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Evaluation of different formulation studies on air-filled polymeric microcapsules by multivariate analysis

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Abstract

Air-filled polymeric microcapsules have been prepared by freeze-drying of emulsions containing the wall-forming polymer in the organic phase of oil in water emulsions. Echogenic air-filled microcapsules were prepared from emulsions containing either (−)-camphene, cyclohexane or cyclooctane as the solvent in the organic phase. Formulation studies have been performed to improve the yield and acoustic quality of the microcapsule suspensions. The yield was measured as particle concentration or efficacy, i.e. normalised attenuation at 3.5 MHz, related to the amount of polymer used.

No overall conclusion could be made for all the variables when visually comparing the results from the different investigations. Multivariate analyses (PCA and PLS) were therefore necessary to be able to reveal any relevant systematic information from all the investigations. Different parameters describing the formulation, the production process and parameters describing the characterisation of the intermediates and the final product were set as independent *X*-variables.

Three to four percent (w/v) of polymer was found to be the appropriate concentration of wall forming polymer. Including PEG 3000 resulted in improved freeze-dried product and suspension. Quenching of the emulsions by freezing in dry ice/methanol prior to freeze-drying was not necessary.

Process parameters for homogenising and freeze-drying should be optimised with regard to the single systems, due to the different physico-chemical properties of the different solvents, especially melting point and vapour pressure.

Keywords: Polymeric microcapsules; Emulsion; PLS; PCA; Freeze-drying; Ultrasound contrast agent

1. Introduction

Air-filled polymeric microcapsules have been successfully prepared by freeze-drying oil in water emulsions containing the wall-forming polymer in the organic phase and the surfactant and mannitol or polyethylene glycol 3000 (PEG 3000) in the water phase (Bjerknes et al., 1997, 2000, 2001). The composition of the emulsion has primarily been investigated in these formulation studies. In addition, other variables were improved in parallel to the formulation and various studies of 5–20 samples have been performed. The molecular (M_w) weight and polydispersity (M_w/M_n) of the wall-forming polymer varied in the different polymer batches due to a new method of synthesis. The improved synthesis resulted in less degradation, reflected in the increased molecular (M_w) weight and decreased polydispersity (M_w/M_n), due

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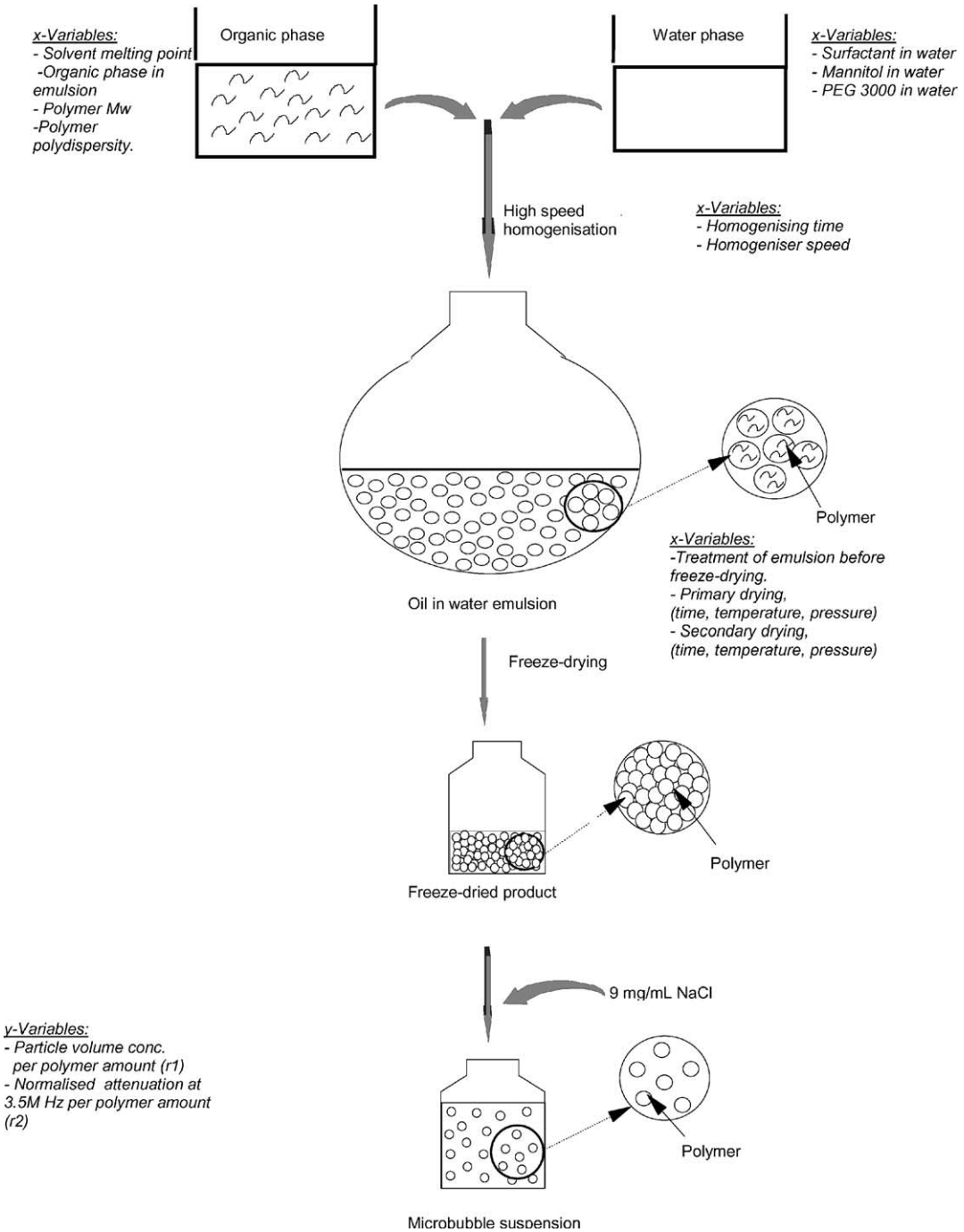

Fig. 1. Process flow scheme showing the different variables included in the multivariate analyses.

to removal of chloride ion. Process variables for the emulsification, such as homogenising time, temperature and speed were varied and the freeze-drying cycle was changed. Different treatment of the emulsion prior to freeze-drying was also studied. The emulsion was either stirred at room temperature (RT), or immediately frozen in a mixture of dry ice and methanol. A schematic overview of the variables is shown in Fig. 1.

