

NEW INSIGHTS INTO STRUCTURES AND COMPOSITION OF PLANT FOOD MATERIALS

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Review



ABSTRACT

The aim of this paper is to review principles and potential application of micro-proton induced X-ray emission (micro-PIXE), synchrotron-based micro-X-ray fluorescence (micro-XRF) and inductively coupled plasma-mass spectrometry (ICP-MS) hyphenated with pulsed laser ablation (LA) for analysing plants and plant materials to elucidate feature of constituents of nutritional value. These are required in order to develop novel high-quality functional and other food products in view of the variability of properties of plant tissues and products.

Keywords: micro-PIXE, micro-XRF, SEM, transmission electron microscopy, laser ablation, food composition, plant structures

INTRODUCTION

Plants consist of different parts, organs, tissues and cells. There are considerable differences in structure and chemical composition - not only between the major plant parts (e.g. roots, tubers, stems, leaves, fruits, seeds), but also between and within individual tissues. There is no uniformity even on the cellular level, as cells display compositional differences between organelles (Cvitanich *et al.*, 2010; Regvar *et al.*, 2011). Numerous differences emerge during differentiation and developmental processes of plants. During germination, for example, metabolic transformation takes place in an embryo and a plantlet, this impacts the relative distribution of elements and metabolites, and the digestibility, availability for humans when consumed as a food (Lintschinger, 1997; Kreft and Kreft, 2000; Kim *et al.*, 2001; Kim *et al.*, 2004; Kim *et al.*, 2009; Pongrac *et al.*, 2013a,b; Nelson *et al.*, 2013; Merendino *et al.*, 2014; Zhang *et al.*, 2015; Zhou *et al.*, 2015; Pongrac *et al.*, 2016a, Kreft, 2016). During growth and development some substances are degraded, while others are synthesised. Ecological and agrotechnical factors, most notably UV-B radiation, influence the structure and composition of plants, thereby provoking the synthesis of secondary metabolites, especially valuable antioxidants (Germ *et al.*, 2010; Nemcova *et al.*, 2011). A further criterion favours low-input plants (Kreft and Luthar, 1990). During cultivation and crop growth some elements or metabolites may be eliminated from plants via leakage or volatilisation (e.g. Se substances, Germ *et al.*, 2007). The structural and compositional features of plant parts are connected to the physiological role of structures and compounds, they are also associated with plant metabolism and reproduction.

Heat and hydrothermal treatments are of special importance among the post-harvest treatments which influence structures and chemical composition (Vogrinčič *et al.*, 2010; Lukšič *et al.*, 2016). The traditional methods of analysing the chemical composition include milling of plant samples, extraction of interesting metabolites and further chemical analysis. However, this masks differences within plant tissues, as well as between and within plant cells (Škrabanja *et al.*, 2004).

An important limitation in the research of plant materials by conventional light microscopy is the wavelength. In principle, it is impossible to distinguish structures which are much smaller than the wavelength of visible light or other

light (e.g. ultraviolet) used in microscopy research. A further limitation concerns distinguishing the chemical or structural composition, even though autofluorescence techniques exist and numerous dyes are available for specific staining of structures. In fluorescence microscopy of plant specimens the structures and compounds often need to be labeled with various fluorescent markers. However, artefacts may emerge during treatment of preparations, and the wavelength is again a limitation.

Transmission electron microscopy enables analyses of plant structures at much higher magnifications even at subcellular level; yet the problem is that most plant structures are translucent for electrons. It is possible to use heavy-element staining (e.g. via osmium tetroxide), but the deposit of such dyes poorly specifies cellular structures and artefacts may occur. Scanning electron microscopy (SEM) is a convenient imaging method; it is often used to reveal details at tissue and cell levels, it produces attractive images, but yields no information on the chemical composition of the observed structures. SEM is consequently used mainly to illustrate or reveal details of structures with known chemical composition which are subsequently investigated by other methods.

