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Fabrication of Food grade Vitamin E nanoemulsion by low energy approach, characterization and its application

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ABSTRACT

The present study was carried out to fabricate the food grade vitamin E acetate nanoemulsion (NE) using edible mustard oil and to evaluate its improved bioactivities. A food-grade vitamin E acetate nanoemulsion was fabricated using the edible mustard oil and surfactant tween-80 and flocculation was not observed for 15 days. The nanoemulsion was characterized for droplet morphology and size distribution using atomic force microscope and zetasizer respectively. We observe a stable nanoemulsion of spherical morphology and a size distribution of 86.45 ± 3.61 nm. Further, the HPLC method was used to determine the vitamin E acetate concentration and encapsulation efficiency for the stable nanoemulsion. These nanoemulsions showed improved bioactivity - antioxidant and antimicrobial activity and could be potentially used to increase the shelf life of fruit juice.

Key words: Vitamin E acetate, Food grade nanoemulsion, Encapsulation efficiency, Antioxidant, Shelf life.

INTRODUCTION

Recently, the interest to use NEs in food, beverage, and pharmaceutical industries are growing due to their potential advantages over conventional emulsions. These NEs contain droplets size varied from 20-200 nm [1, 2]. Additionally, NEs can act as a carrier system for delivering various functional lipophilic compounds - i.e. nutraceuticals, drugs, antioxidants, flavors and anti-microbial agents, thus increases their bioavailability [3, 4]. The food and beverage industries are interested in preparing such colloidal delivery systems where functional components can be delivered through easy and efficient method. NEs are designed to possess high stability towards particle aggregation and gravitational separation, showing improved and efficient activity, mainly antimicrobial activity. Therefore, it can be used in in food industries to extend the shelf life of commercially available food products. Based on the need to design products with novel textural attributes, NEs can also be modified by using different types of emulsifiers and oil phase or by opting different fabricating protocols [5]. Several bioactive compounds were nano-encapsulated to get higher bioactivity but vitamin E based food grade NE is least explored. α-tocopherol is the main component of vitamin E, which possesses antioxidant property and is fat-soluble. These properties are responsible for protecting cell membranes against peroxidation. The antioxidant property of vitamin E can be exploited to prevent chronic diseases such as cardiovascular diseases, arthrosclerosis and cancer[6].It scavenges the free radicals and molecular oxygen, thus protects polyunsaturated fatty acids (PUFAs) and lipoproteins from peroxidation.

Being lipophilic, it accumulates in lipoproteins, cell membranes and fatty deposits [7]. Vitamin-E (α-tocopherol) is prone to oxidation because of its polyunsaturated structure **(Figure 1)** and thus it is also used in its esterified form: Vitamin-E acetate (α-tocopherol acetate) [5].

NEs are thermodynamically stable system of two phases consisting of at least two immiscible liquids with droplet size in nano range. One phase is dispersed throughout the other in the form of small droplets with the help of an emulsifying agent. The dispersed liquid is known as the internal or discontinuous phase, whereas the dispersion medium is known as the external or continuous phase [4, 8]. High energy and low energy approaches are the two major ways for the formulation of NE. Industries are preferably using high energy equipments that are capable of generating intense mechanical forces which can disrupt and fuse the oil and water phases. Usually, homogenizers or ultrasonicators are used to impart external forces that help in formulation of NE[9]. Main factors that influence the size of NE include energy intensity and duration, the surfactant type and concentration, and the physicochemical properties of the oil and water phases [8, 10]. Moreover, low energy approaches make use of spontaneous formation of emulsions either by altering the concentration of oil, water or surfactant or physical parameters such as temperature or pH. However, low energy approaches are not fully exploited at industrial use, even though it is regarded as better than the high energy approaches at laboratory scale [11].

NE based systems are valuable delivery vehicles as long as they are stable and have acceptable release property at target site. Thus, it is a necessary step to analyze the encapsulation efficiency and release property while emulsifying active compounds. In the present study, vitamin E acetate was nanoemulsified using food grade mustard oil and characterized by dynamic light scattering

(DLS) and atomic force microscopy (AFM). Encapsulation efficiency and release property of vitamin E nanoemulsion was further analyzed by HPLC method to calculate free and bound Vitamin E. Further, the antioxidant and antimicrobial properties were studied and the NE was used to increase the shelf life of fruit juice.

