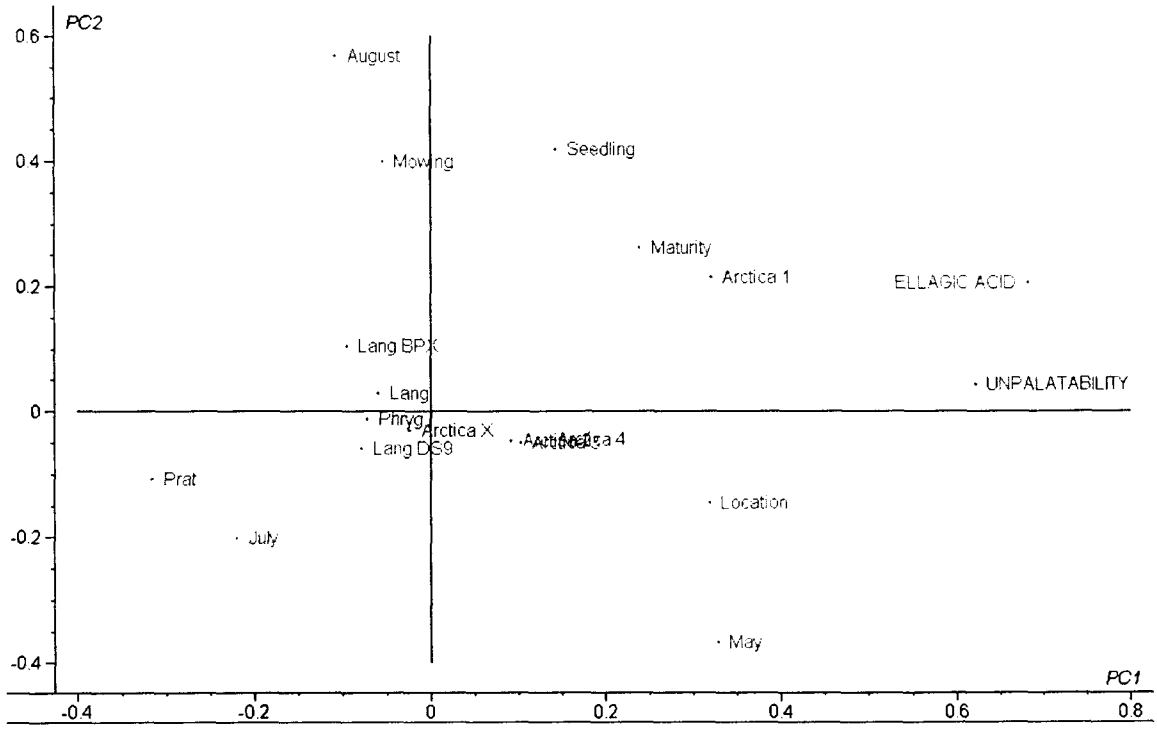


Figure 44. X loading weights and y loadings for the nitrosylation assay. This plot shows the results of a multivariate analysis done on the nitrosylation assay results using The Unscrambler 6.11 software. The x axis is the first principal component, and the y axis is the second principal component. Note how ellagic acid and unpalatability are near each other. This indicates a strong positive correlation. Most of the *P. arctica* genotypes lie clustered near the origin, while Arctica 1 (*P. arctica* genotype 1) lies much nearer to ellagic acid and unpalatability. This shows that the ellagic acid levels in genotype 1 are different from the other *P. arctica* genotypes. The explained variance for the model is X-explained 12%, 16% and Y-explained 82%, 7%.

that resulted from a PLS1 regression analysis. Ellagic acid levels were shown to have a strong correlation with unpalatability. That is, the *P. arctica* grass samples tended to have higher ellagitannin levels than the palatable grass samples. As was seen in Section 2.4.4. this was the opposite of the trend for gallotannins, and makes sense from a biochemical standpoint. Ellagitannin and gallotannin are both derived from the same intermediate, β-1,2,3,4,6-pentagalloyl-D-glucose.[115] It seems that the *P. arctica* grasses are shunting resources to produce ellagitannin at the expense of gallotannin. The *P. pratensis*, *P. langeana*, and *P. phryganodes* grasses in turn are favoring production of gallotannin over ellagitannin.

