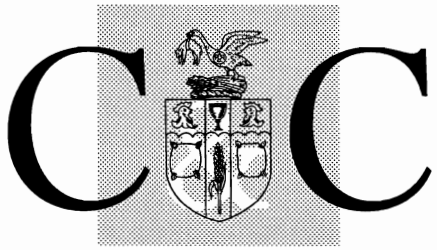
R&D REPORT NO.10

Detecting Irradiation of Seeds Using Microgel Electrophoresis (A Collaborative Trial)

June 1995



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Detecting Irradiation of Seeds Using Microgel Electrophoresis (A Collaborative Trial)

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EXECUTIVE SUMMARY

Preservation of certain foods by irradiation is permitted in the UK. However, all irradiated foods must be labelled as such, to ensure consumer choice. To help enforce labelling, a variety of methods have been developed for distinguishing between irradiated and non-irradiated foods. In preliminary trials, microgel electrophoresis - a simple method of assessing DNA damage - has shown considerable promise in this respect. This report describes microgel electrophoresis, and details results obtained in a blind trial carried out in collaboration with the Swedish University of Agricultural Sciences.

Microgel electrophoresis facilitates analysis of the leakage of DNA from cells extracted from food material. In irradiated samples, the DNA is fragmented and will leak from cells in an electric current. This leakage can be seen as a 'comet' when the stained gel is viewed with a microscope. The size and shape of the comet can be used to estimate the irradiation dose administered to the sample. In non-irradiated samples the DNA is less fragmented, will tend not to leak from the cells and will not form a comet.

The findings of this project are as follows:

- Twenty-two samples were analysed 'blind' by microgel electrophoresis to determine whether they had been irradiated;

- The samples included lentil, sunflower seed, almond, sesame seed, linseed, rose pepper, fig and soya bean;

- In all cases the samples were correctly identified as irradiated or not irradiated;

- Correct estimates of dose administered (+/- 1kGy) were made for 20 of the 22 samples;

- Most of the samples were relatively straightforward to analyse, though the soya bean and rose pepper were more difficult due, in part, to the presence in the preparation of cellular debris.

These findings reinforce the conclusions from two previous blind trials - based on meat samples - that DNA microgel electrophoresis has considerable promise as a means of testing food raw materials to determine whether or not they have been irradiated. It is intended to take the method forward to achieve MAFF "collaboratively trialled non-statutory method" status.

CONTENTS

1. INTRODUCTION

The irradiation of food, by gamma rays or electron beam, can be used as a food preservation technique. It reduces the viable microbiological load of food and can therefore reduce the number of incidents of illness caused by foodborne pathogens, extend shelf life, minimize food loss through spoilage and reduce damage caused by insect pests. Food irradiation is permitted in the UK within a control framework that includes certification procedures and labelling of all irradiated food products (Statutory Instruments 2489 and 2490, 1990).

Methods for determining whether food has been irradiated are required to uphold these controls. Currently there is no method available which is applicable to all foods, not least because of the diversity of food matrices that might be irradiated (Delincée 1991; Raffi and Belliardo 1991; Leonardi *et al.* 1992). However, tests have emerged for application to specific food matrices. These methods are based on the effect of irradiation on some physical, chemical or biological property of the food in question and have been reviewed extensively (see Delincée 1991; Raffi and Belliardo 1991; Leonardi *et al.* 1992; Haine *et al.* 1994). Several of these methods have now been approved as "collaboratively trialled non-statutory methods" by the UK Ministry of Agriculture, Fisheries and Food, including:

- thermoluminescence (TL) for use on herbs and spices (MAFF Report V27);
- electron spin resonance (ESR) for use on meat that contains bone (MAFF Report V28);
- the limulus amoebocyte lysate (LAL) test for use on poultry (MAFF Report V29).

This report outlines further evaluation of microgel electrophoresis as a method for detecting irradiation treatment of foods. The ultimate aim is to achieve "collaboratively trialled non-statutory method" status for this technique, so that it can be made available for routine screening of samples.

