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OPTIMIZATION OF THE FORMULATION OF EMULSIONS PREPARED WITH A MIXTURE OF VITAMINS D AND E BY MEANS OF AN EXPERIMENTAL DESIGN SIMPLEX CENTROID AND ANALYSIS OF COLOCALIZATION OF ITS COMPONENTS

OPTIMIZACIÓN DE LA FORMULACIÓN DE EMULSIONES ELABORADAS CON LA MEZCLA DE VITAMINAS D Y E A TRAVÉS DE UN DISEÑO DE MEZCLAS SIMPLEX CENTROIDE Y ANÁLISIS DE COLOCALIZACIÓN DE SUS COMPONENTES

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Abstract

A suitable alternative for satisfying the demand of healthy and targeted compounds such as vitamins, is the preparation and consumption of capsules formed from emulsions The objective of the present work was the preparation of emulsions of vitamins D and E based on a simplex centroid mixture design, using three wall materials: gum arabic (GA) maltodextrin (MD) and modified starch (MS) that represented 20% of total solids, as well as a colocalization analysis to know the distribution of compounds in the micelle. The most stable formulation according to three response variables: particle size (PS), zeta potential (ZP) and Turbiscan stability index (TSI) was obtained. Data were analysed by using an ANOVA test. Results showed that wall materials have a synergic influence on PS and TSI and antagonic for ZP. PS increased with the proportion of GA while ZP and TSI were affected by the charge of the wall materials. Optimum emulsion resulted in a mixture of 3.28% GA, 13.33% MD and 3.37% MS; Predicted and experimental values of the response variables were very similar among them. Also, colocalization analysis showed that once emulsified, the vitamin D was distributed in the centre of the micelle and a minimum part on its surface.

Keywords: emulsion, vitamins D and E, mixture design, colocalization analysis.

Resumen

Una alternativa adecuada para satisfacer la demanda de compuestos nutritivos como las vitaminas, es la preparación de cápsulas a partir de emulsiones. El objetivo de este trabajo fue la preparación de emulsiones de vitaminas D y E basados en un diseño experimental simplex centroide aumentado y utilizando tres materiales de pared: goma arábiga (GA), maltodextrina (MD) y almidón modificado (MS) cuya concentración total en la emulsión fue 20%. Asimismo, se efectuó un análisis de colocalización para conocer la distribución de los compuestos en la micela. Se obtuvo la formulación más estable en términos de tres variables de respuesta: Tamaño de partícula (PS), potencial zeta (ZP) e índice de estabilidad del Turbiscan (TSI). Los datos fueron analizados a través de un ANOVA. Los resultados mostraron que los materiales pared tienen una influencia positiva en el PS y TSI y antagónica para ZP. El PS incrementó con el aumento de la concentración de GA, mientras que el ZP y el TSI fueron afectados por la carga de los tres materiales de pared. La composición de la emulsión óptima fue 3.28% de GA, 13.33% de MD and 3.37% de MS. Los valores predichos y experimentales de las variables de respuesta fueron muy similares entre sí. Los análisis de colocalización mostraron que la vitamina D, una vez emulsionada, se distribuyó en el centro de la micela y una mínima parte en su superficie.

Palabras clave: emulsión, Vitaminas D y E, diseño de mezclas, análisis de colocalización.

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1 Introduction

The demand for healthy and targeted foods has increased over the last years (Boyer, *et al*. 1999; Ziani, *et al*. 2012; Tavano, *et al*. 2014; Janaswamy, *et al*. 2014). A suitable alternative for satisfying such demand is the preparation and consumption of capsules formed from emulsions which can deliver specific substances during their passage through the gastro-intestinal tract (McClements, *et al*. 2007).

Emulsions are colloidal systems in which one or more immiscible fluids are dispersed in the form of micelles protected by wall materials and sometimes, surfactants are used to stabilize the interphase (Dalgleish, 2004). The properties of the emulsions are determined by their composition and by the production method (Leal, *et al*. 2007). Characterization of emulsions is carried out by evaluating the size and stability of the emulsion by means of the zeta potential and by using other parameters such as the Turbiscan stability index. Particle size is important given the fact that, in general, it has been reported that large micelles coalesce faster than small ones (Turan, *et al*. 2015, Tadros, *et al*. 2004). Zeta potential is related to the charge of the surface of the micelles (Das, *et al*. 1990) which, depending on their value, will provoke that micelles repel or attract to each other. The higher the absolute value of the zeta potential, the more stable the system will be and vice versa (Dickinson, 2009). On the other hand, the Turbiscan stability index measures stability of the system by means of light backscattering, so detecting creaming or otherwise of the emulsion (Formulaction, 2013).

