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LIQUID CHROMATOGRAPHIC DETECTOR FOR ORGANOSULPHUR COMPOUNDS BASED ON A LIGAND-EXCHANGE REACTION

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SUMMARY

A method is described for the post-column reaction detection of organosulphur compounds in liquid chromatography, which is based on a ligand-exchange reaction between the palladium(II)–calcein complex and the sulphur-containing compounds. The release of free calcein provides an indirect measure of the amount of organosulphur compounds via fluorescence detection.

The kinetics of the reaction and the signal intensity have been studied as a function of the structure of selected organosulphur compounds and of parameters such as carrier- and reagent-stream composition and temperature. Optimal conditions are as follows: an aqueous mobile phase of pH 5–7, to which may be added up to 20% of methanol; an elevated reaction temperature of around 60°C and a palladium(II)–calcein reagent solution of *ca.* 10^{-5} *M*, which should contain some zinc(II) to enhance the fluorescence intensity. Detection limits, which are dependent on the structure of the compounds, typically are between 0.5 and 1.0 ng. Good linearity is observed over a two- to three-order concentration range.

The method has been applied to the determination of ethylene thiourea in *e.g.* wash-water of apples and tomatoes, and to the detection of penicillamine in spiked serum and urine.

INTRODUCTION

Divalent palladium and other metal ions such as copper(II) and nickel(II) are known to quench efficiently the fluorescence of suitable ligands such as calcein (fluorescein-2,7-bismethyliminodiacetic acid) upon complex formation[1,2]. In the presence of a reactive sulphur group which competes for the palladium(II), the palladium(II)–calcein complex is destroyed and calcein is released. Its fluorescence —which is maximal at pH values of *ca.* 7 ($\lambda_{ex.}$ and $\lambda_{em.}$ are 492 and 512 nm, respectively)— is a measure of the amount of organosulphur compound present. This technique, which has earlier been used in thin-layer chromatography (TLC)[3], has been adapted in this study to post-column reaction detection in liquid chromatography (LC). Twenty sulphur-containing compounds, such as thiols, thioethers, thioketones and disulphides, were chosen for the investigation. Since the ligand-exchange reaction

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is a relatively slow process, it was necessary to use segmented flow systems and elevated temperatures. The final aim was to apply this detection mode to the selective determination of relevant sulphur-containing compounds in complex matrices.

EXPERIMENTAL

Apparatus

Fig. 1 shows schematically the detection system set up for monitoring the effluent from the LC system. A Technicon (Tarrytown, NY, U.S.A.) Model II pump equipped with standard Tygon tubing was used to deliver the air-segmented reagent streams as well as to control the flow passing through the fluorometer; the latter was either a Kontron (Zürich, Switzerland) SFM 22 or a Perkin-Elmer (Norwalk, CT, U.S.A.) 3000 spectrofluorometer. Reaction occurred in 2-mm I.D. glass coils which were heated in a Technicon oil-bath. A standard Technicon air-injection piece, A-10 T-piece and debubbler were used.

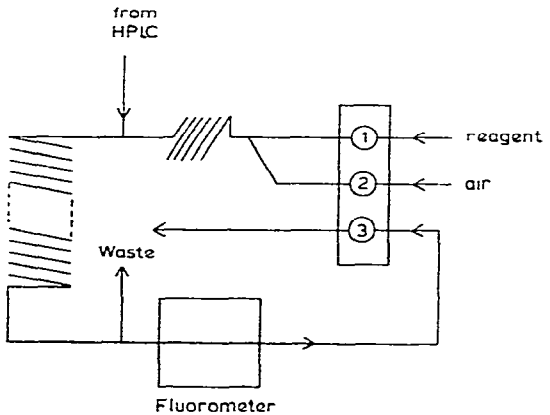


Fig. 1. Auto-analyzer arrangement for post-column ligand-exchange reaction. 1 = Palladium–calcein solution (0.32 ml/min; black). 2 = Air (0.32 ml/min; black). 3 = Debubbled stream through detector (0.43 ml/min; orange). HPLC, 0.3–0.6 ml/min.

For LC, a Perkin-Elmer Series II pump and a Rheodyne (Berkeley, CA, U.S.A.) six-port valve with a 100-μl loop were used. A 25 cm $\times$ 4.6 mm I.D. stainless-steel column prepacked with 5-μm RP-18 Supelcosil (Supelco, Bellefonte, PA, U.S.A.) served for the separation of the thioureas. For the separation of the amino acids a 25 cm $\times$ 4.6 mm I.D. column pre-packed with 10-μm Partisil 10 SCX (Chrompack, Middelburg, The Netherlands) cation exchanger was employed. A Perkin-Elmer LC-55 variable-wavelength detector was placed between the outlet of the analytical column and the post-column reaction detector.

