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Quantification of triazine herbicides using chloroplasts in conjunction with thin-layer chromatography

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ABSTRACT

We present a video-densitometric quantification method for the triazine herbicides atraton, terbumeton, simazine, atrazine and terbutylazine. Triazine herbicides were separated on silica gel using methyl-t-butyl ether, cyclohexane (1+1, v/v) as mobile phase. The quantification was based on a bio-effective-linked analysis using chloroplast and 2,6-dichlorophenolindophenol. Within 1-2 minutes *HILL*-reaction inhibitor substances show blue-grey zones on a pale yellow-green background. To increase the contrast, the moist plate can be dipped into a solution of PEG-600 (10% PEG-600 in methanol) for 2s. Measurements were carried out using a 16 bit ST-1603ME CCD camera with 1.56 megapixels (from Santa Barbara Instrument Group, Inc., Santa Barbara, USA). A white LED was used for illumination purposes. The range of linearity covers more than one magnitude using the (1/R) - 1 expression data transformation. The method can be used for herbicide screenings in environmental samples, because not spectral sensitivity but herbicide activity is measured. The separation method is cheap, fast and reliable.

INTRODUCTION

CCD-cameras

Modern TLC-scanners can measure in absorption, fluorescence as well as in transmittance. TLC-scanners cover the whole wavelength range from 200 up to 1000nm. The disadvantage of TLC and HPTLC scanners is their high purchase price and maintenance costs. The high price of modern TLC-scanners makes CCD image analysis in thin-layer chromatography (TLC) so advantageous. Most TLC-applications are designed to work in the wavelength range from 400 to 800nm, using human eyes as detectors. Scanning equipment like CCD-cameras (charge coupling device-cameras) or flatbed-scanners that work in the visible range are cheap, easily available and can be used for plate evaluation. The term video-densitometer has also been introduced for such scanning devices (Broszat et al. 2010). The disadvantage of a video-densitometer is that spectral information is not available. This makes spectral peak identification and spectral peak-purity tests impossible. To make a separation more specific, we recommend a staining step. It is also possible to measure a specific bioactivity which often makes spectral identification and peak-purity testing unnecessary.

What features should be taken into consideration when buying a CCD-camera for analytical purposes? Quantitative video densitometric measuring needs a detector which can linearly digitalize light intensity measurements. Double-fold light intensity must result in a double signal value, which can easily be checked by changing the measurement time. Therefore double measurement time must result in double measured values (Spangenberg 2010). The digital resolution of commonly used cameras is 8 bits. A signal is rendered in $2^8=256$ different increments (grey levels), which is not sufficient for quantification purposes. At least a 12 bit capacity is necessary for quantifying ($2^{12}=4096$ increments). CCD-cameras with a resolution of 16 bits are much better because such cameras render $2^{16}=65536$ grey scales. Relatively inexpensive cameras with suitable software that meet these requirements are available for astronomy observations. These cameras produce TIFF-pictures, because the TIF-Format (Tagged Image File Format) supports 16 bit data storage.

Triazine herbicides

Herbicides include many substances with different chemical structures that attack various parts of a plant organism thus

blocking its growth. The most important types by quantity are triazines, such as atrazine, simazine, and terbuthylazine, which are colourless compounds detectable only in UV. They all cause underground water contamination.

Triazine herbicides inhibit photosynthesis. This ability can be used in a specific TLC-quantification method (Jork and Roth 1977). The basis of this chloroplast assay for the inhibition of photosynthesis is the inhibition of the *HILL*-reaction (Kováč and Henselová 1977; Kováč et al. 1978, 1983; Lawrence 1980; Sackmauerová and Kováč 1978). To detect herbicidal active substances, isolated chloroplasts or chloroplast fragments from leaves are applied to the thin-layer plate after development. The blue red/ox-indicator 2,6-dichlorophenolindophenol (DCPIP) is discoloured by active chloroplasts. DCPIP acts as an electron acceptor and loses its blue colour. The following method can determine about 40-45% of all commercially available herbicides (Kováč and Henselová 1977).

The purpose of this work is to show that video-densitometric measurements provide a powerful tool for inexpensive quantitative thin layer chromatography using a CCD-camera in conjunction with a bio-effective-linked assay.