There are various works dealing with the inclusion of a number of components in emulsions such as: essential fatty acids, flavonoids and vitamins among others (Habib, *et al*. 2012; Akhtar, *et al*. 2013; Salvia, *et al*. 2013; Relkin, *et al*. 2009) which main objective is their characterization (Mandal, *et al*. 2010), optimization (Zorba, *et al*. 2006); (Bendjaballah, *et al*. 2010) and evaluation of the bioavailability of trapped substances (Ting, *et al*. 2015). Also, there are works dealing with emulsification of liposoluble vitamins, in particular D and E. Vitamin D, can be found in two main forms: Vitamin D2 (ergocalciferol) and Vitamin D3 (colecalciferol). The former is found in very small amounts in foods and is obtained by light irradiation of ergosterol; vitamin D3 is synthetized in human skin by the action of UV irradiation that acts over 7-dehydrocolesterol (Zanchetta, *et al*. 2009; Holick, 2007) which is hydroxylated and triggers a series of metabolic reactions. This is the reason why this vitamin is also considered as a hormone that aids in the absorption of Ca and P by the bones among other functions (Zanchetta, *et al*. 2009). There are reports stating that there are deficiencies of this vitamin due to factors such as diet, skin colour, age of individual, time of exposition to the sun, etc. (Holick, *et al*. 2006). Vitamin E, on the other hand, is widely distributed in nature and refers to a family of 4 tochoferols and 4 tochotrienols (Febles, *et al*. 2002). In particular, α-tochoferol is the most potent and active form of the vitamin which also acts as a strong antioxidant (FAO, 2017) and it has been used to protect vitamin D from oxidation (DSM, 2015). Also, there are a number of works that have reported the preparation of emulsions of vitamin E (Monroy-Villagrana, *et al*. 2014; Cano-Sarmiento, *et al*. 2014; Quintanilla-Carvajal, *et al*. 2011).

During the formulation of an emulsion, it is important to know if the mixture of all protected compounds provides the desired characteristics in the micelle (Villalobos-Castillejos *et al*., 2017) as compared to the function of the single compounds. A number of statistical methods have been implemented to determine the best combination of wall and bioactive materials for a given emulsion (Cornell, 2002; Flores-Martínez *et al*., 2016).

In particular, the design simplex centroid is a particular kind of experimental design in which the response depends only on the relative proportions of factors (ingredients of the mixture) in such a way that changes in the proportion of any of the components will affect the whole design. One of the important features of the emulsions is the relative location of bioactive compounds in relation to the wall materials and in this respect, a number of works there have been published (Quintanilla-Carvajal *et al*., 2011) dealing with the quantification of the relative amounts of surface and core bioactive material. In this respect, Confocal Laser Scanning Microscopy (CLSM) has proved to be a good tool for finding the specific location (Espinosa-Velázquez *et al*., 2016) of the bioactive compounds. Cano-Sarmiento, *et al*., 2014, used this technique to localize tocopherol trapped in a maltodextrin and gum arabic matrix. In particular, colocalization is the presence of the signal of fluorescence at the same pixel location when analysing multichannel fluorescence images. The channels are produced by different fluorochromes when visualizing bioactive and wall materials in the same sample region (Agnati *et al*., 2005; Cario *et al*.,

2006; Criscuoli *et al*., 2005; Patel *et al*., 2006; Petrescu *et al*., 2003; Scriven *et al*., 2005; Zinchuk *et al*., 2004; Hak *et al*., 2015). Colocalization of the components of the emulsion does not provide direct proof of their functional relationship, it gives valuable information regarding structural characteristics so extending the applicability of qualitative observations (Criscuoli *et al*., 2005; Zinchuk *et al*., 2007; Yue *et al*., 2011). The aim of this work was to prepare a stable-small size emulsion of vitamins E and D by following a design simplex centroid methodology and to apply colocalization tools to identify relative location of bioactives (vitamins D and E) and wall materials.

2 Materials and methods

2.1 Material

Vitamin D was supplied by DMS (France); vitamin E (±α-tocopherol) from Sigma-Aldrich Toluca-México; gum arabic (GA) from Alfred L. Wolf, S.A de C.V (México City, México); maltodextrin (MD) from Food Supplements (Naucalpan, State of México, México); modified starch (MS) (Hi Cap 100) from Ingredion (USA) and type I water were used for preparing the emulsions.

