Trends in Chromatography

Original Communication

Characterization of a microemulsion system with AOT as pseudostationary phase in MEEKC for the analysis of estrogens

Sabrina Flor[1], Valeria Tripodi[2,3], Pablo Estevez[1] and Silvia Lucangioli[2,3,*]

[1]Department of Analytical Chemistry and Physicochemistry, [2]Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina,
[3]Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICET, Argentina

ABSTRACT

Microemulsion electrokinetic chromatography (MEEKC) is an electrodriven separation technique, which employs a microemulsion (ME) as pseudo-stationary phase (PSP) in capillary electrophoresis. In recent years, MEEKC has become an important tool for achieving the separation of a diverse range of solutes. Microemulsions are composed of nanometer-sized oil droplets suspended in aqueous buffer, which is commonly referred to as oil-in-water ME. The aim of this work was the characterization of a ME based on sodium bis(2-ethylhexyl)sulfosuccinate (AOT) as pseudostationary phase, developed for the simultaneous determination of natural and synthetic estrogens by MEEKC. The ME system consisted of 1.4% w/w AOT, 1.0% w/w octane, 7.0% w/w 1-butanol and 90.6% w/w 20 mM sodium salt of 3(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) and 10 mM phosphate buffer at pH 12.5. The AOT MEEKC system was characterized electrophoretically, physicochemically and morphologically and parameters such as microdroplets phase residence times (t_{mic}) and microdroplets proportion ($t_{prop,mic}$) were calculated for the first time in order to study the interaction between the solutes and the PSP. In order to characterize the AOT microemulsion regions, a phase diagram was constructed, and two regions, a W/O and another O/W were determined. Dynamic viscosity was determined and dynamic light scattering and transmission electron microscopy were performed to complete the characterization of the PSP.

KEYWORDS: microemulsion, AOT, pseudostationary phase, estrogens

*Corresponding author: Dr. Silvia Lucangioli, Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Buenos Aires, Argentina.
slucangi@ffyb.uba.ar

1. INTRODUCTION

Electrokinetic chromatography (EKC) is a mode of capillary electrophoresis (CE) where the selectivity is performed by the partition of the analytes between the mobile phase and a pseudostationary phase (PSP) as well as by their electrophoretic mobilities. In these systems, the mobile phase is normally an aqueous buffer and the PSP may be micelles (MEKC), vesicles (VEKC) or microdroplets (MEEKC) [1-2].

Microemulsion electrokinetic chromatography (MEEKC) uses a microemulsion as PSP and it was introduced by Watarai in 1991 [3]. Microemulsiones (MEs) are dispersed systems consisting of nanometer size droplets of an immiscible liquid, stabilized by a surfactant and/or co-surfactant molecule (short-chain alcohol). There are two principal types of MEs, oil-in-water and water-in-oil. MEs prepared as water droplets

in an oil phase are named O/W and MEs obtained as oil droplets in a water phase, W/O [1, 4-5].

The mechanism of separation of the compounds in MEEKC is based on the interaction between the analytes and the microdroplets. Therefore, differences in selectivity can be obtained in MEEKC systems using different types and concentrations of surfactants. The main advantage of using microemulsions as PSP is that they can be applied to the simultaneous analysis of structurally related compounds with different hydrophobicity in a single run [6-8].

MEs based on sodium dodecyl sulfate (SDS) as tensioactive agent are the most used MEEKC systems and their applications in various research fields have been reported [1]. However, separation of hydrophobic compounds may be unsuccessful [9]. In this case, for example, the use of tensioactive agents with double chain as phosphatidylcholine achieved a better selectivity with respect to traditional MEEKC system based on SDS [10-11].

In a previous work, we developed a MEEKC system using a novel ME based on sodium bis(2-ethylhexyl)sulfosuccinate (AOT) as tensioactive agent to determine natural and synthetic estrogens in pharmaceutical formulations [12]. AOT is a double chain, anionic and hydrophobic surfactant agent employed as vesicle in EKC [13] and as tensioactive agent in nonaqueous media [14].

In EKC system, the characterization of the PSP helps to understand the behavior of the analytes and their interaction with the PSP with special emphasis in the study of the relationship between retention and hydrophobicity. Different parameters have been reported to characterize the behavior of the PSP when it acts as a micellar system. These parameters have been previously evaluated for different MEKC systems [15-16] using a set of probe molecules.

