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Formulation and Evaluation of a Topical Antioxidant Preparation

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ABSTRACT:-

The aim of this work was to prepare the stable vitamin C water/oil/water (w/o/w) multiple emulsions, to investigate the usage of poloxamine 908, to observe the influence of surfactant percentage on the properties of multiple emulsions. Multiple emulsions were prepared by liquid paraffin, cetyl dimethicone copolyol and poloxamine 908 by a two – step emulsification procedure. Vitamin C was used as an antioxidant and water – soluble model. The viscosity, conductivity and globule size of multiple emulsions were followed over time. The formulations containing 1% cetyl dimethicone copolyol and 1 or 2% poloxamine 908 were the most stable systems. The globule size of multiple emulsions ranged from 20 to 37 μm and did not change during time. The yield of multiple emulsions was between 99.6% and 98.7%. The conductivity increased and the viscosity of systems decreased during time. Increase in poloxamine 908 influenced the viscosity of the system, with the viscosity decreasing as the hydrophilic surfactant concentrations were increased. Vitamin C release from the multiple emulsions was slow; the release was affected by both surfactant concentrations. Poloxamine 908 could be used as a hydrophilic surfactant for formulation of w/o/w multiple emulsions. The concentration of poloxamine 908 was a very important parameter in preparing stable multiple emulsions. It was concluded that vitamin C might be transported out by molecular diffusion and through a reverse micellar mechanism controlled by the viscosity of the system.

دراسة و تقييم الصيغة التركيبية لمستحضر موضعي مضاد للأكسدة

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المستخلص:-

من هذه الدراسة هو تحضير مستحلب متعدد و مستقر لفيتامين سي (ماء/زيت/ماء)، للتحقيق في استخدام البولاكسامين 908 وملاحظة تأثير نسبة عامل الاستحلاب على صفات المستحلب المتعدد. لتحضير المستحلب المتعدد باستخدام البارافين السائل، سيتايل دايميثيكون كوبوليول و بولاكسامين 908 بطريقة الخطوتين للاستحلاب. فيتامين سي تم استخدامه كمضاد للتأكسد و ذو ذوبانية عالية في الماء. اللزوجة، التوصيلية و حجم كريات المستحلب المتعدد تم متابعتها طوال الوقت. الصيغة التي تحوي 1% سيتايل دايميثيكون كوبوليول و 1 او 2% بولاكسامين 908 هي الاكثر استقرارا. حجم كريات المستحلب المتعدد تتراوح من 20 الى 37 ميكروميتر و لا يطرأ اي تغير على حجمها خلال الوقت. ناتج المستحلب المتعدد يتراوح بين 99,6% و 98,7%. التوصيلية تزداد و اللزوجة تقل خلال الوقت. الزيادة في البولاكسامين 908 ينتج عنها قلة في اللزوجة. تحرر فيتامين سي من المستحلب المتعدد ببطء و عملية التحرر تتأثر بالتراكيز المستخدمة من عوامل الاستحلاب. البولاكسامين 908 يمكن استخدامه كعامل استحلاب لتصييغ المستحلب المتعدد (ماء/زيت/ماء). تركيز البولاكسامين 908 المستخدم هو عامل جدا مهم لتحضير مستحلب متعدد مستقر. تحرر فيتامين سي يكون بالتنافذ الجزيئي و يتأثر بلزوجة النظام.

INTRODUCTION:-

Vitamin C (ascorbic acid) has been widely used in formulations of skin care products. Due to its effects on collagen biosynthesis, it is considered as moisturizing and anti – aging active ingredient. Instability problems such as oxidation susceptibility have made incorporating vitamin C in topical formulations a challenging issue [1]. Multiple emulsions can be considered as emulsions of emulsions and have shown promise in cosmetic [2,], pharmaceutical and separation sciences [3]. Their potential pharmaceutical applications include uses such as taste masking, adjuvant vaccines, an immobilization of enzymes and sorbent reservoir of overdose treatments, and for enhancement of enteral or dermal absorption [4]. Multiple emulsions have been formulated as cosmetics, such as skin moisturizer [5].

Prolonged release can also be obtained by means of multiple structures [6]. These systems have some advantages, such as the protection of the entrapped substances [7] and the incorporation of several actives in the different compartments [8]. Despite their potential usefulness, applications of multiple emulsions have been limited because of thermodynamic instability and their complex structure. Water/oil/water (w/o/w) multiple emulsions consist of dispersed oil globules containing smaller aqueous droplets; each inner aqueous droplet is separated from the outer aqueous phase by an oil phase layer. The presence of at least two surfactants is required. One of them is predominantly lipophilic for stabilizing the primary water/oil (w/o) emulsion and the other is hydrophilic for the secondary oil/water emulsion. To produce a w/o/w emulsion, the lipophilic and hydrophilic surfactants are dissolved in oil and continuous aqueous phase, respectively. The most common preparations of w/o/w double emulsions are based on the two – step emulsification process. The stability and release characteristics of multiple emulsions are influenced by different factors, such as surfactant type, surfactant ratio and some physical properties of the system (globule size, viscosity, conductivity, phase volume ratio, etc) [9]. The poloxamine 908 used as a hydrophilic surfactant is nonionic and a block copolymer [10]. Cetyl dimethicone copolyol used as a lipohpilic surfactant is the siliconic polymeric surfactant, and shows good trapping capacity, prolongs the release of active molecules, and produces a w/o emulsion with strong interfacial film [11]. The purposes of this research were (i) to investigate the usage of poloxamine 908 for w/o/w multiple emulsion formulation, (ii) to evaluate the formation and stability of the multiple emulsion system, (iii) to investigate the properties of the system and to observe the influence of hydrophilic and lipophilic surfactant percentage on the characteristic properties of multiple emulsions.

EXPERIMENTAL:-

Chemicals

The following substances were used for the preparation of multiple w/o/w emulsions: The oil used was liquid paraffin (*SDI*). Abil®EM90 (*cetyl dimethicone copolyol, Goldschmidt, France*) was used as a lipophilic surfactant. Tetronic®908 (*poloxamine 908, BASF – New Jersey, USA*) was used as a hydrophilic – nonionic surfactant. Sodium chloride (NaCl) was incorporated into the aqueous internal phase and used as a conductimetric tracer. Vitamin C (*SDI*).

Preparation of w/o/w multiple emulsion

Multiple emulsions were prepared by a two – step emulsification process [12]. The first emulsification was to prepare the w/o primary emulsion and the second emulsification step provided the formation of the w/o/w multiple globules. The compositions of formulations are shown in (Table 1). Electrolyte or both electrolyte and vitamin C were dissolved merely in the aqueous phase of the primary w/o emulsion. The concentrations of paraffin oil and electrolyte in primary emulsions were fixed at 30 and 0.3%, respectively. The percentage of the primary emulsion was also constant at 80%. Water/oil primary emulsions were prepared with different concentration of a lipophilic surfactant (Abil®EM90). Two concentration of Abil®EM90 were used in the primary emulsion (2 and 4%). The oily mixture of Abil®EM90 and paraffin was heated to 75°C and then aqueous solution at the same temperature was added to this mixture by stirring at 1500 rpm at 75°C for 30 minutes. The first step was carried out using mechanical mixer (*Maxi – Mix III™ 65800, Thermolyne, USA*) to produce fine droplets. The emulsion prepared was then cooled to room temperature. The stability of the w/o primary emulsion prepared at the first emulsification was an important factor determining the stability of the w/o/w emulsion. Therefore, the accelerated physical stability of primary emulsions was tested under centrifugation (*Table Top Centrifuge, Triup International Corp, Korea*) during 1 hour at 3500 rpm, and the occurrence of phase separation was examined. The stability of w/o emulsions under centrifugation reflects the strength of the interfacial film between the aqueous and oil phases. No change was macroscopically observed in any of the primary emulsions in this study, which were

quite stable. Moreover, no phase separation was observed during 6 months at room temperature. Water/oil/water multiple emulsions containing vitamin C were prepared by the same emulsification procedure under the same conditions. Both vitamin C and NaCl were dissolved into the inner aqueous phase. For the preparation of the w/o/w multiple emulsion, the freshly prepared w/o primary emulsion (80 g) was emulsified further in an external aqueous phase (20 g) inside which the hydrophilic surfactant was dissolved at room temperature. The mixture (100g) was stirred using mechanical mixer at 700 rpm until the formation of multiple globules. To avoid bursting the multiple globules, stirring was moderate and was stopped after their formation. During the mixing procedure, a small drop of the emulsion sample was placed on a glass slide with a small cavity in the center, the slide was mounted in an optical microscope, and the formation of w/o/w emulsion structure was confirmed. The formation times of multiple globules were determined, and this experiment was done with multiple emulsions containing vitamin C. The vitamin C concentration of multiple emulsions was 15%.

