National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 870

Column Performance Test Mixture for Liquid Chromatography

This Standard Reference Material (SRM) is a mixture of five organic compounds in methanol and is intended for use in characterizing general aspects of liquid chromatographic (LC) column performance, including efficiency, void volume, methylene selectivity, retentiveness, and activity toward chelators and organic bases. Other possible uses include (1) column classification to aid column selection during method development, (2) as a control material for monitoring LC column performance over time, and (3) in quality control for column manufacturing. SRM 870 consists of a mixture of the following five organic compounds in methanol: uracil, toluene, ethyl benzene, quinizarin, and amitriptyline (added as amitriptyline hydrochloride; see Figure 1 for structures). A unit of SRM 870 consists of five ampoules each containing approximately 1.1 mL of the mixture.

Certified Mass Fraction Values: The measurand is the certified mass fraction values for uracil, toluene, ethyl benzene, quinizarin, and amitriptyline hydrochloride are provided in Table 1. 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 taken into account [1]. Values were derived from the combination of results from liquid chromatography with ultraviolet absorbance detection (LC-UV) and from the gravimetric preparation of the solution. The associated uncertainties are expanded uncertainties at the 95 % level of confidence. The expanded uncertainty is calculated as $U = ku_c$, where u_c incorporates the observed difference between the results from the methods and their respective uncertainties, as well as uncertainty related to purity correction, consistent with the ISO/JCGM Guide and its Supplement 1, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte [2–4]. Values are reported on an as-received basis in mass fraction units. Metrological traceability is to the derived SI unit for mass fraction (expressed as milligrams per kilogram).

Information Values for Detection Response: Relative detection responses of the components are listed 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 or only a limited number of analyses were performed [1]. Information values cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 870** is valid, within the measurement uncertainty specified, until **30 September 2025**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Handling, Storage, and 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 or register online) will facilitate notification.

Preparation and analytical determinations were carried out by L.C. Sander and B.A. Benner, Jr. of the NIST Chemical Sciences Division.

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

Carlos A. Gonzalez, Chief
Chemical Sciences Division

Gaithersburg, MD 20899
Certificate Issue Date: 07 July 2016
Certificate Revision History on Last Page

Steven J. Choquette, Acting Director
Office of Reference Materials

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INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

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

Chromatographic Conditions: This test mixture is intended primarily for the characterization of C_{18} columns used in reversed-phase liquid chromatography. To compare columns on the same basis, the user should evaluate column performance by separating the five components of the mixture isocratically under the following conditions: mobile phase 80 % methanol and 20 % aqueous buffer (volume fractions), flow rate 1 mL/min, column temperature 23 °C, injection volume 1 µL to 2 µL. The recommended buffer composition is 20 mmol/L potassium phosphate adjusted to pH 7.0 (final phosphate concentration in the mixed methanol/buffer mobile phase is 4 mmol/L). This buffer can be prepared by mixing 20 mmol/L monobasic potassium phosphate (KH_2PO_4) and 20 mmol/L dibasic potassium phosphate (K_2HPO_4) solutions to obtain the desired pH 7.0 solution, as indicated by a pH meter. Because changes in absolute retention, selectivity, and peak shape can occur with changes in temperature and composition, these conditions should be used for all column evaluations. Figure 1 shows the structures and properties evaluated for components in SRM 870.

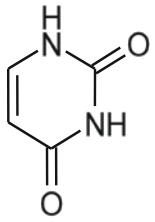
PREPARATION AND ANALYSIS[1]

A methanol solution of uracil, toluene, ethyl benzene, quinizarin, and amitriptyline hydrochloride (see Table 2 for sources) was prepared gravimetrically from individual compounds. The solution was stirred for approximately 13 h and then aliquoted into 2-mL amber glass ampoules, which were purged with argon prior to addition of the solution. Levels of the constituents were determined by liquid chromatography with absorbance detection at 210 nm, using a 25 cm × 4.6 mm ACE 5 C_{18} column (Mac-Mod Analytical Inc., Chadds Ford, PA) and with the conditions listed above (column temperature was maintained at 30 °C to improve the resolution of quinizarin and amitriptyline for purposes of quantitation).