THEORY OF TLC-EVALUATIONS

In planar chromatography light is used for detecting separated sample spots by illuminating the TLC-plate from the top with light of known intensity. A clean illuminated plate will absorb a share of this illuminating light by the layer. The share of light, which is not absorbed but reflected by the surface, should be J_0. If this reflected light shows higher intensity than the reflected light (J) from a sample zone, a fraction of light must be absorbed by the sample (the analyte). The difference between these light intensities is absorbed by the analyte and defines the analyte absorption coefficient a:

$$I_{abs} = J_0 - J = aJ_0 \qquad (1)$$

Increasing sample amounts will induce a decreasing light reflection (J). Therefore a transformation algorithm is needed which turns decreasing light intensities into linear increasing signal values.
With the abbreviation

$$R = \frac{J}{J_0} \qquad (2)$$

following equation for transformation purposes shows linearity between the transformed measurement data (TMD) and the absorption coefficient [Spangenberg 2006].

$$TMD(k) = k\left(\frac{1}{R} - R\right) + (R-1) = \frac{a}{(1-a)} \qquad (3)$$

k: backscattering factor ($k \geq 0$ and $k \leq 1$)
a: absorption coefficient

The back-scattering factor k is the new element in this theory of quantitative remission measurements. The basic idea behind this theory can be explained with the observation that opaque TLC-plates show a bright shine when light from the surface is reflected at a low angle. This observation from all TLC- and HPTLC-plates led to the conclusion that remission is not only influenced by the incident light intensity but also by the reflection angle. The introduced back-scattering factor k exactly describes this observation, the orientation of particles in relation to incident and emission light.

The value of the so called back-scattering factor k lies in the range between 0 and 1 and depends on the scattering quality of the stationary phase. In trace analysis, it is mostly sufficient to use a k-factor of $k=1$ for obtaining linearity for calibration curves.

$$TMD(k=1) = k\left(\frac{1}{R} - 1\right) = \frac{a}{(1-a)} \qquad (4)$$

For $k=0$ no incident light is reflected to the plate top and the resulting expression can be used for fluorescence evaluation (Spangenberg 2006).

EXPERIMENTALS

Preparation of standards and application on HPTLC-plates

All the chemicals used were of analytical reagent grade. Terbumeton, simazine, atrazine, terbutylazine, atraton had a purity of ≥98% and had been purchased from Ehrenstorfer, Augsburg. Potassium iodide was from Riedel-de Haën, Seelze, Germany, and methanol and ethanol from Roth, Karlsruhe, Germany. Cyclohexane, starch (according to Zulkowsky), HCl, PEG-600, glycerine, KH_2PO_4, $KMnO_4$, 2,6-dichlorophenolindophenol and methyl-t-butyl ether were purchased from Merck, Germany, as well as silica gel K60 Lichrospere® (with a fluorescent dye) used as the stationary phase.

Stock solutions were prepared by dissolving 4.000mg of standard triazine herbicides in 25ml of methanol. For calibration purposes the stock solution was subsequently diluted with methanol in order to apply amounts of 1 to 20µl.

Samples and standards were spotted dash-like (7mm) on an HPTLC silica gel Lichrospere® plate (10·10cm, with fluorescent dye) using a DESAGA AS 30 device. The plates were developed in a vertical developing chamber without vapour saturation to a distance of 70mm from the starting point, using methyl-t-butyl ether, cyclohexane (1+1, v/v) as the mobile phase.

The plate was dried in a gentle stream of air for 5 minutes and placed in a chlorine containing chamber for 5 minutes. Chlorine was produced from 10ml $KMnO_4$ solution (3g $KMnO_4$ dissolved in 100ml of water) and 10ml HCl (25ml 32% HCl dissolved in 50ml of water). Five minutes after mixing, the chamber was filled with chlorine and the TLC-plate could be placed.

Staining with starch-iodine reagent

The starch-iodine staining reagent was found to be sufficiently sensitive. To produce the starch-iodine reagent, 800mg of potassium iodide was dissolved in 20ml of water. The amount of 800mg starch (according to Zulkowsky) was dissolved in 20ml of water. Both solutions were mixed and dissolved with 10ml of ethanol. This mixture remains stable for one day.

Red-brown zones are formed on a slightly dark background if the chlorinated plate is dipped for 1s in starch-iodine reagent. The colours remain stable for days. A densitogram of such a staining is shown on Figure 1.

