1 Introduction

1.1 HPLC: A POWERFUL SEPARATION METHOD

A powerful separation method must be able to resolve mixtures with a large number of similar analytes. Figure 1.1 shows an example. Eight benzodiazepines can be separated within 70 seconds.

Such a chromatogram provides directly both qualitative and quantitative information: each compound in the mixture has its own elution time (the point at which the signal appears on the screen) under a given set of conditions; and both the area and height of each signal are proportional to the amount of the corresponding substance.

This example shows that *high-performance liquid chromatography* (HPLC) is very efficient, i.e. it yields excellent separations in a short time. The 'inventors' of modern chromatography, Martin and Synge,[1] were aware as far back as 1941 that, in theory, the stationary phase requires *very small particles* and hence a *high pressure* is essential for forcing the mobile phase through the column. As a result, HPLC was sometimes referred to as *high-pressure liquid chromatography*.

1.2 A FIRST HPLC EXPERIMENT

Although this beginner's experiment described here is simple, it is recommended that you ask an experienced chromatographer for assistance.

It is most convenient if a HPLC system with two solvent reservoirs can be used. Use water and acetonitrile; both solvents need to be filtered (filter with < 1 μm pores) and degassed. Flush the system with pure acetonitrile, then connect a so-called reversed-phase column (octadecyl ODS or C_{18}, but an octyl or C_8 column can be used as well)

[1] A.J.P. Martin and R.L.M. Synge, *Biochem. J.,* **35**, 1358 (1941).

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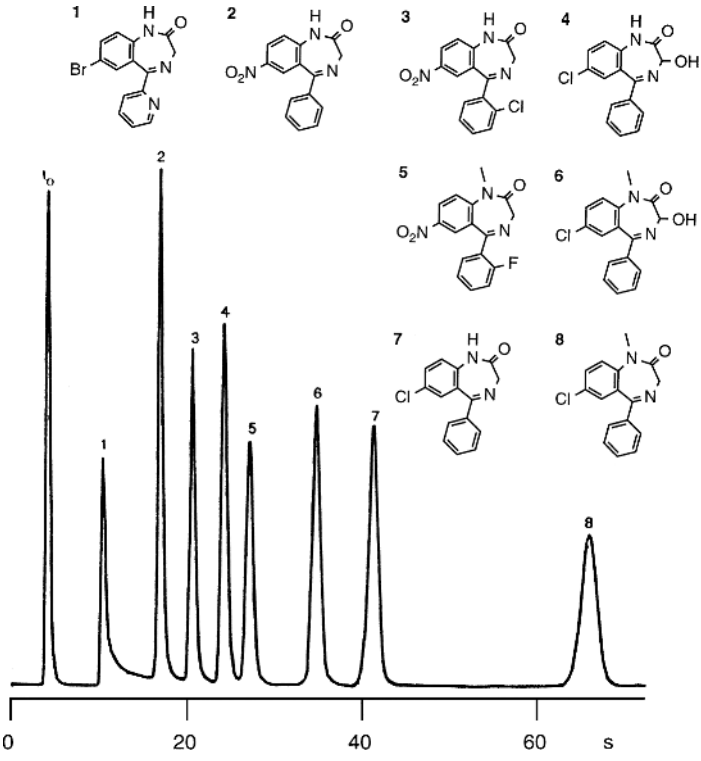

Figure 1.1 HPLC separation of benzodiazepines (T. Welsch, G. Mayr and N. Lammers, *Chromatography*, InCom Sonderband, Düsseldorf, 1997, p. 357). Conditions: samples: 40 ng each; column: 3 cm × 4.6 mm i.d.; stationary phase: ChromSphere UOP C18, 1.5 µm (nonporous); mobile phase: 3.5 ml min^{-1} water–acetonitrile (85 : 15); temperature: 35 °C; UV detector 254 nm. Peaks: 1 = bromazepam; 2 = nitrazepam; 3 = clonazepam; 4 = oxazepam; 5 = flunitrazepam; 6 = hydroxydiazepam (temazepam); 7 = desmethyldiazepam (nordazepam); 8 = diazepam (valium).

with the correct direction of flow (if indicated) and flush it for *ca.* 10 min with acetonitrile. The flow rate depends on the column diameter: 1–2 ml min^{-1} for 4.6 mm columns, 0.5–1 ml min^{-1} for 3 mm and 0.3–0.5 ml min^{-1} for 2 mm columns. Then switch to water–acetonitrile 8 : 2 and flush again for 10–20 min. The UV detector is set to 272 nm (although 254 nm will work too). Prepare a coffee (a 'real' one, not decaffeinated), take a small sample before you add milk, sugar or sweetener and filter it (< 1 µm). Alternatively you can use tea (again, without additives) or a soft drink with caffeine (preferably without sugar); these beverages must be filtered, too. Inject 10 µl of the sample. A chromatogram similar to the one shown in Figure 1.2 will

