*Dedicated to my Grandmother:*
*Elizabeth Swimley Wright*

# Acknowledgments

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## List of Abbreviations

**ADF**: acid detergent fiber

**αTTP**: alpha-tocopherol transfer protein

**BHT**: butylated hydroxy-toluene

**CoQ**: coenzyme Q

**DOXY**: 1-deoxy-D-xylose-5-phosphate

**DM**: dry matter

**EDTA**: ethylenediaminetetraacetic acid

**GGDP**: geranylgeranyl diphosphate

**GSH**: reduced glutathione

**GSSG**: oxidized glutathione

**HGA**: homogentisic acid

**HDL**: high density lipoprotein

**HPLC**: high performance liquid chromatography

**IVOMD**: *in vitro* organic matter disappearance

**LDL**: low density lipoprotein

**MDHA**: monodehydroascorbate

**NDF**: neutral detergent fiber

**2MPG**: 2-methyl-6-phytylquinol

**SDS**: sodium dodecylsulfate

**VLDL**: very low density lipoprotein

# Chapter 1: Introduction and Objectives

Weeds and shrubs can rapidly invade poorly managed pastures. Multiflora rose, autumn olive, and bush honeysuckle now dominate many Appalachian hill-land pastures. Infestation of pastures with weedy, shrub species limits the ability of the pasture to support production of sheep or cattle because the shrubs decreases the pasture quality and decreases the area available for grazing. Goats can be an effective alternative to herbicide control of invasive species. In addition to renovating the pasture, meat goats provide a salable, commercial meat product that can diversify farm enterprise.

Vitamin E is a necessary nutrient in ruminant diets. This study examined concentrations of vitamin in the leaf tissue of three invasive, browse species common to underutilized Appalachian pastures to assess the value of these plants as a source of vitamin E for goats. The hypothesis of this study was that the value of shrubs as a dietary source of vitamin E varies with species and time during the growing season. Experiments addressed two questions.

1. Do differences in the concentration of the individual forms of tocopherol exist in the leaf tissue of the three species?
2. Do changes in the concentration of the tocopherols in the leaf tissue occur during the growing season in the three species?

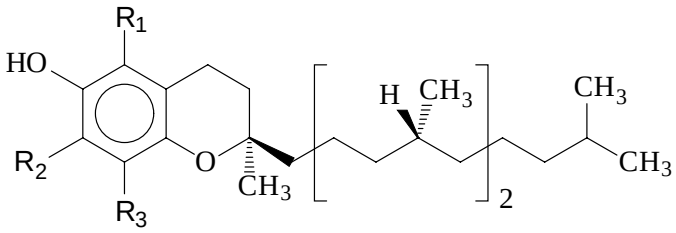
This research was conducted in collaboration with scientists at the USDA, ARS, Appalachian Farming Systems Research Center (AFSRC) in Beaver, WV, who are developing a system for producing meat goats on hill-land pastures overgrown with invasive shrubs. The results of the examination of the tocopherol concentrations present in browse leaf tissue will be used in conjunction with the findings of other scientists at the AFSRC to define the quantity and timing of nutrient supplementation.

# CHAPTER 2: Literature Review

## Biochemistry of Vitamin E

Vitamin E (tocopherol) was first isolated in 1936 from wheat germ oil. The common name vitamin E was given because the vitamin was identified shortly after the discovery of vitamin D. Historically, vitamin E was known for it's essential role in maintaining pregnancy, as the name indicates. "Tocopherol" derives from the Greek words *tocos* (to birth) and *pherein* (carry) (Eitenmiller and Landen, 1994).

**Figure 1. Structure of vitamin E (tocopherol)[a]**




Tocopherols

| Common Name | Chemical name | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| Tocol | -- | H | H | H |
| Alpha-Tocopherol | 5,7,8-Trimethyltocol | $CH_3$ | $CH_3$ | $CH_3$ |
| Beta-Tocopherol | 5,8,-Dimethyltocol | $CH_3$ | H | $CH_3$ |
| Gamma-Tocopherol | 7,8-Dimethyltocol | H | $CH_3$ | $CH_3$ |
| Delta -Tocopherol | 8-Methyltocol | H | H | $CH_3$ |

[a]Vitamin E refers collectively to natural and synthetic derivatives of the tocopherols and tocotrienols.

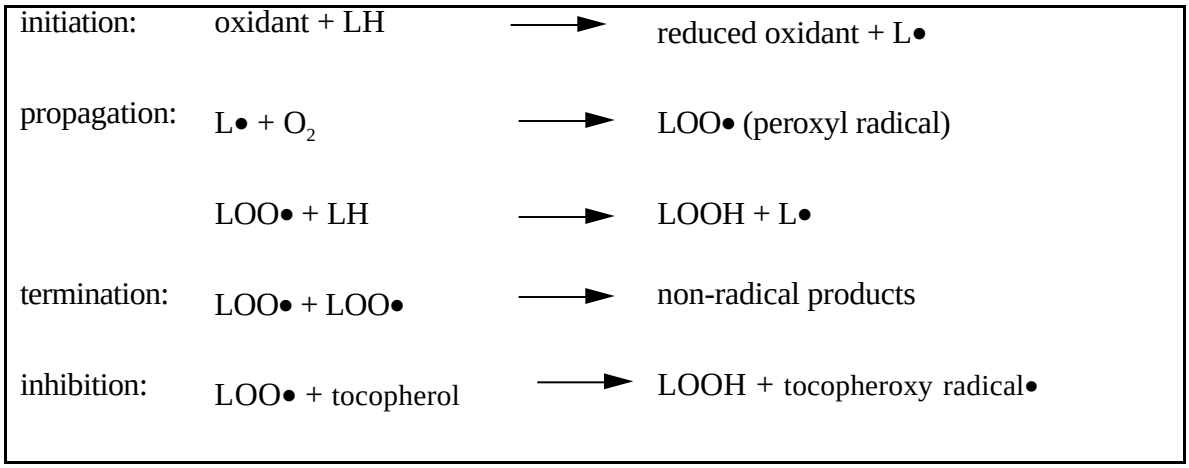
Vitamin E is the collective name for a group of biologically active 6-hydroxychroman compounds that includes four different methylated tocopherols and four analogous methylated tocotrienols, which have 3', 7', and 11' unsaturations (See Figure 1). The vitamin E compounds are freely soluble in lipids and organic solvents such as hexane, but

are practically insoluble in aqueous solutions. Eight stereoisomers can be formed during synthetic production of vitamin E due to the chiral centers at the 2 position of the chroman ring and the 4' and 8' positions on the chain. Only *RRR*-stereoisomers occur in nature (Eitenmiller and Landen, 1994). Tocopherol acetate, which has acetate ester-linked at the 6 position of the chroman ring, is not found in nature but is sold commercially as a stable form of vitamin E.

Vitamin E is most recognized today as the principle lipid-soluble antioxidant in both plant and animal systems (Fryer, 1992; Diplock, 1983). Antioxidants such as vitamin E are a crucial part of plant and animal defenses against oxidative stress. Adequate dietary vitamin E intake has numerous beneficial effects in animal systems that include reducing the risk of cataracts and cardiovascular disease (Packer and Landvik, 1989), enhancing immune surveillance, and suppressing tumor growth (Factor *et al.,* 2000). Some effects such as regulatory interaction with protein kinase C are distinct from the antioxidant function of vitamin E (Ricciarelli *et al.,* 1998).

Vitamin E functions primarily as a peroxyl radical scavenger to disrupt lipid peroxidation chain reactions (Burton and Ingold, 1989). The generalized path of lipid peroxidation is illustrated in Figure 2. Lipid peroxidation begins with production of a carbon-centered radical by some oxidant. The reaction is propagated when the carbon-centered radical then reacts with dissolved molecular oxygen to form a highly reactive peroxyl radical. The peroxyl radical reacts with any peroxidizable molecule, especially polyunsaturated fatty acids, to produce another carbon-centered radical. This chain reaction continues until two peroxyl radicals react producing non-radical species or the reaction is inhibited by chain-breaking antioxidants such as vitamin E. Vitamin E is also capable of reducing reactive oxygen species such as singlet oxygen or superoxide (Liebler, 1998).

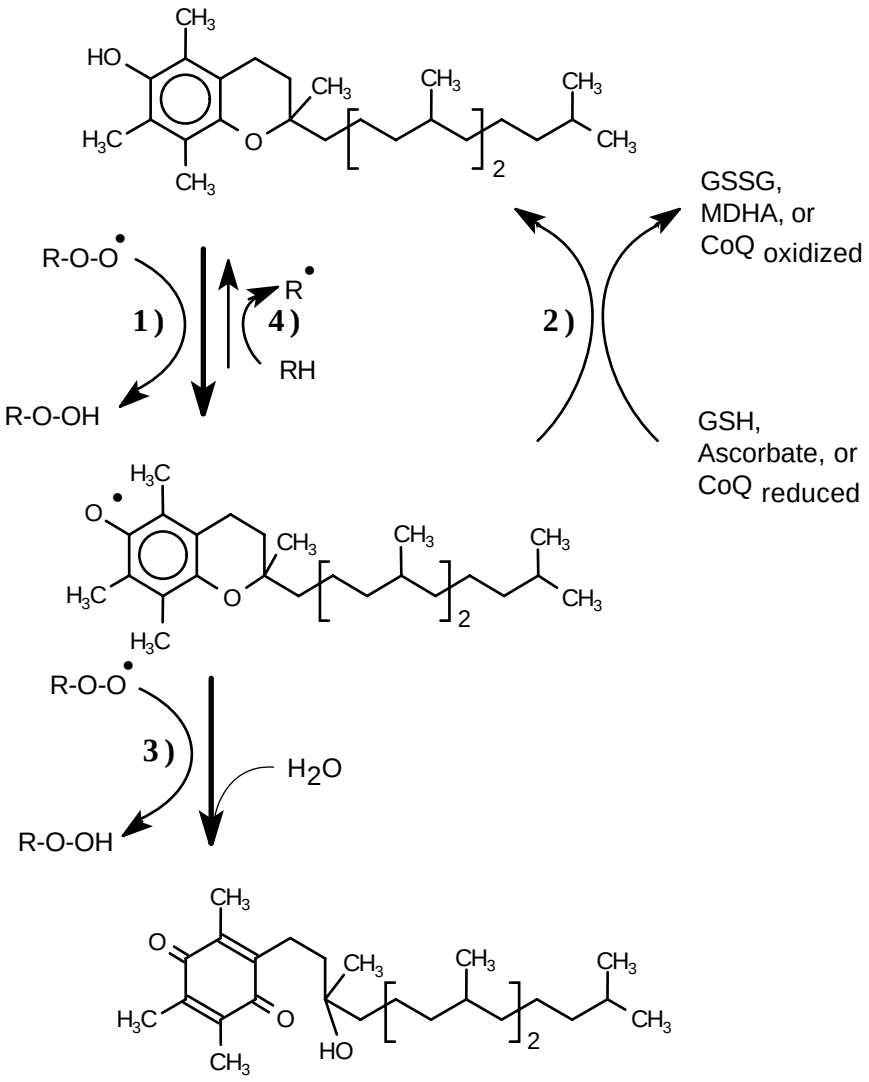
**Figure 2. Path of lipid peroxidation and inhibition by vitamin E[a]**




[a]The initial oxidant reacts with an acyl lipid (LH) to produce a carbon-centered radical (L•) which reacts with oxygen to produce highly reactive peroxyl radicals (LOO•). Peroxyl radicals chain react producing additional carbon-centered and peroxyl radicals until two radical species react or the reaction is inhibited by antioxidants such as tocopherol.

Figure 3 illustrates the mechanism for generation of peroxyl radicals and some of the potential fates of tocopherol. The oxidized tocopheroxy radical (reaction 1) faces several possible fates (Leibler, 1998). The radical can be reduced back to the quinol form (reaction 2) by accepting a hydrogen ion from ascorbate (vitamin C) (Packer *et al.,* 1979), reduced glutathione (McCay *et al.,* 1989), or coenzyme Q (Kagan *et al.,* 1998). Alternatively, the tocopheroxy radical can react further with a second peroxyl radical (reaction 3) or other oxidant to form non-reactive products that include tocopherolquinone (Leibler, 1998). Lastly, if local concentrations of tocopheroxy radicals are high, then the reverse, pro-oxidant reaction can occur by removing a hydrogen from another lipid molecule (reaction 4; Witting *et al.,* 1998). This generates alpha-tocopherol and the lipid radical.

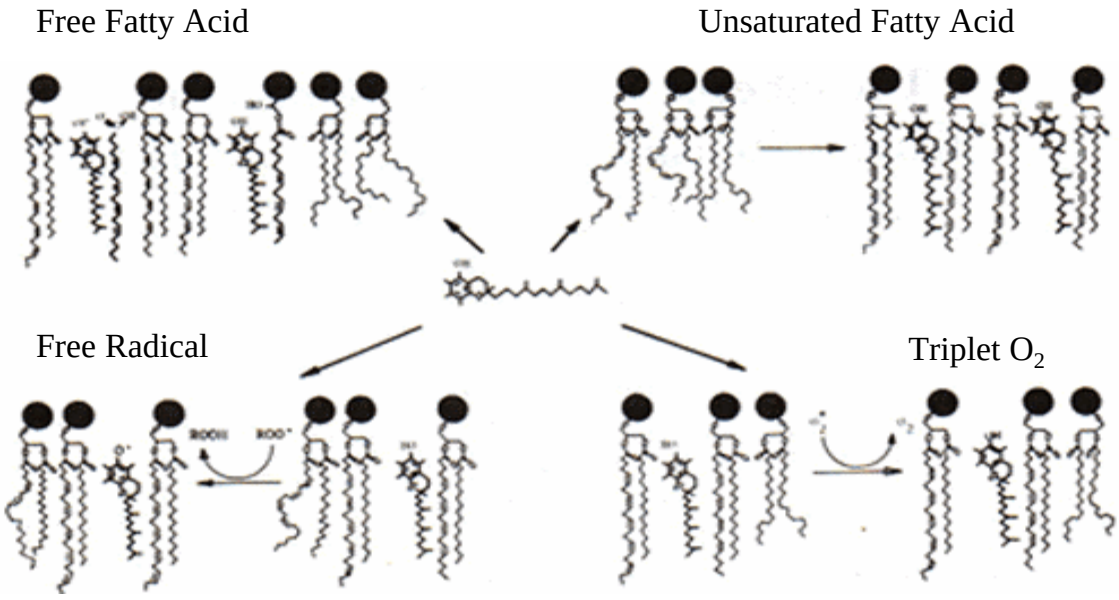
**Figure 3. Reactions of vitamin E**




Tocopherol reacts with peroxyl radicals generating the tocopheroxy radicals (1); under certain conditions the reverse reaction can occur (4). Tocopheroxy radicals can be oxidized further to generate stable tocopherol quinones (3). Regeneration of tocopherol from tocopheroxy radicals occurs through reduction by other cellular antioxidants (2).