2.2 Experimental design

A design simplex centroid was applied by using the software Design Expert 9.0.4 with three components (wall materials, gum arabic, maltodextrin and modified starch), the total concentration of solids was 20% (Krishnan, *et al*. 2005; Quintanilla-Carvajal *et al*., 2011). Optimum emulsion was then obtained by considering the following response variables: Size of micelle (PS), zeta potential (ZP) and Turbiscan stability index (TSI) for a total of 14 experimental runs (Table 1). The design was applied for a wall materials:mixture of vitamins ratio of 2.5:1. Response variables were: average size of the micelle, zeta potential and Turbiscan stability index (TSI) of the emulsion. All concentration figures are given in %w/w.

2.3 Preparation of pre-emulsion and emulsion

Pre-emulsion was prepared with the mixture of the wall materials (Table 1) and the mixture of vitamins D and E and type I water in a nitrogen environment given by an anaerobiosis chamber Cole Parmer model EW-3488-10 (USA) and by using a propeller mixer, model Oster 2612 (México). Pre-emulsion was then subjected to microfluidization in a Microfluidics M110Y microfluidizer, Microfluidics, UK fitted with interaction chambers Y and Z and by using a processing pressure of 69 MPa and 2 microfluidization cycles. Wall materials represented 20% of total solids of the prepared emulsion (see Table 1) (Quintanilla-Carvajal, *et al*., 2011).

2.4 Zeta potential

ZP was evaluated in a 1:1000 dilution of the prepared emulsion by using a ZetaPlus Analyzer (Brookhaven, USA) (Cano-Sarmiento, *et al*., 2014).

Table 1. Experimental design with centroid (Design Expert 9.0.4) including results obtained for PS, ZP and TSI.

RUN	(GA) (%)	(MD) (%)	(MS) (%)	(PS) nm	(ZP) mv	(TSI)
1	0	5	15	308.26	-32.29	0.32
2	10	5	5	350.86	-35	1.47
3	10	0	10	334.8	-33.04	3.23
4	3.33	13.33	3.33	315.3	-38.28	0.2
5	13.33	3.33	3.33	470.1	-36.1	0.36
6	10	10	0	583.16	-38.64	2.17
7	20	0	0	542.9	-38.44	1.39
8	10	10	0	579.76	-37.18	2.04
9	0	0	20	268.9	-35.19	6.45
10	0	0	20	272.46	-32.08	7.7
11	3.33	3.33	13.33	293.05	-31.04	0.6
12	6.66	6.66	6.66	285.45	-34.34	1.95
13	0	5	15	300.66	-32.19	0.32
14	20	0	0	565.63	-36.31	1.83

2.5 *Stability of the emulsion*

Resulting emulsion was tested for stability by using a TURBISCAN Lab expert equipment (Formulation Smart Scientific Analysis, France). Each run was carried out during 6 h. Turbiscan Stability Index (TSI) was then computed by using the software of the equipment (Formulaction, 2013).

2.6 *Colocalization analyses*

Images of the smallest micelle found in the experimental design were used for colocalization analyses by using a confocal laser scanning microscope (CLSM) (LSM 710, Carl Zeiss, Germany). The frame size was 512 x 512, 8 bit, with a 9.6 zoom and minimal scan pixel time of 12.6 μsec to achieve a proper image quality. The confocal pinhole size/Airy size was set to 34 μm. The laser intensity, gain and offset was adjusted to achieve maximum signal with minimal saturation and background disturbance (Manders *et al*. 1993; Agnati *et al*., 2005; Cario *et al*., 2006)).

All images from CLSM experiments of the optimal emulsion were handled and processed by using the ImageJ v.1.50f (National Institutes of Health, Bethesda, USA). To obtain minimal background disturbance, the fluorescence was subtracted by using the plugin: "background subtraction from ROI (region of interest)", considering a number of standard deviations from the mean of 3.0. Further, the images of the different channels were stored in the .lsm format. Files were split and merged to obtain images with overlays from the different signals. To perform a quantitative analysis of CLSM images, the plugin "Colocalization Analysis" of the ImageJ software was used as well as the colocalization tool from the CLSM microscope.

