Project number: BBT-M420

Is a Precursor to the Anti-Malarial Drug, Artemisinin, Glycosylated *in planta*?

A Major Qualifying Project submitted to the Faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science

By:

_____

Martin Dagoberto Driggs

Date: April 26, 2006

Approved:

_____

Dr. Pamela Weathers, Advisor

Abstract: Strains of the malaria parasite, *Plasmodium falciparum*, have become resistant to conventional quinoline-based drugs. The ancient Chinese remedial herb, *Artemisia annua*, produces a sesquiterpene, artemisinin, which has been used to treat otherwise resistant strains of malaria. Low levels of *in planta* production of the compound, however, have not been able to meet the demand for the product. *In planta* production of the anti-malarial drug, artemisinin (**AN**), is enhanced in shoots of *Artemisia annua* when roots are present. Roots, however, do not produce either AN or its artemisinic precursors. On the other hand, a biochemical precursor, artemisinic acid (**AA**), can be glycosylated *in vitro* by suspension cells of *A. annua*. The stimulatory role of roots in the production of AN could, thus, be related to the production of glycosylated terpenoids. This project sought to confirm the presence of conjugated AA in both the shoots and the roots of whole plants of *A. annua*. Bulk extracts of shoots and roots were purified in an attempt to isolate the putative compounds. Also, extracts were hydrolyzed in order to test for increases in the cleaved terpenoids. Existing AA was first removed from aqueous plant extracts which were subsequently hydrolyzed with acid, esterase, or β-glucosidase. Cleavage of an AA conjugate should result in increased pools of the cleaved product. Increases were observed in treated shoots after acid hydrolysis and glucosidase treatment. AA was not observed after any treatment of root residues. Analysis of shoots of *A. annua* showed that putative conjugates may be present in the plant, localized in the shoots. The purified fractions of extracts from bulk amounts of shoots and roots of whole plants analyzed at this time do not indicate the presence of detectable amounts of the conjugate, although further analysis is required.

Acknowledgments

Thanks to:

Dr. Karen Erickson at Clark University – For her guidance with organic techniques, for the use of her laboratory, for all the NMR work, and for her thoughtful insights.

Dr. Melissa Towler – For all the HPLC work, for teaching me lots of laboratory skills, for always knowing where that thing with the thing is, and for being a constant source of ideas.

Dr. Jo-Ann White-fleet Smith – For letting me use the teaching lab equipment and being a forgiving person.

Jess Caron – For killing our biological waste with a smile.

Eileen Dagostino- For her cheery disposition and quickness when replacing nitrogen.

Chris Dacunha – For helping me to locate proper lab equipment in a bind, and for drying residues for me when things got sticky (thanks, brother).

Yi Wang - For always being interested in what I have to say, for watering my plants when I'm away, and for her kind words and cute smile.

Jason Fuller- For talking loud with his hands, wearing really big pants to plant symposiums, and for always knowing the right numbers.

Joslyn Foley- For late-night moral support and helping me stylize my poster.

Shaoshan "Sam" Tang, Research Assistant, Clark University – For teaching me about Mitsunobu reactions and playing good music while I worked in the lab at Clark.

Custodian Wayne Atchue – For being someone with a conscience, doing something because it's the right thing to do, not because you're getting paid to do it; helping a few others with a conscience to recycle to at least some extent. (Hey WPI, RECYCLE!)

Dr. Pam Weathers – For her endless enthusiasm and creativity. Thanks for believing in me, understanding me, and giving me my space to think and breath. And if there is an award for word-smithing, if that's even a word, you should get it.

NIH Grant # 1R15GM069562-01 – For a summer stipend and for all the solvents and supplies.

Table of Contents

List of Tables

List of Figures

Background/Introduction

Importance and shortage of artemisinin

The long-time widespread use of quinolines and the other conventional anti-malarials has resulted in the selection of drug-resistant strains of *Plasmodium falciparum*, one of the malaria parasites (Wernsdorfer, 1994). The ancient medicinal knowledge of the Chinese has fortunately presented a "novel" treatment, traditionally made as tea or tincture from the weed-like herb qing hao (青蒿) (a.k.a. sweet annie, sweet wormwood, *Artemisia annua*) and used to treat dozens of ailments, including hemorrhoids and many fevers (Abdin et al., 2003). The active compound, the sesquiterpene lactone, artemisinin[1] (Figure 1), has met no resistance from the malaria parasites despite widespread application in recent years (Abdin, et al. 2003), and presents a possible solution to the world's malaria problem. The greatest obstacle, however, to the worldwide application of this drug is its relatively low concentration *in planta.* Worldwide demand currently exhausts traditional production (Abdin et al., 2003) and ironically, it is difficult to cultivate in the tropical areas where it is most needed (Klayman, 1989). With complete chemical synthesis being economically unfeasible, extensive research has focused on maximizing the biological production of the compound through various avenues of plant biotechnology and metabolic engineering.

[1] Abbreviations: AN, artemisinin; AA, artemisinic acid; AB, arteannuin B

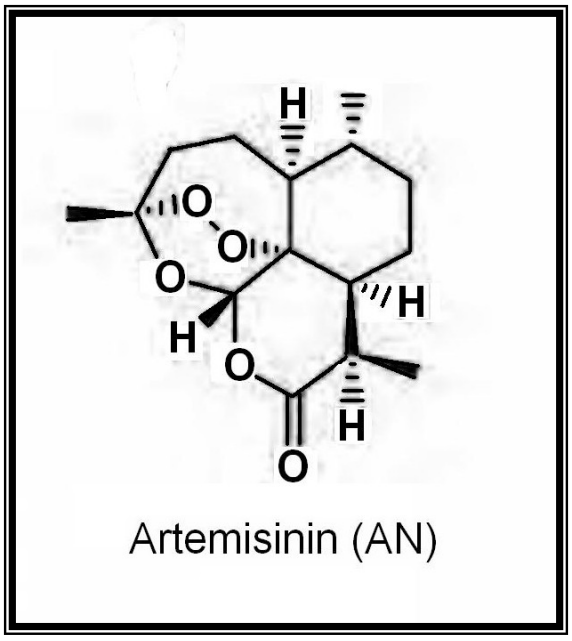


Figure 1: The chemical structure of Artemisinin

Terpenoid Metabolism in A. annua

Like other useful aromatic plants, *A. annua* contains a rich assortment of secondary metabolites, terpenoids including artemisinin, and many structurally related compounds (Tan, et al., 1998). There are four major classes of secondary products: the terpenoids, phenolic compounds, glycosides and alkaloids.

The terpenoids, also known as isoprenoids, include about 15,000 known compounds produced by many forms of life (Hopkins and Huner, 2004). One thing that all terpenoids have in common is that they are derived by repetitive fusion and cyclization of isopentyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAP). Terpenoids are classified by the number of five carbon units (isoprenes) they contain: hemi-, mono-, sesqui-, di-, tri- and tetra terpenes for 1,2,3,6, and 8 isoprenes, respectively. There are two pathways for the production of isopentyl diphosphate, the precursor to all terpenoids. Plants use both (Martin et al., 2003). The mevalonate pathway converts acetyl coenzyme A to mevalonate and then to IPP and DMAPP. This occurs in the cytosol. The non-mevalonate pathway (DXP) is in the plastids and in prokaryotic organisms like green algae and eubacteria. This pathway uses glucose-3-phosphate and pyruvate to make the precursors (Martin et al., 2003). Prenyl groups (IPP/DMAP) from either pathway may be condensed to farnesyl diphosphate, a sesquiterpene that can then be

directed towards a number of pathways, including triterpene and polyterpene production by squalene synthase (SQS), or it can be committed to sesquiterpene production by a sesquiterpene cyclase (SQC) (Martin et al., 2003).

The Artemisinin Pathway

The biochemical steps after amorpha-4,11-diene synthase, the SQC that directs carbon to artemisinin, are still being elucidated, but cyclization is known to be the first committed step in artemisinin synthesis (Figure 2; Bouwmeester et al., 1999). A number of artemisinic precursors have been isolated, and some corresponding enzymes have been demonstrated in cell-free extracts. Artemisinic acid has been identified as a precursor to artemisinin in more than one possible way. Cell-free extracts were shown by several teams to convert artemisinic acid to arteannuin B and then to artemisinin (Abdin et al., 2003). Dihydroarteannuin B has been isolated, and suggested as an intermediate in this step (Abdin et al., 2003). Alternatively, dihydroartemisinic acid has been transformed into artemisinin by cell-free extracts (Kim and Kim, 1992) and has also been photochemically converted to artemisinin *in vitro* with dihydroartemisinic acid hydroperoxide as an intermediate (Wallaart et al., 1999a, b). Artemisinic acid is considered to be the direct precursor to dihydroartemisinic acid (Wallaart et al., 2000), and it appears that there are at least two paths leading from artemisinic acid to artemisinin.

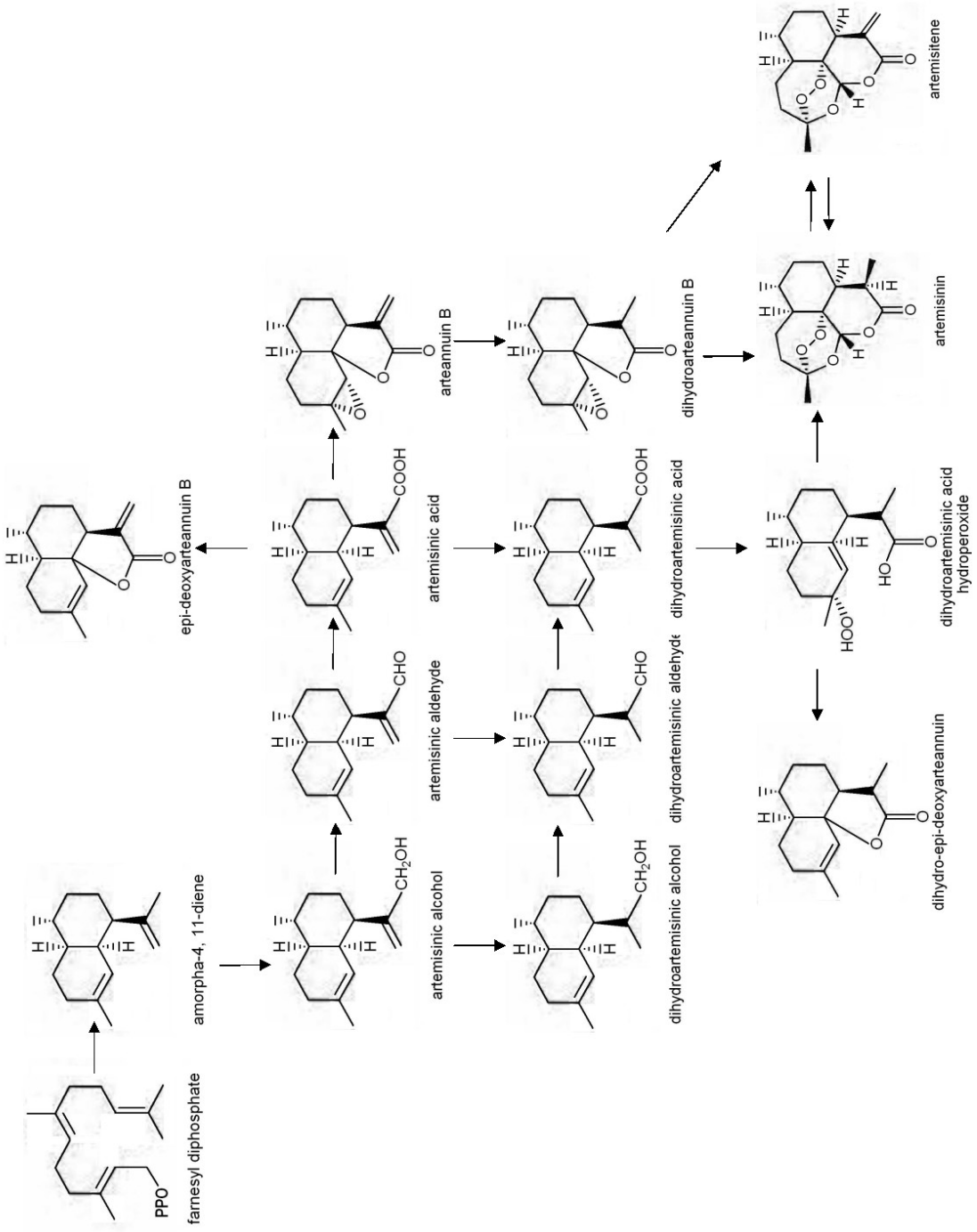


Figure 2: Proposed biosynthetic pathway of artemisinin – A composite of the pathways proposed by Wallart et al. 2000, Abdin et al. 2002 and Bertea et al. 2005.

In a survey of phenotypically identical *A. annua* plants from different geographical regions, Wallaart et al. (2000) discovered that there are two distinct chemotypes with differing relative amounts of artemisinin and its direct precursors artemisinic acid and dihydroartemisinic acid (Wallaart et al., 2000). They found that high levels of artemisinin occur with low levels of artemisinic acid and high levels of dihydroartemisinic acid. In a corollary fashion, geographical samples with low levels of artemisinin had high levels of artemisinic acid and low levels of dihydroartemisinic acid. This difference suggested to Wallaart et al. (2000) that the reduction of artemisinic acid is a rate-limiting step in the production of artemisinin (Wallaart et al., 2000). The team also noted high levels of arteannuin B and epi-deoxyartennuin B in the chemotype with increased artemisinic acid, as well as high levels of a product of dihydroartemisinic acid in the other chemotype. These data further demonstrated a chemotypical difference in artemisinic acid reduction.

The final production of artemisinin appears to be sequestered in the glandular trichomes of the plant. Correlating with the presence of trichomes, observed levels of artemisinin are highest in flowers, then buds and leaves, and lowest in green stems and seeds (Duke et al. 1994; Abdin et al., 2003). A biotype of *A. annua* without glandular trichomes exhibits no production of artemisinin or a possible precursor, artemisitene (Duke, et al, 1994). There are no glandular trichomes on the roots of *A. annua*. Although no artemisinin or artemisitene has been detected in the roots, it was observed in transformed roots (Liu et al., 1997; Weathers et al., 1994). Shoot cultures initiated from leaf tissue callus do not produce artemisinic compounds until roots are also initiated (Fulzele, et al., 1991), and shoots grown in hormone-free medium after root removal show a 53% decrease in artemisinin content (Ferreira and Janick, 1996). Although they contain no artemisinin, roots appear to play some role in its production. The nature of any possible precursor transported within the plant is still unknown.

Conjugation

The diversity of the thousands of secondary metabolites produced by plants is enhanced by their ability to decorate the molecules with many different attachments. The ability to conjugate sugars to endogenous and exogenous organic molecules is such a modification employed by many organisms. In a review of glucosyltransferases, Jones and Vogt (2001) succinctly describe plant secondary conjugates:

> "The addition of a carbohydrate-moiety to endogenous and exogenous organic molecules has a wide range of effects including increased water solubility, improved chemical stability, reduced chemical reactivity and altered biological activity. Secondary plant metabolites are glycosylated to O (OH- and COOH-), N, S and C atoms by glycosyltransferases using nucleotide-activated sugars as donor substrates. Hydroxylated molecules are the most common acceptors, whilst UDP-glucose is the most common donor. A broad range of different carbohydrate moieties can be added employing all forms of sugars, independently (monoglycosides), in parallel or in chains (di, tri-glycosides, etc). This gives rise to a broad spectrum of glycosidic structures for any given aglycone. For example, out of a total of 5,000 different flavanoids, 300 different glycosides of one single flavonol, quercetin, have already been identified" (Jones and Vogt, 2001)

Plants are able to glycosylate harmful secondary metabolites as a means of detoxification (Pedras et al., 2000). The electron draw of the sugar residue serves to convert a reactive and toxic aglycones to a more stable and less reactive form, sequestering the compound from other cellular components (Jones and Vogt, 2001). The addition of sugars to terpenoids makes them less volatile (Aharoni et al., 2003).

The glycosylation of secondary metabolites may have a biological effect in signal recognition, transport and metabolism, affecting the molecule's interactions with other molecules, signal receptors, transport proteins or degradative enzymes (Jones and Vogt, 2001). It plays a role in plant defense, allowing toxic chemical weapons to be pre-made, put on "stand-by", often localized within the point of environmental interaction: the surface cells (Osbourn, 1996). For

example, oats (*Avenus*) produce glycosylated saponins in the root epidermal cells which yield antifungal aglycones upon cleavage, which is stimulated by wounding of the cells (Osbourn, 1996).

Glycosylation also plays a role in intraspecies and interplant signaling. Cleavage of the flavanol glycosidic forms of kaempferol and quercetin is necessary in order to induce pollen germination in maize and petunia (Jones and Vogt, 2001). Glycosylation plays a role in plant hormones, as well, not only mediating turnover but also allowing for their temporary deactivation, conserving resources and decreasing response time (Jones and Vogt, 2001).