The structure of vitamin E is well suited for reaction with peroxyl radicals (Burton and Ingold, 1989). As a lipophilic molecule, vitamin E is not freely distributed in the cell, but localized within phospholipid membranes. Within the membrane, vitamin E can protect the peroxidizable unsaturated fatty acids from degradation. The tocopherol molecule is oriented with the chroman ring in association with the phospholipid head groups of the bilayer while the phytyl tail is oriented toward the hydrophobic core of the bilayer (Kagan, 1989). The tocopherol molecule can diffuse laterally at a rate equal to the diffusion of phospholipids, and vitamin E can rotate around the axis of the phytyl tail (Quinn, 1998).

**Figure 4. Functions of vitamin E within membranes**[a]
Source: Kagan, 1989




[a]upper right: vitamin E stabilizing disruption caused by unsaturated fatty acids. upper left: vitamin E stabilizes membrane against free fatty acids. lower right and left: vitamin E functionining as antioxidant within the membrane.

Vitamin E helps to stabilize membranes (Figure 4), which is a critical role unrelated to its function as an antioxidant (Fryer, 1992). The presence of vitamin E stabilizes the membrane by decreasing the permeability to small ions caused by polyunsaturated fatty acids (Quinn, 1998). The phytyl chain of Vitamin E can bind to free fatty acids and minimize their disruptive effect on the membrane (Kagan, 1989). Additionally, vitamin E in

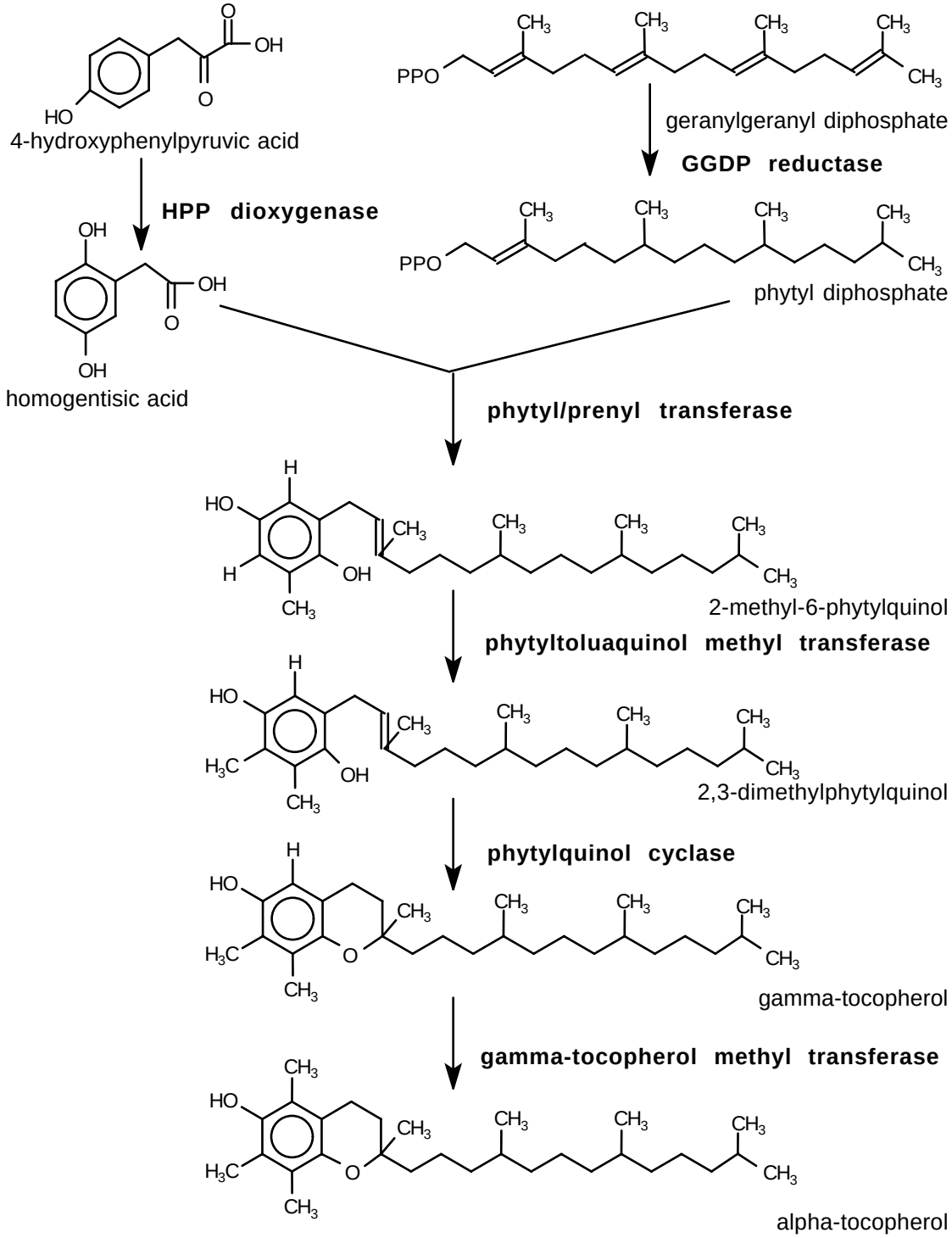
the membrane stabilizes the membrane from temperature changes by broadening the gel-to-liquid crystalline phase transition curve (Wassal *et al.,* 1986).

Vitamin E is synthesized exclusively by photosynthetic organisms. Synthesis occurs in the chloroplast envelope at the inner membrane (Schulze-Siebert *et al.,* 1987). Synthesis can also occur in non-chlorophyll containing tissues such as roots and seeds (Hess, 1993). The isoprenoid pathway and the chorismate pathway provide precursors for tocopherol (Figure 5). Homogentisic acid (HGA), a tyrosine derivative from the chorismate pathway, combines with phytyl-diphosphate from the isopreniod pathway to yield 2-methyl-6-phytylquinol (2MPQ). Availablity of phytyl-diphosphate appears to regulate tocopherol accumulation in *Arabidopsis* (Tanaka *et al.,* 1999). With S-adenosyl methionine as the methyl donor, 2MPQ is then methylated at the 3-position and cyclized to generate gamma-tocopherol. Additional methylation at the 5-position generates alpha-tocopherol (Hirschberg, 1999). The synthesis of beta- and delta-tocopherol is not fully understood but is thought to result from cylization of 2MPQ prior to methylation (Hirschberg, 1999). Synthesis of the tocotrienols is thought to proceed similarly to the tocopherols except geranylgeranyl-diphosphate (GGDP) is not reduced to phytyl-diphosphate by GGDP reductase before combining with homogentisic acid (Hess, 1993).

The phytyl-diphosphate isoprenoid side chain was believed to be synthesized in the cytosol by the classical acetate/mevalonate pathway. Recent elucidation of an alternative, mevalonate-independent, 1-deoxy-D-xylulose-5-phosphate (DOXY) pathway that is plastid-specific raises questions about the origin of the tocopherol phytyl side chain. The DOXY pathway is known to provide the isoprenoid units for carotenoid biosynthesis, chlorophyll biosynthesis, and for plastoquinone-9 (Lichtenthaler, 1999). Although it has not been demonstrated, it is highly likely that the DOXY isoprenoid pathway supplies the isoprenoid units for tocopherol biosynthesis as well.

**Figure 5. Alpha-tocopherol biosynthetic pathway**

Source: Hirschberg, 1999




Homogentisic acid supplied by the chorismate pathway; phytyl-diphosphate supplied most likely by the mevalonate-independent pathway.

The tocopherol synthetic pathway shares some intermediates with other important metabolic pathways. Geranyl-geranyl-diphosphate is an intermediate in both chlorophyll biosynthesis and carotenoid biosynthesis (Shewmaker *et al.*, 1999). Homogentisic acid is a precursor for quinone biosynthesis, and phytyl/prenyl transferase catalyzes both the reaction of HGA and phytyl-diphosphate for tocopherol biosynthesis and the reaction of HGA with solanyl pyrophosphate in plastoquinone biosynthesis (Norris *et al.*, 1995).

Mechanisms that regulate tocopherol biosynthesis are not fully understood and are complicated by interconnection with other pathways. Recently, use of molecular biology techniques has helped increase our understanding. Most notably, Shintani and DellaPenna (1998) were able to shift the form of tocopherol produced in *Arabidopsis* seed oils from 97% gamma-tocopherol to 95% alpha-tocopherol by overexpressing the final enzyme in the pathway, gamma-methyl transferase. These researchers noted that gamma-methyl transferase would also convert delta-tocopherol to beta-tocopherol, and more importantly, that shifting from gamma-tocopherol to alpha-tocopherol did not increase the total tocopherol content. They concluded that gamma-methyl transferase plays a key role in determining the form of tocopherol present but not the total amount. Arango and Heise (1998) reported that phytolquinol cyclase was not the rate-limiting step in tocopherol synthesis as previously thought. They documented a very rapid conversion from homogentisate and phytyl-diphosphate to gamma-tocopherol, an observation which agrees with the findings of Shintani and DellaPenna (1998). While Arango and Heise suggest some regulatory role for gamma tocopherol (1998), the steps that supply HGA and phytyl-pyrophosphate are currently thought to control the total levels of tocopherol (Hirschberg, 1999). Two studies on GGDP are in agreement with this prediction. First, overexpression of phytoene synthase, which decreases the pool of GGDP available for tocopherol synthesis, decreased the levels of tocopherol significantly (Shewmaker *et al.*, 1999). Second, reducing the activity of geranylgeranyl reductase with transgenic expression of

antisense mRNA reduced tocopherol levels compared to untransformed controls (Tanaka *et al.,* 1999).

The different forms of vitamin E have different bioactivities (Pryor, 1995; Table 1). The bioactivites of the tocopherol forms have been investigated using several tests: the classical standard, prevention of rat fetal resorption (Bliss and Gyorgy, 1967), in vivo defense against lipid peroxidation in iron loaded rats (Dillard et al., 1983), in vitro inhibition of autooxidation of styrene (Burton and Ingold, 1989), and various measures of storage in animal tissue (Dicks and Matterson, 1961; Hiditoglou et al., 1988). One international unit (IU) of vitamin E is standardized to equal the bioactivity of 1 mg of *all-rac*-tocopherol acetate. Naturally occuring d-alpha-tocopherol has a greater biological activity, 1.49 IU/mg, than *all-rac*-tocopherol acetate. The measures of bioactivity do not necessarily equal measures of antioxidant activity. Some experiments have indicated that gamma-tocopherol is the more potent in vitro antioxidant (Duthie et al., 1991). Other results from in vitro and in vivo measures of antioxidant activity have identified alpha-tocopherol as the most active antioxidant, which correlates with measures of the bioactivity (Burton and Ingold, 1989). In animal systems, the bioactivities are believed to result from the affinity of alpha-tocopherol transfer protein ($\alpha$TTP) for the various forms of vitamin E (Hosomi et al., 1997).

**Table 1. Bioactivities of different forms of vitamin E**[a]
source: Pryor *et al.*, 1995

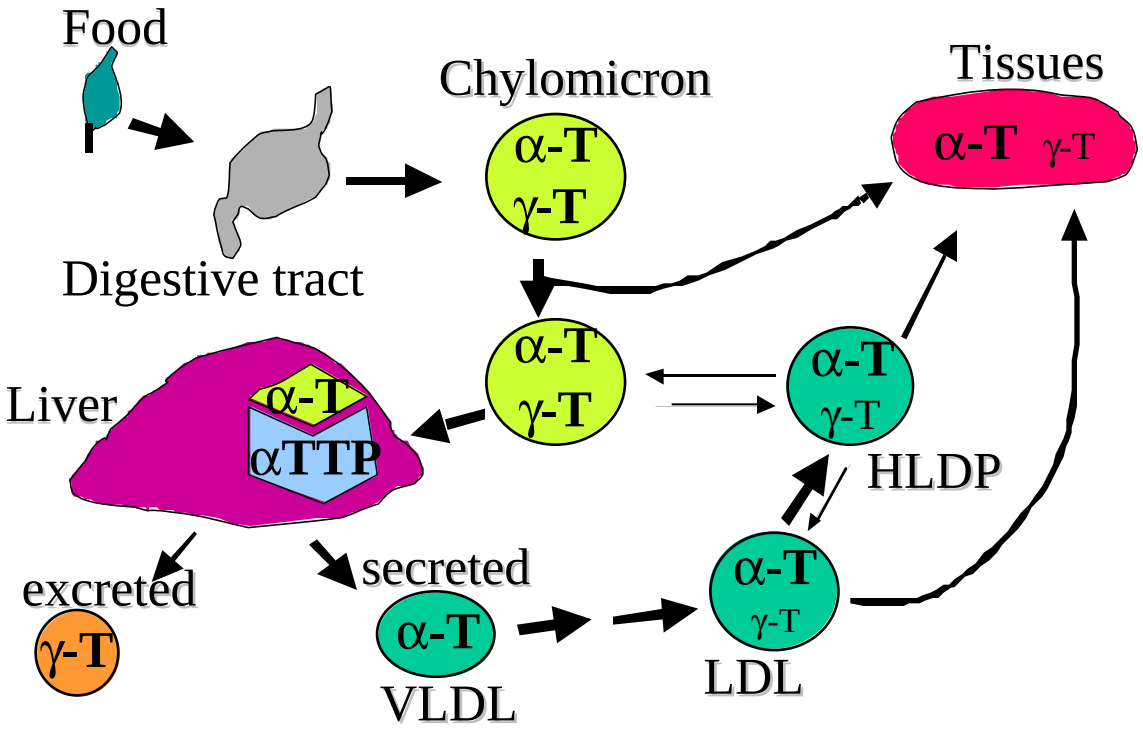
| Forms of vitamin E | Bioactivities | |
|---|---|---|
| | IU/mg[b] | Compared to *RRR*-alpha-tocopherol[c], % |
| **Natural (*RRR*-)** | | |
| alpha-tocopherol | 1.49 | 100 |
| beta-tocopherol | 0.75 | 50 |
| gamma-tocopherol | 0.15 | 10 |
| delta-tocopherol | 0.05 | 3 |
| **Synthetic forms** | | |
| *2R4'R8'R* alpha-tocopherol | 1.49 | 100 |
| *2S4'R8'S* alpha -tocopherol | 0.46 | 31 |
| *all-rac*- alpha -tocopherol | 1.10 | 74 |
| *2R4'R8'S* alpha -tocopherol | 1.34 | 90 |
| *2S4'R8'S* alpha -tocopherol | 0.55 | 37 |
| *2R4'S8'S* alpha -tocopherol | 1.09 | 73 |
| *2S4'S8'S* alpha -tocopherol | 0.31 | 21 |
| *2R4'S8'R* alpha -tocopherol | 0.85 | 57 |
| *2S4'S8'S* alpha -tocopherol | 1.10 | 60 |
| *RRR*- alpha -tocopheryl acetate | 1.36 | 91 |
| *all-rac*- alpha -tocopheryl acetate | 1.00 | 67 |

[a]Bioactivites based on rat fetal resorption studies from several laboratories compiled by Pryor *et al.* (1995). [b]IU is defined as the bioactivity of 1 mg of *all-rac*-tocopherol acetate. [c]*RRR*-alpha-tocopherol is the most biologically active form.