To characterize the retention in MEEKC, parameters such as microdroplets phase residence times (t_{mic}) and microdroplets proportion ($t_{prop,mic}$) were determined in this work. t_{mic}, is the time spent by the analyte in the microdroplet phase and the $t_{prop,mic}$ characterizes the interaction between the analytes and the PSP.

The aim of this work was to characterize the developed MEEKC system in terms of retention, hydrophobicity and morphology, for the resolution of natural and synthetic estrogens. Dynamic light scattering analysis (DLS) was used to determine the diameter of the micelles and their morphology was characterized by transmission electron microscopy (TEM). To complete the characterization, parameters like dynamic viscosity and a phase diagram were also determined.

To our knowledge, this is the first time the characterization of a microemulsion system based on AOT as tensioactive agent is reported.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Acetophenone, propiophenone, butyrophenone, hexanophenone, octanophenone, dodecaphenone, estradiol (1,3,5(10)-estratiene-3,17-ßdiol) (E2), estradiol 17-hemisuccinate (E2-HS), estradiol 17-valerate (E2-V), estradiol 3-benzoate (E2-B), estriol (1,3,5(10)- estratiene-3,16α,17-ßtriol) (E3), estrone (1,3,5(10)-estratien-3-ol-17-one) (E1), etinilestradiol (17ß-ethylnyl-1,3,5(10)-estratiene-3,17-ßdiol) (Et-E2), bis (2-ethylhexyl) sodium sulfosuccinate (AOT), sodium dodecyl sulfate (SDS), sodium salt of 3-(cyclohexylamino)-2-hydroxy-1-propane-sulfonic acid (CAPSO) and1-((4-(fenildiazenil) fenil)diazenil) naftaleno-2-ol (Sudan III) were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide, sodium monohydrogen phosphate, hydrochloric acid, n-octane, 1-butanol and methanol were HPLC grade and supplied by E. Merck (Darmstadt, Germany). Ultrapure water was obtained from an EASY pure™ RF equipment (Barnstead, Dubuque, IA, USA). All solutions were filtered through a 0.45 µm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and degassed before use.

2.2. Instrumentation and electrophoretic conditions

Analysis was carried out with a P/ACE™ MDQ Capillary electrophoresis system (Beckman, Fullerton, CA, USA). Uncoated fused silica capillaries (Microsolv technology, Eatontown, NJ, USA) of 50 cm (40 cm length to the detector) and 75 µm i.d., were used. The capillary temperature was maintained at 25 °C, and UV detection was set at two different wavelength, 214 nm and 254 nm. Samples were injected under 0.5 psi pressure for 3 s and electrophoretic system was operated under positive polarity and a constant voltage of 18 kV.

The separations were performed using a AOT microemulsion system (MEEKC-AOT) consisting of 1.4% w/w AOT, 1.0% w/w octane, 7.0% w/w 1-butanol and 90.6% w/w 20 mM CAPSO and 10 mM phosphate buffer in a range of pH from 8.0 to 12.5.

A SDS microemulsion system (MEEKC-SDS) consisting of 1.44% w/w SDS, 0.81% w/w octane, 6.61% w/w 1-butanol and 91.14% w/w sodium phosphate buffer was adjusted to pH values in the range from 9.0 to 12.5. MEEKC-SDS system was prepared for comparison of CE systems.

2.3. Microemulsion preparation

The amounts of surfactants, octane and 1-butanol as indicated in section 2.2 were accurately weighted. Then SDS microemulsion was mechanically stirred while AOT based on microemulsion was manually stirred until complete dissolution. The buffer solution was then slowly added and clear systems were finally obtained. These microemulsion systems were allowed to stand at room temperature for 10 minutes before use.

2.4. Stock and standard solutions

Stock solutions of natural and synthetic estrogens and test molecules containing 1.0 mg/mL of each one were prepared in methanol. The selected test molecules were acetophenone, propiophenone, butirophenone, hexanophenone, octanophenone and dodecaphenone. Sudan III and methanol were used as microdroplet and EOF marker, respectively.

