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LINEARITY OF PLOTS OF GAS CHROMATOGRAPHIC RETENTION DATA FOR OXYGEN-CONTAINING ORGANIC COMPOUNDS ON POROUS POLYMERS

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SUMMARY

The linearity of the relationship between retention data and carbon number for homologous series of oxygen-containing organic compounds has been investigated for the series of Porapak porous polymers in gas chromatography. A marked deviation from linearity occurs with the lower members of normal alcohols, aldehydes, acetates and methyl ketones.

INTRODUCTION

Since the mid-1960's and the pioneering work of Hollis[1,2] the use of porous polymers for gas chromatographic (GC) separations has become widespread. These polymer beads are considered to have separating powers normally associated with both gas–solid and gas–liquid chromatographies[3]. Hollis[1] considered the solubility of the compound in the polymer to be the most important factor determining retention behaviour, whereas Smith and Waddington[4] reported that a linear relationship exists between the logarithm of the retention times and the boiling points of compounds in a homologous series. Johnson and Barrall[5] showed that surface characteristics such as pore size distribution, pore volume and surface area are important in determining the separating power of the polymer.

Of major concern when using porous polymer packings in gas chromatography is the irreproducibility of retention behaviour. This may result from various factors, the most important being the methods used to condition the column[4] and variations between batches of polymer[6,7]. The extent of the anomalous behaviour of these packings can be large. For example, Hollis[8] found that the elution order of C_2 hydrocarbons was dependent on the type of Porapak and temperatures employed. Recently, Castello and D'Amato[9] showed that the adjusted retention times of compounds vary with column temperature, in different ways, depending on the type of Porapak.

Porous polymers are often used for the separation of highly polar compounds such as alcohols[10] and for the determination of water in organic systems[2]. Therefore

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it is necessary to characterize the retention behaviour of oxygen-containing organics as well as hydrocarbons.

Linearity of the plot of corrected retention time *versus* carbon number is a basic assumption in the methods of reporting retention behaviour. In a recent publication[11] we have shown that the method of Ševčík[12,13] provides a sensitive test of the linearity of such plots for the *n*-alkanes. This method employs the ratio, A, of the differences in uncorrected retention times for neighbouring *n*-alkanes in a homologous series, *i.e.*, the ratio of the antilogarithms of the slopes of the lines between successive pairs of *n*-alkanes; hence it gives a measure of the constancy of the slope of the *n*-alkane line.

Until recently this test of linearity had only been performed on the retention time data obtained for *n*-alkanes on stationary phases of different polarities. In a previous publication[14] we determined the linearity of plots of retention data for *n*-alkanes on porous polymers and found non-linearity for methane and ethane. In this paper we have investigated the linearity of retention time *versus* carbon number for series of normal alcohols, aldehydes, acetates and methyl ketones using the same range of porous polymer packings.

EXPERIMENTAL

The equipment used consisted of a Hewlett-Packard 5750 research chromatograph interfaced to a 16K PDP 11/40 digital computer. Interfacing was achieved by the use of an LPS 11 Laboratory Peripheral System comprising a 12-bit analog-to-digital converter, a programmable real-time clock with two Schmitt triggers and a display controller with two 12-bit digital-to-analog converters. All on-line programming was written in CAPS II Basic with LPS options. The sampling rate was 0.5 sec.

The retention times measured for the alkanes were used to calculate the dead times by the method of Grobler and Balizs[15]. This method has been shown to be simple and accurate[16]. The dead time, t_m, the slope, b, and intercept, C, were then used in eqn. 1 to estimate the uncorrected retention times, t_r, of the various compounds of carbon number Z:

$$\log (t_m - t_m) = bZ + C \tag{1}$$

The ratio, A, of the differences in retention times was calculated as an alternative method of assessing the linearity for the alkanes.

Samples of Porapak (N, P, PS, Q, QS, R, S and T) polymer beads (80–100 mesh) were obtained from Waters Assoc. They were packed in PTFE columns (6 ft. × 1/8 in. O.D.). A mixture of *n*-alkanes from methane to heptane in nitrogen was obtained from Commonwealth Industrial Gases Ltd. It was introduced to the column by a gas sampling valve. The oxygen-containing compounds were injected as liquids. The column oven was operated isothermally at temperatures below the limits recommended by the manufacturer.

RESULTS AND DISCUSSION

The results for *n*-alkanes are presented in Table I to aid discussion of the results

for normal alcohols, aldehydes, acetates and methyl ketones which are given in Table II–V respectively.

It is apparent from the data in Table I that the plot of retention time *versus* carbon number for the lower alkanes is non-linear. This is evident from the values of A calculated for the C_1–C_3 and C_2–C_4 *n*-alkanes. The values of A calculated for higher alkanes are essentially constant. On all porous polymers the A value is lower for the C_1–C_3 *n*-alkanes and higher for the C_2–C_4 *n*-alkanes than the constant value observed for the series of *n*-alkanes with carbon number $\geqslant 3$. These results indicate that the retention times for methane and ethane are different from those expected for *n*-alkanes having carbon numbers of one and two, respectively. This suggests that there is curvature in the plot of corrected retention time *versus* carbon number for *n*-alkanes.

The values of A in Table II suggest that the non-linearity in the plot of retention time *versus* carbon number for the lower *n*-alcohols is not the same as for the corresponding *n*-alkanes. This is evident from the A values calculated for the C_1–C_3 and C_2–C_4 compounds in both series. In the case of the *n*-alcohols the A value for the C_1–C_3 compounds is higher than the constant value observed for higher alcohols. In addition, the value of A becomes essentially constant for the series of *n*-alcohols of carbon number $\geqslant 2$.

The results for *n*-aldehydes (Table III) and acetates (Table IV) do not show a particular trend in A values with carbon number. The values of A calculated for C_2–C_4 methyl ketones are higher than the constant values observed for compounds of greater carbon number in this series.

Two major effects can be noted from the results presented in Tables I–V. First, the plots of retention time *versus* carbon number show non-linearity for *n*-alkanes, *n*-alcohols, *n*-aldehydes, acetates and methyl ketones of low carbon numbers. The origin of the non-linearity varies from one group of compounds to another. Since the boiling points of the oxygen-containing compounds are significantly greater than for the *n*-alkanes of the same carbon number, it is suggested that the non-linearity of the plots for lower members of these homologous series may result from the time of elution. That is, peaks eluated early will not conform to a linear relationship between corrected retention time and carbon number. This suggests that physical rather than chemical effects make a large contribution to the non-linearity.

Secondly, constant values of A are obtained from the retention times of ethanol and higher alcohols (Table II). Therefore, the injection of a mixture of C_2–C_5 *n*-alcohols should provide a good estimate of the column dead time for porous polymers. Estimation of dead time by this method may be preferable to the use of four *n*-alkanes of carbon number $\geqslant 4$. The main advantages of the use of *n*-alcohols is that they can be injected as a liquid (whereas the vapours must be used if *n*-butane is to be included in the *n*-alkane mixture), and the retention times of the C_2–C_5 *n*-alcohols are shorter than those of C_5–C_8 *n*-alkanes if liquid mixtures are injected.

Since one of the main applications for porous polymer packings is to the separation of polar compounds, it is suggested that mathematical estimation of dead time using the retention times of C_2–C_5 *n*-alcohols is a useful method of determining system dead time for these columns. The use of homologous series of compounds other than *n*-alkanes for this purpose has also been shown to be effective[17].

TABLE I
RETENTION TIME DATA FOR C_1–C_7 *n*-ALKANES

Column conditions	*Alkane*	*t (sec)*	t_{calc} *(sec)*	*t′ (sec)*	*A*
Porapak N	Methane	39.9	39.0	13.7	
Temperature, 100°C	Ethane	60.0	59.3	33.8	2.49
Carrier gas (helium),	*n*-Propane	110.0	111.3	83.8	2.64
52 ml/min	*n*-Butane	242.0	244.9	215.8	2.54
Dead time, 26.2 sec	*n*-Pentane	577.0	588.6	550.8	2.59
	n-Hexane	1443.8	1472.0	1417.6	2.58
	n-Heptane	3680.0	3743.3	3653.8	
Porapak P	Methane	78.4	79.6	12.0	
Temperature, 110°C	Ethane	95.8	95.8	29.4	1.88
Carrier gas (helium),	*n*-Propane	128.5	131.7	62.1	2.46
48 ml/min	*n*-Butane	209.1	211.6	142.7	2.20
Dead time, 66.4 sec	*n*-Pentane	382.0	389.2	315.6	2.23
	n-Hexane	767.5	784.3	701.1	2.32
	n-Heptane	1660.5	1662.9	1594.1	
Porapak PS	Methane	61.5	61.8	8.2	
Temperature, 110°C	Ethane	72.5	72.2	19.2	1.95
Carrier gas (helium),	*n*-Propane	94.0	95.3	40.7	2.47
50 ml/min	*n*-Butane	147.0	146.5	93.7	2.20
Dead time, 53.3 sec	*n*-Pentane	263.8	260.0	210.5	2.19
	n-Hexane	519.5	511.6	466.2	2.19
	n-Heptane	1080.3	1070.8	1027.0	
Porapak Q	Methane	71.5	70.1	25.0	
Temperature, 220°C	Ethane	90.5	87.7	44.0	1.42
Carrier gas (helium),	*n*-Propane	117.5	118.4	71.0	1.88
48 ml/min	*n*-Butane	168.3	172.2	121.8	1.77
Dead time, 46.5 sec	*n*-Pentane	258.0	266.1	211.5	1.79
	n-Hexane	419.0	430.3	372.5	1.79
	n-Heptane	707.9	717.1	661.4	
Porapak QS	Methane	58.2	58.3	15.7	
Temperature, 230°C	Ethane	69.5	69.4	27.0	1.64
Carrier gas (helium),	*n*-Propane	88.0	88.5	45.5	1.81
44 ml/min	*n*-Butane	121.5	121.1	79.0	1.69
Dead time, 42.5 sec	*n*-Pentane	177.5	177.0	135.0	1.70
	n-Hexane	272.8	272.4	230.2	1.71
	n-Heptane	436.0	435.7	393.5	
Porapak R	Methane	44.5	43.4	12.8	
Temperature, 180°C	Ethane	55.3	53.3	23.6	1.45
Carrier gas (helium),	*n*-Propane	71.0	71.8	39.3	2.01
40 ml/min	*n*-Butane	102.5	105.9	70.8	1.90
Dead time, 31.7 sec	*n*-Pentane	162.5	169.0	130.8	1.89
	n-Hexane	275.8	285.8	244.1	1.90
	n-Heptane	491.0	502.0	459.3	
Porapak S	Methane	58.5	57.7	15.4	
Temperature, 180°C	Ethane	72.3	70.7	29.2	1.59
Carrier gas (helium),	*n*-Propane	94.3	95.4	51.2	2.05
40 ml/min	*n*-Butane	139.5	142.0	96.4	1.91
Dead time, 43.1 sec	*n*-Pentane	225.8	230.3	182.7	1.90
	n-Hexane	389.8	397.1	346.7	1.91
	n-Heptane	703.3	712.7	660.2	
Porapak T	Methane	43.0	40.4	15.4	
Temperature, 120°C	Ethane	56.5	53.0	28.9	1.60
Carrier gas (helium),	*n*-Propane	76.5	77.9	48.9	2.20
38 ml/min	*n*-Butane	120.5	127.1	92.9	2.00
Dead time, 27.6 sec	*n*-Pentane	208.25	224.4	180.7	2.04
	n-Hexane	387.0	417.0	359.4	2.09
	n-Heptane	761.0	797.7	733.4	

TABLE II

RETENTION TIME DATA FOR ALCOHOLS

Column conditions	*Alcohol*	*t(sec)*	t_{calc}*(sec)*	*t'(sec)*	*A*
Porapak	Methanol	79.5	85.4	34.2	
Temperature, 180°C	Ethanol	122.5	129.6	77.2	2.51
Carrier gas (helium),	*n*-Propanol	230.5	222.2	185.2	2.01
33 ml/min	*n*-Butanol	448.0	416.6	402.7	2.01
Dead time, 45.3 sec	*n*-Pentanol	886.0	824.7	840.7	1.99
	n-Hexanol	1756.5	1681.5	1711.2	
Porapak P	Methanol	76.0	78.0	16.5	
Temperature, 190°C	Ethanol	88.0	92.0	28.5	2.58
Carrier gas (helium),	*n*-Propanol	119.0	116.4	59.5	1.60
30 ml/min	*n*-Butanol	168.5	159.1	109.0	1.64
Dead time, 59.5 sec	*n*-Pentanol	249.75	233.8	190.3	1.50
	n-Hexanol	371.75	364.8	312.3	
Porapak PS	Methanol	61.5	63.4	13.1	
Temperature, 180°C	Ethanol	71.5	75.1	23.1	2.70
Carrier gas (helium),	*n*-Propanol	98.5	95.8	50.1	1.59
35 ml/min	*n*-Butanol	141.5	132.5	93.1	1.66
Dead time, 48.4 sec	*n*-Pentanol	212.75	197.8	164.3	1.53
	n-Hexanol	321.5	313.6	273.1	
Porapak Q	Methanol	63.0	66.9	28.1	
Temperature, 200°C	Ethanol	97.0	101.6	62.1	2.40
Carrier gas (helium),	*n*-Propanol	178.5	173.8	143.6	2.03
37 ml/min	*n*-Butanol	343.75	324.2	308.9	2.03
Dead time, 34.9 sec	*n*-Pentanol	679.0	637.3	644.1	1.96
	n-Hexanol	1335.5	1289.4	1300.6	
Porapak QS	Methanol	73.0	74.7	34.0	
Temperature, 200°C	Ethanol	113.0	115.8	74.0	2.40
Carrier gas (helium),	*n*-Propanol	209.0	204.1	170.0	2.03
30 ml/min	*n*-Butanol	403.5	393.7	364.5	2.11
Dead time, 39.0 sec	*n*-Pentanol	813.0	801.1	774.0	2.17
	n-Hexanol	1700.0	1676.6	1661.0	
Porapak R	Methanol	56.5	59.5	22.6	
Temperature, 200°C	Ethanol	83.0	86.7	49.1	2.42
Carrier gas (helium),	*n*-Propanol	147.0	142.9	113.1	1.96
35 ml/min	*n*-Butanol	272.5	258.6	238.6	2.05
Dead time, 33.9 sec	*n*-Pentanol	529.25	497.3	495.4	1.92
	n-Hexanol	1022.9	989.7	989.0	
Porapak S	Methanol	58.5	62.3	19.3	
Temperature, 200°C	Ethanol	82.5	87.1	43.3	2.56
Carrier gas (helium),	*n*-Propanol	143.5	138.3	104.3	1.95
32 ml/min	*n*-Butanol	263.5	244.4	224.3	2.01
Dead time, 39.2 sec	*n*-Pentanol	504.5	464.0	465.3	1.90
	n-Hexanol	963.5	918.6	924.3	
Porapak T	Methanol	62.0	65.5	14.7	
Temperature, 180°C	Ethanol	73.75	79.8	26.5	3.13
Carrier gas (helium),	*n*-Propanol	110.5	105.4	63.2	1.56
34 ml/min	*n*-Butanol	168.0	151.1	120.7	1.58
Dead time, 47.3 sec	*n*-Pentanol	259.0	232.7	211.7	1.49
	n-Hexanol	395.0	378.6	347.7	

TABLE III

RETENTION TIME DATA FOR ALDEHYDES

Column conditions	*Aldehyde*	*t (sec)*	t_{calc} *(sec)*	*t' (sec)*	*A*
Porapak N	*n*-Propanal	82.5	82.7	80.5	
Temperature, 180°C	*n*-Butanal	156.1	155.2	154.1	1.76
Carrier gas (helium),	*n*-Pentanal	285.5	292.7	283.4	2.17
36 ml/min	*n*-Hexanal	566.6	554.0	564.5	1.71
Dead time, 2.1 sec	*n*-Heptanal	1048.3	1050.1	1046.3	
Porapak N	*n*-Propanal	104.8	105.9	64.0	
Temperature, 180°C	*n*-Butanal	143.2	146.3	102.4	1.78
Carrier gas (helium),	*n*-Pentanal	211.6	211.6	170.7	1.65
40 ml/min	*n*-Hexanal	324.6	317.5	283.7	1.54
Dead time, 40.9 sec	*n*-Heptanal	498.2	489.0	457.3	1.55
	n-Octanal	767.1	766.8	726.2	
Porapak PS	*n*-Propanal	37.5	38.1	23.9	
Temperature, 180°C	*n*-Butanal	50.6	52.2	37.0	1.75
Carrier gas (helium),	*n*-Pentanal	73.5	74.6	59.9	1.80
34 ml/min	*n*-Hexanal	114.75	109.9	101.1	1.38
Dead time, 13.6 sec	*n*-Heptanal	171.6	165.8	158.0	1.44
	n-Octanal	235.5	254.1	239.8	
Porapak Q	*n*-Propanal	60.9	62.4	50.3	
Temperature, 230°C	*n*-Butanal	97.25	100.2	86.7	1.86
Carrier gas (helium),	*n*-Pentanal	165.0	165.5	154.4	1.81
38 ml/min	*n*-Hexanal	287.3	278.2	276.7	1.63
Dead time, 10.6 sec	*n*-Heptanal	486.75	473.0	476.2	1.64
	n-Octanal	813.25	809.6	802.7	
Porapak QS	*n*-Propanal	44.9	46.0	38.8	
Temperature, 240°C	*n*-Butanal	69.1	71.5	62.9	1.78
Carrier gas (helium),	*n*-Pentanal	112.25	113.4	106.1	1.78
45 ml/min	*n*-Hexanal	189.1	182.2	183.0	1.52
Dead time, 61.1 sec	*n*-Heptanal	306.1	295.2	299.9	1.49
	n-Octanal	480.7	480.5	474.6	
Porapak R	*n*-Propanal	48.5	49.5	40.7	
Temperature, 220°C	*n*-Butanal	71.5	74.6	63.7	1.91
Carrier gas (helium),	*n*-Pentanal	115.5	114.7	107.7	1.58
40 ml/min	*n*-Hexanal	185.0	178.9	177.2	1.50
Dead time, 7.8 sec	*n*-Heptanal	289.5	281.7	281.7	1.50
	n-Octanal	446.5	446.3	438.7	
Porapak S	*n*-Propanal	85.6	86.9	67.2	
Temperature, 230°C	*n*-Butanal	127.7	130.7	109.3	1.81
Carrier gas (helium),	*n*-Pentanal	204.1	203.1	185.7	1.62
35 ml/min	*n*-Hexanal	328.1	322.2	309.8	1.61
Dead time, 18.4 sec	*n*-Heptanal	527.5	518.0	509.1	1.51
	n-Octanal	840.7	840.0	822.5	
Porapak T	*n*-Propanal	36.9	37.4	34.6	
Temperature, 190°C	*n*-Butanal	55.4	57.1	53.1	1.75
Carrier gas (helium),	*n*-Pentanal	87.7	87.7	85.4	1.61
40 ml/min	*n*-Hexanal	139.6	135.6	137.3	1.43
Dead time, 2.2 sec	*n*-Heptanal	213.7	210.2	211.5	1.55
	n-Octanal	326.2	326.5	324.0	

TABLE IV

RETENTION TIME DATA FOR ACETATES

Column conditions	*Acetate*	*t(sec)*	t_{calc}*(sec)*	*t'(sec)*	*A*
Porapak N	Methyl	85.9	86.2	59.6	
Temperature, 180°C	Ethyl	140.4	140.9	114.1	1.93
Carrier gas (helium),	*n*-Propyl	245.4	245.6	219.1	1.95
36 ml/min	*n*-Butyl	450.1	445.9	423.8	1.85
Dead time, 26.3 sec	*n*-Pentyl	829.6	829.2	803.1	
Porapak P	Methyl	55.6	55.7	18.7	
Temperature, 200°C	Ethyl	66.1	66.5	29.2	1.67
Carrier gas (helium),	*n*-Propyl	83.7	83.6	46.7	1.60
35 ml/min	*n*-Butyl	111.7	110.6	74.7	1.47
Dead time, 37.0 sec	*n*-Pentyl	152.9	153.2	115.9	
Porapak PS	Methyl	43.6	43.6	18.7	
Temperature, 190°C	Ethyl	54.6	54.7	29.7	1.60
Carrier gas (helium),	*n*-Propyl	72.25	72.3	47.3	1.62
36 ml/min	*n*-Butyl	100.8	100.4	75.9	1.54
Dead time, 24.9 sec	*n*-Pentyl	144.9	145.0	120.0	
Porapak Q	Methyl	65.3	65.6	41.5	
Temperature, 230°C	Ethyl	98.0	98.8	74.2	1.86
Carrier gas (helium),	*n*-Propyl	158.8	158.3	135.0	1.81
40 ml/min	*n*-Butyl	268.5	265.2	244.7	1.72
Dead time, 23.8 sec	*n*-Pentyl	456.9	456.9	433.1	
Porapak QS	Methyl	49.25	49.4	31.6	
Temperature, 240°C	Ethyl	72.25	72.9	54.6	1.82
Carrier gas (helium),	*n*-Propyl	114.1	113.6	96.5	1.72
40 ml/min	*n*-Butyl	186.25	184.3	168.6	1.68
Dead time, 17.6 sec	*n*-Pentyl	307.25	307.2	289.6	
Porapak R	Methyl	66.8	66.5	43.7	
Temperature, 200°C	Ethyl	104.5	104.6	81.6	1.88
Carrier gas (helium),	*n*-Propyl	175.6	175.8	152.7	1.94
40 ml/min	*n*-Butyl	313.2	308.8	290.4	1.77
Dead time, 22.6 sec	*n*-Pentyl	557.8	557.3	534.7	
Porapak S	Methyl	152.3	153.8	91.8	
Temperature, 200°C	Ethyl	236.0	240.2	175.9	2.07
Carrier gas (helium),	*n*-Propyl	410.4	406.6	350.3	1.91
38 ml/min	*n*-Butyl	743.8	724.7	683.4	1.80
Dead time, 60.1 sec	*n*-Pentyl	1341.6	1336.9	1281.5	
Porapak T	Methyl	39.5	39.7	19.6	
Temperature, 190°C	Ethyl	52.5	53.2	32.6	1.85
Carrier gas (helium),	*n*-Propyl	76.5	76.0	56.6	1.66
40 ml/min	*n*-Butyl	116.35	114.4	96.4	1.57
Dead time, 19.9 sec	*n*-Pentyl	178.9	179.1	158.9	

TABLE V

RETENTION TIME DATA FOR METHYL KETONES

Column conditions	*Ketone*	*t(sec)*	t_{calc}*(sec)*	*t'(sec)*	*A*
Porapak N	Methyl ethyl	159.5	162.0	112.3	
Temperature, 180°C	Methyl *n*-propyl	265.75	271.3	218.5	2.15
Carrier gas (helium),	Methyl *n*-butyl	494.0	484.6	446.8	1.86
36 ml/min	Methyl *n*-pentyl	919.0	901.1	871.8	1.89
Dead time, 47.2 sec	Methyl *n*-hexyl	1722.25	1714.3	1675.0	
Porapak P	Methyl ethyl	173.5	174.4	102.6	
Temperature, 180°C	Methyl *n*-propyl	244.1	247.8	173.2	1.90
Carrier gas (helium),	Methyl *n*-butyl	377.6	373.2	306.7	1.63
40 ml/min	Methyl *n*-pentyl	595.6	587.5	524.7	1.64
Dead time, 70.7 sec	Methyl *n*-hexyl	953.4	953.6	882.5	
Porapak PS	Methyl ethyl	64.2	64.5	34.0	
Temperature, 180°C	Methyl *n*-propyl	87.2	88.6	57.0	1.94
Carrier gas (helium),	Methyl *n*-butyl	131.9	129.8	101.6	1.60
34 ml/min	Methyl *n*-pentyl	203.6	199.9	173.1	1.62
Dead time, 30.1 sec	Methyl *n*-hexyl	319.6	319.2	289.0	
Porapak Q	Methyl ethyl	103.75	104.5	76.7	
Temperature, 230°C	Methyl *n*-propyl	161.6	164.2	134.6	1.91
Carrier gas (helium),	Methyl *n*-butyl	272.0	269.7	245.0	1.75
35 ml/min	Methyl *n*-pentyl	464.75	456.3	437.7	1.67
Dead time, 27.0 sec	Methyl *n*-hexyl	786.75	786.6	759.7	
Porapak QS	Methyl ethyl	75.9	76.3	55.2	
Temperature, 240°C	Methyl *n*-propyl	115.6	117.1	94.9	1.88
Carrier gas (helium),	Methyl *n*-butyl	190.0	187.9	169.3	1.67
40 ml/min	Methyl *n*-pentyl	314.0	310.7	293.3	1.69
Dead time, 20.7 sec	Methyl *n*-hexyl	523.8	523.7	503.1	
Porapak R	Methyl ethyl	71.9	72.3	49.9	
Temperature, 230°C	Methyl *n*-propyl	107.7	109.2	85.6	1.90
Carrier gas (helium),	Methyl *n*-butyl	175.5	173.2	153.6	1.65
40 ml/min	Methyl *n*-pentyl	287.4	284.2	265.5	1.70
Dead time, 22.0 sec	Methyl *n*-hexyl	476.9	476.6	454.9	
Porapak S	Methyl ethyl	134.6	135.6	96.0	
Temperature, 230°C	Methyl *n*-propyl	200.2	203.9	161.6	1.91
Carrier gas (helium),	Methyl *n*-butyl	325.2	320.3	286.6	1.61
35 ml/min	Methyl *n*-pentyl	526.6	519.0	488.0	1.64
Dead time, 38.6 sec	Methyl *n*-hexyl	857.4	857.5	818.8	
Porapak T	Methyl ethyl	62.4	62.7	40.9	
Temperature, 190°C	Methyl *n*-propyl	88.0	89.5	66.5	1.88
Carrier gas (helium),	Methyl *n*-butyl	136.0	133.8	114.5	1.53
40 ml/min	Methyl *n*-pentyl	209.25	207.0	187.7	1.62
Dead time, 21.5 sec	Methyl *n*-hexyl	327.6	327.7	306.0	

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PHOTOIONIZATION DETECTOR RESPONSE

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SUMMARY

A number of studies of the factors involved in photoionization detector response are compared, contrasted and tested using published data. It is concluded that ionization potential is the most important single factor and that the relative number of π electrons is not a significant additional factor.

INTRODUCTION

The increasing importance of the photoionization detector (PID), both as a gas chromatography detector and as a selective monitor of the concentration of species of interest in a gas, has resulted in a number of studies of the factors involved in its response[1–4].

Freedman[1] proposed a mechanism for PID response and from this derived an expression for the PID signal. This treatment was used by Senum[2] as the basis of a discussion of the effect of using different carrier gases on PID response.

Casida and Casida[3] have reported an entirely different intuitive approach to the theory of PID response. In addition, Langhorst[4] has determined the relative molar response of a large number of compounds using a PID and has drawn empirical conclusions. This paper contrasts, compares and tests these different approaches.

THEORY

Freedman derived the following expression for PID response:

$$i = I^{\circ}F\eta\sigma NL\,[\mathrm{AB}] \qquad (1)$$

where i is the PID ion current, I° is the initial photon flux, F is the Faraday, η is the photoionization efficiency (the probability that a molecule will absorb a photon to give an excited state), σ the absorption cross-section (the probability that the excited state will ionize), N is Avogadro's number, L is the path length and [AB] the concentration of an ionizable substance. Thus for a particular detector and lamp this expression can be simplified to:

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$$i/[AB] = k\sigma\eta = R \tag{2}$$

where R is the molar response and k is a proportionality constant. The product $\sigma\eta$ is the photoionization cross-section σ_i.

Casida and Casida's intuitively obtained formula for PID response is:

$$R = kn\,F(IP) \tag{3}$$

"where k is a proportionality constant which varies (*sic*) with the probability that a given type of electron will be ionized, *i.e.* with the ionization cross-section of an orbital type", n is the number of ionizable electrons and F(IP) the number of photons with energies greater than or equal to the ionization potential (IP). For the 10.2 eV PID n is taken as the number of π bonding electrons only. Carbon–hydrogen bonding electrons are ignored since methane (IP = 12.98 eV) gave no response in an argon discharge ($h\nu$ = 11.83 eV) PID[5]. Carbon–carbon σ bond electrons are ignored since "paraffins have relatively small R values compared to hydrocarbons with carbon–carbon π bonds... probably due to the fact that IP's of paraffins are usually very close to or above the photon energy limit of 10.2 eV."

DISCUSSION

The function F(IP) in eqn. 3 must be considered in three separate IP ranges, *viz.* above 10.9 eV, below 10.2 eV and between 10.2 and 10.9 eV. Above 10.9 eV the standard MgF_2 lamp window is opaque hence $F(IP) = 0$. Below 10.2 eV "the number of photons emitted into the PID ionization chamber which have energies greater than or equal to IP" will be I°, which is constant for all species. Between 10.2 and 10.9 eV two other factors are important. The energy gap between molecules in excited vibrational states and the ground state of the ion can be up to 0.4 eV less than the IP. In addition, impurities in the lamp may give rise to small side-bands with photon energies between 10.2 and 10.9 eV. These factors will cause species with IP values between 10.2 and 10.9 eV to give a small PID response. The actual response will depend on such factors as temperature and the particular lamp used.

For species with IP < 10.2 eV eqn. 3 can be written as:

$$R \propto Kn \tag{4}$$

where K includes the photoionization cross-section, σ_i, and eqn. 2 can be written:

$$R \propto \sigma_i \tag{5}$$

Thus the difference between the equations of Casida and Casida eqn. 4 and Freedman eqn. 5 is that the former include the number of π electrons.

The theoretical relationship between σ_i and IP is very complex[7] and includes such factors as molecular geometry and symmetry. This can be seen from Langhorst's data for aromatic hydrocarbons[4]. *o*- and *m*-xylene both have an IP of 8.56 eV and R of 1.14 and 1.15, respectively. The more symmetrical *p*-xylene has IP = 8.445 eV and R = 1.2. In contrast, isomeric ethylbenzene has a similar R to *o*- and *m*-xylene of 1.16

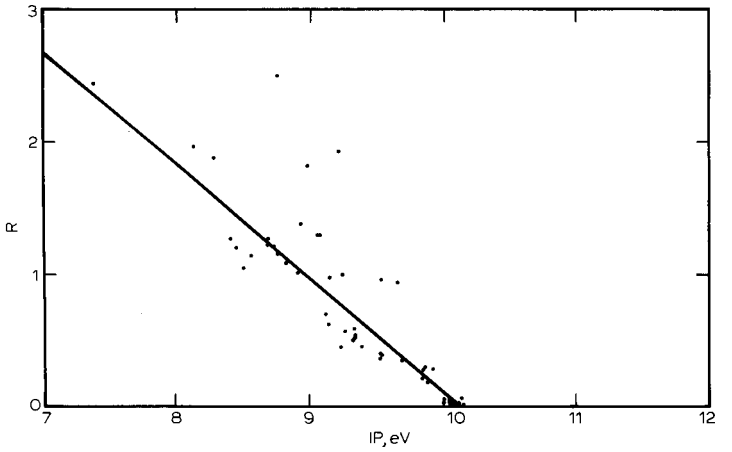

Fig. 1. Response per mole against IP (Langhorst's data).

but a much higher IP of 8.76 eV. The stability of the ion is also important. Aniline, with a very stable ion, has an IP of only 7.7 eV with a relatively low R of 1.13. Freedman suggested that although these other factors were involved, IP was the primary influence on σ_i for most molecules and hence R. The linear regression line for the plot of R against IP using Freedman's data for hydrocarbons gave a correlation coefficient of 93%.

Casida and Casida[3] suggest that these plots will be "more scattered" and "less well behaved" than plots of R/n against IP, where n is the number of π electrons. It is not clear why they limit their theory by restricting n to carbon–carbon π-bonded electrons (they only consider hydrocarbons) since this clearly leads to erroneous results. For all alkanes n is zero yet the varying response of the PID to n-alkanes has been reported[1,4,5]. In addition, if $n = 0$, R/n is infinite which clearly cannot be correct. Freedman's data[1] for eight hydrocarbons which have π electrons gave a

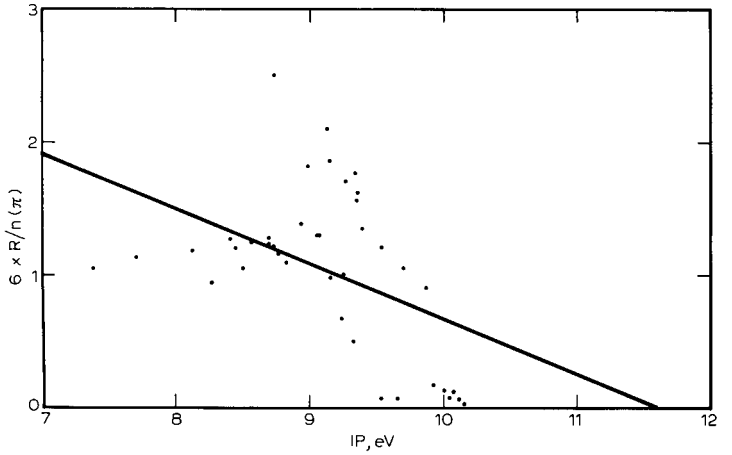

Fig. 2. Response per mole/$n(\pi)$ against IP (Langhorst's data).

correlation coefficient of 54% for R against IP, which fell to 8% for R/n against IP. The equivalent figures using Langhorst's data[4] for thirteen hydrocarbons is a correlation coefficient of 82% for R against IP falling to 1% for R/n against IP. Langhorst's data for 50 widely differing compounds, the IPs of which are known, gave a correlation coefficient of 89% for R against IP (Fig. 1). The correlation coefficient for R/n against IP, where n is the number of π electrons from C=O as well as C=C bonds, for the same 50 compounds fell to 25% (Fig. 2). Casida and Casida[3] show plots of R against IP and R/n against IP for some compounds and claim that these verify their theory. Unfortunately no data is given.

Casida and Casida state that "α-pinene, a compound with no π-electrons (*sic*), gave a response as large as benzene" and draw some conclusions from this. The compound whose structure is shown on their plot is in fact pinane which indeed has no π electrons. α-Pinene has two π electrons and in addition has a four-carbon strained ring which would result in a further reduction in IP, hence it would not be surprising to obtain a similar response to that of benzene.

Langhorst[4] draws a number of empirical conclusions regarding the relative sensitivity of the PID to different groups of compounds. In all cases they reflect the changes in IP between the different groups and confirm the proposal that the IP is the most important factor affecting PID response.

Casida and Casida develop their theory further to produce a quotient, Q, for the ratio response of the PID and flame ionization detector (FID) which has been the subject of previous discussion (Freedman[1]; Driscoll *et al.*[6]). They give

$$\text{PID/FID} = Ln\ F(\text{IP})/N \tag{6}$$

where N is the number of carbon atoms and "L is a constant of proportionality and is chosen so that PID/FID = 1.0 for n-octane". This would appear to be a difficulty since according to their theory $n = 0$ for n-octane; however, L is assigned the value 10. F(IP) is taken as "proportional to the difference between IP and the energy cut-off

TABLE I

PID/FID RELATIVE RESPONSE

Compound	*Exptl. ratio*	*Prediction* $10n(10.2-IP)/N$	*Prediction* $(10.2-IP)/N$	*Ref.*
Toluene	10.0	10.0	10.0	9
Benzene	11.2	8.1	8.1	9
p-Xylene	10.0	11.2	11.2	9
Hexane	2.4	0	0.2	9
Cyclohexane	1.9	0	2.7	1
1-Nonene*	4.6	1.6	4.8	1
n-Decane*	1.3	0	2.0	1
Cyclohexane	2.1	0	2.7	6
n-Octane*	1.0	0	1.8	6
cis-2-Octene*	5.3	2.5	7.6	6
Benzene	8.6	8.1	8.1	6
$\Sigma(x_{exp} - x_{pred})^2$		44.6	23.8	

* Estimated IP values used.

of the lamp window" and shown as $(10.2-\mathrm{IP})$. Since the energy cut-off of the lamp window is 10.9 eV, presumably the photon energy of 10.2 eV is meant. This leads to a formula for the ratio, $Q = 10n(10.2-\mathrm{IP})/N$. This equation can be tested using previously reported data[1,6,9] where IPs are known. In addition, it is possible to estimate IPs for higher members of an homologous series by extrapolation of the IPs of the lower members. Table I shows data for PID/FID response. All data have been normalized to toluene = 10. It is clear from Table I that ignoring the number of π electrons gives a much better fit of the data.

Casida and Casida complete their paper by stating that the correlation between reactivity with hydroxyl radicals and R or PID/FID relative response is excellent. No supporting data are provided. We have determined the correlation coefficient for a linear regression of the relative reactivity of hydrocarbons with hydroxyl radicals reported by Darnall *et al.*[10] with: (i) the values of R given by Langhorst[4]; (ii) the IPs, (iii) Casida and Casida's calculated values for PID/FID response. The coefficients are 16%, 27% and 3%, respectively. This lack of any correlation is not surprising since hydroxyl reaction with hydrocarbons is by hydrogen atom abstraction[11] and not by ionization.

CONCLUSIONS

(1) Ionization potential is the most important single factor determining PID response.

(2) The relative number of π electrons is not a significant additional factor.

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INFLUENCE OF THE PACKING MATERIAL AND THE COLUMN FILTERS ON THE RELIABILITY OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPH–MASS SPECTROMETER INTERFACE BASED ON THE DIRECT LIQUID INLET PRINCIPLE

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SUMMARY

The direct liquid introduction interface for coupled high-performance liquid chromatography (HPLC)–mass spectrometry consists of a 1–3-μm pinhole in a nickel diaphragm. This interface produces an axial jet of HPLC eluent into the ionization source. Variations in the jet affect the mass fragmentation pattern. Physical modifications of the pinhole also induce substantial changes in the jet. The influence of the packing material and the column filters has been examined and solutions are given for preventing plugging of the pinhole.

INTRODUCTION

The use of a mass spectrometer as a detector for liquid chromatography is now well established. Combined high-performance liquid chromatography–mass spectrometry (HPLC–MS) is considered to be the best tool for the analysis of polar, non-volatile or thermolabile compound that are not amenable to gas chromatography–MS.

The interface that we used has been described elsewhere[1,2] and is based on the direct liquid introduction (DLI) principle developed by McLafferty and co-workers[3,4], in which a fraction of the liquid coming out of the HPLC column is introduced directly into the ion source of a mass spectrometer through a fine pinhole. Chemical ionization of the solute is obtained by using the solvent vapour as a reactant gas.

Although the DLI technique is intrinsically simple, a lack of reproducibility of results can be observed under certain conditions. The cause of this effect has been attributed to changes in the jet direction[1].

The analysis of several thermolabile molecules using the Nermag LC–MS interface has shown that the probability of observing the molecular or pseudo-molecular ion is low when the liquid jet impacts on the walls of the ion source. When no

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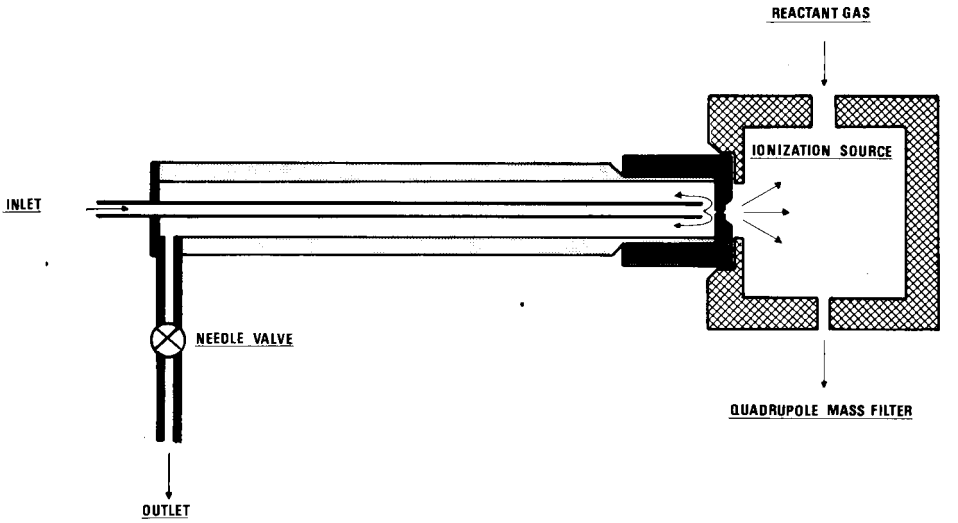

Fig. 1. Schematic diagram of the HPLC–MS interface allowing direct liquid introduction.

impact occurs, chemical ionization without fragmentation can be observed. Thus, for optimal HPLC–MS analysis, the jet must be axial for several hours. In practice, we have observed under certain experimental conditions substantial changes in the jet direction.

The purpose of this paper is to demonstrate that it is material from the HPLC column which is responsible for these changes.

EXPERIMENTAL

We observed the pinhole diaphragm after several HPLC–MS runs with a Cameca MEB 07 scanning electron microscope; the diaphragms were glued to an aluminium stub with silver lac and metallized by gold–palladium evaporation. Colloidal silica was detected on a Jeol JSM 35C stereoscan, with an Ortex X analyser. The HPLC pump (6000 A; Waters Assoc., Milford, MA, U.S.A.), the injector (U6K Waters Assoc.), tubing and HPLC–MS probe were the same throughout the experiments; only the HPLC column and solvents were varied. The HPLC–MS interface is shown in Fig. 1, and has been described elsewhere[1,2].

RESULTS

The best conditions of analysis are obtained when the solvent jet is axial (Fig. 2). Fig. 3 shows a new diaphragm before use. The internal diameter of the pinhole is about 2 μm. Fig. 4 shows a diaphragm after several hours of use. The pinhole appears partially plugged by packing particles and by another material. Small cracks are visible on the surface of the diaphragm, looking like dry mud, which are due to the packing material itself and not to fissures in the diaphragm. It can be concluded that at least two phenomena are able to modify the geometry of the pinhole and, consequently, the characteristics of the jet.

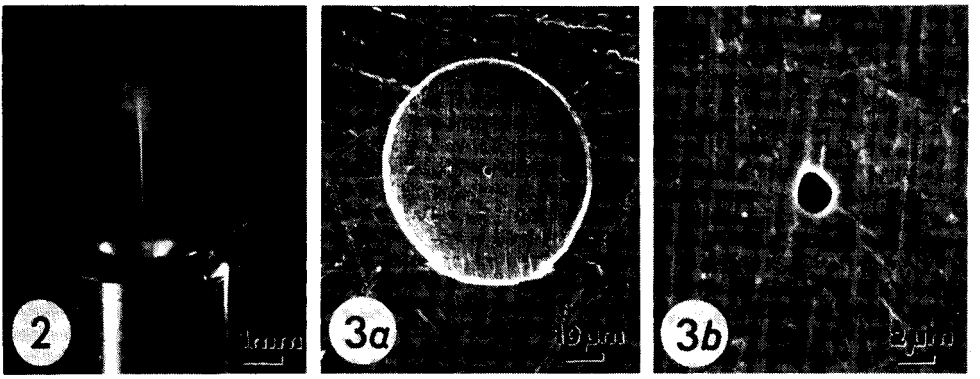

Fig. 2. Axial liquid jet on coming out of interface, expanding into a droplet in the ionization source, observed under atmospheric conditions.

Fig. 3. A clean pinhole before use. (a) Primary groove in 3 mm diameter diaphragm in which pinhole was bored; (b) pinhole observed at high magnification.

Loss of stationary phase from the column through the frit

The first phenomenon is due to the deposition or fixation of packing particles on the walls of the pinhole. These particles leave the column through the frit. As an example, Fig. 5 shows the particles collected on a diaphragm when using a Hibar HPLC column packed with LiChrosorb RP-18 (5 μm) stationary phase. This packing is shown in Fig. 6 with a factor magnification of 1600. The shape and dimensions of the collected particles are clearly representative of the packing material. Similar results can be obtained when using spherical particles as packing material (Figs. 7 and 8). The dimensions of these collected particles are in the micron range and generally below 2 μm, which corresponds to the diameter of the pinhole. This value also corresponds to the limit of efficiency of the classical filters (2 μm porosity).

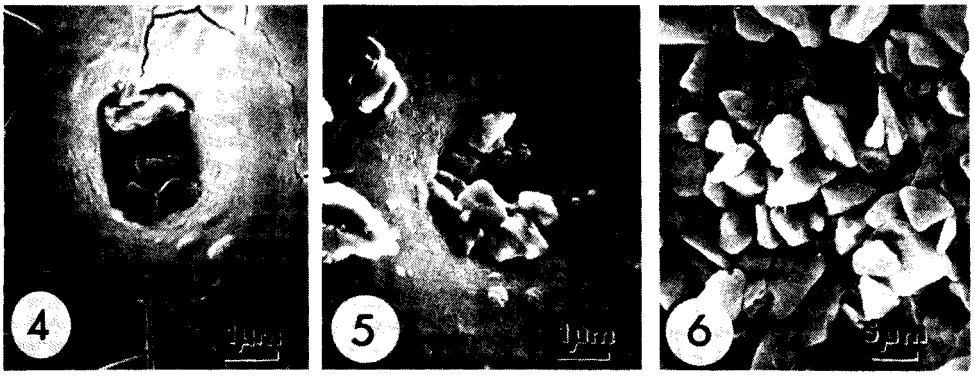

Fig. 4. The same diaphragm observed after several hours of use. Solvent, acetonitrile–water; stationary phase, LiChrosorb RP-18 (5 μm).

Fig. 5. Pinhole observed after use of a Hibar Column (Merck) packed with LiChrosorb RP-18 (5 μm); 2 μm porosity outlet and filter.

Fig. 6. Reversed-phase LiChrosorb RP-18 (5 μm) (Merck).

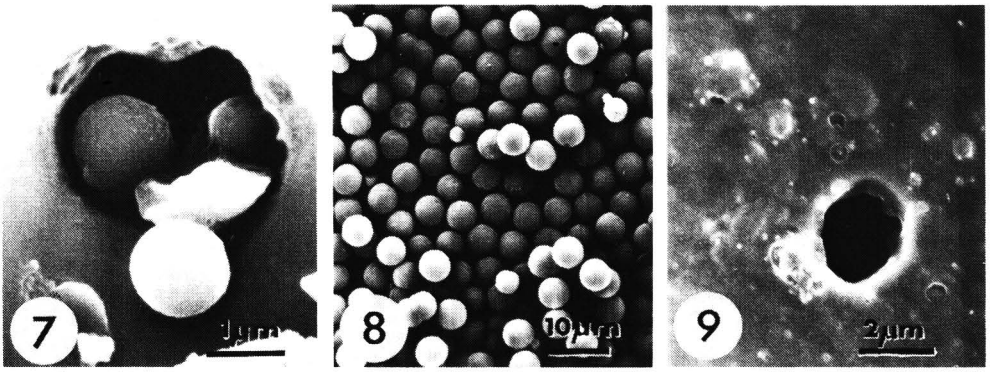

Fig. 7. Pinhole observed after use with a column packed with Zorbax-Sil as stationary phase.

Fig. 8. Zorbax-Sil stationary phase.

Fig. 9. Influence of a 0.5 μm porosity frit on plugging of the pinhole. The filter was introduced between the column and the diaphragm.

Different packing materials are shown in Figs. 6 and 8. However, numerous observations were made with other commercial packings. It can be concluded that, when the particles are not spherical, the particle size distribution can be broad, *e.g.*, from 0.5 to 25 μm. In that event, the frit is not effective in preventing a loss of the column packing. Such a phenomenon may also cause the formation of a dead volume in the column itself and, consequently, a decrease in efficiency.

The second consequence of these particles passing through the frit is that they may cause partial or a total plugging of the pinhole during HPLC–MS runs. Hence changes in the jet direction can be explained by the adhesion of one or more particles on the walls of the pinhole (Fig. 7).

To prevent such an effect, we inserted a stainless-steel frit of 0.5 μm porosity, 1/16 in. O.D. and 1 mm thickness (Chrompack, Middelburg, The Netherlands) between the column and the HPLC–MS probe. The influence of this filter is shown in Fig. 9, where the packing material in the column was LiChrosorb RP-18 (5 μm). Comparison of Figs. 5 and 9 clearly indicates the importance of using a 0.5 μm instead of a 2 μm porosity frit; the shape of the pinhole remains unchanged after several days of use.

The porosity of the filter was satisfactory, and the back-pressure of the system was not significantly increased.

The probability a filter becoming plugged increases as its porosity decreases. However, we used a 0.5 μm porosity stainless-steel frit inserted in a microbore column (stationary phase Partisil 10 μm) for 3 months without observing any plugging of the filter.

The solvents used were *n*-hexane, dichloromethane and isopropanol–*n*-hexane mixture.

Dissolution of silica

Even when using an efficient frit, a pinhole can become plugged after several

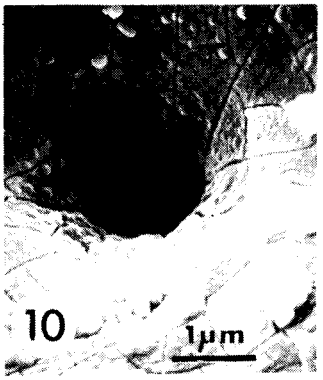

Fig. 10. Pinhole after several days of use; structureless material is observed on the wall of the pinhole.

days of use. This effect appears as due to the deposition of a structureless material on the walls of the pinhole (Fig. 10).

This material was analysed with an Ortex X analyser. The results obtained are shown in Fig. 11. The small amounts of silica are detected with an energy of 1.74 keV. The other main peaks correspond to the gold and palladium used for metallization, which were detected at different energy levels.

Analyses have shown that this type of plugging effect was due to dissolution of silica in the mobile phase and its deposition on the wall of the pinhole.

Silica deposition can be observed when using either reversed- or normal-phase conditions (Fig. 12). This result is not surprising when we consider that the silica in the stationary phase can dissolve in the mobile phase and consequently be deposited in the diaphragm region. The deposition of this colloidal silica is favoured by the presence of stationary phase particles on the pinhole wall. These two phenomena are

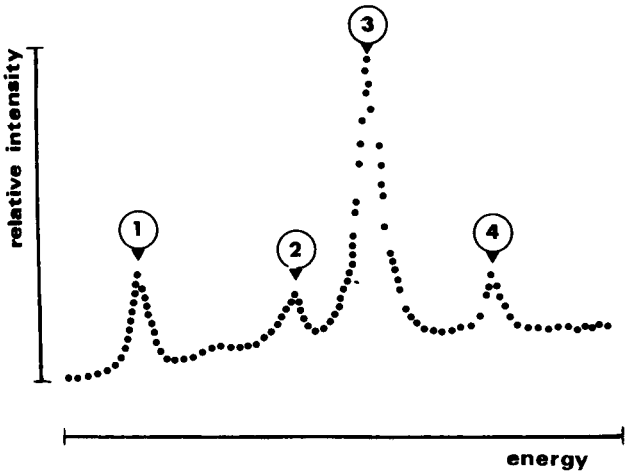

Fig. 11. Determination of compounds observed by scanning electron microscopy with an Ortex X analyser. Each compound is characterized by its energy level: 1, 0.93 keV (copper); 2, 1.74 keV (silica); 3, 2.12 keV (gold); 4, 2.89 keV (palladium).

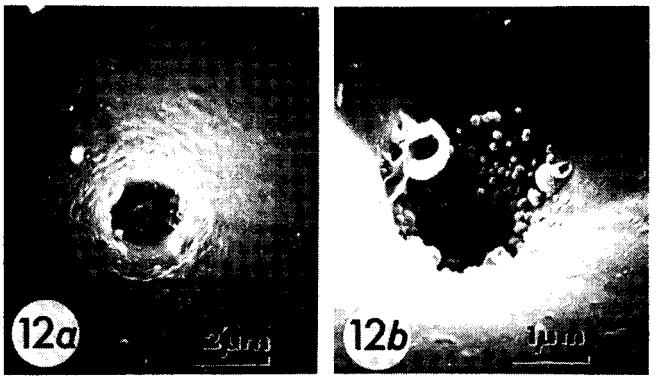

Fig. 12. Diaphragms observed after several days of use: (a) reversed phase; (b) normal phase.

linked. In fact, dissolution of silica leads to the presence of very small stationary phase particles in the column that consequently are carried through the frit. Examination of the stationary phases used confirmed this increase in the smallest particles relative to a fresh packing.

DISCUSSION

The shape and size distribution of a column packing material are critical parameters with respect to the reliability of HPLC–MS coupling when using a DLI interface with a diaphragm. However, this method of column degradation should also not be neglected when working with the usual detectors as a loss of material in the column itself can produce either an increased dead volume in the HPLC column itself with an apparent decrease in the efficiency of the analysis and a reduction in the lifetime of the column, or plugging of the frit.

From a technical point of view, it would be easier to obtain a uniform distribution with spherical particles rather than with irregular shaped particles. It can be concluded that totally spherical particles are the better choice for HPLC–MS purposes. However, we have observed that initially spherical particles can be modified in shape during column packing owing to breakage. Hence, the probability to finding particles with dimensions smaller than 2 μm cannot be neglected. In addition, the specific surface area is higher with particles of irregular shape than with those of spherical shape.

The difficulties increase when using smaller particles, *e.g.*, 3 μm[5,6], although a higher efficiency, lower solvent consumption, increased sensitivity and higher rate of separation can be obtained[7,8]. With such a packing material and a 2 μm porosity column outlet frit, we can predict very rapid plugging of a pinhole of 2–5 μm diameter. The simplest solution would be to replace the usual 2 μm frit with a 0.5 μm porosity frit. The latter is commercially available but should be chosen carefully to minimize the dead volume.

An alternative solution consists in inserting a 0.5 μm porosity filter between the column and the detector, but this does not prevent the loss of packing material in the column itself. The advantage of this solution is the possibility of removing and clean-

ing the filter without removing the column outlet frit, thus avoiding possible disturbance of the column bed.

Another cause of the loss of packing material in the column is dissolution of silica, which has long been recognized[9]. The rate of dissolution depends on the solvent mixture, the pH and the salt concentration in solution[2,10]. For this reason, many experiments are carried out in the pH range 2–8. At pH > 7, dissolution of the silica destroys the backbone support of the organic-modified packing. At pH < 2, acid cleavage of the Si–O–Si bonds can occur[11]. Silica dissolution can occur even in neutral mobile phase, as indicated by the experimental results we have obtained.

To prevent a loss of silica in the column, two approaches are possible. The first is to use silica pre-columns, which saturate the mobile phase with silica[11,12], to reduce the dissolution of particles in the analytical column. The second, more efficient method, is to use a non-siliceous phase such as a synthetic macroporous copolymer[13].

Only with the latter method can the deposition of silica on the wall of the pinhole be completely avoided. In practice, the effect of silica on the dimensions of the pinhole is small, and is negligible in comparison with the action of micron-sized particles. When only silica deposition from solution occurs, the lifetime of diaphragm is at least 1 week. This means that dissolution of silica in the mobile phase is not critical for HPLC–MS purposes, except that it shortens the lifetime of the column.

It is also advisable to immerse the mounted interface in solvent to prevent the deposition and desiccation of silica on the diaphragms when the apparatus is not being used.

CONCLUSION

With conventional HPLC detectors (UV, RI, etc.) a loss of material from the column has negligible influence on the system. The only consequence is a slow decrease in the efficiency of the column due to the creation of a dead volume.

With a mass spectrometer as a detector and especially with an interface based on the DLI principle (even with a diaphragm or a capillary tubing), the consequences can be more dramatic and a substantial decrease in the reliability of the system can be observed under certain conditions. This means that for HPLC–MS purposes the HPLC column itself is an important parameter. The packing material and column outlet frit have to be chosen carefully in order to avoid plugging of the pinhole whith particles from the column.

In practice, the use of a 0.5 μm porosity filter instead of the usual 2 μm porosity filter gives a reliable system. However, it is necessary, for HPLC–MS purposes, to bear in mind that the reliability of the system mainly depends on the reliability of the HPLC column.

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PYROLYSIS–GAS CHROMATOGRAPHY OF SEPARATED ZONES ON THIN-LAYER CHROMATOGRAMS

I. APPARATUS AND METHOD

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(Received September 9th, 1981)

SUMMARY

An apparatus is described for the pyrolysis–gas chromatographic determination of amounts of substances in the approximate range 0.1–10 μg. It consists of a tubular, externally wound, electric furnace attached to the column of a gas chromatograph at one end and fitted with a sampling port and carrier gas entry tube at the other. Although primarily intended for the determination of substances separated by thin-layer chromatography without removal from the substrate, with minor changes to the entry port and furnace hot-zone, samples in liquid form can also be accommodated. The apparatus was tested using poly(vinyl chloride) and partially hydrolysed poly(vinyl acetate).

INTRODUCTION

Among the separation techniques available to the chemist, thin-layer chromatography (TLC) is one of the most versatile and inexpensive in terms of financial outlay on equipment. The versatility is reflected in the range of sample size accommodated, for example, relative to paper chromatography, linear (one- or two-dimensional) and radial development of chromatograms, ease of location of separated zones and wide ranges of stationary and mobile phases available. However, quantitative determination of a component separated by TLC is less convenient than in gas–liquid or liquid–liquid chromatography. Existing measurement techniques applied to thin-layer chromatograms are generally less sensitive and frequently less accurate than those available in gas–liquid or liquid–liquid chromatography[1].

Pyrolysis–gas chromatography (Py–GC), initially applied largely to the study of the thermal degradation of polymers, has, more recently, been extended to the determination of a variety of substances. Where applicable, it offers a rapid and sensitive route to an analytical determination[2]. In an attempt to extend the range of techniques available for quantitative measurement of separated zones on thin-layer chromatograms, work has been carried out on the application of Py–GC to such determinations. The separated zone, after location, is cut, along with its portion of

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backing plate, from the dried, developed chromatogram and transferred to the pyrolysis chamber. Pyrolysis products are swept by the carrier gas into the gas chromatograph for separation and to complete the determination. A furnace, meeting specifications determined by the properties of the samples to be pyrolysed and convenience of operation, was designed and tested; it is described here along with ancillary equipment and validation experiments using poly(vinyl chloride) (PVC) and partly hydrolysed poly(vinyl acetate) (PVA) as test substances.

EXPERIMENTAL

The apparatus, as developed, consists of a tubular electric furnace attached at one end to a gas chromatograph. The other end has facilities for sample introduction and entry of the carrier gas.

Furnace

A scale diagram of the furnace is presented in Fig. 1. It consists of a quartz tube, 15 cm × 0.95 cm O.D. × 0.70 cm I.D. At one end it is fused to a small bore (0.15 cm I.D.) thick-walled quartz tube for connection to the GC column. The other end of the furnace tube is closed by a sampling port and carrier gas entry unit (Fig. 2a). The furnace and tube connecting it to the top of the GC column are heated by an externally wound heating filament consisting of a 2-m length of Vacrom ribbon (0.15-cm wide) having an electrical resistance of 10 Ω m^{-1} at ambient temperature. The number of turnings, per unit length of tube, is arranged so that the furnace hot-zone with 3.5 turns cm^{-1} is located between 10.5 and 12.5 cm from the entrance end of the quartz tube. The heating element, which is heavily lagged on the outside with asbestos cord, is energised by a variable-voltage (0 to 260 V, 5 A rating) a.c. Variac unit

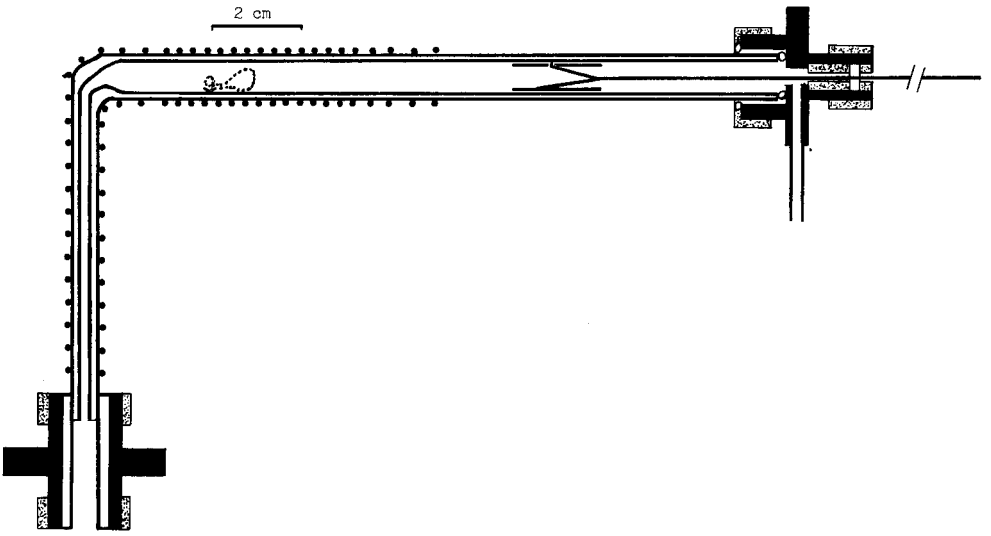

Fig. 1. Scale drawing of the furnace with push-rod arrangement for introduction of solid samples, sample entry and gas exit ports. The heat-insulating layer over the hot furnace winding has been omitted. The scale (2 cm) marking is positioned over the furnace hot-zone and the quartz obstructor (broken line) for injection of liquid samples.

supplied by the Zenith Electric Co. (London, Great Britain). Within the hot-zone the heating coil is capable of giving temperatures up to 1100°C. The temperature drop 1 cm distance along the tube to either side of the centre of this zone is 7°C at 530°C. The heating time for a sample to reach 530°C is *ca.* 0.5 sec.

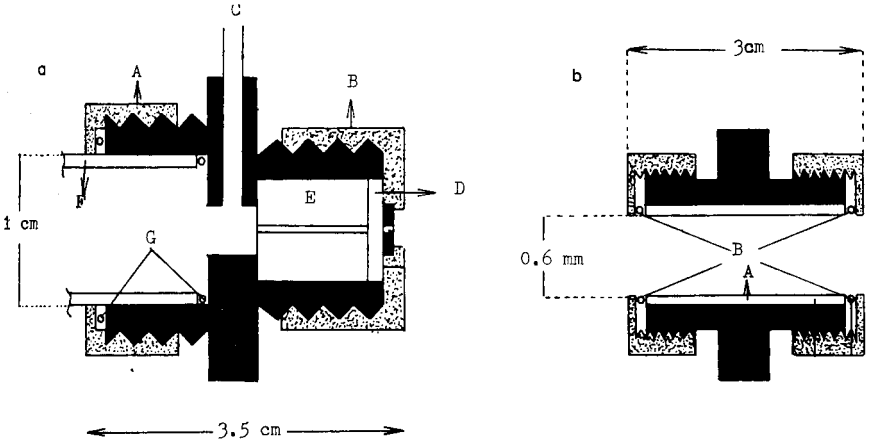

Fig. 2. (a) Entry port showing demountable nuts A and B, carrier gas inlet (2 mm I.D.) C, septum D, metal needle and push-rod guide E, furnace tube F and O-rings G. (b) Furnace coupling to GC column showing the teflon coupling A and positions of O-ring seals B. In (a) and (b) the nuts (speckled cross-section) and main bodies (mat black) of the couplings are all made from brass.

Sample introduction

Sample adsorbed on the detached portion of the thin-layer chromatogram or dried on small pieces of quartz plate are introduced into the furnace through a demountable entry port (Fig. 2a) and moved to the hot-zone by a push-rod. The push-rod is constructed from stainless-steel rod (20 cm × 0.20 cm) to which is attached a piece of quartz tube (2 × 0.60 cm O.D.). The attachment is made by means of a steel syringe needle bent to a V-shape and silver-soldered at its apex to the end of the rod. The end of one limb of the V is bent and passed into a small hole drilled in the inner quartz tube as shown in Fig. 1. The push-rod passes through a cylindrical metal guide and a septum at the entrance to the furnace. The entry port is shown in greater detail in Fig. 2a. It is constructed of brass with O-rings, and the septum is placed as shown to give gas-tight seals. The stainless-steel tube admitting the carrier gas is silver-soldered to the brass coupling. In order to introduce a solid sample, the entry port is detached and removed with the push-rod by undoing the nut at A, and the sample is placed inside the furnace tube in the cool zone. After replacement of the port and push-rod and waiting for 2.5 min for restoration of the baseline of the GC output signal, the sample is pushed to the centre of the furnace by the push-rod which is then immediately withdrawn to the cool zone. Liquid samples are introduced into the furnace using a Hamilton syringe. To facilitate the handling of such samples, the push-rod is removed and a new silicone rubber septum and a brass-cylinder having a narrower guide channel is inserted by uncoupling at B (Fig. 2a). The syringe needle is inserted through the septum and the contents are discharged 1 cm from the furnace centre. To prevent the discharged liquid from being propelled too rapidly through the

hot-zone to cooler regions down-stream before efficient pyrolysis can occur, a pear-shaped piece of quartz (broken line, Fig. 1) is inserted as an obstruction at the centre of the furnace. (This is not required when dealing with solid samples).

Gas chromatograph

The coupling between the pyrolyser unit and the top of the gas chromatographic column is shown in Figs. 1 and 2b. A Pye series 104 chromatograph fitted with a flame ionisation detector maintained at the column oven temperature and a glass column (160 × 0.35 cm I.D.) packed with Porapak Q (50–80 mesh), was used in the work described here. The carrier gas through the furnace and chromatograph was nitrogen at 50 ml/min controlled by a needle valve, flow restrictor and damping coil.

Standard solutions

PVC was dissolved in cyclohexanone to give solutions containing 875 ng/μl and 87.5 ng/μl. PVA was dissolved in water to give solutions containing 1.00 μg/μl and 100 ng/μl.

Pyrolysis and measurement

A known volume of polymer solution was transferred on to the surface of a small square (0.5 × 0.5 cm) of quartz 0.1 cm in thickness and the solvent carefully removed by evaporation. The piece of quartz carrying the polymer sample was then introduced into the furnace, following the procedure for solid samples described above. The optimum temperature for pyrolysis of either polymer was 440°C in this furnace. The oven temperature for gas chromatography was set at 180°C.

RESULTS AND DISCUSSION

Considerable attention was given to the type of furnace to be used, particularly with regard to Py–GC of substances *in situ* on portions of thin-layer chromatograms. A Curie-point pyrolyser of conventional design was considered unsuitable because of the limitation on sample sizes. Various hot-filament devices were constructed and tested but their use revealed major problems. These related to support of the sample on the filament, large temperature gradients due to the nature of the heat source and expansion of the carrier gas during the heat-up period. The last-mentioned phenomenon was particularly serious because of its effect on the GC detector output signal. Other problems, related to the size and geometry of the pyrolysis chamber, gas leakage and deposition of sample debris in the chamber and connecting tubes, were also experienced. On the other hand, the externally heated tubular furnace resulted in relatively fast heat-up times for samples, particularly those on thin-layer substate or other solid support. Problems relating to disruption of the carrier gas temperature and flow were overcome and good temperature control during pyrolysis was made possible. Furnaces of various dimensions were tested before selection of the version described here.

Typical pyrograms for PVC and PVA pyrolysed at 440°C in the hot-zone of the furnace under the conditions described are presented in Figs. 3 and 4, respectively. Pyrolysis products are known[2,3] for these substances, and hence to relate peaks in the pyrogram to particular products it is only necessary to check retention times for GC.

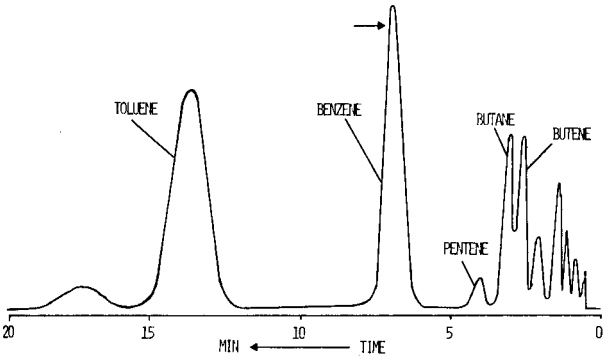

Fig. 3. Pyrogram for PVC showing the peak (marked by the arrow) on which the measurement was based.

In this way, the peaks attributed to particular substances have been identified (Figs. 3 and 4). Thus one or more well-resolved peaks in the pyrogram can be selected for use in quantitative analysis. When the peak(s) have been selected, the yield can be optimised by varying the temperature of pyrolysis. The amount of polymer pyrolysed is compared with peak height or area; in the present work the former was found to be adequate. The benzene and crotonaldehyde peaks were selected for the measurements on PVC and PVA, respectively, and relevant data are presented in Tables I and II. From the standard deviations obtained for replicate determinations, it is seen that reproducibility of results is good. This can be attributed in part to the small amounts of polymer pyrolysed in each experiment. The concentrations of reactive pyrolysis products are kept low as they enter the carrier gas stream. Towards the upper end of the sample ranges investigated (Tables I and II) there is some indication that the standard deviations increase somewhat with sample weight, and this is attributable,

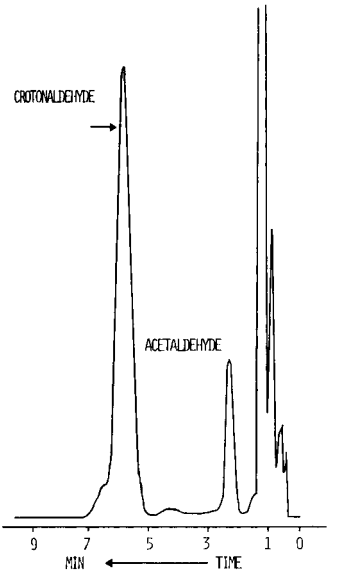

Fig. 4. Pyrogram for PVA showing the peak (marked by the arrow) on which the measurement was based.

TABLE I

PYROLYSIS OF PVC ON QUARTZ AT 440°C

Sample weight (ng)	*Peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
350	10.0	0.0	3
525	14.5	1.3	3
875	25.0	0.0	3
1750	47.0	0.0	3
2625	70.0	2.8	3
3500	89.0	0.7	5
4375	114.0	1.4	5
5250	135.0	0.6	3
6125	163.0	2.0	3
7000	180.0	2.1	3
7875	206.0	4.7	3

at least in part, to secondary reactions before stabilisation in the carrier gas stream[2]. When peak height is plotted against sample weight, good rectilinear relations are obtained for each polymer over the weight ranges investigated and, as noted, the reproducibility is such that a single measurement can be expected to give a reliable result.

TABLE II

PYROLYSIS OF PVA ON QUARTZ AT 440°C

Sample weight (μg)	*Peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.10	4.0	0.0	3
0.30	9.5	0.0	3
0.50	17.0	0.0	3
0.70	26.0	0.5	3
0.90	28.5	0.5	3
1.00	33.0	0.6	3
2.00	64.0	1.0	4
3.00	101.0	2.4	10
4.00	133.0	2.8	3
5.00	163.0	2.1	5
6.00	204.0	1.4	5
7.00	238.0	1.0	3
8.00	264.0	1.6	3

Experiments in which substances adsorbed on thin-layer substrate are pyrolysed and determined using this apparatus are described in Part II[4].

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PYROLYSIS–GAS CHROMATOGRAPHY OF SEPARATED ZONES ON THIN-LAYER CHROMATOGRAMS

II. APPLICATION TO THE DETERMINATION OF SOME WATER-SOLUBLE VITAMINS

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SUMMARY

Using the furnace and gas chromatograph described previously, it is shown that amounts down to *ca.* 0.2 μg of each of the vitamins B_5, B_6 and C, separated from each other by thin-layer chromatography, can be determined with a relative error of 2–3%. Advantages and limitations of the technique as applied to quantitative thin-layer chromatography are discussed.

INTRODUCTION

The pyrolysis–gas chromatographic (Py–GC) technique described in Part I[1] has been applied to the determination of calcium D-pantothenate (vitamin B_5), pyridoxine hydrochloride (vitamin B_6) and L-ascorbic acid (vitamin C). Taking solutions of each vitamin separately, suitable conditions for pyrolysis and separation of volatile pyrolysis products by GC have been determined. Then it is shown that each vitamin can be determined by the Py–GC technique while adsorbed on a thin-layer stationary phase which may or may not have undergone chromatographic development. Mixtures of vitamins are also determined following their separation by thin-layer chromatography (TLC). Advantages and limitations of the Py–GC technique revealed by this work are discussed. GC coupled with mass-spectrometry (MS) was used as an aid to identification of the pyrolysis products on which determination of each vitamin was based.

EXPERIMENTAL

Chemicals and solutions

The vitamins were obtained from Sigma (St. Louis, MO, U.S.A.) and reagent-grade solvents from Fisons (Loughborough, Great Britain). Standard solutions of each vitamin were prepared in double distilled water; they contained either 0.100

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μg/μl or 1.00 μg/μl of the appropriate vitamin. Mixtures, in which the concentration of each vitamin was from 0.1 μg/μl to 1.0 μg/μl, were also prepared in water. The solutions were kept cool and in the dark when not in use and renewed every few days.

Thin-layer plates

The plates were prepared in our laboratory from silica gel G (Merck, Darmstadt, G.F.R.) on a backing of aluminium (0.1 mm thick) sheet (British Aluminium, London, Great Britain). Before coating, the aluminium sheets were heated at 600°C for 12 h to remove any surface organic matter. A coating 0.25 mm thick was used. The plates were dried and activated at 100°C for 30 min before use.

Apparatus

The furnace for pyrolysis and the gas chromatograph including the column are described in Part I. A Pye-Unicam series 104 chromatograph connected through a flow-splitter membrane separator to an AEI MS 20 mass spectrometer (GEC-AEI (Electronics), Manchester, Great Britain) was used in the GC–MS studies. The furnace for pyrolysis was connected to the top of the GC column which was the same as that used in Py–GC.

Thin-layer chromatography

The solvent system for separation of mixtures of the vitamins was glacial acetic acid–acetone–methanol–benzene (5:5:20:70, v/v) ternary mixture as used by Gaenshirt and Malzacher[2]. The development was by ascending mode in a closed tank saturated with solvent vapour at 25°C. The developed plates were air-dried and a revealing agent was applied; iodine vapour was used to locate vitamins B_6 and C and after their zones were removed, ammoniacal silver nitrate (0.1–0.5 *M*) was sprayed on the plate to reveal vitamin B_5.

Pyrolysis–gas chromatography

Known amounts of each vitamin in solution or adsorbed on thin-layer substrate were introduced into the pyrolyser hot-zone, and the volatile products of pyrolysis were swept onto the chromatography column by the nitrogen carrier gas, flow-rate 50 ml/min. The separate procedures for liquid or solid sample introduction and the furnace modifications to accommodate either type are described in Part I. The solid samples for pyrolysis were prepared in one of two ways.

(1) Standard solution of vitamin was spotted on to a small square (*ca.* 6 × 6 mm) of thin-layer plate (silica gel G with aluminium backing) which was then air-dried before insertion into the furnace.

(2) Standard solutions of mixtures of the vitamins were separated by TLC as described above, the zones located by the appropriate revealing agent and each zone punched out from the dried plate as a disc, 6 mm in diameter, using a lever-operated punch.

Pyrolysis–gas chromatography with mass spectrometric detection

Each vitamin (*ca.* 5 mg) was introduced into the furnace in solid form in a shallow aluminium cup (6 mm diameter, 1 mm deep). The pyrolysis temperature depended on the compound and was that considered to be optimum in the Py–GC

studies. The carrier gas was helium, flow-rate 50 ml/min, and the gas chromatograph oven temperature was 180°C. The mass separator was maintained at 200°C, and the mass range 20–200 was scanned. The electron impact energy was 68.2 eV and the current 50 μA.

RESULTS AND DISCUSSION

Pyrograms obtained by direct injection of aqueous solutions differ from those adsorbed and dried on adsorbent for TLC (Fig. 1). However, with the present experimental arrangement and test substances the major differences in the number of resolved peaks and their relative areas is largely confined to products with short retention times, *i.e.* less than *ca.* 2 to 3 min. The differences among the products are partly due to differences in the amount of water present and its accessibility to the decomposing compound. Water will produce a peak of low retention time on a Porapak Q column but it may also, through interaction with reactive intermediate pyrolysis products, give rise to others. Pyrolysis products from vitamin C with retention times greater than 3 min appear to be more affected by the change in form of sample introduction, perhaps partly due to a more significant role for water in its pyrolysis. However, the yields of all substances undergoing pyrolytic decomposition can be expected to be a function of heat-up time[3]. This in turn is determined by the thermal conductivity of the material introduced into the furnace. Fortunately any differences in this property of the system due to changes in the amount of adsorbed substance to be determined on thin-layer substrate are negligible at the microgram level. (The same is true for the substance introduced in liquid form if the volume of solvent is constant.)

From the GC–MS studies, the products of pyrolysis, on which quantitative measurement by Py–GC was based, were identified as isobutanal, acetaldehyde and acetic acid for vitamins B_5, B_6 and C, respectively. Confirmation was obtained by checking their retention times in the Py–GC apparatus. However, from the pyrograms for vitamin C it is evident that the acetic acid peak contains a second, only partially resolved, still unknown, component. Fortunately its presence is not deleterious to the determination of the vitamin using the peak height.

The experiments conducted with standard solutions provide a convenient means of finding the optimum conditions for pyrolysis of a specified compound so as to maximise the yield of a well-resolved pyrolysis product in the gas chromatograph. This is done by studying the change in yield as the furnace temperature is altered by means of the Variac controller. Carrier gas flow-rate, column packing and oven temperature are all amenable to convenient study by this method. Optimum operating conditions thus found for the determination of each vitamin are set out in Table I. Furthermore the range of weights of substance to be determined can readily be delineated and the relation between peak height or area and amount established. In this way the data collected in Tables II–IV were obtained for the three vitamins. A good rectilinear relation exists between peak height and amount of each vitamin, and the standard deviations indicate good reproducibility for replicate determinations.

Results of experiments in which the vitamins have been pyrolysed on thin-layer substrate are collected in Tables V–VII. It is seen that they are not significantly influenced by the history of the vitamin on the thin-layer substrate. The determi-

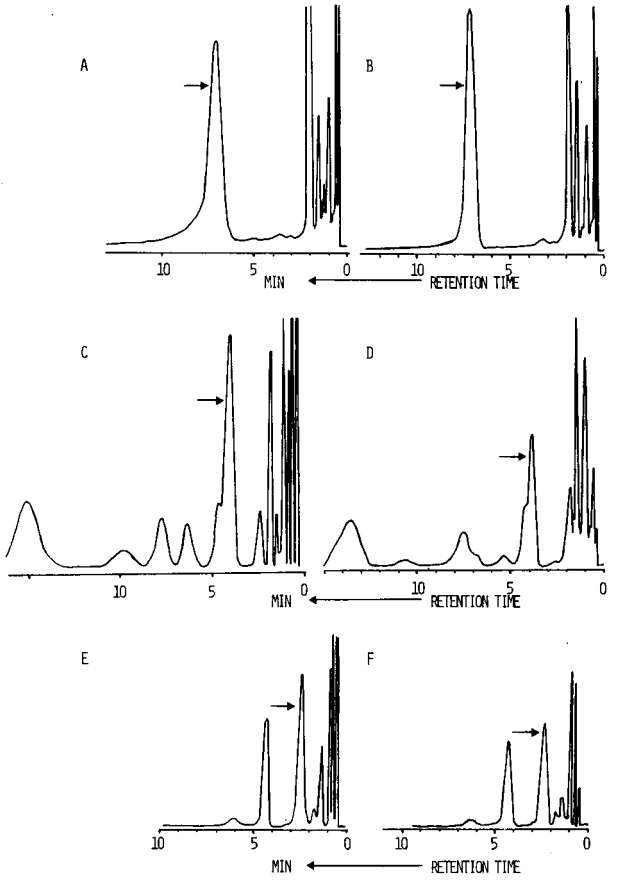

Fig. 1. Pyrograms for samples of calcium D-pantothenate (A and B), L-ascorbic acid (C and D) and pyridoxine hydrochloride (E and F). A, C and E were obtained for samples introduced in aqueous solution; B, D and F for samples adsorbed on thin-layer substrate. The peaks marked by arrows represent those used for measurement purposes.

nations carried out on developed chromatograms relate to mixtures of the vitamins separated by TLC before pyrolysis. (The mean R_F values for vitamins B_5, B_6 and C were 0.55, 0.11 and 0.35, respectively, for solvent travel of 10 cm.) In these experi-

TABLE I

CONDITIONS AND DATA RELATING TO THE DETERMINATION OF THE VITAMINS BY Py–GC

Vitamin	*Optimum pyrolysis temp. (°C)*	*GC oven temp. (°C)*	*Product* determined*	*GC retention time (min)*
B_5	580	150	Isobutanal	7.2
B_6	580	180	Acetaldehyde	2.1
C	530	150	Acetic acid	3.9

* Pyrolysis product on which the determination of the vitamin is based.

