Separation and analysis of biological probes: methods and instruments

Basic principles of centrifugation and chromatography techniques

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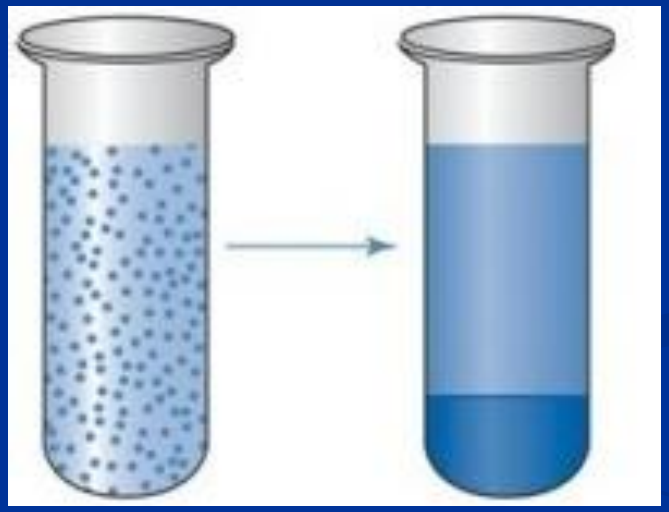
Темы лекций

1. Методы центрифугирования
2. Основные принципы хроматографии
3. Жидкостная хроматография
4. Электрофорез
5. Капиллярный электрофорез

Basic principles of centrifugation

Natural sedimentation of big particles in solution - "centrifugation" with earth gravity



The original idea of centrifugation technique is to increase locally gravity and
to accelerate sedimentation of particles

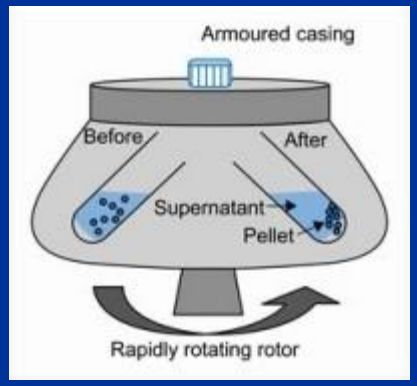
Increased gravity force can be generated during rotation

Centrifuge for fun

Centrifuge to dry clothes

Centrifuge for the cosmonaut training

More exactly, a centrifugal force is generated during fast rotation



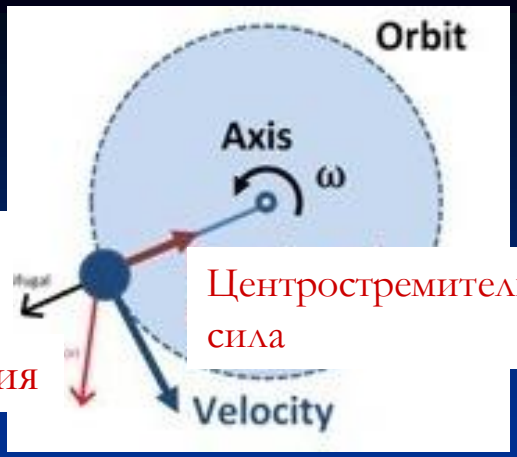
A device that separates particles from a solution using **centrifugal force** is called **a centrifuge**

Centrifugation is a technique used for the separation of particles/molecules from a solution according to their **size, shape and density**.

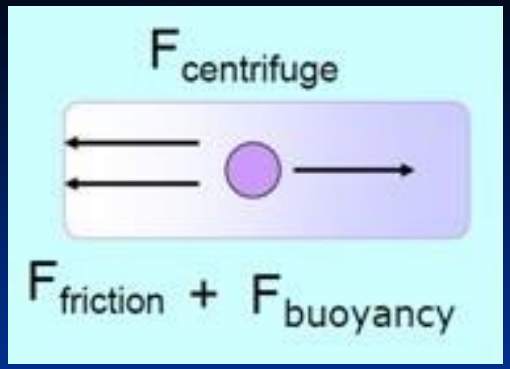
In biology, the particles are usually cells, subcellular organelles, or large molecules

