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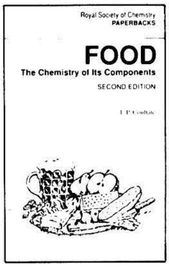
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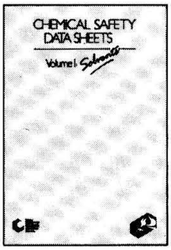

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Column "Dead Volume" in Liquid Chromatography

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The physical and chromatographic volumes associated with a liquid chromatography column have been considered theoretically and their inter-relationship is discussed. The two chromatographically important dead volumes, the kinetic dead volume and the thermodynamic dead volume, have been identified and it is shown that, whereas the kinetic dead volume (the volume of the moving phase) was common to all solutes, the thermodynamic dead volume was unique to each solute. The solute dependence of the thermodynamic dead volume resulted from any exclusion characteristics that the stationary phase possessed. Stationary phases based on silica exhibited strong exclusion properties and hence the thermodynamic dead volume for silica-based phases was strongly dependent on solute size. It followed that the pertinent thermodynamic dead volume had to be determined strictly for each solute. It also followed that correlation of retention with solvent composition could not be carried out employing *k′* (solute capacity factor) values, as these were dependent on accurate dead volume measurements. It is recommended that for such a correlation, corrected retention volumes are employed and then only where the solute is eluted at sufficiently high *k′* values. Under such circumstances any errors that would result from incorrect dead volume measurements are minimised. Experimental methods are given for the evaluation of all chromatographically pertinent volumes and, as an example, the procedures were applied to a Zorbax C_8 reversed-phase column. The data obtained are discussed in the light of the theory presented.

Keywords: *Dead volume; liquid chromatography*

The dead volume of a liquid chromatography column has been the subject of a considerable number of publications[1–7] and, consequently, has been studied extensively from both a theoretical and an experimental point of view. The dead volume of a liquid chromatography column is important in both kinetic and thermodynamic measurements and is particularly so when attempts are made to correlate solute retention with solvent composition. The pertinent column volumes that have been considered to be important in the past have been the column interstitial volume, the column pore volume and the volume of the stationary phase in the column. However, certain assumptions made tacitly in previous work may not be chromatographically acceptable. For example, it is often assumed that all the interstitial volume of the column contains moving phase and that none of the interstitial volume is static. In some instances it has also been assumed that the pore contents have the same composition as the mobile phase, whereas there is considerable evidence that this is not so.[8–10] Another assumption that has been made is that all of the stationary phase in the column is chromatographically available and, probably most important, the exclusion characteristics of the packing material can be totally ignored. The purpose of the work described herein is two-fold. Firstly, to examine the liquid chromatography column from both a physical and chromatographic point of view and to develop expressions for the various chromatographic parameters without employing unrealistic assumptions. Secondly, to describe experimental methods that can be used to determine these parameters and to discuss the implication of the data on chromatographic measurements.

Theory

The internal volume of any liquid chromatography column is occupied by three substances, the mobile phase, the fixed stationary phase and the support. To date, the term mobile phase is a misnomer as it implies that all of the mobile phase is moving, which is not so. Nevertheless, it is now such a well established term that it will still be used even though much of the mobile phase is contained in the pores of the packing material and is therefore not moving. The term "moving phase" will be employed for that portion of the mobile phase that actually moves. The mobile phase may be a single solvent or a solvent mixture. The support is usually silica gel, the surface of which can also constitute the fixed stationary phase. However, the most common form of chromatography employs a bonded phase, in which instance the fixed stationary phase consists of silica gel with an organic moiety bonded on to the surface. The organic moiety can be aliphatic, aromatic or heterocyclic in nature and can consist of a single organic species or a mixture of species.

It follows that,

$$V_c = V_m + V_s + V_{Si} \quad \ldots \quad (1)$$

where V_c is the total volume of the column, V_m is the volume of the mobile phase in the column, V_s is the volume of the fixed stationary phase in the column and V_{Si} is the volume of silica in the column.

In this paper the interaction of the solutes between the mobile and stationary phases will be considered to be liquid - liquid in nature. Whether, in fact, a reversed phase constitutes a liquid-like phase or a solid surface is a moot point and a subject of some controversy. In any event, the results obtained and the conclusions drawn in this paper are independent of the exact nature of the solute - stationary phase interactions and either form can be assumed. The volume of the stationary phase, V_s, can be replaced by Ad_f (A is the surface area of the stationary phase in the column and d_f is the "film" thickness) if so desired and the arguments and conclusions will remain the same. It should also be pointed out that the mass of the bonded phase in the column will, in any event, occupy a given volume in the column and must be included in the total volume of the column.

Now, the total volume of the column is given by

$$V_c = \pi r^2 l \quad \ldots \quad (2)$$

where r is the radius of the column and l is the length of the column.

Hence,

$$V_m + V_s + V_{Si} = \pi r^2 l \quad \ldots \quad (3)$$

As defined classically, a chromatographic separation is achieved by the distribution of the solute between two phases, a moving phase and a stationary phase and hence, it is the volume of the moving phase and the volume of the stationary phase that are chromatographically important. Unfortunately, not all of the mobile phase as defined in equation (1) is moving and not all of the fixed stationary phase can be chromatographically available.

Consider the volume of mobile phase in a column. This is contained partly in the interstitial volume of the column between the particles of silica or bonded silica and partly in the pores of the packing material that are not occupied by the fixed stationary phase.

Consequently,

$$V_m = V_i + V_p \quad \ldots \quad (4)$$

where V_i is the interstitial volume of the packing material and V_p is the pore volume of the packing material.

The interstitial volume of the column is also made up of two parts: that portion of the volume containing the moving phase, $V_{i(m)}$, and that volume close to the points of contact between the particles and away from the flow-stream that is essentially not in motion, $V_{i(s)}$.

Hence,

$$V_i = V_{i(m)} + V_{i(s)} \quad \ldots \quad (5)$$

Where mixed solvents are employed as the mobile phase, it has been shown[10] that one component can be adsorbed preferentially on, or associated somehow with, the surface of the stationary phase. Consequently some of the solvent contents of the pore in proximity with the stationary phase may not necessarily have the same composition as that of the original mobile phase.

Therefore,

$$V_p = V_{p(1)} + V_{p(2)} \quad \ldots \quad (6)$$

where $V_{p(1)}$ is that part of the pore volume having the same composition as the bulk mobile phase and $V_{p(2)}$ is that part of the pore volume having a different composition to that of the mobile phase. Substituting for V_i and V_p from equations (5) and (6) in equation (4) gives

$$V_m = V_{i(m)} + V_{i(s)} + V_{p(1)} + V_{p(2)} \quad \ldots \quad (7)$$

Owing to the nature of the bonded phase and as a result of its method of manufacture some of the smaller pores may be blocked completely by the reversed phase itself and some may therefore be chromatographically unavailable. It follows that,

$$V_s = V_{s(A)} + V_{s(U)} \quad \ldots \quad (8)$$

where $V_{s(A)}$ and $V_{s(U)}$ are the volumes of the available and unavailable stationary phases, respectively. Substituting for V_m and V_s from equations (7) and (8) in equation (1) gives

$$V_c = V_{i(m)} + V_{i(s)} + V_{p(1)} + V_{p(2)} + V_{s(A)} + V_{s(U)} + V_{Si} \quad (9)$$

Now the basic equation for the retention of a solute (N) is commonly, but erroneously, assumed to be

$$V_{r(N)} = V_0 + K_N V_s \quad \ldots \quad (10)$$

where $V_{r(N)}$ is the retention volume of solute N, V_0 is the "dead volume," K_N is the distribution coefficient of the solute N between the two phases and V_s is the volume of the stationary phase; V_s can be taken as Ad_f if it is considered that adsorption is taking place and then, $V_{r(N)} = V_0 + K_N'A$, where K_N' is the equilibrium constant of the solute between the surface and the bulk liquid. In the following arguments, equation (10) will be employed and hence partition assumed. The same results would be obtained if adsorption were assumed.

As can be seen from equation (9) however, equation (10) is an over-simplified expression for solute retention in liquid chromatography. Some of the stationary phase may not be chromatographically available and not all of the pore contents have the same composition as the mobile phase and, therefore, being static, can act as a second stationary phase. This is, in effect, akin to the original and well known development of liquid - liquid systems by adsorbing suitable liquids in the pores of an appropriate support.

Consequently a more accurate form of the retention equation would be

$$V_r = V_{i(m)} + KV_{i(s)} + K_1V_{p(1)} + K_2V_{p(2)} + K_3V_{s(A)} \quad (11)$$

where K is the distribution coefficient of the solute between the moving phase and the static portion of the interstitial volume, K_1 and K_2 are the distribution coefficients of the solute between the moving phase and the static pore volume contents $V_{p(1)}$ and $V_{p(2)}$, respectively, and K_3 is the distribution coefficient of the solute between the moving phase and the fixed stationary phase.

All static phases will affect retention and, consequently, there are a number of distribution coefficients effective in the chromatographic system. However, as the static interstitial volume $V_{i(s)}$ and the fraction of the pore volume $V_{p(1)}$ represent a mobile phase of the same composition as the moving phase then, $K = K_1 = 1$, and hence equation (11) reduces to

$$V_r = V_{i(m)} + V_{i(s)} + V_{p(1)} + K_2V_{p(2)} + K_3V_{s(A)} \quad \ldots \quad (12)$$

Unfortunately, the practical chromatographic system is even more complicated as all silica-based stationary phases exhibit exclusion properties and the pores have diameters ranging from, perhaps, 2 or 3 Å to as much as several hundred Å. Consequently, some otherwise open pores are accessible to the solute while others are not, depending on the size of the solute molecule. Therefore, only those pores that have a diameter equal to or greater than that of the solute molecule are accessible and only the stationary phase inside those pores can effect retention. In addition, the static interstitial volume between the particles can also exhibit exclusion properties and some of the static interstitial volume may also be inaccessible to the larger solutes. As a consequence equation (12) must be modified further to give

$$V_{r(N)} = V_{i(m)} + \Phi_N V_{i(s)} + \alpha_N V_{p(1)} + \alpha_N K_2 V_{p(2)} + \beta_N K_3 V_{s(A)} \quad (13)$$

where $\Phi_N V_{i(s)}$ is that part of the static interstitial volume accessible to solute N, $\alpha_N V_{p(1)}$ and $\alpha_N V_{p(2)}$ are those parts of the pore volumes accessible to solute N and $\beta_N V_{s(A)}$ is that part of the volume of the available fixed stationary phase accessible to solute N. Under some circumstances α_N can equal β_N but a general case will be assumed where they are not equal.

There are two dead volumes that are chromatographically important, *viz.*, the kinetic dead volume and the thermodynamic dead volume. The former is employed in the study of peak variance and in calculating column velocities for use in equations that describe the variance per unit length of a column. The latter is used in making thermodynamic measurements from chromatographic data. The kinetic dead volume is represented by $V_{i(m)}$ in equation (13). The kinetic dead volume must be confined solely to that volume of the mobile phase that is moving. The thermodynamic dead volume is represented by $V_{i(m)} + \Phi_N V_{i(s)} + \alpha_N V_{p(1)}$ in equation (13). The thermodynamic dead volume includes those static portions of the mobile phase that have the same composition as that of the moving phase and hence do not contribute to retention. It is seen that, in contrast to the kinetic dead volume, which by definition can have no static portion and which is consequently independent of the solute chromatographed, the thermodynamic dead volume will vary from solute to solute depending on the size of the solute molecule (*i.e.*, is dependent on Φ_N and α_N). Moreover, the amount of the stationary phase accessible to the solute will also vary with the size of the solute molecule (*i.e.*, is dependent on β_N). Consequently, for a given stationary phase, it is not possible to compare the retentive properties of one solute with those of another in thermodynamic terms unless Φ_N, α_N and β_N are known accurately for each solute. This is particularly so if the two solute molecules have significantly different molecular volumes. The experimental determination of Φ_N, α_N and β_N would also be extremely difficult, if not impossible in practice, as it would necessitate a separate series of exclusion measurements for each solute that could be extremely tedious to carry out.

The two functions involving either K_2 or K_3, or both, can, in theory, contribute to solute retention. This will depend on whether the solute interacts with the adsorbed mobile phase component on the surface of the stationary phase or interacts with the stationary phase proper. In either instance the accessibility of the retentive phase is governed by the magnitudes of α_N and β_N.

Another important aspect of equation (13) is its effect on the measurement of the solute capacity factor (k') as this also depends on the values of Φ_N, α_N and β_N. Now k' is normally calculated by

$$\begin{aligned} k' &= [V_{i(m)} + \Phi_N V_{i(s)} + \alpha_N V_{p(1)} + \alpha_N K_2 V_{p(2)} + \beta_N K_3 V_{s(A)} - \\ &\quad V_{i(m)} - \Phi_N V_{i(s)} - \alpha_N V_{p(1)}]/[V_{i(m)} + \Phi_N V_{i(s)} + \alpha_N V_{p(1)}] \\ &= [\alpha_N K_2 V_{p(2)} + \beta_N K_3 V_{s(A)}]/[V_{i(m)} + \Phi_N V_{i(s)} + \alpha_N V_{p(1)}] \end{aligned} \quad (14)$$

Consequently, values of k' will carry the same errors as those associated with the measurement of the dead volume and will change with solute size.

However, provided that $\alpha_N K_2 V_{p(2)} + \beta_N K_3 V_{s(A)} \gg V_{i(m)} + \Phi_N V_{i(s)} + \alpha_N V_{p(1)}$ then, under the conditions given above, the "corrected retention volume" can be correlated with solvent composition or other chromatographic operating parameters with acceptably low errors.

Experimental

Apparatus

The liquid chromatography system consisted of a Waters solvent pump (Milford, MA, USA, Model 510), a sample injection valve (Rheodyne, Cotati, CA, USA, Model 7125), a variable-wavelength UV absorbance detector (Waters, Model 401) and a potentiometric recorder (Linear, Model 1210). The column was maintained at constant temperature by means of a constant-temperature circulator (NesLab, Newington, NH, USA).

Reagents and Chemicals

All chemicals were of analytical-reagent grade or better. High-performance liquid chromatography grade methanol and water were obtained from J. T. Baker (Phillipsburg, NJ, USA). Prior to use all liquids were filtered through a membrane filter (0.2 mm average pore size, Millipore, Bedford, MA, USA).

Chromatographic Procedure

A Zorbax C_8 column (25 cm × 4.6 mm i.d.) packed with 5-mm silica gel having a specific surface area of 330 $m^2 g^{-1}$ was used. The column contained *ca.* 3.0 g of a material that had a carbon content of 10.0% *m/m*. The column was situated inside a plastic tube jacket through which water flowed at a temperature of 30 ± 0.02 °C.

The different mobile phases were prepared by mixing known volumes of methanol and water and were de-gassed by stirring under vacuum prior to use. Samples to be injected on to the column were dissolved in the mobile phase with which the column was in equilibrium. The concentration of the sample was adjusted so that the height of the peak produced was approximately one order of magnitude greater than the noise level of the detector.

The retention volume was measured by means of a burette connected directly to the outlet of the detector. A burette reading was taken at injection and another at the peak maximum. The difference between the two readings was recorded. All retention measurements were made in triplicate and the mean was taken as the correct retention volume provided that the difference between the extreme values was less than 2% of the mean. The extra column volume was measured by replacing the column with a union and measuring the retention volume of the system devoid of a column. All retention measurements were corrected for the extra column volume.

Determination of the Volume of the Column, Total Volume of the Mobile Phase and the Volumes of Silica and Stationary Phase in the Column

The total volume of the mobile phase in the column was determined by a weighing procedure employing solvent pairs of widely different densities. The column was equilibrated with the first solvent and after capping, the column and its contents were weighed on an analytical balance to the nearest 0.1 mg. The procedure was repeated with the second solvent and the total volume of the mobile phase in the column was calculated from the following equation:

$$V_m = (m_1 - m_2)/(d_1 - d_2) \quad \ldots \ldots \quad (15)$$

where m_1 is the mass when the column is filled with solvent 1, m_2 is the mass when the column is filled with solvent 2, d_1 is the density of solvent 1 and d_2 is the density of solvent 2. Three pairs of solvents were employed and the results are given in Table 1.

The volume of silica and the stationary phase was calculated from the dimensions of the column using

$$V_x = V_c - V_m \quad \ldots \ldots \ldots \quad (16)$$

where V_x is the volume of silica plus the stationary phase in the column and V_c is defined by equation (2).

The volume of silica, V_{Si}, is given by

$$V_{Si} = (V_c - V_m)/(1 + J) \quad \ldots \ldots \quad (17)$$

where $J = Cd_{Si}/d_S(100 - C)$ and d_{Si} is the density of silica gel, d_S is the density of the bonded phase and C is the mass-% of the bonded phase in the total packing material; d_{Si} was taken as 2.28 g ml^{-1} from reference 11.

The volume of the stationary phase in the column, V_s, was calculated using

$$V_s = V_x - V_{Si} \quad \ldots \ldots \ldots \quad (18)$$

The results obtained for V_{Si} and V_s are given in Table 1.

The bonded phase on the Zorbax column was a C_8 chain made from dimethyl(octyl)silyl chloride. The chain was, in fact, equivalent to isodecane and hence $d_s = 0.726$ g ml^{-1} was used in equation (17).

Determination of the Components of the Interstitial Volume

The interstitial volume consists of two parts, the fraction that is moving, $V_{i(m)}$, and the fraction that is essentially static, $V_{i(s)}$. The two parts must be determined separately.

Table 1. Volume, determined using the Zorbax column (25 cm × 4.6 mm i.d.; carbon loading, 10.0% *m/m*; equivalent loading of isodecane, 11.8% *m/m*); values of V_m were determined by weighing

	V_m/ml
Solvent mixture—	
CH_3OH - CCl_4	2.80
CH_3CN - $CHCl_3$	2.79
CH_3OH - $CHCl_3$	2.77
Mean	2.79
Calculated volumes—	
V_{Si}/ml	0.96
V_s/ml	0.40
V_c/ml	4.15

Table 2. Retention volumes of different solutes; V_m* is calculated according to Martire and Boehm[12]; data taken for methanol concentrations below 20% *V/V*. Concentrations below this value are not included, as over this concentration range methanol is being adsorbed on to the surface of the stationary phase[10]

	Retention volume/ml						
Methanol, % *V/V*	"Silica smoke"	Sodium nitroprusside	Sodium nitrate	Sodium sulphite	CD_3OD	D_2O	V_m*/ml
0	1.41	1.62	1.84	1.87	—	2.76	2.76
1	—	—	—	—	—	—	—
20	1.41	1.62	1.81	1.87	2.76	2.78	2.78
30	—	—	—	—	—	—	—
40	1.42	1.61	1.80	1.82	2.78	2.73	2.76
50	1.41	—	1.94	1.90	2.79	2.70	2.75
60	—	—	—	—	—	—	—
70	1.40	1.65	1.84	1.82	2.77	2.81	2.80
80	1.42	—	1.92	1.80	2.62	2.82	2.78
90	—	1.66	—	—	—	—	—
100	—	1.65	1.83	1.86	2.75	2.76	2.75
Mean . .	1.41	1.64	1.85	1.85	2.75	2.77	2.77

Solute	Methanol, % *V/V*	Retention volume/ml
Sodium thiosulphate . .	50	1.83
Potassium dichromate . .	50	1.62

Determination of $\mathrm{V}_{i(m)}$

To measure selectively the moving portion of the interstitial volume only, a "solute" must be chosen that has a very low diffusivity such that it does not have time to diffuse into the static portions of the interstitial volume during passage through the column. Further, it must be of such a size that it cannot enter any of the pores. The "solute" chosen was "silica smoke," a material condensed from silica vapour and having a particle diameter of <0.02 µm. Such grains were sufficiently small to allow their passage between the particles of the packing material, but, at the same time, were large enough to exhibit very low diffusivity in the mobile phase. Consequently, the silica smoke could not enter the static portions of the interstitial volume. The silica was glassy in nature and had a very low specific surface area and hence did not exhibit any significant retentive properties. The solute was detected by a refractive index detector. In order to ensure adequate dispersion of the silica smoke for sample injection, the material was dispersed in a sample of the mobile phase by ultrasonication for 5 h. This extensive dispersion procedure was found to be necessary to eliminate the natural agglomeration of the particles that would prevent their passage between the interstices of the support. The retention volume of the silica smoke gave a direct "value" of the moving portion of the interstitial volume. It could be argued that the silica smoke could either diffuse slightly into the static regions of the interstitial volume or could not explore all the moving phase due to its size. Both these arguments could be valid but the error involved in either is likely to be small. Diffusion into the static portions of the interstitial volume would probably produce peak asymmetry or increased peak dispersion and would not affect the position of the peak maximum of the silica smoke. The volume (if any) not penetrated by the silica smoke (200 Å in diameter) would be a very small fraction of the volume of the moving phase and would not result in a significant error of measurement. The results obtained, using a range of methanol - water mixtures are shown in Table 2.

Determination of $\mathrm{V}_{i(s)}$

The static portion of the interstitial volume was determined as the difference between the total interstitial volume and the moving portion of the interstitial volume. The total interstitial volume was taken as the retention volume of appropriate salts. As salts are excluded from the pores by the Donnan potential,[3] their retention volumes should be equivalent to the interstitial volume. To determine whether the interstitial volume exhibited exclusion properties, five salts were used that differed in both ionic charge and in ionic size: sodium nitrate, sodium sulphite, sodium thiosulphate, potassium dichromate and sodium nitroprusside. The results are included in Table 2, together with the retention volumes of deuteriated methanol and deuteriated water. Also included are values of the mobile phase volume, V_m*, calculated using

$$V_m{}^* = y_{(1)}V_{r(1)} + y_{(2)}V_{r(2)} \quad \ldots \quad (19)$$

where $y_{(1)}$ and $y_{(2)}$ are the volume fractions of water and methanol in the mobile phase and $V_{r(1)}$ and $V_{r(2)}$ are the retention volumes of deuteriated water and methanol, respectively. According to the unified theory of Martire and Boehm[12] and the work of Knox and Kaliszan[3] and Ha *et al.*[13] the volume fraction average of the retention volumes of deuteriated methanol and deuteriated water should give the total volume of the mobile phase as determined by the weighing procedure. The volume of the mobile phase in the column obtained in this way was indeed close to that obtained by the weighing method, provided that the concentration of methanol was above that required to complete the surface coverage, *i.e.*, the flat portion of the adsorption isotherm had been reached.

Table 3. Measurement of pore volume

Alkane carbon number	Retention volume/ml	
	At 30 °C	At 50 °C
6	2.75	—
7	2.75	—
9	2.74	2.69
11	2.63	2.67
14	2.58	2.63
16	2.55	2.57
20	2.51	2.52
24	2.44	2.44
28	2.40	—
32	2.36	2.37
36	2.32	2.33

Measurement of Pore Volume

The chromatographic volume of the mobile phase ($V_{m'}$) may or may not be the same as the total volume of the mobile phase in the column, V_m, depending on the extent of solute exclusion that takes place. The larger the solute molecule the larger the difference between V_m and $V_{m'}$ might be. Hence an alternative method for measuring V_m must be used based on a chromatographic procedure. The column was operated with a mobile phase of octane, a solvent having the same chain length as that of the bonded phase. In order to confirm that pore exclusion did, indeed, take place, the retention volume of a number of n-alkanes ranging from hexane to hexatriacontane was measured and the results obtained are shown in Table 3. These results are also shown in Fig. 1 relating retention volume to the number of carbon atoms in the alkane.

Determination of that Fraction of the Pore Volume Containing Solvent of the Same Composition as the Mobile Phase and that Fraction Containing One Component of the Mobile Phase Only

A bonded phase, situated in an aqueous organic solvent environment adsorbs a certain amount of the organic component into or on to the surface of the stationary phase.[8–10]

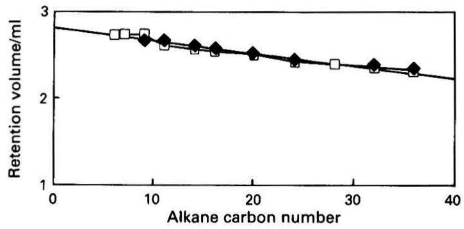

Fig. 1. Retention volume *versus* alkane carbon number; $y = 2.8238 - 0.0149x$; $r = 0.98$. Temperature: □, 30; and ◆, 50 °C

Consequently, the average concentration of the organic component in the total volume of the mobile phase in the column will be greater than that in the original solvent mixture. The volume of methanol adsorbed from 20% *V/V* methanol - water was determined in the following manner. The column was equilibrated with the solvent by passing 200 ml of a mobile phase containing 20% *V/V* methanol and 80% *V/V* water through it. The contents of the column were then stripped with 20 ml of acetonitrile into a 25-ml calibrated flask. A small amount of propan-1-ol was then added as an internal standard and the flask was made up to the mark with acetonitrile. The amount of methanol in the flask was determined by gas chromatography (GC). The concentration of methanol was calculated from the average of three determinations and found to be 0.022 g ml^{-1}.

If the concentration of methanol in the acetonitrile solution is given by c and the density of methanol given by d_{Me} then the volume of methanol in the column (V_{Me}) is given by

$$V_{\mathrm{Me}} = 25c/d_{\mathrm{Me}} \quad \ldots \quad (20)$$

Now

$$V_{\mathrm{Me}} = dV + (V_{\mathrm{m}} - dV)x \quad \ldots \quad (21)$$

where dV is the volume of methanol adsorbed and x is the volume fraction of methanol in the solvent mixture. Consequently,

$$V_{\mathrm{p(2)}} = dV = (V_{\mathrm{Me}} - V_{\mathrm{m}}x)/(1 - x) \quad \ldots \quad (22)$$

and

$$V_{\mathrm{p(1)}} = V_{\mathrm{p}} - V_{\mathrm{p(2)}} \quad \ldots \quad (23)$$

In this manner the volume of methanol adsorbed on the reversed phase was found to be 0.18 ml. To be precise, this volume is an excess of methanol in the pores above that of the mobile phase proper, but is usually attributed to a layer adsorbed on the surface of the stationary phase as demonstrated previously.[10] This assumption is justified further by the fact that 0.18 ml of methanol is stoicheiometrically close to a monomolecular layer of methanol over the surface of the stationary phase.

Measurement of the Volume of the Chromatographically Available Stationary Phase

The chromatographically available stationary phase must also be determined by a chromatographic procedure. The method described here will not take into account exclusion effects and so to minimise the errors involved a solute is chosen that is relatively small in size.

Equation (13) assumes that the system is liquid - liquid in nature. However $\beta_{\mathrm{N}}K_3V_{\mathrm{s(A)}}$ can be put in the form $\beta_{\mathrm{N}}K_3A_{\mathrm{A}}d_{\mathrm{f}}$ if so desired where A_{A} is the surface area of the available stationary phase in the column, in which instance $V_{\mathrm{s}} = A_{\mathrm{A}}d_{\mathrm{f}}$ and K_3 must be defined in appropriate terms.

Under conditions where there is no exclusion and assuming that $K_3 \gg K_2$ then $\alpha_{\mathrm{N}} = 1$, $K_2 = 1$, $V_{\mathrm{i(m)}} + V_{\mathrm{i(s)}} + V_{\mathrm{p(1)}} + V_{\mathrm{p(2)}} = V_{\mathrm{m}}$ and $\beta_{\mathrm{N}} = 1$.

Hence,

$$V_{\mathrm{r(N)}} = V_{\mathrm{m}} + K_3V_{\mathrm{s(A)}} \quad \ldots \quad (24)$$

Although V_{m} is already known, to determine $V_{\mathrm{s(A)}}$ the value of K_3 must be obtained by a separate experiment. The solute chosen was benzene and the mobile phase was pure water. Benzene has a molecular diameter of *ca.* 5 Å and therefore will still suffer some slight exclusion. However, as the mean diameter of the pore distribution of the parent silica is *ca.* 80 Å the error involved will be very small.

The retention volume of the solute on the Zorbax column was determined as the average of three replicates to be 115 ± 1 ml. The value of K_3 was measured as the distribution coefficient of the solute between the liquid - liquid system consisting of octane and water. The solvent, octane, was chosen to simulate the C_8 chain of the bonded phase. Equal volumes of octane and water (the octane containing 0.1% *m/m* of benzene) were shaken well and left to equilibrate at 30 °C for 2 h. Three replicate samples of the octane and water, after equilibrium, were analysed by GC using an internal standard. To simplify the quantitative analysis, the solute peaks were cut out and weighed to provide a proportional measure of the peak areas. The distribution coefficient of the benzene was calculated using

$$K_3 = M_{\mathrm{o}}/M_{\mathrm{w}} \quad \ldots \quad (25)$$

where M_{o} is the mass of the benzene peak in the octane after equilibrium and M_{w} is the mass of the benzene peak in water after equilibrium. This assumes that the physical nature of the distribution of the solute between the bulk liquids is the same as that between the mobile phase and the bonded surface. This assumption could well be debatable, but, as will be seen later, the data obtained validate this assumption. The distribution coefficient of benzene between octane and water, taken as the average of three replicates, was found to be 229 ± 7. The volume of the chromatographically available stationary phase in the column was calculated using

$$V_{\mathrm{s(A)}} = [V_{\mathrm{r(A)}} - V_{\mathrm{m}}]/K_3 \quad \ldots \quad (26)$$

and found to be 0.49 ml; this is slightly greater than the total volume of the stationary phase as determined gravimetrically (0.40 ml). It would appear that there was little if any stationary phase that was not chromatographically available. The discrepancy between the volumes determined gravimetrically and chromatographically is only 90 µl. Further, the accuracy of the data from which this difference is calculated depends on an accurate knowledge of the packing density, the hydrocarbon content of the packing, the effective density of the hydrocarbon moiety and the determination of the distribution coefficient of benzene between octane and water which takes a value in excess of 200. Moreover, an alternative calculation based on the mass of packing material (*ca.* 3 g), carbon content of the packing (*ca.* 10%) and the effective density of the bonded hydrocarbon moiety (0.726 g ml^{-1}), yields V_{s} = 0.49 ml. The significance of the difference between the two values taken for the volume of stationary phase in the column at this stage must, therefore, be considered with some circumspection.

The experiment also validates the use of the water - octane liquid - liquid system to simulate interactions between water and the bonded octyl reversed phase. The value obtained for the total volume of stationary phase in the reversed-phase column is very close to that determined by other means and this could only occur if the liquid - liquid system closely modelled that of the reversed-phase system.

Discussion

The results shown in Table 1 indicate that over two thirds of the volume of the column is occupied by the mobile phase and

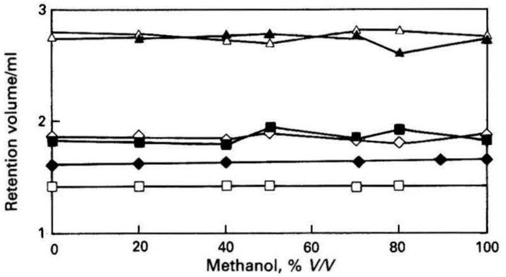

Fig. 2. Retention volumes of different solutes *versus* composition of the solvent. Solute: □, silica smoke; ◆, sodium nitroprusside; ■, sodium nitrate; ◇, sodium sulphite; △, CD_3OD; and ▲, D_2O

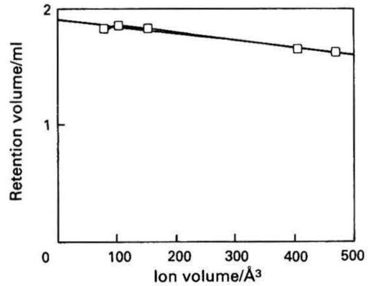

Fig. 3. Retention volume of salt *versus* ion volume; $y = 1.906 - 6.137 \times 10^{-4}x$; $r = 0.99$

less than 10% by the bonded stationary phase. The remaining 23% is taken up by the silica support.

The volume of the interstitial moving phase, given by the retention volume of the "silica smoke," is seen to be *ca.* 50% of the total mobile phase in the column and *ca.* 35% of the total volume of the column. The retention volumes of the different salts are shown as a function of solvent composition in Fig. 2. It can be seen that the largest ion (sodium nitroprusside) gives the smallest retention volume and that sodium nitrate, the smallest ion, gives the largest retention volume. The retention volume of each salt is plotted against its ionic volume in Fig. 3 and it can be seen that the relationship is linear. The lower retention of the larger ions indicates that the interstitial volume also exhibits some exclusion properties. The charge on the ion, be it single or double, has little or no effect on the solute retention. Extrapolation of the linear graph provides an intercept of 1.91 ml for a solute of zero ion volume and is therefore equivalent to the interstitial volume. The retention volume of 1.85 ml obtained with sodium nitrate is close to the extrapolated value and therefore this salt could be used for the measurement of interstitial volume without incurring too great an error. Similarly, the retention volume of sodium nitroprusside is reasonably close to that of "silica smoke" and hence could be used, circumspectly, to measure the kinetic dead volume. Taking the total interstitial volume to be 1.91 ml, then three quarters of the interstitial volume is in fact moving whereas the remainder, probably situated near the points of contact of the particles of the packing material, is essentially static.

The retention volumes of the different alkanes, when using octane as the mobile phase, decrease with the molecular size of the solutes as shown in Fig. 1. This decrease is contrary to that expected from normal retention mechanisms resulting from molecular interactions between hydrocarbons (*e.g.*, the interaction of the normal hydrocarbons with squalane in GC). This is also supported by the fact that temperature has no significant effect on solute retention as the data taken at 50 °C fall on the same straight line as those at 30 °C. Consequently, the exclusion properties of the reversed phase are affirmed and the need to take molecular size into account when measuring thermodynamic dead volumes and corrected retention volumes is demonstrated. If the linear equation given in Fig. 1, obtained by applying a curve fitting procedure to the data, is used to calculate the retention volume of a C_3 organic moiety (the mean carbon number for the solvents employed in the gravimetric measurement of the mobile phase in the column) a value of 2.78 ml is obtained at 30 °C. This value is in excellent agreement with that obtained from the gravimetric measurements (2.79 ml) and the volume fraction average of the deuteriated solvents (2.77 ml). The mean retention volume of deuteriated methanol, 2.75 ml for all solvent mixtures except that of pure water, was also close to the retention volume of a C_3 alkane. The data taken for calculating the mean value for methanol did not include retention values taken between pure water and 20% *V/V* methanol for the reasons given previously. Over this concentration range the methanol is being adsorbed on to the stationary phase as a monolayer and consequently, will be retained significantly.[10]

Table 4. Summary of the physical and chromatographic properties of the Zorbax column

Column length	25 cm
Column radius	2.3 mm
Column packing	Zorbax C_8 reversed phase
Carbon content of reversed phase	10.0% *m/m*
Equivalent isodecane content (2,2-dimethyloctane)	11.8% *m/m*
Total column volume	4.15 ml
Total volume of silica in the column	0.96 ml
Total volume of the stationary phase in the column	0.40 ml
Volume of the chromatographically available stationary phase	0.49 ml
Volume of the chromatographically unavailable stationary phase (by difference)	—*
Total volume of the mobile phase in the column:	
By weighing	2.79 ml
From the retention of the alkanes in octane extrapolated to an alkane carbon number of three	2.78 ml
From the volume fraction average of the isotopes retention volume	2.77 ml
Total interstitial volume; value extrapolated from the retention volumes of ions of different size	1.91 ml
Interstitial moving phase volume (from the retention of "silica smoke")	1.41 ml
Interstitial static phase volume (by difference)	0.50 ml
Total pore volume (by difference)	0.87 ml
Pore volume containing components of the mobile phase of different composition to that of the moving phase (by difference)	0.18 ml
Pore volume containing mobile phase of the same composition as the moving phase (by difference)	0.70 ml

* See text.

Conclusions

The results obtained are summarised in Table 4 and depicted diagramatically in Fig. 4. They indicate that the different physical and chromatographic volumes associated with a liquid chromatography column are far more complex than was originally envisaged.

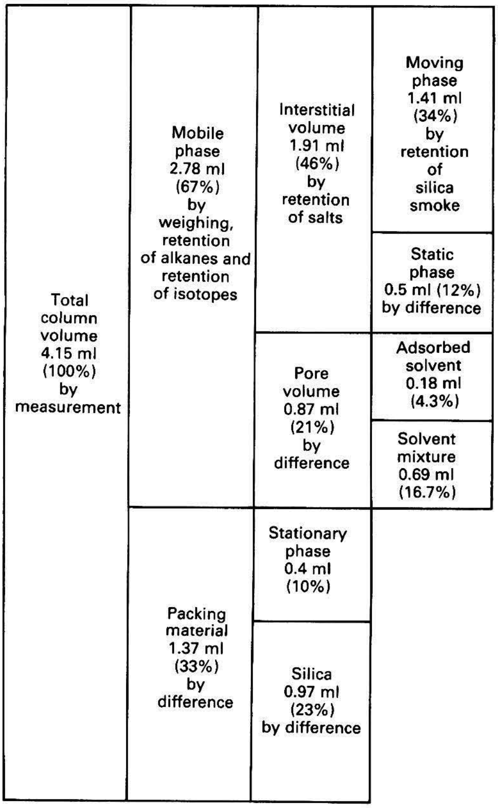

Fig. 4. Contents of a liquid chromatography reversed-phase column; stationary phase volume determined from carbon content and retention measurements, adsorbed solvent determined by extraction and analysis and solvent mixture content of pores determined by difference

The total volume of the mobile phase in a column consists of seven parts. The interstitial volume consists of three components, namely the moving phase, the available static phase and the excluded static phase. The pore volume consists firstly of two components: the chromatographically available and unavailable fractions, each of which can be divided into two further components: those that contain mobile phase of the same composition as the moving phase and those that contain an excess of selectively adsorbed components from the mobile phase.

The volume of the stationary phase can also be composed of three different fractions: that fraction which consists of blocked pores and is therefore completely unavailable, that fraction that is unavailable due to exclusion of the solute and that fraction that is chromatographically available to the solute. In this work the fraction consisting of blocked pores was not identified.

The complex distribution of the different fractions of the phases makes the measurement of retention data for thermodynamic purposes extremely complicated. Although for kinetic studies the kinetic dead volume can be obtained from the retention volume of a single "solute" and be applicable to all solutes, the thermodynamic dead volume will vary from solute to solute depending on the molecular size. Consequently the pertinent dead volume and the chromatographically available volume of the stationary phase appropriate to each solute must be determined before any thermodynamic measurements can be made. This can be extremely difficult in practice and may not, under some circumstances, be possible.

The exclusion properties of the column must also be taken into account when attempting to correlate retention with solvent composition. The use of capacity factors for correlation is likely to lead to serious errors as the values will have the same proportional error as the measurement of the dead volume. Corrected retention volumes would provide more precise data provided that the magnitude of the solute capacity factor was greater than four or five but this would not reduce the error associated with the unknown stationary phase availability. However, for a given solute the available stationary phase will be independent of the solvent composition and hence correlation between solvent composition and solute retention for a single solute would be practical.

The best method for determining the kinetic dead volume is probably from the retention volume of a solute of extremely low diffusivity. The use of "silica smoke" is recommended but care must be taken to ensure that it is well dispersed so that it is eluted through the interstices of the packing material and does not block the column. The use of sodium nitroprusside would give a value close to the volume of the moving phase and would be more practical. However, a method for measuring the thermodynamic dead volume or the volume of the chromatographically available stationary phase for a particular solute, is not, at present, apparent.

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High-performance Liquid Chromatography of Acidic Dyes on a Dynamically Modified Polystyrene - Divinylbenzene Packing Material With Multi-wavelength Detection and Absorbance Ratio Characterisation

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A chromatographic system utilising a dynamically modified 5-μm polystyrene - divinylbenzene packing material for the analysis of acidic dyes under isocratic conditions is described. When used in conjunction with a multi-wavelength UV - visible diode array detector an absorbance ratio technique (absorbance ratioing) provided a method for the characterisation of the 52 acidic dyes chromatographed under these conditions.

Keywords: *High-performance liquid chromatography; polystyrene - divinylbenzene packing material; acidic dye; multi-wavelength detection; absorbance ratio*

Synthetic acidic dyes are used to colour an extensive range of materials including certain fibres, *e.g.*, wool, cotton, viscose and nylon, foods, drinks, cosmetics and water-soluble paints and inks. The ability to analyse and characterise these compounds in a forensic science laboratory is important as they can provide useful evidence.

Acidic dye molecules contain either sulphonic or carboxylic acid functional groups, with the former being the most predominant. It is these anionic sites that present major chromatographic problems, and several modes of separation have been reported in the literature. All of these have been performed with silica-based packing materials and have included anion-exchange,[1–5] reversed-phase[6–8] and ion-pair chromatography on silica[9,10] or modified silica.[8–18]

The samples analysed by these methods were mainly single dyes and related impurities or a restricted number of dyes of one particular class, *i.e.*, food dyes. The majority of these separations were performed under isocratic conditions, with two eluent systems occasionally being used to improve sample identification.

The largest number of dyes that have been analysed using a single isocratic eluent was 33.[10] All of these were food dyes and the results indicated that three of them were not eluted even after 40 min; several dyes co-eluted and the over-all chromatographic efficiency of the system was not ideal. An attempt to improve solute identification by monitoring at several selected wavelengths was only partially successful.

In order to improve chromatographic efficiency and analyse a wider range of acidic dye types, gradient elution systems have been investigated.[16,17] In both studies, spectra of the dyes obtained with multi-wavelength detectors aided sample characterisation, but only limited numbers of these dyes were analysed. Problems with gradient elution were experienced in these investigations, *i.e.*, increased analysis time, irreproducible eluent gradients and hence irreproducible retention time data, and limited selectivity and sensitivity of detection. Concerning this last problem it was shown that, due to the changing background absorbance of the eluent, spectra could only be used for comparison purposes if samples produced absorbances of greater than 50 mA.[17] It is also interesting to note that the narrow-bore column (1.0 mm i.d.) used in this study did not appear to improve either the efficiency or sensitivity of the chromatographic system. As forensic casework sample sizes are typically 1–5 ng and produce absorbances of less than 10 mA it is unlikely that gradient elution techniques would be useful for forensic analysis.

These investigations, together with extensive studies carried out in this laboratory,[19] indicate that the chromatographic separation problems are due to the activity and instability of the silica-based packing materials. Silica is slightly soluble in aqueous eluents and is only stable if employed within a pH range of 2–8. Unfortunately, many dyes are incompletely ionised below pH 8, resulting in poor chromatographic efficiencies.

Recently, there has been considerable interest shown in using macroporous and highly cross-linked polystyrene - divinylbenzene (PSDVB) copolymer packing materials in high-performance liquid chromatography (HPLC). These materials are chemically inert and can be operated over the entire pH range. Reversed-phase systems have been used to analyse the following compounds: carboxylic acids and chlorophenols,[20] sulphur drugs,[20,21] organic acids,[22] alkaloids, nucleosides and nitrogen heterocyclics,[23] peptides and amino acids,[24] drugs in pharmaceutical formulations[25,26] and inorganic anions.[27] The results indicated that a similar method might be applicable to the analysis of acidic dyes, and this paper describes the development of a suitable chromatographic system and the use of absorbance ratioing for characterisation of the analytes.

Experimental

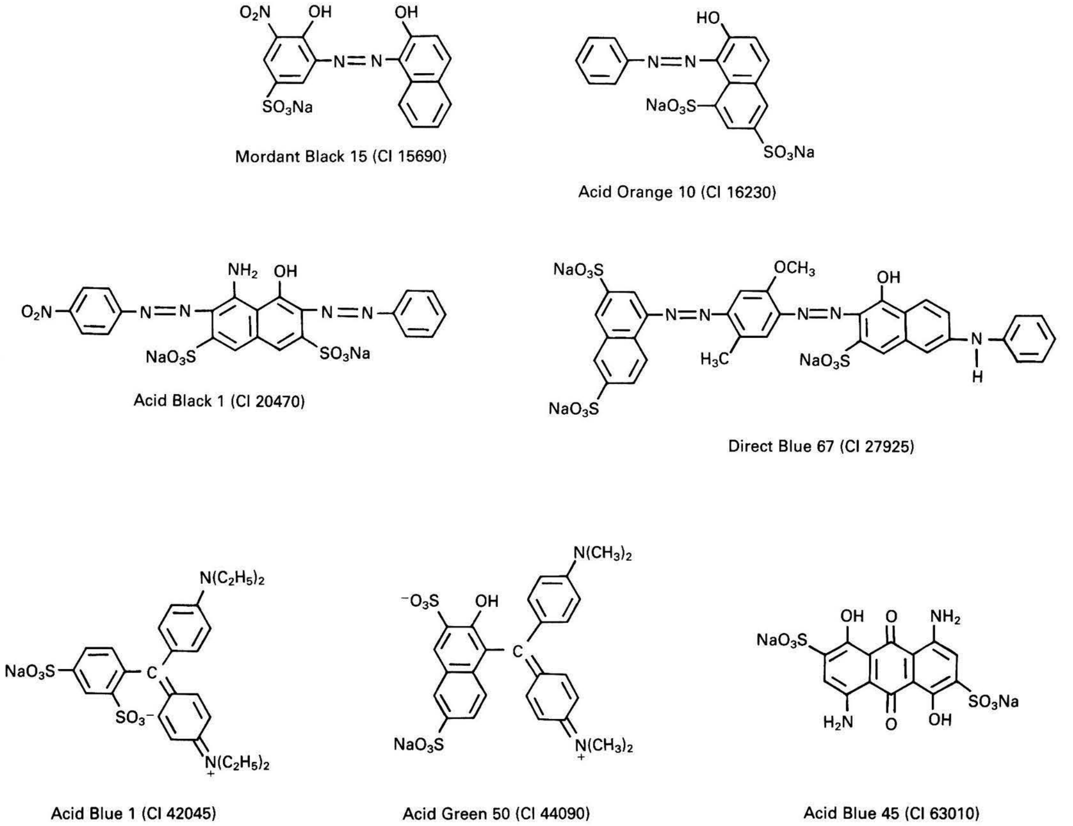
Dye Samples

The dyes investigated are listed in Table 1, together with their Colour Index (CI) number, generic name and chemical grouping. They were dissolved in the HPLC eluent prior to analysis.

During development of the eluent system and assessment of the effect of various parameters, *e.g.*, pH and ionic strength, all the dyes listed in Table 1 were analysed after each change of the eluent conditions. Based on the major dye classes and range of functional groups encountered with these dyes (Fig. 1) seven were selected to indicate the retention trends with changes in the eluent conditions.

Chromatographic Conditions

An Eldex pump (Model B100-S2; Owens Polyscience, Macclesfield, UK) was used to deliver the eluent at 0.7 ml min^{-1} through a 150 × 4.9 mm i.d. stainless-steel tube packed with PLRP-S (Polymer Laboratories, Shrewsbury, UK). The optimised eluent consisted of acetonitrile - water (50 + 50) containing 0.7 g l^{-1} of citric acid and 3.396 g l^{-1} (0.01 M) of tetrabutylammonium hydrogen sulphate (TBAH), and was adjusted to pH 9.0 with concentrated ammonia solution. Samples were introduced on to the column via an injection



Mordant Black 15 (CI 15690)

Acid Orange 10 (CI 16230)

Acid Black 1 (CI 20470)

Direct Blue 67 (CI 27925)

Acid Blue 1 (CI 42045)

Acid Green 50 (CI 44090)

Acid Blue 45 (CI 63010)

Fig. 1. Chemical structure, reference number, generic name and CI number of the seven selected acidic dyes that were examined in detail

valve (Negretti Zambra, Southampton, UK) fitted with a 5-µl loop.

The column was prepared by slurry-packing 3 g of the polymer beads [pre-swollen in acetonitrile - water (30 + 70) and packed with the same solvent] by downward displacement at 6000 lb in^{-2}.

Detection Conditions

Single wavelength detection

Samples were initially monitored with a fixed-wavelength UV-visible detector (Knauer, FRG). The monitoring wavelength in the visible region was selected by using a 400-, 500-, 550- or 600-nm filter, the choice being determined by the colour of the dye solution. The detector sensitivity was in the range 20–190 ma.u.f.s.

Multi-wavelength detection

Samples were also monitored with a linear diode array multi-wavelength UV - visible (190–600 nm) detector (Model HP1040; Hewlett-Packard, Walbrom, FRG). Details of this system and the technique of absorbance ratioing have been described elsewhere.[28]

In order to analyse the dye samples the detector was set to monitor the following eight wavelengths simultaneously: 590, 550, 500, 450, 400, 350, 300 and 250 nm, each with a band width of 20 nm (*i.e.*, ±10 nm). During the chromatographic run one of these was selected as the pilot wavelength and was monitored on the plotter. This wavelength was selected according to the colour of the dye solution as follows: yellow or green, 400; orange, pink or red, 500; blue, 550; and purple, 590 nm.

In order to obtain absorbance ratio data the pilot wavelength was used as the reference wavelength and the ratios were determined using a computer program developed in this laboratory.[28] If, for example, the reference wavelength of 500 nm had been selected for a particular dye, then seven absorbance ratios would be obtained, *e.g.*, A_{500}/A_{590}, A_{500}/A_{550}, A_{500}/A_{450}, A_{500}/A_{400}, A_{500}/A_{350}, A_{500}/A_{300} and A_{500}/A_{250}, where A is the absorbance (mA) as determined by peak-height measurement of the component at the particular wavelength.

Results and Discussion

Optimisation of Chromatographic Conditions

Macroporous polymer packing materials have been used in the reversed-phase mode with and without the use of an ion-pairing reagent in the eluent.[27] Both methods were investigated for the analysis of acidic dyes.

Separation without an ion-pairing reagent

In the study mentioned above, acetonitrile-based eluents provided the highest chromatographic efficiency with a 5-µm polystyrene - divinylbenzene copolymer packing material (Polymer Laboratories: PLRP-S). The same column packing material was used in this investigation and the dyes were chromatographed initially with the following eluent, *viz.*, acetonitrile - water (30 + 70) containing 0.7 g l^{-1} of citric acid, adjusted to pH 10.5 with concentrated ammonia solution. All other conditions were as given under Experimental.

Table 1. Acidic dyes analysed by HPLC on a polymeric packing material

CI No.	CI generic name	Dye structure
13020	Acid Red 2	Monoazo
13025	Acid Orange 52	Monoazo
14720	Acid Red 14	Monoazo
15510	Acid Orange 7	Monoazo
15690	Mordant Black 15	Monoazo
15850 : 1	Pigment Red 57 : 1	Monoazo (Ca salt)
15850 : 2	Pigment Red 57 : 2	Monoazo (Mn salt)
15880 : 1	Pigment Red 63 : 1	Monoazo (Ca salt)
15880 : 2	Pigment Red 63 : 2	Monoazo (Mn salt)
15985	Food Yellow 3	Monoazo
16185	Acid Red 27	Monoazo
16230	Acid Orange 10	Monoazo
16255	Acid Red 18	Monoazo
17200	Acid Red 33	Monoazo
18050	Acid Red 1	Monoazo
18965	Acid Yellow 17	Monoazo
19140	Acid Yellow 23	Monoazo
20285	Food Brown 3	Bisazo
20470	Acid Black 1	Bisazo
24100	Direct Red 7	Bisazo
24140	Direct Blue 8	Bisazo
24410	Direct Blue 1	Bisazo
26905	Acid Red 66	Bisazo
27165	Acid Red 71	Bisazo
27925	Direct Blue 67	Bisazo
27940	Direct Blue 55	Bisazo
28440	Food Black 1	Bisazo
30235	Direct Black 38	Trisazo
34180	Direct Black 74	Trisazo
34215	Direct Blue 81	Trisazo
42045	Acid Blue 1	Triarylmethane
42650	Acid Violet 17	Triarylmethane
42685	Acid Violet 19	Triarylmethane
42700	Direct Blue 41	Triarylmethane
42755	Acid Blue 22	Triarylmethane
44025	Acid Green 16	Triarylmethane
44090	Acid Green 50	Triarylmethane
45170 : 3	Pigment Red 173 : 3	Xanthene (Al salt)
45350	Acid Yellow 73	Xanthene
45380	Acid Red 87	Xanthene
45400	Acid Red 91	Xanthene
45430 : 1	Pigment Red 172	Xanthene (Al salt)
45445	Mordant Violet 25	Xanthene
50090	Acid Red 103	Azine
50315	Acid Blue 59	Azine
51030	Mordant Blue 10	Oxazine
58005	Mordant Red 3	Anthraquinone
60730	Acid Violet 43	Anthraquinone
61211	Reactive Blue 2	Anthraquinone
63010	Acid Blue 45	Anthraquinone
73015	Acid Blue 74	Indigoid
00000*	—	Triarylmethane

* Dye CI No. 00000 is an indicator (Bromocresol Green) and is not listed in the Colour Index.

In order to determine the effect of pH on retention, all dyes were subsequently analysed with eluents buffered at pH values of 1.8, 5.0, 6.5, 8.2, 10.5 and 12.5. The results obtained for the seven selected dyes are presented in Fig. 2.

Of the dyes that were eluted many of these could exist as zwitterions and their retention passed through a minimum. A similar retention effect for zwitterions chromatographed on XAD polymeric packing materials has been reported by Rotsch *et al.*[21]

Based on the chromatographic efficiency observed [*i.e.*, capacity ratio (k') values and peak shape] for all the dyes analysed, a pH of 8.2 was considered to be the most favourable. However, many of the dyes were still either non-retained or not eluted within 25 min under these conditions. Changes in composition or ionic strength of the eluent were unlikely to improve this situation and so the use of an ion-pairing reagent was investigated.

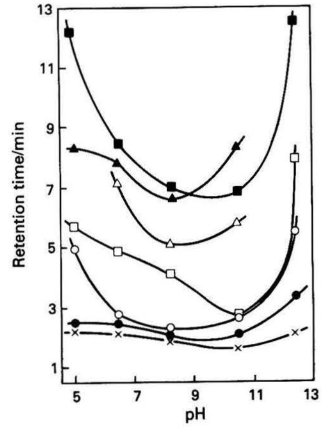


Fig. 2. Effect of pH on retention: ×, CI 63010; ○, CI 16230; ■, CI 20470; □, CI 44090; ▲, CI 42045; ●, CI 15690; and △, CI 27925

Table 2. Effect of eluent composition on retention time

	Retention time/min		
	Acetonitrile : water ratio		
CI No.	30 + 70	40 + 60	50 + 50
63010	NE*	3.70	3.10
20470	NE	NE	8.70
42045	NE	6.70	3.90
16230	NE	7.50	3.90
44090	NE	4.85	3.50
15690	NE	NE	4.70
27925	NE	NE	8.50

* NE = not eluted within 25 min.

Separation with an ion-pairing reagent

The effects of adding a commonly used ion-pairing reagent, TBAH, to the eluent were studied. The eluent conditions for the initial experiment were acetonitrile - water (30 + 70) containing 0.7 g l^{-1} of citric acid and 3.396 g l^{-1} (0.01 M) of TBAH adjusted to pH 8.2 with concentrated ammonia solution.

Following the addition of the ion-pairing reagent to the eluent no dye was eluted within 25 min. The composition, ion-pairing reagent concentration, ionic strength and pH were therefore investigated to reduce the retention times and optimise the separation.

Eluent composition. The non-elution within 25 min on the addition of the ion-pairing reagent indicated that the ion-paired dyes had a very low affinity for the eluent and were therefore strongly retained on the packing material. The organic component (*i.e.*, acetonitrile) of the eluent was therefore increased to enhance the solubility of the ion-paired dyes and thereby reduce their retentions. Acetonitrile - water compositions of 40 + 60 and 50 + 50 were investigated. The eluent composition was as follows: acetonitrile - water (variable) containing 0.7 g l^{-1} of citric acid and 3.396 g l^{-1} (0.01 M) of TBAH adjusted to pH 8.2 with concentrated ammonia solution.

The results obtained for the seven selected dyes are shown in Table 2. As expected an increased acetonitrile content reduced the retention times of the dyes. Based on the retention data obtained for all the dyes analysed, the eluent

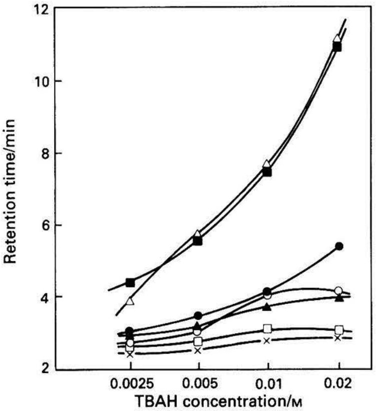


Fig. 3. Effect of ion-pairing reagent concentration on retention. Symbols used to represent the different dyes examined are as shown in Fig. 2

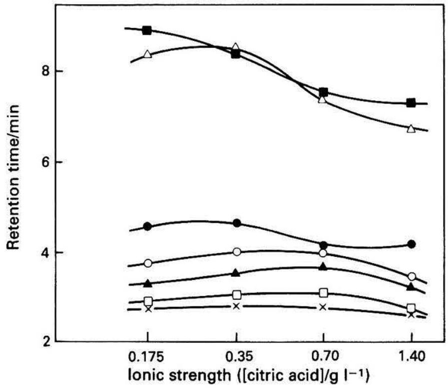


Fig. 4. Effect of ionic strength on retention. Symbols used to represent the different dyes examined are as shown in Fig. 2

containing 50% acetonitrile was considered to be the most favourable as no dye was non-retained and with the exception of one dye they were all eluted within 22 min. Any further increase in the acetonitrile content to reduce the retention of the longer retained dyes would have reduced the retentions of the shortest retained dyes to an unacceptable degree.

Ion-pairing reagent concentration. The effect of the ion-pairing reagent was investigated by using eluents containing 0.0025, 0.005, 0.01 and 0.02 M TBAH. The eluent composition was as follows: acetonitrile - water (50 + 50) containing 0.7 g l^{-1} of citric acid and TBAH (variable), adjusted to pH 8.2 with concentrated ammonia solution.

The results for the seven selected dyes are shown in Fig. 3. These results are typical of a reversed-phase system in that increased ion-pairing reagent concentrations increased the retention times. Retention data from the analysis of all the acidic dyes examined indicated that a concentration of 0.01 M TBAH was the most favourable. Lower concentrations reduced the retention of the longest retained dyes to less than 20 min but reduced the separation of many of the shorter retained dyes.

Ionic strength. The effect of ionic strength (*i.e.*, citric acid concentration) was investigated by using 0.175, 0.350, 0.70 or 1.40 g l^{-1} of citric acid in acetonitrile - water (50 + 50) containing 0.01 M TBAH, and adjusted to pH 8.2 with concentrated ammonia solution.

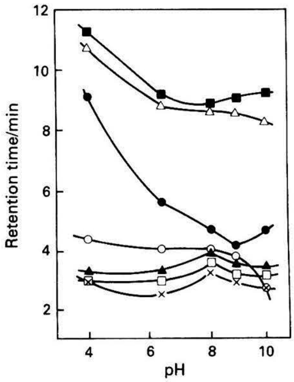


Fig. 5. Effect of pH on retention. Symbols used to represent the different dyes examined are as shown in Fig. 2

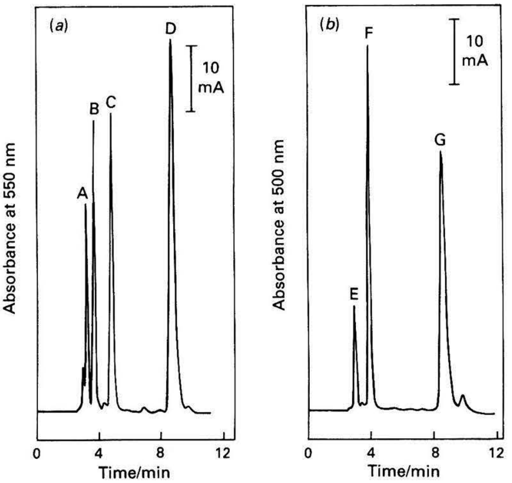


Fig. 6. Chromatograms obtained for the seven selected acidic dyes analysed on the polymeric packing material using the finalised eluent conditions described under Experimental. A, CI 44090; B, CI 42045; C, CI 15690; D, CI 20470; E, CI 63010; F, CI 16230; and G, CI 27925

The results for the selected dyes are shown in Fig. 4. Examination of the retention time data obtained for all the dyes showed that an increase in ionic strength gave an over-all reduction in retention time, a typical finding in reversed-phase ion-pair HPLC. An ionic strength of 0.70 g l^{-1} was considered to be the optimum.

Effect of pH. The dyes were analysed with an eluent buffered to pH 4.0, 6.5, 8.2, 9.0 or 10.0. The eluent composition was acetonitrile - water (50 + 50) containing 0.70 g l^{-1} of citric acid and 0.01 M TBAH, adjusted to the required pH with concentrated ammonia solution. The results obtained for the seven selected dyes are presented in Fig. 5.

Of the parameters investigated the pH of the eluent had the greatest effect on the retentions and chromatographic efficiency. For all the dyes examined the underlying trend observed with the retention time data was that an increase in the basicity of the eluent produced a decrease in dye retention. An eluent buffered to pH 9.0 was considered to be the optimum as all the dyes studied were eluted within 25 min, with acceptable resolution between many of the shorter retained dyes.