Ionising radiation breaks DNA strands into fragments (von Sonntag 1987). Microgel electrophoresis facilitates assessment of the degree of strand fragmentation so that this DNA damage offers a possible marker for the irradiation treatment (Haine *et al.* 1994). The procedure, which is illustrated in Figure 1, involves preparing a suspension

of cell nuclei from intact cells of the sample under investigation. These cells (or nuclei) are embedded in agarose on a microscope slide, lysed using a detergent, and electrophoresed for a short time (e.g. 2 minutes) at a set voltage. During lysis the membranes are disrupted so that the DNA can leak out of the cell and nuclei. When an electrical current is applied, this DNA travels towards the anode at a rate determined by its average size. Thus, if the DNA in the sample has a high number of strand breaks, the average fragment size will be smaller and the DNA will travel relatively quickly through the agarose matrix, spreading further from the cell and forming a long 'comet' (Figure 2). However, if the DNA is relatively intact, fragments will be larger and hence move more slowly through the matrix, forming a smaller 'comet' (Figure 2). The DNA is visualised by staining with a DNA intercalating fluorescent dye (e.g. acridine orange) and viewed under a fluorescence microscope. The technique is fairly simple to perform and does not require particularly costly equipment.

The method was first applied to the detection of irradiation of foods by Cerda *et al.* (1992) who used it to distinguish between irradiated and non-irradiated samples of raw chicken, potatoes and onions. This led to a small BCR blind trial of the method involving three European Laboratories, including CCFRA. Irradiated and non-irradiated samples of frozen chicken could be distinguished and encouragingly accurate estimates of the irradiation doses administered were obtained (Haine and Jones 1992, 1994; Delincée and Marchioni 1993). This work was therefore followed with a further blind trial, organised by the National Food Administration of Sweden (through Dr. Humberto Cerda) and the ADMIT DNA group (through Dr. Henri Delincée), involving nine European Laboratories. Again the potential of the method for distinguishing between irradiated and non-irradiated meat samples (chicken muscle, pork muscle and chicken bone marrow) was demonstrated (Haine *et al.* 1994, 1995). Of the 18 samples analysed, all were correctly identified as irradiated or non-irradiated and in many cases good dose estimates were achieved.

In parallel with the above, further work established that the method can be applied to a wider range of foods including meats, fish, vegetables and seeds (Cerda - unpublished). This collaborative trial was therefore conducted to further evaluate the method for distinguishing between irradiated and non-irradiated samples of plant materials, namely lentil, almond, sesame seeds, linseed, sunflower seeds, rose pepper, figs and soya beans.

