Avenanthramides as a means to slow rancidity development in oats

Wing Tung LO
June 1st, 2022

KBTM01 Master Thesis in Biotechnology, LTH Faculty of Engineering at Lund University

LUNDS UNIVERSITET

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Supervisor: Cecilia Tullberg
Examiner: Carl Grey

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Abstract

Cereal grains are an important staple in many diets across the globe. Being one of the most cultivated cereal crops worldwide, especially in Nordic countries, oats are known to have a high dietary fibre content and a nutritional value that makes them highly beneficial for human health. Avenanthramides are a group of phenolic compounds that, among other cereal grains, are only produced in oats. Avenanthramides are known to have strong antioxidant activity and, in this present study, avenanthramides were investigated in relation to rancidification in oats. Avenanthramide-rich oat extracts were added to milled oat flour in accelerated storage trials with a water-methanol mixture containing copper sulphate, and rancidity markers such as peroxide value (PV), lipase activity and hexanal detection were followed over time to investigate the potential of avenanthramides as a means to slow rancidity development. In the two later storage trials conducted, avenanthramide enrichment was found to result in statistically significant decreases in PV, lipase activity and hexanal levels after 24 hours compared to the controls. The findings of this study serve to provide an overview of avenanthramide enrichment as a means of delaying rancidification in oats and can be used to guide further research into how avenanthramide extracts can be used to optimise oxidative stability in oats and other lipid-based foods. Interestingly, in addition to delaying lipid oxidation, avenanthramides also seem to be effective lipase inhibitors, which is perhaps something that can be further investigated in later studies.

Popular Science Summary

As society becomes more and more health conscious, demand for foods that are beneficial to human health has also increased. One area of particular interest has been antioxidants, a class of nutritional compounds that, when ingested, help neutralise free radicals in our bodies, thereby helping prevent health conditions ranging from heart disease, to cancer. In oats, antioxidants also play an important role during storage by preventing lipid oxidation, a process that ultimately results in the rancidity of oats. Avenanthramides are a type of antioxidant that, among cereals, are only produced in oats.

Apart from antioxidants, oats also contain many other nutritional compounds that are beneficial for human health, including many essential amino acids, as well as dietary fibres such as β-glucan. Oats also contain a lot of healthy fats, which consequently makes them susceptible to rancidification and, subsequently, lowered nutritional quality. While it is common practice to heat treat oat products in order to deactivate enzymes responsible for the degrading of these healthy fats and speeding up the rancidification process, heat treatment can often lead to the breakdown of nutritional compounds. This includes antioxidants that are already present in the oats, which can be counterproductive as antioxidants can help prevent rancidification as well.

This present study aims to uncover the role of avenanthramides in the rancidification of oats, with the hope of using avenanthramides as preservatives in oats and other fat-rich foods. Avenanthramides were first extracted from oats, and then added into fresh oat flour to observe their effect on storage stability. The findings of the study demonstrate a promising potential of avenanthramides to be used as preservatives not only in oats, but in other fat-based foods as well.

Acknowledgements

This project was carried out at the division of Biotechnology, the Faculty of Engineering at Lund University (LTH) as a part of ScanOats, an Industrial Research Centre funded by the Swedish Foundation for Strategic Research, SSF. My deepest gratitude goes to my supervisor, Cecilia Tullberg, without whom this project would not have been possible. Thank you for your patience, support and guidance. I have learnt so much from you throughout this process, and it has pushed me to become a better student and researcher as a result. I would like to thank the members of the BioOrg group as well, who have been incredibly welcoming and helpful whenever I had questions in the lab. I would also like to acknowledge and give thanks to ScanOats, especially Johanna Wetterlind and Anders Jonsson from the Swedish University of Agricultural Sciences (SLU), for supporting the project and providing the oat material used in this study.

My final thank you goes to my family and friends. To my parents, thank you for supporting me and believing in me at times when I didn't believe in myself. To my friends, thank you for all the support, company, laughter and memories throughout this journey that will last a lifetime.

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Introduction

Overview

Cereal grains are an important staple in many diets across the globe. Being one of the most cultivated cereal crops worldwide, especially in Nordic countries (Velvert et al., 2021), oats are known to have a high dietary fibre content and a nutritional value that makes them highly beneficial for human health. Nutritional compounds that can be found in the oat grain include many essential amino acids, dietary fibres such as β-glucan, unsaturated fatty acids as well as anti-oxidative compounds (Rasane et al., 2015). Recent studies have also found numerous health effects linked with the consumption of oats, including gut microbiota and cardiovascular benefits (Kristek et al., 2018).

Compared to other cereals, oats have a relatively high oil content, making them a good source of mono- and di-unsaturated fatty acids (Lehtinen et al., 2011). However, oats also contain many lipolytic enzymes (Lehtinen et al., 2011), making them susceptible to rancidification and subsequently, lowered nutritional quality. It is therefore common practice to inactivate lipolytic enzymes by heat-treating to prolong storage (Ganßmann, W. et al., 1995). Although this ensures the stability of oat products, this heat-inactivation process may also lead to the breakdown of nutritional compounds that may be of benefit to human health. Hence, there has been an increased interest in research surrounding the development of milder treatment processes to better preserve the nutritional quality of oat food products. One particular area of interest is avenanthramides, a group of phenolic compounds that are oat-specific that have been shown to possess strong antioxidant activities (Wise et al., 2014). This present study focuses on avenanthramides and their role in reducing rancidity of oats.

Oat lipids and rancidification

Although oats are generally known to have a relatively high oil content, the total lipid and fatty acid content of oat groats can vary greatly depending on the cultivar, growing conditions as well as methods used for lipid extraction and analysis (Lehtinen et al., 2011). The total lipid content of oats can vary between 2-13%, and while palmitic, oleic and linoleic acids make up 90-95% of the fatty acid composition in oats, the levels of each fatty acid can also vary between

oat cultivars depending on their oil content (Lehtinen et al., 2011). It is estimated that 50-85% of oils in oats are neutral acylglycerols, particularly triacylglycerols, with another 20-40% being glycolipids and phospholipids (Lehtinen et al., 2011).

