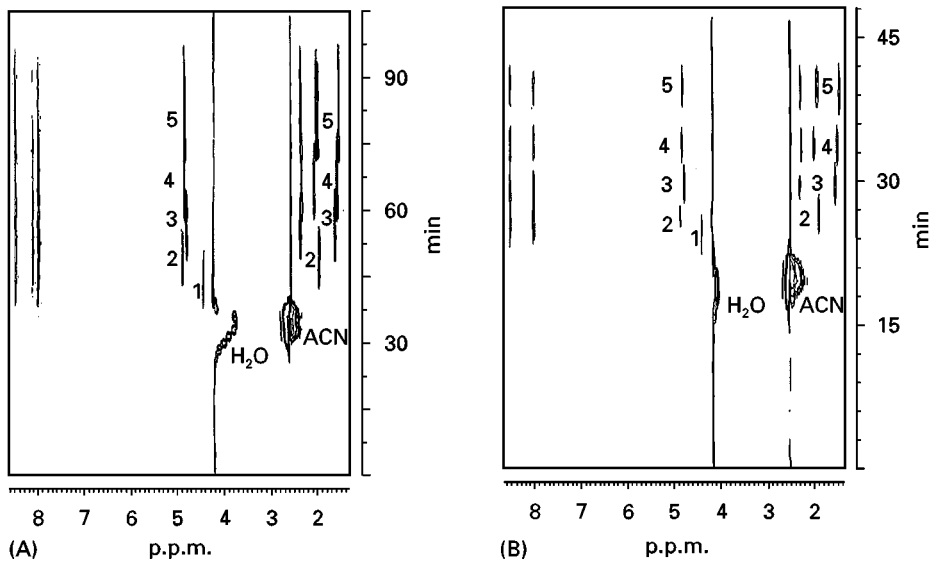


**Figure 17** Contour plots of (A) CHPLC and (B) CEC separation of alkylbenzoates: 1, methyl; 2 ethyl; 3, propyl; 4, butyl; 5, pentyl benzoate; ACN, acetonitrile.

(signal line width) at the height of the $^{13}C$ signals must be further optimized.

A further example is the online CE-NMR and online CEC-NMR separation of alkylbenzoates. **Figure 17** shows the contour plot of the separation performed in the CHPLC and the CEC mode. It is evident from the CEC-NMR contour plot that all compounds are baseline-separated, resulting in distinct NMR rows in the two-dimensional display.

This example shows the great power of CHPLC, CE and CEC-NMR to derive unambiguous information of substances in complex organic molecules. The first steps towards a high-throughput separation system have already been made. For the successful performance of real-life applications, NMR sensitivity must be improved. If NMR probes with 1 ng sensitivity become available, an increasing number of capillary separations coupled with nanoscale NMR will be performed in many applications.

*See also:* **II / Chromatography: Supercritical Fluid:** Instrumentation. **Electrophoresis:** Detectors for Capillary Electrophoresis. **III/Carotenoid Pigments: Supercritical Fluid Chromatography. Gradient Polymer Chromatography: Liquid Chromatography. Natural Products:** Liquid Chromatography-Nuclear Magnetic Resonance. **Pigments:** Liquid Chromatography. **Polyethers: Liquid Chromatography.**

## Further Reading

Albert K (1995) *Journal of Chromatography A* 703: 123–147.

Albert K (1997) *Journal of Chromatography A* 785: 65–83.

Braumann U, Händel H, Albert K et al. (1995) *Analytical Chemistry* 67: 930–935.

De Koenig JA, Hogenboom AC, Lacker T et al. (1998) *Journal of Chromatography A* 813: 55–61.

Hatada K, Ute K, Kitayama T et al. (1989) *Polymer Bulletin* 21: 489–495.

Lindon JC, Nicholson JK and Wilson ID (1996) *Progress in NMR Spectroscopy* 29: 1–49.

Pusecker K, Schewitz J, Gförer P et al. (1998) *Analytical Chemistry* 70: 3280–3285.

Schlotterbeck G, Tseng L-H, Händel H et al. (1997) 69: 1421–1425.

Strohschein S, Schlotterbeck G, Richter J et al. (1997) *Journal of Chromatography A* 765: 207–214.

Wu N, Peck TL, Webb AG et al. (1994) *Analytical Chemistry* 66: 3849–3857.

# Partition Chromatography (Liquid–Liquid)

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## Introduction

Partition or liquid–liquid chromatography (LLC) is a powerful separation technique which has been successfully used for the separation and analysis of

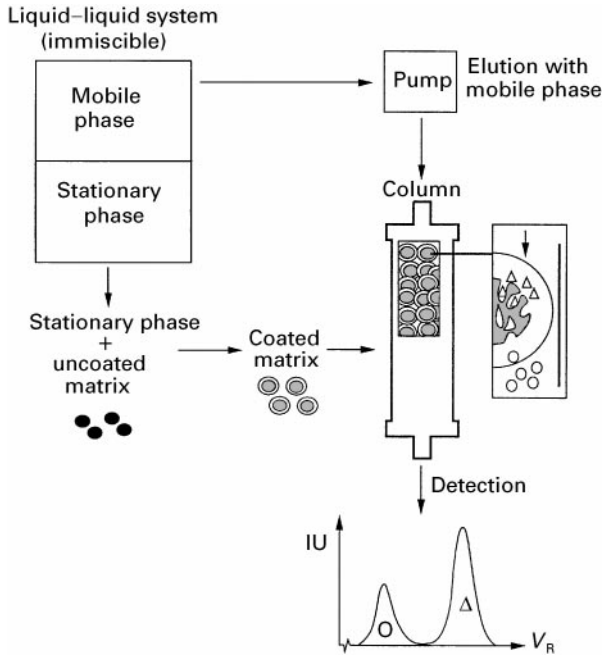



**Figure 1** Schematic illustration of LLC. IU, international units; $V_R$, retention volume.

a wide variety of sample types, including water-soluble and oil-soluble compounds, ionic and nonionic compounds, as well as biopolymers such as nucleic acids and proteins. The basis of LLC is the distribution of sample molecules between two immiscible liquid phases, a stationary phase and a mobile phase (**Figure 1**). In conventional LLC, the stationary phase is mechanically held to a support by adsorption. In liquid chromatography (LC), the most widely used supports are those with chemically bonded organic stationary phases. This may be described as a separate LC method: bonded-phase chromatography (BPC).

