Journal of Chromatography, 360 (1986) 105–117
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 555

RADIOMETRIC DETECTION OF BETA-LABELLED POLAR COMPOUNDS USING AN EFFLUENT STORAGE PRINCIPLE BASED ON SOLVENT SEGMENTATION

H. J. VAN NIEUWKERK and H. A. DAS
ECN, P.O. Box 1, 1755 ZG Petten (The Netherlands)
and
U. A. Th. BRINKMAN and R. W. FREI*
Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)
(Received February 10th, 1986)

SUMMARY

A new method is presented for the on-line detection of beta-labelled compounds of high polarity in reversed-phase column liquid chromatography. The aqueous column effluent containing the ^{14}C-labelled amino acids alanine, valine, isoleucine and/or leucine is segmented with a non-water-miscible liquid scintillator. This two-phase system is introduced into the beta-detector without phase separation, where the betas emitted in the aqueous phase are detected in the scintillator phase. The detector effluent is collected in a storage capillary tube. At the end of the chromatographic process, the contents of the storage capillary tube are introduced into the beta-detector by reversing the direction of the flow. The two phases are homogenized by adding a water-miscible liquid scintillator. By lowering the flow-rates the signal increases, depending on the flow-rates used.

INTRODUCTION

In the on-line detection of compounds containing low levels of radioactivity following a column liquid chromatographic (LC) separation, one is faced with the problem that, owing to the rapid separation, the residence time of the compounds in the detector cell is too short to yield a chromatographic signal that can be distinguished from the background. The residence time may be increased by an increase of the detector cell volume or a decrease of the eluent flow-rate. However, if the detector cell volume is chosen too large (larger than about one third of the volume standard deviation of the peaks of interest[1]), unacceptable peak broadening occurs. By placing a number of detectors in series and summing their signals[2] it is possible to increase the effective cell volume without a noticeable increase in band broadening, but problems with the synchronization of the detector signals and the cost of the apparatus may be prohibitive. If the eluent flow-rate is lowered, much time is wasted

0021-9673/86/$03.50

on regions in the chromatogram where either no or a high level of radioactivity is present. This can be prevented if only the regions of interest are counted at a lower flow-rate or in a stop-flow mode. Changing the flow-rate during the chromatographic run is technically possible, but then detailed information on the nature of the sample is required. In order to be able to deal with unknown samples, we decided to build a system where the effluent of the radioactivity detector is stored in a capillary tube, from which regions of interest can be recalled to the same radioactivity detector and counted at a lower flow-rate or in stop-flow mode.

In a previous paper[3] we described how aqueous column effluents containing medium- or low-polarity compounds labelled with ^{14}C are segmented by a non-water-miscible liquid scintillator to prevent band broadening in the detection and storage system. The ^{14}C-labelled compounds are extracted into and detected in this scintillator. Although the system was originally developed for aqueous column effluents containing compounds of low to medium polarity, *e.g.*, [^{14}C]carbaryl and [^{14}C]parathion, it should be possible to use this system or adapt it for compounds of high polarity. This is desirable for metabolism studies, as many metabolites are relatively polar and are present in low concentrations, hence posing problems of ultimate detection. In contrast to compounds of low or medium polarity, compounds of high polarity are not or only poorly extracted into the scintillator phase. Although they can still be detected by this scintillator, the counting efficiency decreases, as not all betas that are emitted in the aqueous phase reach the scintillator phase.

In this study we describe how the storage principle is applied to aqueous column effluents containing ^{14}C-labelled compounds of high polarity.

EXPERIMENTAL

Apparatus

The basic configuration of the apparatus is shown in Fig. 1. The hardware, software and system constants[4,5] and the performance with aqueous effluents containing ^{14}C-labelled compounds of low or medium polarity[3] have been described elsewhere. In the basic configuration a Model 3B dual-head pump (Perkin-Elmer, Norwalk, CT, U.S.A.) is used. One of the heads delivers the aqueous eluent, which flows via a Rheodyne (Cotati, CA, U.S.A.) Model 7126 injector with the Rheodyne 7136 solenoid valve kit, a Brownlee (Santa Clara, CA, U.S.A.) 10-cm cyano cartridge column and a Uvikon 725 (Kontron, Zürich, Switzerland) UV detector. After the UV detector a non-water-miscible liquid scintillator is added to the effluent stream via the second pump head of the Perkin-Elmer Model 3B. Thus a solvent-segmented stream is formed, which passes an in-line solvent filter (Upchurch Scientific, Oak Harbor, WA, U.S.A.), a Valco (Houston, TX, U.S.A.) Model 12611 eight-port switching valve with an air activator and a Rheodyne 7136 solenoid valve kit, a 40 cm × 0.5 mm I.D. stainless-steel extraction capillary and is introduced into the beta-detector without phase separation for monitoring the effluent during the chromatographic step.

