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A modified Picro-Sirius Red (PSR) staining procedure with polarization microscopy for identifying collagen in archaeological residues

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ABSTRACT

Use-wear and residue analyses of stone artefacts are widely used to better understand the behaviour and resource utilization of past peoples. There are numerous ethnographic reports describing the processing of animal parts, but identification of collagen, the principle component of animal protein, can be difficult because of the similarity in appearance to other non-collagenous residues (e.g. Lombard and Wadley, 2007). Additionally, damage to collagen structure caused by processing and taphonomic factors can alter collagen's microscopic morphological features and further prevent microscopic identification. This paper describes the trialling, blind testing and application of a modified Picro-Sirius Red (PSR) staining protocol not reliant on intact morphology which can be used to identify archaeological collagenous residues. The protocol allows for differentiation of Types I, II and III collagen and can detect the minuscule amounts in archaeological samples. The application of this staining protocol to ten grindstones from arid and semi-arid regions in south and central Australia supports the view that lack of collagen heretofore reported in residue studies is more likely the result of under-detection than its absence (see Monnier et al., 2012). The staining protocol represents an inexpensive, efficient and reliable process which can be added to contemporary use-wear and residue analyses. This addition allows more inclusive assessments of past function and promotes wider understandings of the behaviour of past peoples.

1. Introduction

Collagen is the most abundant mammalian protein and is commonly found in tendons, skin, ligaments, bone, the gut and blood vessels (Di Lullo et al., 2002; Junqueira et al., 1979:447). There are over 28 types of collagen but those most commonly found in the archaeological record are Type I (fibres), Type II (hyaline and elastic cartilage) and Type III (reticulin fibres or fibrillar collagen). Collagen can survive for long periods of time in archaeological contexts (Montes et al., 1985), especially in the matrices and voids of ground stone artefact surfaces (Buonasera, 2005:1379; Field et al., 2009:228; Quigg, 2003:16). Animal processing with particular reference to grinding and pounding is well documented (see Gould et al., 1971:163; Peterson, 1968:366; Yohe et al., 1991:660). Observed in cross-polarized light, collagen fibres in their natural state have a 'wavy' appearance and have little or no birefringence (Langejans and Lombard, 2015; Robertson et al., 2009; Viidik and Ekholm, 1968). However, damage caused by processing and taphonomic factors (such as contact with water) can alter the microscopic morphological features of collagen and, as a result, it is often under-detected, and therefore underestimated, in residue studies (Monnier et al., 2012:3284). This may be further compounded by interpretative difficulties associated with the morphological similarity of some plant and animal fibres (Lombard and Wadley, 2007:155).

The difficulties of identifying collagen based on morphology alone highlight the need for a reliable method of microscopic residue identification that is less dependent on visual features. The aim of this paper is to present a method for the detection and identification of archaeological related collagen residues using a modified Picro-Sirius Red (PSR) biochemical staining procedure. The phases of trialling the biochemical staining protocol are described and a case study demonstrating its practical application to a sample of Australian millstones is presented.

2. Biochemical staining and the Picro-Sirius Red (PSR) stain

Biochemical staining is a simple and inexpensive approach that can overcome the limitations associated with reliance on gross

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morphology for residue identification, especially in cases where residues have been altered by processing. The approach is based on a chemical reaction between a substrate and a stain that selectively colours components of the substrate. In archaeological terms, the substrate is the residue sample extracted from the working surface of an artefact which may contain a number of constituents, including proteins, lipids and carbohydrates, as well as environmental contaminants.

Biochemical staining is widely used in biology and medicine, although, with the exception of iodine–potassium-iodide (IKI) for the detection of starch (see Babot and Apella, 2003; Balme et al., 2001; Barton, 2007; Crowther, 2005; Haslam, 2004; Loy, 1994; Wallis, 1994), the technique has infrequently been used in archaeology for residue analysis. Stains which have been employed on a limited basis in archaeological residue studies include phloroglucinol for the detection of lignin (Hall et al., 1989:137), Toluidine blue for plant fibres and cellulose (Barton, 1990:59; Florian et al., 1990:37), Congo red for gelatinized starch (Crowther, 2009:39; Lamb and Loy, 2005) and Sudan 111 for lipids (Briuer, 1976:482).

Several staining protocols for identifying collagen are routinely employed in pathology studies: van Giesons, Picro-Sirius Red (PSR) and a variety of trichrome dyes (Masson, Mallory and Heidenchain). The most sensitive and specific, however, is PSR (Rich and Whittaker, 2005:97; Whittaker et al., 1994:397). Despite its frequent use in medical investigations PSR has rarely been employed in archaeological studies (see Montes et al., 1985).

