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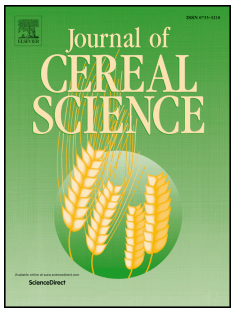

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Isolation of Wheat Endosperm Cell Walls: Effects of non-Endosperm Flour Components on Structural Analyses

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Abstract:

We report the isolation of a pure form of cell walls from wheat endosperm 'popped' out from the whole, enzyme deactivated and soaked grain, and compare them with cell walls isolated from milled flours of extraction rates from 45% to 75%, at physiological (37 °C) and elevated (70 °C) temperatures. Cell walls isolated from flours all contained non-endosperm walls whereas walls from popped endosperm were apparently pure. The monosaccharide composition of 'popped' cell walls was different to that of cell walls isolated from flour, particularly glucose and mannose contents (34 and 7 % for 'popped' cf 29 and 3% for flour respectively) and arabinose to xylose ratios (0.45 for 'popped' cf 0.58 for flour). Total phenolic content of popped endosperm cell walls were three to four times lower than for cell walls from flour. Elevated isolation temperature also had a solubilising effect, altering the cell wall composition. This study provides a novel method of isolating pure wheat endosperm cell walls, and demonstrates how contaminating (thick cell walled) non-endospermic material in milled flours can have a major influence on cell wall compositional analyses.

1. Introduction

The starchy endosperm of wheat grains is covered by multi-layered peripheral tissues of different composition and thickness. The external tissues include the outer pericarp, the inner pericarp, seed coat, hyaline layer and the aleurone layer. The seed coat is the thinnest layer (5-8 μm) while the aleurone is the thickest layer (up to 65 μm in thickness). Besides these layers, the grain also contains the germ comprised of the embryonic axis and the scutellum. Each of the layers differ in their physical and chemical properties (Barron et al., 2007). The starchy endosperm (~85% of whole grain by weight) is contained in cells confined by cell walls that make up only about 2-3% of the total grain dry weight (Lafiandra et al., 2014).

Although wheat is a major cereal, its nutritional functionality as refined flour is lower compared to e.g. barley and oats. This is attributed mainly to the components of the cell walls, arabinoxylans and (1,3;1,4)-β-D-glucans. Wheat endosperm cell walls are composed of about 70 % arabinoxylan and about 20 % (1,3;1,4)-β-D-glucan (hereafter referred to as beta glucan) while barley and oat endosperm cell walls contain about 75% beta glucan and 20% arabinoxylan (Bacic and Stone, 1980; Collins et al., 2010). Compared to arabinoxylan, beta glucan is more soluble and swellable thereby producing viscous effects in the digestive tract which are reported to impart physiological benefits such as lowering of postprandial glucose and cholesterol level (Dikeman and Fahey, 2006; Jenkins et al., 2002). Arabinoxylans are often cross-linked with ferulic acid which are proposed to limit cell wall hydration, swelling and solubility (Fry, 1986; Grabber et al., 1998; Kamisaka et al., 1990).

Most of the studies that have characterised endosperm cell walls or their components have used milled flour as their starting raw material (Bacic and Stone, 1980; Mares and Stone, 1973; Ordaz-Ortiz and Saulnier, 2005), with isolations typically carried out at either high

temperatures or using various solvents. As evident from microscopic imaging and chemical studies (Delcour et al., 1999; Pussayanawin et al., 1988; Ramseyer et al., 2011), milled flour contains particles other than the starchy endosperm. As the composition of cell walls from the endosperm and other layers are different (Antoine et al., 2003; Barron et al., 2007; Piot et al., 2000), this could affect the characterisation of endosperm cell walls. A source of endosperm cells and walls devoid of other tissue types would clearly be useful. Furthermore, higher temperature influences the extraction of arabinoxylans and beta glucans (Anderson et al., 1978; Beresford and Stone, 1983) and, therefore, may influence the components that would be present in real food situations. For studies aimed at understanding behaviours relevant to nutritional value, acid, alkali or solvent treatments should be avoided as these may alter some molecular structures or lead to depolymerisation of the components thus affecting both characterisation and properties. Acidic environments may release arabinose from side chains, and can solubilise hemicelluloses whereas alkali treatment may cause the depolymerisation of polymers. Similarly, alcohol or other solvents may promote aggregation of polysaccharides (Comino et al., 2013). As a result, various properties inherent to the components may be changed, which in turn may affect our understanding of their role(s) in human nutrition, as these changes would not occur during processing and digestion of foods. Hence the aim of this study is to isolate the purest possible form of wheat endosperm cell walls that can be used as a raw material for characterisation studies as well as for investigation of functional properties relevant to nutritional value.

2. Materials and methods

2.1. Materials

White wheat flour (White Wings brand) was obtained from a local supermarket in Brisbane, Queensland, Australia. Whole wheat grains (*Triticum aestivum* var Lincoln) were gifted by Pacific Seeds, QLD, Australia. Wheat flours (*T. aestivum* var Lincoln) of four different extraction rates viz. 75%, 65%, 55% and 45% were obtained by milling the grains as described in section 2.2.

Thermostable alpha amylase from *Bacillus licheniformis* (E-BLAAM; EC 3.2.1.1) and amyloglucosidase from *A. niger* (E-AMGDF; EC 3.2.1.3) were purchased from Megazyme International, Ireland. Protease from *B. licheniformis* (P4860; EC 3.4.21.62) was purchased from Sigma-Aldrich, USA. Phosphate buffered saline (PBS) tablets were purchased from ThermoFisher Scientific Australia Pty Ltd. All other chemicals and reagents were purchased from Sigma-Aldrich, USA and Merck, Germany.

2.2 Milling of wheat

Wheat flour of different extraction rates were produced on a Buhler MLU-202 laboratory test mill, fitted with 160 µm sieves. The grains were pre-conditioned to a moisture content of 15.5% approximately 24 h prior to milling, and the feed rate was set at 100 grams/minute. The flour was collected as separate streams from each of the mill passes (1st, 2nd, 3rd break, and 1st, 2nd, 3rd reduction) and weighed to give total flour. Bran and pollard was also collected and weighed. The total flour plus bran and pollard was defined as the total products. Straight run flour extraction was calculated as: (total flour/total products)*100. The sample milled gave a straight run flour extraction of 75%. The extraction rate (ER) is the percentage weight of the flour obtained from the grain. Normally, white

wheat flour is of 70-80% extraction rate, and patent flours of lower extraction rates are associated with less contamination from non-endosperm components. To produce the flours of lower extraction, the amount of flour required for each was calculated by using the formula: (extraction required*total products)/100. This was further divided by four to obtain 4 different extraction rates viz. 75%, 65%, 55% and 45%. Flour was then taken from the streams in sequence (1st break, 1st reduction, 2nd break, 2nd reduction and so on) until the required total was reached. The number of break and reduction system flour streams used decreases with the extraction rate.

