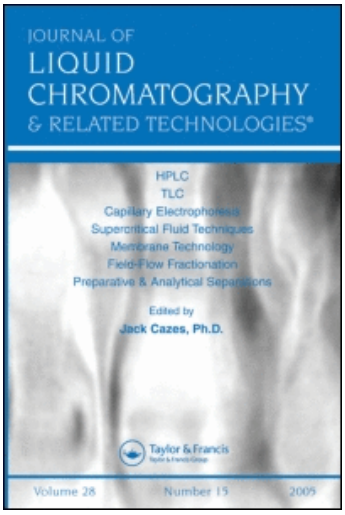

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UTILITY OF REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR ON-LINE YIELD DETERMINATION OF RADIOCHEMICAL SEPARATIONS: STUDIES WITH COBALT

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ABSTRACT

This article indicates the potentials of high performance liquid chromatography (HPLC) as a radiochemical technique for mulitielement separation of neutron irradiated samples. The focus lies on the convenience to use the detector signal of the eluted components to indicate the chemical yield (percentage recovery) of the analyte, which has often proved to be a crucial step in radiochemical separations.

Two signals have been utilised. The UV signal of the metal-ligand complexes separated by reversed phase HPLC and the radioactive response as a result of sample irradiation or carrier-tracer addition. Change in ratio is discussed between the two signals, if any, for a specific sample. Losses of metal as much as 65% were simulated and corrected using the individual UV response.

The method promises improved accuracy for elemental analysis despite losses suffered during the various chemical steps. The procedure omits the necessity of additional analytical steps for yield determination. The present article aims at the chromatographic part of the study. Cobalt as cobalt diethyldithiocarbamate has been used to demonstrate the viability of the concept. The separation was developed on a C18 reverse phase analytical column and optimised on a semi preparative one.

INTRODUCTION

Among the variety of analytical tools currently available for trace element determinations, neutron activation analysis (NAA) is often used because of its multielement capability and sensitivity. NAA involves the bombardment with neutrons, from a nuclear reactor, on the sample material converting atoms into radioactive nuclides. The type and energy of the radiation of the radionuclides and their decay rate are specific identifying characteristics. After irradiation and subsequent decay period the sample can be directly measured for its induced radioactivity using suitable radiation detectors. The amount of induced radioactivity is related to the quantity of the original isotope and therefore, to the element present in the original material. This type of analysis is termed as instrumental neutron activation analysis (INAA). For some trace elements purely instrumental measurement may not be sufficient to obtain accurate results because of the high matrix activity induced which hinders the measurement of the targeted element. On such occasions a post-irradiation radiochemical step is warranted. This radiochemical neutron activation analysis (RNAA), involves the actual separation of the analyte of interest from the radioactive matrix. This is usually done by the addition of elemental carriers, which are known amounts of trace elements of interest in non-radioactive form, having the same chemical form and valence state to that of the activated analyte. Since the analytical signal is obtained from one

specific radionuclide of the element, any amount of carrier can be added depending upon the separation chemistry. Due to the macro amounts of addition as compared to the microlevel concentration of the element of interest in the sample, the carriers facilitate chemical separation and enable the determination of the percentage recovery (yield) of the element. This is an advantage enjoyed by RNAA and IDMS (isotope dilution mass spectrometry). While in case of IDMS, the probability of contamination with stable elements from the glassware and chemicals is noticeable, absence of blank values from reagents added after the end of irradiation (since they do not interfere with the radioactive measurement) makes RNAA a preferred analytical reference method particularly for low levels of trace elements.

Most RNAA separation procedures are designed for a high and reproducible yield. Therefore a yield determination on each individual sample is generally not practised. However, when ultimate accuracy and precision is required , eg for certification of reference materials, individual yield determinations may become mandatory (1). Yield assessment for single element determination is usually performed by classical analytical methods with superior accuracy and precision, such as gravimetry, titrimetry or spectrophotometry. Reactivation of the counted fraction for one or two elemental chemical yield determination has also been reported (2), but in general multi-element chemical yield using classical analytical methods can be very tedious and often impossible.

So far HPLC has seldom been exploited in RNAA for elemental separation and determination of its chemical yield. Nevertheless, in other radioanalytical fields, major advantages of this technique has been explored, namely multi-element analysis in a single run, real-time detection of the separated individual elements, possibility of preparative scale separation and the ease of decontamination when using radioactivity. These features make this system also suitable for RNAA. In HPLC the separation and yield determination can be combined into one single step ("on-line"), thereby eliminating the inherent errors of a second analytical method.

