



Certificate of Analysis

Standard Reference Material[®] 869b

Column Selectivity Test Mixture for Liquid Chromatography (Polycyclic Aromatic Hydrocarbons)

This Standard Reference Material (SRM) is a mixture of three polycyclic aromatic hydrocarbons (PAHs) in acetonitrile: benzo[*a*]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN, alternate name, dibenzo[*g,p*]chrysene), and phenanthro[3,4-*c*]phenanthrene (PhPh) (see Figure 1 for structures). SRM 869b is useful for characterizing liquid chromatographic (LC) column selectivity for separation of PAHs [1,2]. Depending on the elution order of the three components, column selectivity can be predicted for complex PAH mixtures (particularly isomeric PAHs). Even though the primary use of this mixture is to characterize columns for PAH separations, applications to the assessment of column selectivity for other classes of compounds such as carotene isomers have also been demonstrated [4]. A unit of SRM 869b consists of 5 ampoules, each containing 1.1 mL of the PAH mixture. The concentrations and relative responses of the components at 254 nm are listed in Tables 1, 2, and A-1.

Certified Mass Fraction Value: The certified value of benzo[*a*]pyrene is given in Table 1. The uncertainty is the expanded uncertainties at the 95 % level of confidence [1]. The uncertainty includes components ascribed to the calibration of the chromatographic method, measurement of selected samples using the chromatographic method, and purity of the reagents used to prepare the material. The certified value and expanded uncertainty defines a range of values within which the true concentration is expected to lie with 95 % confidence. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST.

Table 1. Certified Values for Benzo[*a*]pyrene in SRM 869b

Compound	CAS Registry No. ^(a)	Mass Fraction ^(b) (mg/kg)
Benzo[<i>a</i>]pyrene (BaP)	50-32-8	2.06 ± 0.05

^(a) Chemical Abstracts, Fourteenth Collective Index Guide, American Chemical Society, Columbus, Ohio, 2001.

^(b) The results are expressed as the certified value ± the expanded uncertainty. The certified value is the mean of the concentrations determined by gravimetric and chromatographic measurements. The expanded 95 % uncertainty uses a coverage factor of 2 and includes both correction for estimated purity and allowance for differences between the concentration determined by gravimetric preparation and chromatographic measurements. The measurand is the certified mass fraction value in Table 1 and is metrologically traceable to the derived SI unit for mass fraction expressed as milligrams per kilogram.

Expiration of Certification: The certification of SRM 869b is valid, within the measurement uncertainties specified, until **30 September 2027**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see “Instructions for Use”). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The coordination of the technical measurements leading to certification was performed under the direction of L.C. Sander and S.A. Wise of the NIST Chemical Sciences Division.

Carlos A. Gonzalez, Chief
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Certificate Issue Date: 27 July 2016
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Steven J. Choquette, Acting Director
Office of Reference Materials

Preparation and analytical determinations were carried out by L.C. Sander and C.A. Rimmer of the NIST Chemical Sciences Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

Information Mass Fraction Values: Information concentration values are provided for dibenzo[*g,p*]chrysene, and phenanthro[3,4-*c*]phenanthrene in Table 2. An information value is a value that may be of interest to the SRM user, but insufficient information is available to assess the uncertainty associated with the value therefore no uncertainty is provided. Information values cannot be used to establish metrological traceability.

Table 2. Information Values for PAHs in SRM 869b

Compound	CAS Registry No. ^(a)	Mass Fraction ^(b) (mg/kg)
Dibenzo[<i>g,p</i>]chrysene (TBN)	191-68-4	11.27
Phenanthro[3,4- <i>c</i>]phenanthrene (PhPh)	187-83-7	3.20

^(a) Chemical Abstracts, Fourteenth Collective Index Guide, American Chemical Society, Columbus, Ohio, 2001.

^(b) The information value is based on gravimetric measurements.

NOTICE AND WARNINGS TO USERS

Toxicity: This test mixture contains small amounts of polycyclic aromatic hydrocarbons, some of which have been reported to have mutagenic and/or carcinogenic properties. Therefore, care should be exercised during handling and use (see “Instructions for Use” and Safety Data Sheet). Use proper methods for disposal of waste.

INSTRUCTIONS FOR STORAGE AND USE

Storage: Sealed ampoules, as received, should be stored in the dark at temperatures between 10 °C and 30 °C.

Conditions: This test mixture is intended primarily for the characterization of C18 columns used in the reversed-phase liquid chromatographic separation of PAHs. To compare columns on the same basis, the user should evaluate column selectivity by chromatographing the mixture isocratically under the following conditions: mobile phase, 85 % acetonitrile/water (volume fraction) at 25 °C ± 2 °C. Changes in both absolute retention and selectivity can occur with changes in temperature [5] and composition [6]; thus these conditions should be used for all column evaluations to enable comparisons with the results reported in this certificate.

