Alpha tocopherol

stability and availability in swine tissue

by

Richard Lincoln Fredrickson Jr.

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Pathology
Interdepartmental Major: Toxicology

Iowa State University
Ames, Iowa

1990

TABLE OF CONTENTS

INTRODUCTION

Vast quantities of information are becoming available on the beneficial properties of vitamin E. Vitamin E is a member of the fat soluble vitamins and is important in preventing specific clinical syndromes. The quantitation of vitamin E concentration is important in the diagnosis of deficiency and assessment of response and therapy. Complications between sampling and analysis can result in erroneous concentrations reported. The primary area of interest is concerned with the antioxidant properties of vitamin E. Literature currently available on vitamin E provides limited information on stability, and its effect on mycotoxins.

This study was divided into three phases. Phase one involved testing different extraction methods found in the literature, and comparing them to the method used at the Iowa State Veterinary Diagnostic Laboratory.

The objective of phase two was to evaluate the stability of alpha tocopherol in serum and tissue samples, as affected by time, exposure to light, temperature variations, and autolysis.

The final phase of the project was to determine the effects of injectable alpha tocopherol (vitamin E) when aflatoxin was fed to swine at levels of 400 micrograms/kilogram (ppb) during a five week period.

Weekly feed efficiency, liver enzymes, and serum alpha tocopherol concentrations were analyzed. At the conclusion of this phase, alpha tocopherol concentrations of serum, heart, and liver tissues were determined. Histopathologic examination of all major tissues was performed.

The purpose of this study was to first produce an extraction method that would quantitate the concentration of alpha tocopherol in serum and tissue samples. This extraction technique should be rapid, straight forward, reproducible, and efficient. Secondly, the study was designed to determine the stability of alpha tocopherol in tissue and serum samples when subjected to conditions that simulate field and laboratory conditions. Finally this research involved feeding aflatoxin at 400 micrograms/kilogram to 10 kilogram pigs. Alpha tocopherol was administered parenterally to determine its effects on liver and serum concentrations in vivo. The effect of alpha tocopherol on weight gain, feed consumption, and feed efficiency was also examined.

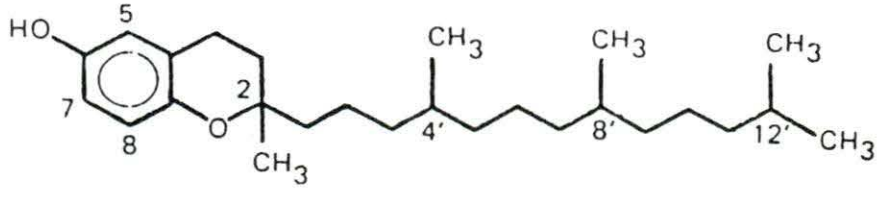
A decrease of alpha tocopherol activity is reported to occur between the time of sampling and when the analysis is performed if proper storage conditions are not maintained. This loss of alpha tocopherol leads to erroneous concentrations reported, and provides false

information for the samples tested. Therefore, the results of this study may be used as a basis for interpretation of alpha tocopherol concentration dependant on the transport and storage technique. The information from this study can be used as background for further research on alpha tocopherol extraction on tissue and serum samples.

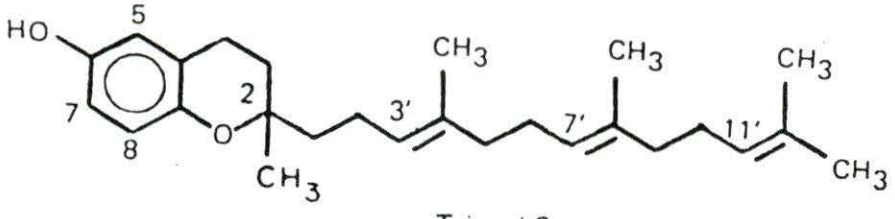
LITERATURE REVIEW

General review of vitamin E

Vitamin E is one of the fat soluble vitamins, and is a generic term for its components; tocopherols and tocotrienols (Diplock, 1985). Currently eight compounds have been isolated having vitamin E activity. Each compound has a 6-chromonal ring with corresponding side chain (Machlin, 1984). The tocotrienols have 3 double bonds (unsaturated) located at 3', 7', and 11' positions, while the tocopherols have a saturated side chain. There are four isomers of both the tocopherols and tocotrienols. Each group contains an alpha, beta, delta, and gamma isomer (Bieber-Wlaschny, 1987). Alpha tocopherol is the most active isomer of vitamin E (Machlin, 1984).



Tocol Structure



Trienol Structure

Figure 1. Chemical structures of tocopherol and tocotrienol.

Two principal sources of vitamin E currently used are RRR-alpha-tocopherol and all-rac-alpha-tocopherol. The all-rac-alpha-tocopherol is synthetic, produced as an acetate ester, and has 60% of the activity of RRR-alpha-tocopherol (natural tocopherol) (Diplock, 1985 and Ewan, 1987). The acetate esters are more stable than natural tocopherol when subjected to oxidation. The RRR-alpha-tocopherol is produced by molecular distillation and methylation of natural sources such as plant oils and whole cereal grains. Any modification of the tocopherol or tocotrienol structure can result in loss of biological activity.

Each isomer of tocopherol has its own individual level of activity. RRR-alpha-tocopherol (d-alpha-tocopherol) has 1.49 International units/mg and all-rac-alpha tocopherol (dl-alpha-tocopherol acetate) has 1 International unit/mg. Their racemic counterparts, dl-alpha-tocopherol and d-alpha-tocopherol acetate, have activity concentrations of 1.1 and 1.36 International units/mg respectively (Roche, 1987b).

Alpha tocopherol is insoluble in water, but completely soluble in fats, oils, acetone, alcohol, chloroform, and other fat solvents (Machlin, 1984). It is a colorless to yellow viscous material, has a boiling point at 200-220 degrees, and a molecular weight of 430.69. Alpha tocopherol remains stabile in heat and alkali materials in the absence of oxygen. They are also unaffected by acids up to 100

degrees centigrade, but alpha tocopherol can be oxidized by atmospheric oxygen. When in the presence of oxygen and light, heat, or alkaline material, the oxidation process is accelerated (Machlin, 1984).

The importance of vitamin E is its in vivo antioxidant properties (Bieber-Wlaschny, 1987). Vitamin E stabilizes membrane phospholipids to prolong the biologic life of unsaturated fatty acids and prevent formation of free superoxide radicals (Philip and Manz, 1982). Other functions include metabolism of arachidonic acid, assisting in cellular respiration, and promoting certain hormone production (Bieber-Wlaschny, 1987). Increases in humoral response and antibody efficiency are enhanced by vitamin E. The function of phagocytic cells, neutrophils, and macrophages increase by improving neutrophil killing action, increasing chemotaxis and adherence, and enhancing resistance to certain infectious agents (Machlin, 1984; Van Vleet, 1987).

Absorption

The level of vitamin E required by the body is determined in part by the daily intake of polyunsaturated fatty acids in the diet (Tyopponen et al., 1984; Losowsky et al., 1972). Tappel (1980) indicated the approximate molar ratio of unsaturated fatty acids to alpha tocopherol in the membrane is 1000:1. Increasing levels of alpha tocopherol in the diet results in a smaller percentage of dietary alpha

tocopherol absorbed. When the unsaturated fatty acid content of feed increases, intestinal uptake of alpha tocopherol decreases (Tyopponen et al., 1984).

The major pathway for alpha tocopherol absorption is similar to fat, via the lymphatics in the small intestine. Alpha tocopherol absorption is biphasic, with a peak absorption at 4 hours post ingestion, and a larger peak 9 hours after administration (Diplock, 1985). After absorption, alpha tocopherol can circulate in lymph bound to proteins of the very low density lipoprotein (VLDL) fraction. In plasma, there are no specific lipoprotein carriers, and it can be transported on both high density and low density lipoproteins (Machlin, 1984). Losowsky et al. (1972) determined that absorption of alpha tocopherol is not affected by body deficiencies or vehicle of administration. It was noted that alpha tocopherol levels in plasma and livers could not be used as a measure of absorption (Losowsky et al., 1972).

Storage

Alpha tocopherol is stored in many tissues in the body. It is concentrated in tissue membrane cell fractions such as the mitochondria and microsomes (Diplock, 1985).

Most tissue serves as storage for alpha tocopherol, however, not all stored alpha tocopherol is accessible for use. The main storage site of retrievable alpha tocopherol

is the liver (Jensen et al., 1988; Gallo-Torres et al., 1970). Other major storage sites are serum, kidneys, adrenals, muscle (both skeletal and cardiac) and adipose tissue. Past research estimates that 1% of the body alpha tocopherol is located in serum (Bieri et al., 1979; Horwitt et al., 1972). Hidiroglou et al. (1988) reported liver and adrenal glands of beef cattle had the highest concentrations of alpha tocopherol, while skeletal muscle and thyroid tissue had the lowest. Adipose tissue was not analyzed in this experiment.

Machlin et al. (1979) and Horwitt et al. (1972) reported adipose tissue as a major depot for alpha tocopherol, but the release and accessibility from adipose tissue is poor. When guinea pigs were fed diets deficient in vitamin E, the half life in cardiac muscle was 3-4 weeks, fat was longer than 8 weeks, and liver produced a biphasic loss (Machlin et al., 1979). The first phase (mobile phase) in liver, lasted 5-6 days, while the second phase (fixed phase) was greater than seven weeks in length (Machlin et al., 1979). It was determined that the release of alpha tocopherol from adipose stores was not sufficient to maintain plasma or muscle levels.

Metabolism

The most common form of alpha tocopherol metabolism is oxidation (Machlin, 1984). Gallo-Torres et al. (1970)

reported the adrenal glands could play an important role in alpha tocopherol metabolism. After feeding, the most abundant compound appearing in the adrenals was free alpha tocopherol, which increased with time (Gallo-Torres et al., 1970).

Excretion

Excretion of alpha tocopherol is primarily by the fecal route. Less than 1% of ingested alpha tocopherol is excreted in urine. Alpha and gamma tocopherol are also secreted in milk. Gamma tocopherol in milk has 10-37% of the activity of alpha tocopherol and potentially could assist in antioxidant activities in human milk (Lammi-Keefe, 1986). Delta tocopherol and beta tocopherol are present only in trace amounts (Lammi-Keefe, 1986).

Stability

Alpha tocopherol stability is environmentally dependent. Exposure to oxygen, heat, or alkali will produce oxidation of alpha tocopherol. In feedstuffs, the natural alpha tocopherol concentration depends on length of storage time, presence of trace minerals, levels of unsaturated fats, presence of mold, and treatments during curing and storage (Reese, 1987).

Deficiency

Vitamin E deficiency has been associated with many clinical presentations and syndromes. Deficiencies can be

caused by minimal amounts of the vitamin itself, high levels of unsaturated fats in the diet, or diets low in sulfur containing amino acids or protein (Van Vleet, 1987).

Vitamin E deficiency in swine can cause hepatosis dietetica, white muscle disease or muscular dystrophy, mulberry heart disease, or microangiopathy (Jensen et al., 1988). To prevent these, producers supplement swine with vitamin E at 10,000-20,000 International units/ton in their diet (Van Vleet and Kennedy, 1987).

In cattle and sheep, the major deficiency syndrome is white muscle disease or muscular dystrophy. This disease can occur in animals of all ages, however, primarily young growing animals are affected (Van Vleet and Kennedy, 1987).

In horses, equine degenerative myeloencephalopathy (EDM) is a diffuse degenerative lesion of the spinal cord and brain stem seen when serum alpha tocopherol concentrations decreased to 0.6-0.8 ug/ml or less (Craig et al., 1989).

Preservation of pork and poultry products used for human consumption can be a problem in meat from vitamin E deficient animals. Low dietary vitamin E concentrations allow for peroxidation of lipids which cause damage to cellular membranes (Adams, 1989). This results in rapid deterioration of packaged products. Dietary vitamin E levels of 200 International units/head/day given one month prior to slaughter improved the shelf life and quality of products.

