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Composition analysis of detergents of the polyoxyethylene type: comparison of thin-layer chromatography, reversed-phase chromatography and matrix-assisted laser desorption/ionization mass spectrometry

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Abstract

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can be used to determine the distribution of single polymer species of non-ionic detergents of the polyoxyethylene type (Triton X-100 and 114, Tween 20 and Brij 35). Thin-layer chromatographic (TLC) and reversed-phase chromatographic (RPC) methods are presented which may separate single polymer species as verified by MALDI-MS. Comparison of chromatographic and MALDI-MS data show Poisson-like distributions for Triton X-100 without marked differences between different methods. Distribution parameters obtained with Triton X-100 charges from different suppliers are very similar. The RPC method used can be scaled up for preparation of pure detergent species. © 1997 Elsevier Science B.V.

Keywords: Detergents; Polyoxyethelyene; Triton X-100; Triton X-114; Tween 20; Brij 35

1. Introduction

Non-ionic detergents of the polyoxyethylene type e.g., Triton X-100, Triton X-114, Nonidet, or of the Tween family belong to the tools most often used in protein biochemistry for solubilization of membrane proteins, for selective identification and traffic studies of GPI-proteins and as prerequisites for solubilization of water insoluble substrates for enzymes, especially for lipases [1]. Despite the overwhelming literature on detergent applications, micelle formation [2] and protein–detergent interactions [3], the known molecular heterogeneity [4] of this group of detergents is taken into account only superficially in practical as well as theoretical considerations [5–10]. To our knowledge, this is due mainly to the fact that at present there exists neither economical methods for producing pure single species nor practicable assays for determination of different molecular species within heterogeneous detergent populations.

MALDI-MS has been developed only recently [11] and has demonstrated its applicability for analytical investigations of nearly all polymeric compounds [12–15]. At present it seems especially interesting to increase the field of application of MALDI-MS by using this technique as an analytical tool for on-line [16] and off-line [17] follow-up of chromatographic procedures. In the present contribution it is shown that MALDI-MS is well suited for

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characterization of heterogeneous detergent preparations by comparison of this method with resolution of single molecular species by thin-layer chromatography (TLC) and reversed-phase chromatography (RPC).

2. Experimental

2.1. MALDI-MS

Mass determinations were performed with a LaserTec Research time-of-flight mass spectrometer (Perseptive Biosystems, Wiesbaden, Germany). The instrument is equipped with a nitrogen laser (λ=337 nm, pulse duration 3 ns, 20 pulses/s). Laser desorbed positive ions were analyzed in the reflectron mode after being accelerated by 20 kV. External calibration was performed using the ions $[M+H]^+$ and $[M+2H]^{2+}$ of melittin (M_r/z=2847.5 and 1424.25, respectively). The matrix solutions applied were: α-cyano-4-hydroxycinnamic acid and sinapic acid in 33% acetonitrile–H_2O containing 0.1% TFA and 2,5-dihydroxybenzoic acid in alcohol–water (1:1, v/v). α-Cyano-4-hydroxycinnamic acid produced the spectra with the best signal-to-noise ratio. Solutions of detergents (0.01–0.02%, w/v) in water or isopropanol were mixed with saturated solutions of the matrix (1:1, v/v). A 2-μl volume of the mixture was pipetted onto the roughened 2.5-mm diameter sample positions on a stainless steel plate. After drying at room temperature, the plate was inserted into the mass spectrometer.

2.2. Thin-layer chromatography (TLC)

Twenty-μl volumes of solutions of detergents in isopropanol (1–1.5 mg) were separated on TLC silica-gel 60 plates (0.25 mm thickness; Merck, Darmstadt, Germany). The TLC plates were developed in the upper phase of ethylacetate–isooctane–acetic acid–H_2O (13:2:3:10, v/v) [18]. After two runs with intermittent drying, spots were visualized by iodine vapour, scraped off the plates, extracted with 0.25 ml isopropanol and measured by their absorbances at 278 nm in a photometer DU 70 (Beckman, München, Germany). After drying in a Speedvac concentrator and addition of 50–100 μl H_2O, the mass spectrum of the molecules contained in each sample was determined by MALDI-MS.

