Journal of Chromatography, 298 (1984) 21–40
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,786

INVESTIGATION OF TWO FLUORINATED REAGENTS FOR THE ANALYSIS OF SELENIUM BY GAS CHROMATOGRAPHY

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(Received April 2nd, 1984)

SUMMARY

As potential reagents for the determination of traces of selenium by gas chromatography, the compounds 4-fluoro-*o*-phenylenediamine and 4-trifluoromethyl-*o*-phenylenediamine have been examined and compared with seven *o*-diamines previously reported for analytical purposes. Retention times of the fluoro-piaselenols are shorter than all other compounds but, with the electron-capture detector, their responses differ widely. However, the detection limit of the trifluoromethylpiaselenol compares favourably with the commonly used derivative, 5-nitropiaselenol.

The analytical requirements of 4-trifluoro-*o*-phenylenediamine as a reagent, and the results of its application to the determination of selenium in various biological matrices, are presented.

INTRODUCTION

At this point in time, selenium can, with surprisingly few other elements (notably chromium, aluminium, nickel, beryllium, copper and vanadium), be determined at the ultra-trace level by gas chromatography (GC). This is possible because selenium forms very stable and volatile piaselenols with a number of substituted *o*-diamines. Sensitive detection is also readily achieved with the electron-capture detector because of the variety and number of substituents which can be introduced into the aromatic ring of the diamine.

A unique feature of the GC method is that the reaction conditions for piaselenol formation make the method essentially specific for the element. These conditions are readily obtained and require only that selenium be present as selenium(IV) for reaction with the *o*-diamine, at *ca.* pH 2, to form the piaselenol. Although high concentrations of other elements, including iron(III), chromium(III), tin(IV), and vanadium(V), may react under these conditions to form coloured species, this does not present any problem in the GC method, other than to consume the reagent,

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because of the extraction of the piaselenol into an organic phase prior to GC.

Many substituted aromatic *o*-diamines have now been examined in an effort to identify the most suitable reagent for this analysis. Of the fourteen reagents[1] which have been used for this analysis (including halo-, alkyl-, alkoxy- and nitro-substituents), the most sensitive of the mono-substituted reagents in its response to the electron-capture detection (ECD) is 4-nitro-*o*-phenylenediamine (4-NO_2-*o*-PDA). Other factors which have contributed to its importance as the preferred reagent[1] are its volatility and thermal stability, and a large distribution ratio favouring partitioning into an organic phase.

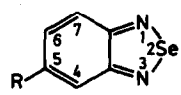
We report here the results of an investigation of two new reagents containing fluorine, an element whose presence in many compounds is associated with enhanced volatility and sensitive detection by ECD. These reagents are 4-fluoro-*o*-phenylenediamine (4-F-*o*-PDA) and 4-trifluoromethyl-*o*-phenylenediamine (4-CF_3-*o*-PDA) which readily form stable, volatile piaselenols with useful chromatographic properties. When compared with other derivatives, 5-trifluoromethylpiaselenol demonstrates many of the properties suitable for analytical applications and is superior in many respects to those of 5-nitropiaselenol.

EXPERIMENTAL

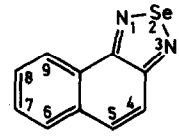
General

Purification of o-diamines. All nine diamines* were purified[2] by decolourizing with activated carbon after conversion into the hydrochlorides. For highly impure diamines, decolourizing was also carried out before acidification. The resulting products were colourless to pink compounds (as solid mono- or dihydrochlorides, or in solution), except for 4,5-dichloro-*o*-phenylenediamine which, as the free base[3], was obtained as a brown material. All purified compounds were microanalytically pure.

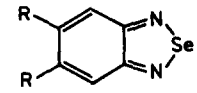
Synthesis of piaselenols. Piaselenols were formed** in good yields by adding



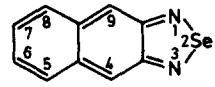
5-substituted piaselenols
(R = H, CH_3, F, Cl, NO_2 or CF_3)



Naphtho [1,2-c][1,2,5] selenadiazole
(4,5-Benzopiaselenol)



5,6-di-substituted piaselenols
(R = Cl)



Naphtho [2,3-c][1,2,5] selenadiazole
(5,6-Benzopiaselenol)

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* In addition to the two fluorinated compounds, other diamines studied were *o*-phenylenediamine (*o*-PDA), 4-methyl-*o*-PDA (4-CH_3-*o*-PDA), 4-chloro-*o*-PDA (4-Cl-*o*-PDA), 4-nitro-*o*-PDA (4-NO_2-*o*-PDA), 5,6-dichloro-*o*-PDA (5,6-diCl-*o*-PDA), 1,2-diaminonaphthalene (1,2-DAN) and 2,3-diaminonaphthalene (2,3-DAN).

** Optimal acidity for all reactions is provided by pH 1–1.5.

selenious acid (or selenium dioxide), in a minimum volume of dilute hydrochloric acid, to a solution containing an equimolar amount of the purified diamine, also in hydrochloric acid. After standing for *ca.* 1 h in an ice-bath, the solid was filtered off, then washed free of acid with cold distilled water and recrystallized from ethanol–acetone (95:5, v/v). The microanalytically pure products were stored over silica gel, in the dark, when not required.

Microanalysis after 2 years showed negligible change except for the 5,6-benzopiaselenol, which was low in both carbon and nitrogen.

Gas chromatography. A Packard-Becker model 427 gas chromatograph, equipped with a ^{63}Ni electron-capture detector and recorder, was used. High purity nitrogen, thoroughly dried over silica gel (5–10 mesh) and molecular sieve 3A, was used as carrier (12 ml/min) and by-pass (28 ml/min) gas. Such a gas allowed sensitivity settings of 5–7 and detector current settings of 8–128 to be used.

The non-polar column was a glass coil (200 cm × 0.2 cm I.D. × 0.6 cm O.D.) packed with Chromosorb W (80–100 mesh, AW, DMCS-treated). Loadings of SE-30 were 5% for determining retention and detection limits, or 10% for other purposes. Detector and injection port temperatures were held at 260 and 240°C, respectively.

Retention times of the piaselenols were measured at 148°C. However, column temperatures were varied to obtain constant retention times for the determination of detection limits from piaselenol peak heights.

Thermal analyses. Thermoanalytical behaviour was observed with a Rigaku Denki Thermoflex unit (Model M8076), as combined DTA/TG data. Samples (7.5 mg) were heated in open aluminium cups at 10°C/min, in a moving environment of pure nitrogen (100 ml/min).

Ultraviolet spectra. Standard solutions of the piaselenols ($5 \cdot 10^{-5}$ *M*) were prepared in cyclohexane and toluene. Cyclohexane solutions were examined at 210–400 nm, and toluene solutions at 280–400 nm, in 1-cm quartz cells using a Cary UV-17 spectrophotometer.

Developmental aspects of the analytical procedure

Distribution ratios of the piaselenols. Distribution ratios of the new 5-fluoro- and 5-trifluoromethylpiaselenols were determined according to Shimoishi's method[4]. The 5-nitropiaselenol was included in this study for comparison.

Briefly, the procedure involved taking a known amount (1 ml) of a standard piaselenol solution in toluene, (and in hexane also), then equilibrating the solution with 200 ml of 1 *M* hydrochloric acid saturated with toluene (or hexane). The distribution ratio was determined from the reduction in the peak heights of the corresponding chromatograms, obtained under the same experimental conditions given above.

