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Microstructure of Bacterial Poly(β -hydroxybutyrate-co- β -hydroxyvalerate) by Fast Atom Bombardment Mass Spectrometry Analysis of the Partial Pyrolysis Products

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ABSTRACT: The microstructure of poly(β -hydroxybutyrate-co- β -hydroxyvalerate) (P(HB-co-HV)) has been studied by mass spectrometry. We describe a novel method for the structural analysis of P(HB-co-HV) copolymers, based on the direct FAB-MS analysis of the partial pyrolysis products. The FAB spectra obtained allow the identification of the copolymer microstructure up to the hexads level, and a comparison between the experimental and calculated peak intensities makes it possible to discern clearly if the sample is made of a single random copolymer or of a mixture of two random copolymers. In addition, an algorithm has been developed that also allows the determination of both the amount of the random copolymers in the binary P(HB-co-HV) mixtures, as well as the copolymer composition of the individual copolymers. The highly discriminating power of the method developed here is due to the possibility of fitting the calculated statistical abundances with the MS experimental values corresponding to higher oligomers, i.e., hexamers as compared to diad and triad monomer sequences seen by ^{13}C NMR.

Introduction

The microstructure of poly(β -hydroxybutyrate) (PHB) and poly(β -hydroxybutyrate-co- β -hydroxyvalerate) (P(HB-co-HV)) has been recently studied.¹⁻⁵

These polymers have attracted much attention because of their microbial origin and of their unique properties.⁶⁻⁸

The sequence distribution of P(HB-co-HV) copolymers has been deduced from diad and triad analysis by ^{13}C NMR spectrometry, and it has been concluded that some of the bacterially synthesized P(HB-co-HV) samples are random copolymers of HB and HV units, but others are mixtures of P(HB-co-HV) random copolymers with different compositions.⁵

However, NMR can hardly discern beyond the triad level, and the distinction between a single copolymer and a mixture of copolymers is sometimes too difficult.⁵

Mass spectrometry is able to look at the mass of individual molecules in a mixture, and it is therefore an interesting alternative to NMR.

We have described how such copolyesters can be analyzed for sequence distribution by fast atom bombardment mass spectrometry (FAB-MS) of the oligomers formed by partial methanolysis of the copolymers.⁴

In this report we describe a novel method for the structural analysis of P(HB-co-HV) copolymers, based on the direct FAB-MS analysis of the partial pyrolysis products.

The FAB spectra obtained allow the identification of the copolymer microstructure up to the hexads level, and a comparison between the experimental and calculated peak intensities makes it possible to discern clearly if the sample is made of a single random copolymer or of a mixture of two random copolymers.

The highly discriminating power of the method developed here is due to the possibility of fitting the calculated statistical abundances with the experimental values corresponding to higher oligomers (hexamers as compared to diads and triads, which are seen by ^{13}C NMR).

We have recorded the negative FAB mass spectra of residues from partial pyrolysis of PHB and P(HB-co-HV), using a standard thermogravimetric (TG) apparatus to monitor the pyrolysis and to stop it at a predetermined weight-loss level.

Once the partial pyrolysis is performed, the residue consists of a mixture of oligomers, and FAB-MS provides an excellent means to detect intense molecular ions corresponding to the higher oligomers present in the mixture. This method is fast and accurate. Its advantage being that it simply requires one TG run followed by one FAB mass spectrum, thus avoiding the time-consuming partial methanolysis and HPLC fractionation described previously.⁴

Experimental Section

Materials. PHB (MW 800 000), P(HB-co-19% HV) (MW 800 000, code 28,248-0, lot no. 1034560, catalog 1986-87) and P(HB-co-28% HV) (code 28,248-0, lot no. 0728469, catalog 1988-89) were obtained from Aldrich Chemical Co. P(HB-co-6% HV) (MW 750 000) and P(HB-co-13% HV) (MW 750 000) were obtained from Marlborough Biopolymers Ltd. (Biopol).

