Designation: D 6156 – 97

An American National Standard

Standard Practice for Use of Reversed-Phase High Performance Liquid Chromatographic Systems[1]

This standard is issued under the fixed designation D 6156; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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, pyrethriods, 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 that 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.

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[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:*

D 1129 Terminology Relating to Water[3]

D 1193 Specification for Reagent Water[3]

E 682 Practice for Liquid Chromatograph Terms and Relationships[4]

E 685 Practice for Testing Fixed-Wavelength Photometric Detectors Used in Liquid Chromatography[5]

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:

$$N = 16\left(\frac{t_{r1}}{w1}\right)^2$$

where:

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

$w1$ = 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] *Annual Book of ASTM Standards*, Vol 11.01.

[4] *Annual Book of ASTM Standards*, Vol 14.02.

[5] *Annual Book of ASTM Standards*, Vol 14.01.

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 chromatic 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 though 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.

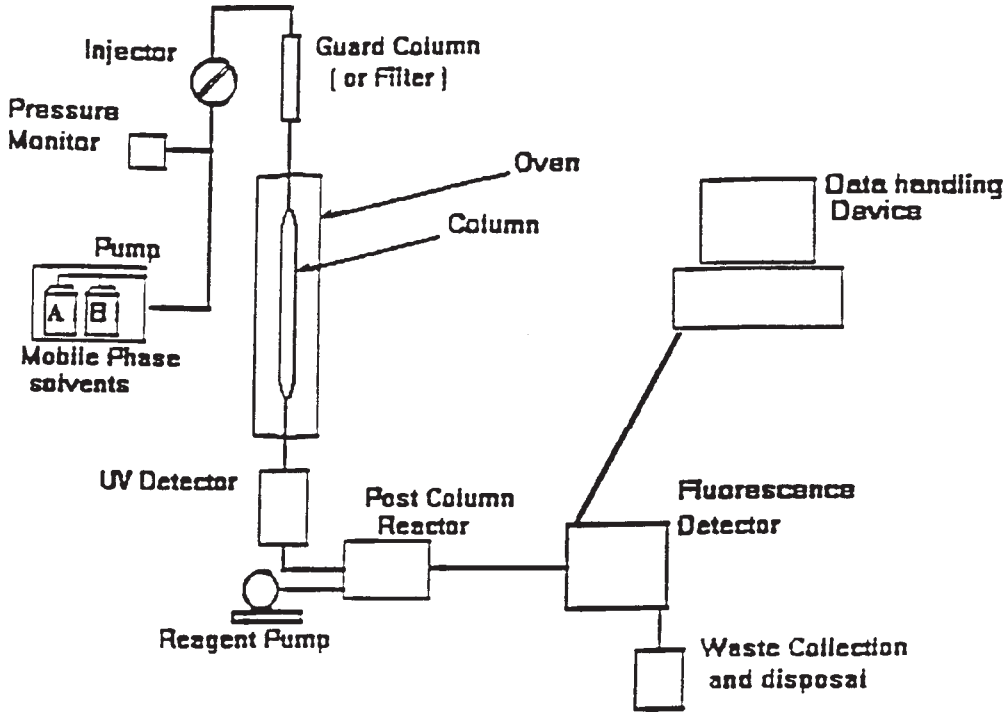
5.2 *Major Components*—The mobile phase composition and required purity depend on the method of analysis, detector used, and type of elution employed. The mobile phases most commonly used in reversed-phase HPLC are mixtures of either methanol or acetonitrile with water.

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 pulseless flow.



NOTE 1—Note that the guard column, column oven, or post column reactor, or all of these, may be included or excluded as required by the analytical method. The column reactor may be located just ahead of the column and is then referred to as a pre-column reactor.

FIG. 1 Schematic Diagram of an HPLC System

5.2.2 The injection device most commonly used is a valve and loop in which the sample contained in a syringe is introduced into an ambient chamber, or loop, and is subsequently displaced into the pressurized flowing mobile phase by means of a rotary or sliding motion.

NOTE 4—Septum and septumless injection devices may be used. The process of injection is often automated.

5.2.3 A guard column may be placed between the injector and column to protect the latter from loss of efficiency because of the presence of particulate matter or strongly absorbed material in the sample.

NOTE 5—A filter should be added if a guard column is not present.

