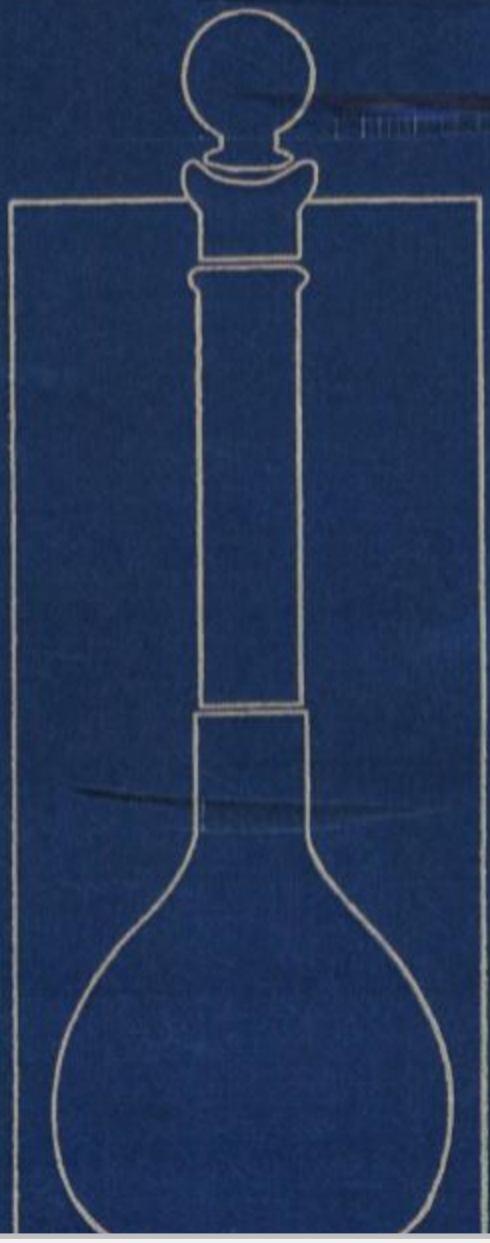
FOOD ANALYSIS

THIRD EDITION



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Ligands for affinity chromatography may be either **specific** or **general** (i.e., group specific). Specific ligands, such as antibodies, bind only one particular solute. General ligands, such as nucleotide analogs and lectins, bind to certain classes of solutes. For example, the lectin concanavalin A binds to all molecules that contain terminal glucosyl and mannosyl residues. Bound solutes then can be separated as a group or individually, depending upon the elution technique used. Some of the more common general ligands are listed in Table 27-4. Although less selective, general ligands provide greater convenience.

Elution methods for affinity chromatography may be divided into **nonspecific** and **(bio)specific** methods. Nonspecific elution involves disrupting ligand analyte binding by changing the mobile-phase pH, ionic strength, dielectric constant, or temperature. If additional selectivity in elution is desired, for example in the case of immobilized general ligands, a biospecific elution technique is used. Free ligand, either identical to or different from the matrix-bound ligand, is added to the mobile phase. This free ligand competes for binding sites on the analyte. For example, glycoproteins bound to a concanavalin A (lectin) column can be eluted by using buffer containing an excess of lectin. In general, the eluent ligand should display greater affinity for the analyte of interest than the immobilized ligand.

27-4 table **General Affinity Ligands and their Specificities**

Ligand	*Specificity*
Cibacron Blue F3G-A dye, derivatives of AMP, NADH, and NADPH	Certain dehydrogenases via binding at the nucelotide binding site
Concanavalin A, lentil lectin, wheat-germ lectin	Polysaccharides, glycoproteins, glycolipids, and membrane proteins containing sugar residues of certain configurations
Soybean trypsin inhibitor, methyl esters of various amino acids, D-amino acids	Various proteases
Phenylboronic acid	Glycosylated hemoglobins, sugars, nucleic acids, and other *cis*-diol-containing substances
Protein A	Many immunoglobulin classes and subclasses via binding to the F_C region
DNA, RNA, nucleosides,	Nucleases, polymerases,

In addition to protein purification, affinity chromatography may be used to separate supramolecular structures such as cells, organelles, and viruses; concentrate dilute protein solutions; investigate binding mechanisms; and determine equilibrium constants. Affinity chromatography has been useful especially in the separation and purification of enzymes and glycoproteins. In the case of the latter, carbohydrate-derivatized adsorbents are used to isolate specific lectins, such as concanavalin A, and lentil or wheat-germ lectin. The lectin then may be coupled to agarose, such as concanavalin A- or lentil lectin-agarose, to provide a stationary phase for the purification of specific glycoproteins, glycolipids, or polysaccharides.