The number of variables made it difficult to directly compare the results from the different formulation investigations. By applying multivariate analyses, such as partial least square regression (PLS) and principal component analysis (PCA), it is possible to include results from several investigations and the number of samples and variables can be significantly increased. The preparation of air-filled polymeric microcapsules includes several process steps and intermediates as outlined in Fig. 1, and the batch-to-batch variations in the suspension could therefore be significant.

2. Materials and methods

2.1. Materials

The biodegradable double-ester polymer with ethylidene units, poly[ethyl-1,1-bis(16-oxohexadecanoate)-1,6-dioxohexamethylene], used as the microcapsule wall-forming material, was synthesised at Nycomed Imaging AS, Norway (Patent nos.: WO 96/07434 and WO 93/17718). The molecular weight average, M_w, was 16,000–55,800 Da and the polydispersity index, M_w/M_n (number molecular weight average), was 2.0–2.3.

The amphiphilic poly(ethylene glycol)-based block copolymer, α-(16-hexadecanoyloxyhexadecanoyl)-Ω-methoxypolyoxyethylene ester used as the surfactant, was synthesised at Nycomed Inc., Philadelphia, USA (Patent no.: WO 95/06518). The M_w was approximately 10,000 Da and the polydispersity index, M_w/M_n, approximately 1.

Cyclooctane (≥99% purity), (−)-camphene (ca. 85% purity) and poly(ethylene glycol) 3000 were from Fluka Chemie AG, Switzerland, while cyclohexane (>99% purity) was from Merck, Germany. The characteristics of the solvents are outlined in Table 1. Mannitolum ad usum parenterale (Ph.Eur.) was purchased from Norsk Medisinal Depot in Norway, and NaCl (9 mg/ml) injection, was from Kabi Pharmacia AB, Sweden. Water for injection was produced at Nycomed Imaging AS, Norway.

2.2. Production methods

The block copolymer (surfactant) was dispersed in cold water by stirring, dissolved at approximately 80 °C for 30 min and swelled at 4 °C over night. Mannitol or PEG 3000 was added to the water phase

Table 1
Characteristics of the different organic solvents included in PLSR1

Organic solvent	Structure	Melting point[a] (°C)	Vapour pressure[a] (°C)
(−)-Camphene		52 (33.5–34.6[b])	47.2s[c] (10 mmHg) 97.9 (100 mmHg) 160.5 (760 mmHg)
Cyclohexane		6.5 (min 6[b])	−15.2s[c] (10 mmHg) 25.5 (100 mmHg) 80.7 (760 mmHg)
Cyclooctane		14.3 (14[b])	37.7 (16 mmHg)[d]

[a] From Lide (1991).
[b] Fluka Catalogue (Chemika, BioChemica, Analytika) 1997/98, Fluka Chemie AG, Switzerland (1997).
[c] s, solid.
[d] Taken from Material Safety Data Sheet, Fluka Chemical Corp., USA (1995).

Table 2
X-variables in the PLSR1 models

	X-variable	Unit	Interval
A	Amount of emulsion	ml	40–80
B	Organic phase in emulsion	%, v/v	10–40
C	Solvent melting point	°C	7–34
D	Polymer in organic phase	%, w/v	2.5–7.5
E	Polymer M_w	Da	30716–55800
F	Polymer polydispersity, M_w/M_n	–	2.03–2.30
G	Surfactant in water phase	%, w/v	1–2.5
H	Mannitol in water	%, w/v	0 or 5
I	PEG 3000 in water	%, w/v	0 or 5
J	Homogenising speed during emulsification	rpm	8000–15000
K	Homogenising time of emulsion	min	1.0–5.5
L	Treatment of emulsion prior to freeze-drying: 1: quenched; 2: 20 min stirring at RT	–	1 or 2
M	Freeze drier: 1: Virtis; 2: Edwards	–	1 or 2
N	Primary drying temperature	°C	−35 to −25
O	Primary drying time	h	20–52
P	Primary drying pressure	mbar	0.1–0.5
Q	Secondary drying temperature	°C	15–20
R	Secondary drying time	h	2–10
S	Secondary drying pressure	mbar	0–0.3
T	Particle mean volume diameter	μm	3.6–11.4

before heating to 60 °C, prior to emulsification. The wall-forming polymer was dissolved in the specific solvent at 60 °C. The organic phase was added to the water phase, and the emulsion was prepared by high shear stirring at 60 °C with an Ystral Rotor Stator T1500 (Ystral GmbH, Germany) in a round bottom flask with heating jacket until a volume mean droplet size of approximately 5 μm was reached. The emulsions and homogenising process parameters are outlined in Table 2.

Prior to freeze-drying, the emulsion was either stirred for 20 min at RT, or immediately frozen in a dry ice/methanol bath for approximately 10 min (quenched). Units of 5 ml emulsions were filled 20 ml vials after stirring at RT, or immediately before quenching treatment. The vials were placed directly on prefrozen shelves (−40 °C) in the freeze-dryer. The emulsion was freeze-dried in a Virtis Genesis 25 SL or an Edward Lyofast S12/NS, com. 8521 freeze-dryer as outlined in Table 2.

The aqueous microcapsule suspension was prepared by adding 10 ml of 9 mg/ml NaCl to the freeze-dried substance. The suspensions were shaken at 250 rpm overnight on an IKA KS 501D laboratory shaker (IKA-Laboratorieteknik GmbH, Germany).

2.3. Characterisation

The size distributions of the emulsion and the microcapsule suspension were measured using a Coulter Multisizer Mrk II E (Coulter Electronics Ltd., UK) with Accucomp for Windows software, version 1.15. The volume mean diameter and the volume concentration were calculated from the size distribution data (Bjerknes et al., 1997).

The echogenic properties were characterised as the decrease in signal intensity of the echogenic attenuation (dB/cm) of an ultrasound pulse passing through a diluted suspension of the sample. A detailed description of these characterisation methods for the microcapsule suspension has been presented earlier (Bjerknes et al., 1997).

