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HOW TO FORMULATE A STABLE AND MONODISPERSE WATER-IN-OIL NANOEMULSION CONTAINING PUMPKIN SEED OIL: THE USE OF MULTIOBJECTIVE OPTIMIZATION

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Abstract - The multiobjective optimization method was applied in order to improve the droplet size distribution and stability of water-in-oil emulsions composed of sunflower and pumpkin seed oils as continuous phase, polyglycerol polyricinoleate as emulsifier, water as dispersed phase and sodium chloride as co-stabilizer (lipophobe). Three composition factors were varied based on the three level Box-Behnken design and three characteristics of the obtained emulsions were measured for each experimental run. The mean volume diameter of water droplets and the span of the droplet size distribution, both determined immediately upon preparation of the emulsion, as well as the stability index over a three-month period were interrelated by regression functions with the surfactant concentration, oil composition and the salt content in the water phase of the emulsion. Also, the fourth objective function based on a difference in the prices of pumpkin seed and sunflower oils was considered for optimization. The multiobjective optimum was calculated by using the minimal loss method with weight factors.

Additionally, effects of the continuous phase composition and the salt content on the equilibrium interfacial tension of water-oil systems and the changes of the droplet size distribution over time were studied.

Keywords: Water-in-oil emulsion; Multiobjective optimization; Pumpkin seed oil; Polyglycerol polyricinoleate; Sodium chloride; The equilibrium interfacial tension.

INTRODUCTION

Tasty, healthy, nutritious and more convenient food products with enhanced stability and shelf life are imperative for today's increasingly demanding markets. The production of water-in-oil (W/O) and water-in-oil-in-water (W/O/W) emulsions is one possible step towards employing the novel idea that biologically active substances and active ingredients in the food industry should be entrapped in some carrier material to form microcapsules or nanoparticles in order to achieve the controlled release of active ingredients and flavour retention, to mask the bad taste or smell of some components, stabilize food ingredients, prevent their oxidation or hydrolysis, and adjust their properties and/or increase their bioavailability (Nikolovski *et al.*, 2011).

Different dispersing methods have been employed to generate emulsions such as conventional simple agitation, colloidal mills, static mixers and high shear mixers, as well as novel methods like membrane emulsification (Dragosavac *et al.*, 2012,

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Vladisavljević and Williams, 2005) and ultrasound cavitation (Sivakumar *et al*., 2014, Tang *et al*., 2013, Tang *et al*., 2012). For production of nanoemulsions intense shear should be applied in order to overcome the Laplace pressure and break up droplets into smaller (nanometre scale) dimensions (Sivakumar *et al*., 2014). The developed high-energy input techniques adequate for production of nanoemulsions include the use of high-pressure homogenizers, ultrasonicators and microfluidizers (Sivakumar *et al*., 2014, Landfester 2006). Also, low-energy input techniques adequate for the production of nanoemulsions have been developed such as phase inversion temperature, solvent-diffusion and spontaneous emulsification (Sivakumar *et al*., 2014). However, low-energy input techniques have their own limitations (Sivakumar *et al*., 2014) including the use of a large quantity of surfactant, usually not of the food grade type, and instability after long-term storage, which can be improved when the droplet disruption is provided predominantly by high-energy input techniques (Santana *et al*., 2013, Sivakumar *et al*., 2014, Tang *et al*., 2013).

The stability problems in food emulsions cannot be improved by increasing the concentration of the emulsifying agent (like in cosmetic and pharmaceutical emulsions) due to limitations in the permitted dose for human consumption (Dickinson, 2011, Jiménez-Colmenero, 2013). The polyglycerol ester of polyricinoleic acid (PGPR) (low HLB value) is a synthetic non-ionic and the most effective hydrophobic emulsifier, commonly used in the food industry as a chocolate thickening agent with excellent water-binding characteristics (Gülseren and Corredig, 2012, Wilson *et al*., 1998a). Used as a food additive, it is recognised as generally safe with a maximum *per capita* mean daily intake of 2.64 mg/kg body weight/day (Wilson *et al*., 1998b).

Although PGPR reduces the interfacial tension very well, thereby facilitating droplet break-up, and prevents coalescence of newly formed water droplets via the Gibbs-Marangoni effect (Walstra, 1993) and by steric stabilisation (Landfester, 2006), diffusional degradation remains the destabilising mechanism, which has to be precluded by osmotic pressure regulation. There are claims that the presence of salt is crucial for emulsion formation and the stability of primary W/O emulsions (Aronson and Petko, 1993). Despite the fact that some authors claim that a stable emulsion can be obtained without electrolytes in the emulsion and/or that the addition of salt (NaCl) or sodium phosphate buffer destabilizes the emulsion (Su *et al*., 2008), it is well known that the stability rating of primary W/O emulsions is markedly affected by the addition of electrolytes in the inner aqueous phase (Moguet *et al*., 2001, Srinivasan *et al*., 2000). Actually, salt is considered to be a co-stabilizer, a lipophobe that builds-up the osmotic pressure to counterbalance the Laplace pressure, and consequentially stabilizes emulsions against diffusional degradation known as Ostwald ripening (Capek, 2010, Colmán *et al*., 2014, Landfester, 2006). Therefore, the addition of an osmotic agent that cannot interdiffuse between two droplets, and the use of an appropriate hydrophobic surfactant, could both be essential for preparation of stable and monodisperse water-in oil emulsions, with droplet sizes ranging from 50 to 500 nm, well known as inverse miniemulsions (Landfester, 2000, Landfester, 2003). For inverse miniemulsions, relations between surfactant content and droplet size, as well as particle size and the coverage of the particles by surfactant would additionally depend on the amount of the osmotic agent (Landfester, 2006).