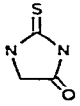
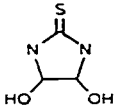
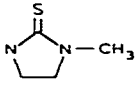
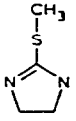
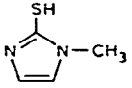
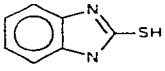
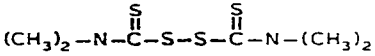
Reagents

Table I lists the compounds studied, together with their chemical structures. The thioureas were gifts from Dr. F. Iverson (Health Protection Branch, Ottawa, Canada); penicillamine was purchased from Serva (Heidelberg, G.F.R.) and the

TABLE I

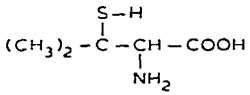
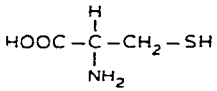
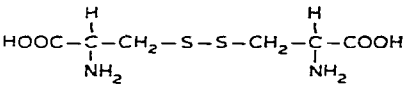
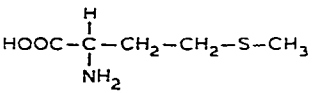
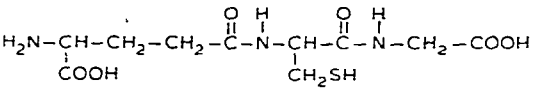
STRUCTURES AND DETECTION LIMITS OF THE INVESTIGATED COMPOUNDS

Detection limits were determined by plug injection using a 9-min reaction time in a pH 7 phosphate buffer and a temperature of 55°C.

Name	*Structure*	*Detection limit (ng)*
Ethylenethiourea (ETU)		8
Thiohydantoin		7
4,5-Dihydroxy-ETU		9
N-Methyl-ETU		10
S-Methyl-ETU		300
Methimazole		0.8
Thioimidazole		0.3
Mercaptobenzimidazole		3
Thiram		1.5

(Continued on p. 168)

TABLE I *(continued)*

Name	*Structure*	*Detection limit (ng)*
Penicillamine		2
Cysteine		0.5
Cystine		3
Methionine		3
Glutathion (red)		5

other amino acids from B.D.H. Chemicals (Poole, Great Britain). The model compounds were dissolved in water at concentrations of 0.1–10 μg/ml. Glutathion and thiohydantoin solutions were stored at −25°C; all other solutions were kept at 4°C. Calcein (Merck, Darmstadt, G.F.R.) was dissolved in 0.01 *M* NaOH. Palladium chloride (Drijfhout & Zn, Amsterdam, The Netherlands) was dissolved in 0.1 *M* HCl. The palladium(II)–calcein reagent was prepared[3] by mixing equal volumes of $1 \cdot 10^{-3}$ *M* solutions of both components; the mixture was allowed to stand overnight and then diluted *ca.* 100-fold (see below) with a phosphate buffer of pH 7. If required, a buffered solution of zinc sulphate was added to the mixture before dilution. Distilled demineralized water and analytical-grade methanol (Baker, Deventer, The Netherlands) were used as solvents. For the separation of the amino acids, a 0.09 *M* citric acid–0.022 *M* Na_2HPO_4 (5:3) buffer[4] of pH 2.2 was employed. All mobile phases for LC were filtered over a 0.8-μm Millipore filter in an all-glass Millipore (Bedford, MA, U.S.A.) filter apparatus prior to use.

Sample pretreatment

Spiked urine samples were centrifuged and filtered over a 0.8-μm Millipore filter. Spiked serum samples were deproteinated by adding 100 μl of acetonitrile–70% $HClO_4$ (4:1) to 0.5 ml of serum. Apart from centrifuging, no further pretreatment was necessary.

RESULTS AND DISCUSSION

Optimal conditions for the ligand-exchange reaction between palladium(II)–calcein and reactive sulphur groups were studied with ethylene thiourea (ETU) as model compound using plug injections.

Reagent solution

Contrary to earlier observations made in TLC[3], it was found that optimal signal-to-noise ratios are obtained by using a reagent solution containing equimolar amounts of both components instead of a 25–50% excess of palladium(II). Linear calibration curves (for ETU) having $R = 0.988$–0.996 were obtained with reagent concentrations of 10^{-4}–10^{-5} *M*. Since it is advisable to use a palladium(II)–calcein concentration as low as possible in order to minimize light-absorption and/or quenching effects (see below), 10^{-5} *M* solutions were routinely used.

Carrier stream

The influence of the pH of the reaction medium (carrier stream plus reagent solution) on the fluorescence signal revealed an optimum value of *ca.* 7 both with a purely aqueous and with a water–methanol (4:1) mixture. Lowering the pH to 5 resulted in a minor loss in sensitivity; however, the linearity was less satisfactory than at neutral pH conditions.

