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RADIO-GAS CHROMATOGRAPHY ON WIDE-BORE CAPILLARY COLUMNS EQUIPPED WITH A SYNCHRONIZED ACCUMULATING RADIOISOTOPE DETECTOR

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SUMMARY

A gas chromatographic system with wide-bore capillary columns and synchronized accumulating radioisotope detector (SARD) was developed. A direct injection method and constant-flow regulation were applied. The performance of wide-bore capillary columns was good and the correspondence of the resolution obtained with SARD and that with mass detection was excellent. It was proved that the apparatus could detect of the order of 1 pg of ^{3}H-labelled substances (3.7 TBq/mmol) and compete in terms of sensitivity with gas chromatography–mass spectrometry.

INTRODUCTION

Radio-gas chromatography (radio-GC) in which the radioactivity eluting from a gas chromatograph is continuously detected, is an important technique because of not only the high resolution, high sensitivity for soft β-emitters, high sensitivity of mass detection, ease of handling and rapidity, but also the excellent correspondence with gas chromatography–mass spectrometry (GC–MS).

Polar and unstable biologically active substances have recently become detectable as a result of the introduction of capillary columns into GC analyses. Hence the development of radio-GC on capillary columns is important. In our laboratory, a synchronized accumulating radioisotope detector (SARD) having high resolution and sensitivity was developed and connected to a GC apparatus on packed columns (packed GC–SARD) [1]. Subsequently a GC–

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SARD on capillary columns (capillary GC–SARD) was manufactured, and the performance is discussed in this paper.

EXPERIMENTAL

Radioactive samples and reagents

n-[^{14}C]Hexadecane (specific activity 2.3 GBq/mmol; radiochemical purity >97%) and [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (specific activity 3.1 TBq/mmol; radiochemical purity >90%) were purchased from the Radiochemical Centre (Amersham, U.K.). DL-2-[4-(2-Thienylcarbonyl)phenyl]propionic-[methin-^{14}C] acid (DL-[^{14}C]suprofen; specific activity 48 MBq/mmol; radiochemical purity >95%) was synthesized as in the previous paper [2]. The labelled substances were used with dilution by mixing with them the corresponding non-labelled substances (carriers) to a specified specific activity or without dilution by the carriers as described later. [^{14}C]Hexadecane, [^{3}H]androstenedione and DL-[^{14}C]suprofen were used as cyclohexane, toluene–ethanol (9:1, v/v) and methanol solutions, respectively. Hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Peptide Institute (Osaka, Japan) and (*S*)-(−)-1-(1-naphthyl)ethylamine from Norse Labs. (Newbury Park, CA, U.S.A.), and these reagents were used as dichloromethane solutions (1 mg/ml). Copper oxide (wire, for elemental analysis, dimensions ca. 5×1 mm) and iron (sponge, 20–30 mesh) were purchased from Wako (Tokyo, Japan) and Aloka (Tokyo, Japan), respectively. Iron was stored in a vessel filled with nitrogen. Other reagents were purchased from Wako and were of analytical-reagent grade.

Capillary radio-GC system

Fig. 1 shows a schematic diagram of the complete unit. A Shimadzu GC-14A gas chromatograph was equipped with a single injection unit for direct injection and a wide-bore fused-silica capillary column (12 m×0.53 mm I.D.) coated with apolar CBP1 (1 μm film thickness) by use of a wide-bore adapter. The column effluent could be split to an oxidation–reduction tube and a flame ionization detector (FID). The former effluents were subjected to oxidation ($^{14}C \rightarrow {}^{14}CO_2$) or oxidation–reduction ($^{3}H \rightarrow {}^{3}H_2O \rightarrow {}^{3}H_2$), and the resulting gas was introduced into the SARD after mixing with counting gas (methane) in the mixing joint as described previously [3]. The oxidation–reduction tube was packed with about 10 g of copper oxide, or about 5 g of copper oxide plus about 4 g of iron, and heated to about 800°C. The splitting ratio was predetermined by the lengths of empty fused-silica capillary tubes (0.2 mm I.D.) between the splitter and the FID or the oxidation–reduction tube. The carrier gas and make-up gas were helium. All the flow-rates of helium except the make-up gas for FID were subjected to constant-flow regulation. The flow-rate of the carrier gas was 5–20 ml/min. Make-up gas (10–15 ml/min) was introduced immedi-