Table 3. Retention time and absorbance ratio data for acidic dyes analysed on the polymeric packing material

CI No.	Colour	Retention time/min	Absorbance ratio							
			590 nm	550 nm	500 nm	450 nm	400 nm	350 nm	300 nm	250 nm
42755	Blue (FD*)	2.40	2.40	R†	2.83	—‡	—	—	1.68	—
45445	Magenta (FD)	2.52	4.09	0.51	R	3.36	8.69	5.41	1.29	1.21
45350	Green (FL§)	2.64	—	—	R	4.82	40.82	35.53	7.69	3.04
42685	Pink (FD)	2.67	5.32	0.57	R	7.04	—	—	2.10	1.55
51030	Blue	2.74	0.65	R	1.20	2.98	—	4.52	1.74	0.99
42700	Blue (FD)	2.77	1.31	R	2.21	—	—	—	1.99	1.63
63010	Blue	2.80	0.55	R	3.41	9.80	20.62	10.06	1.38	0.34
19140	Green (FD)	2.82	—	—	—	0.96	R	3.57	—	0.98
15985	Yellow	2.96	—	20.51	R	1.37	2.17	5.38	2.12	0.96
44090	Turquoise	3.03	0.31	R	3.13	2.67	1.71	2.24	0.78	0.32
73015	Blue	3.03	0.46	R	4.37	5.38	8.52	0.93	0.31	0.41
14720	Pink - red	3.09	5.77	1.11	R	2.42	4.21	4.05	2.22	0.72
17200	Magenta	3.13	7.46	1.35	R	2.30	5.80	2.14	1.47	1.78
44025	Dark green	3.17	0.03	0.12	R	0.11	0.14	0.23	0.07	0.06
58005	Magenta	3.21	1.59	0.92	R	2.46	3.69	0.62	0.86	0.24
16185	Pink - red	3.23	5.94	1.10	R	2.42	4.24	4.09	2.22	0.72
50090	Magenta	3.31	2.98	1.06	R	2.49	3.56	0.67	0.89	0.56
27940	Green	3.35	—	—	—	1.91	R	1.79	4.46	1.80
42045	Turquoise	3.40	0.26	R	—	—	0.94	4.02	0.23	1.56
13020	Yellow	3.40	—	—	R	0.19	0.16	0.67	0.81	0.37
16230	Yellow	3.46	—	48.32	R	1.43	2.64	3.77	2.69	0.78
16255	Pink - orange	3.50	29.69	1.70	R	2.41	4.74	3.43	2.48	0.85
18050	Pink	3.55	—	1.27	R	3.77	5.28	3.66	2.25	1.12
28440	Purple	3.88	0.84	R	2.78	5.71	3.22	3.48	1.97	1.19
15850 : 2	Yellow - orange	4.00	—	6.56	R	1.49	2.31	6.47	3.19	0.94
15850 : 1	Yellow - orange	4.02	—	6.45	R	1.49	2.32	6.68	3.18	0.95
15690	Purple	4.41	0.94	R	2.49	6.13	6.04	8.31	2.77	1.00
45380	Pink (FL)	4.49	—	4.05	R	9.67	23.83	9.46	3.26	1.30
42650	Purple	4.77	0.72	R	6.04	—	—	—	4.12	5.54
34180	Blue	4.78	0.67	R	1.82	1.50	1.94	2.13	0.76	0.52
45400	Pink (FL)	5.30	—	1.08	R	6.60	6.30	6.11	2.23	1.53
13025	Yellow	5.41	—	—	R	0.39	0.56	3.95	1.75	1.31
15880 : 1	Yellow - orange	5.50	—	5.74	R	1.65	3.02	7.72	2.57	0.92
15880 : 2	Yellow - orange	5.51	—	5.76	R	1.65	3.01	7.50	2.57	0.92
15510	Yellow	5.74	—	—	R	1.41	2.24	6.54	2.49	1.46
24410	Blue	5.81	0.53	R	2.79	5.75	5.48	3.30	2.00	1.41
00000	Blue	6.38	0.40	R	3.68	11.27	1.83	3.68	1.26	1.68
24100	Pink - orange	6.85	1.20	1.89	R	1.89	3.61	1.86	2.20	1.59
26905	Pink - orange	6.85	—	1.98	R	2.34	3.54	2.39	3.49	2.61
45430 : 1	Pink	7.18	—	0.92	R	11.06	—	6.65	2.98	1.04
45170 : 3	Pink	7.67	—	0.21	R	—	—	3.02	1.45	0.82
27925	Blue	7.73	0.72	R	2.63	6.21	7.62	7.13	2.05	2.18
20470	Blue - grey	7.84	0.56	R	2.67	2.75	2.55	1.99	1.61	1.58
20285	Orange - brown	8.00	—	2.63	R	0.98	1.97	3.95	4.20	2.04
27165	Pink - orange	8.83	—	2.05	R	2.57	3.67	2.20	3.94	1.41
61211	Blue	9.89	0.57	R	3.85	10.26	1.45	0.78	0.23	0.19
34215	Blue	10.25	0.63	R	2.21	3.97	5.22	3.98	1.04	1.14
50315	Purple	12.55	0.71	R	3.82	—	—	2.74	1.46	1.47
24140	Purple	12.95	0.81	R	2.60	6.60	5.27	—	2.79	2.74
60730	Purple	13.14	0.93	R	2.40	—	8.33	1.82	1.06	0.36
27940	Purple	17.42	0.84	R	2.30	6.47	8.70	6.57	2.48	1.81
30235	Blue	20.55	0.62	R	1.17	1.04	1.47	1.91	1.91	1.44

* FD = colour of solution fades on standing.
† R = reference wavelength.
‡ — = absorbance below 1.0 mA threshold.
§ FL = solution fluoresces.

Finalised HPLC Conditions

From these investigations the optimum eluent conditions for the chromatography of the acidic dyes on the PSDVB packing material were found to be as follows: acetonitrile - water (50 + 50) containing 0.7 g l^{-1} of citric acid and 3.396 g l^{-1} (0.01 M) TBAH adjusted to pH 9.0 with concentrated ammonia solution.

The chromatograms obtained from the analysis of the seven selected dyes using these HPLC conditions are shown in Fig. 6.

Characterisation of Acidic Dyes

Traditionally, with single wavelength HPLC detection, qualitative analysis of a solute is based on the measurement of its retention time. However, with a large group of compounds such as these acidic dyes it can be observed from the retention data presented in Table 3 that the discrimination between many of the dyes is poor if this method is used. From the results that are presented it can be seen that even with the standard dye samples some dyes with the same colour exhibit very similar retention times, *e.g.*, CI Nos. 45445 and 42685, and 51030, 42700 and 63010.

Previous work carried out in this laboratory has shown that it is possible to achieve a much higher degree of sample discrimination using relative retention times and absorbance ratio data.[28–32] This technique is called absorbance ratioing and to obtain the absorbance ratio data the sample must be monitored with a detector that is capable of monitoring several wavelengths simultaneously, *i.e.*, a multi-wavelength detector.

All the dye samples were analysed and monitored with a multi-wavelength detection system as described under Experimental. The retention times and absorbance ratios obtained for the dyes are shown in Table 3.

For the purposes of solute discrimination, if the absorbances are greater than 1.0 mA, absorbance ratios are compared on the basis of a 2% relative standard deviation. A window of $\pm 2\sigma$ is applied to each pair of ratios. If for a pair of dyes the subsequent ranges overlap for all the ratios, it is considered that discrimination is impossible.[28] Using this approach, inspection of the results in Table 3 shows that apart from different salts of the same dyes (*i.e.*, CI Nos. 15850:1 and 15850:2, and 15880:1 and 15880:2), discrimination can be achieved with the absorbance ratio data in all instances, even where the samples display similar retention times and colours.

Blind Trial of Acidic Dyes

Ten dye samples selected from the reference collection were analysed in a blind trial. Nine samples contained a single dye and one sample contained two dyes. Their retention times and absorbance ratios were compared with those shown in Table 3 and all the dyes were identified correctly.

Conclusions

The PSDVB packing material, dynamically modified with TBAH, provided a stable and efficient isocratic chromatographic system for the analysis of the 52 acidic dyes studied.

The technique of absorbance ratioing provided an excellent method for discriminating between all the dye samples analysed.

Further work is in progress to assess the application of this method to acidic dye samples encountered in casework and to correlate the retention properties of this packing material with the chemical structures of the acidic dyes.

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Separation of Cationic Surfactant Homologues by High-performance Liquid Chromatography

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Reversed-phase ion-pair high-performance liquid chromatography on a polystyrene - divinylbenzene analytical column was applied to the qualitative analysis of cationic surfactants. Fingerprint chromatograms of industrial fatty amines and fatty quaternary ammonium salts permitted identification of the homologues present.

Keywords: *Reversed-phase high-performance liquid chromatography; fatty amine; fatty quaternary ammonium salt; cationic surfactant analysis*

Both normal-phase ion-pair high-performance liquid chromatography (HPLC)[1–4] and reversed-phase HPLC[1,5–7] have been used for the analysis of cationic surfactants (*viz.*, fatty amines and fatty quaternary ammonium salts) with some success. Complications tend to exist in all these methods because of interactions of the quaternary nitrogen in the analytes with unprotected silanol groups present on the silica and chemically modified silica stationary phases. The consequence of these interactions is that long solute retention and severe peak tailing occur. Further difficulty arises because of the lack of UV absorbance in the majority of the analytes, and unless UV absorbing ions are present in the mobile phase,[5–9] some alternative, less simplistic detection strategies are required.[10,11]

MacDonald[12] recommended that the concept of indirect photometric detection IEC[9] ("ion-exchange chromatography") could be applied to the separation of cationic surfactants. This system uses benzyltrimethylammonium chloride or benzylhexadecyldimethylammonium chloride in the mobile phase to act as both counter ion and UV-absorber for a Partisil SCX cation-exchange system. Our experience showed that there was a flaw in this system arising from column conditioning. The column requires gradual conditioning with analyte amines to mask the silica backbone before analytes can be eluted, and dynamic conditioning continues in use. We have observed this effect with other silica ion-exchange columns.

This paper describes an alternative method for the qualitative analysis of some common industrial cationic surfactants used in domestic products. The method uses polystyrene - divinylbenzene reversed-phase columns for the ion-pair separation of cationic surfactants.

Experimental

Reagents

HPLC grade acetonitrile was obtained from Rathburn Chemicals.

All standard reagents and chemicals were obtained in the highest available purity from the following suppliers: Aldrich, BDH, Fisons and Lancaster Synthesis.

Apparatus

The HPLC system consisted of a Philips PU 4015 solvent delivery pump, a Rheodyne 7125 injection valve with a 20-μl loop, a Philips PU 4025 UV detector (λ = 262 nm) and a Polymer Laboratories PLRP-S HPLC column, 250 × 4.7 mm i.d. (containing 5-μm polystyrene - divinylbenzene packing). The apparatus was operated isocratically with an eluent flow-rate of 1.0 cm³ min⁻¹.

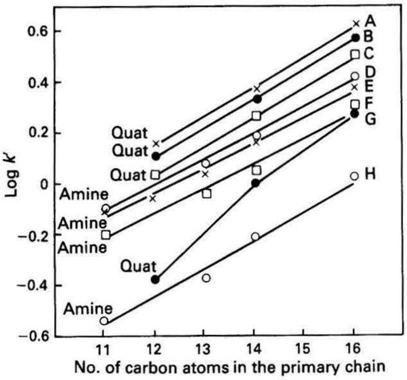

Fig. 1. Graph of the logarithm of the phase capacity ratio (k') *versus* number of carbon atoms in the solute chain, showing the variation of the PLRP-S system with different concentrations of pairing ion and sodium *p*-xylenesulphonate (SXS). Mobile phase, acetonitrile - water - acetic acid (60 + 38 + 2) containing: A, 5×10^{-3}; B, 2×10^{-3}; C, 1×10^{-3}; D, 5×10^{-3}; E, 2×10^{-3}; F, 1×10^{-3}; G, 5×10^{-4}; and H, 5×10^{-4} M SXS. Quat = quaternary ammonium salts; amine = amines

Procedure

All samples were prepared as a 0.2% *m*/*V* solution in the eluent.

Results and Discussion

Sodium *p*-xylenesulphonate (SXS) was chosen as the counter ion. Consequently, the background absorbance was high.

The effect of changing the mobile phase composition on the separation of the test solutes was examined for this system. Three parameters were varied, these being the concentration of the pairing ion, the concentration of the organic modifier and the addition of glacial acetic acid to the system.

Varying the pairing-ion concentration in the mobile phase had a considerable impact on the chromatography. Concentrations of sodium *p*-xylenesulphonate from 5.0×10^{-4} to 5.0×10^{-3} M were studied. Fig. 1 presents the recorded data as a

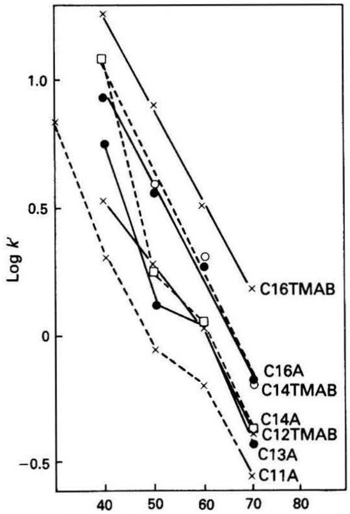

Fig. 2. Graph of the logarithm of the phase capacity ratio (k') *versus* organic modifier concentration, illustrating the subsequent variation of solute retention. C11A = undecylamine; C13A - tridecylamine; C14A = tetradecylamine; C16A - hexadecylamine; C12TMAB = dodecyltrimethylammonium bromide; C14TMAB = tetradecyltrimethylammonium bromide; and C16TMAB = hexadecyltrimethylammonium bromide

Table 1. Resolution of cationic surfactant solute peaks. [SXS] = 5.0×10^{-3} M. C11A = undecylamine; C13A = tridecylamine; C14A = tetradecylamine; C16A = hexadecylamine; C12TMAB = dodecyltrimethylammonium bromide; C14TMAB = tetradecyltrimethylammonium bromide; and C16TMAB = hexadecyltrimethylammonium bromide

	Concentration of modifier in mobile phase, % V/V			
	70	60	50	40
Solute pair	R_s	R_s	R_s	R_s
C12TMAB - C14TMAB	0.55	0.60	0.88	0.96
C14TMAB - C16TMAB	0.70	2.13	0.99	—
C11A - C13A	0.53	0.99	0.60	1.04
C13A - C14A	0.31	0.57	0.39	0.84
C14A - C16A	0.82	1.35	0.83	—
C11A - C12TMAB . .	0.59	1.55	1.08	0.56
C13A - C12TMAB . .	0.20	0.49	0.54	0.57
C14A - C12TMAB . .	0.05	0.11	0.13	1.39
C16A - C14TMAB . .	0.03	0.24	0.04	—
C16A - C16TMAB . .	0.79	1.48	0.91	—

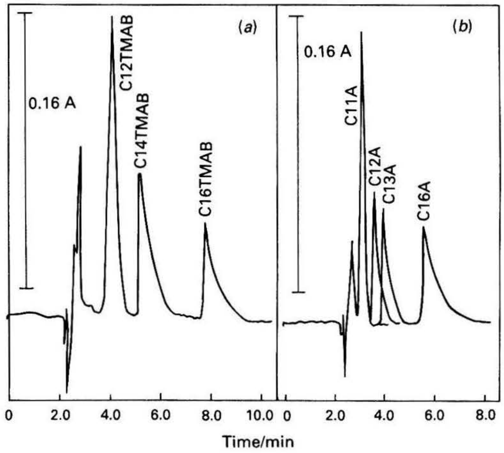

Fig. 3. Chromatograms of standard cationic surfactants obtained with the reversed-phase ion-pair HPLC system. Abbreviations as in Fig. 2

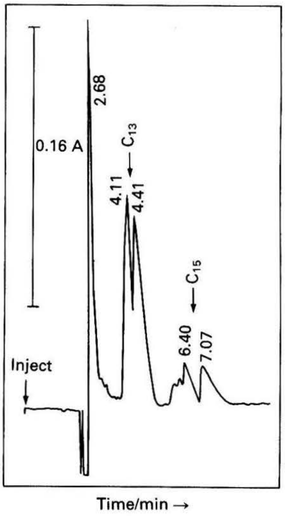

Fig. 4. Chromatogram of an industrial C$_{13}$/C$_{15}$ quaternary ammonium chloride recorded on the reversed-phase ion-pair HPLC system

plot of the logarithm of the phase capacity ratio (k') *versus* the number of carbon atoms in the primary chain; 5.0×10^{-3} M sodium *p*-xylenesulphonate was preferred for the pairing-ion concentration, as this gave the best resolution.

The modifier concentration was adjusted from 70 to 30% *V/V* acetonitrile : mobile phase. The variation of the phase capacity ratio with the modifier concentration is presented in Fig. 2. It has been demonstrated that for octadecylsilica stationary phases with high concentrations of organic modifier in water - modifier mobile phases, a layer of modifier will form on top of the stationary phase and produce a significant variation in the chromatography of such a system.[13,14] Similar behaviour was observed for the polystyrene - divinylbenzene stationary phase employed in this system. The resolution, R_s, between the pairs of adjacent homologues appeared to be greatest at a 60% *V/V* acetonitrile concentration. This was confirmed from the calculation of R_s[15] for different pairs of solutes, displayed in Table 1. (This decision is subjective according to the solute pairs of interest.)

$$R_s = \frac{t_B - t_A}{1/2(Wt_B - Wt_A)}$$

where t_A is the retention time of source A (min), t_B is the retention time of solute B (min), Wt_A is the peak width at the base line for solute A (min) and Wt_B is the peak width at the base line for solute B (min). Peak widths were estimated by extrapolating the tangents on the peak shoulders to the baseline. If R_s = 1, then the peaks are reasonably well resolved.

The addition of acetic acid to the mobile phase was also shown to be essential to achieve a reasonable separation. If the mobile phase contained no acetic acid, then infinite retention of the solutes was seen. This was presumed to be due to either incomplete protonation of the solutes or competition for ion pairs by the acetate ion. The addition of acetic acid to

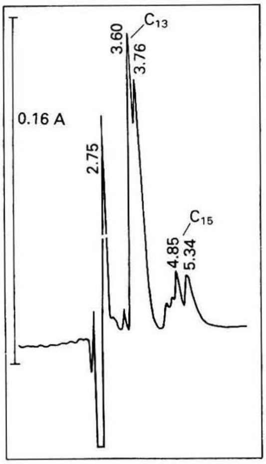

Fig. 5. Chromatogram of an industrial C_{13}/C_{15} alkylamine recorded on the reversed-phase ion-pair HPLC system

the mobile phase was essential and 2% *V/V* was shown to be the optimum. Therefore, it is recommended that for the separation of the solutes a mobile phase of acetonitrile - water - acetic acid (60 + 38 + 2) containing 5.0×10^{-3} M sodium *p*-xylenesulphonate is used. Peak tailing was very pronounced for this system. Chromatograms of the standard cationic surfactants are presented in Fig. 3.

Commercial surfactants tend to be complex and difficult to fingerprint. Two representative samples, an alkylamine and a quaternary ammonium salt, were injected into the system. The quaternary salt consisted of $(C_{13}H_{27})(CH_3)_3NCl$ and $(C_{15}H_{31})(CH_3)_3NCl$. The alkylamine consisted of $C_{13}H_{27}NH_2$ and $C_{15}H_{31}NH_2$. Each sample contained two respective homologues in a characteristic proportion, with a characteristic mixture of linear and branched chain material. The chromatograms obtained for each solute are presented in Figs. 4 and 5. The C_{13} and C_{15} components of each homologue are well separated for each sample. The linear and branched chain isomers are not well resolved; however, the resolution is sufficient to ascertain that the component types are present. Therefore a very characteristic fingerprint is obtained. The proportions of the homologues, $C_{13}:C_{15}$, are correctly recorded.

The analytical column was very stable and the chromatograms were highly reproducible over 3 months of regular used.

Conclusions

This paper describes a reversed-phase ion-pair HPLC method suitable for the qualitative analysis of certain industrial cationic surfactants. A drawback of this system is that should quaternary ammonium salts and amines be present together, insufficient resolution is likely to prevent qualitative analysis. The use of a polystyrene - divinylbenzene column has overcome the drawbacks associated with silica-based stationary phases interacting with cationic solutes. However, the polystyrene-based stationary phase system does not offer high quality peak shapes. Further work is needed in this field to allow better chromatography of large organic cationic molecules.

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High-performance Liquid Chromatographic Technique for the Determination of the Polyether Antibiotic Lysocellin Sodium*

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A high-performance liquid chromatographic technique is described for the polyether antibiotic lysocellin sodium. The technique was developed because the methods currently used for polyether antibiotics are not applicable to lysocellin sodium. This is because lysocellin sodium either lacks a chromophore, does not react with vanillin or lacks the stability needed for derivatisation. The proposed technique involves the use of a UV-absorbing ion pair and is rapid and specific for lysocellin sodium. A method for the determination of lysocellin sodium in fermentation broth which uses the UV-absorbing ion-pair technique is discussed.

Keywords: *Lysocellin sodium; high-performance liquid chromatography; ion-pair chromatography*

Lysocellin sodium, hereafter referred to as lysocellin, is a polyether antibiotic that is currently being examined for its potential in improving feed efficiency and mass gain in cattle. The structure of the compound is shown in Fig. 1. For a product of this type a wide variety of analyses are required in several matrices. Typical matrices include fermentation broths, pre-mixes, feeds and environmental samples.

As with most antibiotics the method first developed for the determination of lysocellin was a microbiological agar diffusion assay. This method was soon replaced by another microbiological method, a turbidimetric assay; the turbidimetric method was eventually automated. Even with automation this procedure, including extraction, sample preparation and determination of lysocellin, requires 24 h for its completion. Therefore, this assay is incapable of adequately handling large numbers of samples rapidly. In addition, the turbidimetric assay lacks specificity and future applications of the product require that a more specific procedure be developed.

Chromatographic methods would provide the speed and specificity needed to supplement the microbiological assay procedure; however, the detection of lysocellin is the major problem with a chromatographic analysis. Lysocellin does not have a useful chromophore for UV absorbance or fluorescence, it does not react with vanillin as do many polyether antibiotics nor does it lend itself readily to derivatisation because of its acid instability[1] which precludes the use of many common derivatisation procedures. Comparative data shown in Table 1 indicate that the type of methods used with other polyether antibiotics[2–5] would not be applicable to lysocellin. As lysocellin does not behave in the same way as other polyether antibiotics, a high-performance liquid chromatographic (HPLC) technique was developed using a UV-absorbing ion pair which solved the detection problem. This technique may also be used with other polyether antibiotics.

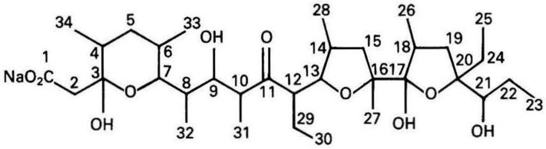

Fig. 1. Structure of lysocellin sodium

Table 1. Methods for the assay of polyether antibiotics

	Assay type			
Polyether	Vanillin[2]	UV - visible[3]	Fluorescence[4]	Derivatisation[5]
Monensin	Yes	No	No	Yes
Lasalocid	Poor	Yes	Yes	Yes
Narasin	Yes	No	No	Yes
Salinomycin	Yes	No	No	Yes
Lysocellin	No	No	No	No

Experimental

Apparatus

The HPLC separations were monitored using either a Varian 5560 chromatograph with a Varian UV 200 variable-wavelength detector or a Waters M-6000 chromatograph with a Waters 481 variable-wavelength detector. The data were collected through a Spectra-Physics 4270 integrator and processed with Spectra-Physics Labnet-XT software.

Reagents and Procedures

All reagents and chemicals used were of analytical-reagent grade except for the polyethers and HPLC solvents; the latter were of HPLC grade. The lysocellin sodium was obtained from Pitman-Moore, the monensin sodium was a gift and the lasalocid sodium was obtained from Aldrich.

The chromatographic conditions and procedures for the HPLC method for the determination of lysocellin in fermentation broth were as follows: column, Zorbax ODS (15 cm × 4.6 mm i.d.); solvent, methanol - water (80 + 20) containing 80 mg l^{-1} of cetylpyridinium chloride (CPC) and 14 mg l^{-1} of NaH_2PO_4; flow-rate, 1.5 ml min^{-1}; temperature 35 °C (column only); detection, UV at 259 nm; and injection volume, 20 µl.

Sample preparation

Samples were dissolved in ethanol and filtered through 0.2-µm Acrodisc CR filters before injection.

Standard preparation

The standard was prepared at a concentration of 0.5 mg ml^{-1} in ethanol and filtered through 0.2-µm Acrodisc CR filters before injection.

Results and Discussion

The UV-absorbing ion-pair reagent allowed the lysocellin to be detected easily, thereby eliminating the difficulties of

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Table 2. Precision and accuracy of the determination of lysocellin in fermentation broth

Precision*		Accuracy			
Sample No.	Peak area, arbitrary units	Sample No.	Actual concentration/ mg ml^{-1}	Concentration found/ mg ml^{-1}	Recovery, %
1	8.818	1	0.50	0.54	108
2	9.292	2	0.50	0.46	92
3	9.413	3	0.50	0.61	122
4	8.940	4	1.00	1.06	106
5	9.563	5	1.00	1.04	104
6	9.518	6	1.00	1.04	104
7	9.398	7	1.50	1.41	94
8	8.184	8	1.50	1.41	94
9	9.310	9	1.50	1.56	104
10	9.159				
Mean:	9.160			Mean:	103%
SD:	0.148			SD:	9.22%
CV†:	4.56%			CV†:	8.95%

* The concentration of the fermentation broth used for determination of the precision was approximately 1 mg ml^{-1}.
† CV, coefficient of variation.

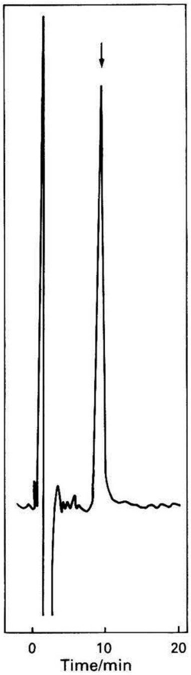

Fig. 2. Lysocellin standard (0.5 mg ml^{-1}) analysed by HPLC; 20-μl injection, 0.04 a.u.f.s.

derivatisation and the problems associated with the lack of a chromophore. In addition to offering selectivity, because the pairing reagent only interacts with acids and acid salts, the technique is rapid in that minimum sample preparation is usually required and a separation can be completed in less than 15 min. Lysocellin has an elution time of approximately 10 min. The limit of detection for the technique is 500 ng on-column. Because of the limit of detection, the technique is usually applied to samples of high concentration.

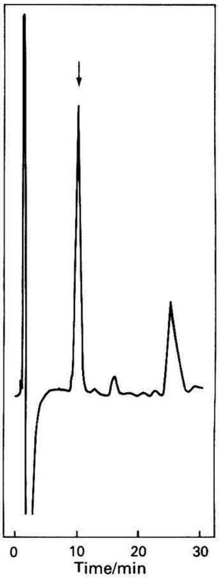

Fig. 3. Lysocellin in diluted (5 + 20 in ethanol) fermentation broth determined by HPLC. Concentration of lysocellin in broth, *ca.* 1 mg ml^{-1}; 20-μl injection; 0.04 a.u.f.s.

Although the technique has been applied to the determination of lysocellin in production streams, the recovery process and feed supplements, its greatest utility is in the assay of fermentation broth. This is because of the high volume of samples and the need for rapid analytical results. Therefore, data and chromatograms obtained using the fermentation broth method will be used to demonstrate the utility of the UV-absorbing ion-pair technique for the determination of polyether antibiotics.

The simple sample preparation and short assay time combine not only to make the fermentation broth method rapid, but also to allow the method to be easily automated. Using only two instruments with autosamplers, one operator can assay a variety of samples, as many as 60 samples prepared in duplicate per day.

An example of a chromatogram obtained for a lysocellin standard using the proposed method is shown in Fig. 2. The lysocellin peak is well formed and elutes sufficiently after the large solvent front caused by the strong UV absorbance of the ion-pair reagent. The chromatography is equally as good in the fermentation broth matrix. Fig. 3 shows a chromatogram of lysocellin in the presence of other components in the fermentation broth.

The few components visible in the fermentation broth is indicative of the selectivity of the method. A fermentation broth contains a wide variety of materials, both those added as nourishment for the micro-organisms and those produced by these organisms. However, as can be seen in Fig. 3, only a few of these materials are visible on the chromatogram and this is because they have an acid or acid salt functionality.

The accuracy and precision data shown in Table 2 are from the validation of the HPLC method for the assay of lysocellin in fermentation broth. Data from this validation provide worst-case precision and accuracy information when using the UV-absorbing ion-pair technique with a polyether both because of the complexity of the matrix and because of the difficulty in sampling the heterogeneous broth. Precision was determined by preparing ten samples of a fermentation broth, then assaying each of the ten preparations. Accuracy was determined from the standard deviation (SD) of the percentage recovery of lysocellin added at three levels to a fermentation broth sample. Samples for ascertaining the accuracy of the method were prepared in triplicate at each level of lysocellin added.

The data in Table 2 show that the method is precise and accurate even at low levels of lysocellin in a very complex matrix. Because of this, the ion-pair technique can be used for most lysocellin assays. Typical samples assayed using this technique were fermentation broth, several process steps which provide a variety of matrices, bulk product and pre-mix. Similar HPLC procedures have been developed for samples such as feed supplements.

It is important to ascertain whether other polyether antibiotics interfere with the determination of lysocellin when the UV-absorbing ion-pair technique is used. It was determined that the retention times of monensin sodium and lasalocid sodium relative to lysocellin were 0.72 and 1.40, respectively. Hence, these two compounds do not interfere with the determination of lysocellin.

In conclusion, the UV-absorbing ion-pair technique provides a method for the determination of lysocellin sodium that is rapid, accurate and selective. Although conditions were not optimised, the technique may also be applicable to other polyether antibiotics, for example, monensin sodium and lasalocid sodium.

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High-performance Liquid Chromatographic Assay of Chondroitin Sulphate in Food Products

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A high-performance liquid chromatographic method for the determination of chondroitin sulphate (ChS) in foods is described based on the detection of two unsaturated disaccharides liberated from ChS by enzymatic digestion with chondroitinase ABC. Chondroitinase ABC hydrolyses ChS to produce two unsaturated disaccharides (6-sulphonated and 4-sulphonated disaccharides), which were retained on an amino-phase column with retention times of 6.2 and 7.3 min, respectively. The peaks resulting from these disaccharides were used to determine the amount of ChS in the sample. The method was applied successfully to the analysis of commercial mayonnaise, dressing and fish sausage.

Keywords: *Chondroitin sulphate in food; chondroitinase ABC; high-performance liquid chromatography*

Chondroitin sulphates (ChSs) belong to a group of related heteropolysaccharides that can be degraded by specific enzymes to produce unsaturated disaccharides. Almost all of the disaccharides produced by the action of chondroitinase ABC on chondroitin 4-sulphate (Ch4S) and chondroitin 6-sulphate (Ch6S) are 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose (ΔDi-6S), respectively.[1] Various structural analyses of ChS from various sources revealed the presence of several types of heterogeneity.[1] In Japan, only Ch4S and Ch6S are employed as emulsifiers, with limits set on their use in certain foods[2]; the permitted levels are usually in the range 0.3–2%. It is therefore necessary to determine the ChS content in foods to ensure that it is within the permitted levels.

The current method of determining ChS in foods is based on spectrophotometry.[3] However, this method requires tedious pre-treatment of the sample and is very prone to interference from other polysaccharides.[3]

An enzymatic method based on the quantitative production of unsaturated disaccharides by digestion of ChS isomers with chondroitinase ABC has been shown to be specific for the determination of ChS isomers. In the field of clinical chemistry, high-performance liquid chromatography (HPLC) has been employed to determine these unsaturated disaccharides.[4–6]

Surprisingly, even with the advent of HPLC, the chromatographic assay of ChS with an enzymatic digestion step has generally been ignored in the area of food chemistry.

Such a method is described here for the determination of ChS in food products. Mayonnaise, dressing and fish sausage samples were studied to demonstrate the feasibility of the proposed method.

Experimental

Apparatus

The chromatographic system consisted of a Model 3A liquid chromatograph (Shimadzu, Kyoto, Japan) with a 20-μl loop injector (Rheodyne Cotati, CA, USA, Model 7125), a UV detector (Shimadzu Model SPD-2A) with an 8-μl flow cell (10 × 1 mm) and a data processor (Shimadzu Model C-R3A). A pre-packed Zorbax NH_2 column (25 cm × 4.6 mm i.d.) was used and separations were performed at ambient temperature (25 °C). Eppendorf micropipettes (100–1000 μl and 1.0–10.0 ml) and a Hamilton microsyringe (25 μl) were employed for the preparation and injection of solutions.

Reagents

The commercially available Ch4S and Ch6S preparations were obtained from Seikagaku Kogyo (Tokyo, Japan). The ΔDi-4S and ΔDi-6S (HPLC grade) and chondroitinase ABC (E. C. 4.2.2.4) were also obtained from Seikagaku Kogyo.

Tris buffer, pH 8.0, 0.4 M. Dissolve 48.4 g of tris(hydroxymethyl)aminomethane (Tris) (Sigma, St. Louis, MO, USA) and 32.8 g of sodium acetate in about 800 ml of water, adjust to pH 8.0 with 1 M hydrochloric acid and dilute to 1 l with water.

Phosphate buffer, pH 5.0, 0.01 M. Dissolve 1.56 g of sodium dihydrogen orthophosphate dihydrate and 3.96 g of ammonium sulphate in about 800 ml of water, adjust to pH 5.0 with orthophosphoric acid and dilute to 1 l with water.

Procedure

Preparation of standard solutions

Standard solutions of Ch4S and Ch6S were prepared by dissolving each in water (1000 μg ml^{-1}). Specified volumes (0.2–5.0 ml) of each standard were pipetted into several 50-ml calibrated flasks and diluted to volume to provide suitable working solutions (4–100 μg ml^{-1}).

Assay of the standard ChS solution

A 2.0-ml volume of working standard solution was added to a test-tube containing 2.0 ml of pH 8.0 Tris buffer. After the addition of 0.1 ml of chondroitinase ABC (2 U ml^{-1}), the mixture was incubated at 30 °C for 30 min in order to digest the ChS. After ultrafiltration through a Centriflow unit (Amicon, Lexington, MA, USA), 10.0 μl of the filtrate were injected on to a column operated at ambient temperature and with a mobile phase of 0.01 M phosphate buffer (pH, 5.0; flow-rate, 1.0 ml min^{-1}). The detector was operated at 235 nm. The areas of the two peaks corresponding to ΔDi-4S and ΔDi-6S were calculated using a data processor.

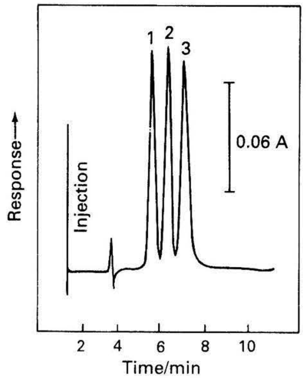

Fig. 1. HPLC trace of authentic standard compounds. 1, ΔDi-6S (50 μg ml^{-1}); 2, ΔDi-4S (50 μg ml^{-1}); and 3, sorbic acid (10 μg ml^{-1}) and benzoic acid (10 μg ml^{-1}). Conditions: column, Zorbax ODS (25 cm × 4.6 mm); flow-rate, 1.0 ml min^{-1}; UV detector, 235 nm; eluent, 0.01 M phosphate buffer (pH 5.0) containing ammonium sulphate (3.96 g l^{-1}); volume injected, 10 μl

Assay of the ChS content in foods

The food sample (10.0 g) was homogenised in an electric blender with 50 ml of water and then the homogenised sample was de-fatted with two 50-ml portions of hexane. The aqueous layer was then made up to 100 ml with water and the resulting mixture was filtered through a filter-paper. Aliquots of this solution (2.0 ml) were then analysed for ChS as described under Assay of the standard ChS solution.

Results and Discussion

Chromatographic Conditions

The principle of this assay method is to measure the amounts of disaccharides liberated from ChS by chromatographic separation. For the determination of ChS isomers in biological fluids, various chromatographic conditions have been proposed.[4-6] We attempted to evaluate the suitability of these methods for the determination of ChS in food products. However, it was found that the separation of ΔDi-4S and ΔDi-6S in food additives such as sorbic acid and/or benzoic acid could not be achieved under these chromatographic conditions. Hence we have evaluated alternative chromatographic conditions using an amino-phase column. When 0.01 M phosphate buffer (pH 5.0) was used as the mobile phase, ΔDi-4S and ΔDi-6S were retained on the column and could not be eluted within 20 min. The effect of ionic strength modifier on the retention behaviour of these disaccharides was then examined. The presence of >0.05 M ammonium sulphate in the mobile phase gave complete resolution of the two disaccharides from sorbic acid although they were not resolved from each other. Accordingly, 0.01 M phosphate buffer (pH 5.0) containing 3.96 g l^{-1} of ammonium sulphate (0.03 M) was adopted as a compromise between good resolution and speed of analysis. Using this mobile phase, ΔDi-4S and ΔDi-6S each gave a single peak. In addition, the two disaccharides were resolved both from each other and from food additives such as sorbic and benzoic acids (Fig. 1).

The choice of detection wavelength is also a critical factor for successful quantification and the most rigorous method would be to calibrate with each disaccharide individually and then to determine each one, as described by Lee *et al.*[6] However, a more convenient procedure utilising the total response of both disaccharides is valid under certain circumstances. Fortunately, ΔDi-4S and ΔDi-6S gave the same response at 235 nm (in terms of peak area) under the proposed chromatographic conditions.

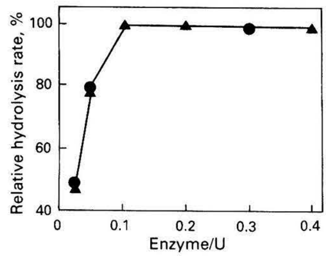

Fig. 2. Effect of chondroitinase ABC activity on the rate of hydrolysis of ChS. Concentrations of Ch4S and Ch6S, 50 μg ml^{-1}. Incubation time, 30 min; temperature, ambient. ●, ΔDi-6S; and ▲, ΔDi-4S

Table 1. Recovery of Ch4S and Ch6S from several foods

Sample	Amount added/mg g^{-1}	Recovery,* % Ch4S	Recovery,* % Ch6S
Mayonnaise	1	94.8	94.6
	10	98.1	97.7
Dressing	1	98.2	98.6
	10	98.8	99.2
Fish sausage	1	97.7	98.1
	3	98.4	98.3

* Results are the means of duplicate determinations.

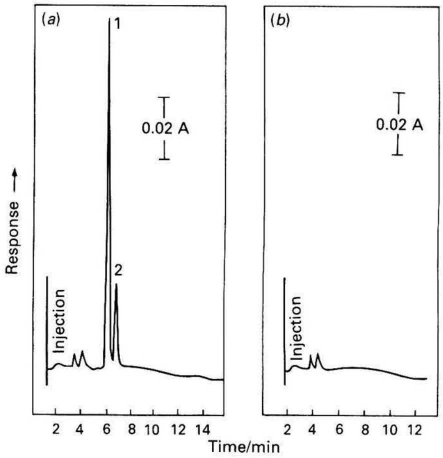

Fig. 3. Typical HPLC traces of (*a*) an extract from mayonnaise containing 1 mg g^{-1} of Ch6S; and (*b*) an extract from blank mayonnaise. 1, ΔDi-6S; and 2, ΔDi-4S. Conditions are as in Fig. 1

Optimisation of the Enzyme Reaction

To achieve maximum sensitivity with the proposed method, several experimental parameters such as the pH for optimum enzyme activity, the reaction time and the enzyme concentration were optimised. Chondroitinase ABC has been shown to achieve its maximum activity at pH 8.0.[7] In the work described here the pH for optimum enzyme activity was determined to be 8.0 using Tris buffer, which agrees well with previously reported results.[7]

With respect to the speed of analysis, the optimum concentration of the enzyme chondroitinase ABC was determined at a fixed reaction time (30 min). As shown in Fig. 2, the rates of hydrolysis of Ch4S and Ch6S increased significantly up to about 0.1 U of enzyme, at which point the

Table 2. Determination of ChS content in commercial foods

Sample	ChS content*/mg g^{-1}	
	Proposed method	Spectrophotometric method
Mayonnaise A	8.3	12.2
Mayonnaise B	6.5	4.3
Dressing A	2.2	1.6
Dressing B	3.5	3.8
Fish sausage A	ND†	ND†
Fish sausage B	ND†	0.2

* Data are expressed as the total amount of Ch4S and Ch6S.
† Not detected.

reaction became nearly zero order with respect to the enzyme and was independent of the enzyme activity.

Calibration Graph, Precision and Detection Limit

Calibration graphs were constructed by plotting the sum of the peak areas of ΔDi-4S and ΔDi-6S against the concentration of ChS (μg ml^{-1}); these were rectilinear in the range 4–100 μg ml^{-1}, regardless of whether Ch4S or Ch6S was used as the standard. The coefficient of variation at the 20 μg ml^{-1} level was 2.2% (five injections) and the minimum detectable amount of ChS was 2 μg ml^{-1}.

Recovery Study and Comparison with a Spectrophotometric Method

Recovery studies were performed on each of the mayonnaise, dressing and fish sausage samples. Known amounts of Ch4S or Ch6S were added to the sample prior to the initial homogenisation step. Hence potential losses occurring during sample treatment could be assessed, together with interferences occurring during the chromatographic separation. The results are given in Table 1 and these indicate that satisfactory recoveries were achieved for the samples tested. Typical chromatograms of spiked and blank mayonnaise extracts are shown in Fig. 3. Comparison of these chromatograms clearly indicates that the proposed method is relatively free from interferences caused by food ingredients. This is also true for the dressing and fish sausage samples.

Table 2 compares the results obtained for the determination of ChS in several commercial foods using the proposed method and a spectrophotometric method.[3] Agreement between the two methods was poor, especially for the mayonnaise sample, and this situation persisted when the analysis was repeated several times. The recovery data given in Table 1 suggest that this may be attributed to deficiencies, such as poor specificity, in the spectrophotometric method. As Ch4S and Ch6S each produced very different ratios of ΔDi-4S and ΔDi-6S by enzymatic digestion,[8] it was difficult to confirm whether it was Ch4S or Ch6S that was detected in a particular food.

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Application of High-performance Liquid Chromatography - Inductively Coupled Plasma Mass Spectrometry to the Investigation of Cadmium Speciation in Pig Kidney Following Cooking and *In Vitro* Gastro-intestinal Digestion

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The speciation of cadmium in retail pig kidney has been examined by size-exclusion chromatography (SEC) coupled directly to inductively coupled plasma mass spectrometry (ICP-MS). Approximately 35% of the cadmium from uncooked kidney was soluble after aqueous extraction at pH 8 and SEC - ICP-MS revealed three discrete peaks whose retention times corresponded to estimated relative molecular masses of 1.2×10^6, 7.0×10^4 and 6×10^3–9×10^3. In the cooked kidney, 35% of the Cd was soluble and was all associated with a peak of a relative molecular mass (M_r) of 6×10^3–9×10^3. After simulated gastric digestion of cooked pig kidney at pH 2.5, 60% of the cadmium was solubilised and associated with a species of $M_r < 1 \times 10^3$. When the digest was also subjected to simulated intestinal digestion at pH 6.8, a single peak, which corresponded to 20% of the original cadmium, was eluted. This peak co-eluted with the single peak extracted at pH 8.0 from the cooked kidney. It was also of similar estimated M_r to the single broad peak observed after simulated gastro-intestinal digestion of equine renal metallothionein ($M_r = 1.1 \times 10^4$). The results suggest that the majority of soluble cadmium in retail pig kidney is associated with a metallothionein-like protein that survives both cooking and simulated *in vitro* gastro-intestinal digestion.

Keywords: *High-performance liquid chromatography - inductively coupled plasma mass spectrometry; cadmium speciation;* in vitro *gastro-intestinal digestion; pig kidney; metallothionein*

The occurrence of cadmium in foodstuffs is of concern due to the well established toxicological properties of this metal.[1] Fortunately the large majority of ingested cadmium is excreted in the faeces and for orally administered cadmium only approximately 6% is absorbed from the intestinal tract.[2] When cadmium is consumed as part of a meal, the degree of uptake is generally lower and in the range 2–3%.[3,4] Investigations into the chemical speciation of cadmium in food have revealed that the metal is not present in the ionic form but complexed to various binding proteins.[5,6] The degree to which cadmium is absorbed by the gastro-intestinal tract will depend upon factors such as the chemical form of the element, the action of digestive enzymes and the effect of ligands originating both from the food and the gut itself. One of the hindrances to an understanding of the possible role of speciation in this area has been the lack of suitably sensitive and selective analytical instrumentation. The recent introduction of inductively coupled plasma mass spectrometry (ICP-MS)[7] interfaced to high-performance liquid chromatography (HPLC)[8] provides such a technique.

This paper describes the use of HPLC - ICP-MS to study the changes in the chemical speciation of cadmium that occur during cooking and simulated gastro-intestinal digestion of pig kidney. It is known[9,10] that administration of various forms of cadmium and certain other heavy metals to experimental animals will induce formation of a metal binding protein, metallothionein (MT), in both liver and kidneys. Here we describe a method to determine if such a protein exists in retail pig kidney, where cadmium levels are low compared with experimental animals. Studies have also been performed to determine whether the nature of the cadmium species is affected by cooking and enzyme treatment.

Experimental

Reagents

De-ionised, distilled water was used throughout. Aristar grade sodium chloride, ammonia solution, sodium hydroxide, hydrochloric, nitric, formic and acetic acids, AnalaR sodium hydrogen carbonate and sucrose and laboratory-reagent grade chelating agents [ammonium tetramethylenedithiocarbamate (1% *m/V*), diethylammonium diethyldithiocarbamate (1% *m/V*)] and 4-methylpentan-2-one were obtained from BDH Chemicals.

Cadmium solutions were prepared from SpectrosoL cadmium nitrate solution (1000 μg ml^{-1}, BDH Chemicals) and serially diluted with water acidified to 1% *V/V* nitric acid.

Ethanol was obtained from James Burroughs (Fine Alcohol Division), propan-2-ol (HPLC grade) from Rathburn Chemicals and Lipoclean from Hoechst UK.

A gel filtration standard (relative molecular masses of marker proteins in the range 1.35×10^3–6.7×10^5) was purchased from Bio-Rad Laboratories. Blue Dextran, bovine serum albumin, β-lactoglobulin, tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) and equine renal MT were obtained from Sigma Chemicals. Buffers and column cleaning solutions were made up with water (the pH was adjusted with hydrochloric acid or ammonia solution as necessary) and filtered (0.22-μm Millipore filter) under vacuum.

The *in vitro* enzymes are detailed elsewhere.[11–13] The pig kidney was purchased, frozen, from a retail outlet in Norwich.

Procedures

All glassware and plastic ware were acid cleaned (25% *V/V* nitric acid) prior to use.

Preparation of pig kidney

The pig kidney was homogenised in a Kenwood food blender and divided into two equal portions. One portion was placed in Pyrex dishes, covered with foil and baked in a domestic electric oven at 190 °C for 1.5 h. The cooked kidney plus juices were homogenised, placed in polythene bags and stored frozen (−15 °C) until required. The remaining portion, *i.e.*, uncooked pig kidney, was stored similarly.

Determination of total cadmium in pig kidney

Sub-samples (2.5 g fresh mass) of uncooked or cooked kidney were weighed into Tecator tubes and digested with nitric and sulphuric acids followed by hydrogen peroxide. Reagent blanks and spiked samples were taken through the entire procedure. After chelation and extraction the total Cd was determined by graphite furnace atomic absorption spectrometry (Thermo Electron "IL"157/AAS with Fastac). The method has been described in detail elsewhere.[11–13]

Extraction of uncooked and cooked pig kidney

An extraction procedure based on the methods employed by Cherian[14] and Taguchi and Nakamura[15] was used to extract MT from the kidney matrices. A frozen sub-sample (2.5 g) of uncooked or cooked kidney was weighed into a glass beaker (10 ml) and homogenised with extractant (7.0 g of 0.02 M Tris HCl, pH 8.0 containing 0.25 M sucrose) for 90 s using a Janke and Kunkel electric homogeniser. The homogenate was transferred into stoppered centrifuge tubes (Oakridge, polysulphone) and centrifuged in a Damon/IEC Centra-4X centrifuge (20 min at 10 000 *g*). The resulting supernatant was transferred into a glass beaker and then filtered successively (3, 1.2 and 0.45 µm). The filtered extract was diluted 1 + 1 with Lipoclean (a chlorofluorocarbon used to clear turbid samples such as sera and plasma), mixed for 1 min on a vortex mixer and then centrifuged in a glass vial (10 min at 3000 rev min^{-1}). Following centrifugation, the aqueous homogenate was removed, diluted 1 + 1 with 0.24 M Tris HCl (pH 7.5) and finally filtered (0.22 µm) prior to measurement by HPLC - ICP-MS. Blank extractions were carried through the entire procedure.

Gastro-intestinal digestion of cooked pig kidney and metallothionein

A two-stage, *in vitro* enzymolysis procedure which has been described previously[12,13] was used. Briefly, sub-samples (10 g) of the cooked kidney were weighed into screw-top polythene bottles (250 ml). Equine renal MT was also taken through the complete digestion. For this purpose 1 mg of the equine renal MT was dissolved in water (5 ml) and 0.5 ml placed in the polythene bottles. Pepsin (1% *m*/*V*, 20 ml) in 154 mM saline acidified to pH 1.8 with hydrochloric acid was added to the digest bottle and incubated at 37 °C for 4 h, then half of the digests were adjusted to neutral pH (pH 6.8 for pig kidney, pH 7.2 for equine renal MT) by adding saturated sodium hydrogen carbonate and "intestinal" juice (20 ml). The "intestinal" juice consisted of equal volumes of (i) pancreatin (3% *m*/*V*) plus α-amylase (1% *m*/*V*) in saline and (ii) bile salts (1.5 g l^{-1}) in saline. The digests were maintained at 37 °C for a further 4 h in a shaking water-bath.

The remaining "gastric" digests were adjusted to pH 2.5 and centrifuged for 30 min (MSE Centrikon TCO45 centrifuge) at 4 °C and 40 000 rev min^{-1}. Centrifugation was carried out using partially or wholly filled capped polycarbonate tubes (38.5 ml). The supernatant was filtered off (0.22 µm) prior to HPLC - ICP-MS analysis. The digests that had undergone simulated gastro-intestinal treatment were treated similarly except that the pH remained at *ca.* 7 and centrifugation was for 5 h. Enzyme blanks (for gastric and gastro-intestinal digests) were taken through the entire procedure.

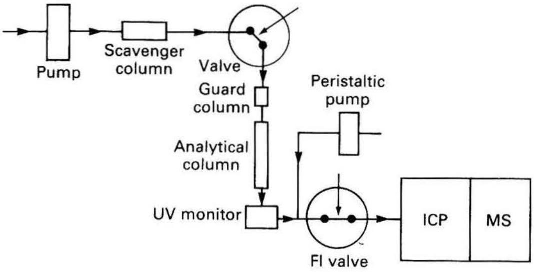

Fig. 1. Schematic diagram of the HPLC - ICP-MS system

HPLC - ICP-MS system

The HPLC - ICP-MS system has been described in detail elsewhere[8] and is shown schematically in Fig. 1. Briefly the system consisted of a Superose-12 (Pharmacia-LKB) size-exclusion chromatography (SEC) column interfaced directly to an ICP-MS instrument (PlasmaQuad, VG Elemental). A scavenger column, packed with Chelex 100 (Bio-Rad Laboratories), was placed between the pump and injection valve. Small (2 cm × 5 mm i.d.) columns were packed with Superose-12 "prep grade" gel and used as guard columns for the analytical columns. Chromatographic components (Pharmacia-LKB) such as valves, syringes and sample loops were such that no metal came into contact with the buffers or samples. A valve for flow injection (FI) was situated between the outlet of the UV monitor and ICP-MS instrument. Operating conditions for the ICP-MS measurements were the same as described previously[8] except that the nickel sample cone had an orifice i.d. of 1.00 mm, not 0.75 mm.

SEC - UV - ICP-MS of standards and calibration of Superose-12

The Superose-12 column was calibrated using proteins of known relative molecular mass (M_r). A plot of retention time against log M_r yielded a straight line for the following standards: Blue Dextran (M_r, 2×10^6), thyroglobulin (6.7×10^5), γ-globulin (1.58×10^5), bovine serum albumin (6.6×10^4), ovalbumin (4.4×10^4), β-lactoglobulin (1.84×10^4), myoglobulin (1.7×10^4) and vitamin B_{12} (1.35×10^3). Additionally, equine renal MT was used as an SEC marker. Standards containing equine renal MT were always the last samples to be run prior to cleaning the column.

SEC - ICP-MS of pig kidney extracts and enzyme digests

For each injection of the kidney extracts and digests a new guard column was used. An injection (100 µl) of the filtered extract or digest (each had been diluted 1 + 1 with 0.24 M Tris HCl, pH 7.5) was eluted from the pre-equilibrated Superose-12 column with 0.12 M Tris HCl (pH 7.5) at a flow-rate of 0.75 ml min^{-1}. The UV signal (254 nm) was recorded on a pen recorder while counts for ^{114}Cd were collected on the multi-channel analyser of the ICP-MS instrument using the single-ion monitoring mode against time. During the period prior to the void volume of the column being eluted, injections (100 µl) were made, using the FI system, of standards (10 ng ml^{-1} Cd in 1% nitric acid and/or 10 ng ml^{-1} Cd in 0.12 M Tris HCl, pH 7.5) and of the kidney extract or digest. This allowed determination of the total Cd in the extract or digest and subsequent calculation of the recovery of Cd from the column. At the end of the chromatographic run, repeat FI injections were made that also served to monitor the system for any change in count rate. The total area counts for each chromatographic run and any ^{114}Cd peaks were noted.

Table 1. Estimated M_r values and Cd content of fractions isolated by SEC - ICP-MS from pig kidney and equine renal MT

Extract or digest	Estimated M_r of Cd-containing peaks	Concentration of Cd in extract or digest/ ng ml^{-1}	Extracted Cd species as % of total Cd from pig kidney*
Tris HCl - sucrose extracts, pH 8.0—			
Uncooked kidney	1.2×10^6	2.1	7
	7.0×10^4	2.5	8
	6.3×10^3	6.5	20
Cooked kidney	8.9×10^3	10.2	30
Gastric digest, pH 2.5—			
Cooked kidney	$<1 \times 10^3$	25	60
Gastro-intestinal digest—			
Cooked kidney, pH 6.8	1.2×10^4	7.5	20
Equine renal MT, pH 7.2	1.1×10^4	30	40
Standard MT—			
No extraction or digestion; equine renal MT (10 ng ml^{-1} Cd) in 0.12 M Tris HCl, pH 7.5	2.5×10^4	10	—
	1.1×10^4	10	—

* For equine renal MT, extracted Cd is expressed as % of total Cd from the MT at the start of digestion (6.3 µg). Total Cd: 0.22(1) and 0.22(8) mg kg^{-1} of fresh mass for uncooked and cooked pig kidney, respectively.

Results and Discussion

Analytical Criteria

There are several potential pitfalls that can be encountered with the use of HPLC for inorganic speciation studies, particularly at trace levels where very small additions or losses become crucial. Inorganic ions or species, from equipment, buffers and samples can be retained on the column. Any organic species that adhere to the column can also bind inorganic species giving rise to anomalous peaks in subsequent runs. Finally, the speciation of the analyte can be altered during its passage through the column. Therefore, essential criteria for meaningful and reliable chromatography were decided upon with regard to the behaviour of samples eluted from the column. These criteria were that recoveries of Cd from the column must be acceptable (ideally 80–100%), that the UV chromatography must be reproducible and that the ^{114}Cd chromatograms must also be reproducible. In practice, various problems had to be overcome to achieve these criteria.

To minimise contamination of the analytical column by trace elements, buffers were passed through Chelex 100 columns prior to use and also during chromatographic runs. The UV chromatography of samples was reproducible but, initially, the corresponding ^{114}Cd chromatograms were not. For example, ^{114}Cd profiles for both uncooked and cooked pig kidney extracts changed with time, although the recoveries of Cd were acceptable. There was no evidence that sample treatment prior to SEC - ICP-MS caused the changes. The problem was resolved by using a new guard column for each injection, of pig kidney extract or digest, to prevent adsorption by ligands with a high affinity for cadmium that would otherwise interfere with subsequent injections. It was also necessary to employ an extensive column clean-up procedure for the Superose-12 column after each batch of samples and standards. This involved sequential washing with two bed volumes of each of the following: formic acid (60% *V/V*), ethanol (20% *V/V*), sodium hydroxide (0.1 M), Millipore water (organics free, de-ionised, distilled water), pepsin (1% *m/V* in 0.1 M acetic acid, 0.05 M sodium chloride), hydrochloric acid (0.05 M), propan-2-ol (20% *V/V*) and finally Millipore water. Filters in the pre-filter and on top of the column were changed before cleaning.

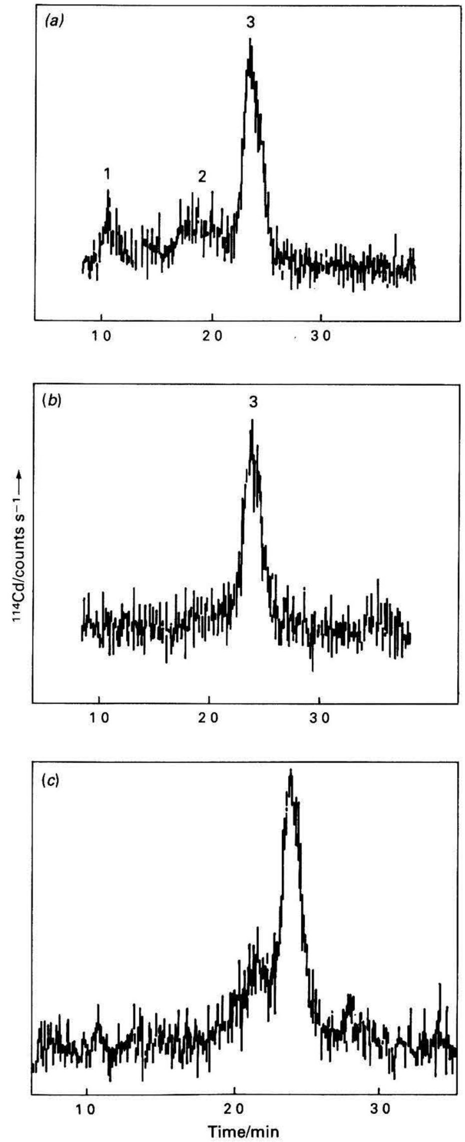

Fig. 2. SEC - ICP mass spectra of (*a*) uncooked and (*b*) cooked pig kidney following extraction with sucrose (0.25 M) and Tris HCl (0.02 M), pH 8.0; (*c*) equine renal MT in Tris HCl (0.12 M, pH 7.5). Superose-12 column; flow-rate, 0.75 ml min^{-1}; buffer, Tris HCl (0.12 M), pH 7.5. 1, 2 and 3 are high, medium and low M_r ^{114}Cd-containing peaks, respectively. For details see text

In addition, the use of ethylenediaminetetraacetic acid, which had been used previously as a scavenger for residual Cd between runs, was discontinued as it gave rise to an anomalous Cd peak in subsequent runs.

Finally, misleading Cd peaks were also encountered when ionic Cd was injected on to a column from which equine renal MT had eluted previously. The ionic Cd eluted at retention times equivalent to those of the equine renal MT. It was therefore possible to obtain totally misleading peaks for ^{114}Cd if a sample containing ionic Cd was eluted after a sample or standard containing equine renal MT. This type of situation serves to emphasise the need for care in inorganic speciation studies. Using the modifications and techniques described, Cd recoveries from the column were consistently 75–100%. The ^{114}Cd chromatograms and determinations of Cd in extracts and digests were reproducible, with coefficients of variation for peak retention times (volumes) being less than 5% and for determinations of Cd, less than 10%.

SEC - ICP-MS of Uncooked and Cooked Pig Kidney Extracts

The results of the SEC - ICP-MS of the kidney extracts are given in Table 1. Of the total Cd (0.22 and 0.23 mg kg^{-1} of fresh mass for uncooked and cooked pig kidney, respectively), 30–35% was extracted by sucrose - Tris HCl at pH 8.0. Other workers[16,17] using experimental animals (rats and mice) have found that 50–80% of Cd can be solubilised using similar extraction procedures to that cited here. In each instance, however, the animals had been administered various forms of Cd and the levels of kidney Cd were subsequently elevated. As Cd - MT is induced under these conditions[9,10] extractable levels would probably be higher. Our results suggest that, for retail kidney, there is a significant amount of Cd that remains insoluble under these conditions.

The ^{114}Cd chromatograms, for uncooked and cooked pig kidney following SEC - ICP-MS, are shown in Fig. 2. The soluble Cd was associated with three peaks of high, medium and low M_r [Fig. 2(*a*)], *i.e.*, 1.2×10^6, 7.0×10^4 and 6.3×10^3, respectively. For cooked kidney, only one Cd-containing peak [Fig. 2(*b*)] was extracted, corresponding to an estimated M_r of 8.9×10^3. It is clear that heat treatment removed the components of high and medium M_r. The former probably consisted of Cd associated with protein aggregates from blood, *e.g.*, α_2-macroglobulin (M_r *ca.* 9×10^5), and a variety of γ-globulins (M_r 1.5×10^5–9.5×10^5). The fraction of medium M_r may have contained Cd bound to plasma proteins such as albumin ($M_r = 6.9 \times 10^4$). Albumin is thought to be the largest protein that can pass through the renal glomerulus.[17] Heat denaturisation can be used to remove components of high M_r during the preparation of MT extracts.[10] Removal of *ca.* 90% of the total protein of the tissue-soluble fraction can be achieved by heating (80 °C for 10 min) without loss of MT. It is likely that the species of low M_r for uncooked and cooked kidney were the same as injection of a 1 + 1 mixture of the two extracts revealed three peaks: the high and medium M_r peaks (1.2×10^6 and 7.0×10^4, respectively) from uncooked kidney and a single peak of low M_r of 6.9×10^3. There was no evidence to suggest that this single peak (low M_r) consisted of two poorly resolved peaks. Additionally, the range of elution volumes (17.2–18.2 ml) for the peaks of low M_r overlapped with the range of the elution volumes (16.7–17.6 ml) for the major equine renal MT ^{114}Cd peak [see Fig. 2(*c*); the smaller equine renal MT peak corresponding to an estimated M_r of 2.5×10^4 was reproducible and may be a dimer of the major equine renal MT peak]. These findings suggest that the low M_r bound Cd in both uncooked and cooked pig kidney extracts may be associated with an MT-like protein, which would, as referred to above, have survived heating.

However, SEC and the position of a known MT peak are insufficient to identify an unknown MT.[18] The interpretation of data from SEC is limited as molecular markers are primarily globular proteins. As the column separates on the basis of size rather than M_r, MT, which has a non-globular shape, elutes earlier than an equivalent mass globular protein. Webb[10] and others[9] reviewed the literature with regard to MT and reported that the M_r of mammalian MT has been consistently derived as 1×10^4–1.2×10^4 by gel-filtration studies. We report 1.1×10^4 for equine renal MT. Under denaturing conditions the M_r of the apoprotein can be as low as 6×10^3.[19]

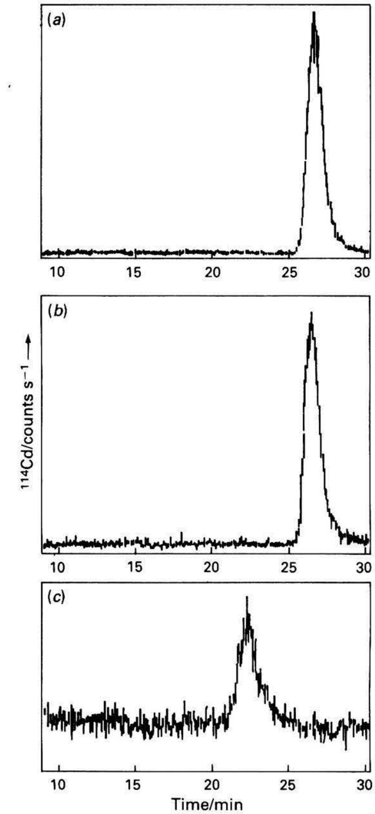

Fig. 3. SEC - ICP mass spectra of (*a*) gastric digest of cooked pig kidney, (*b*) a 10 ng ml^{-1} aqueous Cd standard and (*c*) gastro-intestinal digest of cooked pig kidney. Conditions as in Fig. 2 except for (*a*) and (*b*) where the buffer pH was 2.5

Hence, it is not possible to say with certainty that the low M_r Cd from these pig kidney extracts is associated with an MT, but it is likely. If this is so then the dietary implications may be important as offal contains high levels of Cd. Cherian[14] has shown that Cd - MT is apparently absorbed intact by the gastro-intestinal tract and then degraded during renal uptake. Cadmium would be released, possibly causing some toxic effects prior to being combined with more induced MT. We therefore examined the fate of the MT-like protein in the cooked pig kidney after simulated gastro-intestinal digestion.

SEC - ICP-MS of Enzyme Digest of Cooked Pig Kidney

The ^{114}Cd chromatograms for the gastric digest and gastro-intestinal digest of cooked pig kidney are shown in Fig. 3. The ^{114}Cd chromatogram of the gastric digest [Fig. 3(*a*)] is compared with that of an aqueous Cd standard [Fig. 3(*b*)] injected on to the column. The estimated relative molecular masses for the Cd-containing peaks found after enzyme treatment are given in Table 1.