Chloroplast reaction (HILL-reaction)

The chloroplasts were isolated from spinach leaves. Rinse 300g fresh spinach leaves with distilled water and remove the leaf stems and veins. Place about 140g of rinsed leaves and 20g of ice in a container (wrapped in aluminium foil) in ice water and homogenise. Gradually add 30ml of buffer solution (phosphate buffer pH 7.5, for which 700mg KH_2PO_4 were dissolved in a 1 litre of water). Filter this homogenised mixture through fine gauze (e.g. bandage fabric) with slight hand pressure and then divide the filtrate between three falcon tubes (wrapped in aluminium foil). Centrifuge at 3600 r.p.m. for 10min at 14°C. Dispose of the supernatant. The chloroplast pellets were suspended in 30ml phosphate buffer containing 3g glycerine and then frozen in 5ml portions. The chloroplast solutions must be stored in the dark at –20°C. The chloroplast solution keeps its activity for more than six months.

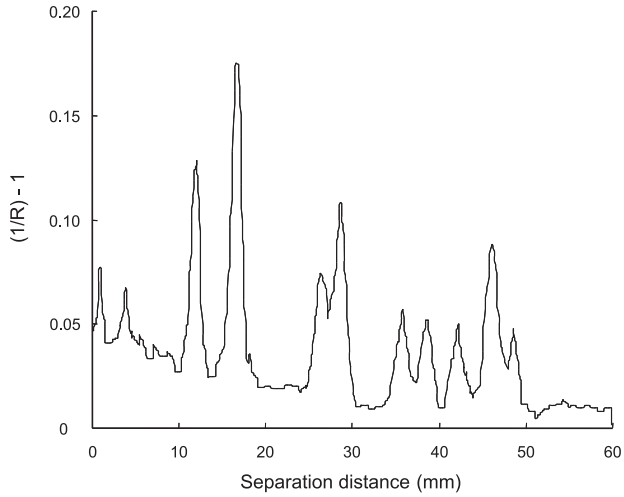

Figure 1. Separation of nine triazine herbicides over the distance of 7cm and stained with iodine/starch reagent (extracted from an 8 bit JPG-picture). The mobile phase was methyl-t-bythyl ether, cyclohexane (1+1, v/v). Separated with rising Rf-values were atraton (10ng), prometone (20ng), desmetryn (10ng), simazine (10ng), atrazine (10ng), ametryn (10ng), terbutryn (10ng), prometryn (20ng) and metribuzine (100ng). The application point is at 3mm and the front signal at 60mm separation distance.

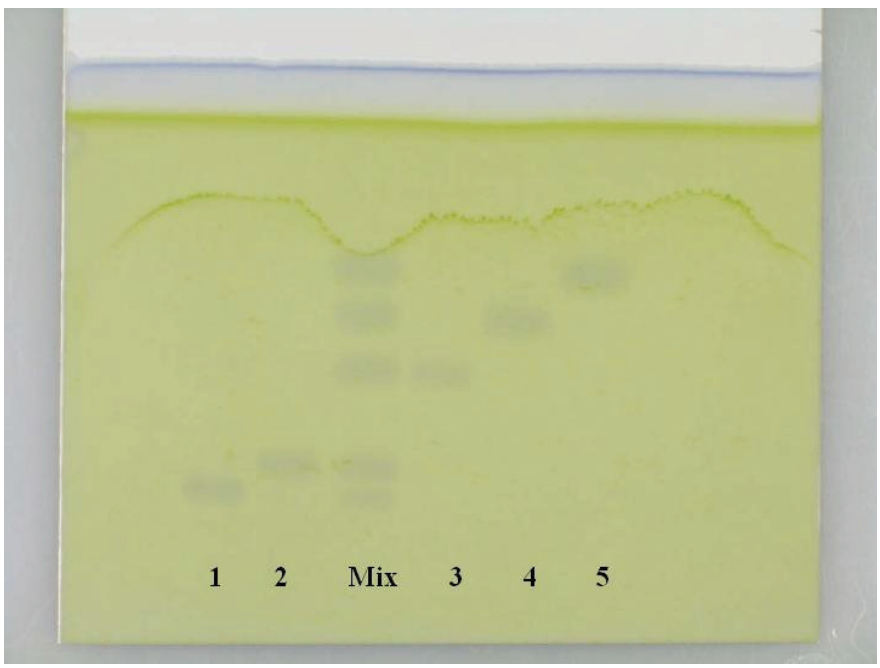

Figure 2. Separation of triazine herbicides after HILL-reaction and 2,6-dichloro-phenolindophenol staining, white light: (1) Atraton (2ng per zone), (2) terbumeton (1ng per zone), (Mix) mixture off all herbicides, (3) simazine (2ng per zone), (4) atrazine (1ng) per zone), and (5) terbuthylazine (1ng per zone) on silica gel with cyclohexane-methyl-tert.-butyl ether (1+1, v/v) as mobile phase