MATERIALS AND METHODS

NE was prepared using Mustard oil, Tween-80 (surfactant) and Vitamin-E acetate. Mangoes were purchased from the local market of Vellore. All the chemicals used in the experiment were as per the national institute of standards and technology (NIST). The following solvents; methanol, iso-propanol, tetrahydrofuran (THF) were purchased from Sigma Aldrich (Bangalore, India). All other chemicals, drugs and solvents were of analytical grade and procured from Merck (India) and HiMedia (India).

Preparation of Nanoemulsion

NE was fabricated by wash-out method [12]. In this method, water phase wasadded continuously to the mixture of oil phase (mustard oil), surfactant (tween-80) and the functional compound (Vitamin E acetate). Various concentrations of surfactant and oil were used as described in the **table 1** and further the conditions were optimized for stability. Initially, a mixture of surfactant tween-80 (5.0%w/w) and vitamin E acetate (2.0 % w/w) were dissolved in the mustard oil phase (3.0 % w/w) such that vitamin E completely gets solubilized in the oil phase with the help of the surfactant. The preparedmixture and water were preheated separately at 75± 2℃. Water phase

was slowly added drop by drop to the oil phase containing surfactant and functional compound using magnetic stirrer. To obtain homogeneity throughout the NE,the mixture was centrifugedat 400 rpm for 15 min at 25℃.

Stability study

Intrinsic stability of NEs was investigated by storing them at room temperature. It was then observed visually for instability -phase separation or creaming or flocculation. The stable sample was further analyzed for physicochemical characterization.

Droplet size distribution and zeta potential

Particle size distribution was determined by dynamic laser light scattering method using Malvern ZetasizerNano ZS90 (Malvern Instruments, UK). Zeta potential, the electrical charge on the oil droplets in the emulsions was determined under holder temperature of 25° C and electrical voltage 3.9 V.

Atomic Force Microscope (AFM)

To examine the detailed morphologyof the NE, a thin film of the samples were prepared on a glass slide by dropping 100 µL of the sample on the slide, and was allowed to dry. The slides were then scanned with the AFM (NanosurfEasyscan 2, Switzerland)

pH Measurement and absorption spectrum

The pH of formulated NE was measured as per the protocol described by Mayes et al.[5]. The absorption spectrum of NEs was recorded between 200 and 400 nm using UV-VIS-NIR Spectrophotometer (UV-3600, Shimadzu scientific instruments, Kyoto, Japan). UV scan testing was performed in order to verify the maximum absorption wavelength (λmax) for vitamin E acetate, mustard oil (oil phase), Tween-80 (surfactant) using methanol, iso-propanol and THF as solubilizing media. The objectives of this were to: (i) determine the strongest λ maximum which would then be used for the HPLC method, since different values were reported in the literature [13 – 16] (ii) quantify the vitamin E acetate by drawing the HPLC calibration curve; and (iii) verify possible interfering peaks (overlapping) of the oil phase constituents (which contains *e.g.* α-tocopherol) [1, 17] and of the surfactants on the drug detection peak. Samples were prepared by dissolving known amounts of vitamin E acetate, mustard oil, Tween-80 in methanol, iso-propanol and THF. The results were reported as mean ± SD (n = 3).

Calibration curve for determining vitamin E acetate

Solvent was selected as suggested and verified by Diane and Burgess, 2014**[1]**. Linearity of the detector response was verified using increasing amounts of vitamin E acetate (with three different initial standard solutions). Calibration solutions of formulated NE of different concentrations(0.5 to 100.0 mg/mL) were prepared. Peak areas and retention times were measured and a calibration curve was plotted. Linear regression analysis was carried out. The selectivity of the RP-HPLC method for the determination of vitamin E acetate NEs was

investigated at the retention times of the analyte[13]. The data are presented as mean values ± SD (n = 3). The significance difference was evaluated using Student's t-test and one way ANOVA (single factor) with the level of significance at p-value ≤ 0.05.

