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Vicki Schlegel  
*University of Nebraska-Lincoln*, vschlegel3@unl.edu

Richard Zbasnik  
*University of Nebraska-Lincoln*, rzbasnik2@unl.edu

Tammy Gries  
*University of Nebraska-Lincoln*, tgries2@unl.edu

Bo Hyun Lee  
*University of Nebraska-Lincoln*, blee2@unl.edu

Timothy Carr  
*University of Nebraska-Lincoln*, tcarr2@unl.edu

See next page for additional authors

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Characterization of potential health promoting lipids in the co-products of de-flossed milkweed

Vicki Schlegel,1 Richard Zbasnik,1 Tammy Gries,1 Bo Hyun Lee,1 Timothy Carr,2 Ji-Young Lee,2 Curtis Weller,3 and Susan Cuppett1

1. Department of Food Science and Technology, 143 Filley Hall, University of Nebraska–Lincoln, Lincoln, NE 68583-0919, USA
2. Department of Human Health and Nutrition, 316 Ruth D. Leverton Hall, University of Nebraska–Lincoln, NE 68583-0806, USA
3. Department of Biological Systems Engineering, 210 Chase Hall, University of Nebraska–Lincoln, NE 68583-0726, USA

Corresponding author — V. Schlegel, tel 402 472-4694, fax 420 472-1693, email vschlegel3@unl.edu

Abstract
The floss and oil of the common milkweed (Asclepias syriaca L.) seeds are currently used to produce comforters/pillows and skin care products, respectively. As an outcome of these products, copious quantities co-products (pressed seed meal and pod biomass) are disposed of each year despite the presence of potential health benefiting lipids. The objective of this project was to determine the feasibility of developing the lipid fraction from of these co-products for the fast growing dietary human health market. Although certain types of lipids were affected by the extraction solvent used (hexane and diethyl ether) as were overall amounts, analysis of the each extract showed novel lipid profiles with several potential health benefiting agents present at levels comparable to or exceeding those present in other typically consumed dietary oils or food systems (vitamin E, carotenoids, sterols and unsaturated free fatty acids, particularly the both omega 7-fatty acids).

Keywords: milkweed, Asclepias syriaca L. co-products, lipids, functional foods, alternative crops

1. Introduction

Alternative crops produced in North America offer growers and processors the opportunity to capitalize on high value technologies and to develop new products. In particular, the National Center for Agriculture Utilization Research has identified the common milkweed (belonging to the Asclepias genus) as a highly potential alternative crop. Because most Asclepias species are indigenous to North America, the plant grows in many types of agro-regional conditions. Milkweed can also improve the quality of farm life because, as a perennial plant, it is not a labor-intensive crop. Studies completed during and post-World War II has further shown that that milkweed had the greatest potential for commercial marketing than most native plants (Timmons, 1946). Yet, on-going attempts to develop milkweed as an economical viable crop have met with little success (e.g., rubber from the latex of the plant and biodiesel from the seed’s oil). Moreover, milkweed has been historically viewed as a noxious weed because it can easily invade other crops due to the floss-covered seeds that are carried by the wind to different areas.

Nonetheless, the milkweed species, Asclepias syriaca L., has received industrial crop status because of the silky seed floss that is currently being used to produce hypoallergenic pillows, comforters, and insulating fiber. The de-flossed seeds have in turn been used to develop skin care products and nematicides/pesticides (Harry O’kuru et al., 1999) but large quantities of dried plant biomass (PB) and cold pressed seed meal (SM) end up as co-products that are typically disposed. However, studies completed in our laboratory as well as by other researchers have shown the presence of potential health benefiting lipids (e.g., specifically the sterols and fatty acids) in the seeds. Considering that functional foods and the dietary supplement industry are expected to grow from 18 billion in 2002 to 30 + billion by 2012 (McCrorie & Bone, 2008), we proposed that the Syriaca L. co-products could be potential source of human health ingredients for the niche but fast growing health promoting dietary market and thus add value to this currently underutilized crop. Therefore, the objective of this project was to determine the feasibility of developing these co-products for this market by characterizing for select lipid based components that have been linked to one or more health promoting benefits.