The 250-MHz ^1H NMR spectra of each sample gave the composition indicated above on the basis of the ratio of the methyl triplet at 0.895 ppm and the methyl doublet at 1.274 ppm. The peak assignments are according to the literature.^{1,3} Manufacturer compositions and compositions determined from NMR and from FAB mass spectra (see below) are indicated in Table I. The mixtures indicated in Table I were obtained by dissolving

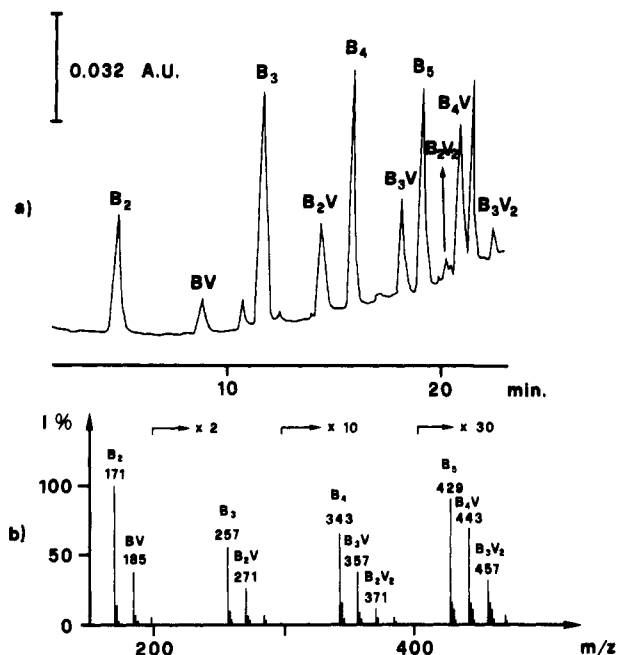


Figure 2. HPLC separation of the partial methanolysis products (a) and negative ion FAB mass spectrum of the partial pyrolysis products (b) of P(HB-co-15% HV) (sample 3).

Table III
Experimental^{a,b} and Calculated^c Relative Amounts of the Oligomeric Degradation Products from Sample 3 by HPLC and TG FAB/MS Analyses

oligomers	HPLC ^a	TG FAB-MS ^b	calcd ^c for B/V	
			87/13	85/15
B ₂	74	72	77	74
BV	26	28	23	26
B ₃	72	68	69	65
B ₂ V	28	32	31	35
B ₄	62	57	59	53
B ₃ V	31	33	34	37
B ₂ V ₂	7	10	7	10
B ₅	53	48	51	46
B ₄ V	40	36	38	40
B ₃ V ₂	7	16	11	14

^a Normalized HPLC areas of the oligomeric species produced by methanolysis (see Figure 2). ^b Normalized intensities of (M - H)⁻ ions of the pyrolysis products in the FAB mass spectrum. ^c Relative intensities of the degradation products, calculated by eq 2 for two copolymer compositions.

have been subtracted). The assignments for the oligomers identified are reported in Table II.

The spectrum in Figure 1 shows little evidence of the molecular ions fragmentation and might therefore be used for quantitative analysis, i.e., to estimate the copolymer composition and sequence distribution.

To test this hypothesis, we have compared the above FAB-MS data with those obtained in the HPLC analysis of the partial methanolysis products of the same copolymer sample, as shown in Figure 2.

In Figure 2a a section of the HPLC trace is reproduced, which was previously obtained from the methanolysis products of the P(HB-co-15% HV) copolymer,⁴ and the corresponding section of the TG-FAB/MS spectrum is shown in Figure 2b (which is a portion of Figure 1).

The relative amounts for each set of the oligomeric products, from dimers to pentamers, obtained by the two partial degradation methods, are compared in Table III. It can be noticed that the level of agreement can be regarded as satisfactory, considering that the oligomers

Table IV
Experimental^a and Calculated^b Normalized Abundances of the Partial Pyrolysis Products from FAB-MS Analysis of P(HB-co-HV) Sample 3

oligomers	exptl ^a	calcd ^b		
		85/15	80/20	90/10
B ₂	69	73	64	81
BV	27	25	32	18
V ₂	4	2	4	1
B ₃	64	61	52	73
B ₂ V	30	33	38	24
BV ₂	6	6	10	3
B ₄	55	52	41	66
B ₃ V	32	37	41	29
B ₂ V ₂	10	10	16	5
BV ₃	3	1	2	
B ₅	45	45	33	60
B ₄ V	35	39	41	33
B ₃ V ₂	16	14	21	7
B ₂ V ₃	4	2	5	
B ₆	40	38	27	53
B ₅ V	38	40	40	36
B ₄ V ₂	17	18	25	10
B ₃ V ₃	5	4	8	1

^a Normalized intensities of the peaks in the FAB mass spectrum.