Центробежная сила

Вектор ускорения



Центростремительная сила



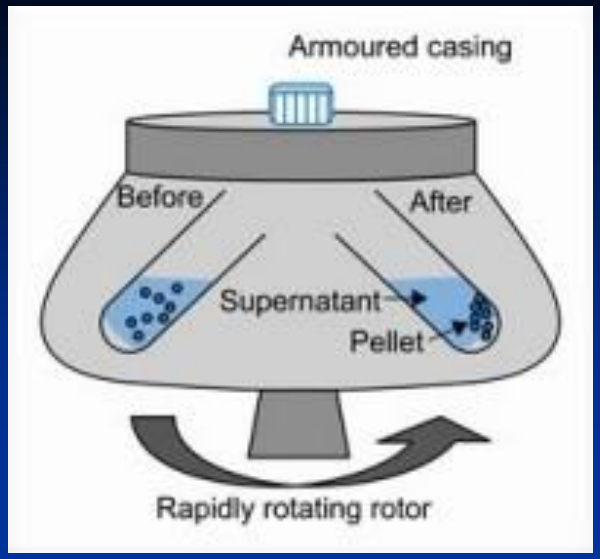
Силы, противодействующие центробежной силе:
Выталкивающая сила - сила, с помощью которой частицы должны вытеснять жидкую среду, в которой они оседают.
Сила трения - сила, создаваемая частицами при их миграции через раствор.

Частицы осаждаются, если **$F_{центробеж.} > F_{трения}+F_{выталк}$**.

Закон Стокса описывает движение сферы в гравитационном поле:

$$v=d^2(p-L)\times g/(18n),$$

where v –sedimentation rate (velocity of the sphere), d -sphere diameter, p –particle density, L-medium density, *n* –viscosity of medium, g –gravitational acceleration



Relative Centrifugal Force (RCF) is expressed in ×*g* (multiple of earth gravitational acceleration)

$$RCF = 1.118 \times R \times (rpm\ /1000)^2$$

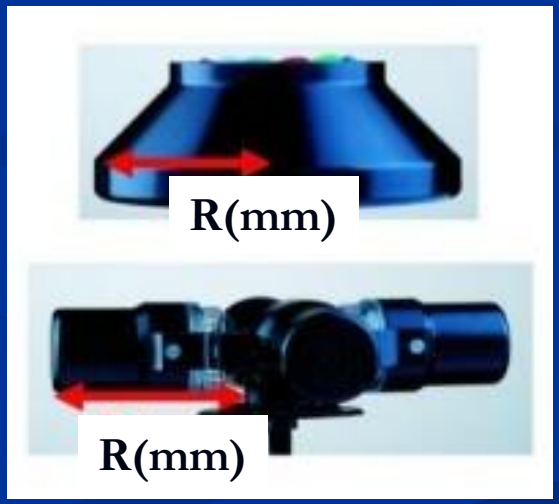
R is a rotor radius in mm

rpm : speed in revolutions per minute

Centrifugation process is characterized by

often: speed (rpm) and time

correctly: RCF (×*g*) and time



Factors that affect centrifugation :

Density of both samples and solution

Temperature/viscosity of solution

Distance of particles displacement

Rotation speed

Методы центрифугирования:

(i) дифференциальное центрифугирование;

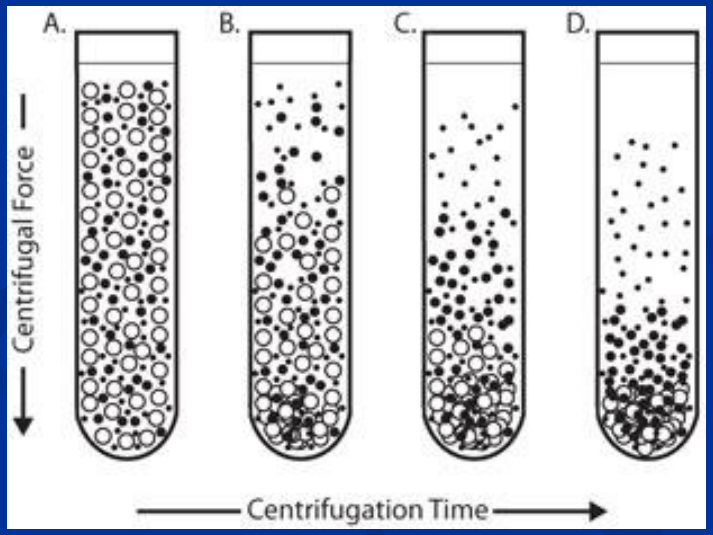
(ii) центрифугирование в градиенте плотности, включая зонально-скоростное центрифугирование, изопикническое центрифугирование