Colocalization Coefficients is the relative number of colocalizing pixels in channel 1 or 2, (Ch1, 2) respectively, as compared to the total number of pixels above threshold. The value range 0-1(0 =: no colocalization, 1: all pixels colocalize (Manders *et al*. 1993).

$$C_{1,2} = \frac{pixels_{Ch1,2;coloc}}{pixels_{Ch1,2;total}} \quad (1)$$

Weighted colocalization coefficients is the sum of the intensities of colocalizing pixels in channel 1 or 2, respectively, as compared to the overall sum of pixel intensities above threshold and in this channel (Manders *et al*., 1993).

$$M_{1,2} = \frac{\Sigma_i Ch1,2;coloc}{\Sigma_i Ch1,2;total} \quad (2)$$

Overlap coefficient is another parameter used to quantify colocalization in image pairs, in which insensitive to differences in signal intensities between the two channels, photo-bleaching or amplifier settings (Manders *et al*., 1993).

$$r = \frac{\Sigma_i Ch1_i * Ch2_i}{\sqrt{\Sigma_i (Ch1_i)^2 * \Sigma_i (Ch2_i)^2}} \quad (3)$$

3 Results and discussion

A total of 14 experimental runs as determined by the experimental design were carried out for determining the dependence of the average size of the micelle, zeta potential (ZP) and Turbiscan stability index (TSI) of the emulsion (Table 1) with the wall materials used.

3.1 *Particle size*

A significant cubic special model was obtained (Eq. 4) with a R^2 = 0.989. It was observed that GA had the largest positive influence on particle size, followed by MS and MD. Interactions GA and MD as well as MD and MS and interaction GA, MS and MD had a negative influence over PS as observed in Fig.1

$$\begin{aligned} PS &= 27.768 * GA + 8.886 * MD + 13.627 * MS \\ &+ 2.1491 * GA * MD - 0.7063 * GA * MS \\ &- 0.8048 * MD * MS - 0.4616 * GA * MD * MS \end{aligned} \quad (4)$$

In Fig. 1, it is possible to observe the behaviour found in Equation (4).

The above described behaviour was due to the fact that GA is the polymer with the highest (1700 kDa) molecular weight (Pedroza *et al*. 2002) which gave place to large particles as supported by runs 7 and 14 in which emulsions only have GA as wall material. Also, the smallest particles were obtained when using MD and MS which have relatively low (1.8 and 12.33 kDa respectively) molecular weights (Pedroza *et al*. 2002). Also, the mixture of GA and MD (runs 6 and 8) gave place to emulsions with relatively high PS which can be due to coalescence of particles when micelles do not have enough polymer (GA) to form an efficient network as reported by Matsumura *et al*., 2000 who stated that the absence of

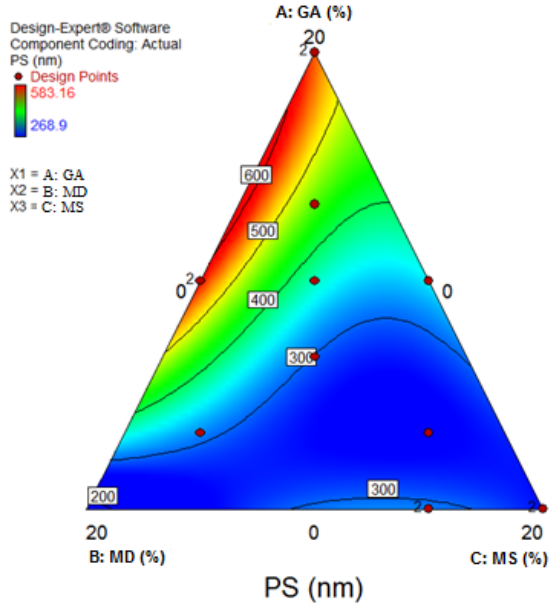

Fig. 1. Graphic response model for PS.

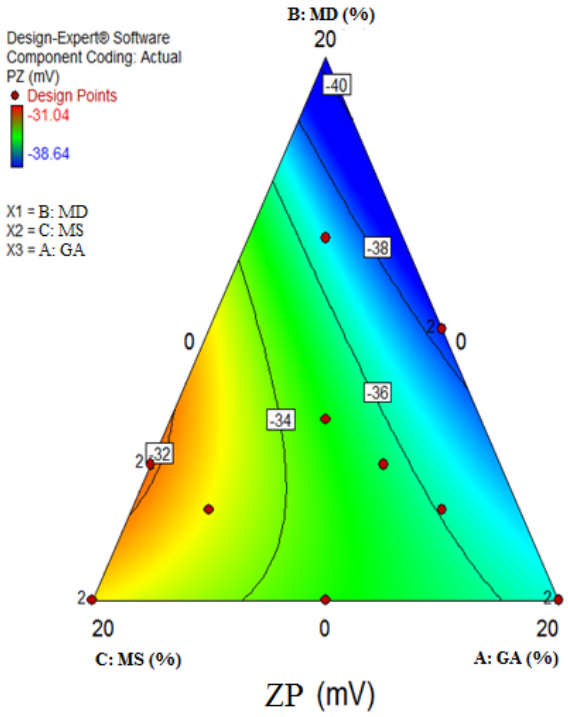

Fig. 2. Graphic response model for ZP.