Characteristics of multiple emulsions

After preparation of the multiple emulsions not containing vitamin C, several physical tests were carried out in order to determine their characteristics, such as viscosity, mean multiple globule size and conductivity. In this study, the physical stability of w/o/w multiple emulsions prepared was also examined using viscometric, conductimetric and granulometric methods. In addition, formulations were evaluated by visual observation of the phase separation. Macroscopic analysis was carried out to observe the homogeneity of the systems. The multiple emulsions were stored at 25 and 40°C, and the characteristic properties of the systems were followed until phase separation was observed. At a definite time, the emulsions were allowed to return to room temperature before observation, and then the viscosity, mean multiple globule size and conductivity values were determined. The data were evaluated by statistical analysis according to the Student's – t test. In addition, standard error (SE) was computed for every mean value.

Microscopic analysis, measurement of globule and droplet size

Microscopic analysis was carried out using an optical immersion microscope (*Soif, Model 4GX*), and observations were made at 100X16 magnification after diluting in the appropriate external phase of emulsion. This examination provided direct information on the multiple structures. We could see the internal aqueous phases as droplets in a w/o/w emulsion structure. The existence of multiple globules was checked microscopically during all experiments until the phase separation was observed. Granulometric analysis (*Sympatec GmbH, HELOS, Germany*) was carried out in order to characterize the globule size of multiple emulsions and the droplet size of w/o primary emulsion. As primary emulsions were diluted with liquid paraffin, the samples of multiple emulsions were diluted both in the distilled water and in iso – osmotic NaCl solution with regard to the internal aqueous phase. Fresh dilutions were made for all measurements. The dilution with water did not affect the size of multiple globules; in fact, the globule size of multiple emulsions was the same whether distilled water or iso – osmotic solution was used.

Measurement of viscosity

For the viscometric measurements, the samples of multiple emulsions were examined using (*J. P. Selecta, s. a., Abrera "Barcelona" Spain*) at 2.5, 5, 10 and 20 rpm. Each reading was taken after equilibration of the sample, at the end of two minutes. The flow curves of all multiple emulsions were obtained by directly reading the viscosity (mPa.s) and shear stress (rpm) from the viscometer. The measurements at 2.5, 5, 10 and 20 rpm were also carried out during storage time at 25 and 40°C, and all measurements were performed three times. The viscosity values that were only at 5 rpm were selected for tables (Table 1 and 4) and figures (Figures 4 and 6).

Conductimetric analysis

The conductivity was measured with conductimeter (*HI – 9033, Hanna Inst.*). It was necessary to dilute multiple emulsions

with water or iso – osmotic NaCl solution to measure conductivity. The preliminary studies were performed to establish whether further dilution could influence the measurement. The measurements were made at room temperature on samples of the multiple emulsion diluted 1/20 in distilled water. The dilution with water did not affect the conductivity of the emulsion, as confirmed by using the NaCl solution that is iso – osmotic with the internal aqueous phase of multiple emulsions. The entrapment percent or yield value ($E_{\%}$) was calculated according to the following equation:

$$E_{\%} = 100 \text{ X } (C_i - C_t) / C_i \ldots \text{(Eq. 1)}$$

where C_i is the conductivity of the internal aqueous phase (0.3% NaCl solution, 4800 μS) and C_t is the conductivity value of multiple emulsion at a given time t.

In vitro release studies

Release study of vitamin C from multiple emulsions was investigated by a dialysis method. Before this experiment, the saturation solubility of vitamin C in buffer solution (pH 5.2) at 32°C was determined in triplicate. An excess amount of vitamin C was added into 50 ml of buffer solution stirred. After 24 hours, the supernatant was filtered and vitamin C was assessed spectrophotometrically. Dialysis tubing (*seamless, D – 0405, lot: 01H0713, Sigma*) was used as a membrane for the release study and was washed several times with distilled water and left soaking in buffer solution (pH 5.2) overnight before use. Immediately after preparation, 2 g of the multiple emulsions containing 15% vitamin C was introduced into the dialysis tubing double – tied at each end and dialyzed in 300 ml buffer solution of pH 5.2 as the release medium at 32±1°C under constant stirring. Two milliliters were withdrawn from the dialysis medium and replenished with the same volume of fresh buffer solution at an hour interval. The concentration of the vitamin C dialyzed was analyzed spectrophotometrically at 280nm (*UV – Visible Recording Spectrophotometer, UV – 160 Shimadzu, Japan*). Release studies were carried out over times up to 6 hours, in triplicate.

RESULTS AND DISCUSSIONS:-

In the development of w/o/w multiple emulsions, it is necessary to estimate their physical stability experimentally. Viscosity, multiple globule size and conductivity measurements are convenient methods for this purpose [13]. In this study, we tried to formulate a stable multiple emulsion with Tetronic®908 in the external phase as the hydrophilic surfactant and with Abil®EM90 in the inner aqueous phase as the lipophilic surfactant. Two different concentrations of the lipophilic surfactant and three different concentrations of the hydrophilic surfactant were used in order to evaluate the influence of the surfactant percentage on the characteristics of multiple emulsions (Table 1). It was shown that the stability of the w/o emulsion requires the presence of electrolyte in the aqueous phase; it is well known that the electrolyte concentration or osmotic pressure of the internal aqueous phase of w/o/w emulsions plays a critical role in the physical stability of the system [14]. Thus, the optimum balance should be ensured between the internal and external aqueous phase of w/o/w multiple emulsions [15]. Therefore, we examined the literature regarding the formulation of w/o/w multiple emulsions, and it was decided to use NaCl at a concentration of 0.3% (0.0513 molar) as electrolyte [16]. Viscosity, mean globule size and conductivity were determined to investigate the characteristics of multiple emulsions not containing vitamin C as a function of time. Measurements were made up to five months at monthly intervals following preparation of the emulsions. In addition, phase separation and phase inversion of multiple emulsions were also investigated, and the results of visual observations are summarized in (Table 3). The beginning of phase separation is shown, which revealed a destruction of multiple globules of the system. During storage time, no phase inversion was observed in any multiple emulsions. It is well known coalescence is amplified with temperature. In this study, the beginning of phase separation at 40°C occurred earlier than at 25°C, except for formulations containing 4% Abil®EM90. For example, phase separation was observed at the fourth month for the multiple emulsion containing 2%

Abil®EM90 at 40°C, whereas phase separation was observed at the fifth month at 25°C. When the physical stability of multiple emulsions containing varying amounts of Tetronic®908 and a constant of Abil®EM90 was evaluated, it was observed that the stability decreased as Tetronic®908 concentration increased. At high Tetronic®908 concentration (4%) coalescence occurred, resulting in phase separation within three or four months. On the contrary, no phase separation or physical stability problem occurred over five months at 25°C for multiple emulsions with prepared lower concentrations of Tetronic®908 (1 and 2%). ***Matsumota*** et al, [17] explained the effect of an increase amount of hydrophilic surfactant on the physical stability of multiple emulsions as: During the second emulsification, some of the molecules of the lipophilic surfactant from the oil layer may be solubilized in the external aqueous phase due to amount of hydrophilic surfactant that is above critical concentration. This phenomenon may be a very important cause of the rupture of the oil layer covering the internal aqueous phase droplets after preparation of the system.