2.3. Reversed phase-chromatography (RPC)

Detergent populations were separated by an HPLC apparatus (Type 6A, Shimadzu Corporation, Duisburg, Germany) using a silica-based Vertex column (Eurospher, Knauer, Berlin, Germany) with integrated guard column (250×4.6 mm I.D.). Particles of RP 100-C_{18} 5 μm in diameter with 100 Å pores were used as bonded phase. Data evaluation was done by a software package from Kontron (Berlin, Germany). A 100-μl volume of sample containing 100 μg detergent in water was injected. Runs were performed isocratically with 45% isopropanol in water containing 0.1% TFA, at a flow-rate of 0.4 ml/min at room temperature. Absorbance was measured at 270 nm.

2.4. Chemicals

Triton X-100 and Triton X-114 (both especially purified for membrane research), Tween 20 (aqueous solution 10%), Tween 80, Genapol X-080 and Nonidet P 40 (aqueous solutions 10%, especially purified for membrane research), sulfobetaine SB14, Mega 8 (detergents set 1124 714), Chaps (detergents set 1124 714), Chapso (detergents set 1124 714), Thesit (especially purified for membrane research, detergents set 1124 714), *n*-octylglucoside (detergents set 1124 714) were from Boehringer (Mannheim, Germany); Triton X-100 (for molecular biology), sinapic acid, >98% were from Fluka Chemie (Buchs, Switzerland); Triton X-100 (Sigma Ultra), Triton X-100 (Lot 16H1421), α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid were from Sigma Aldrich (Deisenhofen, Germany); Brij 35, melittin (research grade) were from Serva (Heidelberg, Germany).

All further reagents used were of analytical grade and either from Aldrich (Germany) or Fluka (Switzerland).

3. Results

3.1. Heterogeneity of detergents as determined by MALDI-MS, TLC and RPC

Results of MALDI-MS analyses of polyoxyethylene-type detergents are presented in Fig. 1. These detergents are very heterogeneous mixtures of polymer species containing different numbers of ethoxy groups. From the known stoichiometric schemes of these detergents (see Scheme 1), one identifies the main peaks as Na^+ adducts and their right neighbours as K^+ adducts. Observed and calculated masses agree within 0.08%. The Tween and Brij spectra are more symmetrical than those of the Triton family and exhibit additional peaks which correspond to Na^+ adducts of species containing two additional CH_2 groups. The Triton species contain markedly fewer ethoxy groups than Brij 35 and Tween 20, thus the peaks of their smallest species are overshadowed by matrix peaks. Besides polyoxyethylene type detergents, also the non-ionic detergents Mega 8 and *n*-octylglucoside and the zwitterionic detergents Chaps, Chapso and sulfobetaine SB 14 could successfully be analyzed by MALDI-MS (spectra not shown).

To test if detergent species with different numbers of ethoxy groups can be separated by chromatography, TLC and RPC were performed. The degree of resolution obtained was examined applying MALDI-MS to the fractions. TLC results for Triton X-100 are shown in Fig. 2a, the MALDI mass spectra of the TLC fractions are shown in Fig. 2b. Most of the TLC fractions contain unique polymer species except the