Formation of 5-trifluoromethylpiaselenol

Optimal conditions for the formation of this piaselenol were obtained by examining the effects of acidity, reagent concentration, reaction time and temperature, and foreign ions in solution.

Effect of acidity. Progress of the reaction under conditions of controlled acidity

was determined from the peak height* of the piaselenol formed during reaction between selenium(IV) and 4-CF_3-*o*-PDA. This involved adjusting the pH of the reagent stock solution (25 ml), adding a known amount of selenium(IV) [0.5 ml solution containing 80 ng of selenium(IV) in distilled water], then extracting the piaselenol into 1 ml of toluene. The conditions were as follows: total reaction time, 5 h; molar ratio [reagent]/Se(IV)], $5 \cdot 10^4$ using 80 ng of Se(IV); reaction temperature, 22°C (±1°C); extraction time, 2 min; acidity range, pH 1–5 (in increments of 0.5 units) and between 0.2 and 5 *M* hydrochloric acid; extraction ratio [volume (ml) toluene/volume (ml) aqueous phase], 1/25.5; extract volume for GC, 1 μl.

Molar ratio of the reagents. The general conditions under "Effect of acidity", including requirements for optimum acidity (*ca.* pH 0.5), were applied. Solutions of the *o*-diamines were prepared in 0.3 *M* hydrochloric acid so that each millilitre provided a $5 \cdot 10^4$ molar excess over 80 ng of selenium(IV). A 20-ml volume of 0.3 *M* hydrochloric acid containing this amount of selenium(IV) was then transferred to each of a series of 25-ml volumetric flasks. Increasing amounts (0.1-ml increments) of the *o*-diamine solution were added to each flask, and the volume adjusted. After 5 h, each mixture was extracted with toluene (1 ml, 2 min). Chromatograms were obtained on 1-μl volumes of the extract.

Effect of reaction time. Conditions were maintained as the previous paragraph except that the molar ratio [reagent]/[Se(IV)] was held at $5 \cdot 10^4$ excess over 400 ng of selenium(IV). At 10-min intervals, one reaction mixture was taken and, as before, extracted with toluene (1 ml, 2 min). Aliquots (1 μl) of the extracts were examined by GC.

Effect of temperature. Using the same conditions as in the study of reaction time, this effect was determined at room temperature (22°C), and at 42°C and 62°C in temperature-controlled water-baths. At 10-min intervals (plus 2 min allowed for transfer and rinses), the contents of a flask were extracted with toluene (1 ml), and aliquots (1 μl) of the extracts were examined by GC.

Effect of foreign ions. Formation of 5-trifluoromethylpiaselenol in the presence of added ions [in amounts varying up to $4 \cdot 10^4$ in excess of 80 ng of selenium(IV)] was observed using the optimal conditions found previously. Stock solutions of the ions were prepared so that a small volume (2 ml) gave the maximum amount of the particular ion. The ions were iron(III), manganese(VII), chromium(III), molybdenum(VI), tin(IV), vanadium(V) and nickel(II), prepared from suitable salts.

Reactions were carried out in 25-ml flasks, in 0.3 *M* hydrochloric acid and excess of the diamine ($5 \cdot 10^4$ molar excess). The reaction temperature was 20°C, and extraction and GC, as before, were carried out after allowing the reaction mixture to stand for 2 h.

Effect of acid concentration on the piaselenol. Because protonation can occur in a strong acid medium[5], the effect of increasing acidity on the peak heights given by a known amount of the piaselenol was studied. For this, a solution (25 ml, $1 \cdot 10^{-6}$ *M*) in toluene was equilibrated with aqueous solutions of 1–10 *M* hydrochloric acid and of pH 1 to 14. As before, a volume ratio of 25:1 (aqueous phase:toluene) was

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* Chromatograms were obtained on the 10% SE-30 column at 138°C, with sensitivity settings 5/32. Under these conditions, the retention time of the piaselenol was 4 min.

maintained, with an equilibration time of 1 min. An aliquot (1 μl) of the clear toluene phase was taken for GC.

Removal of excess diamine

Since the retention time of free *o*-diamine and the corresponding piaselenol is closer than is desirable for an analytical procedure, three methods[6–8] of removing excess reagent were examined.

A measured amount (1 ml) of a solution of the purified reagent (0.65% by weight in 0.1 *M* hydrochloric acid) was added to a solution (10 ml) containing 80 ng of selenium(IV). After standing for 3 h at 22°C, the mixture was shaken with toluene (1 ml, 2 min). The toluene phase was then washed once with (i) 2 ml of 7.5 *M* hydrochloric acid[6], or (ii) aqueous ammonia followed by adsorption on Florisil–magnesium sulphate[7], or (iii) 3 ml of perchloric acid[8] (72%, 1:1 by volume with water), and examined by GC (1-μl volumes).

Analysis of biological materials

The following measures were employed for the analysis of total selenium in a variety of biological materials, including plant materials, milks, blood and plasma, hair, finger-nails, urine, and saliva.

Preliminary treatment of samples. Plant materials, as obtained, were ground in a mortar and pestle, dried in an oven (1 h at 70°C) and stored over silica gel in a desiccator. Some spices, already in powder form, only required mixing and drying.

Homogenized milks, human milk, urine and saliva were processed directly, without delay or storage. Urine samples were collected as single, early-morning eliminations. Saliva was centrifuged to separate any suspended debris. Nails and hair were processed without washing. Animal plasma and blood samples were processed as soon as possible after delivery.

Sample processing. Solid samples (0.1–0.5 g) were accurately weighed and transferred to small (100 ml) Kjeldahl flasks. Volumes of 3–5 ml were taken for most liquid samples, except blood or plasma (0.3–0.5 ml). Samples were treated with concentrated nitric acid (10 ml) and allowed to stand for *ca.* 1 h to reduce frothing later. At this stage, perchloric acid (72%, 2 ml) was added and the mixture heated gently over an electric heating unit for *ca.* 1 h (or until the dense brown fumes were no longer evolved) then, at higher temperature, to the appearance of fumes of perchloric acid (*ca.* 3 h). Heating was continued beyond this point until the final volume was *ca.* 0.5 ml (*ca.* 30 min). To the cool, colourless residue was added a solution of urea (1 *M*, 2 ml) and the mixture heated for a further 10 min to decompose any residual traces of nitric acid.

Reduction of selenium(VI) to selenium(IV) was carried out by adding concentrated hydrochloric acid (1 ml) and re-heating this mixture for 10 min over a boiling water-bath. The cooled digest was transferred to a 25-ml volumetric flask with the aid of dilute hydrochloric acid (0.01 *M*) and made to volume. An aliquot (10–20 ml) of this solution was shaken for 3 min with toluene (10 ml) in a small (100 ml) separatory funnel to remove any toluene-soluble material. Freshly prepared *o*-diamine solution (1 ml of *ca.* 1% w/v in 0.1 *M* hydrochloric acid) was added to the aqueous phase, the mixture shaken briefly, then stood for 1 h at room temperature.