LLC offers unique selectivity for various samples since a wide range of liquid phases can be used (**Table 1**). In normal-phase LLC, the support is coated with a polar stationary phase, whereas a relatively nonpolar solvent is used as mobile phase. In reversed-phase LLC, the two LLC phases are interchanged so that the less polar liquid is now the stationary phase and the polar liquid is the mobile phase. LLC systems composed of aqueous/aqueous, aqueous/organic or organic/organic solvents can be used, depending on the particular separation problem. A variety of supports have been used (**Table 2**), but silica-based matrices appear to be best suited for LLC.

The sample molecules are distributed between the stationary and mobile phases depending on their physicochemical properties and the properties of the

**Table 1** Liquid–liquid chromatographic systems commonly used for LLC and LLPC of compounds, nucleic acids and proteins

| *Sample* | *Chromatographic system* | *Stationary phase*[a] | *Mobile phase* |
|---|---|---|---|
| Compounds | Normal-phase LLC | $\beta,\beta'$-Oxydipropionitrile | Pentane, cyclopentane, hexane, heptane or isooctane. Same, but with 10–20% of chloroform, dichloromethane tetrahydrofuran, acetonitrile, dioxane, etc. |
| | | 1,2,3-Tris(2-cyanoethoxy)propane | See above |
| | | Triethylene glycol | See above |
| | | Trimethylene glycol | See above |
| | | Ethylene glycol | Di-*n*-butyl ether or nitromethane |
| | | Dimethylsulfoxide | Isooctane |
| | | Water/ethylene glycol | Hexane/$CCl_4$ |
| | | Water | *n*-Butanol |
| | | Nitromethane | $CCl_4$/hexane |
| | Reversed-phase LLC | Cyanoethylsilicone | Methanol/water |
| | | Dimethylpolysiloxane | Acetonitrile/water |
| | | Hydrocarbon polymer | Methanol/water |
| Nucleic acids | LLPC | 4.4% PEG 8000/6.2% dextran 500[a] | |
| Proteins | LLPC | 4.4% PEG 8000/5.2% dextran 500[a] | |
| | | 4.4% PEG 8000/6.2% dextran 500[a] | |
| | | 5.2% PEG 8000/10.0% dextran 40[a] | |
| | | 2.7% PEG 20000/4.5% dextran 500[a] | |
| | LLPC | 10% PVP 30/10% dextran 40[a] | |
| | LLPC | PVA 10/dextran 40[a] | |
| | LLPC | 11.25% PEG 1500/12.75% potassium phosphate[a] | |
| | | 16.75% PEG 1500/16.0% sodium citrate[a] | |

[a] Or complete system.

LLC, liquid–liquid chromatography; LLPC, liquid–liquid partition chromatography; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; PVA, polyvinyl alcohol.

**Table 2** Supports commonly used for LLC of compounds, nucleic acids and proteins

| *Sample* | *Support type* | | *Name* | *Particle size (μm)* | *Pore size (nm)* |
|---|---|---|---|---|---|
| Compounds | LLC | Diatomaceous earth | Chromosorb LC-1 | 37–44 | – |
| | Porous | Silica, spherical | Spherosil XOA 600 | 5–8 | 8 |
| | | | Zorbax | 6 | 7 |
| | | Silica, irregular | Porasil | 10 | – |
| | | | LiChrosorb Si-60 | 5, 10 | 6, 10 |
| | Pellicular | 'Inactive' silica, spherical | Zipax | 25–37 | 80 |
| | | | Liqua-Chrom | 44–53 | – |
| | | | Corasil I | 37–50 | – |
| | | 'Active' silica, spherical | Perisorb A | 30–40 | – |
| | | | Vydac | 30–44 | – |
| | BPC | | | | |
| | Corasil modified with octadecylsilane | | Bondapak $C_{18}$/Corasil | 37–50 | – |
| | Perisorb A modified with dimethylsilane | | Perisorb-RP-2 | 30–40 | – |
| | Zipax modified with octadecylsilane | | Permaphase-ODS | 25–37 | 80 |
| | Vydac modified with ethylnitrile | | Vydac SC-polar | 30–44 | – |
| Nucleic acids/proteins | Polyvinyl modified with polyacrylamide | | LiParGel 650 | 25–40 | n.a.[a] |
| | | | LiParGel 750 | 25–40 | n.a.[b] |
| | Silica modified with polyacrylamide | | LiChrospher-Diol 100 | 10 | 10 |
| | | | LiChrospher-Diol 1000 | 10 | 100 |
| | | | LiChrospher-Diol 4000 | 10 | 400 |
| | Agarose modified with dextran | | Superdex 200 prep. grade | 24–44 | n.a.[c] |

[a] Data not available. Exclusion limit for globular proteins of $5 \times 10^6$ Da and for dextrans of $10^6$ Da.
[b] Data not available. Exclusion limit for globular proteins of $5 \times 10^7$ Da and for dextrans of $10^7$ Da.
[c] Data not available. Exclusion limit for globular proteins of $10^6$ Da and for dextran of $10^5$ Da.
LLC, liquid–liquid chromatography; BPC, bonded-phase chromatography.

phases (and support). In the case of compounds, the separation is generally based on the type and number of substituent groups and by differences in molecular weight (up to about 2000 Da). Hence, LLC is very useful for the separation of homologues and mixtures of compounds of different functionality. Biopolymers (nucleic acid fragments) can be separated mainly owing to differences in molecular weight (up to about 4000 base pairs). In the case of proteins, conditions can be designed in such a way that differences in their overall exposed surface properties are detected. In fact, LLC has proved to be a unique tool for protein analysis, that is for purification and fractionation, detection and separation of conformational isomeric forms, examination of surface properties related to biological specificities and for providing information about the events upon binding of ligand and about possible ligand-induced conformational changes. In this article, we describe the use of LLC for the separation and analysis of compounds, nucleic acids and proteins, focusing on the latter group.