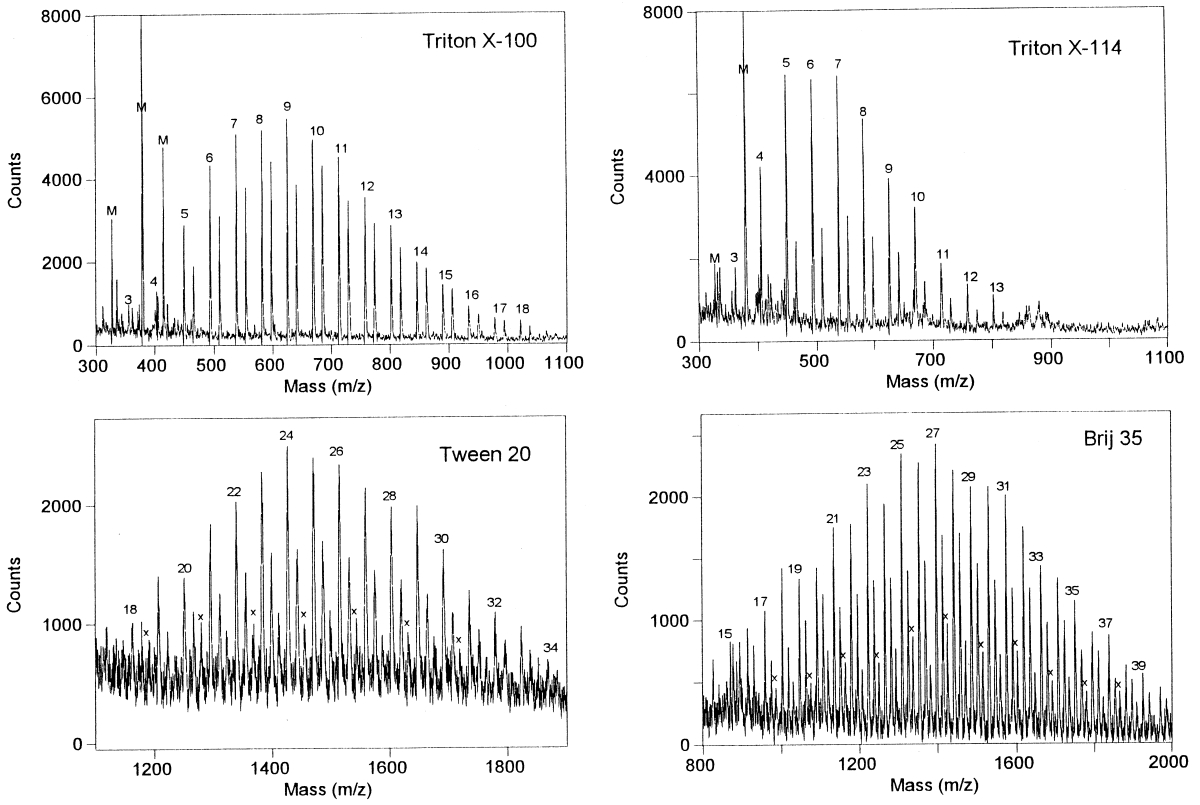
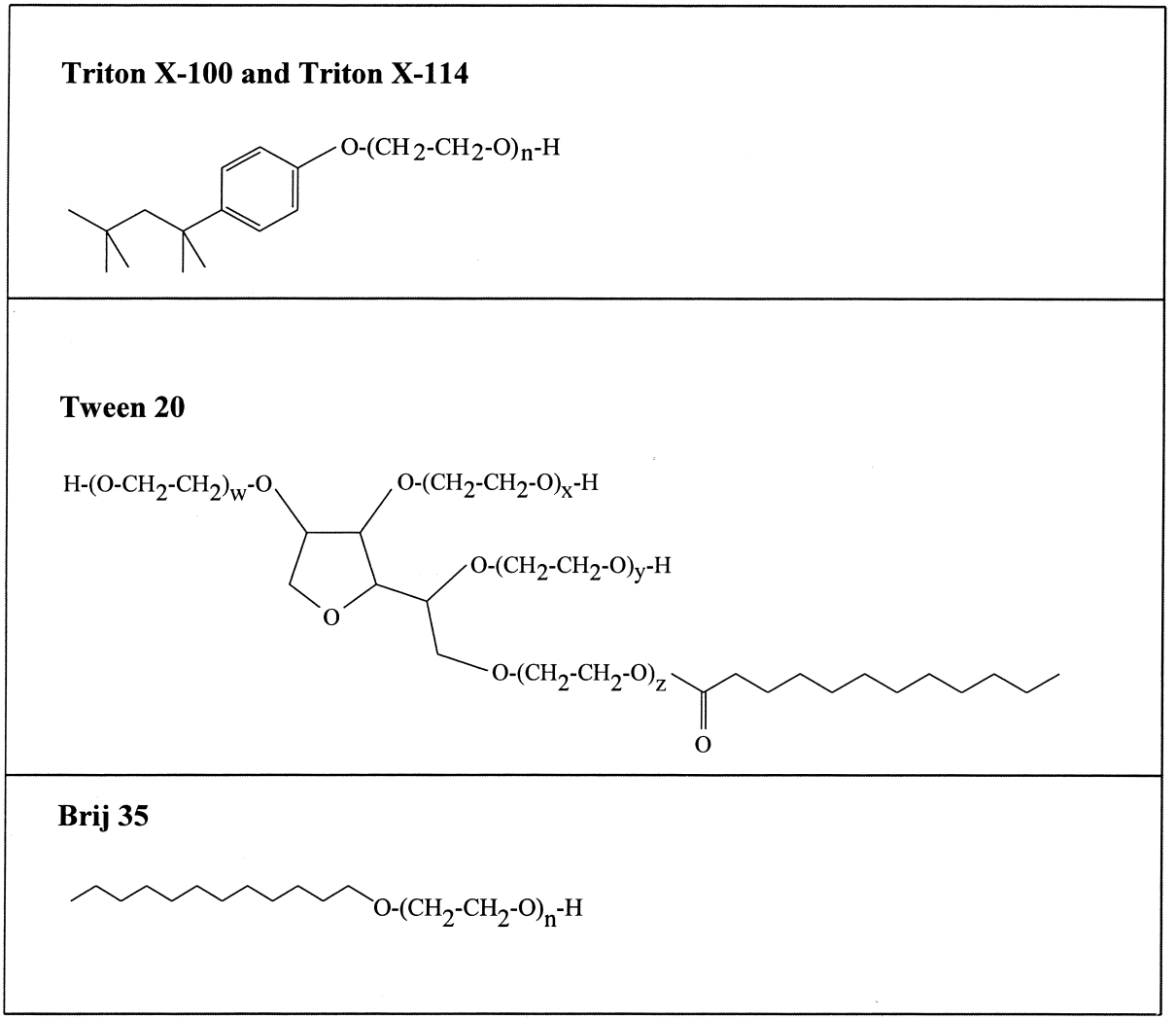