Representative separations of the test mixture are illustrated in Figure 2 (A-E) for several different C18 columns and separation conditions. These chromatograms are examples of the five possible cases for solute elution order, and are provided as an aid for component identification. In cases where complete separation is achieved (Figures 2A, 2C, and 2E), BaP is the smallest peak, PhPh is intermediate in size, and TBN the largest peak when using UV detection at 254 nm (see the Appendix, Table A-1 for peak height and peak area data).

Interpretation of Results: The elution order of the three components changes with C18 stationary phase type [7] as shown in Figure 3. Monomerically bonded (“monomeric”) C18 phases (prepared using monofunctional silane reagents) have been shown to give the elution order BaP ≤ PhPh < TBN (Figures 2A and 2B). Stationary phases prepared using polymeric surface modification procedures (“polymeric”, i.e., using trifunctional silanes in the presence of water) have the elution order PhPh < TBN ≤ BaP (Figures 2D and 2E). Stationary phases with intermediate properties (i.e., densely loaded monomeric or lightly loaded polymeric C18 phases) are indicated by the elution order PhPh < BaP < TBN (see Figure 2C).

A measure of stationary phase selectivity can be calculated to enable relative comparisons between different C18 phases. The selectivity factor $\alpha_{\text{TBN/BaP}}$ (defined as $k'_{\text{TBN}} / k'_{\text{BaP}}$, where $k' = (t_{\text{R}} - t_0) / t_0$, and t_{R} and t_0 are the retention times of the analyte and void volume marker, respectively) has been shown to correlate with PAH selectivity and stationary phase type [2,3,7-9]. It should be noted that while the separation of isomeric PAH mixtures is often achieved with polymeric C18 phases (or other stationary phases with component elution PhPh < TBN < BaP), this type of stationary phase may not be the best for a given separation problem. This column evaluation test mixture enables columns to be grouped into well characterized classes to facilitate method development. A classification scheme has been proposed, based on measurement of $\alpha_{\text{TBN/BaP}}$ values for experimental and commercial C18 columns [2]. Values for $\alpha_{\text{TBN/BaP}} \leq 1$ reflect polymeric C18 phases, and values for $\alpha_{\text{TBN/BaP}} \geq 1.7$ reflect monomeric C18 phases. For values $1 < \alpha_{\text{TBN/BaP}} <$

1.7, the synthesis scheme is less certain, and may indicate light polymerization with di- or trifunctional reagents, or densely loaded monomeric phases.

Relationship Between Retention Order and PAH Selectivity: Two examples of changes in stationary phase selectivity are illustrated in Figures 4 and 5. The separation of PAH isomers of molecular weight 278 (see also Figures 7 and 8) is shown in Figure 4. Selectivity factors for four commercial and experimental columns range from 0.38 to 1.80. The best separation of these isomers was achieved with the most heavily loaded polymeric C18 phase, with $\alpha_{\text{TBN/BaP}} = 0.38$. The lowest selectivity for these isomers was found with a monomeric phase ($\alpha_{\text{TBN/BaP}} = 1.80$). Another example of stationary phase selectivity differences is provided in Figure 5. SRM 1647a Priority Pollutant Polycyclic Aromatic Hydrocarbons in Acetonitrile (the current release is designated SRM 1647f and is similar in composition) was separated using gradient elution conditions on the four columns with $\alpha_{\text{TBN/BaP}}$ selectivity factors which ranged from 0.38 to 1.80. Separation of this mixture (required by EPA Method 610) should be possible with C18 columns having $\alpha_{\text{TBN/BaP}}$ values in the range $\approx 0.5 < \alpha_{\text{TBN/BaP}} < \approx 0.9$. Separation can also be achieved for $\alpha_{\text{TBN/BaP}} < \approx 0.4$. Complete separation of all 16 components was possible with the two polymeric C18 columns ($\alpha_{\text{TBN/BaP}} = 0.65$ and 0.38), but with a reversal in the elution of dibenz-[a,h]anthracene and benzo[ghi]perylene on the heavily loaded polymeric C18 phase. Separation of the components occurred over the widest time interval, with the heavily loaded polymeric C18 phase ($\alpha_{\text{TBN/BaP}} = 0.38$). Separation of all components is generally not possible for $\alpha_{\text{TBN/BaP}} > \approx 0.9$; however, by modifying column selectivity by changing chromatographic conditions (e.g., reducing column temperature, see “Temperature”), baseline separation of all 16 components can be achieved.

DISCUSSION

The selectivity factor $\alpha_{\text{TBN/BaP}}$ has been shown to vary with stationary phase type [7], substrate properties [10], alkyl chain length of bonded ligand [11], stationary phase density [8], mobile phase composition [6] and column temperature [5]. In general, column selectivity for complex PAH mixtures (such as PAH isomers) increases with decreasing $\alpha_{\text{TBN/BaP}}$. Furthermore, separations often can be reproduced under different conditions (i.e., different combinations of stationary phase type, alkyl chain length, column temperature, and mobile phase composition) as long as $\alpha_{\text{TBN/BaP}}$ is held constant. Thus by adjusting these chromatographic variables, differences in column selectivity among columns from different manufacturers may be compensated.

