Classroom

In this section of *Resonance*, we invite readers to pose questions likely to be raised in a classroom situation. We may suggest strategies for dealing with them, or invite responses, or both. "Classroom" is equally a forum for raising broader issues and sharing personal experiences and viewpoints on matters related to teaching and learning science.

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Loading Effects on Resolution in Thin Layer Chromatography and Paper Chromatography

Chromatography is one of the separation methods based on differential migration of components of a mixture. This is usually included in the biology curriculum at the university level. The technique of chromatography was introduced as early as 1906 by a Russian scientist Michael Tswett. In general, if two solvent phases are in contact with one another and if a solute is added, it will distribute itself between the two phases. The ratio of the concentrations of the solute in the two phases is called partition coefficient. Among the two phases one can be static and the other mobile. The sample mixture, introduced into the mobile phase undergoes repeated interactions (partitions) between the stationary and mobile phase while being carried through the system by the mobile phase. Different components of the sample mixture interact with the two phases differentially on the basis of small differences in their physicochemical properties. Since these different rates of interactions govern the migration of sample components through the system, each one of the components migrate at a different rate. The compound that interacts more with the mobile phase and least with the stationary phase migrates fast. The components showing least interactions

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with the mobile phase while interacting strongly with the stationary phase migrates slowly (retarded).

The distribution of the solute between the stationary and the mobile phases is a result of the balance of forces between the solute molecules and the molecules of each phase. Partition coefficient, therefore, reflects the relative attraction or repulsion that the molecules of the two phases show for the solute molecules and for themselves. These attractive or repulsive interactions are accompanied by a release or intake of energy. Partition chromatography is usually carried out in paper or thin layer (TLC). It may also be carried out in a column.

In paper chromatography the stationary phase is supported by cellulose fibers of the paper sheet. In thin layer chromatography, the stationary phase is coated onto a glass or plastic surface. The paper commonly used consists of highly purified cellulose. Cellulose, a homopolysaccharide of glucose, contains several thousand anhydro-glucose units linked through oxygen atoms. Cellulose fibers in the paper hold moisture tightly through formation of hydrogen bonds.

Cellulose takes a negative charge in the presence of water. Therefore paper exhibits weak ion exchange and adsorptive properties. The mobile phase is generally a solvent poorly miscible with water but saturated with water before use. Thin layer chromatography (TLC) is more or less similar to paper chromatography. In TLC a thin layer of a finely divided substance such as cellulose powder is deposited on to a flat glass plate. The sample to be separated is spotted at one end. The plate is dipped into the solvent in glass jar and the development carried out by the ascending technique. After the development, the layer can be dried and the components detected by various methods available.

Legume seeds contain oligosaccharides, majority of which belong to the raffinose family sugars such as raffinose, stachyose, verbascose and ajugose. These oligosaccharides contain one, two, three and four galactose units respectively joined to sucrose by α(1-6) linkages (*Figure 1*).