Vitamin E acetate analysis by HPLC

Vitamin E acetate was analyzed using the RP-HPLC method. The optimal HPLC conditions determined were as follows: mobile phase iso-propanol: methanol: THF (47.6:47.6:4.8) with 1% of phosphoric acid solution (10% v/v); flow rate = 0.7 mL/min; λ = 294.1 nm, injection volume = 20 µL; running time: 10 min. A Ascentis® C18 column (25cm x 4.6 mm) was used. Vitamin E acetate was detected using a UV/Vis detector. The mobile phase was filtered using a 0.45 mm filter (Type HVLP, Millipore) and sonicated for 30 minutes [1].

Determination of encapsulation efficiency

The encapsulation efficiency (EE) of vitamin E acetate in the NE was determined by ultafiltration, the method established by Diane and Burgess, 2014**[1]**. NE was subjected to centrifugal forces by submitting samples to 5000 × g for 30 minutes at 4 °C (Remi laboratory instruments, India). 4 mL was withdrawn from the pellet and diluted using iso-propanol. Subsequently, the samples were filtered using a microfilter of size (0.45 µm) and injected in the HPLC (as described above) for the estimation of vitamin E acetate concentration.

Anti-oxidant activity

Anti-oxidant activity test of the vitamin E acetate NE was undertaken to check whether it can act as an antioxidant if incorporated in food. To accomplish this, DPPH (α, α- diphenyl β-picrylhydrazyl)radical scavenging assay was performed. In this assay, 10 mL of DPPH (0.001 M) was prepared using methanol. 2mL of NE is mixed with 4mL of DPPH solution. The control contained 2 mL of methanol instead of NE. Both the test and the control were incubated in dark place for 30 min. to allow the reaction to occur. After this, absorbance was checked at 517nm [18, 19]. Free radical scavenging activity or antioxidant activity was calculated using formula 1:

$$\text{Antioxidant activity (\%)} = (AD - AE) * 100/AE \qquad \textbf{/Formula 1/}$$

Where AD = Absorbance of sample, AE = Absorbance of control

Shelf life of fruit juice

The influence of the NE on the preservation of fruit juice against microbiological spoilage was evaluated. Fresh mango juice (control) was prepared in the laboratory, and its microbial load was observed by plating one µL of juice onto nutrient agar plates to mark the population of microbes in the small amount of the juice[20]. The same was done for fortified nanoemusified mango juice (samples in 1:9, 2:8, 3:7, 4:6, 5:5 NE and control ratio). After every6 h,streaking was done until microbial growth was observed. Microbial growth was observed after incubation for 24 h at 37° C. Control and samples were kept in programmable environmental test chamber (Remi laboratory instruments, India) at 37°C with 70% humidity.

RESULTS AND DISCUSSION

Preparation of NE and its stability

Vitamin E is a lipophilic molecule, consists of α, β, γ, and δ derivatives of tocopherol and tocotrienol. However, its application in food and beverage industry as an active ingredient is limited due to its poor bioavailability. Vitamin E is highly sensitive to heat and oxygen and sparingly soluble in water. NE based systems can be used as a relatively safe method to overcome this problem. Emulsion-based systems can be fabricated using relatively simple processing operations and commercially viable ingredients [21]. Wash out method is an appreciable process for the preparation of NE. Vitamin-E acetate is only moderately soluble in water as it is a fat soluble vitamin. It has a high affinity for the internal oil phase due to its high partition coefficient [11]. Therefore to formulate o/w NEs, vitamin E is first completely dissolved in oil and surfactant tween-80 aids in this process. Then addition of water is accompanied by magnetic stirring to obtain a homogeneous solution of NE. This method provides uniformity and also allows the NE to be stable for 15 days at room temperature ($27^{O}C$). Vitamin E acetate o/w NEs were formulated and found to be stable when centrifugation was carried out at 400rpm. While for higher speed separation of phases were observed. No instability phenomena such as creaming or phase separation or flocculation were observed visually for a period of 15 days. This preparation was analyzed for further studies.

Droplet size distribution and zeta potential

The droplet diameter greatly affects the *in vitro* release capacity from the dispersed to continuous phases [12]. The average diameter for formulated NE (z-average) was found to be 86.45 ± 3.61 nm with the polydispersivity index (PDI) as 0.391 ± 0.43. The size distribution graph has been shown in the **Figure 2**. We observed that vitamin E acetate incorporated mustard oil NEmaintained a mean droplet size of 86.45 ± 3.61 nm before phase separation.