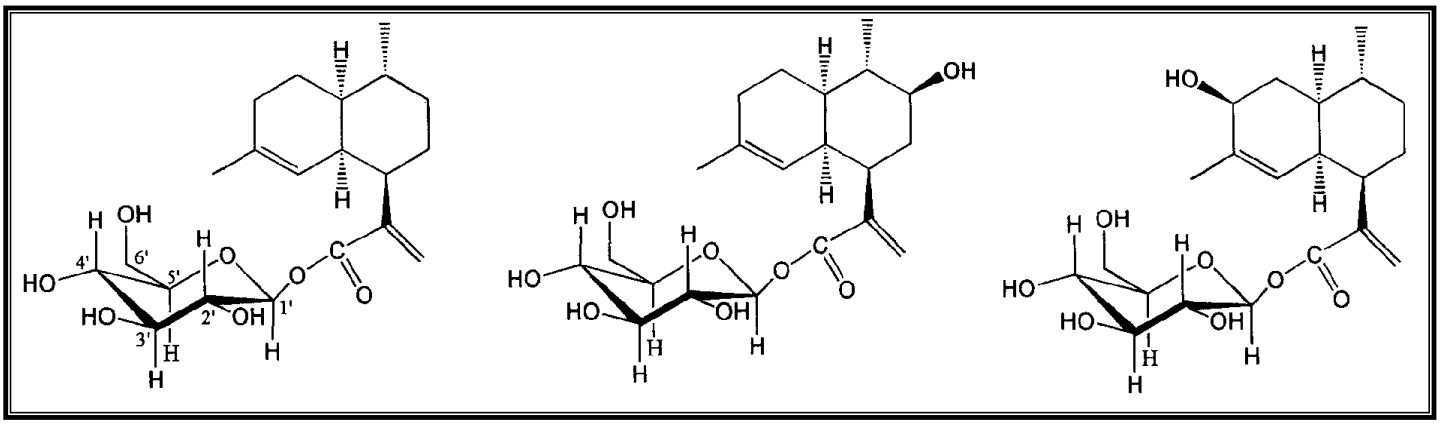
Understanding the glucosyltransferase (GT) responsible for modifying a secondary metabolite is critical to understanding the role that glycosylation has to play in the plant. How specific is the activity of the particular enzyme, and how is the enzyme regulated? Jones and Vogt (2001) report that "several putative and verified GT-encoding genes have been found to be induced by methyl jasmonate, salicylic acid and wounding. The induction was both rapid and transient in several of these cases" (Jones and Vogt, 2001). A light response has also been observed (at the level of mRNA) to play a role in glycosylation, and developmental responses as well (Jones and Vogt, 2001). Further, GTs may exist as part of the enzymatic complex (metabolon) of a metabolic pathway (Jones and Vogt, 2001).

The sesquiterpene lactone artemisinin has an endoperoxide bridge. This is the functional unit that provides the mechanism of action against the *Plasmodium* parasites, but also confers a degree of plant inhibition, or phytotoxicity (Dayan et al., 1999). The phytotoxic nature of artemisinin and prevalence of its production in *A. annua* suggests that some methods of sequestration might exist. It has already been established that artemisinin is physically sequestered in the trichomes, but it is not confirmed if all the final stages of synthesis occur solely in the trichomes. If precursors are transported within the plant, then some means of decreasing compound volatility would be needed. The transportation of glycosylated secondary metabolites is documented, and in some plants, some secondary metabolites are transported only if they are glycosylated (Jones and Vogt, 2001). For example, the iridoid (terpene) glycoside, a defense compound in *Asarina scandens* has been shown to be transported in the phloem (Gowan et al. 1995). In *Arabidopsis*, radiolabelled glycosides applied to the leaf surface of plants was shown to be distributed to all parts of the plant through source-sink transport (Chen, et al. 2001).

Prior Studies

Three preliminary studies have been performed at WPI in a search for a glucose conjugate of artemisinin. Knapp (2001) performed chemical hydrolysis (acid/base) and enzymatic hydrolysis (esterase, β-glucosidase, and β–glucuronidase) of aqueous extracts of transformed roots of *A. annua.* If conjugates of artemisinin were present, then such cleavage activities should have increased available levels of the aglycon. However, because he failed to use a co-injection of an untreated artemisinin standard in his HPLC, the variation of replicate assays rendered the results inconclusive. Bullis (2002) repeated much of this work, but because of the overall dismal production of artemisinin in the transformed roots at the time, no observable increase was observed after enzymatic treatment. In work performed by Driggs (2004, unpublished) aqueous extracts of whole plant shoot tissues, with significant levels of endogenous artemisinin, showed no increase after treatment with β-glucosidase, and β–glucuronidase. These data suggested that if there were conjugates of artemisinin in *A. annua*, they were not susceptible to cleavage and/or were not present in any detectable amount. Upon considering the chemical structure of artemisinin, it was observed that there isn't a conjugate that would be chemically likely. With a closed lactone ring in artemisinin, there is no active functional group that would react with a sugar. On the other hand, precursors of artemisinin, including artemisinic acid, exhibit a hydroxyl group that could allow condensation with sugar.

Kawamoto et al., (1998) found that *A. annua* has the enzymatic ability to glycosylate artemisinic acid. When a suspension culture of *A. annua* cells was fed artemisinic acid, three glucopyranosyl esters (Figure 3) of artemisinic acid were isolated. AA-G1(Figure 3) was exuded by the cells into the media, while the other two (AA-G2 and 3) were extracted from the cells. The production pathway for artemisinin in these cells was not fully functional, however, because no artemisinin or artemisinic acid was detected before addition of artemisinic acid, and no artemisinin was detected with the three glucosyl esters. Kawamoto et al. (1998) suggested this glycosylation is a possible detoxification mechanism. Unfortunately Kawamoto did not search for the compound in whole plant tissue (Kawamoto 2005, personal communication, Appendix A).



AA-G1 AA-G2 AA-G3

Figure 3: The biotransformation products of artemisinic acid:
AA-G1, artemisinic acid B-D-glucopyranosyl ester; AA-G2, 9-B-hydroxyartemisinic acid B-D-gluco-pyranosyl ester; AA-G3, 3-B-hydroxyartemisinic acid B-D-glucopyranosyl ester (Kawamoto et al., 1998)

Taken together with the possibility that the reduction of artemisinic acid is a rate-limiting step in artemisinin production (Wallaart et al., 2000), sequestration of this precursor could play an important role in the regulation of artemisinin production. Since the final two steps in the transformation of dihydroartemisinic acid to artemisinin have been shown to occur spontaneously through photooxidation, control at the point of artemisinic acid reduction would be the last biochemical opportunity to influence final production of artemisinin in this pathway.

From Kawamoto's work, it is clear that glycosylated precursors of artemisinin could also exist in the whole plant, and along with the role that roots appear to have in stimulating artemisinin production (Ferreira and Janick, 1996), it may be possible that such glycosylated precursors are manufactured both in the roots and the shoots. Therefore this study will include a search for glycosylated artemisinic acid in both the roots and shoots of whole plants.

Hypotheses

Prior research conducted at WPI, the work of Kawamoto et al. (1998) and other information in the primary literature has led to the following hypotheses:

1. If conjugated forms of artemisinic acid exists in whole plant tissues of *A. annua,* then chemical or enzymatic hydrolysis of an extract residue will increase free artemisinic acid.
2. If the glucosyl esters identified by Kawamoto et al. (1998) exist in whole plant material of *A. annua*, then they can be isolated and identified using the same methods.

Objectives

This investigation of whole plant tissues of *A. annua* has three objectives:

1. To confirm the presence of conjugated forms of artemisinic acid..
2. To confirm the presence of glucosyl esters AA-G1, AA-G2, AA-G3.

Methods

Plant Growth

Artemisia annua (strain YU16) plants were grown from seed originally supplied by Dr. Nancy Acton, Walter Reed Army Institute for Research. Plants were grown in a soil-less media (SunGro MetroMix 360) under high intensity cool white fluorescent lights running on a flower-inhibiting photoperiod (short night; 16 hr light:8 hr dark) in a temperature-controlled growth chamber (24˚C). Plants received regularly weekly fertilizations (Miracle Grow complete fertilizer, half-strength).

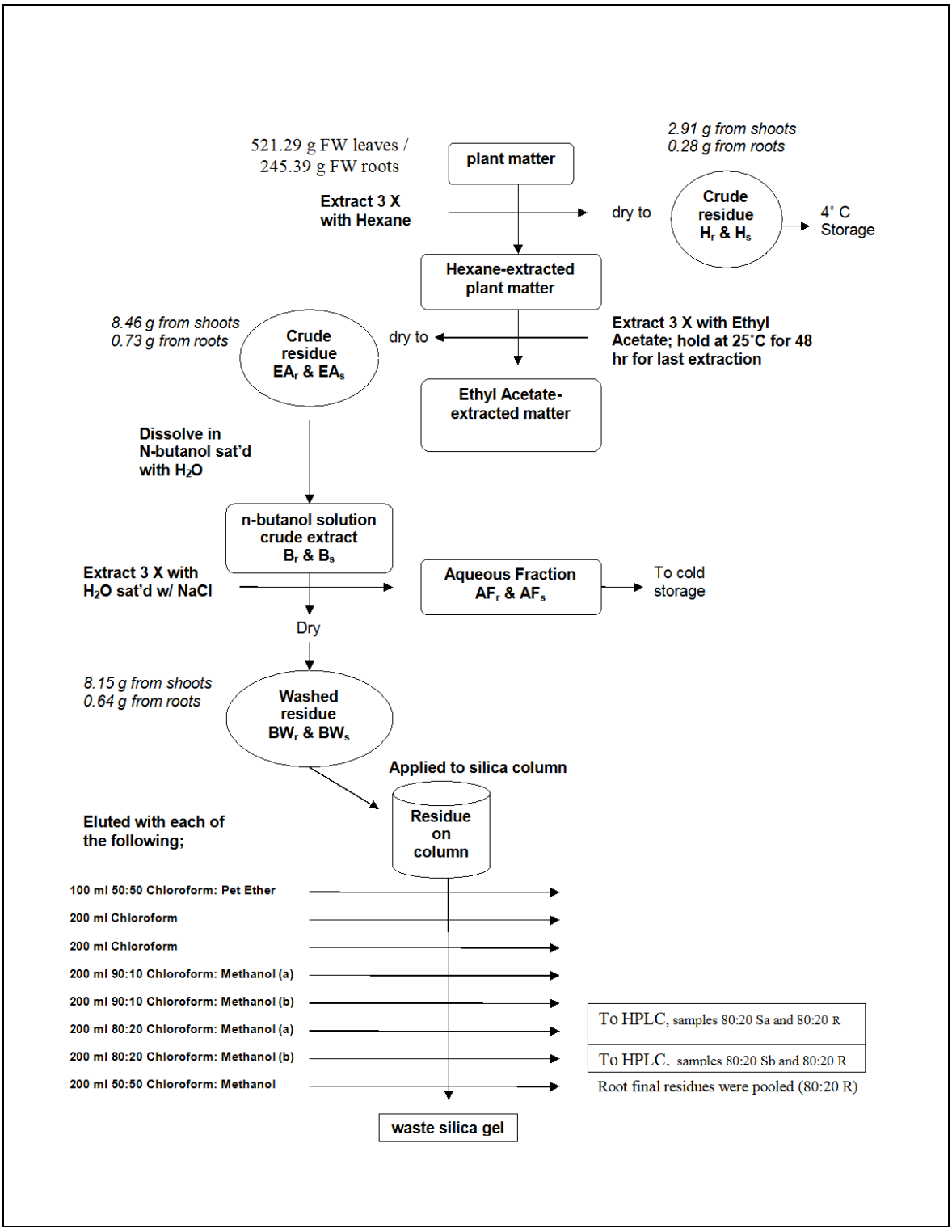


Figure 4: Flowchart depicting the purification procedure as explained in the text.

Putative Glycoside Isolation from Shoots and Roots of A. annua Plants

The isolation of putative glyco-conjugates of artemisinic acid was carried out as described by Kawamoto et al. (1998) and as clarified through personal communications with Kawamoto in 2005 (See Appendix A). Specific separation methods were performed as interpreted by Dr. Karen Erickson, a natural products chemist at Clark University, Worcester, MA.

Fresh material from 53 mature plants of *A. annua* was harvested. Shoots were separated from roots, and the latter cleaned of growing media. Leafs with petioles were removed from the stems of the plant. Total fresh mass of 521.29 g of leaves and 245.39 g of roots were obtained and lyophilized to yield 126 and 36.17 grams dry weight (DW), respectively. This mass is well above the 55 g DW mass of suspension cells of *A. annua* from which 10 mg to 58 mg of conjugate were previously isolated by Kawamoto et al. (1998).

The dried leaves (shoots) were crumbled to a coarse powder in a Ziploc bag. The roots were ground to a dust in liquid nitrogen with a mortar and pestle. The roots and shoots were separately extracted three times with hexane, sonicating in ice for 30 minutes with fresh solvent each time. This was done in order to remove very non-polar molecules from the matter, including free terpenoids. These three hexane extracts were pooled (Fig. 4, H_r and H_s). The extracted plant material was then extracted again using ultrasonics, but with ethyl acetate solvent (Kawamoto et al, 1998). After the third extraction, the plant material sat in the solvent of the third extraction for 48 hours at about 25 ˚C on the bench-top (in the lab under the room's fluorescent lights). Extracts (Figure 4, EA_r and EA_s) were then pooled. All extracts were evaporated under reduced pressure at 35 °C to obtain the following crude residues: 0.28 g hexane-extracted root residue (H_r), 0.73 g ethyl acetate-extracted root residue (EA_r), 2.91 g hexane-extracted shoot residue (H_s) and 8.46 g ethyl acetate extracted shoot residue (EA_s). Samples were stored at 4°C until further analysis.

The crude ethyl acetate residues, EA_r and EA_s, were dissolved in n-butanol saturated with water, and washed three times with sodium chloride saturated water to remove very polar molecules. The aqueous fraction was frozen for later analysis, and the butanol layer was evaporated under reduced pressure to obtain two washed residues (8.15 g washed shoot residue, BW_s, and 0.64 g washed root residue, BW_r).

Both fractions (BW_s and BW_r) were further purified using vacuum liquid chromatography, as follows. A 150 ml fritted disc funnel (dimensions 4.5 cm H x 7.5 cm W) was packed with

type 60 H silica gel and rinsed with petroleum ether. A newly packed column was used for the separation of each 1.5 g aliquot of shoot residue (BW_s). The entire BW_r root residue was applied at once. Each BW sample was dissolved in chloroform with a few drops of petroleum ether, in about 5 ml, and quickly applied to the column, with the vacuum off, and then dried by removing the solvent by vacuum suction. A series of solvents, increasing in polarity, were sequentially run through the column, with aliquots collected separately in vacuum flasks in the following order: a 200 ml wash of chloroform/petroleum ether wash, two 200 ml aliquots of 100% chloroform, two 200 ml aliquots of 90:10 chloroform/methanol, two 200 ml aliquots 80:20 chloroform/methanol, and one final 200 ml wash of 50:50 chloroform/methanol. This process was repeated until all of each of the BW_s had been applied and eluted. The corresponding fractions for each separation, for example, all of the first fractions with 100% chloroform, were pooled and evaporated under reduced pressure.

Kawamoto et al. (1998) reported that the conjugates found in cell tissues were eluted with the 80:20 solvent mix, so both of these fractions were further purified by HPLC, using a 150 X 4.6 mm C18 Column, eluted isocratically with acetonitrile/water (1:4) mobile phase at 1 ml/min and monitored at 220 nm. According to Kawamoto, the two glucosyl esters found in suspension cells, AA-G2 and AA-G3, were expected to elute at 22 and 29 minutes (ml), respectively. A fraction collector was set up to collect the eluent in 30 second (0.5 ml) fractions. Fractions representing peaks of interest were pooled and lyophilized. The fractions of interest were transferred with chloroform to sealable containers and dried under nitrogen. These samples were then transferred to Karen Erickson at Clark University for NMR analysis.

Enzymatic and Acid Hydrolysis of Putative AA Glycosides

Any putative terpenoid glycosides like those reported by Kawamoto et al. (1998), (see Figure 3), should be susceptible to hydrolysis of the terpenoid from its sugar using a number of different methods, including the enzymes, β-glucosidase (E.C.3.2.1.21, Fluka Prod. No. 49290) and carboxyl esterase (E.C. 3.1.1.1, Sigma Prod. No. E-3019), or a strong acid (1M HCl). A positive result would show an increase in free artemisinic acid once the sugars are cleaved. An

artemisinic acid and an arteannuin B standard treated with any experimental hydrolysis methods should provide a control for any aberrant affects on the terpenoids.

