PhD Summary Report No. 5

November 2008

Project No. RD-2003-2975

Effect of processing on the nutritional value of oats

Laura Stenhouse (nee Wylie)

January 2005 - January 2008

Nottingham University, School of Biosciences

Sutton Bonington Campus

Loughborough EL12 5RD

ABSTRACT

The consumption of cereals has been associated with a reduced risk of several chronic diseases such as cancer and cardiovascular diseases such as heart attacks and strokes. The reason for these protective effects is not clear but could be related to the high antioxidant content of these cereals which is primarily dictated by their content of phenolics and various forms of vitamin E or E-vitamers (tocols).

Cereals are most often consumed in the processed state and this processing is likely to have an impact on the content or solubility of these antioxidants. This project examined the effect of industrial processing of oats on their phytonutrients. Oat samples were collected from a commercial processor, extracted with methanol and the soluble antioxidant activity, phenolics content and tocol levels measured. The industrial processing of oat gains to oat flakes resulted in a significant reduction in extractable antioxidant capacity and the content of phenols and tocols. Further processing of the oat flakes was carried out by heating at 180^{o}C and the extractable antioxidant activity again measured. Following this "secondary processing", antioxidant capacity, phenols and tocols were all significantly increased. To determine whether or not these observations are significant in the diet, a gastrointestinal pH simulated digestion experiment was undertaken on oat grains, oat flakes and the heated oat flakes. The results from this *"in vitro"* digestion mirrored findings obtained in the methanolic extracts.

The project also examined the effect of this processing on the composition of the material solubilised from oat. Industrial processing, whilst reducing the total level of extractable phenolics, had very little effect on the actual composition of these phenolics. In contrast the secondary processing step resulted in a pronounced change in the phenolics profile of the extracts. Industrial and secondary processing both impacted on the types of E-vitamer that were being solubilised. It was interesting to note that in this case there seems to be an increase in the solubility of the α-tochopherol

form of vitamin E, the most nutritionally beneficial form of this vitamin and as such this observation may be of significance in terms of nutritional value. These studies have demonstrated the potential benefit of "secondary" processing in the release of antioxidants for the oat samples.

Whether or not this has any actual nutritional benefit remains to be tested. However, phenolics, as well as acting as antioxidants, may also stimulate (or suppress) genes thought to impact on human health. The last stage of the project thus examined the effect of extracts from oat grains, oat flakes and heated oat flakes on the expression of some genes thought to be involved in cardiovascular health. These preliminary results demonstrated an increase in the expression of endothelial nitrous oxide synthase (eNOS). Since the product of this enzyme NO is a vasodilator (acting to reduce blood pressure) this could be beneficial in protection against heart attacks and strokes. Other genes such as endothelin-1, GATA-2 and vascular endothelial growth factor were also effected and again some of these changes could result in improved cardiovascular health. However, these studies have been carried out in cell cultures of Human umbilical vein endothelial cells (HUVEC) and the significance if any for the Human remains to be elucidated.

INTRODUCTION

Consumer awareness of the role of nutrition in health promotion and disease prevention is on the increase. Epidemiological evidence has supported the hypothesis that consumption of whole grains is associated with a reduced incidence of several chronic diseases such as cardiovascular disease (Jacobs et al 1998; Lui et al 2000) diabetes (Fung et al 2002; Lui et al 2000a; Meyer et al 2000) and cancer (Chatenoud et al 1999). This is often attributed to their antioxidant content. The potential nutritional benefit of wholegrain food is not as well established as fruit and vegetables, and consumption of whole grain foods in several western countries is less that one serving per day (Lang and Jebb 2003). Whole grains, agreed upon by the American Association of Cereal Chemists (AACC) in 1999, are defined as "consisting of intact, ground, cracked or flaked caryopsis, whose principal anatomical components; the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact caryopsis." The beneficial health effects of whole grains are that they deliver a unique package such as vitamins, mineral, unsaturated fatty acids, pytosterols, lignans and especially many antioxidants such as phenolic acids, tocotrienols and tocopherols (vitamin E) (Slavin 2003; Marquart et al 2002; Miller et al 2000). Antioxidants are known to combat oxidative stress in the body by maintaining a balance between oxidants and antioxidants. This is important because oxidative stress must be minimised. Under severe oxidative stress, damage to important biomolecules such as DNA, lipids and proteins may occur resulting in disease states (Young and Woodside 2001).

However, in many circumstances, cereals are not eaten as the harvested seed. Cereals need to be processed, using methods that make them more "consumer friendly". Commercially cereals may be "extruded, puffed or flaked" in order to improve product quality (Slavin et al 2001). They are subject to certain industrial processes in order to inactivate enzymes such as lipases to ensure rancidity is avoided during storage. These industrial processes however, can separate the endosperm from the germ and the

bran, leaving many processed cereal products nutrient poor (Slavin 2003), losing a substantial amount of dietary fibre, vitamins, minerals essential fatty acids and also important disease preventing nutrients and phytochemicals.

Many anti-oxidative phenolic compounds in plant based foods are also most frequently found in the bound form, associated with cell wall polysaccharides (Adom and Lui 2002). Different stages of processing may have a detrimental or beneficial effect on the capacity and bioavailability of antioxidants in the cereal. Although desirable nutrients such as antioxidants may be lost, there is potential for processing to enhance the desirability of cereals by increasing the availability of antioxidants, by liberating bound forms, especially the phenolics. This may increase the nutritional benefit of oat products.

Evidence is accumulating to suggest that phenolic components of the diet, as well as providing antioxidant activity, may have more direct nutritional benefits by being able to alter the expression of genes implicated in diseases such as cancer and cardiovascular diseases. In the case of cardiovascular health control of blood pressure is one major aspect. The cells lining the blood vessels (endothelial cells) are known to secrete factors which have either vasodilatory (relaxing) or vasoconstricting (contracting) effects on the smooth muscles that control the diameter of the blood vessels. In this study expression of the endothelial nitrous oxide synthase (eNOS) gene has been investigated since the product of this enzyme activity- NO- is a potent vasodilator (Porter et al, 2004). The expression of the gene for a major vasoconstrictor- Endothelin-1- (Porter et al, 2004) has also been investigated along with GATA-2 a transcription factor implicated in controlling the expression of this gene (Tsai et al. 1994).

The final gene investigated was vascular endothelial growth factor (VEGF) this is secreted by the endothelium and it is a major regulator of both physiological and pathological angiogenesis (Ferrara, 1991). VEGF thus plays a central role in the onset and progression of thrombosis and

atherosclerosis (Oak, 2003). VEGF is also produced by several tumour cell types and plays a role in the growth and metastasis of cancer through the promotion of neovascularization (Kim et al., 1993).

The hypothesis to be tested in this project was that whilst industrial and other processing (domestic heating to form biscuits and flapjacks for instance) is thought to result in a loss of antioxidants the reverse may be true for nutritionally beneficial compounds such as the phenolics which may be released from their bound form within the oat by such processing and as such become more available for digestion. Preliminary studies have also been carried out to determine whether the phenolics found in oats could have any beneficial; effect on the expression of genes implicated in cardiovascular health.

MATERIALS AND METHODS

Chemicals

The compounds 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu phenol reagent, 1,4 – Dioxan (purity grade ≥ 97%) were all purchased from Sigma Aldrich Chemical Co. (Poole, Dorset, UK). Fluorescein, sodium carbonate, sodium hydroxide, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate and all solvents were purchased from Fisher Scientific (Loughborough, UK).

Materials

Oat samples were supplied by Quaker Oats Industrial Mill (Fife, Uk). Oats were collected during one production day at the industrial plant. Raw oat Grains, milled oats, kilned oats, Steel cut oats and oat flakes were all produced at Quaker Oats which is referred to as primary processing. Secondary processing was undertaken at The University of Nottingham, whereby oat flakes were processed into heated oat flakes by taking 1g oat sample and baking at 180ºC in a preheated oven for 30 min prior to solvent extraction

Sample Preparation and Extraction

Oat samples were milled to a fine powder using a Braun coffee grinder. Oat samples (1g) were stirred continuously with 100% Methanol (50mL) for 1h at ambient temperature. Samples were centrifuged at 2000g for 5 min. The resulting supernatant was collected and used for total antioxidant capacity, total phenolics and total tocol analysis.

