ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 263–271

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Polyoxyethylene-$\Delta^{9,11}$-didehydrostearate and glycerol-polyoxyethylene-$\Delta^{9,11}$-didehydrostearate: two new components of the non-ionic emulsifier Cremophor® EL☆

Th. Meyer [a], D. Waidelich [b], A.W. Frahm [a,*]

[a] *Chair of Pharmaceutical Chemistry, Albert-Ludwigs-University, Albertstr. 25, D-79104 Freiburg im Breisgau, Germany*
[b] *Applied Biosystems, PE Germany GmbH, Paul-Ehrlich-Str. 17, 63225 Langen, Germany*

Received 14 January 2001; received in revised form 17 April 2002; accepted 28 April 2002

Abstract

The polyethoxylated heterogeneous components of the so far poorly characterised non-ionic emulsifier Cremophor® EL (polyoxyl 35 castor oil) (CrEL) were fractionated by cyclodextrin modified micellar electrokinetic capillary chromatography (CD-MEKC) combined with indirect UV detection. The resulting peaks were assigned to the corresponding components by delayed extraction matrix-assisted laser desorption/ionization time of flight mass spectrometry (DE–MALDI–TOF–MS) as detection device. In order to combine CE and MS the fractionating robot Probot was employed which enables both the online fractionation of the CE eluate on a MALDI target during the electrophoretic separation and the simultaneous dosage of the MALDI matrix. The obtained mass spectra were evaluated by comparing the residue masses of the homologue peak series of the polyethoxylates with the calculated residue masses of potential CrEL-components. The overlapping of homologue peak series with isobaric residue masses was detected by using the residue mass plot, the newly developed evaluation method. Combining theses techniques, both the first detailed structure elucidation and a semiquantitative analysis of the polyethoxylated CrEL-components was achieved. Together with the polyethoxylate series of yet elucidated structures two additional series were observed the corresponding components of which could not be identified at the beginning. Systematic investigations showed that the elimination of water from ricinoleic acid during the synthesis of the emulsifier leads to the polyethoxylates glycerol POE-$\Delta^{9,11}$-didehydrostearate and POE-$\Delta^{9,11}$-didehydrostearate so far unknown in CrEL.

Keywords: Cremophor EL; Non-ionic polyethoxylated surfactant; Capillary electrophoresis; Delayed extraction matrix assisted laser desorption/ionization time of flight mass spectrometry; Probot

1. Introduction

Cremophor® EL (CrEL), a heterogeneous polyethoxylated non-ionic surfactant, is obtained from the reaction of 1 mol castor oil (referring to

☆ Dedicated to Prof. Dr. W. Fleischhacker on the occasion of the 70th anniversary of his birthday.

* Corresponding author. Tel.: +49-761-203-6335; fax: +49-761-203-6351
E-mail address: august.wilhelm.frahm@pharmazie.uni-freiburg.de (A.W. Frahm).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00333-3

the postulated main component glycerol triricinoleate) and 35 mol ethylene oxide (EO) [1]. Müller who performed the first identification approach in 1966 [2] postulated glycerol polyoxyethylene (POE) ricinoleates, POE ricinoleates, glycerol POE ether and at least unsubsituted polyethylene glycol (PEG) to be components of CrEL. The exact composition especially of the ricinoleic acid containing esters was not given.

As CrEL is extensively used as solubilizer of hydrophobic drugs like the immunosuppressant ciclosporin (Sandimmun®) [3] but causes serious side effects [4–7], detailed information about both the chemical structures and the pharmakokinetic properties of its components were required. Therefore, we elaborated methods for the quantitation of CrEL in plasma of patients undergoing Sandimmun® therapy [8–10] as well as a cyclodextrin modified micellar electrokinetic capillary chromatography method (CD-MEKC) with indirect UV detection [11] for the fractionation of the CrEL-components. However, for an assignment of the CE peaks to the corresponding polyethoxylates the CE separation technique had to be combined with delayed extraction matrix-assisted laser desorption/ionization time of flight mass spectrometry (DE–MALDI–TOF–MS), generally a powerful method for the characterization of high-mass synthetics and biopolymers [12–15].