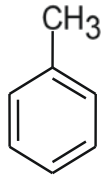
Table 1. Certified Mass Fraction Values for Constituents of SRM 870

Compound	Mass Fraction (mg/kg)	Coverage Factor, *k*
Uracil	27.1 ± 1.3	2
Toluene	1430 ± 40	2
Ethyl Benzene	1730 ± 40	2
Quinizarin	90.8 ± 2.5	2
Amitriptyline HCl	2740 ± 150	2

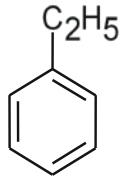
[1] Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.



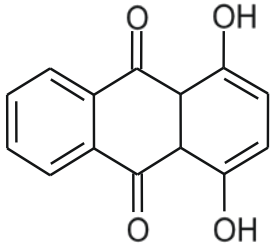
uracil - void volume marker



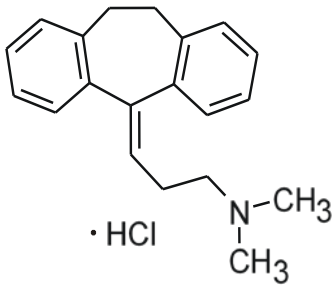
toluene - hydrophobic retention, methylene selectivity



ethyl benzene - hydrophobic retention, methylene selectivity



quinizarin - activity towards chelating reagents



amitriptyline hydrochloride - activity towards bases

Figure 1. Structures and Properties Evaluated for Components in SRM 870

Information Values for Detection Response: The values listed in Table 2 are provided for information only and may be of use as an aid in peak identification. These values are not intended for use in quantitation.

Table 2. Sources and Information Values for Detection Responses of Components in SRM 870

Component	CAS Number[(a)]	Source	Lot	Relative Area, 254 nm	Relative Area, 210 nm	Relative Area, 480 nm
Methanol	67-56-1	J.T. Baker	L30330			
Uracil	66-22-8	Aldrich	MS15011BS	0.02	0.00	
Toluene	108-88-3	Burdick and Jackson	AH700	0.02	0.18	
Ethyl benzene	100-41-4	Aldrich	PS10785MS	0.03	0.20	
Quinizarin	81-64-1	Aldrich	03116HS	0.10	0.01	1.00
Amitriptyline HCl	549-18-8	Sigma	48H0468	0.83	0.61	

[(a)] Chemical Abstracts Service

INTERPRETATION OF RESULTS

Separations of the test mixture are illustrated in Figures 2A through 2F for several different C_{18} columns. These chromatograms are examples of possible types of retention behavior. The most typical elution order is shown in Figure 2C. Uracil elutes near the void volume, followed by toluene and ethyl benzene. The elution order for quinizarin and amitriptyline varies with column properties. Quinizarin may elute before, after, or coelute with amitriptyline.

In most instances, peak identification can be made on the basis of elution order (uracil, toluene, ethyl benzene) and detector response (quinizarin, amitriptyline). Relative peak areas are dependent on the detection wavelength (see Table 2). Quinizarin has significant absorbance at 480 nm, and separations of SRM 870 carried out at this wavelength are selective for this single component. Conversely, quinizarin exhibits reduced absorbance at 210 nm, permitting measurement of amitriptyline in the presence of quinizarin. A comparison of separations carried out with detection at 210 nm, 254 nm, and 480 nm is provided in Figure 3. In the event of coelution of quinizarin and amitriptyline, data for each component can often be obtained by selective detection at 210 nm and 480 nm (see Table 2). At 210 nm, the area of quinizarin is approximately 2 % of the area of amitriptyline, making the interference to amitriptyline small.

The retention behavior of reversed-phase LC columns often differs in a variety of ways. The components in this test mixture were selected as indicators of several types of chromatographic properties. The determination of peak width (efficiency, theoretical plates), peak asymmetry (A_s), absolute retention (k′), and selectivity factor (α, i.e., relative retention k'_1/k'_2) for these components may provide useful measures of these properties [5].

Uracil: This component is commonly used as an indicator of the void volume (unretained volume) in an LC column. The measurement of void volume is somewhat controversial; however, uracil provides an acceptable approximation of this property.