^b Normalized abundances of partial pyrolysis products calculated by eq 2.

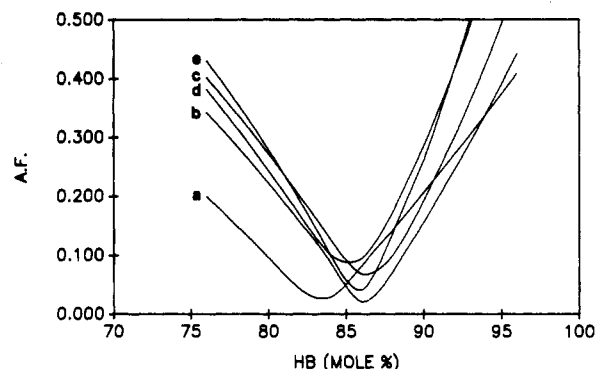


Figure 3. Agreement factor (AF) versus HB content (mol %) corresponding to each set of peak intensities for (a) dimers, (b) trimers, (c) tetramers, (d) pentamers, and (e) hexamers from sample 3.

V₂, BV₂, BV₃, and B₂V₃ are present in very low quantity and are not seen in the HPLC trace. Furthermore, by comparing the two experimental results with the calculated ones, it can be seen that the best fit value is 87/13 for the HPLC and 85/15 for the TG-FAB/MS analysis. Therefore, the results are not very far from each other. Similar results were found by comparing HPLC traces with the corresponding TG-FAB/MS spectra for the other samples investigated here.

Assuming that the same response factor to FAB-MS can be used for each set of oligomers produced in the partial pyrolysis, then the normalized intensities of the peaks corresponding to dimers, trimers, tetramers, pentamers, and hexamers ought to reflect the copolymer composition; this can be calculated on the assumption of a random distribution of the HB and HV units in the copolymer.¹³

The statistical probability $P_{x,y}$ of finding a given B_xV_y sequence, assuming Bernoullian (random) statistics for each oligomer, is given by

$$P_{x,y} = \binom{x+y}{x} P_B^x P_V^y \quad (2)$$

where P_B and P_V are the molar fractions of HB and HV in the copolymer. The binomial coefficient in this equation is the number of possible sequence arrangements of the

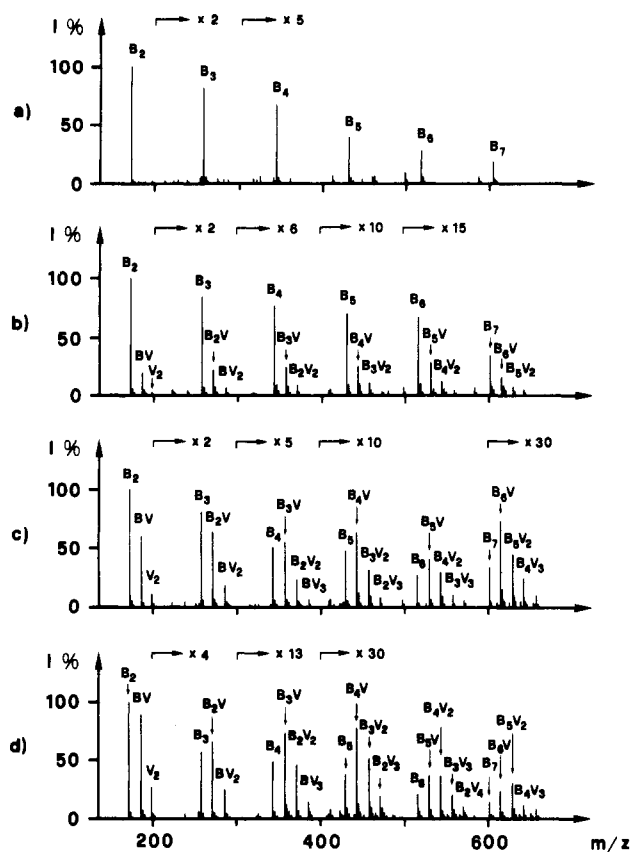


Figure 4. Negative ion FAB mass spectra of the TG residues from the partial pyrolysis of samples 1 (a), 2 (b), 4 (c), and 5 (d) (Table I).

B_xV_y oligomers. Starting with an arbitrary set of P_B and P_V values, it is possible to calculate the statistical abundances, $P_{x,y}$, for the oligomers actually present in the pyrolyzed copolymer. The best match between the experimentally observed oligomer distributions and the calculated statistical abundances identifies the copolymer composition.