Animal systems use mechanisms involving tocopherol binding proteins to preferentially retain alpha-tocopherol (Dutta-Roy, 1999). In the gut, the absorption properties are the same for each of the forms of vitamin E. After dietary consumption, all forms of vitamin E, along with other fat soluble compounds, are absorbed into aggregates of lipoproteins called chylomicrons. The chylomicrons are transported through the circulatory system and eventually are taken up by the liver. In the liver, alpha-tocopherol is transferred from the chylomicrons to very low density lipoproteins (VLDL) by the 30 kiloDalton hepatic alpha-tocopherol transfer protein (αTTP). The affinity of this protein is much higher for alpha-tocopherol than for the other forms. The other forms of tocopherol, which are not transfered to VDLP by αTTP, are removed from the body most likely through the bile.

In circulation, tocopherols can exchange among several carriers. First, the liver secretes VLDL containing tocopherol. These VLDL are metabolized to low density lipoproteins (LDL). The LDL can freely exchange tocopherols with high density lipoprotien (HDL). In turn, HDL and chylomicrons can exchange tocopherol (Traber, 1994). This process of tocopherol absorption and transport is illustrated in Figure 6.

**Figure 6. Absorption and transport of vitamin E in animal systems**[1]
Source: Traber, 1994




[1]Alpha-tocopherol (α-T) is preferentially retained. For simplicity gamma-tocopherol (γ-T) is the only other form depicted. Both alpha- and gamma-tocopherols are equally absorbed in chylomicrons (green) and move to tissues or liver. In liver, gamma- and other forms are removed while alpha-tocopherol is bound by alpha-tocopherol transport protein [(αTTP) (blue) and packaged in nascent VLDL for secretion back into circulation. Exchange between chlylomicrons and HDLP (aqua) can occur in circulation as well as exchange between HDLP and LDL. Smaller γ-T symbol represents lower concentrations of other tocopherols compared to alpha-tocopherol.

Tocopherol binding proteins have been identified in numerous animal tissues (Dutta-Roy *et al.,* 1993; Gordon *et al.,* 1995; Sato, 1993). The 30 kDA alpha-tocopherol binding protein is found exclusively in liver cytosol. A different 15 kDA alpha-tocopherol binding protein is found in all major animal tissues and is thought to transfer alpha-tocopherol intracellularly, although the mechanism is unknown. In addition, some cells may have an alpha-tocopherol binding protein on the plasma membrane to facilitate the uptake of alpha-tocopherol (Dutta-Roy, 1999). Such a protein has been identified in human red blood cells (Bellizzi *et al.,* 1997).

Tocopherol binding proteins have not been identified in plants. Most of the tocopherol in plants is thought to be localized in the thylakoid membrane (Yerin *et al.,* 1983) with excess stored in plastoglobuli (Lichtenthaler, 1968). However, tocopherols have been detected outside of the cell in epicuticular wax (Shepherd *et al.,* 1999), which indicates that some mechanism for transport exists. Possible candidates for a tocopherol transport molecule in plants are the 9 kDA non-specific lipid transport proteins (LTP). These proteins have been shown to enhance the movement of phospholipids and bind acyl chains. Moreover, lipid transfer proteins have been hypothesized to play a role in cutin formation and in stress adaptation (Kader, 1996).

Because different forms of vitamin E have different bioactivites, the value of a feed as a source of vitamin E depends not only on the quantity of tocopherols present but also on the form of tocopherol present. The quantity and forms of tocopherol in plants vary among species (Hess, 1993). Table 2 lists some typical concentrations for alpha-tocopherol in plant species. The variation in tocopherol content in seed oils is distinct enough to provided an effective means to evaluate the taxonomy of certain plant families (Goffman *et al.*, 1999). For instance, within the Boraginaceae family, tocopherol profiles of seed oils ranged from 100% alpha- to 100% gamma- to 90.4% delta-tocopherol for different species (Velasco and Goffman, 1999). Despite these wide variations, it is

**Table 2. Alpha-tocopherol concentrations in several plants**

| Species, tissue description | alpha-tocopherol, ppm DM | Reference |
|---|---|---|
| *Abies alba* | | |
| 1 yr needle | 200 | Finckh & Kunert, 1985 |
| 4 yr needle | 700 | |
| *Amaranthus retroflexus* cotyledon | 600 | Finckh & Kunert, 1985 |
| *Chenopodium album* cotyledon | 120 | Finckh & Kunert, 1985 |
| *Datura stramonium* cotyledon | 830 | Gapor *et al.,* 1986 |
| *Elaeis gueneensis* leaflet | 3200-5600 | Kunert & Ederer, 1985 |
| *Fagus silvatica* | 300 | Green, 1958 |
| 1 month leaf | 2300 | |
| 5 month leaf | | Kandil *et al.,* 1990 |
| *Helianthus annum* seed | 860-950 | |
| *Hordeum sativum* | 67 | Green, 1958 |
| 74 day plant | 10 | |
| ripe seed head | | |
| *Pisum sativa* | 127 | Green, 1958 |
| 31 day plant | 6.5 | |
| seed | 526 | Demo *et al.,* 1999 |
| *Rosmarinus officinalis* leaves | | |
| *Solanum tuberosum* fresh tuber | 0.10 | Booth, 1964 |
| *Trifolium ripens* whole plant | 160 | Green, 1958 |
| *Zea mays* | 5.6 | Green, 1958 |
| 87 day plant | 54 | |
| seed | | |

commonly assumed that over-all gamma-tocopherol is the most common tocopherol in plants (Goffman *et al.,* 1999; Haddarainen and Pehrson, 1987).

Changes in tocopherol quantity can occur in a plant as part of stress responses or due to physiological changes in the plant tissue (Hess, 1993), changes in tocopherol content are not the same in all plants, or in all plant tissues (Franzen and Haab, 1991). Due to the role of vitamin E as an antioxidant, it is not surprising that vitamin E increases have been reported in response to high light intensity (Garcia-Plazaola *et al.,* 1998), drought stress (Munne-Bosch *et al.,* 1999), and chilling stress (Wildi and Lutz, 1996). Tocopherol levels in many species also change with maturity (Franzen *et al.*, 1991*;* Robowsky and Knabe, 1970). In leaf tissue of some annual crops (Tramontano *et al.*, 1992) and in trees (Kunert and Ederer, 1985) alpha-tocopherol levels increased with maturity. In forage grasses, however, tocopherol levels in the whole plant decrease with maturity (Lynch, 1991).

## Importance of Vitamin E in Goat Production

Vitamin E is an essential component of ruminant diets (Tal Huber, 1988). Adequate dietary vitamin E is important in goats for the prevention of nutritional muscular dystrophy (white muscle disease). White muscle disease, the most common manifestation of vitamin E or selenium deficiency, is caused by an accumulation of peroxides in the muscle tissue and can be fatal (Smith and Sherman, 1994). Selenium deficiency contributes to white muscle disease because selenium is a cofactor in the peroxide metabolizing enzyme, glutathione peroxidase. White muscle disease is a concern of local goat producers (G. Groot, 1999, personal communication). The Appalachian region has selenium-deficient soils which limit the amount of selenium livestock can obtain from forage (Ammerman and Miller, 1975). Low levels of selenium in the available forage increase the importance of adequate dietary intake of vitamin E because deficiencies in both micronutrients significantly increase fatality of white muscle disease (Bickhardt *et al.*, 1999).

The absolute dietary requirement of Vitamin E for non-nursing goats is 0.1-0.3 IU/kg body weight; however, daily intake of 25 to 50 IU/kg body weight (B.W.) is recommended (Smith and Sherman, 1994). Goats require higher levels of vitamin E than do sheep or cattle to prevent vitamin E-related myopathies (Jones *et al*., 1988). High vitamin E intake is also required by young horses and lactating dairy cows (McDowell, 1985). The high vitamin E requirement in goats may be linked to the high rate of passage of forage through the rumen (Luginbuhl *et al.*, 1995).

Nutrient supplementation with vitamin E prior to slaughter can improve the shelf life of the meat from cattle (Faustman et al., 1989), sheep (Wulf *et al.*, 1995) and other livestock (Monahan *et al.*, 1992; Lin *et al.*, 1989; Lopez-Bote *et al.*, 1997). The increased level of Vitamin E in the meat prevents discoloration during storage by decreasing lipid

peroxidation and maintaining hemoglobin in the reduced form, yielding a more appealing meat for the consumer (Liu, 1995).

Vitamin E is one of the more expensive supplements to purchase (McDowell, 1985). Considering the high vitamin E requirement for goats, obtaining vitamin E from the available forage is desirable in a low-input, pasture-finished, goat production system.

Goat production may be a viable agricultural enterprise on many underutilized, Appalachian, hill-land pastures. Pastures overgrown with invasive, weedy, browse species that are unsuited for raising cattle or sheep can potentially be used for goat production. Goats prefer rough and steep land over flat land (Luginbuhl *et al.*, 1997) and will consume browse and weedy species that cattle or sheep refuse (Harrington, 1982; Ramirez *et al*., 1990). Goats are also effective in suppressing growth of invasive shrubs which can help improve pasture quality (Luginbuhl *et al.*, 1999). Using overgrown pastures in a meat-goat production system offers the combined benefits of pasture renovation and production of a marketable product.

## Biology of Invasive Shrubs on Appalachian Farms

Multiflora rose (*Rosa multiflora* Thunb.), bush honeysuckle (*Lonicera morowii* Gray and related species), and autumn olive (*Elaeagnus umbellata* Thunb), once promoted for conservation use, have become invasive weeds in many Appalachian pastures (Darlington and Loyd, 1994). At the time these shrubs were being promoted for conservation use, many farmers clipped their pastures annually or kept sheep along with cattle. These practices, which prevented establishment of the invasive species in pastures, are practiced less commonly on farms today (Bryan and Mills, 1988). Once established, these weedy shrubs decrease pasture quality and decrease the area available for grazing (Kay, 1995). These shrubs are now classified as invasive species by the Natural Resource Conservation Service, and in West Virginia, mutliflora rose and autumn olive are considered noxious weeds (USDA, NRCS, 1999).

In conservation programs from the 1930's to as late as the 1960's, planting of these species was encouraged because of their value in preventing soil erosion, providing food for wildlife, and/or acting as a "living fence." Characteristics such as the ability to form dense hedge rows and to become established in poor fertility soil make them ideal for such uses (Darlington and Loyd, 1994). Unfortunately, these same characteristics allow these species to overtake pastures if not properly managed. The spread of these shrubs is enhanced because all three produce attractive, reddish fruit that is eaten, scarified, and widely dispersed by birds. A brief description of each species follows.

Multiflora rose (Figure 7) was introduced into the US from East Asia in the late 1800's as a root stock for ornamental roses (Uva *et al.*, 1997). This prickly stemmed shrub can grow up to 4 m in height. Leaves are alternate and compound with seven to nine oblong, serrate leaflets, 2 to 4 cm long. The presence of a fringed stipule distinguishes multiflora rose from other roses. Reproduction is both by seed and vegetative propagation. Flowers appear in May and June; fruit is produced in late summer and can remain on the

plant through the winter (Kay, 1995). Vegetative reproduction occurs when canes bend over, contact the ground, and develop roots for new plants. Multiflora rose grows rapidly and can form nearly impenetrable thickets.

**Figure 7. Multiflora rose images**

Multiflora rose berries and habit

The name bush honeysuckle refers collectively to several closely related species including the tartarian honeysuckle (*Lonicera tatarica* L.) and Morrow's honeysuckle (*L.* morrowii Gray) (USDA, NRCS, 2000). These exotic species were introduced from Eurasia in the late 1700's as ornamentals and later planted for conservation. These species are common in most eastern states. Bush honeysuckles are branching, deciduous, hardwood shrubs that range in height from 2 to 5 m. Leaves are opposite , simple, oblong with entire margins, and pointed tips. Flowers bloom in pairs on a common stalk from the leaf axis from May to early June. The plants develop attractive red berries in mid-summer (Uva *et al.*, 1997).

**Figure 8. Bush honeysuckle images**

a bush honeysuckle resprouting after cutting

bush honeysuckle (tartarian variety) in flower

Autumn olive (Figure 9) was introduced from East Asia around 1830 and now ranges from Maine to Virginia and West to Wisconsin. Autumn olive is a non-legumninous, nitrogen fixing species that lives in association with *Frankia spp.* (Gardner, 1958; Hensley and Carpenter, 1984). The species tolerates drought and infertile, clayey, low-pH soils (Sharp, 1977; Dirr, 1990). Autumn olive grows up to 5 m tall with a deep taproot. Leaves are alternate, oblong with wavy edges, and have a silvery cast. Fragrant flowers bloom in April and May, and an abundance of small drupes are produced in early fall. These fruits ripen from silvery-brown to speckled red (Strausbaugh and Core, 1978). The fragrance of the flower is attributed to a high concentration of 4-methyl phenol (Potter, 1995). In Japan, immature fruits are used for pickling and to make an alcoholic beverage; once mature the sweet fruits are eaten raw (Sakamura and Suga, 1987)

**Figure 9. Autumn olive images**


autumn olive in flower


autumn olive habit

## Methods of Extracting and Analyzing Vitamin E

Vitamin E is oxidized by heat, light, oxygen, or other oxidizing conditions (Eitenmiller and Landen, 1994); therefore, specific protocols have been developed to minimize loss of vitamin E during extraction and analysis. The AOAC method for analysis of tocopherols calls for saponification of tissues followed by esterification and quantification by thin layer chromatography (AOAC International, 1995). Alternative methods are commonly employed because the heat and alkali conditions called for in saponification can result in substantial degradation of tocopherols (Ueda and Igarashi, 1987).