Microscopic and globule size analysis

Microscopic technique is the useful direct method to assess the formation and to follow the stability of multiple globules. The various stability problems, such as globule deformation and phase inversion to simple emulsion, may be detected using this method. All multiple emulsions were prepared successfully using the method described. The structure of w/o/w multiple emulsions was observed under the microscope during manufacturing. Many multiple globules were observed very well and many small droplets were seen in the internal phase of multiple globules. The microscopic view of a multiple emulsion is shown in (Figure 1). To evaluate the type of multiple emulsions, a drop of a water – soluble dye (methylene blue) solution was added to the system and dissolution of dye solution was observed. The colored mixture was then examined by optical microscopy. This procedure revealed that water was the continuous phase and that the sample was a w/o/w emulsion. The size of globules is the most important parameter in rheology and physical stability of any emulsion. The globule size of multiple emulsions as a function of time is shown in (Figure 2). When change in multiple globule size was investigated, no significant increase or decrease was observed in the mean size during storage time at 25 and 40°C; this may not be evidence of non – coalescence. A similar result was also reported by ***Florence*** and ***Whitehill*** [18] and ***Omotosho*** [19]. They reported that an increase in globule size of multiple emulsions due to coalescence might be offset by a decrease in size due to water loss from the internal to the external aqueous phase [18]. This may explain why no significant change in globule size of multiple emulsions was observed. Although the globule size of multiple emulsions was stable during storage time, the viscosity significantly decreased and unfortunately, phase separation was observed in some of the multiple emulsions. The mean multiple globule sizes of emulsions were recorded in the range of 20±0.025 μm and 37±0.031 μm (mean ± confidence interval). The particle size measurements of w/o primary emulsions demonstrated a mean droplet size (±CI) of the aqueous phase, 3.38±0.0216 μm and 3.44±0.0541 μm for emulsions prepared with 2% Abil®EM90 and 4% Abil®EM90, respectively [20]. These values were statistically the same ($p>0.05$). This result showed that there was no significant effect of high lipophilic amount (4%) on the interfacial tension between water and oil phase; the effective reduction of interfacial tension was probably ensured by 2% of Abil®EM90. The globule size of primary emulsions did not change, and no visual sign of physical destabilization was observed in systems at 25 and 45°C during storage time. As expected, the mean globule size of multiple emulsions depended on the surfactant concentrations. When the effect of Tetronic®908 concentration on globule size of multiple emulsions was investigated, a small increase with increasing surfactant amount was observed; the higher concentration of hydrophilic surfactant most likely caused the coalescence of globules due to rupture of oil layer between two aqueous phases during manufacturing [17]. However, there was no

significant effect of Tetronic®908 concentration (for low concentration, 1 and 2%) on globule size of multiple emulsions containing the same percentage of Abil®EM90 (Table 2) ($p>0.05$). When the percentage of Abil®EM90 was increased from 2% to 4%, the globule size logically increased. The excess of Abil®EM90 was located in a molecular form in the oil phase between external and internal aqueous phase, and this caused swelling of the oil globules (Table 2). On the contrary, the globule size of multiple emulsions containing vitamin C decreased with increasing Abil®EM90 percentage and the same concentration of hydrophilic surfactant (Table 4). It was concluded that vitamin C influenced the tension of the second interface during the second emulsification step.

Conductimetric analysis

This analysis was carried out in order to directly measure the entrapped electrolyte within the internal aqueous phase and to confirm the emulsion type. Conductivity data generated good information about the emulsion type. Both drying method and low positive conductivity values demonstrated the w/o/w type emulsion. Multiple emulsions yielded low conductivity values immediately after preparation of formulations. The yield of the emulsions (entrapment percent, $E_{\%}$) was between 99.6% and 98.7%, meaning that a portion of the NaCl in the inner aqueous phase, approximately between 0.4% and 1.3%, leaked into the external aqueous phase during the preparation of multiple emulsions. Although the yield values were high, unfortunately phase separation was observed in some formulations during storage time at 25 and 40°C (Table 3). During storage of w/o/w emulsions, the conductivity values increased with time (Figure 3). The increase in conductivity during storage time can be ascribed to the increase in NaCl in the external aqueous phase due to (i) a diffusion of NaCl through the mineral oil film [21] or (ii) the coalescence of internal and external aqueous phases [22], destruction of oil film because of osmotic pressure and then expulsion of internal aqueous phase. This most likely explains the phase separation observed in this study. Due to rising conductivity, declining viscosity and the approximately stable globule size of multiple emulsions during storage time, it was concluded that the solute and water molecules were transported from the oil membrane by a diffusional mechanism. The conductivity profiles showed that the transport rate of NaCl molecules from inner to outer aqueous phase decreased with time (first order kinetic) (Figure 3). The cause for the decreasing rate was interpreted as: The inner aqueous droplets of w/o/w emulsions probably grow with time due to coalescence of smaller droplets because of the higher percentage of hydrophilic surfactant. However, in this study, the volume of the oil phase was constant, and therefore the oil layer covering inner growing aqueous droplets thickened. Thus, the thickened layer delayed the transport of electrolyte molecules. The temperature accelerated the diffusion or transport of NaCl molecules from inner to external aqueous phase. The calculated conductivity rate constants (ln μS/month) were significantly different when compared with the formulations having the same composition at 25 or 40°C ($p<0.05$). For example, the conductivity rate constants of multiple emulsions containing 1% Tetronic®908 and 2% Abil®EM90 were calculated as 0.229±0.0183 ln μS/month and 0.339±0.03 ln μS/month at 25 and 40°C, respectively. The effect of Abil®EM90 concentration on conductivity of the system was investigated, and no significant difference was obtained among formulations containing 1% Tetronic®908. Conductivity values were similar with 2 or 4% Abil®EM90 concentration at 25°C. At higher concentrations of Tetronic®908 (2 and 4%), the two – fold increase in lipophilic surfactant did not hinder the increase in conductivity (Figure 3). Unfortunately, the increase in concentration of lipophilic surfactant could not provide the physical stability of the system (Table 3). This finding was in contrast with previous literature [17, 23]. There was a direct relationship between the percentage of Tetronic®908 and the conductivity of multiple emulsions. As could be seen in (Figure 3), the conductivity of systems containing the same amount of lipophilic surfactant increased with

increasing Tetronic®908 concentration. This result was also observed by the other investigators [17, 24]. As mentioned previously, the excess amount of Tetronic®908 in the external aqueous phase probably caused the formation of micelles and then the solubilizing of Abil®EM90 molecules, thus weakening or rendering unstable the interface of the w/o system. Therefore, NaCl molecules migrated more easily from the internal to external aqueous phase.

Viscometric analysis

Measurement of viscosity gives us useful data, especially in quality assurance of emulsions. The variation in viscosity of systems may explain some differences between formulations and at the same time provide important information about coalescence or globule size change. The viscosity of the w/o/w multiple emulsions was measured at four different rpm to follow the time dependence of viscosity. The viscosity values only at 5 rpm were selected for tables and figures. The viscosities of emulsions at first day after preparation are shown in (Table 2). There are many factors affecting the viscosity of multiple emulsions; one is the multiple globule size. There was a direct relationship between the viscosity and globule size of multiple emulsions. As expected, the minimum viscosity was observed with the higher globule size (Figure 4). Higher viscosity may be attributed to the small globule size of multiple emulsions prepared with the same concentration of Abil®EM90. Increased hydrophilic surfactant influenced the viscosity of system, with the viscosity decreasing as the Tetronic®908 concentrations were increased (Table 2), whereas no significant difference in viscosity as a function of Abil®EM90 percentage was observed ($p>0.05$). All the multiple emulsions exhibited non – Newtonian flow and shear thinning behavior. It means the viscosity of system decreased with increasing shear stress. The viscosity of multiple emulsions reduced with increasing shear stress from 5 to 20 rpm. Only one example of the plots of viscosity versus shear stress (rpm) is shown (Figure 5). In some literatures, the bursting of multiple globules [25] or phase inversion under shear stress [26] have been shown, but we did not observe any destruction of multiple globules or phase inversion after viscosity measurement using optical microscopy. It was concluded that shear stress used in this study did not induce irreversible structural changes in multiple emulsions, such as coalescence or phase inversion. Viscosities of all the multiple emulsions decreased continuously during storage with time (Figure 6). This may be due to (i) diffusion of water molecules from the inner to the outer aqueous phase and then the volume decrease of globules in the w/o/w emulsions, or (ii) bursting of multiple globules due to osmotic pressure. When all multiple emulsions were compared considering phase separation at 25°C, systems containing 2% Abil®EM90 and 1 or 2% Tetronic®908 were stable at least five months, although increasing conductivity and decreasing viscosity with time were observed. In these formulations, water molecules probably migrated through the liquid paraffin layer without affecting the entirely of multiple globules. This has been described by (i) micellar transport mechanism, (ii) the hydrated surfactant mechanism or (iii) diffusion of water molecules across the oil layer between internal and external oil phase [22, 27].

Formation time of multiple globules

The effect of surfactant concentration on formation time of multiple globules is shown in (Figure 7). As can be seen, the required formation time of multiple emulsions prepared with 2% Abil®EM90 was similar with multiple emulsions prepared with 4% Abil®EM90 for the same concentration of Tetronic®908. In contrast, the formation time was significantly affected by the percentage of Tetronic®908; the formation time of multiple globules increased with an increase in hydrophilic surfactant. It was considered that Tetronic®908 may influence the interphase between the external aqueous phase and oil layer, which has been described by *Garti* [28]. Abil®EM90 was probably solubilized via the micelles formed by part of the Tetronic®908 molecules. Thus, as Tetronic®908 concentration increased, formation of multiple globules was delayed; the higher the Tetronic®908 concentration, the longer the formation time.

