July 2022

Project Report No. 641

Production of ferulic acid from wheat bran

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This is the final report of an 18-month project (21140013) that started in March 2015. The work was funded by a contract £235,500 from AHDB.

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1. Abstract

This project aimed to develop a scalable and efficient process to produce ferulic acid from wheat bran. A phenolic acid, ferulic acid has many industrial applications, including a precursor for the synthesis of natural biovanillin through fermentation, and an active agent in sun creams and anti-ageing skin care products, due to its high antioxidant activity and UV absorbing capacity. It also has several potential applications in the flavour/aroma, pharmaceutical and chemical industries, as a precursor for the synthesis of various aromatic molecules, through processes based on synthetic chemistry or microbial and enzyme transformations.

Ferulic acid is currently produced commercially from rice bran pitch, the waste stream generated during the production of rice bran oil. Due to the increased commercial applications of ferulic acid, the milling industry is interested in obtaining ferulic acid from alternative sustainable sources. The fact that wheat bran contains high amounts of ferulic acid (5–15 g/kg), which can potentially be obtained from a simpler fractionation process compared to corn bran, makes this abundant material a potentially good source of ferulic acid.

This project addressed limited knowledge: (i) on the ferulic acid content of various wheat cultivars from various locations in the UK, (ii) on the effect of mechanical processing of wheat grains, such as conventional milling and debranning (pearling), on the ferulic acid content of wheat bran, (iii) on the development of a scalable bioconversion process, based on the enzymatic deconstruction of wheat bran using a mixture of hydrolases, capable of releasing the ferulic acid linked to the arabinoxylans of wheat bran and (iv) on the cost and scalability of such a process.

We determined the ferulic acid contents in various bran fractions from two commercial wheat mills that do not currently use these bran fractions as a source of ferulic acid or of other high value products. We showed that the yield of ferulic acid is particle-size dependent and it is possible to increase the yield by reducing the particle size of the starting material. We compared the phenolic acid content to the rate of debranning and showed that the outer 5% of the wheat grain has the highest content of ferulic acid. There is significant potential to increase the ferulic acid yield, if the particle size of the highest yielding bran fraction is reduced, which may have implications for the cost-benefit analysis of ferulic acid extraction from waste bran fractions from wheat. An optimised process for the enzymatic deconstruction of wheat bran was developed and a ferulic acid production equal to that obtained by chemical methods was achieved. This research demonstrated that the development of a sustainable process to produce natural ferulic acid from wheat bran has significant potential. The purity of the ferulic acid produced was significantly improved through scalable and cost-effective approaches, although further research will be needed to ensure complete removal of impurities.

2. Introduction

Ferulic acid (4-hydroxy-3-methoxy-cinammic acid) (Figure 1) is one of the most abundant phenolic acids in plants, especially cereals and vegetables. Industrially, ferulic acid is produced in Japan from rice bran pitch, a blackish waste oil generated during the production of rice bran oil, which contains around 10-20 g/kg of ferulic acid (Rose *et al*., 2010). In the process, rice bran pitch is hydrolysed by incubating it at ~ 100 ^{o}C under alkaline conditions (pH 10, with sodium hydroxide) for about 8 hours, resulting in the production of ferulic acid, with a degree of purity between 70 – 90 %. For obtaining the ferulic acid, the solution is then precipitated by acidification (Taniguchi *et al*., 2005). Ferulic acid could also be purified from other abundant raw materials that contain high amounts, such as corn bran (25-30 g/kg), sugar beet pulp (~8 g/kg) and wheat bran (5-15 g/kg) (Ou and Kwok, 2004; Anson *et al*., 2012). The advantage of wheat bran over corn bran is that the former is a simpler raw material that can be more easily degraded, especially enzymatically, liberating ferulic acid (Rose *et al*., 2010; Shin *et al*., 2006).

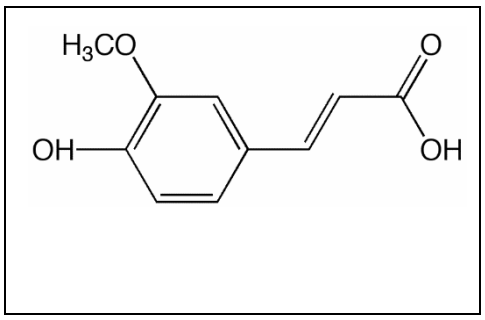

Figure 1: Structure of ferulic acid

Research carried out at Rothamsted as part of the EU FP6 HEALTHGRAIN project demonstrated a wide range of levels of phenolic acids, of which ferulic acid makes up around 80%, depending on the wheat variety (26 genotypes grown) as well as some effect of the environment (four European countries for up to 3 years) upon phenolic acid content (Fernandez-Orozco *et al*., 2010). An analysis of the most common wheat varieties currently used in the UK is, therefore, important in order to obtain a better understanding of the potential of these grains for producing ferulic acid. We, therefore, analysed grain from current wheat cultivars from various locations in the UK for this project.

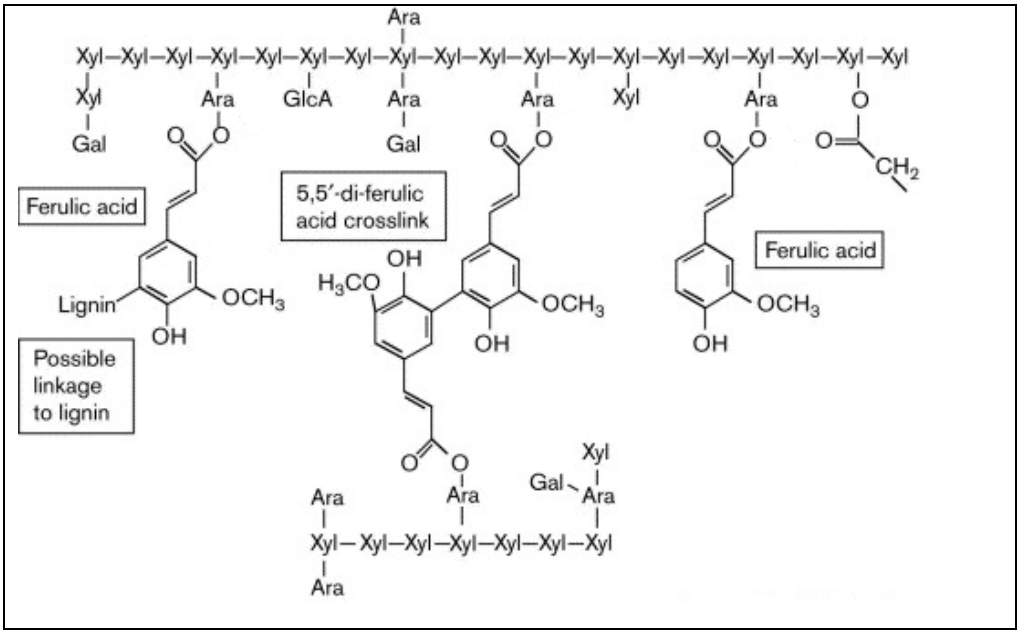

Figure 2: A theoretical feruloylated cereal arabinoxylan. Ara, arabinose; Gal, galactose; GlcA, glucuronic acid; Xyl, xylose (from Walton *et al*., 2000).

