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Liquid chromatographic–mass spectrometric studies on the in vitro degradation of a poly(ether ester) block copolymer

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Abstract

A detailed study on the in vitro degradation of a poly(ethylene glycol) and poly(butylene terephthalate) (PEOT/PBT) segmented block copolymer was carried out using liquid chromatography/electrospray–mass spectrometry. Accelerated hydrolysis of PEOT/PBT was achieved by placing the material for 14 days in a refluxing phosphate buffered saline (pH 7.4) solution. All major degradation products and several side-products were identified using both the positive and the negative ion mode. The data indicate that degradation does not only occur in the "soft", but also in the "hard" segments of the polymer. Liquid chromatographic separation is required to distinguish between degradation products with different sequences but identical molecular mass. The addition of ammonium and sodium ions provided important complementary information on the number of monomer units present in the degradation products.
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Keywords: Degradation; Hydrolysis; Poly(ether ester) block copolymer; Polymers

1. Introduction

Favorable thermal and mechanical properties are distinct features of aromatic polyesters as, e.g., poly(ethylene terephthalate) (PET) and poly(butylene terephthalate) (PBT). These polymers are biocompatible [1] and are used as biomaterials [1,2]. They are quite stable under physiological conditions and cannot be used as biodegradable materials [3,4]. In contrast to aromatic polyesters, aliphatic polyesters turned out to be biodegradable. However, these lack sufficient mechanical strength for many applications. Therefore, aliphatic–aromatic copolyesters have been developed to serve as biodegradable polymers with relatively good mechanical properties [4]. Block and random copolymers based on ethylene terephthalate and lactic acid, ε-caprolactone or ethylene oxide (EO) have been investigated mainly [5].

The in vitro degradation of the latter polymer was investigated by Nagata et al. [6,7] and Reed and Gilding [8]. They found that in vitro degradation occurred via hydrolysis of the ester bonds, and that the degradation process was enhanced by the addition of enzymes. The group of poly(ethylene oxide) (PEO) and PBT poly(ether ester) copolymers has been thoroughly studied with regard to degradation processes and potential medical applications [9,10]. The in vivo degradation of PEOT/PBT segmented copolymers was described [11]. Two degradation pathways are expected in vivo. First, hydrolysis of ester bonds in the PBT part or ester bonds connecting PEO segments and terephthalate units. Second, oxidative degradation of PEO based on a radical mechanism [12,13]. Both mechanisms were observed for in vitro degradation. While oxidation of PEO was performed in $H_2O_2/CoCl_2$ solution [14], hydrolysis of PEOT/PBT took place in aqueous media under non-oxidative conditions [14,15].

The rate of degradation of a polymer can be estimated by following the molecular mass as a function of time when no overall weight loss occurs. Depending on the type of polymer, weight loss can start already in an early

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phase and is a parameter which gives information about the degradation process [7,8]. During degradation the molecular mass as well as the composition of the polymer will change, which process will influence properties like glass transition, swelling in water and intrinsic viscosity [14,15]. The hydrolytic degradation of the polyesters involves cleavage of ester bonds. Therefore, this reaction can be followed by measuring the increasing amount of carboxyl end groups. Zang and Ward determined the rate of formation of carboxyl end groups by infrared spectroscopy [16]. Furthermore, ^{1}H NMR has turned out to be a helpful tool for the determination of degradation products in solution [14,15].

However, all these methods have in common that they give information about the remaining products but not on the variety of degradation products that will dissolve in the buffer solution. To overcome this limitation, mass spectrometric (MS) methods may be applied. MS has turned out to be a versatile tool for the analysis of copolymers [17]. Matrix assisted laser desorption/ionization (MALDI)-MS and electrospray ionization (ESI)-MS are the techniques mainly applied for polymer characterization. In order to enhance selectivity, these MS methods may also be used in conjunction with liquid chromatography (LC) [18,19].