2. Materials and methods

2.1. Preparation of lipid extracts

Samples of dried pod biomass (PB) and seed meal (SM) that had been subjected to a cold pressing process to obtain the oil were obtained from Natural Fibers, Inc., (Ogallala, NE). Lipids were extracted from 105 to 120 g samples ground to fine parti-
cles by recirculating 150–200 ml of hexane or diethyl ether for 3–4 h using a bench-top Soxhlet apparatus. The solvent was collected, removed by a rotovap, and the residue was stored at 4–10 °C until analyzed. Total lipid yields were determined based upon the final weight of the lipid residue respective to the weight of the starting material. The sample was visually inspected for color and phase characteristics.

2.2. Lipid characterization

The lipid classes present in each extract were initially identified and quantitated using thin layer chromatography (TLC). The lipid extract was suspended in hexane at a concentration of 25–30 mg/ml. The samples were spotted onto Whatman silica 60 Å TLC plates (60 general purpose, 20 × 20 cm, 250 μm, Maidstone England) as 10 and 5 μl aliquots. Along with the samples, a cocktail of standards containing cholesterol, fatty acid methyl esters, δ-α-tocopherol acetate, and 3 polyoxyethylene sorbitan monooleate, octacosanol, monooacylglycerides of oleic acid, 1,2-diacylglycerols of oleic acid, 1,3-diacylglycerides of oleic acid, and triacylglycerols of oleic acid was spotted on the same TLC plates. The samples/standards were resolved with hexane, diethyl ether, and acetic acid (85:15:2 (v/v/v)). The lipids were visualized by submerging the plate in a 10% cupric sulphate, 8% phosphoric acid solution and charring at 165 °C for 10 min. A Kodak Gel Logic 440 Imaging System interfaced to Kodak ID Image Analysis software (Carestream Health, New Haven, CT) was used to image the developed plate and the simple lipid classes were identified based on their Rf values compared to the standards.

Levels of total free fatty acids and triacylglycerides were completed as described by AOCS (American Oil Chemists’ Society) (2006) and Vishwanath & Manning (1968), respectively. The results are reported as the mean ± standard deviation (SD) (in mg oleic acid/g of co-product (free fatty acids) or in mg of olive oil/g of co-product (triacylglycerides) of duplicate or triplicate analyses.

Vitamin E, carotenoid, and Co-enzyme Q10 levels were determined by combining 0.05–0.10 g of lipid extract with 1–2 mL of methanol with mixing for 1–2 h. The methanol phase was passed through a 0.45 μm syringe filter and analyzed for the vitamin E isomers (α-, β-, and γ-tocopherols), carotenoids, and co-enzyme Q10 with a Waters 600 S HPLC (Milford, MA) system interfaced to a Waters Millennium 32 Chromatography Manager workstation. The analytes were resolved with an Agilent Zorbax 300SB C-18 (Santa Clara, CA) column and a mobile phase of methanol, acetonitrile, and triethanolamine (90:10:1) under isocratic conditions at a flow rate of 1 mL/min. A Waters 996 photodiode array detector set at 450, 309, and 295 nm was used to detect the carotene, Co-enzyme Q10, and tocopherols, respectively. These analytes were identified based on their retention times with external standards and quantitated against calibration curves generated from standards. Results are reported as the mean ± SD in μg of carotene or Co-enzyme Q10/ g of co-product of triplicate analyses. Spectral patterns of the individual HPLC bands in the 450 nm window, (other than the carotene bands) were monitored against a calibration curve prepared with lutein standards. Total carotenoid levels were determined by adding the absorbance of the individual bands and expressing the final results as the mean ± SD in μg lutein/g of co-product of triplicate analyses.

