trifluoroacetylated *S*-benzyl or butyl ETU derivatives that can be analysed by GC-NPD, GC-ECD or GC-MS. In real samples EBDCs and ETU content decrease with storage time. To prevent this, the addition of cysteine hydrochloride has been recommended.

See also: **II/Chromatography: Gas:** Detectors: Selective; Detectors: Mass Spectrometry. **Extraction:** Solid-Phase Extraction; Supercritical Fluid Extraction. **III/Pesticides:** Gas Chromatography. **Herbicides:** Gas Chromatography; Solid-Phase Extraction.

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Liquid Chromatography

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Introduction

There are some groups of fungicides of wide use (benzimidazoles, ethylenebisdithiocarbamates) whose thermal instability, high polarity and low volatility make them difficult to determine by gas chromatography (GC) unless derivatization methods are employed. This usually makes the process longer and introduces new errors. These compounds are easily measured by high performance liquid chromatography (HPLC) as are many pesticides that were typically analysed by GC in the past. Integrated systems of solid-phase extraction sample cleanup and on line HPLC allows multiple options, not only by including fungicides of very different polarity in the same analysis but also by achieving very high concentration factors and, at the same time, analysing a large number of samples. The use of pre- or post-column derivatization reactions allows the analysis of compounds that are very difficult to determine or have a low sensitivity.

Given these advantages HPLC not only complements GC in fungicide residue analysis but is tending to displace it for many applications. Some considerations related to the use of HPLC are summarized below, with more attention being paid to the groups of fungicides most frequently determined by this technique.

Technique Selection

Most applications are based on the use of reversed-phase HPLC, nevertheless for some fungicides ion-pair HPLC (ethylenebisdithiocarbanates) (EBDC), micellar HPLC (Thiram) or chiral HPLC (Metalaxyl) are used. Normal phase HPLC, with amino-bonded stationary phases, is sometimes recommended, mainly for the benzimidazole group.

Chiral HPLC is very important for the determination of enantiomeric purity, mainly for large-scale synthesis. Resolution of C–chiral enantiomers seems to be easier than that of axial–chiral enantiomers (atropoisomers).

Columns

The most widely used stationary phases for fungicide residue analysis are the n-octyl and n-octadecylsilica because they allow the separation of compounds with a wide range of polarity. Some fungicides, mainly EBDCs, are easily ionized and because of this some

methods propose the use of ion-exchange phases. It may, however, be better to use C_{18} in the ion-pairing mode, adding a counter ion to the mobile phase. When it is necessary to separate enantiomers, then chiral columns are preferred although there is also the possibility of using the C_{18} with a chiral mobile phase.

Usually columns with a diameter of 4.6 mm, packed with 5 μm material are employed, but nowadays it is possible to use shorter columns or even narrow bore, microbore or packed capillary columns. These later make the coupling to an MS detector easier. In both cases the lower mobile phase flow rate provides a big reduction in reagent consumption.

Several manufacturer's offer equivalent columns. Attention must always be paid to batch-to-batch reproducibility. The use of a pre-column helps to preserve the life of the column, and, if it is possible to work at room temperature, the column will last longer than when used with higher temperatures.

Mobile Phase

The selection of the stationary phase and the mode of detection is determined by the characteristics of the analytes to be separated; both, also, control the selection of the mobile phase. As C_{18} is usually employed, the mobile phase is frequently composed of a mixture of water with an organic solvent, mainly methanol or acetonitrile. To improve the peak shape or to separate compounds with acidic or basic character the addition of acid or buffer to vary the pH can be very useful. Also the temperature at which the separation is made, must be established when looking for the best resolution. The reagents used to prepare the mobile phase must be compatible with the detection mode. It is very important when a UV detector is used, because not all commercial methanol or acetonitrile are transparent enough in the low UV region. Attention must be paid to the transmission spectrum of the solvents and to the changes in batch or manufacturer. When there is a great difference between the polarity of the (aqueous) mobile phase, and the organic solvent used to inject the sample, it is possible that the first peaks will be distorted and in this case it is better to reconstitute or dilute the sample in the mobile phase.