Standard solutions of 75 µg/mL of each estrogen and 10 µg/ml of each test molecule were obtained by appropriate dilution with 3 mM CAPSO-phosphate buffer.

2.5. Determination of characterization parameters

Microdroplets phase residence times and microdroplets proportion have been evaluated in order to study the selectivity of the proposed MEEKC system. These parameters were calculated for MEEKC-SDS and MEEKC-AOT systems through the adaptation of work reported for the characterization of micellar systems [15-16].

Briefly, microdroplets phase residence time, t_{mic} is calculated as follows:

$$t_{mic} = t_{mc} k'' \quad (1)$$

where t_{mc} is the migration time of the microdroplets and k'' is the normalized retention factor.

The normalized retention factor is calculated by the equation 2:

$$k'' = \frac{(t_m - t_0)}{(t_{mc} - t_0)} \quad (2)$$

where t_m is the migration time of the analyte and t_0 is the migration time of EOF.

Microdroplets proportion, $t_{prop,mic}$ is given by the following equation:

$$t_{prop,mic} = \frac{t_{mic}}{t_m} \quad (3)$$

The values of the calculated LogP (CLogP) used as estimates of the hydrophobibity of the test molecules and estrogens were taken from United States National Library of Medicine (HSDB) [17].

2.6. Dynamic viscosity

Viscosity measurement were performed at 25 °C ± 0.1 °C using a Brookfield digital viscometer MODEL DV-I+ (Middleboro, MA).

2.7. Phase diagram

The behavior of a phase system comprising an aqueous phase, an oil phase, a surfactant and a cosurfactant agent can be described through a tetrahedral diagram whose vertices represent the pure components of the mixture. However, it is more convenient to describe the behavior of these complex systems with the aid of pseudo-ternary phase diagrams. In order to construct the pseudoternary phase diagram, a fixed ratio of two of the three components (weight, volume or mole) must be established, whereas the third component is slowly added. Titrimetric method was employed to build the pseudoternary phase diagram. To this, buffer solution was added drop by drop to a surfactant-cosurfactant-oil mixture under stirring at 25 °C. Phase separation was visually detected by the appearance of cloudiness or clearly separated phases [18-19].

2.8. Transmission electron microscopy (TEM)

The morphology of the microemulsion based on AOT prepared by sonication and without sonication was studied by means of transmission electron

microscopy (Philips CM-12 TEM instrument, FEI Company, Eindhoven, The Netherlands). The sample was prepared according to Moretton *et al.* [20]. Briefly, samples of 5 µL of each microemulsion were placed on a grid covered with Fomvar film. After 30 s, the excess was carefully removed with filter paper and 5 µL of a 2% w/v uranyl acetate solution was added. After 30 s, the excess was removed and 5 µL of distilled water was added, maintained for 30 s, and removed. Finally, samples were dried in a closed container filled with silicagel and analyzed.

2.9. Dynamic light scattering (DLS)

The average hydrodynamic diameter (D_h) and size distribution of the microemulsion based on AOT system was performed using Zetasizer Nano ZS (Malvern Instruments, UK). The equipment includes a 4 mW, 633 nm He-Ne laser with a back-scattering detector (173 degrees). The particle hydrodynamic diameter was calculated from the translational diffusion coefficient (D) using the Stokes-Einstein relationship (4).

$$d_H = \frac{k_B T}{6\pi\eta D} \quad (4)$$

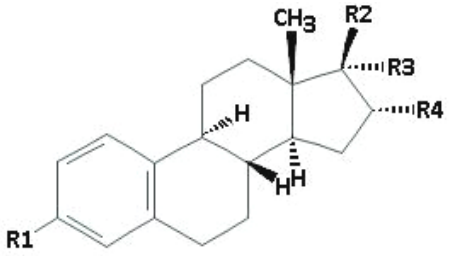
where d_H is the hydrodynamic diameter, k_B is Bolztmann's constant, T is the absolute temperature and η is the solvent viscosity. D_h was expressed as size distribution by number. Data are expressed as the average of at least six measurements. Samples were filtered (0.45 µm) prior to each assay.