Fig. 1. MALDI mass spectra of different detergents. Peaks of the Na^+ adducts of detergent molecules are labeled by numbers indicating the number of ethoxy groups contained in the molecule. Their right neighbours belong to K^+ adducts. Peaks labeled by × correspond to Na^+ adducts of detergent molecules containing two additional CH_2 groups. In the Brij 35 and Tween 20 spectra, only every second peak has been labeled for clarity. M indicates peaks of matrix molecules and their decay products.



Scheme 1. Structure formulae for the detergents Tween 20, Triton X-100, Triton X-114 and Brij 35; *n* denotes the number of ethoxy groups contained in the respective detergent molecule. For Tween 20, $n=w+x+y+z$. Usually, detergents are delivered as mixtures of oligomers with different *n* values. For Triton X-100, the mean *n* is 10, for Triton X-114, the mean *n* is 7 (producer information).

first two fractions immediately after the start position. With Triton X-114, the situation is similar. The same method produced well separated chromatogram peaks also with the polyoxyethylene type detergents Genapol, Nonidet and Thesit (spectra not shown). As shown in Fig. 3, RPC is also suited for separation of detergent species. The MALDI mass spectra of the peak fractions demonstrate that species with 3–16 ethoxy groups per molecule are separated by RPC (mass spectra not shown). From the results it is seen that MALDI-MS can be used to detect the presence of molecules with different numbers of ethoxy groups in detergent populations as well as to examine the purity of chromatographic fractions.