A recent review (3) shows a growing interest of trace metal analysis by HPLC . The ability of diaklyldithiocarbamates to form complexes with a large number of metals and the properties of these complexes, make diakyldithiocarbamates the most preferred reagents in trace element analysis (4-6). A variety of separation conditions, detectors, dithiocarbamates and columns have been utilised (7-20). As far as element specific detection is concerned, electrochemistry (19,21,22) and atomic spectrometry (23) appear to be the universal choice. While HPLC has been widely utilised for metal-chelate separations, there has been no routine application of this chromatography technique for analyzing neutron activated samples presumably because of the increased background count rate due to induced radioactivity of the matrix and the need to inject high sample volume to enjoy sufficient sensitivity of radioanalysis. However, prevailing upon all the shortcomings, we wish to report the first use of HPLC with UV

detection for the simultaneous elemental separation and yield determination for RNAA.

The aim of the present study is twofold : firstly to demonstrate the capability of reversed phase HPLC for radiochemical separations in RNAA and secondly to use the UV absorbance signal of the eluted component to determine on-line the chemical yield. The magnitude of UV absorbance signal of the organo-metal compound (enhanced due to the addition of non-radioactive carrier of the analyte) indicates the chemical yield. The radioactive response (as a result of sample irradiation or radioactive tracer addition to mimic sample irradiation) determines the elemental assay and the concentration of the analyte. The constant ratio between the two signals, UV absorbance and radioactive response, for a specific sample has to be validated. The metal complex is formed by the addition of the known amounts of both the carrier of the target element and a ligand. The elemental behaviour is independently determined by gamma ray spectrometry of the radionuclide.

This paper deals with cobalt as a model element, selected because of its analytical challenge (24) and favourable nuclear properties of its (radio)nuclides. Sodium diethyldithiocarbamate (NaDDTC) was used as a complexing agent since its chelating properties are well documented. As the main extractable DDTC components, having high extinction coefficient, of a biological sample are zinc, iron and copper, the prime aim was to ensure the separation of CoDDTC from these metal dithiocarbamates. Although in

an irradiated sample, the presence of these elements do not pose a problem when measuring cobalt, separation of cobalt from these elements should result in better detection limit

EXPERIMENTAL

Chemicals and Equipment

Water from a Millipore Milli-Q Plus Water Purification system was used for all solutions and dilutions. Individual standard solutions of Zn, Cu and Co metal salts were prepared by dilution of the 1000 mg/L standard solutions (Tritisol, E.Merck, Germany). ^{60}Co and ^{65}Zn were produced by irradiation of their salts with thermal neutrons at a flux of 10^{13} neutrons cm^{-2} s^{-1} for 12 hours in the Institute nuclear reactor. 50 mL of carrier-tracer solutions (100 μg/mL) were prepared by diluting a known amount of ^{60}Co or ^{65}Zn with its respective standard solution. Aliquots were taken from these stock solutions for all further experiments. The ligand stock solution (50 mM) was prepared by dissolving 85.6 mg of sodium diethyldithiocarbamate (Fluka AG, Switzerland) in 10 mL water. This reagent was freshly prepared before complexation and stored in a refrigerator to be used on the same day. H_2SO_4 (96%), HNO_3 (65%), H_2O_2 (30%), $HClO_4$ (70%) and

CH_3COOH (96%) used were of Analytical Grade obtained from Merck. Sodium acetate (99.5% purity) was obtained from Riedel- de Haën AG, Germany.

The buffer solution consisted of 50 mM acetic acid (0.294 mL of 17 M acetic acid diluted to 100 mL with water) added to 50 mM sodium acetate (410 mg in 100 mL water) until a pH of 6.0 ± 0.1 was reached. Solvents used like methanol, chloroform and acetonitrile were of HPLC grade (J.T.Baker B.V. The Netherlands) and were used without further purification. The mobile phases were filtered through 0.45 μm Millipore filters and vacuum degassed prior to use.