In a preliminary study on the degradation of 1000 PEOT71/PBT29 (a copolymer based on PEG with a molecular weight of 1000 g/mol and 71 wt.% of PEO-containing soft segments) degradation products which are soluble in PBS buffer were analyzed by LC with subsequent photometric and MS detection [15]. Atmospheric pressure chemical ionization (APCI) was applied both in the positive and the negative ion mode. In APCI(−), terephthalic acid and the monoester of terephthalic acid and butanediol were identified. In APCI(+), PEG and PEG linked to one molecule of terephthalic acid were found as degradation products. Although the formation of more degradation products was shown by photometric detection, these products were not identified at that time. As APCI requires high temperatures for evaporation of eluent and analyte molecules, some drawbacks were observed while analyzing PEOT/PBT degradation products. On the one hand, application of low probe temperatures (below 400 °C) is insufficient for the evaporation of terephthalic acid linked to PEG and of degradation products with even a higher molecular mass. On the other hand, high temperatures in the range of 500 °C cause thermal degradation. Thermal fragmentation of both the PEG starting material and the terephthalate-PEG species, respectively, was observed. Whereas the latter predominantly revealed a loss of one terephthalate unit, the fragmentation of PEG was mainly characterized by the loss of ethylene glycol moieties. Thus, identification of degradation products with higher molecular mass was not possible. In contrast to APCI, ESI is performed at much lower temperatures. Therefore, ESI-MS detection was applied for the identification of degradation products of the 1000 PEOT71/PBT29 block copolymer with a relatively high molecular mass. The aim of this paper is to further elucidate the type of bonds cleaved (ether or ester bond) and the part of the polymer where cleavage takes place (soft or hard segments).

2. Experimental

2.1. Chemicals

Poly(ethylene glycol) of average molecular weight 1000 g/mol (PEG 1000) (Fluka, Switzerland), poly(butylene terephthalate) (PBT) (Aldrich, Milwaukee, WI, USA), titanium tetrabutoxide ($Ti(OBu)_4$) (Merck, Darmstadt, Germany), dimethyl terephthalate (Merck, Darmstadt, Germany), 1,4-butanediol (Acros organics, Geel, Belgium) and Irganox 1330 from (Ciba-Geigy, Basel, Switzerland) were used without further purification. All solvents used were of analytical grade (Biosolve, Valkenswaard, The Netherlands). As a buffer for degradation experiments, phosphate buffered saline of pH 7.4 (PBS, NPBI, Emmer-Compascuum, The Netherlands) was applied. Solvents for LC were acetonitrile (elution grade) and methanol (elution grade) from Merck Eurolab (Fontenau S/Bois, France). Water for LC was purchased from Merck (Briare le Canal, France). Terephthalic acid (Acros organics, Geel, Belgium) was used as a standard for HPLC measurements.

2.2. Polymer synthesis and processing

The PEOT/PBT multiblock copolymers were synthesized on a 50 g scale by a two-step polycondensation of PEG, 1,4-butanediol and dimethyl terephthalate in the presence of titanium tetrabutoxide as catalyst and Irganox 1330 as antioxidant [14,15]. Polymer films were prepared by compression molding (laboratory press THB008, Fontijne, The Netherlands) at a temperature of 140 °C. For the degradation experiments, discs with a thickness of 400–600 μm and a diameter of 15 mm were prepared.

2.3. In vitro degradation

Accelerated hydrolysis of the 1000 PEOT71/PBT29 copolymer was performed by placing the material for 14 days in refluxing phosphate buffered saline (pH 7.4). The polymer discs became very brittle and were recovered from the PBS solution by filtering. Soluble degradation products were characterized by LC/ESI-MS as described in detail below.

2.4. Analytical instrumentation

For HPLC/MS measurements, the following equipment from Shimadzu (Duisburg, Germany) was used: SCL-10Avp controller unit, DGU-14A degasser, two LC-10ADvp pumps, SIL-10A autosampler, SPD10AV UV-Vis detector,

LCMS QP8000 single quadrupole mass spectrometer with ESI probe and Class 8000 software version 1.20.

2.5. HPLC conditions

All separations were performed using a Merck LichroSpher RP-18ec column with ChromCart cartridges (Macherey Nagel, Düren, Germany) of the following dimensions: particle size, 5 μm; pore size, 100 Å; 125 mm × 2 mm i.d. Flow rate of the mobile phase was 300 μl/min.

