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STRUCTURAL ANALYSIS OF *W/O/W* MULTIPLE EMULSIONS BY MEANS OF DSC

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The properties of the inner and the external aqueous phases, were studied in *w/o/w* multiple emulsions with light microscopic image analysis and differential scanning calorimetry (DSC).

The importance of multiple emulsions lies in the presence of these aqueous phases, making them available for sustained, controlled drug delivery systems. Differentiation of these two aqueous phases, studying the effect of manufacturing technology on droplet structure, quantitative determination of phase volumes and any changes occurring during storage are essential when planning *w/o/w* emulsions. The present study uses microscopic observations combined with DSC measurements in order to identify the formed structure, at developmental stage in case of different components, preparation methods, and stirring rates. These tools are beneficial during manufacturing as in process controls, or to ensure product quality.

Keywords: *different polarity oils, droplet size analysis, DSC, formulation, w/o/w multiple emulsions*

Introduction

Multiple *w/o/w* emulsions are composed of aqueous droplets, which are dispersed inside oily drops. These oily drops are themselves dispersed in an external aqueous phase (Fig. 1). There are two interfaces in these complex systems. A hydrophobic emulsifier with a low HLB value stabilizes the first one, and a hydrophilic emulsifier with a high HLB value is present on the second interface.

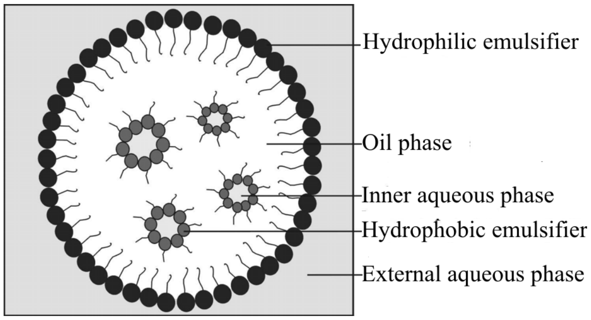

Fig. 1 Schematic representation of *w/o/w* multiple emulsions; structure of a multiple emulsion droplet showing the role of the various components

W/o/w emulsions have numerous advantages arising from their structure, achieving e.g. sustained drug delivery and simultaneous use of active ingredients incompatible with each other. They are widely used both in cosmetic and in pharmaceutical industry.

Studying their complex structure is quite a difficult task, which entails great care. The presently available methods give information about the presence of the multiple droplets, about their structure and size distribution directly or indirectly. Microscopic techniques can be used as direct method for droplets analysis; indirect methods involve e.g. use of indicator compounds, rheological measurements [1].

The aim of the present study was to apply, adopt and combine different methods, which are well known, but not widely used for emulsion systems, specially not in case of multiple ones. Microscopic observations combined with differential scanning calorimetry (DSC) measurements were used in order to identify the formed structure, at developmental stage in case of different components, preparation methods, and stirring rates.

Thermoanalytical examinations have increasing role in structural investigation of different pharmaceutical substances and dosage forms, including multiple emulsions [2–6].

The majority of the publications are limited to the examination of solid pharmaceuticals, the number of publications relating to semisolid medications and cosmetics is insignificant in comparison. Therefore our research group, in line with its research profile dating back to decades, performed thermogravimetric examinations to verify the microstructure of *o/w* creams and to detect the factors contributing to structure formation [7].

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The present experiments involve DSC as a method for the description of more complex, so-called secondary disperse and coherent structures. One of the most critical problems during studying the structure of *w*/*o*/*w* emulsions is the verification of the presence of the inner aqueous phase and its quantitative determination. The formation of this inner aqueous phase is influenced by several factors, such as the type of the emulsifier used, its concentration, the presence of a viscosity increasing agent, the manufacturing technology, nature of the oil phase and presence of active substance. The examination of the latter three factors was given special emphasis during our research. First the presence, type and size distribution of multiple droplets in the emulsions prepared with oils of different polarities were studied with light microscopic image analysis. The further structure examination was carried out with the DSC method. The compositions used are model ones, components widely accepted by the cosmetic and pharmaceutical industry were used.

