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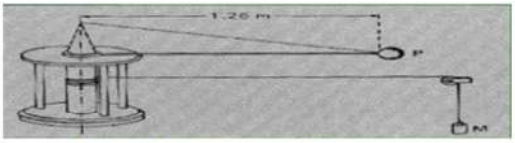
SEPARATION TECHNIQUES - CENTRIFUGATION

Centrifugation is a technique used for the separation of particles from a solution according to their size, shape, density, viscosity of the medium utilizing the principles of centrifugal force. The device which is used to achieve separation of particles through the use of centrifugal force is called **centrifuge**. In a centrifuge, particles are suspended in a liquid medium in a tube known as **centrifuge tube** that is placed in a rotor of a centrifuge and spun to a defined speed. The separation of particles actually involves their attributes of sedimentation which is itself guided through the principles of gravity and physical characteristics of the particles such as shape, size, viscosity, etc. For lighter particles and biomolecules, it is impossible to separate without the help of any specialized device and it would take ages to separate from one another naturally and therefor centrifuge came into existence to separate such particles much faster. Therefore, we can say that a centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity of the medium.

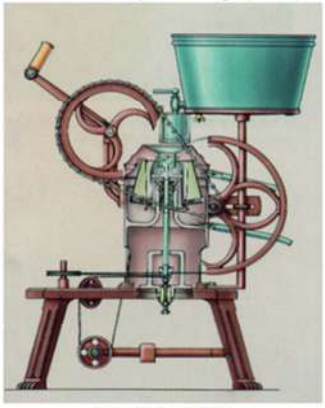
In this line, English military engineer Benjamin Robins (1707 – 1751) invented a whirling arm apparatus to determine drag; however, he used this device in inventing the principles of gunnery. Later, the first dairy centrifuge was developed in 1864 in order to separate cream from milk and in 1879, the first continuous centrifugal separator with 750 revolutions per minute, making its commercial application feasible was invented the Swedish engineer Gustav de Laval and formed a company named Alfa Laval Separator Company to produce this milk cream separated. Again, in 1895, a businessman Franz Ramesohl and cabinetmaker Franz Schimdt, also brother-in-law, made their hand-operated milk separator and named it as 'Westfalia' in Germany. Therefore, a series of works began to continuously upgrade the machine and till now up to 100 types of centrifuge machines have been developed.



Benjamin Robins' Whirling Arm Apparatus


First Dairy Centrifuge (1864)


Continuous Centrifugal Separator (1879)


Westfalia, 1895

Principles of a Centrifuge

As described above, it separates the biomolecules from a solution due to centrifugal force exerted on the biomolecules in the solution thus it separates it according to their size, shape, density, viscosity and speed of rotor. For centrifugation, solution containing biomolecules are suspended in a centrifuge with a balance of opposite centrifuge tube on a rotor and spun at a defined speed. A centrifuge is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward).There are many factors which influence this process described above, such as;

- Density of both samples and solution
- Temperature and viscosity
- Distance of particles displacement
- Rotation speed

There are two types of centrifuge procedures; one is preparative, and the other is analytical. **Preparative centrifuge** is a type of centrifuge which involves only the isolation of specific particles, while the **analytical centrifuge** is a type of centrifuge which involves measuring physical properties of the sedimenting particles. As a rotor spins in a **centrifuge**, a centrifugal force is applied to each particle in the sample; the particle is then sedimented at the rate that is proportional to the centrifugal force applied to it. At a fixed centrifugal force and liquid viscosity, **the sedimentation rate of a particle is proportional to its size** (molecular weight) and to the difference between the particle density and the density of the solution. The movement of higher or heavier particles under the influence of gravitational force or centrifugal force, also called as g-force, is called sedimentation and the rate of sedimentation of each particle in a solution is greatly affected by the viscosity of the sample solution and the physical properties of the particles. Under the influence of g-force, substances separate according to their density. According to the sedimentation of particles in a centrifuge, separation is categorized into isopycnic, density gradient, ultrafiltration, phase separation, and pelleting.

- **Isopycnic separation:** It is a type of density gradient separation, also known as buoyant or equilibrium separation, where particles move until their density is the same as the surrounding medium. Here, particle size only affects the rate of particle movement during the separation. The sample is loaded into the tube with the gradient-forming solution (on top of or below pre-formed gradient, or mixed in with self-forming gradient). The solution of the biological sample and cesium salt is uniformly distributed in a centrifuge tube and rotated in an ultracentrifuge. Under the influence of centrifugal force, the cesium salts redistribute to form a density gradient from top to bottom. Particles move to point where their buoyant density equals that part of gradient and form bands. It is a "true" equilibrium procedure since it depends on bouyant densities, not velocities. Generally, CsCl and NaI gradients are used in the centrifugation as "self-forming" gradients.