Дифференциальное центрифугирование

Скорость осаждения

$$v = d^2(p-L) \times g/(18n)$$

Время осаждения $t \sim 1/v$



The simplest form of separation by centrifugation

Particles of different densities or sizes in a suspension will sediment at different rates, with the larger and denser particles sedimenting faster.

Typical **differential centrifugation (pelleting)** applications:

(i) harvesting cells

(ii) producing crude subcellular fractions from tissue homogenate

Examples.

A suspension of cells subjected to a series of increasing centrifugal force cycles will yield a series of pellets containing cells possessing different sedimentation rates.

A rat liver homogenate: nuclei, mitochondria, lysosomes, and membrane vesicles.

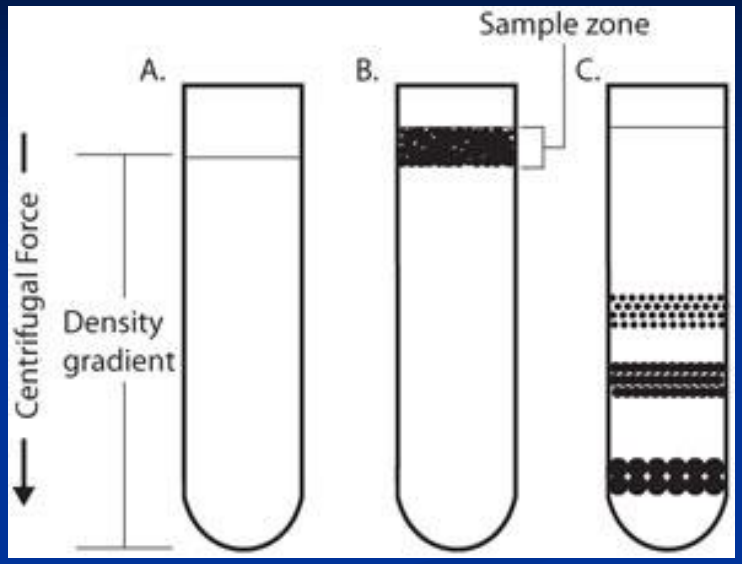
Restrictions:

- Time consuming
- Not more than 4 sedimentation cycles
- Cross contamination of fractions
- Poor recoveries

Rate-Zonal Centrifugation in a Gradient Density
Зонально-скоростное центрифугирование в градиенте плотности

Principle:

1. A density gradient is formed in a tube by using two solutions with different density.
2. A layer of the sample is placed as a narrow zone on the top of a density gradient.
3. Centrifugation



$$v = d^2(p-L) \times g / (18n)$$

Создание градиента плотности:

соли тяжёлых металлов (рубидия или цезия),
растворы сахарозы.

Виды градиента:

Ступенчатый градиент;
Непрерывный градиент

Rate-Zonal Centrifugation in a Gradient Density
Зонально-скоростное центрифугирование в градиенте плотности

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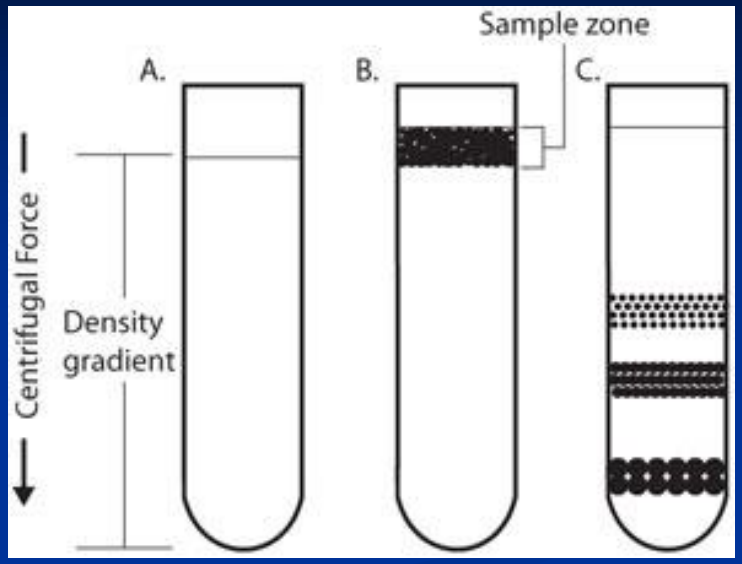