2.4. Mathematical methods

The results were treated by PCA and PLS (Esbensen et al., 1998) by use of Unscrambler software (CAMO ASA, Oslo, Norway). All the data were autoscaled prior to analysis.

In PCA, the data matrix *X* is decomposed to a "structural part" and a "noise part" by placing axis along

the direction that explain most mathematical variation in the data matrix seen as a swarm of data points in a space of p dimensions. No Y-matrix is included. The first axis, principal component 1 (PC1), will have the highest degree of explanation of the variation in the data matrix. The second PC will explain less of the variation than PC1, but more than PC3, and so on. We assume that the directions with maximum variance are more or less directly related to "hidden phenomena" termed properties. The axis has zero point, a direction and a unit of length. The variation in the data points orientated far from the zero point when projected orthogonally down on the PC, is well-explained by that specific PC.

In PLS, Y is used to achieve a guided decomposition of X prior to the estimation of regression coefficients. PLS uses the information in Y to find the Y-relevant information in X. X and Y may be chosen as desired. Measured values are plotted against the values predicted by the mathematical model obtained in PLS, to describe the ability of the model to explain the variation in the data swarm. An ideal plot should therefore be a straight line with the correlation coefficient of 1.00. In addition, the degree of explanation of the variation in the data swarm is important. Several PCs are necessary to be able to explain all of the variation (100%) resulting in a complex and noisy model. Therefore, fewer PCs are included to make the model less complicated, but still allowing most of the variation to be explained. Significant variables with regard to Y in the model are placed away from the zero point at the axis. The position on the PCs is reflected in the regression coefficients. Variables with high regression coefficients are well explained and have high influence on the mathematical model, and thereby influence Y. All parameters with regression coefficients below 0.2 with no influence on the mathematical model were removed during modelling.

Predicting new values with the model and then comparing it to the measured value indicates the quality of the PLS model. Some of the samples from the studies could be retained as a test-set to validate the model. On the other side, retaining some values make the modelling poorer due to less results during modelling. Therefore, validation methods as full cross validation and Unscrambler Jack-knife validation were applied. These validation methods allow the inclusion of all data during modelling.

In the full cross validation (Martens and Næs, 1989) each sample was taken out of the calibration set once. The remaining $n - 1$ data vectors made up the model and predicted the Y-value temporary left out sample. This is carried out n times until all samples had been left out one time. As a result the correct number of PCs is found.

In the stability testing of the regression coefficients (Martens and Martens, 2000), Student's t-test was performed for the regression coefficients from the n models in the full cross validation as a rough significance test.

Double Jack-knife regression is a recently developed method to investigate covariations between the variables when all the models are included (Anderssen et al., submitted). To avoid covariation, the parameters were transformed to orthogonal variables by using the scores obtained in PCA. In that way, the scores in different PC could be used as representing different variables. The yield was calculated as particle volume concentration per mg polymer (r1) and attenuation at 3.5 MHz per mg polymer (r2) to be able to separate the effect of increased amount of polymer in the suspension from the degree of utilisation of the polymer added. Significant PCs could then be found in PLS by modelling the different PC-scores from PCA with regard to r1 or r2. By evaluating the corresponding loading for the significant PC-variables found by the PLS, the important process variables were elucidated.

The independent X-variables and the dependent Y-variables are outlined in Tables 2 and 4.

3. Results and discussion

The dataset consisted of several studies with different main objectives and included a total of 105 samples. Many variables have been varied in formulation investigations, or as a result of other improvements during the investigation phase. The variations in the emulsion process and formulation parameters are quantitatively described in Table 2.

Two different variables were chosen to describe the yield; the particle mean volume concentration per mg polymer (r1) and attenuation at 3.5 MHz per mg polymer (r2). r1 included all the particles in the suspension. In ultrasound imaging, the particles must have an echogenic effect to provide image enhancement. The

attenuation included in r2 indicated the imaging effect of the suspension. The correlation between r1 and r2 was 0.82, showing that the particle concentration strongly influenced the echogenic effect of the suspension, but that not all the particles in the suspensions were echogenic. This could be due to presence of particles with an insufficient content of air. In addition, the quality of the microcapsule, such as the overall density and elasticity of the polymeric wall influenced the echogenic effect (Morse and Ingard, 1968).

When investigating the dataset by PCA, the samples containing different solvents had different scores as seen in Fig. 2. The variation in the dataset was mainly described by PC1 (91%). The yield, expressed both as r1 and r2, had relatively high scores on PC1 and samples nearby r1 and r2 would have high values for the yield. The samples containing cyclohexane or cyclooctane were placed nearest r1 and r2 on PC1, indicating that the samples containing these solvents had higher yield than (−)-camphene systems. The remaining variation (9%) was described by PC2. This PC described the difference in r1 and r2. The samples containing cyclohexane and cyclooctane had different orientations with respect to r1 and r2 indicating that the cyclooctane samples oriented closer to r2 had better echogenic properties than the cyclohexane samples.

The dataset was investigated by PLS to be able to find the variables with significant influence on the yield expressed as r1 and r2. All the variables must be described in a quantitative manner to be included in the mathematical model. The different solvents were described by their melting point, because the preparation of the emulsion was influenced by the consistency of the organic phase. Creaming occurred when the organic phase solidified (Bjerknes et al., 1997).

The polymer weight and polydispersity were included to describe the variation in the quality of the polymer because the main difference between the polymer batches was the final content of free Cl^-, due to addition of an amine during synthesis that bound most of the Cl^-. The removal of free Cl^- mainly resulted in lower degrading rate of the polymer and thereby higher molecular weight and lower polydispersity.