Determination of Total Antioxidant Capacity using Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC was determined according to the method of Huang *et al* (2002), in 75mM phosphate buffer (pH 7.4) at 37 ºC, fluorescein as the probe and AAPH as the radical generator. A FLUOstar Optima microplate reader (BMG Labtechnologies GmbH, Alyesbury, UK) equipped with FLUOstar OPTIMA evaluation software version 2.0 and black, polystyrene,

fluorescence sterile 96-well microplates (Nunc, supplied by Fisher Scientific, Loughborough, UK). The excitation and emission filters were 485 ± 20nm and 530 ± 25nm, respectively.

The final reaction mixture (225 µL) consisted of the extraction sample/trolox standard (25 µL), 100% methanol or phosphate buffer (75mM, pH 7.4), fluorescein (150 µL, 81.6nM) and AAPH (25 µL, 153mM). A standard curve was prepared using 6.25 – 50µM trolox (final concentration). A gain adjustment was performed on the plate before starting the program in order to optimise signal amplification. Fluorescence was determined and recorded every minute for 80 min. All measurements were expressed relative to the initial reading.
Raw data were exported from the Fluostar Optima Software to an excel (Microsoft) worksheet for further calculations. ORAC values are determined by calculating the net area under the curve (AUC) of the standards and samples. The AUC was calculated as:

$$\text{AUC} = 1 + \sum_{1}^{80} fn / f0$$

Where f_0 = initial fluorescence reading at 0 min and fn = fluorescence reading at time n. The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Final ORAC values are calculated using the regression equation between trolox concentration and the net AUC.

The results were calculated using the differences in area under the fluorescence decay curve (AUC) between the blank and the sample and expressed as trolox equivalents (TE) per 100 grams (µmols/100g) of sample.

Determination of Total Phenolics using Folin-Ciocalteu Assay

Total phenolic content was measured as gallic acid (GA) equivalents (mg/100g). One-millilitre samples were taken and Folin-Ciocalteu reagent (0.5mL) Na_2CO_3 (3mL, 200g L^{-1}) were added in the given order. The mixtures were vortexed and reaction allowed to proceed for 15 min at room temperature. The mixtures were then diluted with deionised water

(10mL) and centrifuged for 5 min at 2000g. Absorbance was measured at 725nm. Gallic Acid equivalents were determined from a standard concentration curve.

Identification of Vitamin E Tocols using High-Performance Liquid Chromatography (HPLC)

Extracts were used to determine concentration of tocols using high performance liquid chromatography (HPLC). Samples (20mL) were dried in a rotary evaporator and residues were redissolved in hexane (2mL) and filtered using a 0.3μM nylon syringe filter. Tocols were separated by HPLC on a Hyperclone C18 varian column at 25 ºC. Briefly, a 4.6 x 150 mm column was used with an isocratic Gilson HPLC, using 5% 1,4-Dioxan in hexane as the mobile phase. Flow rate was 1.5 mL min^{-1}, and peaks were detected fluorimetrically using an excitation wavelength of 294nm and emission wavelength of 326nm. Peaks were identified by retention time compared to tocopherol and tocotrienol standards. Total tocols are reported as the sum of α-tocopherol, β-tocopherol, α-tocotrienol and β-tocotrienol.

^{1}H NMR spectroscopy

Samples were prepared for ^{1}H NMR spectroscopy by drying under nitrogen for ca. 3 hours prior to addition of trimethylsilyl [2,2,3,3-$^{2}H_4$] propionate (TSP) in deuterium oxide (600 μL) where the deuterium oxide/TSP mixture was prepared by dissolving *ca.* 5 mg TSP in 10 mL deuterium oxide. All samples except HOF were used in their entirety for analysis. On the other hand, a 1/6 (v/v) dilution of sample HOF was made because the high turbidity of this sample hindered the acquisition of good quality spectra at higher concentrations. Spectra were obtained using a Bruker AVANCE 400 spectrometer operating at 400.13 MHz ^{1}H-resonance frequency and equipped with quadruple resonance (^{1}H, ^{13}C, ^{19}F, ^{31}P) probe with z-gradients. Spectra were measured at 298 K after solvent suppression of the residual HOD signal, achieved by use of a selective presaturation pulse sequence (Bruker Analytik, Rheinstetten, Germany), where secondary irradiation of the water resonance causes saturation. The pulse sequence comprises, RD-[90°-t1-90°-tm-90°-]acquire FID, where:

RD is a relaxation delay of 1.5 s, during which the water resonance is selectively irradiated; t1 is a very short delay, set to 3 □s; and tm has a value of 150 ms, during which the water resonance was again selectively irradiated. A total of 128 transients were collected into 64k data points with a spectral width of 8000 Hz. Prior to Fourier transformation (FT) the FIDs were multiplied by an exponential weighting function corresponding to 0.3 Hz line broadening and zero-filled by a factor of 2. Spectra were referenced to the primary internal chemical shift reference, TSP at δ 0.0 and manually baseline corrected using XWINNMR (version 2.1, *Bruker GmbH, Germany*

Culture of Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics as a frozen pooled 2mL sample. Cells were defrosted at 37ºC for 15min. Cells (2mL) were transferred to culture flasks containing 12mL EBM-2 medium (Cambrex Inc) and cultured at 37ºC and 5% CO_2 for 48h. Media was then discarded and replaced with fresh media. Once the surface of the flasks were at least 90% confluent with cells, HUVECs were harvested by removing media from each flask. HUVEC cultures were washed with 10mL phosphate buffer saline (PBS). The PBS was then discarded before a further 10mL of PBS and 0.25mL Trypsin-EDTA (TE) buffer was added to the flasks. Flasks were then incubated for 10min at 37ºC to allow detachment of cells from the flask surface. After 10min, the flask contents were pipetted up and down the surface of the flask to ensure all cells were detached. Flask contents, along with 11mL of EBM-2 medium was added to a falcon tube and centrifuged at 2500rpm for 10min at 4ºC. The supernatant was then removed and the cell pellet was resuspended in 10mL EBM-2 media and cell number was measured under a microscope, using a haemocytometer. Cell cultures were then diluted with the appropriate volume of EBM-2 media to provide 1,450,000 cells/mL.

Oat Grain, Oat Flake and Heated Oat Flake phenolic treatment of HUVECs for Gene Expression Studies

HUVECs used in this experiment were cultured and harvested as method described above. Harvested cell suspension (2mL) were added to six well plates and incubated for 24 h at 37ºC. Media was then removed before 1.75mL of each treatment was added. Treated plates were left to incubate for 24h (37ºC).

RNA was isolated from treated cells following the RNA extraction protocol supplied by Invitrogen Life Technologies. Cells were lysed in TRIZOL® Reagent by repetitive pipetting up and down the well surface. Homogenised samples were incubated at 20ºC for 5min. Chloroform (200µL/1mL TRIZOL® reagent) was added to the homogenate and mixed by inversion then incubated at 20ºC for 3min. Tubes were centrifuged at 13,000rpm for 15min at 4ºC. Following centrifugation the top aqueous phase was conveyed into a fresh microcentrifuge and isopropyl alcohol (0.5mL/1mL TRIZOL® Reagent) was added to each tube. Samples were incubated at 20ºC for 10min and then centrifuged at 13,000rpm for 10min at 4ºC. The supernatant was removed and the pellet was washed in 75% (v/v) ethanol (1mL/1mL TRIZOL® Reagent). Samples were stored at -80ºC.

Sample were defrosted and centrifuged at 13,000rpm for 15min at 4ºC. The ethanol was removed and samples were spun again for 3 mins. The remaining drops of ethanol were removed. The RNA pellet was left to dry for 5 mins and was then resuspended in 40µL RNase free water, 5µL DNase 10x buffer and 5µL of DNase. Samples were vortexed then centrifuged at 13,000rpm for 2min. RNA extracts were incubated at 37ºC for 30min. Each sample was mixed with 150µL of RNase-free water and 200µL of phenol:chloroform:isoamyl alcohol (25:24:1) and then centrifuged at 13,000rpm for 5min at 4ºC. The top aqueous phase was transferred to a sterile microcentrifuge tube and mixed with 15µL of sodium acetate (3M, pH 5.5) and 375µL of absolute ethanol and left to precipitate overnight at -80ºC. The precipitated RNA was centrifuged at 13,000rpm for 15min at 4ºC to form a pellet. The supernatant was

discarded and the pellet was washed in 1mL of 75% (v/v) ethanol. The pellet was mixed vigorously on a vortex and centrifuged again at 13,000rpm for 15min at 4ºC. The supernatant was removed and the pellet was left to dry for 5 min. RNase-free water (20µL) was used to resuspend the pellet before samples were stored at -80ºC.