However, the application of DE–MALDI–TOF–MS to polymer systems containing varying end groups proved to be rather difficult [16,17], as a high number of polyethoxylated components leads to an overlapping of homologue peak series with isobaric residue masses. Therefore, our group developed two new evaluation techniques, the residue mass plot and the abundance plot, in order to detect such overlapping series [18].

Coupling CE with DE–MALDI–TOF–MS is not established yet since the so far described offline techniques are troublesome due to the hydrodynamic elution of the analytes, especially if the resolution of consecutive peaks is too small [19,20]. On the other hand, approaches towards online coupling are susceptible to interferences and so far not suitable for the commercial use [21–23]. We used the fractionating robot Probot which enables both the online fractionation of the CE eluate directly on a MALDI target without interrupting the CE separation and a simultaneous addition of the MALDI matrix solution [24,25].

This combination of CE and DE–MALDI–TOF–MS including the new evaluation techniques allowed the first detailed structure analysis of the CrEL-polyethoxylates [25] (Fig. 1). In this paper, we will focus on a semiquantitative analysis of these components and the structure elucidation of two polyethoxylates not described so far for CrEL.

2. Experimental

2.1. Chemicals

CrEL was donated by BASF AG (Ludwigshafen, Germany). Polyethylene glycol (PEG) 1000 was purchased from Aldrich-Chemie GmbH & Co KG (Steinheim, Germany). γ-Cyclodextrin (γ-CD, Cavamax® W8 Pharma) was of pharma grade (Wacker-Chemie GmbH, Burghausen, Germany) and sodium dodecylsulfate (SDS) of CE grade (BIO-RAD Laboratories GmbH, München, Germany). Phenobarbital and Phenobarbital-sodium (both pharma grade) were purchased from Synopharm GmbH (Barsbüttel, Germany). Water was bidestillated with a Seralpur Delta system (SERAL Erich Alhäuser GmbH, Ransbach-Baumbach, Germany). Methanol (MeOH) was of HPLC-grade (Janssen Chimica, Geel, Belgium). Sodium acetate (NaOAc) and 2,5-dihydroxybenzoic acid (DHB) were of analytical grade (Aldrich-Chemie GmbH & Co KG, Steinheim, Germany). All reagents and solvents were used without further purification.

2.2. CE and fractionation of the CE eluate

CE was performed on a Crystal 310 CE instrument (ATI Unicam GmbH, Kassel, Germany) with a high voltage power supply (0–30 kV). On-column detection was carried out with a Unicam 4225 UV detector in the indirect UV mode ($\lambda = 260$ nm). The running buffer contained

20 mM phenobarbital (corresponding to 0.5071 g phenobarbital sodium and 0.0012 g phenobarbital per 100 ml buffer), 10 mM SDS, and 20 mM γ-CD as aqueous solution (pH*10.0). The outlet buffer merely contained 20 mM phenobarbital as aqueous solution (pH*10.0). Untreated fused silica capillaries (50 μm internal diameter, 64.5 cm effective length, 80.0 cm total length) obtained from Polymicro Technologies (Phoenix, AZ, USA) were employed. New capillaries were first conditioned by flushing with 1 N NaOH for 10 min, 2 N HCl for 10 min, water for 2 min, and finally with running buffer for 20 min. During all conditioning steps a pressure of 1000 mbar was applied. Between runs, the capillary was flushed with 0.1 N NaOH (10 min, 2000 mbar) followed by running buffer (5 min, 2000 mbar). For the separation, a 8.0% (m/V) CrEL-solution was used, the injection was carried out with a pressure of 20 mbar during 0.2 min. The temperature of the capillary was maintained at 20 °C, the voltage during CE separation was adjusted to 15 kV resulting in a current of 6.4 μA. The run time was 20 min. Chromatographic data were collected by means of an UNICAM 4880 version 2.04 data system.