Quantification of sterols (free and esterified) was achieved with gas chromatography (GC) and flame ionization detection (FID). Each co-product lipid extract (10–20 mg) was prepared and analyzed according to published reports (Schneider et al., 2000). Briefly, the samples were injected (1 μl) onto an HP 5890 Series II + GC with a DB-1 column (15 m × 0.25 mm) (J&W Scientific, Folsom, CA) under the following conditions: initial temperature 190 °C for 1 min, increased to 220 °C at 3 °C/min; injector temperature, 270 °C; flame ionization detector temperature, 300 °C; helium carrier gas; and split ratio of 20:1. External standards of stigmasterol, campesterol, and β-sitosterol were used to identify and quantify the peaks. Results are reported as the mean ± SD of a sterol/lipid extract (μg/g of co-product) of triplicate analyses.

Fatty acid profiles were determined combining boron trifluoride (14%) in methanol to an extract sample followed by heating to a 100 °C for 30 min in sealed reaction vials. The samples were allowed to cool to room temperature before extraction with 1 ml of hexane and three rinses of 2 ml of water. The hexane fraction and fatty acid standards were resolved with a Hewlett Packard Co. 6890 series GC System Plus + (Wilmington, DE) using a DB-Wax column (30 m × 0.25 mm) by J&W Scientific and detected with a flame ionization detector. Injections were achieved with a split ratio of 10:1 with the temperature set at 185 °C initially for 12 min, the temperature was increased to 210 °C at 10 °C/min and held for 0 min, and then increased to a final temperature of 230 °C at 10 °C/min, which was maintained for the remaining 15 min of the run. Results are expressed as the mean ± SD of the relative percentage of each fatty acid of triplicate analyses.

2.3. Statistical analysis

Results were analyzed with R:A language and environment for statistical computing software (Vienna, Austria) and/or by StatsGraphic Plus, version 4 (Statpoint Technologies, Inc., Warrenton, VA). The Cochran C test and the Bartlett’s test were performed to determine whether the variability between different lipid levels were significantly different at the 95% confidence interval (p < 0.05). If not, the Tukey HSD mean separation test was completed to determine whether the means from the lipid data were significantly different at the 95% confidence interval, (p < 0.05). Statistical comparisons of the results were reported based on the Tukey HSD mean separation test. The final results were expressed as the mean ± SD of at least triplicate analyses. Potential outliers were assessed with the Grubbs test and were eliminated at a 5% risk for rejection.

3. Results and discussion

Asclepias syriaca L. is currently used to produce comforters/pillows and skin care products from its seed floss and seed oil, respectively, resulting in large quantities of pressed SM and dried PB that at present are disposed. To determine the feasibility of developing these co-products as sources of dietary human health ingredients, the lipid fraction was analyzed for specific components that have been linked to health promoting benefits. Such applications are particularly conducive to lipid fraction of Asclepias syriaca L. as studies have shown that the oil is not contaminated with the potentially dangerous cardiac glycosides common to the milkweed plant (Harry-O’kuru & Abbott, 1997).

To ensure the recovery of simple lipids with slightly different polarities, each co-product (pressed SM and PB) was extracted with a Soxhlet method using either hexane (HX) or diethyl ether (DE) as the solvent system. These results were expected as other researchers have reported lipid yields surpassing 20% from unprocessed milkweed seeds (Harry-O’kuru & Carriere, 2002). For both co-products, statistically lower yields were obtained with HX compared to DE. The overall appearance also differed depending on the co-product as well as the extraction solvent.
Yields and appearance of lipids extracted from each *Asclepias syriaca* L. co-product with either hexane or diethyl ether.

