

Nickel distribution in *Stackhousia tryonii* shown by synchrotron X-ray fluorescence micro-computed tomography

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ABSTRACT

Context. Hyperaccumulator plants are of considerable interest for their extreme physiology. *Stackhousia tryonii* is a nickel (Ni) hyperaccumulator plant endemic to ultramafic outcrops in Queensland (Australia) capable of attaining up to 41 300 $\mu\text{g g}^{-1}$ foliar Ni. **Aims.** This study sought to elucidate the distribution of Ni in *S. tryonii* by using synchrotron X-ray fluorescence micro-computed tomography (XFM-CT), complemented with elemental maps acquired from physically sectioned plant organs. Its Ni-enriched cylindrical photosynthetic stems make them particularly well suited samples for synchrotron XFM-CT. **Methods.** XFM-CT enables ‘virtual sectioning’ of a sample, avoiding artefacts arising from physical sample preparation. The method can be used on fresh samples that are frozen during the analysis, which preserves ‘life-like’ conditions by limiting radiation damage. It also prevents/minimises other artefacts. **Key results.** The results showed that Ni is mainly concentrated in the apoplastic space surrounding epidermal cells, and in some epidermal cell vacuoles. This finding is significant because this ‘free’ solute Ni is likely to be lost during physical sectioning. **Conclusions and implications.** This case study has highlighted the utility of the XFM-CT approach for visualising metals within intact plant organs, which may be used across the plant sciences.

Keywords: apoplastic space, artefact, hyperaccumulator, nickel, Queensland, sectioning, synchrotron, X-ray fluorescence micro-computed tomography.

Introduction

Hyperaccumulators are unusual plants that accumulate metals or metalloids in their living tissues to orders of magnitude greater concentrations than other plants growing in similar soils (van der Ent et al. 2013). Trace element hyperaccumulation is rather rare, with only ~700 hyperaccumulator species identified globally to date (Reeves 2003; Reeves et al. 2018). *Stackhousia tryonii* (Celastraceae) is an herbaceous nickel (Ni) hyperaccumulator endemic to ultramafic outcrops in Queensland, Australia (Batianoff et al. 1990; Bhatia et al. 2005b). It was discovered from a test of field specimens using dimethylglyoxime-impregnated paper (Fig. 1). It is the only species in the genus *Stackhousia* known to hyperaccumulate and can attain up to ~40 000 $\mu\text{g g}^{-1}$ Ni (i.e. 4 wt%) in its leaves (Batianoff et al. 1990; Burge and Barker 2010; van der Ent et al. 2015). In *S. tryonii*, the dominant ligands in the aqueous shoot extract is a mixture of carboxylic acids, especially malate (Bidwell 2001; Bhatia et al. 2005a), which aligns with many other (tropical) Ni-hyperaccumulator species in which Ni-citrate is the main complex (for example, van der Ent et al. 2017). Particle-induced X-ray emission (PIXE) analysis has shown that Ni is primarily localised in the epidermal cells and also in vascular tissue in the leaves and stems of *S. tryonii* (Bhatia et al. 2003, 2004).

Synchrotron X-ray fluorescence microscopy (XFM)-based techniques are capable of elucidating the distribution of metals and metalloids from the whole plant down to tissue and cellular level (Kopittke et al. 2018, 2020). However, one of the major challenges with XFM is sample preparation. Appropriate sample preparation is difficult to achieve without causing artefacts that can modify internal structures and elemental

composition (van der Ent et al. 2018). Typically, physical sectioning is performed to visualise the internal distribution of metals/metalloids in tissues, cells and plant organs, such as roots or leaves. However, this has the potential to cause elemental deportment and redistribution. Synchrotron X-ray fluorescence microscopy computed tomography (XFM-CT) avoids the need for making physical thin sections and eliminates these sectioning artefacts by producing ‘virtual’ sections of a specimen from much larger plant organs (de Jonge and Vogt 2010; van der Ent et al. 2018). XFM-CT can be used on frozen-hydrated samples to preserve ‘life-like’ conditions and avoid radiation damage (Jones et al. 2020). In hyperaccumulator plants, for example, XFM-CT has been applied to show Ni in *Odontarrhena chalcidica* (formerly *Alyssum murale*) (McNear et al. 2005), thallium in *Iberis linifolia* (formerly *I. intermedia*) (Scheckel et al. 2007) and arsenic in *Pteris vittata* (van der Ent et al. 2020). There remains much potential for this method to be used on both native and cultured plants to show the cellular-level distribution of a wide range of trace elements. *Stackhousia tryonii* has reduced leaves and photosynthetic stems, and the spherical nature and small diameter (1–3 mm) of these organs make them well suited for XFM-CT. This study aimed to acquire XFM-CT data of *S. tryonii* and compare this with physically sectioned specimens and published data on elemental distribution in this species.