The piaselenol was extracted by shaking with toluene (1 ml, 2 min), then the

TABLE I

SOME PHYSICAL AND OTHER PROPERTIES OF THE PIASELENOLS

Piaselenol	*Physical state and melting point (°C)*		R_F[a]	λ_{max} *(nm) (Molar absorptivity,* $\varepsilon \times 10^4$*)*				*DTA, Procedural temperature (°C)*		
	Observed	*Literature*		*Solvent*		*Literature*		*Fusion*[d]	$T_{0.5}$[e]	*Volatilization*[f]
				Cyclohexane	*Toluene*	*Toluene*[b]	*Water*[c]			
H	white needles, 73	white, 73–74	0.36	329(1.70)	334(1.39)	334(1.70)	331.5(1.74)	75	151	175
5-Methyl	yellowish needles, 70	white, 69–70.5	0.32	331(1.63)	337.1(1.56)	337(1.65)	335(1.70)	75	164	190
5-Fluoro	white needles, 104		0.38	330(1.51)	334.7(1.24)			112	139	163
5-Chloro	white needles, 119	pale yellow, 118–119	0.44	336(1.72)	342.9(1.65)	344(1.75)	338(1.69)	120	171	194
5-Nitro	yellow crystals, 222	yellow, 223–224	0.34	341(1.67)	351.6(1.47)	353(1.51)	344(1.65)	229	230	252
5-Trifluoromethyl	white crystals, 91		0.43	332(1.66)	337.5(1.43)			93	140	166
5,6-Dichloro	pale-buff crystals, 163	pale yellow, 162–162.5	0.51	348(1.95)	353.8(1.94)		347(1.93)	166	196	221
4,5-Benzo[g]	greenish crystals, 129	129	0.41	358(1.44)	361.2(1.21)	361(1.28)		1.30	225	250
5,6-Benzo[h]	bright red needles, 265, decomposed	red, 270, decomposed	0.33	259[i]	380[i]		378.5(1.82)	264 (exothermic peak at 148°C)	262?	[j]

[a] Determined on silica gel with pure benzene as solvent. Observed as dark spots under UV light, except for the red fluorescence of the 5,6-benzo-derivative. The corresponding *o*-diamines have small R_F values and are readily detected by overspraying with ninhydrin.

[b] See ref. 23.

[c] See ref. 24.

[d] Temperatures correspond to maxima of the endothermic peaks.

[e] Temperatures corresponding to 50% weight loss.

[f] Colours of sublimates were as previously stated in this Table. Thermal stability was confirmed by examining sublimates by TLC. Residues in cups were negligible.

[g] *Chemical Abstracts* name is naphtho[1,2-*c*][1,2,5]selenadiazole.

[h] *Chemical Abstracts* name is naphtho[2,3-*c*][1,2,5]selenadiazole.

[i] Not determined because of the poor solubility in this solvent.

[j] A small amount of this compound was recovered. The temperature was not known. Residue was 47.3%.

toluene phase was washed by shaking with perchloric acid (1:1 by volume with water, 3 ml, 1 min), and, finally, briefly with water (10–15 ml). Volumes (1 μl) of this solution were examined by GC.

For preparing calibration curves, a stock solution (1 mg/ml selenium(IV) obtained by dissolving selenium dioxide in 0.1 *M* hydrochloric acid) was diluted to obtain the working standard (100 ng/ml selenium(IV). Aliquots of this solution were taken to prepare a working range of 10 to 150 ppb selenium(IV) and transferred to 0.3 *M* hydrochloric acid (10–20 ml). The reagent (1 ml) was then added to the selenium and the procedure for recovery of the piaselenol repeated, as in the previous paragraph.

Reliability of the method was determined by repeated analysis of the same matrix, and by recovery of a "spiked" matrix. Thus, for the determination of precision, samples of urine, hair and orchard leaves (NBS SRM 1571) were used. For recovery efficiency, one of the standards (orchard leaves) was spiked with 50 ppb of selenium(IV). Other samples were blood containing 40 ppb of added selenium(IV), and plasma with 30 ppb of added selenium(IV).

RESULTS AND DISCUSSION

In order to compare the two new piaselenols with those already advocated for use in analytical applications, a total of nine compounds were prepared (see Table I and structures). As with other piaselenols, reaction with 4-F-*o*-PDA and 4-CF_3-*o*-PDA was rapid and copious amounts of the compounds precipitated during their synthesis provided the pH was controlled. Physical and other properties of the piaselenols, including R_F values, molar absorptivities and some thermoanalytical data, are presented in Table I.

Thermogravimetric behaviour of the piaselenols (see Fig. 1) show that all are volatile and thermally stable over the temperature range to 300°C, with the exception of the 5,6-benzopiaselenol which undergoes marked decomposition. Moreover, the presence of fluorine* in the piaselenol raises the volatility of the two new derivatives above all others in the group. On the basis of 50% weight loss, volatility of the piaselenols decreases in the order: 5-fluoro > 5-trifluoromethyl > H > 5-methyl > 5-chloro > 5,6-dichloro > 4,5-benzo > 5-nitro $\gg$ 5,6-benzopiaselenol. Although this trend is in good agreement with available dipole moments[9] which suggest the volatility order: 5-chloro (0.50 Debye) > H (1.19 D) > 5-methyl (1.84 D) > 5-nitropiaselenol (3.59 D), anomalies remain if it is accepted that the electrophilic substituents ought to enhance volatility in the parent compound.

As indicated in Table I, the 5,6-benzopiaselenol melts with extensive decomposition. In fact, differential thermal analysis (DTA) points to decomposition at temperatures as low as 148°C and closer examination of sublimates by thin-layer chromatography (TLC) revealed the presence of three compounds.

Gas chromatographic properties of fluoro-piaselenols

Elution of the piaselenols was examined only on the non-polar stationary phase

* Note that the molecular weight of 5-trifluoromethylpiaselenol (251.07) is only exceeded by that of the dichloro-compound (251.96).

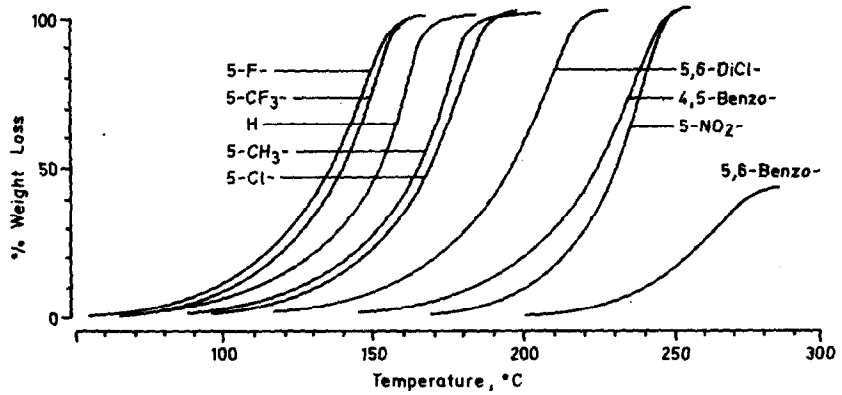

Fig. 1. Thermogravimetric curves for the piaselenols.

SE-30 because of its common use for the purpose and, equally important, because it also gives reasonably short retention times for these compounds. Table II lists the relative retention data for the piaselenols and shows them to be in agreement with the volatility order found by thermogravimetry.