Mannitol was added as a filling agent to the formulations containing (−)-camphene in previous work. This resulted in the improved visual appearance of the dried product and the suspension, but did not prevent the formation of aggregates sufficiently (Bjerknes et al., 2001). Another excipient often used in freeze-dried products is PEG. PEG has a high collapse temperature (−13 °C, M_w not specified) which

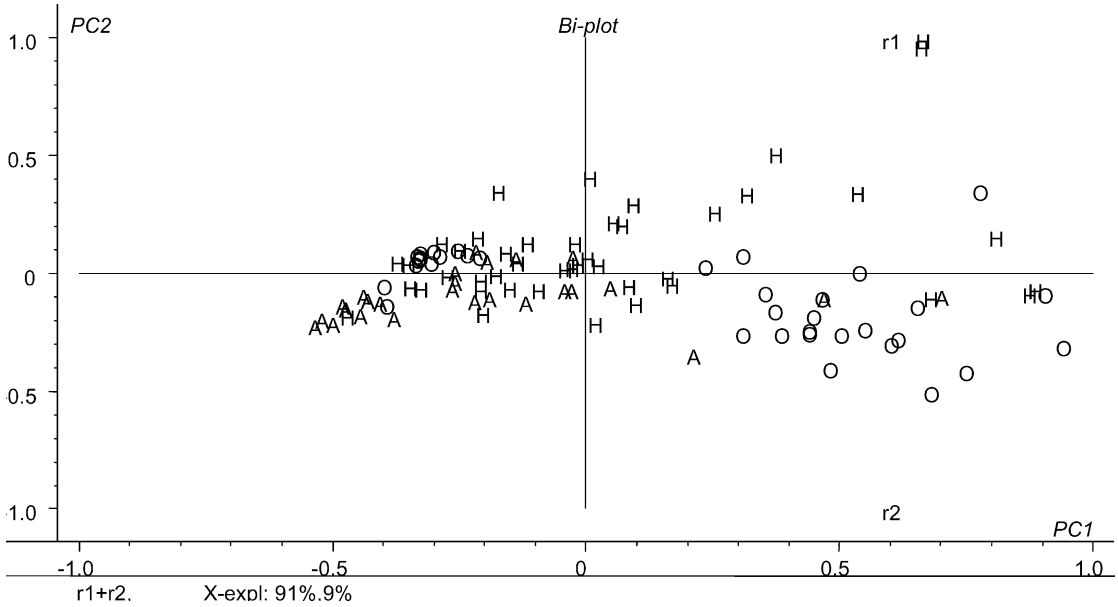

Fig. 2. PCA of r1 and r2 for the data matrix containing O: cyclooctane, A: cyclohexane and H: (−)-camphene.

allows a relatively high primary drying temperature and thereby a faster drying cycle than other well-known excipients for freeze-drying (Snowman, 1993). Different amounts of PEG with different molecular weights (1500–6000 Da) were screened as outlined in Table 3. Also emulsions containing both PEG and mannitol were freeze-dried. The best visual freeze-dried product with no cracks and little shrinkage was obtained with 5% (w/v) of PEG 3000 in the water phase in the emulsion. Adding PEG 3000 also resulted in a product that was easy to disperse, and the resulting suspension contained few and small aggregates. PEG 3000 was therefore chosen for further investigations.

No robust model was found with regard to r1 or r2 when investigating the total dataset by PLS. Relatively low prediction ability for r1 and r2, seen by the correlation coefficients for predicted values plotted against measured values (0.64 and 0.80, respectively), and low explained variance (41 and 54%, respectively) were seen. The poor models could be due to covariation between the different variables, spreading the effect on several variables making each of them small, and hence lower the regression coefficients and making them insignificant. To avoid covariation, the parameters were transformed to orthogonal variables by using principal component decomposition of the response matrix. By evaluating the corresponding loadings and the corresponding interaction terms and square terms for the significant PC-variables found by the PLS, the important process variables were elucidated. No robust model could be found by this method, verifying that an overall model for all the samples would not be reliable. Different submodels representing each of the solvents were therefore prepared by PLS. The estimated values plotted against measured values of r1 and r2 for each solvent are shown in Figs. 3 and 4, respectively. The correlation coefficients for these models are relatively high 0.93–096, indicating precise models including the factors necessary to explain the variation in the *X*-data. Also the scattering of the samples included may be seen. The variables for the different systems are outlined in Table 4 and the regression coefficients for the significant variables are outlined in Table 5.

The effect of PEG 3000 was most thoroughly investigated in the formulations containing cyclohexane. The positive effect on the yield when including PEG 3000 can be seen in the PCA plot in Fig. 5. The plot is the same as in Fig. 2, but the content of PEG 3000 has been included in the sample name. Most of the samples containing PEG 3000 had relatively high scores on PC1, as was the case for r1 and r2. The scores on PC2 were less evident placing the samples containing PEG 3000 closer to r1 than r2, indicating that PEG 3000 contributed less to an increased ratio of echogenic particles. The positive effect of PEG 3000 was also seen in PLS. The regression coefficients outlined in Table 5 show the strong positive effect of this substance on the cyclohexane-formulation, by the significant values of the main effect on r1 and r2, 0.53 and 0.32, respectively. In addition, several interplay including PEG 3000 were significant (Table 5), verifying the influence of PEG 3000.

For the other systems, the low number of samples with PEG 3000 made the modelling less robust. For the (−)-camphene formulations, only two samples contained PEG 3000. These two samples influenced the PLS-models seen by the regression coefficients of 0.32 and 0.51, for r1 and r2, respectively. PEG 3000 was also included in some significant interplay (amount of surfactant × PEG 3000 and polymer % × PEG 3000).

The two cyclooctane-samples containing PEG 3000 influenced the PLS-model for r1 significantly. These samples did not fit into a model with the rest of the samples, due to high r1. The samples were therefore left out as outliers for r1. The samples also had high r2, but the PEG 3000 did not have significant effect on the model for this *Y*-parameter. This is in accordance with the PCA plot in Fig. 5, where the PEG 3000 samples were closer to r1 than r2.

Low amount of polymer in the organic phase was found to be favourable in previous work with (−)-camphene (Bjerknes et al., 1997, 2000). Excess polymer did not form echogenic particles. Especially high amount of polymer in the oil phase (7.5%, w/v) combined with high amount of solvent gave lumpy suspension which could not be analysed by Coulter Counting.

This conclusion was verified by the extended model for the (−)-camphene samples with the significant regression coefficient of −0.25 on r2.

The polymer interval (2.5–7.5%, w/v) was also included for the cyclohexane samples. Lower concentrations of polymer in the organic phase was beneficial also for this system, shown by the negative regression coefficients for r1 and r2, −0.48 and −0.36, respectively.