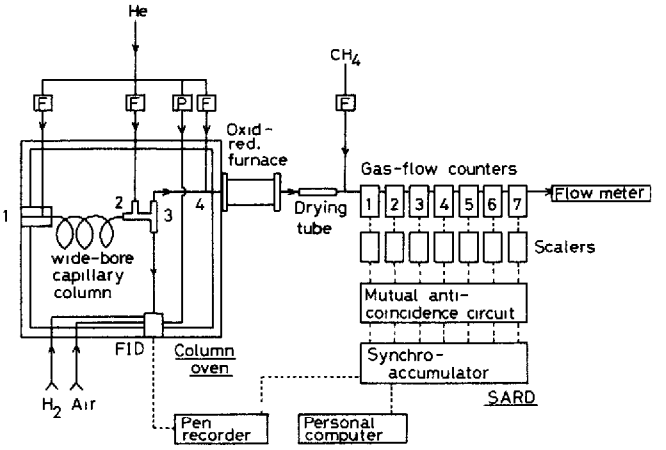

Fig. 1. Schematic diagram of radio-GC with a wide-bore capillary column: 1 = injection port (direct injection); 2 and 4 = addition of make-up gas; 3 = column effluent splitter; F = constant-flow regulator; P = constant-pressure regulator.

TABLE I

PARAMETERS FOR THE DIFFERENT COUNTING CONDITIONS

The sampling time equals the transit time of gas particles in one counter tube.

Counting condition	Flow-rate (ml/min)		Sampling time (s)	High voltage (V)
	Helium	Methane		
A	50	100	4	2.78
B	50	150	3	2.95
C	50	250	2	3.06
D	50	550	1	3.20

ately before the splitter in order to not only stabilize the splitting ratio but also to reduce the disorder of gas flow in the splitter. Further make-up gas was added immediately before the oxidation–reduction tube so that the total flow-rate in the tube was 50 ml/min in order to reduce the disorder of gas flow in the tube.

The construction and operating conditions of the SARD were the same as in the preceding paper [1]. It was possible to select the sampling time (the transit time of gas particles in one counter tube) between 1 and 4 s by regulating the total flow-rate of counting gas and helium in the counter tubes. Various parameters for each counting condition are summarized in Table I. The SARD could be connected also to the GC instrument with packed columns by chang-

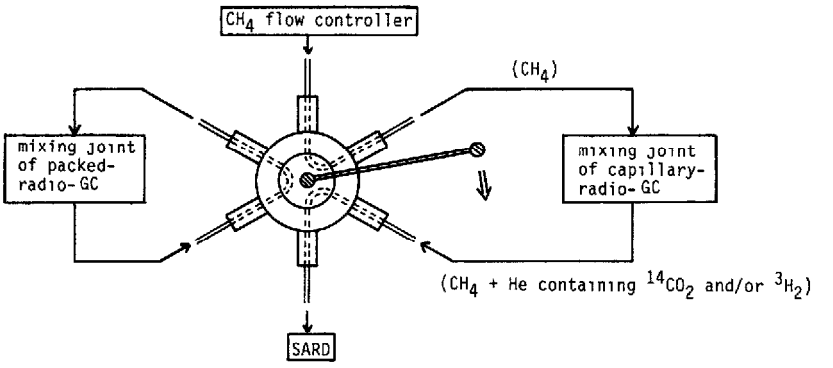

Fig. 2. Six-way valve to select gas flow lines for either capillary GC–SARD or packed GC–SARD. Here the flow lines for capillary GC–SARD are shown. If the lever is shifted down by an angle of 60° as indicated by the arrow, the flow lines for packed GC–SARD are obtained.

ing the gas flow lines by means of a six-way valve as shown in Fig. 2. The signals from the FID and SARD were recorded by a two-pen recorder set at a chart speed of 10 mm/min. SARD signals were memorized and quickly processed by a personal computer. Samples were injected with a Hamilton 10-μl microsyringe or 5-μl zero-dead-volume microsyringe. The latter microsyringe was used for the calibration of the system using [^{14}C]hexadecane [4]. The sample radioactivity was measured in 10 ml of toluene base scintillation cocktail with a liquid scintillation counter (Aloka LSC 903 or 1000).

