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Short communication

In vivo incorporation of radioactive ^{36}Cl, a method for monitoring chloro compounds in biological material

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Abstract

A new method for fast and easy monitoring of the presence, isolation, and separation of natural chloro compounds in plants is described. The method relies on the in vivo incorporation of radioactive ^{36}Cl and new enhancement methods in autoradiographic technology. The method allows the time of exposure to be limited to 4 days and is thus suitable for routine purposes.

Keywords: Plant materials; *Brassica napus*; Autoradiography; Halogenated compounds

1. Introduction

Labelling of plant chloro metabolites with radioactive ^{36}Cl in vivo by addition of aqueous sodium [^{36}Cl]chloride to the nutrient solution 2 days before harvest makes it possible to reveal and selectively isolate the content of natural chloro compounds. The radioactivity is used as a marker during the purification procedure using liquid scintillation counting to determine the amount of radioactivity and autoradiography to determine the number of chlorinated compounds the fraction in question contains. The method has general applicability in the screening for chloro compounds in biological samples. It offers a fast, reliable, and easy way for monitoring the isolation and purification of such compounds and serves to differentiate between natural and anthropogenic sources of chlorinated compounds.

The half-life of 301 000 years of ^{36}Cl resulting in a relatively low specific radioactivity and the limited loading capacity of thin-layer chromatographic plates has previously prevented the use of this method for screening purposes. In 1971 Wei et al. incubated *Aspergillus ochraceus* in a medium containing sodium [^{36}Cl]chloride this resulted in an incorporation into the mycotoxin ochratoxin A [1]. In 1975 Engvild observed chloro compounds in extracts of 15 immature seeds labelled with radioactive chloride [2]. Using direct autoradiography of thin-layer chromatographic plates at room temperature with unflashed films, without a scintillator or an intensifying screen, Engvild had to expose the films for 4 months. In 1992 Kay et al. isolated a chlorinated compound from the slime mould *Dictyostelium discoidium* by incorporation of ^{36}Cl [3]. The radioactivity was detected by autoradiography at −70°C, on preflashed

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films sprayed with the scintillator En^{3}Hance, and an intensifying screen was not applied. These films required exposure for at least 2 months.

Using an intensifying screen and exposing at −70°C it is now possible to obtain sufficient blackening of a film after 4 days of exposure and consequently allows for a larger utilisation of this isotope in biological experiments.

2. Experimental

2.1. Materials

An aqueous solution of 100 μCi sodium [^{36}Cl]chloride with a radiochemical purity >99.5% and a specific activity of 17.2 mCi/g Cl corresponding to 500 MBq/gCl, Hyper MP films, the intensifying screen Hyperscreen, the flashgun Sensitize, and the radiographic cassette Hypercassette were all purchased from Amersham (Bucks., UK). Merck preparative thin-layer chromatographic plates (commercially precoated Kieselgel 60 plates, F_{254}, 20×20 cm, 2 mm layer) were used. The scintillant Ultima Gold was used for liquid scintillation counting.

2.2. Cultivation, incorporation, and extraction

A total of 150 rape seeds (*Brassica napus* L. cv. Sputnik), treated with the seed dressing Vitavax, were sown directly in a tank filled with Grodan granulate, 5 seeds each in 30 equidistant holes. The plants were grown in a Conviron growth chamber under controlled environmental conditions with a light–dark cycle of 16–8 h, a day–night temperature of 17–15°C, a relative humidity of 40% and illuminated with Osram daylight lamps (HQI-T 250 W/D) providing a photosynthetically active photon flux density of 500–600 μmol/(m^2 s). To allow the plants to absorb as much of the added radioactive chloride as possible the plants were grown under suboptimal chloride concentrations using Johnson's chloride free nutrient solution [4]. The nutrient solution was replenished as needed. After 13 days, thinning of the plants left 30 plants, one in each hole, of approximately equal size. At the same time the surface was covered with polyethylene beads to minimise evaporation. Two days before harvest an aqueous solution of 0.9 ml 100 μCi sodium [^{36}Cl]chloride corresponding to 12.8 mg Cl was added to the nutrient solution. Plants were harvested at 3 months and weeks old. The stems were cut into 4–5 cm short pieces and the silicules were cut into two pieces. The rape material was kept frozen at −20°C until extraction with 96% EtOH.

2.3. Preparation of autoradiograms

A 200-μl volume of the concentrated extracts was applied on a thin-layer chromatographic plate and afterwards developed in a vapour saturated tank with the mobile phase *n*-butanol–acetic acid–water (4:4:1, v/v). After development the plate was air dried, wrapped in Vitawrap to avoid contamination, and placed in a radiographic cassette together with an intensifying screen. The cassette was kept at −70°C for 4 days. The film was developed following standard procedures.

