



Radiochromatography and Its Applications

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Radioactivity is used for labelling in a range of chromatographic techniques. This article gives an overview of what to take into account when using each particular method.

The most suitable detection method for nuclear radiation is mainly determined by its pathlength in different forms of matter and its interaction with the detection medium. The nuclear radiation particle can only be detected when it is invading the detection medium and produces some interaction with it. Nuclear radiation that passes the matter without any measurable interaction cannot be detected. Radioactive labels used in chromatography can only be detected in instances where the radiation interacts with the matter of the detection medium.

In radio LC, the radioactive labelled sample is dissolved in a pure or mixed eluate and separated by the column into various fractions.

Liquid Chromatography (LC)

β-liquid scintillation, homogeneous solution: Detection of beta-minus particles of ^3H (tritium) in LC is particularly difficult because both the range of the particle and the penetration through matter is so small that it can only be detected in instances where there is almost no material between the emitting nuclide and the detection medium.

A homogeneous solution in a liquid scintillation cocktail is almost ideal. The matter between the emitting nuclide and the detecting medium liquid scintillator is zero. The soft beta-minus



particle can reach and deposit its energy in the very immediate neighbourhood of solvent molecules. This can transfer the excitation through the homogeneous solution until a scintillator molecule is reached and the transferred energy produces a scintillation effect — a spherical emission of photons.

In situations where the primary solvent does not dissolve the sample at all or adequately enough, a secondary or even third solvent may be used to finally form a homogeneous solution. Sometimes solubilizing agents — which “digest” the sample first and bring it in a homogeneous solution after that — are used.

The energy transfer from one molecule to the neighbouring molecule can be affected by such molecules, which absorb the excitation and do not transfer it to its neighbour. This effect is called “chemical quench”. There are many well-known chemical quench agents. One of the most prominent agents is oxygen dissolved in the homogeneous liquid scintillator solution.

The emitted photons of the scintillator molecules can be absorbed by the colour of the dissolved sample itself and is lost for the detection. This effect is called “colour quench”.

The remaining photons of the scintillation effect can finally be detected by a photomultiplier. If two photomultipliers are used the geometry can be extended to almost 4π and the noise of the photomultipliers can be considerably reduced by coincidence. Coincidence is a co-registration of two independent events with the coincidence time.

β -liquid scintillation, suspension: Quite often the radioactive-labelled sample cannot be brought in a stable, homogeneous solution of a liquid scintillation cocktail. As soon as phase separation appears the detection is critical if not impossible.

Some gelling agents are producing such small volumes of the sample phase in such close proximity to the liquid scintillator phase that the beta particle emitted by the sample can reach the liquid scintillator phase. Providing this very complex suspension is stable, this method can be a suitable and efficient detection method.

β -internal solid scintillation: The medium energy beta-minus radiation is less critical for detection because the pathway of the particle is longer and the penetration through some matter is stronger, which makes the detection much more easy.

Homogeneous solution of the sample in a liquid scintillation cocktail is still the most sensitive method, but the path length of the beta-minus particle is long and the penetration through the matter is high enough that even internal solid scintillator particles can be used for detection. The eluate of an LC column can flow through a quartz glass tube filled with solid scintillator beads. The beta-minus particle can penetrate through the eluate solvent, reach the solid scintillator bead and create a scintillation effect there. The emitted photons of this scintillation effect are detected by a photomultiplier. Two photomultipliers in coincidence enable an almost 4π geometry and a low noise level to be achieved. There is almost no difference in sensitivity between liquid scintillator and internal solid scintillator detection in the case of ^{14}C or higher beta energies. The major disadvantage of liquid scintillator use is the high price for purchasing the cocktail and the disposal of the sample. The principal disadvantage of a potential deposition of the sample molecule on the solid scintillator surface can be easily avoided by adding a low flow-rate of a suitable cleaning solution.