For LC/ESI(+)-MS, a binary gradient of acetonitrile and buffer (either aqueous 20 mM ammonium formate/formic acid or aqueous 20 mM sodium formate/formic acid) was used. The profile was:

Time (min)	CH_3CN (%)
0.01	10
1	10
100	70
108	100
112	100
114	10
120	Stop

A binary gradient of methanol and water was used for LC/ESI(−)-MS. The profile was:

Time (min)	CH_3OH (%)
0.01	20
1	20
100	80
108	100
112	100
114	20
120	Stop

The injection volume was 5 μl and the detection wavelengths were 251 and 300 nm, respectively.

2.6. MS conditions

ESI(+)-MS was performed using the following conditions: probe voltage 3 kV, curved desolvation line (CDL) voltage −55 V, CDL temperature 250 °C, nebulizer gas (N_2) flow rate 4.5 l/min, deflector voltages 35 V and detector voltage 1.7 kV. For the SCAN mode, the mass range *m/z* 200–1600 was chosen, and the integration time was 1 s.

ESI(−)-MS was carried out applying the following conditions: probe voltage −5 kV, curved desolvation line (CDL) voltage 50 V, CDL temperature 250 °C, nebulizer gas (N_2) flow rate 4.5 l/min, deflector voltages −55 V and detector voltage 1.7 kV were used. For the SCAN mode, the mass range of *m/z* 100–500 was chosen, and the integration time was 1 s.

3. Results and discussion

The general structure of PEOT/PBT segmented block copolymers is presented in Fig. 1. The composition of the block copolymer is abbreviated as *a*PEOT*b*/PBT*c*, in which *a* is the starting PEO molecular weight, *b*, the weight percentage of PEOT soft segments and *c*, the weight percentage of PBT hard segments. It has to be noted that terephthalic acid ester units are present in both the soft and the hard segment. Therefore, the notation PEOT (T for terephthalate) is used to refer to the soft part. The abbreviation PEO is used to refer to the repeating segment in the copolymer, whereas PEG is used to refer to the material applied for the synthesis.

The PBT hard segments are relatively hydrophobic and it is therefore expected that hydrolysis of ester bonds in these domains will be relatively slow. The PEOT soft segments are hydrophilic. Cleavage of ester bonds between PEO and terephthalic acid will be relatively fast. For this reason, initial experiments focused on a comparison of PEG 1000, which is used for the synthesis of the block copolymer with the soluble products obtained from the in vitro degradation of the block copolymer. These experiments were carried out by direct injection of diluted aqueous PEG solutions (0.5 mM) as well as the diluted degradation product solutions in PBS buffer into the mass spectrometer. The latter were diluted 1:10 with water and filtered through a 0.45 μm membrane filter. For these experiments, the single quadrupole mass spectrometer was operated with ESI(+) under conditions typically used for instrument tuning with PEG standards.

It is obvious from Fig. 2 that there are strong similarities between the main peak patterns of the PEG standard and the solution containing the degradation products. The peak distribution of the main pattern is almost identical for both samples, indicating that a substantial amount of PEG is released during the hydrolysis experiment. Due to the doubly charged individual substances (see below for interpretation of the mass spectra), the mass difference between the individual major peaks is $m/z = 22$, corresponding to one EO unit. Interestingly, there is no degradation of the ether groups in the PEG chains: that degradation should result in a peak distribution shifted to lower maxima at lower *m/z* values. On the other hand, it can be seen that several peak distributions with lower intensities are observed for the solution containing the degradation products. This is a first indication that different ester bonds are broken to a significant extent during the degradation.

With a single quadrupole mass spectrometer, it is not possible to resolve a sufficient number of peak distributions.

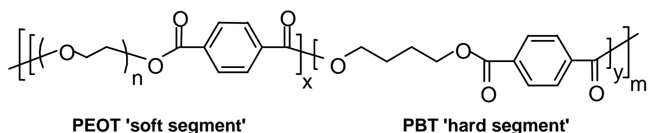

Fig. 1. General structure of PEOT/PBT segmented block copolymers.