Shape analysis by Atomic Force Microscope (AFM)

The nanoemulsion formed was spherical in morphology as confirmed by AFM (**Figure 3)**. This shape is preferable while using for food applications.

pH measurement for formulated NE

The pH of the formulated NE was 5.45 which suggest that it was slightly acidic in nature. The NE can be successfully employed in fruit juice and beverage industry for the fortification of liquid foods having pH in the range 5-6. Further, the formulation properties of vitamin E acetate NE were determined using HPLC.

Determination of absorption spectrum

The λ maximum values were 286.0 ± 0.1 nm, 294.1 ± 0.2 nm and 285.0 ± 0.1 nm for vitamin E acetate in methanol, iso-propanol and THF solutions, respectively. Mustard oil and surfactants do not have absorption peaks (λ) in the spectrum range evaluated, demonstrating that mustard oil

constituents (e.g. α-tocopherol) in their usual concentrations do not have detectable absorption peaks that could overlap and interfere with vitamin E acetate detection. Accordingly, 294.1 nm was chosen for further HPLC analysis of vitamin E acetate.

Calibration curve for determining vitamin E acetate

The calibration curve was drawn from the HPLC graphs got for the standards (data not shown). The variance among the standard solutions was not significantly different and linearity was indicated by r^2-values close to unity. The selectivity of the method was indicated by vitamin E acetate retention times, 3.40 ± 0.02 min in iso-propanol solution.

Vitamin E acetate analysis by HPLC

The total concentration ofvitamin E acetate in o/w NE was identified using calibration curve which was found to be 17.57 ± 1.54 mg/mL.Thus, it can be inferred that the formulated NEcan be used for fortification in beverage industries as a health supplement.

Determination of encapsulation efficiency

For the industrial application of NE as a drug or active component; it is necessary to check for the free and bound drug. For this property we have analyzed encapsulation efficiency. Thus the matrix can be formulated accordingly to achieve desired release properties.Similarly, the free vitamin E acetate was determined by HPLC calibration curve and 0.06 ± 0.03 mg/mL of free vitamin E acetate was detected in o/w NE. Thus, the encapsulation efficiency was calculated as 99.65%.

Anti-oxidant activity

The Anti-oxidant activity was 62.55% as obtained by the DPPH radical scavenging assay. These NEs can act as preservatives for the liquid foods because of their higher antioxidant property as compared with mustard oil and vitamin E acetate in their native form. Antioxidant activity is attributed to the presence of vitamin-E acetate in the NEand can thus scavenge reactive oxygen species formed in the body during oxidation of the fats which can cause damage to the cells.

Shelf life of fruit juice after adding formulated nanoemulsion

Another important aspect of these NEs which make them fit for fortification of liquid foods is their ability to increase the shelf life of the fruit juices. Microbial growth was found at 6 h in control as well as fortified sample with ratio 1:9. Similarly, 12 h and 18 h were observed for fortified sample with 2:8 and 3:7 ratios respectively. Interestingly, shelf life of fortified sample (4:6 ratios) was found as 30 h and the same for 5:5 fortifications. Thus it suggests that NEs possess anti- bacterial properties. The reduction in microbial growth can be attributed because of use of mustard oil. The essential oil extracted from mustard comes under GRAS category (Generally Recognized as Safe) for food application based on 21 CFR (Code of Federal Regulations) part 182.20. The main constituent of mustard oil is allylisothiocyanate. It is a non-phenolic volatile compound which significantly inhibits growth of variety of pathogenic microorganisms even when used at low concentrations [22, 23, 24]. Notably, authors have detected more antioxidant and antimicrobial activities for vitamin E encapsulated NE than the mustard oil/water NE without vitamin E.