Experimental

Materials

The oil phase was constituted by different kinds of oil derivatives of various polarities. Mineral oil derivatives: liquid petrolatum /Paraffinum liquidum/ (Paraffin oil, Gustav Hess GmbH, Ph.Eur.4th), 2,2,4,4,6,6,8-heptamethylnonane /Isohexadecane/ (Arlamol HD, Uniqema, Uniqema grade) and vegetable oil derivatives: avocado oil /Persea Gratissima/ (Symrise, Cosmetics grade), corn germ oil /Zea Mays/ (Naturol, Cosmetics grade) and esters: isopropyl myristate /Isopropyl Myristate/ (Oleon NV, Ph.Eur.4th), 2-ethylhexyl stearate /Octyl Stearate/ (Cetiol 868, Cognis, Ph.Eur.4th), (Table 1).

The surfactants used during the preparation of *w*/*o*/*w* emulsions were: poly(oxy-1,2-ethanediyl) distearate /Steareth-2/ (Brij 72, Uniqema, Uniqema grade), poly(oxy-1,2-ethanediyl) distearate /Steareth-21/ (Brij 721, Uniqema, Uniqema grade), polyoxyethylene (30) dipolyhydroxystearate /PEG-30 Dipolyhydroxystearate/(Arlacel P135, Uniqema, Uniqema grade), block copolymer of polyethylene oxide and polypropylene oxide /Poloxamer 407/ (Synperonic PE/F 127, Uniqema, Uniqema grade) (Table 2).

Table 1 Lipophilic agents used during the preparation of *w*/*o*/*w* emulsions

Lipophilic agents	mass/mass%	Polarity index[1]/ mN m^{-1}
Paraffinum liquidum	4.00	42.5
Isohexadecane	5.00	38.6
Isopropyl myristate	4.00	24.3
Octyl stearate	5.00	21.0
Avocado oil	4.00	11.5
Corn germ oil	5.00	9.6

[1] Polarity index: interfacial tension between oil and water

Viscosity increasing agents in external aqueous phase: sodium alginate (Manugel DJX, ISP Alginates Ltd., Cosmetics grade), carbomer (Carbopol Ultrez-10, BF. Goodrich, Ph.Eur.4th).

Six different compositions were prepared during the experiments (Table 3). Urea (Honeywell Co. Belgium, Ph.Eur.4th) was used as a model substance in the inner aqueous phase of composition 2/C (8 mass/mass%) in order to simulate the osmotic pressure change induced by the active ingredient, which influences the proportion of the inner aqueous phase and the stability of the multiple emulsion [8].

Methods

Emulsion preparation

Multiple *w*/*o*/*w* emulsions were formulated with the one-step and the two-step technology [2, 9, 10]. The oil phase containing the surfactant and the aqueous phase were heated separately to 75°C, in case of the one-step technology. The oil phase was then added to the aqueous phase. The emulsion was homogenized for 5 min and cooled down to 25°C with gentle stirring, and thus a multiple $w_1/o/w_1$ emulsion was obtained which was stabilized by the liquid crystal phase [11]. The stirring rates used were: 1000, 4000, 8000, 13500 rpm (BIOMIX LE-402/LABORMIM, Hungary, DI 25 IKA-VERKE GmbH, Germany).

The two-step technology started as follow: the simple w_1/o emulsion was prepared by adding the w_1 aqueous phase to the oil phase containing the hydrophobic surfactant. Both phases were heated separately to 75°C and then mixed. After the homogenization

Table 2 Surfactants used in the formulations

Emulsion	Surfactants			
	Stabilization of 1st interface		Stabilization of 2nd interface	
	name	HLB value	name	HLB value
one-step technology	Steareth-2	4.9	Steareth-21	15.5
two-step technology	PEG-30 dipolyhydroxystearate	5.0–6.0	Poloxamer 407	high (exact value not presented by the manufacturer)

Table 3 Components of the multiple emulsions

Manufacture	Ingredients of *w*/*o*/*w* emulsions					
	(1) one-step			(2) two-step		
	(A)	(B)	(C)	(A)	(B)	(C)
Oil phase						
Paraffinum liquidum	4.00	–	–	4.00	–	–
Isohexadecane	5.00			5.00		
Isopropyl myristate	–	4.00	–	–	4.00	–
Octyl stearate		5.00			5.00	
Avocado oil	–	–	4.00	–	–	4.00
Corn germ oil			5.00			5.00
Surfactants						
Steareth-2	3.00	3.00	3.00	–	–	–
Steareth-21	2.00	2.00	2.00	–	–	–
PEG-30 dipolyhydroxystearate	–	–	–	4.00	4.00	4.00
Poloxamer 407	–	–	–	2.00	2.00	2.00
External aqueous phase						
Viscosity increasing agents						
Sodium alginate	0.30	0.30	0.30	–	–	–
Carbomer	–	–	–	0.30	0.30	0.30
Model substance						
Urea	–	–	–	–	–	8.00