The methanol content of the carrier stream was varied between 0 and 60%. Addition of up to 20% methanol did not result in a significant decrease of fluorescence intensity. However, a further increase to *ca.* 40% led to a drop in signal of 30–40%. The addition of methanol had no noticeable effect on baseline noise. Probably, increasing the methanol content of the reaction medium considerably decreases the speed of the ligand-exchange reaction.

Addition of metal ions (10^{-3} *M*) other than palladium(II) to a purely aqueous carrier stream was studied in view of possible signal enhancement. As is to be expected[2], addition of copper(II) caused considerable quenching; cadmium(II) had no noticeable influence, and aluminium(III) and chromium(III) effected a slight increase in signal intensity. A significant (25–40%) increase in fluorescence intensity was noted when adding zinc(II) to either the carrier stream or the reagent solution. Addition of the metal ions tested did not exert influence on the retention behaviour of the organosulphur compounds in LC. Over the range 10^{-3}–10^{-8} *M*, the zinc(II) concentration was found not to be critical; addition of zinc sulphate to obtain a final concentration of 10^{-5} *M* in the reagent solution was used routinely.

Temperature

Using pure water as carrier stream, a 10^{-4} *M* reagent concentration and a 7-min reaction time, the influence of temperature on fluorescence intensity was studied for seven model compounds. Over the range 25–75°C a considerable signal enhancement was invariably found, obviously as a result of more rapid ligand exchange at elevated temperatures. For thiols this enhancement typically was two- to three-fold, and for thioketones five- to eight-fold. A temperature of *ca.* 60°C was used in all further studies.

Reaction kinetics

Residence times were varied between 1 and 8 min, using ETU, thiohydantoin and methimazole as test compounds. In all cases, a linear relationship between signal intensity and time of reaction was observed over a two- to three-order concentration range ($R = 0.9967$–0.9993), which indicates that the reaction is far from complete even after 8 min. Next, reaction times of 1 and 16.5 h were studied using a stopped-flow technique. On the basis of the detection limit determined for free calcein (using the same experimental conditions), ligand exchange between palladium(II)–calcein and ETU was calculated to be 12, 35 and 85% complete after 7 min, 1 h and 16.5 h, respectively. Obviously, even at elevated temperatures, the kinetics of the reaction is rather slow —which is in agreement with earlier observations[3]. Fortunately, this does not detract from the analytical potential of the detection principle: provided there is a linear relationship between signal intensity and amount of solute[5] (as was indeed observed; see above), a post-column reaction does not have to go to completion in order to be analytically useful.

For the rest we should note that air segmentation was an efficient means to reduce band-broadening in the post-column reactor, even for the prolonged reaction-time studies mentioned above (*cf.* ref. 6). Using a reaction time of 9 min the contribution made by the reactor to total band-width was $\sigma = 4.4$ sec. This contribution was mainly caused by the debubbler, as is apparent from the fact that band-broadening was found to be essentially the same after 3- and 9-min reaction time, and even after the 1- and 16.5-h stopped-flow-mode experiments.

Detection limits

For all compounds tested, detection limits are given in Table I. They were determined using a 9-min reaction time, and plug injection. Inspection of the data for the thiourea derivatives shows that thiols display slightly lower detection limits than do thioketones, while the single thioether studied lags far behind. In the case of the amino acids, there is no clear correlation between structure and detection limit.

As regards detection, one should keep in mind that, unfortunately, the native fluorescence of calcein is diminished due to an inner filter effect and possibly to quenching phenomena caused by the excess of palladium(II)–calcein present in the carrier stream. To quote an example, the detection limit of calcein in a neutral aqueous medium is $3 \cdot 10^{-8}$ mol/l; in the same medium with $0.9 \cdot 10^{-5}$ *M* reagent and 10^{-5} *M* Zn(II) added, a value of $1.5 \cdot 10^{-7}$ mol/l was determined. In order to keep the loss in sensitivity as small as possible, one should use a low reagent concentration.

Chromatography

The thiourea derivatives mentioned in Table I, excepting S-methyl-ETU, were separated on a RP-18 Supelcosil column using a 0.1 *M* phosphate buffer of pH 7 as mobile phase. Six out of the seven compounds eluted with capacity factors, k', ranging from 0.45 (4,5-dihydroxy-ETU) to 8.2 (N-methyl-ETU). For the elution of mercaptobenzimidazole ($k' = 6.0$) pH 7 buffer–methanol (4:1) was a suitable mobile phase. Detection limits for the relatively weakly retained thioureas were about the same as those recorded when using plug injections. Repeatability was satisfactory (relative standard deviation = 2.3%; $n = 10$).

