INVESTIGATION OF COMMON MILKWEED AS A NOVEL FOOD CROP FOR NEW ENGLAND

Colter Allen Flynn
University of New Hampshire

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INVESTIGATION OF COMMON MILKWEED AS A NOVEL FOOD CROP
FOR NEW ENGLAND

By

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Bachelor of Arts, Case Western Reserve University, 2018

THESIS

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in
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<td>ATPα</td>
<td>Animal Ion Pump Na⁺/K⁺-ATPase</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance Unit</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross Domestic Product</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance Liquid Chromatography</td>
</tr>
<tr>
<td>NUCS</td>
<td>Neglected and Underutilized Crop Species</td>
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<tr>
<td>TLC</td>
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ABSTRACT

Neglected and underutilized crop species (NUCS) represent a reservoir of biodiversity that can be leveraged to help develop a more robust and sustainable food production system. This investigation sought to identify an edible crop species with potential as a novel food crop for the New England food system and conduct a subsequent evaluation of the chosen species based on agronomic traits of interest. A series of interviews were conducted between August and December 2020 with regional experts and specialists with experience in the subject matter. Based on interview data and a subsequent literature review, Asclepias syriaca (common milkweed) emerged as a species of interest due to its agricultural merits, long history of use, and ecological service as an endemic, pollinator-friendly perennial.

Cardenolide content was selected as a priority trait of interest due to a limited understanding of the human health effects of cardenolide ingestion from culinarily prepared milkweed. Cardenolides, steroidal toxins that interrupt transmembrane ion exchange, are produced defensively by common milkweed to deter specialized herbivorous insect species. To evaluate the cardenolide content of regional milkweed populations, a spectrophotometric assay was investigated, drawing upon existing knowledge in the field of biochemical ecology. Despite the ability of the assay to produce replicable results when measuring reference samples of digitoxin in ethanol, the same was not observed when the assay was applied to milkweed tissue extracts.

Chlorophyll pigments and other photoactive compounds were implicated as a major potential confounding variable. Subsequent troubleshooting efforts to remove these compounds from experimental samples included the implementation of a lead acetate depigmentation step, a simple ethyl ether fractionation, and the use of heat as a pigment denaturing agent. Despite these efforts, a functional assay was not achieved.
CHAPTER 1
IDENTIFYING NEGLECTED AND UNDERUTILIZED CROP SPECIES OF POTENTIAL
IN NORTHEAST FOOD SYSTEMS

1.1 Global Food Supply Challenges

Modern agriculture faces a myriad of challenges. The global population is experiencing unprecedented improvements in average life expectancy due, in part, to medical advances beginning in the 1940s that resulted in substantial health improvements in countries with high mortality rates due to infectious diseases (Acemoglu et al., 2020). Gains in life span, in turn, have led to ever increasing population growth with the result that the global population is expected to increase from the current estimated 8.0 billion people to 9.7 billion by 2050 (United Nations, 2022).

Global population growth and an expected corresponding 130% increase in real global mean gross domestic product (GDP) per capita by 2050 will create increased burdens on the food supply (Clarke et al., 2015). Not only will there be more people in need of nourishment, but the global population as a whole will have a marked increase in the capacity to purchase goods and services. Most of this population and economic growth will occur in less industrialized nations (Sadigov, 2022). These trends imply that the demand for food will rise as both population and income per capita growth drive food consumption (Lanz et al., 2018). To feed 9+ billion people by 2050, global food production will need to increase 70-100% from 2018 levels (Raven et al, 2020). To meet this demand, food production needs to grow by 2.5-3.0% annually, even though current annual production growth is only 0.4-1.0% (Fischer, 2020).

While demand for food increases, the available resources to produce that food continue to shrink. The rapid decline of accessible freshwater resources due, among other reasons, to climate change, extended droughts, population growth, demand increases, and previous management
decisions, have resulted in current water shortages that are expected to worsen (Salehi, 2022). By 2050, it is anticipated that a total of 2.2 billion people (24.4 percent of the estimated global population) will suffer from water scarcity (Dhakal et al., 2022). A similarly challenging picture can be painted for arable land and energy. Rapid urbanization will result in an estimated 1.8-2.4% decrease in global cropland coverage and a potential 3-4% loss in worldwide crop production by 2030 (Wang, 2022). Modern crop production relies on energy inputs required to power farms (electricity and fuel) and support production (energy used to generate or supply seeds, fertilizers, pesticides, irrigation water, and machinery). Because energy-intensive agricultural systems are necessary for maintaining and increasing food production in the face of increasing demand, the future food system also faces the challenge of competing with increased energy demands both within and from other sectors (Clarke et al., 2015; Wang, 2020).

1.2 Losses in Food-Crop Biodiversity

Future food-crop biodiversity is also at risk. Current food demands in more industrialized nations are largely fulfilled by the intensive cultivation of a small cohort of highly productive crop species (Diaz et al., 2019; Stobl, 2021). This model of production in which a small suite of crops/crop cultivars are cultivated was further narrowed during the “Green Revolution” (1961-1980), in which the development of high-yielding crop varieties coupled with a newfound accessibility to agricultural inputs caused a worldwide adoption of industrial (i.e., standardized and input intensive) agricultural practices (Evenson & Gollin, 2003). Food production systems in the least industrialized nations are expected to continue to adopt these high-energy, monocultured grains, pulses, and oilseed crops (Pimm & Vijay, 2020). The Green Revolution and the further refining of crop cultivars and growing practices resulted in a substantial increase in food production and has been essential in averting
widespread famine and supporting an urbanizing global population (Pinstrup-Andersen & Haxell, 1985; Evenson & Gollin, 2003). But alongside the adoption of highly productive crop cultivars came the associated rise in inputs needed for their cultivation. As foreseen even by the architects of the Green Revolution, this model of agricultural expansion came with environmental consequences. Over-farming or mismanagement of marginalized land, excessive use of pesticides and fertilizers, and the overtaxing of limited natural resources continuously threaten the food production systems developed in the wake of the Green Revolution (Pinstrup-Andersen & Haxell, 1985; Pimm & Vijay, 2020; Wang, 2020). Now in the face of post-Holocene climatic shifts and ever-increasing demand, the negative consequences of displacing traditional, relatively diverse, regional food-crop cultivation are becoming widely acknowledged (Pimm & Vijay, 2020). In short, decreased crop biodiversity is now understood to threaten crop resilience and human food security (Stobl, 2021).

Plant genetic resources are required to maintain our modern, highly productive cropping systems. The genetic diversity needed for this can be pulled from two distinct reservoirs: (a) the lesser collection of genetic resources contained within domesticated populations and their regional varieties, and (b) the greater genetic reservoir of traits and genes held by wild relatives of modern crop cultivars. Agronomic advances have relied heavily on the introduction of important traits to crop cultivars from the biodiversity within both of these reservoirs. But in terms of nutritional security more broadly, a third reservoir of relevant plant genetic resources exists in the form of the estimated 7,000 edible plant species whose development has gone relatively overlooked before, during, and since the Green Revolution (Diazgranados et al., 2020; Gori et al., 2022). This third bulwark of diversity dwarfs the other two in sheer size and is comprised of edible plant species all along the domestication continuum that are adapted to a wide range of environments, exhibit tolerances to a diverse range of biotic and abiotic stresses, and possess clear nutritional value.
This reservoir of currently underdeveloped edible species represents an opportunity to diversify the food system, such that commercially viable cultivars of these species could be employed by farmers in response to changing environmental conditions and market demands. Yet, as more of the world’s landmass comes under production every year, biodiversity is ultimately undercut as all three genetic reservoirs erode (Khoury et al., 2014). As E. Strobl (2021) observes, “It is widely recognized that agricultural expansion and intensification due to rising demands for food from growing populations has been the primary driver of the loss of biodiversity globally.” In the midst of consequential climate change, this loss of biodiversity reduces the ability of researchers and farmers to leverage this diversity as a resource to feed the planet.

Greenhouse gas emissions have driven an increase in global temperatures by 0.9 °C since the 19th century and will potentially increase to 1.5 °C by 2050 and 2-5 °C by 2100 (Eftekhari, 2022). Climate change will have various consequences for different geographical locations and the biological systems that inhabit them. Within the context of agriculture, growing regions at higher latitudes may experience longer growing seasons due to higher temperatures while lower latitudes may face limited crop yields due to increased drought and/or heat stress (Eftekhari, 2022). Environmental change in combination with decreasing biodiversity poses a major hurdle for agricultural systems whose improvement and expansion will be needed to meet the projected population growth and demand for food. In the New England region of the US, the mean annual temperature has risen 4.32 °C between the years 1901 and 2011 (Janowiak et al., 2018); and this trend is projected to continue, with a potential increase in mean annual temperatures of 5.4-14.4 °C by 2100 (Eftekhari, 2022). Future climate-related challenges faced by farmers in New England include increased instances of heat stress and drought, combined with higher rates of precipitation (when it occurs) and flooding (Janowaik et al., 2018). Agricultural researchers, policymakers,
funders, and farmers in the region will need to take these changes into account when selecting crop species and specific cultivars to develop and grow in the coming decades.

1.3 The Potential of Novel Food Crops

In light of these changes and challenges, various technological advances can aid in the development of a more sustainable food system—yet adjusting existing agricultural systems will likely not be achieved through any one solution. One of these many solutions is the development of neglected and underutilized crop species (NUCS) (Padulosi et al., 1999). NUCS exist at varying levels of cultivation, ranging from wild edible species with potential for further development to semi- or fully domesticated species that, for a wide variety of reasons (cultural, agronomic, sociopolitical, etc.), either never achieved adoption outside a small historical range or had their niches supplanted by competitors. NUCS can be the close relatives of more widely accepted crops or the first in their family to achieve modern recognition as a crop.

Research initiatives worldwide have identified more than a thousand NUCS of potential value across the globe and the value that NUCS may provide to the food system can come in many forms (Hossain et al., 2021). Some NUCS may be adopted for intensive development because they are nutritionally valuable, e.g., *Amaranthus dubius* or *Cleome gynandra*—natives of Africa that are rich in antioxidants and phenolic compounds (Conti et al., 2019). Other NUCS may be cultivated to ensure continued food security in response to climate change, e.g., hardy *Cistus ladanifer* L., which does well in degraded soils, or Andean grains adapted to harsh conditions like *Chenopodium quinoa* and *Chenopodium pallidicaule* (Libiad et al., 2021).

Perennial NUCS are especially valued as their development may help address some fundamental environmental concerns related to modern agricultural systems. Deep-rooted perennials
can help thwart land degradation caused by agriculture as they help prevent erosion and limit soil disturbance due to their multi-year lifespan, drastically reducing tillage or even eliminating it altogether (Van Tassel et al., 2017). As constraints on energy and crop inputs continue to rise alongside demand, perennials can also increase the efficiency of resources used in cultivation. Because perennials allocate more resources into belowground organs, established fields of perennials retain soil moisture and are better equipped than annuals for agrochemical uptake—meaning that they can potentially utilize farmer inputs to a greater extent than their annual counterparts (Ferchaud et al., 2015; Van Tassel et al., 2017). *Thinopyrum intermedium*, a species of wheatgrass commercialized under the trade name kernza, serves as an exemplary model of perennial NUCS development and commercial production of this crop has recently started after two decades of intensive breeding and selection (Fangant et al., 2023).

Due to the broadness of the category, NUCS are described in ways that attempt to reflect their current agricultural status. NUCS that were once cultivated previously in human history are commonly referred to as “orphan” or “neglected” crops while those still farmed or foraged at some scale are referred to as “under-researched” or “traditional.” A species with no known prior cultivation may be described as a “novel,” “experimental,” “emerging,” or “potential” crop. Oftentimes these descriptors (and others) are treated as interchangeable in the literature. For example, marama bean (*Tylosema esculentum*), an edible legume native to southern Africa traditionally utilized as a forage crop, is referred to in many ways including “orphan,” “alternative,” “underutilized,” and “potential” (Cullis et al., 2018; Iyiola et al., 2022; Alabi et al., 2022; Chongtham et al., 2022). It is likely that some of the inconsistent language surrounding NUCS research is the result of a swelling in recent interest and publications on the subject from different authors worldwide.
NUCS development is not a new concept but for much of the 20th century these novel crops did not receive the same level of scientific attention as more established commercial crops. As a result, the peer-reviewed and other literature on such crops from the 20th century tend to be sparse, outdated, and/or focused primarily on the botanical aspects or historical/ethnobotanical use of the species in question (Cannarozzi et al., 2014). Over the past 30 years, however, interest has grown in exploring NUCS for agronomic viability due to their stress resistance (e.g., marama bean, tef), unique nutritional profiles (e.g., gluten-free grains), and/or the wealth of genetic diversity so far unrepresented in agroecosystems (Mal, 1994; Padulosi et al., 1999; Cannarozzi et al., 2014; Cullis et al., 2019; Hossain et al., 2020; Roberto Marceddu et al., 2022). Broadly, studying NUCS has the potential to expedite the process of crop domestication or further improve domesticated crops that have been overlooked and allow plant breeders to dust off neglected human-plant relationships or forge new ones.

Another reason that NUCS are starting to receive more attention is that the accumulated knowledge base surrounding the improvement of conventional crops and the accessibility of analytical tools for breeding traits of interest into productive lines has reached a point where biochemical and genetic trait analysis can now be readily applied to non-conventional crops and inform breeding efforts. Adapting existing food production practices and adopting innovative technologies can aid in the development of more flexible, robust, and sustainable food systems at scales ranging from regional to global. Employing regional knowledge, in particular, to guide the selection of NUCS for further study can be key to the successful development of new crops (Hunter et al., 2019). Additionally, identifying and investigating important agronomic traits of those species could transform otherwise unproductive plants into commercially viable food sources (Raybould, 2019).
1.4 Novel Food-Crop Research Questions

The first goal of this research project was to identify NUCS with potential for adoption in New England\(^1\) as profitable agricultural enterprises. While New England agriculture may appear less impressive compared to more agriculturally productive regions of the US (e.g., the Midwest, California’s Central Valley), at least in terms of acreage, the region offers a unique composition of small farms and sustainability-minded farmers that enjoy relatively more direct marketing avenues to their consumers. New England leads the nation in direct-market sales and boasts a relatively high percentage of organic farms, compared to more productive regions (Hird & Deade, 2020). Due to this unique agricultural landscape of New England, there is an opportunity to develop high-value markets for small niche crops such as NUCS.

The two primary research activities of this thesis were (1) to conduct interviews to acquire regional specialists’ knowledge as a guide for the selection of a NUCS whose cultivation could be agronomically and economically feasible for producers, and (2) through phenotypic analysis, begin to investigate the selected species’ potential as a novel food crop for New England.

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\(^1\) New England is comprised of Maine, New Hampshire, Vermont, Massachusetts, Connecticut, and Rhode Island.
The research questions underlying these activities were:

a) What underutilized species have potential as economically viable food crops in New England?

b) What trait(s) of interest should be considered when evaluating the feasibility of these species as crops?

c) How should these traits be explored to evaluate regional variation and in turn inform future study?

1.5 NUCS Selection: Acquiring Knowledge from Regional Experts

In terms of peer-reviewed scientific literature, there is relatively little formal information available about the relative promise of potential NUCS as commercially viable crops in New England. Although there is an extensive body of peer-reviewed literature addressing neglected or underutilized crops in many parts of the world, a comparable search from September 2020 through June 2023 for formal publications of such crops in New England, a region of one of the largest crop-producing nations, produces no comparable literature. Despite this lack of more peer-reviewed investigations, the cultivation and uses of many potential NUCS exists regionally among more informal knowledge networks. To understand the potential of NUCS to enhance and diversify the food supply in the region, it is worth exploring the extent of this local knowledge. Such knowledge resides with local experts engaged with commercial seed companies and nurseries, smaller growing communities, and backyard gardeners/cultivators, including people who self-identify across indigenous communities and as agricultural educators and researchers, community garden enthusiasts, and homesteaders. These experts are familiar with the lay of the land, growing conditions, and observed shifts associated with climate change and crop resistance. Although
outside of conventional academic or scientific circles, many of these people have relevant expertise in plant husbandry and have experimented with or know others who have experimented with various aspects of crop diversity and production systems. Additionally, regional experts are familiar with local markets and marketing networks, as well as local attitudes toward different foods and food requirements, preferences, and food preparation customs.