The chemical structure of the oil phase, i.e., the chain length of the fatty acids, molecular configuration and the number of unsaturated bonds, is crucial for the stability of the emulsion. Particularly, the polarity of the oil phase can affect the interfacial tension of the W/O interface and the allocation of the components at the interface (Ushikubo and Cunha, 2014). W/O and W/O/W emulsions were usually prepared with sunflower, corn, soybean oil, canola, olive and rapeseed oils (Jiménez-Colmenero, 2013), with olein and miglyol (Bonnet *et al*., 2009), and sometimes with specialty oils like *Moringa oleifera* oil (Khalid *et al*., 2013).

Pumpkin seed oil is a complex mixture of fatty acids, fatty acid esters, monoglycerides (~1.5%), diglycerides (~0.4%), triglycerides (~95%), and minor components, among which the most prominent are: vitamins (tocopherols and tocotrienols 0.03-0.09%), sterols (0.2-0.8%), squalene (0.2-0.8%) phospholipids (1%) and pigments. Therefore, this oil should be considered as a valuable natural source of essential fatty acids and biologically active micronutrients like sterols (Hrabovski *et al*., 2012, Neđeral Nakić *et al*., 2006, Nikolovski, 2009), tocopherols, and especially squalene (Neđeral Nakić *et al*., 2006, Nikolovski, 2009). Moreover, phospholipids and mono- and diglycerides reduce the interfacial tension between phases (Kalvodova, 2010, Mezdour *et al.* 2011), while squalene, due to its intrinsic insolubility in water, prevents oil-in-water emulsion instability caused by the Ostwald ripening mechanism (Fox *et al*., 2011). Thus, pumpkin seed oil might be employed to support the action of the emulsifier in order to eventually decrease the concentration of the emulsifying agent. Nonetheless, emulsions with pumpkin seed oil were rarely the subject of investigation (Nikolovski *et al.*, 2011, Dragosavac *et al.*, 2012). Therefore, the incorporation of pumpkin seed oil into stable W/O emulsions, which could be eventually used for preparation of double W/O/W emulsions, was one of incentives behind our work. For stabilization of W/O/W food emulsions, beside a lipophilic emulsifier, a proper water-soluble food-grade surfactant must be chosen among non-ionic small-molecular-weight emulsifiers (Tweens) (Das and Kinsella, 1990), block copolymer emulsifiers (Pluronics) (Torcello-Gómez *et al.*, 2013, Dragosavac *et al.*, 2012) or biopolymer emulsifiers (protein and hydrocolloid emulsifiers) (Dickinson, 2011). Also, W/O/W emulsions are frequently stabilised by conjugates and complexes of hydrocolloids with food proteins (Dickinson, 2009).

On the one hand, this work aimed to give an insight into an inverse miniemulsion system composed of two oils with different physicochemical characteristics (refined sunflower and unrefined pumpkin seed oil) as continuous phase, polyglycerol polyricinoleate as emulsifier, water as dispersed phase and sodium chloride as co-stabilizer. The influence of three composition factors on three characteristics of the obtained W/O emulsions was studied. On the other hand, optimization of the formulation of this system was attained by the use of a multiobjective optimization technique involving the Box-Behnken experimental design, which is considerably cheaper than the three-level full factorial designs and is considered to be very efficient, when efficiency is estimated as the number of coefficients in the estimated model divided by the number of experiments (Ferreira *et al.*, 2007).

MULTIOBJECTIVE OPTIMIZATION

Since we needed to take into account and eventually simultaneously optimize three different quality parameters of emulsions: the mean volume diameter of water droplets, the span value of the droplet size distribution and the stability index of the emulsions that were studied over a three-month period, it was necessary to resort to some type of multiobjective optimization technique. Our aims had different dimensions, different orders of magnitude and might potentially be of different importance in the decision making.

Multiobjective optimization was performed in three steps. Firstly, each aim had to be described mathematically by its unique objective function f_i = (X_1, X_2, X_3) (i=1, 2 and 3). However, the three aforementioned aims depended on the same independent variables/ factors - in our case polyglycerol polyricinoleate (X_1) and pumpkin seed oil (X_2) contents in the oil phase and sodium chloride (X_3) content in the water phase of the emulsion. In order to calculate the multiobjective optimum by taking into account all the aims simultaneously, some compromises had to be made. Therefore, in the second step, the importance of the objectives was considered by defining the weighting factors (w_i). The weighting factors influence the minimum of the loss function and the set of compromise optimum values of the independent variables $X_{1,opt}$, $X_{2,opt}$, and $X_{3,opt}$. As the third step, the multicriterion optimization method had to be chosen. For our purposes we applied the loss-minimization method (Osyczka, 1984, Gergely *et al.*, 2003). This method calculates the minimum of the sum of the weighted relative deviations:

$$L = \sum_{i=1}^{m} w_i \left[\frac{f_i\left(X_1, X_2, X_3\right) - f_i^*}{f_i^*} \right]^2 \rightarrow min\ ! \qquad (1)$$

where L is the loss function, and f_i^* individual optimum value of the i objective function.

A constrained minimization of the multivariate scalar function (L) was done by using the Scipy.optimize module of The Scientific Computing Tools for Python (ScyPy 0.14.0.). The algorithms that gave good results were: L-BFGS-B (Limited-memory Broyden-Fletcher-Goldfarb-Shanno Bound-constrained) algorithm, the Constrained Optimization BY Linear Approximation (COBYLA) method, and a Truncated Newton Conjugate-gradient (TNC) algorithm.