Detection

Most of the fungicides that are analysed by HPLC can be detected in the UV region. In multiresidue methods it is more convenient to employ a diode array detector (DAD) which allows multiple wavelengths to be employed and peak purity to be checked.

The fluorescence detector gives higher sensitivity and selectivity, so it is preferred for residue analysis (e.g. benzimidazoles, bitertanol). It is also possible to programme the excitation and emission wavelengths to optimize the signal for all eluted compounds.

Occasionally the use of an electrochemical detector is recommended (Phthalimides, Thiram, Disulfiram, etc.); although it gives great sensitivity, it is more difficult to operate, and frequently the electrodes are contaminated; sometimes, for example for EBDC determination, it is coupled on-line after the UV detector.

Nowadays there is an increasing trend to MS detection but some difficulties are still encountered when coupling it to HPLC. It is advisable to use micro-HPLC and avoid, if possible, the presence of salts in the mobile phase. This, in addition to its high cost, means that only a few applications of its use to fungicides have been published.

Derivatization

A very useful option, in HPLC, is derivatization made pre- or post-column, which facilitates compound detection. A great number of derivatizing reagents lead the formation of products with a high absorbance or fluorescence, and in fungicide analysis they are often used in post-column reactions but care is needed to minimize band broadening, particularly for slow reactions. The present use of solid phase reactors has several advantages such as the simplicity in the instrumentation and compatibility with most mobile phases. A clear example is the monitoring of the carbamate pesticides.

As pre-column derivatization can be carried out with an automatic injector and post-column derivatization can be automated with modern devices, this facilitates improved precision.

Sample Treatment

Many applications of fungicide determination require a preliminary sample extraction using an organic solvents such as ethyl acetate followed by clean up by liquid–liquid partitioning. Obviously, the matrix has a great influence on the method. The heavy pigment content in many crops and vegetables has made the popular UV detector almost unusable; even when analysing fungicides in, for example, citrus, celery heart, mint and coriander. A large amount of fluorescent coextractives can appear, causing inference in detection. In these cases the sample treatment must be optimized, including for example a clean up with Florisil or changing the mobile phase polarity, so the coextracted interference elutes together with the solvent front. If the sample preparation step can be

carried out using solid-phase extraction, this favours direct coupling to HPLC and overall automation, facilitating routine multiresidue analysis.

Analysis of some specific fungicide groups

Phthalimides (Captan, Captafol, Folpet)

For formulation analysis extracting the compound with diethylphthalate in methylene chloride and chromatography on silica gel using degassed CH_2Cl_2 as mobile phase is recommended while for residue analysis GC is usually preferred. Nevertheless, recently an isocratic HPLC method using electrochemical detection with single and dual glassy-carbon electrodes has been evaluated, showing good recoveries and precision and with detection limits of about 4 $\mu g\,L^{-1}$.

Benzimidazoles (Benomyl, Carbendazime, Thiabendazole, Methyl Thiophanate)

The high use of these post harvest fungicides means that many methods have been proposed for the determination of their residues.

It is possible to evaluate the total content (benomyl, carbendazime, methyl thiophanate) by transforming them into carbendazime, by refluxing at pH = 6.8. Multiresidue methods have been proposed, extracting the sample with HCl and analysing on LiChrosorb Si 60. Recently a clean up on strong cation exchange cartridges and analysis on C_{18} with UV and water-acetonitrile as mobile phase has been proposed, although ion-pairing HPLC coupled to UV or fluorescence detection can be used. Normal phase HPLC for carbendazime can be employed after extraction with methanol, partitioning in n-hexane-dichloromethane and fluorescence detection at 285/315 nm. Nevertheless, the majority of the proposed methods are devoted to the study of the pair benomyl–carbendazime, using reversed-phase HPLC.