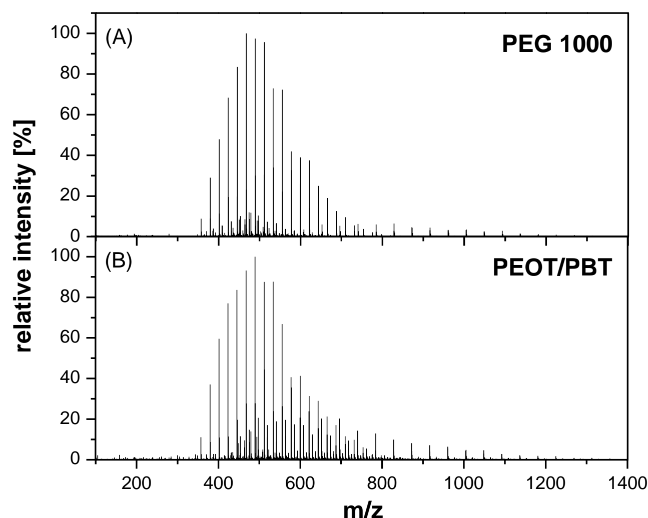

Fig. 2. ESI(+)-mass spectra of PEG 1000 standard (A) and soluble products derived from 1000 PEOT71/PBT29 block copolymer (B).

For this reason, reversed-phase LC was carried out using an endcapped C_{18} stationary phase in combination with a gradient of acetonitrile and aqueous formic acid/ammonium formate buffer. As excellent resolution between the individual oligomers was intended, a long gradient with a total duration of 2 h was selected. In Fig. 3, the total ion current (TIC) chromatogram is presented. For the substances eluting between 12 and 30 min, an excellent separation is observed already for the individual substances within one distribution. The inserted figures contain the complete mass spectra of a combined time period, e.g., 12–30 min. Analogous to Fig. 2, all major peaks in the mass spectra are associated with doubly charged ions, resulting in variations of $m/z = 22$, which correspond to individual EO units. Comparison with a PEG 1000 standard proves that peak group A corresponds to unmodified PEG 1000, while peak groups B–F correspond to degradation products with a higher molecular mass, which are discussed below.

The TIC chromatogram in Fig. 4 is a blow-up of the 12–30 min part of the chromatogram in Fig. 3. Two series of individual mass traces are presented below the TIC chromatogram. All peaks correspond to the PEG oligomers with $n = 12$–32 EO units. Due to the mobile phase used, the ammonium adducts have the highest abundance. For the lower oligomers with $n = 12$–15, the singly charged adducts of one NH_4^+ are most abundant. For the higher oligomers, the doubly charged adducts of two ammonium ions are observed with the greatest intensity. The lower oligomers are baseline

Fig. 3. LC separation of the soluble degradation products derived from 1000 PEOT71/PBT29 with ESI(+)-MS detection ($m/z = 200$–1600). Inserted are extracted mass spectra representing the peak series eluting at different retention times.

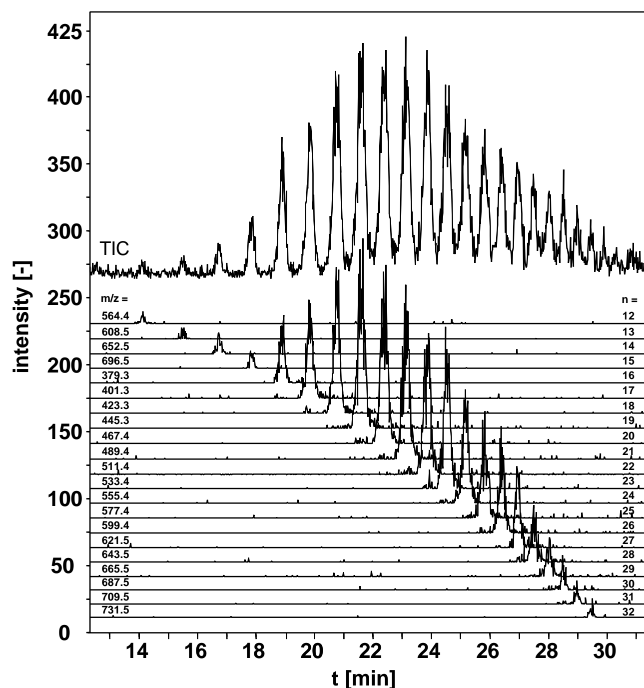

Fig. 4. Part of the LC separation (12–31 min) of the soluble degradation products derived from 1000 PEOT71/PBT29 with ESI(+)-MS detection (m/z = 200–1600). Displayed are the base-shifted mass traces corresponding to the PEO oligomers. Peaks correspond to numbers of EO units from 12 to 32.