β -external solid scintillator: The penetration of high-energy beta-minus particles is sufficient to pass through a thin wall of plastic (PTFE). The radioactive-labelled sample with a high-energy beta radiation in an eluate of a column can flow through a plastic (PTFE) tube, penetrate the wall of the tube and reach an external solid scintillator. The created photons are detected efficiently by a photomultiplier. Two photomultipliers can be arranged to achieve an almost 4π geometry and reduce the noise rate considerably.

β -Cerenkov counting: If the emission energy of the beta-minus particle is high enough, the Cerenkov-effect can be used for detection without any scintillator at all. The detection sensitivity is quite high. The flow cell is formed by a suitable plastic tube coil without any scintillator.

γ -external scintillator crystal: For gamma-emitting nuclides of low energy (0–60 keV) a 1×1 in. NaI(Tl) crystal is most suitable. The shielding for the detector may be 15 or 35 mm lead.

For gamma-emitting nuclides of medium energy (60–600 keV) a 2×2 in. NaI(Tl) crystal is recommended. The central pin hole holds the flow cell in an almost 4π geometry.

For gamma-emitting nuclides of high energy (600–2000 keV) a 3×3 in. NaI(Tl) crystal is recommended. The central pin hole holds the flow cell in an almost 4π geometry.

Energy calibration can be performed by a firmware programme and suitable sources.

Thin-Layer Chromatography (TLC)

In radio TLC the radioactive labelled molecules are transported by the migrating solvent along the thin-layer trace and are finally resting on a certain place along the trace of the TLC. The radioactive fraction is distributed equally in the depth of the thin-layer material (silica gel) between surface and bottom.

Open window gas detection: Gas counters in general, such as ionization chambers, proportional gas counters and GM counters, have the advantage that even a very low-energy beta-minus particle can ionize the counting gas easily and be detected. The condition is that the low-energy beta-minus particle has to get into the counting gas environment and produce sufficient ions.

^3H -beta-minus particles have such a low energy that they cannot penetrate even a very thin window foil in order to get into the counting gas environment. That means ^3H can only be detected in an open window proportional gas flow-through counter. The open window working condition can produce several difficulties for routine operation. Uneven samples may deteriorate the uniform gas supply along the counting tube. Dust from the sample surface can be attracted by the high voltage on the counting wire and produce arcing. Static electricity deposited on the sample surface can affect the counting conditions. Volatile radioactive labelled compounds can contaminate the detector.

The counting conditions of a scanning detector along the chromatogram trace or a linear analyser detector measuring the total trace at once have the same difficulties, besides the advantage of the linear analyser, that it is about 100 times more sensitive than a scanning detector.

Closed window gas counting: The medium-energy beta-minus particle of ^{14}C can penetrate a 0.9 mg/cm^2 Mylar-foil used to close the proportional gas flow-through counter easily.

Even a GM-counter with a window of 1.5 mg/cm² Mika can be used for detecting medium energy beta-minus particles.

Phosphor imager: Imaging plates are very suitable radioactivity detectors for 1- or 2-dimensional TLC plates, providing suitable calibration standards and programmes are applied.

The "detection efficiency" of the imaging plate compared with proportional gas flow-through counters is low, but the exposition time can be quite long (for example, overnight). The total sensitivity is the product of detection efficiency multiplied by the exposition time.

The uniformity of an imaging plate and the image plate reader needs to be calibrated and may be comparable to proportional gas flow-through counters, but the resolution of the phosphor imager is much higher.

Positron-GM scanner: The most simple QC test for radioactive labelled compounds is a single trace scanner with a positron sensitive detector. The end-window Geiger-Müller counter is used to detect the high-energy beta-minus and beta-plus (positron) particle emissions along the single trace scan.

During a scan along a TLC plate the radioactive material may decay because of the short half life time of the nuclide. To avoid the decay along the length of the TLC trace, the detector moves relatively fast forwards and backwards many times from origin to solvent front while measuring and creating a chromatogram, which has no loss of radioactivity by decay from origin to solvent front.

γ-scintillation scanner: If the QC test has to be made for only gamma-emitting nuclides, a V-shape BGO scintillation probe is used. The scintillation detector is mounted on a single-strip scanner, which moves relatively fast forwards and backwards to compensate for radioactive decay along the trace. For low-, medium- and high-energy gamma radiation, various collimators are available to improve the local resolution on the TLC trace.