Toluene/Ethyl benzene: The retention of these compounds can be considered to result primarily from solvophobic interactions. The selectivity factor $\alpha_{E/T}$ is the k′ ratio of ethyl benzene and toluene, and this value has been used to characterize differences among C_{18} and C_8 columns. Absolute retention of a nonpolar component such as ethyl benzene provides a measure of column retentiveness (column strength). Toluene and/or ethyl benzene are also useful markers for calculation of column efficiency (theoretical plates, N).

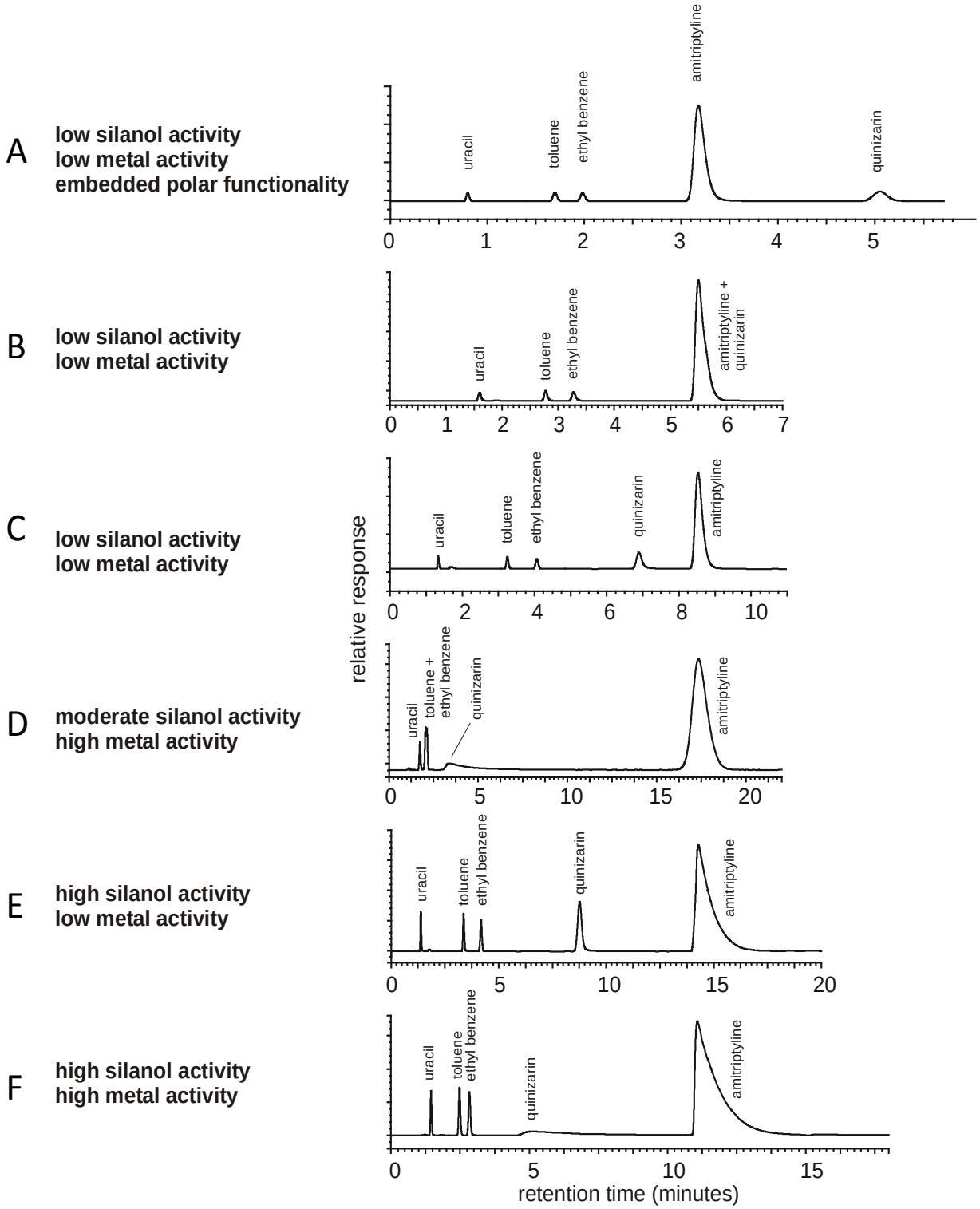


Figure 2. Examples of Separations of SRM 870 on Commercial C_{18} Columns

Quinizarin: Quinizarin (1,4-dihydroxyanthraquinone) is a metal chelating reagent (see Figure 1). The retention behavior of this component is expected to be indicative of the presence or absence of metals in the chromatographic system. Columns demonstrate one of two types of retention behavior. Low activity toward chelating reagents is indicated by symmetric peak shape, and high activity toward chelating reagents is indicated by tailing, asymmetric peak shape. Quinizarin typically elutes after ethyl benzene and before amitriptyline. It is interesting to note that for columns known to contain certain embedded polar functional groups, quinizarin elutes last, with good peak symmetry. Peak asymmetry is not strongly correlated with retention for quinizarin.