TABLE II

GAS CHROMATOGRAPHIC PROPERTIES OF THE PIASELENOLS

Piaselenols	*Relative retention time*	*Relative sensitivities* This work (literature)*	*Minimum detectable** amounts of selenium (pg) (literature)*
H	1.0 (2 min 36 sec)	(1)	86 (90)
5-Methyl	1.6	1.1 (1.4)	55
5-Fluoro	0.9	3.6	13
5-Chloro	1.7	7.4 (17) (8.2)	7
5-Nitro	5.2	27.6 (128, 38.8)	<2 (1)
5-Trifluoromethyl	0.9	23.6	>2
5,6-Dichloro	3.6	25.9 (102)	<2 (1)
4,5-Benzo	5.1	11.6 (25)	4
5,6-Benzo	N.D.	N.D.	N.D. (20)

* Relative sensitivities are averages of four measurements. Literature values are quoted from refs. 4 and 10. N.D. = not determined.

** This is the actual amount injected onto the column which produced a peak height equal to twice the average baseline noise. Literature values are from ref. 2.

Relative sensitivities of the nine piaselenols, determined under the same experimental conditions, are also included in Table II. ECD responses are shown in two additional forms as chromatograms (in Fig. 2) and calibration plots (in Fig. 3). Other than for the fluorinated compounds, these results are in general agreement with previously published[4,10] data. From these results, the order for increasing ECD response is: H < 5-methyl < 5-fluoro < 5-chloro < 4,5-benzo < 5-trifluoromethyl < 5,6-dichloro < 5-nitropiaselenol; this establishes the fluorinated derivatives as outstanding for two reasons. Firstly, both compounds elute at the lowest column temperature and have the shortest retention times. Secondly, the detector responses for the two compounds differ widely, with the detection limit for 5-trifluoropiaselenol approaching closely that of 5-nitropiaselenol. Thus, beyond this point, there was no reason to consider the 5-fluoropiaselenol as a possible analytical reagent.

It may be noted that data for 5,6-benzopiaselenol are absent from Fig. 3 and Table II because of its apparent on-column decomposition to give the two* GC peaks seen in Fig. 2. Yet, this compound formed the basis of an earlier analytical procedure for selenium[12], with ECD. Evidently, its thermal instability was not recognized then, or in a later[2] study of four piaselenols, including this compound.

Method development based on 5-trifluoromethylpiaselenol

For the reasons given above, further work was restricted to assessing the suitability of the reagent 4-CF_3-*o*-PDA for determining trace amounts of selenium. It

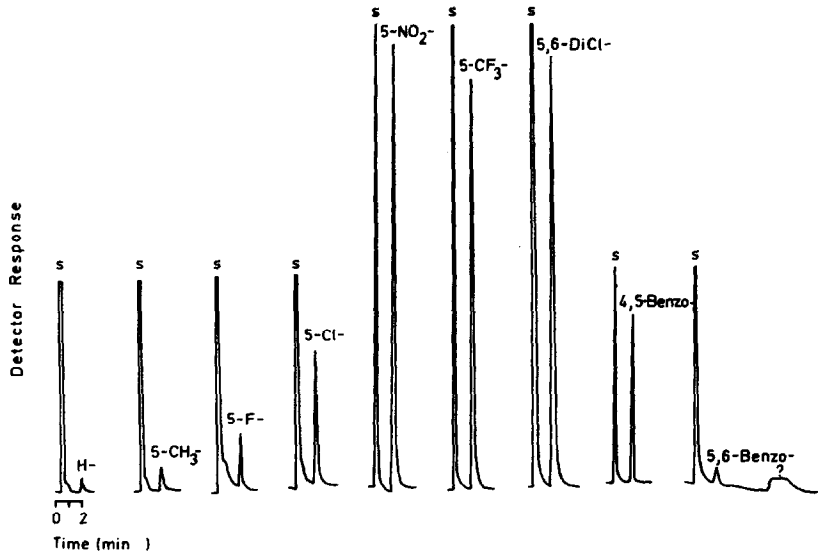

Fig. 2. Electron-capture detector response of piaselenols (0.79 ng of selenium in each chromatogram). Retention time 2 min in each case. (S = solvent in all figures.)

* The white insoluble dimer[11] of 5,6-benzopiaselenol, which rapidly forms in toluene, was removed from the solution prior to GC and is probably unrelated to either of the peaks. Neither peak is due to the diamine (2,3-DAN).

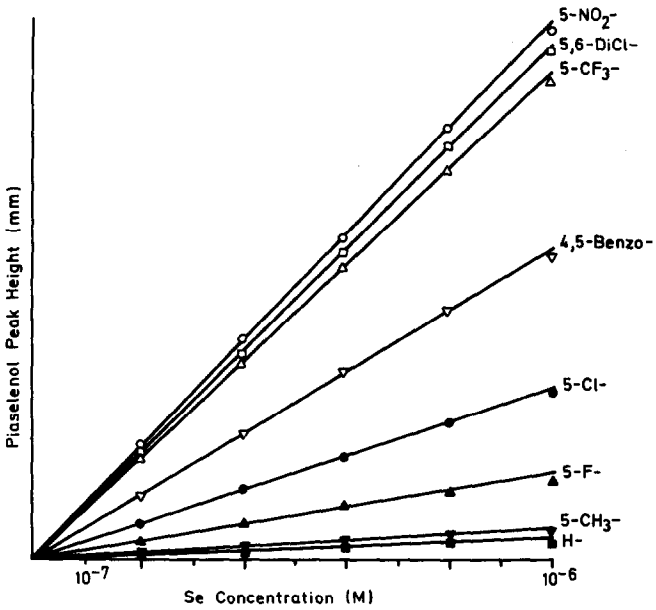

Fig. 3. Calibration curves for the piaselenols.

was established, however, that distribution ratios into toluene for both fluorinated piaselenols were comparable with 5-nitropiaselenol. Values for 5-fluoro-, 5-trifluoromethyl- and 5-nitro-piaselenols[4,13,15] were 339, over 500 and 430, respectively. In hexane, values for the fluorinated piaselenols were, suprisingly, 26 and 402, respectively.

The approach involved three main considerations, namely, the control of spurious chromatographic peaks, optimal reaction conditions, and the removal of excess reagent. These and other factors are now discussed.

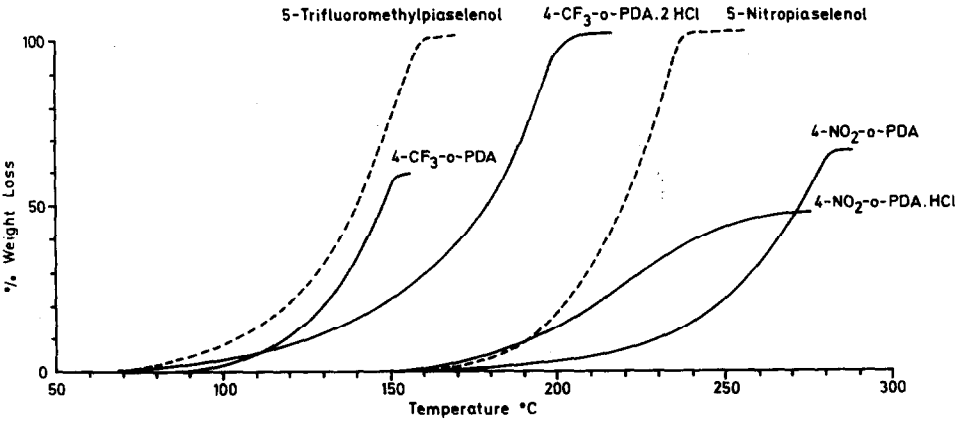

Fig. 4. Thermogravimetric curves of 4-trifluoromethyl- and 4-nitro-*o*-phenylenediamines and derivatives.