enough peptide residues reduced the surface activity properties. Similar findings have been reported by Alaei *et al*., 2016 and Bae *et al*., 2008 when emulsifying ginger oil with green tea extract.

On the other hand, the antagonist effect of GA and MS may be due to the decrement in the amount of the polymer having the largest molecular weight. Also the presence of MS gave place to low PZ due to its relatively low molecular weights as compared with that of GA.

3.2 *Zeta potential*

Results gave place to a quadratic model with an R^2 = 0.78 as shown in Eq. (5)

$$ZP = -1.8219 * GA - 2.022 * MD - 1.642 * MS + 0.038 * MD * MS \quad (5)$$

The three polymers had a negative effect (Fig. 2) on ZP which stabilized the emulsion by moving ZP towards high negative values thus favouring electrostatic repulsion (Zeta-meter Inc, 2012; Duffy *et al*., 2011; Ostolska *et al*., 2014) whereas the combination of MD and MS increased its value as can be observed in Table 2 and Fig. 2. It is noteworthy that ZP values for each wall material are negative thus indicating that particles are anionic (Clogston *et al*., 2011).

In Fig. 2, it is possible to observe the effect of each of the three wall materials on ZP. As stated earlier, the combination of the three materials produced the lowest ZP values due to the electrical charges of GA, MS and MD (Saloko *et al*., 2013; Albert *et al*., 2016; Klinkesorn *et al*., 2004; Castro, 2014). The electrical charge generated by GA can be attributed to the protein component of this substance which contains charged aminoacids on its surface such as arginine, aspartic and glutamic acids (Jayme *et al*. 1999) as well as other acid groups that can be ionized such as carboxyl groups of the D-glucoronic acid and residues of the 4-O-metil D glucoronic acid (Mc Clements, 1999). The MS has a negative charge due to carboxyl groups of the octenil-succinic chains (Shih *et al*. 2003; Castro, 2014). All ZP values were negative and lower than -30 mV and indicated emulsion stability (Maron *et al*. 1993). Results agree with those reported by Monroy-Villagrana, 2014; Panteloglou *et al*, 2010; Chanamai *et al*. 2001; Dokic *et al*. 2008.

3.3 *Turbiscan Stability Index (TSI)*

Results gave place to a quadratic model with an R^2 = 0.88 as shown in Eq. (6)

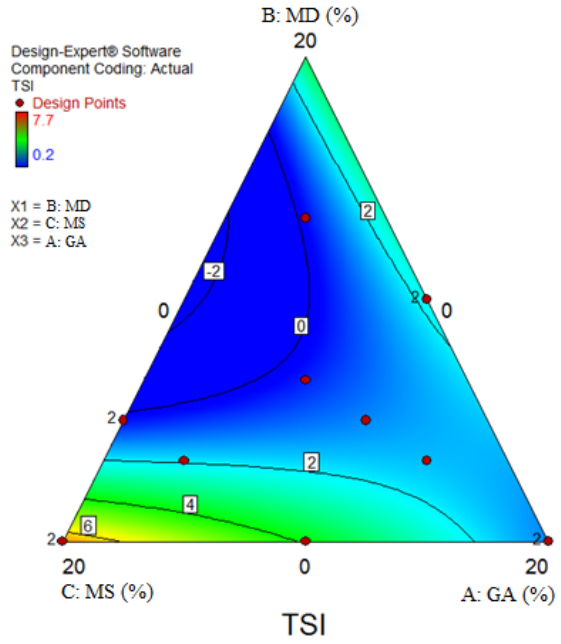

Fig. 3. Graphic response model for TSI.

Table 2. Experimental values of ZP for Wall materials used in the experimental design.

