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The Extraction and Quantification of Resveratrol from Powder and Liquid Supplements

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Abstract

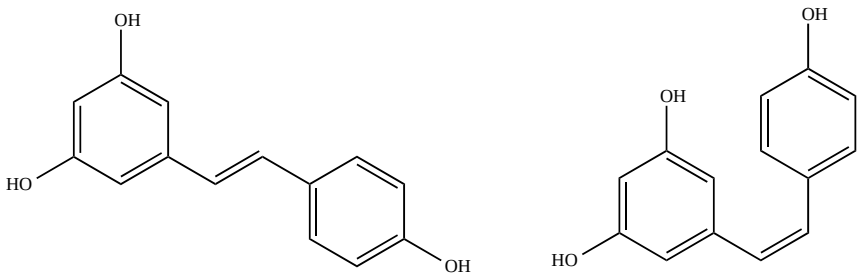
Resveratrol is a natural product and a member of a group of compounds called polyphenols. Resveratrol exists as the both cis and trans isomers and is found in very small quantities in specific plants, both edible and non-edible. There are claims in both the scientific literature and the popular press that resveratrol can combat high risk health problems such as heart disease and cancer and prevent diabetes by decreasing insulin resistance. Resveratrol is a popular supplement for both humans and animals and it can be bought from pharmacies, Walmart, nutrition stores and online. Available as a loose powder, pills, or compounded in oils, prices for resveratrol range from $1 a milligram for pure samples from suppliers like Sigma-Aldrich Chemical Company, to less than $60 for 100 grams from veterinary supply companies and on Amazon. The goal of this research was to develop a method for the extraction, identification, and quantification of resveratrol from powder supplements and work towards qualitatively and quantitatively isolating resveratrol in liquid supplements. While optimizing *trans*-resveratrol isolation, an unusual color change was observed when deprotonating an ethanolic resveratrol solution, with the solution changing from water white to a dark green. Upon adding acid, the solution turned back to clear and with additional acid to a bright red. Possible explanations for this color change continue to be explored as this research continues.

Keywords: Resveratrol, dietary supplements, natural product isolation

1. Introduction

3,5,4'-trihydroxy-*trans*- stilbene or *trans*-resveratrol (Fig1) is a polyphenol found in plants and fruit including grapes, raspberries, mulberries, lingonberries, pistachios, and peanuts.[1] It is also a major component in the invasive Japanese knotweed plant roots.[2] As a polyphenol, resveratrol is a free radical scavenger. It is a phytoalexin protecting the plants from microbial attack and enables the organism to survive drought or other environmental hardships.

Resveratrol has been claimed to increase cardiovascular health in populations that consume red wine (the French Paradox), prevent neurodegenerative disorders and act as an antiaging agent all attributed to its antioxidant capabilities.[3] Although the molecular mechanism of its protective action is unclear[4] it has been characterized as an LDL antioxidant and anti-inflammatory. More recent work suggests that resveratrol protects phosphatidylcholine from phospholipases. [5] Although polyphenolic, resveratrol is poorly soluble in water and the water: octanol partition coefficient (3.06) would indicate that the molecules are associated with the lipid part of cellular membranes rather than the plasma in the body.[6] Although little research has been done to determine bioavailability of resveratrol in humans, a small study found that at least 70% of a 25-mg dose of resveratrol could be found in the plasma (half-life 9.2 ± 0.6 h) of which a very small amount was unchanged resveratrol. Most of the resveratrol was found in epithelial cells in the aerodigestive tract.[7]



trans-resveratrol *cis*-resveratrol

Figure 1: Resveratrol isomers

Research suggests that although both the cis and trans isomer are present in natural products, the trans isomer is the bioactive compound. Cis resveratrol quickly degrades when exposed to light and acidic or basic conditions, while trans resveratrol remains stable under many conditions and is therefore the predominant species in an isomeric mix.[8] A brief survey of the literature suggests that the highest levels of resveratrol in common food products are in red wines containing anywhere from 0.32 to 15.35 □g/g of material.[9]

As a non-modified natural product, the FDA does not strictly monitor the efficacy or purity of resveratrol supplements.[10]

Resveratrol is available for use by humans and animals (dogs, cats and horses) and can be obtained as the pure, loose powder or with other materials in an oil. Most of these sources obtain resveratrol from Japanese knotweed root (*Polygonum cuspidatum*) a highly invasive perennial herbaceous plant. Traditional chemical companies such as Sigma-Aldrich or Cayman Chemical Company charge from $0.36 to $1.15 per mg for pure resveratrol, while Amazon prices range from $0.10 and up per dose and the dose often contains other additives.