Materials and methods

Plant material and specimen preparation for XFM analysis

Plant stock, in the form of fresh stems, and soil were collected from a roadside close to the Bruce Highway (near the Eden Bann turnoff at –23.093298, 150.275231) north of Rockhampton, Queensland, Australia (Fig. 1). The stems were rooted as cuttings with rooting hormone gel (Clonex Red, Yates, contains 8 g L^{–1} indole-3-butyric acid) in perlite-vermiculite mix, and subsequently planted in the natural ultramafic soil collected from the habitat. Plants were grown to mature size over 6 months in the glasshouse in Brisbane, and fresh plant material was brought to DESY in Germany for the XFM analysis described below. Sections were prepared from the root and the stem by hand-cutting with a stainless-steel razor blade (utilizing the ‘dry knife method’) and the sections were immediately mounted between two sheets of 4 µm Ultralene thin film in a tight sandwich to limit evaporation. They were analysed (at room temperature) within 20 min. after excision. An intact leaflet was excised and similarly mounted and analysed. The stem specimen for XFM-CT was mounted inside a Kapton capillary tube and analysed in frozen-hydrated state under a liquid nitrogen cryostream (operated at –140°C).



Fig. 1. *Stackhousia tryonii* plant in the field near the Eden Bann turnoff, Rockhampton, Queensland, Australia, and test paper impregnated with dimethylglyoxime showing the typical pink/purple colour reaction to nickel when pressed against leaves.

X-ray fluorescence microscopy (XFM) and data analysis

The X-ray fluorescence microscopy experiments were undertaken at PETRA III (Deutsches Elektronen-Synchrotron; DESY), a 6 GeV synchrotron radiation source, specifically at the hard X-ray microprobe experiment at the undulator

Table 1. Parameters used in the synchrotron XFM analysis of the *Stackhousia tryonii* specimens.

Parameter	Root (Fig. 2)	Stem (Fig. 3)	Leaf (Fig. 4)	Stem (Figs 5, 6)
Beam size (nm × nm)	700 × 530	700 × 530	700 × 530	530 × 440
Beam energy (keV)	12	12	12	14
Lateral step size (µm)	5	5	10	0.5
Effective angular step size (°) ^A	–	–	–	0.2
Total angles ^A	–	–	–	1804
Subscans ^A	–	–	–	4
Dwell time (ms)	10	10	8	7
Total scan time (min)	10	32	40	590
Cryostream	No	No	No	Yes

^AThe tomographic scan comprised four subscans, each of 451 angles covering 360°, offset from each other for even distribution. Multiple scans of the rotation axis better ensures full angular coverage in the case of data loss during the scan.