<table>
<thead>
<tr>
<th>Co-product</th>
<th>Lipid yields (%)</th>
<th>Lipid yields (%)</th>
<th>Appearance*</th>
<th>Appearance (diethyl ether)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed Meal</td>
<td>10.1 ± 0.1\textsuperscript{a}</td>
<td>13.2 ± 0.6\textsuperscript{b}</td>
<td>Viscous yellow liquid</td>
<td>Viscous yellow liquid</td>
</tr>
<tr>
<td>Pod Biomass</td>
<td>4.0 ± 0.2\textsuperscript{d}</td>
<td>5.0 ± 0.0\textsuperscript{d}</td>
<td>Waxy yellow solid</td>
<td>Waxy green solid</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD of three analyses. Values with different superscript letters are statistically different (p < 0.05).

* At room temperature.
the SM and PB extracts showed differences in several fatty acids. The PB generally contained significantly higher levels of α-tocopherol with respect to the SM co-product extracted with either hexane or diethyl ether. Analysis of the samples for vitamin E showed significantly higher levels of α-tocopherol compared to the HX-SM but the latter sample contained lower risk factors for heart disease and obesity (Wang et al., 2008). These results were attributed in part to vaccenic acid’s ability to reduce chylomicron production, which is considered to play a critical role in the onset of metabolic disorders. While other studies have shown that phytoestrogens and their esters are effective in lowering total and low-density lipoprotein cholesterol in blood by inhibiting the absorption of cholesterol in the small intestine (Ostlund, 2004). As shown by Table 2, the highest levels of sterols were obtained from the SM using either DE or HX. Although the total levels were not significantly different between the two SM extracts, the sterol profiles were affected by the solvent system as the DE-SM contained higher relative percentages of sterol compared to the HX-SM but the latter sample contained higher stigmastanol and campesterol (Table 4). In addition, different compositional profiles were obtained for the PB extracts depending on the solvent system used (Table 4) but the DE-PB contained higher total levels compared to the HX-PB sample (Table 2). Nonetheless, the milkweed sterol levels were 25–95% lower than other typically consumed seeds and nuts (Phillips et al., 2005).

Analysis of the samples for vitamin E showed significantly higher levels in the SM co-product (~70 µg/g of product) relative to the PB (~30 µg/g of product) regardless of the solvent extraction system used (Table 1). α-Tocopherol was the most abundant tocopherol present in all the extracts followed by γ tocopherol (Table 5). δ-Tocopherol was not present in either the HX- or DE-PB extracts but these samples contained significantly higher levels of α-tocopherol with respect to the SM samples. Relative to many other seed oils, the milkweed

### Table 2. Simple lipid classes present in each Asclepias syriaca L. co-product extracted with either hexane or diethyl ether.

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Seed meal (hexane)</th>
<th>Seed meal (diethyl ether)</th>
<th>Pod biomass (hexane)</th>
<th>Pod biomass (diethyl ether)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene (µg/g)</td>
<td>1.2 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>8.2 ± 1.3</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>Carotenoids (µg/g)</td>
<td>11.8 ± 0.3</td>
<td>12.8 ± 0.7</td>
<td>37.7 ± 2.5</td>
<td>37.8 ± 2.3</td>
</tr>
<tr>
<td>Co-enzyme Q10 (µg/g)</td>
<td>64.9 ± 10.4</td>
<td>37.6 ± 6.4</td>
<td>60.3 ± 22.3</td>
<td>230.3 ± 33.6</td>
</tr>
<tr>
<td>Free fatty acids (mg/g)</td>
<td>542.4 ± 29.5</td>
<td>3.7 ± 0.6</td>
<td>28.5 ± 6.9</td>
<td>29.2 ± 3.8</td>
</tr>
<tr>
<td>Polysaccharides (µg/g)</td>
<td>143.0 ± 43.4</td>
<td>26.3 ± 20.3</td>
<td>29.8 ± 15.7</td>
<td></td>
</tr>
<tr>
<td>**Sterols (µg/g)</td>
<td>542.4 ± 29.5</td>
<td>686.6 ± 90.1</td>
<td>60.3 ± 22.3</td>
<td>230.3 ± 33.6</td>
</tr>
<tr>
<td>*Vitamin E (µg/g)</td>
<td>65.9 ± 10.4</td>
<td>73.9 ± 4.8</td>
<td>28.5 ± 6.9</td>
<td>29.2 ± 3.8</td>
</tr>
<tr>
<td>Triacylglycerides (mg/g)</td>
<td>143.0 ± 43.4</td>
<td>130.9 ± 63.2</td>
<td>26.3 ± 20.3</td>
<td>29.8 ± 15.7</td>
</tr>
</tbody>
</table>