Rancidity can be defined as the spoilage or deterioration of lipids leading to a detectable "off-odour" as a result of oxidation (Coe, Mayne R., 1938) and hydrolysis (Gibson and Newsham, 2018). While lipid oxidation is not thermodynamically spontaneous, it is an autocatalytic process that, once initiated, is both self-propagating and self-accelerating (Schaich *et al.*, 2013). Lipid oxidation is commonly understood to involve three main stages: initiation, propagation and termination (Schaich et al., 2013; Barden et al., 2016). In the initiation stage, a hydrogen is typically abstracted from an unsaturated fatty acids, resulting in the formation of a alkyl radical (R•). This stage typically requires initiators such as enzymes, light and high temperature to initiate the autocatalytic process. This is then followed by propagation, where oxygen reacts with the alkyl radical to form a reactive peroxyl radical (ROO•). The resulting lipid peroxyl radical then reacts with an adjacent fatty acid, resulting in the formation of a lipid hydroperoxide (ROOH) and a new alkyl radical (R•), with the process repeating and propagating indefinitely, until either the substrate (fatty acids) is used up, or two radicals react to form more stable products (Schaich *et al*., 2013; Barden *et al.*, 2016). The resulting lipid hydroperoxides (ROOH) from the oxidation process may further degrade into end-products, some of which are volatile (e.g. hexanal) and are responsible for the smell and taste that are characteristic of rancid foods (Pajunen T., 2008). Figure 1 shows a schematic of the three stages of lipid oxidation, while Figure 2 shows a schematic illustrating the levels of polyunsaturated lipids, lipid hydroperoxides, non-volatiles and non-volatile end products over the course of the oxidation process.

Initiation:

$$RH \xrightarrow{\text{initiator}} R^{\bullet} + H^{\bullet}$$

Propagation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

Termination:

$$R^{\bullet} + R^{\bullet} \rightarrow RR$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$$

Figure 1 - Schematic of the 3 stages in the lipid oxidation process taken from Mahne (2018).

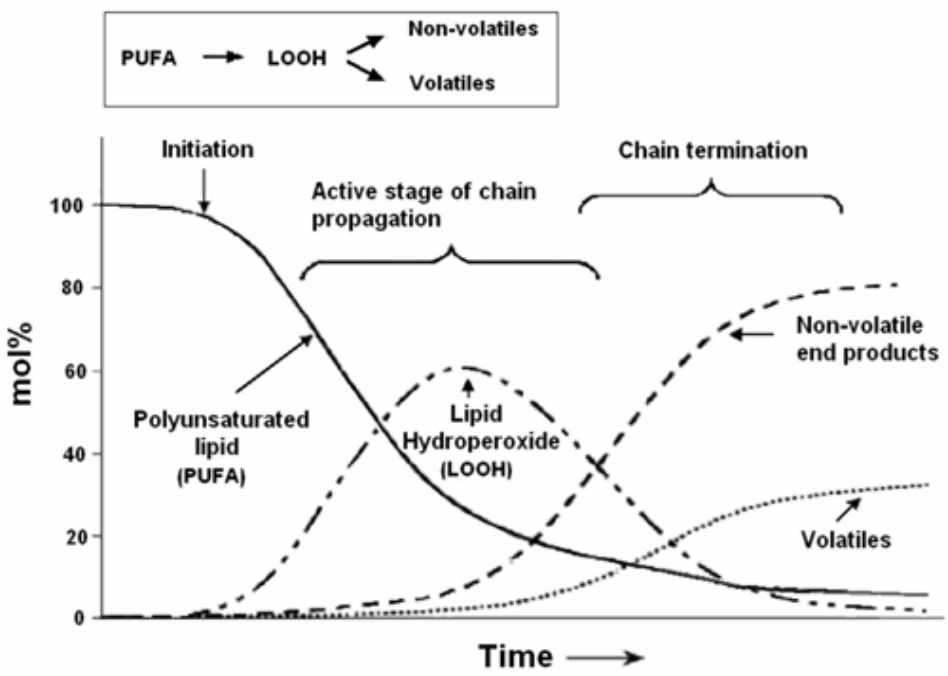


Figure 2 - Schematic illustrating the levels of polyunsaturated lipid, lipid hydroperoxides, non-volatile end products and volatiles over the course of lipid oxidation, taken from Pajunen (2008), redrawn from Gardner (1987).

Lipid oxidation primarily occurs in unsaturated fats, and studies have shown that the addition of one double bond to a fatty acid can increase its oxidation rate by at minimum a factor of two (Holman & Elmer, 1947). The unsaturated linoleic and oleic acids that make up most of the fatty acid composition in oats thereby makes them susceptible to lipid oxidation and, subsequently, rancidification (Mahne, 2018). Oats also contain a high amount of lipid-related enzymes, such as lipases, lipoxygenases and lipoperoxidases, that are meant to degrade the

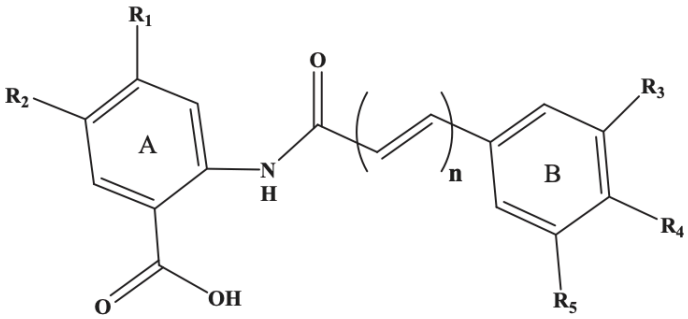
lipids for use as energy sources during germination (Lehtinen et al., 2011; Mahne, 2018). During oat processing, particularly dehulling and milling, breakage of cell structures in the oat kernel exposes the oat lipids to such lipid-degrading enzymes (Mahne, 2018). Lipase hydrolyses lipids such as triglycerides into free fatty acids that are much more susceptible to oxidation, while the oxidative enzymes lipoxygenase and lipoperoxidase further promotes oxidation (Lehtinen et al., 2011; Mahne, 2018). The end products from lipid oxidation, along with free fatty acids from hydrolysed lipids result in deteriorations in the taste and quality of the oats, and studies have also correlated toxic lipid oxidation products with various health implications such as inflammatory disease, cancer, and ageing (Vieira et al., 2017; Gibson and Newsham, 2018).