The multivariate analysis also yielded another interesting result. The ellagic acid levels in *P. arctica* genotypes 2,3,4, and X were all comparable. However, the ellagic acid levels in *P. arctica* genotype 1 were higher than all of the other genotypes. Not counting the August Prudhoe samples, genotype 1 had the highest mean ellagic acid level with a mean of 0.95% dry weight. Genotypes 3 and 4 were very similar with mean ellagic acid levels of 0.82% and 0.83% dry weight respectively. Genotype 2 had the lowest mean ellagic acid level at 0.77% dry weight.

Since the mean ellagic acid level of the *P. arctica* samples was statistically higher than the mean of the palatable samples, it appears that ellagitannins may play a role in the chemical defense of *P. arctica*. Additionally, *P. arctica* genotype 1 has higher ellagitannin levels than the other genotypes. However, it should be noted that the presence of these moderate levels of tannin does not necessarily protect *P. arctica* from herbivory.[64]

2.5. Conclusions

The secondary metabolite profiles of four grasses were compared against one another to look for possible defensive compounds present in *P. arctica* but not in the other species. There were no volatile or non-volatile compounds that were present in *P. arctica* but not in the palatable species. No alkaloids were detected in any of the plant samples. It is also possible, though unlikely, that a minor but highly active compound which was not detected by this analysis could be defending *P. arctica.*

The results from the Bate-Smith and acid butanol condensed tannin assays correlated with one another. The samples that tested positive for condensed tannin in the Bate-Smith assay contained the highest amounts of condensed tannin as measured by the acid butanol assay.

In the acid butanol assay, there was no statistical difference between the mean absorbance values of the *P. arctica* and palatable grass samples. Because of this, it is concluded that condensed tannins are not responsible for chemical defense in *P. arctica.* There was some evidence that seedling and mown *P. arctica* samples may have had elevated condensed tannin levels. However, more work needs to be done to determine if this is indeed so and if the effect is ecologically significant.

The mean esterified gallic acid level in the palatable samples was statistically higher than in the *P. arctica* samples. It thus appears that gallotannins are not responsible for the chemical defense of *P. arctica.* The levels of gallotannin and ellagitannin, derived from the same metabolic precursor, had a strong negative correlation. The *P. arctica* grasses seemed to be shunting resources to produce ellagitannin at the expense of

gallotannin. The *P. pratensis*, *P. langeana*, and *P. phryganodes* grasses in turn are favoring production of gallotannin over ellagitannin.

Since the mean ellagic acid level of the *P. arctica* samples was statistically higher than the mean level of the palatable samples, it appears that ellagitannins may play a role in the chemical defense of *P. arctica*. *P. arctica* genotype 1 had higher ellagitannin levels than the other genotypes. However, the presence of these moderate levels of tannins does not necessarily protect *P. arctica* from herbivory.[64] Additionally, differences in plant tannin levels may be due to differences in tannin extractability as well as to differences in actual tannin levels.[15]

Herbivores selectively browse plants based upon the plants chemical and mechanical defenses, in addition to its nutritional value.[13] Thus, *P. arctica* may be unpalatable to geese due to a single factor or to several factors acting in conjunction. *P. arctica* may be tough and thus difficult for the geese to eat. The grass may be of low nutritional value to the geese, and they may be foraging on more nutritional species. It is known that geese selectively browse plants that are high in both total protein content and in specific amino acids.[116]

The nutritional value of the four grasses, including both total protein and specific amino acid content, should thus be investigated. Additionally, the tannin assays should be performed on grass samples from next season. This would provide further evidence to support the conclusion that ellagitannins are responsible for chemical defense in *P. arctica*. For conclusive proof, the tannins would need to be isolated and used in feeding trials.

These results suggest that *P. arctica* is, barring the existence of some trace and highly toxic substance, appropriate to plant in areas such as playing fields, lawns, and playgrounds.

Chapter 3. Experimental Section

3.1. Plant Material

Peter Scorup of Northern Native Seeds provided the *P. arctica*, *P. phryganodes*, *P. langeana*, and *P. pratensis* plant samples. Only the portion of the plants growing above ground was collected for analysis. Samples were placed in coolers and chilled with freezer packs for transport to the laboratory. Once in the laboratory, plant material was stored in a freezer at -20°C.