CONCLUSION

The present study identified a simple and convenient method to formulate spherical vitamin E NE using food-grade oil. The NE was stable at room temperature for 15 days. The average size diameter was found to be 86.45 ± 3.61 nm with the polydispersivity index (PDI) as 0.391 ± 0.43. Maximum absorption spectrum (λ max) was found at 294.1 nm by using UV/Vis spectrophotometer which was considered for further HPLC analysis and calibration curve was drawn for standard. From calibration curve a concentration of 17.57 ± 1.54 mg/mL vitamin E acetate was determined. Accordingly, this analytical method might also be applied for the detection and quantification of vitamin E, its esters and potentially other lipophilic vitamins in lipid formulations, and for quality control purposes. To use this NE at industrial level, it is necessary to analyze free and bound bioactive compounds; with this focus encapsulation efficiency has been analyzed by using HPLC and was found as 99.65% (~ 100%). DPPH radical scavenging assay shows the higher anti-oxidant activity of 62.55% for mustard oil-vitamin E acetate based NE; also, it has increased anti-bacterial activity and thus can be implemented for increasing shelf life of fruit juices. The formulated NE can be used in beverage industry as a health supplement. Additionally, this method gives the formulation, stability analysis, concentration determination by HPLC, encapsulation efficiency – which can also be studied for other lipophilic NEs. Further the results not only reveal how to formulate, characterize and analyze the free/bound bioactive compounds but also provideinformation on the applications of NEs which can be carried out for other NEs. Additionally, these properties have added new information to the current food grade mustard NE stability data, and will assist on the

development of vitamin E (NEs) and *in vitro* release testing methods for these formulations along with the applications.

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Table 1: Different trials to determine optimum concentration of surfactant and mustard oil to fabricate Nanoemulsion based on the flocculation and phase separation

Trials	Tween-80 (surfactant) (%w/w)	Vitamin-E acetate (%w/w)	Mustard oil (%w/w)	Water (5%w/w)	Phase separation	Flocculation
1.	3	2	5	90	yes	no
2.	4	2	4	90	yes	no
3.	5	2	3	90	no	no
4.	6	2	2	90	no	yes
5.	7	2	1	90	no	yes

Fig A.1: Structure of α-tocopherol

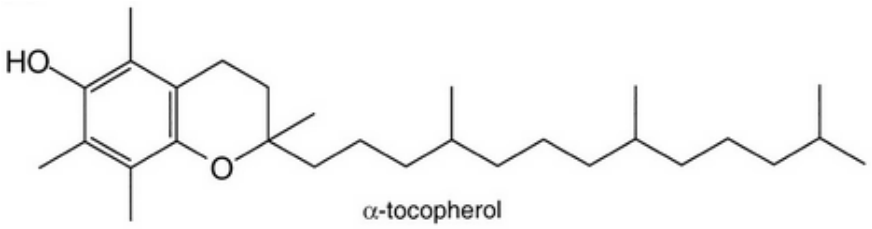


Fig A.2 Droplet size distribution of vitamin E acetate nanoemulsion formulated.

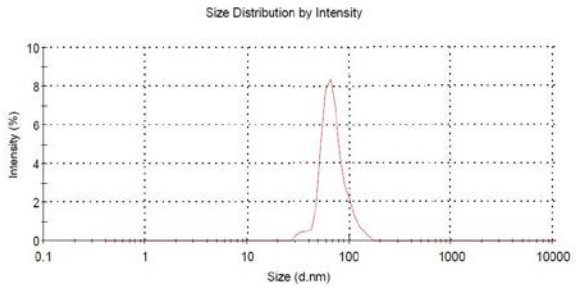
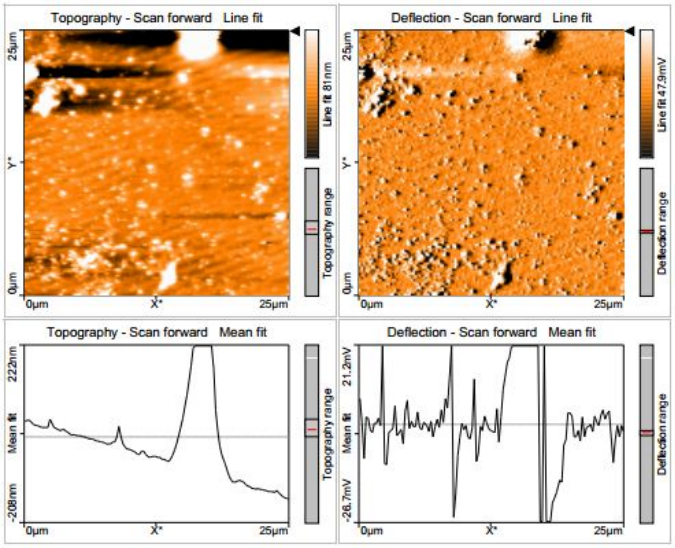


Fig A.3 AFM images of the formulated vitamin E acetate nanoemulsion.



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