While acquiring regional food knowledge can be done in a variety of ways, one effective means is to conduct interviews/focus groups. Qualitative regional data supporting the selection of an appropriate NUCS for further study was collected for this research project through individual interviews with specialists experienced in novel crop cultivation. Initial interview participants were selected by the research team based on prior knowledge of relevance, but the participant pool was then allowed to snowball, as interviewees referred the team to additional specialists known by them. The team recruited all participants via email and telephone. In total, 23 people were approached and all agreed to participate in an interview or focus group.

Sought after for their knowledge of regional agricultural practices, the group of participants included Native American community representatives with specialized plant knowledge, small-scale farm/nursery managers, gardening/permaculture hobbyists, journalists and authors with agronomic interests, and university professors specializing in plant science. Between August and December 2020, the team conducted 20 separate interviews (19 individual and 1 focus group), either over the telephone or videoconference (e.g., Zoom®). Detailed written notes were developed from interview audio recordings. The notes were then analyzed using systematic text condensation to identify key themes (e.g., strengths, weaknesses) and subthemes (e.g., scalability, invasiveness) around the most promising crop species discussed (Malterud, 2012).
Interview questions and discussion prompts were designed to elicit “rich points” or responses about participants’ interests in, beliefs about, and opinions of specific NUCS and their firsthand experiences cultivating them (Agar, 1980) (Table 1).

**Table 1.** Interview questions (general and crop specific) used to identify and collect qualitative data on potential NUCS for future study. For crop specific questions, “X” represents a crop species identified by the interviewee.

<table>
<thead>
<tr>
<th>General Questions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Which underutilized crops do you have first-hand experience within our region?</td>
</tr>
<tr>
<td>Which underutilized crops do you think are promising targets for focused research and investment?</td>
</tr>
<tr>
<td>If multiple crops are indicated: Can you prioritize them in terms of their scalable potential?</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crop Specific Questions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>In your opinion, why does X have potential in the northeast?</td>
</tr>
<tr>
<td>How long, where, and at what scale have you grown X?</td>
</tr>
<tr>
<td>Do you cultivate a specific variety of X? What is your source of germplasm?</td>
</tr>
<tr>
<td>What is your typical yield/productivity?</td>
</tr>
<tr>
<td>What do you do with the product? If you sell X, what do you charge?</td>
</tr>
<tr>
<td>What have been your greatest challenges growing X?</td>
</tr>
</tbody>
</table>

The team also sought information about current plant food preferences held by participants and members of their respective communities. The team encouraged participants to discuss crops that they viewed as having scalable potential, meaning species with direct avenues of improvement that could be readily incorporated into existing food systems. Conversations were wide ranging, as illustrated by the small selection of paraphrased statements in Table 2.
Table 2. Selection of paraphrased statements made by interviewees illustrating the breadth of the comments and insights gathered during the interview process.

<table>
<thead>
<tr>
<th>Statement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interviewee placed an emphasis on leaf crops that are resilient, adapted to the margins, require minimal inputs, and peak when nothing else is growing (e.g., very early spring, mid-to-late fall). Such attributes are particularly important for market gardeners.</td>
</tr>
<tr>
<td>Interviewee recognized potential pitfalls in developing crops with cultural significance to indigenous peoples. If a cultivar/species has an indigenous name, one risks infringing on it and devaluing it by making an “improved version.” If one wishes to release a cultivar through a nursery, for example, they will want to know the line’s history to ensure there was no appropriation, people are being compensated, etc.</td>
</tr>
<tr>
<td>Interviewee emphasized the great potential presented by the “Northeast Megalopolis,” an urban corridor running from Boston to Washington, D.C., and housing approximately 56 million people. Despite covering only about 5% of the nation's land area, this region holds 25% of the nation's wealth. This has attracted numerous immigrants seeking job opportunities, making it an area to focus on crop cultivation for these communities.</td>
</tr>
<tr>
<td>Because seaberry is a wind pollinated NUCS, the interviewee is able to carry out crossbreeding efficiently in their location as there are no indigenous stands of seaberry and therefore no contamination from outside sources.</td>
</tr>
<tr>
<td>The interviewee shared their personal transformative experience with schisandra and its medicinal (particularly anti-fatigue) properties. Local retailers have expressed a strong interest in acquiring as much schisandra as possible for both personal use and inclusion in their own tincture products.</td>
</tr>
<tr>
<td>The interviewee lauded the potential profitability of growing novel fruit trees in the region: &quot;You could make a zillion dollars if you can figure out how to grow figs up here.&quot; They also mentioned medlar and pawpaw as options but noted that both require significant work to scale effectively. Medlar would need production research for quality fruit, while Pawpaw requires postharvest best practices and processing systems.</td>
</tr>
<tr>
<td>Establishing a sustainable funding model remains a challenge when working with minor crops. However, the interviewee recognized the potential benefits of creating a center dedicated to these crops, for example at UNH. They believe that such a center would not only generate significant interest but also provide UNH with a distinct niche in the industry, setting it apart from other states.</td>
</tr>
</tbody>
</table>

A list of 48 NUCS of potential interest was generated from interview discussions (Table 3). These crops were highly diverse and ranged from, among others, niche medicinals (e.g., elderberry, schisandra, Jerusalem artichoke, ashwagandha) to leafy greens (e.g., amaranth, Chinese toon, heblitzia, collard raab), vegetables (winter sprouting broccoli, bitter melon), and oilseed crops (e.g., yellowhorn, hulless pumpkin seed).
Table 3. Common and scientific names of NUCS mentioned in interviews with regional experts.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aji dulce</td>
<td>Capsicum chinense</td>
</tr>
<tr>
<td>Amaranth</td>
<td>Amaranthus spp.</td>
</tr>
<tr>
<td>Ashwagandha</td>
<td>Withania somnifera</td>
</tr>
<tr>
<td>Beach plum</td>
<td>Prunus maritima</td>
</tr>
<tr>
<td>Beechnut</td>
<td>Fagus grandifolia</td>
</tr>
<tr>
<td>Bitter melon</td>
<td>Momordica charantia</td>
</tr>
<tr>
<td>Che fruit</td>
<td>Machura tricuspida</td>
</tr>
<tr>
<td>Cherokee skunk bean</td>
<td>Phaseolus vulgaris</td>
</tr>
<tr>
<td>Chestnut</td>
<td>Castanea spp.</td>
</tr>
<tr>
<td>Chinese toon</td>
<td>Toona sinensis</td>
</tr>
<tr>
<td>Chinese yam</td>
<td>Dioscorea polystachy</td>
</tr>
<tr>
<td>Chokeberry</td>
<td>Aronia spp.</td>
</tr>
<tr>
<td>Common milkweed</td>
<td>Asclepias syriaca</td>
</tr>
<tr>
<td>Cornelian cherry</td>
<td>Cornus mas</td>
</tr>
<tr>
<td>Elderberries</td>
<td>Sambucus spp.</td>
</tr>
<tr>
<td>Gooseberries</td>
<td>Ribes uva-crispa</td>
</tr>
<tr>
<td>Goumi</td>
<td>Elaeagnus multiflora</td>
</tr>
<tr>
<td>Tomatillo</td>
<td>Physalis philadelphica</td>
</tr>
<tr>
<td>American groundnut</td>
<td>Apios americana</td>
</tr>
<tr>
<td>Haskap / Honeyberry</td>
<td>Lonicera caerulea</td>
</tr>
<tr>
<td>Hazelnuts / Filberts</td>
<td>Corylus spp.</td>
</tr>
<tr>
<td>Heblitzia / Caucasian mountain spinach</td>
<td>Hablitzia tamnoides</td>
</tr>
<tr>
<td>Hulless pumpkin</td>
<td>Cucurbita pepo</td>
</tr>
<tr>
<td>Japanese millet</td>
<td>Echinochloa esculenta</td>
</tr>
<tr>
<td>Jerusalem artichoke</td>
<td>Helianthus tuberosus</td>
</tr>
<tr>
<td>Juneberry</td>
<td>Amelanchier spp.</td>
</tr>
<tr>
<td>Intermediate wheatgrass</td>
<td>Thinopyrum intermedium</td>
</tr>
<tr>
<td>Kohlrabi</td>
<td>Brassica oleracea</td>
</tr>
<tr>
<td>Korean pine nut</td>
<td>Pinus koraiensis</td>
</tr>
<tr>
<td>Lambquarter</td>
<td>Chenopodium album</td>
</tr>
<tr>
<td>Mulberry</td>
<td>Morus alba</td>
</tr>
<tr>
<td>Nanking cherry</td>
<td>Prunus tomentosa</td>
</tr>
<tr>
<td>Native black raspberry</td>
<td>Rubus occidentalis</td>
</tr>
<tr>
<td>Pawpaw</td>
<td>Asimina triloba</td>
</tr>
<tr>
<td>Pepino dulce</td>
<td>Solanum muricatum</td>
</tr>
<tr>
<td>Perennial leak</td>
<td>Allium porrum</td>
</tr>
<tr>
<td>Persimmon</td>
<td>Diospyros kaki</td>
</tr>
<tr>
<td>Pine nut</td>
<td>Pinus cembroides</td>
</tr>
<tr>
<td>Radicchio</td>
<td>Cichorium intybus</td>
</tr>
<tr>
<td>Schisandra</td>
<td>Schisandra chinensis</td>
</tr>
<tr>
<td>Sea kale</td>
<td>Crambe maritima</td>
</tr>
<tr>
<td>Seaberry</td>
<td>Hippophae rhamnoides</td>
</tr>
<tr>
<td>Siberian pea shrub</td>
<td>Caragana arborescens</td>
</tr>
<tr>
<td>Sweet sorghum</td>
<td>Sorghum bicolor</td>
</tr>
<tr>
<td>Teff</td>
<td>Eragrostis tef</td>
</tr>
<tr>
<td>Turkish rocket</td>
<td>Bunias orientalis</td>
</tr>
<tr>
<td>Winter sprouting broccoli</td>
<td>Brassica oleracea</td>
</tr>
<tr>
<td>Yellowhorn</td>
<td>Xanthoceras sorbifolium</td>
</tr>
</tbody>
</table>
1.6 NUCS Selection: Merits and Concerns

A pattern of recurring themes surfaced during the interviews that aided in the process of evaluating the potential viability of crop candidates. These themes were articulated as a list of merits that would support the argument for selecting a particular crop species for research (Table 4). In terms of each potential merit, any given NUCS may perform well or poorly as the opposite of each merit (e.g., not regionally adapted) can be understood as a concern that argues against investment in a particular crop species.

Table 4. Merits that emerged as a framework for evaluating the suitability of NUCS for future investigation.

<table>
<thead>
<tr>
<th>Merits</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amendable to Research</td>
<td>Crop characteristics that facilitate efficient cultivation/study under research conditions (e.g., short generation times, genetic tractability, easily observable phenotypes, etc.).</td>
</tr>
<tr>
<td>Scalable Potential</td>
<td>Crop characteristics that enable the successful transition from small scale cultivation for research to large scale cultivation for profit.</td>
</tr>
<tr>
<td>Regionally Adapted</td>
<td>Suitability of a candidate crop species to New England growing conditions, both current and future.</td>
</tr>
<tr>
<td>High Nutritional Value</td>
<td>Crop products contain high concentrations of important dietary nutrients.</td>
</tr>
<tr>
<td>Marketable</td>
<td>Crop characteristics are attractive to would-be consumers and/or a target demographic exists that are familiar with/interested in the product.</td>
</tr>
<tr>
<td>Ecological Value</td>
<td>Crop characteristics that support services such as pest control, nutrient cycling, pollinator conservation and soil health (e.g., perenniality).</td>
</tr>
<tr>
<td>Low Capital Investment</td>
<td>Growers require minimal investment in production equipment, specialized processing infrastructure, and/or distribution facilities to successfully produce and market the crop.</td>
</tr>
<tr>
<td>Non-invasive</td>
<td>The species exhibits no invasive risk to endemic regional ecosystems including forest and grassland habitats.</td>
</tr>
<tr>
<td>Non-weedy</td>
<td>The species’s growth habit and/or seed dispersal strategies are manageable by cultivators so that intentional plantings of the species do not result in its dispersal to undesired locations (e.g., neighboring farm plots).</td>
</tr>
</tbody>
</table>
Some common merits mentioned by the experts interviewed included nutritional benefits (e.g., high vitamin content and protein), adaptation to regional abiotic or biotic stresses, and/or the availability of untapped genetic diversity which could be leveraged to develop productive cultivars. For example, a crop species either native to the region or tolerant of anticipated challenges (e.g., drought or heat stress) would serve as a viable candidate for consideration. NUCS with unique culinary characteristics, such as having an uncommon taste or nutritional profiles, were prioritized, as were those with at least some history of systematic research and/or demonstrated proof of economic viability. Also favored in the selection process was the aforementioned trait of perenniality.

Some prohibitive crop concerns remarked on were the general risk of invasiveness upon introduction to New England (e.g., seaberry) and prohibitively lengthy/onerous growing cycles that make practical cultivation and study unwieldy (e.g., perennial leek). Because legal and functional definitions of the term can vary, invasiveness is broadly defined here as the ability for a nonendemic species to self-establish and persist within healthy natural ecosystems of New England. This is quite different from the concept of weediness, which is defined here as the tendency of the species to persist and/or disperse within agricultural fields beyond its deliberate planted. This was a relevant consideration for species that did not pose a risk of establishing in natural ecosystems but may unintentionally spread to neighboring farm plots and/or resist eradication efforts.

The 48 candidate NUCS were then scored based on their status vis-à-vis the broad list of attributes (Table 5). Attribute scores were designed to indicate the relative level of merit or concern depending on each crop’s characteristics. The averaged attribute scores were used to rank the candidate crops for their potential value as NUCS of interest for further study.
Table 5: Ranking NUCS research candidates from highest to lowest based on identified attributes. Crop ranks are based on the averaged attribute scores of each crop. Attribute scores range from 5 (major merit) to 1 (major concern) based on interview results and subsequent review of the literature (superscripts). If no information was available pertaining to the specific attributes of a crop, no score was given (unknown) and the attribute was not factored into the average.

<table>
<thead>
<tr>
<th>Attribute Scores</th>
<th>Attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major Merit:</strong></td>
<td>Amendable to Research</td>
</tr>
<tr>
<td>Minor Merit:</td>
<td>5</td>
</tr>
<tr>
<td>Neutral:</td>
<td>4</td>
</tr>
<tr>
<td>Minor Concern:</td>
<td>3</td>
</tr>
<tr>
<td>Major Concern:</td>
<td>2</td>
</tr>
<tr>
<td>Unknown:</td>
<td>-</td>
</tr>
</tbody>
</table>

Common milkweed$^{32}$
Hulless pumpkin$^{33, 146}$
Cherokee skunk bean
Bitter melon$^{33}$
Kohlrabi$^{34}$
Amaranth$^{34, 35}$
Intermediate wheatgrass$^{36, 37}$
Elderberries
Beach plum$^{111}$
Radicchio
Aji dulce$^{38, 136}$
Winter sprouting broccoli
Chinese yam
Native black raspberry
Chokeberry$^{39}$
Ground cherry$^{39, 137, 147}$
Heblitzia
Ashwagandha
Cornelian cherry$^{33}$
Schisandra
Lamb'squarter
Hazelnuts
Chestnut
Haskap$^{40}$
Sea kale
Telf$^{41}$
Gooseberries
Seaberry$^{42, 14}$
Chinese toon
Jerusalem artichoke$^{43}$
Juneberry
Mulberry
Pepino dulce
Sweet sorghum
Japanese millet$^{44}$
Turkish rocket
Perennial leek
Che fruit
Yellowhorn
American groundnut$^{16, 121}$
Persimmon
Pawpaw
Goumi$^{35}$
Korean pine nut
Pine nut
Beechnut$^{46}$
Nanking cherry
Siberian pea shrub$^{37}$

3.38
3.38
3.33
3.29
3.25
3.25
3.20
3.17
3.17
3.17
3.14
3.14
3.13
3.13
3.00
3.00
3.00
3.00
2.88
2.86
2.86
2.86
2.86
2.86
2.86
2.86
2.75
2.71
2.71
2.67
2.63
2.57
2.43
2.33
2.33
2.33
2.17
2.17
The crop rankings depicted in Table 5 were established based on two sources. The interviews provided largely anecdotal accounts of the cultivation of specific NUCS, which provided some basis for their rough rankings. Additionally, a literature review investigated the available information surrounding each NUCS’s biological characteristics, production/processing requirements, and research feasibility. There was a high level of agreement between the accounts from interviews and the available literature. Due to the anecdotal nature of the interview data and the varying availability of information on the cultivation of any given candidate NUCS in New England, the ranking provided in Table 5 is intended to be an initial ordering rather than an authoritative or final passing of judgement on the overall potential of any given crop.