Table 3
Screening of different excipients for freeze-drying

Oil/water ratio	Polymer in oil phase (%, w/v)	Freeze drying excipient in water (%, w/v)	Visual inspection of the freeze-dried product	Visual inspection of the suspension after manually shaken for 1 min	Particle volume mean diameter (μm)	Particle volume concentration	Attenuation at 3.5 MHz
0.25	5.0	–	Shrinkage and collapse	No aggregates	6.7	7.7	54.5
0.25	5.0	5% mannitol, 5% PEG 6000	Smooth top surface, some humidity seen in the bottom of the glass	Aggregates present	6.4	5.3	58.7
0.25	5.0	5% PEG 3000	Smooth top surface, slightly coarser longitudinal surface	Few and small aggregates	4.5	7.6	30.1
0.25	5.0	5% mannitol, 5% PEG 1500	Smooth top surface, slightly coarser bottom surface	Some aggregates	5.3	7.2	44.2
0.15	2.5	–	Cracks	Solid substance at the glass wall	4.3	1.0	3.2
0.15	2.5	–	Lifted product cut in two	Solid substance at the glass wall	5.1	1.5	5.0
0.15	2.5	10% PEG 3000	Collapsed and wet	Few aggregates, but few microcapsules	5.6	0.9	1.1
0.15	2.5	5% PEG 6000	Small cracks at the edge	Few aggregates	4.5	1.4	2.9
0.15	2.5	10% PEG 6000	Collapsed and wet	Few aggregates	4.3	0.7	0.6
0.15	2.5	10% PEG 3000	One vial with collapsed product and two vials with visual acceptable product	Few aggregates	4.4	2.6	6.9

Polymer concentrations of 1.5–5.0% (w/v) in the organic phase were investigated in the cyclooctane samples. This polymer interval was somewhat lower, but overlapping with the interval investigated for the systems containing (−)-camphene and cyclohexane. The negative effect of the polymer concentration above 5.0% (w/v) was removed and the effect of lowering the concentration below 2.5% (w/v) was

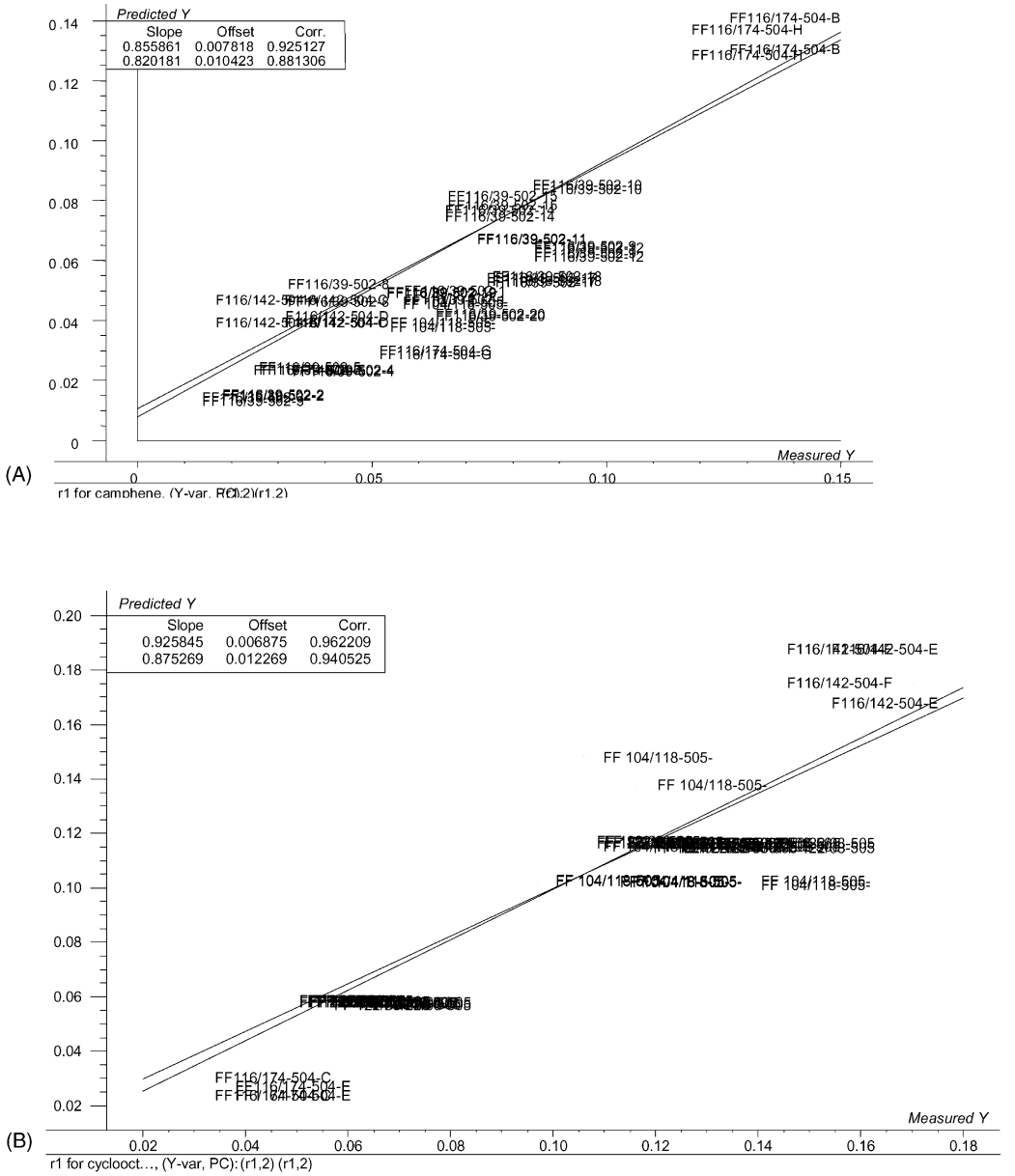

Fig. 3. Estimated values of r1 (the particle mean volume concentration per mg polymer) by PLS vs. measured values of r1. The samples are labelled by their respective batch no. Both modelled values and values from samples kept out of the modelling by Jack-knife validation are shown (upper and lower line in the text box, respectively). (A) (−)-Camphene as solvent, (B) cyclooctane as solvent, and (C) cyclohexane as solvent.

