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Countercurrent Distribution

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IN COUNTERCURRENT distribution a mixture of substances is fractionated by repeated partition or distribution between two immiscible liquid phases. The distributions are performed in a discontinuous and quantitative manner so that the process is adaptable to mathematical analyses.

Apparatus for countercurrent distribution has evolved from the small metal instruments described by Craig in 1944 (1) to completely automatic glass equipment with many tubes. With such equipment separations are made that would be practically impossible if the distributions were performed individually in separatory funnels.

The subject of countercurrent distribution has been reviewed by Craig (2), Weisiger (3), and Hecker (4). Its applications to lipids have been described by Dutton (5, 6).

Modern instruments consist of a train of glass cells connected in series in such a way that they are suitable for automatic operation (7). The operation of the cells is illustrated in Fig. 1 (7). Each cell contains lower phase solvent to bring the interface to *a* in position C. The amount of upper layer may be varied to suit the partition coefficients of the solutes being resolved. Equilibration of the phases is accomplished by rocking between positions A and B. The phases separate in position B. On tilting to C the upper layer decants through *c* to *d*. On tilting to A the contents of *d* flow through *e* to the adjoining cell.

An instrument with 220 such tubes and with a robot for automatic operation has been described by Craig (7). The 200-tube automatic apparatus used at this Laboratory is shown in Fig. 2.

Craig (1) has also described the operation of countercurrent distribution instruments using fundamental, single withdrawal, and recycle procedures. In

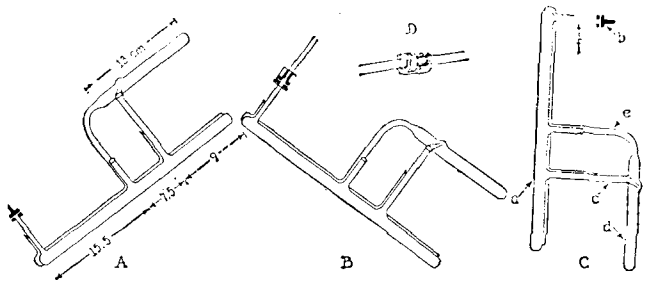

FIG. 1. Operation of countercurrent distribution cell.
Courtesy of Analytical Chemistry.

the fundamental operation procedure the sample is placed in the first tube (numbered tube 0) and rocked as described above to bring it into equilibrium with the upper and lower solvent phases. The upper phase is then transferred to the next tube (tube 1) containing fresh lower phase. Fresh upper phase is added to tube 0, and the process is repeated until the original upper phase reaches the last tube of the instrument. The upper and lower phases are then removed together from each tube, and the amounts of solute are measured, usually by evaporation of solvent and weighing of residue.

The distribution of a compound among the tubes may be represented by a binominal expansion. If the partition coefficient, the concentration in the upper phase divided by the concentration in the lower phase, is represented by K, then the fraction of the compound in any tube, r, after n transfers is

$$T_{n,r} = \frac{n!}{r!(n-r)!}\left(\frac{1}{K+1}\right)^n K^r \qquad (1)$$

The calculation of partition coefficients and of theoretical distribution curves has been described by Williamson and Craig (8).

For large numbers of transfers, equations based upon the approach of a binominal distribution to the normal curve of error may be used. Craig (9) gives the equation

$$y = \frac{1}{\sqrt{2\pi nRK/(RK+1)^2}}\, e^{\frac{-x^2}{2nRK/(RK+1)^2}} \qquad (2)$$

where x = number of tubes from the tube with maximum weight

y = amount of substance in tube x
n = number of transfers
R = volume of upper phase/volume of lower phase
K = partition coefficient

The position of the maximum, N, or the partition coefficient, K, may be calculated from the formula

$$N = n\left(\frac{RK}{RK+1}\right) \qquad (3)$$

where N = number of the tube with maximum weight—the point where x = 0.

Single withdrawal operation is carried out in the same way as fundamental operation until the first upper phase reaches the last tube of the instrument. The distribution is then continued allowing the upper phase from the last tube to pass from the instrument and collecting it in tubes in a fraction collector. The amount of material in individual collector tubes is measured and plotted against the transfer number at which it left the last tube of the apparatus.