27.3.4 Chromatographic Techniques

The same general principles of chromatography apply, regardless of the specific method or technique used. Paper, thin-layer, and column liquid chromatography all utilize a liquid mobile phase, but the physical form of the stationary phase is quite different in each case. SFC is an analytical technique similar to LC, except that a supercritical fluid, instead of a liquid, is used as the mobile phase.

27.3.4.1 Paper Chromatography

Paper chromatography was introduced in 1944. Although adsorption by the paper itself has been utilized, paper generally serves only as a support for the liquid stationary phase (**partition chromatography**). To carry out this technique, the dissolved sample is applied as a small spot or streak one half inch or more from the edge of a strip or square of filter paper (usually cellulose) and is allowed to dry. The strip is then suspended in a closed container, the atmosphere of which is saturated with the **developing solvent** (mobile phase), and the paper chromatogram is **developed**. The end closer to the sample is placed in contact with solvent, which then travels up or down the paper by capillary action (depending on whether **ascending** or **descending** development is used), separating sample components in the process. When the solvent front has traveled the length of the paper, the strip is removed from the developing chamber and the separated zones are detected by an appropriate method.

The stationary phase in paper partition chromatography is usually water. However, the support may be impregnated with a nonpolar organic solvent and developed with polar solvents or water (reversed-phase paper chromatography). In the case of complex sample mixtures, a two-dimensional technique may be

dried, turned 90°, and developed again, using a second solvent of different polarity. Another means of improving resolution is the use of ion-exchange papers. Both paper that has been impregnated with ion-exchange resin and paper in which cellulose hydroxyl groups have been derivatized (with acidic or basic moieties) are available commercially.

In paper and thin-layer (planar) chromatography, components of a mixture are characterized by their relative mobility (R_f) value, where:

$$R_f = \frac{\text{Distance moved by component}}{\text{Distance moved by solvent}} \quad [3]$$

Unfortunately, R_f values are not always constant for a given solute/sorbent/solvent, but depend on many factors, such as the quality of the stationary phase, layer thickness, humidity, development distance, and temperature.

27.3.4.2 Thin-Layer Chromatography (TLC)

TLC, first described in 1938, has largely replaced paper chromatography because it is faster, more sensitive, and more reproducible. (Both of these techniques may be referred to as **planar chromatography**.) The resolution in TLC is greater than in paper chromatography because the particles on the plate are smaller and more regular than paper fibers. Experimental conditions can be easily varied to achieve separation and can be scaled up for use in column chromatography. (Thin-layer and column procedures are not necessarily interchangeable, due to differences such as the use of binders with TLC plates, vapor-phase equilibria in a TLC tank, etc.) Some distinct advantages of TLC include high sample throughput and low cost; the possibility to analyze several samples and standards simultaneously; and minimal sample preparation (since the stationary phase is disposable). In addition, a plate may be stored for later identification and quantitation.

TLC has been applied to the analysis of lipids (see Chapter 14). HPLC of lipids is complicated by the lack of chromophores that permit ultraviolet–visible (UV–Vis) detection, and most GC analyses require prior derivatization; however, many good lipid detection reagents are available for TLC. TLC is applied in many fields, including environmental, clinical, forensic, phamaceutical, food, flavors, and cosmetics. Within the food industry, TLC may be used for quality control. For example, corn and peanuts are tested for aflatoxins/mycotoxins prior to their processing into corn meal and peanut butter, respectively. Applications of TLC

27.3.4.2.1 General Procedures TLC utilizes a thin (ca. 250 μm thick) layer of **sorbent** or **stationary phase** bound to an **inert support** in a planar configuration. The support is often a glass plate (traditionally, 20 cm × 20 cm) but plastic sheets and aluminum foil also are used. Precoated plates, of different layer thicknesses, are commercially available in a wide variety of sorbents, including chemically modified silicas. (Plates are seldom hand-coated today.) Four frequently used TLC sorbents are silica gel, alumina, diatomaceous earth, and cellulose. Many separations achieved by paper chromatography can be transferred to TLC on cellulose. Modified silicas for TLC may contain polar or nonpolar groups, analogous to bonded phases for column chromatography (see section 27.3.3.2.3.), and both normal and reversed-phase thin-layer separations may be carried out. **High performance thin-layer chromatography** (HPTLC) simply refers to TLC performed using plates coated with smaller, more uniform particles. This permits better separations in shorter times.