For reactivation, dissolve 20mg 2,6-dichlorophenolindophenol in 50ml of water. Mix 5ml of the chloroplast solution with 45ml of water, then mix this suspension with the 2,6-dichlorophenolindophenol solution in the ratio 5:1.

Dip the dried plate in the 5:1 mixture (see above) for 4s (Lawrence 1980). Cover the TLC plate with another glass plate to keep the layer moist, and then expose the plate to intense white light (ca. 40 Watt) at a short distance for about 2-5 minutes. Halogen floodlights are suitable for this purpose, placed at about 20cm above the plate. The light exposure should be stopped when the background ceases fading. HILL-reaction inhibitor substances will appear as blue-grey zones on a pale yellow-green background within 1-2 minutes. In order to increase the contrast, the moist plate can be dipped into a solution of PEG-600 (10% PEG-600 in methanol) for 2 s. After dipping, the plate should be scanned as soon as possible. PEG-600 enhances the red chlorophyll fluorescence. The reagent can be used on silica gel and cellulose plates.

Figure 2 illustrates the separation of 5 triazine herbicides. On track 1 and 2 as well as on tracks 3 to 5 standards were separated. The standard mixture was separated on a track marked with Mix.

Apparatus

For direct video-densitometric evaluation a ST-1603ME CCD camera with 1.56 megapixel from Santa Barbara Instrument Group, Inc., Santa Barbara, USA was used. The camera was mounted with a Kodak KAF-1603ME CCD pixel array containing 1530·1020 pixel. The array size is 13.8·9.2mm with a pixel size of 9·9 microns. The camera uses a 16 bit A/D converter and a high speed USB interface. The camera was used in combination with a Schneider SKR KMP Xenoplan 28/2.0-M30.5 lens. For plate evaluation the CCD-array was cooled to –5°C. After separation and staining the HPTLC-plate were placed below the camera at a distance of 30cm. This distance was adjusted so that 8.5cm were detected by 1020 pixel providing a resolution of 83.3μm per pixel. A single mm separation distance is measured by 12 diodes producing 12 data points. The plate was measured in the dark using two LEDs emitting white light. The time of 6 seconds was necessary to measure the full 16 bit range.

RESULTS AND DISCUSSION

Figure 1 shows the result of a video-densitometric evaluation of different triazines (10ng each), separated on silica gel with methyl-t-butyl ether and cyclohexane (1+1, v/v) as a mobile phase. The plate was stained with iodine-starch reagent after treatment with chlorine. The application band-width of different analyte amounts was 7mm. The detection limits were in the range of 10ng per triazine. The densitogram in Figure 1 shows sharp bands and a baseline separation of all these trianzines. Figure 3 shows the calibration graph for atrazine, measured by using the iodine/starch-reagent. Expression (4) is used for evaluation, rendering a linear calibration graph in the range from 10 to 1000ng atrazine per band.

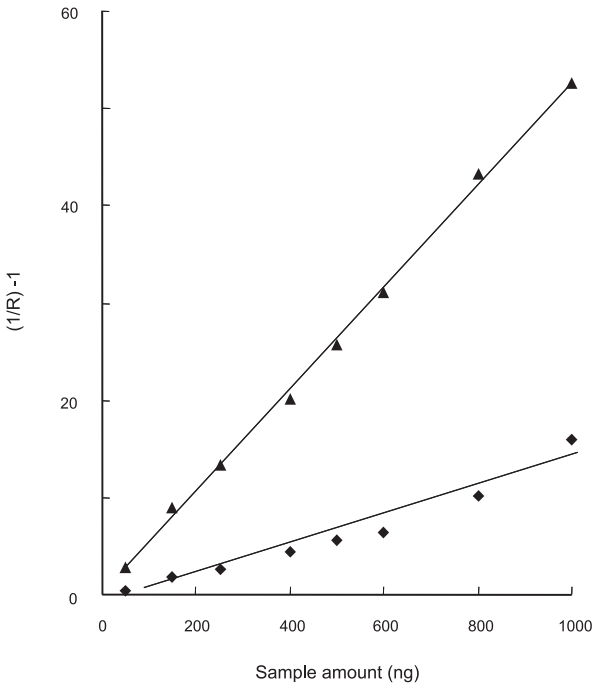

Figure 3A. The plot showing the range of linearity for triazine herbicides (stained with iodine/starch-reagent): atrazine (top) and simazine (bottom) in the range from 50ng to 1000ng per zone (from top to bottom) (Broszat et al. 2010).