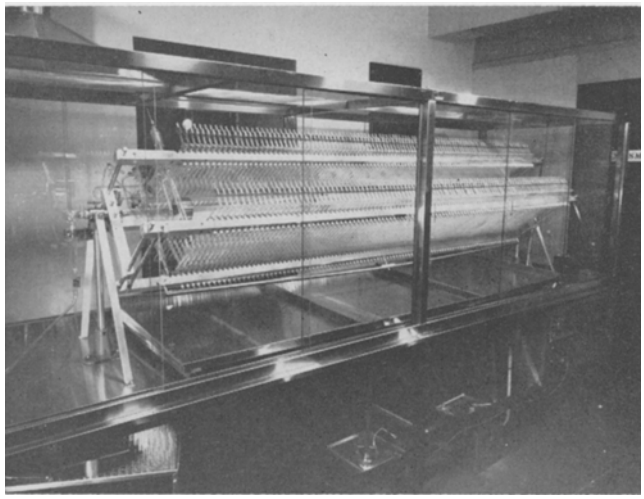

FIG. 2. Automatic 200-tube countercurrent distribution apparatus.

For single withdrawal operation

$$y = \frac{1}{\sqrt{2\pi n/RK}}\, e^{\frac{-x^2}{2n\,RK}} \qquad (4)$$

In this equation n is the transfer number for the fraction with maximum weight and x is the number of transfers from this fraction. The partition coefficient can be calculated from the formula

$$RK = u/n\text{-}u$$

where u is the number of tubes in the train.

If a mixture consists of components which pass through the instrument as one band with only partial separation, better fractionation is obtained by recycling. In this process each upper phase from the last tube is reintroduced into tube 0. Recycling may be continued until the band spreads so that it occupies the whole instrument. Further recycling would cause the leading and trailing edges of the band to mix. The distribution may either be stopped at this point or the upper layers collected in a fraction collector in a single withdrawal operation.

For the performance of a successful countercurrent distribution the choice of a suitable solvent system is essential. The materials to be separated must be soluble in both phases and have partition coefficients in a useful range. The phases should separate rapidly, and the interface should not change greatly with temperature changes. The solvent system should be selective; that is, the partition coefficients for the substances to be separated should differ as much as possible. Several lists of solvent systems used for different compounds have been published (4,10-12). Specific solvent systems used for various lipids will be discussed under applications.

The equations presented depend upon the assumption of a constant value for K. This assumption is usually valid for the dilute solutions used in countercurrent distribution. With higher concentrations or with compounds where the association of the solute

molecules varies, changes in K will occur. In practice this variation places a limit upon the sample size that can be used. In preparative runs with large samples it is common practice to put the sample in several beginning tubes instead of only in tube 0 in order to lower the concentration (2).

At the Northern Laboratory use was recently begun of a recording differential refractometer to locate bands as they emerge form the instrument in a single withdrawal operation. After passing through the refractometer, the upper layers either go to a fraction collector or are pumped back into the instrument for recycling. The use of this instrument will be described in a later publication.

Applications of Countercurrent Distribution to Lipid Analysis

The following section describes some applications of countercurrent distribution to the fractionation and analysis of lipids. It illustrates the problems for which countercurrent distribution is useful, but it is not intended to be complete.

Fatty Acids. Ahrens and Craig (13) proposed solvent systems containing acetic acid to prevent skewed distribution curves caused by association of fatty acid molecules. Such systems have been used extensively for the isolation of highly unsaturated acids. Recently, Will (14) proposed petroleum ether and dimethyl sulfoxide-1-octanol (9:1) for fatty acid separations.

Methyl Esters. The distribution of methyl esters of fatty acids between petroleum ether and 80% ethanol (15) and between petroleum ether and nitroethane-nitromethane (4:1) (16) has been studied by Cannon, Zilch, and Dutton. More recently, petroleum ether and acetronitrile have proved quite selective for esters of different unsaturation (17). At this Laboratory this system is preferred for such separations.

Nichols (18) showed that it should be possible to separate methyl oleate and methyl elaidate by distribution between methanolic silver nitrate and isooctane. This separation has been confirmed in this laboratory, and the use of such systems for separating esters containing *cis* and *trans* bonds will be reported in detail at a later time.

Triglycerides. Countercurrent distribution has been very effective for the separation of the more highly unsaturated triglycerides. The distribution of fatty acids among the triglyceride molecules has been shown to approximate a random pattern for linseed, soybean, safflower, and corn oils (19-22). Cocoa butter departs from the random pattern (23,24). However, countercurrent distribution does not provide information about the position of the acids on the triglyceride molecule. Other methods employing enzymatic hydrolysis show that there is some departure from randomness in the position of the fatty acids on the glycerol molecule (25).

Monoglycerides. Countercurrent distribution has been used in the isolation of monoglycerides from bread and lard (26) and from the intestinal tract of rats (27) and of humans (28). Perry and Brokaw (29) measured the partition coefficients of several monoglycerides between petroleum ether and 85% methanol.