The PSR staining procedure was originally developed by Puchtler et al. (1973) and improved upon by Junqueira et al. (1979:447). PSR selectively stains Type I collagen fibres, as well as Type III reticular fibres (the latter are not stained by van Giesons and are obscured by the trichrome dyes) (Junqueira et al., 1986). It reacts strongly with collagen to produce distinctive staining when viewed under cross-polarized light and also enhances collagen birefringence (Junqueira et al., 1979:447; Rich and Whittaker, 2005). With PSR staining under cross-polarized light, Type I collagen appears as thick, strongly birefringent red or yellow fibres, Type III collagen appears as thin, weakly birefringent green fibres and Type II collagen displays a weak birefringence of varying colour (Kiernan, 1999). Birefringence is a property usually associated with plant cellulose rather than collagen (Loy, 1993; Langejans and Lombard, 2015). When collagen is stained with PSR, however, the elongated dye molecules attach their long axes in parallel with collagen fibres to refract the light and create birefringence (Junqueira et al., 1979). The biochemical staining reaction takes place at a cellular level, and, therefore, the structure (intact or damaged) of collagenous residues does not affect the reaction (Dapson et al., 2011). These qualities suggest that PSR might be applicable for collagen detection in archaeological residue analysis.

3. Phase 1: development of a protocol for staining archaeological collagen

When used in biology and medicine, the PSR protocol involves applying the PSR solution to thin paraffin tissue sections that have been de-waxed and hydrated (see Junqueira et al., 1979). The procedure requires three solutions and reagents:

1) PSR solution, comprising 0.5 g of Sirius red F3B (C.I. 35782) and 500 mL of saturated aqueous solution of picric acid (1.3% in water);
2) Acidified water, comprising 5 mL of glacial acetic acid and 1 L of distilled or tap water; and,
3) Weigert's haematoxylin stain.

To ascertain whether PSR would be suitable for archaeological samples, an experimental test program was carried out. A sample of periosteum fibres, the membranous covering of bone, and bone fragments, ground butchered beef and ground lizard were combined and pulverised on the surface of a sandstone slab and allowed to dry for a week. Following the principles of commonly used residue extraction techniques (Field et al., 2009:231) 80 μL of purified water was added in 20 μL aliquots to the slab surface and allowed to soak into the matrix. A second pipette containing 20 μL of purified water was used to scrape the soaked surface and to extract a sample from the matrix. This was repeated at 15 discrete points across the slab surface. The extracted samples were transferred to glass slides which were allowed to dry overnight under a laminar flow hood and clean glass dish covers to prevent air-borne contamination. Meanwhile, 50 mL of PSR solution was prepared by adding 0.05 g of Sirius red F3B (C.I. 35782)[1] to 50 mL of saturated aqueous solution of picric acid (1.3%).

In contemporary medical applications, Weigert's haematoxylin is applied to a sample before the PSR solution to facilitate the staining of nuclei. Cell nuclei are not usually a focus of archaeological investigations; hence the step involving Weigert's haematoxylin was omitted for the archaeological protocol. Should the differentiation of cell nuclei be desired, this earlier step could be retained.

Initial staining of the experimental samples with the standard PSR solution produced a uniform deep red colour which lacked differentiation and obscured all details. As the collagen found in archaeological samples will be more degraded, fragmented and in very small amounts compared to the collagen in thin paraffin tissue sections (Brown, 1978:323; Flint, 1994:28), it was recognised that it would be necessary to significantly modify the PSR solution reagent and protocol.

A series of progressively diluted PSR solutions were prepared and trialled to reduce the staining intensity and thereby promote more selective staining. Staining the trial samples with the weaker 0.25% (one part PSR solution to three parts deionised water) and 0.125% (one part PSR solution to seven parts deionised water) PSR solutions produced results that most closely matched those of published PSR tissue section stains in cross-polarized light (cf. Junqueira et al., 1979; Kiernan, 1999) (see Table 1). Using the diluted 0.25% and 0.125% PSR solutions, all collagen appeared pink in plane-polarised light, with the expected colour changes being consistently observed in cross-polarised light. The birefringence of all collagen types was enhanced using all diluted PSR solutions (Table 1).

These results indicated that a 0.25% PSR solution was optimal for staining collagen in the experimental trial, and the following protocol was adopted for use in the subsequent experimental trial phases:

Step 1 With a clean pipette tip, apply 20 μL aliquots of ultra-pure water to the areas of interest on the stone and allow to soak into the matrix. Reapply as necessary to ensure the matrix is saturated. Time will vary depending on to the porosity of the matrix.

Step 2 Use a second pipette to apply another 20 μL of ultra-pure water and agitate the soaked area with the tip. Inject the 20 μL of ultra-pure water into the matrix, then extract it. Repeat the action creating a venturi effect until a suitable amount of sample is extracted from the stone matrix. Sample size varies depending on the porosity of the matrix and the number of slides being prepared. Note that the

[1] Various Sirius reds are available. It is important to use C.I. 35782 as other Sirius reds undergo different manufacturing processes and as such the resulting colour intensities of the stains may differ.