The results of these calculations are reported in Table IV. The experimental data used are the normalized intensities of the oligomeric species appearing in the FAB spectrum in Figure 1. The agreement between the observed and calculated values was a function of the P_B/P_V ratio, and the best fit was for a ratio 85/15 (Table IV), i.e., almost the same value (86/14) obtained for the same sample when FAB mass spectra were obtained from lyophilized fractions of the methanolysis products.

The difference between observed and calculated values can be expressed in terms of error by means of the Hamilton¹⁴ agreement factor (AF):

$$AF = \left[\sum_i (I_{\text{exp},i} - I_{\text{calcd},i})^2 / \sum_i I_{\text{exp},i}^2 \right]^{1/2} \quad (3)$$

where $I_{\text{exp},i}$ and $I_{\text{calcd},i}$ are the normalized experimental and calculated abundances of partial pyrolysis products.

Figure 3 illustrates the calculated AF curves as a function of the copolymer composition for sample 3. Each curve corresponds to a specific set of oligomers (dimers, trimers, etc.), and it can be noticed that the minima indicate almost the same composition of about 15 mol % HV, whereas the AF value at the minimum is roughly constant (below 10%).

In Figure 4 are shown the partial pyrolysis FAB mass spectra of the remaining three P(HB-co-HV) copolymers investigated, together with the spectrum of the pure PHB homopolymer. Careful inspection of the spectra in Figure

Table V
Experimental and Calculated Normalized Abundances of Partial Pyrolysis Products for P(HB-co-HV) Samples

sample	copolymer ^a compn B/V										mixture compn A/B/X													
	B ₂	BV	V ₂	B ₃	B ₂ V	BV ₂	B ₄	B ₃ V	B ₂ V ₂	BV ₃	B ₅	B ₄ V	B ₃ V ₂	B ₂ V ₃	B ₆	B ₅ V	B ₄ V ₂	B ₃ V ₃	B ₂ V ₄					
2	expt ^b	84	15	1	79	18	3	75	22	3	70	23	6		65	26	9							
	calcd ^c	86	13	1	80	18	2	75	20	5	70	26	4		65	29	6							
3	expt ^b	69	27	4	64	30	6	55	32	10	3	45	35	16	4	40	38	17	5					
	calcd ^c	73	25	2	61	31	6	58	34	7	1	45	39	14	2	43	39	15	3	0.06				
4	expt ^b	59	35	6	49	40	11	38	40	17	5	32	42	20	6	24	39	27	10					
	calcd ^c	61	34	5	48	41	11	37	42	17	4	29	41	23	7	23	39	27	11	0.07				
5	expt ^b	49	41	10	39	44	17	28	40	25	7	21	41	28	10	18	30	30	15	7				
	calcd ^c	52	40	8	38	45	17	27	42	24	6	20	39	30	11	14	32	32	16	5				
6	expt ^b	70	24	6	65	25	10	60	22	14	4	54	22	16	8	56	16	19	9	0.10				
	calcd ^c	74	24	2	64	31	5	55	35	9	1	47	38	12	2	40	40	16	3	1/0.74/0.4 ^a				
7	expt ^b	76	20	4	69	22	9	64	21	12	3	60	19	15	6	59	17	16	8	0.50 ⁿ				
	calcd ^c															0.31				1/0.72/0.5 ^a				
7	expt ^b	66	30	4	59	30	11	48	31	18	3	42	32	18	8	39	31	20	10	0.07				
	calcd ^c	69	28	3	58	35	7	48	39	12	1	40	40	17	3	33	41	21	5	0.95/0.73/0.4 ^a				
	expt ^b	69	27	4	60	31	9	51	32	14	3	45	32	17	6	40	32	19	9	0.22				
	calcd ^c															0.15				0.93/0.72/0.5 ^a				
	AF ^d															0.06				0.04				

^a B/V best-fit values calculated by eqs 2 and 3. ^b Relative intensities of the peaks in FAB mass spectrum. ^c Relative intensities calculated by eqs 2 and 3 for the B/V best-fit composition values. ^d Agreement factor calculated by eq 3. ^e Relative intensities calculated by eqs 4-8 using the known values of A, B, and X from Table I. ^f B/V content (mol %) in the mixture from Table I. ^g A, B, and X best-fit values calculated with algorithm described in the text.