Heat-treatment of oats to reduce lipid oxidation

In order to minimise lipid oxidation and prolong storage, it is common practice for oats to be heat-treated to inactivate native lipid-degrading enzymes (Ekstrand *et al.*, 1993; Ganßmann, W. *et al.*, 1995). As mentioned previously, while this ensures the stability of oat products, the heat-inactivation process may also lead to the breakdown of nutritional compounds that may be of benefit to human health. This includes antioxidants that are naturally occurring in oats, which can help prevent the autoxidation of oat lipids (Bryngelsson, 2002). Under certain conditions, heat inactivation has been found to promote lipid oxidation, rather than prevent it. A study by Lehtinen (2003) found that, in dry oat fractions kept in long-term storage, heat-treatment resulted in an increase in lipid oxidation and hexanal formation compared to non-heat treated fractions. This was possibly due in part to the heat inactivation of heat-sensitive antioxidants. It was also concluded that, while heat inactivation of lipase prevents the hydrolysis of storage lipids into free fatty acids, it also renders polar lipids more susceptible to oxidation. Although the findings of such studies still recognise the need for heat-inactivation of native lipases in oats, especially for oat fractions mixed with and stored in water, they also provide valuable insight into the complexities surrounding heat-treatment and highlights a need for milder treatments to optimise oat products for both storage and nutritional purposes.

Oat antioxidants and avenanthramides

In addition to essential amino acids, dietary fibres such as β-glucan, as well as unsaturated fatty acids, oats also contain a variety of antioxidants, such as tocols, phytic acid, and avenanthramides (Lehtinin *et al*., 2011). Produced in response to pathogen infection such as crown rust, avenanthramides are a group of phenolic compounds that, among other cereal grains, are only produced in oats (Wise, 2014). Their structure consists of a phenylpropanoid (either p-coumaric, ferric, or caffeic acid) conjugated with an anthranilic acid. In certain congeners, the anthranilic acid subunit can be hydroxylated or methoxylated (Wise, 2014). Avenanthramides are known to possess strong antioxidant properties that are primarily due to their radical scavenging activities, particularly for avenanthramide congeners containing hydroxyl and ortho methoxy groups (Fagerlund, 2009). While phenolic compounds are typically known to be connected to bitterness in foods (Soares *et al.*, 2013), studies have also found a positive correlation between avenanthramides and the fresh taste of oats, as well as a negative correlation between avenanthramides and rancid odor and flavour of oat porridge (Box, 2015). Meydani, M. (2009) summarised the potential health benefits of avenanthramides due to their strong antioxidant activities.



Nomenclature							
Dimberg	Collins	n	R_1	R_2	R_3	R_4	R_5
1p	D	1	H	H	H	OH	H
$1p_d$	L	2	H	H	H	OH	H
1f	E	1	H	H	OCH_3	OH	H
$1f_d$	M	2	H	H	OCH_3	OH	H
1c	F	1	H	H	OH	OH	H
$1c_d$	N	2	H	H	OH	OH	H
*1a		1	H	H	H	H	H
*1s		1	H	H	OCH_3	OH	OCH_3
2p	A	1	H	OH	H	OH	H
$2p_d$	**O	2	H	OH	H	OH	H
2f	B	1	H	OH	OCH_3	OH	H
$2f_d$	P	2	H	OH	OCH_3	OH	H
2c	C	1	H	OH	OH	OH	H
$2c_d$	Q	2	H	OH	OH	OH	H
*2a		1	H	OH	H	H	H
*2s		1	H	OH	OCH_3	OH	OCH_3
3p	X	1	OCH_3	OH	H	OH	H
$3p_d$	U	2	OCH_3	OH	H	OH	H
3f	Y	1	OCH_3	OH	OCH_3	OH	H
$3f_d$	V	2	OCH_3	OH	OCH_3	OH	H
3c	Z	1	OCH_3	OH	OH	OH	H
$3c_d$	W	2	OCH_3	OH	OH	OH	H
*3a		1	OCH_3	OH	H	H	H
*3s		1	OCH_3	OH	OCH_3	OH	OCH3
4p	G	1	OH	H	H	OH	H
$4p_d$	**R	2	OH	H	H	OH	H
4f	H	1	OH	H	OCH_3	OH	H
$4f_d$	S	2	OH	H	OCH_3	OH	H
4c	K	1	OH	H	OH	OH	H
$4c_d$	T	2	OH	H	OH	OH	H
5p	AA	1	OH	OH	H	OH	H
$5p_d$	OO	2	OH	OH	H	OH	H
5f	BB	1	OH	OH	OCH_3	OH	H
$5f_d$	PP	2	OH	OH	OCH_3	OH	H
5c	CC	1	OH	OH	OH	OH	H
$5c_d$	QQ	2	OH	OH	OH	OH	H

Figure 3 - Structure and nomenclature of the different avenanthramide congeners, taken from Wise (2014).