The soluble Cd in the gastric digest, pH 2.5, represented 60% of the total Cd from the kidney prior to cooking. It was all present as small species of $M_r < 1 \times 10^3$. It is known that, on lowering the pH, Cd bound to MT is released.[9] The pH at which 50% of the metal is dissociated is between 2.5 and 3.5 for Cd.[9] In this simulated gastric digest the soluble Cd behaves, chromatographically, in the same way as the inorganic Cd standard, indicating that the element is present as very small M_r species.

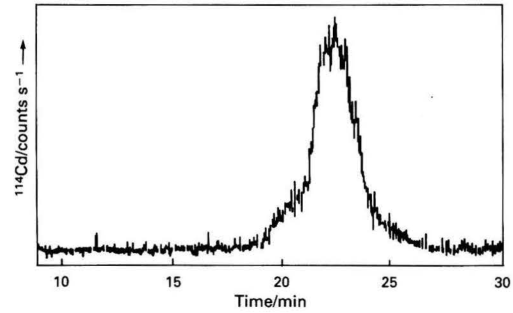

Fig. 4. SEC - ICP mass spectrum of gastro-intestinal digest of equine renal MT. Conditions as in Fig. 2

In contrast, only 20% of the total Cd was soluble after complete enzymolysis (pH 6.8) of the cooked kidney. All of this Cd was associated with low M_r species of 1.2×10^4 [Fig. 3(*c*)]. Similarly, when equine renal MT was subjected to complete enzymolysis the ^{114}Cd (40% of the total Cd from the equine renal MT) was present as a broad peak with a shoulder [*cf.* Fig. 4 with Fig. 2(*c*)], of estimated M_r 1.1×10^4. Further, when a 1 + 1 mixture of the cooked pig kidney extract and gastro-intestinal digest was injected on to the column a single ^{114}Cd peak, whose retention corresponded to an M_r of 1.2×10^4, was eluted. This suggests that the components of the enzyme digest cause the low M_r proteins in the cooked extract to elute slightly earlier from the column. It may be due to a slight change in shape, which also causes the peak broadening seen for equine renal MT after simulated gastro-intestinal digestion. In order to check that the MT-like species was not derived from the enzymes, a gastro-intestinal blank (*i.e.*, no sample was involved) was spiked with Cd (100 ng ml^{-1}; pH 7.5) and subjected to SEC - ICP-MS. No peaks corresponding to Cd associated with MT were eluted.

It appears from this study that at least some of the renal equine MT and the MT-like protein isolated from cooked pig kidney survived the *in vitro* enzyme treatment. This possibility is supported by the work of Cherian[14] in which it was found that orally administered Cd - MT can be absorbed intact. Other workers[20] have investigated the stability of MT (from rabbit liver) in pepsin solutions and *in vitro* in human gastric juice. They concluded that at pH 2.5 in both solutions some of the protein was digested resulting in the formation of peptides. The levels of Cd were measured after chromatographic separation and they found that for the pH 2.5 digests, Cd was associated with both MT and peptides. However, the pH of the mobile phase was considerably higher (a little less than pH 6) than that of the pepsin digest or human gastric juice. It is likely, therefore, that any Cd which was dissociated from the rabbit liver MT during proteolysis at pH 2.5, would recombine at the higher pH. A similar phenomenon is also likely to occur for digests carried out at pH 1.7 and chromatographed at a higher pH.[20] In the work reported here the pH of the buffer was the same as that of the gastric digest, pH 2.5, and no Cd eluted in the range of M_r associated with equine renal MT or MT-like proteins (Table 1). The exclusion limit of the Superose-12 column prevents distinction between what might be Cd bound to small peptides[20] and inorganic Cd.

It is probable that, in the study of Klein *et al.*,[20] if an intestinal digest had been added, no further hydrolysis of the rabbit liver MT would have occurred while MT-derived peptides would have been hydrolysed further to amino acids and dipeptides, all the Cd being associated with the remaining MT. This type of sequence is the most likely explanation for the behaviour of Cd in the work reported here, with Cd that was not bound to the proposed MT-like protein being associated with insoluble components of the pig kidney gastro-intestinal digest.

Conclusions

Many previous workers have studied cadmium, and other elements, in tissues from animals that had been administered abnormally high levels of these elements. Using SEC - ICP-MS we have been able to study the speciation of Cd in retail pig kidney at normal "background" levels of this element. Aqueous extraction, at pH 8.0, released a small amount of Cd species of high and medium M_r (1.2×10^6 and 7.0×10^4, respectively) from uncooked pig kidney that was presumably rendered insoluble during cooking. A protein of low M_r, containing 20–30% of the total Cd from pig kidney was found in both cooked and uncooked kidney and also in the *in vitro* gastro-intestinal digest of cooked kidney. The M_r of this protein was similar to that of equine renal MT (1.2×10^4 and 1.1×10^4, respectively) after *in vitro* gastro-intestinal treatment. The major equine renal MT peak had the same M_r before and after digestion indicating that the protein could survive gastro-intestinal treatment. Under *in vitro* gastric conditions all of the soluble Cd (60%) from cooked pig kidney was associated with a species of $M_r < 1 \times 10^3$. It is probable that retail pig kidney contains an MT-like protein which is able to survive cooking and *in vitro* gastro-intestinal digestion. Further work is underway to try and characterise the protein fractionated from pig kidney after enzyme treatment.

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Dissolution of Soils and Geological Materials for Simultaneous Elemental Analysis by Inductively Coupled Plasma Optical Emission Spectrometry and Atomic Absorption Spectrometry

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A method has been developed using hydrogen peroxide, 6 M HCl and dilute HF for the dissolution of soils and geological materials. Boron, Si, Se, As and other elements often volatilised by other methods were retained by using closed containers at relatively low temperatures and by avoiding the use of perchloric acid. Six United States Geological Survey (USGS) standard rock samples and additional soil and sediment samples were dissolved and analysed by simultaneous multi-element inductively coupled plasma optical emission spectrometry to test the effectiveness of the method. The results compared favourably with other methods used for the analysis of USGS standards. Analyses of soil and sediment samples indicated that Se and As were retained and measured accurately.

Keywords: *Acid dissolution; volatilisation; multi-element analysis; inductively coupled plasma optical emission spectrometry; atomic absorption spectrometry*

A method for the complete dissolution of soils and geological materials which minimises losses of volatile constituents and contamination from reactants should be of interest to those engaged in agricultural, geological and related environmental research. Analysis of the resulting solutions by inductively coupled argon plasma optical emission spectrometry (ICP-OES) offers the advantages of fast, simultaneous, multi-element analysis.

Minerals, rocks and soil samples can be decomposed chemically by fusion and/or dissolution in acids. Decomposition by fusion is relatively rapid; however, there are some disadvantages. (1) Samples cannot be analysed for elements comprising the added flux. This may include Li, B, Ba, Na, K, S, F, etc., depending on the choice of flux. These elements are often of particular interest in environmental studies and in soil genesis and classification, especially in arid regions of the world. (2) Some elements such as Se, As, Sb and Hg[1] may be partially lost by volatilisation during fusion, while compounds such as CaF_2 may be precipitated during acid dissolution of the fusion residue. (3) High concentrations of the elements comprising the flux often cause matrix problems in the final analyses.

The dissolution of rocks and minerals in acids is a relatively successful process when the type and concentration of acid(s), the temperature and the duration of heating and agitation have been optimised. Hydrogen haloacids, sulphuric acid (H_2SO_4), perchloric acid ($HClO_4$), orthophosphoric acid (H_3PO_4), nitric acid (HNO_3) and some organic acids have been used individually or in combination in dissolution processes.

Although there are conflicting reports in the literature, we conclude, both from our own experience and from the work of others, that if volatile elements are to be determined the use of high boiling-point acids such as $HClO_4$ and H_2SO_4 should be avoided and hydrofluoric acid (HF) should be used only in a specified sequence and dilution.

Langmyhr and Sveen[2] state that Berzelius was the first to use a mixture of HF and H_2SO_4 as a decomposing agent for rock samples. After the dissolving reactions were complete, excess of HF had to be removed by evaporation. Silicon was lost during this operation and had, therefore, to be determined using a separate sample. Hillebrand[3] experienced difficulties in completely removing HF by evaporation and advised against its use as a decomposing agent. Case[4] used HF, in addition to other common mineral acids, to increase the rate of dissolution of certain non-ferrous alloys and silicon was finally determined spectrophotometrically as yellow silicomolybdic acid. Case[4] used boric acid to complex the excess of HF. Odegard[5] digested rock samples with 40% HF and later added 4.5% H_3BO_3 solution to complex free HF and introduce B as a reference element.

A survey of the extensive literature on the analysis of rocks and minerals showed that HF is often applied in combination with other acids when used as a decomposing agent. However, some rock-forming minerals are very resistant to dissolution when treated with mixtures of HF and H_2SO_4, HNO_3, and/or $HClO_4$. Langmyhr and Sveen[2] investigated the ease with which 28 major and some trace minerals of silicate rocks could be decomposed with a mixture of HF and $HClO_4$. After 20 min of treatment with the acid mixture, quartz (SiO_2), enstatite ($MgSiO_3$), anthophyllite [$(Mg,Fe)_7Si_8O_{22}(OH)_2$], beryl ($Be_3Al_2Si_6O_{18}$), kyanite ($Al_2SiO_5$), topaz [$Al_2SiO_4(F,OH)_2$], staurolite [$Fe(OH)_2Al_4O_2Si_2O_8$], andradite [$Ca_3Fe_2(SiO_4)_3$], epidote [$Ca_2(Al,Fe)_3(SiO_4)_3(OH)$], magnetite ($Fe_3O_4$), pyrite ($FeS_2$), chalcopyrite ($CuFeS_2$) and pyrrhotite ($Fe_{1-n}S$) were only partially decomposed. When the reaction time was increased to 40 and then 60 min, beryl, kyanite, topaz, staurolite, pyrite, chalcopyrite and pyrrhotite were still only partially decomposed. With the exception of topaz, these minerals were found to decompose completely at higher temperatures in bombs lined with fluorinated ethylene-propylene resin (FEP). Langmyhr and Sveen also indicated that HF alone was more effective as a decomposing agent than a mixture of HF with another mineral acid. Antweiler[6] reported that the rate of reaction of silicates with HF was slowed down by the addition of another mineral acid. Dolezal *et al.*[7] reviewed the techniques used for the decomposition of rocks and minerals with mineral acids for analytical purposes. They stated that "according to current opinions, addition of mineral acid hinders the hydrolysis of silicon fluoride, binds the reaction water and causes a shift of equilibrium, leading to a complete decomposition of the rock."

Dolezal *et al.*[7] also studied the volatilisation of the fluoride forms of some elements during decomposition with HF. They concluded that decomposition with HF was not suitable for the determination of As in ores, shale or silicates. In a study of the volatilisation of Se tracers, Chau and Riley[8] spiked portions of marine sediments with ^{75}Se and left them to digest overnight in a water-bath with 1 + 1 HNO_3 - HF. Excess of HF was evaporated in the presence of HNO_3 under an infrared heater and the residue was dissolved in 4 M HCl, boiled to reduce Se^{VI} to Se^{IV}, diluted and then counted for the ^{75}Se activity remaining. It was found that 100% of the radioactive Se was present in the final solution. Chapman *et al.*[9] reported on the results of evaporating $HClO_4$ - HF solutions containing compounds of 37 elements at 200 °C. Analysis of the residues showed that appreciable amounts of B, Si, Ge, As, Sb, Cr, Se, Mn and Rh were lost during such treatment. They concluded that the loss was due, in most instances, to the volatilisation of the fluorides of the elements. Bajo[10] studied the possible volatilisation of As^{III}, As^{V}, Sb^{III}, Sb^{V}, Se^{IV} and Se^{VI} when granite was digested with $HClO_4$ - HF using tracer solutions of ^{76}As, ^{122}Sb, ^{124}Sb and ^{75}Se. It was shown that there were no losses of Sb and Se, irrespective of the initial oxidation state. Arsenic(III) was partially or totally lost by volatilisation. The oxidation state of all three elements after the $HClO_4$ - HF treatment was determined. As naturally occurring Se is known to be associated mainly with organic matter,[11] recovery tests with spiked additions to mineral samples may not give a valid comparison with soils. Soltanpour *et al.*[1] recommended the use of an HNO_3 - $HClO_4$ - HF treatment based on data reported by Bajo[10] for the digestion of rocks and minerals for total elemental analysis in order to avoid losses of As, Se and Sb in the presence of silicates.

As part of our work on the distribution of Se and As in soils and sediments from the west side of the San Joaquin Valley, California, we used the HNO_3 - $HClO_4$ - HF method[1] for dissolution of the samples. In order to detect and measure Se and As with maximum sensitivity, the samples were analysed using ICP-OES with continuous hydride generation after portions of the above samples had been heated with 6 M HCl in a water-bath. Detection limits of 1 $\mu g\,l^{-1}$ were obtained and no interferences were observed. However, the concentrations of Se in the samples seemed to be much lower than those expected based on published reports. This suggested that volatilisation may have been a problem. When $HClO_4$ is added to a solution containing Se, Se^{IV} is oxidised to Se^{VI}; the addition of HF leads to the formation of SeF_6, which has a boiling-point of −35 °C. The following fluorides and their boiling-points indicate the potential for volatilisation losses of several other elements during heating of the sample to the boiling-point of $HClO_4$ (203 °C): SbF_5, 149.5; AsF_5, −53; OsF_6, 45.9; PF_5, −75; PuF_6, 62; ReF_6, 47.6; SF_4, −40; WF_6, 17.5; UF_6, 56.2; VF_5, 111.2; BF_3, −65; and SiF_4, −100 °C.

These volatilisation losses of the elements of interest, which were observed in our laboratory and which have been reported by other workers,[12,13] prompted us to develop an alternative dissolution method. The aim of developing this alternative method was to minimise losses and to provide a solution from which appropriate aliquots could be taken for analysis by ICP-OES with pneumatic nebulisation and/or continuous hydride generation.

The rate of decomposition of a sample of rock or mineral in an acid depends on the physical and chemical properties of the solid materials, the decomposing acid, the temperature of the system and the time of reaction. At low diffusion rates, products accumulate on the solution - sample interface and reduce the rate of decomposition. The slow decomposition of some rocks by H_2SO_4 may be attributed to the formation of sulphate films on the sample surface. Limestones are decomposed with difficulty by H_2SO_4 because of the formation of a film of $CaSO_4$, but they are readily decomposed with dilute HCl and HNO_3.[7] Intensive agitation helps to overcome diffusion problems by maintaining uniform concentrations throughout the reaction medium. In other instances, the rate of decomposition may be increased by the presence of an oxidising or reducing agent in the reaction medium.

Based on preliminary results of soil analyses obtained in our laboratory and on reports in the literature, we decided to investigate the possibility of developing a dissolution procedure using H_2O_2 to oxidise the organic matter, followed by treatment with HCl and digestion of the residue with dilute HF at low temperature in closed FEP centrifuge tubes to dissolve the inorganic material. If this approach were to prove successful it would allow the complete digestion of both the organic and inorganic material, with minimum volatilisation and precipitation losses, and with the advantage that the final solution could be analsyed directly by ICP-OES with either direct nebulisation or hydride generation. The 6 M hydrochloric acid can be conveniently prepared by distillation as a constant-boiling mixture. The procedure would also have an additional advantage in that no special fume hood for $HClO_4$ would be required.

Dolezal *et al.*[7] reviewed the decomposition of rocks with HCl as the principal decomposing agent and made the following observations. (*i*) All of the carbonates including the fluorocarbonates of the rare earth elements, *e.g.*, parisite $\{Ca(La,Ce...)[F_2(CO_3)_2]\}$, are soluble in HCl. (*ii*) Iron oxides (FeO and Fe_2O_3) and hydroxides [$Fe(OH)_2$ and $Fe(OH)_3$] and magnesite ($MgCO_3$) are also soluble in HCl. (*iii*) Sulphates do not dissolve easily, although gypsum ($CaSO_4$) goes into solution in HCl when heat is applied. (*iv*) Fluorite (CaF_2) is partially decomposed by dilute HCl. (*v*) The majority of the boron ores are completely decomposed by HCl. (*vi*) Hydrochloric acid easily decomposes the natural phosphates. (*vii*) Natural iron(III) arsenates ($2FeAsO_3.Fe_2O_3.5H_2O$) dissolve completely in HCl. (*viii*) Hydrochloric acid decomposes tungstates such as scheelite ($CaWO_4$) forming insoluble tungstic acid. (*ix*) The majority of silicates are not dissolved by HCl; their resistance to dissolution depends largely on the ratio of SiO_2 to bases. The greater the basicity of the cation and the lower the SiO_2 to bases ratio, the more easily the silicate dissolves. Sodium silicate is water soluble, $CaSiO_3$ is soluble in acids and Al_2SiO_5 is insoluble in HCl. Silicates containing heavy metals as the cation decompose totally even in dilute mineral acids. (*x*) Chlorides of all the metals are soluble in water, except those of the univalent heavy metals Ag, Cu, Hg and Au and the chlorides of the divalent metals Pb and Pt. The chlorides of multi-valent metals often form hydrates which dissolve in water; the anhydrous chlorides are sometimes insoluble.

In the decomposition of rocks and minerals, concentrated HF has an effect that differs greatly from that of other mineral acids. It reacts with SiO_2 or silicates to form the very unstable, volatile silicon tetrafluoride (SiF_4). The direct reaction between boron trioxide (B_2O_3) and concentrated HF leads to the formation of the very unstable, volatile boron trifluoride (BF_3). It is found, however, that if SiF_4 or BF_3 is formed in a dilute aqueous solution containing an excess of HF, then hydrofluorosilicic acid, H_2SiF_6, or hydrofluoroboric acid, $H_2B_2F_8$, will be formed, both of which are fairly strong acids. Most salts of these types of acid are highly soluble. The system then contains these acids plus boric acid and gelatinous silicic acid. Concentration of H_2SiF_6 and $H_2B_2F_8$ by evaporation results in partial decomposition and volatilisation of SiF_4 and BF_3.[14]

Langmyhr and Graff[15] discussed the ternary system HF - H_2SiF_6 - H_2O, which was first investigated by Munter *et al.*[16] They obtained a constant-boiling ternary solution having the composition 10% HF, 36% H_2SiF_6 and 54% water (boiling-point 116.1 °C at 759.7 mmHg). Considering the chemical reactions $SiO_2 + 4HF \rightarrow SiF_4 + 2H_2O$ and $SiF_4 + 2HF \rightarrow H_2SiF_6$, we used the liquid - vapour equilibrium diagram for the system HF - H_2SiF_6 - H_2O[16] to calculate that, if 1 g of soil or geological material containing 70% SiO_2 was mixed with 6 ml of H_2O and then dissolved in approximately 4 ml of 49% HF, a ternary solution containing approximately 5% HF, 15% H_2SiF_6 and 80% H_2O with a boiling-point of about 106 °C would be produced. Such a small acid to solid ratio (2 : 1) eliminates the need for the removal of excess of HF and, as a result, Si, B and several other elements are retained in soluble forms, particularly when the temperature is maintained at 90–95 °C during the reaction. Addition of water to the siliceous material before HF treatment prevents formation of

Table 1. Wavelengths for ICP-OES and calculated instrumental detection limits

Element	Wavelength/nm	Calculated detection limit*/mg l^{-1}
Aluminium	308.21	0.03
Arsenic	193.69	0.001†
Antimony	206.83	0.001†
Barium	493.40	0.002
Beryllium	234.86	0.0005
Boron	249.67 II‡	0.005
Bismuth	223.06 II	0.001†
Cadmium	228.80 II	0.004
Calcium	393.36	0.005
Calcium	317.80	0.050
Chromium	267.71	0.002
Cobalt	228.61	0.005
Copper	324.75	0.010
Gold	242.80	0.005
Germanium	199.82	0.001†
Iron	259.94	0.005
Lead	220.35	0.020
Lithium	670.70	0.005
Magnesium	279.55	0.020
Manganese	257.61	0.005
Mercury	253.65	0.001†
Molybdenum	202.03 II	0.008
Nickel	231.60	0.010
Potassium	766.40	1.0
Phosphorus	214.90	0.050
Selenium	196.02	0.001†
Scandium	341.38	0.001
Silicon	251.61	0.010
Silver	328.06	0.005
Sodium	588.90	0.20
Strontium	421.50	0.008
Tellurium	214.20 II	0.001†
Thallium	190.86 II	0.100
Tin	284.00	0.100
Titanium	334.90	0.010
Uranium	385.96	0.20
Vanadium	292.40	0.010
Zinc	206.20 II	0.005

* Detection limits vary with the sample matrix.
† Continuous hydride system with ICP-OES.
‡ II = second-order lines.

the fluorides of Ca, Mg, Ba and Sr. Because the solution is dilute, the compounds will tend to form a sol, which dissolves readily when agitated or shaken.

Experimental

In order to develop and test the proposed dissolution method, six United States Geological Survey (USGS) reference samples,[17] a sample of colemanite ($Ca_2B_6O_{11}.5H_2O$) and a sample of pure quartz were decomposed and analysed for mineral elements as follows.

Quadruplicate samples of each material were weighed in FEP centrifuge tubes and, except for quartz, treated with H_2O_2 and HCl according to steps 1–4 of the procedure described under Dissolution. Colemanite dissolved completely in 6 M HCl. The residues from the reference and quartz samples were decomposed with HF according to steps 5 and 6 of the dissolution procedure. The HCl and HF digests were each made up to 100 ml with 6 M HCl and then diluted further prior to analysis by ICP-OES. Aqueous solutions were analysed using a pneumatic nebulisation technique. A Jarrell-Ash Atomcomp Series 800 ICP spectrometer was used; however, atomic absorption spectrometry (AAS) and/or other instrumentation and analytical methods can be used in the final analyses. For system operation the argon flow-rates were as follows: coolant, 14 l min^{-1}; and sample, 0.5 ml min^{-1}. The remaining parameters were the following: plasma, 0 l min^{-1}; argon pressure to nebuliser, 345 kPa; observation height, 13 mm above the coil; incident power, 1.25 kW; reflected power, <10 W; nebuliser type, cross-flow; integration time, 17 s on-line, 17 s background; and sample aspiration rate, 2.5 ml min^{-1} via a peristaltic pump. Analytical lines (nm) and approximate detection limits are shown in Table 1.

The USGS standards were analysed for Se and As, but these elements were not positively identified. In order to test and confirm the validity of the proposed dissolution method for the analysis of soils and geological materials for Se and As, two different approaches were used. First, four standard sediment samples, prepared by the US Bureau of Reclamation, were digested and analysed. The samples were distributed to several laboratories to assess the accuracy of different analytical techniques for the measurement of Se in sediments of the San Joaquin Valley, California, USA. Second, known amounts of Se and As were added to a soil sample, a rock sample and a sediment sample and the blanks and spiked samples were digested and then analysed by ICP-OES.[18]

The dissolution technique described below has been tried and tested extensively. Its effectiveness in retaining volatile elements is due to the fact that violent chemical reactions at high temperatures are avoided. An alternative and more rapid dissolution procedure for soils high in organic matter, where elements such as Se are mainly concentrated, has been developed by the authors using concentrated HNO_3 in closed containers and will be the subject of a future paper.

We encountered a number of problems when working with samples that had been processed to a very fine particle size so that they passed through a 200-mesh sieve (0.074 mm). A fine dust was produced and moisture was absorbed during the sieving, weighing and other handling operations. Very fine particle size samples formed dense, moist lumps similar to clay balls, which were difficult to disperse and dissolve in H_2O_2 or acids. Langmyhr and Sveen[2] encountered similar problems during a study of the effect of particle size on HF dissolution. Antweiler[6] recommended the use of HF for the decomposition of silicate fragments that had been neither crushed nor ground. Other disadvantages of preparing fine particle size samples are the increased time required for sample preparation and the increased potential for contamination by metals from steel ball mills and other metallic grinding apparatus. Pulverisation may be necessary, however, for samples to be brought into solution by fusion, especially for minerals such as chromite and zircon and others that are resistant to acid dissolution. With USGS samples containing chromite, fine grinding did not prevent incomplete acid dissolution.

Dissolution Technique

Equipment

Quartz mortar and pestle.
Plastic 60-*mesh* (<0.25 mm) *sieve or screen.*
Digestion block adjustable to 90 °C *or sand-bath.*
Oak Ridge FEP centrifuge tubes (50 ml) *with sealing caps.*
Reciprocal two-speed shaker.
High-speed centrifuge with rotor that holds 50-ml *Oak Ridge centrifuge tubes.*
Plastic calibrated containers (100 ml).
FEP pipettes or plastic-tip dispensers.

Reagents

Hydrogen peroxide, 30%.
Hydrochloric acid, concentrated (38%) *and* 1 + 1.
Hydrofluoric acid, 49%.
De-ionised, distilled water (*DDW*).

Dissolution

Crush a 50-g sample of air-dried soil or rock into small pieces using a non-metallic tool. For further grinding, use a quartz mortar and pestle until all of the particles finer than 0.25 mm pass through a plastic 60-mesh sieve or screen.

1. Weigh 1.0 g of the ground sample in a centrifuge tube.
2. Slowly add 5 ml of H_2O_2 and allow the mixture to stand for 10 min. If there is a vigorous effervescence, shake the unsealed tube to prevent the accumulation of gaseous material. When the reaction has ceased, gradually add an additional 10 ml of H_2O_2 and shake the mixture slowly for 16–18 h. Place the tube on the digestion block (90 °C) and evaporate the solution over a period of several hours to a volume of about 5 ml, but not to dryness. If the soil is still dark, repeat the addition of H_2O_2 and heat again. The volume of the final solution at the end of this step should not exceed 4–6 ml.
3. Add 5 ml of concentrated HCl gradually. Exercise care as this is a vigorous reaction with calcareous materials. After effervescence has ceased, place the unsealed tube on the shaker and shake slowly for 16–18 h. Remove the tube from the shaker, cap loosely and place on the digestion block (90 °C). Heat the tube for at least 4 h, remove, cool and centrifuge the mixture at 11 000 rev min^{-1}, for 20 min. Decant the supernatant into a 100-ml plastic calibrated container, seal and store at room temperature (HCl fraction).
4. Add 10 ml of 6 M HCl to the centrifuge tube and seal tightly. After shaking for 4 h, place the tube on the digestion block (90 °C) overnight. Then, centrifuge and decant the supernatant into the storage container used in step 3. Repeat this step at least five times or until a colourless solution is obtained.
5. Add 5–6 ml of distilled water to the residue, seal the centrifuge tube and shake vigorously for 2 h. Add 4 ml of 49% HF, seal the tube and shake vigorously for a further 1 h. Place the tube on the digestion block overnight. Dissolution of siliceous materials should be completed within 24 h. Centrifuge the mixture and decant the supernatant into a new plastic storage container (HF fraction).
6. Add 10 ml of distilled water to the centrifuge tube, seal and shake for 1 h. Centrifuge the mixture and transfer the supernatant into a storage container. If organic residues are observed in the tube, add 1 ml of HNO_3, cap the tube and place it on the digestion block; reddish fumes will be observed. After 2 h, unseal the tube and heat the contents until no more reddish fumes are observed. Add 2 ml of H_2O_2 and place the tube on the digestion block until no more bubbling occurs. Transfer the final solution into the first storage container using 6 M HCl (HCl fraction). If any undissolved residue is present at this step of the procedure it must be subjected to a separate fusion and analysis.
7. Dilute the HCl and HF fractions to give a final volume of 100 ml using 6 M HCl and set aside for analysis. Alternatively, the HF fraction may be diluted to volume with DDW if this fraction is not to be analysed by hydride generation.

Final Analyses

Using the proposed dissolution method, major elements, trace elements and silica are present in the HCl and HF fractions. The relative proportion in each fraction depends on the unique mineral composition of the sample. Low standard deviations for replicate determinations of some of the elements in the two acid fractions indicate that complete dissolution of a particular type of mineral has been accomplished by each acid. The method may therefore be of value for distinguishing different mineral types within a sample.

Dilution of the HCl and HF fractions is necessary to avoid viscosity effects in the nebuliser of the ICP system and to

Table 2. Concentration of mineral elements in USGS standard rock sample GSP-1. The UCR columns represent values obtained using H_2O_2 - HCl - HF dissolution and analysis by ICP-OES. The USGS column represents the range of values reported by Flanagan.[17] The Abbey column represents usable values given by Abbey[19]

	Granadiorite GSP-1				
	UCR			USGS	
Element*	HCl	HF	Total	Range	Abbey
Si	0.065 ± 0.010	31.4 ± 0.096	31.5	31.4–31.4	31.4
Al	3.15 ± 0.016	4.98 ± 0.013	8.13	8.08–8.21	8.08
Fe	2.92 ± 0.059	0.082 ± 0.005	3.00	2.78–3.23	2.99
Mg	0.567 ± 0.005	0.010 ± 0.000	0.577	0.540–0.594	0.582
Ca	0.538 ± 0.005	0.953 ± 0.026	1.49	1.36–1.48	1.45
Na	0.255 ± 0.030	1.82 ± 0.024	2.08	200–2.09	2.08
K	2.71 ± 0.019	1.79 ± 0.021	4.50	4.57–4.65	4.57
Ti	0.325 ± 0.044	0.02 ± 0.000	0.345	0.389–0.408	0.396
B	<2.50	3.27 ± 1.13	3.27	—†	—‡
Ba	687.0 ± 5.20	879.0 ± 6.40	1566	1000–1600	1300
Be	<0.25	1.03 ± 0.045	1.03	<2–<2	1?§
Cd	<2.00	<2.00	<2.00	—†	—‡
Co	6.47 ± 0.620	<2.50	6.47	6–<10	7.8
Cr¶	5.17 ± 0.737	<1.00	5.17	9–18	12
Cu	34.1 ± 2.27	<5.00	34.1	38–60	33
Li	21.8 ± 2.66	<2.50	21.8	27–50	30
Mn	265.0 ± 33.4	4.68 ± 0.156	270.0	309.0–387.0	309
Mo	<4.00	<4.00	<4.00	<2–10	1.5?§
Ni	7.49 ± 1.15	<5.00	7.49	4–12	9
P	988.0 ± 126.0	<2.50	988.0	1222–1397	1222
Pb	—‖	—‖	—‖	50–90	54
Sc	4.80 ± 0.600	0.262 ± 0.097	5.06	6–13	6.6
Sr	53.4 ± 6.83	167.0 ± 1.92	220.0	200–430	240
V	42.3 ± 5.25	10.68 ± 0.146	53.0	30–70	54
Zn	79.0 ± 9.84	6.45 ± 0.469	85.5	—†	105

* Values for Si, Al, Fe, Mg, Ca, Na, K and Ti given in %; values for the other elements given in mg kg^{-1}.

† Not determined.

‡ Usable values not reported.

§ ? implies a relatively greater degree of uncertainty.

¶ Incomplete dissolution.

‖ Values not reported because of the high coefficient of variation as a result of the low concentration near the detection limit.

Table 3. Concentration of mineral elements in USGS standard rock sample G-2. The UCR columns represent values obtained using H_2O_2 - HCl - HF dissolution and analysis by ICP-OES. The USGS column represents the range of values reported by Flanagan.[17] The Abbey column represents usable values given by Abbey[19]

	Granite G-2				
	UCR			USGS	
Element*	HCl	HF	Total	Range	Abbey
Si	0.060 ± 0.000	32.3 ± 0.050	32.4	32.2–32.3	32.3
Al	2.95 ± 0.017	5.35 ± 0.086	8.30	8.16–8.31	8.15
Fe	1.86 ± 0.008	0.020 ± 0.000	1.88	1.80–2.00	1.87
Mg	0.452 ± 0.005	<0.001	0.452	0.408–0.456	0.450
Ca	0.422 ± 0.005	1.00 ± 0.022	1.42	1.29–1.43	1.40
Na	0.512 ± 0.005	2.58 ± 0.214	3.09	2.82–3.07	3.01
K	2.49 ± 0.008	1.12 ± 0.037	3.61	3.69–3.82	3.70
Ti	0.260 ± 0.000	0.010 ± 0.000	0.270	0.282–0.294	0.288
B	4.75 ± 1.09	2.24 ± 0.329	6.99	—†	—‡
Ba	1263 ± 5.00	683.0 ± 10.61	1946	1400–2100	1900
Be	<0.25	2.21 ± 0.194	2.21	2–3	2.4
Cd	<2.00	<2.00	<2.00	—†	0.039?§
Co	5.81 ± 0.539	<2.50	5.81	2–<10	5
Cr¶	4.83 ± 1.56	<1.00	4.83	7–12	8
Cu	11.0 ± 1.05	<5.00	11.0	9–14	10
Li	28.0 ± 0.311	<2.50	28.0	30–75	35
Mn	242.0 ± 1.29	2.65 ± 0.186	245	309–620	232
Mo	<4.00	<4.00	<4.00	<2–<10	—‡
Ni	<5.00	<5.00	<5.00	2–<7	3.5
P	483.0 ± 8.62	<2.50	483.0	567.0–699.0	568
Pb	—‖	—‖	—‖	20–50	30
Sc	3.32 ± 0.460	<0.500	3.32	<2–<7	3.5
Sr	118.0 ± 0.816	340.0 ± 28.4	458.0	340–400	480
V	31.4 ± 2.47	<5.00	31.4	30–50	36
Zn	77.1 ± 0.45	6.62 ± 0.657	83.7	—†	84

* Values for Si, Al, Fe, Mg, Ca, Na, K and Ti given in %; values for the other elements given in mg kg^{-1}.
† Not determined.
‡ Usable values not reported.
§ ? implies a relatively greater degree of uncertainty.
¶ Incomplete dissolution.
‖ Values not reported because of the high coefficient of variation as a result of the low concentration near the detection limit.

measure elements within a linear concentration response range. Each fraction should therefore be analysed separately.

The HCl and HF sample solutions prepared in step 7 are 1 + 99 *m/V* dilutions. These solutions must be diluted further depending on the sample composition. For routine samples, we suggest a 1 + 4 dilution of each acid fraction from step 7 except when determining silica which is best analysed in a 1 + 499 dilution of the HF fraction. Samples such as peridotite and dunite which are high in Mg require a separate 1 + 99 dilution. The diluted HCl fractions are ready for analysis by ICP-OES using pneumatic nebulisation and/or hydride generation.

The sequence of using an oxidising agent first to destroy any organic matter followed by a final treatment with HCl has the added advantage that hydride-forming elements are kept in their reduced state and hence can be analysed by hydride generation in contrast to the more conventional final treatment with a strong oxidising agent such as $HClO_4$.

It should be noted that 6 M HCl suppresses the intensity of the signals from most elements by 15–20% compared with a <5% HCl matrix. The standard solutions used to calibrate the ICP-OES system were therfore prepared in the same HCl matrix and approximate major salt matrix as the samples. The zero concentration calibration standard was prepared by diluting distilled, constant-boiling HCl with resin-purified distilled water to the sample acid concentration. The high-concentration calibration standard was prepared to contain the same concentration of HCl plus 200 mg l^{-1} of Ca, 10 mg l^{-1} each of Mg and P, 100 mg l^{-1} each of Na and K and 1–8 mg l^{-1} each of the trace elements depending on the sensitivity of the element. Mixed calibration standards were prepared fresh for each sample set. If the indicated sample concentration values for an element exceeded the known linear response for that element, then the sample was diluted and re-run until the concentrations in two two-fold dilutions agreed to within 3%. A reagent blank was processed with each set of samples to correct for contamination. The computer software corrected for spectral interferences by subtracting the interference signals from those of the analytes.

The following steps are suggested for hydride determination. Pipette a 30-ml aliquot of the 1 + 99 *m/V* solution from step 7 into a 40–50-ml glass test-tube. Place the tube in a boiling water-bath for 45 min to ensure reduction of the hydride-forming elements and analyse the sample by continuous hydride generation using ICP-OES.[18]

Results and Discussion

Analytical data for six USGS standard silicate rock samples are presented in Tables 2–7. The columns headed "HCl," "HF" and "Total" show the results obtained for the two acid fractions. The column headed "Range" indicates the range of measured values reported by Flanagan[17] using the complete, conventional rapid rock and spectrochemical analyses recommended by the USGS.

As noted by Shapiro and Brannock,[20] for the rapid analysis of silicate rocks by the USGS laboratories, Si and Al are determined spectrophotometrically using aliquots of a solution prepared by fusing the samples with NaOH: a Molybdenum Blue method is used for SiO_2, whereas Alizarin Red S is used for the determination of Al_2O_3. A second portion of the sample is digested with HF - H_2SO_4 - HNO_3 in FEP beakers and the solution is used for the spectrophotometric determination of total Fe with 1,10-phenanthroline, TiO_2 with Tiron, P_2O_5 with molybdovanadophosphoric acid, MnO as

Table 4. Concentration of mineral element in USGS standard rock sample PCC-1. The UCR columns represent values obtained using H_2O_2 - HCl - HF dissolution and analysis by ICP-OES. The USGS column represents the range of values reported by Flanagan.[17] The Abbey column represents usable values given by Abbey[19]

Peridotite PCC-1

	UCR			USGS	
Element*	HCl	HF	Total	Range	Abbey
Si	0.140 ± 0.000	19.70 ± 0.096	19.8	19.5–19.8	19.6
Al	0.382 ± 0.005	0.042 ± 0.000	0.424	0.344–0.408	0.386
Fe	5.67 ± 0.013	0.140 ± 0.000	5.81	5.64–5.83	5.8?†
Mg	25.5 ± 0.245	0.548 ± 0.005	26.0	25.8–26.0	26.1
Ca	0.352 ± 0.005	0.040 ± 0.000	0.392	0.286–0.343	0.393
Na	<0.01	<0.01	<0.01	0.000–<0.040	0.007
K	<0.05	<0.05	<0.05	0.000–<0.050	0.00
Ti	0.0005	<0.005	<0.0005	0.000–0.006	0.006
B	<2.50	3.09 ± 0.829	3.09	—‡	—§
Ba	<1.00	<1.00	<1.00	<4–20	4?†
Be	<0.25	<0.25	<0.25	<2–<4	—§
Cd	<2.00	<2.00	<2.00	—‡	—§
Co	104.0 ± 0.577	3.41 ± 0.264	107.0	66–110	110
Cr¶	773 ± 12.5	295 ± 0.577	1066.0	1200–3600	2800
Cu	9.13 ± 1.41	<5.00	9.13	5–16	8
Li	<2.50	<2.50	<2.50	<2–<2	3?†
Mn	790.0 ± 0.957	23.42 ± 0.250	813	852.0 ± 929.0	929
Mo	<4.00	<4.00	<4.00	<2–<10	0.5?†
Ni	2218 ± 9.57	24.1 ± 0.629	2242	600–2300	2400
P	15.2 ± 3.42	<2.50	15.2	0.000–<218.0	44
Pb	—‖	—‖	—‖	<20–<20	11
Sc	5.97 ± 0.530	<5.00	5.97	<9–<20	9?†
Sr	<4.00	<4.00	<4.00	10–50	0.40
V	<5.00	<5.00	<5.00	20.38	29
Zn	<2.50	7.17 ± 0.716	7.17	—‡	41

* Values for Si, Al, Fe, Mg, Ca, Na, K and Ti given in %; values for the other elements given in mg kg^{-1}.

† ? implies a relatively greater degree of uncertainy.

‡ Not determined.

§ Usable values not reported.

¶ Incomplete dissolution.

‖ Values not reported because of the high coefficient of variation as a result of the low concentration near the detection limit.

Table 5. Concentration of mineral elements in USGS standard rock sample DTS-1. The UCR columns represent values obtained using H_2O_2 - HCl - HF dissolution and analysis by ICP-OES. The USGS column represents the range of values reported by Flanagan.[17] The Abbey column represents usable values given by Abbey[19]

Dunite DTS-1

	UCR			USGS	
Element*	HCl	HF	Total	Range	Abbey
Si	0.040 ± 0.000	18.7 ± 0.189	18.7	18.9–19.3	18.9
Al	0.01 ± 0.000	0.020 ± 0.005	0.030	0.132–0.164	0.132
Fe	5.55 ± 0.055	0.090 ± 0.001	5.64	5.74–6.30	6.10?†
Mg	29.5 ± 0.263	0.160 ± 0.001	29.7	29.6–29.9	29.9
Ca	0.112 ± 0.005	0.020 ± 0.000	0.132	0.000–40	0.100
Na	<0.01	<0.01	<0.01	0.030–50	0.007?†
K	<0.05	<0.05	<0.05	0.000–<0.042	0.00
Ti	<0.0005	<0.005	<0.0005	0.000–0.006	0.00?†
B	<2.50	<2.50	<2.50	—‡	—‡
Ba	<1.00	<1.00	<1.00	<4–<20	5?†
Be	<0.25	<0.25	<0.25	<2–<4	—‡
Cd	<2.00	<2.00	<2.00	—‡	—‡
Co	124.0 ± 1.83	1.65 ± 0.481	126.0	85–130	135
Cr§	—‡	—‡	—‡	—‡	—‡
Cu	1.20 ± 1.30	<5.00	5.200	4–9	5
Li	<2.50	<2.50	<2.50	<1–<2	2?†
Mn	833 ± 8.06	<2.50	833	939–1006	929
Mo	<4.00	<4.00	<4.00	<2–<10	1?†
Ni	9210 ± 18.3	8.83 ± 0.950	2219	1700–2000	2300
P	32.5 ± 4.28	<12.5	13.5	0.000–175.0	0?†
Pb	—¶	—¶	—¶	<20–<20	11.0
Sc	1.09 ± 0.46	<0.50	1.09	<2–<7	3.8
Sr	<4.00	<4.00	<4.00	<2–<4	0.4?†
V	<5.00	<5.00	<5.00	9–<20	11
Zn	<2.50	3.68 ± 0.129	3.68	—‡	46

* Values for Si, Al, Fe, Mg, Ca, Na, K and Ti given in %; values for the other elements given in mg kg^{-1}.

† ? implies a relatively greater degree of uncertainty.

‡ Not determined.

§ Incomplete dissolution.

¶ Values not reported because of the high coefficient of variation as a result of the low concentration near the detection limit.

Table 6. Concentration of mineral elements in USGS standard rock sample AGV-1. The UCR columns represent values obtained using H_2O_2 - HCl - HF dissolution and analysis by ICP-OES. The USGS column represents the range of values reported by Flanagan.[17] The Abbey column represents usable values given by Abbey[19]

Andesite AGV-1

Element*	UCR			USGS	Abbey
	HCl	HF	Total	Range	
Si	0.045 ± 0.006	27.7 ± 0.129	27.7	27.5–27.6	27.8
Al	5.05 ± 0.025	3.87 ± 0.008	8.92	9.05–9.21	9.10
Fe	4.67 ± 0.037	0.130 ± 0.000	4.80	4.52–4.90	4.77
Mg	0.908 ± 0.005	0.010 ± 0.000	0.918	0.840–0.960	0.912
Ca	3.30 ± 0.137	0.325 ± 0.006	3.63	3.36–3.49	3.53
Na	1.36 ± 0.008	1.35 ± 0.006	2.71	3.12–3.15	3.21
K	0.627 ± 0.005	1.76 ± 0.014	2.39	2.32–2.32	2.42
Ti	0.490 ± 0.000	0.070 ± 0.000	0.560	0.599–0.659	0.635
B	<2.50	6.56 ± 1.42	6.56	—†	6?‡
Ba	396.0 ± 3.16	496.0 ± 2.06	892.0	1200–1800	1200
Be	<0.25	1.10 ± 0.102	1.11	<2–<4	2?
Cd	<2.00	<2.00	<2.00	—†	—§
Co	10.5 ± 1.13	0.698 ± 1.12	11.2	10–18	16
Cr¶	—†	—†	—†	—†	—†
Cu	61.4 ± 1.58	<5.00	61.4	55–100	59
Li	<2.50	<2.50	<2.50	9–20	12
Mn	586.0 ± 4.35	6.00 ± 0.329	592.0	774.0–852.0	774
Mo	<4.00	<4.00	<4.00	4–<10	3?‡
Ni	13.1 ± 0.450	1.46 ± 0.550	14.5	7–18	15
P	1605.0 ± 19.2	<2.50	1605	2139–2314	2227
Pb	—‖	—‖	—‖	20–50	33
Sc	7.79 ± 0.079	<0.500	7.79	9–22	12.5
Sr	360.0 ± 3.16	138.0 ± 0.577	498.0	610–1000	660
V	99.9 ± 4.66	<5.00	99.9	80–150	125
Zn	49.0 ± 0.316	5.68 ± 0.250	54.7	—†	86

* Values for Si, Al, Fe, Mg, Ca, Na, K and Ti given in %; values for the other elements given in mg kg^{-1}.

† Not determined.

‡ ? implies a relatively greater degree of uncertainty.

§ Usable values not reported.

¶ Incomplete dissolution.

‖ Value not reported because of the high coefficient of variation as a result of the low concentration near the detection limit.

Table 7. Concentration of mineral elements in USGS standard rock sample BCR-1. The UCR columns represent values obtained using H_2O_2 - HCl - HF dissolution and analysis by ICP-OES. The USGS column represents the range of values reported by Flanagan.[17] The Abbey column represents usable values given by Abbey[19]

Basalt BCR-1

Element*	UCR			USGS	Abbey
	HCl	HF	Total	Range	
Si	0.080 ± 0.005	25.2 ± 0.082	25.28	25.2–25.3	25.4
Al	4.21 ± 0.180	2.96 ± 0.013	7.17	7.22–7.41	7.26
Fe	8.28 ± 0.010	1.14 ± 0.006	9.12	9.06–9.52	9.41
Mg	1.52 ± 0.059	0.420 ± 0.000	1.94	2.08–2.16	2.09
Ca	3.93 ± 0.024	1.08 ± 0.006	5.01	4.86–4.94	4.98
Na	1.19 ± 0.053	1.21 ± 0.005	2.40	2.30–2.46	2.46
K	0.387 ± 0.005	0.945 ± 0.006	1.33	1.33–1.41	1.41
Ti	1.27 ± 0.054	0.100 ± 0.000	1.37	1.32–1.35	1.35
B	<2.50	<2.50	<2.50	—†	4
Ba	157.0 ± 6.95	565.0 ± 2.39	722	400–900	680
Be	<0.25	0.281 ± 0.025	0.281	<2–<4	1.6?‡
Cd	<2.00	<2.00	<2.00	—†	0.09?‡
Co	<2.50	4.63 ± 0.281	4.63	26–40	36
Cr§	24.4 ± 3.12	<1.00	24.4	9–27	15
Cu	<5.00	<5.00	<5.00	19–35	16
Li	16.5 ± 1.25	<2.50	16.5	14–27	14
Mn	1065 ± 43.6	<2.50	1065	1471–1471	1394
Mo	<4.00	<4.00	<4.00	6–<10	1.5?‡
Ni	0.91 ± 2.77	1.87 ± 0.457	12.8	6–29	10
P	1322 ± 60.2	<2.50	1322	1528–2052	1572
Pb	—¶	—¶	—¶	32–50	14
Sc	23.2 ± 2.33	6.07 ± 0.412	29.3	<20–<20	33
Sr	207.0 ± 8.85	104.0 ± 0.500	311.0	300–400	330
V	379.0 ± 20.2	30.3 ± 2.84	409	170–490	420
Zn	58.8 ± 2.47	9.42 ± 0.403	68.2	—†	125

* Values for Si, Al, Fe, Mg, Ca, Na, K and Ti given in %; values for the other elements given in mg kg^{-1}.

† Not determined.

‡ ? implies a relatively greater degree of uncertainty.

§ Incomplete dissolution.

¶ Vaues not reported because of the high coefficient of variation as a result of the low concentration near the detection limit.

Table 8. Results of statistical comparison of compositional elements of USGS standard samples using the H_2O_2 - HCl - HF method and data given by Abbey[19]

	Element measured in USGS reference samples							
	Si	Al	Fe	Mg	Ca	Na	K	Ti
t-Value*	0.0001	0.449	1.35	2.06	2.65	0.919	2.61	1.50
Correlation coefficient	1.00	1.00	0.998	1.00	1.00	0.990	1.00	0.998
	B	Ba	Be	Cd	Co	Cr	Cu	Li
t-Value*	—†	0.084	—†	—†	1.67	—†	0.583	208
Correlation coefficient	—†	0.973	—†	—†	0.978	—†	0.951	0.930
	Mn	Mo	Ni	P	Sc	Sr	V	Zn
t-Value*	2.54	—†	1.49	2.12	4.05	1.48	2.94	3.88
Correlation coefficient	0.988	—†	1.00	0.994	0.991	0.985	0.998	0.850

* The tabulated *t*-values for five degrees of freedom and a two-tailed test with $\alpha = 0.01$ is 4.03 and for $\alpha = 0.05$ it is 2.57.

† Not listed because of insufficient data (see footnotes to Tables 2–7).

Table 9. Selenium content of US Bureau of Reclamation standard reference samples prepared for quality assurance and assessment of analytical techniques. All values are given in mg kg^{-1}

Standard reference sample	USGS*	LBL†	CSUF‡	UCR§	UCR¶ (ICP-OES)
KS-1-S	63	58	53	56 ± 7.39	58 ± 1.57
K-3-S	85	87	82	82 ± 4.47	89 ± 2.50
K-6-S	22	24	19	19 ± 0.39	21 ± 0.67
KS-12-E	2.5	4.2	2.5	2.7 ± 0.06	2.1 ± 0.10

* USGS Laboratory Geological Division using AAS (ten replicate analyses).

† Lawrence Berkeley Laboratory using an X-ray fluorescence technique (three replicate analyses).

‡ California State University at Fresno using AAS.

§ University of California, Riverside, using H_2O_2 - HCl - HF and AAS (three replicate analyses).

¶ University of California, Riverside, using H_2O_2 - HCl - HF and ICP-OES (four replicate analyses).

permanganate after a persulphate oxidation and low-level MgO with Thiazol Yellow. Automatic spectrophotometric titrations are used for the determination of CaO, MgO and CaO plus MgO. The conventional data are obtained from single analyses whereas the rapid rock data are the average of two analyses. The spectrographic data were obtained by the method of Barston *et al.*[21] The general steps of the procedure employed in this method follow the conventional d.c. arc analysis of powdered material where graphite electrodes are used.

Abbey[19] applied the "select laboratories method" and presented the compositional data for the USGS rock samples. He used the term "usable value" to imply that the value may be used, but with more caution than that exercised by other workers who used much more assertive terms such as "best," "recommended," "accepted," "certified" and "guaranteed." The use of a question mark in Tables 2–7 implies a relatively greater degree of uncertainty. The "select laboratories method" was designed by Abbey[22] to approach the most probably correct value for the concentration of each constituent of proposed reference samples in instances where the available results were dispersed over a broader range than would normally be expected from the imprecision of the analytical methods. The method assumes that the work of some of the contributing laboratories is less subject to systematic error than that of others. The method was only applied fully to constituents for which ten or more results were reported. Where fewer than ten results are available, relatively uncertain estimates may be based on the median.

An examination of the data presented in Tables 2–7 indicates that, in general, the values obtained lie within the ranges reported by Flanagan[17] and Abbey.[19] However, some differences are apparent, particularly for Cr in peridotite and dunite which contain the mineral chromite. According to Thompson[23] chromite does not dissolve in acid mixtures when these are used under the conditions normally employed for geochemical analyses. Our observations during the acid dissolution of peridotite and dunite indicated that chromite was only partially dissolved as evidenced by the presence of a small amount of a black insoluble residue, which remained in the centrifuge tube during step 6 of the dissolution procedure. When the black residues from USGS samples DTS-1 and PCC-1 were fused with sodium carbonate and the mixtures dissolved and analysed, the total Cr values were within the ranges reported by Flanagan.[17] We have dissolved several hundred soil and geological samples and only with USGS samples DTS-1 and PCC-1 was there any evidence of an undissolved residue at this step in the procedure. If an undissolved residue is present, it must be subjected to a separate fusion and analysis. Although this adds an extra step to the procedure for some samples, many of the disadvantages encountered in fusing the original sample are avoided.

The value for Al in DTS-1 is significantly lower than the reported values (Table 5). This is the only instance of a low Al value among the six USGS samples. After a detailed discussion of the differences in the CaO concentration found in DTS-1 by different methods in different laboratories during the period 1967–1983, Abbey[19] concluded "a possible object lesson from the foregoing might be that even the best and most reliable analytical methods have limitations, and that the knowledgeable analyst must exercise vigilance in using them."

The evaluation of the proposed method is based on the application of chemical principles and comparison of the results with published data from other laboratories. Separation of most of the elements from siliceous materials prior to treatment with HF, and formation of the HF - H_2SiF_6 - H_2O ternary system to prevent volatilisation and/or precipitation of several elements supports this argument.

The H_2O_2 - HCl - HF method was designed to be used with a range of soil samples and geological materials and its validity was tested using many different types of sample. We used a statistical method, *viz.*, comparison of sample means and meaningfully paired observations, to compare our data with those of Abbey for a large number of elements. Steele and Torrie[24] state that if members of the pair tend to be positively correlated, an increase in the ability of the experiment to detect a small difference is possible. The information in pairing is used to eliminate any source of extraneous variance that exists from pair to pair. This is carried out by calculating the variance of the differences rather than that among individuals within each sample. The number of degrees of freedom on which the estimate of variance is based is one less than the number of pairs. If Y_{1_j} and Y_{2_j} ($j = 1, \ldots, n$) represent pairs of a particular element shown in the columns headed "Total" and "Abbey" in Tables 2–7, n is the number of paired values and is equal to 6 and t is the test criterion, then $t = \bar{D}/S_{\bar{D}}$, where $\bar{D} = (\Sigma D)/n$, $D = Y_{1_j} - Y_{2_j}$ and $S_{\bar{D}} = S_D/\sqrt{n}$ (D is the arithmetic difference of the paired values for an

Table 10. Analysis of a soil, a sediment and a rock sample for Se and As to assess the accuracy of the H_2O_2 - HCl - HF dissolution method followed by analysis using ICP-OES with hydride generation. All values given in mg kg^{-1} unless indicated otherwise

	Se					As				
Sample	Blank	Added	Expected	Measured	Recovery, %	Blank	Added	Expected	Measured	Recovery, %
Soil 266	0.471	4.00	4.47	5.01 ± 0.02	112	9.90	3.00	12.9	13.0 ± 0.435	101
	0.471	—*	—*	—*	—*	9.90	12.0	21.9	22.1 ± 0.451	101
Sediment 308	21.2	20.0	41.2	40.7 ± 0.30	98.8	3.85	3.00	6.85	6.95 ± 0.180	101
Rock 331	0.337	0.400	0.737	0.699 ± 0.051	94.8	4.58	0.400	4.98	5.11 ± 135	103

* Selenium was not added.

element in a sample and S_D is the standard deviation referred to D values of an element for n samples).

Table 8 presents the correlation coefficients and calculated t-values for measurements of Y_{1_j} and Y_{2_j}. It should be noted that the test is performed independently of sample type. The high positive correlation coefficients indicate that a significant linear relationship exists between the data for the elements measured by the proposed dissolution method and those reported by Abbey.[19] The t-values are indicative of insignificant differences between the measured and reported data. It is almost impossible to expect a better agreement when the values obtained by ICP-OES are compared with those obtained from the spectrophotometric analysis using NaOH fusion techniques and/or with the values obtained from spectrochemical analysis of the solid inorganic forms using d.c. arc techniques.

Samples of colemanite ($Ca_2B_6O_{11}.5H_2O$) were digested and analysed by the proposed method. The measured value for B was 15.6 ± 0.01% compared with a calculated value of 17.5%, while the measured value for Ca was 19.7 ± 0.13% compared with a calculated value of 19.5%. Dissolution and analysis of samples of pure quartz (SiO_2) gave a value of 46.4 ± 0.1% for Si or an SiO_2 equivalent of 99.4%.

Table 9 presents data for the analysis of US Bureau of Reclamation standard reference samples. The column headed "USGS" shows the accepted values for Se based on ten replicate analyses by the USGS Laboratory Geological Division. The column headed "UCR (ICP-OES)" shows the results of the analysis of the sediment samples digested using the proposed method in our Trace Element Laboratory at the University of California, Riverside (UCR). Table 10 presents data for the digestion and analysis of a soil, a sediment and a rock sample with and without added Se and As. Both tables indicate good recovery and measurement of Se and As.

Separate analyses of the HCl and HF digests indicated that less than 2% of the Si was released by HCl and that dissolution was actually completed by treatment with HF. Boron in USGS samples was released and measured in the HF fractions only, whereas in colemanite it was completely dissolved by the HCl treatment. Addition of HF to this solution resulted in the precipitation of CaF_2.

Based on the above observations and a comparison of data, we recommend the two-step H_2O_2 - HCl and HF dissolution method followed by separate elemental analysis of each fraction of soil and geological material. However, for some applications, analysis of the HCl fractions may be sufficient.

To correct for problems caused by volatilisation of SiO_2 and B from glass components of the nebuliser assembly by dilute HF solutions, these components were reconstructed from poly(vinyl chloride) plastic pipes and used in the assembly for all the analyses. No dissolution of the glass torch by the dilute HF sample aerosol was observed.

Conclusion

Oxidation of organic matter breaks the structural units of soils and results in a more dispersed system. Hydrochloric acid then dissolves the salts and disperses the system further. Separation of dissolved materials from residues followed by repeated treatment with acids at low temperatures in capped containers with agitation provides an optimum solid - liquid interface for a complete dissolution process with minimum losses of volatile constituents.

The time per sample treatment can be conveniently minimised by processing a large number of samples (50–100) simultaneously. Most steps in the procedure can be left unattended which reduces further the actual time spent in sample treatment. The procedure is easily followed in most chemical laboratories including those not equipped with a costly $HClO_4$ fume hood. The concentrations of elements found in six USGS standard rock samples compare favourably with values reported by other laboratories.

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Determination of Aluminium Levels in Tea and Coffee by Inductively Coupled Plasma Optical Emission Spectrometry and Graphite Furnace Atomic Absorption Spectrometry

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Aluminium levels in tea leaves from different origins were determined by wet ashing followed by inductively coupled plasma optical emission spectrometry. Coffee beans were found to contain comparatively small amounts of Al. Typical tea and coffee infusions were also assessed by means of graphite furnace atomic absorption spectrometry.

Keywords: *Aluminium determination; tea leaves; coffee beans; inductively coupled plasma optical emission spectrometry; graphite furnace atomic absorption spectrometry*

The amount of Al present in tea (*Camellia sinensis*), both in the dry leaves and in tea infusions, has recently generated considerable interest.[1,2] The potential toxicity of Al to mammals has been widely studied in the past decade,[3] particularly as a result of reports that Al intoxication appears to be linked with a variety of neurological[4,5] and behavioural disorders.[6] Although the tea plant has long been known to be an Al accumulator species,[7] Coriat and Gillard[1] have drawn attention to the relatively large amounts of Al that may be present in tea infusions (up to 100 μg cm^{-3} were reported). Although the human gut (in healthy individuals) is known to be a formidable barrier to the absorption of inorganic forms of Al, it has been found that significant increases in the urinary levels of Al occur in patients who have been treated with Al containing antacid preparations.[8] Recently, we also observed an increase in the urinary Al levels in human volunteers, following the consumption of relatively large amounts of tea.[9]

Because of the surprisingly large range of Al concentrations reported in both dry tea leaves and tea infusions,[1,2] we undertook a survey of Al levels in commercially available teas from different origins, and for the purposes of comparison, assessed the Al levels in coffee beans. The Al was determined by means of inductively coupled plasma optical emission spectrometry (ICP-OES), as the technique has been shown to be well suited for the determination of Al in biological matrices.[10,11] For trace levels of Al, graphite furnace atomic absorption spectrometry (GFAAS) was found to be more satisfactory.[12,13] Further, we attempted to validate our analyses by determining the amount of Al in the National Institute for Environmental Studies (NIES) Certified Reference Material (CRM) No. 7 Tea Leaves (Japan Environment Agency), in view of the risk of accidental contamination as a result of the ubiquitous presence of Al in the lithosphere (dust), in addition to possible matrix effects which may play a role in the determination of Al.

Experimental

All glassware was scrupulously cleaned by soaking overnight in a good quality phosphate-free detergent (Contrad), followed by overnight soaking in 10% *V/V* nitric acid and subsequent rinsing with Al-free water obtained from a Milli-Q system (Millipore).

Apparatus

An Il-Plasma 200 ICP spectrometer and a Perkin-Elmer 5000 atomic absorption spectrometer equipped with an HGA-500 graphite furnace and AS 40 autosampler were used. In the latter instance pyrolytic graphite coated graphite tubes were used. Table 1 shows the relevant instrumental conditions for the determination of Al.

Table 1. Instrumental conditions

ICP-OES—

R.f. power supply	27.12 MHz
Operating power	1.2 kW
Nebuliser	Cross flow
Sample aspiration rate	1 cm^3 min^{-1}
Argon flow-rate	15 dm^3 min^{-1}
Peak search window width	0.033 nm
Integration time	3 s
Al emission line	308.22 nm

GFAAS—

Al hollow cathode lamp current	9 mA
Wavelength	309.12 nm
Slit width	0.7 nm
Absorbance	Peak-height
Tubes	Pyrolytic graphite-coated graphite
Aliquot volume	20 μl

Furnace settings	Dry	Ash	Atomise	Clean
Temperature/°C	120	1200	2700	2700
Ramp time/s	30	10	1	1
Hold time/s	35	20	4	4
Ar flow-rate/cm^3 min^{-1}	300	300	10	300

Procedures

Grind all samples of tea leaves and coffee beans in a ball-mill to a fine powder. Weigh, in duplicate, 250-mg portions of a sample that has been dried at 85 °C to constant mass, into a microKjeldahl flask. Digest the sample with 4 cm^3 of specially pure concentrated HNO_3 and 1 cm^3 of $HClO_4$ (Aristar, BDH) by heating for *ca.* 30 min until no more brown oxides of nitrogen are evolved and the solution begins to give off white fumes. After cooling, transfer the digest quantitatively into a 50-cm^3 calibrated flask and dilute to volume with Al-free water. Store these solutions in clean polyethylene bottles. For each batch of five digestions prepare a blank solution.

Prepare tea/coffee infusions by allowing the desired amount (500 mg) of dry sample (*not* ground) to infuse with 45 cm^3 of water at *ca.* 95 °C. Do not add acid to the infusion as this leads to precipitation of some organic material.

Prepare standard solutions of Al by diluting a commercially available 1000 μg cm^{-3} stock solution of $Al(NO_3)_3$ (Spectro-

Table 2. Aluminium content of tea leaves and coffee beans as determined by ICP-OES ($n = 2$)

Sample	Al/µg g^{-1}
Tea leaves (origin)—	
South African Netul	942 (2)*
Malawi	1009 (1)
Mauritian	555 (2)
Sri Lanka (Ceylon)	526 (3)
Bangladesh	473 (1)
India (Darjeeling)	434 (2)
China	789 (2)
Blend A†	768 (5)
Blend B†	780 (5)
NIES CRM No. 7‡	729 (2)
Coffee beans (unroasted)—	
Columbia	19 (10)
Costa Rica	21 (9)
Guatemala	19 (30)
South African	11 (40)

* Values in parentheses are the relative standard deviations (%).

† Blends A and B are different commercially available "tea-bag" contents of unknown composition.

‡ NIES CRM No. 7 quoted value 775 ± 20 µg g^{-1}.