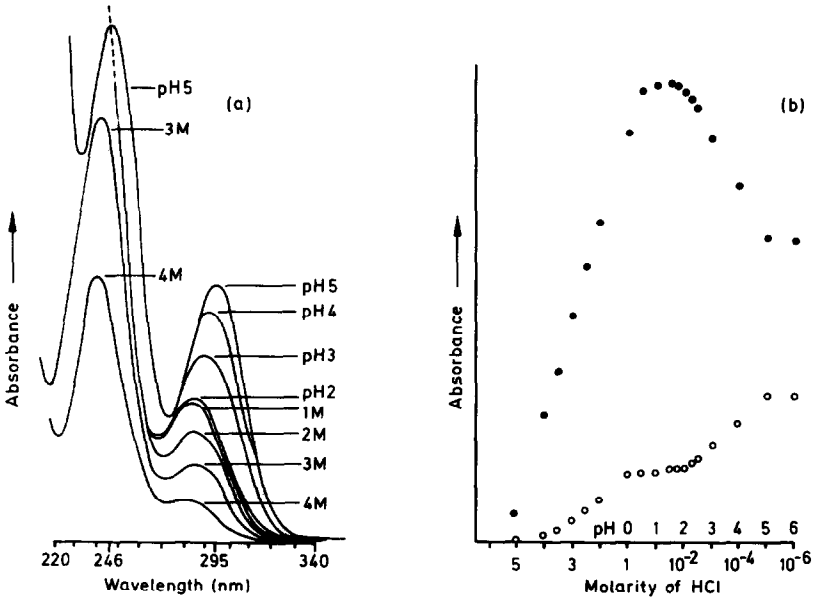

Fig. 5. Effect of acidity on 4-trifluoromethyl-*o*-phenylenediamine as seen in (a) UV absorption spectra (off-scale and incomplete at lower wavelengths) and (b) a plot of absorbance *versus* acidity at two wavelengths (including some points not shown in (a)); ● at 246 nm, ○ at 295 nm.

Optimal conditions for piaselenol formation

Spurious chromatographic peaks. The major source of interference in the GC determination of selenium is the appearance[1] of spurious chromatographic peaks close to (or, sometimes, overlapping) the piaselenol peak. In other instances, the peaks may have long retention times which delay subsequent injections and sample output.

Before proceeding to examine this piaselenol, the volatility of species related to the reagent was examined, where possible. Fig. 4 shows, with others, the thermogravimetric curves for 4-CF_3-*o*-PDA and, for comparison, the corresponding nitro-compound also. As free bases, both reagents undergo thermal decomposition* whereas the salt** 4-CF_3-*o*-PDA · 2HCl is thermally stable. Consequently, spurious peaks may appear in chromatograms where any excess reagent is co-extracted with the piaselenol into the organic phase, since volatile species can form almost simultaneously with the volatilization of the piaselenol. However, although the dihydrochloride is both volatile and thermally stable, interference is probably small for the reasons that its solubility in a solvent such as toluene should be low, and because it requires distinctly acidic conditions to exist. Thus, the free base seems the more likely source of the spurious peaks.

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* DTA curves show exothermic peak maxima at 155°C and 288°C for 4-CF_3-*o*-PDA and 4-NO_2-*o*-PDA, respectively.

** Also in DTA curves, an exothermic peak maximum at 225°C for 4-CF_3-*o*-PDA. HCl is indicative of decomposition.

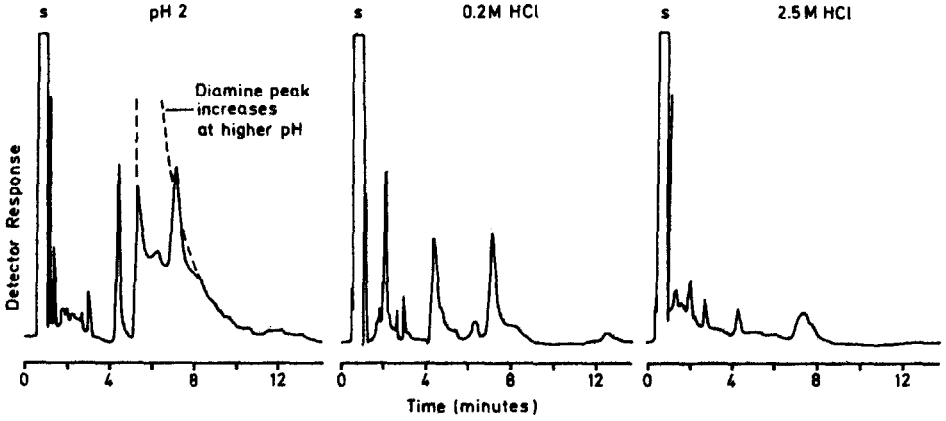

Fig. 6. Chromatograms showing ECD response to extracts of 4-trifluoromethyl-*o*-phenylenediamine obtained at different acidities.

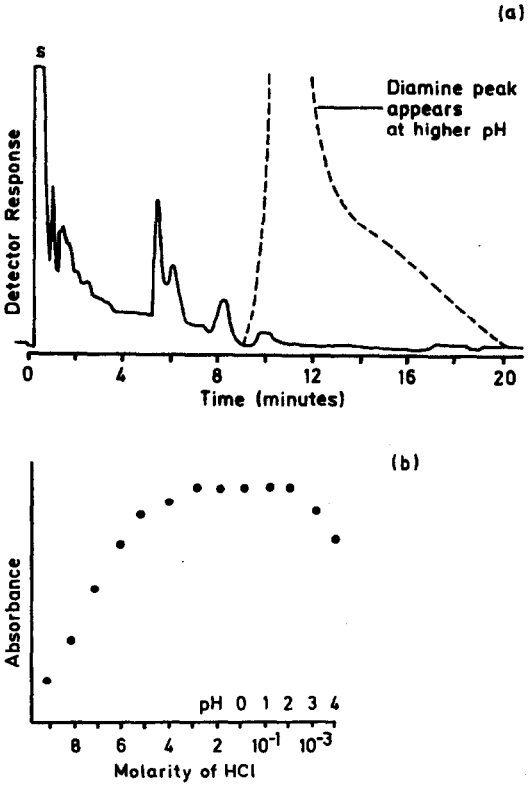

Fig. 7. Effect of acidity on 4-nitro-*o*-phenylenediamine. (a) ECD response of extract of diamine dissolved in 1 *M* hydrochloric acid and (b) plot of absorbance at 403 nm *versus* acidity.

The importance of the latter is clarified in the data of Figs. 5 and 6. Fig. 5 shows the effect of acid concentration upon the *o*-diamine, as measured by UV absorption. Here, in Fig. 5a, the maxima at *ca.* 295 nm and 246 nm correspond to the bands for the free base and its mono-protonated form, respectively. As the acidity increases (see Fig. 5b), the absorbance at 295 nm is reduced, passing through a plateau in the region of pH 0–2, then falling to zero absorbance in *ca.* 5 *M* hydrochloric acid. Simultaneously, absorbance at the lower wavelength increases to a maximum at pH 0–2, where the mono-protonated form is at its highest concentration.