TABLE II

PYROLYSIS OF CALCIUM D-PANTOTHENATE USING THE DIRECT INTRODUCTION PROCEDURE FOR SOLUTIONS

For operating conditions see Table I.

Sample weight (μg)	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	16	0.6	3
0.4	29	0.5	8
0.6	46	1.2	7
0.8	59	0.8	6
1.0	74	0.9	8
1.4	102	2.1	6
1.8	135	0.6	3
2.0	148	1.4	8
2.5	183	1.0	3
3.0	223	0.6	3

ments the vitamins were located on the chromatogram using a revealing agent, and in each instance it can be concluded that the latter did not affect the yield of the pyrolysis product that was made the basis of the determination. The standard deviations obtained from replicate determinations indicate that good reproducibility can be expected over the sample ranges investigated. The day-to-day reproducibility depends on careful control of all operating temperatures and gas flow-rates including, of course, those to the flame ionisation detector (FID), and it can be checked by injection of a standard mixture such as methanol–isopropanol into the furnace[3]. The main sources of experimental error then relate to sample handling and volumetric transfer of solutions to the thin-layer plates.

Introduction of a sample on a solid support into the furnace takes *ca.* 30 sec and *ca.* 2.5 min is needed to restore the baseline of the detector output signal; hence

TABLE III

PYROLYSIS OF PYRIDOXINE HYDROCHLORIDE BY INJECTING STANDARD AQUEOUS SOLUTION INTO THE FURNACE

For operating conditions see Table I.

Sample weight (μg)	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	25	0.0	3
0.4	51	0.3	3
0.6	72	0.3	3
0.8	100	0.6	3
1.0	128	2.6	8
1.5	172	1.7	8
2.0	236	2.6	8
2.5	296	3.4	5
3.0	352	4.1	8

TABLE IV

PYROLYSIS OF VITAMIN C BY INJECTION OF AQUEOUS SOLUTIONS INTO THE FURNACE

For operating conditions see Table I.

Sample weight (μg)	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	16	0.6	6
0.4	33	1.4	8
0.6	47	2.2	8
0.8	64	1.4	10
1.0	82	1.2	10
1.2	96	1.2	3
1.4	113	2.3	3
1.6	129	1.5	3
1.8	144	1.5	3
2.0	160	0.8	5
2.5	201	3.0	3
3.0	241	2.5	3

these operations are not particularly time-consuming. The next sample preferably should not be introduced into the pyrolyser until the least volatile product has passed through the detector, otherwise valuable qualitative information from the profile of pyrolysis products (pyrogram) may be lost. Thus the retention time of the least volatile pyrolysis product will determine the sampling rate, which would be *ca.* 5, 6 and 3 per hour for vitamins B_5, B_6 and C in that order. Sometimes more than one peak in the pyrogram can be made the basis of the determination, *e.g.* for vitamins B_6 and C, and the ratios can be used as a check on the purity of the zone from the thin-layer chromatogram.

The sensitivity of the method is dependent on the limit of detection of the

TABLE V

PYROLYSIS–GAS CHROMATOGRAPHY OF CALCIUM D-PANTOTHENATE ON THIN-LAYER SUBSTRATE WITH AND WITHOUT CHROMATOGRAPHIC DEVELOPMENT

For operating conditions see Table I.

Sample weight (μg)	*Without development*			*With development*		
	Mean peak height (mm)	*Standard deviation (mm)*	*Number of measurements*	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	25	1.0	3	26	1.5	3
0.5	69	1.2	4	69	1.2	3
0.8	110	2.5	5	113	2.6	3
1.0	138	1.5	3	139	1.5	3
1.3	181	4.6	4	180	5.5	3
1.7	236	5.6	3	238	4.9	3
2.0	275	5.5	3	275	4.0	3

TABLE VI

PYROLYSIS–GAS CHROMATOGRAPHY OF PYRIDOXINE HYDROCHLORIDE ON THIN-LAYER SUBSTRATE WITH AND WITHOUT CHROMATOGRAPHIC DEVELOPMENT

For operating conditions see Table I.

Sample weight (μg)	*Without development*			*With development*		
	Mean peak height (mm)	*Standard deviation (mm)*	*Number of measurements*	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	27	0.6	3	28	0.6	3
0.4	46	1.0	3	45	1.5	3
0.6	66	1.0	4	66	0.8	4
0.8	90	1.5	3	90	1.7	3
1.0	115	1.3	7	114	2.0	3
1.5	177	2.5	4	177	3.1	3
2.0	230	2.2	7	230	3.1	3
2.5	289	3.5	5	288	3.5	3
3.0	341	3.4	4	340	4.5	3

separated constituent of a mixture on the thin-layer chromatogram; this, in turn, depends on the chemical revealing agents or physical methods available. The preferred method will generally be a physical one such as fluorescence in UV light. A chemical revealing agent must be such that it will not undergo pyrolysis to give products which would obscure those from the substance to be determined.

The technique described here may be compared with long-standing techniques for quantitative TLC based on elution or *in situ* densitometric scanning procedures. Problems associated with reproducible, preferably quantitative, elution of the zone from the chromatogram in a state of purity are avoided and unlike densitometry, the surface uniformity and grain density of the thin-layer are unimportant[4]. Further-

TABLE VII

PYROLYSIS–GAS CHROMATOGRAPHY OF VITAMIN C ON THE THIN-LAYER SUBSTRATE WITH AND WITHOUT CHROMATOGRAPHIC DEVELOPMENT

For operating conditions see Table I.

Sample weight (μg)	*Without development*			*With development*		
	Mean peak height (mm)	*Standard deviation (mm)*	*Number of measurements*	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.1	15	0.5	4	13	1.5	3
0.3	40	1.0	4	40	0.6	3
0.5	63	2.1	6	64	1.0	3
0.8	106	0.8	4	106	1.1	3
1.0	127	2.2	4	128	1.5	3
1.4	179	3.1	4	179	3.6	3
2.0	260	3.4	6	260	5.0	3

more, a single measurement can give a result of good accuracy whereas replicate determinations are needed to give mean values of comparable reliability in applying methods based on elution or densitometry. This can be ascribed, at least in part, to the mode of measurement which is based on the total constituent and not just a fraction of it as in reflective densitometry and in some elution methods. Two general points can be made in comparison with the Iatroscan instrument[5,6] in which TLC is carried out on adsorbent-coated thin silica rods and the developed rods are passed through a hydrogen flame to produce gaseous decomposition products which are determined by the FID principle. The rods have a very low loading capacity so that sample loading can present problems not experienced in operating the technique described here. Furthermore, unlike the present technique, the detector is non-discriminating with respect to the pyrolysed substance relying entirely on TLC for discrimination.

Apart from restrictions on location of zones on developed chromatograms, the main additional limitations to general application of the technique described here relate to materials for TLC and to the need to generate at least one volatile pyrolysis product. Backing plates, stationary phases and binding agents for TLC made from organic substances obviously cannot be used. Materials for backing plates are further restricted to those readily cut with minimum disturbance to the supported thin layer. However, thin-layer material could be removed mechanically, but less conveniently, from a glass backing to a spoon device for insertion into the furnace. Lack of suitable volatile decomposition products produced by pyrolysis might be overcome by supplementary use of suitable chemical reagents, *e.g.* oxidising agents, as an aid to break-up of stable molecules but, as yet, this possibility remains unexplored.

The proposed technique uses a furnace of simple construction to interface TLC and GC so that a quantitative analysis can be performed on mixtures containing involatile constituents for which either GC or Py–GC alone would be insufficient. Its application is illustrated by the separation and determination of vitamins B_5, B_6 and C and it follows that it should be applicable also to the determination of one or all of these substances in any matrix provided each can be separated[7] from other constituents of the sample by TLC.

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AUTOMATED QUARTZ INJECTOR/TRAP FOR FUSED-SILICA CAPILLARY COLUMNS

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SUMMARY

An automated quartz injector/trap was developed for a Perkin-Elmer 3920 gas chromatograph. The injector/trap serves to concentrate gaseous or liquid samples without introducing organic contaminants. The concentrated sample is volatilized and transferred to a second trap made by cooling a small section of a fused-silica capillary column. Reconcentration in the capillary column preserves band-shape and provides a system which delivers quantitative results on a variety of samples. Liquid samples show relative standard deviations of 1 % and 2 %, respectively, for isothermal and temperature-programmed analyses. A detection limit of less than 1 ppb is expected, with a flame ionization detector, for samples of *n*-octane in air.

INTRODUCTION

The growing interest in the monitoring of air pollution, in the diagnosis of disease states, and in the characterization of aromas and odors, demands rapid and accurate measurement of organic compounds at parts-per-billion (ppb*) levels. The measurement of concentrations at the ppb level can be performed with gas chromatographs equipped with flame ionization detectors (FIDs). However, because of limitations in FID sensitivity, the analysis requires injection of several milliliters of a gaseous sample or several microliters of a liquid sample into a column.

The introduction of these large volumes into a capillary column requires special techniques to preserve the efficiency of the column: gaseous samples are concentrated in traps[1–6], while liquid samples are concentrated via the solvent effect[7] or by means of extracolumnar devices[8].

Concentration techniques are frequently plagued by artifacts caused by the surfaces contacting the samples. Traps packed with solid materials may retain compounds strongly enough to introduce large quantitation errors, or in extreme cases, cause the total loss of some compounds. Another problem associated with concentration techniques is the introduction of significant levels of contaminants, along with

* Throughout this article, the American billion (10^9) is meant. Concentrations of gaseous samples are expressed as v/v.

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the sample, into the analytical system. This problem is particularly important when measuring compounds present at ppb levels and below.

Inert traps for gaseous samples[9,10] and injectors for liquids[11,12] have been reported; however, these devices are designed to handle only one type of sample: either gaseous or liquid. Rijks *et al.*[13] reported a capillary trap for condensing material contained in gaseous or liquid samples. The authors reported analyses on 1-ml and 0.2-μl volumes of gaseous or liquid samples directly charged into the trap by means of syringes, but long waiting times were necessary to allow the unwanted solvent to pass through the column. The authors suggested the use of an appropriate exhaust between the the trap and column to minimize this drawback.

In this report we describe an automated injector/trap for fused-silica capillary columns. The system is designed to perform rapid, artifact-free, quantitative measurements of nanogram and subnanogram amounts of materials contained in large volumes of either gaseous or liquid samples.

EXPERIMENTAL

Modified gas chromatograph

A block diagram of this unit is shown in Fig. 1. A Perkin-Elmer 3920 with dual FIDs was modifed by the installation of a quartz injector/trap, a heated four-port valve and actuator, and a second trap for the concentration of materials at the head

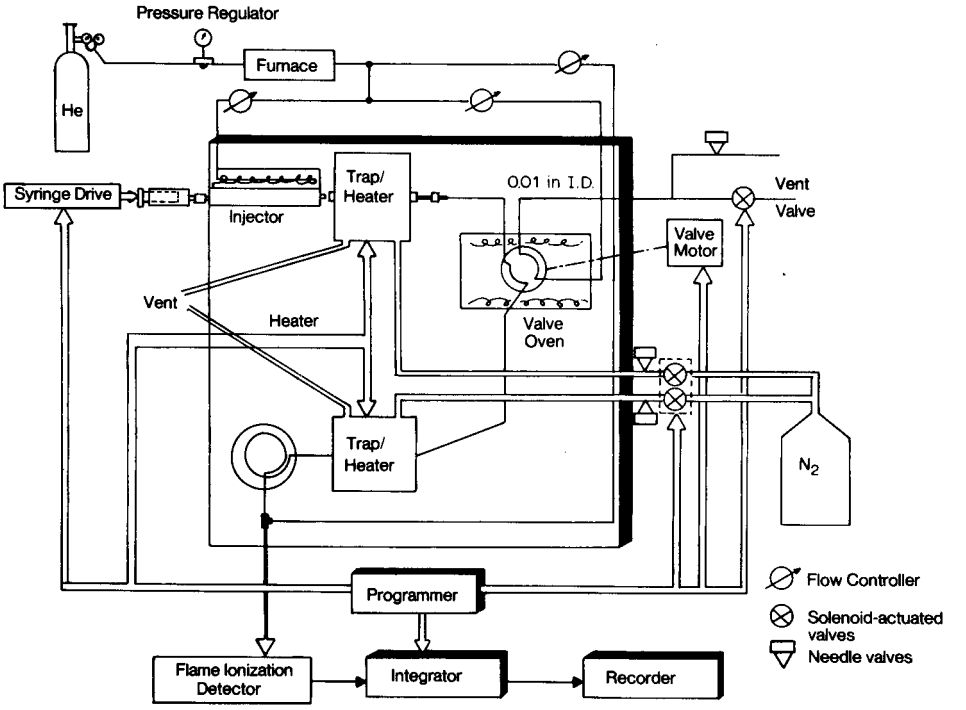

Fig. 1. Functional diagram of a gas chromatograph equipped with injector/trap system. Four-port valve is shown in the "inject" position.

of the column. The unit was also equipped with "ultra clean" grade flow controllers (Model 8744, Brooks, Hatfield, PA, U.S.A.), and a double pattern metering valve (Nupro, Willoughby, OH, U.S.A.) to match pressures when the four-port valve is switched. Organic materials present in the helium carrier gas were converted into carbon dioxide and water by flowing the gas over copper (II) oxide heated to 700°C by means of an electric furnace (Lindberg, Watertown, WI, U.S.A.).

The quartz injector/trap (first trap) is shown in Fig. 2. This unit was made by wrapping 24 loops of 2.68 Ω/ft. glass-insulated nichrome wire (Pelican Wire, Naples, FL, U.S.A.) around a 6-cm section of a quartz tube (part D) 28.5 cm long (Scientific Quartz, Fairport Harbor, OH, U.S.A.). This tube is 3 mm O.D. × 1 mm I.D. The wrapped section corresponds to the trapping section of the injector and is packed with quartz fibers. The trapping section is enclosed in a PTFE jacket (C) which serves to contain the liquid nitrogen used for cooling the trap. The trapping temperature is monitored and controlled via an iron-constantan thermocouple (Thermoelectric Co., Saddle Brook, NJ, U.S.A.) attached externally at the midpoint of the trap. The PTFE jacket is connected to the injection port (A) by means of a brass barrel (B) fitted at both ends with Swagelok nuts. The injection port was made by drilling a 1/16-in. hole on the side of a reducing union (1/8 to 1/16 in.) followed by insertion of a 1/16 in. O.D. piece of tubing. After soldering, the tube connects the injection port to the carrier gas source.

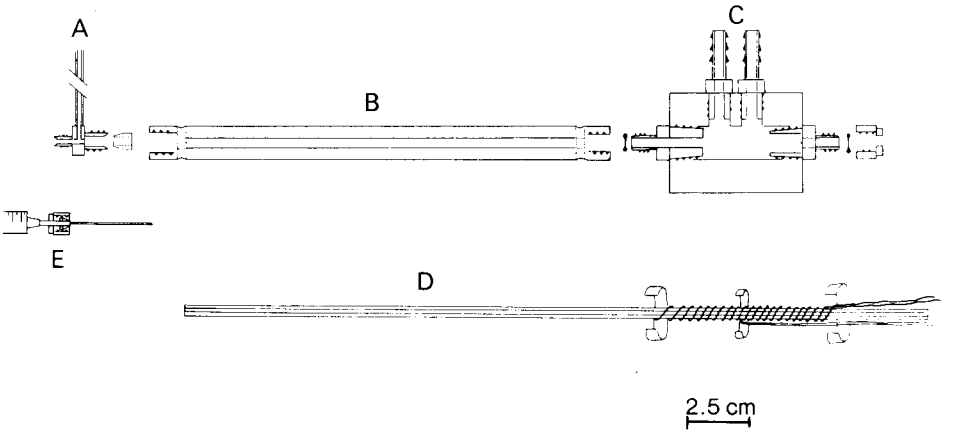

Fig. 2. Diagram of injector/trap for a Perkin-Elmer 3920 gas chromatograph. A = Modified injection port; B = brass barrel; C = PTFE jacket; D = quartz tube with thermocouple and heating wire; E = adapted syringe.

The injection port is sealed by a Vespel ferrule (Altech, Arlington Heights, IL, U.S.A.). The samples are introduced by means of gas-tight syringes equipped with a 1.5-cm section of 1/16 in. stainless-steel tubing and a Swagelok nut (E). This fitting mates with the body of the reducing union used as the injection port. Gaseous samples of less than 1 ml are injected by hand. Larger volumes are introduced by means of an infusion pump (Sage Instruments, Cambridge, MA, U.S.A.) mounted so that the syringe needle is aligned with the injection port. The infusion rate is 10 ml/min or less.

A heated four-port valve (Carle Instrument, Fullerton, CA, U.S.A.) serves to vent unwanted solvents or to transfer the contents of the trap to the column. The valve is mounted inside the column oven and is switched by a motorized actuator

mounted externally to the chromatograph (Fig. 1). An auxilliary heater is necessary for quantitative transfer of some materials. The heater was made by wrapping nichrome wire around a three-section piece of insulated aluminium sheet and mounting this unit so that it fits around the valve. The surface temperature of the valve was monitored by a thermocouple attached to the valve body.

Materials transferred through the valve are reconcentrated in the fused-silica column by making the column part of a second cold trap (Fig. 1). This trap is similar to the trap described above except for the replacement of the quartz tube by an insulated 1/16 in. O.D. × 0.02 in. I.D. stainless-steel tube *ca.* 15 cm long. The column is passed through the tube prior to connection to the valve. A 25 m × 0.2 mm I.D. SP-2100 fused-silica column (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used throughout this work.

The system was operated under a constant pressure of 20 p.s.i. The column and injector pressures were adjusted to 15 p.s.i. by means of the needle valves built into the flow controllers. The controllers only served as variable restrictors because the system flow-rates (less than 1 ml/min) are below their operating range. The column back-pressure on the injector/trap was simulated by adjusting the double pattern needle valve connected at the end of a 1 m × 0.01 in. I.D. tubing.

The chromatograms were integrated by a Varian CDS 111 integrator and displayed by a Perkin-Elmer 056 recorder.

Programmer

All the necessary operations are controlled by a programmer built around an Intel 8010 single board computer. Communication between the programmer and operator is by a keyboard and an alphanumeric display, while the control of the instrumentation is done with opto-isolated relays. The relays are switched according to a time sequence determined by the operator. Four relays can be driven from analogue comparators used to sense the cooling and heating cycles of the traps. These temperatures are set by potentiometers accessible to the operator.

The software is written in PL/M and is designed to perform the interactions with the operator in the background while carrying the systems operations in the foreground. Therefore, timing and controlling functions have the highest priority and yet the computer is free, most of the time, to allow the operator to monitor the status of the experiment or to change parameters during the run.

System operation

The operations performed by the automated system are: trapping of materials of interest in the quartz injector/trap and venting of the solvent, desorption and transfer of materials from the quartz trap to the column, reconcentration in the column followed by desorption and elution. These operations are shown in Fig. 3. The analysis begins by injecting a sample while the four-port valve is in the "load" position. In this position the injector/trap is connected to the atmosphere via the 0.01-in. I.D. tubing and vent valve. The materials of interest are retained in the trap section of the injector while most of the solvent is vented, the solvent injected being either gaseous or liquid.

The trapping of liquids uses the solvent effect to concentrate materials in the trap: the temperature of the trap is controlled a few degrees below the boiling point of

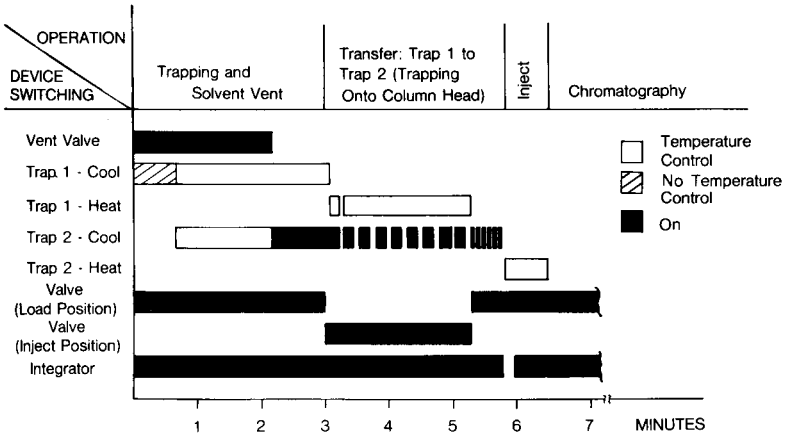

Fig. 3. Typical program for the injection of gaseous and liquid samples.

the solvent. Gases are trapped in a similar fashion except that the first trap temperature is low enough to prevent losses of any material of interest. This temperature depends on the mass of quartz fibers used to pack the first trap, the smaller masses require lower temperatures for quantitative trapping. Throughout this work the trap was packed with a 2.5-cm long bundle of fibers weighing *ca.* 5 mg. When the trapping is complete (Fig. 3), the valve is switched from the "load" to the "inject" position and the trap heated to 270°C in *ca.* 9 sec. The temperature is allowed to drop to 190°C and is controlled at this temperature for the remaining time required to transfer materials from the first trap to the column. The transfer time can be programmed by the operator to accommodate materials that may adsorb to the internal parts of the valve. The transfer was optimized for some compounds by the use of the valve heater. For example, both band-shape and recovery of *n*-hexadecane improved when the valve was heated to 210°C during transfer.

The materials transferred to the head of the column (second trap) are reconcentrated by keeping a small section of column at −150°C or below by flowing liquid nitrogen into the PTFE jacket. The materials are desorbed by heating the stainless steel tubing encasing the column to 300°C in 10 sec. This step marks the beginning of the gas chromatographic run. To insure complete desorption, the programmer keeps the temperature of the tube at 220°C for an additional 30 sec.

RESULTS AND DISCUSSION

The collection efficiency of the quartz injector/trap (first trap) depends on trap temperature, mass and type of packing material, and linear velocity of the sample as it travels through the trap. The desorption depends primarily on the first two factors mentioned.

The internal temperature of the trap was measured by a thermocouple placed inside the quartz tube. This thermocouple was positioned opposite to the external thermocouple used to monitor and control the trap temperature. The instantaneous temperature recorded by the thermocouples agree to ±10°C over the range −100 to

200°C. Therefore, the trapping and desorption temperatures measured by the external thermocouple are a good indication of the actual conditions experienced by the sample.

The conditions required to collect and desorb materials contained in the column (second trap) were established empirically because of the difficulty in measuring the actual temperature inside the column.

The temperature of the first trap and its heating profile with respect to time were measured with packings of quartz fibers ranging in mass from 23 to 5 mg. The lower mass was selected because of the favorable desorption profiles measured with highly surfacephilic materials (free carboxylic acids and alcohols). However, the low mass limits the linear velocity through the trap to 30 cm/sec for maximum collection efficiency. Consequently, the maximum infusion rate for a gaseous sample is 10 ml/min.

Although the cooling and heating of the traps are reproducible, cooling produces unavoidable changes in oven temperature. These changes, and the changes in pressure due to valve switching, combine to produce variations in the retention time: the range of standard deviations is 0.03–0.05 and 0.06–0.12 min for isothermal and temperature-programmed runs, respectively. However, the retention times are independent of the solvent used. We observed no differences in the retention times of hydrocarbon mixtures in *n*-pentane, isopentane, dichloromethane or air. An exception is the injection of gaseous samples exceeding 10 ml and having high relative humidity: the retention time of materials present in these samples decreased and the standard deviations increased compared to values obtained by injecting smaller volumes. This behavior is caused by the formation of an ice plug in the capillary column which obstructs the gas flow and increases the back-pressure when the column is cooled to −150°C. When the column is heated to start the chromatography, the ice plug is vaporized. This results in an increase in the linear velocity of the carrier gas and a reduction of retention times. An air volume of 20 ml, at 50 % relative humidity, is the largest practical sample volume for the 0.2-mm I.D. column when the first trap temperature is below 0°C.

The analysis of gaseous samples was performed by keeping the first trap at −100°C. The experimentally determined composition of four dilutions of an *n*-hydrocarbon mixture (C_6, C_7 and C_8) in air agrees to ±10 % relative to the nominal composition of the mixture for injections containing a total mass of hydrocarbons ranging from 210 to 1.2 ng. The results are shown in Table I. A chromatogram of the 1.2-ng sample is shown in Fig. 4, where the masses of *n*-hydrocarbons (C_6, C_7 and C_8) injected are 400 pg per compound. These masses correspond to a concentration of 5 ppb per compound when a 20-ml sample is injected. The signal-to-noise ratio of the *n*-octane peak is better than 20. Therefore, the estimated detection limit for this compound is *ca.* 40 pg or 0.5 ppb. Residual contaminants in the syringe or air are apparent in the chromatogram. These contaminants increased the detection limit of the *n*-hexane and *n*-heptane and are responsible for the results reported in Table I for the 0.7-ng sample (*ca.* 250 pg of mass per compound).

The quantitative analysis of liquid samples of hydrocarbons is shown in Table II. The relative standard deviation is 1 % when the analysis is performed under isothermal conditions and increases to *ca.* 2 % for temperature-programmed runs. These results were obtained while injecting liquid samples ranging in volume from less than

TABLE I

DISTRIBUTION OF *n*-HYDROCARBON (C_6, C_7 AND C_8) STANDARDS IN AIR SAMPLES

Volume taken (ml)	*Total mass injected (ng)*	*Composition (% found)*		
		C_6	C_7	C_8
0.1	210	31.7 ± 0.6	35.5 ± 0.2	32.9 ± 0.8
1.0	11.6	32.1 ± 0.4	35.2 ± 0.0	32.8 ± 0.4
10.0	0.7	37	35	29
20.0	1.2	29	37	34
Nominal composition (%)		32	34	34

1 to *ca.* 4 μl. The peak area increased linearly with volume injected as shown in Fig. 5. The relative deviation of an individual value from the "best fit" value is less than 10%. The smallest volume injected is an exception. The larger relative error is probably caused by the reading error of the syringe. A typical chromatogram is shown in Fig. 6.

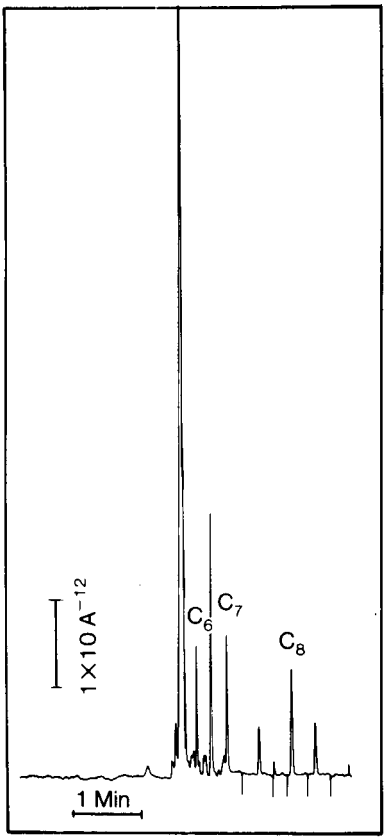

Fig. 4. Chromatogram of *n*-hydrocarbons (C_6, C_7 and C_8) in a 20-ml air sample. The amounts injected (400 pg) correspond to concentrations of *ca.* 5 ppb per compound.

TABLE II

REPRODUCIBILITY OF HYDROCARBON ANALYSES UNDER ISOTHERMAL AND TEMPERATURE-PROGRAMMED CONDITIONS

A: Isothermal analysis: 170°C, injection port 200°C. B: Temperature-programmed analysis: 140°C isothermal hold 4 min, 8°C/min to 190°C, final hold 2 min. Injection port 200°C.

Conditions	*Injected volume (μl)*	*Composition (% found)*			
		C_{10}	C_{12}	C_{14}	C_{16}
A	1.6		25.0	34.0	41.0
	0.5		24.9	34.7	40.4
	0.8		25.1	33.7	41.2
	0.3		25.1	34.5	40.4
	1.1		24.9	34.1	41.0
Mean			25.0	34.2	40.7
Relative standard deviation (%)			0.4	1.3	1.0
Nominal composition (%)			26	34	40
Sample concentration		25 ng total mass of standards per microliter of pentane			
B	3.8	14.8	22.8	29.0	33.5
	2.5	14.8	22.7	29.1	33.5
	0.5	14.8	21.9	28.9	34.4
	1.0	14.2	22.4	29.3	34.1
	1.2	14.5	22.4	29.3	33.7
	0.8	14.5	21.3	29.0	35.3
	0.7	14.7	21.9	29.0	34.4
	1.3	14.0	22.5	29.1	34.3
Mean		14.5	22.2	29.1	34.1
Relative standard deviation (%)		2.0	2.2	0.5	1.7
Composition by independent GC		14.7	21.3	29.3	34.7
Nominal composition (%)		17	22	28	33
Sample concentration		30 ng total mass of standards per microliter of pentane			

The remaining solvent, pentane, does not interfere with the *n*-decane peak since the boiling point difference between these two compounds is *ca.* 140°C. We have successfully performed analyses of *n*-C_8 in pentane solutions where the boiling point difference is 90°C. Compounds having even smaller boiling point differences with the solvent could be analyzed by improving the temperature control of the injector/trap.

All liquid samples were injected by the use of a solvent flush technique where the sample was followed by an air segment and a small amount of solvent. The volume of solvent is comparable to the needle volume, and suffices to displace the sample from the needle. Low recoveries were obtained when a hot needle injection, akin to that described by Grob and Neukom[14], was used to introduce the sample. The syringe needle was equilibrated in the injection port for over 30 sec prior to the injection of a sample. Except for the 5-mm section of needle joining the syringe barrel and the Swagelok adapter, the lowest needle temperature is 120°C. This temperature was measured at the point where the adapter and injector mate, the rest of the needle

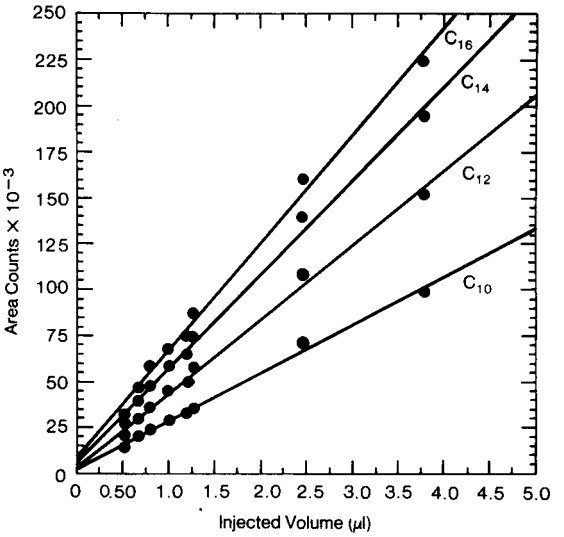

Fig. 5. Dependence of integrated area on volume of hydrocarbon standard injected.

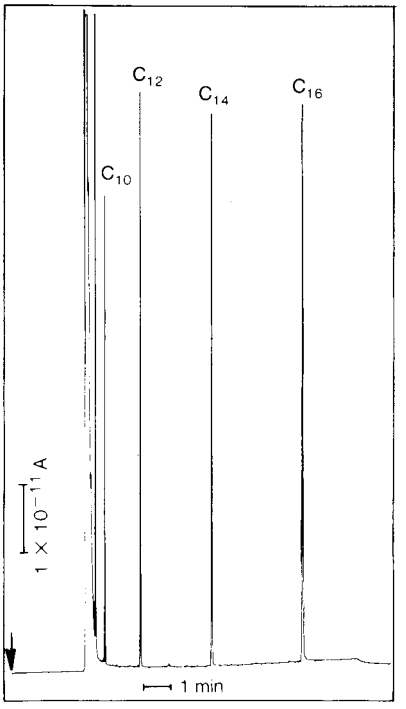

Fig. 6. Temperature-programmed analysis of *n*-hydrocarbons. Temperature program: 140°C isothermal hold 4 min, 8°C/min to 190°C, final hold 2 min. Sample volume, 3.8 μl.

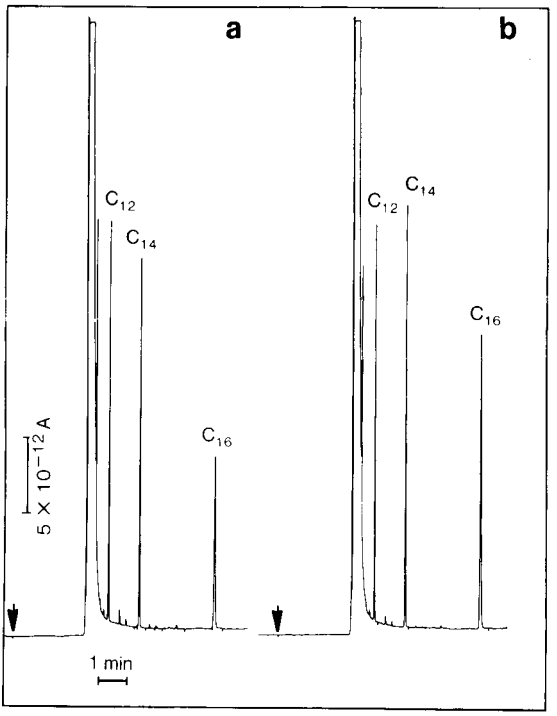

Fig. 7. Influence of injection method on quantitation. Injector temperature, 200°C. Isothermal run (170°C): a, hot needle injection; b, solvent flush injection. Injection of 1-μl sample containing a total C_{12}–C_{16} mass of 25 ng.

(*ca.* 3 cm) is at the injector temperature (200°C). Discrimination of *n*-hexadecane (b.p. 288°C) is apparent in the chromatogram shown in Fig. 7, and the chromatogram suggests a slight loss of *n*-tetradecane. The extent of the losses are shown in Table III. The area ratio of $C_{12}/(C_{12} + C_{14})$ is higher for the hot needle injection, indicating a small loss of C_{14}. However, the loss is small enough to preclude a significant change in the standard deviation of this ratio. The standard deviation for the ratio $C_{12}/(C_{12} +$

TABLE III

EFFECT OF INJECTION METHOD ON QUANTITATION OF A HYDROCARBON MIXTURE

Injector temperature, 200°C, isothermal run, 170°C.

Injection method	*Composition (% found)*			*Area ratio*	
	C_{12}	C_{14}	C_{16}	$\frac{C_{12}}{C_{12} + C_{14}}$	$\frac{C_{12}}{C_{12} + C_{16}}$
Solvent flush	25.0	34.2	40.8	0.422 ± 0.005	0.380 ± 0.005
Hot needle	30.1	37.2	32.6	0.448 ± 0.005	0.48 ± 0.03
Nominal composition	26	34	40	0.43	0.39

C_{16}) is greater for the hot needle injection compared to the solvent flush injection and the ratio is higher than expected, corresponding to losses of C_{16} averaging 35%.

The injector/trap fused-silica column system presented here has been applied to the quantitation of known materials present in headspace samples. Equipment calibration is conveniently done by the injection of standards dissolved in liquid solvents. Demonstration of inertness and applicability to the analysis of different classes of compounds will be the subject of subsequent communications.

ACKNOWLEDGEMENTS

We thank G. J. Fischesser and F. B. Schroeder for the construction of trap prototypes and T. P. Franz for the construction of the programmer. We also thank R. J. Lloyd for valuable suggestions and discussions.

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FACTORS INFLUENCING THE RETENTION OF INSULINS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS

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SUMMARY

The effects of the concentrations of methanol, buffer and neutral salt, of pH and temperature, as well as of the type, chain length and concentration of ion-pair reagents added to aqueous phosphate buffer–methanol eluents upon the retention of bovine and porcine insulins have been investigated in detail. A possible explanation for the retention-influencing effects of both the cationic and anionic ion-pair reagents is proposed.

INTRODUCTION

Traditionally, gel chromatography has been used for the analysis of proteins, and large poly- and oligopeptides including insulins, *e.g.*, refs. 1–4. Chemically bonded controlled porosity glass[5], deactivated silica, etc., have also been tried with various degrees of success[6]. These techniques, at best, allow the separation of insulins from other much larger or much smaller polypeptides, but not the separation of insulins of various species, and of related compounds. Since then, reversed-phase (RP) and ion-pair high-performance liquid chromatography (HPLC) have been introduced for the analysis of proteins and polypeptides (for a comprehensive review see, *e.g.*, ref. 7). Most RP separations of insulins published so far are based on one of two principles: (a) control of the degree of ionization of the insulins; (b) use of ion pair reagents.

In the first group the extent of retention is controlled by altering the degree of ionization of the insulin molecule. It was shown[8,9] that at pH < 2 all terminal and side-chain carboxyl groups of insulins are protonated, along with the terminal and side-chain NH groups. Between pH 2 and 4 only the side-chain carboxyl and the terminal and side-chain NH groups are protonated. Above pH 4 even the side-chain carboxyl groups become dissociated (the isoelectric point of insulins is at 5.3). Therefore, adjustment of the eluent pH allows control of the ionization of the insulin molecule, *i.e.*, its actual hydrophobicity. This is the basis of the separation schemes described in refs. 10–15. Hancock and co-workers[10–13] postulated that insulins formed hydrophilic ion pairs with $H_2PO_4^-$ present in the eluent. Similar separations were obtained with sulphuric acid[14], hydrochloric acid[15] and perchloric acid[16] even though

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they are generally not considered strong hydrophilic ion-pairing agents. Low pH and high salinity were mandatory in each case for good peak shape and reproducible retention. Acetate and formate anions were not as effective. The organic solvent added to the eluent also had a profound effect upon the capacity factor (k') of insulin: k' values increased in the somewhat unexpected order acetonitrile, tetrahydrofuran, dioxan and methanol.

Dinner and Lorenz[14] described an elegant and simple isocratic method for the analysis of bovine and porcine insulins, and their monoarginine and monodesamido derivatives.

In the second general method, insulins are separated on a hydrophobic packing (alkyl silicas) by a buffered hydroorganic eluent containing either cationic[16–18] or anionic[11,19] ion pair reagents. Rivier[17] used a trialkylamine–phosphoric acid buffer system in acetonitrile, Biemond *et al.*[16] added a tetramethylammonium hydroxide–phosphoric acid buffer to methanol and Damgaard and Markussen[18] used an ethanolamine–phosphoric acid system. It was claimed that the small tetraalkylammonium ion could block the residual OH groups of the RP packing and also possibly act as an ion-pairing agent interacting with the carboxylate groups of the insulins. Identical retentions were obtained with a pH 2 phosphate eluent without and with tetramethylammonium ions. However, in the last case the peak shape was better[16].

Terabe *et al.*[19] added butanesulphonate anions to pH 3.0 tartrate buffer–acetonitrile as eluent. Again, the peak shape of the insulins was better in the presence, rather than in the absence, of the butanesulphonate anion. Changes in the eluent pH had only a slight effect upon the k' values. It was assumed that the RSO_3^- anions blocked the residual OH groups of the RP packing, and also possibly formed ion pairs.

Apparently, acceptable separations can be obtained with both cationic and anionic ion pair reagents. However, to our knowledge, there are no quantitative data relating to the effects of the type and concentration of the ion pair reagent, the ionic strength, pH, temperature and methanol concentration of the eluent upon the retention of insulins. The aim of the work reported here was the procurement of such data, and the explanation of the somewhat unexpected effects of the cationic and anionic ion pair reagents.

EXPERIMENTAL

Experiments were carried out on a Varian LC 5020 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with an LC55 variable-wavelength detector (Perkin-Elmer, Norwalk, CT, U.S.A.) set to 220 nm, and custom-made stainless-steel columns (250 × 4.0 mm I.D.) packed with 10-μm RP-18 silica (E. Merck, Darmstadt, G.F.R.). The columns were jacketed and thermostatted[20] by a circulating U10 water-bath (MLW, Medingen, G.D.R.).

In preliminary experiments it was found that the k' values of insulins were extremely sensitive to the methanol concentration of the eluents. Therefore, great care was taken in eluent preparation. The calculated amount of methanol was weighed into a 1000-ml volumetric flask followed by the calculated amount of buffer components, ion-pair reagent and salt used for adjustment of the ionic strength. Then the contents of the flask were brought almost to the mark with double distilled water,

leaving a free space of about 500 μl. The temperature of the flask was adjusted to 25 ± 0.2°C, and the pH of the eluent was measured with a combined glass electrode, calibrated with aqueous buffers, and a precision digital pH meter (Radelkis, Budapest, Hungary). If necessary, (minor) final pH adjustment was achieved by adding a few drops of concentrated phosphoric acid. Then the flask was brought to the mark with distilled water. As an ultimate check on the actual elution strength of the eluent, members of the nitroalkane homologous series, benzene and toluene were also separated and their k' determined, since their retentions are primarily dependent on the methanol content of the eluent. With these precautions, the k' values of insulins were reproducible within 2% relative.

The ion-pair reagents were obtained from BDH (Poole, Great Britain), Fluka (Buchs, Switzerland) and Ferak (West Berlin, G.F.R.). Methanol, phosphoric acid, sodium bromide and sodium dihydrogen phosphate were obtained from Reanal (Budapest, Hungary). The insulin samples tested were from Gedeon Richter (Budapest, Hungary) and NOVO (Copenhagen, Denmark).

RESULTS AND DISCUSSION

Effect of the methanol concentration of the eluent

Fig. 1 shows the k' values of porcine and bovine insulins and the neutral reference compounds as a function of the methanol concentration in a pH 3.2 eluent containing 0.05 mole/l tetramethylammonium hydroxide and 0.1 mole/l phosphoric acid at 30°C. The dead volume of the column was determined by injecting a saturated KI solution as described in ref. 21. A 5% (w/v) increase in the methanol concentration causes a ten-fold decrease in the k' values of insulins, while the k' of "regularly behaving" nitroalkanes and other small molecules changes only by a factor of 1.1–1.3. In the narrow methanol concentration range where insulin separation can be achieved,

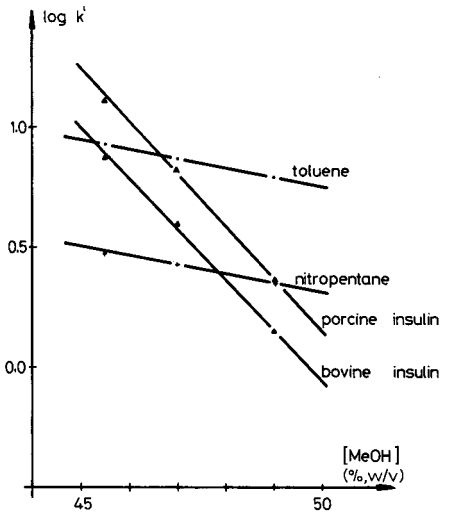


Fig. 1. Dependence of the k' values of bovine and porcine insulins upon the methanol (MeOH) concentration in 0.05 mole/l tetramethylammonium hydroxide, 0.1 mole/l phosphoric acid eluent, pH 3.2, at 30°C.

there is a linear relationship between log k' and the methanol concentration. The slopes obtained for the bovine and porcine insulins and their monodesamido derivatives are identical, -0.22, and differ significantly from those of the nitroalkanes, -0.04.

Similar curves and similar slopes were obtained when the methanol concentration of pH 3.0 0.05 mole/l tetraethylammonium hydroxide–0.1 mole/l phosphoric acid and pH 2.6 0.005 mole/l tetrabutylammonium hydroxide–0.01 mole/l phosphoric acid eluents was changed, -0.19 and -0.21. When heptanesulphonic acid was used as ion pair reagent the slope was -0.25.

Thus, it can be concluded that, independently of the type of ion-pair reagent used, the k' values of insulins are extremely sensitive to the methanol concentration of the eluent. Therefore, the methanol concentration range available for practical insulin separations is very narrow and reproducible separations require extreme care in the preparation of the eluents.

Effect of the salt concentration of the eluent

A number of papers[8–19] have emphasized that high salinity is mandatory for good insulin peak shape. Therefore, eluents which contained 49% (w/v) methanol, 0.05 mole/l NaH_2PO_4, 0.05 mole/l H_3PO_4 and increasing amounts of sodium bromide (0–0.8 mole/l) were prepared.

The k' of insulins is plotted against the sodium bromide concentration in Fig. 2. The increase in k' caused by the 0–0.8 mole/l increase of the sodium bromide concentration is equivalent to that caused by a 1% decrease in the methanol concentration of the eluent. Such a change in methanol concentration would result in an increase the k' of benzene, toluene and the nitroalkanes as described above. However, no such change can be detected, *i.e.*, the increase in the k' of insulins can be attributed to the presence of NaBr.

It was noted that the pH of the eluents decreased about 0.3 units from pH 3.2 when the concentration of NaBr increased from 0 to 0.8 mole/l. This is due to a change in the dissociation of phosphoric acid with the sodium bromide concentration. The apparent first pK values of H_3PO_4 taken from potentiometric titration

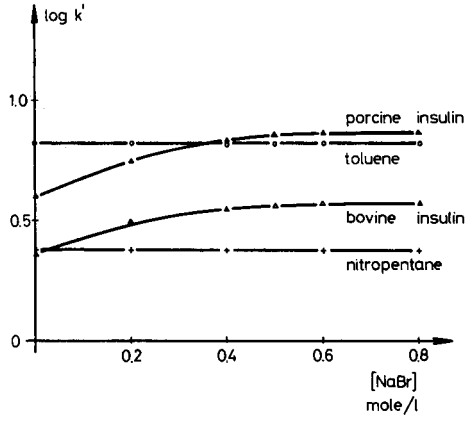


Fig. 2. Dependence of the k' values of bovine and porcine insulins upon the NaBr concentration in a 49% (w/v) methanol, 0.05 mole/l H_3PO_4, 0.05 mole/l NaH_2PO_4 eluent at 30°C.

curves of 0.05 mole/l H_3PO_4 dissolved in the different eluents were: 2.25 in 0.4 mole/l NaBr in water; 2.95, 3.05 and 3.30 in 1.0, 0.5 and 0.1 mole/l NaBr in 49% (w/v) methanol, respectively. It will be shown later that the change in k' caused by a pH change of 0.3 units is negligible compared with the overall change observed.

Effects of the ion pair reagents

Anionic ion pair reagents

Phosphate anion. In preliminary experiments we found that the k' of insulins was sensitive to the concentration of the phosphate buffer. Therefore, the effects of the overall phosphate concentration of the eluent upon the k' of insulins was investigated. The concentration ratio of NaH_2PO_4 and H_3PO_4 was kept at about 1:1, and the overall phosphate concentration was changed from 0.04 to 0.7 mole/l in pH 3.2, 49% (w/v) eluents. The results obtained are shown in Fig. 3.

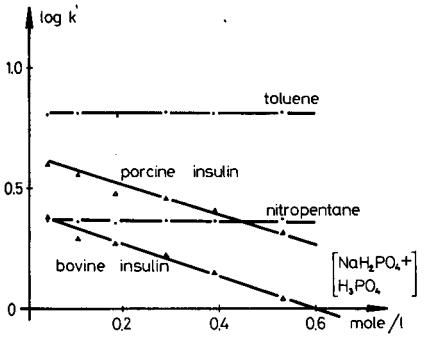


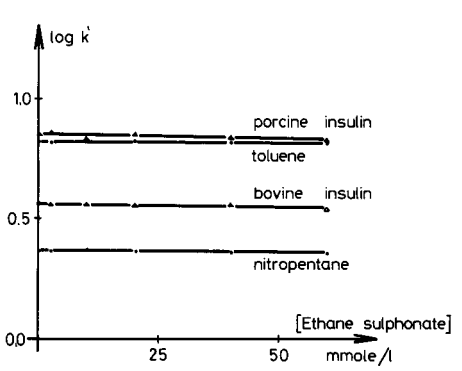
Fig. 3. Influence of the overall phosphate concentration of the eluent upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, pH 3.2, at 30°C.

The log k' of insulins decreases strongly with increasing overall phosphate concentration, slope −0.6. The increase in overall phosphate concentration also means an increase in ionic strength, which, as shown above, results in greater retention of insulins. Thus, the overriding effect is the decrease in retention caused by $H_2PO_4^-$. Since the eluent pH was constant, 3.2, and changes in the methanol concentration could also be ruled out (as indicated by the constant k' values of nitroalkanes, benzene and toluene), the most plausible explanation is that the $H_2PO_4^-$ anion interacts with the positive charges of the insulin molecule and forms hydrophilic ion pairs. The effect of the concentration of $H_2PO_4^-$, about 0.1 log k' units, is much larger than in the case of other ion pair reagents (see below). This means that the rôle of phosphate is dual: it acts as pH-controlling buffer, and also as a retention-controlling ion pair reagent. Therefore, its concentration has to be kept strictly constant for reproducible separations.

Alkanesulphonic acids. As mentioned in the Introduction, butane-, hexane- and heptanesulphonic acids have been used in various eluents, but no data relating to the effects of chain length and concentration of the ion-pair reagent are available. Therefore, a water-soluble alkanesulphonic acid, ethanesulphonic acid, and a much less

soluble acid, heptanesulphonic acid, were selected and their concentration effects were studied.

The pH 3.1 eluents contained 49% (w/v) methanol, 0.5 mole/l NaBr–0.05 mole/l phosphate buffer (1:1) and various amounts of ethanesulphonic acid and heptanesulphonic acid. The results are shown in Figs. 4 and 5. It is seen that the k' values of the neutral components are practically constant (or decrease very slightly) over the reagent concentration range tested. The retention of insulins is practically constant with ethanesulphonic acid as ion pair reagent. With the more hydrophobic heptanesulphonic acid, the k' of insulins first increases, then begins to level off. Unfortunately, the *n*-heptanesulphonic acid concentration could not be increased past the point indicated, because our supply ran out.



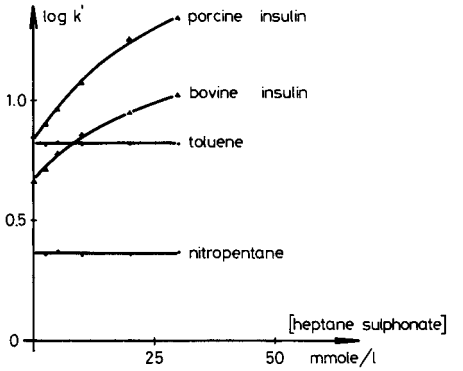


Fig. 4. Influence of the ethanesulphonate concentration of the eluent upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1, at 30°C.

Fig. 5. Influence of the heptanesulphonate concentration of the eluent upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1, at 30°C.

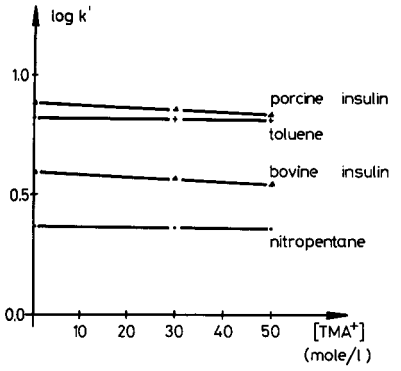
In contrast to the effect of the similarly negatively charged $H_2PO_4^-$ anion, the ethanesulphonate anion apparently does not influence k', while the heptanesulphonate anion increases the retention of insulins. This means that the hydrophobic anions interact with the positive charges of the insulins (which, at this pH, are in excess with respect to the negative charges) and increase their retention in the usual way.

The peak shapes obtained with the base eluent in the presence and absence of alkanesulphonate anions were practically identical.

Changing the methanol concentration of the eluent resulted in a log k' *vs.* methanol concentration relationship of slope -0.25 a value similar to those obtained without ion-pair reagents and with cationic ion-pair reagents.

Cationic ion pair reagents. Tetramethylammonium (TMA) bromide and tetrabutylammonium (TBA) bromide were used as positively charged ion pair reagents. Once again 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1, was the base eluent. The log k' *vs.* reagent concentration relationships obtained are shown in Figs. 6 and 7 for TMA^+ and TBA^+, respectively.

It is seen that, contrary to the effects of the alkanesulphonates, the k' of in-



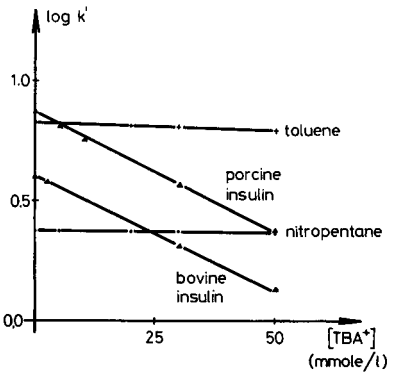


Fig. 6. Influence of the tetramethylammonium bromide concentration upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1 at 30°C.

Fig. 7. Influence of the tetrabutylammonium bromide concentration upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1 at 30°C.

sulins decreases with increasing tetraalkylammonium ion concentration. The decrease is much more pronounced for the tetrabutylammonium ion (Fig. 7).

A possible explanation of this behaviour is that the reversed-phase material adsorbs the positively charged tetraalkylammonium ions, so the packing repels the insulin molecules on which positive charges are in excess at this pH. This behaviour is similar to that noted by Knox and Hartwick[22] for smaller zwitterions, the opposite charges of which were sufficiently separated in space. Melin *et al.*[23] and Sokolowski and Wahlund[24] also noted that the tetrabutylammonium cation decreased the retention of amines in phosphate buffers.

Effect of the eluent pH

Using the 49% (w/v) methanol, 0.5 mole/l NaBr and 0.05 mole/l phosphate eluent, the effects of the pH upon the retention of insulins was investigated. Successive eluents contained $H_2PO_4^-$ only, heptanesulphonate and tetrabutylammonium as ion pair reagents. The results obtained are shown in Fig. 8. For the sake of clarity, only the k' values of porcine insulin are shown, but those of bovine insulin run parallel.

Independently of the type of ion-pair reagent, $\log k'$ decreases with pH, and the slopes are identical, -0.05. Since all three eluents contained identical amounts of the phosphate buffer, and the ratio of $H_2PO_4^-/H_3PO_4$ changed in the same manner, the apparent pH dependence was attributed to the effects of increasing $H_2PO_4^-$ concentration. To verify this, another eluent was prepared which contained twice as much phosphate buffer. The k' values obtained are also shown in Fig. 8. This time the slope was -0.10. This indicates that in the pH range 2.3–4.2 the change in the k' of insulins is not due to the change in pH, but to the increasing concentration of $H_2PO_4^-$ as ion-pairing agent.

Effect of the eluent temperature

The effects of eluent temperature upon the k' values of insulins and nitroal-

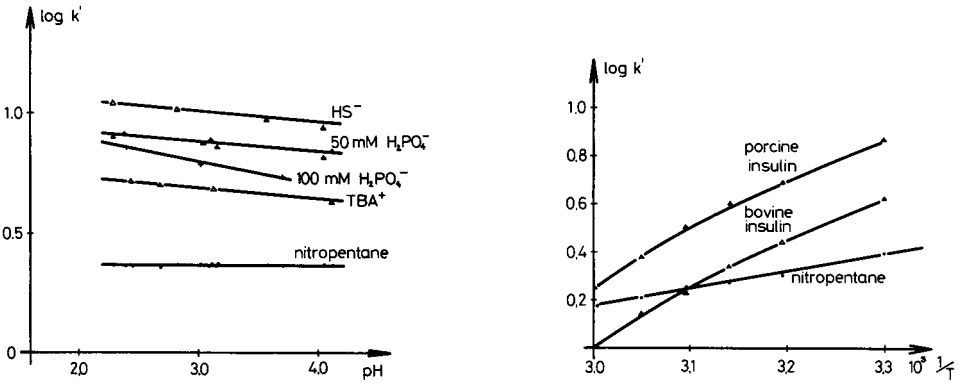


Fig. 8. Apparent influence of eluent pH upon the k' of porcine insulin. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l NaH_2PO_4, 0.05 mole/l H_3PO_4, 0.005 mole/l heptanesulphonic acid (HS^-) and 0.01 mole/l tetrabutylammonium bromide (TBA^+).

Fig. 9. Effect of the eluent temperature upon the k' values of nitropentane, bovine and porcine insulins. Eluent: 45.9% (w/v) methanol, 0.05 mole/l tetramethylammonium hydroxide, 0.1 mole/l H_3PO_4, pH 3.2.

kanes are shown in Fig. 9. The log k' *vs.* $1/T$ curves of insulins are not linear as those of the nitroalkanes, or those of other simple, non-ionic solutes examined earlier[25]. The curvature can be attributed to changes with temperature of the protonation constants of both the insulins and the components of the buffer[26].

CONCLUSIONS

A review of the RP-HPLC separations of insulins shows that although a number of successful separations have been published in the last 3 years, quantitative data relating to the retention-influencing parameters are lacking. Therefore, we investigated the effects of methanol, phosphate buffer, neutral salt and ion pair reagent concentration upon k' of bovine and porcine insulins. All experiments were carried out in the pH range 2.4–4.1.

The practical methanol concentration range is extremely narrow; a 5% (w/v) change results in a ten-fold increase in log k'. Increasing NaBr concentration results in an increase of log k' which, at high molarity, tends to level off. The $H_2PO_4^-$ anion greatly influences the k' of insulins; k' decreases with increasing phosphate concentration, indicating the formation of hydrophilic ion pairs. Alkanesulphonate ion-pair reagents increased the retention of insulins, the magnitude of the increase depending on the chain length of the reagent. The relationship between log k' and heptanesulphonate concentration is not linear. Tetraalkylammonium ion pair reagents greatly decrease the k' of insulins, the extent of the decrease again depending on the chain length of the reagent. The slight apparent pH dependence could be traced back to the dependence of k' upon the $H_2PO_4^-$ concentration.

All these results indicate that reproducible separations require extreme care in the preparation of the eluents.

ACKNOWLEDGEMENT

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IONENAUSTAUSCH BEI HOHEN KONZENTRATIONEN DER LÖSUNG

VII. EINFLUSS VON ORGANISCHEN SOLVENZIEN AUF DIE SORPTION VON ANIONISCHEN KOMPLEXEN DURCH STYROL–DIVINYLBENZOL-COPOLYMERISATE

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(Eingegangen am 15. September 1981)

SUMMARY

Ion exchange at high concentrations of the mobile phase. VII. The influence of organic solvents on the sorption of anionic complexes by styrene–divinylbenzene copolymers

The influence of different solvents such as methanol, ethanol, isopropanol, *n*-butanol, glycol, glycerol, acetone, methyl ethyl ketone, acetonitrile, dioxane, pyridine and glacial acetic acid upon the sorption of $TlBr_4^-$ complexes by styrene–divinylbenzene copolymer is investigated.

In the presence of a solvent with a concentration $\geqslant 2$ *M* a decrease of the distribution coefficient and of the selectivity of the sorption of the $TlBr_4^-$ complexes can be observed.

The mechanism is discussed.

EINLEITUNG

Die Sorptionseigenschaften von Ionenaustauschern können durch organische Solvenzien beeinflusst werden. Dabei gibt es charakteristische Unterschiede im Verhalten der verschiedenen Ionen, die in vielen Fällen zur Verbesserung von Trennprozessen genutzt werden können[1–5]. In wässrig-organischen Systemen führt eine Erhöhung des Solvensgehaltes generell zu einer Zunahme der Ion–Ion-Wechselwirkung, d.h. der Wechselwirkung der zu sorbierenden Ionen mit den Coionen in der Lösung (Komplexbildung) bzw. mit den Festionen im Ionenaustauscher. Ausserdem erfolgt unter dem Einfluss des Lösungsmittels die Zerstörung der Wasserstruktur[6,7].

In Systemen, in denen die Wechselwirkung der zu sorbierenden Ionen mit den Coionen vernachlässigt werden kann, beobachtet man im allgemeinen einen Anstieg des Verteilungskoeffizienten K_d mit den Solvensgehalt infolge Zunahme der Ion–Festion-Wechselwirkung.

In Systemen, in denen die Komplexbildung der zu sorbierenden Ionen mit den Coionen der Lösung eine Rolle spielt, beobachtet man je nach der Stärke der Kom-

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plexbildung eine unterschiedliche Abhängigkeit des Verteilungskoeffizienten vom Solvensgehalt. In Kationenaustauschern durchläuft K_d für eine Reihe von Ionen mit schwacher Komplexbildung bei konstanter Coionenkonzentration und wachsendem Solvensgehalt ein Maximum, im Fall starker Komplexbildung nimmt K_d generell ab. In Anionenaustauschern beobachtet man in Systemen mit schwacher Ion–Coion-Wechselwirkung eine Zunahme von K_d mit dem Solvensgehalt, oder es wird ein Maximum von K_d durchlaufen. Bei starker Ion–Coion-Wechselwirkung nimmt die Sorption mit dem Solvensgehalt ab. Zur Interpretation des Solvenseinflusses wird in diesem Fall die Bildung von Assoziaten zwischen den Komplexionen und den Solvensmolekülen[3,4] bzw. die Selektivitätsänderung durch Zerstörung der Wasserstruktur[7,8] angenommen.

Zum Studium des Solvenseinflusses auf das Sorptionsverhalten grosser hydrophober Anionen, speziell zur Rolle der Wasserstruktur bei der Selektivität, sind Copolymerisate auf Basis Styrol–Divinylbenzol (DVB) besonders geeignet, da sie keine funktionelle Gruppe haben und deshalb die Ion-Festion-Wechselwirkung vernachlässigt werden kann. In der vorliegenden Untersuchung wurde die Sorption von $TlBr_4^-$-Ionen durch ein makroporöses Copolymerisat vom Typ Wofatit Y29/1 in Gegenwart verschiedener organischer Solvenzien studiert. Auf Grund der hohen Stabilität der $TlBr_4^-$-Ionen kann auch der Einfluss des Solvens auf die Komplexbildung vernachlässigt werden.

In der Literatur sind Daten über die Sorption anionischer Komplexe durch Polymere aus solvenshaltigen Lösungen nicht bekannt.

EXPERIMENTELLES

Als Sorbens wurde Wofatit Y29/1, ein makroporöses Copolymerisat auf der Basis Styrol–DVB, verwendet, mit einem DVB-Anteil von 60%, einer spezifischen Oberfläche von 470 m^2/g, einer relativen Porosität von 0.65–0.8 cm^3/g und einem mittleren Porendurchmesser von 2.8 nm. (Versuchsprodukt vom VEB Chemiekombinat Bitterfeld, D.D.R., Forschungsgruppe Ionenaustauscher, siehe auch Lit. 10.)

Die Verteilungskoeffizienten wurden mittels batch-Technik und mit ^{204}Tl als Tracer bestimmt. 200 mg des lufttrockenen Sorbens wurden in einen 100-ml Erlenmeyerkolben eingewogen und über Nacht mit 20 ml Methanol vorgequollen. Anschliessend wurde vom Methanol restlos abdekantiert, 25 ml der zu untersuchenden Lösung sowie 1 ml getracerter Tl(III)-Lösung (0.1 mg Tl/ml) zupipettiert und zur Gleichgewichtseinstellung eine Woche geschüttelt. Nach Abfiltrieren des Harzes wurde die Tl(III)-Konzentration der Lösung gegen einen Aktivitätsstandard mit einem Flüssigkeitszählrohr VA-Z-430 und einem Strahlungsmessplatz, bestehend aus VA-V-100, VA-G-120 und VA-G-24A (VEB Messelektronik Dresden, D.D.R.) gemessen.

Die Messung des statischen Wertes der Oberflächenspannung erfolgte mit einem Tensiometer nach Dognon-Abribat (Prolabo, Paris), das nach dem Prinzip der Adhäsionswaage mit einem Platinblech als Abreisskörper arbeitet. Zur Messung wurde ein Flüssigkeitsvolumen von 50 ml eingesetzt.

ERGEBNISSE UND DISKUSSION

Frühere Untersuchungen hatten gezeigt, dass Tl(III) aus HBr durch Styrol–DVB-Copolymerisate je nach deren spezifischer Oberfläche mit K_d-Werten von 10^3–10^4 ml/g aufgenommen wird. Die Sorption hat bei einer HBr-Konzentration von ≈ 2 *M* ein Maximum. Zur Interpretation wurde angenommen, dass die hydrophoben $TlBr_4^-$-Ionen an der Polymeroberfläche in Form von $HTlBr_4$-Molekülen adsorbiert werden[9,10]. Die Selektivität dieses Prozesses wird im wesentlichen vom Grad der Störung der Wasserstruktur der äusseren Lösung bestimmt.

Zum Studium des Einflusses von organischen Solvenzien auf die Sorption an Polymeroberflächen wurde eine Reihe von Solvenzien eingesetzt, die in einem weiten Konzentrationsbereich mit 2 *M* HBr mischbar sind.

Die Ergebnisse sind in den Fig. 1 und 2 graphisch dargestellt. Daraus ergeben sich die folgenden Befunde:

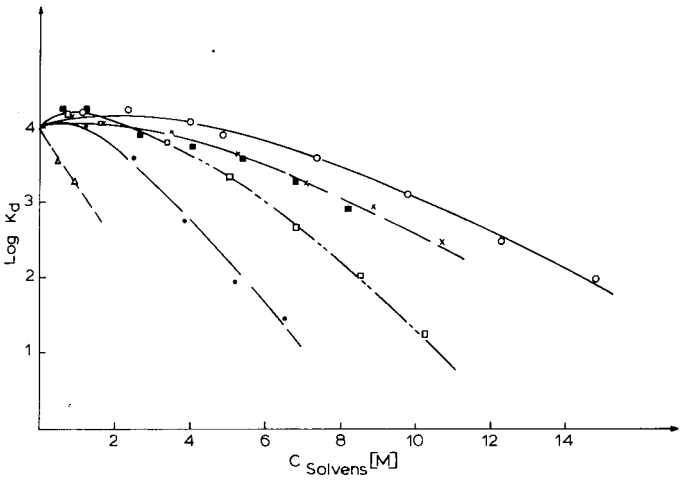

Fig. 1. Sorption von Tl(III) aus 2 *M* HBr durch Wofatit Y29/1 in Gegenwart verschiedener Alkohole. ⊙ = Methanol; △ = *n*-Butanol; ⊡ = Äthanol; × = Glykol; ● = Isopropanol; ■ = Glycerin.

(1) Im Bereich geringer Solvenskonzentrationen von $\leqslant 2$ *M*, entsprechend einem Molenbruch des Solvens von $x_s \leqslant 0{,}04$, nimmt mit Ausnahme von Isopropanol, *n*-Butanol, 1,4-Dioxan und Eisessig die Sorption zu.

(2) Bei Solvenskonzentrationen von > 2 *M* beobachtet man in allen Fällen eine starke Abnahme von K_d, d.h. der Selektivität der Sorption von $TlBr_4^-$-Ionen.

(3) In der homologen Reihe der Alkohole nehmen die K_d-Werte mit der Kettenlänge des organischen Restes ab, das gilt auch für die untersuchten Ketone.

Die Übereinstimmung dieser Befunde mit den für die Sorption von $TlBr_4^-$-Komplexen an sulfonsauren Kationenaustauschern bzw. für die Sorption der Chlorokomplexe verschiedener Metallionen an Anionenaustauschern erhaltenen Ergebnissen weist darauf hin, dass die Ursachen für den Selektivitätsverlust dieser Systeme bei hohen Solvenskonzentrationen die gleichen sind[2,5].

Betrachten wir zunächst den Zustand der Sorbensphase. Die Donnankoeffizienten des Grundelektrolyten HBr von ≈ 1 im Konzentrationsbereich von 0.1–7.6

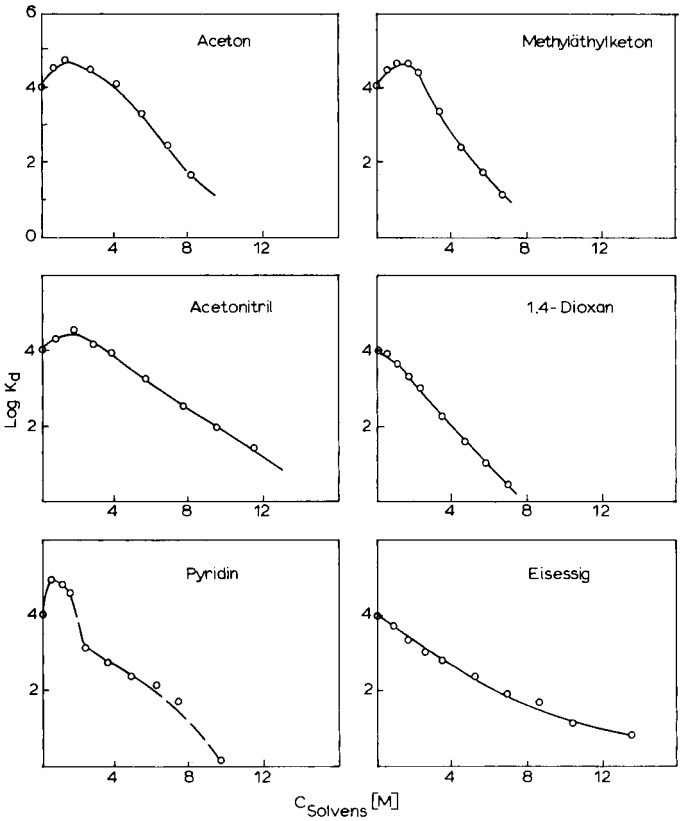

Fig. 2. Sorption von Tl(III) aus 2 *M* HBr durch Wofatit Y29/1 in Gegenwart verschiedener Solvenzien.

M HBr zeigen, dass an der Polymeroberfläche keine Anreicherung von HBr stattfindet[9].

Über die Sorption von Alkoholen aus wässrig-alkoholischen Lösungen durch makroporöse Copolymerisate auf Basis von Styrol–DVB wurde in der Literatur berichtet[12]. Das dabei verwendete Sorbens hatte mit einer spezifischen Oberfläche von 505 m^2/g ähnliche Eigenschaften wie Wofatit Y29/1. In Fig. 3 sind die Sorptionsisothermen für einige Alkohole dargestellt, die auf der Grundlage der in dieser Arbeit angegebenen Daten errechnet wurden. Man erkennt, dass die Sorption von Äthanol zu *n*-Butanol stark zunimmt. Die K_d-Werte betragen für Äthanol 1.0–1.6 ml/g, *n*-Propanol 10-15 ml/g und *n*-Butanol 66–90 ml/g. Die Sättigungskapazität von 3.4–3.9 mmol/g, die bei den drei untersuchten Alkoholen gleich ist, wird dementsprechend bei *n*-Butanol bereits bei Solvenskonzentrationen von 0.1 *M* ($\approx$ 1 %, v/v), bei *n*-Propanol von $\leq$ 1 *M* ($\approx$ 8 %, v/v) und bei Äthanol erst bei $\approx$ 5 *M* ($\approx$ 30 %, v/v) erreicht. Bei den angegebenen Konzentrationen ist die Sorbensoberfläche dann mit einer monomolekularen Schicht von Solvensmolekülen bedeckt (22–23 $Å^2$/Molekül). Die Sorbensphase geht mit Erreichung dieser Sättigungskonzentration in einen stationären Zustand über, der durch konstante Werte für die HBr-Konzentration, die Solvenskonzentration an der Oberfläche und die Quellung charakterisiert ist.

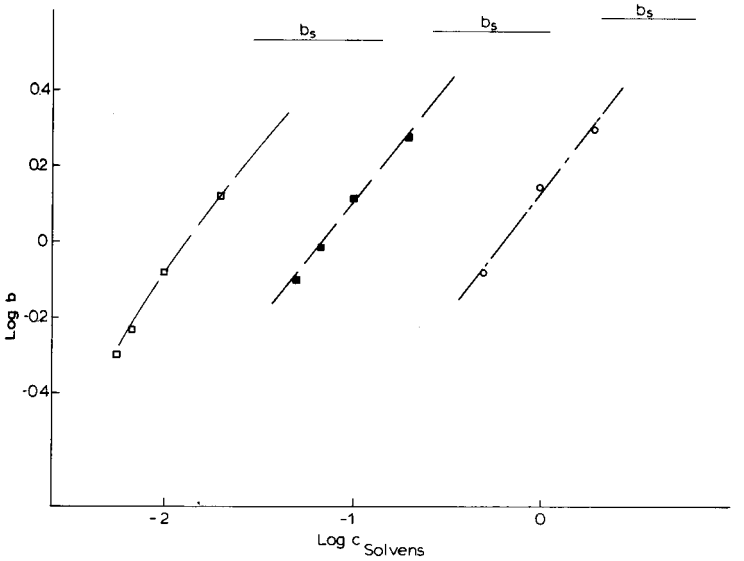

Fig. 3. Sorption von Alkoholen durch ein makroporöses Styrol-DVB-Copolymerisat nach Schneider *et al.*[12]. ⊙ = Äthanol; ■ = *n*-Propanol; ⊡ = *n*-Butanol.

Ähnliche Ergebnisse wurden auch für Anionenaustauscher erhalten. Anionenaustauscher nehmen in Kontakt mit wässrig–alkoholischen Lösungen bei einem Molenbruch des Solvens von $x_S < 0.2$ bevorzugt Alkohol auf. Bei Solvenskonzentrationen von x_S 0.2–0.8 tritt auch hier eine gewisse Sättigung der Harzphase mit Alkohol ein[13].

In Fig. 4 sind die σ-Werte der eingesetzten Solvensgemische dargestellt. Daraus folgen die oberflächenaktiven Eigenschaften der verschiedenen Solvenzien. In der homologen Reihe der Alkohole erkennt man die Korrelation der Oberflächenspannung mit der Sorption des Solvens auf der Polymeroberfläche bzw. der desorbierenden Wirkung auf die Tl(III)-Sorption.

Aus dem bisher Gesagten ergeben sich für den Zustand der Sorbensphase zwei Bereiche.

Bei geringen Solvenskonzentrationen wird das Solvens auf der Sorbensoberfläche bis zur Sättigung angereichert, wobei die Sättigungskonzentration solvensspezifisch bei unterschiedlichen Gleichgewichtskonzentrationen der Lösung erreicht wird. In diesem Bereich beobachtet man eine Zunahme des K_d-Wertes von Tl(III), da dabei infolge der gegenüber 2 *M* HBr geringeren Oberflächenspannung der solvenshaltigen Lösungen die sorbierende Polymeroberfläche geringfügig zunimmt, was sich z.B. in einem erhöhten Quellungswert ausdrückt[10]. Ausserdem wird durch den Aufbau einer Solvensschicht auf der Polymeroberfläche der Einbau der hydrophoben $HTlBr_4$-Moleküle begünstigt. Bei Solvenzien, wie Isopropanol und *n*-Butanol, bei denen die Sättigungskonzentration infolge ihrer stark oberflächenaktiven Eigenschaften bereits bei Solvenskonzentrationen von $\leqslant 5\%$ (v/v) erreicht wird, konnte ein Anstieg von K_d experimentell nicht gefunden werden.

Nach Erreichen der Sättigungskonzentration des Solvens auf der Polymerober-

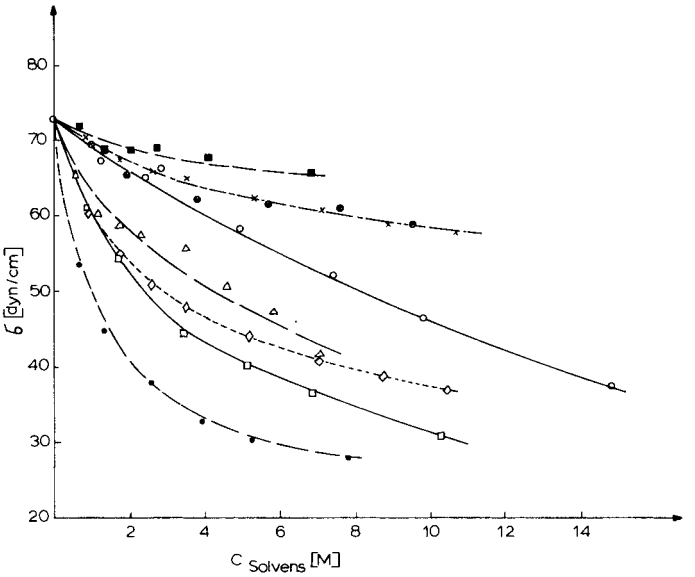

Fig. 4. Abhängigkeit der Oberflächenspannung von 2 *M* HBr von der Konzentration verschiedener Solvenzien. ⊙ = Methanol; ■ = Glycerin; ⊡ = Äthanol; △ = Dioxan; ○ = Acetonitril; × = Glykol; ◇ = Eisessig.

fläche, d.h. des stationären Zustandes, müssen alle Sorptionseffekte den Strukturveränderungen in der äusseren Lösung zugeordnet werden. Mit zunehmender Solvenskonzentration wird in diesem Bereich infolge der fortschreitenden Zerstörung der Wasserstruktur der Einbau der hydrophoben $TlBr_4^-$-Ionen in die äussere Lösung erleichtert. Das führt zwangsläufig zur Abnahme von K_d, was gleichbedeutend mit einer Verringerung der Selektivität des Systems ist. In Anionenaustauschern erfolgt unter diesen Bedingungen eine Selektivitätsumkehr, vor allem auch durch die Zunahme der Ion–Festion-Wechselwirkung[14].

Dieses Ergebnis macht noch einmal deutlich, dass die Aufnahme von anionischen Komplexen vom Typ MX_4^- (M = Tl(III), Au(III), Fe(III), X = Cl^-, Br^-, J^-) durch makroporöse Copolymerisate aus Styrol–DVB und Kationenaustauscher ein Adsorptionseffekt ist, der in erster Linie durch den hydrophoben Charakter dieser Anionen bedingt ist.

Zur Interpretation der desorbierenden Wirkung organischer Solvenzien in den hier untersuchten Systemen ist es nicht notwendig, die Bildung von Assoziaten zwischen $HTlBr_4$ und dem Solvens anzunehmen, deren Existenz insofern wenig wahrscheinlich ist, da experimentelle Untersuchungen in den Systemen HCl–Äthanol und HCl–*n*-Propanol gezeigt haben, dass bis zu Gehalten von 80 % (v/v) Alkohol HCl vollständig dissoziiert vorliegt[15,16].

Für den praktischen Einsatz von organischen Polymeren zur Sorption und Trennung von Anionen und anionischen Komplexen ergibt sich die Schlussfolgerung, dass die Elution mit wässrig-solvenshaltigen Lösungen besonders effektiv ist.

ZUSAMMENFASSUNG

Der Einfluss verschiedener Solvenzien, wie Methanol, Ethanol, Isopropanol, *n*-Butanol, Glykol, Glycerol, Aceton, Methylethylketon, Acetonitril, Dioxan, Pyridin und Eisessig auf die Sorption von $TlBr_4^-$-Komplexen durch Styren–Divinylbenzen-Copolymerisate wird untersucht.

