



Research Article

STABILIZATION OF VITAMIN A USING VITAMIN E AS ANTIOXIDANT IN LYOPHILIZED AUTOLOGOUS SERUM AND ITS ANTIBACTERIAL PROPERTIES

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ABSTRACT

Dry eye syndrome is defined as an ocular surface disease caused by a lack of tear production or increased tear evaporation. Dry eye treatment with conventional drugs were less effective due to the nature and composition of the drugs that less akin to tears. Autologous serum is used as eyedrops in dry eye disease because it is natural and does not cause allergic reactions, as well as having biomechanical and biochemical properties that resemble normal tears. In liquid form, the content of serum didn't last long, so the storage method of the serum was developed by using lyophilization with sucrose as lyoprotectant. The use of sucrose in lyophilization can only stabilize the protein content in serum so that the content of vitamin A in serum still decreased. In an effort to increase the stability of vitamin A in serum, this research was conducted to prepare freeze-dried autologous serum with the addition of vitamin E as an antioxidant of vitamin A. The purpose of this study was to determine the effect of vitamin E in stabilizing the concentration of vitamin A in freeze-dried autologous serum and checking its antibacterial properties by total plate count method. In this method, the separated blood serum was diluted with 0.9% sodium chloride followed by adding sucrose 60mM and vitamin E in different concentrations 0.5; 1; 2.5; 5% then this preparation was lyophilized. After that the serum was stored at 4°C and the content of vitamin A was analyzed every month by HPLC. Freeze-dried autologous serum with 1% addition of vitamin E showed the best results in stabilizing the vitamin A content up to 2 months. The content of vitamin A left in the 2-month freeze-dried serum was 0.0191-0.0761 ppm.

Keywords: Autologous serum, vitamin A, vitamin E, lyophilization

INTRODUCTION

Dry eye syndrome is one of the most common ocular morbidities. The disease is defined as an ocular surface disease resulting in a lack of tear production or increased tear evaporation. The prevalence of dry eye in Indonesia is 27.5%, this study is based on the island of Sumatra¹. The classification of dry eye is divided into two, namely the lack of tear fluid and dry eye evaporation. There are two subclasses, namely Dry eye Sjogren and non Sjogren¹.

Tears have anti-microbial properties, nutritional, mechanical and optical because they have various components, such as growth factors, fibronectin, and vitamins to support the proliferation, migration and differentiation of the corneal epithelium and conjunctiva. These factors are essential for maintaining corneal health and conjunctival epithelium². Reduced isotropic epithelial factor and epithelial surface integrity, will promote damage to maintenance and reduce wound healing³.

Autologous serum has a lot of component that resemble tears as shown in table 1.

Table 1: Comparison of normal tears component and serum component^{3,4}

	Tears	Serum
pH	7.4	7.4
Osmolality	298 (10)	296
EGF (ng/mL)	0.2 – 3.0	0.5
TGF-β (ng/mL)	2-10	6-33
Vitamin A (μg/mL)	0.016	0.806
Lysozyme (mg/mL)	1.4(0.2)	6
IgA (μg/mL)	1190(904)	2
Fibronectin (μg/mL)	21	205

The use of autologous serum in the form of eye drops has been reported as a treatment for a variety of serious and chronic eyeball surface disorders^{5,6,7}. Autologous serum is used as an eye drop for dry eye disease because it is natural and does not cause allergic reactions and has biomechanical and biochemical properties that resemble normal tears^{3,5}.

The easily damaged nature of the autologous serum results in the need for storage at low temperatures to maintain the activity of the components present in the serum. To improve the stability of autologous serum in order to last longer, autologous serum was made in dried form by freeze drying method with 60mM sucrose lyoprotectant⁸, the result of the average autologous serum content can be maintained for longer. At 4°C temperature serum can last for 2-3 months and at temperatures of -20°C to 6 months⁸⁻¹¹. However, vitamin A levels in freeze-dried autologous serum

didn't last long because of its easy to oxidize properties⁹, so to stabilize the content it was necessary to add another antioxidant which can be present in serum to maintain its properties.

Vitamin A itself has a role in bio signaling of some retinoid dependent protein transcription¹² so that it needs to be stabilized.