3. RESULTS AND DISCUSSION

In a previous work, we presented a new method applied to the simultaneous determination of natural and synthetic estrogens by EKC using a novel ME based on AOT as tensioactive agent [12]. AOT is a double chain anionic highly hydrophobic tensioactive compound. In this work, the MEEKC-AOT system allowed the simultaneous and complete resolution of estriol, estradiol, estrona, ethynilestradiol, estradiol hemisuccinate, estradiol valerate and estradiol benzoate (Fig. 1) in a short time, with adequate stability, employing low AOT concentration and easy preparation.

3.1. Electrophoretic parameters

The selectivity of the proposed MEEKC-AOT system was compared with a MEEKC system using SDS as tensioactive agent. SDS is by far the mostly used tensioactive agent in MEKC and MEEKC. However, many works have demonstrated that SDS would be useless in the separation of some highly hydrophobic compounds [1, 9]. We compared both systems MEEKC-AOT and MEEKC-SDS at pH 9.0 to avoid the effect of the charge of the analytes (except E2Hs which is charged at alkaline pH values) and thus evaluate only hydrophobic interaction between the analytes and the microdroplets. First of all, the selectivity of each system was evaluated with a set of test molecules. By plotting the microdroplet time proportion ($t_{prop,mic}$) values vs. the hydrophobicity



	E1	E2	E3	E2-HS	E2-V	E2-B	E2
R1	OH	OH	OH	OH	OH	$C_7H_5O_2$	
R2	=O	OH	OH	$C_4H_5O_3$	$C_5H_9O_1$	OH	OH
R3		H	H	H	H	H	=CH
R4	H	H	OH	H	H	H	H

Fig. 1. Chemical structure of natural and synthetic estrogens.

(CLogP) (Fig. 2, Table 1), two different regions can be distinguished, one from CLogP 1.6 to CLogP 3.5, with a slope reaching the maximum value, and another from CLogP 3.5 to CLogP 6.8, with the slope approaching zero. This fact could imply that the ME´s had only an appropriate selectivity for analytes with a CLogP under a value of 3.5.

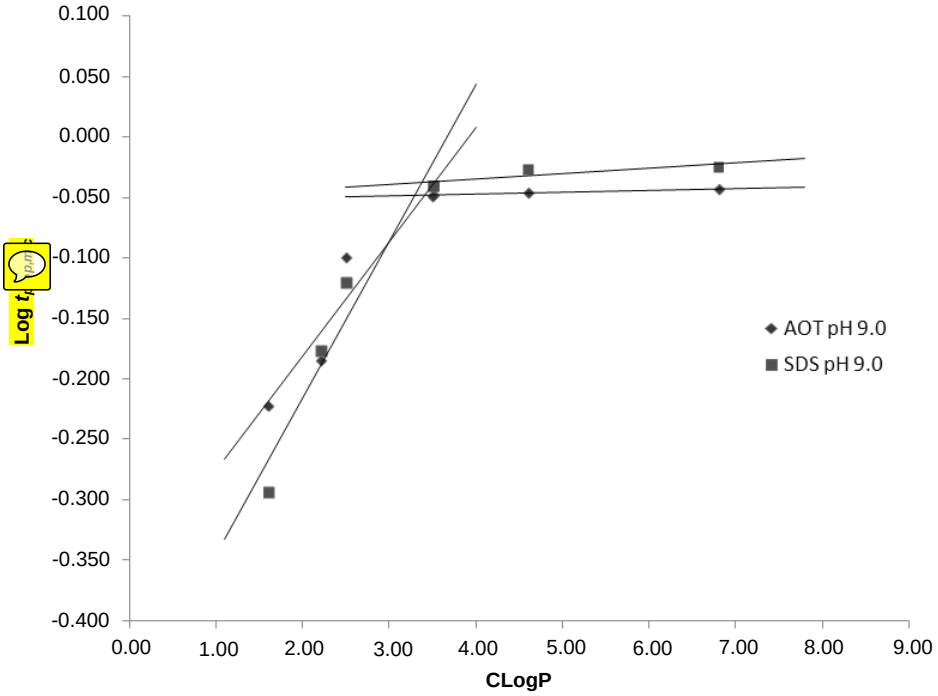


Fig. 2. Relationship between hydrophobicity (CLogP) and microdroplet times proportion ($t_{prop,mic}$) for test molecules calculated on Table 1.

Table 1. Comparison of electrophoretic parameters for test molecules.