Table 3. Aluminium levels in tea infusions as determined by GFAAS ($n = 2$ unless indicated otherwise)

Tea infusion	Al concentration/ µg cm^{-3}
South African* (5 min)	1.6 ± 0.1
South African (10 min)	1.8 ± 0.1
South African (20 min)	1.9 ± 0.1
Bangladesh	0.9 ± 0.1
India (Darjeeling)	1.0 ± 0.1
China	2.0 ± 0.1
Malawi	3.0 ± 0.1
Blend A	2.5 ± 0.1
Blend B	3.2 ± 0.1
Blend B†	5.0 ± 0.4‡
Local tap water	0.05–0.27
Coffee infusion	0.23–0.34

* Infusions prepared with 500 mg of tea leaves per 45 cm^3 of Al-free water unless stated otherwise (infusion time 5 min).

† Infusion prepared from one "tea bag" (2 g of tea) per 150 cm^3 of tap water (infusion time 2–4 min).

‡ $n = 8$.

soL, BDH) with Al-free water and HNO_3. Store these solutions in polyethylene bottles. Attempt to match the matrix of the analyte and standard solutions as closely as possible by adjusting the final acid concentration.

Determine the Al content by ICP-OES for solutions likely to contain an Al concentration >20 µg cm^{-3}. Use GFAAS for solutions containing less than 20 µg cm^{-3} of Al, using the method of standard additions. Note that in the standard additions method employed here, an aliquot of the standard solution was added directly to the graphite tube, immediately after the sample, using the AS 40 autosampler.

Results and Discussion

The amounts of Al found in various dry tea leaves are shown in Table 2. Also given are the Al levels found in coffee beans. Although detailed studies of the applicability of ICP-OES to the determination of Al in biological matrices have been carried out previously,[10,11] we assessed the possible spectral interferences that might be anticipated in tea-leaf digests. In order of sensitivity, some of the spectral lines that may be employed for the determination of Al are 395.15, 309.27, 308.22 and 226.92 nm. It was found that potential interference from Ca, Mg, V, Fe and Co may be anticipated for most of the above emission lines, with the line at 308.22 nm being subject to the least spectral interference. Only V at levels of >10 µg cm^{-3} was found to interfere at 308.22 nm. The determined amount of Al in the NIES CRM No. 7 Tea Leaves was 729 ± 16 µg g^{-1} ($n = 3$), which is slightly lower than the quoted value, 775 ± 20 µg g^{-1}, although at the 99% confidence level the difference is not significant, $t_{\text{exp.}}$ (4.98) < $t_{\text{calc.}}$ (5.84). The consistently low amounts of Al found in coffee beans are in marked contrast to those found in tea leaves. The low levels of Al in the former could not be quantified satisfactorily using the method employed for tea leaves (wet ashing, then ICP-OES) as is reflected in the large relative standard deviations observed. No attempts were made to obtain more accurate values for the Al levels in coffee beans.

It is interesting to note the considerable variation in the Al levels in teas from different origins. We found that teas of African origin contained considerably more Al than samples from India, Bangladesh and Sri Lanka, although we did not find levels as high as 20 000 p.p.m. as reported elsewhere.[14] It is possible that the variation in Al levels in the teas analysed here may reflect different soil conditions and hence Al uptake by *Camellia sinensis* in different locations, although to our knowledge, this hypothesis has not been tested systematically.

In view of the discrepancies between the reported Al levels in tea infusions,[1,2] tea infusions were prepared with Al-free water and also local tap water, and the Al concentrations in *drinkable* tea determined by GFAAS. Clearly, the actual amount of Al in a typical tea infusion may be expected to depend on the "strength" of the tea, *i.e.*, the infusion time and the amount of dry tea per unit volume used to prepare the infusion, in addition to the Al content of the water used to prepare the infusion. It was found that for typical infusions (0.5 g of tea leaves per 45 cm^3 of Al-free water), the Al concentration is a function of time, with the amount of Al leaching into the infusion levelling off after 20–25 min standing at temperatures near to the boiling-point of water (*ca.* 95 °C). Hence, for example, the Al concentration of a typical infusion prepared from an African tea using Al-free water was found to increase from 1.4 to 1.8 µg cm^{-3} for an infusion time of 5 and 20 min, respectively. This corresponds to the leaching of between 140 and 174 µg of Al per gram of dry tea leaves into the infusion (representing *ca.* 15–20% of the total Al in the tea leaves). Further, an approximate correspondence between the Al content of the dry tea leaves and the Al concentration of the resulting infusion was observed (for fixed amounts of tea leaves after 5 min of infusion), Table 3.

In order to calculate the Al levels in a realistic "cup of tea," commercially available "tea bags" (containing *ca.* 2 g of finely divided tea per bag) were allowed to infuse into 150 cm^3 of local tap water for 2 min and the Al content was then determined (Table 3). The mean concentration of Al was found to be 5.0 ± 0.4 µg cm^{-3} ($n = 8$). The local tap water was found to have an Al concentration of between 0.046 and 0.267 µg cm^{-3}, hence the major source of Al in the tea infusions was the tea leaves, as mentioned above. It is noteworthy that the mean Al concentration of a tea infusion prepared in a new aluminium tea-pot approximately doubles (10.2 and 9.1 µg cm^{-3}) relative to an infusion prepared in a glass vessel. The latter point is intended to illustrate the possible effect of using Al containers to prepare tea; however, this possibility was not examined further. The Al concentrations found here compare well with those reported by Fairweather-Tait *et al.*,[2] although we did not find concentrations as high as 100 µg cm^{-3} as has been reported elsewhere.[1]

In conclusion, it was found that wet ashing followed by ICP-OES allows for the reasonably accurate determination of Al in dry tea leaves. On the other hand, for tea/coffee infusions, which contain up to two orders of magnitude less Al, GFAAS is more suitable as a means of quantification. Further, it is evident that the Al concentrations found in tea infusions depend on the origin of the tea and the infusion time in addition to the fineness of the tea (*vide infra*, tea bags contain finer tea and a greater portion of tea "dust" compared with tea leaves that are not pre-packed). In contrast, coffee infusions prepared from ground coffee beans contain only small amounts of Al. Typical tea infusions contain between 10 and 20 times more Al per unit volume than do coffee infusions. We have previously reported[9] that the consumption of relatively large amounts of tea relative to tap water or coffee leads to a significant increase in urinary Al levels in a small sample of healthy volunteers.

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Determination of Total Selenium in Nutritional Supplements and Selenised Yeast by Zeeman-effect Graphite Furnace Atomic Absorption Spectrometry

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A method for the determination of total selenium in nutritional supplements and selenised yeast is described. The samples were ashed in nitric acid. Hydrochloric acid was used to prevent precipitation of, in particular, iron salts. After appropriate dilutions, the selenium was determined by Zeeman-effect background corrected graphite furnace atomic absorption spectrometry. A furnace ashing step at 1100 °C was necessary in order to obtain a total recovery of selenium when present in the organic form. Palladium nitrate - magnesium nitrate was used as a matrix modifier. Independent methods were used to determine the content of selenium in a selenised yeast check sample. Accuracy was assured using this sample and by recovery experiments. Between-day random error showed a coefficient of variation of 4.2%. Results from the analysis of eight different commercial supplements were in good agreement with declared contents.

Keywords: *Selenium determination; Zeeman-effect atomic absorption spectrometry; nutritional supplement; selenised yeast; standard additions calibration*

It has been known since 1975 that selenium is an essential element to humans,[1] and recently it has been discussed whether the intake of selenium via the diet is adequate.[2] Consequently, a wide range of nutritional supplements containing selenium, frequently in combination with other minerals and vitamins, has appeared on the market. At present, 35 supplements containing selenium are sold on the Danish market. The majority of these are in the form of tablets and only a few as capsules. In 17 of the formulations the source of selenium is sodium selenite, whereas in 16 formulations the source is selenised yeast. Two preparations contain selenium as L-selenomethionine. The amount of selenium added ranges from 20 to 125 μg per dose. In selenised yeast the selenium species are less well known compared with inorganic selenite and selenomethionine. It has been shown however, that selenium in yeast occurs mainly as selenomethionine.[3] Besides the added selenium and other nutrients, the supplements also contain additives such as talc, silicon dioxide and magnesium trisilicate. These compounds are not dissolved by acid digestion procedures unless hydrofluoric acid is used.[4] Other additives can also remain undissolved during wet ashing. These are the colorants titanium dioxide and various less well defined iron oxides and hydroxides. Only a few papers describe specifically the determination of selenium in nutritional supplements. Thompson and Allen[5] determined selenium in supplements and selenised yeast by injection of a slurry into the graphite furnace of an atomic absorption spectrometer. Nickel was used as a matrix modifier and a deuterium arc was used for background correction. They reported good reproducibility, but we suspect that possible insufficient homogenisation of sugar-coated formulations in particular, can cause errors. Possible spectral interferences using the deuterium-arc background corrector were not discussed. Other methods commonly used for the determination of selenium in various matrices are spectrofluorimetry[6] and hydride generation atomic absorption spectrometry.[7–9] Both of these methods require the total conversion of all the selenium species present into inorganic selenite for accurate determinations. Hence the use of strong oxidising agents such as nitric and perchloric acids during the wet ashing procedure seems mandatory.[10] Selenium can also be determined by graphite furnace atomic absorption spectrometry.[11] This technique is less prone to errors due to the presence of different selenium species, but the possibility of spectral interferences from iron[12] and phosphorus[13,14] must be considered.

This paper describes a method for the determination of total selenium in nutritional supplements and selenised yeast using Zeeman-effect background corrected graphite furnace atomic absorption spectrometry. The method omits the use of perchloric acid in the wet ashing step and is equally sensitive to the selenium species present and insensitive to possible concomitant interferents.

Experimental

Reagents

Nitric acid and hydrochloric acid were of analytical-reagent grade (Merck). Selenite standards were prepared daily from a Titrisol standard solution (Merck). D,L-Selenomethionine (Sigma) was used for the preparation of a standard after drying in an exsiccator. Palladium metal (Ventron) was of analytical-reagent grade and was used in a mixture with magnesium nitrate (Suprapur, Merck) as a matrix modifier. Water was obtained from a Millipore Super Q apparatus.

Procedure

Ten units (tablets or capsules) of the nutritional supplement were ground to a fine powder using a pestle and mortar. The powdered sample (0.5–1 g) was weighed accurately into a round-bottomed flask, two boiling chips and a few drops of water were added and a water-cooled condenser was attached. Concentrated nitric acid (5 ml) was added through the condenser and the mixture was boiled gently for 2 h with occasional swirling. The mixture was allowed to cool and 20 ml of concentrated hydrochloric acid were added through the condenser. The mixture was boiled for 15 min to dissolve any precipitated iron compounds and other minerals from the sample matrix. Finally 25 ml of water were added followed by boiling for 5 min. The mixture was allowed to cool and then transferred quantitatively into a 100-ml calibrated flask and diluted to volume with water. The digestion mixtures were

Table 1. Graphite furnace programme and other instrumental settings

Graphite furnace—

Step	Temperature/°C	Ramp time/s	Hold time/s	Ar flow-rate/ ml min^{-1}
1 Dry	130	1	10	300
2 Dry	150	20	40	300
3 Ash . .	1100	20	20	300
4 Atomise . .	2300	0	5	0
5 Clean . .	2500	1	3	300

Autosampler and spectrometer—

Sample volume	20 µl
Standard volume	10 and 20 µl
Matrix modifier	10 µl
Se electrodeless discharge lamp light source	6 W
Wavelength	196.0 nm
Spectral band width	0.7 nm
Peak evaluation	Peak area

often turbid due to insoluble silicious matter. The digestates from selenised yeasts were clear and colourless. Before measurement, the digestates were diluted with water to give approximate concentrations of 50 µg l^{-1} of selenium. Palladium nitrate - magnesium nitrate was used as a matrix modifier as suggested by Schlemmer and Welz[15] and was prepared by mixing a 3000 mg l^{-1} solution of Pd dissolved in nitric acid with an equal volume of a 2000 mg l^{-1} solution of $Mg(NO_3)_2$. For each 20 µl of the sample solution injected, 10 µl of the matrix modifier were used.

Blanks were taken through the entire procedure and diluted in the same manner as the samples.

Apparatus and Instrumental Settings

The content of selenium was determined using a Perkin-Elmer Zeeman 3030 atomic absorption spectrometer with an HGA-600 graphite furnace, an AS-60 autosampler and a PR-100 printer. Pyrolytic graphite coated graphite tubes and L'vov platforms were used throughout. The instrumental settings are given in Table 1.

Some of the highly resolved absorption profiles obtained by the Zeeman 3030 instrument showed a small peak just before the main peak. This was also observed by Carnrick *et al.*[11] and emphasises the use of peak-area measurements.

Optimisation of the Graphite Furnace Programme and Calibration

In order to assure equal responses from selenium that originated from selenite, selenomethionine and selenised yeast, optimisation of the furnace ashing step was performed. A selenite standard measured according to the settings in Table 1, but ashed at 900 °C, gave a characteristic mass of *ca.* 25 pg of Se per 0.0044 A s. This value is in accordance with that reported by Schlemmer and Welz.[15] Increasing the ashing temperature to 1100 °C did not alter the characteristic mass found. However, standards of selenomethionine ashed at 900 °C gave recoveries of only 92–94% relative to selenite. We found that the minimum ashing temperature necessary for the complete recovery of selenium from this compound was 1100 °C. When determining selenium that originated from the selenised yeast sample (see below) we again found, as for selenomethionine, that the necessary ashing temperature was 1100 °C. Consequently, this setting was used for all analyses.

When selenite standards of 50 and 100 µg l^{-1} were added to sample digestates prepared as described under Procedure, a characteristic mass of *ca.* 30 pg of Se per 0.0044 A s was found. This is a poorer value compared with the characteristic mass of 25 pg of Se per 0.0044 A s obtained for a selenite standard, and indicates a negative matrix effect. Consequently, calibration was carried out by the standard additions technique.

Table 2. Determination of selenium in eight nutritional supplements marketed in Denmark. All results are in µg per unit

	Selenium source	Declared content	Found ± SD ($n = 2$)
A	selenite	125	111 ± 0
B	selenite	125	127 ± 1
C	selenite	125	152 ± 6
D	Se yeast	125	129 ± 1
E	Se yeast	125	124 ± 2
F	Se yeast	45	40 ± 1
G	Se yeast	125	135 ± 1
H	L-selenomethionine	100	97 ± 3

Table 3. Determination of selenium in a selenised yeast check sample. All results are given in µg g^{-1} of Se

Method	Found (mean) ± 1 SD	n
Neutron activation analysis . .	2005 ± 59	2
Spectrofluorimetry	1967 ± 108	6
Weighted average	1977 ± 95	8
Proposed method	1940 ± 50	7

Results

Selenium in Commercial Supplements

Table 2 shows the results for eight different commercial supplements. They were selected to represent different selenium sources and tablet matrices occurring on the market. Hence, samples A–C (selenite) and D–G (selenised yeast) included selenium supplements with high added composite mineral contents and some with no minerals added. The results obtained show good agreement with the manufacturers declaration of selenium content, except for preparation C. The higher result found for this preparation is due to the fact that the manufacturers target dose is actually 150 µg of Se per unit in spite of the declared content of 125 µg of Se per unit.

Accuracy Experiments

In order to assure accuracy of the analytical work a check sample of *ca.* 100 g of selenised yeast was analysed by two independent methods. The yeast was donated by Consult Pharma, Birkerød, Denmark, and was a light brown fine uniform powder. The content of selenium was determined by neutron activation analysis (Danish Isotope Centre, DK-1717 Kbh.V, Denmark) and by spectrofluorimetry. The latter method is a modified AOAC method[6] as the wet ashing was carried out using nitric acid and hydrogen peroxide, but without perchloric acid. The weighted average selenium content was 1977 ± 95 µg g^{-1} of Se. The selenium content was also determined by the proposed method. Table 3 shows the results for selenium in the yeast.

The accuracy was also investigated by performing recovery experiments on selenium from two blank (no selenium) research supplements donated by Ferrosan, Søborg, Denmark. The recovery of 125 µg of selenium added as selenite, selenomethionine or selenised yeast is shown in Table 4. The blank research preparations contained no detectable endogenous selenium.

Discussion

Blank Values

Selenium was not detectable in the blanks, *i.e.*, < 1.5 ng l^{-1} of Se.

Accuracy and Precision

The accuracy of the method was investigated by the use of independent techniques and by recovery experiments. Table 3

Table 4. Recoveries of selenium added to two blank nutritional research supplements. Recoveries are in % ± 1 SD

Supplement	Selenite	D,L-Seleno-methionine	Selenised yeast
A	104 ± 1 ($n = 2$)	105 ± 2 ($n = 4$)	101 ± 2 ($n = 2$)
B	105 ± 4 ($n = 2$)	101 ± 2 ($n = 3$)	103 ± 1 ($n = 2$)

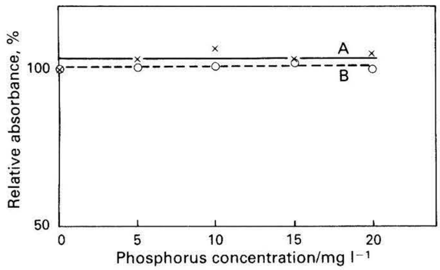

Fig. 1. Influence of phosphorus on the absorbance of a 50 µg l^{-1} Se selenite standard. Phosphorus was added as $(NH_4)_2HPO_4$. Instrumental settings were as indicated in Table 1. Similar settings were used on a Perkin-Elmer 703 atomic absorption spectrometer with an HGA-76B graphite furnace equipped with a deuterium-arc background corrector. A, Deuterium-arc background correction (peak-height absorbance) and B, Zeeman-effect background correction

shows that the determination of selenium in a selenised yeast check sample by neutron activation analysis and by spectrofluorimetry gave the same results taking into consideration the reported standard deviations. Results for selenium in the yeast sample analysed by the proposed method were the same as those obtained using the two independent methods.

However, as digestates of the yeast have a relatively simple matrix as a result of a large dilution factor, we also performed recovery experiments on selenium from two research nutritional supplements. These were in the form of tablets with iron and phosphate, which are possible interferents in the determination of selenium. Table 4 shows that all recoveries are close to 100%.

From duplicate analyses of 11 samples a between-day random error (coefficient of variation) of 4.2% was found.

Interferences

Numerous workers have discussed the spectral interferences from iron[12] and phosphorus,[13,14] when determining selenium using graphite furnace atomic absorption spectrometry. They reported overcompensation of the selenium signal when using the deuterium-arc background corrector on the 196.0-nm selenium line. Many of the selenium supplements appearing on the Danish market contain iron and phosphorus. Digestates of these supplements, diluted as described under Procedure, are up to 20 mg l^{-1} in both iron and phosphorus. The minimum concentration of iron that causes a spectral interference on the measurement of selenium, when using the deuterium-arc background corrector, has been calculated by Welz *et al.*[16] to be 1 mg l^{-1} of Fe (20-µl injected volume). However, it has been shown[11,13,16] that the Zeeman-effect background corrector is able to eliminate the interference from iron. Welz *et al.*[16] found no influence from iron up to at least 50 mg l^{-1} on the selenium signal. Therefore no spectral interference from iron is expected even in the nutritional supplements high in iron, when using Zeeman-effect background correction. This is supported by our recovery experiments which show complete recovery.

The influence of phosphorus on the selenium signal at the 196.0-nm line can also be eliminated by Zeeman-effect background correction[13] or by matrix modification with nickel.[14] Welz *et al.*[16] found that up to 5 mg l^{-1} of phosphorus did not affect the selenium signal using palladium nitrate - magnesium nitrate as the matrix modifier and Zeeman-effect background correction. As the nutritional supplements are up to 20 mg l^{-1} in phosphorus in the final dilutions we investigated the influence of phosphorus on the selenium signal. Fig. 1 shows that the absorbance of selenium is not influenced by phosphorus up to this level either when Zeeman-effect or deuterium-arc background correction is used.

Conclusions

An accurate and precise method has been developed for the determination of total selenium in nutritional supplements on the Danish market and in selenised yeast. Careful optimisation of the furnace ashing step is important in order to recover all the organic selenium present.

Evidence from the literature indicates that the use of Zeeman-effect background correction is necessary to compensate for the spectral interference on the selenium signal from concomitant iron in nutritional supplements.

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Determination of Arsenic and Selenium in Vegetable and Herbage Samples by X-ray Fluorescence Spectrometry Using Coprecipitation

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A procedure is described for the determination of both arsenic and selenium in vegetable and herbage samples. Organic material was destroyed by nitric acid in the presence of magnesium and nickel nitrates, prior to the separation of the analytes. The arsenic and selenium were separated from solution by reduction to the elements using tellurium as a carrier and hypophosphorous acid as reductant. The precipitated analytes together with the carrier were collected by filtration and the filter was examined directly in the wavelength-dispersive X-ray fluorescence spectrometer. For a sample mass of 1 g the procedure has a detection limit of 0.1 µg of arsenic and selenium and the calibration is linear up to at least 25 µg of the analytes. The accuracy and precision of the procedure are better than 3% relative at the 4 µg g^{-1} level.

Keywords: *Arsenic and selenium determination; coprecipitation with tellurium; vegetable and herbage samples; X-ray fluorescence spectrometry*

The Central Electricity Generating Board produces about 12 million tonnes of ash each year as a direct result of the production of electricity from coal. Over half the ash is disposed of through land reclamation schemes and much of the land thus reclaimed may ultimately be used for agricultural and, to a lesser extent, horticultural purposes. The Board's evaluation of the environmental implications of these operations necessitates the determination of the trace element content of the vegetable and herbage species grown on various substrates, including those containing pulverised fuel ash. Two elements of particular interest are arsenic and selenium. Samples of these are expected to have an analyte content of <10 µg g^{-1}.

Usually, trace elements are determined by authenticated procedures that are found in the literature. For selenium, the procedures advocated are based on either molecular fluorescence or hydride generation atomic absorption spectrometry. It was considered undesirable to adopt the molecular fluorescence procedures as those procedures use aromatic diamines, which are suspected carcinogens. Owing to the conflicting information in the literature regarding the suppressive effect of transition metals on hydride generation it was thought desirable to develop an alternative procedure.

One available technique was wavelength-dispersive X-ray fluorescence spectrometry, which would permit the determination of both arsenic and selenium in the same sample (subject to an appropriate sample presentation). Direct examination of the sample (in the form of a pressed disc) was rejected for two reasons. Firstly, the inadequate sensitivity of the technique with respect to the expected analyte content (<10 µg g^{-1}) and secondly, the restricted amount of sample available in some instances. One way to overcome these limitations is to separate the analytes from the sample matrix. For the determination of selenium, this is usually carried out by destroying the organic component of the matrix with an oxidation system that includes perchloric acid. As a large number of samples were to be analysed and because of the hazardous nature of perchloric acid, it was necessary to avoid the use of that compound, if possible. After destruction of the sample matrix, it is then necessary to separate the analytes for presentation to the spectrometer; frequently, this separation is achieved by precipitation or coprecipitation. It was thought advantageous to adopt a procedure in which both analytes were separated simultaneously. The literature reports the use of tellurium as a coprecipitant for selenium,[1–3] selenium for arsenic[4,5] and arsenic for both tellurium[6] and selenium.[7,8] Procedures for the simultaneous coprecipitation of both arsenic and selenium do not appear to have been reported.

Experimental

Apparatus

All glass apparatus was made from Pyrex glass.

The following apparatus was used: 50-ml narrow-necked conical flasks and watch-glasses to suit; small conical filter-funnels; an electrically heated hot-plate, boiling water-bath and muffle furnace (set at 450 °C); Whatman 9-cm 541 filter-papers; borosilicate glass membrane filter holder (to suit 25-mm filters); cellulose nitrate membrane filters, 8-µm pore size (Sartorius SM11301); a Philips 1410 X-ray spectrometer with associated generator and counting electronics.

Reagents

Distilled or de-ionised water was used throughout.

Nitric acid, 70% m/m (Aristar grade, BDH).

Hydrochloric acid, 36% m/m (Aristar grade, BDH).

Hydrochloric acid (4 + 1) *(prepared from hydrochloric acid, 36% m/m).*

Magnesium nickel nitrate solution. Prepared by dissolving 90 g of magnesium nitrate hexahydrate (AnalaR, BDH) and 12 g of nickel nitrate hexahydrate (AnalaR, BDH) in water and diluting to 100 ml.

Copper(II) sulphate pentahydrate (AnalaR, BDH) solution, 6% m/V.

Tellurium solution. Prepared by dissolving 0.26 g of sodium tellurite (GPR grade, BDH) in 50 ml of 10% *V/V* hydrochloric acid and diluting to 500 ml with water.

Hypophosphorous acid, 50% m/m (GPR grade, BDH). Filtered before use.

Procedure

Place 1 g of sample in a 50-ml conical flask and add 3 ml of the magnesium nickel nitrate solution and 5 ml of nitric acid; mix well and heat gently on a hot-plate until the initial reaction has subsided. Slowly increase the temperature until a solid residue remains in the flask. At this stage bake the flask at the maximum temperature of the hot-plate (*ca.* 260 °C) for at least 2 h after the evolution of fumes has ceased. Transfer the flask into a muffle furnace (450 °C) and allow it to remain there for 1 h. Remove the flask from the furnace and cool. When cold,

Table 1. X-ray spectrometer operating conditions

Tube	Chromium anode operated at 60 kV, 50 mA
Path	Vacuum
Crystal	Lithium fluoride (LiF, $2d = 4{\cdot}028$ Å)
Spinner	On
Collimator	Fine setting (150-μm spacing)
Detector	Flow + scintillation

Angles and counting times—

Element	Angle/ °2θ	Counting time/s	Identification
Te	12.91	20	Te Kα
	12.41	20	Background
Se	31.89	200	Se Kα
	31.39	100	Background
	32.39	100	Background
As	34.00	200	As Kα
	33.50	100	Background
	34.50	100	Background

moisten the residue with water (1 ml) followed by 10 ml of hydrochloric acid (4 + 1) and cover with a watch-glass. When any reaction has subsided add 1 ml of copper(II) sulphate solution and 1 ml of sodium tellurite solution, mixing between additions. Heat the covered flask on a boiling water-bath for 30 min. Remove any insoluble material remaining after cooling by filtration through a pre-washed Whatman 541 filter-paper. It is important that the filter-paper is not washed after the filtration, as this would result in the acidity of the filtrate being reduced, which affects the subsequent reduction. Finally, add 5 ml of hypophosphorous acid to the filtrate and mix. Cover the flask and heat on a boiling water-bath for 30 min then allow to cool.

The precipitated tellurium together with the coprecipitated analytes are collected on a membrane filter. The filter is then dried at a moderate temperature (35–40 °C) prior to presentation to the X-ray spectrometer. The filter is introduced into the X-ray spectrometer, clamped between two masks, preferably copper, in a suitable holder (precipitate adjacent to the X-ray tube). The arsenic, selenium and tellurium Kα intensities together with the appropriate backgrounds are measured using the conditions specified in Table 1.

The nett analyte count is adjusted to compensate for any variation caused by the initial filtration (prior to the coprecipitation stage) and filter placement in the sample holder according to the equation

$$C_{\text{corr.}} = \frac{\text{nett analyte count}}{\text{nett tellurium count}}$$

where the nett counts are the actual count values obtained after correction for the background counts.

The nett corrected analyte count is directly proportional to the amount of analyte in the sample, when due allowance has been made for the analyte content of the reagents used.

Results and Discussion

Sample Matrix Oxidation

The oxidation procedure proposed for the destruction of the organic material in the sample is basically that of Holak,[9] modified in two ways. Firstly, nickel nitrate, which is frequently used in electrothermal atomisation atomic absorption spectrometry to prevent the loss of volatile analytes (particularly arsenic, selenium and tellurium) during pyrolysis stages,[10] was added to the digestion mixture to prevent loss of analyte during the ashing stage. Secondly, the muffle furnace temperature was reduced to 450 °C. The oxidation procedure left a residue consisting of magnesium and nickel oxides and the inorganic material remaining from the sample oxidation together with the analytes. Subsequent work has indicated that analyte recoveries are not reduced by omitting the nickel nitrate from the oxidation media. The main advantage of this sample matrix oxidation was the avoidance of perchloric acid and the hazards associated with its use. The described procedure was found to be effective for all sample types examined (grass, wheat, barley, fruit and leaf and root vegetables).

Separation of the Analytes

Dissolution of the residue

During the development of this procedure it was assumed that the analytes could be present in either of their common oxidation states (*i.e.*, As^{III} or As^{V} and Se^{IV} or Se^{VI}) and the calibration of the procedure must be independent of such oxidation state variations. Examination of the literature[7,11] indicated that considerable difficulties would arise during the reduction to elemental selenium if any selenium was present in the higher oxidation state (Se^{VI}). Selenium(VI) is very difficult to reduce to elemental selenium without prior converison to the lower oxidation state (Se^{IV}). This conversion was achieved by dissolving the residue in hydrochloric acid and digesting on a boiling water-bath as described under Procedure. Prior to the addition of the hydrochloric acid the residue was moistened with approximately 1 ml of water in order to moderate the vigorous reaction between the magnesium nickel oxide and hydrochloric acid. A small amount of material remains undissolved after the hydrochloric acid digestion (primarily silicaceous material from the original sample), which was easily removed by filtration. The advantage of adding the tellurium carrier prior to the hydrochloric acid digestion stage was that any filtration losses [due to solution retention by the filter-paper (typically *ca.* 10%)] were easily corrected for and it was unnecessary to wash the filter (hence the effects of any possible changes in acidity were avoided).

Precipitation of the analytes and tellurium

The quantitative reduction of tellurite to elemental tellurium in hydrochloric acid media was reported[7] to be catalysed by the presence of copper. The effect of copper on the separation (by coprecipitation) of the analytes and the carrier (tellurium) was investigated and found to be advantageous. The effect on arsenic and tellurium was considerable, whereas selenium was only affected to a small extent. At low copper concentrations [those equivalent to 1 ml of a copper(II) sulphate solution containing less than 2.5% *m*/*V* of copper] the main effect was that very variable results were obtained. In addition, the rate of precipitate formation and coagulation was much greater with higher copper(II) sulphate concentrations. When separated on a membrane filter the tellurium precipitate is uniform with the 6% *m*/*V* copper(II) sulphate solution in comparison to the filters obtained with lower concentrations of copper(II) sulphate. Indeed, the over-all result of using lower copper(II) sulphate concentrations was to worsen the mean calibration precision by a factor of up to 7 [1.5% *m*/*V* copper(II) sulphate solution compared with 6% *m*/*V* copper(II) sulphate solution]. Accordingly, a 6% *m*/*V* solution was selected as being appropriate.

The precipitate formed in the presence of 6% *m*/*V* copper(II) sulphate was found to contain, in addition to tellurium and the analytes, approximately 110 μg of copper. An X-ray diffraction study of the precipitate indicated the presence of a copper - tellurium compound. This would suggest that the actual carrier is a copper - tellurium species and not tellurium alone.

Amount of tellurium used as carrier

It was found that the tellurium Kα count rate was linearly related to the amount of tellurium taken in the range 100–2500

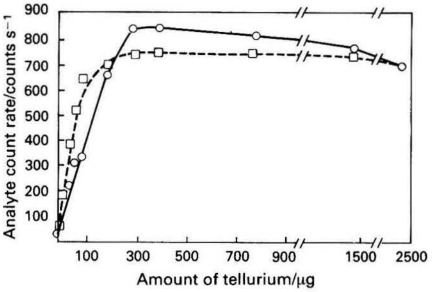

Fig. 1. Effect of the amount of tellurium on the analyte count rate. Analytes, *ca.* 20 µg. ○, Arsenic; and □, selenium

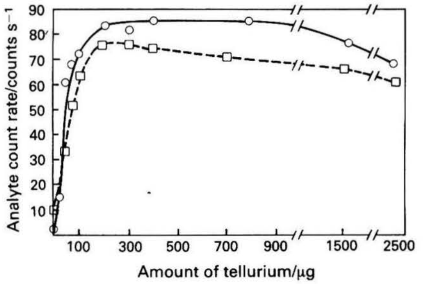

Fig. 2. Effect of the amount of tellurium on the analyte count rate. Analytes, *ca.* 2 µg. ○, Arsenic; and □, selenium

µg. Below 100 µg the results obtained were very erratic. The linear relationship between count rate and the amount of tellurium indicated that the tellurium deposit (on the membrane filter) was behaving as an X-ray thin film. This type of behaviour was expected from theoretical considerations based on mass absorption coefficient calculations. The recovery of the analytes was investigated for various amounts of tellurium with both 2 and 20 µg of the analytes and the results are shown graphically in Figs. 1 and 2. The analyte recovery increased until 200–300 µg of tellurium had been added and thereafter remained constant. Accordingly, 350 µg of tellurium were selected as being appropriate.

Variation of acidity and amount of reductant

Variation of the hydrochloric acid strength between 60 and 100% *V/V* (concentrated) had no effect on the recovery of the analytes. Similarly, variation of the amount of hypophosphorous acid by ±2 ml had no effect on the analyte recovery.

Separation of the precipitated analytes and tellurium

The precipitated carrier (tellurium) together with the coprecipitated analytes were separated on a cellulose nitrate membrane filter. It was found advisable to dry the filter (at *ca.* 40 °C for 1 h) prior to presentation to the X-ray spectrometer. If the filter was not pre-dried, the background count rate varied as the filter dried while in the instrument. An alternative procedure was to allow complete vacuum drying to occur in the spectrometer. If neither approach was adopted, the nett count rates could be significantly lower than the true values (particularly for those filters containing small amounts of the analytes). This effect was due to the wet filter having a considerably greater X-ray scattering capability than a dry filter. Significant reductions in background count rates were achieved by using copper masks to hold the filter in preference to the more usual aluminium masks. This again was due to the lower X-ray scattering by the copper compared with the aluminium. When the filters were introduced into the spectrometer, no loss of precipitate or cockling was observed. If the filters were exposed to the X-ray beam for longer than 30 min then disintegration of the filter was often observed. However, this caused no problem in the procedure described here as the exposure time was approximately 15 min.

Table 2. Results obtained for certified reference materials

	Arsenic content/µg g^{-1}		Selenium content/µg g^{-1}	
Sample	Found	Certified	Found	Certified
NIST SRM 1571				
Orchard Leaves	9.1		≤0.1	
	9.6	10 ± 2*	≤0.1	0.08 ± 0.01*
	9.7		≤0.1	
NIST SRM 1575				
Pine Needles . .	0.20		≤0.1	
	0.15	0.21 ± 0.04*	≤0.1	0.049 ± 0.004‡
	0.19		≤0.1	
NIST SRM 1573				
Tomato Leaves	0.21		≤0.1	
	0.24	0.27 ± 0.05*	≤0.1	0.06 ± 0.02‡
	0.30		≤0.1	
NIST SRM 1572				
Citrus Leaves . .	3.3		≤0.1	
	3.1	3.1 ±0.3*	≤0.1	(0.025)*
	2.8		≤0.1	
NIST SRM 1567				
Wheat Flour . .	≤0.1		1.2	
	≤0.1	(0.006)*	1.2	1.1 ± 0.2*
	≤0.1		1.0	
NIST SRM 1568				
Rice Flour . .	0.49		0.39	
	0.37	0.41 ± 0.05*	0.44	0.4 ± 0.1*
	0.28		0.34	
Bowens' Kale . .	0.12		0.19	
	≤0.1	0.14 ± 0.02†	0.16	0.14 ± 0.01†
	≤0.1		0.15	
NIST SRM 1566				
Oyster Tissue . .	14.8		2.1	
	12.7	13.4 ± 1.9*	2.2	2.1 ± 0.5*
	12.3		1.9	

* Values taken from the NIST certificates supplied with the materials. (Values in parentheses are uncertified.)
† Values from reference 13.
‡ Values from reference 14.

Calibration, precision and accuracy

The sensitivity of the procedure is independent of the oxidation state in which the analytes are added, thus confirming that the procedure adequately handles both oxidation states. Further, the sensitivity is unaffected by the stage at which the analytes are added (if prior to the hydrochloric acid digestion stage); this confirms that the sample oxidation stage with the magnesium nickel nitrate - nitric acid mixture results in no analyte loss when inorganic arsenic and selenium species are taken through the entire procedure. The calibration graph of the tellurium corrected analyte count *versus* analyte amount is linear up to 500 µg and passes through the origin. The mean calibration constant over the range 0.5–25 µg has a precision of better than 3%. The blank value is approximately 0.1 µg with a standard deviation of *ca.* 0.04 µg (based on ten results) for both analytes. Ten replicate determinations were carried out on a grass sample for both analytes: the precision obtained for arsenic was 0.09 µg g^{-1} (at the 0.4 µg g^{-1} level) and for selenium 0.12 µg g^{-1} (at the 4 µg g^{-1} level). This is comparable to that obtained by hydride generation methods.[12]

Results Obtained With Certified Reference Materials

Certified reference materials were analysed for both analytes

Table 3. Results obtained by NAA and the recommended procedure. All results are reported for air-dried (60 °C) samples; the fresh mass results are considerably lower (*ca.* four times for grass and *ca.* ten times for leaf vegetables)

	Arsenic content/ μg g^{-1}		Selenium content/ μg g^{-1}	
Sample	Proposed method	NAA*	Proposed method	NAA*
Grass A	0.43		1.6	
	0.44	0.42	1.7	1.5
	0.52		1.7	
Grass B	0.81		4.9	
	0.91	0.78	4.8	4.5
	0.88		4.6	
Grass C	0.41		≤0.1	
	0.44	0.40	≤0.1	<0.3
	0.40		≤0.1	
Grass D	0.46		≤0.1	
	0.41	0.44	≤0.1	<0.2
	0.51		≤0.1	
Grass E	0.85		7.6	
	0.71	0.63	7.6	7.5
	0.79		7.7	
Grass F	1.7		2.6	
	1.7	1.7	2.8	2.7
	1.7		2.7	
Grass G	1.0		3.6	
	0.9	<0.4	3.6	3.8
	1.0		3.5	
Grass H	1.1		3.0	
	1.3	0.9	2.9	2.6
	1.0		3.0	
Grass I	1.6		4.0	
	1.6	1.5	4.0	3.9
	1.6		4.2	
Leaf broccoli† . .	0.42		17.4	
	0.32	0.20	16.9	17.3
	0.32		16.9	
Spring cabbage†	0.12		8.8	
	0.28	0.14	9.4	9.2
	0.18		8.8	

* Single determination.

† Results expressed on a dry mass basis; results for vegetables are more usually expressed on a fresh mass basis (in which case the results will be approximately ten times lower).

in triplicate; the results are given in Table 2 from which it can be seen that excellent agreement was obtained. Additionally, a number of grass and green leaf vegetables were analysed both by the recommended procedure and by a completely independent procedure [neutron activation analysis (NAA)]. Although NAA is not a reference method, the results obtained (Table 3) with both techniques are in agreement and support the validation of the proposed procedure. Samples were also analysed by the standard additions technique and the results obtained confirmed the reliability of the procedure.

Interferences

From an examination of X-ray wavelength tables, the only significant instrumental interference was thought to be from the lead Lα_1 line on the arsenic Kα line. Both 1000- and 200-μg amounts of lead were taken through the complete procedure and the apparent arsenic content was measured. With 1000 μg of lead a value of *ca.* 0.2 μg of arsenic was obtained, whereas for 200 μg of lead the corresponding arsenic value was <0.1 μg. In this procedure, no specific separation of lead was performed. Clearly, lead is not precipitated during the final separation of the analytes. As the lead content of the samples for which the procedure is proposed is well below 200 μg, the effect of lead can be ignored.

Although the silicaceous content of samples can vary (depending on the climatic conditions during growth), it was not expected to affect the method. The validity of this assumption was confirmed by the results obtained for fly ash. Fly ash is a material that is highly silicaceous (*ca.* 50% SiO_2) and also contains 72 μg g^{-1} of lead. The arsenic and selenium contents found using the proposed procedure (141 ± 3 μg g^{-1} of arsenic and 10.6 ± 0.3 μg g^{-1} of selenium compared to the certified values of 145 ± 15 and 10.3 ± 0.6 μg, respectively) indicate that the presence of a large amount of silicaceous material has no adverse effect. Accordingly it is reasonable to assume that the variable silicaceous content of the grass and vegetable samples will have no effect on the procedure.

Conclusion

The proposed procedure for the determination of arsenic and selenium in herbage and green leaf vegetables (based on separation of the analytes by coprecipitation with tellurium followed by X-ray spectrometric measurement in the form of a thin film) gives good results for samples containing upwards of 0.1 μg g^{-1} of the analytes. The results obtained for reference samples are in agreement with the certified values. The procedure has a detection limit of less than 0.1 μg g^{-1} (two standard deviations of the blank) and a linear calibration up to at least 25 μg g^{-1}. The accuracy and precision of the method are better than 3% relative and are comparable to those of other techniques.

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Alternate Sample/Standard Exchange Method Using a Pair of Identical Ion-selective Electrodes for the Improvement of Precision in an Automated Potentiometric Analysis System

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The use of an automated potentiometric analysis system with a pair of identical ion-selective electrodes as the detector is described. The sample and standard solutions were introduced simultaneously into the flow cell, which was equipped with a pair of identical ion-selective electrodes. The sample and standard streams were then exchanged with each other by using a four-way valve controlled by a microcomputer. The potential difference between the two electrodes was measured before and after switching of the four-way valve. Hence the effect of the potential shift of the ion-selective electrodes could theoretically be cancelled. The characteristics of the system were investigated by using nitrate or chloride ion-selective electrode pairs. A precision of <1% was achieved for five successive measurements when the sample concentration was close to that of the base standard solution.

Keywords: *Nitrate-selective electrode; chloride-selective electrode; automated analysis system; potentiometric analysis; calibration graph drift*

For potentiometric measurements with an ion-selective electrode (ISE) problems may arise due to malfunction of the reference electrode.[1,2] To avoid this, an ISE itself has been utilised as the potential standard[3–7] and in addition an analysis system using two identical ISEs has been designed.[8,9] However, the problem of shift in the standard electrode potential ($E°$) of the ISE has not yet been solved, even in the system without the reference electrode because one electrode of the electrode pair has to maintain a constant potential.

To enhance the reliability of measurement by an ISE, the shift in $E°$ must be eliminated. Before discussion of this problem, the terms used are now defined. "Drift" is the gradual change of a measured potential in one direction and where there is drift no reliable determination will be possible, as the true equilibrium potential cannot be obtained. Even in the absence of drift a "potential shift" might be observed; this is the difference in $E°$ obtained during several calibration runs having a constant slope. The potential shift can be divided into two types, *i.e.*, systematic and non-systematic. Systematic potential shift describes that situation for which the change of $E°$ occurs in one direction; for a non-systematic potential shift no such systematic change is observed. If the $E°$ and slope values change at random, the situation will be described as a "fluctuational variation of a calibration graph." Although the term drift has been used not only as the long-term variation of a measured potential but also as the monotonous variation of $E°$ for repeated calibration runs, these two concepts should be clearly distinguished.

In this paper, the use of a potentiometric measurement system with a pair of identical ISEs is proposed. The main feature of the system is that each electrode alternately plays the role of the reference electrode by exchanging the flows of the sample and base standard solutions by means of a four-way valve. Consequently, the shift of $E°$ can theoretically be cancelled. A microcomputer was used to control the four-way valve and data acquisition and the characteristics of the system were investigated by using two nitrate or chloride ISEs.

Experimental

Apparatus

The following four combinations of electrodes were used: nitrate ion-selective electrode (Orion Model 93-07) pairs; laboratory-made tubular silver - silver chloride electrode pairs for the alternate sample/standard exchange method; nitrate or chloride ISE and reference electrode (Orion Model 90-02-00) pairs for the flow method or batchwise measurement.

The silver - silver chloride electrodes were prepared by electro-oxidation of the inner surface of a silver tube (20 × 1 mm i.d.) in 1 M potassium chloride solution. The configuration of the flow cell was the same as that used previously[9] (transparent acrylic resin, 7.5 cm long, 7 cm high and 3 cm wide). No common metallic electrode was used in this system. For the batchwise measurement of nitrate, the internal filling solution of a double-junction type reference electrode was 2 M ammonium sulphate.[10] The potentials were measured at room temperature (25 ± 1 °C) using a digital ion meter (Orion Model 701A) connected through a differential amplifier (precision, 0.1 mV)[9] and were recorded by an analogue pen recorder (Rikadenki Model R-11). The values of potential were transmitted every 0.6 s into a microcomputer (NEC PC8801 MkII; CPU, 8-bit μPD780C-1, Z80A compatible) through an 8-bit parallel I/O interface. The switch-over of an automatic four-way valve (Kusano Kagakukikai Model KAV-4L) was controlled through the general I/O port of the microcomputer. A digital printer (Brother Model HR-5X) was used to record the response time and the equilibrium potential and a four-channel peristaltic pump (Atto Model SJ1220) was used for the sample and standard solutions. Single-channel peristaltic pumps (Atto Model SJ1221H and SJ1221L) were used for solutions of the ionic strength adjuster (ISA) and the flow-rates were adjusted so that they were identical for both lines of ISA.

Reagents

Both sample and standard solutions were prepared from guaranteed-reagent grade potassium nitrate or chloride from Nacalai tesque Chemicals (Kyoto, Japan). They differed only in that the concentration of the sample was varied whereas that of the standard was fixed. The ISAs were 0.44 M ammonium sulphate for nitrate and 1 M potassium nitrate for chloride. For the batchwise measurement, 1 ml of 2 M ammonium sulphate was added to 50 ml of sample solution.[10]

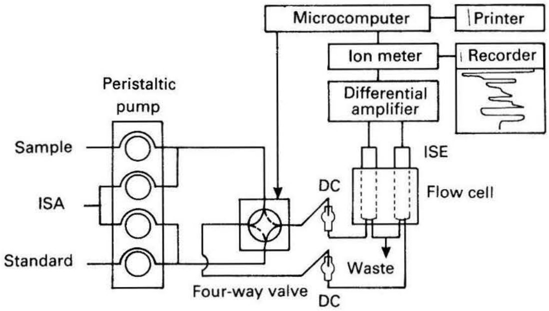

Fig. 1. Schematic diagram of the alternate sample/standard exchange system. ISA, Ionic strength adjuster; DC, drop chamber; and ISE, ion-selective electrode

Alternate Sample/Standard Exchange System

Fig. 1 shows a schematic diagram of the alternate sample/standard exchange system for a microcomputer-controlled automated analysis system. The sample and standard solutions were introduced into the flow cell alternately via an automatic four-way valve. The potential difference between the electrodes was measured every 0.6 s by the ion meter, which was connected through the differential amplifier. When the potential change over an approximately 30-s period fell below 0.3 mV, the mean of the last five potential values was printed out as an equilibrium potential and switch-over of the four-way valve occurred. The potential difference was then measured again in a similar manner. The two apparent equilibrium potentials obtained for the same standard/sample pair comprised one data set; the difference between the potential values was used to construct a calibration graph. The sample was then changed and the equilibrium potentials were measured twice, both before and after the switch-over.

Principle of the System

A microcomputer was used to handle the measured potentials and to control switch-over of the four-way valve. The entire procedure was coded by the N88 Disk BASIC.

The electrode potential (E) of each ion-selective electrode (A and B) can be expressed by the usual Nernstian equations

$$E_A = E^{\circ}_A - S_A \log c_A \quad \ldots \quad (1)$$

and

$$E_B = E^{\circ}_B - S_B \log c_B \quad \ldots \quad (2)$$

where S is the slope and c the concentration. In this system, it is the potential difference between each ion-selective electrode that is measured and not the electrode potentials themselves, measured against the reference electrode

$$dE^{I} = E_A - E_B = (E^{\circ}_A - E^{\circ}_B) - S_A \log c_A + S_B \log c_B \quad (3)$$

After the switch-over, the concentrations of the two solutions are exchanged

$$E_A' = E^{\circ}_A - S_A \log c_B \quad \ldots \quad (4)$$

$$E_B' = E^{\circ}_B - S_B \log c_A \quad \ldots \quad (5)$$

where E_A' and E_B' are the electrode potentials after switch-over.

The measured potential difference is as follows:

$$dE^{II} = E_A' - E_B' = (E^{\circ}_A - E^{\circ}_B) - S_A \log c_B + S_B \log c_A \quad (6)$$

(Superscripts I and II refer to the potentials of the sample solution before and after switch-over, respectively.)

Finally, the potential difference between dE^{I} and dE^{II} is

$$\Delta E = dE^{I} - dE^{II} = (S_A + S_B)\log(c_B/c_A) \quad \ldots \quad (7)$$

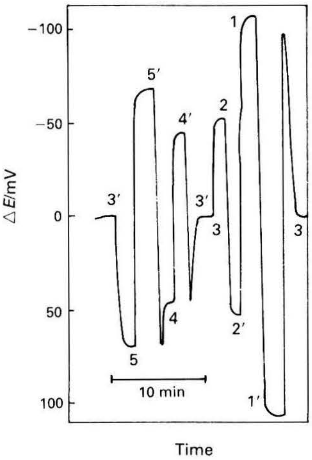

Fig. 2. Continuous-flow diagram of the alternate sample/standard exchange system with two nitrate electrodes. Concentration of the base standard solution, 10^{-3} M. Sample concentration: 1 and 1′, 1×10^{-1} M; 2 and 2′, 1×10^{-2} M; etc. Flow-rate: nitrate solutions, 3 ml min^{-1}; and ISA [0.44 M $(NH_4)_2SO_4$], 0.33 ml min^{-1}

The plot of the logarithmic concentration of sample B ($\log c_B$) against ΔE should be linear with a slope about twice as large as that of a normal Nernstian slope. The value of ΔE is independent of E° and this is the most important feature of the system. It should be noted that, for both electrodes, the constancy of E° and the slope was expected both before and after the switch-over.

Calculation of Concentration

The calibration graph was constructed using the measurements obtained from five standard solutions in the concentration range 10^{-5}–10^{-1} M. The concentration of a sample solution was calculated by the Spline interpolation method using a plot of ΔE *versus* $\log c_{NO_3}$.[11] The calibration procedure was repeated six times and the first run was chosen as the calibration graph for the next five runs. The accuracy and precision of the potentials measured at each concentration level were calculated from the error and standard deviation of five replicate measurements for each concentration.

Results and Discussion

Calibration Graph

Fig. 2 shows the continuous-flow diagram of the proposed system with two nitrate electrodes. In this diagram, a potential difference of zero indicates that the sample solution was 10^{-3} M in nitrate because the concentration of the base standard solution was chosen to be 10^{-3} M. Pairs of peaks in opposite directions were used to construct a calibration graph. The undesirable peaks observed, for example, the first peak of 10^{-4} M nitrate (Fig. 2, peak 4), were caused by the time delay in new solutions reaching the electrode surfaces. Fig. 3 shows the calibration graph, which was constructed from this flow diagram (Fig. 2). Although the limit of the linear response region was not necessarily improved, the slope was −107.1 mV decade^{-1}, which was lower than the sum of the slope values (−114.8 mV decade^{-1}) obtained for the batch-wise measurements.

Effect of Flow-rate

Table 1 gives the potential differences (ΔE) obtained with various flow-rates (2–6 ml min^{-1}). No significant dependence

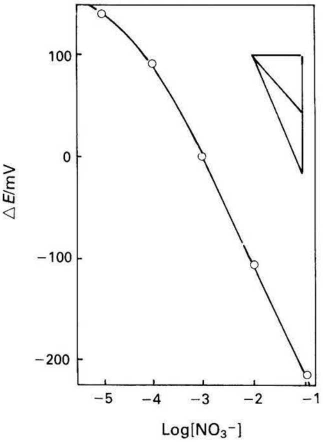

Fig. 3. Calibration graph obtained using the alternate sample/standard exchange system. The conditions are as in Fig. 2. The small triangles inset indicate Nernstian (59 mV decade^{-1}) and bi-Nernstian (118 mV decade^{-1}) slopes

Table 1. Effect of flow-rate on the measured potential difference (in mV) between two nitrate electrodes before and after switch-over of the four-way valve. Concentration of the base standard solution of nitrate, 10^{-3} M

Concentration of the sample solution/M	Flow-rate/ml min^{-1}				
	2	3	4	5	6
10^{-5}	135.5	141.3	143.1	139.9	143.9
10^{-4}	91.2	91.3	92.9	90.6	92.1
10^{-3}	−0.1	−0.2	0.2	0.0	−0.2
10^{-2}	−108.0	−107.2	−107.7	−104.6	−107.1
10^{-1}	−216.3	−214.3	−216.6	−211.2	−215.0

Table 2. Effect of concentration of the base standard solution of nitrate on the measured concentration of the sample and standard deviation (SD) of the alternate sample/standard exchange system with two nitrate electrodes. Flow-rate: standard, 3 ml min^{-1}; ISA [0.44 M $(NH_4)_2SO_4$], 0.3 ml min^{-1}

Concentration of the base standard solution/M	Concentration of the sample solution/M	Measured concentration (mean ± SD)/M	Error, %
10^{-1}	10^{-5}	$(0.862 \pm 0.186) \times 10^{-5}$	−13.8
	10^{-4}	$(1.080 \pm 0.0283) \times 10^{-4}$	+8.0
	10^{-3}	$(1.020 \pm 0.0141) \times 10^{-3}$	+2.0
	10^{-2}	$(1.018 \pm 0.0045) \times 10^{-2}$	+1.8
	10^{-1}	$(1.000 \pm 0.0009) \times 10^{-1}$	0.0
10^{-2}	10^{-5}	$(1.072 \pm 0.193) \times 10^{-5}$	+7.2
	10^{-4}	$(1.038 \pm 0.0892) \times 10^{-4}$	+3.8
	10^{-3}	$(1.014 \pm 0.0238) \times 10^{-3}$	+1.4
	10^{-2}	$(0.992 \pm 0.0041) \times 10^{-2}$	−0.8
	10^{-1}	$(0.992 \pm 0.0208) \times 10^{-1}$	−0.8
10^{-3}	10^{-5}	$(0.662 \pm 0.107) \times 10^{-5}$	−33.8
	10^{-4}	$(0.898 \pm 0.0460) \times 10^{-4}$	−10.2
	10^{-3}	$(0.978 \pm 0.0031) \times 10^{-3}$	−2.2
	10^{-2}	$(1.044 \pm 0.0089) \times 10^{-2}$	+4.4
	10^{-1}	$(0.986 \pm 0.0097) \times 10^{-1}$	−1.4
10^{-4}	10^{-5}	$(1.027 \pm 0.0773) \times 10^{-5}$	+2.7
	10^{-4}	$(0.999 \pm 0.0031) \times 10^{-4}$	−0.1
	10^{-3}	$(0.975 \pm 0.0184) \times 10^{-3}$	−2.5
	10^{-2}	$(0.975 \pm 0.0241) \times 10^{-2}$	−2.5
	10^{-1}	$(1.012 \pm 0.0516) \times 10^{-1}$	+1.2
10^{-5}	10^{-5}	$(1.005 \pm 0.0246) \times 10^{-5}$	+0.5
	10^{-4}	$(0.950 \pm 0.0231) \times 10^{-4}$	−5.0
	10^{-3}	$(0.912 \pm 0.0611) \times 10^{-3}$	−8.8
	10^{-2}	$(0.913 \pm 0.0575) \times 10^{-2}$	−8.7
	10^{-1}	$(0.900 \pm 0.0749) \times 10^{-1}$	−10.0

Table 3. Potential shift (mV) for six successive calibration runs for nitrate solutions obtained with the batch method, the flow method with a nitrate - reference electrode pair and the alternate sample/standard exchange method with two nitrate electrodes

	Concentration of the sample solution/M	n–(n − 1)*				
		2 − 1	3 − 2	4 − 3	5 − 4	6 − 5
1. *Batch method—*						
Electrode						
A ..	10^{-1}	−2.7	+1.1	0.0	−0.9	+0.5
	10^{-2}	−3.4	+1.1	+0.1	−1.3	+0.9
	10^{-3}	−3.1	+1.1	−0.3	−2.4	+2.1
B ..	10^{-1}	−2.1	−1.6	+2.9	−0.1	−2.4
	10^{-2}	−1.5	−1.8	+2.0	−0.6	−1.1
	10^{-3}	−1.6	−1.8	+2.2	−0.6	−1.0
2. *Flow method with nitrate - reference electrode pair—*						
Electrode						
A ..	10^{-1}	−0.6	−0.1	+0.1	0.0	0.0
	10^{-2}	−0.6	+0.1	+0.3	0.0	+0.3
	10^{-3}	0.0	−0.1	+0.4	+0.2	+0.5
B ..	10^{-1}	+0.4	0.0	+0.2	+0.3	−0.4
	10^{-2}	+0.8	0.0	+0.2	+0.1	−0.1
	10^{-3}	+1.1	+0.1	+0.3	+0.1	−0.1
3. *Alternate sample/standard exchange method with two nitrate electrodes†—*						
Flow-rate/ ml min^{-1}						
3	10^{-1}	+1.8	+0.2	−0.5	+0.6	+1.2
	10^{-2}	−0.3	+0.2	−0.1	+0.1	+1.1
	10^{-3}	+0.1	−0.1	0.0	0.0	+0.5
5	10^{-1}	+1.2	−0.8	0.0	+1.4	+0.7
	10^{-2}	+0.3	+0.1	−0.2	+0.3	+1.3
	10^{-3}	−0.4	−0.2	+0.2	+0.2	+0.4

* Potential difference between nth and (n − 1)th calibration runs.
† Concentration of base standard solution, 10^{-3} M.

of ΔE on flow-rate was observed at any concentration. A flow-rate of 3 ml min^{-1} was used in all further experiments unless stated otherwise.

Effect of Base Concentration

Table 2 gives the standard deviation (SD) and error for five replicate measurements of standard solutions of nitrate at five different base concentrations of standard solution. The best reproducibility of the measured potentials was observed when the concentration of the sample solution was the same as that of the base standard solution except for a base standard solution concentration of 10^{-5} M. In this instance, the measured potential before and after switch-over of the four-way valve should always be almost zero, as found previously in so-called "null-point potentiometry."[12] The standard deviation increased with an increase in the concentration difference between the sample and the base standard solutions. This effect is most pronounced in the lower concentrations as shown typically for a 10^{-3} M solution. The error was also affected by the reproducibility of the measured potentials; the lowest error was observed at the same concentration as that of the base standard solution. Relatively good precision and accuracy can be obtained in the concentration range 0.1–10 times the concentration of the base standard solution. The precision was less good when the ratio of the concentrations of the sample and base solutions exceeded 100 and it became more significant in the direction of the lower concentrations.

Comparison With the Batch Method or Flow Method Using a Reference Electrode

In order to understand the type and degree of the potential

Table 4. Comparison of the results obtained by the different methods. The calibration was performed six times with standard solutions of nitrate in the range 10^{-5}–10^{-1} M. The concentration of each standard solution was calculated from the first calibration graph; the mean and SD of five replicate measurements are given. Concentration of the base standard solution, 10^{-3} M

Concentration of the sample solution/M	Electrode A Measured concentration (mean ± SD)/M	Error, %	Electrode B Measured concentration (mean ± SD)/M	Error, %
1. *Batch method—*				
10^{-5}	$(1.146 \pm 0.0963) \times 10^{-5}$	+14.6	$(0.971 \pm 0.0835) \times 10^{-5}$	−2.9
10^{-4}	$(1.182 \pm 0.0517) \times 10^{-4}$	+18.2	$(1.001 \pm 0.0341) \times 10^{-4}$	+0.1
10^{-3}	$(1.126 \pm 0.0503) \times 10^{-3}$	+12.6	$(1.094 \pm 0.0404) \times 10^{-3}$	+9.4
10^{-2}	$(1.116 \pm 0.0279) \times 10^{-2}$	+11.6	$(1.090 \pm 0.0387) \times 10^{-2}$	+9.0
10^{-1}	$(1.082 \pm 0.0228) \times 10^{-1}$	+8.2	$(1.086 \pm 0.0532) \times 10^{-1}$	+8.6
2. *Flow method with nitrate - reference electrode pair—*				
10^{-5}	$(1.154 \pm 0.138) \times 10^{-5}$	+15.4	$(0.834 \pm 0.0568) \times 10^{-5}$	−16.6
10^{-4}	$(1.001 \pm 0.0449) \times 10^{-4}$	+0.1	$(0.888 \pm 0.0100) \times 10^{-4}$	−11.2
10^{-3}	$(0.984 \pm 0.0179) \times 10^{-3}$	−1.6	$(0.938 \pm 0.0095) \times 10^{-3}$	−6.2
10^{-2}	$(1.013 \pm 0.0127) \times 10^{-2}$	+1.3	$(0.960 \pm 0.0054) \times 10^{-2}$	−4.0
10^{-1}	$(1.026 \pm 0.0022) \times 10^{-1}$	+2.6	$(0.977 \pm 0.0083) \times 10^{-1}$	−2.3
3. *Alternate sample/standard exchange method with two nitrate electrodes—*				
10^{-5}	$(1.246 \pm 0.158) \times 10^{-5}$*	+24.6	$(1.240 \pm 0.0628) \times 10^{-5}$†	+24.0
10^{-4}	$(1.054 \pm 0.0251) \times 10^{-4}$*	+5.4	$(1.044 \pm 0.0371) \times 10^{-4}$†	+4.4
10^{-3}	$(0.998 \pm 0.0043) \times 10^{-3}$*	−0.2	$(1.005 \pm 0.0067) \times 10^{-3}$†	+0.5
10^{-2}	$(0.998 \pm 0.0110) \times 10^{-2}$*	−0.2	$(0.986 \pm 0.0139) \times 10^{-2}$†	−1.4
10^{-1}	$(0.957 \pm 0.0133) \times 10^{-1}$*	+2.6	$(0.973 \pm 0.0187) \times 10^{-1}$†	−2.7

* Flow-rate, 3 ml min^{-1}.
† Flow-rate, 5 ml min^{-1}.

Table 5. Results for the alternate sample/standard exchange system with two nitrate electrodes in the low-concentration range. Concentration of the base standard solution of nitrate, 10^{-4} M. Flow-rate: standard, 3 ml min^{-1}; and ISA, 0.3 ml min^{-1}

Concentration of the sample solution/M	Measured concentration (mean ± SD)/M	Error, %
10^{-5}	$(1.034 \pm 0.0363) \times 10^{-5}$	+3.4
10^{-4}	$(1.006 \pm 0.0055) \times 10^{-4}$	+0.6
10^{-3}	$(0.971 \pm 0.0108) \times 10^{-3}$	−2.9

shift, the potential differences were measured between the *n*th and $(n-1)$th calibration run for each concentration; these are listed in Table 3 for three different methods. In the batch method (1), the sign of the potential difference for two successive calibration runs was independent of the sample concentration in almost every instance. The sign was changed from one calibration run to the next. This result indicated that a non-systematic potential shift had occurred; however, this shift was <3 mV. The mean values of $E°$ and the slope for concentrations in the range 10^{-1}–10^{-3} M were −58.62 mV (SD = 0.863 mV) and −58.99 mV decade^{-1} (SD = 0.383 mV decade^{-1}), respectively, for electrode A and −54.28 mV (SD = 1.602 mV) and −59.05 mV decade^{-1} (SD = 0.279 mV decade^{-1}), respectively, for electrode B. Although the response of both electrodes was completely Nernstian, a small fluctuation was observed in the values of the slope.