This research details our isolation and identification procedures of *trans*-resveratrol in readily available over the counter supplements. The reported quantity of the natural product varied over the range of dry and liquid trans-resveratrol supplements that were analyzed. Two solvent extraction methods of dry material were compared based on the literature partition coefficients and the percent *trans*-resveratrol recovered was determined. The samples were purified via column chromatography and compared to analytical standards using MS and ^{1}H-NMR. It was noted the purity of supplements were variable compared to the labeled quantities. The two methods resulted in comparable recovery. In order to extract *trans*-resveratrol from an oil-based supplement, an acid-base liquid-liquid method of isolation of the polyphenol was developed with unexpected but reproducible results.

2. Experimental:

Trans-resveratrol samples were purchased from Sigma-Aldrich Chemical (> 99% HPLC Grade), Ark-Pharm Inc (>98%) and Cayman Chemical (>98%). Unless otherwise stated, all other solvents and reagents were used as received from Sigma-Aldrich and Fisher Scientific without further purification.

Equine Factor Powder Supplement (EFP) was obtained from Equinefactor.com as a loose, powder in a polyethylene bottle (100 g). ZHOU Nutrition Trans-Resveratrol supplement (Zhao Nutrition, Kansas City, MO from Amazon) came as 500 mg gelatin capsules (60 per bottle) in an amber plastic bottle. Both supplements were dark brown powder as received. Advantage Bioscience Resvantage and Resvantage Equine supplement capsules (resveratrol, micronized) were obtained from Amazon (15 and 200 mg). According to the manufacturer these capsules were filled under nitrogen and heat sealed.

Soxhlet extraction glassware was obtained from ChemGlass and disposable Whatman/GE cellulose thimbles were used to contain the crude sample. A BUCHI Rotavapor R-124 was used to remove solvents under reduced pressure. Proton NMR spectra were obtained on a Varian iNOVA 600 MHz nuclear magnetic resonance spectrophotometer (20 Hz spin rate at 25 °C). d_6-Acetone with 0.03% TMS was used for all ^{1}H NMR analysis of *trans*-resveratrol standards. Mass spectrometry (MS) was performed on a Thermo Fisher Scientific LTQ XL using positive ion mode. Methanol was used to flush the instrument between each sample and to insure baseline integrity and reproducibility.

2.1 Isolation of *trans*-Resveratrol from Commercial Powdered Samples

2.1.1 . room temperature, 1-hour extraction with ethanol sop

Approximately 0.5 g Equine Factor Powder (or ZHOU supplements) was suspended in 75 mL of 95% ethanol and allowed to stir for approximately 1 h at room temperature. The solution was filtered through fluted filter paper and the filtrate was evaporated under reduced pressure and allowed to air dry overnight. This process was repeated for a total of 8 times (n=8) for each supplement sample. In a similar manner, this process was repeated with the Ark Pharm *trans*-resveratrol standard (purity of >98 %) five times to determine the percent recovery. Extracts of all samples were characterized using LC-MS and ^{1}H NMR.

2.1.2 . soxhlet extraction, 4-days sop

EFP supplement (2.55 g) was loaded into a cellulose Soxhlet thimble and extracted for 96 h using 400 mL of 95% ethanol. At the end of the extraction period, the ethanol solution was filtered (fluted filter paper), cooled and evaporated to dryness under reduced pressure and allowed to air dry overnight. The extract was characterized using LC-MS and ^{1}H NMR.

2.1.3 purification of extracts using column chromatography

Flash column chromatography using a silica stationary phase and a 80:20 mixture of hexanes:ethyl acetate as the mobile phase was used to purify the crude isolates. Solvents were removed under reduced pressure and the resulting material was verified by LC-MS and ^{1}H-NMR.