For example, hulless pumpkins were suggested by one interviewee as a potential crop of interest for their dual uses as an oilseed and fruit crop. Hulless pumpkins lack an outer seed coat and are easier to process into oils. The seeds can therefore be harvested as one product while the fruit may have culinary or livestock feed uses (Charaya et al., 2023). The interviewee also highlighted the potential of cucurbitacin expression as a trait of interest for hulless pumpkins as it may act as a deworming agent for livestock. As an annual crop, hulless pumpkins become productive within a single growing season which means a research population could be generated and subsequent generations evaluated relatively quickly when compared to NUCS that may take years to reach sexual maturity. This, alongside the potential evaluation of cucurbitacin expression as a trait of interest suggests that hulless pumpkin could be feasible to research within this context. Also acknowledged were the potential shortcomings and pitfalls of hulless pumpkins, such as their susceptibility to pest pressure and the relative difficulty of adopting the crop by small farming operations. A subsequent literature review supported observations about pest issues but also suggested that hulless pumpkins could be an economically viable crop for organic farms in
neighboring Quebec (Beleize et al., 2014; Richard et al., 2014). Additionally, while not widespread, hulless pumpkins have been adopted in Quebec and marketed as a seed crop for human consumption (Quebec Pumpkin Seeds, 2021). Because of these factors, hulless pumpkin ranks favorably with respect to research amenability and capital investment.

The dominant concern that barred most candidate NUCS from serious consideration was the issue of scalable potential (Table 5). Just about any NUCS can be grown for research purposes without concern for profitability, but to ultimately transition from NUCS to an economically viable crop, the cultivation (not to mention harvesting, processing, marketing, and distribution) of an edible species needs to be feasible and profitable at a commercial scale. Many of the candidate crops discussed also would require various forms of equipment and infrastructure investments for harvesting (e.g., specialized machinery, handling, and storage facilities) and processing (e.g., washing, husking, slicing, milling); and the development and adoption of such equipment would only be feasible if growing the NUCS in question was economically viable. Moreover, New England states are among those with the highest share of what the US Department of Agriculture defines as small family farms that operate with a “gross cash farm income under $250,000” (USDA, 2021). Small family farmers’ interest in a novel crop may be dampened by any high fiscal risk involved.

1.7 Broadening Selection Criteria Based on Interview Data

When evaluating the interview data, identification of a NUCS appropriate for research investment was found to be challenging, in large part due to the rigorous demands for proven scalable potential, economic feasibility, and ready availability of plant genetic resources. Scalability, especially for NUCS with complicated or extended growing cycles, was a major factor
in decision making. If a species is difficult to cultivate at a scale required for research, then those same challenges would be magnified when brought to commercial production systems. While many of the species mentioned in interviews enjoy varying degrees of success as backyard plants, the majority have unclear potential in commercial agriculture.

An unexpected idea that emerged during the interviews was a challenge to the assumption that NUCS development only makes sense in the context of commercial farms, especially in a state like New Hampshire. According to the 2019 USDA Agricultural Statistics Annual Bulletin for New England, only 1.3% of New Hampshire’s land is utilized for crop production (Hird & Deade, 2020). In stark contrast to this, roughly 5% of the state’s land is used for turfgrass (Milesi et al., 2005). Largely maintained for aesthetic and recreational purposes, turfgrass is highly resource intensive (water, energy, nutrients, etc.), is responsible for significant leaching of nutrients and toxins into the environment, is an unsupportive habitat for most New England wildlife species, and confers no benefit vis-à-vis food production (Steir et al., 2013; Hatfeild, 2017). In terms of NUCS development, the data on New England land usage and data on New Hampshire in particular suggest that a focus on commercially viable agricultural crops may be artificially narrow. Indeed, the potential use of NUCS on lands managed by homeowners would perhaps lead to a greater positive impact than a purely farm-centric one.

What ultimately led to the selection of the chosen species of interest was a broadening of scope during data analysis to include crops intended for non-commercial cultivation, particularly those with spillover environmental and nutritional benefits, for relevant stakeholder groups including dedicated hobbyists, permaculturalists, and sustainability-minded homeowners interested in alternative land management practices. This change in perspective relaxes the strict criteria based around scalability and better reflects the current status of many NUCS already grown
in New England. Many of the edible species described in the interviews and focus groups are locally cultivated and recommended based on their preexisting status as edible ornamentals (e.g., common milkweed, schisandra), backyard oddities (e.g., pawpaw, tomatillo), or home garden experiments (e.g., Jerusalem artichoke, groundnut) whose success was worth mentioning. This approach also alleviates the strict conditions for a new species to become cultivated as they are not competing for space with proven crops, an inherent financial risk and opportunity cost that could severely limit uptake by even the most open-minded farmers.

While infrastructure requirements may be a prohibitive factor for small farms when adopting new crops, these same farms may still find value in adopting a novel low-input crop for use in home gardens or otherwise unproductive plots of land. Assuming much of the turfgrass in New England exists as groundcover for private property, the case can be made that by promoting an edible species for backyard growers, one can help encourage the conversion of turfgrass into productive home gardens, replete with various environmental benefits. This mindset aligns strongly with the vision of diversified land use advocated by regional, sustainably minded farmers, researchers, hobbyist, community leaders, and interest groups such as the permaculture and edible landscaping movements.

With this perspective in mind, common milkweed (*Asclepias syriaca*) was selected as the crop of interest for this project. Common milkweed was first introduced as a candidate for study by an interviewee who lauded the species as an unsung hero with excellent culinary attributes and an exceptional nutritional profile. As the name would imply, common milkweed is a commonly-found native species of New England—meaning that any qualms over potential invasiveness or poor adaptation to the region can be dismissed. Due to its hardy nature and quick development, the inputs and time required for cultivating and evaluating this species were deemed to be within
the scope of this study. The species is a deep-rooted perennial and so shares the intrinsic agronomic merits of perennials outlined in the previous section. And lastly, as an important pollinator plant and a crucial player in endangered species conservation, the claim for common milkweed as a NUCS of interest is supported by a strong argument for the ecological service provided by the crop in tandem with its agricultural benefits. The inedible portions of the plant provide sustenance and a habitat for many New England species, and cultivators may choose to leave a portion of their yield unharvested to the benefit of local wildlife.

But just as there are aspects of common milkweed that would-be growers may find attractive, there are also potential challenges to introducing the species as a food crop. One obvious hurdle is to convince the general public of milkweed’s edibility. It is likely that the vast majority of New Englanders have never been exposed to the idea that milkweed is edible and convincing regional populations to adopt the crop over similar vegetable products (asparagus or broccolini) may prove difficult. Complicating this further is the apparent widespread public knowledge that milkweed contains toxic secondary metabolites that are sequestered by specialized pest species for their own defenses. While educating the public on the ecological interactions of milkweed has been pivotal for conservation efforts, that same narrative becomes an obstacle when trying to market the species as safe for consumption. This is further hindered by the lack of formal research surrounding common milkweed from an agricultural or culinary perspective, and the potential toxicity of milkweed to humans will need to be addressed upfront before further investigations are made. In a similar vein, due to common milkweed’s resistance to manual excavation and ability to spread clonally, many farmers consider it a weed and may resist deliberately planting it. But despite these qualms, milkweed ranked favorably out of the 48 species considered (Table 5) and a subsequent literature review of milkweed (see Chapter 2) solidified the species as a NUCS of
potential use as a food crop for backyard growers in New Hampshire. This research contributes to the evidence base by investigating the food uses of milkweed and the research challenges associated with those uses.
CHAPTER 2

THE CASE FOR *ASCLEPIAS SYRIACA* AS A CROP SPECIES OF INTEREST

2.1 Introduction to *Asclepias*

*Asclepias* is a genus of herbaceous, perennial Gentianales known collectively as milkweeds. They are members of the family Apocynaceae, commonly referred to as the “dogbanes,” and are typified as being unpalatably poisonous but useful in traditional medicine and as potential sources of pharmaceutical compounds (Roberts, 1998; Agrawal, 2017). The family contains a total of 366 genera distributed across tropical and temperate regions of every continent except Antarctica (Endress et al., 2014). Members of the *Asclepias* genus grow throughout three geographically distinct regions: Africa (250 spp.), North America (130 spp.), and South America (9 spp.) with each region containing distinct evolutionary clades (Agrawal et al., 2014). Phylogenetic studies suggest that the genus originated in Africa, later spread to North America, and that the South American lineage arose from a single dispersal event from a North American ancestor (Fishbein et al., 2011). As the North American clades diversified over time, directional trends in either the increase and/or decrease of defensive traits within members of the clade emerged (Agrawal et al., 2014). This has resulted in a wide range of diversity for defensive traits within the North American clades, even though many members of this clade are morphologically similar.

Some *Asclepias* species have adapted an arsenal of structural and chemical defenses against herbivory and are commonly referred to as “milkweeds” due to the milky-white latex most species exude from specialized stem and leaf tissues when ruptured (e.g., swampy milkweed, silky milkweed, tropical milkweed, etc.) (Agrawal, 2005). Considered to be a purely defensive trait with no known role in plant metabolism or resource management/allocation, latex physically dissuades
herbivorous chewing insects by gumming up their mouthparts and acts as a medium for releasing defensive chemicals produced throughout the plant. These chemicals include saponins, pregnanes, phenolics, alkaloids, specialized cysteine proteases (enzymes that degrade the peritrophic membrane of herbivorous insects), and cardenolides (steroids that inhibit ion channel function) that can disrupt the feeding of or outright kill herbivorous insects (Malcom et al., 1989; Brower et al., 1982; Agrawal, 2014; Agrawal et al., 2021).

Some associated insect herbivores of *Asclepias* have developed an insensitivity to the chemical defenses of the genus. Common herbivores of *Asclepias* native to North America include the red milkweed beetle (*Tetraopes tetraophthalmus*), milkweed weevil (*Rhyssomatus lineaticollis*), small milkweed bug (*Lygaeus kalmii*), milkweed leaf miner (*Liriomyza asclepiadis*), oleander aphid (*Aphis nerii*), and monarch butterfly (*Danaus plexippus*) (Figure 1a-f). The monarch butterfly (hereafter monarch) actively seeks out milkweeds on which to lay its eggs. The resulting monarch larvae feed on their *Asclepias* host and internally sequester its toxins, thereby retaining the bitter-tasting and poisonous compounds into adulthood (Brower et al., 1982; Malcom et al., 1989; Agrawal, 2005). It is thought that these sequestered toxins, in combination with the bright warning colorations of monarchs, help deter predators. Similarly colored butterflies like the viceroy butterfly (*Limenitis archippus*) and queen butterfly (*Danaus gilippus*) are widely recognized examples of mimicry, an evolutionary adaptation that benefits from a visual association with the monarch’s defense strategy.
Figure 1. Some common insect herbivore species of North American *Asclepias* spp.: (a) red milkweed beetle, (b) milkweed stem weevil, (c) milkweed bug, (d) the damage caused by milkweed leaf miner larvae (four separate mines), (e) oleander aphids and three *Formicidae* spp. symbionts, and (f) monarch butterfly (larvae).

There is a strong ecological connection between the renowned annual monarch migrations and the distribution of *Asclepias* spp., as the former species’ northernmost range depends almost entirely on the presence of the latter (Agrawal et al., 2012). This co-evolutionary relationship between the charismatic monarch and *Asclepias* spp. is known by much of the general public. Because of this interaction, milkweed species native to New England are widely regarded as environmentally important due to their integral connection to monarch/pollinator conservation efforts. Also because of this interaction, these same *Asclepias* spp. are popularly believed to be unpalatable, if not outright dangerous, for human consumption, if they are considered for
consumption at all (Penn et al., 2018). This perception of milkweed as inedible could potentially hinder the acceptance of the species as a food crop.

### 2.2 Distribution of *Asclepias syriaca*

*Asclepias syriaca* ("common milkweed," hereafter referred to simply as "milkweed") is a dicotyledonous perennial species belonging to the North American *Asclepias* lineage. Its native range is wide, from the east side of the Rockies to the Atlantic coast and from Quebec in the north to Florida in the south (Malcom et al., 1989; Agrawal et al., 2014). Adapted to a diverse range of climatic and edaphic conditions, milkweed is present in many areas but is most commonly found in well-drained, loamy soils and areas where the soil has been recently disturbed or tilled (Hartzler & Buhler, 2000; Bagi, 2008). Because of this, milkweed is a common sight alongside roads and railways where large clonal patches can establish, develop, and spread without the hindrances of canopy cover or pre-established competition (Bagi, 2008). Due to its ubiquity and hardy nature, milkweed is a common pest plant for North American farmers and gardeners who view it as a well-entrenched weed able to shade out smaller cultivated species. A survey conducted by Hartzer and Buhler (2000) measuring milkweed infestations in Iowa cropland from June to July of 1999 found the species to be present in approximately 50 percent of corn and soybean fields. A similar survey in Nebraska found that over 70 percent of soybean, oat, and sorghum fields had milkweed infestations (Cramer & Burnside, 1982). Despite their hardy nature, milkweed can be eradicated through repeated treatment with glyphosate. And the introduction of this herbicide, coupled with glyphosate-resistant corn and soybean cultivars, has been implicated in greatly reducing the presence of milkweed in agricultural fields (Hartzler, 2010). Ten years after his initial survey,
Hartzler (2010) replicated his 1999 survey of milkweed infested cropland in Iowa and observed that the presence of milkweed had dropped from 50 percent in 1999 to only 8 percent in 2009.

Milkweed has long since spread from its native range by human intervention and was imported to Europe from North America in the 17th century as an ornamental. By the end of the 18th century, both cultivated and naturalized milkweed were widely distributed across West and Central Europe and had reached as far east as Russia (Whiting, 1943; Gaertner, 1979). In modern Hungary, milkweed is now the most widespread invasive grassland plant species, spreading from pasture-to-pasture via roads, railways, and abandoned farmland (Bagi, 2008; Bakacsy & Bagi, 2020). While it does not appear to affect the total species richness in Hungarian grasslands, milkweed does outcompete some native species for sunlight and potential pollinators (Szilassi et al., 2019).

2.3 Milkweed Physiology and Reproduction

Milkweed in New England typically emerges from its perennial root system in late May and flowers in late June through most of July (Bagi, 2008; Woods et al., 2012). Milkweed grows 80-150 cm high and reproduces both sexually through perfect flowers such as in Figure 2 and asexually via rhizomes (Jeffery & Robinson, 1971; Bagi, 2008). Milkweed forms a wide network of rhizomes which can expand as much as 3 meters in a single season as well as a taproot which can penetrate nearly 4 meters underground (Bagi, 2008). This combination of extensive clonal propagation and a deep-set taproot makes eradication of milkweed by farmers and gardeners difficult, as rootstalk cuttings as short as 2.5 cm can regrow into new plants and the lifespan of well-established plants is thought to be well in excess of a hundred years (Bagi, 2008).
The plant’s broad lanceolate leaves are arranged on opposite sides of the stem and are 7-25 cm in length, 5-7 cm wide, and have well-defined vasculature (Jeffery & Robinson, 1971; Bagi, 2008). The leaf and stem tissues contain a system of specialized canals called laticifers in which latex is stored under pressure (Agrawal, 2005). When a leaf is torn open, one can observe the near-instant formation of latex along the tear with large droplets forming at the terminus of each damaged vein.