Burton and Ingold (1985) developed an efficient, rapid extraction for vitamin E from animal tissue using a combination of sodium dodecylsulfate (SDS), ethanol, and heptane. The SDS disrupts the membranes and liberates the tocopherols, the ethanol precipitates the proteins, and the heptane is a non-polar solvent into which the tocopherol will readily partition. Tramontano and colleagues (1993) adapted this protocol for use with plant tissue with good results.

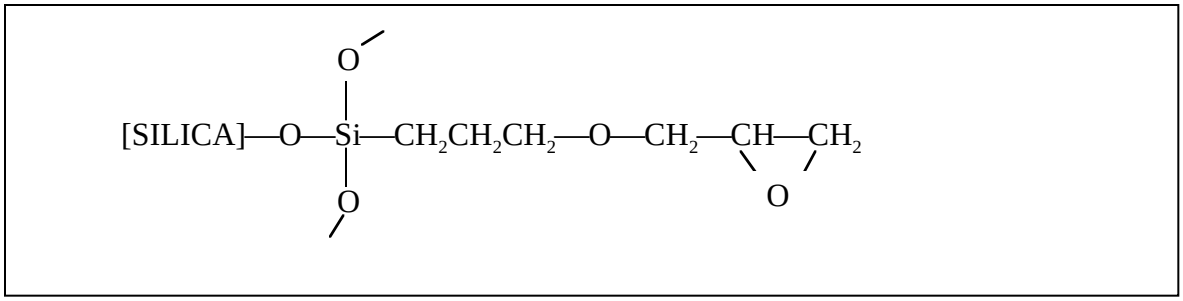
Other factors can influence the recovery of vitamin E during extraction. Coexisting fats can decrease the recovery of tocopherols, particularly delta-tocopherol and tocol, the tocopherol derivative with an unmethylated chroman ring (Ueda and Igarashi, 1987). Ueda and Igarashi (1990) also noted that loss of alpha-tocopherol can occur during evaporation of organic solvents.

High performance liquid chromatography (HPLC) is an effective means to separate tocopherols following extraction. Both normal-phase and reverse-phase HPLC are commonly employed, however each type of chromatography has some drawbacks. Reverse-phase HPLC cannot resolve beta- and gamma-tocopherols (Tan and Brzuskiewicz, 1989). In normal-phase HPLC, reproducibility is often poor when using a silica stationary phase (Kramer *et al.*, 1999). In normal phase chromatography, the forms of vitamin E are

separated based on the degree of methylation of the chroman ring and are eluted in order of increasing polarity (Tan and Brzuskiewicz, 1989). Kramer and collegues (1999) recently used a normal phase diol column to separate tocopherols with good results. Through NMR analysis they determined that the stationary phase was not a diol but actually an epoxide (Figure 10). The epoxide structure gives the column stability to polar and nonpolar solvents and moderate polarity. They report that the diol column was stable over hundreds of assays and results were reproducible (Kramer *et al.,* 1999)

Both UV and fluorescence detection are appropriate for quantification of vitamin E, but fluorescence detection is significantly more sensitive (Thompson and Hatina, 1979). The chroman ring absorbs maximally between 280 nm and 300 nm. The most suitable excitation wavelengths for fluorescence detection are around 290 nm to 295 nm; emission is measured at 320 nm (Eitenmiller and Landen, 1994).

**Figure 10. Structure of the bonded phase of the diol column**
Source: Kramer *et al.,* 1999



# CHAPTER 3: Materials & Methods

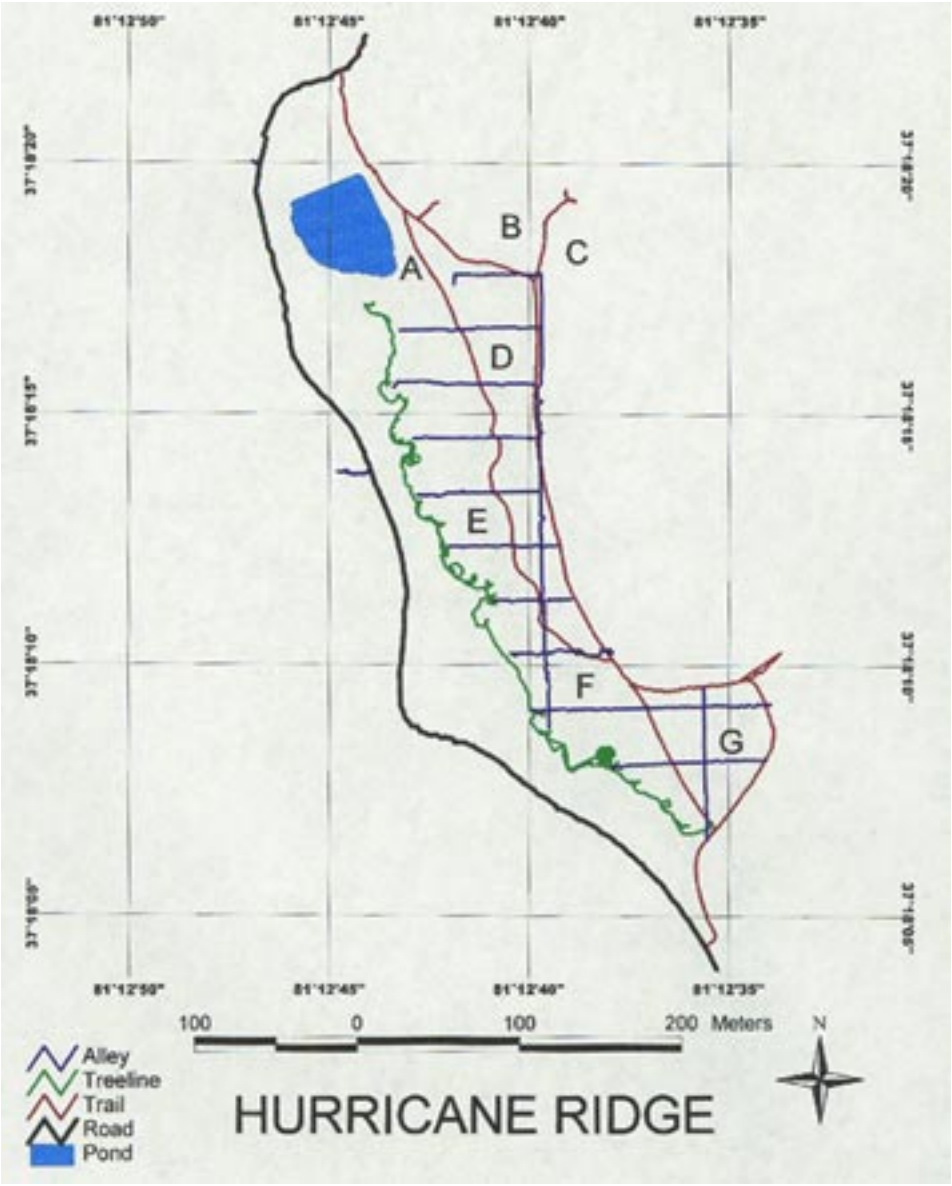
## Sampling

Leaf tissue was harvested from a 15-acre site in southern West Virginia that had been unmanaged for ten years. The site, located on Hurricane Ridge (37° N, 81° W, 880 m above sea level), lies outside of the town of Princeton, WV, near the Bluefield Mercer County Airport. Multiflora rose, Morrow's honeysuckle, and autumn olive occupy up to 42.8%, 23.3% and 8.7% respectively of the site (J. Fedders [USDA, ARS], personal communication).

The site, a fully enclosed watershed, was divided into seven replicate plots based on the topography (Figure 11). Plots F-G were located at the top of the watershed; plots A and D near the pond were at the bottom of the watershed. Leaf tissue samples from each of the three browse species were harvested from each plot five times during the 1999 growing season (Table 3). Collection of samples from all plots required two days, and samples were harvested between 9 A.M. and 4 P.M. Leaf tissue from only one plant per species was harvested for each plot on a given sampling date. Different plants were sampled on each collection date. The harvested leaf tissue consisted of the portion of plant tissue most likely to be consumed by a browsing goat. At different times in the growing season the harvested leaf tissue contained on or more of the following tissue types: leaves, green stems, flower or leaf buds, and/or immature or mature fruits. Table 3 gives a description by species of the size of leaf that was harvested at each harvest date.

The tissue was frozen on dry ice within 5 min after removal from the plant. Tissue samples were stored at -90° C for up to 3 weeks prior to lyophilization. After lyophilization, frozen samples were ground to 0.5 mm using a UDY cyclone mill (UDY Corp, Fort Collins, CO) and then stored at -90° C for up to 10 months until analyzed.

**Figure 11. Map of Hurricane Ridge[a]**




[a]Letters A-G in grid indicate plot locations. Total size of the watershed is approximately 15 acres.

**Table 3. Size of leaf tissue from browse species on harvest dates during the growing season.**

| Date of Harvest | Range of leaf tissue length at Harvest | | |
|---|---|---|---|
| | multiflora rose leaflet, cm | bush honeysuckle leaf, cm | autumn olive leaf, cm |
| **4/16, 4/19** | <2 | 3-5 | 2-4 |
| **5/18, 5/19** | 2-3 | 4-6 | 4-6 |
| **6/2, 6/3** | 2-3 | 4-7 | 5-7 |
| **6/24, 6/25** | 3-4 | 4-7 | 5-8 |
| **8/12, 8/13** | 3-4 | 4-7 | 5-8 |

## Vitamin E Extraction Procedure

Procedures for extraction and analysis of vitamin E were adapted from existing protocols (Tramantano *et al.,* 1993, Kramer *et al.,* 1999). Sixty-five mg of ground, lyophilized tissue were rehydrated in 5 ml with an aqueous solution of 0.5 mM EDTA containing 20 mg ascorbic acid (final pH = 3.1) in a 28 ml polypropylene centrifuge tube (Sorvall, Newtown, CT). Tocol (150 µl), a tocopherol without methylation around the chroman ring, was added as an internal standard to the extraction tube. Tocol has not been found in nature and is an appropriate internal standard for tocopherol extractions (Kramer *et al.,* 1999). The centrifuge tube was flushed with argon gas for 3 to 5 s, sealed with a screw cap, and mixed with a Vortex Genie (Fisher Scientific, Pittsburg, PA). Two ml 100 mM SDS were added, mixed by swirling once, and the solution was placed on ice for 6 min. Ice-cold ethanol (5 ml) and 3 ml of hexane containing 0.2% BHT (w/w) were added to the centrifuge tube. The solution was again mixed with the vortex mixer under argon gas for 6 min, and then centrifuged for 3 min at 1149*g* at 18°C to achieve a phase separation. Two ml of the upper organic phase containing the tocopherols was removed with a glass syringe and filtered through a 0.45 µm nylon filter with glass fiber prefilter (Osmonics, Inc.,Minnetonka, MN). One ml of the filtrate was transferred to a 2 ml amber

glass sample vial. The organic phase was evaporated to dryness under argon gas and redissolved in hexane containing 0.2% (w/w) BHT. Oxygen was flushed from the sample vial with argon, and the vial was sealed with a screw cap and placed on ice, in the dark until analyzed. The beta-tocopherol (approximately 99% purity) standard and tocol were supplied by Matreya, Inc (Pleasant Gap, PA). Gamma-tocopherol (pure), delta-tocopherol (approximately 90% purity), and two alpha-tocopherol standards (95% and 1000 IU/gm) were supplied by Sigma Chemical Co. All other chemicals were obtained from Fisher Scientific except for BHT which came from Sigma Chemical Co. (St. Louis, MO).

## HPLC Analysis

Vitamin E was analyzed by HPLC using: a Rainin Instrument Co. (Woburn, MA) Dynamax SD-200 pump, a Rheodyne (Cotati, CA) 7725 manual injector with 100 µl sample loop, a normal-phase Supelcosil LC-Diol column 250 x 4.6 mm, fitted with a 2 cm diol guard column from Supelco (Bellefont, PA), and a Perkin Elmer (Oak Brook, IL) LS-3 fluorescence detector. Isocratic chromatography was performed at room temperature with a mobile phase of hexane:isopropanol (99:1, v/v) flowing at 1.5 ml/min. An excitation wavelength of 295 nm and an emission wavelength of 320 nm were used for fluorescence detection. Integration of peak areas was performed with Dynamax HPLC Method Manager Software (Rainin Instrument Co., Woburn, MA).

Tocopherol standard curves were generated several times during the weeks of extractions to ensure proper quantification of tocopherols following replacement of the xenon lamp and for changes in flow rate of the mobile phase. Quantities of individual forms of tocopherol were corrected for loss during extraction based on the recovery of tocol. For routine calculations, percent recovery of tocol was calculated as follows:

$$\text{Peak Area}_{obs}/\text{Peak Area}_{exp} \times 100 = \text{Percent Recovery}$$

Where $\text{Peak Area}_{obs}$ is the measurement obtained from integration from fluorescence detection and $\text{Peak Area}_{exp}$ is the peak area measured from direct injection of the tocol standard at the proper concentration. The average quantity of tocopherol from two

extractions per sample was used for statistical analysis. The general linear model comparing plant species, harvest date, and plot location and the means ± standard error were calculated using the SAS system (SAS Institute, Cary, NC).

# CHAPTER 4: Standardization of Protocols

Experiments were performed to establish the reproducibility of the extraction method and to refine published analytical protocols for vitamin E measurement in plant tissue samples.

## Variability of Repeat Injections

To assess the variation in peak area areas obtained from repeat injections, a solution of BHT was made in hexane, dispensed into nine tubes that were sealed and put on ice until analyzed. Sample from each tube was loaded manually into the 100 µl sample loop and then injected onto the column. The mean peak area (6770000) and standard deviation (± 37000) was calculated from the results of the nine injections. Hence, variations due to equipment and manual injection technique limit the values to three significant figures.

