

3.2. DETERMINATION OF THE DENSITY OF SOLIDS

Table 3.2.2.1. Possible substances for use as gradient-column components

| Hydrophobic components | | | Hydrophilic components | |
|-----------------------------|--|-----------|------------------------------|---|
| Liquid | Approximate density at 298 K (g ml ⁻¹) | α | Solute | Approximate maximum density of concentrated aqueous solution at 298 K (g ml ⁻¹) |
| Isooctane (2-methylheptane) | 0.69 | -0.8 | Sodium chloride | 1.20 |
| Kerosene | 0.79 | -0.5 -0.8 | Potassium chloride | 1.40 |
| <i>m</i> -Xylene | 0.86 | -0.85 | Potassium iodide | 1.63 |
| Chlorobenzene | 1.10 | -1.1 | Iron(III) sulfate | 1.80 |
| Bromobenzene | 1.49 | -1.3 | Zinc bromide | 2.00 |
| Carbon tetrachloride | 1.60 | -1.9 | Zinc iodide | 2.39 |
| Methyl iodide | 2.28 | -2.7 | Thallium(I) formate | 3.5 |
| Bromoform | 2.89 | -0.9 | Thallium(I) formate-malonate | 4.3 |
| <i>s</i> -Tetrabromoethane | 2.96 | -2.2 | | |
| Methylene iodide | 3.32 | -2.6 | Ficoll* (60% w/w in water) | 1.25 |

The density at temperature T K can be computed by substituting the values of the density at 298 K and α in the formula $d_T = d_{298} + 10^{-3}\alpha(T - 298)$. *Trade name for a synthetic high-molecular-weight polysaccharide derivative.

inclusions still remain, or if the sample is truly a mixture, a stable distribution of material will be observed. The density of the material of interest can then usually be obtained by measurement of the appropriate layer, generally the most dense, without further treatment of the sample. This is the only technique by which the homogeneity of the sample can be tested simply. All other methods provide an average density value. A satisfactory technique for removing crystalline powders from the gradient column has not been devised. If a precision of

± 0.002 g ml⁻¹ is adequate, it is simplest to prepare a new wide-range column for each determination in a 10 ml test tube.

Detailed specifications for the preparation of large density-gradient columns are contained in the records of the British Standards Institution (1964). In the experience of the author, for ordinary laboratory use, the procedures described are unnecessarily complicated as is the large scale of the system. The large columns are not suitable for centrifuging and the settling times tend to be many hours. However, if extreme sensitivity (*i.e.* use of a shallow gradient) is required, the large column may be useful, as it was in the original studies of Linderstrom-Lang (Linderstrom-Lang, 1937; Linderstrom-Lang & Lanz, 1938).

In the specific application of this technique to protein crystals, where a gradient of organic liquids is used, it is necessary to have available crystals sufficiently large that they can individually be quickly wiped free of adhering mother liquor with dampened filter paper before insertion. The uncertainty of successful cleaning combined with rapid evaporation of liquid from the pores within the crystal always affect the estimated accuracy of the measurement. An important improvement in the technique has been made by Westbrook (1976, 1985) through the use of concentrated aqueous solutions of the water-soluble polymer Ficoll. This very high molecular weight polysaccharide can be dissolved in water to concentrations of at least 60% by weight. The solutions are very viscous but do provide satisfactory water-based gradient columns. The polymer is both too large to enter the solvent-filled pores of the protein crystals and too high in molecular weight to develop a significant osmotic pressure. An aqueous suspension of crystals can be added directly to the column. This procedure has been adapted for measurements of protein-crystal density under hydrostatic pressures from 1 to 2000 atm (1 atm \equiv 101 325 Pa) (Kundrot & Richards, 1988). The general principle of using high-polymer-based gradients can presumably be extended to other porous materials.

3.2.2.1.2. Suitable substances for columns

Some representative liquids are listed in Table 3.2.2.1; all are readily available. For further information, see Meyrowitz, Cuttitta & Hickling (1959), and for very heavy liquids Sullivan

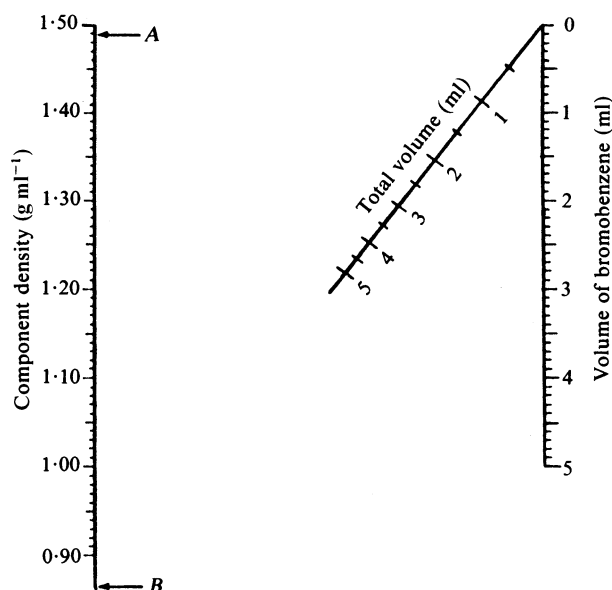


Fig. 3.2.2.1. Nomogram for the preparation of bromobenzene-xylene gradient column components at room temperature. From the desired component density and total volume, the required amount of bromobenzene is read from the chart, the volume difference being made up with xylene. To adapt this chart to any other pair of liquids, it is only necessary to change the component density scale. A uniform scale is drawn up such that the density of the heavy liquid lies at the point *A* while that of the light liquid is at *B*. The volume scales may be multiplied by any constant factor in order to change their range.