Figure 2. A blooming *Asclepias syriaca* inflorescence and the anatomy of an individual flower (drawing adapted from Morgan & Schoen, 1997). The major features of the flower include the nectar-containing hoods, which attract pollinators, and the pollen-containing pollinia.
Milkweed is well known as an attractive flowering plant for pollinators. The inflorescence is a large pink-white umbel with perfect flowers growing from the upper leaf axils and tips of the stem (Jeffery and Robinson, 1971; Woods et al., 2012) (Figure 2). The flower structure and its associated pollinating mechanism are notably complex, as Morgan and Schoen (1997) describe:

Inflorescences consist of many flowers, each of which has a ‘corona’ made up of five cup-shaped ‘hoods’ and associated ‘horns’ extending from the base of the hood toward the center of the flower (gynostegium). Pollen grains are packaged into pollinia, each pollinium consisting of two pollen sacks joined by translator arms and a corpusculum; only the corpusculum is exposed to pollinators. Hoods accumulate copious nectar, and the arrangement of the hoods around the gynostegium directs hairs of foraging pollinator limbs and other body parts toward a small groove in the corpusculum. The hairs become wedged in the groove, and the corpusculum and associated pollinia become dislodged as the pollinator leaves the flower. During pollinator flight, the translator arms [the stalks upon which the pollinia are connected to the corpusculum] dehydrate and the orientation of the pollinia changes so that when pollinators visit another inflorescence the pollinium may insert into the stigmatic slit and juxtapose against the stigmatic surface.

While milkweed’s bright, aromatic flowers and notably pronounced nectar production make it an attractive resource for pollinators, the small grooves within the corpusculum have been known to trap the legs of smaller pollinators (Gaertner, 1979; Bagi, 2008) (Figure 2). Like many members within Asclepias, milkweed is self-incompatible, meaning that flowers must be fertilized by pollen from a genetically compatible mate (Wyatt & Broyles, 1994; Weitemier et al., 2019). Lipow and Wyatt (2000) suggest that the late-acting, postzygotic self-incompatibility in A. exaltata, a close relative of milkweed, is determined by a single gene and that crosses between individuals sharing even one allele are incompatible. Interestingly, this self-incompatibility trait has potentially broken down in invasive and genetically isolated milkweed populations in Hungary (Agrawal, personal communication, 2021), an outcome consistent with Lipow and Wyatt’s manual crosses between self-fertile and self-infertile accessions of A. exaltata.

In New England, milkweed follicles, commonly referred to as “seed pods,” develop by late September (Bagi, 2008). Seeds are easily germinated via conventional methods and all seeds from
a single follicle represent a full-sibling genetic family (Woods et al., 2012). A single stem from a mature plant typically produces four to six follicles, each containing as many as 300 seeds (Figure 3a-b). Each seed has a plume of remarkably fine silky hairs (or “floss”) which allow for wide dispersal via air currents (Whiting, 1943; Jeffery & Robinson, 1971).

Figure 3. Common milkweed (*Asclepias syriaca*) at different stages of development, ranging from (a) seeds being harvested from a follicle, (b) germinating seeds beginning to sprout, (c) young seedlings reared under greenhouse conditions, (d) the bound roots of a potted seedling, and (e-f) mature plants growing in a farm plot.
2.4 Human Utilization of Milkweed

Previous studies involving *Asclepias* spp. predominantly focused on biochemical-mediated ecological interactions between members of the genus and their respective herbivores and pollinators. Despite a long historical relationship with people, much less has been written about the human utilization of milkweed. Milkweed is described by botanist E.E. Gaertner as North America’s “greatest underachiever among plants” due to the disconnect between the potential economic importance of the species and the simple fact that it has never been consistently cultivated for commercial purposes (1979). That is not to say that there has been a lack of human interest or utilization of milkweed throughout the ages. Members of the Omaha and Sioux nations are known to have used milkweed shoots, roots, and buds as food and medicine (Shane, 2013). The leaves and latex are thought to be valuable as both a diuretic and anti-asthmatic as well as being useful in treating bronchitis, pneumonia, rheumatism, and kidney stones (Gaertner, 1979; Rosu et al., 2011). Milkweed stem fibers are notably robust and easily processed into yarn, and their use by Indigenous people is found in both archeological evidence and historical accounts (Whiting, 1943; Berkman, 1949; Shane, 2013). Milkweed was listed by botanist L. Herbert in 1635 as one of the first plant species of potential medicinal importance identified by European colonizers of the Americas (Gaertner, 1979). Milkweed seeds were later introduced to Europe, where the seed floss was briefly used for clothing and as a stuffing material in the 18th century (Whiting, 1943; Dréan, 1993) (Figure 3a). By the 19th century, numerous small-scale attempts to cultivate milkweed had been made in France, Germany, and Russia, with mixed reception (Whiting, 1943; Berkman, 1949). In 1838, M. Lichenstein summarized the shortcomings of milkweed fibers, remarking that the stem fiber was weak and brittle as a spinning fiber, difficult to dye, and that the seed floss tended to lump when used for padding (Whiting, 1943).
Throughout the 20th century, milkweed was explored as a source of industrial fibers in several countries. American interest in milkweed during this time included two major endeavors to evaluate and utilize the species as a fiber crop. Milkweed was cultivated on a nearly industrial scale at the Iowa Agricultural Experimental Station from 1925 to 1929 in order to assess leaf tissue composition, fiber production, and the potential uses of fibers. The study concluded that milkweed was productive despite overcrowding and severe drought conditions but that the seed floss and stem fibers lacked the tensile strength and qualities to compete with more popular hemp, ramie, and cotton fibers (Gerhart, 1928).

The most notable utilization of milkweed floss was as a replacement for kapok fiber in life vests worn by Canadian and US service members during World War II (Whiting, 1943; Berkman, 1949; Gaertner, 1979). In 1943, the US government opened a specialized milkweed seed floss extracting plant coined the “Milkweed Floss Corporation of America” in Petoskey, Michigan, which produced two million pounds of seed floss collected from milkweed populations across 26 US states for the armed forces during the single year of its operation (Berkman, 1949). Postwar interest in milkweed as a fiber crop continued but never again approached the level of investment of resources in 1943, and the same can be said for its use as a source of rubber and oil (Campbell, 1983). While milkweed fibers can be blended with other fibers such as cotton, the smooth and brittle nature of milkweed fiber hinders mechanical spinning (Louis & Andrews, 1987; Dréan, 1993).

Despite its many limitations, interest in milkweed as a fiber crop has continued into the 21st century, as manufacturers consider natural alternatives to synthetic materials (Rosu et al., 2011; Hassanzadeh & Hasani, 2015; Rasile-Digrindakis, 2019). Modern research may yet find a place for milkweed in the textile or fashion industries, but for now milkweed continues to embody
Gaertner’s poignant title of “greatest underachiever” and is mainly grown and sold as a flowering ornamental for use in landscaping and occasionally in honey production (1979; Bagi., 2008).

2.5 Milkweed as a Food Crop

In contrast to the research investments in milkweed as a potential fiber crop, we were unable to find evidence of research efforts that explore milkweed as a culinary crop for human consumption. Despite the knowledge gap in the scientific literature on the subject, a modern body of grey literature exists on the cultivation/preparation of milkweed as a foodstuff (Elias & Dykeman, 1990; Craft, 2011; Bergo 2020). In aggregate, these sources suggest that milkweed is not only edible as a perennial vegetable but is tasty, nutritious, and has potential as a productive, highly stress tolerant, edible crop. Perennial cropping system expert Eric Toensmeier described milkweed’s vitamin C content to be eight times greater than oranges (Toensmeier, 2020). A nutritional analysis of underserved vegetable crops led by Toensmeier (2020) found milkweed to be markedly high in calcium, vitamin A, and vitamin C when compared to commonly grown reference crops. In that analysis, the leaves of milkweed were used as representative samples; but while the young leaves of milkweed are edible, they are not commonly consumed and rapidly decrease in nutritional value as they mature (Gerhart, 1928; Gaertner, 1979; Bergo, 2020; Toensmeier et al., 2020). Instead, young shoots are typically harvested in late spring and prepared in a similar fashion to asparagus, while the immature inflorescences can be collected throughout the summer and parboiled, steamed, or fried (Gaertner, 1979; Bergo, 2020). Toensmeier described the taste of milkweed as “somewhere between asparagus and spinach” (personal communication, 2021). Other reported culinary uses for milkweed include the use of milkweed flowers as a colorant or sweetener for wines, teas, and syrups, as well as the cooking and consumption of young seed
pods, with the incipient seed floss imparting a “cheesy” consistency (Gaertner, 1979; Berkman, 1949; Bergo, 2020).

**Milkweed’s Ecological Services**

Alongside culinary uses, the grey literature on milkweed carries a strong ecological narrative that would be attractive to sustainability-minded growers in New England. Because the species is endemic to the region, the concern of unintentional introduction/invasiveness is not an issue as it might be for some exotic species. As a native species, milkweed is also a long-established member of the region’s plant guilds, especially in pasture ecosystems where milkweed provides an ecological service as a natural food source and habitat for native species of insects, arachnids, birds, mammals, and amphibians, all of which have been observed to utilize or be attracted to milkweed over the course of this study. Additionally, milkweed was observed to grow in a myriad of diverse locations around New England, including flat grasslands, the shaded edges of wooded areas, private/public landscaping projects, and rising from the cracks in sidewalks and asphalt on unkempt streets. Milkweed is known to be a hardy plant and will readily grow on marginal land with poor soil (Berkman, 1949). The extensive root system and the perennial nature of milkweed support its consideration as a climate-ready vegetable crop, as these traits help buffer drought stress while promoting soil health and increasing carbon sequestration at a rate higher than non-perennials (Toensmeier et al., 2020).

Milkweed’s current popularity as an ornamental is in large part due to its well-known status as a key player in monarch and other pollinator conservation efforts and it is marketed as such by nurseries across North America. Milkweed developed and cultivated for culinary reasons has the opportunity to share and build upon these same compelling narratives. Under favorable cultivation
conditions, harvested portions of the plant readily grow back within a single season, and monarch larvae consume the leaves as opposed to the shoots and buds more commonly eaten by humans. A milkweed cultivar developed for culinary uses may inhabit a niche within human agriculture as a nutritious vegetable crop grown on otherwise unused turf or unproductive land, making it potentially attractive to would-be growers as an adapted and nutritious crop with ecological merits for smaller New England farms and productive backyards.

2.6 Milkweed-Specific Agricultural Challenges

Despite its many merits, the development of milkweed as a food crop is not without its challenges, namely: 1) The actual (objectively true) biological characteristics of the species; and 2) The perceived (subjectively true) characteristics of the species by the public. Regarding the first challenge, as a NUCS milkweed faces all the challenges inherent to developing a new crop whether for commercial agricultural or homeowner use. Agronomic/breeding challenges include: characterizing extant diversity; developing reliably performing varieties for target environments; overcoming potential issues due to milkweed’s complex pollination system; and developing production best management practices, whether in the field or under controlled conditions (e.g., inducing flowering under greenhouse conditions) (Campbell, 1983). Moreover, herbivore/disease pressure in the form of aphid infestations and viral infections are common (Gerhart, 1928; Gaertner, 1979), with milkweed seedlings grown under greenhouse conditions being notably susceptible to thrips and struggling when potted, as they appear to invest primarily in the development of a vertical taproot (Figure 3c-d).

Regarding the second major challenge, there are a number of common perceptions of milkweed that present a challenge to its future as an acceptable food crop, even if its production
challenges can be overcome. From the perspective of those engaged in agriculture, milkweed is often viewed as a noxious weed whose eradication from farm fields either requires a labor intensive excavation due to its deep perennial root system or the application of non-selective herbicides such as glyphosate. In terms of the broader public, conservation education campaigns have successfully established milkweed as a pollinator-friendly species boasting a particularly beneficial cocktail of plant toxins that protect the charismatic monarch butterfly. Although this understanding of the species supports its planting in ornamental and pollinator gardens, it also suggests it to be inedible to humans, with little distinction being made between latex-rich leaves and stem versus edible shoots and immature inflorescences. To this point, rhubarb presents a promising precedent, being a plant whose uneaten leaves are well-known to be toxic to humans. Even without toxic associations, however, marketing and achieving popular uptake of new, unfamiliar crops is a formidable challenge. In this case, the challenge is complicated by the name “milkweed” itself, a name that would likely require rebranding to build public interest, as was found necessary for promoting the abundant “trash fish” known as dogfish (*Squalus acanthias*) for New England markets (Anthony, 2014).
Addressing Milkweed’s Agricultural Challenges

Despite these obstacles, milkweed has potential to be adopted as a crop by backyard growers. The species’s agronomic traits of interest, prior historical utilization, and allure to consumers as an ecologically friendly planting option support the possibility of a revitalized relationship between humans and this would-be domesticate for New England. While already well adapted to New England growing conditions, milkweed’s wide natural range and the diversity of closely related members within *Asclepias* suggests ample genetic diversity is available for researchers interested in selectively breeding the species. As an already popular flowering ornamental with a low level of necessary inputs, strong connections to current conservation efforts, and status as an historical forage crop, a productive milkweed cultivar could be readily implemented for culinary purposes by backyard growers already familiar with the species with limited fiscal risk involved. By marketing milkweed as a backyard food crop, milkweed’s transition into the modern food system may skirt the concerns of agronomic scalability that impede the feasibility of many other NUCS while providing a means for New England cultivators to convert otherwise unused land into stands of nutritious, pollinator friendly, perennial milkweed.

Barring milkweed’s agricultural potential from being realized as presented above are concerns related to the species’s biological characteristics that undermine human cultivation/reproductive control efforts and the aforementioned widespread public perception that milkweed is inedible. Agronomic traits of interest, both positive and negative, must be identified and variability within those traits measured in order to truly determine their effects on yield and quality. It is possible that the impact of negative traits can be reduced or negated entirely with targeted breeding or species-specific cultivation practices. However, while easily said, the
breeding and introduction of a novel crop is rife with unforeseen obstacles but these obstacles will remain unseen unless efforts are made to study milkweed within this context.

Although the biological and social obstacles to developing milkweed will take concerted effort and time to overcome, there is no reason not to start work on both. The misconception of milkweed’s unpalatability is based on the presence of a class of bitter-tasting, steroidal toxins called cardenolides that are sequestered by monarch larvae (Agrawal et al., 2012). By measuring the concentrations of these toxins within local populations of milkweed, an opportunity exists to initiate agronomic research on the species while concurrently addressing qualms over its toxicity.

As explained in detail in Chapter 3, research on cardenolide content within milkweed has been ongoing since the 1960s but within the context of chemical ecology and never from an agricultural perspective (Brower et al., 1967). And while the relative content of cardenolides within some milkweed tissues is known and the palatability of the species evident by its extensive history of use, the amount of cardenolides present in a serving size of young shoots or flower buds prepared for human consumption is not well understood. Illuminating the possible amount of cardenolides that are transferred from field to plate is an important component for challenging milkweed’s social obstacles. Additionally, because cardenolides are a large and diverse class of compounds exhibiting a wide range of physical properties, different culinary techniques (e.g., blanching or sautéing) may aid in the removal of some cardenolides before consumption. When boiled, milkweed is often cooked in three changes of water, becoming creamy and losing bitterness as residual latex and metabolites are boiled away (Toensmeier, 2020). Investigating the effects of different preparation methods may help to reduce cardenolide intake and improve taste. By sampling from populations around New England, the regional genetic diversity for this trait can be evaluated and may help inform future research on the location and availability of potential
breeding stock. Identifying the levels of certain toxins within milkweed tissue, possibly reducing their levels with certain culinary practices, and understanding their relative expression within regional milkweed populations is a first step in investigating this NUCS’s potential in New England.
CHAPTER 3

CARDENOLIDE CONTENT AND QUANTIFICATION IN *Asclepias syriaca*

3.1 Introduction to Cardenolides

Cardenolides are a diverse group of bitter-tasting, steroidal secondary metabolites produced by a wide range of botanical families, such as Moraceae and Plantaginaceae, including members of the genus *Asclepias* (Seiber et al., 1982; Petschenka et al., 2013; Agrawal et al., 2021). They are thought to be produced solely for defensive purposes and have been long studied as a mediator of antagonistic ecological interactions (Agrawal et al., 2021). Digitoxin, a well described cardenolide, is commonly used to treat arrhythmia and heart failure (Raghavan et al., 2023). For the purposes of this research, variable cardenolide concentrations in milkweed represent a trait of agronomic interest due to their potential toxicity to humans, making necessary the quantitation of their relative concentrations across accessions.