When vitamin E was supplemented in swine for short periods of time the flavor of bacon, fresh and frozen backfat, and lard was improved (Adams, 1989).

Abetalipoproteinaemia, fat malabsorption syndromes, retrolental fibroplasia, and intravascular hemorrhages have been associated with vitamin E deficiency in humans (Diplock, 1985). Other problems associated with deficiencies are fetal resorption and testicular atrophy in rats, and encephalomalacia and exudative diathesis in chickens (Machlin, 1984).

Analysis

With more information on the importance of alpha tocopherol in nutrition and disease, a valid, rapid, and repeatable procedure for quantitation of vitamin E in serum, tissues, and feed is needed.

There has been great concern over the variability in alpha tocopherol concentrations measured in the same animal, as well as between testing periods and testing laboratories. Alpha tocopherol levels in serum can fluctuate greatly when compared to tissue levels. For example, Craig et al. (1989) indicates that a single analysis of alpha tocopherol is not a satisfactory indicator of an animals status, and it has been shown that alpha tocopherol concentration can vary up to 20% within a short period of time.

Recently there have been great progress in analytical

techniques for alpha tocopherol. Currently two forms of alpha tocopherol analysis are used. First are the bioassays which monitor the biological activity of alpha tocopherol to reverse or alter symptoms incurred from alpha tocopherol deficiencies. Fetal resorption, red blood cell hemolysis, muscular dystrophy and muscle degeneration, encephalomalacia, and liver storage tests are the most common forms of bioassays (Parrish, 1980). The second form of analysis is by physical-chemical analysis. The Emmerie-Engel reaction is a spectrophotometric procedure that results in a complex formed between ferrous ions and dipyridyl after reduction of ferric ions to ferrous ions by alpha tocopherol (Machlin, 1984; Diplock, 1985). This complex turns red to purple in color, and can be measured colorimetrically. One disadvantage of this procedure is that ferric chloride can be reduced photochemically.

Thin layer chromatography is another form of purification and quantitation. Two problems that occur with thin layer chromatography are decreased sensitivity in high ambient humidity, and destruction of alpha tocopherol by ultraviolet light (AOAC Manual, 1980). Gas chromatography utilizing argon or hydrogen flame ionization has also been utilized. This technique has the advantage of identifying free and protein bound alpha tocopherol, and can separate sterols from fat soluble vitamins (Parrish, 1980).

Disadvantages are the need for pure extracts, cost of equipment, temperature, gas flow rate, and problems with column overloading (Parrish, 1980).

Current emphasis is towards the use of high performance liquid chromatography (HPLC) for identification. Reverse and normal phase systems are available for use, with reverse phase used most often (Parrish, 1980). High performance liquid systems can be coupled with ultraviolet, fluorescent, and photodiode array detectors. Fluorescent detectors have an advantage over ultraviolet detectors because many impurities do not fluoresce, thus reducing interference with detection.

Extraction, purification, and detection are the three basic principles that are important when performing an analysis. There have been many different extraction techniques attempted. The matrix in which alpha tocopherol is extracted dictates the technique selected (Parrish, 1980). Feed and tissue samples use either direct extraction or a saponification technique, while plasma and serum use direct extraction techniques.

Saponification involves degradation of tissue with the strong alkali potassium hydroxide and heat (AOAC Methods, 1980; Machlin et al., 1979; Diplock et al., 1966). This procedure produces loss of alpha tocopherol from oxidation, if an antioxidant such as ascorbic acid or pyrogallol is not

included (Diplock et al., 1966). Modifications of saponification have been attempted (including variations of temperature, alkali concentration, exposure time, and extraction solvent) without much success. Even in tissue samples containing small amounts of fat, the antioxidant pyrogallol could not prevent loss of alpha tocopherol (Diplock et al., 1966). The purpose of saponification is to digest tissue or feed in order to allow easier extraction of alpha tocopherol. When saponification was used on fatty samples, it did not appear to affect the cholesterol levels recovered, but did result in loss of alpha tocopherol (Greenspan et al., 1988).

Current emphasis is on simple extraction with solvents such as hexane, heptane, methylene chloride, acetone, and petroleum ether. Direct extraction decreases the amount of time required for analysis, and reduces the loss of alpha tocopherol during the procedure. Many variations of this technique are currently employed and include: variations in extraction solvents, solvents used in mobile phase, and type of detector used. Variations in the extraction procedure have been summarized in Table 1. Mostow et al. (1985) performed homogenization of rat liver in acetone to extract alpha tocopherol. He compared acetone, ethanol, and hexane/water as extraction solvents. In this study, acetone recovered 31% more alpha tocopherol than the other solvents.

Table 1. Comparison of HPLC analytical techniques used in the extraction of alpha tocopherol from tissue and serum summarized from the literature

Author	Type	Extraction	Mobile Phase
Greenspan et al. 1988	Homogenized	Methanol Chloroform	Hexane
Miller and Yang 1985	Vortex	Hexane	Methanol Acetonitrile
Hidiroglou et al. 1988	Homogenized	Hexane	Methanol Water
Bieri et al. 1979	Vortex	Hexane	Methanol Water
Chou et al. 1985	Vortex	Heptane	Methanol
Cohen and LaPointe 1980	Wrist Shaking	Dichloro-methane	3-Way[a]
Cort et al. 1983	Shaking	Pet. Ether	Isooctane
McMurray et al. 1980	Shaking	Ethyl Ether	Methanol Water
Mostow et al. 1985	Homogenized	Acetone	Methanol Water
Biesalski et al. 1986	Shaken	Hexane	Hexane
Catignani 1986	Vortex	Hexane	Methanol Water
Craig et al. 1989	Vortex	Acetonitrile	Methanol
Mino et al. 1977	Homogenized	Ethyl Ether	Ether/Hexane

[a] Hexane-Dichloromethane-Isopropanol.

Another study conducted by Zaspel and Csallany (1983) homogenized frozen tissue in the extraction solvent to facilitate removal of alpha tocopherol. They compared acetone, hexane, and methanol/chloroform to determine their extracting abilities. Acetone recovered 25% more alpha tocopherol than hexane, and 15% more than chloroform/methanol. Huang et al. (1986) used ethanol and hexane to extract alpha tocopherol from plasma samples, using HPLC with an ultraviolet detector. Catignani (1986) used hexane extraction to detect alpha tocopherol from plasma and serum. To facilitate extraction, he vortexed vigorously to allow better contact of the extraction solvent to help remove alpha tocopherol. Biesalski et al. (1986) used a similar technique as Catignani (1986), but they included ethanol to denature specific binding proteins.

In summary, extraction techniques for alpha tocopherol are quite varied with each researcher, or group of researchers attempting to produce their own technique. Many attempts have been made to duplicate some of these procedures with inconsistent results. Currently, there is no one technique that has been standardized and implemented on a widespread basis. The only point that seems to be in agreement is that high performance liquid chromatography is the instrument of choice for analysis. The methods of extraction to prepare a sample for analysis, however, are

many and varied.

There is need for more research in this area to produce a quick, rapid, and repeatable procedure which can be universally used.

SECTION I. COMPARISON OF ALPHA TOCOPHEROL EXTRACTION TECHNIQUES

INTRODUCTION

Phase one of this project involved evaluation of various analytical techniques to determine the alpha tocopherol concentration in serum and tissue samples. Alpha tocopherol levels can be determined using various techniques (Table 1). Gas chromatography, thin layer chromatography, high performance liquid chromatography, colorimetry, and bioassays are a few of the methods used (Machlin, 1984; Diplock, 1985). These methods have been used separately, in combinations, and combined with various forms of extraction and purification techniques to determine the alpha tocopherol content of samples.

The current area of interest in alpha tocopherol quantitation is using high performance liquid chromatography (HPLC) for separation, coupled with an ultraviolet, fluorescent, or photodiode array detector. To prepare samples, researchers have attempted different techniques to facilitate extraction and purification of alpha tocopherol. Some found success using saponification of tissues and feeds with the strong base potassium hydroxide (Diplock, 1985; Cohen and LaPointe, 1980; Roche, 1987a). Others have used direct extraction with solvents such as acetone, hexane, heptane, methylene chloride, or petroleum ether (Mino et al., 1977; Chou et al., 1985; Bieri et al., 1979).

MATERIALS

Chemicals

All chemicals used in this study were purchased from Fisher Scientific, Pittsburgh, Pennsylvania (U.S.A.). These chemicals include; reagent grade hexane, methylene chloride, acetone, petroleum ether, and HPLC grade chloroform and methanol.

Standard

The alpha tocopherol standard was obtained from Fluka Biochemika, Sweden. The standard was labeled as greater than 98% pure according to HPLC analysis.

Instrumentation

A Waters 600A solvent pump[1] was coupled with a Micrometrics Chromonitor 785 variable ultraviolet detector.[2] A Shimadzu SIL 9A autosampler[3] allowed analysis of multiple samples; and a Shimadzu CR501 integrator[3] was utilized to record the data.

The mobile phase was mixed in two steps. Step one was a 90:10 ratio of methanol and chloroform. Step two involved mixing the methanol-chloroform mixture with deionized water at the ratio of 95:5.

---

[1]Waters Associates Inc., Milford, MA. 01757.

[2]Micrometrics Instrument Corp., Norcross, GA 30071.

[3]Shimadzu Corp., Analytical Division, Kyoto, Japan.

METHODS

Swine liver samples for the extraction comparisons were collected from the Iowa State University Meat laboratory. Immediately after slaughter, samples were placed in clean plastic bags, and transported packed in ice. The samples were divided so analysis could be performed on uniform samples. The samples not used for immediate analysis were frozen at -70 celsius. The following are brief descriptions of each extraction technique used in the comparison. A summary of these descriptions is listed in Table 2.

Method A was the technique used by the Chemistry Laboratory at Iowa State University Veterinary Diagnostic Laboratory. Two ten gram samples were accurately weighed and placed in individual 250 ml erlenmeyer flasks. Two grams of celite were placed in each flask, and the flasks covered with aluminum foil to reduce light exposure. Fifty milliliters (ml) of methylene chloride was added to each flask. Next, 100 ul of 0.1 ug/ul (micrograms per microliter) alpha tocopherol was added to one flask as an internal standard. These flasks were covered with aluminum wrapped rubber plugs and agitated using a wrist action shaker for 20 minutes. The extract was suction filtered, and the recovered solvent volume measured. The extract was then concentrated under low heat and a stream of nitrogen. The residue was rinsed with methylene chloride, transferred to a one dram vial, and

Table 2. Summary of alpha tocopherol extraction methods for HPLC

Method	Type	Solvent
A	Direct extraction, wrist action shaking. Celite addition	Methylene chloride
B	Direct extraction, wrist action shaking	Hexane, Heptane Acetone
C	Direct extraction, blending tissue with solvent	Methylene chloride Acetone
D	Saponification, digestion with potassium hydroxide, ascorbic acid, and ethanol	Methylene chloride Hexane
E	Saponification, variations in temperature, KOH and Ascorbic acid concentrations	Hexane, Petroleum Ether
F	Direct extraction Vortex mixing Ethanol Added	Methylene Chloride Petroleum Ether
G	Direct extraction Volume of ethanol 10, 20, and 30 ml compared	Petroleum Ether
H	Comparison of saponification, direct methylene chloride extraction and petroleum ether and ethanol	Petroleum Ether Methylene Chloride Hexane
I	Direct extraction with methylene chloride and petroleum ether with and without ethanol.	Methylene Chloride Petroleum Ether Ethanol

References cited in descriptive section.

concentrated to dryness. The extract was resuspended in one ml of HPLC grade methanol.

Method B is similar to A, but the extraction solvent was changed. Solvents tried were hexane, heptane, and acetone (Biesalski et al., 1986; Miller and Yang, 1985; Bieri et al., 1979; Chou et al., 1985). These solvents were used separately to compare their alpha tocopherol extracting ability.