Table 6 summarizes the plant sample designators used throughout this thesis and their associated collection information. Mature and young samples of *P. arctica* genotypes 1-4 were collected from the Northern Native Seeds nursery in Palmer, AK in May of 1999. The mature samples were from seedrows that were originally planted from seed collected on Alaska's North Slope some five years previous. The young samples were plants that later developed in the nursery from seed dispersed between the seed rows.

Mown and unmown samples of *P. arctica* genotypes 1-4 were collected from Palmer in July of 1999. Samples of *P. arctica* genotypes 2, 3, and X were collected from Prudhoe Bay in August of 1999. Samples of *P. arctica* genotypes 1-4 seedlings were collected from Anchorage in August of 1999. This seedling analysis was done after Northern Native Seeds suggested that *P. arctica* may be less palatable in the seedling stage and then become more palatable when the grass is older.

The *P. langeana*, *P. phryganodes*, and *P. pratensis* samples used as palatable controls for this study were collected at approximately the same times and from the same locations as the *P. arctica* samples. Three *P. langeana* samples that were collected from

Table 6. Summary of plant samples and collection information.

Plant Sample	Species	Date Collected	Collection Location	Comments
arc/1mature/may	*P. arctica*	5/27/99	Palmer	mature genotype 1
arc/2mature/may	*P. arctica*	5/27/99	Palmer	mature genotype 2
arc/3mature/may	*P. arctica*	5/27/99	Palmer	mature genotype 3
arc/4mature/may	*P. arctica*	5/27/99	Palmer	mature genotype 4
arc/1young/may	*P. arctica*	5/27/99	Palmer	young genotype 1
arc/2young/may	*P. arctica*	5/27/99	Palmer	young genotype 2
arc/3young/may	*P. arctica*	5/27/99	Palmer	young genotype 3
arc/4young/may	*P. arctica*	5/27/99	Palmer	young genotype 4
arc/1mow/july	*P. arctica*	7/12/99	Palmer	mown genotype 1
arc/2mow/july	*P. arctica*	7/12/99	Palmer	mown genotype 2
arc/3mow/july	*P. arctica*	7/12/99	Palmer	mown genotype 3
arc/4mow/july	*P. arctica*	7/12/99	Palmer	mown genotype 4
arc/1nomow/july	*P. arctica*	7/12/99	Palmer	unmown genotype 1
arc/2nomow/july	*P. arctica*	7/12/99	Palmer	unmown genotype 2
arc/3nomow/july	*P. arctica*	7/12/99	Palmer	unmown genotype 3
arc/4nomow/july	*P. arctica*	7/12/99	Palmer	unmown genotype 4
arc/2prud/august	*P. arctica*	8/6/99	Prudhoe Bay	genotype 2
arc/3prud/august	*P. arctica*	8/5/99	Prudhoe Bay	genotype 3
arc/Xprud/august	*P. arctica*	8/5/99	Prudhoe Bay	genotype X
arc/1seed/august	*P. arctica*	8/19/99	Anchorage	genotype 1 seedlings
arc/2seed/august	*P. arctica*	8/19/99	Anchorage	genotype 2 seedlings
arc/3seed/august	*P. arctica*	8/19/99	Anchorage	genotype 3 seedlings
arc/4seed/august	*P. arctica*	8/19/99	Anchorage	genotype 4 seedlings
lang/septem	*P. langeana*	9/17/98	Prudhoe Bay	D.S. #9 plants
lang/DS9/august	*P. langeana*	8/5/99	Prudhoe Bay	D.S. #9 plants
lang/BPX//august	*P. langeana*	8/6/99	Prudhoe Bay	BPX plants
phryg	*P. phryganodes*	9/19/98	Prudhoe Bay	
prat/mow/july	*P. pratensis*	7/12/99	Palmer	mown
prat/nomow/july	*P. pratensis*	7/12/99	Palmer	unmown
prat/seed/august	*P. pratensis*	8/19/99	Anchorage	seedlings

Prudhoe Bay were analyzed. One sample, a D.S. #9 genotype, was collected in September of 1998. The other twc, D.S. #9 and BPX genotypes, were collected in August of 1999. The *P. phryganodes* sample was collected from Prudhoe Bay in September of 1998. The mown and unmown *P. pratensis* samples were collected from Palmer in July of 1999. The *P. pratensis* seedlings were collected from Anchorage in August of 1999.