The beta detector consists of the following parts: an Isoflo flow cell (Nuclear Enterprises, Edinburgh, UK) with a geometrical volume of 63 μl, two Model 8575 photomultiplier tubes (RCA, Harrison, NJ, U.S.A.) and two Model 218 magnetic shields (Ortec, Oak Ridge, TN, U.S.A.) in a home-made light-tight housing, a high-

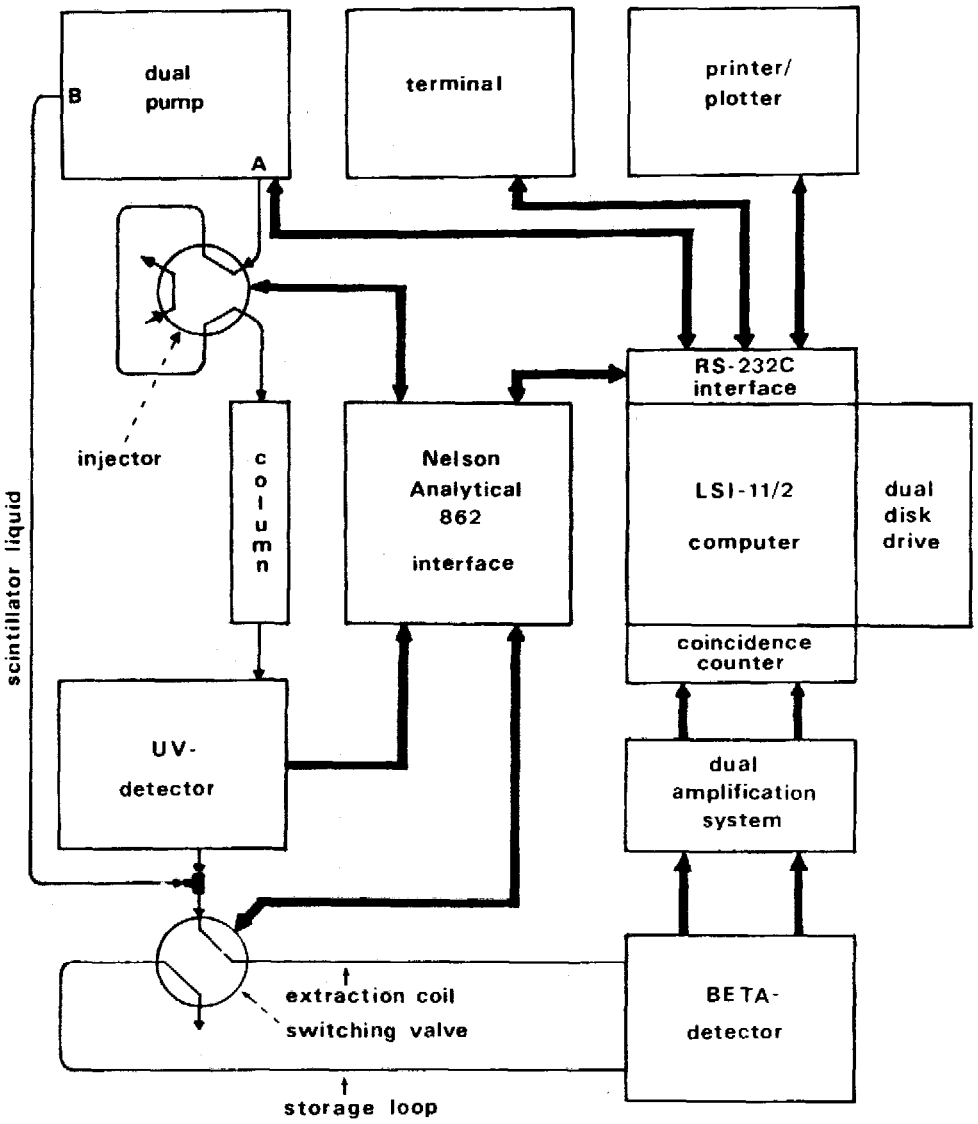

Fig. 1. Schematic diagram of LC equipment with on-line beta-detector and extraction/storage system for aqueous column effluents containing ^{14}C-labelled extractable compounds of low to medium polarity.

voltage power supply (Ortec 456), two photomultiplier bases (Ortec 265), two scintillation pre-amplifiers (Ortec 113), two Model 2110 timing filter amplifiers (Canberra, Meriden, CT, U.S.A.) and two constant fraction discriminators (Canberra 1428 A). The segmented effluent of the beta-detector flows into a stainless-steel storage capillary of 50 m × 1/16 in. O.D. × 1.0 mm I.D. After the chromatographic process the contents of the storage capillary tube can be reintroduced into the beta-detector by rotation of the switching valve.