2.3. Isolation of endosperm from whole grain

Whole wheat grains were heated in a hot air oven at 130 °C for 90 min in order to inactivate the endogenous enzymes (Comino et al., 2013). After cooling, they were soaked in excess water in a beaker (200 ml/100g grains) and kept in a refrigerator (2 °C) for 5 days changing the water on a daily basis. It was found that soaking the grains for 5 days was sufficient to make them soft enough to press using two fingers. One end of the soaked grain towards the brush (the distal end) was cut carefully with a pair of scissors and the proximal end pressed such that the white endosperm 'pops' out from the grain. This method is thus named as 'popping' and is illustrated in Fig. 1(a).

Wheat flours were also heated in a hot air oven at 130 °C for 90 min in order to inactivate endogenous enzymes.

2.4 Isolation of cell walls

Popped endosperm and heat-treated flour (both 100 g, as is basis) were each mixed in 1L deionised water in a 2L beaker at 37 °C for 60 min on a hot plate using a magnetic stirrer at

500 rpm. The slurry was then centrifuged at 3200g for 10 min and the supernatant discarded. Prior to centrifugation, the popped endosperm was subjected to wet-sieving through a 250 micron sieve to remove any peripheral parts of grains that may have been collected with the popped endosperm while cutting.

To the residue, PBS buffer was added (40 ml per gram). The residue was mixed thoroughly in the buffer manually to ensure there were no clumps. The enzymatic treatments were carried out at two different temperatures- one at 70 °C to gelatinise starch and maximise enzyme activity, and the other at 37 °C to approximate human physiological temperature. In the first set of experiments, the slurry was heated to 70 °C on a hot plate for 1h to gelatinise the starch and denature proteins. This was followed by the addition of thermostable alpha-amylase (50 µl per gram residue) and incubation for a further 1.5 h (after which the slurry was starch negative, as shown by iodine addition). The temperature was then lowered to 60 °C and the slurry further incubated for 4h with addition of protease (60 µl per gram) and amyloglucosidase (100 µl per gram) to hydrolyse proteins and residual starch oligosaccharides respectively.

In another experiment, instead of 70 °C, the slurry was incubated at 37 °C for 48 h, the proportions of enzymes being the same. Sodium azide was added at 0.02% to the buffer to prevent microbial spoilage.

The extent of starch and protein digestion was checked with iodine solution and light microscopy. 1 ml of the slurry was taken and centrifuged, with both residue and supernatant collected. The solid residue was observed under the light microscope where the extent of degradation of starch and proteins can be assessed. A few drops of iodine solution was added to the supernatant, with a blue colour indicating the presence of starch. After complete digestion (7h at higher isolation temperature and 48 h at 37 °C), the mixture was

allowed to cool and was wet-sieved through a 20 micron screen under running water to remove smaller particulate matter. The non-digestible cell walls remain on the screen and were centrifuged at 3200 g for 10 min. The residue was washed twice with 70% ethanol and acetone and left to air dry at room temperature. The isolation process is shown schematically in Fig. 1 (b).

2.5. Microscopic analysis

The efficacy of starch and protein removal during the isolation process was examined using an Olympus BX-61 light microscope (LM) (Tokyo, Japan).

Confocal laser scanning microscopy (CLSM) observations were carried out using a Zeiss LSM700 confocal microscope under 10X or 20X objective lens at an excitation wavelength of 405 nm to observe the auto-fluorescence of phenolic acids present in the cell walls. Images were taken from at least 10 different regions of each sample.

Scanning electron microscopic (SEM) images of the cell wall samples were also acquired. The samples were cleaned by plasma before coating with approximately 10 nm of iridium (Bal-Tec Coater, Leica microsystems, Wetzlar, Germany). Images were taken using a JSM-7100F scanning electron microscope (JEOL, Tokyo, Japan) at 5kV and 10 mm working distance. At least three different locations were chosen for each sample. All images were taken from the top side of the sample, which is the one in contact with air during production.

2.6. Monosaccharide analysis

Monosaccharides were estimated following the alditol acetate method (Pettolino et al., 2012). In brief, about 5 mg of the sample was hydrolysed with 72% sulphuric acid, reduced

193 with sodium borodeuteride in dimethyl sulphoxide (DMSO) and acetylated by adding 1-
194 methylimidazole followed by acetic anhydride. The alditol acetate thus formed was
195 extracted in dichloromethane to run on a GC-MS (QP2010 Ultra, Shimadzu, Japan) using a
196 high polarity BPX70 column. Myo-inositol was used as the internal standard. Analyses were
197 performed in duplicate.
198
199 *2.7. Microanalysis*
200 Elemental microanalyses were conducted in duplicate on a FLASH2000 Elemental
201 Microanalyser (ThermoFisher Scientific, Waltham, MA, USA).
202
203 *2.8. Total phenolics*
204 The extraction of phenolics was done by alkali treatment of the sample (Waldron et al.,
205 1996) with some modifications. Briefly, about 10 mg of the cell wall sample was taken and
206 treated with 800 µl of 2M NaOH (25 °C / 24 h). After the incubation, the content was
207 acidified to pH 2 with concentrated HCl and the supernatant collected (16000xg/ 10min,
208 Sigma 1-14, Germany). The phenolics were extracted in ethyl acetate, dried under vacuum
209 and re-dissolved in 50% MeOH.
210 The total phenolic content was determined according to the Folin-Ciocalteu assay (Singleton
211 and Rossi, 1965) with some modifications. Briefly, 25 µl of blank, ferulic acid standards or
212 the diluted (1:4) methanolic extracts were reacted with 200 µl of 10% (v/v) freshly prepared
213 Folin-Ciocalteu reagent. The mixture was then neutralised with 800 µl of 0.7 M sodium
214 carbonate. The absorbance of the mixture was read at 725 nm with a UV/Vis
215 spectrophotometer (UV-1700, Shimadzu Corporation, Japan) against 50% MeOH as a blank.
216 Ferulic acid (0-250 µg/ml) was used as a reference standard, and the results were expressed

as micrograms of ferulic acid equivalent (FAE) per gram of the cell wall (µg of FAE/g of cell wall). The analyses were carried out in triplicate.