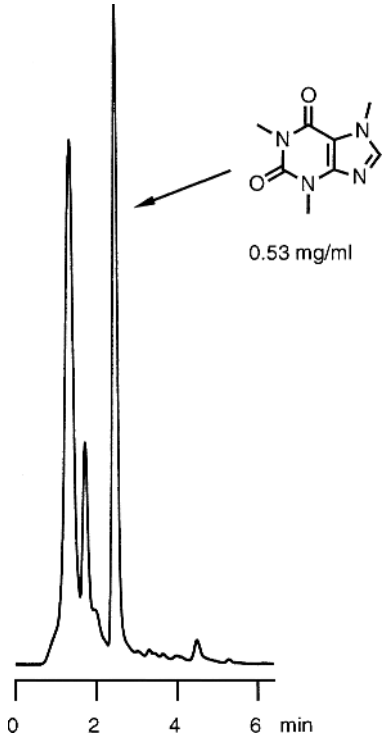

Figure 1.2 HPLC separation of coffee. Conditions: column, 15 cm × 2 mm i.d.; stationary phase, YMC 120 ODS-AQ, 3 μm; mobile phase, 0.3 ml min^{-1} water–acetonitrile (8:2); UV detector 272 nm.

appear. The caffeine signal is usually the last large peak. If it is too high, inject less sample and vice versa; the attenuation of the detector can also be adjusted. It is recommended to choose a sample volume which gives a caffeine peak not higher than one absorption unit as displayed on the detector. If the peak is eluted late, e.g. later than 10 min, the amount of acetonitrile in the mobile phase must be increased (try water–acetonitrile 6 : 4). If it is eluted too early and with poor resolution to the peak cluster at the beginning, decrease the acetonitrile content (e.g. 9 : 1).

The caffeine peak can be integrated, thus a quantitative determination of your beverage is possible. Prepare several calibration solutions of caffeine in mobile phase, e.g. in the range 0.1–1.0 mg ml^{-1}, and inject them. For quantitative analysis, peak areas can be used as well as peak heights. The calibration graph should be linear and run through the origin. The caffeine content of the beverage can vary within a large range and the value of 0.53 mg ml^{-1}, as shown in the figure, only represents the author's taste.

After you have finished this work, flush the column again with pure acetonitrile.

1.3 LIQUID CHROMATOGRAPHIC SEPARATION MODES

Adsorption Chromatography

The principle of adsorption chromatography (normal-phase chromatography) is known from classical column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are also often used. The mobile phase is relatively nonpolar (heptane to tetrahydrofuran). The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. A nonpolar solvent such as hexane elutes more slowly than a medium-polar solvent such as ether.

Rule of thumb: polar compounds are eluted later than nonpolar compounds.

Note: polar means water-soluble, hydrophilic; nonpolar is synonymous with fat-soluble, lipophilic.

Reversed-Phase Chromatography

The reverse of the above applies:

(a) The stationary phase is very nonpolar.
(b) The mobile phase is relatively polar (water to tetrahydrofuran).
(c) A polar solvent such as water elutes more slowly than a less polar solvent such as acetonitrile.

Rule of thumb: nonpolar compounds are eluted later than polar compounds.

Chromatography with Chemically Bonded Phases

The stationary phase is covalently bonded to its support by chemical reaction. A large number of stationary phases can be produced by careful choice of suitable reaction partners. The reversed-phase method described above is the most important special case of chemically bonded-phase chromatography.

Ion-Exchange Chromatography

The stationary phase contains ionic groups (e.g. NR_3^+ or SO_3^-) which interact with the ionic groups of the sample molecules. The method is suitable for separating, e.g. amino acids, ionic metabolic products and organic ions.

Ion-Pair Chromatography

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain problems inherent in the ion-exchange method. Ionic sample

molecules are 'masked' by a suitable counter ion. The main advantages are, firstly, that the widely available reversed-phase system can be used, so no ion exchanger is needed, and, secondly, acids, bases and neutral products can be analysed simultaneously.

Ion Chromatography

Ion chromatography was developed as a means of separating the ions of strong acids and bases (e.g. Cl^-, NO_3^-, Na^+, K^+). It is a special case of ion-exchange chromatography but the equipment used is different.