MATERIAL AND METHODS

Refined sunflower oil (“Vital”, Vrbas, Serbia) and unrefined cold-pressed pumpkin seed oil (“Suncokret”, Hajdukovo, Serbia) were used in their original form without further purification. The contents of saturated fatty acids, monounsaturated oleic and polyunsaturated linoleic fatty acids in the sunflower oil were 14.3, 24.2 and 63.1%, respectively. The sunflower oil density measured at 25 °C was 0.921 $g{\cdot}cm^{-3}$. The fatty acid composition of the pumpkin seed oil (density at 25 °C 0.9169 $g{\cdot}cm^{-3}$), expressed as weight percentage of the total, was: 18.09% saturated fatty acids (11.72% palmitic acid, 5.58% stearic acid, 0.42% arachidic acid, 0.14% behenic acid, 0.12% myristic acid, 0.08% heptadecanoic acid, and

0.03% lignoceric acid); 37.73% monounsaturated fatty acids (37.43% oleic acid, 0.16% cis-11-eicosenoic acid and 0.14% palmitoleic acid) and 43.98% polyunsaturated fatty acids (43.75% linoleic acid and 0.23% linolenic acid). The oil soluble emulsifier polyglycerol polyricinoleate (PGPR 90) was kindly provided by "Jaffa" a.d. (Crvenka, R. Serbia). NaCl was purchased from Sigma Aldrich (UK). Demineralised water with a conductivity of about 4 μS·cm^{-1} was used as the aqueous phase.

Emulsion Preparation

Water-in-oil (W/O) emulsions were prepared by homogenization using a high speed homogenizer (UltraturraxT-25, IKA, Germany) at 24000 rpm for 10 min. Continuous phases were prepared by dissolving a certain amount of PGPR (1, 3 and 5% (w/w)) in an oil phase (the sunflower oil, the pumpkin seed oil and a mixture of the oils (mass ratio 1:1)) at 50 °C, by mixing on a magnetic stirrer for 30 minutes. Final 20% (v/v) W/O emulsions were prepared by careful addition (drop by drop) of the dispersed phase (water and water solutions of NaCl (0.15 and 0.3 M) into the continuous oil phase stirred by the high-speed homogenizer. The emulsification temperature was maintained at 25 °C by means of a water bath. After preparation all emulsions were initially analysed and then stored in a refrigerator at 4 °C.

Sedimentation Stability

For the stability test, the prepared emulsions were transferred into 10ml graduated glass cylinders and stored at room temperature for three months. During storage, the emulsions separated into an opaque layer of emulsion and a transparent serum layer consisting of oil at the top or water at the bottom of the cylinders. The stability index (the creaming stability) was measured by the height of the serum layer (H_S) with the storage time. The stability index (*SI*) (Perrechil *et al.*, 2014) was reported as:

$$SI(\%) = (H_S / H_E) \times 100 \tag{2}$$

where H_E represents the initial height of the emulsion.

Droplet Size Analysis

The mean droplet size and droplet size distribution of the primary W/O emulsions were measured immediately after the formation using a laser light scattering instrument ZetasizerNano ZS (Malvern Instruments, U.K.). To avoid multiple light scattering, samples of the emulsions were diluted with sunflower oil at a dilution ratio of 1:100, and analysed in triplicate. The optical parameters selected were: dispersed phase refractive index 1.33, dispersant liquid viscosity (sunflower oil) 51.32 mP·s and refractive index 1.4723. The stability of W/O emulsions was determined weekly during a month of storage.

The results of the measurements are shown as the droplet size distribution and the volume-weighted mean droplet diameter, $d_{4,3}$, given by Eq. (3):

$$d_{4.3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \tag{3}$$

where n_i is the number of droplets with diameter of d_i.

The width of the droplet size distribution and polydispersity are expressed through the span value, defined by Eq. (4):

$$span = \frac{(d_{90} - d_{10})}{d_{50}} \tag{4}$$

where d_{10}, d_{50} and d_{90} are standard percentile readings from the cumulative droplet volume distribution curve, meaning the droplet diameters below which 10%, 50% and 90% of the sample lies, respectively (Ushikubo and Cuncha, 2014).

Surface/Interfacial Tension

A digital tensiometer KSV – Sigma 703D (Finland) was used and the *Du Noüy* ring method was employed for interfacial tension measurements between the water and oil phases. Before the measurement, the ring was immersed in the water phase, the oil phase was added slowly and the surface was left for 10 min to equilibrate. The reported values of the interfacial tension were the average values of at least three measurements. All measurements were performed at 25 °C.

Viscosity Measurements

An RV20 rotational viscometer (cone plate geometry) with a SVI measuring sensor (Haake, Germany) was used for viscosity measurements of the oil phase. The samples were transferred to the instrument and allowed to equilibrate to 25 °C for 5 min prior to measurement. Shear stress τ (Pa) was determined with continually changing shear rates D (s^{-1}) from zero to 500 s^{-1} and the reverse. The apparent viscosity was calculated as:

$$\eta = \frac{\tau}{D} \tag{5}$$

Statistical Analysis

Multivariate optimization schemes involve designs for which the levels of all the variables (the significant factors) are changed simultaneously. The optimum operational conditions are attained by using more complex experimental designs such as the three-level Box-Behnken design, which has proven to be slightly more efficient than other experimental designs in use (Ferreira *et al.*, 2007).

The Box-Behnken designs are a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. The required number of experiments (*N*) is defined as $N = 2 \cdot k \cdot (k - 1) + C_0$, (where *k* is number of factors and C_0 is the number of central points) (Ferreira *et al.*, 2007). The Box-Behnken designs give the possibility to estimate all linear effects, all quadratic effects, and all linear 2-way interactions between factors.

In order to investigate the effect of emulsion composition parameters on the droplet size distribution and the sedimentation stability, the Box-Behnken design for three factors was applied. The three input variables were the content of PGPR in the continuous phase (1-5% (w/w), X_1), pumpkin seed oil content in the continuous phase (0-100% (w/w), X_2) and NaCl concentration in water phase (0-0.3 M, X_3), where three levels were chosen (−1, 0, +1) as shown in Table 1. The experimental design consisted of 15 runs as shown in Table 2. The experimental values were expressed as the means of three determinations and the standard deviation. All statistical analyses were performed using STATISTICA 12 software (StatSoft, Inc., 2012).

Table 1: Treatment levels and coded values for each of the independent variables used in developing the experimental data to optimize the W/O emulsion content.