In practice, stationary phase type, alkyl chain length, and pore size are most easily varied by changing columns. As column manufacturers begin to provide $\alpha_{\text{TBN/BaP}}$ data on their C18 columns, column selection and method development will be facilitated. A few trends, however, can be summarized.

Stationary Phase Type: The single most important parameter affecting column selectivity is bonded phase chemistry [7]. As described above, stationary phases prepared using polymeric surface modification chemistry will usually have $\alpha_{\text{TBN/BaP}}$ values < 1 , and will exhibit increased selectivity for PAH isomers compared to monomeric C18 phases ($\alpha_{\text{TBN/BaP}} > 1.7$, Figure 3). Stationary phases prepared using limited polymeric syntheses, or prepared using highly reactive monomeric syntheses may result in intermediate properties, (i.e., $1 < \alpha_{\text{TBN/BaP}} < 1.7$).

Pore Size: Stationary phase selectivity is also dependent on pore size of the silica substrate. For polymeric C18 phases, the ability to separate PAH isomers (shape selectivity) increases with increasing pore size [10]. For monomeric C18 phases, little dependence is observed between column selectivity and pore size. For polymeric C18 phases, however, selectivity shifts with changes in pore diameter, becoming more shape selective (more polymeric-like) with increasing pore size. Benz[a]anthracene and chrysene coelute on 6 nm pore diameter polymeric C18 columns, but are fully resolved on 30 nm pore diameter polymeric C18 columns. Similarly, benzo[ghi]perylene and indeno[1,2,3-cd]pyrene are unresolved on the narrow pore polymeric C18 columns, but are resolved on the wide pore polymeric C18 columns. For both monomeric and polymeric C18 phases, these trends are reflected in the retention behavior of the column selectivity test mixture.

Stationary Phase Length: Changes in column selectivity are also observed for stationary phases with different alkyl chain lengths [11]. In general, shape selectivity increases with increasing alkyl chain length. The elution order for the selectivity test mixture is as expected for the monomeric and polymeric C18 phases. Benzo[a]pyrene elutes earlier than normal on stationary phases shorter than C18 and longer than normal on phases longer than C18. This trend is summarized in Figure 6 as a plot of selectivity ($\alpha_{\text{TBN/BaP}}$) VS. stationary phase length. It is interesting to note that stationary phase selectivity of monomeric and polymeric phases merge at short and long alkyl chain lengths. Monomeric and polymeric C8 phases have similar selectivity toward PAH, as do monomeric and polymeric C30 phases. Again, the selectivity factor $\alpha_{\text{TBN/BaP}}$ is indicative of overall stationary phase selectivity for more complex PAH mixtures [11].

Stationary Phase Coverage: Among polymeric C18 phases, selectivity has been shown to vary with the density or coverage of the polymeric layer [8]. A similar but more subtle trend has been demonstrated for monomeric C18 phases [12]. Shape selectivity for PAH isomers increases with increasing stationary phase density. This trend is illustrated for a series of PAH isomers (MW 278) in Figures 7 and 8, for which retention increases with increasing solute length-to-breadth [13]. Baseline resolution of the isomers was achieved on a heavily loaded polymeric C18 phase, but only partial resolution was possible on lower loaded polymeric C18 phases. Very little resolution of the isomers was achieved on monomeric C18 columns.

Temperature: Temperature can have a significant effect on stationary phase selectivity [5]. PAH shape recognition increases dramatically with decreasing temperature. Conversely, shape recognition decreases with increasing temperature. These trends are reflected in the retention behavior of the selectivity test mixture as illustrated in Figure 9. The relative retention of BaP increases with decreasing temperature, indicating an increase in shape selectivity. This behavior has been verified for several complex PAH mixtures, including SRM 1647a and the six methylchrysene isomers [5]. Although Figure 9 illustrates temperature effects for a polymeric C18 column, the change in selectivity with temperature is a general phenomenon that is not dependent on stationary phase type or column manufacturer.

The dependence of selectivity on temperature for a typical monomerically bonded and polymerically bonded column is summarized in a plot of $\alpha_{\text{TBN/BaP}}$ versus temperature in Figure 10. Changes in selectivity are relatively uniform for the polymeric C18 phase. In contrast, changes in selectivity for the monomeric C18 phase are most significant at subambient temperatures; selectivity is nearly constant above 25 °C. Two possibilities are suggested by this plot. It should be possible to duplicate “polymeric-like” selectivity with a monomeric column, by performing the separation at reduced temperature. This possibility has been demonstrated for the separation of SRM 1647a [5]. Furthermore, it should be possible to achieve column selectivity characteristics not possible with available columns, (i.e., by cooling polymeric C18 columns). This possibility has been demonstrated for a separation of methylchrysene isomers which were previously not separable by liquid chromatography [5]. Thus, column selectivity may be adjusted as required by altering column temperature. Although the temperature dependence of $\alpha_{\text{TBN/BaP}}$ will vary for individual columns, the plots in Figure 10 provide a basis for tentative predictions.

