FINAL REPORT

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Livestock feed preservatives based on antioxidant enzymes extracted from animal blood

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Duration of Project: 12 months

Lay Overview of Project and Goals: Auto-oxidation of unsaturated fats is one of the primary mechanisms of quality deterioration in animal feed. These alterations in quality are manifested through adverse changes in flavor, color, texture, and nutritive value and there is some concern that toxic compounds are produced during the deterioration process. Most currently used antioxidants are synthetic chemicals, and there is global concern among regulatory bodies and customers regarding the safety of these compounds (e.g., ethoxyquin). Naturally-derived antioxidants are available (e.g., tocopherols), but are expensive, and are often not as effective as their synthetic counterparts. Thus, there is a clear need for development of novel, inexpensive, and efficient natural antioxidants. Erythrocytes are readily available as a major component of animal blood. Being natural oxygen carriers, they are equipped with highly efficient antioxidant machinery, which utilizes a number of antioxidant enzymes and compounds. Here, we propose the continuing development of a cost effective, natural antioxidant derived from animal blood.

Previously, we have achieved the following milestones:

- Established a simple protocol to produce an antioxidant enzyme concentrate from animal blood
- We compared the efficacy of our enzyme cocktail to commercially available antioxidants, such as Ethoxyquin, PetOx® (BHA + BHT) and NaturOx® (mixed tocopherols), and completed accelerated shelf-life studies.
- Evaluated the efficacy of our antioxidant cocktail in comparison to the current industry standards, ethoxyquin and BHA/BHT, using food models relevant to those utilized by the industry.
- Performed more detailed efficacy studies with commercial pet food samples, kibbles, turkey protein meal and rendered fat models using FOX-II assay to measure Peroxide Value of the samples, and performed antimicrobial studies.
- Prepared and submitted new AAFCO Ingredient Definition “Erythrocyte Protein Extract (preservative)” for our antioxidant.
- Performed testing for possible viral contamination, including Porcine Epidemic Diarrhea Virus;
- Organized a meeting with FDA CVM officers in order to determine the required steps to receive an approval for the new ingredient petition.

This year we focused our research efforts on the following key issues necessary for successful commercialization and use of our technology by rendering industry: 1) perform testing for possible viral contamination, including Porcine Epidemic Diarrhea Virus; 2) perform tests of antioxidant activity after thermal treatment to evaluate whether or not our product can be applied before rendering process.

Objective (s):

The objectives of this research are:

- To identify and test for possible viral contamination. Although our product is made of erythrocytes, which lack DNA and therefore are unlikely to carry a viral load, this question appears to be of a significant concern for AAFCO and FDA due to recent viral outbreaks. Therefore, we propose to meet with veterinarians, who will help us to identify several most important viruses to be tested for and perform the tests for these viruses either in the lab or using an independent third party;

- We will also perform experiments comparing antioxidant properties of our product, when applied prior the heat treatment. Application of antioxidants at the early stage of the rendering process will minimize oxidative deterioration in edible animal fats during rendering process and is of significant interest for the rendering industry. Therefore, we propose to test our antioxidant in such conditions, and compare its performance to that of commercial antioxidants.

Project Overview: During the last year our team was working on viral testing, long-term efficacy of the antioxidants, and preparing data for the regulatory submission. We also working on optimization of scaling up of the process. We met with FDA CVM officers and discussed with them steps necessary steps for the regulatory submission. Furthermore, we have organized in 3rd party laboratory testing of our product – Eurofins Labs has performed accelerated shelf life study (up to 2 months at 50ºC) with sunflower oil.

Risk of viral contamination is deemed to be extremely low if EPE is used in feeds intended for species other than pig, which has been confirmed by Dr. Gauger, a swine virologist at Iowa State University Veterinary Diagnostic Lab. In the case of EPE used in feeds intended for pig species, quality control will be used to detect five viruses that can potentially be present in porcine blood and pose risk of infection for pigs. These blood-born five viruses include: Porcine parvovirus: PPV; Porcine Circovirus: PCV2; Porcine Deltacoronavirus: PDCoV; Porcine Epidemic Diarrhea virus: PEDV; and Porcine reproductive and respiratory syndrome virus: PRRSV. Testing for these five viruses is routinely performed by the others as a part of the quality control for products made from porcine blood and intended for uses in pig species (such as porcine plasma protein). 5 existing batches of EPE were sent for testing of viral loads to Iowa State University Veterinary Diagnostic Lab. The testing is performed using PCR technique, which is extremely sensitive to extremely low concentrations of viral genes. All batches tested negative for any of the above listed viruses, the complete result of the testing will be included in the final report(due to page limitations), and here we demonstrate results of PCR testing for PCV2 and PEDV(see Table I).