Table 1
Summary of observations of experimental residue samples stained with varying concentrations of PSR solution.

Concentration of PSR solution	Observations
100% (undiluted)	All residues stained deep red without differentiation or discrimination. Although staining was indiscriminate, enhanced birefringence of microscopically identified collagenous structures was noted.
0.75%	All residues stained deep red without differentiation or discrimination. Although staining was indiscriminate, enhanced birefringence of microscopically identified collagenous structures was noted.
0.50%	Residues stained a strong pink. Some pink to yellow colour change was observed in cross-polarized light (xp). No differentiation of reticulum fibres. Bone fragments displayed some varied birefringence on the margins.
0.25%	Vivid colour changes from pink to yellow, orange and green observed in cross-polarized light. Small collagenous pieces easily discernible. Bone fragments displayed varied birefringence on the margins. Hyaline cartilage displayed a weak birefringence of a varying colour.
0.125%	Vivid colour changes from pink to yellow, orange and green observed in cross-polarized light. The hue changes from of green to yellow in cross-polarized light were not as distinctive as those observed with the 0.25% dilution. Small collagenous pieces easily discernible. Bone fragments displayed varied birefringence on the margins. Hyaline cartilage displayed a weak birefringence of a varying colour.

water is not being used as a solvent; instead it is the venturi effect of water removal from the matrix which extracts the residues regardless of their lipophilic or hydrophilic nature.

Step 3 Transfer extracted sample to microcentrifuge tube for storage.

Step 4 Using a Pasteur glass pipette, place one drop of the sample onto a glass slide and allow to dry overnight, avoiding airborne contamination by covering with a clean inverted glass petri dish, lid or similar.

Step 5 Freshly prepare 0.25% PSR solution.

Step 6 Apply one drop of 0.25% PSR solution using a 230 mm glass pipette to the dried sample.

Step 7 Leave the slide for one hour under a clean cover such as a glass dish lid.

Step 8 After one hour apply one to two drops of acidified water to the stained substrate. Excess PSR should wash out easily and not remain affixed to the slide.

Step 9 Place a cover-slip on the stained area. Do not seal the cover-slip so the dry stain can be rehydrated later as needed.

Step 10 Gently remove excess liquid using a lint-free wipe.

Step 11 Transfer the stained slide to a bright-field transmitted light microscope with polarizing filters for examination.

4. Phase 2: testing the diluted PSR solution and modified protocol on experimental samples

4.1. Methods

To test the efficacy of the diluted PSR solution and the modified protocol for use in archaeological contexts, replicative grinding of a variety of materials ethnographically recorded as being used by indigenous people in Australia (Table 2) was undertaken. Assuming that grindstones were multifunctional tools, combinations of raw materials listed below were ground on separate slabs for 15–30 min (see Table 3) on ten untreated, ethanol cleaned sandstone slabs[2] until a groove was macroscopically visible. Two residue samples were extracted from the middle of each experimental grinding groove and prepared for staining and examination following the protocol described above. It is noted that the staining solution and reagents were freshly prepared and staining viability was checked periodically on collagen control slides that were prepared earlier.

5. Results

In all the trials the diluted PSR consistently highlighted collagenous residues and allowed for differentiation between morphologically similar plant and animal material (see Fig. 1). Additionally, when the stain dried, slides were able to be rehydrated with acidified water without loss of staining integrity. This allowed results to be revisited (Stephenson, 2011).

All of the extracted samples of known residues (Phase 2) which included collagenous materials displayed the expected colour changes and increased birefringence. The collagenous residues observed included fibres, amorphous collagen, sheet collagen and collagen fibrils. Some non-collagenous residues, such as matrix minerals, appeared green or yellow in cross-polarised light; however, these colours were unchanged in plane-polarised light and thus were easily differentiated from collagen. The intact mineralized hydroxyapatite matrix of bone fragments does not take-up PSR; however, when bone was associated with collagenous fibrous material, colourimetric changes associated with PSR staining were observed along the bone margins. Hyaline cartilage (Type II collagen) was weakly stained. The cartilage displayed a weak birefringence of varying colour and did not present the dramatic colour change between plane and cross-polarised light. Unlike Type I and III collagen, hyaline cartilage does not form fibres (Montes and Junqueira, 1991:3), but has a mesh-like or netted form.

In plane-polarized light the majority of residues (both animal and plant, excluding starch) were stained pink by the PSR (Fig. 1). With non-collagenous residues, however, a colour change to yellow, green or orange was not observed when switching from plane to cross-polarized light (Fig. 1). Intact starch granules did not stain with PSR (Fig. 1); however, bordered pits and plant annular rings, grossly similar to starch granules, partially stained, allowing clear differentiation. Further, the visual diagnostic properties of starch and plant material such as plant fibres and amorphous cellulose, were unaltered by the staining process (Fig. 1). Starch granules, plant fibres and amorphous cellulose continued to display birefringence in cross-polarized light, and the extinction cross associated with starch granules remained clearly visible and rotated when moving from cross-polarized to plane polarized light (Fig. 1).

