Journal of Cereal Science **37** (2003) 311–318
doi:10.1006/jcrs.2002.0511

Determination of the Distribution of Sulphur in Wheat Starchy Endosperm Cells Using Secondary Ion Mass Spectroscopy (SIMS) Combined with Isotope Enhancement

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Received 1 March 2002

ABSTRACT

Secondary ion mass spectrometry (SIMS) is a sensitive, high resolution technique capable of determining the location of elements or ions in a sample down to sub-cellular levels. The distribution of elements or ions are superimposed onto an image of the plant cell or tissue generated by ion-induced secondary electrons, allowing a visual representation of their positions in the sample. The elements are separated by their mass/charge ratios which can lead to a loss of sensitivity when separating signals from elements with very similar masses such as $^{16}O_2$ (31·990 a.m.u.) and ^{32}S (31·972 a.m.u.). The abundance of ^{34}S isotope was, therefore enriched, allowing sulphur to be located at the starch granule/protein interface of the mature wheat grain. This demonstrates that isotopic enrichment can be used to extend the applications of SIMS technology to biological systems.

Keywords: secondary ion mass spectroscopy (SIMS), ion microanalysis, sulphur, wheat seed.

INTRODUCTION

The identification of the location of individual components at the sub-cellular level is one of the major challenges facing plant cell biologists. Proteins can often be located in fixed (i.e., dead) material using specific antibodies for microscopy or in living tissues using laser confocal microscopy using specific fluorescence labels. However, the localisation of inorganic components is more problematic with all currently available methods (e.g., energy dispersive X-ray analysis (EDX)[1] X-ray photoelectron spectroscopy (XPS)[2] and electron energy loss spectroscopy (EELS)[3] having significant limitations in terms of sensitivity, resolution and range of detectable elements and isotopes.

Secondary ion mass spectroscopy (SIMS) is a highly sensitive technique capable of mapping elements on a surface of a sample with sub-micron resolution and parts per million sensitivity[4]. It is based on the bombardment of samples with a primary ion beam to liberate secondary particles, some of which are ionised and can be captured and analysed to determine their mass to charge ratio. A wide range of elements and isotopes can be detected and identified provided that they differ in mass.

ABBREVIATIONS USED: HMW = high molecular weight; LMW = low molecular weight; SEM = secondary electron microscopy; SIMS = secondary ion mass spectroscopy; ^{34}S = sulphur isotope mass 34 a.m.u.; ^{32}S = sulphur isotope mass 32 a.m.u.; ^{16}O = oxygen isotope mass 16 a.m.u.

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0733–5210/03/030311 + 08 $35.00/0

We have recently used an improved SIMS technique to map the distribution of minerals (K^+, Na^+, Ca^{2+}, Mg^{2+} and PO_2^-), proteins (identified by CN^- signals) and carbohydrates (identified by C_2 and O^- signals) in aleurone and starchy endosperm cells of mature wheat grain, with excellent resolution and sensitivity[5]. However, we were unable to map the distribution of sulphur reliably. The major isotope (^{32}S) has a mass of 31·972 which, is close to that of $^{16}O_2$ (31·990) and although the present instrument is capable of resolving these species (whose mass difference of 0018 is higher than the maximum resolution limit of mass differences of 0·016) this is accompanied by an unacceptable loss of sensitivity.

Furthermore, although the naturally occurring isotopic form of sulphur ^{34}S is distinguishable from $^{16}O_2$ by the SIMS technique, it is only present at about 4·3% of all the sulphur atoms in the environment[6] and hence in the wheat grain[7].

The distribution of sulphur is of particular interest to cereal chemists as sulphur-rich seed proteins contribute to important functional properties for milling (grain texture) and baking (visco-elasticity) and it has been shown that increasing sulphur concentration in wheat grain results in increased dough extensibility[8]. We have therefore enriched the abundance of ^{34}S in developing grain of wheat and determined its distribution in mature starchy endosperm cells by SIMS.