Independent variable	Symbol		Level	
	Uncoded	Coded	Uncoded	Coded
PGPR content, % (w/w)	E	X_1	1 3 5	-1 0 +1
Pumpkin seed oil content in the continuous phase, % (w/w)	PO	X_2	0 50 100	-1 0 +1
NaCl concentration in the water phase, M	S	X_3	0 0.15 0.30	-1 0 +1

$X_1 = (E-3)/2$, $X_2=(PO-50)/50$, $X_3=(W-0.15)/0.15$

RESULTS AND DISCUSSION

Varying PGPR and PO content in the oil phase and NaCl content in the water phase, thirteen W/O emulsions of different formulations were prepared and investigated in a such a way as to determine the mean volume diameter ($d_{4,3}$) and span value of the freshly prepared emulsions and the stability index over a three-month period. All emulsions in the experimental design contained 20% (v/v) of water and the results obtained are shown in Table 2. The stability index determined after 90 days of storage was labelled as *SI*90.

Table 2: Experimental design and results of production of W/O emulsions.

Experiment	PGPR, E, % (w/w)	Pumpkin seed oil, PO, % (w/w)	NaCl concentration, S, M	Mean volume diameter, $d_{4,3}$, nm	*span*	*SI* (%)		
						day 1	day 30	day 90
	X_1	X_2	X_3					
1	−1	−1	0	201.2±12.13	0.74	0	17.5	33
2	+1	−1	0	187.0±9.18	0.76	1	4	10.5
3	−1	+1	0	562.8±45.75	1.22	1.5	10	22
4	+1	+1	0	174.8±5.65	0.65	0.5	3.5	5.5
5	−1	0	−1	990.7±63.17	0.65	24	41	50
6	+1	0	−1	598.3±40.45	0.70	1	16	22
7	−1	0	+1	264.9±22.28	1.04	1.5	6	30
8	+1	0	+1	185.1±6.55	0.66	0	3	5.5
9	0	−1	−1	699.4±6.09	0.60	1	8	25
10	0	+1	−1	843.6±75.02	0.80	1	6	15
11	0	−1	+1	205.2±7.13	0.80	0	7	14
12	0	+1	+1	186±13.22	0.77	1	3.5	7
13	0	0	0	203.2±18.97	0.86	0	2	5.5
14	0	0	0	192.4±10.90	0.89	0	3	8
15	0	0	0	204.6±13.30	0.86	1.5	4	8

Statistical analysis of the results (Main effects ANOVA, (StatSoft, Inc. 2012)) indicates that both the mean volume diameter of the water droplets and the stability rating of the emulsions were markedly affected by the addition of salt in the aqueous phase, even when the concentration of salt was 0.15M. Smaller droplets (Probability for Post Hoc Dunnett test (StatSoft, Inc. 2012) was 0.00004, and the corresponding result of Fisher tests was 0.00002) and lower values of *SI*90 (Dunnett test p=0.00009, Fisher test p=0.00007) were obtained when the comparison was made between emulsions with added salt at a concentration of 0.15 M and emulsions without added salt. Further increase of the salt content could not be statistically justified.

Figure 1 shows the droplet size distribution of the emulsion without NaCl (a) and with the salt (b) during storage time. As can be seen, the composition without salt in the water phase showed significant coalescence after only 7 days of storage. A typical bimodal distribution curve was formed even after only one day, where about 86.2% of the volume of the aqueous phase was in droplets with a mean droplet size of 614.9 nm, and the rest was droplets with an average diameter of 133.2 nm (13.8 vol%). A bimodal droplet size distribution curve of W/O emulsions without salt was reported by other authors who have studied the same emulsifier for the stabilization of water-in-sunflower oil emulsions (Wolf *et al.*, 2013). The coalescence of water droplets after 7 days of storage resulted in a droplet size distribution which consisted of three peaks with a proportion of about 21 vol% in small droplets (size 169.5 nm), a proportion of 25 vol% in medium droplets (869.4 nm), and a proportion of large droplets with a size of about 5.2 µm occupying 53.8 vol%. Further analysis showed that the percentage of large droplets in the emulsion increased constantly (data not shown). Phase separation in the composition without the salt was also investigated visually since a layer of water appeared at the bottom of the observed cylinders, unlike other different formulations where only a small percentage of oil layer was detected at the top of the glass cylinders. On the other hand, the composition with salt in the water phase was stable during a period of one month (Figure 1b), followed by an insignificant increase in droplet size from 185.1 nm to 227.2 nm. Eventually, the salt took the role of a co-stabilizer in the system of pumpkin seed and sunflower oils given that the absence of salt always led to the formation of rather big droplets. Results of this work are in a good accordance with the well-established theory of inverse miniemulsion formation that gives a detailed explanation of the effect of salt in a W/O emulsion system. Namely, salt builds-up the osmotic pressure and prevents Ostwald ripening, a physicochemical phenomenon whereby emulsified droplets increase in size due to diffusion of molecules from smaller to larger droplets based on differences in interfacial Laplace pressure. Also, the droplet size depends on the amount of the osmotic agent. (Capek, 2010, Colmán *et al.*, 2014, Landfester, 2006). Some authors explained the quantitative influence of salt on emulsion stability through so called surfactant activity difference (Salager, 1990) or its dimensionless equivalent expression (the hydrophilic-lipophilic deviation) (Rondon *et al.*, 2006). A non-ionic surfactant in a W/O emulsion exhibits more affinity for the oil phase, meaning that the surfactant affinity difference (the hydrophilic-lipophilic deviation) has a positive value. According to this generalized formulation concept, an increase in salinity results in an increase in the surfactant activity difference (the hydrophilic-lipophilic deviation), producing a “decrease of hydrophilicity of the emulsifier in the system”, and consequently stabilizing the W/O emulsion (Salager, 1990).