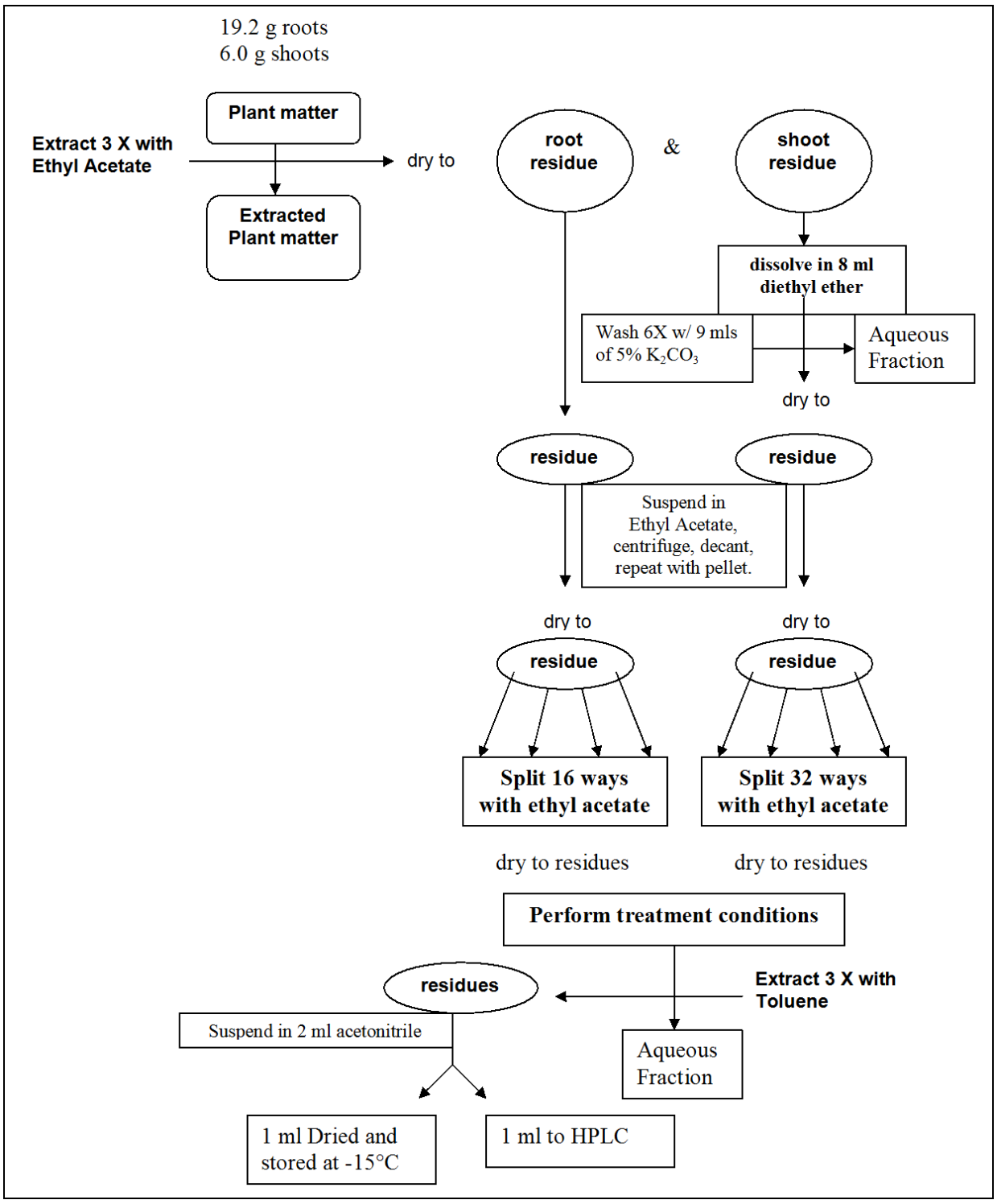


Figure 5: Flowchart depicting the preparation of hydrolysis samples.

As displayed in Figure 5, fresh biomass from 7 mature plants of *A. annua* was harvested. Shoots were separated from roots, and the latter were cleaned of growing media. Leaves with petioles were removed from the stems. Total fresh mass of 6 g of leaves and 19.2 g of roots were obtained and lyophilized. These plant fractions were separately extracted three times with ethyl acetate, sonicating in ice for 30 minutes with fresh solvent each time. The extracts were pooled and then evaporated under reduced pressure at 35 °C to obtain the following residues: 0.125 g shoot residue, 0.085 g root residue. Samples were stored at -18°C until further analysis.

Because this experiment measured an increase in free artemisinic acid, and *A. annua* leaves normally contain endogenous pools of artemisinic acid (AA), a method was developed to remove free AA to facilitate measurement of AA that was produced as a result of enzyme or acid cleavage of any putative glycosides. A modified version of the separation techniques of Vonwiller et al. (1993) was used to remove AA and other acidic compounds from the shoot residue. The prior ethyl acetate extracts were first dissolved in 8 mls of diethyl ether and then washed 6 times with 9 ml of a 5% (w/v) potassium carbonate aqueous solution. The appropriateness of the wash procedure for separating AA from terpene glycosides was confirmed previously using a mixture of standard AA and the triterpene glycoside, digitoxin (data not shown). The ether fraction was dried to obtain the washed shoot residue. The washed shoot residue was split 32 ways by completely dissolving it in 65 ml of ethyl acetate and removing 1 ml at a time. This was done twice. The root residue was split similarly into 1 ml aliquots, 16 ways, twice, with 32.5 ml ethyl acetate.

Each of the separated residues was treated according to the methods of Enyedi et al. (1992) as follows. Using a 2 minute pulse of ultrasonics, residue fractions were resuspended in 4 ml of the respective solvent. As shown in Table 1, twenty-two paired aliquots of the washed shoot residue were treated with 11 conditions. A non-treated condition served as a reference; the sample was immediately analyzed by HPLC for AB and AA. A suspension in a neutral buffer (0.2 M sodium phosphate, pH 7.0), or in each of the two enzyme buffers (see below), was extracted immediately to determine the effect of that pH and buffer on the extract. Enzymatic hydrolysis was performed for 90 minutes at 37°C in 4.0 ml of leaf extract resuspended in hydrolysis buffer (10 mM borate buffer, pH 8.0 for esterase; 100 mM sodium acetate buffer, pH 5.0 for glucosidase, per Sigma-Aldrich quality control test procedures, available as part of the

on-line catalog) containing 20 units of each of the respective enzyme. Residue fractions were also resuspended in each of the enzyme buffers without the enzyme, and incubated at 37°C for 90 minutes concurrent with the other samples. Acid hydrolysis was performed in 4 ml 1N HCl at 80°C for 30 minutes. To determine the effect of the strong acid and the high temperature, independently, residue fractions were subjected to each condition separately (one at room temperature, and one with neutral buffer). Seven of these conditions, as explained in Table 1, were also carried out in duplicate on aliquots of the root residue. To end incubation for each condition, the sample was placed on ice and immediately extracted three times with toluene. Each of the conditions listed in Table 1 was also tested on a 20 ug standards of artemisinic acid and arteannuin B, in order to determine the effects of each treatment on existing artemisinic acid and arteannuin B. One-tenth fractions of these test residues were injected into the HPLC.

To develop a reasonable method, the cleavage activity of the three different treatments was first investigated using a glycoside standard, digitoxin, a steroid (triterpene) attached by an ether bond to a chain, of three sugar molecules, and analyzed with TLC (data not shown). Use of this terpenoid glycoside enabled somewhat of an optimization of the methods of hydrolysis conditions, especially for the acid hydrolysis method.

The specific activity of each of the enzymes used in this study was confirmed in the respective experimental conditions. The activity of glucosidase was confirmed by the cleavage of a known substrate, p-nitrophenyl-B-D-glucopyranoside, causing an increase in absorbance at 410 nm (per Sigma Aldrich). Esterase activity was confirmed in a similar fashion, with fluorescein-diacetate as a substrate causing increased absorbance at 495 nm (Weathers and Kim, 2001).

Quantitative analysis of AA and AB

Assay of AA and AB was performed using HPLC, according to the modified method of Roth and Acton (1989). Samples in acetonitrile were separated with a C-18 column (25 cm x 4.6 cm l x d) in acetonitrile-water (60:40) and detected at 220nm. Peaks of interest were compared to authentic AA and AB standards.

Table 1: Experimental treatment conditions for washed shoots, roots, arteannuin B standard and artemisinic acid standard.

Treatment Name	Substrate Amount	Solvent	pH	Volume (ml)	Temp (°C)	Incubation period (min.)	# of replicates
Washed Shoots	(mass represented by residue)						
No Treatment	0.188 g FW	(none)	(n/a)	(n/a)	(n/a)	(n/a)	2
Immediate extract of buffer (pH 7.0)	0.188 g FW	0.2 M Sodium Phosphate	7.0	4	25°	0	2
Immediate extract of buffer (pH 8.0)	0.188 g FW	10 mM Boric Acid	8.0	4	25°	0	2
Esterase buffer only	0.188 g FW	10 mM Boric Acid	8.0	4	37°	90	2
Esterase	0.188 g FW	10 mM Boric Acid	8.0	4	37°	90	2
Immediate extract of buffer (pH 5.5)	0.188 g FW	100 mM Sodium Acetate	5.5	4	25°	0	2
Glucosidase buffer only	0.188 g FW	100 mM Sodium Acetate	5.5	4	37°	90	2
Glucosidase	0.188 g FW	100 mM Sodium Acetate	5.5	4	37°	90	2
Hot Neutral Buffer	0.188 g FW	0.2 M Sodium Phosphate	7.0	4	80°	30	2
Acid Room Temp.	0.188 g FW	1 N Hydrochloric Acid	0.0	4	25°	30	2
Hot Acid	0.188 g FW	1 N Hydrochloric Acid	0.0	4	80°	30	2
Roots	(mass represented by residue)						
RT No Treatment	1.20 g FW	(none)	(n/a)	(n/a)	(n/a)	(n/a)	2
RT Buffer pH 7.0, immed.extract	1.20 g FW	0.2 M Sodium Phosphate	7.0	4	25°	0	2
RT Esterase buffer (8.0) only	1.20 g FW	10 mM Boric Acid	8.0	4	37°	90	2
RT Esterase	1.20 g FW	10 mM Boric Acid	8.0	4	37°	90	2
RT Neutral 80°C	1.20 g FW	0.2 M Sodium Phosphate	7.0	4	80°	30	2
RT Acid Room Temp.	1.20 g FW	1 N Hydrochloric Acid	0.0	4	25°	30	2
RT Acid 80°C	1.20 g FW	1 N Hydrochloric Acid	0.0	4	80°	30	2
Arteannuin B Standard	(amount of standard)						
AB No Treatment	20 ug	(none)	(n/a)	(n/a)	(n/a)	(n/a)	2
AB Immediate extract of buffer (pH 7.0)	20 ug	0.2 M Sodium Phosphate	7.0	4	25°	0	2
AB Acid Room Temp.	20 ug	1 N Hydrochloric Acid	0.0	4	25°	30	2
AB Hot Acid	20 ug	1 N Hydrochloric Acid	0.0	4	80°	30	2
AB Hot Neutral Buffer	20 ug	0.2 M Sodium Phosphate	7.0	4	80°	30	2
AB Esterase	20 ug	10 mM Boric Acid	8.0	4	37°	90	2
AB Glucosidase	20 ug	100 mM Sodium Acetate	5.5	4	37°	90	2
Artemisinic Acid Standard	(amount of standard)						
AA No Treatment	30 ug	(none)	(n/a)	(n/a)	(n/a)	(n/a)	1
AA Immediate extract of buffer (pH 7.0)	30 ug	0.2 M Sodium Phosphate	7.0	5	25°	0	1
AA Hot Acid	30 ug	1 N Hydrochloric Acid	0.0	5	80°	30	1
AA Hot Neutral Buffer	30 ug	0.2 M Sodium Phosphate	7.0	5	80°	30	1
AA Esterase	30 ug	10 mM Boric Acid	8.0	5	37°	90	1
AA Glucosidase	30 ug	100 mM Sodium Acetate	5.5	5	37°	90	1

Results

In order to determine whether or not a naturally occurring AA glycoside is present in *A. annua* plants, two different experimental approaches were used. First, large masses of roots and shoots from vegetativly grown plants were extracted and separated into fractions for NMR analysis. Alternatively, if any AA glycosides exist *in planta* they should be susceptible to hydrolysis of the glycosidic bond linking the sugar to the terpenoids, resulting in increased pools of free AA. Results from both experimental approaches are described.

Putative Glycoside Isolation from Shoots and Roots of A. annua Plants

Based on the work of Kawamoto et al. (1998), the putative AA conjugates were expected to be found in the 80:20 Chloroform-methanol fraction (Figure 4). The 80:20 fractions (shoots and roots) were the only fractions pooled and analyzed by NMR (as shown in Figures 6 and 7).

The residue (74 mg) from the purified shoot fraction, (80:20 S_a), when separated by HPLC resulted in the chromatogram displayed in Figure 6. The sample was applied in two injections of 200 ul each. The eluted fractions gathered using the fraction collector were pooled into 6 groups, labeled “c” through “i" in Figure 6. The six pooled fractions from the 2 injections (c through i), represented the peaks detected at 220 nm between 13.5 minutes and 31.5 minutes, and were transferred to Dr. Karen Erickson at Clark University for NMR analysis. The final mass of each pooled residue is listed in Table 2 and in total represented 9 mg of the total 74 mg of 80:20 S_a.

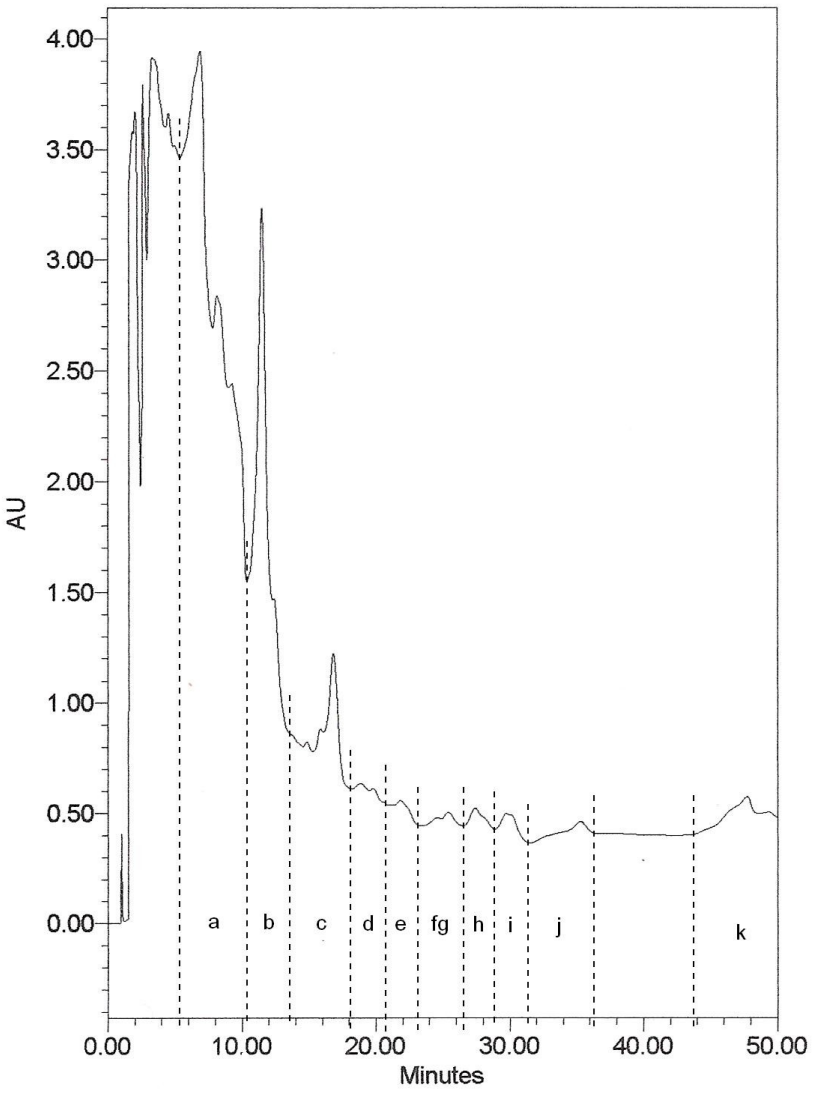


Figure 6: Chromatogram of shoot fraction 80:20a separated by HPLC. The letters represent the eluted fractions that were pooled from two identical injections.

Table 2: Masses of pooled residues after separation into 6 peaks eluted off the HPLC column.

Fraction letter	Final mass
c	2 mg
d	2 mg
e	1 mg
f/g	2 mg
h	1 mg
i	1 mg

The purified root fractions, 80:20 R_a and 80:20 R_b, were pooled together yielding a combined weight of 42 mg. Separation by HPLC resulted in the chromatogram displayed in Figure 7. The sample was applied in two injections of 100 ul each. The eluted fractions were collected and pooled as labeled in Figure 7. The six pooled fractions, labeled "m" through "q" in Figure 7, representing the peaks detected between 15 minutes and 43 minutes, were transferred to Dr. Karen Erickson at Clark University for NMR analysis. The final mass of each pooled residue was unfortunately barely detectable (≤ 1 mg).

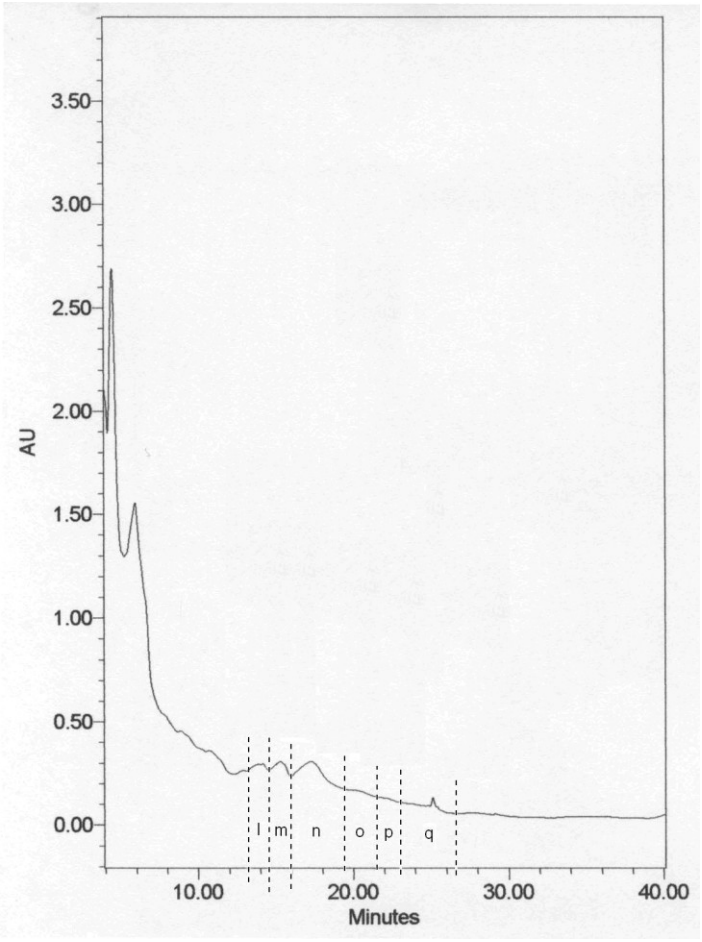


Figure 7: Chromatogram of root fraction 80:20 separated by HPLC. The letters represent the eluted fractions that were pooled from two identical injections.