3. Results and Discussion

3.1. Morphology of cell walls

Microscopy of cell walls isolated from flour (irrespective of extraction rate) revealed contamination with non-endosperm cell walls visually assigned to aleurone layer, pericarp and brush/hairs (Fig. 2 A1, B1 and C1). This was confirmed with images of the different anatomical parts that were separated by hand-dissection (Fig. 3 A, B and C), showing that the white flours contained other peripheral parts of wheat grain. In contrast, cell walls isolated from popped endosperm did not show any of these features as illustrated in Fig. 2 D1. Fig. 2 A2, B2, C2 and D2 are the images in auto-fluorescence mode. It can be observed that the aleurone, pericarp and brush have greater auto-fluorescence than the endosperm cell walls. This is consistent with endosperm cell walls having a lower amount of cell wall phenolic acids compared to the peripheral layers of wheat (De Brier et al., 2015; Jääskeläinen et al., 2013; Piot et al., 2000). The endosperm cell walls are very thin and appear to be like a transparent sheet, as shown in Fig. 2 D1. Similar micrographs of cell walls with thin and large size are found in previous reports using different isolation methods (Mares and Stone, 1973; Toole et al., 2013). Endosperm cells are as large as 100 µm with walls as thin as 1 µm or less, whereas aleurone cells are around 20 µm in diameter with 2-3 µm thick walls (Dornez et al., 2011; Jääskeläinen et al., 2013). The outer layers are somewhat elongated structures while the aleurone cells are cuboidal in shape as shown in Fig. 3 A, B and C and previously reported by other workers (Antoine et al., 2003; Barron et al., 2007).

241 It was shown in a previous study (Henry, 1987) that flour obtained from pearled grain
242 contains non-endosperm tissues as well. This has been supported by the recent study of De
243 Brier et al. (2015) that pearled kernels with up to 12% grain weight removed, still showed
244 the auto fluorescence characteristic of peripheral layers. Hence, an alternative was sought
245 to pearling and we studied cell walls isolated from flour of four different extraction rates,
246 viz. 75%, 65%, 55% and 45%. However, it was found that even flour with an extraction rate
247 as low as 45% was not free of non-endosperm tissues (Fig. 2). Hence, a novel, yet simple
248 method called 'popping' was developed (Fig. 1a) to isolate a pure form of starchy
249 endosperm which was used as the starting material for cell wall isolation. The popping
250 process is manually intensive. On average, it takes about 8 working hours to pop the
251 endosperm out from 100g grains. The cell walls isolated from 'popped' endosperm are
252 however very clean and apparently free from peripheral layers as shown in Fig. 2 D1.
253 Fig. 4 shows SEM images of cell walls isolated from milled flours of four different extraction
254 rates and from the popped endosperm. The cell walls obtained from flours include rods, flat
255 chunks and honey-comb shaped structures that are characteristic of brush, pericarp and
256 aleurone layers respectively. They are all present in flours of the four different extraction
257 rates as shown in Fig. 4 A, B, C and D. It is, however, difficult to estimate how much of these
258 anatomical structures enter into the flour. In contrast, the cell walls isolated from popped
259 endosperm have thin cell walls only as shown in Fig. 4 E.
260 The cell wall preparations were distinctly different to the naked eye as well. The popped
261 endosperm cell walls were white while the cell walls from flour all had some brown
262 colouration- the degree of brownness increasing with the increase in extraction rate (Fig. 5).
263 Furthermore, there was a difference in the percentage yield (dry weight basis based on
264 popped endosperm/flour) of the cell walls. The popped cell walls had a yield of about 0.2%

while for flours this increased with extraction rate, the maximum being 1.2% for 75% ER flour, presumably reflecting the greater proportion of non-endosperm walls with increasing extraction rate. These numbers, however, may be an underestimate the true content since some of the smaller fragments are lost during the wet sieving steps.

Hence this study confirmed that milled flour even at 45% extraction rate is not free of peripheral layers of the grain, and popping is a better method to source the purest form of endosperm for isolation and characterisation studies of wheat endosperm cell walls. In succeeding sections, we compare the cell walls obtained from popped endosperm to those obtained from flours by different analytical methods.

3.2. Monosaccharide analysis

The monosaccharide content of the acid-hydrolysed cell walls varied depending upon the source of endosperm and temperature of isolation as shown in Table 1.

The dominant sugars present in cell walls of wheat endosperm are arabinose, xylose and glucose. There is a compositional difference between the cell walls isolated from popped endosperm and those from different extraction rate flours at both isolation temperatures of 37 °C and 70 °C. As seen from Table 1, cell walls from popped endosperm isolated at 37 °C are composed of polysaccharides containing 57% arabinoxylans (assuming that all arabinose and xylose are from arabinoxylan), 34% glucose and 7% mannose while that isolated at 70 °C has 73% arabinoxylan, 20% glucose and 6% mannose, with minor amounts of rhamnose and galactose. Although the cell walls isolated from flours at the two temperatures have almost similar compositions, the effect of temperature is marked in the case of popped endosperm cell walls, primarily the percentage of glucose. This could be due to the solubilisation of β-glucans at the higher temperature. The glucose value is higher than

289 reported in some earlier studies (Bacic and Stone, 1980; Comino et al., 2013; Gruppen et al.,
290 1989), but is in line with the report of Ordaz-Ortiz and Saulnier (2005). The high glucose
291 content in the popped endosperm cell walls is presumed to be due primarily to a higher
292 content of β-glucans, as no starch was detected. Apart from β-glucans, the glucose could
293 also arise from (galacto)glucomannan or cellulose. The mannose content in popped
294 endosperm cell walls is 6-7% (Table 1). Mares and Stone (1973) have reported the glucose
295 to mannose ratio of wheat glucomannan to be 1:3. Hence 2-3% of the glucose is likely to be
296 contributed by glucomannan. The composition of cell walls from flours and A/X ratio are in
297 line with the work of Ordaz-Ortiz and Saulnier (2005) in which the cell walls are obtained
298 from white flour by a dough-washing method and incubating the residue with enzymes up
299 to a maximum temperature of 95 °C. The A/X ratios of popped endosperm cell wall is ca
300 0.45 while those in cell walls from flour is 0.56-0.60. The higher ratio in the latter could be
301 due to the inclusion of aleurone and other peripheral layers (Barron et al., 2007). Hence,
302 based on our analysis, the composition of cell walls isolated from popped endosperm cell
303 walls at 37 °C represents the most likely true values for wheat endosperm cell walls as these
304 are apparently free of non-endosperm tissues as revealed by microscopy (Fig. 2 D and 4 E).
305 The A/X ratio of cereal arabinoxylans can be functionally important, as solution stability is
306 decreased at low A/X ratio (Collins et al, 2010). However, extractability from cell walls may
307 not be so affected by A/X ratio (Comino et al, 2013).
308
309 *3.3. Microanalysis*
310 The nitrogen content of all the samples was less than 0.5% by weight, some of them below
311 the instrument's sensitivity (Table 1). This indicates that the protease digestion was
312 sufficient to remove almost all of the proteins from the flour and endosperm. Previous