The aim of the present paper is to present modern approaches to analysis of plant materials which enable elucidation of their technological and nutritional value in detail (down to the cell-type level) in order to develop novel food products based on the variability and peculiarities of plant species, cultivars and genotypes.

PLANT BREEDING

Plant breeding is the basic approach to improve the technological and nutritional value of plants and plant materials (Pinson *et al.*, 2015). Plant breeding is focused on increasing the yield. However, it has been realised that during breeding the quality of a product often plummets. In wheat, for example, breeding increased the amount of starch per grain, plant and field area. This, however, is accompanied by lower concentrations of the substances which are important for technological value (e.g. proteins) or nutritional value (e.g. carotenoids, minerals) of wheat (Zhao *et al.*, 2009). Old species and varieties of wheat were characterised by a pronounced yellow colour, which has largely disappeared in the last hundred years of bread wheat breeding. Similar arguments

hold true for proteins important for bread quality, and for elements of nutritional importance in wheat kernels (Regvar et al., 2011).

Recently, more attention has been paid to the composition and utilisation quality of plants and plant materials. It starts at the pre-breeding stage by evaluating plant genetic material in variety collections, seed banks and other gene banks. Effective plant breeding aimed at quality requires in-depth analysis of utilisable plant parts with regard to the distribution of elements and metabolites. In this way, breeding may be used to increase the number of structures with convenient compositions and avoid those with less desirable compositions (Holaseva et al., 2002; Kim et al., 2009).

Besides analysing and selecting plant breeding starting material, it is also necessary to monitor the compositions throughout the process of plant breeding.

EXPERIMENTAL APPROACHES TO REVEAL THE DETAIL OF PLANT STRUCTURES AND THEIR COMPOSITION

Micro-proton induced X-ray emission (micro-PIXE)

Our group initiated PIXE (proton induced X-ray emission) research using the van de Graaff particle accelerator to accelerate protons. X-ray emission, characteristic for elements in the sample and proportional to the concentration of elements (e.g.

sulphur), was used for elemental quantification (Kump et al., 1976; Kump et al., 1977).

Over the years the micro-PIXE technique was improved and a better lateral discrimination in screening the elemental composition was obtained (Vogel-Mikuš et al., 2009; Pongrac et al., 2011). In the last decade significant progress has been made in the development and application of various 2D imaging techniques, to be used in complex biological systems. Lateral resolution and sensitivity have been much improved. The capabilities of the PIXE technique were greatly extended for different applications with the development of the focused proton beam — micro-PIXE. High-energy focused proton beam set-ups are often referred to as nuclear microprobes. The magnetic quadrupole lenses are generally used to focus the proton beam down to the sub-micrometer level. By rastering the focused proton beam over the sample, and by the scoring of the induced X-ray fluorescence (XRF), spatially resolved element distribution maps of the samples can be obtained (Figure 1). The advantage of micro-PIXE over other 2D imaging techniques is in its wide elemental range (from sodium (Na) to uranium (U)), high elemental sensitivity (sub-micron spatial resolution) and fully quantitative element concentration analysis. A review of recent developments has been published by Vogel-Mikuš et al. (2014).