Figure 1 - The microgel electrophoresis method

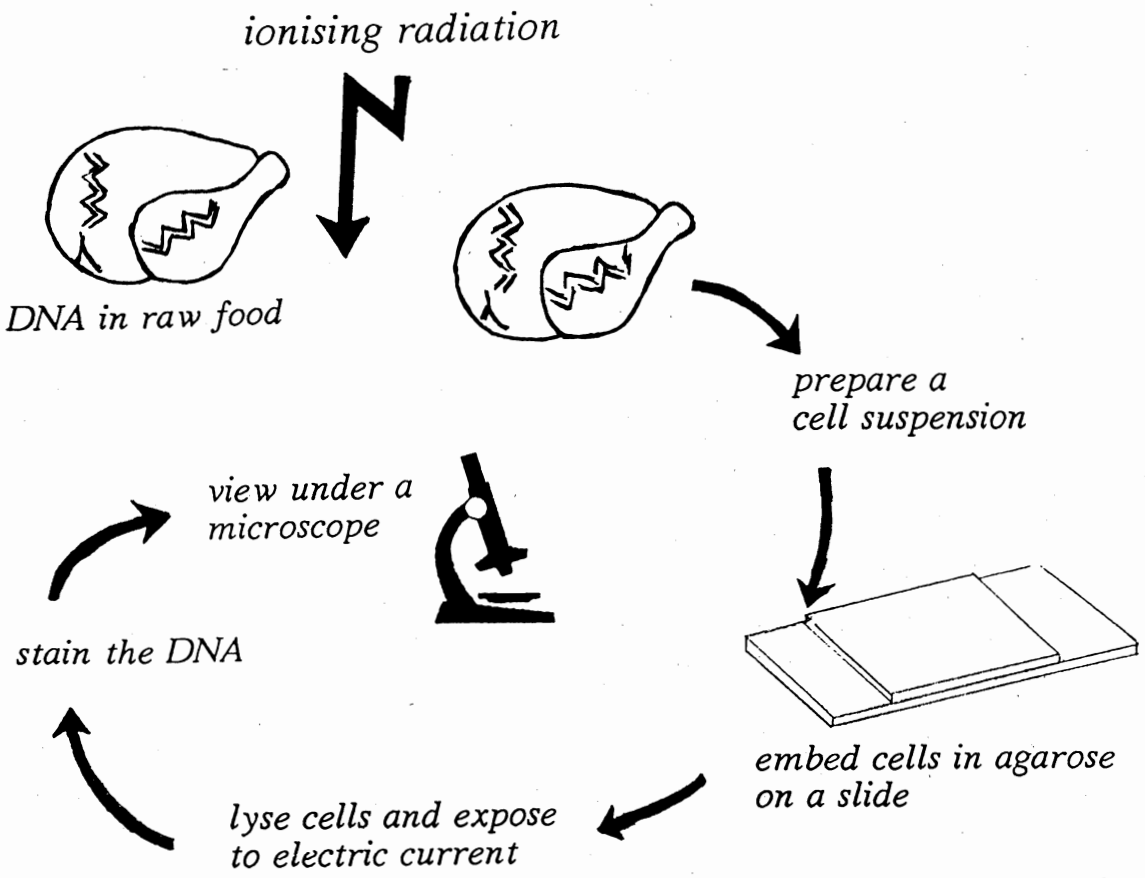


Figure 2 - Typical 'comet' shapes obtained with irradiated and non-irradiated tissues

(a) Non-irradiated

(b) Irradiated at 5.0 kGy

2. MATERIALS AND METHODS

2.1 Abbreviations

DMSO - Dimethylsulphoxide
LMP agarose - Low melting point agarose
Na_2EDTA - Ethylenediaminetetraacetic acid, disodium salt
SDS - Sodium dodecyl sulphate
Tris - Tris(hydroxymethyl)aminomethane

2.2 Materials

Unless otherwise stated chemicals were purchased from Sigma Chemical Company (Poole, UK) or BDH (Poole, UK). Solutions were made with deionised water.

Grant water bath (BDH, Poole, UK)
Hot plate stirrer (BDH, Poole, UK)
Microwave oven - Philips M734, 700 watts (Philips, Croydon, UK)
Gilson pipettes (Anachem, Luton, UK)
Power pack model 500/200 (BioRad, Hemel Hempstead, UK)
Horizon 58 minigel system (Gibco-BRL, Paisley, UK)
Leitz Dialux 22 fluorescence microscope (Leica, Milton Keynes, UK)
Nylon mesh (Lockertex, Warmington, UK)
SeaKem LE agarose (FMC-Flowgen, Sittingbourne, UK)
LMP agarose (BioRad, Hemel Hempstead, UK)
Acridine orange (USB - Cambridge BioScience, Cambridge, UK)

Stock solutions

1mg/ml Acridine orange in water - store 4°C
5x TBE (450mM Tris-borate, 10mM Na_2EDTA pH 8.0)
PBS (8% sodium chloride, 0.02% potassium chloride, 0.144% disodium hydrogen orthophosphate, 0.024% potassium dihydrogen orthophosphate, pH 7.4) - autoclave and store 4°C
0.5% agarose in water - autoclave and store 4°C
0.8% LMP agarose in PBS - autoclave and store 4°C

2.3 Methods

Only minor alterations have been made to the original protocol (Johanson 1991; Cerda *et al.* 1992). Firstly, 0.5x TBE was used as the electrophoresis buffer rather than tris-acetate EDTA buffer. Also, it was found that better separation was routinely obtained if 0.1% SDS was included in this electrophoresis buffer. Secondly, lysis was improved by increasing the SDS concentration in the lysis buffer from 0.1% to 2.5% and the lysis time from 5 to 10 minutes.