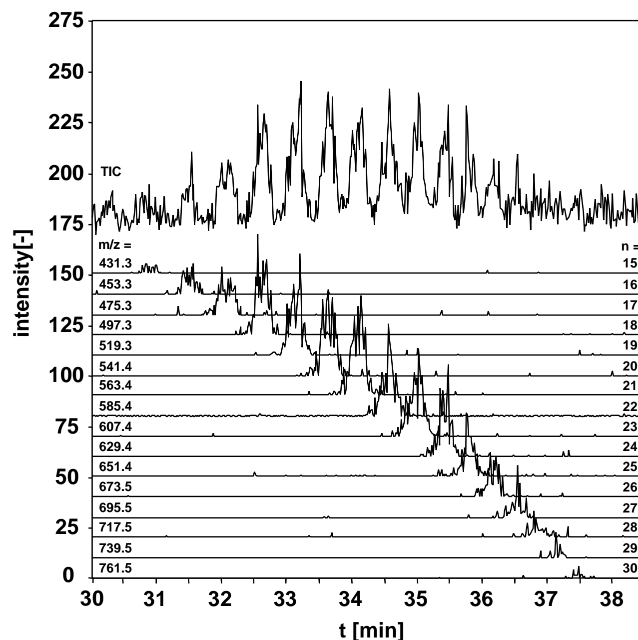

Fig. 5. Part of the LC separation (30–39 min) of the soluble degradation products derived from 1000 PEOT71/PBT29 with ESI(+)-MS detection (m/z = 200–1600). Displayed are the base-shifted mass traces corresponding to one terephthalate linked to PEO molecules. Peaks correspond to numbers of EO units from 15 to 30.

separated by LC, and there is still a very good separation for the higher oligomers.

Additional experiments were carried out with a mobile phase containing sodium formate instead of ammonium formate. To elucidate the nature of the adducts (kind and number of the associated cations), the comparison between the mobile phases consisting of sodium or ammonium salts is very helpful. Together with an investigation of the ^{13}C satellite peak to obtain the charge state, these experiments help to obtain the molecular weight information of the species. Regarding the PEG distribution itself, charge state assignment is not required, because a comparison with the commercially available PEG starting material together with the retention time does already allow an unambiguous identification.

Series B in Fig. 3 comprises peaks eluting between 30 and 38 min, and the peaks are presented in an expanded manner in Fig. 5. The peak distribution indicates that PEG is involved, and the m/z ratios allow the conclusion that this series comprises the adducts of the PEG oligomers with one molecule of terephthalic acid (PEG + T). The m/z difference of 22 indicates that doubly charged ions are observed again. All extracted mass traces in Fig. 5 correspond to the respective $[M + 2NH_4]^{2+}$ ions. In Fig. 6, the mass spectra of the (PEG + T) oligomer with $n = 20$ are presented with ammonium ions (left) and sodium ions (right) in the mobile phases. For this compound, the mass spectra are very similar

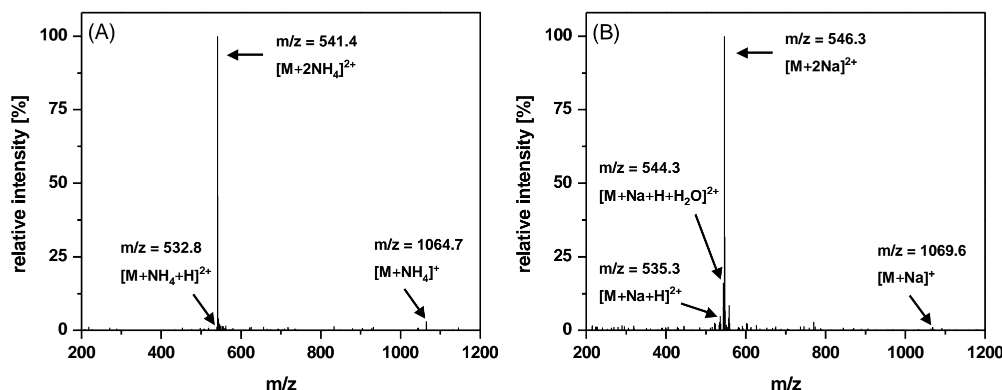

Fig. 6. ESI(+)-mass spectra of terephthalic acid linked to one PEO oligomer ($n = 20$) with ammonium ions (A) and sodium ions (B) in the mobile phases.