This pair of compounds is normally analysed by monitoring carbendazime, although using light petroleum ether and a special drying step it is possible to analyse benomyl without conversion to carbendazime. The analysis involves an extraction with an organic solvent (methanol, ethyl acetate or acetone) followed by partitioning with n-hexane or an alkaline solution, using C_{18} columns and UV detection at 224 nm or better by fluorescence at 285/317 nm. The type of matrix, even quite similar ones, strongly conditions the sample preparation. In **Figure 1** some schemes for the determination of carbendazime in apiarian samples are shown. In the case of pollen an additional partition with n-hexane is required because of the intense colour of the extracts. As is shown, pollen or beeswax are better extracted with methanol because ethyl acetate extraction gives an emulsion that makes the separation difficult. Another problem that must be taken into account is the influence of the fortification level on the carbendazime–benomyl recoveries. Thus when analysing vegetable samples, higher fungicide concentrations added to the samples results in lower recoveries, even for smaller samples. With samples bigger than 2 g, problems also appear because the pigments are extracted, giving a greenish-yellow colour and therefore the determination of carbendazime is hindered. Some relevant data are shown in **Table 1**.

Thiabendazole has also received special attention. Several HPLC methods have been proposed for its determination, usually employing ethyl acetate as extractant and C_{18} columns and acetonitrile–water or methanol/aqueous buffer mixtures as mobile phases. A mixture of n-hexane/ethanol/0.2 N HCl, with cation exchange columns or ion pairing HPLC has also been used. Detection can be made by UV (298 nm) although usually fluorescent detection is preferred. Changes in the extractant or of the chromatographic parameters require selection of the wavelength to achieve the best sensitivity. The use of several wave-lengths, mainly the couples 285/350 nm and 305/345 nm have been proposed. In some cases, the metabolite 5-hydroxythiabendazole has also been determined.

Dicarboximides (Iprodione, Vinclozolin)

There are not many HPLC methods to determine residues of these fungicides. Vinclozolin is perhaps the most frequently used compound and so, some methods have been proposed for its analysis, and of its metabolite (3,5-dichloroaniline) using reversed-phase HPLC with UV detection. In complex mixtures analysis it has also been proved that SPE cartridges are more selective than extraction with organic solvents and they provide simpler chromatograms.

Triazoles (Bitertanol, Triadimefon, Triadimenol, Tryciclazole)

Residues of Triadimefon and its metabolite Triadimenol are seldom determined by HPLC, but they can be analysed by reversed-phase HPLC on C_{18} columns with UV detection. The same stationary phase is recommended for Bitertanol, with acetonitrile–water as mobile phase and fluorescent detection at 254/322 nm. The selection of the extracting solvent (acetone/water, acetone, methanol) is very important in order to achieve high recoveries. If a cleanup is required, SPE on C_{18} cartridges eluted with cyclohexane–ethyl acetate seems to be the most adequate.