In vitro release studies

To investigate the release characteristics of multiple emulsions, vitamin C was used as the model solute. When vitamin C was introduced in the internal aqueous phase of multiple emulsions, the viscosity and multiple globule size of emulsions changed (Table 4). The solubility of vitamin C was found to be 300±0.97 mg/ml (saturation value, C_s) (pH: 5.2). During release experiments, the release rate should not be influenced by the concentration gradient between the vitamin C concentration of release medium (C_{rm}) and the saturation concentration of vitamin C (C_s) in release medium. If the C_{rm} is less than or equal to "C_s X 10/100" for every sampling time, it is said that a sink condition is maintained. So, when we used 300 ml of release medium, even if all of the vitamin C (o.3 gm) in two grams of multiple emulsion released, C_{rm} could not be higher than "C_s X 10/100" value (= 300 X 10/100 = 30 mg/ml). Therefore, it was concluded that the volume of dialysis medium was not rate limiting in diffusion of vitamin C and the experiments were conducted under sink conditions. The cumulative percent release of vitamin C from multiple emulsions is shown in (Figures 8 and 9). Points and bars in each figure represent mean value±standard error (SE). When the SE was smaller than the size of symbol, no bar was shown. All release profiles exhibited an initial rapid release phase and then a slower release of molecules. First phase of release profiles (the first hour of profile) might be due to vitamin C existence in the external aqueous phase during the manufacturing; during the second emulsification, vitamin C molecules probably leaked out of the internal aqueous phase. Therefore, the secondary emulsification was stopped immediately after observation of multiple globules on microscope. The vitamin C molecules at the external aqueous phase were free molecules that were released immediately. The release of vitamin C in the external phase of w/o/w emulsions was completed within approximately 2 hours. Negligible vitamin C release from multiple emulsions was observed between the 1st and 2nd hours. This may be attributed to the slow transport of vitamin C from the internal aqueous phase; a period of time was probably required for vitamin C molecules to transport from internal droplets to the dialysis membrane. After 2 hours, vitamin C in the internal aqueous phase was released slowly at a rate governed by the interphase barrier between the inner and outer aqueous phases because the hydrophilic vitamin C molecules could not pass freely through the oily layer. So, the second phase might be attributed to a prolonged release of vitamin C from the internal aqueous phase. The release rates presented in (Table 4) were determined from slopes of the release percentage versus time between the 2nd and 6th hours. These rates were used the explanation and comparison of the effect of surfactant concentration on the release profiles.

The effect of hydrophilic surfactant concentration

Profiles shown in (Figures 8 and 9) indicate that emulsions containing 4% Tetronic®908 enhanced the drug released in comparison with the other concentration of this surfactant. It was also observed that multiple emulsions containing 1 or 2% of Tetronic®908 gave almost similar patterns of vitamin C levels. When the release percentages at the end of the release experiment were compared, the difference between values was not very pronounced, except for the emulsions containing 4% Tetronic®908. In fact, the release percentages of vitamin C from formulations containing 1 or 2% Tetronic®908 after 6 hours were not significantly different (p=0.849). On the contrary, when the release percentages for multiple emulsions prepared with 4% Tetronic®908 were compared after 6 hours, the difference was statistically significant (p=0.0297), 41.1 and 54.2% of the total dose. When we used the ANOVA, it was found that there was the significant difference between the release rates of multiple emulsions containing different amount of hydrophilic surfactant (p=0.017). Although the release rate or release % of vitamin C for formulation containing 4% Tetronic®908 was relatively large, the physical stability of those emulsions was not better than the other multiple emulsions containing 1% or 2% Tetronic®908 as shown in (Table 3); the high concentration

of hydrophilic surfactant did not improve the physical stability of system. This finding agrees with those of *Matsumoto* et al, [17] and *Jiao* and *Burgess* [29]. These authors suggested that the usage of a high concentration of hydrophilic surfactant caused the solubilization of lipophilic surfactant and then disruption of multiple globules. In our study, the higher concentration of Tetronic®908 in the outer aqueous phase may have served to solubilize the molecules of the lipophilic surfactant, which probably led to the rupture of the oil layer and then the rapid release of molecules of vitamin C to the external aqueous phase. In addition, this situation probably caused the phase separation. In this study, Tetronic®908 was used in concentrations of 1, 2 and 4% and these percentages are much higher than critical micelle concentration defined by *Attwood* et al, [30] and *Dong* et al, [31], 0.020% at 40°C and 0.16% at 25°C, respectively. Therefore, Tetronic®908 chains probably formed micelles in both aqueous and oil phases and this facilitated the transport of vitamin C molecules through the oil phase, as described *Omotosho* et al, [32].

The effect of lipophilic surfactant concentration

Previous literatures have shown that the percentage of the lipophilic surfactant plays an important role in the release amount of active molecule. In some of these reports, when the lipophilic surfactant concentration was increased, the release of solute molecules decreased due to maximum viscosity of the system. The cause of increasing viscosity has been explained as the maximum swelling of multiple globules of the system [23, 33]. In contrast, in other reports, an increase in lipophilic surfactant amount caused an increase in the release rate of solute and water [32, 34]. This has been explained by the authors as follows: During the first emulsification, the reverse micelles form in the oil phase of w/o primary emulsion due to the higher concentration of lipophilic surfactant that is above critical value. In addition, during the second emulsification, the existence of hydrophilic surfactant may cause the formation of mixed inverse micelles. Thus, both water and water – soluble molecules in the micelles could be easily carried across the oil layer between the internal and external aqueous phase of w/o/w emulsion. In this study, the effect of Abil®EM90 concentration on the release rate or release amount of vitamin C is shown in (Table 4). It can be seen that as the concentration of lipophilic surfactant in the primary w/o emulsion increased, the release rate of vitamin C from multiple emulsions increased. In addition, the percentage of vitamin C released from multiple emulsions containing 4% Abil®EM90 was greater than that of the other multiple emulsions containing 2% Abil®EM90 prepared with same concentration of hydrophilic surfactant. The release rates of multiple emulsions containing 2% of lipophilic surfactant were compared to the release rates of multiple emulsions containing 4% of lipophilic surfactant using ANOVA test. It was found that the difference was not statistically significant ($p=0.114$). In our study, the droplet size of w/o primary emulsion was approximately the same for two concentrations of Abil®EM90, which likely indicates that the two – fold increase of lipophilic surfactant no longer significantly decreased the interfacial tension. Thus, it is plausible to assume that a concentration of 2% Abil®EM90 was just sufficient to cover the inner aqueous phase of the primary emulsion; the excess amount was likely located in a molecular and micellar form (containing water, vitamin C and NaCl) in the oily phase of the primary emulsion. So, when a higher concentration of Abil®EM90 is used in multiple emulsions, vitamin C molecules in the inner aqueous phase may be transported by micelles in addition to the molecular diffusion. The release studies showed that the release rate of vitamin C was faster for those systems with lower viscosity at the same percentage of Abil®EM90; this also depended on increasing Tetronic®908 concentrations. The viscosity of systems was more effectual than the globule size of multiple emulsions, because emulsions with smaller globules were unable to generate the faster release rate. Therefore, although the globule size did not affect the rate of drug released, the viscosity of multiple emulsions did due to consistency of the system. Based on results of release

experiments, it was concluded that vitamin C might be transported out by molecular diffusion and through a reverse micellar mechanism controlled by the viscosity of the system.

The possible kinetic model for release of vitamin C from multiple emulsions

In the previous studies, *Higuchi*'s mechanism for release of molecule from dispersed polymeric matrix was modified and adapted for multiple emulsions [8, 34]. *Magdassi* and *Garti* applied this model to w/o/w type of multiple emulsions and defined the following equation [35]:

$$3/2\,(1-(1-F)^{2/3}) - F = [(3 \times D \times C_s) / (r_0^2 \times C_0)] \times t \quad \text{...(Eq. 2)}$$

where **F** is the fraction of the drug release from globule; **D** is the diffusion coefficient of drug through oil membrane; C_s is the drug solubility in the oil membrane between the inner and outer aqueous phases; r_0 is the radius of the mean globule size of multiple emulsion; C_0 is the initial concentration of the drug. For simplification the value of "$3/2\,(1-(1-F)^{2/3}) - F$ " was called *B*. In our study, we used this equation and the calculated *B* values were plotted against time, t (Figure 10). The vitamin C solubility in the paraffin film and "D" value were assumed to be constant. The swelling of the globules of multiple emulsions was also cancelled, because as mentioned before the mean size of multiple globules was not changed during time (Figure 2). As shown in (Figure 10), the profiles exhibited two phase for all the multiple emulsions. This was the unexpected situation; the relationship between *B* and t could be linear. *Sela* et al, [34] explained the first phase of profile as resulting from the time required for the formation of reverse micelles and solubilization of water and vitamin C in these micelles. The second and linear phase was the straight line as expected; this confirmed the validity of the equation and the r^2 values was higher than 0.978 (Tablet 5). As a result, it can be concluded that the release of the vitamin C molecules through the paraffin film probably was controlled by diffusion mechanism.