The chromatographic apparatus is controlled and data from the UV and beta-

detectors are collected via home-made software[4,5] by a Dataram computer (Technitron, Schiphol, The Netherlands) with a DEC LSI-11/2 microprocessor Model KD11-HA, a DLV 11-J four-line asynchrone serial interface, a DSD 430-2 single/double density floppy disk, a DR 115 S 23 K × 16 memory module, a home-made coincidence counter/timer and a Nelson Analytical (Cupertino, CA, U.S.A.) Model 862 interface. For user communication with the computer and hard copies, a Tele Video (Sunnyvale, CA, U.S.A.) Model 910 terminal with UT-52 modification and graphics board installed, and an Epson (Nagano, Japan) Model MX-100 III printer/plotter are used, respectively. The apparatus required to adapt this system for storing effluents containing compounds of high polarity is discussed further under Results and Discussion.

Chemicals

The amino acids L-[U-^{14}C]alanine, L-[U-^{14}C]valine, L-[U-^{14}C]isoleucine and L-[U-^{14}C]leucine (specific activities 6.48, 10.7, 19.2 and 12.9 GBq mmole^{-1}, respec-

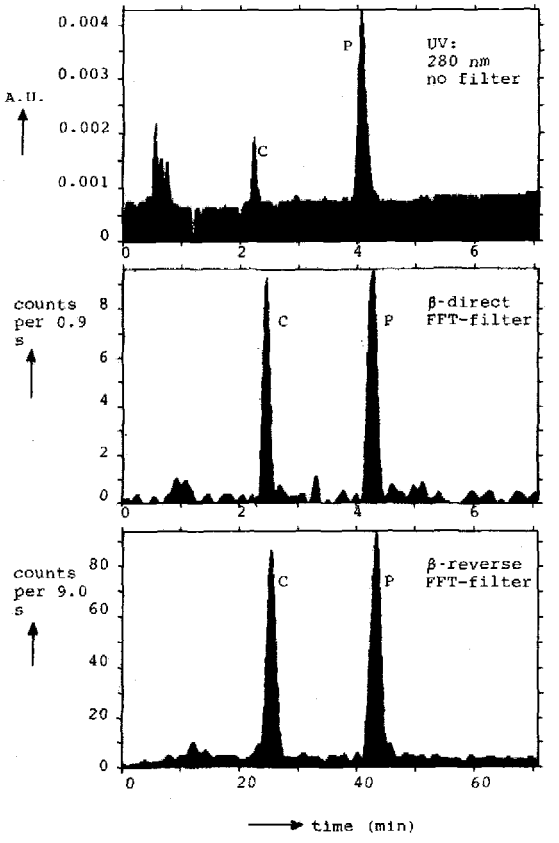

Fig. 2. Chromatograms of [^{14}C]carbaryl (C) and [^{14}C]parathion (P). Upper chromatogram: column, 10 cm × 4.6 mm I.D. cyanobonded; eluent, acetonitrile–water (30:70) at a flow-rate of 1 ml min^{-1}; UV detection at 280 nm. Middle chromatogram: conditions as in upper chromatogram, but with 1 ml min^{-1} of Ready-Solv NA added; beta-detection with FFT-filtered signal. Lower chromatogram: contents of 50 m × 1.0 mm I.D. stainless-steel storage capillary tube; eluent 0.2 ml min^{-1} flow-rate; FFT-filtered beta signal.

tively) were obtained from Amersham International (Amersham, Buckinghamshire, U.K.). LC was carried out on a 10 cm × 4.6 mm I.D. Brownlee 5-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.) cartridge, with an eluent consisting of doubly distilled, demineralized water, 0.05 *M* sodium hexyl sulphate (Eastman Kodak, Rochester, NY, U.S.A.) and 0.1 *M* phosphoric acid (Baker, Deventer, The Netherlands) adjusted to pH 4.3 with sodium hydroxide (Baker). At a later stage, the phosphoric acid was replaced with acetic acid (Baker) and 4% of methanol (Baker) was added. The liquid scintillators used were non-water-miscible Ready-Solv NA and water-miscible Ready-Solv CP (Beckman, Palo Alto, CA, U.S.A.).

RESULTS AND DISCUSSION

Two-pump systems

When extractable compounds such as [^{14}C]carbaryl (C) and [^{14}C]parathion (P) are separated with the system described under *Apparatus*, the results shown in Fig. 2 are obtained[3]. The top chromatogram represents the UV absorption signal. The middle chromatogram is the direct measured beta-signal, which has been smoothed by a technique using fast Fourier transform (FFT) as described elsewhere[4]. At the end of the chromatographic run the switching valve (Fig. 1) is rotated and the total flow-rate is lowered from 2.0 ml min^{-1} to 0.2 ml min^{-1}. Thus the chromatogram that is stored in the storage capillary tube is reintroduced into the beta-detector (reverse run) at a 10 times lower flow-rate. The signal then obtained is presented in the lower part of Fig. 2. As can be seen, no additional band broadening occurs in the storage capillary, and the signal increases by a factor of 10. More details can be found in ref. 3.

