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EVALUATION OF ^{35}S-RESIDUES IN GRAINS AND GRAIN FRACTIONS FUMIGATED WITH ^{35}S-LABELLED CARBONYL SULFIDE (COS)

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ABSTRACT

^{35}S-labelled carbonyl sulfide (COS) was used to measure the amount of sorbed ^{35}S residues and converted ^{35}S residues in grains and grain fractions. Hard wheat, soft wheat, paddy rice, brown rice, polished rice, sorghum, maize, canola, barley, oats and peas were exposed for 4 days to 50 mg L^{-1} of COS^{35} with a total radioactivity of 20m*Ci*. After exposure, the samples were aired. The levels of ^{35}S residues varied with commodity, eg. 0.003-0.02 mg (COS equivalents) kg^{-1} (grain) in lipid extractions and 0.09-0.38 mg kg^{-1} in water extractions. More than 90% of ^{35}S (COS equivalents) residues were in the water extractions. The total uptakes of radioactivity were below 0.4 mg kg^{-1} for all 11 commodities. The total radioactivity determined by phosphorus imaging of extractions and sectioned commodities ranged from 0.1-0.4 mg kg^{-1}. The radiation image shows that more than 90% of ^{35}S residues was located or distributed in the embryo, testa, pericarp and husk, and the ^{35}S was still slowly desorbing from grains after 2 days aeration.

INTRODUCTION

Fumigation of stored products with chemicals such as phosphine (PH_3) or methyl bromide (MeBr) is used to prevent insect damage. However, MeBr is being withdrawn as an ozone depleting substance under the Montreal Protocol (UNEP, 1996) and insect resistance to PH_3 is increasing (Chaudhry, 1997). PH_3will then be the only registered fumigant available for grains around the world. The over-reliance on PH_3 has resulted in the increasing levels of insect resistance to PH_3 in the world (Collins *et al.,* 2002, 2003). There is, therefore, an urgent requirement for the development of an alternative fumigant for MeBr and for management of PH_3 resistance on stored product insects. Carbonyl sulfide (COS) is a potential fumigant to replace MeBr and PH_3 for stored products (Desmarchelier, 1994; Ren, 1997; Zettler *et al.*, 1997; Plarre and Reichmuth, 1997; Weller, 1999), and has been patented by CSIRO Entomology (Banks *et al.,* 1993).

Carbonyl sulfide is a colourless, odourless gas with a boiling point of -50.2°C. Its chemistry has been reviewed (Ferm, 1957). Carbonyl sulfide is stable, but can undergo decomposition, hydrolysis, oxidation and reduction. Many natural sources of COS have been identified, including oceans, soils, volcanoes and marshes (Khalil and Rasmussen, 1984; Brown *et al.,* 1986; Adams *et al.*, 1981). Therefore, COS is

naturally present in the atmosphere, in water, soil, and plants, as well as many raw and processed foodstuffs, including cereals and oilseeds. The natural levels of COS in grains and oilseeds were found to be 0.02-0.07 mg kg^{-1} (Ren 1997; Desmarchelier *et al*., 1998; Ren and Desmarchelier, 2002). As part of a study to evaluate COS as grain fumigants, information about the COS residue in stored products is important for its safety to consumers and relevant to the establishment of Maximum Residue Limits (MRL) and to the acceptance of COS as fumigants of stored products. Studies on the fate of ^{14}C labelled COS in grain has shown that ^{14}C was undetectable in sugars, protein, starch, amino acids, protopectines and hemicelluloses extracted from wheat, paddy rice, polished rice, mungbean, and safflower (Annis et al., 2000). The total uptake of ^{14}C determined after fractionation was in the range of 0.036-0.053 mg kg^{-1} (COS equivalents).

The report in this paper measures uptake of ^{35}S-labelled COS on representatives of three major grain groups, cereals, legumes, and oilseeds. It describes the determination of the total radioactivity extracted by water, acid and lipid. The total radioactivity, ^{35}S in grain fractions and distributions are measured by phosphorus imaging. This study is complimentary to those conducted earlier with ^{14}C labelled COS (Annis *et al*., 2000) and without $^{14}CO_2$ interference.