- **Density gradient separation:** It is used to separate particles on the basis of s and ρ (size and density) by employing a medium of graded densities, therefore different particles are found to contain in different bands containing different sizes and densities of particles

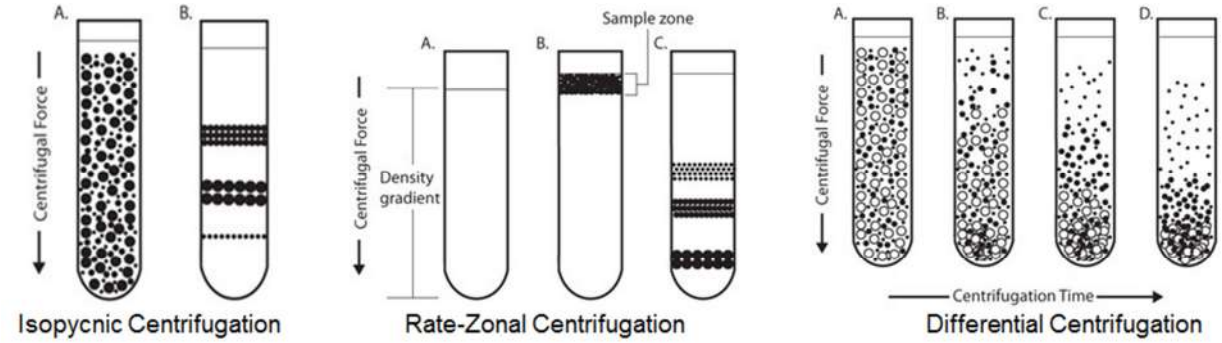
layered over a density. It is mainly applied for the separation of viruses, ribosomes, membranes, etc. In this method, a sucrose density gradient is created by gently overlaying lower concentrations of sucrose on higher concentrations in centrifuge tubes. The samples are placed on top of the gradient and spun in ultracentrifuges. The particles travel through the gradient until they reach a point at which their density matches the density of surrounding sucrose. The fraction is then easily removed and analyzed.

Density gradient media and their principle uses

Gradient medium type	Principle uses
Polyhydric alcohols	
Sucrose	Sucrose Organelles, membrane vesicles, viruses, proteins, ribosomes, polysomes
Glycerol	Mammalian cells (infrequent), proteins
Sorbitol	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)

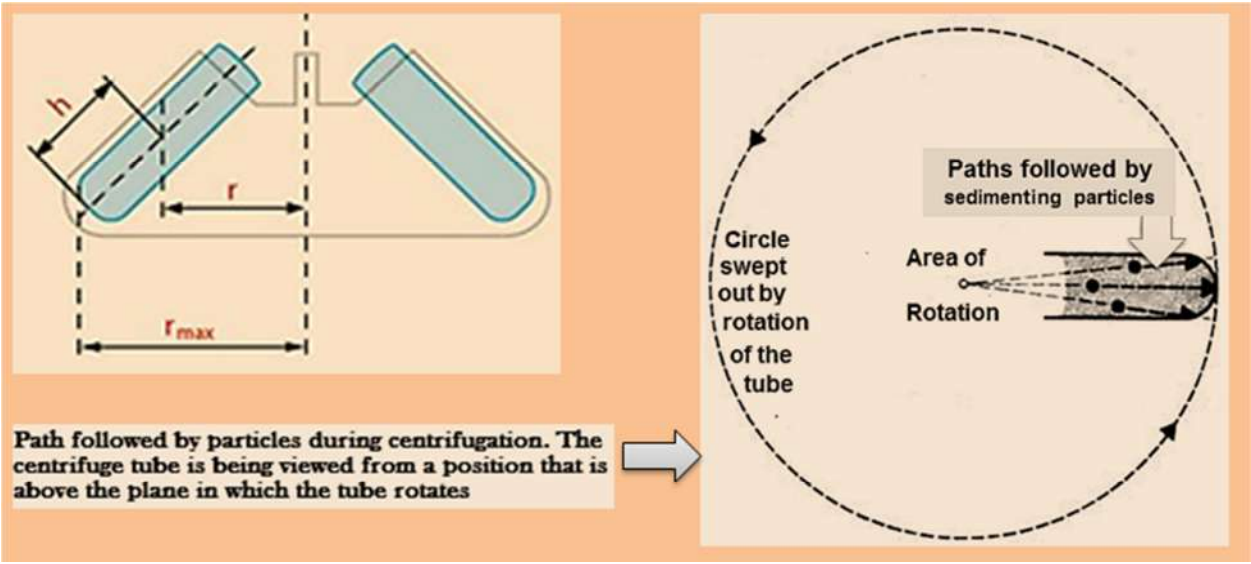
- **Rate-Zonal density gradient centrifugation:** It is also known as band or gradient centrifugation which relies on the concept of sedimentation coefficient (i.e. movement of sediment through the liquid medium). It is generally utilized in the purification of protein. In this method, a density gradient is created in a test tube with sucrose and high density at the bottom. The sample of protein is placed on the top of the gradient and then rotor is spun. With centrifugation, faster sedimenting particles in sample move ahead of slower ones i.e. sample separated as zones in the gradient. The protein sediments are collected by creating a hole at the bottom of the tube according to their sedimentation coefficient.
- **Ultrafiltration separation:** It is a pressure-driven separation process separation process that is governed by a screening principle dependent on particle size. The ultrafiltration membrane has a pore size between 1nm and 100nm, thus allowing retention of compounds with a molecular weight of 300 to 500,000 Dalton.
- **Phase separation:** It is the creation of two distinct phases during separation from a single homogeneous mixture. The most common type of phase separation is between two immiscible liquids such as oil and water.
- **Pelleting:** Pelleting and/or differential pelleting is a method of separation in which particles are concentrated as a pellet at the bottom and separated from the remaining fluid in the centrifuge tube known as supernatant due to centrifugal force. It is most common