The three chromatograms reproduced in Fig. 6 are consistent with this argument and show a pattern of peaks due to various volatile species. For pH 2, several unresolved peaks with a retention time between 5 and 8 min are observed. At higher pH, this group of peaks rapidly goes off-scale owing, as is easily demonstrated, to the free base. In more acidic conditions (0.2 *M* hydrochloric acid) when the reagent

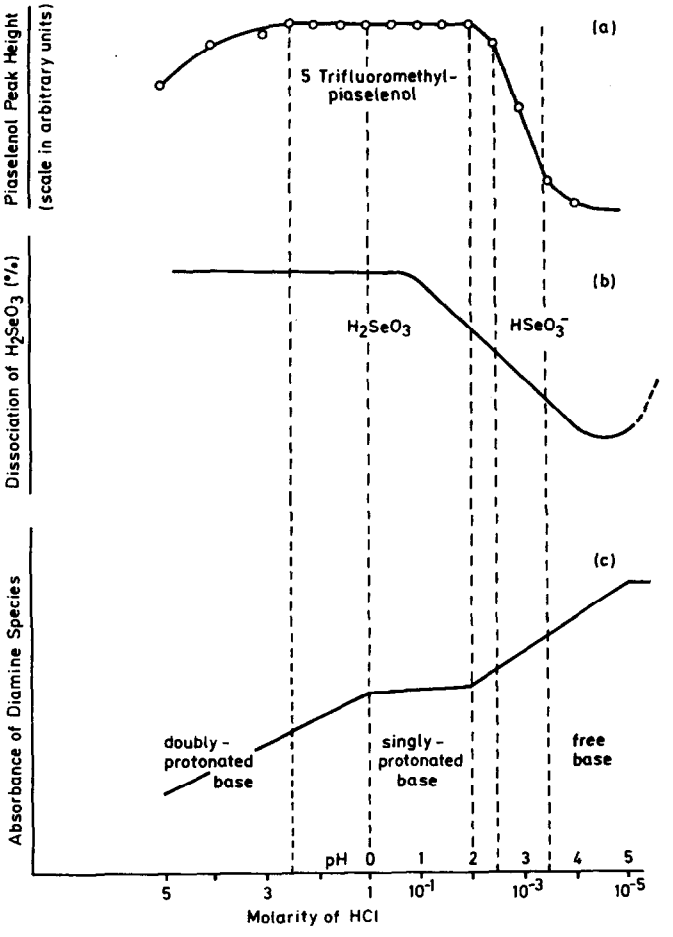

Fig. 8. Formation of 5-trifluoromethylpiaselenol at various levels of acidity, as determined by ECD response (a). Included in figure are (b) the dissociation curve for selenious acid, and (c) the UV absorbance plot for the reagent.

exists only as the singly-protonated species, the number and height of peaks are reduced. Finally, in 2.5 *M* hydrochloric acid still fewer and smaller peaks are seen in the chromatogram. Although the thermal behaviour of the singly-protonated form of the reagent was not determined, if unstable and soluble in the extracting solvent then its decomposition products could be expected to add to the confusion of spurious peaks in chromatograms.

A parallel mechanism is also observed with the reagent 4-NO_2-*o*-PDA, as shown in Fig. 7a, where the free base peak (at 10–12 min) increases rapidly as the pH rises. However, because the nitro-compound (see Fig. 7b) is more difficult to fully protonate than 4-CF_3-*o*-PDA, formation of the mono-hydrochloride is indicated (and supported by microanalysis). As this salt is thermally unstable, any transferring to the organic phase can also be expected to produce spurious peaks. Compounds responsible for the peaks eluting at *ca.* 6 min are difficult to remove, and appear to be identical with those reported[1] in the literature.

Effect of acidity. The effect of acidity on the formation of 5-trifluoromethylpiaselenol is summarized in Fig. 8. Plotted as a function of the concentration of hydrochloric acid, Fig. 8a shows the variation in peak height (constant for acid concentrations ranging from 10^{-2} to 2.5 *M*), together with related plots for the dissociation[13] of selenious acid (Fig. 8b), and the absorbance of the three diamine species (Fig. 8c). These data confirm that at pH 3.5, where selenious acid exists predomi-

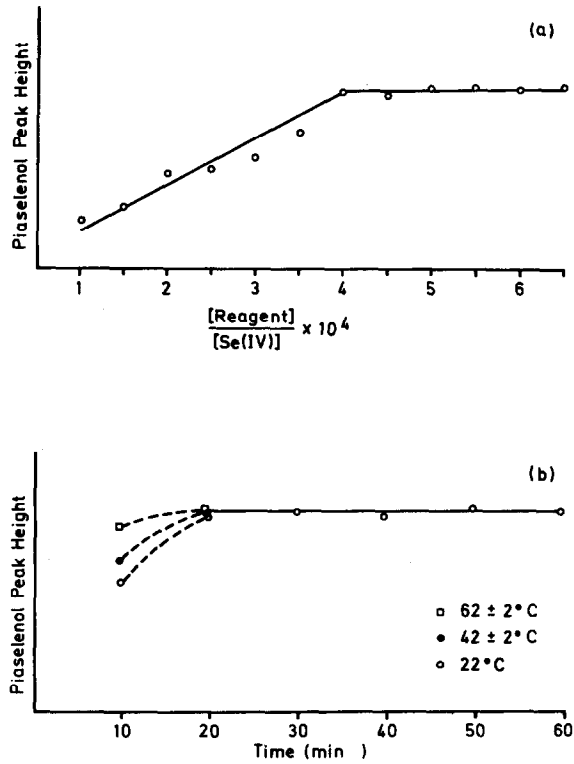

Fig. 9. Effect on 5-trifluoromethylpiaselenol formation of (a) the molar concentration ratio of reactants, and (b) the reaction time and temperature.

nantly as $HSeO_3^-$ and the diamine is present in equal concentrations of singly-protonated and free base, only a small peak results for the piaselenol. At pH 2.5, where the concentration of undissociated selenious acid is higher and the diamine is principally in the mono-protonated form, the GC peak height is correspondingly higher and approaching its maximum for the experimental conditions. From pH 2.0 to pH 0, where full protonation of the reagent begins, the height of the piaselenol peak is at a constant maximum height. This situation is maintained until the acid concentration is 2.5 *M* when the height of the piaselenol peak again decreases as the doubly-protonated diamine steadily increases in concentration. Overall, these results support and extend previous observations made by UV measurement of other piaselenols[14] and highlight the essential mechanistic features of the reaction.

From the analytical standpoint, since the formation of the piaselenol is clearly dependent on acid concentration, it is obvious that the reagent 4-CF_3-*o*-PDA offers a relatively wide working range of acidity for the reaction (in this case, from 10^{-2} to 2.5 *M* hydrochloric acid). However, compared with the dissociation constants of other *o*-diamines (for which adjustment of the acidity prior to the reaction is more critical), 4-NO_2-*o*-PDA offers a still wider and more flexible range of acid concentrations (see Fig. 7b). Of course, kinetic factors in the reaction need not, and do not[14], follow this trend.

Reagent concentration. Examination of the amount of reagent needed to form the piaselenol within an acceptable reaction time was intended to cover selenium levels in most samples while using no more than *ca.* 2 ml of a reagent solution of 0.5–1% concentration.