The NMR analysis of pooled fractions from both the HPLC-purified shoots and the roots did not indicate the presence of glycosylated terpenoids. Rather, the results suggested that only fats were present in the samples (data not shown).

The majority of the purified shoot and root extracts were in the 90:10 fractions, which were not separated by HPLC or analyzed by NMR. The shoot fractions weighed 5.1 grams (90:10As, Figure 4) and 0.619 grams (90:10Bs, Figure 4), in total representing 70% of the semi-crude residue applied to the chromatography column in the last step of purification. The 90:10 root fractions were 0.18 and 0.11 g (90:10 Ar and Br, respectively, representing 45% of the total root residue applied). In contrast, the residues that were analyzed in this report, the 80:20 fractions, were only 74 mg and 18 mg (shoots and roots, respectively) before being separated by HPLC. If an error was made in the interpretation or the application of the isolation methods of

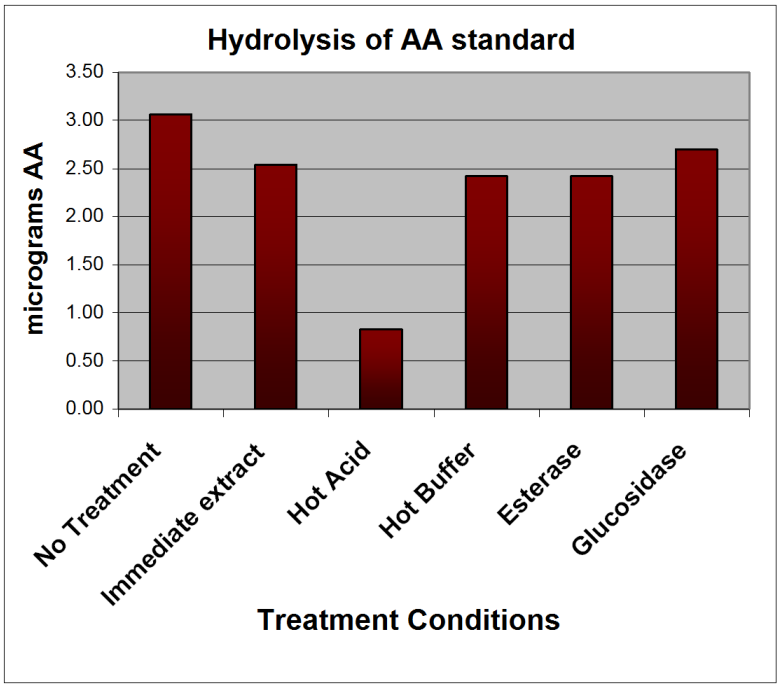
Kawamoto et al. (1998), then the compounds of interest may have ended up in a different fraction. The 90:10 fractions should be the next place to search, but time restricted the extent of this project.

Enzymatic and Acid Hydrolysis of Putative AA Glycosides

If any artemisinic acid glycosides exist *in planta* they should be susceptible to hydrolysis of the glycosidic bond linking the sugar to the terpenoids, resulting in increased pools of free AA. The hydrolysis conditions used in this study were first tested on standards of AA and AB to determine the extent of degradation due to these treatments. The shoot and root extracts were subsequently subjected to the same conditions in a search for those increased pools of AA. The results of HPLC analysis of these experiments are presented.

Hydrolysis of Standards AA and AB

The different extractions clearly showed some specific and degradative effects on the pure standards AA and AB as shown in Figure 8 and 9, respectively. When 20 ug of AA (an aliquot representing 2 ug was analyzed) was exposed only to a 0.2 M sodium phosphate buffer, pH 7.0, 17.3% of AA was lost (Figure 8). However, it did not appear that prolonged exposure to heat (80°C for 30 min) resulted in any further loss. The standard was not further degraded by esterase, and appeared to be slightly more



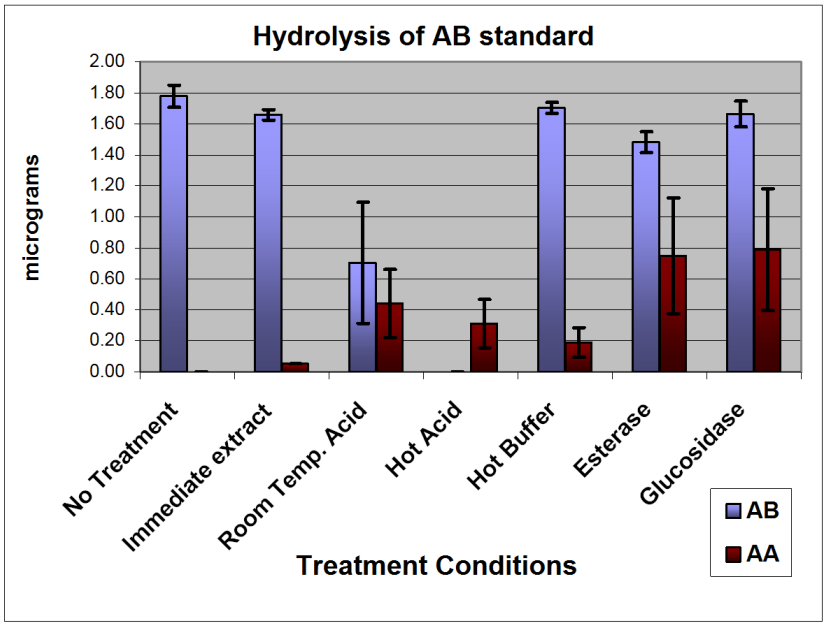
Hydrolysis of AA standard
starting mass of 3.0 ug

Treatment	*pH*	*Temp.*	*Time (min.)*	*ug AA*	*% AA remaining*
No Treatment	n/a	n/a	n/a	3.07	100.0
Immediate Extract	7.0	25°	0	2.54	82.7
Hot Acid	0.0	80°	30	0.83	27.0
Hot Buffer	7.0	80°	30	2.42	78.8
Esterase	8.0	37°	90	2.42	78.8
Glucosidase	5.5	37°	90	2.70	87.9

*only one sample analyzed, hence, no error bars

Figure 8: Effects of hydrolysis conditions on artemisinic acid standard

stable in the glucosidase buffer. A treatment with hot acid, however, resulted in the loss of 73% of the standard. Although this sample set was not replicated, similar results were obtained in the past (data not shown).



Hydrolysis of AB standard
starting mass of 2.0 ug

Treatment	*pH*	*Temp.*	*Time (min.)*	*ug AB*	*% AB remaining*	*ug AA*	*% AA appearing*
No Treatment	n/a	n/a	n/a	1.78	100.0	0.00	0.0
Immediate Extract	7.0	25°	0	1.66	93.3	0.05	2.9
Room Temp Acid	0.0	25°	30	0.70	39.3	0.44	24.8
Hot Acid	0.0	80°	30	0.00	0.0	0.31	17.5
Hot Buffer	7.0	80°	30	1.70	95.5	0.19	10.7
Esterase	8.0	37°	90	1.48	83.1	0.75	42.0
Glucosidase	5.5	37°	90	1.66	93.3	0.79	44.3

% AA appearing was calculated based on the total AB added to the reaction, assuming AB was the only compound converted.

Figure 9: Effects of hydrolysis conditions on arteannuin B standard

The treatment of arteannuin B standard (Figure 9) with the hydrolysis conditions resulted in the apparent formation of artemisinic acid. Degradation of AB could thus yield the chemically related AA. For the purpose of comparison, the observed quantities of AA (calculated based on the absorbance value of AA standard) were given a percent value based on the amount of AB originally present. No AA peak was detected in the untreated sample, and a small amount (2.9%) was detected in the immediate extract which also had a 6.7% decrease in AB. The room temperature acid (1M HCl for 30 min.) treatment degraded the AB by 60.7% and caused the appearance of 24.8% AA. The hot acid treatment (80°C) completely degraded the AB, but yielded 17.5% AA. This yield was similar to the 21.2% of the AA remaining after the same treatment of the pure AA standard (Figure 8). It is as if the AB was completely converted into AA and subsequently degraded as before. The hot buffer (pH 7.0, 80°C, 30 min) caused little degradation of AB (4.5%) and the formation of 10.7% AA. Curiously, the increase in putative AA after the enzymatic treatments

(42% and 44.3% for esterase and glucosidase, respectively) was much greater than the decrease in AB (16.9% and 6.7%, respectively) for those treatments.

These changes in AA and AB standards that were observed after the various treatments were used to better quantitate AA and AB remaining in the experimental plant extracts.

Hydrolysis of Root and Shoot Samples

The root and shoot extracts were prepared and treated as explained in the methods. The HPLC data obtained from the resulting samples were analyzed for changes in AA and AB. The analysis of chromatograms was complicated by the presence of a double peak at the retention times for both standards. While no AA or AB was observed upon treatment of root residues, changes were observed in the levels of AA across the conditions, as previously explained.

The HPLC chromatograms displayed in Figure 10 are representative of the samples treated with the 3 hydrolysis conditions. The complete set of chromatograms is contained in Appendix B. The retention times for the AB and AA standards are displayed in Figure 10A. The chromatograms of AA samples in this trial were irregular in baseline and area (Appendix B), but the retention time of the apparent AA peak was consistent along with the variations noted in the AB standard retention times as shown in Figure 10A. The chromatograms of the regular pure AB standard injections exhibited single-peaks (Figure 10A, B). The non-treated and treated AB standards, however, exhibited double-peaks; compare Appendix B-4 with B-22.

Analysis of the chromatograms was complicated by the occurrence of an unresolved double peak at the retention times for both AA and AB. This shouldered peak was not observed with a more polar mobile phase (80:20 water-acetonitrile). It was determined that the double-peak most likely represents a slightly modified form of the respective artemisinic compound that is visible as a single peak in the more polar mobile phase. Considering that the double peak was observed in samples that contained only authentic standard (Figure 10C), the area of the entire double-peak was used for consistent analysis of results. The trends obtained from this method of analysis correlated strongly with those obtained by estimating the value of the single peak, separately. The retention times, peak areas, and corresponding equivalence of AA and AB are detailed and calculated for both single and double peaks in Appendix C.

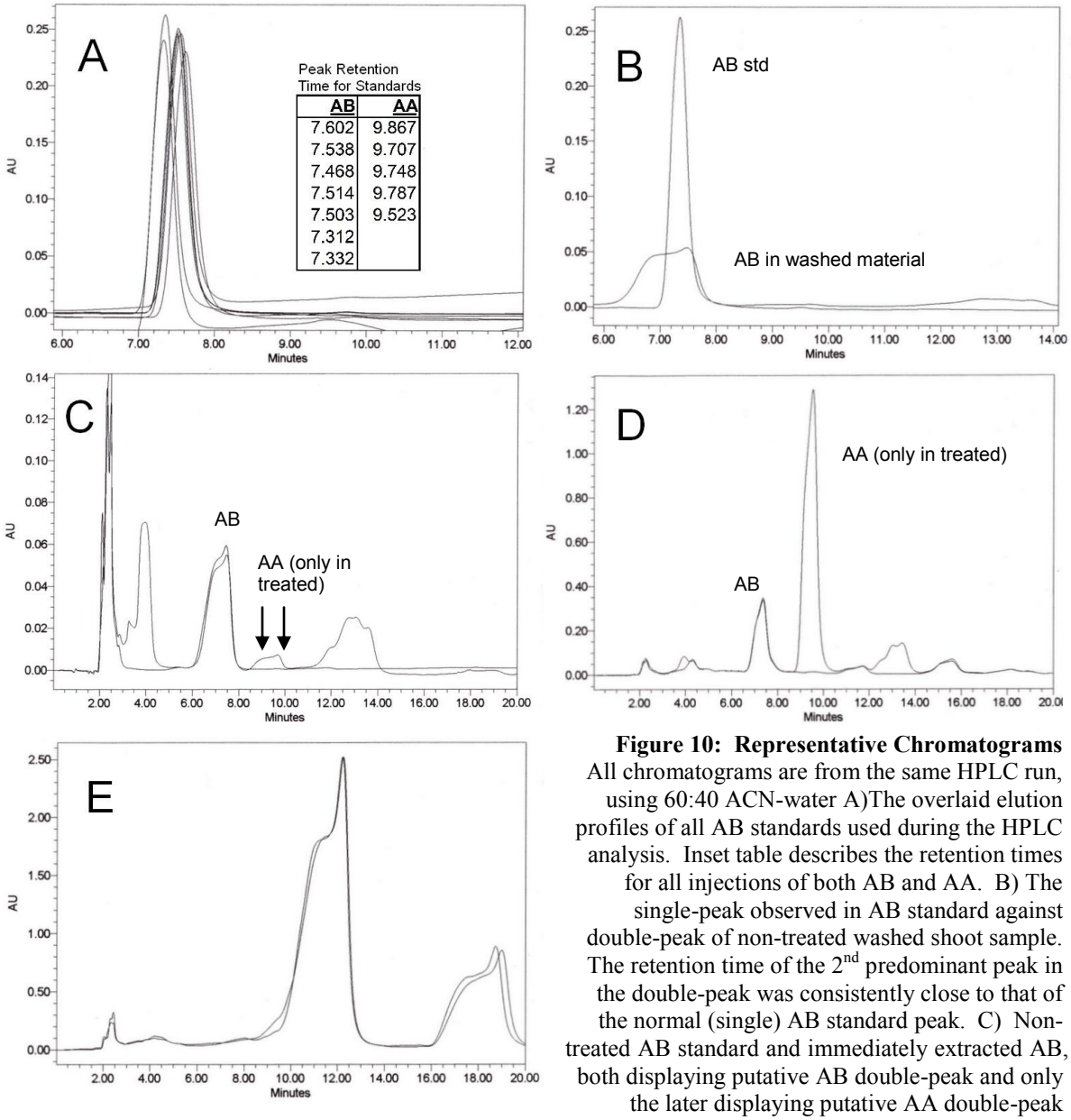


Figure 10: Representative Chromatograms All chromatograms are from the same HPLC run, using 60:40 ACN-water A)The overlaid elution profiles of all AB standards used during the HPLC analysis. Inset table describes the retention times for all injections of both AB and AA. B) The single-peak observed in AB standard against double-peak of non-treated washed shoot sample. The retention time of the 2nd predominant peak in the double-peak was consistently close to that of the normal (single) AB standard peak. C) Non-treated AB standard and immediately extracted AB, both displaying putative AB double-peak and only the later displaying putative AA double-peak D) Non-treated and immediately extracted washed shoot residues. The dramatic increase in the putative AA peak is evident after resuspension in pH 7.0 buffer. E) The root treatments displayed no new peaks at the retention times for either AA or AB.

Hydrolysis of washed shoot residues: effects on AA

In order to determine the effect of the 3 hydrolysis methods on any putative AA glycosides, one has to be able to quantitatively measure any increases in free AA pools within the test samples. Considering that it is likely that only small amounts of glycosides may be present, removal of native free AA would facilitate detection, thus samples first had free AA pools removed before proceeding with any of the hydrolysis methods. The free carboxylic acid groups on AA make it very soluble in the carbonate wash used to remove endogenous pools of AA.

The hydrolysis of shoot residues as listed in Table 1 produced some dramatic changes in artemisinic acid (Figure 11). The washed shoot residue showed minimal endogenous AA (8.32 ug AA/g FW ± 0.27) remaining, demonstrating that the washing procedure was reasonably effective (Figure 11) and that only about 2% of the original AA remained. Un-washed, air-dried shoot tissues contained 390 ug/g FW (data not shown). Surprisingly, however, the immediate extraction of the buffer (pH 7.0) suspension of washed shoot residue resulted in an apparent dramatic increase in AA to an average of 2269 ug AA/g FW (Figure 10D). Immediate extraction of residues suspended in the other two buffers used in the enzyme experiments (esterase buffer, pH 8.0 and glucosidase buffer, pH 5.5) also resulted in increased levels of AA compared to the untreated washed control, but considerably less than that observed at pH 7.0. Clearly, this apparent pH effect needs to be investigated further in order to fully understand these results. When the washed shoot extracts were incubated for 90 minutes in glucosidase buffer or esterase buffer only (without enzyme), the amount of extractable AA decreased compared to extraction under the same conditions immediately extracted (Figure 11). No apparent increases in AB occurred in these samples. Responses of AB to these treatment conditions of plant extracts are discussed later.

Although one of the esterase-treated washed shoot residues displayed AA levels that nearly doubled compared to the amount detected in the enzyme-free control (10 mM boric acid buffer, pH 8.0), the replicate sample indicated degradation of AA, resulting in an averaged 11% increase in AA due to the enzyme (Table 3). The apparent increase in AA due to glucosidase, when compared to the enzyme-free control (100 mM sodium acetate buffer, pH 5.5), was

substantial and repeatable (average 72.3%, an increase of 167 ug AA/g FW). The washed residues treated with hot (80°C) pH 7.0 sodium phosphate buffer resulted in a 51.9% decrease in AA when compared to the immediate extract with the same buffer. The acidic treatment (1M HCl) at room temperature (25°C, for 30 min) displayed lower levels of AA (32% lower than the hot buffer).