MATERIAL AND METHODS

Ethical Approval

The Health Research Ethics Committee Faculty of Medicine Universitas Padjadjaran Bandung, in order to protect the rights and welfare of the health research subject and to guaranty that the research using survey questionnaire/ registry/ surveillance/ epidemiology/ humaniora/ social-cultural/ archived biological materials/stem cell/other non-clinical materials, will be carried out according to ethical, legal, social implications and other applicable regulations, has been thoroughly reviewed in this research with no. reg. 0416010011.

Sample Preparation

A total of 100 mL of blood was taken and allowed to stand for 2 hours to coagulate, after coagulation of blood, centrifuged at 5000 g for 10 minutes. After the serum is formed accommodated in the tube. A total of 25mL of serum obtained was dissolved with 0.9% sodium chloride (w / v) with a final concentration of 20% (v / v). Serum was then added 0.5134 g sucrose (60 mM) and α -tocopherol with a concentration of 0; 0.5; 1; 2.5; 5%(w/v). The solution was then dried by freeze dry method. Solution was stored at 4°C for the period of 0, 1, 2 months for analysis of vitamin A levels by HPLC method.

Vitamin A Content Analysis by HPLC

For preparation of the sample, first serum was taken as much as 100 μ L added 100 μ L *n*-hexane then mixed it and centrifuged at a rate of 800 g for 5 minutes and taken the organic phase. Then the organic phase was evaporated in water bath with temperature 60 °C. Afterwards the residue was taken and added 25 μ L diethyl ether and 75 μ L methanol. The sample was ready to inject into the injector, the detector was set at 325 nm wavelength, and the flow rate of 1 mL/min. The HPLC motion phase was used methanol and water (methanol: water = 95: 5, v/v), where the solvent must be sonicated before being used as an eluent. The stationary phase was used C-18 columns. Standard retinol was dissolved with ethanol. A total of 20 μ L samples were injected into columns. Then the results of the chromatogram data were interpreted.

Total Plate Count

Samples were prepared for analysis. 5 mL samples were introduced into 5 ml diluent solution phosphate buffer (obtained dilution 10^{-1}). Serial dilution was made, by inoculation of 1 ml of the above suspension into a 9 ml dilution solution (obtained by dilution 10^{-2}). Created serial dilution like this until the solution obtained with dilution 10^{-5} . Note, before being inoculated into the next diluent tube should be homogenized. Aseptically, each dilution series was taken as 1 ml of culture suspension from the result of 10^{-1} - 10^{-5} dilution, which was then put into a sterile cup. For the record, the sterile cup should be labeled according to the last dilution series. Poured sterile NA medium temperature ± 45 °C or other media approximately 15 mL. (Usually 100 mL of medium to be used for 8 cups). Immediately before the media becomes hard, it was homogenized by wiggling the cup with the direction of movement like writing the number 8 and then let up to solidify. All the saucers were incubated in an incubator at a

temperature of 35-37 °C for 18-24 hours with the reversed position. Growths of colonies were observed and calculated on each medium of the cup. An observation table was prepared.

RESULTS AND DISCUSSION

Vitamin A Content in Autologous Serum by HPLC

The content of autologous serum usually differs from one person to another^{6,13-15}, so in this research we made a serum from four volunteers to reach conclusion. The chromatogram resulted by HPLC can indicate the concentration of retinol by calculating and comparing the peak area of sample with standard, moreover it can indicate the character of retinol by seeing the retention time of the peak. The best chromatogram obtained is shown in this paper as Figure 1.

From the chromatogram, we can see that the character of retinol remains the same in serum that being added the vitamin E or not. As shown from the chromatogram, we can see there was an effect of vitamin E addition to the serum for stabilizing the retinol content in serum. We can see it when we compare (2a) with (3a) and (2b) with (3b), there were a huge different in peak area between them.

To see which concentration of vitamin E addition best used, we can see it by comparing the result of one volunteer to another and take a conclusion out it. The results are shown in Figure 2.

In the four individuals, after serum was dried with lyophilization then it was treated with addition of vitamin E and there was a difference between dry serum added vitamin E and untreated dry serum. Non-treated serum dry retinol levels decreased more than those given vitamin E addition. In the lyophilization process, dehydration occurred in serum content, be it protein or vitamin, dehydrated so that retinol oxidized into other forms and damaged⁹. A significant difference between serum retinol levels added with vitamin E and untreated serum proves that retinol actually undergoes oxidation in the lyophilization process and vitamin E can maintain retinol levels.