Fig. 9a shows that for 4-CF_3-*o*-PDA, the minimum molar concentration ratio of the reactants needed to produce a constant piaselenol peak height with 80 ng selenium is $4 \cdot 10^4$, at 22°C. This represents *ca.* 1 ml of the reagent solution and is well within the limit set.

Reaction time and temperature. The time required for complete reaction between 400 ng of selenium(IV) and a reagent molar concentration ratio held constant at $5 \cdot 10^4$ is shown in Fig. 9b. Although the reaction is relatively fast at the three temperatures studied, a reaction time of 1 h was established for analytical purposes to cater for the variation in selenium levels of samples. It is important to note that despite the apparent advantage in time of the higher reaction temperatures, the appearance of spurious peaks in chromatograms was then increased and their elimination became more difficult. For these reasons, reactions for the analytical work were conducted at ambient room temperatures.

An indication of the effect exerted by substituents in the reagent upon reaction time (and referred to under *"Effect of acidity"*), is illustrated by the fact that whereas a constant peak height is attained after 20 min with 4-CF_3-*o*-PDA (Fig. 9b), with the reagent 4-NO_2-*o*-PDA under identical conditions, the time is increased to 35 min.

Effect of foreign ions. Interference problems associated with the presence of foreign ions have been noted[15,16] since the early development of *o*-PDA as a reagent for determining selenium. Reactions, especially at higher pH, lead to stable compounds[17] nickel(II), or the formation of diaminophenazines[18] with iron(III). Other common interfering ions[15,16] are vanadium(V), chromium(III) and molybdenum(VI). Using 4-CF_3-*o*-PDA, interference is sometimes apparent but, since the piaselenol is formed in a highly acidic medium, decomposition through oxidation

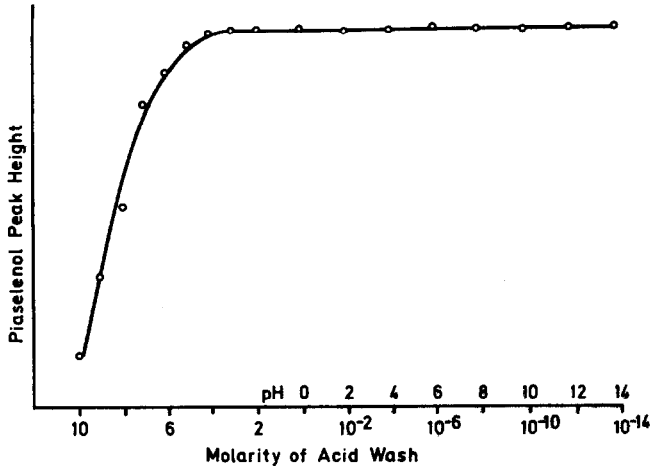

Fig. 10. Effect of acid washing on the loss of 5-trifluoromethylpiaselenol.

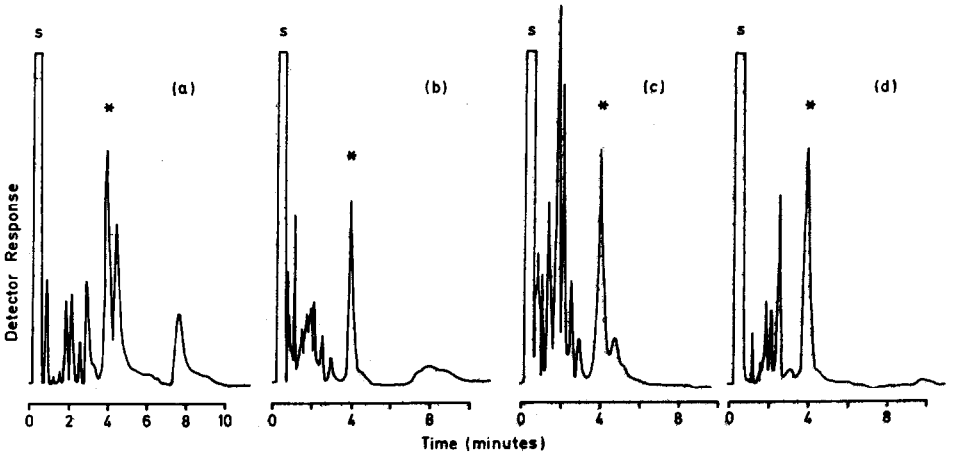

Fig. 11. Chromatograms showing the effect of clean-up procedures on 5-trifluoromethylpiaselenol (identified by *) separation. Chromatograms are for (a) control (*i.e.* no clean-up), (b) after Florisil–magnesium sulphate treatment, (c) after a hydrochloric acid (2 ml, 7.5 *M*) wash, and (d) after washing with perchloric acid (3 ml, 1 + 1 by volume with water).

is reduced and formation of stable complexes (such as with nickel[17]) is largely prevented.

A few examples illustrate the levels needed for interference by these ions. Iron(III) present at a level of $4 \cdot 10^3$ in excess of nanogram amounts of selenium was without effect. For ratios between $0.5 \cdot 10^4$ and $4 \cdot 10^4$, the height of the piaselenol peak (retention time, 4 min) was constant but an unknown peak (retention time, 19 min) appeared in the chromatogram.

With chromium(III), nickel(II) and molybdenum(VI) at a level 10^4 times greater than the selenium concentration, the height of the piaselenol peak was constant and no additional peak was observed. On the other hand, vanadium(V) and

manganese(VII) interfered more seriously, perhaps as a result of oxidation of the reagent. For a ratio of $4 \cdot 10^3$, vanadium(V) gave a peak overlapping with the piaselenol, and another at longer retention time similar to that observed with iron(III). With concentration ratios greater than $5 \cdot 10^4$, manganese(VII) produced a still greater number of unknown peaks in the chromatograms.

In most cases, the presence of excessive amounts of foreign ions is characterized, depending on the concentration, by the pink to violet colour of the reaction mixture and the extract. However, although the amount of added foreign ion was intentionally large in these tests, the effect can be minimized by the judicious use of a masking reagent (such as EDTA), so that the problem is not a serious one in practice.

Removal of excess reagent. Of the spurious peaks appearing in chromatograms under the GC conditions used, one peak was particularly troublesome because of the closeness of its retention time (*ca.* 4.5 min) to that of the 5-trifluoromethylpiaselenol (*ca.* 4 min). Although there are at least two alternatives to resolving this problem, namely, improvement of column resolution or the use of a clean-up procedure, the latter approach was favoured. The methods examined consisted of two which used acid washing of the extract[6,19], and a third employing a mixed adsorbent[7] of Florisil and anhydrous magnesium sulphate.

For the experimental conditions specified, Fig. 10 shows that washing of the extract with moderately strong hydrochloric acid may be safely effected without loss through protonation of the piaselenol[5], provided the acid concentration does not exceed 4 *M* hydrochloric acid. The effectiveness of the three clean-up procedures is shown in the chromatograms reproduced in Fig. 11. When compared with the control, this revealed that simply swirling the extract with adsorbent was attractive (being simple, effective and rapid) but, as in earlier work[20], some piaselenol was retained and lost. Washing with hydrochloric acid failed to eliminate completely the interfering peak close to the piaselenol peak and generated others at shorter retention time. Perchloric acid, however, gave best results and, therefore, was incorporated into the analytical procedure together with adequate water washes to remove all acid.