Online fractionation of the CE eluate was performed with the fractionating robot Probot. Controlling of the system was carried out with PROBOT control version 3.23 (both BAI GmbH,

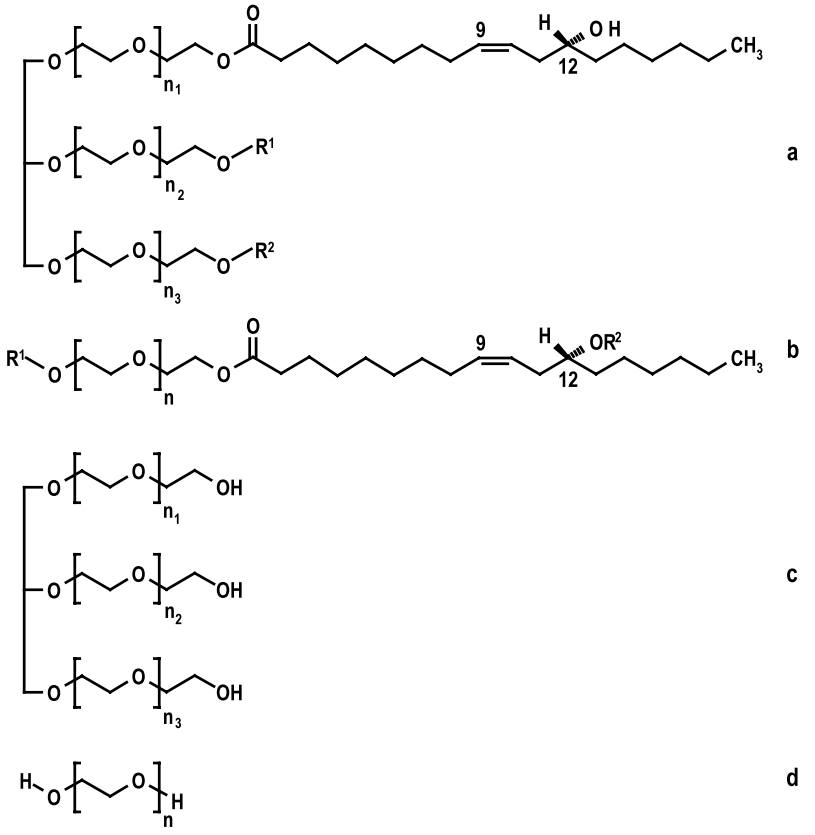

Fig. 1. CrEL-components identified by coupling CE with DE–MALDI–TOF–MS[25]. (a) Esters of ricinoleic acid and glycerol POE ethers: glycerol POE monoricinoleate ($R^1 = R^2 = H$); $n = n_1 + n_2 + n_3 = 29$; glycerol POE diricinoleate (R^1 = ricinoleate; $R^2 = H$); $n = n_1 + n_2 + n_3 = 28$; glycerol POE triricinoleate ($R^1 = R^2$ = ricinoleate); $n = n_1 + n_2 + n_3 = 30$. (b) Esters of ricinoleic acid and PEG: POE monoricinoleate ($R^1 = R^2 = H$); $n = 20$; POE diricinoleate (R^1 = ricinoleate; $R^2 = H$); $n = 16$; POE triricinoleate (R^1 = ricinoleate; R^2 = ethoxylated ricinoleate); $n = 23$. (c) Glycerol POE ether; $n = n_1 + n_2 + n_3 = 26$. (d) Polyethylene glycol; $n = 19$, glycerol POE monooleate (not shown); $n = 26$, POE monooleate (not shown); $n = 17$.

Lautertal, Germany). The matrix solution was manufactured by dissolving 750 mg DHB in 20 ml MeOH, mixing with 5.0 ml of a 1 mM solution of NaOAc and filling up with water to 50.0 ml. The fractionation of the CE eluate started with a delay time of 10 min, meanwhile the cannula with the capillary outlet dips in a vial filled with outlet buffer. The collecting time per sample point was 30 s, thus 20 fractions per run were collected. 1.0 μl of matrix solution were added per sample point (flow rate, 12 μl min^{-1}).

2.3. DE–MALDI–TOF–MS

DE–MALDI–TOF–MS was performed with the Voyager DE STR Workstation (Applied Biosystems, Framingham, MA, USA). The mass spectra were evaluated with DATAEXPLORER version 4.0.0.0 (Applied Biosystems, Framingham, MA, USA). The reflectron positive ion mode was used for all experiments. The total acceleration voltage was set to 20 kV, the voltage on the first grid to 68.5%. All experiments were carried out with a delay time of 200 ns between ion production and extraction. Six hundred single laser shots were accumulated for each mass spectrum.