## Separation of Compounds

A variety of compounds including detergents, drugs, insecticides, metal chelates, pesticides, phenols, saccharides, steroids and vitamins, some of which are shown in **Table 3**, have been successfully separated using LLC. LLC provides a wide range of separation selectivity since several different liquid–liquid systems can be used. In the case of normal-phase LLC, a number of separations have been performed using either $\beta,\beta'$-oxydipropionitrile (BOP), or one of the various polyethylene glycols (PEGs) as stationary phase. Both liquids are useful for the same type of compounds, although the PEGs may be more selective for alcohols, and BOP is preferred for basic compounds such as amines. Normal-phase LLC is used for more polar, water-soluble compounds, and the solute elution order is similar to that observed in adsorption chromatography. In reversed-phase LLC, cyanoethylsilicone, hydrocarbon polymers and squalene, among others, have been used as stationary phases. Reversed-phase LLC is generally used to separate compounds with poor water solubility, i.e. non-polar and organic-soluble compounds.

Besides the binary liquid systems normally used in LLC, ternary liquid–liquid systems have also been found useful (Table 1). Such systems are obtained from binary liquid systems by adding a third component (cosolvent) that is miscible with both phases. Either of the two resulting phases can be used as

**Table 3** Applications of LLC for separation of compounds

| *Approach* | *Application* | *Examples* |
|---|---|---|
| Normal-phase LLC | Separation of compounds containing metals | Radioactive metals |
| | | Isomers of cobalt complexes involved in synthesis of vitamin $B_{12}$ |
| | | Metal-β-diketonates |
| | | Platinum–metal complexes |
| | Determination of compounds in biological samples | Vitamins $K_1$ and E in serum |
| | | Phosphatidylcholine in serum |
| | | 2,4,6-Triiodophenol and other iodinated derivatives in serum |
| | | Flumequine and 7-flumequine in serum |
| | Analysis of drugs in food products | Flumethasone pivalate in serum |
| | | Methyl prednisolone in milk |
| | | *N*-Nitrosamines in extract of pork |
| | Determination of compounds in environmental samples | Pesticides, e.g. mosquito larvacide in salt ponds |
| | | Insecticides, e.g. imidacloprid in water and soil |
| | General separations and determinations | Penicillin derivatives |
| | | Steroids, e.g. derivatized urinary 17-ketosteroids |
| | | Coumarins |
| | | Phenols |
| | | Dinitrophenylhydrazine derivatives |
| | | Ethylene oxide oligomers |
| Reversed-phase LLC | Determination of compounds in biological samples | Ritonavis |
| | | Enoxacin and 4-oxyenoxacin in plasma and tissue |
| | | Tacrine and its metabolites in plasma and urine |
| | | Nucleotides in different tissues and body fluids |
| | Separation of important organic chemical | Antioxidants |
| | | Plasticizers |
| | | Nonionic organic dyes |
| | General separations and determinations | Fat-soluble vitamins, e.g. vitamins A, D, E and K |
| | | Aromatics |
| | | Paraffins |
| | | Olefins and diolefins |

LLC, liquid–liquid chromatography.

stationary phase, with the other as mobile phase. As an example, the ternary system formed by water/ethanol/2,2,4-trimethylpentane has been found to be very useful for the separation of many compounds including metal chelates, pesticides and steroids.

Several different supports can be used, but silicas and diatomaceous matrices appears to be best suited. These supports are inert, and have relatively large pores so as to allow ready access of the sample molecules to the stationary phase contained within the porous structure. It may be more convenient to use bonded-phase packings, the main advantage being that they are quite stable, since the stationary phase is chemically bound to the support and subsequently cannot be easily removed in use.

The separation of compounds by LLC is generally based on the type and number of substituent groups and by differences in molecular weight. This is exemplified by the fact that ethylene oxide oligomers (7–14 ethylene oxide units) can be separated by differences in number of functional groups, naphthalene derivatives by differences in compound type, and 2,4-dinitrophenylhydrazine derivatives ($C_2$ to $C_6$) by differences in molecular weight (Table 3).

Modern LLC have been used to separate several biologically important natural and synthetic compounds, including cortisol, derivatized urinary 17-ketosteroids and free underivatized steroids. Many compounds containing metal atoms have been separated using LLC, such as radioactive metals, isomers of cobalt complexes involved in the synthesis of vitamin $B_{12}$, metal-$\beta$-diketonates and platinum metals complexes. Moreover, LLC has also been frequently used for determination of specific compounds in biological samples, as for example vitamins $K_1$ and E in serum, phosphatidylcholine in serum, 2,4,6-triiodophenol and other iodinated derivatives in serum, and flumequine and 7-flumequine in plasma.

In addition, LLC has been used to analyse drugs in various food products, e.g. flumethasone pivalate in cream, methyl prednisolone in milk and *N*-nitrosoamines in extract of pork. Various derivatives of

penicillin have also been separated using LLC. Moreover, LLC has been used to separate and determine a number of pesticides and insecticides, such as imidacloprid in water and soil.

Reversed-phase chromatography has been used to separate mixtures of fat-soluble vitamins (vitamins A, D, E and K); mixtures of aromatics, paraffins, olefins and diolefins; chlorinated benzenes; and fused-ring aromatics. A wide variety of important organic chemicals such as antioxidants, plasticizers and nonionic dyes has been separated using LLC. Reversed-phase chromatography has also been used to determine compounds in biological samples, including ritonavis, an HIV protease inhibitor, enoxacin and 4-oxyenoxacin in plasma and tissue, and tacrine and its metabolites in plasma and urine. Another important area of application is the separation of nucleotides, nucleosides and bases that are used to examine a variety of biomedical problems.