Determination of RNA Yield and Purity

Total RNA yield was measured using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. Total RNA yield was determined by measuring the absorbance at 260nm. A ratio of the absorbance at 260/280nm was determined for each sample to determine the purity of RNA and values between 1.8 and 2.0 were generally accepted as 'pure' RNA for the purpose of reverse transcription PCR. Based on these values, each sample was appropriately diluted to a concentration of 50ng/µL with RNase-free water.

Determination of RNA Integrity

RNA integrity was assessed to determine its suitability for reverse transcription PCR. Electrophoresis of RNA through an agarose gel matrix stained with ethidium bromide usually confirms RNA integrity. Intact RNA should produce two bright predominant 28S and 18S rRNA (ribosomal RNA) bands. The ratio of the intensities of these two bands should be 2:1. A decrease in the intensity ratio or a low molecular weight smear indicates degraded RNA.

A 1% agarose gel was prepared by fully dissolving 1g of agarose in 100mL of 1 x TAE buffer (0.04M tris, 0.01M glacial acetic acid, 0.7mM EDTA pH 8.0) using a microwave oven. The dissolved agarose was poured into a gel mould and left to set at ambient temperature for ~ 1h. Five microlitres of each RNA sample was loaded onto the gel and run at 100V for 90 min in 1 x TAE buffer. The gel was stained in ethidium bromide (0.5µg/mL) for 20min, rinsed briefly in water and the gel image captured using GelDoc system and multi-analyst program (BioRad, Hemel Hempstead, UK).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA extracted from cells was transcribed into complementary first strand DNA (cDNA). The initial reaction involved annealing random hexadeoxynucleotide primers to the RNA. This process involved the addition of 1µL of random hexamer primers, 10µL of total RNA (50ng/µL) and RNase-free water to a final volume of 15µL into a sterile thin walled microcentrifuge tube. The mixtures were heated to 70ºC for 5 min. Extension of the annealed primers to form cDNA was then undertaken. This process involved the addition of 5µL MMLV (Moloney Murine Leukemia Virus) 5 x reverse transcriptase buffer, 1.25µL dNTP (10mM ATP, CTP, GTP , UTP), 0.5µL RNase inhibitor, 1µL MMLV reverse transcriptase and 2.25µL RNase –free water into each tube. Reactions were incubated at 42ºC for 1h. The synthesised single stranded cDNA was diluted by a factor of 4 to a final volume of 100µL and stored at -80ºC ready for use in real time PCR analysis.

Real-Time PCR Analysis

Real time quantitative PCR was carried out using the Roche LightCycler® 480 System whereby the quantification of expression of specific mRNA transcripts was determined. mRNA expression of GAPDH (Glyceraldehyde -3- Phosphate Dehydrogenase) was used as an internal standard. Quantification of transcripts was carried out using the relative standard curve method as described by Applied Biosystems (1997). An equal aliquot of undiluted cDNA from each sample was pooled together. This cDNA was serially diluted (neat, 1:2, 1:4, 1:8, 1:16, 1:24, 1:32, 1:64) to produce a set of standards. cDNA was diluted 5 fold to ensure samples fell within the standard curve range.

A mastermix was prepared for each of the sequences being studied. Mastermix solution contained 6.25µL of 2 x PCR mastermix, 0.625µL Gene expression assay (This contained forward primer, reverse primer and probes) and 3.125µL RNase-free water for each sample. The mixture was then vortexed to ensure vigorous mixing of all the constituents. Each well of 384 well plate contained 10µL mastermix, 5µL cDNA (sample/standard). Non-template controls were loaded in triplicate for

each primer set and were prepared by replacing the cDNA fraction of the PCR reaction with an equivalent volume of RNase-free water. The target genes for this reaction were endothelial nitric oxide synthase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF) and GATA-2 (ET-1 transcription factor).

Statistical Analysis

Data are reported as mean ± standard error of the mean (SEM), unless otherwise stated. The statistical analyses were performed using General Analysis of Variance (ANOVA) using Genstat for windows (version 7.2) (Hemel Hempstead, UK). Post-hoc Dunnets test were conducted to identify differences among means. The Pearson correlation coefficient (R) and P value was used to show correlations and their significance. Statistical significance was declared as $p<0.05$.

RESULTS

The first aim of this project was to assess the impact of processing on the nutritional value of oat grains. Samples were obtained from a commercial supplier these were taken at various stages of the process these being - Oat grains (OG), milled oats (MO), kilned oats (KO), steel cut oats (SCO) and oat flakes (OF). In addition the effect of secondary processing was also examined by taking oat flakes and heating these at 180^0C to mimic biscuit manufacture, these were termed heated oat flakes (HOF). All samples were extracted into methanol and tested for total antioxidant capacity (TAC), total phenolics content (TPC) and total E-vitamer content. The results are shown in Figures 1,2 and 3 for antioxidants, phenolics and E-vitamers, respectively. .

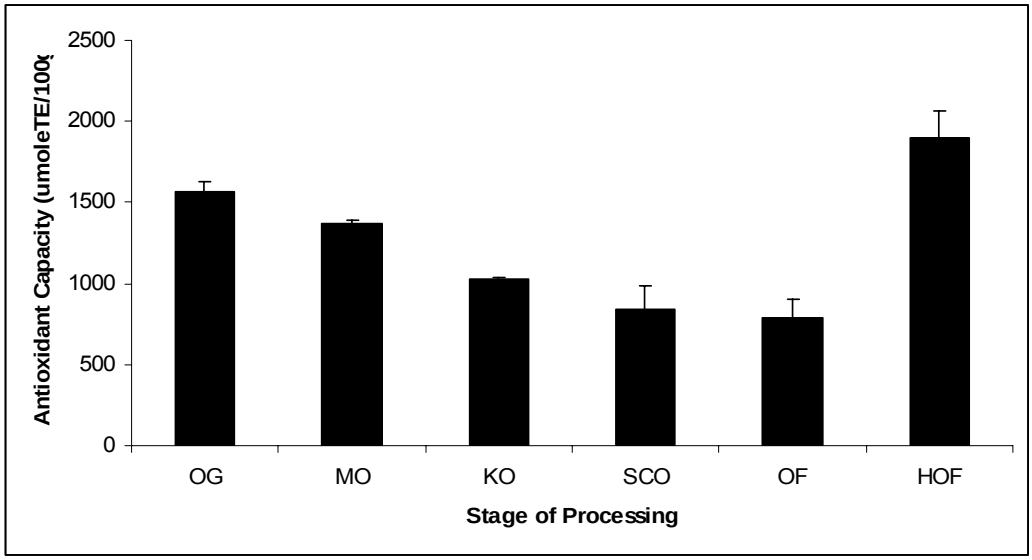

Figure 1 Effect of Primary and Secondary Processing on Total Antioxidant Capacity in Oats.

Primary processing resulted in a significant decrease in total antioxidant capacity when processed from OG (1611 ± 47 µmol TE/100g) to the final product OF (870 ± 21 µmol TE/100g) ($p<0.001$) (**Figure 1**). This represents a 54% reduction in TAC once oats have completed the primary process. When OF were secondary processed to HOF, a significant 84% increase in total soluble antioxidants was observed (1900 ± 84 µmol TE/100g) ($p<0.001$). Secondary processing also resulted in a significant

18% increase in total antioxidant when compared to the original raw product, OG ($p<0.01$).

Phenolics and vitamin E are likely to represent the major antioxidant components in the oat grain. Thus the impact of processing on these two groups of compounds was also assessed. The results for total extractable phenolics are shown in figure 2.

