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Water-Soluble: Thin-Layer (Planar) Chromatography

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As a tool, chromatography has long been important for the separation of vitamins from complex mixtures and their initial isolation and identification would have been greatly hampered without the use of paper, column or thin-layer chromatography (TLC). While more sophisticated chromatographic techniques are now widely available, TLC has great advantages in terms of its simplicity and flexibility of use.

The vitamins classified as water-soluble are all compounds important in human metabolism either as coenzymes or their precursors which the body cannot make for itself (**Figure 1**). The recommended daily allowance (RDA) of each vitamin ranges from hundreds of milligrams to just a few micrograms a day (**Table 1**). These compounds have few properties in common apart from their water-solubility, but this fact alone makes TLC an excellent technique for their separation, particularly in pharmaceutical preparations and food products. Even at physiological concentrations, TLC is widely used after extraction of the vitamins from tissues or body fluids. This generally needs to be under acid conditions. Since most of these compounds are unstable at high pH. Some are in addition very light-sensitive. Following TLC separation, special methods of detection may also be required, since tissue levels of most water-soluble vitamins are low or very low.

Thiamin (Vitamin B_1)

Thiamin occurs in plant and animal tissues and the richest sources are seeds and nuts, peas and beans, cereals and yeast. Fish and meat, notably pork, are also good sources. Thiamin is commonly available as its monohydrochloride, but it also forms acid salts and esters with nitric and phosphoric acids. Metabolically, thiamin is required as the coenzyme thiamin pyrophosphate for the mitochondrial metabolism of glucose and pyruvate.

Thiamin may be extracted from tissues, foodstuffs or pharmaceutical preparations with aqueous alcohol mixtures at a pH of 4–6 and separated from closely related compounds and metabolites by TLC on cellulose or silica gel. Various mobile phases have been successfully used, including isopropanol–water–trichloracetic acid–ammonia (71 : 9 : 20 : 0.3) and butan-1-ol–acetic acid–water (40 : 10 : 50). Thiamin may be separated from its hydrolysis and oxidation products by TLC/densitometry and other chromatographic techniques have been reviewed. Sandwich-type chambers afford rapid separation of thiamin from other water-soluble vitamins by TLC on silica gel GF254 and the spots then located under UV light. An alternative technique for the quantitation of thiamin in pharmaceutical products involves the use of high performance TLC (HPTLC) and post-separation derivatization with a hexacyanoferrate (III)-sodium hydroxide reagent and fluorodensitometry, sensitive down to 500 pg per spot. Other modifications include the use of a fibreoptic probe