MATERIALS AND METHODS

Reagents and Apparatus

All chemicals were obtained from Tech Ajax, Sydney, Australia. Scintillation vials (20 mL) and scintillation fluid was obtained from Edwards Instruments Company. Elementary [^{35}S] sulphur in toluene solution was supplied by Amershanm Biosciences International plc, U.K. The specific activity was 20 m*C*i (740 MBq) and concentration was 2-10 *Ci* mg^{-1} (74-370 Bq mg^{-1}). All reagents were analytical grade, unless otherwise specified.

The purity of synthetic $CO^{35}S$ was determined on a GOW-MAC (Model 40-001) gas density detector (GOW-MAC Instrument Co., Madison, NJ), after separation on a 1 m × 5mm (i.d.) Porapak Q 100/120 mesh (Alltech Associates, Sydney, Australia, Cat. No. 2702) at 105°C and carrier (N_2) flow of 150 mL min^{-1}. The reference gas was tetrafluoroethane (> 99.9 % pure).

During exposure period, the concentration of $CO^{35}S$ was determined on a Shimadzu GC6AM GC (Shimadzu Seisakusho, Kyoto, Japan), equipped with a flame photometric detector (FPD). Separation was achieved on a 1 m × 3 mm (id) glass column packed with HayeSep Q (Alltech Associates, Cat. No. 2801) at 140°C and carrier (N_2) flow of 40 mL min^{-1} at 0.8 psi.

A model LS 200 Beckman (Beckman Instrument Co., USA) liquid scintillation analyser was used for scintillation counting, operating at the appropriate wavelength for the radioisotope.

A model of FLA-5000 Fluorescent Image Analyzer (Fuji Photo Film Co. Ltd. Japan) was used for scanning radiation images of extraction and sectioned commodities.

Synthesis of 35S-labelled COS

^{35}S-labelled COS was synthesized by the method as described by Kluczewski *et al.*, (1984). Elementary ^{35}S-labelled sulphur in toluene (0.4 mL) reacted with carbon monoxide (CO) to produce ^{35}S-labelled COS (Eq 1). After generation, the reaction chamber (135 mL sealed glass tube) containing ^{35}S-labelled COS, ^{35}S-labelled CS_2 and CO was inserted into dry ice to crystallize ^{35}S-labelled CS_2. Therefore, 56% pure of ^{35}S-labelled COS and balance gas was CO which not contain radioactivity) was obtained.

$$^{35}S + CO \rightarrow CO^{35}S \qquad \text{Eq 1}$$

Commodities Conditioning and Fumigant Dosing

Eleven representative grains (hard wheat, soft wheat, barley, oats, maize, sorghum, paddy rice, brown rice, polished rice, peas and canola) were used. All commodities (187 g) were placed into a sealed chamber (2.25 L) and allowed to equilibrate at 25°C and 65% relative humidity (RH). After a period of 6 weeks the commodities were removed and moisture content and equilibrium relative humidity (Table 1) were checked. Moisture content (wet basis) was measured from loss of mass in ground samples after oven drying at 130°C for 2 hours. Relative humidity was calculated from measured equilibrium dew point observed on a cooled mirror dew point meter (MBW Elektronic AG, Model DP3-D).

TABLE 1
Moisture content of commodities at 25°C and equilibrium relative humidity of 65%

Commodity	Moisture content (%, w/w, wet basis)
Oats	9.9
Hard wheat	11.2
Soft wheat	11.0
Barley	11.2
Paddy rice	11.0
Brown rice	12.0
Polished rice	12.2
Sorghum	11.9
Peas	11.1
Canola	5.1
Maize	12.3

The conditioned grains (10 g for each variety) were separately transferred to Petri dishes (1 × 5cm i.d.), and all samples were placed in a desiccator (2.25 L), equipped with a septum. ^{35}S-labelled COS (50 mL) containing 16.7 m*C*i of total radioactive was injected into the desiccator by gastight syringe to give an initial concentration of 60 mg L^{-1}, which is the maximum recommended dosage of the fumigant

(Desmarchelier, 1994). The grain samples were fumigated for a typical exposure period (4 days) at 25±2°C. Following the initial dose the fumigant was circulated in the desiccator by a magnetic stir bar. After 4 days exposure, the desiccator was opened and the samples were transferred to a fume hood and aired for 2 days at 25±2°C.

Determination of Radioactivity in the Extractions

The method of fractionating nutrients relies on the sequential solubilising of one fraction while leaving the residue as the substrate for the next extraction. The procedures listed in Fig. 1 were carried out to determine the fate of applied $CO^{35}S$. Untreated grains were used as controls. All samples were prepared for duplicate testing.

