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Developing an Emulsifier System To Improve the Bioaccessibility of Carotenoids

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Food emulsion designs, with the aim of delivering lipophilic bioactive compounds, should include an estimate of their bioaccessibility to support the claimed effect. With this goal in mind, in vitro digestion models and experimental design of mixtures were used as analytical tools to measure this parameter and to optimize the formulation of an O/W emulsion, including carotenoids as functional ingredients. Two experimental stages were applied. First, a screening phase was completed to detect the critical factors that exerted a significant effect on the response (bioaccessibility). During this phase, we observed that the response was modified mainly by secondary effects such as synergies and antagonisms of the emulsifying mixture. A group of four emulsifiers was selected at this phase to perform the second experimental stage, the optimization phase. This allowed us to obtain the mixture that produced the maximum carotenoid bioaccessibility. This formulation had emulsifying properties of the liposugars, acyl- and polyacyl-glycerides, as well as the synergistic effect arising from the combination of materials; this maximized the response. The analytical approach applied in this work is of interest for food designers for screening and controlling the bioaccessibility of bioactive compounds in a given matrix and, consequently, selecting the formulation conditions for higher bioaccessibilities.

KEYWORDS: Carotenoid; bioaccessibility; emulsion; formulation; optimization

INTRODUCTION

New food preferences, dietary recommendations, and the presence of bioactive compounds (all with the aim of improving our health) have introduced new challenges for food science and industry. Although a natural food may be considered functional, usually the bioactive compounds are isolated from the appropriate source, their concentration is increased to the required level, and they are then added to a suitable food matrix. This process requires integrated action in several stages, such as the identification of a bioactive compound, its toxicological evaluation, application of the appropriate isolation technology, incorporation of the functional ingredient into the food matrix, and measurement of its stability and bioaccessibility (*1*). Once these factors have been evaluated and optimized, the final product is produced. However, each stage requires varying degrees of consideration. Thus, the essential aspects include toxicological evaluation to assess the health risks of the new product and the technology required to produce it. Production optimization must also be considered. Improving bioaccessibility was not initially a goal in the development of functional foods, although it should be considered as such (*2*). It is possible to find designed functional foods where the bioaccessibility of some bioactive compounds is diminished by some other ingredients, and thus, antagonisms are produced. Some studies have dealt with this subject (*3, 4*). Even in natural foods, bioaccessibility of the bioactive compounds that characterize the product may be diminished by other interfering components of the diet, and carotenoids are one example of this problem.

Carotenoids have attracted the interest of industry because they may be used for the formulation of functional foods, having both positive impacts on human health and economic benefits (*5*). Dietary carotenoids present different biological actions such as pro-vitamin A activity and antioxidants for radical species, and they act as enhancers of the immune system (*6*). Bioaccessibility of these compounds from natural sources (mainly fruits and vegetables) is often low and is conditioned by different factors, mainly the processing state of the food and the matrix composition (*7*). Several lines of evidence point to the positive effects (increasing accessibility) of homogenization and heat treatment of the food and/or extractability of carotenoids (*8*), while fiber diminishes the assimilation of these compounds (*9*). One of the key factors affecting the accessibility is the amount and type of fat present in the compound. A minimum amount of fat is required for increasing absorption, so formulation of carotenoids in an oily matrix (vegetable oils) may provide high bioaccessibility (*10*). However, it has been shown that the

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bioaccessibility of carotenoids from fatty food formulations is not as high as expected. For example, bioaccessibility is lower in tomato oleoresin (concentrated source of lycopene) and red pepper oleoresin (rich source of capsanthin) than the bioaccessibility provided by other food formulations containing carotenoids, such as tomato and paprika juices (*11*−*14*).

Therefore, hydrophilic matrices should be considered as a suitable formulation with significant bioavailability value. In addition, because the tendency consumers have to eat foods with bioactive compounds is conditioned by the medium through which compounds are delivered, hydrophilic matrices may provide an efficient source of carotenoids (*15*). Hydrophilic matrices with lipophilic carotenoids, incorporated by means of the use of the appropriate technique, use the traditional dietary sources of carotenoids (raw fruits and vegetables or oily sources like margarines, sauces, and oils) and may result in higher consumer acceptance. In this sense, the application of either encapsulation or emulsions is an adequate strategy that may be applied to achieve several objectives, including improving bioaccessibility and stability of the bioactive compounds. With this aim, different emulsion formulations for improving the incorporation of carotenoids into hydrophilic food matrices were evaluated (*16*) with the idea that better incorporation into the hydrophilic environment should also improve the bioaccessibility (although this parameter was not measured). It must be emphasized that these techniques may improve carotenoid bioaccessibility if the selection and formulation of the emulsifiers or encapsulated agents are optimized while taking bioaccessibility into consideration. Indeed, the influence of the food matrix on the bioaccessibility of bioactive compounds is a required criterion to support the claimed effect of functional foods. It has been proposed that in vitro digestion models should be developed and used as analytical tools to estimate the bioaccessibility of bioactive ingredients in different food matrices (*17*).