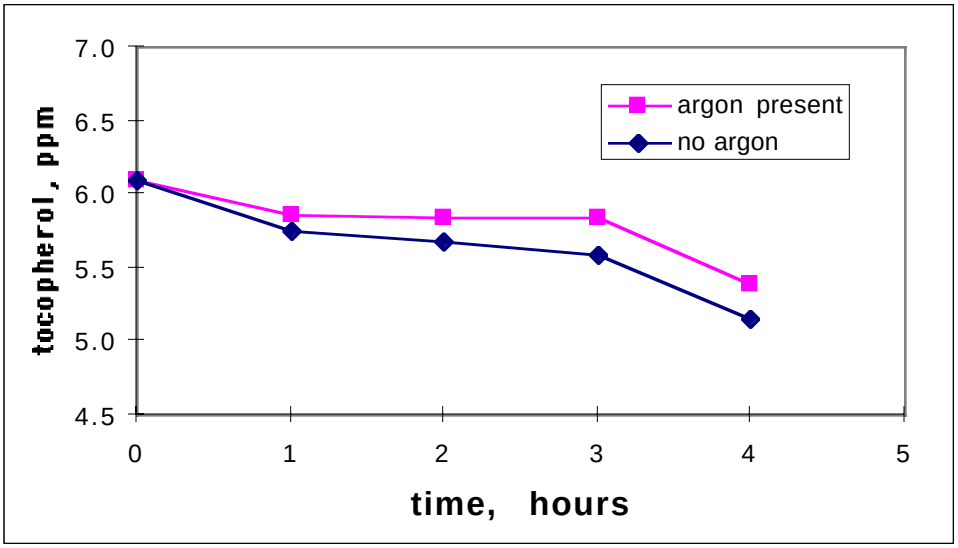
## Recovery of Tocol in Absence of Plant Tissue

The extraction procedure was performed twice in absence of plant tissue to assess the maximum recovery of tocol and to determine whether concentration of the hexane phase occurs during flushing with argon. Tocol was added prior to mixing with hexane. The average recovery for the two extracts was 101% indicating that full recovery is possible and that the effect of solvent evaporation during flushing is minimal.

## Effect of Argon on Tocopherol Stability

After dissolving a sample in hexane, the vial is flushed briefly with argon gas to eliminate any oxygen that may oxidize the tocopherols. After an aliquot is withdrawn for analysis the sample vial is again flushed with argon gas. The effect of flushing the tubes with argon on tocopherol stability was examined by comparing the level of tocopherol in tubes that were flushed with argon to the level of tocopherol in tubes that were not flushed (Figure 12). There is decreased loss of tocopherols over time in the samples with argon present. Although the loss is minimal, argon was used to exclude oxygen from the sample vials prior to analysis.

**Figure 12. Effect of argon on the stability of alpha-tocopherol[a]**




[a]Ten tubes were filled with the same sample and sealed with an airtight cap. Five of the tubes were flushed with argon prior to being sealed; five were not flushed with argon. All tubes were placed on ice until analyzed. A new tube was used at each time. SE for data points is ± 0.05 based on variability of repeat injections.

## Reproducibility of Tocopherol Extraction

A ground, lyophilized sample of black locust leaf tissue was prepared in the same manner as the browse samples and used for development of the vitamin E extraction procedure. To determine the reproducibility of the tocopherol extraction procedure four extractions were performed each day for three days and the alpha tocopherol content measured. The results of the extractions are listed in Table 4.

**Table 4. Reproduciblity of quantifying alpha-tocopherol in extracts of locust leaf tissue[a]**

| Date of Extraction[a] | ppm DM[b] alpha tocopherol extracted |
|---|---|
| 1/25 | 250 |
| 1/25 | 249 |
| 1/25 | 256 |
| 1/25 | 236 |
| 1/26 | 219 |
| 1/26 | 218 |
| 1/26 | 218 |
| 1/26 | 220 |
| 1/27 | 301 |
| 1/27 | 300 |
| 1/27 | 251 |
| 1/27 | 267 |
| **Average** | **249** |
| **Standard Deviation (SD)** | **29** |
| **SD as Percentage of Mean** | **12%** |
| **Standard Error** | **8.3** |

[a]The sample was treated in the same manner as invasive plants during harvest. The sample was stored at -90°C until thawed for analysis, a portion was removed for analysis, and then the sample was returned to the freezer. For analysis on subsequent days the same sample was removed from the freezer, thawed, dispensed, and then returned to the freezer.
[b]Dry matter (DM)

## Stability of Tocopherol in Storage

To confirm the stability of tocopherols in plant tissue stored at -90°C, extracts were made of a multiflora rose sample at four different times. The sample was brought to room temperature and the amount required for extraction was removed. The remaining sample was sealed and returned to the freezer. After an initial measurement (6 months after harvest), a second extraction of alpha tocopherol was made one week later, followed by additional extractions at six and ten weeks after the initial measurement (Table 5). No change ($P>0.1$) in the alpha-tocopherol concentration was observed over ten weeks. The differences in mean alpha-tocopherol concentration are within the standard deviation of the mean estimated for repeat extractions (Table 4).

**Table 5. Contencentration of alpha-tocopherol extracted from multiflora rose leaf tissue after various periods of storage**

| Storage Time, weeks | Alpha-tocopherol, ppm DM | | Mean alpha-tocopherol, ppm DM |
|---|---|---|---|
| | Extract 1 | Extract 2 | |
| Initial[a] | 262 | 287 | **275** |
| 1 | 281 | 300 | **291** |
| 6 | 320 | 315 | **318** |
| 10 | 295 | 280 | **288** |

[a]Initial measurement was made after sample had been stored for 6 months at -90°.

## Effect of Second Extraction on Tocol Recovery

Experiments were performed to assess if additional tocopherols could be extracted by following the first hexane extraction of tocopherols from a plant sample with a second hexane extraction on the same sample. Two ml of the 3.15 ml of the hexane layer were removed following the initial extraction and replaced by 2 mls of fresh hexane with 0.2% (w/w) BHT for an additional extraction. The total recovery obtained in the first extraction and the total tocopherol recovered in the first and second extractions are shown in Table 6 for four separate extracts. The results indicated that the first extract removed nearly all the tocopherol and that a second extraction does not improve recovery. The difference in alpha-tocopherol extracted between the first and first plus additional extracts is not significant ($P$ <0.01).

**Table 6. Recovery of alpha-tocopherol from locust leaf after first and first plus additional extraction[a]**

| Sample | Recovery from first extract, ppm DM | Recovery from first plus additional extract, ppm DM |
|---|---|---|
| **1** | 13.3 | 13.8 |
| **2** | 12.9 | 13.6 |
| **3** | 15.7 | 15.6 |
| **4** | 15.9 | 14.8 |

[a]Difference between recovery from first and first plus additional extraction is not significant ($P$<0.01).

## Stability of Tocopherol in Plant Extracts

Although multiple extracts were carried out at one time, chromatography could only be performed one extract at a time. With up to 20 min required per sample for chromatography, considerable time could pass between extraction and analysis of all samples from a group of extracts. To assess the stability of alpha-tocopherol in the plant extract over time, two extracts were taken from a sample of locust leaf tissue. Each of the two extracts was divided into five individual tubes that were held on ice and analyzed over the course of six hours. There was no significant ($P$>0.1) loss of alpha-tocopherol over 6 hours (Figure 13).

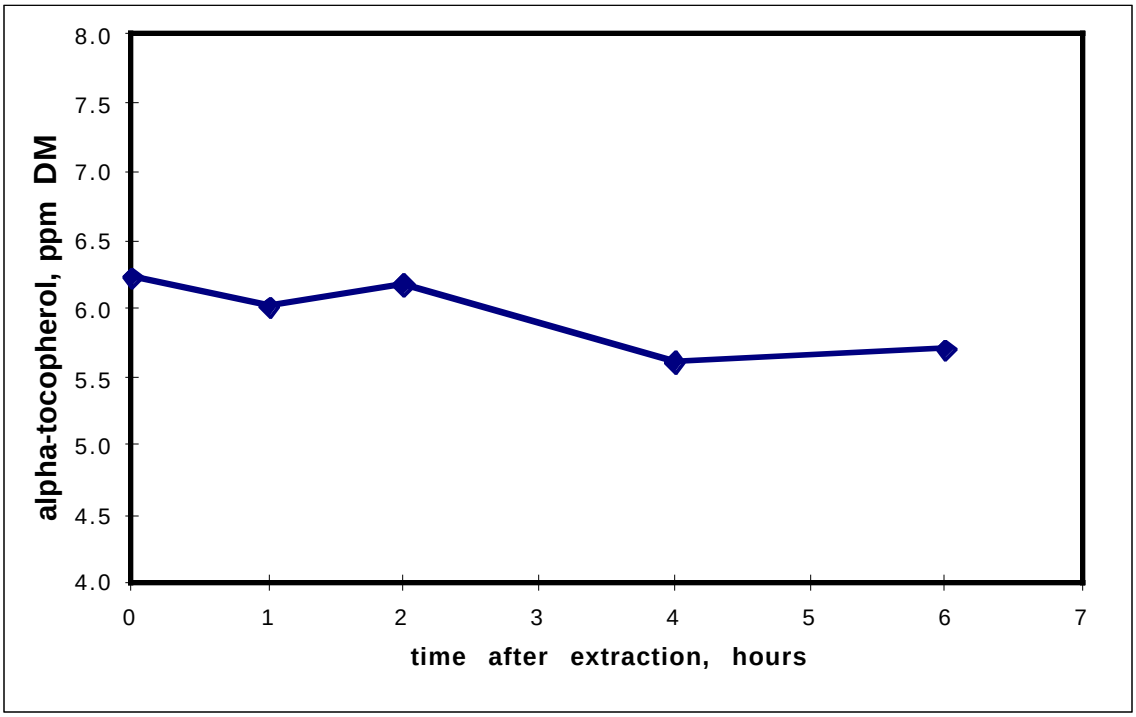


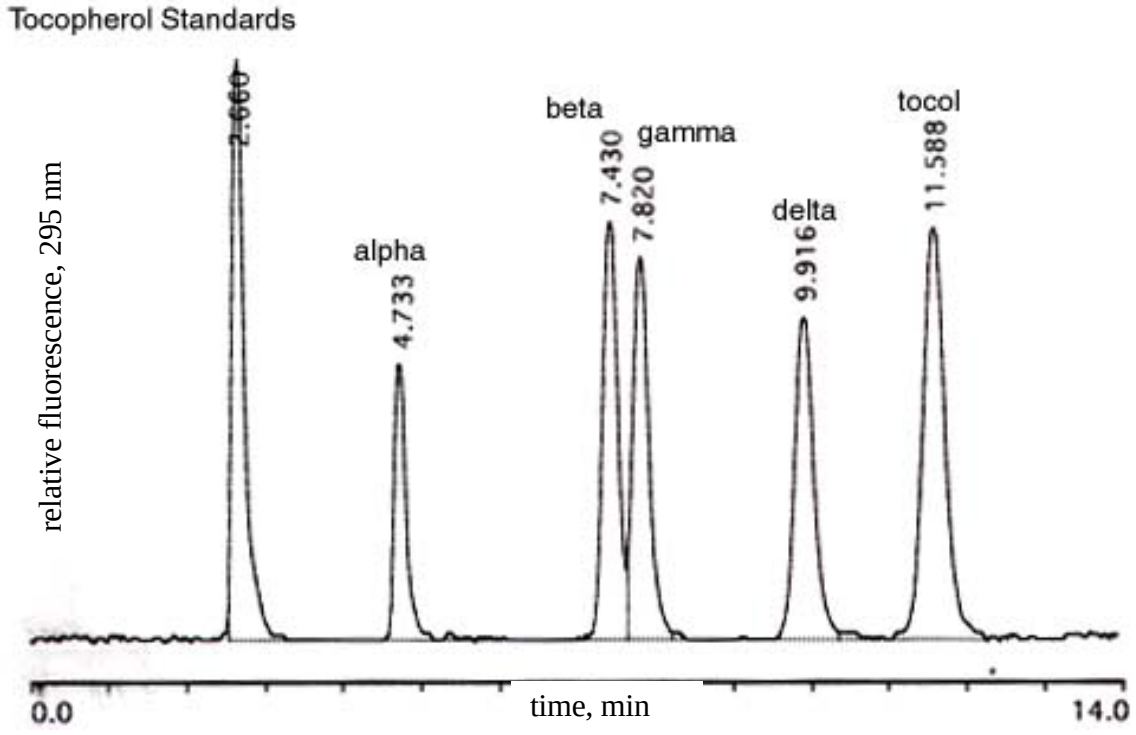

**Figure 13. Stability of alpha-tocopherol in plant extracts over time[a]**

[a] Two extracts were divided into five individual tubes that were held on ice and analyzed over the course of six hours. Data points are the average alpha-tocopherol concentration from the two extracts. SE for data points is ± 0.05 based on variability of repeat injections.

## Standard Curves and Calculations

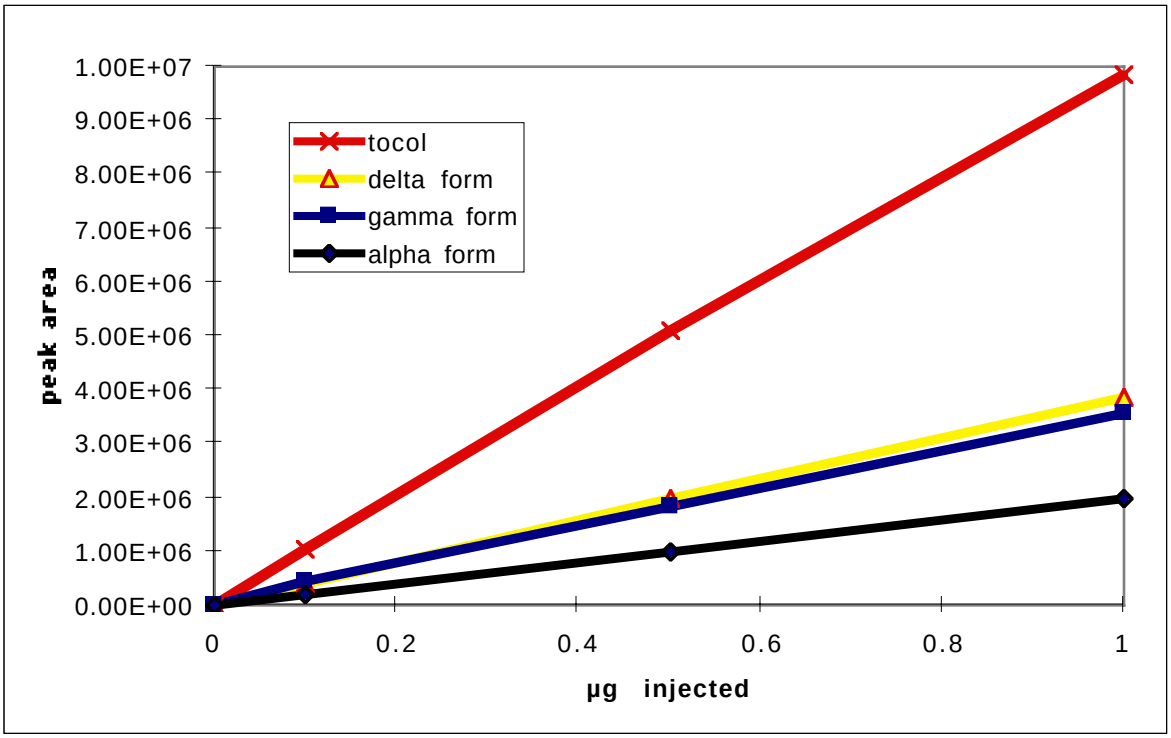
Standard curves were produced from tocopherol standards several times during the study. New standard curves were needed after replacing the xenon lamp in the fluorescent detector and for different flow rates of the mobile phase. The tocopherol concentrations measured for the standard curve were made by serial dilutions. A typical chromatogram (Figure 14) of tocopherol standards is shown along with a standard curve (Figure 15). Zero was used as a value in generating the curve, but the line was not forced through the origin. All lines are linear at $R^2 \geq 0.998$. Curves were routinely generated with standard concentrations ranging from 0 to 1.0 µg. When concentrations of ranging up to 2.5µg were analyzed the linear trend continued ($R^2=0.997$). Typically alpha-tocopherol was detected at levels below 1.5 µg in plant extracts.