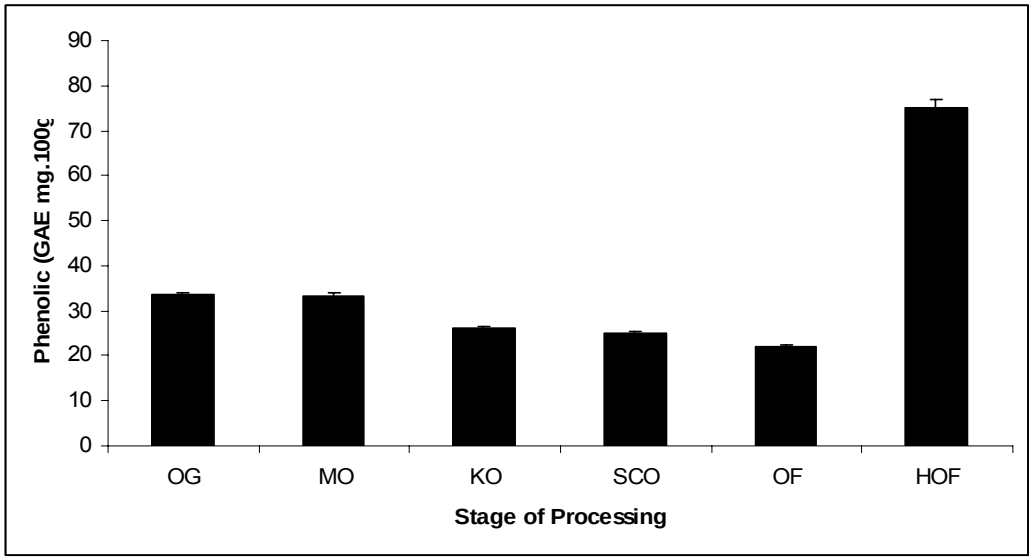

Figure 2 Effect of Primary and Secondary Processing on Total Phenolic Content in Oats

Total phenolic content was significantly reduced from the raw OG (33 ± 0.80 mg/100g) product to OF (26.9 ± 0.48 mg/100g) as oats progress down the production line ($p<0.001$) (**Figure 2**). This represents an 18% reduction in TPC. As oats were secondary processed to HOF, total phenolic content was significantly increased by 64% (75 ± 2.10 mg/100g) ($p<0.001$), again to a content significantly greater than the primary raw product ($p<0.001$).

The effect of processing on the extractability of the various E-vitamers is shown in figure 3.

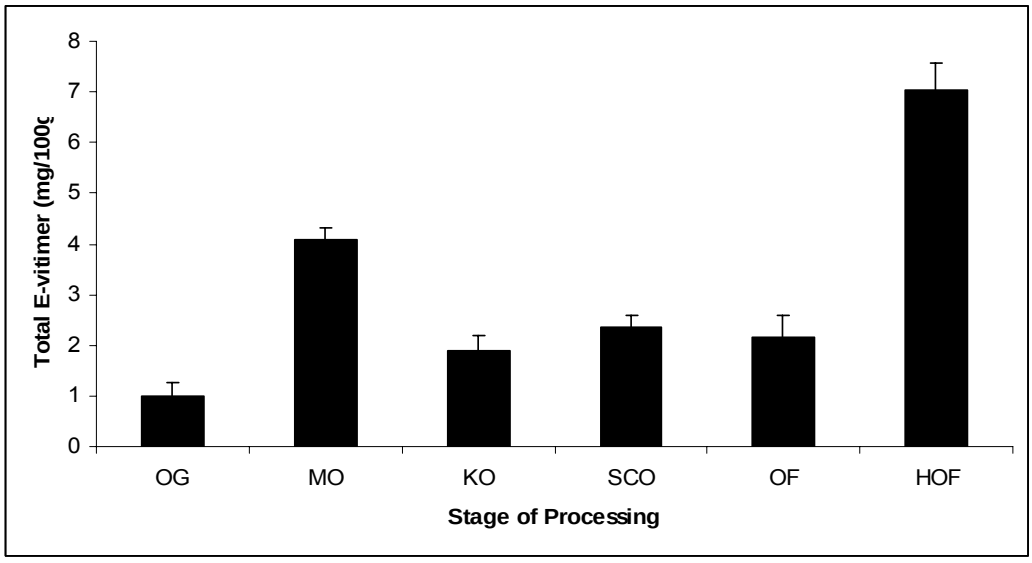

Figure 3 Effect of Primary and Secondary Processing on Total Vitamer-E Content in Oats

In this instance there was an increase in extractable tocols as oats were milled. This is likely to be the result of the fact that the vitamin E content of the husk and bran is relatively low. Thus when taking a one gram sample of milled oats a greater proportion of vitamin E rich germ is present than in a gram of oat grains. The results do indicate however, that there was a significant decrease in tocols from the MO to the OF stage of processing (4.081 ± 0.205 mg/100g) ($p<0.001$) (**Figure 3**). Once again secondary processing has significantly increased the tocol content from OF (1.182 ± 0.162 mg/100g) ($p<0.001$) to HOF (7.05 ± 0.53 mg/100g), resulting again in a greater content of tocols being extracted than from the primary raw product, OG ($p<0.001$).

It was clear from these results that that processing of oats has a significant effect on their extractable antioxidant composition and that this is mirrored by changes in the total content of phenolics and E-vitamers. The previous results measured total phenolics and E-vitamers but in each case this represents a complex mixture of individual chemicals. Since it is known that these can vary in their relative nutritional benefits it would be useful to examine the profile of these compounds in the oat extracts and

determine the impact, if any, on this profile of the various processing steps.

Phenolic compounds in plants are known to exist in either free (soluble) or bound (insoluble) forms. In the first instance the total (free and bound) phenolic composition of the oat flakes was determined. This was achieved by treating oat flakes with alkali to break the ester bonds responsible for binding the phenolics to other components of the plant cell (mainly sugars). These were then extracted into methanol and analysed by high performance chromatography (HPLC). The results are shown in figure 4.

This technique is only semi-quantitative and serves primarily as a means to determine the profile of the individual phenolics. The profile shows the presence of 16 clear peaks. Some of these have been tentatively identified by comparison with standards. For instance the major peak eluting with a retention time of 10 minutes is likely to be ferulic acid, a major component of plant cell walls. These phenols represent the total potentially available in the diet. However, to be absorbed by the body during digestion these must be "solubilised". Thus of more immediate nutritional significance are the soluble phenolics.

The total phenolic profile in figure 4 can thus be compared to that in figure 5 which represents the fraction of the total phenolic freely extractable into the methanolic extract. It can be noted by comparing the scales on the y-axes of figures 4 and 5 that, as expected, the "soluble" phenolics represent only a minor fraction (estimated at about 15-20%) of the total phenolics present in the oat flakes. That said the profiles appear to be very similar in that on a comparison by retention time there seem to be the same relative peaks in each case. The major difference is in their relative concentrations. It can be seen that the five major peaks eluting between 5 and 9 minutes are all of similar intensities in both traces. In comparison the two major peaks eluting between 10 and 12 minutes are greatly reduced.

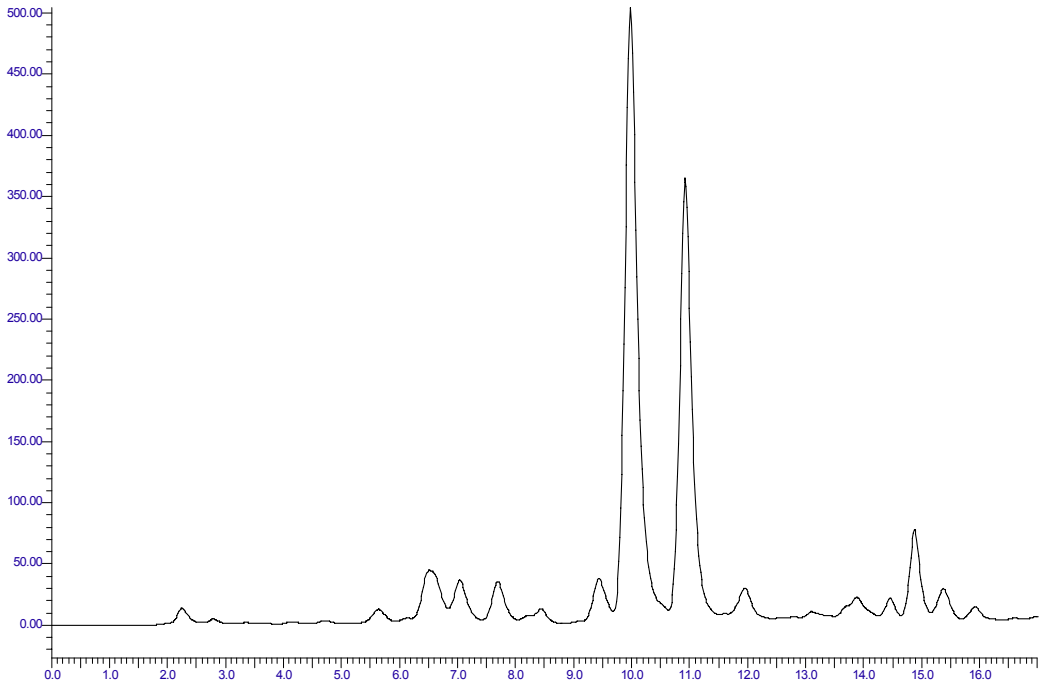

Figure 4. Profile of Total Phenolics extracted from Oat Flakes as determined by High Performance Liquid Chromatography (HPLC)