In vitro digestion models have been used for the estimation of bioaccessibility of minerals, vitamins, and lipophilic compounds such as cholesterol and carotenoids from different foodstuffs and to control both dietary factors and components that modulate this parameter (*11, 18*). These models may be considered useful tools for screening the factors and components that modify the bioavailability of bioactive compounds in a given matrix composition and for selecting the formulation conditions that produce the highest availability.

Consequently, it is possible to better understand the emulsions that are used to deliver carotenoids into hydrophilic matrices by screening the bioaccessibility of these compounds during design and evaluation of the formulations. This screening may be used to optimize the composition of the formulation to make a hydrophilic matrix with improved bioaccessibility of carotenoids. To develop a scientific method for this screening, two experimental set-ups were considered. While a factorial experiment studies the effect of some observable response of two or more varying factors, such as temperature and raw material origin, this proves to be a problem in a mixture, where the measured response is assumed to depend only on the proportion of the factors (in this case, ingredients) present in the mixture and not on the absolute amount of the mixture (*19*). Thus, if we represent the proportion of the *i*th constituents in the mixture by x_i, then

$$x_i \geq 0 \text{ for } i = 1, 2, ..., q$$

and

$$\sum_{i=1}^{q} x_i = x_1 + x_2 + ... + x_q = 1.0$$

The aim of the present study was to apply the experimental design of mixtures to optimize the formulation of emulsions O/W, where the oily phase contained carotenoid pigments as functional ingredients, to obtain a formulation that provides the highest bioaccessibility of carotenoids. The application of an in vitro digestion method allowed the comparison of the bioaccessibility that each matrix provided, using this response as a discriminating tool for optimizing the composition of the emulsion. Thus, the contribution of the emulsifying agents was analyzed, discarding the factors that had a negative effect on bioaccessibility and optimizing the presence of those that improved bioaccessibility.

MATERIALS AND METHODS

Raw Materials. Paprika oleoresin (*Capsicum annuum* L.) was used as an oily extract, as it is rich in carotenoid pigments. The sample was kindly supplied by Extractos Vegetales S.A. (La Línea de la Concepción, Cádiz, Spain). The set of emulsifying agents used in the experiments was selected from different products that function as liquid bioavailability enhancers or as oily carriers. These products were supplied by Gattefossé (Gennevilliers, France): saccharose distearate, CAS 25168-73-4 (EMF-1); saccharose monodistearate, CAS 25168-73-4 (EMF-2); saccharose monopalmitate, CAS 26446-38-8 (EMF-3); 2,3-dihydroxypropyl docosanoate, CAS 30233-64-8 (EMF-4); glyceryl palmitostearate, CAS 8067-32-1 (EMF-5); polyglyceryl oleate, CAS 76009-37-5 (EMF-6); propylene glycol laurate, CAS 27194-74-7 (EMF-7); glyceryl monostearate, CAS 31566-31-1 (EMF-8); and hexaglyceryl distearate, CAS 34424-97-0 (EMF-9).

Reagents and Solvents. Pepsin, bile extract, and pancreatin were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC) grade acetone and methanol were from Romyl (Teknokroma, Barcelona, Spain). Purified water was obtained with a Milli-Q water-purifying system (Millipore, Milford, MA). All other reagents were of analytical grade.

Experimental Design. Resolving scientific problems of a factorial nature is well-described in the scientific literature, and some tutorial examples have been published (*19*). We begin with a number of factors to be examined and identify the most important (critical) factors that exert a significant effect on the output variable/s or response/s that define the problem. The best approach to take is to use a screening experimental design (for example, the Plackett−Burman experimental design), because it is most economical and efficient for determining the most critical factors during the screening phase. Once the critical factors have been identified, they may be optimized by means of other specific experimental designs, such as the Box and Behnken or Box and Wilson central composite experimental designs.