3. Results and discussion

3.1. CE separation of CrEL and evaluation of the mass spectra

The CD-MEKC of CrEL yields the five peaks *CrEL* 1–5 (Fig. 2). The reason for the appearance of positive peaks (*CrEL* 4 and 5) despite of the low UV absorbance of the CrEL-components and the indirect UV detection mode is not fully understood yet. Probably, complexes are formed between CrEL-components, γ-CD, and the background absorber phenobarbital resulting in different UV profiles.

The homologue peak series in the mass spectra of the Probot fractions were assigned to the corresponding polyethoxylates [26] by comparing the residue mass of a potential component $m_{res,theo}$ (see Eq. (1)) with the experimental determined residue mass $m_{res,aver}$ of the series.

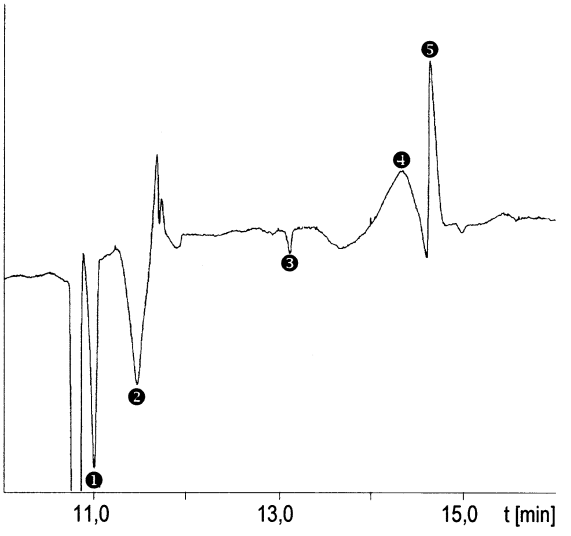

Fig. 2. CD-MEKC fractionation of CrEL ($w = 8.0\%$ (m/V)) with the Probot system as outlet. Inlet buffer: phenobarbital (20 mM)-SDS (10 mM)-γ-CD (20 mM) (pH*10.0). Outlet buffer: phenobarbital (20 mM) (pH*10.0). Capillary: total length 80.0 cm; effective length 64.5 cm. Voltage 15 kV; resulting current 6.3 μA. Separation temperature 20 °C. Indirect UV detection ($\lambda = 260$ nm) 1–5: CE peaks *CrEL* 1–5.

$$m_{res,theo} = m_{end,theo} + m_{cat} - n \cdot m_{mon,theo} \quad (1)$$

where $m_{end,theo}$ is the mass of the end group of a polyethoxylate, e.g. ricinoleic acid in case of POE ricinoleate, m_{cat} the mass of the sodium cation complexed by the polyethoxylate, $m_{mon,theo}$ the mass of a C_2H_4O-monomer (44.026215 Da), and n the number of this monomer leading to a $m_{res,theo}$ value between 0 and 44.026215 Da.

The experimental residue masses $m_{res,aver}$ were obtained by calculating the residue mass $m_{res,calc}$ of every single peak within a series by means of Eq. (2) [27,28].

$$m_{res,calc} = m_{signal} - n^* \cdot m_{mon,theo} \quad (2)$$

where m_{signal} is the mass of every single oligomer peak and n^* corresponds to n in Eq. (1). Finally, all $m_{res,calc}$ values are averaged leading to the mean $m_{res,aver}$.

The main requirement for the applicability of this calculation procedure and of the newly developed evaluation methods for overlapping series is a high mass accuracy achieved by internal calibration with polymer standards. However, the addi-

tion of an internal standard to the matrix solution completely suppressed the polyethoxylate series of the CrEL-components due to their low intensities.