Gas Chromatography (GC)

In radio GC the radioactive labelled compound is in gas- or vapour phase, transported by the carrier gas and separated by partial adsorption on to the GC packing material in various fractions.

Proportional gas flow-through counting with sample

conversion: In this instance the radioactive-labelled compound is volatile, gas chromatography (GC) needs to be applied. Depending on the GC method packed or capillary columns are used.

When ³H- and/or ¹⁴C-labelled compounds are applied there is no other way than counting the beta-minus radiation in a internal gas flow mixture mode. The penetration of the beta radiation of ³H and ¹⁴C is too low to try to measure the GC gas eluate with an external detector. Moreover, all labelled compounds have different electrical and chemical reactivities in a gas mixture phase. Some compounds are strongly electronegative and suppress the formation of a plateau in a proportional gas flow-through counter. Therefore the volatile sample compound has to be converted in a reproducible form suitable for gas flow-through counting.

There are two procedures: reduction to CO₂ and H₂O or hydration to CH₄.

Hydration to CH₄ is preferred, because it can be used for ³H as well as ¹⁴C; the form of CH₄ is a good counting gas itself and can easily be mixed with the counting gas flow. For

hydration the GC eluate is directed through a quartz tube filled with Platinum particles heated to about 800 °C under continuous admixture of hydrogen gas. The automatic plateau scan demonstrates the reproducible counting conditions.

Gamma gas flow detection: In quality control (QC) of the PET-compound ¹⁵OH₂, the eluate of a GC contains a PET-compound with the nuclide ¹⁵O which emits a positron and consequently 2 × 511 keV gamma radiation. After the separation in the dual column the gas eluate is directed into a transfer line to the gamma sensitive scintillation detector. The transfer line between the GC oven, gamma gas flow detector and FID is heated to a selectable temperature to avoid any condensation. The gamma gas flow detector is a 2 × 2 in. NaI(Tl) full crystal with a suitable gas flow loop. A special programme controls the GC operation as well as the recording and evaluation of the gamma-measurement and the flame ionization detector (FID) or thermal conductivity detector (TCD).

Capillary Electrophoresis

Capillary electrophoresis (CE) is operating with an internal very high voltage from the entry to the exit of the column at many thousands of volts. For nuclides, which require "internal counting" because of the low energy of the emitted particles, that is an insurmountable problem. The only method described is the spotting of the CE-eluate on a sheet of adsorbing paper and detection by exposition to an imaging plate. The evaluation programme of the imaging plate is deconvoluting the detected spots to a chromatogram trace and treats the obtained radio-CE-chromatogram the same way as a TLC single trace evaluation.

Gamma-labelled compounds in CE are relatively easy to detect. The CE capillary is conducted through a small scintillation detector and the radio-CE chromatogram is recorded and evaluated.

Single event counting or true measurement value

acquisition: The unit of the radioactive activity is defined as Bq (Becquerel, desintegrations per second). The number of events registered by any radioactivity detector is defined as CPS (counts per second) and the relation is

$$\text{activity (Bq)} = \text{obtained count/s} \div \text{efficiency}$$

This clearly points out, that radioactivity detection is a "digital" number of counts per time. While UV measurements are naturally analogue and the diode current is not truly the absorption, the measurement of radioactivity does not need converters, more than that: converters destroy important portions of information.

To obtain a "mean value of the obtained radioactivity" a ratemeter has been developed, which has an "analogue output of 0–1 V" of the mean value of counts per time. The advantage of this is the compatibility to standard chromatography equipment, the price for this is a signal covered by noise by some reason, for example:

- Background and zero offset become indistinguishable.
- The effect of smoothing is impacting the signal unpredictable.
- The dynamic decreases from 1:5000000 to 1:50000 (two decades) because of FVCs.
- The relation between true counts and signal is lost.

The ambiguity of background and zero offset is worse because the background reduces the sensitivity while a zero offset does not. If background is subtracted, the original information is lost. When the obtained background is lower than the subtracted background (by statistical variations) "negative activity" is created, which does not exist. If zero offset is used, sensitivity of radioactivity determination is impossible because the base of zero activity is lost.