## Separation of Biopolymers

The classical version of LLC uses combinations of an organic and an aqueous or two organic liquid phases, which limits its application to small molecules without defined secondary or tertiary structure. However, the use of LLC for separation of large active biomolecules such as nucleic acids and proteins became possible (at least in theory) when P.-Å. Albertsson succeeded in developing aqueous–aqueous two-phase systems made up of two 'incompatible' water-soluble polymers such as PEG and dextran. Finding suitable supports for these systems was for a long time a major problem, however. Several attempts were made to adsorb the bottom phase onto supports made of agarose beads, polyethers immobilized on Sepharose, silicates and cellulose. The problem was solved by W. Müller in 1986 by combining the affinity of polyacrylamide for the dextran-rich bottom phase of the PEG/dextran system with the mechanical strength of hydrophilic vinyl (LiParGel) or silica (LiChrospher Diol) particles (Table 2). LLC, or LLPC (liquid–liquid partition chromatography), was then introduced as a method for the separation of biopolymers. This technique was later developed further with respect to sensitivity, selectivity and reproducibility by U.-B. Hansson and co-workers, turning it into a powerful tool for protein analysis in particular. It has recently also been shown that dextran-grafted agarose beads (Superdex) can be used as a support for LLPC. LiParGel 650 has been used for the analysis of small as well as of large proteins while LiParGel 750 has mostly been used for the analysis of nucleic acids. LiChrospher-Diol 100 has been used for the analysis of small proteins ( $< 100\,000$ Da) while proteins of a wide range of sizes have been analysed on LiChrospher-Diol 1000 or 4000 columns. Superdex is suitable for the analysis of small proteins ( $< 100\,000$ Da).

Buffered phase systems formed by polyvinyl pyrrolidone (PVP)/dextran, polyvinyl alcohol (PVA)/dextran and PEG/salt solutions have been used for LLPC, but the PEG/dextran systems are by far the most frequently used (Table 1). A serious drawback of the PEG/dextran systems is, however, the incompatibility between PEG and some proteins such as immunoglobulins. Concentrations of PEG greater than 3% can be used to precipitate antibodies. However, this incompatibility can be partly overcome by adding appropriate salts. IgG can, for example, be solubilized in PEG/dextran systems at pH 7.0, i.e. near its isoelectric point, in the presence of $100\,\text{mmol}\,\text{L}^{-1}$ betaine ( $\leq 0.8\,\text{mg}\,\text{mL}^{-1}$) or $0.1\,\text{mol}\,\text{L}^{-1}$ glycine and $0.1\,\text{mol}\,\text{L}^{-1}$ sodium chloride ( $\leq 2\,\text{mg}\,\text{mL}^{-1}$). Hence, the PEG/dextran systems are, for the moment, the obvious choice of two-phase system for LLPC of biopolymers, whereas the usefulness of other phase systems remains to be elucidated.

## Nucleic Acids

LLPC in aqueous two-phase systems has been successfully used for DNA fractionation by size, topology and by base sequence, as well as for the fractionation of 'soluble' and ribosomal RNAs (**Table 4**). Hence, LLPC may provide resolutions unattainable by any other method.

**Table 4** Separation of nucleic acids by LLPC

| *Separation according to:* | *Examples* |
|---|---|
| Size | DNA fragments, linear relationship for fragments up to about 4000 base pairs<br>Various RNAs from natural sources, e.g. soluble RNAs from tomato plants<br>Ribosomal RNA up to about 30 S |
| Base composition | *Eco* RI generated fragments from λ DNA<br>Calf thymus DNA |
| Topology | Fractionation of circular DNAs |

LLPC, liquid–liquid partition chromatography.

Fractionation of DNA restriction fragments by size can be performed with DNA fragments up to at least 4000 base pairs (Table 4). Mainly two-phase systems formed by PEG 8000/dextran 500 have so far been applied (Table 1). The separation can be greatly improved by applying a salt-exchange gradient. Side effects, possibly caused by slightly hydrophobic properties of the supports, may strongly influence the fractionation of DNA. Although the influence of the supports on DNA may be obvious, as in the case of proteins (see below), the general applicability of the matrices is not invalidated as the interactions may be exploited as an additional parameter for separation.

DNA may be fractionated according to base composition or sequence using PEG-bound AT- or GC-specific DNA ligands consisting of basic dyes (Table 4). In this way, these ligands will prefer the PEG-rich mobile phase and are subsequently able to shift the partitioning of matching DNA towards this phase. As in the case of DNA, RNA may also be fractionated by LLPC in aqueous two-phase systems due to differences in size. Also large (ribosomal) RNA ranging up to 30 S can be fractionated by LLPC. The high solubility of RNAs in the PEG/dextran systems is also advantageous.

## Proteins

The way in which a protein molecule is distributed between the two phases in a given PEG/dextran system depends on its three-dimensional structure and general surface properties and is described by its partition coefficient, $K_c$ (the ratio of the concentration of the molecule in the top phase to that in the bottom phase). Thus, partitioning in aqueous PEG/dextran two-phase systems offers a unique means of separating proteins in solution with respect to their overall exposed surface properties. In addition, the method is mild so that the conformation of a partitioned protein is not likely to be disturbed.

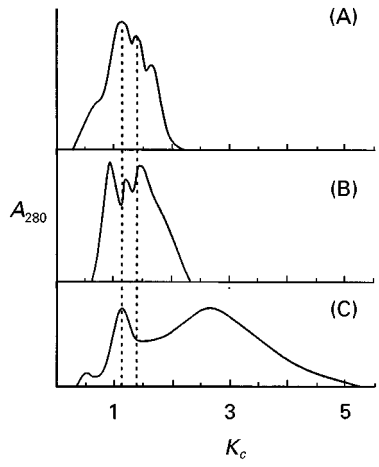



**Figure 2** Influence of the support on LLPC of polyclonal IgG: (A) LiParGel, (B) LiChrospher, (C) Superdex. Data adapted from Wingren *et al., Journal of Chromatography* 603, 73 (1992); *Journal of Chromatography* 668, 65 (1994), with permission from Elsevier Science.