The effect of vitamin E on the stability of blood serum retinol levels tends to vary in each individual because in blood serum it already contains natural antioxidants such as vitamin C, vitamin E, provitamin A, lycopene, lutein and zeaxanthin¹⁶, that is why vitamin E concentration in the dried autologous serum resulting different effect.

At 1-month storage time, it was shown that in non-vitamin E-added dry serum there was a considerable decrease in retinol levels. There was another research that shown the retinol levels of untreated lyophilized autologous serum still exist but the levels were too small so it was difficult to analyze⁹. In volunteer A (figure 2(a)), the decreased levels of retinol that occurred quite large whether serum was given vitamin E or not, but the decreased serum levels treated by vitamin E was smaller than that the untreated one, except in addition of concentrations of 2.5 and 5%. In volunteer B, C and D (figure 2(b);(c);(d)), the decreased serum retinol levels treated vitamin E showed better results than those not. From these data, by looking at the pattern of decreased retinol levels the effect of vitamin E addition on each individual, the four individuals showed different results but there were some similarities in the pattern. The decreased levels of retinol of A with D and B with C are almost similar. The serum itself in the medical world is often used for medical analysis for individuals, so there is the possibility of individuals who show almost similar results having similar physical conditions. The effectiveness of antioxidant will vary with each individual because of other factors

in the serum of each individual that may interfere with the antioxidant ability of transferring radical hydrogen atoms. Serum with vitamin E addition of 1% showed the best results in maintaining the serum retinol levels followed by 0.5%, 2.5%, and 5% in 1-month storage period. At concentrations of 2.5% and 5% additions in the four individuals the average showed a lower yield than the 1% despite excessive concentration. The higher concentrations of α -tocopherol used, the antioxidant activity of α -tocopherol itself decreases because when the α -tocopherol concentration is high, the compound will be more likely to experience side reactions which less stabilize the target compound to be stabilized¹⁷.

At 2-month storage time, serum without vitamin E has lost all retinol levels, whereas in serum treated with vitamin E persisted but in some individual's retinol levels have disappeared. The loss of retinol levels in some individuals suggests that the α -tocopherol compound has decreased in the dry serum so that the retinol level was not stabilized anymore. In individuals A and B, serum retinol levels can still be maintained in the overall concentration of vitamin E supplementation, but in individual C retinol that persist only at 1 and 2.5% additions and in individual D retinol levels are completely depleted. In individuals B, a decrease in retinol levels in a 1-month storage period showed the best results but at the time of 2 months individual 3 shots showed the best results at a 1% vitamin E addition concentration. This

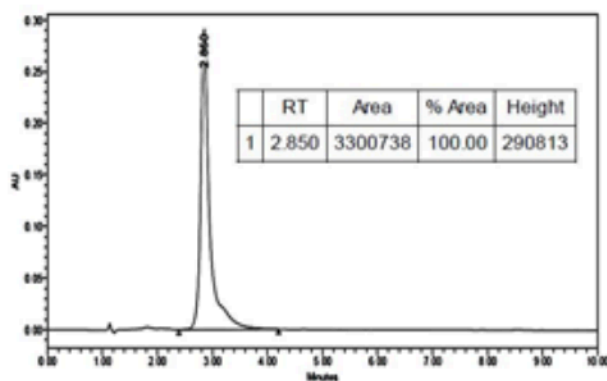
suggests that the possibility of serum endogenous antioxidants and α -tocopherol decreased in individual B was lesser than in individual C.

At the concentration of 1% addition of vitamin E, ranges of retinol content of dried serum preparation stored for 2 months period successfully maintained 0.0191-0.0761 ppm. The level of retinol in tears was 0.016 ppm⁴, so autologous serum retinol content of dry preparation with addition of vitamin E 1% still fulfill retinol level for serum usage requirement as an eye drops until 2 months.