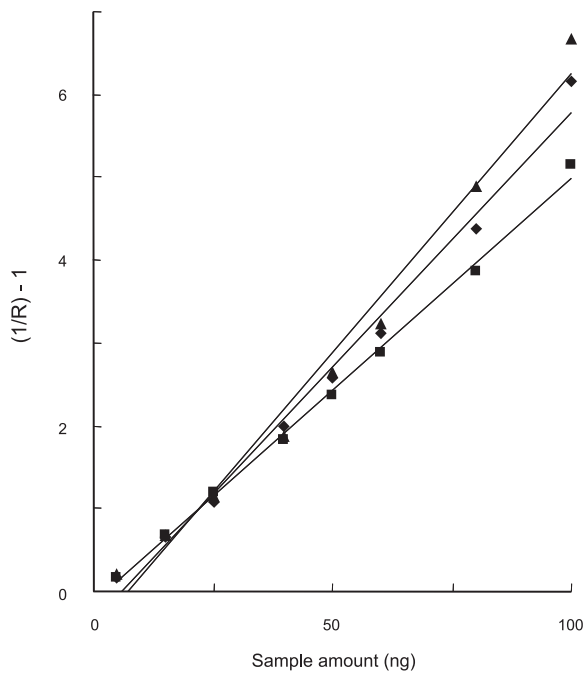

Figure 3B. The plot showing the range of linearity for triazine herbicides (stained with iodine/starch-reagent): terbumeton, atraton and terbuthylazine in the range from 5 to 100ng per zone (from top to bottom).

The plate stained by cloroplast/2,6-dichlorophenolindophenol reagent (Figure 2) shows a baseline separation of only five triazines. The separation of atraton, simazine (2ng per zone, each) and terbumeton as well as atrazine (1ng per zone, each) was degraded in comparison with the same separation in Figure 1. This was surprising, because the amounts separated in Figure 1 were much larger (10 to 20ng) than the separated amounts in Figure 2 (1 to 2ng).

Figure 2 shows a strong longitudinal diffusion (vertical diffusion) which dramatically reduced peak resolution. This was a result of the separation, because the diffusion is dependant on the Rf-values. Zones of higher Rf-values showed broader peaks. The diffusion was not the result of the staining process, in which the plate was kept wet for some minutes. Diffusion during this staining process was negligible, because no horizontal diffusion (latitude diffusion) was observed.

The bands in Figure 1 and Figure 2 show widths of 7mm, independent of the separation distance. The rectangular shape of the application was clearly observable, which was an argument against diffusion in all directions. Diffusion in all directions would render a more oval band shape.

The longitudinal diffusion in Figure 2 was a result of the low detection limit of the bio-effective-linked analysis. In other words, the nice separation of all triazine herbicides in Figure 1 was the result of a less sensitive staining process. Figurally speaking, the starch/iodine reagent just stained the top of the peaks. Sample molecules in-between remained unstained, "pretending" that the resolution was good. This effect was shown in Figure 4, where densitogram of five triazine herbicides had been measured by iodine/starch reagent and chloroplast assay. Each application band was measured using 64 data points resulting in 64 densitograms. These 64 densitograms measured from each track were combined in a single densitogram. This data-averaging improved the signal-to-noise ratio by a factor of 8 in comparison with the signal-to-noise ratio of a densitogram registered only by a single data point. All tracks were evaluated with the same number of measurement data. They were located in the centre of the application band. To achieve reliable evaluation, all tracks were evaluated using the same position within the track and using the same evaluation widths (Broszat et al. 2010).

Figure 5 shows the atrazine and simazine calibration graphs of the chloroplast assay, which showed linearity in the range from 0.5ng to 26.0ng. The data were evaluated using expression (4). The graphs for terbumeton, atraton and terbuthylazine showed similar results. Especially, the limits of detection for all five were nearly identical and lied at 1ng per spot.