Table I. Results of PCR testing for PCV2 and PEDV.

PCR - PCV2

Animal ID	Specimen	Ct / Result	Comment
VRM1, Tube #1	Fluid	>37 / Negative	
VRM2, Tube #2	Fluid	>37 / Negative	
VRM3, Tube #3	Fluid	>37 / Inconclusive	ICF WILL RETEST
VRM4, Tube #4	Fluid	>37 / Negative	
VRM5, Tube #5	Fluid	>37 / Negative	

PCR - PCV2 RETEST

Animal ID	Specimen	Ct / Result	Comment
VRM3, Tube #3	Fluid	>37 / Negative	

PCR - Porcine epidemic diarrhea virus N gene

Animal ID	Specimen	ct / Result	Comment
VRM1, Tube #1	Fluid	>35 / Negative	
VRM2, Tube #2	Fluid	>35 / Negative	
VRM3, Tube #3	Fluid	>35 / Negative	
VRM4, Tube #4	Fluid	>35 / Negative	
VRM5, Tube #5	Fluid	>35 / Negative	

Our second goal was to check the ability of the antioxidant product to withstand high temperatures. For this purpose, samples of Erythrocyte Protein Extract and 150 ppm Ethoxyquin were mixed with Cod oil and incubated at 90, 110 and 120°C for the period of 3 hours, 18 minutes and 6 minutes correspondingly. Our hypothesis is that according to kinetics of chemical reactions, the 10°C temperature increase will triple the reaction rates. After incubation, TBARS assay was performed for all the samples and controls. For all the temperature, the EPE samples performed better or similar to 150 ppm ethoxyquin, and better than oxidized control, though due to small sample number of samples and large error bars, at 110 and 120°C the difference between levels of oxidation was statistically insignificant. The results of TBARS assay are presented in the Figure 1.

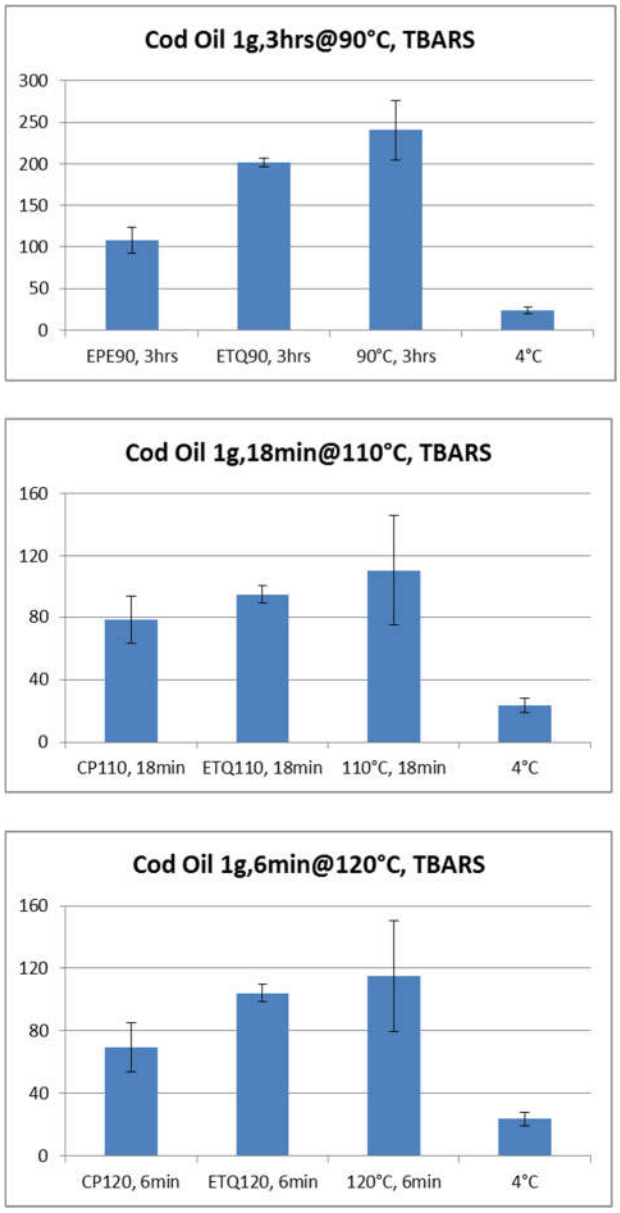

Fig. 1. Efficacy of EPE compared to that of ethoxyquin in a Cod Oil model at different temperatures (90, 110,120°C). EPE showed similar or better protection with Ethoxyquin 150 ppm.