5.2.4 The column used depends on the analytical method. It should be selected for optimum separation of the analytes.

NOTE 6—Bonded-phase silicas are widely used as packing (stationary phase and solid support).

5.2.4.1 For reversed-phase HPLC, octadecylsilyl is the most common functional group used for the stationary phase although alkyl, phenyl, and nitrile may also be used. Two types of packing are generally used, porous packing in which the stationary phase is found throughout each porous particle and pellicular packing in which the stationary phase is found only on the outer layer of an otherwise impermeable particle.

5.2.5 Pre column or post column reactor is used to derivatize analytes prior to detection.

5.2.6 *Detectors:*

5.2.6.1 UV/visible spectrophotometric detectors may be used for measuring the absorbance of light by each analyte as it elutes from the column. The absorbance measured may be related to the concentration of the analyte. Single (fixed) wavelength detectors typically use a low-pressure mercury light source at wavelengths determined by the mercury emission lines and an associated optical filter. Appropriate variable wavelength detectors include those having, a grating monochromator to select one or more wavelengths and, a photodiode array with optics to select a range of wavelengths simultaneously. Deuterium, tungsten, or pulsed xenon lamps are usually used as the light sources to cover the wavelength range for both variable-wavelength detector types.

5.2.6.2 Fluorescence detectors may be used for measuring the fluorescence of the analytes as they elute from the column. The fluorescence measured may be related to the concentration of the analytes. In a fluorescence detector, light of a specific wavelength is selected using an excitation monochromator and directed onto a flow cell. Any fluorescence that results is directed at right angles through an emission monochrometer to a photo multiplier type light sensor. The excitation monochrometer is typically a tunable grating type monochrometer. The emission monochrometer may be a simple optical filter to select the emission wavelength range of interest, or it can be a tunable grating monochrometer. Some fluorescence detectors allow both the excitation and emission wavelengths to be set and changed under program control during the course of a chromatogram.

5.2.6.3 Electrochemical detectors may be used for measuring the concentration of analytes that can be oxidized or reduced at an electrode surface. A fixed or program controlled

potential is applied to the detector cell working electrode and a current results when an electroactive analyte elutes from the column. The current is proportional to the analyte concentration.

5.2.6.4 Refractive Index detectors can be used for measuring samples since they are a bulk property or universal detector that responds to all solutes that have a refractive index (RI) different from the mobile phase. The differential refractometer measures the deflection of a light beam due to the difference in refractive index between the sample and reference liquids in a single compact cell.

NOTE 7—For additional information about detectors, see Refs **(4)** and **(6)**.

5.2.7 The data system provides a means for recording and displaying the output signal of an HPLC system detector as a function of time. A potentiometric strip chart recorder, computing integrator or a computer system may be used for this purpose. More advanced data systems may be available that include automatic control of sample injection, instrument operation, detector, and integrating and report parameters. Functional testing of chromatographic software as part of computer/instrument validation is recommended.

6. Metrological Requirements

6.1 *Pumps:*

6.1.1 The pump shall be capable of performing in the pressure range of 0 to 40 MPa.

6.1.2 The pump shall be capable of delivering flow rates from, 0.1 to 5 mL/min. When tested with water, its flow rate shall be repeatable within ± 3 % as determined for a flow rate of 1 mL/min and collected over a 10 min interval at an overall system pressure of at least 14 MPa.

6.1.3 The pump's pulsation shall be less than ± 2 % peak to peak in pressure when monitored for water at a back pressure of 14 MPa and for a flow rate of 1 mL/min of water over a 10 min interval. This measurement shall be carried out at the pump (just before the injector) at the operating pressure.

NOTE 8—The pressure of the pulsations at the detector will be much less than that measured above, since the detector is at the lowest pressure of the system. Pump performance may affect the detection limit of a detector for some sample components. To optimize system performance, the detector noise generated by the pump shall be minimized.

6.2 *Injectors*—Injectors shall be capable of delivering a specific sample volume with a repeatability of +/–1 % or +/–0.5 µL, whichever is greater, for a volume within the range from 1.0 to 1000 µL. Injectors shall meet these requirements for pressures up to 34 MPa.