3.2 Classification and Structural Diversity of Cardenolides

Cardenolides are categorized as part of a greater class of compounds called cardiac glycosides, which derive their name from their observed effect on heart muscle and are produced as defensive chemicals by various plant, insect, and toad species (Bartos & Pesez, 1976). Cardiac glycosides are C-23 steroidal toxins comprised of three structural elements: (1) a pregnane backbone consisting of four fused carbon rings with (2) a lactone ring at C-17 and (3) a sugar linked glycosidically to a 3-hydroxyl group on C-3 (Bartos & Pesez, 1976) (Figure 4). Cardiac glycosides with five-membered lactone rings are classified as cardenolides, while compounds with 6-member rings are called bufadienolides (Bartos & Pesez, 1976).
In both cardenolides and bufadienolides, the sugar moieties which branch off the pregnane backbone can vary in size, chemical composition, and polarity. Additional monosaccharide units can also be linked to the sugar attached to the steroid to form complex polysaccharide chains (Bartos & Pesez, 1976). Because of this variety in sugar moieties, cardenolides as a group display a wide range of chemical and physical properties. Plant species that deploy cardenolides for defensive measures are thought to utilize this range of properties to modify the effectiveness of their chemical defenses in response to environmental stimuli (Malcolm, 1994; Agrawal et al., 2012).

**Figure 4.** Cardenolide structural elements including (a) the basic anatomy of the pregnane backbone with distinctive functional groups at C-3 and C-17, (b) a comparison of the C-17 lactone rings that distinguish cardenolides from bufadienolides, and (c) cardenolides desglucouzarin and digitoxin, each with distinctive sugar moieties.
3.3 Biosynthesis, Toxicology, and Ecological Importance of Cardenolides

Cardenolide biosynthesis has been studied primarily in foxglove species (*Digitalis* spp.) and thale cress (*Arabidopsis thaliana*) due to their use in cardiovascular treatments and potential use as tumor suppressants (Perez-Bermudez et al., 2009; Kreir & Muller-Uri, 2018). While little work has been done to elucidate cardenolide biosynthetic pathways in *Asclepias* spp., it is thought that the orthologues responsible for cardenolide production are highly conserved across five different angiosperm orders, including those containing both *Asclepias* and *Digitalis* species (Bauer et al., 2010). Cardenolides and other C23 steroids can be derived from a wide range of cholesterol precursors or pregnane intermediates (Agrawal et al., 2012). In 1998, Kreis et al. argued that due to the varied routes utilized, cardenolide biosynthesis should be imagined as a “complex multidimensional metabolic grid” rather than a set of defined pathways. Since then, much has been done using radiolabeled precursors to elucidate the pathways leading to cardenolide biosynthesis, including identification and characterization of key enzymes and their underlying genes (Perez-Bermudez et al., 2009; Bauer et al., 2010; Kreis & Muller-Uri, 2018). Two putative pathways involving either (1) progesterone or (2) 23-nor-4,20(22)E-choladienic acid-3-one as pregnane intermediates are suggested as the main routes for cardenolide synthesis (Agrawal et al., 2012).

In *Digitalis* species, it is thought that the intracellular management and storage of cardenolides is facilitated by the addition/removal of glucose to the tail end of the sugar moiety. This glucose molecule increases the overall polarity of the cardenolide and prevents its passive efflux through vacuolar membranes (Kreis et al., 1993; Agrawal et al., 2012). While an organizational mechanism has not been described in *Apocynaceae* (the family of milkweeds), it is thought that a similar system is used and could help explain the organization and release of
cardenolides in the tissues of many milkweeds (Seiber et al., 1982; Agrawal et al., 2012). Milkweeds get their namesake from the production of latex – a milky-white substance containing a wide array of specialized metabolites that exudes from a network of specialized cells called laticifers in stem, leaf, and pod tissues when damaged (Hagel et al., 2008; Agrawal et al., 2012). Non-articulated laticifers are elongated channels of multinucleated cells that hold cytoplasmic latex under pressure until ruptured (Hagel et al., 2008; Huber et al., 2016). Latex quickly coagulates when exposed to air and acts as both a physical deterrent for chewing herbivores and a delivery system for defensive chemicals, including cardenolides (Agrawal, 2005). Although cardenolide concentration and latex exudation are commonly associated together as defensive traits, there is no evidence of genetic correlation between them and different Asclepias species exhibit varying levels of expression for both traits (Agrawal et al., 2014; Agrawal & Hastings, 2019).

Members within Asclepias produce up to 200 structurally different cardenolides that all appear to share a similar, well-defined pharmacological effect (Zust et al., 2019). When ingested by an herbivore, the cardenolides bind to and inhibit the membrane extruding part of the ubiquitous animal ion pump Na⁺/K⁺-ATPase (hereafter ATPα) (Petschenka et al., 2013; Zust et al., 2019), thereby limiting the ability of affected cells to maintain proper ionic concentrations across membranes. The result can be especially disruptive to cardiovascular and nervous system function (Yamane et al., 2010). The symptoms and timing of acute cardenolide poisoning depend on species (plant and herbivore), the age and type of milkweed tissue consumed, and the overall amount of tissue consumed (Keener & Tu, 1983; Rahnama-Moghadam et al., 2015). Initial symptoms such as nausea may emerge within minutes, while the presentation of more serious symptoms like dangerous dysrhythmias may take up to 72 hours (Rahnama-Moghadam et al., 2015). Fatal
cardenolide poisonings in mammals typically include tetanic seizures, second/third-degree heart block, and cardiac arrest (Keener & Tu, 1983; Rahnama-Moghadam et al., 2015). *Asclepias* species native to the western United States, including narrowleaf milkweed (*A. mexicana*), whorled milkweed (*A. galioides*), showy milkweed (*A. speciosa*), horsetail milkweed (*A. subverticilata*), Utah milkweed (*A. labriformis*), Indian milkweed (*A. eriocarpa*), and plains milkweed (*A. pumila*), are thought to be responsible for intermittent livestock poisonings due to lack of adequate forage or from contaminated feed (Marsh and Clawson, 1921; Keener & Tu, 1983). For example, 250 sheep died in 1975 after ingesting hay potentially contaminated with Indian milkweed in Sonoma County, CA (Keener & Tu, 1983).

Despite the physical and chemical obstacles milkweed presents to many would-be herbivores, a diverse group of specialized insects have co-evolved to not only ingest but in some cases substantively benefit from cardenolide-rich milkweed tissue (Fordyce & Malcolm, 2000; Agrawal et al., 2012; Petschenka et al., 2013). Adaptations by herbivorous insects to overcome milkweed’s defenses vary, and it is within the context of antagonistic pest-host interactions that various *Asclepias* species have been studied by chemical ecologists seeking to understand the co-evolutionary pressures that drive these relationships. Some of these adaptations are simply behavioral, enacted throughout the life history of the insect species. For example, stem weevils avoid latex-rich tissues during feeding and oviposition while monarch larvae intentionally puncture lactificers to drain latex from the section of tissue they intend to consume (Fordyce & Malcolm, 2000; Agrawal, 2005). Foliage-feeding red milkweed beetles leverage the induced defensive response of milkweeds to improve the chances of their root-feeding offspring (Erwin et al., 2014).
Many milkweed herbivores have also co-evolved to resist the chemical defenses of their hosts. One such adaptation is the development of proteins with increased target-site insensitivity to cardenolides (Petschenka et al., 2013). Because the relative ATPα affinity of any cardenolide binding site is governed by its residues, different ATPα protein sequences result in different binding affinities for cardenolides. Agrawal et al. (2021) found the monarch butterfly ATPα homologue to be 50 to 100 times more resistant to cardenolide binding than its porcine equivalent, and the evolution of target-site insensitivity is known to have occurred multiple times in unrelated lineages. Both Dobler et al. and Zhen et al. reported in 2012 on the high level of molecular convergence in ATPα substitutions in Apocynaceae-feeding insects from four different orders, suggesting that these adaptations have high payoffs for a low evolutionary cost. And some insects, such as the aforementioned monarch butterfly, take this cardenolide insensitivity a step further and are able to sequester these toxins for use in their own defenses (Agrawal et al., 2012).

While there is little published on cardenolide expression in milkweeds from a potential crop (i.e., human food) perspective, the extensive literature on cardenolides as defensive compounds within Asclepias provide useful insights into cardenolide expression as a trait of agronomic relevance. For example, work has been done to categorize and quantify the different cardenolides expressed by Asclepias species, with a particular aim to identify those that are preferentially sequestered by monarchs (Roeske et al., 1976; Malcolm, 1994). The varied chemical structures of milkweed-produced cardenolides are a major factor in their effects once consumed, with nonpolar cardenolides thought to be more effective at passing through gut membranes and into the bloodstream than polar ones (Rasmann et al., 2009; Agrawal et al., 2012). This knowledge is useful in establishing a base-level understanding of what kinds of cardenolides may be transferred from field to plate and thus relevant to the development of A. syriaca as a perennial
vegetable crop, including how culinary preparation methods may impact direct gut uptake (Agrawal et al., 2012).

3.4 Chemical Profile of Cardenolides in Asclepias syriaca.

Early interest in Asclepias spp. and their cardenolides emerged in 1914 when British evolutionary biologist E. B. Poulton challenged American chemists to explain why some aposematically colored butterflies are toxic to birds (Malcolm, 1994; Agrawal et al., 2012). Starting in the late 1950s and in response to Poulton, researchers published potential explanations of the toxicity of monarch butterflies, citing the presence of cardenolides in both monarchs and Asclepias spp. and the capacity for monarchs to sequester cardenolides from their host plants (Brower, 1958; Parsons 1965; Rothchild et al., 1966).

Starting in the late 1960s, researchers began outlining the chemical characterization of cardenolides found in Asclepias spp. Milkweed produces a wide array of cardenolides with different sizes, shapes, and polarities (Agrawal et al., 2012). Studies reporting on the cardenolides observed within milkweed tissue reflect this diversity but are sometimes conflicting as separate researchers identify different cardenolides in various tissues and in populations both within and beyond the species’ natural range (Roeske et al., 1976). In 1989, Malcolm et al. detected the presence of 27 different cardenolides using thin-layer chromatography (hereafter TLC). By comparing known TLC cardenolide mobilities, seven of these 27 cardenolides were identified as syriogenin, usarigenin, uzarin, labriformidin, and labriformin. Additional cardenolides observed in milkweed tissue include desglucouzarin and xysmalogenin (Roeske et al., 1976; Sikorska & Matławska, 2000). Uzarigenin is the most concentrated cardenolide in stem and foliar tissue (Nelson at al., 1981). And just as type can vary, total cardenolide concentrations
within milkweed can also differ greatly between tissue types within a single individual, among members within a genetic family, and across genetic families within a population (Roeske et al., 1976; Fordyce & Malcolm, 2000; Agrawal et al., 2012).

Given their wide variety in form and levels of expression, the separate identification and quantification of cardenolides in a tissue sample can be technically challenging, if not outright impossible, depending on the methods employed. A common approach to quantifying total cardenolide concentration in a sample is to compare the aggregated chemical activity (such as light absorbance) of all cardenolides to that of a standard reagent (generally digitoxin) at a known quantity. Because of this, total cardenolide content is often expressed in terms of milligrams of an equivalent standard reagent per milligram of dry tissue (\( \frac{\text{mg of equivalent standard}}{\text{millgram of dried tissue}} \)), hereafter abbreviated as mg/mg.

3.5 Cardenolide variation within Asclepias syriaca

Cardenolide production is controlled genetically and therefore subject to variation across different Asclepias species and genetically isolated populations of common milkweed. Throughout their regions of natural distribution, Asclepias species typically display a geographical trend in cardenolide content in which lower latitude species/populations from tropical regions display higher concentrations, with up to threefold differences when compared to those at higher, more temperate latitudes (Vannette & Hunter, 2011; Agrawal et al., 2012). But common milkweed is an outlier in this regard, with seemingly no latitudinal trend in cardenolide concentration. Instead, a slight trend in greater latex exudation at higher latitudes within the continental United States has been observed (Agrawal et al., 2012).
Roeske et al. (1976) proposed three general groupings for North American *Asclepias* spp. based on similar cardenolide profiles, geographical distribution, and population density: (1) low cardenolide concentration, wide distribution, and high density, (2) medium cardenolide concentration, narrow distribution, and low density, and (3) high cardenolide concentration, narrow distribution, and low density. Common milkweed belongs to this first grouping with its wide geographical range of densely clustered populations and relatively low concentration of cardenolides when compared to close relatives (Table 6).

**Table 6.** A summary of four publications reporting cardenolide content within different *Asclepias* species. Data from ¹Malcolm et al. (1989); ²Martin et al. (1992); ³Malcolm & Browler (1989); ⁴Martin & Lynch (1988).

<table>
<thead>
<tr>
<th><em>Asclepias</em> species</th>
<th>Cardenolide Content (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td><em>A. syriaca</em>¹</td>
<td>0.05</td>
</tr>
<tr>
<td><em>A. humistrata</em>²</td>
<td>0.417</td>
</tr>
<tr>
<td><em>A. viridis</em>³</td>
<td>0.376</td>
</tr>
<tr>
<td><em>A. asperula</em>⁴</td>
<td>0.245</td>
</tr>
</tbody>
</table>

For common milkweed, cardenolide concentrations vary widely at the population level. In the most comprehensive study to date, Malcolm et al. (1989) collected 158 foliar tissue samples from 28 collection sites across much of common milkweed’s northern and eastern distribution from northern North Dakota in the west to Vermont in the east and then Virginia in the south (Table 6, Table 7). They observed cardenolide content to range from 0.0004-0.299 mg/mg with a mean of 0.05 mg/mg. While differing in the absolute levels detected, other studies reflect this wide variation in total cardenolide content (Table 7).
Table 7. Summary of works measuring foliar tissue cardenolides of common milkweed (Asclepias syriaca) collected from various locations within the United States. Data from 1Malcolm et al. (1989); 2Roeske et al. (1976); 3Vannette & Hunter, (2011); 4Woods et al. (2012). 5Note that Woods et al. (2012) includes two separate measurements of the same sample group.

<table>
<thead>
<tr>
<th>Name of study</th>
<th>Collection Range</th>
<th>N</th>
<th>Results (mg/mg)</th>
</tr>
</thead>
</table>
| Cardenolide fingerprint of monarch butterflies reared on common milkweed, Asclepias syriaca L. | ND, MN, MI, ON, NY, VT, CT, NJ, PA, MD, VA | 158 | $\bar{x} = 0.004$-0.299  
$R = 0.05$ |
| Milkweed cardenolides and their comparative processing by monarch butterflies (Danaus plexippus) | MN | 100  | $\bar{x} = 0.006$-0.264  
$R = 0.114$ |
| Genetic variation in expression of defense phenotype may mediate evolutionary adaptation of Asclepias syriaca to elevated CO2 | MI | 188 | $\bar{x} = ND$  
$R = 0.00113$ |
| Adaptive geographical clines in the growth and defense of a native plant | ON, MI, NY, VA, NJ, PA, VT, MA, | 170 | $\bar{x} = 0.0001$-0.0006  
$R = 0.0003$ |

High in-family variation in cardenolide content has also been observed, spanning two- to threefold differences in total cardenolides under similar growth conditions (Bingham & Agrawal, 2010). Couture et al. (2013) further supported this finding, reporting a concentration range of 0.0001-0.00031 mg/mg in leaf samples from the progeny of a single seed pod in Wisconsin.

