Independent project (15hp)

The Effect of Different pH During Steeping on Avenanthramide Content in Germinated Oats

Fristående project (15hp)

Effekten av olika pH Under blötläggning på Avenanthramide Innehåll på grodda Havre

Agata Mackevic

Department of Food Science, Swedish University of Agricultural Sciences, P.O Box 7051, SE-750 07 Uppsala, Sweden;

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Swedish University of Agricultural Science

Faculty of Natural Resources and Agricultural Sciences

Department of Food Science

Agata Mackevic

Supervisor: Lena Dimberg, Swedish University of Agricultural Science, Department of Food Science, Cereal Science division.

Examiner: Annica Andersson, Swedish University of Agricultural Science, Department of Food Science, Cereal Science division.

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Oats, pH, germination, avenanthramides, roots, shoots

The Effect of Different pH During Steeping on Avenanthramide Content in Germinated Oats

Agata Mackevic

Departments of Food Science, Swedish University of Agricultural Sciences, P.O Box 7051, SE-750 07 Uppsala, Sweden; October 2009

Abstract

Oat *(Avena sativa L.)* grains from the cultivar Ivory were steeped at pH 2, 4, 6, 8, 10 and 12 in 0.01M phosphate buffer solution and in tap water: The pH effect during steeping on avenanthramides **2c, 2p, 2f, 2pd**, **2fd** and **3f** was analysed and compared between germinated samples and with a control. Additional unidentified compounds **X, Y and Z** in oat grains were included in the analysis. Statistically significant differences were obtained between control and pH2, pH4, pH6 and pH8 treatments for **3f** ($p<0.001$) while **2c, 2p** and **2f** did not show any significant difference from the control. Avenanthramides **2pd** and **2fd** together with compounds **X, Y** and **Z** seemed to peak at higher pH ranges with statistically significant differences found between control and pH8 and pH10 ($p<0.001$). Analysis of compounds found in roots (**A, B, C**) and shoots (**D, E, F**) was also performed using the same experimental method as for avenanthramides. No pattern regarding concentration of unidentified compounds between different pH values was observed for shoot samples; root samples showed increase of compound **D** in pH2. However, there was no clear trend of increase or decrease.

Keywords

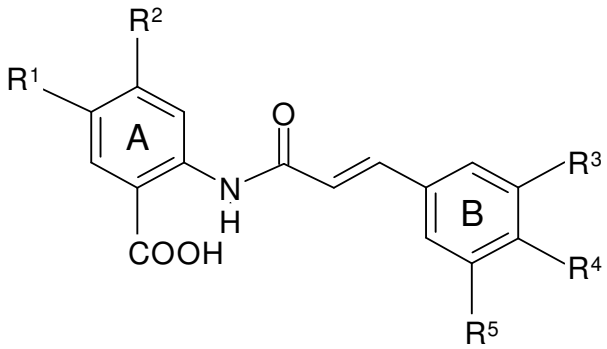
Oats, pH, germination, avenanthramides, roots, shoots

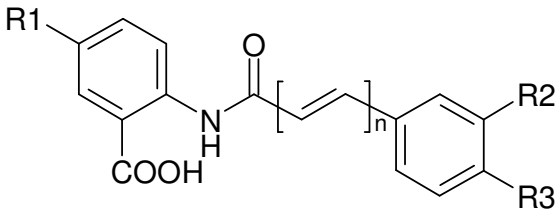
pH values within the text relate to the treatments of oat grains with the particular phosphate buffer pH value.

Introduction

Oats are important cereals due to high nutritional value and also dietary fibre content. The grains contain well-balanced protein concentration with a higher nutritive value than that of other cereals. Biel (2009) found that content of essential amino acids such as methionine, cysteine, threonine, isoleucine, tryptophan, valine, leucine, histidine, phenyalalnine and tyrosine was high according to human needs. Lysine, however, was found to be the first limiting amino acid in naked oat grains. Bioavailability of proteins in oats is increased due to absence of a protein matrix which is present in other cereal varieties (Klose et al., 2009). Oats also have the highest lipid content among the common cereals which determines high energy intake as well as nutritional quality through fatty acid composition. Oats contain high proportion of essential fatty acids and are especially rich in linoleic acid (Zhou et al. 1999; Forsberg et al. 1974). Banas et al. (2007) showed that 86-90% of total oat lipids are concentrated in the endosperm of oat grain making it a concentrated source of nutrients. However, high fat content results in enzymatic and non-enzymatic oxidation and rancidity leading to off flavours over a period of time. High antioxidant activity in oats is considered to prevent non-enzymatic oxidation of lipids (Peterson, 2000). In addition, oat grains are valued as a natural antioxidant in food industry versus synthetic alternatives with undesirable health effects i.e. carcinogenic activity (Peterson, 2000). Avenanthramides (AVAs) are phenolic compounds unique for oats which were shown to exert anti-oxidative activity. They are mainly found in the aleurone layer of oats with

the most abundant ones being derived from caffeic acid (**2c**), p-coumaric acid (**2p, 2pd**) and ferulic acid (**2f, 3f, 2fd**) (Figure 1).





Avenanthramide	R1	R2	R3	R4	R5
2c	OH	H	OH	OH	H
2p	OH	H	H	OH	H
2f	OH	H	OCH_3	OH	H
3f	OH	OCH_3	OCH_3	OH	H

a)

Avenanthramide	n	R1	R2	R3
$2p_d$	2	OH	H	OH
$2f_d$	2	OH	OCH_3	OH

b)

Figure1. Chemical structure of avenanthramides **2c, 2p, 2f, 3f** (a) and **$2p_d$** and **$2f_d$** (b).