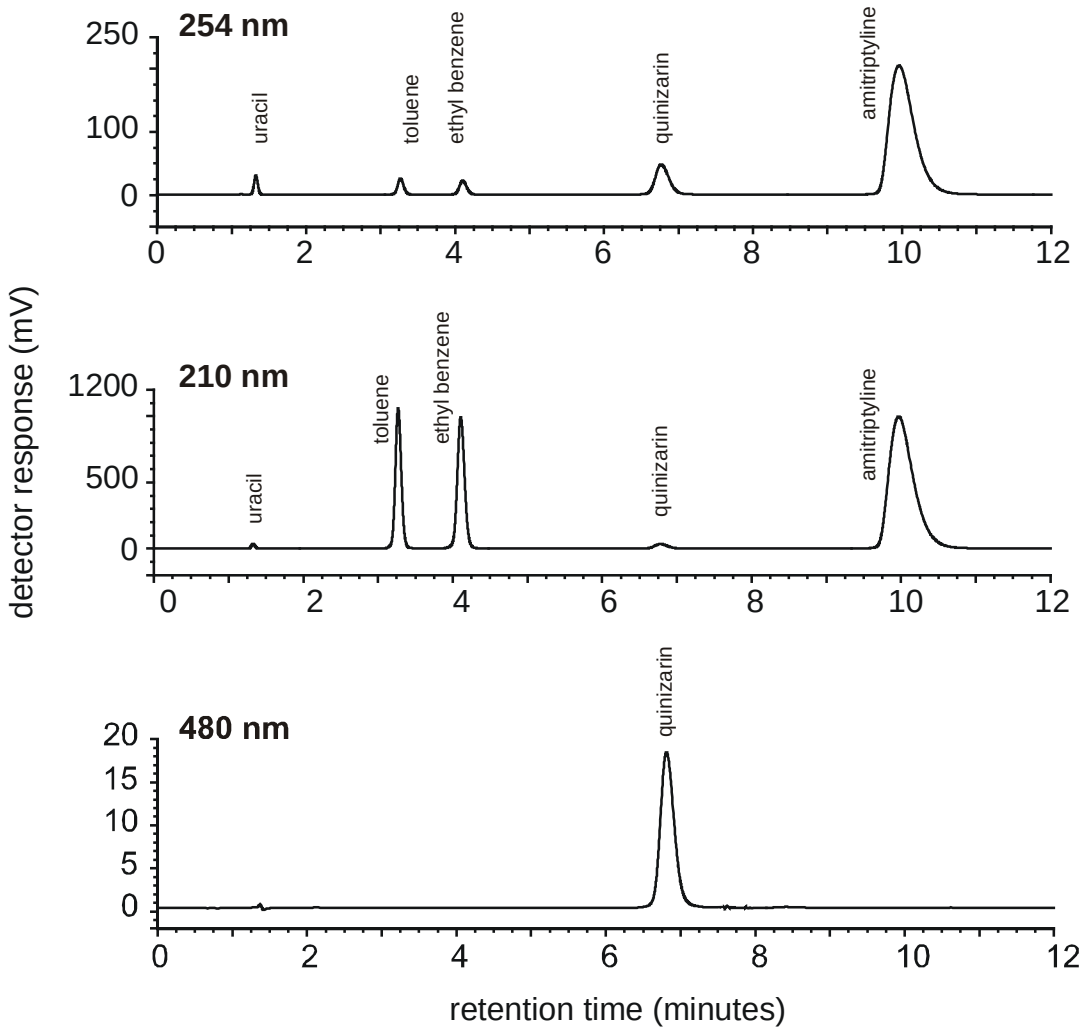


Figure 3. Separations of SRM 870 with Detection at 254 nm, 210 nm, and 480 nm

Amitriptyline: Amitriptyline is a basic (pKa = 9.4) pharmaceutical drug (tricyclic antidepressant) commonly used by column manufacturers for column characterization. Elution of organic bases with severe peak tailing is often associated with high silanol activity; however, the elution of such compounds with symmetrical peak shape is considered indicative of column deactivation. Because peak tailing is the most objectionable property associated with silanol activity, A_s is an appropriate measure of this property. Peak asymmetry is not strongly correlated with retention for amitriptyline.

DISCUSSION

Selection of the components in SRM 870 was based on published testing protocols [6,7] and commercial column literature [8]. An effort was made to provide a simple, easy-to-evaluate test with a limited number of components. Component concentrations were adjusted to facilitate identification. This test is not intended for column classification as "good" or "bad;" however, columns that exhibit certain properties may be more suitable for a given application than others.

Test Conditions: The influence of chromatographic conditions on test results was examined for several different parameters. Relative changes in retention have been evaluated in reference 4 for pH, temperature, buffer concentration, and mobile phase composition. Because retention, efficiency, and peak shape