Application to Carotenoid Separations: Many of the selectivity trends described above for PAH isomers are also observed for carotenoids [4, 14]. As with PAH isomers, better separations of carotenoid isomers usually result from the use of polymeric C18 phases compared with monomeric phases. In addition, stationary phases prepared with long (C30) alkyl chains have enhanced selectivity toward carotenoids [14]. These properties are indicated by $\alpha_{\text{TBN/BaP}} < 1$. It should be noted that other stationary phase properties not indicated by SRM 869b may also be important in the assessment of column suitability for carotenoid separations. For example, silanol activity has been shown to influence the separation of xanthophylls (polar carotenoids) [14].

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Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>.

APPENDIX

The following supplementary information is supplied for the convenience of the user of this material. This information does not meet the requirements for certification by the National Institute of Standards and Technology.

Descriptive Information

This information is provided as an aid for peak identification. The values listed are not intended for quantitative comparisons.

Table A-1: Relative Responses at 254 nm

Compound	Relative Response ^(a)									
	A		B		C		D		E	
BaP	1	[1]	1 ^(b)	[1] ^(b)	1	[1]	1 ^(b)	[1] ^(b)	1	[1]
PhPh	1.55	[1.76]	1 ^(b)	[1] ^(b)	2.09	[1.96]	0.54	[0.64]	3.56	[2.24]
TBN	2.63	[4.29]	1.05	[1.55]	2.91	[4.35]	1 ^(b)	[1] ^(b)	6.66	[5.63]

^(a) These measurements refer to the separations in Figure 2 (A-E). Values represent peak heights and peak areas (areas are shown in brackets) relative to BaP, and are listed only as an aid for peak identification.

^(b) Note that for cases B and D, BaP coelutes with PhPh or TBN, respectively, and the peak heights and peak areas reported are relative to these coeluting components.

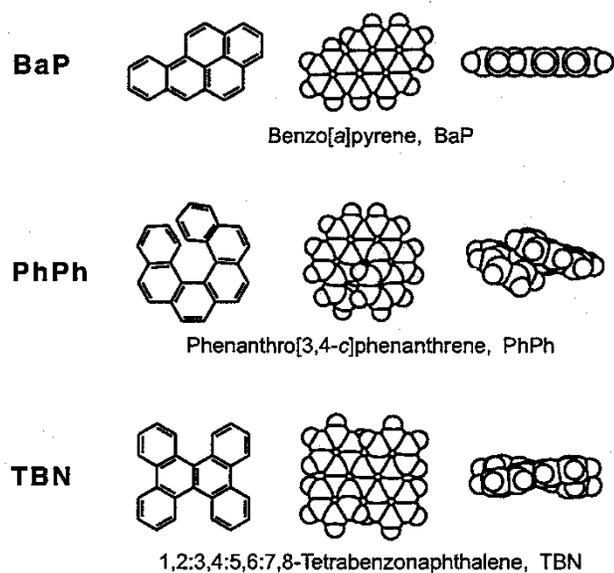


Figure 1. Structures and space filling models for compounds in SRM 869b.