process (5 min at 1000–13500 rpm), the emulsion was cooled down to room temperature with gentle stirring. This w_1/o emulsion was dispersed – at a low stirring rate (500 rpm) – in the w_2 aqueous phase at room temperature.

Light microscopic image analysis

A computerized image analysing device was used for the microscopic observations, which was connected to a light microscope (LEICA Q500MC Image Processing and Analysis System, Leica Cambridge Ltd, UK). The type and size distribution of the multiple emulsion droplets were examined at 100× magnification. The oil droplets and the inner water droplets were counted and the diameter of droplets was determined in all of the cases.

Thermoanalytical measurements

Thermoanalytical measurements were performed with a DSC821^e (Mettler-Toledo GmbH, Switzerland) DSC instrument. The samples were first cooled down from 25 to –60°C, and then they were heated steadily up to 25°C in hermetically sealed aluminum pan. The heating/cooling rate was 5°C min^{-1}. The mass of the samples was 10±1 mg, the measurements were performed in a nitrogen medium. The peak areas were evaluated with STAR^e Software.

Results and discussion

Light microscopic image analysis

The light microscopic images revealed that the type of the multiple systems and the size of the droplets depended on the preparation method. Florence and Whitehill classified multiple droplets into three groups. Type A droplets contain one single inner droplet. In type B droplets there are several small inner droplets independent of each other, while type C droplets are characterized by a great number of inner droplets, which are in interaction with each other [12]. The microscopic images showed two types of multiple emulsions: in the case of one-step emulsions several small inner drops of water were seen in the oil droplets (type B), while in two-step emulsions only one greater inner water droplet could be seen (type A). Droplet size decreased with the increase of the stirring rate used during preparation. The average diameter of oil droplets in one-step emulsions varied between 5–30 μm, while the diameter of inner drops of water ranged between 0.5–1.5 μm. On the other hand, the diameter of the oil droplets in two-step emulsions ranged between 5–8 μm – depending on the stirring rate – and an inner drop of water with a greater diameter of about 0.8–2.8 μm could be seen (Figs 2a and b).

Moreover, the microscopic examination revealed that in compositions prepared with Paraffinum liquidum and isohexadecane (samples: 1A and 2A) – non-polar lipophilic substances – the number of multiple droplets was considerably lower, thus these were not examined with DSC.

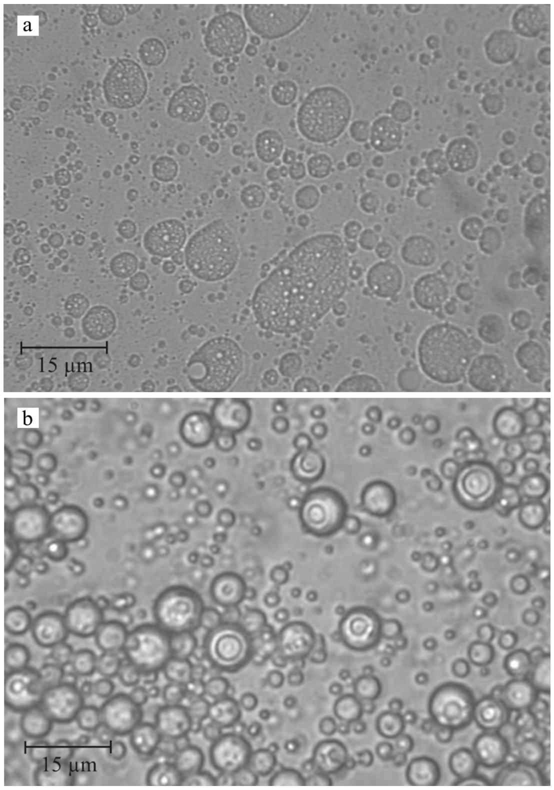

Fig. 2 Microscopic photograph of multiple emulsion made by a – one-step and b – two-step procedures. Magnification 100× was used in the microscopic observation

DSC measurements

During the examination of the structure of *w*/*o*/*w* emulsions, the presence of the inner aqueous phase was deduced from the shape of the DSC curves [13]. This was indicated by the appearance of a second peak in the DSC curve and the area under the curve gives the extent of the total enthalpy change. In the case of one aqueous phase, only one peak can be seen in the DSC curve.