Thus, a first measurement series was taken with internal monomeric calibration by means of the Na^+ adduct signal of the buffer component γ-CD ($m = 1319.4126$ Da). However, this one point calibration allows the determination of exclusively the nominal residue masses $m_{res,nom}$ of the series and not of $m_{res,aver}$ values. Therefore, the polymer series were indirectly assigned by comparing them with the corresponding polyethoxylate series of two CrEL-fractions previously obtained by cation exchange column chromatography the $m_{res,aver}$ values of which have already been determined [18]. In a second measurement series the Probot fractions on the MALDI target were spiked with the small amount of 10.0 ng PEG 1000 standard each in order to determine the $m_{res,aver}$ values of the series and thus to verify the results obtained from the $m_{res,nom}$ values. However, due to their very low intensities the Na^+ series of the minor components POE-triricinoleate and glycerol-POE monooleate could not be verified by $m_{res,aver}$ values and by the corresponding residue mass plots.

3.2. Structure elucidation of the new CrEL-components

Table 1 shows the assignment of the CrEL-components to the CE peaks identified with indirect UV detection. A multitude of components are observed in the range between the peaks *CrEL* 2 and 4, whereas the electropherogram only shows the weak peak *CrEL* 3 surrounded by the intervals 2/3 and 3/4. Probably, negative and positive peaks are overlapping each other in this range resulting in the baseline intervals 2/3 and 3/4, respectively.

POE monoricinoleate and glycerol POE monoricinoleate are the most conspicuous components since they appear in nearly all fractions. This behaviour is caused by their hydrophilicity on the one hand resulting in an elution together with the hydrophilic components PEG and glycerol POE ether. On the other hand, they are able to interact with the more hydrophobic polyethoxylates due to their lipophilic part ricinoleic acid. Thus, they are also contained in the CE peaks *CrEL* 3 and 4.

Furthermore, two components with the nominal residue masses 25 and 39 Da are observed (Table 1) with so far not assignable endgroups. A comparison of these series with the other CrEL-components showed that the series with the nomi-

Table 1
CrEL components contained in CE peaks identified by indirect UV detection

CE peak		CrEL component	
CrEL 1	Glycerol POE ether	Glycerol POE monoricinoleate	PEG
CrEL 2	POE monoricinoleate	Glycerol POE ether	PEG
CrEL 2/3[a]	POE monoricinoleate	Glycerol POE monooleate	
	POE monooleate	Glycerol POE monoricinoleate	
CrEL 3	POE triricinoleate	POE monoricinoleate	
	Glycerol POE monooleate	POE diricinoleate	
	POE monooleate	Glycerol POE monoricinoleate	
	Series with $m_{res,nom} = 25$ Da	Series with $m_{res,nom} = 39$ Da	
CrEL 3/4[a]	POE monooleate	Series with $m_{res,nom} = 39$ Da	
CrEL 4	POE monoricinoleate	Glycerol POE monooleate	
	POE monooleate	Glycerol POE monoricinoleate	
	Series with $m_{res,nom} = 39$ Da		
CrEL 5	Glycerol POE diricinoleate	POE diricinoleate	
	Glycerol POE triricinoleate		

[a] *CrEL* 2/3, 3/4 are the baseline interval zones between the peaks *CrEL* 2 and 3 and *CrEL* 3 and 4, respectively.

Table 2
Specifications of glycerol POE monoricinoleate, POE monoricinoleate and the two new components glycerol POE-$\Delta^{9,11}$-didehydrostearate and POE-$\Delta^{9,11}$-didehydrostearate

$m_{res,nom}$[a,b]	Component	Distribution range[a]; n[c]	Distribution max.[a]; n[c]
13	POE monoricinoleate	937–1422; 14–25	1113; 18
25	Glycerol POE-$\Delta^{9,11}$-didehydrostearate	1214–2050; 19–38	1610; 28
39	POE-$\Delta^{9,11}$-didehydrostearate	831–1580; 12–29	1095; 18
43	Glycerol POE monoricinoleate	1364–2068; 22–38	1628; 28

[a] Masses given in Da.
[b] $m_{res,nom}$ is the nominal residue mass of a series.
[c] n is the number of EO units.

