Research Article

OPTIMIZATION OF SOLVENTS FOR THE EXTRACTION AND METHODS FOR QUANTIFICATION OF VITAMIN E FROM SOYMEAL

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ABSTRACT

Extraction of vitamin E from soymeal (the product obtained after oil extraction) was carried out using six different solvents viz., Methanol, n-hexane, 2-Methyltetrahydrofuran, Methanol:n-hexane:2-MeTHF (80:10:10), 0.1 N H_2SO_4 followed by ethanol extraction and Petroleum ether: ethanol (2:1.6) and two spectrophotometric methods: Bathophenanthroline and Ammonium Phosphor Molybdate (APM) methods. The results showed significantly higher yield of Vit-E (7.5 gm/100gm) with a recovery percentage of 83.3% when extracted using 0.1N sulfuric acid treatment followed by ethanol. These values were further validated by HPLC quantification. Whereas bathophenanthroline method was found more suitable method to quantify Vit-E extracted from oil samples using non polar solvents (data not shown). Thus, our study helps to select the suitable solvents for the extraction of Vit-E and the selection of solvents depends on the type of the sample (oil or powder form). Further our study also revealed that, methods for the estimation of Vit-E should be carefully selected as the extraction of Vit-E is related to the type of solvents (polar and non-polar) used.

INTRODUCTION

Vitamin E (Vit-E) is a lipid soluble antioxidant, essential for health as it interrupts the propagation of reactive oxygen species that spread through biological membrane (Choe eunok *et al.,* 2009). Vit-E which protects cell membranes against damage caused by free radicals thus acts as a radical scavenger by delivering an H atom to free radicals. Vit-E composed of both tocopherols and tocotrienols, which occurs naturally in eight different forms i.e., α-, β-, γ-, δ-tocopherols and tocotrienols respectively (Brigelius-Flohe and Traber, 1999). Each of these different compounds has distinct chemistries and biological effects. Vit-E is necessary for structural and functional maintenance of skeletal, cardiac and smooth muscle and helps in preventing diseases of the heart and blood vessels including hardening of the arteries, heart attack, chest pain, leg pain due to blocked arteries, and high blood pressure (Miller *et al.,* 2005). It also assists in the formation of red blood cells and helps to maintain the stores of vitamins A and K, iron, and selenium (Bjelakovic *et al.,* 2013). Vit-E is sold as dietary supplements either by itself or incorporated into a multi-vitamin product (Sidgwick *et al.,* 2015). Vit-E deficiency can cause peripheral neuropathy, myopathies, ataxia, retinopathy, impartment of immune response and red blood cell destruction (Whitney and Rolfe's, 2011). α tocopherols is the most common form of Vit-E present in most of vegetable oils, nuts, oil seeds, egg yolk, margarine, cheese, soya beans, wheat germ (Micronutrient Information Center, 2017; Bieri and Evarts, 1974; Reboul *et al.,* 2006*).* The daily recommended dietary allowance of Vit-E for adults is 15mg/day (22.4 International units) (Institute of Medicine, 2000*).* Market size generated for natural Vit-E is over USD 820.18 million in 2017 and is set witness over 5.1% CAGR (Compound Annual Growth Rate) during 2017-2022 period (Global natural Vit-E market, 2017). Tocopherol is expected to lead in terms of demand as well as in terms of being the highest revenue contributor due to its needed for heart and cognitive health. Since tocotrienols are difficult to absorb during digestion and are poorly distributed to blood cells, tocopherols are in high demand use in dietary supplements. Asia-pacific is expected to have the highest growth rate, during 2017-2022, due to the growing preference for healthy foods. The North American region has the highest share of natural Vit-E market, followed by Europe. The natural Vit-E has a variety of applications, such as it is used as a dietary supplements, fortified/ functional food and beverages, pharmaceuticals, cosmetics, animal feed.