However, this very well-established two-step method for factorial problems is not available for situations involving mixtures. Authors generally describe only the optimization phase, assuming that the identification of the main effects has already been done. It may be deduced that the screening phase is performed empirically, on the basis of the researchers' practical experience. In essence, there are two main experimental designs for working with mixtures: simplex-lattice (SL) and simplex-centroid (SC) (*20*). In all cases, the minimum number of runs is higher for SC experimental designs, with fewer runs for fewer ingredients (six and seven for three ingredients for SL and SC experimental designs, respectively) but much more for higher numbers of ingredients number (45 and 511 for nine ingredients for SL and SC experimental designs, respectively). For this reason, our experimental design was conducted in two phases: first to identify the most critical effect on the response using an SL experimental design (screening phase) and then to optimize the ingredients selected to obtain the maximum response value using an SC experimental design (optimization phase).

Screening Phase: Detection of Main Effects. The aim of this phase was to identify the compounds that provided the most relevant effects

Table 1. Phase 1: SL Experimental Design Used to Identify the Critical Emulsifier

emulsifier[a]	levels (%)						
1−9[a]	0	1.7	2.5	3.3	5		
water	80	85	86.7	87.5	0.90	0.95	98.3

[a] Key: 1, saccharose distearate; 2, saccharose monodistearate; 3, saccharose monopalmitate; 4, 2,3-dihydroxypropyl docosanoate; 5, glyceryl palmitostearate; 6, polyglyceryl oleate; 7, propylene glycol laurate; 8, glyceryl monostearate; and 9, hexaglyceryl distearate.

(those with a high positive absolute value) on the response, that is, the micellization percentage of carotenoid pigments. To achieve this, we applied a SL experimental design for 10 ingredients (nine emulsifiers and water) augmented with interior points and a centroid. According to previous experiments and both the manufacturer's and our own experiences, the restrictions were considered as follows: (i) for 10 g of O/W emulsion, 0.1 g of the carotenoid source sample was used (the equivalent of 6.26 mg of carotenoid pigments), and (ii) the minimum amount of water considered was 8 g, and the remainder of the emulsion (up to 10 g) was filled with each of the 65 prototype mixtures included in the experimental design. **Table 1** shows the experimental design used at this phase. The components of the emulsion were added to a mixing vessel while applying a melt-coating process. The mixture was incubated in a water bath at 40 °C and stirred at maximum speed for 1 h to obtain a carotenoid emulsion. Finally, the emulsion was homogenized again with a liquid homogenizer (Ultra-Turrax, model T-25, IKA Labortechnik, Staufen, Germany) for 2 min at maximum speed.

Mixture Ingredient Optimization. From the results obtained in the screening phase, four emulsifiers were selected as most relevant for obtaining the most effective micellization of carotenoid pigments (optimization), and we maintained an 80% water content (w/w), according to a SC experiment with the following levels of the selected emulsifiers: 0, 25, 33.3, and 100%. The homogenization process of components for the production of emulsions was the same as described in the previous experimental phase.

Independent of the phase, all experiments (runs) were performed in a random order (trial order) because randomization eliminates erroneous conclusions due to extraneous sources of variability. During both phases, response surface methodology (RSM) was used to analyze and graphically present the results.

In Vitro Digestion Model. The response of interest that was used as a discrimination variable was the carotenoid bioaccessibility of the emulsion. This was defined as the amount of carotenoid that was incorporated into micelles after undergoing an in vitro digestion process using the initial amount of carotenoid content as the reference. Thus, the percentage of micellization was obtained, and this value depended on the capacity of the emulsion components to facilitate the transfer of carotenoids from the matrix to the micelles. The challenge was to obtain an emulsion that provided the highest micellization percentage and thus higher bioaccessibility. The in vitro digestion was applied to all emulsions formulated using compositions. The experimental conditions were similar to those used in previous studies (*18, 21*). Briefly, the emulsion sample (0.25 g) was mixed in a 50 mL test tube with 5 mL of pepsin solution (0.05%, pH 2). The mixture was incubated in a water bath with orbital shaking at 37 °C for 1 h. The pH was then adjusted to 7, and 5 mL of phosphate buffer (0.1 M, pH 7.4), 30 mg of bile extract, and 250 μL of saline solution (3 M NaCl, 75 mM $CaCl_2$) were added. After incubation at 37 °C for 30 min, 100 μL of a lipase suspension (50 mg protein extract/mL, 5 mM $CaCl_2$) was added, and the mixture was kept in the orbital shaker for 2 h at 37 °C. Once the digestion was complete, a centrifugation procedure was applied to isolate the micellar fraction from the rest of the emulsified components. The digested mixture was centrifuged at 49300 rpm for 100 min at 4 °C in a 70.1 Ti rotor (L8-70 M Beckman Instruments, Inc., Palo Alto, CA). A 10 mL aliquot was withdrawn for subsequent analysis.