are influenced by testing conditions, column evaluation should be carried out under standardized conditions to facilitate column comparisons. The largest changes in retention behavior occur with changes in the mobile phase composition. As specified in the "Instructions for Handling, Storage, and Use" section of this certificate, the recommended composition of the mobile phase is 80 % methanol and 20 % buffer (volume fractions), where the buffer composition is 20 mmol/L potassium phosphate adjusted to pH 7.0 ± 0.1. The retention of quinizarin and amitriptyline is strongly dependent on the pH of the potassium phosphate buffer solution (see Figure 4). The retention of quinizarin is reduced at high pH, whereas the retention of amitriptyline is reduced at low pH. At pH 7.0, both solutes exhibit significant retention. The ionic strength of the buffer is less significant. Only slight changes in retention, efficiency, and peak asymmetry are

measurable with changes on the phosphate buffer concentration at pH 7.0. The presence of the buffer is essential, however. At levels below 1 mmol/L (buffer concentration before dilution with methanol), A_s and k′ increase dramatically for amitriptyline. The absolute retention of the polar and nonpolar components increase with the percentage of buffer in the mobile phase (at pH 7.0 and constant ionic strength in the mixed solution). A composition of 80 % methanol and 20 % buffer was selected to provide appropriate retention for a broad range of column types.