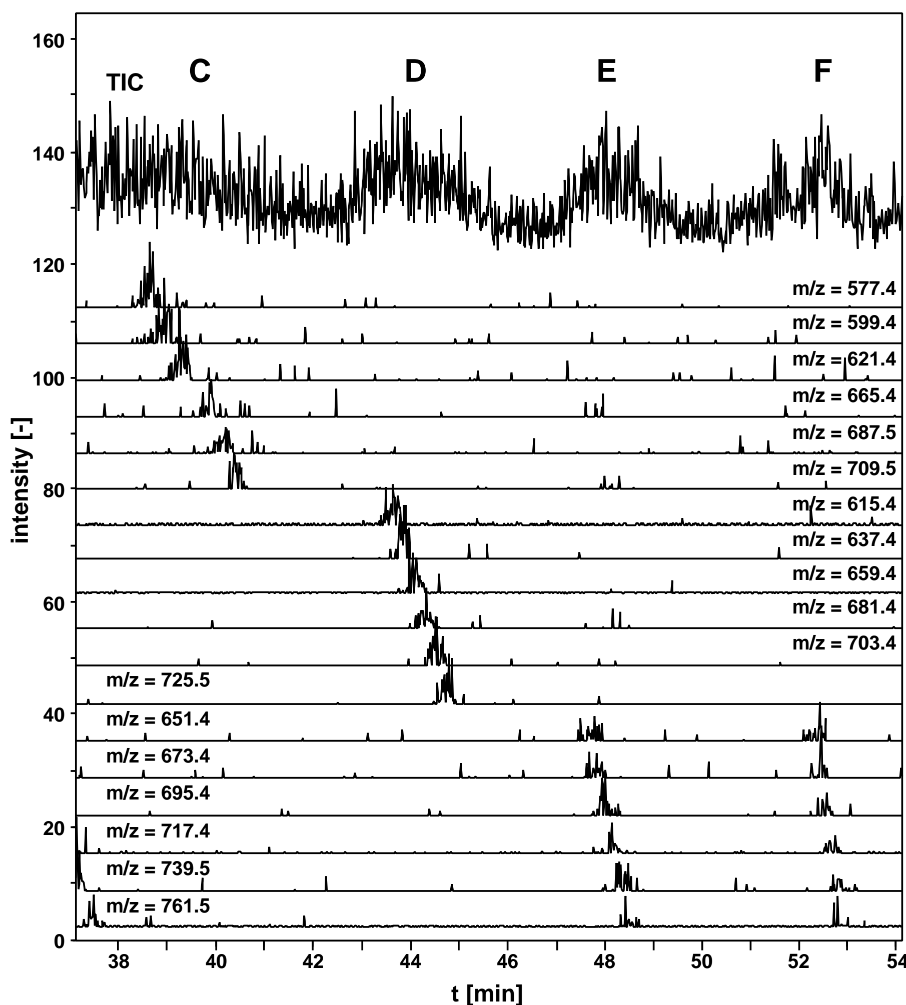

Fig. 7. Part of the LC separation (37–54 min) of the soluble degradation products derived from 1000 PEOT71/PBT29 with ESI(+)-MS (m/z = 200–1600). Displayed are the base-shifted mass traces corresponding to the linked PEO molecules: PEO + T + Bu (series C); T + PEO + T (series D); and PEO + T + Bu + T or Bu + T + PEO + T (series E and F). For the four series, the peaks correspond only for the numbers of EO units from 20 to 25.

to the mass spectra for the PEG oligomer with $n = 20$. However, regarding the (PEG + T) sequence, the sodium adducts are observed with much lower intensity than the ammonium adducts. Therefore, the mobile phase including ammonium formate was applied for all further measurements of degradation products with a higher molecular mass.