$$v=d^2(p-L)\times g/(18n)$$

Advantage:
cross-contamination of sedimented fractions is avoided;
faster than differential centrifugation

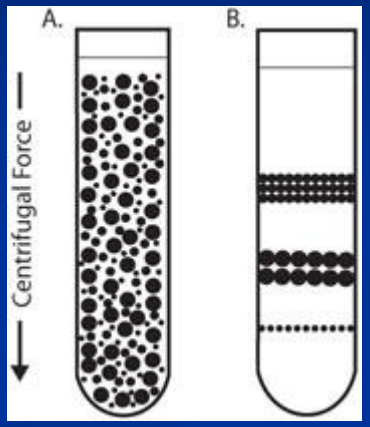
Restrictions:
the narrow load zone limits the volume of sample (typically 10%)

Isopycnic Centrifugation in a Gradient Density

Principle:

$$v = d^2(p-L) \times g / (18n)$$

1. A density gradient is formed in a tube by using two solutions with different density.
2. Maximal density of the gradient media **is higher** than the density of the particles.
3. Sample is mixed with the gradient medium
4. Centrifugation



Feature:
the particles will never sediment to the bottom of the tube, no matter how long the centrifugation time

Advantages:

cross-contamination of sedimented fractions is avoided;
faster than differential centrifugation;
high volume of the sample undergoes separation

Properties of an ideal density gradient media for biological samples

- Sufficient solubility to produce the range of densities required
- Does not form solutions of high viscosity in the desired density range
- Is not **hyperosmotic or hypoosmotic** (for osmotically sensitive particle/cells)
- Solutions of the gradient should be adjustable to the pH and the ionic strengths that are compatible with the particles being separated
- Does not affect the biological activity of the sample
- Nontoxic and not metabolized by cells
- Does not interfere with assay procedures or react with the centrifuge tubes
- Easily removed from the purified product
- Autoclavable
- Reasonable cost

No single compound can satisfy all of the above criteria.

A wide range of gradient media are used for the different types of samples.

Осмолярность раствора

Осмотическая концентрация — суммарная концентрация всех растворённых частиц.

Осмолярность выражается в единицах осмоль на литр раствора.
Осмоляльность выражается в единицах осмоль на килограмм растворителя.

Осмоль — единица осмотической концентрации, получаемой при растворении в одном литре (или в 1 кг) растворителя одного моля неэлектролита.

Т.е. раствор неэлектролита (сахар) с концентрацией 1 моль/л имеет осмолярность 1 осмоль/л.

Осмолярность **электролита** зависит от его концентрации, коэффициента диссоциации и числа ионов, на которые он диссоциирует:

$$Osm = \Phi n C,$$

где Ф — коэффициент диссоциации, принимает значения от 0 (для неэлектролита) до 1 (полная диссоциация), C — молярная концентрация раствора, n — количество частиц, на которые диссоциирует молекула.

Для NaCl n = 2, для H_2SO_4 n = 3.

Osmolality and Biological Particles

The osmolality of most mammalian fluids and balanced salt solutions (e.g., 0.85-0.9% NaCl) for cellular studies is **290-300 mOsm**.

Either high or low osmolality of solutions will disturb membrane-surrounded biological particles (cells, mitochondria, lysosomes, etc),
affect their size and density,
thereby changing their buoyancy and sedimentation rate.

The small osmotic effect is usually reversible,
though it is a possible source of errors.