4 reveals that the relative intensities of the peaks within each cluster change sensibly with the HV content in the copolymer.

In Table V are reported the experimental and calculated normalized abundances of the partial pyrolysis products for the samples investigated and the corresponding AF for the best fit values.

From Table V it can be seen that for the pure copolymers (samples 2–5) the best-fit copolymer compositions, calculated by eqs 2 and 3, are in excellent agreement with those determined by ^1H NMR (reported in Table I).

In Figure 5b,c the FAB mass spectra are shown for the partial pyrolysis products of two physical mixtures of P(HB-co-HV), having an overall B/V composition very similar to that of the pure copolymer shown in Figure 5a.

Significant differences in the relative intensities of the peaks are discernible among the three spectra in Figure 5. When we tried to calculate the average copolymer compositions by means of eq 2, the AF values for the two mixtures (samples 6 and 7 in Table V) revealed an increasing error level going from lower to higher oligomers. This is shown in Figure 6, where the AF values calculated according to eq 3 for sample 6, are plotted as a function of the copolymer composition.

Contrary to the case reported in Figure 3, which pertains to a pure copolymer (sample 3, Table I), for the mixture in Figure 6 the minimal AF values show a drastic increment going from lower to higher oligomers, and furthermore the minima do not fall at the same composition value. This is because eq 2 assumes a Bernoullian (random) comonomer distribution, whereas sample 6 is actually a mixture of two polymers. Evidently, higher oligomers are more sensitive in discriminating a pure copolymer from a mixture because they carry more specific sequential information. This example indicates the high discriminating power of the present MS method, which is capable of determining the relative abundances of oligomers up to the hexamers.

Furthermore, we have verified the absence of ester exchange processes in several poly(β -lactones) and in P(HB-co-HV) mixtures, when they are heated in a TG experiment, and have concluded that the rate of the hydrogen-transfer process described in eq 1 is much higher than that of the transesterification reaction, below 220 $^{\circ}\text{C}$, for poly(β -lactones).¹⁵

Determination of the Copolymer Composition in the Case of Two-Component Mixtures

Having developed a rapid method for determining the composition of a random copolymer and for discriminating a pure copolymer from a mixture of copolymers, we wish now to discuss an algorithm for determining the composition of the two copolymers that constitute the blend.

Given a mixture of two Bernoullian random P(HB-co-HV) copolyesters, let A be the molar fraction of HB in the copolyester 1, B the molar fraction of HB in the copolyester 2, and X the molar fraction of copolyester 1 in the mixture. Then the following equations can be written for each set of oligomers:

dimers

$$\begin{aligned} F_{B_2} &= A^2X + B^2(1-X) \\ F_{B_1V} &= 2[A(1-A)X + B(1-B)(1-X)] \\ F_{V_2} &= (1-A)^2X + (1-B)^2(1-X) \end{aligned} \quad (4)$$

trimers

$$\begin{aligned} F_{B_3} &= A^3X + B^3(1-X) \\ F_{B_2V} &= 3[A^2(1-A)X + B^2(1-B)(1-X)] \\ F_{B_1V_2} &= 3[A(1-A)^2X + B(1-B)^2(1-X)] \\ F_{V_3} &= (1-A)^3X + (1-B)^3(1-X) \end{aligned} \quad (5)$$

tetramers

$$\begin{aligned} F_{B_4} &= A^4X + B^4(1-X) \\ F_{B_3V} &= 4[A^3(1-A)X + B^3(1-B)(1-X)] \\ F_{B_2V_2} &= 6[A^2(1-A)^2X + B^2(1-B)^2(1-X)] \\ F_{B_1V_3} &= 4[A(1-A)^3X + B(1-B)^3(1-X)] \\ F_{V_4} &= (1-A)^4X + (1-B)^4(1-X) \end{aligned} \quad (6)$$

pentamers

$$\begin{aligned} F_{B_5} &= A^5X + B^5(1-X) \\ F_{B_4V} &= 5[A^4(1-A)X + B^4(1-B)(1-X)] \\ F_{B_3V_2} &= 10[A^3(1-A)^2X + B^3(1-B)^2(1-X)] \\ F_{B_2V_3} &= 10[A^2(1-A)^3X + B^2(1-B)^3(1-X)] \\ F_{B_1V_4} &= 5[A(1-A)^4X + B(1-B)^4(1-X)] \\ F_{V_5} &= (1-A)^5X + (1-B)^5(1-X) \end{aligned} \quad (7)$$