Determination of the Carotenoid Content in Emulsions or Micelles. The carotenoid fraction was extracted by mixing emulsion samples (0.1 g) or micellar solutions (4 mL) with 2 mL of *N*,*N*-dimethylformamide, 4 mL of hexane, and 4 mL of Na_2SO_4 (2%, w/v) or 26 mL of hexane and 4 mL of Na_2SO_4 (2%, w/v), respectively. The mixture was vortexed for 2 min, placed in an ultrasound bath for 5 min, and centrifuged at 4400 rpm for 5 min. An aliquot of the organic layer was withdrawn, and the carotenoid content was determined by measuring the absorbance value at 456 nm with $E_{1cm}^{1\%} = 2654$ (*22*).

RESULTS AND DISCUSSION

Screening Phase. Results obtained in the screening phase are shown in **Table 2**, and the coefficients of regression were estimated by the least-squares method shown in **Table 3**. It should be noted that traditionally it has been thought that the confidence level needed to achieve statistical significance in scientific research is 95%, and so, the α-level is usually set to 5% ($p \leq 0.05$); however, this criterion is a convention only. In the screening phase of our study, most of the tested factors would not have been considered significant (according to the Pareto principle, trivial many and vital few), and the noise level may have affected the results, so when a p value ≤ 0.05 was used, none of the analyzed factors (**Table 3**) would have been selected for the optimization phase. A threshold of $p \leq 0.15$ has been previously used for the screening phase with optimal results (*23, 24*). In this case, even that p value is not enough to pinpoint the critical factors (vital few) as only one factor value passed that threshold (**Table 3**, term number 28, 3×4, -374.7, $p = 0.08$). There was a high correlation between the absolute value of the term and its p value (**Table 3**, $r = 0.82$, $p \leq 0.05$). This correlation results from the calculation used to obtain statistical significance of the coefficients. Thus, a model reduction may be accomplished in several ways, but the most obvious approach to model reduction is to remove the terms according to a statistical criterion (*25*). We used a t test and divided the absolute value of each coefficient by its associated standard error to test whether the coefficient was different from zero, rejecting the null hypothesis. Because similar standard errors were achieved for different coefficients obtained in the model (**Table 3**), at the end of this process, the higher absolute coefficients were closer to values that would require rejecting the hull hypothesis and, thus, were considered the critical factors. Therefore, the criterion that we followed for model reduction was determined exclusively by the absolute values of the terms: We selected those with higher absolute values to begin the process of locating the critical ingredients to be considered for the optimization phase.

During the screening phase, we found a relationship between the structure of an emulsifying agent and its micellization index. From a chemical structure point of view, emulsifiers considered in this study may be classified into three different groups: saccharose esters or liposugars (EMF-1, EMF-2, and EMF-3), acylglycerides (EMF-4, EMF-5, EMF-7, and EMF-8), and polyacylglycerides (EMF-6 and EMF-9). The saccharose ester group showed varying results on the micellization index. Considering the individual primary effects of compounds that were individually produced by each component alone (**Table 3**), it seemed that the micellization index depended on the lipophilic character of the saccharose ester. Thus, higher effects were achieved with EMF-3 (term value, 169.3), but when the length of the lipophilic tail and the esterification degree increased, the term value of the micellization index decreased (22.6 and -64.7 for EMF-1 and EMF-2, respectively). Fanun observed that the solubility of lipophilic compounds in emulsions tended to be lower when the lipophilic character of the emulsifier increased (*26*). This led to a negative effect on the micellization index because low solubilization reduced the efficiency of the interchange of carotenoids to micelles. In this