(a) A weighed proportion of commodity (5 g) was removed and placed in a stainless steel mortar. The sample was crushed (not finely ground), and transferred to a Soxhlet extraction thimble.

(b) The extraction thimble (containing the 5 g sample) was placed into the Soxhlet apparatus and the sample extracted with 30 mL chloroform overnight. After extraction, the solvent (containing lipid, fat-soluble vitamin, pigments, etc) was transferred into a 25 mL volumetric flask for analysis of ^{35}S in total lipid. The thimble was then placed in a fumehood to allow complete evaporation of residual chloroform. The sample was then transferred to a 25 mL centrifuge tube.

(c) The sample (remaining from step b) was resuspended in 10 mL distilled water. The sample was vortexed and allowed to soak at 25°C for 3 hours. The sample was centrifuged for 10 minutes at 3500 r.p.m and the supernatant transferred to a 25 mL volumetric flask. The pellet was resuspended twice in 5 mL distilled water for 15 min soak between each washing. The centrifuged washings were collected in the 25 mL volumetric flask for analysis of ^{35}S in this solution which contained sugars, amino acids, inorganic acids and sulfide. The pellet was retained (for step d).

(d) The pellet (from step c) was resuspended in 10 mL 1*N* HCl. The sample was vortexed and allowed to soak at 25°C for 1 hour. The sample was centrifuged for 10 minutes at 3500 r.p.m and the supernatant transferred to a 25 mL volumetric flask. The pellet was resuspended twice in 5 mL distilled water for 15 min soak between each washing. The centrifuged washings were collected in the 25 mL volumetric flask for analysis of ^{35}S in this solution which contained sugars, amino acids and sulfide. The pellet was retained (for step e).

(e) The remaining pellet was resuspended with 20 mL 1*N* HCl in a 25 mL volumetric flask for subsequent analysis of ^{35}S in remainings.

Scintillation Counting

Liquid samples (2 mL each, collected from steps b-k) were mixed with the scintillation fluid (5 mL) in a scintillation vial and then was placed in the dark. Precautions were taken to avoid complication due to photo luminescence. Four replicate samples were prepared and each was counted four times, and averaged. Quenching was determined on each fraction to allow correction for counting efficiency.

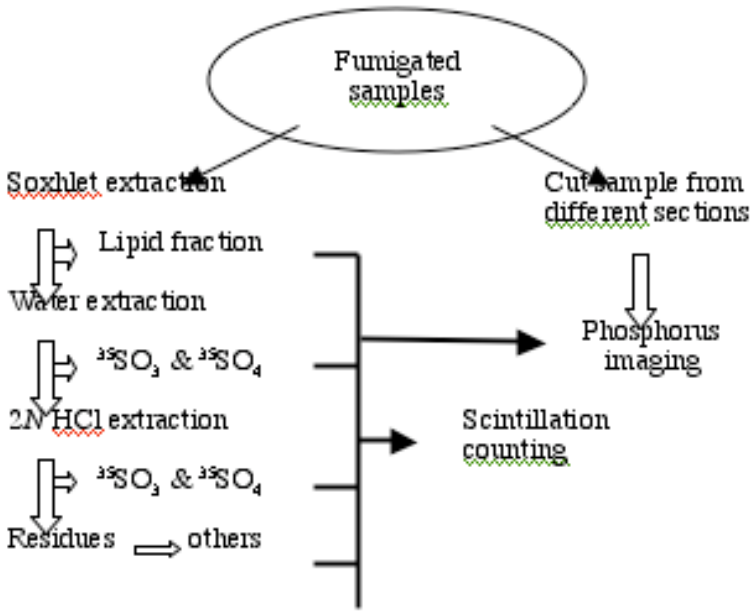

Figure 1. Scheme for the fractionation of commodities and procedures of radioactive measurement

Radiation Image

Commodity kernels treated with $CO^{35}S$ were cut in half section (cross and longitudinal). The sections were held by BLU TACK Bostik (Made in Australia by Bostik Pty. Ltd.) and then placed the BLU TACK on the sample holder of Fluorescent Image Analyzer for scanning the radiation images.