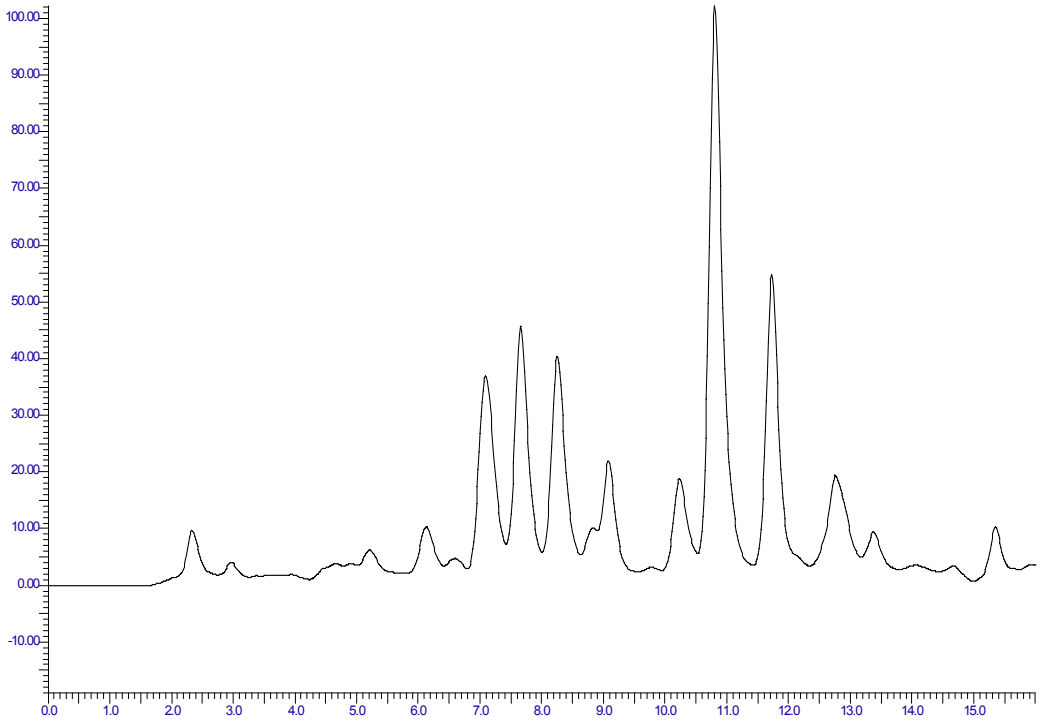

Figure 5 Profile of "soluble" phenolics from oat flakes as determined by High Performance Liquid Chromatography (HPLC)

The profile of the "soluble" phenolics extracted into methanol from heated oat flakes is shown in figure 6.

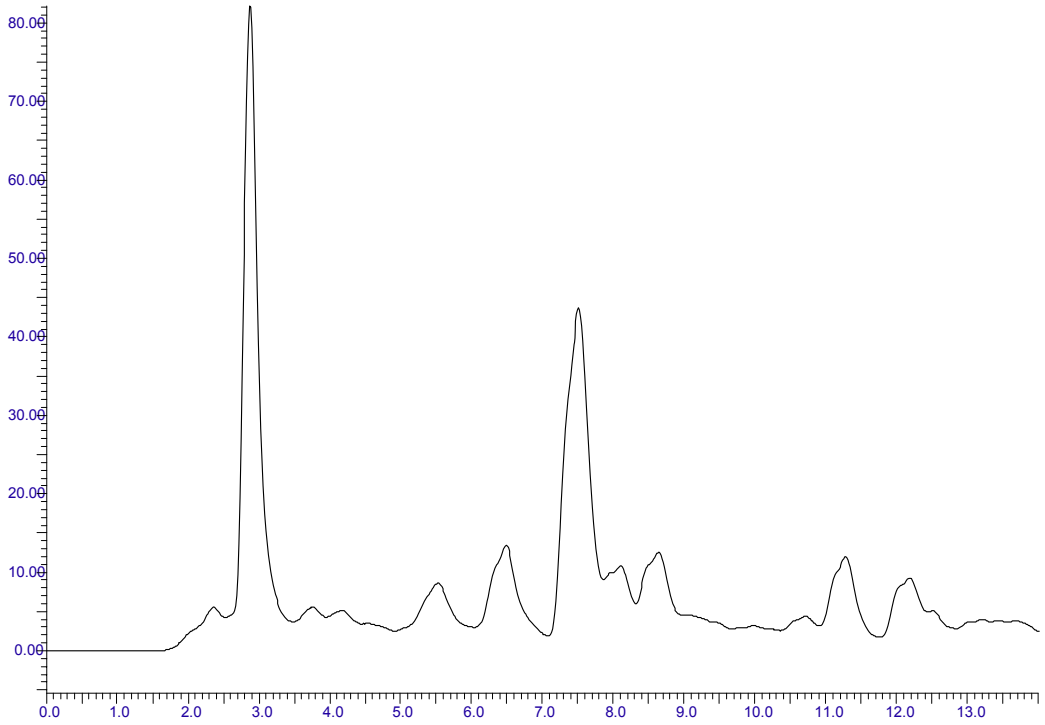

Figure 6 Profile of "soluble" phenolics from Heated Oat Flakes as determined by High Performance Liquid Chromatography (HPLC)

A comparison of figures 5 and 6 clearly shows that the heating has resulted in a major change to the observed phenol profile. The minor peak eluting at around 3 minutes in the extract from oat flakes has become the major peak in the extract from heated oat flakes. This is accompanied by a reduction in many of the other peaks in figure 5 with perhaps the exception of that eluting at around 7.5 minutes.

It was not possible, within the scope of this current project, to identify the actual compounds associated with the majority of the peaks in these HPLC traces. It is clear however, that the heating associated with the secondary processing is having a pronounced effect on the nature of the phenols that can be subsequently extracted from the oat flakes.

A similar study was undertaken to assess the effect of processing on the profile of extractable E-vitamers. In this instance the methanolic extract was again analysed by HPLC and the major E-vitamers identified and

quantified by comparison with suitable standards. The results are summarised in figure 7.

The extract from oat grains and milled oats contained four of the possible E-vitamers- α and β tochopherol and α and β tochotrienol. The loss of E-vitamer during primary processing to oat flakes was principally associated with the loss of α-tochotrienol and this was accompanied by the appearance of γ-tochopherol at the kilned oat stage.