6.3 *Detectors*—Within an HPLC system, a detector's performance depends upon interactions among many variables including the choice of solvent, the column efficiency, the detector's cell volume, and the type elution. Because of such interactions, the metrological requirements of the detectors are defined in terms of performance tests. An overall performance test for a HPLC system having either a UV/Visible or fluorescence detector is provided in Annex A2, and Annex A3, which should be considered for an initial test of a HPLC system. The following procedures are examples of how to establish detector performance and instrument detection limits.

NOTE 9—An initial performance test should include a measurement of the instruments sensitivity and its working range using a test sample that may represent the compound or compounds to be analyzed as well as the intended measurement method of application. The requirements of this section and the Annex reflects using an isocratic elution mode; however, the gradient elution profile response of the detector should also be determined when it too will be applied.

6.3.1 *UV/Visible Spectrophotometric Detector:*

6.3.1.1 *Type*—Concentration and cell length dependent selective and widely applicable,

6.3.1.2 *Test Compound*—Anthracene,

6.3.1.3 *Wavelength*—254± 5 nm,

6.3.1.4 *Test Conditions*—The test conditions should be compatible with those provided in Annex A1.

6.3.1.5 *Peak Width at Half Height*—≤(5× tr^2/L × Dp)1/2 0.02 min at a signal to noise ratio >100,

where:

tr = retention time,

L = column length, and

Dp = particle diameter of the packing of the column (see A1.2.3),

6.3.1.6 *Instrument Detection Limit*—3 ng anthracene injected, and

6.3.1.7 *Linear Range*—10^4.

NOTE 10—For additional details about tests, see Annex A3 and Ref **(6)**.

6.3.2 *Fluorescence Detector*

6.3.2.1 *Type*—Concentration dependent, selective and compound specific, cell volume dependent,

6.3.2.2 *Test Compound*—Anthracene,

6.3.2.3 *Wavelength*—Excitation, 250 ± 5 nm, and emission, 600+/–5 nm,

6.3.2.4 *Test Conditions*—Same as 7.3.2 for internal diameter, flow rate, and retention time range for a specific column length. (See Annex A1.)

6.3.2.5 *Instrument Detection Limit*—1 ng anthracene injected, and

6.3.2.6 *Linear Range*—10^3.

6.3.3 *Electrochemical Detector:*

6.3.3.1 *Type*—Concentration dependent, selective,

6.3.3.2 *Test Compound*—Hydroquinone (oxidation), quinone (reduction),

6.3.3.3 *Test Conditions*—The test conditions should be compatible with those provided in Annex A2,

6.3.3.4 *Peak Width at Half Height*—≤(5× tr^2/L × Dp)1/2 0.04 min at a signal to noise ratio >100,

6.3.3.5 *Detection Limit*—50 ng hydroquinone injected, and

6.3.3.6 *Linear Range*—10^4.

NOTE 11—The sensitivity of the detector depends on the flow rate in the detector cell (see 3.2.15).

6.3.4 *Refractive Index Detector:*

6.3.4.1 *Type*—Concentration dependent, universal,

6.3.4.2 *Test Compound*—Anthracene,

6.3.4.3 *Test Conditions*—The test conditions should be compatible with those provided in Annex A1,

6.3.4.4 *Detection Limit*—1 µg anthracene injected, and

6.3.4.5 Linear Range—10^4.

6.4 *Temperature Control:*

6.4.1 The instrument's detector shall be capable of meeting the requirements of 6.3 within a range from 10 to 35°C when the temperature of the detector is stable to within ± 1°C during a complete measurement.

6.4.2 The temperature of the column compartment shall be adjustable in 1°C increments, when such an oven is provided.

NOTE 12—When an HPLC system column is operated at temperatures of 10°C or more above ambient, preheating of the mobile phase may be required to avoid bubble formation.

7. Technical Requirements

7.1 The mobile phase should contain reagent (HPLC) grade chemicals which may require further purification for some measurements. Water shall be of high purity meeting the requirements of Specification D 1193, especially with respect to organics and shall require filtration in addition to chemical purification. Before use, filtration shall be carried out using the filter porosity and media recommended by the instrument manufacturer. Use only fresh solvents.

7.2 Immediately before use, the mobile phase shall be degassed to prevent bubble formation.

7.3 A pressure monitor and a pressure limit switch shall be provided in the system at least between the pump and the column.

