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Standard Practice for Use of Reversed-Phase High Performance Liquid Chromatographic Systems¹

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1. Scope

1.1 This practice covers requirements for defining, testing, and verifying the performance of high performance liquid chromatographic (HPLC) systems when used for trace analysis of pesticides and toxic substances in carrying out pollution control programs and in assessing the quality of food products as mandated by national laws and regulations. As a practical matter, this practice is intended to cover requirements of reversed-phase (adsorption) HPLC systems. Microbore column and ion-exchange column HPLC systems are not covered here. It is not intended to exclude any other equivalent means of analysis. An HPLC system can successfully be applied in the analysis of a variety of sample types including ground and surface water, municipal and industrial effluents, workplace air, soils and sediments, plant and animal tissue, and food products (1, 2, 3).² Collection and extraction techniques, appropriate to the sample type, are required prior to analysis. Sampling techniques and measurement methods are not covered in this recommendation; however, some relevant measurement methods may be found in references listed in Appendix X1.

NOTE 1—High performance liquid chromatography is synonymous with high pressure liquid chromatography.

1.2 Metrological and technical requirements are provided for the major components of an HPLC system including the pump(s), injector(s), column(s), detector(s), and temperature control and data handling systems. The conditions of operation of a single integrated instrument, or one combined from separate components, are intended to cover the application for trace analysis.

1.3 Basically four types of packed columns for liquid chromatography exist: partition, adsorption, ion exchange, and gel permeation. Other terms are used to refer to each type. For many separations, however, the actual separation may not be

clearly defined and may involve a combination of retention mechanisms. Furthermore, the polarity of the stationary phase can be greater or less than the mobile phase. The separation method is called normal-phase HPLC when the stationary phase is more polar than the mobile phase, and the separation method is called reversed-phase HPLC when the reverse condition exists. The reversed-phase HPLC system, using an adsorption column, has become the more frequently used technique for separation and analysis of organic compounds. It can separate a broad spectrum of nonionic, ionizable, and ionic compounds, and its columns are usually stable and separations may be performed with good repeatability since the stationary phases are chemically bonded.

1.4 The detector type selected for use with an HPLC system depends generally on the concentration as well as the chemical and physical properties of the sample matrix and the analyte to be measured. The following detectors are covered in this practice. UV/Visible spectrophotometric, fluorescence, electrochemical, and refractive index.

NOTE 2—The mass spectrometer is a highly specific and sensitive detector appropriate for most applications. It is normally coupled to the HPLC system through an appropriate interface. The use of a mass spectrometer as a detector is not covered in this practice because of its specialized nature.

1.5 The following are examples of classes of analytes that may be measured by an HPLC system: carbamates, pyrethroids, organophosphates, polycyclic aromatic hydrocarbons, phenolics, isocyanates, aflatoxins, chlorophenoxy-acid herbicides, triazine herbicides, and amines. An advantage of HPLC over gas chromatography is that it may be used for the direct measurement of thermally labile compounds, compounds of low volatility, and strongly polar compounds without conversion to derivatives.

1.6 Optimizing the performance of each major component of the measuring system may achieve performance better than the criteria prescribed for these applications. Success in this respect depends on the knowledge, skill, and experience of the analyst.

1.7 The values stated in SI units are to be regarded as the standard.

¹ This practice is under the jurisdiction of ASTM Committee D-19 on Water and is the direct responsibility of Subcommittee D19.06 on Methods for Analysis for Organic Substances in Water.

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² The boldface numbers given in parentheses refer to a list of references at the end of the text.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazard statements, refer to Section 8 for precaution.

2. Referenced Documents

2.1 ASTM Standards:³

D 1129 Terminology Relating to Water

D 1193 Specification for Reagent Water

E 682 Practice for Liquid Chromatograph Terms and Relationships

E 685 Practice for Testing Fixed-Wavelength Photometric Detectors Used in Liquid Chromatography

3. Terminology

3.1 Definitions:

3.1.1 For definition of terms used in this practice refer to Terminology D 1129 and Practice E 682.

3.2 Definitions of Terms Specific to This Standard:

3.2.1 *analytes*—the dissolved components of a mixture that are to be separated and detected by the HPLC system.

3.2.2 *baseline*—the portion of a chromatogram recording the detector response when only the mobile phase emerges from the column.