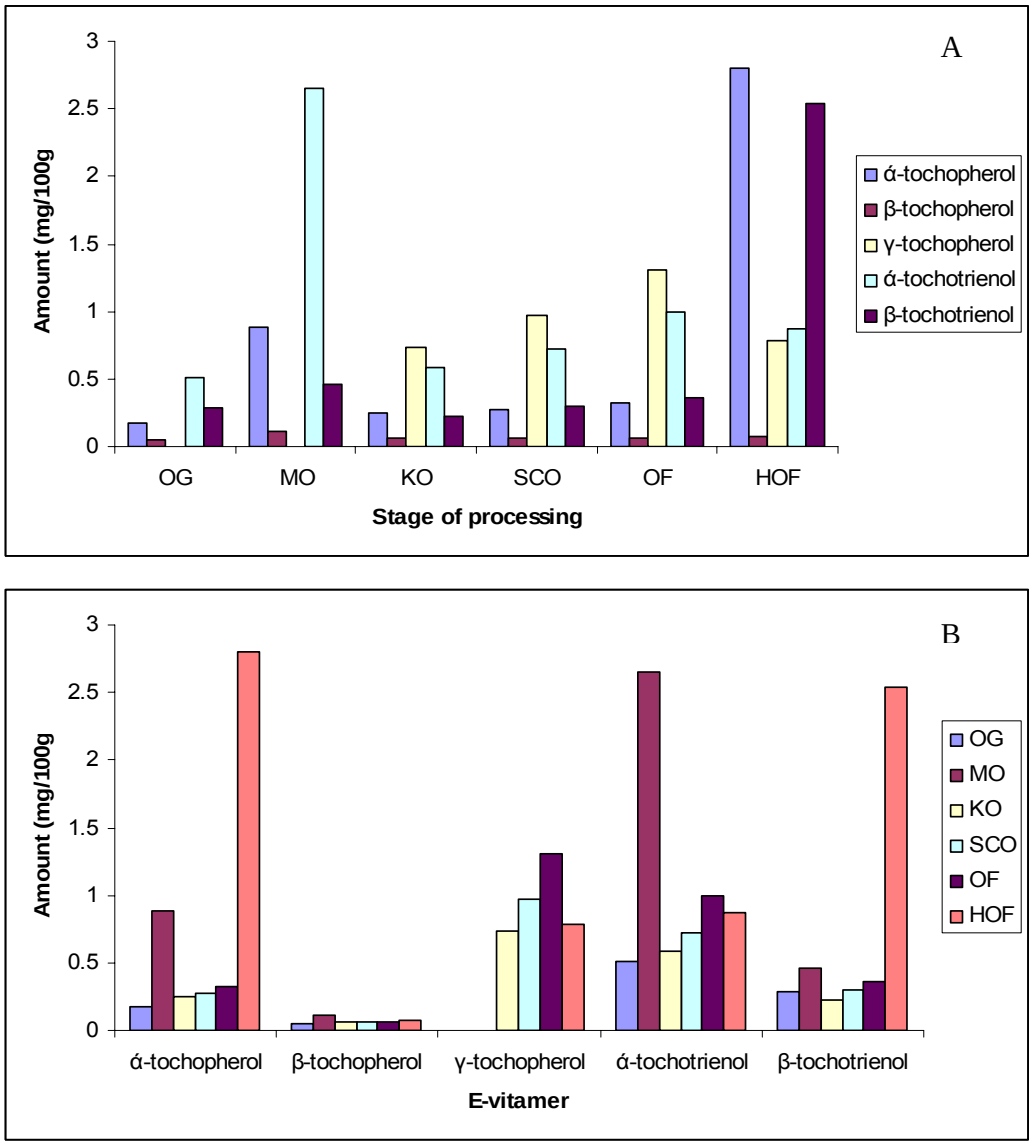

Figure 7. Effect of processing on the E-vitamer composition of methanolic extracts from oats.

Total E-vitamers were extracted into methanol from oats at various stages of processing: Oat grains (OG); Milled oats (MO); Kilned oats (KO); Steel cut oats (SCO); Oat flakes (OF) and heated oat flakes (HOF). These were separated by HPLC and individual vitamers identified and quantified. Trends are shown in terms of processing (A) or vitamer (B).

Processing from the milled oats to oat flakes also resulted in a loss of extractable α-tochopherol. This is important as it is thought that this vitamer is the most nutritionally significant antioxidant.

The significant increase in total extractable E-vitamer during secondary processing of oat flakes could be largely attributed to the increased solubility of both α-tochopherol and β tochotrienol. This increase in α-tochopherol could be nutritionally significant.

These results clearly demonstrate a major impact of secondary processing on the extractable E-vitamers from oat flakes. An interesting question was whether or not a similar phenomenon would be observed following the secondary processing of milled oats, i.e, before any losses consequent on the primary processing of the oats. To examine this milled oats were subjected to secondary processing, total E-vitamers extracted and the profile determined by HPLC. The results are shown in figure 8.

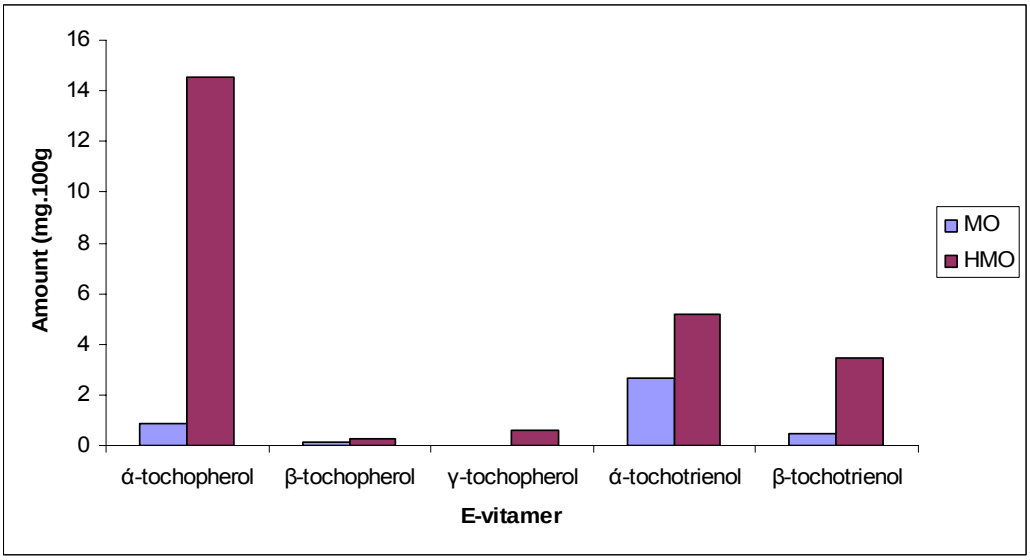

Figure 8. Effect of heating on the E-vitamer composition of milled oats.

Total E-vitamers were extracted into methanol from Milled oats (MO); Kilned and heated milled oats (HMO). These were separated by HPLC and individual vitamers identified and quantified.

As before secondary processing has significantly increased the total extractable E-vitamer for the milled oats. As with the oat flakes this increase is largely attributable to an increased extractability of α-tochopherol and to a lesser extent α and β tochotrienols. The appearance of γ-tochopherol is also apparent after heating. These observations support those for the oat flakes and confirm the potential nutritional benefit of the enhanced α-tochopherol solubility in heated products. It should be noted however, that this may be bioavailable even in the "non-soluble" form

Processing, particularly secondary heating, clearly has an impact on the composition of the oat extracts especially in terms of their antioxidant components. It is entirely possible that the impact may be more widespread and effect several other components of the oat. To examine this potential a more general metabolite analysis was carried out using NMR. Methanolic extracts from oat grain, oat flakes and heated oat flakes were compared in this study since these represent the three stages at which the most significant changes in antioxidant components had been identified. NMR spectra were obtained and compared.