type of separation of cells from tissue or cell debris. In this method of centrifugation, tissue such as the liver is homogenized at 32°C in a sucrose solution that contains buffer, and then homogenate is placed in a centrifuge and spun at constant centrifugal force at a constant temperature.



The centrifugation is largely depending on the gravitational force that is acting upon the molecules in the centrifuge tube. Since, the gravitational force is accelerated by the rotation of arms holding centrifuge tube, the accelerated gravitational field is known as **centrifugal force**. The centrifugal force varies depending upon the radius of the rotor or length of the arms holding centrifuge tube and is readily calculated as **relative centrifugal force (rcf)**, which is actual representation of force that acts upon the particles.

Therefore, protocols for the centrifugation typically specify the relative centrifugal force (rcf) and the degree of acceleration in multiples of g (g-force), as the working with the revolutions per minute (rpm) is improper and imprecise for the separation to occur. Because, two rotors with different diameters running at the same rotational speed (rpm) may result in different accelerations (rcf). Therefore, the distinction between rpm and rcf is important. The acceleration is typically given in gravity (× g or multiples of × g where g is the standard acceleration value due to gravity at the Earth's surface i.e. 9.81 m/s^2). The calculation of rcf is carried out by following formula;



$$rcf = r_{max} \times \frac{(2 \times \pi \times n)^2}{g}$$

Where, ***'r'*** is the maximum rotational radius, ***'n'*** is the rotating speed, measured in revolutions per minute, and ***'g'*** is the earth's gravitational acceleration.

When defining the rotational speed in revolutions per minute (rpm) and the rotational radius is given in centimeters (cm), the above formula becomes;

$$rcf = \frac{4 \times \pi^2 \times r \times n^2}{g}$$

$$= \frac{39.478 \times r \times n^2}{9.80\ m/s^2}$$

$$= (4.028\ s^2/m) \times r \times n^2$$

$$= (0.00001119\ min^2/cm) \times r \times n^2$$

and finally,

$$rcf = (1.119 \times 10^{-5} \times r_{cm} \times n^2{}_{rpm}$$

where ***'r_{cm}'*** is the rotational radius measured in centimeters (cm), and ***'n_{rpm}'*** is the rotating speed measured in revolutions per minute (rpm).

Types of Centrifuge

On the basis of speed of rotors and requirements, there are mainly three types of centrifuge, namely low speed centrifuge, high speed centrifuge, and ultracentrifuge.

Low speed centrifuge

For routine work applying sedimentation of heavy particles, most laboratories use a standard low-speed centrifuge, which has a maximum speed of 4000 – 5000rpm. These instruments usually operate at room temperatures with no means of temperature control. This type of centrifuge has two types of rotors; fixed angle rotor and swinging bucket rotor. It is used for sedimentation of red blood cells until the particles are tightly packed into a pellet and supernatant is separated by decantation.