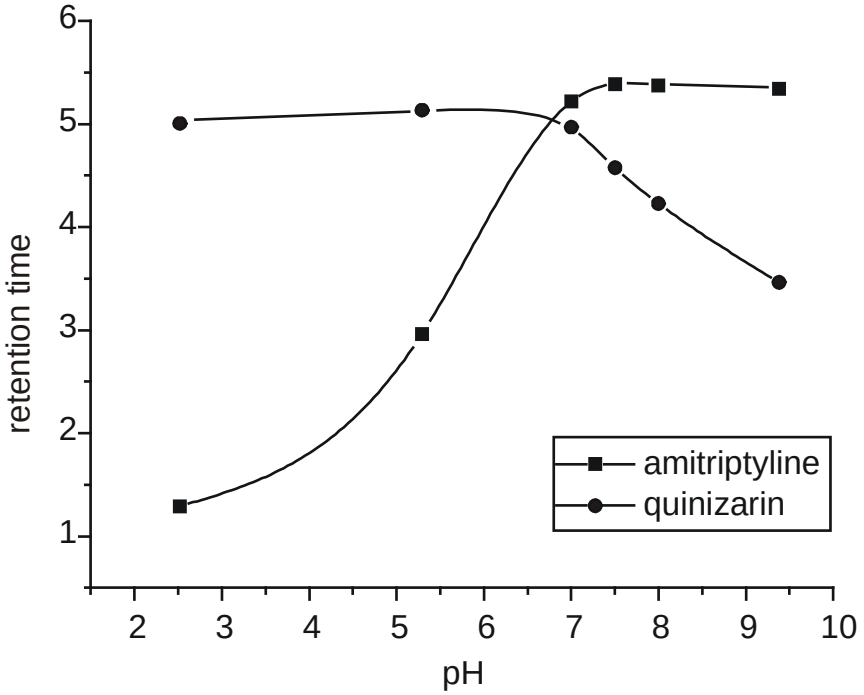


Figure 4. Plot of Retention vs. pH for Amitriptyline and Quinizarin

Injection volume can also significantly influence test results (see Figure 5). Separation efficiency typically decreases with increased injection volume. Injection overload results in degraded peak shape and, in some instances, reduced retention. An injection volume of 1 µL to 2 µL is recommended for 4.6 mm i.d. columns. Proportionately smaller injection volumes may be required for smaller i.d. columns.

Changes in column temperature influence the absolute retention of the components in SRM 870; however, relatively small effects are observed in the peak shape of quinizarin or amitriptyline. It is recommended that column temperature be controlled to 23 °C ± 1 °C.

Column Comparisons: Forty-one commercial C_{18} columns were utilized in the development of SRM 870. Columns were selected to represent a broad sampling of chromatographic retention properties, and included alkyl phases prepared with embedded polar functional groups. No two columns exhibit identical retention behavior; however, similarities do exist among several columns. Among columns utilized, values of k′ for ethyl benzene ranged from 0.2 to 2.8. In contrast, only slight differences were observed for methylene selectivity ($\alpha_{E/T}$; range, 1.26 to 1.45). The retention of quinizarin ranged from k′ = 1 to k′ = 23.6. In two instances, no elution of this compound was detected. Peak asymmetry values ranged from A_s = 1.1 to A_s = 5.7 (peaks were not defined well enough in two instances to permit determination of A_s). Finally, the retention of amitriptyline ranged from k′ = 1.4 to k′ = 72.9 (A_s = 1.0 to A_s = 11).

Figure 2 illustrates typical elution patterns for SRM 870. Five of the columns utilized are known to contain embedded polar functional groups within the stationary phase to improve chromatographic performance toward bases. The separation of SRM 870 was similar for these columns. In each case, quinizarin eluted last, and both amitriptyline and quinizarin exhibited symmetrical peak shape (e.g., Figure 2A).

Peak asymmetry data for quinizarin and amitriptyline are plotted in Figure 6. The scatter in the data indicates independence of the two terms. Thus, it is possible for a column to exhibit high activity toward chelating agents and low activity toward bases, or other combinations (e.g., Figures 2C through 2F).