In order to review the changes in AA and AB caused by the different treatment conditions of the shoot residues, the values are summarized in Table 3. Percent changes are displayed for meaningful pairs of conditions, as listed in the column labeled "% change compared to."

Interestingly, the condition combining high heat and low pH (1M HCl at 80°C for 30 minutes) resulted in levels of AA which were higher than the hot buffer by an average of 7.3%. The acid appears to cause an increase in AA when combined with the high temperature. Treatment of AA standard with the hydrolysis condition (Figure 8) shows that AA is degraded significantly by the hot acid treatment. If the amount of AA detected in washed shoot residues after the hot acid treatment is adjusted for this amount of degradation detected in the standard, the resulting amount of AA is actually 4337 ug AA/g FW, which is even higher than the amount of AA detected in the immediate extract with neutral buffer.

Hydrolysis of washed shoot residues: effects on AB

The resulting quantities of AB detected after hydrolysis of shoot residues are displayed below, in Figure 12. The peaks were identified as AB based on their retention time. Changes in retention times over the course of sample injections were accounted for by regular injections of authentic standards. After the shoot residue was essentially washed to remove free AA, rather large endogenous AB (646 ug/g FW ± 7.55) remained. There was no increase in AB upon treatment of washed shoot residues with aqueous buffers. In contrast to what was observed for AA, there was no difference in the level of AB in the immediate extracts at any of the three different pHs used (Figure 12). Similar to the treated AB standards, AB decreased by less than 10% in all conditions except those that were acidic. The room temperature acid resulted in a 17% decrease in AB compared to the pH 7.0 buffer incubated at 80°C for the same amount of time. Similar to what was observed for AB standard (Fig. 9), AB was fully degraded by the hot acid condition.

Table 3: Hydrolysis effects on AA and AB in washed shoots: The percent increase is the increase in AA or AB in each condition in comparison to the appropriate reference as listed in the "compared to" column. Values marked "*" are no different than 0% due to replicate variation. Relative increases in AA are compared to corresponding decreases in AB in order to consider the possibility of a conversion between them.

Treatment	*pH*	*°C Temp.*	*Time (min.)*	*ug AA/ g FW*	*% change*	*% change compared to*	*Increase in AA covered by decrease in AB?*	*ug AB/ g FW*	*% change*	*% change compared to*
No Treatment	n/a	n/a	n/a	8.32	0	no treatment		646	0	no treatment
Immediate Extract	7.0	25°	0	2269	27171.6	no treatment		615	-4.8	no treatment
Hot Buffer	7.0	80°	30	1091	-51.9	pH 7.0 immediate		616	0.2	pH 7.0 immediate
Immediate Extract	8.0	25°	0	1176	-48.2	pH 7.0 immediate		618	0.5	pH 7.0 immediate
Buffer only	8.0	37°	90	378	-67.9	pH 8.0 immediate		623	0.8	ph 8.0 immediate
Esterase	8.0	37°	90	420	11.1*	esterase buffer only	42 ug AA from 41 ug AB	582	-6.6	esterase buffer only
Immediate Extract	5.5	25°	0	1200	-47.1	pH 7.0 immediate		611	-0.7	pH 7.0 immediate
Buffer only	5.5	37°	90	231	-80.7	pH 5.5 immediate		599	-2	ph 5.5 immediate
Glucosidase	5.5	37°	90	398	72.3	glucosidase Buffer only	167 ug AA from 14 ug AB	613	2.3	glucos. Buffer only
Hot Buffer	7.0	80°	30	1091	-51.9	pH 7.0 immediate		616	0.2	pH 7.0 immediate
Room Temp Acid	0.0	25°	30	676	-38	Hot Buffer		512	-16.9	Hot Buffer
Hot Acid	0.0	80°	30	1171	7.3*	Hot Buffer	80 ug AA from 616 ug AB	0	-100	Hot Buffer
Hot Acid (values adjusted for degradation)				4337	297.5	Hot Buffer	3246 ug AA from 616 ug AB			

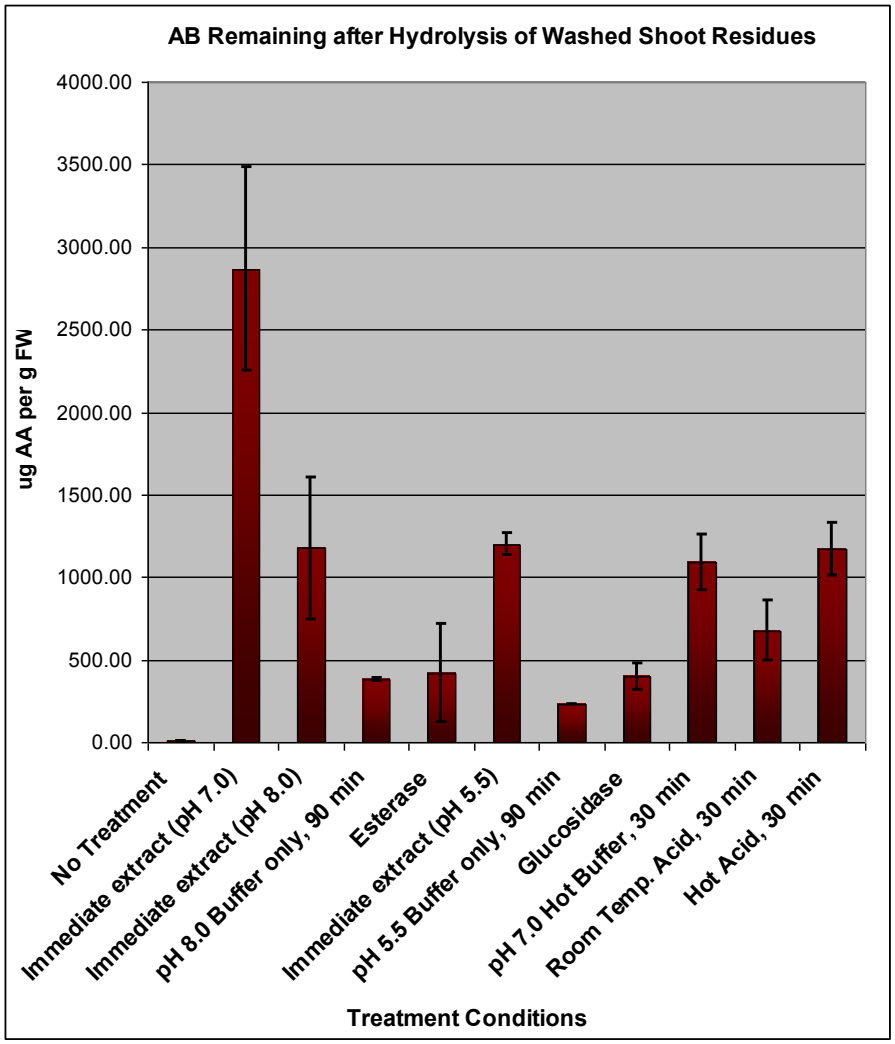


Figure 11: Effects of hydrolysis conditions on AA in washed shoot residues.

Although the possibility of the conversion of AB to AA due to hydrolysis conditions was seen in the standard treatments, such an effect can not account for the sometimes large increases in AA seen in the washed shoot residues (Table 3). The decreases in AB resulting from the hydrolysis of the shoot extracts was much lower than the increases in AA resulting from the corresponding conditions. The glucosidase treatment caused an apparent (but minor) increase in AB, while also causing an increase in AA. The amount of AA detected after hot acid treatment of shoot extract could not be fully accounted for as the product of degraded AB, since the detected level of AA was much higher than the endogenous levels of AB. The average increase in AA due to esterase, when compared to the esterase buffer alone (42 ug AA/g FW) does correlate with the apparent decrease in AB due to esterase (41 ug AB/g FW), however, the two

values used to obtain the average increase in AA were highly divergent, with one value showing a large increase of 337 ug and the other showing a 253 ug decrease.

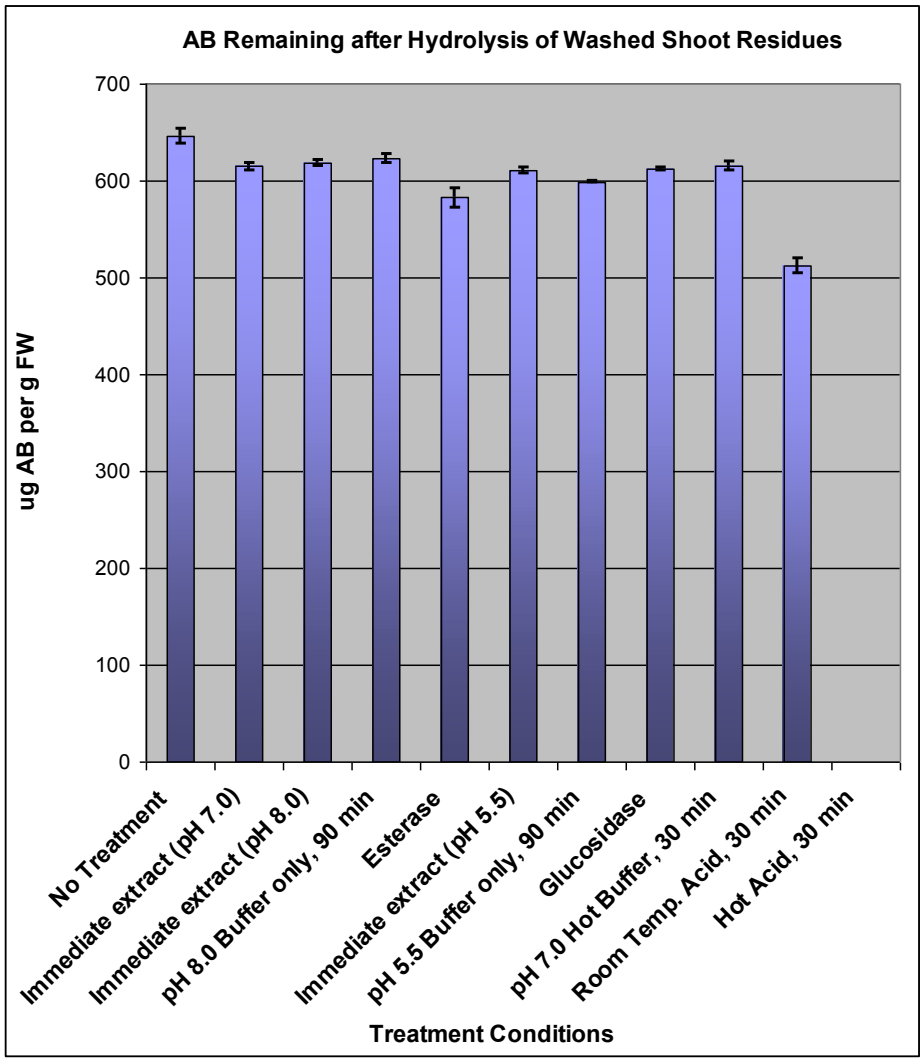


Figure 12: Effects of hydrolysis conditions on AB in washed shoot residues.

Hydrolysis of root residues

There was no increase in absorbance at the respective retention times for AA or AB in any of the root extracts treated with acid, esterase, or β-glucosidase. No peaks existed at the corresponding retention times either before or after hydrolysis treatment, although subtle increases may have been obscured by neighboring peaks. The only noteworthy modification in chromatogram profiles resulted from treatment with hot acid, where all the HPLC peaks decreased in absorbance, and some new peaks appeared at retention times not related to the elution times of known standards.

Discussion

Using two different analytical approaches, this project sought to confirm the presence of conjugated forms of artemisinic acid in whole plant tissues of *A. annua*. Bulk extracts were purified according to the methods of Kawamoto et. al (1998), who isolated three glycosides of AA from suspension cells. NMR analysis of the purified fractions of shoot and root extracts which corresponded to the fraction in which the glycosides were previously found did not at present result in the detection of any of the glycosides described by Kawamoto. Results from the alternative experiments, however, indicated that under some conditions hydrolysis of shoot extracts apparently resulted in the liberation of AA, supporting the hypothesis that conjugated forms of AA exist *in planta*. The results of both these experiments are discussed below.

Putative Glycoside Isolation

The isolation of putative glycosides from bulk plant extracts was a daunting challenge from the start. The procedure, as described in the literature by Kawamoto et al. (1998) was incomplete, vague, and (at least) partially incorrect, as was later discovered upon correspondence with the author (communications are compiled in Appendix A). After multiple communications with the author via email, it seemed that the details of the more specific procedures (CC and HPLC) were clarified. The procedure itself proved to be a time and resource-consuming endeavor, and the final NMR analysis of fractions was unfortunately limited by the duration of the project and the lack of a prep HPLC.

The chemical procedure up to the point of column chromatography was consistent with a sequence of actions which would conserve glycosylated terpenoids, and it is unlikely that putative glycosides were removed from the extract before this point. If the glycosides were in the residue when it was applied to the column, it is quite possible that they were eluted before the 80:20 chloroform-methanol wash, for the majority of the applied residue was eluted in the previous fraction (90:10A) in both the shoot and root extracts. Further, this was the fraction in which Kawamoto et al. (1998) observed the third glycoside which was only found in the spent cell culture medium.

The NMR analysis took much more time than expected, limiting the number of samples that could be analyzed. Analysis was prioritized, and since the two glycosides extracted from suspension cells by Kawamoto et al. (1998) were found in the 80:20 fraction, this was the first and ultimately the only fraction to be analyzed. The small amount of residue resulting from the isolation and purification of these fractions necessitated extensive analysis in order to produce interpretable data. Dr. Erickson was unable to confirm anything that looked like terpenoids, or the compounds described by Kawamoto. At the time of this writing, not all of the pooled fractions listed in the report have been fully analyzed.

To complete this part of the study, the major residues from the 90:10 fractions should be purified and examined by NMR. The second part of this study (the hydrolysis of extracts) indicated that glycosides may indeed be present, but only in the shoot tissues. The 90:10 shoot fraction represents a large portion shoot biomass, and could contain the glycosides. It is unlikely that glycosides were present only in undetectable amounts, because the starting plant biomass was much higher than the cell biomass extracted by Kawamoto et al. (1998), but this remains a possibility. Alternatively, the glycosides may simply not exist *in planta*. This however, challenges the results of the hydrolysis study, so the previous possibilities are more likely. Further, it is conceptually not possible to prove a negative result.

Samples of the AA glycosides isolated by Kawamoto et al. (1998) would have been extremely helpful in the search for the compounds *in planta*. Unfortunately the compounds were discarded some time after Dr. Kawamoto left the research facility. There was no glycoside reference standard available for any of the targeted artemisinic acid glycosides. Although organic synthesis of the glycosides is feasible (Mitsunobu Protocol, Batovska et al., 2005), it was not pursued in this project. The synthesis was described by Sam Tang, a graduate-student organic chemist, as "relatively easy," and should be considered in future studies of these glycosides.

Enzymatic and Acid Hydrolysis of Putative AA Glycosides

Although the results of the isolation procedure are as of yet, inconclusive, the hydrolysis study appears to support the presence of glycosylated artemisinic acid in shoots of *A. annua*. The hydrolysis of the authentic AB standard revealed interesting degradative effects which merit

further investigation. Similarly, an apparent pH effect on the AA levels of washed shoot extracts must also be examined. Hydrolysis of the washed shoot residues with glucosidase appeared to increase levels of AA. The data indicates the presence of glycosylated forms of AA, as further discussed.

Standard Treatments

The hydrolysis of the AA standard was performed to determine the effects of the treatment condition on the product (Figure 8). When a small amount of the compound, 20 ug, was exposed to each of the conditions, only the acidic treatment caused much more degradation than the immediate extract. The percent decrease in AA due to hot acid treatment was later used to adjust the value of AA seen in hot acid treated washed shoot residues, in order to obtain an estimate of how much AA was freed by the acid hydrolysis but subsequently destroyed by the procedure.

The treatment of AB standard yielded interesting and perplexing results. It appears that AB is partially converted to AA upon solution in any of the aqueous conditions used in this experiment (Figure 9). AA was not detected in the untreated sample, but was present in amounts which, in all the conditions without enzymes, could be accounted for by the amount of decrease in detected AB. Upon inspection of the chemical structures of the two compounds, acid reduction of AB to AA seems quite plausible. Further, the reaction that is known to convert AA to AB is a photo-oxidation reaction, which can occur *in vitro* (Roth and Acton, 1989). One encounters a complication, however, when interpreting the AB standard treatment results, because the apparent increase in AA due to the enzyme treatments is far greater than the corresponding decrease in AB. Further, the standards were not treated with the enzyme buffers alone, so the increase in putative AA can not be positively attributed to the enzymes. The absorbance of AA and AB at 220 nm was roughly the same (within 10%), so it does not appear that that the areas of the HPLC chromatograms were misinterpreted. Although the extent and nature of AB degradation to AA must be investigated further, it is clear that it is occurring to some extent, and must be taken into consideration when interpreting later results.

Hydrolysis of Whole Plant Residues

The salt water wash procedure, performed in order to remove endogenous AA from a diethyl ether solution of shoot extract, appeared to be largely effective. Previous studies had yielded concentrations in the range of 300 to 400 ug AA/g FW AA in unwashed air-dried leaf tissues. Although the majority of endogenous AA appears to have been removed before hydrolysis treatment, all of the conditions involving an aqueous phase seem to have made more AA available. The dramatic increase in AA upon solution and immediate extract with the neutral buffer was unexpected, and the source of this AA is unknown. As explained in the results, the increases in AA can not be accounted for by the decreases in AB, except in the case of the averaged value of the esterase treatment (as explained in the results). Since the AA was identified solely by its retention time, it still needs to be confirmed as AA using NMR or MS.