2.1.4 . acid-base liquid-liquid extraction of trans-resveratrol from an oil containing supplement sop

In order to develop a SOP for extracting *trans*-resveratrol from an oil-based supplement, 100 mg of resveratrol was blended with 5 mL of sesame oil and added to a separatory funnel. The oil mixture was extracted with 10 mL of 5% NaOH (3X). The organic layer was removed, and the aqueous layers were extracted again this time with 20 mL of ethyl acetate. The aqueous layer was made acidic with concentrated HCl. The resulting solid was removed by vacuum filtration and verified using LC-MS and ^{1}H NMR.

3. Results

In spite of the multiple phenolic groups, the solubility of *trans*-resveratrol in water is quite low (about 3 mg per 100 mL). In polar organic solvents such as ethanol, DMSO and DMF, the solubility is approximately 65 mg/mL. Ethanol (95%) was chosen for its ease of removal.

Supplements are available in multiple forms including loose powder, powder in gelatin capsules, and compounded with other additives including oils. The supplements available ranged from pure *trans*-resveratrol to a mixture of *trans*-resveratrol with other health promoting additives. Initial studies focused on the pure *trans*-resveratrol powder supplements in order to establish an efficient, reproducible extraction method without interference from other compounds.

Although *trans*-resveratrol has a rather high melting point (253-255 °C), it does decompose with heating. This research kept the extraction temperatures at or below that of the boiling point of ethanol to minimize potential decomposition.

The commercially available resveratrol supplements that were in an oil matrix contained multiple additives such as sesame oil, lecithin, mixed tocopherols, Atlantic kelp, flaxseed oil, biotin, vanilla extract, titanium dioxide and caramel coloring. In order to develop an isolation procedure, a 2% resveratrol in oil mixture was used to model the oil containing supplements.

The gravity filtered supplement separated into visible bands (yellow and orange in color) during column chromatography elution. The yellow band eluted first and was proceeded by the desired resveratrol product.

4. Characterization of trans-resveratrol by 1H-NMR, MS

4.1 Proton NMR analysis:

It was determined that polar aprotic d_6-acetone (including 0.03% TMS) would work well as it allowed full spectral resolution and limited the deuterium exchange from the phenolic functional groups. This analysis was performed on all standards (Sigma-Aldrich, Cayman and Ark Pharm) and a representative spectrum with its proton assignment is shown in Figure 2.

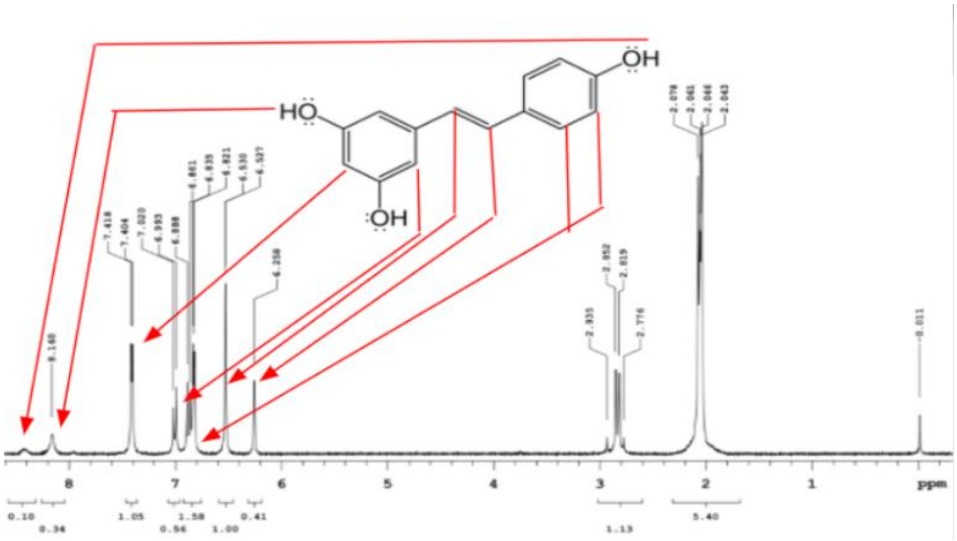


Figure 2: ^{1}H NMR spectra of the Ark Pharm *trans*-Resveratrol standard in d_6-acetone with peak assignments. Peaks at 2.8 ppm and 2.1 ppm are due to the presence of water and acetone solvent respectively.