AOT 9.0	CLogP	k''	t_{mic}	$t_{prop,mic}$	log $t_{prop,mic}$
Acetophenone	1.60	0.35	5.65	0.60	-0.222
Propiophenone	2.20	0.36	5.93	0.65	-0.185
Butirophenone	2.50	0.54	8.85	0.80	-0.099
Hexanophenone	3.50	0.72	11.78	0.89	-0.048
Octanophenone	4.60	0.73	11.97	0.90	-0.045
Dodecaphenone	6.80	0.75	12.16	0.91	-0.043
SDS 9.0					
Acetophenone	1.60	0.14	6.01	0.51	-0.294
Propiophenone	2.20	0.24	10.23	0.67	-0.177
Butirophenone	2.50	0.34	14.19	0.76	-0.120
Hexanophenone	3.50	0.62	26.28	0.91	-0.040
Octanophenone	4.60	0.72	30.33	0.94	-0.026
Dodecaphenone	6.80	0.74	31.26	0.95	-0.024

However, it can be observed that the behavior of the seven estrogens in both systems were quite different. The plots did not show two defined regions, which suggest that the interaction between the analytes and the microdroplets is not entirely due to hydrophobic interactions (Table 2).

Comparing both MEs systems, it was observed that AOT at pH = 9.0 was capable of resolving the seven estrogens, but the ME system based on SDS showed the same retention time for E2-V and E2-Bz, which means that both analytes spend exactly the same time inside the microdroplet ($t_{prop,mic}$ = 0.842) (Table 2).

Although MEEKC-AOT system at pH = 9.0 could solve the seven estrogens, the system was evaluated over a range of pH 8 to 12.5 in order to optimize selectivity and achieve complete baseline separation. Comparing the $t_{prop,mic}$ of each estrogen the effect of pH is noticeable, and therefore it demonstrates that the ionization of the analytes influences selectivity. The pKa values for these estrogens are in the range of 10.25 to 15.05 except for E2Hs which has a carboxylic group with a pKa value of 4.40 (Fig. 3).

The obtained results point that the interaction between the analytes and the microdroplets which leads to the separation is not given only by the hydrophobic interaction and the intrinsic mobility of the analytes, but instead it is the result of a combination of different types of interactions like hydrogen bond donor or acceptor.

3.2. Dynamic viscosity

The dynamic viscosity obtained was 1.85-1.90 Cpoise for ME based on AOT. A rheological profile performed showed that the ME behaves as a Newtonian fluid at high speeds (50, 60 and 100 rpm).

3.3. Phase diagram

ME based on AOT as delivery system has been widely reported [21-23]. In these applications, the AOT concentration is in the range of 30-60% w/w. However ME based on AOT are used in electrophoretic application at concentrations between 0.7 to 2.7% w/w, obtaining stable microemulsions [24]. In this case, the construction of the pseudoternary phase diagram shows two regions where it is possible to obtain a stable ME. One of the two regions corresponds to oil in water ME systems, which includes the proportion used as PSP, and the other corresponds to water in oil ME (Fig. 4).

Table 2. Comparison of electrophoretic parameters for natural and synthetic estrogens.

AOT pH 9.0	CLogP	k''	t_{mic}	$t_{prop,mic}$	log $t_{prop,mic}$
Estriol	2.50	0.282	6.311	0.611	-0.214
Estradiol hemisuccinate	4.00	0.396	8.867	0.724	-0.140
Estrona	3.10	0.371	8.311	0.702	-0.153
Etinyl estradiol	3.70	0.421	9.422	0.744	-0.129
Estradiol	4.00	0.376	8.422	0.707	-0.151
Estradiol benzoate	4.50	0.480	10.756	0.787	-0.104
Estradiol valerate	6.00	0.629	14.089	0.871	-0.060
SDS pH 9.0					
Estriol	2.50	0.35	7.73	0.678	-0.169
Estradiol hemisuccinate	4.00	0.51	11.33	0.804	-0.095
Estrona	3.10	0.47	10.53	0.780	-0.108
Etinyl estradiol	3.70	0.50	11.20	0.800	-0.097
Estradiol	4.00	0.49	10.93	0.792	-0.101
Estradiol benzoate	4.50	0.57	12.80	0.842	-0.075
Estradiol valerate	6.00	0.57	12.80	0.842	-0.075