313 studies (Comino et al., 2013; Mares and Stone, 1973) have reported somewhat higher
314 values which might be due to residual or cytoplasmic proteins that escaped the treatments
315 (Ballance and Manners, 1978).
316
317 *3.4. Total phenolics*
318 The total phenolic contents in the cell walls from popped endosperm were 3-4 times lower
319 than those present in the cell walls obtained from flours of different extraction rates (Table
320 1). There was a small increase in phenolic contents with increasing extraction rates. The
321 total phenolics of 75% ER flour cell walls was the highest indicating that there was more
322 contamination of non-endospermic tissues (Figs. 2 and 4) that have a high content of
323 phenolic acids. The phenolic compounds are located mostly in the outer layers of the grain
324 which is evident from the auto-fluorescence in micrographs (De Brier et al., 2015;
325 Jääskeläinen et al., 2013). Various authors have investigated the phenolic acid contents in
326 different anatomical parts of wheat and have found that they are very low in the starchy
327 endosperm (Antoine et al., 2003; Barron et al., 2007; Ndolo et al., 2013). The effect of
328 extraction rate of flour and degree of pearling on the dietary fiber content, total
329 arabinoxylan content and the phenolic acids have also been reported by various researchers
330 (De Brier et al., 2015; Delcour et al., 1999; Ramseyer et al., 2011). Hence our results
331 demonstrate that milled low extraction rate flour is not free of peripheral layers and the
332 phenolics content of the flour does not represent the content in the endosperm.
333
334 **4. Conclusions**
335 An apparently pure form of endosperm cell walls is obtained by a novel 'popping' method. It
336 is shown that milled flour even at low extraction rates contains aleurone and other

peripheral tissues of wheat. The chemical composition of popped endosperm cell walls differs from cell walls isolated from white flour, and it is shown that a low extraction rate of flour is not an indicator of endosperm purity. Availability of endosperm cell walls devoid of walls from other tissues will allow investigation of the molecular and microscopic factors responsible for cell wall integrity and functionality during both food manufacture and digestive processing.

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354 **References**

355 Anderson, M., Cook, J., Stone, B.A., 1978. Enzymatic determination of 1, 3: 1, 4-β-glucans in barley
356 grain and other cereals. Journal of the Institute of Brewing 84, 233-239.
357 Antoine, C., Peyron, S., Mabille, F., Lapierre, C., Bouchet, B., Abecassis, J., Rouau, X., 2003. Individual
358 contribution of grain outer layers and their cell wall structure to the mechanical properties of wheat
359 bran. Journal of Agricultural and Food Chemistry 51, 2026-2033.
360 Bacic, A., Stone, B.A., 1980. A (1→3)- and (1→4)-linked β-d-glucan in the endosperm cell-walls of
361 wheat. Carbohydrate Research 82, 372-377.
362 Ballance, G.M., Manners, D.J., 1978. Structural analysis and enzymic solubilization of barley
363 endosperm cell-walls. Carbohydrate Research 61, 107-118.
364 Barron, C., Surget, A., Rouau, X., 2007. Relative amounts of tissues in mature wheat (< i> Triticum
365 aestivum</i> L.) grain and their carbohydrate and phenolic acid composition. Journal of Cereal
366 Science 45, 88-96.
367 Beresford, G., Stone, B.A., 1983. (1→ 3),(1→ 4)-β-d-Glucan content of Triticum grains. Journal of
368 Cereal Science 1, 111-114.
369 Collins, H.M., Burton, R.A., Topping, D.L., Liao, M.-L., Bacic, A., Fincher, G.B., 2010. Review: Variability
370 in fine structures of noncellulosic cell wall polysaccharides from cereal grains: Potential importance
371 in human health and nutrition. Cereal Chemistry 87, 272-282.
372 Comino, P., Shelat, K., Collins, H., Lahnstein, J., Gidley, M.J., 2013. Separation and purification of
373 soluble polymers and cell wall fractions from wheat, rye and hull less barley endosperm flours for
374 structure-nutrition studies. Journal of Agricultural and Food Chemistry 61, 12111-12122.
375 De Brier, N., Hemdane, S., Dornez, E., Gomand, S.V., Delcour, J.A., Courtin, C.M., 2015. Structure,
376 chemical composition and enzymatic activities of pearlings and bran obtained from pearled wheat
377 (Triticum aestivum L.) by roller milling. Journal of Cereal Science 62, 66-72.
378 Delcour, J.A., Van Win, H., Grobet, P.J., 1999. Distribution and structural variation of arabinoxylans in
379 common wheat mill streams. Journal of Agricultural and Food Chemistry 47, 271-275.

380 Dikeman, C.L., Fahey, G.C., 2006. Viscosity as related to dietary fiber: A review. Critical Reviews in
381 Food Science and Nutrition 46, 649-663.
382 Dornez, E., Holopainen, U., Cuyvers, S., Poutanen, K., Delcour, J.A., Courtin, C.M., Nordlund, E., 2011.
383 Study of grain cell wall structures by microscopic analysis with four different staining techniques.
384 Journal of Cereal Science 54, 363-373.
385 Fry, S.C., 1986. Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annual
386 Review of Plant Physiology 37, 165-186.
387 Grabber, J.H., Hatfield, R., Ralph, J., 1998. Diferulate cross-links impede the enzymatic degradation
388 of non-lignified maize walls. Journal of the Science of Food and Agriculture 77, 193-200.
389 Gruppen, H., Marseille, J.P., Voragen, A.G.J., Hamer, R.J., Pilnik, W., 1989. Mild isolation of water-
390 insoluble cell wall material from wheat flour: Composition of fractions obtained with emphasis on
391 non-starch polysaccharides. Journal of Cereal Science 9, 247-260.
392 Henry, R., 1987. Pentosan and (1→ 3),(1→ 4)-β-Glucan concentrations in endosperm and wholegrain
393 of wheat, barley, oats and rye. Journal of Cereal Science 6, 253-258.
394 Jääskeläinen, A.-S., Holopainen-Mantila, U., Tamminen, T., Vuorinen, T., 2013. Endosperm and
395 aleurone cell structure in barley and wheat as studied by optical and Raman microscopy. Journal of
396 Cereal Science 57, 543-550.
397 Jenkins, D.J., Kendall, C.W., Augustin, L.S., Franceschi, S., Hamidi, M., Marchie, A., Jenkins, A.L.,
398 Axelsen, M., 2002. Glycemic index: Overview of implications in health and disease. The American
399 Journal of Clinical Nutrition 76, 266S-273S.
400 Kamisaka, S., Takeda, S., Takahashi, K., Shibata, K., 1990. Diferulic and ferulic acid in the cell wall of
401 Avena coleoptiles—Their relationships to mechanical properties of the cell wall. Physiologia
402 Plantarum 78, 1-7.
403 Lafiandra, D., Riccardi, G., Shewry, P.R., 2014. Improving cereal grain carbohydrates for diet and
404 health. Journal of Cereal Science 59, 312-326.