MATERIALS AND METHODS

Plant growth

An S-deficient soil was collected from Woburn, Bedfordshire, air-dried and sieved to < 5 mm. The soil is a sandy loam, containing 1·0% organic C and 2·0 mg kg^{-1} of extractable sulphate-S. Soil pH was 6·5. Two kg air-dried soil was weighed and placed in a 15 cm plastic pot. Each pot received a basal nutrient dressing of 400 mg N (NH_4NO_3), 100 mg P (KH_2PO_4), 126 mg K (KH_2PO_4), 75 mg Mg ($MgCl_2 \cdot 6H_2O$), 5 mg Mn ($MnCl \cdot 4H_2O$), 1 mg Cu ($CuCl_2 \cdot 2H_2O$), 2 mg Zn ($ZnCl_2$), 1 mg B (H_3BO_3) and 0·5 mg Mo (Na_2MoO_4). The nutrients were dissolved in water and mixed thoroughly into the soils. Soils were then adjusted to 70% water holding capacity using deionised water. Seeds of the transgenic wheat (line B73-6-1) and its parent line (L88-6) were germinated on moistened filter paper in the dark and then planted into the pots (2 seedlings per pot). At the tillering stage, 20 mg of S (K_2SO_4) labelled with 30 atom % of the stable isotope ^{34}S was added in solution to each pot, together with 100 mg N (KNO_3) containing 99·9 atom % ^{15}N. The ^{34}S labelled K_2SO_4 was produced from elemental S (90 atom % of ^{34}S; Sigma Chemical Company, St Louis, USA)[7] Plants were grown in a glasshouse under controlled environment conditions: day/night durations of 14/10 h and day/night temperatures of 20 °C/16 °C. The natural light was supplemented with 1 kW SON-T lamps to maintain a minimum photon flux of 250 μmol m^{-2} s^{-1}. The pots were watered with deionised water and the grains harvested at maturity (110 days after planting).

Sample preparation

Transverse sections of dry mature wheat grains were cut using a razor blade and fixed using a solution of 3·7% paraformaldehyde/2·5% glutaraldehyde in 25 mM PIPES buffer pH 6·9[9]. The samples were vacuum infiltrated with the fixation solution for 30 min followed by 4 h incubation with continuous rotation at atmospheric pressure and then washed in 25 mM PIPES buffer for 3×15 min periods. The fixed samples were dehydrated in an increasing ethanol:water series (30, 50, 70, 80, 90, 100 and 100% ethanol), incubating for 30 min in each solution with continuous rotation.

A Polyscience Inc JB-4^{TM} embedding kit was used to embed the samples in resin. The samples were incubated with the catalysed infiltration resin for 2×30 min, $1 \times$ overnight and 1×30 min after which the samples were placed in the embedding solution (as per manufacturer's protocols) in aerobically sealed containers for 60 min. The embedded samples were removed from their containers and a glass knife mounted on an ultramicrotome (Ultracut Reichert-Jung) provided a flat surface for analysis.

Surface coating the sample

Prior to analysis, the samples were attached to steel microscope stubs and coated with gold in an Edwards 'Scancoat six' plasma-coating unit, coating for 90 s to produce a film of approximately 100 nm thickness. Flat aluminium plates were then attached to the tops of the samples to maintain a flat surface and to facilitate a uniform electric field for accelerating secondary ions into the mass spectrometer. Secondary electron images were first obtained to give topographical details, and the gold was then sputtered away using the gallium ion beam, monitoring on a secondary ion of interest and sputtering until the signal stabilised, indicating complete removal of the gold.