3.2. Chemicals

All of the chemicals used were reagent grade. Acetone, MeOH, EtOH, CH_2Cl_2, and $CHCl_3$ were purchased from EM Science. The H_2SO_4 and HCl were obtained from Allied Chemical. The (+) catechin, rhodanine, and I_2 were purchased from Sigma. Water was purified by reverse osmosis. The bismuth subnitrate, ellagic acid, gallic acid, KOH, and ammonium iron (III) sulfate dodecahydrate were purchased from Aldrich. The KI, platinum chloride solution, HOAc, and isoamyl alcohol came from Fisher Scientific. The pyridine, $NaNO_2$, and n-BuOH were from the J. T. Baker Chemical Co. Ultra high purity He was provided by Airgas. Querbracho tannin came from Pilar River Plate Corporation.

3.3. General Extraction Procedure

For general extraction, 1.8g portions of plant material were placed in 20ml screw capped disposable scintillation vials along with 20ml of solvent. Plant material was extracted with MeOH to remove polar compounds. Separate portions of plant material

were extracted with CH_2Cl_2 to remove nonpolar compounds. Extractions were done at room temperature and carried out for a period of 30hrs. The extracts were gravity filtered and stored in 20ml screw capped disposable scintillation vials in a freezer at -20°C. These MeOH and CH_2Cl_2 extracts were used for the comparative GC/MS, comparative TLC, and alkaloid screening analyses.

3.4. Comparative GC/MS Analysis

GC/MS analysis of the *P. arctica*, *P. langeana*, *P. phryganodes*, and *P. pratensis* extracts was done using a Hewlett Packard 5890 Series II Plus gas chromatograph connected to a Hewlett Packard 5972 Series Mass Selective Detector. An Alltech EC-5 capillary column measuring 0.25mm internal diameter x 30m was used. The column was coated with a 0.25μm thick film of crosslinked 5% Ph Me silicone.

The inlet temperature was 275°C. The detector temperature was 280°C. The carrier gas was ultra high purity He. Initial oven temperature was 40°C for 4min. Oven temperature was programmed to then rise at 10°C/min to 280°C over the course of 24min. This final oven temperature was held for 5min. The total run time was thus 33min.

A solvent delay of 4.5min before detector activation was employed. Compounds were identified using a probability-based matching library search[117,118] of the G1035A Wiley PBM Library running on Hewlett Packard G1034 MS Chemstation software. The MeOH and CH_2Cl_2 extracts were diluted $x10^{-3}$ and $x10^{-2}$, respectively, prior to injection on the GC/MS. MeOH and CH_2Cl_2 solvent blanks were also run.

3.5. Comparative Thin-Layer Chromatography

Alltech precoated TLC plates with florescent indicator were used for thin-layer chromatography. The plates had an aluminum backing and a 0.2mm thick layer of silica gel 60 F_{254}. The *P. arctica*, *P. langeana*, *P. phryganodes*, and *P. pratensis* extracts were spotted on TLC plates and eluted with either $CHCl_3$, 5% (v/v) MeOH in $CHCl_3$, 10% (v/v) MeOH in $CHCl_3$, or 40% (v/v) MeOH in $CHCl_3$. The MeOH extracts were spotted on separate plates from the CH_2Cl_2 extracts. An appropriate extract of the *P. arctica* mature genotype 3 plant sample from May was spotted on all of the MeOH and CH_2Cl_2 extract TLC plates to serve as a standard. The TLC plates were eluted under normal saturation (NS) conditions.[45]

The TLC plates were allowed to air dry for 10min and then visualized using short wave UV light and sulfuric acid spray reagent. A Spectroline model EF-140C was used to provide UV radiation with a wavelength of 254nm.

The sulfuric acid spray reagent was 5% (v/v) H_2SO_4 in EtOH.[45] The TLC plates were sprayed with the reagent and allowed to air dry for 10min. The TLC plates were then heated on a hot plate at 80°C for 7min. Since the spots faded over time, the TLC plate images were stored on computer by scanning them with a Hewlett Packard ScanJet 6200C color scanner.