Semi-quantitative analysis of radiation image was carried out by serial dilutions, using sodium hydroxide ethanol solution containing known volume of $CO^{35}S$ as a reference. Serial dilution was performed by adding 0.5 mL of ^{35}S labelled solution (containing 0.05 Mbq/mL) in water to 0.5 mL of water. Each dilution (2 μL) was spotted on a TLC plate (Art. 5554, DC-Alufolien, Kieselgel 60 F_{254}) (3 replicates at each dilution) for scanning the radiation images. The sample extractions were also spotted on the TLC plate for scanning. Radioactivity was compared with serial diluted references and calculated as COS equivalents.

Preparation of Quenched Standards

In scintillation counting, quench correction was carried out by calibrating a series of progressively quenched standards with reference to an external standard of $CO^{35}S$ in sodium hydroxide ethanol solution. Samples were replicated 3 times, and each was counted 4 times, and averaged. All radioactive residue data were converted/calculated from scintillation counting data by calibrating with the quenching standard curve.

RESULTS AND DISCUSSION

Method Evaluation

The quenching standard curve (Fig. 2) was linear over the tested region. The counts increased with increasing dose of ^{35}S mixing with scintillation fluid, and the standard error (SE) between replicates at a minimum was < 8%.

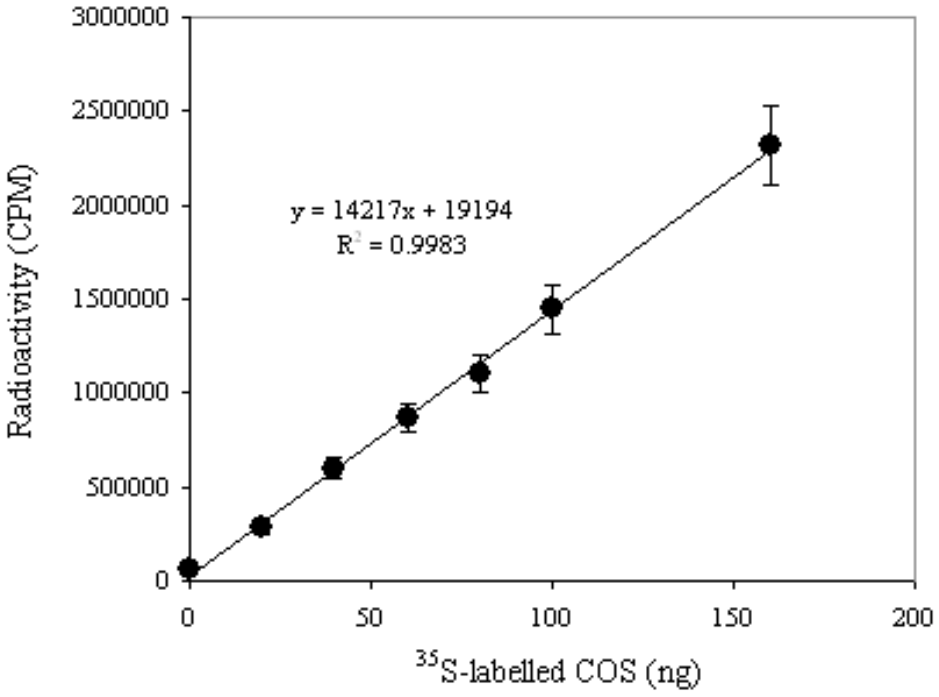

Figure 2. Quenching standard curve. Plot from scintillation counting data (CPM) against series of progressively added $CO^{35}S$. Error bars indicate SD, n=12.

Uptake and Distribution of 35S-containing Substances

The residues of ^{35}S in the extractions of commodities after treatment with $CO^{35}S$ are shown in Fig. 2. Amounts were calculated as mg COS equivalents per kilogram of commodity (kg^{-1}). Residues of COS or alteration products (calculated as COS equivalents) were detected at low levels in a) total lipids, 0.003-0.02 mg kg^{-1} COS equivalents; b) water extractions, 0.09-0.38 mg kg^{-1} COS equivalents; c) 2N HCl extractions 0.005-0.026 mg kg^{-1} COS equivalents and, d) remained residues 0.001-0.003 mg kg^{-1} COS equivalents. The largest readings were in water extractions. That is, almost of 90% ^{35}S residues were presented in water extractions. The total uptakes of radioactive residues (calculated as COS equivalents) were summed the COS equivalents in each extractions. The total residue levels were 0.1-0.16 mg kg^{-1} in

canola, brown rice and polished rice; 0.2-0.22 mg kg^{-1} in maize, oats, barley, soft wheat, paddy and peas; and 0.3-0.4 mg kg^{-1} in hard wheat and sorghum.