3. PREPARATION AND EXAMINATION OF SPECIMENS

(1927). Standardized solutions or mixtures from one list in Table 3.2.2.1 may be used as calibrating drops in gradients made from those of the other.

For rapid preparation of mixtures from stock solutions of the basic compounds, a nomogram is very useful, such as is given in Fig. 3.2.2.1 for the system bromobenzene-xylene at room temperature. In the construction of the nomogram, it has been assumed that the volumes of the liquids are additive. In general, this assumption is not valid, but it is a sufficiently good approximation for the purpose.

3.2.2.1.3. *Sensitivity*

The range of density covered by a column, and thus the accuracy of the determination, is controlled by the liquids or liquid mixtures chosen for the top and bottom components. A precision of about $\pm 0.002 \text{ g ml}^{-1}$ can easily be obtained without any special precautions. If narrow-range columns are carefully protected from temperature changes and vibration, the accuracy of the measurement may be increased 10- to 100-fold.

3.2.2.2. *Flotation method*

Although historically used much earlier, this technique is essentially an approximation to the gradient-tube method. The specimen is immersed in a liquid, and a denser or less dense liquid miscible with the first is added until the sample neither rises nor sinks in the solution (Wulff & Heigl, 1931). The density of the immersion medium is then determined immediately by standard techniques such as pycnometry, by the Westphal balance, or by refractive index (Midgley, 1951). The method is reported as capable of a probable accuracy as great as 0.02%.

The compounds listed in Table 3.2.2.1 are also useful in this method. With slurries or with specimens smaller than 1 mm^3 , a centrifuge must be used to achieve a reasonable rate of settling. As little as 0.05 mg of material has been used with good results (Bernal & Crowfoot, 1934). A modification of this method has been described in which the density of the immersion medium is varied by altering the temperature (Reilly & Rae, 1954; Wunderlich, 1957).

3.2.2.3. *Pycnometry*

This is one of the most demanding of the available techniques. A previously calibrated pycnometer containing the sample is weighed. A liquid of known density is then introduced, air bubbles are removed by reducing the pressure, and the filled bottle is reweighed. The volume of the sample and its mass may thus be determined. With care, a probable accuracy of 0.02% may be achieved (Johnston & Adams, 1912). Contrary to many published statements, the accuracy of this technique is not dependent to any significant extent on the use of immersion media of high density.

Liquids with low surface tension will facilitate the removal of air bubbles. In some cases, it is advantageous to fill the bottle with the mother liquor from which the crystal grew. Powders or many small crystals may be used as well as large single specimens. There is no restriction on the density of the materials for which this technique is suitable.

A micropycnometer for use with samples of total volume as small as 0.01 ml has been described (Syromyatnikov, 1935). An accuracy of better than 1% has been achieved with this instrument.

3.2.2.4. *Method of Archimedes*

The specimen is weighed in air and again in a liquid of accurately known density. From the apparent loss of weight the volume is computed, and thence the density (Reilly & Rae, 1954). The technique requires little special equipment and is capable of great accuracy when used with large, well formed crystals. The accuracy is maximized by using immersion liquids of density as close to that of the crystals as possible. For precise work, correction must be made for the interfacial tension between the supporting wire and the upper surface of the suspending medium.

A torsion microbalance has been adapted to the determination of crystals as small as 25 mg (Berman, 1939). A probable accuracy of better than 1% may be achieved with this micro-method.

A densitometer based on Archimedes principle with control of the composition of the gas phase and a wide temperature range has been described by Graubner (1986). The method is not suitable for finely divided materials.

3.2.2.5. *Immersion microbalance*

Some crystals, such as those of globular proteins grown from alcohol-water mixtures, rapidly change their composition, and thus their density, when removed from the mother liquor in which they were grown. The density may then be computed from the weight of the crystal immersed in its mother liquor, the density of the latter, and the volume of the crystal (Low & Richards, 1952*b*, 1954; Richards, 1954).

A horizontal quartz fibre, free at one end, is mounted in a glass case that can be filled with liquid. After calibration, the deflection of the fibre gives the weight of an immersed crystal suspended on the free end. The volume is computed from the crystal dimensions as determined from two photomicrographs of the immersed crystal taken at right angles to each other. The density of the mother liquor is measured by one of the standard techniques for liquids.

The method is suitable for single, well formed crystals having a volume of about 0.1 mm^3 or greater. The accuracy is related inversely to the difference in density between the crystal and its mother liquor.

3.2.2.6. *Volumenometry*

This is the only technique not requiring immersion of the sample in a liquid medium. The technique is therefore used in instances where the specimen would be attacked by the customary immersion media, or where one wishes to work over a temperature range where liquid media would be inappropriate.

The gas-pressure change caused by altering the volume of a calibrated vessel by a given amount is determined when the vessel is empty, and again after the weighed specimen has been introduced (Reilly & Rae, 1954).

Any gas inert to the crystal may be used. Powders and crystal fragments may be employed. A probable accuracy as great as 0.1% may be attained. Samples with an aggregate volume as low as 0.01 ml have been measured with a probable accuracy of 1% (Hauptmann & Schulze, 1934).

3.2.2.7. *Other procedures*

A novel procedure that may be useful in special circumstances is based on measuring the frequency of a vibrating string of the material in question. If the length of the string is fixed and the transverse deformation is small, the various harmonic frequen-