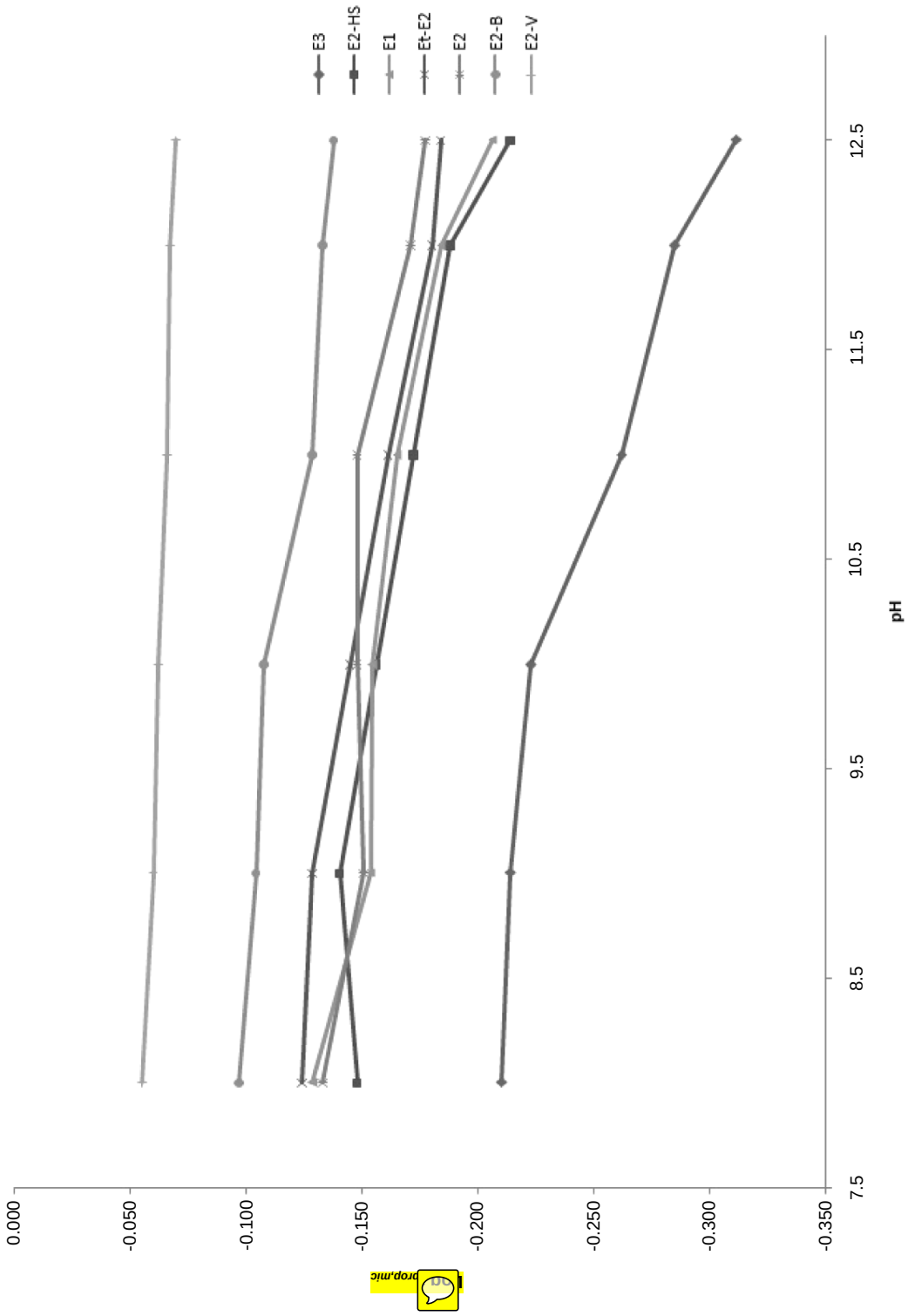


Fig. 3. Comparison of the pH effect in a range 8-12.5 on the $t_{prop,mic}$ of E1, E2, E3, E2-V, E2-HS, E2-B and Et-E2.