3.2. Molecular mass distribution: comparison of different charges and of MALDI-MS, TLC and RPC results

Fig. 4 shows molecular mass distributions of

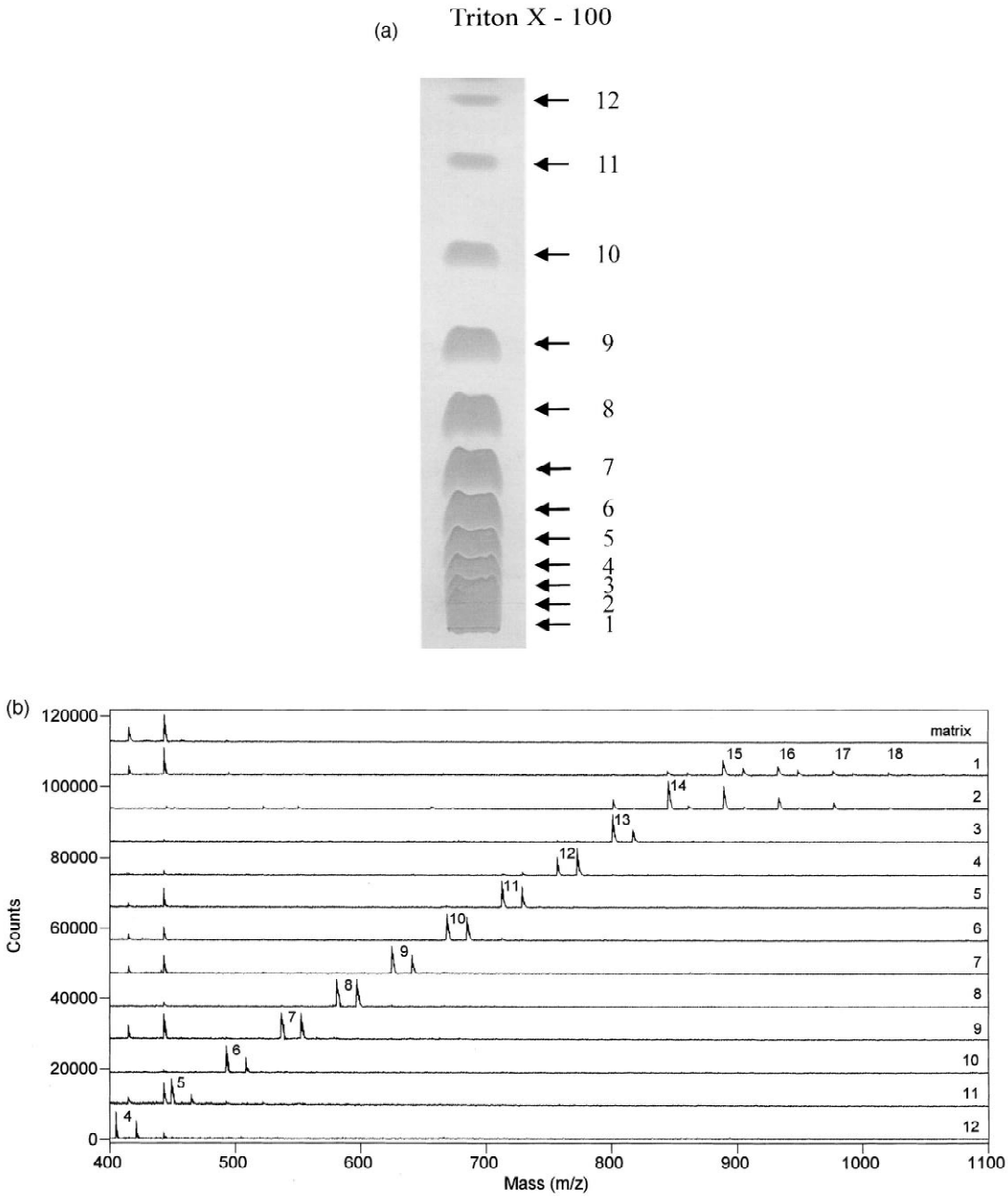


Fig. 2. TLC fractions and their mass spectra. (a) TLC of Triton X-100. For obtaining fractions for MALDI-MS analysis, the spots labeled by numbered arrows were totally scraped off the TLC plates and eluted (cf. Section 2.2). (b) MALDI mass spectra of Triton X-100 fractions; fraction numbers are indicated at the right ordinate; numbers of ethoxy groups are indicated at the corresponding pairs of Na^+ and K^+ adduct peaks.