There are several methods available to estimate Vit-E from various food, pharmaceuticals and plant samples. Out of them are HPLC, Gas Chromatography, Colorimetric, Spectrophotometric, fluorometric and Gas-Liquid

Chromatography (Moszczyński and Pyć, 1999), still burdened with various faults and limitations. Although HPLC method of Vit-E estimation is quite precise and reliable but is also an expensive and not readily available in every laboratory. Colorimetric and fluorometric methods are easily carried out using common laboratory equipment but it consumes more time (Desai, 1984). Spectrophotometry based method of Vit-E are quick, inexpensive and efficient as well as applicable to semi-micro scale tests (Rutkowski and Grzegorczyk, 2007). The extraction of Vit-E and its estimation by phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate /Mo (V) complex at acidic pH. (Prieto *et al.*, 1999). Another spectrophotometric method for estimation of Vit-E is bathophenanthroline method which is based on reduction of ferric chloride to ferrous state by the formation of red color (Tsen, 1961; Desai and Machlin, 1985; Hashim and Schuttringer, 1966). The above two methods are selected for determination of Vit-E because of it selective, fast, accurate and simultaneously not excessively time- and work consuming, being used for small sample volume. Solvents are usually volatile organic compounds (VOCs) sourced from non-renewable resources. They are also usually harmful to health and the environment (Hansen *et al.*, 2007). Research of greener, biodegradable and non-dangerous solvents for tocopherol has become a major concern for researcher/ industrialists (Fine *et al.*, 2013). Me, produced from biomass like corncobs, sugar cane bagasse or oat hulls, is one of these "green" and bio-based solvents and can be degraded by solar light and air (Pace *et al.*, 2012). It possesses a polar aprotic chemical characteristic and also the boiling point and evaporation heat of MeTHF are similar to hexane (PennAKem., 2013). Studies show that 2-methyltetrahydrofuran (2-MeTHF), appears to be a promising alternative to n-hexane for the extraction of Vit-E from vegetable oils (Pace, 2012). Depending upon their solubility and polarity, six different solvents namely 2-methyl tetra hydro furan (2-MeTHF, green solvent), methanol, 0.1 N Sulphur acid treatment followed by ethanol extraction, hexane, petroleum ether: ethanol (2:1.6) and Methanol: Hexane: MeTHF (80:10:10) were chosen for extraction of Vit-E from soy meal to estimate its content further by using two spectrophotometric methods viz. phosphomolybdate and bathophenanthroline. Further the results were validated by a standard HPLC method.

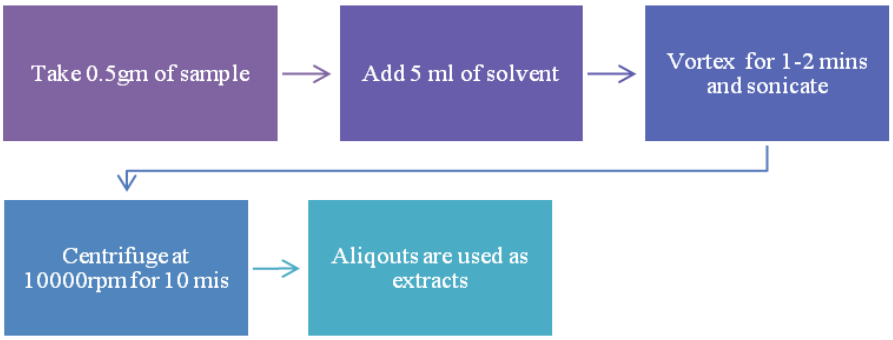

Figure 1: Schematic diagram for the extraction of vitamin E using different solvents

MATERIALS AND METHODS

Chemical and Reagents

Tocopherols standards, ammonium molybdate, Bathophenanthroline reagent, sulphuric acid were purchased from Sigma (St. Louis, MO). All HPLC grade solvents including methanol, acetronitrile, n- hexane, MeTHF, ethanol and other chemicals were purchased from Merck.

Sample and Standards Preparation

Soy meal (oil distillate) was obtained after oil extraction from soybean seeds in the lab. α, δ and γ-tocopherol standards were dissolved in HPLC grade ethanol to get 1000 ppm stock concentration and different dilutions were further prepared from them.