7.4 The pump shall be designed to deliver a single mobile phase at a constant rate through the column for isocratic elution. If gradient elution is to be employed, solvent gradients may be generated either by controlled mixing of the mobile phase components on the inlet side of a single pumping system or by combining the outputs of two or more pumping systems prior to the injector.

7.5 Additional filters of from 2 to 10 µm may be used before the pump. A 0.5 µm filter and guard column, shall be used after the pump and the injector in order to remove particulate matter and chemical interferences.

7.6 The columns shall be tubular and packed, may be constructed of metal, glass, or plastic and shall be capable of withstanding the pressure generated by the pump.

NOTE 13—The column connections should be designed for easy assembly and disassembly.

7.7 All plumbing connections of the HPLC system shall be arranged to minimize the pre-column and post-column dead volume of the system.

NOTE 14—The connections should be selected for compatibility in sealing against high-pressure fluids and for their resistance against being dissolved or corroded by the applicable mobile phase.

7.8 A means shall be provided for temperature control for detectors if the ambient temperature is not sufficiently stable to meet the requirements of 6.4.1.

NOTE 15—A metal block for a heat sink or a resistance type heater are examples of devices used for temperature control.

7.9 The data system shall accurately record and display the output signal of an HPLC system detector as a function of time.

7.10 Markings shall be attached conspicuously to all major components of the HPLC system as follows:

7.10.1 Name of manufacturer,

7.10.2 Instrument model, serial number, and date (year and month) of manufacture,

7.10.3 Voltage, frequency, and current requirements, and

7.10.4 Compliance with national regulations concerning personnel safety and radio frequency interference emission.

8. Practical Instructions

8.1 HPLC systems use high voltage and potentially toxic and flammable liquids under high pressure during normal operation. Warning labels shall be conspicuously placed on the instrument to alert the user to these and other potential hazards. Instrument installation and operation, particularly with respect to ventilation and a means for disposal of sample and solvent wastes, shall be consistent with national and local safety regulations.

8.2 Manufacturers of HPLC systems or their components shall supply a manual that describes the installation, operation, and routine maintenance of the systems or components. (See also Ref **(7)**.) Service manuals shall be available upon request.

8.3 Before installation, all laboratory environmental factors shall be considered. Manufacturers shall provide operating specifications for the HPLC system that include the power consumptions, the upper and lower rated voltage and frequency and the range for ambient temperature and humidity.

9. Metrological Controls

9.1 *General Considerations:*

9.1.1 *Calibration*—It is essential to calibrate the measuring system to ensure that the performance is acceptable, particularly that the range over which the output of the device, whether expressed in peak area or peak height, is linear with respect to analyte concentration. Failure to perform this calibration may introduce substantial errors into the results.

9.1.1.1 External standard calibration begins with preparation of standards of known and increasing concentration. Working standards should be made from individual weighings and dilutions into volumetric flasks, if a component's concentration is large enough. If not, make working standards from a stock solution. The concentration range for the working standards should cover the range of interest.

9.1.1.2 External standard calibration curve is generated by plotting for each working standard concentration (*X*-axis) the peak area response (*Y*-axis). After verification of a linear multiple calibration, a one-point calibration curve using a single working standard can be used to generate a response factor. The legitimacy of using a one-point curve for analysis of unknowns rests on method validation which is performed at the outset.

9.1.2 Calculation of results using an external standard is determined by the expression:

$$RF_{(X_i)} = Concentration\ (X_i)std/Response\ (X_i)std$$

$$Unknown\ Concentration\ (X_{unk}) = RF_{(X_i)} \times Response\ (X_{unk})$$

where:

$RF_{(Xi)}$ = response factor

9.1.3 An HPLC system is an instrument comprised of a pump(s), injector(s), column(s), detector(s), and data system

for a complete measurement. The specific components used depend on the analytical method determined to be appropriate for the analyte(s) by the responsible national body for monitoring and control of environmental pollution. Therefore, traditional legal metrology controls for instrument validation may not be enough for these instruments. However, the responsible national body usually provides the quality control procedures like those described in 9.2, as a means of assuring the continued metrological integrity of an HPLC system.

9.1.4 Quality control procedures for specific analytical methods are usually established by the responsible national body. Once such quality control procedures are in place, the national body might consider means for assessing laboratories that use HPLC systems. Such means include: accreditation of the user laboratory, self certification by the user laboratory, proficiency testing through intercomparison of measurements among user laboratories, or a combination of these procedures.