The increased pools of AA must have been derived from the degradation of products other than AB which were also present in the washed extract. Other sources of AA could include related artemisinic compounds or even the putative glycosides which are the subject of this study. There was an apparent pH effect on the amount of AA detected on immediate extracts, with pH 7 buffer resulting in levels twice those found after treatment with pH 5.5 or pH 8.0 buffers. The liberation of AA in aqueous extracts of shoot tissues and the effect of pH must be investigated, as these effects may complicate other studies of this compound. More importantly, if this "new" AA truly is AA, then perhaps a novel reservoir has indeed been accessed.

When enzyme treatments of washed shoot residues were compared to the enzyme-free incubated controls, the difference in AA suggested that the compound was made available because of treatment with the enzymes. Although the β-glucosidase treatment was the only enzyme to show a repeated increase in AA (average 72%), one of the esterase samples suggest a substantial (88%) increase due to the enzyme (while the other sample exhibited degradation). The β-glucosidase used in this study (EC 3.2.1.21) hydrolyses terminal, non-reducing β-linked D-glucose residues. This enzyme is more specific than the general carboxylic esterase also used in this study (EC 3.2.1.21). In a similar hydrolysis study of salicylic acid glucosyl esters, Enyedi et al. (1992) reported cleavage due to β-glucosidase and no detectable change due to carboxyl esterase.

The acid treatments of washed shoot residues indicate that some compound or group of compounds is being modified by the hot acid to yield an increase in AA. The apparent increase

(especially when adjusted for AA degradation) is much larger than that seen in the enzyme treatments. It appears that that whatever chemical species are being converted by acid, they are not entirely susceptible to enzymatic cleavage. This makes sense, since a strong acid would be much more of a general reducing agent compared to an enzyme, and is likely degrading related artemisinic compounds. Again, however, these increases in putative AA must be confirmed by NMR or MS.

Although the effect of esterase on *A. annua* shoot extracts is unclear, the glucosidase treatment appears to have freed AA in washed shoot extracts, supporting the hypothesis that glycosylated forms of AA exist *in planta*. If the increase in AA due to glucosidase is an indicator of the amount of glycosides found in the plant, then the increase indicates the presence of about 170 ug of glycoside per g FW. If this figure is adjusted for the apparent degradation of AA due to the 90 minute incubation period (80.7%, from Table 3), then the value is 880 ug glycoside per g FW. If this range of values is taken into consideration with the bulk of biomass extracted in the glycoside isolation procedure (521 g FW), then 89 to 460 mg of the glycoside should have been present in the crude extract. As previously explained, not all fractions from the purification procedure have yet been analyzed.

Because no increases in AA were observed upon hydrolysis of root extracts with either esterase or acid, the hypothesis that AA glycosides occur in root tissues of *A. annua* can not be supported. However, the analysis of the root tissues may have been less than complete. A subtle increase in absorbance at the retention time of AA may have been obscured by neighboring peaks. To be sure that no AA was liberated due to hydrolysis treatments, the samples should be examined using a different mobile phase, or alternatively, using thin layer chromatography.

As of this time, the hypothesis that AA glycosides exist in shoot tissues is supported, although the hypothesis of root production and transport of the glycosides is not. The glycosides have not yet been isolated *in planta*, and the only indication of their presence is their apparent cleavage by β-glucosidase and acid hydrolysis, as indicated by increased pools of AA.

Works Cited

Abdin MZ, Israr M, Rehman RU, Jain SK. 2003. Artemisinin, a novel anti-malarials drug. Planta Medica 69:289-299.

Aharoni A, Giri AP, Deuerlein S, Griepink F, de Kogel WJ, Verstappen FWA, Verhoeven HA, Jongsma MA, Schwab W, Bouwmeester HJ. 2003. Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. Plant Cell 15: 2866-2884.

Batovska DI, Kishimoto T, Bankova VS, Kamenarska ZG, Ubukata M. 2005. Synthesis of some phyenylpropanoid monoglycerides via the Mitsunobu protocol. Molecules 10:552-558.

Bertea CM, Freije JR, van der Woude H, Verstappen FWA, Perk L, Marquez V, De Kraker JW, Posthumus MA, Jensen BJM, de Groot Ae, Franssen MCR, Bouwmeester HJ. Identification of intermediates and enzymes involved in the early steps of Artemisinin Biosynthesis in *Artemisia annua*. Planta Medica 71: 40-47.

Bouwmeester HJ, Wallaart TE, Michiel HA, Janssen, MHA, van Loo B, Jansen BJM, Posthumus MA, Schmidt CO, De Krakera JW, König WA, Franssenc MCR. 1999. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry 52: 843-854.

Bullis S. 2002. Is artemisinin conjugated to sugar in hairy roots of *Artemisia annua*? WPI MQP Report

Chen A, Petersen BL, Olsen CE, Schulz A, Halkier BA. 2001. Long distance phloem transport of glucosinolates in *Arabidopsis*. Plant Physiology 127:194–201.

Dayan F, Hernandez A, Allen S, Moraes R, Vroman J, Avery M, Duke S. 1999. Comparative phytotoxicity of artemisinin and several sesquiterpene analogues. Phytochemistry 50: 607-614.

Duke MV, Paul RN, ElSohly HN, Sturtz G, Duke SO. 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua L*. International Journal of Plant Science 155:365-372.

Enyedi AJ, Yalpani N, Silverman P, Raskin I. 1992. Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proceedings National Academy of Sciences 89: 2480-2484.

Ferrira JF, Janick J. 1996. Immunoquantitative analysis of artemisinin from *A. annua* using polyclonal antibodies. Phytochemistry 41: 97-104.

Fulzele DP, Sipahimalani AT, Heble MR. 1991. Tissue cultures of *Artemisia annua*: organogenesis and artemisinin production. Phytotherapy Research 5:149-153.

Gowan, E, Lewis, B, Turgeon, R. 1995. Phloem transport of antirrhinoside, an iridoid glycoside in *Asarina scandens* (Scrophulariaceae). Journal of Chemical Ecology 21 (11): 1781-1788.

Hopkins WG, Huner NP. 2004. Introduction to plant physiology (3rd ed.). Hoboken, John Wiley and Sons. p 322.

Jones P and Vogt T. 2001. Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. Planta 213 (2):164-174.

Kawamoto H, Asada Y, Sekine H, Furuya T. 1998. Biotransformation of artemisinic acid by cultured cells of *Artemisia annua*. Phytochemistry 48 (8): 1329-1333.

Kim NC, Kim SU. 1992. Biosynthesis of artemisinin from 11,12-dihydroarteannuic acid. Journal of the Korean Agricultural and Chemical Society 35:106-9.

Klayman DL. 1989. Weeding out malaria. Natural History 10: 18-26.

Knapp J. 2001. Analysis of hypothesized artemisinin conjugates in *Artemisia annua*. WPI MQP Report.

Liu C, Wang Y, Ouyang F, Ye H, Li G. 1997. Production of artemisinin by hairy root cultures of *Artemisia annua L*. Biotech. Lett. 19: 927-929.

Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nature biotechnology 21(7); 796-801.

Osbourne AE. 1996. Pre-formed antimicrobial compounds and plant defense against fungal attack. Plant Cell 8:1821-1931.

Pedras MSC, Zaharia IL, Gai Y, Zhou Y, Ward DE. 2001. *In planta* sequential hydroxylation and glycosylation of a fungal phytotoxin: Avoiding cell death and overcoming the fungal invader. PNAS 98(2):747-752.

Roth RJ, Acton N. 1989. A simple conversion of artemisinic acid into artemisinin. J. Nat. Prod. 52:1183-1185.

Tan RX, Zheng, WF, Tang HQ. 1998. Biologically active substances from the genus *Artemisia*. Planta Medica 64:295-302.

Vonwiller SC, Haynes RK, King G, Wang, HJ. 1993. An improved method for the isolation of qinghao (artemisinic) acid from *Artemisia annua*. Planta Medica 6:59 662.

Wallaart TE, Pras N, Quax WJ. 1999a. Isolation and identification of dihydroartemisinic acid hydroperoxide from *Artemisia annua*. J. Nat. Prod. 62: 1160-1162.

Wallaart TE, van Uden W, Lubberink HGM, Woerdenbag HJ, Pras N, Quax WJ. 1999b. Isolation and identification of dihydroartemisinic acid from *Artemisia annua* and its possible role in the biosynthesis of artemisinin. J. Nat. Prod. 62: 430-433.

Wallaart TE, Pras N, Beekman AC, Quax WJ. 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. Planta Medica 66: 57-62.

Weathers P, Cheetham R, Follansbee E, Teoh K. 1994. Artemisinin production by transformed hairy roots of *Artemisia annua*. Biotech Lett. 16: 1281-1286.

Weathers P, Kim YJ. 2001. Transformed roots of *Artemisia annua* exhibit an unusual pattern of border cell release. In Vitro Cell. Dev. Biol. –Plant 37:440-445.

Wernsdorfer WH. 1994. Epidemiology of drug resistance in malaria. Acta Trop. 56:143-56

Appendix A- Full record of correspondence with Professor Kawamoto

From: **Marty <mdriggs@gmail.com>**
To: **info@nisr.or.jp**
Date: **May 14, 2005 7:12 PM**
Subject: **Contact information for Mr. Hiroshi Sekine**

Greetings,
My name is Martin Driggs. I am a student at Worcester Polytechnic Institute in Massachussetts. I am trying to contact Hiroshi Sekine. A 1998 publication in Phytochemistry lists him as a contact for correspondence, and I have some questions for him. If he is still at your institute, or if you have his information, please help me contact him (email is preferable).

Thank you sincerely,
Martin Driggs
Worcester Polytechnic Institute

From: 河本 啓 **<hkawamoto@mail.kikkoman.co.jp>**
To: **mdriggs@gmail.com**
Date: **May 16, 2005 3:31 AM**

Dear Mr Driggs,

Thank you for your e-mail of 14 May which has been forwarded to me. My name is Hiroshi Kawamoto. I had researched on artemisinin until 1999 at Noda Institute for Scientific Research. I am now working at Kikkoman Corp. which is related to the Institute. Since Mr Hiroshi Sekine had retired from the Institute, would you ask me questions about the publication ?

Yours sincerely,
Hiroshi Kawamoto
Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp

From: **Marty <mdriggs@gmail.com>**
To: **hkawamoto@mail.kikkoman.co.jp**
Date: **May 16, 2005 6:16 PM**
Subject: **Greetings Professor Kawamoto**

Greetings Professor Kawamoto,

Thank you for replying so quickly to my email. I am performing research on Artemisia annua in the Plant Tissue Culture Laboratory of Prof. Pam Weathers at WPI. We are investigating the distribution of artemisinin and its precursors within tissues of the whole plants and in transformed roots. I found your 1998 publication on sugar conjugates of artemisinic acid quite interesting, and I would like investigate the presence of such conjugates in the whole plants and transformed roots. However, I have several questions regarding your publication and would greatly appreciate your help.

1. Did you look for these compounds in any whole plant tissues?

2. Must the cells used for extractions be lyophilized, or will airdrying suffice?

3. In the creation of the C-BuOH fraction, are the cells extracted with EtOAc and then is that EtOAc fraction subsequently extracted with n-BuOH satd with H2O? Or are these separate fractions? Please describe this process in more detail.

4. What was the procedure for purification of the fractions using CC on silica gel? We are not organic chemists and need a bit more detail.

5. For the HPLC, what kind of detector was used, and at what wavelength? What where the retention times and what standards wereused?

Thank you for your time and any help you can supply. I am looking forward to reading your responses and pursuing this investigation.

Sincerely,

Martin Driggs
Worcester Polytechnic Institute

From: 河本　啓 **<hkawamoto@mail.kikkoman.co.jp>**
To: **mdriggs@gmail.com**
Date: **May 26, 2005 4:23 AM**
Subject: **Sorry for delay**

Dear Mr Driggs,

Thank you for your interesting in my 1998 publication. I am not performing research on Artemisia now but I can help you by E-mail with pleasure.

>1. Did you look for these compounds in any whole plant tissues?
No, I did not and can not now. I hope you to do !

>2. Must the cells used for extractions be lyophilized, or will air drying suffice?
Air drying will be OK.

>3. In the creation of the C-BuOH fraction, are the cells >extracted with EtOAc and then is that EtOAc fraction >subsequently extracted with n-BuOH satd with H2O? Or are these >separate fractions? Please describe this process in more detail.
These are separate fractions. After EtOAc extraction, the residue was evaporated and extracted by n-BuOH satd with H2O.

>4. What was the procedure for purification of the fractions >using CC on silica gel? We are not organic chemists and need a >bit more detail.
This is a classical separation method. Silica gel (about 500 ml) was packed in a column. The compound "3" was eluted by Chloroform-MeOH (9:1)at the first 500 ml fraction. "4" and "5" were eluted by Chloroform-MeOH (8:2)at 500 ml - 900 ml fraction. But this separation step will not be necessary for purification (analysis) of a small amount of the compounds.

>5. For the HPLC, what kind of detector was used, and at what >wavelength? What where the retention times and what standards >were used?
The absorbance at 220 nm was always measured. RT of "3" was 20 minute. RT of "4" and "5" were 29 and 22 minute, respectively. No standard was used.

Sincerely,

Hiroshi Kawamoto

Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp
From: **Marty <mdriggs@gmail.com>**
To: 河本 啓 **<hkawamoto@mail.kikkoman.co.jp>**
Date: **Jun 20, 2005 11:31 AM**
Subject: **Re: Sorry for delay**

Dear Professor Kawamoto,

Thank you for your willingness to help me search for the artemisinic acid conjugates in whole plants. I am still confused by some of the protocol and I hope you can take some time to answer several more questions for me. I appreciate your help greatly. Since I am looking for these compounds in the whole plants, with no liquid media, I will be looking for all three of the compounds in the tissue. In your opinion, if they exist in the tissue, will I be able to isolate 3 and 4/5 sequentially using a step gradient of elution buffer or must these isolations be performed on separate gels with separate extracts?

1. Was the silica column chromatography wet (traditional) or dry?
2. What were the dimensions of the column?
3. What was the loading solvent for each silica column chromatography?
4. What were the sample volumes for each column chromatography?
5. What were the flow rates for each HPLC run?
6. What were the injection solvents for each HPLC run?
7. What was the injection volume for HPLC?

Again, thank you much for your time.

Sincerely,
Martin

From: **Marty <mdriggs@gmail.com>**
To: **hkawamoto@mail.kikkoman.co.jp**
Cc: **"Weathers, Pamela" <weathers@wpi.edu>**
Date: **Dec 19, 2005 2:10 PM**
Subject: **URGENT QUESTION on artemisinic glycosides**

Dear Professor Kawamoto,

If you recall, I am searching for the artemisinic glycosides in whole plant tissues and earlier this year you helped me with some information on your analysis of biotransformation products in Artemisia annua. For that I am very grateful and my project is proceeding well. However, I have one more very crucial question.

I am about to perform the HPLC purification step. However, our HPLC will not accommodate a prep-size column (injection loop size is limited to 0.2 ml), thus we have to scale down the separation to use a smaller column (longer, but much narrower) and do multiple injections to collect adequate product for NMR analysis. To do this my advisor, Professor Weathers and I have to do some calculations that will enable us to estimate the approximate elution VOLUME of the putative artemisinic glycosides using your HPLC method as a guide. Although you gave me the elution times, I can't translate them into volumes because I do not know the flow rate you used. Could you, therefore, please tell me what mobile phase flow rate (ml/min) you used on your prep column, so that we may calculate the expected elution volumes for the putative conjugates? I am trying to do the HPLC work in the next 2 weeks while I am on holiday break.

Thank you for your reply,

Martin Driggs, student
and
Professor Pamela J. Weathers
Professor Biology and Biotechnology

From: 河本　啓 **<hkawamoto@mail.kikkoman.co.jp>**
To: **mdriggs@gmail.com**
Date: **Dec 26, 2005 12:45 AM**
Subject: **Re:URGENT QUESTION on artemisinic glycosides**

Dear Mr Driggs,

I'm sorry to be late reply.
The mobile phase flow rate for HPLC was always 1 ml/min.

Good luck to your work and holiday.

Sincerely,

Hiroshi Kawamoto
Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp

From: **Weathers, Pamela <weathers@wpi.edu>**
To: **hkawamoto@mail.kikkoman.co.jp**
Cc: **Marty <mdriggs@gmail.com>**
Date: **Jan 5, 2006 3:48 PM**
Subject: **URGENT question!!**

Dear Dr. Kawamoto,

We are now running our HPLC using a column that is 1/10 the volume of yours in all of its proportions. So we are scaling down everything to parallel your HPLC separation. However, it appears that all of your artemisinic glycosides eluted BEFORE the void volume!

What solvent did you use to resuspend your samples for injection into the HPLC?

Please let us know quickly so we can continue and thank you again for all of your help!

Best regards,

Pamela J. Weathers
Professor Biology and Biotechnology
Worcester Polytechnic Institute

Worcester, MA 01609

From: **hkawamoto@mail.kikkoman.co.jp**
To: **Weathers, Pamela**
Date: **Monday, January 09, 2006 9:26 PM**
Subject: **Re:URGENT question!!**

Dear Dr. Driggs,

I always used MeOH to resuspend samples for HPLC injection. But the solvent should have little influences on the retention time. I recommend you to change the elution solvent (ratio of MeCN:H2O).