4.2 Mass Spectral Analysis:

MS provided the expected parent peak of 229.08 m/z (M + 1). The base peak was observed at m/z = 134.92 and is assumed to be the fragment shown in Figure 3. A smaller peak at m/z = 211.03, attributed to the loss of H_2O from the M+1, was also observed.

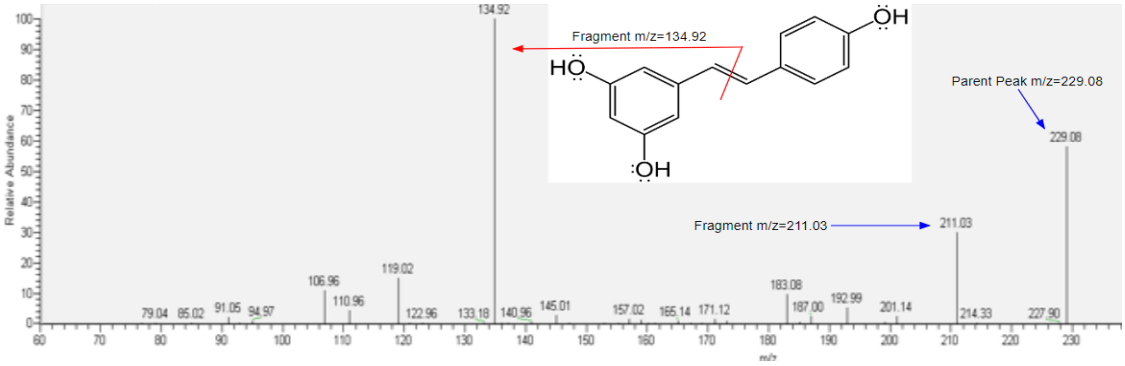


Figure 3: The mass spectra of Ark Pharm *trans*-Resveratrol standard.

5. Room temperature, 1-hour extraction with Ethanol

As noted, the EFP and ZHOU supplements were dark brown in color as received. *Trans*-resveratrol samples received from Sigma-Aldrich, Caymen Chemical and Ark Pharm were consistently white to slightly off white in color indicating that the EFP and the ZHOU were either contaminated or decomposed. The average percent recovery from the room temperature, 1-hour ethanol extraction of the Ark Pharm was 93.5%. The Equine Factor Powder (EFP) supplement was labeled as 99.87% *trans*-resveratrol and the average percent recovery of 67.6% per gram. The ZHOU *trans*-resveratrol supplement was labeled as 50% *trans*-resveratrol and an average of 80.4% of the available resveratrol was obtained for the ZHOU supplements. Proton NMR and LC-MS were used to confirm the identity and determine the purity of the extracts.

All of the ^{1}H NMR spectra displayed the characteristic peaks for *trans*-resveratrol (Figure 4). The spectra for the Equine Factor supplement showed minor amounts of deuterium exchange with the phenolic functional groups that are present on resveratrol (Figure 4a).

Characterization of the ZHOU and EFP supplements via MS analysis resulted in expected spectra (Figure 5). The values for m/z at 134 and 211 as well as the parent peak were observed for both of these samples.