Wall material	Dilution	ZP (mV)
MD	1:1000	-17.36
GA	1:1000	-28.97
MS	1:1000	-19.20

$$TSI = 0.058 * GA + 0.163 * MD + 0.332 * MS - 0.073 * MD * MS \quad (6)$$

A synergic effect of GA, MD and MS was observed, together with an antagonic influence of the combination MD and MS. Results were due to the stability given by each one of the wall materials as well as stability provided by steric mechanisms (Chanamai *et al*. 2001; Bouyer *et al*. 2012) which is enhanced by the viscosity of the media (Bouyer *et al*. 2013).This can be graphically seen in Fig. 3, in which it is noted that the lowest value of TSI was given by the mixture of MD and MS. On the other hand, a slight increment of GA increased values of TSI and the contrary was observed with MD which provoked steric repulsion between particles and did not have emulsifying properties (Bouyer *et al*. 2012; Matsumura *et al*. 2000).

Table 3. Predicted and experimental values of PS, ZP and TSI for optimum emulsion

Parameter	Predicted values	Experimental values
PS	310.06 nm	311.33 ± 8.58 nm
ZP	-36.7 Mv	-34.10 ± 1.03 mV
TSI	0.2	0.65 ± 0.08

The optimum emulsion having the lowest PS, ZP and TSI resulted to have 3.28% GA, 13.33% MD and 3.3% MS. This can be observed in Fig. 4 for each parameter with a desirability of 0.868. Fig. 4a presents the prediction for the three parameters, while Figs. (4b)-(4d) show predictions for PS, ZP and TSI respectively.

Predicted and experimental results for PS, ZP and TSI for optimum emulsion are given in Table 3.

Predicted and experimental results were very close so that this formulation can be considered as optimum.

3.4 Colocalization of the vitamins in the emulsion

Interaction and distribution of the vitamins D and E over the wall materials (GA, MD, MS) were studied by using CLSM and colocalization analysis (Agnati *et al*., 2005; Cario *et al*., 2006; Criscuoli *et al*., 2005; Patel *et al*., 2006; Petruscu *et al*., 2003; Scriven *et al*., 2005; Zinchuk *et al*., 2004; Hak *et al*., 2015). From inspections of the signals of images in which the vitamin D (green, Fig. 5b) and vitamin E with wall materials (red, Fig. 5a) resulted that channels were superimposed (Fig. 5c) and only partial colocalization of vitamins D and E within wall materials can be subjectively identified by the appearance of yellow-orange colour (around the red channel in Fig. 5c). This indicated partial coexistence and distribution of both vitamins and wall materials with an efficiency of 71.72% of the distribution of vitamin D and E in the emulsion and showing that the stability was not affected by the surface density. This indicated that once emulsified, the vitamin D was distributed in the centre of the micelle and a minimum part on the surface and perimeter of each micelle. Furthermore, 1.7% of the total area of the vitamin D was not colocalized in the micelle.

This was confirmed by quantitative colocalization analysis (Fig. 5d). The colocalization showed a correlation between the intensities in the two channels for both vitamins and wall materials.