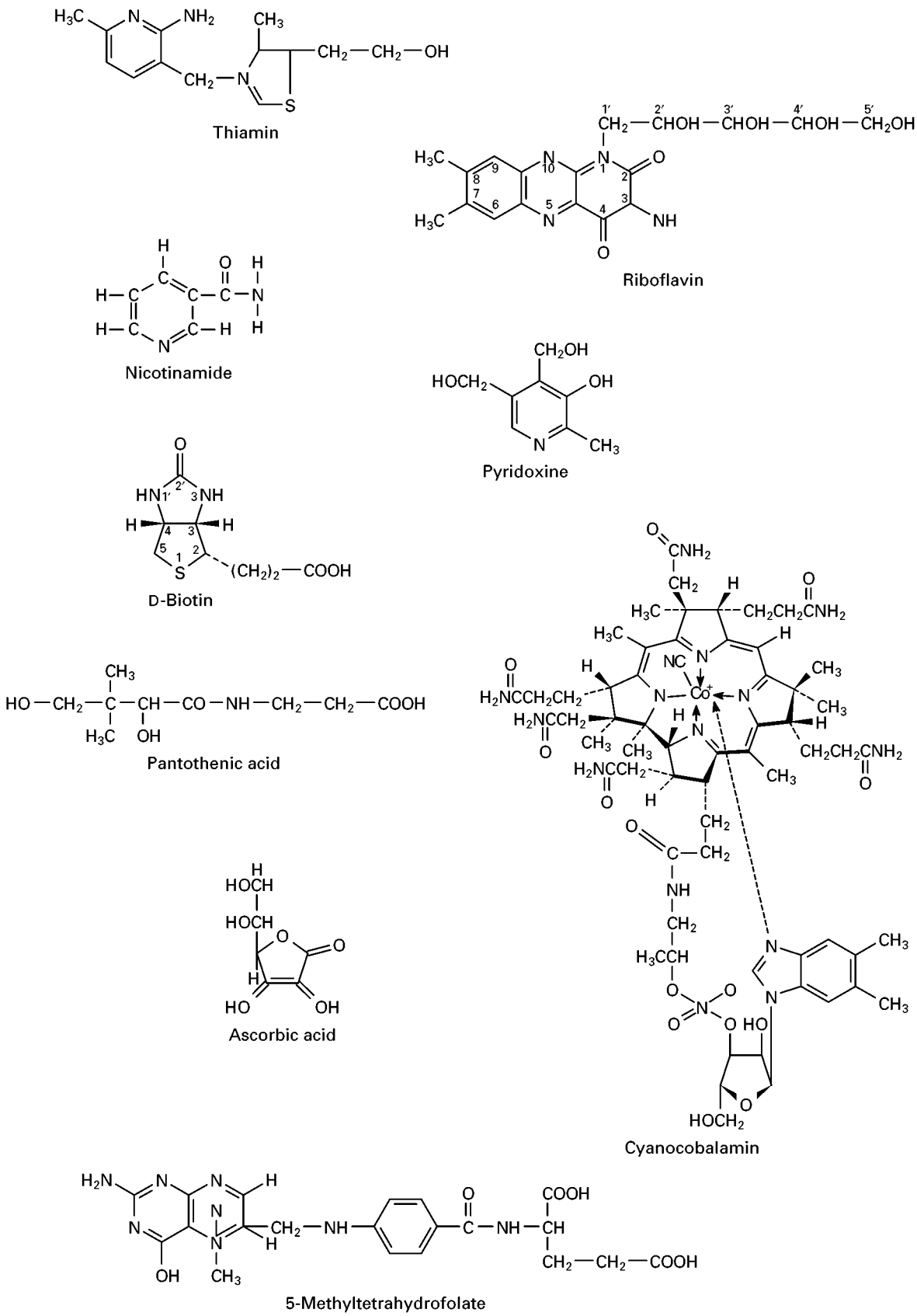

Figure 1 Structural formulae of water-soluble vitamins.

Table 1 Recommended daily allowances[a] water-soluble vitamins

Vitamin	*RDA*
Ascorbic acid	30–75 mg
Nicotinic acid	15–20 mg
Pyridoxine	1–3 mg
Riboflavin	1.5–2.0 mg
Thiamin	1–2 mg
Folic acid	300 μg
Cobalamin	1–2 μg

[a]There is no quoted RDA for biotin or pantothenic acid.

to improve measurement of thiamin in the nanogram range.

Riboflavin (Vitamin B_2)

Riboflavin and other flavinoids occur in dairy produce, meat and to a lesser extent in cereals. Flavins are stable to heat and acid but are destroyed by exposure to light. Ultraviolet irradiation of riboflavin in acid or neutral solution gives rise to the fluorescent compound lumichrome, whereas in alkaline solutions irradiation produces lumiflavin. Flavins are required in the body as their coenzymes flavin mononucleotide and flavin adenine dinucleotide, which are involved in redox reactions involving one- and two-electron transfers and linked to many energy-dependent processes in the body.

Pharmaceutical preparations containing riboflavin may be analysed by applying concentrated ethanolic extracts to silica gel TLC plates developed in butanol–benzene–acetic acid–water (8 : 7 : 5 : 3) or butanol–acetic acid–water (9 : 4 : 5). Foods, tissue samples and urine each require particular methods of sample preparation and these methods and the solvent systems successfully employed have been reviewed elsewhere. A dark room is required for sample preparation and chromotography of flavins to prevent photolytic degradation. The fluorescent property of flavins provides a convenient means of detection and spots may be located under radiation at 254 and 366 nm. HPTLC followed by fibreoptic fluorimetry has been used to measure riboflavin in vitamin mixtures and can detect 48–320 ng. Separation is also effective on mixed-layer plates of GDX-102 and silica gel G (1 : 1) pre-coated with hexadecyltrimethylammonium bromide, developed in 60–70% ethanol.