Fatty Acid Oxidation Products. Oxidized methyl oleate was separated by Fugger and coworkers (30) into methyl oleate, methyl oleate hydroperoxide, and secondary oxidation products by countercurrent distribution between hexane and 80% ethanol. Similar results were obtained by Cannon *et al.* (31) with oxidized methyl linoleate. Using 400 transfers Johnston *et al.* (32) fractionated the decomposition products of methyl linoleate hydroperoxide. With oxidized methyl linolenate, monomeric hydroperoxides were not resolved in a 29-transfer distribution (33) but were separated by Frankel *et al.* using a 600-transfer distribution (34).

Phospholipids. If hexane and aqueous methanol are used, the alcohol-insoluble phospholipids from soybean, corn, and linseed oils can be separated with only a 25-tube instrument into two types of inositol containing phospholipids (35-37). Partial separation of phosphatidyl choline and phosphatidyl ethanolamine in the alcohol-soluble fractions can also be accomplished.

However, countercurrent distribution of phospholipids is hindered by slow separation of solvent phases caused by emulsion formation, and by poor separations caused by association of the phospholipids. Carter *et al.* (38) used a heptane: butanol: methanol: water system, which had less tendency to form emulsions for distribution of corn inositol lipids. This solvent system was also used by Mason and Johnston (39) in the study of wheat flour phosphatides. Cole, Lathe, and Ruthven (40) used solvent systems containing light petroleum, methanol, water, and chlorinated solvents for distribution of lipids from brain. Lovern and Olley have used hexane and 85% ethanol to fractionate haddock flesh lipids, and Olley (41) has reviewed this work as well as the application of countercurrent distribution to phospholipids.

Pigments. Lancaster *et al.* (42) showed that chlorophyll a, chlorophyll b, and carotene could be separated by distribution between hexane and 90% ethanol. Curl (43) used countercurrent distribution to separate orange juice carotenoids into six classes based upon the hydroxyl and cyclic ether groups present. The classes were then further separated by chromatography. Burnett *et al.* (44) used countercurrent distribution to study sorghum leaf and stem lipids. Five pigment bands—carotene, pheophytin, chlorophyll a, chlorophyll b, and carotenoids—were separated. In addition, fractionation among the other lipid constituents was observed.

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Paper Chromatography of Lipids: Methods, Applications, and Interpretations

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THIS REPORT describes methods for the paper chromatography of lipids and shows some of the ways the methods can be applied. The proper use of paper chromatography requires a clear understanding of the limitations of the method, and the limitations and uses are best appreciated against a background of interpretations concerning the mechanism of the chromatographic process at the molecular level. An effort is made to describe the probable basis for paper chromatographic separations in this article, and for column chromatographic methods in another report.

Paper chromatography is useful for the comparisons of compounds as an aid in identification, for the detection and tentative identification of impurities, reaction products and intermediates, and for the exploration of variables before undertaking column chromatography. The apparatus is simple and the process is not difficult. A complete and reliable characterization of a lipid by paper chromatography is not always possible. Even when compounds are compared with different types of solvent systems, one should consider the results tentative until confirmed (spectral properties, hydrolysis products, formation of derivatives). Paper chromatography has set new, high standards for the purity of lipids and allied substances, and has been used extensively in the development of new column procedures and for monitoring of column fractions.

Paper chromatography is not generally as useful as column chromatography for the isolation of pure lipids. The ease with which pure substances can be isolated in large amounts suitable for complete characterization makes column procedures more desirable. Exposure to air and autoxidation are easily avoided in column chromatography.

Attempts to use paper chromatography for quantitative determinations are generally not entirely satisfactory. Fairly precise quantitative paper chromatographic methods can be developed for authentic compounds. When the methods are applied to biological samples however a number of difficulties are encountered. A single spot on a chromatogram may represent more than one substance. Another difficulty is the selection of a sensitive, specific assay method for small amounts of material. Color reactions are difficult to control and the sensitivity may vary. When an elution method is used, impurities eluted from the paper along with the sample may influence the determinations. Paper chromatography is sometimes selected for quantitative determinations because of the mistaken impression that column chromatographic procedures can not be made to handle extremely small samples. Although the ease with which small samples may be examined by column chromatography does vary, the extremely small amounts of lipids that can be detected and determined by gas chromatography make it quite clear that column chromatography can be even more sensitive than paper chromatography, or modifications such as thin-layer chromatography.

Types of Paper Chromatography

The chromatographic process is recognized as being one of adsorption or partition. Adsorption columns have been used widely, but the process is seldom used

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