Results expressed as the mean ± standard deviation (wt of lipid/wt of co-product) of two–three analyses. Values with different superscript in the same row are statistically different (p < 0.05). Reported as the summation; * of α, γ, δ-tocopherol, or as ** of free or esterified sterols.

† Lipid class was not detected.

### Table 3. Compositional profiles of fatty acids extracted from each Asclepias syriaca L. co-product with either hexane or diethyl ether.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Seed meal (hexane) (%)</th>
<th>Seed meal (diethyl ether) (%)</th>
<th>Pod biomass (hexane) (%)</th>
<th>Pod biomass (diethyl ether) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 (Palmitic)</td>
<td>8.4 ± 1.2</td>
<td>7.7 ± 0.3</td>
<td>23.6 ± 8.6</td>
<td>11.9 ± 0.3</td>
</tr>
<tr>
<td>C16:1 (Palmitoleic)</td>
<td>16.0 ± 0.6</td>
<td>17.0 ± 0.4</td>
<td>2.9 ± 1.3</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>C16:2 (Hexadecadienoic)</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>3.5 ± 0.5</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>C18:0 (Stearic)</td>
<td>2.0 ± 0.0</td>
<td>1.8 ± 0.0</td>
<td>2.4 ± 0.4</td>
<td>4.6 ± 3.6</td>
</tr>
<tr>
<td>C18:1 (Vaccenic)</td>
<td>11.8 ± 0.3</td>
<td>11.1 ± 0.5</td>
<td>14.8 ± 3.2</td>
<td>17.6 ± 1.1</td>
</tr>
<tr>
<td>C18:1 (Oleic)</td>
<td>21.4 ± 0.4</td>
<td>22.4 ± 0.5</td>
<td>6.6 ± 3.0</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td>C18:2 (Linoleic)</td>
<td>34.6 ± 0.4</td>
<td>34.6 ± 0.3</td>
<td>39.8 ± 6.0</td>
<td>46.0 ± 2.4</td>
</tr>
<tr>
<td>C18:3 (Linolenic)</td>
<td>2.1 ± 0.8</td>
<td>1.6 ± 0.1</td>
<td>8.6 ± 3.8</td>
<td>4.1 ± 0.0</td>
</tr>
</tbody>
</table>

Results expressed as the mean ± standard deviation (in relative% fatty acid) of duplicate or triplicate analyses. Values with different superscript in the same row are statistically different (p < 0.05).

### Table 4. Compositional profiles of sterols of each Asclepias syriaca L. co-product extracted with either hexane or diethyl ether.

<table>
<thead>
<tr>
<th>Sterol class</th>
<th>Seed meal (hexane) (%)</th>
<th>Seed meal (diethyl ether) (%)</th>
<th>Pod biomass (hexane) (%)</th>
<th>Pod biomass (diethyl ether) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campesterol</td>
<td>33.7 ± 0.2</td>
<td>28.4 ± 0.7</td>
<td>1.0 ± 0.0</td>
<td>17.5 ± 1.9</td>
</tr>
<tr>
<td>Stigmastanol</td>
<td>31.3 ± 0.1</td>
<td>13.2 ± 0.1</td>
<td>100.0 ± 0.0</td>
<td>17.2 ± 0.5</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>35.0 ± 0.1</td>
<td>58.3 ± 0.6</td>
<td>67.1 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as the mean ± standard deviation (in relative% sterol) of duplicate or triplicate analyses. Values with different superscript in the same row are statistically different (p < 0.05).