Direct Solvent Extraction

Direct Solvent extraction is a simple procedure to extract Vit-E from various crop samples. The extract may be used directly after being dissolved in the mobile phase and filtered, or after purification step. There are no universal solvents that would yield optimal results in all materials. Solvent extraction is one of major commercial importance to the chemical and biochemical industries, as it is often the efficient method of separation of valuable products from commercial feed stock.

0.5 gm of soymeal sample was weighed and 5ml of solvents (Hexane, Methanol, 2-MeTHF, Petroleum ether: ethanol (2:1.6), Methanol: n-hexane: MeTHF (80:10:10) were added individually into each tubes and vortexed, followed by sonication at medium frequency at 15 sec intervals for 5 min in ice and the sample was incubated overnight at 4 ^{o}C.

Centrifugation was carried out at 10,000 rpm at 4ºC for 10 minutes and the supernatant was collected, resuspended the pellet in each of the above solvents mentioned and stored at 4ºC till further use.

Extraction using 0.1N H_2SO_4

Sample (soymeal–2.5 g) was homogenized in small volume of 0.1N H_2SO_4 and volume was made upto 50 ml by adding 0.1N H_2SO_4 slowly without any shaking in a conical flask to allow stand for overnight at room temperature. Contents of the flask were shaken vigorously using shaker on the next day for 15 min. and filtered through Whatman No.1 filter paper. Aliquots were stored in 4˚C for further estimation. During estimation, 1.5 ml of extract of sample was taken and 1.5ml ethanol was added. Then centrifuged at 10000 rpm for 10 mins.1 ml of upper layer was used for vitamin E estimation.

Estimation of Vitamin E

The extracts were estimated for the amount of vitamin E present in each extract by following two spectrophotometric methods (i) Ammonium phosphor molybdate method and (ii) bathophenanthroline method. Ammonium phosphomolybdate reagent: Dissolve 0.494gm of ammonium molybdate in little amount of water. Add 28ml of 100mM PO_4 buffer followed by addition of 3.26ml of H_2SO_4 drop by drop in the above solution and make up the volume to 100 ml. This method is based upon the reduction of Mo(VI) to Mo(V) by the sample analyte (Vit E) and subsequent formation of green phosphate / Mo(V) complex at acidic pH. 2.0 ml of the Phosphomolybdenum reagent was added to 1.0 ml of the extracted sample and different standards amount in test tubes, mixed and vortexed briefly. The tubes were shaken vigorously for 90mins in dark at room temperature (37˚C). Absorbance of the aqueous phase was measured at 695nm against the appropriate reagent blank containing 1 ml of the solvent and 2 ml of the reagent solution incubated under same condition as for the samples.

Different sample and standards aliquots were taken in test tubes and volume was made to 1 ml. using absolute ethanol followed by addition of 0.5ml each of 6mM alcoholic bathophenanthroline reagent and 1mM anhydrous ferric chloride ($FeCl_3$) to vortex briefly. 0.5 ml of 40mM orthophosphoric acid was added after 15 sec to stabilize the red colour produced. Absorbance of solutions was taken at 534nm after 3 min against a reagent blank.