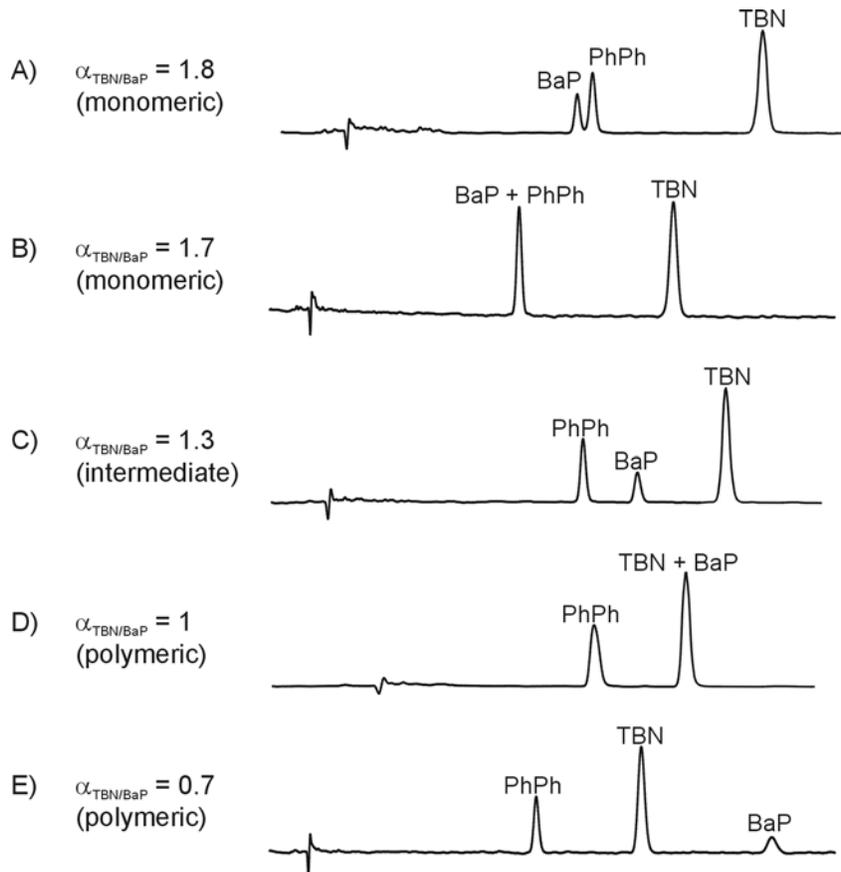


Figure 2. Separation of SRM 869b on LC columns with dissimilar properties. Monomeric and polymeric designations refer to stationary phases prepared with monomeric or polymeric surface modification chemistry; intermediate designation refers to a stationary phase (possibly monomeric or polymeric) with intermediate properties. These chromatograms are provided to aid peak identification.

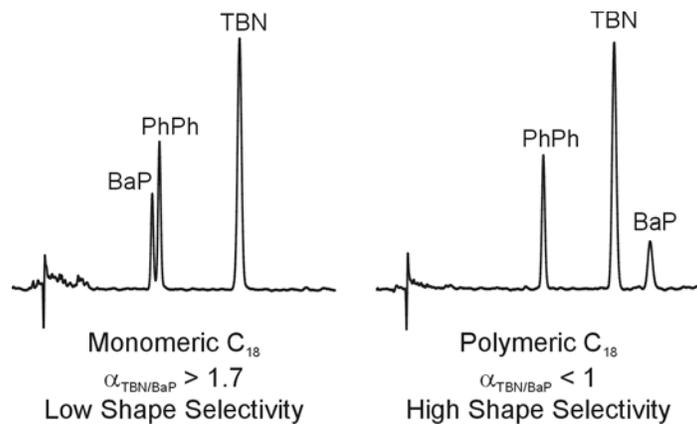


Figure 3. Separation of SRM 869b on C18 columns prepared with monomeric and polymeric surface modification chemistry.