For the sulpur-containing amino acids an ion-exchange system modified from

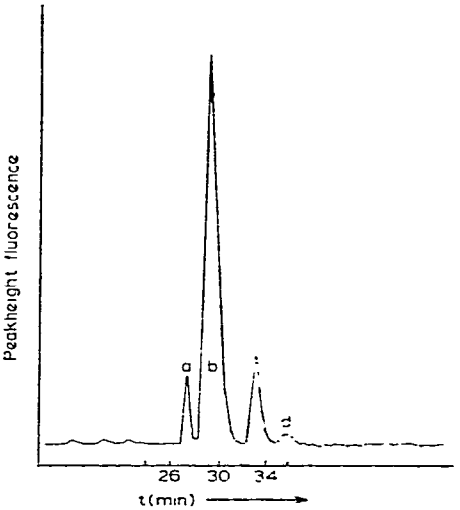


Fig. 2. Separation of (a) glutathion (red), (b) cystine, (c) cysteine and (d) methionine on a Partisil 10 SCX column. Mobile phase, pH 2.2 buffer; reaction time, 20 min; temperature, 55°C. For further details, see text.

a paper by Eggli and Asper[4] was selected. A satisfactory separation was obtained on a Partisil 10 SCX cation exchanger, with a pH 2.2 buffer as mobile phase; the flow-rate was 0.3 ml/min (Fig. 2). Due to the low pH value of the mobile phase, the palladium(II)–calcein reagent solution had to be diluted with 0.4 *M* NaOH–0.4 *M* NaH_2PO_4 (29:50) instead of the usual pH 7 buffer in order to adjust the final pH in the reaction coil to *ca.* 5.5.

Application

Samples of urine and of wash-water of apples and tomatoes were spiked with ETU, which is an important metabolite of ethylene-bisdithiocarbamate fungicides and is suspected to be carcinogenic[7]. With the wash-water, the presence of *ca.* 50 ng of ETU on 250 g of fruit could be detected. The selectivity of the ligand-exchange detection principle allowed direct injection of the water samples, whereas detection by means of UV absorption at 254 nm (detection limit of ETU, 5 ng) did not yield a positive result due to the interference of a broad UV-absorbing band. In the case of spiked urine samples, a rather high detection limit of 18 ppm of ETU was obtained, which was caused by the incomplete separation of ETU from the broad "solvent" peak; this problem has not adequately been solved as yet.

In another application, penicillamine —at the 1–10-ppm level— was determined in serum and urine samples; the sample preparation is described in the experimental section. A chromatogram of a spiked serum sample is shown in Fig. 3. On the basis of retention-time data, at least some of the peaks which precede penicillamine can be attributed to sulphur-containing amino acids. In this application, and also with the urine samples, the detection limit of penicillamine was found to be *ca.* 20 ng. This relatively high value (*cf.* Table I) is caused by the rather low pH value of 5.5 of the reaction medium —which in its turn is due to the demands of the mobile phase that has to be used in chromatography (see above)— and the rather high contribution to band-broadening effected by the cation-exchange column used.

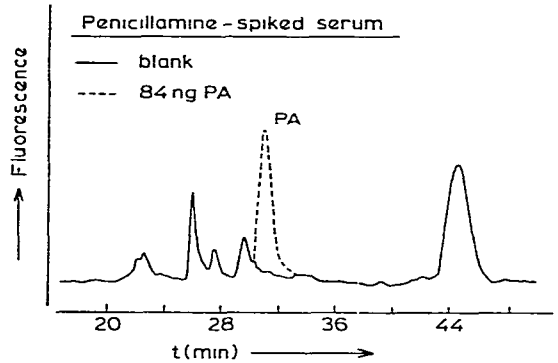


Fig. 3. Chromatogram of penicillamine–spiked serum sample. Conditions as in Fig. 2.

CONCLUSION

The principle of ligand-exchange based on a reaction with the palladium(II)–calcein complex has proven useful for the detection of thiols, thioketones, thioethers and disulphides. Its selectivity will often permit detection of traces of these compounds in complex matrices with a minimum of sample handling. The method appears to be particularly attractive for compounds which display poor UV-absorbing or fluorescence properties, such as the sulphur-containing amino acids. The detection limits are comparable to those obtained by means of electrochemical detection with a mercury-drop[8] or mercury-pool[4,9,10] detector based on complex formation on the mercury surface. By extending the residence times in the reactor the detection limits can be improved still further.

Generally speaking, it can be said that the ligand-exchange principle can well be expanded to the detection of other classes of compounds, *e.g.* those having nitrogen- or oxygen-containing reactive groups. The selection of suitable metal-complex reagents should lead to more or less specific detection techniques for such types of compounds.

ACKNOWLEDGEMENTS

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