405 Mares, D., Stone, B.A., 1973. Studies on wheat endosperm I. Chemical composition and
406 ultrastructure of the cell walls. Australian Journal of Biological Sciences 26, 793-812.
407 Ndolo, V.U., Beta, T., Fulcher, R.G., 2013. Ferulic acid fluorescence intensity profiles and
408 concentration measured by HPLC in pigmented and non-pigmented cereals. Food Research
409 International 52, 109-118.
410 Ordaz-Ortiz, J., Saulnier, L., 2005. Structural variability of arabinoxylans from wheat flour.
411 Comparison of water-extractable and xylanase-extractable arabinoxylans. Journal of Cereal Science
412 42, 119-125.
413 Pettolino, F.A., Walsh, C., Fincher, G.B., Bacic, A., 2012. Determining the polysaccharide composition
414 of plant cell walls. Nature Protocols 7, 1590-1607.
415 Piot, O., Autran, J.C., Manfait, M., 2000. Spatial distribution of protein and phenolic constituents in
416 wheat grain as probed by confocal raman microspectroscopy. Journal of Cereal Science 32, 57-71.
417 Pussayanawin, V., Wetzel, D.L., Fulcher, R., 1988. Fluorescence detection and measurement of
418 ferulic acid in wheat milling fractions by microscopy and HPLC. Journal of Agricultural and Food
419 Chemistry 36, 515-520.
420 Ramseyer, D.D., Bettge, A.D., Morris, C.F., 2011. Distribution of total, water-unextractable, and
421 water-extractable arabinoxylans in wheat flour mill streams. Cereal Chemistry 88, 209-216.
422 Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-
423 phosphotungstic acid reagents. American Journal of Enology and Viticulture 16, 144-158.
424 Toole, G.A., Selvatico, E., Salt, L.J., Le Gall, G., Colquhoun, I.J., Wellner, N., Shewry, P.R., Mills, E.N.C.,
425 2013. Effect of dough mixing on wheat endosperm cell walls. Journal of Agricultural and Food
426 Chemistry 61, 2522-2529.
427 Waldron, K.W., Parr, A.J., Ng, A., Ralph, J., 1996. Cell wall esterified phenolic dimers: Identification
428 and quantification by reverse phase high performance liquid chromatography and diode array
429 detection. Phytochemical Analysis 7, 305-312.
430

Table

Table 1. Monosaccharide composition, total arabinoxylan, protein content and total phenolics of cell walls

Cell walls	Monosaccharides[a], mol%								Protein content, % (%N[c] x 5.7)	Total Phenolics (µg FAE/g)
	Rha	Ara	Xyl	Man	Gal	Glc	A/X	total AX[b], %		
Popped endosperm-37	1.9	17.4	39.6	6.9	0.6	33.8	0.44	57.0	ND	1679
45% ER flour-37	1.1	24.7	41.7	3.6	1.4	27.7	0.59	66.4	2.51	5296
55% ER flour-37	1.8	24.1	42.2	2.8	0.8	28.3	0.57	66.3	1.14	5370
65% ER flour -37	1.1	24.8	42.1	2.8	1.2	28.2	0.59	66.9	2.79	6111
75% ER flour -37	0.8	24.7	41.0	2.8	1.4	29.5	0.60	65.7	2.28	6766
Popped endosperm-70	1.0	23.0	49.6	6.3	0.5	19.5	0.46	72.6	ND	1825
45% ER flour -70	0.6	24.1	42.4	3.6	2.2	27.3	0.57	66.5	ND	6122
55% ER flour -70	0.5	22.3	39.9	3.3	1.7	32.5	0.56	62.2	ND	6206
65% ER flour -70	1.1	23.3	41.0	3.0	1.4	30.3	0.57	64.3	ND	7107
75% ER flour -70	0.5	23.1	41.3	2.7	1.3	31.4	0.56	64.4	0.86	7514

[a] *The monosaccharides are rhamnose (Rha), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc). A/X represents the arabinose to xylose ratio. The monosaccharide contents are expressed as a percentage of the total sugars present in the cell walls.*

[b] *Total arabinoxylan % contents have been calculated by adding the arabinose % and xylose %.*

443 [C] *The nitrogen content of some of the samples were below the instrument's sensitivity (less*

444 *than 0.1% in 2 mg sample). Hence the protein content was not detected (ND).*

445

Figure captions:

Fig.1 (a). Popping of endosperm from soaked wheat grains

Fig.1 (b). Overview of cell walls isolation methods

Fig.2. Micrographs of cell walls obtained from flour (A, B, C) and popped endosperm (D). A, B and C show the presence of peripheral layers as hair/brush, aleurone and pericarp while D contains endosperm cell walls only. The blue colour in A2, B2, C2 and D2 is due to the auto-fluorescence of phenolic acids. The apparently greater blue intensity for endosperm cell walls in D2 and A/B/C 2 are not comparable. The subdued fluorescence of endosperm cell walls in latter is due to the dominating fluorescence of other structures.

Fig.3. Micrographs of peripheral layers of wheat obtained by hand-dissection. A= External pericarp B=Intermediate layer, C=Superimposition of intermediate layer and aleurone cells, D=Aleurone layer

Fig.4. Scanning electron micrographs of cell walls isolated from different sources. A, B, C and D are cell walls obtained from flour of 45%, 55%, 65% and 75% ERs respectively. The presence of hairs/brush, pericarp and aleurone can be seen in those images. E shows the images of cell walls, free from non-endosperm tissues, isolated from popped endosperm.

Fig. 5. Images of cell walls isolated from different sources. The cell walls from popped endosperm are white in colour while those from flours are more brown.