Apart from food industry applications, AVAs are becoming of interest in medical studies on human cardiovascular health. Inhibition of LDL oxidation by antioxidants in oats was conferred by Handelman et al., (1999) who stated that aleurone layer of oats showed the highest antioxidant activity on LDL compared to other oat groat fractions analysed in the study. Chen et al. (2004) examined interaction of phenolic compounds in oats with vitamin C on hamster and human LDL oxidation. In both hamsters and humans they have found that oat phenolics decreased LDL oxidation in a dose dependent manner. They have also detected a synergy between vitamin C and phenolic compounds in oats indicating antioxidant properties of the latter. Antioxidant properties of avenanthramides were shown by Bratt et al. (2003) to decrease in such or order: sinapic > caffeic > ferulic > p-coumaric. AVAs are also considered to have anti-inflammatory properties. Guo et al. (2008) showed that synthetic compounds prepared from avenanthramide called 'methyl ester avenanthramide-c' decreased inflammation of blood cell walls and therefore incidence the risk of atherosclerosis.

Several studies have shown an increase in AVA content in intact oat grains following germination (Skoglund et al., 2008; Bryngelsson et al., 2002). This primarily occurred due to activation of hydroxyanthranilate *N*-hyroxycinnamoyltransferase (HHT) enzyme involved in AVA synthesis. Germination of oats also improves nutritional properties of the grain by increasing free amino acid content and therefore protein bioavailability (Tian et al., 2009). Starch content was shown to decrease during germination due to development of shoots and roots (Tian et al., 2009) and correlated negatively with protein, free amino acids and free and reducing sugars content, indicating an increase in enzymatic activity during steeping and germination. A positive correlation was observed by Tian et al. (2009) between starch and phytic acid content indicating decrease of

both during germination. Phytic acid was shown as a natural antioxidant by Graf et al. (1987) due to shift of iron redox potential ensuring rapid removal of Fe^{2+} and therefore preventing $^{\cdot}OH$ radical formation. Phenolic compounds, however, showed an increase in concentration during germination. These compounds are also regarded as possessing antioxidant activity especially phenolic acids as discussed by Peterson (2001).Unidentified compounds were also determined in roots and shoots of germinated oat samples in a previous study by Vitalisson (2007). In addition, during germination, β-glucanase activity is elevated and the degradation of β-glucan occurs (Tiwari & Cummins, 2008).

β-Glucan is a partly soluble fibre found in oats in the bran fraction. It is a linear molecule composed of glucose residues with β-(1-3) and β-(1-4) linkages. Since humans do not possess enzymes that degrade β-links, the entire polysaccharide molecule appears in colon where it is degraded by gut microflora maintaining healthy digestive system. The effect was studied by Van Hai (2009) on prawns fed with β-1, 3-D-glucan and showed an increase in colonic bacteria population. Additionally β-glucan is thought to regulate blood glucose and insulin levels. Behall et al. (2006) compared barley β-glucan with resistant starch and found a significant decrease in glucose and insulin concentrations for consumption of β-glucan, while resistant starch had lower effect. By increasing food viscosity and decreasing transport of nutrients across the gut β-glucan also contributes to slow energy release from foods and therefore satiety for a longer period thus helping to fight obesity issues in the population (Wood, 2004). Low molecular weight (LMW) β-glucans used in a study performed by Biorklund et al. (2008) did not show significant effect on the decrease in serum LDL-cholesterol in hyperlipidemic subjects when either soup fortified with β-glucan or without it was used. Consumption of the same control and treatment soup in the study also did not show significant differences in postprandial glucose and insulin levels between subjects who received β-glucan and who did not. However, when Braaten et al. (1994) examined β-glucan effect on hyperlipidemic subjects, they found a significant decline in serum total and LDL cholesterol. They also observed an increase of cholesterol to the baseline on cessation of β-glucan consumption. Wood et al. (2000) found an inverse relationship between β-glucan and blood glucose indicating that β-glucan consumption lowers blood glucose concentration. They also detected that higher MW β-glucan molecules are responsible for this effect. Haraldsson et al. (2003) showed that activity of β-glucanase in mated barley is reduced when steeping oat grains at low pH and completely suppressed when low pH is combined with high temperature which in their case was 48^{o}C.

The aim of the study was to determine pH effect on avenanthramide content during steeping in germinated oat samples, and to analyse the unidentified compounds present in shoots and roots of the oat samples subjected to buffer solutions of different pH values. The main idea of the present study was to determine the pH range at which the highest increase in avenanthramides is achieved, and to construct vision for the following studies to test the effect of pH during germination on the molecular weight and overall content of β-glucan.

Material and Methods

Oat material

Husked oat *(Avena sativa L.)* samples of the cultivar NS Ivory, EU were used in the current study. Oats were grown in South West of Sweden in Vreta Kloster in Östergötland. They were sown 2006-05-10 and harvested 2006-08-19. Nitrogen and phosphorus fertilizer (NP 27-5) was used. Oats were treated with herbicides but not fungicides. The ordinary agronomical data i.e. yield, water and protein content and weight of 1000 grains for this variety sample was of average value compared to other varieties in the same experimental batch (from 'sortprovning's' protocol plan L7-501, 2006). No diseases were identified in harvested oat samples.

Avenanthramide standards

Synthetic standards were supplied by Dr, K. Sunnerheim, Dep. Of Chemistry, Mid University, Sweden.

Steeping and germination

Oats were surface sterilised using a 1% chlorine solution, washed with distilled autoclaved water and steeped at 4^{o}C for 14h, then at 20^{o}C for 8h in a 0.01M phosphate buffer using a range of pH: 2, 4, 6, 8, 10 and 12 and tap water. Then grains were germinated in Petri dishes for 5.5 days in 20^{o}C. Each Petri dish had filter paper moistened with 5ml of distilled water on both sides within it. The germination process was stopped by freezing (-20^{o}C) and the samples were freeze dried. The dried samples were stored at room temperature in the Petri dishes kept in a plastic box with granites to absorb excess water for 20 months until analysis. Control samples were ungerminated freeze dried grains.