Nicotinic Acid (Vitamin B_3)

Nicotinic acid (niacin) and various nicotinamides are sources of the coenzyme nicotinamide adenine dinucleotide, synthesized in the mitochondria and vital for oxidative energy production in many metabolic reactions. Niacin is normally acquired from a balanced diet of meat, fish, whole cereals and yeast. Peas, beans, nuts, fruit and vegetables are also good sources of this vitamin.

Analysis of powdered preparations containing nicotinic acid has been achieved on silica gel plates impregnated with zinc acetate, developed in butanol–benzene–acetic acid–water (8 : 7 : 5 : 3) or butanol–acetic acid–water (9 : 4 : 5) to provide a self- indicating system. An overpressure chromatographic procedure using HPTLC silica gel plates and a mobile phase of butan-1-ol–pyridine–water (50 : 35 : 15) is also effective. This method uses photodensitometric detection to separate nicotinamide from other vitamins and the method is fast, accurate and specific. Other methods based on HPTLC and fibreoptic fluorometric quantitation have been described in which nicotinic acid is converted to a fluorescent derivative before chromatography. After separation, the plate is scanned by a bifurcated fibreoptic which transmits the excitation radiation and collects the signal emitted from the plate. Good calibration curves have been obtained in the range 10–100 ng nicotinic acid.

Pantothenic Acid (Vitamin B_5)

Pantothenic acid is required in the formation of acetyl coenzyme A which holds a key position in many metabolic pathways. Only the natural dextrorotatory form is active. Pantothenic acid is found in most foods of plant and animal origin and good sources include liver, kidney, wheat germ, royal jelly, peanuts, spinach, cheese and peas. There is no quoted RDA, though most diets provide at least 10 mg per day.

Panthenol and pantothenic acid have been identified and quantified in pharmaceutical preparations by extraction with ethanol or benzyl alcohol and separated by TLC on silica gel plates developed in propan-2-ol–water (85 : 15). Spots are measured by spectrodensitometry. Postaire has applied the over-pressure derivatization technique following separation of calcium pantothenate from other hydrophilic vitamins on silica gel HPTLC layers developed in butan-1-ol–pyridine–water (50 : 35 : 15).

Pyridoxine (Vitamin B_6)

Pyridoxine is found chiefly in animal tissues; pyridoxal and pyridoxamine occur in plant tissues. Together these three forms of the vitamin are of vital importance in the body for the synthesis of pyridoxal 5-phosphate which acts as coenzyme to amino

transferases, facilitating more than 60 amino group transfers and other reactions, including formation of neurotransmitters. The RDA is 1–3 mg but may increase on a high protein diet. Good sources are yeast, liver, peanuts, bananas, grapes and pears, beef and fish.

Chromatographic analysis of the vitamin B_6 complex, including sample preparation and pre-TLC extraction, have been well reviewed. Separation of pyridoxine from other water-soluble vitamins in pharmaceutical preparations can be improved by impregnating silica gel plates with zinc acetate to provide a self-indicating system after separation. Impregnation of plates with hexadecyltrimethylammonium bromide has similarly been used to improve the TLC analysis of vitamin B_6 in foods. Postaire has reported better separation and resolution of B_6 from other compounds using the overpressure layer technique than by HPTLC.

Cobalamin (Vitamin B_{12})

Vitamin B_{12} is the generic name for a group of vitamins known as cobalamins. The basic molecule consists of a corrin ring enclosing a central cobalt atom subtending axial ligands which determine the form and function of each individual cobalamin. Cyanocobalamin (CNCbl) was the first form of the vitamin isolated in 1948, independently by two groups. Both relied heavily on chromatography for the final separation and purification of CNCbl. Its complex three-dimenstional structure was elucidated in 1956 by Dorothy Hodgkin using elegant X-ray crystallographic techniques.

The cobalamin molecule can only be synthesized by microorganisms, but all mammalian cells are equipped to covert the vitamin into its coenzymes. Cobalamin is without known function in plants and, if present, is only associated with the metabolic activity of microorganisms. Hence, unlike folate, dietary sources of the vitamin are exclusively animal in origin and include fish, meat – particularly liver and kidney – eggs and milk. Cobalamin is acid- and heat-stable but, like other hydrophilic vitamins, is destroyed by exposure to high pH. Notable features of cobalamin are that it is a much larger molecule (mol wt of OHCbl is 1346) than any other B-group vitamin and tissue levels are lower than any other, with total amounts in the body amounting to only a few milligrams. The low RDA of 1–2 μg is a reflection of the efficient means employed by the body to retain the vitamin. The low tissue levels of cobalamins naturally cause analytical problems and this has led to the development of enhanced methods of detection, discussed below.