The chromatography was performed on a modular system comprising of the following components: A LKB 2150 HPLC pump with ceramic lined pump heads, a LKB SuperRac fraction collector (LKB Produckter AB, Sweden), a Rheodyne 7125 titanium injector with 20 μl and 1.0 mL loops (Rheodyne Inc., USA), a Waters 484 Tunable Absorbance Detector (Waters Chromatography Division, Millipore Corporation, USA) and a Model 1020 Personal Integrator (Perkin Elmer Nelson Systems Division, Perkin Elmer Corp., USA).

The columns used were a Nova-Pak C18, 3.9 mm x 150 mm stainless steel analytical column with 4 μm particle size and 7% carbon load and a Nova-Pak C18 HR stainless steel semi-preparative column, 7.8 mm x 300 mm with 6 μm particle size and 7% carbon load. Both columns were supplied by Waters Chromatography.

Sample mineralisation

Most of the experiments reported refer to the optimisation of the radiochemical separation using non-irradiated materials unless mentioned otherwise. These materials were spiked with radiotracers and used as samples so as to mimic real-world matrixes. 200 mg of the biological reference material, NIST-SRM-1577A Bovine Liver, obtained from National Institute of Standards and Technology (USA) was taken in an Erlenmeyer flask. 1 mL each of Co and Zn carrier-tracer solution (i.e. ^{60}Co and ^{65}Zn with their respective standard solution) and 100 μg of Cu carrier were added. 2 mL of concentrated H_2SO_4 was added and the sample was allowed to charr at room temperature for 5 minutes. 2 mL of H_2O_2 was added dropwise to oxidise the contents. The sample turned pale yellow in colour. It was subsequently boiled for 10 minutes on a hot plate and a further 2 mL of H_2O_2 was added. 1 mL of HNO_3 was added to oxidise any remaining organic content, thereby averting violent reaction due to the addition of $HClO_4$. The sample was boiled for 5 minutes. After 10 minutes cooling time, 1 mL of $HClO_4$ was added and the sample was boiled until no more dense white fumes appeared. The flask was cooled and the colourless contents transferred to a 100 mL beaker, after repeated washings with water.

Extraction

To the mineralised solution, ammonia was added to bring the pH to 6.0 ± 0.1. The final volume was made to 75 mL with water. 5.0 mL of 50 mM NaDDTC was added and the mixture was quantitatively transferred to a 100 mL separating funnel with stop cock. The complex was extracted with a mixture of 70/30 (v/v) chloroform/acetone (3 x 5 mL) by vigourous shaking. The two phases were allowed to settle and then separated. Each was counted for radioactivity for 30 minutes on a GeLi detector to measure the extraction yield (between 85 to 97%). The organic phase was transferred to a clean 50 mL beaker and evaporated under gentle stream of nitrogen. The residue was dissolved in 200 μl chloroform and a mixture of 65/35 (v/v) acetonitrile/water was added to make the volume to 3.0 mL. This was then filtered using a Millex-HV, 0.45 μm filter unit (Millipore). An aliquot of 20 μL or 1000 μL was injected into the chromatographic system. As for processing the irradiated material only 100 μg of standard Co(II) carrier solution was added, and identical steps were followed.

Chromatographic method development

Initial work of developing the separation of CoDDTC complex from other DDTC complexes was tried using the analytical column. Having

decided to use reversed phase chromatography, the ideal choice for an organic modifier was between methanol and acetonitrile or both. It was observed that a binary mixture of water and one of the organic components did not give adequate resolution of the metal dithiocarbamate peaks. Hence, an acetate buffer(25) was used along with the organic component. 75/25 Acetonitrile/Buffer gave a satisfactory peak shape of cobalt dithiocarbamate, but the separation factor was only 1.1 between cobalt- and copper dithiocarbamate. By replacing acetonitrile with methanol, keeping the 75/25 ratio constant, the resolution between the metal dithiocarbamates improved (1.97) with increased retention time. Since it is known that the DDTC complexes may be unstable (21), 0.01 mM ligand was added to the mobile phase to reduce the decomposition of the complex. Tailing of the peaks was stopped by the addition of triethylamine (TEA) to the mobile phase. Although the CoDDTC complex has a high absorbance in the visible range, it was decided to select 254 nm, so as to be able to study the retention behaviour of the Zn- and CuDDTC complexes as well. It was observed that the resolution between zinc and cobalt was very poor ($\alpha < 1$) without base line separation. This was improved ($\alpha = 1.38$, see Table 1) by modifying the mobile phase to its final composition of 65/35 methanol/buffer. At a flow of 0.5 mL/min. the dithiocarbamates of Zn, Co and Cu were well separated on analytical column.