Sample preparation and extraction

Roots and shoots were separated from the grains of each sample. Grains, shoots and roots were weighted separately. Grains were milled using a Retsch mill (<0.5mm particle size) and frozen for the analysis. Two experimental duplicates were prepared for each sample. Avenanthramides were extracted from 1 g of milled oats using 10 ml of 80% ethanol (pH=2) at 52^{o}C for 20min. Acidic ethanol was used as a more effective than neutral solvent in avenanthramide extraction (Dimberg, personal communication). Extraction was followed by centrifugation at 2500 rpm for 10 min. The samples were re-extracted twice. Supernatants from 1st, 2nd and 3rd extractions were pooled together. Mixtures were evaporated at 50^{o}C under vacuum. Residues were dissolved in 2 ml methanol, transferred to an ependorf tube and centrifuged at 13000 rpm for 10 min. Extract were transferred to vials and analysed using HPLC (see below). Shoots and roots were milled separately with 5 ml of 80% ethanol (pH=2) using Heidolph DAX 600 mill (milling speed adjusted to 20000-24000 loads/min) and were subjected to the same extraction procedure as used for grain samples, however instead of 3x10 ml extracts, 2x5 ml extracts were obtained.

HPLC analysis

Reversed-phase C-18 column chromatography with 0.01M formic acid (pH=2.9, 5% acetonitrile) solution and acetonitrile as a mobile phase was used to analyse oat extracts on an Agilent 1100 system. A linear gradient from 0-40% of acetonitrile was used and an injection volume of 10µl with a flow rate of 1ml min^{-1} was used. Detection was performed at the wavelength of 340nm and chromatograms obtained were compared between samples with the areas having been analysed as a comparable variable (for example refer to Figure2 a & b). UV spectra were obtained for the unknown compounds X, Y and Z (see Figure 3 a-c). Due to similarity in UV spectra and retention time these compounds are assumed to be avenanthramides (Dimberg, personal communication). For HPLC analysis of root and shoot sample the same technique was used. UV spectra for detection was produced for each compound A-F (see Figure 4 a-f) with an assumption that D and E are flavonoids (Dimberg, personal communication).Compound chromatograms were used to compare samples (see Figure 5 for chromatogram example).

Peak areas of avenanthramides **2c, 2p, 2f, 2pd, 2fd** and **3f** and of compounds **X**, **Y** and **Z** were obtained from HPLC analysis of all grain samples. Two experimental duplicates with an analytical duplicate for each were analysed except for pH 4 treatment which had only one experimental duplicate. Areas were adjusted for 1g of each sample. Concentration (µmol/g) was calculated for **2c, 2p, 2f** and **3f** using equations obtained from avenanthramide standards.

Due to inconsistency between sample duplicates repeated analysis was performed for both experimental duplicates of tap water and pH2 treatment; repeated analysis for only one experimental duplicate was performed for pH4, pH6, pH8, pH10 and pH12 treatments due to lack of material . The new results were added to previous ones and the mean area or concentration for each treatment per gram of sample was calculated (see Table 1).

Percentage values for weight of shoots and roots from the total weight of grains used were obtained for every experimental duplicate to minimise variation between avenanthramide content due to differences in germination ability. Compound areas/g of sample were determined using sample weights adjusted for the percentage weights.

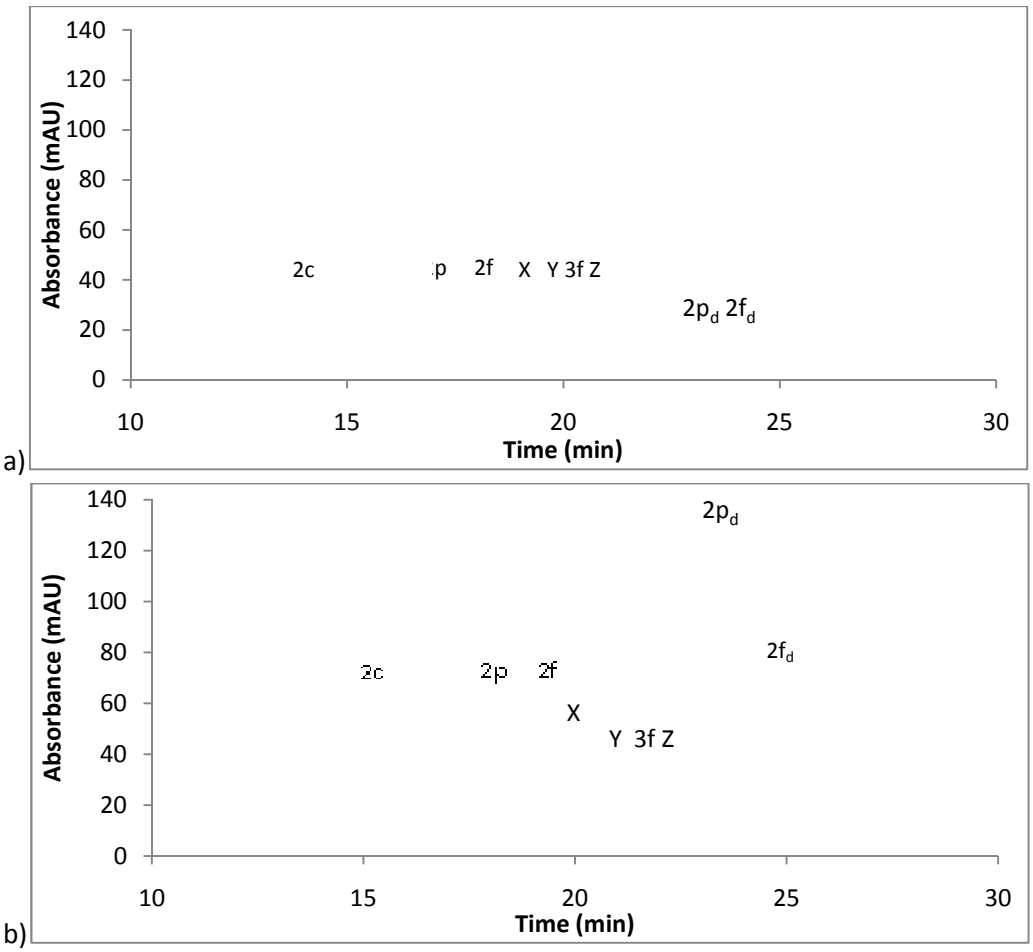
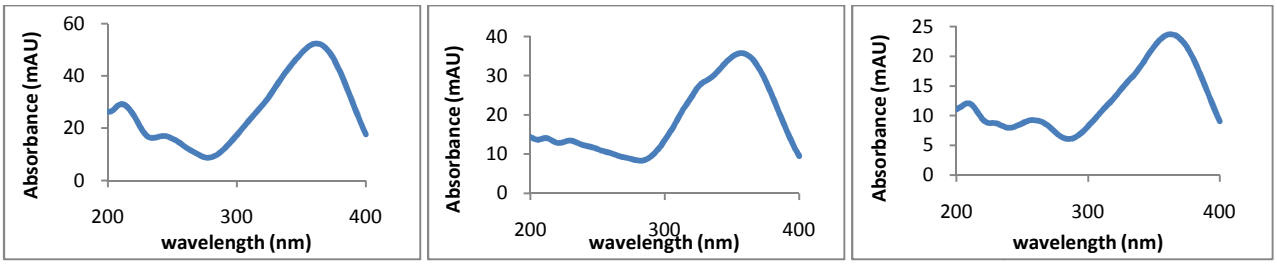