**Figure 14. Chromatogram of tocopherol standards**[1]




[1]Fluorescent detection of tocopherol standards in hexane with BHT (2.66 peak). Concentrations were 0.67 µg alpha-tocopherol, and 1.0µg for the other forms of tocopherol. Mobile phase 99:1 hexane: isopropanol flowing at 1.5ml/min.

**Figure 15. Standard curve of tocopherols[a]**




[a]Standard curve for fluorescent detection of tocopherols on 3/22/00. Flow rate of 1.5ml/min. Beta-tocopherol is not shown, but the slope is similar to gamma-tocopherol.

**Sample calculation of tocopherol concentration in a plant extract.**

Given: equation of the line: peak area=1.95 x $10^6$X µg tocopherol + 1.81 x $10^4$
detected peak area: 3,000,000
injection volume: 100 µl
sample volume: 1 ml
total volume of extract: 3.15 ml
recovery based on tocol: 90%
initial weight of plant tissue: 0.065g DM

1) adjust peak area for recovery: 3,000,000 / 0.9=3,330,000
2) convert peak area to amount:3,330,000=1.95 x $10^6$X µg tocopherol + 1.81 x $10^4$
X µg tocopherol= 1.70 µg tocopherol injected
3) calculate amount in total volume of extract:
1.70µg x 10 injection volumes/sample volumes x 3.15 sample volumes/total extract
=53.6µg tocopherol/total extract
4)divide total tocopherols by amount of plant tissue:
53.6 µg / 0.065 g=825 ppm DM

## Recovery of Tocopherols

Tocopherols are sensitive to oxygen, light, and temperature, and care must be taken to avoid their degradation (Eitenmiller & Landen, 1994). Tocol, a tocopherol-derivative not found in nature, was used as an internal standard to correct for any loss of tocopherols during the extraction procedure. Tocol is equally, if not more, sensitive to oxidizing conditions than the naturally occurring forms of tocopherol (Ueda and Igarishi, 1987). The average recovery of tocol during the extractions was 90 $\pm$ 7.1 (mean ± SD; n=146). The recovery ranged from 68% to 100%. No significant difference ($P< 0.001$) in recoveries existed between species based on tocol.

One factor critical to the efficiency of the extraction depends upon the vigorousness of the vortex mixing. The recoveries from samples that were not mixed at full vortex were significantly lower and more variable compared to samples that were mixed at full vortex.

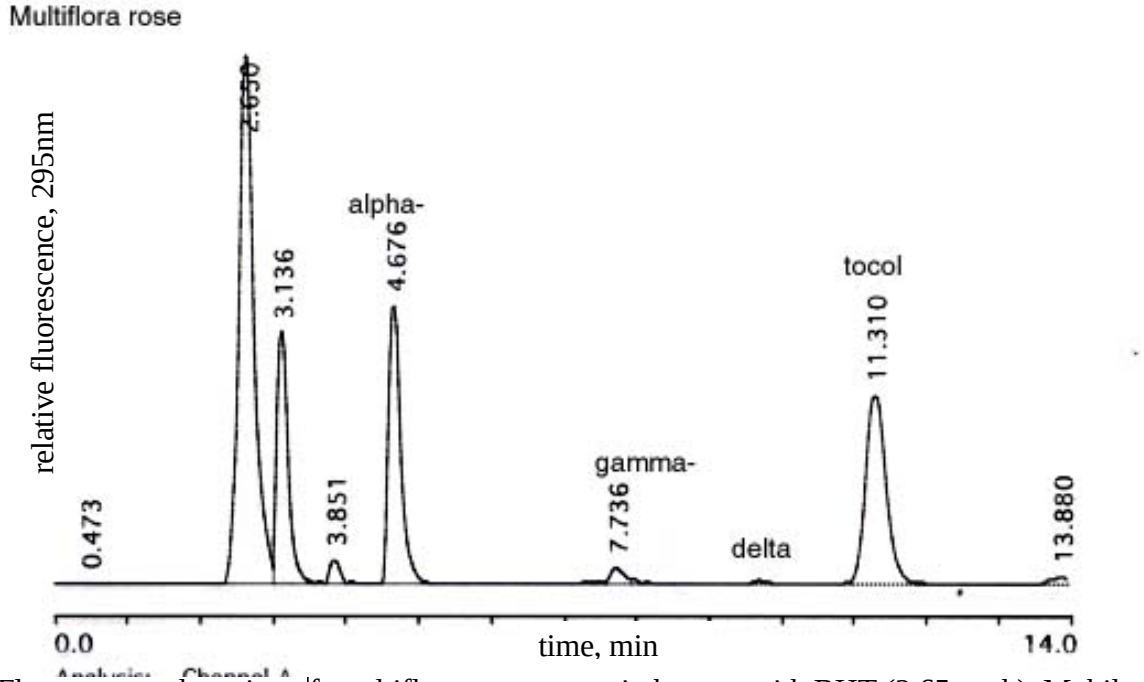
## Chromatograms of Tocopherols from Plant Extracts

Representative chromatograms of vitamin E extracts from multiflora rose, autumn olive, and bush honeysuckle are shown in Figures 17-18. The initial peak is attibuted to BHT. The tocopherols elute in order of increasing polarity. Mobile phase was 99:1 hexane: isopropanol flowing at 1.5ml/min except for analysis of bush honeysuckle. In bush honeysuckle the flow rate was adjusted to 1.0ml/min to separate the gamma-tocopherol and unknown peaks.

Typical retention times at a flow rate of 1.5 ml/min ranged from 4.5 to 5.0 min for alpha-tocopherol, 7.0 to 7.9 min for beta-tocopherol, 7.5 to 8.3 min for gamma-tocopherol, and 9.6 to 10.6 for delta-tocopherol. Peak determination was based on similarity of retention times with standards. The identity of the alpha- and gamma-

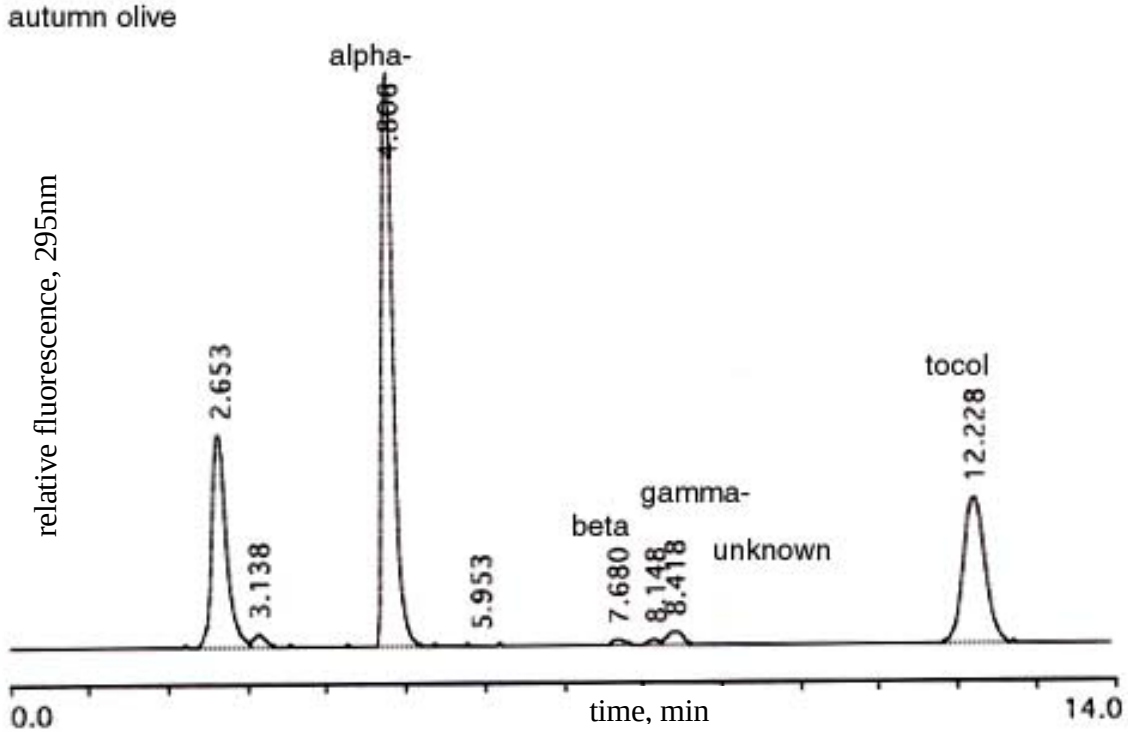
tocopherol peaks was confirmed using gas chromatography-mass spectroscopy (GC-MS; K. Harich, personal communication, 2000)

**Figure 16. Chromatogram of tocopherols from multiflora rose leaf extract[a]**



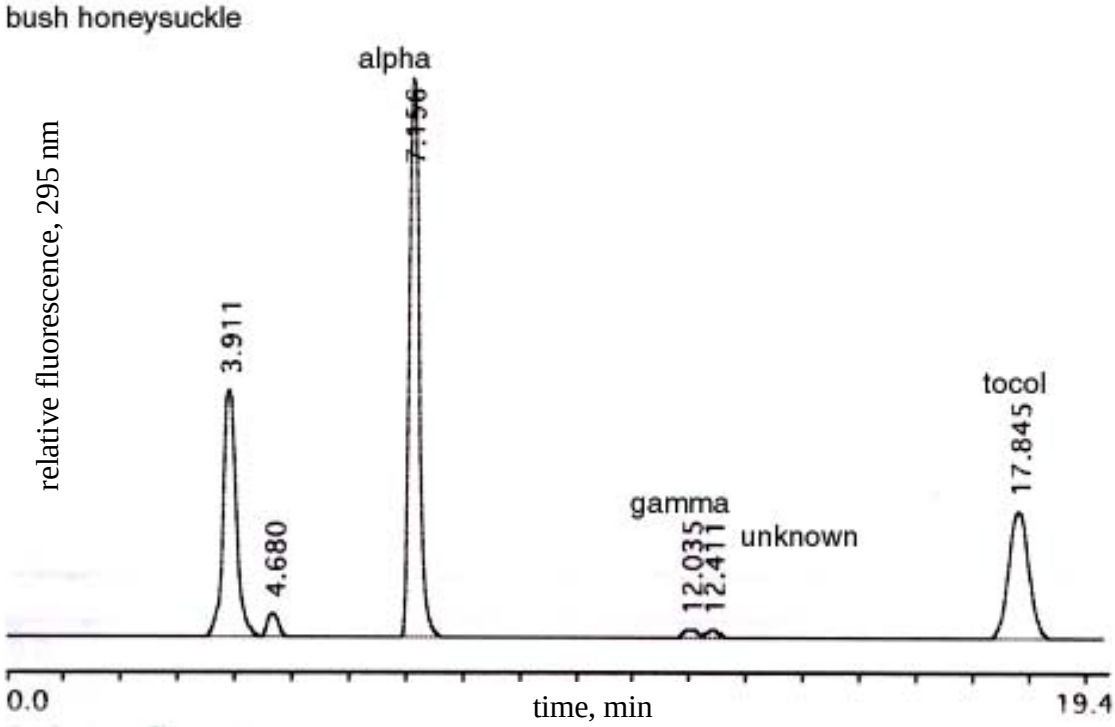

[a]Fluorescence detection of multiflora rose extract in hexane with BHT (2.65 peak). Mobile phase is 99:1 (v/v) hexane: isopropanol flowing at 1.5 ml/min.

**Figure 17. Chromatogram of tocopherols from autumn olive leaf extract[a]**




[a]Fluorescence detection of autumn olive extract in hexane with BHT (2.65 peak). Mobile phase is 99:1 (v/v) hexane: isopropanol flowing at 1.5 ml/min.

**Figure 17. Chromatogram of tocopherols from bush honeysuckle leaf extract[a]**




[a]Fluorescence detection of bush honeysuckle extract in hexane with BHT (3.9 peak). Mobile phase is 99:1 (v/v) hexane: isopropanol flowing at 1.0ml/min.

# Chapter 5: Results and Discussion

## Vitamin E in Invasive Shrubs

Significant differences ($P<0.001$) in the vitamin E content were found among autumn olive, bush honeysuckle, and multiflora rose. Seasonal averages for tocopherols are given in Table 7, illustrating the overall differences in tocopherol content. Autumn olive had the highest total tocopherols and alpha-tocopherol, followed by Morrow's honeysuckle, followed by multiflora rose. Alpha-tocopherol predominated in all species comprising greater than 99% of the total tocopherols in autumn olive, 98% in Morrow's honeysuckle, and 92% in multiflora rose. The alpha-tocopherol concentrations are within the range of those typically reported for plant leaf tissue (Table 2). Total tocopherol content increased during the growing season in all species (significant at $P<0.001$; Figure 19). The species do not change during the growing season in the same way. The general linear model (SAS, 1996) indicated that the increase in tocopherols is in part linear and in part quadratic, suggesting an increase in tocopherols with increasing rate over time. There was no difference ($P>0.1$) among species in the linear trend however the non-linear trend was different for the species ($P<0.01$). The change in tocopherols during the growing season in autumn olive appears more sigmoidal than the change in bush honeysuckle and multiflora rose. The more rapid increase in autumn olive may be related to the increased nitrogen that autumn olive gains through symbiosis.