3.6. Alkaloid Screening

Alkaloid screening involved spotting the *P. arctica*, *P. pratensis*, *P. langeana*, and *P. phryganodes* extracts on TLC plates and eluting the plates as in Section 3.5. The MeOH

extracts were spotted on separate plates from the CH_2Cl_2 extracts. After elution, the plates were allowed to air dry for 10min and visualized with UV light and an alkaloid spray reagent. The alkaloid spray reagents used were Dragendorff's reagent, iodoplatinate, and iodine-potassium iodide. Since the spots faded over time, the TLC plate images were stored on computer by scanning them with a Hewlett Packard ScanJet 6200C color scanner.

Commercially available long cut tobacco was extracted and used as a positive control, since tobacco is known to contain several alkaloids.[32] Two separate extractions were done using the procedure in Section 3.3., one with MeOH and one with CH_2Cl_2, to verify that the procedure would extract alkaloids from the plant material. The MeOH tobacco extract was spotted on the plates with the MeOH plant extracts and the CH_2Cl_2 tobacco extract was spotted on plates along with the CH_2Cl_2 plant extracts. The tobacco extracts contained alkaloids and yielded positive reactions with all of the alkaloid spray reagents.

(+) Catechin was spotted on the TLC plates and used as a negative control. It does not contain nitrogen and did not yield a positive reaction with the alkaloid spray reagents.

Dragendorff's reagent[36] was prepared by mixing 0.15g bismuth subnitrate in 0.5ml conc. HCl and 2.5ml H_2O. A second solution consisting of 1.5g KI in 2.5ml H_2O was also prepared. The two solutions were mixed together and combined with 1.8ml conc. HCl and 3.8ml H_2O. The resulting mixture was then diluted with 100ml H_2O.

The iodoplatinate spray reagent[36] was prepared by mixing 2ml of a 10% (w/v) aqueous platinum chloride solution, 2ml H_2O, and 2ml conc. HCl. The resulting mixture was then added to a solution of 1.9g KI in 94ml H_2O.

The iodine-potassium iodide spray reagent[32] was prepared by first dissolving 10g KI in 50ml H_2O. A 1g quantity of I_2 was then added to this solution. A 2ml volume of glacial HOAc was added and the resulting mixture was diluted to 100ml with H_2O.

3.7. Tannin Assays

3.7.1. Bate-Smith Assay

To assay for condensed tannin, a 0.40g portion of lypholized plant material was placed in a centrifuge tube along with 3ml of 2M HCl.[119,120] The tube was loosely capped and heated in a 100°C water bath for 20min. The tube was then removed from the water bath and allowed to cool. Upon cooling, 0.5ml of isoamyl alcohol was added to the centrifuge tube and the tube was agitated. A faint pink to deep crimson color in the upper isoamyl alcohol layer indicated the presence of condensed tannins.

3.7.2. Acid Butanol Assay

To extract condensed tannins, a 0.20g portion of lypholized plant material was extracted with 3ml of 50% (v/v) MeOH in H_2O for 1hr at room temperature.[46] The extraction was repeated twice, and the aqueous MeOH extracts were then combined.

For analysis, a 1ml portion of the aqueous MeOH extract was placed in a 20ml screw capped disposable scintillation vial along with 6ml of 5% (v/v) conc. HCl in n-BuOH and 0.2ml of 2% (w/v) ammonium iron (III) sulfate dodecahydrate in 2M HCl.[85] The vial was then capped and placed in an oven at 95°C for 40min.

Upon cooling, a 3ml aliquot of the solution was placed in a quartz cuvette with a path length of 1cm. The absorbance was then measured at 550nm using a Hewlett Packard 8452A UV-Visible diode array spectrophotometer with a spectral range of 190-820nm, and a sampling interval of 2nm. The instrument was blanked with an aliquot of a solution containing 4ml 50% (v/v) MeOH in H_2O, 24ml of 5% (v/v) conc. HCl in n-BuOH, and 0.8ml of 2% (w/v) ammonium iron (III) sulfate dodecahydrate in 2M HCl.