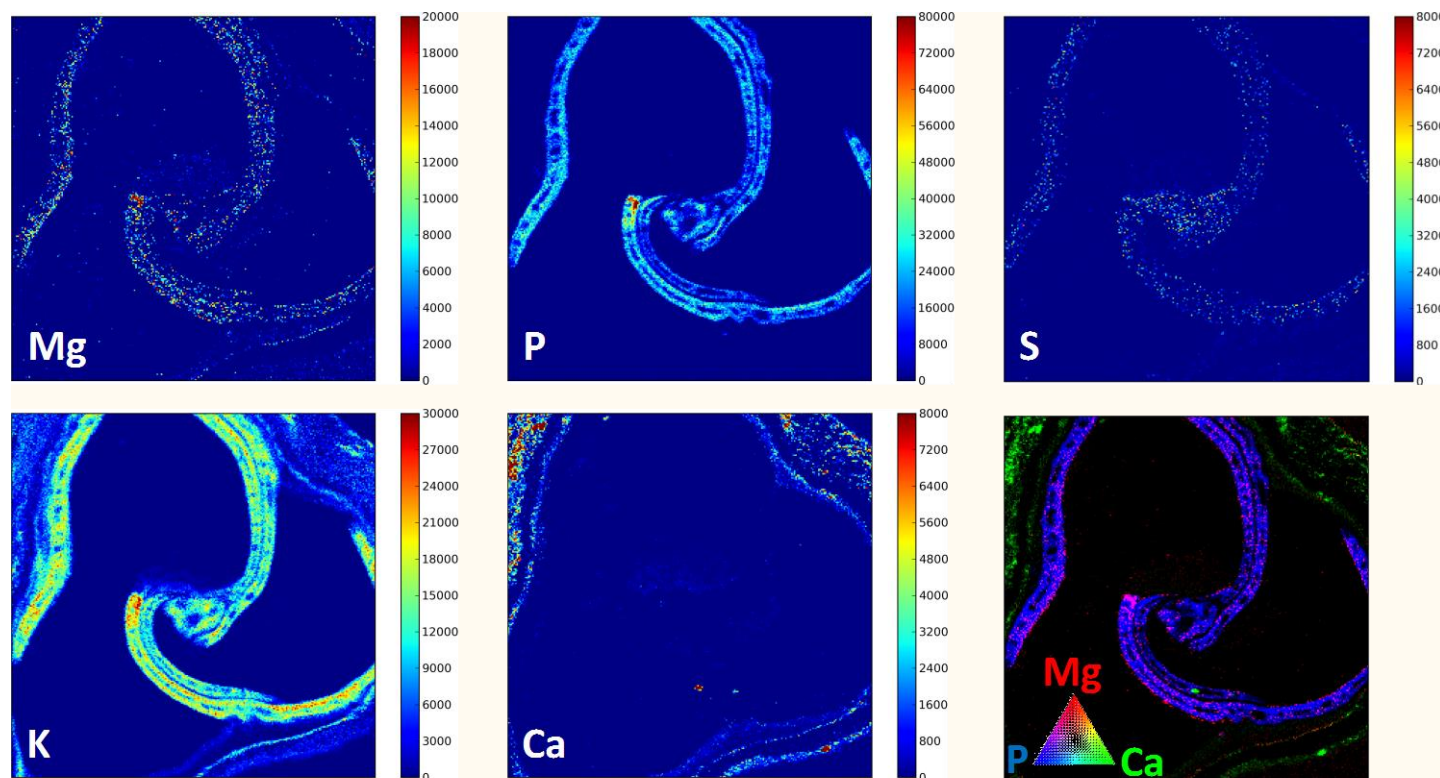


Figure 1 Quantitative distribution maps of magnesium (Mg), phosphorus (P), sulphur (S), potassium (K) and calcium (Ca) in a cross-section of Tartary buckwheat grain. The bottom right panel is a co-localisation image in which distributions of Mg (red), Ca (green) and P (blue) are merged to identify co-localisation pixels. Scan size: 2.0 mm × 2.0 mm.

Synchrotron-based micro-X-ray fluorescence (micro-XRF) and X-ray absorption near-edge spectroscopy (XANES)

Groats and sprouts of Tartary buckwheat were analyzed for their mineral element distribution using μ -XRF (Pongrac et al., 2016b). Groats and unfolded cotyledons of 7-day-old sprouts were frozen in liquid propane and sectioned at -25°C in a cryotome to 25 μm thick sections that were freeze dried for 3 days at 0.240 mbar and -30°C (Vogel-Mikuš et al., 2014). Freeze-dried sections were analyzed for the distribution of Mg, P, S, K, Ca, Mn, and Fe using 7200 eV at the ID21 beamline (ESRF, Grenoble, France; Koren et al., 2013). Quantitative element data were retrieved (Koren et al., 2013) and quantitative element maps were generated using PyMCA software (Solé et al., 2007).