Method C changed the method of agitation. Wrist action shaking was replaced with more aggressive blending of samples in an explosion proof blender. Each sample was blended with the extraction solvent, to facilitate alpha tocopherol recovery (Mino et al., 1977; Mostow et al., 1985; Zaspel and Csallany, 1983).

Method D involved saponification to assist releasing alpha tocopherol from tissue membranes. Our saponification technique used a strong alkaline solution of potassium hydroxide (KOH), along with ethanol and an antioxidant, to facilitate alpha tocopherol extraction (Cohen and LaPointe, 1980; Roche, 1987a; McMurray et al., 1980). This procedure involved accurately weighing two ten gram samples of tissue in a 250 ml erlenmeyer flasks. To one flask, 100 ul of the alpha tocopherol internal standard was added. Next 3.3 ml of a 25% ascorbic acid solution was added, then 6.6 ml of absolute ethanol. This solution was mixed briefly and then

incubated at 70 degrees celsius for 5 minutes. After incubation, 6.6 ml of 10 N KOH was added slowly. This was saponified for 30 minutes, then placed in a cold water bath. Once cooled, 50 ml of extraction solvent was added and vortexed for 1 minute. The contents were transferred to a centrifuge bottle and centrifuged for 5 minutes. The top solvent layer was removed and concentrated to dryness under nitrogen. The extract was resuspended in one ml of HPLC grade methanol.

Method E involved variations in the quantities of KOH, ascorbic acid, and extraction solvents used. These were altered to determine if they had any effect on recovery.

Method F involved simple extraction with petroleum ether after specific volumes of absolute ethanol were added. Ethanol assists in disruption of the protein membrane, allowing release of alpha tocopherol. Methylene chloride and petroleum ether were compared, with and without the addition of ethanol.

Method G was designed to determine the volume of ethanol required to recover the highest alpha tocopherol concentration. Levels of 10 ml, 20 ml, and 30 ml of ethanol were used on similar tissue samples to allow comparison of recovered alpha tocopherol concentrations.

Method H compared saponification using hexane for extraction (method D), direct methylene chloride extraction

(method A), and direct extraction using petroleum ether and ethanol.

The final comparison method I, involved analyzing direct extraction with methylene chloride and petroleum ether. These were compared with and without the addition of 20 ml of ethanol.

RESULTS

Results from the comparison of different extraction techniques provided useful information. The following are brief descriptions of the results of each comparison.

Methylene chloride was the solvent used in method A, while heptane, hexane, and acetone were used for method B. The data indicate that hexane and heptane extracted the smallest amount of alpha tocopherol. Alpha tocopherol concentration using acetone extraction was significantly greater ($p<0.05$), however, many samples had interfering material obstructing ultraviolet detection (Tables 3 and 4). A purification step would be required to clean the extracts prior to detection, if acetone were used. Methylene chloride provided intermediate concentrations that were significantly higher than hexane ($p<0.05$), but significantly lower than acetone (Tables 3 and 4).

The next comparison was to determine if aggressive agitation liberated more alpha tocopherol than wrist action shaking. Acetone and methylene chloride were chosen as the extraction solvents. Acetone, again extracted contaminants which would not allow quantitation using an ultraviolet detector. The levels recovered in methylene chloride were significantly greater after blending with the extraction solvent, compared to wrist action shaking (Table 5). Because

Table 3. Direct solvent extraction of liver tissue. Solvent comparison for determination of alpha tocopherol concentration in liver tissue samples (micrograms/gram)

Solvent	N	Alpha Tocopherol Concentration Mean +/- Standard Error
Acetone	5	0.48 +/- 0.976*
Methylene Chloride	5	0.23 +/- 0.156[a]
Hexane	5	0.05 +/- 0.005

[a] Significantly different from hexane, $p < 0.05$.

* Significantly different from hexane and methylene chloride, $p < 0.05$.

Table 4. Direct solvent extraction of liver tissue. Comparison of three extraction solvents for determination of alpha tocopherol concentration in liver tissue samples (micrograms/gram)

Solvent	N	Alpha Tocopherol Concentration Mean +/- Standard Error[a]
Acetone	5	No recovery-contaminants
Methylene Chloride	5	0.4 +/- 0.21
Heptane	5	0.18 +/- 0.077

[a]No significant difference.

of the extreme risk of explosion when blending volatile solvents, vortexing provided the same rapid mixing action and a greater margin of safety.

Next was the comparison between methylene chloride and saponification using hexane. Alpha tocopherol concentration from saponification with hexane extraction was not significantly different than extraction with methylene chloride ($p>0.05$) (Table 6). A problem that developed with saponification, was the increased interference from cholesterol. Potassium hydroxide and heat appears to assist liberation of cholesterol from tissue. The cholesterol found in the extraction solvent is eluted from the HPLC column a few seconds after alpha tocopherol. If a large amount of cholesterol was released, it would cover up the alpha tocopherol eluted from the column. Even though saponification values were slightly higher than methylene chloride, recovered levels were less than average alpha tocopherol levels reported for swine liver tissue (Van Vleet, 1987; Bieber-Wlaschny, 1989).

The next area analyzed was the effect of varying concentrations of ascorbic acid and potassium hydroxide on alpha tocopherol recoveries. Ascorbic acid was added at 2x and 4x the original level. No significant change in the extraction occurred when ascorbic acid levels were increased. Increased levels of KOH also produced no significant changes

Table 5. Comparison of alpha tocopherol concentration from two agitation techniques. A comparison of wrist action shaking and blending. Methylene chloride was the extraction solvent used (micrograms/gram)

Agitation Technique	N	Alpha Tocopherol Concentration Mean +/- Standard Error
Blender[a]	3	0.69 +/- 0.18*
Wrist action shaker	3	0.15 +/- 0.05

[a] An explosion proof air driven blender was used.

* Significantly different from wrist action shaking, $p < 0.05$.

Table 6. Saponification with KOH vs. direct extraction with methylene chloride to determine alpha tocopherol concentration in liver tissue (micrograms/gram)

Extraction Technique	N	Alpha Tocopherol Concentration Mean +/- Standard Error[b]
Saponification[a]	5	0.25 +/- 0.10
Methylene Chloride	5	0.16 +/- 0.06

[a] Saponification used hexane as extraction solvent.

[b] No significant difference between methods.

in the recovery level. The last alteration in the saponification process was to compare two different extraction solvents; petroleum ether and hexane. Petroleum ether recovered concentrations of alpha tocopherol significantly higher than hexane (Table 7).

After careful analysis of the different extraction techniques, it was decided to compare the Diagnostic Laboratory analysis (method A) to two other methods. One method was saponification using petroleum ether as the extraction solvent. The other method selected was direct extraction with petroleum ether and ethanol. Saponification and methylene chloride extracted similar alpha tocopherol concentrations, and were not significantly different from each other. Direct extraction with petroleum ether and ethanol resulted in alpha tocopherol concentrations that were significantly higher (Table 8).

The final comparison was designed to determine if there were significant changes when methylene chloride replaced petroleum ether, and if ethanol was an important component in release and extraction of alpha tocopherol from animal tissues. Data indicate that ethanol was important in the extraction process and produced levels significantly higher than straight solvent extraction without ethanol (Table 9). The comparison of extraction solvents indicates that petroleum ether and methylene chloride result in similar

Table 7. Comparison of the extraction solvents petroleum ether and hexane used in saponification, to determine alpha tocopherol concentration in liver tissue (micrograms/gram)

Extraction Solvent	N	Alpha Tocopherol Concentration Mean +/- Standard Error
Petroleum Ether	4	0.23 +/- 0.106*
Hexane	4	0.07 +/- 0.009

* Significantly different from hexane, $p < 0.05$.

Table 8. Comparison of direct extraction using methylene chloride and petroleum ether with ethanol, vs. saponification. Mean values of alpha tocopherol concentration (micrograms/gram)

Treatment	N	Alpha Tocopherol Concentration Mean +/- Standard Deviation
Methylene chloride	10	0.272 +/- 0.091
Petroleum ether/Ethanol	10	0.502* +/- 0.217
Saponification	10	0.277 +/- 0.089

* Significantly different from methylene chloride and saponification, $p < 0.05$.

alpha tocopherol concentrations when ethanol was added (Table 9).

Next, volumes of 10 ml, 20 ml, and 30 ml of ethanol were compared during extraction of tissue samples. Data show that 20 ml and 30 ml volumes of ethanol resulted in similar recoveries and there was no benefit when the larger 30 ml volume was used (Table 10).

Table 9. Means values of alpha tocopherol concentration comparing methylene chloride and petroleum ether, with and without ethanol (micrograms/gram)

Treatment	N	Alpha Tocopherol Concentration Mean +/- Standard Deviation
Methylene chloride	10	0.284 +/- 0.376
Methylene chloride/ethanol	9	0.82 +/- 0.57*
Petroleum ether/ethanol	10	0.91 +/- 0.527[a]
Petroleum ether	6	0.34 +/- 0.17

*[a] There is no significant difference between methylene chloride and petroleum ether when ethanol is added.

*[a] Petroleum ether/ethanol and methylene chloride/ethanol are significantly different from methylene chloride and petroleum ether without ethanol ($p < 0.05$).

Table 10. Mean values of alpha tocopherol concentration resulting from the comparison of ethanol volume effects on alpha tocopherol concentration recovered (micrograms/gram)

Treatment	N	Alpha Tocopherol Concentration Mean +/- Standard Deviation
10 ml ethanol	4	0.81 +/- 0.31
20 ml ethanol	4	1.26 +/- 0.56*
30 ml ethanol	4	1.23 +/- 0.46[a]

[a]* No Significant difference between 20 and 30 ml of ethanol, $p > 0.05$.

* Significantly different from 10 ml of ethanol, $p < 0.05$.

DISCUSSION

Information on the analytical techniques evaluated above was found in the literature except for the direct extraction with petroleum ether and ethanol (Table 1). Currently alpha tocopherol quantitation is performed using HPLC coupled with ultraviolet, fluorescent, or photo-diode array detectors. These systems allow for rapid separation of compounds and are sensitive enough to detect microgram quantities (Huang et al., 1986; Mostow et al., 1985). Another advantage is they can be coupled with autosamplers, permitting many samples to be analyzed in a shorter period of time with less involvement of lab personnel.

Highest alpha tocopherol recovery levels occurred with direct solvent extraction of tissues, after the addition of absolute ethanol. Ethanol assisted the degradation of protein, thereby allowing for more complete extraction of alpha tocopherol (Fabianek et al., 1968). Alpha tocopherol concentrations recovered from swine liver samples using this technique were similar to those reported in the literature and samples sent to other labs for comparative studies (Van Vleet, 1987; Bieber-Wlaschny, 1987; Michigan State University Diagnostic Lab, 1990). Other advantages of the direct extraction technique were minimal contamination and interference when fresh samples were used, and the extraction solvent petroleum ether concentrated rapidly under nitrogen.

Direct extraction with petroleum ether and ethanol consistently produced higher recovery concentrations. Tissue samples spiked with the alpha tocopherol internal standard produced recoveries of 85% and higher, indicating there is little degradation or loss. Another advantage is that one person can extract and analyze one sample in approximately 40 minutes. This provides an advantage over the saponification technique, which requires 2-6 hours depending on technique and procedure followed (Cohen and LaPointe, 1980; Roche, 1987a).

There were many problems associated with other extraction techniques attempted. Saponification was a long, time consuming process, and recovery levels of internal standards were poor. Researchers have reported that saponification can assist the release of alpha tocopherol from tissue, and that it has been used to remove interfering fats prior to analysis (McMurray et al., 1980; Cohen and LaPointe, 1980; Zaspel et al., 1983; Parrish, 1980). Saponification also can result in alpha tocopherol loss from tissues, thus giving poor representation of actual tissue levels (Greenspan et al., 1988). These losses could be attributed to oxidation, heat degradation, and inadequate antioxidant protection of alpha tocopherol. McMurray et al. (1980) indicated there was no significant concentration change associated with length of time for saponification, or

the type of antioxidant used.