Additionally, as PSR is collagen specific, the contrasting outline of non-collagenous residues, in particular, phytoliths, enhances observations of these residues in cross-polarized light (Fig. 2).

[2] The experimental sandstone slabs obtained from a commercial quarry were examined at 40× magnification after cleaning to ensure that the grinding surfaces were free of pre-existing residues.

Table 2
Summary of ground materials as documented in Australian ethnographic sources.

Category	Ethnographically documented material	Material used for replicative grinding	References
Bone	Desert rat kangaroo (*Caloprymnus campestris*) Lesser bilby (*Macrotis leucura*) Feral cat (*Felis catus*) Small mammals	Assorted animal bones including Kangaroo (*Macropod*) vertebrae and long bones	Gould et al. (1971:164), Kamminga (1982:49) and Veth et al. (1990:51)
Meat	Gould's goanna (*Varanus gouldii*)	Lizard/skink; Raw beef	Gould (1969), Tonkinson (1978) and Veth et al. (1990:51)
Seeds	Mulga (*Acacia aneura*) Millet (*Panicum decompositum*)	Mulga (*Acacia aneura*) Millet (*Panicum decompositum*) Woollybutt (*Eragrostis eriopoda*)	Allen (1974), Badman (2000:6), Juluwarlu Aboriginal Corporation (n.d.), Latz (1995:52), O'Connell et al. (1983) and Smith (1986:29)
Fruit	Desert raisin (*Solanum centrale*)	Banana and honey	Gould (1969), Moore (2005), O'Connell et al. (1983) and Tonkinson (1978)
Wood	Artefact shaping and smoothing	Lancewood (*Acacia shirleyi*)	Hayden (1979:114) and Kamminga (1982:63);
Pituri	Pituri leaves (*Duboisia hopwoodii*)	Dried *Duboisia* sp. plant Crushed tobacco	Cox (1881) and McBryde (1987:258)
Resin	*Triodia* spp.	Unidentified species of Spinifex (*Triodia*) from the Western Desert, Western Australian	Brokensha (1978:64), Pitman and Wallis (2012) and Sheridan (1979);
Stone	Axe grinding	Hammerstones—sandstone Quartzite and basalt stones	Dickson (1981), McCarthy (1967:67) and Spencer (1982:89)
Ochre	Yellow and red ochre	Ochre from Camooweal, Queensland	McBryde (1987:261), Smith and Fankhauser (2009) and Tacon (1991)

Table 3
Raw material combinations used in the Phase 2 experimental program.

Allocated grindstone number	Raw material combination
1	Bone, Seed and Wood
2	Lizard, Seed and Pituri
3	Fruit, Ochre and Seed
4	Stone, Lizard and Seed
5	Ochre, Wood and Bone
6	Resin, Pituri and Wood
7	Ochre, Seed and Meat
8	Resin, Meat and Bone
9	Wood, Ochre and Seed
10	Seed, Bone and Meat

While the highlighted colourimetric changes are collagen specific, the outlining of non-collagenous residues and the absence of colourimetric changes allows for the additional identification of non-targeted plant and mineral residues.

6. Phase 3: blind testing

Blind trials of the PSR staining protocol were conducted by residue analyst Dr Gail Robertson of the University of Queensland. A second set of residue samples was extracted via the method outlined above from another series of experimental grinding exercises. These samples were placed on numbered slides one week prior to trials; no information was provided to Dr Robertson about what materials had been ground or what residues might be expected to occur. On each prepared slide was a unique, known residue