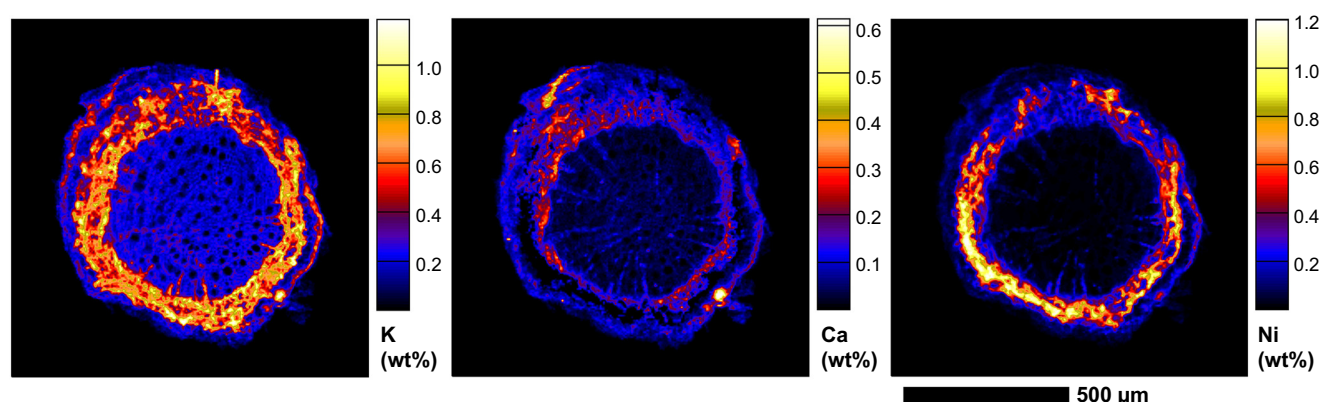


Fig. 2. Synchrotron XFM elemental maps of K, Ca and Ni in a fresh-hydrated *Stackhousia tryonii* root that was physically sectioned.

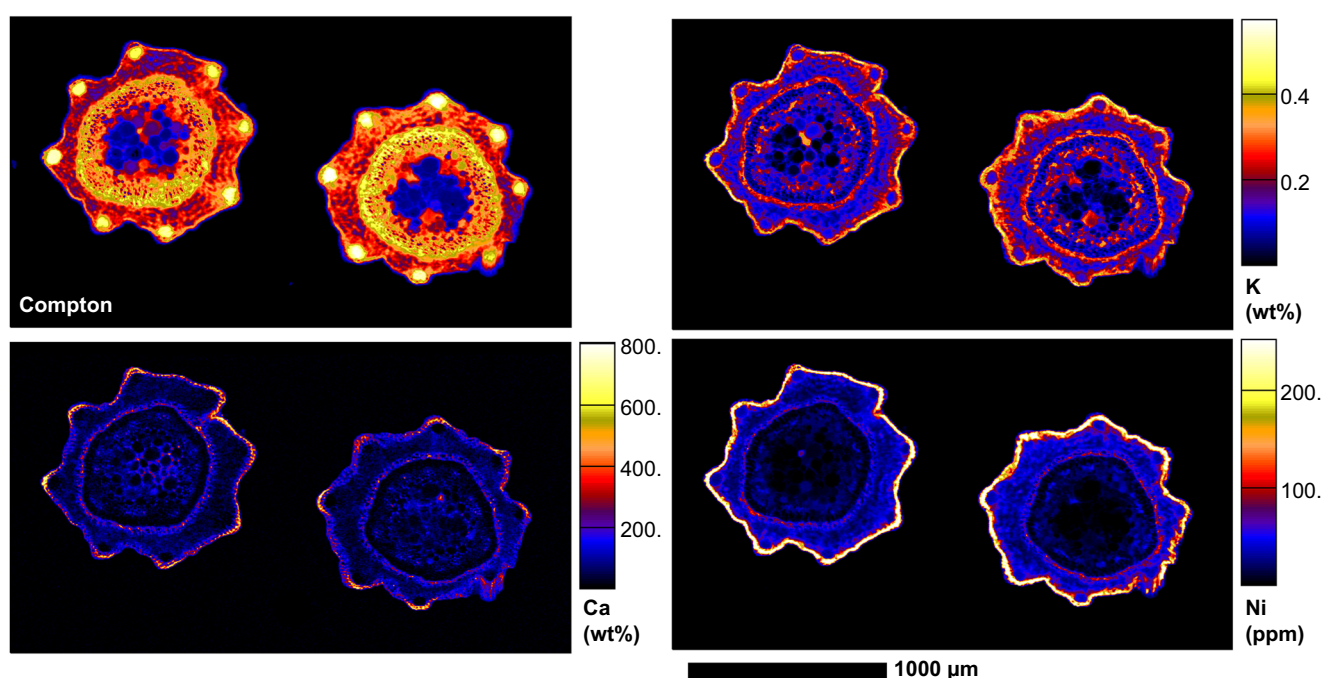


Fig. 3. Synchrotron XFM Compton map and elemental maps of K, Ca, and Ni in fresh-hydrated *Stackhousia tryonii* stems that were physically sectioned.