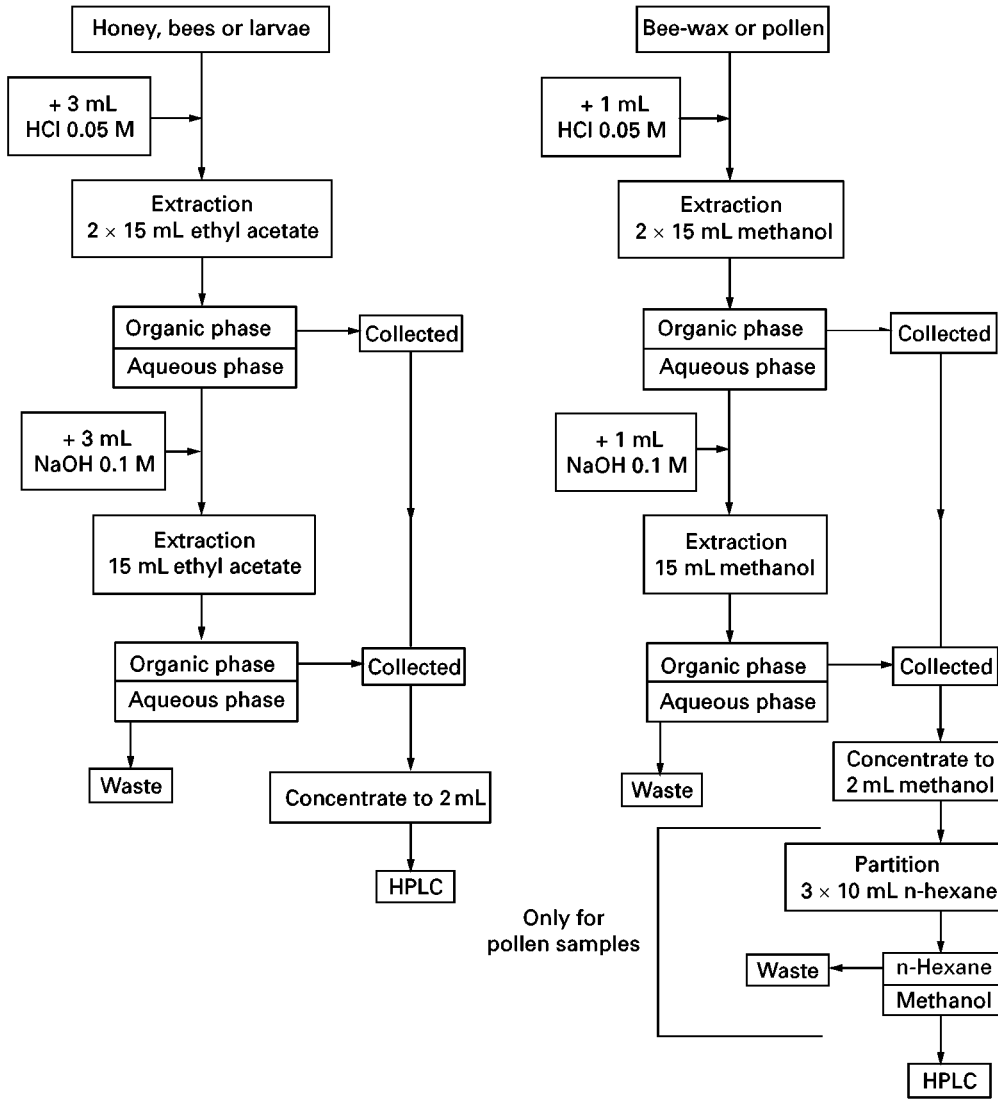

Figure 1 Flow charts showing the sample preparation procedures used in the analysis of carbendazime in apiarian products. (Reproduced from Bernal JL, del Nozal MJ, Toribio L, Jiménez JJ and Atienza J (1997) *Journal of Chromatogrphy A* 787: 129–136, with permission from Elsevier Science.)

Dithiocarbamate fungicides

The US Food and Drug Administration (FDA) has recommended the evaluation of new uniresidue methods for the analysis of dithiocarbamates in vegetables, because of problems which have arisen from the application of the carbon disulfide method. This lacks specificity because naturally occurring carbon disulfide and degradation products of the EBDCs, such as dialkyldithiocarbamate and thiuram disulfide, can give serious interference.

EBDCs have little or no solubility in water and, because of this, in many methods the compounds are converted into their soluble sodium salt by means of EDTA and subsequently hydrolysed to form carbon disulfide, but if the hydrolysis is not carried out the final product is the fungicide Nabam. This is water soluble so some methods are based on this transformation. On the other hand, ethylene thiourea (ETU), ethylene urea and 2-imidazoline are decomposition products of the EBDCs. The parent compounds have a relatively low toxicity but ETU has been demonstrated to be goiterogenic, carcinogenic and teratogenic, so there is a great interest in determining this compound.