There has not been a lot of studies investigating avenanthramides in oats under different storage conditions and pre-treatment processes, and it is therefore of strong interest to study avenanthramides and their role in the oxidative stability of oats. Due to antioxidant levels already present, lipid oxidation in oats can occur over a long period of time, and storage trials involving raw oat flour can span months before meaningful changes are observed (Molteberg

et al., 1996). In order to study the lipid oxidation process under shorter periods of time, accelerated storage trials can therefore be conducted by adding prooxidants such as copper sulphate. Like other transition metal ions such as iron, copper sulphate can promote oxidation via Fenton-type reactions where, in its reduced form (Cu^{+}), copper ions act as electron donors and react with hydrogen peroxide, oxidising to Cu^{2+} leading to the formation of hydroxyl radicals (Figure 4; Thanonkaew *et al*., 2006).

$$H_2O_2 + \text{Fe(II) or Cu(I)} \rightarrow \bullet OH + OH + \text{Fe(III) or Cu(II)}$$

Figure 4 – Fenton-type reaction retrieved from Thanakaew et al. (2006)

Phenolic compounds such as avenanthramides typically exhibit antioxidant activity as a result of the resonance stabilization of phenoxyl radicals generated from oxidation, allowing them to serve as radical scavengers in the reaction matrix (Zhou and Elias, 2013). However, while phenolic compounds from plant extracts have been widely used as antioxidants in lipid-based foods, it should be emphasized that lipid oxidation is highly complex and, depending on factors such as pH and concentration, extracts that are originally added with the intent of delaying oxidation have been found to exhibit prooxidant activity instead (Chedea *et al.*, 2010; Zhou and Elias, 2013). Such a shift from antioxidative to prooxidative activity can also be exacerbated by the presence of transition metal ions. Metal-catalysed oxidation of phenolic compounds can lead to the regeneration of transition metal ions to their catalytically active reduced forms, further promoting oxidation (Figure 5; Zhou and Elias, 2013). This antioxidative/prooxidative balance should therefore be taken under careful consideration during experimental design and data interpretation with regards to this present study.

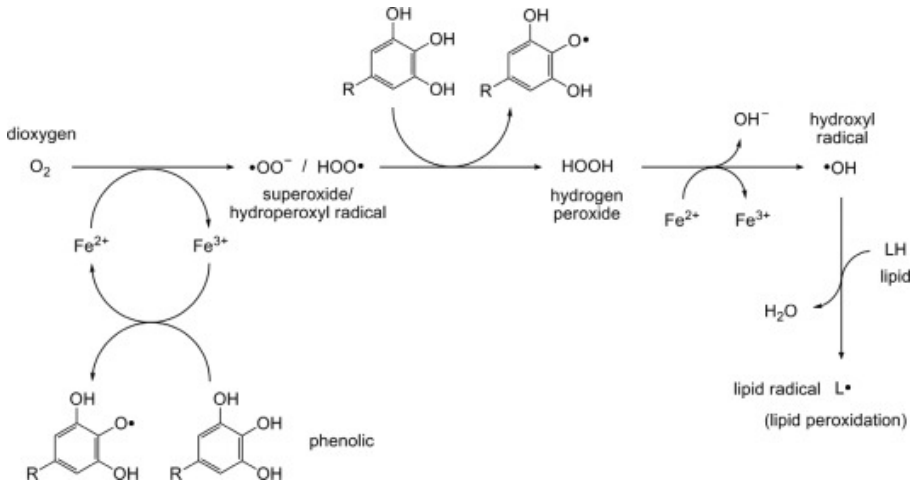


Figure 5 -Prooxidant activity of phenolic compounds in food lipids (Zhou and Elias, 2013)

Avenanthramides as a means to slow rancidity development in oats

Over the years, natural plant extracts have been used in both research and in industry to delay lipid oxidation and prolong storage capabilities in various types of food. Oats, for example, have been shown to increase the stability of foods and oils at frying temperatures (Wise, 2014). In this present study, avenanthramide-rich oat extracts were added to oat flour in accelerated storage trials. Oxidation and rancidity markers such as peroxide value (PV), lipase activity and hexanal detection followed over time to investigate the role of avenanthramides in oat rancidification, as well as their potential to work as preservatives in oats and possibly other foods as well.

Materials and Methods

Materials

Oat samples used in this study were from the cultivar “Belinda” from the year 2017 (Alnarp) and 2020 (Skara). Lantmännen provided the oat variety, which was then cultivated by researchers from ScanOats. The hulled oats were stored at room temperature, and were transferred to -80 ºC storage after dehulling and milling. Initially, the oats were first dehulled by hand and milled into flour with the Precellys 24 Bead-Mill Homogenizer, but as sample size increased over the progression of the project, they were dehulled with a dehuller and milled into flour with the Wondermill Electric Grain Mill. The MiliQ Purification System was used to purify the water used in the storage trials and all the analytical procedures. All other solvents used in this project were of analytical grade. Avenanthramide standards A, B and C were obtained from ReseaChem (Switzerland). Standards from lipid analysis were obtained from Larodan (Sweden). For Hexanal detection, Aldehyde/Ketone-DNPH stock standard at 15 µg/ml was obtained from Supelco (USA).

Methods

Avenanthramide extraction and analysis

Avenanthramides were extracted from oat samples based on a method previously developed by Pridal *et al.* (2018). Milled oat flour was suspended in citrate-phosphate buffer (pH 2.8, 80% EtOH) at a ratio of 1:10, vortexed (10 seconds), sonicated (10 minutes), and placed on a rotary shaker (20 minutes) before centrifugation at 10 000 rpm for 5 minutes to collect the supernatant. This process was repeated 3 times in total and pooled before evaporation and resuspension in MeOH, such that the ratio between the starting material and final extract is 1:1. Avenanthramide extractions performed during preliminary characterisations were evaporated with nitrogen gas, while later extractions used a rotary evaporator at 20ºC and 50ºC. The resulting extracts were then analysed with liquid chromatography-UV detection/Mass spectrophotometry (LC-UV/MS), with H_2O (0.1% formic acid) as mobile phase A and acetonitrile (0.1% formic acid) as mobile phase B. The column used was Phenomenex Kinetex® 2.6 µm C18 100 Å, 50x2.1 mm. The column was heated to 40ºC with an injection

volume of 5 µl and flow rate of 0.5 ml/min. The gradient program used is shown Table 1. In addition to analysis with LC-UV/MS, the avenanthramide-rich oat extracts were also added to milled oat flour in accelerated storage trials to observe their effect on peroxide value (PV), lipase activity and aldehyde levels. MS measurements were taken using Velos Pro mass spectrometer using electrospray ionisation (ESI) in positive and negative mode, at a range of 110 to 2000 m/z. The heating temperature was 230 ºC, with a sheath, auxiliary and sweep gas flow rate of 60, 30 and 1 arb respectively. Spray voltage was set to 3 kV, capillary temperature was set to 380 ºC and S-Lens RF level was kept at 35%.