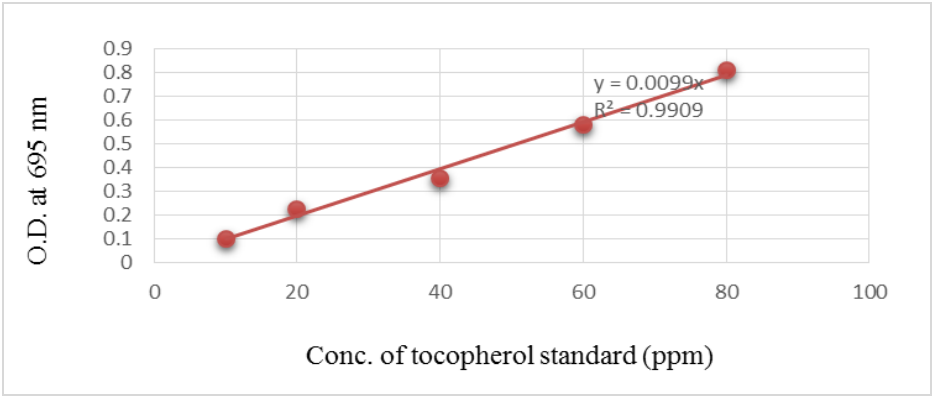

Figure 2: Standard graph for ammonium phosphor molybdenum method

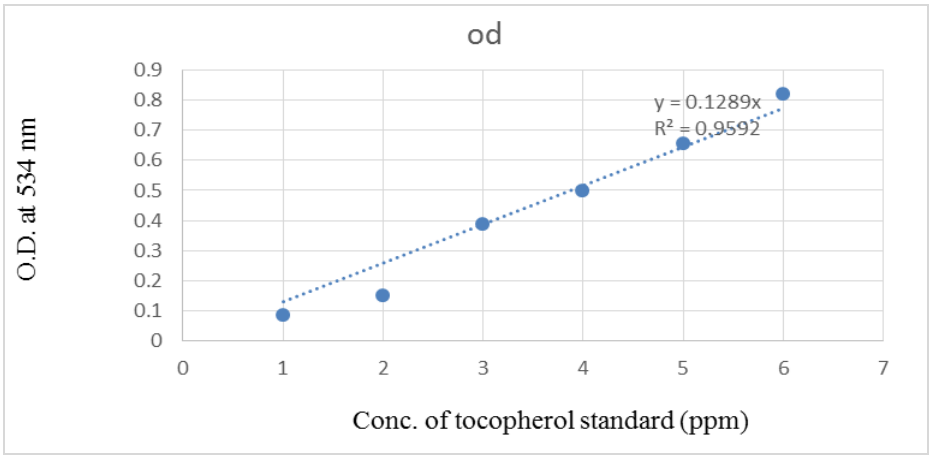

Figure 3: Standard graph for bathophenanthroline method

HPLC Quantification:

Reverse phase HPLC (C-18) system (Agilent) was used to quantify the vit E content and also in validating the results obtained by two spectrophotometric methods. Mix of acetonitrile and methanol (50:50 v/v) was used as a mobile phase at flow rate of 1 ml.min with column temperature 40˚C. Separations of peaks were achieved by injecting 20µl of the sample at wavelength of 295 nm.