Another problem that occurred when using KOH was the release of cholesterol which was soluble in petroleum ether. Cholesterol eluted from the HPLC column shortly after alpha tocopherol, and the cholesterol peak covered up the smaller alpha tocopherol peak. The identity of cholesterol was confirmed using mass spectroscopy (Section two). In order to correct this problem, an additional purification step would be required to remove or separate the alpha tocopherol and cholesterol. Sep-pak[4] clean up was attempted, but did not provide any separation of alpha tocopherol and cholesterol. Tandem columns and a larger column were also used in an attempt to separate these compounds with no success.

---

[4] Sep-pak, Waters Chromatography, Milford, MA 01757.

CONCLUSION

The data indicate a straight extraction with petroleum ether and absolute ethanol (Method I) provided the highest alpha tocopherol recovery, was the quickest to perform, and highly reproducible (Table 8). Recovery of alpha tocopherol internal standards were greater than 85%. This suggests that this procedure does not destroy alpha tocopherol during extraction. Due to problems associated with other techniques attempted, method I was used throughout the remainder of this research project. This method provided uniform analysis of samples tested and allowed statistical comparisons to be performed.

SECTION II. ALPHA TOCOPHEROL STABILITY IN SWINE SERUM, LIVER, AND HEART TISSUE

INTRODUCTION

Phase two of this study examined the stability of alpha tocopherol in serum, liver, and heart tissue samples. Tissues submitted for alpha tocopherol analysis may reach their destinations in various stages of autolysis. Poor sampling, improper packaging, and various exposures to heat and light can result in lower tissue alpha tocopherol concentration. This study was designed to help explain what conditions result in loss of alpha tocopherol from liver and serum tissue samples. Tissues were subjected to a variety of conditions to study the stability and recovery of alpha tocopherol. These conditions include 23 degrees celsius (room temperature), 4 degrees celsius (refrigeration), -20 celsius (deep freeze), and -70 celsius (ultra low freezer) were selected for analysis.

Time periods were chosen to simulate field and laboratory conditions. Temperature, light exposure, and autolysis were selected to simulate field conditions. To provide these conditions, 4 degrees C. and 23 degrees C. with various time periods were used. The 23 degrees C. and 4 degrees C. samples were analyzed at 0, 12, 24, 48, and 96 hours post mortem. To simulate laboratory conditions, freezer temperatures and time limits were used to determine storage and freeze/thaw effects. Samples stored at -20

degrees C. and -70 degrees C., were analyzed at 0 and 72 hours, as well as 1, 2, 3, and 4 weeks post mortem. The results were compared to determine if alpha tocopherol levels decrease, with increased exposure to conditions described above.

MATERIALS

Chemicals

All chemicals used for this study were purchased from Fisher Scientific, Pittsburgh, Pennsylvania (U.S.A.). The solvents used for extraction of alpha tocopherol were reagent grade petroleum ether and ethanol. HPLC grade methanol and chloroform were used for the HPLC system mobile solvent.

Nitrogen gas for concentrating the extracts, was purchased from Iowa State University Chemistry Stores.

Standard

Alpha tocopherol standard was purchased from Fluka Biochemika, Sweden. This standard was labeled greater than 98% pure according to HPLC analysis.

Instrumentation

A Waters 6000A HPLC pump[1] was coupled with a Micrometrics Chromonitor 785 ultraviolet detector[2] and a C-18 (3 cm x 3 mm) column. A Shimadzu SIL 9A autosampler[3] was used to allow programming multiple samples per analysis. Data were recorded using a Shimadzu CR501 chromatopac integrator.[3]

---

[1]Waters Corp., Milford, MA 01757.

[2]Micrometrics Instrument, Norcross, GA 30071.

[3]Shimadzu Instrument, Kyoto, Japan.

A Finnigan Mass Spectrum and Gas Chromatography system[4] was used to identify unknown compounds. For separation of cholesterol from alpha tocopherol a second HPLC was used. It consisted of a Waters 6000A pump,[5] C-18 column, Waters 991 Photo-Diode array detector,[5] NEC Power-Mate computer for data collection, and a Waters 5200 Printer-Plotter[5] for data printing.

---

[4]Finnigan Corp., Sunnyvale, CA.

[5]Waters Corp., Milford, MA 01757.

METHODS

Preparation

Two groups of pigs weighing 18 kg +/- 5 kg were used in this study. Group one consisted of five animals and group two contained six animals. Both groups were offered a 16% grower ration ad libitum for ten days prior to euthanasia, to provide uniform dietary intake of alpha tocopherol at 22 I.U. per kg of feed.

Sample Collection

Immediately after euthanasia, blood was collected in Corvac serum separator tubes.[6] Samples were allowed to clot for 1 hour at room temperature, then centrifuged and the serum transferred to plastic serum tubes with plastic caps. These samples were labeled and placed in their appropriate testing condition. Livers and hearts were collected and separately homogenized for two minutes to provide uniform sampling. Fresh tissues collected at time zero were used as control samples and were analyzed in triplicate to determine baseline levels. Twenty gram samples were placed in clear plastic bags and appropriately labeled. Each experimental group was placed in its respective testing environment (23 degrees C., 4 degrees C., -20 degrees C., or -70 degrees C.).

---

[6]Corvac, Sherwood Medical, St. Louis, MO 63103.

Extraction Technique

The analytical technique for alpha tocopherol was chosen based on data presented in section one of this thesis. Two, six to eight gram samples were placed in 50 ml screw top tubes wrapped in aluminum foil. Six ml of absolute ethanol was added, followed by 8 ml of petroleum ether. To one tube, 100 ul of 0.1 ug/ul (micrograms per microliter) of the alpha tocopherol internal standard was added. Each tube was vortexed twice for one minute. Tubes were centrifuged at 5,000 rpm (revolutions per minute) for 5 minutes. The top solvent layer was removed and its volume recorded. This was concentrated to dryness under nitrogen. The extract was resuspended in one ml of HPLC methanol, and 20 ul of this was injected in the HPLC for analysis.

Calculation

Data were calculated by measuring peak heights of alpha tocopherol standards injected. The data were used to calculate the unknown concentration of alpha tocopherol in samples. The following formula was used to calculate alpha tocopherol concentration in liver and heart tissue. For serum calculations, the dilution factor of 200 ul/20 ul, replaces 1,000 ul/20 ul used for tissues.

Calculation Formula:

0.4 ug of alpha tocopherol x 1,000 ul/20 ul x Unknown peak/standard peak x 1.0/weight of sample adjusted for solvent recovery levels = ug/g concentration of alpha tocopherol.

The standard injected was 20 ul of 0.02 ug/ul to produce standard peaks from the HPLC.

RESULTS

The control liver concentration of 0.957 +/- 0.172 ug/g was similar to normal values reported by previous researchers (Van Vleet, 1987; Bieber-Wlaschny, 1987; Jensen et al., 1988). Alpha tocopherol concentrations in liver samples stored at 23 degrees C. and 4 degrees C. remained stabile for 12 hours post mortem. At 24 hours, there was a significant decrease in alpha tocopherol concentration (Tables 11 and 12). After 24 hours, the alpha tocopherol concentration increased slightly while stored at 4 degrees C., however, at 96 hours the concentration was still significantly lower than controls. The concentration of samples at 23 degrees C. remained significantly lower than the controls, but the concentrations were stabile between 24 and 48 hours, and then declined at 96 hours. Complications developed in detecting alpha tocopherol in room temperature samples after 24 hours. A peak of unknown identity, obscuring the smaller alpha tocopherol peak, eluted off the HPLC column. This problem occurred only in liver samples, while serum and heart tissues were not affected. This unknown peak did not appear in fresh tissue or tissue not autolyzed (refrigerated).

Twenty-four hours exposure to 23 degrees C. was the point when autolysis became obvious. As indicated by a necrotic odor and gas production, evidenced by swollen plastic bags. Once this occurred, alpha tocopherol detection

Table 11. Mean concentration of alpha tocopherol from liver tissue exposed to 23 degrees C. (micrograms/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	0.957	0.172
12 hours	11	1.07	0.316
24 hours	11	0.572*	0.374
48 hours	11	0.636*	0.156
96 hours	11	0.536*	0.250

* Significantly different from control tissue, $p < 0.05$.

Table 12. Mean concentration of alpha tocopherol from liver tissue 4 degrees C. (micrograms/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	0.957	0.172
12 hours	11	0.9909	0.270
24 hours	11	0.591*	0.408
48 hours	11	0.727*	0.346
96 hours	11	0.791*	0.266

* Significantly different from control tissue, $p < 0.05$.

was obscured by an interfering compound identified by gas chromatography/mass spectroscopy and HPLC with photo diode array detection, as cholesterol. The C-18 column on the photo diode array system was the same as the column on the ultraviolet system used in this study. The photo diode array system was sensitive enough to detect alpha tocopherol and cholesterol. Five samples were analyzed in this manner and compared to the ultraviolet detection system. The photo diode array system produced good separation of alpha tocopherol and cholesterol.

The alpha tocopherol concentration of control serum was 0.655 +/- 0.191 micrograms/gram. There was no significant change until after 24 hours, however, the concentration dropped significantly ($p < 0.05$) at 48 hours at 23 degrees C. (Table 13). Refrigerated samples (4 degrees C.) had no significant changes ($p < 0.05$) in concentration through 96 hours (Table 14).

Because of limited heart tissue, there was not enough available for all phases of the test. The 4 degrees C. and 23 degrees C. were complete except, for the 96 hour analysis, which was omitted. The control level of alpha tocopherol in heart tissue was approximately 1.6 ppm. There was a significant decrease in concentration between the control samples and the 12 hour samples in both groups. Concentrations at 0, 24, and 48 hours were not significantly

Table 13. Mean concentration of alpha tocopherol in serum samples stored at 23 degrees C. (microgram/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	0.655	0.191
12 hours	11	0.672	0.162
24 hours	11	0.663	0.186
48 hours	11	0.481*	0.194
96 hours	11	0.472*	0.228

* Significantly different from the control concentration, $p < 0.05$.

Table 14. Mean concentration of alpha tocopherol in serum samples stored at 4 degrees C. (microgram/gram)

Time Period	N	Mean Concentration [a]	Standard Error
Control-0	11	0.655	0.186
12 hours	11	0.700	0.219
24 hours	11	0.636	0.128
48 hours	11	0.563	0.092
96 hours	11	0.691	0.197

[a] No significant differences from control concentration.

different between refrigerated and room temperature samples (Tables 15 and 16).

Freezer samples showed less variability than the refrigerated or room temperature samples. Control liver concentration was 0.957 +/- 0.172 micrograms/gram. Tissue frozen at -20 degrees celsius had no significant changes through 4 weeks (Table 17). Tissue samples stored at -70 degrees celsius had no significant changes ($p<0.05$) through three weeks. The concentration after the fourth week was significantly higher than the control levels (Table 18).

Alpha tocopherol concentration in frozen serum was variable. The control concentration of alpha tocopherol was 0.655 +/- 0.186 micrograms/gram. There were no significant changes in concentration in the first week when stored at -20 celsius (C.). The two week concentration was significantly lower than the control value (Table 19). Samples stored 3 and 4 weeks at -20 C. were not significantly different from the control values. Serum stored at -70 degrees celsius (C.) was not significantly different from the control concentration through week four (Table 20).

Table 15. Mean concentration of alpha tocopherol in heart tissue stored at 4 degrees C. (refrigerated) (micrograms/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	1.63	0.615
12 hour	5	0.88*	0.554
24 hour	11	1.5	0.501
48 hour	11	1.89 [a]	0.933

*,[a] Significantly different between the 12 and 48 hour levels, $p < 0.05$.