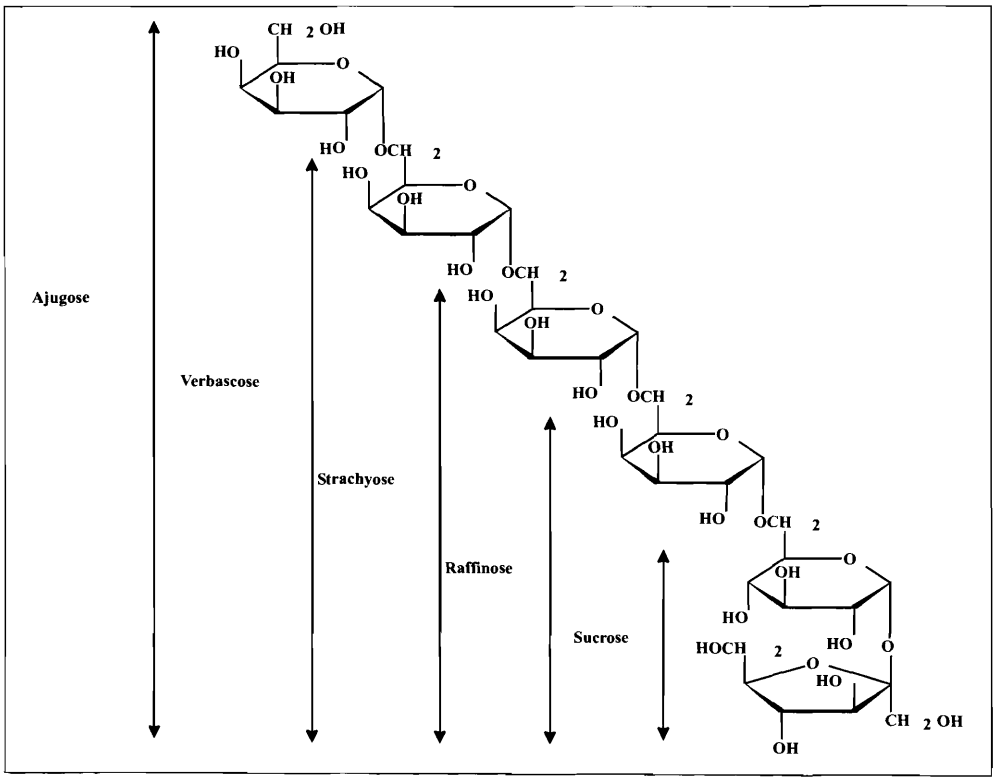

Figure 1. Structural relationship of the raffinose family sugars.

In this article, we present a simple method to separate oligosaccharides of black gram and study the effect of increasing loading concentration. It is simple, inexpensive and less time consuming of all TLC and paper chromatography techniques. This experiment can be performed in any laboratory with minimum facilities.

Extraction of Oligosaccharides from Blackgram

The black gram seeds were milled to flour and the fraction, which passes through a 400 mm sieve, was used for the present study. 5 g of flour was extracted with 50 ml of 70% (v/v) aqueous ethanol and kept on an orbital shaker at 130 rpm for 13 hours. The contents of the flask were filtered through Whatman No.1 filter paper and the residue was further washed with 25 ml of 70% ethanol. The filtrate was evaporated in a rotary vacuum evaporator at 45 °C. The concentrated sugar syrup was dissolved in 5 ml of distilled water.

Preparation of TLC plate

The TLC plate was prepared by suspending 30 g of cellulose-G powder in 45 ml of distilled water; the slurry was poured on to the glass plate to 0.2 mm thickness. The plates were then air-dried.

Paper Chromatography

Whatman No.1 filter paper (14×20 cm) was selected for the experiment.

Loading the Sample

Residue dissolved in 5 ml distilled water was spotted on to TLC plate and paper with different concentrations. 5, 10, 15, 20 and 25 μl sample was spotted with a 100ml glass micropipette with their standard oligosaccharides and dried using a hair drier.

Solvent System

The TLC plate was developed with the solvent propanol: ethyl acetate: water (6:1:3) ratio and air dried. Paper was developed with n-butanol: ethyl acetate: acetic acid: water (40:30:25:40) ratio.

Spraying Reagent

The TLC plate and paper was sprayed with the spraying reagent (1% α-napthol in 95% ethanol containing 10% orthophosphoric acid) and the plate was air dried and kept in an oven at 100 °C for 10-15 min.

Determination of R_f values

R_f values of different oligosaccharides were determined by measuring the movement of solvent and movement of solute molecules with a ruler. Movement of solute divided by movement of solvent gives the R_f value.

Discussion

When 5ml sample was spotted, some of the developed spots were

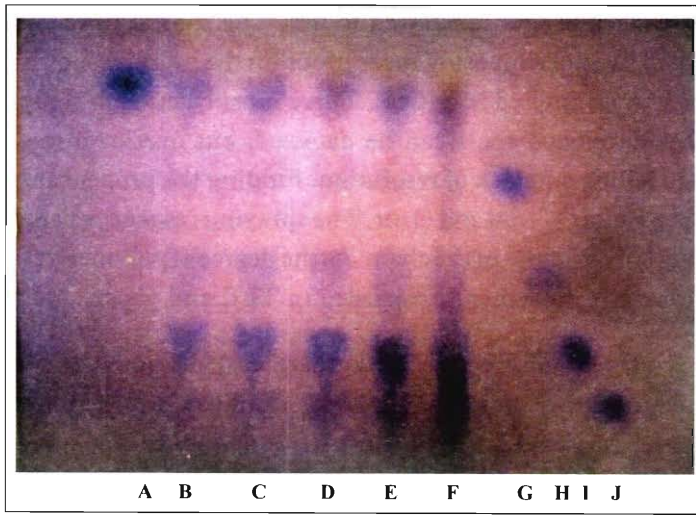

Figure 2. Thin layer chromatographic pattern of separation of oligosaccharides from black gram. A. Sucrose (50 µg); B. Black gram extract (5 µl); C. Black gram extract (10 µl); D. Black gram extract (15 µl); E. Black gram extract (20 µl); F. Black gram extract (25 µl); G. Raffinose (50 µg); H. Stachyose (50 µg); I. Verbascose (50 µg); J. Ajugose (50 µg)