beamline P06. P06 is equipped with a cryogenically cooled double-crystal monochromator with Si(111) crystals. For these experiments, a KB mirror pair was used to focus the X-ray beam to 700×530 nm or 530×440 nm, resulting in flux on the sample in the order of 10^{10} photon s^{-1} . An incident X-ray energy of 12 or 14 keV was used for these experiments (full details on scan parameters are given in Table 1). The modalities utilised were standard 2D (x - y) planar and 2D (x - θ) single-slice tomography (CT) scans. Located upstream of the sample position was the 384-element Maia detector array system (Model C) in backscattering geometry, for fast and efficient acquisition of X-ray fluorescence data (Kirkham *et al.* 2010; Siddons *et al.* 2014). The XRF event stream was analysed using the Dynamic Analysis

method (Ryan and Jamieson 1993; Ryan 2000) as implemented in GeoPIXE (Ryan *et al.* 1990). The tomographic data were aligned using consistency and cross-correlation methods to correct for unwanted sample movement. The abundant angular sampling and statistics of the tomographic data allowed the use of the time-efficient filtered back-projection (FBP) method for reconstruction (Bruyant 2002) without suffering from too low signal-to-noise ratio.

Results and discussion

The physical cross-section of a fresh-hydrated root shows that potassium (K) is enriched in the cortex, and the distribution of

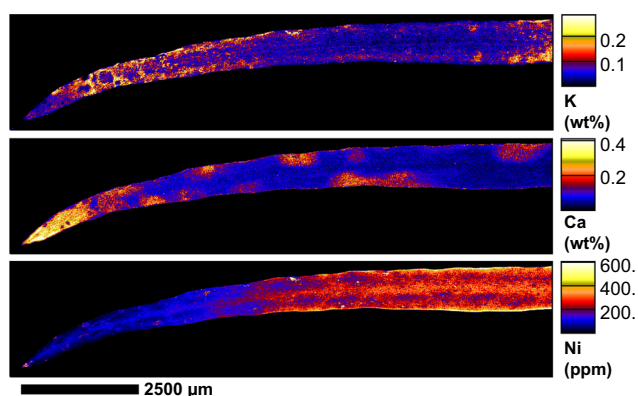


Fig. 4. Synchrotron XFM elemental maps of K, Ca, and Ni in a fresh-hydrated *Stackhousia tryonii* leaf.

Ni has a similar distribution (Fig. 2). Physical cross-sections of fresh-hydrated stems shows K concentrations in the epidermal

cell layer and in the cortex surrounding the pith, whereas calcium (Ca) occurs in the epidermal area and Ni is located in the epidermal cells (Fig. 3). Planar 2D elemental maps of a leaf blade show K enrichment towards the apex, Ca concentration to be very low in the leaf blade and indicate that Ni concentration is lowest towards the apex and highest in the central part of the blade, with distinct enrichment along the margins (Fig. 4). The XFM-CT reconstructions through the stem (Fig. 5) showed that Ni is mainly concentrated in the apoplastic space surrounding epidermal cells, and in the vacuoles of some epidermal cells. The Ni localisation in the apoplastic space would likely have been disturbed (because of losses of Ni from sectioning) if commonly used physical sample-sectioning techniques had been employed; however, this situation has been avoided here by using the XFM-CT approach. The Compton scatter and Absorption maps clearly show the sclerenchyma rays that provide structural support, epidermal cells surrounding the spongy mesophyll and the