Table 16. Mean concentration of alpha tocopherol in heart tissue stored at 23 degrees C. (room temperature) (microgram/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	1.63	0.615
12 hours	5	0.86*	0.517
24 hours	11	1.11 [a]	0.516
48 hours	6	1.88 [b]	0.823

[a,b] Significantly different between 24 and 48 hours, $p < 0.05$.

*,[b] Significantly different between 12 and 48 hours, $p < 0.05$.

* Significantly different between control and 12 hours, $p < 0.05$.

Table 17. Mean concentration of alpha tocopherol in liver tissue stored at -20 degrees C. (micrograms/gram)

Time Period	N	Mean Concentration [a]	Standard Error
Control-0	11	0.957	0.172
72 hours	11	0.80	0.357
1 week	11	0.80	0.282
2 week	11	0.75	0.225
3 week	11	0.90	0.346
4 week	11	0.936	0.58

[a]No significant differences.

Table 18. Mean alpha tocopherol concentration of liver tissue stored at -70 degrees C. (micrograms/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	0.957	0.172
72 hours	11	0.854	0.383
1 week	11	0.863	0.283
2 week	11	0.800	0.223
3 week	11	0.818	0.235
4 week	11	1.22*	0.334

* Significantly different from control (higher), $p < 0.05$.

Table 19. Mean concentration of alpha tocopherol in serum stored at -20 degrees C. (microgram/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	0.655	0.191
72 hours	11	0.7545	0.191
1 week	11	0.59	0.186
2 week	11	0.445*	0.246
3 week	11	0.736	0.297
4 week	11	0.609	0.273

* Significantly different from control (lower), $p < 0.05$.

Table 20. Mean concentration of alpha tocopherol in serum stored at -70 degrees C. (microgram/gram)

Time Period	N	Mean Concentration	Standard Error
Control-0	11	0.655	0.191
72 hours	11	0.773	0.195
1 week	11	0.664	0.168
2 week	11	0.60	0.077
3 week	11	0.718	0.391
4 week	11	0.564*	0.246

* Significantly different from control, $p < 0.05$.

DISCUSSION

Control and 12 hour concentrations of liver alpha tocopherol were not significantly different at either 4 degrees C. or 23 degrees C. samples. Concentrations at 24, 48, and 96 hours were significantly decreased. After 24 hours, the tissue started to autolyze and gas production began to occur. By 36 hours, there was necrotic odor present from the room temperature samples. Storage at 4 degrees C. did not protect liver tissue from significant loss of alpha tocopherol, although it was smaller than 23 degrees C. loss. Refrigerated samples lost alpha tocopherol at a similar rate as room temperature samples from 12 to 24 hours. This loss could result from exposure to oxygen during the collection and storage, and the ability of autolytic bacteria to survive these conditions.

Another problem with aged and decomposed tissue samples was interference by cholesterol. It appears cholesterol is released with increased degradation of the tissue (Greenspan et al., 1988). The cholesterol interference occurred as well when saponification techniques were used. Saponification involved the use of heat and KOH to degrade and destroy membranes to allow the release of membrane associated alpha tocopherol. Cholesterol is released when tissue membranes are damaged or destroyed (Greenspan et al., 1988). This could be related to the location of cholesterol in the

membrane, and the increased degradation of the membrane during autolysis. The cholesterol peak masked the alpha tocopherol peak when it eluted from the HPLC column, when using ultraviolet detection, after extraction of autolytic tissue. HPLC analysis with photo diode array detection produced separation of alpha tocopherol and cholesterol peaks (Figures 1 and 2).

The concentration of alpha tocopherol in frozen liver tissue remained stable through the study. When frozen at -20 celsius, there were no significant changes through four weeks. Tissue frozen at -70 celsius, had no significant changes in alpha tocopherol concentration through three weeks. At four weeks, there was a significant increase in the alpha tocopherol concentration. This increase might result from increased membrane destruction as a result of extremely low temperatures and period of time tissues were stored.

The stability of serum alpha tocopherol at 23 degrees C. was similar to that seen in liver tissue. No significant change in concentrations was observed between control values and those at 12 and 24 hours. A significant loss of alpha tocopherol occurred after 24 hours. Serum stored at 4 degrees C. did not have significant changes from the controls in alpha tocopherol concentration.

Serum frozen at -20 and -70 degrees celsius had no

Figure 1: Spectrum of alpha tocopherol standard from high performance liquid chromatography system using photo diode array detection. Alpha tocopherol peak is identified by a.

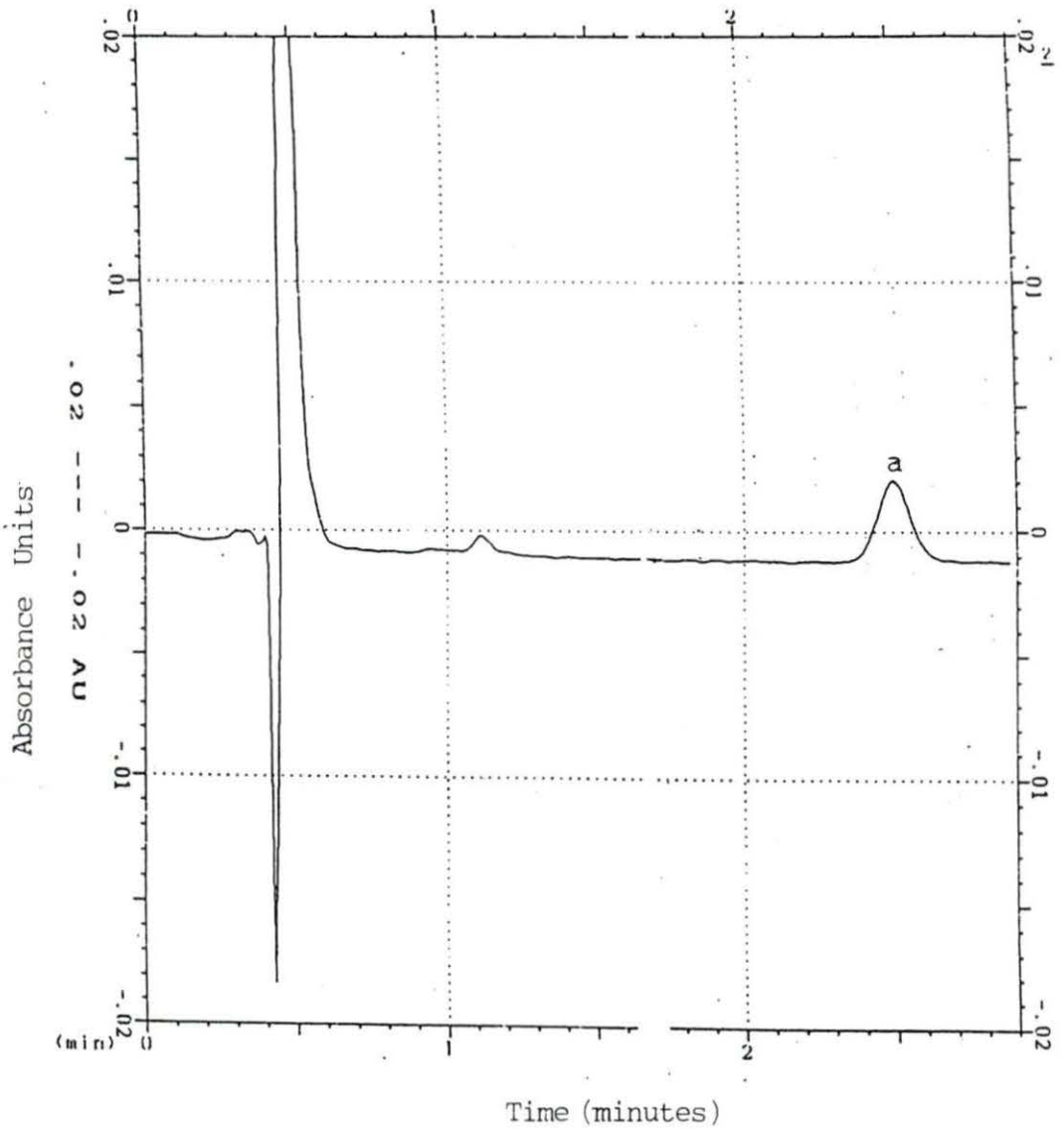
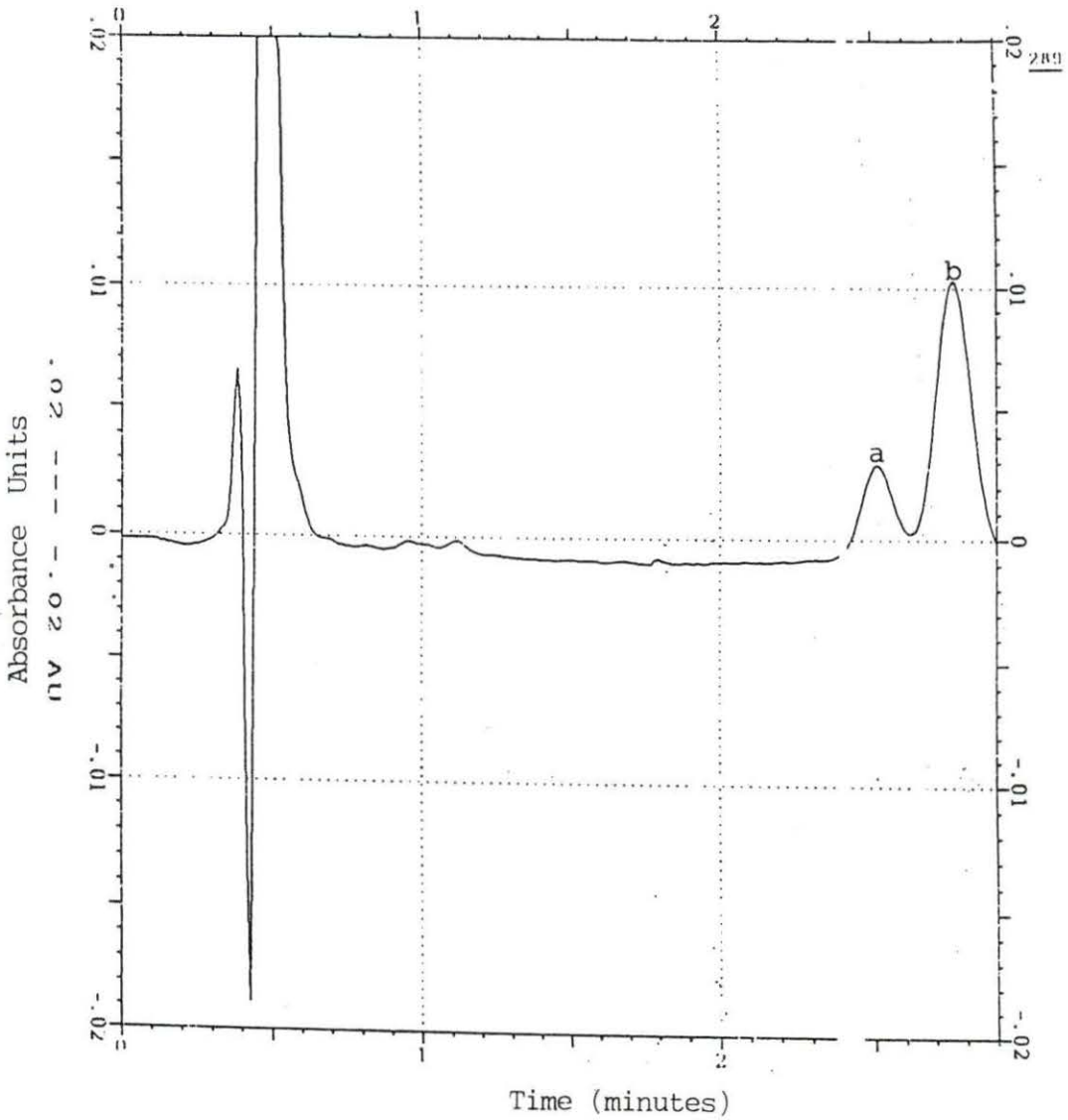

Absorbance Units
.02 --- --- -.02 AU
a
(min)
0
1
2
Time (minutes)

Figure 2: Spectrum from tissue extracted for alpha tocopherol using high performance liquid system with photo diode array detection. This spectrum shows separation of alpha tocopherol (a) and cholesterol (b).