If **adsorption** TLC is to be performed, the sorbent is first **activated** by drying for a specified time and temperature. Sample (in carrier solvent) is applied as a spot or streak 1–2 cm from one end of the plate. After evaporation of carrier solvent, the TLC plate is placed in a closed **developing chamber** with the end of the plate nearest the spot in the solvent at the bottom of the chamber. Traditionally, solvent migrates up the plate (**ascending development**) by capillary action and sample components are separated. After the TLC plate has been removed from the chamber and solvent allowed to evaporate, the separated bands are made visible (**visualized**) or detected by other means. Specific **chemical reactions (derivatization)**, which may be carried out either before or after chromatography, often are used for this purpose. Two examples are reaction with sulfuric acid to produce a dark charred area (a **destructive chemical method**), and the use of iodine vapor to form a colored complex (a **nondestructive method** inasmuch as the colored complex is usually not permanent). Common **physical detection methods** include the measurement of absorbed or emitted electromagnetic radiation (e.g., fluorescence) and measurement of β-radiation from radioactively labeled compounds. **Biological methods** or biochemical inhibition tests can be used to detect toxicologically active substances. An example is measuring the inhibition of cholinesterase activity by organophosphate pesticides.

Quantitative evaluation of thin-layer chromatograms may be performed: (1) in situ (directly on the

27.3.4.2.2 Factors Affecting Thin-Layer Separations In both planar and column liquid chromatography, the nature of the compounds to be separated determines what type of stationary phase is used. Separation can occur by adsorption, partition, ion-exchange, size-exclusion, or multiple mechanisms as previously discussed in section 27.3.3. Table 27-5 lists the separation mechanisms involved in some typical applications on common TLC sorbents.

Although selection of both mobile and stationary phases determines the success of a given TLC separation, the rationale behind choice of mobile phase for a particular fractionation often is not described. Solvents for TLC separations should be selected on the basis of their chemical characteristics and **solvent strength** (a measure of interaction between solvent and sorbent; see section 27.3.3.1). In simple adsorption TLC, the higher the solvent strength, the greater the R_f value of the solute. One usually tries to use a mobile phase such that R_f values of 0.3–0.7 are obtained. (Although single solvent mobile phases may provide adequate mobility, they often do *not* give adequate separation.) Fortunately for the beginner, mobile phases have been developed for the separation of various compound classes on specific sorbents; see, for example, table 7.1 in reference (12).

In addition to the sorbent and solvent, several other factors must be considered when performing thin-layer (or paper) chromotography. These include the **type of developing chamber** used, **vapor phase conditions** (saturated versus unsaturated), **development mode** (ascending, descending, horizontal, radial, etc.), and **development distance**.

27-5 table **Thin-Layer Chromatography Sorbents and Mode of Separation**

Sorbent	*Chromatographic Mechanism*	*Typical Application*
Silica gel	Adsorption	Steroids, amino acids, alcohols, hydrocarbons, lipids, aflatoxins, bile acids, vitamins, alkaloids
Silica gel RP	Reversed phase	Fatty acids, vitamins, steroids, hormones, carotenoids
Cellulose, kieselguhr	Partition	Carbohydrates, sugars, alcohols, amino acids, carboxylic acids, fatty acids
Aluminum oxide	Adsorption	Amines, alcohols, steroids, lipids, aflatoxins, bile acids, vitamins, alkaloids
PEI cellulose[1]	Ion exchange	Nucleic acids, nucleotides, nucleosides, purines, pyrimidines
Magnesium silicate	Adsorption	Steroids, pesticides, lipids, alkaloids

27.3.4.3 Column Liquid Chromatography

Column chromatography is the most useful method of separating compounds in a mixture. Fractionation of solutes occurs as a result of differential migration through a closed tube of stationary phase, and analytes can be monitored while the separation is in progress. This section of the chapter will cover general procedures, theory, and the quantitation of data from column liquid chromatography.

27.3.4.3.1 General Procedures A system for **low-pressure** (i.e., performed at or near atmospheric pressure) column liquid chromatography is illustrated in Fig. 27-9. (While the procedure outlined below is applicable to column chromatography in general, the reader is referred to subsequent chapters for details specific to HPLC or GC).

Having selected a stationary and mobile phase suitable for the separation problem at hand, the analyst must first prepare the **stationary phase** (**resin**, **gel**, or **packing material**) for use according to the supplier's instructions. (For example, the stationary phase often must be **hydrated** or **preswelled** in the mobile phase.) The prepared stationary phase then is **packed** into a column (usually glass), the length and diameter of which are determined by the amount of sample to be loaded, the separation mode to be used, and the degree of resolution required. Adsorption columns may be either dry or wet packed; other types of columns are wet packed. The most common technique for wet packing involves making a slurry of the adsorbent with the solvent and pouring this into the column. As the sorbent settles, excess solvent is drained off and additional slurry is added. This process is repeated until the desired bed height is obtained. (There is a certain art to pouring uniform columns and no attempt is made to give details here.) If the packing solvent is different from the initial eluting solvent, the column must be thoroughly washed **(equilibrated)** with the starting mobile phase.

The sample to be fractionated, dissolved in a minimum volume of mobile phase, is applied in a layer at the top (or head) of the column. Classical or low-pressure chromatography utilizes only **gravity** flow or a **peristaltic pump** to maintain a flow of **mobile phase** (**eluent** or **eluting solvent**) through the column.

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