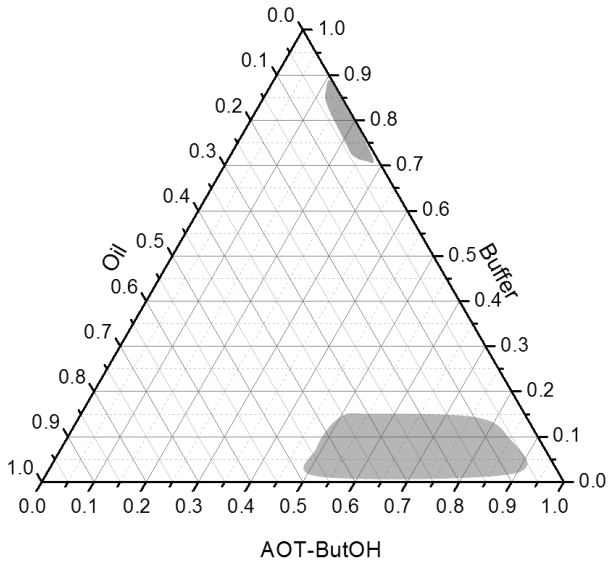


Fig. 4. Pseudoternary phase diagram.

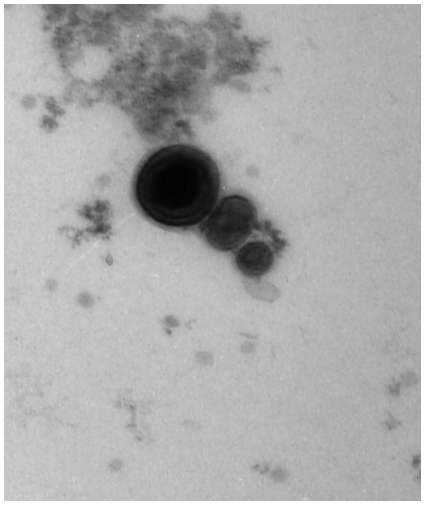


Fig. 5. ME prepared by manual stirring (30 kX).

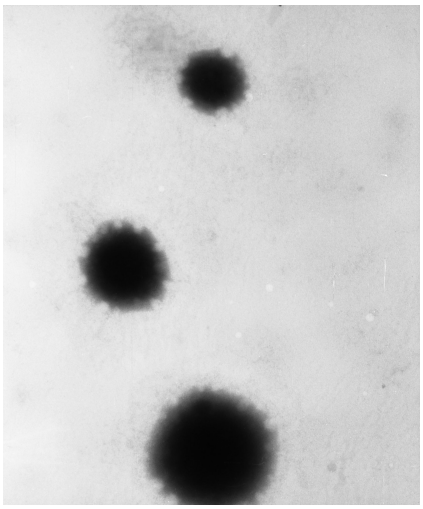


Fig. 6. ME prepared by sonication (30 kX).

3.4. TEM

The preparation of a ME usually consists in the dissolution by sonication of the hydrophobic tensioactive agent with the co-tensioactive and the oil phase. Afterwards, the aqueous phase is added to the mixture, drop by drop. But in the case of a ME based on AOT, significant differences were observed between a ME prepared by sonication, and a ME prepared by manual stirring. ME prepared by sonication was useless as PSP, due to the fact that the electrophoretic system was unstable, baseline was too noisy, and the peak shape was not good.

TEM images of ME sonicated and without sonication showed morphological differences between both microdroplets which could explain their different behavior as PSP's. The image of the ME without sonication showed a microdroplet with well-defined contours, in which a double layer distribution resembling a lipid membrane with a defined core can be clearly distinguished (Fig. 5). On the other hand, the microdroplets of the sonicated ME appear with irregular contours (Fig. 6).

3.5. DLS results

The hydrodynamic diameter of the microdroplets of the ME based on AOT without sonication was 12.5 nm and the diameter of the microdroplets obtained by sonication was 9.0 nm. These results were in agreement with those obtained by TEM.