The mass fraction of the inner aqueous phase can be calculated from this value. The decrease of the area under the peak and therefore the decrease of the values of enthalpy change, and the disappearance of the second peak indicate, that inner water loss occurs during storage and multiple droplets breakdown [2, 3, 14].

The DSC measurement carried out in *w*/*o*/*w* emulsion samples with steady cooling demonstrated the presence of the two types of water, as the solidification of the external aqueous phase and that of the inner aqueous phase took place at different temperatures. Figure 3 shows two different exothermic peaks typical of *w*/*o*/*w* multiple emulsions.

During the thawing of the frozen sample, recorded between –60 and +25°C, only one endothermic signal is observed: all the water (inner and external) melts at 0°C.

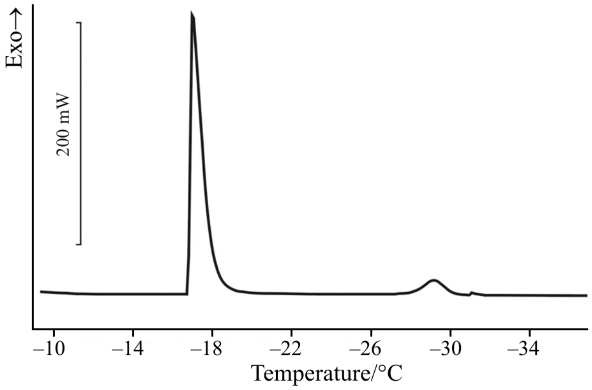

Fig. 3 Typical DSC curve of *w*/*o*/*w* multiple emulsion. The first peak represents external aqueous phase (–18°C) and the second peak shows the inner aqueous phase (–30°C)

During the evaluation of the results of the DSC measurements, the mass fraction of inner aqueous phase was calculated on the basis of the following equations:

$$\Delta h_c^{II}=[-443.17+4.5026T_c^{II}-(1.3832\cdot 10^{-2})(T_c^{II})^2+ +(1.5963\cdot 10^{-5})(T_c^{II})^3]\cdot 4.1868 \quad (1)$$

$$m_c^{II}=\Delta H_c^{II}/\Delta h_c^{II} \quad (2)$$

$$m_f=\Delta H_f/\Delta h_f \quad (3)$$

$$X_i^{CII}\ [\%]=m_c^{II}/m_f \quad (4)$$

where T_c^{II} [K] is the freezing temperature belonging to the 2nd peak (inner aqueous phase), $\Delta H_{c,norm.}^{II}$ [J g^{-1}] is the normalized freezing enthalpy of the inner aqueous phase, ΔH_c^{II} [J] is the freezing enthalpy of the inner aqueous phase, $\Delta H_{f,norm.}^{II}$ [J g^{-1}] is the normalized melting enthalpy, ΔH_f [J] is the melting enthalpy, m_c^{II} is the mass of the frozen water of inner aqueous phase, m_f is the mass of the melted water, Δh_c^{II} [J g^{-1}] is the latent heat of freezing of inner aqueous phase, Δh_f is the latent heat of melting, it is taken from literature (79.9 cal g^{-1} (334.5253 J g^{-1}) for pure water at 0°C), X_i^{CII} [%] is the mass fraction of the inner frozen aqueous phase.

Equation (1) describes the empiric relationship between the mass latent heat of freezing (Δh_c^{II}) and the freezing temperature of dispersed aqueous phase (T_c^{II}) in the undercooled region (223 K<T_c<273 K) [3].

Tables 4a, 4b, 5a and 5b present the values calculated with this relationship, where the temperatures measured in °C by the DSC equipment were converted into Kelvin (K).

The proper stirring rate can be chosen on the basis of the results presented in Tables 4a, 4b and 5a, 5b. The greatest enthalpy change (ΔH_c^{II}) was measured at the stirring rate of 8000 rpm both with the one-step and the two-step technologies, so the mass fraction of the inner aqueous phase (X_i^{CII}) was the greatest in this case.