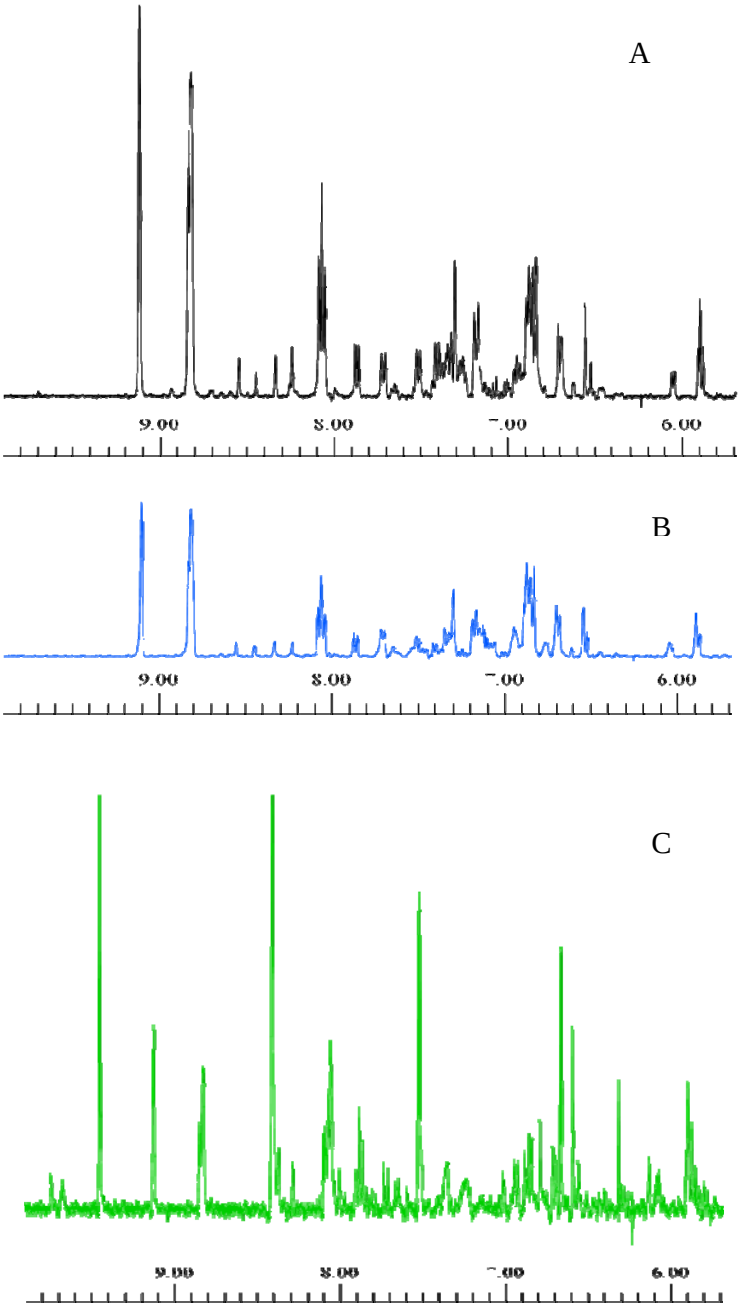

Figure 9 – Partial 400 MHz NMR Spectrum of the Aromatic Region (δ 5.7-10.0ppm) for Oat Grain (A) ; Oat flakes (B) and Heated oat flakes (C).

The NMR spectra within the aromatic range for these three oat extracts are shown in figure 9. This is the region containing signals from the phenolics components of the extract. Oat grain and oat flake spectra can be seen to be very similar the major differences being primarily in the strength of the signals. This would suggest that the lost of phenolics observed during primary processing is due to a general reduction in all the aromatics present and not to a selective loss of individual components. In comparison the spectrum from heated oat flakes is very different to that from the other two oat extracts. The signal intensities are higher which may reflect the increase solubilisation of phenolics observed in the earlier results. Also there are several new peaks appearing which again supports the observed changes in the phenolic profiles observed with the HPLC.

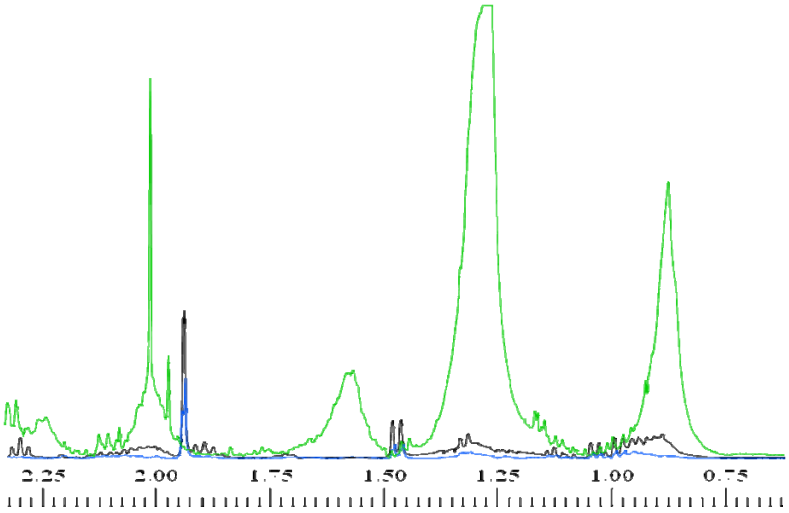

Figure 10 – Partial 400 MHz NMR Spectra of Oat Grain, Oat Flakes and Heated Oat Flakes (Methyl and Methylene Region) (δ 0.5-2.3ppm)

Combined Nuclear Magnetic Resonance (NMR) spectra of Oat Grain (black spectra), Oat Flake (blue spectra) and Heated Oat Flake (green spectra) extracts.

The "methyl and methylene region" of the NMR spectra also showed major differences between the three oat extracts (Figure 10). Again oat grains and oat flakes exhibited quite similar profiles the major difference being in

the spectrum obtained from the heated oat flakes. In this instance the new peaks that appear following secondary processing could be attributed to the solubilisation of lipids. This could account for the significant changes previously observed in the E-vitamers at this stage of processing as these molecules are inherently lipid soluble. Thus secondary processing may be influencing the structure of the lipid components of the oat, making them less stable and as a consequence lipids and their associated E-vitamers are becoming more soluble.

The previous work employed a methanol solution to extract antioxidants from the oats. Obviously the digestive process is aqueous based and as such it was of interest to study the impact, if any, of the solvent on the solubility of these antioxidants. It was not possible to directly mimic the Human digestive system so oat grains (OG), oat flakes (OF) and heated oat Flakes (HOF) were analysed to determine the effect of pH simulated stomach digestion on antioxidant solubility.

Oat samples (10g) were incubated at ambient temperature for 30 min in 150mL phosphate buffer (pH 6.5). The pH of the samples was decreased to 2 using 6M HCL and incubated for a further 30 min at 37ºC. The pH was raised to 6 using 4M NaOH and incubated for another 30 min at 37ºC. The resulting extracts were analysed for total antioxidant capacity (figure 11).

Primary processing has still resulted in a significant 58% reduction in antioxidant capacity, from 2330 ± 202 µmole TE/100g in OG to 968 ± 14 µmole TE/100g in OF ($p<0.01$). However, secondary processing to HOF again resulted in a very significant increase in extractable antioxidant capacity (6965 ± 156 µmoleTE/100g) ($p<0.001$). These results were similar to those obtained with methanolic extracts.

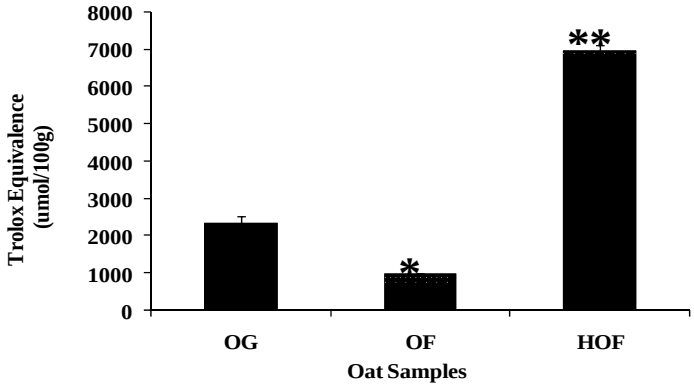

Figure 11. Effect of pH simulated gastrointestinal digestion on total antioxidant solubility.

Oat Grains (OG), oat flakes (OF) and heated oat Flakes (HOF). Data expressed as mean ± sem. Data significantly different from oat grain (*$p<0.05$, **$p<0.01$, ***$p<0.001$), from oat flake (†$p<0.001$).

The results for the total phenolic content are shown in figure 12. The effect on TPC also mirrored the findings found with methanolic extracts, processing from OG (130 ± 5.50 mg/100g) to OF (58 ± 2.03 mg/100g) had a significantly detrimental impact on total phenolic content ($p<0.001$) whilst secondary processing to HOF (208 ± 3.75 mg/100g), increased total phenolic content by 72%.

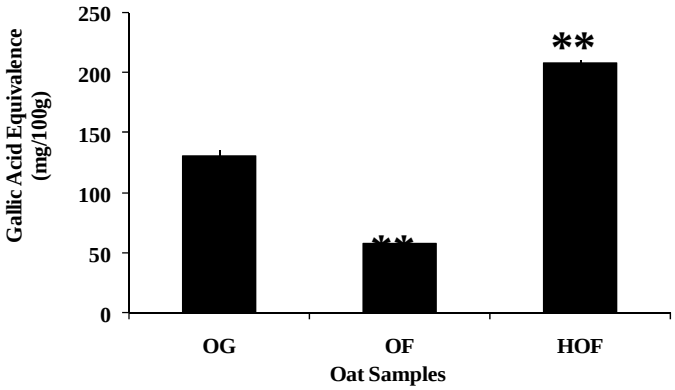

Figure 12. Effect of pH simulated gastrointestinal digestion on total phenolic content.

Oat Grains (OG), oat flakes (OF) and heated oat Flakes (HOF). Data expressed as mean ± sem. Data significantly different from oat grain (*p<0.05, **p<0.01, ***p<0.001), from oat flake (†p<0.001).

The results above demonstrated a significant effect of processing on the extractable antioxidant activity from oats. In the case of secondary processing this was accompanied by a marked change in the composition of the extractable compounds and in particular to a change in the observed phenolic profile.