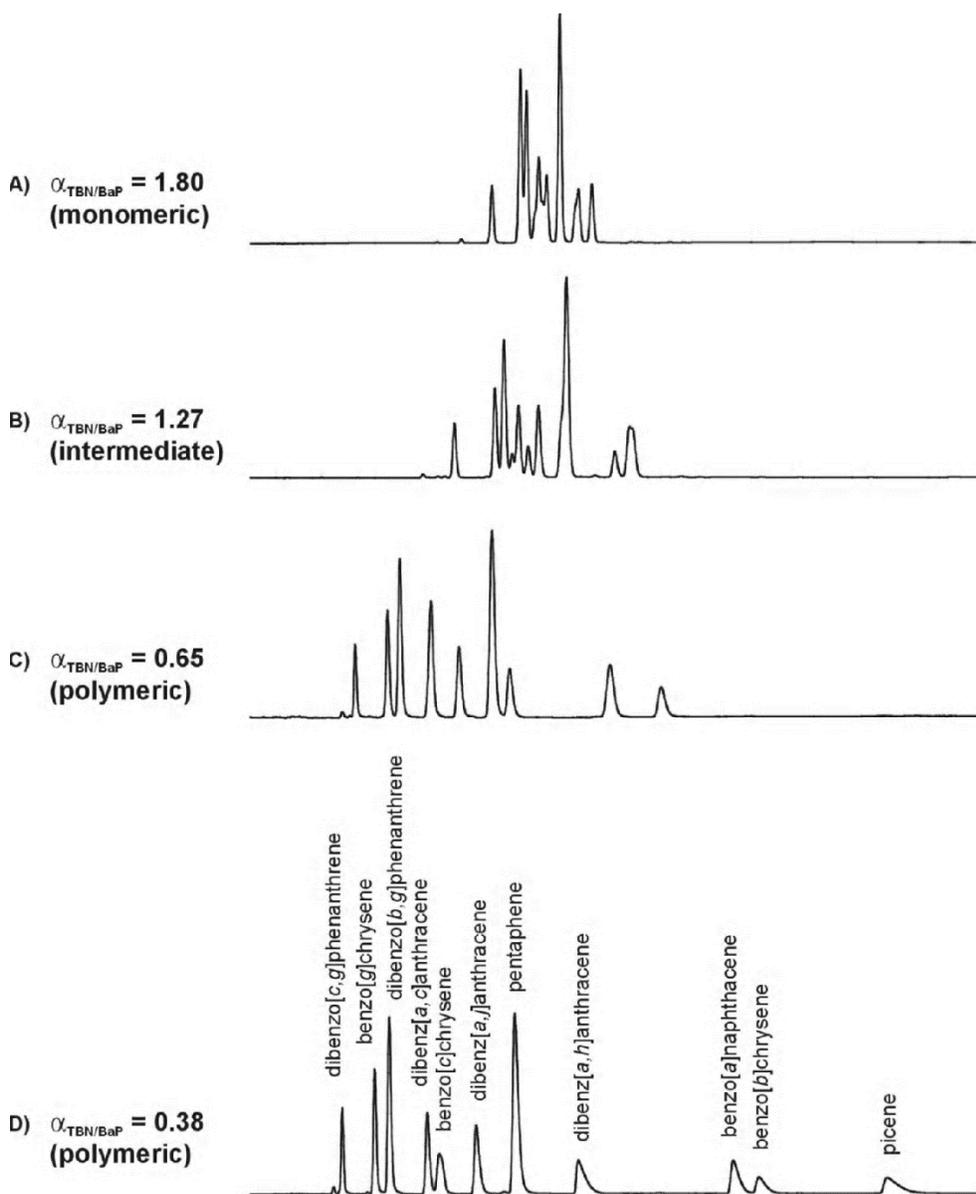


Figure 4. Separation of 11 PAH isomers of molecular weight 278 on C18 columns prepared using monomeric and polymeric surface modification chemistry.

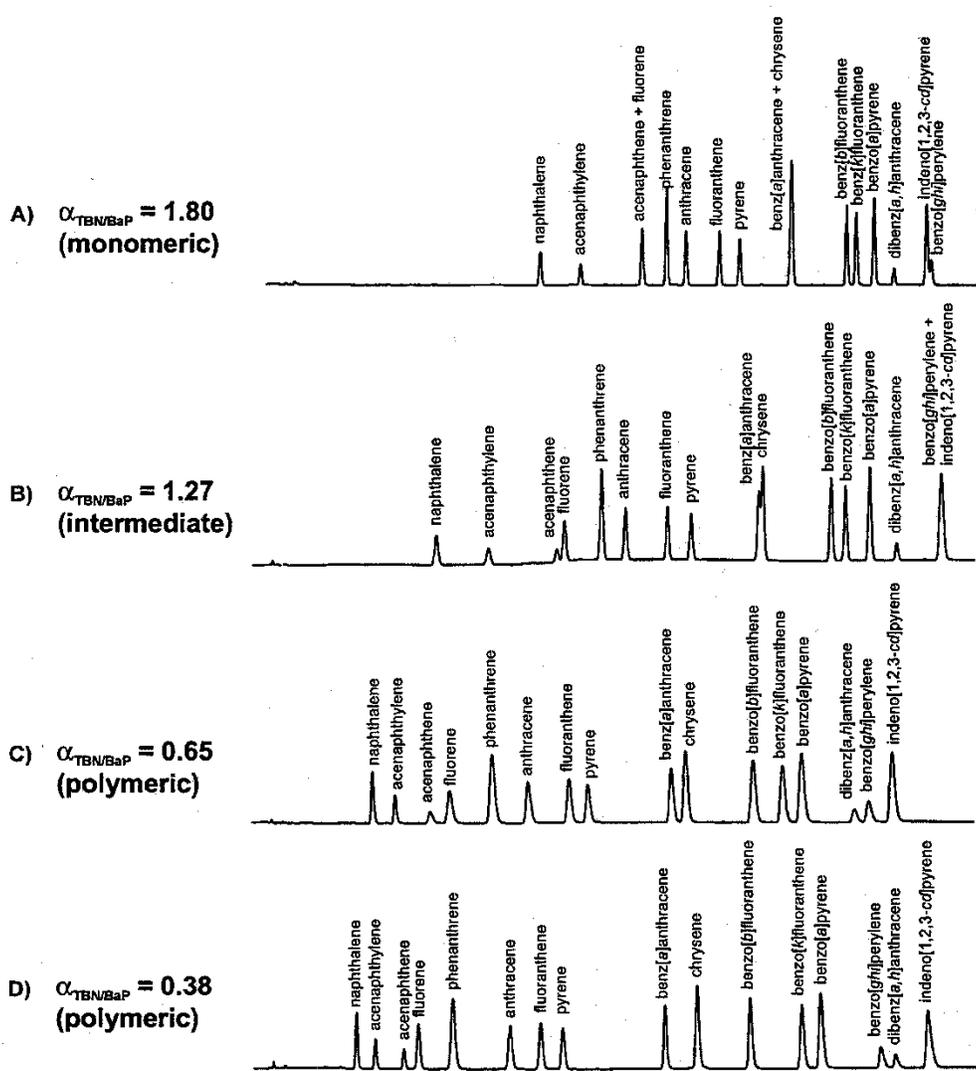


Figure 5. Separation of SRM 1647a on the same C18 columns as in Figure 4. Complete separation of all 16 components was only possible with the C18 columns prepared using polymeric surface modification chemistry ($\alpha_{TBN/BaP} = 0.65$ and 0.38).