In Gegenwart der Solvenzien beobachtet man bei Konzentrationen von $\geqslant 2\ M$ eine Abnahme des Verteilungskoeffizienten bzw. der Selektivität der Sorption der $TlBr_4^-$-Komplexe.

Der Mechanismus wird diskutiert.

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AFFINITY CHROMATOGRAPHY ON IMMOBILIZED TRIAZINE DYES

POST-IMMOBILIZATION CHEMICAL MODIFICATION OF TRIAZINE DYES

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(Received September 14th, 1981)

SUMMARY

Sepharose-immobilized Procion dyes were subjected to simple chemical modifications in an attempt to assess the significance of certain parts of the dye structure in the dye-enzyme interaction, and whether these modifications would alter the chromatographic properties of the adsorbents towards several model enzymes such as pig heart lactate dehydrogenase, yeast glucose-6-phosphate dehydrogenase and yeast hexokinase. In general terms, cleavage or hydrogenation of the azo-linkages by treatment with sodium dithionite or borohydride, respectively, resulted in weakened enzyme–dye interactions. This is a consequence of the removal of sulphonated polycyclic parts from the dye structure which presumably interact with the enzymes. However, this effect has proved useful in dye chromatography since, for modified dye gels, non-specific proteins appear in the buffer washings in higher recoveries, and the enzymes bound were eluted at lower eluent concentrations and with higher enzyme recoveries than for the native dye-gels.

INTRODUCTION

Reactive triazine dyes are currently becoming established as useful preparative and analytical tools available to the biochemist[1–3]. These sulphonated polyaromatic triazine molecules have proved to be effective in the purification of NAD^+-dependent dehydrogenases, kinases, glycolytic enzymes, blood proteins and a number of other enzymes and proteins[1–5].

The ability of triazine dyes to bind to a wide variety of enzymes could not at first be explained. Inevitably, in addition to the purely empirical data derived from actual purifications, many direct studies of the interaction of various proteins with the dye ligands have been made. Electrophoretic[6,7], enzyme inhibition[4,8–16], absorption spectral difference[17–20], induced circular dichroism[21,22], X-ray crystallographic[23], affinity labelling[24–26] and chromatographic[4,27] techniques have been employed. In particular, in an attempt to define the structural requirements of the enzymes for the

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binding of dyes, a variety of cellular enzymes have been tested for their ability to bind a number of Cibacron Blue F3G-A analogues[10,14,28]. However, to my knowledge, no detailed studies have been reported on the use of chemically modified immobilized triazine dyes as a means of investigating the mode of binding to enzymes or the use of such modified dyes in enzyme purification. It was briefly reported[29] that reduction of immobilized Procion Red HE-3B with 0.2 *M* sodium dithionite in alkaline solution is accompanied by marked spectral changes in the dye and an alteration in chromatographic properties towards yeast glucose-6-phosphate dehydrogenase and *Escherichia coli* IMP dehydrogenase.

On the basis of that report I proposed a series of experiments to yield information on (i) the functional groups or parts of the dye structure which appear to be important in the binding of the enzyme, and (ii) how to improve the chromatographic behaviour of immobilized dyes in terms of increased enzyme recovery under mild elution conditions, and of increased specific activity of the eluted enzyme.

In the present work I have employed three well-documented enzymes: an NAD^+-dependent oxidoreductase, pig heart lactate dehydrogenase; an $NADP^+$-dependent dehydrogenase, yeast glucose-6-phosphate dehydrogenase and a typical ATP-dependent enzyme, yeast hexokinase. The Sepharose-immobilized native and chemically modified triazine dyes tested were: Procion Blue H-B, Blue HE-RD, Green HE-4BD, Green H-4G, Yellow H-5G and Red HE-3B.

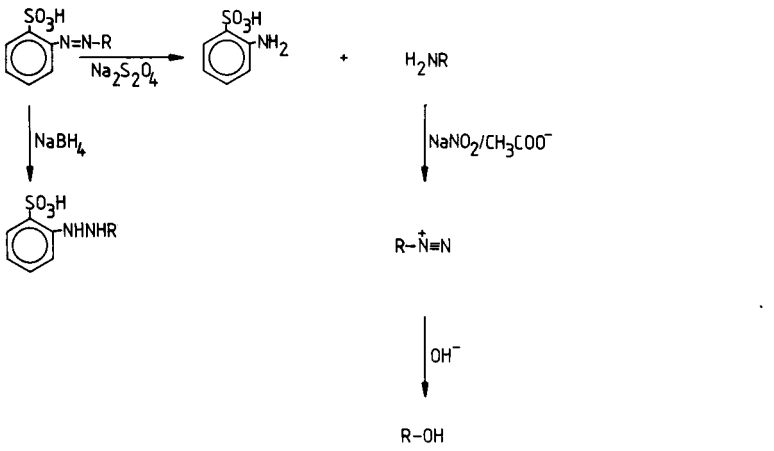

Fig. 1. Anticipated effects of the post-immobilization chemical modification of triazine dyes. R represents the remaining structure of a Procion dye immobilized to Sepharose.

The post-immobilization chemical modification of the triazine dyes involved (i) treatment of the immobilized native dye with sodium dithionite, (ii) treatment of the dithionite-reduced dye with sodium nitrite in acetic acid followed by sodium hydroxide and (iii) treatment of the native dye with sodium or lithium borohydride. Fig. 1 illustrates the anticipated effect of these facile chemical manipulations on the structure of a typical Procion dye.

The chromatographic behaviour of the chemically modified and native immobilized triazine dyes was studied in terms of their ability to bind the three enzymes and bovine serum albumin.

EXPERIMENTAL

Materials

Pig heart lactate dehydrogenase (220 units per mg) was obtained from Boehringer (Lewes, Great Britain), whilst yeast glucose-6-phosphate dehydrogenase (315 units per mg) and yeast hexokinase (310 units per mg) were from Sigma (London, Great Britain). ATP disodium salt and NADH were from Boehringer, $NADP^+$ and glucose-6-phosphate from Sigma and sodium pyruvate, bovine serum albumin (BSA, fraction V) and all other chemicals were from BDH (Poole, Great Britain).

The triazine dyes were a gift from I.C.I. Organic Division (Blackley, Manchester, Great Britain). Dyes are referred to in this paper by their commercial names (I.C.I.). Procion Blue H-B (I.C.I.) is chemically identical to Cibacron Blue F3G-A (Ciba-Geigy).

Sepharose 4B was purchased from Pharmacia (G.B.) (Hounslow, Great Britain).

Enzyme assays

Enzyme assays were performed at 25°C and 340 nm, unless stated otherwise. The reaction mixture contained the following in a total assay volume of 1 ml:

(i) Pig heart lactate dehydrogenase (LDH): potassium phosphate buffer, 50 μmol, pH 7.0; sodium pyruvate, 0.73 μmol; NADH, 0.2 μmol and LDH, 0–0.05 units. One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol NADH per min at 25°C.

(ii) Yeast glucose-6-phosphate dehydrogenase (G6PDH): Tris–HCl buffer, 30 μmol, pH 7.5; glucose-6-phosphate, 2.0 μmol; $NADP^+$, 0.3 μmol; $MgCl_2$, 6 μmol and G6PDH, 0–0.05 units. One unite of enzyme activity was defined as the amount of enzyme required to produce 1 μmol NADPH per min at 25°C.

(iii) Yeast hexokinase: Tris–HCl buffer, 0.1 mmol, pH 7.5; D-glucose, 5 μmol; ATP, 3 μmol; $NADP^+$, 0.7 μmol; $MgCl_2$, 10 μmol; yeast glucose-6-phosphate dehydrogenase, 3 units and yeast hexokinase, 0–0.03 units. One unit of enzyme activity was defined as in (ii).

All enzyme assays were initiated by adding the enzyme to be assayed and the following molar extinction coefficients (ε_m, $1 \cdot mol^{-1} \cdot cm^{-1}$) were used[30]: NADH, 6220; $NADP^+$, 18,000 and ATP, 15,400.

Chromatographic procedures

All chromatographic procedures were performed on an analytical scale (0.5 g moist weight gel) and at 25°C. Sepharose-immobilized triazine dyes were prepared as described before[4] with the following dye concentrations (μmol dye per g moist weight gel): Procion Blue H-B, 0.8; Blue HE-RD, 1.7; Green HE-4BD, 2.5; Green H-4G, 2.6; Yellow H-5G, 2.7; Red HE-3B, 1.0.

Pig heart lactate dehydrogenase. A sample containing pig heart lactate dehydrogenase, 150 units, and bovine serum albumin, 5.0 mg, in a total volume of 0.4 ml was dialysed overnight at 0–4°C against 1 l of 30 m*M* potassium phosphate buffer, pH 7.0. A sample of the dialysed enzyme (50 μl; 8.8 units LDH; 0.43 mg BSA) was applied to a column (3.2 × 0.45 cm) containing Sepharose-bound triazine dye (native or modified; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) equili-

brated with the same phosphate buffer. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected with a linear gradient of NADH (0–20 μM; total volume 20 ml). Fractions (1.5 ml) were collected at a flow-rate of 23 ml/h and assayed for enzyme activity. Bovine serum albumin was determined from its absorbance at 280 nm, and the progress of the gradient determined by absorbance at 340 nm (Fig. 2).

Yeast glucose-6-phosphate dehydrogenase. A sample containing yeast glucose-6-phosphate dehydrogenase, 70 units, and BSA; 5.0 mg, in a total volume of 0.5 ml was dialysed overnight at 0–4°C against 500 ml of 30 mM Tris–HCl buffer, pH 7.5, containing 1.5 mM $MgCl_2$. A sample of the dialysed enzyme (100 μl; 3.6 units G6PDH; 0.82 mg BSA) was applied to a column (3.2 × 0.45 cm) containing Sepharose-bound triazine dye (native or modified; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) equilibrated with the same buffer. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected with a linear gradient of $NADP^+$ (typically 0–0.1 mM or as specified; total volume 20 ml). Fractions (1.5 ml) were collected at a flow-rate of 23 ml/h and assayed for enzyme activity. Bovine serum albumin was determined from its absorbance at 280 nm, and the progress of the gradient determined by absorbance at 260 nm.

Yeast hexokinase. A sample containing yeast hexokinase, 31 units, and BSA, 5.0 mg, in a total volume of 0.5 ml was dialysed overnight at 0–4°C against 500 ml of 30 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. A sample of the dialysed enzyme (100 μl; 4.2 units hexokinase; 0.66 mg BSA) was applied to a column (3.2 × 0.45 cm) containing Sepharose-bound triazine dye (native or modified; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) equilibrated with the same buffer. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected either by omitting the Mg^{2+} from the irrigating buffer or with a pulse of ATP (20 mM; total volume 5 ml). Fractions (1.4 ml) were collected at a flow-rate of 23 ml/h and assayed for enzyme activity. Bovine serum albumin was determined from its absorbance at 280 nm, and the eluting nucleotide (ATP) determined by absorbance at 260 nm (Fig. 3).

Post-immobilization chemical modification of Sepharose-bound triazine dyes

Cleavage of azo linkages of immobilized triazine dyes (treatment with sodium dithionite). A Sepharose-bound dye column (3.2 × 0.45 cm; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) was washed with distilled water (5 ml) and then a solution (*ca.* 10 ml) of sodium dithionite (29 mM; 5 mg/ml) in 1% sodium carbonate was slowly passed through at 24°C until a constant colour was observed. The column was then washed with at least 10 ml of 1% sodium carbonate, to remove the excess of sodium dithionite, followed by 10 ml of distilled water. The gel was then ready for equilibration in the buffer to be used in the subsequent studies.

Replacement of aryl-amino groups with phenolic hydroxyl groups of immobilized triazine dyes via diazonium formation. A sodium dithionite-reduced dye column was equilibrated with 0.5 M acetic acid and the gel transferred to a glass vial. The vial was placed in an ice-bath and when the temperature of the gel slurry (*ca.* 2 ml; 0.5 M acetic acid) had fallen to 0°C a total of 140 mg (2 mmol) sodium nitrate was added in two equal portions over a period of 20 min under gentle stirring. The gel suspension

was left under gentle stirring at 0°C for another 10 min and then washed with 1 m*M* NaOH (20 ml) at 24°C followed by distilled water (20 ml).

Reduction of the azo linkages of immobilized triazine dyes with sodium or lithium borohydride. A Sepharose-bound bye column (3.2 × 0.45 cm; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) was washed with distilled water (5 ml) and 1% sodium bicarbonate until the pH of the washings was 8.0. The gel was then transferred to a glass vial. To the gel slurry (*ca.* 2.0 ml in 1% sodium bicarbonate) was introduced, at 24°C, a total of 40 mg (1 mmol) sodium borohydride or lithium borohydride (1.7 mmol) in portions of 10 mg over a period of 1 h, under gentle stirring. The gel was then washed with water (100 ml) on a sintered funnel under mild suction.

RESULTS AND DISCUSSION

Post-immobilization chemical modification of triazine dyes by treatment with sodium dithionite has been briefly reported[29]. The author reports that a marked spectral change accompanied the treatment of immobilized Procion Red HE-3B by sodium dithionite in alkaline solution. Indeed, Table I reveals the changes in colour and λ_{max} that occurred to Sepharose-immobilized and free triazine dyes when treated with several different reagents (see Experimental section) (Fig. 1). Sodium dithionite is known to be a strong reducing agent for azo-linkages[31] capable of cleaving an azo bond to (aryl) amino groups, thereby disrupting or cleaving part(s) of the dye structure (Fig. 1). The colour changes observed were in agreement both with the structure of the dyes[2,32,33] and the known chemical properties of sodium dithionite. For example, since Procion Blue H-B contains no azo bonds one would expect that sodium dithionite should have no permanent effect on this dye, as was indeed observed. An amber shade developed temporarily but was soon re-oxidized in air to the original blue. On the other hand, Procion Blue HE-RD was converted into a greenish gel. In this case sodium dithionite presumably removed an azo-bonded aromatic moiety. Furthermore, Procion Green HE-4BD was reduced to a colourless gel on treatment with sodium dithionite. This suggests a serious disruption of the dye structure and consequent loss of the chromophore. This dye consists of several polysulphonated aromatic moieties linked together via azo bonds. It is obvious that cleavage of the linking azo bonds would decimate the dye and leave only a small part of the original structure immobilized. The effect of sodium dithionite was not as dramatic on immobilized Procion Red HE-3B where only a terminal benzosulphonyl group was removed, resulting in an orange-yellow shade. Procion Green H-4G changed from brilliant green to turquoise on treatment with sodium dithionite. Treatment of sodium dithionite-reduced immobilized triazine dyes with acetic acid and sodium nitrite in order to convert arylamino groups into diazonium groups, and subsequent treatment with alkali, leads to an overall replacement of the arylamino groups with phenolic hydroxyl groups[31]. In terms of colour changes, the above treatment had no effect since a hydroxyl group is unlikely to change significantly the overall chromophoric properties of the triazine dye.

Treatment of immobilized triazine dyes with sodium or lithium borohydride would lead to hydrogenation of the azo bonds (Ar–N = N–Ar) to the corresponding hydrazino analogues (Ar–NHNH–Ar)[31]. For Procion dyes with large and complex

TABLE I

SHADES OF NATIVE AND CHEMICALLY MODIFIED SEPHAROSE-IMMOBILIZED TRIAZINE DYES

The shades referred to are gels equilibrated at neutral pH or in distilled water. The absorption maxima (nm) are of the free dye in distilled water.

*Treatment with**	*Blue H-B*	*Blue HE-RD*	*Green HE-4BD*	*Green H-4G*	*Yellow H-5G*	*Red HE-3B*
(Native)	Blue (620)	Dark blue (620)	Dark green (630)	Brilliant green (675)	Brilliant yellow (410)	Brilliant red (530)
$Na_2S_2O_4$	Amber (490, amber)	Grey-green	Colourless (520, pale pink)	Turquoise	Brilliant yellow (< 340, colourless)	Orange-yellow (420, orange-yellow)
$Na_2S_2O_4/HNO_2/NaOH$	–	Brown-green	Colourless	–	Brilliant yellow	Orange-yellow
$NaBH_4$ or $LiBH_4$	Pale violet	Dark blue	Very dark green	Brilliant green	Brilliant yellow	Orange-yellow

* See Experimental section and Fig. 1.

chromophores such as Blue HE-RD, Green HE-4BD and Green H-4G the reduction of the azo bonds had a moderate effect on the shades (Table I). In contrast, dyes belonging to the azo-class such as Procion Red HE-3B are profoundly affected on reduction with sodium borohydride, confirming that the azo linkages contribute significantly to the chromophoric properties of these dyes. In general, either cleavage at the azo bonds by sodium dithionite or reduction by sodium borohydride would lead to some loss of the chromophoric properties of the immobilized dye because of the elimination of the azo bonds and the removal of (poly) aromatic moieties of the dye structure. Accordingly, we observed (Table I) a hypsochromic shift of the shades. However, Procion Yellow H-5G maintained its normal shade when subjected to these chemical treatments. This dye contains a single azo bond[33] which forms the chromophore along with a sulphonated aromatic and a heterocyclic moiety; therefore, cleavage of this bond should alter the dye's shade. Perhaps the immobilized dye is less susceptible to modification than the free one since the free dye was decolourized on treatment with sodium dithionite and displayed no absorption in the 340-nm region.

Chemical modification of immobilized triazine dyes also alters their chromatographic properties towards a number of enzymes. Table II illustrates the binding properties of pig heart lactate dehydrogenase for immobilized native and chemically modified Procion dyes under identical experimental conditions. It is evident in all cases that chemical treatment of the immobilized Procion dyes with sodium dithionite resulted in less strong binding of the enzyme than with the native dye, as judged by the lower concentrations of eluent required to desorb peak activity of the enzyme. As a consequence of the weaker binding, higher recoveries of enzyme were obtained. Further treatment of sodium dithionite-treated immobilized triazine dyes with HNO_2 followed by NaOH, in order to replace $-NH_2$ groups by –OH groups, resulted in adsorbents exhibiting slightly weaker binding strengths than the sodium dithionite-treated gels (Table II). It seems that the dramatic changes which occurred in the chromatographic behaviour of immobilized triazine dyes after chemical treatment were due to the elimination of (poly)sulphonated (poly)cyclic part(s) from the native molecules rather than to simple group substitutions such as replacement of $-NH_2$ with –OH. Interestingly, chemically modified dye-gels exhibited, in general, higher recovery of bovine serum albumin than the native gels, suggesting a decrease in non-specific interactions for the chemically treated gels. These observations are logical since removal of some structural parts from the native dye molecule containing hydrophobic (aromatic or other cyclic moieties) and ionic (sulphonic acid) species should lead to some weakening of the dye-macromolecule interaction. In this context, it is known that both hydrophobic[17,34] and ionic[35] interactions occur in dye–macromolecule binding. Furthermore, dyes and complementary enzymes interact with a fair degree of specificity[4,25,26] for the nucleotide binding site, thus, disruption of the native dye structure should, in general, lead to weakened dye–enzyme interaction.

Reduction of the azo linkages of immobilized triazine dyes with either sodium or lithium borohydride resulted in gels with slightly higher (Green HE-4BD and Green H-4G) or slightly lower (Blue H-B, Blue HE-RD, Yellow H-5G and Red HE-3B) binding strengths than the native gels for pig heart lactate dehydrogenase (Table II). These observations suggest that the azo linkages are probably not as significant as other structural parts of the dye chromophore in dye–enzyme binding. Similar results were obtained with yeast glucose-6-phosphate dehydrogenase (Table III). Chemical

TABLE II

BINDING STRENGTH OF PIG HEART LACTATE DEHYDROGENASE (LDH) TO NATIVE AND CHEMICALLY MODIFIED IMMOBILIZED TRIAZINE DYES

Columns (0.5 g moist gel) were equilibrated in 30 m*M* potassium phosphate buffer, pH 7.0. A sample (50 μl) of dialysed LDH (8.8 units) and bovine serum albumin (BSA) (0.43 mg) was applied to each column. Non-adsorbed proteins were washed off with buffer and elution of the bound LDH was effected with a linear gradient of NADH (0–20 μM; total volume 20 ml).

*Immobilized dye treated with**	*LDH activity (%) eluted in the buffer washings and bound and subsequently desorbed*											
	Blue H-B		*Blue HE-RD*		*Green HE-4BD*		*Green H-4G*		*Yellow H-5G*		*Red HE-3B*	
	Eluted	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*
(Native)	29	47	0	72	0	71	0	80	0	76	0	68
	(–)	[2.1]**	(24)***	[243]	(73)	[11.4]	(93)	[1.6]	(116)	[5.6]	(70)	[2.7]
$Na_2S_2O_4$	68	9	0	89	0	90	47	47	26	65	46	46
	(–)	[1.4]	(15)	[16.1]	(106)	[1.3]	(116)	[1.0]	(120)	[1.2]	(114)	[1.2]
$Na_2S_2O_4/HNO_2/NaOH$		–	0	77	48	23		–	57	27	21	47
			(29)	[10.2]	(108)	[1.0]			(103)	[0.5]	(80)	[0.9]
$NaBH_4$ or $LiBH_4$	67	17	0	14	0	45	14	51	0	84	70	7
	(105)	[1.4]	(29)	[34.1]	(33)	[15.9]	(109)	[2.7]	(112)	[3.2]	(102)	[1.3]

* See Experimental section and Fig. 1.

** Concentration (μM) of NADH required to elute LDH with maximal activity on a linear gradient of NADH.

*** Bovine serum albumin (%) in buffer washings.

TABLE III

BINDING STRENGTH OF YEAST GLUCOSE-6-PHOSPHATE DEHYDROGENASE TO NATIVE AND CHEMICALLY MODIFIED IMMOBILIZED TRIAZINE DYES

Columns (0.5 g moist gel) were equilibrated in 30 m*M* Tris–HCl buffer, pH 7.5, containing 1.5 m*M* $MgCl_2$. A sample (100 μl) of dialysed enzyme (3.6 units) and bovine serum albumin (0.82 mg) was applied to each column. Non-adsorbed proteins were washed off with buffer and elution of the enzyme was effected with a linear gradient of $NADP^+$.

*Immobilized dye treated with**	*G6PDH (%) eluted in buffer washings and bound and subsequently desorbed*							
	Blue H-B		*Green H-4G*		*Yellow H-5G*		*Red HE-3B*	
	Eluted	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*
Native)	0	101	0	0	0	110	0	105
	(101)**	[0.11]***	(93)	[–]	(112)	[0.12]	(68)	[0.60]
$Na_2S_2O_4$	0	104	0	77	0	120	0	35
	(93)	[0.01]	(49)	[0.70]	(118)	[0.05]	(110)	[0.01]
$NaBH_4$ or $LiBH_4$	0	102	0	62	0	107	0	90
	(104)	[0.03]	(109)	[0.90]	(108)	[0.05]	(105)	[0.09]

* See Experimental section and Fig. 1.

** Bovine serum albumin (%) in buffer washings.

*** Concentration (m*M*) of $NADP^+$ required to elute glucose-6-phosphate dehydrogenase with maximal activity in a linear gradient of $NADP^+$.

modification of the immobilized triazine dyes, in all cases, was accompanied with weaker binding of the enzyme than with the native adsorbents.

The different binding properties of the native and chemically modified immobilized triazine dyes may prove to be useful in practical dye chromatography. Fig. 2 illustrates the elution profiles of pig heart lactate dehydrogenase from native and chemically modified immobilized Procion Green HE-4BD under identical experimental conditions. The enzyme was quantitatively adsorbed to the native gel (Fig. 2a), while bovine serum albumin was recovered (73%) in the buffer washings. The enzyme was recovered (71%) with maximum enzyme activity at 11.4 μM on a linear gradient of NADH. The binding of the enzyme to sodium dithionite-treated gel was weaker than to the native gel. Fig. 2b shows that leaking of the enzyme occurs in the early fractions before the application of eluent. Furthermore, higher recoveries were obtained for both BSA (106%) in the buffer washings and for LDH (90%) at 1.3 μM on a linear gradient of NADH, than with the native gel. The latter results (see also Table II) compare favourably to those of native dye gels. Replacement of aryl-amino group(s) with phenolic hydroxyl group(s) resulted in an adsorbent exhibiting very weak binding properties for lactate dehydrogenase. In this case the enzyme appeared with 71% recovery in the early fractions prior to application of the eluent (Fig. 2c). Finally, reduction of the azo linkages of immobilized Procion Green HE-4BD with borohydride yielded a gel with a higher binding strength than the native gel for lactate dehydrogenase. It might be that reduction of the double bonds increases the flexibility of the dye, allowing orientation to a more favourable position for binding with the macromolecule. In this case (Fig. 2d) the enzyme was recovered (45%) with peak activity at 15.9 μM of eluent (NADH).

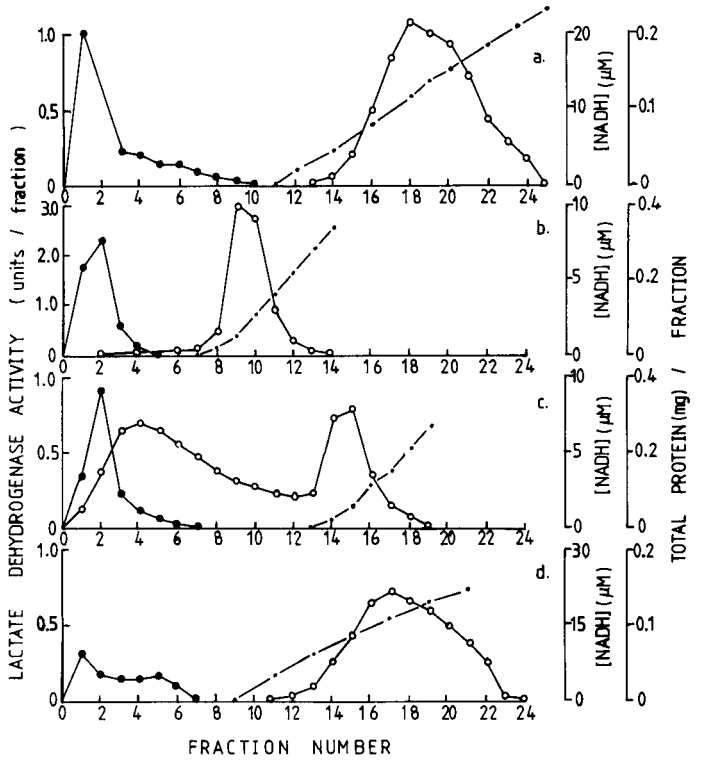

Fig. 2. Elution of pig heart lactate dehydrogenase from Sepharose-bound native and chemically modified Procion Green HE-4BD. A sample (50 μl) containing dialysed enzyme (8.8 units) and bovine serum albumin (0.43 mg) was applied to each column (3.2 × 0.43 cm) of immobilized dye (0.5 g moist gel; 2.5 μmol dye per g moist gel) equilibrated in 30 m*M* potassium phosphate buffer, pH 7.0. Non-adsorbed proteins were immediately washed off the column with buffer (10–12 ml) and elution of lactate dehydrogenase activity was effected with a linear gradient of NADH (0–20 μ*M*; total volume 20 ml). Fractions (1.5 ml) were collected at a flow-rate of 23 ml/h and assayed for lactate dehydrogenase activity (○—○), bovine serum albumin (●—●) and eluent (— · — ·). a, Native or unmodified gel; b, gel treated with sodium dithionite; c, gel treated with sodium dithionite followed by HNO_2 followed by NaOH; d, gel treated with sodium (or lithium) borohydride.

A similar general picture was obtained on chromatography of yeast glucose-6-phosphate dehydrogenase on native and chemically modified immobilized Procion dyes. For example, treatment of native Procion Yellow H-5G with sodium borohydride resulted in gels with significantly lower binding strengths for yeast G6PDH as judged from the concentrations of $NADP^+$ required to elute peak activity of the enzyme (Table III). The inert protein, bovine serum albumin, was (quantitatively) recovered in the void volume which, along with the low concentrations of $NADP^+$ required to elute the enzyme, suggests that chemically treated Procion Yellow H-5G may prove useful in the purification of G6PDH.

Yeast hexokinase was also tested for its ability to bind to immobilized native and chemically modified Procion dyes. At first, this enzyme was not adsorbed onto any of the four different Sepharose-bound triazine dyes tested. However, in the pres-

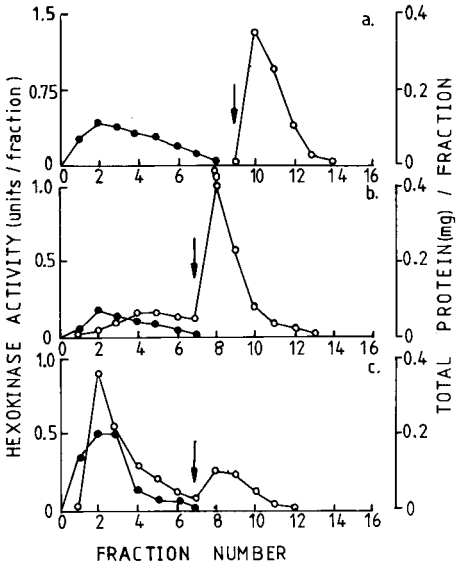

Fig. 3. Elution of yeast hexokinase from Sepharose-bound native and chemically modified Procion Green H-4G. A sample (100 μl) containing dialysed enzyme (4.2 units) and bovine serum albumin (0.66 mg) was applied to each column (3.2 × 0.43 cm) of immobilized dye (0.5 g moist gel; 2.6 μmol dye per g moist gel) equilibrated in 30 m*M* Tris–HCl buffer, pH 7.5, containing 10 m*M* $MgCl_2$. Non-adsorbed proteins were immediately washed off the column with buffer (10–12 ml) and elution of the hexokinase activity effected by omitting the Mg^{2+} from the irrigating buffer (↓). Fractions (1.4 ml) were collected at a flow-rate of 23 ml/h and assayed for hexokinase activity (○) and bovine serum albumin (●). a, Native gel; b, gel treated with sodium dithionite; c, gel treated with sodium (or lithium) borohydride.

ence of 10 m*M* $MgCl_2$ in the irrigating buffer, quantitative binding of yeast hexokinase only onto Procion Green H-4G was achieved, whereas the enzyme passed unretarded through all the other dye-adsorbents, native or chemically modified. These observations are in agreement with previous reports[3,26]. The enzyme bound native Procion Green H-4G–Sepharose could be desorbed in good yield (70%) when Mg^{2+} was removed from the irrigating buffer (Fig. 3a). Chemical treatment of the native immobilized dye has a profound effect on its ability to bind yeast hexokinase. Treatment with sodium dithionite weakens the dye–enzyme interaction (Fig. 3b) and the enzyme is recovered (*ca.* 20%) in the buffer washings. This is probably due to removal of a terminal sulphonated polycyclic moiety. Furthermore, treatment of the native gel with borohydride produces a gel in which the enzyme is largely unretarded and appears in the early fractions (Fig. 3c) with *ca.* 70% recovery of enzyme activity. In each case the enzyme retained on the Procion Green H-4G adsorbent was recovered by removal of Mg^{2+} from the irrigating buffer. Alternatively, yeast hexokinase quantitatively adsorbed to native immobilized dye could be recovered by applying a pulse of 20 m*M* ATP (100% recovery of enzyme activity) or 20 m*M* D-glucose (60% recovery) both in the presence of 10 m*M* $MgCl_2$. The unique behaviour of yeast hexokinase towards Procion Green H-4G has been discussed earlier[26].

It appears, therefore, that removal of sulphonated (poly) cyclic parts from the

dye structure results, in general, in weakened dye–macromolecule interactions. This may prove useful in dye chromatography because:

(i) Non-specific proteins such as bovine serum albumin appear in the buffer washings in higher recoveries than from unmodified dye-gels

(ii) The binding of enzymes on the modified adsorbents is weaker, allowing higher recovery at lower eluent concentrations

(iii) Points (i) and (ii) should lead to enzyme preparations with higher yields and specific activities than the unmodified dye-gels

Finally, chemical treatment of immobilized Procion dyes is economically feasible because of the cheap reagents utilized and the ease with which the modified gels are prepared in a short time.

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THE ADSORPTION OF SIMPLE TRACE METAL CATIONS ON AMBERLITE XAD-1 AND XAD-2

A STUDY USING A MULTICHANNEL NON-DISPERSIVE ATOMIC FLUORESCENCE DETECTOR WITH QUANTITATION BY BATCH MEASUREMENTS

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SUMMARY

The macroreticular resins Amberlite XAD-1 and XAD-2 are unsuitable for quantitative studies of trace metal speciation in natural waters since significant amounts of the simple cations of copper, iron and zinc are readily adsorbed by the resins. These cations are not desorbed by methanol although they are eluted by methanolic HCl, methanolic NH_3 and sodium dihydrogen ethylenediaminetetraacetate. The interactions have been studied using Cheminert chromatographic equipment directly coupled to a multichannel non-dispersive atomic fluorescence detector, and the results quantitated by batch measurements using graphite furnace atomic absorption spectroscopy. XAD-2 resin adsorbed more trace metals than XAD-1, and the adsorption capacity could be reduced by methylation. The adsorption of copper and zinc was not strongly dependent on pH, and there were differences in the behaviour of XAD-1 and XAD-2 toward competing trace metal ions. Contrary to its usual role in the analysis of natural waters, XAD-2 was capable of removing copper and zinc from acetonitrile, and at least some of this adsorbed metal could be eluted with distilled, deionized water.

INTRODUCTION

The cross-linked polystyrene resins Amberlite XAD-1, XAD-2 and XAD-4 have been widely used to adsorb organic compounds from natural waters at concentrations down to the parts per billion (10^9) level and, in contrast to activated charcoal, this adsorption is often reversible. These resins are therefore suitable both for the quantitative determination of some ecologically important man-made compounds (*e.g.* DDT) as well as for the extraction of naturally occurring organics such as the humic acids. These resins would seem to be ideally suited for separating trace metal organic compounds from seawater and thereby providing a quantitative estimate of the extent of organic complexation of a given metal. However, not all organic com-

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plexes are retained by these resins, and some metal–organic compounds may be irreversibly adsorbed. If these affects are important, this would preclude the use of these resins for quantitative measurements of the concentration of naturally occurring metal–organic compounds in seawater, although the adsorption of a trace metal from seawater and its subsequent elution from the resin could provide an estimate of the minimum concentration of such compounds.

Sugimura *et al.*[1,2] passed seawater through a column of XAD-2 resin and found that, for all thirteen metals investigated, between 7% and 100% of the metal was retained by the resin and could be eluted with either methanol or dilute aqueous ammonia. However, their conclusion —that most of the minor elements in seawater are present in organic forms— is valid only if other chemical forms of the elements are not reversibly retained by the XAD-2. In particular, it was assumed that simple inorganic ions were not retained by the resin. Lyons *et al.*[3] made the same assumption in their investigation of iron in nearshore pore fluids. Although there have been numerous reports in the literature that simple cations are not adsorbed[1,2,4–6], the use of these resins to investigate trace metal speciation requires that the resins have a cation exchange capacity of less than 10^{-2} μequiv g^{-1}, and such a low value may well be considered negligible in many applications of these resins.

In an earlier paper[7] it was found that Amberlite XAD-1 was unsuitable for the quantitative determination of the speciation of copper, zinc and iron in seawater. The amount of metals removed from seawater was not reproducible and did not vary linearly with the volume of seawater passed through a column of the resin. The behaviour suggested that the column capacity was being exceeded and it was suggested that the resin adsorbed simple inorganic ions as well as metal–organic species. It was thought that these inorganic ions were adsorbed on to a small number of polar impurity sites, as have been observed in closely related resins used as supports for gas chromatography[8,9]. This paper reports on the adsorption of simple inorganic ions of copper, zinc, iron and magnesium on to XAD-1 and the chemically identical XAD-2.

Although batch techniques are useful in providing quantitative information on the adsorption and desorption of trace metals, the procedure provides low resolution and is very time consuming, particularly if several metals are being investigated. For this reason, a multichannel non-dispersive atomic fluorescence detector (AFD) has been built and its use, in conjunction with batch experiments, provides more detailed information on the interaction between trace metals and the resins XAD-1 and XAD-2.

EXPERIMENTAL

Reagents

Amberlite XAD-1 and XAD-2 resins (20–50 mesh) were Soxhlet extracted with analytical-reagent grade methanol, acetonitrile and diethyl ether and stored under quartz-distilled methanol. Distilled deionized water (DDW) from a Milli-Q system was used in all experiments. XAD-2 resin (25 ml) was washed with DDW, soaked in DDW–tetrahydrofuran (THF) (1:1), vacuum filtered and drained at the pump. It was then resuspended in 80 ml of DDW–THF (1:1) to which were added 3 g of NaOH and 10 ml of dimethyl sulphate. The mixture was then refluxed gently for 5 h. The methylated resin was collected by vacuum filtration, washed and stored under methanol.

The columns were eluted and preconditioned with 10% Merck Suprapure HCl in double-quartz-distilled methanol (H), 5% Merck Suprapure NH_3 in the same methanol (N) and a 10^{-3} *M* solution of disodium dihydrogen ethylenediaminetetraacetate in DDW (Na_2H_2Y). Metal stock solutions contained 1 g l^{-1} of metal and were BDH standard solutions for atomic absorption spectroscopy or were prepared by dissolving analytical-reagent grade zinc in Merck Suprapure HCl or by dissolving the appropriate analytical-reagent grade metal sulphate in DDW. The pH of the solutions was not controlled, so there was no interference from potential organic complexing agents (except acetate from the BDH standard zinc solution). All glassware was acid soaked and, unless otherwise stated, all organic solvents were double quartz distilled.

Chromatographic equipment

A Laboratory Data Control Cheminert CMP-3K pump was used in conjunction with 6 and 9 mm I.D. columns. All fittings were of the Cheminert type, and the only materials in contact with the solutions were borosilicate glass, PTFE and Kel-F. The pressure gauge was stainless steel and was isolated from the rest of the system by a film of PTFE tape inserted between the Cheminert fittings connecting the gauge. The tape tended to leak after a while and the gauge was removed from the system when quantitative measurements were being made. A diagram of the apparatus is shown in Fig. 1. The valve system allowed the two columns to be loaded and eluted separately or jointly depending on the experiment. The eluant could either be collected for batch analysis or pass directly to the nebulizer of the AFD.

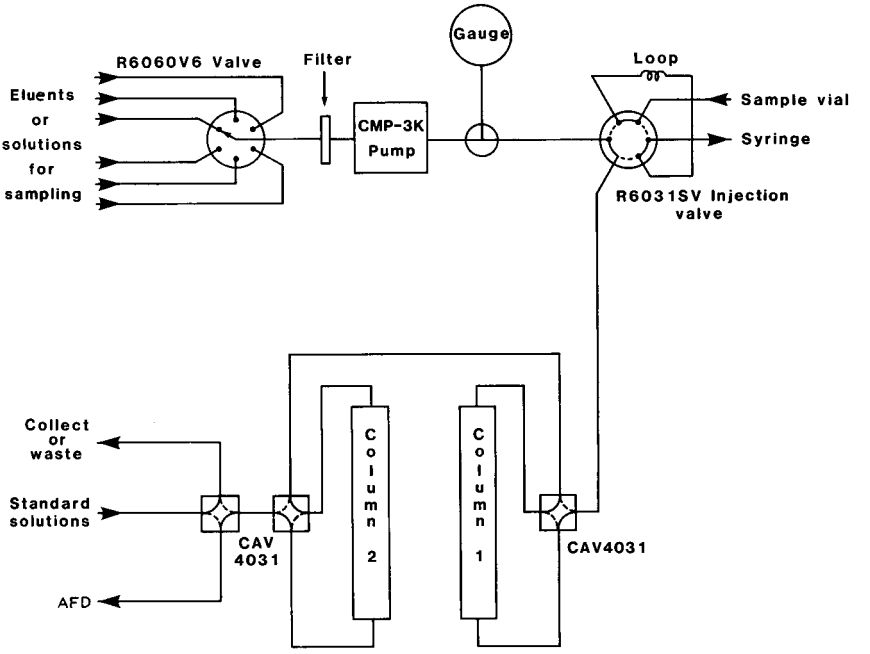

Fig. 1. Schematic diagram of the Cheminert chromatography equipment. The CAV 4031 valves enable the columns to be loaded and eluted separately or jointly while the output can be sampled by batch techniques or directly using the AFD. The detector response can be calibrated via standard solutions.

Metal analyses

For quantitative measurements, aliquots (usually 10 ml) of eluant were collected in acid-soaked polyethylene vials and were analysed by graphite furnace atomic absorption spectroscopy (GFAAS) using a Varian AA6 fitted with a CR-63 furnace. These analyses were performed in a Class-100 clean room.

Atomic fluorescence detector

A four-channel non-dispersive AFD was designed and built along the general lines of those of Larkins[10] and Van Loon *et al.*[11]. The amplifiers and power supplies were of two different types. For the detection of copper, zinc and iron, separate Varian AA4 indicator modules (IM-1) and lamp power supplies (MLS1-A) were used. The modulation frequencies of two of the indicator modules were altered to 263 Hz (Cu) and 313 Hz (Fe) while the third operated at the standard frequency of 285 Hz (Zn). In addition, a three-channel indicator unit, lamp supply and photomultiplier high-voltage source were specially made by GBC Scientific Equipment Pty. The frequencies could be easily varied from 180 to 330 Hz. For the experiments described here only one channel was used for the detection of magnesium at 217 Hz. No interference was observed between the lock-in amplifiers at the frequencies used. The Varian hollow cathode lamps were operated at their maximum rated currents of 15 mA (Cu and Zn), 20 mA (Mg) and 25 mA (Fe). The copper fluorescence was detected by a Hamamatsu R106 photomultiplier operated at 490 V and shielded by a Schott UG11 filter and a 6-mm iris. The iron, zinc and magnesium fluorescence was detected by a single Hamamatsu R166 solar blind photomultiplier operated at 595 V and shielded by a 9-mm iris. The lenses were made from Suprasil I, were 40 mm in diameter and had a focal length of 50 mm. The light intensity within the flame was approximately doubled by using mirrors to reflect the light back through the flame. These mirrors were 35 mm in diameter, had a 103 mm radius of curvature and were coated with aluminium and a protective layer of either UV-transmitting glass or magnesium fluoride. The lenses, filter, irises, mirrors, radiation shields and photomultiplier housings were mounted on an aluminium disc and all metal parts were painted matt black to reduce stray reflections. The lamps were supported on a second aluminium disc rigidly bolted to the first one. The positions of the lamps, lenses and mirrors were separately adjustable over a limited range. The mirrors could be replaced by lenses and additional lamps mounted on the bottom disc to produce an eight-channel detector. The overall sensitivity of the system would be reduced by a factor of *ca.* 2 owing to the loss of reflected light and a small loss due to splitting the photomultiplier outputs over more channels.

The nitrogen-sheathed burner assembly was made of titanium and was identical to that of Larkins[10]. The burner bowl was a Varian AA4 model, rhodium-plated to reduce contamination, and the bakelite plug at the bottom of the bowl was replaced by a PTFE plug. An air–acetylene flame was used and the mixture controlled by a Varian GCU-2 unit. The output from the chromatography equipment was connected directly to a Varian adjustable tantalum nebulizer. A constant pressure of air (100 kPa) was applied and the uptake rate of the nebulizer was adjusted to be identical to the flow-rate through the chromatography columns (100 ml h^{-1}).

With inflammable organic solvents in the nebulizer the supply of acetylene had to be reduced otherwise the flame became much too rich. There were difficulties

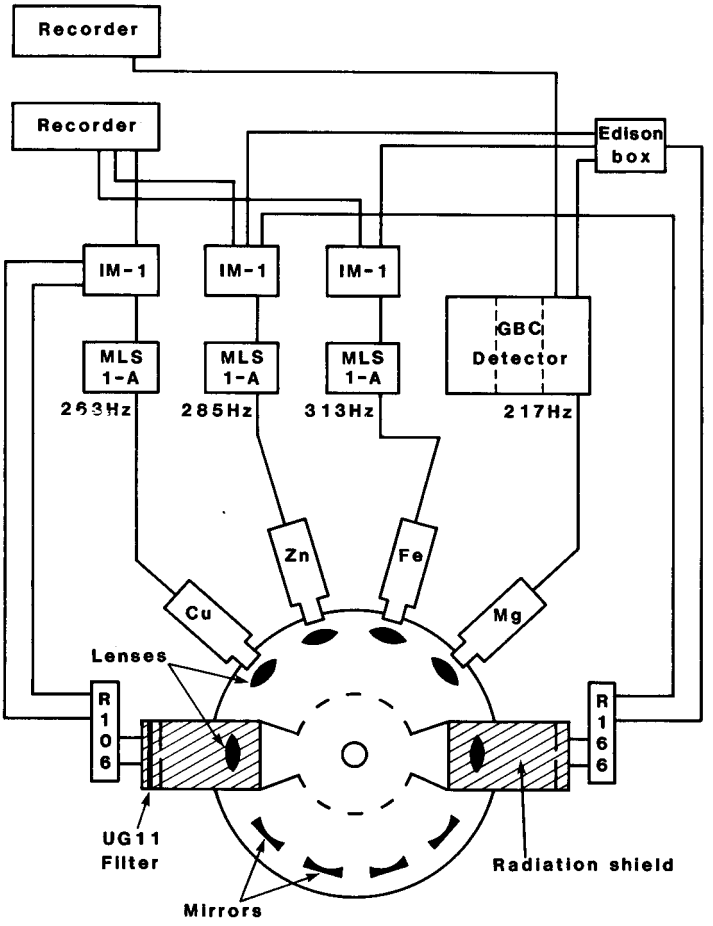

Fig. 2. Schematic diagrams of the non-dispersive multichannel AFD. For copper, zinc and iron, the lamp supplies (MLS1-A) and amplifiers (IM-1) are Varian AA4 units while for magnesium the lamp supply and amplifier are part of a three-channel unit supplied by GBC Scientific P/L. The copper fluorescence is detected by a R-106 photomultiplier and a UG11 filter, while the iron, zinc and magnesium signals come from a R-166 photomultiplier. The signal is split into three by an edison box. The recorders are YEW-3056 models, and Varian hollow cathode lamps are used. The silica lenses have a focal length of 50 mm while the mirrors have a radius of curvature of 103 mm.

involved in changing solvents since the fluorescence signals became very noisy for lean mixtures and the flame tended to go out for rich mixtures. The acetylene flow-rate either had to be changed continuously with the composition of the eluting solvents or maintained at a compromise level with a subsequent increase in noise, particularly when an oxidizing flame was used. The latter approach was adopted here. The iron, copper and zinc fluorescence signals were recorded on a 3-pen recorder while the magnesium signal was recorded on a separate 2-pen recorder. A diagram of the apparatus is shown in Fig. 2.

TABLE I

RECOVERY OF ADSORBED INORGANIC COPPER AND ZINC FROM TWO COLUMNS OF XAD-1 CONNECTED IN SERIES

See text for conditions of elution.

Load (μg)	*Sample composition**	*Sample volume (ml)*	*pH (approx.)*	*DDW wash volume (ml)*	*Copper*		*Zinc*	
					Col. 1 (μg)	*Col. 2 (μg)*	*Col. 1 (μg)*	*Col. 2 (μg)*
50	$Zn(OAc)_2$	50	2.7	50	0.97	1.15	0.61	0.66
100	$CuCl_2$							
50	$Zn(OAc)_2$	10	2.3	75	1.18	1.02	0.94	0.75
50	$CuCl_2$							
50	$Zn(OAc)_2$	10	2.3	150	0.58	2.35**	1.39	7.62**
50	$CuCl_2$							
5	$Zn(OAc)_2$	10	3.3	75	0.53	0.40	1.03	0.57
5	$CuCl_2$							
5	$Zn(OAc)_2$	10	3.3	75	0.58	0.27	2.1	0.87
5	$CuCl_2$							
500	$CuCl_2$	10	1.3	75	1.20	1.03	–	–
500	$ZnCl_2$	10	–	75	–	–	0.92	0.94

* HOAc = acetic acid.

** Samples were apparently contaminated.

Batch experiments on XAD-1

DDW was spiked with copper and zinc using BDH standard solutions for atomic absorption spectroscopy. The solutions were cupric chloride in 1 *M* HCl and zinc acetate in 1 *M* acetic acid. The pH of the solutions ranged from *ca.* 1.3 to 3.3. In order to eliminate the possibility that acetate complexes may be involved in the adsorption, some experiments were conducted using only metal chlorides. An aliquot of solution, containing zinc or copper or both, was passed through two 9-mm columns connected in series. The first and second columns contained 6.5 ml and 10 ml of XAD-1 resin, respectively, and before each experiment the columns were preconditioned by washing them successively with 200 ml (H), 200 ml methanol, 200 ml (N) and 50 ml of DDW. The columns were loaded and preconditioned at a flow-rate of 250 ml h^{-1}. The columns were eluted with 6 × 10 ml (H), 3 × 10 ml (methanol) and 3 × 10 ml (N) at 50 ml h^{-1} and the aliquots analysed separately as described earlier. The results are summarized in Table I, and only the total amounts of copper and zinc recovered are listed.

Experiments on XAD-2

Atomic fluorescence detector. Samples of DDW were simultaneously spiked with BDH standard solutions of copper, zinc and iron and were aspirated into the nebulizer at 100 ml h^{-1}. The fluorescence peak heights are plotted as functions of concentration in Fig. 3.

The adsorption of copper, zinc, iron and (sometimes) magnesium on XAD-2 was investigated using the chromatographic arrangement shown in Fig. 1. A stock solution containing these metals (and also nickel and manganese) at a concentration

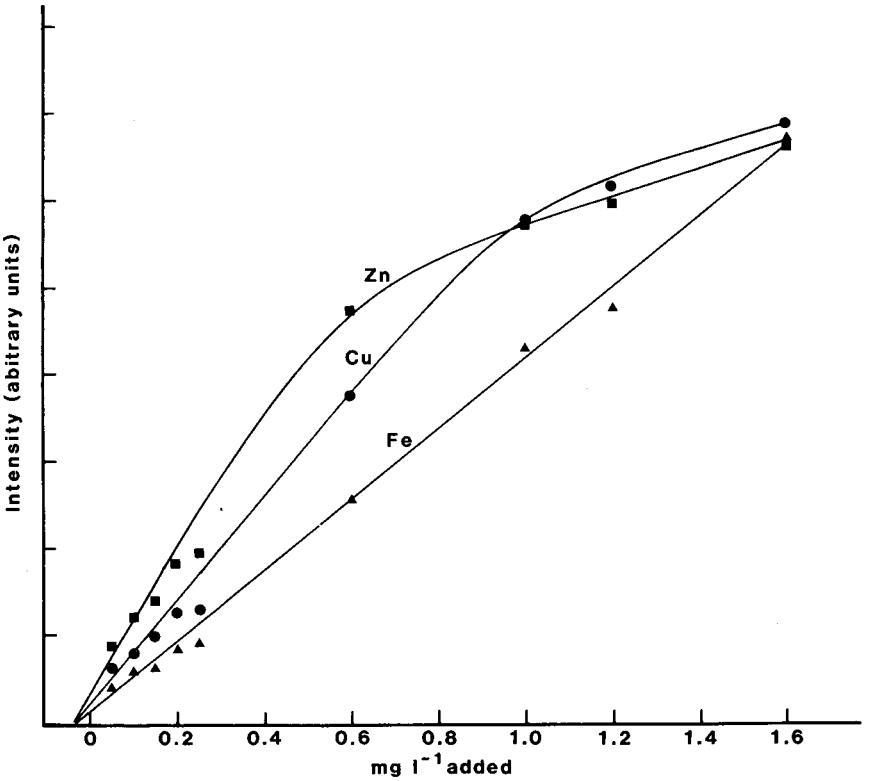

Fig. 3. The AFD response (peak heights) for the simultaneous determination of iron, copper and zinc in aqueous solution.

of 10 mg l^{-1} each was made from BDH standard solutions. The pH of the solution was *ca.* 1.5, although the effective pH on the column may have been significantly higher owing to retention and dilution effects. A 6-mm column, containing 4 ml of XAD-2 resin, was washed with 40 ml of Na_2H_2Y and DDW. The flow-rate of the DDW was 100 ml h^{-1} and, after 20 ml had passed through the column, 500 μl of stock solution (containing 5 μg of each metal) was injected on to the column while the effluent was monitored for copper, iron and zinc. When the fluorescence signals had returned to background levels, another sample was injected on to the column and the whole procedure was repeated until the column had been loaded five times. The column was then eluted with methanol (analytical-reagent grade), acetonitrile (analytical-reagent grade) and Na_2H_2Y, and the results are shown in Fig. 4a. The higher noise level during the elutions was due to a compromise fuel–air mixture being used as the solvent system was changed.

The experiment was repeated using the same column but eliminating the methanol and acetonitrile elutions and monitoring the magnesium fluorescence as well (Fig. 4b). Finally the previous experiment was repeated using a column which contained 4 ml of XAD-2 which had been methylated with dimethyl sulphate (Fig. 4c).

Batch experiments. The atomic fluorescence experiments described above

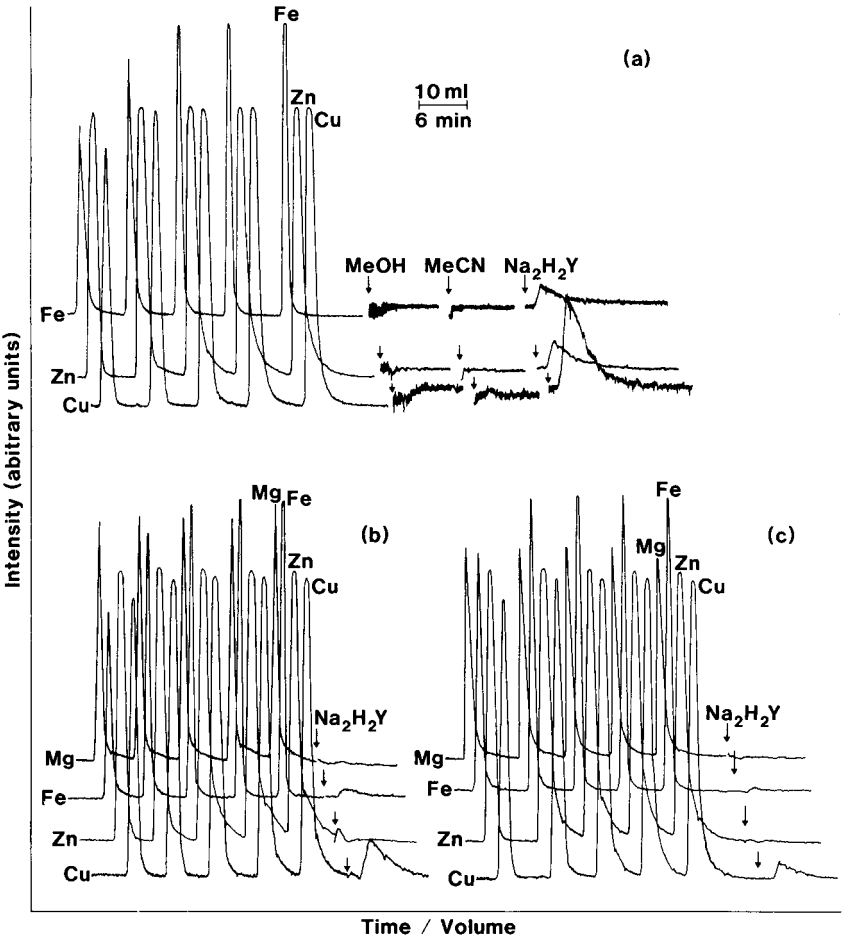

Fig. 4. (a) Iron, zinc and copper fluorescence signals from the effluent from a column of XAD-2 after five successive 500-μl injections of stock solution containing 5 μg of iron, copper, zinc, magnesium, manganese and nickel. The column was then eluted successively with methanol (MeOH), acetonitrile (MeCN) and 10^{-3} *M* disodium dihydrogen ethylenediaminetetraacetate. (b) Same as (a), except that the magnesium fluorescence was also monitored and the MeOH and MeCN elutions were eliminated. (c) Same as (b), except that the resin had been methylated with dimethyl sulphate. The signals were recorded simultaneously but have been offset for clarity.

showed that XAD-2 adsorbs trace metals not only from aqueous solution but also from organic solvents, and this effect was investigated further. The copper and zinc levels of DDW, methanol (analytical-reagent grade) and acetonitrile (analytical-reagent grade) were measured before being pumped through the same 6-mm column of XAD-2 used in the atomic fluorescence experiments. The pump and lines were then flushed out with the appropriate solvent, and 25 ml were passed through the column in the order methanol, acetonitrile and DDW. The copper and zinc levels were measured in the solutions prior to being passed through the column and in successive 5-ml aliquots of the effluent, and the results are given in Table II in terms of absorbance units.

TABLE II

GFAAS MEASUREMENTS (ABSORBANCE UNITS*) ON 5-ml ALIQUOTS OF METHANOL, ACETONITRILE AND DDW AFTER BEING PUMPED THROUGH A COLUMN OF XAD-2 RESIN

	Methanol		*Acetonitrile*		*DDW*	
	Cu	*Zn*	*Cu*	*Zn*	*Cu*	*Zn*
Analytical-reagent grade solvent***	0.003	0.085**	0.017	0.390	0.003	0.060
Analytical-reagent grade solvent §	0.003	0.020	0.020	0.405	0.002	0.065
(Double quartz distilled)	<0.001	0.020	<0.002	0.045	–	–
Aliquot 1	0.004	0.280**	0.009	0.260	0.018	0.210
Aliquot 2	0.003	0.045	0.006	0.045	0.008	0.210
Aliquot 3	0.003	0.025	0.008	0.035	0.009	0.155
Aliquot 4	0.003	0.025	0.009	0.035	0.004	0.080
Aliquot 5	0.003	0.025	0.013	0.055	0.004	0.085

* Approximate conversion factors: 0.100 absorbance units are equal to 120 $\mu g\, l^{-1}$ (Cu) and 2 $\mu g\, l^{-1}$ (Zn).

** These fractions appear to be contaminated.

*** Sampled from solvent container.

§ Sampled directly prior to the column.

The adsorption capacity of methylated and unmethylated XAD-2 were compared in batch experiments in which two columns of XAD-2 (6-mm diameter containing 4 ml of resin) were connected in series and loaded with inorganic copper and zinc. The first column contained methylated resin. The columns were loaded with a 500-μl sample loop with metal concentrations of 10, 100 and 500 mg l^{-1} giving metal loadings of 5, 50 and 250 μg, respectively. The columns were loaded with copper and zinc using diluted BDH standard solutions or analytical-reagent grade metal sulphates.

The columns were preconditioned by washing successively with Na_2H_2Y (100 ml), DDW (25 ml), methanol (25 ml), methanolic NH_3 (100 ml) and finally DDW (100 ml). The columns were loaded at 250 ml h^{-1} by passing 10 ml of DDW through the sample loop. The column was eluted at the same flow-rate with 6 × 10 ml fractions of Na_2H_2Y, and the results are given in Table III.

RESULTS AND DISCUSSION

There are two mechanisms whereby simple inorganic ions could be adsorbed on to XAD-1. In the first case, adsorption could occur via polar impurity groups in the resin itself, such as occur on styrene–divinylbenzene copolymers used as supports for gas chromatography[8,9,12]. In the second case, organic ligating agents could be strongly adsorbed on to the resin and these adsorbed molecules could then interact with the inorganic species. The adsorbed molecules would probably contain aromatic rings, since these are known to bind strongly to XAD-1, and polar functional groups such as –OH, $-NR_2$ or –COOH. It has been reported[7] that methanolic HCl removed virtually all of the copper, zinc and iron from XAD-1. This solution would be expected to remove most neutral polar molecules and particularly molecules that could be protonated to acquire a positive charge, but it may not remove strongly bound

TABLE III

RECOVERY OF ADSORBED COPPER AND ZINC FROM TWO COLUMNS OF XAD-2 CONNECTED IN SERIES

The columns were eluted separately with 6 × 10 ml aliquots of Na_2H_2Y and only the total amounts recovered are listed. The resin in column 1 had been methylated with dimethyl sulphate. The columns were loaded by injecting 500 μl samples of standard solution (SS).

SS conc. (mg l^{-1})	*Load (μg)*	*Sample* composition*	*pH (approx.)*	*Copper*		*Zinc*	
				Col. 1 (μg)	*Col. 2 (μg)*	*Col. 1 (μg)*	*Col. 2 (μg)*
10	5	$Zn(OAc)_2$ + $CuCl_2$	2	1.96	3.11	**	**
10***	5						
10	5	$Zn(OAc)_2$ + $CuCl_2$	2	2.85	3.61	0.37	0.57
10***	5						
100	50	$Zn(OAc)_2$ + $CuCl_2$	1	5.96	7.36	0.60	0.85
100	50						
500	250	$Zn(OAc)_2$ + $CuCl_2$	0.3	5.15	6.57	0.68	0.37
500	250						
500	250	$ZnSO_4$ + $CuSO_4$	4	3.62	7.35	0.61	0.61
500	250						
500	250	$ZnSO_4$	5	§	–	§	3.03
500	250	$CuSO_4$	4	§	6.00	§	–

* HOAc = Acetic acid.
** Samples were contaminated.
*** Total copper recovered exceeds load amount.
§ Column 1 was not used.

organic molecules that remain electrically uncharged in strongly acid solutions (*e.g.* aromatic carboxylic acids). These would probably be removed by methanolic NH_3 and it is unlikely that any organic molecule capable of binding inorganic ions would remain adsorbed on XAD-1 after washing with both methanolic HCl and methanolic NH_3. However, inspection of Table I shows that in all experiments, significant amounts of copper and zinc were recovered from both columns, and it must be concluded that the coordinating sites are on the resin itself and not on adsorbed organic molecules. Virtually all of the copper and zinc recovered from the columns was found in the first two methanolic HCl aliquots, but there was occasionally some tailing observed for zinc as was reported earlier[7]. Although methanolic NH_3 was capable of eluting zinc and copper from XAD-2, no copper or zinc was observed in the methanolic NH_3 fractions, implying that these metals had been removed previously by the methanolic HCl.

Both columns appear to be saturated with copper and zinc at loadings of less than 5 μg because there is no significant increase at loadings of 500 μg. The recovery rate shows no significant correlation with pH. The results for the second column in one run are anomalous. It is thought that these samples were contaminated. When these values are excluded, the average recovery of copper and zinc from the second column (0.77 ± 0.41 μg and 0.76 ± 0.15 μg, respectively) is less than that from the first column (0.84 ± 0.31 μg and 1.17 ± 0.52 μg, respectively). This result is surprising since the second column contained 50% more XAD-1 resin than the first and may

be due to an inherent variability in the amount of metal retained by the resin since the results have larger standard deviations than expected. Alternatively, it may be due to contamination of the system by metal–organic complexes which are trapped on the first column. The Milli-Q Water system had a mixed cation–anion exchange resin bed in the final position, and it is possible that organic complexing agents could bleed from the resin and complex copper and zinc with the resultant compounds being adsorbed by the resin. However, this is unlikely since there is no correlation between the amount of copper and zinc recovered from the columns and the amount of DDW passed through the columns.

The amount of copper recovered is independent of the amount of zinc in the solution and *vice versa*. This implies that the two metals are competing for different sites. Although copper generally forms stronger complexes than zinc, the two metals tend to form similar complexes with the types of donor groups likely to exist as impurities on XAD-1 resin. It seems improbable that the resin would contain approximately equal numbers of copper- and zinc-specific coordination sites, and a more likely explanation is that similar functional groups are involved but that the specificity is due to the stereochemical requirements of the two metals since copper prefers square planar (or tetragonal) coordination while zinc prefers tetrahedral coordination.

The AFD, although good for qualitative monitoring of the adsorption and desorption of metals from various chromatographic packing materials, is less useful for quantitative measurements since the response is linear with respect to concentration over a limited range. From Fig. 3, the iron fluorescence signal remains linear up to at least 1600 $\mu g\ l^{-1}$ while the copper and zinc signals deviate from linearity at *ca.* 1000 and 600 $\mu g\ l^{-1}$, respectively. The departure from linearity occurs at a metal concentration of *ca.* 20 times the detection limit as defined by Larkins[10]. While this restricted linear range is not desirable for quantitative measurements, it is advantageous to its use as a chromatographic detector since the recorder sensitivity does not have to be adjusted as often when samples of unknown concentration are being studied.

In the experiments with XAD-1, it was found that the trace metals could be completely removed with methanolic HCl. However, this solvent is volatile and corrosive and when used with the AFD it causes the magnesium fluoride coatings on the mirrors to become opaque. Batch experiments showed that Na_2H_2Y released copper and zinc as well as methanolic HCl, and hence this solution was used in the atomic fluorescence experiments. The use of Na_2H_2Y, rather than methanolic HCl, also eliminated the possibility of hydrolysis of the methylated XAD-2 resin.

The method of loading the columns of XAD-2 differs from the one used in the batch experiments on XAD-1 in that the local metal concentration is much higher but there is less time for reaction to occur. The hydrogen ion concentration is also higher when the BDH standard solutions are used but, as mentioned earlier, the pH of the solution on the column is probably not well defined.

The low sensitivity of the AFD relative to GFAAS batch methods precludes any measurement of the tailing effects at elution volumes of 20 ml or more, but it is apparent that most of the metals are eluted within a volume of *ca.* 10 ml. Although Larkins[10] reported a short-term variability of *ca.* 20% and a long-term variability of about a factor of two, the traces shown in Fig. 4a and b are virtually

identical for the separate 5-μg loadings of iron, copper and zinc.

Much information about the interaction between trace metals and XAD-2 can be obtained from the size and shape of the peaks in the effluent from successive injections of 500-μl aliquots of trace metal solution on to the XAD-2 column. Since magnesium cannot compete with the other metals for coordination sites, there is no difference between the first and last effluent peaks nor is there any significant difference between the methylated and unmethylated resin. In addition, no magnesium is observed in the Na_2H_2Y elution. The iron effluent peaks become successively larger and reach a constant height at the third injection, and this is interpreted as being due to ferric ions being adsorbed on to the resin up to the third injection. In agreement with this, an iron fluorescence signal is observed when the column is eluted with Na_2H_2Y. The signal is smaller than expected and this could be due to the slow kinetics of the reaction between ferric ions and Na_2H_2Y[13] leading to the gradual release of ferric ions at concentrations too low to be detected by atomic fluorescence. Copper appears to interact much more strongly with the resin since the peaks show a successively greater tailing with subsequent injections of stock solution. The effluent peaks grow in intensity up to the third injection and, as expected, a strong asymmetric copper fluorescence signal is observed on elution with Na_2H_2Y. In contrast to the behaviour of copper, no zinc fluorescence is observed in the Na_2H_2Y eluate and the peak heights in the column effluent remain constant over the five injections. While this suggests that zinc is not adsorbed by the resin there is obviously some interaction since the peaks show even more tailing than is observed for copper. The increased tailing of copper and zinc with successive injections suggests that these metals interact more strongly with the resin as the metal loading increases, but the reason for this is not clear.

Although unreacted vinyl groups have been observed on the chemically similar Chromosorb 100 series resins[8], such groups are unlikely to contribute significantly to the complexation and binding of heavy metals. The donor sites on XAD resins are probably carboxyl groups, phenolic groups or similar entities which are capable of being methylated. The resultant esters and ethers would be weaker Lewis bases and hence should not interact so strongly with heavy metals. In agreement with this, it is found that methylated XAD-2 adsorbs significantly less copper, zinc and iron than the unmethylated resin, as can be seen in the smaller elution peaks in Fig. 4c. However, the methylated resin still adsorbs significant amounts of these ions, and it seems that some coordination sites remain after methylation.

If XAD-2 does contain polar coordinating sites that are capable of removing inorganic ions from aqueous solution at low pH then it is unlikely that the adsorbed ions would be readily removed by organic solvents. This is confirmed in Fig. 4a where no copper, iron or zinc is observed in the methanol or acetonitrile elutions. However, the amount of iron and copper eluted by Na_2H_2Y is much greater in this experiment where the elution was preceded by methanol and acetonitrile elutions. Moreover, zinc is observed in the Na_2H_2Y eluate whereas no zinc fluorescence was observed in the Na_2H_2Y eluate when the column had not been previously eluted with organic solvents (Fig. 4b and c). These organic solvents had not been quartz distilled and it was known that the acetonitrile contained appreciable amounts of heavy metals. The most likely explanation was that the XAD-2 resin was removing heavy metals from the organic solvents and that these were being eluted by Na_2H_2Y.

The adsorption of trace metals from organic solvents was investigated by batch experiments on XAD-2 and the results are shown in Table II. There appears to be no contamination of any of the solvents from the pumping system, and the analytical-reagent grade methanol was essentially free of copper and zinc. Apart from one fraction of methanol eluent which appears to have been contaminated, the concentration of copper and zinc in methanol was uniformly low and was not affected by the XAD-2 column. On the other hand, the analytical-reagent grade acetonitrile was contaminated with copper and zinc but the levels decreased on passing through the XAD-2 column. When DDW was subsequently passed through the column, large amounts of copper and zinc appeared in the first aliquot and then the levels gradually decreased toward background values. Thus XAD-2 can remove copper and zinc from an organic solvent (acetonitrile), and at least some of this adsorbed metal can be subsequently eluted using only DDW. This behaviour is in direct contrast to the use of this resin to remove trace metals from seawater followed by elution with organic solvents (*e.g.* methanol) to remove the organically complexed trace metals.

Experiments using the AFD suggested that methylation of XAD-2 reduced, but did not eliminate, adsorption of inorganic ions. This effect was investigated by batch experiments and the results are summarized in Table III. It is seen that methylated XAD-2 (column 1) adsorbs significantly less copper than the unmethylated resin, in agreement with the atomic fluorescence results. At pH <2 and at loadings high enough to ensure saturation of the column, methylation decreases the amount of copper adsorbed by XAD-2 resin by *ca.* 20%. This effect is not nearly so pronounced for zinc, and in one experiment the unmethylated resin adsorbed less zinc than the methylated column. When the unmethylated resin (column 2) was loaded separately with 250 μg of zinc or copper, the amounts of metal eluted (6.0 and 3.0 μg, respectively) should represent the maximum amounts that can be retained by the column. The recovery of up to 7.4 μg of copper from column 2, is probably due to uncertainties in the measurements, and the amount of copper adsorbed by the resin does not appear to be affected by the presence of zinc. The amount of zinc that can be retained by column 2 is approximately half the amount of copper that can be retained but the amount decreases to one-fifth in the presence of an equivalent amount of copper. This behaviour is different from that observed for XAD-1 (see Table I), but it does offer an explanation for the absence of adsorbed zinc in the atomic fluorescence experiments where zinc had to compete with ferric, nickel, manganese and magnesium ions in addition to copper. The different adsorption capacities of XAD-2 for copper and zinc suggest that there is more than one type of adsorption site on the resin. While this was also postulated for XAD-1 resin, XAD-2 behaves differently in that the adsorption of zinc is strongly dependent on the amount of copper present. An alternative explanation is that there is only one type of site present but that zinc reacts more slowly with the resin than does copper and that the lower capacity of the resin for zinc is an artifact. This could be significant in the present experiment where a concentrated solution is passed rapidly through the column, but it implies that the adsorption capacity for copper is best represented by combining the copper and zinc values from those experiments, using these metals together (neglecting the difference in atomic weights). This would then increase the discrepancy between these experiments and the one where only copper was loaded on to the column. The true situation is probably a combination of these effects.

At loadings of 5 μg each of copper and zinc the reproducibility of the results is not very good, and in one case a total of 6.5 μg of copper was recovered from both columns. Despite the poor reproducibility it is apparent that the adsorption of copper and zinc is not very dependent on pH in agreement with the results on XAD-1.

The surface areas of XAD-1 and XAD-2 are *ca.* 100 and 300 $m^2\ g^{-1}$, respectively, and one would expect that XAD-2 would therefore have about three times the adsorption capacity. If it is assumed that any metal–organic impurities would be trapped on the first column then, from Table I, 10 ml of XAD-1 is capable of adsorbing 1.0 μg of copper and 0.9 μg of zinc. For XAD-2, the same volume of resin could adsorb *ca.* 15.0 μg of copper and 7.5 μg of zinc. The column packings were not weighed but it is clear that XAD-2 adsorbs far more copper and zinc, relative to XAD-1, than would be expected on the basis of surface area alone. The adsorption capacity of XAD-2 for inorganic copper and zinc is appreciable, and it is interesting to compare the figures given above with the results of Puon and Cantwell[14] who found that XAD-2 was capable of adsorbing 1 μmole of H_3O^+ per gram of resin. The observed copper adsorption could be accounted for if *ca.* 5% of the hydrogen binding sites were capable of binding copper, and it is surprising that other authors[1,2,4] have reported that XAD-1 and XAD-2 do not adsorb inorganic ions. This could be due to batch variations, so each batch should be tested to determine its adsorption capacity for each ion. The procedure would be further complicated by possible kinetic effects and the fact that different ions would be competing for the limited number of polar donor groups on the resin. It is obvious that this testing cannot be accomplished on a natural water sample of unknown composition. Elution of columns of XAD-1 and XAD-2 resins by organic solvents such as methanol or acetonitrile probably removes only metal–organic complexes from the resin but it is unlikely that adsorbed naturally occurring metal–organic compounds would be quantitatively removed by organic solvents. Any elutions with hydrochloric acid, ammonia or complexing agents would also remove inorganic ions. It is also apparent that some electrically charged metal–organic compounds are not retained by XAD-1 and XAD-2 since iron, copper and zinc can be rapidly and completely removed from these resins by elution with Na_2H_2Y, presumably as the CuY^{2-}, ZnY^{2-} and FeY^- complexes. This is in agreement with the results of Sakai[5]. Since metal–organic complexes can be neither totally adsorbed nor totally eluted from the resins without contamination from inorganic species, these resins cannot be used to obtain quantitative information on metal speciation in natural waters.

CONCLUSIONS

(1) Contrary to earlier reports in the literature, XAD-1 and XAD-2 resins adsorb appreciable amounts of inorganic iron, copper and zinc. It is logical to assume that many other inorganic ions can also be adsorbed.

(2) Compared with XAD-1, XAD-2 adsorbs larger amounts of copper and zinc than would be expected on the basis of their relative surface areas.

(3) The adsorption of copper and zinc ions is not strongly dependent on pH even down to values as low as 0.3.

(4) For XAD-1, the amount of zinc adsorbed is independent of the amount of copper present and *vice versa*. This is not true for XAD-2 and the results using the

AFD suggest that zinc is not adsorbed in the presence of iron, copper, nickel, manganese and magnesium.

(5) Ethylenediaminetetraacetate complexes are not adsorbed and Na_2H_2Y can rapidly and completely remove iron, copper and zinc from XAD-1 and XAD-2. This is probably true for many other electrically charged metal–organic species.

(6) Methanol and acetonitrile do not remove adsorbed inorganic copper, zinc and iron from these resins.

(7) The adsorption of inorganic ions by XAD-2 can be reduced, but not eliminated, by methylation with dimethyl sulphate.

(8) XAD-2 is capable of removing iron, copper and zinc from acetonitrile. Some of this adsorbed metal can be eluted using only DDW.

(9) The multichannel, non-dispersive AFD provides a rapid and convenient means of investigating the interaction between trace metals and XAD resins or any other chromatographic support.

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DETERMINATION OF PROSTAGLANDIN PRECURSORS IN FROG TISSUE USING SELECTED-ION MONITORING IN GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS

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SUMMARY

Eicosatrienoic acid, eicosatetraenoic acid (arachidonic acid), and eicosapentaenoic acid are unsaturated fatty acids that are possible precursors for prostaglandin synthesis in amphibians. These compounds have been quantitatively identified in tissues of the American bullfrog by using methods of selected-ion monitoring for fatty acid methyl esters. Results from comparison of yield for four methylation techniques showed that boron trifluoride in methanol gave the best yield and reproducibility. Resolution values of 1.2 and 2.7 for these methyl esters were achieved by using a cyanosilicone liquid phase, and the limits of detection based on ion 79.1 a.m.u. were 0.5 ng absolute. These acids were extracted and methylated with overall recoveries of 47, 37 and 28%, respectively. Methyl esters were detected in extracts of all tissues in concentration ranges 12 to 912 ng/μl of extract, or 30 to 2810 μg/g of tissue for 0.1- to 1.3-g samples.

INTRODUCTION

Prostaglandins (PGs) are a family of biologically active compounds that are synthesized from polyunsaturated fatty acid precursors released from plasma membrane phospholipids[1]. In well-described mammalian pathways, arachidonic acid (AA) ($C_{20:4}$, ω-6) is utilized in the synthesis of bisenoic prostaglandins (*e.g.*, PGE_2). However, production of monoenoic (*e.g.*, PGE_1) or trienoic (*e.g.*, PGE_3) prostaglandins from eicosatrienoic acid ($C_{20:3}$, ω-6) or eicosapentaenoic acid ($C_{20:5}$, ω-3), respectively, might also contribute, in some organisms, to biochemical or physiological regulation[2,3]. Eicosapentaenoic acid (EPA) and eicosatrienoic acid (ETA) are readily incorporated into tissue phospholipids of mammals if diets are supplemented with these fatty acids[4,5]. For example, Eskimos have elevated levels of EPA from their fish-based diet, and AA is predominant in mammals with meat-based diets[6]. EPA is largely unused in mammals for synthesis of trienoic prostaglandins and acts as a

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competitive inhibitor in prostaglandin synthesis involving AA[7,8]. Prostaglandin synthesis has not been extensively studied in non-mammalian vertebrates. Although utilization of AA and EPA particularly has been suggested from recent evidence[9], the natural availability of these precursors in tissues of amphibians has been unknown.

Gas chromatographic (GC) methods have been used in separation of ETA, AA and EPA on both packed[10] and capillary columns[11], and most analyses have been based on comparison of relative retention times and detection by ionization detectors[12–14]. Although liquid phases used in these analyses were ester-based materials, such as diethylene glycol succinate, cyanosilicone phases with properties of high temperature stability and low bleed have also been used[15,16]. In this paper, quantitative techniques based on GC–mass spectroscopy (MS) with selected-ion monitoring (SIM) have been developed and used for determining ETA, AA and EPA in representative tissues of the American bullfrog, *Rana catesbeiana*. In addition, heart and urinary bladder tissue from the related leopard frog, *R. pipiens* were analyzed for comparative purposes. Several derivatization procedures have been examined quantitatively for use with these unsaturated fatty acids.

EXPERIMENTAL

Instrumentation

A Hewlett-Packard model 5880A gas chromatograph was equipped with a splitless inlet, a flame ionization detector (FID), and a 0.2 mm I.D. × 25 m OV-101 wall-coated open tubular (WCOT) column. Conditions of analysis were: initial temperature, 150°C; final temperature, 250°C; temperature-programme rate, 4°C/min; final time, 10 min; detector and injection-port temperature, 250°C; attenuation, $16 \cdot 10^{-12}$ A full scale; carrier gas, helium; average linear velocity 20 cm/sec; and splitless time, 0.5 min.

A Hewlett-Packard model 5992A GC–MS instrument was equipped with splitless inlet, glass-jet separator, and 0.2 mm I.D. × 30 m OV-101 WCOT column for use in scanning GC–MS; the chromatographic conditions were as described above. The MS conditions were: lower mass range, 45 a.m.u.; upper mass range, 500 a.m.u.; scan rate, 330 a.m.u./sec; electron multiplier voltage, 2000 V; and solvent time out, 3 min. Other MS conditions were optimized by using Autotune software provided by the manufacturer.