For accelerated shelf-life study we used 3rd party laboratory – Eurofins located in Des Moines, Iowa.

Briefly, 2 kg of unstabilized sunflower oil were split into four 250g aliquots in 500 mL beakers and ten 100 g aliquots in test tubes. The aliquots were labeled and fortified as follows:
#A 250 g was fortified with VRM antioxidant to a final level of 1250PPM;
#B 250 g was fortified with ethoxyquin to a final level of 150PPM. Made a concentrate in oil and added it to the sample;
#C 250 g was fortified with ethoxyquin to a final level of 500PPM. Made a concentrate in oil and added it to the sample;
#D 250 g received no fortification;
#E 100 g vials with no fortification. They were flushed with N_2 prior to storage.

The beakers containing #A - #D were covered with filter paper and placed in a 50°C oven with no humidity monitoring or control capabilities. The 10 control vials (#E) were stored in a freezer. The Peroxide and p-Anisidine values were tested after 5, 12, 24 and 60 days. Photos of the samples were taken to monitor possible phase separation:

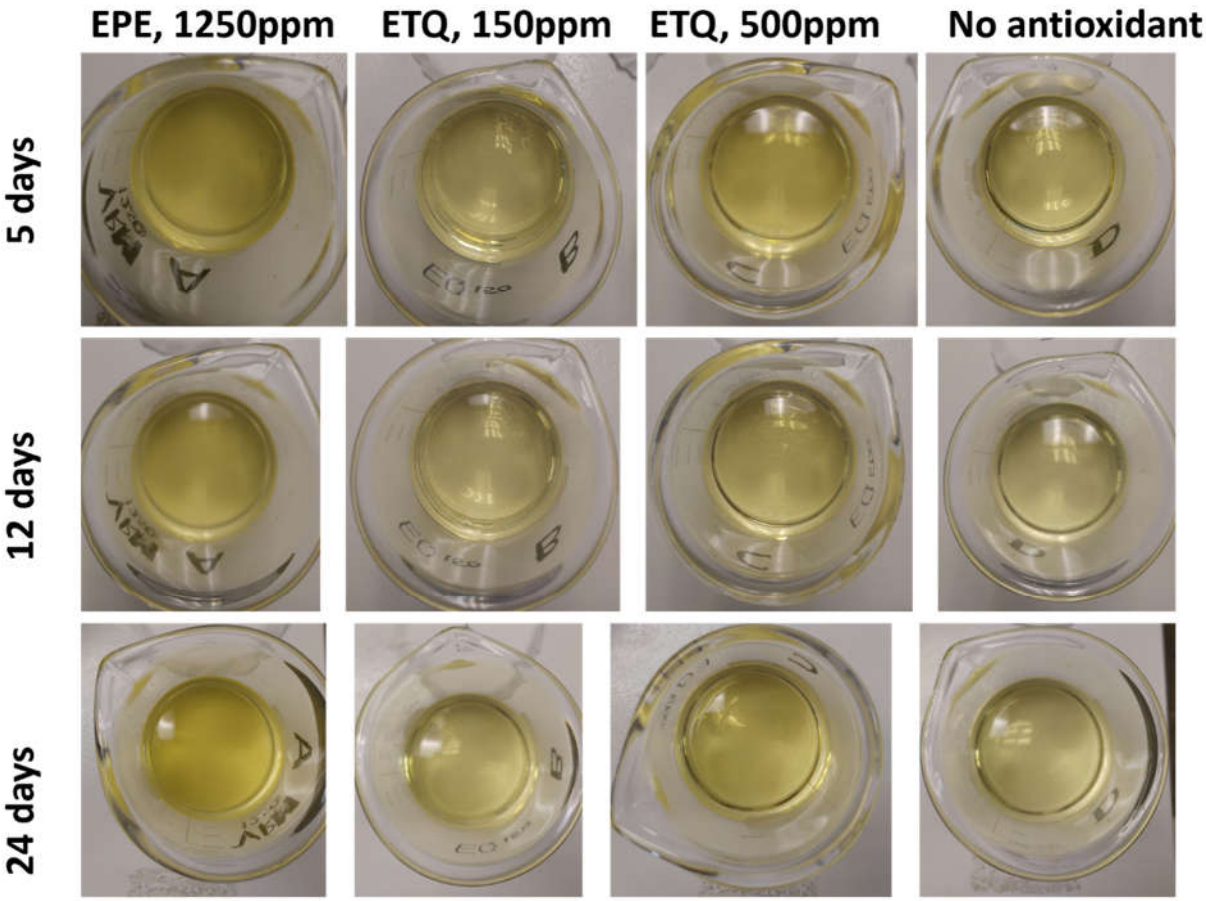

Fig.2. Photos of sunflower oil samples incubated for 5, 12, 24 days demonstrate no evidence of phase separation.