4. CONCLUDING REMARKS

The electrophoretic, physicochemical and morphological characterization of a MEEKC system based on AOT as tensioactive agent is described for the first time. Using a set of test molecules, different parameters have been calculated not only for characterization of the MEEKC-AOT system but also for MEEKC using SDS as a tensioactive agent. A good correlation between t_{mic}, $t_{prop,mic}$ and hydrophobicity of test molecules has been demonstrated for both MEEKC-AOT and MEEKC-SDS systems, but only for test

molecules with a CLogP between 1.6 to 3.5. On the other hand, the estrogen groups showed significant differences in $t_{prop,mic}$ values in the CLogP range 2.5 to 6.0 specially in MEEK-AOT systems. This demonstrates the higher selectivity of MEEKC-AOT system with respect to MEEKC-SDS on molecules with high CLogP values. However, to optimize the resolution a higher pH value was necessary.

Pseudoternary phases diagram showed that the AOT microemulsion presented two regions where it is possible to obtain a stable ME. In addition, the diameter of the particles measured by DLS as well as the morphology presented by TEM could demonstrate that the ME based on AOT as tensioactive agent prepared by sonication had microdroplets with different shape and size which make them unsuitable as PSP in capillary electrophoresis.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Jorge Muse for assistance.

REFERENCES

1. Ryan, R., Altria, K., McEvoy, E., Donegan, S. and Power, J. 2013, Electrophoresis, 34, 159.
2. Tripodi, V. and Lucangioli, S. 2008, Microemulsions as pseudostationary phases in electrokinetic chromatoraphy: I. Estimation of physicochemical parameters. II. Analysis of drugs in pharmaceutical and biofluidics matrices. Progress in Colloid and Interface Science Series, Vol. 144: Microemulsion: Properties and Aplications. Ed. Taylor and Francis.
3. Watarai, H. 1991, Chem. Lett., 10, 391.
4. Trotta, M., Gallarate, M., Pattarino, F. and Carlotti, M. 1999, Int. J. Pharm., 190, 83.
5. Yu, L., Chu, K., Ye, H., Liu, X., Yu, L., Xu, X. and Chen, G. 2012, Trends in Analytical Chemistry, 34, 140.
6. Miola, M., Snowden, M. and Altria, K. 1998, J. Pharm. Biomed. Anal., 18, 785.
7. Marsh, A., Clark, B., Broderick, M. and Power, J. 2004, Electrophoresis, 25, 3970.
8. Huie, C. 2006, Electrophoresis, 27, 60.
9. Lin, J., Nakagawa, M., Uchiyama, K. and Hobo, T. 2002, Electrophoresis, 23, 421.
10. Lucangioli, S., Carducci, C., Scioscia, S. and Carlucci, A. 2003, Electrophoresis, 24, 984.
11. Lucangioli, S., Kenndler, E., Carlucci, A. and Tripodi, V. 2003, J. Phar. Biomed. Anal., 33, 871.
12. Tripodi, V., Flor, S., Carlucci, A. and Lucangioli, S. 2006, Electrophoresis, 27, 4431.
13. Klotz, W. L., Shure, M. R. and Foley, J. P. 2002, J. Chromatogr. A, 962, 207.
14. Pascoe, R. J. and Foley, J. P. 2003, Electrophoresis, 24, 4227.
15. Dobos, Z., Kiss, É., Hallgas, B., Kéri, G. and Idei, M. 2005, Electrophoresis, 26, 849.
16. Varga, A., Huszár, M., Dobos, Z., Kiss, É., Horváth, A. and Idei, M. 2009, Electrophoresis, 30, 1923.
17. http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
18. Fanun, M. 2010, Journal of Colloid and Interface Science, 343, 496.
19. Fu, X., Feng, F. and Huang, B. 2006, International Journal of Pharmaceutics, 321, 171.
20. Moretton, M., Glisoni, R., Chiappetta, D. and Sosnik, A. 2010, Colloids and Surfaces B: Biointerfaces, 79, 467.
21. Skodvin, T., Sjöblem, J., Saeten, J. O. and Gestblom, B. 1993, Journal of Colloid and Interface Science, 155, 392.
22. Trotta, M., Gasco, M. R. and Pattarino, F. 1990, Acta. Pharm Technology, 36, 226.
23. Varshney, M., Khanna, T. and Changez, M. 1999, Colloids and Surfaces B: Biointerfaces, 13, 1.
24. Bustamante-Rangel, M., Delgado-Zamareño, M. M, Sánchez-Pérez, A. and Carabias-Martinez, R. 2006, Journal of Chromatography A, 1125, 270.