High speed centrifuge

This type of centrifuge has high speed of rotors, and is used in more sophisticated biochemical applications. High speed centrifuge has temperature control mechanism in the rotor chamber because most of the biochemical has optimal thermal requirement. It has a maximum speed of 15,000 – 20,000rpm. Generally, it has three types of rotors; fixed angles, swinging bucket, and vertical rotors. In this centrifuge, speed and temperature that are required for sensitive biological samples can be controlled.

Ultracentrifuge

It is the most sophisticated instrument which has a maximum speed of 65,000 rpm (100,000's × g). Since, intense heat is generated due to its ultra-high speed, the spinning or rotor chambers must be refrigerated and kept at a high vacuum. It is used for both preparative and analytical work.

However, according to their applicability, there are so many categorization of centrifuge, such a micro centrifuge, small bench top centrifuge, general purpose centrifuge, and large capacity centrifuge.

Types of rotors

Rotors of centrifuge fall into three categories; swinging-bucket rotors, fixed angle rotors, and vertical rotors. Each category is designed to address three key factors which are type of centrifugation (differential, rate-zonal, or isopycnic), speed, and volume range. Of these categories, fixed-angle and swinging-bucket rotors are the most common styles for bench top, low-speed, and high-speed floor-model centrifuge applications. Vertical rotors are used primarily in ultracentrifugation. *The size of the rotor is inversely proportional to its maximum speed capability i.e. the larger the rotor, the lower the maximum speed.*

Swinging bucket rotors: These types of rotors are ideal for separating large-volume samples (up to 12 liter) at low speeds. It consists of three parts, which are as follows;

- *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,
- *Trunnion pins* are used to hold the buckets in place.

Additional accessories can be added as needed to tailor the rotor for a specific application or sample format. For example, large-volume rotors frequently offer a wide variety of adapters (plastic inserts) that can be placed into the buckets to hold the desired tube size. Certain buckets offer sealing lids, which provide biocontainment for potentially hazardous samples.

This type of centrifuge allows the tubes placed in the cups of the rotor to assume a horizontal plane when the rotor is in motion and a vertical position when it is at rest. During centrifugation particles travel uniformly and constantly along the tube while the tube is at right angle to the shaft of centrifuge; thus the sediment is distributed uniformly against the bottom of the tube and remains there when rotor stops, with liquid above it. This liquid can be decanted off and both liquid and sediment can be separated for analysis. The spinning rotor offers considerable resistance to rotation and generates heat due to air friction.

Fixed angle rotors: This type of rotors is most commonly used rotors or ubiquitous rotors used in centrifugation, mostly used in pelleting application either to collect pellet or to discard the excess debris. It has centrifuge tube cavity which have volume ranging from 0.2ml to 1lit, with speeds ranging from single digits to 1,000,000 × g. Its requirement is determined by two factors, which are;

- Desired g-force
- Desired volume

Here tubes are held in a fixed position at angles from 250 to 400 to the vertical axis of rotation. Upon centrifugation particles are driven outward horizontally but strike the side of the tube so that the sediment packs against the side and bottom of the tube with the surface of sediment parallel to the shaft of the centrifuge. As rotor slows down or stops, gravity causes the sediment to slide down the tube, usually a poorly packed pellet is formed.

An important specification when selecting a fixed-angle rotor is the K factor, which indicates the pelleting efficiency of the rotor at top speed, taking into account the **maximum and minimum radius (pathlength) of the rotor cavity**. *A low K factor indicates a higher pelleting efficiency*; therefore, the K factor can be a useful metric for comparing the speed at which particles will pellet across a range of rotors.