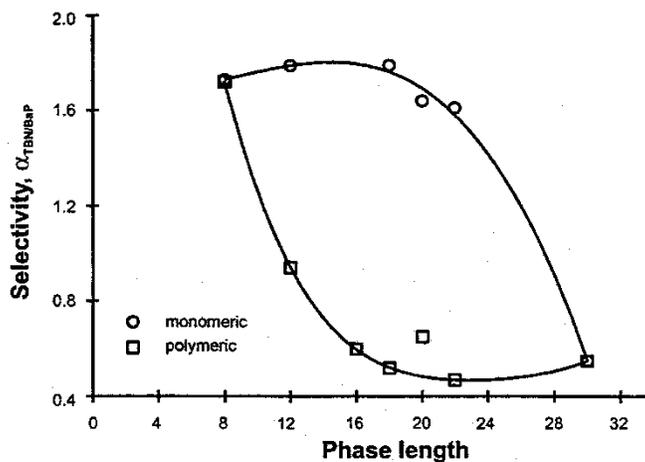


Figure 6. Plot of column selectivity ($\alpha_{TBN/BaP}$) vs. alkyl chain length for stationary phases prepared using monomeric and polymeric surface modification chemistry.

	Structure	Name	L/B
1		Dibenzo[c,g]phenanthrene	1.12
2		Benzo[g]chrysene	1.32
3		Dibenzo[b,g]phenanthrene	1.33
4		Dibenz[a,c]anthracene	1.24
5		Benzo[c]chrysene	1.47
6		Dibenz[a,j]anthracene	1.47
7		Pentaphene	1.73
8		Dibenz[a,h]anthracene	1.79
9		Benzo[a]naphthacene	1.77
10		Benzo[b]chrysene	1.84
11		Picene	1.99

Figure 7. Structures and associated length-to-breadth ratios¹⁰ for PAH isomers of molecular weight 278.

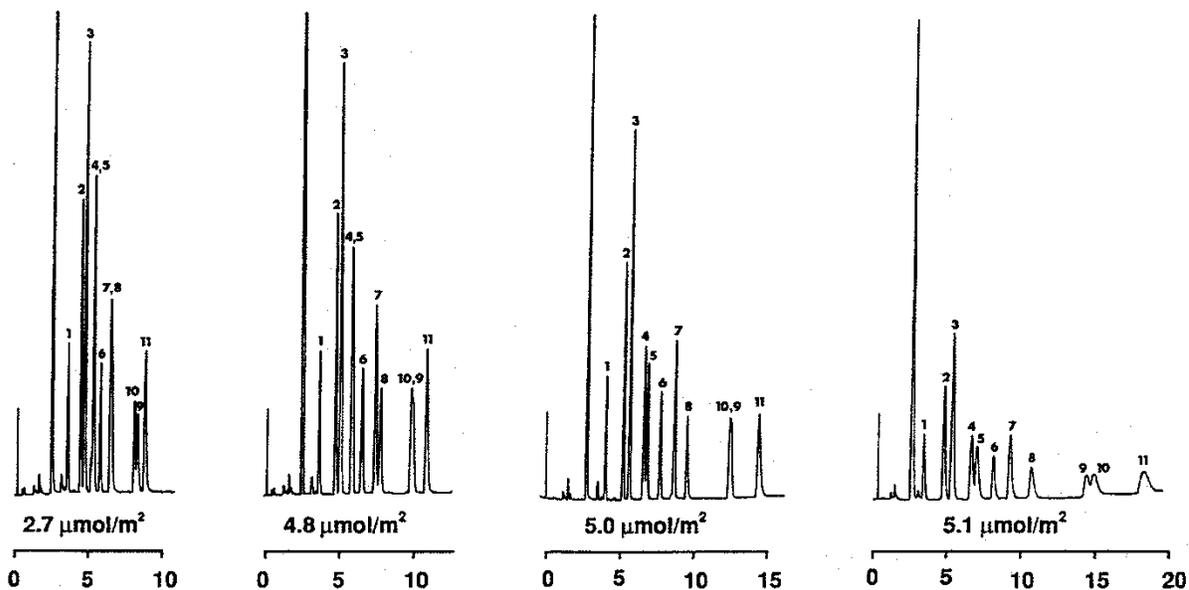


Figure 8. Separation of molecular weight 278 isomers on polymeric C18 columns with different stationary phase loadings. Loadings are indicated in terms of surface coverage values, $\mu\text{mol}/\text{m}^2$. See Figure 7 for compound identification.