Peroxide value was measured by AOCS Method Cd 8b-90 (isooctane) and consolidated results are shown on the Fig.3.

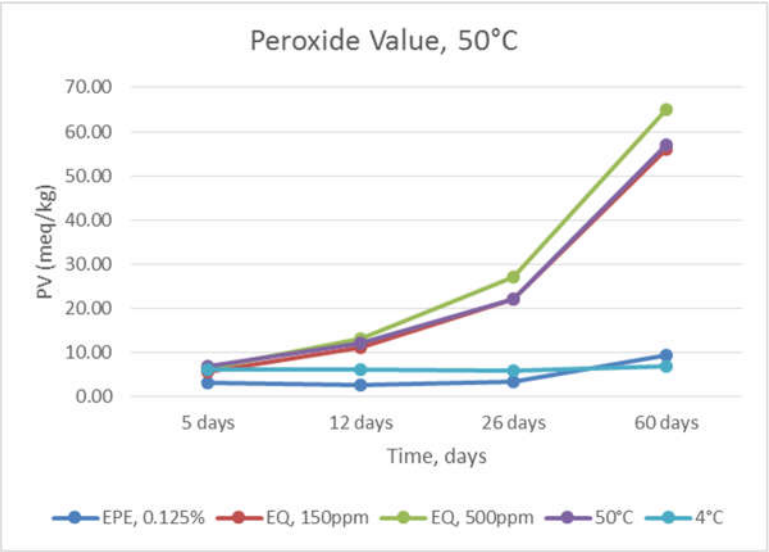

Fig.3. Combined results of PV measurements of sunflower oil samples, stabilized with antioxidants, incubated at 50°C for 5, 12, 24, and 60 days. Notably, even after 60 days the PV value of sample protected by EPE is on par with sample store in the refrigerator.

In addition to Peroxide value, one more oxidation marker has been studied – p-Anisidine value. This chemical analysis method for p-Anisidine Value determines the amount of aldehydes (principally 2-alkenals and 2,4-dienals) in animal and vegetable oils and fats by reaction of these compounds with the p-Anisidine. Briefly, samples of oil were dissolved in iso-octane and reacted with p-anisidine for 10 minutes, after that the absorbance of solution was measured at 350nm. The results of this analysis are provided below:

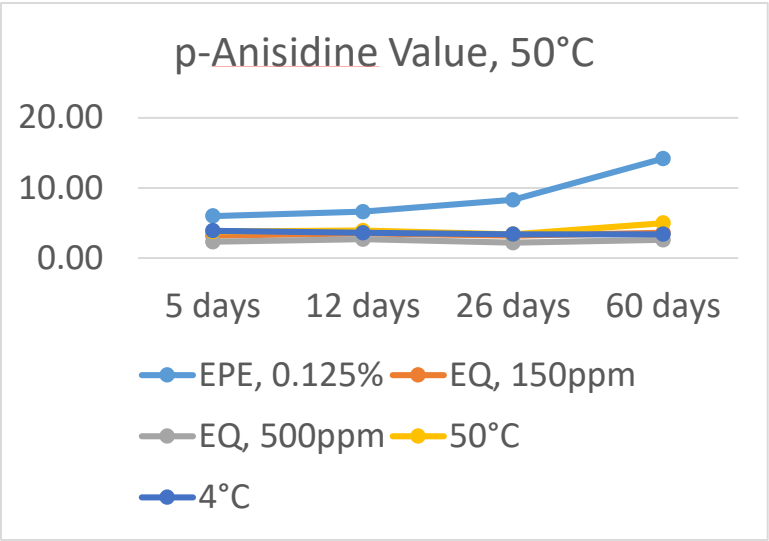

Fig.4. Results of pAV measurements of sunflower oil samples.

The PV is a measure of primary oxidation products and pAV specifically measures levels of aldehydes – secondary oxidation products. Most customers will require a pAV of less than 30 in marine oils. Since PV and pAV characterize two different oxidation values, there is a third parameter, TOTOX (TOTal OXidation), which combines both values of PV and AnV, giving a total oxidation assessment. Many people consider the TOTOX value to be the most important evaluation in determining an oil's freshness, because PV indicates its actual oxidative status and AnV its oxidative history. The combination of both values gives a good indication of the overall rancidity or quality. This value is determined by calculation: TOTOX = AnV + (2 x PV).