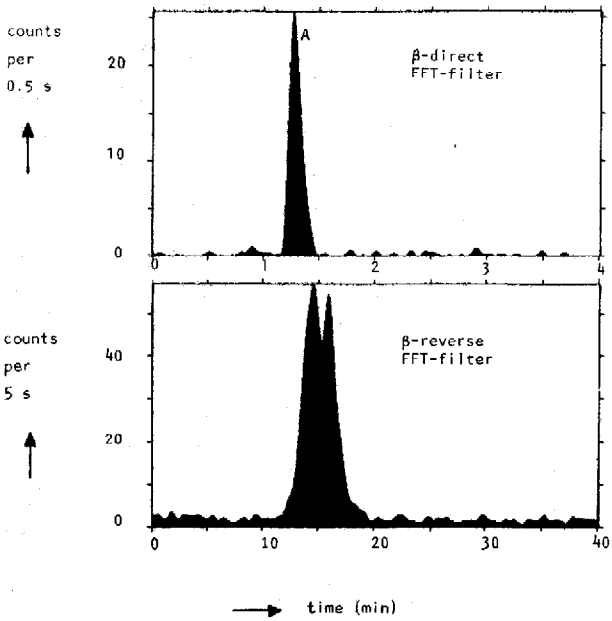

Fig. 3. Beta chromatograms of [^{14}C]alanine (A). Upper chromatogram: conditions as in middle chromatogram of Fig. 2. Lower chromatogram: conditions as in lower chromatogram of Fig. 2.

When this system is used for polar non-extractable compounds, results such as those shown in Fig. 3 are obtained. The top chromatogram represents the direct beta signal of [^{14}C]alanine and the lower chromatogram the signal of the reverse run; both have been smoothed. Fig. 3 shows that (1) the peak in the reverse chromatogram does not have the same position as the peak in the direct chromatogram; this was caused by a malfunctioning pump and, therefore, requires no further discussion; (2) the total peak area in the reverse run is less than ten times the peak area in the direct run (see also Table I); the reason for this is that during the direct run the segments are smaller, so that a larger amount of beta-radiation reaches the scintillator phase; (3) the reverse peak is broadened; as the inner wall of the stainless-steel storage tube is wetted by the polar aqueous phase, alanine, which resides in the aqueous phase, leaks to other segments via the inner wall of the storage tube; (4) the peak has been split; this is caused by a combination of peak broadening, counting statistics and the filtering procedure.

TABLE I

COMPARISON OF STORAGE SYSTEMS USED IN FIGS. 3, 4 AND 6

System	*Compound*	*Area (counts)*		*Area ratio**	
		Direct	*Reverse*	*Found*	*Expected*
Fig. 3	Alanine	409	2123	5.2	10
Fig. 4	Valine	332	98	0.3	10
Fig. 6	Alanine	553	4412	8.0	5
Fig. 6	Isoleucine	182	1326	7.3	5
Fig. 6	Leucine	250	1873	7.5	5

* Area ratio = area reverse/area direct.

Band broadening due to wetting can be avoided by replacing the stainless-steel capillary storage tube with a PTFE tube of 60 m × 0.8 mm I.D. (Chrompack), as can be seen in Fig. 4. Still, however, a large proportion of the signal is lost (Table I) by recombination of segments, even more than in Fig. 3, because here a more polar eluent was used. The reverse peak area now is even smaller than the direct peak area, although the counting time is ten times longer. In other words, in this instance peaks that are not or poorly detectable during the direct chromatographic run, certainly cannot be detected in the reverse run. The fact that valine was used as a test compound, whereas in Fig. 3 alanine was used is of no significance, as will be shown later.

To overcome the problems, a system was set up in which a water-miscible scintillator was added to the column effluent and segmentation took place with air, as suggested by Snyder[6]. It appeared that such a system cannot be used, because, owing to the emulsifiers present in the scintillator, segment formation is very poor, and compressibility problems occur when the flow-rate is changed or the flow direction is reversed.

Another approach was therefore followed: the effluent was segmented with hexane and a solid scintillator was used (yttrium silicate, nominal particle diameter 18 μm). Although with separate streams of effluent and hexane no pressure problems