There are five main classes of density gradient media enhancing the separation process and overcoming osmolality and viscosity problems :

- Многоатомные (сахарные) спирты (этилен гликоль, глицерин)
- Полисахариды
- Неорганические соли
- Йодированные соединения
- Коллоидная окись кремния (silica), кремнезем

Density gradient media types and their principle uses

Gradient medium type	Principle uses
Polyhydric alcohols	
Sucrose	Organelles, membrane vesicles, viruses, proteins, ribosomes, polysomes
Glycerol	Mammalian cells (infrequent), proteins
Sorbitol	Nonmammalian subcellular particles
Polysaccharides	
Ficoll®, polysucrose and dextrans	Mammalian cells (sometimes in combination with iodinated density gradient media), mammalian subcellular particles (infrequent)
Inorganic salts	
CsCl	DNA, viruses, proteins
Cs_2SO_4	DNA, RNA
KBr	Plasma lipoproteins
Iodinated gradient media	
Diatrizoate	Mainly as a component of commercial lymphocyte isolation media
Nycodenz®, Histodenz™	Mammalian cells, organelles, membrane vesicles, viruses
Iodixanol	Mammalian cells, organelles, membrane vesicles, viruses, plasma lipoproteins, proteins, DNA
Colloidal silica media	
Percoll®	Mammalian cells, organelles, membrane vesicles (infrequent)

Typical clinical and biomedical applications of centrifugation technique

- Separation of blood plasma and serum for conservation
- Separation of urine components
- Separation of blood cells
- Centrifugation stages in many clinical assays

Rotor – the main component of a centrifuge
Types of rotors

Fixed-angle rotors

The cavities range from **0.2 mL to 1 L.**
Acceleration ranges from several to **1,000,000 × g**

Swinging-bucket rotors

- Separation of large-volume samples (up to 12 L)
- Low accelerations.

A swinging-bucket rotor system consists of three parts:

- **The rotor body** attaches to the centrifuge drive and has four or six arms to support the buckets,
- **The buckets** are placed onto the arms of the rotor body,
- **Plastic inserts-** to fit properly the tubes of different size

Types of centrifuges

- Microcentrifuges
- Small Benchtop Centrifuges/General Purpose Centrifuges
- Large Capacity Centrifuges
- Ultracentrifuges

Microcentrifuges

- Compact, safe and easy-to-use
- Relatively lost cost
- Support all micro volume protocols
- Can be used with small volume (0.2-2 ml) tubes, minipreps, spin columns, PCR tubes and strips, and hematocrit capillaries.
- With or without thermo-stabilization option

Small Benchtop Centrifuges/General Purpose Centrifuges

- maximized capacity and flexibility in a compact footprint
- changeable fixed-angle and swinging-bucket rotors
- with (usually) or without thermo-stabilization option

Research Applications: Cellular Biology, Microbiology, Genomics / Molecular Biology, Proteomics, Biochemistry, Pharmaceutical Studies.

Clinical Applications: Clinical Chemistry, Clinical Microbiology, Hematology, Immunology, Clinical Studies.

Large Capacity Centrifuges

provide reproducible separations for high-throughput applications such as blood banking and bioprocessing.

Ultracentrifuges

to maximize productivity with **impressive acceleration rates (up to 1,000,000 ×*g*)**.

Applications: separation of micro-organisms, cellular debris, cellular organelles, proteins.

Classes of centrifuges and their applications

Parameters	Low speed	High speed	Ultracentrifuge
Speed ranges (r.p.m. x 10^3)	**2–6**	**18–22**	**35–120**
Maximum RCF (x 10^3)	**8**	**60**	**700**
Bacteria	—	Yes	Yes*
Animal and plant cells	Yes	Yes	Yes*
Nuclei	Yes	Yes	Yes*
Precipitates	Some	Most	Yes*
Membrane organelles	Some	Yes	Yes
Membrane fractions	Some	Some	Yes
Ribosomes/polysomes	—	—	Yes
Macromolecules	—	—	Yes
Viruses	—	Most	Yes

*Possible, but used infrequently

Basic principles of chromatography

Chromatography is a set of laboratory techniques for the separation of **analyte**, i.e. a mixture of compounds/particles present in gases or in liquids.

Chromatography is a physical method of separation that is based on the distribution of components between **two phases**, **stationary phase** and **the mobile phase** moving in a definite direction.

Stationary phase is usually a solid substance (often called sorbent) or a liquid film immobilized by adsorption on a solid support matrix.