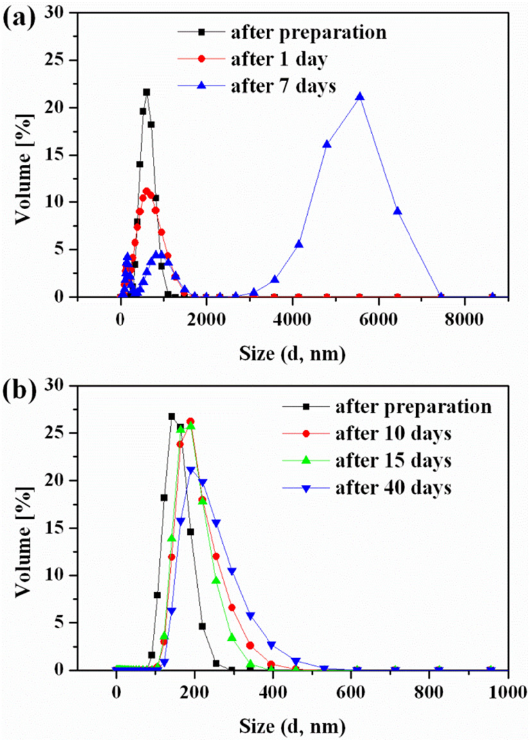

Figure 1: Droplet size distribution of W/O emulsions containing 5% of PGPR and the mixture of pumpkin seed oil and sunflower oil (1:1, w/w): (a) without NaCl in the water phase, (b) with NaCl in the water phase (0.3M).

The stability of W/O emulsions also depends on the composition of the continuous phase. Statistical analysis of the results (Main effects ANOVA) indicates that there is a difference between results with and without pumpkin seed oil in the continuous phase. While addition of the salt was obviously favourable for the production of stable nanoemulsions, the presence of the pumpkin seed oil in the continuous phase caused an increase in the mean droplet diameter and a decrease in the *SI*90 value. The influence of pumpkin seed oil is less pronounced compared to the influence of salt considering the fact that the probability values for the Post Hoc Dunnett and Fisher tests, although lower than 0.05, are much higher than the corresponding probability values obtained for the influence of salt. Pumpkin seed oil in the continuous phase influences the diameter of the water droplets when the mixture of oils and sunflower oil were compared (Dunnett test p = 0.038) and when pumpkin seed and sunflower oils were compared (Dunnett test p = 0.010). The corresponding results of Fisher tests were p = 0.044 and p = 0.012. On the other hand, a more prominent influence of pumpkin seed oil on the stability index could be observed, with probabilities for the Post Hoc Dunnett test of 0.00003 and the Fisher test of 0.00004. If the oil phase was made of sunflower oil with PGPR and NaCl in the water phase, the emulsion was stable during one month. However, if the continuous phase was made of pumpkin seed oil, considerable coalescence of water droplets was noted after just 15 days (Figure 2). In this formulation after the observed time, 71.5% of the volume was formed by droplets with an average droplet size of 584.2 nm, while smaller droplets made up a proportion of 24.4 vol% with an average droplet size of 148.5 nm. This effect can be avoided by mixing pumpkin seed oil with sunflower oil. The emulsion with two mixed oils was stable during a whole month, having a mono-modal droplet size distribution curve pattern, similarly to the formulation with the refined sunflower oil only. Considering all the facts, there was a need to optimize the pumpkin seed oil content in the continuous phase.

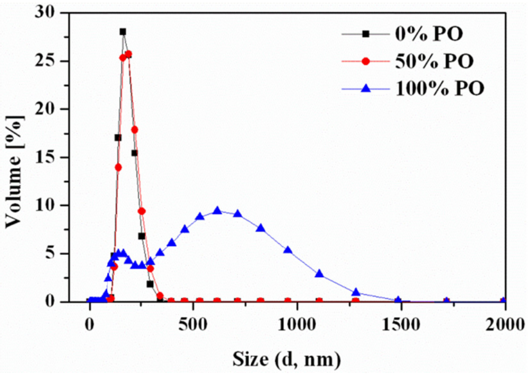

Figure 2: The effect of the composition of the continuous phase on the stability of the droplet size of W/O emulsions containing salt in the water phase after 15 days of storage; 5% PGPR, 0.15 M NaCl.