Iron insufficiency is a worldwide problem in human diets (White and Broadley, 2009; Lombi et al., 2011; Mamo et al., 2014; Pongrac et al., 2016a). In cereals like wheat, the bran layer of the grains is an important source of iron. However, the dietary availability of iron (Fe) in wheat flour is limited due to the loss of the iron-rich bran during milling and processing and the presence of anti-nutrients like phytic acid that keep iron strongly chelated in the grain. The study of Singh et al. (2013) investigated the localization of iron and phosphorus in grain tissues of wheat genotypes with contrasting grain iron content using synchrotron-based micro-X-ray fluorescence (micro-XRF) and micro-proton induced X-ray emission (micro-PIXE). X-ray absorption near-edge spectroscopy (XANES) was

employed to determine the proportion of divalent and trivalent forms of Fe in the grains. It revealed the abundance of oxygen, phosphorus, and sulphur in the local chemical environment of Fe in grains, as their Fe-O-P-R and Fe-O-S-R coordination. Contrasting differences were noticed in tissue-specific relative localization of Fe, P, and S among the different genotypes, suggesting a possible effect of localization pattern on iron bioavailability. The study reports the shift in iron distribution from maternal to filial tissues of grains during the evolution of wheat from its wild relatives to the present-day cultivated varieties, and thus suggests the value of detailed physical localization studies in varietal improvement of cultivated plants.

Laser ablation (LA) - inductively coupled plasma-mass spectrometry (ICP-MS)

Inductively coupled plasma-mass spectrometry (ICP-MS) hyphenated with pulsed laser ablation (LA) is a microanalytical technique based on direct sampling of solid material and subsequent elemental analysis of the particles generated. Compared to conventional techniques it offers high sensitivity with a dynamic range of eleven orders of magnitude, very little or no sample preparation, low detection limits (ng g^{-1}), and it is considered "quasi" non-destructive. LA-ICP-MS generates spatially resolved quantitative information on major, minor, and trace element levels, offering a depth resolution in the order of 150 nm per pulse and a lateral resolution in the order of the laser beam size (5-

250 μm). In its current implementation for imaging, LA-ICP-MS can be used in three modes, viz. elemental depth profiling (1D) and elemental mapping (2D and 3D) via laser drilling and/or rastering. The characteristics of the LA-ICP-MS system that determine the element maps in terms of analysis time, noise, sensitivity and spatial resolution comprise the LA device parameters (cell design, laser beam size, dwell time, fluence, repetition rate, background gas flow rate, ablation mode, etc.) and the MS operational parameters (number of elements, acquisition time, settling times, etc.). Quantification is generally performed using matrix-matched standards and if possible an internal standard to correct for ablation rate differences between sample and standard.

A LA-ICP-MS method based on a 213 nm Nd:YAG laser and a quadrupole ICP-MS has been developed for mapping of mercury in root cross-sections of maize (*Zea mays* L.) to investigate the mechanism of mercury uptake from soil and its potential translocation to the edible parts (Debeljak et al., 2013; Lefevre et al., 2014).

Synchrotron radiation-based low-energy X-ray fluorescence

The composition and distribution of elements inside the aleurone cell layer of cereals reflect their biogenesis, structural characteristics and physiological functions. It is therefore of primary importance to understand the mechanisms underlying metal-ion accumulation, distribution, storage, and bioavailability in

aleurone subcellular organelles for seed fortification purposes. Direct imaging methods reveal the accumulation patterns between the apoplast and symplast, and highlight the importance of globoids with phytic acid mineral salts and walls as preferential storage structures. C, N, and O chemical topographies are directly linked to the structural backbone of plant substructures. The combination of high spatial and chemical X-ray microscopy techniques highlights how in situ analysis can yield new insights into the complexity of the plant cells and tissues (Regvar et al., 2011).