Table 1 - Gradient program for LC-UV/MS analysis of avenanthramides in oat sample, with with H_2O (0.1% formic acid) as solvent A and acetonitrile (0.1% formic acid) as solvent B.

Time (min)	Solvent A (%)	Solvent B(%)
0	85	15
3	85	15
12	35	65
15	35	65
16	0	100
18	0	100
19	85	15

Lipid extraction

Lipids were extracted from oat samples using a chloroform:methanol extraction method adapted from Bligh and Dyer (1959). For 100-200 mg oat flour, 3.75 ml chloroform: methanol (2:1 v/v, 0.05% BHT w/v) was added in glass tubes for the extraction of oat lipids. Widely considered as the quintessential universal lipid solvent, chloroform has been used for decades for lipid extraction purposes, and works by disrupting the hydrogen bonding between lipids and their surrounding proteins, thereby promoting their release and subsequent extraction (Schaich, 2016). Methanol is added to chloroform to reach a chloroform:methanol ratio of 2:1 in order to aid extraction of polar lipid fractions (Schaich, 2016). In order to create a two-phase system for lipid extraction, this was then followed by the addition of 1 ml 0.15M acetic acid, 1.25 ml chloroform and 1.25 ml water, with the tubes being vortexed between each addition. After centrifugation at 2000 g for 6 minutes, the resulting lower chloroform phase was then extracted using a glass pipette, and transferred into a separate vials for further analysis. The

whole procedure was performed on ice in order to prevent lipids from oxidising during the extraction process.

Lipid class separation, methylation and analysis with gas chromatography (GC)

After transferring part of the extracted chloroform phase for other analyses (e.g. 1 ml for PV, 250 µl for TLC analysis), the remaining chloroform phase was evaporated with nitrogen gas at room temperature until dryness, and resuspended in 1 ml chloroform. This was then fractionated using Telos NH2 1ml SPE columns (Kaluzny *et al*. 1985). The columns were first washed with 1 ml hexane twice, before loading the samples to the column. Neutral lipids were eluted from the column with 1 ml chloroform:2-propanol (2:1), free fatty acids were eluted with 1 ml 2% acetic acid in diethyl ether, glycolipids were eluted with 1 ml chloroform:methanol (9:1), and finally, phospholipids were eluted with 1 ml methanol. The resulting fractions were evaporated with nitrogen gas at room temperature until dryness.

For GC analysis, the fractions were first methylated by adding 2ml H_2SO_4 (2% in methanol) and placing on a heat block at 90 ºC for 30 minutes. This was followed by adding 2 ml each of water and cyclohexane, after which the fractions were vortexed for 10 seconds. After centrifugation at 2500 g for 5 minutes, the resulting upper phase was transferred into new tubes and evaporated under nitrogen gas until dryness. Samples were then resuspended in 250 µl cyclohexane and transferred to vials for analysis with GC. Settings for GC were used based on Lindberg-Yilmaz *et al.* (2020).

Analysis of lipid composition by high-performance thin-layer chromatography (HPTLC)

Lipid composition of extracted oat lipids and avenanthramides were analysed using HPTLC. 3 µl of each sample, including standards, were injected onto the TLC plate twice, totalling 6 µl for each sample. The plate was then developed manually in a developing chamber, with heptane, diethylether and acetic acid (70:30:1) for neutral lipid separation and chloroform, methanol and water (95:20:2.5) for polar lipid separation. After development, the plate was then sprayed with primulin and visualised under UV light.

Determination of peroxide value (PV)

PV was determined based on a ferric thiocyanate method previously developed by Shantha and Decker (1994) and with minor additional modifications in the lab for the oat material. Lipid hydroperoxides present in the extracted oat lipids oxidise ferrous ions to ferric ions, which subsequently react with ammonium thiocyanate to produce ferric thiocyanate, which is detectable at 500 nm (Frankel, 2005; Undeland et al., 2002). A proportion of extracted lipids (1 ml) in chloroform (see Lipid Extraction) was combined with 1 ml methanol to reach a chloroform:methanol ratio of 1:1. This was followed by the addition of 1.33 ml ice-cold chloroform:methanol 1:1 to dilute the sample, after which 33.4 µl each of ammonium thiocyanate and iron (II) chloride respectively, with vortexing in between additions. Following incubation for 20 minutes at room temperature, hydroperoxide levels in the extracted oat lipids was determined by spectrophotometric detection at 500 nm. A standard curve of 0, 4, 8, 12, 16 and 20 µM cumene hydroperoxide dissolved in chloroform:methanol 1:1 was used to calculate final peroxide values.

Determination of lipase activity

Lipase activity was determined with a p-nitrophenyl butyrate (pNPB) assay. Lipase hydrolyses p-NPB into p-nitrophenol and butyric acid, the former of which can be detected spectrophotometrically at 400 nm (Shirai and Jackson, 1982). Enzyme solution was prepared by suspending 100-150 mg oat flour in 900 µl sodium phosphate buffer. The pNPB assay was performed using 100 mM sodium phosphate buffer (pH 7.2), at a starting concentration of 0.5 mM pNPB, and 10% (v/v) enzyme solution. The assay was conducted in a 96-well plate, where each individual reaction had a total volume of 200 µl. Reaction rate was determined using a plate reader measuring absorbance at 400 nm, with measurements taken every second for 2-5 minutes at 37ºC. All preparations for the assay were conducted on ice, with p-NPB added immediately prior to activity measurement.