The chloroplast assay maked the detection of herbicides more specific because a biological activity was measured and was not just an unspecific measure in terms of light absorption or fluorescence. The slope of the herbicide array results were a measure for the biological activity. Figure 5 describes that in its biolocical activity terbutylazin was as effective as atrazine. Terbumeton and simazine were less effective whereas atraton showed the lowest biolocical activity from all five investigated triazines.

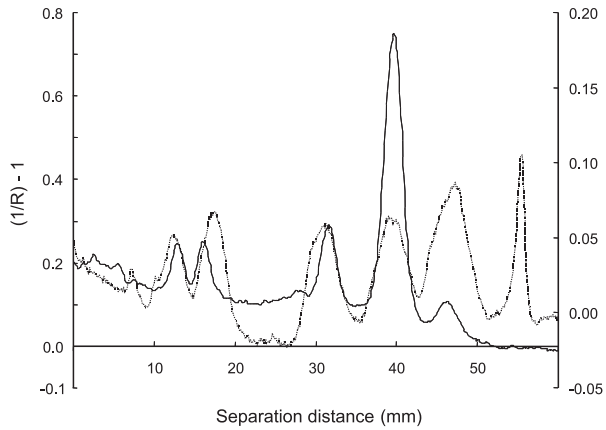

Figure 4. Densitograms of five triazine herbicides, measured by iodine/starch reagent (solid line) with the concentrations 400ng per spot for atrazine and simazine and 40ng per spot for terbumeton, atraton and terbuthylazine. The chloroplast assay results in a densitogram showing broader peaks (dotted line) for the concentrations 5ng per spot for atrazine, terbumeton, atraton and terbuthylazine and 10ng per spot for simazine.

The herbicide assay also provided much lower detection limit than the iodine/starch reagent. The detection limit was improved by a factor of nearly 20. In some cases the evaluation in fluorescence at 366nm showed advantages concerning signal-to-noise ratio and detection limits. All in all, both detection methods (UV 366nm or white light) provided comparable results.

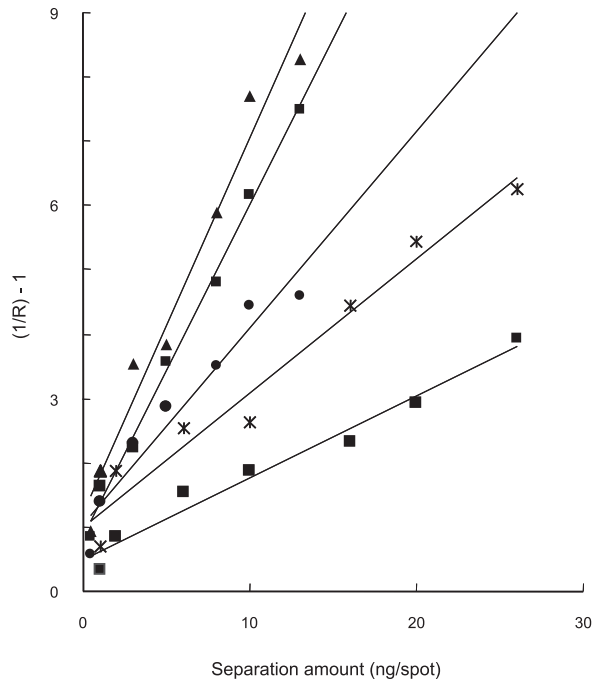

Figure 5. Range of linearity for terbutylazine, atrazine, terbumeton, simazine and atraton (from left to right) for the chloroplast assay in the range from 0.5ng to 26.0ng per spot.

CONCLUSION

Herbicides can be quantified by using a TLC-separation in combination with different staining methods. The iodine/starch reagent provides an excellent zone resolution and a separation of nine triazines on a single track over a separation distance of 70mm. The detection limit was at 10ng. The disadvantage of this method was the unspecific staining of all triazine molecules. The chloroplast assay measures the biological effect of herbicides. This assay provided very low detection limits (below 1ng per zone) and specifically measured a blocking of chloroplast activity. The disadvantage of this assay was the reduced zone resolution which was caused by the low detection limits. In summary, the method can be used for herbicide screenings in environmental samples, because not spectral sensitivity but herbicide activity is measured. The chloroplast solution keeps its activity for more than six months. This makes it possible to use the method as a commercially available screening test for all herbicide active substances.

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