CONCLUSIONS:-

The investigations presented lead us to conclude that the multiple emulsions prepared with Tetronic®908 were highly uniform and high yields of w/o/w multiple emulsions were obtained. Relatively stable systems were formed using Tetronic®908 and Abil®EM90 surfactant pair. It is possible to formulate an optimal multiple emulsions consisting of Tetronic®908 (1%) in the outer aqueous phase and Abil®EM90 (2%) in the oil phase. The results indicated that Tetronic®908 as hydrophilic surfactant might be good emulsifier for the preparation of w/o/w type emulsions. Stability measurements showed that the concentration of hydrophilic and lipophilic surfactant were very important parameter. The release of vitamin C as model hydrophilic substance from system studied; an initial rapid release followed by a much slower rate of release was obtained. Multiple w/o/w emulsion systems can be utilized as potential prolonged release dosage forms. It was concluded that surfactant concentrations affected the release rate. Further in – vitro examinations such as different type of oil phase or the different combinations of aqueous phase are still required and it is recognized that further formulation studies are needed to obtain the most stable formulations using Tetronic®908.

Table 1: Composition of the w/o/w multiple emulsions.

First emulsification (for primary w/o emulsion)
Without vitamin C
Oil phase
Liquid paraffin 30%
Abil®EM90 ..... 2 or 4%
Internal aqueous phase
Sodium chloride 0.3%
Distilled water to 100
With vitamin C
Oil phase
Liquid paraffin 30%
Abil®EM90 ..... 2 or 4%
Internal aqueous phase
Sodium chloride 0.03%
Vitamin C 15%
Distilled water to 100
Second emulsification (for w/o/w multiple emulsion)*
Oil phase
Primary w/o emulsion 80%
External aqueous phase
Tetronic®908 ..... 1, 2or 4%
Distilled water to 100
**The vitamin C concentration of multiple emulsions = 15%*

Table 2: Main properties of multiple emulsions after immediately preparation.

Abil®EM90 (%)	Tetronic®908 (%)	Viscosity* at 5 rpm (mPa.s)	Globule size (µm)	Conductivity (µS)
2	1	60000	20.6	21.3
2	2	58000	20.6	26.8
2	4	46000	34.6	28.4
4	1	60000	29.5	28.9
4	2	54000	30.2	53.9
4	4	44000	36.8	64.0

Table 3: Effect of surfactant concentration on physical stability of multiple emulsions.

Abil®EM90 (%)	Tetronic®908 (%)	1 month	2 months	3 months	4 months	5 months
2	1	-	-	-	-	no PS PS at 40°
2	2	-	-	-	no PS at 25° PS at 40°	no PS at 25° PS at 40°
2	4	-	-	-	no PS at 25° PS at 40°	PS at 25° PS at 40°
4	1	-	-	-	-	PS at 25° PS at 40°
4	2	-	-	-	PS at 25° PS at 40°	PS at 25° PS at 40°
4	4	-	-	PS at 25° PS at 40°	PS at 25° PS at 40°	PS at 25° PS at 40°

"-" indicates phase separation neither at 25°C nor at 40°C; PS, phase separation

Table 4: Release rate constant of vitamin C and some physical characteristics of multiple emulsions containing vitamin C.

	2% Abil®EM90			4% Abil®EM90		
Tetronic®908, %	1%	2%	4%	1%	2%	4%
K_r°	2.31	2.89	5.66	2.56	3.82	6.95
(Released%/hour)[a]	(0.369)[b]	(0.496)[b]	(0.511)[b]	(0.198)[b]	(0.331)[b]	(0.552)[b]
Viscosity (mPa.s)	54000	32000	30000	56000	48000	32000
Globule size (µM)	25.5	34.6	36.6	19.6	20.6	35.8
Released% (±SD)[c]	14.8	15.9	18.3	17.2	19.0	30.9
(at first two hours)	(±1.17)[c]	(±1.55)[c]	(±0.673)[c]	(±0.708)[c]	(±0.154)[c]	(±1.76)[c]
Released% (±SD)	26.3	26.8	41.1	26.4	27.6	54.2
(at 6th hours)	(±1.09)	(±1.09)	(±1.00)	(±0.462)	(±1.51)	(±0.905)

[a] $r^2 \geq 0.997$, [b] SE of regression, [c] ±standard dev., n = 3

Table 5: Calculated parameters of the kinetic model suggested by ***Magdassi*** and ***Garti*** [35] for release of drug from multiple droplets.

	2% Abil®EM90			4% Abil®EM90		
Tetronic®908, %	1%	2%	4%	1%	2%	4%
Slope	0.00211	0.00234	0.00714	0.00198	0.00330	0.0123
Intercept	0.000205	-0.000796	-0.00978	0.00127	-0.00297	-0.00839
r^2	0.993	0.998	0.978	0.998	0.978	0.989

Slope = $(3XDXC_s) / (r_0^2XC_0)$

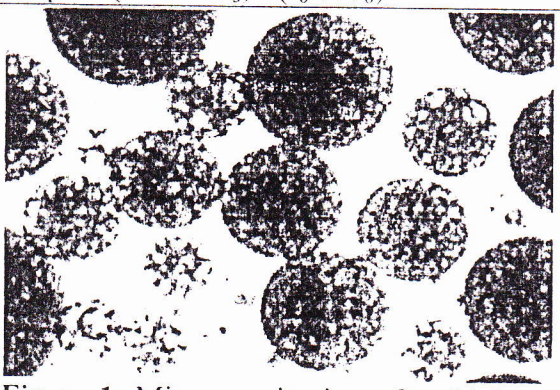

Figure 1: Microscopic view of multiple emulsion (100x16 magnification).

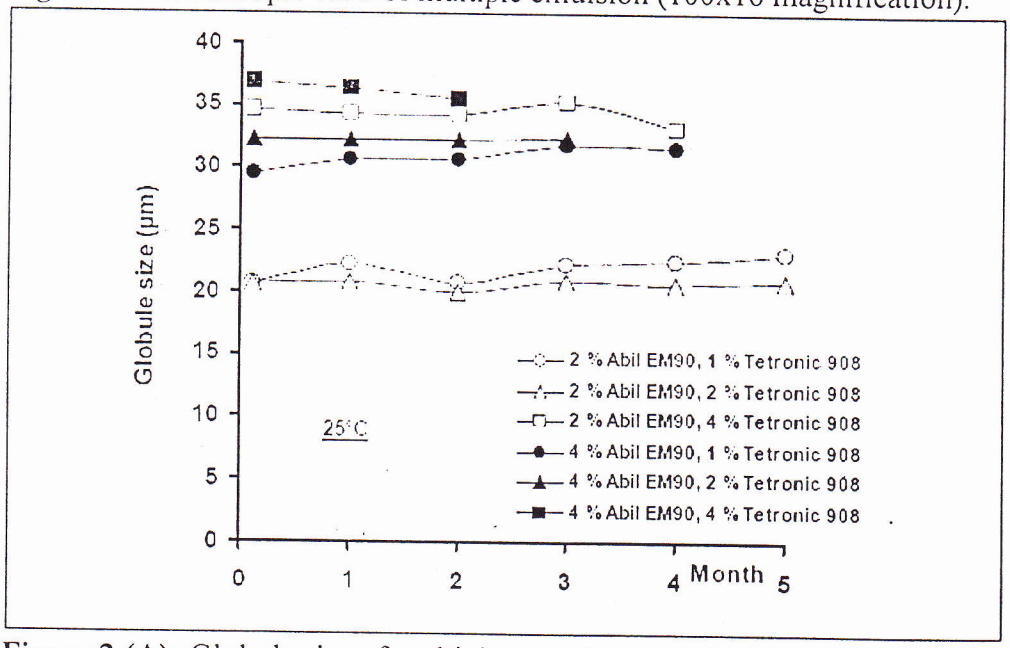

Figure 2 (A): Globule size of multiple emulsions as a function of time at 25°C.