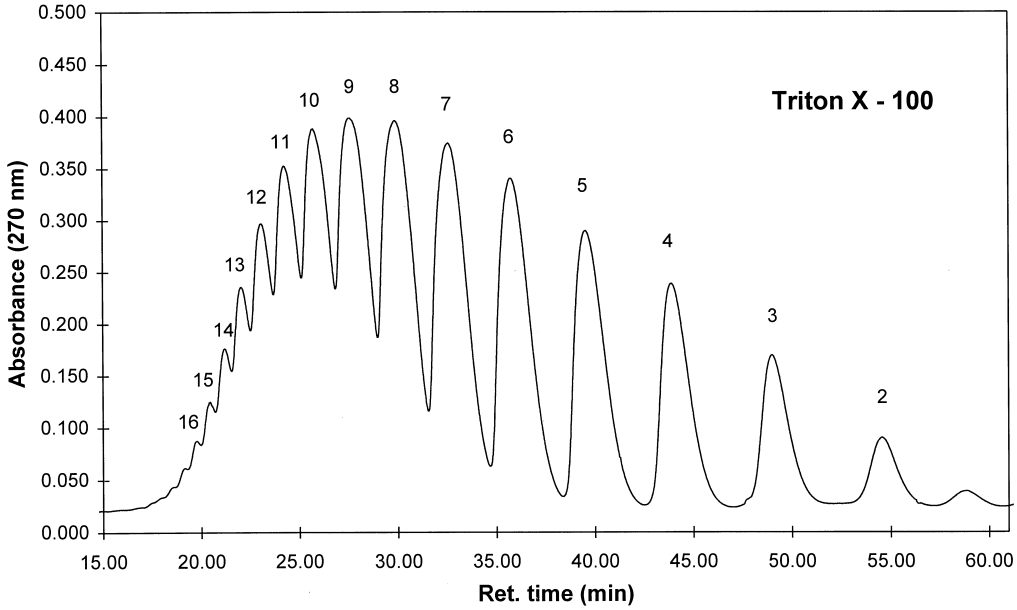


Fig. 3. Reversed-phase chromatogram of Triton X-100. At each fraction, the number of ethoxy groups contained in the dominating detergent species is indicated.

Triton X-100 calculated from MALDI-MS peak heights and from the absorbances of TLC and RPC fractions. Because of the incomplete separation of single species in the first two TLC fractions, their contributions (less than 10%) could not be assigned to definite masses, therefore they were omitted in the

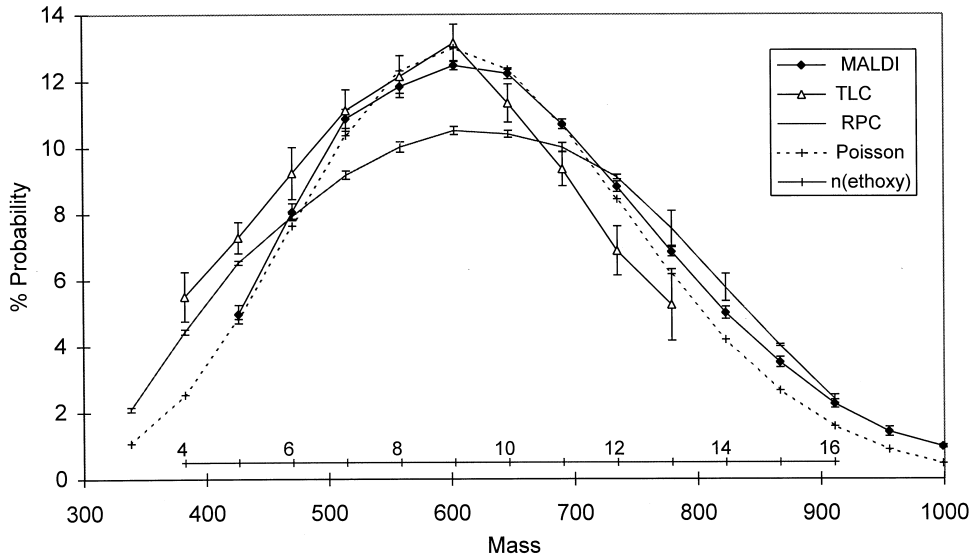


Fig. 4. Molecular mass distribution of Triton X-100. Error bars indicate ±standard error of mean values; for the number of spectra averaged see Table 1. Points connected by dashed lines correspond to a Poisson-distributed number of ethoxy groups, *n*. The corresponding molecular masses were calculated from *n* (indicated at the secondary abscissa) according to the stoichiometry $C_{14}H_{22}O(C_2H_4O)_n$.

diagram but were taken into account for calculating the percentages shown. For RPC, the absolute peak heights were taken throughout as measures of the concentrations of single polymer species. For the heavier fractions, this might introduce an error because of the small depth of the valleys separating neighbouring peaks. However, at the peak top the contribution of neighbour peaks cannot be severe because only one dominant polymer species was found there by MALDI-MS.