Instructions for preparing cell extracts

Lightly crush approximately 0.25g of the sample with a mortar and pestle. Add 2-3ml of cold PBS and grind a little more until the solution becomes cloudy. Filter sequentially through 200μm and 100μm nylon mesh filters. Leave to settle on ice for 20-60 minutes. Do not filter the soya extracts nor allow them to settle. Transfer 1ml of the supernatant to a labelled tube and add DMSO to 10%.

All extracts must be stored on ice until use and storage time must be kept to a minimum. Extracts can be stored frozen at -20°C for extended periods. These may be used until they have been frozen/thawed up to 5 times, after which they should be discarded.

The nylon filters were prepared by cutting the end off a disposable 5ml syringe, heating the cut end in a bunsen flame until soft, and pressing this firmly onto a piece of the appropriate nylon mesh. For filtration, the filters must be used wet and washed immediately after use, but may be reused many times.

Instructions for coating slides

To attach the single cell layer firmly to the slides, the slides must first be pre-coated with a thin layer of agarose. Carefully smear 70μl of molten 0.5% agarose, held at 45°C, across two thirds of a clean warm microscope slide with the side of a pipette tip. Place the slide on ice for 3 minutes to allow the agarose to set and then air dry. Coated slides may be stored (covered) at 4°C for a month.

Instructions for slide preparation

Thaw cell extracts at 45°C and re-freeze any spare extract immediately. To prepare

a thin layer of sample cells first mix 500μl of molten 0.8% LMP agarose, held at 45°C, with 50μl of thawed cell extract, in a microcentrifuge tube. Onto a warm, coated slide pipette 100μl of the cell suspension in agarose. Carefully lower a clean rectangular coverslip onto the slide such that the sample is spread evenly beneath the coverslip. Make sure that no air bubbles are trapped in the agarose, and place slide on ice for 3 minutes. Remove the coverslip by pushing one corner to the side with a needle or scalpel blade tip, and gently sliding the coverslip off the agarose.

Instructions for cell lysis

Submerge the slides completely in lysis buffer (0.5x TBE containing 2.5% SDS), for 15 minutes, taking care not to touch the agarose layer. Wash the slides by gently blotting the edge of the slide and submerging in 0.5x TBE for 5 minutes.

Electrophoresis

Place the slides (two per run) in the horizontal electrophoresis tank, ensure that they are submerged (2-4mm) in the electrophoresis buffer (0.5x TBE containing 0.1% SDS) and apply 2 volts/cm for 2 minutes exactly. Carefully remove the slide from the tank, keeping it horizontal as the agarose becomes quite soft and delicate during electrophoresis. Place the slides in ice cold water for 5 minutes, fix the preparations by submerging in 70% ethanol for 3 minutes, and then air dry the slides. These slides may be stored covered at 4°C for several months.

Staining procedure

The slides must be stained immediately prior to observation, as the dye fades during storage. Acridine orange was found to stain samples well, and is the least toxic of the dyes that can be used. Prepare a working solution of acridine orange at 5μg/ml in sterile PBS from the stock. This can be stored for up to one week at 4°C. Submerge the slide for 5-10 minutes in stain and then wash by submerging in water for 1-5 minutes. Before viewing, place a coverslip on the wet slide and blot off excess water. View the slides under a fluorescence microscope within 1 hour of staining, using a blue filter.

Problems with staining include fading and over-staining. Fading can occur with prolonged exposure to light, and makes sample identification difficult. Over-staining causes a high background fluorescence which can be reduced by further washing.

This is less of a problem with acridine orange which stains DNA green but stains the background and debris orange.

Dose allocation / standard slides

Assessment of the irradiation dose administered is aided by referring to a set of standard slides. These may be prepared from any sample treated with a known dose of irradiation, but the slides should ideally be prepared alongside the unknown samples to ensure identical lysis and electrophoresis conditions. The reference slides used in this work were: soya (0kGy), lentil (0.2kGy, 1kGy), sunflower (0kGy, 1kGy), linseed (1kGy, 5kGy), almond (1kGy), sesame (1kGy, 5kGy) and rose pepper (5.0kGy).