To analyse ETU by HPLC an extraction with methanol, a clean up on a mixture of sorbents and a mobile phase of ethanol–isooctane has been frequently used. The detection can be electrochemical or by HPLC/MS, with similar detection limits. Extraction of ETU from vegetables is preferred with methanol and analysis on a CN column, with a mobile phase of

Table 1 Recovery of carbendazime obtained by using an SFE-hplc procedure on spiked lettuce samples ($n = 5$). (Reproduced from Jiménez JJ, Atienza J, Bernal JL and Toribio L (1994) *Chromatographia* 38: 395–405, with permission from Vieweg-Publishing.)

Sample amount (*g*)	*Fortification level* ($mg\,kg^{-1}$)	*Recovery* (%)	σ_{n-1}
0.20	1.0	98.4	3.0
0.20	6.0	98.3	2.9
0.20	12.0	96.4	3.3
0.50	0.3	98.3	3.2
0.50	6.0	98.0	3.4
0.50	12.0	83.3	3.5
1.00	0.3	98.2	3.3
1.00	0.6	98.0	3.3
1.00	12.0	72.4	3.9
2.00	0.3	88.3	3.7
2.00	0.6	68.4	5.5
2.00	12.0	53.7	7.0

methanol in chloroform/cyclohexane and detection at 240 nm. Another possibility is to extract ETU and react it with dihaloquinones to produce a yellow derivative that can easily be detected at 385 nm.

There is always a problem with fungicide determination because they are very similar in chemical structure and behaviour. Ferbam and Ziram have the same organic moiety and the difference is in the metallic ion. Nabam, Maneb, Zineb, Mancozeb and Propineb, frequently used in agriculture, have in common the ethylenebisdithiocarbamate group and therefore it is very difficult to separate them from their mixtures. So in some situations it is easier to determine the residue of only one fungicide. Another approach is to try to separate mixtures of three fungicides belonging to different chemical groups and the third and most difficult one is to try to separate all of them.

Some methods attempt to distinguish between compounds using both HPLC and atomic absorption methods. This can cause problems because in EBDC manufacture there is always an excess of the metallic ion that has not been incorporated into the compound, so if the extraction of the compound is not specific, the extract will contain not only the metal belonging to the fungicide but also the remaining metal coextracted. As a consequence, atomic absorption data are usually very much higher compared with those from HPLC.

To analyse individual fungicides, transition metal salts are frequently employed as ion-pairing reagents for reversed-phase HPLC with detection in the UV region. According to the complex used the wavelength selected is obviously different, so for Ziram, Maneb and Zineb forming as 1 : 1 Cu(II)-dithioligand the wavelengths are in the 260–287 nm range, with detection limits at the nM level.

Sometimes the problem arises of the presence of Thiram and Disulfiram which could interfere with the dithiocarbamate determination. This situation is usu-