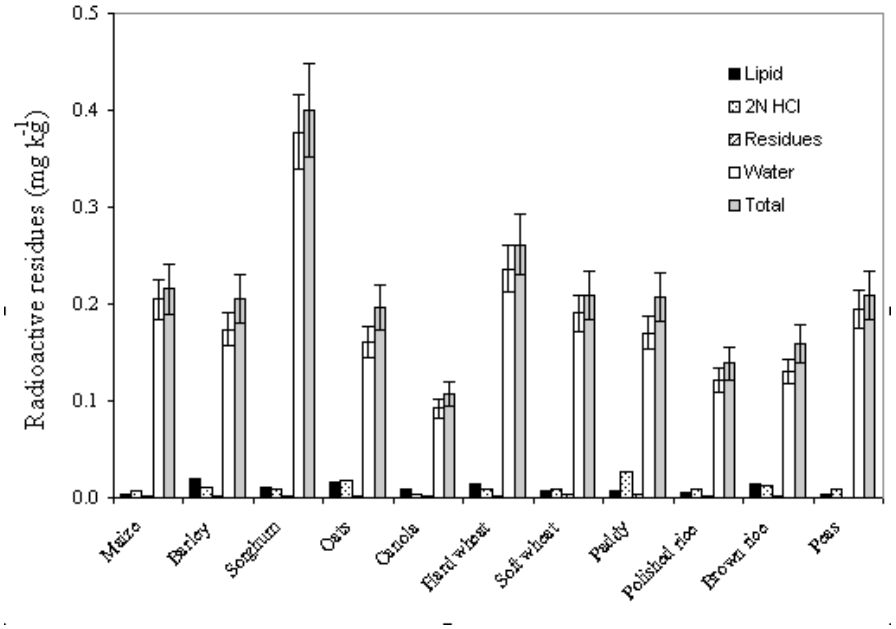

Figure 3. ^{35}S residues in different extractions from grain fumigated with $CO^{35}S$. Error bars indicate SE, n=12

Additional evidence of the uptake of ^{35}S by commodities was obtained from the radiation image (Figs 4 and 5). The method detected radioactivity down to a level equivalent to 0.075 mg kg^{-1} COS equivalents (Fig. 4). ^{35}S residue levels in water extractions were approx 0.3 mg k^{-1} in hard wheat, sorghum, peas and oats; approx 0.15 mg kg^{-1} in maize, barley and soft wheat; and less than 0.075 mg kg^{-1} in paddy, polished rice, brown rice and canola (Fig. 4). ^{35}S residues in lipid and 2*N* HCl extractions were less than 0.075 mg kg^{-1} (Fig. 4). The total uptake of radiolabel at the cut face was approximately 0.3 mg kg^{-1} COS equivalents (Fig. 5). Thus results from three methods for uptake of ^{35}S were similar.

The total amount of $CO^{35}S$ used in fumigation was 112.5 mg (2.2.5 L x 50 mg L^{-1}), which was applied to 187 g of commodity (11 x 17g). Thus the maximum possible uptake of ^{35}S was 600 mg kg^{-1} COS equivalents. The total uptake of radioactivity was less than 0.4 mg kg^{-1} COS equivalents, that is, less than 0.07% of total possible uptake. If all labels were present as COS, this would represent a residue of approx 0.04 mg kg^{-1}. However, as the label is distributed among several biochemical fractions, the total amount of intact COS is much lower than this figure.

These results on low residues and alteration products are consistent with other evidence. For example, COS is less sorbed on grain than is methyl bromide, and can be blown more easily through grain (Desmarchelier 1994; Ren 1997). Exposure to COS also resulted in no loss of germination (Ren *et al.*, 1996), and no observed loss of thiamine, niacin, lysine, maltose, riboflavin, pyridoxine or α-tocopherol (Ren 1997). There were no irreversible reactions between COS and lipids in wheat germ

oil and canola oil, and no effect on total lipids (Ren *et al*., 1997). The reversible partitioning of COS into lipid (Ren *et al*., 1997) provides a useful insight into the behaviour of residual COS on grain. The results from ^{35}S study are consistent with ^{14}C (Annis *et al*., 2000).