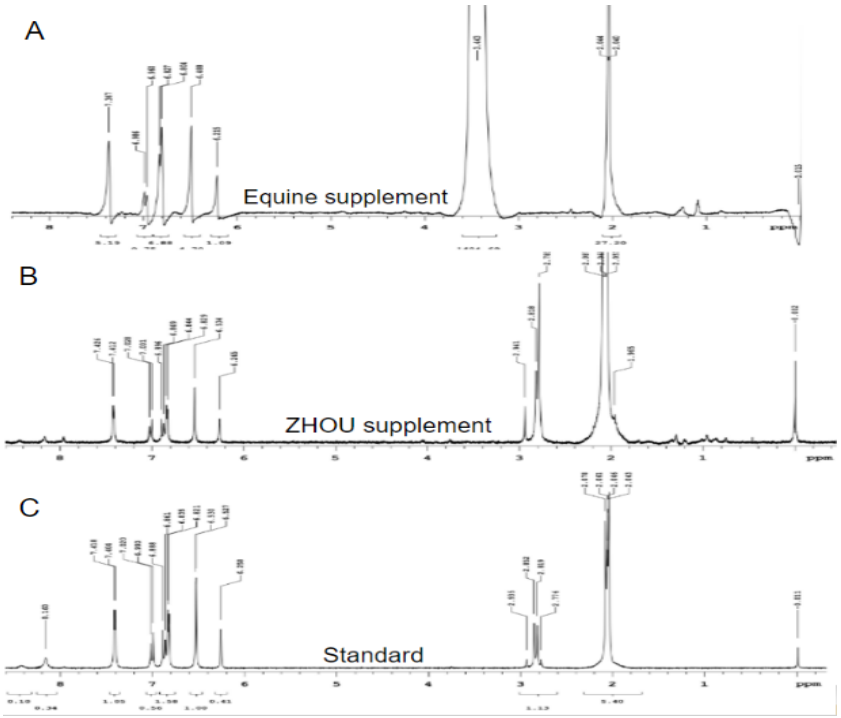


Figure 4: The H^{1}NMR spectra for *trans*-resveratrol are included for the Equine Factor Supplement (A), the ZHOU Supplement (B), and the Ark Pharm standard (C).

Comparison of the extracts to the Ark Pharms standard that was not subject to extraction or heat resulted in matching spectra (NMR, MS). Although both the EFP and ZHOU resveratrol samples looked very similar, the available *trans*-resveratrol recovery was very different for the two sources with the ZHOU material more accurately matching the labeled quantity. The remaining material isolated was not further characterized.