Table 2. Phase 1 Results Obtained for the SL Experimental Design

		emulsifier (%) [a]										response micellization
run	trial	1	2	3	4	5	6	7	8	9	water	(%)
57	1	0	5	0	0	3.3	0	5	0	0	86.7	8.00
20	2	0	0	0	0	0	0	0	5	0	95	4.27
8	3	5	5	5	0	0	0	0	0	5	80	9.22
17	4	5	0	5	0	5	0	0	5	0	80	12.1
7	5	5	5	0	5	0	0	0	5	0	80	5.08
32	6	0	5	0	5	5	5	0	0	0	80	7.59
30	7	0	0	3.3	1.7	0	5	5	0	5	80	11.2
29	8	5	5	0	0	5	5	0	0	0	80	2.83
27	9	5	0	0	0	5	5	5	0	0	80	3.39
59	10	0	0	5	2.5	5	0	0	0	0	87.5	13.6
9	11	0	5	5	5	5	0	0	0	0	80	8.59
3	12	0	0	0	0	5	0	5	5	5	80	17.3
12	13	5	0	0	0	0	5	0	5	5	80	9.60
63	14	0	0	0	5	5	2.5	0	2.5	5	80	9.73
1	15	0	1.7	0	0	0	0	0	0	0	98.3	7.15
62	16	0	0	5	0	5	5	0	0	5	80	9.68
52	17	0	0	2.5	2.5	5	5	0	5	0	80	8.49
46	18	0	3.3	0	5	1.7	0	5	0	5	80	12.0
24	19	0	0	5	0	5	5	0	0	5	80	13.2
4	20	0	5	5	0	0	5	0	5	0	80	9.75
5	21	0	5	0	5	0	5	0	0	5	80	3.21
43	22	0	0	0	5	5	2.5	0	2.5	5	80	16.4
38	23	0	2.5	5	0	0	2.5	5	0	5	80	14.5
23	24	5	5	0	0	0	5	5	0	0	80	7.70
21	25	5	0	5	0	0	0	5	5	0	80	4.00
61	26	5	0	0	5	0	0	0	0	0	90	12.2
60	27	0	5	0	2.5	0	0	0	0	5	87.5	4.16
2	28	5	0	5	5	0	5	0	0	0	80	4.18
31	29	0	5	0	0	5	0	5	0	5	80	6.91
13	30	0	0	0	5	0	5	5	5	0	80	1.72
64	31	5	0	0	0	0	5	0	0	0	90	6.79
39	32	0	0	5	0	1.7	5	1.7	3.3	3.3	80	16.7
19	33	0	0	0	0	5	0	0	0	5	90	8.98
58	34	0	0	0	0	0	5	3.3	5	0	86.7	6.50
55	35	5	5	0	0	2.5	2.5	0	0	5	80	7.08
44	36	0	0	5	5	2.5	0	2.5	0	5	80	15.4
49	37	0	2.5	5	0	0	0	0	5	0	87.5	9.06
16	38	0	0	5	5	0	0	0	5	5	80	6.86
35	39	0	5	0	5	0	0	0	5	0	85	11.4
56	40	5	0	0	0	5	0	0	3.3	0	86.7	17.3
26	41	0	0	0	5	5	0	5	5	0	80	16.3
22	42	0	5	0	0	0	0	5	5	5	80	15.8
54	43	3.3	5	1.7	0	0	0	1.7	5	3.3	80	9.37
42	44	1.7	0	0	3.3	1.7	5	0	5	3.3	80	14.1
37	45	0	0	5	5	0	5	0	0	0	85	10.3
28	46	0	5	0	0	5	0	0	5	5	80	11.4
47	47	5	0	5	0	0	0	0	0	0	90	12.2
25	48	5	5	0	0	0	0	0	0	0	90	5.82
11	49	0	0	0	0	0	0	5	0	0	95	3.49
34	50	0	5	0	0	5	0	5	5	0	80	13.3
50	51	5	0	0	0	0	0	3.3	0	5	86.7	14.6
18	52	0	0	0	0	0	5	0	0	5	90	13.5
33	53	0	5	5	0	0	5	0	0	0	85	2.34
40	54	5	1.7	5	1.7	0	0	0	3.3	3.3	80	5.82
45	55	5	2.5	2.5	0	5	0	5	0	0	80	12.1
41	56	2.5	0	5	0	0	0	5	2.5	5	80	16.0
6	57	0	5	5	5	0	0	5	0	0	80	8.11
36	58	5	0	0	5	0	0	0	0	0	90	13.8
15	59	5	0	0	5	5	0	0	0	5	0.8	9.07
53	60	5	0	0	5	0	2.5	5	0	2.5	80	11.3
65	61	2.5	0	5	0	0	0	5	2.5	5	80	12.4
51	62	5	0	0	0	0	5	0	0	0	90	4.86
10	63	0	0	0	0	5	5	0	0	0	90	13.4
14	64	0	0	5	0	5	5	5	0	0	80	3.33
48	65	5	5	0	2.5	5	0	2.5	0	0	80	9.39

[a] Key: 1, saccharose distearate; 2, saccharose monodistearate; 3, saccharose monopalmitate; 4, 2,3-dihydroxypropyl docosanoate; 5, glyceryl palmitostearate; 6, polyglyceryl oleate; 7, propylene glycol laurate; 8, glyceryl monostearate; and 9, hexaglyceryl distearate.

sense, the use of acylglycerides in the formulation of emulsions may allow for better solubilization and interchange of carotenoids because the presence of glycerol in the composition, instead of saccharose, would provide higher interface flexibility (*27*).