faintly visible, whereas 10µl and 15µl sample loading resulted in clear spot development. As it is evident from the figure (*Figure* 2). 20ml and 25µl loading resulted in tailing or overlapping of the spots, whereas in the case of paper chromatography 5µl sample loading shows clears separation of spots, as the concentration increases the overlapping appears (*Figure* 3). Therefore optimization of concentration of loading is crucial in TLC and paper chromatography.

Usually a very small drop of sample solution is spotted on to the TLC and paper chromatography with a disposable microcapillary

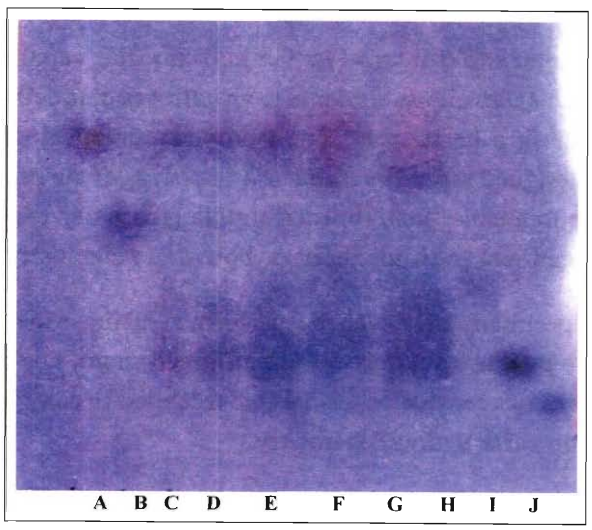

Figure 3. Paper chromatographic pattern of separation of oligosaccharides from black gram. A. Sucrose (50 µg); B. Raffinose (50 µg); C. Black gram extract (5µl); D. Black gram extract (10 µl); E. Black gram extract (15 µl); F. Black gram extract (20 µl); G. Black gram extract (25 µl); H. Stachyose (50 µg); I. Verbascose (50 µg); J. Ajugose (50 µg).

Suggested Reading

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[3] A Upadhyay, K Upadhyay, N Nath, *Biophysical Chemistry Principles and Techniques*, Himalaya Publishing House, pp.344, 2003.

pipette and the spot is allowed to dry. The spotting process is repeated by superimposing more drops on the original spot. The exact sample applied is critical. There must be enough samples so that developed spots can be detected, but overloading will lead to tailing and lack of resolution. Finding the proper sample size is a matter of trial and error. The spraying reagent α-napthol detects only fructose in the chain. As the degree of polymerization increases the movement is retarded in TLC and paper.

The R_f values of different oligosaccharides determined by paper chromatography were sucrose 0.36, raffinose 0.25, stachyose 0.17, verbascose 0.12, ajugose 0.06. R_f values by TLC for sucrose, raffinose, stachyose, verbascose and ajugose were 0.72, 0.57, 0.42, 0.30 and 0.18 respectively.

We have used this method to identify and separate oligosaccharides from different legumes. The method discussed here is a simple one. Even though many other forms of chromatography like gel permeation chromatography, gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC) work by the same principle it cannot be demonstrated vividly because, the separated components cannot be distinguished by simple procedures. Moreover all these methods need expensive equipment, chemicals and maintenance.

The simple and illustrative method for the separation of oligosaccharides allows students to visualize and understand the technique in a better way. The laboratory practical described here is designed for undergraduate and postgraduate life science students to enable them to learn simple laboratory techniques, encourage them to think about carbohydrate chemistry.

This experiment may also be performed using other locally available legumes such as red gram, green gram, cowpea, etc. This easy, inexpensive experiment is intended to complement a lecture on carbohydrate chemistry.