Counting 'comets'

The comet pattern and distribution is first assessed under low magnification (x200) and then examined in closer detail at higher magnification (x400). For difficult samples (those where the dose is not apparent, or where a range of comet patterns is present due to background DNA degradation) an initial dose estimation is made by examination of the sample under lower magnification, and this is then revised using a 'comet count'. In this, a count is made of the different comet patterns at higher magnification, and the following table is used to revise the dose estimate, on the basis of the relative proportions of each type of comet: the dose allocated to the sample is the lowest dose containing 5% or more of total comets counted. This is because in our experience a 'damaged' but non-irradiated sample will always contain a proportion of comets of the '0 kGy type', whereas in a 'damaged' irradiated sample there will be relatively few (if any) of these.

For example, on first examination a sample may be adjudged to have been irradiated at 2kGy. However, a detailed assessment of comet patterns in a comet count across the slide (e.g. covering at least 100 comets) revealed that more than 5% of the comets were of the non-irradiated type (0kGy), and so the sample was reclassified as non-irradiated (Table 1).

Table 1 - Example of results from a comet count

	Detailed comet analysis				
	Estimate of 0kGy	Less than initial estimate of dose	Equivalent to initial estimate of dose (2kGy)	Greater than initial estimate of dose	Total comets counted
Number of comets	29	11	304	12	356
% of comets	8.1%	3.1%	85.4%	3.4%	100%

Lowest dose category which has at least 5% of the comets => 0kGy
Revised dose estimate following comet count => 0kGy

Samples analysed

The trial involved analysis of 11 known reference samples (see above) and 22 unknown experimental samples covering lentil, sunflower seeds, linseed, almond, sesame seed, rose pepper, fig and soya bean. The soya bean samples were analysed using the revised protocol, as described.

3. RESULTS AND DISCUSSION

The aim of the trial was to determine whether or not each of a series of seed samples had been irradiated, and, if possible, to estimate the irradiation dose that had been administered to the samples. No guidance was given on possible doses administered and all samples were analysed blind. The estimates obtained are detailed in Table 2 and summarised in Table 3.

The twenty-two samples, covering lentil, sunflower seeds, linseed, almond, sesame, rose pepper, fig and soya bean, were all correctly identified as irradiated or non-irradiated (Table 2). Moreover, in twenty of the twenty-two cases, estimates of dose administered were correct to within 1kGy (Table 2). The two estimates that were less accurate comprised an almond sample, where the actual dose administered was 1kGy but the estimate was 2.5kGy, and a sunflower seed sample, where the actual dose administered was 5kGy but the estimate was 1-2kGy.

For one of the samples of lentil seeds (C3) it was not possible to determine whether or not it had been irradiated. It was therefore decided to reserve judgement on the basis that if the sample had been irradiated then it would have been at a very low dose (<1kGy). In the event, this estimate also proved to be accurate as the sample had been irradiated at 0.2kGy. In the light of this, it is envisaged that if the method is used for the routine analysis of samples for irradiation treatment, then instances will arise where the dose administered is too low to be reliably detected using microgel electrophoresis. However, this problem is only likely to arise in a minority of cases, where the dose is extremely low. It is suggested that further work be carried out to establish the lowest detectable dose for specific food materials, so that this can be compared with the lowest dose likely to be of use for that material (e.g. minimum dose for sterilisation, minimum dose to inhibit sprouting, maximum legally permitted doses).

The two samples of soya bean were analysed using a modified protocol. This is because the initial analyses of the soya bean, using the standard protocol, were not successful: no comets of any description were obtained, so that it was impossible to determine whether or not the samples had been irradiated. The repeat analyses were conducted using a protocol in which the extracts were not filtered nor allowed to settle prior to mixing with the molten agarose. In these repeat analyses, comets were obtained but, not surprisingly, the samples also contained much more cellular debris. This made the analysis of the comet patterns more difficult, as distinguishing

between cellular debris and 'tail-less comets' was not easy. Nevertheless, the samples were correctly identified as irradiated or not irradiated, and again irradiation dose was estimated correctly.