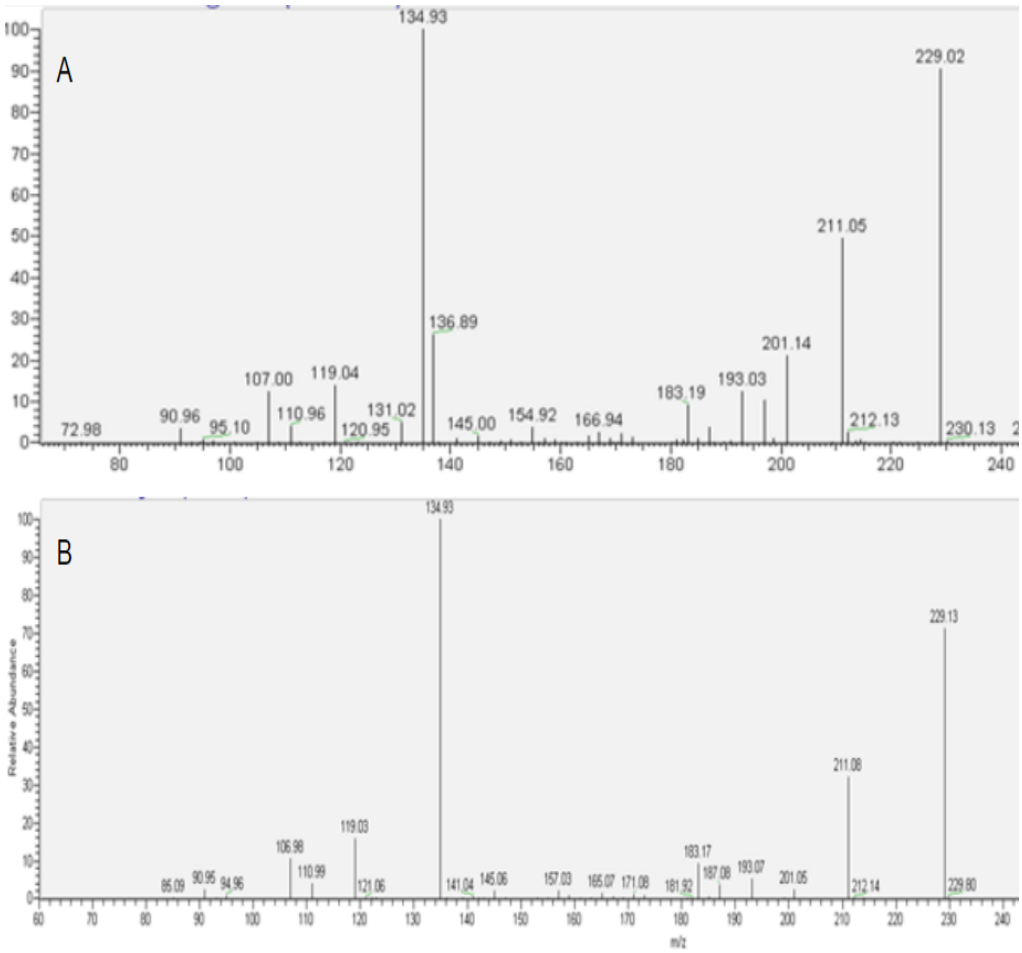


Figure 5: The mass spec results from the gravity filtered ZHOU Supplement (A), and the Equine Factor Supplement (B).

6. Results, Soxhlet extraction

The Soxhlet extraction of the EFP resulted in only a slightly higher recovery (approximately 10%) compared to the extraction procedure using benchtop extraction with stirring. The recovered product was identical spectroscopically to the material obtained in 1-2 h benchtop extraction. Although the greater recovery could be attributed to longer contact times, it could also be attributed to sample size error. As this technique was only investigated once, the benefits of using Soxhlet extraction of the raw materials for recovery is not conclusive.

7. Extraction of *trans*-resveratrol from oil containing supplements using acid base separation

Although resveratrol with a partition coefficient of P_{OW} =3.10 is not highly soluble in oils, the addition of an oil complicates the isolation of the desired product. As a polyphenol, resveratrol should be deprotonated and thus water soluble when exposed to pH's above 10. Following the above protocol, the ^{1}H NMR of the isolated is shown in Figure 6. The phenolic protons were sharper and more intense, but the spectra are essentially identical.