Size-Exclusion Chromatography

This mode can be subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solutions).

Size-exclusion chromatography separates molecules by size, i.e. according to molecular mass. The largest molecules are eluted first and the smallest molecules last. This is the best method to choose when a mixture contains compounds with a molecular mass difference of at least 10%.

Affinity Chromatography

In this case, highly specific biochemical interactions provide the means of separation. The stationary phase contains specific groups of molecules which can only adsorb the sample if certain steric and charge-related conditions are satisfied (cf. interaction between antigens and antibodies). Affinity chromatography can be used to isolate proteins (enzymes as well as structural proteins), lipids, etc., from complex mixtures without involving any great expenditure.

1.4 THE HPLC INSTRUMENT

An HPLC instrument can be a set of individual modules or elements, but it can be designed as a single apparatus as well. The module concept is more flexible in the case of the failure of a single component; moreover, the individual parts need not be from the same manufacturer. If you do not like to do minor repairs by yourself you will prefer a compact instrument. This, however, does not need less bench space than a modular set.

An HPLC instrument has at least the elements which are shown in Figure 1.3: solvent reservoir, transfer line with frit, high-pressure pump, sample injection device, column, detector, and data acquisition, usually together with data evaluation. Although the column is the most important part, it is usually the smallest one. For

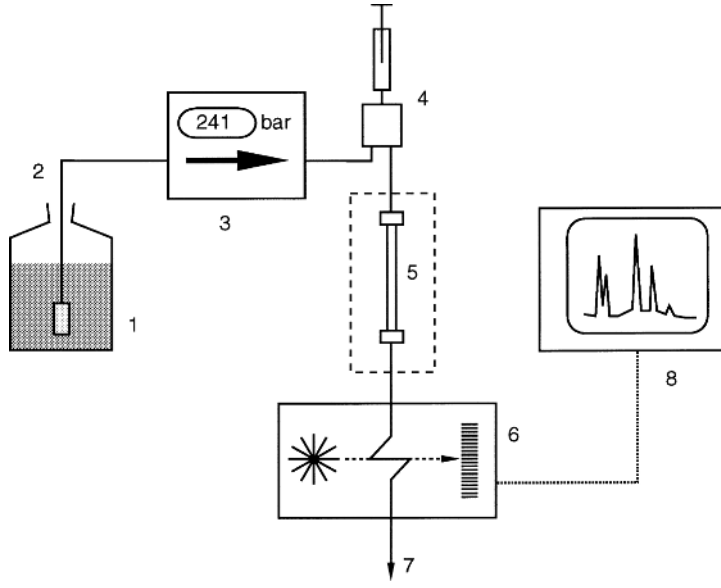

Figure 1.3 Schematic diagram of an HPLC unit. 1 = Solvent reservoir; 2 = transfer line with frit; 3 = pump (with manometer); 4 = sample injection; 5 = column (with thermostat); 6 = detector; 7 = waste; 8 = data acquisition.

temperature-controlled separations it is enclosed in a thermostat. It is quite common to work with more than one solvent, thus a mixer and controller are needed. If the data acquisition is done by a computer it can also be used for the control of the whole system.

1.5 SAFETY IN THE HPLC LABORATORY

Three health risks are inherent in HPLC, these being caused by:

(a) Toxic solvents,
(b) Pulmonary irritation from the stationary phase, and
(c) Dangers resulting from the use of high pressures.

Short- and long-term risks of exposure to solvents and vapours are generally known but too little attention is paid to them. It is good working practice to provide all feed and waste containers with perforated plastic lids, the hole being just large enough to take a PTFE tube for filling or emptying purposes, so that no toxic vapours can escape into the laboratory environment and no impurities can contaminate the highly pure solvent. A good ventilation system should be provided in the solvent handling areas.

The fact that particles of 5 μm and less, as used in HPLC, may pass into the lungs (they are not retained by the bronchial tubes but pass straight through) is less well known and the potential long-term risk to health has not yet been adequately researched. Amorphous silica, as used for stationary phases, is not hazardous[2] but inhalation should be avoided anyway. As a safety precaution, any operation involving possible escape of stationary phase dust (opening phials, weighing etc.) must be carried out in a fume cupboard.