In the flow method (2), the potential shift appeared to be systematic, although its magnitude was small. The mean values of $E°$ and the slope were −9.26 mV (SD = 0.494 mV) and −54.70 mV decade^{-1} (SD = 0.270 mV decade^{-1}), respectively, for electrode A and −8.28 mV (SD = 0.195 mV) and −53.69 mV decade^{-1} (SD = 0.177 mV decade^{-1}), respectively, for electrode B. The reproducibilities of $E°$ and the slope were better than those achieved with the batch method. The reference electrode potential drift also appeared to be insignificant in this instance. The slope values obtained were sub-Nernstian for both electrodes. However, electrode B gave better reproducibilities for both $E°$ and the slope than electrode A.

In the alternate sample/standard exchange method (3), the potential shift tended to fluctuate. The mean values of $E°$ and the slope were −336.84 mV (SD = 1.426 mV) and −112.2 mV decade^{-1} (SD = 0.467 mV decade^{-1}), respectively, for a flow-rate of 3 ml min^{-1} and −314.74 mV (SD = 1.375 mV) and −105.0 mV decade^{-1} (SD = 0.413 mV decade^{-1}), respectively, for a flow-rate of 5 ml min^{-1}. The reproducibilities of $E°$ and the slope fell between those obtained with methods 1 and 2 as judged from the values of the SDs [in the last method (3) the slope is twice that of the other two methods]. The effect of flow-rate on the SDs of both $E°$ and the slope could also be ignored.

Table 4 gives the results of the calibration experiment using the three different methods. Each calibration was performed six times in succession and the concentration of each sample was calculated from runs 2–6 using the calibration graph constructed from the data of the first calibration run. The mean values with the SDs for five replicate determinations at five different concentrations are listed in Table 4.

In the batch method (1), the dependence of the SD on the concentration is not as marked as for the other two methods. The fluctuation of the measured potentials appeared to occur at each concentration and to be highest in the most diluted samples (*i.e.*, 10^{-5} M).

In method 2, the dependence of the SD on the concentration was clearly observed, *i.e.*, the higher was the concentration, the lower was the value of the SD.

In method 3 also, there existed a dependence of the SD on concentration; however, in this instance the minimum value occurred at 10^{-3} M. In the alternate sample/standard exchange method, the reproducibility of the potential was optimum when the sample concentration was the same as that of the base standard solution (see Table 2). In method 2, the optimum concentration was always the most concentrated. Hence the optimum concentration could be chosen in the alternate sample/standard exchange method according to the range of sample concentrations; this is the most significant characteristic of the method.

Results in the Low-concentration Range

Table 5 gives the results obtained when the base standard solution concentration was 10^{-4} M. The accuracy and precision of five successive measurements were improved compared with the results presented in Table 4. The alternate sample/

Table 6. Comparison of the results obtained using the flow method with one tubular silver - silver chloride electrode - reference electrode pair and the alternate sample/standard exchange method with two tubular silver - silver chloride electrodes (A and B). Concentration of base standard solution of chloride, 10^{-3} M. Flow-rate: standard, 3 ml min^{-1}; and ISA (1 M KNO_3), 0.3 ml min^{-1}

Concentration of the sample solution/M	Electrode A Measured concentration (mean ± SD)/M	Electrode A Error, %	Electrode B Measured concentration (mean ± SD)/M	Electrode B Error, %
Flow method with chloride - reference electrode pair—				
10^{-5}	$(1.091 \pm 0.0837) \times 10^{-5}$	+9.1	$(1.206 \pm 0.0808) \times 10^{-5}$	+20.6
10^{-4}	$(1.054 \pm 0.0329) \times 10^{-4}$	+5.4	$(1.056 \pm 0.0391) \times 10^{-4}$	+5.6
10^{-3}	$(1.034 \pm 0.0134) \times 10^{-3}$	+3.4	$(1.031 \pm 0.0454) \times 10^{-3}$	+3.1
10^{-2}	$(1.052 \pm 0.0277) \times 10^{-2}$	+5.2	$(1.036 \pm 0.0422) \times 10^{-2}$	+3.6
10^{-1}	$(1.058 \pm 0.0363) \times 10^{-1}$	+5.8	$(1.023 \pm 0.0480) \times 10^{-1}$	+2.3
Alternate sample/standard exchange method with two chloride electrodes (A and B)—				
10^{-5}	$(1.088 \pm 0.0526) \times 10^{-5}$	+8.8		
10^{-4}	$(1.025 \pm 0.0514) \times 10^{-4}$	+2.5		
10^{-3}	$(0.998 \pm 0.0073) \times 10^{-3}$	−0.2		
10^{-2}	$(0.987 \pm 0.0081) \times 10^{-2}$	−1.3		
10^{-1}	$(0.987 \pm 0.0087) \times 10^{-1}$	−1.3		

Table 7. Potential shift (mV) for six successive calibration runs in the flow method with a tubular chloride - reference electrode pair and alternate sample/standard exchange method with two tubular chloride electrodes. Concentration of the base standard solution of chloride, 10^{-3} M

	Concentration of the sample solution/M	$n-(n-1)$* 2 − 1	3 − 2	4 − 3	5 − 4	6 − 5
Flow method with tubular chloride - reference electrode pair—						
Electrode						
A	10^{-1}	−0.5	−0.4	−0.1	−1.5	−0.5
	10^{-2}	−0.5	−0.2	−0.8	−0.7	+0.8
	10^{-3}	−0.4	−0.2	−0.8	+0.3	0.0
	10^{-4}	−0.3	−0.4	−1.1	+0.4	0.0
B	10^{-1}	0.0	−2.4	+1.4	+1.4	−0.2
	10^{-2}	−0.4	−1.5	0.0	+2.2	−0.5
	10^{-3}	−0.4	−1.1	−0.6	+2.8	−0.9
	10^{-4}	−1.3	−0.2	−0.4	+1.5	+0.1
Alternate sample/standard exchange method with two tubular chloride electrodes—						
	10^{-1}	+1.1	−0.9	+0.9	−0.6	−0.2
	10^{-2}	+0.6	−0.4	+1.1	−0.8	+0.1
	10^{-3}	+0.4	−0.6	+0.5	−0.6	+0.6
	10^{-4}	−0.5	+1.4	−1.9	+1.2	−4.5

* Potential difference between the *n*th and $(n-1)$th calibration runs.

standard exchange method appears to compensate not only for the shift but also, at least in part, for the fluctuational variation of each calibration run. The results obtained in the low-concentration range clearly indicate the usefulness of the alternate sample/standard exchange method.

Results Obtained With Tubular Silver - Silver Chloride Electrodes

In order to demonstrate the general applicability of the method another electrode system, which consisted of tubular silver - silver chloride electrodes, was used. Six calibration runs were performed using the flow method with a reference electrode and the alternate sample/standard exchange method with two tubular chloride electrodes. The mean values of E° and the slope for the flow method with a reference electrode were −63.46 mV (SD = 1.053 mV) and −56.98 mV decade^{-1} (SD = 0.224 mV decade^{-1}), respectively, for electrode A and −63.12 mV (SD = 1.242 mV) and −56.87 mV decade^{-1} (SD = 0.253 mV decade^{-1}), respectively, for electrode B in the flow method using a reference electrode. The mean values of E° and the slope were −345.06 mV (SD = 1.206 mV) and −114.61 mV decade^{-1} (SD = 0.616 mV decade^{-1}), respectively, in the alternate sample/standard exchange method. For E°, the SD was apparently lower in the latter method; however, the SD of the slope was higher.

Tables 6 and 7 show the results of the determination using both of these methods and the potential shift between the two successive calibration runs, respectively. For the flow method, no significant dependence of the SD on the sample concentration was observed except for the 10^{-5} M chloride solution and the potential shift in this instance was non-systematic. The reproducibility of the measurements was clearly improved in the alternate sample/standard exchange method. This result may also demonstrate the usefulness of the alternate sample/standard exchange method.

The alternate sample/standard exchange method may be effective enough to improve the reproducibility of the potential measurements even for a non-systematic potential shift. However, the magnitude of the potential shift was small for the results given in Tables 3, 4, 6 and 7, which utilise nitrate or chloride electrodes together with a reference electrode. Further applications of this method to the typical situation for which the potential shift is systematic may be necessary to prove the usefulness of the method.

Possibly, the disadvantage of the method is that the time required for calibration is almost twice that of the flow method with a reference electrode because of switch-over of the standard and sample solutions. However, there is the advantage of the relatively high precision for direct potentiometric measurements.

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Hybrid Multiple Standard Additions - Analyte Addition Method for Ion-selective Electrodes With Integral Calibration

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A hybrid multiple standard additions - analyte addition method for ion-selective electrodes is described in which the calibration of the potentiometric cell and the determination of the unknown concentration of the sample are carried out in a single experiment. This ensures maximum constancy of the calibration characteristics, $E^{\circ\prime}$ and S, of the cell. The proposed method allows the determination of trace amounts (100 µg) of a sample. The data treatment includes an iterative minimisation procedure using the least-squares sum method, which improves the accuracy and the statistical reproducibility of the results. The method can be automated by means of an automatic titrator, controlled by an HP-85B microcomputer. A BASIC program was constructed for this purpose. The method was tested by applying it to the determination of sodium, potassium, chloride and fluoride ions and the errors of the results were less than 1%. The limit of determination of the method was investigated by determining decreasing concentrations of fluoride ions. It was found that the method gives satisfactory results over the entire linear range of the ion-selective electrode.

Keywords: *multiple standard additions; analyte addition; automatic potentiometric determination; integral calibration method; ion-selective electrode analysis*

Ion-selective electrodes (ISEs) have been widely used in recent years due to the high quality of the commercially available ISEs and to the large number of ions that can be determined with these electrodes. These are the basic pre-conditions for the future development and improvement of the analytical methods involving ISEs. In addition to classical direct potentiometry, the incremental methods have now found extensive application.[1] Their principles are encompassed in most of the commercially available ion analysers (*e.g.*, Metrohm Titroprocessor, Mettler DL 40 GP Memo-Titrator, Orion 960 Autochemistry System, Radiometer Ion-85 Ionanalyzer). The incremental method most used is the standard (or known) additions method. This method usually requires preliminary information about the electrode slope (calibration) and about the influence of the ionic strength adjustor ("blank" sample). The need for a preliminary calibration can be prevented if two or more additions are made (the so-called "double" and multiple known additions—MKA, also known as the Gran titration[2]). In situations where a Gran titration is performed, an overdefinite set of equations for the potential of the potentiometric cell is obtained. The calibration constants of the cell, $E^{\circ\prime}$ and S, and the unknown concentration of the sample, c_x, can be determined by applying a non-linear fitting procedure to the experimental data by means of a computer.[3,4] All of the standard additions methods, however, require evaluation of the "blank" sample volume, which is carried out in a separate experiment.

In this paper a hybrid multiple standard additions - analyte addition (MSA-AA) method for analysis with ISEs is proposed, which allows calibration of the cell and determination of the sample concentration in a single experiment without the need for a "blank" sample determination. Basically, the method is carried out as follows: in a vessel, containing a suitable ionic strength adjustor, the titration with a standard solution of the ion to be determined is started, then the sample is dosed and the titration is continued. The experimental data are treated according to the Nernst equation by means of a computer until the best fit is obtained. An automatic variant of the method is also proposed, which is performed by means of an automatic titrator controlled by an HP-85B microcomputer.

Experimental

Reagents

Analytical-reagent grade sodium chloride, sodium nitrate, potassium chloride, potassium fluoride, potassium nitrate and calcium nitrate (Merck) were used after drying at the appropriate temperatures (105, 105, 105, 150, 105 and 170 °C, respectively).

Apparatus

Potentiometric measurements were carried out with a Radiometer PHM-84 digital pH meter, accurate to 0.1 mV, and with the following electrodes, also manufactured by Radiometer: G502Na, F2312K, F1521Cl and F1052F indicator selectrodes and K401, K701 and K801 reference electrodes. A Radiometer ABU-80 automatic burette, accurate to 0.001 ml, and an Eppendorf 4710 Varipette piston pipette, accurate to 0.6%, were employed for solution dosing. The measurements with the ISEs were performed according to the conditions recommended in the manufacturer's instructions.[5] The experimental data were treated by means of a Hewlett-Packard HP-85B microcomputer. The automatic titrations were carried out with the same apparatus, controlled by the computer by means of a BCD interface, according to the configuration specified by the manufacturer.

Outline of the Method

A known volume V_0 of a suitable ionic strength adjustor (in which a zero concentration of the analyte ion is assumed) is titrated by adding equal amounts of a standard solution of the ion to be determined, with a concentration c. After several additions, the sample, with an unknown concentration c_x, is dosed using a piston pipette (volume V_x) after which further standard additions are made. The potential of the cell, which consists of a suitable sensing - reference electrode pair, follows the Nernst equation.

(*i*) For the measurements made before sample dosing

$$E_i = E^{\circ\prime} + S\log\left[\frac{c(iV)}{V_0+(iV)}\right] \quad \ldots \quad (1)$$

where E_i is the measured potential; $E^{\circ\prime}$ is the conditional standard potential of the electrode pair; S is the slope of the indicator electrode; and $i = 1,2, \ldots, n_1$ is the number of standard additions made before sample dosing.

(*ii*) For the measurement made immediately after the sample has been dosed

$$E_x = E^{\circ\prime} + S\log \frac{c(n_1V) + c_xV_x}{V_0 + (n_1V) + V_x} \quad \ldots \quad (2)$$

(*iii*) For the measurements made after sample dosing

$$E_j = E^{\circ\prime} + S\log \frac{c(n_1V) + c_xV_x + c(jV)}{V_0 + (n_1V) + V_x + (jV)} \quad \ldots \quad (3)$$

where $j = 1,2, \ldots, n_2$ is the number of standard additions made after sample dosing.

The experimental data, ml/mV (m values, where $m = n_1+1+n_2$), are treated in the following manner. (*i*) For the initial n_1 pairs, subjected to equation (1), a least-squares procedure is used so that the calibration constants of the cell, $E^{\circ\prime}$ and S, can be determined. As the linear regression is only performed on part of the experimental data, the results obtained for $E^{\circ\prime}$ and S are "rough" values and they are used as starting values in the following operations. (*ii*) By means of the "rough" $E^{\circ\prime}$ and S values, and from the potential measured immediately after the sample has been dosed, a "rough" value for the unknown concentration of the sample, c_x, is calculated from equation (2). (*iii*) An iterative minimisation procedure using all the experimental data is performed, equation (3). The "rough" value obtained for c_x is used as a starting value and is varied in decreasing steps, the first of which is 5% of c_x. The number of variations in each direction is five, which gives 11 varied values for c_x in one iteration. For each varied value of c_x a linear regression is performed on all the experimental data, equation (3), and the error-square sum is calculated as follows:

$$U = \sum_{i=1}^{m} (E_{\text{meas.}} - E_{\text{calc.}})^2 \quad \ldots \quad (4)$$

This value for c_x, which gives the smallest error-square sum, is taken as a starting value, the step is decreased five times and a new iteration is performed as described above. Iterations are repeated until the step for the variations decreases to 0.01% of the "rough" value initially found for c_x. The results obtained for $E^{\circ\prime}$, S and c_x after the last iteration and their standard deviations are taken as the final values.

A BASIC program for a Hewlett-Packard HP-85B microcomputer was constructed, which performs the calculations within 1–2 min and presents the final results in numerical and graphical form. The flow-chart of the program is shown in Fig. 1.

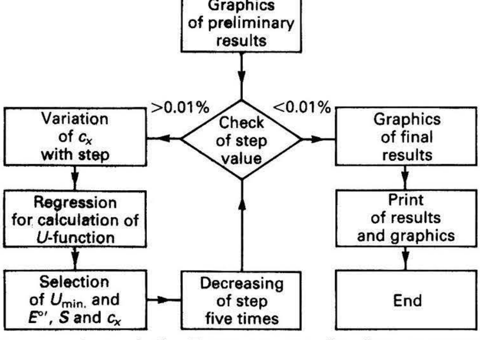

Fig. 1. Flow-chart of the BASIC program for data treatment according to the proposed method

Results and Discussion

The proposed method was tested by determining sodium, potassium, chloride and fluoride ions, where the same solutions were used both as the sample and the standard. In order to illustrate the method, the determination of K^+ is discussed. The experimental data and the conditions of the determination are presented in Table 1. The third column shows the time after which the potential was read. Fig. 2 gives the "rough" values found for $E^{\circ\prime}$ and S after regression of the experimental data before sample dosing [see equation (1)]. Also shown is the 'rough" value for c_x, obtained from equation (2), which was used as the starting value in the procedure of consecutive approximations.

The "rough" value of c_x differs from the true value in all instances. In this instance the difference is 1.54%, which can be explained by the fact that the determination of c_x is based on a single measurement. Table 2 presents the development of the iterative minimisation procedure, which consists of decreasing the error-square sum, found by means of a regression of all the experimental data. Fig. 2 gives the final results obtained for $E^{\circ\prime}$, S and c_x and their standard deviations. It can be seen that the final value for c_x differs from the real value by 0.13%. We have found that more than three standard additions do not improve the accuracy of the determination significantly.

Similarly, Na^+, K^+, Cl^- and F^- were determined at concentrations of 1.0 and 0.1 M. In order to compare the proposed method with the incremental method, which is used most frequently, the same solutions were also determined by the MKA method. The results of these determinations are shown in Table 3; each result is an average of three determinations. It was found that good results were obtained when sample dosing caused a change in the potential of the cell of about 5–10 mV for the monovalent ions determined. As can be seen from the last two columns of Table 3, the errors of the determinations performed with the proposed method are less than 1% and in almost all instances they are less than the corresponding errors given by the conventional MKA method. This is undoubtedly due to the fact that with the proposed method it is possible to calibrate the cell in the same experiment in which the determination of the sample concentration is carried out, thus ensuring maximum constancy of the calibration characteristics of the cell.

Table 1. Data for the determination of potassium ions according to the proposed method. Conditions: [K^+], 0.01 M; initial volume (V_0), 100 ml; $NaNO_3$, 0.1 M; indicator electrode, F2312K; and reference electrode, K701

V/ml	E/mV	t/s
0.500	−44.0	30
0.550	−41.8	15
0.600	−39.8	15
Sample		
0.100	−36.2	30
0.650	−34.5	15
0.700	−33.0	15
0.750	−31.6	15

The applicability of the method was tested when the ratio of the concentration of the sample to that of the standard solution was other than 1:1. For this purpose a series of solutions with concentrations of 1.0, 0.1, 0.01 and 0.001 M were prepared. These solutions were analysed in different combinations so that the ratio of the concentration of the sample to that of the standard solution varied from 1:100 to 100:1. Good results were obtained when the difference between the concentration ratio was up to one decade, *i.e.*, from 1:10 to 10:1.

The sensitivity of the method was assessed by determining a series of decreasing fluoride concentrations, under the conditions described by Liberti and Mascini.[6] The average results of three determinations for each concentration are shown in Table 4 and are compared with the results obtained by Liberti and Mascini. It must be pointed out that when the concentration of the sample was lower, the sample volume was increased so that the change in the potential due to sample dosing was always about 5–10 mV. In each instance the concentration obtained differs from the true value by less than 1%. Attempts to analyse solutions with a concentration of 10^{-6} M gave unsatisfactory results.

The analyses performed here show that the proposed MSA - AA method gives reliable results in the concentration range 0.01–1.0 M for most of the ISEs tested. The satisfactory results obtained at lower concentrations of fluoride ions are very promising; further work is in progress to investigate the sensitivity of the method with other ISEs.

Automation

The proposed method was automated by employing an HP-85B microcomputer, which controlled the pH meter and the autoburette by means of a standard BCD interface and a BCD module for the autoburette. A computer program was constructed for this purpose, written in BASIC and expanded with commands for control of the pH meter and the autoburette, according to the BCD interface protocol. The algorithm of this program is virtually the same as that shown in Fig. 1, with one exception. The sub-routine "Input of data" was replaced by the sub-routine "Data acquisition," which activates the computer to add the standard solution, read the experimental data (ml, mV) and signal a manual addition of the sample at a corresponding moment. The time for a single determination is about 5 min.

In conclusion it should be emphasised that the proposed hybrid MSA - AA method allows simultaneous calibration of the galvanic cell and determination of the sample concentra-

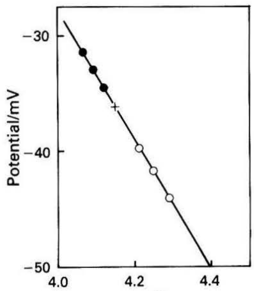

Fig. 2. Final results for the determination of potassium ions (0.01 M) as shown on the CRT display of the HP-85B microcomputer. ○, Measurements made before sample dosing; ×, measurement made immediately after sample has been dosed; and ●, measurements made after sample dosing. Rough values: $E^{\circ\prime} = 185.51 \pm 0.25$; $S = -53.34 \pm 0.06$; and $c_x = 1.0154 \times 10^{-2}$. Final results: $E^{\circ\prime} = 189.74 \pm 0.75$ mV; $S = -54.33 \pm 0.18$ mV pX^{-1}; and $c_x = 0.9987 \times 10^{-2} \pm 2.25 \times 10^{-5}$ M

Table 2. Course of the iterative minimisation procedure for the determination of potassium ions (see also Table 1)

Number of the iteration	Step for variation/ 10^{-4} M	$U_{\text{min.}}$/ 10^{-4} mV^2	Best fitting concentration/ 10^{-2} M
1	3.9821	403.32	1.0154
2	0.7964	385.29	0.9915
3	0.1593	379.65	1.0011
4	0.0319	379.40	0.9986
5	0.0064	379.37	0.9987

Table 3. Comparative determination of K^+, Na^+, Cl^- and F^- ions according to the proposed method and with the conventional MKA method. Conditions: $V_0 = 100$ ml, $V_{\text{sample}} = 0.100$ ml

			Found/µg		Error, %		Relative standard deviation, %	
Determined ion	Sample concentration/M	Sample taken/µg	Proposed method	MKA method	Proposed method	MKA method	Proposed method	MKA method
Na^+	1.0	2299.0	2288.2	2289.1	−0.47	−0.43	0.40	0.37
	0.1	230.0	229.6	227.2	−0.17	−1.23	0.15	1.02
K^+	1.0	3910.2	3900.4	3870.1	−0.25	−1.02	0.17	0.76
	0.1	391.0	388.9	385.3	−0.53	−1.45	0.37	1.11
F^-	1.0	1899.8	1902.7	1872.6	+0.15	−1.43	0.13	0.98
	0.1	190.0	191.0	186.8	+0.50	−1.70	0.36	1.24
Cl^-	1.0	3545.3	3548.9	3574.7	−0.10	+0.83	0.09	0.55
	0.1	354.5	353.3	352.0	−0.33	−0.71	0.27	0.49

Table 4. Assessment of the sensitivity of the proposed method by determination of decreasing concentrations of fluoride ions, compared with the results obtained using the conventional MKA method (values taken from reference 6)

	Sample taken				Results			
	Volume/ml		Amount/µg		Found/µg		Error, %	
Fluoride ion concentration/M	Proposed method	MKA method	Proposed method	MKA method	Proposed method	MKA method	Proposed method	MKA method
1.0×10^{-2}	0.1	20	19.00	3800	18.96 ± 0.03	3820	−0.2	+0.5
1.0×10^{-3}	0.1	20	1.900	380	1.888 ± 0.003	382	−0.6	+0.5
1.0×10^{-4}	1.0	20	1.900	38.0	1.904 ± 0.005	38.2	+0.2	0
1.0×10^{-5}	5.0	20	0.950	3.80	0.948 ± 0.009	3.88	−0.2	+2.0
5.0×10^{-6}	5.0	20	0.475	1.90	0.479 ± 0.008	1.97	+0.9	+3.5

tion, without the need for a "blank" sample determination. The latter is the main advantage of the proposed method over the conventional MKA method. The method can be automated, which makes it convenient for routine analysis.

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Pre-concentration of Vitamin K_1 (Phylloquinone) at Carbon Paste Electrodes and its Determination in Plasma by Adsorptive Stripping Voltammetry

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Linear sweep voltammetry was used to study the accumulation behaviour of vitamin K_1 at carbon paste electrodes prepared with different types of graphite and pasting agents. The vitamin was found to undergo strong accumulation, but this depended on the type of graphite and pasting agent used. A carbon paste electrode containing Nujol - Ultra Carbon Ultra Superior Purity graphite (25 + 75 *m/m*) gave the highest sensitivity with adsorptive stripping voltammetry; the optimum accumulation time was 15 min at an open circuit. A variety of procedures were investigated in order to separate vitamin K_1 from plasma prior to adsorptive stripping analysis. These procedures were evaluated for plasma levels of the vitamin that are likely to be encountered in pharmacokinetic studies. A solvent extraction method using hexane and ethanol gave the best recovery (91%) and detection limits [180 ng ml^{-1} in the supporting electrolyte (450 ng ml^{-1} in plasma)]. However, the analysis time could be reduced by 50% (with some loss of sensitivity) by using ethanol to deproteinate the plasma with the measurement being made directly on the resulting supernatant. As the calibration graphs are linear, quantification can be performed by the method of single standard additions; therefore, relatively short analysis times are feasible.

Keywords: *Vitamin K_1; phylloquinone; adsorptive stripping voltammetry; plasma analysis*

Vitamin K_1 (phylloquinone; 2-methyl-3-phytyl-1,4-naphthoquinone) is known to be necessary for the production of certain blood clotting factors;[1] it has also been shown to be an essential cofactor in the formation of a protein called osteocalcin, which is found in bone and is involved in its calcification.[2] It is, therefore, of interest to determine the levels of this vitamin in certain biomedical studies. Valuable information concerning the possible role of vitamin K_1 in fracture healing was obtained by measuring the clearance rates from the plasma of patients with fractures following a 20-mg intravenous injection.[3] Therefore, sensitive methods of analysis are required for such determinations.

Studies have been initiated to exploit the accumulation of organic compounds at electrode surfaces for enhancing the sensitivity and selectivity for their voltammetric measurement;[4–6] this technique has been referred to as adsorptive stripping voltammetry.[6] In one particular study[4] a fat-soluble vitamin, vitamin E, was found to accumulate strongly on carbon paste with the surface species retaining their characteristic electroactivity. As vitamin K_1 is also a fat-soluble vitamin, and has been shown to be electroactive at carbon electrodes[7–9] we considered that this might also be a suitable candidate for determination by adsorptive stripping voltammetry. This paper describes our findings. The possibility of using adsorptive stripping voltammetry for vitamin K_1 has recently been reported;[10] however, no systematic studies on the adsorption process were carried out nor was the application to plasma analysis reported.

Experimental

Chemicals and Reagents

All chemicals were of analytical-reagent grade, and obtained from BDH, unless stated otherwise. Vitamin K_1 was obtained from Sigma and used without further purification.

The graphite powders were a gift from Unilever Research and were as follows: Ultra Carbon Ultra Superior Purity (USP) (Carbon Corporation, Bay City, MI, USA); Ultra Carbon Ultra "F" Purity (UFP) (Carbon Corporation); Natural Graphite UF2/99.5 (Graphitwerk Kropfmühl, Munich, FRG); and Synthetic Graphite, KS25/6 (Lonza, Italy).

Nujol was obtained from Aldrich.

Standard solutions of vitamin K_1 were prepared in ethanol and diluted with ethanol as required. All glassware containing vitamin solutions was protected from light throughout the experimental procedures.

Working standards, for optimisation studies, were prepared by mixing ethanolic solutions of vitamin K_1 with 0.5 M acetate buffer of pH 5.0 and de-ionised water to produce a final composition of 50% ethanol in 0.05 M acetate buffer (pH 5.0).

Instrumentation and Equipment

Linear sweep voltammetry was performed with a Bruker E310 polarograph connected to a Gould 60 000 series *X - Y* recorder. The voltammetric cell contained a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and a carbon paste working electrode. The last was based on a previously reported design[5] and consisted of a Kel-F [poly(chlorotrifluoroethylene)] barrel with an inner threaded brass rod arranged in a piston-like configuration so that the paste could be extruded easily. The carbon paste eletrodes were prepared by adding the required amount of either Nujol or silicone grease to the graphite powder (20–40% by mass) and grinding this with a pestle and mortar for 30 min. This was then packed into the barrel of the electrode to a depth of 3 mm and the surface smoothed on a computer card. It was found that the best precision was obtained when the piston was rotated four complete turns and the recess was packed with paste to leave a protruding mound; the excess of graphite was then removed by wiping on a computer card. The piston was then rotated clockwise one turn to leave a depth of 3 mm and repolished on a computer card (note that one turn of the piston was equivalent to 1 mm). After each measurement the old paste was removed and the barrel was rinsed thoroughly with ethanol, then dried with a tissue; the packing procedure was then repeated.

The cell used for the voltammetric measurements was obtained from Metrohm; a small magnetic stirrer bar was placed inside the cell and a magnetic stirrer facilitated the required stirring rate during the pre-concentration step. Unless stated otherwise, linear sweep voltammetry was performed using the following instrumental parameters: scan rate, 20 mV s^{-1}; initial potential, 0 V; final potential, −1.4 V; and current range, variable.

Procedure for Adsorptive Stripping Voltammetry

All solutions were initially de-aerated with a stream of nitrogen for 5 min. For the pre-concentration step, the carbon paste electrode was immersed in a stirred solution of the vitamin (10 ml) for a given period of time; the accumulation proceeded at an open circuit. The stirring was then stopped and after a 30-s quiescent period the voltammogram was recorded using a cathodic linear scan with a 15-s capacity current decay; the scan was terminated at −1.4 V *versus* SCE. Further adsorptive stripping voltammograms were recorded at freshly prepared electrodes.

Extraction Procedures for Separating Vitamin K_1 from Plasma

Solvent extraction

Two different solvent extraction methods were investigated. The first involved an extraction on a 5-ml aliquot of plasma using hexane and ethanol; this extraction method has been reported previously.[7] The second extraction involved the use of chloroform and methanol and has also been described previously.[3] The organic phases which contained the vitamin were evaporated to dryness at 60 °C under nitrogen; the residues were reconstituted in the supporting electrolyte, 50% ethanol in 0.05 M acetate buffer (pH 5.0), prior to analysis by adsorptive stripping voltammetry.

Simple protein precipitation

A 5-ml aliquot of plasma was treated with 6.0 ml of ethanol and 1 ml of 0.6 M acetate buffer (pH 5); after shaking and centrifugation, a 10-ml aliquot of the supernatant was transferred into the voltammetric cell for analysis.

Medium exchange

Two variations were investigated. In the first a 5-ml plasma sample was treated with an equal volume of 0.2 M phosphate buffer of pH 7.4. The carbon paste electrode was immersed in the solution and pre-concentration performed; following this step, the electrode was transferred into the cell containing ethanolic acetate buffer and a voltammogram recorded. In the second method the carbon paste electrode was simply immersed in a 10-ml aliquot of plasma for the pre-concentration step; after the accumulation the electrode was again transferred into the voltammetric cell for analysis.

Calibration, Recovery and Precision of the Procedures

Solvent extraction

Spiking plasma. Aliquots of plasma (5 ml) were transferred into conical centrifuge tubes. Various amounts of an ethanolic solution (up to a maximum volume of 50 µl of ethanol) of vitamin K_1 were added to all except one of the tubes so that the final concentration in plasma was in the range 600–4000 ng ml^{-1}. In addition, five aliquots of plasma were spiked with 2.9 µg ml^{-1} of vitamin K_1 to determine the precision. The two separate solvent extraction procedures were then performed and the residues were dissolved in 12.5 ml of the electrolyte prior to analysis by adsorptive stripping voltammetry.

Standard additions. The residue from the control plasma was dissolved in 12.5 ml of supporting electrolyte and a 10.0-ml aliquot was transferred into the cell. After the first adsorptive stripping voltammogram had been recorded standard additions of vitamin K_1, dissolved in the supporting electrolyte, were made to the extracts; the voltammograms were recorded after each addition. The final concentration range of the vitamin added to the control solution was similar to that of the spiked solution.

Calibration graphs were constructed from the results obtained above and the mean recovery was calculated from the ratio of the slopes (described later).

Simple protein precipitation

Spiking plasma. The procedure was the same as that described above except that the concentration range was 1000–4000 ng ml^{-1}. Protein precipitation and adsorptive stripping voltammetry were performed as described earlier. The precision was determined on five samples spiked with 0.3 µg ml^{-1} of vitamin K_1.

Standard additions. An unspiked plasma sample was subjected to the adsorptive stripping technique; standard additions of the vitamin were made to this sample in the same range as for the previous study.

The recovery was determined in a similar manner to that described above.

Medium exchange

Spiking plasma. A series of plasma samples (5.0 ml) were treated with 5.0 ml of phosphate buffer of pH 7.4. To these was added standard vitamin K_1 so that the final concentration of the vitamin in the plasma was in the range 12–50 µg ml^{-1}. The carbon paste electrode was immersed in the solutions for the required accumulation time and then transferred into the cell for voltammetric measurement. The precision was tested on five replicate additions to five plasma samples.

Standard additions. The carbon paste electrode was immersed in an unspiked plasma - phosphate buffer solution for the required accumulation period; this was then transferred into the voltammetric cell for analysis by standard additions.

Results and Discussion

Optimisation of Conditions for the Pre-concentration of Vitamin K_1 and Linear Sweep Voltammetry

The optimum composition of the electrolyte used for adsorptive stripping voltammetry, where pre-concentration and linear sweep voltammetry were performed in the same solution, was found to be 50% ethanol in 0.05 M acetate buffer of pH 5.0; this was found to give peak currents with the largest magnitude and also did not affect the stability of the carbon paste electrode; higher concentrations of ethanol resulted in disintegration of the carbon paste electrode (results not shown).

Adsorptive stripping voltammetric studies were carried out with UFP grade graphite containing 25–40% *m/m* Nujol and with various accumulation times; the solution contained 1×10^{-5} M vitamin K_1 in 50% ethanolic acetate buffer. Fig. 1 shows that the peak height increases with accumulation time for each concentration of Nujol; this indicates that phylloquinone undergoes pre-concentration at the carbon paste electrodes. It is also clear from Fig. 1 that the highest peak currents were obtained when the electrode contained 25% *m/m* Nujol. This behaviour indicates that the magnitude of the peak current is not determined solely by partitioning of high concentrations of the vitamin into the Nujol phase as might be expected. However, at a critical Nujol concentration electrode resistance plays a major role; it was found that high concentrations of Nujol dramatically increased the electrode resistance (22 Ω at 20% *m/m* Nujol increasing to 250 Ω at 40% *m/m* Nujol) and might, therefore, be expected to inhibit the electron-transfer process.

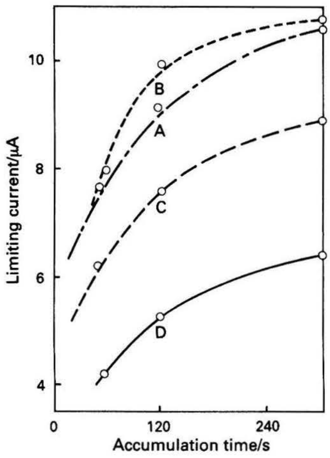

Fig. 1. Effect of pre-concentration time and percentage of Nujol in the electrode on the linear sweep voltammetric peak current for 1 × 10^{-5} M vitamin K_1: A, 20; B, 25; C, 30; and D, 40% *m/m* Nujol. The electrolyte was 50% ethanol in 0.05 M acetate buffer of pH 5.0

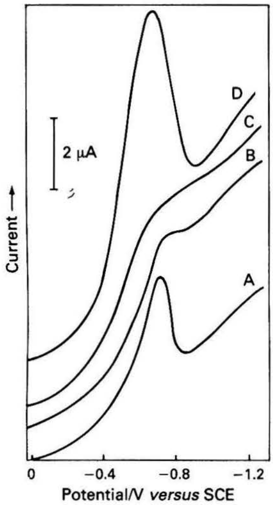

Fig. 2. Linear sweep voltammograms for vitamin K_1 after pre-concentration using different types of graphite to fabricate the electrode: A, UFP Graphite; B, Natural Graphite; C, Synthetic Graphite; and D, USP Graphite. The percentage of Nujol in each electrode was 25% *m/m*. The electrolyte and vitamin concentrations were as in Fig. 1; accumulation time 2 min with stirring and 30 s quiescence

As other workers had found that silicone grease carbon paste electrodes gave improved performance[4,5] we also investigated this pasting material. However, for vitamin K_1 no improvement in peak sensitivity was observed and the electrode was prone to disintegration. Therefore, in further studies only Nujol was employed in the fabrication of the electrodes.

The effect of various types of graphite on the voltammetric behaviour of vitamin K_1 was investigated using 25% *m/m* Nujol as the pasting agent. Fig. 2 shows the voltammograms obtained for four types of graphite from which it can be seen that the peak of greatest magnitude was obtained with the USP grade graphite.

From the above studies it appeared that the most sensitive voltammetric peak for phylloquinone was obtained with a carbon paste electrode containing 25% *m/m* Nujol and USP grade graphite; therefore, this composition was used in subsequent studies.

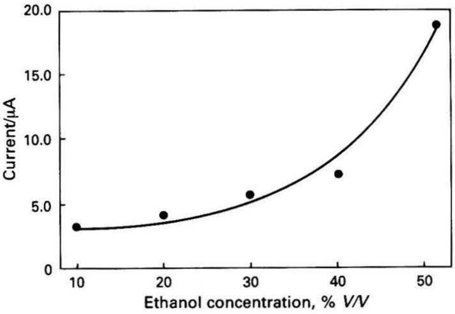

Fig. 3. Effect of the ethanol content of the electrolyte on the voltammetric peak current of vitamin K_1 following medium exchange from a phosphate buffer of pH 7.4. The electrolyte contained a constant concentration of 0.05 M acetate buffer of pH 5.0

As it was intended to investigate the medium exchange technique for plasma analysis (described later) it was considered important to ascertain whether the solution on which voltammetric measurements were to be made needed to contain 50% ethanol, and indeed whether lower levels might be better. A phosphate buffer of pH 7.4 was prepared to mimic plasma, and in which it was intended to perform pre-concentration; a series of ethanolic acetate buffers was then prepared for voltammetric measurements following transfer from the phosphate buffer. Fig. 3 shows that the maximum currents were obtained with 50% ethanol in the electrolyte. These results indicate that an increased ethanol concentration improves the efficiency of the electron-transfer process, because the same amount of the vitamin should be accumulated at each electrode transferred. This is analogous to the situation reported previously for determinations at mercury electrodes.[11] Therefore, in further studies the ethanol concentration of the buffer was kept constant at 50%.

In order to improve the sensitivity of the procedure to that required for real analyses it was found necessary to extend the accumulation time to 15 min. The initial studies with UFP purity graphite had indicated that a maximum current was produced with an accumulation time of about 5 min, but a further study on USP grade graphite showed an increased current even beyond 15 min (results not shown). This may be due to diffusion of the vitamin further into the electrode body as the mean particle size of USP graphite was found to be greater than that of UFP graphite (36.4 and 23.1 μm, respectively); this has been reported previously for similar species by Wang and Freiha.[4,5] Therefore, this would permit a greater degree of accumulation of vitamin K_1 for USP graphite paste. This concept is substantiated by the observation that the peak currents are reduced for both Natural and Synthetic graphites (Fig. 2) where the particles are even smaller (5.8 and 7.1 μm, respectively); these particles might, therefore, form a barrier against penetration of the vitamin into the electrode body.

A series of standard solutions containing between 500 and 3000 ng ml^{-1} of vitamin K_1 were then subjected to the adsorptive stripping procedure, where pre-concentration and voltammetry were performed on the same solution. The graph was found to be rectilinear over this range; the precision was determined on six electrodes at a vitamin concentration of 2.5 μg ml^{-1} and was found to be 2.9%. The limit of detection, based on twice the height of the base-line ripples, was 130 ng ml^{-1}. Therefore, the method was considered suitable for investigations on plasma samples.

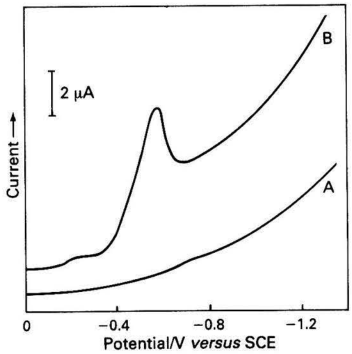

Fig. 4. Adsorptive stripping voltammograms following hexane extraction of A, control plasma; and B, plasma spiked with 2.9 μg ml^{-1} of vitamin K$_1$. The pre-concentration time was 15 min at an open circuit

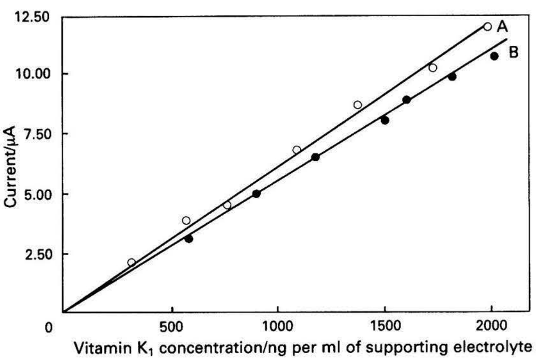

Fig. 5. Calibration graphs for vitamin K$_1$: A, by standard additions to control plasma; and B, by spiking plasma

Calibration, Recovery and Precision of Adsorptive Stripping Voltammetry on Plasma Samples

The adsorptive stripping voltammograms, obtained for plasma extracts following extraction with hexane, exhibited well defined peaks at a potential of −0.58 V as shown in Fig. 4; also shown for comparison is the voltammogram obtained for the control plasma. The small peak that occurred at a potential of −0.25 V was found to be present in all the plasma samples analysed and is probably due to ubiquinone.[3] Clearly, this compound did not interfere with the measurement of the peak currents of vitamin K$_1$; in addition there were no other interferences in the potential region where reduction of phylloquinone occurred.

The calibration graphs obtained by the method of standard additions and by spiking plasma were found to be linear in the concentration range 300–2000 ng ml^{-1} in the supporting electrolyte (Fig. 5). The mean recovery of the vitamin based on the slope ratio (slope *b*/slope *a* × 100) was 91%. The coefficient of variation at a concentration of 2.9 μg ml^{-1} was 3.6% ($n = 5$). The detection limit was about 180 ng ml^{-1} in the supporting electrolyte (450 ng ml^{-1} in plasma). It should be noted that in this work a fairly large voltammetric cell was used for the measurement step, which consequently required reconstitution of the plasma residues in 12.5 ml of electrolyte; therefore, lower detection limits are feasible by simply reconstituting in smaller volumes and using a smaller voltammetric cell.

Similar observations were made with chloroform as the extraction solvent except that the mean recovery of the vitamin from plasma was only 71%. The reason may be related to the lower solubility of the vitamin in chloroform and/or to the residual chloroform remaining in the upper aqueous ethanolic phase. Therefore, hexane was found to be superior to chloroform for the extraction of vitamin K$_1$ from plasma under the conditions described here.

Although the hexane extraction was considered suitable for the separation of the vitamin prior to voltammetry, an alternative method was investigated in an attempt to reduce the analysis time. This involved simple deproteination of the plasma with ethanol, followed by adjustment of the pH with an acetate buffer. The mean recovery using this means of extraction, calculated by the method described above, was 81%; the coefficient of variation at a vitamin concentration of 2.3 μg ml^{-1} was 3.6% ($n = 5$). There were no interfering peaks from the plasma at the potential where the vitamin was reduced. The detection limit was found to be 510 ng ml^{-1} in the supporting electrolyte (1300 ng ml^{-1} in plasma). The poorer detection limit, compared with the hexane extraction, is the result of the slightly greater noise and lower sensitivity (slope of the calibration graph). This may be due to pre-concentration of lipids and other material, from plasma, with the vitamin at the electrode.

It has been reported[6] that the selectivity of voltammetric procedures can sometimes be enhanced by carrying out the pre-concentration of the analyte in one solution and then transferring the electrode into a different solution; this is known as medium exchange. This method should eliminate any interferences from plasma compounds that remain in the same solution as the analyte and which give peaks at similar potentials. It was considered that this might also have the added advantage of reducing the analysis time if protein precipitation could be avoided. Therefore, this approach was investigated for plasma samples which were not deproteinised, but which were treated with phosphate buffer after spiking with various amounts of phylloquinone. The addition of phosphate buffer of pH 7.4 was found to be necessary because in its absence no voltammetric signals were observed. Although satisfactory voltammetric peaks were obtained at fairly high concentrations, the background current was high and the detection limit was only 12 μg ml^{-1} in plasma. The high background may be the result of interferences (discussed above) which may also pre-concentrate with the vitamin. In addition, it is possible that the partition of the vitamin into lipoprotein plays a role in the poor detection limit.

These investigations indicate that the highest extraction efficiency is to be expected with hexane, following protein precipitation with ethanol. Further, this separation method also results in improved sensitivity and gives the lowest detection limit. However, provided that there are no interferences in the plasma, a more rapid separation prior to analysis can be carried out by simply deproteinating with ethanol; this reduces the analysis time by about 50% but there is some loss of sensitivity.

A medium exchange procedure is possible but the sensitivity is low, possibly due to the lack of separation of the vitamin from lipids and lipoproteins; this could probably be improved by using a protein precipitation step prior to pre-concentration/solution transfer. This approach might prove useful in situations where *in situ* pre-concentration results in interference from exogenous substances such as drugs; no interferences from endogenous substances were observed in the samples analysed.

Analytical Applications

As mentioned earlier, the analytical utility envisaged for these methods is in their application to patients who have been given oral or intravenous doses of the vitamin; hence concentration ranges that were of clinical relevance were selected.[3] As it has been shown that calibration graphs, obtained by the method of multiple standard additions to an extract of plasma, are linear

(Fig. 5) it should be possible to analyse plasma samples by the single standard additions technique;[12] this would result in fairly short analysis times, particularly when used with a simple ethanol protein precipitation step and direct pre-concentration of vitamin K_1 into the electrode. It should be added that we investigated pre-concentration followed by differential-pulse voltammetry, using these procedures, but found no significant improvement in sensitivity or selectivity (results not shown); as this is also a slower technique, the use of linear sweep voltammetry was preferred.

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Voltammetric Assay of Methadone in Urine

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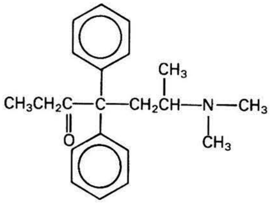
A simple, rapid and sensitive voltammetric method for the determination of methadone in human urine is proposed, which permits the detection of concentrations of methadone as low as 0.3–0.4 μg ml^{-1}. The proposed method was compared with methods normally used in clinical trials, such as the enzyme multiplied immunoassay technique, and was found to be superior with regard to cost and to possess high sensitivity.

Keywords: *Methadone; human urine; voltammetry*

Methadone [6-dimethylamino-4,4-diphenylheptan-3-one (**I**)] is a narcotic analgesic that has been tested as an active agent in the treatment of heroin and morphine addicts. However, reports show that methadone itself has created its own addicts and that methadone dependence is on the increase. This has led to the search for a rapid and reliable method for the detection of methadone in biological fluids, mainly serum and urine. A further problem is the appearance on the market of a new morphine-like drug, buprenorphine, which is potentially dangerous. There is, therefore, a need for new drug-screening methods to detect and differentiate opiate compounds in biological fluids.



(I)

Electroanalytical techniques are widely used for the determination of important molecules in biological fluids because of their low cost and also because it is possible to select the most suitable technique for the analyte oxidation - reduction requirements.[1] The use of carbon paste electrodes for electroanalytical measurements has increased in recent years. One of the reasons for this is that these electrodes are suitable for the determination of substances that undergo oxidation reactions: mercury electrodes do not have this versatility. One problem associated with carbon paste electrodes is the gradual fouling of their surfaces; however, several methods for activating these surfaces have been proposed.[2,3] When active, these electrodes show excellent reproducibility of the analytical signal and can be used for any analytical purposes. The electrochemical behaviour of methadone at carbon paste electrodes has been discussed previously[4]: methadone undergoes a complex oxidation process that is irreversible and depends strongly on the pH. The nature and mechanism of the process were also discussed. The mechanism is characterised by a Nernstian-type behaviour involving a two-proton - two-electron process resulting in the formation of a secondary amine and a ketone. The oxidation peak is analytically significant and the best signal is achieved at pH 7.00. There are several methods available for the determination of methadone; however, difficulties in testing all kinds of drugs of abuse, including methadone, have been reported.[5] Various chromatographic techniques have been suggested: thin-layer chromatography has been found to be useful in combination with a previous extraction[6] and is sufficiently sensitive to detect concentrations of methadone as low as 0.5 μg ml^{-1}. Several detection systems combined with gas - liquid chromatography (GLC) have also been proposed[5,7–9] and, in fact, GLC is the most widely used technique for the determination of methadone in different types of biological fluids. When mass spectrometry is used, concentrations as low as 40 ng ml^{-1} can be detected in serum or saliva.[10]

Ferrara *et al.*[11] have shown that GLC with thermionic detection is more sensitive than the conventional enzyme multiplied immunoassay technique (EMIT) and can detect concentrations of 8 μg ml^{-1}.

A sensitive fluorescence assay for methadone has been proposed[12] and, more recently, high-performance liquid chromatography with UV or on-line fluorescence detection. This latter technique can detect methadone concentrations as low as 20–30 ng ml^{-1} in serum or urine. Pre-treatment of biological fluids (mainly extraction) is always required because of matrix difficulties.[13–15]

This paper describes the development of a sensitive voltammetric method for detecting methadone in human urine. The method may be applied to the detection and differentiation of opiates in human urine.

Experimental

Instrumentation and Reagents

The experiments were carried out in an all-glass cell designed for a three-electrode potentiostatic circuit. A Metrohm 663-VA stand equipped with a carbon paste disc electrode (18 mm^2) and coupled to a Metrohm VA scanner was connected to a Graphtec WX 4221 *x* - *y* recorder for voltammetric measurements. Alternatively, differential-pulse voltammetric measurements were performed using a Metrohm E-506 polarograph coupled to a Metrohm polarographic stand.

A calomel electrode served as the reference electrode and was separated from the assay solution by a salt bridge filled with background electrolyte. A platinum wire was used as the auxiliary electrode. Britton - Robinson buffer (pH 7) was used as the background electrolyte. All chemicals used were of analytical-reagent grade, and methadone hydrochloride was purchased from Sigma. Water was purified by using a Milli-Q (Millipore) system.

Procedures

Electrode activation

As reported previously[4] activation of carbon paste electrodes by a high anodic potential is highly effective because the electrode transfer characteristics are similar to those shown by "dry" graphite immersed in an aqueous solution. Consequently, the following *in situ* pre-treatment was employed to activate the surface of the electrode.

The electrode was kept in a quiescent solution for 30 s at a

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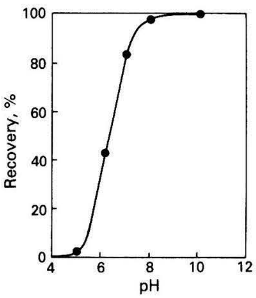

Fig. 1. Recovery of methadone in cyclohexane as a function of pH

potential of +1.500 V. Then, the current was switched off and, at the same time, the solution was stirred for 15 s. Before starting the potential scan, the current was established for 15 s at the required potential.

By following this procedure, excellent reproducibility of the voltammetric signals is obtained with a relative standard deviation (RSD) of 0.8% (n = 10).

Differential-pulse voltammetry (DPV)

The influence of the pulse amplitude on the voltammetric peak current was checked. As expected, for a methadone concentration of 5.0×10^{-5} M at pH 7 and at a scan rate of 10 mV s^{-1}, the DP signal increased linearly as the pulse amplitude rose from 5 to 100 mV, as shown by the following equation: $i/\mu A = 0.01574 \Delta E/mV - 0.009$ ($n = 8, r = 0.9993$).

The activation procedure described above was also checked by DPV and a slightly lower reproducibility was observed. In fact, an RSD of 1.5% (n = 10) was found for the voltammetric signal when a pulse amplitude of 100 mV was used. This amplitude was used in all subsequent experiments.

Assay of methadone in urine

A 5-ml sample of human urine was mixed with 0.5 ml of 1 M sodium hydroxide solution and 4 ml of cyclohexane were used as the extracting solvent. The mixture was shaken and, after separation, the organic phase was evaporated to dryness at 40 °C under an atmosphere of argon. The residue was re-dissolved by adding 5 ml of the Britton - Robinson buffer (pH 7.0) supporting electrolyte and the voltammetric signal [linear sweep voltammetry (LSV) or DPV] was recorded.

Extraction procedure

As methadone has a marked non-polar character, several non-polar organic solvents were tested as extractants. Cyclohexane was found to be highly efficient for the extraction of methadone from human urine. Fig. 1 shows the influence of pH on the percentage of methadone extracted when the described procedure is followed. As can be seen, a pH of 8 or higher results in a recovery close to 100%.

Results

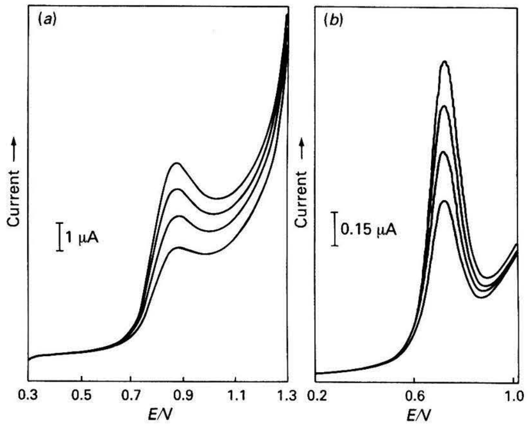

Fig. 2. Standard additions method: sample and successive additions of 3.1 μg of methadone. (*a*) LSV responses: sample methadone concentration, 4×10^{-6} M; scan rate, 50 mV s^{-1}. (*b*) DPV responses: sample methadone concentration, 6×10^{-4} M; scan rate, 10 mV s^{-1}; ΔE = 100 mV

Table 1. Calibration equations for urine spiked with methadone

	Range/M	Equation*	n	r
LSV ..	10^{-6}–10^{-5}	$i = 4.004 \times 10^5 c + 0.023$	6	0.999
	10^{-5}–10^{-4}	$i = 2.627 \times 10^5 c + 0.88$	7	0.999
DPV ..	10^{-6}–10^{-5}	$i = 1.303 \times 10^5 c - 0.01$	6	0.999
	10^{-5}–10^{-4}	$i = 7.330 \times 10^4 c + 0.53$	6	0.9995

* i is expressed in μA.

Table 2. Comparative results obtained by EMIT and voltammetric methods

	Methadone/μg ml^{-1}		
Sample	EMIT assay	LSV*	DPV*
1	0.37	0.37 (0.04)†	0.32 (0.01)†
2	0.79	0.83 (0.05)	0.92 (0.10)
3	1.98	1.94 (0.03)	1.95 (0.10)
4	2.37	2.32 (0.11)	2.2 (0.4)

* Results are the means of three replicate determinations.
† Figures in parentheses are the standard deviations.

The described procedure was checked by adding a known amount of methadone to a pool of human urine which was then processed. Fig. 2 shows the successive voltammetric responses obtained when increasing amounts of methadone were added to the reconstituted sample and indicates that a standard additions procedure can be used to measure the concentration of methadone in human urine. It should be noted that both DPV and LSV gave a similar recovery of 99.2 ± 0.7% when five samples ranging from 10^{-6} to 10^{-5} M were analysed. The DPV signals were more suitable for measurements although, as mentioned above, the reproducibility was not as good as that obtained with LSV. Nevertheless, both recording systems can be used. When DPV was used, a half-peak width of 95 mV was obtained; this must be taken into account when an evaluation of the resolution is carried out. In fact, some of the reconstituted samples produced interferences of the signal due to the existence of matrix-derived substances capable of oxidising at potentials close to that of methadone. This interference can be eliminated by reducing the pulse amplitude, hence improving the resolution of the voltammetric signal.

Calibration of Methadone in Urine

Good calibration graphs, covering two orders of magnitude (10^{-4}–10^{-6} M), were obtained with both DPV and LSV. The calibration graphs are described by the equations given in Table 1.

The reproducibility of both systems was examined by analysing five samples containing 10^{-5} M methadone. In both instances excellent results were obtained, with standard deviations of 2.49 and 2.69% for LSV and DPV, respectively.

Methadone in Real Human Urine Samples

Samples from four patients undergoing methadone treatment were analysed and both voltammetric methods used were checked by EMIT. The results obtained are summarised in Table 2. As shown in the respective columns, the results obtained with both methods are in good agreement with those obtained by EMIT and, in fact, are probably more specific.

Work is in progress in our laboratory with the aim of analysing mixtures of opiates in biological fluids by voltammetric measurements.

We thank the Consejeria de Sanidad del Principado de Asturias (Spain) for providing the samples used in this study and J. M. Cabeza González de la Fuente for performing the EMIT experiments.

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Determination of Salicylic Acid in Pharmaceutical Formulations and Foods by Differential-pulse Voltammetry Using a Glassy Carbon Electrode

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A simple and rapid voltammetric method for the determination of salicylic acid in pharmaceutical preparations and foods has been developed. The sample was extracted using either diethyl ether or ethanol - diethyl ether and then back-extracted into 0.015 M disodium hydrogen phosphate solution. The peak current was measured using a glassy carbon electrode at 0.85 V. The working range was 0–25 p.p.m. The average recovery for ointment was 99% with a standard deviation of 5.0%. Results are reported for eight different pharmaceutical formulations.

Keywords: *Salicylic acid determination; differential-pulse voltammetry; pharmaceutical; food*

Salicylic acid is a compound used as an antimicrobial agent in many pharmaceutical preparations and foods. The pharmaceutical preparations include ointments, dusting powder and lotion. The techniques described for the determination of salicylic acid include colorimetry,[1–4] titrimetry,[5–7] UV spectrophotometry,[8,9] spectrofluorimetry,[10] gas chromatography,[11,12] direct potentiometry[13] and high-performance liquid chromatography (HPLC).[14–17] Of these, the titration methods are non-selective and subject to interference by reducing agents. The colorimetric methods, which involve the use of iron(III) to complex with salicylic acid, do not have sufficient sensitivity to determine salicylic acid down to p.p.m. levels. The HPLC methods are simple and suitable for routine analysis where many samples must be analysed.

Few voltammetric methods have been proposed for the determination of salicylic acid; Woodson[18] reported that it could be reduced in acetonitrile medium and detected down to 10 p.p.m. The purpose of this work was to develop a voltammetric method for the determination of salicylic acid in drugs and foods. The sample, such as an ointment, is first extracted by an organic solvent and then back-extracted by an alkaline medium before being analysed by direct electrochemical oxidation using a glassy carbon electrode. The results show that the method is selective, simple and salicyclic acid can be determined down to p.p.m. levels with satisfactory precision.

Experimental

Apparatus

Voltammograms were recorded with a PAR Model 174A polarographic analyser equipped with a Houston *x* - *y* recorder (RE0089). A laboratory-made working electrode made from 3 mm diameter polished glassy carbon (Atomergic Chemical), a saturated calomel reference electrode (SCE), separated from the electrolyte by a saturated potassium chloride solution, with acid resistant Vycor ends, and a platinum wire counter electrode were used. The modulation amplitude, scan rate and current range were 50 mV, 5 mV s^{-1} and 20–50 μA, respectively. The scan direction was positive and with an initial potential of +0.4 V.

Reagents

The reagents used were of analytical-reagent grade.

Standard salicylic acid solution, 300 μg ml^{-1}. Prepared by dissolving 0.0300 g of salicylic acid in 95% ethanol and diluting to 100 ml in a calibrated flask with ethanol.

Supporting electrolyte (disodium hydrogen phosphate) solution, 0.015 M. $Na_2HPO_4.12H_2O$ (5.4 g) was dissolved and diluted to 1 l with distilled water.

Hydrochloric acid solution. Prepared by mixing concentrated hydrochloric acid and distilled water (1 + 2 *V*/*V*).

Procedure

Preparation of sample solutions

An amount of the drug sample equivalent to 2–4 mg of salicylic acid was weighed accurately in a 250-ml beaker. Ethanol - diethyl ether (1 + 1; 100 ml) was added. Using an ultrasonic shaker, the salicylic acid was extracted from the sample. After extraction, the suspension was transferred completely into a 250-ml calibrated flask and diluted to the mark with 1 + 1 ethanol - diethyl ether. As most of the undissolved solids had settled, 25 ml of the mixture were pipetted into a 125-ml separating funnel and extracted with three portions (20, 10 and 10 ml) of the supporting electrolyte. These three portions were combined and transferred totally into a clean, dry cell for analysis.

For the food samples, 4.5 ml of the sample were pipetted into a 125-ml separating funnel and 0.5 ml of hydrochloric acid was added together with 25 ml of diethyl ether. The separating funnel was stoppered and shaken. The aqueous phase was discarded and the diethyl ether fraction was washed using two portions (2 ml) of distilled water. The diethyl ether layer was then back-extracted with three portions (20, 10 and 10 ml) of the supporting electrolyte. These three portions were combined and transferred totally (40 ml) into a clean, dry cell for analysis.

Determination of Salicylic Acid

The combined electrolyte was stirred using a magnetic stirrer and the voltammogram was recorded. The peak height of salicylic acid at 0.85 V was measured.

With the analyser at the "off" position, 0.5 ml of the standard solution was pipetted into the cell. The voltammogram was then recorded again. The addition of the standard solution and the recording of the voltammogram were repeated until 1.5 ml of the standard solution had been added. The amount of salicylic acid in the sample was determined by the standard additions technique.

Result and Discussion

In selecting the solvent for extracting the analyte into the organic solvent, those solvent systems recommended by standard methods[3,6] were adopted. Therefore ethanol - diethyl ether and diethyl ether were used for the drugs and foods, respectively. The dissociation constant for salicylic acid is 1.07×10^{-3} and it dissociates completely at a pH above 5.[19] The pH of the solution used to back-extract the salicylic acid should therefore be greater than 6. To select the pH of the back-extraction solution, a known amount of the analyte was added to either diethyl ether or ethanol - diethyl ether and then extracted with aqueous solutions of different pH. The results showed that above pH 7, the extraction efficiency was >90% and a pH of *ca.* 9 (0.015 M disodium hydrogen phosphate) was therefore chosen as the working medium for extraction and voltammetric determination.

In scanning from 0.0 to 1.0 V using the glassy carbon electrode as the working electrode, two peaks (0.4 and 0.85 V) were observed for salicylic acid. The first had no analytical value because the precision was poor and the response was non-linear with respect to concentration. As a result the second peak (0.85 V) was chosen because on scanning three times at the 11.3 p.p.m. level, the relative standard deviation was 3.8%. A typical voltammogram is shown in Fig. 1.

Calibration Graph, Recovery and Precision

The calibration graph for salicylic acid was linear up to 25 p.p.m. The slope was 0.87 µA p.p.m.$^{-1}$ and gave a correlation coefficient of 0.9993. The lowest concentration of salicylic acid detected was 0.5 p.p.m.

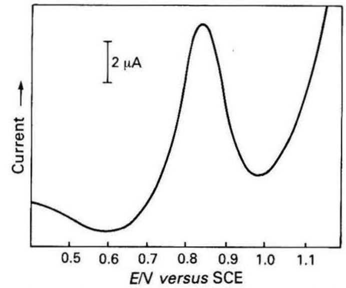

Fig. 1. Typical voltammogram of salicylic acid (9 p.p.m.)

Table 1. Interference studies; concentration of salicylic acid, 9 p.p.m.