9.2 *Quality Control Procedures:*

9.2.1 *Records*—A record shall be maintained that contains the following information in chronological order for each HPLC system:

9.2.1.1 Results of initial and overall performance test,

9.2.1.2 Results of routine tests that document, at a minimum, the retention time, peak area or height, and peak width,

9.2.1.3 Identification of the reference sample for each analysis performed, of the mobile phase(s), injector(s), column(s) (including particle diameter), elution procedure, detector(s), and data system(s),

9.2.1.4 Description of malfunctions and corrective actions taken, and

9.2.1.5 Extent of maintenance or repair, or both.

9.2.2 *Initial Test*—An initial test of the HPLC system shall be performed according to the manufacturer's instructions. The results of this test shall be within the specifications provided by the manufacturer.

9.2.3 *Overall Test*—A test of the entire HPLC system shall be carried out frequently (for example, once per work period) using reference materials, or specifically prepared samples, that are appropriate for the analytical method for the class of analytes normally determined. This test should specifically characterize the instruments detection limit, sensitivity, and operating range.

NOTE 16—Procedures for routinely checking the operational conditions and the overall performance of an HPLC system are given in Annex A2 and Annex A3, respectively. Appropriate reference materials may be available as indicated in Refs **(8, 9, 10)**.

9.2.4 *Routine Test*—Test procedures based on the relevant measurement method should be considered for routine testing of a HPLC system because of the possible extreme variation in an instrument's performance in measuring sample components of interest.

9.2.5 *Published Procedures*—Quality control procedures are often published by the responsible national body and usually specify appropriate HPLC system performance tests, calibration procedures, and routine tests that apply for the analytical methods for specific pollutants. The time interval between tests should also be specified.

10. Keywords

10.1 electrochemical detector; fluorescence detector; food products; high performance liquid chromatography; industrial effluents; pesticide; refractive index detector; reversed-phase; soils; spectrophotometric detector; surface water; toxic substances; workplace air

ANNEXES

(Mandatory Information)

A1. TEST CONDITIONS FOR HPLC DETECTORS

A1.1 This annex provides the recommended conditions under which the requirements for detectors covered in 6.3 can be tested.

A1.2 *UV/Visible Spectrophotometric Detector:*

A1.2.1 The nominal flow rate for a selected internal diameter (I.D.) of a column should be as follows:

I.D., mm	Flow Rate, mL/min
4.6	1.0
3.9	0.7
2.0	0.2

NOTE A1.1—For other IDs, flow rate = (ID^2/21.2 mL/min.)

A1.2.2 The retention time range for selected length of column should be as follows:

Column Length, cm	Retention Tme Range, min
30	10.6 to 14.0
25	8.8 to 11.7
20	7.0 to 9.3
15	5.3 to 7.0
10	3.5 to 4.7
7.5	2.7 to 3.5
6	2.1 to 2.8

A1.2.3 The peak width at half height for various packing particle diameters and various lengths of a column should be the following.

Column Length, cm	tr, min	3 µm, min	5 µm, min	10 µm, min
30	12.3	0.17	0.22	0.32
25	10.25	0.16	0.205	0.29
20	8.15	0.14	0.18	0.26
15	6.15	0.12	0.16	0.22
10	4.1	0.10	0.13	0.18
7.5	3.1	0.09	0.11	0.16
6	2.5	0.08	0.102	0.14

NOTE A1.2—The indicated retention time (t_r) is the mean value of the

expected retention time range.

A1.3 *Fluorescence Detector*—The test conditions recommended shall be equivalent to or compatible with those of A1.2.

A1.4 *Electrochemical Detector:*

A1.4.1 For the mobile phase, use 0.1 M acetate buffer (pH = 4.9 ± 0.1) in 15 % (V/V) methanol/water.

NOTE A1.3—Prepare buffer, adjust pH, and then add methanol to bring volume composition to 15 % methanol. The buffer serves as a supporting electrolyte.

A1.4.2 *Working Electrode*—Use either glassy carbon or porous graphite.

A1.4.3 *Working Electrode Potential*—For oxidation use +0.7 V vs. Ag/AgCl reference electrode or +0.5 V vs. Pd reference electrode and for reduction use –0.2 V vs. Ag/AgCl reference electrode or +0.4 versus Pd reference electrode.