It is also necessary to understand the effect of the mechanical processing of the wheat grains, in particular, conventional milling and pearling on the ferulic acid content of the produced wheat. Recent analyses at Rothamsted of milling fractions from wheat *cv* Hereward showed the presence of 3 g/kg of ferulic acid in the bran (based on conventional alkaline hydrolysis), while previous analyses of whole grain from the same cultivar showed that about 12% of the total ferulic acid is present as dehydrodimers (Shewry *et al*., 2012)). However, there is currently little information on the ferulic acid content of bran fractions from several tail-ends from conventional roller milling, consisting of break and reduction systems (personal communication, Peter Skeggs, Premier Foods). Pearling is a debranning method which is based on abrasion, and is often used by millers prior to milling, with the view to decrease microbial contamination and to increase the quality of the flour. It has been suggested that up to 25%-30% of the wheat grains in the UK are pearled prior to milling (personal communication, Martin Savage, NABIM/UK Flour Millers). Previous research has shown that pearling fractions corresponding to about 5 and 10% of the wheat kernel have the highest antioxidant activity and the highest total phenolic acid content (Beta *et al*., 2005). This is most likely due to the fact that such fractions would include a significant proportion of the aleurone layer, which is very rich in ferulic acid. Preliminary pearling experiments have also shown that it is possible to remove mineral-rich outer layers from the grain for analysis (Figure 3), although from this preliminary analysis, it seemed that the content of the pearled fractions had lower ferulic acid content than wheat bran from conventional milling. The above preliminary analyses demonstrate the potential of using wheat bran as a feedstock for ferulic acid production, and the importance of defining the most appropriate starting material from either conventional milling or debranning, in relation to the separation method used.

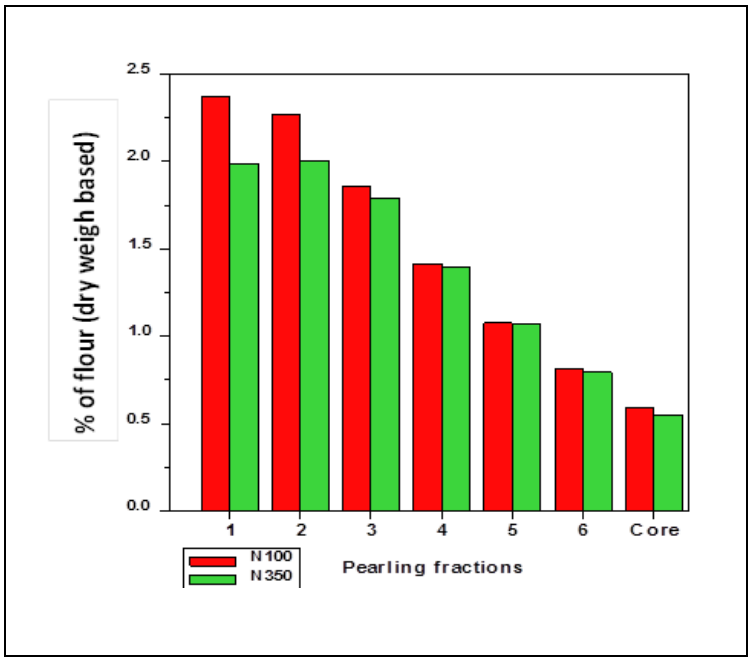

Figure 3: Total minerals content of 6 pearling fractions (accounting for 50% of the grain dry weight) from grain of Hereward grown at 100 and 350 kg N/Ha.

In this project, a process based on the enzymatic deconstruction of wheat bran followed by the separation of ferulic acid, will be developed. Most research up to now has focused on the enzymatic hydrolysis of wheat bran and corn bran, particularly the second, by endoxylanases (either research enzymes or commercial formulations) and feruloyl esterases (either research enzymes or commercial crude multi-enzyme preparations). These two types of enzymes are used in combination as they act synergistically, hydrolysing arabinoxylans to allow the breakdown of the feruloyl ester bonds. The feruloyl esterases are classified into four types, A, B, C and D, based on their substrate specificity. Types B and C only release ferulic acid monomers, whereas types A and D also release ferulic acid dimers (Fazary and Ju, 2007). Most of the published research has been conducted with the type A enzymes, most commonly produced from *Aspergillus niger* and *Humicola insulens*, which produce ferulic and diferulic acids (Bartolome *et al.,*1997; Faulds *et al*., 2004). In this project, two enzymes, provided by the industrial partner (Biocatalysts Ltd) will be used for the first time in combination targeting ferulic acid production from wheat bran. The first is a commercial endoxylanase (Depol 761P) and the second, a pure feruloyl esterase from *Talaromyces stipitatus*. The advantage of this enzyme mixture is that it is likely to result in a high yield of monomeric ferulic acid. Also, the fact that both of these enzymes are commercial, makes the potential commercialisation of such a process considerably easier. The advantage of an enzymatic method over a potential alkali treatment of wheat bran (a standard treatment process at laboratory scale) is that in the latter case, the environmental impact is higher due to the generation of large amount of hazardous waste. Moreover, it may be easier to purify ferulic acid from an

enzyme-based process as other interfering substances, such as proteins and lignins are not liberated, as would be the case following alkaline hydrolysis.

Of considerable importance for a process producing ferulic acid using as the primary step either an enzymatic- or an alkali-based method is the purification of ferulic acid from the hydrolysate mixture. This can be done either by solvent extraction (such as ethanol) or by selective absorption onto charcoal or polymeric resins. Although some work has been carried out with a range of agricultural waste materials (Tilay *et al*., 2008; Salgado *et al*., 2012), there has been little work carried out with wheat bran, and there is in particular, a lack of understanding of the effect of the primary extraction step (enzymatic or alkali hydrolysis), or the type of feedstock (milled or pearled fractions) may have on the efficiency of the purification process.

The aims of the project were to determine whether wheat bran, and more specifically, which specific fractions, are a good source of ferulic acid and to develop a scalable, efficient and cost-effective process for producing ferulic acid from commercially available, low value wheat bran fractions.

Objectives:

1. Determine the ferulic acid content of different wheat cultivars to identify possible trends in composition.
2. Evaluate, in lab-scale experiments, the effect of mechanical processing of wheat grains, i.e., conventional milling and debranning (pearling), on the ferulic acid content of in-process bran fractions. This will provide a fundamental understanding on the relationship between mechanical processing and the ferulic acid content of specific wheat bran fractions.
3. Generate data regarding the ferulic acid content of various bran fractions taken from industrial milling operations, including both conventional milling and debranning operations.
4. Develop a one-step process for the enzymatic deconstruction of wheat bran, using commercial endo-xylanases and feruloyl esterases, to avoid the use of high alkaline conditions, and ensure that the maximum amount of ferulic acid is extracted.
5. Develop a downstream process for the isolation of ferulic acid from the complex hydrolysates using either extraction with ethanol or absorption onto polymeric resins.
6. Develop process flow schemes for the production of ferulic acid from wheat bran and carry out a process cost analysis.

3. Materials and methods

3.1. Raw materials

Raw materials from two commercial mills (Hovis Manchester and Southampton), wheat bran and wheat flour samples were stored at -20°C before analysis for phenolic acids.

3.1.1. Sample milling

Fractions with the highest phenolic contents were additionally milled for 5 minutes using a Tema mill (Tema Machinery UK) to determine the effect of reducing the particle size on ferulic acid extractability. Samples were fractionated using a set of sieves (>250 µm, 250 – 150 µm and <150 µm) before and after Tema milling to determine the particle size distribution.

3.1.2. Sample pearling

Four wheat varieties (Cadenza, Hereward, Viscount, Yumai-34) were pearled using a AEVF (Streckel & Schrader KG, Germany) laboratory-scale pearling machine for different time intervals (up to 56s) depending on the wheat variety, to remove the outer layers accounting for 3, 5 and 10% of the grain weight.