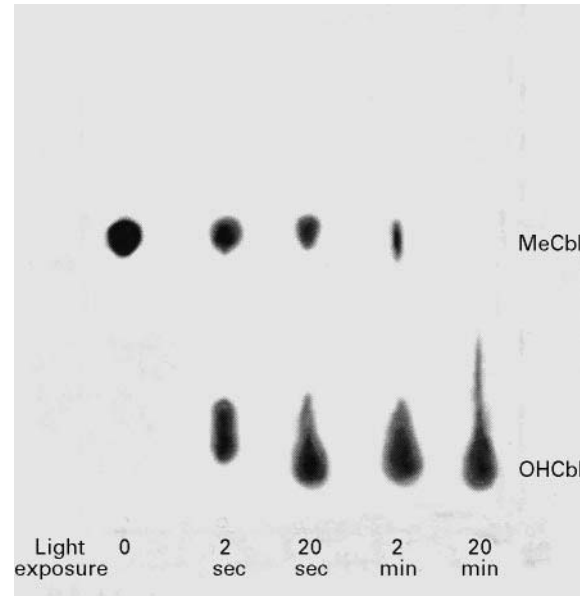

Figure 2 Photolysis of methylcobalamin (MeCbl) in extracts of normal human plasma exposed to daylight. Most of the MeCbl was converted to hydroxocobalamin (OHCbl) in 2 min.

In humans, the two coenzyme forms of vitamin B_{12} are adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) and each is required in specific reactions involving, respectively, isomerization and transmethylation. Both coenzymes are very light-sensitive and are readily converted to hydroxocobalamin (OHCbl) by exposure to white light, as may be demonstrated (**Figure 2**). MeCbl was first synthesized in the laboratory by Lester Smith and detected in human plasma by Lindstrand in 1963 as an unidentified zone on paper chromatograms. Using large quantities of liver, this compound was isolated using chromatographic methods and characterized as MeCbl.

The bulk of cobalamin in the body occurs as AdoCbl in cells and MeCbl in plasma, but other forms detected include OHCbl, CNCbl and suphitocobalamin, which may be a breakdown product of glutathionylcobalamin, possibly an important metabolic intermediate. A variety of adsorbents may be used for cobalamin TLC but none has been found to better a mixed layer of Whatman CC41 microgranular cellulose and silica gel G (**Figure 3**). A sensitive two-dimensional TLC method has been developed which allows small blood and tissue samples to be used (**Figure 4**) to investigate cobalamin metabolism in health and a wide range of diseases, including cobalamin deficiencies and genetic errors of B_{12} metabolism. The sensitivity of the method relies on the bioautography organism which is a selected strain of *Escherichia coli*, which has a cobalamin growth response down to 1–2 pg. Growth zones are

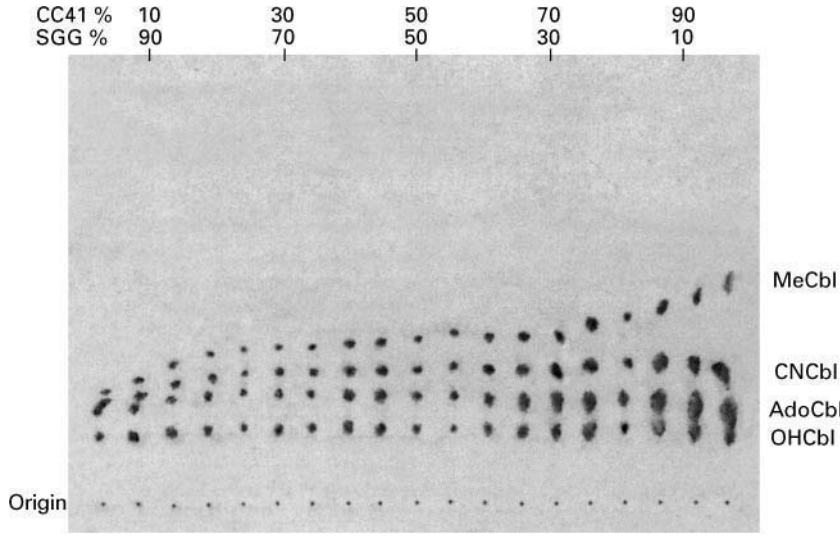

Figure 3 Separation of an aqueous mixture of four cobalamins by TLC on a gradient layer of cellulose (CC41) and silica gel (SGG), showing the influence of varying adsorbent mixtures on separation of the cobalamins. The mobile phase was butan-2-ol–water–0.880 ammonia (75 : 25 : 2).