We have assessed the distributions of C, O, Mg, P, Mn, Fe, Cu and Zn at the cellular and subcellular levels using synchrotron radiation-based low-energy X-ray fluorescence (Figure 2), (Pongrac et al., 2016a,b,c). The relative mineral-element distributions calculated on dry weight basis confirm the observed mineral distribution profiles. In the cotyledons, P clearly partitions to the mesophyll and is mainly ascribed to phytate. In the mesophyll, the P spatial distribution strongly correlates with Mg and, in decreasing order, with Cu, Fe, Mn and Zn. These spatial distributions of the mineral elements, their concentrations and their co-localisation are important also in other pseudocereal and cereal grain.

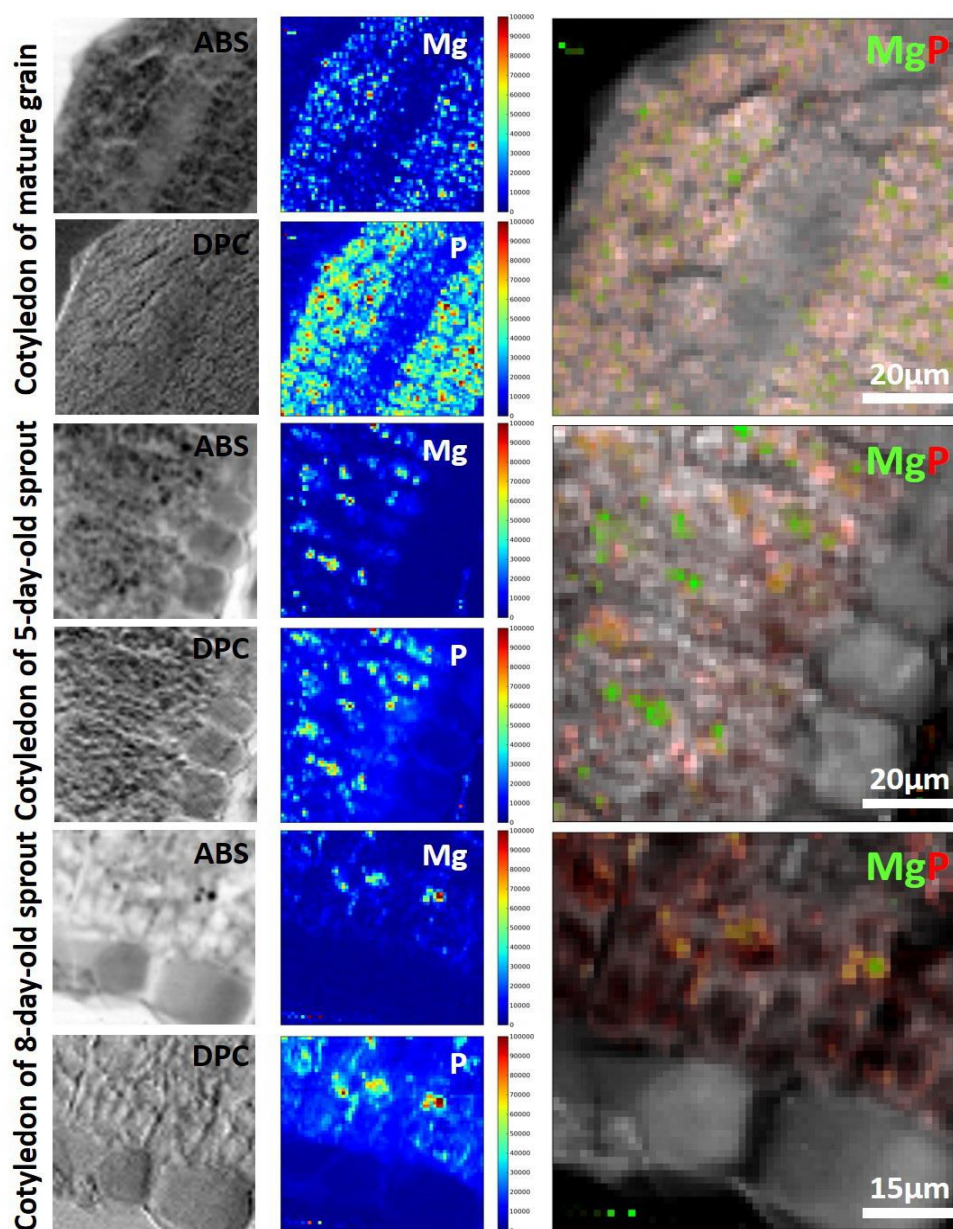


Figure 2 Mg and P distribution in the developing Tartary buckwheat cotyledon. Selected figures of morphology (left part of fig.), quantitative estimation of distribution of P and Mg (middle part), and colocalisation (right part) of cotyledons of the respective development phase (mature grain cotyledon, 5-days old sprout and 8-days old sprout). Morphology is showed with absorption picture (ABS) and with differential phase contrast (DPC). On colocalisation pictures localisation of P is marked with red and Mg with green colour. Their colocalisation on the same pixel is shown with a mixture of red and green – namely with yellow. The bitmap images in the centre and on the right hand side are not photographs but were obtained by scanning the sample pixel by pixel using the low-energy X-ray fluorescence microscope at TwinMic beamline, Elettra synchrotron, Trieste, Italy. The pixellation of the images on the right hand side is a result of the pixel size (1.2 x 1.2 μm) displayed at the dimension, where individual pixels (i.e. small single-coloured squares) can be seen.

Chemical imaging by MeV-SIMS

The distribution of organic molecules with masses between 200 and 1000 Da in the grains and in the plant tissue sections are measured with a newly developed MeVSIMS method (Jenčič *et al.*, 2016). This procedure is used to investigate secondary metabolites in plant samples for the first time, and the dedicated sample preparation method is under development for different types of plant material.