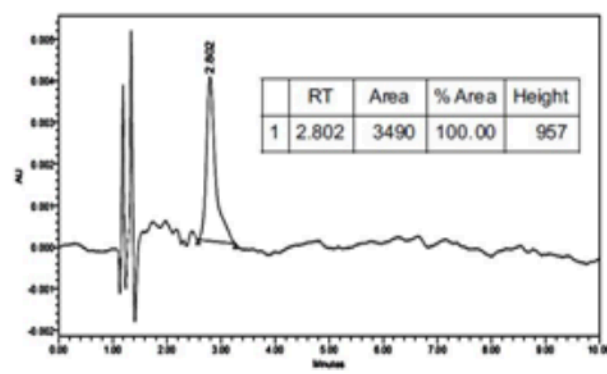
Antimicrobial Properties

Antimicrobial properties of the serum were conducted only at 2-month storage time without the addition of vitamin E and with 1% addition of vitamin E. The results are shown at Table 2.

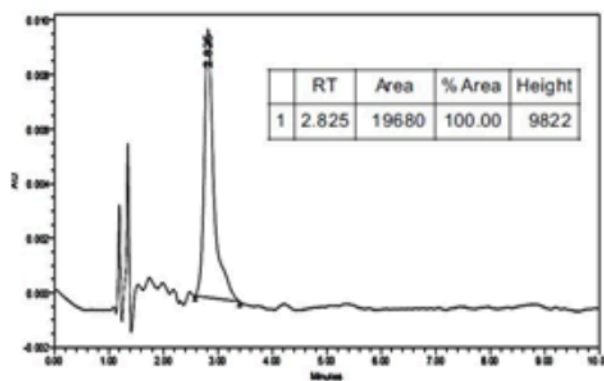
From table 2, we can see in 2-month storage period, the serum still had an antimicrobial activity. From four individuals, all of them shown a little, mostly zero, colony forming unit (CFU). From table 1, we know that in serum which contain enzyme called lysozyme that has antibacterial activities. Lysozyme in freeze-dried autologous serum still present up until 6 month¹⁰, so from that we know addition of vitamin E didn't inhibit the function of lysozyme and the serum will still be sterile.



(1a)



(2a)



(3a)