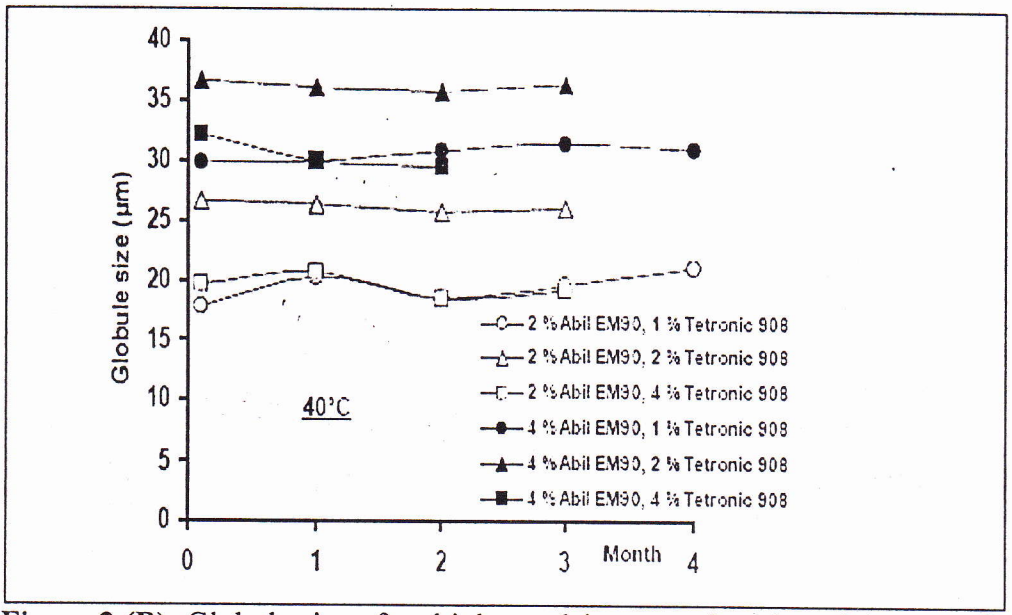

Figure 2 (B): Globule size of multiple emulsions as a function of time at 40°C.

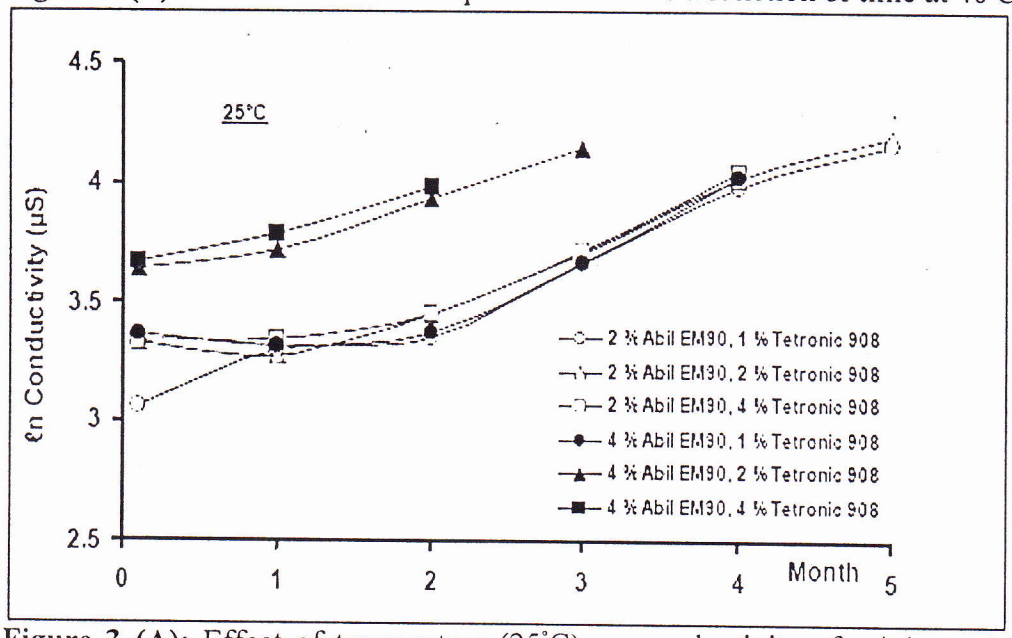

Figure 3 (A): Effect of temperature (25°C) on conductivity of w/o/w multiple emulsions during storage time.

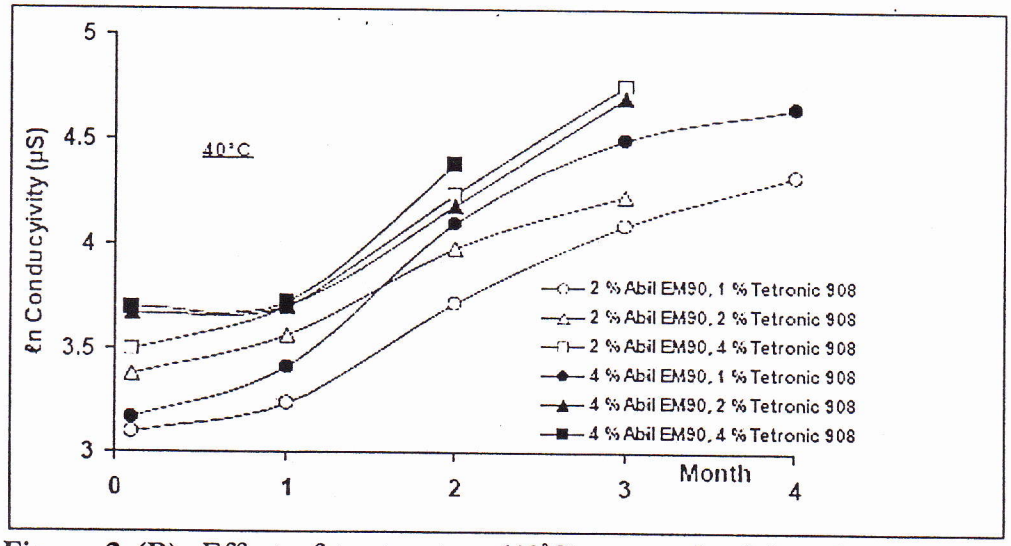

Figure 3 (B): Effect of temperature (40°C) on conductivity of w/o/w multiple emulsions during storage time.

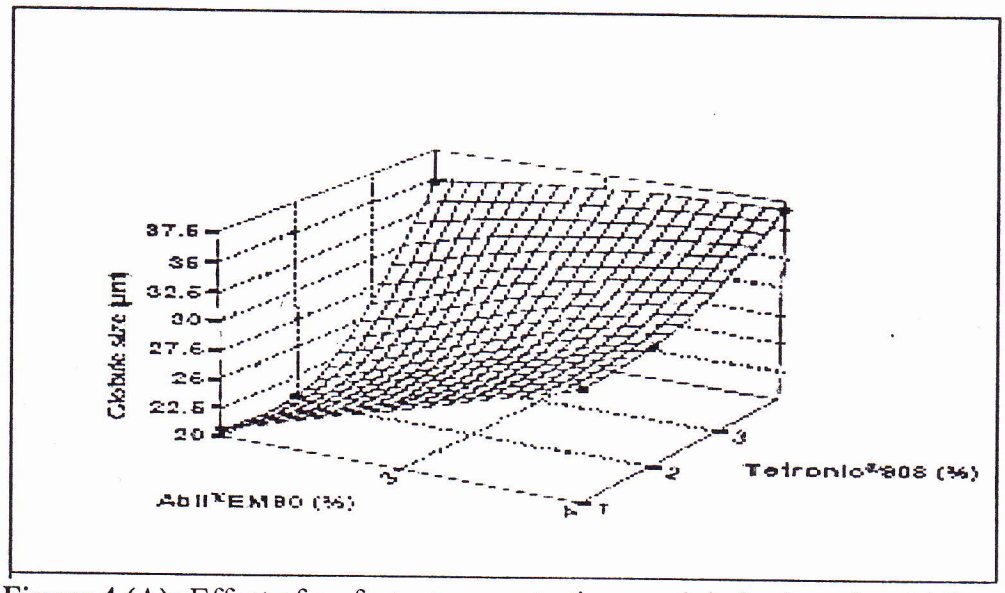

Figure 4 (A): Effect of surfactant concentration on globule size of multiple emulsions.