Swing-bucket rotor

Fixed-angle rotor

Vertical tube rotor

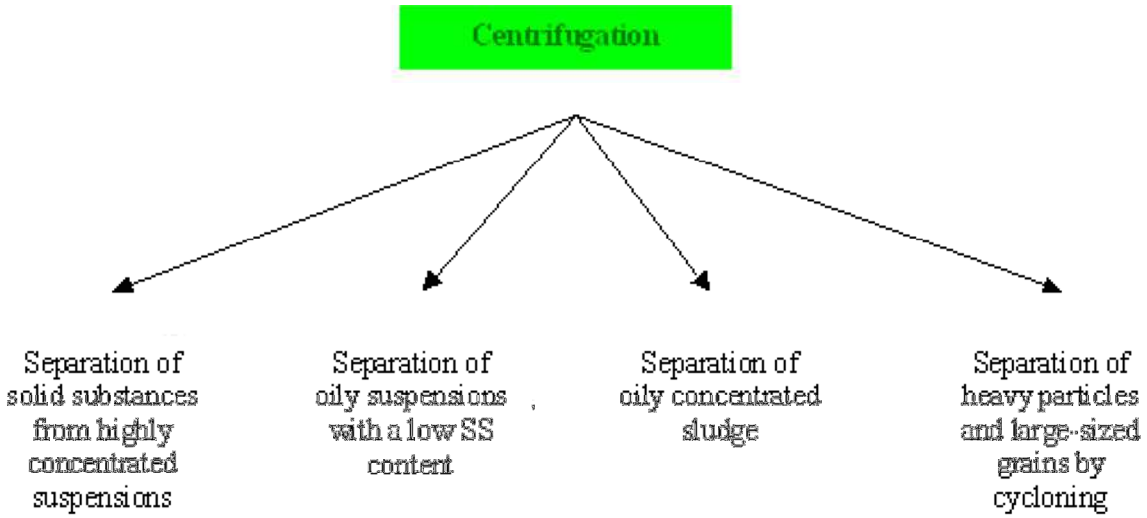
Vertical tube rotors: These are considered as zero angle, fixed angle rotors in which the tubes are aligned vertically in the body of the rotors at all times. It is mostly commonly used during ultracentrifugation for isopycnic separations, especially for the banding of DNA in cesium chloride. This type of rotors have very low K factors (typically in the range of 5 – 25), indicating that the particle must only travel a short distance to pellet or to form a band. Therefore, run time in this type of rotors is greatly minimized. Once it is determined that a vertical rotor is appropriate for the end-user application, **volume** and **speed** become the **deciding factors** for which rotor to use.

Applications of centrifugation

It has wide variety of application in laboratory investigation to industrial practices. Some common applications of centrifugation are given below;

- *Production of bulk drugs:* It has greater role in the bulk drug industry. Whenever a crystalline material is to be separated from suspension, it is done by centrifugation e.g. aspirin is separated from its mother liquor.
- *Production of biological products:* It is used in the separation of blood cells, purification of insulin by selectively precipitating other fraction of proteins, and separation of most of the proteinaceous drugs and macromolecules.

- *Biopharmaceutical analysis of drugs*: Drugs present in the blood, tissue fluids and urine are normally present in the form of colloidal dispersion. Centrifugation is used for separating the drugs which is essential for the evaluation of pharmacokinetic parameters and bioequivalence studies.



- *To separate tow miscible substances*.
- *To analyze the hydrodynamic properties of macromolecules*.
- *Fractionation of subcellular organelles (including membranes/membrane fractions) as well as membrane vesicles.*
- *Separating chalk powder from water.*
- *Removing fat from milk to produce skimmed milk.*
- *Separating particles from an air-flow using cyclonic separation.*
- *Clarification and stabilization of wine.*
- *Separation of urine components and blood components in forensic and research laboratories.*
- *Separation of proteins using purification techniques such as salting out, e.g. ammonium sulfate preceipitation.*

Calculation of relative centrifugal force (rcf)

What is the maximum relative centrifugal force applied when red blood cells are sedimented at 1000 rpm in a rotor of maximum sample radius equal to 10 cm?

$$rcf = (1.119 \times 10^{-5} \times 10 \times 1000^2{}_{rpm}$$

$$= (1.119 \times 10^{-5} \times 10 \times 10^6$$

$$= (1.119 \times 10 \times 10$$

$$= (111.9 \times g$$

For a centrifuge, if rotating speed of rotor A **&** B **is 14, 000 rpm but have different radius; 5.98 cm for A and 9.50 cm for B and then what are the** rcf **for** rotor A **and** B**?**

Therefore, from the equation,

$rcf(A) = (1.119 \times 10^{-5} \times 5.98 \times 14,000^2{}_{rpm}$ & $rcf(B) = (1.119 \times 10^{-5} \times 9.50 \times 14,000^2{}_{rpm}$

$rcf(A) = (1.119 \times 10^{-5} \times 5.98 \times 196 \times 10^6{}_{rpm}$ $rcf(A) = (1.119 \times 10^{-5} \times 9.50 \times 196 \times 10^6{}_{rpm}$

$rcf(A) = (1.119 \times 5.98 \times 1960)$ $rcf(A) = (1.119 \times 9.50 \times 1960)$

$rcf(A) = 13104 \times g$ $rcf(A) = 20817 \times g$

Reference

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