Mobile phase is a liquid or gas moving through a stationary phase

Chromatography techniques allows **separation** of multicomponent mixtures, identification of components and their **quantification**.

History of chromatography

First employed in Russia by the Italian-born scientist Mikhail Tsvet in 1900 for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls. Since these components have different colors, they gave the technique its name.

Chromatography techniques were further developed by Archer John Porter Martin and Richard Laurence Millington Syng (1940-1950s) and marked with the Nobel Prize in 1952.

Classification of chromatography techniques

By aggregation state:

gas chromatography
- gas-liquid chromatography
- gas-solid phase chromatography

liquid chromatography
- liquid-liquid chromatography
- liquid-solid phase chromatography
- liquid-gel chromatography

Classification of chromatography techniques

By interaction mechanism between sorbent and analyte:

Distribution chromatography (Распределительная хроматография) separates molecules according to their solubility in the stationary phase (gas-liquid chromatography) or according to their solubility in both mobile and stationary phases. Стационарная фаза химически связана с основой/субстратом. Подвижная фаза – жидкость или газ. Разделение за счет различий в полярности веществ. Частный случай - обращённо-фазная хроматография.

Size-exclusion chromatography (гель-фильтрация или эксклюзионная хроматография) separates molecules according to their size (or more accurately according to their hydrodynamic diameter or hydrodynamic volume). Вещества разделяются по размеру за счёт их разной способности проникать в поры неподвижной фазы. Стационарная фаза инертна и с разделяемыми веществами не взаимодействует.

Classification of chromatography techniques

By interaction mechanism between sorbent and analyte:

Ion exchange chromatography (Ионообменная хроматография, разновидность ионной хроматографии**)** uses an ion exchange mechanism to separate analytes based on their respective charges. Разделяет практически любые заряженные молекулы, включая белки, нуклеотиды и аминокислоты. Часто это первый этап очистки белков.

Adsorbtion chromatography (Адсорбционная хроматография) разделяет соединения за счёт их различной способности сорбироваться (физисорбция или хемисорбция) и десорбироваться на поверхности адсорбента с развитой поверхностью, например, силикагеля.

Affinity chromatography (Аффинная хроматография) is based on selective non-covalent interaction between an analyte and specific molecules. Например, высокоспецифичные взаимодействия антигена и антитела, фермента и субстрата, рецептора и лиганда, белка и нуклеиновой кислоты.

Classification of chromatography techniques

По типу субстрата/основы:

Column chromatography - the stationary bed is within a tube
The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole volume of the tube (packed column)
or be concentrated on or along the inside tube wall leaving a free channel for the mobile phase along the axis of the tube (open tubular column).

Planar chromatography is a separation technique in which the stationary phase is present as a plane or applied on a plane.
The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (**paper chromatography**)
or a layer of solid particles spread on a support such as a glass plate (**thin-layer chromatography**).

Glossary for liquid chromatography

Term	Definition
Mobile phase or carrier	solvent moving through the column/plane substrate
Stationary phase or adsorbent	substance that stays fixed inside the column/on the surface of plane substrate
Eluent	fluid entering the column/plane substrate
Eluate	fluid exiting the column (that is collected in flasks)
Elution	the process of washing out a compound through a column (plane substrate) using a suitable solvent
Analyte	mixture whose individual components have to be separated and analyzed

Classification of chromatography techniques

<u>Special techniques</u>:

Reversed-phase chromatography is any liquid chromatography procedure in which the mobile phase is significantly more polar than the stationary phase. (in normal-phase liquid chromatography, the mobile phase is significantly less polar than the stationary phase).

Two-dimensional chromatography - when one chromatographic mode is insufficient to separate all compounds

In planar chromatography - The sample is spotted at one corner of a square plate, developed, air-dried, then rotated by 90° and usually redeveloped in a second solvent system.

In column chromatography - a series of unresolved peaks eluted from the first column is directed onto a second column with different physico-chemical properties.

Classification of chromatography techniques

By aim:

Analytical chromatography – quantitative and qualitative analysis.

Preparative chromatography – production of pure compounds

Industry chromatography - to control industrial processes.

Chromatography is widely used to study solutions, catalytic processes, kinetics of chemical processes and etc.