Interferent	Concentration added, p.p.m.	Concentration of salicylic acid found, p.p.m.	Error, %
Methyl paraben	1.5	8.51	−5.5
Methyl salicylate	6.9	8.46	−6.0
Phenol	14.8	9.57	6.3
Tartrazine	15.0	9.45	5.0

The recovery and precision were studied by adding a known amount of the analyte to simple cream (3.12% *m/m*) and an apple drink (334 p.p.m.), which were free of salicylic acid and then analysed five times using the proposed procedure. The recovery was *ca.* 99% with a precision of 5%. An ointment sample was also assayed four times and gave a salicylic acid content of 7.78 ± 0.17%, which was *ca.* 97.2% of the claimed value.

Interference Studies

Various substances commonly found in drugs and foods were investigated. Voltammograms of a number of substances were scanned. Substances including boric acid, camphor, cetyl alcohol, ethanol, mercury(II) chloride, menthol, saccharin, sodium citrate, starch, sulphur, titanium dioxide and zinc oxide did not exhibit a peak within the working potential range and are therefore not expected to cause significant interference. Methyl paraben, methyl salicylate, phenol and tartrazine all exhibited peaks within the working potential range. Their concentrations that caused a 5% deviation in the peak current at 0.85 V for 9 p.p.m. of the analyte were determined. The results are summarised in Table 1. Phenol and tartrazine can be tolerated up to a 1:1 ratio. The method is affected by methyl paraben and methyl salicylate. However, these compounds seldom co-exist with salicylic acid and hence should not affect the determination of salicylic acid using a glassy carbon electrode.

Real Sample Analysis

The results for drug analysis using the proposed method (Table 2) are in close agreement with the claimed values and HPLC methods. The titration methods have a lower accuracy, especially for ointments D and E and prickly heat powder. In part, this can be explained by a matrix effect. Ointment D contained 30% zinc oxide and the prickly heat powder contained an appreciable amount of boric acid (4.5%). Therefore when these samples were titrated directly with standard sodium hydroxide, the interferent caused a negative deviation for ointment D and a positive deviation for the powder.

Although the spectrophotometric method is more accurate and sensitive than titration, the solvent system used was not effective enough to extract the analyte. As for ointments D and E in the first few trials, the results obtained were much lower than the claimed values. Satisfactory results could only be obtained when heat was applied to melt the ointment during the extraction.

In Hong Kong salicylic acid was once found to be added illegally to fish ball to improve its palatability.[20] This indicated the necessity to have more control over its application. As it has been banned therefore, the validity of the method could only be tested by known addition. The results (Table 3) show that the proposed method can be applied successfully to various types of food samples such as wine and oil. This is

Table 2. Drug sample analysis

Sample	Claimed value*	Concentration found*			
		Proposed method	Titration†	Spectrophotometry[8]	HPLC[14]
Ointment A	2.0	1.76	1.78	—	1.89
B	3.0	3.08	2.77	—	3.11
C	8.0	7.78	7.55	7.77	8.09
D	2.0	2.09	0.80	2.26	—
E	2.5	2.39	4.15	2.49	—
Backache pill	13.9	12.90	—	12.90	—
Prickly heat powder	0.5	0.47	0.82	0.49	—
Eye lotion	0.025	0.026	—	0.072	0.026

* Concentration is expressed as % of salicylic acid except for the backache pill, which is expressed as % of sodium salicylate.

† The BP method[6] was used for ointments A and D and the prickly heat powder and iodimetry[7] was used for the other ointments.

Table 3. Food sample analysis

		Concentration found, p.p.m.	
Sample	Salicylic acid added, p.p.m.	Proposed method	AOAC method[3]
Apple drink	334	329	325
Beer	171	160	174
Blackcurrant drink	148	138	151
Fish ball	277	291	250
Lemon tea	160	154	182
Orange juice	319	303	275
Peanut oil	246	244	251
Soya sauce	239	227	213
Vinegar	186	176	174
Table wine	266	271	302

much superior to the AOAC method, in which the colour development is affected by ethanol,[3] and illustrated by the analysis of the table wine. In addition, the AOAC method involves more tedious clean-up steps that are simplified in the proposed method.

Conclusion

The proposed voltammetric method is simple, specific, economical and sensitive for the determination of salicylic acid in pharmaceutical formulations and foods. Although it is not as convenient as the HPLC method, it offers the advantage that little preliminary preparative work is needed. In the HPLC method the column has to be flushed for 1–2 h before analysis and HPLC grade solvent is required for the preparation of the mobile phase; in the proposed method no such tedious work is required and the reagents are much cheaper. The HPLC method is suitable for routine work where many samples are to be analysed but when samples are to be analysed on an occasional basis, the proposed method is to be prefered as little preliminary work has to be done and no expensive solvents or reagents are required.

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Solid-state Detector for Gas-phase Flow Injection of Nitrogen Dioxide at Ambient Temperature

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An Au - $RbAg_4I_5$ - Ag galvanic detector, consisting of a disc 13 mm in diameter and 1.3 mm thick has been developed by using a rubidium silver iodide solid electrolyte. The working electrode (25 nm thick) was made by sputtering gold in an argon atmosphere. When the sample (10 ml) containing NO_2 was injected into the nitrogen carrier stream flowing at a rate of 45 ml min^{-1}, the current flowing in the external circuit was linearly related to the concentration of NO_2 in the range 10 p.p.b.–1 p.p.m. Twelve samples could be analysed per hour. After 200 samples (50 p.p.b. of NO_2 - N_2, 10 ml) had been analysed, the response decreased by about 10% of its original value.

Keywords: *Solid-state detector; gas sensor; nitrogen dioxide; flow injection; rubidium silver iodide*

It is now recognised that even trace amounts of NO_2 can have an adverse effect on ecological systems. The permitted level of NO_2 in atmospheric air in Japan is less than 40 p.p.b. This recognition has emphasised the need to develop increasingly more sensitive devices to measure such low concentrations. The chemiluminescence method, which is based on the reaction of NO with ozone, is widely used and its limit of detection is about 50 p.p.b. However, this method requires a convertor ($NO_2 \rightarrow NO$), oxygen cylinder, ozone generator, photomultiplier and the disposition of large amounts of ozone. Several solid-state gas sensors[1–4] have been shown to be useful for the detection of trace pollutants because of their small size, ease of use and low cost.

We have previously reported a galvanic solid-state Pt - AgI - Ag detector[5] for the monitoring of NO_2. The detector must be thermostated at 90 ± 0.1 °C because of the low conductivity of silver iodide at ambient temperature. In order to provide an effective detector for monitoring NO_2 at ambient temperature, $RbAg_4I_5$[6–10] was used for the detector (Au - $RbAg_4I_5$ - Ag).[11] As the solid electrolyte $RbAg_4I_5$ has high conductivity at ambient temperature, the resistance of the cell was only 150 Ω. When the carrier gas containing NO_2 impinges on the working electrode of the cell[11] at a flow-rate of 20 ml min^{-1}, the current flowing in the external circuit is linearly related to the concentration of NO_2 in the range 2 p.p.b.–1 p.p.m. The response time was 17 min for 40 p.p.b. of NO_2; therefore, only two samples could be analysed per hour.

In order to increase the number of analyses per hour and to prolong its lifetime, the detector was used in conjunction with flow injection. This paper describes the results of the investigation.

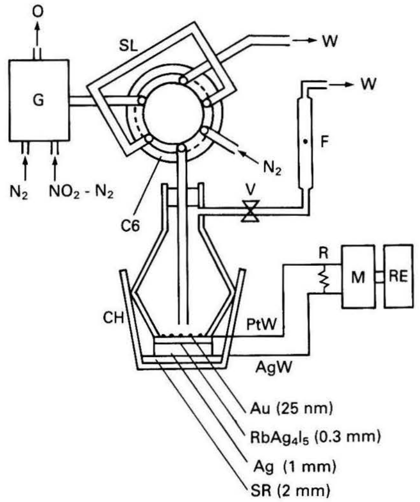

Fig. 1. Gas flow system and cell assembly. O, Overflow; G, standard gas generator for NO_2; SL, sample loop; C6, six-way sample valve; W, waste; CH, cell holder; V, flow control valve; F, flow meter; PtW, Pt wire; AgW, Ag wire; SR, silicone rubber; R, resistance (50 kΩ); M, voltmeter; RE, recorder; and N_2, carrier gas

Experimental

Apparatus

The configuration of the gas flow system and cell assembly is shown in Fig. 1. The instrumentation consisted of the galvanic solid-state detector, a cell holder, flow control system for the carrier gas (45 ml min^{-1}), six-way sample valve for injection of the sample (inner diameter of the sample loop 2.0 mm, sample size 10 ml), external 50-kΩ resistance, voltmeter and recorder.

Construction of the Cell

The cell shown in Fig. 1 was prepared as follows.[11] Firstly, the solid electrolyte, $RbAg_4I_5$, was prepared[6] by combining stoicheiometric amounts of AgI and RbI, melting the mixture at 400 °C for 10 min under a pressure of *ca.* 100 Pa and annealing the intermediate product at 165 °C for 17 h. The product was ground in an agate mortar; 1 g of silver powder was placed in a die (13 mm i.d.) and levelled off and a pressure of 1.1 tonnes cm^{-2} was applied. Any residual silver powder on the surface of the plunger and on the inner wall of the die was removed with a clean tissue. Rubidium silver iodide powder (0.3 g) was placed on top of the silver disc and a pressure of 5.5 tonnes cm^{-2} was again applied for 5 min. The thickness of the $RbAg_4I_5$ - Ag disc was about 1.3 mm. After removal from the die, the working electrode was formed on the surface of the $RbAg_4I_5$ by vaporisation of gold sputtered under reduced pressure in an atmosphere of argon. A thickness of gold on the working electrode of about 25 nm was obtained using the following sputtering conditions: current 5 mA, time 2 min (Quik Coater SC-701, Sanyu Electron, Tokyo, Japan). A pad of silicone rubber was placed in the bottom of a Pyrex glass cell holder. A silver wire was placed on this pad and the other end of the wire was threaded through a small hole in the cell holder. The cell was placed on this silver wire over the pad and the other contact was made by attaching the gold layer to a platinum wire threaded through another hole in the cell holder. The two small holes for these connections were sealed with silicone adhesive.

Generation of NO_2 Standards

The standard concentrations of NO_2 were prepared by controlling the flow-rates (SGGU-72; Stec, Kyoto, Japan) of streams of primary standard NO_2, 24.0 and 476 p.p.m. (nitrogen balance, Takachiho, Tokyo, Japan), and nitrogen. The primary standards were diluted 120000–450 times with pure nitrogen (2 l min^{-1}). The flow-rate of the primary standard was indicated by the difference in pressure. Various concentrations of NO_2 (0.2 p.p.b.–1 p.p.m.) were obtained by adjusting the dilution ratio.

Procedure

The base line for the detector became constant about 5 min after the nitrogen carrier gas began to flow at a rate of 45 ml min^{-1} and remained constant at 6.0 ± 0.4 nA for 10 h. The current produced by the detector was converted to a voltage via an external 50 kΩ resistance and the voltage was recorded. The external circuit was made at all times, even when the detector was not in use. The sample gas (10 ml) was injected by changing the position of the six-way sample valve and was then carried and impinged on the detector. The peak appeared after a few seconds and the peak height (current) was measured from the base line to obtain a value for the response.

Results and Discussion

Effect of Thickness of Gold Layer on Response

The relative responses obtained by using cells with a gold layer 3, 13, 25, 40 and 50 nm thick were 1.00, 0.89, 0.61, 0.33 and 0.06, respectively. As the thickness of the gold layer decreased, the effective three-phase contact area[12,13] increased, hence the sensitivity for NO_2 increased. However, as the thickness of the gold layer decreased the physical stability of the cell also decreased; a cell with a gold layer 25 nm thick was used in all subsequent work.

Table 1. Relationship between concentration of NO_2 and detector sensitivity for each cell

	NO_2, p.p.b.				
	30	80	130	170	220
Mass of electrolyte used/g	Relative sensitivity*				
0.1	1.01	1.09	1.10	1.15	1.27
0.3	1.01	1.00	1.00	1.00	1.00
0.5	1.00	0.97	0.94	0.89	0.82
0.7	1.00	0.96	0.88	0.74	0.63

* The sensitivity (nA p.p.b.$^{-1}$) of the 0.3-g cell for 130 p.p.b. of NO_2 was taken as 1.00.

Table 2. Detector resistance and background current as a function of temperature

Temperature/ °C	Resistance/Ω	Background current/nA
5	300	1
10	260	2
20	150	6
30	80	15
38	60	53
43	50	82

Effect of Electrolyte Mass on Sensitivity

Four cells were made by using 0.1, 0.3, 0.5 and 0.7 g of electrolyte in order to investigate the dependence of the sensitivity on the mass of the electrolyte taken. The results are shown in Table 1. The sensitivity of the cell when 0.3 g of electrolyte was used was constant up to *ca.* 1 p.p.m. of NO_2. Therefore, 0.3 g was chosen as the optimum mass of the electrolyte.

Effect of Temperature on Response

The relative responses obtained at cell temperatures of 10, 20, 35, 40 and 45 °C were 0.67, 1.00, 1.50, 1.67 and 1.40, respectively. As the temperature of the cell increased, the response also increased; however, above 38 °C the response decreased because the adsorption efficiency of NO_2 on the working electrode decreased. In addition, at elevated temperatures the background current became unstable. Therefore, the optimum temperature of the cell was in the range 15–35 °C.

Temperature Dependence of Background Current and Cell Resistance

The detector resistance and background current as a function of temperature are shown in Table 2. The electrical resistance of the detector at each temperature was measured with an a.c. (1000 Hz) bridge.

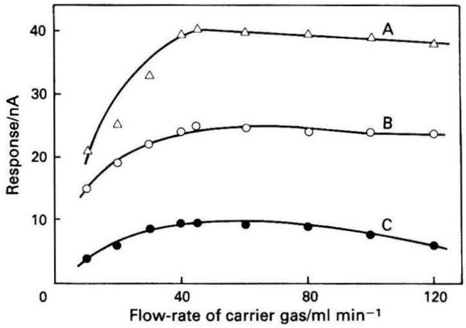

Fig. 2. Effect of carrier gas flow-rate on response for different sample sizes. A, 17; B, 10; and C, 2.6 ml. [NO_2], 225 p.p.b.

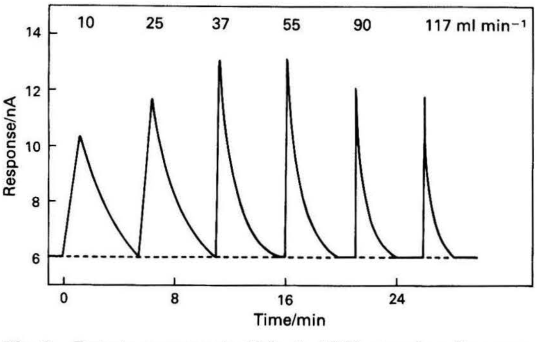

Fig. 3. Detector response to 10.0 ml of NO_2 at various flow-rates. [NO_2], 72 p.p.b.

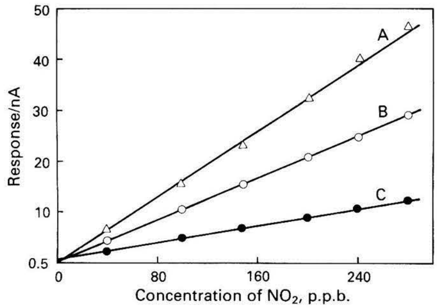

Fig. 4. Calibration graphs. Carrier gas flow-rate, 45 ml min^{-1}. Sample size: A, 17; B, 10; and C, 2.6 ml

Effect of Flow-rate of Carrier Gas on Response

When different sample sizes of NO_2 were injected into different flow-rates of the carrier gas, a graph of output current (peak height) *versus* carrier gas flow-rate (Fig. 2) showed that the maximum response was obtained at a flow-rate of 45 ml min^{-1}, independent of the sample size. As the carrier gas flow-rate increased, the response of the detector increased because the rate of diffusion of NO_2 into the pure nitrogen carrier gas decreased at high flow-rates until the sample reached the detector. However, above 50 ml min^{-1}, the response decreased because the adsorption efficiency of NO_2 on the working electrode decreased. Therefore, 45 ml min^{-1} was chosen as the optimum flow-rate of the carrier gas. A typical recorder trace for 10 ml of sample is shown in Fig. 3. There was no evidence for the absorption of NO_2 in the sampling valve as no tailing of the peaks was observed. The peak areas correspond to the amount of current produced by the reaction of NO_2 with the detector. Fig. 3 shows that as the flow-rate of the carrier gas decreases the reaction efficiency increases. Hence 12 samples can be analysed per hour.

Calibration Graphs

The relative slopes of the calibration graphs obtained with sample sizes of 17, 10 and 2.6 ml were 1.00, 0.67 and 0.36, respectively. The results are shown in Fig. 4. The relative standard deviations of the responses for 200 p.p.b. of NO_2 using 17-, 10- and 2.6-ml samples were 5, 3 and 3%, respectively (n = 4). Considering both sensitivity and relative standard deviation, a sample size of 10 ml was chosen as the optimum. When 10 ml of sample were used, the calibration graph was linear up to *ca.* 1 p.p.m. and the detection limit was calculated to be about 12 p.p.b. (signal to noise ratio = 5) of NO_2. After 200 samples (50 p.p.b. of NO_2 - N_2, 10 ml) had been analysed, the detector response decreased by about 10% of its original value.

The response for 100% oxygen was approximately equivalent to that for 10 p.p.b. of NO_2.

Mechanism of Response

When the sample gas containing NO_2 impinges on the working electrode (Au) of the detector, the NO_2 reacts with the silver ions of the rubidium silver iodide to produce silver oxide at a three-phase contact area, NO_2 - Ag^+ (in $RbAg_4I_5$) - Au. The reaction $NO_2 + 2e^- + 2Ag^+ \rightarrow NO + Ag_2O$ proceeds at the gold cathode. The activity of the silver ions at the working electrode side of the solid electrolyte becomes less than that on the opposite side (counter electrode side). The e.m.f. of the detector is based on the difference between the silver activities at the two sides of the solid electrolyte layer. The reaction $Ag \rightarrow Ag^+ + e^-$ then occurs and the silver ions pass through this layer from the silver anode to the gold cathode. The rate at which the electrons flow through the external circuit is proportional to the concentration of NO_2 provided that the carrier gas flow-rate and sample size are constant.

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Determination of Bromide Using Flow Injection and Chemiluminescence Detection

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A method is described for the determination of bromide ion in the range 1–100 μg ml^{-1} (correlation coefficient 0.9996) with a detection limit of 3.75 pg and a coefficient of variation of ≤1%. The optimum parameters were studied and were found to be the following: HNO_3, 0.25 M; luminol, 0.01 mM; H_2O_2, 10 mM; $KBrO_3$, 100 mM; and flow-rate, 3 ml min^{-1}. The interference from several cations was overcome by using batch and continuous exchange with a strong cation-exchange resin. Up to 100 μg ml^{-1} of chloride ion and 1 μg ml^{-1} of iodide ion did not interfere with the determination of 10 μg ml^{-1} of bromide; 113 samples can be injected per hour.

Keywords: *Chemiluminescence detection; homogeneous catalysis; bromide determination; luminol; flow injection*

Most chemiluminescence applications involve trace metal analysis because of the catalytic effects of many metals on different chemiluminescence reactions.[1,2] Much less attention has been paid to the direct determination of anions. This may be attributed to the nature of some chemiluminescence reactions, which are not affected by anions, or to the non-linear calibration effects of some anions on a particular chemiluminescence reaction.[2] However, some examples of anion determination using either chemiluminescence alone[3,4] or in combination with flow injection[5–8] have been reported. The most recent indirect method involves the displacement by analyte anions of a chemiluminescence anion bound to an ion-exchange resin, followed by a reaction that produces chemiluminescence.[8] The major problem associated with these methods, which utilise the metal-catalysed chemiluminescence of luminol, was the lack of selectivity in that many cations and anions interfered seriously with the determinations. The interferences from cations were due to the fact that the luminol chemiluminescence reaction was not selective. An additional problem with the method of Melanie and Stanford[8] was non-selective displacement from the ion-exchange resin which led to serious interferences from anions because any anionic species could simply displace the anionic derivative bound to the resin. In order to eliminate the effect of a particular anion, specific conditions have to be established which might be incompatible with the chemiluminescence reaction conditions. Therefore, the previously reported conditions for the metal - luminol - hydrogen peroxide chemiluminescence reaction were not suitable for such an analysis. In the present work a flow injection chemiluminescence method was developed for the determination of bromide using conditions for the chemiluminescence of luminol based on the homogeneous catalysis of the bromine generated from the bromate - bromide - acid decomposition of hydrogen peroxide, which is necessary for luminol chemiluminescence.

Experimental

Apparatus

A multi-channel peristaltic pump (Desaga), a six-way injection valve, a 60-μl sample loop, a laboratory-built electronic photon detector (sensitivity 10 nA–1 mA, amplification ×1, ×3 and ×10, time constant 30 ms, output level 0–1 V, impedance 1 kΩ, non-linearity ±1% and output filter rise time to remove low frequency chopping 20 ms, 200 ms and 2 s) with wavelength scanning facilities (0.6–53 nm s^{-1}) covering the range 199–990 nm, a high radiance monochromator (1200 nm mm^{-1} blazed at 300 nm), an EMI IP28 photomultiplier tube, output filter rise time 2 s, and three-way valves (Vygon) were used.

Reagents

The following stock solutions were prepared.

Nitric acid, 1 M. Dilute 63 ml of HNO_3 (70% *m/m*, sp. gr. 1.42) to 1 l with water.

Sodium carbonate decahydrate solution, 0.1 M. Dissolve 28.617 g of $Na_2CO_3.10H_2O$ in 1 l of water and adjust the pH of the solution to 10.5 using 0.1 M HNO_3.

Luminol solution, 1 mM. Dissolve 0.17717 g of luminol in 1 l of carbonate buffer solution.

Hydrogen peroxide solution, 100 mM. Dilute 10.3 ml of 35% H_2O_2 to 1 l with water.

Potassium bromate solution, 100 mM. Dissolve 16.701 g of $KBrO_3$ in 1 l of water.

Cobalt(II) solution, 100 μg ml^{-1}. Dissolve 0.40372 g of $CoCl_2.6H_2O$ in 1 l of water.

Copper(II) solution, 100 μg ml^{-1}. Dissolve 0.46531g of $Cu(NO_3)_2.6H_2O$ in 1 l of water.

Cadmium(II) solution, 100 μg ml^{-1}. Dissolve 0.27442 g of $Cd(NO_3)_2.4H_2O$ in 1 l of water.

Chromium(III) solution, 100 μg ml^{-1}. Dissolve 0.76937 g of $Cr(NO_3)_3.9H_2O$ in 1 l of water.

Potassium chloride solution, 100 μg ml^{-1}. Dissolve 0.21315 g of KCl in 1 l of water.

Potassium iodide solution, 100 μg ml^{-1}. Dissolve 0.13081 g of KI in 1 l of water.

Potassium bromide solution, 1000 μg ml^{-1}. Dissolve 1.48941 g of KBr in 1 l of water.

A strong cation-exchange resin (Dowex HCR-W) (16–40 mesh) was used. It was washed with 2 M HCl, then with distilled water until the chloride washings were chloride free.

General Procedure

The schematic diagram in Fig. 1 shows the system used, in which bromide solution (60 μl through the sample loop) is injected into a stream of BrO_3^- and H_3O^+ ions, either passing through the by-pass tube to a delay coil or through the mini-column where cationic interferents pass through the cation-exchange resin prior to the solution reaching the delay column. Emission was monitored at 417 nm, using a 40-μl cell with an inlet slit of 20 nm.

Effect of Acid Concentration

Two bromide concentrations were studied, 3 and 6 μg per 60-μl sample. Various acid concentrations (0, 0.01, 0.05, 0.1, 0.25 and 0.5 M HNO_3) were prepared by appropriate dilution of the stock solution, either directly or by serial dilution. It was found that 0.25 M HNO_3 was the most suitable. Fig. 2 shows

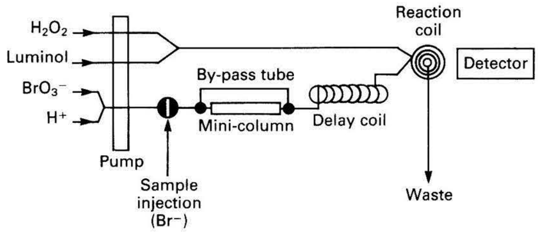

Fig. 1. Schematic diagram of the flow injection system used for the determination of bromide

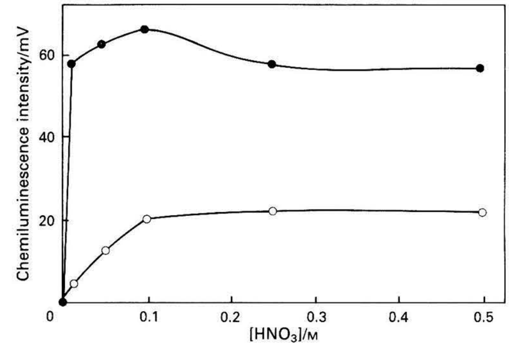

Fig. 2. Effect of HNO_3 concentration on chemiluminescence intensity. Luminol, 0.01 mM; H_2O_2, 10 mM; and $KBrO_3$, 100 mM; 0.25 M HNO_3 was used as the optimum acid concentration. ○, 3; and ●, 6 µg of Br^-

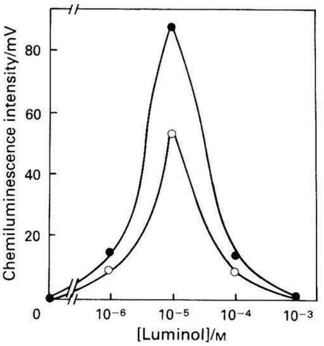

Fig. 3. Effect of luminol concentration on chemiluminescence intensity. HNO_3, 0.25 M; H_2O_2, 10 mM; and $KBrO_3$, 100 mM; 0.01 mM luminol was used as the optimum luminol concentration. ○, 3; and ●, 6 µg of Br^-

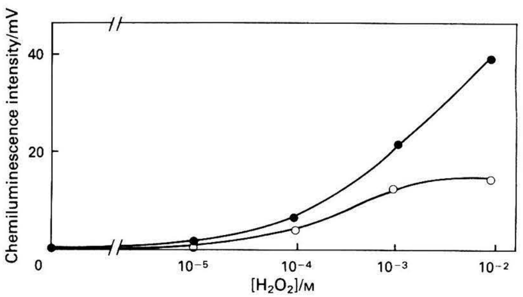

Fig. 4. Effect of H_2O_2 concentration on the chemiluminescence intensity at the optimum acid and luminol concentrations. $KBrO_3$, 100 mM; 10 mM H_2O_2 was used as the optimum peroxide concentration. ○, 3; and ●, 6 µg of Br^-

the chemiluminescence intensity expressed as peak height (mV) (average of four successive measurements) *versus* nitric acid concentration (M).

Effect of Luminol Concentration

Luminol solutions of different concentrations were prepared (0, 0.001, 0.01, 0.1 and 1 mM). The maximum chemiluminescence intensity was obtained with a 0.01 mM luminol solution. Fig. 3 shows the variation of the chemiluminescence emission intensity expressed as peak height (mV) with the concentration of luminol solution (M). Two different concentrations of bromide solution were studied as above and the average of four successive peak-height measurements was used.

Effect of Hydrogen Peroxide Concentration

By appropriate dilution of the stock solution of hydrogen peroxide a series of solutions was prepared (0, 0.01, 0.1, 1 and 10 mM). Fig. 4 shows the variation of the chemiluminescence intensity expressed as peak height (mV) with the hydrogen peroxide concentration (M); 10 mM H_2O_2 was found to be suitable. In this study, 3 and 6 µg of bromide ion per 60-µl sample were used.

Effect of Flow-rate

The experimental parameters for maximum chemiluminescence intensity (determined above) were used, *viz.*, bromate, 100 mM; acid, 0.25 M; luminol, 0.01 mM; and H_2O_2, 10 mM. Flow-rates of 0.5, 1, 1.5, 2, 3, 4 and 5 ml min^{-1} were employed. Fig. 5 shows the variation of the chemiluminescence intensity (mV) (average of four successive measurements) with the flow-rate (ml min^{-1}). A flow-rate of 3 ml min^{-1} was found to give the best sensitivity. Fig. 6 shows the chemiluminescence intensity - time profile for various flow-rates. As a compromise between the clarity of response, its maximum and the time required for a single measurement, 3 ml min^{-1} was selected as the optimum flow-rate.

Calibration Graph

Various concentrations (0, 1, 5, 10, 20, 50, 75, 100, 150, 200 and 250 µg ml^{-1}) of bromide were prepared using the parameters established above. Each measurement was repeated four times. The logarithm of the average peak height (mV) of the chemiluminescence intensity was plotted against the logarithm of the concentration of bromide ion, a straight-line graph (Fig. 7) from 1 to 100 µg ml^{-1} of bromide was obtained. Regression analysis gave the following equation: $\log CL = 0.415\log[Br^-] + 1.1586$, with a correlation coefficient of 0.9996. [CL = chemiluminescence intensity in mV (average of four successive measurements).]

Detection Limits

Successive dilutions of 1 µg ml^{-1} bromide solution (as KBr) were carried out for this study. It was possible to observe a clear signal for 6.25×10^{-5} µg ml^{-1} of bromide (above the signal for distilled water, which gave a zero base line). Each measurement was repeated 15 times. The coefficient of variation was ⩽1%. Fig. 8 shows typical responses obtained for 3.75 and 75 pg of bromide and for 12 µg of bromide, and illustrates the base line obtained using different amplifications and recorder outputs.

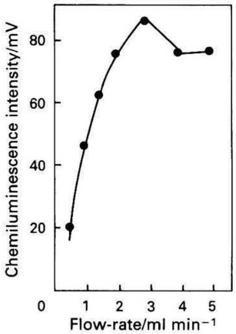

Fig. 5. Effect of flow-rate on chemiluminescence intensity. HNO_3, 0.25 M; luminol, 0.01 mM; H_2O_2, 10 mM; and $KBrO_3$, 100 mM. A flow-rate of 3 ml min^{-1} gave the best signal

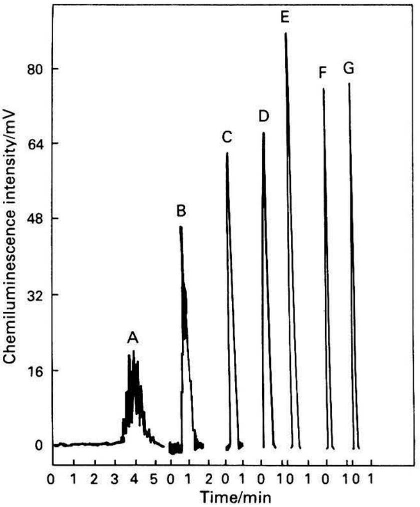

Fig. 6. Chemiluminescence emission time course for different flow-rates used with the optimum fixed parameters. Flow-rate: A, 0.5; B, 1; C, 1.5; D, 2; E, 3; F, 4; and G, 5 ml min^{-1}

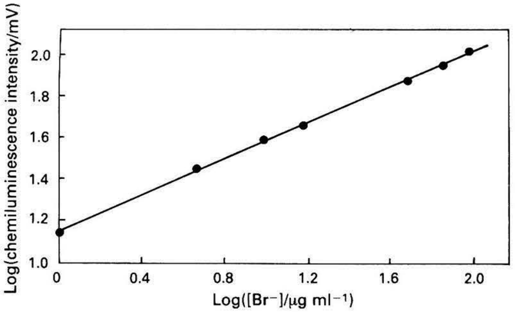

Fig. 7. Linear calibration graph for the determination of bromide using flow injection with chemiluminescence detection

Interferences

The optimum conditions (described above) were used. A 10 μg ml^{-1} solution of bromide was used and 0.1, 1, 10 and 50 μg ml^{-1} of interfering ion (*i.e.*, 0.01, 0.1, 1 and 5-fold excesses of Co^{II}, Cr^{III}, Cu^{II} and Cd^{II}) were added (*i.e.*, prepared in the same flask). Four successive measurements were made for each concentration. The mini-column (Fig. 1),

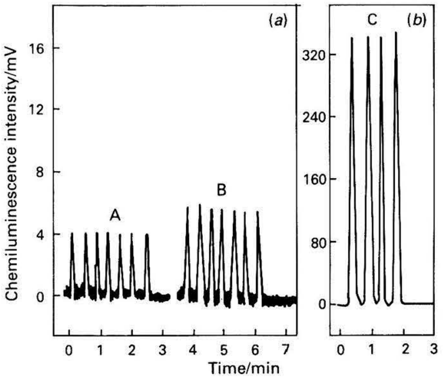

Fig. 8. Chemiluminescence - time profile for various bromide concentrations showing the background noise and the signal obtained. Amount of bromide: A, 3.75 pg; B, 75 pg; and C, 12 μg

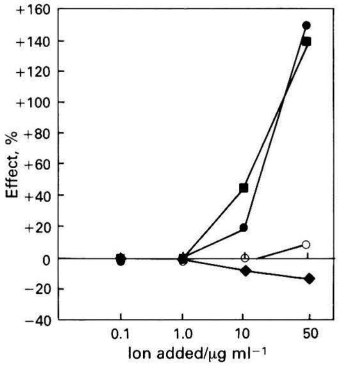

Fig. 9. Effect of added cations on 10 μg ml^{-1} of bromide. ●, Co^{2+}; ■, Cr^{3+}; ○, Cu^{2+}; and ◆, Cd^{2+}

Table 1. Effect of interfering ions on the chemiluminescence intensity of 10 μg ml^{-1} of Br^- (0.6 μg of Br^-) as KBr

Ion added	Treatment	Effect, %*
Cd^{II}	A†	+8.0
	B‡	+2.0
	C§	0.0
	D¶	0.0
Cu^{II}	A	+20.0
	B	+8.8
	C	+1.4
	D	0.0
Cr^{III}	A	+32.0
	B	+22.6
	C	+18.3
	D	+3.8
Co^{II}	A	+63.0
	B	+51.6
	C	+3.8
	D	0.0

* Average of four successive measurements.

† Standing with the cation-exchange resin for 15 min after the solution had been prepared; the emission was then measured via the by-pass tube (see Fig. 1).

‡ As for A but with a standing time of 30 min.

§ As for A but with a standing time of 45 min; the emission was measured via the mini-column (see Fig. 1).

¶ As for C except that 5 μg ml^{-1} of interfering ion were used rather than 10 μg ml^{-1} as for A–C.

Table 2. Effect of added chloride (1–100 μg ml^{-1}) and iodide (1–100 μg ml^{-1}) ions on the chemiluminescence intensity of 1, 10 and 50 μg ml^{-1} of bromide ion (all as potassium salts)

	Effect on chemiluminescence intensity, %		
Chloride ion added/μg ml^{-1}	1 μg ml^{-1} Br$^-$	10 μg ml^{-1} Br$^-$	50 μg ml^{-1} Br$^-$
0.0	0.0	0.0	0.0
1.0	0.0	0.0	0.0
10.0	0.0	0.0	0.0
50.0	11.3	0.0	0.0
100.0	20.1	0.0	0.0

	Effect on chemiluminescence intensity, %		
Iodide ion added/μg ml^{-1}	1 μg ml^{-1} Br$^-$	10 μg ml^{-1} Br$^-$	50 μg ml^{-1} Br$^-$
0.0	0.0	0.0	0.0
1.0	0.0	0.0	0.0
10.0	−67.2	−56.5	−77.7
50.0	−67.2	−79.8	−83.1
100.0	−69.0	−75.0	−81.9

10 cm × 2 mm i.d., containing the strong cation-exchange resin was used. Fig. 9 shows the plot of the percentage increase - decrease effect of the interfering ion on 10 μg ml^{-1} of bromide. Up to 1 μg ml^{-1} of the metal ion studied can be removed effectively, while up to a 150% increase was observed for Co^{II} and Cr^{III} ions. As metal ion catalysis was avoided, the aim was to remove the effect completely. Another set of solutions was prepared as described above, but 0.35770 g of the resin was added to the same flask after the solution had been prepared (batch exchange). Standing times of 15 and 30 min (treatments A and B, respectively) were allowed for the exchange of the ions, then the samples were injected and passed through the by-pass tube (Fig. 1). Table 1 shows the results obtained with treatments A and B. A noticeable effect can be seen. By allowing a standing time of 45 min and passing the solution through the mini-column (Fig. 1 and Table 1, treatment C), a better result was obtained. When the experiment was repeated, using 5 instead of 10 μg ml^{-1} of bromide, the interferences were completely removed by coupling both the batch and continuous flows through the exchange column.

In order to study anionic interferences, chloride and iodide were used. Various bromide solutions containing 1, 10 and 50 μg ml^{-1} of bromide were prepared and to each solution were added 0, 1.0, 10.0, 50.0 and 100.0 μg ml^{-1} of chloride ion. Another set of solutions was prepared similarly for the iodide ion (same concentrations as used for chloride ion). Table 2 presents the results obtained. It can be seen that chloride does not interfere at 1, 10 and 50 μg ml^{-1}, whereas iodide interferes seriously at concentrations >1 μg ml^{-1}.

Discussion

The proposed luminol chemiluminescence method involving the *in situ* generation of bromine has been applied successfully to the determination of bromide with a detection limit of 3.75 pg. The method is highly reproducible. For all the measurements performed, the coefficient of variation does not exceed 1% at the bromide concentrations studied. The blank always gives a zero base line, because the combination of BrO_3^- - Br^- - H_3O^+ causes the release of bromine; hence the absence of any one of these three ions causes the detector response to give a zero base line because the luminol luminescence fails to give any emission, as there is no metal to catalyse the reaction. The fact that an appreciable amount of some cations, *e.g.*, Co^{II} and Cr^{III}, produces an enhancement of the bromide signal may enable the detection limit for bromide to be lowered in some situations. A high flow-rate enhances the chemiluminescence intensity but reduces the effectiveness of the cation-exchange resin. The use of both batch and continuous exchange gives a better exchange process for the cations. The method can be used for the determination of the hydronium ion without employing a pH glass electrode. Further work on the determination of acidity is in progress, using the same principle, and also on the determination of the bromate ion.

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Flotation - Spectrofluorimetric Determination of Phosphate in Natural Water Using Rhodamine B as an Ion-pair Reagent

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A sensitive and accurate spectrofluorimetric method is described for the determination of phosphate in natural water using Rhodamine B as an ion-pair reagent. A Rhodamine B - molybdophosphate ion pair was floated as a membrane-like substance at the phase boundary between the aqueous phase and a diethyl ether phase. After the aqueous phase had been discarded, the excess of Rhodamine B extracted into the diethyl ether was removed by shaking with 25 ml of 1.0 M hydrochloric acid. The floated ion pair was dissolved by the addition of methanol to the diethyl ether phase. The determination of phosphate was performed by measuring the fluorescence intensity of the organic phase at 573 nm with excitation at 554 nm. The calibration graph was linear up to 0.25 μg of phosphorus with a correlation coefficient of 0.999. Although arsenic(V) caused a positive error, no ions commonly existing in natural water, including silicate, interfere with the determination of phosphate. The proposed method was applied to the determination of phosphate phosphorus in some natural water samples.

Keywords: *Phosphate determination; flotation; spectrofluorimetry; Rhodamine B; natural water*

As the determination of trace amounts of phosphate in natural samples is important for environmental chemistry and geochemistry, a sensitive and accurate method is needed for its determination. A number of sensitive methods have been reported for the determination of phosphate using a dyestuff such as Malachite Green.[1,2] We have also developed methods using flotation - spectrophotometry and indirect atomic absorption spectrometry with Malachite Green.[3] These methods are suitable for the determination of phosphorus at levels of >0.01 p.p.m. In environmental analysis, however, a method suitable for its determination at the p.p.b. level is also required. Spectrofluorimetry is a highly sensitive method used for the determination of many substances, including phosphate.[4,5]

In this paper, a spectrofluorimetric method is described for the determination of phosphate using Rhodamine B (C.I. 45170, Basic Violet 10) as an ion-pair reagent. Rhodamine B is a fluorescent cationic dyestuff that is used for the determination of trace amounts of some elements. The ion pair of molybdophosphate, which is formed in acidic solution in the presence of phosphate and molybdate, with Rhodamine B is floated at the phase boundary between the aqueous phase and a diethyl ether phase. The floated ion pair is dissolved by the addition of methanol to the diethyl ether phase. The fluorescence intensity of the solution is proportional to the concentration of phosphate and p.p.b. levels of phosphorus can be determined by measuring its fluorescence intensity.

Spectrofluorimetric[4] and spectrophotometric[6] methods have been reported for the determination of phosphate using Rhodamine B. In the former, which used butanol - chloroform (4 + 1 *V*/*V*) as an extractant,[4] the excess of Rhodamine B extracted into the organic phase was removed using two 10-ml portions of chloroform, prior to extraction of the Rhodamine B - molybdophosphate ion pair. In the latter, which used 4-methylpentan-2-one - cyclohexane (1 + 4 *V*/*V*),[6] the excess of Rhodamine B extracted into the organic phase was also removed using 10 ml of chloroform followed by washing with 25 ml of water. The proposed method is superior to these methods because a toxic solvent is not used to remove the excess of Rhodamine B extracted into the diethyl ether phase and the procedure is simple.

Experimental

Reagents

A standard 1 mg ml^{-1} stock solution of phosphorus was prepared by dissolving 4.394 g of potassium dihydrogen phosphate (Wako Pure Chemical Industries, Osaka, Japan; special grade) in water and diluting to 1000 ml with water.

A 0.2 M sodium molybdate solution was prepared by dissolving 24.2 g of sodium molybdate dihydrate (Wako Pure Chemical Industries; special grade) in 500 ml of water. To remove phosphorus from this solution, a 250-ml portion was washed with three 100-ml portions of butan-1-ol - chloroform (1 + 4 *V*/*V*) by shaking in a separating funnel. The butan-1-ol dissolved in the aqueous phase was removed with four 100-ml portions of chloroform by shaking the solution.

A 0.12 mM Rhodamine B solution was prepared by dissolving 57.5 mg of Rhodamine B (Wako Pure Chemical Industries, special grade) in 1000 ml of water.

A 2 M sulphuric acid solution was prepared by diluting concentrated sulphuric acid (Wako Pure Chemical Industries, special grade) nine times.

A 1.0 M hydrochloric acid solution was prepared by diluting concentrated hydrochloric acid (Wako Pure Chemical Industries, special grade) 12 times.

De-ionised, distilled water was used throughout.

Apparatus

The fluorescence intensity measurements and spectra were obtained using an Hitachi Model 650–10S spectrofluorimeter equipped with an Hitachi Model 056 recorder. Quartz cells (1 cm) were used.

The spectral band pass of the spectrofluorimeter was set at 3 nm for both excitation and emission.

Procedure

A sample solution containing up to 0.25 μg of phosphorus is placed in a 50-ml beaker, 5 ml of 2 M sulphuric acid, 3 ml of 0.2 M sodium molybdate solution and 2 ml of 0.12 mM Rhodamine B solution are added and the mixed solution is diluted to 25 ml with water. After leaving to stand for 15 min at 25–30 °C to allow the reaction to occur, the solution is transferred into a separating funnel and 10.0 ml of diethyl ether are added. The solution is then shaken for 1 min to float the Rhodamine B - molybdophosphate ion pair. After a few minutes, and by careful manipulation of the tap, virtually all of the aqueous phase can be discarded, leaving only a very small volume above the tap bore. In this way the floated ion-pair at the phase boundary will not be discarded accidentally.

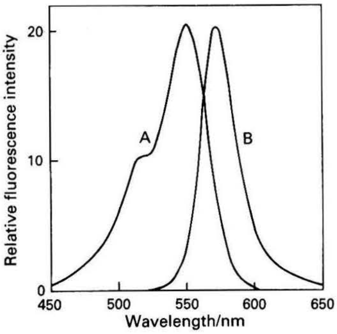

Fig. 1. Excitation and emission spectra of the Rhodamine B - molybdophosphate ion pair. [H_2SO_4] = 0.4 M; [Na_2MoO_4] = 24 mM; and [Rhodamine B] = 9.6 µM. A, Excitation; and B, emission spectra of 0.25 µg of phosphorus

To remove the excess of Rhodamine B extracted into the diethyl ether phase, the organic phase is shaken for 1 min with 25 ml of 1.0 M hydrochloric acid. The aqueous phase is discarded (again apart from a very small volume left above the tap), 15 ml of methanol are added to the organic phase and the solution is shaken for 30 s to dissolve the ion pair.

The solution is then transferred into a 25-ml calibrated flask and made up to volume with diethyl ether. The fluorescence intensity is measured at 573 nm with excitation at 554 nm. The standard solution for fluorescence intensity measurement was a 3.0 µM Rhodamine B solution, the intensity of which was set to 30.

Results and Discussion

Excitation and Emission Spectra

Fig. 1 shows the uncorrected excitation and emission spectra of the Rhodamine B - molybdophosphate ion pair. The maximum fluorescence intensity was observed at the excitation wavelength of 554 nm and the emission wavelength of 573 nm. For the determination of phosphate, these wavelengths were used.

Selection of Solvent for Flotation or Dissolution of the Ion Pair

Several organic solvents were examined for the extraction or flotation of the Rhodamine B - molybdophosphate ion pair. The results are shown in Table 1. When benzene or hexane was used, the ion pair floated at the phase boundary between the organic phase and the aqueous phase as a membrane-like substance. The ion pair was extracted into isoamyl alcohol or 4-methylpentan-2-one. Excess of Rhodamine B, however, was extracted into these solvents and it could not be removed by shaking with 1.0 M hydrochloric acid.

When diethyl ether was used the ion pair also floated at the phase boundary. Although the excess of Rhodamine B was extracted into diethyl ether, it could be removed by shaking with 1.0 M hydrochloric acid. Diethyl ether was therefore adopted as the solvent for flotation of the ion pair.

For dissolution of the floated ion pair, methanol was used for its convenience of handling.

Selection of Acid and the Effect of its Concentration

For the adjustment of acid concentration in the aqueous phase, sulphuric, hydrochloric, nitric and perchloric acids were examined. Of these, the largest fluorescence intensity of the ion pair and the smallest fluorescence intensity of the reagent blank were observed when sulphuric acid was used.

The effect of the concentration of sulphuric acid is shown in Fig. 2. The fluorescence intensity of the ion pair was constant above a concentration of 0.3 M. Therefore, the acid concentration of the aqueous phase was adjusted to 0.4 M with sulphuric acid.

Table 1. Selection of the solvent* for flotation of the ion pair; [H_2SO_4] = 0.4 M; [Na_2MoO_4] = 24 mM; and [Rhodamine B] = 9.6 µM

Solvent	Ion pair	Rhodamine B	Removal of excess of Rhodamine B
Diethyl ether	F	E	P
Benzene	F	E	I
Hexane	F	E	I
Isoamyl alcohol	E	E	I
4-Methylpentan-2-one	E	E	I
Chloroform	N	E	—

* F, Floated; E, extracted; N, neither floated nor extracted; P, possible; and I, impossible.

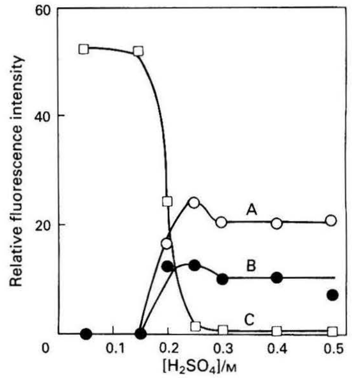

Fig. 2. Effect of the concentration of sulphuric acid on the fluorescence intensity of the ion pair. [Na_2MoO_4] = 24 mM; and [Rhodamine B] = 9.6 µM. A, 0.25; and B, 0.125 µg of phosphorus against the reagent blank; C, reagent blank

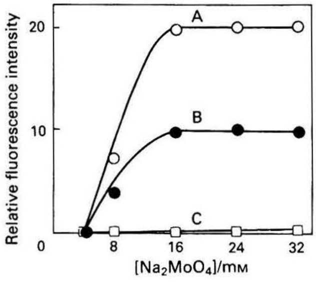

Fig. 3. Effect of the concentration of sodium molybdate on the fluorescence intensity of the ion pair. [H_2SO_4] = 0.4 M; and [Rhodamine B] = 9.6 µM. A–C as in Fig. 2

Effect of Concentration of Sodium Molybdate

Fig. 3 shows the effect of the concentration of sodium molybdate on the fluorescence intensity of the ion pair. The fluorescence intensity was constant above concentrations of 16 mM. A concentration of sodium molybdate of 24 mM was chosen for the procedure.

Effect of Concentration of Rhodamine B

The effect of the concentration of Rhodamine B on the fluorescence intensity is shown in Fig. 4. The fluorescence intensity was constant above concentrations of 9.6 μM. Therefore, 9.6 μM was chosen for the concentration of Rhodamine B in the procedure.

Removal of Excess of Rhodamine B

To remove the excess of Rhodamine B extracted into the diethyl ether phase, water and various concentrations of hydrochloric acid were examined. The results are shown in Fig. 5. When 1.0 M hydrochloric acid was used, the fluorescence intensity of the reagent blank was small and that of the ion pair was proportional to the concentration of phosphate.

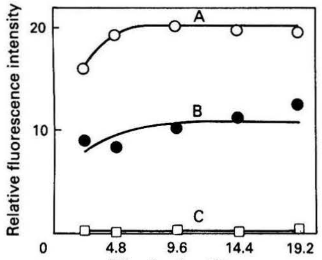

Fig. 4. Effect of the concentration of Rhodamine B on the fluorescence intensity of the ion pair. [H_2SO_4] = 0.4 M; and [Na_2MoO_4] = 24 mM. A–C as in Fig. 2

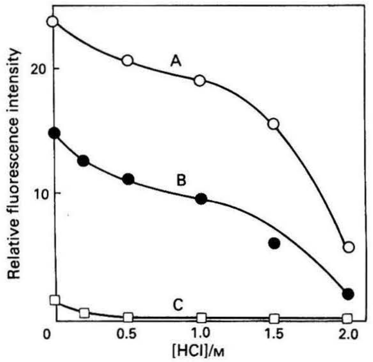

Fig. 5. Effect of the concentration of hydrochloric acid, used to remove the excess of Rhodamine B, on the fluorescence intensity of the ion pair. [H_2SO_4] = 0.4 M; [Na_2MoO_4] = 24 mM; and [Rhodamine B] = 9.6 μM. A–C as in Fig. 2.

The effect of the volume of 1.0 M hydrochloric acid on the fluorescence intensity is shown in Fig. 6. The use of one 25-ml portion of 1.0 M hydrochloric acid gave a good result. When one 10-ml portion was used, poor linearity was obtained between the phosphate concentration and the fluorescence intensity.

Therefore, 25 ml of 1.0 M hydrochloric acid were employed to remove the excess of Rhodamine B.

Calibration Graph and Precision

The calibration graph obtained was linear up to 0.25 μg of phosphorus with a correlation coefficient of 0.999. The relative standard deviations were 4.0 and 5.4% for 0.25 and 0.125 μg of phosphorus (n = 10 from several different batches), respectively. The detection limit was 0.4 μg l^{-1}, which was determined for twice the standard deviation of the reagent blank.

Effect of Foreign Ions

Table 2 shows the effect of foreign ions on the determination of phosphate. Arsenic(V) showed a large positive error for the determination of phosphate as it forms molybdoarsenate and is also floated as an ion pair with Rhodamine B.

Sodium chloride up to 0.7 M had no effect on the determination of phosphate. Silicon showed no interference

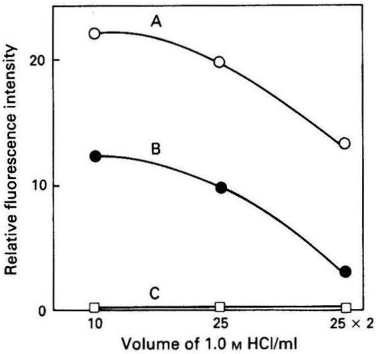

Fig. 6. Effect of the volume of 1.0 M hydrochloric acid on the fluorescence intensity of the ion pair. [H_2SO_4] = 0.4 M; [Na_2MoO_4] = 24 mM; and [Rhodamine B] = 9.6 μM. A–C as in Fig. 2

Table 2. Effect of foreign ions on the determination of phosphate

Species	Added as	Taken/μg	Fluorescence intensity	
			0.125 μg of P	0.25 μg of P
—	—	—	9.1 ± 0.5†	18.7 ± 0.8†
As^{III} . .	. . $NaAsO_2$	0.125	9.6	19.5
		0.250	9.4	19.3
As^{V} . .	. . Na_2HAsO_4	0.125	12.0	22.0
		0.250	13.6	23.7
Cr^{III} . .	. . $K_2Cr_2(SO_4)_4$	0.125	8.8	18.0
		0.250	9.2	18.0
Cr^{VI} . .	. . K_2CrO_4	0.125	9.0	18.2
		0.250	—	18.2
Tartrate . .	$Na_2O_2CCH(OH)CH(OH)CO_2$	0.125	9.0	17.2
Citrate . .	$Na_3O_2CCH_2C(OH)(CO_2)CH_2CO_2$	0.125	8.9	18.4
		0.250	8.7	18.8
EDTA* . .	Na_2H_2EDTA	0.125	8.8	17.5
SiO_2 . .	. . Na_2SiO_3	100	—	19.4

* Ethylenediaminetetraacetate.
† Mean ± standard deviation (n = 10).

Table 3. Recovery of phosphate phosphorus in a synthetic river water. Composition (mg l^{-1}): Na^+, 9.2; K^+, 1.2; Mg^{2+}, 1.9; Ca^{2+}, 8.0; Cl^-, 7.1; SO_4^{2-}, 7.7; and HCO_3^-, 24.4 (pH 7.33)

Phosphorus/µg		
Added	Found	Recovery, %
0	0.000	—
0.125	0.130	104.0
0.250	0.246	98.4

Table 4. Recovery of phosphate phosphorus in natural water. Sample taken, 10.0 ml

	Phosphorus/µg		
Sample	Added	Found	Recovery, %
Sea water	0	0.107	—
	0.125	0.249	113.6
	0.250	0.348	96.4
Snow 1	0	0.052	—
	0.125	0.162	88.0
	0.250	0.296	97.6
Tap water	0	0.032	—
	0.250	0.289	102.8
Ground water . .	0	0.054	—
	0.250	0.301	98.8
Snow 2	0	0.024	—
	0.250	0.284	104.0

Table 5. Comparison of the proposed method with other methods for the determination of phosphate phosphorus in natural water

	Phosphorus*/µg l^{-1}		
Sample	Proposed method ($n = 4$)	Co-5-Cl-PADAP method[8] ($n = 3$)	Molybdenum Blue method[9] ($n = 3$)
River water	2.1 ± 1.0	4 ± 1	
Ground water . .	17.5 ± 0.7	18 ± 1	
Snow	1.8 ± 0.1		<2

* Mean ± standard deviation.

when co-existing as silicon dioxide in an amount 400 times that of phosphorus.

The results of recovery tests for phosphate in a synthetic river water are shown in Table 3. The composition of the synthetic river water was taken as the mean of the rivers in the district.[7] Satisfactory recoveries were obtained.

Applications

The proposed method was applied to the determination of phosphate phosphorus in some natural water samples. Samples were filtered through a filter-paper (No. 5A, Toyo Roshi Kaisha, Tokyo, Japan), which was washed with hydrochloric acid, or filtered through a 0.45-µm pore size membrane filter. The results are given in Table 4 together with the results of recovery tests. Phosphate phosphorus down to p.p.b. levels can be determined by the proposed method.

Table 5 shows the results of the determination of phosphate phosphorus in other natural water samples using the proposed method compared with flotation - spectrophotometry using bis[2-(5-chloro-2-pyridylazo)-5-(diethylamino)phenolato]cobalt(III) chloride as the ion-pair reagent (Co-5-Cl-PADAP method)[8] and spectrophotometry using Molybdenum Blue after extraction with 2,6-dimethylheptan-4-one (Molybdenum Blue method).[9] A satisfactory agreement was obtained.

The proposed method is sensitive enough to determine phosphate phosphorus at p.p.b. levels. Washing with one 25-ml portion of 1.0 M HCl is sufficient to remove the excess of Rhodamine B whereas two portions of chloroform are necessary for the method proposed by Kirkbright *et al.*[4] No ions commonly present in natural water interfere with the determination of phosphate using the proposed method.

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Spectrophotometric Determination of Total Cyanide, Iron - Cyanide Complexes, Free Cyanide and Thiocyanate in Water by a Continuous-flow System

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A combination of three automated methods has been developed to determine total cyanide, free cyanide [*i.e.*, HCN(aq) + CN^-], iron - cyanide complexes and thiocyanate. The methods are based on a continuous-flow technique, combined with spectrophotometric detection, and have a detection limit of 0.6 µg l^{-1}. The distinction between iron - cyanide complexes and free cyanide plus thiocyanate was made by UV irradiation and distillation with citric acid. The distinction between free cyanide and thiocyanate was made by masking free cyanide with formaldehyde. Rapid and complete recovery of iron - cyanide complexes was obtained; the interference from nitrate was minimised. Shielding the samples from diffuse daylight appeared to be necessary to prevent rapid photolysis of the iron - cyanide complexes.

Keywords: *Cyanide determination; hexacyanoferrate determination; thiocyanate determination; continuous-flow technique; spectrophotometry*

The differences in toxicity and environmental behaviour between free cyanide [*i.e.*, HCN(aq) + CN^-], iron - cyanide complexes [$Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$] and thiocyanate make it desirable to distinguish these species analytically.

Small amounts of cyanide are usually determined after distillation by spectrophotometric methods based on a method first described by Aldridge.[1] In this method the CN^- ion is first halogenated (with bromine) whereafter it reacts with a mixture of pyridine and benzidine to form a stongly coloured polymethine dye. Modifications of this method are based mainly on the replacement of pyridine, benzidine or bromine with less toxic equivalents. The most important modifications are the use of isonicotinic acid instead of pyridine,[2] the replacement of benzidine by pyrazolone[3] or barbituric acid[4] and the replacement of bromine by chloramine-T. Some of these methods are used in continuous-flow systems.[5,6]

The rate of cyanide recovery from iron - cyanide complexes after distillation depends on the distillation conditions. The distinction between these complexes and free cyanide is made by varying these conditions so that in one instance only free cyanide and in the other both free cyanide and iron - cyanide complexes are decomposed and distilled. This strategy is followed in both manual and automated methods.

In the manual procedures, which are used as standards,[7,8] the total decomposition of iron - cyanide complexes is achieved by prolonged boiling of the samples under acidic conditions. Different acid mixtures such as HCl, H_3PO_4 - H_3PO_2[4] or H_2SO_4, sometimes with the addition of $MgCl_2$ or Cu_2Cl_2 are used.[7,8]

Milder distillation conditions (*e.g.*, pH 4 and room temperature) are employed in order to minimise decomposition of these complexes. Further, the dissociation of iron - cyanide complexes is reduced by enhancing the formation of their precipitates by adding zinc or lead salts.[9,10]

The results of these methods show that it is difficult to distil all the free cyanide without partly decomposing the iron - cyanide complexes and that to obtain complete recovery of iron - cyanide complexes a time-consuming distillation under vigorous conditions is required. These conditions can, however, cause decomposition of thiocyanate, which is not distilled, to yield cyanide.

In the automated method proposed by Goulden *et al.*[5] the decomposition of iron - cyanide complexes is accelerated by the use of UV irradiation. Decomposition thus proceeds much faster, but is, under the experimental conditions used, not complete.[4] Moreover, with this treatment thiocyanate is also decomposed to yield cyanide and sulphide. Without UV irradiation, not only free cyanide but also about 20% of the iron - cyanide complexes are distilled. These results make accurate determination of the amounts of free cyanide and iron - cyanide complexes impossible.

The aim of this work was to develop an automated method without the drawbacks mentioned above. For this purpose three independent determinations were optimised. (A) The total concentration of cyanide plus thiocyanate was determined spectrophotometrically by a modification of Aldridge's method after destruction of the sample by UV irradiation and subsequent distillation with citric acid at pH 3.8. (B) The concentration of free cyanide plus thiocyanate was determined with the same spectrophotometric method but without previous UV irradiation and distillation. (C) The thiocyanate concentration was determined with method B after masking the free cyanide with formaldehyde. From the results of these three methods it was possible to calculate the total concentration of cyanide (A − C) and the distribution of free cyanide (B − C) and iron - cyanide complexes (A − B).

Experimental

Apparatus

A Skalar 5101 continuous-flow system equipped with a UV irradiation unit, a closed distillation system and a spectrophotometric detector was used. The sampling rate was 22 samples per hour and the sample to wash ratio was 1:1. Other conditions were as follows: UV irradiation time, approximately 3 min; chlorination time, 1.5 min; and colour development time, approximately 2.5 min at 40 °C.

Reagents

All chemicals used were of analytical-reagent grade.

Distillation reagent. Dissolve 42.02 g of citric acid in 600 ml of water, add 200 ml of 1 M sodium hydroxide solution and dilute to 1000 ml with water. Add 259.5 ml of 0.1 M hydrochloric acid to 240.5 ml of this solution. Adjust the pH to 3.8 if necessary with 1 M sodium hydroxide solution or hydrochloric acid. Add 1.08 ml of concentrated (12 M) hydrochloric acid. The extra amount of acid will compensate for the amount of hydroxide in the samples at pH 12.

Glycylglycine solution. Dissolve 40.0 g of glycylglycine in 100 ml of water. Dilute this solution to 1000 ml with water.

Buffer solution (pH 5.2). Dissolve 2.3 g of sodium hydrox-

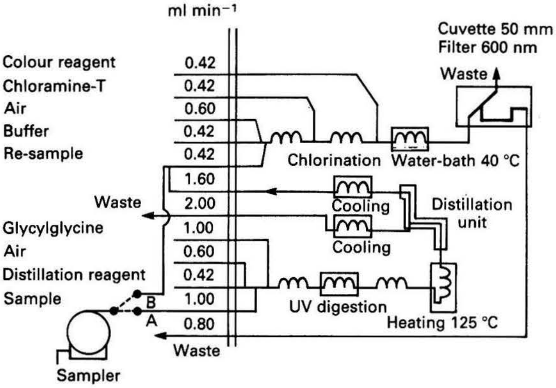

Fig. 1. Schematic representation of the apparatus used

ide in 500 ml of water. Add 20.5 g of potassium hydrogen phthalate to this solution and make up to 1000 ml with water. Adjust the pH if necessary to 5.2 with 1 M sodium hydroxide solution or hydrochloric acid. Add 1 ml of wetting agent (Brij 35, Skalar Chemicals) shortly before use. (After addition of the wetting agent, the solution is only stable for about 2 d.)

Chloramine-T solution. Dissolve 2.0 g of chloramine-T in 100 ml of water and dilute to 1000 ml with water.

Colour reagent. Dissolve 8.8 g of sodium hydroxide in 500 ml of water. To this solution add 16.8 g of 1,3-dimethylbarbituric acid and 13.6 g of isonicotinic acid and dilute to 100 ml with water. Adjust the pH if necessary to 5.2 with 1 M sodium hydroxide solution or hydrochloric acid.

Formaldehyde reagent. Dilute 10 ml of 37% formaldehyde to 100 ml with water.

Standard solutions. Stock solutions of potassium cyanide, potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) and potassium thiocyanate, containing 1000 mg l⁻¹ of cyanide, were prepared in 0.01 M sodium hydroxide solution. The concentrations of these solutions were checked by measuring the potassium concentrations. From these solutions, mixtures with different ratios of the above mentioned cyanide species were prepared. Standard cyanide solutions were prepared from the potassium cyanide solution in the concentration range 25–200 µg l⁻¹ in 0.01 M sodium hydroxide solution. These solutions were analysed to establish a calibration graph for each of the three methods.