SIMS equipment

The SIMS instrument was constructed at the University of Bristol comprising a focused gallium ion gun (FEI electronically variable aperture type) fitted to a Vacuum Generators model 7035 double-focusing magnetic sector mass analyser. A Thornley-Everhard electron detector allows the acquisition of secondary electron images from the sample at spatial resolutions determined by the diameter of the ion beam. The sample potential is 4 kV and secondary ions are accelerated into the mass spectrometer through an ion extraction lens system. They then travel through an electrostatic energy filter with adjustable pass energy, a magnetic mass filter, and a slit and into a channeltron detector. A diagram of the instrument is presented in Heard *et al.* (2001). The spectrometer has a high transmission efficiency of secondary ions from the sample to the channeltron, with the option of high mass resolution (at a cost of lower transmission), allowing a spectral resolution, $M/\Delta M$ ratio of several thousand. For routine analyse where mass interference for the elements of interest is minimal, low spectral resolution with high transmission was selected, with resulting sensitivities in the parts per million range, depending on the element to be analysed.

The PC control software was written in house to allow control of spectral acquisition, beam scanning, image acquisition, and depth profiling, operating under the Windows system to allow easy transfer of data and images to standard spreadsheet and image manipulation packages (Dayta System Ltd, Thornbury, UK). False colour display images were created using UTHSCSA image tool version 2.03 software.

Spectrum and image acquisition

Typically mass spectra are obtained on the system by scanning through the mass range 0–100 Daltons in 0·05 Dalton steps, spending 100 ms per step and taking 200 s in all. While obtaining spectra, the ion beam is scanned over the area of interest, usually at low magnification to limit the damage caused to the specimen by sputtering.

Secondary ion images are obtained by selecting the mass/charge ratio of the ion of interest and raster scanning of the ion beam over the sample. The images presented in this paper contain 640×480 pixels and were acquired in 90 s each. For atomic species present at low concentrations, the acquisition time could be increased in order to obtain low noise images.

Analysis of S and N concentrations, and ^{34}S and ^{15}N abundance

The abundance of ^{34}S in wheat grain was determined using a continuous flow isotope ratio mass spectrometer[7,10]. The instrumentation used was an ANCA-SL sample converter and a 20-20 IRMS (Europa Scientific Ltd, Crewe, UK). Total S concentration was determined using inductively coupled plasma atomic emission spectrometry, after the samples were digestion with $HNO_3/HClO_4$ (85/15 v/v). Total N concentration and ^{15}N abundance in the grain samples were quantified using a mass spectrometer (Integra, Europa Scientific Ltd, Crewe, UK).

RESULTS AND DISCUSSION

Two lines of wheat were grown with enriched $^{34}SO_4$ to increase the abundance of ^{34}S in the grain. These were L88-6, which forms part of a near isogenic series developed from Olympic × Gabo[11] and B73-6-1, which is a transgenic derivative of L88-6 in which the addition of multiple copies of the *Glu-1D* gene encoding HMW glutenin subunit 1Dx5 leads to an increase of about four fold in the amount of this protein[12].

The results obtained with the two lines were qualitatively the same and only data for B73-6-1 are presented. The samples of B73-6-1 studied contained $0{\cdot}085 \pm 0{\cdot}001\%$ total sulphur with a ^{34}S isotopic enrichment of $12{\cdot}1 \pm 0{\cdot}04$ atom %. The grain contained $1{\cdot}72 \pm 0{\cdot}05\%$ total nitrogen, and was also enriched with ^{15}N to $12{\cdot}2 \pm 0{\cdot}2$ atom % but, this isotope was not followed in the present study.

Figure 1 shows the images obtained of a section from the central part of the starchy endosperm of a mature grain of B73-6-1 at two magnifications. The ion induced secondary electron images of the tissues clearly show the presence of cell walls and starch granules embedded in a matrix, with some fissures arising from shrinkage during sample preparation. Superimposition of the locations of C_2 and ^{16}O show clear labelling of the starch granules while the superimposition of CN^- on the matrix between the starch granules is consistent with its proteinaceous nature.