The high-pressure pump does not present too much of a risk. In contrast to gases, liquids are almost incompressible (approximately 1 vol% per 100 bar). Hence, liquids store very little energy, even under high-pressure conditions. A jet of liquid may leak from a faulty fitting but there is no danger of explosion. However, this liquid may cause serious physical damage to the body. A column under pressure which is open at the bottom for emptying purposes must not be interfered with in any way. The description of an accident resulting from this type of action is strongly recommended for reading.[3]

1.6 COMPARISON BETWEEN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY

Like HPLC, gas chromatography[4] (GC) is also a high-performance method, the most important difference between the two being that GC can only cope with substances that are volatile or can be evaporated intact at elevated temperatures or from which volatile derivatives can be reliably obtained. Only about 20% of known organic compounds can be analysed by gas chromatography without prior treatment. For liquid chromatography, the sample must be dissolved in a solvent and, apart from cross-linked, high-molecular-mass substances, all organic and ionic inorganic products satisfy this condition.

The characteristics of the two methods are compared in Table 1.1. In comparison with gas chromatography there are three important differences:

(a) The diffusion coefficient of the sample in the mobile phase is much smaller in HPLC than in GC. (This is a drawback because the diffusion coefficient is the most important factor which determines the speed of chromatographic analysis.)
(b) The viscosity of the mobile phase is higher in HPLC than in GC. (This is a drawback because high viscosity results in small diffusion coefficients and in high flow resistance of the mobile phase.)

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[2] C.J. Johnston *et al. Toxicol. Sci.*, **56**, 405 (2000).
[3] G. Guiochon, *J. Chromatogr.*, **189**, 108 (1980).
[4] H.M. McNair, J.M. Miller and F.A. Settle, *Basic Gas Chromatography*, Wiley-Interscience, New York, 2009.

TABLE 1.1 Comparison of GC AND HPLC

Problem	GC	HPLC
Difficult separation	Possible	Possible
Speed	Yes	Yes
Automation	Possible	Possible
Adaptation of system to separation problem	By change in stationary phase	By change in stationary and mobile phase
Application restricted by	Lack of volatility, thermal decomposition	Insolubility
Typical number of separation plates	Per column	Per metre
GC with packed columns	2000	1000
GC with capillary columns	50 000	3000
Classical liquid chromatography	100	200
HPLC	5000	50 000

(c) The compressibility of the mobile phase under pressure is negligibly small in HPLC whereas it is not in GC. (This is an advantage because as a result the flow velocity of the mobile phase is constant over the whole length of the column. Therefore optimum chromatographic conditions exist everywhere if the flow velocity is chosen correctly. Moreover, incompressibility means that a liquid under high pressure is not dangerous.)

1.7 COMPARISON BETWEEN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

Capillary electrophoresis[5] (also termed capillary zone electrophoresis, CZE) is suited for electrically charged analytes and separates them, simply speaking, according to their ratio of charge to size. In addition, the shape of the molecues is another parameter which influences their speed, therefore the separation of isomers or of analytes with identical specific charge is possible. Cations (positively charged molecules) move faster than anions (negatively charged molecules) and appear earlier in the detector. Small, multiply charged cations are the fastest species whereas small, multiply charged anions are the slowest ones.

[5] P. Schmitt-Kopplin, *Capillary Electrophoresis*, Humana Press, Totowa, 2008; S. Wren, *Chromatographia Suppl.*, **54**, S-15 (2001).

The separation is performed at high voltage. An electric field of up to 30 kV is applied between the ends of the separation capillary. As a consequence, the buffer solution within the capillary moves towards the negatively charged cathode. The capillaries have a length of 20–100 cm and an inner diameter of 50–250 µm. In contrast to HPLC they are not packed with a stationary phase in the chromatographic sense but in some cases with a gel which allows the separation of the analytes by their size (as in size-exclusion chromatography).

The separation performance can be of much higher order of magnitude than in HPLC (up to 10^7 theoretical plates), making CE an extremely valuable method for peptide mapping or DNA sequencing. However, small molecules such as amino acids or inorganic ions can be separated as well. The absolute sample amounts which can be injected are low due to the small volume of the capillaries. A major drawback is the lower repeatability (precision) compared to quantitative HPLC. Preparative separations are not possible.

Electrokinetic chromatography (see Section 23.6) is a hybrid of HPLC and CE. For this technique the capillaries are packed with a stationary phase and the separation is based on partition phenomena. The mobile phase acts as in CE; it consists of a buffer solution and moves thanks to the applied electrical field.