Problems also arose with the analysis of one of the rose pepper samples, namely C15. The comets obtained in this sample appeared to contain very faint 'tail shapes' but it was not clear whether these were actually comet tails or some artefact arising from sample preparation. A further batch of the same sample (C15-CR) was therefore re-analysed. On this occasion clearer comets with distinct tails were obtained, and it was decided on this basis to record the sample as not irradiated. Although correct results were thus obtained with the rose pepper samples, it is suggested that application of the method to this material be further evaluated before the method is offered as a means of detecting irradiation of rose pepper.

The problems arising from the presence of background debris, faint comet tails and DNA degradation are illustrated in Figure 3. One further point worth noting is that the comet patterns obtained in this trial generally differed from those obtained in the previous trials which were based on meat (e.g. pork and chicken): in this trial the comet 'heads' were generally smaller and, for comparable doses, the tails generally longer than previously observed with meats.

Overall, however, the estimates obtained in this trial from microgel electrophoresis analysis of seed samples were extremely encouraging. All the samples were correctly identified as irradiated or not irradiated, and correct estimates of irradiation dose administered were obtained for over 90% of the samples (Table 3). These results are particularly encouraging as they were obtained despite the problems described above - namely the presence of debris, differences in comet shape, and the presence of faint comets in the rose pepper sample.

The trial demonstrates that the method has considerable potential as a means of distinguishing between irradiated and non-irradiated seed samples. It also demonstrates the usefulness of including parallel analysis of blank (i.e. non-irradiated) standards, though these need not be of the same material as the unknown samples. Parallel analysis of irradiated standards of similar material to the unknown sample is useful if estimates of dose are to be achieved, but again, standards of the same material as the unknown sample are not an absolute requirement.

Table 2 - Microgel electrophoresis analysis of seeds for detection of irradiation treatment

			Estimates from analyses		Accuracy of estimates	
Sample Type	Sample Code	Actual Dose	Has it been irradiated?	Dose estimate	Correct Identification	Correct Dose Estimate? (+/- 1kGy)
Lentil	C3*	0.2kGy	Possibly*	0-1kGy	Yes	Yes
	C4	0kGy	No	0kGy	Yes	Yes
	C5	1kGy	Yes	0.5-1kGy	Yes	Yes
Sunflower	C2	1kGy	Yes	1-2kGy	Yes	Yes
	C6	5kGy	Yes	1-2kGy	Yes	No
	C8	0kGy	No	0kGy	Yes	Yes
Linseed	C2	0kGy	No	0kGy	Yes	Yes
	C3	1kGy	Yes	1-5kGy	Yes	Yes
	C5	5kGy	Yes	5kGy	Yes	Yes
Almond	C3	1kGy	Yes	2.5kGy	Yes	No
	C7	0kGy	No	0kGy	Yes	Yes
Sesame	C1	0kGy	No	0kGy	Yes	Yes
	C4	1kGy	Yes	1kGy	Yes	Yes
	C7	5kGy	Yes	3-5kGy	Yes	Yes
Rose pepper	C15-CR^	0kGy	No	0kGy	Yes	Yes
	C17/1	5kGy	Yes	5-6kGy	Yes	Yes
	C17/2	5kGy	Yes	5kGy	Yes	Yes
Fig	C2/1	5kGy	Yes	3-5kGy	Yes	Yes
	C2/2	5kGy	Yes	5kGy	Yes	Yes
	C5	0kGy	No	0kGy	Yes	Yes
Soya bean	C2	1kGy	Yes	2kGy	Yes	Yes
	C4	0kGy	No	0kGy	Yes	Yes

*Comet analysis suggested that this sample may have been irradiated at a very low dose (i.e. less than 1kGy) but it was decided that it was not possible to conclude reliably whether or not the sample had been irradiated.
^Rose pepper - Problems arose with the analysis of sample C15 and so a further batch (CR) of this sample was despatched for analysis. This batch was deemed not to have been irradiated. However, it is recommended that further assessment of the applicability of the method to rose pepper is undertaken.