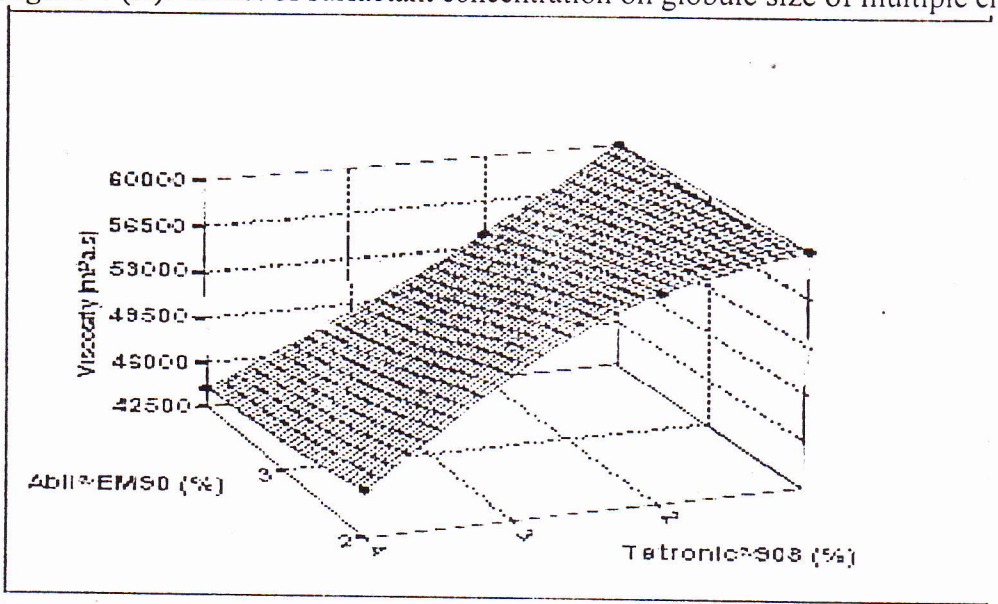

Figure 4 (B): Effect of surfactant concentration on viscosity of multiple emulsions.

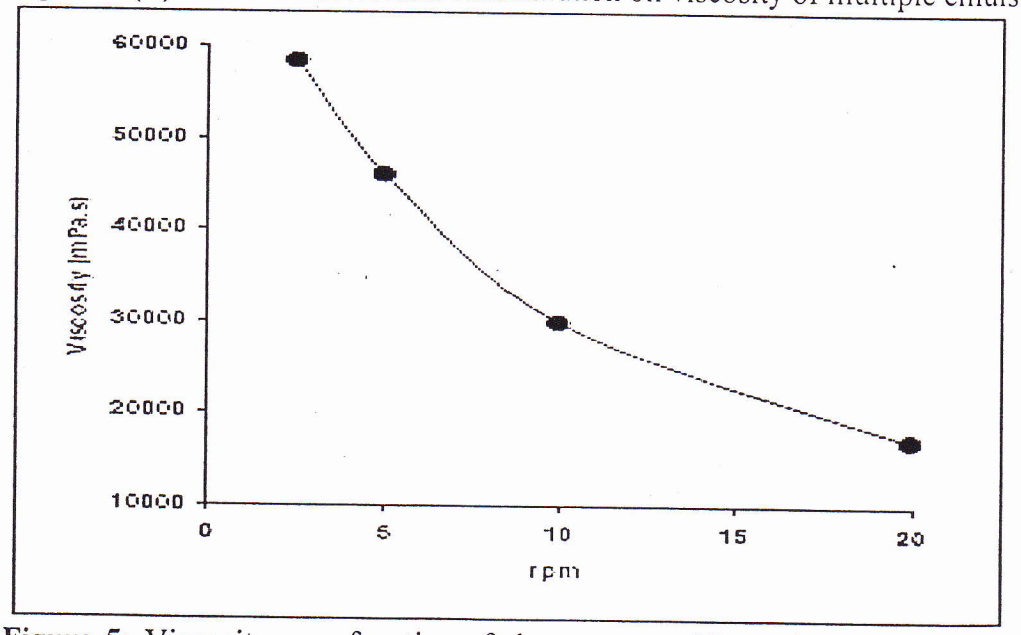

Figure 5: Viscosity as a function of shear stress – Non – Newtonian flow behavior of multiple emulsion containing 2% Abil®EM90 and 2% Tetronic®908.

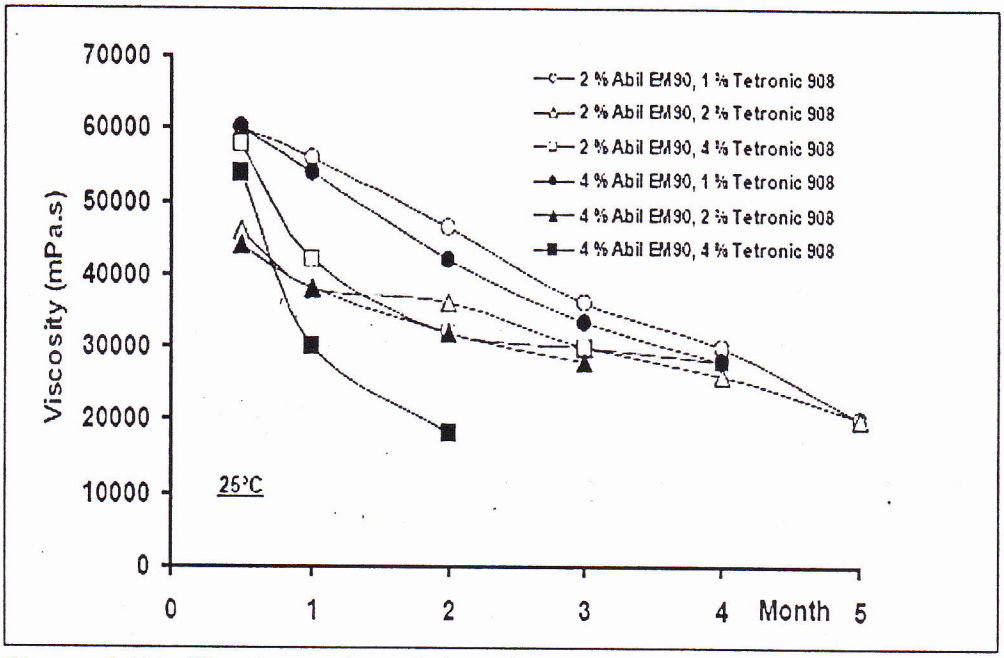

Figure 6 (A): The decreasing of viscosity as a function of time at 25°C (shear stress: 5 rpm).

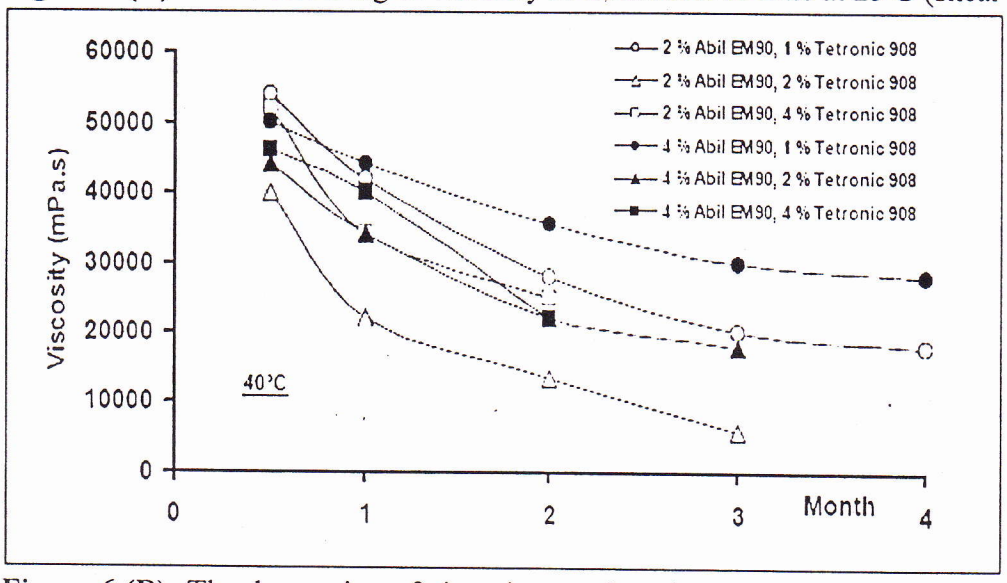

Figure 6 (B): The decreasing of viscosity as a function of time at 40°C (shear stress: 5 rpm).