Absorbance Units
AU .02 --- .01 --- 0
.02
.01
0
-.01
-.02
0
1
2
289
a
b
Time (minutes)

significant changes through one week. At two weeks, tissue frozen at -20 celsius had a significant decrease in concentration, but concentrations at three and four weeks were not significantly different from the control concentration. This decrease at two weeks could be from improper sampling, extraction, or detection. Biesalski et al. (1986) determined that alpha tocopherol was stabile in serum greater than two weeks, when stored at -34 degrees celsius.

For heart tissue stored at 4 degrees C., there was a decline in alpha tocopherol concentrations at 12 hours, however, there was a rapid, steady increase in the concentrations between 12 and 48 hours. The only significant difference noted was between the 12 and 48 hour samples. There was no significant difference between the control concentrations and those recovered at 12, 24, and 48 hours. Heart tissue stored at 23 degrees C. produced different results. There was a significant loss of alpha tocopherol at 12 hours, but no significant difference was observed between the 24 and 48 hour samples and the control concentrations. These results were slightly different than those observed in liver and serum. One reason could be variation in the extraction and/or analysis with the HPLC system used. In addition, after tissue autolysis and production of gas occurred, there was no cholesterol interference in heart

tissue, similar to that which occurred in liver tissue. Heart tissue stored at -20 and -70 degrees celsius had no significant changes in alpha tocopherol concentration until the third week of storage. The increase in alpha tocopherol concentration recovered at week three remained stabile through the end of the study (week 4).

CONCLUSIONS

Data on swine liver tissue show that alpha tocopherol remains stabile for 12 hours when subjected to 23 degrees C. and 4 degrees C.. At 24 hours, there was a significant drop in alpha tocopherol concentration at both 4 degrees C. and 23 degrees C. (Tables 11 and 12). Swine liver tissue stored at -20 and -70 degrees C. did not have any significant change in alpha tocopherol concentration during 4 weeks of storage (Table 17 and 18). This indicates that alpha tocopherol can be lost if tissue samples are not fresh or properly stored until analyzed.

Serum stored at 23 degrees C. did not have a significant loss of alpha tocopherol until after 24 hours. When stored at 4 degrees C., there was no significant loss of alpha tocopherol through 96 hours. Frozen serum (-20 and -70 degrees C.) remained stabile through three weeks when stored at -70 C. When stored at -20 C., there was a significant drop in alpha tocopherol concentration at 2 weeks, but the three and four week samples were similar to control concentrations.

To minimize tocopherol loss in tissue between the time from collection to analysis, sampling of fresh tissue and transporting or storing frozen at either -20 or -70 C. is recommended.

SECTION III. INFLUENCE OF PARENTERAL ALPHA TOCOPHEROL ON SWINE CONSUMING AFLATOXIN CONTAMINATED FEED

INTRODUCTION

The final phase of this project was a feeding study to determine the effects of an injectable alpha tocopherol product on pigs consuming feed containing 400 parts per billion (ppb) aflatoxin. Aflatoxin, a mycotoxin produced by Aspergillus flavus and Aspergillus parasiticus, can be found most frequently in animal feedstuffs such as corn, cottonseed, grain sorghums, and peanuts (Coppock and Swanson, 1986). Aflatoxin is a potent liver toxin and its effects vary with dose, length of exposure, species, breed, diet, or nutritional status (C.A.S.T., 1989). Aflatoxin has produced great public health concern, because of its widespread occurrence in many human dietary foodstuffs (C.A.S.T., 1989). The ingestion of this toxin by humans has been associated with primary liver cancer in certain populations (Emerole et al., 1984). Young animals of most species appear to be more susceptible than older animals, and mature males are more susceptible than mature females, except pregnant females (Blood and Radostits, 1989; Coppock and Swanson, 1986). Dietary intake levels of 300-600 ppb are required over an extended period of time for clinical signs to become apparent. Large concentrations consumed in short periods of time can be lethal (C.A.S.T. 1989; Osweiler et al., 1985). Two common problems associated with aflatoxin are decreased feed intake, which produces a smaller rate of gain and poor

feed efficiency, and reduced resistance to infectious diseases (Coppock and Swanson, 1986). Other clinical problems associated with aflatoxin in swine are hepatocellular damage and necrosis, nephrosis, and systemic hemorrhages (Osweiler et al., 1985; Coppock and Swanson, 1986).

Alpha tocopherol is a natural antioxidant and is required to prevent white muscle disease, mulberry heart disease, and hepatosis dietetica. Alpha tocopherol may act as a protective antioxidant in the gastrointestinal tract, similar to its affect in tissue (Parrish, 1980). Because aflatoxin is often found associated with moldy corn, where heat and moisture are present, it was decided to use parenteral supplementation of alpha tocopherol instead of dietary supplementation. Ewan (1987), indicates that alpha tocopherol decreases in the diet during storage, and alpha tocopherol destruction can be accelerated by heat, moisture, and presence of trace minerals. This could limit the beneficial effect of dietary supplemented alpha tocopherol. Emerole et al. (1984) indicated that dietary vitamin E could have significant effects on the metabolism of aflatoxin B1 in vivo. Swine require this vitamin in their diet. Due to its antioxidant capabilities, this vitamin could potentially reduce the effects of dietary aflatoxin.

MATERIALS

Chemicals

All solvents used in this study were purchased from Fisher Scientific, Pittsburgh, Pennsylvania. Solvents used were reagent grade petroleum ether, ethanol, HPLC methanol, and chloroform.

Alpha Tocopherol Supplement

Alpha tocopherol (d-alpha-tocopherol) was obtained from Stuart Products, Bedford, Texas. This product contained 300 International units of alpha tocopherol per milliliter, and was labeled for intramuscular injection.

Instrumentation

A Waters 6000A HPLC pump[1] was coupled with Micrometrics Chromonitor 785 ultraviolet detector.[2] A Shimadzu SIL 9A autosampler[3] was used to program multiple samples per analysis. Data were collected and recorded using a Shimadzu CR501 Chromatopac integrator.[3]

---

[1] Waters Corp., Milford, MA 01757.

[2] Micrometrics Instrument Corp., Norcross, GA 30071.

[3] Shimadzu, Kyoto, Japan.

Alpha Tocopherol Standard

Alpha tocopherol standard was purchased from Fluka Biochemika, Sweden. This product was greater than 98% pure according to HPLC analysis.

Animals

Twenty-four cross bred pigs initially weighing 9-15 kilograms were purchased from Laboratory Animal Resources, located at the Iowa State University Veterinary Medicine Complex.

METHODS

Animal Preparation

Twenty-four pigs were randomly assigned to four groups of six each. Experimental groups were: 1) control, 2) dietary aflatoxin, 3) alpha tocopherol injected, and 4) dietary aflatoxin and alpha tocopherol injected.

The pigs were initially weighed to determine base line weights for group feed efficiency analysis (Table 21). At weighing, each pig was ear tagged for identification. Blood samples were collected using Corvac serum tubes,[4] to provide serum for initial alpha tocopherol levels and baseline clinical pathology data. Initial serum alpha tocopherol concentrations were repeated in triplicate to produce baseline control values.

At the conclusion of the study, post mortem samples of liver and heart were collected from three pigs in both the control and the alpha tocopherol groups. The remaining liver and heart were left in the animals and the abdominal openings were closed with sutures. These carcasses were left at room temperature conditions for an additional 24 hours. Then another liver and heart tissue sample was removed and alpha tocopherol concentrations were determined.

---

[4] Corvac, Sherwood Medical, St. Louis, MO. 63101.

Clinical Pathology

Serum clinical chemistry analyses were performed every two weeks to monitor liver function. The four tests selected were bilirubin, total protein, albumin, and aspartate aminotransferase.

Albumin was selected because it is synthesized by the liver. Damage to the liver would result in decreased levels of albumin in the serum. Albumin levels were determined using Abbott Laboratories A-Gent Albumin test.[5] This test is based on albumin's ability to bind to bromcresol green. This complex is then analyzed using a spectrophotometer.

Aspartate Aminotransferase (AST) is an enzyme that is released from liver tissue when hepatic cells are damaged. AST reagent was purchased from Sigma Diagnostic Services.[6] The AST reagent allows for quantitation of AST using a spectrophotometer. Hemolyzed serum can produce inaccurate results due to the high levels of AST found in red blood cells.

Total protein was monitored to evaluate the protein level in serum. The biuret reagent used for quantitation was purchased from Abbott Laboratories.[5] Polypeptides with two or more peptide bonds react with the biuret reagent, which forms

---

[5] Abbott Diagnostics, Abbott Park, IL 60064.

[6] Sigma Chemical, Co., St. Louis, MO, 63178.

a cupric ion-protein nitrogen complex. This is analyzed with a spectrophotometer.

Total bilirubin was determined using A-Gent bilirubin purchased from Abbott Laboratories.[7] Bilirubin levels increase in serum as a result of decreased bile flow and/or liver disease. Bilirubin levels are determined using the A-Gent reagent and a spectrophotometer.

---

[7] Abbott Diagnostics, Abbott, IL 60064.

Alpha Tocopherol Supplementation

Two groups were supplemented with injectable alpha tocopherol obtained from Stuart Products.[8] This was administered at a dosage of 60 International Units per kilogram body weight and was given intramuscularly in the perineal region. Alpha tocopherol was given on day 0 and 21, to maintain serum levels above 1 ppm.

Feed

Feed was mixed and weighed before being placed in the respective pens. It was estimated that each pig would eat approximately 7% of its body weight. Feed was made available ad libitum in self feeders. Only enough feed was mixed and fed for one week at a time. Feed left in the feeders at the weekly weighing was weighed back to calculate net feed consumption and feed efficiency (Table 22).

Ration

The ration was a 16% grower ration using Master Mix[9] 40% soybean meal concentrate, as the supplement. This ration contained added macronutrients and micronutrients to meet National Research Council requirements. Two groups were fed corn contaminated with aflatoxin. Corn that contained low levels of aflatoxin was ground and supplemented with corn

---

[8] Stuart Products, Bedford, Texas.

[9] Master Mix, Central Soya and Subsidiaries, Ft. Wayne, IN.

containing 30,000 ppb to attain intended concentrations of 200-600 ppb (Table 23). These feedstuffs were sampled immediately after mixing and submitted to the Chemistry Laboratory at the Iowa State University Veterinary Diagnostic Laboratory to determine the aflatoxin concentration present. Actual aflatoxin concentrations analyzed are shown in Table 23. Two samples were also submitted for analysis to determine if other mycotoxins were present which might interfere with this study. Water was available ad libitum, from pressurized nipple waterers.

Animals were observed twice daily for clinical signs throughout the study.

RESULTS

Weight gain and feed efficiency was monitored and calculated weekly in all four groups of pigs. Weekly weights for these four groups are listed in Table 21. The control group had the highest daily gain of 0.61 pounds per day, and the alpha tocopherol group gained 0.52 lbs per day. The group fed aflatoxin and supplemented with alpha tocopherol, had the lowest gain at only 0.4 lbs per day. Groups two and four each had one pig that did not respond similarly to the others in the group. Statistical analysis was performed to compare the ratio of ending weight to starting weight (Table 24). These statistics were run with and without the two smaller pigs in groups 2 and 4. In both cases, there was no significant difference in weight gain among the four groups. Comparisons shown are for 6 pigs per group (Table 24).