As in the case of DNA separation, the supports may (strongly) influence the fractionation of proteins. This is illustrated in **Figure 2**, where LLPC on these supports resulted in different elution profiles for polyclonal IgG, i.e. the supports influenced the partitioning of immunoglobulins in different ways. The exact nature of the observed interactions is still not clear, but they may be determined by the conformation of the molecules together with the properties of the support with respect to pore size and polyacrylamide/dextran coating. Although ideal partitioning is desirable, the general applicability of the matrices is not invalidated as the interactions can be exploited as previously mentioned.

### Separation

LLPC can be used to separate a wide variety of proteins including enzymes, hormones, immunoglobulins, serum proteins and transport proteins, some of which are shown in **Table 5**. LLPC has been successfully used for separations that have previously been difficult, for instance the separation of molecules with similar molecular weight and pI values such as α-1-antitrypsin and albumin, and corticosteroid-binding globulin and a sex hormone-binding globulin. Moreover, LLPC may replace the need of other conventional separation techniques, since LLPC, in contrast to most of these methods, is able to separate proteins owing to differences in several physicochemical properties. As for example, proteins in human sera, which can be separated in a multistep procedure by using a combination of several other techniques such as precipitation, ion exchange chromatography, gel filtration and electrophoresis, can be separated in a single step by LLPC. Furthermore, proteins that can be separated by differences in pI values, as for example isoforms of lactate dehydrogenase and malate dehydrogenase, can be separated by LLPC. In addition, monomeric and dimeric forms of HSA that can be separated by gel filtration and proteins that may be separated by hydrophobic chromatography can also be separated by LLPC. Thus, LLPC in aqueous polymer two-phase systems offers new possibilities to separate proteins in a single step and/or to obtain fractionation that is not readily achieved by other techniques.

### Fractionation

LLPC can be used to fractionate proteins and may provide a selectivity and sensitivity unattainable by any other chromatographic method. Some

**Table 5** Separation of proteins by LLPC

| *Separation problem* | *Example* | *LLPC results/comments* |
|---|---|---|
| Complex system | Proteins in human serum | A single step separation achieved; previously separated in a multistep procedure using a combination of several other methods |
| | Proteins in goat, sheep, dog and horse serum | |
| | Proteins in mouse ascites | |
| Proteins with similar $M_r$ and pI | α-1 antitypsin and HSA | |
| | CBG and SHBG from human serum | |
| Proteins with different pI | Isoforms of LDH | LLPC is able to separate proteins due to differences in 'several' physicochemical properties and may thus replace the need of many conventional separation techniques |
| | Isoforms of MDH | |
| Proteins with different $M_r$ | Monomers and dimers of HSA | |
| Proteins with different hydrophobicity | Human IgG with different relative hydrophobicity | |
| General separations | Myoglobin, peroxidase, BSA and OVA | |
| | Cyt *c*, HSA, β-lactoglobulin, lysozyme, MB, ovotransferrin, OVA and peroxidase | |
| | ADH, GDH and ferritin | |
| | ADH, FDH and LDH | |
| | Transferrin, IgG and HSA | |

ADH, alcohol dehydrogenase; BSA, bovine serum albumin; CBG, corticosteroid-binding globulin; Cyt *c*, cytochrome *c*; FDH, formate dehydrogenase; GHD, glucose 6-phosphate dehydrogenase; HSA, human serum albumin; LDH, lactate dehydrogenase; LLPC, liquid–liquid partition chromatography; MB, myoglobin; MDH, malate dehydrogenase; OVA, ovalbumin; SHBG, sex hormone-binding globulin.

representative examples, including enzymes and antibodies, are given in **Table 6**. The usefulness of LLPC for this purpose was highlighted in recent studies where several enzymes known to exist in equilibrium between two allosteric forms were analysed. Yeast alcohol dehydrogenase, liver alcohol dehydrogenase, the heart and muscle isoforms of lactate dehydrogenase and two types of hexokinase (fraction I and II of Kaji), are all eluted as two components by LLPC, in spite of the fact that the enzyme preparations appear to be homogeneous when analysed by other conventional methods such as isoelectric focusing, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and high performance liquid chromatography–size exclusion (HPLC-SE). Remarkably, an equilibrium between the two components with different surface properties for each of these enzymes can be demonstrated by LLPC. These results show that LLPC can be used to detect and even separate different conformational isometric forms of unliganded enzymes. It

**Table 6** Fractionation of proteins by LLPC

| *Proteins* | *LLPC results/comments* |
|---|---|
| LADH<br>YADH<br>LDH-$H_4$<br>LDH-$M_4$<br>Hexokinase[a] | An equilibrium between two components with different surface properties detected (i.e. conformational isomeric forms) |
| sMDH<br>mMDH | Two components with different surface properties detected |
| Polyclonal IgG<br>Polyclonal IgA<br>Polyclonal IgM | Several components detected |
| Monoclonal IgG | An equilibrium between at least two components with different surface properties detected (for six of 57 antibodies; conformational isomeric forms?) |

[a]Similar results were obtained for two types of hexokinase, fraction I and II of Kaji. LADH, liver alcohol dehydrogenase; mMDH, mitochondrial malate dehydrogenase; sMDH, cytoplasmic malate dehydrogenase; LDH-$H_4$, heart lactate dehydrogenase; LDH-$M_4$, muscle lactate dehydrogenase; LLPC, liquid–liquid partition chromatography; YADH, yeast alcohol dehydrogenase.