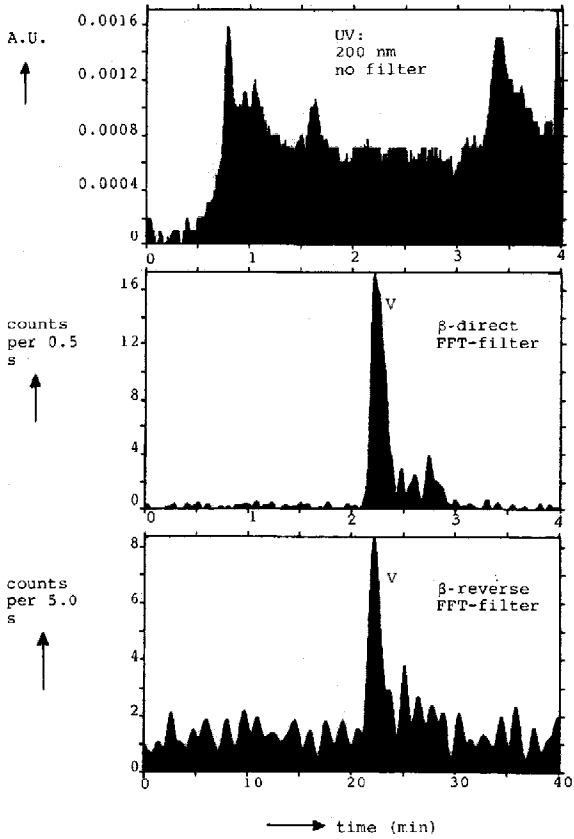

Fig. 4. Chromatograms of [^{14}C]valine (V). Upper chromatogram: column, 10 cm × 4.6 mm I.D. Li-Chrosorb RP-18; eluent, 0.1 *M* phosphate buffer + 0.03 *M* sodium hexylsulphate (pH 4.27); at a flow-rate of 1 ml min^{-1}; UV detection at 200 nm. Middle chromatogram: conditions as in upper chromatogram, but with 1 ml min^{-1} of Ready-Solv NA added; beta-detection with FFT-filtered signal. Lower chromatogram: contents of 60 m × 0.8 mm I.D. PTFE storage capillary tube; eluent flow-rate: 0.2 ml min^{-1}; FFT-filtered beta signal.

were observed in the detector cell, with a segmented system of effluent and hexane the pressure increased so rapidly that no measurements could be performed, and eventually the glass wall of the cell broke.

Three-pump systems

From the previous section it can be concluded that in the forward (direct) mode the solvent segmentation principle can be used for radioactivity detection. With reversed flow, however, band broadening, peak distortion and very low counting efficiency occur, probably as a result of a breakdown of the solvent segmentation pattern.

The reverse signals can be enhanced while still maintaining the band-broadening suppression effect of the segmented system, by homogenizing the contents of the storage tube just before reintroduction into the beta-detector. The highest count-

ing yields can be expected if this is done by adding a water-miscible liquid scintillator. For this we used Ready-Solv CP, because of its capability to dissolve a large amount of water (up to 50%) and in order to avoid high back pressures due to gel formation. Ready-Solv CP is added to the contents of the storage tube in a 1:1 ratio. Fig. 5 shows how the system in Fig. 1 is modified to allow the addition of the second scintillator with a Gilson (Villiers-le Bel, France) Minipuls 2 peristaltic pump. In Fig. 6 and Table I the performance of the modified system is shown for the amino acids alanine, isoleucine and leucine. From Table I it can be seen that in the reverse mode the peak areas now are distinctly higher than in the direct mode. The increase is however, higher (7–8 fold) than can be expected on the basis of the increased counting time (5-fold). This will be explained in the next section.

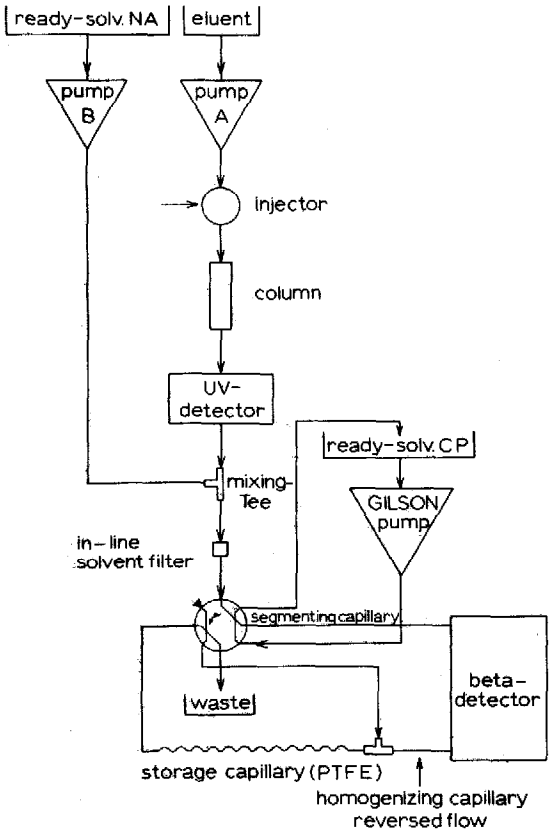

Fig. 5. Schematic diagram of LC equipment with on-line beta-detector and storage system for aqueous column effluents containing non-extractable ^{14}C-labelled compounds of high polarity. For connections to computer, see Fig. 1.