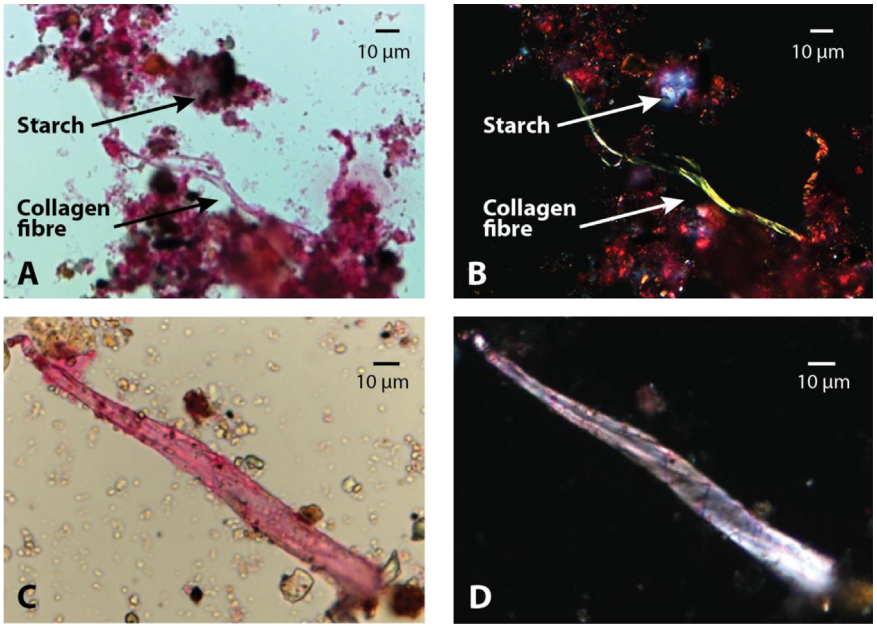

Fig. 1. Examples of colour and diagnostic features in plane (pp) and cross-polarized (xp) light A). PSR stained collagen fibre and starch granule (pp). B). PSR stained collagen fibre and starch granule (xp). C). PSR stained plant fibre and mineral (pp). D). PSR stained plant fibre with unaltered birefringence and mineral (xp). All images at 400× magnification.

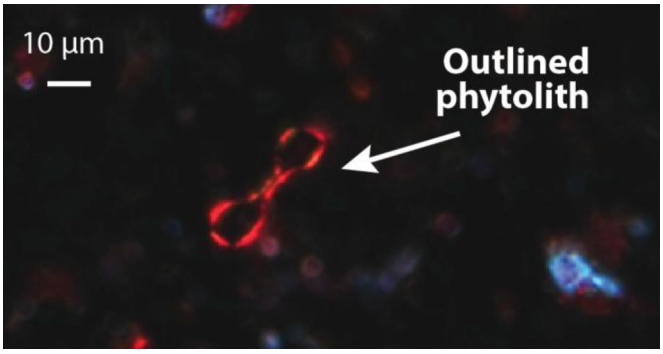

Fig. 2. PSR stained bilobate phytolith in cross-polarized light 400×.

combination. Residue materials included ground plant, seeds, bone, lizard and beef collagen, ochre and minerals. After training in the staining protocol, Dr Robertson stained the pre-prepared slides and undertook the blind test using an Olympus BX50 microscope with polarising filters at magnifications of 200× and 400×. A third independent person compared Dr Robertson's observations with the known correct answers; 100% accuracy was obtained, with all residues being correctly identified and no false positive collagen results being obtained, which conferred confidence in the modified protocol.

7. Phase 4: testing of archaeological samples

Following the blind testing of experimental residue samples, a pilot study designed to examine 10 basal grinding stones was undertaken using the modified PSR protocol. The stone artefacts were selected from amongst those in the Queensland Museum collection, and most came from surface contexts in the Cooper Creek/Lake Eyre Basin south central Australia. Each grindstone was labelled with an alpha code (A, B, C etc.). Two grinding fragments which could be conjoined were G and H. The individual grooves were each assigned a number and hence each groove had a unique reference, e.g. A1.

7.1. Methods

Residue extractions were undertaken following the modified protocol established for diluted PSR staining. Three to six extractions were taken from each groove depending on the length of the groove. The extractions were taken along the length of the groove and incorporated the deepest point of the groove as well as the margins. Residues were identified based upon staining reactions and morphological comparisons with known residue reference collections.

Samples were also taken from adjacent non-ground surfaces and the residue densities between those and samples from the