In theory, the observed differences between emulsions prepared with pumpkin seed oil and sunflower oil might be attributed to the fatty acid composition and the minor constituent content of the oils. The mass ratio of linoleic to oleic acids was higher for the sunflower oil (2.5) then for the pumpkin seed oil (1.2). Viscosities of the refined sunflower and the unrefined pumpkin seed oils were 51.32 and 53.98 mPa·s. Since the viscosity reflects the fatty acid composition or, more precisely, the triglyceride composition, the minor viscosity difference between the oils can be attributed to the difference in fatty acid composition. A higher viscosity would prevent the sedimentation of water droplets (Knoth *et al.*, 2005) and should influence only slightly the droplet size distribution (Walstra, 1993). Yet, the major difference between the oils used lies in the contents of the minor compounds. Contrary to the refined sunflower oil, the pumpkin seed oil was not subjected to any refining process, keeping important components like phospholipids, mono- and diglycerides, sterols and free fatty acids, all being able to adsorb at the water/oil interface (Kiosseoglou and Mourlidis, 1991; Hildebrandt *et al.*, 2013). It is known that the lower interfacial tension value of olive oil can be attributed to the presence of minor surface-active components common to natural olive oil (Kiosseoglou and Mourlidis, 1991). The more complex composition of the unrefined pumpkin seed oil compared to the refined sunflower oil was emphasised through the difference in values of the water-oil interfacial tension and the interfacial tension changes when PGPR was added to the systems (Table 3). The measured interfacial tension of pure water-sunflower oil was 27.49±0.33 $mN{\cdot}m^{-1}$, while the interfacial tension of water-pumpkin seed oil was 8.91±0.08 $mN{\cdot}m^{-1}$. The interfacial tension between water and pure pumpkin seed oil was low enough to create an emulsion without emulsifier, but the stability of such an emulsion during the storage period was not satisfactory. The addition of 5% PGPR in sunflower oil causes a reduction of interfacial tension to 3.03±0.05 $mN{\cdot}m^{-1}$, while in pumpkin seed oil it was reduced to 4.46±0.02 $mN{\cdot}m^{-1}$ and in the mixture of two oils it was reduced to 5.34±0.12 $mN{\cdot}m^{-1}$. It is obvious that a significant reduction of interfacial tension was accomplished when the continuous phase was sunflower oil, which confirms the hypothesis that the complex composition of pumpkin oil has an impact on the adsorption kinetics of PGPR. An investigation of the forces generated by adsorption of PGPR-phospholipid mixtures on particle surfaces indicated that large aggregates of PGPR and phospholipid mixtures had been formed and adsorbed on the surfaces. When these aggregates come into contact with water molecules, the water molecules associated with the polar parts of phospholipids and PGPR, changing the aggregation state of phospholipids, modifying the interactions between phospholipids and PGPR and thus affecting the properties of the adsorbed layer (Dedinaite and Campbell, 2000). The addition of PGPR in a range between 4% and 5% did not produce a significant change of interfacial tension. Table 5 indicates that the addition of NaCl slightly reduces interfacial tension between the water and oil phases in the presence of PGPR from 5.3±0.12 to 4.4±0.22 $\text{mN}\cdot\text{m}^{-1}$; nevertheless, it provides better stability of the emulsion during storage. Very stable droplets could be formed shortly after preparation of inverse miniemulsions due to the fact that the added salt enables the occurrence of a real zero-effective-pressure situation (i.e., the osmotic pressure counterbalances the Laplace pressure) (Landfester, 2006). This study confirms the hypothesis of Pawlik *et al.* (2010) that the addition of salt in the water phase strengthens the interaction between adsorbed molecules, and provides better packing of the PGPR in the interfacial layer. Therefore, increasing the elasticity of the layer decreases the interfacial mobility and the rate of film drainage between approaching droplets, leading to increasing emulsion stability by "stiffening" the interface (Lutz *et al.*, 2009). Moreover, the typical triangular relation between the amount of surfactant, resulting particle size, and surface coverage, existing as a result of droplet break-up and the recoalescence mechanism of minidroplet formation was recognized to depend on the amount of added salt (Landfester, 2006).

Response surface methodology was applied in order to obtain relationships between the emulsion quality parameters ($d_{4,3}$, *span* and *SI*90) and the composition of W/O emulsions (X_1, X_2, and X_3). The proportion of variance accounted for by the whole model, with 9 degrees of freedom, for $d_{4,3}$, *span* and *SI*90 was 0.994 (adjusted r^2=0.982), 0.958 (adjusted r^2=0.881) and 0.979 (adjusted r^2=0.942), respectively. However, all parameters of the whole model were by no means statistically significant, and the whole model was reduced to obtain regression functions with all the significant parameters. The developed regression models for $d_{4,3}$, *span* and *SI*90 and the coded values of the independent variables X_1, X_2, and X_3 and their interdependence are shown in Eq. (6), (7) and (8), respectively. The values of the appropriate coefficients of the regression models are listed in Table 4.

Table 3: The effect of composition of the continuous phase and NaCl on the equilibrium interfacial tension (25 °C) of the water-oil system.

Water	PO (%)	PGPR (%)	NaCl (M)	Interfacial tension ($\text{mN}\cdot\text{m}^{-1}$)
+	0	-	-	27.49±0.33
+	100	-	-	8.91±0.08
+	0	3	-	3.87±0.03
+	0	5	-	3.03±0.05
+	100	3	-	6.13±0.05
+	100	5	-	4.46±0.02
+	50	4	-	5.3±0.12
+	50	4	0.15	4.3±0.22

$$d_{4,3} = a_0 + a_1 \cdot X_1 + a_3 \cdot X_2 + a_5 \cdot X_3 + a_6 \cdot X_3^2 + a_7 \cdot X_1 \cdot X_2 + a_8 \cdot X_1 \cdot X_3 \tag{6}$$

$$span = b_0 + b_1 \cdot X_1 + b_3 \cdot X_2 + b_5 \cdot X_3 + b_6 \cdot X_3^2 + b_7 \cdot X_1 \cdot X_2 + b_8 \cdot X_1 \cdot X_3 \tag{7}$$

$$SI90 = c_0 + c_1 . X_1 + c_2 \cdot X_1^2 + c_3 \cdot X_2 + c_5 \cdot X_3 + c_6 \cdot X_3^2 \tag{8}$$

Table 4: The values of the coefficients in Eq. (6), (7) and (8) and the proportion of variance accounted for by the models (r^2).

***d*$_{4,3}$ (Eq. (6))**			***span* (Eq. (7))**			***SI*90 (Eq. (8))**		
r^2= 0.976			**r^2= 0.916**			**r^2= 0.963**		
Parameter	**Estimate**	**Standard error**	**Parameter**	**Estimate**	**Standard error**	**Parameter**	**Estimate**	**Standard error**
a_0	246.6	21.7	b_0	0.854	0.0235	c_0	8.08	1.37
a_1	-109.3	20.3	b_1	-0.110	0.0220	c_1	-10.6	1.01
a_3	59.3	20.3	b_3	0.068	0.0220	c_2	9.24	1.48
a_5	-286.4	20.3	b_5	0.065	0.0220	c_3	-2.69	1.01
a_6	250.1	29.7	b_6	-0.102	0.0322	c_5	-7.31	1.01
a_7	-93.4	28.7	b_7	-0.148	0.0311	c_6	8.12	1.48
a_8	78.2	28.7	b_8	-0.108	0.0311			

Eqs. (6-8) are regression models for the relationship between the mean droplet diameter ($d_{4,3}$), *span* and the stability index after 90 days of storage time (*SI*90) and the coded values of the independent variables for PGPR content (X_1), pumpkin seed oil content in the continuous phase (X_2), and NaCl content in the water phase (X_3).