Analysis of aldehydes

Aldehyde levels in the oat samples were analysed according to Matthew et al. (2011) and Tullberg et al. (2016). Water was added to 100-200 mg oat flour in order to reach a 500 µl sample. This was followed by the addition of 20 µl 25% Butylated hydroxytoluene (BHT, w/v) dissolved in ethanol, 40 µl 0.02 M EDTA dissolved in water, and 1 ml 0.25 M hydrochloric

acid in water. The oat samples were then vortexed and left for 5 minutes in room temperature, before centrifuging the samples and transferring the supernatant into new vials. 100 µl 2,4-Dinitrophenylhydrazine (DNPH) was added to the transferred supernatant for derivatization. DNPH has an aromatic hydrazine group, and, under acidic conditions, reacts with aldehydes and ketones to form hydrazones, which can be separated and detected using LC-UV/MS. DNPH was incubated with the supernatant for 1 hour at room temperature, after which 500 µl dichloromethane was added to create a 2-phase system. The lower phase was collected and evaporated under nitrogen gas at room temperature until dryness, and resuspended in 300 µl acetonitrile for analysis with LC-UV/MS. For LC-UV/MS analysis, water was used as mobile phase A and acetonitrile as mobile phase B. The column used was Kinetex® 2.6 µm C18 100 Å, 150x2.1 mm, and was kept at 20ºC with an injection volume of 5 µl and flow rate of 0.3 ml/min. The gradient program used is shown in Table 2. MS measurements were taken using Velos Pro in positive and negative mode, at a range of 110 to 1000 m/z and 110 to 2000 m/z respectively. The heating temperature was 250 ºC, with a sheath, auxiliary and sweep gas flow rate of 22, 10 and 1 arb respectively. Spray voltage was set to 3 kV, capillary temperature was set to 275 ºC and S-Lens RF level was kept at 66%.

Table 2 - Gradient program for LC-UV/MS analysis of aldehydes in oat samples, with water as solvent A and acetonitrile as solvent B.

Time (min)	Solvent A (%)	Solvent B(%)
0	55	45
10	45	55
20	48	62
25	8	92
26	8	92
28	55	45
33	55	45

Experimental design and workflow

Prior to conducting the accelerated storage trials, preliminary characterisations were performed on reference oat samples to gain a better understanding of oat lipid composition. Oat lipids were extracted for class separation and GC analysis. Avenanthramides were also extracted and analysed with LC-UV/MS to verify extraction technique used for the study.

After preliminary characterisations, two accelerated storage trials were conducted with the milled oat flour under room temperature conditions. Samples were enriched with avenanthramide-rich oat extracts to observe the effect of avenanthramide enrichment on lipid oxidation. The methanol-dissolved avenanthramide extracts were added so that the quantity of starting material used to make the oat extract was equal to the quantity of the milled oat flour the extracts were added to (e.g. 150 µl of avenanthramides in methanol extracted from 150 mg oats was added to 150 mg of milled oat flour). Storage trials were conducted with a final concentration of 0.67 mM copper sulphate ($CuSO_4$) in order to accelerate the lipid oxidation process. As negative controls, the milled oat flour was also stored dry, with a water-methanol mixture, and finally with a water-methanol mixture with 0.1 mM $CuSO_4$ to achieve a final concentration of 0.67 mM.

In the first storage trial, time points were taken at 0, 5 and 72 hours for analysis of PV, lipase activity and aldehyde levels. This was decreased to 0, 2 and 24 hours in the second storage trial. All samples were placed in -80 ºC storage after the storage trial and sample preparation until analysis, with the exception of samples prepared for analysis of PV and lipase activity in the first storage trial. Samples used for PV analysis in the first storage trial were placed in -20ºC storage after lipid extraction for 1 week prior to analysis. Samples used for lipase activity in the first storage trial were placed in -20ºC storage after enzyme solution preparation overnight prior to analysis. This was later rectified in the second storage trial, where all samples were placed either on dry ice or in -80ºC storage whenever possible to prevent oxidation and enzymatic activity during sample preparation. In the second storage trial, avenanthramide extracts were concentrated five fold, and total liquid volume was increased to 300 µl, thereby reducing the methanol concentration of the liquid added to 10% in order to prevent methanol inhibition when determining lipase activity. Details of the different storage conditions used for the first and second storage trial are shown in Table 3 and 4, respectively.

Table 3 - Dtails of different storage conditions used for first storage trial. All concentrations mentioned are calculated prior to mixing the system with 0.15g milled oat flour.

Sample	Storage conditions	Concentration of MeOH (% v/v)	Concentration of $CuSO_4$ (mM)	Avenanthramide enrichment
CT1	0.15 g dry oat flour	0	0	N

CT2	0.15 g oat flour in 200µl water-methanol mixture	75%	0	N
CT3	0.15 g oat flour in 200µl water-methanol mixture	75%	0.1	N
AE2	0.15 g oat flour in 200µl water-methanol mixture	75%	0	Extracted from 0.15 g oats, dissolved in 150 µl MeOH
AE3	0.15 g oat flour in 200µl water-methanol mixture	75%	0.1	Extracted from 0.15 g oats, dissolved in 150 µl MeOH

Table 4 - Details of different storage conditions used for second storage trial. All concentrations mentioned are calculated prior to mixing the system with 0.15g milled oat flour.

Sample	Storage conditions	Concentration of MeOH (% v/v)	Concentration of $CuSO_4$ (mM)	Avenanthramide enrichment
CT1	0.15 g dry oat flour	0	0	N
CT2	0.15 g oat flour in 300µl water-methanol mixture	10%	0	N
CT3	0.15 g oat flour in 300µl water-methanol mixture	10%	0.1	N
AE2	0.15 g oat flour in 300µl water-methanol mixture	10%	0	extracted from 0.15 g oats, dissolved in 30 µl MeOH
AE3	0.15 g oat flour in 300µl water-methanol mixture	10%	0.1	extracted from 0.15 g oats, dissolved in 30 µl MeOH

The data from the second storage trial was then analysed with the statistical software Jamovi, where analysis of variance (ANOVA) tests were conducted to determine the significance of differences observed (p-value < 0.05) as well as the effective strength of each factor investigated (F-value).