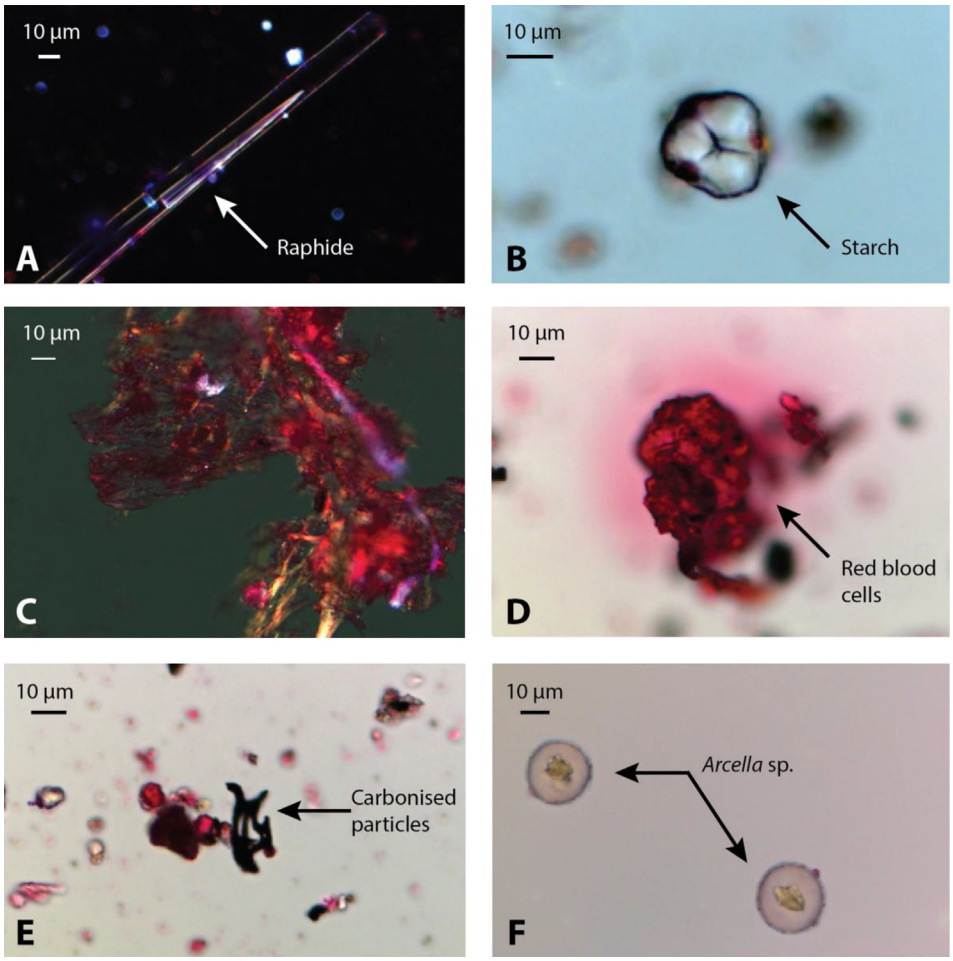

Fig. 3. Examples of residue categories: A). Raphide (C3–5) in cross-polarised (xp) light. B). Starch granule (B1–5) plane polarized (pp) light. C). Collagen fibres (C1–5) associated with large blood vessels (xp). D). A cluster of red blood cells (F3–5) (pp). E). Carbonised material (F3–5) (pp) F). *Arcella* sp., an amoeba contaminant often found in freshwater or mosses (F2–5) (pp). All images 400× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adjacent ground surfaces compared. If the densities of similar types of residues were notably higher within a groove, they were considered to be use-related. Density differences were also a useful measure by which to assess possible contamination from surrounding sediments (Atchison and Fullagar, 1998:121; Barton et al., 1998:1233).

Previous studies (e.g. Barton, 2007:1758; Loy and Hardy, 1992) have demonstrated that organic residues survive well on archived museum artefacts, even when their curation history may have included washing. The specific history of the grinding stones examined in this study was not known though they were stored in plastic covering and the artefacts were not washed or otherwise altered during the investigation. To assess the potential of air-borne contamination during storage, control slides were positioned on shelves in the Queensland Museum Hendra Annexe and left in place for a 14 month period. They were then examined at 400× magnification and stained with diluted PSR solution to identify possible contaminants. A similar control study was undertaken in the residue laboratory on benches adjacent to the work areas for a period of six months.

Solutions and reagents were also regularly examined for possible environmental or handling contamination. This included PSR staining of the purified water to test for collagenous contaminants. Stain viability was periodically tested to ensure that colour reactions remained consistent over time, using the Phase 2 slides of known collagenous residues.

7.2. Results

Varying amounts of residue were extracted from the grooves of the ten grindstones (Fig. 3 and Table 4). The PSR stain allowed the grouping of residues into five broad categories: plant; animal;

Table 4
A summary of observed residues per grinding groove.