A 2 mm I.D. × 2.5 m glass column containing 1.8663 g of 10% SP-2330 on Supelcoport (100–120 mesh; Supelco, Bellefonte, PA, U.S.A.) was installed in the GC–MS system for SIM analysis. Chromatographic conditions were: oven temperature, 250°C (isothermal); injection-port temperature, 250°C; and helium carrier gas flow-rate, 24 ml/min. Conditions for MS during SIM analysis were: ions monitored, 55.0, 67.0, 79.1, 91.1, 320.1 and 318.1 a.m.u.; dwell time, 166 msec; electron-multiplier voltage, 2000 V; and solvent time out, 8 min. Response in SIM analysis was characterized through preparation of calibration curves for the methyl esters of ETA, AA and EPA at approximate mass levels of 50, 25, 12, 5 and 0.5 ng.

Derivatization study

The yield (%) and reproducibility of derivatization of four methylation techniques were determined for ETA, AA and EPA. These methods involved use of

diazomethane, BF_3 in methanol, Methylute (Alltech Assoc., Deerfield, IL, U.S.A.) or Methyl-8 (Alltech). Stock solutions of each fatty acid [8,11,14-eicosatrienoic acid (Sigma, St. Louis, MO, U.S.A.), 5,8,11,14-eicosatetraenoic acid (AA) (Sigma) and 5,8,11,14,17-eicosapentaenoic acid (Analabs, North Haven, CT, U.S.A.)] were prepared at 50 mg/ml in ethanol and stored at −20°C. Five replicate samples were prepared from stock solutions for use in each derivatization method so that final concentrations were *ca.* 1000 ng/μl; the absolute amount of acid varied between 25 and 500 μg, depending on the procedure.

(a) Diazomethane was prepared by using 133 mg of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; Aldrich, Milwaukee, WI, U.S.A.) freshly prepared in 0.5 ml of water and placed in an apparatus (Aldrich) containing 3 ml of anhydrous ethyl ether (Mallinkrodt, St. Louis, MO, U.S.A.). Diazomethane was generated for 45 min at 1°C after 0.6 ml of 5 *N* NaOH had been slowly added to the MNNG solution. A 2-μl volume of stock solution was placed in a 5.0-ml Wheaton mini-vial (Southland Cryogenics, Carrolton, TX, U.S.A.), and evaporated to dryness in a stream of nitrogen. Then 0.9 ml of the ether solution was added, and the vial was capped and gently shaken. After 60 min, the solution was evaporated to dryness and reconstituted in 100 μl of hexane (Burdick and Jackson, Muskegon, MI, U.S.A.) for analysis by GC.

(b) A 10-μl volume of sample was placed in a mini-vial and evaporated to dryness. Then 2 ml of BF_3 in methanol (Supelco) were added and the vial was sealed and stored at 60°C for 20 min in a Temp-Blok Module Heater (Scientific Products, Tempe, AZ, U.S.A.). Deionized water (2.0 ml) was added, and the mixture was vigorously shaken for 1 min with 1 ml of hexane. The aqueous phase was removed with a pipet, and hexane was condensed to 500 μl and analyzed by GC.

(c) A 12.5-μl volume of a new sample solution (2 mg/ml) was evaporated to dryness in a mini-vial. Methylute (25 μl) was added, and the mixture was injected directly into the GC system.

(d) New sample solution (150 μl) was evaporated to dryness in mini-vials. Methyl-8 (300 μl) was added to each sample, and each mixture was heated at 60°C for 10 to 15 min in sealed mini-vials before analysis by GC.

Since methyl arachidonate (Sigma) was the only methyl ester of known purity available, and since weight-response factors for FID are affected only slightly or not at all by the numbers (*e.g.*, 2 *vs.* 3, or 3 *vs.* 4) of double bonds in a particular chain length fatty acid[17], yield was quantified for each acid *vs.* methyl arachidonate.

In a second derivatization study, solutions of AA and ETA at 100 ng/μl and 10 ng/μl were derivatized by using BF_3 in methanol (the preferred method) to measure any effects of concentration on the percentage yield.

Extraction of tissues

Rana catesbeiana or *R. pipiens* (Nasco Biological, Fort Atkinson, WI, U.S.A.) were pithed, and tissues were quickly excised and weighed. They were extracted with 40% KOH solution in methanol with 0.1 ml of hydroquinone solution (1 mg/ml) as described by Kent *et al.*[18]. The mixture was heated under reflux at 90°C for 1 h, acidified to pH 3.0 and extracted three times with 6 ml of diethyl ether. The combined extracts were evaporated to dryness under nitrogen, and the residues were methylated with 2.0 ml of BF_3 in methanol as described above. Final volumes for these samples were 1.0 ml in hexane.

"Spiking" studies

Absolute recovery efficiencies were determined for tissue extraction and the entire analytical method. Samples of tissue were divided into equal parts, and one part was "spiked" with 100 μg of each acid from stock solutions. Samples were separately extracted, derivatized and analyzed by SIM as described above. Comparison of the actual increase in concentration with the expected increase corrected for derivatization efficiency gave the yield from extraction. Values were determined for three replicates for ETA, AA and EPA.

RESULTS AND DISCUSSION

Results from studies on yield from the four methylation techniques with EPA, AA and ETA are listed in Table I. Values for the percentage yield with use of BF_3 in methanol were generally as good or better than those for the other methods, including the use of diazomethane. On the basis of percentage yield and acceptable standard deviations, BF_3 in methanol was adopted for use in subsequent analyses. The percentage yield for AA at two other concentration levels with this reagent were: 10 ng/μl, 67; and 100 ng/μl, 69.8. Thus, under our experimental conditions, the efficiency of derivatization of AA did not vary substantially within the concentration range expected in these tissue extracts. The value for derivatization of ETA at 100 ng/μl also remained nearly constant at 57.5%; however, efficiency was lost at the 10-ng level, for which the value was 31%.

TABLE I
YIELD FOR METHYLATION TECHNIQUES

Reagent	*Yield (%)*		
	ETA	*AA*	*EPA*
Diazomethane	57.7 ± 8.5	40.9 ± 6.0	56.7 ± 6.6
BF_3–methanol	56.0 ± 1.3	77.4 ± 7.9	59.1 ± 1.7
Methyl-8	50.0 ± 1.8	43.8 ± 3.2	57.8 ± 3.1
Methylute	24.4 ± 6.7	5.7 ± 0.5	7.0 ± 0.9

The chromatographic performance of the GC–MS system is shown in Fig. 1a as a SIM plot from the analysis of a standard solution of the methyl (Me) esters of ETA, AA and EPA. Retention times and specific retention volumes at 200°C for these compounds were: Me-ETA, 10.7 min, 1.86 l/g; Me-AA, 11.8 min, 1.52 l/g; and Me-EPA, 15.1 min, 1.94 l/g. Reproducibility of retention time was better than ±0.1 min, and values for resolution were 1.2 and 2.7 for Me-ETA/Me-AA and Me-AA/Me-EPA, respectively. MS patterns of these acids were similar, and major ions common to each were 55.0 a.m.u., C_4H_7; 67.0 a.m.u., C_5H_7; 79.1 a.m.u., C_6H_7; and 71.1 a.m.u., C_7H_7. These four most abundant ions were chosen for monitoring in SIM analysis, together with the molecular ions for Me-ETA (320.1 a.m.u.) and Me-AA (318.1 a.m.u.). Because four ions were monitored and ratios of abundance values were used, instances of false identification based only on single-ion monitoring were reduced in tissue analysis. Results from the SIM analysis of two tissue extracts are

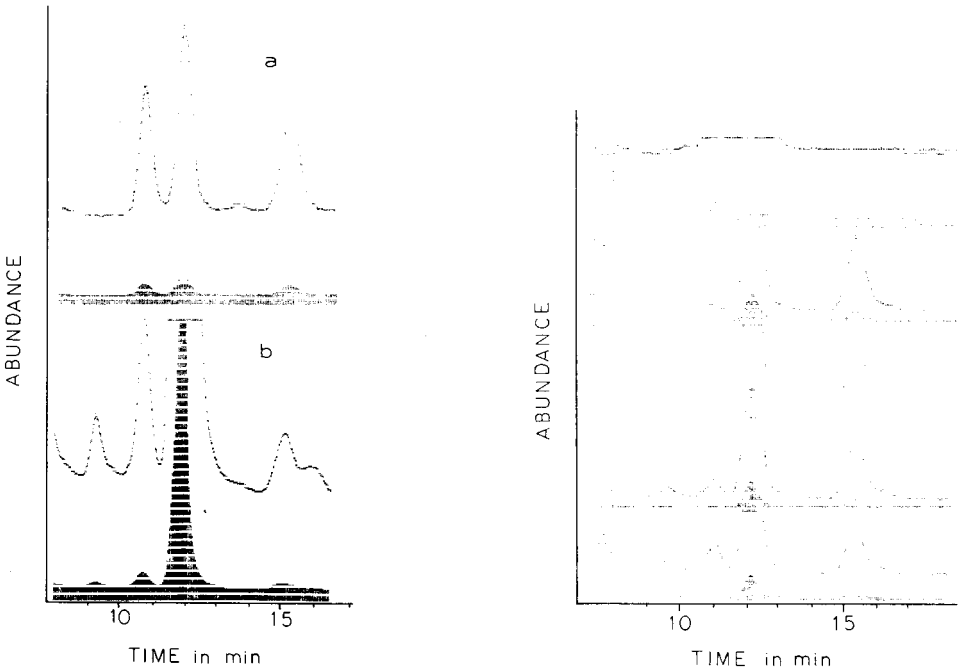

Fig. 1. Plots from SIM analysis of (a) standard and (b) tissue extract. Abundance values are the sum of the values for six ions. Order of elution is Me-ETA, Me-AA and Me-EPA on 10% SP-2330.

Fig. 2. Plots of individual ions from SIM analysis of lung-tissue extract.

shown in Figs. 1b and 2. In Fig. 1b, the summed abundance of the total ions monitored is plotted from analyses of tissue extract and standard solution for comparison of retention behavior. Retention times for the standards and for several large components in the extract were the same. Resolution of SIM data into individual ions is shown in Fig. 2 as plots of individual ion abundance *vs.* time from analysis of another similar tissue extract. Full scale (FS) values, which indicate relative concentrations, clearly show the ion 79.1 a.m.u. as being the ion of choice for the best limits of detection. The major ions for each methyl ester are also seen in these plots. The probability of false identification is further reduced by positive matches between mass spectra from this sample and standard spectra. Also present in these plots is a molecular ion for Me-ETA. Throughout these analyses, interferences when present were detected in plots of ion 55.0 a.m.u. and, in diminishing degree, in plots of ions of higher mass. Consequently, the ion 79.1 a.m.u. was chosen for quantitative analysis. Calibration curves for SIM analysis using 79.1 a.m.u. are shown in Fig. 3. The response of the entire GC–MS system is linear on a log–log plot, and the sensitivities for Me-ETA, Me-AA and Me-EPA are comparable.

However, absolute limits of detection will vary very slightly and were between 0.3 to 0.7 ng for all methyl esters. Instrumental blanks consisting of 2 μl of fresh hexane were used periodically to determine residual contaminations from syringe, septum and column. No components were ever detected in these blanks. Procedure blanks, which were treated as samples (but without tissue) were used to determine

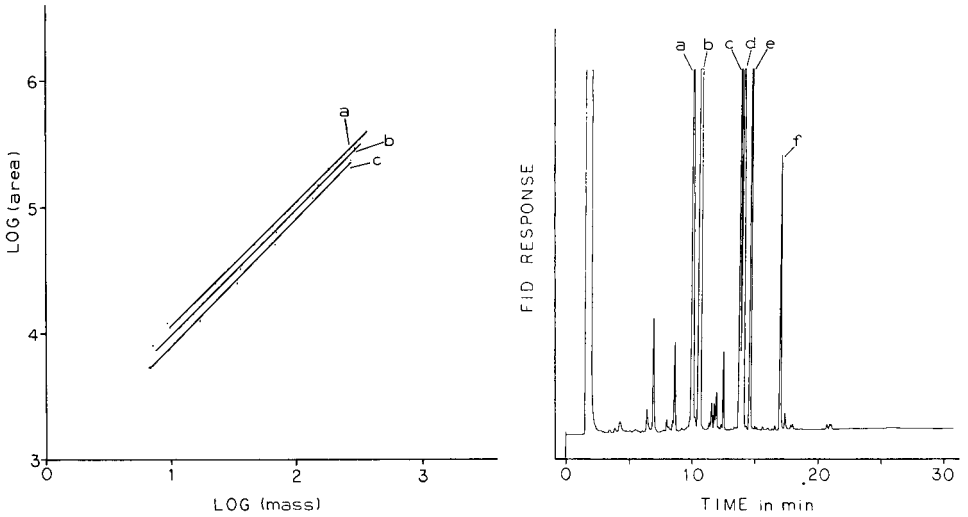

Fig. 3. Calibration curve for Me-ETA (c), Me-AA (b) and Me-EPA (a) based on the 79.1 a.m.u. ion from SIM analysis. Values for mass are in nanograms absolute.

Fig. 4. Chromatogram from GC–FID analysis of lung-tissue extract. For identities of major components (a–f) see Table II.

levels of contamination from glassware, vials, extraction apparatus and sample handling. Small amounts (less than 1 ng/μl) of Me-AA were detected in these blanks; other methyl esters were detected, but below 1 ng for 1-μl injections. Although the origins of these small amounts of contaminants are unknown, by comparison with the natural abundance (12 to 912 ng/μl), these errors in quantitative analyses are negligible.

Extracts of these tissues were relatively free from interfering components, and results from GC–FID analysis of the extract of the lung sample are shown in Fig. 4. Less than twenty major components were detected and were tentatively identified by using mass spectra. These compounds, which are listed in Table II, were present in every extract and thus may also be expected to be widely abundant. The three unsaturated fatty acids were not completely resolved on this capillary column.

TABLE II

COMPOUNDS IDENTIFIED USING MASS SPECTRA FROM ANALYSIS OF LUNG TISSUE

Reference to Fig. 4	*Retention time (min)*	*Compound*	*Molecular ion (a.m.u.)*	*Major ions (a.m.u.)*
a	12.8	Me-Palmitoleate	268	55, 74, 69, 236
b	13.5	Me-*n*-Hexadecanoate	260	74, 87, 143
c	16.4	Me-Linoleate	294	55, 67, 71, 95
d	16.6	Me-Oleate	296	55, 69, 74, 264
e	17.2	Me-*n*-Octadecanoate	298	74, 87, 143, 267
f	19.3	Me-Arachidonate	N.D.	79, 67, 91, 55

Results from the "spiking" study showed average extraction efficiencies and standard deviations for each acid were: ETA, 84 ± 14; AA, 48 ± 6; and EPA, 48 ± 1 %. When corrected for average derivatization efficiency the overall net recoveries and standard deviations* for the entire analytical procedure were: ETA, 47 ± 8; AA, 37 ± 6; and EPA, 28 ± 10 %. Results from analysis of several types of tissue are given in Table III. These values are expressed in units of μg/g and have been corrected for overall recovery efficiency. Every tissue extract contained more than 2 ng/μl of ETA, AA and EPA. However, with values lower than 10 ng/μl, automated integration failed, and triangulation was unreliable for peaks of this low abundance, especially with large full-scale values for Me-AA. All three unsaturated fatty acids were found in representative frog tissues. The data suggests a natural abundance, in order of

TABLE III

ANALYSIS OF PROSTAGLANDIN PRECURSORS IN FROG TISSUES

Sample No.	*Tissue type*	*Weight (g)*	*Concentration in extract (ng/μl)*			*Concentration in tissue (μg/g)*		
			ETA	*AA*	*EPA*	*ETA*	*AA*	*EPA*
1	Heart	0.96	<5	525	<5	Tr*	1330	Tr
2	Heart**	1.08	12	759	219	30	2280	870
3	Heart**	0.42	<5	347	95	Tr	2680	970
4	Heart**	0.74	20	457	110	60	1670	530
5	Bladder	0.29	<5	174	<5	Tr	2110	Tr
6	Bladder	0.13	<5	87	<5	Tr	2350	Tr
7	Bladder	0.56	20	416	<5	60	2810	Tr
8	Bladder**	0.66	13	263	100	50	1290	650
9	Bladder**	0.18	<5	93	110	Tr	1670	2620
10	Bladder**	0.18	<5	79	75	Tr	1420	1780
11	Gastric mucosa	1.26	71	912	117	130	2150	360
12	Gastric mucosa	1.26	63	870	107	120	2050	330
13	Gastric mucosa	1.34	53	794	72	100	1920	230
14	Lung	0.99	46	603	59	130	2140	280
15	Atria	0.19	<5	96	<5	Tr	1640	Tr
16	Atria	0.14	<5	96	<5	Tr	2220	Tr
17	Ventricle	0.38	<5	200	<5	Tr	1560	Tr
18	Ventricle	0.51	<5	437	30	Tr	2540	230
19	Conus arteriosus	0.15	<5	87	<5	Tr	1880	Tr
20	Conus arteriosus	0.10	<5	17	<5	Tr	550	Tr

* Tr indicates trace amount, insufficient for quantification.

** Pooled tissue from five to seven frogs (*Rana pipiens*); all other values are individual tissues from *R. catesbeiana*.

* $s_{total} = \bar{x}(\sqrt{s_1^2 + s_2^2})$ where $\bar{x}$ is average net recovery, s_1 is relative standard deviation of derivatization and s_2 is relative standard deviation of extraction. s_{total} is given in absolute standard deviation.

decreasing concentration: AA, EPA, ETA. Thus, AA is the most abundant prostaglandin precursor in *R. catesbeiana* tissues, and undoubtedly offers a major pool of substrate for bisenoic prostaglandin synthesis. The other fatty acid precursors are also available in significantly smaller quantities. These amounts are several orders of magnitude greater than the physiological concentrations of prostaglandins and could therefore be utilized for synthesis of monoenoic and trienoic prostaglandins. Interestingly, in the *R. pipiens* tissue examined, both AA and EPA represented major fatty acid components.

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STUDY OF ALICYCLIC ETHYL *cis*- AND *trans*-2-HYDROXYCARBOXYLATES BY GAS CHROMATOGRAPHY

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(First received June 4th, 1981; revised manuscript received September 15th, 1981)

SUMMARY

The structure and conformation of alicyclic *cis*- and *trans*-2-hydroxycarboxylates were studied by gas chromatography. As these substances serve as starting materials for the synthesis of stereospecific alicyclic compounds, it was of major importance to establish the stereochemical uniformity of each isomer. Further, the study of the structure and conformation of the iosmers was important *per se*, as these compounds, containing hydroxy and carbethoxy groups in vicinal positions, are prone to form intramolecular and intermolecular hydrogen bonds. Gas chromatography was the method of choice for the study of these hydrogen bonded structures.

INTRODUCTION

The synthesis of *cis*- and *trans*-2-hydroxycyclopentanecarboxylic acid[1], *cis*- and *trans*-2-hydroxycyclohexanecarboxylic acid[2], *cis*- and *trans*-2-hydroxycycloheptanecarboxylic acid[3,4] and *cis*- and *trans*-2-hydroxycyclooctanecarboxylic acid[4] has been reported. In the study of these compounds, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy[5,6] were primarily used for the conformational analysis of intramolecular and intermolecular hydrogen bonds and the elucidation of the preferred conformation of the substituents. In addition to spectroscopic studies, Castells and Palau[5] also used viscosity measurements to confirm that the *cis*-isomer of 2-hydroxycyclohexanecarboxylic acid is less associated than the *trans*-isomer. In the course of conformational analyses, Baumann and Möhrle[7,8] determined the equilibrium constants of the isomers[9] and found that equatorial hydroxy groups promote a more pronounced association than axial groups. The preferred conformation of Ia and Ib (Fig. 1) was established, in agreement with literature data[10], by Baumann and Möhrle on the basis of NMR spectra[8] and by Bernáth *et al.*[4] on the basis of both IR and NMR spectra, and the measurement of the rate of chromic acid oxidation and dissociation constants[6]. According to NMR spectra, in *cis*-2-hydroxycyclohexanecarboxylic acid the equatorial position may be assigned to the carboxy group and the axial position to the hydroxy group[6].

By calculating the dipole moments of compounds I–III (Fig. 1), a means was

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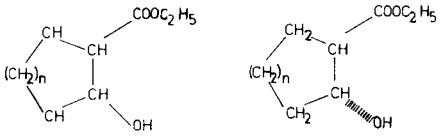
established for establishing configuration–physical data relationships[11]. In earlier studies the retention index–molecular structure relationship for alicyclic *cis*- and *trans*-2-hydroxycarboxylates was studied by gas chromatography with the help of empirical equations[12] on the basis of the additivity of gas chromatographic interactions.

In this study the configuration of isomers of compounds I–IV (Fig. 1), the interactions of vicinal hydroxy and carbethoxy groups and the interactions of the stationary phase and individual groups were examined in detail.



Ia: n=1 Ib: n=1
IIa: n=2 IIb: n=2
IIIa: n=3 IIIb: n=3
IVa: n=4 IVb: n=4

Fig. 1. Model compounds

EXPERIMENTAL

Materials and reagents

The silylating agents hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCMS) were obtained from Pierce (Rockford, IL, U.S.A.) and the stationary phases SE-30 and Reoplex 400 and the carrier Chromosorb W AW DMCS (80-100 mesh) from Applied Science Labs. (State College, PA, U.S.A.). The synthesis of model compounds was reported in refs. 4 and 11.

Gas chromatography

An HP 5830 gas chromatograph equipped with a flame-ionization detector was used. The glass columns (2 m × 4 mm I.D.) were packed with the stationary phases SE-30 (5%) and Reoplex 400 (20%) (columns A and B, respectively). The injection temperature was 240°C, the column temperatures were (A) 120°C and (B) 160°C and the detector temperature was 240°C. The carrier gas (nitrogen) flow-rate was 45 ml/min.

Derivatization

Amounts of 1 mg of compounds I–IV were made to react with the silylating reagent pyridine–HMDS–TMCS (2:2:1) at room temperature for 1 h. A 1-μl volume of each sample was injected into the apparatus.

RESULTS AND DISCUSSION

The physical data for ethyl *cis*- and *trans*-2-hydroxycyclopentanecarboxylate

(Ia and b), ethyl *cis*- and *trans*-2-hydroxycyclohexanecarboxylate (IIa and b), ethyl *cis*- and *trans*-2-hydroxycyclopheptanecarboxylate (IIIa and b) and ethyl *cis*- and *trans*-2-hydroxycyclooctanecarboxylate (IVa and b) are compiled in Table I. The physical data for the *cis*-isomers are generally higher than those for the *trans*-isomers, except for their boiling points and viscosities. These types of compounds follow Van Alkel's rule[11], and the higher boiling points of the *trans*-isomers may be due to associated molecules. This property of the molecules is retained under gas-chromatographic conditions, also as is apparent from the relative retention data in Table II, the *trans*-isomers being eluted from both columns with higher retention times than the *cis*-isomers. Compared with the more hindered *cis*-isomers, in the *trans*-isomers the equatorial hydroxy group can interact freely with the stationary phase, resulting in higher retention times. Möhrle and Baumann[9], in assessing the equilibrium constants of the compounds, concluded that association is much better promoted by equatorial than by axial hydroxy groups. Under gas chromatographic conditions no significant association occurs, but it remains an inherent property of the *trans*-isomers to interact more intensely than *cis*-isomers with the stationary phase. Evaluating the separation

TABLE I

PHYSICAL DATA FOR ALICYCLIC ETHYL *cis*- AND *trans*-2-HYDROXYCARBOXYLATES

Compound	*Isomer*	*B.p. (°C)*		n_D^{25}		η^{25} *(lit.)*
		Measured	*Literature*	*Measured*	*Literature*	
Ethyl 2-hydroxy-cyclopentane-carboxylate	*cis*	129–130 (42 mmHg)	54–56 (0.1–0.2 mmHg) (ref. 10)	1.4551	1.4551 (ref. 10)	0.075 (ref. 10)
	trans	139–140 (42 mmHg)	57.5–60 (0.1–0.2 mmHg) (ref. 10)	1.4536	1.4534 (ref. 10)	0.084 (ref. 10)
Ethyl 2-hydroxy-cyclohexane-carboxylate	*cis*	134–135 (39–40 mmHg)		1.4600	1.4600 (ref. 17)	0.109 (ref. 17)
	trans	143–144 (39–40 mmHg)	120–121 (30 mmHg) (ref. 17)	1.4596	1.4596 (ref. 17)	0.191 (ref. 17)
Ethyl 2-hydroxy-cycloheptane-carboxylate	*cis*	142–143 (40 mmHg)		1.4684	1.4683 (ref. 11)	
	trans	148–149 (40 mmHg)		1.4670	1.4675 (ref. 17)	
Ethyl 2-hydroxy-cyclooctane-carboxylate	*cis*	147–148 (40 mmHg)		1.4750		
	trans	151–152 (40 mmHg)		1.4749		

TABLE II

RELATIVE RETENTIONS AND SEPARATION FACTORS OF ALICYCLIC ETHYL *cis*- AND *trans*-2-HYDROXYCARBOXYLATES

Compound	*Relative retention**		*Separation factor (α)***	
	5% SE-30, 120°C	*20% PEGA, 160°C*	*5% SE-30, 120°C*	*20% PEGA, 160°C*
Ia	4.50	4.74	1.18	1.62
Ib	5.30	7.70		
IIa	7.10	6.70	1.12	1.33
IIb	7.90	8.90		
IIIa	13.70	9.82	1.09	1.33
IIIb	15.00	13.10		
IVa	26.77	17.83	1.00	1.28
IVb	26.77	22.86		
Cyclohexanol	1.00 (2 min)	1.00 (2.2 min)		

* t' relative to cyclohexanol = 1.00.

** $\alpha = \frac{t'_{trans}}{t'_{cis}}$.

factors in Table II, it is apparent that the highest value was obtained in the gas chromatography of compounds Ia and b. This is in good agreement with spectroscopic data[10], confirming that there is a strong intermolecular interaction in the Ib isomer. At the same time we failed to achieve the separation of isomers IVa and b on SE-30. Compounds with a cyclooctane skeleton are the first representatives of medium-sized rings. The cyclooctane ring is flexible and the apolar stationary phase fails to promote the preferential positioning of the isomers which would permit separation. There exists a complex equilibrium as regards isomer conformation. The bulky carbethoxy group assumes preferentially an equatorial position, while the smaller hydroxy group may be either equatorial or axial. However, there is no question of an exclusively equatorial or axial orientation, only of a major proportion of one of the conformers. In Fig. 2 a representative example of the conformational equilibrium of compounds Ia and b is demonstrated.

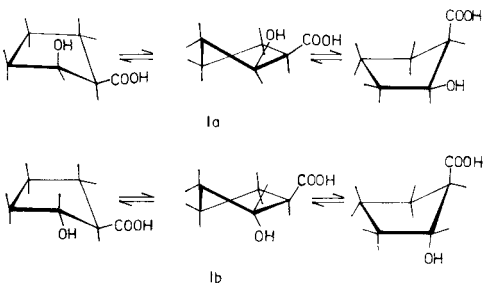


Fig. 2. Conformational equilibrium of ethyl *cis*- and *trans*-2-hydroxycyclopentanecarboxylate.

ABLE III

ELATIVE RETENTIONS AND SEPARATION FACTORS OF TRIMETHYLSILYLATED ALICYCLIC THYL *cis*- AND *trans*-2-HYDROXYCARBOXYLATES

mpound	*Relative retention**					*Separation factor (α)***				
	5% SE-30			*20% PEGA*		*5% SE-30*			*20% PEGA*	
	120°C	*140°C*	*160°C*	*120°C*	*160°C*	*120°C*	*140°C*	*160°C*	*120°C*	*160°C*
a	9.83			2.56		0.81			0.66	
b	8.00			1.71						
yclohexa-	1.00			1.00						
nol	(2.4 min)			(6.4 min)						
Ia		10.00		4.27			1.00		0.84	
Ib		10.00		3.55						
yclohexa-		1.00		1.00						
nol		(1.4 min)		(6.4 min)						
IIIa			11.00	6.25				1.00	1.00	
IIIb			11.00	6.25						
Cyclohexa-			1.00	1.00						
nol			(0.65 min)	(6.4 min)						
IVa			15.00		7.51			1.00		1.00
IVb			15.00		7.51					
Cyclohexa-			1.00		1.00					
nol			(0.52 min)		(4.6 min)					

* t' relative to cyclohexanol = 1.00.

** $\alpha = \frac{t'_{trans}}{t'_{cis}}$.

If the compounds are trimethylsilylated and subsequently submitted to gas chromatography (Table III), the elution order of compounds Ia and b and IIa and b is reversed, and compounds IIIa and b and IVa and b could not be separated on either of the stationary phases. The existence of an intramolecular hydrogen bond between the carbethoxy and hydroxy groups, reducing the polarity of the parent molecule, which is consequently eluted at a lower retention time, is confirmed by the reversal of the elution order following derivatization. In the presence of the protective group no intramolecular hydrogen bond is formed, and the components are eluted according to their physical properties. The rate of interaction between the trimethylsilyl *cis*- and *trans*-isomers and the stationary phase is reduced, in the case of compounds IIIa and b and IVa and b, to such an extent that the isomers cannot even be separated (see the separation factors in Table III). The α values in Table II decrease with increasing ring size; as the strength of intramolecular hydrogen bonds is lowered even the *cis*-isomers may show stronger interactions with the stationary phase.

The separation may be affected, in addition to hydrogen bond interactions, by the existence of different conformers. Under the conditions of gas chromatography the bulky trimethylsilyl group is in an equatorial position whereas the carbethoxy

group of compounds Ia and b and IIa and b may be either in an axial or an equatorial position. In compounds IIIa and b and IVa and b the bulky trimethylsilyl group is always equatorial and the carbethoxy group connected to the carbon atom may easily take on a conformation where there is practically no difference between the angle of the two groups in the *cis*- and *trans*-isomers.

In Fig. 3 the conformational equilibrium of compounds IIa and b and IIa and b-TMS is demonstrated.

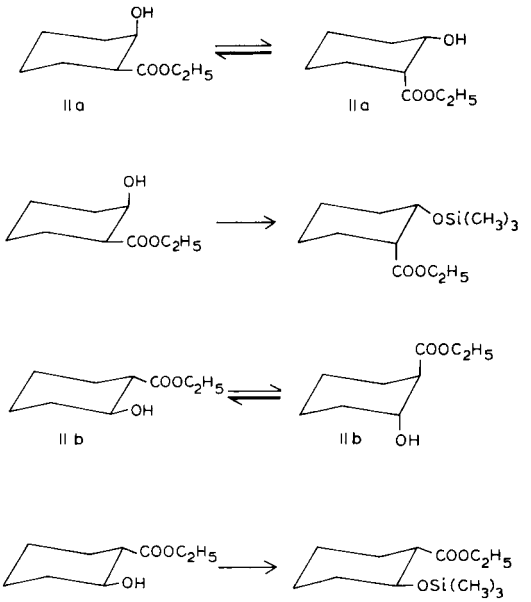


Fig. 3. Conformational equilibrium of compounds IIa and b and IIa- and b-TMS.

In the studies of intermolecular hydrogen bonds the retention indices of the compounds were measured as a function of column temperature. With increasing temperature the retention index increments of the *cis*-isomers were scarcely surpassed by those of the trans-isomers (Table IV). The interaction between the hydroxy and carbethoxy groups was reduced in the *cis*- and *trans*-isomer, respectively, with increasing column temperature and increasing ring size.

The interaction between the stationary phase and the axial and equatorial hydroxyl groups was enhanced.

The increase in retention indices with increasing column temperature indicates that both isomers are prone to enhanced association and interaction with the stationary phase. The importance of solubility differences in the separation of the isomers is well demonstrated by the standard molar free energy differences (Table V). With isomers of compounds I–III it can be concluded that the higher are the negative values the higher are the solubility differences between the pairs of isomers. As the conformation and the standard molar free energy differences are strongly correlated, it is assumed that the stronger the stabilizing effect exerted by the stationary phase on the isomers the better is the separation.

TABLE IV

RETENTION INDICES OF ALICYCLIC ETHYL *cis*- AND *trans*-2-HYDROXYCARBOXYLATES AS A FUNCTION OF COLUMN TEMPERATURE

Stationary phase: PEGA.

Compound	*Isomer*	*Column temperature (°C)*							
		150		*160*		*170*		*180*	
		*I**	*b***	*I**	*b***	*I**	*b***	*I**	*b***
Ethyl 2-hydroxy-cyclopentane-carboxylate	*cis*	1934	0.159	1944	0.165	1964	0.151	1974	0.141
	trans	2074	0.157	2082	0.154	2101	0.146	2110	0.141
Ethyl 2-hydroxy-cyclohexane-carboxylate	*cis*	2003	0.157	2017	0.154	2038	0.146	2048	0.141
	trans	2106	0.157	2116	0.154	2134	0.146	2144	0.143
Ethyl 2-hydroxy-cycloheptane-carboxylate	*cis*	2168	0.157	2180	0.154	2202	0.155	2218	0.148
	trans	2273	0.157	2283	0.160	2306	0.146	2318	0.148
Ethyl 2-hydroxy-cyclooctane-carboxylate	*cis*	2310	0.159	2328	0.161	2353	0.146	2361	0.148
	trans	2377	0.159	2391	0.161	2416	0.160	2422	0.140

* I = retention index (index units).
** b = slope of n-alkane curve.

TABLE V

SEPARATION FACTORS (α) MEASURED AT 120°C AND CALCULATED STANDARD MOLAR FREE ENERGY DIFFERENCES OF ALICYCLIC ETHYL *cis*- AND *trans*-2-HYDROXY-CARBOXYLATES $\Delta(\Delta G)^0$

Compound	*Stationary phase*			
	5% SE-30		*20% PEGA*	
	$\Delta(\Delta G)^0$ *(cal/mole)*	α*	$\Delta(\Delta G)^0$ *(cal/mole)*	α*
Ethyl 2-hydroxy-cyclopentane-carboxylate	−129.0	1.18	−548.1	2.02
Ethyl 2-hydroxy-cyclohexane-carboxylate	−59.9	1.08	−440.7	1.76
Ethyl 2-hydroxy-cycloheptane-carboxylate	−67.2	1.09	−356.6	1.58

* $\alpha = \frac{t'_{trans}}{t'_{cis}}$.

The $\Delta(\Delta G^0)$ values were calculated from the equation

$$\Delta(\Delta G^0) = -RT\ln\alpha$$

The α values are given in Table V.

Vapour pressure differences also play an important role in the separation of the isomers. Isomers of compounds I–III could be separated by fractional distillation on a high-resolution column. The separation of isomers IVa and b required multiple fractionation and a stereochemically uniform product could be obtained only by preparative gas chromatography[4].

On the basis of relative retentions, the temperature dependence of the retention index and standard molar free energy data, it can be concluded that the preferred conformation of the substituents promotes mainly intramolecular and partly intermolecular interactions in the *cis*-isomers, whereas in the *trans*-isomers the intermolecular interactions are uniformly predominant, *i.e.*, hydrogen bonded structures exist even under the conditions of gas chromatography.

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STANDARDIZATION OF AROCLOR LOTS FOR INDIVIDUAL-PEAK GAS CHROMATOGRAPHIC CALIBRATION

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SUMMARY

Commercially available Aroclors were compared to characterized lots of Aroclors to estimate their weight-percent composition, and thus expand the availability of characterized Aroclors required for individual peak calibration. Individual peak calibration is recommended for the gas chromatographic electron capture determination of polychlorinated biphenyls which result from partially degraded Aroclors. An application is described where polychlorinated biphenyls are determined in chemically dehalogenated oil using response factors derived from characterized Aroclor standards. Rapid clean-up using disposable silica cartridges was used to prepare oil samples prior to gas chromatographic analysis.

INTRODUCTION

The concern for widespread polychlorinated biphenyl (PCB) contamination in the environment has resulted in an increasing need for PCB determinations in a variety of samples. The most common and the most sensitive instrumental technique has been gas chromatography with electron-capture detection (GC–ECD). The analysis is complicated since the "PCB concentration" represents the sum of the individual concentrations of mono- through decachlorobiphenyl and their isomers. This amounts to a total of 209 theoretically possible compounds, many of which have different sensitivities at the electron-capture detector.

The ultimate method would provide the separate quantitation of each PCB compound. The rigorous analytical calibration of all 209 compounds has not been reported; however, this capability may eventually be realized since capillary GC provides resolution superior to that obtained using packed GC columns, and *ca.* 80 PCB compounds are commercially available for calibration [Ultra Scientific Inc. (formerly RFR Corp.), Hope, RI, U.S.A.]. In practice, as this goal is approached, the analysis becomes cumbersome and one that would not usually be appropriate for routine use. The analysis can be simplified by making varying levels of approximations, and so the reliability of the PCB analyses depends upon the validity of these assumptions. The limitations of such approximations must be recognized and justi-

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fied according to the type of PCB contamination encountered.

Aroclors® are complex mixtures of PCB compounds which were used in a variety of applications such as product additives, coolants and insulating fluids. Aroclors are identified by the type of molecule (12 = biphenyl) and the total weight-percent of chlorine, *e.g.* Aroclor 1232 is a biphenyl containing 32% chlorine.

Aroclor 1016 is an exception; it is a biphenyl containing 41% chlorine. Aroclors with the prefixes 54, 25, and 44 are chlorinated terphenyls and blends of PCBs with chlorinated terphenyls. The Aroclors 1242, 1254 and 1260 were produced in the largest amounts and are generally considered the most prevalent in the environment.

The GC–ECD analysis is simplified when contamination is due to a single Aroclor and the relative peak intensities in the chromatogram match those of an available Aroclor standard. In this case, quantitation has been accomplished by comparing selected peak heights or total peak areas for the samples to those in Aroclor standards[1–3]. This simplistic approach, however, is not suitable for quantitating PCB contamination arising from: (i) Aroclors which have partially decomposed through biological or chemical action; (ii) PCBs not originating from an Aroclor; (iii) mixtures of Aroclors[4,5].

A GC calibration technique was proposed by Webb and McCall[6] which employed individual-peak response factors. A table was provided for each Aroclor where the weight-percent composition of each peak was listed and the peaks were identified by whole numbers representing their relative retention times *versus* a reference compound, *p,p'*-DDE (1,1-dichloro-2,2'-bis-*p*-(chlorophenyl)ethylene), defined as 100. The weight-percent compositions of Aroclors were determined using GC–mass spectroscopy and a Coulson conductivity (or Hall-type) detector. These tables were used for the individual calibration of each peak, with the precaution that the tables are valid *only* for these specific lots of Aroclors.

Sawyer[7] characterized a set of Aroclors in the same manner as Webb and McCall and used these Aroclors in an interlaboratory comparison of analytical methods. This study concluded that individual peak calibration is the most reliable approach for samples containing a non-Aroclor PCB residue[5].

We have made a peak-by-peak comparison of other lots of Aroclors (available commercially for GC calibration) to characterized lots of Aroclors in order to provide an estimate of their weight-percent compositions. This expands the availability of characterized Aroclor standards required for individual-peak calibration of PCBs, and also provides some insight as to the variation which might be expected from different lots of the same type of Aroclor.

Application of the individual-peak calibration technique was made to monitor a plant-scale process for the economical removal of PCBs from oil (to less than 5 ppm) by chemical dehalogenation with sodium naphthalide reagent[8] (see Fig. 2). Recommendations are made for establishing appropriate response factors required for this determination. Consideration is given to the possibility of variations in the ratio of co-eluting isomers for an individual peak occurring during chemical treatment.

A rapid sample preparation scheme is proposed as an alternative to a more tedious Florisil clean-up, recommended by the U.S. Environmental Protection Agency (EPA).

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5840A gas chromatograph equipped with a ^{63}Ni electron-capture detector (15 mCi) and automatic liquid sampler, Model 7671A, was used.

Sep-Pak® silica cartridges, used for sample clean-up, were obtained from Waters Assoc. (Milford, MA, U.S.A.) part No. 51900.

GC conditions

GC conditions were maintained similar to those reported elsewhere[6,7,9] to retain the order of elution of PCB compounds as listed in the weight-percent composition tables being referenced.

A glass column (183 cm × 2 mm I.D.) was packed with 3% OV-1 on Supelcoport, 100–120 mesh. The carrier gas was methane–argon (5:95) at a flow-rate of 20 ml/min. The column and detector temperatures were maintained at 180°C and 250°C, respectively. The injection volume was 5 μl and the attenuation range was 2^4 to 2^6 (or 16 to 64 ×).

Chemicals

Solvent. Pesticide-grade hexane (Burdick & Jackson, Muskegon, MI, U.S.A.), was used in the preparation of all samples and standards, and as a final rinse for all glassware.

Aroclor standards. The source and lot numbers for each series of Aroclors used in this study are given in Table I. Commercial suppliers, *viz.* Applied Science, Analabs and Ultra Scientific, have indicated through personal communication that they are

TABLE I

LOT NUMBERS FOR AROCLORS USED IN THIS STUDY

Aroclor	*Source**				
	Applied Science	*Analabs*	*Ultra Sci.*	*Sawyer*[7]	*Webb/McCall*[6]
1016	721	F-216A	NA**	77029	NA
1221	101	K-099F	NA	NA	–***
1232	17	NA	–	NA	–
1242	KA-478	J-147C	–	71696	AK55
1248	07771	L-279	NA	71697	–
1254	610	J-147A	NA	71698	AK38
1260	07771	NA	–	71699	–
1268	NA	G-266M	–	NA	NA

* Applied Science Products, State College, PA, Cat. No. 19589; Analabs, North Haven, CT, Cat. No. RCS-066; Ultra Scientific Inc., RI, Cat. No. RPCK-1; Sawyer, these are the same Aroclor lots as characterized in ref. 7, obtained from the Food and Drug Administration, Washington, DC; Webb/McCall, these are the same Aroclor lots as characterized in ref. 6, obtained from Radian Corporation, Austin, TX.

** Not available from this source.

*** No lot number listed on Aroclor sample.

dispensing from single lots of each Aroclor and, as a lot of Aroclor is exhausted, they will discontinue supplying that Aroclor. These Aroclors were produced by Monsanto (St. Louis, MO, U.S.A.), however, no identification such as batch number or time of production is available.

Preparation of Aroclor solutions

Separate solutions of the following Aroclors were accurately prepared to contain *ca.* 1 mg/ml: Aroclors 1016, 1221, 1232, 1242, 1248, 1254 and 1260. These solutions are stable indefinitely provided they are properly sealed, refrigerated and protected from ultraviolet light.

Working standards were prepared daily by diluting 30 μl of the above solutions to 50 ml with hexane. These solutions will contain *ca.* 600 ng/ml or 3 ng/5 μl injection aliquot.

Procedure for comparison of Aroclor lots

The comparison of different lots (or sources) of the same type of Aroclor was made as follows. A single GC run was made for solutions prepared with each available lot, then this sequence was repeated a minimum of nine times. The relative standard deviation of individual peak areas, determined from the repetitive injections of a single Aroclor lot, was generally *ca.* 1–2% for the major Aroclor components. This GC precision was usually well within the precision reported by Sawyer[7] for the weight-percent determinations of these peaks using a Hall detector.

Individual-peak calibration with Aroclors

To calibrate for sample analyses, chromatograms with integrated peak areas were obtained for known injected weights of each of the Aroclors, 1221 through 1260. Each peak in the chromatograms was identified by a whole-number relative retention time (R_{DDE}) *versus p,p'*-DDE, whose retention time is defined as 100 (*ca.* 17 min). This format, which was originally used by Webb and McCall[6], is also used throughout this article. Labeled chromatograms (Fig. 1) show the R_{DDE} and resolution for some of the Aroclor peaks. The response factor for each peak is calculated as follows:

$$R_{F(n)} = \frac{W_{tot} \cdot \%_{on}}{A_n \cdot 100}$$

where $R_{F(n)}$ is the external response factor for a peak with a relative retention time (R_{DDE}) of n; W_{tot} is the total weight of Aroclor injected; $\%_{on}$ is the weight-percent of peak "n" in the Aroclor; A_n is the integrated area for peak "n".

These peak response factors, determined with several Aroclors, are shown in Table II along with a calculated overall (or average) factor. Considerations related to the calculation of these factors are discussed in the text.

Preparation and clean-up of oil samples

An accurately weighed portion of oil sample (*ca.* 1–10 g) was dissolved in 50 ml of hexane. A Sep-Pak silica cartridge was attached to a 5-ml glass syringe and pre-eluted with 5 ml of hexane. An aliquot of the sample solution (1–5 ml) was passed through the cartridge. The PCBs were eluted from the cartridge with three 3-ml portions of hexane while the unwanted constituents were retained on the Sep-Pak. All eluent was collected in a 25-ml volumetric flask and diluted to the mark with hexane.

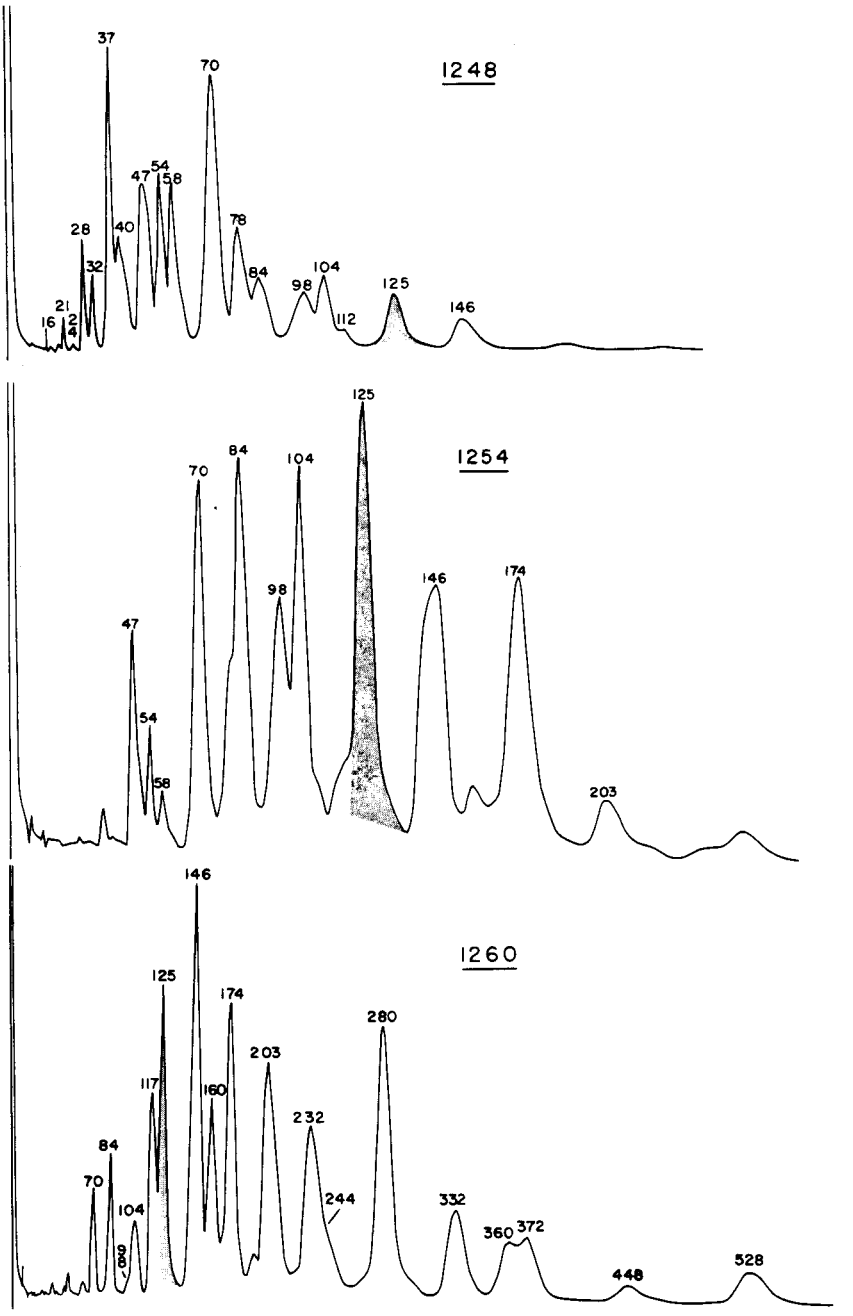

Fig. 1. Chromatograms for selected Aroclors. Peaks are identified by retention times relative to *p,p'*-DDE = 100.

TABLE II

RELATIVE RESPONSE FACTORS* DETERMINED USING CHARACTERIZED AROCLORS

No. of chlorines	*Peak R_{DDE}***	*Overall R_F*§	*Aroclors***						
			1221	*1232*	*1016*	*1242*	*1248*	*1254*	*1260*
	11	23	27	18					
	14	38	45	30					
	16	13	16	12	12	(152)	(1.3)		
	19	2.0	2.0	3.5	–	–	–		
2	21	3.8	4.7		2.8	–	–		
	24	2.9	–	–	2.9	(0.42)	(0.26)		
	28	3.7	4.4	3.5	3.8	4.0	2.6		
	32	2.4	3.6	2.2	2.3	2.5	1.5		
3	37	1.4	1.2	1.3	1.7	1.7	1.0		
	40	1.5		1.5	1.7	1.7	1.4		
	47	1.6		1.2	1.7	1.7	1.8	1.7	
	54	1.5		1.4	1.5	1.6	1.5	1.3	
	58	1.0		1.1	1.2	1.0	1.1	0.77	
4	70	1.0		0.88	0.77	0.86	1.1	1.3	1.1
	78	0.72		0.88		0.46	0.83	–	–
	84	0.96					0.76	1.2	0.88
	98	0.68					0.55	0.83	0.66
5	104	0.74					0.48	1.0	
	112	0.87					0.87	–	–
	117	0.60					–	–	0.60
	125	0.62					0.29	0.65	0.93
	146	0.47					0.23	0.50	0.68
	160	0.63							0.63
	174	0.51						0.39	0.62
	203	0.42						0.23	0.61
6	232	0.55							0.55
	244	0.55							
7	280	0.47							0.47
	332	0.42							0.42
	360	0.41							0.41
	372	0.41							
	448	0.24							0.24
	528	0.28							0.28
	900	0.44	(decachlorobiphenyl)						

* These are relative values, provided to compare peak factors obtained using different Aroclors. Actual response factors must be determined by each analyst.

** Aroclor 1221 and 1232 were portions of the same lots characterized by Webb and McCall[6]. The remaining Aroclors were from lots characterized by Sawyer[7].

*** Relative retention time for Aroclor peaks *versus* *p,p'*-DDE (assumed to be 100).

§ Overall response factors represent the average of values obtained using different Aroclors. Factors were excluded from the average when judged to be inaccurate due to a low abundance of these isomers in the Aroclor.

RESULTS AND DISCUSSION

Commercial and characterized Aroclor lots

Individual-peak calibration using Aroclors is a widely recommended approach for the determination of PCB residues, especially those arising from the degradation of Aroclors. Webb & McCall[6] and Sawyer[7] determined the weight-percent distribution of PCBs in different types of Aroclors according to the resolved peaks in their GC–ECD chromatograms. These lots of Aroclors were then used to calibrate individual peaks. It was emphasized that the distribution of PCBs may vary for different lots of the same Aroclor type, and so these characterizations are valid only for their specific Aroclor lots. The U.S. Food and Drug Administration is currently using Sawyer's lots of Aroclors as standards for their PCB analyses.

Characterized Aroclors for this type of calibration are not commercially available. Such characterization requires an effort and equipment which are beyond the capability of many analytical laboratories. Aroclors generally marketed for GC calibration (Table I) have not been characterized in this manner. These commercial suppliers have indicated that their Aroclors are from single, but randomly obtained, lots originally produced by Monsanto for industrial applications.

When calibrating with non-characterized lots of Aroclors, the assumption is implied in the analysis that Aroclors of the same type have identical compositions. To test this assumption, we have made direct, statistical comparisons of Aroclors from several GC supply houses to the same lots of Aroclors characterized by Sawyer[7] and by Webb and McCall[6]. The peak compositions (weight-percent) were calculated for the commercial Aroclors and are given in Tables III-IX. Sawyer's Aroclor standards were used as the reference in most comparisons. Since Aroclors 1221 and 1232 were not characterized by Sawyer, Webb and McCall's values were used for them.

This indexing of commercially available lots of Aroclors to characterized Aroclor standards expands the availability of standards suitable for individual-peak calibration and provides some insight as to how different lots of the same Aroclor compare.

Comparison of Aroclor lots

Variations in the calculated peak compositions are noted for different lots of the same Aroclor type. These differences were tested for significance using the "*t*-test" at an 80% confidence level[10]. An Aroclor lot could be considered to be the same as the reference Aroclor (Sawyer or Webb and McCall) if the majority of the prominent peaks passed the "*t*-test". This did occur for some lots of Aroclors 1248 and 1016.

Although most Aroclor lots were not statistically identical by this criterion, the differences in the peak compositions between many lots were relatively small. In several cases, this difference was within the precision reported in the original weight-percent characterizations of the reference Aroclors.

From this limited survey, it appears that application of Sawyer's or Webb and McCall's weight-percent composition tables to other lots of Aroclors would not always result in gross errors in the response factor calibration. The best analytical practice, however, would dictate that the calibration be done with Aroclor lots where the weight-percent composition of each peak has been established. The weight-percent composition data for commercially available Aroclor standards are provided in Tables III–IX.

TABLE III

COMPOSITION OF AROCLOR 1016 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1016 STANDARD

Peak R_{DDE}**	*Wt.% reported for standard*		*Wt.%, calculated**	
	Sawyer[7]	*HECD Precision****	*Applied Science* §	*Analabs* §
11	0.2	(±0.05)	0.2	0.2
16	3.8	(0.3)	3.5	3.8
21	8.1	(0.6)	7.5	7.8
24	1.2	(0.1)	1.2	1.2
28	16.8	(1.1)	16.8	16.8
32	7.6	(0.6)	7.5	7.5
37	18.5	(1.3)	18.4	18.2
40	14.6	(1.0)	14.3	14.1
47	11.6	(0.9)	11.6	11.7
54	7.7	(0.5)	7.1	7.4
58	6.4	(0.5)	5.6	6.2
70	3.4	(0.4)	1.7	2.3

* Weight-percent composition for each peak was calculated relative to that in a characterized Aroclor standard. The weight-percent reported for the characterized Aroclor (Wt. $\%_{oStd}$) and the relative peak areas (A_{std}, A_x) for equal amounts of the two Aroclors injected were used.

$$\text{relative wt.}\% = \frac{A_x}{A_{Std}} \cdot \text{Wt.}\%_{oStd}$$

Aroclor lot numbers are given in Table I. Each value represents the average of nine runs.

** Relative retention times for Aroclor peaks *versus p,p'*-DDE (assumed to be 100).

*** Absolute precision for the weight-percents reported[6,7] for the characterized Aroclor standard using a Hall electrolytic conductivity detector (HECD). This is included to show the uncertainty of the standard weight percent values and does not reflect precision for the comparison of Aroclor lots by GC–ECD.

§ Application of the "*t*-test"[10] indicates that the weight-percents for the major Aroclor peaks are statistically equivalent to those of the characterized Aroclor standard, *i.e.* the existence of a difference was not proved with a confidence limit of 80%.

TABLE IV

COMPOSITION OF AROCLOR 1221 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1221 STANDARD

Peak R_{DDE}*	*Wt.% reported for standard*		*Wt.%, calculated**	
	Webb and McCall[6]	*HECD Precision****	*Applied Science*	*Analabs* §
11	31.8	(±5.0)	32.5	31.7
14	19.3	(1.8)	17.9	19.7
16	10.1	(1.0)	9.4	9.7
19	2.8	(0.3)	2.4	2.7
21	20.8	(1.9)	17.2	20.1
28	5.4	(0.8)	5.7	6.3
32	1.4	(0.4)	2.5	2.1
37 and 40	1.7	(0.8)	3.7	2.9

*, **, *** § See footnotes to Table III.

TABLE V

COMPOSITION OF AROCLOR 1232 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1232 STANDARD

Peak R_{DDE}**	*Wt.% reported for standard*		*Wt.%, calculated**	
	Webb and McCall[6]	*HECD Precision****	*Applied Science*	*Ultra Sci*[§]
11	16.2	(±0.6)	15.1	16.8
14	9.9	(0.3)	9.6	10.5
16	7.1	(0.5)	6.0	7.4
20 and 21	17.8	(0.4)	15.7	20.1
28	9.6	(0.3)	9.9	11.1
32	3.9	(0.2)	4.7	4.5
37	6.8	(0.2)	7.4	8.0
40	6.4	(0.2)	7.0	7.6
47	4.2	(0.2)	4.3	4.7
54	3.4	(0.1)	3.6	4.0
58	2.6	(0.1)	2.8	3.1
70	4.6	(0.1)	4.9	5.8
78	1.7	(0.1)	2.0	2.3

*,**,***,§ See footnotes to Table III.

Choice of Aroclor for peak calibration

Individual-peak calibration is required when the distribution of PCBs in the sample does not match that for a specific Aroclor since the electron-capture response for PCB compounds may vary as much as 100-fold.

TABLE VI

COMPOSITION OF AROCLOR 1242 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1242 STANDARD

Peak R_{DDE}**	*Wt.% reported for standard*		*Wt.%, calculated**			
	Sawyer[7]	*HECD Precision****	*Applied Science*[§]	*Analabs*[§]	*Ultra Sci*[§]	*Webb and McCall*[6, §]
16	3.4%	(±0.1)	3.9	3.9	3.0	5.4
21	10.3	(0.3)	10.6	10.0	10.0	10.6
24	1.1	(0.2)	1.1	1.1	1.1	1.1
28	15.8	(0.4)	15.2	15.8	16.7	15.6
32	7.3	(0.2)	6.9	7.2	7.8	7.2
37	17.0	(0.4)	15.3	15.9	16.7	15.9
40	13.0	(0.3)	12.4	12.8	13.5	12.8
47	9.9	(0.2)	10.1	10.7	10.0	10.2
54	7.1	(0.2)	7.0	7.2	7.0	7.1
58	4.4	(0.1)	4.4	4.5	4.3	4.4
70	8.7	(0.2)	8.9	8.7	8.0	8.6
78	1.9	(0.5)	2.0	1.9	1.7	1.9

*,**,***,§ See footnotes to Table III.

TABLE VII

COMPOSITION OF AROCLOR 1248 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1248 STANDARD

*Peak R_{DDE}***	*Wt.% reported for standard*		*Wt.%, calculated**		
	Sawyer[7]	*HECD Precision****	*Applied Science* [§]	*Analabs* [§]	*Webb and McCall*[6, §]
16	0.3	(±0.06)	0.1	0.3	0.2
21	1.1	(0.1)	0.2	1.0	0.8
24	0.2	(0.02)	0.05	0.2	0.2
28	6.0	(0.3)	5.4	5.8	6.3
32	2.6	(0.1)	2.3	2.6	2.7
37	8.7	(0.5)	9.0	9.7	8.6
40	7.4	(0.3)	7.4	7.7	7.4
47	15.7	(0.6)	15.5	14.9	15.6
54	9.3	(0.5)	8.9	8.6	9.2
58	8.3	(0.5)	8.2	7.9	8.2
70	18.2	(0.8)	19.7	18.7	18.2
78	6.4	(0.4)	6.7	6.4	6.3
84	4.6	(0.2)	3.8	3.8	4.5
98	3.4	(0.2)	2.5	2.7	3.3
104	3.3	(0.2)	2.8	2.8	3.0
112	1.0	(0.1)	0.9	1.0	1.0
125	2.3	(0.1)	1.9	1.8	2.1
146	1.2	(0.1)	1.1	1.0	1.2

*,**,***, § See footnotes to Table III.

TABLE VIII

COMPOSITION OF AROCLOR 1254 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1254 STANDARD

*Peak R_{DDE}***	*Wt.% reported for standard*		*Wt.%, calculated**		
	Sawyer[7]	*HECD Precision****	*Applied Science* [§]	*Analabs* [§]	*Webb and McCall*[6, §]
47	7.1	(±0.3)	4.0	6.0	6.6
54	2.7	(0.1)	1.9	2.2	2.5
58	1.2	(0.1)	1.1	0.8	1.0
70	14.7	(0.5)	13.9	12.8	13.9
84	18.6	(0.5)	12.2	18.1	18.1
98	8.3	(0.3)	7.3	7.9	7.9
104	14.1	(0.5)	12.5	13.4	13.4
125	15.6	(0.4)	17.2	15.1	14.9
146	9.0	(0.3)	10.5	8.7	8.5
174	7.4	(0.3)	8.0	7.2	6.7
203	1.3	(0.1)	1.4	1.2	1.2

*,**,***, § See footnotes to Table III.

TABLE IX

COMPOSITION OF AROCLOR 1260 LOTS, CALCULATED BY COMPARISON TO A CHARACTERIZED AROCLOR 1260 STANDARD

Peak R_{DDE}**	*Wt.% reported for standard*		*Wt.%, calculated**		
	Sawyer[7]	*HECD Precision****	*Applied Science* §	*Ultra Sci* §	*Webb and McCall*[6, §]
70	2.4	(±0.1)	2.6	2.6	3.0
84	3.6	(0.4)	3.9	4.1	4.8
98 and 104	2.8	(0.2)	3.0	3.2	3.3
117	4.4	(0.3)	4.3	4.4	4.4
125	11.0	(0.7)	11.2	11.7	12.4
146	13.3	(0.7)	13.8	13.8	14.5
160	5.5	(0.4)	5.2	5.4	5.3
174	10.0	(0.5)	10.8	11.2	12.2
203	10.9	(0.7)	9.6	10.1	10.1
232 and 244	11.2	(0.7)	10.4	10.7	10.5
280	12.5	(1.0)	11.1	11.6	11.3
332	4.2	(0.5)	4.1	4.4	4.4
360 and 372	5.4	(0.5)	4.3	4.6	4.6
448	0.8	(0.1)	0.7	0.7	0.7
528	2.0	(0.2)	1.6	1.6	1.6

*,**,***, § See footnotes to Table III.

Since individual peak response factors often vary when determined with different Aroclor types, it is important to consider the approximations which are implied in this mode of calibration. Peaks in an Aroclor chromatogram usually represent the co-elution of two or more PCB compounds. Peaks having the same relative retention times (R_{DDE}) occur in different Aroclor types. If co-eluting compounds have different electron-capture responses and their relative concentrations vary in the different Aroclors, peak response factors will vary accordingly. For example, the relative response factors determined for peak R_{DDE} 125 were 0.29, 0.65 and 0.93 when calibrating with Aroclors 1248, 1254 and 1260, respectively. Peak 125 comprises the co-elution of penta- and hexachlorobiphenyl in the ratios 9:1, 7:3 and 2:9 for these respective Aroclors. Thus, the selection of the type of Aroclor for peak calibration will affect the quantitation of PCBs.

Webb and McCall[6] proposed a flow-chart scheme to identify residues of Aroclors 1242, 1254 and 1260 in mixtures by the presence (or absence) of certain peaks. This scheme is incorporated in the EPA method for PCBs in oil as a guide to determine which of these Aroclors to use for individual peak calibration[11]. The effectiveness of this procedure was tested, when contamination was not due to the above Aroclors, by analyzing a known amount of Aroclor 1248. The scheme dictated calibration of the first group of peaks ($\leqslant R_{DDE}$ 84) with Aroclor 1242 and the remaining peaks with Aroclor 1254. The recovery of Aroclor 1248 was 116%.

If Aroclors are partially decomposed, the peak compositions may be different from those for Aroclor peaks with the same retention time. To determine PCBs arising from the decomposition of Aroclors, we recommend that individual peaks be

calibrated by averaging peak factors determined from a series of Aroclors. This series includes the original Aroclor (before decomposition) and Aroclors of less chlorination. Aroclor standards with chlorine contents higher than that of the original Aroclor are not used in the calibration. For example, in the analysis of PCBs from decomposed Aroclor 1248, the response factor used for peak R_{DDE} 28 would be the average of the factors for this peak calculated from Aroclors 1221, 1232, 1016, 1242 and 1248.

It is assumed that the peak compositions of the Aroclors reflect a favored distribution of PCB compounds over a wide range of total chlorination. Thus, averaging peak factors from several Aroclors appear to be preferable to using factors from a single Aroclor when degradation has occurred. The improvement in accuracy would vary depending on the distribution of PCBs in the sample; however, the recovery of a known amount of Aroclor 1248 was 102% by this method, whereas Webb and McCall's scheme yielded 116% recovery. A disadvantage in this approach is that more effort is required to calculate and average peak response factors for several Aroclor types rather than using peak factors from a single Aroclor. The decision of which calibration approach to apply must be made by a qualified analyst after considering the type of PCB contamination.

Application to chemically dehalogenated oil

The efficient destruction of PCBs in oil has been accomplished by chemical dehalogenation with sodium naphthalide reagent[8]. Supporting analyses require the determination of non-Aroclor PCB mixtures. It is apparent in Fig. 2 that, as the dehalogenation of Aroclor 1242 proceeds, the higher chlorinated PCBs diminish more rapidly than the lower chlorinated compounds. The calibration and analyses of these samples were performed using average (or overall) peak factors, as described previously.

Sample clean-up prior to GC analysis was accomplished using disposable silica cartridges, as outlined in the Experimental section. This procedure was accomplished in less than 5 min and was effective in removing miscellaneous GC-interfering contaminants and filtering insoluble residues which would accumulate on the GC column. PCBs were quantitatively recovered from the silica cartridge in the first 5 ml of hexane eluent.

If a significant amount of alcohol or tetrahydrofuran (used in the dehalogenation treatment) was present in the oil, the silica failed to remove the soluble impurities. Alcohol and tetrahydrofuran were removed from the oil by warming on a Roto-Vap to 60°C at reduced pressure (25 mmHg) for 60 min. Anhydrous sodium sulfate was added during this process to remove traces of water. This step was required only when poor GC resolution or extraneous peaks were observed in the chromatogram.

The silica cartridge is favored over the Florisil column clean-up because of the shorter preparation time and the smaller amounts of solvents required.

CONCLUSIONS

The compositions of several Aroclor lots marketed for GC calibration are similar to characterized Aroclors previously reported in the literature; however,

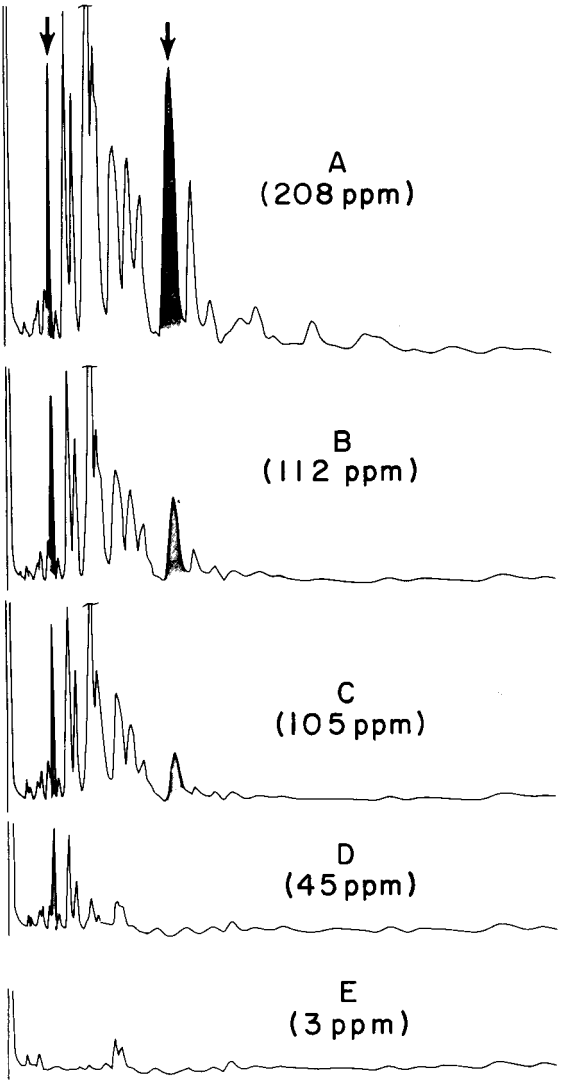

Fig. 2. Chromatograms for oil samples taken during the dehalogenation of PCBs with sodium naphthalide reagent. A, Oil sample before treatment, 208 ppm Aroclor 1242; E, oil sample after treatment, 3 ppm.

characterized materials are preferable for PCB calibration. The weight-percent distribution of PCBs in commercial Aroclors was obtained by direct comparison to characterized Aroclor lots, thus expanding the availability of standards suitable for individual-peak calibration.

Averaging of peak response factors calculated from different Aroclor types is recommended when individual-peak calibration is required.

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ANALYSIS OF POLYBROMINATED BIPHENYLS BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

The structure–sensitivity relationship of polybrominated biphenyls analyzed by an electron-capture detector was investigated. The greatest variations in sensitivity with positional isomerism occurred for the least brominated isomers and for polybrominated biphenyls with bromine substituents on only one ring. The ratio of sensitivity (on a molar basis) of decabromobiphenyl to 2-bromobiphenyl was *ca.* 50, with response increasing approximately linearly with degree of bromination. Increased detector temperature resulted in improved sensitivity. The presence of oxygen in the carrier gas resulted in slightly decreased sensitivity.

INTRODUCTION

Polybrominated biphenyls (PBBs) are compounds manufactured as fire retardants for use in plastics. Industrial PBBs are produced by direct bromination of biphenyl yielding mixtures of compounds differing both in the number of bromines atoms per molecule and by positional isomerism[1]. In 1973 these toxic chemicals were accidentally introduced into the human food chain in Michigan[2–5]. Since this incident, environmental and human contamination by PBBs has also been found near plants in New Jersey where octabromo- and decabromo-biphenyls were manufactured[6].

The initial concern over the biochemical and physiological effects of PBBs was due to recognition of the structural similarities of the PBBs to the polychlorinated biphenyls (PCBs). The ability of PCBs to induce the hepatic mixed-function oxidase (MFO) enzymes has been well studied[7]. On a molar basis, the PBBs are *ca.* five times more potent than the PCBs in the induction of the MFO enzymes[8].

Both PCBs[9] and PBBs[10] accumulate in human adipose tissue and exhibit long half-lives. PCBs have been found to be immunosuppressive agents[11]. PBBs have been shown to be immunosuppresive in mice[12] and in swine[13]. Decreased immune system function was reported in Michigan dairy farmers exposed to PBB[14]. However, no consistent correlation was found between the concentration of PBB in their blood plasma and the degree of immune response alteration.

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Quantification of PCB and PBB residues has been accomplished mainly by gas chromatography (GC) with electron-capture detection[15]. The electron-capture detector (ECD) is utilized because of its sensitivity for halogenated compounds and its wide availability. Where the PCB or PBB pattern of congeners does not exactly match the available commercial formulations, quantification is complex since the response or sensitivity of the ECD depends on the position of the halogen on the biphenyl nucleus as well as the number of halogens[1,16]. A model for calculating the ECD response of the 209 PCBs has been published[17]. No systematic study of structure–sensitivity relationships for PBBs analyzed by a modern pulsed ECD has previously been reported.

The ECD responses to 22 individual PBBs containing 1–6 bromine atoms per molecule were measured. In addition several isomers from FireMaster FF-1 (containing 6 or 7 bromine atoms) and Bromkal 80-9D (containing 8–10 bromine atoms) were utilized after liquid chromatographic pre-separation and microcoulometric quantification. Two ECD temperatures were examined. The effect of the addition of oxygen to the carrier gas was also investigated.

EXPERIMENTAL

PBB standards

Twenty PBB isomers (1–6 bromine atoms) were obtained from RFR Corporation (RI, U.S.A.). The catalogue listed the isomers as 95% pure. Three additional pentabromobiphenyls were supplied by Dr. S. Safe (University of Guelph, Ontario, Canada) FireMaster FF-1 (lot FF1312-FT) was received from the National Institute of Environmental Health Science and FireMaster FF-1 (lot FH7042) was received from the Detroit Laboratories of the U.S. Food and Drug Administration. Bromkal 80-9D was received from Dr. O. Hutzinger (University of Amsterdam, The Netherlands).

Standards were prepared by weighing milligram amounts on an August Sauter 10-mg capacity balance, and transferring to 100-ml flasks. Baker Resi-Analyzed (or comparable interference-free) hexane was used as the solvent. Volumetric dilutions were made as required. Standards were stored in glass test-tubes with PTFE-lined caps under refrigeration and were wrapped in aluminum foil to shield from light.

Gas chromatography–electron-capture detection

A Hewlett-Packard 5730A gas chromatograph with a side-mounted Linear Electron Capture Detector (constant current) was employed during this study. To minimize water and oxygen contaminants in the argon–methane (95:5) carrier gas, a Supelco carrier gas purifier at 600°C with a molecular sieve 5A pretrap was used. Glass columns (6 ft.) packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q were operated isothermally. Area measurements for this study were determined using a Hewlett-Packard 3380A integrator.

An adapter made of inert[18] nickel tubing (Regis Chemical Co., IL, U.S.A.) allowed additional carrier gas flow to be added after the GC column and before the ECD. This permitted oxygen to be added after the column, thus avoiding deterioration of the GC packing. The oxygen flow-rate was limited by a porous stainless-steel flow restricter installed between the oxygen regulator and a "tee" connection to the

gas flow. For pressure difference of *ca.* 30 p.s.i., the restricter allowed a flow-rate of 1 ml/min. An increase in the standing frequency was used as an indication that oxygen was reaching the detector.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was employed to partially purify isomers from FireMaster FF-1 and Bromkal 80-9D formulations, which were then analyzed by an ECD and quantified by microcoulometric detection. The pre-separation was necessary since baseline separation of the components in FF-1 was not achieved by GC. In addition, if the solutions were concentrated for analysis of the minor isomers, the major peaks (2,2′,4,4′,5,5′-hexabromobiphenyl[19] in FF-1 and a nonabromobiphenyl[20] in Bromkal) would overload both the ECD and the microcoulometric detector. The elution order from the Partisil 5 ODS reversed-phase HPLC column employed was favorable for easy separation of isomers which gave overlapping peaks by GC. For FireMaster FF-1 a mobile phase of water–methanol (10:90) was used. For Bromkal 80-9D a mobile phase of water–ethanol (12:88) provided satisfactory separation. The UV detector wavelength was set at 225 nm for optimum sensitivity. Fractions of the HPLC effluent associated with peaks were collected in small vials and the mobile phase was evaporated. Several 20-μl injections of FF-1 and of Bromkal were made, and the corresponding fractions from each injection were pooled. The contents of the vials were then diluted with an appropriate amount of hexane and analyzed by electron-capture detection and by microcoulometric detection. The remaining PBB in each vial was concentrated for analysis by mass spectrometry.

Gas chromatography–microcoulometric detection

A Dohrmann DE-20 halogen-specific microcoulometric GC detector adapted to a Microtek MT-220 gas chromatograph with a Texas Instruments recorder was used to quantify PBB isomers in solution. For detection, the halogen content of the GC effluent is, through oxidative pyrolysis, converted into titratable halides. If the number of bromines per molecule (from mass spectral analysis) for a given GC peak is known, the amount of the particular PBB can be determined.

Mass spectrometry

An AEI MS 30 double-beam mass spectrometer connected via a silicone membrane to a Pye Series 104 gas chromatograph was utilized for GC–mass spectrometric measurements.

RESULTS AND DISCUSSION

Linearity

The constant-current ECD was designed to give linear response over a wide range of concentrations[21]. Linear response has been questioned for strongly electron-attaching compounds[22]. A study of detector linearity was conducted on 2,2′,4,4′,5,5′-hexabromobiphenyl. This isomer was chosen because it is the major component in FireMaster FF-1 (the PBB formulation involved in the Michigan contamination incident) and because a hexabromobiphenyl was the most highly brominated, and

therefore presumably the strongest electron-attaching, PBB isomer available in pure form. Conditions typically used for the analysis of PBBs in environmental samples were employed. Table I shows that the ECD response was linear over a concentration range of 600, from 40 to 24,300 pg.

TABLE I

LINEARITY OF DETECTOR RESPONSE TO 2,2′,4,4′,5,5′-HEXABROMOBIPHENYL

Linear regression, $r^2 = 0.998$. Conditions: column, 6 ft., 3% SE-30 on 100–120 Gas-Chrom Q; column temperature, 240°C; inlet temperature, 250°C; detector temperature, 300°C; carrier gas, methane–argon (5:95); flow-rate, 41 ml/min.

Injected (pg)	*Injected (μl)*	*Retention time (min)*	*Peak area (μV sec)**	*Response (μV sec/ μg sample)*
40	5	12.5	$2.94 \cdot 10^4$	735
405	5	12.1	$3.50 \cdot 10^5$	864
4050	5	11.5	$3.45 \cdot 10^6$	852
24300	5	11.0	$1.91 \cdot 10^7$	786

* Average of two values; duplicate injections varied by less than 5%.

Retention times

The range of retention times (see Tables II and III) varied from 0.8 min for 2-bromobiphenyl with a column flow-rate of 40 ml/min to 73.5 min for decabromobiphenyl with a column flow-rate of 107 ml/min (total flow-rate through the detector, *i.e.* column flow-rate plus adapter flow-rate, was kept at 160 ml/min). Substitution *ortho* to the biphenyl linkage (2,2′,6 or 6′) results in the shortest retention times on the non-polar SE-30 GC column. *Para* bromines (4 or 4′) cause the PBB to be most retained. Addition of bromine always increases the retention time, but positional differences are sufficient that 3,3′,5,5′-tetrabromobiphenyl has a greater retention time than 2,2′,4,5′,6-pentabromobiphenyl.

Utilizing the retention time data shown in Table II for a column temperature of 240°C and a column flow-rate of 40 ml/min, the following empirical equation for retention times (t_R) based on the number of bromines in the *ortho*, *meta*, and *para* positions was produced:

$$\log t_R \text{ (sec)} = 1.44 + 0.14 \Sigma \textit{ortho}\text{-Br} + 0.26 \Sigma \textit{meta}\text{-Br} + 0.29 \Sigma \text{ para-Br}$$

Although this equation is specific for the column and conditions used, a similar polarity column phase should result in a similar ordering of retention times. This is confirmed by the similar results reported for the phase OV-101[20] and by the fact that the non-*ortho*-substituted 3,3′,4,4′,5,5′-hexabromobiphenyl has been found to have a longer retention time than *ortho*-substituted hexabromobiphenyls on the GC phases OV-17[20] and OV-210[1].

The 2,2′,4,4′,6,6′-hexabromobiphenyl standard was found to contain three large peaks. The above equation was used to determine whether any of these peaks could reasonably be assigned as the 2,2′,4,4′,6,6′-hexabromobiphenyl peak. The pre-

TABLE II

ECD RESPONSE TO PBBs

Total flow-rate through detector 160 ml/min; flow-rate through GC column, 40 ml/min.

PBB isomer	*Retention time (min)**	*Response (10^{-6})***	*Relative response****	*Response 350°C/300°C*	*Impurities (%)*§
2-	0.78	14.9 ± 0.5	1.0	1.3	<1
3-	0.89	27.7 ± 0.6	1.9	1.3	<1
4-	0.92	45.8 ± 3.1	3.1	1.3	20–25
2,2′-	1.01	33.4 ± 0.3	2.2	1.5	4
2,4-	1.15	125.5 ± 2.4	8.4	1.2	<1
2,5-	1.13	139.0 ± 0.5	9.3	1.2	<1
2,6-	1.03	137.4 ± 1.8	9.2	1.2	6
4,4′-	1.60	108.7 ± 1.3	7.3	1.2	<1
2,2′,5-	1.58	176.6 ± 2.4	11.9	1.3	<1
2,3′,5-	1.98	177.1 ± 4.9	11.9	1.4	2
2,4′,5-	2.11	209.8 ± 1.0	14.1	1.3	<1
2,4,6-	1.59	255.9 ± 3.5	17.2	1.3	<1
2,2′,4,5′-	2.89	231.1 ± 5.1	15.6	1.3	2
2,2′,5,5′-	2.73	204.9 ± 1.8	13.8	1.3	7
2,2′,5,6′-	2.35	229.8 ± 1.5	15.4	1.3	<1
3,3′,5,5′-	4.33	187.4 ± 2.3	12.6	1.4	3
2,2′,3′,4,5-	6.15	370.1 ± 7.1	24.8	1.3	1
2,2′,4,5,5′-	5.43	341.9 ± 6.5	23.0	1.3	3
2,2′,4,5′,6-	4.14	295.9 ± 6.7	19.9	1.3	7
2,3′,4,4′,5-	8.40	320.8 ± 1.9	21.5	1.4	4
2,2′,4,4′,5,5′-	11.06	377.5 ± 9.0	25.3	1.2	<1
3,3′,4,4′,5,5′-	26.36	289.2 ± 4.9§§	19.4	1.3	1
2,2′,4,4′,6,6′-	6.20	–	–	–	68

* Column temperature, 240°C.