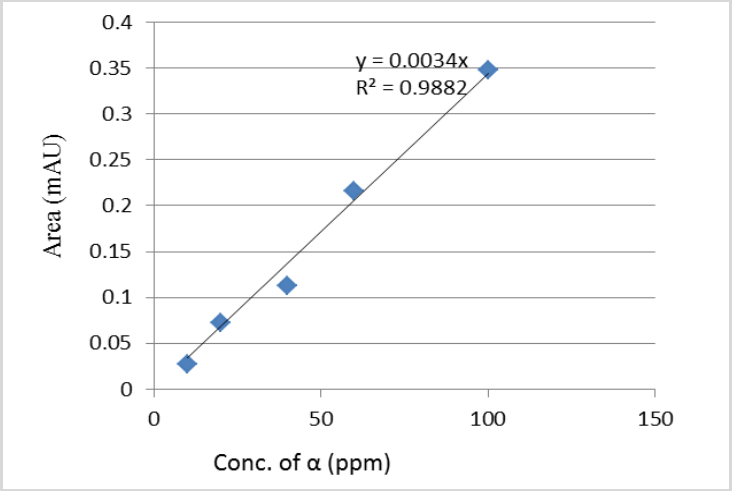

Figure 4: Standard graph for alpha tocopherol

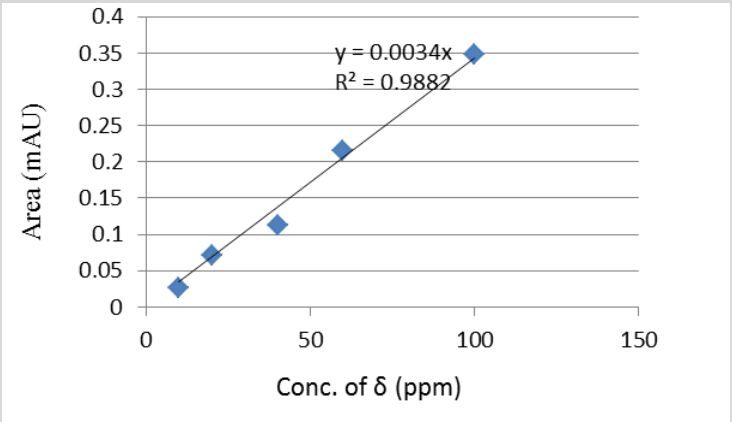

Figure 5: Standard graph for δ-tocopherol

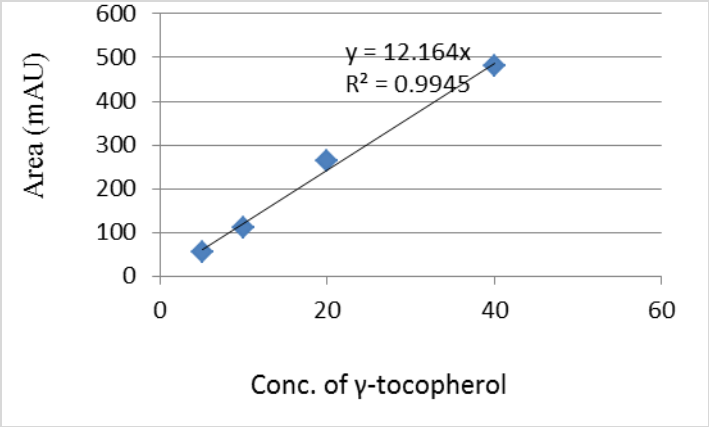

Figure 6: Std graph for γ-tocopherol

RESULTS AND DISCUSSION

Extraction of vit E using various solvents and its estimation using two spectrophotometric methods were carried out in order to found the best solvent to extract Vit-E in high amount as well as in cost and time effective manner. Six different types of solvents n-Hexane, Methanol, Methanol : Hexane : MeTHF (80:10:10, v/v), Petroleum ether : Ethanol (2:1.6), MeTHF and 0.1N sulfuric acid treatment followed by ethanol extraction were used in order to extract Vit-E from soy meal. The results were validated by using HPLC method of quantification and were compared with reported values from other studies.

Table 1: Vitamin E content (gm/100gm)) in soy meal extracted by direct solvent method and estimated by a) Ammonium molybdate method b) Bathophenanthroline method c) HPLC

Method of Vit-E estimation	Report-ted Vit-E values in *gm/100gm*	Vit-E content in soymeal from different solvents in *gm/100gm*					
		MeTHF	Hexane	Methanol	0.1 N sulphuric acid plus ethanol	PE:Ethanol (2:1.6)	M:H:MeTHF (8:1:1)
Phosphomolybdate		6.5	6.8	5.2	7.5	6.2	6.4
Bathophenanthroline	9.0	5.9	6.0	5.4	5.0	5.1	6.1
HPLC		6.2	6.5	5.5	7.8	6.8	6.0