NOTE A1.4—The oxidation potential is for the oxidation of hydroquinone to quinone, and the reduction potential is for the reduction of quinone to hydroquinone.

A1.4.4 The nominal flow rate for a selected internal diameter (I.D.) of a column should be as follows:

I.D., mm	Flow Rate, mL/min
4.6	2.0
3.9	1.4
2.0	0.4

A1.4.5 The retention time range for a selected length of column should be as follows:

Column Length, cm	Retention Time Range, min
30	6.6 to 8.7
25	5.5 to 7.25
20	4.4 to 5.8
15	3.3 to 4.4
10	2.2 to 2.9
7.5	1.6 to 2.2
6	1.3 to 1.7

A1.4.6 The peak width at half height for various packing particle diameters and lengths of the column should be the same as the values given in the table under A1.2.3 after the values provided are multiplied by a factor of two.

A1.5 *Differential Refractometer Detector:*

A1.5.1 The test conditions should be equivalent to or compatible with those of A1.2.

A1.5.2 *Detector Settings:*

Sensitivity: 32
Scale Factor: 20
Time Constant: 1.0
Polarity: +

A2. A PERFORMANCE TEST FOR AN HPLC SYSTEM

A2.1 This test provides a procedure for the conditioning and checking of an HPLC system before operation for an analysis. (See Practice E 685.) The purpose is to provide a routine overall HPLC system check.

A2.2 *Start-Up Procedure:*

A2.2.1 Turn on power to all HPLC system modules.

NOTE A2.1—The time and date should also be set on microprocessor based systems.

A2.2.2 Fill all solvent reservoirs with appropriate degassed mobile phase solvents.

A2.2.3 Flush and de-gas all plumbing lines prior to pumping mobile phase through column at a flow rate of 1 mL/min.

A2.3 *Checking Procedure:*

A2.3.1 Measure the repeatability of the flow rate by collecting the delivered mobile phase in a graduate cylinder over at least three successive 10-min periods.

A2.3.2 Check system pressure and check the pressure fluctuations of the solvent delivery system after the column is conditioned with the mobile phase. Pressure fluctuations should be less than ± 0.2 MPa for a 10 MPa head pressure.

A2.3.3 Check all plumbing for solvent leaks.

A2.3.4 Check the detector stability by observing the digital display or recorder, integrator, or data system response.

NOTE A2.2—This stability should be within ± 1 percent of maximum full scale attenuation.

A2.3.5 Confirm that all temperature controlled components have reached their nominal instrument settings.

A2.4 *Conclusion*—If all conditions under this annex are acceptable, the HPLC system can be considered ready to perform an analysis.

A3. AN OPERATIONAL TEST FOR AN HPLC SYSTEM

A3.1 This test involves measuring anthracene in a methanol/water solution. It is specific to a reversed-phase HPLC system that includes a UV/Visible spectrophotometric and/or fluorescence detector. The purpose of this test is to provide a performance test for an entire HPLC system. The test results should be consistent with the requirements of Section 4 of this practice.

NOTE A3.1—The results of this test may not be reliable in predicting an instrument's performance in detecting sample components required by analytical method. Therefore, in some cases, the analytical method may be a useful means for routine testing of an instrument's performance (see 9.2.4).

A3.2 Prepare a stock solution of anthracene in methanol that has a concentration of 2 mg/mL.

NOTE A3.2—Anthracene may be a potential health hazard; therefore, persons who carry out this test should be made aware of this hazard through a material safety data sheet and should be instructed to follow safe laboratory practices.

A3.3 Prepare three additional reference solutions in 60 % methanol and 40 % water by serial dilution of the stock solution by factors of 0.1, 0.01, and 0.001.

NOTE A3.3—Acetonitrile and water may be used.

A3.4 Set the flow rate appropriate for the column diameter as given in A1.2.1. Adjust the mobile phase composition and/or column temperature to meet the retention time requirements given in A1.2.1 for the appropriate column length. Observe the temperature requirements of 6.4. Measure the peak width at half height. If the peak width at half height does not meet the requirements of 6.3.1 or 6.3.2, replace the column before continuing with the test (see also A1.2.3 and A2.3.3).