** Response = area (μV sec)/nmole ± S.D.; $n \geq 3$ unless noted. Slope sensitivity, mV/m = 0.30.

*** Relative to 2-bromobiphenyl at 300°C detector temperature.

§ Percentage of chromatographic area (exclusive of solvent) resulting from impurity peaks.

§§ $n = 2$.

dicted retention time for this isomer using the above equation is 6.3 min; a peak at t_R 6.2 min was therefore assumed to be the actual isomer peak. Mass spectral data confirmed this peak to be a hexabromobiphenyl.

Impurities

The percentages of impurities in the PBB isomers are listed in Table II. These values are estimations made by comparing areas, assuming that the ECD response to the impurity is the same as the response to the isomer in question. Where the retention times of the impurities are greater than that of the isomer, the impurities may be overestimated since they are likely more highly brominated than the main solution component. Poor electron-capturing impurities, such as biphenyl itself, if present, would be underestimated. Mass spectrometry data on the 4-bromobiphenyl solution and the 2,6-dibromobiphenyl solution indicate the impurities are a dibromobiphenyl

TABLE III

ECD RESPONSE TO PBBs

Total flow-rate through detector, 160 ml/min. Isomers quantified by microcoulometric detector. Identification of isomers in FireMaster from Dannan *et al.*[30].

Isomer	*Origin of isomer*	*Retention time (min)**	*Flow-rate GC column (ml/min)*	*Response (10^{-6})***	*Relative response****	*Response 350°C/300°C*
2,2′,4,5,5′-	FireMaster	5.5	*ca.* 40	348.3 ± 29.3	23.4	1.2
2,2′,4,4′,6,6′-	"Pure" standard	6.2	*ca.* 40	276.6 ± 19.8[§]	18.6	1.2
Hexabromobiphenyl	FireMaster	9.1	*ca.* 40	414.8 ± 39.7	27.8	1.2
2,2′,3,4,4′,5-	FireMaster	13.1	*ca.* 40	371.5 ± 28.9	24.9	1.3
2,3′,4,4′,5,5′-	FireMaster	16.5	*ca.* 40	297.3 ± 53.2	19.9	1.4
2,2′,3,4,4′,5,5′-	FireMaster	26.4	*ca.* 40	326.7 ± 25.1	21.9	1.3
2,2′,3,4,4′,5,5′-	FireMaster	16.4	*ca.* 80	348.0 ± 26.9	23.4	1.4
First octabromobiphenyl	Bromkal	25.1	*ca.* 80	659.9 ± 125.8[§§]	44.3	1.4
Third octabromobiphenyl	Bromkal	37.1	*ca.* 80	542.7 ± 149.0	36.4	1.2
Nonabromobiphenyl	Bromkal	60.2	*ca.* 80	911.7 ± 162.1	61.2	1.2
Nonabromobiphenyl	Bromkal	49.0	*ca.* 107	904.5 ± 182.1[§§]	60.7	1.0
Decabromobiphenyl	Bromkal	74.0	*ca.* 107	786.2 ± 119.5[§§]	52.8	1.1

* Column temperature, 240°C.

** Response = area (μV sec)/nmole ± S.D.; $n = 2$ unless noted.

*** Relative to 2-bromobiphenyl (Table II).

[§] $n = 5$.

[§§] Single injection; S.D. from detector temperature of 350°C used as estimate.

and a tribromobiphenyl, respectively. From the retention time of the dibromobiphenyl impurity it can be identified as 4,4′-dibromobiphenyl and the 4-bromobiphenyl standard can be calculated, using the response factors in Table II, to be 88% pure.

Mass spectral data for the least pure isomer, the 2,2′,4,4′,6,6′-hexabromobiphenyl, revealed a tribromobenzene and a tetrabromobenzene as impurities. The hexabromobiphenyl in the solution was quantified by microcoulometric detection. Samples of 2,2′,4,4′,6,6′-hexabromobiphenyl purchased from RFR and Aldrich prior to 1977 were reported by De Kok *et al.*[20] to consist mainly of 2,2′,4,4′,5,5′-hexabromobiphenyl and to have nearly the same composition as BP-6, the formulation used to make FireMaster FF-1.

ECD responses

For comparisons of ECD response to various PBBs, high concentrations are desirable to minimize column adsorption, or possible sample decomposition effects[23]. The maximum frequency of pulsing was observed with an oscilloscope and the corresponding peak height on the integrator printout noted. The concentrations employed were such that this upper limit of pulsing was not reached during any peak.

The ECD responses to 23 PBBs are listed in Table II. The fifth column shows the ratios of detector response at 350°C to detector response at 300°C. The increased responses with increased detector temperature are indicative of a dissociative electron-capturing mechanism[24]. In Table III the ECD responses to the isomers quantified by microcoulometric detection are given. The large standard deviation (S.D.) of these values reflects the limited precision achieved by the microcoulometric quantification. In Fig. 1 the molar responses, (μV sec/nmole)$\cdot 10^{-6}$, are plotted *versus* the

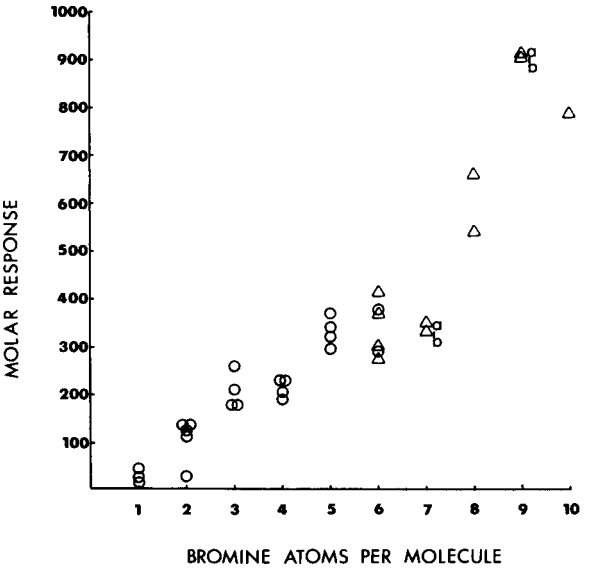

Fig. 1. ECD response to brominated biphenyls. Detector temperature, 300°C. Molar response calculated using the area of the gas chromatographic peak; y-axis is molar response expressed as (μV sec/nmole)$\cdot 10^{-6}$. △, solution quantified by microcoulometric detection; $\triangle_a$ and $\triangle_b$, response of a single isomer quantified from two HPLC fractions.

number of bromine atoms per molecule. Triangles distinguish on the figure those isomers quantified by the microcoulometric detector. The apparent decrease in response of the decabromobiphenyl relative to the nonabromobiphenyl probably reflects the uncertainty in the microcoulometric quantification of these isomers rather than any real decrease in ECD response.

The greatest variation in response attributable to positional isomerism occurs for isomers with few bromine substituents, *e.g.* compare in Table II 2- and 4-bromobiphenyls. In addition, substitution on a single ring enhances response over splitting the same number of bromines between both rings, as is evident if one compares 2,2′- and 2,6-dibromobiphenyls, or 2,4′,5- and 2,4,6-tribromobiphenyls. Commercial PBB formulations, since they are produced from the bromination of biphenyl and are highly brominated mixtures, would be unlikely to contain isomers brominated on a single ring.

A linear regression of the data from Table II for molar response in μV sec/n-mole gives:

$$\text{molar response} = \{-19.7 + 64.8(\text{number Br/molecule})\} \cdot 10^6 \qquad r^2 = 0.87$$

Only the data from Table II were used for the regression equation since the values derived based on microcoulometric quantification of the isomers have a large uncertainty. The predicted response for decabromobiphenyl using this equation is 629, a reasonable agreement with the value found.

Since a linear regression gives a reasonable fit to the data in Fig. 1, the analysis of PBBs in environmental samples can be simplified by assuming the position of the bromine on the ring can be ignored without resulting in large errors in quantification by electron-capture detection. In addition, as previously mentioned, isomers substituted on a single ring are unlikely. The insensitivity of the ECD response to positional isomerism is understandable since the molar responses, from 2-bromobiphenyl to decabromobiphenyl, increase only by a factor of *ca.* 50. In terms of ECD response factors, this is not a large range. The response of CCl_4, for example, is 10^4 times the response of CH_3Cl[25]. For PCBs, Bøe and Egaas[17] found the ratio of the response of decachlorobiphenyl to 2,6-dichlorobiphenyl to be *ca.* 40. In this work, for PBBs the corresponding ratio was *ca.* 6. Since there is an upper limit to response, namely each collision of an electron and sample molecule resulting in an electron capture, the better electron-attaching ability of the bromine atom over the chlorine atom[26] would be expected to result in a compression of the range of sensitivities for the PBBs relative to the PCBs.

Sullivan[27], calculating a collision rate for the sample molecule and an electron based on the electronic polarizability of the molecule, arrived at a theoretical upper limit of electron-capture rate constants for PCBs of varying degrees of chlorination. The highly chlorinated PCBs approached these limits, with decachlorobiphenyl at *ca.* 70% of the theoretical value. The highly electron-capturing compounds which approach the collision limit of ECD response, would also be expected to give coulometric response, *i.e.* one electron captured for each sample molecule, under conditions of excess electrons[28]. In this research, near coulometric response was found for 40 pg of 2,2′,4,4′,5,5′-hexabromobiphenyl when determined as follows:

For the constant-current Hewlett-Packard 5730A ECD, the current is held at

ca. 1 nA. The average current, $\bar{I}_d$, is equal to the electrons collected per pulse times the frequency, f, of pulsing[21]:

$$\bar{I}_d = e^- \cdot f \approx 1 \text{ nA}$$

Substituting in the above equation the measured value for the standing frequency, the number of electrons collected per pulse was determined for the condition when no sample is in the detector. Conversion of the integrator output, for the peak area resulting from the injection of 40 pg of sample, from μV sec to Hz sec gives the number of extra pulses needed to maintain the current at 1 nA when the sample is in the detector. Since at this low sample concentration the change in frequency was a small percentage of the standing frequency, the change in electrons collected per pulse was also small and can be approximated by the value calculated from the standing frequency. Extra pulses were required to keep the current constant since electrons were being captured by the sample. The electrons captured then can be calculated as:

$$(\text{extra pulses}) \cdot (\text{electrons/pulse}) = 1.9 \cdot 10^{10}$$

Since 40 pg is equivalent to $3.8 \cdot 10^{10}$ molecules, the electrons captured per sample molecule is 0.5, confirming that under conditions of excess electrons near coulometric response resulted.

Addition of oxygen

Recently, intentional addition of oxygen to a carrier gas has been used to enhance ECD response to halogenated methanes[25] and other simple chlorinated molecules[29]. In the present study the added oxygen was not quantified, but resulted in an observed 12-fold increase in the response of $ClCH_2CH_2Cl$ at 300°C detector temperature. The PBB responses decreased slightly with the addition of oxygen. The ECD response decreases ranged from 0–38% at 300°C detector temperature and 0–30% at a detector temperature of 350°C. An increased noise level when the oxygen was added, requiring a decreased slope sensitivity for the integrator, may account for the decreases. No large changes in the relative responses of one PBB to another were seen with oxygen addition to the carrier gas.

CONCLUSIONS

The ratio of sensitivity by electron-capture detection (on a molar basis) of decabromobiphenyl to 2-bromobiphenyl is *ca.* 50. Response increases approximately linearly with degree of bromination. The greatest variations in sensitivity with positional isomerism occur for the least brominated isomers and for PBBs with bromine substituents on only one ring. For the highly brominated commercial formulations, if the number of bromines per molecule for a GC peak is known either from mass spectral or retention time data, good response factors can be predicted with only a few standards by assuming a linear increase in response with increasing bromine substitution.

Increased detector temperature resulted in improved sensitivity. This study shows that for the analysis of PBBs by an ECD, the presence of oxygen impurities in

the carrier gas will not significantly affect the absolute response of the PBB or the relative response of one isomer to another.

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SAMPLE PROCESSING FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ECDYSTEROIDS

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SUMMARY

An improved purification procedure is described for high-performance liquid chromatography (HPLC) analysis of ecdysteroids. It can be used for both quantitative analyses of ecdysone and 20-hydroxyecdysone, and for various metabolic studies.

The procedure comprises two steps only: chloroform–water partition and adsorption/purification of the water phase solutes on a SEP-PAK® C_{18} cartridge. The 60% methanol eluate can be used after evaporation for direct HPLC quantitative analysis of ecdysteroids on a C_{18}-bonded column. The detection limit is about 10 ng of hormones for biological extracts. Makisterone A added before extraction is used as internal standard.

Application of this procedure to metabolic studies requires all the metabolites to be retained on the SEP-PAK cartridge, as is the case for the insect system investigated, *i.e.*, *Pieris brassicae* (Lepidoptera) pupae. Examples of separations are given and the limits of the procedure are discussed.

INTRODUCTION

Since the early work of Hori[1], the use of high-performance liquid chromatography (HPLC) for ecdysteroid studies, has become widespread. It is mostly used to separate compounds that are further characterized or quantified by off-line procedures[2,3]. These procedures include radioimmunoassays[4,5], bioassays[6], mass spectrometry[7] or sulphuric acid-induced fluorescence[8]. To our knowledge, there have been only two reports on the use of HPLC for direct quantification of ecdysteroids by UV absorbance monitoring[9,10], as a routine procedure[9] or for further analysis of fractions previously separated by thin-layer chromatography (TLC)[10]. Another recent attempt with rather crude biological extracts proved much less effective than gas–liquid chromatography (GLC) with electron capture detection[11].

The problem of using HPLC for on-line quantification of ecdysteroids appears

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a priori solvable, since it involves favourable factors: first, the strong UV absorbance of the unsaturated ketone ($E_{243} \approx 12{,}000\ cm^2$/mole); secondly, the relative abundance of ecdysteroids in arthropods, compared to vertebrate steroid hormones. The use of a suitable HPLC system allows easy detection of pure compounds in the nanogram range[2,3], and the problem to be solved is connected with the poor specificity of UV detection, which must be counteracted by extremely efficient chromatographic systems and purification procedures. Adequate purification is of course possible by a multi-step procedure, but this is generally too time-consuming for routine analyses. The aim of the present paper is to describe a new purification procedure, which is both efficient and rapid, and allows many analyses per day. Several chromatographic systems will also be compared.

MATERIALS AND METHODS

Animals

Most analyses were performed with extracts of the white cabbage butterfly, *Pieris brassicae* L., using either hemolymph or whole animals (pupae). A few comparative studies were made with other materials, to assess the general applicability of the procedure. These materials included *Locusta migratoria* hemolymph and eggs, and *Carcinus maenas* eggs.

Chemicals

Reference ecdysteroids (ecdysone, 20-hydroxyecdysone and makisterone A) were from Simes SA (Milan, Italy) or Schering (Berlin, G.F.R.). Ponasterone A was a gift from Dr. Courgeon (Paris, France).

High-purity solvents (HPLC grade) were from Fluka (Buchs, Switzerland), Fisons (Loughborough, Great Britain) or E. Merck (Darmstadt, G.F.R.). Water was twice distilled in a quartz apparatus, and all solvents were filtered on a regenerated cellulose filter (pore size 0.45 μm) from Schleicher & Schüll (Dassel, G.F.R.). All solvents were degassed for at least 20 min in an ultrasonic bath, Bransonic B220 (Branson, Stamford, CT, U.S.A.).

Tritiated ecdysone (specific activity 60–68 Ci/mmole) was purchased from New England Nuclear (Dreieich, G.F.R.).

HPLC

The HPLC system (Waters Assoc.) comprised a 6000A module pump, a U6K septumless injector and a M440 UV detector (254 nm). Certain analyses were performed outside the laboratory, by courtesy of Waters Assoc. and DuPont Instruments, in order to test automated injectors, integrators and temperature control systems.

A Flo-One/DR™ radioactivity monitor (Radiomatic Instruments & Chemical Co., Tampa, FL, U.S.A.) was used for metabolic studies.

The solvent systems and columns chosen are described in the Results section.

RESULTS

Choice of HPLC procedure

A primary consideration was the need for isocratic conditions. The use of

gradient elution would cause baseline disturbances which would impede the use of high sensitivity of detection[11]. On the other hand, isocratic conditions and UV detection at 0.01 a.u.f.s. allow ready quantification of $\leqslant$ 10 ng of pure ecdysteroids.

Very efficient separations of pure ecdysteroids have been obtained with both normal-phase and reversed-phase systems[12,13]. However, when biological extracts are injected, various impurities accumulate at the top of the columns. Consequently, periodical elution of the column with a stronger solvent is necessary. Because of their long equilibration time, silica columns cannot be used in series, although they provide very good separations, even with biological extracts.

The column efficiency must be high enough to separate the ecdysteroids from all interfering substances. This necessitates the use of columns filled with 5-μm rather than 10-μm particles and, at least under the chromatographic conditions used in our laboratory[3], eliminates diol-bonded columns because of their low efficiency. Consequently, the choice is limited to reversed-phase columns. Several C_{18}-bonded columns were tested under various chromatographic conditions chosen to give complete and rapid analyses, and the results are summarized in Table I. A flow-rate of 1.5 ml/min can generally be used, except for methanol–water mixtures which have a high viscosity.

The different mobile phase systems (methanol–water, acetonitrile–water and acetonitrile–buffer) and running temperatures allowed the definition of the most suitable conditions. The best results were obtained with acetonitrile rather than methanol, and at 50°C rather than at ambient temperature. The use of acetonitrile–water mixtures was rejected, because it led to very bad results with certain columns (*e.g.*, Ultrasphere-ODS), and water was replaced by a buffer in order to obtain symmetrical peaks. Columns filled with spherical particles were the most efficient ones (Ultrasphere-ODS and Zorbax-ODS). Ecdysone is generally eluted in less than 20 min, allowing three analyses per hour, provided that no other compound is eluted after this hormone.

Sample processing

A large number of purification procedures have been used to prepare biological samples, They usually involve solvent partitioning between hexane–aqueous methanol, hexane–acetonitrile[9] and chloroform–water[8] for lipid removal, and butanol–water partitioning[14] to eliminate polar substances (for further details, see refs. 13, 15). These steps are then followed by chromatography on a silicic acid column or by TLC on silica plates[13,15]. Holman and Meola[9] recently suggested replacing this second step by HPLC purification on Poragel PN, a cheap large-size reversed-phase support. The whole procedure thus comprised two successive partitions, two HPLC injections on Poragel PN and the evaporation of 50 ml acetonitrile for each sample[9]. Our procedure was designed to involve fewer steps and the evaporation of a small volume of solvent. The principle is to obtain ecdysteroids in a water phase, and then to purify this crude extract on a reversed-phase column.

(a) Extraction of ecdysteroids. These compounds can be extracted with hot water[14], a method that eliminates proteins by thermal coagulation, but another possibility is to mix the biological sample with water and an equal volume of an organic solvent not miscible with water, which can extract the non-polar substances. The solvent chosen should: (i) denature proteins and render them insoluble in water; (ii)

TABLE I

COMPARISON OF VARIOUS CHROMATOGRAPHIC SYSTEMS

Suppliers: 1 = Serva (Heidelberg, G.F.R.); 2 = E. Merck (Darmstadt, G.F.R.); 3 = Waters Assoc. (Milford, MA, U.S.A.); 4 = DuPont (Wilmington, DE, U.S.A.); 5 = Beckman Instruments (Berkeley, CA, U.S.A.). Retention time and plate number are given for ecdysone (see Fig. 4A).

Column	*Supplier*	*Length (cm)*	*Particle size (μm)*	*Solvent*	*Temperature (°C)*	*Flow-rate (ml/min)*	*Retention time (min)*	*Plate number*
Servachrom RP-18	1	25	5	Acetonitrile–water (18:82)	Ambient	1.5	20	3700
LiChrosorb RP-18	2	25	5	Acetonitrile–water (18:82)	Ambient	1.5	20	8700
μBondapak C_{18}*	3	30	10	Acetonitrile–buffer (18:82)	Ambient	1.5	19	4600
Radialpak C_{18}*	3	10	10	Acetonitrile–buffer (18:82)	Ambient	1.5	13	2300
Zorbax-ODS**	4	25	5.5	Acetonitrile–buffer (18:82)	50	1.5	19	11,000
Ultrasphere-ODS	5	25	5	Methanol–water (40:60)	Ambient	1	30	8500
					50	1	15	8000
				Acetonitrile–buffer (20:80)	Ambient	1.5	16	10,500
					50	1.5	16	15,500
				Acetonitrile–buffer (25:75)	Ambient	0.5	15	19,000

* Performed with R. Barbès, Waters Associates.

** Performed with J. C. Chambet, DuPont Instruments.

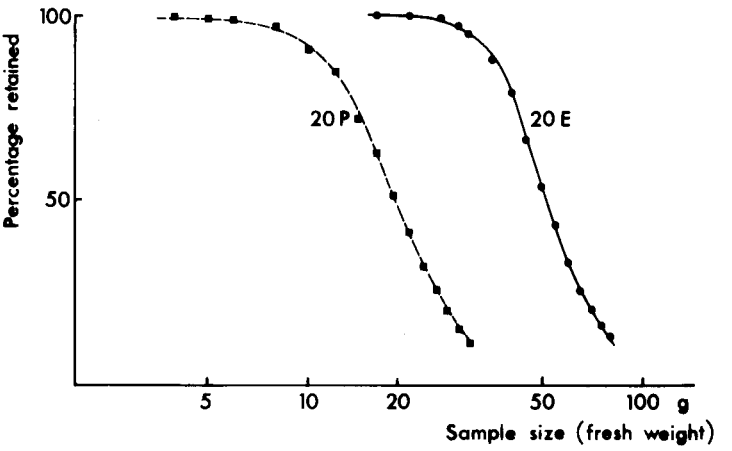

Fig. 1. Saturation experiments with a SEP-PAK cartridge, illustrating the combined retention percentages of 20-hydroxyecdysone (20E) and a polar metabolite (20P) when sample size is increased.

not extract the ecdysteroids, but be as polar as possible in order to extract almost all compounds less polar than ecdysone; (iii) not form unduly stable emulsions with water, so that the two phases can readily be separated by brief centrifugation; (iv) be denser than water, to allow collection of supernatant water phases.

In accordance with these properties, we have chosen chloroform as the organic solvent. The chloroform–water partition coefficient of ecdysone is 7:93, and two successive partitions (v/v) thus allow recovery of more than 99% of the ecdysone in the water phase. This system has an obvious limitation if less polar ecdysteroids are to be recovered. For instance, the partition coefficient of ponasterone A is about 50:50, and this compound is at the limit of the application of the present partition system.

With some samples of whole insects or with crabb eggs, a stable gel appeared in the water phase in our procedure, and this necessitated prior extraction with acetone–ethanol (1:1) and evaporation of the extract to dryness before water–chloroform partition. An alternative possibility would have been to replace chloroform by ethyl acetate, and then to collect the underlying water phase.

(b) Adsorption on a C_{18} phase. When ecdysteroids are contained in a water phase, even in the presence of a few percent methanol or acetonitrile, they are efficiently adsorbed on reversed-phase supports. This was previously shown using a Poragel PN column[9], and we obtained similar results by using a short column (50 × 4.6 mm I.D.) filled with Zorbax-BP-C_8. However, to save time and avoid the need for an HPLC system, we decided to use disposable SEP-PAK C_{18} cartridges (Waters Assoc.). These are currently used to concentrate organic compounds from water[16], but their use in the case of ecdysteroids had been limited to the elimination of non-polar contaminants from methanolic extractions[11]. These cartridges adsorb very efficiently the ecdysteroids contained in the water phase prepared as above. Not only ecdysone and 20-hydroxyecdysone, but also their polar metabolites are retained (Fig. 1). Thus, one SEP-PAK can retain 100% of the 20-hydroxyecdysone in an extract of 20 g *Pieris* pupae. The capacity for the polar metabolites is of course lower, due to the competition by less polar substances in the water extracts. Adsorption was completed by

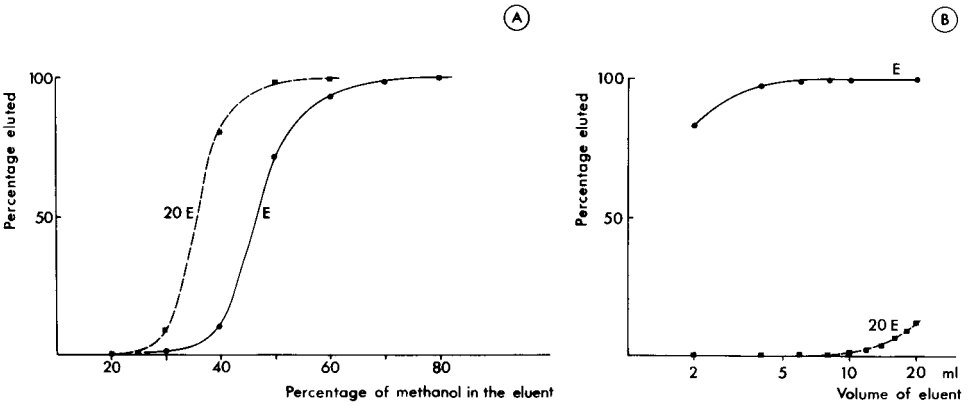

Fig. 2. Elution of ecdysone (E) and 20-hydroxyecdysone (20E) previously adsorbed on a SEP-PAK cartridge. A, Percentage of E and 20E eluted with 3 ml of various methanol–water mixtures; B, percentage of E and 20E eluted with increasing volumes of 60 % methanol (for E) or 25 % methanol (for 20E). See text for further details.

means of a water pump at a flow-rate of *ca.* 2 ml/min. Higher flow-rates would reduce the adsorption efficiency[17].

(c) Elution of ecdysteroids from the SEP-PAK cartridge. We determined standard conditions corresponding to the best use of the SEP-PAK cartridge, by eliminating all substances more polar than 20-hydroxyecdysone, and by retaining all substances less polar than ecdysone. This allowed the selection of compounds of similar polarity, as required for further isocratic HPLC analysis.

Standardized conditions were selected by adsorbing ecdysteroids from four *Pieris* pupae (*ca.* 1.7 g wet weight) on different SEP-PAK cartridges. Tritiated ecdysone or 20-hydroxyecdysone was added as marker. In the first step, 3 ml of various methanol–water mixtures were used, and the percentage of hormone eluted was determined (Fig. 2A). We found that 3 ml of 25 % methanol eluted less than 1 % of 20-hydroxyecdysone, and that 3 ml of 60 % methanol eluted 94 % of ecdysone. In the second step, increasing volumes of 25 % and 60 % methanol were employed (Fig. 2B), and it was found that up to 8 ml of 25 % methanol eluted less than 1 % of the 20-hydroxyecdysone, and that 5–6 ml of 60 % methanol eluted more than 99 % of the ecdysone. These conditions selected were rinsing with 5 ml of 25 % methanol (to elute the polar metabolites of ecdysone) and elution of 20-hydroxyecdysone and ecdysone with 5 ml of 60 % methanol.

(d) Overall design of the procedure. The procedure is shown schematically in Fig. 3. Extraction was performed twice [chloroform–water (1:1)] when ecdysone and 20-hydroxyecdysone were to be analyzed, and three to four times [chloroform–water (1:2)] when samples also contained less polar ecdysteroids (*e.g.*, ponasterone A or 2-deoxyecdysone).

The extraction yield was studied with *Pieris* diapausing pupae (endogenous hormone levels < 5 ng/g) injected with [^{3}H]ecdysone and variable amounts (10–10^3 ng) of unlabelled ecdysone prior to extraction. By using groups of four pupae (*ca.* 1.7 g wet weight), 98.7 $\pm$ 0.4 % of the radioactivity in the two first water extracts (10 ml each) was recovered. Recovery was almost independent of biological sample size between 0.2 and 5 g wet weight.

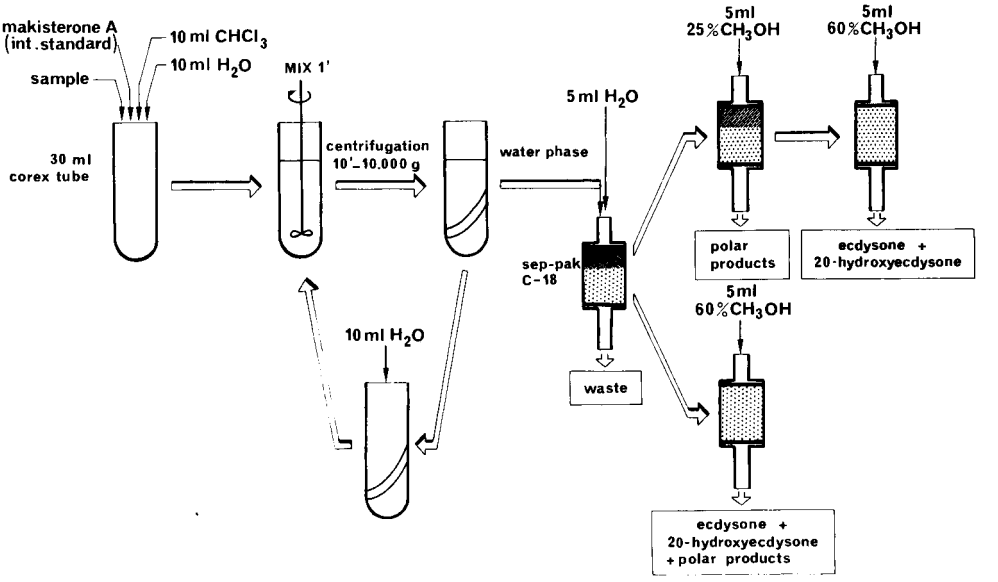

Fig. 3. Purification procedure used for biological samples. The adsorbed ecdysteroids can be eluted in two different ways, depending on the experiment. If ecdysone and 20-hydroxyecdysone are to be quantified, the most polar metabolites are eliminated with 5 ml of 25% methanol. For metabolic experiments in which the entire spectrum of metabolites is analyzed, elution is performed directly with 5 ml of 60% methanol.

The ecdysteroid recovery after extraction and purification may of course vary slightly for each sample, and it is advisable to use an internal standard. Makisterone A, a C_{28} ecdysteroid only found in *Oncopeltus fasciatus* embryos[18], has chromatographic properties intermediate between ecdysone and 20-hydroxyecdysone (Fig. 4A), and appeared suitable for this purpose, if added at the first step of extraction. The ecdysone and makisterone A recovery was checked with diapausing pupae extracts containing 10^3 ng of makisterone A, [^{3}H]ecdysone and 10–10^4 ng unlabelled ecdysone. In all cases, subsequent HPLC analysis showed that the final recovery was the same for both hormones (80 ± 5%) independently of sample size (0.2–4 g) or ecdysone content.

Easy quantification of hormones was performed on HPLC traces by comparing hormone and internal standard peaks.

Application to various biological materials

The above procedures have essentially been used with *Pieris* pupae (hemolymph or whole bodies) without any special difficulty. From the extract of four pupae or 0.5 ml hemolymph, an aliquot corresponding to one pupa or 0.1–0.2 ml hemolymph was injected. The results mostly agreed with those previously reported using radioimmunoassays[19] or mass fragmentography[20]. Similar experiments with *Locusta migratoria* also gave results consistent with available data[21–23]. In addition, it was possible to quantify ponasterone A in *Carcinus maenas* egg extracts, in which this ecdysteroid was recently reported to occur[24]. Examples of such separations are given in Figs. 4–7.

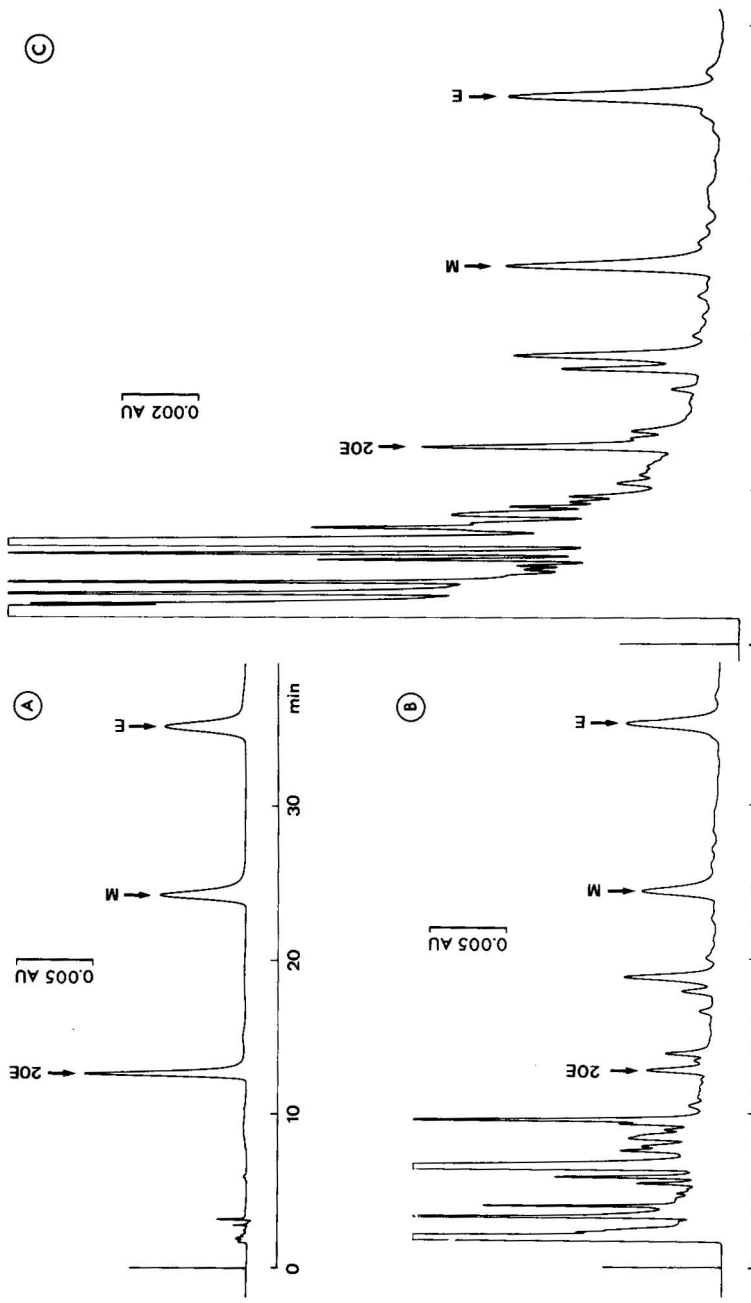

Fig. 4. Analysis of ecdysteroids on an Ultrasphere-ODS column (250 × 4.6 mm I.D.). Solvent: 17% acetonitrile in 20 m*M* Tris–$HClO_4$ buffer, pH 8.5. Flow-rate: 1 ml/min. Temperature: ambient. A, Standard mixture used for calibration (330 ng per compound); B, *Pieris* hemolymph (96 h-old pupae) containing 20E (0.6 μg/ml) and E (2.5 μg/ml); C, *Pieris* hemolymph (108 h-old pupae) containing 20E (1.2 μg/ml) and E (2 μg/ml). M = Makisterone A (internal standard).

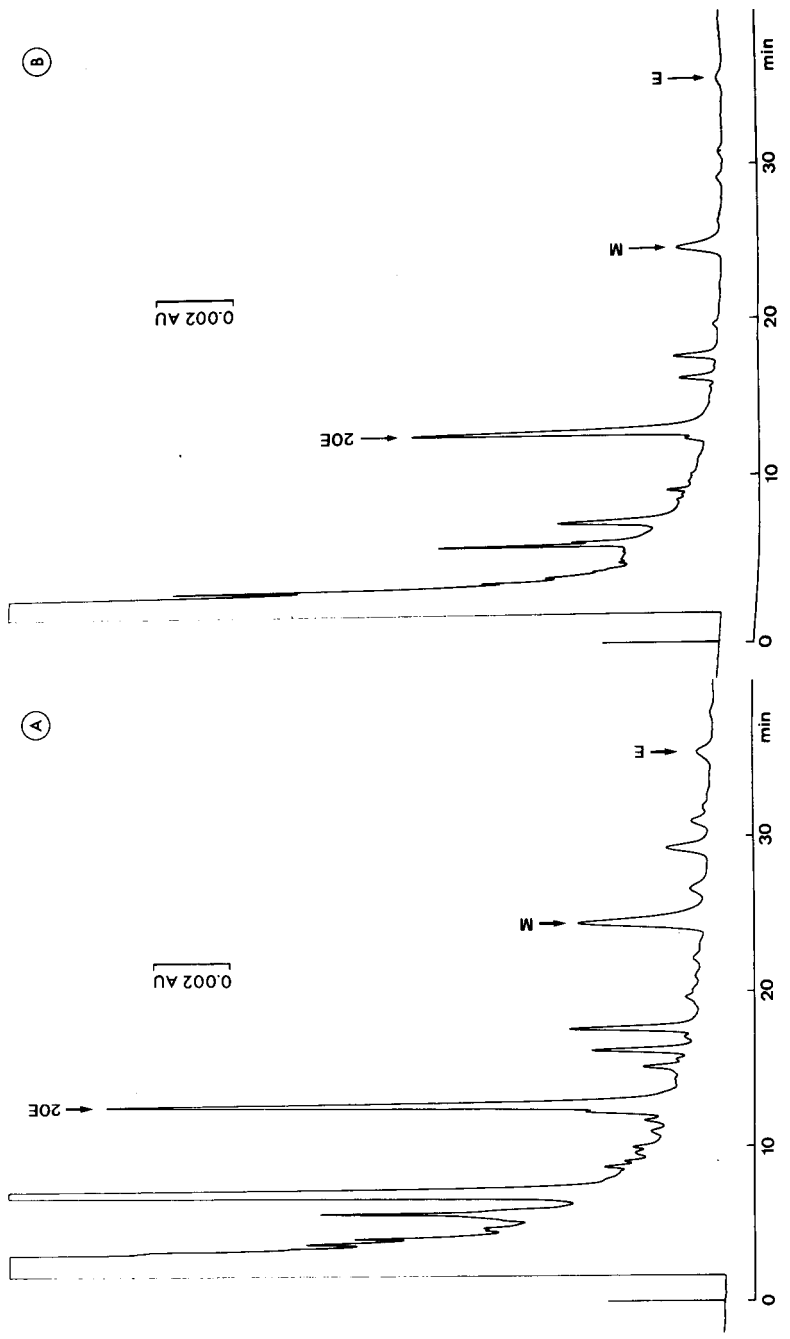

Fig. 5. Analysis of ecdysteroids in last instar larvae *Locusta* hemolymph. Chromatographic conditions as in Fig. 4. A, 7 day-old male larvae; 1.6 ml hemolymph with 1 μg makisterone A added, with injection of a small aliquot. Hormone concentrations: E (0.08 μg/ml) and 20E (1.4 μg/ml). B, 6 day-old female larvae: 0.6 ml hemolymph with 1 μg makisterone A. Hormone concentrations: E (0.24 μg/ml) and 20E (5.8 μg/ml). M = Makisterone A (internal standard).

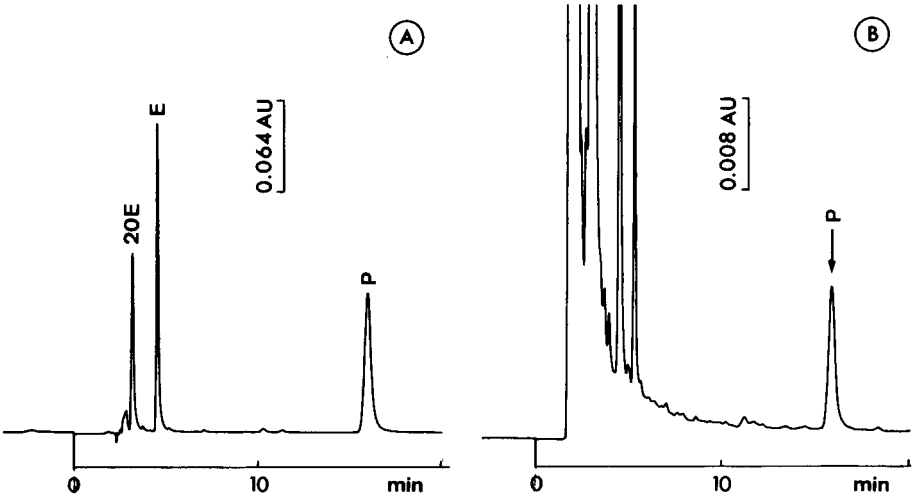

Fig. 6. Analysis of ponasterone A in *Carcinus maenas* eggs. Chromatographic conditions as in Fig. 4 except for the solvent: 26% acetonitrile in buffer. A, Standard ecdysteroid mixture containing 2 μg of ponasterone A (P); B, egg extract (1.5 g wet weight) containing 170 ng/g of ponasterone A.

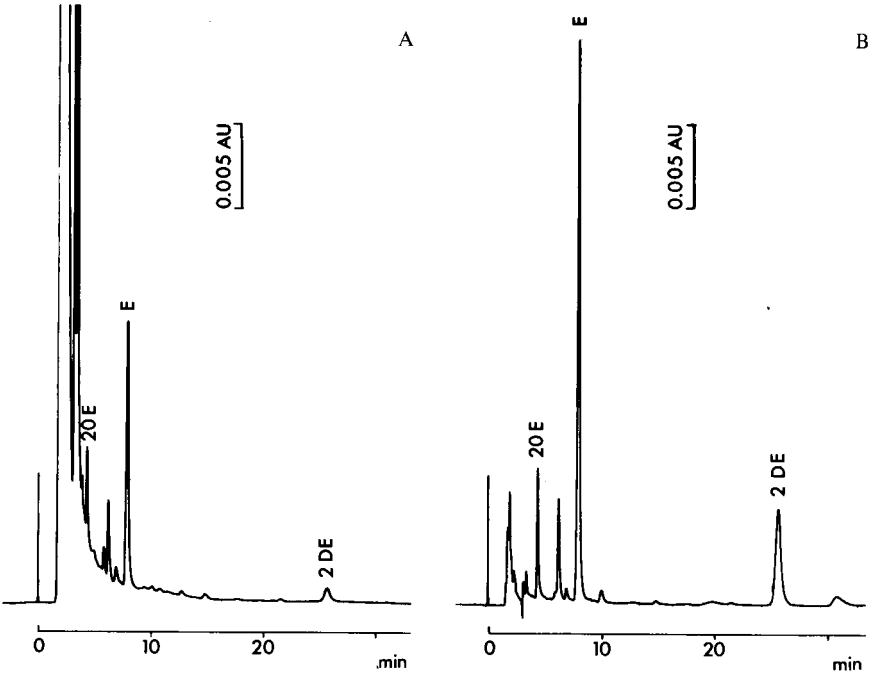

Fig. 7. Analysis of ecdysteroids in *Locusta migratoria* eggs. Conditions as in Fig. 4 except for the solvent: 23% acetonitrile in buffer. A, Free hormones eluted in the 60% methanol fraction (injection of eight eggs); B, hormones released from hydrolysed conjugate fraction (water phase and 25% methanol eluate hydrolysed according to ref. 10 and again processed as in Fig. 3), injection corresponding to one egg. 2DE = 2-Deoxyecdysone.

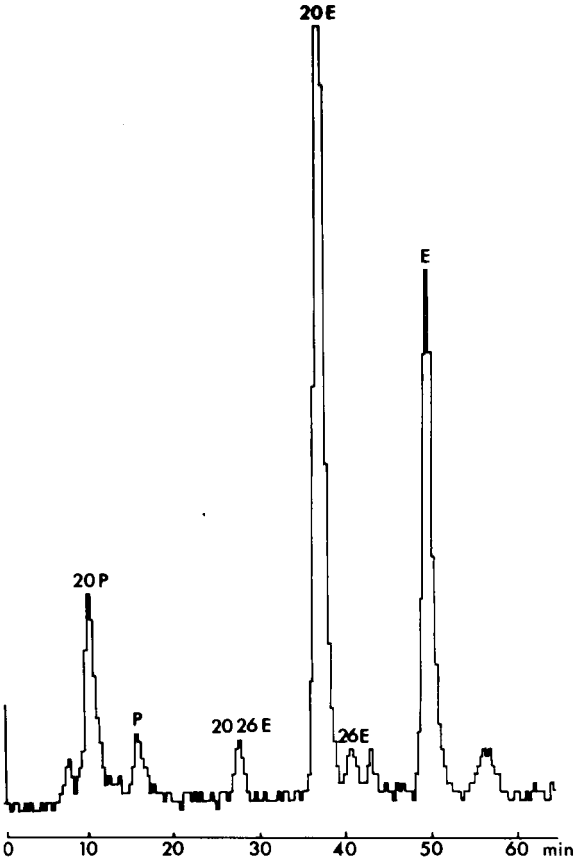

Fig. 8. Analysis of ecdysone metabolites in *Pieris* by on-line monitoring of radioactivity. Column: Zorbax-BP-ODS, 200 × 4.6 mm I.D. (laboratory-made). Solvent: linear gradient (in 85 min) from 13% to 40% acetonitrile in Tris buffer. Flow-rate: 0.3 ml/min. Scintillation cocktail (2,5-diphenyloxazole/1,4-bis(5-phenyloxazolyl-2)benzene/Triton/toluene), flow-rate 1.2 ml/min. Integration time: 20 sec. Major metabolites are ecdysone (E), 26-hydroxyecdysone (26E), 20-hydroxyecdysone (20E) and 20,26-dihydroxyecdysone (2026E); P and 20P refer to the acidic metabolites arising from 26E and 2026E, respectively.

The use of a radioactivity monitor allowed direct analysis of the ecdysone metabolites in *Pieris* pupae (Fig. 8). A conventional column (4.6 mm I.D.) was employed at a reduced flow-rate (0.3 ml/min), and the analysis required 1 h. The use of a small bore column or a splitting device would considerably shorten this period.

DISCUSSION

The purification–HPLC procedure described can be used for the direct quantification of ecdysteroids by monitoring of UV absorbance, or only in combination with an off-line procedure, like radioimmunoassay or fluorometry. This will depend on the relative abundance of ecdysteroids and interfering impurities in biological extracts. When hormone levels are >50 ng/g, direct quantification is generally feasible. Between 10 and 50 ng/g, a control by another method may be needed. Below 10 ng/g, radioimmunoassay of collected fractions is the only solution.

The present procedure is not more efficient than the previous one of Holman and Meola[9], but is simpler and faster. It is suitable for large series of samples and requires no HPLC system for sample preparation, but only a 10-ml glass syringe. From our experience with this procedure, we can draw the following conclusions.

With 1–2 g samples, one SEP-PAK can be used at least four times without noticeable loss of efficiency; rinsing with methanol and reconditioning with water allows repeated use, even if coloured substances such as ommochromes and melanins remain adsorbed on the cartridge.

The procedure may be simplified for studies of organ culture media: even if these contain up to 20% calf serum, they can be directly placed on the SEP-PAK without preliminary chloroform–water partition, without noticeable loss of ecdysteroids (less than 5%). Such treatment is not possible for insect hemolymph, as in this case the major part of the ecdysteroids is not retained on the SEP-PAK. Whether this procedure could be used to study ecdysone-binding compounds in hemolymph remains to be investigated; it could perhaps constitute a faster method than the usual Sephadex G-25 column[25].

The procedure can of course be used for metabolic studies. The catabolism of ecdysone essentially results in the formation of more polar compounds, found in the water phase after partitioning. We obtained evidence (Fig. 1) that, at least in the case of *Pieris*, the acidic metabolites were retained on the SEP-PAK, but this need not be true of all polar metabolites including conjugates. By eluting the SEP-PAK directly with 60% methanol, all the metabolites are eluted together (Fig. 3) and can be analyzed by HPLC in the gradient mode (Fig. 8). If the metabolic study is restricted to one reaction, *e.g.*, conversion of ecdysone into 20-hydroxyecdysone, the mixture eluted from the SEP-PAK can be analyzed isocratically on a short C_{18}-bonded column, with 25% acetonitrile in the mobile phase and on-line radioactivity monitoring. This allows injections every 10 min and easy automation.

By using an integrator and an automated injector, it would be possible to quantify ecdysteroids in 30–50 samples per day, even if a short step-gradient to 60% acetonitrile is needed between successive injections. The number of samples analyzed per day could even be increased by using the recently designed "boxcar" procedure[26], which would eliminate the need for step-gradient cleaning.

There remains, however, the physical impossibility of preparing samples at this rate: in our experience, one person can only process 20 samples a day. As a consequence, sample processing is the limiting factor in this method, and more highly automated sample preparation procedures are being tried, so as to restrict manual operations to the chloroform–water partition step.

CONCLUSIONS

The procedure described appears cheap, rapid and sensitive enough for most samples containing >30 ng/g ecdysteroids. This study was restricted to a few species, but we believe the method could be extended to other cases.

The same procedure can be applied to metabolic studies, and is used in our laboratory for routine analysis of ecdysone 20-mono-oxygenase activity in various *Pieris* tissues during development.

Further experiments are in progress to improve the method's sensitivity and

specificity (by the use of fluorescence detection) and rapidity (by the automation of certain steps).

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CHROM. 14,347

DETERMINATION OF AMINO ACIDS BY MEANS OF GLASS CAPILLARY GAS–LIQUID CHROMATOGRAPHY WITH TEMPERATURE-PROGRAMMED ELECTRON-CAPTURE DETECTION

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SUMMARY

Amino acids were separated as their heptafluorobutyryl isobutyl ester derivatives on a glass capillary support-coated open tubular column coated with OV-101 and Chromosorb R, using temperature programming and the electron-capture detector. The relative molar response values showed that some derivatives were detectable in much smaller amount than others. The method was applied to the determination of amino acids attached to tRNA.

INTRODUCTION

During the past twenty years there have been extensive developments in the separation and analysis of amino acids by gas–liquid chromatography (GLC). Amino acids, being multifunctional, require derivatization of reactive groups to obtain suitably volatile compounds. A large number of different derivatives and techniques for preparing derivatives have been employed (see extensive reviews, refs. 1–3). Separation of the amino acid derivatives was carried out usually with conventional GLC packed columns, but more recently capillary columns have been introduced to obtain improved resolution and sensitivity of detection[4–6]. With few exceptions workers have used the flame ionization detector (FID)[3]. The potential use of the electron-capture detector (ECD) for amino acids derivatives possessing suitable electronegative substituent groups was indicated[7,8]. However, the ECD was used normally under isothermal conditions and it was not possible to separate derivatives showing a wide range of volatilities, and reference was made to the difficulty of using the ECD with temperature programming[8]. Recently, support-coated open tubular (SCOT) capillary columns with the ECD and temperature programming of the column oven were used for trifluoroacetylhexafluoroisopropyl ester derivatives of homovanillic, isohomovanillic and vanillylmandelic acids[9] and N-heptafluorobutyryl (HFB) amino acid isobutyl ester derivatives[10]. We report here on the separation of the N(O)-HFB amino acid isobutyl ester derivatives on a glass capillary SCOT column with temperature programming and the ECD and show the application of the method to amino acids obtained after deacylation of silk-worm tRNA.

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EXPERIMENTAL

Gas chromatography was carried out with a Hewlett-Packard Model 7620A gas chromatograph fitted with a 2-mCi ^{63}Ni ECD. Peak areas were determined with a Hewlett-Packard integrator Model 3370A. A capillary column (25 m × 0.4 mm I.D.) was coated with 15% OV-101 and 5% Chromosorb R by a single-step method[11]. Direct injections onto the column with sample volumes up to 2.0 μl were made without inlet heater[12].

The N(O)-HFB amino acid isobutyl ester derivatives were prepared as previously described[13,14] using 10–20 nmol of each amino acid. Norleucine, pipecolinic acid and homoarginine were used as internal standards. Isooctane was the solvent for sample dilution and injection.

RESULTS AND DISCUSSION

Fig. 1. shows the response–concentration curves for N(O)-HFB amino acid isobutyl ester derivatives. The linear dynamic range applied over a range of 10-fold increase in concentration for each amino acid under the conditions given and with the instrument used. It was claimed that there was a linear response within the 10–400 pg

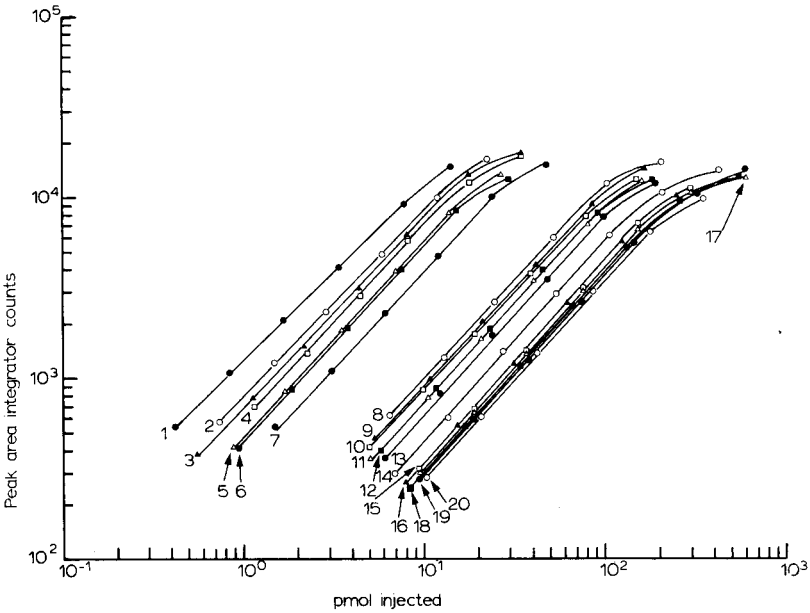

Fig. 1. Response–concentration curves for N(O)-HFB isobutyl ester derivatives of amino acids with the ECD. Preparation of sample: see Experimental. GLC conditions: 25 m × 0.4 mm I.D. coated with 5% Chromosorb R and 15% OV-101 SCOT column; carrier gas, hydrogen at a flow-rate of 3 ml/min; make-up gas argon–methane (90:10) flow-rate, 50 ml/min. Temperatures: detector, 320°C; no inlet heater block; column, 80°C programmed at 4°C/min. Pulse interval, 15 μsec; attenuation, 2 × 10^2; sample size, 1.0 μl. Curves: 1 = tyrosine; 2 = arginine; 3 = ornithine; 4 = threonine; 5 = lysine; 6 = serine; 7 = cystine; 8 = pipecolinic acid; 9 = glutamic acid; 10 = methionine; 11 = aspartic acid; 12 = proline; 13 = phenylalanine; 14 = alanine; 15 = leucine; 16 = 2-aminobutyric acid; 17 = glycine; 18 = norleucine; 19 = isoleucine; 20 = valine.

range for the same amino acid derivatives, but evidence was only presented for histidine[10]. Under our conditions the linear ranges for all the derivatives lay between *ca.* 1 and 100 pmol injected.

Increasing the pulse interval of the detector from 15 to 150 μsec showed a *ca.* 10-fold increase of sensitivity whilst still retaining a linear response over a limited range, as shown for alanine and lysine in Fig. 2. However, in practice it was best to work with a pulse interval of 15 μsec as previously reported[9]. Fig. 3 shows the separation of 27 N(O)-HFB amino acid isobutyl ester derivatives on a column at 80°C and programmed at 4°C/min to 230°C. Occasional "dips" in the baseline were seen. Increasing the pulse interval to gain increased sensitivity resulted in an increased size of the "dips", which made difficult the integration of peak areas. Some increase in sensitivity of detection was shown with an increase of pulse interval from 100 to 2000 μsec[15].

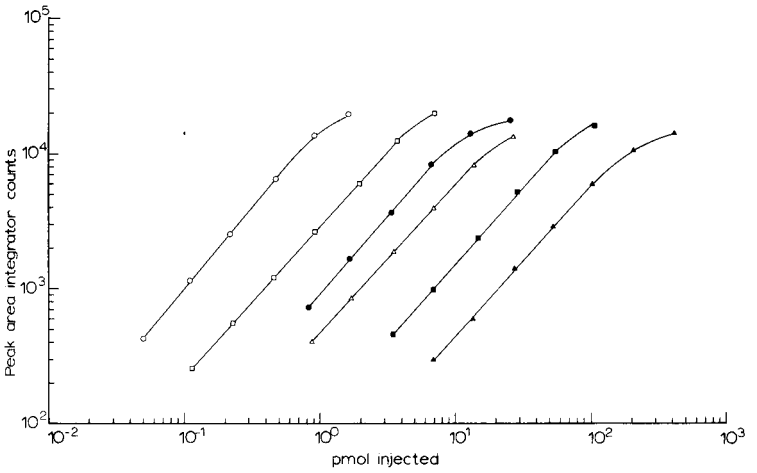

Fig. 2. Response–concentration curves for N(O)-HFB isobutyl ester derivatives of two amino acids with the ECD and different pulse intervals. Preparation of sample: see Experimental. GLC conditions as in Fig. 1. Pulse interval, 15 μsec; ▲ = alanine; △ = lysine. Pulse interval, 50 μsec; ■ = alanine; □ = lysine. Pulse interval, 150 μsec; ● = alanine; ○ = lysine.

Table I shows the relative molar response (RMR) values of HFB-amino acid isobutyl esters. The variation in RMR was *ca.* 36-fold (0.90 for valine to 32.7 for tyrosine). The reason for this wide range of RMR values cannot be fully explained. It is known that the ECD response varies with the type of electron-capturing group and the structure of the carrying molecule, but it is not proportional to the number of electron-capturing groups on a single molecule. Thus, because they carry two HFB groups, serine and threonine gave much higher RMR values than amino acids such as alanine, which carry only one HFB group. The very high value for tyrosine (32.7) was due not only to the presence of two HFB groups, but additionally to the effect of the aromatic ring. Thus, tyrosine showed a higher response than phenylalanine (Table I) as shown for the trifluoroacetyl (TFA) *n*-butyl ester derivatives[8]. Our results for the lower response given by 3,4-dihydroxyphenylalanine compared with that given by tyrosine cannot be explained.

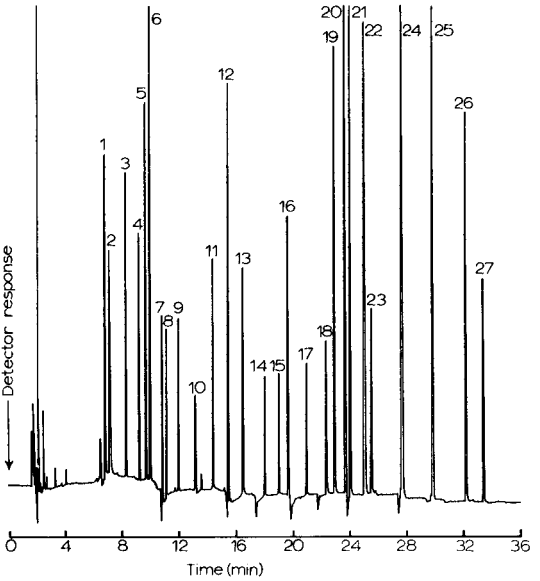

Fig. 3. Separation of N(O)-HFB isobutyl ester derivatives of amino acids with the ECD. Preparation of sample: see Experimental. GLC conditions: as in Fig. 1. The amounts of amino acids represented by individual peaks ranged from 1.4 pmol (tyrosine) to 34.0 pmol (valine). Peaks: 1 = alanine; 2 = glycine; 3 = 2-aminobutyric acid; 4 = valine; 5 = threonine; 6 = serine; 7 = leucine; 8 = isoleucine; 9 = norleucine; 10 = proline; 11 = pipecolinic acid; 12 = hydroxyproline; 13 = methionine; 14 = aspartic acid; 15 = phenylalanine; 16 = ornithine; 17 = glutamic acid; 18 = lysine; 19 = tyrosine; 20 = ε-monomethyllysine; 21 = 3,4-dihydroxyphenylalanine; 22 = arginine; 23 = carboxymethylcysteine; 24 = homoarginine; 25 = lanthionine; 26 = cystathionine; 27 = cystine.

Bengtsson *et al.*[10] rather surprisingly showed an ECD response for HFB-glycine isobutyl ester which was much greater than that for the corresponding lysine derivative, which carried two HFB groups. It was shown with N,O-bis-TFA threonine methyl ester that the ECD response was *ca.* 3500 times greater than the FID response, whilst N-TFA alanine, valine, isoleucine and leucine methyl esters gave an increased sensitivity of detection 100–200-fold over the FID[7]. HFB derivatives gave the best compromise between sensitivity and volatility, compared with TFA or pentafluoropropionyl derivatives[16] and N,O-bis-HFB tyrosine methyl ester could be detected down to *ca.* $0.5 \cdot 10^{-15}$ M[17].

The separation of N(O)-HFB-isobutyl ester derivatives of 27 amino acids on an OV-101 SCOT column with temperature programming from 80 to 230°C and the ECD set at 320°C is shown in Fig. 3. There were fewer extraneous peaks due to impurities than previously reported[10]. The small peak at 13.5 min following proline was due to cysteine which was derived from cystine. Histidine was not included in the mixture, because to ensure complete acylation of the amino acid it was recommended that on-column acylation should be used[14]. The excess acylating reagent affected the ECD response and this method was not applicable in our hands.

Widely differing concentrations of the amino acids were used because of their varying responses to the ECD. Thus there is a disadvantage in using the ECD for the determination of all the protein amino acids in a single sample. Different amounts of

TABLE I

RELATIVE RETENTION TIMES AND RELATIVE MOLAR RESPONSE VALUES OF N(O)-HFB AMINO ACID ISOBUTYL ESTER DERIVATIVES DETERMINED WITH THE ECD AGAINST NORLEUCINE (= 1.0).

GLC conditions as in Fig. 1. The retention time for norleucine in minutes is given in parentheses.

Amino acid	*Relative retention time*	*RMR ± S.D. (n = 8)*
Alanine	0.57	1.55 ± 0.03
Glycine	0.60	1.01 ± 0.03
2-Aminobutyric acid	0.69	1.06 ± 0.02
Valine	0.77	0.90 ± 0.03
Threonine	0.81	17.0 ± 0.31
Serine	0.83	15.1 ± 0.49
Leucine	0.90	1.14 ± 0.05
Isoleucine	0.93	0.95 ± 0.02
Norleucine	(11.90)	1.00
Proline	1.09	2.22 ± 0.08
Pipecolinic acid	1.21	3.05 ± 0.08
Hydroxyproline	1.30	24.3 ± 0.84
Methionine	1.38	2.46 ± 0.08
Aspartic acid	1.51	2.23 ± 0.05
Phenylalanine	1.59	2.02 ± 0.04
Ornithine	1.64	17.8 ± 0.97
Glutamic acid	1.76	2.50 ± 0.05
Lysine	1.87	15.2 ± 0.52
Tyrosine	1.92	32.7 ± 0.95
ε-Monomethyllysine	1.98	19.0 ± 0.73
3,4-Dihydroxyphenylalanine	2.01	25.4 ± 0.50
Arginine	2.09	21.8 ± 0.94
Carboxymethylcysteine	2.13	5.71 ± 0.15
Homoarginine	2.19	20.4 ± 0.87
Lanthionine	2.49	15.9 ± 0.43
Cystathionine	2.69	17.4 ± 0.77
Cystine	2.79	10.8 ± 0.53

the sample might require injecting onto the column for the determination of "low sensitivity" and "high sensitivity" electron-capturing derivatives.

It should be noted that when the ECD was set at 250°C a baseline rise was observed during the programme when the oven temperature exceeded *ca.* 180°C. The difficulty of using the ECD with temperature programming using conventional packed GLC columns was mentioned[8]. However, with capillary columns the carrier gas flow-rate (*ca.* 3 ml/min) is small compared with the 50 ml/min used for the make-up gas, and a steady baseline may be maintained as seen in Fig. 3.

The determination of amino acids attached to tRNA[18] was carried out in this study. Fig. 4a shows the separation of fourteen identifiable amino acids from tRNA detected with the ECD. The advantage of the use of the ECD over that of the FID is seen by comparison with Fig. 4b, where certain amino acids present in small amounts, such as proline, lysine and arginine, were barely detectable by the FID although they were readily determined with the ECD. In addition there were peaks of unknown composition with high electron-capturing properties which eluted after 5, 7, 30 and 32 min. The detection of these compounds was only possible with the ECD, and how they were derived from tRNA is now being investigated.

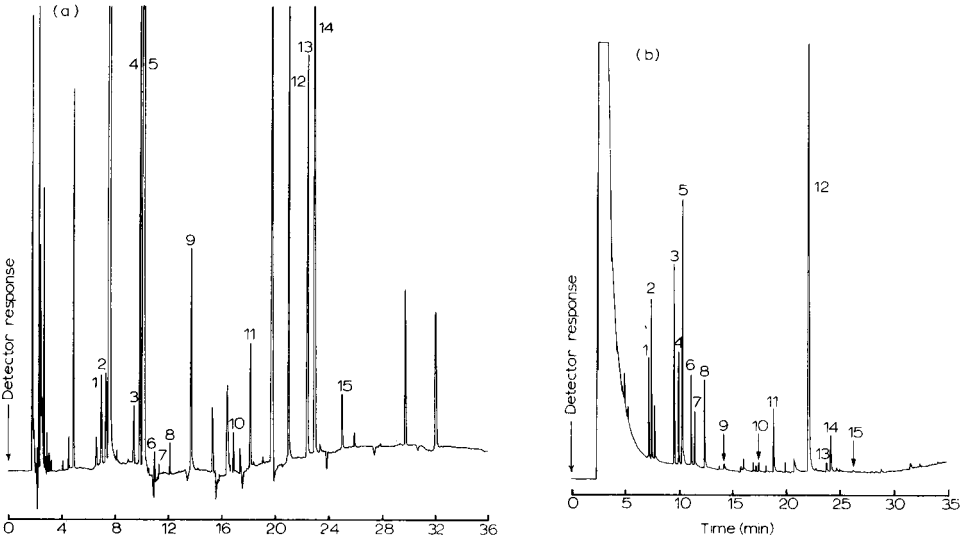

Fig. 4. Separation of N(O)-HFB amino acid isobutyl ester derivatives obtained from silk-worm tRNA after deacylation and analysed with (a) the ECD and (b) the FID. GLC conditions: (a) as in Fig. 1; (b) same conditions except make-up gas nitrogen flow-rate 30 ml/min; hydrogen 27 ml/min; air 350 ml/min; amount injected 20 times greater. Peaks: 1 = alanine; 2 = glycine; 3 = valine; 4 = threonine; 5 = serine; 6 = leucine; 7 = isoleucine; 8 = norleucine (I.S.); 9 = proline; 10 = methionine; 11 = aspartic acid; 12 = glutamic acid; 13 = lysine; 14 = tyrosine; 15 = arginine.

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CHROM. 14,370

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BENZOPHENONE DERIVATIVES FOR THE DETERMINATION OF BENZODIAZEPINES IN CLINICAL EMERGENCIES

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(First received June 22nd, 1981; revised manuscript received September 2nd, 1981)

SUMMARY

A screening procedure for fifteen 1,4-benzodiazepines in biological material (urine), suitable for clinical toxicology, is described. The benzodiazepines and/or their metabolites are hydrolysed to their benzophenones; after alkalinization, the benzophenones are extracted with chloroform. The extract is evaporated to dryness and the dried residue is dissolved in methanol and analysed by high-performance liquid chromatography on a reversed-phase column at 254 nm with methanol–water (1:1) as eluent.

INTRODUCTION

Benzodiazepines are widely used for their sedative and hypnotic activity and for anticonvulsant medication, and are responsible for considerable clinical demands in emergency toxicology. Although benzodiazepines are seldom responsible for fatal cases in toxicology, the danger is enhanced when they are taken in addition to other intoxicants such as alcohol, barbiturates or tricyclic antidepressants.

The analysis of benzodiazepines and their metabolites is often performed by gas–liquid chromatography (GLC)[1–12], mostly with electron-capture detection, high-performance liquid chromatography (HPLC)[13–18] and gas chromatography–mass spectrometry (GC–MS)[19–21]. These very specific and sensitive methods, focused on one or a few benzodiazepines, are very suitable for use in drug monitoring or when the intoxicant is known. In clinical toxicology, however, a less complex and less time-consuming procedure for screening a group of compounds can be very useful. From this point of view, the analysis of benzodiazepines as their hydrolysis products (benzophenones) is frequently used. The hydrolysis breaks down the benzodiazepines and their metabolites, both free and conjugated, to benzophenones, yielding an increased sensitivity. These benzophenones can be analysed by thin-layer chromatography[22–24], GLC[25] or GC–MS[26].

In the proposed method, benzodiazepines are analysed by the HPLC of their benzophenones, which are obtained by acid hydrolysis of biological samples. After

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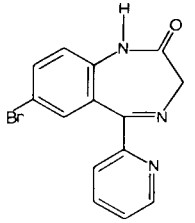
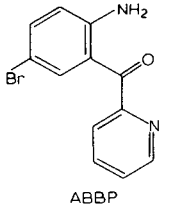
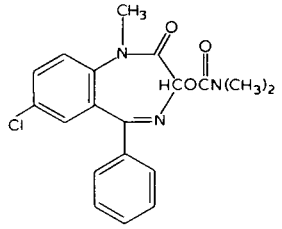
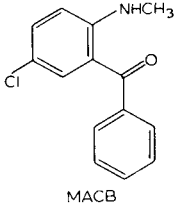
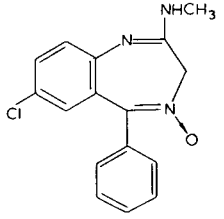
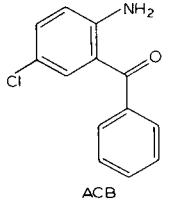
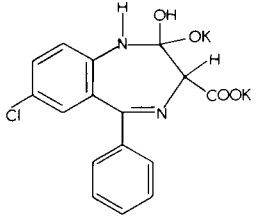
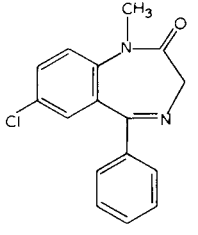
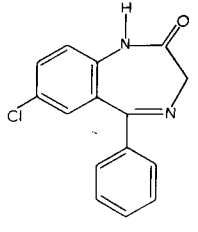
TABLE I

BENZODIAZEPINES, THEIR METABOLITES AND THE RESPECTIVE BENZOPHENONES

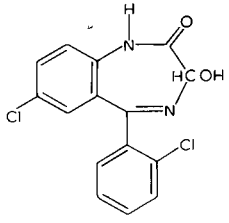
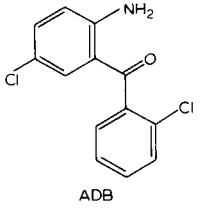
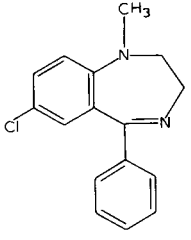
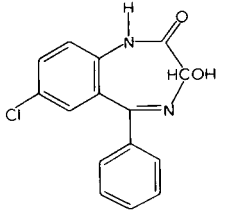
ABBP = 2-(2-Amino-5-bromobenzoyl)pyridine; MACB = 5-chloro-2-(methylamino)benzophenone; ACB = 2-amino-5-chlorobenzophenone; ADB = 2-amino-5-chloro-2′-chlorobenzophenone; ANB = 2-amino-5-nitrobenzophenone; DAB = 2,5-diaminobenzophenone; ANCB = 2-amino-2′-chloro-5-nitrobenzophenone; DACB = 2′-chloro-2,5-diaminobenzophenone; CACB = 5-chloro-2-(cyclopropylmethylamino)benzophenone; PACB = 5-chloro-2′-(propynylamino)benzophenone; ANFB = 2-amino-2′-fluoro-5-nitrobenzophenone; MANFB = 2′-fluoro-2-(methylamino)-5-nitrobenzophenone; MAAFB = 5-amino-2′-fluoro-2-(methylamino)benzophenone.

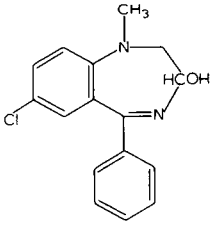
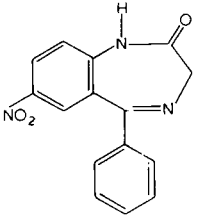
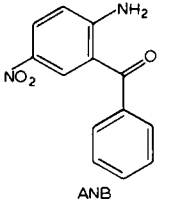
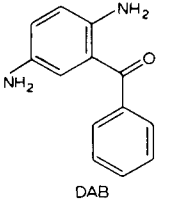
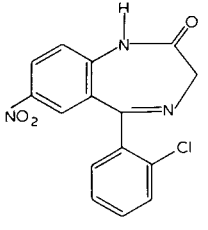
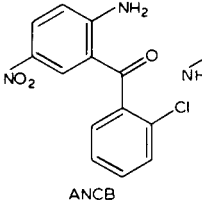
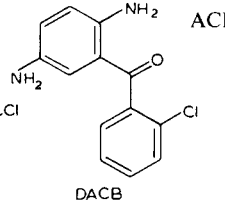
Name	*Formula*	*Urinary metabolites (free or conjugated)*	*Respective benzophenone(s)*	*Benzophenone(s) used*
Bromazepam (Lexotan)		3-Hydroxybromazepam 2-(2-Amino-5-bromobenzoyl)-pyridine 2-(2-Amino-5-bromo-3 hydroxy-benzoyl)pyridine	 ABBP	ABBP
Camazepam (Albego, Paxor)		N-Methyloxazepam	 MACB	MACB
Chlordiazepoxide (Librium)		Demethylchlordiazepoxide Demoxepam Nordiazepam	 ACB	ACB

Dipotassium chlorazepate (Belseren, Tranxène)		Nordiazepam Oxazepam	ACB	ACB
Diazepam (Valium)		Temazepam Nordiazepam Oxazepam	MACB, ACB	MACB, ACB
Demethyldiazepam (Madar)		Nordiazepam Oxazepam	ACB	ACB

(Continued on p. 160)

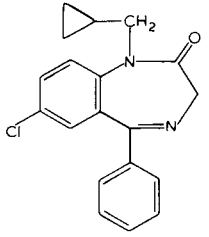
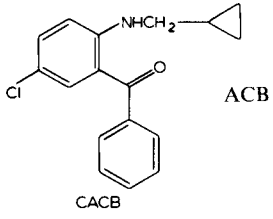
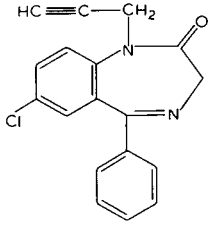
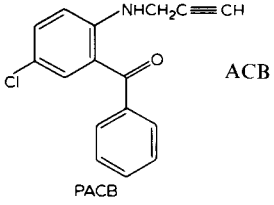
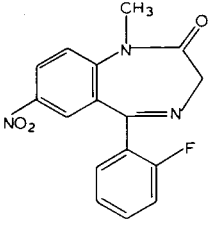
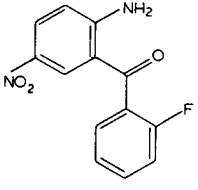
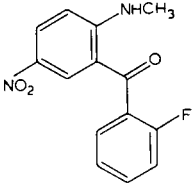
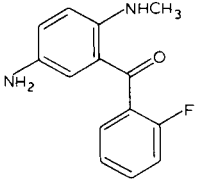
TABLE I *(continued)*

Name	*Formula*	*Urinary metabolites (free or conjugated)*	*Respective benzophenone(s)*	*Benzophenone(s) used*
Lorazepam (Temesta)		Almost not metabolized but excreted as glucuronide	 ADB	ADB
Medazepam (Nobrium)		Oxazepam (major metabolite) Diazepam Normedazepam Nordiazepam Dehydromedazepam	MACB, ACB	ACB, MACB
Oxazepam (Seresta)		Not metabolized but excreted as glucuronide	ACB	ACB

Temazepam (Levanxol)		Oxazepam Temazepam	MACB, ACB	ACB, MACB
Nitrazepam (Mogadon)		2-Amino-5-nitrobenzophenone 2-Amino-3-hydroxy-5-nitro-benzophenone 7-Aminonitrazepam 7-Acetamidonitrazepam	 ANB  DAB	ANB
Clonazepam (Rivotril)		7-Aminoclonazepam 7-Acetamidoclonazepam 3-Hydroxyclonazepam 7-Amino-3-hydroxyclonazepam	 ANCB  DACB	ACB

(Continued on p. 162)

TABLE I *(continued)*

Name	*Formula*	*Urinary metabolites (free or conjugated)*	*Respective benzophenone(s)*	*Benzophenone(s) used*
Prazepam (Lysanxia)		Nordiazepam Oxazepam	 ACB CACB	ACB
Pinazepam (Domar)		Nordiazepam Oxazepam (major metabolite) 3-Hydroxypinazepam	 ACB PACB	ACB
Flunitrazepam (Rohypnol)		Norflunitrazepam (major metabolite) Flunitrazepam 7-Aminoflunitrazepam 3-Hydroxyflunitrazepam	 ANFB  MANFB  MAAFB	ANFB, MANFB

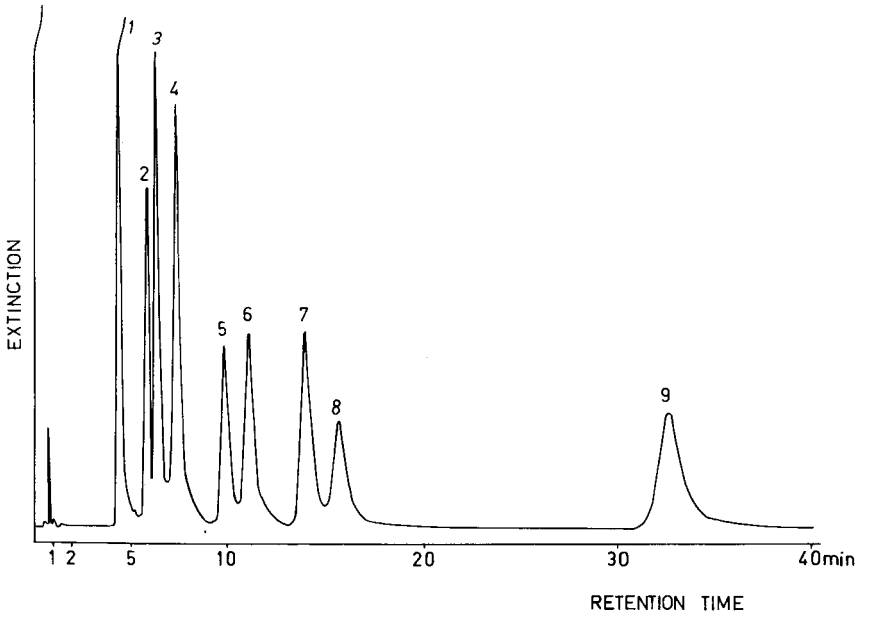


Fig. 1. Chromatogram of eight benzophenones and internal standard: 1 = ABBP; 2 = ANFB; 3 = ANB; 4 = ANCB; 5 = MANFB; 6 = camazepam; 7 = ACB; 8 = ADB; 9 = MACB.

alkalinization of the hydrolysate, the benzophenones are extracted with chloroform, the solution is evaporated to dryness, the residue is dissolved in methanol, an internal standard is added and HPLC is then performed, following a previously described method[27]. Identification and quantification of the benzophenones are performed by calculating relative retention times and relative peak heights. Hydrolysis and extraction yields were calculated for eight benzophenones derived from thirteen benzodiazepines.