3.2. Phenolic acid extraction

Phenolic acids were extracted according to Li *et al*. (2008) with some modifications.

3.2.1. Internal standard preparation

3,5-Dichloro-4-hydroxybenzoic acid (Aldrich) was used as internal standard (IS). Solution A consisted of 1.5 $mg.mL^{-1}$ concentration of IS dissolved in 80% ethanol (v/v). Internal standard solution B was prepared by diluting 20 times solution A.

3.2.2. Sample preparation and washing

20 ± 0.2 mg of sample were weighed into 2 mL SafeLock Eppendorf tubes in triplicate. Five microliters of internal standard (solution B) were added to samples analysed for free and conjugated phenolic acids. One millilitre of 80% ethanol (v/v) was added to the samples and vortexed thoroughly. Samples were sonicated for 10 minutes, heated at 80°C for 15 min and centrifuged at 5,000 x g for 15 min and the supernatants were collected into new Eppendorf tubes. Ethanol washing was repeated twice, omitting the sonication and heating steps. All supernatants of the same sample were combined into the same Eppendorf tubes and dried in vacuo. These were then used to determine the free and conjugated phenolic acids contents.

3.2.3. Sample saponification

Free and conjugated phenolic acids

400 µL of 2M NaOH were added to the free and conjugated phenolic fractions (combined supernatants of ethanol washes), vortexed thoroughly, and shaken in the dark for 4 hours. 125 µL of concentrated HCl were then used to acidify the sample to pH 2; the samples were vortexed, and the pH checked to ensure that it was below 2 using a pH indicator.

Bound phenolic acids

20 µL of IS (solution A) were added to the wet pellet (following removal of free and conjugated phenolic acids) together with 800 µL of 2M NaOH; the samples were vortexed thoroughly and shaken in the dark for 16-18 hours. The samples were then centrifuged at 5,000 x g for 15 min, the supernatants transferred into fresh Eppendorf tubes, and 220 µL of 12M HCl were used to acidify the samples to pH 2; the samples were then vortexed and checked for their pH.

3.2.4. Phenolic acid recovery

800 µL of ethyl acetate were added to each sample, vortexed thoroughly and centrifuged at 16,100 x g for 5 min. 600 µL of the upper layer were carefully removed into new Eppendorf tubes and dried under vacuum. Ethyl acetate extraction was repeated twice more removing 700 and 800 µL of the upper layer, respectively. The upper layers of the same samples were combined into a single tube and dried in vacuo. Samples were stored at -20°C until analysed by HPLC.

3.3. Phenolic acid analysis

3.3.1. Sample preparation

The phenolic acid extracts from wheat flour were suspended in 100 µL of 2% acetic acid (Fisher Scientific) and those from wheat brans in 1mL of 50% methanol (Sigma) with 2% acetic acid. To dissolve the samples, they were vortexed thoroughly, left for an hour at room temperature, sonicated for 10 min and vortexed thoroughly again. Finally, the samples were centrifuged at 16,100 x g for 5 min and the supernatants transferred into low-volume inserts in HPLC vials.

3.3.2. HPLC conditions

20 µL of extract were injected onto a pre-equilibrated Kinetex 5µm Phenyl-Hexyl column (150 x 4.6 mm, 2.6 µm particle size; Phenomenex) and analysed using a Shimadzu HPLC (Kyoto, Japan) using solvents: A = 100% acetonitrile, B = 2% acetic acid. Starting conditions were 100% B, followed by linear decrease of solvent B down to 30% at 12 min; kept isocratic till 14 min and returned back to 100% B at 14.1 min, staying isocratic until the end of the run. Total run time was 18 min with 2 $mL.min^{-1}$ flow rate. The column compartment was heated to 40°C. A Photodiode Array Detector (PDA) detector was used to record signals at between 280 and 320 nm. Two calibration curves were prepared for trans ferulic acid (FA) and *p*-coumaric acid (*p*CA) in the following ranges: FA in 1 – 10 µg and *p*CA in 0.01 – 0.2 µg; and FA in 10 – 150 µg and *p*CA in 0.01 – 1 µg to ensure accurate quantification of free, conjugated and bound FA and *p*CA.

3.2. Enzymatic extraction of ferulic acid

3.2.1. Enzymes

The enzymes used in this project were provided by Biocatalysts (Cardiff, UK). Ferulic acid esterase (Depol 740L) was produced from *Humicola insolens* and standardised on its ferulic acid esterase activity (36 FAE U/g). Xylanase (D761P) from *Bacillus subtilis* had a standardised xylanase activity of 800 U/g.

3.2.2. Optimisation of enzymatic conditions

The enzymatic reaction was carried out in water, using a near-neutral pH (5.5-6.0) and a temperature of incubation equal to 55°C, which were identified as optimal for both xylanase and FAE. Different bran to water ratios were tested initially in order to identify the substrate amount (wheat bran) that allowed for proper mixing of the solution (10% w/v). The enzymes were tested at different combined concentrations ranging from 0.2 to 8.6 U/g of bran for FAE and from 2.2 to 22 U/g of bran for xylanase. The reaction was carried out over a wide range of incubation times, ranging between 2 to 24 h, in order to identify the exact time at which the synergistic effect of the enzymes allowed the maximum release of ferulic acid. The reaction was stopped by incubating the solution at 95°C for 5 min to deactivate the enzymes. The extract was centrifuged and filtered through a 0.20 μm syringe filter before HPLC analysis.

3.2.3. Ultrasound-assisted enzymatic extraction and scaling up

Ultrasound (US) was tested both as pre-treatment and simultaneously with the enzymatic extraction aiming at increasing the FA yield. The scaling up of the extraction process was also carried out (up to 1 L). In order to ensure the efficiency of the US treatment and a proper stirring of the bran sample in the solution, a reduction of the bran concentration from 10% to 4% was used in the extraction medium. The enzyme concentrations were modified accordingly in order to maintain the optimum bran to enzyme ratios that were previously identified. The US pre-treatment was applied at 30W and tested for 30 and 60 min, followed by non-US enzymatic extraction at 55°C. The simultaneous US-assisted enzymatic extraction was carried out by applying 10W over 4 hours and maintaining the temperature constant at 55°C. The reaction was stopped at 95°C (5 min) and the extract was recovered by vacuum filtration, filtered and used for HPLC analysis.

3.2.4. HPLC analysis of ferulic acid

An Agilent Technologies 1260 Infinity HPLC system, equipped with a Diode Array Detector (DAD) (Agilent Technologies LDA UK Limited, UK) was used for the detection of FA. Chromatographic separation was performed on a Zorbax Eclipse Plus C18 column (100 mm × 4.6 mm i.d., 3.5 μm particle size) (Agilent Technologies, UK). The mobile phase for separation of FA consisted of 1% (v/v) acetic acid (A) and 100% (v/v) acetonitrile (B). The initial mobile phase composition was set at

95% A and 5% B and the total run time was 44 minutes. The %B had a gradient increase from 5 to 15% over 0 to 20 min period, followed by an increase to 50% in 13 min time. B% was further increased to 70% in 4 min, until 100% phase B was reached at 40 min. Then, it stayed constant for 1 min to wash out the column and returned to the initial condition (A 95%, B 5%) at 41 min and held constant for 3 min. 10 µL of samples were injected and the solvent flow rate was 1 mL/min. The column temperature was set at 30°C and detection was carried out at between 280 and 320 nm. Identification and quantification of FA was carried out by comparison with commercial FA standard based on the retention time and absorbance spectra.