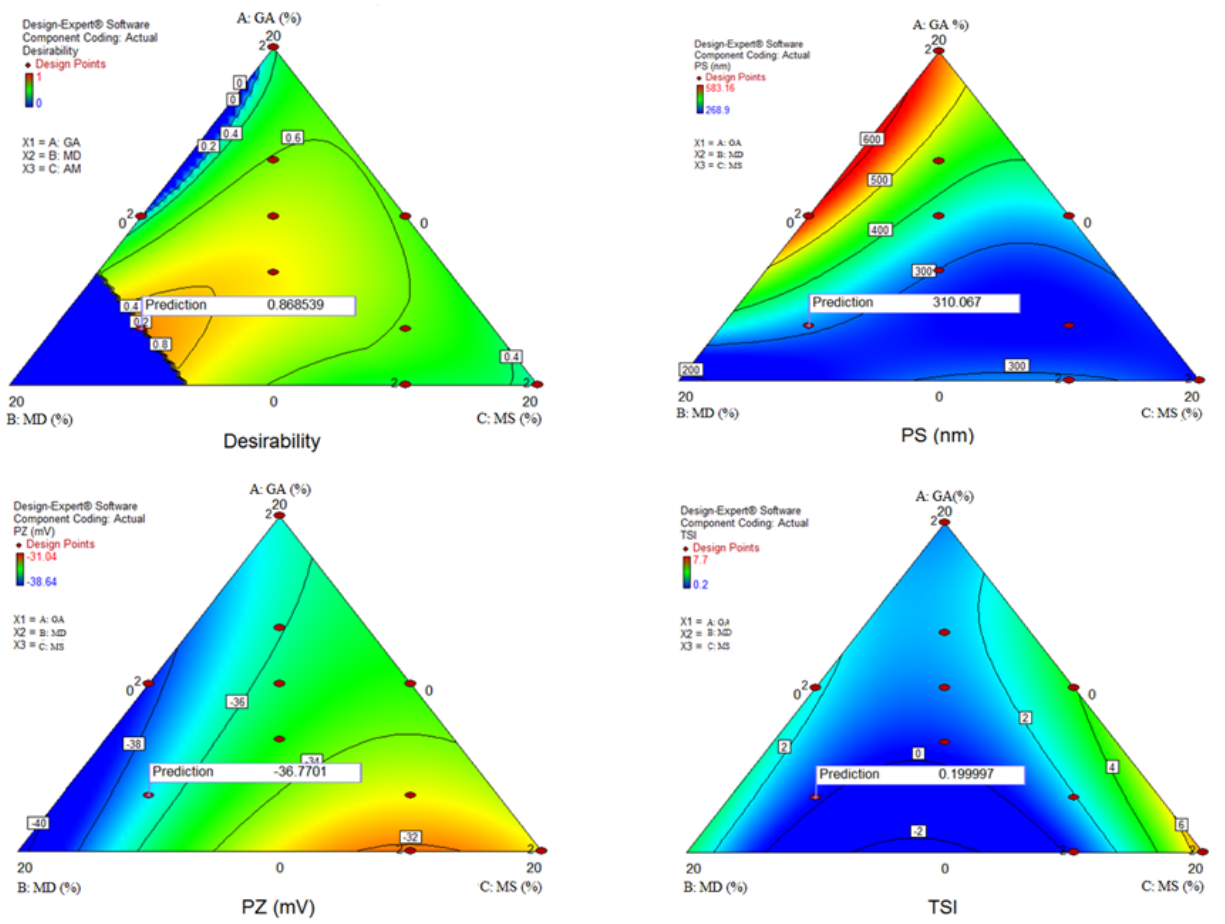

Fig. 4. Responses for optimization of the formulation of the emulsion; a. Optimization of the ternary mixture GA, MD and MS with a desirability of 0.868; b. Prediction for particle size; c. Prediction of ZP and d Prediction of TSI. "Prediction" labels in graphs, correspond to the optimum.

The colocalization coefficient (Eq. (1)) (Manders *et al*. 1993) for the vitamin D and vitamin E with wall materials were 0.41±0.01 and 0.045±0.001 respectively. Moreover, the overlap coefficient (r in Eq. (3)) (Manders *et al*. 1993) which indicated the highest intensity between the two channels was 52.75% showing the difference in the signal intensities between the two channels. The weighted colocalization coefficient (M in Eq.(2)), describing the fraction of Vitamin D colocalized with vitamin E and wall materials, was 0.39±0.01. At the same time, the M for the vitamin E and wall materials over the vitamin D was 0.028±0.001 (due to a low concentration of vitamin D).

Colocalization analysis indicated partial distribution and interaction of vitamin D within wall materials and vitamin E, showing that the optimal conditions for the microencapsulation of vitamin D and E with gum arabic, maltodextrin and starch provides stability and distribution. The average size of the micelle obtained from the colocalization analysis was 1.497±0.012 μm and had a distribution of the components as follow, 6.01% from vitamin D and 93.99% from vitamin E and wall materials. Furthermore, colocalization of all components of the micelle, showed a 95.66% of the total area with a complete interaction of the two analysed channels.

Conclusions

The experimental design showed the effect of each of the three wall materials used to prepare the emulsion on the response variables considered. GA showed the largest positive influence on PS.