The distributions found by the three methods are similar to each other and to a distribution simulated using a medium value for the molecular mass of Triton X-100, 625 u (see Table 1, M_n values) and assuming a Poisson probability distribution of the number of ethoxy groups. This type of distribution has been suggested by Flory [19] for compounds formed by consecutive addition of monomers and should thus apply also to polyoxyethylene type detergents like Triton X-100. Table 1 shows molecular mass distribution parameters calculated from the peak heights of single species of Triton X-100 and Triton X-114. With m_i and c_i denoting molecular mass and concentration of single species i, these parameters are (cf. [20,21])

1. number average molecular mass, $M_n = \Sigma_i\ m_i c_i / \Sigma_i c_i$
2. weight average molecular mass, $M_w = \Sigma_i m_i^2 c_i / \Sigma_i m_i c_i$
3. polydispersity, $P = M_w / M_n$
4. most probable mass, $M_m = m_i$ with maximum c_i
5. most probable number of ethoxy groups, $n_{ethoxy,\,m}$ = number of ethoxy groups contained in the species with maximum c_i

For calculating these parameters, peak heights are taken as measures of the concentrations of the respective oligomers. The average molecular masses, M_n and M_w, as obtained from MALDI-MS results, are up to 9% higher than those obtained by TLC and RPC. This difference is relatively small in comparison with the width of the mass distributions found. If one takes the value of $2\sigma_m$ for the width of a distribution, the latter one can be calculated using the relation

$$\sigma_m^2 = \sum_i (m_i - M_n)^2 c_i / \sum_i c_i = M_n^2 (P - 1).$$

For the widths of the distributions found, one gets about 40% of M_n for each column of Table 1. Because of the above mentioned incomplete mass separation in TLC fractions numbers 1 and 2, the TLC distribution parameters exhibit a small insecurity. This insecurity can be shown to be less than 2%, if one calculates upper and lower limits for the distribution parameters (i) using the highest masses found in fractions numbers 1 and 2 and (ii) using the smallest masses found there.

Higher average masses found by MALDI-MS in comparison with other methods have been reported also by Kühn et al. [22]. These authors analyzed different waxes and interpreted the differences by discrimination of ions below 500 u by MALDI-MS.

To examine the molecular mass distribution for Triton X-100 of different suppliers, MALDI-MS was

Table 1
Molecular mass distribution data for Triton X-100 and Triton X-114 (Boehringer, purified for membrane research) as determined by MALDI-MS, RPC and TLC

	Triton X-100			Triton X-114		
	MALDI	RPC	TLC	MALDI	RPC	TLC
No. of spectra	10	9	6	8	6	4
M_n	643	622	605	567	560	523
M_w	670	655	634	589	586	545
$P = M_w / M_n$	1.042	1.053	1.049	1.039	1.047	1.042
M_m	607±33	613±29	603±28	526±25	515±20	493±25
$n_{ethoxy,m}$	9.10±0.74	9.22±0.67	9.00±0.63	7.25±0.56	7.00±0.45	6.50±0.58

M_n = number-average molecular mass; M_w = weight-average molecular mass; P = polydispersity; M_m = most probable molecular mass; $n_{ethoxy,m}$ = most probable number of ethoxy groups per molecule.
M_n, M_w and P have been calculated after averaging the peak heights of the indicated number of spectra. M_m and $n_{ethoxy,m}$ have been calculated for all single spectra and are given as mean±S.D.