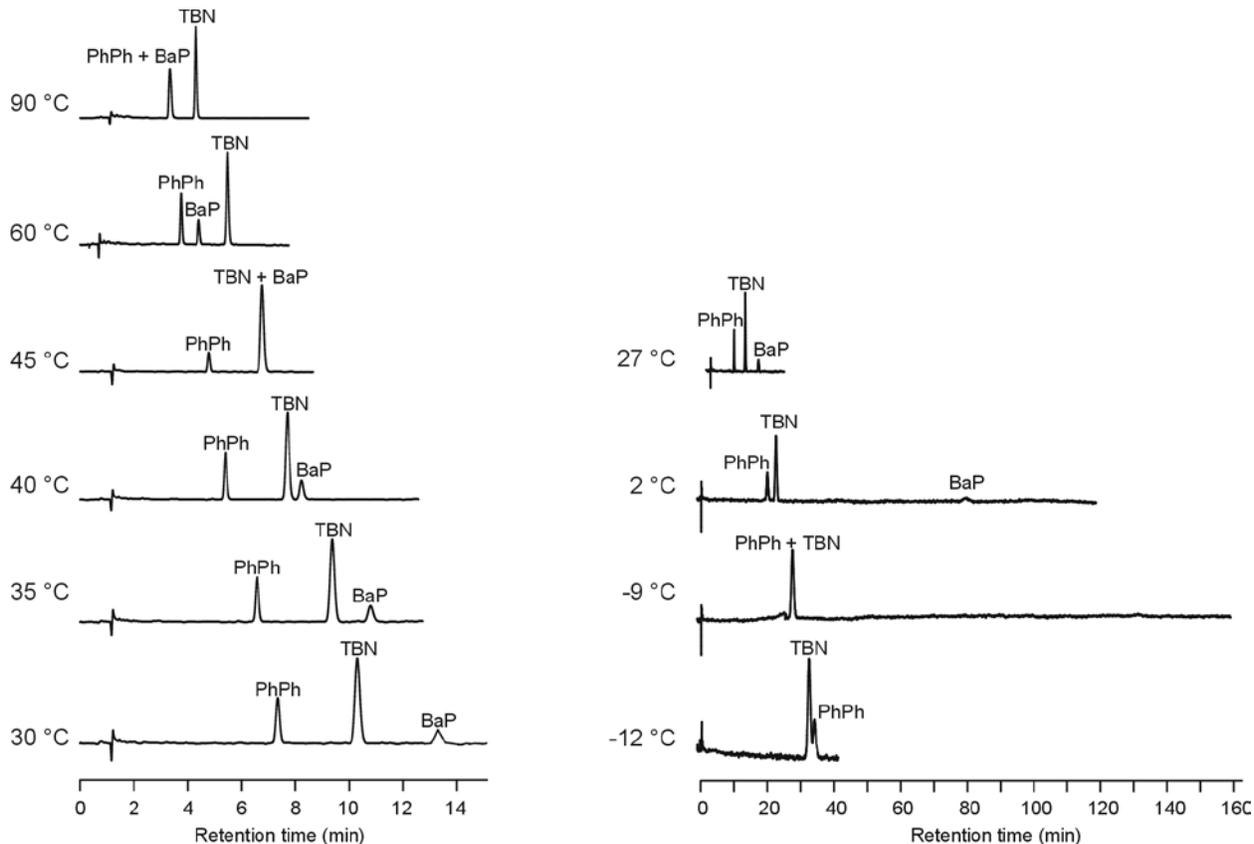


Figure 9. Separation of SRM 869b on a polymeric C18 column at different temperatures. Subambient separations were carried out using 85 % acetonitrile/water; separations at elevated temperatures were carried out using 75 % acetonitrile.

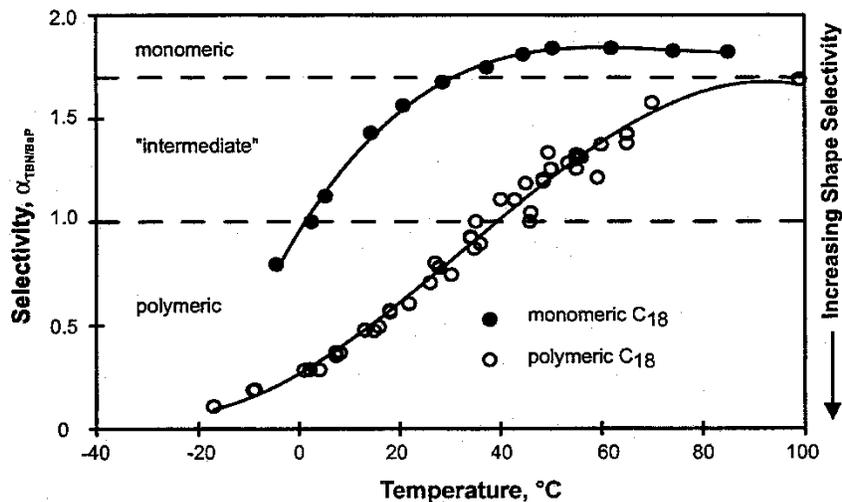


Figure 10. Stationary phase selectivity ($\alpha_{TBN/BaP}$) plotted as a function of temperature for monomeric (●) and polymeric (○) C18 columns. Mobile phase composition for the monomeric C18 column was 85:15 acetonitrile/water; various acetonitrile/water compositions were used with the polymeric C18 column.