Table 4 Mass of sample (m [mg]), specific freezing enthalpy data of inner aqueous phase ($\Delta H^{\mathrm{II}}_{\mathrm{m,c}}$ [J g^{-1}]), freezing enthalpy data of inner aqueous phase ($\Delta H^{\mathrm{II}}_{\mathrm{c}}$ [J]), freezing temperature data of inner aqueous phase ($T^{\mathrm{II}}_{\mathrm{c}}$ [°C]), value of specific melting enthalpy ($\Delta H_{\mathrm{m,f}}$ [J g^{-1}]), value of melting enthalpy (ΔH_{f} [J g^{-1}]) and mass fraction of inner aqueous phase after freezing ($X^{\mathrm{CII}}_{\mathrm{i}}$ [%]) in case of one-step emulsion

Table 4a Isopropyl myristate (IPM), octyl stearate (cetiol)

rpm	Technology: 'one-step' *w*/*o*/*w* emulsion						
	Ingredients: IPM–cetiol						
	m/mg	$\Delta H^{\mathrm{II}}_{\mathrm{m,c}}$/J g^{-1}	$\Delta H^{\mathrm{II}}_{\mathrm{c}}$/J	$T^{\mathrm{II}}_{\mathrm{c}}$/°C	$\Delta H_{\mathrm{m,f}}$/J g^{-1}	ΔH_{f}/J	$X^{\mathrm{CII}}_{\mathrm{i}}$/%
1000	10.43	4.14	0.0432	–45.30	176.72	1.8432	3.50
4000	10.29	5.81	0.0598	–45.35	176.70	1.8182	4.92
8000	10.11	7.30	0.0738	–45.13	166.26	1.6809	6.54
13500	10.30	3.12	0.0321	–45.15	172.67	1.7785	2.69

Table 4b Avocado oil–corn germ oil

rpm	Technology: 'one-step' *w*/*o*/*w* emulsion						
	Ingredients: avocado oil–corn germ oil						
	m/mg	$\Delta H^{\mathrm{II}}_{\mathrm{m,c}}$/J g^{-1}	$\Delta H^{\mathrm{II}}_{\mathrm{c}}$/J	$T^{\mathrm{II}}_{\mathrm{c}}$/°C	$\Delta H_{\mathrm{m,f}}$/J g^{-1}	ΔH_{f}/J	$X^{\mathrm{CII}}_{\mathrm{i}}$/%
1000	10.30	1.85	0.0191	–46.29	211.01	2.1734	1.33
4000	10.92	1.04	0.0114	–45.71	192.87	2.1061	0.81
8000	10.09	2.10	0.0212	–45.46	223.06	2.2507	1.41
13500	10.83	0.84	0.0091	–45.63	198.58	2.1506	0.64

Table 5 Mass of sample (m [mg]), specific freezing enthalpy data of inner aqueous phase ($\Delta H^{\mathrm{II}}_{\mathrm{m,c}}$ [J g^{-1}]), freezing enthalpy data of inner aqueous phase ($\Delta H^{\mathrm{II}}_{\mathrm{c}}$ [J]), freezing temperature data of inner aqueous phase ($T^{\mathrm{II}}_{\mathrm{c}}$ [°C]), value of specific melting enthalpy ($\Delta H_{\mathrm{m,f}}$ [J g^{-1}]), value of melting enthalpy (ΔH_{f} [J g^{-1}]) and mass fraction of inner aqueous phase after freezing ($X^{\mathrm{CII}}_{\mathrm{i}}$ [%]) in case of one-step emulsion

Table 5a Isopropyl myristate (IPM), octyl stearate (cetiol)

rpm	Technology: 'two-step' *w*/*o*/*w* emulsion						
	Ingredients: IPM–cetiol						
	m/mg	$\Delta H^{\mathrm{II}}_{\mathrm{m,c}}$/J g^{-1}	$\Delta H^{\mathrm{II}}_{\mathrm{c}}$/J	$T^{\mathrm{II}}_{\mathrm{c}}$/°C	$\Delta H_{\mathrm{m,f}}$/J g^{-1}	ΔH_{f}/J	$X^{\mathrm{CII}}_{\mathrm{i}}$/%
1000	10.35	1.36	0.0141	–34.54	277.65	2.8737	0.65
4000	11.30	1.42	0.0160	–37.80	268.90	3.0386	0.72
8000	11.05	1.63	0.0180	–35.21	277.30	3.0642	0.78
13500	10.66	1.03	0.0110	–38.22	270.51	2.8836	0.52