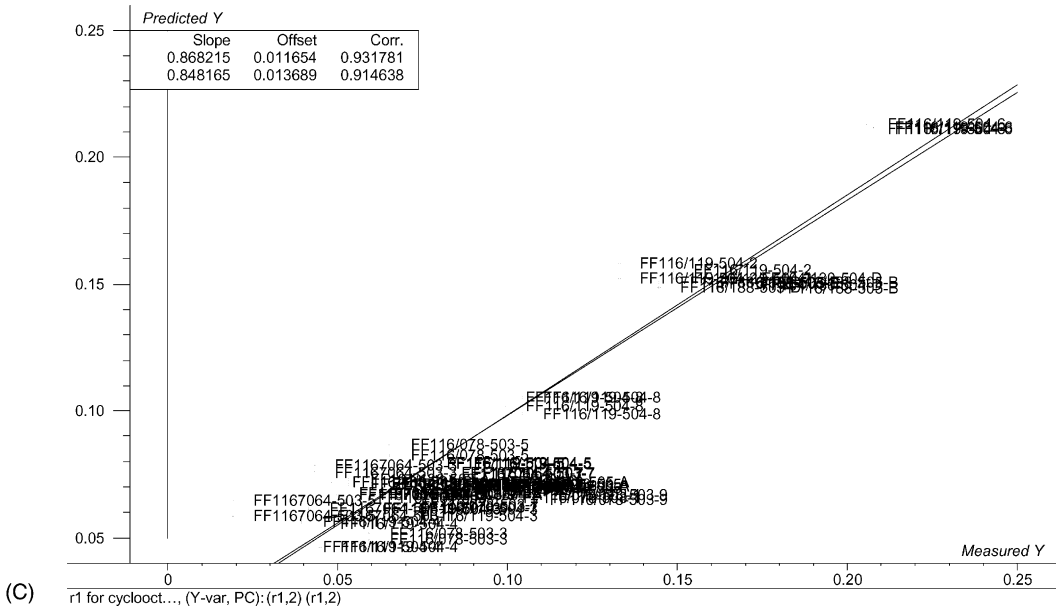

Fig. 3. (*Continued*).

Table 4
Parmeters investigated for formulations containing (−)-camphene, cyclooctane and cyclohexane

Variable	(−)-Camphene (samples)	Cyclooctane (samples)	Cyclohexane (samples)
Amount of emulsion (ml)	40 or 80	40 or 80	40 or 80
Organic phase in emulsion (%, v/v)	10–40	15–25	10–40
Polymer in organic phase (%, w/v)	2.5–7.5	1.5–5.0	2.5–7.5
Polymer M_w (Da)	30716–55800	16000–55800	30716–55800
Polymer dispersity, M_w/M_n	2.06–2.70	2.05–2.70	2.06–2.70
Surfactant in water (%, w/v)	0.5–2.5	1.0–2.0	1.0–2.0
Mannitol in water (%, w/v)	0–5.0	0	0
PEG 3000 in water (%/v)	0 or 5.0	0 or 5.0	0 or 5.0
Homogeniser speed during emulsification (rpm)	10000–12000	10000–12000	8000–15000
Homogenising time of emulsification (min)	2.0–4.0	3.0 or 4.0	1.0–5.5
Treatment of emulsion prior to freeze-drying: 1, Quenched; 2, 20 min stirring at RT	1 or 2	1 or 2	1 or 2
Freeze drier: 1, Virtis; 2, Edwards	1 or 2	1 or 2	1 or 2
Primary drying temperature (°C)	−30 to −35	−30 to −35	−25 to −35
Primary drying time (h)	20–48	20–52	20–30
Primary drying pressure (mbar)	0.08–0.45	0.08–0.45	0.08–0.48
Secondary drying temperature (°C)	15 or 20	15 or 20	15 or 20
Secondary drying time (h)	2.00–9.00	6.00–10.00	2.00–8.00
Secondary drying pressure (mbar)	0.01–0.16	0.01–0.23	0.01–0.27
Particle mean volume diameter (μm)	4.7–10.7	4.6–11.4	3.6–10.1
Particle mean volume concentration	0.36–4.09	0.90–7.28	0.68–7.82
Particle mean volume concentration per amount polymer (r1)	0.010–0.147	0.030–0.018	0.019–0.026
Normalised attenuation at 3.5 MHz	0.18–24.82	0.42–35.73	0.88–24.45
Attenuation at 3.5 MHz per amount polymer (r2)	0.006–0.827	0.029–1.143	0.011–0.945

investigated for this system. A polymer concentration of 1.5% (w/v) was too low, seen by the positive regression coefficient for r1 and r2, 0.30 and 0.49, respectively, indicating that increasing the concentration gave better result. The advantage of increasing the polymer concentration decreased in the higher part of the interval, shown by the negative regression coefficient for [polymer %]^2. This indicate that 5.0% (w/v) might be too high for this system.

An overall conclusion from all the models described is therefore that an adequate concentration of the polymer in the organic phase is in the medium area of the investigated interval for all the solvents (approximately 3–4%, w/v). Previous studies concluding that a polymer concentration of 2.5% (w/v) in the oil phase