Influence of sampling time on maintenance of chromatographic resolution

A 2-μl volume of [^{14}C]hexadecane (20 MBq/mmol) solution (ca. 48 Bq/μl) was injected into the GC–SARD and the radioactivity was measured under the counting conditions A, C and D, simultaneously with FID mass detection. The flow-rate of the carrier gas was set at about 5 ml/min. The temperature of the injection port was set at 220°C and that of the column oven was programmed from 100 to 220°C at 40°C/min.

Comparison of radio-gas chromatogram produced by capillary GC–SARD with that by packed GC–SARD

About 100 μl of DL-[^{14}C]suprofen (48 MBq/mmol) solution (ca. 192 Bq/μl) were flushed by a stream of nitrogen and the residue was reconstituted with 1 ml of dichloromethane. To the solution were added about 100 μl each of hydroxybenzotriazole solution, carbodiimide solution containing 1% pyridine and naphthylethylamine solution. After vortexing, the mixture was allowed to stand for 1.5 h at room temperature. The reagents were extracted from the reaction mixture with two 1-ml portions of water in order to stop the reaction. The organic layer was placed in a 1-ml sample tube with a disposable Pasteur pipette and evaporated to dryness under a stream of nitrogen. The residue was

reconstituted in about 50 μl of ethyl acetate, followed by three 1-μl injections into both GC–SARDs under counting condition C. The temperatures of the injection port and column oven were set at 300 and 280°C, respectively. The flow-rates of carrier gas in the capillary GC–SARD and the packed GC–SARD were set at 20 and 40 ml/min, respectively. A silanized glass column (1 m×3 mm I.D.) packed with 1.5% OV-1 on Shimalite W AW-DMCS (80–100 mesh) (Shimadzu) was used.

Resolution

Two consecutive injections of about 1-μl portions of [^{3}H]androstenedione (37 MBq/mmol) solution (ca. 74 Bq/μl) into the GC–SARD were made at intervals of 10, 20 or 30 s. The temperatures of the injection port and column oven were set at 270 and 250°C, respectively. The flow-rate of the carrier gas was 20 ml/min. Counting condition C was used.

Detection limits

A 2-μl volume of [^{3}H]androstenedione (3.1 TBq/mmol) solution (5.6 or 59 Bq/μl) was injected into the GC–SARD seven times. The temperatures of the injection port and column oven were set at 270 and 250°C, respectively. The flow-rate of carrier gas was 10 ml/min. Counting condition A was used.

RESULTS AND DISCUSSION

In radio-GC, the method in which the radioactivity in the column effluents is continuously monitored should be used, considering the maintenance of chromatographic resolution and the simultaneous detection of mass and radioactivity. Radioactivity detectors in the continuous method have the inherent drawback that the transit time of gas particles in the detector should be as short as possible to maintain the chromatographic resolution, while any reduction in the transit time is accompanied by a corresponding drop in the counting time, i.e., the detection sensitivity, which is based on the integral peak intensity [5]. No capillary radio-GC method reported so far has overcome this drawback [6–12]. In all reports, the widths of the radioactive peaks are much wider than those of the mass peaks, and the correspondence is very poor. The SARD described in the preceding paper [1] made it possible to improve the maintenance of chromatographic resolution obtained in GC on packed columns markedly without appreciably sacrificing the detection sensitivity. Hence SARD seemed to be worthy of application in capillary GC.

Capillary columns of 0.20–0.33 mm I.D. are usually used. However, the applications of such columns to radio-GC are accompanied by three significant problems. The first is the method of sample introduction. Split and splitless injections are generally used in capillary GC. The former is not suitable for radio-GC as the sensitivity is markedly reduced. The application of the latter

leads to an error in the balance of radioactivity owing to the purge. Solid injectors are tedious to use although they have been applied in capillary radio-GC [7,10,11]. The second problem is the amount of component loaded in the samples and the injection volume of the liquid samples. It is generally desirable that the amount loaded is less than 50 ng per component and the injection volume is as small as possible. However, radio-GC has the following features that are contrary to these requirements: (i) large amounts of the carriers can be added to the samples in order to reduce the loss of labelled substances by vaporization, adsorption and thermal decomposition during the analyses; (ii) the injection volume should be relatively large as samples have to be injected precisely for quantitative analyses without internal standards. The third problem is that radioactivity detectors have a certain limitation with regard to the maintenance of chromatographic resolution obtained in capillary columns of 0.20–0.33 mm I.D. as the transit time of gas particles in radioactivity detectors is much longer than that in mass detectors.