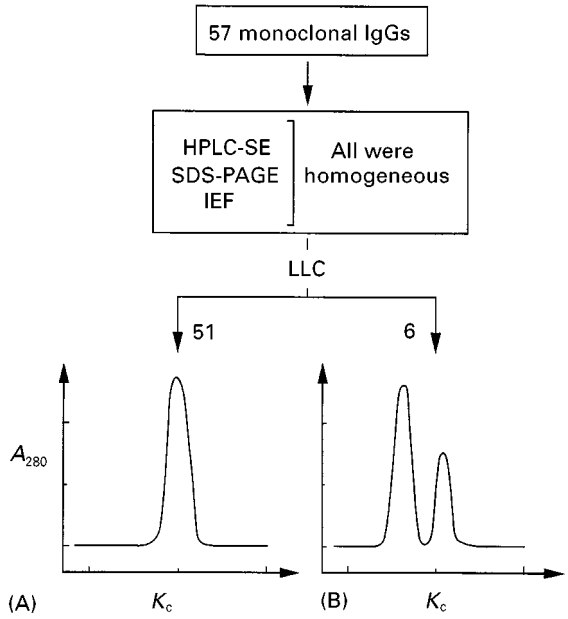



**Figure 3** LLPC of 57 monoclonal IgG antibodies containing 95% IgG and homogeneous with respect to their physicochemical properties as determined by HPLC-SE, SDS-PAGE and IEF. The LLPC chromatograms are schematically illustrated in (A), which represents 51 of the IgGs, and (B), which represents six of the IgGs.

may be of interest to note that the LLPC fractionations of the mitochondrial and cytoplasmic isoforms of malate dehydrogenase, the allosteric mechanisms of which are still unclear, also result in two components with different surface properties.

Immunoglobulins are another group of proteins that have been successfully fractionated using LLPC (Table 6 and see below). Purified polyclonal immunoglobulins of IgG, IgM or IgA class can be fractionated into several components using LLPC. In the case of IgG and IgA, it is of a special interest to note that the fractionation is not subclass-related since the elution profile of each of the subclasses is similar to that of the applied polyclonal antibody population. Surprisingly, some monoclonal antibodies have been found to be fractionated into at least two components by LLPC, in spite of the fact that they are homogeneous with respect to their immunochemical and physicochemical properties (**Figure 3**). This phenomenon was observed not only for IgG antibodies but also for IgA and IgM myeloma proteins. Recent LLPC experiments have demonstrated that six of 57 monoclonal IgG antibodies seem to exist in an equilibrium between at least two components with different surface properties. Thus, LLPC seems to be able to detect and separate conformational isomeric forms of unliganded antibodies. It has been suggested (using different kinetic techniques) that a tenth of all antibodies may indeed display conformational isomerism.

## Specificity

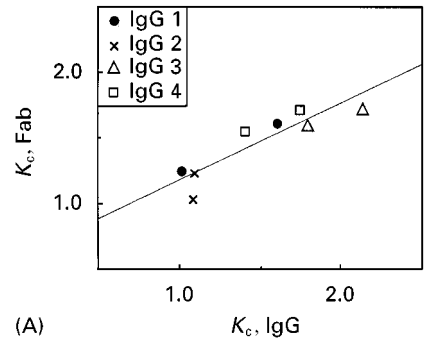
LLPC can be used to examine differences in surface properties of proteins (mainly immunoglobulins) related to their biological specificities; some illustrative examples are given in **Table 7**.

In early LLPC experiments, polyclonal rabbit IgG antibodies with different specificities were found to exhibit different partition properties (expressed as a partition coefficient, $K_c$). Since no IgG subclasses have been reported for rabbit IgG, i.e. their Fc parts are likely to be the same, these results indicated that LLC had detected differences in surface properties between IgGs located on structures in their Fab parts. This exciting finding was followed up by more extensive studies in which large sets of well-characterized monoclonal IgG antibodies were analysed by LLPC. These studies clearly showed that there was no correlation between $K_c$ and a single immunochemical and physicochemical property such as light-chain and

**Table 7** Examination of surface properties of proteins related to biological specificities using LLPC

| *Proteins* | *Example* | | *LLPC results* |
|---|---|---|---|
| pAbs with different specificities | anti-IgG, anti-HSA and anti-transferrin | | Different $K_c$ values |
| mAbs with different affinities for the same Ag | Protein Ag | 5 mAbs against cytolomegalovirus | Different $K_c$ values |
| | | 5 mAbs against albumin | Different $K_c$ values |
| | Hapten Ag | 3 mAbs against DNP | Different $K_c$ values |
| | | 2 mAbs against $T_3$ | Different $K_c$ values |
| | | 3 mAbs against $T_4$ | Different $K_c$ values |
| mAbs with identical affinity for one | Hapten | 2 mAbs against $T_3$ | Identical $K_c$ values |
| | | 2 mAbs against $T_4$ | Identical $K_c$ values |
| | Epitope | 2 mAbs against IgGFc | Identical $K_c$ values |
| | | 3 chimaeric mAbs against NIP | Identical $K_c$ values |

pAbs, polyclonal antibodies; mAbs, monoclonal antibodies; Ag, antigen; HSA, human serum albumin; DNP, 2,4-dinitrophenol; $T_3$, triiodothyronine; $T_4$, thyroxine; NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; $K_c$, the ratio of the concentration of the molecule in the top phase to that in the bottom phase; LLPC, liquid–liquid partition chromatography.




| Subclass $K_c$, Fc | |
|---|---|
| IgG 1 | 1.22 ± 0.01 |
| | 1.23 ± 0.01 |
| | 1.21 ± 0.02 |
| IgG 2 | 1.22 ± 0.01 |
| | 1.22 ± 0.01 |
| | 1.21 ± 0.02 |
| IgG 4 | 1.22 ± 0.01 |
| | 1.22 ± 0.01 |
| | 1.21 ± 0.02 |

(B)

**Figure 4** Comparison of the partition properties of (A) intact IgG with those of the corresponding Fab fragments and (B) Fc fragments from one IgG subclass with those of the other subclasses. The partition properties are expressed as a partition coefficient, $K_c$. The correlation coefficient was (A) 0.80 ($P < 0.05$). (B) The 95% confidence limits of the $K_c$ values are given.

heavy-chain isotypes, charge, size and shape. However, a significant linear correlation between the partition properties of intact IgG1, 2, 3 and 4 and their corresponding Fab fragments was detected (**Figure 4**). In contrast to the Fabs, Fc fragments from IgG1, 2 and 4 displayed almost identical surface properties (Figure 4). Taken together, the results showed that the differences detected by LLPC between intact IgGs were indeed located on their Fabs.