Analysis of the distributions of ^{32}S and ^{34}S gave less sharp images but both isotopes were clearly concentrated at the starch granule surface and the interface between the granules and the matrix

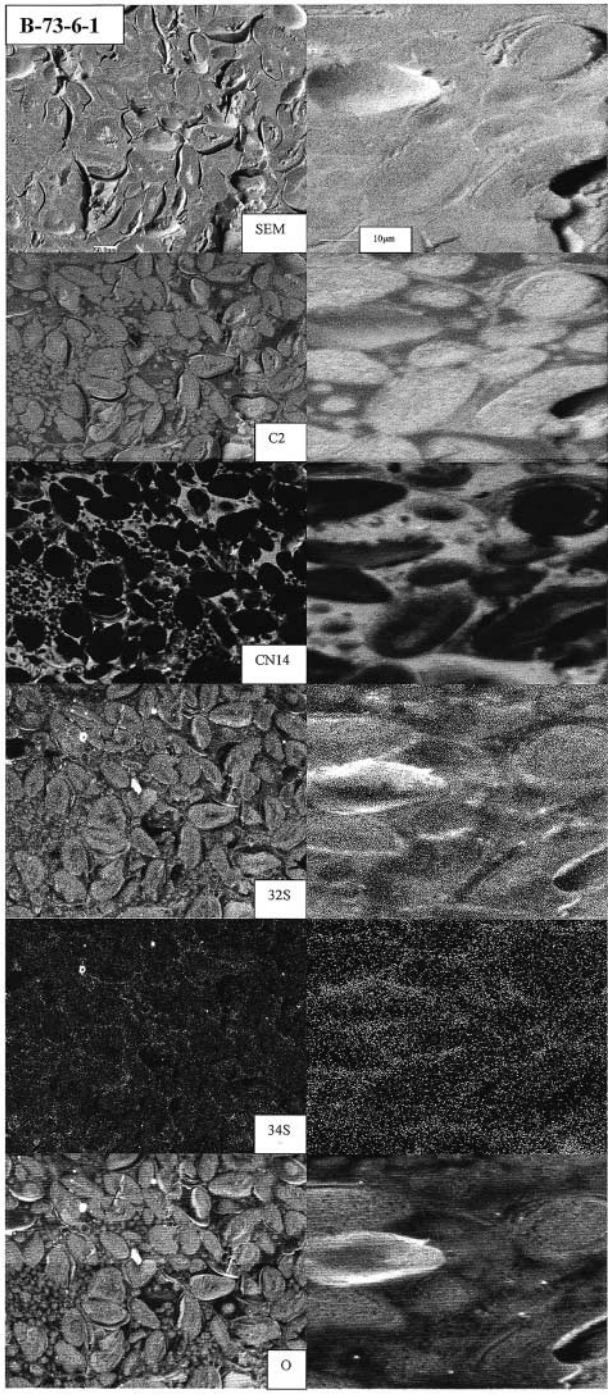

Figure 1 SIMS images of the starchy endosperm cells of B73-6-1 wheat seed at low and high magnifications, showing the distribution of ions as bright areas, showing from top to bottom a secondary electron microscopy image of the samples, the location of the starch granules and protein matrix (C_2 and CN SIMS analysis respectively) and the distribution of ^{32}S, ^{34}S and ^{16}O ions by SIMS analysis.

proteins. This is seen more clearly in the high magnification images shown in Figure 2.

The SIMS images have been normalised in brightness for presentation purposes, and there is no implication that the elements are present in equal quantities. Within each image however, the brightness of each pixel is proportional to the amount of signal received at that point. Analysis of the signal strengths of isotopes ^{32}S, ^{34}S and ^{16}O in different parts of the endosperm cells (see Fig. 3 and Table I) showed a higher concentration of ^{34}S at the outer surfaces of the starch granules compared to the cut interior faces of these structures. The protein matrix also had a lower concentration of ^{34}S isotope than the starch granules.