Figures

Fig. 1.(a)Popping of endosperm from soaked wheat grains

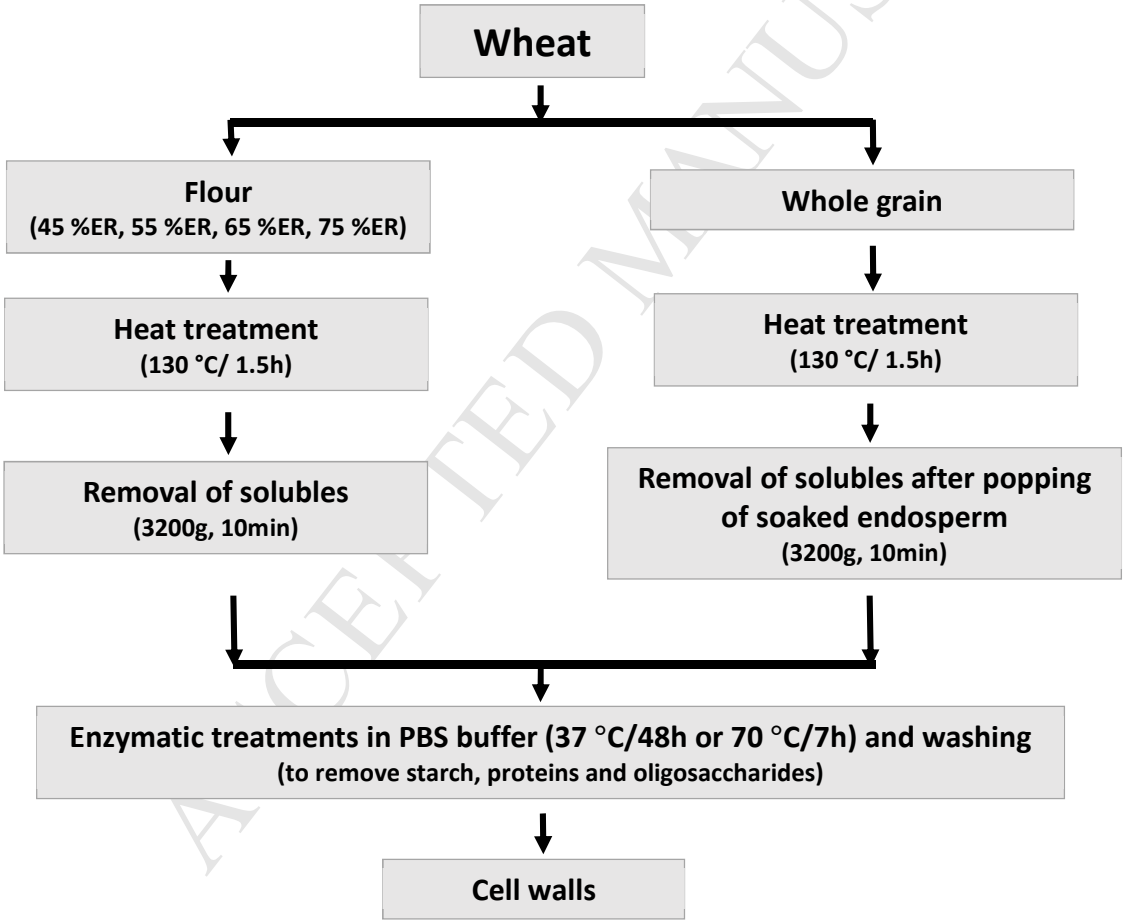

Fig. 1. (b) Overview of cell walls isolation methods

478 **Figure 2**

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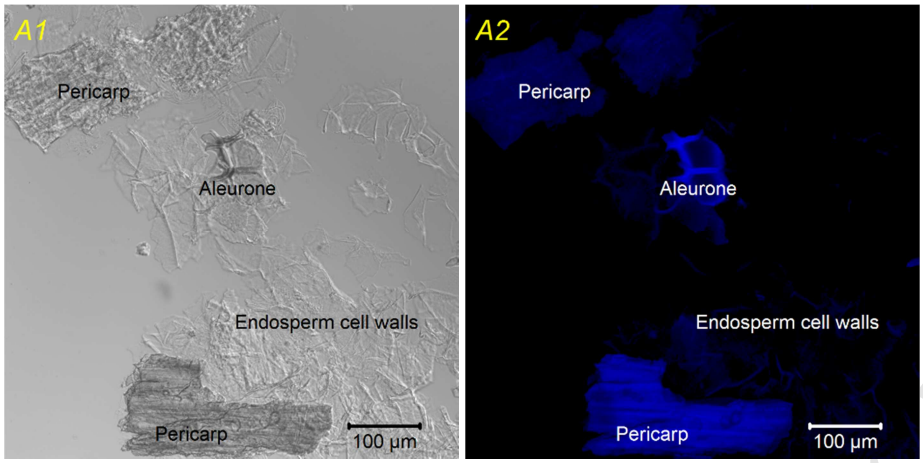

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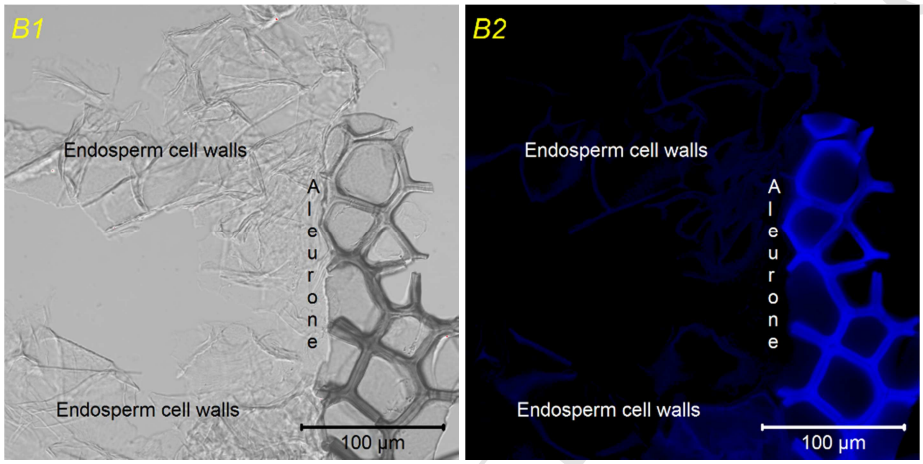

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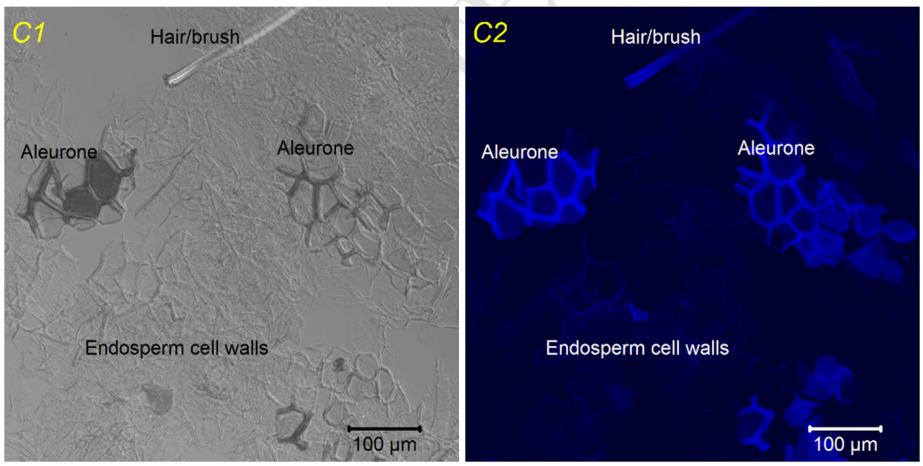