3.3. Isolation of ferulic acid and analysis of purity

3.3.1. Isolation by a polystyrene resin

A polystyrene resin Amberlite XAD16N (Sigma Aldrich, UK) was used to isolate FA from the enzymatic extract. The adsorption/desorption process was performed both in a batch with continuous stirring and in a column packed with the resin. Different amounts of resin were tested, expressed as resin to FA ratios and ranging from 15:1 to 800:1 w/w. The elution was carried out using 30 mL of 96% ethanol heated at 60°C per g of resin. The eluate was recovered, filtered and analysed by HPLC to estimate the FA recovery.

3.3.2. Analysis of protein and sugars

The enzymatic extract and the ethanolic eluate obtained after the desorption from the resin were analysed also for their protein and sugar content. Proteins were determined using the Bradford method. Briefly, 1.5 mL of Bradford reagent (Sigma Aldrich, UK) was mixed with 0.05 mL of sample and the absorbance was recorded at 595 nm after 5 min. Protein concentration was calculated based on a standard calibration curve using bovine serum albumin (BSA). Sugars analysis was carried out by HPLC equipped with a refractive index (RI) detector and an Aminex HPX-87H column (Biorad, UK) (9 µm, 300 x 7.8 mm). The mobile phase was 5 mM sulphuric acid and was used in an isocratic method running at 0.6 mL/min for 30 min. Samples obtained after enzymatic extraction were filtered and directly used for analysis, whereas the eluate samples were vacuum dried and suspended in water before filtration and HPLC analysis. The determination of sugar content was carried out by comparison with retention times and the spectra obtained from standards of glucose, arabinose and xylose.

3.3.3. NMR analysis

The isolated ferulic acid extract was analysed by nuclear magnetic resonance (NMR) using a 400 MHz high resolution spectrometer (Bruker Nanobay 400, Bruker Ltd, UK). ICON NMR 4.2 under TOPSPIN 2.4 was used for the acquisition and elaboration of spectra, performing a proton

detection (^{1}H) followed by water suppression. Samples obtained from the resin purification were concentrated by vacuum drying and dissolved in deuterated water prior to NMR analysis.

4. Results

Phenolic acid content of milling fractions

In-process samples were obtained from two commercial wheat mills (Hovis Manchester and Southampton) which use different debranning procedures (see Figure 4 for details). All fractions were ball-milled to ≥250 µm particle size and analysed for ferulic acid which is present in three fractions: as free acid, as soluble conjugates (to sugars, sterols, etc) and bound to cell wall polysaccharides (primarily arabinoxylans). All obtained data are summarised in Table 1 as the sum of free acid and soluble conjugates ('free and conjugated') and bound *p*-coumaric (pCA) and trans ferulic (FA) acid. In the following text, only the bound ferulic acid content is discussed as it comprises up to 93.4% (M1 sample) and 93.3% (S7 sample) of the total ferulic acid content.

Increasing the yield of phenolic acids

The two fractions with the highest bound ferulic acid yields from each mill (M1 and M9 from Manchester mill; S7 and S8 from Southampton mill) were further processed using a Tema mill (Te) to reduce their particle size. Figure 5 shows the particle size distribution before and after Tema milling. This shows an 8.5% increase in the proportion of the <150 µm fraction in sample M9 after Tema milling and a 14.5% increase in the proportion of the 150-250 µm fraction in sample S7. One fraction from each mill (M9 – 'K Sifter wheat feed'; S7 – 'Wheat feed ex Bran finisher 3') showed a significant increase in the yield of bound ferulic acid after particle size reduction, of 8% for M9 (t-test $p<0.05$) and of 16% for S7 ($p < 0.001$) (Figure 6 and Table 1).

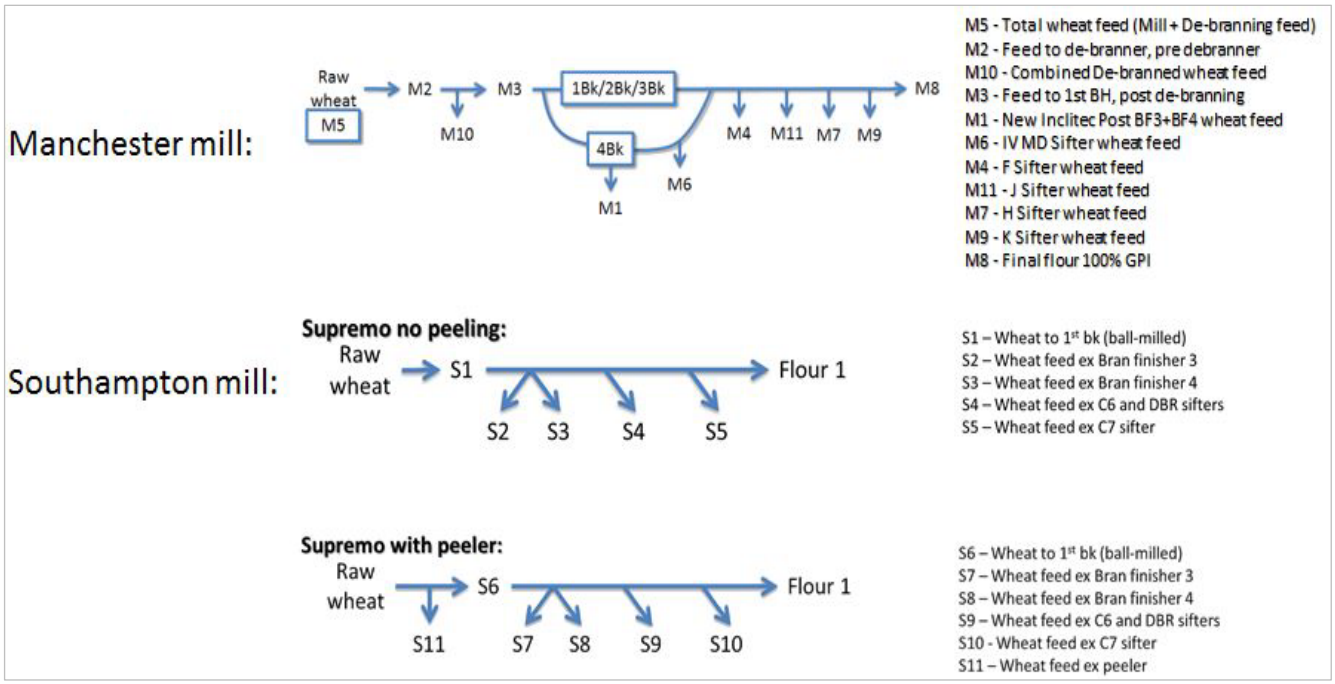

Figure 4: Scheme of milling procedures used in Manchester and Southampton mills.