Eqs. (6) - (8) were used as the objective functions for multiobjective optimization. Table 5 summarizes the individual optima (minima) for the objective functions Eqs. (6-8), and the compromise optima calculated at optimized $X_{1,opt}$, $X_{2,opt}$ and $X_{3,opt}$ values, which were obtained as the result of the multicriterion optimization by the loss-minimization method (Osyczka, 1984; Gergely *et al*., 2003). For the same value of all weighting factors of 1/3, the set of optimized values was (0.75, 1, 0.45) as obtained by L-BFGS-B, TNC and/or COBYLA – constrained optimization methods of Scientific Computing Tools for Python (ScyPy 0.14.0, 2014). The results of the optimization suggested that the smallest and most uniform water droplets in pumpkin seed oil with good sedimentation stability could be obtained at a relatively high value of PGPR content (about 4.5%), in the presence of salt in the water phase at a concentration of 0.21 M.

In order to verify the optimization procedure an emulsion should be prepared according to the recommended optimal combination levels and the experimentally obtained values should be compared to the corresponding predicted values. However, the formulation of a W/O emulsion with only pumpkin seed oil obtained by the optimization was not preferred for two reasons. On the one hand, the occurrence of the bimodal distribution curve during storage indicated the coalescence of water droplets during aging of the emulsion. On the other hand, due to the high price of pumpkin seed oil (at the moment 23 times more expensive than sunflower oil) the economic factor should be also considered as an objective parameter. The price of the oil phase grows linearly with the content of pumpkin seed oil. A very simple objective function that can be derived from the difference in the prices of two oils is as follows:

$$EC = \mathrm{d}_0\left(1 + 22\cdot \mathrm{X}_2\right) \qquad (9)$$

The result of the multiobjective optimization when four objective functions (Eqs. 6-9) were taken into consideration with weighting factors of 0.25, was the set of optimized values of (0.58, 0.03, 0.45), which corresponded to (4.16%, 51.5%, 0.22 M). This formulation with a lower content of pumpkin seed oil in the oil phase (about 50%) was both economically favoured and experimentally justified (Figure 2). Despite the fact that the salt concentration value predicted by multiobjective optimization was 0.22 M, the statistical analysis of the experimental results suggested that a salt content of 0.15 M in the water phase was sufficient to increase the sedimentation stability of the emulsions and significantly decrease the mean volume diameter of the water droplets. Additionally, this result was emphasised by the profiles for the predicted values and desirability function (StatSoft, Inc., 2012) in Figure 3. The objective functions were transformed into individual preferred/desired functions whose values ranged from 0 to 1. The value "0" of an individual desired function and the overall desired function represents the worst value, while the value "1" represents the best value of the observed response.

Considering the results of multiobjective optimization and the profiles for the predicted values and the desirability function given in Figure 3, the set of values for the investigated composition parameters (0.5, 0, 0) was selected to prepare stable W/O emulsions with pumpkin seed oil in the continuous phase. The coded results corresponded to values of 4% (w/w) PGPR, 0.15 M NaCl, and 50% (w/w) pumpkin seed oil. Experimentally determined values of $d_{4,3}$, *span* and *SI*90 for the optimized formulation were 155.8±12.3 nm, 0.70 and 5.5%, respectively, and they corresponded well to the values calculated by the objective functions (Figure 3). Furthermore, the droplet size distribution curves recorded immediately after preparation and after 6 month of storage shown in Figure 4 additionally emphasise the stability of the optimised formulation, which is intended for the preparation of double W/O/W emulsions.

Table 5: Results of multiobjective optimization of the water-in-oil emulsion quality parameters.

Objective parameter	Objective	Individual optimum of the objective function, f_i^*	Weight factor of the objective function, w_i	Values of the objective function at $X_{1,opt}$, $X_{2,opt}$ and $X_{3,opt}$
$d_{4,3}$ (Eq 6)	minimum	59.8 nm	1/3	102 nm
Span (Eq 7)	minimum	0.60	1/3	0.70
*SI*90 (Eq 8)	minimum	0.7%	1/3	0.9%

The compromise optimum values are $X_{1,opt}$=0.98, $X_{2,opt}$ =1 and $X_{3,opt}$ =0.12

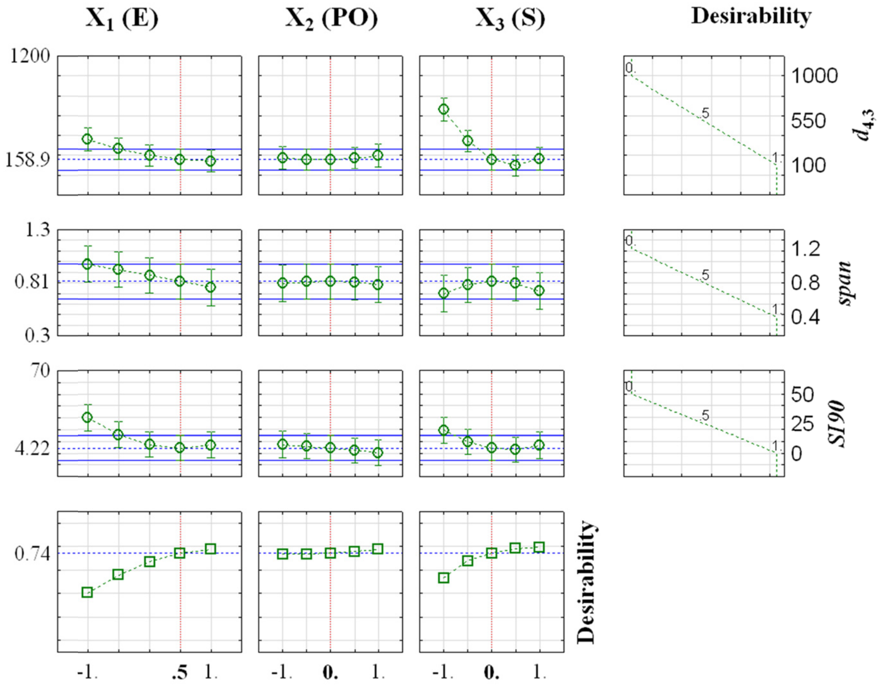

Figure 3: Profiles for predicted values and the desirability of droplet size ($d_{4,3}$), span and stability index after three month of storage ($SI90$) of W/O emulsions.