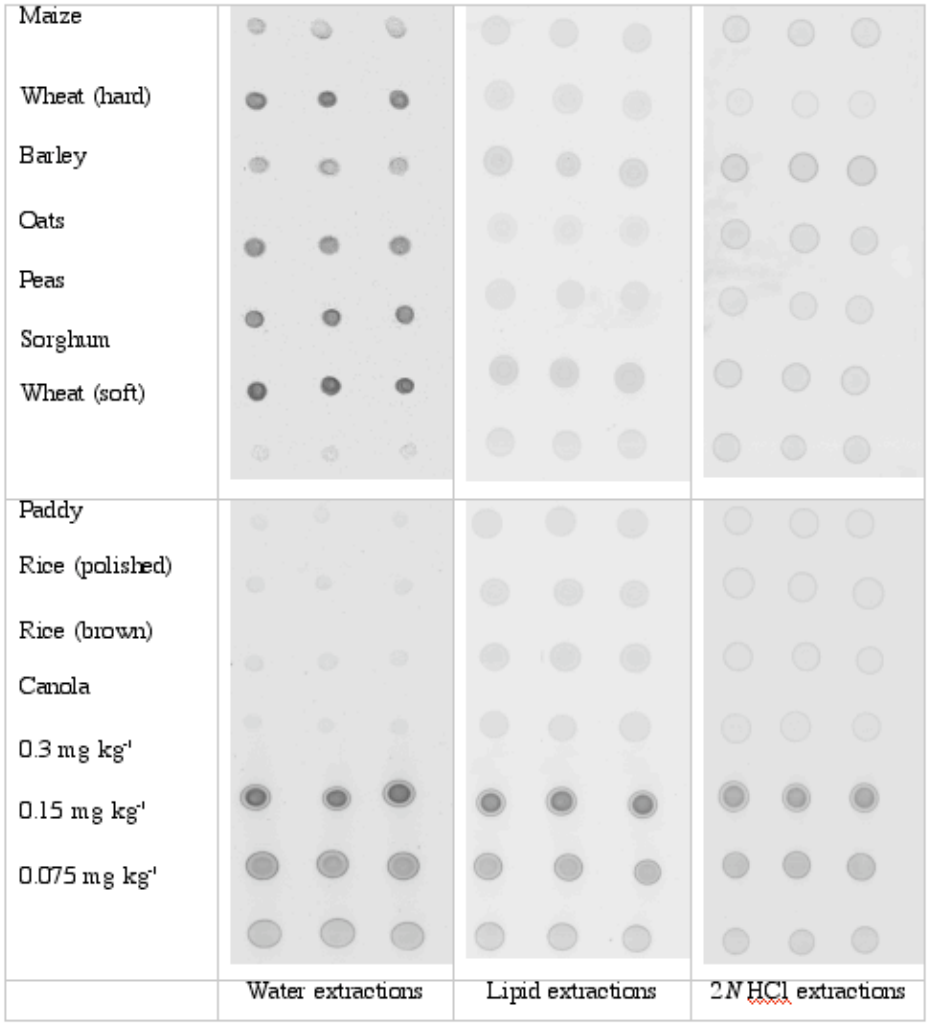

Figure 4. The phosphorus imaging of different extraction from commodities after exposure to $CO^{35}S$ at 25°C, 50 mg/L for 4 days and 2 days aeration.

All these results provide an upper limit of COS residues. Because the experiments here were conducted with eleven different grains, if can be argued that this can be generally expected. In view of high natural COS levels, the radiolabelling demonstrates that only a very low fraction of additional residues can be expected from COS treatment. It is a good fumigant from point view of consumer safety.

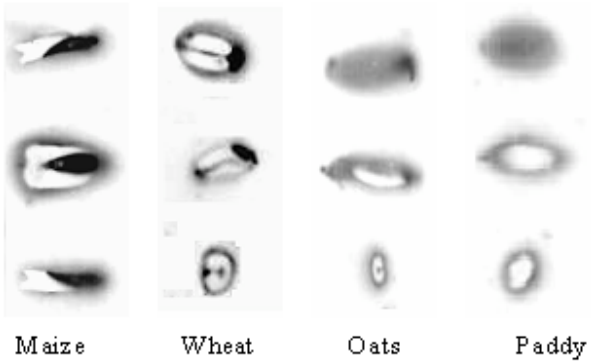

Figure 5. Distribution and position of ^{35}S in the grain fumigated with $CO^{35}S$

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