3. Results and discussion

3.1. Investigation of the autoradiographic enhancement procedures

The use of a scintillator like Amplify or En^{3}Hance will only result in a marked increase in efficiency with weak β-emitters. The gain is a 1000-fold for ^{3}H (18 keV), 15-fold for ^{14}C (156 keV) and ^{35}S (167 keV), and lesser for ^{33}P (249 keV) [5]. Considering these facts it is estimated that experiments with ^{36}Cl (709 keV) will not gain from a scintillator. When long exposure times are necessary it is recommended [6] to use preflashed films, with an absorbance unit of 0.1–0.2 above an unexposed film measured at 540 nm, to correct for the non-linear relationship between radioactivity of the sample and absorbance of the film image. Experiments with films preflashed from heights of 5.5–90 cm above the film in 2.5-cm increments showed that a height of 18 cm resulted in an absorbance unit of 0.18 measured with a conventional UV spectrophotometer at 540 nm. This is specifically related to our darkroom facility and should be adjusted to suit the darkroom concerned. However, comparison of an autoradiogram exposed for 4 days of a preflashed film with an autoradiogram

of a non-flashed film showed no difference in this case, since the exposure time was too short to gain from preflashed films. To account for the amount of radioactivity and thus the amount of chloro compounds in a given fraction, autoradiograms can be combined with liquid scintillation counting of this fraction.

3.2. Investigation of the sensitivity of the method

The sensitivity of the method using a film exposed for 4 days at −70°C together with an intensifying screen was investigated. Two intensifying screens can in some cases be used to increase the efficiency of light detection and consequently lower the exposure time. However, this diminishes the degree of resolution and is not optimal for this kind of experiment. Solutions of radioactive sodium chloride in amounts from 38 to 0.15 μg were applied to a thin-layer chromatographic plate (Fig. 1). An applied amount of 0.15 μg sodium chloride could hardly be detected, however, it was possible to observe sufficient blackening of the film when the radioactivity applied originated from 0.30 μg sodium chloride corresponding to 2.3 nCi.

3.3. Liquid scintillation counting

^{36}Cl has a relatively high β-emission energy allowing the measurement of the radiation directly in the tritium region from 0 to 18.6 keV. Consequently addition of a scintillant is avoided and hence the fraction concerned is not contaminated. This can be done by measuring Čerenkov radiation, which is the phenomenon where β-particles are emitted to a medium at velocities exceeding that of light in the medium concerned. The other radioactive chloro isotopes have higher β-emission energies, however, their short half lives render them impractical for these kinds of experiments. Čerenkov radiation is not affected by chemical quenching i.e. absorption of the UV and visible Čerenkov photons by a chemical prior to detection. Unfortunately, Čerenkov radiation is seriously affected by colour quenching. Bleaching materials can be used, but is not recommended owing to the resulting contamination of fractions. Fractions, in the early purification steps, are often heavily coloured and hence prohibit the measurement of Čerenkov radiation in the beginning of the purification procedure. Instead 50 μl of the fraction concerned and 5 ml scintillant are added to a plastic vial and counted in the direct DPM protocol in the liquid scintillation counter. The dilution results in fractions with a less intense colour and hence with diminished colour quenching. After a few purification steps the fractions lose colour and Čerenkov radiation can then be measured.

Plastic vials contain phthalates, which are extracted into the fractions when apolar solvents are used, demanding the use of glass vials to prevent the

Fig. 1. Autoradiogram of a thin-layer chromatographic plate (Si 60; *n*-butanol–acetic acid–water, 4:4:1) with applied amounts of chloride from 38.0 to 0.15 μg. The starting and end lines of the plate are at the bottom and top of the film, respectively. The exposure time was 4 days.

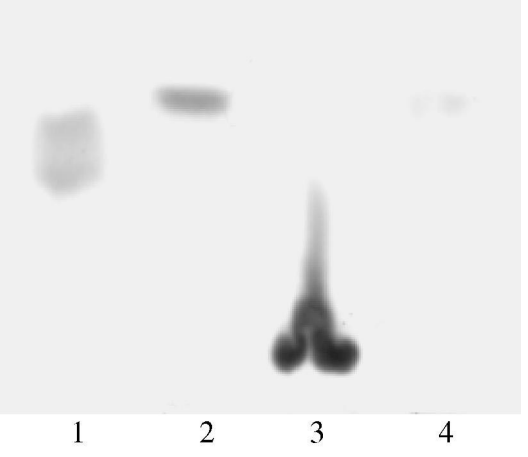

Fig. 2. Autoradiogram of a thin-layer chromatographic plate (Si 60; *n*-butanol–acetic acid–water, 4:4:1), where 200 μl concentrated EtOH extract of rape leaves (1), stems (2), silicules (3) and a standard of sodium [^{36}Cl]chloride (4) was applied. The position of starting and end line of the plate are at the bottom and top of the film, respectively. The exposure time was 4 days.

pollution of the fractions. The counting efficiency of ^{36}Cl using glass vials is very low when counting Čerenkov radiation, only 4.7% in water [7], hence the number of scintillation counts are often in the region of the counts of the background. However, addition of 0.9 ml 100 μCi sodium [^{36}Cl]chloride to 30 rape plants (*Brassica napus*) is enough to reveal the presence of natural chloro compounds from liquid scintillation counts and in autoradiograms with 4 days of exposure (Fig. 2).

4. Conclusions

A new method of selective monitoring chlorinated compounds in extracts is described. It requires a relatively short exposure time of only 4 days. This new method can be useful for revealing the presence of chloro compounds in various extracts and for the monitoring of such compounds during isolation and separation.

Acknowledgements

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