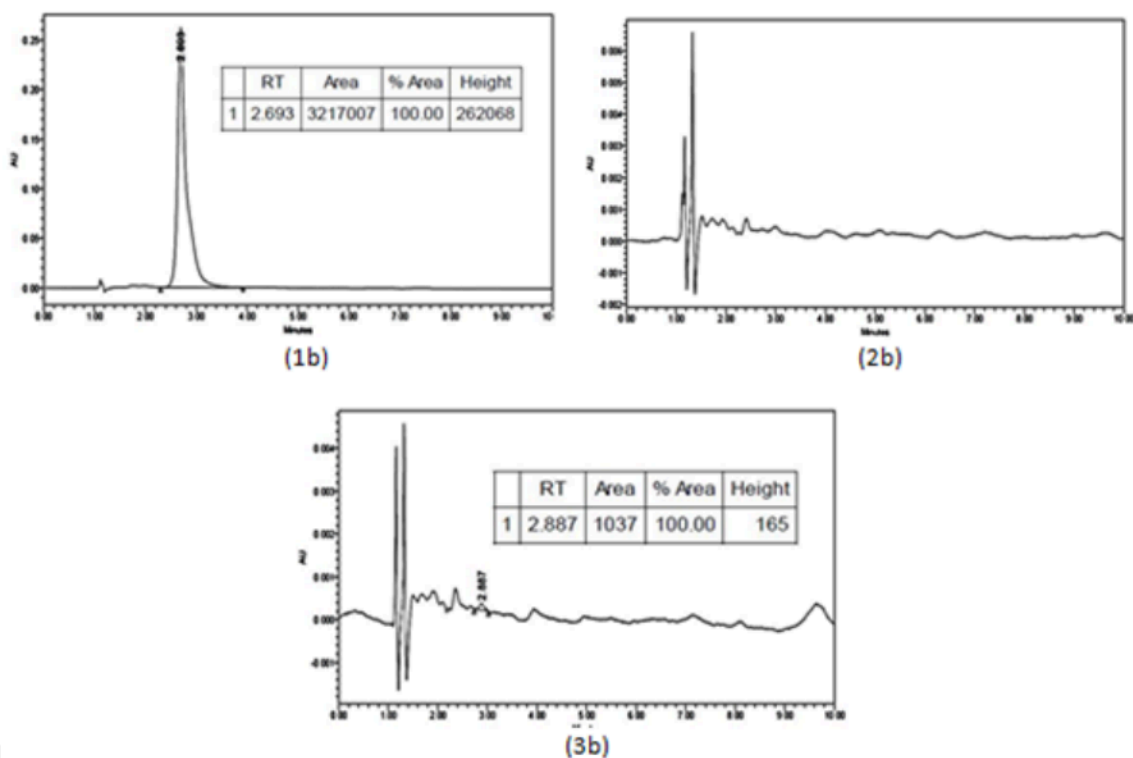
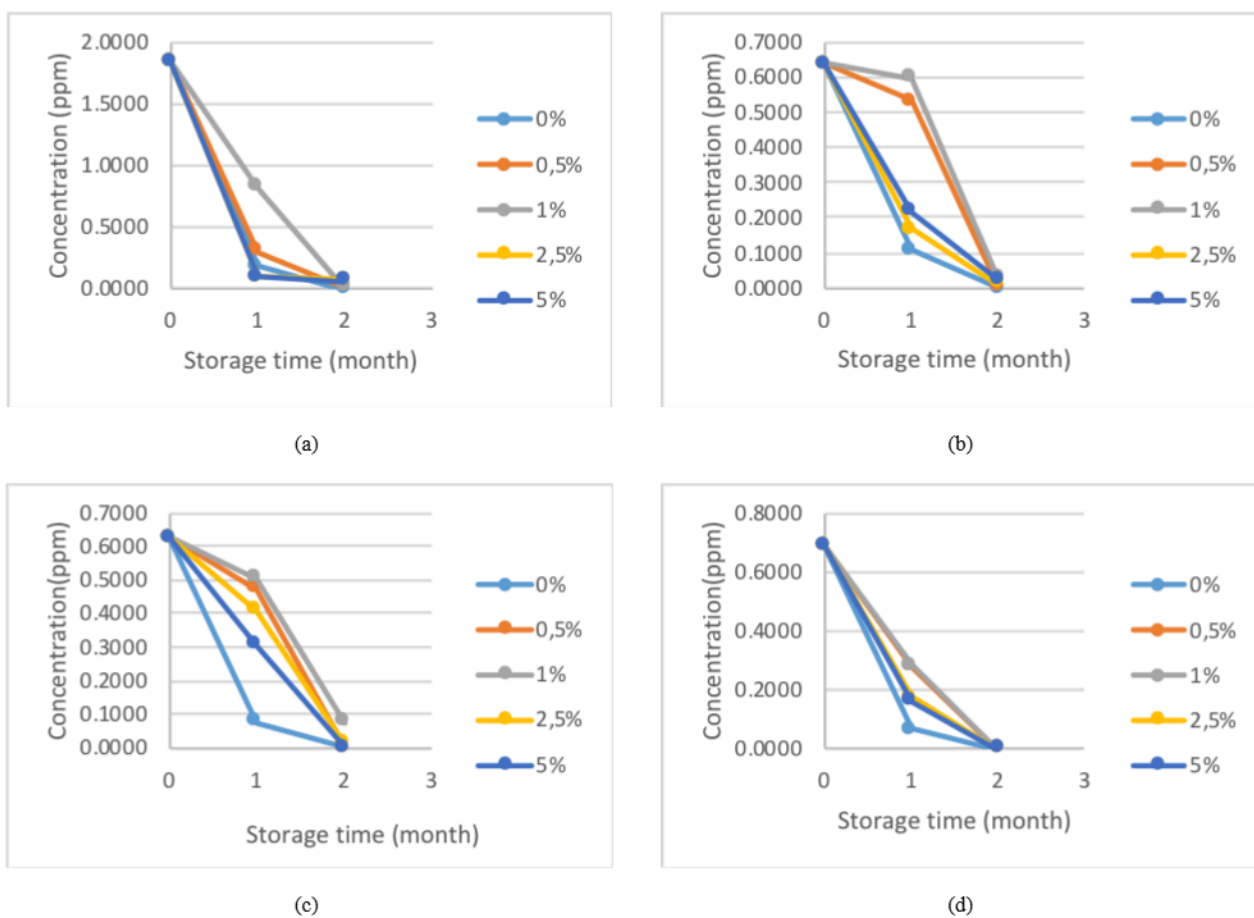


Figure 1: The chromatogram of retinol in autologous serum analyzed by HPLC from volunteer B. (a) indicates the chromatogram from 1-month storage time and (b) indicates the chromatogram from 2-month storage time. 1 indicates the standard retinol, 2 indicates the serum without addition of vitamin E and 3 indicates the serum with 1% addition of vitamin E