Calibration graphs

The linearity of response of the system used in Fig. 6 was checked by injecting amounts of 170–4300 Bq of [^{14}C]valine, 110–4500 Bq of [^{14}C]alanine and 50–4400 Bq of [^{14}C]leucine. Fig. 7 shows that the three amino acids share the same calibration

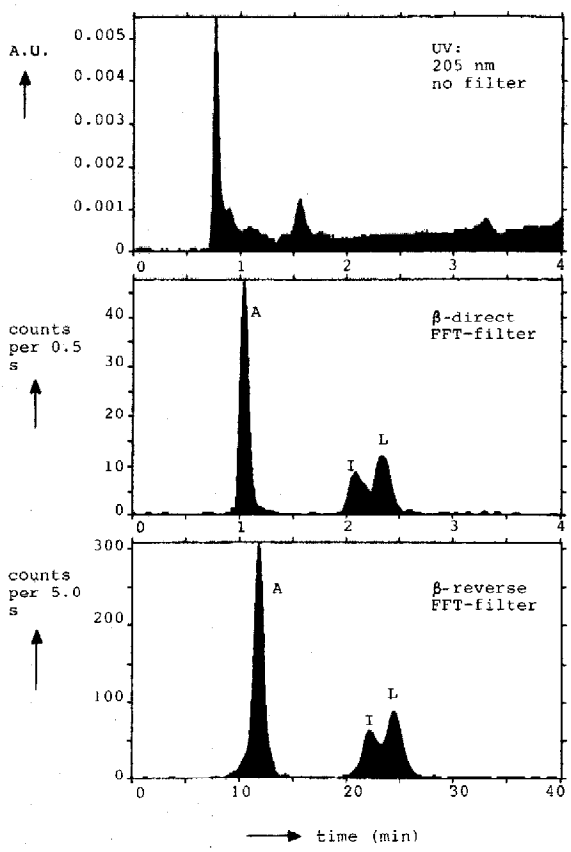

Fig. 6. Chromatograms of ^{14}C-labelled alanine (A), isoleucine (I) and leucine (L). Upper chromatogram: conditions as in Fig. 4, but UV detection at 205 nm. Middle chromatogram: as in Fig. 4. Lower chromatogram: as in Fig. 4 but with 0.2 ml min^{-1} of Ready-Solv CP added to the contents of the storage capillary just before entry into the beta-detector.

graphs for the direct (lower plot) and also for the reverse (upper plot) measurements. Here we see an advantage of radioactivity detection: one calibration graph is valid for different compounds.

Direct measurement. From the plot of the direct measurements one can conclude that very reproducible results are obtained although a two-phase detection system has been used. Usually in liquid scintillation detection these systems are avoided because of their low reproducibility. Obviously, in the present system cross-radiation of the betas from the non-extractable analytes in the aqueous plugs to the organic scintillator plugs is reasonably efficient, provided that small segments are formed. This implies that the technique can be used instead of solid scintillation detection, particularly in situations where dirty samples have to be handled which cause an increase in the background level or a decrease in the counting efficiency due to adsorption phenomena. Because the compounds remain in the aqueous phase, their isolation from the effluent for further analyis is as easy as with solid scintillation detection. The technique may also be used instead of homogeneous detection, where

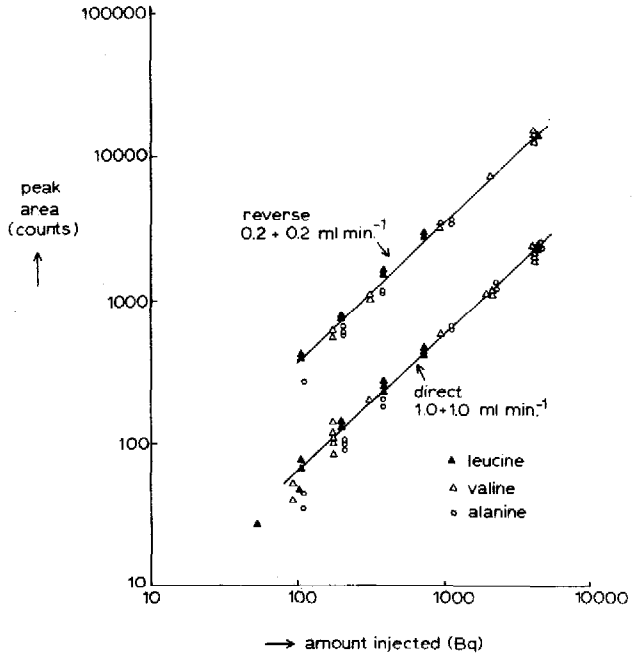

Fig. 7. Calibration graphs of ^{14}C-labelled alanine, valine and leucine under conditions as in Fig. 6. Upper line: slope, 3.24; regression coefficient, 0.998; 35 data points. Lower line: slope, 0.52; regression coefficient, 0.996; 55 data points.

a water-miscible liquid scintillator is added to the column effluent. Using a two-phase system, band broadening, which may be considerable in homogeneous detection because of the high viscosity of the effluent–scintillator mixture, is suppressed. In addition, it may lead to a saving of scintillation liquid, as the mixing ratio of scintillator to effluent usually varies between 3:1 and 1:1 in a homogeneous system. Again, it is possible to isolate the separated compounds as easily as with solid scintillation detection.