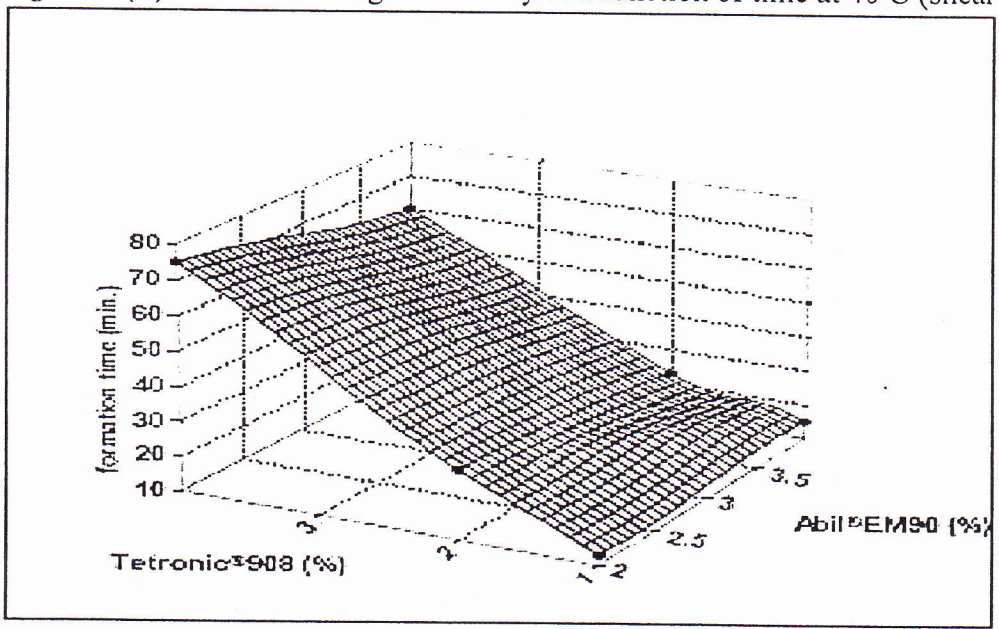

Figure 7: Effect of surfactant concentration on formation time of multiple globules.

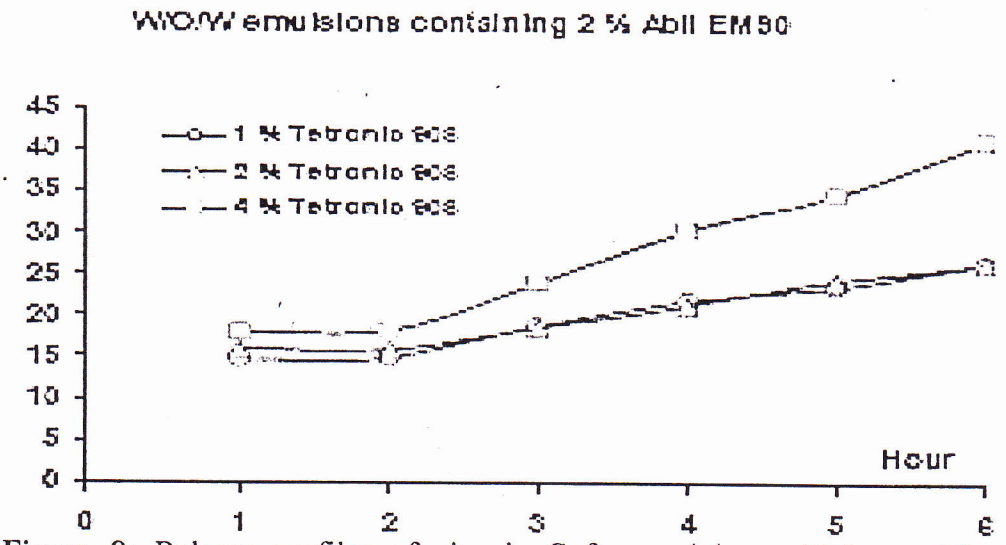

Figure 8: Release profiles of vitamin C from w/o/w multiple emulsions containing different concentration of Tetronic®908 (mean±SE, n: 3) at 2% Abil®EM90.

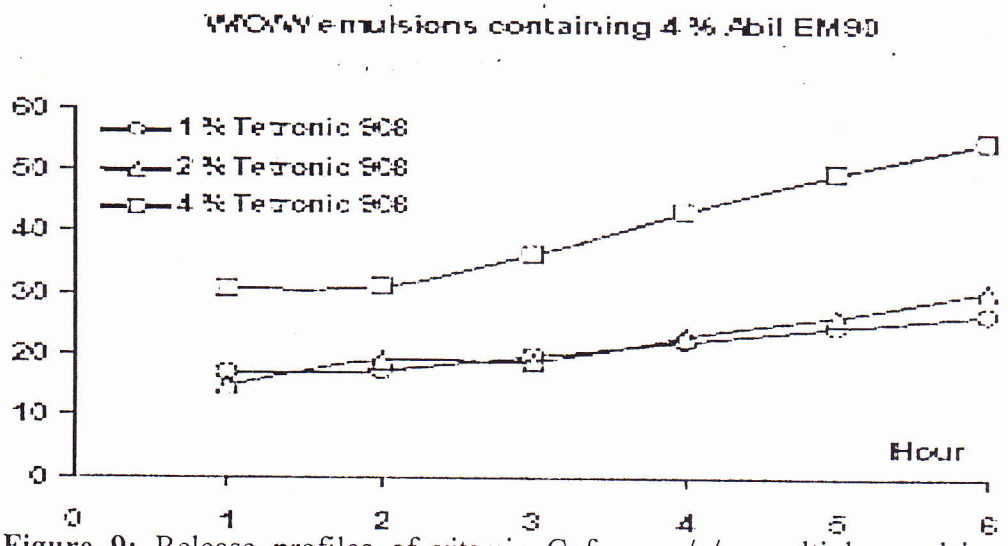

Figure 9: Release profiles of vitamin C from w/o/w multiple emulsions containing different concentration of Tetronic®908 (mean±SE, n: 3) at 4% Abil®EM90.

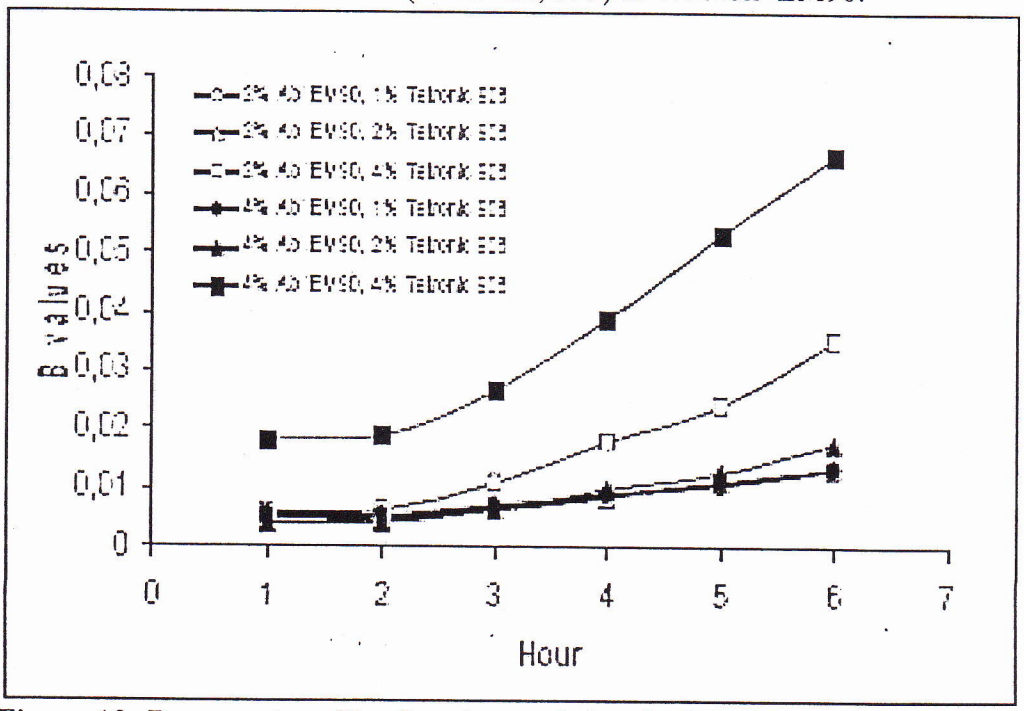

Figure 10: B versus t profiles for release of vitamin C from multiple droplets.

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