Figure 2. Control (a) and pH10 (b) chromatograms obtained from HPLC for oat grain samples



a) UV spectrum - X **b)** UV spectrum - Y **c)** UV spectrum - Z

Figure 3. Typical UV spectra of unidentified compounds X, Y and Z in grains (a-c), for chromatograms see Figure 2.

Statistical analysis

One-way ANOVA along with Tukey's Family Error test in Minitab 15 was used for every sample treatment (Table 1).

Results and Discussion

Avenanthramides 2c and 2f

HPLC analysis gave peak areas for every compound given in Table 1. Areas were corrected for 1g of each sample. Significant differences in areas/g for every compound in germinated samples were determined using one-way ANOVA. Avenanthramides **2c** and **2f** did not show any statistically significant differences between other treatments and control (p-values 0.178 and 0.475 respectively). Similar results for **2c** were also obtained by Skoglund et al. (2007) who studied effect of germination on avenanthramide content in oats but not effect of pH during steeping. SD for **2c** in treatments at pH4, pH8, and pH10 seems to be very high in current study; therefore results cannot be conclusive. Vitalisson (2007), however, found a 23% increase for **2c** of germinated samples when compared to un-germinated samples while pH effect during steeping was not analysed. Although Skoglund et al., (2007) did not find an increase in **2c** concentration; she detected that avenanthramide **2f** showed significant difference with control sample following germination. Vitalisson (2007), within her cultivar range also analysed Ivory oat variety and showed 49% increase for **2f** in germinated samples compared to un-germinated samples. However, results for the same variety can differ from year to year due to other factors such as environmental conditions, e.g. weeds (O'Donnell and Adkins, 2001). Skoglund et al. (2007) found a big difference in AVA concentration between twenty Swedish oat genotypes from the year 2000 and the same genotypes from the year 2001. Total AVA content from the harvest of the year 2000 was 123.1 nmol/g of oats while total AVA content from the harvest of the year 2001 was only 40.9 nmol/g of oats. In the present study **2f** showed an increasing pattern in all samples except for pH12 compared to control (Figure 6 a-b). The reason for the difference between statistical analysis and obtained trend as well as previous findings may be a high standard deviation (SD) for pH2, pH6, pH8, pH10 samples of **2f** (refer to Table 1). Increase in **2f** content following steeping and germination can be noted for pH2, pH4, pH6, pH8 and pH10 (Figure 6 a-b). Another explanation regarding **2f** analysis could lie under normality of the data. Residual plots obtained from the statistical analysis were not consistent, i.e. histogram did not show normal distribution as for **2c** due to outlier in the middle and versus fit plot also showed quite uneven distribution across treatments compared to **2c**. Other graphs looked satisfactory normal (refer to Figure7 a-b): Normal probability plot represented straight line and points in versus order graph were randomly scattered. Such graphical representations obtained from ANOVA analysis indicate data to be normal and suitable for the analysis. On the other hand, obtained results are only representative for the current study and they could differ between years and cultivars. As already noted Skoglund et al. (2007) analysed the same oat varieties grown for two years and found a big differences in AVA content between samples grown in 2000 and 2001. Vitalisson (2007), showed that there are differences in AVA content between different cultivars. There were also differences in AVA content between cultivars used in the study performed by Skoglund and colleagues and Ivory cultivar used in the current study. Avenanthramide **2c** concentration for the year 2000 in Skoglund study was 49.2 nmol/g of oats while in current study **2c** concentration reached 754 nmol/g of oats when ungerminated samples were compared. Big differences were also found for **2p** and **2f** concentration. Avenanthramide **2p** content in ungerminated oat samples from the year 2000 in Skoglund study was 42.9 nmol/g of oats compared to 348 nmol/g of **2p** from ungerminated

control oat sample used in current study. Avenanthramide **2f** concentration from ungerminated cultivar analysed previously by Skoglund et al. (2007) appeared to be 31.0 nmol/g of oats used while in current analysis **2f** concentration for ungerminated control reached 411 nmol/g of oats.