Smoothing makes even the calculation of a noise impossible, which could be a poor representation of the counting error. The loss of dynamic reduces the comfort and a lost relationship between true counts and signal makes it impossible to employ the laws of decay to calculate the counting error.

The system can be nicely explained by the measurement of the water level in a leaking rain water collection drum. The drum has a certain diameter and a particular height. The rain falling on the roof is collected in the eaves and creates a certain flow-rate into the drum. The leaking hole of the rain drum at the bottom has a different drain off flow-rate. The fill height of rain water in the drum can be measured and used as an indication of the amount and the intensity of the rain. The drawback is that the obtained water level in the rain drum does not say anything about the variation of the strength of the rain collected.

The statistical information about the number of rain drops per time over time is completely lost.

The radioactivity measurement by a ratemeter is quite similar and the raw data, the obtained single radioactivity events per time over the time is lost.

Therefore, the requirement of the FDA to store and maintain raw data is disregarded. The reconstruction of measured values at any later time is impossible.

Any chromatography recording and evaluating device using the "converted" analogue output signal 0–1 V is in principle not in agreement with the FDA-requirement of raw data. The original raw data of the radioactivity measurement in flow cannot be reconstructed at any time later.

Conventional chromatography data stations accept only "analogue signals 0–1 V" and are, therefore, not suitable for radioactivity detectors in chromatography according to FDA-rules.

The best evidence of the principle advantage of "single event counting" is the digital integration of a peak. The number of obtained radioactive events over time allows integration (adding up the obtained number of counts per time) over time. $\text{Counts/time} \times \text{time} = \text{counts}$. The statistical variation can be calculated from the integrated number of counts. The mean value of the obtained background can be calculated and the statistical variation of that can be determined. All obtained variations of the background count rate are radioactive fractions, which produce more counts than the mean value of background plus the n -sigma statistical variation. In case the user selects the 3-sigma level, the statistical security that the obtained peak is a radioactive fraction for 99.5% security, when it contains more counts than background mean plus 3-sigma variation.

This statistical calculation is made for every small peak and this gives the user an absolutely clear decision, which small peak is a radioactive fraction and which small peak is random noise.

In many applications — especially in metabolism and pharmacokinetic — this statistical calculation of a small peak

over background is of very high importance because it determines the dynamic range of the method and the limit of detection.

Furthermore, the "single event counting" does not require any more standardization such as analogue peak integration because the relation between obtained counts and radioactivity concentration is a simple factor: efficiency.

The analogue output 0–1 V of any ratemeter can never supply this important information.

Micro-flow- to preparative flow-rates: There is a wide range of applications of radioactivity detection in liquid chromatography. Preparative flow-rates are sometimes litres/min and micro-flow rates are $\mu\text{L}/\text{min}$.

With "single event counting" there is no principle difference in sensitive detection. It is always the statistical difference that has to be determined between background and a potential small peak.

Sometimes the number of counted events is so small that it is hard to detect a peak. In that particular instance again digital counting of events-per-time over time is a huge advantage.

Digital single event counting makes it possible to use four detectors of the same type and size in line after each other and obtain four almost undetectable peak profiles. Digital single event counting allows the peak profiles to be delayed for a given calibrated amount of time; staple the individual peak profiles linearly on top of each other and the detectability of the small fraction improves by a factor of four. Incidentally, the background under those peaks adds up not linearly like the signal but under the square root because of the statistical character of background count rate. The total sensitivity of the radioactivity flow detector is dramatically improved. Analogue output signal of radioactivity flow detectors 0–1 V can never produce the same effect.

Conclusion

Detection of radioactivities in chromatography requires individual methods depending on the nature and energy of the emitted radiation. Sensitivity of radioactivity detection in chromatography requires "single event counting" and "raw data storage" for true measurement values and correct registration, evaluation, calibration and reconstruction of chromatograms at any time later on. Raw data of radioactivity in chromatography are digital and have statistical character.

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