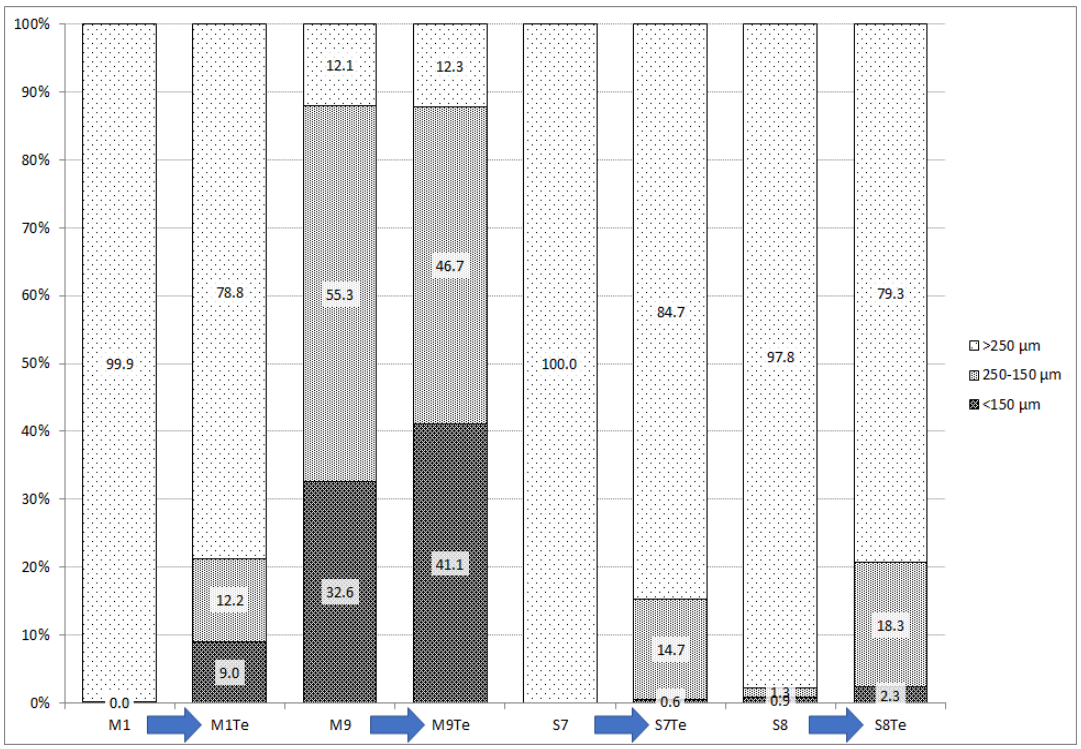

Figure 5: Particle size distribution before and after Tema milling.

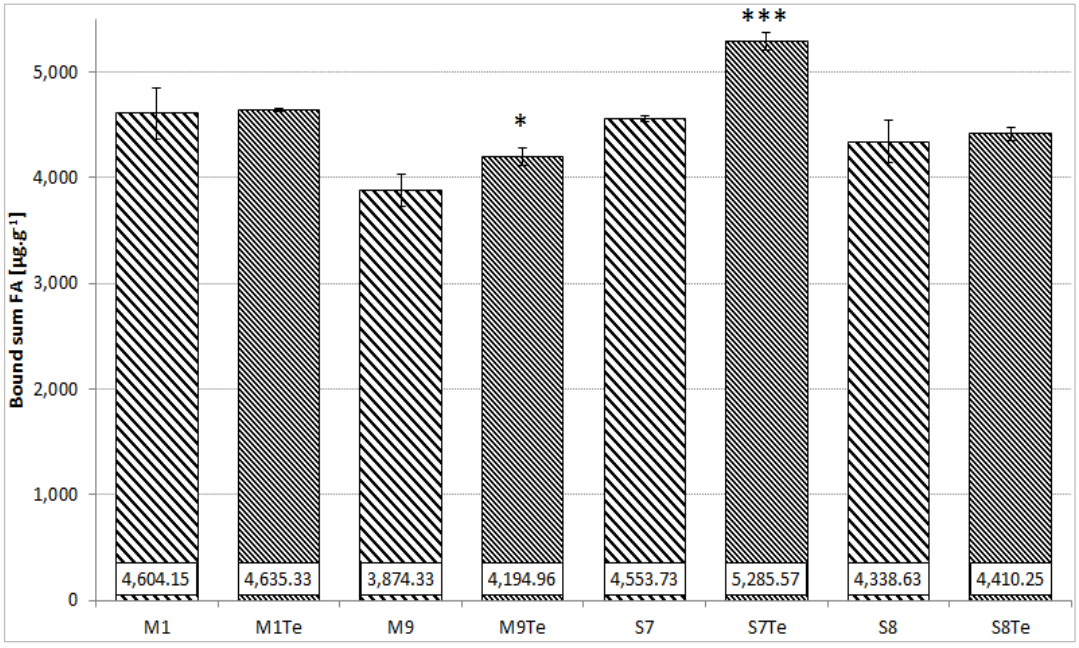

Figure 6: Effects of Tema milling on the yields of bound ferulic acid from the two fractions with the highest contents from each mill. The yields of ferulic acid from M9 (Te) and S7 (Te) were significantly higher after Tema milling due to the reduction of the particle sizes.

Table 1: Summary of 'free and conjugated' and 'bound' *p*-coumaric (pCA) and ferulic (FA) acid content in samples from Manchester (M1-10) and Southampton (S1-11) mill and Tema milled samples (M1Te, M9Te and S7Te, S8Te).

Fraction	*Free and conjugated*		*Bound*	
	*p*CA [µg/g]	tFA [µg/g]	*p*CA [µg/g]	tFA [µg/g]
M1	5.99 ± 0.13	323.20 ± 5.21	48.82 ± 4.22	4,604.15 ± 240.65
M1Te	4.75 ± 0.06	338.66 ± 9.46	51.69 ± 0.43	4,635.33 ± 14.26
M2	1.11 ± 0.10	136.20 ± 7.96	6.99 ± 0.50	783.42 ± 7.85
M3	0.95 ± 0.27	137.90 ± 3.35	3.96 ± 0.85	686.83 ± 15.27
M4	7.97 ± 0.85	474.41 ± 1.24	31.26 ± 2.10	3,014.59 ± 177.96
M5	7.46 ± 0.19	397.51 ± 24.34	44.90 ± 1.93	3,335.66 ± 21.27
M6	6.06 ± 0.86	288.37 ± 21.44	34.42 ± 2.55	3,068.20 ± 117.44
M7	5.92 ± 1.35	374.45 ± 22.05	31.48 ± 1.45	3,279.37 ± 85.18
M8	-	91.27 ± 3.12	-	151.61 ± 5.2
M9	7.77 ± 1.06	428.94 ± 7.41	39.18 ± 0.76	3,874.33 ± 153.16
M9Te	4.42 ± 0.15	330.63 ± 3.80	42.20 ± 1.06	4,194.96 ± 79.58
M10	7.61 ± 1.65	290.75 ± 16.68	60.39 ± 4.54	3,429.01 ± 532.20
S1	1.69 ± 0.32	155.61 ± 12.47	9.92 ± 1.79	1,048.72 ± 53.24
S2	9.51 ± 2.13	317.05 ± 14.06	62.41 ± 3.29	4,103.58 ± 41.51
S3	10.79 ± 2.25	363.34 ± 39.60	52.63 ± 6.57	3,946.54 ± 188.11
S4	10.04 ± 0.93	470.74 ± 13.50	48.57 ± 1.40	3,688.84 ±121.94
S5	10.87 ± 2.49	460.66 ± 13.93	35.13 ± 2.75	2,994.48 ± 299.76
S6	1.32 ± 0.20	142.89 ± 9.78	7.51 ± 0.30	860.38 ± 4.59
S7	7.63 ± 1.22	325.48 ± 23.70	54.43 ±2.26	4,553.73 ± 25.39
S7Te	5.67 ± 0.01	309.88 ± 6.46	69.98 ± 1.44	5,285.57 ± 86.82
S8	9.31 ± 0.15	377.97 ± 30.64	50.11 ± 2.13	4,338.63 ± 202.24
S8Te	5.52 ± 0.06	341.38 ± 29.09	54.84 ± 1.11	4,410.25 ± 63.00
S9	10.92 ± 2.40	430.05 ± 47.22	44.11 ± 1.70	3,957.10 ± 167.37
S10	4.86 ± 0.64	288.54 ± 21.45	25.94 ±0.30	2,551.01 ± 212.43
S11	2.54 ± 0.60	151.68 ±11.28	124.06 ± 18.16	1,289.77 ± 104.92