Group feed efficiency results are listed in Table 22. At the end of week one, group two (alpha tocopherol only) had the best feed efficiency. This indicates that supplementation with the injectable alpha tocopherol in conjunction with a 16% grower ration produced one kilogram of body weight with less feed required than the other three groups. The next lowest was the combination of alpha tocopherol-aflatoxin group. Their efficiency was 2.5 kilograms feed per kilograms body weight. The control group's efficiency was 2.82 (Table 22).

Table 21. Mean weekly weights for each of the 4 test groups (Kilograms)

Date	Group	Mean	Standard Error
5/3/90*	1	14.16	2.69
	2	12.8	2.04
	3	12.41	2.06
	4	11.41	1.71
5/9/90*	1	16.25	2.58
	2	15.5	2.98
	3	14.16	2.73
	4	13.16	2.04
5/16/90[a]	1	20.41	2.90
	2	19.0	4.15
	3	17.16	2.97
	4	15.75	2.85
5/23/90[b]	1	24.9	3.86
	2	22.58	5.98
	3	20.5	4.00
	4	18.37	4.09
6/6/90[c]	1	30.58	11.93
	2	30.5	9.04
	3	28.58	6.35
	4	25.16	6.34

* No significant difference between these two dates.

[a,b,c] Significant difference in these weights between each weighing date, and from the original control weight.

Table 22. Group feed efficiency calculated weekly for each of the 4 test groups kilograms feed/kilogram gain

Group	Week 1	Week 2	Week 3	Week 4
1	2.82	2.36	3.33	2.56
2	2.08	1.96	2.91	2.48
3	2.71	1.89	2.48	2.32
4	2.5	1.66	2.15	1.85

Table 23. Weekly Aflatoxin Feed Levels (ng/g)

Week	Aflatoxin Concentration
1	360
2	200
3	204
4	682
5	250

Table 24. Ratio of starting weight to ending weight in swine

Group	No.	Mean Ratio [a]	Standard Error
1	6	2.4	0.228
2	6	2.35	0.472
3	6	2.28	0.147
4	6	2.2	0.34

[a]No significant differences among groups.

By week five, the feed efficiency results had changed. The control group feed efficiency (kg feed/kg weight gain) was 2.56, followed by group 2 at 2.48, group 3 at 2.32, and group 4 with 1.85 (Table 22). This increased feed efficiency in group four could be attributed to the group consuming less, but average daily gain remaining the same.

Weekly serum alpha tocopherol levels were monitored throughout the study. Control pigs had no significant change ($p < 0.05$) in serum alpha tocopherol concentration through the study (Table 25). The aflatoxin only group (group 3) also had no significant changes in serum alpha tocopherol levels (Table 25).

The two groups that were supplemented with injectable alpha tocopherol had significant increases within one week after injection (Table 25). These concentrations decreased at an average rate of 0.12 parts per million (ppm) per day from week one to week two, and declined 0.05 ppm per day from week two through week three.

The alpha tocopherol groups were injected again during week four and the serum alpha tocopherol concentrations at week five were significantly increased (Table 25). The mean concentration in group 2 at week five (alpha tocopherol only) was similar to the concentration after the initial alpha tocopherol injections. The mean concentration at week five in group 4 (aflatoxin-alpha tocopherol) was slightly less

Table 25. Mean concentration of alpha tocopherol in serum for each of the four experimental swine groups tested (micrograms/gram)

Group	Date	No.	Mean Concentration	Standard Error
1	5/3/90	6	0.73	0.21
2		6	0.83	0.31
3		6	0.77	0.103
4		6	0.83	0.258
1	5/9/90	6	0.78	0.17
2		6	2.46 *	0.28
3		6	0.77	0.16
4		6	2.56 [a]	0.22
1	5/16/90	6	0.75	0.28
2		6	1.58 *	0.25
3		6	0.82	0.20
4		6	1.7 [a]	0.35
1	5/23/90	6	0.75	0.20
2		6	1.28 *	0.31
3		6	0.87	0.22
4		6	1.31 [a]	0.20
1	6/6/90	6	1.11	0.14
2		6	2.26 *	0.33
3		6	1.28	0.31
4		6	1.95 [a]	0.30

[a] Group four alpha tocopherol concentration significantly different from the control concentration on 5/3/90, $p < 0.05$. Group four was significantly different each week from groups one and three, $p < 0.05$.

* Group two alpha tocopherol concentration significantly different from the control concentration on 5/3/90, $p < 0.05$. Group two was significantly different weekly from groups one and three, $p < 0.05$.

than group 2, which may indicate that alpha tocopherol is being used, altered, or lost rapidly from the serum (Table 25).

At the conclusion of the feeding period, pigs were euthanized and liver, heart, and blood collected for analysis. Livers were initially weighed to determine if there was a correlation between liver to body weight ratio and treatment. Statistical data analyzed liver in relation to percent of body weight. No significant change in liver weight was noted in any of the four groups.

Heart and liver tissues were sampled immediately to determine alpha tocopherol concentration. Animals supplemented with alpha tocopherol had significantly higher tissue concentrations, than those not supplemented. This occurred in both liver and heart tissue.

Suturing of the carcasses was not tight, which allowed gas to escape. Liver and heart tissues recovered from the post mortem study were in the autolytic process, and their structures were starting to deteriorate. Also noted was a necrotic odor produced from the carcasses after 12 hours. Liver and heart tissues from these pigs were analyzed to determine the concentration of alpha tocopherol following normal autolysis. The results show that alpha tocopherol levels recovered from both heart and liver tissue from group 2 (alpha tocopherol supplemented) were significantly higher

after 24 hours than when sampled at day 0 (Tables 26 and 27). There was no significant difference in alpha tocopherol concentrations in group 1 (non-supplemented) at 24 hours. However, alpha tocopherol concentrations of group 2 were significantly higher at 24 hours post mortem (Table 28). Cholesterol interference was not a problem at this time.

There were no significant changes in any of the four clinical pathology tests monitored during the study (Table 29). There also were no significant lesions observed on histopathologic examination of tissues.

Table 26. Mean alpha tocopherol concentration in swine liver (microgram/gram)

Group	No.	Mean Concentration	Standard Error
1	6	0.93	0.28
2	6	2.66*	0.71
3	6	1.43	0.32
4	6	2.03*	1.2

* Groups two and four were significantly different than groups one and three. Groups two and four were supplemented with alpha tocopherol at 60 IU/kg on day 0 and 21.

Table 27. Mean alpha tocopherol concentration in swine hearts (micrograms/gram)

Group	No.	Mean Concentration	Standard Error
1	6	1.08	0.23
2	6	2.98*	0.65
3	6	0.85	0.23
4	6	2.38*	0.765

* Groups two and four were significantly higher than groups three and one, $p < 0.05$. Groups two and four were supplemented with alpha tocopherol at 60 IU/kg on day 0 and 21.

Table 28. Mean concentration of alpha tocopherol in liver tissue 24 hours in situ post mortem (micrograms/gram)

Group	No.	Mean Concentration	Standard Error
1-Control	3	0.86	0.37
1-24 hours	3	0.80	0.52
2-Control	3	2.3	0.83
2-24 hours	3	2.86 *	0.40

* Significantly different than 2-Control, $p < 0.05$.

Table 29. Clinical Pathology results for groups 3 and 4 (groups 1 and 2 were not tested)

Group	Pig No.	Date	T. Bili[a]	T.P.[b]	Alb.[c]	AST[d]
3	123	5/4/90	0.1	5.8	2.1	50
	123	5/17/90	0.0	5.7	2.0	42
	123	6/7/90	0.2	6.3	2.2	49
	122	5/4/90	0.1	4.8	2.5	45
	122	5/17/90	0.0	5.7	2.4	50
	122	6/7/90	0.1	6.0	2.5	51
	125	5/4/90	0.1	5.1	2.5	54
	125	5/17/90	0.0	5.6	2.4	51
	125	6/7/90	0.2	6.1	2.8	44
	124	5/4/90	0.1	4.7	2.2	80
	124	5/17/90	0.0	4.7	2.0	69
	124	6/7/90	0.2	5.4	2.5	66
	121	5/4/90	0.2	6.2	2.8	56
	121	5/17/90	0.0	6.4	2.3	41
	121	6/7/90	0.3	6.4	2.3	46
	144	5/4/90	0.1	5.1	2.7	68
	144	5/17/90	0.0	5.4	2.7	47
	144	6/7/90	0.2	6.4	3.3	39
4	325	5/4/90	0.1	5.2	2.2	70
	325	5/17/90	0.0	5.5	2.1	41
	325	6/7/90	0.2	6.1	2.3	37
	12	5/4/90	0.1	5.0	2.4	77
	12	5/17/90	0.0	7.5	2.1	34
	12	6/7/90	0.6	8.2	1.7	190
	90	5/4/90	0.1	5.3	2.6	54
	90	5/17/90	0.0	5.8	2.5	54
	90	6/7/90	0.2	6.4	2.5	44
	297	5/4/90	0.1	4.8	2.3	25
	297	5/17/90	0.0	5.0	2.3	40
	297	6/7/90	0.1	5.4	2.5	58
	224	5/4/90	0.1	4.4	1.8	78
	224	5/17/90	0.0	5.3	2.2	61
	224	6/7/90	0.2	6.2	2.2	58
	324	5/4/90	0.1	5.0	2.2	82
	324	5/17/90	0.1	5.4	2.2	51
	324	6/7/90	0.2	5.8	2.7	42

[a] Total Bilirubin.
[b] Total Protein.
[c] Albumin.
[d] Aspartate Aminotransferase.

DISCUSSION

Two important aspects of swine production are weight gain and feed efficiency (Vinson et al., 1986). Aflatoxin, can produce decreases in feed intake which affects both weight gain and feed efficiency (C.A.S.T., 1989).

These results show parenterally alpha tocopherol did not have a significant protective effect against dietary aflatoxin. The aflatoxin-alpha tocopherol groups average daily gain was smaller than the other three test groups.

Serum alpha tocopherol concentrations significantly changed in the two groups supplemented, however, nonsupplemented groups had no significant change. After injection of 60 International Units/kilogram body weight of alpha tocopherol, serum levels increased approximately 3 fold. This indicates that the injected alpha tocopherol was absorbed from the muscle into blood circulation. At the end of the study, the supplemented groups had significant increases in alpha tocopherol concentrations in liver and heart tissue, compared to nonsupplemented groups (Tables 26 and 27). Tissue concentrations indicate alpha tocopherol is absorbed from the serum and stored in tissue.

Tissues from alpha tocopherol supplemented pigs exposed to room temperature (23 degrees celsius) for 24 hours had liver alpha tocopherol concentrations slightly higher than the controls (Table 28). This could be the result of

beginning autolysis and tissue degradation which allows liberation of alpha tocopherol from tissues. It may be possible that recently supplemented elevated tissue alpha tocopherol reserves are less tightly bound or more readily extractable after autolysis. Twenty-four hour samples produced no interference from cholesterol.

CONCLUSIONS

This study indicates that injectable alpha tocopherol does not significantly protect against aflatoxin consumed by swine. The aflatoxin levels fed appeared to produce a decrease in feed consumption, but did not significantly alter liver function (Table 29).

This study did show that alpha tocopherol can be injected parenterally and will be absorbed into the circulatory system, and that it can be repartitioned to other tissues in the body. This was evidenced by the large alpha tocopherol concentrations found in heart and liver tissue from supplemented groups vs. non supplemented groups. It is presumed that repartitioning could provide antioxidant protection in tissues when needed.

CONCLUSIONS

This study produced a direct extraction technique for alpha tocopherol in both serum and tissue samples. It was shown the addition of absolute ethanol to tissue and serum samples assisted in alpha tocopherol extraction. This technique was found to be rapid, reproducible, and produced minimal alpha tocopherol loss as evidenced by recovery of internal alpha tocopherol standards.