Once the analytical chromatography was developed, the immediate task was to extend this procedure to ascertain the ability to detect

TABLE 1.

Chromatographic properties of metal-DDTC complexes

Column and other conditions refer Figure 1.

Peak identity	ZnDDTC	CoDDTC	CuDDTC
Analytical Separation.			
t_0 = 3.15 min			
t_r (min.)	28.77	34.77	44.61
k'	8.13	10.13	13.16
α		1.38	2.01
Semi preparative separation			
t_0 = 2.45 min.			
t_r (min.)	40.93	48.85	63.33
k'	15.70	18.93	24.84
α		1.20	1.31

t_0 = Unretained sample elution time; t_r = Sample retention time;
k' = Column capacity ratio; α = Resolution of peaks

nanogram amounts of cobalt in neutron activated samples. As earlier work was performed only on synthetic sample i.e. by the addition of ^{60}Co carrier-tracer solution to the sample, facts like radioactive matrix interference on chromatography, radiochemical purity of the cobalt dithiocarbamate peak fraction were concealed. This information can only be obtained by actual neutron irradiation of the sample. By neutron irradiation of Bovine Liver, it was observed by gamma ray spectrometry that the cobalt peak fraction, separated after chromatography, had insufficient detection sensitivity, possibly because of the low injection volume of the sample, only 20 μL out of a total volume of 3 mL. Decreasing the dilution volume from 3 mL was not practicable since that was the minimum amount required to completely dissolve the dried CoDDTC complex, hence the only other alternative was to increase the injection volume. It was observed from earlier studies that the maximum amount that could be injected on the analytical column, without loss of resolution, was only 165 μL, i.e. a mere 5.5% of the sample volume. The only means to utilise all the sample volume was to scale-up to a larger column. Use of a semi-preparative column was the only option to inject larger amounts, thereby improving the efficiency in gamma-ray measurement.

A Nova Pak HR C18 7.8 mm x 300 mm was used, being able to tolerate a injection volume of 1.0 mL. Mobile phase conditions remained the same, the flow was restricted to 3 mL/min since the operating pressure at this flow was 100 bars which could not be exceeded. Figure 1 shows a

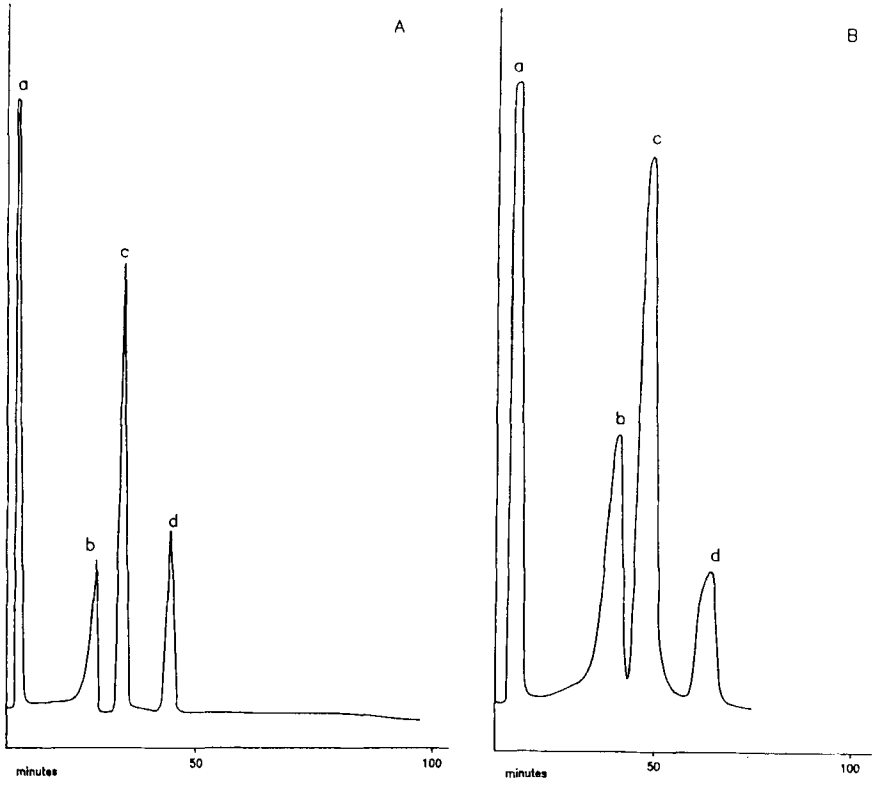

Figure 1 : Chromatographic separation of metal dithiocarbamates.