Cardenolides can be present in any plant tissue and their concentration can vary widely within an individual plant, even among closely allied tissues (e.g., leaves of various ages). Specialist herbivores that target different parts of the plant present a defensive challenge for common milkweed and are thought to influence the varied production of cardenolides across different tissues (Agrawal et al., 2012). Fordyce and Malcolm (2000) reported that cardenolide concentrations within various sections of the stem vary more than fivefold, with the vascular tissues (including the laticifer system) containing 56% of the total cardenolide content within an individual. They observed in 60 untreated milkweed genotypes an average total cardenolide concentration of roughly 0.026 mg/mg, with tissue-specific concentrations of the vasculature, pith, cortex, and epidermis being 0.150, 0.05, 0.047, and 0.025 mg/mg, respectively.
Environmental factors also are known to influence cardenolide content in milkweed species, including seasonal changes/phenostages (Nelson et al., 1981) and the introduction of artificial inputs (Vannette & Hunter, 2011; Agrawal et al., 2012; Couture et al., 2015). Regarding inputs, both N:P:K fertilization and ample waterings appear to reduce foliar cardenolide content, but because cardenolides are measured per mg of sample tissue, such treatments may simply be diluting concentration via increased overall biomass as opposed to directly affecting cardenolide expression itself (Agrawal et al., 2012; Couture et al., 2015). Based on work by Vannette and Hunter (2011), elevated atmospheric CO$_2$ was shown to have a mixed effect, with cardenolide concentrations dropping at CO$_2$ levels below 770 ppm (0.00077 mg/mg) in some families but having no effect in others.

The effects of biotic factors (e.g., pest pressure) on cardenolide content have been much more closely studied than abiotic ones. There are two general mechanisms by which milkweed increases localized cardenolide content in response to tissue damage: (1) a rapid efflux of latex to the affected area, and (2) a relatively delayed induction of increased localized cardenolide concentration in the affected tissue. Malcolm and Zalucki (1996) were the first to suggest that herbivore damage may induce a rise in localized, tissue-specific cardenolide concentration in milkweed. They observed the first mechanisms in action after hole-punching milkweed leaves, which led to a rapid increase in cardenolide content starting within 10 minutes, a threefold increase by 24 hours, and an eventual return to control levels within five days. In contrast, more recent studies indicate that the response caused by abiotic mechanical damage does not appear to result in a significant induction response beyond the initial efflux of latex (Mooney et al., 2008; Agrawal et al., 2012). In contrast, it only takes a small amount of damage from monarchs, as little as 5% leaf tissue loss, to trigger an induction response (Mooney et al., 2008). Such cardenolide induction
is systemic, appears to be genetically variable, and is associated with an endogenous jasmonic acid
burst. Artificial induction triggered by manually applying jasomic acid to the leaves of milkweed
can lead to a 15-30% increase in cardenolides as soon as three days post-damage before eventually
returning to pre-induction levels after about ten days (Agrawal et al., 2012). The extent of
cardenolide induction appears to vary by pest species as well. For example, aphid species *Aphis
nerii* and *A. asclepiadis*, while thought to have some impact on cardenolide levels within
milkweed, do not appear to cause the induction response observed elsewhere (Mooney at al., 2008;
Agrawal et al., 2012).

Again, while not concerned with the potential agricultural value of milkweed, studies
exploring the chemical ecology of cardenolide production in milkweed provide useful insights. In
a 2013 study investigating changes in foliar cardenolide content after purposefully damaging
milkweed leaves, Countre et al. were able to mitigate the efflux of latex-bound cardenolides in
damaged leaves by first notching the petioles. This observation may lead to useful harvesting
practices as stems could be drained of latex before harvesting flower buds. Our current
understanding of cardenolide inducibility suggests that the harvesting of flower buds would not
lead to cardenolide induction in neighboring flower buds or stems but, if necessary, a “resting
period” between harvests in the same location could be one strategy to limit the chances of such
induction. Similarly, if young seed pod consumption generates interest as a fried food, then
evaluating the maturation rate of seed pods and establishing a harvesting period may prove useful
as the mature seeds are exceptionally cardenolide-rich (Agrawal et al., 2012). The studies
referenced above also demonstrate that there is a wide breadth of variation in cardenolide-related
defensive traits within milkweeds and that at least some of this variation is genetically determined.
This suggests that, should the need arise, a breeding program invested in reducing cardenolide
expression in relevant tissues may have natural resources to draw from. And perhaps further downstream, research could potentially lead to productive milkweed lines with natural levels of cardenolides in non-edible tissue but with lowered expression in selected ones.

But before such projects can even be considered, initial research on cardenolide content in milkweed from an agricultural perspective must be conducted. Reliably quantifying the relative concentrations of cardenolides in common milkweed tissue samples is essential to crop development efforts, in terms of: (1) providing insight into regional variation among endemic milkweed populations throughout New England (i.e. germplasm acquisition and characterization), (2) providing a phenotypic assay for guiding parental selection and progeny evaluation (i.e. breeding), and (3) discerning what (if any) agricultural practices and culinary preparations are effective at reducing the cardenolide content while cultivating and preparing milkweed for consumption.

3.6 The History of Cardenolide Quantification via Spectrophotometry

Several analytical chemistry techniques can be used to measure cardenolide content. The predominant strategies for bulk cardenolide analysis employed by researchers fall under the wide umbrellas of spectrophotometry and chromatography. The core concept of the former relies on the quantitative analysis of photoactive compounds interacting with specific wavelengths of light. As light passes through a solution, individual photons may be absorbed or scattered by the constituents of the solution. A spectrophotometer is able to measure the change in light transmission (hereafter “absorbance”) due to these interactions and quantify it in terms of a change in absorbance units (hereafter AU).
Inferences about the chemical composition of a solution can be made based on what is known about the absorptive properties of the sample. When testing for the presence of a single photoactive compound within a solution, samples containing the compound will display higher AU values than those without. When quantifying the relative concentrations of photoactive compounds within a set of samples, a series of stock mixtures comprised of the photoactive compound at known concentrations can be used to establish AU reference points along a gradient of increasing concentration (hereafter referred to as a ladder). The AUs of the experimental samples can then be compared to this ladder and their concentrations mathematically deduced.

Spectrophotometry can also be used to quantify the presence of non-photoactive compounds if such compounds can first be made photoactive via chemical modification by certain conditions or reagents. Generating/enhancing photoactivity in this way can be used to overcome issues caused by background interference. For example, in complex mixtures, non-target contaminants may be photoactive at the very wavelengths required to measure a target. But if the photoactivity of the target compound can be influenced, then the additive effect caused by interference can be accounted for by measuring the difference in AU between a sample treated to increase target photoactivity and a “blank” sample without the treatment. It is through such strategies that spectroscopy can be utilized for a wide range of applications and identify specific compounds, photoactive or not, within complex mixtures.

Despite their incredible diversity, all cardenolides contain a single unsaturated lactone ring and no other light-absorbing features. They can also form charge transfer complexes with other compounds to become photoactive and impact the absorption of certain wavelengths of light in solution (Zust et al., 2019). While limited descriptions exist for the specifics of these reactions and their resultant color changes (i.e., interactions with precise wavelengths of light), it appears that
they generally rely on an introduced reagent interacting with anions belonging to either the sterol group or sugar moiety on the cardenolide in an alkaline environment (Rowson, 1952). It is this ability to colorize cardenolides that allows for their measurement with spectrophotometry. Depending on the reagent employed, the specificity of the reaction can be quite low and may include not only cardenolides but bufanolides and other related compounds as well. A lack of specificity is a major challenge when employing some reagents for this purpose (Rowson, 1952). An additional challenge of this type of assay is the possibility for unrelated photoactive compounds already present in samples to contribute to or interfere with absorbances at specific wavelengths. For example, early iterations of cardenolide analysis in milkweed via spectrophotometry attempted to measure absorbance at suboptimal frequencies as a way to avoid the absorptive interference caused by ubiquitous chlorophyll pigments (Brower et al., 1972). To study cardenolide content in plant tissues, researchers required a reagent with high specificity and preparation methods that reduced absorption from unrelated constituents.

In a series of publications, Bell and Krantz (1948; 1946; 1945) presented the findings of their investigations into the color intensity of cardenolides isolated from *Digitalis purpurea* when treated with the Baljet reaction and how the intensity of the resulting color correlates to their relative concentrations and cardiological effects. In 1952, J. M. Rowson explored different approaches to colorimetrically measure digitoxin in the leaves of *Digitalis purpurea*. Rowson used lead sugars to strip samples of interfering photoactive compounds and then employed various alkaloid reagents as a means of colorizing the resulting cardenolide-containing solutions. Rowson observed complications caused by unintended interactions with some reagents but eventually settled on recommending a process to depigment tinctures of *Digitalis purpurea* with lead sugars and measure the intensity of the colorimetric reaction between cardenolides and 3:5-dinitrobenzoic
acid. Rowson’s 1952 efforts to consistently measure cardenolides via 3:5-dinitrobenzoic acid and other reagents were later applied in 1972 to investigate *Asclepias* chemical ecology by Duffey and Scudder. Instead of depigmenting solutions with lead sugars, Duffer and Scudder utilized a chromatographic column to purify samples before subsequently analyzing cardenolide-containing fractions as they eluted from the column.

Through the late 1960s and concurrent with Duffey and Scudder’s cardenolide analysis work in the 1970s, Brower et al. (1967; 1968) released a series of publications that established a link between the unpalatability of monarch butterflies to blue jays after being raised on certain species of host plants and the presence of cardiac glycosides in the tissues of *Asclepias* species utilized by monarchs. In 1972, Brower et al. released their first paper spectrophotometrically measuring cardenolide content within butterfly tissue. While aware of the works of Rowson, Duffey, and Scudder, Brower et al. developed their assay based on a 1969 paper published by Rabitzsch and Tambor in the German medicine paper *Pharmazie*. Instead of 3:5-dinitrobenzoic acid or other reagents used by their contemporaries, Brower et al. used 2,2’4,4’-tetranitrodiphenyl (hereafter TNDP) as the reagent of choice. This TNDP-based assay can be divided into three general stages: (1) Sample Preparation – solutes from dry powdered tissue are dissolved in ethanol and depigmented, (2) Reaction Mix Creation – a small amount of sample is combined with a TNDP mixture and the reaction catalyzed by sodium hydroxide, and (3) Spectrophotometric Measurement – the absorbance of the reaction mix is measured at a specific wavelength.

Because their 1972 study was conducted using ethanolic tinctures derived from soaking powdered insects, Brower et al. employed a simple preparation method that had no depigmentation step, noting that their “experimental arrangement canceled the absorbance due to plant and animal pigments but still permitted accurate detection of the reaction absorbance.”
In the following decades, Brower and others would further develop their understanding of the milkweed-monarch relationship by improving upon their original TNDP-based assay. Brower’s 1972 version of the assay was modified and used in 1974 and 1975 to measure cardenolides in butterfly tissue (Brower & Moffitt, 1974; Brower et al., 1975). It was not until 1981, however, that Brower and colleagues published on the use of a TNDP-based assay alongside a lead acetate cleanup step based-off of Rowson’s 1952 work to quantify cardenolide content in *Asclepias eriocarpa* tissue (Nelson et al., 1981). Absorbance measurements were taken pre- and post-clean up to assess the recovery rate of the sample treatment. Unfortunately, the recovery rate was variable, ranging from 67% to 107% of cardenolides recovered. A year later, Brower and colleagues would publish a similar paper in which a refined lead cleanup step was utilized, this time with a realized recovery efficiency of 82% (Brower et al., 1982). Brower and colleagues would ultimately rely on this TNDP-based assay, with some variations and in conjunction with other chemical assays, throughout the following decades (Martin et al., 1992).

Interest in a TNDP-based spectroassay of cardenolides in milkweed tissue waned in the late 1990s and early 2000s as researchers began to favor high-performance liquid chromatography (hereafter HPLC). But from 2005 to 2009, a handful of papers were published that utilized a TNDP-based assay derived from Brower and colleagues’ 1982 protocol (Agrawal, 2005; Mooney et al., 2008; Agrawal et al., 2009; Delaney et al., 2009; Rasmann et al., 2009; Agrawal et al., 2021). Deviations from the 1982 assay were minor and generally related to the type of spectrophotometer available. As examples, in 2005 Agrawal adapted the assay for a microplate reader and in 2009 Delaney et al. increased the volume size of the reaction mix for use with 3.5 ml cuvettes.

Beyond the changes to accommodate different spectrophotometers, the 1982 TNDP-based assay and the later versions conducted in the 2000s also differed in terms of starting sample sizes,
extraction step lengths, and the inclusion (or not) of a lead acetate cleanup step. A review of the literature yielded no publications during the 2000s that mentioned the use of a lead acetate cleanup step during sample preparation. A related procedure to the 1982 cleanup step for cardenolide analysis was published in 2021 by Agrawal et al., but this procedure was comparably simpler to Brower’s and samples were prepared for HPLC and not for spectroscopy. In a personal communication, Agrawal (2021) emphasized the usefulness of a lead acetate cleanup step for preparing plant tissue samples but may have adopted the step after switching to HPLC.

While spectrophotometry and HPLC are the most widely utilized methods for quantifying cardenolides in *Asclepias*, other analytical methods exist. Alongside HPLC, there is an array of other liquid or gas chromatography assays used for both quantitative and qualitative cardenolide analysis (Ravi et al., 2020; Klein et al., 2021; Raghavan et al., 2023; Rubiano-Buitrago et al., 2023). In 2020, an assay involving liquid chromatography coupled with mass spectrometry was developed for the first time to both profile and quantify cardenolides specifically from plant extracts and has a 1000-fold increase in sensitivity over HPLC (Ravi et al. 2020). Other cardenolide-related assays have been developed that are not applicable in this setting. Immunoassays for some cardenolides exist but are narrow in their specificity and not applicable for use in tissues where cardenolide amount and type can vary (Yoshimatsu et al., 2004), which is likely the case in milkweed extracts. In a similar vein, an ATPα inhibition assay for measuring the bulk effects of cardenolides on porcine ATPα has also been developed. But since the binding affinity for cardenolides to ATPα varies by type, no clear observations referring to combined concentration of different cardenolides within a sample can be made (Petschenka et al., 2023).
3.7 Troubleshooting a Cardenolide Spectroassay for *A. syriaca*

Despite its long history of utilization, milkweed is generally viewed as unpalatable as its public reputation is strongly connected with monarch conservation and ecology. Before promoting the potential culinary uses of milkweed, the supposition of its inedibility/toxicity must be addressed and an understanding of the amount of cardenolides per serving must be established. Being able to measure cardenolides in edible milkweed tissues also can inform those interested in the agricultural development of this species and aid in the adoption of this NUCS by growers. For example, given the wide range of variation in cardenolide concentrations observed in prior studies, identifying regional populations with relatively low cardenolide expression could serve as a useful starting point in developing the species for New England. To these ends, a method of reliably quantifying bulk cardenolide content in plant tissue samples was required.

3.7a Initial TNDP-based Assay

Due to the relative accessibility of equipment and the costs/expertise requirements for HPLC, a TNDP-based spectroassay was selected as the quantitative method of choice for this work. An initial protocol for the assay provided by Agrawal in a personal communication (2021) included the three general stages of the assay previously mentioned, namely (1) sample preparation, (2) reaction mix formulation, and (3) absorbance measurement. The protocol also included steps for the creation and use of a digitoxin ladder. Elements from other TNDP-based protocols were explored during various aspects of troubleshooting and separate changes were made across all three stages. The initial protocol for the TNDP-based assay is as follows; Freeze-dry tissue samples and grind to a fine powder. Mix 50 mg of fine powdered tissue into 1.6 ml of 95% ethanol, agitate for 90 s with a vortex mixer, and centrifuge at 14000 rpm for 12 min. Load
45 µl of supernatant into two wells of a 96 well-plate. One 45 µl aliquot will be used to create the reaction mix and the other used as the reference blank. Add 90 µl of a 0.15% TNDP solution to the experimental well and 90 µl of 95% ethanol to the blank. The same is done for wells containing the ladder, except that stock solutions of digitoxin are used in lieu of ethanolic plant extracts. Next, the charge-exchange reaction is initiated with the addition of 70 µl of 0.1 M NaOH to ladder, experimental, and blank wells. Afterwards, the well plate is left in a dark space for 20 minutes, and the color is allowed to develop as the sample is incubated.

To prepare the 0.15% TNDP stock solution, weigh out the amount of powdered TNDP required for each experimental well and mix into a corresponding amount of 95% ethanol and 0.5 ml of acetonitrile. The 0.5 ml of acetonitrile helps dissolve the solid TNDP into solution and the volume added remains consistent regardless of the volume of 0.15% stock solution being prepared. While the starting amounts of TNDP and ethanol vary depending on the number of wells being measured per experiment, the 0.5 ml of acetonitrile remained consistent throughout experiments.