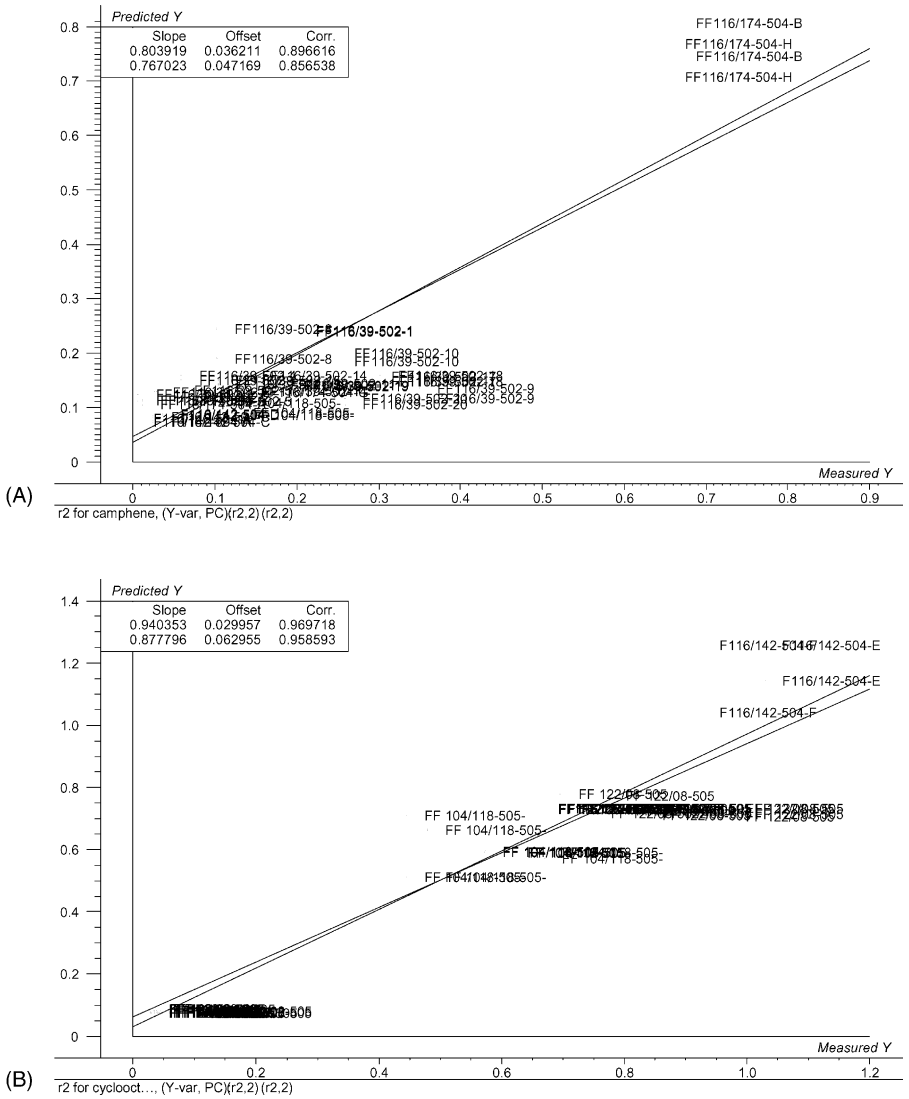

Fig. 4. Estimated values of r2 (attenuation at 3.5 MHz per mg polymer) by PLS vs. measured values of r2. The samples are labelled by their respective batch no. Both modelled values and values from samples kept out of the modelling by Jack–Knife validation are shown (upper and lower line in the text box, respectively). (A) (−)-Camphene as solvent, (B) cyclooctane as solvent, and (C) cyclohexane as solvent.

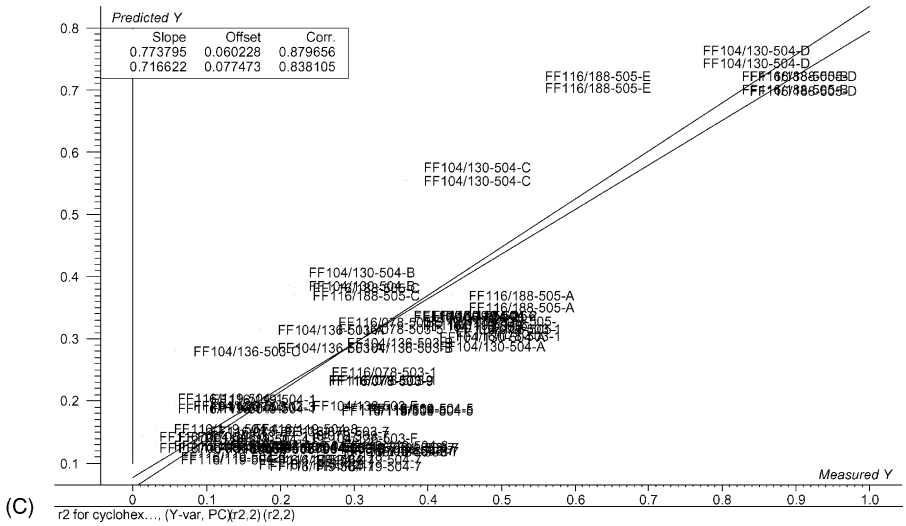

Fig. 4. (*Continued*).

Table 5
Significant regression coefficients for formulations containing (−)-camphene, cyclooctane and cyclohexane in PLSR1

Variable	Cyclohexane		Cyclooctane		(−)-Camphene	
	r1	r2	r1	r2	r1	r2
Amount of emulsion (ml)			−0.50[a]	−0.30		
Organic phase in emulsion (%, v/v)	0.29					
Polymer in organic phase (%, w/v)	−0.48	−0.36	0.30	0.49		−0.25
Surfactant in water (%, w/v)					0.33	
PEG 3000 in water (%/v)	0.53	0.32	[b]		0.32	0.51
Secondary drying temperature (°C)		−0.35	−0.22			
Secondary drying pressure (mbar)		−0.37				
Particle mean volume diameter (μm)					0.37	0.22
Amount of emulsion (ml) × Polymer in organic phase (%, w/v)			0.30	0.49		
Organic phase in emulsion (%, v/v) × PEG 3000 in water (%/v)	0.25					
Polymer in organic phase (%, w/v) × PEG 3000 in water (%/v)	−0.50					−0.34
Amount of emulsion (ml) × PEG 3000 in water (%/v)					−0.46	
Polymer in organic phase (%, w/v) × secondary drying temperature (°C)		−0.34	−0.34			
[Polymer in organic phase (%, w/v)]^2			−0.40	−0.72		
Correlation coefficient	0.93	0.88	0.96	0.97	0.93	0.90
Principal components included in model	2	2	2	2	2	2
Explained variation in the response parameter (%)	86	78	92	94	85	81
No of samples included in the modelling	43	43	38	38	26	26
No of outliers removed during modelling	2	2	2	2	2	2

[a] Not found significant by Jack-knife regression.
[b] Samples containing PEG 3000 were removed as outliers due to high r1.