A3.5 Inject 20 µL of each reference solution of A3.3 in turn. Measure the peak area and peak height of each specific reference solution at least four times. Also measure the peak-to-peak noise.

A3.6 Record retention times, peak areas, peak heights, noise measurements, and the peak widths at half height. Calculate the signal to noise ratio by dividing the peak height by the noise. Calculate the coefficient of variation for the peak areas and retention times.

A3.7 For a signal to noise ratio of greater than 100, the coefficient of variation of the peak areas should be less than 5 %. The maximum measured peak widths at half height shall meet the requirements specified in A1.2.3.

A3.8 Determine the detection limit from a linear plot of the results of A3.5 and A3.6 using peak height versus the amount injected in nanograms as follows:

A3.8.1 Calculate the amount injected of each reference solution,

A3.8.2 Draw a parallel line equivalent to the noise level, and

A3.8.3 Extend the linear plot of the measured data to intersect with the line representing three times the noise level. The intersection on this plot is the detection limit and shall be less than or equal to 3 ng injected for the UV/visible and 1 ng injected for the fluorescence detectors.

A3.9 The sensitivity of the instrument may be calculated according to the equation given in 3.2.15 or may be derived from the linear plot described in A3.8, in which it is equal to the slope of that plot.

APPENDIX

(Nonmandatory Information)

X1. REFERENCE TO MEASUREMENT METHODS

X1.1 U.S. Food and Drug Administration (FDA), *Pesticide Analytical Manual*.

X1.2 U.S. Environmental Protection Agency," Test Methods for Organic Chemicals of Municipal and Industrial Waste Water".

X1.3 U.S. Environmental Protection Agency," Guidelines Establishing Test Procedures for the Analysis of Pollutants Under the Clean Water Act: Final Rule and Interim Final Rule and Proposed Rule," 40 CFR Part 136, U.S. Federal Register Notice, Friday, October 26, 1984.

X1.4 Association of Official Analytical Chemists, *Official Methods of Analysis of the Association of Official Analytical Chemists*, Methods Manual, 14 Edition 1984, Arlington, VA 22209, USA.

X1.5 United Kingdom Health and Safety Executive (HSE), *Methods for Determination of Hazardous Substances*, St. Hugh's House, Stanley Precinct, Bottle L20 3QZ, England.

X1.6 APHA-AWWA-WPFC, American Public Health Association, *Standard Methods for the Examination of Water and Wastewater*, 16th Edition, (revised every three years).

X1.7 *Annual Book of ASTM Standards, Index*, Vol. 00.01, 1986.

X1.8 International Organization for Standardization (ISO), *Catalogue* 1986.

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(**1**) Poole, Coin F. and Schuette, Sheila A., *Contemporary Practice of Chromatography*, Elsevier, New York, 1984.

(**2**) Standing Committee of Analysts, Department of the Environment, U.K., "High Performance Liquid Chromatography, Ion Chromatography, Thin Layer and Column Chromatography of Water Samples," Her Majesty's Stationery Office, 1983.

(**3**) Lawrence, James F., Editor, *Liquid Chromatography in Environmental Analysis*, Humana Press, Clifton, New Jersey, 1984.

(**4**) Snyder, Lloyd R. and Kirkland, Joseph J., *Introduction to Modern Liquid Chromatography*, Wiley, New York (1979).

(**5**) Joint Working Group of ISO, IEC, BIPM, and OIML, *International Vocabulary of Basic and General Terms in Metrology*, ISO, 1984.

(**6**) Scott, Raymond P. W., *Liquid Chromatography Detectors*, second edition, Elsevier, New York, 1986.

(**7**) Runser, D.J., *Maintaining and Troubleshooting HPLC Systems*, John Wiley and Sons, New York, 1981.

(**8**) International Organization for Standardization, *ISO Director of Certified Reference Materials*, Geneva, Switzerland.

(**9**) United States Environmental Protection Agency, Quality Assurance Division, Environmental Monitoring Systems Laboratory, Office of Research and Development, *Analytical Reference Standards and Supplementary Data: Pesticides and Industrial Chemicals*, Repository, EPA-600/4-84-082, October 1984, Las Vegas, NV 89114, USA.

(**10**) United States National Institute of Standards and Technology, NIST Standard Reference Materials Catalogue, NIST Special Publication *260*, Gaithersburg, MD 20899, USA.