Differences in the individual primary effects of the acylglyceride groups were also observed. The highest term value corresponded to EMF-4 (139.2), a lower but still positive value was seen with EMF-8 (37.4), while negative term values were seen in EMF-5 and EMF-7 (−93.2 and −21.7, respectively). In this case, the combination of two structural features, esterification degree, and length of the lipophilic tail seemed to be implicated in the observed effect. It has been reported that the micellization of carotenes is dependent on the acyl chain length of triglycerides present in the medium but not on the number and position of double bonds; thus, by increasing the acyl chain of triglycerides, a greater efficiency of micellization may be achieved (*28*). This result correlates nicely with the trend seen in this study. The emulsifier EMF-4, which contained the longest acyl chain of the tested group (22 carbon atoms), showed the highest term value of this group, and when the length of the acyl chain decreased, (EMF-8 > EMF-5 > EMF-7), the term value also decreased.

With regard to the polyacylglycerides, it has been shown that polyacylglycerides reduce the staggering of the molecules in the emulsion, increasing the definition of the interface (*29*). A better definition of the interface would promote the transfer to micelles. However, the individual primary effects are very different (**Table 3**) and relatively low in comparison with individual primary effects from the other emulsifier groups, so in this case a clear trend could not be obtained on the basis of the structural features of these compounds.

It must be noted that at this stage of the study, hypotheses that describe the individual primary effects should not be considered as definitive for this part of the experiment. Some combinations of different emulsifiers may provide a synergistic or antagonistic effect on the response. The effect of the combination would then exceed the one produced individually by each emulsifier, independent of the value of the individual primary effects. Thus, on the basis of the primary effects (those individually produced by each emulsifier, such as term numbers from 1 to 10, **Table 3**), EMF-3 and EMF-4 appear to be the most effective emulsifier agents (coefficient values 169.3 and 139.2, respectively, **Table 3**). However, this first approach is not adequate because the secondary effects are strongly negative and thus show an antagonistic effect (coefficient value −374.7, **Table 3**), which nullifies the primary effects produced for EMF-3 and EMF-4; these cannot be used individually for the optimization phase.

Our first conclusion is that none of the emulsifiers alone produced the most effective responses; effective responses came mainly from secondary effects (synergies or antagonisms). Therefore, any of the components may produce maximum response levels if they are introduced individually in a formulation that combines diverse emulsifiers.

Considering the secondary effects, we identified higher positive values of coefficients in **Table 3**, such as 267.8 (term number 51 EMF-7 × EMF-9 synergy, **Table 3**), 234.1 (term number 44 EMF-5 × EMF-9 synergy, **Table 3**), 200.7 (term number 43 EMF-5 × EMF-8 synergy, **Table 3**), 198.6 (term number 48 EMF-6 × EMF-9 synergy, **Table 3**), 193.2 (term number 26 EMF-2 × EMF-9 synergy, **Table 3**), 182.6 (term number 22 EMF-2 × EMF-5 synergy, **Table 3**), 178.4 (term number 45 EMF-5 × EMF-10 synergy, **Table 3**), 164.5 (term

Table 3. Phase 1 Coefficients Obtained for Each Term of the Quadratic Model Generated on the Basis of Results Shown In **Table 2**

term no.	term[a]	term value	p	term no.	term[a]	term value	p	term no.	term[a]	term value	p
1	1	22.6	0.88	20	2 × 3	−147.7	0.43	38	4 × 8	−235.3	0.25
2	2	−64.7	0.52	21	2 × 4	−92.5	0.62	39	4 × 9	−80.9	0.67
3	3	169.3	0.18	22	2 × 5	182.6	0.33	40	4 × 10	−124.1	0.34
4	4	139.2	0.17	23	2 × 6	56.05	0.80	41	5 × 6	96.8	0.66
5	5	−93.2	0.40	24	2 × 7	161.7	0.46	42	5 × 7	164.5	0.41
6	6	27.8	0.82	25	2 × 8	121.9	0.52	43	5 × 8	200.7	0.35
7	7	−21.7	0.85	26	2 × 9	193.2	0.39	44	5 × 9	234.1	0.25
8	8	37.4	0.72	27	2 × 10	84.8	0.54	45	5 × 10	178.4	0.22
9	9	−95.8	0.50	28	3 × 4	−374.7	0.08	46	6 × 7	−31.6	0.89
10	10	5.4	0.20	29	3 × 5	−84.9	0.73	47	6 × 8	−29.2	0.88
11	1 × 2	92.0	0.74	30	3 × 6	−253.6	0.34	48	6 × 9	198.6	0.36
12	1 × 3	−200.5	0.44	31	3 × 7	−178.1	0.43	49	6 × 10	−18.3	0.91
13	1 × 4	−218.3	0.39	32	3 × 8	−287.5	0.25	50	7 × 8	−41.3	0.85
14	1 × 5	153.1	0.51	33	3 × 9	−47.7	0.84	51	7 × 9	267.8	0.22
15	1 × 6	−103.2	0.73	34	3 × 10	−206.4	0.24	52	7 × 10	25.8	0.86
16	1 × 7	70.8	0.79	35	4 × 5	49.4	0.81	53	8 × 9	127.4	0.52
17	1 × 8	−126.3	0.58	36	4 × 6	−225.9	0.34	54	8 × 10	−40.7	0.77
18	1 × 9	120.7	0.60	37	4 × 7	−131.6	0.55	55	9 × 10	144.6	0.45
19	1 × 10	−6.5	0.97								