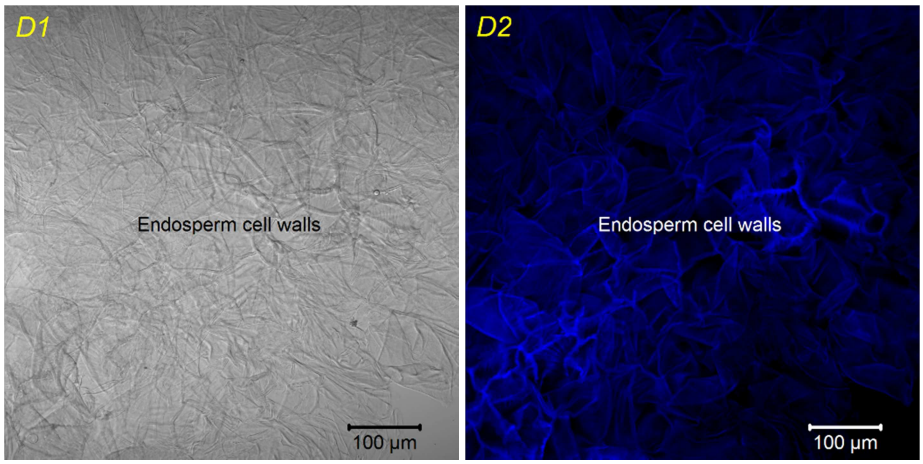

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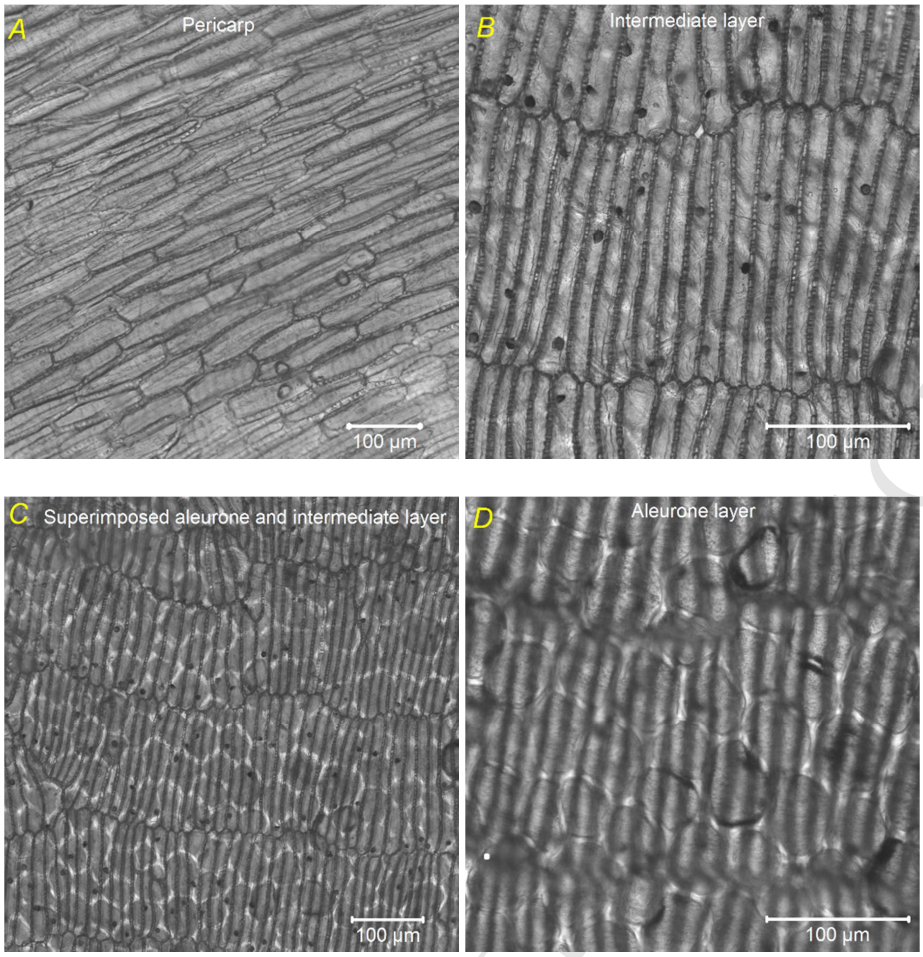
Figure 3



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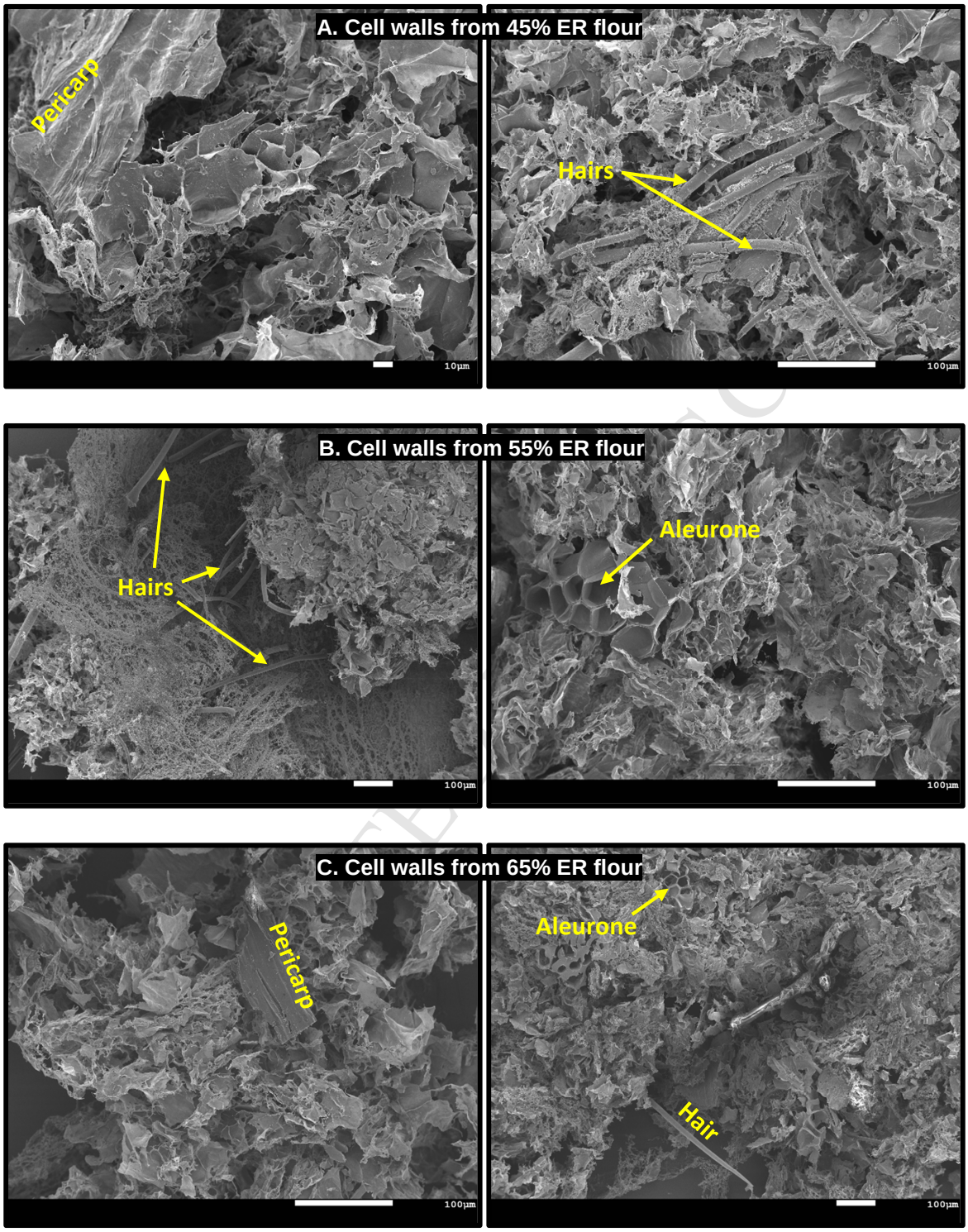
515 **Figure 4**