Grindstone	Groove	Amorphous cellulose	Starch	Phytoliths	Plant fibre	Plant vessel elements	Pollen	Raphides	Spore heads	Wood	Blood	Blood vessels	Bone	Collagen amorphous (a),Fibre (f), reticular (r), periosteum (p)	Fat	Muscle rings	Muscle tissue	Ochre	Various minerals	Charcoal flecks	Charred plant	Sponge - spicules (s), diatoms (d)	Possible contaminants	Starch mimics
A	1	X	X	X	X	X				X				a f r	X					X		s	X	
A	2	X	X	X	X	X	X	X		X				a f r						X		s	X	
B	1	X	X	X	X									a f r								d		X
B	2	X	X	X	X		X			X				a f r	X					X	X		X	X
B	3	X	X		X									a r	X									X
B	4	X	X	X	X	X								a f r	X									X
C	1	X	X	X	X	X		X			X	X		a f r						X			X	
C	2	X	X	X	X	X		X		X				a f						X		d	X	X
C	3	X	X	X	X	X	X	X	X	X			X	a r p						X		d	X	X
C	4	X	X	X	X	X		X	X	X	X		X	a f r	X	X	X			X	X	d	X	
D	1	X	X	X	X	X	X							a r						X		s d	X	
D	2	X	X	X	X									a f r						X			X	
D	3		X	X	X					X				a r			X	X		X		s d	X	
E	1	X		X	X									a				X		X	X			
E	2	X	X		X		X				X	X	X	a f	X					X			X	
E	3	X	X		X	X	X	X					X	a f r						X	X	d	X	
F	1	X	X		X	X		X		X			X	a f		X			X	X	X		X	
F	2	X	X		X	X	X		X	X			X	a f		X				X	X	d	X	
F	3	X	X		X	X				X	X	X	X	a f r p		X				X	X		X	
GH	1	X	X		X				X	X	X		X	a f	X					X		d	X	X
GH	2	X	X	X	X	X			X	X			X	a f r	X					X			X	
GH	3	X	X	X	X	X			X	X			X	a f r						X	X		X	
I	1	X	X	X	X	X								a					X	X	X		X	X
J	1	X	X	X	X	X		X		X				a f r						X		d	X	X
J	2	X	X	X	X	X				X	X			a f r	X					X	X		X	
J	3	X	X	X	X	X				X			X	a f r p			X			X		d	X	

Key:

Residue Category	Plant	Animal	Mineral	Carbonised	Contaminant use-related	Contaminant non-use related

mineral; carbonised material; and 'other materials', which included probable contaminants, such as fungi and lichen. While it is recognised that their presence may be the result of a processing activity, contaminants were not considered to have been purposely processed. The broad categories were further broken down into particular residue types where possible, to allow for more inclusive assessments of function, Examples of these categories presented in Fig. 3.

Collagen in varying density was recovered from all grinding grooves on all grinding stones. It was not however observed in all extracted samples; macerated and irregular amorphous collagen was found in 92% of samples (Fig. 4). Residue combinations indicative of animal processing were also observed. These included blood vessels, blood cells and intact bone (Table 4). On one grindstone, hairs surrounded by a collagenous sheath and morphologically similar to bat hair were found (Fig. 4).

8. Discussion and conclusion

Monnier et al. (2012:3298) argued that, because of damage caused by processing and subsequent taphonomic factors, residue studies often underestimate and misidentify collagenous residues. The results of this study clearly demonstrate the utility of the modified PSR staining protocol for detecting and identifying collagenous material, even when it is degraded and altered, as it is often the case in archaeological contexts. The modified PSR protocol presented here is a simple and inexpensive, yet a specific and sensitive method for the detection and identification of collagenous residues on the working surfaces of stone artefacts, including grindstones. Following PSR staining, the resulting colourimetric changes allow for the differentiation of collagen Types I, II and III, and further facilitate the identification of a range of residues. The method is minimally invasive, as much residue is left on the tool surface for future study. Also, the rehydration of stained slides allows for results to be viewed by other researchers.

The grindstones studied here were proposed previously to be a type of specialised seed grinder not usually associated with animal processing (Smith, 1985, 2004). The extracted residues, however, included abundant collagen fibres and fibrils, muscle tissue, amorphous collagen and folded periosteum. These materials have been infrequently reported in previous studies of other seed grinders from Australia (e.g. Balme et al., 2001), but the results of the current study support the ethnographic and archaeological evidence that suggests during their lifetime they were