In Fig. 7, it is obvious in the TIC chromatogram (extract of Fig. 3 from 37 to 54 min) that the oligomers of the degradation products with a higher molecular mass are not baseline separated by LC. Nevertheless, the extracted mass traces prove that the distributions may still be detected mass spectrometrically. The extracted mass traces are the $[M + 2NH_4]^{2+}$ ions in all cases. Peak series C contains the degradation products consisting of one PEG unit, one terephthalate and one butanediol residue (PEG + T + Bu) and series D are (T + PEG + T). Series E and F are both composed of one PEG unit, two terephthalate and one butanediol residues. However, the two possible different sequences (PEG + T + Bu + T) and (Bu + T + PEG + T) obviously lead to a different chromatographic behavior of the series. It can be assumed that the larger polarity of (PEG + T + Bu + T) results in earlier elution. The LC separation of the two isomers clearly proves that both sequences are present in the sample, thus demonstrating again that the LC separation provides very useful data, which could not be obtained with MS alone.

Furthermore it should be noted that the (PEG + T) peak series (compare to Fig. 5) and the peak series E and F in Fig. 7 differ by 220 mass units. It is obvious from the mass traces of m/z = 739.5 and 761.5 in Fig. 7 that the higher homologues (n = 29, 30) of the (PEG + T) series are detected at retention times between 37 and 38 min. The same m/z values are observed for series E and F, but in this case for n = 24 and 25, because the mass of the additional (T + Bu) in series E and F equals that of five EO units for a doubly charged molecule. Under reversed-phase LC conditions, the decomposition product with higher polarity (more EO units) elutes first. Without LC separation, it would not be possible to distinguish between these series.

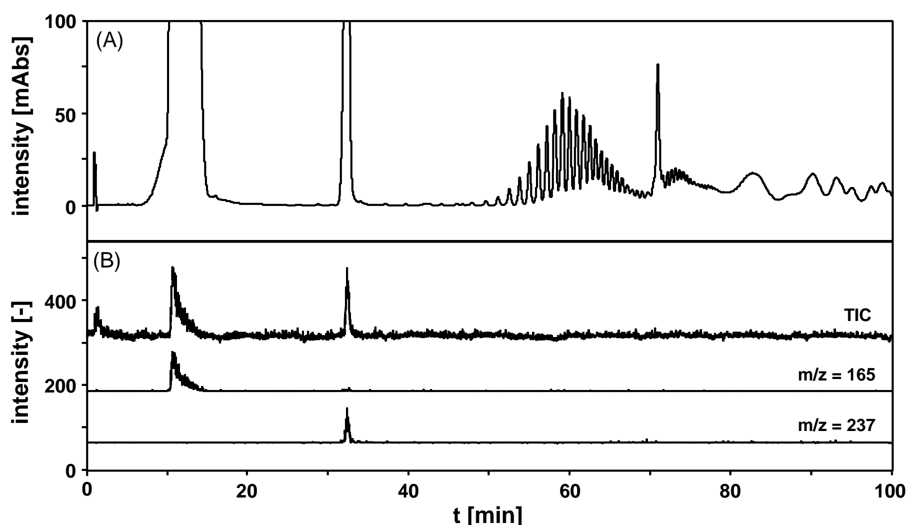

Fig. 8. LC separation of the soluble degradation products derived from 1000 PEOT71/PBT29 with UV detection at 251 nm (A) and ESI(−)-MS detection (m/z = 100–500) (B).

As a significant number of degradation products contains terephthalate, showing strong UV-Vis absorption at ~251 nm, HPLC separation with subsequent UV detection had already been performed in an earlier study [15]. In Fig. 8, a LC separation of the complete mixture of degradation products comparing photometric detection at $\lambda = 251$ nm (Fig. 8A) and ESI(−)-MS detection (Fig. 8B) is presented. In the negative ion mode, deprotonation of terephthalic acid is expected. This had been already shown in a previous study, in which atmospheric pressure chemical ionization was applied in the negative ion mode [15].