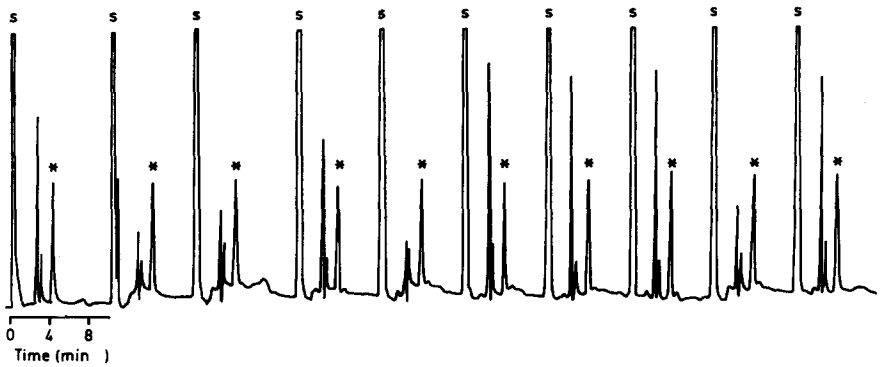

Fig. 12. Chromatograms of ten consecutive injections of a urine extract processed by the 4-trifluormethyl-*o*-phenylenediamine method. The piaselenol is identified by the asterisk (*) in chromatograms run at 10-min intervals.

TABLE III

ANALYTICAL RESULTS FOR TOTAL SELENIUM DETERMINATIONS USING 4-TRIFLUOROMETHYL-*o*-PHENYLENEDIAMINE

Determination	*Samples*	*Number of analyses*	*Selenium present*		*Selenium found*		*Recovery (%)*
			Initial (ppb)	*Added (ppb)*	*Mean (ppb)*	*R.S.D. (%)*	
Precision	Orchard leaves (NBS SRM 1571)	5	80 ± 10*	–	83	1.9	103.2
	Hair	6	–	–	472	4.7	–
	Urine	10	–	–	37.5	2.7	–
Recovery	Orchard leaves (NBS SRM 1571)	5	80 ± 10*	50	133	2.6	102.4
	Sheep's blood (pooled)	8	110	40	151	2.7	100.8
	Sheep's plasma (pooled)	5	143.5	30	175.5	1.2	101.8

* Value indicated is certified value.

TABLE IV

ANALYTICAL RESULTS FOR TOTAL SELENIUM IN PLANT MATERIALS, MILKS AND ANIMAL SPECIMENS

Samples	*Total selenium**,***	
	5-Trifluoromethylpiaselenol method (ppb)	*5-Nitropiaselenol or other method (ppb)*
Plant materials		
Mixed herbs	98.0	99.0
Garlic powder	280.5	275.0
Ginger	93.5	90.0
Oregano leaves	117.0	119.0
Basil leaves	178.5	181.0
Mint flakes	49.1	51.0
Milk		
Bottled	8.33	N.D.
Carton-packed	7.57	N.D.
Carton-packed, "Hi-Lo"	7.75	N.D.
Animal specimens		
Sheep's blood A	319.5	331.7***
B	289.5	283.4***
C	341.5	333.6***
D	67.5	70.0***
E	82.0	76.6***
F	34.5	33.2***
Sheep's plasma A	136.0	133.6***
B	106.5	109.0***
C	151.0	158.1***
D	25.8	21.1***
E	37.8	40.9***
F	16.6	21.5***

* Values are means of two determinations.

** Determined using aliquots of the same sample solutions.

*** Determined by fluorimetric method for all animal specimens. Values were supplied by the C.S.I.R.O.'s Division of Animal Production's Pastoral Research Laboratory at Armidale.

TABLE V

ANALYTICAL RESULTS FOR TOTAL SELENIUM IN HUMAN SPECIMENS USING 4-TRIFLUOROMETHYL-*o*-PHENYLENEDIAMINE

*Subject or group***		*Total selenium (ppb)**				
		Urine	*Hair*	*Nails*	*Saliva*	*Milk*
A,	A_1	31.4	394.5	852.5	7.5	–
	A_2	33.7	689.0	1132.5	6.0	8.0
B,	B_1	28.3	723.0	412.0	–	–
	B_2	30.1	1473.5	377.0	–	–
C,	C_1	11.5	935.5	531.5	–	–
	C_2	28.1	355.5	530.5	–	–
	C_3	27.2	646.5	574.0	–	–
D		26.8	–	–	–	–
E		56.0	726.5	390.5	–	–
F,	F_1	–	127.0***	–	–	–
	F_2	–	90.7***	–	–	–
	F_3	–	63.3***	–	–	–

* Values are means of two determinations.
** Each group denotes a family on the same diet.
*** Subjects on regular use of selenium-based shampoo (2.5% w/v). These values are in ppm.

Analysis of biological material for total selenium using 4-CF_3-o-PDA

Analytical procedure. The choice of method for sample oxidation in this study was based on earlier work[1], and employed wet digestion with nitric–perchloric acid mixtures. Digestions were carried out in Kjeldahl flasks because of the simple, uniform conditions attainable, with continuous refluxing of the boiling acid mixture. Apart from the need for occasional mixing of the digestion mixture and close supervision to avoid any localized or over-heating, the only drawback is the capacity of the heating device. In our case, this was limited to ten flasks or five duplicate analyses, taking between 4 and 5 h. Although this through-put could be increased with little, if any, extra time being involved, shorter digestion times were generally unsatisfactory and produced an increase in the number of spurious peaks.

No difficulty was encountered with the reduction or reaction steps. Here, although adjustment of acidity prior to reaction was not necessary, the total acidity of the solution (including *ca.* 0.5 ml of residual acid from the digestion) was kept near pH 0. With little significant difference in peak height for 1- and 2-h reaction time, the former was selected as the standard condition for all analyses. During this period, the preparation of a calibration curve was usually begun and, as a precaution against the reported[6,21,22], and observed, day-to-day variation in the ECD response, a new calibration curve was prepared for each batch of analyses.

As mentioned previously, the presence of certain foreign ions causes the otherwise colorless reaction mixture to develop pink to violet compounds, some of which are transferred to the toluene layer. Washing with perchloric acid is valuable in removing these as well as in minimizing the appearance of unwanted peaks.

Analytical results. The reliability of the method using 4-CF_3-*o*-PDA is demonstrated by the results presented in Table III. Apart from illustrating typical chromatograms obtained for actual samples, the precision attainable for a series of un-

interrupted, consecutive injections of a processed urine sample is shown in Fig. 12.

A comparison of the selenium content of blood and plasma, determined by GC and an automated fluorimetric method[25], is shown in Table IV. Included in these data for comparison are a number of results obtained with 4-NO_2-*o*-PDA. Finally, Table V gives the selenium levels of specimens supplied by healthy human donors. The subjects included adults (aged 30 ± 3 years) and children (subjects C_3 and F_3) living close to the University campus. Only Group F showed high selenium levels in hair due to the regular use of selenium-based shampoos, a practice which has been reported[26] to elevate normal levels by as much as 40 times and, in some cases, to levels[27] in the range 33–72 ppm. In general, the results agree with the levels in various biological materials, where these have been reported. However, it is emphasized that the analyses are intended only to demonstrate the applicability of the new reagent and not, for example, as a study or assessment of the selenium status in humans.

With real samples, the detection limit of the method is *ca.* 5 ppb and is, therefore, comparable with the value of 1–10 ppb reported[1] for 4-NO_2-*o*-PDA.

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