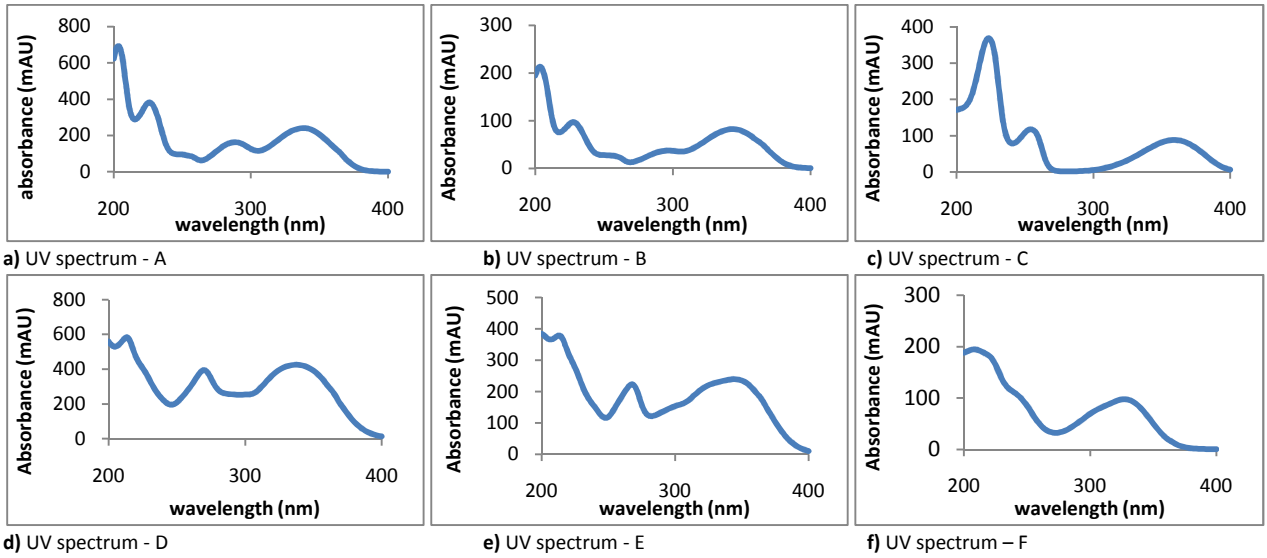


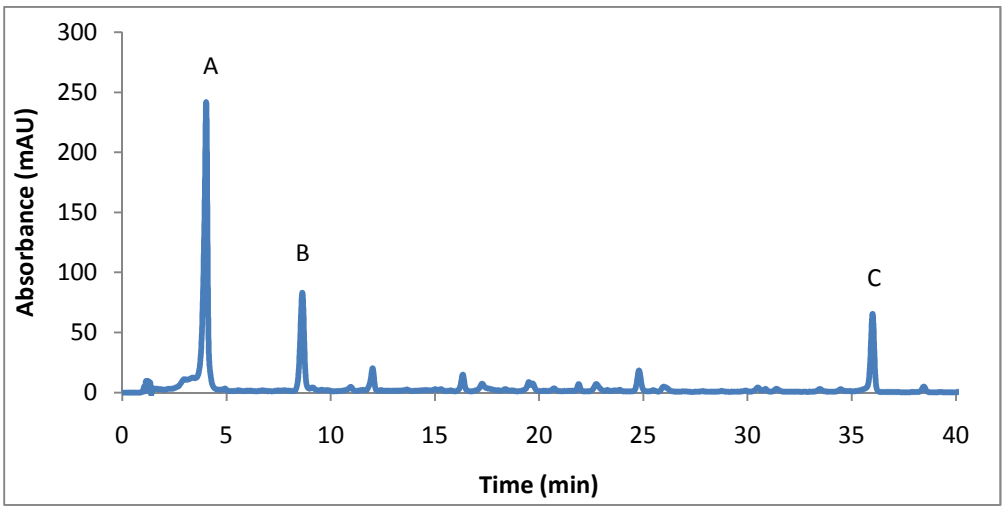
Figure 4. Typical UV spectra of unidentified compounds A, B and C in roots (a-c), (for chromatograms see Figure 5a) and D, E and F in shoots (d-f) (for chromatograms see figure 5b)

Avenanthramide **2p** did not show any statistical significant difference from control sample, however, it was found that **2p** content for pH2 (c=0.62 µmol/g) sample was higher than in pH12(c=0.39 µmol/g); p=0.009 (Table 1). Avenanthramide **3f** showed significant difference between control sample (c = 0.099 µmol/g) and samples steeped at pH2 (c = 0.28 µmol/g), pH 4 (c = 0.25µmol/g), pH6 (c = 0.28 µmol/g) and pH8 (c = 0.31 µmol/g), p<0.001 (Table 1). Samples steeped at pH 8 also showed higher **3f** content than samples steeped at pH12 (c = 0.28 µmol/g); p<0.001(Table 1). All samples showed an increase in **3f** concentration compared to control (Figure 6 a-b). Residual plots obtained from one-way ANOVA seem to be normal. Skoglund et al. (2007) also detected an increase of avenanthramide **3f** during germination. They also noted the biggest increase in **3f** during germination compared to other compounds.

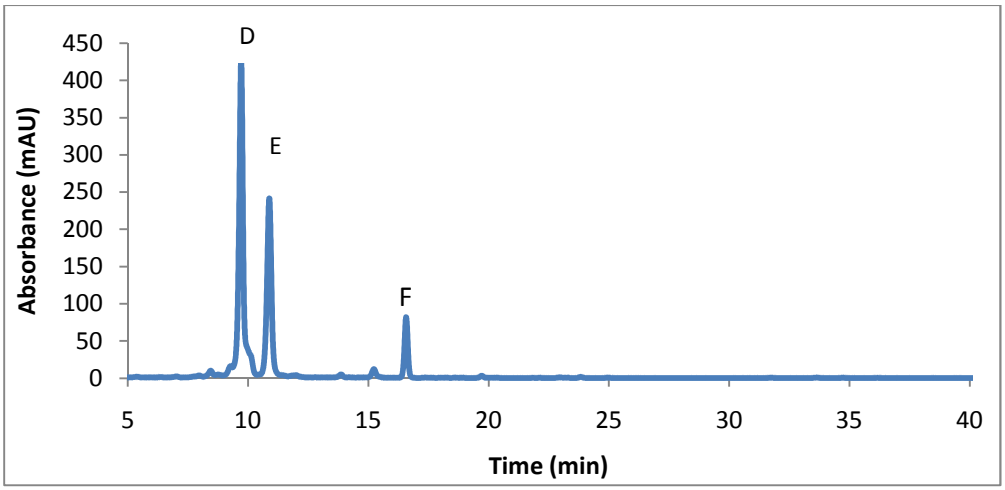
Both **2pd** and **2fd** were significantly higher in samples steeped at pH 10 than control sample (p=0.007). Avenanthramide **2pd** showed a remarkable increase in all samples compared to control where the highest increase was obtained in pH8 and pH10 (for the references see Table 1 and Figure 6 a-b). However, **2pd** showed extremely high SD for pH2, pH4, pH8 and pH10 with the latter being 50% as high as area of the sample; therefore results might not be reliable while residual plots still look normal.