The slope of the calibration graph for the direct measurement was 0.52 counts (Bq injected)$^{-1}$, whereas for extractable compounds such as parathion and carbaryl under similar circumstances a value of 1.48 counts (Bq injected)$^{-1}$ was found[3]. In other words, the counting efficiency is approximately three times lower than observed previously[3]. As the counting efficiency for carbaryl and parathion is 81 ± 1%[5], the value for the amino acids is about 30%.

Reverse measurement. The slope of the calibration graph of the reverse measurement (Fig. 7) was 3.24 counts (Bq injected)$^{-1}$, which is about six times greater than that for the direct run, whereas the residence time was only five times higher*. The main reason for this is that the amount of quenching is lower in the reverse run, as the aqueous phase containing the amino acids is held in micellar form inside the

* This difference (6-*vs.* 5-fold increase) is smaller than that reported in the previous section (7–8-*vs.* 5-fold). The former result must be considered to be more accurate than the latter, because it is based on the high number of experiments used to construct the calibration graphs.

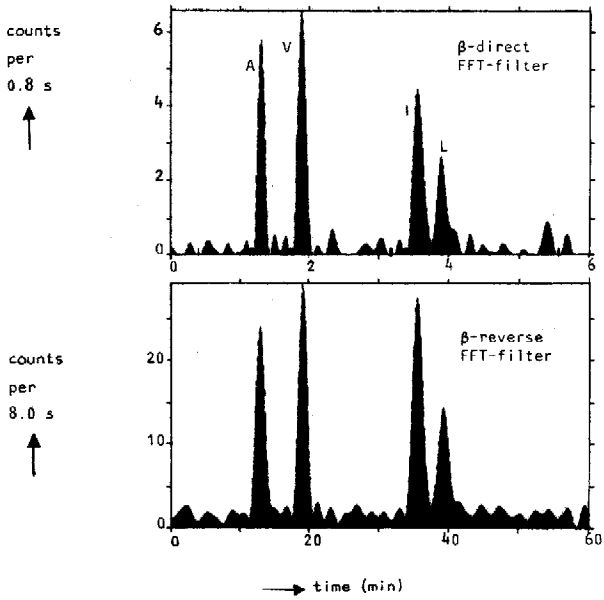

Fig. 8. Chromatogram of 65 Bq of alanine (A), 77 Bq of valine (V), 82 Bq of isoleucine (I) and 41 Bq of leucine (L). Upper chromatogram: column, 10 cm × 4.6 mm I.D. LiChrosorb RP-18; eluent, 0.02 *M* acetate buffer + 0.03 *M* sodium hexylsulphate (pH 4.23) + 4% methanol at a flow-rate of 1 ml min^{-1}; scintillator, 1 ml min^{-1} of Ready-Solv NA. Lower chromatogram: as lower chromatogram in Fig. 6.

scintillator phase, owing to the emulsifiers in the Ready-Solv CP. Another reason is that the cell response volume increases when the flow-rate decreases[4,5].

Influence of eluent composition

The eluent mixture used so far caused rapid deterioration of the column performance. This resulted in a gradual increase in the pressure and a sudden occurrence of double peaks. In order to study the influence of an organic modifier in the eluent, the eluent was slightly modified: the phosphate buffer was replaced with an acetate buffer and 4% of methanol was added. Under these circumstances much better peak shapes and resolution were obtained (Fig. 8) and a slope of 0.708 counts (Bq injected)$^{-1}$ was found for the calibration graph of the direct measurement (Fig. 9), which is 1.4 times greater than that with the phosphate buffer eluent. In a previous study[3] it was observed that the presence of methanol results in a slower separation of the aqueous phase from the liquid scintillator. In other words, the 4% of methanol in the eluent causes the segments to be smaller without a loss of peak integrity, thereby increasing the counting efficiency by a factor of 1.4, as a larger amount of the beta-radiation reaches the sintillator segments.