Results and Discussion

Preliminary Characterisations

Total lipid and fatty acid composition

A total of 0.025 g of lipids was successfully extracted from 16 oat kernels totalling 0.485 g, which translates to a total lipid content of around 5.2 %. Prior to class separation, internal standards (50 µl 5 mg/ml each of C15 in triacylglycerol form for neutral lipids, C17 heptadecanoic acid for free fatty acids) were added to the oat lipids. The lipids were then separated by class into neutral lipids (NL), free fatty acids (FFA), glycolipids (GLY) and phospholipids (PHO) on SPE columns. In addition to the different fractions, the initial flow through from the SPE column was also collected for GC analysis.

Figure 6 shows the chromatograms of the four fractions, while Table 5 shows the peak area (pA*min) of the fatty acids of interest. GC analysis showed that, in all fractions, the fatty acid composition of the extracted oat lipids primarily consists of linoleic, oleic and palmitic acid. Between the four fractions, NL had the highest peak areas for all three fatty acids, suggesting that majority of oat lipids consist of neutral lipids. In both NL and FFA, oleic acid had the highest peak area, followed by linoleic acid and finally palmitic acid. This is slightly different in GLY and PHO, where linoleic acid had the highest peak area. Analysis of the initial flow through from the SPE column showed large quantities of the three main fatty acids (2.68 times more palmitic acid, 3.03 times more oleic acid, and 2.64 times more linoleic acid compared to the NL fraction), as well as the internal standard C15 pentadecanoic acid (8.11 times more compared to the NL fraction). The internal standard C17 (heptadecanoic acid) could also be detected in the NL fraction. It can therefore be speculated that the level of neutral lipids and free fatty acids may be higher than what was detected. A plausible explanation for this could be the overloading of lipids in the column, as well as leftovers in the bottom of the column from previous elutions being collected in the next elution as well. The findings of the GC analysis is in line with what has been already published in literature and summarised in the introduction, particularly with regards to total lipid and fatty acid composition (Lehtinen *et al.*, 2011).

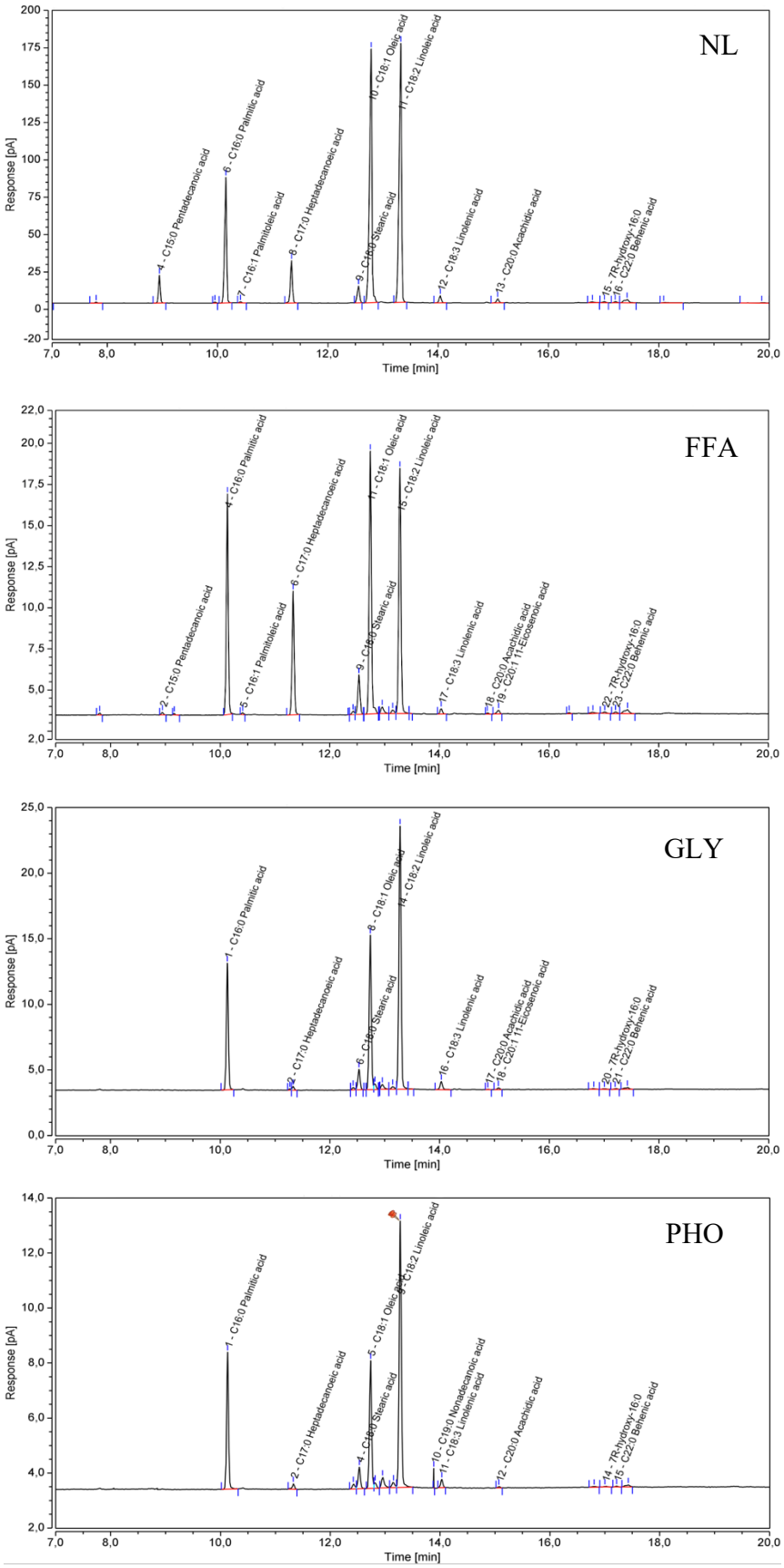


Figure 6 - GC chromatograms of extracted oat lipids. The extracted lipids were class separated on SPE columns into neutralipids (NL), free fatty acid (FFA), glycolipids (GLY) and phospholipids (PHO) before running on GC.