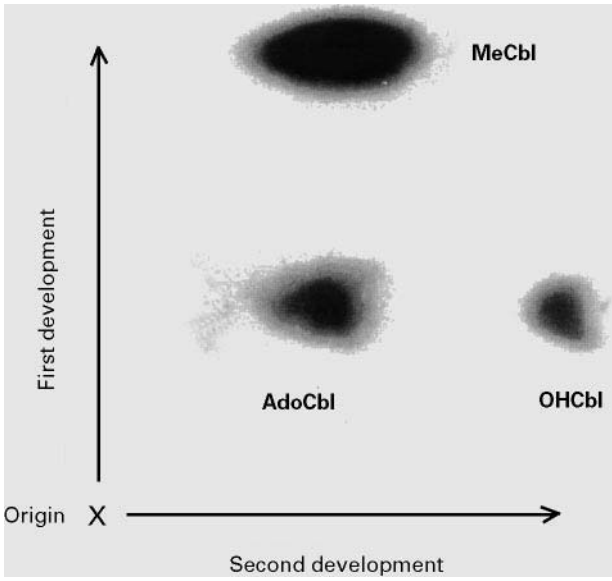

Figure 4 Separation of cobalamins extracted from normal human plasma. The adsorbent was cellulose CC41–silica gel G (3 : 1) developed first in butan-2-ol–water–0.880 ammonia (75 : 25 : 2) and second, in water saturated with benzyl alcohol. The second development was at right angles to the first, after air-drying the plate. Extraction and chromatography were in darkness or by red light. Cobalamin zones were detected bioautographically by over-layering the chromatogram with agar seeded with a cobalamin-senistive *Escherichia coli* mutant and a tetrazolium growth indicator. The 'sandwich' was incubated at 35°C for 18 h. Methylcobalamin (MeCbl) is the main form present in healthy subjects, with smaller amounts of adenosylcobalamin (AdoCbl) and hydroxocobalamin (OHCbl).

enhanced by inclusion of 2,3,5-triphenyltetrazolium chloride in the agar medium which is converted to the red dye fomazan during growth of the organism. The red zones corresponding to cobalamins separated on the TLC plate are then scanned by densitometer or computer and quantitated. A 10–100-fold increase in sensitivity is gained if radiolabelled cobalamins are separated and the bioautogram growth zones excised and their radioactivity measured.

Folic Acid

Folic acid (pteroylglutamic acid) and related compounds are present at high concentrations in liver, but spinach, broccoli, peanuts and fresh fruit are also good dietary sources. Folates are important for the synthesis of tetrahydrofolate, which is important with cobalamin for a series of 1-carbon transfer reactions leading to DNA synthesis, failure of which leads to megaloblastic anaemia.

Chromatographic analysis of folate compounds including methotrexate and other antifolates has been reviewed. Process impurities in the reduced folate compound leucovorin calcium may be monitored using a TLC method with fluorescence detection. An overpressure layer TLC procedure (OPLC) has been used to improve the separation of folic acid from other water-soluble vitamins with good recovery and resolution. The method uses silica gel layers developed in butan-1-ol–pyridine–water (50 : 35 : 15) at a rate of 0.25 mL min^{-1} for baseline separation. Quantitation is achieved without derivatization.

Biotin

Good sources of biotin are liver, pork, nuts, chocolate, pulses, cereals and royal jelly; biotin is widely distributed among all types of food and dietary deficiency is rare. However, biotin is inactivated by avidin, which is present in raw egg white, and severe eczema has been reported from this type of deficiency. This does not arise if cooked eggs are included in the diet, since heat deactivates avidin. Biotin acts as coenzyme to carboxylase enzymes, for example in the catabolism of propionate to methylmalonate. Biotin is stable in acid and neutral solutions and hence may be extracted at low pH before chromatography.