Table 5b Avocado oil–corn germ oil

rpm	Technology: 'two-step' *w*/*o*/*w* emulsion						
	Ingredients: avocado oil–corn germ oil						
	m/mg	$\Delta H^{\mathrm{II}}_{\mathrm{m,c}}$/J g^{-1}	$\Delta H^{\mathrm{II}}_{\mathrm{c}}$/J	$T^{\mathrm{II}}_{\mathrm{c}}$/°C	$\Delta H_{\mathrm{m,f}}$/J g^{-1}	ΔH_{f}/J	$X^{\mathrm{CII}}_{\mathrm{i}}$/%
1000	10.34	1.95	0.0202	–30.46	262.27	2.7119	0.94
4000	10.32	2.58	0.0266	–32.17	273.74	2.8250	1.21
8000	10.93	2.93	0.0320	–29.62	254.30	2.7795	1.45
13500	10.43	2.39	0.0249	–31.66	268.56	2.8011	1.14

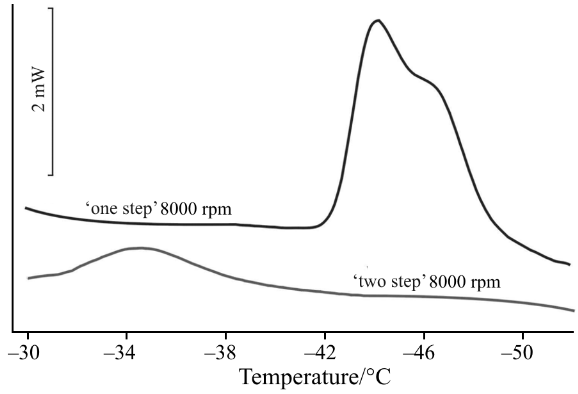

Fig. 4 DSC curve of *w*/*o*/*w* emulsion containing isopropyl myristate (IPM) and octyl stearate (cetiol) ester made by different preparation methods; curves indicate the crystallisation of the inner aqueous phase

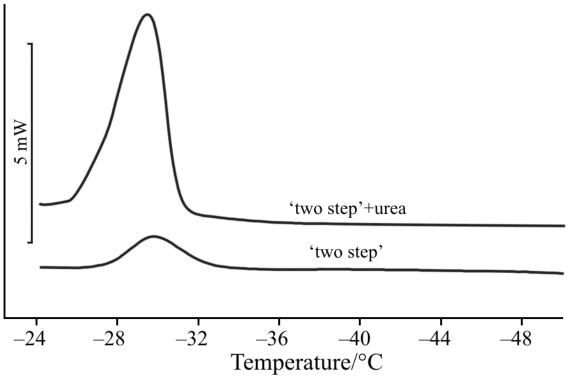

Fig. 5 DSC curve of a multiple emulsion containing avocado oil and corn germ oil with and without urea indicating the freezing of the inner aqueous phase

The shape of the thermoanalytical curves (Fig. 4) is in agreement with the droplet structure seen in the light microscopic images. While in the case of the one-step technology the second peak, indicating the presence of the inner aqueous phase, appeared at about –45°C, in the case of the two-step technology it could already been detected between –30 and –40°C, which is due to the size difference of water droplets, since smaller droplets freeze at lower temperatures [15].

Figure 5 shows how the presence of the dissolved urea influenced the dynamic equilibrium of the *w*/*o*/*w* multiple emulsions. Upon the effect of the dissolved material in the inner aqueous phase, the equilibrium between the inner and external aqueous phases changed as an osmotic pressure difference arose between them. The increase of the second peak indicated the migration of the external aqueous phase into the inner aqueous phase.

The DSC method was used also for studying the stability of the formed systems. The enthalpy data of the inner aqueous phase was determined in urea containing emulsions (sample: 2C) 1 h, 2 weeks, and then 1, 3, 6 months after preparation.