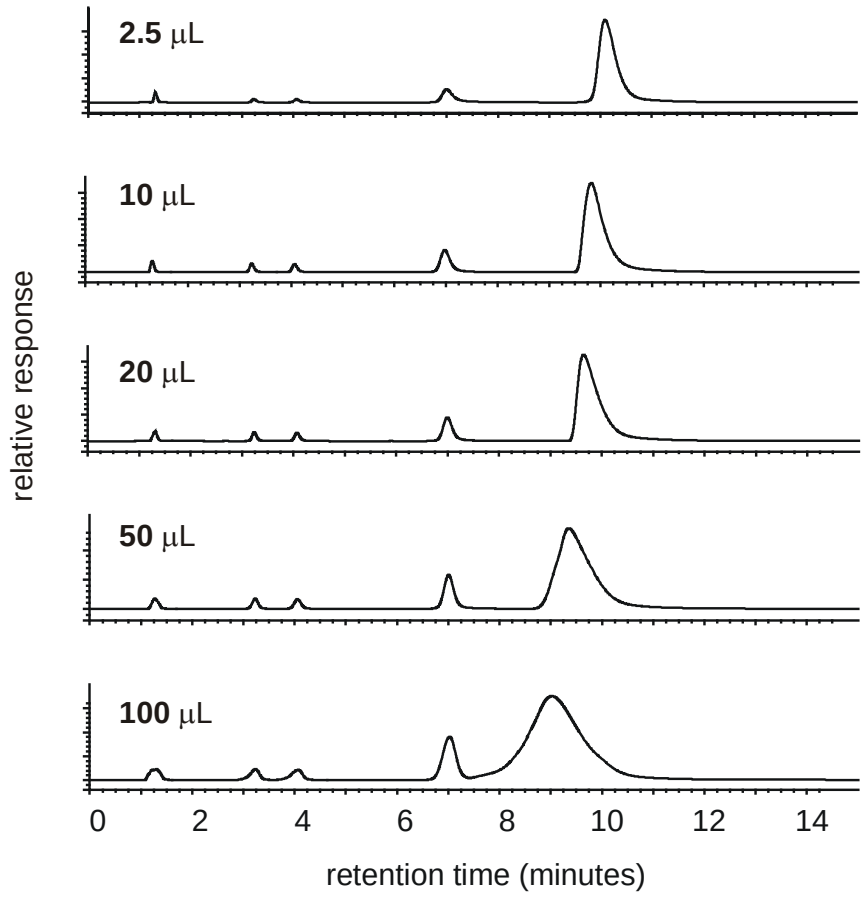


Figure 5. Separations of SRM 870 for Different Injection Volumes

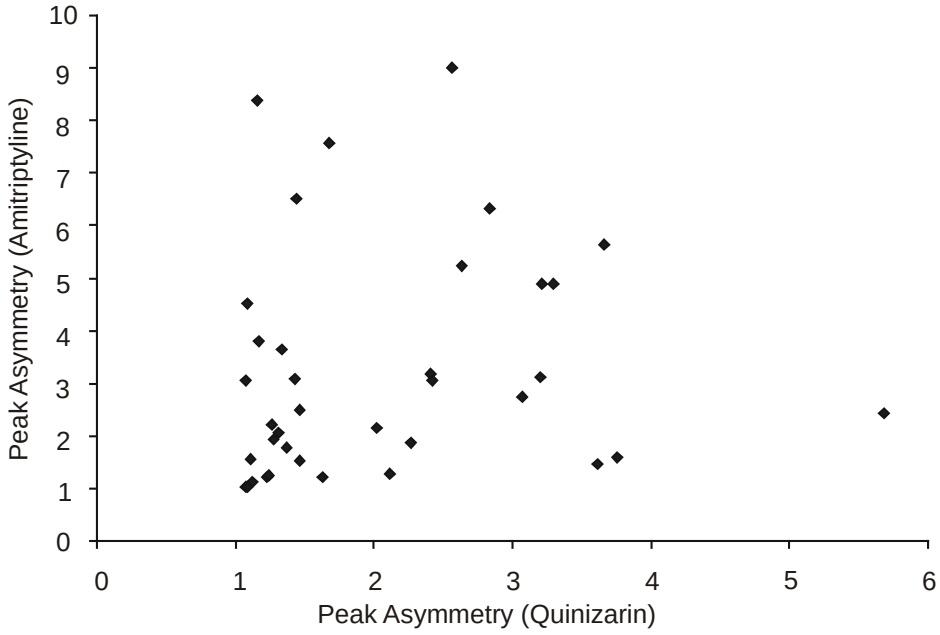


Figure 6. Plot of Peak Asymmetry for Amitriptyline vs. Peak Asymmetry for Quinizarin for Various C_{18} Columns

REFERENCES

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[8] Nacalai Tesque, Inc.; *Product Catalog*; Kyoto, Japan (1998).

Certificate Revision History: 07 July 2016 (Change of expiration date; editorial changes); **11 August 2011** (Addition of certified values for mass fractions of solution constituents; editorial changes; extension of certification period); **19 December 2002** (Updated specifications in the *Chromatographic Conditions* section to expand the utility of the test and removed data collected under previous test conditions); **30 October 2000** (Original certificate date).

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, email srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.