† Sterol was not detected.
co-products contained higher tocopherol levels in μg/g of oil, including sesame seed (~600 μg/g), linseed (~600 μg/g), rapeseed (~340 μg/g), and sunflower, (700 μg/g), (Kamal-Elden & Andersson, 1997). For example, ~680 and 570 μg/g of lipid (data not shown in Table 1) were obtained from the HX- and DE-SM fractions, respectively, whereas lower amounts were obtained from the DE-PB (~585 μg/g of lipid) and the HX-PB extract (~725 μg/g of lipid) extracts. Vitamin E intake has been linked to the prevention of multiple diseases, including cardiovascular disease, cancer, diabetes, etc., due partly to its antioxidative properties. Many studies have indicated that γ-tocopherol is more efficacious in delivering antioxidative as well as non-antioxidative health benefits compared to the other tocopherols (Jiang et al., 2001). Although α-tocopherol was the most abundant vitamin E isomer in the milkweed co-products, the pressed SM co-product contained higher or comparable γ tocopherol to other typically consumed seed oils (Kamal-Elden & Andersson, 1997).

Carotenoids (the oil based pigments in photosynthetic organisms) also act as potent antioxidants by deactivating free radicals and singlet oxygen. Deficiency of dietary carotenes is a major cause of premature death in developing nations but elevated consumption may be harmful to certain populations (Alija et al., 2004). Other types of carotenoids have been shown to exert multiple health benefits including enhancing the immune system function (Bendich, 1988) and inhibiting the development of certain types of cancers (Nishino, 1998). Based upon these benefits, the milkweed co-products were examined for β-carotene and for total carotenoid levels (excluding the β-carotene). As listed in Table 2, β-carotene was present in only the PB samples, which in turn was affected by the extraction solvent with higher quantities obtained with HX compared to DE. Conversely, each co-product contained other carotenoids at levels that were not significantly different regardless of co-product or extraction solvent used (Table 2). Comparison with the literature further showed that the milkweed co-products are good sources of carotenoids with quantities higher than many seeds and plants (Holden et al., 1999).

Co-enzyme Q10 is another ubiquitous oil soluble vitamin that has been studied in regard to heart health, anti-aging, neurological health, anti-cancer, diabetes prevention, etc., (Dhanasekaran & Ren, 2005). Although meat and marine based sources have been identified as containing the highest amounts of co-enzyme Q10 plant-based systems, such as spinach, broccoli, peanuts, wheat germ and whole grains, have also been shown to contain co-enzyme Q10 but at significantly lower levels. The milkweed co-product extracts were thus evaluated for co-enzyme Q10 but the vitamin was not detected in any sample (Table 2).

4. Conclusions

In summary, this study confirmed the presence of several potential health-benefiting simple lipid components in both the pressed SM and PB co-products. In particular, the fatty acid data showed a unique compositional profile that could in combination with the other lipid components deliver optimal but different health benefits compared to other types of lipids. In addition, it is the SM co-product will more readily lend itself as an ingredient source for human health promoting dietary foods or supplements due to the presence of higher lipid levels. More research is needed to develop and optimize extraction processes that do not involve volatile solvents and to directly link specific lipid components in the common milkweed to scientifically supported health benefits.

Acknowledgements

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References


Menendez et al., 2005 ◄ J. A. Menendez, L. Vellon, R. Colomer and R. Lupu, Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/new (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin™) in breast cancer cells with Her-2/new oncogene amplification, Annals of Oncology 16 (2005), pp. 359–371.


Terës et al., 2008 ◄ S. Terës, G. Barceló-Coblijn, M. Benet, R. Alvarez, R. Bressani and Je Halver et al., Oleic acid content is responsible for reduction in blood pressure induced by olive oil, Proceedings of the National Academy of Sciences of the United States of America 105 (2008), p. 13811.