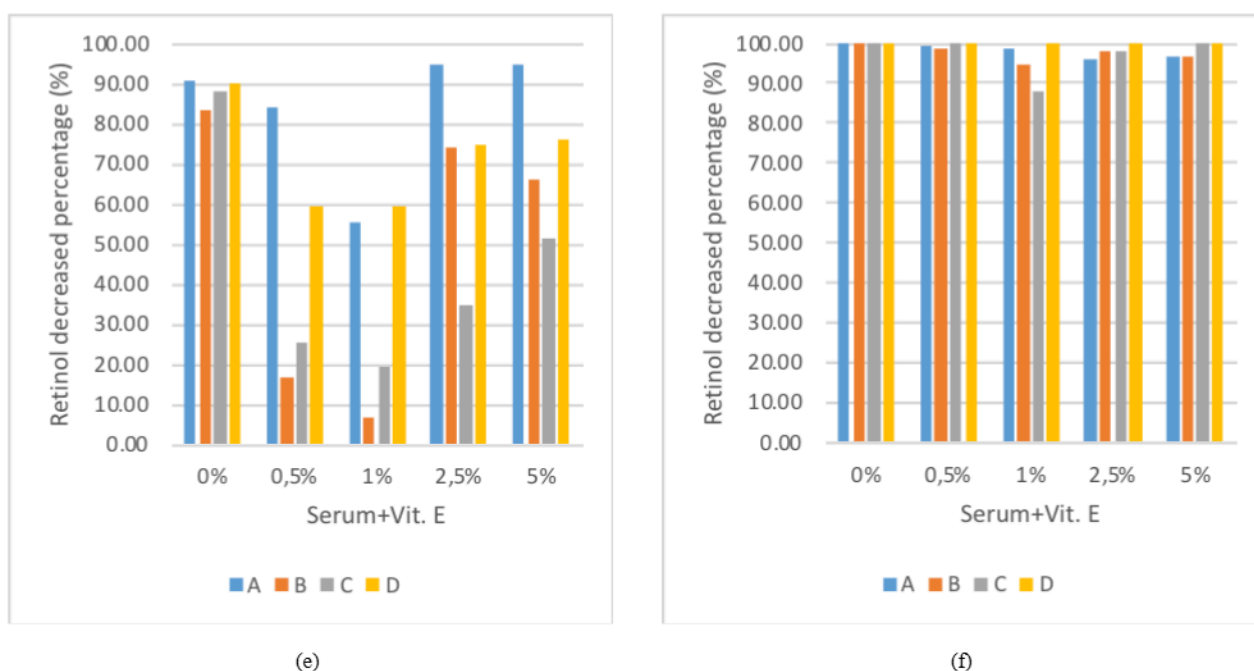


Figure 2: The effect of vitamin E on stabilizing vitamin A content of autologous serum from four volunteer A(a); B(b); C(c); D(d). Each line represents concentration of retinol in autologous serum with and without the addition of vitamin E. (e) shown the decreased percentage of retinol from each individual for 1-month storage period and (f) from 2-month storage period.

Table 2: The total plate count of 4 different serum

Sample Code	Dilution			SPC (CFU/mL)	Notes
	10^{-3}	10^{-4}	10^{-5}		
A 0%	0	0	0	$<1 \times 10^3$	Not growing colonies
	0	0	0		
A 1%	0	0	0	$(0+1000)/2 = 500$	
	1	0	0		
B 0%	2	0	0	$(2000+0)/2 = 1000$	
	0	0	0		
B 1%	0	0	0	$<1 \times 10^3$	Not growing colonies
	0	0	0		
C 0%	0	0	0	$<1 \times 10^3$	Not growing colonies
	0	0	0		
C 1%	0	0	0	$<1 \times 10^3$	Not growing colonies
	0	0	0		
D 0%	0	0	0	$(0+3000)/2 = 1500$	
	3	0	0		
D 1%	0	0	0	$<1 \times 10^3$	Not growing colonies
	0	0	0		

CONCLUSION

Vitamin E supplemented into lyophilized autologous serum successfully maintains vitamin A levels in autologous serum for two months and its antibacterial properties still present.

The most optimal vitamin E concentration was 1%, and the retinol content successfully maintained was in the range 0.0191-0.0761 ppm.

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