1A : Analytical separation, mobile phase used was 65/35 methanol/acetate, column = Nova-Pak C18, 3.9 x 150mm, flow rate = 0.5 mL/minute, sample volume = 20 μL.

1B : Semipreparative separation, mobile phase identical to the analytical separation, column = Nova-Pak C18, 7.8 x 300mm semipreparative column, flow rate 3.0 mL/minute, sample volume = 1.0 mL. Operating wavelength for both the separations was 254 nm at 0.05 a.u.f.s.

Peak a : ligand peak, b : ZnDDTC, c : CoDDTC and d : CuDDTC.

chromatogram of the separation of the dithiocarbamates in Bovine Liver on both the analytical and the semi-preparative column.

Chromatographic parameters

A litre of mobile phase was prepared by adding 650 mL of methanol to 350 mL of acetate buffer (pH=6.0) containing 50 mM of TEA and 0.01 mM NaDDTC. The two components were thoroughly mixed by stirring. The chromatograms were developed at a flow of 0.5 mL/min for the analytical column and 3.0 mL/min for the semi-preparative separation with both columns at ambient temperature. The pressure ranged between 80 and 100 bars for both the columns. The UV absorbance was measured at 254 nm at 0.05 a.u.f.s., and the peak fractions of CoDDTC and ZnDDTC were collected in 50 mL polyethylene containers and counted on a GeLi detector.

RESULTS AND DISCUSSION

The carrier-tracer solution with the fixed isotopic ratio of ^{60}Co (after decay correction) and ^{59}Co in presence of mineralised Bovine Liver was used to study possible experimental errors during pre-concentration and

chromatography. From this solution, varying aliquots were processed as samples, so as to verify the assumption that ratio of the count rate due to ^{60}Co and the UV absorbance signal due to ^{59}Co has not changed during the analytical procedure. Both the $^{59}CoDDTC$ peak area and the collected $^{60}CoDDTC$ fraction were evaluated (respectively by the $^{59}CoDDTC$ UV signal and the ^{60}Co counting result) as a function of different ^{59}Co masses.

Figure 2 shows the relation between the $^{59}CoDDTC$ peak area and varying amounts of $^{59}Co(II)$ in the range from 60 μg to 100 μg. From the scattering around the linearity in the figure (observe UV signal), it is obvious that the analytical control is lost during the process, a triplicate analysis displays a range of 10% in the UV signal. This can possibly be explained by the apparent losses occurring during mineralisation or extraction. The same figure also demonstrates the relation between the ^{60}Co counting result and the mass of ^{59}Co taken for chromatography. The observed range in ^{60}Co activity (9.4%) for an injected mass of 100 μg ^{59}Co, leads to a similar conclusion as drawn for the UV signal. 100 μg of ^{59}Co appeared to be a practical compromise for both extraction and chromatographic purposes.

Figure 3 demonstrates the beneficial effect of normalizing the ^{60}Co signal at any concentration, using the UV signal of 100 μg ^{59}Co as a norm. Considering this linearity study as a deliberate attempt to mimic variations in yield, with losses in Co upto 35%, it is pivotal to observe that variations in one signal is completely reflected by variations by another, $R = 0.991$.