All three unidentified compounds **X**, **Y** and **Z** which peak between **2f** and **2pd** (Figure 2), except **Y** for pH8, showed higher avenanthramide content in pH8 and pH10 than control sample. Compound **X** also showed pH8 and pH10 to have higher concentration than pH2, while compound **Y** showed significant difference between pH2 and pH10 as well as between pH4 and pH10 (refer to Table 1). The content of compound **Y** in pH10 was higher than in pH12 and content of compound **Z** in pH8 treatment also appeared to be higher than in pH12. For the most part all the compounds showed an increase in their concentrations for all the

treatments compared with control (Figure 6 a-b). SD were quite small with some higher values in pH10 and pH8 for **X** and pH10 for **Y**.



a)

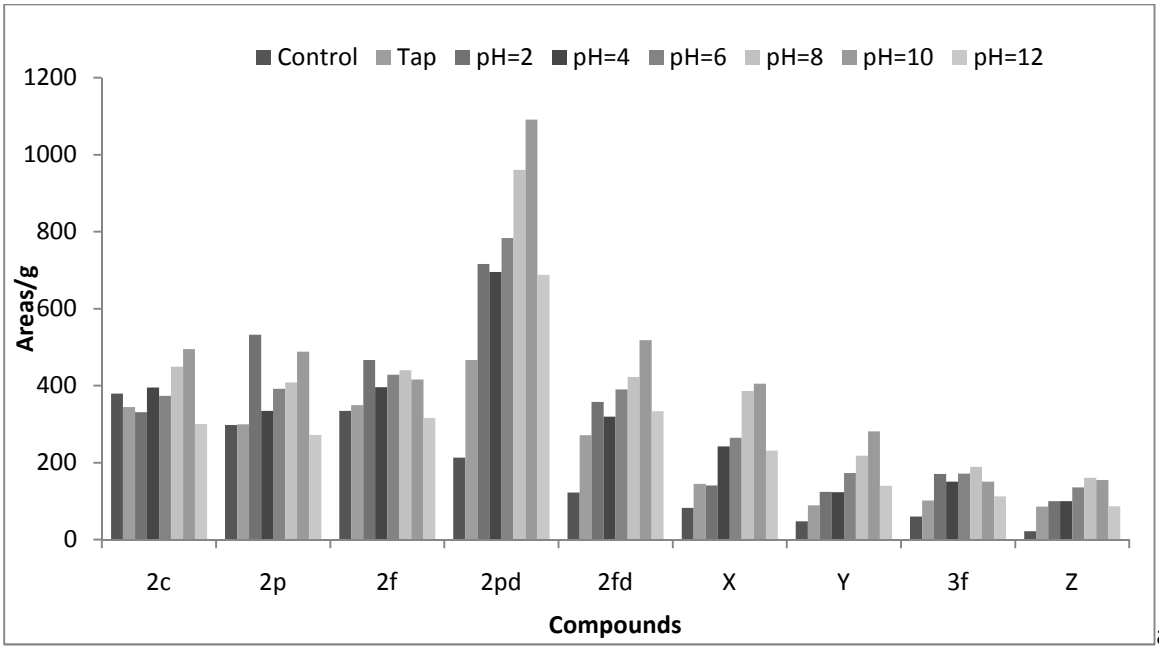


b)

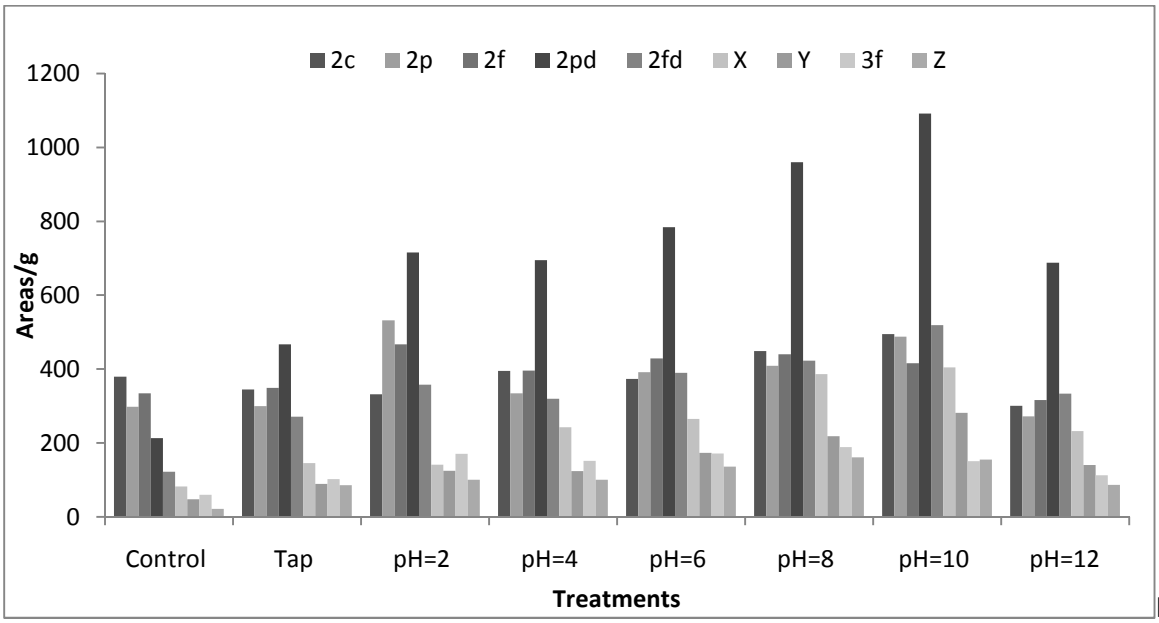
Figure 5. Root (a) and shoot (b) chromatogram obtained from HPLC for samples treated with phosphate buffer at pH 2

Both avenanthramides **2p** and **3f** showed significant differences between samples steeped with tap water and different phosphate buffer pH values. Avenanthramide **2p** content in pH2 (c = 0.62 µmol/g) was higher than in tap water (c = 0.35 µmol/g) (Table 1). Figure 6 a-b showed an increase in **2p** content in all treatments except pH12 compared with tap water where pH2 and pH10 showed the highest increase. However, no statistical significance was found for pH10. **3f** concentration was significantly higher in pH2 (c = 0.283µmol/g), pH6 (c = 0.284 µmol/g) and pH8 (c = 0.313 µmol/g) than in tap water (c=0.064µmol/g) (Table 1).