Sincerely,

Hiroshi Kawamoto
Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp

From: **Weathers, Pamela**
To: **'hkawamoto@mail.kikkoman.co.jp'**
Date: **Tuesday, January 10, 2006 1:09 PM**
Subject: **RE your reply to our question**

Dear Dr. Kawamoto,

Thank you for your helpful reply. When you say change the ratio of the mobile phase, to what? We were going to use your published mobile phase ratios of 1:4 and later 2:3, acetonitrile:water, but your message has given us pause.

If you have other information helpful to us we would truly appreciate if you would share it with us.

Also can you please explain how your retention times for the stated peaks were coming out before the void volume? Which below describes how are you defining the retention time that you gave us for each of the artemisinic glycosides? Given your flow rate was 1 ml/min. then which is true, please?

1) (Rt vol) = (Peak vol) - (void vol)

OR
2) (Rt vol) = (Peak vol)

If it is the latter, then how is it possible for a peak retention volume to be LESS than the void volume of a column? I want to be sure we understand what you have shared with us in our effort to separate these unknown materials from our plant extracts.

Thank you so much for your help!

Regards,
Pam Weathers
Professor

From: **hkawamoto@mail.kikkoman.co.jp**
To: **Weathers, Pamela**
Date: **Thursday, January 12, 2006 1:36 AM**
Subject: **RE your reply to our question**

Dear Dr. Driggs,

I mean (Rt vol) = (Peak vol).
Generally, RT is delicate as you know and can be caused by the column maker,
the use history and so on.
That's simply why I recommended to change a little the elution solvent.

I do not understand how you identified the peaks coming out before the void
volume to be the glycosides we found.
Do you have the authentic samples? Shall I send ?

Sincerely,

Hiroshi Kawamoto
Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp

From: **Weathers, Pamela <weathers@wpi.edu>**
To: **hkawamoto@mail.kikkoman.co.jp**
Date: **Jan 12, 2006 2:20 PM**
Subject: **the confusion**

Dear Dr. Kawamoto,

First I must say that I am Dr. Weathers, the faculty advisor for Mr. Driggs. Mr. Driggs is an undergraduate student in my lab. Sorry for any confusion.

It would be wonderful if you could send us some of your authentic samples. It is very problematic as you know to work without any "standard".

The reason I asked about the Void vol, Vo, is because my calculations of the Vo for the column (100mm height x 25 mm diam) you used (~70% of Vt) equals a Vo of about 35 ml. This is much higher than the Rt volumes that you gave us of 22 ml and 29 ml for # 4 and #5 AA glycosides, respectively, when flow rate was at 1 ml/min. So we were quite confused. Considering that there was no way for us to measure your Vo, we had to base our assumptions on this approximate calculation of Vo from the dimensions of your column. Thus, the reason for our questions (and confusion) is clear...I hope!

Thank you again,
Professor Pamela Weathers

From: **hkawamoto@mail.kikkoman.co.jp**
To: **Weathers, Pamela**
Date: **Friday, January 13, 2006 12:16 AM**
Subject: **RE: the confusion**

Dear Professor Weathers

I am very sorry for the confusion which was maybe caused by my low English ability.

The HPLC flow rate of 1 ml/min and the retention times I informed are measured when samples were analyzed by a smaller analytical column (150mm height X 4.6mm diam).

The flow rate when the samples were injected into the larger separarion column (100mm height X 25mm diam) was 20 ml/min. The retention times are approximately the sames.

Sincerely,

Hiroshi Kawamoto
Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp

From: **"Weathers, Pamela" <weathers@WPI.EDU>**
To: **<hkawamoto@mail.kikkoman.co.jp>**
Date: **Fri, 13 Jan 2006 09:33:35 -0500**

Dear Dr. Kawamoto,

Ahhhh yes now everything makes sense! By the way, I think that your English is fine. You should hear my very poor Japanese!

Do you have any authentic samples left of those AA glycosides you isolated? You mentioned earlier that you did. If you could send us some samples for comparison that would be very helpful! My mailing address is below.

Thank you very much for the clarification.

Best regards,
Professor Pamela Weathers
Biology and Biotechnology
Worcester Polytechnic Institute
100 Institute Rd.
Worcester, MA 01609
USA

From: **hkawamoto@mail.kikkoman.co.jp**
Sent: **Friday, January 20, 2006 4:10 AM**
To: **Weathers, Pamela**
Subject: **Bad news**

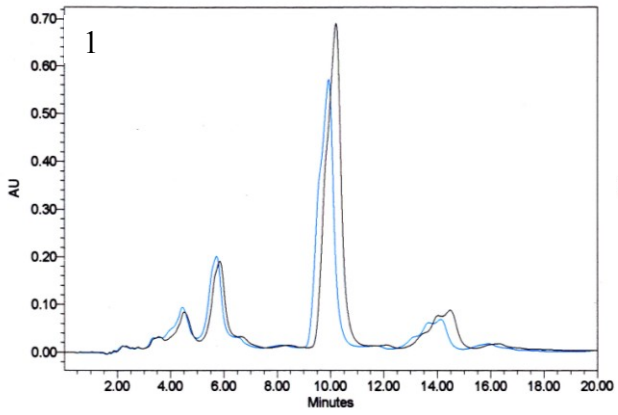
Dear Professor Weathers

I am so sorry that I can not help you.
We quitted the artemisinin research project 7 years ago and I have moved to other section where I work now.
Yesterday, I visited the laboratory I worked before and looked for the authentic samples in the refrigerator.
But I could not find. Who disposed my trasures !!

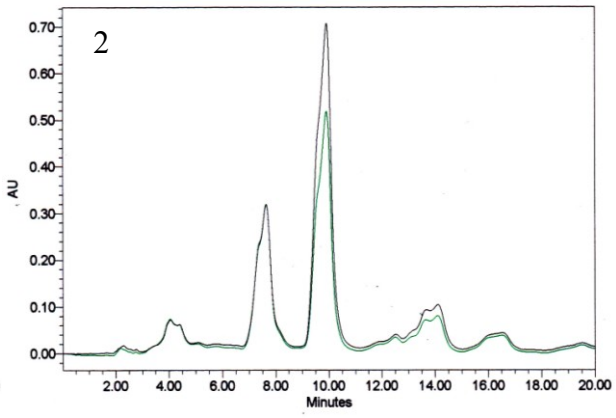
Sincerely,

Hiroshi Kawamoto
Kikkoman Corp.
hkawamoto@mail.kikkoman.co.jp

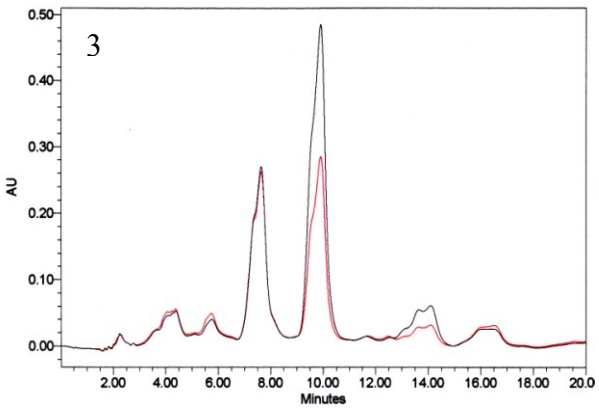
Appendix B- HPLC chromatograms from hydrolysis treatment conditions and replicates, presented in order of injection (left to right)



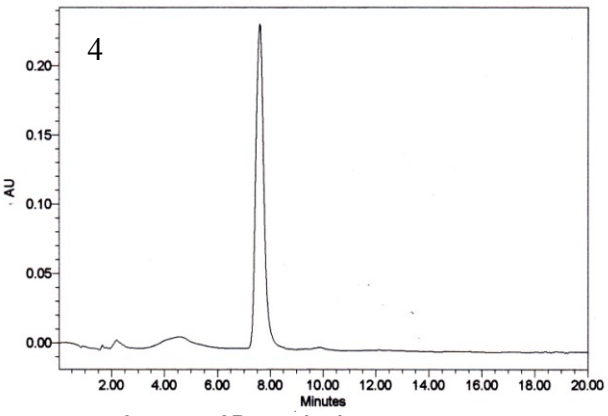
sample name: washed shoots hot acid
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 10.208 (9.94)



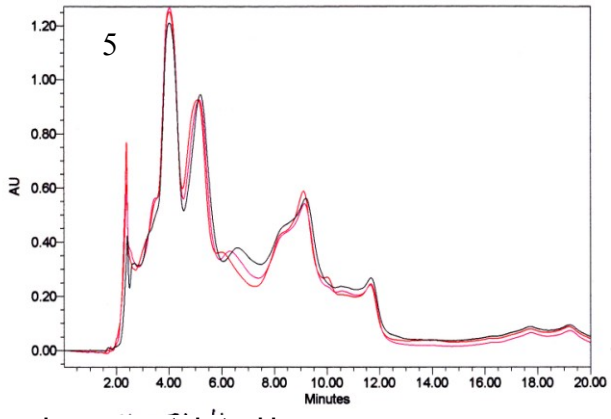
sample name: washed shoots hot buffer (pH 7.0)
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.923 (9.867) and 7.648 (7.649)



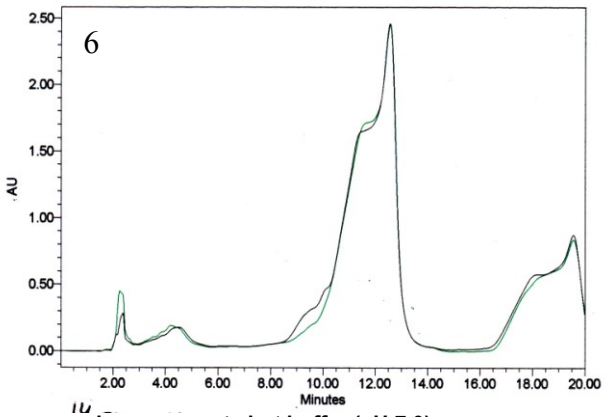
sample name: washed shoots acid room temp.
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.921 (9.914) and 7.649 (7.647)



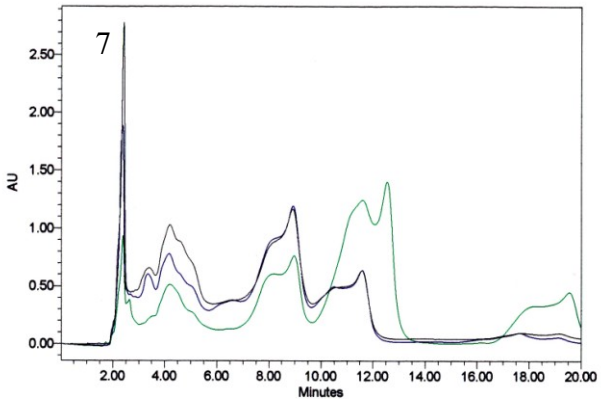
sample name: AB standard
injection volume: 50 ul
injection mass: 2.5 ug
mobile phase: 60:40 ACN-water
retention times of interest: 7.602



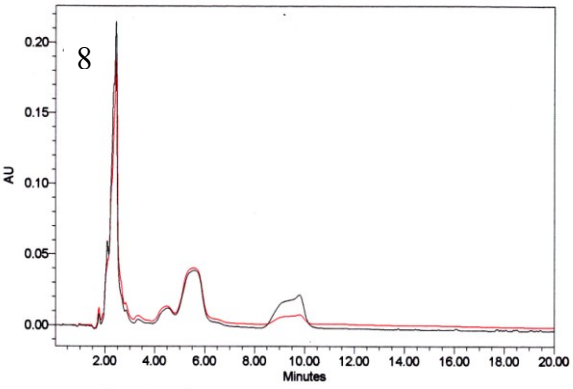
sample name: roots hot acid
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest:



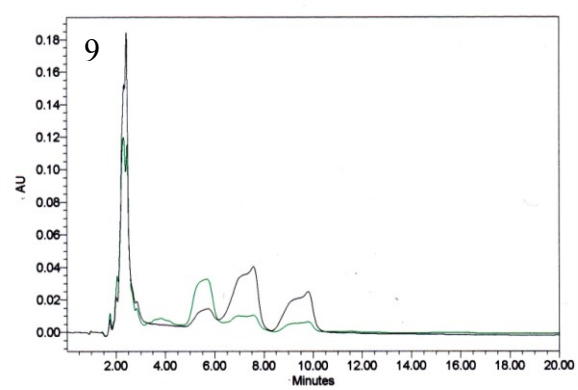
sample name: roots hot buffer (pH 7.0)
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest:



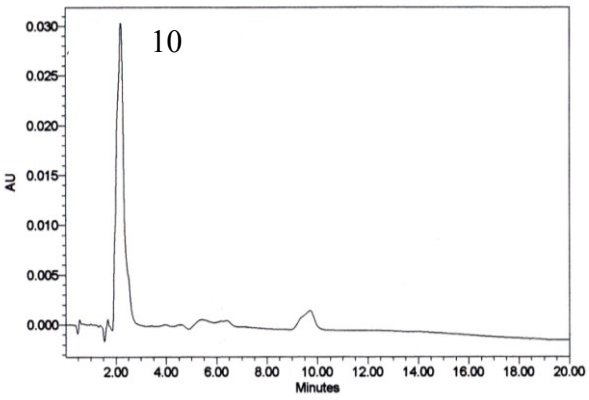
sample name: roots acid room temp.
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest:



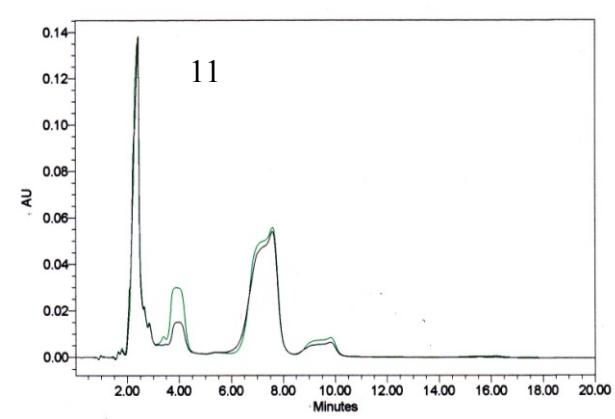
sample name: AB standard hot acid
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.797 (9.810)



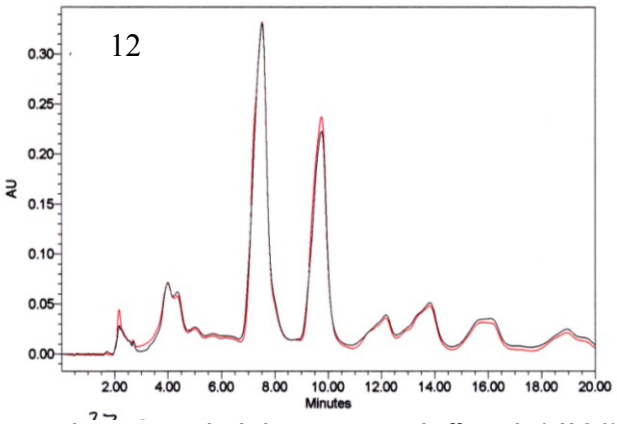
sample name: AB acid room temp.
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.797 (9.807) and 7.571 (7.575)



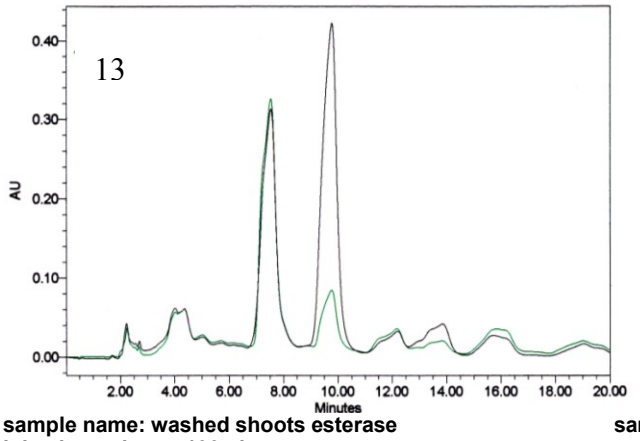
sample name: AA standard
injection volume: 100 ul
injection mass: 1.5 ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.709



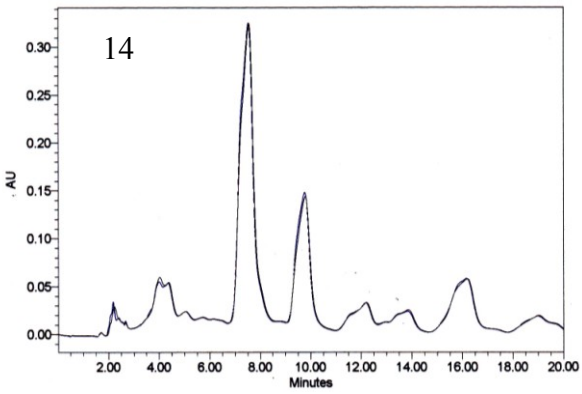
sample name: AB Hot Buffer (pH 7.0)
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.818 (9.835) and 7.585 (7.602)