Recently, much attention has been paid to wide-bore capillary columns of 0.50–0.75 mm I.D. [13]. These columns have the following advantages for radio-GC, although the resolution is inferior to that of capillary columns with the usual I.D.: (i) the thick film makes it possible to analyse samples with high concentrations (up to a few micrograms per component); (ii) the high flow-rate of the carrier gas (up to 20 ml/min) makes it possible to inject relatively large volumes of sample; (iii) direct injection is feasible so that no error in the balance of radioactivity arises. The above considerations imply that wide-bore capillary columns and direct injection are most suitable for radio-GC.

Fig. 1 shows a schematic diagram of a GC–SARD equipped with a wide-bore capillary column. Constant-pressure regulation of the gas flow-rate is usual in capillary GC, but it leads to a serious problem in temperature programming in radio-GC. The flow-rates of the carrier gas and make-up gas are reduced as the column temperature increases, so that the transit time of gas in counter tubes is increased and the radioactivity is overestimated. Therefore, constant-flow regulation was adopted except for the make-up gas for FID.

[^{14}C]Hexadecane was injected, and the radioactivity was measured under the various counting conditions given in Table I in order to examine the maintenance of chromatographic resolution in the apparatus (Fig. 3). Of course, the detection sensitivity is lowered as the sampling time decreases. The maintenance of chromatographic resolution can be determined from the broadening of radioactive peaks compared with the corresponding mass peaks. The maintenance of resolution in all the records is far better than in any radio-GC method so far reported. The maintenance of resolution was improved as the sampling time decreased, and the apparent broadening of SARD peaks compared with FID peaks (half-width <3 s) was almost negligible under counting conditions C and D. Condition C (sampling time 2 s; counting time 7×2 s) is recommended for experiments that require high resolution, and condition A (sam-

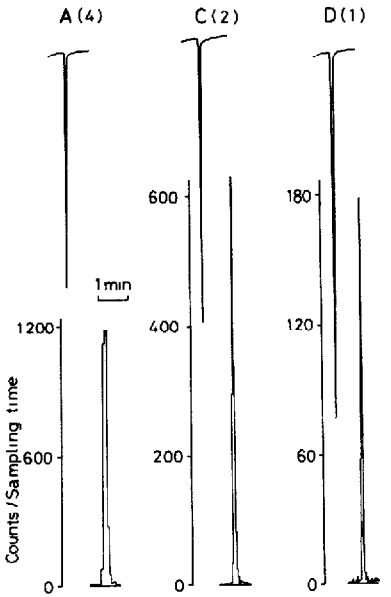

Fig. 3. Influence of sampling time on maintenance of chromatographic resolution. Sample, [^{14}C]hexadecane, ca. 96 Bq. Sampling time (s) is given in parentheses at the top. A, C and D correspond to A, C and D in Table I. Upper and lower traces, FID and SARD, respectively.

pling time 4 s; counting time 7×4 s) is recommended when a high detection sensitivity is required, although which condition should be selected also depends on the injection radioactivity. Theoretically, SARD peaks becomes wider than the corresponding FID peaks by two sampling times at the most [1,14]. Thus the half-widths of the SARD peaks under conditions A, C and D are calculated to be less than 11, 7 and 5 s, respectively. Every half-width shown in Fig. 3, in practice, proved to be less than the calculated values. In the present apparatus, the correspondence of resolution between SARD and FID proved to be excellent, as no FID peaks narrower than this case were obtained using wide-bore capillary columns.