Further studies have revealed a remarkable relationship between the partition properties of an IgG molecule and the structure of its combining site (specificity; see Table 7). In particular, monoclonal IgG antibodies with different affinities for the same antigen or directed against different antigens are eluted with different $K_c$ values, while monoclonal IgGs with identical affinity constants for the same hapten or the same epitope on a protein are found to have identical $K_c$ values. Moreover, chimaeric anti-NIP antibodies with identical variable regions, corresponding to the human IgG1, 2 and 4 subclasses, display identical surface properties, i.e. differences in the constant part of the heavy chains did not affect the partitioning. Thus, the surface properties of IgGs are, as detected by LLPC, dominated by those of their antigen-binding sites. Recent LLPC experiments have indicated that the antigen-binding site dominance observed for IgG is valid for all the other Ig classes and subclasses provided that antibodies only within a given class or subclass are compared with respect to exposed surfaces. Indications of a dominance of ligand-binding sites for other specific proteins, such as enzymes, have been reported. Taken together, LLPC may offer a unique possibility to screen the antigen-binding sites for differences/similarities in exposed surfaces, even in those cases when we do not know the specificity of the antibodies.

This unique property of LLPC was made use of in a recent study that showed that the LLPC profile for polyclonal IgG antibodies isolated from the sera of patients with an autoimmune disease (primary Sjögren's syndrome) differed significantly from those of polyclonal IgG isolated from the sera of healthy individuals. These antibodies with 'unique and deviating' specificities could also be isolated by LLPC. Thus, LLPC may provide us with the means to fractionate and isolate specific antibodies 'of unknown specificities'.

### Ligand Binding

Finally, LLPC can also be used to study the events upon binding of a ligand by specific proteins in solution. Some typical example, including transport proteins, enzymes and polyclonal as well as monoclonal antibodies, are given in **Table 8**. In many cases, LLPC can simply be used to separate the ligand–protein complex from either of the free components. This is, for example, the case for most of the enzyme–ligand and antibody–ligand complexes analysed so far.

In recent studies, the capacity of LLPC for analysis of protein–ligand complexes was demonstrated using well-characterized ligand–protein complexes where ligand-induced conformational changes are known to occur. In addition, the ligands in these model complexes are small and almost completely buried upon binding, i.e. the bound ligands themselves would not be responsible for any changes in surface properties observed upon complexation. Small changes in surface properties were detected when rabbit transferrin bound ferric ions, while no changes were observed when human transferrin (which is known to be less flexible) bound the ions. The ligand-induced transition between two conformational isomeric forms could be detected by LLPC for a large number of enzymes, including alcohol dehydrogenase, citrate synthase, glutamate-oxaloacetate transaminase, hexokinase, lactate dehydrogenase, malate dehydro-

**Table 8** Examination of the events upon binding of ligand by specific proteins in solution using LLPC

| *Protein* | *Ligand* | *LLPC results/comments* |
|---|---|---|
| Rabbit transferrin | $Fe^{3+}$ | Small changes in $K_c$ detected |
| Human transferrin | $Fe^{3+}$ | No changes in $K_c$ detected |
| CS | Oxaloacetate | Ligand-induced transition between two conformational isomeric forms detected |
| Hexokinase | Glucose | |
| GAPDH | NADH or $NAD^+$ | |
| GOT | α-Methyl-aspartate | |
| LADH | NADH or $NAD^+$ | |
| LDH-$M_4$ | NADH or NADH + oxamate | |
| LDH-$H_4$ | $NAD^+$ | |
| mMDH | Citrate + $NAD^+$ | |
| sMDH | Citrate + $NAD^+$ | |
| PGK | GDP + 3-PGA | |
| YADH | NADH or $NAD^+$ | |
| pAbs | IgG, HSA or transferrin | Differences in $K_c$ detected, may be interpreted in terms of conformational characteristics |
| mAb | $T_3$ | |
| 9 chimaeric mAbs | NIP | |
| 5 mAbs | HSA | Differences in $K_c$ detected; different mAbs formed AgAb with identical $K_c$ |
| 18 mAbs | 5 haptens | Linear relationship between $K_c$ of unliganded mAbs and their corresponding hapten–mAb complex |

CS, citrate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOT, glutamate-oxaloacetate transaminase; HSA, human serum albumin; LADH, liver alcohol dehydrogenase; LDH-$H_4$, heart lactate dehydrogenase; LDH-$M_4$, muscle lactate dehydrogenase; mAbs, monoclonal antibodies; LLPC, liquid–liquid partition chromatography; mMDH, mitochondrial malate dehydrogenase; sMDH, cytoplasmic malate dehydrogenase; NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; pAbs, polyclonal antibodies; PGA, 3-phosphoglycerate; PGK, 3-phosphoglycerate kinase; $T_3$, triiodothyronine; YADH, yeast alcohol dehydrogenase.

genase and 3-phosphoglycerate kinase. In the case of antibodies, conformational changes may occur upon binding of either hapten ($T_3$ and NIP) or protein antigens (HSA, IgG and transferrin) that are detectable by LLPC. The surface properties of chimaeric anti-NIP antibodies with identical variable regions, corresponding to the human IgA1, IgA2, IgE, IgG1, IgG2, IgG3, IgG4 and IgM isotypes, have been compared before and after binding of hapten. The hapten–antibody complexes were found to be eluted in a considerably narrower range of $K_c$ values than were the free antibodies. It was concluded that conformational changes, detectable by LLPC, occurred in either IgA1, IgA2, IgE and/or IgM, but not in the IgGs, making the surfaces of the constant regions of the heavy chains of the Ig classes and subclasses more similar. Hence, LLPC may provide us with the means to examine whether ligand binding induces conformational changes in the antibody when in solution.