All reagents except the buffer reagent are stable for at least 1 week when stored at 5 °C and shielded from light.

Procedure

To determine the concentration of total cyanide, free cyanide, iron - cyanide complexes and thiocyanate in a sample, three independent determinations are necessary. (A) The total concentration of cyanide plus thiocyanate is measured after UV irradiation and distillation. The sample must be inserted at position A (Fig. 1) of the apparatus. (B) The concentration of free cyanide plus thiocyanate is measured without previous distillation and UV destruction. The sample must be inserted at position B (Fig. 1). (C) The concentration of thiocyanate is measured with method B after masking the free cyanide with formaldehyde. This is performed by adding 50 µl of 3.7% formaldehyde to a 10-ml sample and either leaving the sample overnight or heating it for 15 min at 50 °C.

With all three methods the samples, at pH 12, are measured against standard solutions of potassium cyanide. It is important to shield the samples from light, not only during storage, but also during all treatment steps prior to the determination. In this work the automatic sampler was covered with a black plastic hood.

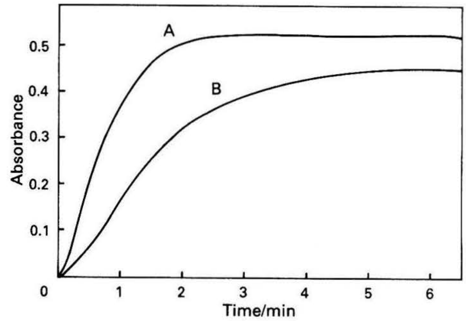

Fig. 2. Rate of colour development for the reaction of 100 µg l⁻¹ of cyanide with A, 1,3-dimethylbarbituric acid; and B, barbituric acid

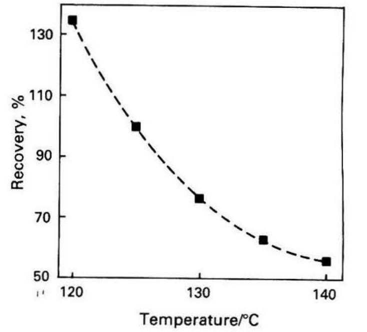

Fig. 3. Effect of distillation temperature on cyanide recovery. Recoveries of 100 µg l⁻¹ of cyanide, added as KCN, were compared with recovery at 125 °C (100%)

Results and Discussion

Spectrophotometric Determination of Cyanide and Thiocyanate

The spectrophotometric method used here is basically the same as that proposed by Nagashima.[2] The two major differences are the use of 1,3-dimethylbarbituric acid instead of barbituric acid and the use of 0.1 M phthalate instead of 1 M phosphate buffer. 1,3-Dimethylbarbituric acid has previously been used in combination with pyridine[11] and appears to give good results in combination with the less toxic isonicotinic acid.

The absorption spectrum of the complex formed by the reaction of cyanide with 1,3-dimethylbarbituric acid has a maximum at 600 nm. Colour development is faster and more intense than with barbituric acid (Fig. 2), resulting in a faster, more sensitive determination.

To prevent the loss of HCN(aq) and to optimise the reaction between formaldehyde and free cyanide, as described below, the pH of the samples must be adjusted to 12. However, for our purpose the rate of the chlorination reaction for cyanide and thiocyanate should be the same: this is the situation at pH values below 5.2,[12] whereas at higher values the reaction rate for thiocyanate is lower than for cyanide resulting in a lower sensitivity for the former. To maintain this pH with a phosphate buffer, a high concentration (1 M) is used.[2] A phosphate buffer, however, is not very efficient at this pH and, further, we have found that precipitates are sometimes formed. Therefore, other buffer systems were tried; phthalate was chosen as the most suitable, and it was capable of maintaining a pH of 5.2 at a concentration of 0.1 M.

Masking Free Cyanide With Formaldehyde

In order to measure only thiocyanate with method C, free cyanide was masked with formaldehyde[7] in samples adjusted to pH 12. In practice, 50 µl of 3.7% formaldehyde are sufficient to cover concentrations of up to 250 µg l^{-1} of cyanide in 10 ml, without any effect on the concentration of thiocyanate measured.

Distillation

The effects of temperature and pH on the distillation were investigated. Results for the temperature dependence are shown in Fig. 3. The adverse effect of a higher temperature is due to the influence of dilution caused by the extra amount of water that is distilled. At 125 °C slightly more fluid is distilled than needed by re-sampling for the spectrophotometric measurement. Under the experimental conditions used, cyanide is distilled quantitatively below pH 6.

Table 1. Recovery of iron - cyanide complexes and interference of nitrate on the distillation according to Goulden *et al.*[5]

Sample	Concentration of cyanide present/µg l^{-1}	Concentration of cyanide measured/µg l^{-1}
$Fe(CN)_6$ (68 µg l^{-1})	50	42
$Fe(CN)_6$ (136 µg l^{-1})	100	86
$Fe(CN)_6$ (203 µg l^{-1})	150	127
$Fe(CN)_6$ (271 µg l^{-1})	200	169
NO_3^- (50 mg l^{-1}) + humic acid (100 mg l^{-1}) . .	0	10
NO_3^- (50 mg l^{-1}) + citrate (50 g l^{-1})	0	45

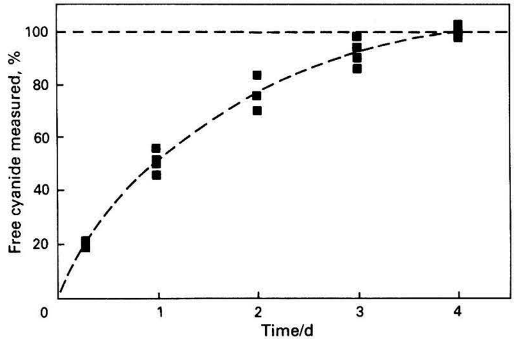

Fig. 4. Rate of photodecomposition of 100 µg l^{-1} of the iron - cyanide complexes in diffuse daylight at pH 12

Decomposition of Iron - Cyanide Complexes

For total cyanide determination iron - cyanide complexes must be decomposed before distillation. In the method described by Goulden *et al.*,[5] UV irradiation in combination with a pH of less than 1 is used for this purpose. According to Prober *et al.*,[6] who used the same method, this decomposition appears not to be complete under the specified conditions. These findings were confirmed by our results with this method, which gave recoveries of iron - cyanide complexes of about 85% (Table 1).

However, the total decomposition of these complexes appeared to be possible under less vigorous conditions, according to the results of the following experiment. It has been reported[8,13] that samples containing iron - cyanide complexes placed in direct sunlight undergo photolysis at a measurable rate. In order to determine the storage conditions, the rate of photodecomposition in diffuse daylight (laboratory conditions) had to be quantified. For this, samples containing 100 µg l^{-1} of cyanide as hexacyanoferrate(III) were adjusted to pH 12 (the usual practice during storage and pre-treatment for analysis) and exposed to diffuse daylight. Free cyanide was then measured at various times (Fig. 4). After 4 d the iron - cyanide complexes were decomposed completely. The initial decomposition rate at this pH is equal to *ca.* 2.5% per hour. This complete decomposition could have been caused by the lowering of the activites of Fe^{2+} and Fe^{3+} by hydrolysis. The observed variation in the reaction rate might be due to small differences in the exposure of the samples to light. The decomposition appeared not to be reversible to a measurable extent after 4 d.

The results of this experiment were used to ascertain the optimum conditions for the automated determination. It is, however, not possible to obtain complete decomposition of iron - cyanide complexes, by simply bringing the sample to pH 12 during UV irradiation, because at pH values above *ca.* 4, UV irradiation destroys the cyanide.[4] An alternative way of achieving low Fe activities below pH 4 is to add complexing agents. Citrate has been used for this purpose because of its

Table 2. Recoveries obtained for the various cyanide species with the different methods

Cyanide added/µg l^{-1}			Cyanide detected/µg l^{-1}			Cyanide calculated/µg l^{-1}		
Species			Method			Total	Free	$Fe(CN)_6$
CN^-	$Fe(CN)_6$	SCN^-	A	B	C	A − C	B − C	A − B
50	—	—	50	51	0	50	51	−1
100	—	—	99	100	1	98	99	−1
200	—	—	203	198	1	202	197	5
—	30	—	29	1	0	29	1	28
—	50	—	50	2	0	50	2	48
—	100	—	98	2	1	97	1	96
—	200	—	195	1	0	195	1	194
—	—	30	31	33	32	−1	1	−2
—	—	50	53	54	52	1	2	−1
—	—	100	98	104	105	−7	−1	−6
—	—	200	198	204	206	−8	−2	−6
50	50	50	152	103	53	99	50	49
—	50	50	101	53	52	49	1	48
50	—	50	103	101	52	51	49	2
50	50	—	102	52	0	102	52	50
—	30	100	127	105	104	23	1	22
30	—	100	127	131	104	23	27	−4
30	100	—	131	30	0	131	30	101
100	30	—	131	103	0	131	103	28

Table 3. Levels of interference of various substances with 100 μg l^{-1} of cyanide

			Cyanide measured/μg l^{-1}	
			Method	
Interfering substance	Added as	Amount added/mg l^{-1}	A	B and C
SO_4^{2-}	Na_2SO_4	1	100	—
		10	99	—
		100	97	—
		1000	98	—
SO_3^{2-}	Na_2SO_3	1	96	94
		10	59	10
S^{2-}	Na_2S	1	101	100
		5	103	99
		10	107	101
		15	110	102
		100	129	—
NO_2^-	$NaNO_2$	1	100	100
		5	107	99
		10	112	99
		25	132	102
NO_3^-	$NaNO_3$	1	100	100
		10	101	101
		100	104	100
		1000	114	99
CO_3^{2-}	Na_2CO_3	1	101	—
		10	104	—
		100	125	—
		1000	206	—
Ca^{2+}	$CaCl_2$	1	100	99
		10	100	101
		100	100	100
		1000	103	102
Humic acid		100	102	101
		1000	105	—

good solubility, its stability under UV irradiation and the high stability of its iron complexes at low pH. At pH 3.8, a concentration of 13 g l^{-1} of citrate improved the decomposition of iron - cyanide complexes exposed to UV irradiation. This decomposition was found to be complete when 16 g l^{-1} of citrate, or more, were present in the distillation reagent.

Recoveries

Samples containing different mixtures of cyanide species were analysed by the three methods (Table 2). The results obtained with method A show an equal recovery for all three cyanide species within 2%. The results of method B show an equal sensitivity for thiocyanate and cyanide. Iron - cyanide complexes give a response of less than 2% when stored in the dark at pH 12. The results of method C show that the masking of free cyanide with formaldehyde is complete without influencing the sensitivity for thiocyanate.

Interferences

Although the influence of most interfering substances is less after distillation, the UV irradiation used in method A causes other substances to interfere. Ultraviolet irradiation will cause oxidation of organic substances by nitrate and nitrite to yield cyanide. In this method citrate is present as a potential reducing agent. Without precautions, 5 mg l^{-1} of nitrate will therefore give a response equal to that of 0.1 mg l^{-1} of cyanide. Under the same experimental conditions, nitrite gives a response about 20 times that of nitrate. This could be an indication that nitrite is the reactive species.

The formation of cyanide does not only occur under the conditions used here, but also under those used by Goulden *et al.*[5] where nitrate and an organic substance (citrate, humic acid) are added (Table 1).

If nitrite is indeed the reactive form, nitrate and nitrite interference could be reduced by masking nitrite. The addition of sulphamic acid, as described by Csikai and Barnhard,[10] is not possible because sulphamic acid is destroyed under UV irradiation to yield cyanide. Therefore, an alternative approach was required. The addition of glycylglycine, a substance that is able to form diazo compounds after reaction with nitrite, was tried. It was found that 17 g l^{-1} of glycylglycine, added before UV irradiation, reduced the response for 50 mg l^{-1} of nitrate to less than that for 0.005 mg l^{-1} of cyanide. The interference from 5 mg l^{-1} of nitrite was reduced from a response equivalent to 2 mg l^{-1} of cyanide to a response equivalent to only 0.007 mg l^{-1} of cyanide.

In addition to nitrate - nitrite, the total cyanide method is also relatively sensitive to interference from sulphite and carbonate (Table 3). However, this was not a problem when analysing soil solutions.

The colorimetric reaction between cyanogen chloride and isonicotinate is a relatively specific reaction which is subject to very few interferences.[14] The results of testing the influence of the most common substances on the measurement of 100 μg l^{-1} of cyanide are shown in Table 3. Because none of the interfering substances studied reacted with formaldehyde, the results for methods B and C are equivalent. Sulphide, a known interferent in this method, gives no interference up to 5 mg l^{-1}, and a positive interference over the range 10–100 mg l^{-1}.

Analytical Performance

The detection limit, defined as three times the uncertainty (standard deviation) of ten measurements of the analytical blank, with the proposed system was found to be 0.6 μg l^{-1} of cyanide. The quantification limit, defined as ten times the standard deviation of the analytical blank, was 2 μg l^{-1} of cyanide. The repeatability, obtained from ten replicate analyses of a solution containing 100 μg l^{-1} of cyanide, was 1%. The absorbance appeared to be proportional to the concentration in the range 1–250 μg l^{-1} of cyanide.

The distillation in method A and the presence of formaldehyde in method C had no influence on the sensitivity or on the standard deviation of the measurements. The analytical performance of the three methods is, therefore, equal.

Conclusions

The automated method proposed here allows the accurate determination of low cyanide concentrations and can readily distinguish between free cyanide, thiocyanate and iron - cyanide complexes. The combination of UV irradiation and citric acid as the complexing agent results in the rapid and complete decomposition of iron - cyanide complexes.

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Selective Spectrophotometric Determination of Canavanine

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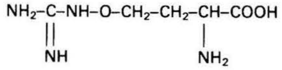
The optimisation of the analytical conditions for the spectrophotometric determination of canavanine (a non-protein amino acid that is important in animal nutrition) with pentacyanoaminoferrate-(II) and -(III) (PCAF) is described. The most influential parameters, *e.g.*, reagent preparation, oxidant, pH, buffer composition, interferences, etc., were studied. The best results were obtained with freshly synthesised PCAF whose aqueous solutions were either kept in the dark or activated with UV light, with pH 7 phosphate buffer, by the addition of potassium persulphate solution (1%) and by measurement at 520 nm. The procedure was applied to the determination of canavanine in an acidic extract of lentil vetch seeds, a legume that is widely used in animal feed.

Keywords: *Spectrophotometry; pentacyanoaminoferrate reagent; canavanine determination; non-protein amino acid; toxic amino acid*

Canavanine [2-amino-4-(guanidinoxy)butyric acid] is an oxyguanidine amino acid with the following formula:



$$NH_2{-}\underset{\underset{NH}{\|}}{C}{-}NH{-}O{-}CH_2{-}CH_2{-}\underset{\underset{NH_2}{|}}{CH}{-}COOH$$

It is a non-protein amino acid with a structure similar to arginine (a protein amino acid) and is the principal free amino acid in many legume seeds, making it important in animal nutrition.[1] As canavanine is high in nitrogen (31.8%), its main function may be to store nitrogen for embryo growth.[2] Studies carried out with micro-organisms, insects, higher plants and animals have demonstrated the adverse biological effects of canavanine, which acts primarily as an arginine antagonist, being incorporated in the cell nucleus and other proteins and hence interfering with desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) formation.[3,4] Its toxic effects in mammals are not fully known, although they seem to be fewer than expected, perhaps because canavanine is degraded by certain bacteria in the digestive tract.[5]

The importance of this amino acid to plants is clear, as is its influence on man and animals; therefore, it is necessary to develop selective analytical techniques for its determination in plant-based foods.

The first studies on canavanine were carried out by Kitagawa and Tomiyama.[6] They isolated canavanine from the seeds of *Canavalia ensiformis* and determined its exact structure by enzyme tests; further studies were carried out by other workers.[7,8] In some instances, in addition to the general procedure for detecting amino acids with ninhydrin, specific guanidine developers such as Sakaguchi reagent or butane-2,3-dione were used for the determination of canavanine. More recently, enzyme degradation techniques have been used.[9] At the same time, selective spectrophotometric methods for the determination of canavanine, which could be applied to plant extracts were developed.[10] These studies independently pointed out the significance of the use of pentacyanoaminoferrate (PCAF) and of the influence of factors such as oxidising agents, pH and reaction time on the colour development. However, as these factors were not studied together, specific methods for canavanine were not developed.

Therefore, given the various, and often contradictory, analytical conditions described by different workers, and the need for a fast, specific and sensitive method for the determination of canavanine that is applicable to plant extracts (which contain many amino acids and related compounds), we carried out a detailed study of the canavanine - PCAF reaction in order to obtain the optimum reaction conditions for the spectrophotometric determination of canavanine.

Experimental

Amber-coloured glassware was used throughout; where this was not available, the glassware used was covered with aluminium foil.

Apparatus

A Perkin-Elmer (Hitachi, 124) double-beam spectrophotometer with 1.0-cm quartz cuvettes was used. Irradiation experiments were performed with a 16-W Electronica Argemi blue - black UV lamp operating in the range 300–350 nm. A Crison micropH 2000 pH meter equipped with a combined glass - calomel electrode was also used.

Reagents and Solutions

All reagents used were of analytical-reagent grade and were obtained from Sigma and Merck.

Pentacyanoaminoferrate reagent. Prepared by the method of Kenney *et al.*[11]

Potassium persulphate solution, 1% *m/V*.

Standard L*-canavanine solution*, 1 mg ml^{-1}.

Aqueous PCAF solutions, 1% *m/V*. Prepared according to three different procedures: solution A was prepared in the dark; solution B was prepared in the dark and exposed to natural light for 1 h before filtering; and solution C was prepared in the dark and then activated with UV light for 15 min.

Phosphate buffer solutions, each 0.2 M. Solutions of pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 were prepared as described by Henry *et al.*[12]

Imidazole buffer solution, *pH* 7, 0.05 M.[12]

Sodium veronal (*sodium* 5,5*-diethylbarbiturate, USP grade*) *- HCl buffer solution*, *pH* 7, 0.0536 M.[12]

Veronal (5,5*-diethylbarbituric acid*) *- sodium veronal buffer solution*, *pH* 7, 0.004 M.[12]

Standard solutions of protein and non-protein amino acids and related compounds. Prepared at a concentration of 2 mg ml^{-1} in 0.1 M HCl.

Preparation of PCAF Reagent

Sodium pentacyanoaminoferrate (PCAF) was prepared by a modification of Hoffman's method.[11] The procedure was as follows. A 10-g amount of sodium nitroprusside was dissolved in 55 ml of concentrated ammonia solution. The solution was kept in the dark at 0 °C for 24 h; a yellow - green precipitate formed containing a mixture of sodium pentacyanoaminoferrate-(II) and -(III) and was filtered off. The filtrate was treated with ethanol until complete precipitation had occurred. This precipitate was combined with the first precipitate and washed with absolute ethanol until all the ammonia had been eliminated. The precipitate was dried over H_2SO_4 and stored in the dark over $CaCl_2$. It must be used within about 48 h of preparation as after this time the PCAF begins to decompose, turning from its characteristic yellow colour to a brownish green.

Preparation of Lentil Vetch Samples

A 5-g amount of lentil vetch (*Vicia ervilia*) seeds (white and red types) were split, finely ground and dried in an oven at 110 °C to constant mass. The powder was de-greased in a Soxhlet extractor with hexane for 5 h and, after drying in air, the seeds were extracted with 0.1 M HCl[13] in the proportion 1 + 10 *m/V*. The mixture was stirred mechanically for 6 h at room temperature and then set aside for 24 h. The operation was repeated and the supernatant liquid was centrifuged at 10 000 rev min^{-1} for 20 min, decanted off and adjusted to exactly pH 7.0 (pH meter) with 0.1 M NaOH solution. The residue from the seeds was subjected to a second extraction under the same conditions as the first and the combined extracts were diluted to a final volume of 100 ml.

Procedure

A 1-ml volume of standard canavanine solution (1 mg ml^{-1}), diluted so that its concentration was within the Beer's law range (0.005–0.08 mg ml^{-1} of canavanine), was placed in a 10-ml calibrated flask and 6.5 ml of pH 7 phosphate buffer solution, 1 ml of 1% potassium persulphate and 0.5 ml of 1% aqueous PCAF (in the dark) were added and the mixture was diluted to 10 ml with distilled water. The mixture was then homogenised and, after 15 min, the absorbance was measured at 520 nm.

Results and Discussion

Influence of Radiation and Oxidants on PCAF

As sodium pentacyanoaminoferrate(II) reacts with canavanine differently from sodium pentacyanoaminoferrate(III), and as these two compounds could not be separated from the synthesised product, the influence of light and the presence of oxidants on PCAF solutions was studied in order to determine which species was the most reactive towards canavanine.

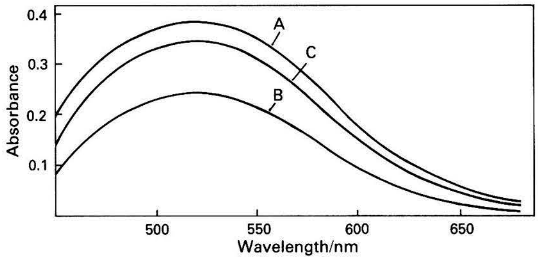


Fig. 1. A–C, Absorption spectra of the different solutions of PCAF - canavanine. For details see text. [Canavanine] = 5×10^{-2} mg ml^{-1}; and [PCAF] = 5×10^{-4} g ml^{-1}

The influence of light was studied by preparing three solutions of PCAF and subjecting one to UV light (solution C), one to sunlight (solution B) and keeping the third in the dark (solution A). Using these three solutions, the colour was developed with aliquots of the same canavanine solution and a spectral scan from 400 to 750 nm was performed against a PCAF solution blank as described under Procedure.

Fig. 1 shows the results and demonstrates that radiation, in particular visible radiation, has an adverse effect on colour development.

The influence of oxidising agents was investigated by adding 1 ml of 1% potassium persulphate to the canavanine solution and developing the colour as described under Procedure. Solutions A, B and C were studied in order to ascertain whether the oxidising agent compensated for the adverse effect of the radiation.

Fig. 2 shows the results. It can be seen that the absorbance values are significantly higher than those obtained in the absence of an oxidant and that the shapes of the absorption spectra are unchanged. The spectrum obtained for solution A was almost identical with that of solution C, indicating that the oxidant compensates for the adverse effect of UV light, but not for that of visible light. The addition of larger amounts of persulphate did not affect the colour development. Therefore, subsequent studies were carried out with either solution A or solution C with 1 ml of 1% potassium persulphate.

Effect of pH

To obtain maximum colour development in the PCAF - canavanine reaction the effect of pH was studied in the range 5–9 using phosphate buffers. Fig. 3 shows that colour development reached a maximum at pH 7 and then decreased rapidly above this value. Hence a pH of 7 was taken as the optimum for this reaction.

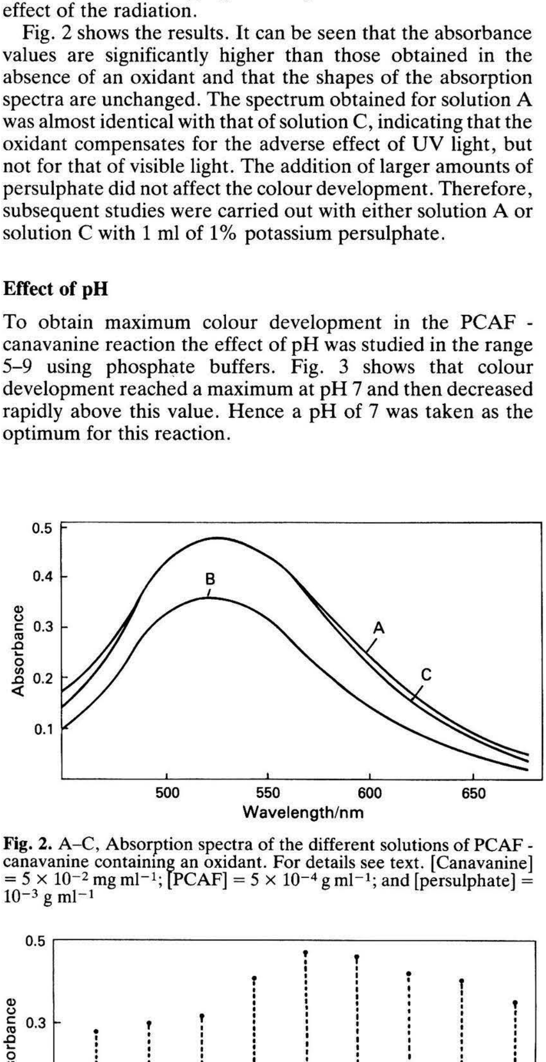


Fig. 2. A–C, Absorption spectra of the different solutions of PCAF - canavanine containing an oxidant. For details see text. [Canavanine] = 5×10^{-2} mg ml^{-1}; [PCAF] = 5×10^{-4} g ml^{-1}; and [persulphate] = 10^{-3} g ml^{-1}

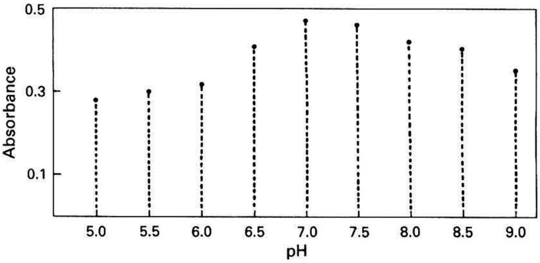


Fig. 3. Absorbance of the PCAF - canavanine solutions at different pH values. [Canavanine] = 5×10^{-2} mg ml^{-1}; [PCAF] = 5×10^{-4} g ml^{-1}; and [persulphate] = 10^{-3} g ml^{-1}

Effect of Buffer Composition

To study the influence of buffer composition on the PCAF - canavanine reaction, various veronal and imidazole buffers were prepared and used instead of the phosphate buffers, following the procedure described above. The imidazole buffer was found to inhibit colour development, whereas the other two types of veronal buffer had no significant effect.

Effect of Time on Colour Development

Under the optimum conditions, the time for maximum colour development was studied for three different concentrations of canavanine. Fig. 4 shows that the colour becomes stable 10 min after mixing the reagents and begins to decompose after 40 min, independently of the canavinine concentration.

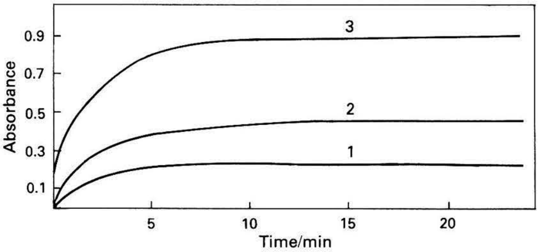


Fig. 4. Kinetics of the PCAF - canavanine reaction. [Canavanine]: 1, 2.5×10^{-2}; 2, 5×10^{-2}; and 3, 10×10^{-2} mg ml^{-1}. [PCAF] = 5×10^{-4} g ml^{-1}; and [persulphate] = 10^{-3} g ml^{-1}

Beer's Law Range

The canavanine absorbance *versus* concentration graph is linear from 0.005 to at least 0.08 mg ml^{-1}; its equation is $A = 9.62c + 0.0002$, where A is the absorbance and c is the concentration expressed in mg ml^{-1}. The correlation coefficient is 0.9988.

Reproducibility

The precision of the determination was established by analysing ten samples of canavanine (5×10^{-2} mg ml^{-1}). The standard deviation of these determinations was 3.3×10^{-3} mg and the standard error was 1.036×10^{-3} mg %.

Study of Interferences

Because of the structural similarity between canavanine and arginine (a protein amino acid), it was necessary to study the possible interference of the latter in the PCAF - canavanine reaction. Therefore, the PCAF - arginine reaction was studied following the procedure established for canavanine, not only at pH 7 but also over the pH range 5–9. This was carried out with pure arginine solutions and with canavanine solutions containing increasing amounts of arginine.

The influence of various amino acids on the PCAF - canavanine reaction at pH 7 was also studied. The amino acids chosen were those found in plants containing canavanine.

No colour development was observed for the PCAF - arginine reaction at any pH value, even at concentrations of 10 mg ml^{-1}. There was no interference on the PCAF - canavanine reaction, even at canavanine to arginine ratios of 1:10.

Table 1. Interference study. Canavanine concentration, 1 mg ml^{-1} (dilution, 1 + 49)

Amino acid	Amount added/ mg ml^{-1}	Amount of canavanine found/ mg ml^{-1}*	Standard deviation/mg
β-Alanine	2	1.1	0.0693
L-Alanine	2	0.95	0.0598
D-α-Aminoadipic acid	2	1.07	0.0674
D-α-Aminobutyric acid	2	1.2	0.0756
γ-Aminobutyric acid	2	0.87	0.0548
L-Arginine	2	1.1	0.0693
L-Arginine	10	1.1	0.0693
L-Aspartic acid	2	1.2	0.0756
DL-Citrulline	2	1.06	0.0668
β-Cyano-L-alanine	2	0.99	0.0624
L-Cysteic acid	2	0.93	0.0586
L-Cystine	2	1.03	0.0649
L-2,4-Diaminobutyric acid dihydrochloride	2	1.1	0.0693
DL-2,3-Diaminopropionic acid hydrochloride	2	1.1	0.0693
DL-β-3,4-Dihydroxyphenylalanine	2	0.90	0.0567
Ethanolamine	2	0.80	0.0504
D-(+)-Glucosamine hydrochloride	2	1.2	0.0756
L-Glutamic acid	2	0.90	0.0567
L-Glutamine	2	1.2	0.0756
Glycine	2	1.3	0.0819
L-Histidine	2	0.8	0.0504
DL-allo-δ-Hydroxylysine hydrochloride	2	0.8	0.0504
4-Hydroxy-L-proline	2	0.9	0.0567
L-Homocarnosine sulphate	2	1.1	0.0693
DL-Homoserine	2	1.2	0.0756
L-Isoleucine	2	0.8	0.0504
L-Leucine	2	0.7	0.0441
L-Lysine	2	0.8	0.0504
L-Methionine	2	1.2	0.0756
L-Ornithine	2	0.9	0.0567
L-Phenylalanine	2	0.9	0.0567
L-Proline	2	0.7	0.0441
L-Serine	2	0.6	0.0378
L-Threonine	2	0.7	0.0441
L-Tyrosine	2	1.1	0.0693
L-Valine	2	1.1	0.0693

* Average of five determinations.

Table 2. Determination of canavanine in lentil vetch samples

Sample*	Amount of canavanine added/ mg†	Amount of canavanine found/ mg‡	Standard deviation/mg
Red lentil vetch	—	5.15	0.33
	5	9.6	0.60
	10	14.6	0.92
	15	19.2	1.21
	20	24.6	1.55
White lentil vetch . .	—	4.93	0.31
	5	9.6	0.60
	10	14.8	0.93
	15	19.4	1.22
	20	24.1	1.52

* Extract from 5 g of seed in 100 ml.

† Amount of canavanine added to 5 g of seed.

‡ Average of five determinations.

The results obtained (Table 1) show that none of the amino acids tested interfere.

Applications

The proposed method was applied to the quantification of canavanine in lentil vetch seeds (*Vicia ervilia*), a legume that is widely used in animal nutrition because of its high protein content. Table 2 presents the results obtained, which show that there are no significant differences in the canavanine content between the two types of lentil vetch. Also, the levels of this amino acid in the seeds are well below toxic levels, bearing in mind that a normal diet with a particular feed would never consist solely of lentil vetch.

Conclusions

A rapid, simple and selective spectrophotometric method for the determination of canavanine using PCAF as the reagent has been developed. The reliability of the procedure was checked by the method of standard additions.

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Determination of Beryllium in Water by Ion-exchange Spectrofluorimetry

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A method for the determination of trace amounts of beryllium has been developed, based on ion-exchange spectrofluorimetry. Beryllium reacts with morin to give a highly fluorescent complex, which was fixed on a dextran-type anion-exchange resin. The fluorescence of the resin, packed in a 1-mm quartz cell, was measured directly using a solid-surface attachment. The concentration range of the method is 0.10–0.70 µg l^{-1}, the relative standard deviation 1.4% and the detection limit 0.02 µg l^{-1}. The method was applied to the determination of beryllium in natural waters. The aluminium and calcium levels can be determined first in order to reduce their interfering effect. The proposed method is more sensitive and selective than that using morin alone.

Keywords: *Morin; beryllium determination; ion-exchange spectrofluorimetry; natural water*

Ion-exchange spectrofluorimetry (IEF) combines the measurement of solid-surface fluorescence with the use of a solid support, *e.g.*, an ion-exchange resin, to pre-concentrate the analyte, which has been rendered fluorescent by the use of an appropriate reagent. This approach can be useful for the analysis of very dilute solutions, such as in water analysis.[1]

Conventional fluorescence with solid-surface methods, which is widely used for the assay of organic substances directly on solid surfaces such as paper chromatograms, thin-layer chromatography plates, KBr discs, electrophoresis strips and silicone rubber pads,[2–4] involves measurement of the diffuse reflected fluorescence (DRF), whereas the method described here is based on the use of diffuse transmitted fluorescence (DTF). The latter was chosen because the dextran-type resin used is more translucent than silica gel or alumina at the 1-mm thickness used.

This paper describes a method for the determination of beryllium using the IEF methodology. The use of beryllium compounds in metallurgy and nuclear technology and their high toxicity[5] suggests that more sensitive and reliable methods for determining trace amounts of beryllium are required.

The use of morin, a known spectrofluorimetric reagent for beryllium,[6–14] allowed the development of an IEF method for the determination of beryllium in natural waters that eliminated interference problems caused by the presence of Al^{III} and Fe^{III} ions. The proposed method is more sensitive and selective than that using morin alone.

Experimental

Reagents

All reagents were of analytical-reagent grade unless stated otherwise.

Sephadex QAE A-25 anion-exchange resin (Pharmacia Fine Chemicals) was used in the chloride form and without pre-treatment in order to avoid contamination.

Morin (2′,3,4′,5,7-pentahydroxyflavone) solution, 0.02% *m/V in absolute ethanol (Merck).* More dilute solutions were prepared by appropriate dilution with absolute ethanol. Morin was purified by the procedure of Laitinen and Kivalo.[7] The dilute morin solutions were prepared fresh every other day and the concentrated solutions weekly.

Beryllium(II) stock solution, 0.1 g l^{-1}. Prepared from $BeSO_4.4H_2O$ (Merck) in 6×10^{-2} M $HClO_4$ and standardised by the phosphate method.[15] Solutions of lower concentration were prepared by dilution with doubly distilled water.

Buffer solutions. Solutions of the required pH were prepared from 0.4 M Na_3PO_4 and 0.4 M H_3PO_4.

Apparatus

All spectrofluorimetric measurements were performed with a Perkin-Elmer LS 5 luminescence spectrometer, equipped with a xenon discharge lamp (9.9 W) pulsed at line frequency, Monk-Gillieron F/3 monochromators, a quantic Rodamine 101 counter to correct the excitation spectra, a Hamamatsu R928 photomultiplier, a Houston Omnigraphic *x* - *y* recorder, a variable-angle solid-surface accessory, designed and constructed by the authors,[16] and a Braun Melsungen Thermomix 1441 thermostat. In order to compare all the spectrofluorimetric measurements and to ensure reproducible experimental conditions, the LS 5 spectrometer was checked daily. A sample of the fluorescent polymer *p*-terphenyl (10^{-7} M) gave a relative fluorescence intensity of 90% at $\lambda_{em.}$ = 340 nm, $\lambda_{ex.}$ = 295 nm, slit widths of 2.5 and 2.5 nm and a sensitivity factor of 0.594. A Crison 501 digital pH meter with calomel and glass electrodes and an Agitaser 2000 rotating agitator were also used.

Fluorescence Measurements

The measured relative fluorescence intensity (RFI) of the resin beads, containing the fluorescent product and packed in a 1-mm quartz cell, was the diffuse transmitted fluorescence (DTF) emitted from the resin at the unexcited surface of the cell. The optimum angle formed between the cell plane and the excitation beam was 45° in all instances.

Procedures

Basic procedure

A 500-ml water sample containing 0.10–0.70 µg l^{-1} of beryllium was transferred into a 1-l polyethylene bottle and 3 ml of 0.0015% morin solution, 10 ml of pH 11.50 HPO_4^{2-} - PO_4^{3-} buffer solution and 80 mg of Sephadex QAE A-25 resin were added. The mixture was shaken mechanically for 30 min after which the resin beads were collected by filtration under suction and, with the aid of a pipette, were packed in a 1-mm cell together with a small volume of the filtrate. A blank solution containing all the reagents except beryllium was prepared and treated in the same way as described for the sample. The fluorescence intensities (20.0 ± 0.5 °C) of the sample and blank were always measured 15 min after loading the samples, at $\lambda_{em.}$ = 525 nm, with $\lambda_{ex.}$ = 457 nm. A calibration graph was constructed in the same way using beryllium solutions of known concentration.

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Procedure for waters

To a 500-ml water sample placed in a 1-l polyethylene bottle were added 2 ml of a 0.01 g l^{-1} BF_4^- solution, 2 ml of a 1 g l^{-1} fluoride solution and the amount of disodium ethylenediaminetetraacetate (Na_2EDTA) necessary to eliminate the interference from Ca^{II} and Mg^{II} ions (up to 0.4 g is usual). When the required amount of Na_2EDTA had been added, 3 ml of 0.0015% morin solution, 10 ml of pH 11.50 HPO_4^{2-} - PO_4^{3-} buffer solution (for raw water 60 ml of buffer solution were necessary to fix the pH) and 80 mg of Sephadex QAE A-25 were added. The mixture was shaken mechanically for 30 min and then treated as described under Basic procedure. The standard additions method was used for calibration.

Treatment of the sample

Natural waters were filtered through a filter-paper with a pore size of 0.45 µm (Millipore), preserved in concentrated $HClO_4$ (0.25 ml of $HClO_4$ per litre of sample) and collected in a polyethylene container that had been carefully cleaned with $HClO_4$.

In order to eliminate the chlorine in tap water, the 500-ml samples were treated for 5 min with 5 g of activated charcoal and filtered before being transferred into a polyethylene bottle.

Results and Discussion

Excitation and Emission Spectra in the Resin and Solution

Morin reacts with beryllium at high pH (>11) to form several fluorescent complexes in solution depending on the morin to beryllium molar ratio. The 1:1, 2:2 and 2:1 (morin:beryllium) complexes have been described.[10] In the presence of Sephadex anion-exchange resin, the 1:1 complex is probably sorbed on the resin, due to the small amounts of morin and morin - beryllium complex present in solution. The complex has an anionic nature due to the ionisation of morin ($pK_1 = -1$; $pK_2 = 4.8$; $pK_3 = 7$; $pK_4 = 9$; and $pK_5 = 13$).[10] Moreover the complex is not fixed on cation-exchange resins. We selected a QAE A-25 dextran-type resin as this led to a lower background fluorescence.

The peak wavelengths in the emission spectra of the morin - beryllium system are identical for the immobilised and solvated systems (525 nm) (Fig. 1). The similarities in the emission spectra for these two systems suggest that the complex is relatively insensitive to its environment. The maxima of the excitation spectra of the two systems differ. They are located at 443 nm in solution and at 457 nm in the resin phase. On the other hand the most noticeable differences between the fluorescence spectra are the better resolution of the resin-phase spectrum and the smaller peak width which characterises the solution spectrum. A similar effect is observed in the spectra of aluminium chelates fixed on silica gel.[17]

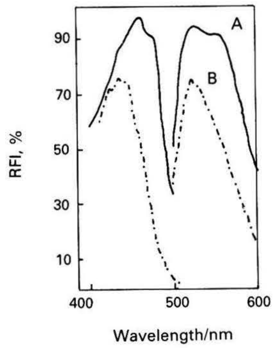

Fig. 1. Fluorescence spectra of the morin - Be^{II} complex. A, Phase resin: [morin] = 2.7×10^{-7} M; [Be^{II}] = 6.6×10^{-8} M; pH = 11.50 (HPO_4^{2-} - PO_4^{3-} buffer solution); 80 mg of Sephadex QAE A-25; 500-ml sample; stirring time = 30 min; $\lambda_{ex.}$ = 457 nm; $\lambda_{em.}$ = 525 nm; f_s = 0.15; $slit_{ex.}$ = $slit_{em.}$ = 2.5 nm; T = 20.0 ± 0.5 °C; ethanol = 0.6%. B, Solution: [morin] = 2.4×10^{-5} M; [Be^{II}] = 5.5×10^{-6} M; pH = 11.50 (HPO_4^{2-} - PO_4^{3-} buffer solution); $\lambda_{ex.}$ = 443 nm; $\lambda_{em.}$ = 525 nm; f_s = 0.8; $slit_{ex.}$ = $slit_{em.}$ = 2.5 nm; T = 20.0 ± 0.5 °C; ethanol = 4%

In addition, we have observed that a decrease in the excitation slit width ($slit_{ex.}$) or an increase in the emission slit width ($slit_{em.}$) increases the fluorescence signal. A similar effect has been reported by García-Vior and Possidoni de Albinati.[18] For optimum excitation and emission, slit widths of 2.5 nm were selected in both instances.

From a study of the half-life of the excited state of the complex in the solid phase at different temperatures, we believe that the luminescence process is fluorescence ($\tau < 5 \times 10^{-6}$ s).

Optimisation of Variables

pH dependence

We tested the buffer solutions commonly used to study the beryllium - morin system in solution (piperidine buffer, 0.1 M NaOH solution, phosphate buffer). The HPO_4^{2-} - PO_4^{3-} mixture gave the best results.

The RFI increases up to pH 11.40, this being the optimum value for the formation and fixation of the species between pH 11.40 and 12.00 (Fig. 2). We used 11.50 as the working pH in the standard procedure. It should be noted that the optimum pH in the resin phase is the same as that in solution.

The fluorescence is independent of the ionic strength, adjusted with the buffer solution, up to 0.5 M.

Morin concentration

The dependence of the RFI on the morin concentration is shown in Fig. 3. The optimum morin concentration for maximum fluorescence intensity is 4.5×10^{-7} M, *i.e.*, 0.000015%, for a morin to beryllium ratio of about 7. Such a dependence is analogous to that found in solution[8] and the optimum concentration is similar to that reported by Laitinen and Kivalo[7] (0.00002%). At higher morin concentrations the quenching effect observed in solution is more marked in the resin phase, probably due to the re-absorption effect by the solid matrix (morin fixed on Sephadex).[19] An increase in the percentage of ethanol increases the fluorescence of both the fixed complex and the blank. We chose 0.6% as the optimum value.

Influence of temperature

The effect of temperature on the ion-exchange process and hence on the fluorescence emission was studied. The dependence of the ion-exchange process on temperature is

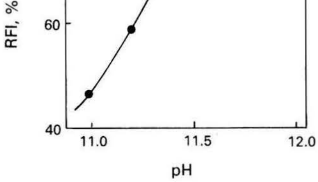

Fig. 2. Influence of pH on RFI. [Morin] = 5.9×10^{-7} M; [Be^{II}] = 1.1×10^{-7} M; 100 mg of Sephadex QAE A-25; 500-ml sample; stirring time = 20 min; $\lambda_{ex.}$ = 457 nm; $\lambda_{em.}$ = 525 nm; f_s = 0.15; $slit_{ex.}$ = $slit_{em.}$ = 2.5 nm, T = 20.0 ± 0.5 °C; ethanol = 0.6%

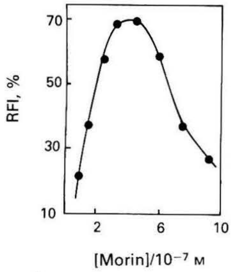

Fig. 3. Effect of the morin concentration on the RFI. $[Be^{II}] = 6.6 \times 10^{-8}$ M; [morin], 7.4×10^{-8}–8.9×10^{-7} M; pH = 11.50 (HPO_4^{2-} - PO_4^{3-} buffer solution); 100 mg of Sephadex QAE A-25; 500-ml sample; stirring time = 20 min; $\lambda_{ex.}$ = 457 nm; $\lambda_{em.}$ = 525 nm; f_s = 0.15; $slit_{ex.} = slit_{em.}$ = 2.5 nm, T = 20.0 ± 0.5 °C; ethanol = 0.6%

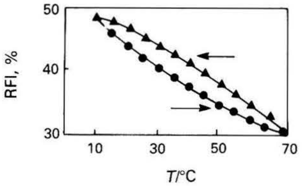

Fig. 4. Influence of the temperature on the RFI. ●, Heating; ▲, cooling. [Morin] = 4.4×10^{-7} M; $[Be^{II}] = 6.6. \times 10^{-8}$ M; pH = 11.50 (HPO_4^{2-} - PO_4^{3-} buffer solution); 100 mg of Sephadex QAE A-25; 500-ml sample; stirring time = 20 min; $\lambda_{ex.}$ = 457 nm; $\lambda_{em.}$ = 525 nm; f_s = 0.05; $slit_{ex.} = slit_{em.}$ = 2.5 nm; ethanol = 0.6%

complicated. In the range 5–50 °C, measuring the RFI at 20 °C, the dependence can be described by a polynomial equation, $RFI = 35.0 + 4.7T - 0.3T^2 + 8.0 \times 10^{-3}T^3 - 6.6 \times 10^{-5}T^4$ (°C). This unusual dependence may be due to an increase in the aerial photo-oxidation of the morin - beryllium complex with temperature.[20] On the other hand the RFI decreases when the temperature of the system increases; this effect is reversible as there is a small hysteresis loop (Fig. 4).

The experimental equations are: $\ln RFI = 3.94 - 0.008T$ (°C) ($r = 0.999$) for heating and $\ln RFI = 4.00 - 0.008T$ (°C) ($r = 0.985$) for cooling. The decrease in the RFI is 12% at 25 °C, 21% at 40 °C and 32% at 60 °C. In the last instance, the fixation of the species was carried out at 20 °C. All measurements reported here were made at 20.0 ± 0.5 °C.

Other experimental conditions

The stirring time necessary for maximum RFI development was 30 min for 250-, 500- and 1000-ml samples. For stirring times longer than 40 min, the RFI decreases slightly (20% at 80 min). The order of addition of the reagents did not affect the results obtained. The order used was as follows: beryllium, morin, buffer and resin. As the use of a large amount of resin lowered the RFI, only the amount required to fill the cell and facilitate handling, *i.e.*, 80 mg, was used for all the measurements. With regard to the stability of the fixed complex, the RFI decreases slightly for the first 15 min (8%) and then remains constant for at least 1 h.

Effect of sample volume on the sensitivity

The sensitivity of ion-exchange spectrofluorimetry should be increased if a larger amount of the sample solution is taken for equilibration with the solid.[21] The increase in sensitivity can be calculated by measuring the RFI of Sephadex equilibrated with different volumes of solutions containing the same concentration of beryllium and proportional amounts of the other reagents. Fig. 5 shows the increase in the fluorescence signal with sample volume (V). The nature of the experimental data (the RFI becomes independent of sample volume at higher samples volumes) suggests an exponential dependence. An equation of the type $RFI = a + be^{-cV}$ was tested. Suppose that $a = b$; if we linearise the equation according to $\ln(RFI/a - 1) = cV$, a good agreement is obtained. For 0.4 µg l^{-1} of beryllium and $a = 56$, $c = -1.95 \times 10^{-3}$ and $r = 0.977$. For 0.2 µg l^{-1} of beryllium and $a = 40$, $c = -1.86 \times 10^{-3}$ and $r = 0.989$. The equation breaks down at higher sample volumes due to fluctuation of the fluorescence measurements. This fluctuation is of the same order of magnitude as the noise of the spectrometer operated under the experimental conditions.

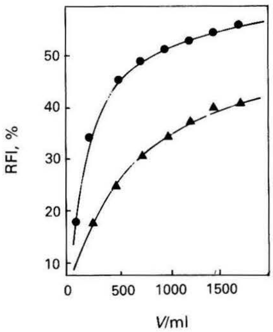

Fig. 5. Influence of the sample volume on the RFI. ●, [Morin] = 3.0×10^{-7} M; $[Be^{II}] = 4.4 \times 10^{-8}$ M. ▲, [Morin] = 1.5×10^{-7} M; $[Be^{II}] = 2.2 \times 10^{-8}$ M. pH = 11.50 (HPO_4^{2-} - PO_4^{3-} buffer solution); 80 mg of Sephadex QAE A-25; sample volume, 100–1750 ml; stirring time = 30 min; $\lambda_{ex.}$ = 457 nm; $\lambda_{em.}$ = 525 nm; f_s = 0.05; $slit_{ex.} = slit_{em.}$ = 2.5 nm; T = 20.0 ± 0.5 °C; ethanol = 0.6%

Table 1. Analytical parameters

	Sample volume/ml		
	250	500	1000
Intercept	−2.2	6.1	−3.1
Slope	117.94	124.08	144.70
Linear dynamic range/µg l^{-1}	0.10–0.70	0.10–0.70	0.10–0.70
Correlation coefficient	0.997	0.992	0.996
Detection limit (n = 3)/µg l^{-1}	0.02	0.02	0.02
Quantification limit (n = 10)/ µg l^{-1}	0.06	0.06	0.06
RSD, %	1.7	1.4	1.2
Analytical sensitivity/µg l^{-1}	0.006	0.005	0.005

Calibration and Precision

The calibration graphs for the water samples are linear in the concentration range 0.10–0.70 µg l^{-1} for 250-, 500- and 1000-ml samples. The analytical parameters are summarised in Table 1.

The reproducibility of the proposed method and of the packing of the resin in the 1-mm cell was determined. The precision was measured for a beryllium concentration of 0.3 µg l^{-1} by performing ten independent determinations using the proposed method. The relative standard deviations (RSDs) were 1.7, 1.4 and 1.2% for 250-, 500- and 1000-ml samples, respectively. On the other hand, the precision (RSD) of the packing operation, calculated from ten measurements, was 1.8% for the resin fixed with the beryllium - morin complex, 1.6% for the resin blank (resin with morin and buffer) and 1.8% for the resin only. It appears, therefore, that one of the main factors affecting the reproducibility is the packing of the resin. Centrifugation of the resin packed in the cell does not lead to better precision. The precision (RSD) of the fluorescence measurements (noise) was about 0.5% in all instances.

Table 2. Methods for the spectrofluorimetric determination of beryllium

Reagent	Detection limit*/ µg l^{-1}	Reference
1-Amino-4-hydroxyanthraquinone + β-cyclodextrin	3	23
o-(Salicylideneamino)benzoic acid	2	24
o-(2-Pyridyl)phenol	1.6	25
1-Hydroxy-6-methylanthrone†	1	26
2-Ethyl-3-methyl-5-hydroxychromone†	1	27
2-Hydroxy-*N*-(2-hydroxy-5-methylbenzylidene)-4-methylaniline	1	28
o-(Salicylideneamino)-3,5-dimethylbenzenearsonic acid†	1	29
2-Quinizarinsulphonic acid	1	30
o-(Salicylideneamino)benzenearsonic acid†	0.9	31
6-(4-Methylsalicylideneamino)-*m*-cresol	0.8	32
o-(Resorcylideneamino)benzenearsonic acid-	0.4	33
2-Hydroxy-*o*-(salicylideneamino)benzenesulphonic acid	0.4	34
2-Ethyl-5-hydroxy-7-methoxyisoflavone†	0.4	35
Morin	0.4	14
2-Hydroxynaphthalene-3-carboxylic acid†	0.18	36
4-Hexyloxysalicylaldehyde 4-ethoxysalicylidenehydrazone‡	0.1	37
Morin - IEF	0.02	This work

* Or minimum concentration used for calibration.
† Extraction procedure.
‡ Synchronous excitation fluorescence.

Sensitivity and Detection Limit

The increase in sensitivity obtained with the proposed method is substantial, particularly with respect to solution methods that use morin as a reagent. In order to compare this increase in sensitivity, the calibration graph for the determination of beryllium with morin in solution was constructed, *i.e.*, for the method of Klemperer and Martin,[6] under the experimental conditions used here. The equation for the calibration graph was RFI = 0.2 + 0.14c (r = 0.996), the ratio of the slopes being 886.

In IEF methods, the sensitivity can be enhanced by increasing the volume of the sample to be analysed. In practice, this increase can be calculated from the slope of the calibration graphs. The calculated values of the sensitivity ratio for the samples analysed here are: $S_{1000/500}$ = 1.17; $S_{500/250}$ = 1.05 and $S_{1000/250}$ = 1.23.

The fluctuation in the background fluorescence measured for the blank, from the average of ten determinations and expressed by the standard deviation is 0.75, 0.78 and 0.79 for 250-, 500- and 1000-ml sample volumes, respectively. The IUPAC detection limit (n = 3) is 0.02 µg l^{-1} of beryllium for 250-, 500- and 1000-ml sample volumes and the quantification limit (n = 10) is 0.06 µg l^{-1} of beryllium for 250-, 500- and 1000-ml sample volumes.

The calculated analytical sensitivity, S_A, defined by García-Sánchez and Cruces-Blanco,[22] is 0.006, 0.005 and 0.005 µg l^{-1} for 250-, 500- and 1000-ml sample volumes, respectively.

The method was compared with the chelate formation methods decribed in the literature for the spectrofluorimetric determination of beryllium. For comparison purposes we selected those methods which we considered to be among the most sensitive reported so far (Table 2).

Effect of Foreign Ions

A systematic study of the effect of foreign ions on the determination of beryllium, at the 0.4 µg l^{-1} level, was undertaken. A 40 000 µg l^{-1} level of potentially interfering ions was tested first, and, if interference occurred, the ratio was reduced progressively until interference ceased. Higher ratios were not tested. Tolerance was defined as the amount of foreign ion that produced an error equal to ±5% in the determination of the analyte. The results are summarised in Table 3.

Table 3. Effect of foreign ions on the determination of 0.4 µg l^{-1} of beryllium

Foreign ion or species	Tolerance level/µg l^{-1}
F$^-$	32 000
SO_4^{2-}	16 000
Cl$^-$	12 000
EDTA	10 000
NO_3^-, CO_3^{2-}	8000
SiO_3^{2-}	7000
BF_4^-	4000
Ca^{II}	400
Mg^{II}	90
Fe^{III}, Al^{III}	5

Table 4. Recovery study of beryllium in natural waters

Water	Amount added/ µg l^{-1}	Amount found*/ µg l^{-1}	Recovery, %
Tap water (Granada town)	—	0.09	—
	0.10	0.19	100
	0.30	0.40	103
	0.50	0.60	102
Raw water (Quentar dam)	0.20	0.19	95
	0.40	0.38	96
	0.60	0.61	102
Mineral water (Lanjaron)	—	0.10	—
	0.10	0.20	100
	0.30	0.39	97
	0.50	0.61	102
Mineral water (Ortigosa del Monte)	0.20	0.19	95
	0.40	0.39	97
	0.60	0.59	98

* Data are the average value of three determinations.

In order to apply the method to the determination of beryllium in natural waters, the interference from those ions commonly found in water was studied. The relative errors for the determination of 0.4 µg l^{-1} of beryllium were: NO_3^- (4 mg l^{-1}) 3%, Cl$^-$ (8 mg l^{-1}) 5%, SO_4^{2-} (20 mg l^{-1}) 5%, CO_3^{2-} (100 mg l^{-1}) 7%, SiO_3^{2-} (4 mg l^{-1}) 2%, Ca^{II} (4 mg l^{-1}) 28%, Mg^{II} (4 mg l^{-1}) 14%, Al^{III} (400 µg l^{-1}) 15% and Fe^{III} (40 µg l^{-1}) 17%.

The most serious interferences were from Al^{III}, Fe^{III}, Ca^{II} and Mg^{II}. Greater tolerances for these ions can be achieved by several methods. In order to eliminate the interference from Al^{III}, sodium tetrafluoroborate can be used as a masking agent.[38] The optimum amount of BF_4^- required to eliminate the interference from Al^{III} is a function of the Al^{III} content because the masking agent interferes slightly (tolerance 4 mg l^{-1}). For an Al^{III} level up to 400 µg l^{-1}, the interference from this element can be eliminated with 40 µg l^{-1} of BF_4^-.

The interference from Fe^{III} can be eliminated by using a 100-fold (*m/m*) excess of fluoride. On the other hand the interferences from Ca^{II} and Mg^{II} can be eliminated with EDTA. As a large amount of EDTA interferes and as large amounts of the EDTA masking agent are necessary, the optimum amount of EDTA is a function of the Ca^{II} and Mg^{II} content. For a Ca^{II} level of 30 mg l^{-1} it is necessary to use a 26-fold (*m/m*) excess and for 2 mg l^{-1} a 50-fold (*m/m*) excess of EDTA is required. This also applies to Mg^{II}.

Determination of Beryllium in Tap and Natural Waters

The method was applied to the determination of beryllium in water samples by the standard additions method. Tap water and raw water from the supply to Granada (Spain) and mineral water from Lanjaron (Granada) and Ortigosa del Monte (Segovia) springs were selected as representative samples. The volume of water used for the analysis was 500 ml in all instances.

The loss of sensitivity caused by the matrix effect can be evaluated from the ratio of the slope of the standard calibration graph to that of the standard additions calibration graph. The ratios were 1.1 for tap water, 1.5 for raw water, 1.3 for Lanjaron water and 1.1 for Ortigosa del Monte water.

Prior to the determination of beryllium, the content of Al^{III} (160, 48, 5 and 15 $\mu g\ l^{-1}$ for tap water, raw water, Lanjaron water and Ortigosa del Monte water, respectively), Fe^{III} (20, 18, 20 and 3 $\mu g\ l^{-1}$, respectively), Ca^{II} (20, 31, 32 and 2 $mg\ l^{-1}$, respectively), and Mg^{II} (6, 16, 12 and 0.2 $mg\ l^{-1}$, respectively) was determined by atomic absorption spectrometry in order to select the amounts of masking agents required.

The average value (three determinations) of the beryllium content was found to be $0.09 \pm 0.01\ \mu g\ l^{-1}$ for the town tap water (experimental quantification limit $0.06\ \mu g\ l^{-1}$), $<0.10\ \mu g\ l^{-1}$ for raw water from a nearby natural supply (Quentar dam) (the beryllium content was lower than the quantification limit in this instance), $0.10 \pm 0.01\ \mu g\ l^{-1}$ for Lanjaron mineral water (experimental quantification limit $0.09\ \mu g\ l^{-1}$) and $<0.06\ \mu g\ l^{-1}$ for Ortigosa del Monte mineral water (beryllium content lower than the quantification limit).

To check the accuracy of the proposed method a recovery study was carried out on the waters mentioned above. For this, various amounts of beryllium were added to 500-ml water samples and the percentage recovery was determined. Table 4 shows the results obtained for all the water samples. The results show good agreement with those obtained by Tao *et al.*[39]

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Spectrophotometric Determination of *N*-Acetylcysteine in Drug Formulations With *o*-Phthalaldehyde and Isoleucine

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A spectrophotometric procedure is proposed for the assay of *N*-acetylcysteine (NAC) in the range 0.5–49 μg ml^{-1}, based on the reaction of the thiol group with *o*-phthalaldehyde and isoleucine at pH 9.5 ($\lambda_{max.}$ = 335 nm). The procedure was applied successfully to the determination of NAC in several pharmaceutical formulations. The recoveries ranged from 98 to 106%, with relative standard deviations of less than 1.5%. The method is free from interferences and is simple, rapid and easy to automate.

Keywords: *N-Acetylcysteine determination; o-phthalaldehyde - isoindole formation; spectrophotometry; drug formulation*

N-Acetylcysteine (NAC) is a mucolytic agent that is used as an adjunct to other types of therapy to reduce the viscosity of pulmonary secretions in cystic fibrosis of the pancreas and other afflictions where a mucolytic therapy is required.

Several methods have been proposed for the determination of NAC; however, the procedures have usually only been applied to aqueous solutions of NAC in the absence of other compounds.

The methods described in the literature are generally based on the quantification of the thiol group and can be classified into three types: (*i*) oxidation of the thiol group; (*ii*) formation of metal mercaptides; and (*iii*) use of chromophoric reagents. The oxidation of the thiol group can be performed with iodine,[1] chlorine or bromine,[2] tetrathionate[3] or Fe^{III}.[4] The metal mercaptides can be obtained by titration of the thiol with Hg^{II} or Ag^{I} [5–7] or Pd^{II}.[8]

Direct UV spectrophotometry has also been used for the quantitative analysis of NAC solutions, but the method has been criticised because it suffers from numerous interferences and does not distinguish between NAC and the inactive degradation product *N,S*-diacetylcysteine.[9] The official method[10] for determining the NAC content in sterile solutions and inhalation solutions containing isoproterenol is based on the separation of NAC by high-performance liquid chromatography (HPLC) and detection at 214 nm.

Other methods are based on the reaction of NAC with 5,5′-dithiobis(2-nitrobenzoic acid)[11] and hydroxylamine.[12] A fluorimetric method with monobromobimane (4-bromomethyl-2,3,5-trimethyl-3a,6a-diazapentalene-1,6-dione has also been reported[13] and the method has been applied to studies of the pharmacokinetic behaviour of NAC by HPLC.

Primary amines react rapidly with *o*-phthalaldehyde (OPA) in the presence of a thiol (usually 2-mercaptoethanol) to give 1-alkylthio-2-alkyl-substituted isoindoles. The reaction has been used for the separation and fluorimetric detection of amino acids.[14] The reaction has also been applied to the fluorimetric determination of thiols.[15–19] Nakamura *et al.*[17] developed a procedure for the determination of thiols (not including NAC) with OPA and tryptophan as reagents. The procedure consists of the addition of 3.2 × 10^{-5} M OPA to a stirred mixture of 6.3 × 10^{-4} M tryptophan, boric acid - borate buffer and the thiol in the concentration range 6.6 × 10^{-7}–6.6 × 10^{-6} M.

More recently, NAC has been used in place of 2-mercaptoamino acid enantiomers with fluorimetric detection.[20–24] The reaction may also be applicable to the assay of NAC.

In a previous paper[25] we studied the formation and stability of the amino acid - OPA - NAC derivatives by using spectrophotometric measurements. The reactions occurred at room temperature and maximum absorbance was attained in the pH range 7.5–10.5. This paper describes a spectrophotometric procedure for the assay of NAC with OPA and isoleucine. The method was applied successfully to the determination of NAC in several pharmaceutical formulations.

Experimental

Reagents and Apparatus

o-Phthalaldehyde (Serva, analytical-reagent grade). Solutions (1.25 × 10^{-2} M) in ethanol were prepared weekly and stored in the dark.

Boric acid - borate buffer (pH 9.5). Prepared by dissolving 6.18 g of H_3BO_3 and 2.8 g of NaOH in 1 l of water. The OPA - buffer reagent was prepared by mixing 200 ml of the OPA solution and 800 ml of the buffer.

N-Acetyl-L-cysteine (Fluka, puriss). Solutions (1 × 10^{-3} M) in water were prepared weekly and stored in a refrigerator.

Isoleucine (Scharlau, puriss). Solutions (1.25 × 10^{-2} M) were prepared in 1 M HCl.

Absorbance measurements were carried out with a Shimadzu UV-240 spectrophotometer.

Procedures

Spectrophotometric determination of NAC with OPA and isoleucine

Suitable volumes of a solution containing 0.012–1.2 mg of NAC were transferred into 25-ml calibrated flasks. Ten millilitres of the 2.5 × 10^{-3} M OPA - buffer solution and 2 ml of 1.25 × 10^{-2} M isoleucine were added and the mixture was diluted to volume with water. The absorbance at 335 nm (ε = 6260 l mol^{-1} cm^{-1}) was measured against a blank, prepared without thiol.