Twenty minutes after starting the reaction, the well plate is measured at 620 nm via microplate reader. The AU values of the experimental samples can be corrected by subtracting the value of their respective blanks. The corrected AU values of the ladder can be plotted (X = the observed AU for each standard, Y = the known \( \frac{mg\ of\ Digitoxin}{mL\ of\ Ethanol} \) concentrations for each standard, Y intercept = 0) to generate a 2nd order polynomial (\( Y = aX^2 + bX + 0 \)). Using this function, the corrected experimental AU results for the bulk cardenolides can then be transformed into their equivalent milligrams of digitoxin. To calculate cardenolide content of dry tissue, the transformed values for each sample (\( \frac{mg\ of\ Digitoxin}{mL\ of\ Ethanol} \)) are then divided by 31.25 \( \frac{mg\ of\ dry\ tissue}{mL\ of\ Ethanol} \), resulting in a value reflecting the amount of cardenolides present in the sample tissue and expressed in terms of \( \frac{mg\ of\ Digitoxin}{mg\ of\ dry\ tissue} \).
3.7b Digitoxin Ladder Design and Results

The ladder for this assay was derived from a stock solution of digitoxin in 95% ethanol and comprised of standards with concentrations of 1.0 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml, and 0.0 mg/ml of digitoxin in 95% ethanol. This ladder was used across all experiments and the color change behavior and AU values from these digitoxin mixtures were consistent throughout.

After the addition of NaOH, the initially clear ladder wells immediately darken and develop a deep blue coloration, visually suggesting that the assay functions as intended for ethanolic solutions containing cardenolides. As full-spectrum light enters the well, a subset of the photons within that spectrum interact with the charge transfer complexes comprised of the dissolved TNDP and digitoxin. In blank ladder wells without TNDP, the addition of NaOH induced no color change. Because the range of the frequencies of light absorbed by the reaction does not include frequencies below 450 nm on the visible spectrum, the remaining unabsorbed photons exiting the well confer a bluish tint to the solution when observed. The intensity of the coloration increases alongside the concentration of digitoxin (Figure 5). This circumstantially indicated that the colorimetric reaction is dependent on the amount of cardenolides present in each well when all other reagents are present across the ladder at the same concentrations.

These empirical observations on the success and the functionality of the TNDP-based assay are supported by the measured AU results for the ladder (Figure 5). Ladder AU results behaved as expected and displayed a consistent gradient in AU values with the highest value corresponding with the highest concentration of digitoxin. Due to the lack of TNDP in the ladder blanks, they typically measured from 0.041-0.045 AU with an average of 0.042 AU, which was observed to be the general result for wells containing solutions with little to no absorptive properties. Polynomials
derived from ladder results also consistently had high coefficients of determination ($R^2 > 0.99$), suggesting a good fit between the real-world behavior of the ladder and the polynomial model (Figure 5).
Figure 5. The results of measuring three technical replicates of the digitoxin ladder. The ladder is generated by measuring the absorbances of a series of digitoxin solutions of known concentrations (0.0-1.0 mg/ml). The absorbances of these solutions at 620 nm is measured twice – one without the TNDP dye (blank wells) and one with the dye (experimental wells). In the experimental wells, the interaction of TNDP with digitoxin generates an observable colorimetric reaction which directly relates to the relative concentration of digitoxin within the wells (a). The absorbance unit values from these measurements (b) can be used to derive a best-fit polynomial (c) which in turn can be used to calculate the concentration of cardenolides in tissue samples measured on the same 96 well plate.
3.7c Plant Extract Results and Troubleshooting

The TNDP-based assay as described previously led to immediate complications when applied to plant tissues. Early trials resulted in nonsensical AU values that either vastly exceeded the 0.0004-0.299 mg/mg range of cardenolide measurements observed by Malcolm et al. (1989) or were sometimes nonexistent or negative due to their respective blanks having nearly the same or more absorbance (Figure 6 and 7). Troubleshooting began by first halving the amount of ethanol used to generate plant extracts from 1.6 ml to 0.8 ml with the idea being that a smaller volume would better concentrate the cardenolides dissolved in solution (Figure 6). But instead of increasing the corrected AU values, the 0.8 ml resulted in lower absorbance. Additionally, the calculated cardenolide concentrations for nearly all the 1.6 ml extracts exceed the observed range for the species, despite having less cardenolides to contribute to the total AU of the sample. This trend broadly persisted in subsequent repeats of this experiment.
The results of measuring absorbances on three sets of 50 mg milkweed leaf extracts made with two starting volumes of ethanol (1.6 and 0.8 mL), with two technical replicates per sample/volume combination. The corrected absorbance results of the digitoxin ladder (a) were used to generate a best-fit polynomial (b). This polynomial was then used to convert the absorbance unit results of the leaf extracts into values that represent the theoretical equivalent of digitoxin contained within each sample (c). While the ladder (a) and derived trendline (b) behaved as predicted, the corrected results for the plant extracts (c) did not.

To further explore the option of concentrating plant extract in order to increase the concentration of cardenolides in each sample, the starting sample mass was scaled up from 50 mg to 250 mg and the starting extract volume was raised from 1.6 ml to 8 ml of 95% ethanol. This was done due to difficulties in fully dissolving 50 mg of powdered plant tissue in 0.4 ml ethanol and recovering sufficient supernatant for technical replicates or various experiments requiring extract from the same sample. Three 250 mg tissue samples from the same source were mixed into 4 ml, 6 ml and 8 ml of 95% ethanol and measured at 620 nm. This experiment was conducted three separate times with different tissue samples each (Figure 7). In this set of experiments, the samples with smaller volume appear to confer higher AU values for both the experimental and blank wells.
This would make sense as a higher concentration of cardenolides should be present in the 4 ml extracts over the 6 and 8 ml. But the corrected AU values lacked adequate consistency and in some cases the 6 ml or 8 ml results exceeded those of the 4 ml in the same experiment.
Figure 7. The results of three identical experiments (a-c) measuring the absorbances of plant extracts made from 250 mg of powdered milkweed leaf tissue combined with 4, 6, and 8 ml of 95% ethanol, with three technical replicates each (left, center, and right plots within each panel). The experimental wells contained TNDP while the blank wells did not. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values. These corrected AU results were intended to reflect the relative contribution of the TNDP-cardenolide complexes to absorbance at 620 nm. As the starting extract volumes increase both experimental and blank AU levels tend to decrease as the extracts become more diluted. But this trend does not extend to the corrected results, as shown by roughly equivalent experimental and blank AU values.
In order to determine whether an extended mixing step would lead to a more thorough extraction of foliar cardenolides, samples were placed on an oscillating table and measured at 0, 20, 60, and 120 minutes after preparing the extract (Figure 8). This was done with three replicates of the same tissue. The results showed no noticeable change in AU values, suggesting that if cardenolides continue to dissolve into solution over time, that change is not measurable due to other variables at play.

![Figure 8](image)

**Figure 8.** The results of measuring the absorbances at 620 nm of three plant extract replications following extraction in 95% ethanol for a range of times. Plant extracts were prepared with 250 mg of powdered milkweed leaf tissue and 8 ml of 95% ethanol. Samples were placed on an oscillating table set to a low speed for 120 minutes and their absorbances at 620 nm repeatedly measured at 20 minutes intervals. Omitted data entry for replicate three at 120 minute due to pipetting error.

Ideally, experimental and blank results should follow a pattern where the TNDP added to the former leads to more absorbance and therefore higher corrected AU results. Replicates derived from the same extract and measured simultaneously also should behave uniformly with relatively minor variation limited to that caused by observational or pipetting errors. Some variation in AU results for both the ladder and experimental samples can occur if the same experiment is conducted on different well plates, or the same well plate is measured at different times. This occurs not only due to the aforementioned sources of error but also due to the variable reaction rate of each well and plate. To ensure that the reaction mixture has reached maximum color development nearly
simultaneously across all wells, NaOH is added as the last step and well plates are measured after a strict 20 minutes of incubation. But despite these steps taken to reduce variation, the AU measurements as they are recorded represent a brief snapshot of a dynamic reaction that can vary across consecutive measurements and different wells. The digitoxin ladder is integral for generating a reference on which the results of unknowns can be evaluated as the ladder will always relate to the known concentrations of digitoxin despite potential variation across different experiments.

Much can be inferred from the general relationships between experimental wells and their reciprocal blanks. In Figure 5, when comparing the sample AU results with their blanks, a relationship between the AU values and the increasing concentrations of digitoxin is apparent. A similar relationship should be clear when measuring varying quantities of cardenolides from the same source of plant tissue. For example, if 100 mg of plant tissue are used to generate the extract instead of 50 mg, then ideally that would be reflected in both the observational relationship between experimental/blanks wells and the cardenolide concentration derived from comparing the corrected AU results with the ladder. Similar predictions can be made for samples containing additional digitoxin, in which AU results for these samples are higher than those without it. Yet while the TNDP-based assay as described above was able to generate consistent ladder AU results, trials with ethanolic plant extract yielded poor and erratic results overall (Figures 6 and 7).

The tendency for sample AU values to behave unpredictably in the above investigations suggested a significant contribution to absorbance at 620 nm by interfering photoactive compounds. These compounds include plant pigments such as chlorophylls a and b that both have a red absorption maxima near 661 and 642 nm (Lichtenthal et al., 2001). As the most common class of pigments in milkweed leaf tissue, chlorophylls are considered a key contributor to this
interference. Chlorophylls also have a broader range of absorbance than that of the target, meaning that shifting the wavelength of measurement from 620 to a lower or higher value is unlikely to improve results. Measurements taken at 600 nm and 630 nm were roughly equivalent to those at 620 nm, while measurements taken at 575 nm and 675 nm yielded uninterpretable results.

In Figure 6, the calculated cardenolide content of extracts prepared at various volumes suggest nonsensically, that somehow higher concentrations of plant extract contains fewer cardenolides. These AU values are not only suspicious as they suggest unrealistically high cardenolide content for *Asclepias syriaca*, but also because the observed downward trend with respect to increasing concentration simply does not behave as predicted. Unlike Figure 6 which presents the calculated cardenolide content based off the corrected AU results, Figure 7 directly presents the AU results from a similar experiment in which an apparent trend can be observed where the experimental and blank AU values of a sample increase with concentration. The corrected AU values, those used to determine cardenolide content, do not follow this trend. From these findings, it was hypothesized that while an increase in concentration may increase cardenolide content, doing so also increased the concentration of interfering photoactive molecules. This hypothesis also may explain the results seen in Figure 8 as, regardless of whether additional cardenolides were dissolving into solution over 120 minutes, the interference may have masked any changes in cardenolide content that might have taken place.

To ascertain the extent of this potential interference, digitoxin tracers of varying concentration were added to sample extracts during preparation. It was hypothesized that the addition of concentrated cardenolides could be discernable despite the interference. In this experiment, 250 mg of powdered plant tissue was mixed into 7 ml of 95% ethanol and 1 ml of digitoxin in 95% ethanol. The digitoxin tracers ranged in concentration from 0-1 mg/ml. This
experiment was conducted with three technical replicates (Figure 9). A general trend of increasing AU values alongside increasing digitoxin concentrations was observed in the experimental absorbances, but this uniformity did not extend to the blanks and corrected values. Repeats of this experiment resulted in similar observed trends. These results suggests that while the effects of adding digitoxin to a sample increase overall AU at 620 nm in the presence of TNDP, the variable interference generated by unrelated photoactive compounds in both the experimental and blank wells reduces the accuracy of the corrected results when measuring cardenolide content.
Figure 9. The results of measuring the absorbances at 620 nm of three plant extract replications (a-c) prepared with varying levels of supplemental digitoxin (0-1 mg/ml). Plant extracts were prepared with 250 mg of powdered plant tissue, 7 ml of 95% ethanol, and 1 ml of appropriately diluted digitoxin solution. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.

As previously mentioned, chlorophyll pigments were suspected to be a major source of interference. This observation was supported by absorbance measurements made of milkweed leaf, milkweed stem, and Spinacia oleracea (hereafter spinach) leaf tissue extracts. Spinach leaves were chosen as they are heavily pigmented and contain negligible levels of cardenolides, making them a representative sample with high chlorophyll but low cardenolide content. In contrast, milkweed stems are less pigmented than either milkweed or spinach leaves, and also contain greater levels...
of cardenolides than spinach leaves, making them a representative sample with low chlorophyll and high cardenolide content. Extracts derived from 500 mg and 250 mg of each tissue type were measured at 620 nm and their relative absorbances compared (Figure 10). Both milkweed and spinach leaf tissues had markedly higher experimental and blank well AU values than milkweed stem tissue, suggesting that the increased concentration of chlorophylls in those samples greatly contributed to absorbance. Stem tissue, being comparably low in chlorophylls, had lower sample AU values.

**Figure 10.** The results of measuring the absorbances at 620 nm of plant extracts made from milkweed leaves, milkweed stem, and spinach leaf tissue. Extracts were prepared with either 250 or 500 mg of each tissue type in 8 ml of 95% ethanol. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.

Due to consistent difficulties in accurately measuring cardenolides in ethanolic plant extracts, troubleshooting efforts turned towards attempting to reduce the AU values of sample blanks as opposed to increasing cardenolide-specific absorbance. To that end, a number of decolorization techniques were evaluated for their ability to selectively remove enough interfering compounds to improve the accuracy of the TNDP-based assay.
3.7d Lead Acetate Cleanup Step Design and Troubleshooting

The depigmentation technique most widely employed by researchers to spectrophotometrically quantify cardenolides in *Asclepias* species is the lead acetate cleanup step. During this process, samples are treated with a water-soluble lead compound, typically lead acetate or lead subacetate, and then washed with an anion donor, typically sodium sulfate (Rowson, 1952). As the dissolved Pb$^{2+}$ ions interact with the various chemical constituents within the solution, some form insoluble complexes and precipitate out as lead salts. Excess Pb$^{2+}$ ions can then be cleared from the solution with the addition of the anion donor which will precipitate the residual lead ions. These lead salts and any interfering compounds bound by the lead can then be centrifuged or filtered out. As previously mentioned, this process inevitably removes some cardenolides with recovery rates dependent on which variation of the technique is employed (Nelson et al., 1981; Rowson, 1952).

The lead acetate cleanup step first pursued here was adapted from Agrawal et al. (2021) based on a recommendation from the author (personal communication, 2021). After preparing 8 ml of plant extract made from 250 mg of ground plant tissue in a 15 ml falcon tube, 1 ml of 1.0 M aqueous lead acetate was added, and the sample incubated for 20 minutes. The sample was then centrifuged at 12,000 x g for 10 minutes and the supernatant transferred to a new tube. To this sample, 1 ml of 1.0 M sodium sulfate was mixed into solution. After another 20-minute incubation the sample was once again centrifuged, and the resulting supernatant measured for cardenolide content using the TNDP-based assay.

Initial observations were promising, as adding lead acetate to ethanolic plant extracts was observed to have an immediate effect, namely the visible formation of solid lead salts within the mixture and their concentration at the bottom of the 15 ml falcon tube. The subsequent addition of
sodium sulfate led to further clearing of the sample as solid lead sulfates formed a milk-white precipitant. These visual observations (Figure 11) suggested that the cleanup step was functioning as intended and that a majority of the interfering chlorophylls had been removed.

**Figure 11.** Milkweed leaf extracts at various stages during depigmentation with lead acetate. Plant extracts were prepared by mixing known quantities of powdered leaf tissue with 95% ethanol. Before introducing lead acetate, the untreated plant extracts exhibited strong pigmentation (a). After the introduction of lead acetate, the sample became clearer as lead compounds precipitated out of solution (b). After the solid lead compounds were removed, the addition of sodium sulfate converted any residual lead ions to insoluble lead sulfates that also were removed (c).