The ^{32}S isotope signals are stronger than the ^{34}S signals in all structures in the B73-6-1 seeds (see Table I) which was expected as ^{32}S accounts for 87·9% of the sulphur atoms in the samples. However, a difference was seen between the distributions of ^{34}S and ^{32}S isotopes in the interior of the starch granules with the ^{34}S signal being proportionally lower than that of ^{32}S. We consider that this could be due to corruption of the ^{32}S signal with that from charged particles of $^{16}O_2$, which would be expected to be formed from the ^{16}O ions released from the carbohydrate molecules present in the starch granules after bombardment with the primary ion beam. As the signal level of ^{16}O is two orders of magnitude higher than that of ^{32}S (see Table I) even a small proportion of ^{16}O ions forming $^{16}O_2$ molecules would have a marked effect on the ^{32}S signal, as shown by the similarity in the ^{16}O and ^{32}S images in Figure 2. The signal for ^{16}O was highest in the centre of the granules where the ^{34}S signal was weakest; a combination of the ^{32}S and $^{16}O_2$ signals would therefore give a strong signal throughout the whole starch granule, as seen with the ^{32}S images.

The distributions of these signals are shown more clearly in Figure 4 where the use of colour display software portrays the interior of the starch granules as green/blue due to a combination of the ^{32}S (shown in green) and ^{16}O (shown in blue) signals whereas the ^{34}S signal (shown in red) is located predominantly at the surface of the starch granules.

This highlights the importance of using ^{34}S to obtain accurate localisation of sulphur at a subcellular resolution using the SIMS equipment.

The high concentration of sulphur at the starch granule surface may result from the presence of a high concentration of sulphur-rich proteins

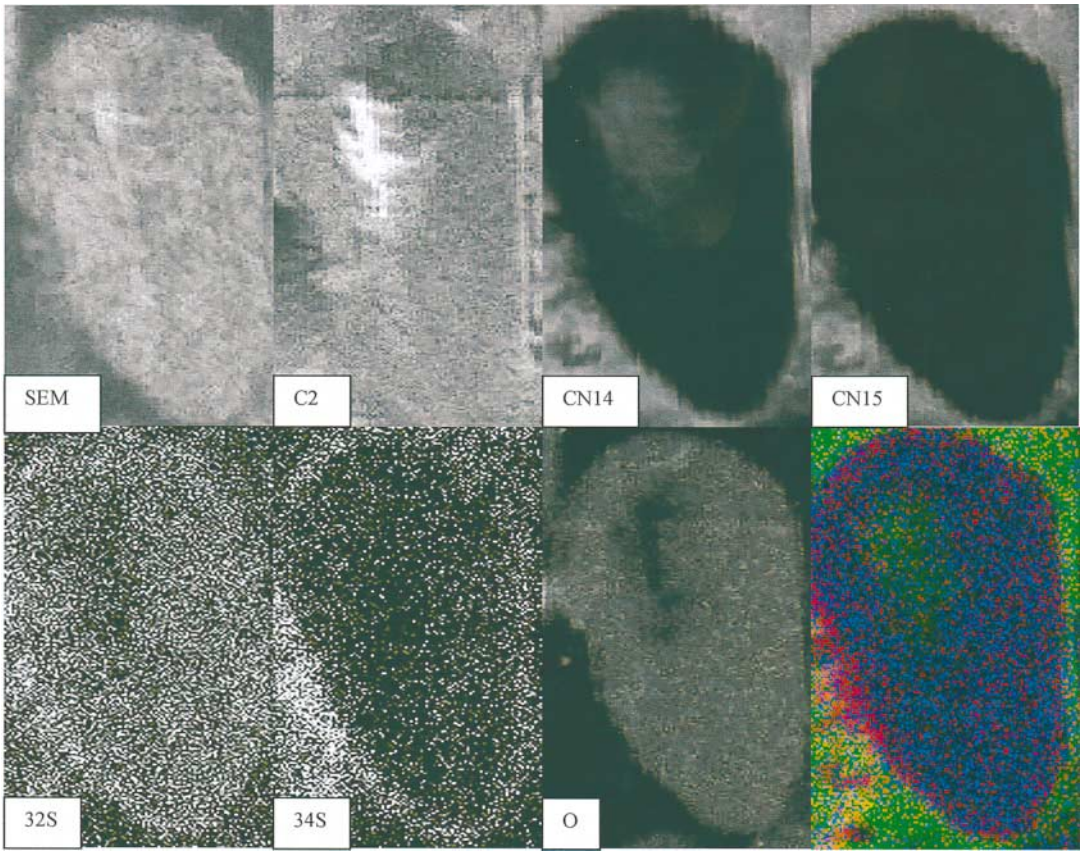

Figure 2 SIMS analysis of a single starch granule from B73-6-1 wheat seed, showing secondary electron microscopy image, starch granule (C_3), protein matrix (CN^{14} and CN^{15}), distribution of ^{32}S, ^{34}S and ^{16}O ions and colour display image showing the positions of ^{32}S (blue), ^{34}S (red) and CN^{14} (green) ions.