Table 2
Molecular mass distribution data for different charges of Triton X-100 as determined by MALDI-MS

	Supplier and product			
	Fluka Triton X-100	Sigma Triton X-100	Sigma Triton X-100 Sigma ultra	Boehringer Triton X-100 purified for membrane research
No. of spectra	7	9	9	10
M_n	631	629	654	643
M_w	662	657	683	670
$P=M_w/M_n$	1.050	1.045	1.043	1.042
M_m	597±40	595±28	625±23	607±33
$n_{ethoxy,m}$	8.86±0.90	8.83±0.64	9.50±0.53	9.10±0.74

M_n = number-average molecular mass; M_w = weight-average molecular mass; P = polydispersity; M_m = most probable molecular mass; $n_{ethoxy,m}$ = most probable number of ethoxy groups per molecule.
M_n, M_w and P have been calculated after averaging the peak heights of the indicated number of spectra. M_m and $n_{ethoxy,m}$ have been calculated for all single spectra and are given as mean±S.D.

chosen because it separates different polymer species completely. Table 2 shows results obtained from four charges. The parameters are very similar, the average masses differing by less than 4%.

4. Discussion

MALDI-MS has been introduced successfully during recent years as an analytical tool for characterization of synthetic polymers [23]. The procedure is attractive because of its high sensitivity and practicability and can easily be employed to determine the molecular mass distribution parameters M_n, M_w, M_m etc. of polymer mixtures. However, the systematic comparison of MALDI-MS results with parameters obtained by alternative standard methods for different kinds of polymers yields conflicting results [20]. Most often MALDI-MS yields lower M_n and M_m values [20,21], but for waxes, higher values were found with MALDI-MS than with supercritical fluid chromatography (SFC) and size exclusion chromatography (SEC) [22]. In some cases, it was shown that the reference method exhibited strong imprecision [24]. With SEC methods, the elution volume depends linearly on the logarithm of the molecular mass. This leads to systematic overestimation of the most probable mass by SEC in comparison with MALDI-MS depending on the degree of polymerization [21].

We use TLC and RPC as reference methods that separate single polymer species and thus do not lead to the above mentioned overestimation. The group of detergents investigated has a narrow range of polydispersity values. The results show acceptable agreement between distributions of detergents provided by different suppliers as well as between results obtained with the two chromatographic procedures and with MALDI-MS (Tables 1 and 2). Separation of single species of mixtures of compounds similar to those studied by us (alcohol ethoxylates and alkylphenol ethoxylates) has also been achieved with normal phase and reversed-phase HPLC by Kiewiet and de Vogt [25].

Detergents formed by consecutive addition of a repeating unit e.g. $-OCH_2CH_2-$ should exhibit a Poisson distribution of the number of repeating units added, provided that the addition of the repeating unit to the initiator is not much slower than succeeding additions. Indeed the distributions observed, especially by MALDI-MS, are similar to this shape (Fig. 4).

TLC and RPC yield good resolution for species with low degrees of polymerization (up to ten pure fractions could be obtained by TLC and RPC). The species with lower degree of polymerization ($M_r <$ 405) are not to be estimated by MALDI-MS because of the matrix cinnamic acid interfering with the lower part of the mass spectrum. Addition of silver salts to the MALDI-MS sample [26,27] shifts the peaks observed to higher mass values by formation of Ag^+ adducts. Thereby, matrix peaks may be identified, however, the spectra obtained are often of poorer overall quality, the isotopes ^{107}Ag and ^{109}Ag

lead to double peaks, and the shape of the molecular mass distributions of polyethylene type detergents is altered. Without silver addition, we found the MALDI-MS mass distributions to be very similar to those obtained by TLC and RPC. Addition of gold chloride to MALDI-MS samples has also been tested, however, this led to significant fragmentation or loss of end-groups of some polymers [22]. A further essential restriction of the MALDI-MS method is its inability to resolve isomeric compounds. If there are isomeric compounds to be expected in the sample, appropriate separation techniques must be applied.

MALDI-MS can help to detect impurities within the preparations as e.g. species containing residues with additional CH_2 groups. The MALDI-MS peak heights belonging to molecules with definite masses can be used for quasi on-line characterization of chromatographic fractions if suitable fractionation and liquid handling techniques are used.

The presented reversed-phase separation method can be scaled up for preparation of single pure detergent species. It should be of interest to reinvestigate the characteristics and behaviour of detergents which have been investigated hitherto only as complex mixtures. So far, the interaction of detergents with hydrophobic proteins has been studied only with mixtures containing different polymer species, but the present report shows that such studies can now be conducted with better defined single polymer species.

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