The regulation of vitamin E content in a plant is poorly understood. However, some factors that are known to influence the vitamin E content of a plant are nutritional status, developmental stage of the plant, tissue type, age of the tissue, and level of oxidative stress. Some of the observations from this study can be interpreted based on previous research which identified these factors known to influence vitamin E levels. Differences in available nutrients may contribute to the differences in vitamin E content between autumn olive and other species. Robowsky and Knabe (1971) reported that

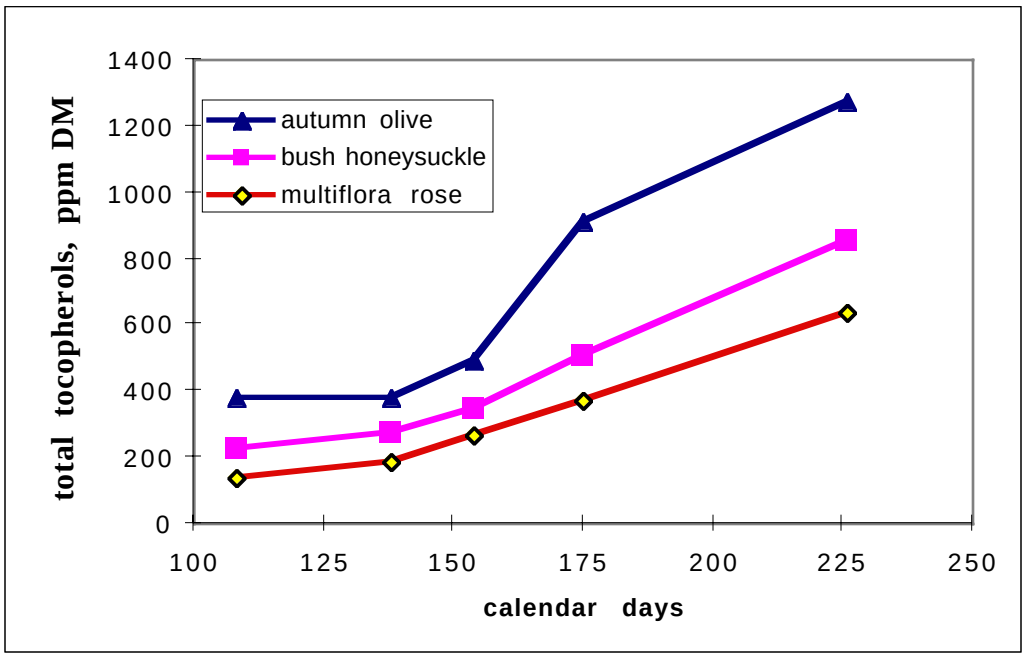
tocopherols in browse species increased in response to nitrogen fertilization. Additional nitrogen available to autumn olive through its association with nitrogen-fixing *Frankia spp.*, is consistent with the higher level of total tocopherols in autumn olive compared to the other non-nitrogen fixing shrubs examined.

**Table 7. Seasonal average concentration of tocopherols in browse leaf tissue**

| Species | Tocopherol Concentration, ppm DM | | | |
|---|---|---|---|---|
| | alpha-[a] | beta-[b] | gamma-[c] | delta-[d] |
| multiflora rose | 295 | 1.9 | 18.0 | 3.8 |
| bush honeysuckle | 431 | ns[e] | 9.8 | ns |
| autumn olive | 685 | 0.8 | 2.0 | ns |

[a]SE±24.6. [b]SE±0.2. [c]SE±1.0. [d]SE±0.2 [e]no significant amount detected.

**Figure 19: Concentration of total tocopherol in browse leaf tissue during the growing season.**



Tocopherol content varies in different tissues (Hess, 1993). In general, alpha-tocopherol is the predominant form in photosynthetic tissues while gamma-tocopherol is more often associated with seeds and fruits (Franzen *et al.*,1991; Molina-Torres and Martinez, 1991). The observation that alpha-tocopherol is the predominant form of tocopherol present in the leaf extracts of the browse species in this study is consistent with other research on samples that are primarily leaf tissue.

Considering the individual forms of tocopherol, the change in concentration during the growing season was significant for alpha-tocopherol ($P<0.001$), gamma-tocopherol ($P<0.05$) and delta-tocopherol ($P<0.001$; only in multiflora rose) but not for beta-tocopherol ($P>0.1$). The changes in concentration for each form of tocopherol in browse leaf tissue during the growing season are reported in Figures 20-23.

**Figure 20. Concentration of alpha-tocopherol in browse leaf tissue during the growing season**

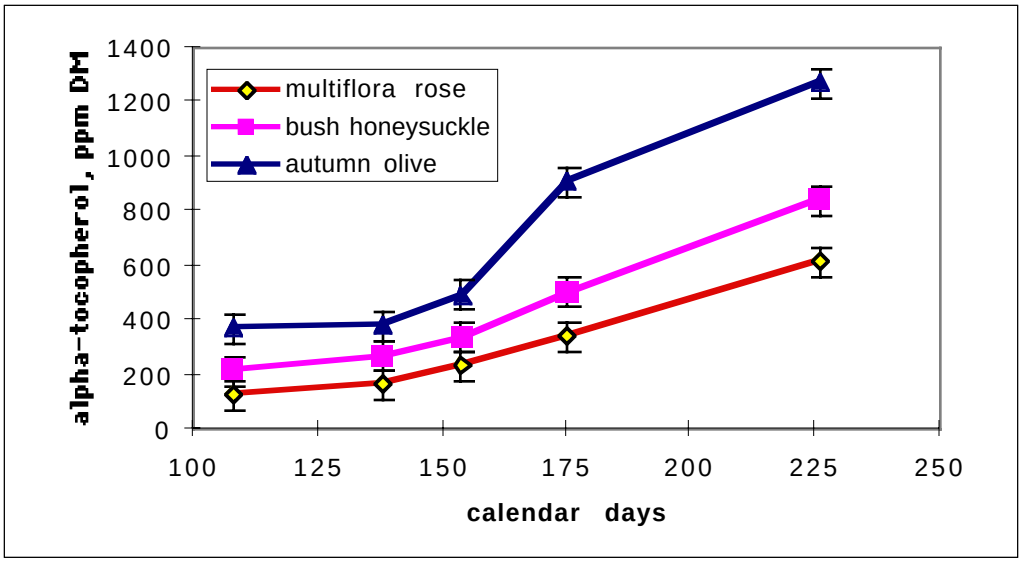


**Figure 21. Concentration of beta-tocopherol in browse leaf tissue during the growing season**

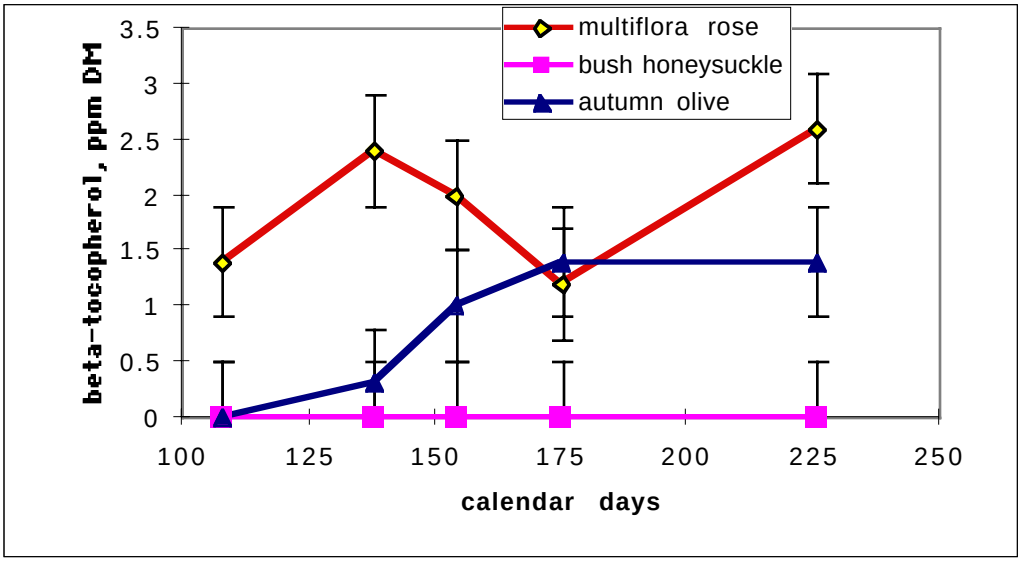



**Figure 22. Concentration of gamma-tocopherol in browse leaf tissue during the growing season**

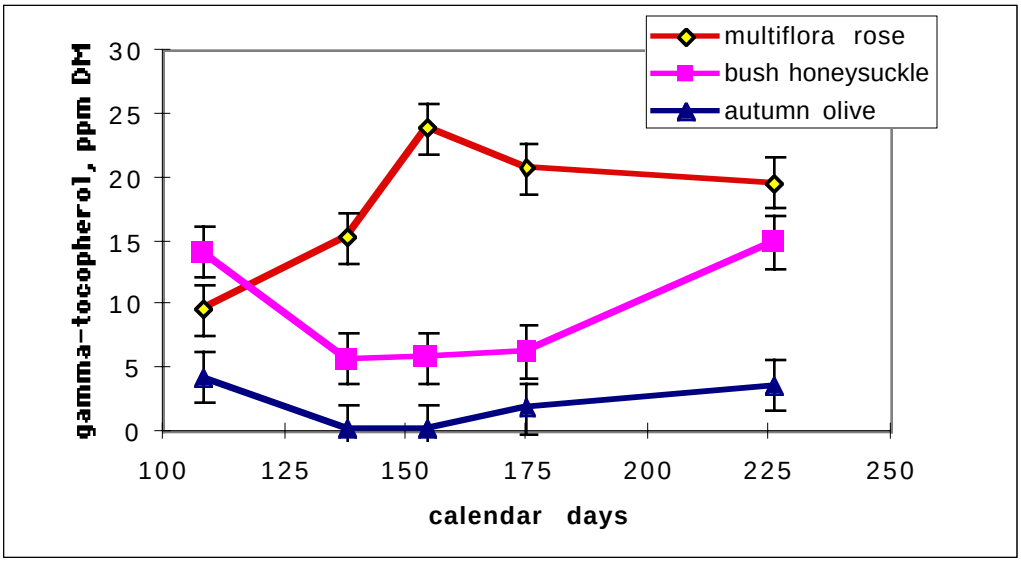
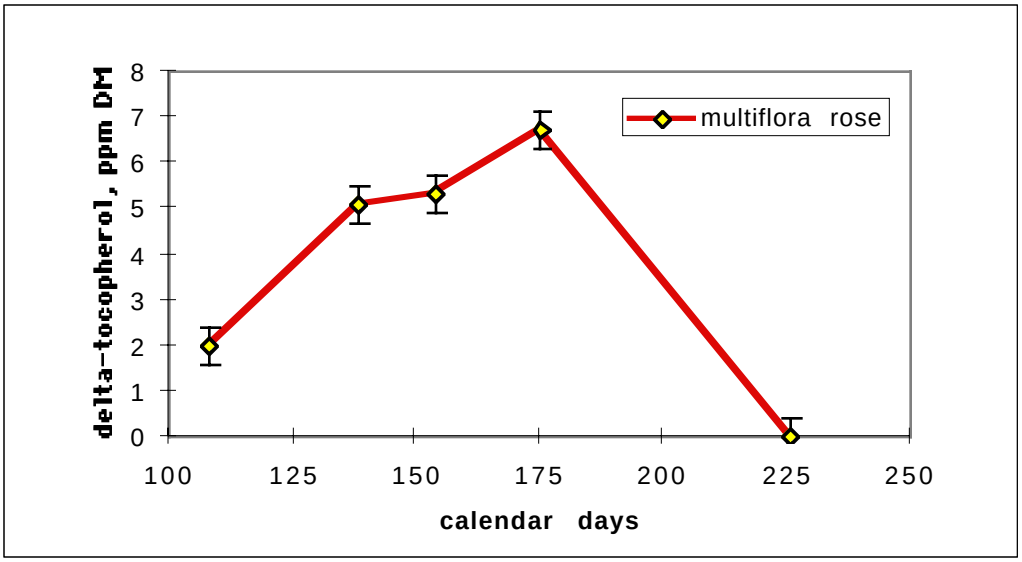


**Figure 23. Concentration of delta-tocopherol in multiflora rose leaf tissue during the growing season[a]**




[a]delta-tocopherol was not detected at any time in autumn olive or bush honeysuckle

In this study, beta-tocopherol (Figure 20) in multiflora rose doubled from the fourth to the fifth sampling date while remaining unchanged in autumn olive; gamma-tocopherol levels in multiflora rose and bush honeysuckle increased then declined (Figure 22). The presence and fluctuations of beta-tocopherol and gamma-tocopherol during the season may reflect the presence of fruit tissue in the sample. Although the browse samples contained primarily leaf tissue, flowers and fruits were occasionally present. Fruits set and matured during the sampling interval.

Fluctuations in tocopherol content similar to the fluctuations observed in this study have been reported during fruit ripening in several species. In ripening tomatoes, the level of beta-tocopherol doubled as the fruit matured from green to red, while gamma-tocopherol increased and then began to decline (Abushita *et al*, 1997). Likewise in New Mexican-type chiles gamma-tocopherol increased and then declined (Osuna-Garcia *et al.*, 1998). The absolute quantity of fruits present in our tissue samples is not known; however, gamma- and beta-tocopherol present in the fruit may contribute to the low levels detected and to the

seasonal fluctations. In the case of multiflora rose, support for this argument comes from an examination of rose hip (*Rosa canina*) seed oils by Zlatanov (1999) who found gamma-tocopherol to be predominant.