nal residue mass of 25 Da resembles glycerol POE monoricinoleate ($m_{res,nom} = 43$ Da) and the series with the nominal residue mass of 39 Da resembles POE monoricinoleate ($m_{res,nom} = 13$ Da) concerning both the distribution range and the distribution maximum (Table 2). Furthermore, glycerol POE monoricinoleate and the series with $m_{res,nom} = 25$ Da differ in their residue masses by 18 Da. The same mass difference is observed between POE monoricinoleate and the series with $m_{res,nom} = 39$ Da provided that a shift of one EO unit is considered (13 Da + 44 Da − 39 Da = 18 Da). This difference of 18 Da exactly corresponds to the mass of a water molecule which could have been eliminated from ricinoleic acid in both cases. Thus, the series with $m_{res,nom} = 25$ Da can be assigned to glycerol POE-$\Delta^{9,11}$-didehydrostearate and the series with $m_{res,nom} = 39$ Da to POE-$\Delta^{9,11}$-didehydrostearate (Fig. 3). The stereochemistry of the resulting double bound in position 11 could not be elucidated, but the *trans*-configuration is most probable due to the mechanism of the elimination which is caused by the extreme conditions of the alkaline polymerization of CrEL. The $m_{res,aver}$ value of glycerol POE-$\Delta^{9,11}$-didehydrostearate could not be determined by internal calibration with PEG 1000 since the corresponding polyethoxylate series was completely suppressed. However, the $m_{res,aver}$ value of POE-$\Delta^{9,11}$-didehydrostearate (39.0621 Da) exactly correlates with the calculated residue mass ($m_{res,theo} = 13.0571$ Da + 44.0262 Da − 18.0106 Da = 39.0727 Da) and thus verifies the structural proposal.

3.3. Semiquantitative determination of the CrEL-components

The quantitative composition of CrEL was not known so far. Indeed, there was only a single identification and quantitation approach in 1966 based on saponification, consecutive extraction and thin layer chromatography [2]. This procedure merely allowed to estimate the content of the hydrophilic polyethoxylates as PEG and glycerol POE ether on the one side and of the ricinoleic acid containing polymers on the other side. CrEL was proposed to contain 7% PEG, 10% glycerol POE ether, 80% polyethoxylated ricinoleic acid esters, and finally 3% non-reacted castor oil.

Thus, there was a need for the quantitation of the content of the single CrEL-components especially in view of pharmacokinetic studies after the structures of all polyethoxylates contained in the Probot fractions had been elucidated. The method we have applied is based on the results obtained from the MALDI-TOF mass spectra of the Probot fractions. The content of a single component was calculated as follows:

The heights of the peaks of the corresponding series observed in the spectra of all Probot fractions were summarized. The resulting total height was divided by the sum of the total heights of all series representing all polyethoxylates. This procedure allows only a semiquantitative determination since differences in the ionization efficiency of components may occur depending on their non-ethoxylated end groups. However, DE–MALDI–TOF–MS is so far the only method allowing a differentiation between the multitude of polyethoxylates contained in CrEL.

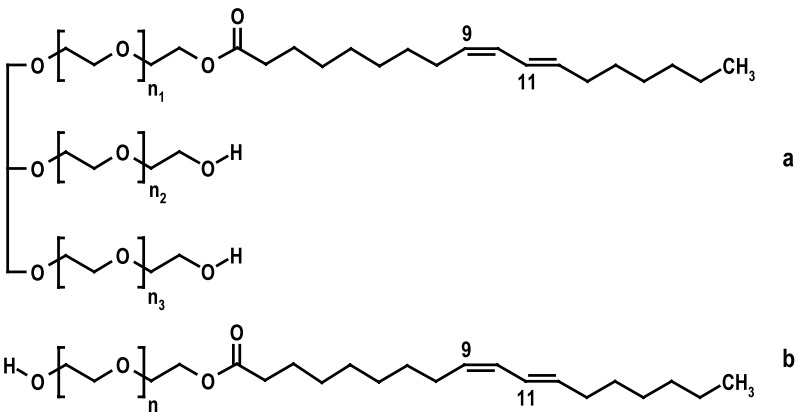

Fig. 3. Glycerol POE-$\Delta^{9,11}$-didehydrostearate (a) and POE-$\Delta^{9,11}$-didehydrostearate (b) originating from glycerol POE monoricinoleate and POE monoricinoleate by elimination of water during the production process of CrEL. The *trans*-configuration of the double bound in position 11 is not proved yet, but most probable due to the mechanism of the elimination reaction.