Initial experiments were carried out with terephthalic acid as a standard in order to optimize the conditions for the ESI(−) detection. Since only low abundant peaks for terephthalic acid could be obtained using acetonitrile as mobile phase constituent, it was replaced by methanol. Methanolate, CH_3O^-, is a stronger gas phase base than the carbanion of acetonitrile $^-CH_2CN$. Thus, the use of the stronger gas phase base methanol leads to increased proton abstraction and improved sensitivity. Owing to the lower elution strength of methanol, the amount of the latter which was used for the gradient had to be increased. Although the retention times were shifted to higher values, the elution order was not affected at all.

The total ion current and two individual mass traces are presented in Fig. 8B. The signals of m/z = 165 and 237 correspond to the $[M - H]^-$ peaks of terephthalic acid and the monoester of butanediol and terephthalic acid, respectively. The full mass spectra of both compounds, recorded in a mobile phase containing methanol and aqueous formic acid/sodium formate, are presented in Fig. 9. In Fig. 9A (terephthalic acid), the $[M-H]^-$ peak is most abundant, but the peaks of m/z = 121 ($[M-CO_2-H]^-$) and m/z = 353 ($2[M-H+Na]^-$) are observed as well. In Fig. 9B (T + Bu), the $[M - H]^-$ signal at m/z = 237 is most abundant. The $[M + HCOONa - H]^-$ peak at m/z = 305 is observed, and the fragment at m/z = 121 ($[M - COO(CH_2)_4OH - H]^-$ is present, too. This ESI-MS data confirm the identification of terephthalic acid and the monoester of terephthalic acid and butanediol obtained with APCI-MS in the earlier study [15].

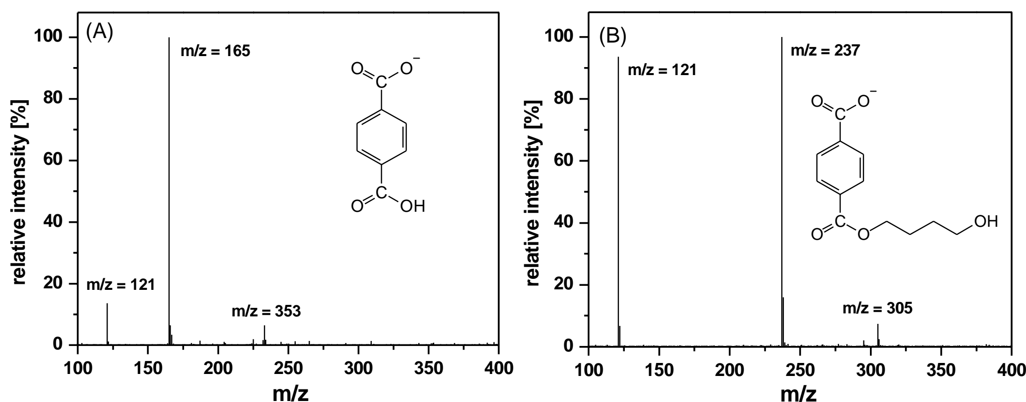

Fig. 9. ESI(−)-mass spectra of terephthalic acid (A) eluting at ~11 min and monoester of butanediol and terephthalic acid (B) eluting at ~32 min.

4. Conclusions

It can be concluded that the ester bonds within the block copolymer are much easier cleaved than the ether bonds of the PEG part. Due to the very similar chemical nature of all ester bonds within the block copolymer, it is likely that the cleavage is observed in a similar way for all ester bonds. However, the hydrolysis of ester bonds is also dependent on chain mobility and permeability [20–22]. The higher grade of cristallinity in the hard segment hinders the attack of water. In contrast, the amorphous and more hydrophilic PEO containing soft segment is much easier accessible by water. Thus, initial degradation is restricted to the amorphous soft segment. The fact that PEG is detected alone as major degradation product proves this assumption. To obtain these main fragments, both neighboring ester bonds of PEO—the one within the soft segment and the one coupling it to other soft segments—have to be cleaved. However, cleavage of the ester bond connecting PEO to the hard segment is also possible. For the ester bond between Bu and T in the hard segment, a final proof for cleavage could only be the direct detection of either Bu or (Bu + T + Bu), as both could not be detected under the selected conditions. However, as both series C (PEG + T + Bu) and F (Bu + T + PEG + T) prove, there is at least a cleavage between Bu and T between a hard and a soft segment.

Acknowledgements

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