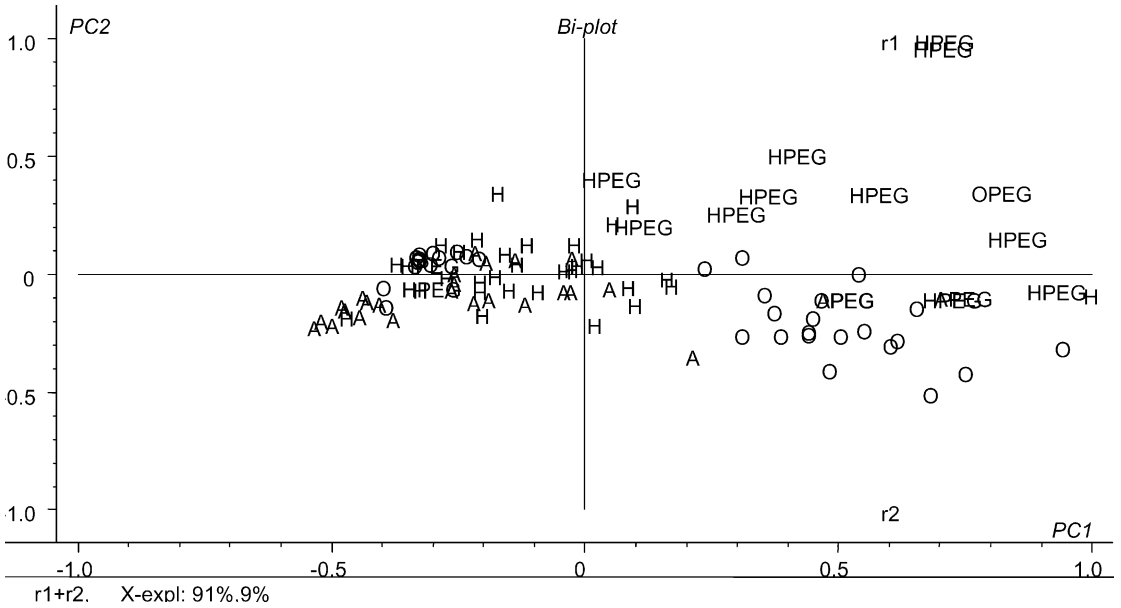

Fig. 5. PCA of r1 and r2 for the samples in the data matrix, containing O: cyclooctane, A: cyclohexane and H: (−)-camphene. The samples containing PEG 3000 have been indicated by "PEG" after the letter identifying the solvent.

gave higher yield than 5% (w/v) is in correlation with this finding.

The various systems were influenced by different parameters shown by the several significant regression coefficients in the PLS-models as outlined in Table 5. This could be partly due to the variation in the interval for the variables in the different systems. The amount of polymer in organic phase and amount of surfactant in the water phase had the largest intervals for the (−)-camphene samples. Mannitol was included only in some of the (−)-camphene samples relatively early in the investigation phase. PEG 3000 was mainly included in the cyclohexane samples later in the investigation phase.

On the other hand, some of the parameters were insignificant within the investigated intervals for all the systems and could be omitted in future investigations. Quenching of the emulsions is unnecessary when compared to stirring at RT for 20 min prior to freezing on shelves at −40 °C. This is promising with regard to develop a more suitable freezing step for large scale production. Also the pressure in the freeze dryer seemed to be acceptable within the investigated range for the primary drying (0.08–0.45 mbar). The pressure variation was acceptable and freeze-drying in the two different freeze-dryers did not give any significant influence on the yield. This is also favourable regarding the up scaling of the process. The molecular weight and the polydispersity of the polymer were insignificant on the yield, indicating that the new polymer synthesis did not significantly affect the suspension.

The emulsification process and freeze-drying process should be improved for the specific formulation systems. This is indicated by the variation in process parameters for the systems containing different solvents. The different physico-chemical characterisation of the solvents as the viscosity, melting point and vapour pressure will influence the process parameters chosen.

None of the process parameters were significant with regard to the yield when (−)-camphene was used as solvent in the organic phase. This could be due to the fact that the process originally was developed with regard to formulations containing (−)-camphene and that the parameters were set based on the previous experience with this system.

Regarding the formulations containing cyclooctane, only one of the process parameters, i.e. the temperature in the secondary drying was significant (−0.22 for r1). It was more favourable with 15 °C than 20 °C.

This process parameter was also included in a significant interplay with the amount of polymer in the emulsion. This could be due to the low amount of polymer investigated in this system and the possibility of collapse of microcapsule with thin polymer wall if dried too fast/with too high temperature. During drying, the solvent inside the organic droplets evaporates or sublimes out of the droplets. If the temperature is raised too fast, the evaporation and sublimation of solvent may be so fast and violent that the microcapsules could be destroyed.

The batch size seemed to influence the cyclooctane systems, but the smaller batch size of 40 ml was represented by only two samples. These batches contained 2.5% polymer in the organic phase in the emulsion. The significant interplay between amount of polymer and batch size then rather reflected the amount of the polymer than the actual batch size. Indeed, Jack-knife regression did not find that the main parameter, batch size, was significant with regard to r1.

Cyclohexane differed from (−)-camphene in regard to melting point and vapour pressure. The variation in secondary drying temperature and secondary drying pressure influenced r2 indicated by the significant regression coefficients, −0.35 and −0.37, respectively. This could be due to the high vapour pressure of cyclohexane making it important to have a slow drying cycle to prevent destruction of the microcapsule by too high internal pressure. On the other hand, a slower freeze-drying cycle applied for some batches to prevent this might have led to incomplete primary drying resulting in poor freeze-dried product.

4. Conclusion

Echogenic air-filled microcapsules could be prepared successfully from emulsions containing either (−)-camphene, cyclohexane or cyclooctane as the solvent in the organic phase. Approximately 3–4% (w/v) was found to be the adequate concentration of the wall-forming polymer (poly[ethyl-1,1-bis(16-oxohexadecanoate)-1,6-dioxohexamethylene]) in the organic phase of the emulsion. Addition of PEG 3000 to the formulations improved the visual appearance of the dry product and the corresponding suspensions. Quenching of the emulsions by freezing in dry ice/methanol prior to freeze-drying was not necessary. The emulsion may be stirred for 20 min before filling into vials and freezing on prefrozen shelves in the freeze-dryer.

Process parameters for homogenising and freeze-drying should be optimised with regard to each single system, due to the different physico-chemical properties of the different solvents.

Multivariate analyses, such as PCA and PLS, were important methods for revealing systematic information from the large dataset with many variables. These methods are powerful tools in the development of new pharmaceuticals.

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