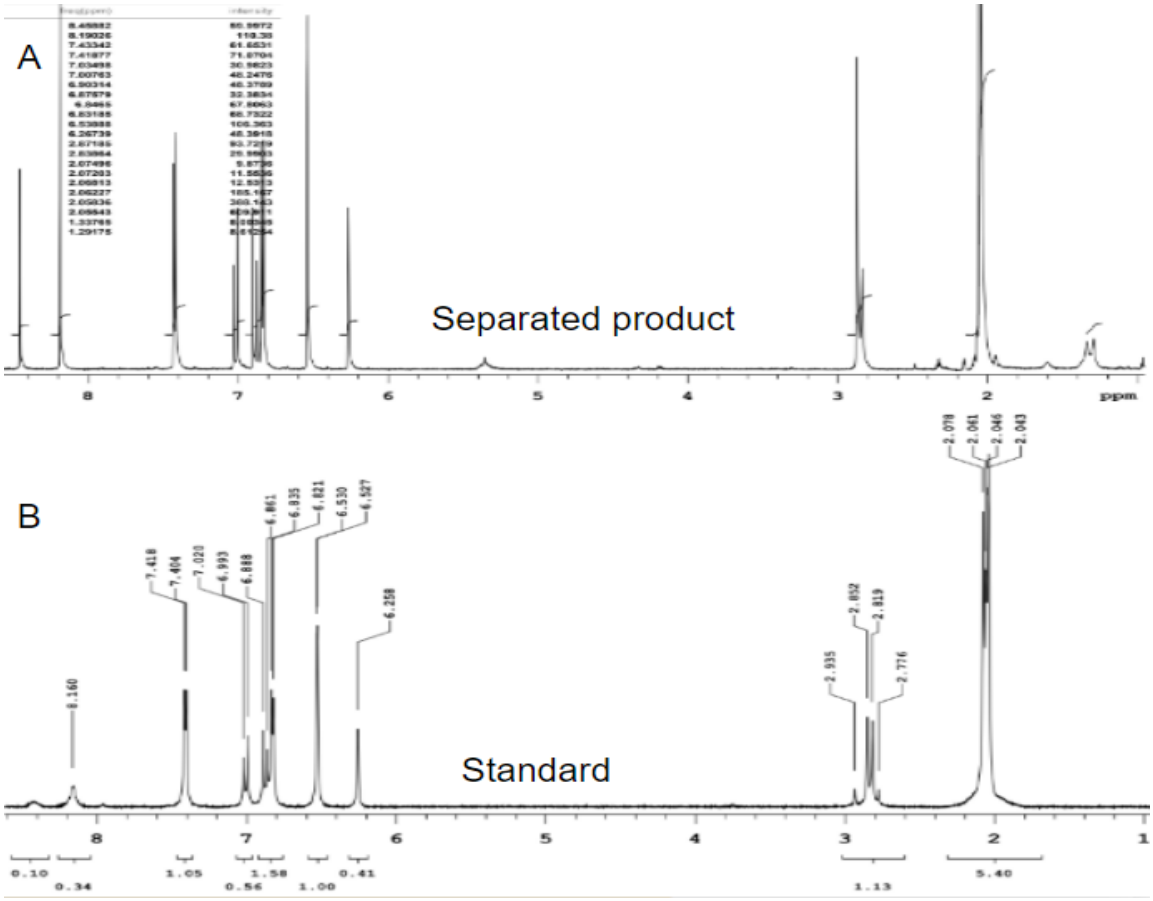


Figure 6: Comparative ^{1}H NMR results of the dried solid from liquid-liquid extraction (A), and Ark Pharm *trans*-resveratrol standard (B).

The dried product from this isolation procedure was a white solid with minimal color. This liquid-liquid the isolation procedure resulted in a recovery of about 32.4% of the available resveratrol. This is a single result and needs to be replicated.

8. Color Change of *Trans*-resveratrol

During liquid-liquid extraction, a very noticeable color change of the aqueous solution occurred that was pH dependent. This color change was reversible. The initial appearance of a 95% ethanol solution of resveratrol was water white. Addition of 10 % NaOH to this mixture turns the solution to a brown and then green color (Figure 7). Addition of concentrated HCl to the presumably deprotonated resveratrol changes the color of the ethanolic solution from green to red in color via the brown color. Direct addition of concentrated HCl to the initial ethanol solution of resveratrol causes a precipitate. Only after resveratrol has been deprotonated by addition of aqueous NaOH, does the solution appear red.

Similar color changes were observed when different acids and bases were used including sulfuric acid, phosphoric acid, acetic acid, and potassium hydroxide. The only observable difference when using different acids and bases was the concentration required to induce color change. Experimenting with different solvents including methanol and acetone showed consistent results. Further investigations are ongoing.

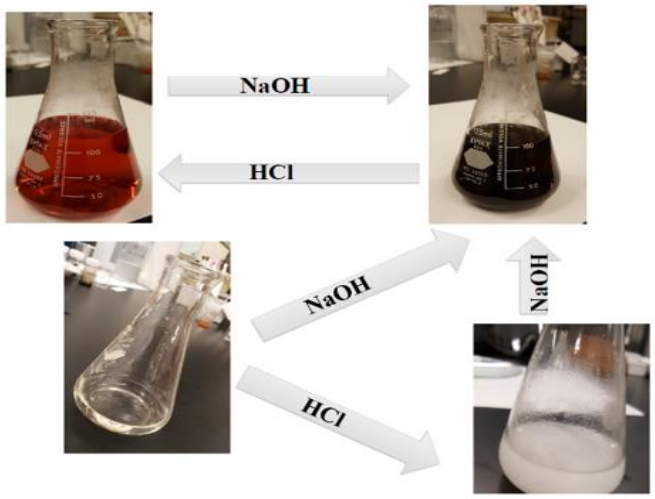


Figure 7: *trans*-resveratrol reacting to changes in pH. Solution of 95% ethanol and Ark Pharm *trans*-resveratrol standard (lower left), direct addition of HCl to resveratrol/ethanol solution (lower right), addition of NaOH to resveratrol/ethanol solution (upper right), addition of HCl to alkaline resveratrol/ethanol solution (upper left).

9. Conclusions

Powdered commercial supplements of *trans*-resveratrol were labeled as containing between 80 and 99% resveratrol. Two solid liquid extraction methods that could reproducibly isolate 93% of a known standard of resveratrol were developed. The isolated resveratrol from the commercial materials was 50-67% of the available resveratrol. Comparing the two solid material extraction methods, it appears that the Soxhlet extraction method does not add significantly to the amount of recovered *trans*-resveratrol. Stirring the powdered extract for one hour at room temperature resulted in approximately the same recovery as extracting the solid samples using Soxhlet extraction (96 h).