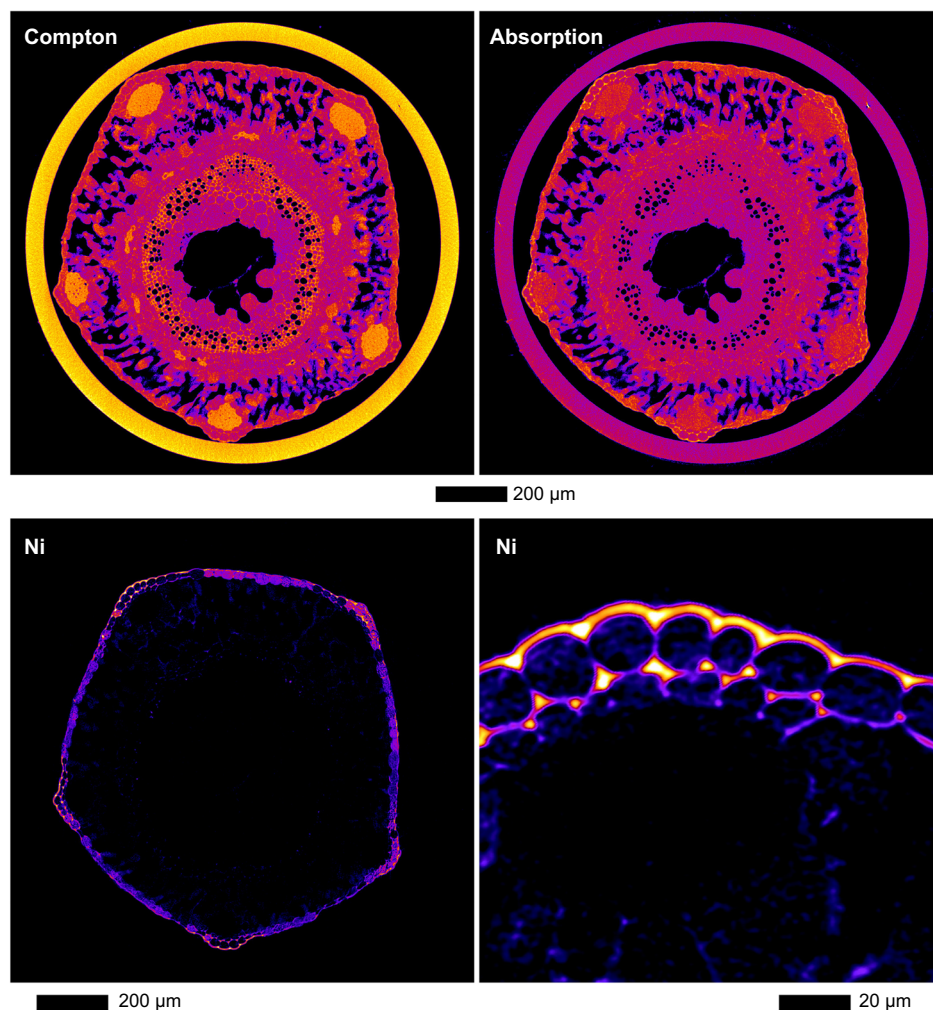


Fig. 5. Synchrotron XRF-CT 3D reconstruction of absorption, Compton scatter and Ni in a frozen-hydrated *Stackhousia tryonii* stem. The colours denote relative elemental concentrations, with brighter shades denoting higher prevailing concentrations.

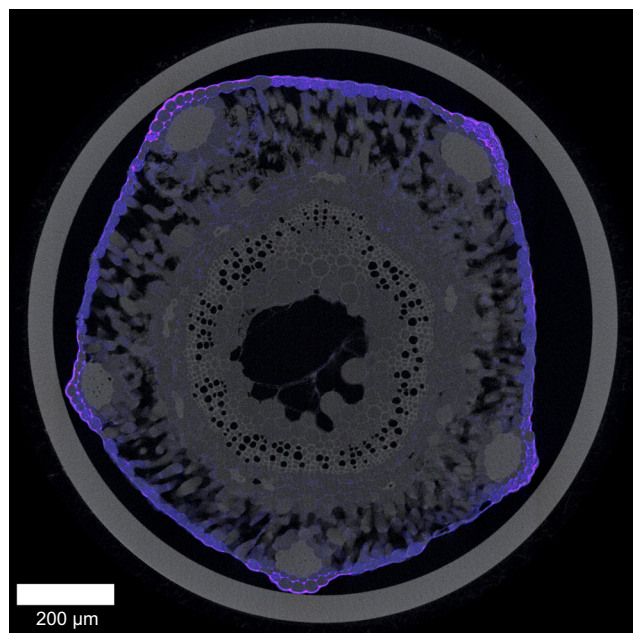


Fig. 6. Synchrotron XRF-CT reconstruction of the same frozen-hydrated *Stackhousia tryonii* stem as shown in Fig. 5, with the Ni signal superimposed over Compton scatter map.

vacuolar bundles and xylem in the pith. These structures are all extremely delicate and would likely not survive physical sectioning (compare with the 2D physical section in Fig. 3, in which this detail is lost). It should be noted that the 2D physical stem section was scanned with a step-size of 5 μm , which, although significantly larger than the beamsize, enables fast scans of fresh samples. In contrast, the XFM-CT single-slice image of the frozen-hydrated stem piece has an order of magnitude higher resolution, having been performed with a lateral step-size of 0.5 μm (closer to the beamsize) and angular spacing of 0.2° in four interleaved subsans with 0.8° step size each.