Both **2pd** and **2fd** were higher at pH10 than in tap water; p=0.002. Compounds named **X**, **Y** and **Z** all showed significant difference between pH8 and tap water as well as pH10 and tap water where pH8 and pH10 treatments had higher concentration of a particular compound (p<0.001).



a)



b)

Figure 6. Areas per gram of various compounds in oat grains of germinated oat samples steeped in different pHs: ordered by compounds (a), ordered by pH (b)

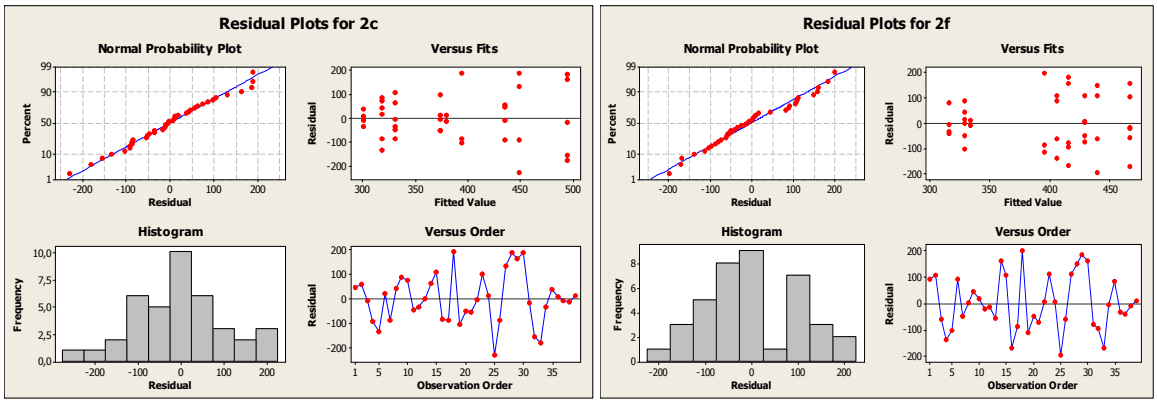
Analysis of avenanthramides obtained from the sample steeped with tap water (pH= 6-7) did not follow the same pattern as pH 6. Content of **3f** in pH6 even showed statistically significant difference from tap water sample. Other samples for avenanthramides **2p**, **2pd, 2fd** and **3f** as well as compounds **X**, **Y** and **Z** showed statistically significant differences from tap water while no such outcome was observed for pH6. If only pH value was responsible for AVA content in samples, pH6 would be expected to show similar results to tap water. Ions and other components present in tap water might account for the differences. It is also unknown whether or not the same result would be obtained if a pH gradient with another buffer was used instead of phosphate buffer.

Generally, despite some outliers and high SD obtained in particular samples mentioned above it appears that avenanthramide content especially for **2p** and **3f** was highest when oat samples were germinated at lower

pH and especially pH2. Unknown compounds (**X**, **Y** and **Z**) together with avenanthramides **2pd** and **2fd**, however, seem to peak at higher pH values. In Figure 6 a & b it might seem that higher avenanthramide concentration also appears in higher pH range due to **2pd** concentration. Although, **2pd** showed significant difference between tap water treated and control samples for pH10 where SD was about half of the area (1092 area/g ± 537), avenanthramides **3f** and **2p** had more significant differences between samples at lower pH ranges and control (see Table 1). Avenanthramides **2c** and **2f** did not show any statistically significant differences between any samples.

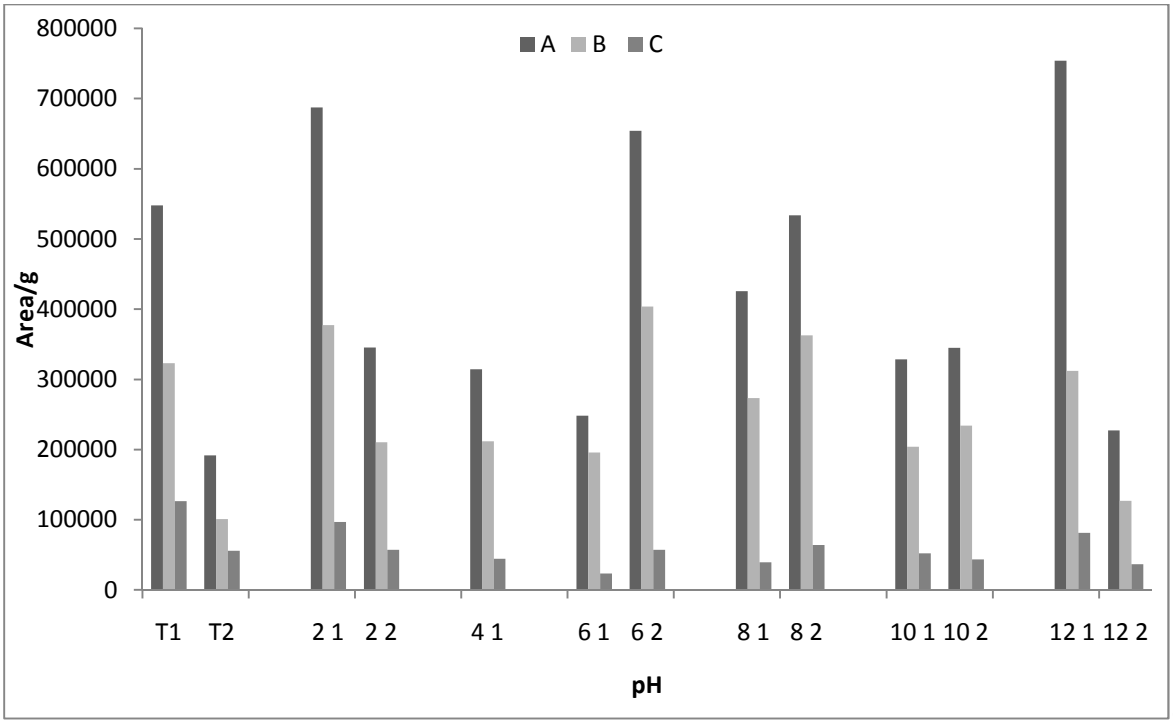
Variation between experimental duplicates might depend on sample germination ability. Differences were found between the percentage amounts for shoots and roots from the grain weight between experimental duplicates. However, these differences might also be due to mass of shoots and roots and not the number of germinated oat grains. This information was not collected during the study. Vitalisson (2007) also obtained high difference in germination ability between experimental duplicates in her experiment with different samples. However, she did not get very big difference for the cultivar Ivory. She conducted a correlation study between avenanthramide peak areas and oat germination ability; however, she did not find any relationship.