Almost all the antigen–antibody pair analysed by LLPC so far have formed complexes that are eluted as single homogeneous peaks by LLPC (**Figure 5**). Moreover, the complexes are eluted with the same value of $K_c$ irrespective of the molar ratio of antigen to antibody at which they are formed (ranging from antigen to antibody excess). Thus, the results imply that each antigen–antibody pair forms one type of complex with respect to exposed dominant surfaces. This is thought to be the first study reporting such a feature for antigen–antibody complexes.

Remarkably, a linear relationship between the surface properties of unliganded IgGs and their corresponding hapten–IgG complexes has been reported, and it was concluded that the surface properties of

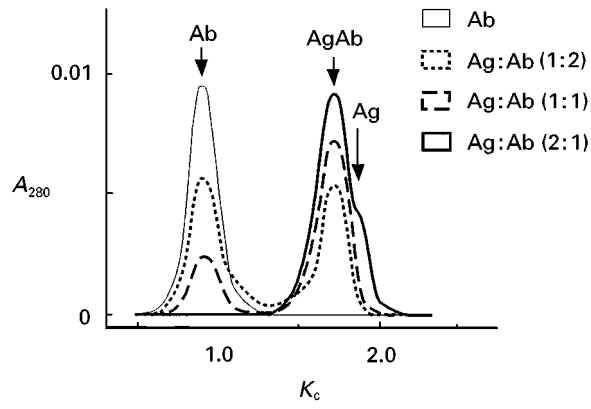



**Figure 5** LLPC of antigen–antibody complexes (AgAb) formed at different molar ratios of antigen (Ag) to antibody (Ab) (Ag : Ab = 2 : 1–0.5 : 1). Representative results obtained for one monoclonal IgG antialbumin antibody are shown.

IgG are dominated by those of its antigen-binding sites even after the specific binding of hapten or hapten-carrier at the combining sites. By contrast, the surface properties of protein–antibody complexes are not related to those of the unliganded antibodies. Instead, the surface properties of protein–IgG complexes are related mainly to those of the antigens. Depending on the type of antigen (hapten or protein), LLPC may thus be used to separate antigen–IgG complexes through differences in exposed surfaces of either the antibody combining sites or the antigen. LLPC may thus provide us with new, interesting information concerning the events upon complex formation in solution and possible ligand-induced conformational changes.

*See also:* **III/Nucleic Acids:** Liquid Chromatography. **Proteins:** Ion Exchange. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins. Essential Guides for Isolation/Purification of Immunoglobulins. Essential Guides for Isolation/Purification of Nucleic Acids.**

## Further Reading

Albertsson P-Å (1986) *Partition of Cell Particles and Macromolecules*. New York: Wiley.

Careri M, Mangia A and Musci M (1996) Applications of liquid chromatography–mass spectrometry interfacing systems in food analysis: pesticide, drug and toxic substance residues. *Journal of Chromatography* 727: 153–184.

Grune T and Siems WG (1993) Reversed-phase high-performance liquid chromatography of purine compounds for investigation of biomedical problems: application to different tissues and body fluids. *Journal of Chromatography* 618: 15–40.

Hansson U-B and Wingren C (1998) Separation of antibodies by liquid–liquid aqueous partition and by liquid–liquid partition chromatography. *Separation and Purification Methods*.

Hansson U-B and Wingren C (1999) Liquid–liquid partition chromatography. In: *Methods in Biotechnology*. Totowa: Humana Press.

Hansson U-B and Wingren C (1999) Liquid–liquid partition chromatography of proteins. In: Kastner M (ed.) *Protein Liquid Chromatography*. Oxford: Elsevier.

Müller W (1986) New phase supports for liquid–liquid partition chromatography of biopolymers in aqueous poly(ethyleneglycol)–dextran systems. Synthesis and application for the fractionation of DNA restriction fragments. *European Journal of Biochemistry* 155: 213–222.

Müller W (1988) *Liquid–Liquid Partition Chromatography of Biopolymers*. Darmstadt: Git-verlag.

Rizzolo A and Polesello S (1992) Chromatographic determination of vitamins in foods. *Journal of Chromatography* 624: 103–152.

Walter H and Johansson G (eds) (1994) Aqueous two-phase systems. *Methods in Enzymology* 228.

Walter H, Brooks DE and Fisher D (eds) (1985) *Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses and Application to Biotechnology*. London: Academic Press.

Wang P and Lee HK (1997) Recent applications of high-performance liquid chromatography to the analysis of metal complexes. *Journal of Chromatography* 789: 437–451.

Wingren C, Hansson U-B, Magnusson CG and Ohlin M (1995) Antigen-binding sites dominate the surface properties of IgG antibodies. *Molecular Immunology* 32: 819–827.

# Physico-Chemical Measurements

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Chromatography theory establishes that the retention volume of a solute is a function of its distribution coefficient and the variance of the eluted peak is a function of solute diffusivity. Consequently, retention volume measurements can provide distribution coefficient data which, if determined over a range of temperatures, will disclose the necessary thermodynamic information that will help reveal the physical nature of the distribution process. In addition, peak dispersion measurements can furnish accurate diffusion coefficients for almost any solute, in any liquid, at any chosen temperature, assuming appropriate solute solubility and temperature stability. Hence, the use of liquid chromatography to measure physicochemical properties of a distribution system hinges on the accurate measurement of retention volumes and peak widths.

Gas chromatography retention volume measurements have been used to provide a significant amount of thermodynamic data, including standard free energies, enthalpies and entropies of distribution, together with activity coefficients. These types of physicochemical measurements were pioneered in the late 1950s. In contrast, the use of liquid chromatography