However, the AU results of experiments evaluating the functionality of the cleanup step revealed that the treatment did not result in improved accuracy. Initial results seemed to suggest that the treatment was actually contributing to interference as experimental and blank AU values were roughly equivalent (Figure 12). Subsequent experiments measuring cardenolide content with varying starting masses of tissue, various tissue types, and with a range of added digitoxin tracers did not yield experimental AU values that were within reported ranges for milkweed foliar tissue as described by Malcolm et al. (1989) or reliably repeatable.
Figure 12. The results of the lead acetate cleanup step when applied to ethanolic extracts of milkweed foliar tissue. In this experiment, four leaf extract replicates were depigmented with lead acetate and cleaned of residual lead ions with sodium sulfate. Three technical replicates (wells) of each extract were measured on the same 96 well plate. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.
Modifications to the lead acetate cleanup step were attempted to improve results. Some steps to improve reaction efficiency were enacted, such as placing samples in ice baths during both 20-minute incubations to encourage the precipitation of lead compounds. Other adjustments were made to the amounts of lead and sodium sulfate employed in the cleanup step, including changes to the relative amounts of each used. In some experiments, the molarities of these reagents were adjusted to mimic the relative amounts described in various papers that employed a lead acetate cleanup step (Agrawal et al., 2021; Brower et al., 1981; Rowson, 1952). But evaluating the molarities of each reagent from 1.0 M to 0.2 M across multiple experiments did little to establish a concentration of lead acetate that was effective at reducing interference and producing consistent results.

Difficulties with the lead acetate cleanup step prompted a screening of experimental reagents to see if any individually, or when combined, would act additively towards absorbance and cause potential interference and lead to results like those observed in Figure 12. To this end, the individual absorbances of various components of the assay (lead acetate, sodium sulfate, sodium hydroxide, and TNDP) were measured in 95% ethanol (Figure 13). Reagent volumes were chosen based on the amount traditionally used per experiment. For example, when evaluating the absorbance of sodium hydroxide, 70 µl of 0.1 M sodium hydroxide was added to a well alongside 135 µl of 95% ethanol for a total volume of 205 µl. When evaluating TNDP, 90 µl of 0.15 % TNDP was combined with 115 µl of ethanol for a total volume of 205 µl. A well filled with 205 µl of de-ionized water was used as a reference blank. In similar experiments, lead acetate and sodium sulfate samples were prepared from 0.22 M and 0.88 M stocks and further diluted to match the amounts assumed to persist through to the measurement step of the assay if they had not been removed during cleanup. Interestingly, it was found that TNDP by itself can contribute to
absorbance, and all samples containing TNDP were observed to have markedly high AU values. It is unclear as to why TNDP behaves this way in the absence of cardenolides as this phenomenon has not been described by previous studies. Lead acetate was observed to uniquely contribute to absorbance in the presence of sodium hydroxide – possibly due to the formation of lead hydroxides that precipitated within the well. Sodium sulfate did not appear to contribute meaningfully to AU values except when combined with TNDP. But because TNDP appears to contribute to absorbance independently, it was unclear whether or not TNDP interacted with lead acetate or sodium sulfate to increase absorbance or did so by itself.

**Figure 13.** A series of experiments evaluating the potential contributions of assay reagents to absorbance unit values at 620 nm: (a) the absorbance results of de-ionized water, sodium hydroxide, TNDP, and a combination of TNDP and sodium hydroxide; (b and c) the absorbance results for lead acetate and sodium sulfate at 0.22 M and 0.88 M combined with various mixtures of other experimental reagents. Lead acetate and sodium sulfate concentrations were diluted to match the amounts that enter the reaction mix under the standard assay. If a chemical reagent was not present in any given sample, it was replaced by an equivalent volume of 95% ethanol.
Due to the unsatisfactory results and lack of progress while troubleshooting the lead acetate cleanup step, a series of additional sample depigmenting methods were explored. Focus was directed solely at leveraging the differences in chemical and physical properties between the allegedly interfering compounds and the target cardenolides. Of the various approaches attempted, two methods showed promise in improving the accuracy of the assay.

3.7e Ether Extraction Protocol Design and Troubleshooting

While different cardenolides and their derivatives may vary in overall polarity due to the diversity of sugar moieties they may contain, cardenolides as a class of steroidal compounds with a nonpolar backbone generally prefer the nonpolar fraction of a heterozygous suspension. Because of this, it was theorized that an ethyl ether extraction could be used to separate nonpolar cardenolides from any interfering polar compounds. The nonpolar fraction could then be evaporated under nitrogen gas and its contents resuspended in ethanol.

To evaluate the potential utility of an ether extraction, six ethanolic plant extracts made from 250 mg of the same plant tissue were prepared. These six extracts were treated with 1.0, 0.5, 0.25, 0.125, 0.0625, and 0 ml of a 1 mg/ml digitoxin tracer respectively. It was hypothesized that the digitoxin would be transferred to the nonpolar fraction and impact any subsequent AU measurements. Each extract was then mixed into 2 ml of deionized water and 5 ml of ethyl ether. The development of a phase separation between different constituents of the plant extract was observed. The chlorophyll pigments behaved as expected, such that the nonpolar fraction became much more heavily pigmented than the polar fraction. Each sample was then dipped in liquid nitrogen to flash-freeze the polar fraction while the ethyl ether remained as a liquid. The nonpolar fraction was decanted into a new container and evaporated to dryness under a stream of nitrogen gas to prevent oxidation. The recovered solutes were then resuspended in 3 ml of 95% ethanol and
measured with the TNDP-based assay (Figure 14). These results were promising as a trend of increasing corrected AU values alongside increasing digitoxin tracer amounts suggested that digitoxin had transferred successfully to the nonpolar fraction, was measurable despite the presence of chlorophyll pigments in the original sample, and that the ether extraction was working as intended. However, despite the apparent trend observed in the samples containing digitoxin, the experimental and blank AU values of the sample with no added digitoxin tracer were relatively high compared to those with the same relative concentrations of interfering compounds and added digitoxin.

**Figure 14.** The results of ether extraction on plant extract replicates with varying levels of digitoxin tracer added. Plant extracts were prepared with 250 mg of powdered plant tissue, 2 ml of deionized water, and 5 ml of ethyl ether. To each sample, an additional 1 ml of digitoxin solution at a known concentration (0-1 mg/ml) was added. The nonpolar ether fraction of the mixture was removed, evaporated to dryness, and the remaining solid residuals resuspended in 3 ml of 95% ethanol. The absorbances of these ethanolic solutions were then measured at 620 nm with the TNDP-based assay. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.

To explore whether the ether extraction method improved the accuracy of the TNDP-based assay when measuring extracts without added digitoxin, two samples of milkweed foliar tissue were used to prepare 250 and 500 mg plant extracts. A series of 250 mg extracts of both samples with added digitoxin tracers (0, 0.5, and 1 ml of a 1 mg/ml digitoxin solution) were also prepared. All samples underwent ether extraction and were measured with the TNDP-based assay as
described previously. The results of this experiment revealed that, while the effects of the digitoxin tracers were generally observable, samples containing no tracer resulted in experimental and blank sample AU values that were roughly equivalent (Figure 15). It was inferred from these results that the ether extraction method alone was not enough to clear the nonpolar interfering compounds that had been transferred to the ethyl ether fraction alongside the nonpolar cardenolides. Subsequent evaluations of the aqueous fractions from this experiment did not yield interpretable results. Experiments in which the lead acetate cleanup step was applied to the ethanolic suspension following ether extraction similarly failed to improve the accuracy of the assay.
Figure 15. Results from two ether extraction experiments on the same milkweed leaf tissue sample. In the first experiment (a), the initial sample mass ranged from 250 to 500 mg. In the second experiment (b), all samples had an initial mass of 250 mg, but before adding ethyl ether or deionized water, 1 ml of digitoxin tracer at concentrations of 0, 0.5, and 1 mg/ml were introduced. The nonpolar fraction was separated, dried, and the residue was resuspended in 3 ml of 95% ethanol. The absorbances of these ethanolic solutions were then measured at 620 nm with the TNDP-based assay. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.

Chlorophyll pigments once again were implicated as a potential source of interference as the ether extraction was observed to collect a majority of the dark green pigments observed in plant extracts in the nonpolar fraction. It was inferred that while the ether extract was able to remove many interfering polar compounds, the same method also was potentially concentrating chlorophyll pigments to the extent that the natural cardenolide content within samples became undetectable. Because of this, troubleshooting turned towards methods to specifically target and remove chlorophylls from sample extracts.
3.7f Heat Degradation Design and Troubleshooting

One major difference between the physical properties of chlorophylls and cardenolides is their stability when exposed to heat. When exposed to temperatures exceeding 60 °C, chlorophylls a and b degrade into derivatives of pheophytin and chlorophyllide (Petrovic et al., 2012). While both pheophytins and chlorophyllides still retain a local maximum absorbance peak around 650-660 nm, their contribution to absorbance at those wavelengths is greatly reduced compared to the absorbance of their precursors (Weemaes et al., 1999; Petrovic et al., 2012).

Cardenolides were presumed to be more heat stable and to evaluate this, 1 ml of a stock solution of 1 mg/ml digitoxin in 95% ethanol was heated to 80 °C over 120 minutes with absorbance measurements taken over 30 minute intervals (Figure 16). The results from this simple heat treatment suggested that digitoxin does not readily degrade at 80 °C.

![Figure 16](image.png)

**Figure 16.** The results of evaluating the potential heat degradation of digitoxin over time. In this trial, digitoxin solution (1 ml of 1 mg/ml digitoxin in 95% ethanol) was heated to 80 °C for 120 minutes and its absorbance at 620 nm repeatedly measured at 20 minute intervals. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.

To evaluate the effect of heat on chlorophylls within ethanolic plant extracts, two extracts derived from 250 mg of *Lactuca sativa* (hereafter iceberg lettuce) foliar tissue were heated to 60 °C for 120 minutes, with measurements taken every 60 minutes (Figure 17). An additional 1 ml of 1 mg/ml of digitoxin in 95% ethanol was added to one of the extracts. Because iceberg lettuce
tissue is relatively low in pigmentation when compared to milkweed, it was hypothesized that the effects of chlorophyll degradation would be more easily observed in this tissue type. The results for this experiment support this claim as there was an observable decrease in overall AU value results over time for the iceberg lettuce extract with no added digitoxin. Conversely, the extract that did contain a digitoxin tracer maintained comparably steady AU value results over the course of the experiment.
Figure 17. The results of evaluating the potential heat degradation of plant pigments and digitoxin over time. In this trial, two separate plant extracts were prepared from 250 mg of powdered iceberg lettuce tissue. One extract was prepared with 8 ml of 95% ethanol (a) and the other prepared with 7 ml of 95% ethanol and 1 ml of digitoxin solution (1 ml of 1 mg/ml digitoxin in 95% ethanol) (b). Both samples were heated to 80 °C for 120 minutes and their absorbance at 620 nm repeatedly measured at 60 minute intervals. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.

These initial results in iceberg lettuce were promising. Also promising was the observed color change that occurred in all plant extracts when heated over time. Extracts would begin to lose color intensity with heating, and both milkweed and high-chlorophyll spinach leaf samples would lose their verdant coloration and become brownish with time. This seemed to suggest that the degradation of chlorophyll pigments was taking place. However, when milkweed and spinach samples were heat treated, no apparent changes in absorbance were observed despite the color change. These results were interpreted to mean that, while the effects of the heat treatment may impact chlorophyll degradation enough to improve the accuracy of the assay for low-chlorophyll
content tissue types (iceberg lettuce), the remaining chlorophylls (or their derivatives) in high-chlorophyll tissue types (spinach and milkweed) were still in high enough concentration to impact results.

To investigate whether additional cleanup measures enacted upon the heat-treated extracts would improve the accuracy of the assay, samples were evaluated for both the singular and combined effects of the heat and lead acetate cleanup treatments. Two sets of eight 8 ml plant extracts using 250 mg of milkweed foliar tissue were prepared and 1.0 mL of 1 mg/ml of digitoxin tracer was added to half of each set of samples. The samples were then separated into four treatment groups, defined by a factorial combination of heat degradation and lead acetate cleanup treatments: (1) samples with no additional treatment, (2) samples treated with the lead acetate cleanup, (3) samples treated with heat, and (4) samples treated with heat and then the lead acetate cleanup. The results suggest that neither treatment, nor their combined effects, were enough to improve the accuracy of the results (Figure 18). In the untreated samples, there was no apparent trend in increased absorbance conferred by the digitoxin tracer. The singular effects of heat or the lead acetate cleanup did little to improve these result. The same was observed for samples in which both treatments were combined.
Figure 18. The results of evaluating the individual and combined effects of the lead acetate cleanup and heat treatments on milkweed leaf extracts from two sources prepared both with and without added digitoxin tracer (1 ml of a 1 mg/ml digitoxin solution). (a) the AU results of neither treatment on ethanolic plant extracts prepared from 250 mg of powdered milkweed leaf tissue, (b) the AU results of samples depigmented with the lead acetate cleanup treatment, (c) the AU results of samples subjected to the heat treatment (60 °C over 120 minutes), and (d) the results of the combined treatments where samples were first depigmented and then heated. The corrected AU values were calculated by subtracting the blank AU values from the experimental AU values.
Conclusion

Due to increasing demands on the global food supply, interest has grown in the utilization of neglected or underutilized crop species for agricultural purposes. This is due to the vast potential for currently under-researched edible plant species to improve the resilience of regional food systems. Common milkweed was selected as NUCS of interest to develop as a productive, edible ornamental for backyard growers in New England. This was done based on the crop’s outstanding merits, prior historical utilization, and ecological value – all selling points that may be used to convince regional growers to adopt the species. Barring that potential from being realized are qualms regarding common milkweed’s palatability, as the public perception of the species is strongly tied to the well-known biochemical exchange of toxic cardenolides between milkweed and its herbivores. To evaluate milkweed cardenolide content in an agricultural context, a spectrophotometric assay was investigated for use in measuring foliar cardenolide content. Due to persistent issues suspected to be cause by interfering photoactive compounds, a functional assay was not achieved despite extended troubleshooting and the use of depigmentation techniques such as a lead acetate cleanup step, an ethyl ether extraction method, and heat treatment. Considerations for future research on this topic include further refinements to the current protocol employed and/or a shift to employing other quantification methods such as HPLC.

The results of this study put the reproducibility of the TNDP-based assay as it was applied by past authors into question. Some ambiguities arise when considering prior publications measuring foliar cardenolide content. The first relates to the use of a lead-based depigmentation step. If and how this step is implemented varies by author with some omitting it entirely and still generating publishable results – suggesting that the lead acetate cleanup step is optional. Yet the results of this study support that some form of depigmentation step is necessary to clear interfering
photoactive compounds because even samples that had been spiked with unnaturally high levels of digitoxin still yielded poor results. Another ambiguity is related to the specificity of TNDP and its functionality. Very little has been described about the behavior of TNDP except that it is thought to interact with the six-membered lactone ring shared by all cardenolides and the resulting complex absorbs light at 620 nm. Indeed this relationship is apparent when measuring digitoxin in 95% ethanol as evidenced by repeatably consistent results of the digitoxin ladder. But considering the vast diversity of compounds present in plant extracts, there is little reassurance that the only relevant interaction conferring absorbance at 620 nm is made by TNDP when specifically forming complexes with the lactone rings present on cardenolides and no other lactone-containing chemical constituents. Unclear or vague protocol steps for both the lead acetate cleanup step (if used) and the TNDP assay also hampered troubleshooting efforts. In multiple cases, authors would simply cite previous publications either written by themselves or their predecessors when describing the methodology of their research. In other cases, authors would mention that refinements to previous protocols were made but offer no further explanation. In conclusion comments on troubleshooting the assay in general were rare and no prior study reviewed has reported the results of tissue sample when measured at varying starting masses or extract volumes.
LIST OF REFERENCES


25. Bell, F. K., & Krantz, J. C. (1948). Digitalis VII. The Effect of Various Alkalies on the Sensitivity of the Baljet Reaction for Digitoxin††The expense of this study was defrayed in part by a grant from the Board of Trustees of the United States Pharmacopeial Convention. Journal of the American Pharmaceutical Association (Scientific Ed.), 37(8), 297–301. https://doi.org/10.1002/jps.3030370802


62. Endress, M. E., Liede-Schumann, S., & Meve, U. (2014). An updated classification for Apocynaceae. *Phytotaxa, 159*(3), 175. [https://doi.org/10.11646/phytotaxa.159.3.2](https://doi.org/10.11646/phytotaxa.159.3.2)


164. Thakur, T., & Hurley, T. (2022). Do farmers need to be paid to grow milkweed for monarchs or will they volunteer if it is easy enough? *Applied Economic Perspectives and Policy, n/a*(n/a). https://doi.org/10.1002/aepp.13290