hexamers

$$\begin{aligned} F_{B_6} &= A^6X + B^6(1-X) \\ F_{B_5V} &= 6[A^5(1-A)X + B^5(1-B)(1-X)] \\ F_{B_4V_2} &= 15[A^4(1-A)^2X + B^4(1-B)^2(1-X)] \\ F_{B_3V_3} &= 20[A^3(1-A)^3X + B^3(1-B)^3(1-X)] \\ F_{B_2V_4} &= 15[A^2(1-A)^4X + B^2(1-B)^4(1-X)] \\ F_{B_1V_5} &= 6[A(1-A)^5X + B(1-B)^5(1-X)] \\ F_{V_6} &= (1-A)^6X + (1-B)^6(1-X) \end{aligned} \quad (8)$$

where $F_{B_nV_m}$ is the normalized intensity of the MS peak corresponding to each B_nV_m oligomer. The normalization factor is the sum of intensities within each set of equations.

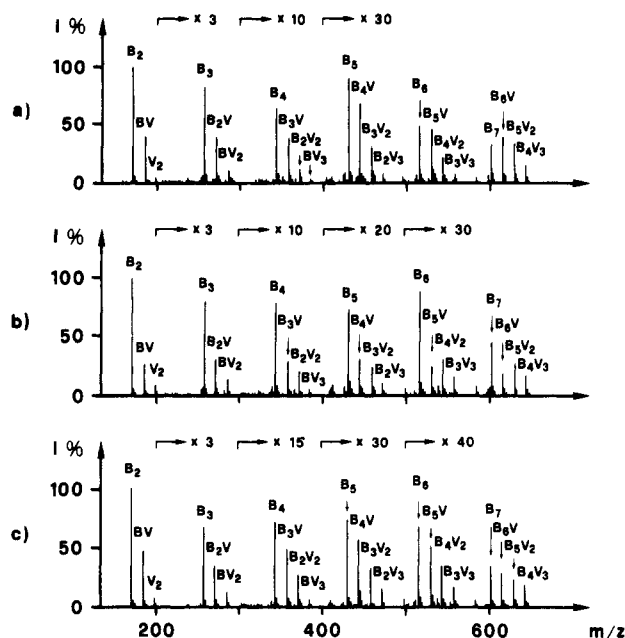


Figure 5. Negative ion FAB mass spectra of the TG residues from the partial pyrolysis of samples 6 (a), 7 (b), and 3 (c) (Table I).

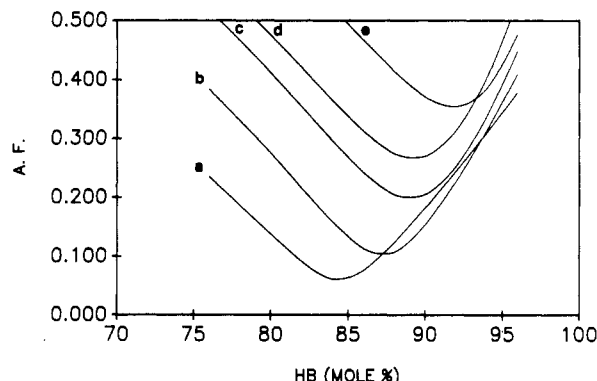


Figure 6. Agreement factor (AF) versus HB content (mol %) corresponding to each set of peak intensities for (a) dimers, (b) trimers, (c) tetramers, (d) pentamers, and (e) hexamers from sample 6.

For each term (A, B, X), these equations yield 25 values. The composition of the copolymer mixture is that which gives 25 values closest to the experimental data.

A FORTRAN-77 best-fit program named Mass Analysis of Copolymers (MACO) has been written that runs on a Microvax computer (MicroVMS4.2). The program finds the composition (A, B, X) that best fits the 25 experimental normalized intensities and employs the minimization routine MINPACK1 belonging to Argonne Library.¹⁶

In Table V the best-fit values of A, B , and X are reported for the two mixtures analyzed.

In Table V are also reported the normalized oligomer abundances calculated by using eqs 4–8 with the known values A, B and X from Table I for each mixture.

It can be seen that these calculated relative intensities are in good agreement with the experimental ones, confirming that the FAB-MS is a suitable technique for determining the relative amounts and copolymer compositions of binary P(HB-co-HV) mixtures.

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