The net stage of the project investigated the effect of the methanolic oat extract on the expression of some target genes thought to be implicated in protection from heart disease. To examine the impact, if any, of processing on these nutrient-gene interactions, extracts from oat grain, oat flakes and heated oat flakes were compared. These experiments utilised Human umbilical cord endothelial cells (HUVEC).

It is essential for these studies that the concentration of the extract used does not result in any cell death. A preliminary dose response was thus carried out that demonstrated that extracts with concentrations of phenolics up to and including XX $\mu g.mL^{-1}$ had minimal effect on the proliferation of HUVEC.

Human umbilical vein endothelial cells (HUVEC) were incubated for 24 hours with oat phenolics extracted from oat grain (OG), oat flake (OF) or heated oat flake (HOF). The extracts were all adjusted to a final concentration of $\mu g.mL^{-1}$ GAE. Total RNA was extracted from the cells and used for real time RT-PCR analysis of target gene expression: eNOS (Endothelial Nitric Oxide Synthase); ET-1 (Endothelin-1); GATA-2 and VEGF (Vascular Endothelial Growth Factor). Expression was expressed relative to GAPDH mRNA adundance (internal standard). The results are shown in table 2.

The results show that all three extracts significantly increased the expression of eNOS. Oat grain extract also significantly increased expression of ET-1. Expression of ET-1 was also enhanced by oat flakes and heated oat flakes but in these cases the increase was not statistically significant. In contrast all three extracts resulted in a significant decrease in the expression of the transcription factor GATA-2 and in the expression of VEGF.

	mRNA Abundance Relative to GAPDH				
	Control	**OG**	**OF**	**HOF**	**P value**
eNOS	**0.79** ± 0.048	**1.584** ** ± 0.133	**1.117** ** ± 0.037	**1.219** ** ± 0.042	<0.001
ET-1	**0.891** ± 0.058	**1.423** ** ± 0.137	**1.046** ± 0.033	**1.058** ± 0.104	0.004
GATA-2	**1.888** ± 0.079	**1.562** * ± 0.134	**1.066** ** ± 0.036	**0.868** ** ± 0.036	<0.001
VEGF	**1.262** ± 0.144	**0.733** ** ± 0.111	**0.692** ** ± 0.015	**0.671** ** ± 0.109	0.002

Table.1 – The Effect of Oat Grain, Oat Flake and Heated Oat Flake Polyphenolic Profiles on Human Umbilical Vein Endothelial Cell Gene Expression of Target Genes

Data was expressed as mean ± S.E.M. Results were statistically analysed using General Analysis of Variance (ANOVA) and significant differences from control was determined according to Dunnets test (*$p<0.05$, **$p<0.01$).

DISCUSSION

Evidence has shown that the awareness of the role of nutrition in health promotion and disease prevention is on the increase. This is mainly attributed to antioxidant content in food and their ability to protect living organisms from oxidative stress. Findings from this present study have shown that processing of oats can alter the content of antioxidants.

It was observed that primary processing had a significant reduction in total soluble antioxidant capacity when oat grains are processed into oat flakes. Primary processing also had a detrimental effect on the level of extractable of phenolics. The impact on total E-vitamers was less pronounced although it was observed that the kilning of the oats resulted in a significant shift in the profile of these antioxidants. However, even oat flakes provided a considerable level of antioxidant activity and phenolics and there is still a case for eating oats as part of a healthy diet. When compared to other food stuff; oats will still provide our body with a significant exogenous source of antioxidants (Wang and Lin 2000; Zdunczyk et al 2006). This data demonstrates the importance of cereals as a nutritionally beneficial food source.

Secondary processing significantly increased soluble antioxidant activity and this was accompanied by increases in both phenolics and E-vitamers. This was also accompanied by a significant change in the profile of these two key antioxidant classes. In the case of the e-vitamers the increase in α-tochopherol observed at this stage may be particularly relevant in terms of nutritional value. The observation that "secondary heating" of the oat flakes results in an increased extractability of antioxidants is consistent with previous studies on citrus peels (Joeng et al 2004; Xu et al 2007) and tomatoes (Dewanto et al 2002). The study by Dewanto *et al* (2002), showed that thermal processing significantly increased the total

antioxidant capacity of tomatoes. However, no significant changes in total phenolics were found in this case. During this study, the highest temperature tomatoes were subject to was 88ºC. This is a lower temperature than our heating regime and may be the reason for no observed change in phenolics. Thermal processing may cause the release of bound phytochemicals from the food matrix. This finding is supported by the study by Xu *et al* (2007) on citrus peel. During their study, an increase in free phenolics was found to accompany the increase in antioxidant activity following thermal processing. This was accompanied by a corresponding decrease in the amount of phenolics involved in ester, glycoside and ester bounds suggesting that the heat treatment had released phenolics from the food matrix. This may also explain the results observed during our oat trial, showing that the increase in antioxidant activity and phenolics following heat treatment could be due to the thermal liberation of phenolics that are not typically readily available for extraction.

Considering total tocols a significant increase was observed during the milling of the oats. This may in part be due to the position of the tocols in the grain. A study by Peterson (1995) reported that hulls are almost devoid of tocols and when the tocols were extracted during this study, the 1g of oat grains (with hulls) may thus have a lower percentage of tocols available than the 1g of milled oats (without hulls) used in the extractions. This may explain the large, apparent, increase in total tocols that were observed at this stage. Subsequent steps in the production line resulted in a smaller (compared to phenols) but still significant reduction in extractable tocols. This may be due to an increase in moisture content following steaming as the oats were kilned. This is supported by Peterson (1995), who observed that when oats had a higher moisture content, tocol concentration was reduced. The increase in total tocols observed following secondary processing, may be due to a decreased availability in some homologues, but increases in others. Peterson (1995) showed a significant loss of α-homologues, but an increase in β-homologue following heating. The increase in β-homologues may outweigh the loss of α-homologues, thus causing an overall increase in total tocols. It must also

be noted, that heating during our research was of a far greater temperature for a shorter period of time. The increase in total tocols may be due to bound forms of tocols being released by heating, similar to the phenolics. However, the NMR analysis showed that secondary heating appears to result in an increase in the "solubilisation" of lipid compounds. Since the E-vitamers are likely to represent the major hydrophopic antioxidant in the oat grain then solubilisation of the lipid fraction may be accompanied by solubilisation of the E-vitamers.

The observation that the processing was also effecting solubility of these phytonutrients under simulated gastrointestinal pH conditions suggests that this could be nutritionally significant. Indeed the effect of processing seems to be even greater under these conditions. It was reported by Baulblis *et al* (2000), that a pH treatment of cereal samples resulted in increased antioxidant extractability. The authors suggested that this may be due to the acid conditions causing alterations in the activity, composition and/or the concentration of water-soluble low molecular weight antioxidants such as the phenolics. These findings suggest that that the amount of antioxidants released by the oat product matrix into the gastrointestinal system, hence the antioxidant capacity recorded for these samples, may be greater than what was expected from data recorded from organic extracts. Similar findings were also reported by Perez-Jimenez & Saura-Calixto (2005). These authors reported that the antioxidant capacities of physiological extracts of all samples were significantly higher than in their aqueous-organic counterparts. Similar findings were also reported by Liyana-Pathirana & Shahidi (2005). During this study, the effect of pH simulated digestion was tested on wheat products and it was shown that treatment enhanced the antioxidant properties of the extracts when compared to non treated extracts.

The fact that processing has been shown to have an effect on the solubility of antioxidants, whilst having a potential benefit, does not in itself indicate that this makes the antioxidants more likely to be absorbed during digestion. This would require a much more detailed investigation than was possible in this project.

The final stage of the project examined the effect of oat extracts on the expression of genes in human umbilical vein endothelial cells. This is used as a model for potential benefits for cardiovascular health. The extracts were shown to increase the expression of eNOS and decrease expression of VEGF and GATA-2 genes. These could have beneficial effects in terms of both cardiovascular health and cancer. Similar findings have been reported for other food commodities for instance red wine (Oak et al 2003). Extracts were also shown to enhance expression of ET-1 this would not be beneficial but was only significant in the extract from oat grains and not from the processed samples. These studies are very preliminary and use extracts prior to any digestion. There is a strong possibility that metabolism by the gut microflora or in the liver, as well as the potential for compounds to be absorbed across the intestine may limit or modify the effects on gene expression observed in this study. Again further studies would be required to fully ascertain the potential "bioactive" potential of oats and to identify the compounds responsible.

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