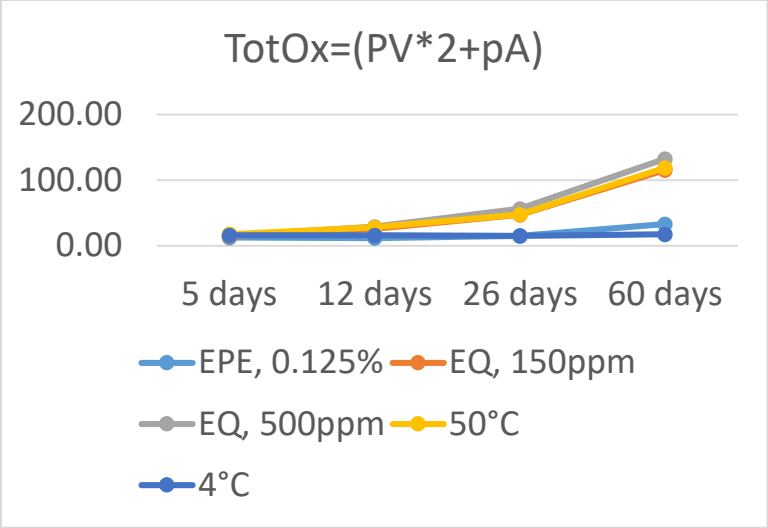

Fig.5. Results of TOTOX measurements demonstrated 0.125% EPE protects sunflower during 60 days of incubation at 50°C.

In addition to completion of proposed research objectives we have also collaborated with Dr.Rafael Garcia from USDA on use of hemoglobin by-product of our process as a flocculants – additives that facilitate the removal of particles or colloidal material from a liquid. In a preliminary study we attempted re-solubilization of hemoglobin precipitate obtained as a by-product of the EPE production process. The precipitate consists of dark-brown slurry with large amount of captured supernatant. Hemoglobin precipitation by Zn^{2+} is reported in the literature to be reversible upon removal of Zn^{2+}, for example by a chelating agent such as EDTA. However, our initial attempts to re-solubilize hemoglobin precipitate by 0.1 M EDTA solution failed, perhaps due to high concentration of zinc chloride in the supernatant captured by the precipitate. We therefore attempted an amended approach, in which hemoglobin precipitate was washed by 3x volume of water prior to treatment with EDTA solution (Fig. 6). This washing allowed us to remove considerable fraction of zinc chloride. Following treatment with excess of 0.2 M EDTA lead to complete re-solubilization of the precipitate (Fig. 7). Final protein concentration in thus prepared solution was 36 mg/mL (as measured by absorbance of diluted solution at 280 nm); calculated EDTA concentration was 0.13 M (49,000 ppm); and Zn^{2+} content was 9,100 ppm (as measured by Inductively Coupled Plasma method for a diluted solution). pH of the solution as 5.8, and zeta

potential was -32 mV. Such a negative value of zeta potential indicates high likelyhood of the effectiveness of this product as a flocculant.
Thus, our preliminary experiments demonstrated the possibility of re-solubilization of hemoglobin precipitate obtained from the EPE production process. However, there is a need to optimize re-solubilization method to reduce the amount of EDTA required to achieve solubilization and concentrations of zinc and EDTA in the final product.

We have recently received USDA SBIR phase I grant to further develop this technology.

In conclusion, our recent data indicates that antioxidant EPE prepared from porcine blood doesn't show any presents of viral contamination and the EPE was effective in protecting sunflower oil for oxidation during 2-month long accelerated study at 50°C.

Fig.6. Hemoglobin precipitate after washing by 3x volume of water

Significance to the rendering industry: Our product can be used as a cost-efficient natural antioxidant to prevent rancidity in rendered products, animal feeds and pet food. We anticipate such product would in great demand by the rendering industry because of high cost of currently used natural antioxidants and somewhat negative perception of synthetic antioxidants by many consumers. By-products of EPE production have high potential as natural flocculants.

Fig.7. Re-solubilized hemoglobin.

Outside funding: VRM Labs have been awarded 2 SBIR Phase I grants from USDA – one to continue development of antioxidant technology and second – for flocculant development, both starting August 15, 2016.

Intellectual Property Development:

The provisional patent application filed in 2012 has been converted into a utility application with PCT (international) option on 07/15/2013. In December 2014, Clemson University executed an exclusive option agreement with VRM Labs, Inc., a company started by Drs. Reukov, Vertegel and Meisinger to seek commercialization of this antioxidant.