Chemical evaluation

Inductively coupled plasma mass spectrometry (ICP-MS) is an elemental analytical technique which is capable of detecting most elements of the periodic system at concentrations as low as one part in 10^{15} (part per quadrillion, ppq). This is achieved by ionising the sample with inductively coupled plasma and then using a mass spectrometer to separate and quantify the ions produced in the ICP torch. Samples usually introduced into ICP-MS instruments are acidified aqueous solutions. First, the solution is converted to an aerosol by a pneumatic nebuliser, then the fine mist is introduced to the torch. In the high temperature argon plasma (5000 – 8000 K) in the ICP torch the sample mist instantly dries, melts, evaporates, atomises and ionises. The ions are then extracted from the plasma by a two-stage differential vacuum system into the MS part of the instrument where they are separated by their mass-to-charge ratio and quantified. Many types of samples are solid or inappropriate for direct introduction into the ICP-MS and have to be digested beforehand. Microwave assisted acid digestion is by far the most commonly used solid sample digestion procedure. Here, the samples are placed in hermetically sealed PTFE autoclaves along with concentrated acid/peroxide mixtures (HNO_3 , HCl , H_2O_2). The autoclaves are heated by microwave energy. Samples are digested at high temperatures ($>180^\circ\text{C}$) and high pressures (>150 bar), yielding clear solutions which are after dilution appropriate for injection into the ICP-MS instrument.

CONCLUSION

In the last decade, completely novel physical and chemical methods have been developed to evaluate minute details of the structures, composition and function of plant parts and plant-based food products. These methods are based mainly on the use of beams generated by the particle accelerators which are located in Ljubljana (Slovenia) and Bazovica (Italy). The present team significantly contributed to the development of the presented methods and their use in the research and evaluation of nutritional value of cereals, pseudocereals and pulses.

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