Table 3 - Summary of the results obtained with the trial samples

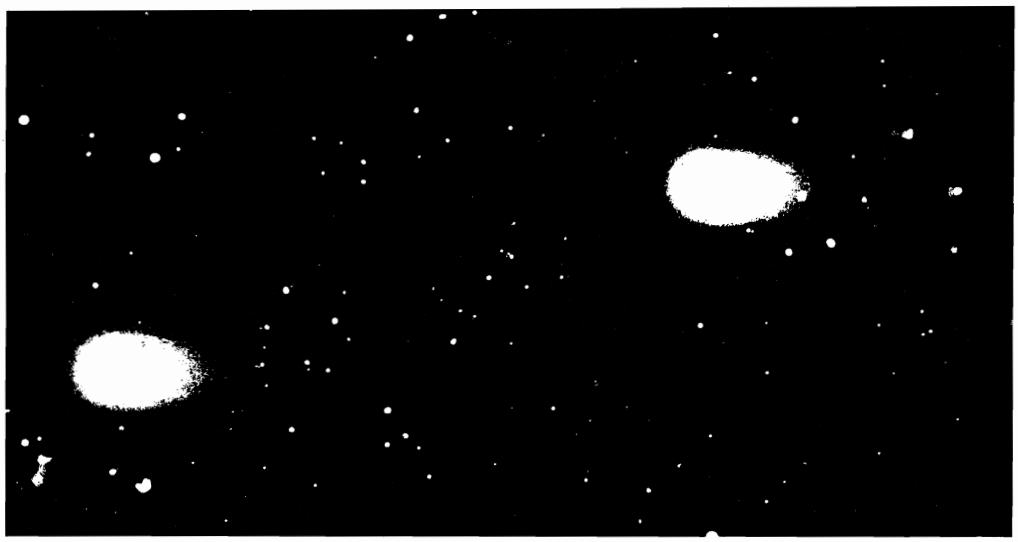
Sample	Total number tested	Identified correctly as irradiated or non-irradiated		Correct estimate of dose (+/- 1kGy)	
		Number	%	Number	%
Non-irradiated	8	8	100%	8	100%
Irradiated	14	14	100%	12	86%
All Samples	22	22	100%	20	91%

With regard to the potential of the method, it is important to stress its limitations and that much work remains to be done. One complication with samples such as small seeds (e.g. linseed) is the potential problem of analysing mixtures of irradiated and non-irradiated seeds. This would require evaluation of the method against a whole series of such mixtures, with detailed analysis of the relative frequencies of the different comet types in the samples. However, identifying such samples should prove possible, especially using a less subjective assessment of comet size and shape - i.e. instrumental image analysis systems. The problem may not arise with larger seeds such as sunflower or soya bean, as analyses could be conducted on single seeds. A second limitation is that the method, although applicable to a wide range of raw materials, is unlikely to be applicable to foods which have been cooked, repeatedly frozen-thawed or subject to high shear forces, as each of these treatments can cause DNA fragmentation and/or loss of cell or nucleus structure.

In summary, the method is relatively rapid, simple and cheap, and on the basis of this collaborative trial and the two previous trials based on meat, looks highly likely to prove useful as a screening method for a wide range of unprocessed food materials. Certainly the technique is worthy of further evaluation to progress it to the stage of a MAFF "collaboratively trialled non-statutory method".