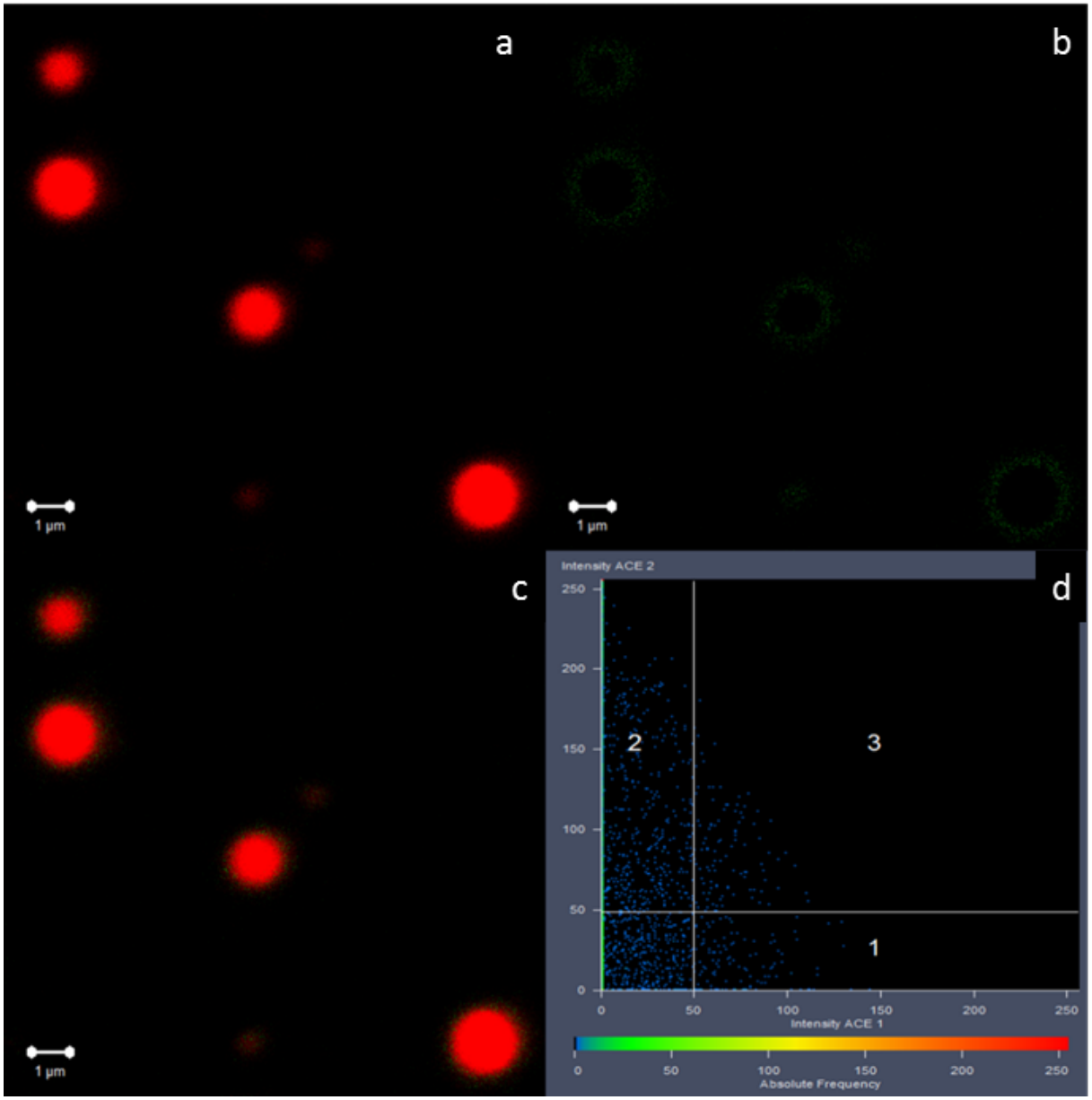

Fig. 5. CLSM micrographs for analysis of the emulsion of vitamins D and E using arabic gum, maltodextrin and modified starch as wall materials. Wall materials and vitamin E (a) (red channel), vitamin D (b) (green channel), overlap channels for colocalization analysis (c) and quantitative analysis of colocalization of the emulsion (d). In (d): Region 1 corresponds to the red channel, region 2 to green channel and region 3 to colocalization area.

Emulsions prepared with MD and MS had the smallest particle size. The three polymers had a decreasing effect on ZP which stabilized the emulsion by moving ZP towards high negative values thus favouring electrostatic repulsion whereas the combination of MD and MS increased its value, the electrical charge generated by GA can be attributed to the protein component of this substance which contains charged aminoacids on its surface. Regarding TSI, a synergic effect of GA, MD and MS was observed, together with an antagonist influence of the combination MD and MS. Results were due to the stability given by each one of the wall materials as well as stability provided by steric mechanisms.

The optimum emulsion having the lowest PS, ZP and TSI resulted to contain 3.28% GA, 13.33% MD and 3.3% MS. Regarding colocalization analysis it was concluded that once emulsified, the vitamin D was distributed in the centre of the micelle and a minimum part on its surface.

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