[a] Key: 1, saccharose distearate; 2, saccharose monodistearate; 3, saccharose monopalmitate; 4, 2,3-dihydroxypropyl docosanoate; 5, glyceryl palmitostearate; 6, polyglyceryl oleate; 7, propylene glycol laurate; 8, glyceryl monostearate; 9, hexaglyceryl distearate; and 10, water.

Table 4. Phase 2 Results Obtained for the SC Experimental Design and Coefficients Obtained for Each Term of the Quadratic Model Generated on the Basis of the Results

		emulsifier (%)[a]				response micellization	term	
run	trial	2	5	7	9	(%)	value	p
6	1	50	0	50	0	20.6	66.9	0.00
11	2	33.3	33.3	33.3	0	13.8	—[b]	—
2	3	0	100	0	0	11.3	10.6	0.02
15	4	25	25	25	25	17.3	—	—
7	5	50	0	0	50	5.71	11.7	0.40
13	6	33.3	0	33.3	33.3	19.9	—	—
10	7	0	0	50	50	13.1	42.2	0.02
3	8	0	0	100	0	7.89	6.9	0.07
14	9	0	33.3	33.3	33.3	11.4	—	—
1	10	100	0	0	0	5.98	5.3	0.14
4	11	0	0	0	100	3.39	2.7	0.41
12	12	33.3	33.3	0	33.3	5.10	—	—
9	13	0	50	0	50	5.96	1.0	0.94
5	14	50	50	0	0	4.30	−10.6	0.45
8	15	0	50	50	0	4.28	−9.9	0.48

[a] Key: 2, saccharose monodistearate; 5, glyceryl palmitostearate; 7, propylene glycol laurate; and 9, hexaglyceryl distearate. [b] Not applied in the quadratic model.

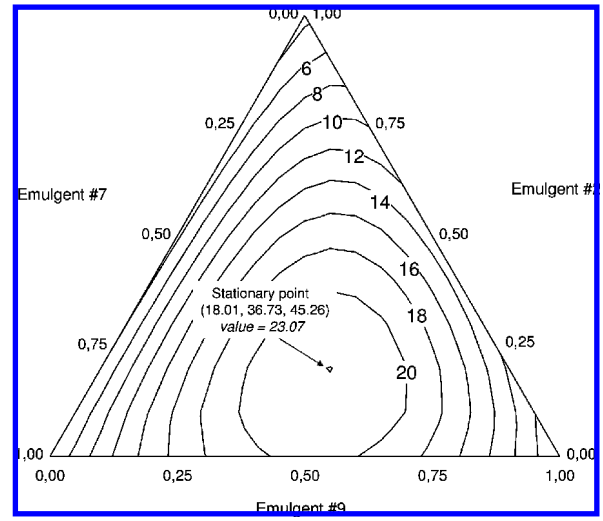

Figure 1. Location of the stationary point (optimized mixture) as a function of EMF-2 (saccharose monodistearate), EMF-7 (propylene glycol laurate), and EMF-9 (hexaglyceryl distearate) emulsifiers.

number 42 EMF-5 × EMF-7 synergy, **Table 3**), 161.7 (term number 24 EMF-2 × EMF-7 synergy, **Table 3**), and 153.1 (term number 14 EMF-1 × EMF-5 synergy, **Table 3**). Therefore, we must select the ingredients to be optimized from the list that includes EMF-1, EMF-2, EMF-5, EMF-6, EMF-7, EMF-8, and EMF-9. According to the values and signs of the two-order interaction for these ingredients (**Table 3**), we decided to eliminate the emulsifiers with strong antagonisms (EMF-1, EMF-6, and EMF-8). Then, we selected EMF-2, EMF-5, EMF-7, and EMF-9 to be considered in the following phase. With regard to water, due to the small coefficient value (5.4, term number 10, **Table 3**), we used a constant value of 80% (w/w) during the optimization phase.