3.2.3 *chromatogram*—a graphic representation of the detector response versus retention time or elution volume as the analytes elute from the column and through the detector.

3.2.4 *column*—for HPLC systems, consist of a tube that contains a packing (a solid support plus stationary phase) through which the mobile phase flows.

3.2.5 *detector*—the device that responds to the presence of analytes eluting from the column.

3.2.6 *drift*—the average slope of the baseline signal measured over a minimum period of one-half hour.

3.2.7 *dynamic range*—the range of concentration or mass of the analyte over which a change in sample amount produces a measurable change in the detector signal output. Its value is the ratio of the upper limit of detection to the instrument detection limit.

3.2.8 *elution*—the removal of an analyte from the stationary phase by the mobile phase.

3.2.9 *flow rate*—the volumetric rate of flow of mobile phase through the chromatographic system.

3.2.10 *injection device*—a means of introducing a sample into the mobile phase at a location just ahead of the column.

3.2.11 *instrument detection limit*—the concentration of the analyte that gives a detector signal output equal to three times the mean short-term noise as determined on a statistical basis.

3.2.11.1 *Discussion*—This term is sometimes called “minimum detectability” in references and manufacturer’s literature. The detection limit is sometimes defined as an output signal equal to some multiple (two or ten) of the noise.

3.2.12 *linear range*—the range of concentration or mass of an analyte over which the sensitivity of the detector is constant to within 5 percent. Its value is the ratio of the upper limit of linearity to the detection limit.

3.2.13 *long-term noise*—all observable random variations of the detector signal over a 5 to 15 min period. It is this noise that can be mistaken for a late-eluting peak.

3.2.14 *mobile phase*—the liquid (solvent) used to elute the analytes through and from the column and may consist of a single component or a mixture of components.

3.2.15 *noise*—a measure of variation in the detector signal and can be divided into three components.

3.2.16 *peak*—the portion of a chromatogram recording detector response when a single analyte, or two or more unresolved analytes, elute from the column.

3.2.17 *pump*—delivers the mobile phase at a controlled flow rate through the chromatographic system.

3.2.18 *repeatability*—the closeness of agreement between results of successive measurements of the same sample and carried out by the same instrument under the same conditions of use during a short period of time.

3.2.19 *retention time* (t_r)—the time between sample injection and the appearance of the peak maximum of the analyte at the detector.

3.2.19.1 *Discussion*—A related term, Capacity Factor (k), measures the degree of retention that is defined as:

$$k = (t_r - t_M)/t_M$$

where:

t_M = the time for the mobile phase to proceed from the point of injection to the detector.

Selectivity (α) is a measure of relative retention of two compounds expressed as:

$$\alpha = k_2/k_1$$

Efficiency (N) or theoretical plates is a number which describes peak broadening as a function of retention. Efficiency is calculated from:

$$N = 16 \left(\frac{t_{r1}}{wI} \right)^2$$

where:

t_{r1} = retention time of Compound 1, and

wI = peak width of Compound 1.

3.2.20 *resolution* (R_s)—the ability of a column to separate chromatographic peaks. It is usually expressed in terms of the separation of two peaks. One attempts to achieve the largest resolution possible.

3.2.20.1 *Discussion*—Resolution can be calculated in two ways:

$$R_s = (t_{r2} - t_{r1}) / 1/2(w_b^1 + w_b^2)$$

where:

t_{r1} = retention time of Compound 1,

t_{r2} = retention time of Compound 2,

w_b^1 = peak width at baseline of Compound 1, and

w_b^2 = peak width at baseline of Compound 2.

or:

$$R_s = 1/4 \left(\frac{\alpha - 1}{\alpha} \right) (\sqrt{N}) \left(\frac{k}{1+k} \right)$$

³ For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard’s Document Summary page on the ASTM website.



where:

α = selectivity or separation factor,

N = efficiency or number of theoretical plates, and

k = capacity factor.

If N is assumed constant for Peaks 1 and 2, then $w_b^1 \approx w_b^2$. Therefore, the expression for resolution becomes:

$$R_s = 1/4 (\alpha - 1) (\sqrt{N}) \left(\frac{k}{1+k} \right)$$

For the usual case of closely adjacent peaks ($\alpha \approx 1$), these two expressions for R_s are not sufficiently different to merit concern. See Ref (4).