To confirm that the assay gave linear results, commercially available quebracho tannin was analyzed. The quebracho tannin standard solution was 0.21mg/ml quebracho tannin in 50% (v/v) MeOH in H_2O. Appropriate aliquots of the quebracho tannin standard solution were made up to 1ml with 50% (v/v) MeOH in H_2O and assayed as above.

3.7.3. Rhodanine Assay

To extract and hydrolyze gallotannins, a 0.20g portion of lypholized plant material was extracted with 3ml of 70% (v/v) acetone in water for 30min at room temperature.[106] The extract was then vacuum filtered and placed in a glass ampule. A 5ml volume of 1M H_2SO_4 was added to the ampule. The ampule was then frozen in liquid N_2, vacuum sealed, and heated in an oven for 26hr at 100°C. After cooling, the ampule was opened and the hydrosylate was diluted to 50ml with H_2O.

To assay for gallic acid, a 1ml volume of the diluted hydrosylate was mixed with 1.5ml of 0.667% (w/v) rhodanine in MeOH. After 5min, 1ml of 0.5M KOH was added. After an additional 2.5min, the solution was diluted to 25ml with H_2O. Five minutes

later, a 3ml aliquot of the diluted solution was placed in a quartz cuvette with a path length of 1cm. The absorbance was then measured at 520nm using a diode array spectrophotometer as in Section 3.7.2. The instrument was blanked with a H_2O.

To measure gallotannin levels with this assay, two separate gallic acid assays must be done on each sample. One portion of the plant sample is assayed as above and provides a measure of total gallic acid, both free and esterified, in the plant. A second portion of the plant sample is extracted, diluted to 50ml with H_2O, and then assayed without hydrolysis to measure the amount of free gallic acid in the plant. The free gallic acid is then subtracted from the total gallic acid to determine the amount of esterified gallic acid in the plant.

Commercially available gallic acid was analyzed to construct a calibration curve. The gallic acid standard solution was 0.20mg/ml gallic acid in 0.1M H_2SO_4. Appropriate aliquots of the gallic acid standard solution were made up to 1ml with 0.1M H_2SO_4 and assayed as above.

3.7.4. Nitrosylation Assay

To extract and hydrolyze ellagitannins, a 0.10g portion of lypholized plant material was placed in a glass ampule with 8ml of 1M H_2SO_4.[88] The ampule was then frozen in liquid N_2, vacuum sealed, and heated in an oven for 24hr at 100°C. After cooling to room temperature, the ampule was opened and placed in an ice bath for 10min. This caused the ellagic acid, which has rather limited solubility,[121] to precipitate out of solution.

The hydrosylate was then vacuum filtered, and the solid ellagic acid was washed with ice-cold 70/30/1 (v/v/v) acetone/H_2O/conc. HCl and air dried. The ellagic acid was then dissolved in 10ml of pyridine.

To assay for ellagic acid, 4.2ml of the dissolved ellagic acid was mixed with 0.2 ml of conc. HCl and warmed to 30°C. A 0.2 ml volume of 1% (w/v) aqueous $NaNO_2$ was added. The absorbance at 538nm was then measured on a diode array spectrophotometer as in Section 3.7.2. The instrument was blanked with pyridine.

After the absorbance measurement, the solution was heated at 30°C for 36min in an oven and the absorbance at 538nm was again recorded. The difference between the initial and final absorbance measurements was proportional to the ellagic acid concentration.

Commercially available ellagic acid was analyzed to construct a calibration curve. The ellagic acid standard solution was 0.01mg/ml ellagic acid in pyridine. Appropriate aliquots of the ellagic acid standard solution were made up to 4.2ml with pyridine and assayed as above.

Appendix: Synthesis of Cyanidin Chloride

Cyanidin chloride, for use as a standard in the acid butanol assay, may be synthesized by the reductive acetylation of quercetin.[122] A 1.0g quantity of quercetin was refluxed at 90°C in 20 ml of acetic anhydride along with 1.0g of 30 mesh zinc powder and 0.5g of anhydrous sodium acetate. After 1hr, an additional 1.0g of 30 mesh zinc powder was added and the solution was refluxed for a further 1hr.