Compounds **A**, **B** and **C** were found in all shoot samples with compounds **D**, **E** and **F** observed in all root samples. However, root and shoot analysis did not give consistent results regarding differences between samples steeped in different pHs (see Figure 8 a & b). There was no pattern of increase or decrease in the concentration of compounds neither in shoot analysis nor in root analysis, while it was still visible from the histogram that there was high concentration of compound **D** at pH2 in root sample. Despite the percentage weight correction for root and shoot samples (see HPLC analysis in experimental methods), high differences between duplicates of samples were obtained for shoots with root samples having lower differences (refer to Figure 8 a & b). Due to these differences results from both experimental duplicates for each sample treatment are displayed in Figure 8 a & b and not the mean of two.

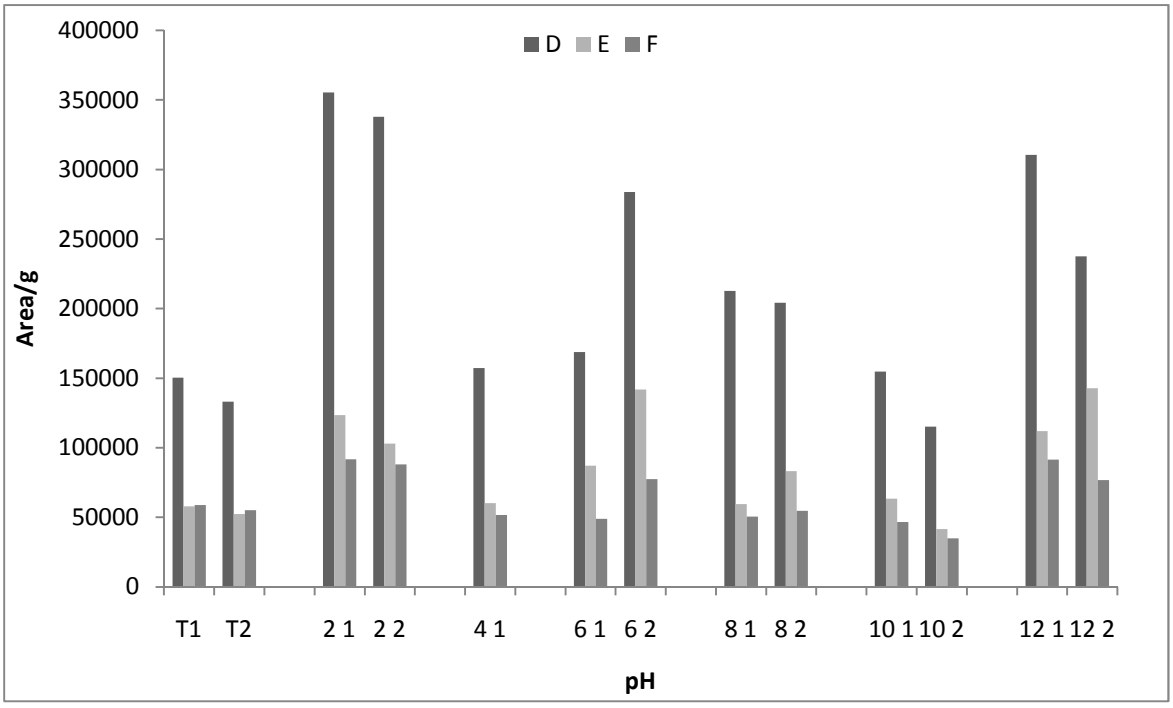


a) b)

Figure 7. Residual plots for 2c (a) and 2f (b) derived from Tukey's Family Error statistical analysis



a)



b)

Figure 8. Area per gram for compounds from each experimental duplicate in shoots (a) and roots (b) of steeped in different pHs and germinated oat grains

Summary

The amount of avenanthramides **2p** and **3f** increased in lower steeping pH ranges ($p=0.009$ and $p<0.001$ respectively) while **2pd** and **2fd** showed an increase in higher steeping pH ($p=0.002$ and $p=0.007$ respectively) compared to control and tap water samples. Similar results regarding germination for **3f** were also found in previous studies by Skoglund et al., (2008), however, she did not examine pH effect. Avenanthramides **2c** and **2f** did not show any statistically significant differences between treatments ($p=0.178$ and $p=0.475$ respectively). Although no pH effect was examined, an increase in **2c** was found

previously by Skoglund et al., (2008) and Vitalisson (2007) in germinated oat samples , however, both of them detected an increase in **2f** following germination**.** Unidentified compounds **X, Y** and **Z** also showed higher concentrations in high pH ($p<0.001$). Substances **A, B** and **C** in shoots and **D, E** and **F** in roots of germinated oat samples were analysed regarding pH of samples, however, no clear pattern between different treatments was observed.

Future studies

- More studies are needed to completely understand the behaviour of avenanthramides during steeping and germination as well as relationship between content of avenanthramides and germination ability of oats.
- Extension studies are required to estimate pH effect on β-glucan with the vision to obtain the highest content of avenanthramides and at the same time retaining high molecular weight β-glucan molecules.
- Careful analysis is needed to determine the importance of activity of avenanthramides which increase in different pH ranges with regards to β-glucan content as an important dietary fibre in reduction of human serum LDL oxidation as well as blood glucose content.

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