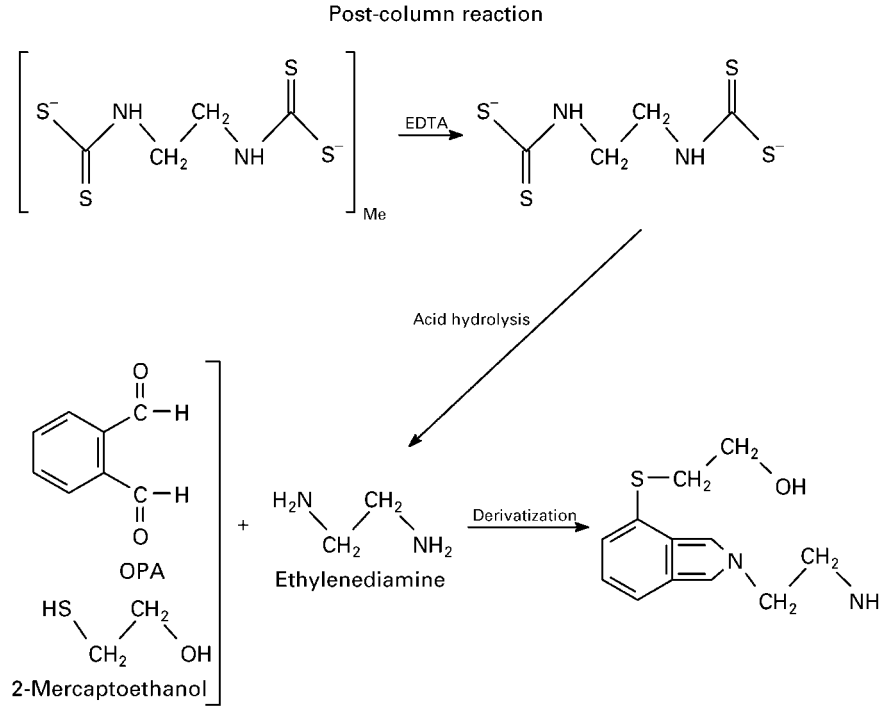

Figure 2 Post-column derivatization reaction for EBDCs.

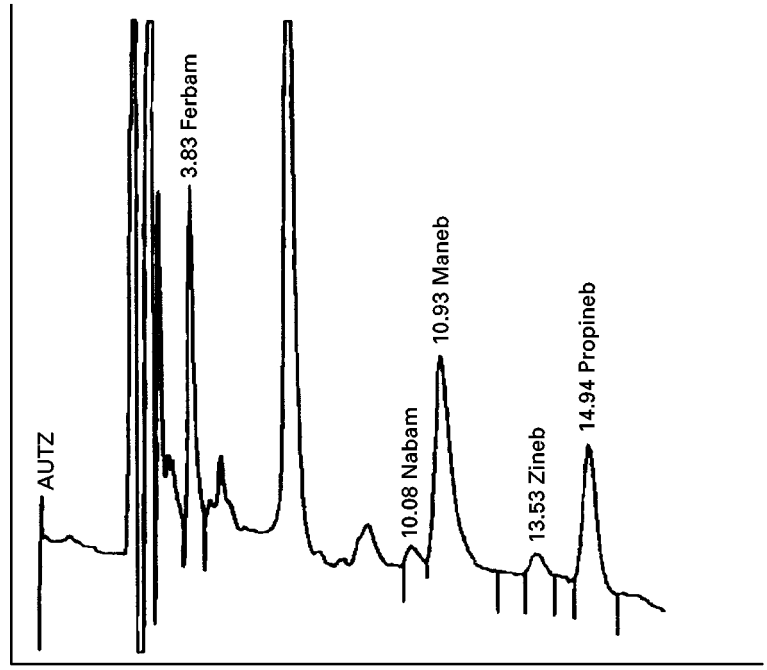

Figure 3 Chromatogram of a mixture of EBDCs. 1 μg L^{-1} each. Detection of 254 nm. Mobile phase: EDTA/MeOH/AcCN (60 : 13 : 27, v/v).

ally solved by using HPLC on a C_{18} column with electrochemical detection or by determining Thiram using micelles of CTAB in the mobile phase, so Nabam, Ziram and Ferbam do not interfere.

Nabam determination is of interest because EBDC fungicides can be converted into this fungicide and so they can be indirectly determined. In this case there is a method, very similar to one devoted to carbamate residues determination, based on a post-column reaction through an acid hydrolysis to form ethylenediamine that is afterwards fluorogenically labelled with o-phthalaldehyde-mercaptoethanol and detected at 356/450 nm. The scheme of the post-column reaction is shown in **Figure 2**.

Attention must be paid to carrying out the separation at the lowest possible temperature. The hydrolysis temperature must be considered because when the temperature is near 100°C the possibility of more by-products and background noise increases, and OPA degrades easily at higher temperatures. Separation of Nabam is carried out by micellar HPLC with a mobile phase of cetylpyridinium (CPC) phosphate buffer/acetonitrile.

The real problem is the difficulty to convert quantitatively EBDCs into Nabam and recoveries lower than 30% are usually obtained although if EDTA is used, the conversion is favoured. Thus a simpler method has been proposed based on the transformation into Nabam by means of an aqueous EDTA solution, followed by reverse-phase chromatography on an NH_2 column with acetonitrile–methanol and detection at 272 nm. However, the lifetime of the column is only about 15 analyses.