For the reverse measurements (Fig. 9) a slope of 3.89 counts (Bq injected)$^{-1}$ was found, which is 1.2 times greater than that with the phosphate buffer eluent. This increase may be explained by assuming that smaller micelles are formed when the methanol is present in the aqueous phase, while the segmentation pattern still effectively suppresses the band broadening in the storage loop. The fact that the counting efficiency increases in both the direct and in the reverse measurements when

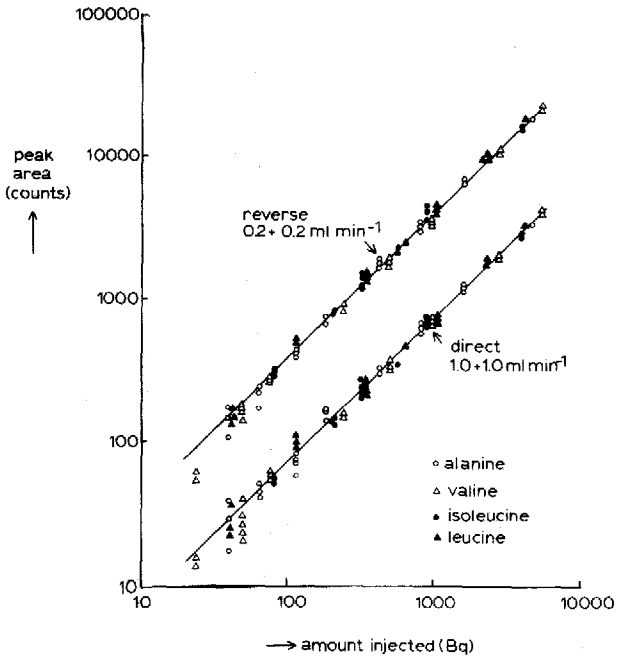

Fig. 9. Calibration graphs of ^{14}C-labelled alanine, valine, isoleucine and leucine under conditions as in Fig. 8. Upper line: slope, 3.89; regression coefficient, 0.999; 89 data points. Lower line: slope, 0.71; regression coefficient, 0.998; 88 data points.

methanol is added to the eluent implies that the system has to be recalibrated when the eluent composition is changed (*e.g.*, with gradient elution).

Detection limits

Under the experimental conditions used the limit of detection is 11 counts per peak in the direct mode and 21 counts per peak in the reverse mode[3]. These values represent injected amounts of 21 Bq (Fig. 7) or 16 Bq (Fig. 9) for the direct mode and 6.4 Bq (Fig. 7) or 5.4 Bq (Fig. 9) for the reverse mode, respectively. These amounts correspond to low (direct) or sub(reverse) picomole levels for the four amino acids used, as is evident from the data summarized in Table II.

CONCLUSIONS

It has been shown that the two-phase storage/detection system, which was originally developed for extractable compounds of low to medium polarity[3], can also be used for non-extractable compounds of high polarity.

In the direct mode the reproducibility and sensitivity of the liquid–liquid system are highly satisfactory and the system may be used as a third detection method besides the conventional detection techniques with solid scintillators or water-miscible scintillators. For reverse-mode usage the stainless-steel storage capillary tube has to be replaced with a small-bore (*ca.* 0.8 mm I.D.) PTFE capillary tube, and an

TABLE II

DETECTION LIMITS OF FOUR AMINO ACIDS UNDER DIRECT- AND REVERSE-MODE CONDITIONS

For details of experimental conditions, see Figs. 7 and 9.

Compound	*Mode*	*Detection limit (pmole)*	
		From Fig. 7	*From Fig. 9*
[^{14}C]Alanine	Direct	3.3	2.4
	Reverse	1.0	0.8
[^{14}C]Valine	Direct	2.0	1.5
	Reverse	0.6	0.5
[^{14}C]Isoleucine	Direct	1.1	0.8
	Reverse	0.4	0.3
[^{14}C]Leucine	Direct	1.6	1.2
	Reverse	0.5	0.4

extra pump has to be installed to add a homogenizing liquid scintillator to the contents of the storage loop just before their reintroduction into the beta-detector.

The counting efficiency is about 30% for the four amino acids in the direct mode, resulting in detection limits at the low (direct mode) or sub- (reverse mode) picomole level. Optimization of the counting efficiency is currently being studied. An alternative way to homogenize the contents of the storage loop just before their reintroduction into the beta-detector may be by means of another high-pressure in-line solvent filter, which causes a homegeneous dispersion of infinitely small non-miscible particles. This possibility will be studied in the near future.

ACKNOWLEDGEMENT

The authors thank A. C. Veltkamp for his assistance with some of the measurements.

REFERENCES

1 H. Oster and E. Ecker, *Chromatographia*, 3 (1970) 220.
2 S. Baba, M. Horie and K. Watanabe, *J. Chromatogr.*, 244 (1982) 57.
3 H. J. van Nieuwkerk, H. A. Das, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 19 (1984) 137.
4 H. J. van Nieuwkerk, *Thesis*, Free University, Amsterdam, in preparation.
5 H. J. van Nieuwkerk, H. A. Das, R. W. Frei and U. A. Th. Brinkman, *J. Radioanal. Chem.*, in press.
6 L. R. Snyder, *J. Chromatogr.*, 149 (1978) 653.