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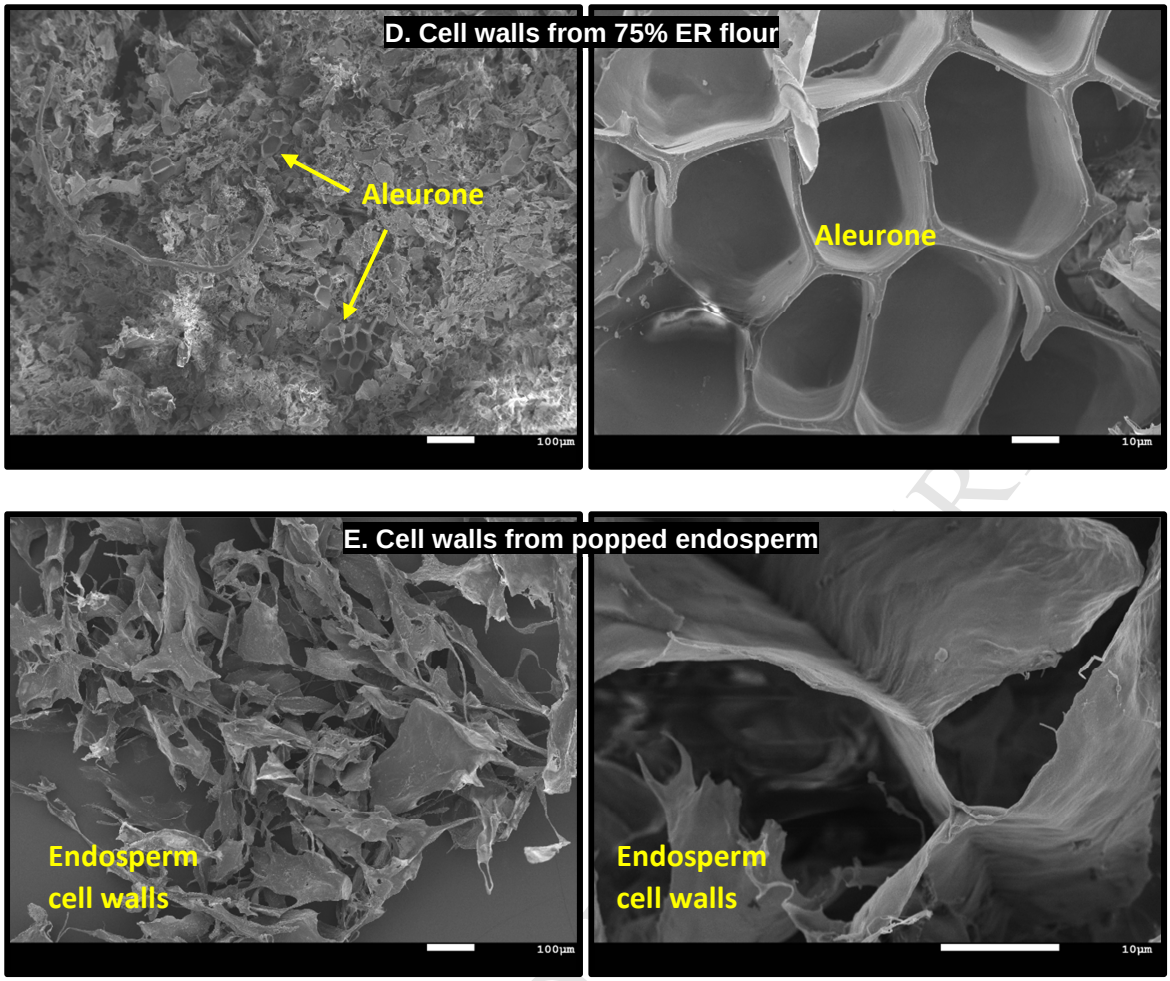

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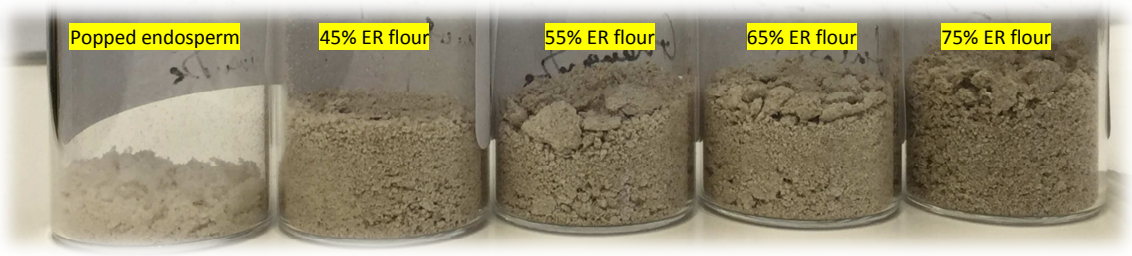
Figure 5



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557 **Graphical abstract**:

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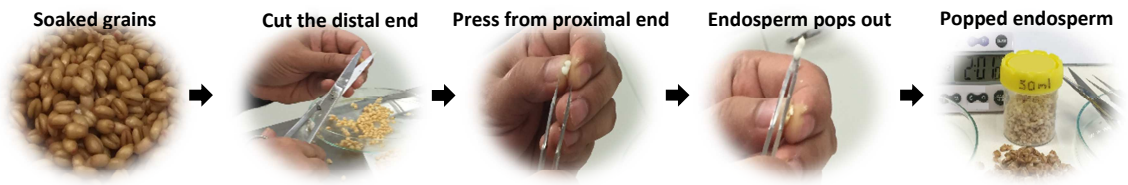

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Highlights:

- A novel method to isolate a pure form of endosperm cell walls is developed
- White flour is not free of non-endospermic tissues even at low extraction rates
- The isolation temperature affects the composition of cell walls
- Non-endosperm cell wall material in milled flours has a major influence on compositional analyses