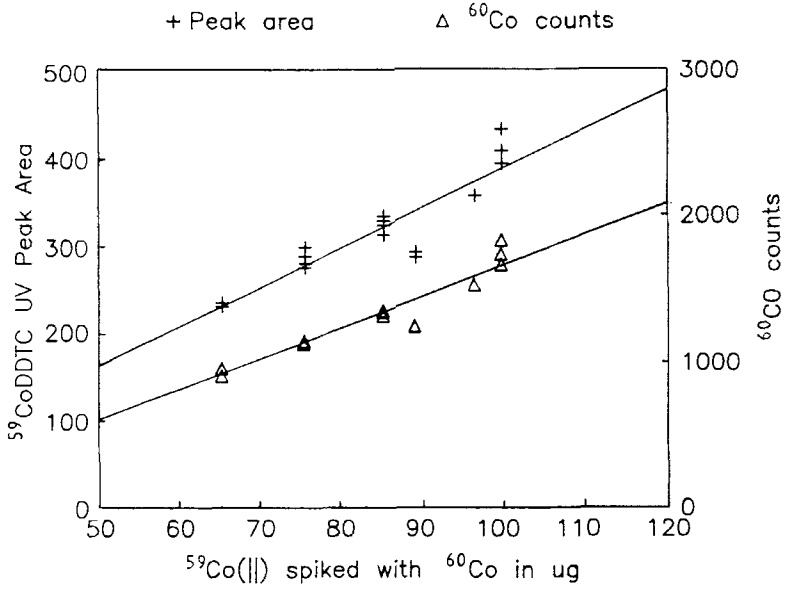

Figure 2 : Signal responses with increasing Co(II). ^{59}Co(II) versus UV signal/^{60}Co counts.

The relative standard deviation in the normalized ^{60}Co counting result of 16 analyses (determinations of Figure 2) in a range from 60 to 100 μg points to only 3.5% which is agreement with the counting statistics. This result suggests that variations in yield can be compensated for.

Not relying only on the simulated samples, linearity was also confirmed using irradiated cobalt as source of ^{60}Co. Increasing amounts of cobalt solution in nanogram level were irradiated to mimic a range of Co, all with the same natural matrix. After processing according to the usual RNAA procedure, the samples were subjected to HPLC after addition of 100 μg ^{59}Co as non-radioactive carrier and complexing with DDTC. The

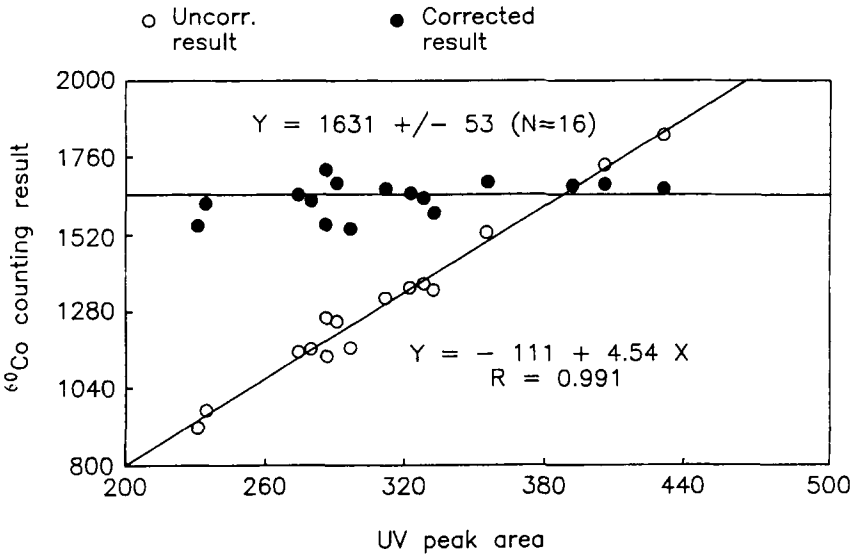

Figure 3 : Corrected and un-corrected ^{60}Co signal as a function of the UV response.

UV ^{59}CoDDTC peak area was fairly constant (s.d. 2.6% ,n = 5). This is as expected since the Co added as part of the sample is negligible compared to the added ^{59}Co (^{59}Co/^{60}Co ≥1000). The UV peak area can therefore be used to correct for variations in yield, also in actual samples after mineralization. Determination of cobalt concentration in various biological materials using this method is elaborated elsewhere (26).

CONCLUSIONS

From the results it can be concluded that a reliable "on-line" yield of the radiochemical separation using UV detection of CoDDTC *after*

separation from other metal complexes by reversed phase HPLC is possible. The correlation between two signals of cobalt, UV and radioactive from ^{59}Co and ^{60}Co respectively, is almost perfect (R = 0.991) regardless of the yield of the chemical process. This means that the signal of one isotope can be used to correct for procedural losses in the analysis of the other. This yield determination can be performed without the inclusion of an extra elemental analytical technique, since the same chromatography is used for both the radiochemical separation and yield determination. As several metal complexes can be separated in one run, the method intrinsically reveals multielement capability.

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