Biotin is separable from other water-soluble vitamins by TLC on silica gel or cellulose layers developed in neutral or acidic butanol–water mixtures. Various detection reagents have been used for biotin, including iodine vapour, 1% potassium permanganate, 1% dimethylaminobenzaldehyde in hydrochloric acid and *p*-dimethylaminocinnamaldehyde in a mixture of methanol and sulfuric acid, which is specific for biotin, yielding intense orange zones with an absorbance maximum at 533 nm. More recently, TLC, HPTLC and OPLC techniques have been compared, using five different mobile phases. Biotin tends to be resolved poorly from pantothenic acid by HPTLC but this is improved by OPLC, although in the systems investigated this led to less than perfect separation of biotin and folic acid.

Ascorbic Acid (Vitamin C)

Ascorbic acid occurs abundantly in fresh fruit, especially blackcurrants, citrus fruit and strawberries, and in most fresh vegetables; good sources are broccoli and peppers. It is destroyed by heat and is not well stored in the body. Ascorbic acid is a good reducing agent and facilitates many metabolic reactions and repair processes.

In pharmaceutical preparations and fruit juices, ascorbic acid is readily separated from other compounds by TLC on silica gel and quantitated directly by absorption at 254 nm. Serum and plasma may be deproteinized with twice the volume of methanol or ethanol. Various ascorbic acid compounds in plant extracts and foods have been separated on cellulose layers and detected by spraying with 2,5-dichlorophenol indophenol. Heulandite, a natural zeolite (particle size 45 μm) has successfully been employed as an adsorbent and ascorbic acid and other hydrophilic vitamins have separated within 5 cm by ascending chromatography in dimethylformamide. HPTLC and OPLC methods have been developed to improve the separation of ascorbic acid from other water-soluble vitamins, with some success.

Conclusion

TLC is a flexible and well-established technique for the separation of water-soluble vitamins, limited only by the stability of the compounds to be separated, the resolving power of the TLC system and the sensitivity of the detection method. In complex biological systems these factors assume greater importance as vitamin concentrations are lower and metabolites may interfere with the separation. A preliminary extraction step or use of a short clean-up column can help remove salts and other interfering substances and may increase the concentration of vitamins to be chromatographed. Recovery experiments will monitor any selective losses at this stage.

The introduction of HPTLC and OPLC with optimized solvent systems has undoubtedly increased the resolving power for a number of vitamins. Gradient or two-dimensional TLC can increase this still further. Ultimately, it is the means of detection which determines the sensitivity of the system. Fluorimetry has become the method of choice for those vitamins forming fluorescent derivatives, but there are alternatives. One is to overlayer the chromatogram with an agar medium seeded with a microorganism whose growth is sensitive to the vitamin. This can detect as little as a few pg of the vitamin. Even higher sensitivity can be achieved using radioactive vitamins detected autographically or with phosphorimagers. In future, the development of an immunoassay technique similar to Western blotting is likely to allow the most sensitive quantitation of vitamins separated by HPTLC.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Instrumentation; Modes of Development: Forced Flow, Overpressured Layer Chromatography and Centrifugal; Spray Reagents. **III/Vitamins:** Liquid Chromatography.

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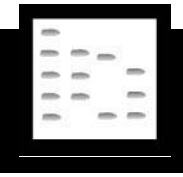
VOLATILE ORGANIC COMPOUNDS IN WATER: GAS CHROMATOGRAPHY



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Introduction

An important class of substances for which it is increasingly necessary to analyse in environmental waters comprises a wide range of volatile organic compounds (VOC). These include aromatics such as methylbenzene (toluene) and the dimethylbenzenes (xylenes), and the environmentally persistent halogenated solvents such as tetrachloromethane and trichloroethene. Many of these compounds are finding their way onto national and international lists of proscribed or regulated compounds, and as a result there is a requirement for robust methods of analysis to monitor both the environment itself and potential sources of discharge to it.

In the aqueous environment, there are a number of sample types that an analyst may be required to examine, each presenting their own problems and challenges and requiring slightly different analytical solutions. Drinking waters, for example, are a relatively straightforward matrix, often with a clearly defined quality standard imposed, such as the requirements of the European Union Drinking Water Directive (see Further Reading). River waters and marine waters may also be required to meet exacting environmental quality standards (EQS), which are frequently much lower than those set for drinking waters where the presence of haloforms, for example, is an accepted by-product of the disinfection process. Monitoring of wastewater effluents is fundamental to environmental quality management, since these are