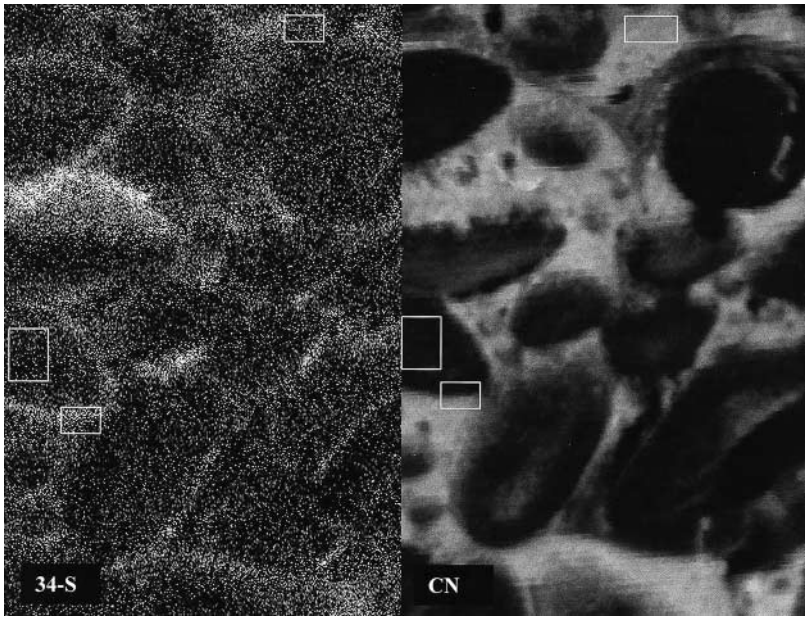

Figure 3 Areas of the endosperm cells analysed for signal strength shown in both ^{34}S and CN^{14} images.

Table I Relative signal strengths for different isotopes at different structures in the endosperm cells

	Mean signal levels			Ratios of the mean signal levels	
B73-6-1	Outer section of the starch granule	Protein matrix	Whole inner section of the starch granule	Outer section/inner section of the starch granules	Outer section of the starch granule/ protein matrix
34-Sulphur	$10{\cdot}66 \times 10^{-7}$	$2{\cdot}83 \times 10^{-7}$	$3{\cdot}33 \times 10^{-7}$	3·20:1	3·70:1
32-Sulphur	131×10^{-7}	74×10^{-7}	90×10^{-7}	1·45:1	1·77:1
16-Oxygen	$2{\cdot}55 \times 10^{-5}$	$1{\cdot}45 \times 10^{-5}$	$3{\cdot}55 \times 10^{-5}$	0·72:1	1·76:1