Optimization Phase. During this stage, experiments were performed with one emulsifier from the liposugars (EMF-2), one from the polyacylglycerides (EMF-9), and two emulsifiers from the acylglycerides group (EMF-5 and EMF-7). Results obtained during the optimization phase are shown in **Table 4**, and the coefficients of regression were estimated by the least-squares method (**Table 4**). Most of the terms of the model were positive (**Table 4**), and terms due to the secondary effects overcame those of the individual effects; that is, the response was greatly affected by synergisms. Although the individual primary effect of EMF-5 was the highest (10.6), this ingredient is involved in two antagonistic effects with EMF-2 and EMF-7. Because of their secondary effects, EMF-5 must be eliminated from the final mixture so that the response value is not diminished.

Consequently, the optimized mixture included EMF-2 (saccharose monodistearate), EMF-7 (propylene glycol laurate), and EMF-9 (hexaglyceryl distearate). The stationary point of the system (mathematically, the point of the quadratic function where the derivative is zero, and from a pragmatic point of view, the value that shows the best combination of the ingredients that achieves the highest maximization of the response) was obtained in a mixture of the following composition: EMF-2 (36.73%), EMF-7 (45.26%), and EMF-9 (18.01%), with a maximum micellization index of 23.07% (see **Figure 1**). Although the experimental design and procedure was optimized

Table 5. Validation of the Regression Model on the Premise That at the Final Model EMF-5 (Glyceryl Palmitostearate) = 0.[a]

	emulsifier (%)[b]			values		IC
mixture type	2	7	9	obtained	estimated	$1 - \alpha = 0.95$
randomized	20.36	15.41	64.23	15.48	12.42	7.11−16.26
randomized	9.58	40.81	49.61	15.45	16.05	10.25−21.85
randomized	30.06	4.58	63.36	7.82	9.72	4.16−14.86
randomized	2.76	25.07	72.17	11.53	11.02	6.47−17.86
optimized	18.01	36.73	45.26	23.75	23.07	17.12−26.34

[a] Emulsifiers are shown as a percentage of the mixture, and values are the micellization response percentages. [b] Key: 2, saccharose monodistearate; 7, propylene glycol laurate; and 9, hexaglyceryl distearate.

to obtain the best mixture, we performed experiments with both randomized and optimized mixtures, including EMF-2, EMF-7, and EMF-9, to validate the regression model shown in **Table 4**. However, we considered the optimized model if EMF-5 levels were zero. **Table 5** shows that the experimental values obtained were very similar to the predicted values. Likewise, for the optimized mixture, the experimental value of the response (23.75%) was very close to the value predicted by the model, 23.07%.

The combination of EMF-2 with EMF-7 and EMF-9 produced the maximum micellization index, so the final optimized mixture included a compound of each emulsifier group (EMF-2 for liposugars, EMF-7 for acylglycerides, and EMF-9 for polyacylglycerides). Emulsifying properties of each one of these emulsifiers and specifically their synergy (**Table 4**) worked together to achieve the highest response value. By only considering the individual primary effects obtained during the screening phase (**Table 3**), this combination would not have been predicted to achieve a maximum response value because of the negative primary effect. However, as mentioned above, during that stage of the experiment, the individual primary effects could not be used as discriminating criteria and the development of an optimization phase was required. Some formulations in dermal delivery systems use similar combinations to the one found in this study to increase the transfer of drugs to the target tissue (*30*). The use of EMF-7 may increase the fluidity of the interface and decrease the polarity of the aqueous environment. This effect may enhance the transfer of lipophilic compounds to the micelles (*31, 32*). The addition of EMF-9 may also facilitate the solubilization of carotenoids and their transfer to micelles. This compound has been used in the formulation of microparticles to increase drug delivery (*33*).

The application of this experimental design allowed for the creation of a formulation with the aim of maximizing the bioaccessibility of bioactive compounds (carotenoids). The development of bioactive food items requires the estimation of bioaccessibility and/or bioavailability as one of the multiple criteria for assessing the functionality of formulated foodstuffs. Therefore, this analytical approach is of interest for food designers for the screening and control of the factors and components that modify the bioaccessibility of bioactive compounds in a given matrix, and consequently, formulation conditions may be selected through which higher bioaccessibility may be achieved.

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