DL-[^{14}C]Suprofen, after derivatization to its diastereomeric mixture with (*S*)-(−)-1-(1-naphthyl)ethylamine, was injected in order to compare the performances of capillary GC–SARD and packed GC–SARD. The results are shown in Fig. 4. The carrier gas in both GC–SARDs was set at the usual flow-rate, and the temperatures were identical. In capillary GC–SARD, the time required for analyses was markedly shorter than that in packed GC–SARD although the resolutions were almost identical (1.2–1.4). The total GC peak yield [4] of D- and L-suprofen derivatives in capillary GC–SARD was approximately 20% higher than that in packed GC–SARD. The GC peak yields in packed GC–SARD had a tendency to increase with repeated injection. The precision of measurement was substantially improved by using capillary col-

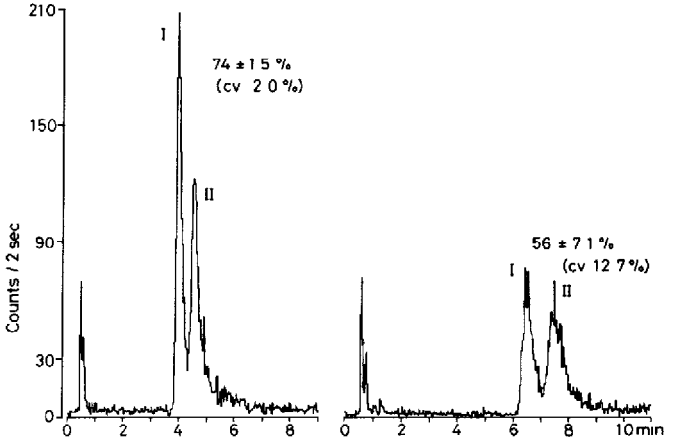

Fig. 4. Radio-gas chromatograms produced by capillary GC–SARD (left) and packed GC–SARD (right). DL-[^{14}C]Suprofen (48 MBq/mmol), after derivatization to its diastereomeric mixture with (*S*)-(−)-1-(1-naphthyl)ethylamine (ca. 192 Bq), was injected without any purification. Peaks I and II are D- and L-[^{14}C]suprofen, respectively. Total GC peak yields of D- and L-suprofen (mean ± S.D., $n=3$) are shown by the peaks; counting condition C.

umns. It can be assumed that the superiority of the GC peak yields and precision in capillary GC–SARD is to be ascribed to the suppression of column adsorption and thermal decomposition of the substances owing to the inertness of the column and the short time required for analyses. The above results demonstrate that capillary GC–SARD could show the characteristics of wide-bore capillary columns, which contributes to the improvements in precision and detection limits.

In recent years, trace amounts of biologically active substances have often been studied. Tracer techniques using ^{3}H-labelled substances with high specific activity are useful in such studies and methods for the determination of ^{3}H-labelled substances by radio-GC with high resolution and sensitivity are therefore important. First, the resolution of the present apparatus was investigated using [^{3}H]androstenedione, which is relatively polar. Underivatized samples were injected at intervals of 10 (I), 20 (II) or 30 (III) s, and the radioactivity was measured under counting condition C. The results are shown in Fig. 5. The resolution in records II and III was 1.2 and 1.8, respectively. It seems that each peak in record III can be quantitated. Karmen et al. [15] added hydrogen immediately before the iron in the oxidation–reduction tube in order to prevent the adsorption of $^{3}H_2O$ and $^{14}CO_2$ on the surface of the deteriorated iron. In contrast, in GC–SARD, no appreciable adsorption was observed although hydrogen was not added. The above results show that a high resolution of ^{3}H-labelled substances in addition to ^{14}C-labelled substances is possible without any adsorption in the apparatus.

Second, detection limits for ^{3}H-labelled substances were investigated.

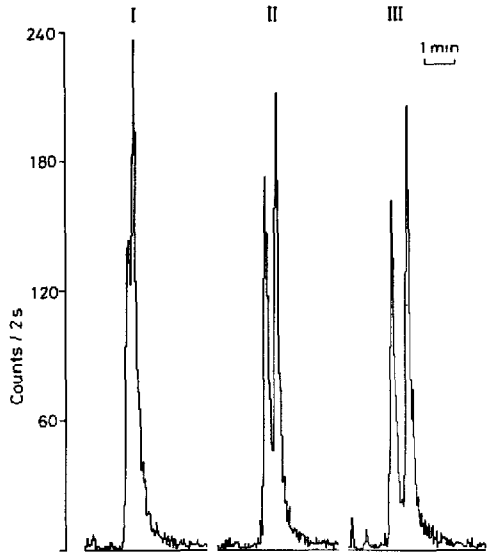

Fig. 5. Radio-gas chromatograms obtained by two consecutive injections of [^{3}H]androstenedione at intervals of 10 (I), 20 (II) and 30 s (III). The radioactive peaks emerged at 1.7 min after injection under the experimental conditions used; counting condition C.