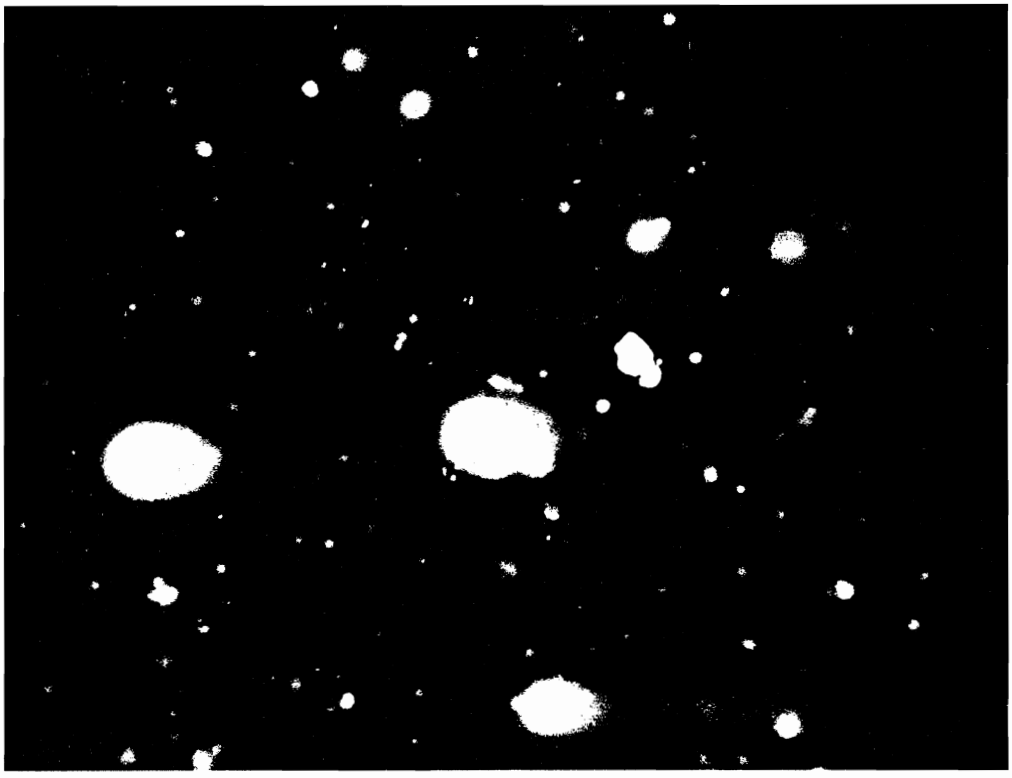
Figure 3

(a) A 'clean' non-irradiated sunflower sample - C8



The comet heads are bright and easy to see. There is very little debris to mask any comet tails.

(b) A 'damaged' but non-irradiated almond sample - C7



There are two bright 0kGy shaped comet heads and some smaller heads with less fluorescence. The more DNA that leaves the 'head' then the larger the tail and the smaller the head. The spread of DNA within the tail reduces the tail's fluorescence. Debris can mask these fainter tails making dose allocation and counting more difficult.

4. CONCLUSIONS AND FUTURE WORK

The results of this trial on a range of plant seeds demonstrate that microgel electrophoresis can provide a reliable screening procedure for the detection of irradiation treatment of these materials. The method can also be used to give a reasonable estimate of irradiation dose administered. These results, together with those obtained from previous trials which focused on meats, strongly suggest that the method is applicable to a wide variety of fresh and frozen raw foods with little or no modification. This is especially the case as the method is relatively rapid, cheap, and simple, requiring a limited amount of training and equipment.

To maximise adoption and use of this method, a standard recommended protocol should be compiled, perhaps initially for use on raw chicken and pork or on seeds, preferably within the current UK framework of MAFF collaboratively trialled non-statutory methods. In addition, the application of the method to a broader range of foods and irradiation treatments merits more extensive assessment. For example, the method should be investigated further with a wide range of relevant high value / low bulk materials including seafoods, and herbs and spices. The effects on method performance of re-irradiation of food samples and of blending irradiated and non-irradiated materials should also be investigated.

Although previous work has indicated that cooking and other processing methods cause DNA damage which can result in comets similar to those of irradiation treatment, microgel electrophoresis still offers a very useful approach to the screening of many raw food materials for irradiation treatment. Furthermore, in addition to its use as a screening method, the application of image analysis and statistical interpretation of comet shape and distribution may enable use of the method to estimate accurately the irradiation dose administered, so that it could become acceptable as a final method for the detection of irradiation treatment of some foods.

5. ACKNOWLEDGEMENTS

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