Contents of phenolic acids in pearling fractions of four wheat cultivars

Fractions enriched in the grain outer layers were isolated from four wheat cultivars (Cadenza, Hereward, Viscount and Yumai-34) using a laboratory-scale pearling mill to remove layers corresponding to 3%, 5% and 10% of the grain dry weight, and their phenolic acid content determined. Small differences in phenolic acid content were observed between the four cultivars, although previous studies (Shewry *et al*., 2013) have shown that the phenolic acid content of wheat grain is strongly affected by the environment. The fractions produced by pearling to remove 5% of the grain dry weight had the highest concentrations of bound ferulic acid, between 840 – 1013.5 $\mu g.g^{-1}$. When only 3% of the grain weight was removed by pearling, the bound ferulic acid yield

was lower, between 771.5 – 957.3 μg.g^{-1}. When 10% of the grain weight was removed, the bound ferulic acid yield was found to be the lowest, only between 587.7 and 702.5 μg.g^{-1} confirming that the inner layers content less bound ferulic acid than the outer layers. Hence, there is a dilution effect on the concentration of ferulic acid per mass (g) as more inner layer material is removed by the pearling mill. Figure 7 shows the cumulative content of bound ferulic acid for each wheat variety tested. Based on these results we estimate that about 2% of the outer layers was removed by debranning in the Southampton mill and about 4% in the Manchester mill.

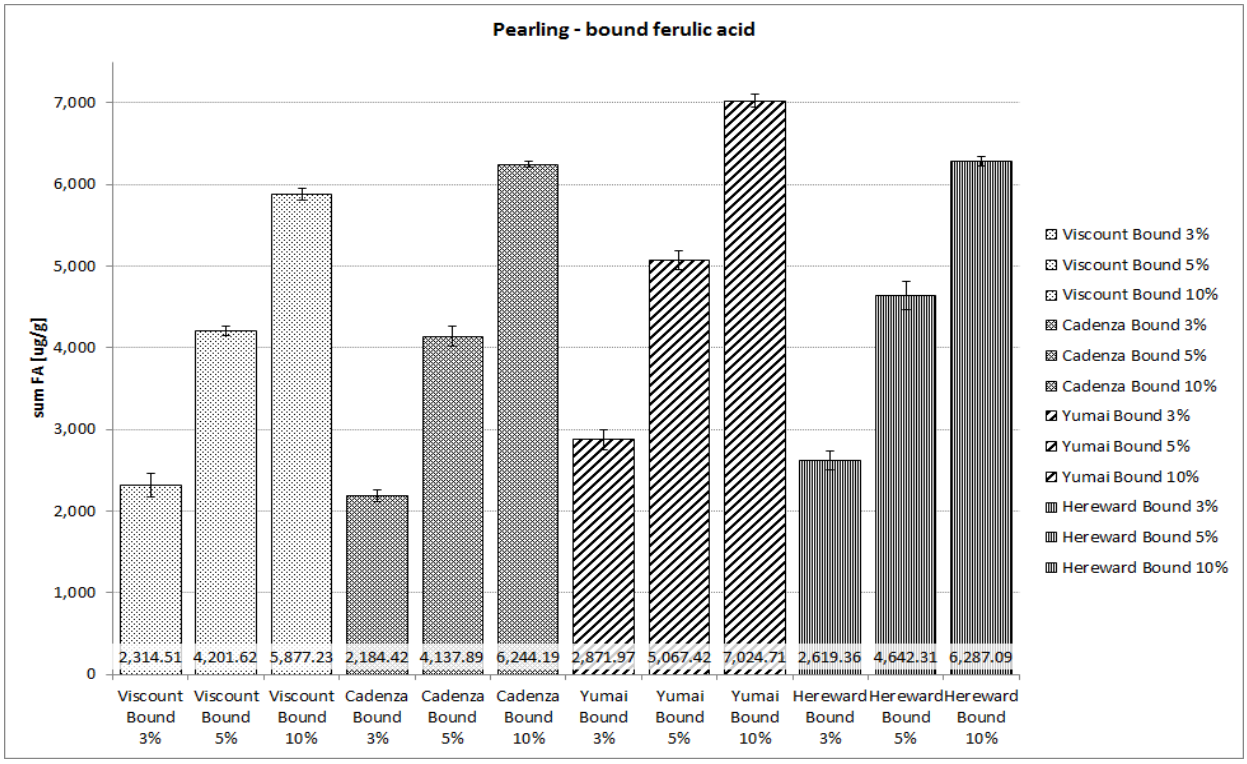

Figure 7: Cumulative content of bound ferulic acid in four wheat cultivars following laboratory pearling to remove 3, 5, and 10% of the grain.

By considering the ferulic acid content of the four different wheat cultivars and that of the Southampton and Manchester milling samples, it can be deduced that in this study, there were no significant findings of a direct relationship between wheat cultivar and ferulic acid content. On the contrary, the level of debranning, as well as the particle size reduction of the raw material (bran) seemed to play a significant role in ferulic acid yield.

Optimisation of the enzymatic conditions

The optimisation of the enzymatic process included the testing of different enzyme concentrations (xylanase 2.2-22 U/g, ferulic acid esterase (FAE) 0.2-8.6 U/g), temperature, pH, bran concentration and time of incubation (2-24 h). A wide range of ferulic acid yield was obtained using the different conditions as evidenced in the contour plot in Figure 8. Overall, considering the time of incubation, the maximum efficiency was identified between 4 and 8 hours, while longer incubations led to a

decrease of the ferulic acid concentration, probably due to oxidation and degradation of the compound. The maximum yield equal to 2.30 µg/mg of bran was obtained when using FAE and xylanase at the concentrations of 4.3 and 8.8 U per g of bran. Increasing the amount of the two enzymes did not lead to a significant higher production. Therefore, the optimum conditions were identified as: pH (5.5), enzyme concentrations (xylanase 8.8 U/g, ferulic acid esterase 4.3 U/g), temperature (55°C) and time (4h) and led to an extraction yield of 71.6% of the total FA content (2.30 µg/mg of bran).