The solution was then cooled to room temperature and vacuum filtered. The filter was washed with a small volume of glacial HOAc, which was added to the filtrate. The filtrate was then mixed into 250ml of H_2O and an orange solid precipitated out of the solution. This orange solid was removed from the liquid by vacuum filtration, redissolved in a small volume of glacial HOAc, and again mixed with 250ml of H_2O. The orange precipitate again formed, and was removed from the liquid by vacuum filtration.

The orange precipitate was then dissolved in 40ml of 17% (v/v) isopropyl alcohol in 3M HCl and refluxed for 40min at 100°C. The reaction mixture was then cooled to room temperature and taken to dryness under vacuum to yield a red solid. This red solid was then dissolved in 30ml of 1% (v/v) HCl in H_2O. The acidic solution containing the red solid was washed three times with 30ml volumes of ethyl acetate. The ethyl acetate washings were discarded, and the acid solution was then extracted five times with 30ml volumes of 1-butanol. The 1-butanol extracts were combined and rotaryevaporated to yield 0.83g of a red solid, cyanidin chloride.

For NMR analysis, 40mg the synthesized cyanidin chloride was dissolved in 0.8ml of DMSO-d_6 along with 500µl of 37% (w/w) deuterium chloride in D_2O. A ^1H NMR spectra was taken using a Varian 300MHz multinuclear NMR spectrometer. Figure 45 displays the spectra that was obtained. Table 7 compares the obtained chemical shift values with those reported in the literature for cyanidin-3-coumaroylglycoside. The obtained values for carbons 6, 8, 2', 3', and 6' are within 0.1ppm of the reported values. The obtained value for carbon 4 is shifted 0.27ppm from the reported value. However, this is expected since the reported value is for cyanidin-3-coumaroylglycoside. This cyanidin derivative has a coumaroylglycoside moiety at carbon 3 instead of a hydroxyl group as in the red cyanidin chloride product. The presence of additional peaks in the spectra indicates that the synthesized cyanidin chloride is not pure.

For UV-Visible analysis, a small quantity of the synthesized cyanidin chloride was dissolved in 0.01% (v/v) HCl in MeOH. The absorbance spectrum was then taken on a diode array spectrophotometer as in Section 3.7.2. The instrument was blanked with 0.01% (v/v) HCl in MeOH. The obtained spectrum is displayed in Figure 46. The notable features are a major peak around 275nm and another large peak at 535nm. The reported spectral features for cyanidin chloride are also peaks at 275nm[123] and 535.[124] However, the literature reports that the peak at 535nm is the major peak and that the peak at 275nm is a minor one with only 55% of the intensity of the major peak.[123] The greater relative intensity of the peak at 275nm indicates that the cyanidin chloride is not pure.