1.8 UNITS FOR PRESSURE, LENGTH AND VISCOSITY

Pressure Units

The common pressure unit of HPLC is bar, but the SI unit is pascal (Pa): $1\,\text{Pa} = 1\,\text{N m}^{-2}$. The atmosphere (atm or at, respectively) should no longer be used. The unit psi (pounds per square inch) is American and is still in use. Note the difference between psia = psi absolute and psig = psi gauge (manometer), the latter meaning psi in excess of atmospheric pressure.

$$1\,\text{bar} = 10^5\,\text{Pa} = 10^5\,\text{kg m}^{-1}\,\text{s}^{-2} = 0.987\,\text{atm} = 1.02\,\text{at} = 14.5\,\text{lb in}^{-2}\ (\text{psi})$$

Conversion data:

1 MPa = 10 bar (megapascal)
1 atm = 1.013 bar (physical atmosphere)
1 at = 0.981 bar (technical atmosphere, $1\,\text{kp cm}^{-2}$)
1 psi = 0.0689 bar

Rule of thumb:

$1000\,\text{psi} \approx 70\,\text{bar},\ 100\,\text{bar} = 1450\,\text{psi}$

Length Units

English units are often used in HPLC to indicate tube or capillary diameters, the unit being the inch (in or ″). Smaller units are not expressed in tenths but as 1/2, 1/4, 1/8, or 1/16 in, or multiples of these.

Outer diameters:

$1'' = 25.40\,\text{mm}$ $1/2'' = 12.70\,\text{mm}$ $3/8'' = 9.525\,\text{mm}$ $1/4'' = 6.35\,\text{mm}$
$3/16'' = 4.76\,\text{mm}$ $1/8'' = 3.175\,\text{mm}$ $1/16'' = 1.59\,\text{mm}$

Inner diameter of capillaries:

$0.04'' = 1.0\,\text{mm}$ $0.02'' = 0.51\,\text{mm}$ $0.01'' = 0.25\,\text{mm}$ $0.007'' = 0.18\,\text{mm}$

Viscosity Units

The SI unit of the dynamic viscosity is the pascal second: $1\,\text{Pa s} = 1\,\text{kg m}^{-1}\,\text{s}^{-1}$. Solvents have viscosities around $1{\cdot}10^{-3}\,\text{Pa s} = 1\,\text{mPa s}$. The old unit was the centipoise (cP): $1\,\text{mPa s} = 1\,\text{cP}$.

1.9 SCIENTIFIC JOURNALS

Journal of Chromatography A (all topics of chromatography) ISSN 0021–9673.

Journal of Chromatography B (biomedical sciences and applications) ISSN 1570-0232.

Until volume 651 (1993) this was one journal with some volumes dedicated to biomedical applications. Afterwards the journal was split and continued with separate volumes having the same number but not the same letter (e.g. 652A and 652B). Elsevier Science, P.O. Box 211, NL-1000 AE Amsterdam, The Netherlands.

Journal of Chromatographic Science, ISSN 0021–9665, Preston Publications, 6600 W. Touhy Avenue, Niles, IL 60714–4588, USA.

Chromatographia, ISSN 0009–5893, Vieweg Publishing, P.O. Box 5829, D-65048, Wiesbaden, Germany.

Journal of Separation Science (until 2001 *Journal of High Resolution Chromatography)*, ISSN 1615–9306, Wiley-VCH, P.O. Box 10 11 61, D-69451 Weinheim, Germany.

Journal of Liquid Chromatography & Related Technologies, ISSN 1082–6076, Marcel Dekker, 270 Madison Avenue, New York, NY 10016–0602, USA.

LC GC Europe (free in Europe, formerly *LC GC International*), ISSN 1471–6577, Advanstar Communications, Advanstar House, Park West, Sealand Road, Chester CH1 4RN, UK.

LC GC North America (free in the USA, formerly *LC GC Magazine)*, ISSN 0888–9090, Advanstar Communications, 859 Willamette Street, Eugene, OR 97401, USA.

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Biomedical Chromatography, ISSN 0269–3879, John Wiley & Sons, Ltd, 1 Oldlands Way, Bognor Regis PO22 9SA, UK.

International Journal of Bio-Chromatography, ISSN 1068-0659, Gordon and Breach, P.O. Box 32160, Newark, NJ 07102, USA.

Separation Science and Technology, ISSN 0149–6395, Taylor & Francis, Mortimer House, 37–41 Mortimer Street, London, W1T 3JH, UK.

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The *Journal of Microcolumn Separations* (John Wiley & Sons, Ltd, ISSN 1040–7865) merged with the *Journal of Separation Science* after issue 8 of volume 13 (2001).

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E. Heftmann, ed., *Chromatography, Part A: Fundamentals and Techniques, Part B: Applications*, Elsevier, Amsterdam, 6th ed., 2004.
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