EXPERIMENTAL

A survey of the benzodiazepines and their urinary metabolites[1,3,5,19,21,28–37], with their respective benzophenones, is given in Table I.

The eight benzophenones used (see Table I, last column) were analysed by HPLC. As described previously[27], the chromatogram permits the identification of methanolic solutions of the benzophenones (for the chromatogram of all of the benzophenones and the internal standard, see Fig. 1) from their relative retention times and peak heights, in the concentration range 0.1–1000 μg/ml.

The technique was applied to the analysis of urine.

Benzodiazepine and benzophenone standards

Benzodiazepine standards were obtained as follows: bromazepam, chlordiazepoxide, diazepam, medazepam, temazepam, desmethylflunitrazepam, flunitrazepam, nitrazepam and clonazepam from Roche (Brussels, Belgium); lorazepam and oxazepam from Wyeth (Münster, G.F.R.); camazepam from Sintesa (Milan, Italy);

dipotassium chlorazepate from Clin-Midy (Brussels, Belgium); and desmethyldiazepam from Willpharm (Brussels, Belgium).

Benzophenone standards were donated by Hoffmann-La Roche (Basle, Switzerland), except for ADB, which was synthesized[22].

A standard mixture of benzophenones (see Table I, last column), each at a concentration of 50 μg/ml in methanol, was prepared, and camazepam was added at the same concentration as an internal standard.

Hydrolysis and extraction

A 5-ml volume of sample (containing benzodiazepines and free or conjugated metabolites) was hydrolysed with concentrated hydrochloric acid for 15 min on a boiling water-bath. The hydrolysates were alkalinized to pH 12 with 30% sodium hydroxide solution. The samples were cooled and 10 ml of chloroform were added. After extraction for 5 min, the aqueous phase was aspirated. The organic layer was filtered through Whatman No. 1 phase-separating filters and evaporated to dryness. The residue was dissolved in 500 μl of methanol containing 25 μg of camazepam as an internal standard.

All reagents were of analytical-reagent grade from UCB.

Chromatographic conditions

The high-performance liquid chromatograph was a Hewlett-Packard Model 1081A with UV detection at 254 nm. The stationary phase was LiChrosorb Hibar (Merck, Darmstadt, G.F.R.) with a particle size of 7 μm, pre-packed in a 250 × 4 mm I.D. column. The mobile phase was methanol–water (1:1).

The column pressure was kept at 350 bar with a solvent flow-rate of 3.02 ml/min. The six-port valve loop injector was provided with a 10-μl loop. All work was carried out at ambient temperature.

RESULTS

Extraction of benzophenones

The percentage extractions of the benzophenones were determined by HPLC. Each benzophenone was extracted from water and blank urine, spiked with benzophenone (5 μg/ml) and the above extraction and HPLC procedure was carried out as described above. The results ($n = 15$, $\sigma = 0.05$) are given in Table II.

Hydrolysis yield of benzodiazepines to benzophenones

Knowing the percentage extraction, the hydrolysis yield to the benzophenone was determined for each benzodiazepine. Benzodiazepines were added to water and

TABLE II

EXTRACTION YIELDS (%) OF BENZOPHENONES FROM WATER AND URINE

Sample	*ABBP*	*MANFB*	*ANFB*	*MACB*	*ACB*	*ANB*	*ADB*	*ANCB*
Water	84	57	79	85	89	93	88	78
Urine	82	73	80	92	88	93	88	82

TABLE III

HYDROLYSIS YIELD OF BENZOPHENONES FROM BENZODIAZEPINES

Benzodiazepine	*Benzophenone*	*Hydrolysis yield (%)*	
		Water	*Urine*
Bromazepam	ABBP	42	45
Flunitrazepam	MANFB	81	78
Demethylflunitrazepam	ANFB	93	92
Diazepam	MACB	74	72
Temazepam	MACB	99	96
Camazepam	MACB	99	100
Medazepam	MACB	0	0
Oxazepam	ACB	83	78
Dipotassium chlorazepate	ACB	30	28
N-Demethyldiazepam	ACB	94	98
Chlordiazepoxide	ACB	60	72
Nitrazepam	ANB	93	96
Lorazepam	ADB	65	61
Clonazepam	ANCB	76	65

blank urine samples in the following concentrations: (mol.wt. benzodiazepine/mol.wt. benzophenone)·5 μg/ml. The above-described procedure was then performed. The results ($n = 15$, $\sigma = 0.06$) are given in Table III.

Using the hydrolysis and extraction yields in water, standard benzophenones for HPLC can be prepared from benzodiazepines and can be used in routine analysis.

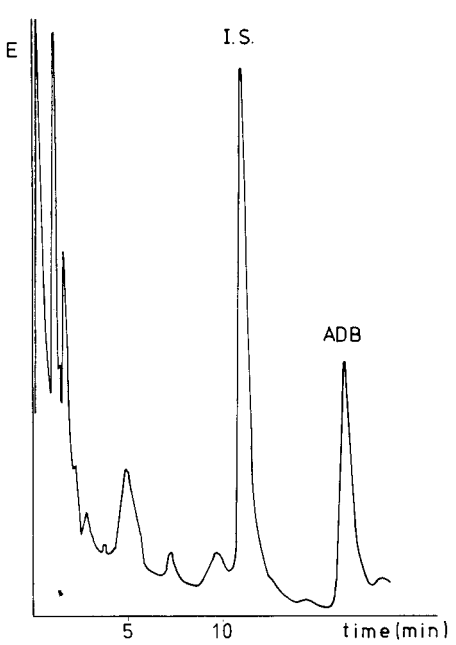
Analysis of benzodiazepines in urine

Fig. 2–6 are examples of chromatograms of benzophenones obtained on applying the described procedure to urine samples. Knowing the percentage extraction and hydrolysis yield, the amount of benzodiazepine equivalent to the amount of benzophenone can be calculated.

The sensitivity of the method, when the screening is performed as described above, is 0.01 μg/ml of benzophenone in urine. When a single dose of 1 mg of benzodiazepine (which is a low dose) is ingested, and assuming that 5% of the dose (minimal value, *i.e.*, for chlorazepate) is excreted in the urine, the benzophenone concentration will be 0.03 μg/ml in 24-h urine. If necessary, the sensitivity can be increased by dissolving the residue in less methanol or by increasing the sample size.

DISCUSSION

In practice, all of the 15 benzodiazepines and/or their metabolites (Table I) yield one or two of the eight benzophenones in acceptable amounts: bromazepam (ABBP), camazepam (MACB), chlordiazepoxide (ACB), dipotassium chlorazepate (ACB), demethyldiazepam (ACB), diazepam (ACB, MACB), lorazepam (ADB), oxazepam (ACB) and temazepam (ACB, MACB) are totally recovered as their benzophenones.



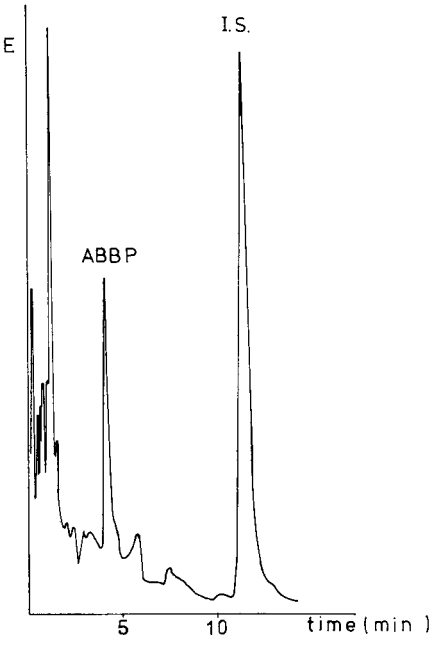
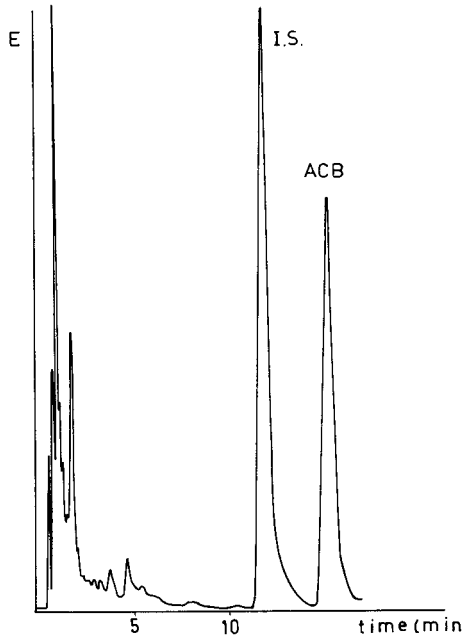


Fig. 2. Chromatogram of urine extract of ADB from a patient taking lorazepam (4 μg/ml of lorazepam in urine).

Fig. 3. Chromatogram of urine extract of ABBP from a patient taking bromazepam (1.2 μg/ml of bromazepam in urine).



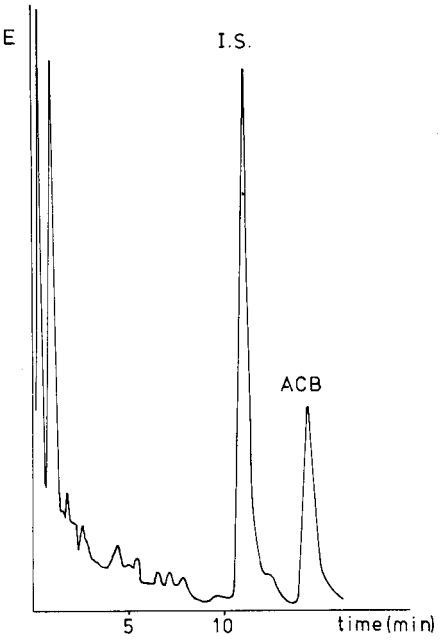


Fig. 4. Chromatogram of urine extract of ACB from a patient taking oxazepam (10 μg/ml of oxazepam in urine).

Fig. 5. Chromatogram of urine extract of ACB from a patient after ingestion of dipotassium chlorazepate (6.4 μg/ml of dipotassium chlorazepate in urine).

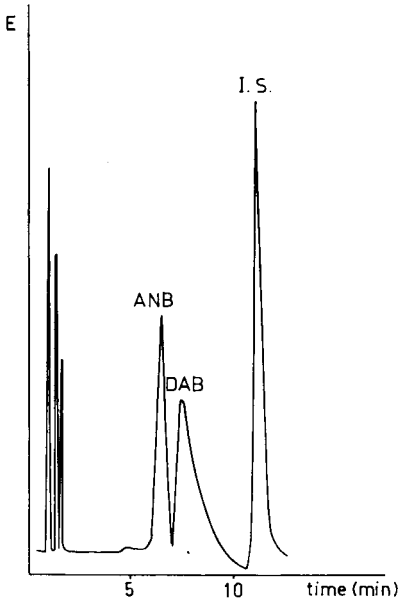


Fig. 6. Chromatogram of a urine extract of ANB and DAB after ingestion of nitrazepam.

Although medazepam cannot be hydrolysed, it is recovered in urine via its metabolization products, diazepam, nordiazepam and, as a major metabolite, oxazepam (MACB, ACB).

Flunitrazepam is quantitated via its own benzophenone MANFB and via ANFB, the benzophenone of the main metabolite.

Nitrazepam and its metabolites yield two benzophenones. ANB and DAB, from which only ANB is quantitated. DAB under these conditions gives too much tailing (see Fig. 6), and can be used only for qualitative purposes. Clonazepam is recovered as ANCB. The other benzophenone, DACB, does not chromatograph under these conditions. As pinazepam and prazepam are readily metabolized to their N-demethyl derivatives, these products can also be quantitated via the benzophenone ACB.

For the specific benzophenones ADB, ABBP, ANB, ANCB, MANFB and ANFB results of analysis in urine can be expressed in μg/ml of lorazepam, bromazepam, nitrazepam, clonazepam and flunitrazepam, respectively. When ACB and MACB are found the results are expressed in equivalents of diazepam; and when ACB is found, in equivalents of demethyldiazepam. If further identification and quantification of the unhydrolysed benzodiazepines and metabolites should be necessary, a supplementary procedure can follow the HPLC, *e.g.*, GLC with electron-capture detection[6]. The analysis time in routine application for total screening is 1 h, according to the retention time of the last eluting peak (MACB). As in some instances the amount of benzodiazepines might not be totally recovered (owing to non-detection of DACB, non-quantification of DAB or imperfect hydrolysis of metabolites), the method provides a semi-quantitative analysis of the benzodiazepines. As the benzodiazepines are analysed as their benzophenone derivatives, there is a loss of

specificity but a gain in sensitivity as benzodiazepines themselves and their metabolites, free or conjugated, yield the same benzophenone(s). This possible decrease in specificity can be offset against the possibility of screening all of these benzodiazepines simultaneously in a reasonable time.

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DETERMINATION OF MIMOSINE AND 3-HYDROXY-4(1*H*)-PYRIDONE IN *LEUCAENA*, AVIAN EXCRETA AND SERUM USING REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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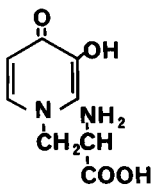
SUMMARY

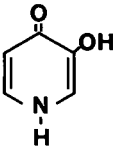
The estimation of mimosine and 3-hydroxy-4(1*H*)-pyridone in *Leucaena leucocephala*, *Leucaena* seeds, chick excreta and chick serum using reversed-phase ion-pair high-performance liquid chromatography was investigated. Isocratic elution of both compounds was achieved in 11 min using sodium octyl sulphate as the pairing agent in a pH 2.25 buffer. Good recoveries of both mimosine and 3-hydroxy-4(1*H*)-pyridone in all but serum samples were obtained.

INTRODUCTION

The legume *Leucaena leucocephala* is grown extensively in semi-arid tropical and sub-tropical areas of the world, and its wide variety of uses have been thoroughly discussed[1–4]. Its use as a protein source (224–344 g kg^{-1} on a dry matter basis)[5,6] for animals is limited because of a number of factors, one of the major constraints being its relatively high content (10–100 g kg^{-1} of dry matter)[6,7] of the unusual and toxic amino acid mimosine ((*S*)-β-[N-(3-hydroxy-4-pyridone)]-α-aminopropanoic acid; I). It is well documented that mimosine is a depilatory agent, and studies on the use of mimosine and its analogues as defleecing agents have been reported in detail[8–10]. The large variety of biochemical, biological and nutritional effects of mimosine, including inhibition of protein synthesis, is also well documented[10–15,22,23].



I



II

The major hydrolysis product of mimosine, 3-hydroxy-4(1*H*)-pyridone (DHP; II), has been found in dried *Leucaena*. It has also been found in ruminant and non-

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ruminant excreta when these animals have been fed diets containing *Leucaena* or mimosine[9,16–18]. DHP has been reported to be goitrogenic, a potent inhibitor of some enzymes[19,20] and a weak inhibitor of thymidine incorporation into mouse bone marrow cells *in vitro*[21].

The methods commonly used for the analysis of mimosine and/or DHP in *Leucaena* include colorimetry[24,25], paper chromatography[16], thin-layer chromatography[26], gas–liquid chromatography[27], ion-exchange chromatography (IEC)[28] and electrophoresis[29]. The use of IEC for the estimation of mimosine in ovine blood has also been reported[9,30]. All of these methods suffer from a variety of disadvantages, not least their inability to estimate mimosine and DHP simultaneously, rapidly, and in the case of colorimetry, specifically.

The use of high-performance liquid chromatography (HPLC) for the separation and estimation of amino acids from various sources is becoming popular[31]. Recently, however, the use of reversed-phase ion-pair HPLC (RP-IP-HPLC) for the separation and quantification of cation (and anion) forming compounds[32–34] has tended to predominate. A logical step in the estimation of both mimosine and DHP would, therefore, appear to be the use of RP-IP-HPLC. Two brief reports on the use of HPLC for the estimation of mimosine and DHP in *L. leucocephala*, ruminant urine, and chick excreta have appeared recently[35,36]. One of these methods involved the use of phosphoric acid as the ion-pairing agent[35], while the other utilised the ion-pairing effects of sodium octyl sulphate[36].

We report here the detailed methodology of RP-IP-HPLC, using the octyl sulphate anion as the pairing ion, for the estimation of mimosine and DHP simultaneously in *Leucaena* leaf meal (LLM), *Leucaena* seeds, chick excreta and serum.

EXPERIMENTAL

Sample preparation

LLM, Leucaena seeds and excreta. Finely ground samples of LLM, *Leucaena* seeds and freeze-dried excreta (or *ca.* 20 g of a homogenous mixture of fresh excreta) were prepared as described previously[28]. An aliquot of the resultant extract was forced through a Sep-Pak C_{18} cartridge (Waters Assoc., Stockport, Great Britain) to remove or reduce contaminants. Washing the Sep-Pak cartridges with 0.1 *M* HCl (2 × 5 cm^3), methanol (2 × 5 cm^3), distilled water (2 × 5 cm^3) followed by 2–3 cm^3 of extract, prior to collection of the eluate from the Sep-Pak produced a cleaner solution for chromatography. This procedure also allowed re-use of the cartridges.

Serum. Whole chick blood was allowed to stand overnight at 4°C and was then centrifuged (2500*g*) for 8 min. The resultant serum was decanted off and stored at −20°C until required for analysis. Protein precipitation in the serum was accomplished using two precipitants: sulphosalicylic acid (SSA; 8 g in 100 cm^3 of distilled water) and phosphotungstic acid (PTA; $H_3PO_4 \cdot 12WO_3 \cdot xH_2O$; 6 g in 100 cm^3 of distilled water). (Both precipitants were obtained from BDH, Poole, Great Britain). Precipitation of protein was also attempted using both saturated $(NH_4)_2SO_4$ and ethanol, but these proved to be unsatisfactory because addition of SSA to the supernatant precipitated further amounts of protein. Precipitation of serum proteins using SSA and PTA was achieved by adding 0.25 cm^3 of either of the precipitants to 1 cm^3 of serum. The resultant mixture was then centrifuged (3000 g) for 5 min, the super-

natant was decanted off and subsequently forced through a C_{18} Sep-Pak cartridge, pretreated as described above, prior to chromatographic analysis. The Sep-Pak cartridges were then discarded.

Preparation of standards

Stock standard solutions of mimosine (0.25 m*M*) and DHP (0.5 m*M*) were prepared by dissolving the dried, crystalline materials in 0.1 *M* HCl. More dilute standards were prepared by dilution of appropriate volumes of the stock standards with 0.1 *M* HCl. Standard solutions with concentrations in the expected working ranges of 6.25 μM to 0.25 m*M* with respect to mimosine and 12.5 μM to 0.25 m*M* with respect to DHP were prepared. Mimosine (Sigma, Poole, Great Britain) was dried to constant weight in an oven prior to dissolution. DHP was prepared by a modification of the method of Hegarty *et al.*[16]. Mimosine (2 g) was refluxed in 0.1 *M* HCl (200 cm^3) for 24 h, and the resultant DHP separated from other impurities as described by Hegarty *et al.* DHP, however, could not be eluted from the ion-exchange resin using 0.1 *M* HCl (2.5 dm^3). Elution of DHP was accomplished by washing the resin with 1 *M* HCl (700 cm^3). Other preparative details are as described by Hegarty *et al.*[16], although vacuum sublimation was not performed. Repeated recrystallisation of DHP from ethanol followed by washing with diethyl ether yielded buff-coloured crystals (m.p. 240–243 dec; lit. 242–244 dec.[16,37]). Infrared spectroscopy produced a spectrum which was consistent with that expected for DHP, while chromatographic analysis did not reveal any impurities in the prepared DHP. The UV spectrum showed a λ_{max} of 269 nm and an extinction coefficient, in 0.1 *M* HCl, of 516 $m^2\ mol^{-1}$ at 269 nm.

Recoveries

Recovery of mimosine from LLM, estimated using IEC, has been previously reported[28]. Recovery of mimosine, added to excreta prior to extraction, also estimated using IEC, has been found to be 102.7% ($\pm$ 3.0) (previously unreported results). A comparison of results obtained using IEC with those obtained using HPLC was regarded as yielding sufficient information on recovery of mimosine from excreta, LLM and *Leucaena* seeds. Recovery of DHP was measured by determining the DHP content of LLM and then adding crystalline DHP to the LLM at two levels. Recovery of DHP from excreta was ascertained by adding crystalline DHP to a freeze-dried DHP-free excreta sample. Extraction and subsequent preparation of the sample was as described in the Sample Preparation section of this report. An aliquot of a standard DHP solution was also added to the prepared LLM extract to measure chromatographic recovery, and give an indication of interference caused by any other components in the extract.

Recovery of both mimosine and DHP from chick serum was determined by the addition of 1 cm^3 of a standard solution (0.125 m*M* mimosine and 0.25 m*M* DHP) to 1 cm^3 of serum. Precipitation of protein was accomplished by the addition of 0.5 cm^3 of either PTA or SSA. Further treatment was as described earlier in this report.

Chromatography

An Altex liquid chromatography system (Scotlab Instrument Sales, Lanark, Great Britain) consisting of an Altex Model 110A pump, a Rheodyne 7120 injection valve with 20-μl loop, and an Altex-Hitachi Model 100-10 variable wavelength detec-

tor was used. An Altex column (25 × 0.46 cm I.D.) packed with LiChrosorb RP-18 ($d_p \approx 10$ μm) was used for all chromatographic separations reported here. The column was packed in this laboratory using a Magnus P6050 column packer (Magnus Scientific, Cheshire, Great Britain). Column efficiency, determined using naphthalene eluted isocratically with aqueous methanol (70 cm^3 CH_3OH made to 100 cm^3 with distilled water) at a flow-rate of 1 cm^3 min^{-1}, was 4321 theoretical plates, (*i.e.* $N = 4321$). The buffer used for chromatography was prepared by mixing 200 cm^3 of 0.01 *M* sodium octyl sulphate in 2% v/v HPLC grade methanol (crystalline sodium octyl sulphate was obtained from Kodak, Liverpool, Great Britain and CH_3OH was obtained from Rathburn Chemicals, Peeblesshire, Great Britain) with 240 cm^3 of HPLC grade methanol. Analytical grade sodium nitrate (5.1 g; BDH) was added and the mixture made up to 2 dm^3 with "in glass" double distilled water. The buffer was filtered through a Whatman GF/F glass fibre filter under reduced pressure, and the pH adjusted to 2.25 with HNO_3 (≈ 7.9 *M*). It was then degassed ultrasonically prior to use.

The volume of extracts and standards loaded was restricted to 20 μl.

The methodology used for ion-exchange chromatographic analyses of samples has been previously reported[28].

RESULTS AND DISCUSSION

The excellent linear response of the system to both mimosine and DHP within their respective, expected working concentrations is shown in Fig. 1. Standards with concentrations ranging from 6.25 μ*M* to 0.25 m*M* for mimosine, and from 12.5 μ*M* to 0.25 m*M* for DHP were analysed in triplicate, and peak area was plotted against concentration. Correlation coefficients and standard errors for mimosine and DHP standards, measured at their λ_{max} wavelengths of 278 and 269 nm, respectively, and at different sensitivities, indicate an excellent linear response and precision of analysis for loaded amounts of mimosine from 0.125 nmol to 5 nmol. Loaded amounts from 0.25 nmol to 5 nmol of DHP produced similarly good results.

Typical chromatograms of a standard mixture of mimosine and DHP, those of a 6 *M* HCl extract of LLM and two deproteinised serum samples are shown in Fig. 2. Near-baseline resolution of mimosine and DHP was obtained. The mean resolution (R_S) obtained from nine samples was 1.62 (± 0.106). The relative standard deviation (R.S.D.; 6.54%) is fairly large presumably because the nine samples consisted of serum, LLM, excreta and standards analysed on different days. The resolution was calculated as follows: $R_S = 2\,(t_{RDHP} - t_{R\ mimosine})/(W_{DHP} + W_{mimosine})$ where $t_{R\ DHP}$ and $t_{R\ mimosine}$ are retention times for DHP and mimosine, respectively, and W_{DHP} and $W_{mimosine}$ are base widths of the DHP and mimosine peaks, respectively. The phase capacity ratios (k') of mimosine and DHP were 3.63 (± 0.33) and 5.56 (± 0.493), respectively. The k' values were calculated from the same nine chromatograms used to calculate the resolution.

A small shoulder appeared at the base of the mimosine peak in some excreta extracts, but had little or no effect on the estimation of recovery of mimosine or DHP (Tables I and II). Mimosine or DHP was not detected in the serum of chicks fed a diet containing LLM. An interesting feature of the chromatograms of serum (Figs. 2c and 2d), however, was the concentration of compounds which eluted prior to

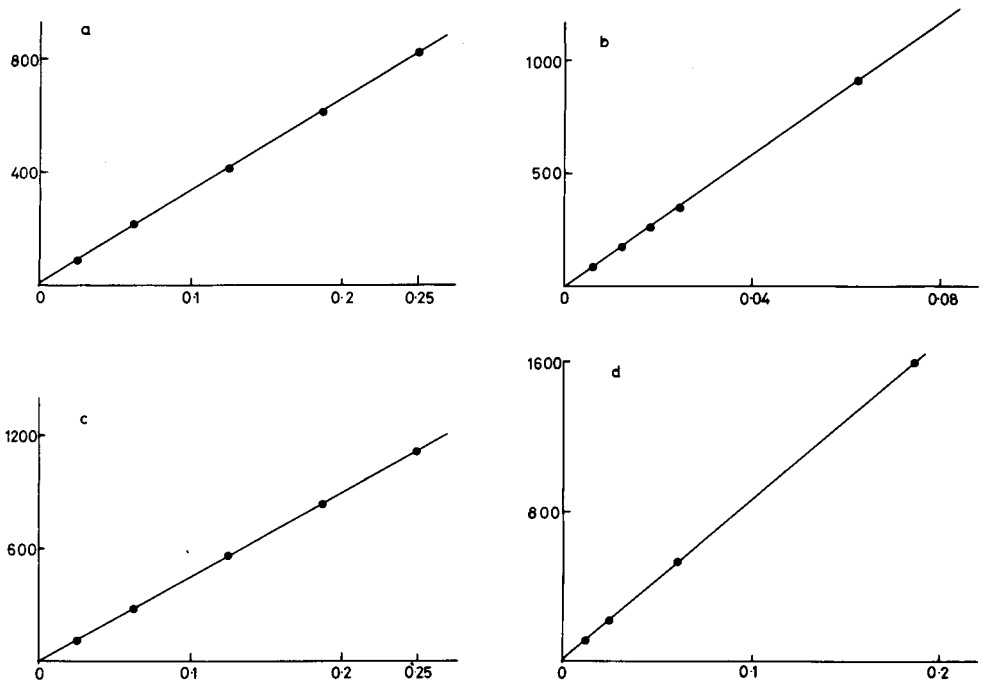

Fig. 1. HPLC response curves for mimosine (a, b) and 3-hydroxy-4(1*H*)-pyridone (c, d) at 278 and 269 nm, respectively. Concentration (m*M*) plotted on abscissa, peak area (mm^2) on ordinate. (a) 0.05 a.u.f.s.: $y = 3271.0x + 6.374$; standard error (S.E.) = 10.3; correlation coeff. (R) = 0.999. (b) 0.01 a.u.f.s.; $y = 14{,}738x - 15.208$; S.E. = 11.2; $R = 0.999$. (c) 0.02 a.u.f.s.: $y = 4486.6x - 0.77$; S.E. = 19.3; $R = 0.999$. (d) 0.01 a.u.f.s.: $y = 8513.5x + 3.57$; S.E. = 10.0; $R = 1.000$.

mimosine. Serum from chicks fed LLM had a higher concentration of these components than had serum from chicks fed LLM-free diets. The identities and significance of these compounds are not known but further study is warranted.

Mimosine values obtained in samples of LLM, chick excreta and *Leucaena* seeds, using IEC, agree well with those obtained using HPLC (correlation coefficient = 1.000) although slightly lower values were obtained for the two excreta samples when determined using HPLC. The percentage R.S.D. values for the seven samples vary somewhat, becoming fairly large when different extracts of the sample were analysed. The R.S.D. values for replicate analyses of the same extract, however, are fairly low even when mimosine was estimated at two different wavelengths. All R.S.D. values are within the limits expected for this type of analysis[38]. The precision, and good agreement with IEC values, obtained for the mimosine content of the LLM sample, analysed at 278 and 269 nm, indicate that no interfering compounds eluted simultaneously with mimosine.

The recovery of DHP (Table II), when added in crystalline form to LLM and excreta, and in solution to extracts of LLM, averaged 98.9%. The excellent recovery of DHP when added to extracts of LLM indicates that no interference from other compounds in the extract had occurred. Losses during clean-up of the sample were also negligible. Recovery of DHP, added in the crystalline form to LLM prior to extraction (Table II), is slightly low being least for the lowest level of addition. The

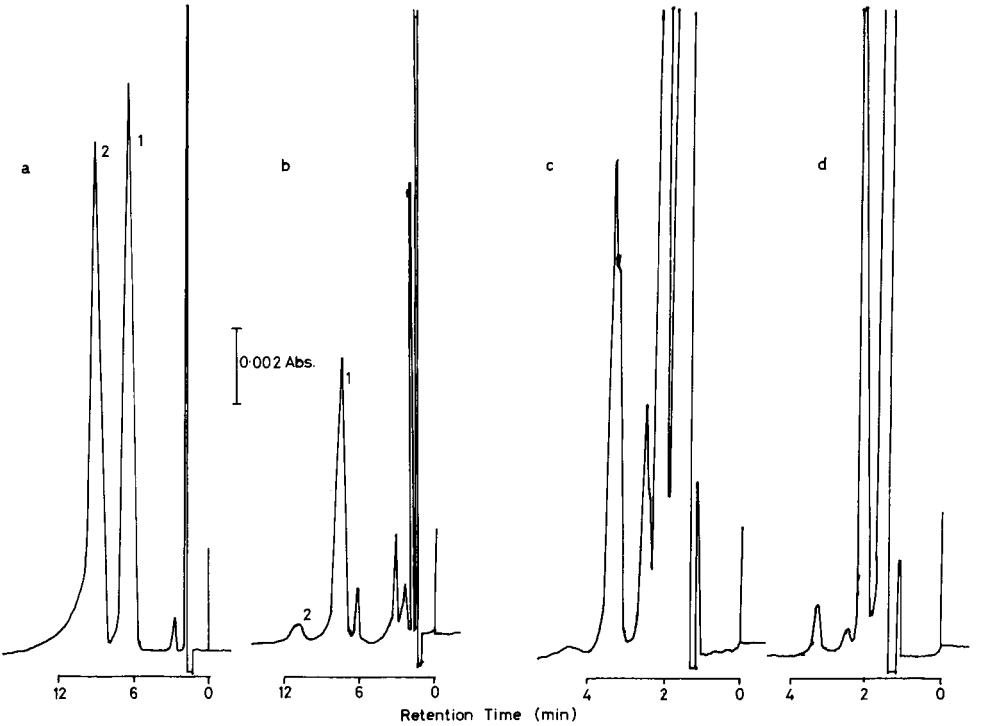

Fig. 2. Typical chromatograms of (a) a standard solution of mimosine and 3-hydroxy-4(1*H*)-pyridone; (b) a *Leucaena* leaf meal (LLM) extract; and serum samples from chicks fed (c) an LLM diet and (d) a diet without LLM. Peaks: 1 = mimosine; 2 = DHP. Flow-rate of pH 2.25 buffer, 1.8 cm^3 min^{-1}; detector 269 nm and 0.02 a.u.f.s.

TABLE I

MIMOSINE CONTENT OF *LEUCAENA LEUCOCEPHALA*, *LEUCAENA* SEEDS AND EXCRETA DETERMINED USING IEC AND HPLC

Sample	*Mimosine concentration (g kg^{-1} dry matter)*		*Percentage R.S.D.*	*HPLC / IEC*
	IEC	*HPLC (± S.D.)*		
Leucaena leaf meal (sun-dried, pelleted) "Peru" cultivar *ex* Malawi 1979	24.27	24.73 (±0.692)*	2.80	1.019
Leucaena leaf meal (sun-dried, unpelleted) "Peru" cultivar *ex* Malawi 1977	10.26	10.42 (±0.260)**	2.50	1.016
Leucaena leaf meal (sun-dried, unpelleted) "Peru" cultivar *ex* Malawi 1979	23.28	23.76 (±0.254)***	1.07	1.021
Leucaena seeds *ex* Mexico 1981	67.54	67.95 (±0.350)§	0.52	1.006
Leucaena seeds *ex* Mexico 1981	73.19	73.93 (±0.052)§	0.07	1.010
Chick excreta (from chicks fed *Leucaena* diets)	3.63	3.43 (±0.027)§	0.79	0.945
Chick excreta (from chicks fed *Leucaena* diets)	1.11	1.01 (±0.007)§	0.69	0.910

* Mean of duplicate analyses of three samples.
** Mean of duplicate analyses of six samples.
*** Mean of four analyses of two samples (two at 278 nm and two at 269 nm).
§ Mean of triplicate analyses of one sample.

TABLE II

RECOVERY OF 3-HYDROXY-4(1*H*)-PYRIDONE (DHP) FROM *LEUCAENA* LEAF MEAL (LLM) AND CHICK EXCRETA (MEASURED AT 269 nm) USING HPLC

Sample	*DHP content by analysis (g kg^{-1} dry matter)*	*Expected DHP content (g kg^{-1} dry matter)*	*Percentage recovery (± S.D.)*	*Percentage R.S.D.*
LLM (sun-dried, unpelleted)* "Peru" cultivar *ex* Malawi 1977 + DHP	3.084	3.069	100.5 (± 2.6)	2.59
LLM (sun-dried, unpelleted)** "Peru" cultivar *ex* Malawi 1977 + DHP	8.484	9.024	98.1 (± 5.4)	5.49
LLM (sun-dried, unpelleted)** "Peru" cultivar *ex* Malawi 1977 + DHP	5.475	5.912	92.6 (± 5.2)	5.64
Excreta (obtained from chicks fed on LLM-free diet)** + DHP	5.197	4.974	104.5 (± 1.3)	1.28

* DHP solution was added to three LLM extracts prior to analysis.

** Crystalline DHP was added to powdered sample prior to extraction. Three samples were taken for extraction of DHP.

R.S.D. values for recovery of DHP from LLM are fairly high, averaging 5.57%. Recovery of DHP from excreta, however, is slightly higher than expected although precision of analysis is good.

Recovery of mimosine and DHP from chick serum (Table III) shows that substantial losses of both mimosine and DHP occur during sample preparation. This is a problem which has been reported[39,41] for analysis of compounds in blood and was not unexpected. Recovery of mimosine was highest when SSA was used as the precipitant with serum levels of mimosine and DHP of 62.5 and 125 nmol cm^{-3}, respectively. Precision of analysis was fairly low however. The use of SSA as the precipitant yielded only *ca.* 58% recovery of DHP at a concentration of 125 nmol cm^{-3}, although precision was fairly good. It was observed that when SSA was used as the precipitant, peak broadening and eventually splitting occurred after the analysis of twelve samples. This condition remained even when standard solutions were subsequently loaded. Removal and replacement of the top 1–2 mm of column packing resolved the problem, indicating that perhaps some proteinaceous material had been adsorbed on to the top of the column. Another possible cause may have been due to adsorption of some SSA on to the column. This possibility, although remote because of the hydrophilic groups on the benzene ring of SSA causing lack of retention, would appear to be confirmed by the fact that peak splitting slowly decreased as the number of injections of a standard solution of mimosine and DHP increased. PTA was used as the preferred precipitant since peak broadening or splitting did not accompany its use. As can be seen (Table III) a reversal in recovery is produced when PTA was used to precipitate the protein from serum containing the same concentrations of mimosine and DHP as those used for SSA precipitation. Precipitation of protein from serum samples containing half the concentration of mimosine and DHP produced an increase in recovery of mimosine but a slight reduction in recovery of DHP. R.S.D. values for the recovery of DHP are fairly consistent but are quite high and variable for mimosine. The combined molar recoveries of both mimosine and DHP are almost identical for both SSA- and PTA-treated serum, although the values obtained for the

TABLE III

RECOVERY OF MIMOSINE AND 3-HYDROXY-4(1*H*)-PYRIDONE (DHP) FROM CHICK SERUM USING HPLC

Serum sample	*Mimosine concentration (nmol cm^{-3})*	*Mimosine recovery (%) (±S.D.)*	*Percentage R.S.D.*	*DHP concentration (nmol cm^{-3})*	*DHP recovery (%) (±S.D.)*	*Percentage R.S.D.*	*Combined recovery (%) of DHP and mimosine*
A*	62.5	81.3 (± 5.5)	6.77	125.0	57.7 (± 1.6)	2.77	69.5
B**	62.5	54.9 (± 1.7)	3.10	125.0	81.8 (± 2.1)	2.57	68.4
C**	31.25	71.1 (± 4.5)	6.33	62.5	75.5 (± 1.2)	1.59	73.3

* Values are the means of triplicate analyses of three samples; sulphosalicylic acid used as protein precipitant.

** Values are the means of duplicate analyses of three samples; phosphotungstic acid used as protein precipitant.

lower concentrations of mimosine and DHP is slightly higher than those for the higher concentrations.

Ion-pairing of mimosine and DHP with the precipitants could have been partially responsible for the low recoveries. If ion-pairing had occurred then it seemed likely that some mimosine and DHP would be eluted simultaneously with either SSA or PTA, both of which were retained only slightly. This premise was tested by adding SSA or PTA to a standard solution of mimosine and DHP (0.125 m*M* and 0.25 m*M*, respectively, in 0.1 *M* HCl) in the same ratio as for precipitation of the protein from serum (*i.e.* 2 cm^3 of standard plus 0.5 cm^3 of precipitant). Analysis of these sep-pak pretreated standards confirmed that losses occurred, possibly by ion-pairing. Recoveries of 85.7% (± 0.7) and 87.9% (±1.9) for mimosine and DHP, respectively, were obtained from triplicate analysis of an SSA treated standard. Analysis of a PTA-treated standard showed that recoveries of 78.1% (± 0.4) and 85.6% (± 0.6) were obtained for mimosine and DHP, respectively. It would appear, therefore, that precipitation of protein from serum using either SSA or PTA causes loss of mimosine by at least two mechanisms: adsorption of mimosine and DHP to the protein, and non-retention of both during chromatography due to ion-pairing with the precipitant. No loss of mimosine or DHP was detected when standard solutions (without added precipitant) were treated with Sep-Pak cartridges prior to analysis. The re-use of Sep-Pak cartridges for LLM and excreta extracts produced cleaner samples than those which had been passed through unused cartridges, without loss of mimosine or DHP.

Recovery of 100.3% (± 1.6) has been reported for mimosine added to ovine plasma (in the range 0.5–1.25 μmol cm^{-3}) when analysed using IEC[30]. It appears, however, that this recovery was obtained from samples with mimosine added after protein precipitation with SSA. Losses during protein precipitation would therefore not have been accounted for. We have not yet been able to detect mimosine or DHP in the serum of chicks fed LLM, although levels of up to 0.4 μmol of mimosine cm^{-3} of ovine plasma have been reported for sheep fed diets containing mimosine[9]. Our inability to detect mimosine or DHP in chick serum may be due to a variety of factors, not least of which may be that mimosine or DHP do not enter the blood system. This possibility is likely since it has been reported that a high proportion of mimosine ingested by the chick is excreted[22]. It is also possible that mimosine and DHP, if present in the blood, is bound/adsorbed to protein and thus lost during serum preparation. Different sample preparation techniques, such as hydrolysis of whole blood or ultra-filtration, may yield information on mimosine and DHP in chick blood. Another possibility is that the "known addition" technique could be used to estimate mimosine and DHP in blood and, perhaps, urine. Further work is continuing on this aspect of sample preparation.

A recent report has shown that mimosine and DHP have been separated in 0.1 *M* HCl extracts of *leucaena* and in ruminant urine using RP-IP-HPLC[35]. The authors reported that, when using orthophosphoric acid as the ion-pairing agent, resolution of DHP and DHP-glycoside was not possible. They subsequently resorted to hydrolysis of the urine to convert the DHP-glycoside to DHP. Retention times for mimosine and DHP were of a similar order to those obtained by us although we used a higher effluent flow-rate. No recoveries for DHP or mimosine were given although negligible levels of mimosine were detected in urine. The very low mimosine levels, compared to DHP, appear to be in direct contrast to work with chicks[22,40] (unpub-

lished data) and sheep[9] which has shown that substantial amounts of mimosine and DHP were excreted.

A total of *ca.* 900 samples consisting of LLM, excreta and serum have been analysed in this laboratory, using the same column, since the work presented here was carried out. During these analyses the top 2–3 mm of column packing has been changed five times and, on two occasions, the column was washed with aqueous methanol (70% v/v). The column efficiencies before (901 $\pm$ 25; $n = 4$) and after (996 $\pm$ 34; $n = 4$) the analyses of the 900 samples were almost identical. On both occasions efficiency was determined on two separate days, using the mimosine peak. The long column life indicates that sample preparation was good.

The current study shows that the use of RP-IP-HPLC, using sodium octyl sulphate as the ion-pairing agent, provides a rapid, accurate and precise technique for the estimation of mimosine and DHP in LLM and excreta with minimal sample preparation, using a column of medium efficiency ($N \approx 4000$ measured using naphthalene). Although analysis of both mimosine and DHP in serum by this method gives low recoveries and variable precision, it seems feasible that the technique can be developed with further study and used for the estimation of mimosine, DHP, other metabolites and related analogues in ruminant and non-ruminant blood.

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LEAF ANALYSIS FOR ABSCISIC, PHASEIC AND 3-INDOLYLACETIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography (HPLC) is described for the purification and analysis of abscisic acid (AbA), phaseic acid (PA) and 3-indolylacetic acid (IAA) extracted from *Sorghum bicolor* leaves. The method is rapid, suitable for automation and capable of accommodating large numbers of samples. Detection limits are less than 1 ng for each hormone. Recovery efficiency is 75% for AbA and PA and 64% for IAA.

After initial extraction and partition, AbA, PA and IAA were purified together as ammonium salts (ion pairs) on polyvinylpyrrolidone. This was followed by further purification and separation of the three hormones on preparative C_{18} reversed-phase HPLC (5-μm spherical particles). Analysis of AbA and PA was by absorption phase silica HPLC (5-μm spherical particles) with detection by UV absorption. Analysis of IAA was by C_{18} reversed-phase HPLC (5-μm spherical particles), with fluorescence detection.

INTRODUCTION

Since hormones are present in low concentrations in most plant tissues, special techniques are needed to measure them accurately. The most sensitive chromatographic techniques for hormone analysis are capillary gas chromatography (GC), gas chromatography–mass spectrometry with selected ion current monitoring (GC–MS–SICM) and high-performance liquid chromatography (HPLC).

Capillary GC is effective for trace analysis due to high column resolution and high sample sensitivity. However the sensitivity is limited by the sample size which can be injected onto the column and hence the samples must be rigorously purified prior to injection. For plant hormones the sample must also be derivatized. GC–MS–SICM is an extremely accurate and sensitive method[1], but has similar disadvantages as capillary GC. As well, the high cost of GC–MS instrumentation, makes this method out of range for routine analysis.

On the other hand HPLC is effective for both purification and analysis of endogenous hormones[2]. The method has several advantages. Preparative and analytical high-resolution columns are available. Run times are short and the samples usu-

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ally do not require derivatization. Variations in selectivity may be obtained by changes in mobile or stationary phases. Thus the method is applicable to the separation of a wide range of chemically diverse compounds. An added advantage over GC methods is that the sample is not destroyed.

Reversed-phase preparative HPLC is well suited for the purification of plant extracts prior to hormone analysis. Efficiency, resolution and recoveries from the columns are high[2]. Polar compounds are not retained, so that the columns do not deteriorate over a period of time. Reversed-phase HPLC has been used for the purification of extracts for analysis of abscisic acid (AbA)[3–6], 3-indolylacetic acid (IAA)[4,6,7], phaseic acid (PA)[4] and dihydrophaseic acid (DPA)[4] and other hormones. As well, high-performance steric exclusion chromatography was effective in purification of plant extracts for the analysis of IAA[8].

Various methods of analysis of plant hormones by HPLC have been reported. The methods for AbA and IAA include ion exchange[9–12], reversed phase[4,6,7,13,14,24], ion-pair reversed phase[15], partition[3], and normal absorption phase[3,5]. Abscisins and IAA can be detected by UV absorption. More selective and sensitive detection of IAA may be achieved by fluorescence[12,13] or by electrochemical means[12].

We have been studying the effects of drought stress on the levels of endogenous hormones in field grown *Sorghum bicolor*[16,17]. HPLC was found to be effective for the purification and analysis of AbA, PA, DPA, IAA, zeatin and zeatin riboside extracted from leaf tissue[4,18]. As HPLC technology has advanced, we have improved our methods. Presented herein is a rapid and sensitive method for the analysis of AbA, PA and IAA in *Sorghum* leaf tissue.

EXPERIMENTAL

High-performance liquid chromatography

The instrument was a Beckman Model 322 microprocessor controlled HPLC system, including two Model 100A pumps, Model 210 sample injector and Model 420 microprocessor controller. A Model 440 absorbance monitor (Waters Assoc.) detected UV absorption at 254 nm. This detector was coupled in series with a Model 420 fluorescence detector (Waters Assoc.).

All solvents were either HPLC grade or fractionally distilled and filtered through Fluoropore (Millipore), pore size 0.5 μm. In addition, all aqueous solvents were boiled to remove dissolved air.

Extraction and initial purification

The top three leaves from 60-day old plants of *Sorghum bicolor* Moench cv. NK 300 were cut and plunged into liquid nitrogen. After measurement of fresh weight, the tissue was freeze dried and stored at -70°C. The equivalent of 5 g fresh weight (about 1.25 g dry weight) of tissue was extracted for analysis of free and conjugated hormones. The tissue was homogenized in pre-cooled (5°C) methanol–water (80:20; 50 ml), which contained 200 mg/l sodium diethyldithiocarbamate as antioxidant. The homogenized tissue was stirred for 4 h at 5°C, the mixture filtered, and the residue resuspended in methanol–water (80:20) and stirred overnight at 5°C. The mixture was again filtered and the two filtrates combined. The methanol was evaporated *in vacuo* and the aqueous solution at pH 8.0 was centrifuged for 1 h (14,000 g). The supernatant was carefully removed.

To release conjugated hormones, half of the aqueous solution was treated with an equal volume of 0.1 M sodium hydroxide solution at 50°C for 30 min. Each of the treated (total hormone) and untreated (free hormone) samples were adjusted to pH 3.0 and extracted (3 ×) with diethyl ether. The residue from the evaporated ether phase was dissolved in 1 ml methanol–diethyl ether (1:1) and treated dropwise with concentrated ammonium hydroxide solution, with shaking, until the ammonia was in excess (6–8 drops). The solution was evaporated *in vacuo*. Polyvinylpyrrolidone (PVP) powder was sieved (100 mesh size) to remove fines and washed with tap, then distilled water. The PVP was mixed thoroughly with distilled water and the fines decanted after settling for 5 min. When five decantations were complete, columns (8 × 1.5 cm) were prepared in distilled water. The ammonium salts prepared above were dissolved in 1 ml water and chromatographed on the PVP columns. The columns were eluted with distilled water containing sodium diethyldithiocarbamate (200 mg/l). The first 25 ml were collected, adjusted to pH 3.0, extracted (3 ×) with diethyl ether, which was evaporated.

High-performance liquid chromatography of extracts

The partially purified extracts from the PVP treatment were dissolved in water–methanol (1:1; 2 ml) and filtered through Fluoropore, pore size 0.5 μm. The solvent was evaporated. The residue was injected onto a 25 × 1 cm I.D. Beckman Ultrasphere ODS column. The mobile phase was a linear gradient of water–methanol–acetic acid starting with composition (60:40:0.5) and ending with composition (30:70:0.5) over a period of 37 min. The flow-rate was 1.6 ml/min. The sample injection solvent (150 μl) was the same as the starting solvent. Three fractions were collected corresponding to PA, IAA and AbA (Fig. 1). These were evaporated *in vacuo*. After completion of each run, the solvent composition was changed to water–meth-

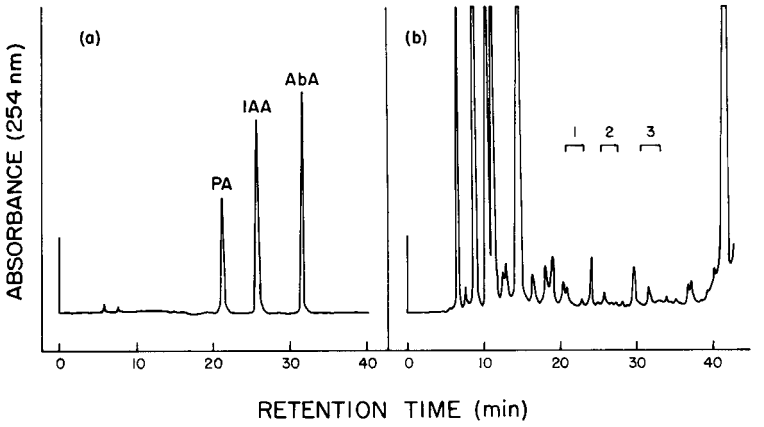


Fig. 1. (a) Separation of AbA, IAA and PA on a reversed-phase preparative HPLC column. Column, 25 × 1 cm I.D. Beckman Ultrasphere ODS; mobile phase, linear gradient over 37 min of water–methanol–acetic acid, starting with composition (60:40:0.5) and ending with composition (30:70:0.5); flow-rate, 1.6 ml/min; detection, UV absorption. *trans,trans*-AbA (not shown) elutes at 29.1 min. (b) Purification of AbA–IAA–PA extract from *Sorghum* leaves on same column and with same conditions as in (a). At the end of gradient, the solvent composition was changed to water–methanol–acetic acid (20:80:0.5) in order to remove impurities still remaining on the column. Three fractions were collected corresponding to the elution of 1 = PA, 2 = IAA and 3 = AbA.

anol–acetic acid (20:80:0.5) and kept for 10 min to remove impurities still remaining on the column. The column required equilibration for 15 min on initial conditions before the next sample was injected.

AbA and PA samples were analysed on a 25 × 0.46 cm I.D. Beckman Ultrasphere Si column. The mobile phase was chloroform–acetonitrile–acetic acid (94:5:1) and flow-rates were 1.0 ml/min for AbA and 1.5 ml/min for PA. Samples were dissolved in 50 μl of mobile phase and either 10 or 20 μl of this solution were injected. Peaks were detected by UV absorption at 254 nm.

IAA samples were analysed on a 25 × 0.46 cm I.D. Beckman Ultrasphere ODS column. The mobile phase was water–acetonitrile–acetic acid (71:28:1) and flow-rate was 1.0 ml/min. Samples were dissolved in 40 μl of the mobile phase and 10 or 20 μl of this solution were injected. Peaks were detected by fluorescence, with excitation at 254 nm and emission at 360 nm using a band pass filter.

The recorder response, in terms of peak height, for both UV absorption and fluorescence (IAA only) was found to be linearly proportional to the amount of hormone injected. Thus hormone levels were estimated by measuring peak heights.

Gas–liquid chromatography

Methyl esters of hormones were prepared as previously described[4]. Glass columns 1.83 m × 2 mm I.D. were packed with 2% OV-1 or 2% QF-1. Helium carrier gas flowed at 50 ml/min and detection was by flame ionization. The retention times at the temperatures indicated were: 2% OV-1: *cis,trans*-AbA methyl ester, 4.8 min (165°C); PA methyl ester, 5.9 min (165°C); and IAA methyl ester, 4.8 min (148°C). 2% QF-1: *cis,trans*-AbA methyl ester, 6.0 min (182°C); PA methyl ester, 6.8 min (182°C); and IAA methyl ester, 5.3 min (150°C).

RESULTS

Batches of 5 g fresh weight of leaves were sufficient for measurement of both free and conjugated AbA, PA and IAA. The antioxidant sodium diethyldithiocarbamate was added to the initial extraction medium and the PVP eluent to reduce oxidation of IAA in aqueous solutions[19]. Efficiency of recovery was improved from 27%, without the antioxidant, to 64%, with the antioxidant.

The extracts were initially purified by treatment with ammonia and chromatography on short PVP columns. The conversion to ammonium salts took about 5 min and the elution of each column with water (25 ml) took about 10 min. The procedure is therefore very rapid, but nevertheless highly effective in reducing extract dry weight (Table I). Acids in the extract form ammonium salts, which rapidly pass through the column as ion pairs. Phenols do not form salts with ammonia and hence are retained on the column. This method, first described for AbA analysis[20] was bound to be more effective in reducing extract dry weight than chromatography of untreated extracts on PVP[21], on Sephadex[4] or on PVP using methanol as eluent[22] (Table I). The PVP method with methanol as eluent was moderately effective in reducing extract dry weight, but did not remove interfering pigments co-chromatographing with AbA and IAA on the HPLC purification column.

After treatment with PVP, the extract was sufficiently pure for reversed-phase preparative HPLC. A high-resolution 25 × 1.0 cm I.D. Beckman Ultrasphere ODS

TABLE I

COMPARISON OF EFFECTIVENESS OF FOUR COLUMN TREATMENTS FOR REDUCING THE DRY WEIGHT OF THE RESIDUE FROM AN ETHER EXTRACT OF *SORGHUM* LEAVES

The ether extract (340 mg) was obtained from 5 g fresh weight (1.25 g dry weight) of leaf tissue (see Experimental). The extract was not hydrolysed. Indicated volume of mobile phase was the retention volume required to elute AbA, PA and IAA in an extract, plus 20% extra mobile phase. Method includes recovery from mobile phase. If the solvent was aqueous, it was extracted at pH 3.0 into diethyl ether (3 ×), and the ether evaporated.

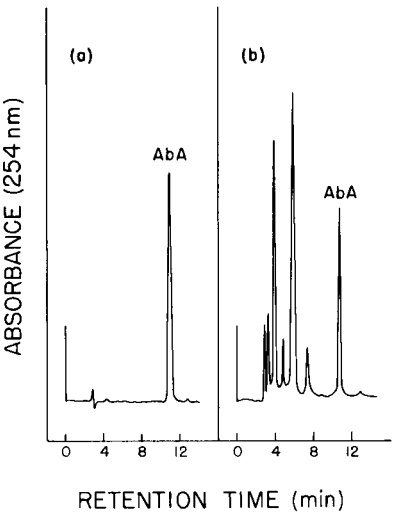
Treatment	*Weight (mg) of extract following treatment (average of duplicates)*
Convert to ammonium salts, 8 × 1.5 cm PVP column, mobile phase: water (25 ml)	61
12 × 1.5 cm PVP column, mobile phase: methanol (50 ml)	89
15 × 1.5 cm PVP column, mobile phase: 0.1 *M* phosphate buffer, pH 7.0 (80 ml)	119
20 × 1.5 cm Sephadex G-10 column mobile phase: 0.25 *M* phosphate buffer, pH 7.0 (150 ml)	129

(C_{18}, 5 μm spherical particles) column was used with a mobile phase of a gradient of acidified water–methanol (Fig. 1). Acetic acid was added to the mobile phase to suppress ionization of the hormones and to minimize the effect of absorption on exposed silica[2]. In this way peak tailing was eliminated. Three fractions were collected, corresponding to the retention volumes of AbA, PA and IAA (Fig. 1). Judging by the UV absorbing peaks, most impurities eluted either before or after the elution of the hormones.

Analysis of AbA and PA was by normal-phase chromatography with a Beckman Ultrasphere Si column (5-μm spherical particles) with a mobile phase of acidified chloroform–acetonitrile. Peaks corresponding to the elution of AbA and PA (Figs. 2 and 3) were well resolved from impurity peaks. Detection was by UV absorption at 254 nm. The detection limit for each hormone was about 1 ng.

Analysis of IAA was by reversed-phase on a Beckman Ultrasphere ODS analytical column)5-μm spherical particles), with mobile phase of acidified water–acetonitrile (Fig. 4). Detection was by fluorescence. The detection limit was about 100 pg.

In order to ensure that peaks were correctly identified and that they did not represent more than one compound, samples corresponding to each hormone peak were collected and examined by gas–liquid chromatography (GLC). The samples were first methylated, and the methyl esters chromatographed on 2% OV-1 and 2% QF-1 GLC columns as previously described[4]. In each case chromatography gave a single peak corresponding to the appropriate standards. Furthermore, by careful comparison of peak areas to those of the standards, the amounts observed on the GLC columns were the same as those observed on the HPLC columns. The overall recovery of the hormones, estimated by spiking five extracts with known amounts of



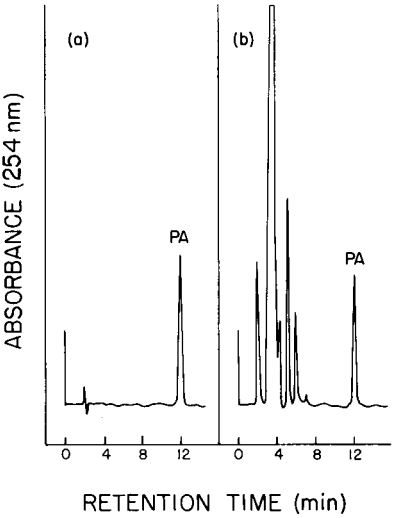


Fig. 2. (a) HPLC of AbA. Column, 25 × 0.46 cm I.D. Beckman Ultrasphere Si; mobile phase, chloroform–acetonitrile–acetic acid (94:5:1); flow-rate 1.0 ml/min; detection, UV absorption. (b) HPLC of residue recovered from fraction 3 (Fig. 1) on same column and with same conditions as in (a).

Fig. 3. (a) HPLC of PA. Column, 25 × 0.46 cm I.D. Beckman Ultrasphere Si; mobile phase, chloroform–acetonitrile–acetic acid (94:5:1); flow-rate 1.5 ml/min; detection, UV absorption. (b) HPLC of residue from fraction 1 (Fig. 1) on same column and with same conditions as in (a).

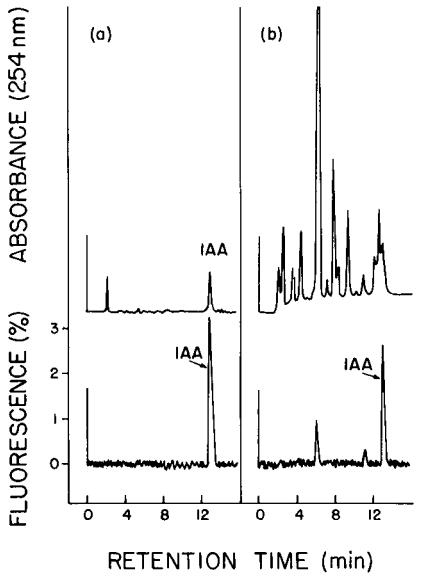


Fig. 4. (a) HPLC of IAA. Column, 25 × 0.46 cm I.D. Beckman Ultrasphere ODS; mobile phase, water–acetonitrile–acetic acid (71:28:1); flow-rate, 1 ml/min; detection, fluorescence, with excitation at 254 nm and emission at 360 nm using a band pass filter. (b), HPLC of residue from fraction 2 (Fig. 1) on same column and with same conditions as in (a).

hormones (and in similar amounts to those found in the tissue) were 75 ± 3% for both abscisins and 64 ± 3% for IAA.

Quantities of hormones (adjusted for extraction losses) in the top three leaves of 60-day old plants are given in Table II. Conjugated hormones, defined as those hormones which were released by 0.1 *M* sodium hydroxide solution at 50°C, were also estimated. These hydrolysed extracts had large dry weights, presumably due to the release of many other compounds by the treatment. In these extracts, UV-absorbing peaks were 2–5 times larger than those of unhydrolysed extracts. The increased dry weight did not affect the performance of the columns or the separation of the hormones from impurities. IAA conjugates were present in much larger amounts than free IAA (Table II and ref. 23).

TABLE II

LEVELS OF HORMONES IN THE TOP THREE LEAVES OF 60-DAY OLD PLANTS OF *SORGHUM BICOLOR* CV. NK300

Values averaged from five extracts. Values for conjugated hormones were calculated by subtracting free hormone from total hormone.

Hormone	*Free (ng/g fresh weight)*	*Conjugated (ng/g fresh weight)*
AbA	65.1 ± 3.0	58.8 ± 4.1
IAA	26.5 ± 2.4	164 ± 21
PA	16.5 ± 1.3	9.9 ± 1.6

DISCUSSION

The HPLC analysis of AbA, PA and IAA reported herein is rapid, efficient and sensitive. Although two purification steps are required, these are relatively simple and could be automated. Thus large numbers of samples could be processed.

The combination of chromatographing the extracted hormones first as ammonium salts on a short PVP column and then as free acids on a preparative HPLC reversed-phase column proved effective for purification. The initial PVP treatment was required before the extract could be chromatographed on HPLC. We have had no success in chromatographing crude extracts directly on high performance columns due to contamination effects and loss of resolution. Preparative HPLC is most effective when peak resolution is high and narrow band collections are made. In a recent report[6] a high degree of purification of hormone extracts from *Solanum tuberosum* leaf and root tissue was claimed by repeated use of PVP and μBondapak C_{18}. In our hands this method tended to concentrate impurities in the hormone zones, rather than separate impurities from hormones.

Preparative HPLC was by reversed-phase, whereas analytical HPLC for AbA and PA was by adsorption phase. These contrasting systems enabled AbA and PA to be effectively resolved from UV-absorbing impurities. For IAA, both purification and analysis were by reversed-phase C_{18} columns. However the mobile phase for the preparative column was acidified methanol–water, whereas for the analytical column it was acidified acetonitrile–water. Thus the two systems had different selectivities,

enabling IAA to be separated from interfering impurities. As well, fluorescence detection was found to be more selective than UV absorption at 254 nm (Fig. 4). Since IAA has a low extinction at 254 nm, fluorescence detection was found to be more sensitive.

An important criterion of trace analysis is that the analytical chromatograms should contain few peaks, and that the hormones should be well resolved from impurities. A large impurity peak close to a hormone peak can distort the latter making quantitation inaccurate. In the present work, excellent separation of AbA and PA peaks from impurity peaks was observed with UV detection at 254 nm. For IAA, however, it was necessary to use a selective fluorescence detector.

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SEPARATION OF THE RADIOLYSIS PRODUCTS OF HYPOPHOSPHITES

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SUMMARY

The formation of hypodiphosphite ($^{2}_{2}$P–P) as a hydrolytic product of the γ-ray decomposition products of ammonium hypophosphite was confirmed and the formation of $^{0}_{2}$P–P was assumed from the anion-exchange chromatographic result. The half-life periods of these products were determined in 10^{-3} *M* hydrochloric acid, assuming that their reactions were first order.

INTRODUCTION

In the study of the γ-ray radiolysis of solid hypophosphite, it has been known that the radiolytic products and the rates of the decomposition of the ligand molecules in metal complexes are affected by the central metal ions and that alkali metal hypophosphite is comparatively sensitive to γ-ray irradiation[1]. Anselmo and Sanchez[2] reported briefly that two new active species attributed to N–P bonded compounds containing oxy-anions were observed in the study of ammonium hypophosphite irradiated with thermal neutrons. Kobayashi *et al.*[3] reported the existence of hypodiphosphite in neutron-irradiated sodium hypophosphite by means of paper chromatography. Our observation showed the formation of hypodiphosphite ion experimentally. Little is known about the chemical properties of this ion except for a report by Baudler *et al.*[4], although the existence of this ion has been known for many years. We have therefore investigated the chemical and chromatographic behaviour of this ion.

EXPERIMENTAL

Ammonium hypophosphite and sodium hypophosphite purified by recrystallization were used for the study of irradiation effect. Both samples, sealed in glass tubes in vacuum, were irradiated at a dose rate of $4.5 \cdot 10^{5}$ R h^{-1} at room temperature using 3500-Ci cobalt-60 at the γ-ray Irradiation Laboratory, Kyushu University. The total absorption dose was $1.0 \cdot 10^{8}$ rad and the irradiated samples were used for chemical separation immediately after the end of the irradiation. The irradiated samples (about 120 mg) were dissolved in 10 ml of 0.01 *M* hydrochloric acid and then diluted to 100 ml with water.

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Anion-exchange chromatography was performed on a strongly basic anion exchanger [Bio-Rad AG 1-X8 (Cl^-), 100–200 mesh] column (66 × 1.54 cm I.D.). A gradient elution technique was applied by mixing 0.2 *M* sodium chloride into 750 ml of 0.075 *M* sodium chloride solution, all eluent solutions being buffered by adding 25 ml of 2 *M* ammonium acetate solution per litre of solution. The effluent was collected in 7-ml fractions with an automatic fraction collector of the weight type and an aliquot of each fraction was used for determination and identification.

Blaser's abbreviated notation[5] was employed for the oxo-anions of phosphorus in the present paper: $PH_2O_2^-$ (^{1}P), PHO_2^{2-} (^{3}P), PO_4^{3-} (^{5}P), $H_2PO_2^-$ ($^{2}_{2}$P–P), $P_2HO_5^{3-}$ ($^{3}_{4}$P–P), $H_2P_2O_6^{2-}$ ($^{4}_{4}$P–P) and $P_2H_2O_5^{2-}$ (^{3}P–O–^{3}P).

Three different methods for the colorimetric determination of each oxo-anion of phosphorus were carried out by using molybdenum (V)–molybdenum (VI) reagent[6], as follows.

Method A: This is useful for the determination of orthophosphoric acid (^{5}P). A 1-ml volume of molybdenum (V)–molybdenum (VI) reagent is added to 2 ml of each fraction in a test-tube and heated at 100°C for 1 h in a water-bath. After cooling and adjusting the volume of the solution to 20 ml with water, the absorbance is measured.

Method B: This is useful for the determination of total phosphorus, including lower oxo-anions. A 1-ml volume of molybdenum (V)–molybdenum (VI) reagent and 0.5 ml of 1 *M* sodium hydrogen sulphite are added to 2 ml of each fraction in a test-tube. Other procedures are as in method A. Elution diagrams were usually drawn for the results measured by this method.

Method C: This is useful for the determination of hypophosphoric acid ($^{4}_{4}$P–P). A 1-ml volume of molybdenum (V)–molybdenum (VI) reagent is added to 2 ml of each fraction in a test-tube. After adjusting the volume of the solution to 20 ml with water, the absorbance is measured.

The absorbance was measured with a Hitachi 101 spectrophotometer, at 830 nm for orthophosphoric heteropoly blue (for methods A and B) and at 630 nm for hypophosphoric heteropoly blue (for the method C). The detailed procedure for the quantitative determination and the technique of anion-exchange chromatography are similar to those described by Tominaga *et al.*[7].

RESULTS AND DISCUSSION

Fig. 1 shows the elution diagram for the hydrolysis products of freshly prepared P_2I_4 in which solution $^{2}_{2}$P–P anions are produced[4]. The elution was started 15 min after the beginning of hydrolysis of P_2I_4. The elution diagram was drawn for the absorption at 830 nm by applying method B. The inset in Fig. 1 represents the elution diagram obtained for the elution started after 53 h after the hydrolysis of P_2I_4. The dotted line was drawn for the absorbance at 830 nm by using method C (This peak is assigned to $^{4}_{4}$P–P and the most coloured position is fraction 113). The component of ^{4}P anions is found to increase with the progress of the hydrolysis. The solid line was obtained on the basis of calculation by substituting the absorbance at 830 nm for the coloured fraction by using method C from the absorbance obtained by using method B (the most coloured position of this peak is fraction 118). The elution positions for ^{1}P, ^{3}P and ^{5}P anions are determined easily.

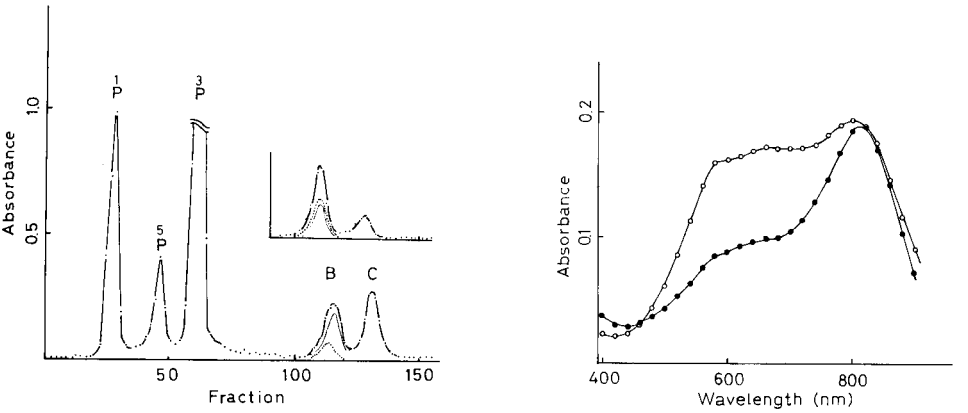

Fig. 1. Chromatographic elution curve for the hydrolysed products of P_2I_4. Peaks: B is assigned to a mixture of $^{4}_{4}$P–P and $^{2}_{2}$P–P anions; C is assigned to $^{2}_{4}$P–P anion. Resin: Bio-Rad AG 1-X8 (Cl^-), 100–200 mesh. Column dimensions: 66 × 1.54 cm I.D. Flow-rate: 1 ml/min. The eluent (pH 6.8) is buffered with 25 ml of 2 *M* ammonium acetate per litre of eluent. A gradient elution technique was applied by mixing 0.2 *M* sodium chloride into 750 ml of 0.075 *M* sodium chloride solution.

Fig. 2. Absorption spectra of (○) fractions 113 ($^{4}_{4}$P–P) and (●) 118 (P) in Fig. 1.

Fig. 2 represents the absorption spectra of fractions 113 (peak B1) and 118 (peak B2). The spectrum of fraction 113 shows the overlapped absorption spectrum of orthophosphoric and hypophosphoric heteropoly blue and that of fraction 118 shows the absorption spectrum of orthophosphoric heteropoly blue. Peak B consists of the overlap of peak B1 assigned to $^{4}_{4}$P–P anions and B2 to $^{2}_{2}$P–P anions. The last peak in Fig. 1 is assigned to $^{2}_{4}$P–P.

Fig. 3 represents the chromatographic elution diagram for the solution in which the irradiated sodium hypophosphite was dissolved under the same conditions as described in the experimental section. The presence of $^{4}_{4}$P–P anions in this solution was also confirmed because peak B in Fig. 3 shows a pale blue colour on applying

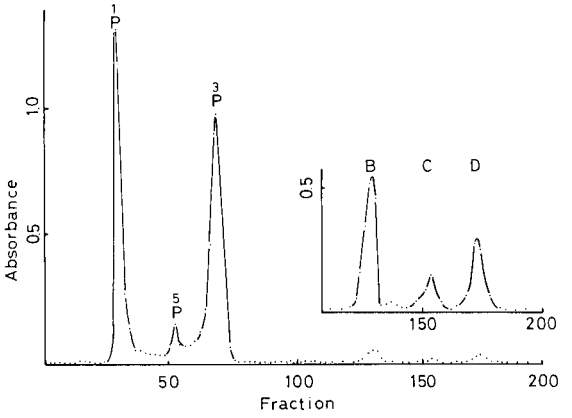

Fig. 3. Chromatographic elution curve for irradiated sodium hypophosphite. Experimental conditions and peaks B and C as in Fig. 1. Peak D is assigned to the $^{3}_{3}$P–O–P anion.

method C. However, the main component of peak B was $^{2}_{2}$P–P. Peaks C and D are assigned to be $^{2}_{4}$P–P and $^{3}_{3}$P–O–P, respectively, as described later.

The solution in which the irradiated ammonium hypophosphite was dissolved under the conditions described in the experimental section was chromatographed to separate each species. Fig. 4 shows the chromatographic elution curve obtained by using method B. Time t shows the time elapsed between the dissolution of the irradiated ammonium hypophosphite and the onset of the elution of the chromatography. The chemical species are denoted by peaks A, B, C and D as shown in Fig. 4. Peak B of the elution curve for $t = 0$ did not show any colouring, even though method C was applied, but that for $t = 261$ showed a pale blue colour. The chemical species of peaks A, B and D are unstable in acidic solution and their concentrations calculated from Fig. 4 decrease with standing time, as shown in Figs. 4 and 5. Fig. 5 shows that the plots for ln K *vs.* time (t) are almost linear. K is ratio peak area ($t = t$)/peak area ($t = 0$). If the hydrolysis reactions of the chemical species A, B, C and D are assumed to be first order, the half-life periods of disappearance for peaks A, B, C and D are calculated to be 75, 230, 820 and 40 h respectively, in 10^{-3} M hydrochloric acid at 20°C.

Peaks C and D are assigned to be $^{2}_{4}$P–P and $^{3}_{3}$P–O–P anions, respectively, because the elution positions C and D are in good agreement with those reported by Tominaga *et al.*[7]. The $^{3}_{3}$P–O–P anions are known to be comparatively stable in a solution

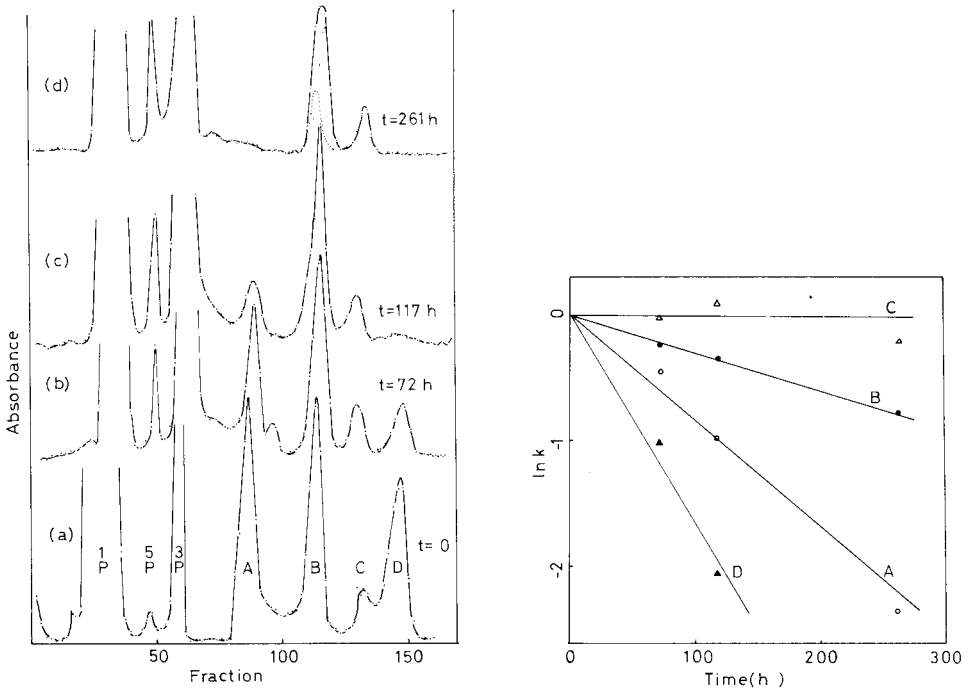

Fig. 4. Variation of the chromatographic elution curves for ammonium hypophosphite with the time elapsed, t, between the dissociation of the sample and the onset of elution. Experimental conditions and peaks as in Fig. 1. Peak A may be assigned to the $^{0}_{2}$P–P anion.

Fig. 5. Plots of K *vs.* time. K is the ratio peak area ($t = t$)/peak area ($t = 0$). Peak A may be assigned to the $^{0}_{2}$P–P anion. Peaks B, C and D are assigned to $^{2}_{2}$P–P (main species), $^{2}_{4}$P–P and $^{3}_{3}$P–O–P anions, respectively.

of pH range 6–8. The half-life period of the hydrolysis of $^{3}_{3}$P–O–P is reported to be 1000 h at pH 5–7, 60 h at pH 4 at 30°C, and is dependent on the pH of the solution[8]. The difference in the half-life periods of the hydrolysis results in a difference in the pH of the reaction solutions because the solution used here contains a large amount of ammonium hypophosphite (140 mg per 100 ml) in 10^{-3} *M* hydrochloric acid.

The fractions for elution peak A were collected and dried. The residue was dissolved in concentrated hydrochloric acid, the precipitate of NaCl produced was filtered off and the residue was dried under mild conditions to evaporate hydrochloric acid. The residue was then dissolved in 5 ml of water and introduced on to an anion-exchange column. Its elution diagram indicates the existence of ^{1}P anion (90%) and ^{3}P anion (10%). One of the possible chemical species for peak A may be $^{0}_{2}$P–P anions because its anions are hydrolysed to ^{1}P anions in acidic solution. This is analogous to the hydrolysis reaction of $^{2}_{4}$P–P in which ^{3}2P anions are produced. Unfortunately, however, the presence of $^{0}_{2}$P–P has not yet been confirmed. The P–N bonded lower oxophosphorus ions might be eluted more rapidly than their non-amidooxophosphorus ions, because mono-, di- and triamidophosphate ions are eluted in front of the elution position of ^{5}P anions[9]. Therefore, it is doubtful whether peak A should be assigned to be P–N bonded lower oxophosphorus. However, this peak appears only on the elution curve of irradiated $NH_4H_2PO_2$, which suggests that the chemical species of peak A may have a P–N bond.

The elution positions of peak B in Figs. 1, 3 and 4 are consistent with each other. The $^{4}_{4}$P–P anions are eluted down the column in front of $^{2}_{2}$P–P anions, and so it is expected that $^{2}_{2}$P–P anions will show stronger bonding with anion exchangers.

Morton[10] recognized a radical $O_2P–PHO_2^{2-}$ in γ-irradiated $NH_4H_2PO_2$ crystals on the basis of ESR spectra. This radical ion can produce $^{2}_{2}$P–P anions in the hydrolysis of the irradiated samples. Disproportionation reactions may take place in the solid because the formation of phosphine and yellow phosphorus is observed during the hydrolysis of the irradiated samples in acidic solution.

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Note

Study of the heterogeneously catalyzed dehydrogenation of secondary alcohols using gas chromatography

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A technique was described[1] in which kinetics of heterogeneously catalyzed reactions could be studied using a simple gas chromatographic (GC) setup. The dehydrogenation of 2-propanol on manganese(II) oxide, a model reaction whose kinetics are well known, was chosen to determine the validity of this method. Results obtained from that study agreed well with literature values[2,3]. As an extension of this work the dehydrogenation of other secondary alcohols on manganese oxides was chosen for investigation.

EXPERIMENTAL

A small amount of catalyst was placed in the front section of a chromatographic column. The injection port, a stainless-steel tube cast in a well-insulated aluminium heat cone, controlled the temperature of the catalyst bed. The catalyst bed was followed immediately by an analytical column for the separation of product from unreacted starting material. In this case the analytical column was a 12 ft. × 0.25 in. O.D. glass column packed with 20% SP2401 + 0.1% Carbowax 1500 on Chromosorb W AW DMCS (60–80 mesh) from Supelco (Bellefonte, PA, U.S.A.). The sieved (60–80 mesh) catalysts, manganese(II) oxide (99%+) and manganese(IV) oxide (99%+) were obtained from Chemetals Corporation (Baltimore, MD, U.S.A.). These were placed in the section of the glass column in the injection port. The temperature of the catalyst bed was monitored by a thermocouple pyrometer with parallax correction mirror. For measurements of retention volume of the reactants on the catalysts, a 15 in. × 2 mm I.D. glass column packed with the respective catalysts was used. A Varian Model 1840-1 gas chromatograph equipped with dual flame ionization detectors was connected to a Vidar Autolab digital integrator. The helium carrier gas was dried and purified over molecular sieve 5A and indicating calcium chloride, and regulated by a differential flow controller.