The results (Table 3) clearly indicate the monoesters glycerol POE monoricinoleate and POE monoricinoleate to constitute the main components of CrEL, whereas glycerol POE triricinoleate so far claimed as lead component by Müller and by the manufacturer BASF (see Section 1) does not really play an important role. However, Müller's estimate for CrEL with approximately 17% hydrophilic components i.e. PEG and glycerol POE ether could be roughly verified. Glycerol POE monooleate is also observed to a remarkable extent of 12.7% of the polyethoxylates in CrEL, although oleic acid is said to be only a minor component of genuine castor oil. Thus, oleic acid might be ethoxylated more easily than ricinoleic acid.

Furthermore, the results show that both the newly identified monoesters glycerol POE-$\Delta^{9,11}$-didehydrostearate and POE-$\Delta^{9,11}$-didehydrostearate must have been formed during the production process of CrEL to a significant extent of 5.4 and 4.6%, respectively.

4. Conclusions

This work describes the application of a new coupling technique between CE and DE–MALDI–TOF–MS for the separation of the heterogeneous non-ionic ethoxylated emulsifier CrEL. To this aim, the Probot system was used which enables both the online fractionation of the CE eluate on a MALDI target during the elec-

Table 3
Semiquantitative determination of the CrEL-components contained in the Probot-fractions

Component	$m_{res,nom}$[a]	Content w (%)[b]
POE triricinoleate	1	3.4
POE monoricinoleate	13	16.1
Glycerol POE diricinoleate	15	6.0
Glycerol POE-$\Delta^{9,11}$-didehydrostearate	25	5.4
Glycerol POE ether	27	11.0
Glycerol POE monooleate	27	12.7
POE diricinoleate	29	7.0
Glycerol POE triricinoleate	31	1.6
POE-$\Delta^{9,11}$-didehydrostearate	39	4.6
PEG	41	8.4
POE monooleate	41	6.8
Glycerol POE monoricinoleate	43	17.1

[a] $m_{res,nom}$ is the nominal residue mass of a series given in Da.

[b] The content of a component is calculated by the ratio of the total heights of the peaks of the corresponding series to the sum of the total peak heights of all series.

trophoretic separation and the simultaneous dosage of the MALDI matrix.

With this technique two new polyethoxylate series were observed in addition to those series the structures of which had been elucidated in a previous work [25]. The corresponding new components could be identified as glycerol POE-$\Delta^{9,11}$-didehydrostearate and POE-$\Delta^{9,11}$-didehydrostearate so far unknown in CrEL. These components originate from the elimination of water from the corresponding ricinoleic acid containing polyethoxylates during the alkaline polymerization of CrEL. The resulting double bound in position 11 is most probably *trans*-configured due to the elimination mechanism.

Furthermore, the evaluation of the MALDI-TOF mass spectra of the Probot fractions allowed a so far missing semiquantitative determination of the single CrEL components showing the monoesters glycerol POE monoricinoleate and POE monoricinoleate to constitute the main components of the emulsifier with a content of 33% in sum, whereas glycerol POE triricinoleate so far claimed as lead component by Müller [2] and by the manufacturer BASF is contained to a significantly less extent. With PEG and glycerol POE ether, CrEL contains approximately 17% hydrophilic components roughly verifying Müller's estimate [2].

In summary, for the first time the complete composition of CrEL with two new polyethoxylates was elucidated by hyphenated Capillary Electrophoresis and DE–MALDI–TOF–mass spectrometry using the fractionating robot Probot.

Acknowledgements

We gratefully acknowledge the financial support from Fonds der Chemischen Industrie (Frankfurt am Main, Germany). We also are thankful to Applied Biosystems GmbH (Langen, Germany) for extensive support in MALDI–TOF–MS and BASF AG (Ludwigshafen, Germany) for the generous gift of CrEL.