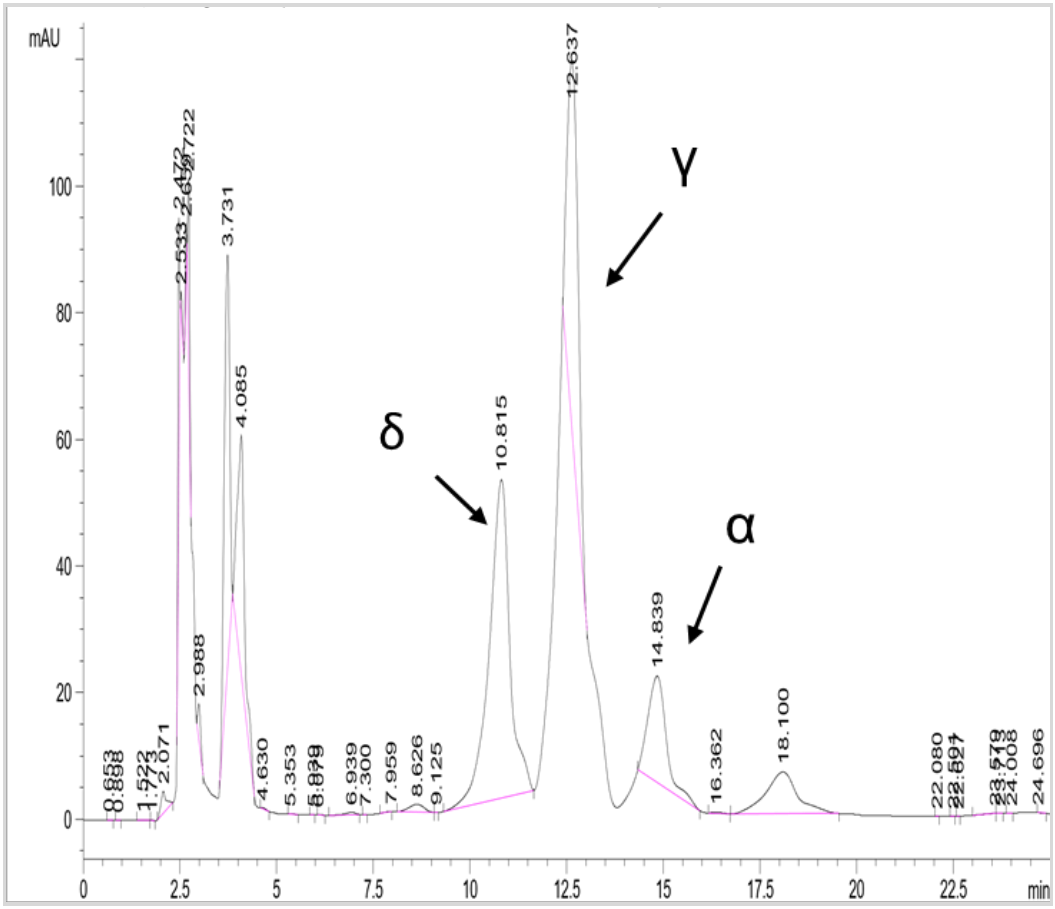

Figure 7: HPLC chromatogram for soymeal tocopherols extracted in 0.1N sulfuric acid plus ethanol

In bathophenanthroline method, formation of red colored complex was used to estimate Vit-E since the analyte Vit-E reduces ferric to ferrous ion which is responsible for color formation. Unlike bathophenanthroline method, Vit-E was estimated by the formation of green coloured complex due to the reduction of Mo (IV) to Mo (V) ion in the ammonium

molybdate complex by Vit-E in ammonium phosphomolybdate method. Amongst the above two methods, ammonium phosphomolybdate method was found better for Vit-E estimation extracted from soymeal whereas bathophenanthroline method was found more suitable method for estimation of Vit-E extracted from oil (data not shown).In APM method, 0.1N sulfuric acid treatment followed by solvent extraction yield highest amount of Vit-E from soymeal (7.5 gm/100gm) with a recovery percentage of 83.3% which was validated by HPLC quantification whereas methanol extraction showed the lowest amount of Vit-E (5.2gm/100gm) with 57.8% recovery. In bathophenanthroline method, M:H:MeTHF extracted the highest amount of Vit-E from soymeal (6.1gm/100gm) in comparison with the reported values.

In ammonium phosphomolybdate method, the formation of green coloured upper phase occurs in solvents such as n-hexane, M:H:MeTHF, 0.1N sulfuric acid treatment followed by ethanol extraction, Petroleum ether: ethanol while there is no occurrence of such phase in methanol and M:H:MeTHF solvents since the methanol was found soluble in ammonium phosphomolybdate complex.

Since ammonium phosphomolybdate complex reacts well polar solvents, high amount of vit-E was obtained in 0.1N sulphuric acid treatment followed by ethanol solvent extraction when estimated using APM method. Also, that, when we used0.1N sulfuric acid treatment followed by solvent extraction, the values obtained by APM method was very high and it might also due to the fact that the ammonium phosphomolybdate complex reacts well under acidic pH conditions.

CONCLUSION

Simple, sensitive, highly accurate spectrophotometric methods for determination of Vit-E from soymeal were optimized. The highest percentage of recovery of Vit-E was achieved in 0.1N sulfuric acid followed by ethanol extraction by ammonium phosphomolybdate spectrophotometric method.

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