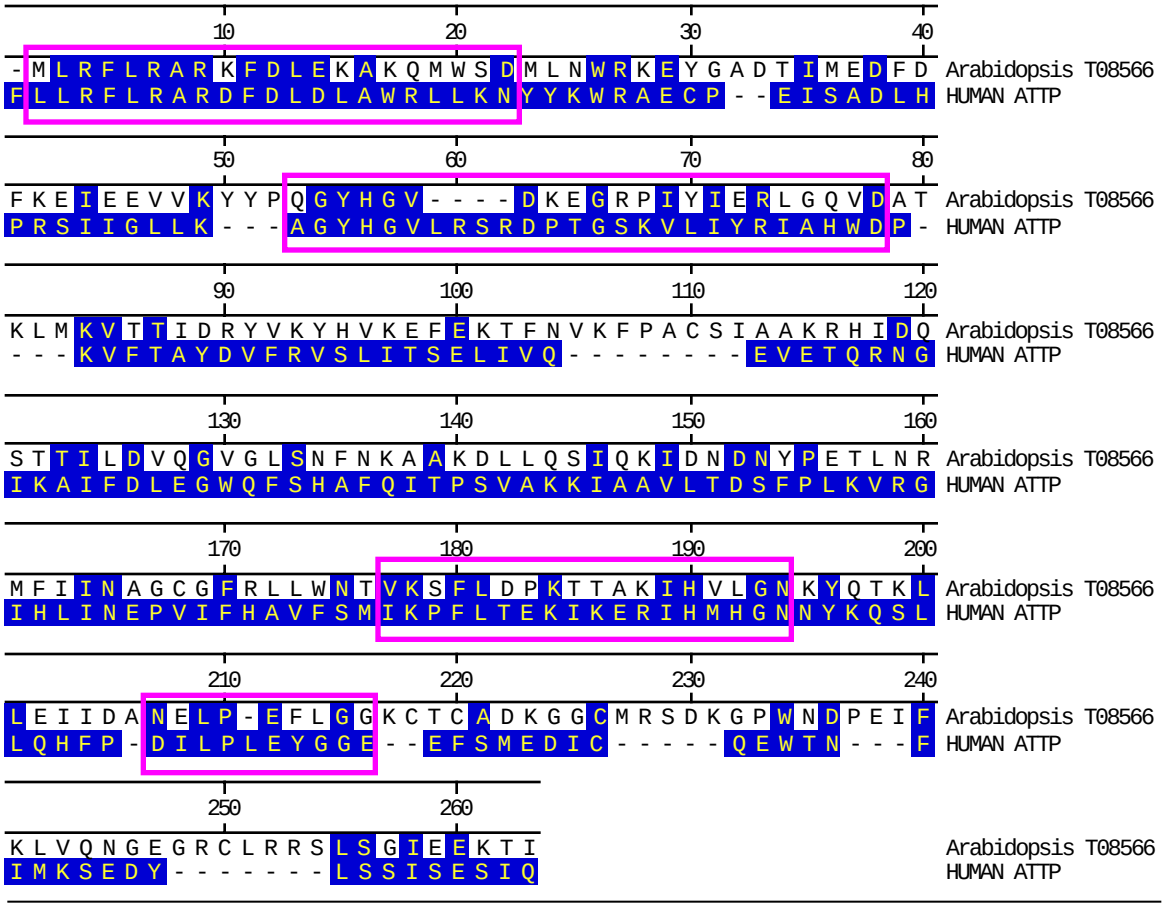
The "free radical theory of aging" predicts an age-related increase in free radicals resulting in increased cell damage in plant and animal cells (Harman, 1981; Leshem, 1988). Age-related increases in vitamin E in the leaf may help counter increases in the production of free radicals in older tissues (Kunert and Ederer, 1985). The observed increase in alpha-tocopherol content with maturity agrees with previous research on annual, herbaceous crops (Tramontano *et al.*, 1992) and in tree crops (Kunert and Ederer, 1985). Using evergreen spruce species, Franzen and collegues (1991) studied the accumulation of tocopherols in leaf tissue over several years. They documented an increase that is asymptotic, leveling off at approximately 2 years. These observations suggest a limited capacity to acquire tocopherols or the fulfillment of the physiological need for tocopherols. Franzen and collegues (1991) speculated that once a critical level of tocopherol is reached further incorportation of alpha-tocopherol will destabilize the membrane. No leveling off was observed in the deciduous browse species examined in this study. Even after a 3-fold increase in tocopherol concentration, the total tocopherol levels in the browse leaf tissue continued to increase indicating that no such critical level was reached.

If a substantial sink for tocopherols existed outside of the thylakoid membrane, then the accumulation would not be limited by the capacity of the membrane to absorb tocopherols. Such a sink in leaves could exist in plastoglobuli as proposed by Lichtenthaler and Becker (1971), in the epiculticular wax (Shewmaker, 1999). Lichtenthaler (1968) observed an age-related increase in the size and number of plastoglobuli, which correlates with tocopherol increases. A mechanism for tocopherol transport has not been identified in plants.

To investigate if any protein sequences in plants share characteristics with known, mammalian tocopherol transport proteins (Sato, 1993) a genomic search was conducted.

Several hypothetical proteins with no known function were identified in *Arabidopsis thaliana*. The protein with the greatest similarity to the known tocopherol binding proteins, Accn: T08566, had significant similarity with 3 of 5 regions that are characteristic of the superfamily of proteins that includes tocopherol binding proteins.

**Figure 24. Sequence alignment of *Arabidopsis thaliana* Accn: T08566 and human αTTP[a]**




[a]Alignments of T08566 and αTTP (Accn: D49488) by Clustal Method. Blue shade indicates amino acid similarity with in 1 distance unit. Blastp search indicates 45% similiarity between the two aligned sequences. Fuschia boxes indicated signature regions for the cellular retinaldehyde binding protein superfamily.

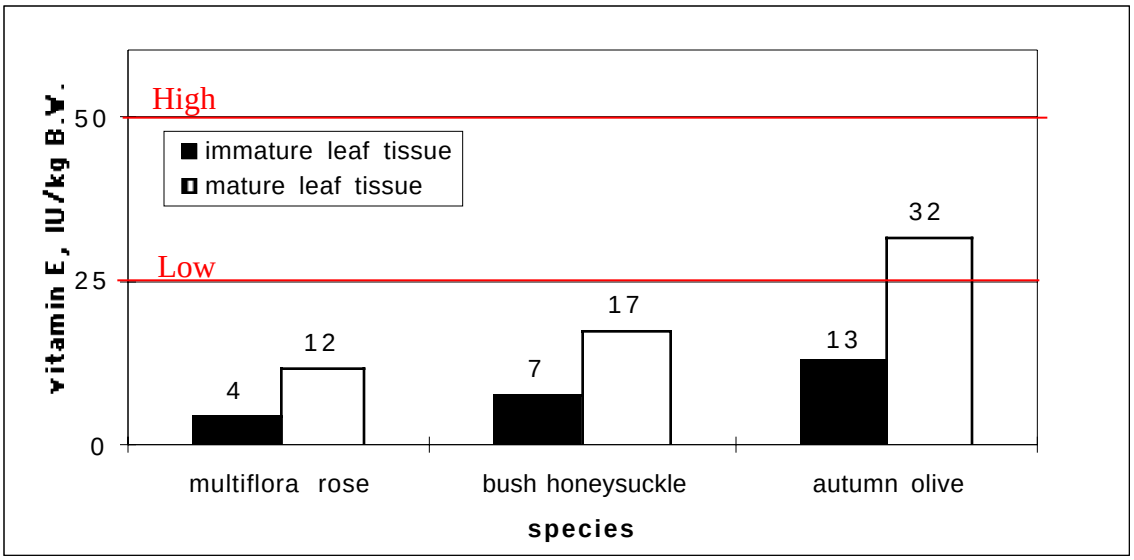
Drought stress can lead to oxidative stress in plants, and increases in total tocopherols from 2- to 15-fold have been reported (Bartoli *et al.*, 1999; Munne-Bosch *et al.*, 1999) and correlate with increased drought tolerance (Price and Hendry, 1989). During the summer of 1999, a drought occurred, which could have affected tocopherol concentrations. For all of the summer months, rainfall was well below the 30-year average, and soil moisture levels were below normal. However, due to the topography of the Hurricane Ridge site certain plots would have experienced the drought more severely than other plots. Although no precise measures of soil moisture were made, it is likely that plot A, at the bottom of the watershed beside the pond, was less affected by the drought than plots located at the top of the watershed, such as plot G (Figure 11). No effect ($P>0.1$) of plot location was observed in the ANOVA analysis nor in a T-test comparing the seasonal mean tocopherol levels in plant from plot A and plot G. These statistical results do not indicate that the drought was not a factor in the increasing tocopherol levels. It is possible that the plant-to-plant variation in tocopherol levels is greater than any increase caused by the drought.

Vitamin E is just one component of an integrated system of antioxidant defenses within the leaf. The effectiveness of the antioxidant system against drought or age-related processes may depend on the interactions among the various antioxidants. For example, Kunert and Ederer (1985) have examined the sensitivity to age-related lipid peroxidation in the context of vitamin C/vitamin E ratios. In their study of fir and beech leaves, Vitamin E increased with maturity while vitamin C decreased in. As the ratio of vitamin C/E dropped from greater than 10:1 to 1:1 the effectiveness of the vitamin C/vitamin E antioxidant system decreased, leading ultimately to cell death. The changes in antioxidants other than vitamin E were not examined in the current study. Further study on the interactions among vitamin E and the other antioxidants may help to elucidate the factors regulating vitamin E concentrations in browse.

## Implications of Vitamin E in Invasive Shrubs for Goat Production

In a pasture-finished goat production system, it is important to know the amount and form of tocopherol available in browse in order to determine the proper level of vitamin E supplementation. The recommended level of supplementation for non-nursing goats is between 25-50 IU/kg B.W. (Sherman and Smith, 1994). The amount of vitamin E that could be provided by a diet of 50% young or mature leaf tissue from multiflora rose, autumn olive, and bush honeysuckle is reported in Figure 25. Calculations were based on a 30kg yearling goat consuming 1.4 kg dry matter per day, 50% of which was derived from the invasive browse species (NRC, 1981). At this level of intake, goats browsing mature autumn olive may obtain adequate vitamin E through the diet. Substantial amounts of vitamin E may also be obtained from young autumn olive as well as mature Morrow's honeysuckle. Hence, goats browsing on these species would have a decreased need for supplemental vitamin E than goats that do not have access to fresh browse.

**Figure 25. Contirbution of browse as a source of dietary vitamin E[a]**




[a]Calculated values for intake are based on a 30 kg yearling goat with a 1.4 kg daily DM intake of which 50% is derived from browse. Red Gridlines indicate the high and low recommended daily intake of vitamin E. Immature leaf tissue from 4/16/99 harvest date; mature tissue from 6/24/99 harvest date.

Many common livestock feeds are poor sources of vitamin E (Lynch, 1991). For example, cereal grains often contain predominately the gamma-tocopherol predominately (Hakkarainen and Pehrson, 1987), but this form of tocopherol has only 10% of the biological activity of alpha-tocopherol (Table 1). In forage grasses, the tocopherol levels decrease on a whole plant basis with maturity (Robowskey and Knabe, 1970). Moreover, hay making and ensiling can have drastic effects on the level of tocopherols present in the feed. Due to the conditions necessary for production of these feeds, the tocopherol levels can be reduced by 30 to 80% (Lynch, 1991). Further losses in tocopherol can occur during prolonged storage of feed (Kivimae and Carpena, 1973). Therefore, fresh herbage from the species of browse studied, which contain greater levels of the most biologically active form of vitamin E, may be more valuable as a source of vitamin E than other common livestock feeds.

Vitamin E is only one component of animal nutrition. Other aspects that influence animal performance or feeding behavior, such as nutritive value or concentrations of antiquality factors, must be considered. High nutritive value and/or low concentrations of antiquality factors may not coincide with high levels of vitamin E. Hence, it is not possible to predict animal performance based solely on vitamin E concentrations in browse. Turner and Foster (in press) performed a preliminary analysis of the nutritive value and *in vitro* organic matter disappearance (IVOMD), a measure of the digestibility, on multiflora rose, Morrow's honeysuckle, and autumn olive. The results, summarized in Table 8, indicate that at certain times during the growing season the browse species can have levels of crude protein in the same range as other common livestock feeds. Like most forage crops, protein levels decreased in all species as tissue matured.

Autumn olive, which showed good levels of both crude protein and vitamin E throughout the growing season, may supply both adequate nutrition and adequate vitamin E. However, the IVOMD of autumn olive was slightly less than for the other species which may limit vitamin E uptake. Since goats have a higher rate of passage of forage

through the rumen (Luginbulh, 1995), they may not sufficiently digest autumn olive or the more mature leaves of other species to take advantage of the high levels of vitamin E.

Goats may select the youngest tissues that provide more digestible, higher quality forage. If goats preferentially browse on young multiflora rose or young honeysuckle, then the animals may need supplemental vitamin E. Further study of the goat's preference for each of these browse species is necessary before conclusions can be drawn regarding the actual intake of vitamin E and the requirements for supplementation.

**Table 8. Nutritive value of browse species**

| Item, | autumn olive | multiflora rose | bush honeysuckle | Alfalfa pasture[1] |
|---|---|---|---|---|
| Crude Protein[2], % | 37-22 | 23-13 | 24-15 | 22-14 |
| NDF[3], % | 33.2 | 27.7 | 26.5 | 44 |
| ADF[3], % | 20.0 | 17.5 | 18.0 | 32 |

[1]Included for comparision, sources: Ball *et al.*, 1996. [2]Range from young to mature plant tissue (Turner and Foster, in press) [3] NDF measures the total carbohydrates present , while ADF indicates amount of the total carbohydrate which is less digestible cellulose, lignin, and cutin.

In summary, autumn olive had the highest concentration of total tocopherols in leaf tissue followed by Morrow's honeysuckle, followed by multiflora rose. Alpha-tocopherol predominated in all species and increased during the growing season. Mature autumn olive and bush honeysuckle shrubs may provide adequate vitamin E in a low-input pasture-finished goat production system and reduce the need for supplemental vitamin E.

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# Chapter 7: Vita for Gabriel Wilmoth

**Date of Birth:** August 11, 1976

**Education:**

| | |
|---|---|
| Jan. 1999-May 2000 | M.S. Biochemistry, Virginia Polytechnic Institute and State University |
| Aug. 1994-May 1998 | B.S. Biochemistry, Virginia Polytechnic Institute and State University |
| July-Aug. 1996 | Field study in tropical rainforest ecology, La Suerte Biological Station, Costa Rica. |
| July 1999 | Field study in ecology of Southwestern arid grazing lands, Texas Tech |

**Research:**

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|---|---|
| Jan.-Dec. 1997 | Undergraduate research, Virginia Polytechnic Institute<br>Advisor: Malcolm Potts<br>Objective: clone SOD gene from *Nostoc commune* |
| May-Nov. 1998 | Agriculture research, Rodale Institute Experimental Farm<br>Advisor: Peggy Wagoner<br>Objective: evaluate organic production methods for food grade soybeans grown in Pennsylvania |
| June-Aug. 1999 | Graduate research, USDA, ARS, Appalachian Farming Systems Research Center<br>Advisor: Joyce Foster<br>Objective: develop method for extraction and quantification of tocopherols |

**Posters:**

Wilmoth, G.; Foster, J.; Hess, J. Tocopherol (vitamin E) content in several shrubs common to browse dominated plant communities in Southern West Virginia. Presented at the VAS meeting in Radford, VA. **2000.**

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**Publications:**

Wilmoth, G.; Foster, J., Hess, J. Tocopherol (vitamin E) content in three invasive browse species on underutilized Appalachian farmland. *Proceedings of the American Forage and Grassland Council.* **2000,** *9.*

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