References

[1] BASF AG, Technisches Merkblatt: Cremophor®, EL, Ludwigshafen, 1987.
[2] K. Müller, Tenside 2 (1966) 37–45.
[3] E. Nürnberg, P. Surmann, Hagers Handbuch der Pharmazeutischen Praxis, fifth ed., Springer, Berlin/Heidelberg, 1991, p. 767.
[4] D. Dye, J. Watkins, Br. Med. J. 280 (1980) 1353.
[5] D.A. Moneret-Vautrin, C. Laxenaire, F. Viry-Babel, Br. J. Anaesth. 55 (1983) 469–471.
[6] L. Howrie, R.J. Ptachcinski, B.P. Griffith, R.J. Hardesty, J.T. Rosenthal, G.J. Burckart, R. Venkataramanan, Drug Intell. Clin. Pharm. 19 (1985) 425–427.
[7] H. Habazettl, B. Vollmar, F. Röhrich, P. Conzen, A. Doenicke, A. Baethmann, Anaesthesist 41 (1992) 448–456.
[8] M. Kunkel, J. Böhler, E. Keller, A.W. Frahm, Pharmazie 53 (1998) 314–321.
[9] M. Kunkel, T. Meyer, J. Böhler, E. Keller, A.W. Frahm, J. Pharm. Biomed. Anal. 21 (1999) 911–922.
[10] Th. Meyer, J. Böhler, A.W. Frahm, J. Pharm. Biomed. Anal. 24 (2001) 495–506.
[11] M. Kunkel, J. Böhler, E. Keller, A.W. Frahm, Pharmazie 52 (1997) 109–121.
[12] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299–2301.
[13] U. Bahr, A. Deppe, M. Karas, H. Hillenkamp, U. Gießmann, Anal. Chem. 64 (1992) 2866–2869.
[14] M. Karas, U. Bahr, A. Deppe, B. Stahl, H. Hillenkamp, U. Gießmann, Macromol. Symp. 61 (1992) 397–404.
[15] M.L. Vestal, P. Juhasz, S.A. Martin, Rapid Commun. Mass Spectrom. 9 (1995) 1044–1050.
[16] A.T. Jackson, H.T. Yates, W.A. MacDonald, J.H. Scrivens, G. Critchley, J. Brown, M.J. Deery, K.R. Jennings, C. Brookes, J. Am. Soc. Mass Spectrom. 8 (1997) 132–139.
[17] G. Montaudo, M.S. Montaudo, C. Pugliesi, F. Samperi, Rapid Commun. Mass Spectrom. 8 (1994) 981–984.
[18] Th. Meyer, M. Kunkel, A.W. Frahm, D. Waidelich, J. Am. Soc. Mass Spectrom. 12 (2001) 911–925.
[19] J. Chakel, E. Pungor, W. Hancock, S.A. Svedberg, J. Chromatogr. B 689 (1997) 215–220.
[20] G. Choudhary, J. Chakel, W. Hancock, A. Torres-Duarte, G. McMahon, I. Wainer, Anal. Chem. 71 (1999) 855–859.
[21] L. He, L. Liang, D.M. Lubman, Anal. Chem. 67 (1995) 4127–4132.
[22] S.Y. Chang, E.S. Yeung, Anal. Chem. 69 (1997) 2251–2257.
[23] J. Preisler, F. Foret, B.L. Karger, Anal. Chem. 70 (1998) 5278–5287.
[24] BAI GmbH, Manual: PROBOT—Sample robot for HPLC and CZE, version 3.2, Lautertal, 1999.
[25] T. Meyer, A.W. Frahm, D. Waidelich, Electrophoresis 23 (2002) 1053–1062.

[26] C.G. de Koster, M.C. Duursma, G.J. van Rooij, R.M.A. Heeren, J.J. Boon, Rapid Commun. Mass Spectrom. 9 (1995) 957–962.
[27] G.J. van Rooij, M.C. Duursma, R.M.A. Heeren, J.J. Boon, C.G. de Koster, J. Am. Soc. Mass Spectrom. 7 (1996) 449–457.
[28] S. Koster, M.C. Duursma, J.J. Boon, R.M.A. Heeren, J. Am. Soc. Mass Spectrom. 11 (2000) 536–543.