The reactants, 2-butanol and 2-pentanol and their respective dehydrogenation products, 2-butanone and 2-pentanone, were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). The same headspace sampling technique as was described previously[1] was used for obtaining samples for injection.

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RESULTS AND DISCUSSION

Bassett and Habgood[4] outlined a treatment for analysis of chromatographic data obtained from on-column catalysis experiments. For a rate-controlled surface reaction it can be shown that

$$\ln [1/(1-x)] = \frac{RTW}{F} \cdot kK \tag{1}$$

where x is the extent of conversion (calculated from chromatographic peaks for reactant and product), R is the gas constant, T is the temperature (°K) of the catalyst bed, W is the total weight of catalyst in grams, F is the flow-rate (ml/sec) of carrier gas in the reactor, k is the rate constant of the surface reaction and K is the adsorption equilibrium constant. Thus, a plot of $\ln [1/(1-x)]$ *vs.* $1/F$ will yield a straight line whose slope is proportional to the product of the rate constant for the surface reaction and the adsorption equilibrium constant. The value of K is needed so that k can be calculated. Values of K and ΔH_{ads} are obtained from retention volume measurements at a number of different temperatures. This data is subsequently plotted in the form of a van 't Hoff plot. The apparent activation energy, E_a, may be obtained from the slope of a plot of $\ln kK$ *vs.* $1/T$, and at constant flow-rate this is equivalent to a plot of $\ln [\ln 1/(1-x)]$ *vs.* $1/T$. The value of the activation energy of the surface reaction is obtained by summing the apparent activation energy and the heat of adsorption.

The catalysis reactions for 2-butanol and 2-pentanol on MnO were carried out at five different flow-rates from 10 to 40 ml/min of helium at a constant injector temperature (192°C).

The catalysis reactions on MnO_2 were carried out at similar flow-rates, but at a temperature of 245°C. For each flow-rate the extent of conversion of each alcohol to its corresponding ketone was calculated from calibration curve and the known amount of reactant injected. Measurements of extents of reaction were also made at constant flow-rate but at different temperatures. For the reactions on MnO the temperature range was 140–200°C at a flow-rate of 27.0 ml/min, while for those on MnO_2 the range was 175–300°C at a flow-rate of 28.0 ml/min. It is felt that rate constants obtained from constant temperature *versus* variable flow-rate experiments are less reliable than those obtained from constant flow-rate *vs.* variable temperature experiments. The limited range of flow-rates due to (1) back pressure from a 12-ft. packed column and (2) the flame detector not staying lighted at high flow-rates is the basis of this reasoning. Results of the constant flow-rate *versus* variable temperature experiments are summarized in Table I.

In addition to the abovementioned alcohols all the primary alcohols through 1-octanol, including 2-methyl-1-propanol (isobutanol), were injected to determine if any of these would undergo dehydrogenation on MnO and MnO_2 to form the corresponding aldehydes. No reaction was observed in any of these cases (T = 192°C, flow-rate = 27.0 ml/min for MnO; T = 245°C, flow-rate = 28.0 ml/min for MnO_2). The secondary alcohols, 2-propanol and 3-pentanol, were also injected under the same conditions. As was mentioned earlier[1] the dehydrogenation of 2-propanol is a well-known reaction, and indeed, dehydrogenation was observed on both MnO and

TABLE I

RESULTS OF CONSTANT FLOW-RATE *VERSUS* VARIABLE TEMPERATURE EXPERIMENTS

Reactant	ΔH_{ads} *(kcal/mole)*	E_a *(kcal/mole)*	*E Surface (kcal/mole)*	$k(sec^{-1})$
2-Butanol/MnO	−2.23	11.7	13.9	$2.05 \cdot 10^5 e^{-13{,}900/RT}$
2-Pentanol/MnO	−2.53	13.4	15.9	$1.25 \cdot 10^6 e^{-15{,}900/RT}$
2-Butanol/MnO_2	−1.44	21.5	22.9	$1.61 \cdot 10^8 e^{-22{,}900/RT}$
2-Pentanol/MnO_2	−2.32	25.4	27.7	$5.34 \cdot 10^9 e^{-27{,}700/RT}$

MnO_2. However, no reaction was observed in either case for 3-pentanol. Its structure corresponds to $R_1-\underset{\displaystyle H}{\overset{\displaystyle OH}{\overset{|}{\underset{|}{C}}}}-R_2$ where $R_1 = R_2 = -CH_2CH_3$. The other secondary alcohols studied, but which do undergo dehydrogenation have a structure $R^1-\underset{\displaystyle H}{\overset{\displaystyle OH}{\overset{|}{\underset{|}{C}}}}-CH_3$

Experiments on other secondary alcohols with structures corresponding to those above may provide an interesting area of study in which structure may be correlated with activity in catalytic reactions.

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Note

Kinetics and yield of the esterification of amino acids with thionyl chloride in *n*-propanol

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The separation and quantitative measurement of amino acids by ion-exchange chromatography has been the standard procedure since its introduction by Moore and Stein[1]. The application of this principle[2] for suitable amino acid derivatives[3,4] to high-performance liquid chromatography has provided faster amino acid analyses with high sensitivity. In this respect gas chromatography offers the same advantages at a lower cost of equipment[4], but derivatization of the amino acids to suitable volatile compounds is essential. Various derivatives have been found to be of value[5], particularly the *n*-acyl esters of alkanols[6], which are usually obtained with an anhydrous hydrochloric acid–alkanol solution, at concentrations varying from 1.25 *M*[7] to saturation[8]. Increasing time, concentration and temperature are required as one goes from lower to higher alcohols, especially for amino acids such as the basics Cys and Ile[9].

The use of thionyl chloride in methanol[10] or thionyl chloride in hydrochloric acid–methanol mixtures[11] in place of hydrochloric acid have been used for methyl ester derivatives with reproducible yields. Based on these results, we have studied the reaction of amino acids with thionyl chloride in *n*-propanol in an effort to develop an alternative approach for the quantitative derivatization with alkanols. Kinetic results for neutral amino acids are reported.

EXPERIMENTAL

Reagents

L-Amino acids were purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, G.F.R.). Thionyl chloride (technical grade) was refluxed with 10% linseed oil and distilled using a 50-cm Vigreaux column. *n*-Propanol was purified through the ternary azeotrope with benzene, and the fraction collected at 94–95°C (710 mmHg) was refluxed for 24 h with calcium oxide and redistilled.

Esterification reagent

Thionyl chloride was added dropwise to *n*-propanol at such a rate that the temperature of the mixture was kept at −5°C. After the addition was completed, the mixture was allowed to stand for 1 h at room temperature and kept at −5°C. The proportions of the reagents used were adjusted to give 1, 2, 3 and 4 *M* solutions.

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Esterification procedure

A 1–2-μmole amount of amino acid and 0.4 ml of reagent were placed in a screw-capped tube (60 × 11 mm) and heated from 30 to 100°C. The amino acids studied were Ala, Gly, Ile, Leu, Pro, Phe, Ser, Thr, Tyr, Val, Asp, Glu, Arg, His, Lys and Cys.

Kinetic measurements

Kinetic measurements were carried out with the neutral amino acids, except those with the third functional group, and were followed with a Beckmann Model 120-B amino acid analyser[12]. Esterification yields at levels above 95% were measured as follows: samples of 1, 3, 10, 20 and 30 nmole of amino acid were applied on a silica gel thin-layer chromatographic (TLC) plate (10 × 20 cm) and eluted with *n*-butanol–acetic acid–water (12:3:5). The plate was dried completely and the spots were detected by spraying with ninhydrin for 5 min at 100°C. The lower limit of detection (very light spot) was 3 nmole, no spot being detected at the 1-nmole level. A 20-μl volume (100 nmole) of the reaction mixture containing 2 μmole of amino acid in 0.4 ml of reagent was applied to a TLC plate, plus spots of 3 and 100 nmole of amino acid standard, and were detected by spraying with ninhydrin after elution. Levels greater than 95% were considered satisfactory when no ninhydrin-positive material was observed at the position corresponding to the free amino acid when compared with the 3-nmole spot.

RESULTS AND DISCUSSION

The order of reactivity established at 40°C with 2 *M* thionyl chloride by kinetic measurement of each individual amino acid is shown in Fig. 1 ($173 \cdot 10^{-3}$ min^{-1} > $k_{obs_{am.ac}}$ > $3.0 \cdot 10^{-3}$ min^{-1}. The same order of reactivity was found under these conditions for the mixture. The dependence on thionyl chloride concentration and tem-

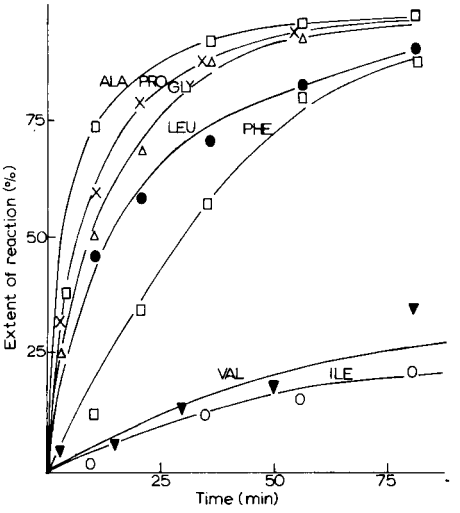

Fig. 1. Order of reactivity established for the neutral amino acids at 40°C using 2 *M* thionyl chloride in *n*-propanol.

TABLE I

DEPENDENCE OF THE RATE CONSTANT ON THE CONCENTRATION OF THIONYL CHLORIDE AND ON TEMPERATURE

Thionyl chloride concentration (M)	$k_{obs} \times 10^{-3}$ *(min)*				
	30°C	*40°C*	*50°C*	*60°C*	*70°C*
1		1.7			
2		3.0			
3		4.5	6.5	12.2	17.7
4	1.7	6.4		9.2	17.8

perature showed that whereas there is only a 3.7-fold increase in the rate constant on going from 1 to 4 *M* solutions, over the temperature range studied the increase was about 10-fold (Table I), so that on a comparative basis increasing the temperature has a greater effect on the reaction than increasing the thionyl chloride concentration (Fig. 2). Similar results have been obtained for the transesterification of methyl to *n*-butyl esters[13]. No significative differences in the rate constants were observed with 3 or 4 *M* thionyl chloride solution. Higher thionyl chloride concentrations were not studied because they are not physically stable (they boil on pipetting). Studies of esterification yields obtained with 3 *M* thionyl chloride solution between 70 and 110°C showed that most of the amino acids are esterified at levels greater than 95% at 80°C with variable times, 20 min for Pro up to 40 min for Val. His, Arg, Lys, Glu, Cys and Ile were only esterified at this level at temperatures of 100°C or higher, His and Arg being the most resistant.

Quantitative recoveries of the amino acids were obtained after alkaline hydrolysis at room temperature as measured by ion exchange. This result, plus the fact that

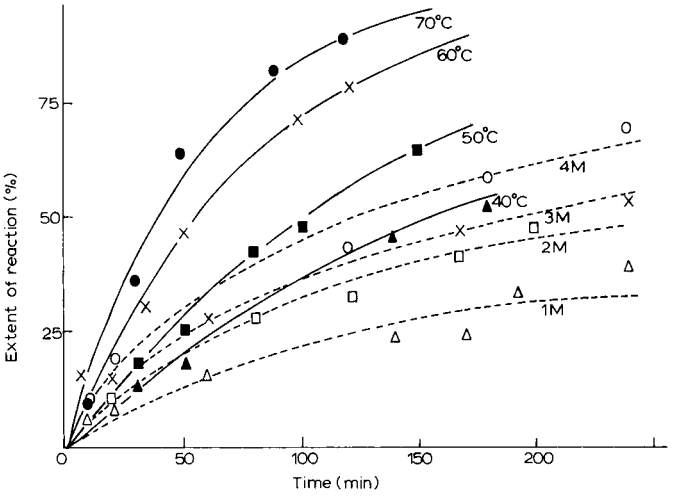

Fig. 2. Effect of temperature (solid lines) using 3 *M* thionyl chloride and of concentration (broken lines) at 40°C on the esterification kinetics of isoleucine.

only the spot corresponding to the ester is observed after complete esterification, suggests that no side-reaction has occurred as far as peptide formation is concerned. Study with Ile at 100°C and using 3 *M* thionyl chloride gave reproducible results with reagents kept for 3 months at −5°C. Incomplete reaction (< 95%) was observed when the reactions were carried out in reaction vessels double the size used here, especially those reactions which had been shown to be more resistant.

In conclusion, 16 amino acids have been esterified at levels greater than 95% using 3 *M* thionyl chloride in *n*-propanol at 100°C for 60 min, with reproducible results, thus offering an alternative approach for the synthesis of O-*n*-propyl esters of amino acids. Studies with lower thionyl chloride concentrations at higher temperatures are in progress.

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Note

Improved derivative of 6-keto-prostaglandin $F_{1\alpha}$ for gas chromatographic–mass spectrometric analysis

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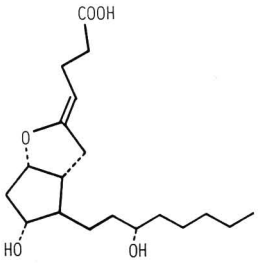
We describe here the properties and facile preparation of an improved derivative (III) of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, II), the stable hydrolysis product of prostacyclin (I). The previously reported[1] methylester–methoxime–tris-trimethylsilyl (TMS) derivative (IV) has the serious disadvantages of susceptibility to hydrolysis by traces of moisture and a mass spectrum which is less than optimum for selected ion monitoring (SIM). The mass spectrum is deficient in high mass ions of high intensity for optimal quantitation by SIM. The improved derivative, a methyl ester–methoxime–tris-*tert.*-butyl dimethylsilyl ether (MeMOTBDMS, III), is easily prepared using a new silylating reagent, N-(*tert.*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), which has not been commercially available,



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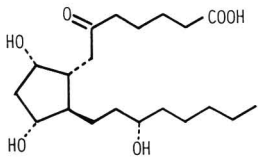

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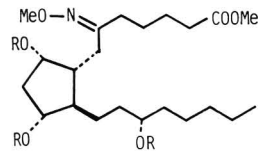

III R = TBDMS
IV R = TMS

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but is readily synthesized[2]*. TBDMS derivatives are sufficiently stable that excess reagent and solvent may safely be evaporated so the derivative may be reconstituted in a minimum volume of solvent for improved sensitivity and reduced contamination of instruments. Alternatively the derivative may be injected in solution with excess reagent. This is a marked improvement over use of the *tert.*-butyldimethylchlorosilane–imidazole–dimethyl formamide (DMF) reagent of Corey and Venkateswarlu[3] which must be removed through extraction or chromatography[4] prior to analysis. The other important advantage of the derivative is the abundance of high mass ions suitable for SIM in the mass spectrum. The gas chromatographic (GC) properties of the derivative also make it highly suitable for GC–mass spectrometric (MS) analysis.

MATERIALS AND METHODS

Reagents and instruments

Ethyl trifluoroacetate was obtained from Eastman, Rochester, NY, U.S.A.; anhydrous monomethylamine was from Matheson, East Rutherford, NJ, U.S.A. NaH dispersion, anhydrous tetrahydrofuran (THF) and *tert.*-butyldimethylchlorosilane were from Aldrich, Milwaukee, WI, U.S.A. Methoxyamine HCl was obtained from Pierce, Rockford, IL, U.S.A. and GC-grade pyridine was from Regis, Morton Grove, IL, U.S.A. Water was glass distilled. 6-Keto-$PGF_{1\alpha}$ was a gift from Upjohn, Kalamazoo, MI, U.S.A.

Evaporation of solvents from samples was accomplished with a Model SVC-100H Speed Vac Concentrator from Savant Instruments, Hicksville, NY, U.S.A. GC analyses were accomplished with a Varian 1400 instrument equipped with a flame ionization detector and a silanized glass column (2 m × 2 mm I.D.) packed with 2% OV-17 on Supelcoport. GC–MS analyses were performed on Finnigan 3200 and Finnigan MAT 212 instruments.

Synthesis of silylating reagent

The method of Kutchinski[2] is here described because it is not readily accessable in the literature.

Preparation of N-methyltrifluoroacetamide. A flask containing ethyl trifluoroacetate (29.7 g, 0.21 mole) was weighed and cooled to 0°C with dry ice. Anhydrous monomethylamine was bubbled through the ester while the temperature was maintained at 0°C until the mass increase was 10.9 g (0.35 mole monomethylamine). The mixture was clear and colorless after standing at room temperature overnight. The product mixture was distilled; material boiling at 156–157°C was collected and crystallized upon cooling, m.p. 50–51°C, lit. 50–51°C[2], yield 78% of theoretical.

Preparation of N-(tert.-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). Baked glassware (250-ml three-neck flask, condensor, and addition funnel) was assembled and flushed with nitrogen. NaH dispersion (50%, 3.8 g, 0.078 mole) was washed with three 12-ml volumes of dry hexane. Anhydrous THF (78 ml) was added to the NaH under nitrogen and N-methyltrifluoroacetamide (10 g, 0.078

* The reagent has recently been made available commercially by Regis Chemical Co., Morton Grove, IL, U.S.A.

mole, in 10 ml of anhydrous THF) was added. The mixture was stirred under reflux and nitrogen for 4 h before adding *tert.*-butyldimethylchlorosilane (11.8 g, 0.078 mole, in 6 ml of THF). Reflux under nitrogen was continued for 24 h. Solvents were removed through rotary evaporation, and the residue was stored overnight in a capped flask. The residue was distilled and the fraction boiling at 174–182°C collected. Infrared (IR) and nuclear magnetic resonance (NMR) spectra were in agreement with literature[2] spectra and amounted to 32% of the theoretical yield. The reagent is stored at room temperature in a desiccator.

Derivatization of 6-keto-$PGF_{1\alpha}$

Methoximation. Methoxyamine HCl (0.1 ml of 0.025 g reagent/ml dry GC-grade pyridine) was added to a residue of the prostaglandin in a silanized centrifuge tube. After capping (PTFE-lined cap) and vortexing, the mixture was either warmed at 38°C for 1 h or left at room temperature overnight. The pyridine was then evaporated with nitrogen while the tube was partially immersed in warm water. Water (0.5 ml) was added, the mixture was acidified with dilute formic acid to pH 3.5 and then extracted twice through vortexing with 1-ml volumes of diethyl ether. The combined extracts were washed with 1 ml of water and the ether was evaporated in a vacuum centrifuge.

Esterification. Etherial diazomethane (0.2 ml *ca.* 0.6 *M*) was added to the methoxime. Dissolution of the methoxime was assured through vortexing. After 5 min excess reagent was dispersed with a stream of nitrogen, and the etherial solution was transferred by pipette to a 1-ml Reactivial®. A small ether wash (0.1 ml) was also transferred to the vial and the ether was evaporated under nitrogen.

Silylation. DMF (25 μl) and MTBSTFA (25 μl) were next added to the vial and complete dissolution was assured through vortexing. The capped vials were heated at 60°C for 1 h, then the solvent and reagent were evaporated with a stream of nitrogen. The residue was reconstituted in 10 μl hexane. Alternatively the reaction mixture could be injected directly.

Instrumental parameters. For GC analysis the carrier gas (nitrogen) flow-rate was 30 ml/min. Injector temperature, 270°C; detector temperature, 300°C; column temperature, 260°C. GC–MS analysis was performed with a helium flow-rate of 12 ml/min and the following parameters: electron energy, 70 eV; injector temperature, 270°C; column temperature, 260°C; separator temperature, 270°C.

RESULTS AND DISCUSSION

In recent years much attention has been devoted to the optimization of the analysis of 6-keto-$PGF_{1\alpha}$. The result has been improvements in the analytical procedure. Most recently Hensby *et al.*[5] have compared the sensitivity and specificity of GC–MS and radioimmunoassay (RIA), and found RIA to be more sensitive but more subject to interferences. They state the importance of the validation of an RIA for a particular prostaglandin by GC–MS, a technique which is both highly specific and sensitive. We now offer a further improvement in the derivatization of 6-keto-$PGF_{1\alpha}$ for analysis by GC–MS.

The original derivatization published by Pace-Asciak[1] involved esterification followed by methoximation and trimethylsilylation to yield a methyl ester–meth-

oxime–tris-TMS (MeMOTMS) derivative. Papers with attention to various details and variations in the procedure were subsequently published[6,7]. A significant variation concerned the order of esterification and methoximation. Like Pace-Asciak, most workers have proceeded to first esterify and then form the methoxime; however, Clayes *et al.*[6] have reported a five-fold increase in derivative yield at the 100-ng level by first executing the methoximation. We have adopted this sequence in our procedure.

The necessarily huge excess of methoxyamine HCl has been dealt with differently by several authors. Frölich[7] in a general derivatization procedure suggests extraction of the derivative with ether from excess solid reagent after evaporation of pyridine. Oliw *et al.*[8] apparently proceeded to esterify and silylate in the presence of excess reagent. We have found these methods to be less than optimum, and prefer evaporation of the pyridine and extraction of the methoxime with ether from an acidified aqueous solution of methoxime and excess reagent. Evaporation of the water-washed ether extracts is accomplished with a centrifugal vacuum evaporator which concentrates the derivative residue in the bottom of a silanized centrifuge tube.

For esterification, the use of freshly prepared etherial diazomethane is specified by many authors, and many use a mixture of methanol–etherial diazomethane. We find it effective and much more convenient to use only an etherial solution of diazomethane prepared in advance and stored at −20°C. The reagent keeps well for weeks under these conditions.

In recent years attention has been focused on improving the mass spectra of prostaglandins for quantitative analysis through SIM. Thus, Watson and Sweetman[9] prepared methyl ester TBDMS derivatives of PGA and PGB, and reported mass

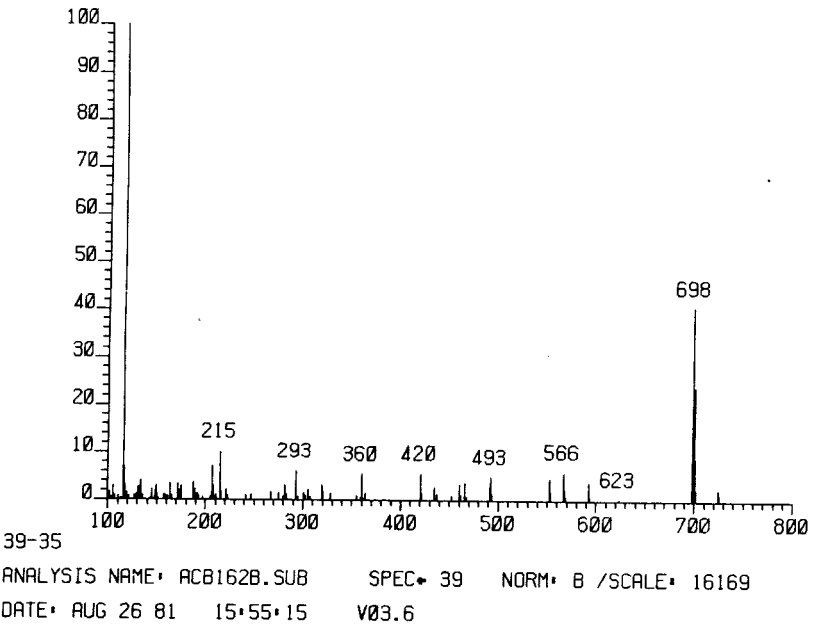


Fig. 1. Mass spectrum of MeMOTBDMS derivative of 6-keto-$PGF_{1\alpha}$.

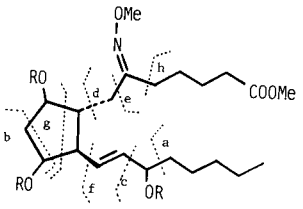
spectra with abundant molecular ions and base peaks at M − 57 from loss of the *tert.*-butyl group. Gaskell and Pike[10] have also emphasized the advantages of TBDMS ethers in their recent paper on the GC–MS of methyloxime-TBDMS derivatives of androstanolones. For silylation all of these authors employed the *tert.*-butyldimethylchlorosilane–imidazole reagent, which must be removed through extraction or chromatography prior to analysis[4].

Pace-Asciak[11] reported that the MeMOTMS derivative of 6-keto-$PGF_{1\alpha}$ had a "multiplicity of ions of high intensity in the high end of the mass spectrum, providing excellent potential for use in mass fragmentography." These results were obtained with a Varian MAT CH-5, a magnetic sector instrument. Unfortunately such multiplicities of high-intensity high-mass ions are often not seen, especially with quadrupole instruments which may discriminate against the high-mass end of the spectrum.

One solution to this problem, that of Morita and co-workers[12,13], is the use of chemical-ionization (CI) mass spectrometry. These authors compared ammonia,

TABLE I

MASS SPECTRA OF MeMOTMS[14] AND MeMOTBDMS (III) DERIVATIVES OF 6-KETO-$PGF_{1\alpha}$



Numbers shown are mass numbers (m/z) with relative abundances given in parentheses.

Ion assignment	*MeMOTMS (IV)*	*MeMOTBDMS (III)*
$[M]^{+\cdot}$	629 (8)	755 (not observed)
$[M-Me]^+$	614 (6)	–
$[M-tert.\text{-}Bu]^+$	–	698 (40)
$[M-OMe]^+$	598 (52)	724 (2)
$[M-HOR]^{+\cdot}$	539 (13)	623 (0.3)
$[M-OMe-HOR]^+$	508 (64)	592 (4)
$[M-OMe-b]^+$	482 (5)	566 (6)
$[M-HOR-a]^+$	468 (34)	552 (5)
$[M-d]^+$	443 (6)	569 (0.7)
$[M-OMe-HOR-b]^+$	392 (10)	434 (3)
$[M-2(HOR)-a]^+$	378 (100)	420 (5)
$[M-d-HOR]^+$	353 (28)	437 (1)
$[M-OMe-3(HOR)]^+$	328 (25)	328 (1)
$[M-2(HOR)-e]^+$	277 (17)	319 (3)
$[M-2(HOR)-d]^+$	263 (17)	305 (2)
$[g]^+$	217 (24)	301 (1)
$[f]^+$	199 (25)	241 (1)
$[d+H]^{+\cdot}$	187 (26)	187 (4)
$[c]^+$	173 (61)	215 (10)
$[h]^+$	115 (49)	115 (100)
$[TBDMS]^+$	–	115 (100)
[TMS]	73 ($\gg$ 100)	–

methane, and isobutane as reagent gases and found ammonia to be the superior reagent. With ammonia the quasimolecular ion (QM^+, *m/z* 630) was weak; but a base peak at *m/z* 540 ($QM^+ - TMSOH$) was used for quantitative mass fragmentography with practically no interference from other endogenous biological substances. These results point out the advantage of monitoring high-intensity high-mass ions. However, since many laboratories are not equipped for GC–CI-MS, an alternative solution would be useful. We offer an easily prepared new derivative as a means of obtaining interference-free high-intensity high-mass ions for quantitative analysis of 6-keto-$PGF_{1\alpha}$ through SIM.

The mass spectral fragmentation of the new derivative of 6-keto-$PGF_{1\alpha}$ closely parallels that of the MeMOTMS derivative which has been well characterized by Cockerill *et al.*[14]. The mass spectrum of the new derivative and a tabular comparison of its fragmentation with that of the MeMOTMS derivative appear in Fig. 1 and Table I, respectively. It should be noted that the data in Table I for the relative intensities of the high-mass ions from IV appear high, but this is due to normalization on the *m/z* 378 peak rather than the most intense *m/z* 73 peak. If the analogous peak (*m/z* 115) from III were omitted, the M − 57 peak (*m/z* 698) would become the base peak. It can be seen from Table I that there are several high-intensity high-mass ions in the mass spectrum of III in which the hydrogen atoms at C-3 and C-4 are retained. This is significant because it allows use of the 3,3,4,4,-$^2H_4$ analogue as an internal standard.

A further simplification of the derivatization of 6-keto-$PGF_{1\alpha}$ is the simultaneous esterification and ether formation through reaction of the methoxime with MTBSTFA to form the MO TBDMS ester TBDMS ether. However, we find the TBDMS ester to be inferior to the methyl ester for GC–MS analysis; GS retention time is longer and the higher mass of fragment ions may present problems for limited mass range instruments. Neither the methyl ester nor the TBDMS ester derivatives showed GC separation of the *syn*- and *anti*-methyloxime isomers.

In conclusion, this new derivative of 6-keto-$PGF_{1\alpha}$ is comparatively easily prepared as described and offers the advantages of hydrolytic stability and superior mass spectrometric properties for GC–MS–SIM quantitation.

ACKNOWLEDGEMENTS

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Note

Separation of prostaglandins on an OV-210 whisker-wall-coated open tubular column

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Prostaglandins present in biological fluids are routinely analyzed by capillary gas chromatography where the columns usually employed are coated with apolar liquid phases[1–5]. Columns coated with the more polar OV-225 and Carbowax 20M phases have been investigated but are not widely used[1]. Adequate resolution of $PGF_{1\alpha}$ and PGE_2 may not be achieved using apolar liquid stationary phases. Although this does not present a problem in the analysis of prostaglandin mixtures when the E and F fractions are previously separated by liquid chromatography on silicic acid[1], a capillary gas chromatographic system capable of this separation would be helpful.

In our experience, packed columns containing OV-210 coated Gas-Chrom Q have found application for the separation of hydroxylated drug metabolites which were not separated on apolar or moderately polar siloxane liquid phase[4]. We felt that this liquid phase would provide a different retention pattern for the prostaglandins than the apolar variety as a consequence of the interaction with the hydroxyl and keto moieties on the prostaglandin molecules. It is very difficult to obtain a stable coating of OV-210 on a glass open tubular column. However, Sandra and Verzele[5] described the successful coating of this liquid phase on whisker-wall-coated open tubular (WWCOT) columns. This paper demonstrates the utility of a WWCOT column containing OV-210 for complete separation of $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGA_1, PGA_2, PGE_1 and PGE_2 as the methyl ester trimethylsilyl ether derivatives.

EXPERIMENTAL

Materials

All solvents were distilled in glass and purchased from Burdick & Jackson

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Labs. (Muskegon, MI, U.S.A.). The prostaglandins were generously provided by Dr. Edward Ham, Merck Sharp & Dohme Research Labs., Rahway, NJ, U.S.A. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Regis Chemical Co. (Morton Grove, IL, U.S.A.). N-Nitrosomethylurea for preparation of ethereal diazomethane was purchased from K & K Labs.–ICN Pharmaceuticals (Plainview, NY, U.S.A.). A splitter injection system and glass-lined tubing (GLT) were purchased from Scientific Glass Engineering (SGE) (Austin, TX, U.S.A.).

Gas chromatography

A Hewlett-Packard Model 5750 gas chromatograph was modified for use with capillary columns. The major modifications were the use of an injection port from an F & M Scientific Model 810 gas chromatograph and a pressure-controlled SGE splitter injection system with needle valve regulation of the split ratio. The capillary column was connected to the injector and flame ionization detector with 1/16 in. O.D. × 0.3 mm I.D. GLT transfer lines. WWCOT columns (25 m × 0.25 mm I.D.) coated (d_f = 0.2 μm) with either SE-30 or OV-210 liquid stationary phases were prepared as described previously[6]. Helium, which was purified over a desiccant of $CaCl_2$–5 Å molecular sieves and an Oxy-Trap (Alltech, Arlington Heights, IL, U.S.A.) was employed as the carrier and make-up gas.

All operating conditions are listed in the appropriate figures.

Derivatization

The methyl esters of the prostaglandins were prepared by reaction of 200 μl of a freshly prepared 0.5 *M* ethereal diazomethane solution with 100-μl aliquots of each prostaglandin (1 mg/ml in methanol). After 10 min, the reaction mixtures were concentrated to dryness under nitrogen at 25°C. The trimethylsilyl ethers were prepared by adding 100 μl of BSTFA to the residue at room temperature and immediately concentrating the sample to dryness under dry nitrogen after brief vortex mixing. The resulting methyl ester trimethylsilyl ether derivatives were reconstituted in hexane prior to capillary gas chromatographic analysis.

RESULTS AND DISCUSSION

A typical chromatographic separation of the six prostaglandins as the methyl ester trimethylsilyl ether derivatives is illustrated in Fig. 1. Adequate resolution was obtained within 14 min for all of the components except $PGF_{1\alpha}$ and PGE_2 at a column temperature of 250°C with the SE-30 WWCOT column. This separation is similar to that reported in the literature when the methyl ester methoxime trimethylsilyl ether derivative of the PGEs was prepared[1]. Although the methyl ester trimethylsilyl ether derivatives of the PGE analogs are less stable than those prepared following oximation of the 9-keto function, the conditions of derivatization were adjusted to minimize degradation of these derivatives.

A chromatogram of the same mixture of the six prostaglandins as the methyl ester trimethylsilyl ether derivatives chromatographed on an OV-210 WWCOT column is presented in Fig. 2. At a column temperature of 230°C, all of the components are well separated within 20 min. Upon comparison of these results with those observed on an SE-30 column (Fig. 1), the retention order has changed such

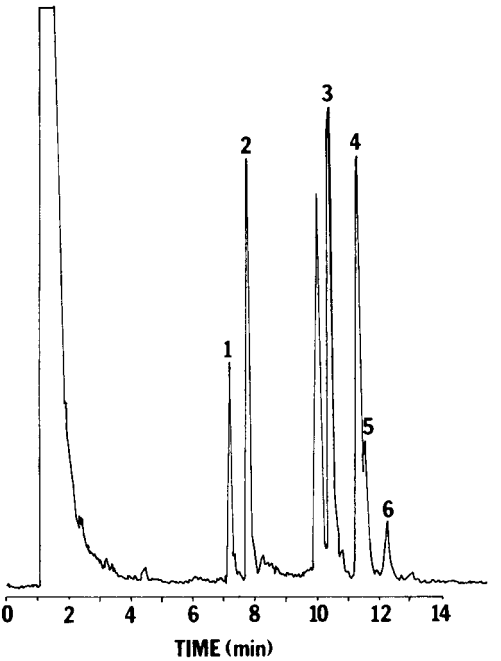

Fig. 1. Chromatogram of a mixture of prostaglandins as their methyl ester trimethylsilyl ether derivatives in hexane. Column: SE-30 whisker glass capillary (d_f = 0.2 μm) (25 m × 0.25 mm I.D.); N = 3378 plates per metre, k = 11.5. Linear velocity: 38.6 cm/sec. Carrier gas: helium, 30 p.s.i. Temperatures: detector, 245°C; injector, 250°C; column, 250°C. Splitting ratio: 10/1. Attenuation: 8 × 1. Peaks: 1 = PGA_2; 2 = PGA_1; 3 = $PGF_{2\alpha}$; 4 = $PGF_{1\alpha}$; 5 = PGE_2; 6 = PGE_1.

that the PGF analogs elute much earlier than the PGA and PGE analogues. Although the resolution of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ has diminished slightly from that observed in Fig. 1, the resolution of PGA_1 and PGA_2, as well as that of PGE_1 and PGE_2, has markedly increased.

It is well known that with the present technology only the non-polar silicone liquid stationary phases may be coated with good stability on fused-silica open tubular columns. Columns containing the moderately polar and polar silicone liquid phases may be prepared with sufficient stability on a roughened glass surface. WWCOT columns have been considered too difficult to prepare, to possess approximately ten times the activity compared to smooth wall glass columns, as well as less efficient due to the severe roughening. This view is not totally correct. It is now possible to deactivate sufficiently the whisker-walled columns for analysis of biological samples as demonstrated in this paper. It is true that these columns do not provide the theoretical plate efficiency of smooth glass columns, but the whisker-walled columns offer the flexibility of supporting almost any liquid phase. This consideration alone may be worth more than the efficiency gained with other types of open tubular columns which may not provide the necessary selectivity. The benefits of selectivity in many cases outweigh the minor loss in efficiency for many applications of WWCOT columns. The effective theoretical plate value listed in the legend of Fig. 1 is comparable to similar columns prepared on a non-whiskered surface, suggesting that WWCOT columns provide adequate efficiency.

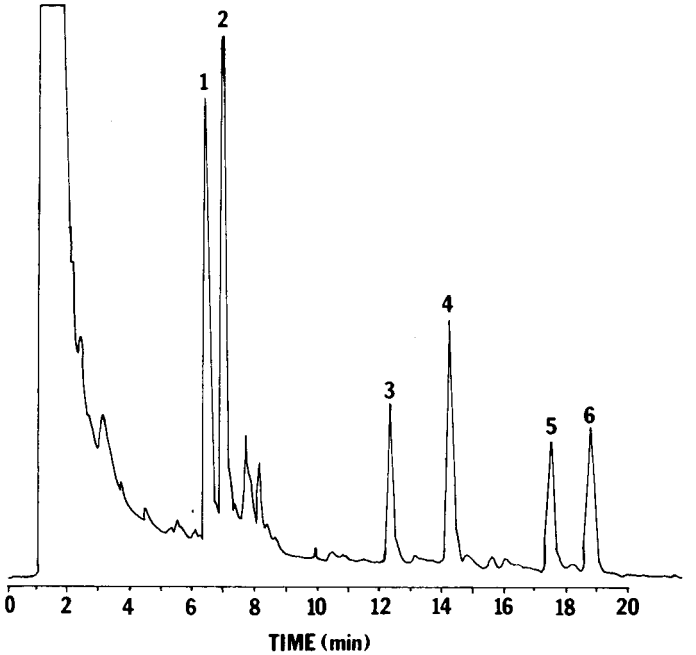

Fig. 2. Chromatogram of prostaglandins as their methyl ester trimethylsilyl ether derivatives in hexane. Column: OV-210 whisker glass capillary (d_f = 0.2 μm) (25 m × 0.25 mm I.D.); N = 1986 plates per metre, k = 21.5. Linear velocity: 37.9 cm/sec. Carrier gas: helium, 30 p.s.i. Temperatures: detector, 245°C; injector, 250°C; column, 230°C. Splitting ratio: 10/1. Attenuation: 8 × 1. Peaks: 1 = $PGF_{2\alpha}$; 2 = $PGF_{1\alpha}$; 3 = PGA_2; 4 = PGA_1; 5 = PGE_2; 6 = PGE_1.

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Note

Utilisation du couplage chromatographie sur gel perméable–diffusion de la lumière pour la caractérisation de résines formophénoliques

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Ce travail s'inscrit dans le cadre de l'étude de la caractérisation des polymères par chromatographie sur gel perméable (GPC) que nous effectuons depuis un certain nombre d'années[1]. D'une manière plus précise, nous utilisons dans ce but un chromatographe GPC muni d'un système à multidétection[2]. C'est ainsi que dans le passé nous avons pu, de sucroît, évaluer le degré de ramification[4]. Ces études ont été et en nombre et l'indice de polydispersité correspondant dans le cas d'homopolymères linéaires ou ramifiés et de copolymères[3]. En ce qui concerne les polymères ramifiés, nous avons pu, de surcroît, évaluer le degré de ramification[4]. Ces études ont été réalisées avec un chromatographe comportant outre le réfractomètre différentiel, un spectrophotomètre UV et un viscosimètre automatique. Récemment nous avons adjoint au système un détecteur constitué par un photodiffusomètre. Ce dispositif a été appliqué à la caractérisation des résines formophénoliques utilisées pour l'amélioration des propriétés du bois de hêtre par imprégnation et stratification[5,6]. Dans ce mémoire nous décrivons les premiers résultats obtenus concernant ce type de résines.

PARTIE EXPÉRIMENTALE

Les résines formophénoliques de type "résol" ont été soumises à un fractionnement par précipitation, en utilisant comme couple solvant–non-solvant le système tétrahydrofuranne (THF)–cyclohexane[7].

Les fractions ont été caractérisées à l'aide essentiellement de la chromatographie en phase liquide sur gel perméable. En raison de la faible adsorption des constituants formophénoliques sur les gels de polystyrène[7,8], nous avons utilisé ce type de support de préférence au gel de silice. Plus précisément nous avons employé deux séries de colonnes, telles que la taille des pores du gel couvre la distribution des tailles des molécules en solution. La première série était constituée de sept colonnes remplies de Styragel de porosité comprise entre 1000 Å et 60 Å. Le deuxième jeu de colonnes

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était formé de quatre colonnes de μStyragel de porosités allant de 1000 Å à 60 Å. Le solvant d'élution était le THF (le débit étant de 1 ml/min).

Nous avons utilisé un chromatographe Waters 200 muni d'un système à détection multiple comprenant, outre le réfractomètre différentiel, un spectrophotomètre UV, un viscosimètre automatique de type Ubbelohde et un photodiffusomètre. Ce dernier détecteur dérivé de l'appareil de diffusion de la lumière Fica-42000 muni d'un laser à gaz hélium–néon (633 nm) a été adapté par Gallot et Strazielle[9] à la sortie du chromatographe Waters 200. Pour cela, nous avons employé une cellule à écoulement qui permet la détection en continu de l'intensité de la lumière diffusée sous un angle de 90°.

Les masses moléculaires moyennes en poids des fractions ont également été mesurées indépendamment à l'aide d'un appareil de diffusion de la lumière Fica-42000 (λ = 633 nm) en utilisant comme solvant le THF.

RÉSULTATS ET DISCUSSION

Les chromatogrammes de la résine W enregistrés avec les séries de colonnes No. 1 et No. 2 sont représentés sur les Figs. 1 et 2. On constate que la résine W est fortement polydisperse, mais que la série No. 2 garnie de μStyragel est nettement plus performante en ce qui concerne le pouvoir de séparation. Cependant l'utilisation de

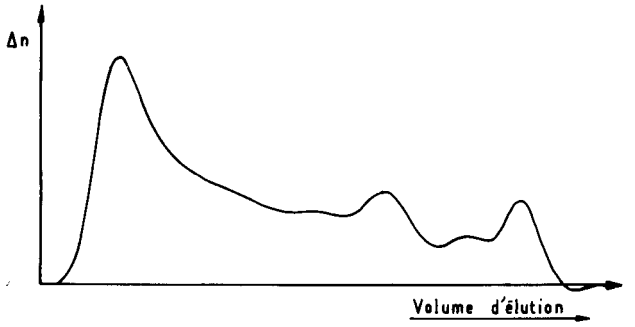

Fig. 1. Chromatogramme de la résine W, obtenu avec une série de sept colonnes de Styragel de porosités respectives 1000, 500 (deux colonnes), 200, 100 et 60 Å (deux colonnes). *Δn* est la différence d'indice de réfraction entre la solution et le solvant.

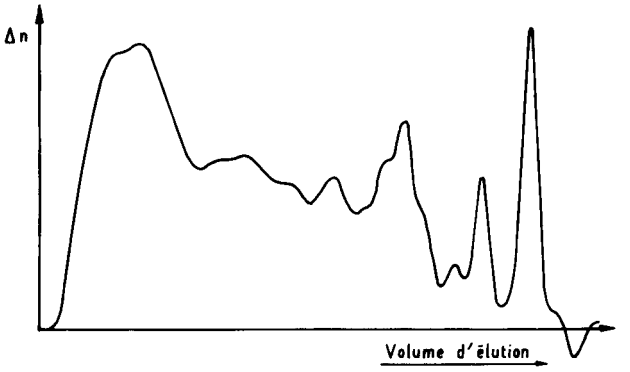

Fig. 2. Chromatogramme de la résine W, obtenu avec une séries de quatre colonnes de μStyragel de porosités respectives 1000, 500 et 60 Å (deux colonnes).

colonnes remplies de μStyragel ne permet d'injecter que des petits volumes de solution; en conséquence, la quantité de polymère introduite étant très faible, les différences de temps d'écoulement entre la solution et le solvant mesurées par le viscosimètre automatique sont très faibles et rendent peu précise la détermination de la viscosité intrinsèque. Or la connaissance de ce paramètre est indispensable pour le calcul des masses moléculaires moyennes à partir de l'étalonnage universel. Pour cette raison, nous avons employé exclusivement la série de colonnes No. 1. La Fig. 3 rassemble les chromatogrammes de la résine W et de ses fractions, à partir desquels nous avons calculé les valeurs des viscosités intrinsèques, des masses moléculaires en poids et en nombre et de l'indice de polydispersité. L'observation des chromatogrammes montre que les sept premières fractions présentent une distribution des masses moléculaires nettement plus étroite que celle de la résine de départ. Cependant les chromatogrammes correspondants sont fortement assymétriques vers les plus faibles masses moléculaires ce qui indique qu'il y a présence de molécules de très faibles masses moléculaires dans chacunes de ces fractions.

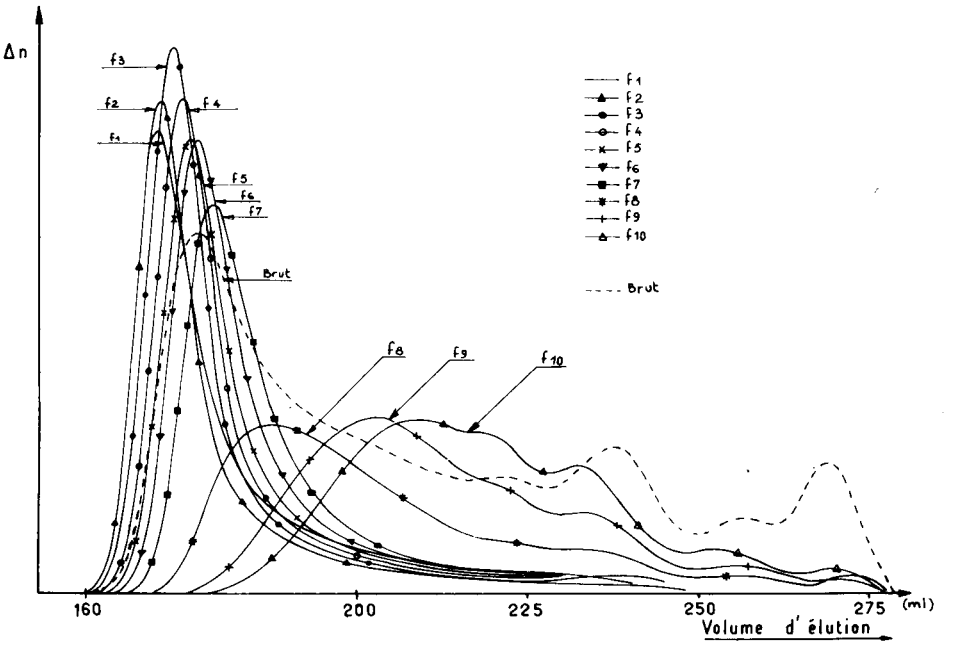

Fig. 3. Chromatogrammes de la résine W et de ses fractions.

Si l'on considère maintenant les valeurs des paramètres moléculaires donnés dans le Tableau I, on observe que si la viscosité intrinsèque diminue progressivement avec le numéro d'ordre des fractions, il n'en est pas de même pour les masses moléculaires moyennes.

Il nous a donc paru nécessaire de vérifier par diffusion de la lumière (DDL) les masses moléculaires moyennes en poids fournies par GPC. Les résultats obtenus par DDL (Tableau I) sont ordonnés de façon logique, mais sont très différents des précédents pour les premières fractions, et deviennent progressivement plus voisins pour les dernières fractions.

TABLEAU I

VISCOSITÉ INTRINSÈQUE ET MASSES MOLÉCULAIRES MOYENNES EN POIDS (M_p) ET EN NOMBRE (M_n) DES RÉSINES

Échantillon	$[\eta]_{(ml/g)_{GPC}}$	$M_{p,GPC}$	$M_{n,GPC}$	M_p/M_n	$M_{p,DDL}$
Brut	4.1	940	100	9.4	7900
F-1	7.3	1800	780	2.3	111000
F-2	7.9	2000	1350	1.5	56000
F-3	6.1	2500	1800	1.4	30400
F-4	6.2	1900	1400	1.4	19400
F-5	4.9	2400	1300	1.8	9900
F-6	4.5	2400	1600	1.5	7500
F-7	4.3	1100	600	1.8	4500
F-8	4.2	1000	190	5.3	1600
F-9	3.5	700	190	3.7	800
F-10	2.9	500	230	2.2	800

Ces résultats étonnants en ce qui concerne les premières fractions nous ont conduit à ajouter au détecteur réfractométrique classique un détecteur de diffusion de la lumière, afin d'examiner si les chromatogrammes obtenus à l'aide de ces deux types de détecteurs sont identiques. La Fig. 4 représente les chromatogrammes de la fraction F-7 enregistrés au moyen du réfractomètre et du photodiffusomètre respectivement. On voit que les deux courbes de réponse sont différentes dans le domaine des plus faibles volumes d'élution, c'est-à-dire des plus grandes masses moléculaires. Le détecteur DDL permet en effet de mettre en évidence la présence en très faible concentration de composés de très grandes masses, indécelables par le réfractomètre différentiel, ce qui explique les valeurs plus faibles des masses moléculaires calculées à partir de la réponse réfractométrique. Le détecteur DDL s'avère donc plus performant (sa sensibilité est plus élevée) que le détecteur réfractométrique, ce qui s'explique

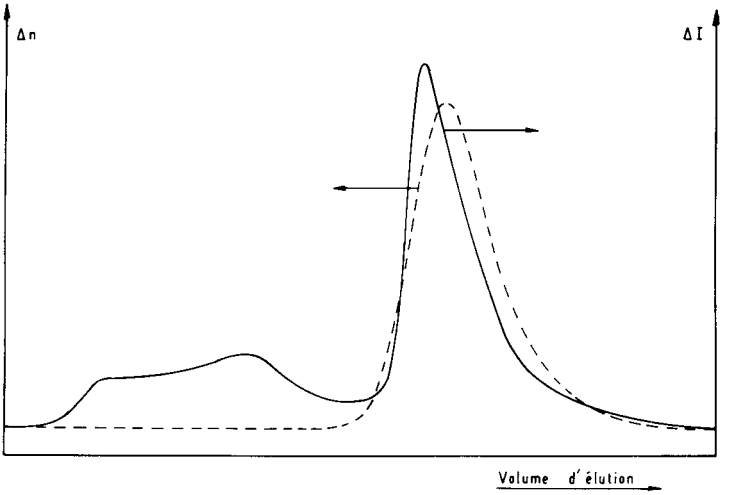

Fig. 4. Chromatogrammes de la fraction F-7 obtenus au moyen du réfractomètre différentiel ($\Delta n = fV_e$) et du photodiffusomètre ($\Delta I = fV_e$). ΔI est la différence d'intensité diffusée entre la solution et le solvant. V_e est le volume d'élution.

de la façon suivante: la réponse du photodiffusomètre est proportionnelle au produit de la concentration en polymère par la masse moléculaire ($c \times M$), tandis que celles du réfractomètre différentiel n'est proportionnelle, qu'à la seule concentration en polymère (c).

Nous avons voulu vérifier que les valeurs élevées des masses moléculaires des premières fractions mesurées dans le THF, étaient bien dues à la présence en très faible quantité d'espèces de très hauts poids moléculaires et non pas à la présence d'agrégats formés par l'association d'un certain nombre de molécules. Pour cette raison, nous avons effectué des mesures de diffusion de la lumière dans plusieurs bons solvants des résines, comme l'acétone et le méthanol. Nous avons obtenu des valeurs analogues à celles mesurées dans le THF, ce qui confirme l'existence de composés de très grande masse plutôt que d'agrégats.

REMERCIEMENT

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Note

Séparation et dosage du L-fucose et du D-xylose par chromatographie liquide à haute pression

Application à l'analyse des polysaccharides sulfurylés des algues brunes

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(Reçu le 4 mai 1981; manuscript modifié reçu le 14 septembre 1981)

L'application de la chromatographie liquide à haute pression (HPLC) à l'analyse des polysaccharides sulfurylés des algues brunes nécessite que l'on puisse identifier et doser par cette technique le fucose, le xylose, le mannose, le galactose, et l'acide D-glucuronique constituants de ces macromolécules[1], ainsi que ceux apportés par d'éventuels contaminants tels que la laminarine (β 1-3 glucane) ou l'algine (copolymère d'acide D-mannuronique et d'acide L-guluronique).

Les techniques de HPLC utilisées pour le dosage des glucides peuvent être réparties en deux groupes, selon qu'elles font appel ou non à une transformation chimique préalable des oses à doser. Parmi les méthodes ne nécessitant aucune transformation des oses avant chromatographie, on peut citer: la chromatographie d'absorption sur silice[2–4], sur phase inverse de silice aminée[5,6] ou amino-alkylée[7,8], ou greffée C_{18}[9]; la chromatographie d'échange d'ions[10–13]; la chromatographie d'exclusion[14], ou encore des méthodes mixtes qui allient plusieurs de ces types de chromatographie[14–16]. Toutes ces méthodes conviennent au dosage des hexoses et des oligosaccharides, mais aucune d'entre elles ne permet une bonne résolution des mélanges de pentoses et méthylpentoses, si l'on excepte la technique récemment mise au point par Heyraud et Rinaudo[9] qui permet, sur phase inverse de silice C_{18} de séparer le fucose du xylose. De plus, leurs applications sont limitées par la faible sensibilité à l'égard des glucides des détecteurs actuellement disponibles.

C'est pour pallier à cet inconvénient qu'ont été développées des techniques comportant une transformation des glucides, avant leur injection ou en sortie de colonne. Les dérivations post-colonne[14] s'accompagnent souvent d'une diminution de resolution des pics chromatographiques[2] et ne peuvent donc pas être utilisées pour doser des oses qui sont mal séparés en sortie de colonne. A l'inverse, les techniques qui font appel à une dérivation pré-colonne des oses par des chromophores aromatiques[17–21] n'abaissent pas la qualité de leur résolution, tout en multipliant par trois ou quatre ordres de grandeur la sensibilité de leur détection. Bien plus, la fixation des chromophores accroît, en modifiant les fonctions et la structure spaciale des oses, les possibilités de séparation chromatographique d'oses très voisins dans l'état non dé-

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rivé. Toutefois, aucune des méthodes de dérivation existantes ne permettait, avec les conditions de phase stationnaire et de phase mobile décrites par leurs auteurs, de doser le fucose et le xylose en mélange.

Nous nous sommes donc attaché à résoudre ce problème particulier, en prenant comme base de départ la méthode proposée par Thompson[20] pour la séparation des dérivés benzyloxime-perbenzoyle des oses simples, non carboxyliques.

PARTIE EXPÉRIMENTALE

Nous avons utilisé un chromatographe en phase liquide LDC (Sopares, Gentilly, France) comprenant une pompe Constametric II G, une vanne d'injection à boucle (100 μl) Valco, une colonne Sphérisorb NH_2 5 μm (200 × 4.7 mm I.D.), ou une colonne Spherosil XOA 800-7 μm (150 × 4.7 mm I.D.) un détecteur UV Spectromonitor III, et un enregistreur Linear.

Les glucides standards utilisés ont été fournis par Sigma (St Louis, MO, U.S.A.) et les produits nécessaires à la dérivation, benzyloxime et chlorure de ben-

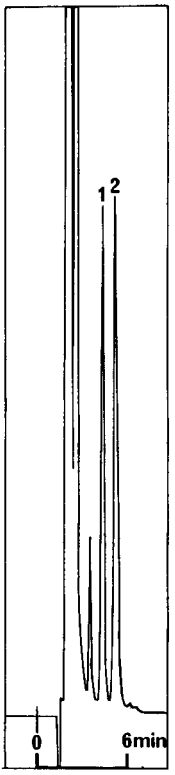

Fig. 1. Séparation par HPLC du fucose et du xylose à l'aide d'un mélange d'hexane et de dichlorométhane (éluant I). Phase mobile, hexane–dichlorométhane (68:32); phase stationnaire, silice aminée; débit, 2 ml/min; détecteur, spectrophotomètre UV, 230 nm. 1 = Benzyloxime–tetrabenzoyle–L-fucose; 2 = benzyloxime–tetrabenzoyle–D-xylose.

zoyle par Aldrich (Milwaukee, WI, U.S.A.). Tous les solvants utilisés (qualité Uvasol ou LiChrosolv) provenaient de chez Merck (Paris, France). Chaque éluant était dégazé avant utilisation à l'aide d'une sonde à ultra-sons (Sourimase 250 T, 220 V) et filtré sur filtre Millipore 0,45 μm.

Les glucides (2–100 μg) ont été dérivés selon la méthode décrite par Thompson[20], et extraits du mélange réactionnel selon le protocole suivant, adapté de la technique utilisée par Lehrfeld[17]. L'excès de réactif est hydrolysé par 500 μl de méthanol. La solution obtenue est évaporée sous un courant d'air comprimé. Les glucides dérivés sont alors repris par 5 ml d'hexane. Après centrifugation (2000 *g*, 2 min), un aliquote (1 ml) du surnageant est évaporé à sec. Le résidu est lavé par 3 ml d'eau saturée en bicarbonate de sodium et repris par 2 ml d'hexane. Un aliquote (10 à 100 μl) de la phase organique est alors soumis à chromatographie.

RÉSULTATS ET DISCUSSION

Choix d'un éluant permettant la séparation du fucose et du xylose

L'éluant utilisé par Thompson (hexane–dioxanne, 80:20) ne permet qu'une résolution médiocre du fucose et du xylose, que ce soit avec une colonne de silice ou avec une colonne de silice aminée.

L'utilisation d'un mélange binaire (éluant I) de dichlorométhane et d'hexane dans le rapport 32:68 conduit à une séparation complète du fucose et du xylose (Fig. 1), mais ne permet pas d'obtenir une séparation satisfaisante des hexoses (Tableau I).

En définitive, nous avons opté pour un solvant ternaire, composé d'hexane, de dichlorométhane et de dioxanne (éluant II) dans les proportions 50:7.5:2.5. Cet éluant

TABLEAU I

TEMPS DE RÉTENTION DE QUELQUES MONOSACCHARIDES POUR UNE PHASE STATIONNAIRE DE SILICE AMINÉE ET UNE PHASE MOBILE

Phase mobile: éluant I, hexane–dichlorométhane (68:32); éluant II, hexane–dichlorométhane–dioxanne (50:7.5:2.5).

	Temps de rétention (min)	
	Éluant I	*Éluant II*
Glycérol	4.10	4.00
Mésoérythritol	5.30	5.40
L-Rhamnose	4.40	5.70
L-Fucose	4.40	5.70
D-Xylose	5.40	6.50
L-Arabinose	4.90	6.40
Xylitol	6.10	8.50
D-Fructose	5.65	9.00
D-Glucose	5.90	9.60
D-Galactose	6.45	10.40
D-Mannose	6.30	10.00 et 10.60*
Mannitol	7.65	12.20

* Deux pics, représentant respectivement 18.5% et 81.5% de la quantité injectée.

permet une bonne séparation du fucose et du xylose avec 98 % environ de retour à la ligne de base, tout en conduisant à une résolution satisfaisante des hexoses (Tableau I et Fig. 2). Toutefois, il n'est pas possible à l'aide de ce solvant de séparer le mannose du galactose.

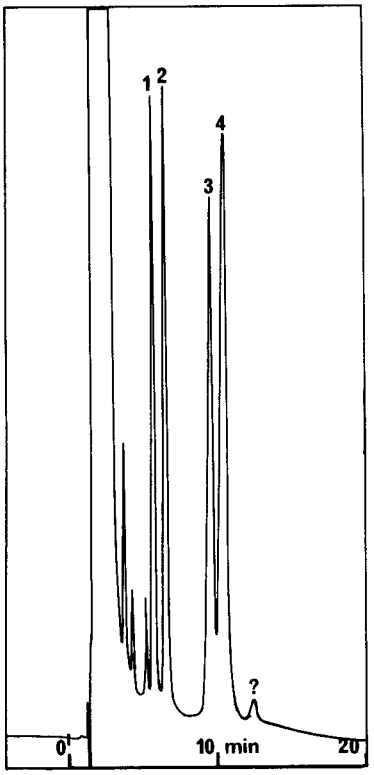

Fig. 2. Séparation par HPLC du fucose, du xylose, du glucose et du galactose à l'aide d'un mélange d'hexane, de dichlorométhane et de dioxane (éluant II). 1 = Benzyloxime–tetrabenzoyle–L-fucose; 2 = benzyloxime–tetrabenzoyle–D-xylose; 3 = benzyloxime–pentabenzoyle–D-glucose; 4 = benzyloxime–pentabenzoyle–D-galactose. Phase mobile, hexane/dichlorométhane/dioxanne (50:7.5:2.5). Autres conditions chromatographiques identiques à celles de la Fig. 1.

Application à l'analyse de la composition osidique des Polysaccharides sulfurylés des algues brunes

La méthode décrite par Thompson pour l'extraction des glucides dérivés à partir du mélange réactionnel ne nous a pas permis d'obtenir des résultats suffisamment reproductibles pour envisager le dosage de ces dérivés par chromatographie liquide.

Nous proposons donc d'utiliser le précédent protocole d'extraction décrit au chapître précédent, qui permet de doser les oses présents dans l'échantillon ainsi que l'indique la Fig. 3. L'utilisation d'un standard interne conduit à une précision de dosage de l'ordre de 1 % (Fig. 3b). La limite de détection du L-fucose —cinq fois le

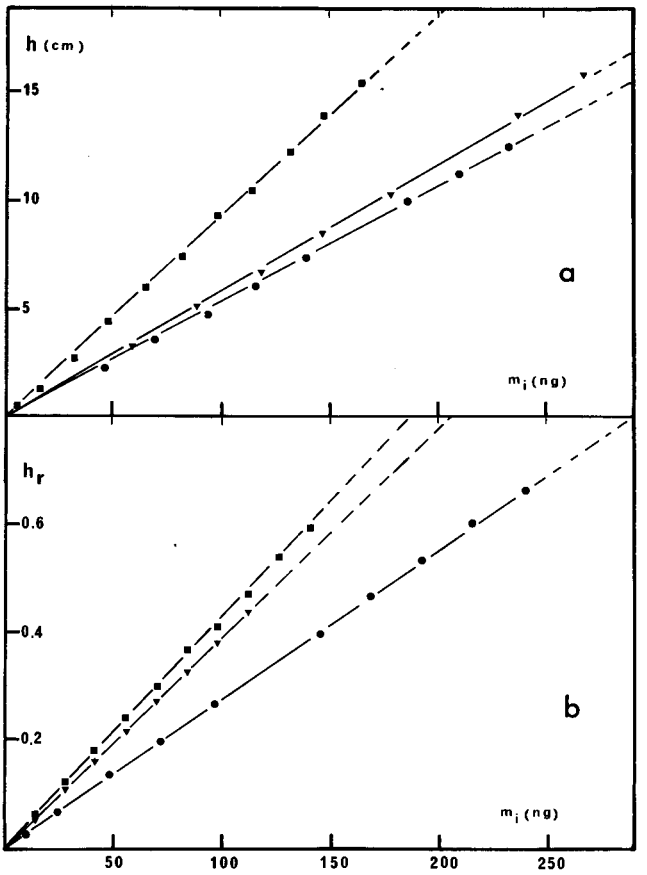

Fig. 3. Courbes d'étalonnage de quelques oses par HPLC. (a) Étalonnage direct; h, hauteur du pic en cm; m_i, masse injectée en ng. ■, Mésoérythritol; ▲, L-arabinose; ●, L-fucose. (b) Étalonnage par référence à un standard interne; h_r, hauteur relative du pic; m_i, masse injectée en ng. ■, Étalonnage du galactose par le xylitol; ▲, étalonnage du fucose par le mésoérythritol; ●, étalonnage du xylose par le mésoérythritol. Conditions chromatographiques identiques à celles de la Fig. 1.

bruit de fond à 0.005 unité d'absorbance pleine échelle— est environ 1.5 ng avec l'appareillage utilisé. La Fig. 4 représente des exemples de chromatogrammes HPLC obtenus à partir d'hydrolysats de fucoidanes extraits de thalles de *Pelvetia canaliculata*, *Fucus vesiculosus* et *Laminaria digitata* (respectivement: P_{cpc}, F_{cpc} et L_{cpc}).

Ces chromatogrammes sont conformes aux résultats précédemment établis par chromatographie d'échange d'ions[1] indiquant que le fucose représente, en masse, environ 90% des oses constituant les fucoidanes purifiés à l'aide de chlorure de cétylpyridinium (cpc).

La méthode de dosage des oses neutres décrite dans cet article apparaît donc comme applicable à l'analyse des polysaccharides sulfurylés des algues brunes.

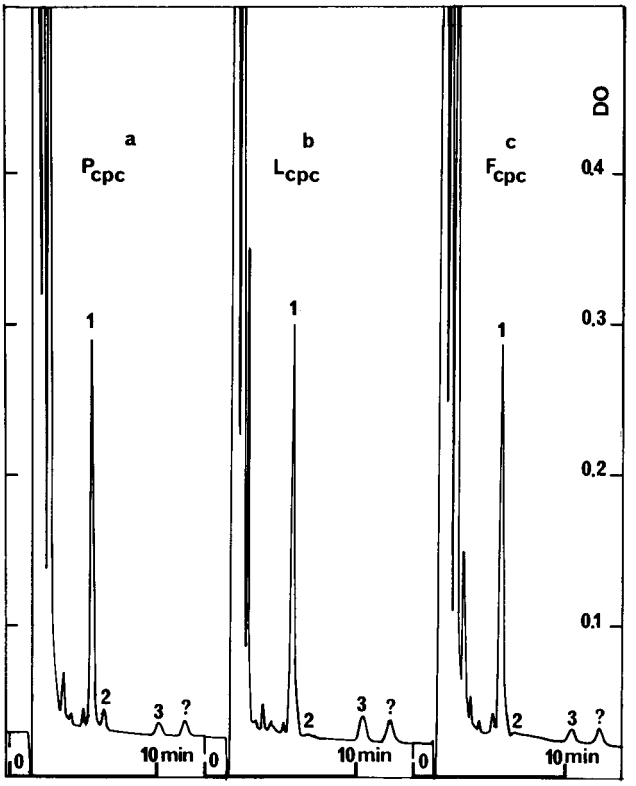

Fig. 4. Exemples de chromatogrammes HPLC obtenus après hydrolyse de fucoïdanes extraits de, *Pelvetia canaliculata* (a); *Fucus vesiculosus* (b); *Laminaria digitata* (c). Conditons chromatographiques identiques à celles de la Fig. 1.

REMERCIEMENT

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Note

Evaluation of some phytoecdysteroids as internal standards for the chromatographic analysis of ecdysone and 20-hydroxyecdysone from arthropods

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In the analysis of trace constituents such as ecdysteroids some losses during sample preparation are likely. In different tissues, or where levels are fluctuating considerably such loss may vary from sample to sample. Use of an internal standard can remove uncertainty resulting from these factors. In addition the percentage recovery of the internal standard may be used to determine the efficiency of the sample preparation.

We have examined the chromatographic properties of a number of common phytoecdysteroids to assess their suitability for use as internal standards in the determination of insect and crustacean ecdysteroid titres.

EXPERIMENTAL

HPLC

High-performance liquid chromatography (HPLC) was performed on a 15 cm × 3 mm I.D. stainless-steel column packed with either 5 μm ODS Spherisorb or 5 μm ODS Nucleosil (HPLC Technology, Wilmslow, Great Britain). Solvents were mixtures of water and methanol or water and acetonitrile, and were degassed before use. Solvents were delivered using an LDC Constametric III pump (Laboratory Data Control, Stone, Great Britain), at 1 ml min^{-1}. Ecdysteroids were detected in the column effluent at 254 nm using a Pye Unicam LC3 UV detector (Pye Unicam, Cambridge, Great Britain). Ecdysteroids were made up in methanol, and injected onto the column via a Rheodyne 7125 loop injector (Magnus Scientific, Stone, Great Britain).

GLC

Samples of ecdysteroids were prepared for gas–liquid chromatography (GLC) by silylation with trimethylsilylimidazole in pyridine (35 and 65 μl, respectively), at 120°C for 5 h contained in a 1-ml Reacti-vial (Pierce and Warriner, Chester, Great Britain). The sample was diluted with ECD-grade toluene prior to injection[1]. Samples

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(1 μl) were then injected onto a 0.9 m × 4 mm I.D. column of OV 101 (1.5%), on Chromosorb W. Chromatography was performed on a Pye series 104 gas chromatograph at 285°C with nitrogen as carrier gas at a flow-rate of 50–60 ml min^{-1}. Derivatives were detected using an ^{63}Ni electron-capture detector (ECD) at 300°C.

Ecdysteroids were gifts.

RESULTS AND DISCUSSION

Miyazaki *et al.*[2] have used cyasterone as a GLC internal standard, as have Lafont and co-workers[3,4]. More recently, Lafont *et al.*[4] have also used makisterone A. At about the same time we investigated a number of alternative compounds in our laboratory to see what advantages, if any, these had over makisterone A and cyasterone. Both HPLC and GLC are used by us for ecdysteroid analysis, depending on levels of ecdysteroid in the sample. Each candidate internal standard has been examined using both techniques. Ideally the internal standard should be compatible with both methods, as it is often useful to analyse the same sample by both techniques. In this case it is important that the internal standard should not interfere with any of the compounds of interest.

Five compounds were examined: cyasterone, inokosterone, makisterone A, polypodine B and ponasterone A. Inokosterone and makisterone A, and more recently ponasterone A, have been detected in some arthropod extracts[5–7], which may complicate their use as internal standards.

The chromatographic and other relevant properties of each compound are discussed below.

Cyasterone

The poor GLC characteristics of this compound (long retention times, multiple product formation on silylation, and poor peak shape) preclude its use as an internal standard for the GLC analysis of ecdysone and 20-hydroxyecdysone (see also ref. 4). Cyasterone may be used for HPLC analysis if care is taken in the choice of organic modifier used in the mobile phase. We have found that water–methanol based solvents give poor resolution of cyasterone and 20-hydroxyecdysone[8]. However, adequate resolution may be obtained with acetonitrile–water systems (Fig. 1 and Table I).

Inokosterone

Differing from 20-hydroxyecdysone only in possessing a C-26 rather than a C-25 hydroxyl, inokosterone has very similar chromatographic properties. On GLC the presumed penta-trimethylsilyl (TMS) ether of inokosterone has a longer retention time (9.7 min) than those of ecdysone and 20-hydroxyecdysone (6.15 and 8.5 min, respectively). Separation from 20-hydroxyecdysone is not large in this system, but could be improved at the expense of an increase in analysis time. The ECD response of this compound is similar to that of ecdysone. On HPLC (as was the case with cyasterone) acetonitrile–water mobile phases, but not methanol–water systems, resolve inokosterone from 20-hydroxyecdysone. Inokosterone exists as a mixture of C-25 *R* and *S* isomers, and under some circumstances (*e.g.* chromatography on ODS Spherisorb) these are resolved, resulting in two peaks for the internal standard.

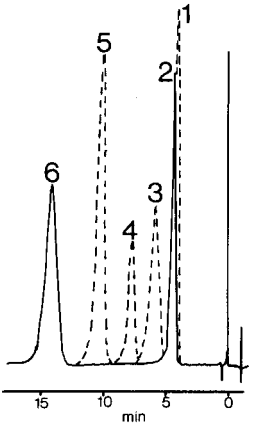

Fig. 1. A composite chromatogram showing the positions of polypodine B (1), 20-hydroxyecdysone (2), inokosterone (3), makisterone A (4), cyasterone (5), and ecdysone (6) on reversed-phase chromatography on ODS Nucleosil with acetonitrile–water (20:80) as mobile phase.

Makisterone A

On GLC this 24-methyl homologue of 20-hydroxyecdysone is well resolved from 20-hydroxyecdysone and ecdysone (Figs. 2 and 3). In addition its response to the ECD is similar to that of ecdysone. On reversed-phase HPLC, makisterone A elutes between 20-hydroxyecdysone and ecdysone, and there appears to be no limitation on the organic modifier used in the mobile phase.

TABLE I

HPLC RETENTION DATA FOR ECDYSTEROIDS WITH ACETONITRILE-BASED MOBILE PHASES

Under these conditions ponasterone A is not eluted from the column. On ODS spherisorb with 20% acetonitrile, ecdysone has retention time (t_R) 5.2 min, 20-hydroxyecdysone has retention time (t_R) 1.6 min, and ponasterone A retention time (t_R) 17.4 min.

Compound	*ODS Spherisorb: acetonitrile–water (15:85)*		*ODS Nucleosil acetonitrile–water (20:80)*	
	t_R *(min)**	t_R *relative to ecdysone*	t_R *(min)**	t_R *relative to ecdysone*
Ecdysone	22.4	1	14.2	1
20-Hydroxyecdysone	6.8	0.3	4.4	0.31
Makisterone A	14	0.63	7.8	0.55
Inokosterone	8.6, 10**	0.38, 0.45	5.8	0.41
Polypodine B	6.4	0.29	4.2	0.30
Cyasterone	13	0.58	9.5	0.67

* Measured from the solvent front.

** C-25 *R* and *S* isomers.

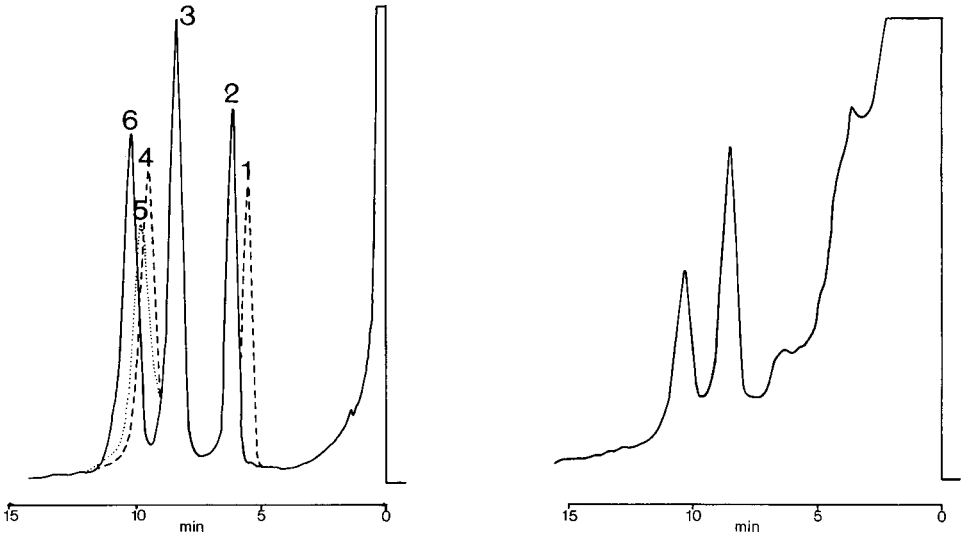

Fig. 2. A composite chromatogram showing the positions of ponasterone A (1), ecdysone (2), 20-hydroxyecdysone (3), polypodine B (4), inokosterone (5), and makisterone A (6), on GLC on 1.5% OV 101 at 285°C.

Fig. 3. A 0.7 μl^{-1} sample from the barnacle *Balanus balanoides* with makisterone A as internal standard. 20-Hydroxyecdysone is present (1.8 ng μl^{-1}). Chromatographic conditions as described in the Experimental section. The area of the makisterone A peak indicates an overall recovery of 86%.

Polypodine B

Polypodine B (5β-20-dihydroxyecdysone) has a similar GLC retention time to inokosterone, eluting after the penta-TMS ether of 20-hydroxyecdysone. However, the ECD response of this compound is only about a fifth of that of ecdysone (Table II), presumably due to the presence of the 5β hydroxyl. On reversed phase HPLC it is very difficult to resolve polypodine B from 20-hydroxyecdysone, even with extended analysis times. This similarity in HPLC properties might be useful in the analysis of

TABLE II

GLC RETENTION DATA FOR TMS ETHERS OF ECDYSTEROIDS

Compound	t_R *(min)*	t_R *relative to*		*ECD response relative to ecdysone*
		Ecdysone	*20-Hydroxy-ecdysone*	
Ecdysone	6.15	1	0.72	1
20-Hydroxyecdysone	8.5	1.38	1	1
Makisterone A	10.1	1.64	1.2	1
Inokosterone	9.7	1.57	1.14	1
Polypodine B	9.5	1.55	1.12	0.22
Cyasterone*	17.6	2.86	2.07	–
Ponasterone A	5.5	0.89	0.65	1

* Cyasterone also gave peaks at 15.5 and 27 min, amounting to *ca.* 20% of the total.

20-hydroxyecdysone if HPLC was used in sample preparations, and GLC for analysis, as only a single peak would need to be collected.

Ponasterone A

The absence of a C-25 hydroxyl in ponasterone A (25-deoxy-20-hydroxyecdysone), greatly reduces the polarity of the compound and increases the volatility of its silyl ether. This results in a short GLC retention time, when the compound is not well isolated from ecdysone, and a long HPLC analysis time.

CONCLUSIONS

The extensive purification, generally required of samples before chromatographic analysis for ecdysteroids is possible, demands the use of an internal standard, if precise and accurate quantitations are to be assured. However, the internal standard chosen depends to some extent on the type of analysis (GLC or HPLC), and the ecdysteroid present in the sample. For the general analysis of ecdysone and 20-hydroxyecdysone by both GLC and HPLC, makisterone A is suitable. Inkosterone is also suitable provided that acetonitrile–water systems are used for reversed-phase HPLC. A disadvantage of makisterone A and inokosterone is that both have been detected in arthropods[6,7]. This should not restrict their use if a preliminary screen shows them to be absent from the species under investigation at that stage of development. Polypodine B is suitable for GLC, but not HPLC where 20-hydroxyecdysone is present. Cyasterone is unsuitable for GLC, but usable on HPLC with an appropriate mobile phase. Ponasterone A is unsuited for use in HPLC because of its long retention time. On GLC it might be useful for the analysis of 20-hydroxyecdysone in the absence of ecdysone.

The candidate internal standards described here have usually been isolated from mixtures of other ecdysteroids, including ecdysone and 20-hydroxyecdysone. Some contamination with other ecdysteroids may therefore be encountered (see for instance Lafont *et al.*[4] on inokosterone). Standards should therefore always be checked for purity, and purified if necessary.

Provided that such precautions are taken, a suitable internal standard can only improve the quality of results obtained by chromatographic analysis. The internal standard chosen will depend on the type of analysis, and the ecdysteroids present in the sample.

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Note

Separation of dolichols and polyprenols by straight-phase high-performance liquid chromatography

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Polyprenols and their 2,3-dihydro derivatives, the dolichols, are widely distributed in nature[1,2]. Recently, as part of a project to prepare substrates for the CTP-dependent dolichol kinase, we developed a procedure for converting polyprenols to dolichols by specific chemical reduction of the α-isoprene unit[3]. To monitor the course of reduction and quantify the final product, we investigated the applicability of high-performance liquid chromatography (HPLC). We found that reversed-phase HPLC, a method used exclusively by previous workers to analyze dolichols[4–6], was unsatisfactory for resolving polyprenols from dolichols.

In this paper we demonstrate the separation and quantitation of polyprenols and dolichols by adsorption (straight-phase) HPLC. We show that the broadened peaks observed for each compound are due to partial fractionation of the individual isoprenolog species. In addition, by employing this technique, we show that commercial laboratory rat chow contains considerable amounts of both polyprenols and dolichols.

EXPERIMENTAL

Materials and methods

Solanesol and pig liver dolichol were purchased from Sigma. Individual isoprenologs of dolichol were prepared by semipreparative HPLC as described below. Polyprenol-16 (hexadecaprenol) and polyprenol-19 (nonadecaprenol) were from Calbiochem. Ficaprenol was prepared from leaves of *Ficus elastica* according to Burgos *et al.*[7]. Pinaprenol (a mixture of polyprenols ranging in length from fourteen to eighteen isoprenes) was prepared from *Pinus elliottii* by a similar procedure. α-*trans*-Polyprenol-16, rat chow prenol, and dolichol-11 were prepared according to Keller *et al.*[3]. Geraniol and nerol were from Tridom/Fluka. Citronellol was from Pfaltz and Bauer, Inc.