The NAC content in several drug formulations was found by using the standard additions method. Appropriate amounts of the preparations were dissolved in distilled water; working solutions were prepared by appropriate dilution. Superpeni mucolytic and Flubiotic solutions were filtered through sintered glass crucibles. Increasing volumes of the NAC standard solution were added to the pharmaceutical solutions, which were then analysed as described above.

Determination of NAC with tetrathionate

Five millilitres of phosphate buffer (pH 7) and 10 ml of 0.025 M tetrathionate (obtained by titration of 0.05 M thiosulphate with 0.05 M iodine) were added to the sample solution containing 16–49 mg of NAC. The reagent solution was purged with nitrogen and stored in a stoppered, dark-coloured bottle. The solution was stirred for 1 min, then 0.5 g of potassium iodide, 25 ml of water, 1 ml of 0.5% starch solution and 3 ml of 0.1 M HCl were added. The liberated thiosulphate was titrated with 0.005 M iodine monochloride (obtained by the reaction between potassium iodide and potassium iodate in HCl) until a blue colour appeared. The iodine monochloride solution was standardised iodimetrically against thiosulphate. Blank determinations were also performed.

Spectrophotometric determination of NAC with Fe^{III} and 1,10-phenanthroline

Five millilitres of 4.8×10^{-3} M Fe^{III}, 2.5 ml of 0.25% 1,10-phenanthroline, 5 ml of 0.1 M acetic acid - 0.3 M acetate buffer (pH 4) and water to a final volume of 25 ml were added to the sample solution containing 0.05–0.13 mg of NAC. The absorbance at 515 nm ($\varepsilon = 10\,800$ l mol^{-1} cm^{-1}) was measured after 20 min against a blank prepared without thiol.

Results and Discussion

Selection of the Amine

Amino acids also react with OPA in the absence of thiols to give a product with a broad absorption band at 335 nm.[26] The rate of the reaction depends on the amino acid involved. The absorbance of the blank in the proposed procedure depends on this reaction.

In previous experiments, tryptophan was used under the conditions given by Nakamura *et al.*[17] (molar ratio of tryptophan : OPA : NAC = 100 : 5 : 1) except that the absorbance rather than fluorescence was measured. It was observed that the blank solutions were highly coloured (yellow to red), and gave very high absorbance values. With lower tryptophan concentrations, the absorbance of the blank was initially lower, but it increased with time. In addition, the absorbance of the NAC - isoindole solutions decreased gradually, indicating that the isoindole formed under these conditions was unstable.

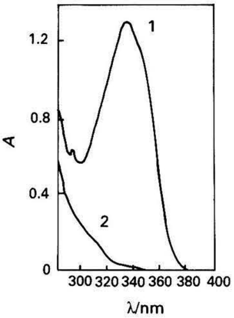

Fig. 1. Absorption spectrum of 1, the isoleucine - OPA - NAC derivative and 2, the blank. $c_{NAC} = 2.1 \times 10^{-4}$ M; $c_{isoleucine} = c_{OPA} = 1 \times 10^{-3}$ M

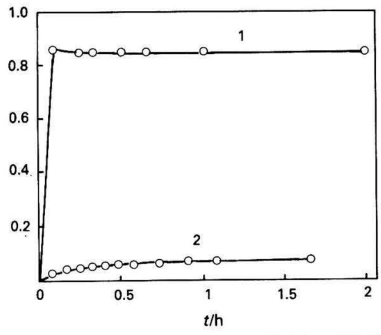

Fig. 2. Solution stability: 1, isoleucine - OPA - NAC and 2, isoleucine - OPA. $c_{NAC} = 1.36 \times 10^{-4}$ M; $c_{isoleucine} = c_{OPA} = 1 \times 10^{-3}$ M

In contrast, isoleucine shows a low reactivity towards OPA in the absence of thiols compared with other amino acids.[26] Hence this compound seems to be more suitable for the derivatisation process. The apparent molar absorptivities with respect to NAC for the isoleucine and tryptophan derivatives obtained with 1×10^{-3} M OPA and 1×10^{-3} M amino acid were *ca.* 6000 and *ca.* 3000 l mol^{-1} cm^{-1}, respectively. The higher value obtained with isoleucine was probably due to the negligible extent of the OPA - isoleucine side-reaction, whereas this reaction is important for tryptophan. The formation of the tryptophan isoindole was also incomplete.

Fig. 1 shows the absorption spectrum of the isoleucine isoindole ($\lambda_{max.}$ = 335 nm) together with the spectrum of the blank. The solutions of the isoleucine - OPA - NAC derivative are very stable, a 2% decrease in the absorbance being observed 2 h after mixing the reagents (Fig. 2).

Optimisation of the Experimental Conditions

Isoleucine and OPA must be in excess but their concentrations must not be too high as this would lead to high blanks. It should be noted that the OPA - isoleucine side-reaction becomes more significant at increasing concentrations of both the OPA and isoleucine reagents.

With 6×10^{-3} M OPA, the blanks were always high, even in the presence of a low concentration of isoleucine (5×10^{-5}–1.0×10^{-4} M). With 1×10^{-3} M OPA and 1×10^{-4}–1×10^{-3} M isoleucine, the blank absorbance was initially low, but it increased very slowly (Fig. 2). Therefore, it is convenient to prepare the blank and sample solutions simultaneously.

With 1.4×10^{-4} M NAC, the absorbance was constant for OPA : NAC molar ratios of 5–25 and isoleucine : NAC molar ratios of 3–25. Higher concentrations of OPA and isoleucine should not be used because of the high blanks that result.

Analytical Figures of Merit

The slope and intercept of the calibration graph obtained by applying a linear least-squares treatment to the absorbance - NAC concentration data were 6260 l mol^{-1} cm^{-1} and 0.003 A, respectively, with a correlation coefficient of >0.9999. The linear dynamic range extended up to 3.0×10^{-4} M NAC (49 µg ml^{-1}) with a limit of detection of 9.1×10^{-7} M (0.15 µg ml^{-1}) (ten blank replicates, 3*s* criterion). The relative standard deviation for 1.26×10^{-4} M NAC was 0.3% (ten replicates).

Determination of NAC in Commercial Formulations

The proposed method was applied to the assay of NAC in a number of different types of drug formulations collected from local pharmacies: Fluimucil (granules), Fluimucil antibiotic (ampoules), Rinofluimucil (nasal drops), Superpeni mucolytic (suspension) and Flubiotic (granules) (see Table 1). The results were compared with those obtained using two other methods: iodimetric titration of the thiosulphate liberated after the reaction of NAC with tetrathionate[3] and the oxidation of NAC with Fe^{III} in the presence of 1,10-phenanthroline.[4] The former method has also been used by other workers[27] for comparison purposes.

Triplicate determinations were performed for each sample. The absorbance at 335 nm of the pharmaceutical solutions at pH 9.5 in the absence of OPA and isoleucine was lower than 0.01 A. The slopes of the straight lines obtained when the standard additions method was applied to the different samples were the same as that obtained from the calibration graph.

Table 1. Determination of NAC in drug formulations

		Determined content as percentage of label claim ± standard deviation*		
Product	Nominal composition	Method 1†	Method 2‡	Method 3§
Fluimucil	NAC, 200 mg; saccharin, 8 mg; and excipient, 5 g (per bag)	103.5 ± 1.6	111.8 ± 1.1	94 ± 2
Fluimucil antibiotic	NAC, 113 mg; and thiamphenicol, 250 mg [per ampoule (lyophilised powder)]	105.7 ± 1.4	445 ± 2	98.7 ± 0.2
Rinofluimucil	NAC, 10 mg; tuaminoheptane sulphate, 5 mg; and benzalkonium chloride, 0.125 mg (per ml)	113.6 ± 0.1	116.6 ± 0.1	110.8 ± 0.3
Superpeni mucolytic	NAC, 12.5 mg; amoxicillin trihydrate, 250 mg; terbutaline sulphate, 2.5 mg; oxolamine citrate, 15 mg; guaiacol glycerol ether, 7.5 mg; and saccharin sodium excipient, 15 mg (per 5 ml)	101.3 ± 0.6	104.5 ± 1.1	109 ± 2
Flubiotic	NAC, 100 mg; amoxicillin trihydrate, 250 mg; saccharin, 4 mg; and excipient, 5 g (per bag)	98.0 ± 0.6	101.1 ± 0.6	100.1 ± 0.6

* Average of three determinations.
† Method 1: formation of the isoleucine - OPA - NAC isoindole.
‡ Method 2: oxidation with tetrathionate.
§ Method 3: oxidation with Fe^{III} in the presence of 1,10-phenanthroline.

The other compounds found in the formulations do not react with the reagents OPA and isoleucine, as they do not contain a thiol group. On the other hand, the presence of primary amine groups in some of the compounds does not seem to interfere with the determination of NAC. Commonly used excipients also do not interfere with the procedure.

The values found with the proposed method agreed well with those declared by the manufacturers and ranged from 98 to 106% (Table 1). Only Rinofluimucil gave results that were too high; however, the percentage of NAC found was similar to that obtained with the other two methods. The results were reproducible with a relative standard deviation of below 1.5% (three replicates).

The comparative methods used were not always appropriate for the assay of NAC in the formulations tested as the reductants present interfered. Hence the tetrathionate method gave a large positive error with the Fluimucil antibiotic, which contains thiamphenicol. This compound has a sulphinic acid group that can be oxidised to give a sulphonic acid. On the other hand, the colour change at the end-point was not permanent with the Superpeni mucolytic and Flubiotic formulations. These drugs contain amoxicillin, which probably reacts with the iodine formed at the end-point of the titration. In these instances, the volume taken for the end-point corresponds to the first appearance of the blue colour.

The Fe^{III} - 1,10-phenanthroline method should give a constant absorbance 20 min after mixing the reagents.[4] However, with the Superpeni mucolytic and Flubiotic formulations the absorbance increased steadily. This may also be due to the presence of amoxicillin, which probably reduces Fe^{III}, but at a slower rate. Measuring the absorbance immediately after 20 min gave good results.

The proposed method, based on the formation of a derivative of NAC after reaction with OPA and isoleucine, allows the sensitive, accurate, precise and reliable determination of NAC in various pharmaceutical formulations. The method is also simple and rapid, taking only a few minutes to perform, which permits it to be automated easily. The reagent solutions used are stable for at least 1 week and the derivative is formed almost instantaneously and remains stable for at least 2 h. The main interferences expected would be from other thiol compounds, but these are usually absent from commercial formulations of NAC; hence it is not necessary to employ a separation step. The method is suitable for the routine analysis of commonly used preparations of NAC in drug control analysis.

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SHORT PAPERS

2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium Tetrakis(1-imidazolyl)borate as a Reagent for the Determination of Nickel, Zinc, Cobalt and Iron by Flame Atomic Absorption Spectrometry

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The precipitated 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium tetrakis(1-imidazolyl)borate formed by the reaction between the respective chloride and sodium salts was found to be an excellent adsorber for heavy metal ions in solution. A procedure is described for the *in situ* solid - liquid chelate adsorptive separation and flame atomic absorption spectrometric determination of trace amounts of Ni, Zn, Co and Fe.

Keywords: *Flame atomic absorption spectrometry; nickel, zinc, cobalt and iron determination; reactive exchange reagent; 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium tetrakis(1-imidazolyl)borate*

Trace elements present in environmental, biological and biochemical samples are usually quantified by atomic absorption spectrometry (AAS) either with an air - acetylene flame or using a graphite furnace. This technique has become very popular because of the minimum sample preparation required and the relative freedom from chemical interferences and simple matrix effects.[1,2] In recent years competitive instrumentation costs have rendered this approach almost indispensable to an analytical laboratory engaged in trace inorganic analysis. However, pre-concentration of the analyte of interest must be performed when its concentration in a sample is so low that direct analytical signals become unreliable. In this context, ion exchange, liquid - liquid extraction, absorption separation, evaporation concentration and solid - liquid extraction steps are incorporated routinely in the experimental protocol.[3–10] This becomes imperative, particularly for flame AAS, where a 4–5-ml sample volume may be required to complete the analysis.

The pre-concentration steps generally require controlled experimental conditions and are invariably slow. However, separation and pre-concentration by solid - liquid extraction is relatively simple and rapid and has proved to be effective in many selective separations in different media.[11–15] This approach is based on the principle of selective metal chelate adsorption on a decomposable substrate form *in situ* in order to achieve homogeneous analyte retention. Unsaturated hydrocarbons, namely naphthalene and benzophenone, have been employed frequently as suitable substrates for metal ion retention. In recent studies carried out in this laboratory and elsewhere, reactive exchange through ion-associated metal co-ordination compounds has been used successfully for pre-concentration purposes.[16] This paper describes the use of a novel ion-associated reagent for multi-element analysis in aqueous samples that does not require the use of a substrate for pre-concentration of the analyte of interest. The insoluble reagent formed by the reaction between 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and sodium tetrakis(1-imidazolyl)borate acts as an efficient collector for a number of heavy metal ions present in aqueous solutions. The species of interest can be recovered from the bulky precipitated reagent by simply shaking for a few minutes with 1 M H_2SO_4 and quantified by flame AAS.

Experimental

Analytical-reagent grade chemicals (Fisher Scientific) or equivalent were used throughout. Stock solutions of metal ions (1000 p.p.m.) were prepared by dissolving accurately weighed amounts of the oven-dried (105 °C) chloride or nitrate salts in 1 l of doubly distilled water. Working standards at 100 p.p.m. were prepared by dilution with doubly distilled water when required. The reagents, sodium tetrakis(1-imidazolyl)borate (TIB) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INPT) were purchased from Aldrich. A 2% solution of the former and a 0.2% solution of the latter were mixed to generate the adsorptive exchange surface. Buffers composed of acetic acid, sodium acetate and sodium hydroxide were used for adjusting the pH. The analytical data were obtained on a Perkin-Elmer 5000 atomic absorption spectrometer fitted with a deuterium flame background corrector operated in the air - acetylene mode.

General Procedure

Amounts of metal ion ranging from 10 to 200 μg were transferred into 100-ml calibrated flasks by withdrawing a suitable aliquot of the diluted standards and the volumes made up to about 40 ml with doubly distilled water. Ten millilitres of acetate buffer (pH 4.5–6.5) followed by 20 ml of 0.2% INPT solution and 30 ml of 2% TIB reagent were added in sequence with thorough shaking after each addition and the volume was made up to the mark with doubly distilled water. The contents of the flask were mixed thoroughly for 2–3 min and allowed to settle for about 30 min. A system blank was prepared in the

Table 1. Recoveries of metal ions at different pH values: TIB, 0.6%; INPT, 0.04%; metal ion, 200 μg; volume, 100 ml; and contact period, 30 min

Metal ion	Recovery, %	
	pH 4.5	pH 6.5
Ni^{2+}	96.4	100.7
Zn^{2+}	98.4	99.7
Co^{2+}	93.8	95.0
Cu^{2+}	92.9	92.1
Fe^{3+}	96.5	92.1
Cd^{2+}	88.5	89.1
Mn^{2+}	89.2	88.3
Cr^{6+}	52.9	72.3
Pd^{2+}	60.0	65.4
Pb^{2+}	55.1	40.8
Au^{3+}	18.3	19.2
Tl^{+}	9.6	12.4

Table 2. Effect of dilution on the recovery of metal ions: pH, 4.5; metal ion, 200 µg. Other conditions as in Table 1

	Recovery, %										
Volume/ml	Ni^{2+}	Zn^{2+}	Co^{2+}	Cu^{2+}	Fe^{3+}	Cd^{2+}	Mn^{2+}	Cr^{6+}	Pd^{2+}	Pb^{2+}	Au^{3+}
100	96.4	98.4	93.8	92.9	96.5	88.5	89.2	52.9	60.0	55.1	18.3
200	95.3	98.9	94.9	89.7	100	86.0	89.3	13.6	50.0	55.9	16.7
250	95.1	98.0	93.4	88.2	97.6	81.9	87.0	7.3	51.9	43.6	16.8
500	71.7	76.4	65.2	65.4	68.4	62.9	62.2	7.4	49.0	32.1	17.6

Table 3. Effect of salinity on the recovery of metal ions: pH, 4.5; metal ion, 200 µg; volume, 100 ml; and contact period, 30 min

	Recovery,* %								
Salinity, %	Ni^{2+}	Zn^{2+}	Co^{2+}	Cu^{2+}	Fe^{3+}	Cd^{2+}	Mn^{2+}	Pd^{2+}	Pb^{2+}
1.0	97.9	99.1	94.9	90.0	97.7	89.0	86.6	65.7	55.0
5.0	97.9	97.3	89.4	87.5	99.7	83.4	87.6	53.1	21.4

* Average of five determinations.

same manner but without metal ions. The precipitate was recovered on a fine filter-paper (No. 542 or equivalent), placed in a glass funnel and was washed three times with doubly distilled water. The precipitate was then treated with four 10-ml portions of 1 M H_2SO_4 and the filtrate collected directly in a 50-ml calibrated flask and diluted to volume with doubly distilled water. The prepared solutions and blank were aspirated directly into the air - acetylene flame and the analytical signals recorded at the appropriate analytical wavelengths. The instrument was adjusted to read zero when a blank solution was aspirated with the deuterium background corrector on-line. The analytical signal in all other instances was therefore a direct measure of the net analyte response. The recovery in each instance was computed by comparison with the analytical signals of the standards at their specific wavelengths.

Results and Discussion

The retention behaviour of 12 metal ions, namely Au^{3+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Cr^{6+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Pd^{2+}, Pb^{2+}, Tl^{+} and Zn^{2+}, on the reagent was investigated. The exchange reagent was fairly soluble in strongly acidic solutions and this prevented its use under more acidic conditions (pH <2); hence only the pH range 4.5–6.5 was examined. In alkaline solutions, the precipitated hydroxides of the metal ions led to uneven retention and hence pH values higher than 7.0 were not considered. The data shown in Table 1 give the recoveries for 200 µg of each metal ion in a 100-ml sample volume. It can be seen that recoveries of over 88% were obtained for Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Cd^{2+} and Mn^{2+}, whereas much lower recoveries were observed for Cr^{6+}, Pd^{2+}, Pb^{2+}, Au^{3+} and Tl^{+}. At pH 6.5, the recoveries were only marginally better than at pH 4.5. Further studies showed that a 30 min contact period was adequate for adsorptive exchange and retention of metal ions on the precipitated reagent. The dilution effect at pH 4.5 demonstrated that metal ions (200 µg) were recovered quantitatively even at a 2.5-fold sample dilution but thereafter the retention decreased significantly (see Table 2). This implies that the reactive exchange surface formed under the experimental conditions described is effective up to a sample volume of about 250 ml. The bulk of the reagent may be increased if larger sample volumes are to be handled. This can be achieved by increasing the initial concentrations of the INPT and TIB reagents. The recoveries for five ions, *viz.*, Ni^{2+}, Zn^{2+}, Fe^{3+}, Co^{2+} and Cu^{2+} were evaluated by performing six replicate determinations at a 1 µg ml^{-1} concentration. The recoveries were within the range ±1.5% for all the ions except Cu^{2+} for which the variation was up to 2.5%. All data reported represent an average of at least three determinations. The data suggest that this approach may be useful for the rapid screening of the contamination from heavy metal ions in aqueous samples at about the 0.8 µg ml^{-1} level.

An interesting feature of this study is reflected in the quantitative recovery of metal ions from saline media. A salinity of up to 5% (composed of sodium chloride) could be tolerated as the recoveries for Zn^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+} and Cd^{2+} were found to deviate only marginally (Table 3). However, recoveries for Pd^{2+}, Pb^{2+}, Au^{3+}, Cr^{6+} and Tl^{+} remained partial to about the same extent as seen earlier for the aqueous system. The data in Table 3 also show incomplete recoveries for Pd^{2+} and Pb^{2+} under saline conditions.

The retention of the metal ions probably occurs through selective exchange on the surface

$$n[\mathrm{INPT:TIB}] + \mathrm{M}^{n+} \rightleftharpoons [\mathrm{M(TIB)}_n] + n[\mathrm{INPT}^+]$$

The degree of exchange and eventual retention by the adsorptive process depends on the solubility of the metal imidazolylborates in aqueous media. Specific values for their solubility products are not available but Chao and Moore[17] have reported that Zn^{2+}, Cd^{2+}, Tl^{+}, Pb^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Mn^{2+} form insoluble imidazolylborates. The data reported here reveal that at microgram levels Pd^{2+}, Tl^{+}, Pb^{2+}, Cr^{6+} and Au^{3+} are not retained as imidazolylborates and hence are sufficiently soluble even under very mild conditions. However, for Zn^{2+}, Fe^{3+}, Ni^{2+} and Co^{2+}, their entrapment through reactive surface exchange even at the sub-microgram level is feasible, which is indicative of the greater insolubility of their imidazolylborates. The proposed reagent offers the advantages of ion exchange and surface sorption, thereby eliminating the need to introduce an additional substrate (naphthalene or benzophenone) for metal ion recovery as was necessary in some of the earlier studies.[11–14]

The procedural simplicity of the method and the quantitative recoveries, even under saline conditions, of Zn^{2+}, Ni^{2+}, Fe^{3+} and Co^{2+} (≥1%) suggest that the method may be applicable to the monitoring of these ions in samples of diverse composition, particularly saline waters or waste effluent waters from industrial operations.

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Adsorption and Pre-concentration of Some Metal Ions From Ethanol on Silica Gel Modified With Pyridinium Ion

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Silica gel modified with pyridinium ions has been developed for the adsorption of metal ions from ethanol. This material was used to remove cobalt, copper, zinc, cadmium and mercury and can be employed for the pre-concentration of these metals by the column technique. These metals are eluted selectively from the column by a C_2H_5OH - H_2O binary mixture. For the elution mixture with a mole fraction of 0.5, the observed order is: Co ~ Cd (*ca.* 96%) > Cu ~ Zn (*ca.* 30%) > Hg (not eluted). All the metals, except Hg can be eluted quantitatively from the column with pure water. Mercury is recovered totally if an aqueous solution of 0.1 M HNO_3 is used as the eluent.

Keywords: *Ethanol; pyridinium ion; pre-concentration; adsorption; silica gel*

Silica gel modified with organofunctional groups has been the subject of considerable interest in recent years. Coating the hydrophilic surface with organic groups through several known coupling reactions,[1,2] has opened up the possibility of using modified silica gel to adsorb metal ions from non-aqueous solvents.[3,4]

The use of an anionic ion exchanger, consisting of a pyridinium-modified silica gel matrix, to adsorb anionic metal complexes from non-aqueous solvents was described recently.[5] This material was demonstrated to be highly selective in the ion-exchange process.[6]

The development of less time-consuming methods for the pre-concentration and separation of metal ions present in ethanol has been investigated as a result of the extensive use of the latter as a fuel.[7,8] It is important to note that the presence of metal ions in ethanol used as a fuel is undesirable as they can induce corrosion in the vehicle components in contact with the liquid.[9,10]

The use of imidazole-modified silica for the pre-concentration of metal ions from ethanol solution has been described.[8] As this organofunctional group can form strong surface complexes, the following conditions for the elution of the metal and regeneration of the adsorbent were necessary: (i) an aqueous acid solution, (ii) an ethanol solution of potassium hydroxide (0.5% *m/V*) and (iii) a large volume of pure ethanol to eliminate the excess of the alkali.

This paper describes the use of silica gel modified with a pyridinium ion to adsorb and separate metal ions from an ethanol solution. As the metal ions are adsorbed at the surface as anionic complexes, a facile method of elution using an ethanol - water mixture has been developed.

Experimental

Preparation and Characterisation

Silica gel with a specific surface area of 500 m^2 g^{-1} was de-gassed for 12 h at about 423 K under high vacuum. 3-Chloropropyltrimethoxysilane (12 g, *ca.* 80 mmol) dissolved in 100 ml of dry toluene was mixed with 50 g of the activated silica gel and the suspension was stirred for 24 h at the temperature of the refluxing solvent. The suspension was filtered off and the silica gel was washed with toluene, ethanol and finally with diethyl ether and then dried and de-gassed at 373 K under high vacuum. The silica gel functionalised with 3-chloropropyl groups was suspended in *ca.* 100 ml of dry pyridine and maintained at *ca.* 353 K for 120 h with constant agitation.

The resulting silica gel, functionalised with the pyridinium salt [$\equiv Si(CH_2)_3N^+C_5H_5Cl^-$], was filtered off and washed consecutively with ethanol, dilute HCl (0.05 M) and then distilled water until all the free chloride ions had been eliminated.

The degree of functionalisation was determined by shaking 0.1 g of the modified silica gel with 30 ml of 0.1 M HNO_3 for 30 min. The material was filtered and the amount of chloride, determined by potentiometric titration with $AgNO_3$, indicated an exchange capacity of 0.31 mmol g^{-1}.

Adsorption Isotherms

Anhydrous MCl_2 (M = Co^{II}, Cu^{II}, Zn^{II}, Cd^{II} or Hg^{II}) salts were used without further purification.

Adsorption isotherms for these metals on the modified silica gel were determined from C_2H_5OH - H_2O solutions of various composition, using the batch technique and a temperature of 298 K. About 0.2 g of the modified silica gel was used for each experimental point on the curve and equilibrated with 50 ml of a solution, whose initial concentration was maintained constant at 1.56×10^{-3} M. Adsorption isotherms from a pure anhydrous ethanol solution were also determined. Concentrations of the metal ion solutions in the range 0.1×10^{-3}–5×10^{-3} M were used.

Pre-concentration and Elution

In the pre-concentration and elution experiments, a small glass column (10 × 0.5 cm i.d.) packed with 1.5 g of the modified silica gel (particle size, 0.06–0.2 mm) was used. The column was loaded with 9–12 μmol of the metal ion and then eluted with 25 ml of C_2H_5OH - H_2O of variable composition. The flux of the solutions in the loading and elution processes was maintained constant in all experiments at 1.25 ml min^{-1}. In order to check the reproducibility of each point on the curve, the experiments were carried out in triplicate.

Chemical Stability

The chemical stability of the organofunctional groups was checked by determining the amount of chloride after various adsorption - elution cycles. The material was shown to be stable as no significant decrease in the amount of chloride was observed after 24 cycles. For Hg, the packing material was changed after about nine runs because the organofunctional groups were lixiviated by the continuous treatment with 0.1 M HNO_3, used to remove this metal from the column.

Chemical Analysis

The metals were determined by spectrophotometric titration using 0.01 M ethylenediaminetetraacetic acid.

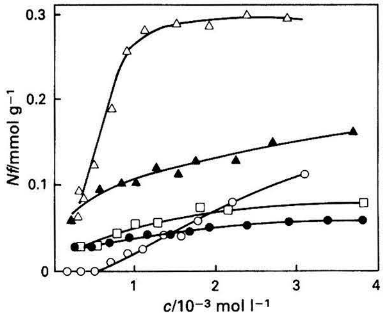

Fig. 1. Adsorption isotherms for MCl_2 from ethanol solution on SiL^+Cl^- at 298 K. M: ●, Co; ○, Cu; □, Zn; ▲, Cd; and △, Hg

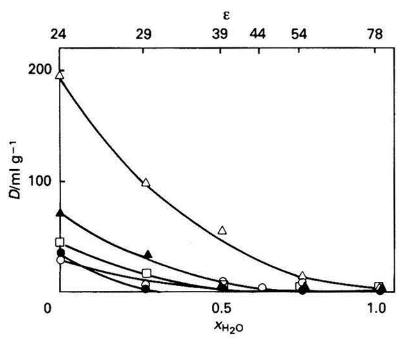

Fig. 2. Distribution coefficient of MCl_2 *versus* mole fraction, x_{H_2O}, of a binary mixture of C_2H_5OH - H_2O and respective dielectric constant, ε, at 298 K. M: ●, Co; ○, Cu; □, Zn; ▲, Cd; and △, Hg

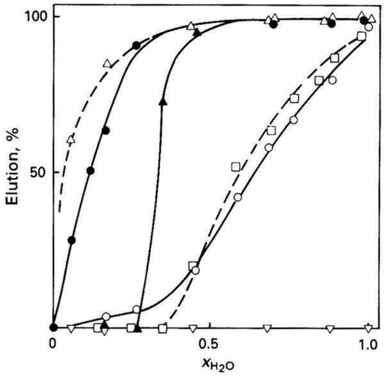

Fig. 3. Elution of $\overline{MCl_{z+n}^{n-}}$ with a C_2H_5OH - H_2O binary mixture. M: ●, Co; ○, Cu; □, Zn; ▲, Cd; and ▽, Hg. Elution of $\overline{HgCl_{z+n}^{n-}}$ with C_2H_5OH - H_2O + 0.1 M HNO_3 is represented by the curve passing through the points △

Results and Discussion

The adsorption of MCl_2 by $\equiv Si(CH_2)_3N^+C_5H_5Cl^-$, hereafter designated as SiL^+Cl^-, from ethanol solution can be expressed by the general equation:

$$n\overline{Cl^-} + M^{z+} + zCl^- \rightleftharpoons \overline{MCl_{z+n}^{n-}} \quad \ldots \quad \ldots \quad (1)$$

where the bars indicate the solid phase.

The amount of metal adsorbed in the anionic complex form, $\overline{MCl_{z+n}^{n-}}$ (in mmol), per mass W of SiL^+Cl^- (in g) is defined by

$$Nf = \overline{MCl_{z+n}^{n-}}/W \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

Table 1. Adsorption and recovery of metal ions adsorbed on SiL^+Cl^-

	Co	Cu	Zn	Cd	Hg*
Metal ion added/μmol ..	11.6	9.75	10.04	9.93	9.10
Metal ion recovered/μmol	11.6	9.70	9.70	9.80	9.00
Recovery, %	100	99.5	97.6	99.4	98.9

* Elution with 0.1 M HNO_3.

and the distribution coefficient by

$$D = Nf/c \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (3)$$

where c is the total concentration of the metal ion (in mmol ml^{-1}) in solution.

The transference of the metal ion from the solvent to the solid phase is strongly dependent on the dielectric constant of the solvent and on the stability constant of the complex species MCl_{z+n}^{n-}. In aqueous solution, anionic metal ion complexes occur with the addition of a large amount of the electrolyte (Cl^- in this instance).[11] In ethanol the metal ions are associated to a much greater extent than in aqueous solution[12] because of the lower dielectric constant and therefore these ions are adsorbed from the solvent without the addition of any electrolyte (see Fig. 1). The observed affinity order closely follows the formation constants of the anionic complexes MCl_{z+n}^{n-} ($z = 2$; $n = 1$ or 2), with Hg^{II} being the most stable followed by Cd^{II} and then the remaining metals with similar formation constants.[13–16]

The adsorption isotherms for the metal ions from a C_2H_5OH - H_2O binary mixture show that (see Fig. 2) the distribution coefficient decreases as the mole fraction, x_{H_2O}, increases (and consequently the dielectric constant, ε, of the mixture also increases). Mercury(II) was observed to be the most adsorbed at all compositions of C_2H_5OH - H_2O except at $x_{H_2O} = 1$, at which no metal is transferred from solution to the solid surface.

Adsorption experiments, carried out using a column packed with the adsorbent material, show that SiL^+Cl^- can be used in the pre-concentration process as shown by the results presented in Table 1. The recovery of the metal ion by elution from the column with pure water is higher than 97% in every instance, except for Hg^{II} where it was necessary to use 0.1 M HNO_3 in order to obtain a recovery of *ca.* 99%.

The results of the above experiments demonstrate clearly that the adsorption process is influenced greatly by the composition of the mixture and in the particular instance of Hg^{II}, the hydrolysis constant of the metal in aqueous solution is also important. The hydrolysis constant pK_1, where $K_1 = [M(H_2O)_{n-1}(OH)^+]/[H_3O^+][M(H_2O)_n^{2+}]$, for Hg^{II} is 2.5 while for all the remaining metals $pK_1 > 7.9$.[16]

Fig. 3 shows the effect of the C_2H_5OH - H_2O composition on the elution of each metal from the column. It is observed that Hg^{II} remains adsorbed on the surface of SiL^+Cl^- unless acid is introduced. Fixing the concentration of HNO_3 at 0.1 M in the C_2H_5OH - H_2O mixture, Hg^{II} is eluted smoothly as shown in Fig. 3. At $x_{H_2O} = 0.5$, the metal ions are eluted selectively from the column in the order: Co ~ Cd (*ca.* 96%) >Cu ~ Zn (*ca.* 30%) > Hg (not eluted).

Conclusion

Considering the relatively high chemical stability and only a small tendency to swell, which is mainly responsible for good selectivity,[6] the use of SiL^+Cl^- in pre-concentration and separation processes is feasible.

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Cerimetric Determination of Antimony in Alloys and Drosses After Hydrazine Reduction

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A simple method is described for the determination of antimony in metals, alloys and drosses, using hydrazine for the reduction of antimony(V) to antimony(III) followed by titration with cerium(IV) sulphate. The advantages of the proposed method over the standard procedures consisting of reduction by sulphur dioxide are discussed.

Keywords: *Cerimetry; antimony determination; alloys and drosses; hydrazine reduction*

Several titrimetric procedures are described in the literature for the determination of antimony.[1–7] Of these the most popular procedure involves suitable digestion of the sample, extraction of the digested mass with hydrochloric acid, the addition of sulphite or sulphur dioxide to convert all the antimony to the trivalent state, the expulsion of excess of sulphur dioxide by careful boiling and finally titration of the trivalent antimony with bromate or cerium(IV) sulphate.[2,3,6] In this procedure, the expulsion of the dissolved sulphur dioxide is critical. Vigorous boiling of the solution may lead to losses of antimony and insufficient boiling will yield higher values of antimony. Further, when metal ions such as copper, tin and iron are present, they are reduced to their lower valent states by sulphur dioxide; hence the solution has to be re-aerated for oxidation of these ions. However, this is at the risk of the possible re-oxidation of some antimony(III), which would result in low values.[2] Also, if arsenic is present, it must be eliminated either during the digestion stage or by careful boiling of the hydrochloric acid solution to low volume before titration. Arsenic interference can be overcome to a large extent by the use of cerium(IV) sulphate instead of potassium bromate.[8,9] For the reasons already mentioned, the use of sulphur dioxide requires that the procedure be followed carefully and in addition makes it prone to operative error. The use of hydrazine sulphate has also been recommended for the reduction of pentavalent antimony to its trivalent state[1,10]; however, no systematic procedure has been reported for the determination of antimony following its use. We have found that the proposed procedure gives more precise and reliable values, is less prone to operative errors and is applicable to the determination of antimony in drosses and alloys without any interference from associated metals.

Experimental

A Varian Model AA-475 atomic absorption spectrometer was used for all absorbance measurements.

Reagents

Cerium(IV) sulphate solution, 0.0895 M. Pure ammonium cerium(IV) sulphate (56.61 g) was dissolved in 10% sulphuric acid and made up to 1 l. The solution was standardised against antimony potassium tartrate (1 ml equivalent to 5.44 mg of Sb). A solution of this strength is used routinely in our laboratory for the determination of iron (1 ml equivalent to 5 mg of Fe).

Potassium bromate solution, 0.0166 M. Prepared from AnalaR grade potassium bromate directly by dissolving 2.78 g of the salt in distilled water and diluting to 1 l (1 ml equivalent to 6.08 mg of Sb).

Methyl Red solution, 0.1% *m/V*.

Procedure

Place a 1- or 2-g sample (containing 50–100 mg of antimony) in a beaker. Add 10–15 ml of sulphuric acid, 2 g of ammonium sulphate and 1.3 g of potassium sulphate, allow to fume strongly until dissociation is complete and cool. Rinse the glass cover with a small volume of distilled water. Add 0.5 g of hydrazine sulphate and again allow to fume until the condensing sulphuric acid rinses down the sides of the beaker and then cover. Allow to cool. About 200 ml of 1 + 1 hydrochloric acid are added slowly, with stirring, to dissolve the solids. The contents of the beaker are warmed if necessary to aid dissolution and again cooled to room temperature. This solution is titrated with cerium(IV) sulphate using Methyl Red indicator until a clear yellow end-point is attained. A blank titration is performed using the same volume of hydrochloric acid solution and indicator.

Results and Discussion

Table 1 gives the values of antimony obtained by the recommended procedure for drosses, antimony alloys and slags. The results are compared with those of the standard method described by Furman,[2] which consists of reduction with sulphur dioxide followed by titration with bromate. The results show that more precise values are obtained with the proposed method compared with the standard method. The relative standard deviations (RSDs) for dross-I and dross-II using the proposed method were 1.06 and 2.00% whereas RSDs of 2.87 and 6.50%, respectively, were obtained with the method described by Furman.[2] Potassium bromate could not be employed in the proposed procedure, because it was observed that small amounts of hydrazine sulphate always remained even after strong fuming. Potassium bromate readily oxidises hydrazine in cold acidic solutions, leading to higher values of antimony. In contrast, cerium(IV) sulphate does not oxidise hydrazine sulphate readily under these conditions.[11] This observation was also confirmed by performing blank titrations (Table 2). However, it should be noted that the final solution must be cooled to room temperature before titration as cerium(IV) sulphate tends to react with hydrazine at higher temperatures.

It is interesting to note that copper, iron and tin are not reduced by hydrazine in strong sulphuric acid solutions, thereby simplifying the determination. The non-interference of arsenic was also confirmed by determining antimony in dross samples to which arsenic had been added (Table 3). Certain methods for the determination of metals avoid the reduction step[7]; however, it is a well established fact that even sulphuric acid digestion of antimony metal itself will not yield entirely antimony(III) species.[12] Moreover, for samples such as drosses, antimony is present both as Sb_2O_5 and Sb_2O_3. Experiments conducted without the addition of hydrazine always yielded low values.

The samples were also analysed by atomic absorption

Table 1. Determination of antimony in drosses and alloys

		Proposed method			Reference method[2]			AAS
Sample	Approximate composition, %	Antimony, %	SD	RSD, %	Antimony, %	SD	RSD, %	Antimony, %
Antimony dross-I . .	Pb: 70–80; Cu: 0.01–0.2; Ag: 0.1	11.09, 10.99, 10.88, 10.77, 10.77, 10.88, 10.88			11.20, 10.84, 10.96, 10.23, 11.08, 10.47, 10.96, 10.59, 10.84			10.46, 11.11, 10.83
		Mean: 10.89	0.12	1.06	Mean: 10.80	0.31	2.87	
Antimony dross-II . .	As for sample 1	5.87, 6.09, 5.77, 5.98, 5.87, 5.98, 5.66			6.51, 5.78, 6.27, 5.60, 5.85, 6.33, 5.66, 6.45, 5.54,			6.12
		Mean: 5.89	0.12	2.00	Mean: 6.00	0.39	6.50	
Antimony dross-III	As for sample 1	1.30, 1.41, 1.50, 1.41, 1.20			—	—	—	1.30
		Mean: 1.36	0.12	8.53				
White metal	Cu: 3.5; Sn: 79.5	9.86, 9.87, 9.50			9.06, 9.24, 9.70, 10.00			9.88
		Mean: 9.74	0.21	2.16	Mean: 9.50	0.43	4.51	
Antimony lead . .	Pb: 80–90; Ag: 0.5	6.77, 6.70, 6.65			6.94, 6.60, 6.92,			6.88, 7.20
		Mean: 6.70	0.06	0.91	Mean: 6.82	0.19	2.79	
Rotary lead	Pb: 80–90; Ag: 0.06; Cu: 0.87	3.89, 3.84, 3.90			4.08, 3.78, 3.90,			3.64
		Mean: 3.87	0.03	0.86	Mean: 3.92	0.15	3.85	
Hard lead	Pb: 95–96; Cu: 0.2; Ag: 0.2	1.12, 1.23			—	—	—	1.13
		Mean: 1.17	0.08	6.67				
Rotary slag	Pb: 5–9; FeO: 26.2; Cu: 4.2	3.10, 3.21, 3.05			—	—	—	3.00
		Mean: 3.12	0.08	2.62				

Table 2. Results of experiments investigating the presence of undecomposed hydrazine after digestion

	Titre value obtained/ ml	
Sample	Cerium(IV) sulphate	Bromate
Hydrazine, 500 mg	0.05	1.3
Hydrazine, 500 mg	0.05	2.0
Hydrazine,* 500 mg	0.1	16.4
	Antimony, %	
Dross-I	10.9	13.9
Dross-II	5.9	7.7

* Solution directly titrated without digestion.

Table 3. Effect of added arsenic and copper on the determination of antimony using the recommended procedure

Sample	Element added	Antimony value obtained, %
Dross-I	None	10.89
Dross-I	Arsenic,* 25 mg	10.77
Dross-I	Arsenic, 50 mg	10.88
Hard lead	None	1.17
Hard lead	Copper,† 40 mg	1.23
Hard lead	Copper, 80 mg	1.12

* Added as sodium arsenite.
† Added as copper(II) sulphate solution.

spectrometry (AAS) after suitable digestion. The drosses and rotary slag were digested with nitric acid followed by fuming with sulphuric acid. The digested mass was extracted with a mixture of 10% hydrochloric acid and 10% tartaric acid and filtered. The lead metal sample and alloys were dissolved in 50 ml of dilute nitric acid - 5 ml of 40% hydrofluoroboric acid. The white metal sample was attacked by concentrated sulphuric acid and taken up in 10% hydrochloric acid - 10% tartaric acid. The solutions were diluted as appropriate and the absorbances measured. These results are also presented in Table 1 for comparison.

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Spectrophotometric Determination of Periodate With Salicylaldehyde Guanylhydrazone Using Flow Injection. Determination of Glycerol in Vegetable Oils

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Periodate has been determined using flow injection with spectrophotometric detection on the basis of the red colour obtained when salicylaldehyde guanylhydrazone (SAG) and periodate were mixed in a basic medium. The carrier stream was de-mineralised water. The reagent stream was an aqueous SAG solution and another stream contained an ammonia buffer (pH 9.2) solution. The calibration graph was linear between 5 and 50 p.p.m. of periodate when the injection volume was 200 µl. The relative standard deviation ($n = 10$) was 1.0% and the detection limit, corresponding to a signal to noise ratio of 3, was 0.35 p.p.m. The proposed method was applied to the determination of glycerol in vegetable oils using the Malaprade reaction.

Keywords: *Flow injection; periodate determination; spectrophotometry; salicylaldehyde guanylhydrazone; glycerol*

Flow injection techniques have been used widely in such fields as environmental, clinical, agricultural and food analysis because they permit the automatic determination of some analytes and the determination is often more rapid, sensitive, precise and simple than the batchwise method. Glycerol has been determined in several samples, for example, honey[1] and fats and fatty oils.[2] Periodate reacts with 1,2-diols through the Malaprade reaction and this reaction can be used for the determination of glycerol.

In a previous paper[3] we established an indirect spectrophotometric method for determining glycerol and other 1,2-diols based on the oxidation of salicylaldehyde guanylhydrazone (SAG) by an excess of periodate added to the diol solution. In this paper, the spectrophotometric method is adapted to a flow injection (FI) system and applied to the determination of glycerol in vegetable oils. The proposed method is more rapid, sensitive, simple and precise than the thermodynamic method.

Experimental

Reagents

All chemicals and solvents used were of analytical-reagent grade.

Salicylaldehyde guanylhydrazone (SAG) solution. Transfer 0.06 g of SAG (synthesised according to the method of Thiele and Bihan[4]) into a 100-ml calibrated flask, add 2 ml of dimethylformamide and shake until the SAG is dissolved, then dilute to volume with water. This solution is stable for at least 24 h.

Buffer solution, pH 9.2. Dissolve 14 g of ammonium chloride and 19 ml of concentrated ammonia solution in water and dilute to 1 l.

Potassium periodate solution, 0.01 M. Dissolve an accurately weighed amount of the salt in water and standardise the solution by iodimetry; dilute as required, just before use.

Stock solutions of glycerol were prepared fresh as required.

Apparatus

A Pye Unicam SP-800 UV - visible spectrophotometer with a 10-mm microflow cell (18 µl) was used to measure absorbance.

A Gilson precision peristaltic pump was used to propel the carrier solution (water), reagent solution (SAG) and buffer solution.

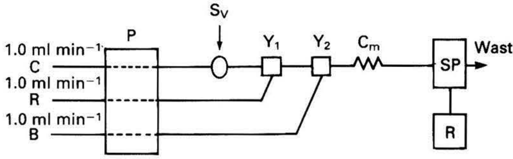

Fig. 1. Schematic diagram of the FI system. C, Carrier solution; R, reagent solution; B, buffer solution; P, peristaltic pump; S_V, sample volume (200 µl); Y_1 and Y_2, Y-connectors (0.5 mm i.d.); C_m, mixing coil (length, 3 m); SP, spectrophotometer; and R, recorder

A six-way injection valve with a variable loop (Omnifit) was used to inject the samples into the carrier stream. Connections were made of PTFE tubing (0.5 mm i.d.). A diagram of the flow system used is shown in Fig. 1.

Procedures

Determination of periodate

By using the flow system shown in Fig. 1, a 200-µl sample containing up to 50 p.p.m. of periodate was injected into the carrier stream through the sample valve and was mixed first with a 0.06% SAG solution and then with a 0.25 M buffer solution through two Y-connectors. The chart signal was obtained by measuring the absorbance at 490 nm.

Determination of glycerol

To a sample aliquot containing between 0.4 and 9.5 p.p.m. of glycerol were added 50 p.p.m. of periodate and water in a 25-ml calibrated flask. After reaction for *ca.* 30 min at room temperature in the dark, the excess of periodate was determined as described under Determination of periodate.

Determination of glycerol in vegetable oil

A 5–8-g amount of the oil was dissolved in 15–20 ml of chloroform and spiked with known amounts of glycerol. Thereafter, the sample was shaken with two 6-ml portions of water in a separating funnel. The aqueous phase was diluted with water in a 25-ml calibrated flask and analysed as described under Determination of glycerol.

Results and Discussion

Optimisation of Parameters

A series of experiments were performed to establish the

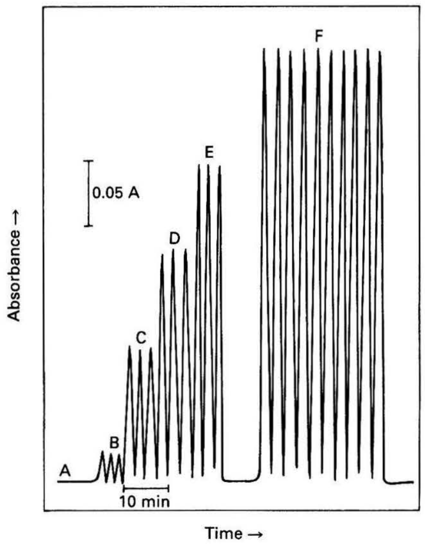

Fig. 2. Flow signals for the SAG - periodate system. Periodate concentration (p.p.m.): A, 0; B, 4.5; C, 16.5; D, 28.0; E, 37.6; and F, 50.1

Table 1. Determination of glycerol in vegetable oils

Sample	Glycerol added, p.p.m.	Glycerol found, p.p.m.	Recovery,* %	RSD,% ($n = 4$)
Olive oil (1.0°) ..	—	24.0	—	3.0
	29.8	55.4	105	1.0
	60.1	83.3	99	1.1
	87.6	113.1	102	1.7
Sunflower oil (0.4°) ..	—	7.4	—	5.4
	27.3	35.9	104	1.9
	54.9	63.6	102	1.7
	109.1	114.7	98	2.0

* Values obtained from glycerol added to the oil before any extraction.

conditions for optimum peak shape. The reagent concentration, the buffer concentration, the sample injection volume, the flow-rate and the mixing coil parameters were investigated.

The buffer concentration was tested by using a pH 9.2 NH_3 - NH_4Cl solution (optimum pH of reaction). The maximum peak height was obtained over the concentration range 0.20–0.40 M. A buffer concentration of 0.25 M was selected.

The maximum peak height was obtained for a 2.5×10^{-3} M SAG solution (0.06% *m/V*).

The volume of the sample injected was varied by changing the length of the sample loop in the injection valve. The peak height increased with increasing sample size. A sample volume of 200 µl was selected as a suitable volume.

The flow-rate of the three streams was varied from 0.6 to 1.3 ml min^{-1}. The maximum signal was obtained between 0.8 and 1.1 ml min^{-1}. A flow-rate of 1.0 ml min^{-1} was selected for the three streams because a higher sampling rate was obtained.

The mixing coil was investigated by changing the length of the tubing (0.5 mm i.d.). The optimum signal was obtained with a length of 3 m.

Periodate Calibration Graph

Under the optimum conditions established, the periodate calibration graph was obtained. It was linear up to a periodate concentration of 50 p.p.m.

Flow signals for the SAG - periodate system at different periodate concentrations are shown in Fig. 2.

The calculated correlation coefficient was 0.9990 and the relative error (95% confidence level) of the method was 1.0%. The detection limit, corresponding to a signal to noise ratio of 3, was 0.35 p.p.m. The sampling rate was 20 samples h^{-1}.

Determination of Glycerol

The most widely used methods for the determination of 1,2-diols and related compounds are based on the Malaprade reaction:

$$-CH(OH)CH(OH)- + IO_4^- \rightarrow 2 > C{=}O + IO_3^- + H_2O$$

followed by the determination of the excess of periodate.

The determination of periodate with SAG can be performed in the presence of a 50-fold (*m/m*) excess of iodate. This allows the indirect determination of glycerol via the Malaprade reaction.

In this work, we determined that the reaction between periodate and glycerol was complete after 20 min (efficiency $\geqslant$95% when $[IO_4^-] \geqslant 3$[glycerol]). A time of 30 min was selected. This time is also recommended by the British Standards Institute.[2]

The efficiency of the oxidation of glycerol with periodate was $\geqslant$95% when $[IO_4^-] \geqslant 3$ [glycerol]. In consequence, the concentration of glycerol could be determined by using the periodate calibration graph, taking into account the stoicheiometry of the oxidation reaction. Also, a calibration graph for 0.4–9.5 p.p.m. of glycerol was established when the samples were oxidised with 50 p.p.m. of periodate. Coincident results were obtained by using both calibration graphs.

Applications

Glycerol was determined in samples of vegetable oils by using the proposed procedure. The results obtained are given in Table 1.

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BOOK REVIEWS

Handbook of Inductively Coupled Plasma Spectrometry. Second Edition

M. Thompson and J. N. Walsh. Pp. xi + 316. Blackie. 1988. Price £50. ISBN 0 216 92264 X.

The second edition of this useful handbook claims to be an extended and revised version. The book has retained the excellent qualities of being easily readable and yet comprehensive in its contents. Of particular merit are Chapters 4 and 5, which explain in detail procedures for the analysis of geological materials. Perhaps this is to be expected as geochemistry is the mainstream interest of the authors. Chapter 4 is very useful in that it describes blow by blow and element by element how to carry out silicate rock analysis and the pitfalls associated with the analysis of such complex materials.

The additional Chapters 11 and 12 really are warranted for the sake of completeness of a book of this kind. Chapter 11, on inductively coupled plasma mass spectrometry, is a fairly standard description of instrumentation, interferences and applications with useful tables of geological applications with appropriate decomposition procedures, calibration strategies and isobaric corrections where required. Chapter 12 presents comprehensive data concerning calibration standards for a variety of metals and alloys together with detection limits for different elements in these materials. Again the tabular form of these data is useful and amenable to quick reference.

The first part of the book (Chapters 1–3) I am less happy about. These chapters are a workmanlike ramble through the history of inductively coupled plasma atomic emission spectrometry (ICP-AES) analytical characteristics and instrumentation for ICP-AES. It is the aim of the authors to keep things simple, as required for an essentially applications text. In this they are successful but only up until 1986. There are few references to work published after this date. For example, in the field that I am familiar with, *i.e.*, the development of torches and Fourier transform spectroscopy, there are no references after 1986 and yet there have been several important papers in these fields published in 1986, 1987 and 1988. The second edition purports to be a 1989 edition and while I accept that it may take a year to publish I do not accept that it takes two years!

There are also some niggling inaccuracies in this section of the book, for example, Figure 3.4 of a typical side window photomultiplier is grossly misleading. I cannot conceive of this device ever working in practice.

Notwithstanding these minor criticisms, the book is well worth having as a primer and quick reference book on the subject, especially if you are a geochemist.

R. D. Snook

Soils Contaminated by Petroleum: Environmental & Public Health Effects

Edited by Edward J. Calabrese and Paul T. Kostecki. Pp. xiii + 458. Wiley-Interscience. 1988. Price £65. ISBN 0 471 85106 X.

This book is based on the proceedings of a multi-disciplinary conference on "Environmental and Public Health Effects of Petroleum-contaminated Soils," held at the University of Massachusetts in October 1985. The conference took place not long after the publication of the preliminary findings of the US Environmental Protection Agency programme on leaking underground storage tanks (LUST). Consequently, soil contamination from the estimated 2 million such tanks containing petroleum products in the USA is a major theme in the book. It is likely that the US experience in this area will have longer-term implications for other countries where environmental protection has not reached the level of regulatory control present in the USA.

The papers in the book are grouped into four chapters, corresponding to the working sessions of the original conference.

Chapter 1, Chemistry and Modelling, in fact gives scant consideration to the influence of the chemistry of petroleum components (as distinct from their physical properties) and the balance of the chapter is disrupted further by the inclusion of an 80-page contribution on mathematical modelling, some 4–5 times longer than other papers in the book. Certain authors have tended to stray from the specific topic of petroleum contamination into more general and, at times, esoteric discussion of techniques for modelling the behaviour of chemicals in soil. The over-all message would appear to be that there is a lack of field data to validate the current models and that there is considerable difficulty in applying these to complex mixtures of petroleum hydrocarbons.

Chapter 2, Public and Environmental Health and Safety, provides a concise introduction to risk assessment and the public health implications of petroleum-contaminated soils, including the thorny question of how clean is clean? This is well balanced by an interesting case study concerning contamination of soil and groundwater in a residential area of Long Island.

Chapter 3, Engineering Remedial Action, continues on the essentially practical theme, with emphasis on case studies illustrating possible remedial solutions, including microbial degradation, soil venting of volatile hydrocarbons and direct vacuum recovery of liquids, together with methods for the clean-up of contaminated groundwater and for the reclamation of contaminated sites for redevelopment. The importance of finding both appropriate and cost-effective solutions is stressed repeatedly.

Chapter 4, Regulatory and Legal Aspects, summarises the US situation at the state and federal levels towards the end of 1985, with pointers to possible future developments. This is material which rapidly becomes outdated and is even now likely to be of limited use to the majority of readers.

A feature of the book is the inclusion (verbatim) of discussions from the original meeting. This unscripted dialogue adds relatively little to the useful content of the book and often does not read well.

An important consideration in assessing the value of such a book is whether the material is presented in a form that will facilitate the interchange of information and concepts between workers in the various disciplines involved. In this respect, Chapters 2–4 should represent fairly straightforward reading. Chapter 1, however, will probably be of more restricted value, except to those with a firm grounding in mathematics.

D. I. Gasking

Modern Methods of Plant Analysis. New Series Volume 8. Analysis of Nonalcoholic Beverages

Edited by H. F. Linskens and J. F. Jackson. Pp. viii + 412. Springer-Verlag. 1988. Price DM320; £105 (hardback). ISBN 3 540 18820 7.

This book contains 15 chapters written by 20 workers from nine countries under the guidance of two editors within a series on different analytical methods (Volumes 1–5), different classes of analyte (*e.g.*, alkaloids, drugs or hormones) and different plant materials, including Wine, Beer and this volume on Nonalcoholic Beverages. Here the editors do not appear to have exercised sufficient discipline, as the authors have evidently been allowed freedom not only of style and spelling, but also of emphasis and content. Each chapter meets a satisfactory standard in its own way, making the book a collection of unnumbered isolated papers, which are considered in order.

The general description of "Enzyme Linked Immunosorbent Assay (ELISA)" was obviously intended to appear in the volume on Beer rather than Nonalcoholic Beverages, but nevertheless introduces an exciting technique slowly being recognised by food analysts. "Carboxylic Acids in Soft Drinks" contains protocols in strained English selected without critical appraisal and includes inappropriate details and applications. "Analysis of Fruit Juice Authenticity," which describes patterns of free amino acids in different juices, demonstrates relevance but falls short of specific conclusions. "The Spectral Characteristics of Citrus Products" also suffers from natural variation, the chapter concluding negatively by recommending against direct application of this approach. "Bitterness in Fruit Juices" details, uncritically, chromatographic methods for the citrus analytes responsible. "Analytical Methods for Orange Juice Volatiles" involves isolation and gas - liquid chromatography. "Stable Carbon Isotope Ratios" covers the principle of the method and its application to the detection of sugars and citric acid added to fruit juices. A general overview of "Analytical Methods for Tomato Products" claims that they are too innumerable to elaborate, and selects parameters commonly measured (*e.g.*, total solids, sugars or polysaccharides) in unripe and ripe fresh tomatoes, concentrates, juices and soups. One method based on reversed-phase high-performance liquid chromatography is detailed in "Quinine Beverages" and, like the following chapter on "Formaldehyde in Coffee," it is brief and limited in scope, suggesting suitability for a journal rather than a book. On the other hand, the next two chapters are overlong: "Metabolism and Analysis of Caffeine" and "Colour and Flavour Characteristics of Made Tea" contain material of marginal relevance to the over-all title or that overlap with following chapters, in particular with "Analysis of Tea Volatiles," which emphasises detailed data rather than methodology. "Cocoa Flavour Components and Precursors" is a comprehensive, if lengthy, account of the current position in this field. The final chapter considers briefly "The Importance of Plant Analysis to Food Regulations," and concludes that it will continue to provide the primary protection of public health.

A few misprints were noted, but more serious is an illogical policy for the index that will not help the casual reader: for instance, entries occur under Amino acids in cocoa but Tomato amino acids; under Cocoa, steam distillates (p. 376) but Steam distillates of cocoa (p. 381). Water, milk, yogurts, nonalcoholic beers, herbal teas, pear juice, blackcurrant juice and cola drinks are not included. Overall, this volume contains much of interest to the plant analyst, unspoilt by the loose editorial control that one has come to expect in such multi-authored books.

C. H. S. Hitchcock

Compositional Analysis by Thermogravimetry

Edited by Charles M. Earnest. Proceedings of the Symposium on Compositional Analysis by Thermogravimetry, held March 16–17, 1987. STP 997. Pp. x + 293. American Society for Testing and Materials (ASTM). 1988. Price $59. ISBN 0 8031 1177 0.

The symposium on Compositional Analysis by Thermogravimetry was sponsored by ASTM Committee E-374 (Thermal Measurement). Dr. Earnest served as Chairman of the symposium and subsequently edited the publication under review, which includes all the papers presented.

As would be expected of a symposium of this type and with ASTM sponsorship, the papers are predominantly from workers in the United States, although Canada, West Germany, India, The Netherlands, Switzerland and the UK are also represented. Each paper was evaluated by three referees and the quality of all the papers reflects the efforts of both the authors and referees.

The text is divided into four sections: general papers (five), polymeric materials (five), thermogravimetric techniques and new instrumental concepts (four) and inorganics, fuels, minerals and raw materials (eight). This was truly a landmark symposium and the text must be essential reading for all those either involved in or contemplating the use of thermogravimetry for compositional analysis. In his opening remarks, the Chairman commented that the symposium was "an historic occasion, as at no time in history has an international group of scientists gathered together for two entire days to discuss one application area (that is, compositional analysis) of thermogravimetry." I would like to hope that history will repeat itself and that such symposia will, in future, be held on a regular basis.

As is to be expected of an ASTM publication, printing errors are extremely rare, the quality of the publication is high and the price is reasonable.

Cyril J. Keattch

Analytical Techniques for Material Characterisation. Proceedings of the International Workshop, Baton Rouge, USA, 11–16 May, 1987

Edited by W. E. Collins, B. V. R. Chowdari and S. Radhakrishna. Pp. xiv + 407. World Scientific. 1987. Price £55.30. ISBN 9971 50 511 8.

The book presents state of the art reviews on different techniques used in characterising important materials for a wide range of applications. Several techniques are described for semiconductive materials, fast-ion conducting glasses, optical fibres used in communication and nuclear materials, etc. A theoretical treatment of charge density is discussed and compared with experimental results for Be, V, Cr, Fe, Cu, Si, Ge and GaAs. Pitfalls in the numerical integration of differential equations are included.

Shorter contributions include investigations of resistivity variations in metal films deposited on substrates with temperature and different excitations such as electron, X-ray and laser beam, *e.g.*, Au films on Si or SrF_2 crystals. Application of the Rutherford back-scattering technique to depth profile studies is described. Creation and elimination of In islands on clean InP surfaces has been studied by electron energy loss spectroscopy. Additional electron beam induced voltage contrast for mercury cadmium telluride has been ascribed to skin resistance. This additional contrast mechanism has proved helpful for judging the surface electrical property of the sample.

Earlier X-ray photoelectron spectroscopy (XPS) researches on silicate, borosilicate, germanosilicate, phosphate and fluoride glasses are extended to fluorophosphate glasses. Studies on the structure of the $Mg(PO_3)_2$ - BaF_2 - AlF_3 glass system are supplemented by infrared (IR) and Raman spectroscopy. Metallic glasses have become important in recent years because of their electrical properties, high tensile strength and excellent corrosion behaviour. X-ray studies of the crystallisation behaviour and thermal stability are described for glasses containing Fe, Co, Ni with B, Si and C.

In the search for superionic conducting materials to be used as electrolytes in solid-state batteries and analogue memory devices, efforts are being made to develop vitreous electrolytes with an ionic conductivity of 0.1 ohm^{-1} cm^{-1} at room temperature. For silver-based ternary systems with Ag_2O as a glass modifier and V_2O_5 and P_2O_5 as glass formers, ionic conduction was enhanced when much of the Ag_2O was replaced by AgI. Another ionic conductor based on silver iodide in a solid solution with lead iodide is described. The silver-ion conducting solid electrolyte $Ag_4I_2WO_4$ in the mixed system AgI - Ag_2WO_4 is also characterised. Solid-state electrolytes and intercalation compounds of high-valence ions are reviewed briefly, *e.g.*, exchange of higher valence cations into some minerals such as montmorillonite and mordenite. Also, sodium yttrium fluoride, $NaYF_4$, exhibits somewhat unusual electrical and dielectric properties in both crystalline and thin film forms. These properties have been investigated for application to solid-state lasers and batteries.

The hardening of austenitic stainless steels by gas nitriding is examined by scanning electron microscopy, WDX, scanning Auger spectroscopy, X-ray diffraction and XPS. In the analysis of austenitic stainless steels, the performance of capacitance discharge plasma (spark), inductively coupled plasma and X-ray fluorescence is compared. The brittleness of Fe - Mn - Al - Cr alloys is elucidated by X-ray diffraction.

Advances in Fourier transform IR spectroscopy are reviewed, with their application to polymeric systems using pyrolysis. The application of NMR spectroscopy for probing the characterisation of polymeric materials and blends is developed by a composite pulse magic echo sequence for the excitation of three level systems. A new analytical tool is thermoluminescence induced by thermally stimulated field emission (TSFE). This gives glow curves for CaF_2 similar to those from γ-radiation. A sensitive method has been developed for the determination of sulphate at p.p.m. levels using atomic absorption spectrometry.

The book is therefore very useful, both in illustrating the scope of many analytical techniques and their co-ordination for the characterisation of materials.

D. R. Glasson

CUMULATIVE AUTHOR INDEX

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