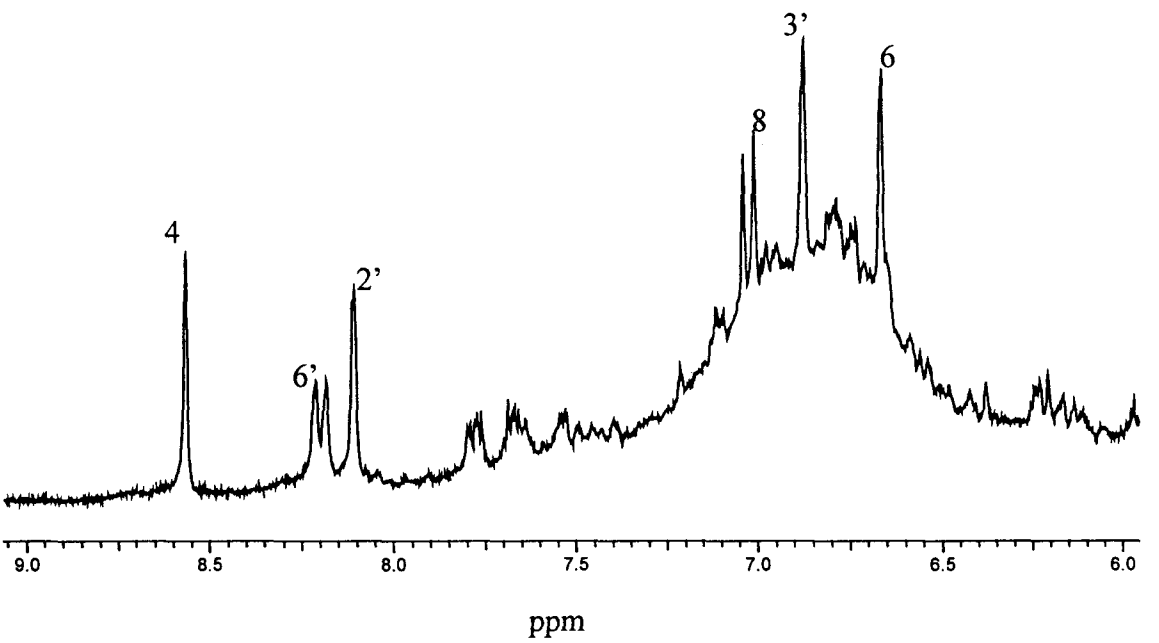


Figure 45. ^{1}H NMR spectra of cyanidin chloride product. The chemical shift is in ppm from TMS, and the solvent is DMSO-d_6 along with deuterium chloride in D_2O. The carbon atoms assigned to the peaks are shown. See Figure 14 for a description of position numbering in flavanoids.

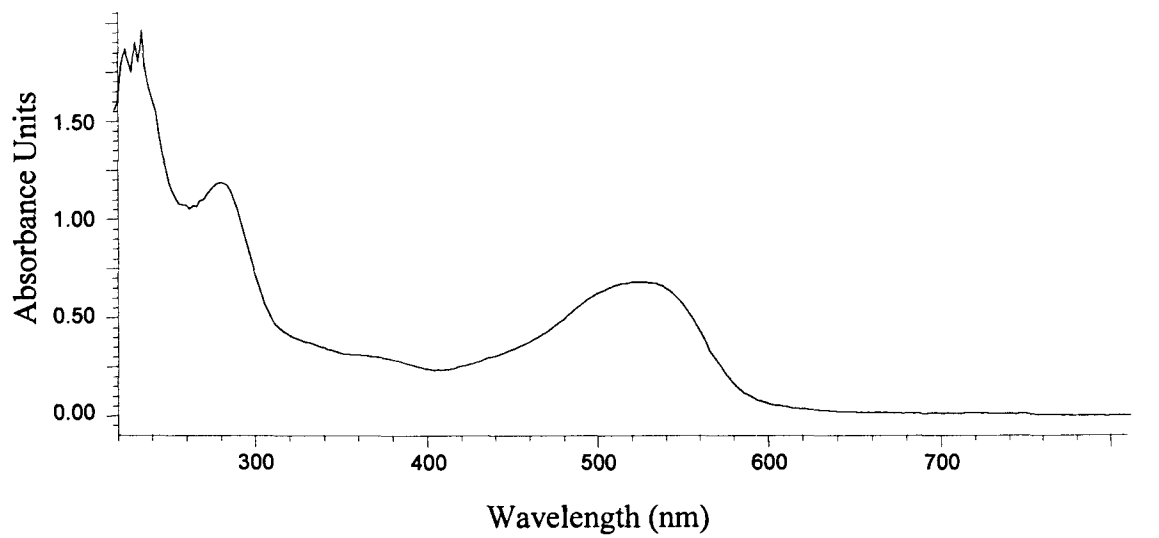


Figure 46. UV-Visible spectra of cyanidin chloride product. The solvent was 0.01% HCl in MeOH.

Table 7. Obtained and reported ^{1}H NMR chemical shifts for cyanidin chloride. The obtained values are for the cyanidin chloride product taken on a 300MHz instrument. Reported values are for cyanidin-3-coumaroylglycoside taken on a 100MHz instrument.[125] See Figure 14 for a description of position numbering in flavanoids.

Carbon Atom	Obtained Chemical Shift in ppm	Reported Chemical Shift in ppm
4	8.57	8.84-8.92
6	6.67	6.72-6.78
8	7.00	6.83-6.90
2'	8.11	8.00-8.02
3'	6.88	6.80-6.82
6'	8.21	8.20

On the basis of the NMR and UV-Visible spectral data, it was concluded that the red solid was impure cyanidin chloride. Further attempts to purify the compound for use as a standard in the acid butanol assay were unsuccessful.

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