High-performance liquid chromatography

HPLC was carried out at room temperature on a Laboratory Data Control Constametric II chromatograph equipped with a variable wavelength detector. UV monitoring was at 210 nm. UV signals were integrated on-line with a Perkin-Elmer Minigrator. Straight-phase (adsorption) chromatography was performed at a flow-rate of 2 ml/min on a 25-cm Whatman Partisil-5 column using mixtures of diethyl

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ether in hexane as described in the figure legends. Reversed-phase chromatography was carried out on a 25-cm Whatman Partisil-5 ODS column using a mobile phase of isopropanol–methanol (1:1 v/v) and a flow-rate of 1.5 ml/min.

RESULTS AND DISCUSSION

Initial experiments on the chromatographic behavior of prenols used the model compounds nerol, which has an α-*cis*-isoprene unit, geraniol, which is α-*trans*, and citronellol, which has a saturated α-isoprene unit. All three compounds have the same chain length. Fig. 1 shows the separation of these three compounds on straight-phase HPLC. The elution order nerol, citronellol, geraniol suggests that the bulky hydrocarbon group *cis* to the alcohol moiety sterically hinders association of nerol with the silica support, leading to a relatively early elution time. Apparently the steric effect overcomes the increased polarity due to the double bond. Steric hindrance is apparently not a factor in the association of the hydroxyl groups of citronellol and geraniol with the support and thus these two compounds fractionate according to their respective polarities.

When polyprenols and dolichols were tested on straight-phase HPLC, the same order of elution was found. As shown in Fig. 2, the naturally occurring α-*cis*-polyprenol-16 emerged earlier than dolichol-16, which in turn emerged earlier than synthetically prepared α-*trans*-polyprenol-16. The baseline separation of the *cis*-polyprenols and dolichols allowed us to monitor purity of these compounds during the chemical conversion of polyprenols to dolichols. Detector response was linear from 0.5 to 50 μg.

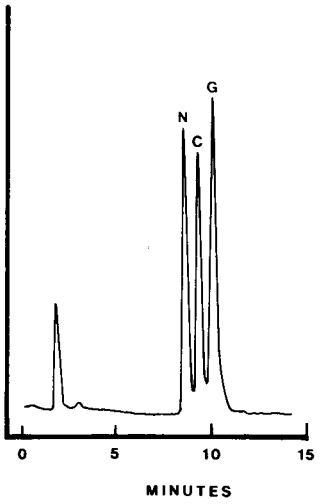

Fig. 1. Straight-phase HPLC of nerol (N), citronellol (C), and geraniol (G). The mobile phase employed was hexane–diethyl ether (90:10).

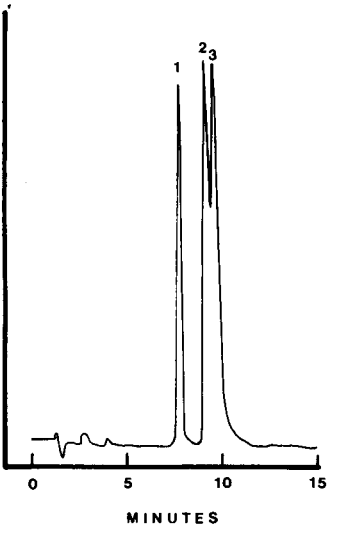

Fig. 2. Straight-phase HPLC of α-*cis*-polyprenol-16 (1), dolichol-16 (2) and α-*trans*-polyprenol-16 (3). The mobile phase was hexane–diethyl ether (93:7).

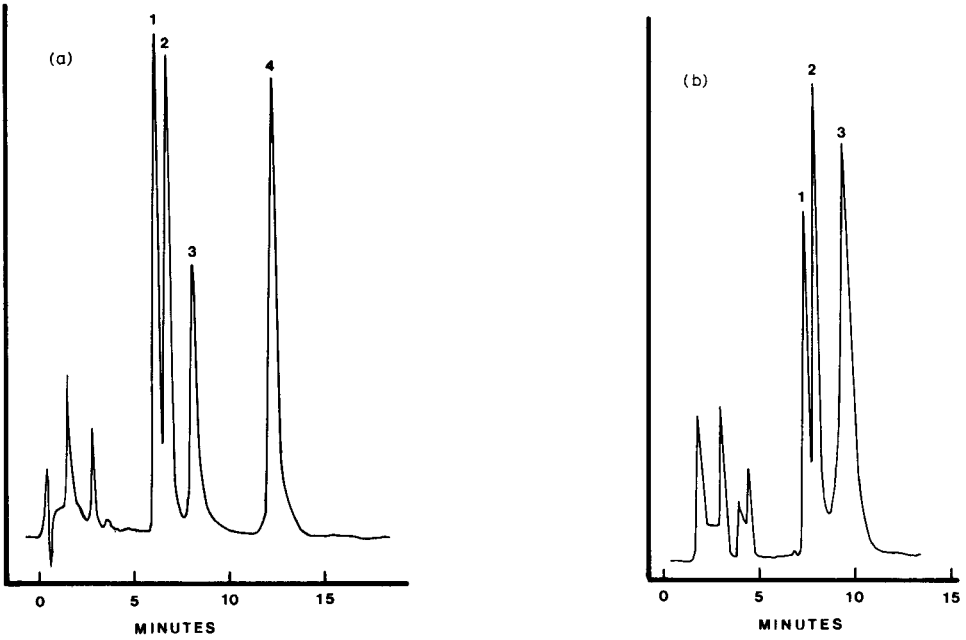

Fig. 3. Straight-phase HPLC of: (a) polyprenol-19 (1), polyprenol-16 (2), ficaprenol (polyprenol-11) (3) and solanesol (4); (b) dolichol-20 (1), dolichol-17 (2), and dolichol-11 (3). The mobile phase was hexane–diethyl ether (93:7).

Straight-phase HPLC of pig liver dolichol, containing a mixture of isoprenologs, gave a broadened peak compared with that of pure dolichol-16 or -19. Similar results were obtained with a polyprenol fraction from pine needles. These results suggested that partial fractionation of prenols into their individual isoprenolog compounds was taking place. To examine this possibility, dolichols and polyprenols of defined chain length were chromatographed. The results show that significant separation of isoprenologs is achieved for each class of prenol (Fig. 3). When the relative elution times were plotted as a function of isoprene number, a linear relationship was obtained (Fig. 4). Interestingly, all-*trans*-solanesol (nine isoprenes) eluted considerably later than would have been predicted based on chain length and the effect of the α-*trans* isoprene. Apparently, internal *trans* residues can also contribute to a lengthened retention time. Although the 25-cm straight-phase column that we have employed was not able to resolve prenols differing by a single isoprene, it may be possible to achieve such separation using tandem columns. However, for the purpose of quantitation, it is convenient to obtain a single broad peak rather than a a resolved isoprenolog pattern since, in the latter case, each peak must be integrated and summed to give the total. Indeed, it is for this reason that straight-phase HPLC is preferable to the reversed-phase mode in simplicity of quantitation.

The ability to resolve polyprenols and dolichols on straight-phase HPLC proved useful in examining the prenol content of commercial laboratory rat chow. Fig. 5 shows that chow contains polyprenols and dolichols in a ratio of 1:4. Quantitation of these pea,s indicates that 11.5 μg prenols are present per g of chow. By

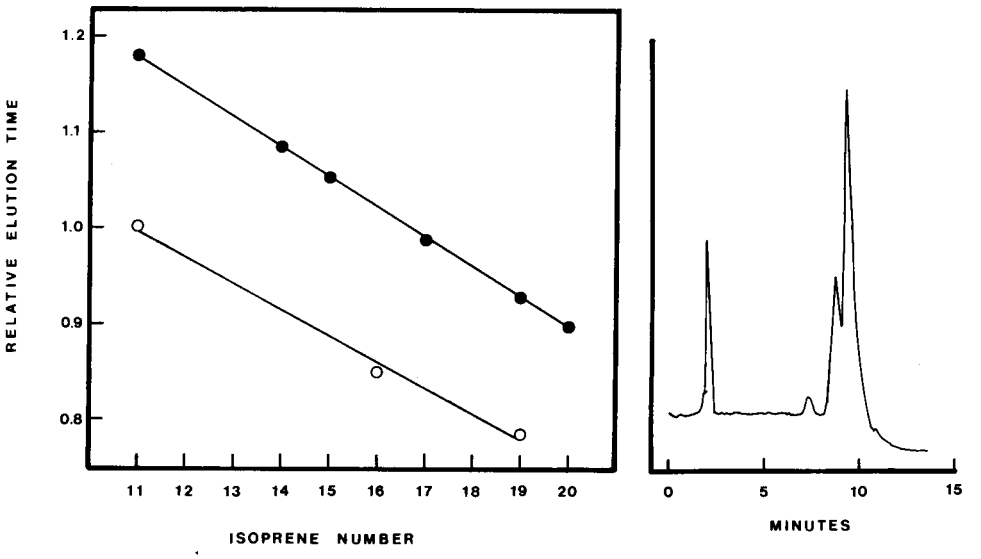

Fig. 4. Plot of relative elution time *vs.* isoprene number for dolichols (●) and polyprenols (○). Data are plotted relative to the elution time of ficaprenol, which was added as an internal standard in all experiments.

Fig. 5. Straight-phase HPLC of high-molecular-weight prenol fraction from rat chow. A high-molecular-weight prenol fraction was prepared using Fractogel 6000[8] and subjected to straight-phase HPLC in hexane–diethyl ether (93:7).

reversed-phase HPLC, the dolichols and polyprenols were found to contain isoprenologs ranging from thirteen to eighteen isoprenes in length[3].

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Note

Hydrophobic chromatography of 28S and 18S ribosomal RNAs on a nitrocellulose column

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It is known that nitrocellulose interacts specifically with single-stranded DNA, poly(A) + mRNA and viral RNAs (for reviews, see refs. 1–3). However, there is little information on the retention of rRNAs on a nitrocellulose column. We have observed that 28S rRNA may be selectively retained on a nitrocellulose column at high ionic strength. Thus, at a sodium chloride concentration of approximately 0.8 *M*, it was possible to achieve a clear separation of 18S polymer from 28S rRNA. 28S rRNA undergoes reversible adsorption, possibly associated with thermal gelation. Under these conditions 18S rRNA elutes in the void volume of the nitrocellulose column.

EXPERIMENTAL

RNA was isolated from liver microsomes of the Novi Sad variant of Wistar rats, as described previously[4], and freed from 4–6S RNAs and other contaminants by precipitation with 1 *M* sodium chloride solution[5]. Poly(A) + mRNA are separated from 28S and 18S rRNAs using poly(U) Sepharose, as described previously[6]. The solution of purified rRNAs in buffer A containing 0.8 M sodium chloride was kept frozen until used [buffer A: 0.1 % sodium dodecyl sulphate (SDS)–0.0025 *M* EDTA, disodium salt, pH 7.5–0.02 *M* Tris-HCl, pH 7.5]. Buffer A was kept at room temperature until used, in order to prevent precipitation of the SDS.

Nitrocel S (Serva, Heidelberg, G.F.R.) was packed in jacketed glass columns; 10–50 ml beds were used with 2–10 mg of RNAs. The columns were carefully equilibrated with 0.8 *M* sodium chloride solution in buffer A before packing. All column operations were carried out at temperatures above 21°C because of the tendency of the SDS to salt out at the relatively high salt concentration employed. The concentration of rRNAs in the range 2–5 mg/ml did not influence the separation. Elution was carried out at 25°C, with a flow-rate of 20–25 ml/h per 100-ml column. Higher temperatures resulted in elution of increasing amounts of 28S rRNA together with 18S rRNA. After elution of 18S rRNA in the void volume of the nitrocellulose column, 28S rRNA was eluted by changing the concentration of sodium chloride in buffer A to 0.1 *M*. Slightly more than one column volume of this buffer was needed for complete elution of the larger rRNA. The nucleic acids were recovered from the eluates by ethanol–ether diethyl concentration and precipitation[4]. The average recovery of the total A_{260} absorbing material applied to the column was 97–99 %. In

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addition, we did not detect UV-absorbing material eluted with 0.1 *M* sodium hydroxide solution after the chromatography of RNAs.

Electrophoresis was performed in 1.25% agar gels prepared with phosphate–citrate buffer (pH 8.0) as described by Tsanev[7]. The electrophoretic bands in the dry agar film were located by direct spectrophotometric analysis at 260 nm.

The nucleotide compositions were determined by the method of Petrović and Brkić[8].

RESULTS AND DISCUSSION

Fig. 1a shows the standard separation of rRNAs using a nitrocellulose column. The electrophoretic mobilities of rRNAs isolated from peaks A and B in Fig. 1 a are shown in Fig. 1b. This analysis suggests that the electrophoretic mobilities of peak A and peak B RNA are similar to those of cytoplasmic 18S and 28S RNA, respectively. Cross-contamination was not observed. The ratio of 28S rRNA to 18S rRNA was in the range 2.15–2.2 (Fig. 1a), which is in agreement with the ratio of their molecular weights and suggests their total separation. The nucleotide composition revealed significant differences between the two isolated rRNAs, especially in overall G–C content (Table I). In this respect the nucleotide composition of rRNA isolated by nitrocellulose chromatography gives results similar to those of Hirsch[9].

The methods most commonly used for the separation of rRNAs are density gradient centrifugation[10] and agar or acrylamide gel electrophoresis[7,11]. Although both provide good separations, they have limitations and are not easily used for the

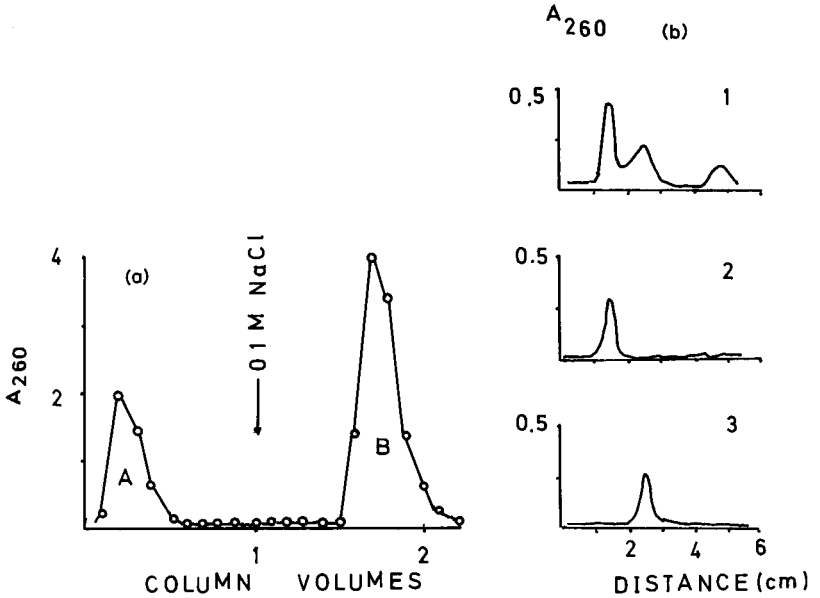

Fig. 1. (a) Fractionation of 3.1 mg of rat liver 28S and 18S mixtures on a 50-ml bed of Nitrocel S. Elution with 0.8 *M* NaCl in SDS–Tris–EDTA buffer (see text) and then with 0.1 *M* NaCl in the same buffer. (b) Electrophoretic mobility of diethyl ether–ethanol precipitation of 28S and 18S rRNAs, separated by nitrocellulose chromatography run as in (a). Electrophoretic patterns of rRNAs isolated from peak A (3) and peak B (2) from Fig. 1 left, compared with cytoplasmic rat liver rRNAs (1).

TABLE I

NUCLEOTIDE COMPOSITION OF rRNAs ISOLATED BY NITROCELLULOSE CHROMATOGRAPHY

The data are algebraic means of four determinations. Numbers in parentheses are standard deviations.

RNA	*Amount (moles per 100 moles of nucleotide)*				*G + C (mole-%)*
	Cytidylic acid	*Adenylic acid*	*Uridylic acid*	*Guanylic acid*	
18S	27.5 (1.1)	24.8 (1.5)	21.5 (1.3)	27.1 (1.8)	56
28S	32.1 (1.8)	18.9 (1.4)	17.1 (1.7)	31.2 (1.2)	64

separation of large amounts of material. Other methods have been reported that have not achieved the same popularity, including column chromatography on methylated albumin on Kieselguhr[12], reversed-phase chromatography[13] and selective immobilization of rRNA on agarose[14] or agar[4] gel chromatography at high ionic strength. The nitrocellulose chromatography of rRNAs has same advantages in comparison with agarose or agar chromatography: (1) nitrocellulose columns give higher and constant flow-rates; (2) the flow-rate does not decrease with increasing concentration of RNA as it does in gel chromatography; (3) the nitrocellulose column does not contain UV-absorbing material, whereas in gel chromatography traces of UV-absorbing material owing to resin destruction and resin pigments; (4) 18S rRNA is eluted in the void volume of the nitrocellulose column, so that 28S rRNA may be eluted immediately; this is not so in gel chromatography, where a molecular sieving phenomenon exists (18S rRNA has a K_d value in the range 0.55–0.65). This is why the nitrocellulose column is faster.

Experiments at elevated temperatures revealed that the adsorption of the larger rRNA progressively decreased as the temperature increased. At 40°C nearly all rRNAs eluted within the void volume. This observation seems to support the idea that the established separation results from preferential thermal-dependent gelation of the larger rRNA under conditions of high ionic strength.

In conclusion, this paper has presented a potentially useful method for the rapid separation the two rRNA species found in eucaryotic ribosomes.

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Note

Isocratic reversed-phase high-performance liquid chromatographic separation of underivatised tyrosine-related peptides of thymopoietin$_{32-36}$ pentapeptide

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Thymopoietin is a immunoregulatory thymic hormone whose 49 amino acid sequence is known[1]. This polypeptide hormone has been shown to influence prothymocyte to thymocyte differentiation[2,3] as well as the functioning of peripheral cells involved in immunity[4,5]. A pentapeptide (TP5), comprising the amino acid residues 32–36 of thymopoietin, was shown to have the biological properties of the parent thymic hormone both *in vitro* and *in vivo*[6]. This pentapeptide (NH_2-Arg-Lys-Asp-Val-Tyr-COOH) is currently under pharmaceutical development and is being evaluated clinically for the treatment of rheumatoid arthritis[7].

Because of the low plasma levels of TP5 anticipated following administration of a therapeutic dose of the pentapeptide, a tritiated peptide ([^{3}H]TP5, radiolabelled on the tyrosine residue) was synthesized to study the *in vitro* stability of the pentapeptide in human plasma as well as the disposition of the drug-related radioactivity in animals[8]. High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) were developed to assay for the [^{3}H]TP5 as well as to serve as a method to purify the labelled peptide from radioactive preparations. The TLC method, used in an *in vitro* stability study, was previously reported[8].

In this communication an HPLC method is described for the separation of underivatised tyrosine-related peptide and amino acid fragments of TP5 (*i.e.*, the pentapeptide, tetrapeptide, tripeptide, dipeptide, and tyrosine). The method utilizes a reversed-phase, ion-paired chromatography similar to that reported by Rivier[9]. Discussion of the pentapeptide stability and the resolution of the reference compounds under various chromatographic conditions is presented.

MATERIALS AND METHODS

Synthesis of the pentapeptide (TP5), tetrapeptide, tripeptide and dipeptide was performed by Peninsula Labs. (San Carlos, CA, U.S.A.). The amino acid L-tyrosine was obtained from Calbiochem (San Diego, CA, U.S.A.). Structures of these compounds are given in Fig. 1.

High-performance liquid chromatographic system

A Model ALC 202/401 HPLC system (Waters Assoc., Milford, MA, U.S.A.) operated at ambient temperatures, equipped with Model M-6000A pumps, a Model

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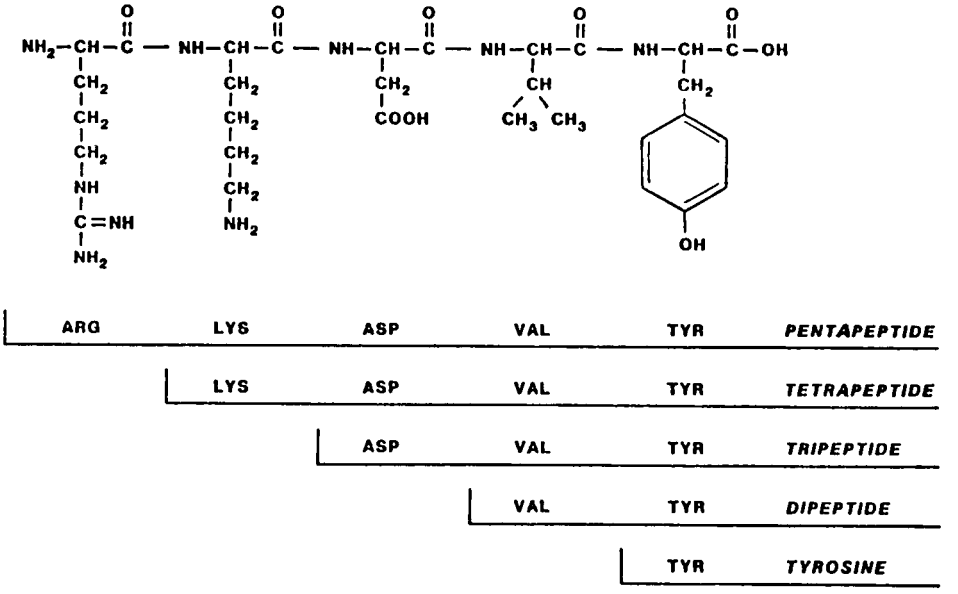

Fig. 1. Structures of the reference compounds.

440 absorbance detector (280 nm) and a U6K septumless injector, was employed. Chromatograms were traced on a dual-pen strip chart recorder (Linear Model 391, Linear Instruments, Irvine, CA, U.S.A.) maintained at a speed of 6 in./h.

Column and solvent system

Separation of all the tyrosine-containing reference compounds was achieved using a 1 ft. × 1/4 in. O.D. stainless-steel column with chemically bonded μBondapak C_{18} reversed-phase support (Waters Assoc.) and an aqueous triethylammonium phosphate (TEAP)–acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A.) (96:4, v/v) mobile phase, 1 ml/min flow-rate. The TEAP buffers (0.13–0.002 *M* phosphate) were prepared by diluting phosphoric acid (85%) with distilled water to give the appropriate molarity of phosphate. The pH of the solution was then adjusted by addition of triethylamine (reagent grade, Mallinkrodt, St. Louis, MO, U.S.A.). Retention times of the reference compounds were determined with the Model 3352B computer (Hewlett-Packard, Avondale, PA, U.S.A.), and quantitation of TP5 was achieved using peak-area integration performed by the same computer.

Stability of the pentapeptide under the conditions used to chromatograph the compound was evaluated by recycling the TP5 peak eluting from the column. The resulting chromatograms of the recycled TP5 peaks were then examined for signs of instability, such as shouldering or the appearance of secondary peaks.

Calibration curves were generated which compared detector response (computer integration peak areas) to the amount of TP5 applied to the HPLC system (microlitres of a stock 1 mg/ml TP5 aqueous solution).

RESULTS AND DISCUSSION

HPLC separations of peptides based on ion exchange, gel permeation, reversed-phase and affinity chromatographies have been reviewed[10]. The isocratic HPLC method described in this report for the separation of underivatised tyrosine-related compounds of TP5 was developed for use in the analysis and purification of radiolabeled TP5 (tritiated in the tyrosine residue). The method employs reversed-phase paired-ion methodology reported by Rivier[9]. Specifically, a μBondapak C_{18}

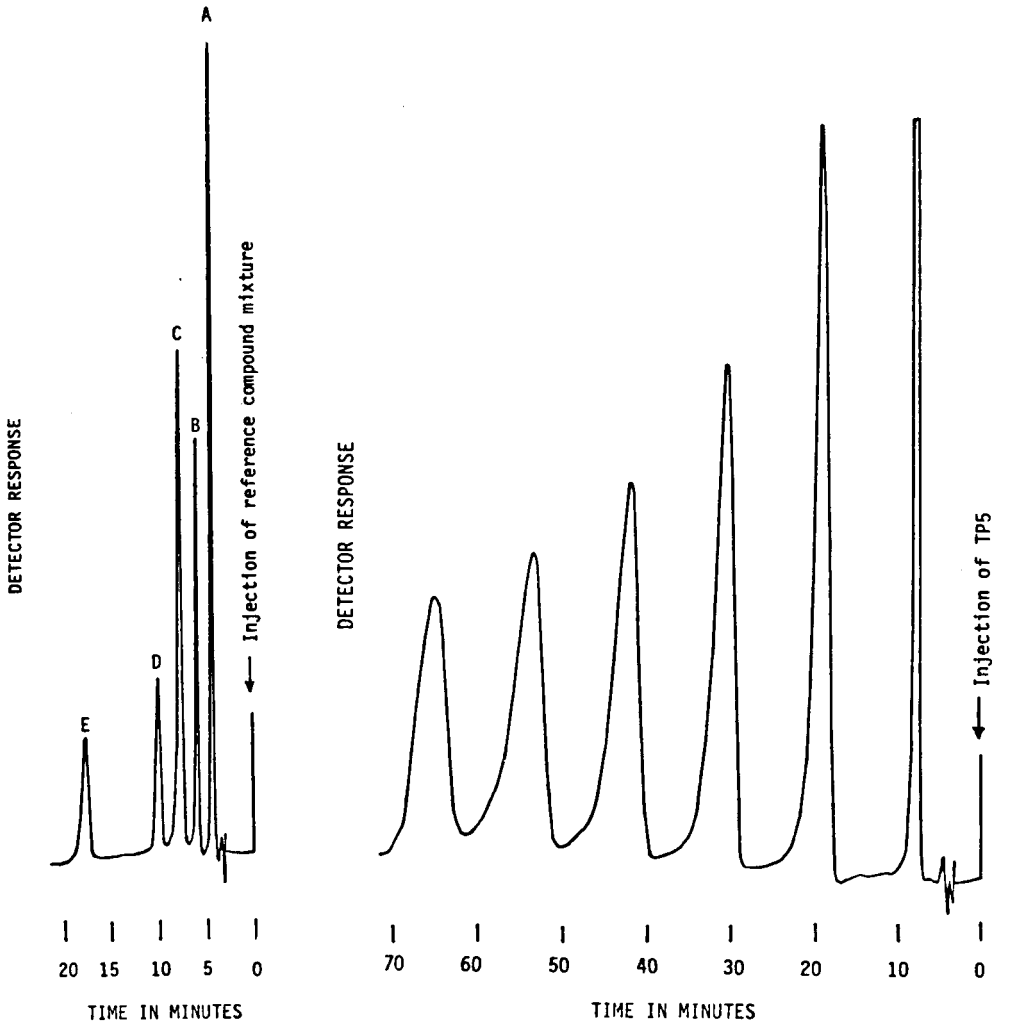

Fig. 2. Detector response *versus* time. HPLC method (μBondapak C_{18} reversed-phase support with 0.08 *M* TEAP (pH 4.0)–acetonitrile, (96:4 v/v) mobile phase) where the pentapeptide, tetrapeptide, tripeptide, dipeptide and tyrosine are peaks C, D, E, B and A, respectively.

Fig. 3. Detector response *versus* time. Chromatogram of HPLC method (μBondapak C_{18} reversed-phase support with 0.08 *M* TEAP (pH 4.0)–acetonitrile (96:4, v/v) mobile phase) recycling eluate containing TP5.

reversed-phase support and a triethylammonium phosphate (TEAP) buffer–acetonitrile mixture (96:4, v/v) as the mobile phase were employed.

Separation of reference compounds

Resolution of the pentapeptide, tetrapeptide, tripeptide, dipeptide and tyrosine was obtained with several different molar phosphate buffer concentrations. A representative separation is shown in Fig. 2 (0.08 *M* TEAP, pH 4.0–acetonitrile (96:4 v/v) mobile phase and μBondapak C_{18} reversed-phase support). The retention times (in minutes) of the pentapeptide, tetrapeptide, tripeptide, dipeptide and tyrosine were 7.5, 9.8, 17.5, 5.9 and 4.3, respectively. Similar chromatograms were generated with the 0.03 *M* and 0.02 *M* TEAP (pH 4.0) buffer systems. The remaining pH 4.0 phosphate buffers (*i.e.* the 0.13 *M* and 0.002 *M* TEAP) did not adequately separate the

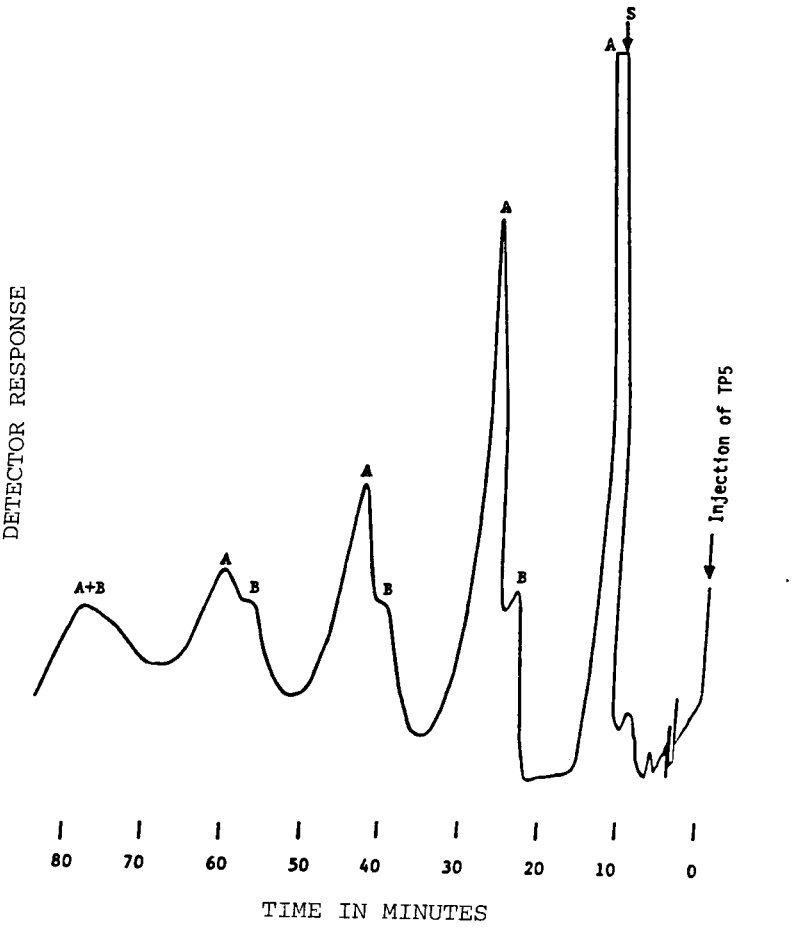

Fig. 4. Detector response *versus* time. Chromatogram represents the recycling of eluate containing TP5 (peak A) using HPLC method, μBondapak C_{18} reversed-phase support with 0.02 *M* TEAP (pH 6.7)–acetonitrile (96:4, v/v) mobile phase. Peak B is "apparent degradation product" and S indicates start of recycling mode of HPLC.

reference compounds. The elution sequence, however, was maintained in the pH 4.0 TEAP mobile phase systems over the entire molarity range investigated (0.13 *M* to 0.002 *M* phosphate): tyrosine (first), dipeptide, pentapeptide, tetrapeptide and tripeptide (last).

Stability

The procedure of recycling the HPLC eluate containing the compound of interest to evaluate the stability of the compound under chromatographic conditions[11] was employed in this study. The stability of the pentapeptide at pH 4.0 (0.13 *M* to 0.002 *M* TEAP) buffer conditions was demonstrated by the lack of secondary peaks of shouldering appearing in the chromatograms upon recycling the eluate

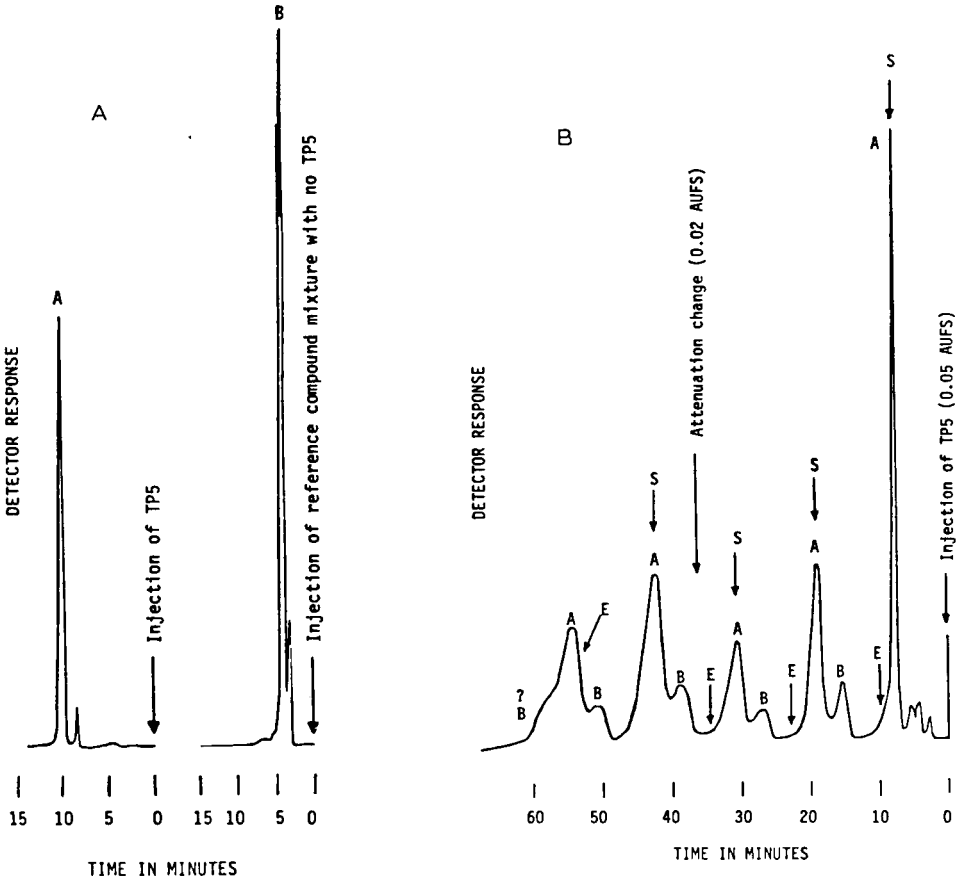

Fig. 5. A, Detector response *versus* time where peak A represents TP5 (15 μg) and peak B represents a mixture of the tetrapeptide, tripeptide, dipeptide and tyrosine using a second HPLC system (0.005 *M* ammonium acetate, pH 6.7, mobile phase and a μBondapak CN reversed-phase support). B, Detector response *versus* time (HPLC method: μBondapak CN reversed-phase support with 0.005 *M* ammonium acetate, pH 6.7, mobile phase). Chromatogram represents recycling of eluate containing TP5 (peak A), where B is "apparent degredation product(s)" of TP5, S indicates start of recycle mode and E indicates end of recycle mode of HPLC.

containing TP5 (Fig. 3 illustrates a representative chromatogram of a TP5 recycle run).

Interestingly, other HPLC systems, where adequate separation of all the tyrosine-related compounds was not demonstrated, exhibited conditions in which TP5 appeared unstable. For example, under 0.02 *M* and 0.002 *M* TEAP buffer (but not at 0.13 *M*, 0.08 *M* or 0.03 *M* TEAP) pH 6.7–acetonitrile (96:4, v/v) and C_{18} reversed-phase conditions, obvious shoulder patterns of the TP5 peak and secondary peaks were generated upon recycling the HPLC eluate containing the pentapeptide (see Fig. 4). Also, a similar phenomenon was exhibited with a μBondapak CN reversed-phase support and a 0.005 *M* ammonium acetate (pH 6.7) mobile phase HPLC system (see Fig. 5B) in which TP5 was adequately separated from the other unresolved reference compounds (Fig. 5A). This shouldering and secondary peak formation observed upon recycling the HPLC eluate containing TP5 suggest instability of the pentapeptide under low ionic strength and/or neutral pH chromatographic conditions. Alternatively, a mixture of retention mechanisms, as suggested by Hancock *et al.*[12], such that simulataneous action of two retention mechanisms, ion exchange and ion pair formation, may be causing the multiple peaks for a single compound upon recycling. In addition, the appearance of multiple peaks has been interpreted as being

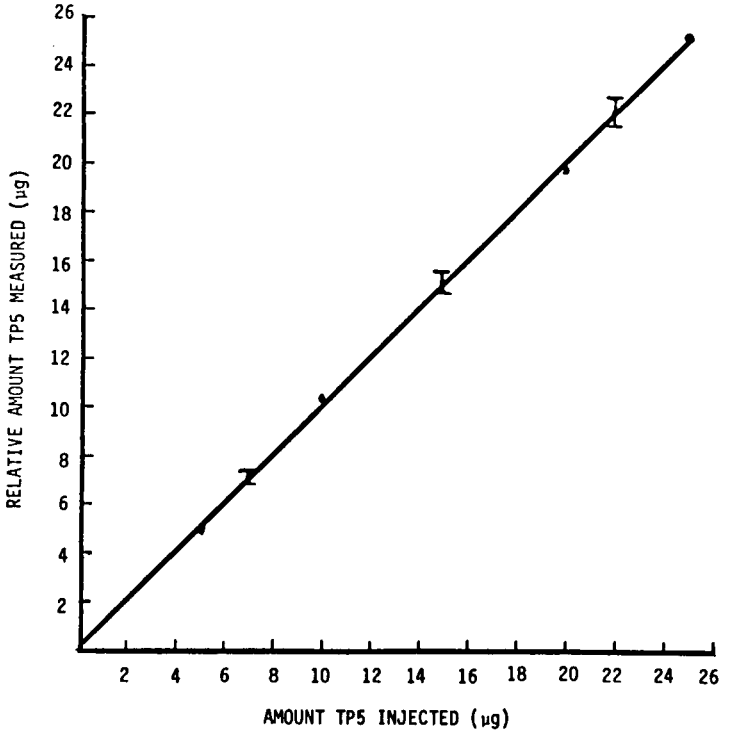

Fig. 6. Pentapeptide calibration curve (computer integrated peak area in relative micrograms measured *versus* micrograms of pentapeptide injected) generated at 0.08 *M* TEAP (pH 4.0)–acetonitrile (96:4, v/v) on a μBondapak C_{18} support. Linear regression line is $y = 0.996x + 0.226$ with a 0.9990 correlation coefficient.

a consequence of polymeric complexes formed by ionic or hydrogen bonds of the peptides (or amino acids)[13]. Whatever the reason for the multiple peak phenomenon, improved chromatographic performance regarding precision and accuracy was attained by increasing the ionic strength and/or decreasing the pH of the mobile phase.

Linearity

A calibration curve for TP5, with 0.08 *M* TEAP (pH 4.0)–acetonitrile (96:4, v/v) used as the mobile phase, is shown in Fig. 6. (*N.B.* $n = 1$ for 5, 10, 20 and 25 μl injections; $n = 5$ for 7 and 15 μl injections; and $n = 3$ for 22 μl injection.) Linearity was demonstrated from 5 to 25 μg TP5 with a correlation coefficient of 0.9990 and a calculated linear regression of $y = 0.996x + 0.226$.

CONCLUSION

An HPLC method is described for the separation of the related tyrosine-containing compounds of TP5. The method employs reversed-phase paired-ion chromatography under isocratic conditions. Linearity of sample recovery over the range 5–25 μg of TP5 was demonstrated by monitoring at 280 nm. In addition, increasing the ionic strength and/or decreasing the pH of the mobile phase improved the stability of the pentapeptide under the chromatographic conditions employed.

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Note

Use of ion-paired, reversed-phase thin-layer chromatography for the analysis of peptides

A simple procedure for the monitoring of preparative reversed-phase high-performance liquid chromatography

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(Received October 5th, 1981)

Recent studies have demonstrated that the use of C_{18}-microparticulate silica, which is packed in flexible-walled cartridges that can be subjected to radial compression, allows the rapid and effective separation of peptide and protein mixtures[1–6]. One problem with these studies was the lack of a suitable thin-layer chromatographic (TLC) system for the monitoring of the separation. However, a number of reversed-phase TLC plates have recently become available and used for the analysis of phenols[7], benzo[*a*]pyrene metabolites[8], dyes and plant pigments[9], cephalosporin intermediates[10], bile acids[11], peptides[12–15] and amino acids[16–17]. The purpose of this study was therefore to develop a reversed-phase TLC system which allowed the efficient anlysis of high-performance liquid chromatographic (HPLC) fractions. The system described here uses Whatman $KC_{18}F$ plates with a mobile phase which contained an aqueous solution of sodium chloride (3%) and sodium dodecylsulphate (SDS, 0.2%)–acetonitrile–methanol (50:10:10, apparent pH = 2.50).

EXPERIMENTAL

Materials

The solvents used were purified as described previously[1] and the peptides studied were obtained from the sources described in Table I. The octadecapeptide was prepared by standard solid phase synthetic techniques used in this laboratory[19] The Whatman $KC_{18}F$ reversed-phase TLC plates were obtained from Whatman (Clifton, NY, U.S.A.).

TLC procedures

The TLC plates were unwrapped just prior to spotting samples, and the chromatography tanks were allowed to equilibrate in the solvent for 30–60 min prior to the development of the chromatogram. For impregnation the reversed-phase plates were immersed in ethanol–solvent II (1:1) for 30 sec and then dried overnight at room temperature or in an oven at 80°C for 5 min.

Peptides were dissolved in 50% acetic acid at a concentration of approximately

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10 mg/ml and small spots of a maximum 1.5 mm diameter (usually 0.3 μl) were introduced on the plate. After drying in a stream of air at room temperature, the plates were developed over a distance of 50 mm from the origin. The plates were then dried in an oven at 80°C, sprayed with a ninhydrin solution (1 % w/v, acetone–acetic acid, 96:4) and heated again to develop the colour. The resulting spots were of a good shape and compact (usually 3 mm diameter at an R_F of 0.5).

Solvent systems

I: water (50 ml), NaCl (1.5 g), SDS (0.1 g), acetonitrile (10 ml) and methanol (10 ml). To this mixture acetic acid was added to adjust the apparent pH to 2.5.

II: same composition as solvent I except that the amount of acetonitrile was increased to 20 ml.

III: same composition as solvent I except that the amounts of both acetonitrile and methanol were increased to 20 ml.

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used, which consisted of two M6000A solvent delivery units, an M660 solvent programmer and a

TABLE I

THE ANALYSIS OF PEPTIDES BY REVERSED-PHASE TLC

*Peptide**	R_F *in solvent***		
	I	*II*	*III*
Dipeptides			
Arg–Phe[1]	0.42		
Leu–Trp[1]	0.16	0.28	0.43
Phe–Ala[1]	0.54		
Gly–Phe[2]	0.52		
Tripeptides			
Arg–Phe–Ala[1]	0.44		
Leu–Trp–Met[1]	0.08	0.2	0.32
Met–Arg–Phe[1]	0.26	0.42	0.50
Gly–Leu–Ala[3]	0.50		
Gly–Leu–Tyr[4]	0.40		
Tetrapeptides			
Leu–Trp–Met–Arg[2]	0.12	0.30	0.43
Met–Arg–Phe–Ala[2]	0.26	0.42	0.52
Pentapeptide			
Leu–Trp–Met–Arg–Phe[2]	0.01	0.12	0.16
Octapeptide			
Phe–Val–Gln–Trp–Leu–Met–Asp–Thr[5]	0.00	0.13	0.16

* The peptides were obtained from the following suppliers: 1, Research Plus Lab. (Denville, NJ, U.S.A.); 2, BDH (Poole, Great Britain); 3, Nutritional Biochemicals (Cleveland, OH, U.S.A.); 4, Sigma (St. Louis, MO, U.S.A.); 5, Mann Research Labs. (New York, NY, U.S.A.).

** The TLC system used is described in the Methods section.

U6K universal liquid chromatograph injector, coupled to a Model RCM-100 Radial Compression Module equipped with a Radial-PAK-C_{18} cartridge. This system was coupled to an M450 variable-wavelength UV spectrophotometer (Waters Assoc.) equipped with a 1-cm flow-through cell, and an Omniscribe two-channel recorder (Houston Instruments, Austin, TX, U.S.A.). The sample was injected using a Microliter 825 syringe (Hamilton, Reno, NV, U.S.A.).

RESULTS AND DISCUSSION

Brinkman and De Vries[18] found that Whatman $KC_{18}F$ TLC plates developed rapidly with aqueous–organic solvent mixtures and, relative to other reversed-phase TLC plates, demonstrated superior wettability with mobile phases which contained more than 20–40% water. Also these authors found that mobile phase with proportions of water of over 40% must contain 3% NaCl to avoid flaking of the precoated reversed-phase layers. In our studies 3% NaCl was always added to the mobile phases, as it had been previously demonstrated that a high concentration of an electrolyte suppresses unwanted interactions between silanol groups present in the reversed-phase and ammonium groups present in the peptide[5].

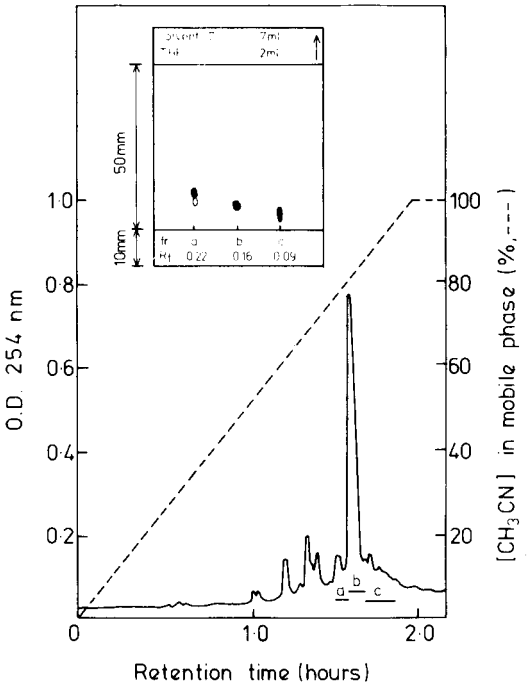

Fig. 1. The semipreparative purification of a synthetic octadecapeptide. Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp(CHO)–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala. The chromatographic conditions were described in the Methods section. Solvent A consisted of aqueous triethylammonium phosphate (1.5 m*M*, pH 3.2) and solvent B of propan-2-ol–acetonitrile–aqueous triethylammonium phosphate (7.5 m*M*), (40:40:20, v/v/v). The flow-rate was 1.0 ml/min and the gradient (A to B) shown by the dotted line was used. A 5-mg sample of the peptide dissolved in buffer A (0.2 ml) was used. The inset shows the corresponding reversed-phase TLC separation of the fractions.

Several authors[7,8,13,15] have found that a suitable hydrophobic ion-pairing reagent must be added to the mobile phase before polar samples can be satisfactorily chromatographed on a reversed-phase TLC plate. The ion-pairing reagent is particularly useful in reducing the mobility of polar solutes and in minimizing spreading of the spots. For example, Lepri *et al.*[7,15,16] used dodecylbenzenesulphonic acid in the TLC of amino acids and peptides. In this study we found that a significant improvement in the shape of the spots we obtained by the addition of SDS (0.2%) to the mobile phase, and by decreasing the apparent pH to 2.5. SDS was found to give better results than octylnaphthyl- or dodecylsulphonic acid or the triethylammonium phosphate that was used in the analytical reversed-phase HPLC separation shown in Fig. 1. At higher concentrations of SDS (up to 0.6%) a small decrease in R_F was observed, a result which agreed with concentration effects described by Lepri *et al.*[7]. The size of the spots was minimised and resolution was improved if the $KC_{18}F$ plates had been previously impregnated with the mobile phase (see Methods section).

The separation of a number of peptides with the TLC system is shown in Table I. As is often observed in reversed-phase HPLC[20], mobile phases which contained acetonitrile were found to give more efficient separations of peptides in reversed-phase TLC, than was achieved with methanol. As was found in earlier studies[15], the presence of hydrophobic and basic residues in the peptide lead to increased interaction of the sample with the stationary phase and thus lower R_F values. Conversely an increase in the amount of methanol or acetonitrile in the mobile phase (see solvent systems II and III in Table I) resulted in a higher R_F value.

Fig. 1 shows use of the reversed-phase TLC system to follow the semipreparative purification of a synthetic octadecapeptide, Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp(CHO)–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala. The TLC results provided a rapid check of the HPLC separation and also verified that the optical density peaks actually consisted of peptidic material (ninhydrin reactive). This peptide contained a large number of hydrophobic residues and therefore it was strongly retained on a reversed-phase TLC plate even with solvent III as the mobile phase. However, satisfactory results were obtained when tetrahydrofuran was added to the mobile phase (2 ml of THF to 7 ml of solvent II) and the narrow spots shown in Fig. 1 were obtained. Despite differences in the mobile phases there is a clear correlation between the HPLC and TLC systems, for example the early eluting material in pool A of the HPLC fractions gave the highest R_F in the reversed-phase TLC system.

In conclusion, it was found that a reversed-phase TLC system based on Whatman $KC_{18}F$ plates and a mobile phase which contained 3% sodium chloride and 0.2% sodium dodecylsulphate, allowed the efficient separation of both peptide standards and fractions from a HPLC separation.

ACKNOWLEDGEMENTS

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Note

Thin-layer chromatographic method for the identification of natural polyisoprene and synthetic polyisoprene rubber

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(Received September 14th, 1981)

It has been reported[1,2] that natural rubber can be determined both qualitatively and quantitatively by means of identification of β-sitosterol via thin-layer chromatography (TLC), Refs. 1 and 2 gave a TLC method for a relatively simplified system of compound ingredients. Our method was applied to more complex commercial compounds such as tire compounds, and automobile motor mounts. Furthermore, it was stated[1] "that relative concentration of β-sitosterol expressed as the spot area per gram of natural rubber was reasonably constant, irrespective of the natural rubber grade...". For the following natural rubber grades: No. 1 SSR, No. 3 SSR, No. 5 SSR, SMR Hevea crumb 5-50 etc., this statement is correct; however, SMR 20 or SIR 20 grade, natural gum stock had a β-sitosterol concentration of 1.7 times less than the others. (This is explained by the fact that this gum stock is prepared differently from other grades of natural rubber, and in turn shows different physical characteristics.) Our studies developed a two-stage solvent system, in one dimension, as well as utilizing a different visualization agent. The plates are high-efficiency plates, HETLC-GHL made by Analtech (Newark, DE, U.S.A., Cat. No. 56077). This type of plate is of utmost importance.

The solvent system is carbon tetrachloride followed by a mixture of 100 ml of chloroform and 5 ml of acetone; visualization agent, 3 g of cupric acetate in 100 ml of 10% H_3PO_4. Blue-gray spots on a white background will appear for β-sitosterol, with no background interferences of other compounding ingredients. Fig. 1 shows a chromatogram of model compounds based on known and unknown tire compounds investigated by us. The spots above the β-sitosterol are the oils, antioxidants, antiozonants, waxes, accelerators, etc. The spots below are those of the different surfactants.

EXPERIMENTAL

Rubber compound samples of known composition (containing natural rubber), were thinly milled on a cold mill, then 5 g were analytically weighed and Soxhlet extracted for 16 h (overnight) with ACS-grade acetone. (All solvents used are ACS grade or better.) The acetone was evaporated on a low-heat hot-plate under the hood, and the last 10-ml portion of the acetone extractables were transferred carefully

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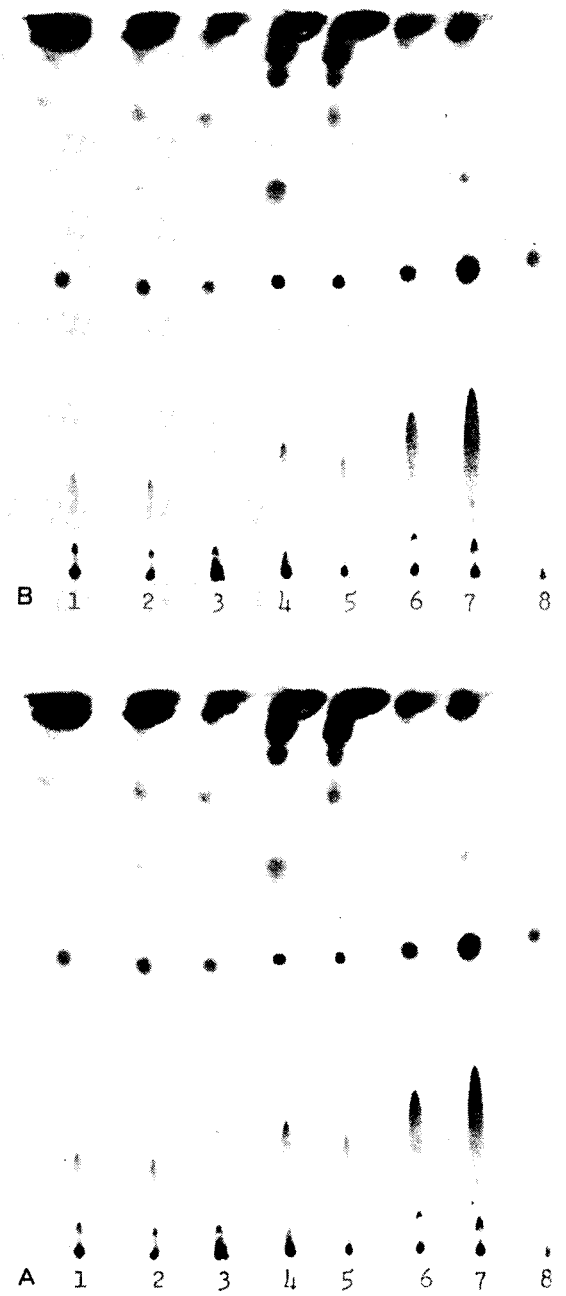


Fig. 1. A, Actual photocopy of plate, using bright light. B, Actual photocopy of plate, using intermediate light intensity (what the human eye actually sees on the plate). Spots: 1 = model based on No. 1 SSR rubber; 2 = Model based on No. 1 SSR different ratio of natural to synthetic; 3 = manufacturer No. 1, tire stock based on No. 1 SSR rubber; 4 = manufacturer No. 2, tire stock based on No. 1 SSR rubber; 5 = manufacturer No. 3, tire stock based on SMR 20 rubber; 6 = SMR 20; 7 = No. 1 SSR; 8 = β-sitosterol.

into a 20-ml glass vial and evaporated to complete dryness in an air oven at 75°C. The vial, after the acetone evaporation, was kept for 10 min at 105°C in the air oven. The vial was cooled, and exactly 5 ml of methylene chloride were added to the residue in the vial, the vial was capped, and then shaken to dissolve all the residue. Next, 2 μl of the extracted methylene chloride solution were spotted onto the HETLC-GHL plate (10 × 10 cm). This type of plate is one of the keys to the success of the analysis. The spotting is done 1 cm from the bottom of the plate, and at least 1.5 cm from the edge of the plate. We used TLC multi-spotter syringes (Analytical Instrument Specialties, Libertyville, IL, U.S.A.).

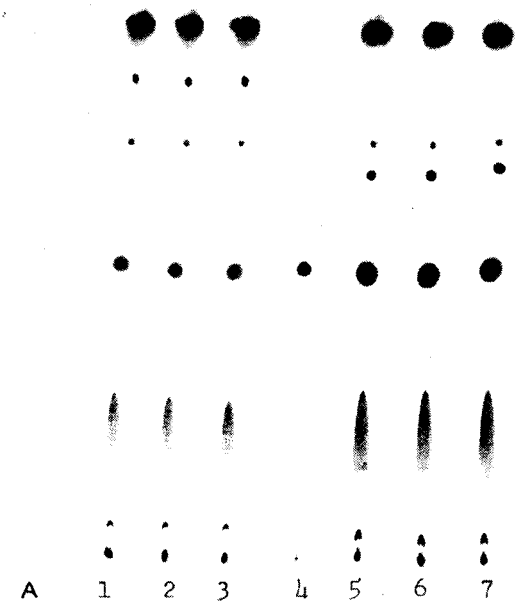
The plate is first developed in carbon tetrachloride. Let the solvent front reach the end of the plate. We always spot a pure β-sitosterol as a control. An efficiency developing chamber assembly, made by Analtech, Cat. No. 050A, was used. No saturating pads or filter paper were used, enough solvent was added so it just touched the bottom of the plates below the applied spots.

After the carbon tetrachloride reached the top of the plate, we removed the plate and let the solvent evaporate in an air oven at 105°C. We let the plate completely cool in the open air, and then developed the same plate in the same direction, in a solvent system consisting of 100 ml of chloroform and 5 ml of acetone mixed well and freshly prepared and introduced into the chamber 2 min prior to the insertion of the plate. After the solvent front reaches the top of the plate, the plate is removed and is dried in an air oven at 105°C. The plate, after cooling down enough to be handled by hand, is dipped for a few seconds, not sprayed, in a solution of 3 g of cupric acetate in 100 ml of 10% H_3PO_4. The plate is heated in an air oven for 10 min at 125°C. Blue-gray spots on a white background will appear for the β-sitosterol.

RESULTS AND DISCUSSION

Fig. 2 shows the TLC plate obtained for SMR 20 and/or SIR 20 gum stock not compounded, and No. 1 SSR gum stock not compounded. The naked eye can see the difference in the size of the β-sitosterol. In our laboratory, we also photocopied the plate and with a planimeter measured the area of the β-sitosterol spot. We compared these results with the density counts obtained on the same spots by a Joyce Loebl scanning densitometer, and in both cases the No. 1 SSR rubber had a 1.7 factor bigger than that of the SMR 20 or SIR 20 for the β-sitosterol. Before further discussing the results, I would like to emphasize the importance of the plates we were using. They contain inert inorganic binder (not inert organic binder) which is the key to the good separation, and the dipping technique that gives it a uniform layer of visualization reagent, with no flaking or washing off of the TLC layer.

In Fig. 1, we show the TLC plate of several tire compounds being manufactured by several different companies. We have determined the ratios of natural to synthetic polyisoprene in the rubber. The procedure was confirmed by the analysis via TLC of model compounds and comparing it to known formulations, using both No. 1 SSR stock and SMR 20 stock rubber. It should be mentioned that those model compounds as well as the unknown, contained natural rubber, synthetic polyisoprene, polybutadiene and SBR blends. The composition of the models were obtained using thermal gravimetric analysis (TGA), which gave us percent volatiles, percent polymer and percent carbon black and ash. The ratio of the polymer types was



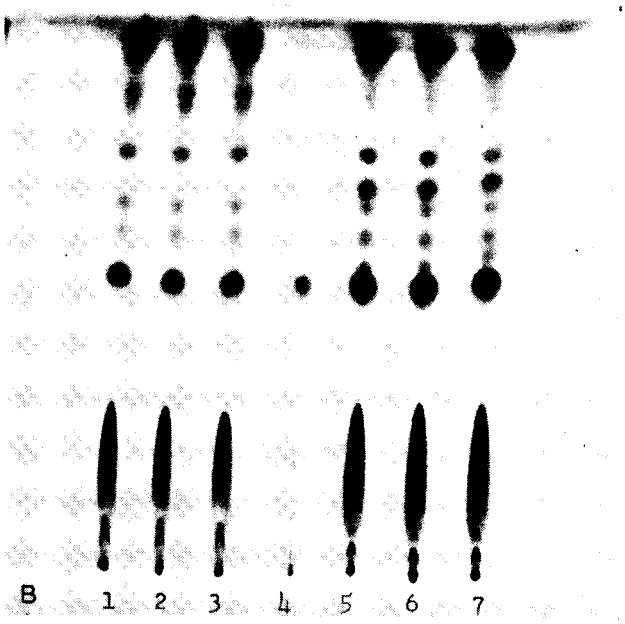


Fig. 2. A, Actual photocopy of plate, using bright light. B, Actual photocopy of plate, using intermediate light intensity (what the human eye actually sees on the plate). Spots: 1, 2, 3 = 2 μl of 5 g SMR 20 stock rubber extract in 5 ml of methylene chloride; 4 = 2 μl of 0.5% (w/w) β-sitosterol in methylene chloride; 5, 6, 7 = 2 μl of 5 g No. 1 SSR stock rubber extract in 5 ml of methylene chloride.

obtained using infrared analysis. Within the model, we have changed the ratio of natural polyisoprene to synthetic polyisoprene. Figure 1 shows extractables in the model (based on the unknowns). A typical formulation was found to be: natural polyisoprene; polybutadiene and SBR; carbon black; paraffinic type oil; paraffin wax; Santocure (an accelerator); Flectol-H (an antiozonant); Wingstay 300 (an antiozonant); stearic acid and rosin acid.

The model compounds were the basis for a calibration curve via β-sitosterol measurements. This paper emphasizes the qualitative aspect of the separation, but when the square root of the intensity of the spots obtained for β-sitosterol on the TLC plates was plotted *versus* $\log_{10}$ of the concentrations of natural rubber to synthetic, a straight line was obtained[3]. A Joyce Loebl densitometer was utilized to determine the intensity of the TLC spots. A TLC scanner utilizing our method no doubt will give better results for the quantitative measurement of natural rubber as a function of its β-sitosterol content.

ACKNOWLEDGEMENT

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Note

Separation and detection of Propamocarb by thin-layer chromatography*

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(First received June 10th, 1981; revised manuscript received September 8th, 1981)

Propamocarb, propyl-(3-dimethylaminopropyl) carbamate monohydrochloride, $[(CH_3)_2N–CH_2–CH_2–CH_2–NH–CO–O–C_3H_7 \cdot HCl]$, is a synthetic organic product belonging to the dialkylcarbamate class, recently introduced by Schering (Berlin/Bergkamen, G.F.R.) under code SN 66752. Its action characteristics make it particularly interesting for the control of horticultural and ornamental plant diseases caused by *Oomycetes*.

As with other carbamate substances, Propamocarb suffers from some analytical problems associated with molecular instability. Generally, the analytical procedures applicable are limited and spectrophotometry, gas chromatography and other techniques are not sufficiently responsive and/or specific in all instances. High-performance liquid chromatography (HPLC) seems to be more promising but, in absence of such sophisticated facilities, we have attempted to devise a thin-layer chromatographic (TLC) method that is simple, rapid and sufficiently sensitive for application to low Propamocarb levels. It is our intention to use this technique for the detection and determination of Propamocarb in plant tissues.

EXPERIMENTAL

Silica gel 60G plates (Merck), 200 μm thick, were used after heating in an oven at 110°C for 20 min. Six 3-cm wide tracks were marked on each plate and 100-μl volumes of different concentrations of the active ingredient (94% standard for analysis, supplied by courtesy of Schering) were deposited on the starting line at the centre of each track.

The plates were developed in a saturated chamber using as mobile phase the series of solvent systems listed in Table I. After development, the chromatograms were dried in air.

The active ingredient was located using the detection reagents and methods cited in Table II.

In detection method No. 6 a fluorescent TLC plate, obtained by adding 0.04% sodium fluoresceinate solution to the silica gel 60G, loses its fluorescence in the presence of the carbamate following exposure to bromine vapour. Because during

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development of the plate in the chosen solvent the sodium fluoresceinate is carried along by the solvent, thus making any further treatment pointless, we modified the method as follows: the sodium fluoresceinate solution (1 ml, 0.04 %) was added to the mobile phase and, after development, the plates were dried in air, sprayed with 20 % aluminium sulphate solution and exposed to bromine vapour. When viewed under ultraviolet light in these conditions, the compound appears as a blue spot against a yellow-green background.

The quantitation of Propamocarb on the chromatograms was accomplished in two ways:

(1) by measuring the absorbance of the coloured spots with a Quick Scan R & D densitometer (Helena Laboratories) at 420 nm;

(2) by using the linear relationship between the logarithm of the weight of compound in the spot and the square root of the spot area and then calculating the amount of product present by reference to a calibration graph (Fig. 1).

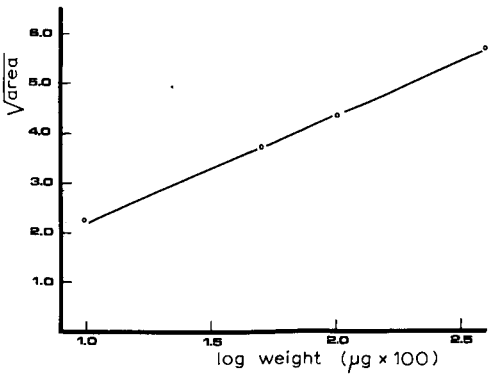

Fig. 1. Spot area *versus* weight relationship for Propamocarb.

Recovery procedure for vegetable samples

Pepper plant samples (20 g), to which 0.5–2.5 ml of 0.1 % Propamocarb monohydrochloride solution and 40 ml of 0.1 *N* hydrochloric acid were added, were ground in a Sorvall Omnimixer (J. Norwack, U.S.A.) for about 2 min. The slurry thus obtained was immediately filtered through cheese-cloth and the sample tissue was re-extracted in the same way. The combined filtrates were centrifuged at 14,000 *g* for 10 min and the acidic supernatant was transferred into a separating funnel and made alkaline with 10 *N* sodium hydroxide solution. Finally, the Propamocarb was extracted twice from this basic solution using 20-ml volumes of chloroform. The organic phase, concentrated to 10 ml in a rotary vacuum evaporator at 40°C, was used for analysis.

RESULTS AND DISCUSSION

The best solvent system among those tried for the separation of Propamocarb was methanol–25 % ammonia solution (30:1); the development time was about 45 min and the R_F value was 0.2 (Table I).

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF PROPAMOCARB USING VARIOUS SOLVENT SYSTEMS

Solvent system	*Development time (min)*	$R_F \times 100$
Methanol	59	7
Methanol–water (1:1)	125	14
Methanol–water (7:3)	105	13
Methanol–water (9:1)	75	7
Water–formic acid–methanol (4:1:5)	126	1
Water–acetone (6:4)	122	35
Methanol–25% ammonia solution (30:1)	45	16
Acetone	26	1
Methanol–diisopropyl ether (1:1)	68	4
Methanol–diisopropyl ether (1:4)	65	3
Hexane–acetone (9:1)	33	0
Hexane–acetone (7:3)	30	0
Chloroform	57	0
Chloroform–acetone (7:3)	47	0
Chloroform–acetone (9:1)	48	0
Diethyl ether	35	0

With the other solvents, either the development time was too long, the spots had tails and poor shapes or the R_F values were either too high or too low. The solvent system chosen was excellent even for the separation of the active ingredient from vegetable tissues as it avoids interferences resulting from co-extracted pigments, which have very different R_F values.

Regarding the detection of Propamocarb on the chromatograms, many of the reagents used gave negative results or showed poor sensitivity (Table II) for the product sought. Reagents 8 and 9 are highly sensitive, although not specific, with a

TABLE II

DETECTION OF PROPAMOCARB WITH VARIOUS REAGENTS

No.	*Method of detection*	*Minimal determinable amount (μg)*	*Colour*	*Colour stability (min)*
1	Rhodamine B–UV[1]	0.0	–	–
2	*p*-Dimethylaminobenzaldehyde[2]	0.0	–	–
3	Potassium permanganate[3]	0.0	–	–
4	Silver nitrate + bromophenol blue[4]	100	Blue against yellow background	>60
5	Pinacriptol yellow–UV[1]	10	Blue against fluorescent background	>60
6	Sodium fluoresceinate–UV[5]	10	Black against blue background	>60
7	Sodium fluoresceinate–UV (modified method)	5	Blue against yellow-green background	>60
8	Iodine vapour	0.01	Brown	<5
9	Iodine spray	0.01	Brown	<5

minimal determinable amount of 0.01 μg, but their action is reversible and as their coloration disappears in a few minutes the subsequent quantitative determination is difficult. Reagent 7, although not very sensitive (minimal determinable amount 5 μg), results in persistent coloration and hence allows subsequent determination without difficulty.

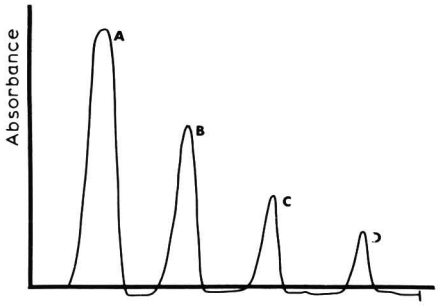

Fig. 2. Densitometric measurements after detection with iodine vapour (λ = 420 nm). Each peak represents a spot of propamocarb: A = 50 μg; B = 25 μg; C = 10 μg; D = 5 μg.

The densitometric analysis (Fig. 2) provided results that are comparable to those obtained by applying the linear relationship between the square root of the spot area and the weight of compound present in the spot.

By applying the procedure described above, the separation, detection and determination of the active ingredient in the vegetable tissue were achieved. The mean recovery (24 determinations) was 95.75% with a standard deviation of 2.25%.

ACKNOWLEDGEMENTS

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CHROM. 14,414

Letter to the Editor

Comment on the proposed R_Q transformation method for optimizing mobile phase composition in high-performance liquid chromatography

Sir,

Toon and Rowland have suggested recently[1,2] a simple graphical method for the rapid selection of an optimal mobile phase composition for the high-performance liquid chromatographic (HPLC) analysis of a multi-component mixture. If correct, their treatment has exciting implications not only in the design of, for example, optimal gradient elution systems, but also in the developing use of HPLC for providing solute physico-chemical data. However, a number of points arise from their treatment that require comment.

(1) Taking the suggested[1,2] transformation term R_Q as

$$R_Q = \log (1 - R_O/R_T) \tag{1}$$

where R_O and R_T are non-retained and retained solutes respectively, it follows that, as

$$R_Q = \log \left(\frac{R_T - R_O}{R_O} \cdot \frac{R_O}{R_T} \right) \tag{2}$$

then

$$R_Q = \log \left(\frac{k}{k+1} \right) \tag{3}$$

where k is the capacity factor, equal to $(R_T/R_O) - 1$. These deductions show that R_Q is always negative, which is at variance with Figs. 1, 3, 5 and 8 in ref. 1.

(2) Fig. 8 in ref. 1 is based on the data of Crechiolo and Hill[3] for thiamphenicol determined in an octadecylsilane HPLC system using aqueous methanol eluents, and purports to show how a found curvilinear relationship between log k and the percentage of organic modifier in the eluent can be linearized using the R_Q transformation. Assuming eqn. 1 to be correct, then Toon and Rowland[1,2] are suggesting a plot of log[log $(k/1+k)$] *versus* percentage composition as the normalization step. Least-squares regression of the data of Crechiolo and Hill[3] (Table I), according to log$(k/1+k)$ *versus* percentage composition, gives a correlation coefficient (r) of 0.994 whereas using log[log$(k/1+k)$], r = 0.955 [the sign of log$(k/1+k)$ has been reversed to accomplish regression].

(3) It therefore appears that the authors intend R_Q to be equal to $k/1+k$, which we shall call R_Q^*.

(4) R_Q^* cannot be 1.0 or greater, which means that the ordinate intercepts of R_Q^*

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TABLE I

HPLC RETENTION DATA FOR THIAMPHENICOL AND CHLORAMPHENICOL DETERMINED IN AN ODS/METHANOL–WATER SYSTEM (ESTIMATED FROM THE PAPER BY CRECHIOLO AND HILL[3])

Methanol in mobile phase (%)	*k*	
	Thiamphenicol	*Chloramphenicol*
10	17	
15	12	
20	6.2	19
25	4.5	13
30	3.2	10
35	2.7	7.2
40	2.2	5.5
45	2.1	4.5
50	1.8	3.5

versus percentage mobile phase composition also should not be equal to or greater than 1.0.

(5) Let us now attempt to use R_Q^* for linearization of retention data with respect to mobile phase composition. To do so we have simply returned to the paper of Crechiolo and Hill[3] using, this time, data for chloroamphenicol that were published in the same figure as for thiamphenicol. Fig. 1 shows that the R_Q^* transformation does not linearize the retention data, with regressions of log R_Q^* *versus* percentage composition giving $r = 0.991$, whereas simply plotting log k *versus* percentage composition gives $r = 0.996$.

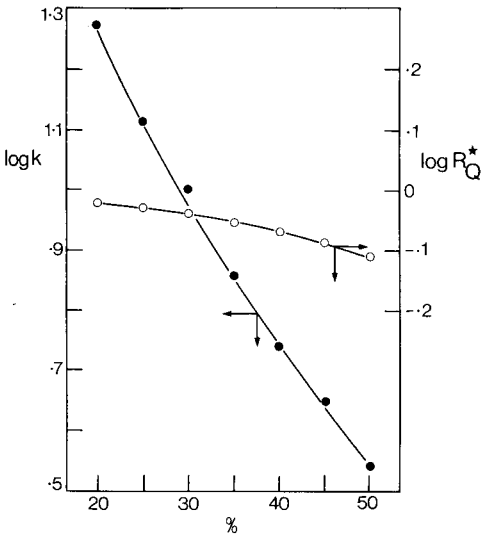

Fig. 1. Plots of log k and log R_Q^* for chloramphenicol against the percentage by volume of methanol in the eluent using the data of Crechiolo and Hill[3] (Table I).

TABLE II

GROUP EXTRA-THERMODYNAMIC TERMS (ASSUMING k_i = 10.0) OBTAINED FROM CAPACITY FACTOR DATA USING EQNS. 4 AND 5

k_j	τ *(eqn. 4)*	τ^*_Q *(eqn. 5)*
10.1	0.004	0.0004
10.2	0.008	0.0008
10.5	0.021	0.0019
11.0	0.04	0.0036
15.0	0.17	0.013
20.0	0.30	0.020
100	1.00	0.037
500	1.70	0.041
1000	2.0	0.041

(6) Structural effects in liquid chromatography are well described using simple extra-thermodynamic terms such as[4]

$$\tau = \log k_j - \log k_i \tag{4}$$

where j and i are solutes differing by a structurally discrete function. Toon and Rowland have claimed[2] that R^*_Q values obtained by extrapolation to zero percentage of organic modifier are useful in describing drug physico-chemical characters. One would assume that if this were so, a group extra-thermodynamic term could be obtained from R^*_Q, *i.e.*,

$$\tau^*_Q = \log (R^*_{Q_j}/R^*_{Q_i}) \tag{5}$$

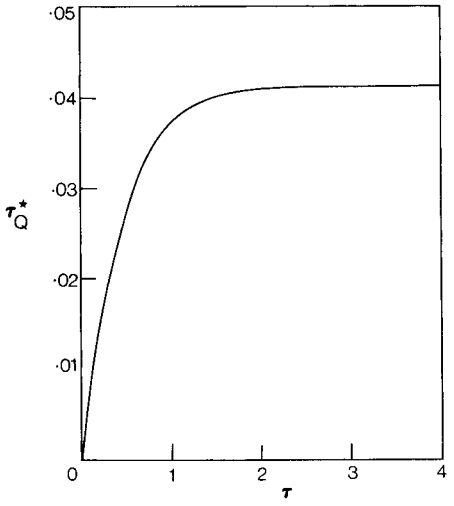

Fig. 2. Relationship between extra-thermodynamic group terms τ and τ^*_Q, where τ is obtained from log (k_j/k_i) and τ^*_Q from log$(R^*_{Q_j}/R^*_{Q_i})$ using a k_i value of 10 and k_j values from 10.1 to 1000 (Table II).

Table II and Fig. 2 shows that τ_Q^* is quasi-linear with respect to τ only between values of 0 and approximately 0.25. This arises because with k_j values between 100 and infinity the range in τ_Q^* is only 0.037 to approximately 0.042 (Fig. 2).

In conclusion, neither R_Q^* nor R_Q can be described as being appropriate for the linearization of HPLC retention data with respect to mobile phase composition, nor can extrapolated intercept values be of any general use for describing structural characteristics. Other transformations based on log–log plots[5], log k *versus* organic modifier volume fraction[6] or log k *versus* organic modifier thermodynamic activity[7] seem more useful.

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CHROM. 14,416

Book Review

Dictionnaire contextuel anglais–français de la chromatographie, by Robert Serré, published by Robert Serré, 1057 Riviera Drive, Ottawa K1K 0N7, Canada, VI + 106 pp.

This dictionary was prepared for translators and aims to give all the information necessary for a successful translation. Thus for each term there is a definition in English and the reference where this definition was coined, then a French sentence in which the equivalent French term (underlined) is used correctly and the reference to this sentence (sometimes more than one!), and then often also references to further reading. Altogether 375 terms are explained and translated and 528 French terms proposed. The reference list contains 91 entries.

I would say that both the approach and the execution are the best I have seen yet, and the author should be congratulated for having undertaken a task which will surely influence future technical dictionaries. Of course, not all of the definitions are perfect or exhaustive; for example, the definition for "affinity chromatography" is too narrow, but there are very few instances where the author was not successful in finding the equivalent expression and how it can be used.

This dictionary will be invaluable for French-speaking authors who need to write papers in English. Finally, it gives a list of abbreviations in both English and French; most readers know GLC and HPLC, but few will immediately know what HAP, LBOT or CPL mean.

Lausanne (Switzerland) MICHAEL LEDERER

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CHROM. 14,306

Book Review

Practice and applications of thin layer chromatography on Whatman KC_{18} reversed phase plates, (TLC Technical Series, Vol. 1), by J. Sherma, Whatman Chemical Separation Inc., Clifton, NJ, 1981, 24 pp., free of charge.

With this 24-page review, Whatman plan to initiate a new *TLC Technical Series.*

This first number in the series is excellent and provides a very welcome summary of work done with reversed-phase bonded C_{18} thin layers. The author cites 52 papers on the topic and provides unpublished material from the Whatman Applications Laboratory.

Some minor faults and omissions can be mentioned. In the Introduction the author states correctly that reversed-phase TLC was carried out previously with paraffin-impregnated plates or silanized silica gel, but he does not mention a bonded phase, acetylated cellulose, which has been used extensively.

When talking about "a typical efficiency" of TLC plates (on p. 3) an example is chosen which gives 1000 plates. Yet if one looks at the various figures in this review it is evident that the efficiency is usually much higher. When one of the better separations (Fig. 3) is taken, about 7000 plates are calculated for the linolenic acid ester spot. We hope that the low value given on p. 3 was not intentional.

Lausanne (Switzerland) MICHAEL LEDERER

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CHROM. 14,308

Book Review

The analysis of explosives, by J. Yinon and S. Zitrin, Pergamon Press, New York, Oxford, 1981, XII + 310 pp., price £9.35, US$ 22.50, ISBN 0-08-023845-9 (F); or £25.00, US$ 60.00, ISBN 0-08-023846-7 (H).

The analysis of explosives is a field that has recently become of rapidly increasing importance, and this timely publication gives the first complete review of the available literature on the subject citing references up to and including 1979. The fifteen chapters describe explosive compositions, chromatographic separation techniques, detection methods (including spectroscopy, thermal analysis, polarography and colour reactions), and applications of these methods to quality control, residue analysis, and the detection of hidden explosives. Each chapter begins with a short description of the principles of a particular technique followed by a well written summary of all the relevant work described in the original literature. Finally, the advantages, limitations and applications of the techniques are briefly discussed.

The book is a valuable objective reference work which summarizes the current state of explosives analysis and will provide a powerful stimulus for further research. The style is clear, concise and very readable and the content reflects the authors' considerable professional experience. The publication of this book will be welcomed by everyone involved in the analysis of explosives.

London (Great Britain) J. M. F. DOUSE

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH		J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography		234/1 234/2 235/1 235/2	236/1 236/2	The publication schedule for further issues will be published later.									
Chromatographic Reviews													
Biomedical Applications		227/1	227/2										

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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