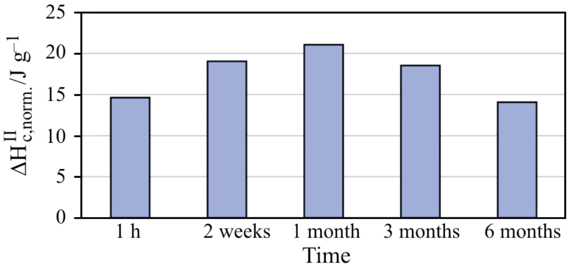

Fig. 6 Normalized enthalpy data (determined by DSC) of *w*/*o*/*w* multiple emulsion 2C (urea incorporated) made by the two-step technology measured after different storage time

The value of $\Delta H^{II}_{c,norm.}$ obtained during the DSC measurement increased with time for 1 month, and then it decreased, as it can be seen in Fig. 6. The reason for this is, that after 1 month, the water migration from the external water due to the dissolved material in the inner aqueous phase resulted in such a great increase in the diameter of the inner droplets that the swelling droplets bursted the oil membrane [16]. The breakage of the oil film led to the mixture of the two aqueous phases, thus the number of multiple droplets decreased, which was indicated by the decrease in enthalpy change.

Conclusions

It can be concluded, that multiple emulsions prepared with non-polar lipophilic agents contained much less multiple droplets than those ones containing polar ones, at constant surfactant concentration. This observation was confirmed by the microscopic analysis and the DSC studies either.

In the present multiple emulsion compositions DSC method was found to be adequate tool:

- Quantitatively measure the mass fraction of different aqueous phases,
- to detect the relationship between the stirring rate and the mass fraction of inner aqueous droplets,
- to give a feedback for the formulation technology, by characterizing the structure of multiple emulsions,
- to follow the microstructural changes occurring during storage.

DSC method should be combined with other methods for a complete quantitative examination of the mass fraction of the inner water phase. Simultaneous application of DSC method with microscopic examinations is a useful help in observing the structure of *w*/*o*/*w* emulsions [2]. The next phase of these experiments is, to analyse the rheological behaviour and the drug release process of these compositions.

The joint use of above mentioned methods provides a rapid and efficient procedure for the formulating expert, and the best composition and production technology can be chosen.

References

1 I. Erős, I. Csóka and J. Balázs, Gyógyszerészet, 37 (1993) 135.
2 J. L. Grossiord and M. Seiller (Eds), Multiple Emulsions: Structure, Properties and Applications, Editions de Santé, (1998).
3 S. Raynal, I. Pezron, L. Potier, D. Clausse, J. L. Grossiord and M. Seiller, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 91 (1994) 191.
4 L. Potier, S. Raynal, M. Seiller, J. L. Grossiord and D. Clausse, Thermochim. Acta, 204 (1992) 145.
5 P. Relkin, S. Sourdet and O.-Y. Fosseux, J. Therm. Anal. Cal., 71 (2003) 187.
6 A. Raemy, C. Appolonie Nouzille, P. Frossard, L. Sagalowicz and M.-E. Leser, J. Therm. Anal. Cal., 80 (2005) 439.
7 M. Kónya, M. Sorrenti, F. Ferrari, S. Rossi, I. Csóka, C. Caramella, G. Bettinetti and I. Erős, J. Therm. Anal. Cal., 73 (2003) 623.
8 D. Clausse, I. Pezron and A. Gauthier, Fluid Phase Equilib., 110 (1995) 137.
9 K. Pays, J. Giermanska-Kahn, B. Pouligny, J. Bibette and F. Leal-Calderon, J. Controlled Release, 79 (2002) 193.
10 Th. F. Tadros, C. Py, J. Rouviere, M. C. Taelman and P. Loll, SÖFW-Journal, 121 (1995) 714.
11 M. C. Taelman and P. Loll, Multiple emulsions in cosmetics ICI Surfactants, (1994) 2.
12 A. T. Florence and D. Whitehill, J. Colloid Interface Sci., 79 (1981) 243.
13 S. H. Gohla and J. Nielsen, SÖFW-Journal, 121 (1995) 707.
14 A. Kovács, M. Kónya, N. Zajc, I. Csóka, S. Srcic and I. Erős, Farm. Vestn., 54 (2003) 465.
15 D. Clausse, I. Pezron and L. Komunjer, Colloids Surf. A, 152 (1999) 23.
16 J. L. Grossiord and M. Seiller, S. T. P. Pharma Sci., 11 (2001) 331.

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