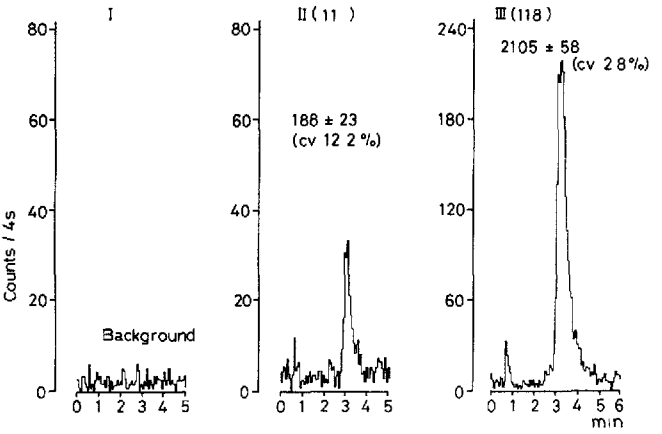

Fig. 6. Detection limits: sample, [^{3}H]androstenedione (3.1 TBq/mmol); counting condition A. Injection radioactivities (Bq) are given in parentheses at the top. Counts (mean ± S.D., $n=7$) are given above the peaks.

[^{3}H]Androstenedione of 11 Bq (record II) or 118 Bq (record III) was injected into the GC–SARD and the radioactivity was measured under counting condition A. The resulting chromatograms are shown in Fig. 6. The amounts of II and III injected were calculated to be 1 and 10 pg, respectively, based on the specific activity. The reproducibility in III was excellent [coefficient of varia-

tion (C.V.) 2.8%]. It was easy to detect the peak in II although the reproducibility was relatively poor (C.V. 12.2%). The above results indicate that the quantitative and qualitative limits of analyses of this labelled substance under the conditions used were less than 10 and 1 pg, respectively.

Detection limits in SARD can be represented by the following expression [16]:

$x \times$ (GC peak yield) $\times$ (oxidation–reduction efficiency)

$\times$ (counting efficiency) $\times$ (transit time in SARD)

$\times$ ($TCSF_{n,max}$/total counts of peak)

$= 3 \times$ (S.D. of $TCSF_{n,back}$)

where $TCSF_n$ = total counts of n successive fractions, $TCSF_{n,back} = TCSF_n$ in the background region and $TCSF_{n,max} = TCSF_n$ that gives the highest value in the region of the peak under consideration (corrected for the background). In this paper, n is defined as the number of fractions making $TCSF_{n,max}$ approximately 70% of the total counts under the peak in question. In the present experiments, the ratio of $TCSF_{10,max}$/total peak counts was 0.73 ± 0.01, and then $TCSF_{10}$ was used as the criterion for the detection limit. $TCSF_{10,back}$ was 25.3 ± 4.75 (mean $\pm$ S.D., $n = 100$). The product of the GC peak yield and oxidation–reduction efficiency of androstenedione and counting efficiency was 0.591. The counting time was 7×4 s. The detection limit for [^{3}H]androstenedione was therefore calculated as follows:

$x \times 0.591 \times 28 \times 0.73 = 3 \times 4.75$

$x = 1.2$ Bq (0.1 pg)

The detection limit defined here has a detection level $100(1-\beta)$ of 50%. Hence detection limits can clearly be calculated by using such a definition. Similar detection limits are also considered to be obtained for other ^{3}H-labelled substances as the specific activity of ^{3}H-labelled substances is usually of the order of 3.7 TBq/mmol. The above results show that the apparatus corresponds well with GC–MS as regards sensitivity.

In conclusion, it has been demonstrated that the apparatus could clearly show the characteristics of wide-bore capillary columns, such as inertness of columns and short analysis times, there is excellent correspondence of radioactivity detection with mass detection as regards resolution and highly sensitive analysis of trace amounts of ^{3}H-labelled substances are possible. The apparatus is useful for preliminary examinations in stable isotope tracer techniques using GC–MS and for metabolism studies with radioisotope tracer techniques.

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