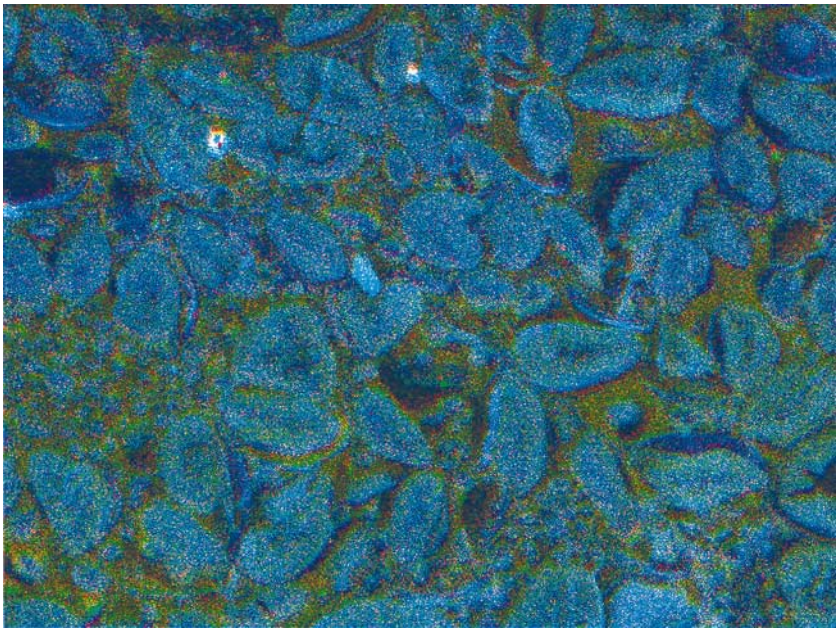

Figure 4 Colour display images of the oxygen (blue) interference on the ^{32}S signal (green) with ^{34}S shown in red.

including puroindolines, grain softness proteins (both of which are components of friabilin) and inhibitors of α-amylase[13–18]. In particular, the puroindolines contain a total of 10–11 mol % of sulphur-containing amino acids (cysteine and methionine)[19] compared with a maximum of 5 mol % in the most sulphur-rich gluten proteins (α-gliadins, γ-gliadins and LMW subunits of glutenin)[20].

Advantages of SIMS technology

The study reported here illustrates the advantages and drawbacks of the SIMS technology.

Firstly, it is capable of detecting all elements, which contrasts with X-ray photoelectron spectroscopy (XPS) and energy–dispersive X-ray analysis (EDX) which have limitations with respect to hydrogen, helium and, with EDX, also lithium and beryllium. It is also unique in being able to detect all isotopes, but varies in sensitivity. For example, concentrations of electropositive elements such as sodium, potassium and lithium in the ppm to ppb range can be detected, allowing us to identify of sodium in phytin granules of wheat for the first time[5]. However, the sensitivity for less easily ionisable elements may be in the 10–100 ppm range. Of the other currently available methods only electron energy-loss spectroscopy (EELS) combined with TEM has comparable sensitivity, with EDX/WDX (wavelength dispersive X-ray spectroscopy) and XPS having sensitivities in the 0·01–0·1 wt % range.

However, SIMS is only able to resolve ions with significant differences in their mass/charge ratios. Sulphur presents a difficult challenge in this respect, with the major isotope ^{32}S only being resolved from

$^{16}O_2$ with reduced sensitivity and the minor isotope ^{34}S being insufficiently abundant (only 4·3% of total sulphur) to give reliable results. We have overcome this problem by enriching grains with ^{34}S and comparing the distribution of ^{34}S, ^{32}S and $^{16}O_2$.

In terms of spatial resolution SIMS (<100 nm) compares well with EDX/WDX with SEM (1–2 μm) and XPS (10 μm) but is gives lower resolution than EDX/WDX with TEM/STEM and EELS/TEM (both <10 nm). In addition quantitative analysis is more difficult than with other systems due to a number of factors including the enhancement of signals by oxygen or oxides. Nevertheless, quantitative analysis can be achieved by comparisons with control samples of similar but known composition.

Finally, SIMS does not require the preparation of sections but only a flat surface, which is similar to EDX/WDX with SEM and XPS. In contrast, EDX/WDX with TEM/STEM and EELS/TEM both require material to be cut into 50–100 nm sections.

We consider, therefore, that the range, sensitivity, spatial resolution and ease of sample preparation and handling make SIMS an attractive addition to more widely used system for the surface analysis of cereals and other biological materials.

We have not attempted to use SIMS to determine the distribution of sulphur in other plant tissues but foresee no technical problems provided freeze substitution is used to preserve inorganic sulphate.

Acknowledgements

Long Ashton Research Station and Rothamsted Research receive grant-aided support from the Biotechnology and Biological Science Research Council of the UK.

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