Comparing the elemental maps of the physical stem cross-sections (Fig. 2) with the XFM-CT reconstructions (Fig. 5) shows that, overall, the distribution of Ni is similar, with distinct enrichment in the epidermal apoplastic space. However, in the 2D physical section, it cannot be seen whether Ni is present in the apoplast (as is clear from the XFM-CT data) or in the vacuoles of the epidermal cells. Even though great care was undertaken to limit elemental losses by dry-cutting and immediate cryo-fixation of the stem sections (followed by freeze-drying), the Ni elemental maps obtained by Bhatia *et al.* (2004) were very different from the 2D and XFM-CT data shown here. The earlier micro-PIXE-based study showed Ni enrichment in the broader epidermal area and around vascular tissues. In part this can be attributed to the ostensibly different sensitivity of the methods used (although this is not necessarily a limitation of PIXE, but rather of the experimental setup,

detectors used, etc.), with relatively poor pixel statistics compared with the synchrotron XFM data. The XFM data presented here has megapixel definition and the use of the Maia detector-array results in exceptional detection sensitivity. There is also a distinct possibility that there are differences in the distribution of Ni within cell types depending on the age of the stem and the prevailing level of Ni accumulation. It is conceivable that in younger stems, or at lower or higher Ni concentrations, distribution differs among storage sites, with allocation in the apoplastic space or epidermal vacuoles. This might also (partly) explain the differences in the results of this study and those of Bhatia *et al.* (2004).

Of interest is that Bhatia *et al.* (2004) tested sections that were frozen and freeze-dried after sectioning, and sections that were soaked in water. The latter lost nearly all K and 88% of Ni, pointing to the great risks involved with sample preparation to introduce catastrophic artefacts. It is therefore highly likely that sectioning under liquid (water), as is common when using a vibratome, would have led to very substantial losses of Ni and other elements from our specimens. This will confound the data obtained from subsequent elemental mapping of these tissues, because those elements would have been lost from the specimen before the analysis even takes place. Of course, a fully cryogenic pathway in which specimens are (flash) frozen, cryo-microtomed and subsequently analysed with XFM in this frozen state, would minimise or eliminate such problems. However, it is extremely challenging to prepare and cryo-transfer specimens for XFM analysis, and the required equipment for doing so at synchrotron facilities, for samples of millimetre dimensions and larger, while of significant interest, is not currently widely available. The main limitations of the XFM-CT technique are its highly restrictive availability at synchrotron facilities. Furthermore, the method is limited by the size of the specimen that can be examined, as absorption of the escaping fluorescence X-rays is dependent on their energy and, hence, chemical element in question. Elements with a higher K-absorption edge energy (>7 keV), such as Ni, Zn, As, Se, are therefore best suited for tomographic analysis of plant organs up to ~2 mm in diameter, such as roots, stems, and seeds (van der Ent *et al.* 2018).

This study on *S. tryonii* has provided an example of how the XFM-CT approach can be used to visualise metals within intact plant organs while minimising sample preparation artefacts. The methodology described here is applicable to plant species and plant organs for a wide range of metals and metalloids. We hope that more plant scientists will use this technology, which is available at many synchrotron facilities, including at the Australian Synchrotron, to visualise metals and metalloids. This technology is not limited to hyperaccumulators, but could equally be used to probe elements of interest in a wide range of wild and crop plants.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare no conflicts of interest relevant to the content of this manuscript.

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Author contributions. PDE collected the plant material and AVDE cultivated the plants and prepared the samples for analysis. KMS, DB and AVDE conducted the synchrotron experiment. DB and KMS processed the synchrotron data. All authors contributed to writing the manuscript.

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