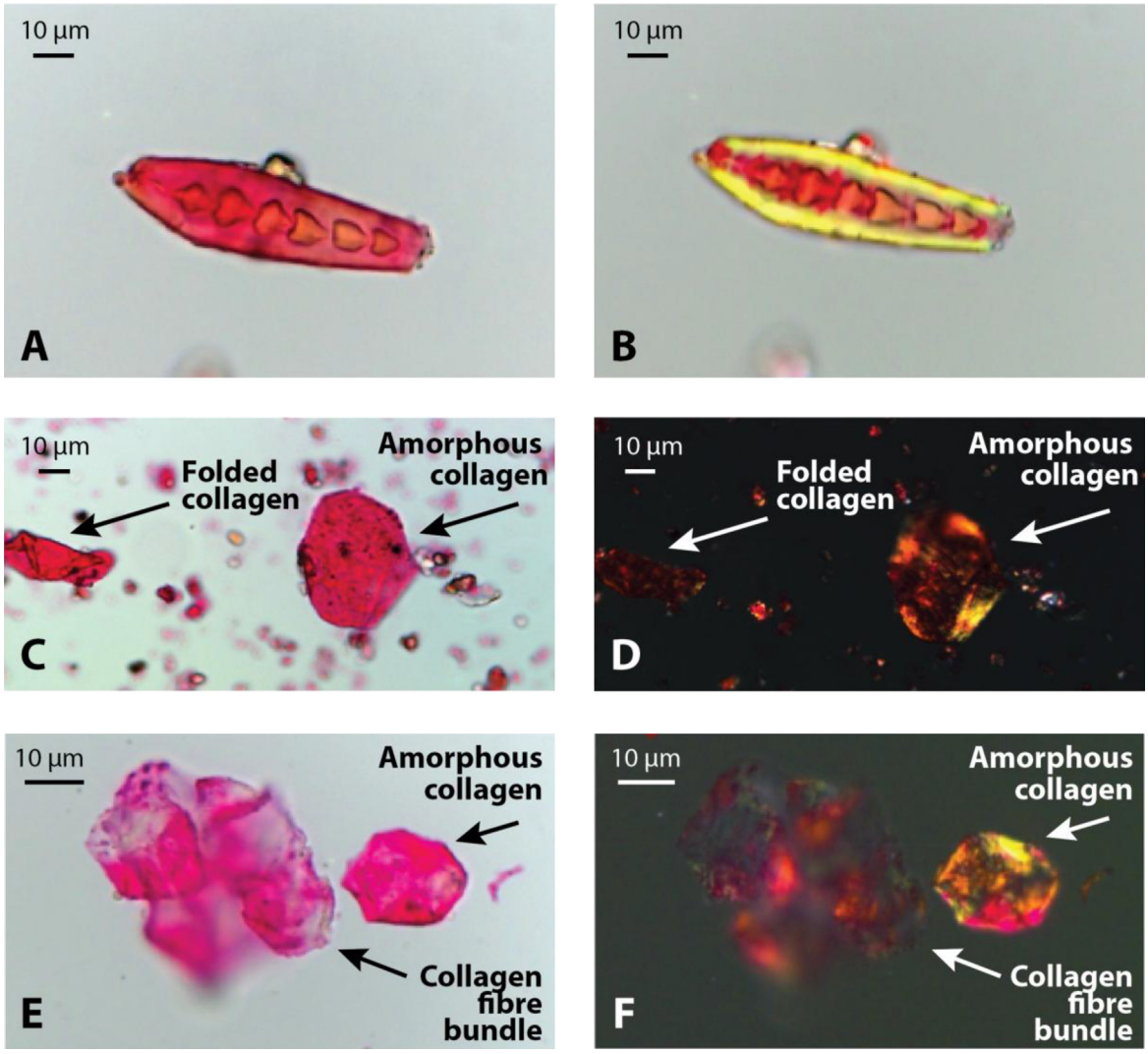

Fig. 4. Examples of collagenous residues: A). PSR stained collagenous sheath surrounding bat hair (D1–5) (pp). B). PSR collagenous sheath surrounding bat hair (D1–5) (xp). C). PSR stained amorphous collagen (GH3–5) (pp). D). PSR stained amorphous collagen (GH3–5) (xp). E). PSR stained collagen fibres and amorphous collagen (D2–2) (pp) F). PSR stained collagen fibres and amorphous collagen (D2–2) (xp). All images 400× magnification.

multifunctional (Table 2; see also Yohe et al., 1991). Kangaroo tail, goannas, small rodents, mammals and cooked lizards were often pounded on grinding slabs in the Western Desert (e.g. Gould, 1969; Gould et al., 1971:163; Hayden, 1979:141; Peterson, 1968:366; Tonkinson, 1978). Also, the vertebrae of reptiles, rabbits and feral cats were ground to facilitate their consumption by the very young and the elderly (Cane, 1989:113; Gould et al., 1971:164), and animal long bones and skulls were cracked on ground surfaces to extract their marrow and brains (Peterson, 1968:366; Walshe, 1999, 2000). There is also extensive documentation of ground surfaces being used to sharpen and shape macropod and bird bone points (Boot and McBryde, 1994:5; Fullagar et al., 1992:45). The fashioning of possum skin coats in southeast Australia has been extensively noted (Horton, 1994:993), and the use of sinew for binding has been observed throughout Australia (Napier and van Leeuwen, 2008). It is therefore possible that collagenous residues have been unrecognised by conventional use-wear and residue analyses.

PSR is only one of a number of existing, well-established yet under-utilised biochemical protocols which might be incorporated into the array of use-wear and residue analysis approaches to better understand past human behaviour (see Stephenson, 2011). Biochemical staining techniques, generally, and PSR plus polarization specifically, have the great advantage of not depending on residue morphology being intact or pristine to enable detection and identification (Fig. 4). These techniques allow detection of residues that are otherwise overlooked or misidentified, such as collagen, which can often appear similar to other common residues, such as plant cellulose. PSR staining can be incorporated into the current array of use-wear and residue analysis approaches, to permit mapping and quantitative assessments of collagen across worked surfaces, and thereby better understand the mechanics and complicated biography of individual artefacts. The addition of PSR plus polarization allows for more inclusive and therefore comprehensive understandings of the behaviour and resource utilization of past peoples.

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