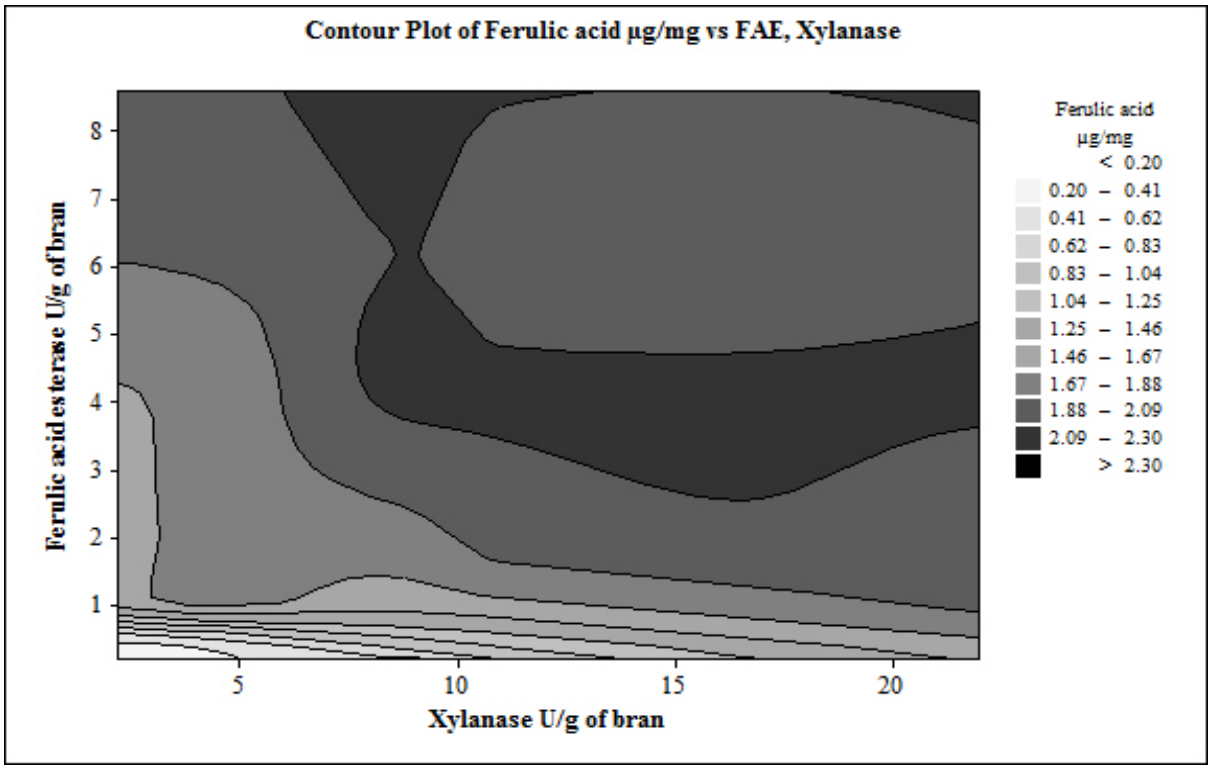

Figure 8. Contour plot of the ferulic acid amount obtained from the enzymatic hydrolysis using different concentrations of ferulic acid esterase and xylanase.

Ultrasound-assisted enzymatic extraction of ferulic acid

Ultrasound (US) was proven to efficiently aid the deconstruction of the wheat bran matrix and the release of ferulic acid. Results reported in Table 2 show that the use of US as pre-treatment of the bran (30-60 min) allowed a 10-18% increase of the extraction efficiency, but the highest productivity was observed when US was applied at low intensity (10 W) along with the enzyme (4 h), allowing a complete deconstruction of the bran matrix and a total recovery of 100% of the total ferulic acid content (~3.30 µg/mg of bran).

Table 2. Effect on the FA yield of the ultrasound as pre-treatment or simultaneously with enzymes.

TREATMENT	Time	FERULIC ACID YIELD %
Enzymes (control)	4 h	72.2%
30W US + enzymes	30 min US +4 h enzymes	82.3%
30W US + enzymes	60 min US + 4h enzymes	90.8%
10W US + enzymes	4 h (simultaneous US and enzyme treatment)	100.7%

Purification and isolation of ferulic acid

The purification and isolation of FA from the enzymatic extract was achieved by using a polystyrene resin (Amberlite XAD16N). A resin to FA ratio equal to 800:1 was found to be the most efficient one among various ratios tested and allowed the isolation and recovery of 92.1% of the ferulic acid present in the solution (Figure 9).

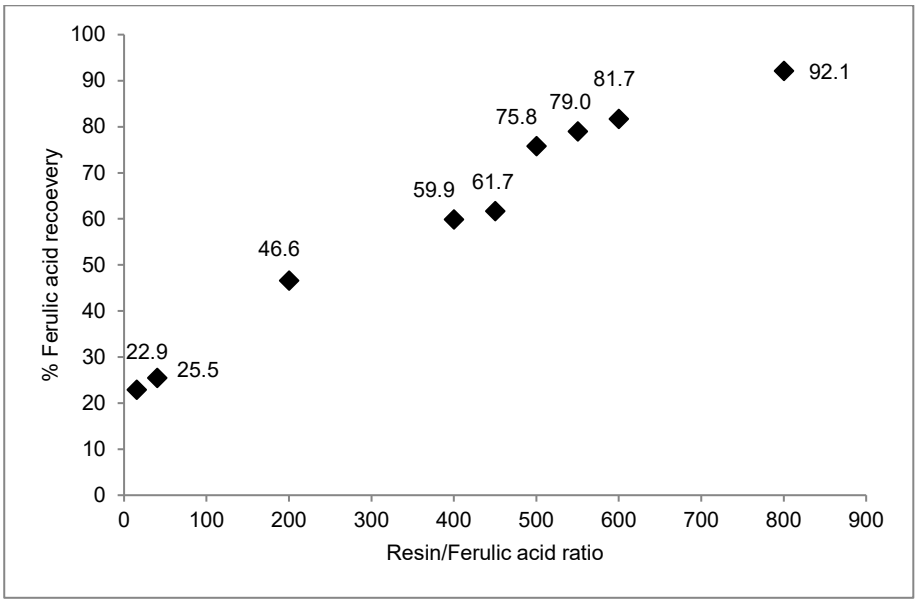

Figure 9. Percentage recovery of FA after the adsorption/desorption process as a function of different resin:ferulic acid ratios.

The enzymatic extract was also analysed for the presence of other components such as sugars and proteins. As shown in Table 3, the deconstruction of the bran matrix liberated high amounts of sugars, namely arabinose (104.9 µg/mg of bran), glucose (98.0 µg/mg of bran) and xylose (82.8 µg/mg of bran) along with some protein (13.0 µg/mg of bran). The adsorption/desorption process into the resin was proven to be an effective method for increasing the purity of the extract, with complete removal of sugars and a substantial decrease of the protein amount (Table 2).

Table 3. Composition of the extract and eluate obtained after the adsorption/desorption process.

	Ferulic acid	Glucose	Xylose	Arabinose	Protein
Extract composition µg/mg	3.30	98.0	82.8	104.9	13.0
Recovery percentage					
Eluate after adsorption/desorption	92.1%	0.0%	0.0%	0.0%	23.8%

NMR analysis of the purified extract

The NMR analysis was performed to confirm the presence of ferulic acid in the purified extract and obtain information about the purity. Figure 10 shows the NMR spectra obtain from the commercial standard of ferulic acid and the purified enzymatic extract.