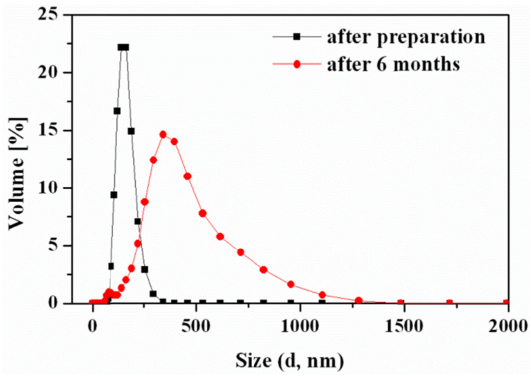

Figure 4: Droplet size distribution curves of W/O emulsions containing 4% PGPR, 50% pumpkin seed oil in the oil phase and 0.15 M NaCl in water after preparation and after 6 months of storage.

CONCLUSIONS

We successfully incorporated pumpkin seed oil, as a valuable source of biologically active ingredients, into W/O emulsions. A multiobjective optimization method combined with response surface methodology and the Box-Behnken experimental design for three factors, was implemented to create an optimal formulation for a stable monodisperse nanoemulsion. Regression models were developed for predicting the mean volume diameter of water droplets, the span of the droplet size distribution and the sedimentation stability index after 90 days based on the coded values of three independent variables: polyglycerol polyricinoleate content, pumpkin seed oil content in the oil phase and sodium chloride content in the water phase of the water-in-oil emulsion. The multiobjective optimum was calculated by using the minimal loss method with a weight factor value of 1/3 for three objective functions. Additionally, the final optimal formulation was designed taking into consideration the costs of the oil phase containing pumpkin seed oil by using the minimal loss method with a weight factor value of 1/4 for four objective functions. The emulsion of the optimized composition contained 0.15 M NaCl in the water phase and a continuous phase that was a mixture of pumpkin seed and sunflower oil at a mass ratio of 1:1, with a PGPR concentration of 4% (w/w). The addition of salt to the aqueous phase had a great importance for the reduction of the droplet diameter and the stability increase of the obtained emulsions, giving evidence that the salt stabilized the emulsions against Ostwald ripening. When the continuous phase was a mixture of pumpkin seed oil and sunflower oil at a mass ratio of 1:1, the sedimentation stability of the emulsion was improved compared to emulsions without pumpkin seed oil. Experimentally determined values of the mean volume diameter of water droplets, the span of the droplet size distribution and the sedimentation stability index after 90 days of storage were 155.8±12.3 nm, 0.70 and 5.5%, respectively, and corresponded well to the values calculated by the objective functions. Droplet size distribution of the emulsion after six months of storage was recorded and confirmed good stability of the optimized formulation, which is intended for preparing double W/O/W emulsions.

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NOMENCLATURE

Symbols

a_0, a_1, a_3, a_5, a_6, a_7, a_8	adjustable parameters in Eq. (6)
b_0, b_1, b_3, b_5, b_6, b_7, b_8	adjustable parameters in Eq. (7)
C_0	the number of central points in the Box-Behnken design
c_0, c_1, c_2, c_3, c_5, c_6	adjustable parameters in Eq. (8)
D	shear rate (s^{-1})
d_0	parameter in Eq. (9)
d_{10}, d_{50}, d_{90}	the droplet diameters below which 10%, 50% and 90% of the sample lies, respectively (nm)
$d_{4,3}$ (Eq. (3))	the volume-weighted mean droplet diameter (nm)
d_i	diameter of droplet i (nm)
E	PGPR content % (w/w)
EC	Objective function for the price
f_i	the *i* objective function
f_i^*	individual optimum value of the *i* objective function
H_E	initial height of an emulsion in a cylinder (nm)
H_S	height of a serum layer in a cylinder (nm)
k	the number of factors in the Box-Behnken design
L (Eq. 1)	the loss function (-)
N	the required number of experiments in the Box-Behnken design
n_i	number of droplets of diameter d_i
PO	pumpkin seed oil content in the continuous phase % (w/w)
S	NaCl concentration in the water phase $mol \cdot dm^{-3}$
SI	the stability index (%)
*SI*90	the stability index after 90 days of storage time (%)
span	the span of droplet size distribution (-)
w_i	the weighting factor of the *i* objective function
X_1	coded value of the emulsifier content in the continuous phase (X_1 = (E-3)/2), -
$X_{1,opt}$, $X_{2,opt}$, and $X_{3,opt}$	the set of compromise optimum values of the independent variables
X_2	coded value of pumpkin seed oil content in the continuous phase, (X_2=(PO-50)/50), -
X_3	coded value of sodium chloride content in the water phase, X_3=(W-0.15)/0.15, -

Greek Letters

τ	Shear stress, Pa
η	apparent viscosity (Pa·s)

Abbreviations

COBYLA	the constrained optimization by linear approximation method
L-BFGS-B	Limited-memory Broyden-Fletcher-Goldfarb-Shanno bound-constrained algorithm
PGPR	polyglycerol polyricinoleate
TNC	a truncated Newton conjugate-gradient algorithm
W/O	water-in-oil
W/O/W	water-in-oil-in-water

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