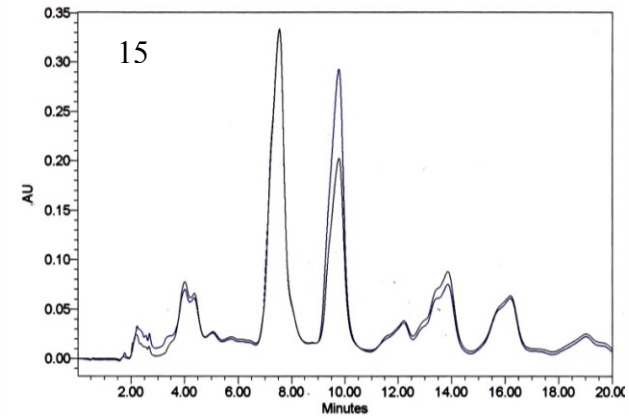
sample name: washed shoots esterase buffer only (pH 8.0)
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.760 (9.764) and 7.528 (7.533)



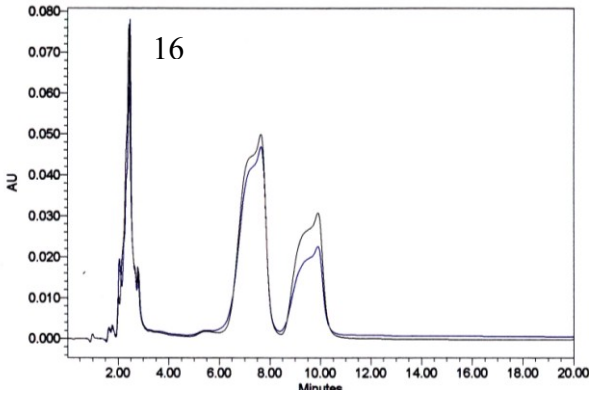
sample name: washed shoots esterase
injection volume: 100 ul
injection mass: 0.009 g FW ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.781 and 7.543 (7.533)



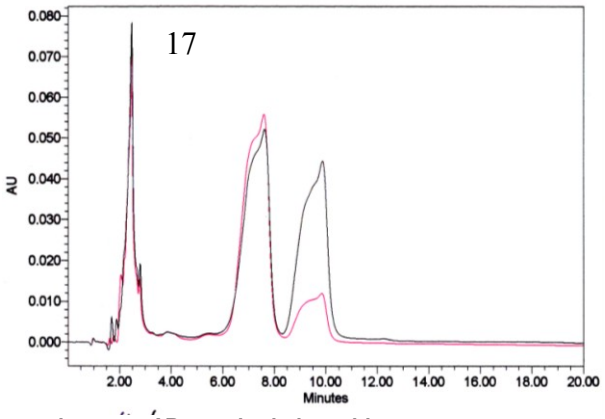
sample name: washed shoots glucosidase buffer only (pH 5.5)
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.78 (9.794) and 7.544 (7.557)



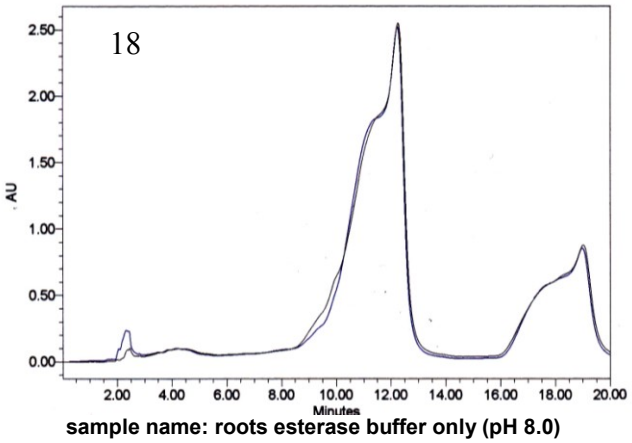
sample name: washed shoots glucosidase (pH 5.5)
injection volume: 100 ul
injection mass: 0.009 g FW ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.783 (9.788) and 7.548 (7.551)



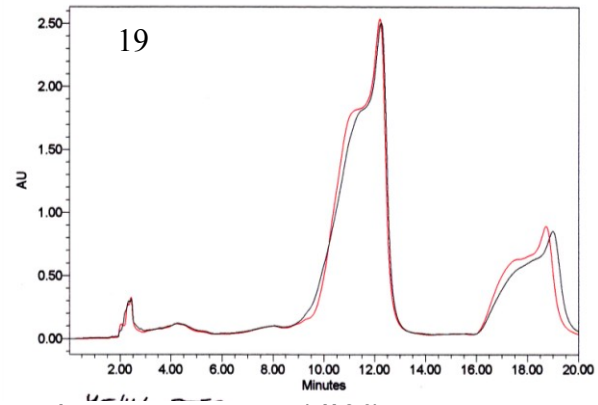
sample name: AB standard esterase (pH 8.0)
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.903 (9.912) and 7.651 (7.660)



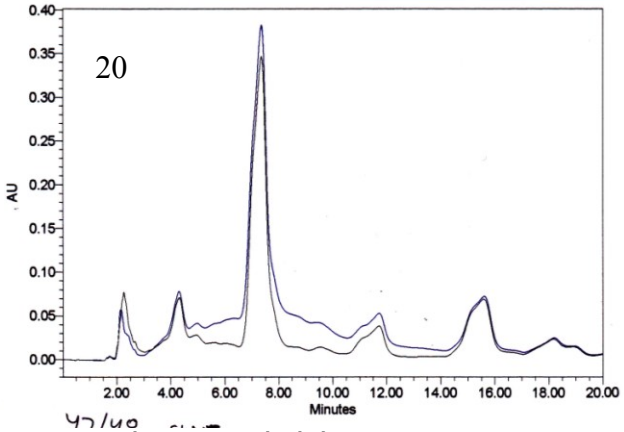
sample name: AB standard glucosidase
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.874 (9.857)



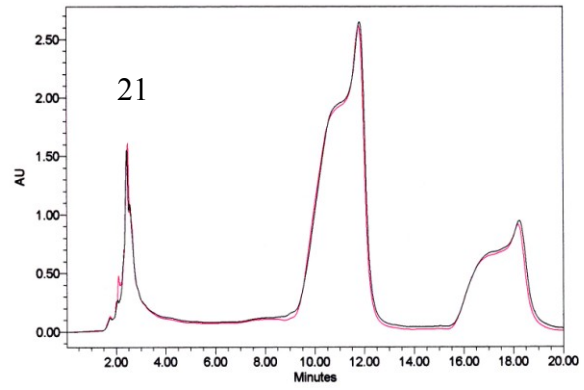
sample name: roots esterase buffer only (pH 8.0)
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest:



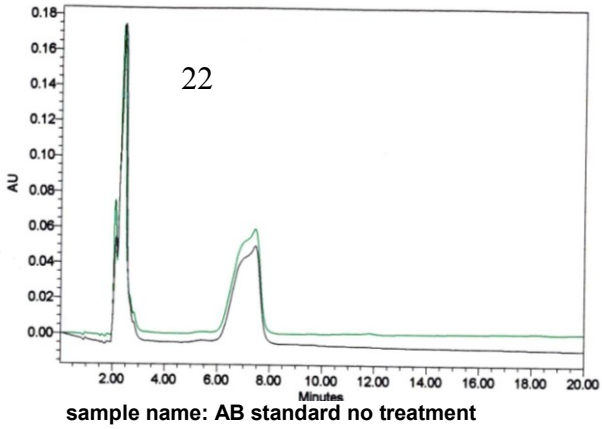
sample name: roots esterase (pH 8.0)
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest:



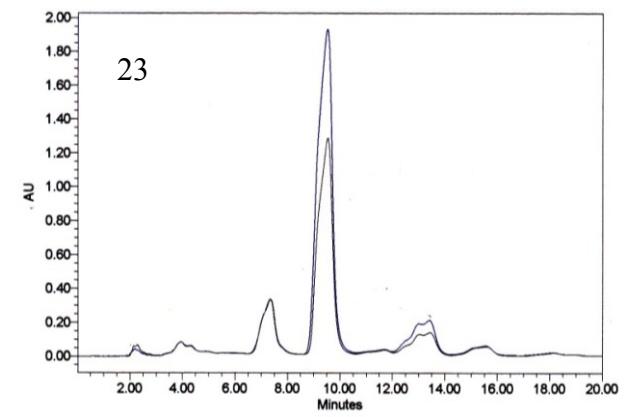
sample name: washed shoots no treatment
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.583 (9.539) and 7.373 (7.364)



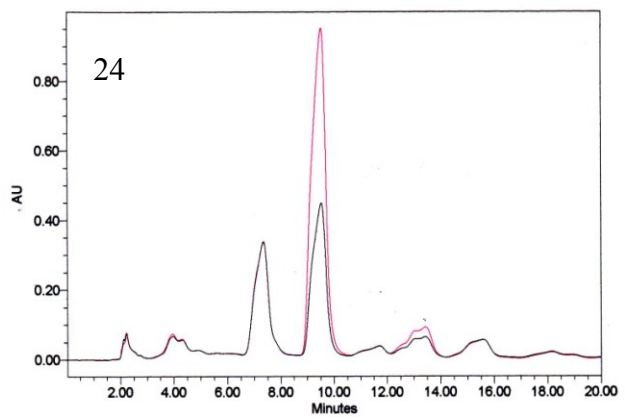
sample name: roots no treatment
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.874 (9.857)



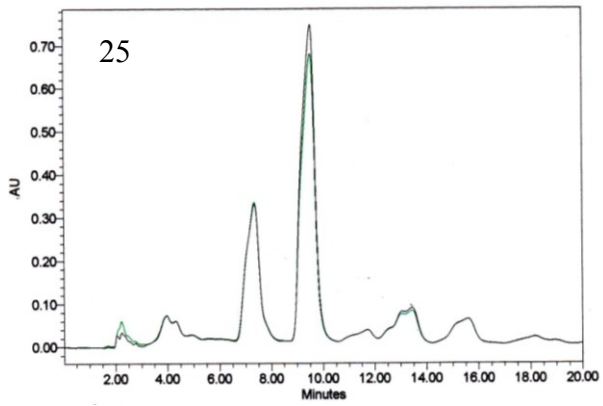
sample name: AB standard no treatment
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 7.438 (7.446)



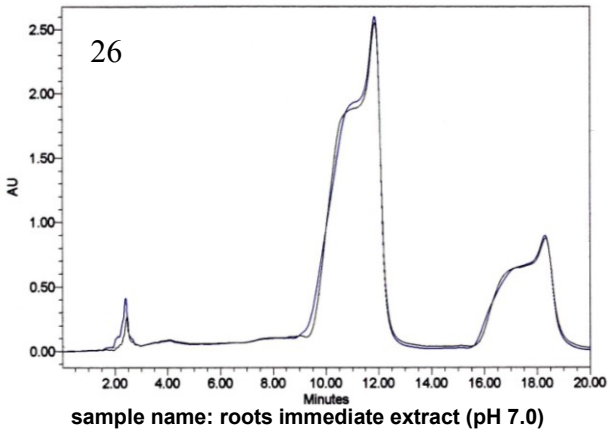
sample name: washed shoots immediate extract (pH 7.0)
injection volume: 100 ul
injection mass: 0.009 g FW ug
mobile phase: 60:40 ACN-water
retention times of interest:9.524 (9.534) and 7.353 (7.362)



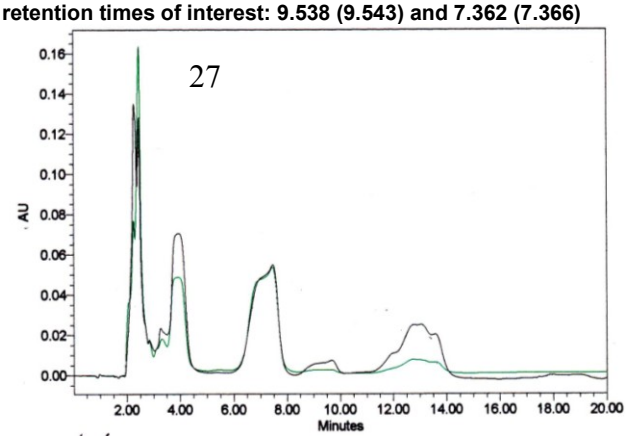
sample name: washed shoots immediate extract (pH 8.0)
injection volume: 100 ul
injection mass: 0.009 g FW
mobile phase: 60:40 ACN-water
retention times of interest: 9.533 (9.539) and 7.359 (7.362)



sample name: washed shoots immediate extract (pH 5.5)
injection volume: 100 ul
injection mass: 0.009 g FW ug
mobile phase: 60:40 ACN-water
retention times of interest: 9.538 (9.543) and 7.362 (7.366)



sample name: roots immediate extract (pH 7.0)
injection volume: 200 ul
injection mass: 0.12 g FW
mobile phase: 60:40 ACN-water
retention times of interest:



sample name: AB standard immediate extract (pH 7.0)
injection volume: 200 ul
injection mass: 2 ug
mobile phase: 60:40 ACN-water
retention times of interest: 7.472 (7.474)

Appendix C

The full set of data resulting from the analysis of HPLC chromatograms of hydrolysis treatment condition experiments, including the values for single-peak and double peak calculations, are presented in the following table.

Shoot samples representing 0.009375 g FW
Root samples representing 0.120 g FW

Name	Retention Time	Area	ug AB	ug AA/g FW	Ave. ug/g	Variance	% of No Treatment	Retention Time	Area	ug AA	ug/g FW	Ave ug/g	Variance	% of 7.0 instant
No Treatment - single peak value	7.373	9242145	4.55	486	482	3.26	100.00	9.583	182464	0.08	8.59	8.32	0.27	0.40
double-peak value		12435177	6.13	653	646	7.55	100.00		182464	0.08	8.59	8.32	0.27	0.29
No Treatment	7.364	9118021	4.49	479				9.539	171029	0.08	8.05			
		12147822	5.98	638					171029	0.08	8.05			
Immediate extract (pH 7.0)	7.353	8303231	4.09	436	434	2.36	90.04	9.524	53531214	23.62	2519.31	2086	432.85	100.00
		11637459	5.73	611	615	3.75	95.26		74139806	32.71	3489.21	2869	620.09	100.00
Immediate extract (pH 7.0)	7.362	8213584	4.05	432				9.534	35136436	15.50	1653.61			
		11780255	5.80	619					47788125	21.08	2249.03			
Immediate extract (pH 8.0)	7.359	8608692	4.24	452	471	18.42	97.59	9.533	11894051	5.25	559.76	909	349.30	43.57
		11824698	5.82	621	618	3.10	95.72		15794091	6.97	743.31	1176	432.42	40.98
Immediate extract (pH 8.0)	7.362	9309829	4.59	489				9.539	26737980	11.80	1258.36			
		11706776	5.77	615					34170631	15.08	1608.16			
Buffer only, 90 minutes	7.528	11769439	5.80	618	622	3.11	128.85	9.76	7840381	3.46	368.99	379	10.46	18.19
		11769439	5.80	618	623	4.58	96.46		7840381	3.46	368.99	378	9.15	13.18
Buffer only, 90 minutes	7.533	11887975	5.86	625				9.764	8284911	3.66	389.91			
		11943651	5.88	628					8229235	3.63	387.29			
Esterase	7.543	8730759	4.30	459	442	17.03	91.57	9.78	1975318	0.87	92.96	293	200.31	14.06
		11278360	5.56	593	582	10.19	90.18		2661046	1.17	125.24	420	294.84	14.64
Esterase	7.545	8082445	3.98	425				9.781	10487923	4.63	493.59			
		10890368	5.36	572					15190784	6.70	714.92			
Immediate extract (pH 5.5)	7.362	8456318	4.17	444	448	3.59	92.86	9.538	19794973	8.73	931.60	931	0.89	44.61
		11553433	5.69	607	611	3.75	94.58		26856183	11.85	1263.92	1200	63.46	41.84
Immediate extract (pH 5.5)	7.366	8593059	4.23	452				9.543	19757151	8.72	929.82			
		11696132	5.76	615					24159290	10.66	1137.00			
Buffer only, 90 minutes	7.544	8636454	4.25	454	468	14.35	97.05	9.78	3605419	1.59	169.68	168	1.51	8.06
		11405275	5.62	599	599	0.12	92.77		4980526	2.20	234.40	231	3.74	8.04
Buffer only, 90 minutes	7.557	9182558	4.52	482				9.794	3541125	1.56	166.65			
		11400690	5.62	599					4821627	2.13	226.92			
Glucosidase	7.548	9273087	4.57	487	486	1.01	100.80	9.783	5481179	2.42	257.96	324	66.03	15.53
		11688663	5.76	614	613	1.50	94.86		6805362	3.00	320.28	398	78.18	13.89
Glucosidase	7.551	9234677	4.55	485				9.788	8287174	3.66	390.02			
		11631403	5.73	611					10127774	4.47	476.64			
Hot Buffer	7.648	8161754	4.02	429	421	8.05	87.24	9.923	17833865	7.87	839.31	745	94.67	35.69
		11638199	5.73	612	616	4.12	95.32		26770279	11.81	1259.88	1091	168.95	38.02
Hot Buffer	7.649	7855329	3.87	413				9.925	13810833	6.09	649.97			
		11794989	5.81	620					19590581	8.64	921.98			
Room Temp. Acid	7.647	6737462	3.32	354	357	3.09	74.03	9.921	7448458	3.29	350.54	471	120.03	22.55
		9599713	4.73	504	512	7.59	79.28		10518081	4.64	495.01	676	181.28	23.57
Room Temp. Acid	7.642	6855196	3.38	360				9.914	12549498	5.54	590.61			
		9888625	4.87	520					18221854	8.04	857.57			
Hot Acid						0	0	10.208	19174725	8.46	902.41	540	125.08	
						0.00	0.00		26389025	11.64	1241.93	1127	157.79	
Hot Acid								9.94	15274172	6.74	718.84			
									21521881	9.50	1012.87			