A good separation between compounds of the three families (EBDC, PBDC and DMDC) can be obtained using reverse-phase HPLC on a C_{18} column, with a mobile phase of EDTA 0.05 M, pH = 7.7, and detection in series (UV at 280 nm and amperometric at 400 mV), but it is not possible to distinguish between compounds of the same group.

A method that allows the separation of five compounds (see **Figure 3**) uses ion-pair HPLC with tetrabutylammonium bromide as counterion on C_{18} columns, with a mobile phase of EDTA/methanol/acetonitrile and detection at 254 nm. However, there are still some problems because the separation is strongly dependent on the analyte concentration, achieving only the overall separation for very low concentrations.

As a conclusion, it can be said that the analysis of these fungicides is very difficult when there are several of them in the sample and that further work is necessary.

See also: **II/Chromatography: Liquid:** Derivatization. **III/Fungicides:** gas chromatography.

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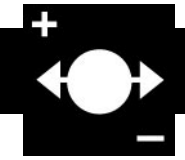
FUSED SALTS: ELECTROPHORESIS



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The interest in this technique is mainly centred around the solution chemistry of molten salts, which had its renaissance in the nuclear field and in the study of nonhydrated ions for the purpose of separating isotopes.

Techniques

Moving Boundary Method

Migrating boundaries can be observed using a cell like that shown in **Figure 1**.

Flat Bed Methods

Electromigration in a support to eliminate convection is carried out much as in normal electrophoresis,

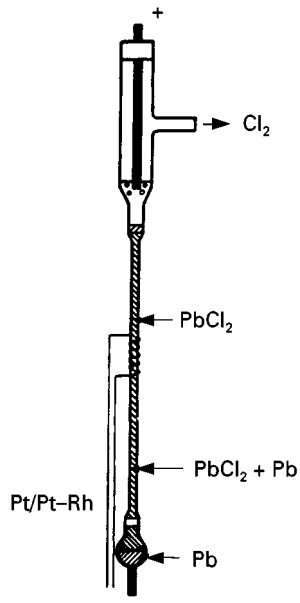

Figure 1 Cell for the determination of mobilities by observation of migrating boundaries. Reproduced with permission from Herzog and Kelmm (1961).

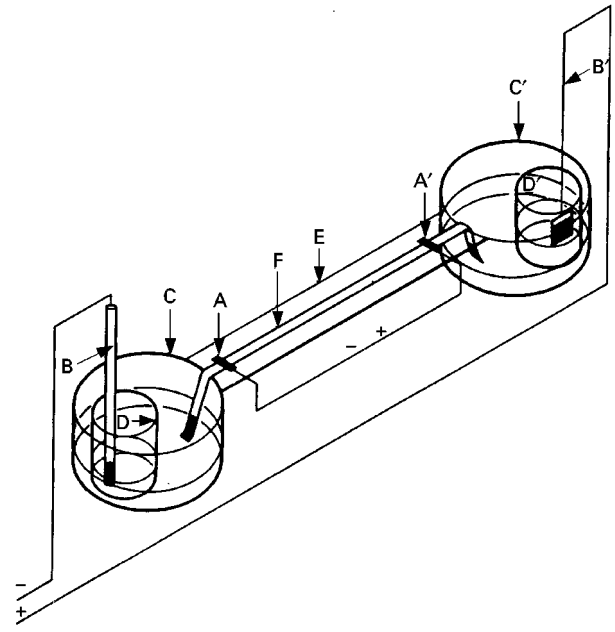

Figure 2 Apparatus for zone electrophoresis in molten salts. A and A′, platinum wires for measurement of potential difference; B and B′, electrodes; C and C′, reservoirs; D and D′, electrode compartments provided with sintered discs at the bottom; E, supporting glass plate; F, electrophoretic strip. Reproduced with permission from Alberti *et al.* (1964).