Alpha tocopherol appears to remain stabile and extractable in serum and tissue samples when frozen at -20 and -70 degrees celsius. This was supported because of minimal loss (not significant) of alpha tocopherol. Tissue and serum samples exposed to 23 degrees celsius (room temperature) for time periods longer than 12 hours had significant losses of alpha tocopherol. Serum samples that were stored at 4 degrees celsius (refrigerated) remained stabile through 96 hours, while tissue samples refrigerated had significant losses after 24 hours.

This data suggests that samples collected fresh, placed in airtight containers, and stored or shipped frozen at -20 degrees C. or colder, will provide the best samples for alpha tocopherol extraction.

Finally, section three suggests that parenterally administered alpha tocopherol is absorbed into the blood

circulation, and can be repartitioned from blood to tissue. This was indicated by tissue samples from pigs supplemented with alpha tocopherol had higher alpha tocopherol concentrations than the non-supplemented group.

This research provides some fundamental information on alpha tocopherol stability in swine serum and liver tissue. Further research needs to be done to facilitate more information on alpha tocopherol stability and its effects on aflatoxin.

BIBLIOGRAPHY

Adams, C. R. 1989. Effect of Supplemental Vitamin E on Preservation of Poultry Meat and Pork Products. Roche Corporation, Nutley, New Jersey.

Association of Analytical Chemistry Methods. 1980. Analytical Methods of alpha tocopherol and alpha tocopherol acetate. 13th ed. Association of Analytical Chemistry Methods, Washington, D.C.

Bieber-Wlaschny, M. 1987. Vitamin E in Swine Nutrition. Roche Corporation, Nutley, New Jersey.

Bieri, J. G., T. J. Tolliver, and G. L. Catignani. 1979. Simultaneous determination of alpha tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. American Journal of Clinical Nutrition 32:2143-2149.

Biesalski, H., H. Greiff, K. Brodda, G. Hafner, and K. H. Bassler. 1986. Rapid Determination of Vitamin A (Retinol) and Vitamin E (alpha Tocopherol) in Human Serum by Isocratic Adsorption HPLC. Int. J. Vit. Nutr. Res. 56:319-327.

Blood, D. C., and O. M. Radostits. 1989. Veterinary Medicine. 7th ed. Bailliere Tindall, Philadelphia, PA.

Catignani, G. L. 1986. An HPLC Method for the Simultaneous Determination of Retinol and Alpha Tocopherol in Plasma or Serum. Methods in Enzymology 23:215-219.

Chou, P. P., P. K. Jaynes, and J. L. Bailey. 1985. Determination of Vitamin E in Microsamples of Serum by Liquid Chromatography with Electrochemical Detection. Clinical Chemistry 31(6):880-882.

Cohen, H., and M.R. LaPointe. 1980. Determination of Vitamin E in Animal Feeds by Normal Phase High Pressure Liquid Chromatography. J. Assoc. Off. Anal. Chem. 63(6):1254-1257.

Coppock, R. W., and S. P. Swanson. 1986. Current Veterinary Therapy, Food Animal Practice 2. W. B. Saunders, Philadelphia, PA.

Cort, W. M., T. S. Vicente, E. H. Waysek, and B. D. Williams. 1983. Vitamin E Content of Feedstuffs Determined by High-Performance Liquid Chromatographic Fluorescence. Journal of Agricultural and Food Chemistry 31:1330-1333.

Council for Agricultural Science and Technology. 1989. Mycotoxins, Economic and Health Risks. Task report No. 116. Council for Agricultural Science and Technology, Ames, Iowa.

Craig, A. M., L. L. Blythe, E. D. Lassen, K. E. Rowe, R. Barrington, and M. Slizeski. 1989. Variations of serum vitamin E, cholesterol, and total serum lipid concentrations in horses during a 72-hour period. Am. J. Vet. Res. 50(9):1527-1531.

Diplock, A. T. 1985. Fat Soluble Vitamins, Their Biochemistry and Applications. Heinemann: London.

Diplock, A. T., J. Green, J. Bunyan, and D. McHale. 1966. The analysis of alpha tocopherol and ubiquinone in rat liver. Br. Food Nutr. 20:95-100.

Emerole, G. O., M. I. Thabrew, and H. O. Kwanashie. 1984. Effect of dietary vitamin E (alpha tocopherol) on aflatoxin B metabolism. European Journal of Drug Metabolism and Pharmacokinetics 9(4):295-300.

Ewan, R. C. 1987. Selenium-Vitamin E Deficiency: Causative Factors. Department of Animal Science, Iowa State University, Ames, Iowa.

Fabianek, J., J. DeFilippi, T. Rickards, and A. Herp. 1968. Micromethod for Tocopherol Determination in Blood Serum. Clinical Chemistry 14(5):456-462.

Gallo-Torres, H. E., O. N. Miller, J. G. Hamilton, and C. Tratnyek. 1970. Distribution and Metabolism of Two Orally Administered Esters of Tocopherol. Lipids 6(5):318-325.

Greenspan, M. D., Chia-Yee Lee Lo, D. P. Hanf, and J. B. Yudkovitz. 1988. Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, alpha tocopherol, and retinol by high performance, liquid chromatography with a diode array detector. Journal of Lipid Research 29(7):971-976.

Hidiroglou, N., L. F. Laflamme, and L. R. McDowell. 1988. Blood Plasma and Tissue Concentrations of Vitamin E in Beef Cattle as Influenced by Supplementation of Various Tocopherol Compounds. J. Anim. Sci. 66:3227-3234.

Horwitt, M. K., C. C. Harvey, C. H. Dahm Jr., and M. T. Searcy. 1972. Relationship Between Tocopherol and Serum Lipid Levels for Determination of Nutritional Adequacy. Annals New York Academy of Sciences 96:223-236.

Huang, May-Lynn, G. J. Burckart, and R. Venkataramanan. 1986. Sensitive High-Performance Liquid Chromatographic Analysis of Plasma Vitamin E and Vitamin A Using Amperometric and Ultraviolet Detection. Journal of Chromatography 380:331-338.

Jensen, M., J. Hakkarainen, A. Lindholm, and L. Jonsson. 1988. Vitamin E Requirement of Growing Swine. J. Animal Sci. 66:3101-3111.

Keating, R. W., and P. R. Haddad. 1982. Simultaneous Determination of Ascorbic Acid and Dehydroascorbic Acid by Reversed-Phase Ion-pair High-performance Liquid Chromatography with Pre-Column Derivatisation. Journal of Chromatography 245:249-255.

Lammi-Keefe, C. J. 1986. Tocopherols in Human Milk: Analytical Method Using High-Performance Liquid Chromatography. Journal of Pediatric Gastroenterology and Nutrition 5:934-937.

Losowsky, M. S., J. Kelleher, and B. E. Walker. 1972. Intake and Absorption of Tocopherol. Annals New York Academy of Sciences 96:212-221.

Machlin, L. J. 1984. Handbook of Vitamins. Nutritional, Biochemical, and Clinical Aspects. Marcel Decker, Inc., New York and Basel.

Machlin, L. J., and J. Keating, J. Nelson, M. Brin, R. Filipski, and O. N. Miller. 1979. Availability of Adipose Tissue Tocopherol in the Guinea Pig. J. Nutr. 109:105-109.

Manz, U., and K. Philipp. 1989. Analytical Methods for Vitamins and Carotenoids in Feed. ROCHE: Department of Vitamin Research and Development, Nutley, N.J.

McMurray, C. H., W. J. Blanchflower, and D. A. Rice. 1980. Influence of Extraction Techniques on Determination of alpha Tocopherol in Animal Feedstuffs. J. Assoc. Off. Anal. Chem. 63(6):1258-1261.

Michigan State University Diagnostic Laboratory. 1990. A-152 Veterinary Clinical Center, East Lansing, Michigan, 48824-1314.

Miller, K. W., and C. S. Yang. 1985. An Isocratic High-Performance Liquid Chromatography Method for the Simultaneous Analysis of Plasma Retinol, Alpha Tocopherol, and Various Carotenoids. Analytical Biochemistry 145:21-26.

Mino, M., H. Nishino, T. Yamaguchi, and M. Hayashi. 1977. Tocopherol Level in Human Fetal and Infant Liver. J. Nutr. Sci. Vitaminol. 23:63-69.

Mostow, N. D., R. O'Neill, D. L. Noon, and B. R. Bacon. 1985. Determination of Alpha Tocopherol in Rat Liver by High Performance Liquid Chromatography Utilizing Ultraviolet Detection. Journal of Chromatography 344:137-143.

Osweiler, G. D., T. L. Carson, G. Van Gelder, and W. B. Buck. 1985. Clinical and Diagnostic Veterinary Toxicology. 3rd ed. Kendall Hunt Publishing, Dubuque, IA.

Parrish, D. B. 1980. Determination of Vitamin E in Foods -- A Review. Critical Reviews in Food Science and Nutrition 13(2):161-187.

Reese, D. E. 1987. Selenium/Vitamin E Nutrition of Swine. George A. Young 27th Annual Conference, Lincoln, Nebraska.

Philip, K. and U. Manz. 1982. Vitamin E and its effects on Chicken, Turkey, and Pork Products. Roche Corporation, Nutley, New Jersey.

Roche. 1987a. Method for the Determination of Vitamin E in Feed and Tissue by HPLC. Roche Corporation, Nutley, New Jersey.

Roche. 1987b. Vitamin E Overview. Roche Corporation, Nutley, New Jersey.

Tappel, A. L. 1980. Vitamin E and Selenium Protection From In Vivo Lipid Peroxidation. Annals New York Academy of Sciences 104:18-31.

Tyopponen, J., J. Hakkarainen, T. Juokslahti, and P. Lindberg. 1984. Vitamin E requirement of mink with special reference to tocopherol composition in plasma, liver, and adipose tissue. Am. J. Vet. Res. 45(9):1790-1794.

Van Vleet, J. F. 1987. Monensin and Selenium Toxicity in Swine. George A. Young 27th Annual Conference, Lincoln, Nebraska.

Van Vleet, J. F., and S. Kennedy. 1987. Selenium-Vitamin E Deficiency in Swine. George A. Young 27th Annual Conference, Lincoln, Nebraska.

Vinson, R. A. 1986. Diseases of Swine. 6th ed. Iowa State University Press, Ames, IA.

Zaspel, B. J., and A. S. Csallany. 1983. Determination of Alpha Tocopherol in Tissues and Plasma by High Performance Liquid Chromatography. Analytical Biochemistry 130:146-150.

ACKNOWLEDGEMENTS

I would like to express my thanks to the many individuals who contributed to the completion of this degree. The following are those that contributed, supported, and encouraged me the most during this experience.

I want to express my sincere gratitude to Evon Reinsch, for all her help, encouragement, and understanding for the last two years. A special thanks to my mother, Barbara Fredrickson, and brother Ken Fredrickson, for all their help and support, with the farm, home, and school.

To my major professor, Dr. Gary D. Osweiler, I thank you for allowing me the rare opportunity to continue my pursuit of both advanced degrees. You have contributed significantly to assist my research and writing efforts. I have valued greatly all your instruction and constructive criticism. I would also like to thank you for the financial support of this project.

To my committee members, Dr. Thomas Carson, and Dr. Donald Dyer, the continued encouragement and support of my abilities in my pursuit of this degree.

A special thanks to Paula Imerman, for all her time and patience during this project with all the questions that I asked. To Dr. Mike Stahr, thanks for giving your time, help and use of equipment which greatly assisted in the completion of this project. Thanks to Dr. Scott Witte, for all his

computer assistance and late night entertainment.

To Cathy Lindstrand, thanks for all her assistance, understanding, and patience during those often long hours and late nights in the laboratory.

Finally, I would like to dedicate this thesis to my father, Richard L. Fredrickson Sr., who passed away when I started my advanced degrees. I wish to thank him for 25 years of help, understanding, instruction, love, and determination that he instilled in me, which I will carry with me the rest of my life. Thanks Mom and Dad.