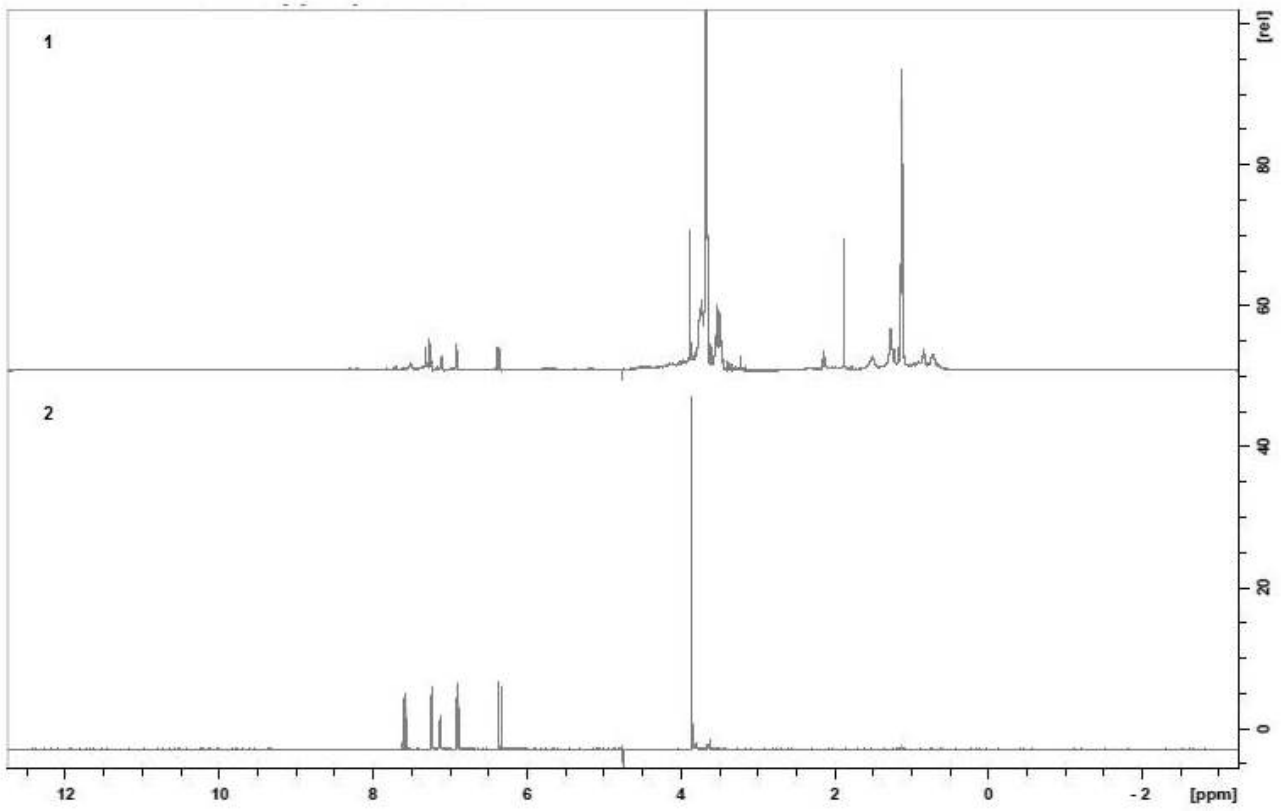

Figure 10. NMR spectra of the purified enzymatic extract (1) and ferulic acid commercial standard (2).

The ^{1}H-NMR spectrum of ferulic acid standard (Figure 10, 2) shows the characteristic signal for a methoxy group at δ 3.98 as a major peak. The compound spectrum also shows three aromatic protons between δ 6.97 and 7.09, characteristics for the H-6, H-5 and H-3 atoms of the aromatic part of FA. The presence of further two proton doublets representing the H-2' and H-1' in the side chain of the compound was also observed. The comparison of the standard spectrum with that obtained from the purified extract confirmed the presence of ferulic acid, as all the characteristic

peaks were detected in the sample (Figure 10, 1). However, the spectrum also showed the presence of several impurities which caused noise disturbance and did not allow a proper calculation of ferulic acid purity. Attempts of purity improvement were made using membrane ultrafiltration with molecular cut-off of 3 KDa (Vivaspin 500, GE Helathcare, UK), although high loss of ferulic acid was observed during the process. The removal of these impurities (most probably proteins) will require further investigation for further purification of the obtained extracts.

Process flow scheme for ferulic acid recovery and process cost

Based on the results of this study, a process flow scheme can be proposed, as depicted in Figure 11. The enzyme-assisted ultrasonication process is expected to recover the total amount of ferulic acid contained in the previously milled wheat bran. The absorption/desorption step was found to significantly purify the FA extract, through the removal of the sugars present in the extract. At this stage, the FA recovery is estimated at 92%, roughly around 3 kg of FA per ton of wheat bran. A nanofiltration step would be necessary in order to further purify FA, by removing any residual proteins.

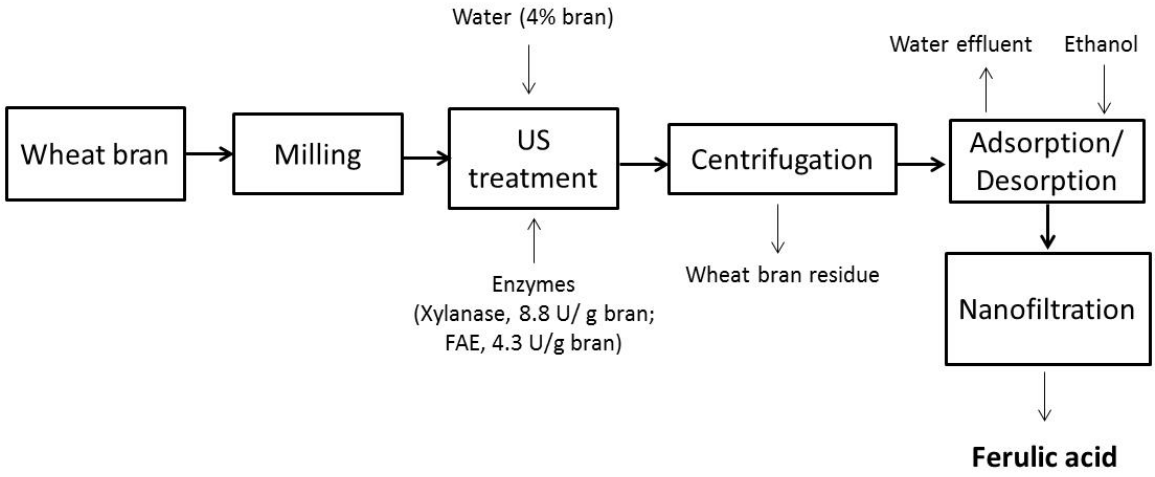

Figure 11. Proposed process flow scheme for the extraction and recovery of ferulic acid

However, further investigation is required in order to test the suitability of the nanofiltration step and be able to provide a full cost breakdown of the proposed process.

5. Discussion

Fractions from two commercial mills which use different debranning processes (Hovis Manchester and Southampton) were analysed for their contents in free, conjugated and bound ferulic acid. The two fractions from each mill with the highest content of ferulic acid were milled in a Tema mill to reduce their particle size, which resulted in significant increases in the yield of ferulic acid by 8% and 16%, respectively. Fractions produced by pearling to remove the outer part of the grain corresponding to 5% of the dry weight, gave higher yields of phenolic acids than fractions corresponding to 3% and 10% of the grain dry weight. We, therefore, suggest that the isolation of bran fractions corresponding to the outer 5% of the grain could provide an economically viable source of ferulic acid for commercial production. There were no associations shown between wheat cultivar and ferulic acid content.

Some of the investigated wheat bran fractions showed high potential as natural sources of ferulic acid and their bio-deconstruction was successfully achieved by using an ultrasound-assisted enzymatic extraction, based on the synergistic enzymatic activity of xylanase and ferulic acid esterase. Previous studies involving similar enzymes for the extraction of ferulic acid from different cereal sources (wheat bran, corn bran, barley spent grain) showed significantly lower percentage of recovery (50-70%) compared to the total 100% recovery achieved in this study (Bartolomé and Gomez-Cordovés, 1999; Faulds *et al*. 2003; Barberousse *et al*. 2009). The suggested optimised process for the enzymatic production of ferulic acid is an effective and sustainable alternative to the alkaline hydrolysis approach which involves the use of harsh chemicals. The purity of the obtained extract was highly improved by the adsorption/desorption process onto a polystyrene resin, which can be easily scaled up and applied at industrial level and allowed the removal of the sugars and most of the proteins. However, depending on the final use, further purification procedures maybe needed to allow the commercial use of the enzymatically produced ferulic acid, particularly for high value applications.

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