A standard protocol to determine the recovery of resveratrol from an oil supplement was initiated. Using acid base/chemistry and extraction, resveratrol could be isolated from a sample and quantified. Isolated *trans*-resveratrol from these supplements was approximately 32% of the labeled resveratrol content. The recovery of resveratrol from an oil-based sample was not optimized at this time. During the extraction procedure, a series of reproducible color changes dependent on pH were observed. Further studies are ongoing.

10. References

1. King, Robert E.; Bomser, Joshua A.; Min, David B. Comprehensive Reviews in Food Science and Food Safety **2006**, *5*, 65-70. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1541-4337.2006.00001.x

2. Chen, H.; Tuck, T. Ji, X., Xin, Z.; Kelly, G.; Cuerrier, A.; Zhang, J. Quality Assessment of Japanese Knotweed (Fallopia japonica) Grown on Prince Edward Island as a Source of Resveratrol *Journal of Agricultural and Food Chemistry* **2013** *61* (26), 6383-6392.

3. Neves, A. R.; Lucio, M.; Lima, J.L.C.; Reis, S.; Resveratrol in medicinal chemistry: a critical review of its pharmacokinetics, drug-delivery, and membrane interactions, *Curr. Med. Chem*. **2012**, *19*, 1663–1681.

4. a.) de Ghellinck, A., Shen, C., Fragneto, G. & Klosgen, B. Probing the position of resveratrol in lipid bilayers: A neutron reactivity study. *Colloids and surfaces. B, Biointerfaces,* ***2015****,134*, 65–72. b.) Longo, E., Ciuchi, F., Guzzi, R., Rizzuti, B. & Bartucci, R. Resveratrol induces chain interdigitation in DPPC cell membrane model systems. *Colloids and surfaces. B, Biointerfaces,* **2016**, *148*, 615–621. c.) Neves, A. R., Nunes, C., Amenitsch, H. & Reis, S. Effects of resveratrol on the structure and fluidity of lipid bilayers: a membrane biophysical study. *Soft matter* **2016**, *12*, 2118–2126.

5. a.) Jensen, M. D. *et al*. Involvement of oxidative pathways in cytokine-induced secretory phospholipase A2-IIA in astrocytes. *Neurochemistry International* **2009,** *55*, 362–368. b.) Sun, G. Y. *et al*. Phospholipases A2 and in ammatory responses in the central nervous system. *Neuromolecular medicine* **2010**, *12*, 133–148. c.) Yarla, N. S. *et al*. Targeting arachidonic acid pathway by natural products for cancer prevention and therapy. *Seminars in Cancer Biology* **2016**, *40-41*, 48–81.

6. a.) PubChem Data Base https://pubchem.ncbi.nlm.nih.gov/compound/resveratrol - section=Top b.) Fei, Q.; Kent, D.; Botello-Smith, W. M.; Nur, F.; Nur, S.; Alsamarah, A.; Chatterjee, P.; Lambros, M.; Luo, Y. Molecular Mechanism of Resveratrol's Lipid Membrane Protection. Scientific Reports, **2018**, *8*, Article number: 1587.

7. Walle, T.; Hsieh, F.; DeLegge, M.H.; Oatis Jr, J. E.; Walle, U. K. High Absorption But Very Low Bioavailability of Oral Resveratrol in Humans, Drug Metabolism and Disposition, **2004**, *32*, 1377-1382.

8. Trela, B. C.; Waterhouse, A. L. Resveratrol: Isomeric Molar Absorptivities and Stability. *Journal of Agricultural and Food Chemistry* **1996,** *44*, 1253-1257.

9. Burns, J.; Yokota, T.; Ashiroshi, H.; Lean, M. E. J.; Crozier, A. Plant, Foods and Herbal Sources of Resveratrol, J. Agric. Food Chem. 2002, 50, 3337−3340.

10. U.S. Food & Drug Administration. *Dietary Supplements Guidance Documents & Regulatory Information.* https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/DietarySupplements/default.htm (9/6/2018).