3.2.21 *sensitivity—of a detector*, is the signal output per unit concentration of the analyte and may be expressed as:

$$S = (A * F)/M$$

where:

S = sensitivity,

A = the integrated area of the analyte,

F = the flow rate, and

M = the mass of the analyte injected.

3.2.21.1 *Discussion*—This equation does not apply to an electrochemical detector of the coulombic type.

3.2.22 *short-term noise*—all observable random variations of the detector signal over a 1 to 2 min and should be measured peak to peak.

3.2.23 *solid support*—ideally the inert material within the column to which the stationary phase is bonded (together providing the packing material) and through which the mobile phase flows. The solid support may be characterized by the particle diameter, D_p , which can be measured in μm .

3.2.23.1 *Discussion*—For more discussion of relevant terms, see Refs (2, 5, 6).

3.2.24 *stationary phase*—the active immobile material within the column attached to the solid support or the solid support itself that delays the passage of analytes by one of several possible mechanisms or by a combination of such mechanisms.

4. Significance and Use

4.1 High performance liquid chromatography provides a simultaneous separation for both qualitative and quantitative analysis of trace level pesticides and toxic substances in a variety of sample matrices, from a single analytical operation requiring only a few millilitres of sample. Compound confirmation can be determined by performing a second analysis on a column that has a different stationary phase than the primary column. The mobile phase may also be different, creating a different selectivity for the analyte. Proof of confirmation occurs if the target analyte is detected on both columns.

4.2 A photodiode array detector can be used to determine peak “purity” by determining spectral homogeneity across the peak. Peak purity or homogeneity is used to help with chromatographic methods development and as an indication that a peak may not be a single compound.

4.3 Positive compound identification can be determined by using a mass spectrometer detector coupled to the HPLC system.

4.4 Analysis time is usually 20 to 30 min depending upon the retention times of the analytes.

5. Description of an HPLC Instrument

5.1 General:

5.1.1 A diagram of an HPLC system is shown in Fig. 1. The mobile phase from a solvent reservoir is filtered and then pumped through the injector, analytical column and detector. The injector, column, and/or detector may be enclosed in a thermostatically controlled oven. A sample is introduced at the top of the column through an injection device and the analytes separated while passing through the column. A guard column may be required for some methods to retain materials which could degrade the analytical column performance. A post column reactor may be necessary for derivitization of analytes prior to detection. The eluate from the column is monitored by a detector that responds to analytes. The detector output signal is displayed instantaneously and/or stored in a data system. The eluate is finally collected in a waste container for proper disposal.

5.1.2 Separation of a sample's components by a column depends on the interaction of the column packing (stationary phase) with the mobile phase. The term isocratic elution is used when the composition of the mobile phase is kept constant during the chromatographic procedure. The term gradient elution is used to specify that a deliberate change is made in the composition of the mobile phase during the chromatographic procedure. The term gradient elution is synonymous with mobile phase or solvent programming, which is somewhat more descriptive of the process taking place within the HPLC system.

5.1.3 The individual analytes eluting from the column are monitored by a detector. The detector signal output versus time is called a chromatogram which has peaks associated with the components. Peak areas or peak heights are related to the concentration of sample components.

5.1.4 The overall performance of an HPLC system may be characterized by the repeatability of measurements of the retention time and peak height (or area) for specific analytes under controlled measurement conditions.

5.2 *Major Components*—The mobile phase composition and required purity depend on the method of analysis, detector used, and type of elution employed. The mobile phases most commonly used in reversed-phase HPLC are mixtures of either methanol or acetonitrile with water.

5.2.1 Pumps:

5.2.1.1 Syringe pump utilizes solvent displacement of the mobile phase by a mechanically controlled piston advancing at a constant rate in a fixed volume chamber.

5.2.1.2 Reciprocating pump with one or more heads uses small volume chambers with reciprocating pistons or diaphragms to facilitate the flow of the mobile phase against back pressure. Check valves are synchronized with the piston (or diaphragm) drive to alternate the filling and emptying of the mobile phase from the chamber.

NOTE 3—Mechanical pulse dampers and/or electronic or pneumatic transducers are often incorporated to ensure pulsation-free flow.