*Table 5 - Peak area (pA*min) of fatty acids of interest for each lipid class, including the initial SPE column flow through. "nil" denotes cases where fatty acids were not detected in the lipid fraction.*

	Peak area (pA*min)				
	Flow Through	NL	FFA	GLY	PHO
C15 Pentadecanoic acid (internal standard NL)	5635	695	nil	nil	nil
C17 Heptadecanoeic acid (internal standard FFA)	nil	1238	327	nil	nil
C16 Palmitic acid	9550	3562	546	399	209
C18:1 Oleic acid	27080	8924	728	525	209
C18:2 Linoleic acid	23084	8743	674	910	444

Avenanthramide extraction and analysis

Figure 7 shows the LC-UV chromatogram of an external standard mix (5 µg/ml) of avenanthramide A, B and C, as well as the corresponding mass spectra of each peak. Avenanthramide C is the first to pass through the column, with a retention time of around 2.5 minutes, followed by avenanthramide A at around 4.4 minutes, and finally avenanthramide B at 5.6 minutes. The identity of the avenanthramide congeners were confirmed with mass spectrometry, where avenanthramide C has a m/z of 314.14, avenanthramide A a m/z of 298.15, and avenanthramide B a m/z of 328.10.

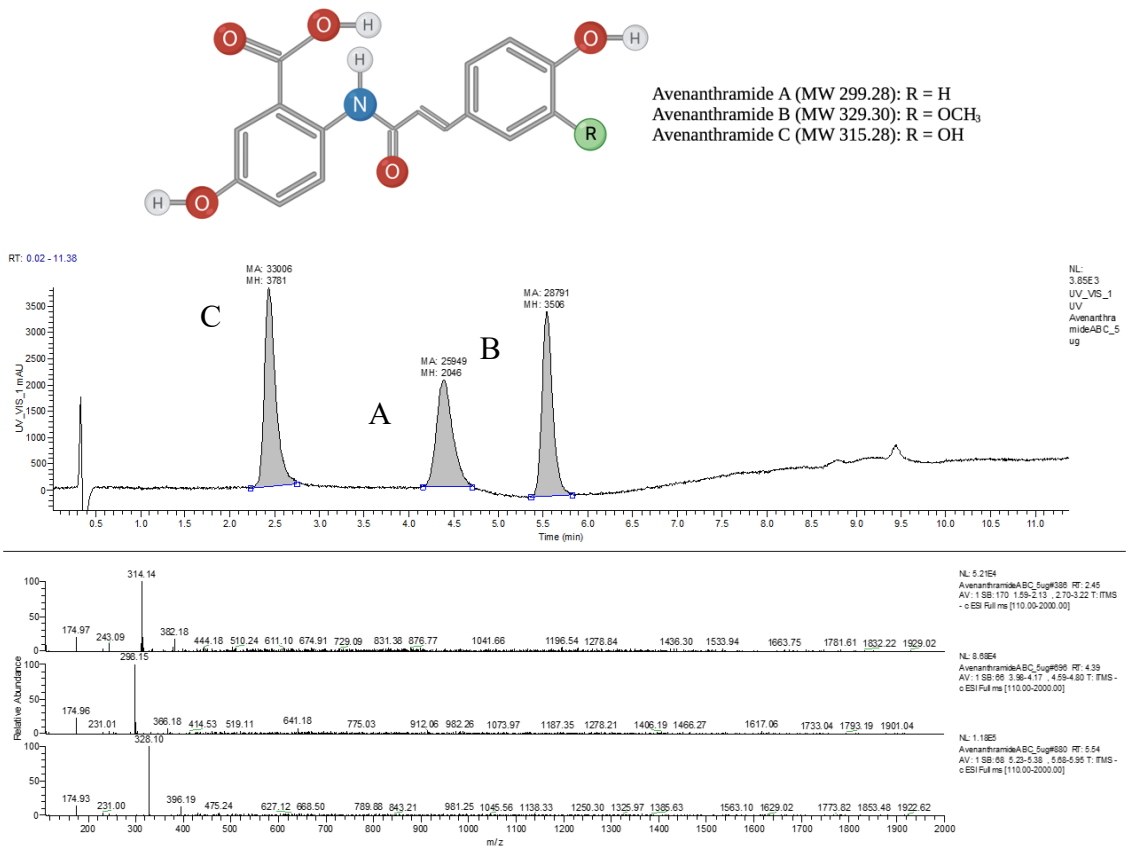


Figure 7 - LC-UV chromatogram of external standard mix of 5 µg/ml of avenanthramide A, B and C (labelled), with the corresponding mass spectra of each peak. UV was detected at 340 nm. The chemical structure of avenanthramides A, B and C, including their respective molecular weights, are also included at the top (sketched with the software Biorender). Molecular sketch of the avenanthramide molecule was created with BioRender.com

Avenanthramide extract from 0.1 g fresh oat flour was evaporated and resuspended in 100 µl methanol prior to analysis on LC-UV/MS. Figure 8 shows the LC-UV chromatogram of the extract. Three peaks (shaded in grey) can be seen in the chromatogram, with very similar retention times (2.5, 4.5 and 5.6 minutes respectively) and m/z (314.03, 298.09 and 326.10 respectively) to those of the external standard mix previously described, thereby supporting the identity and presence of the three avenanthramide congeners (A, B and C) in the oat extract. Other peaks can also be visualised in the LC-UV chromatogram, suggesting that, in addition to avenanthramides A, B and C, perhaps other compounds were also extracted in the process, possibly other avenanthramide congeners or phenolic compounds in general.

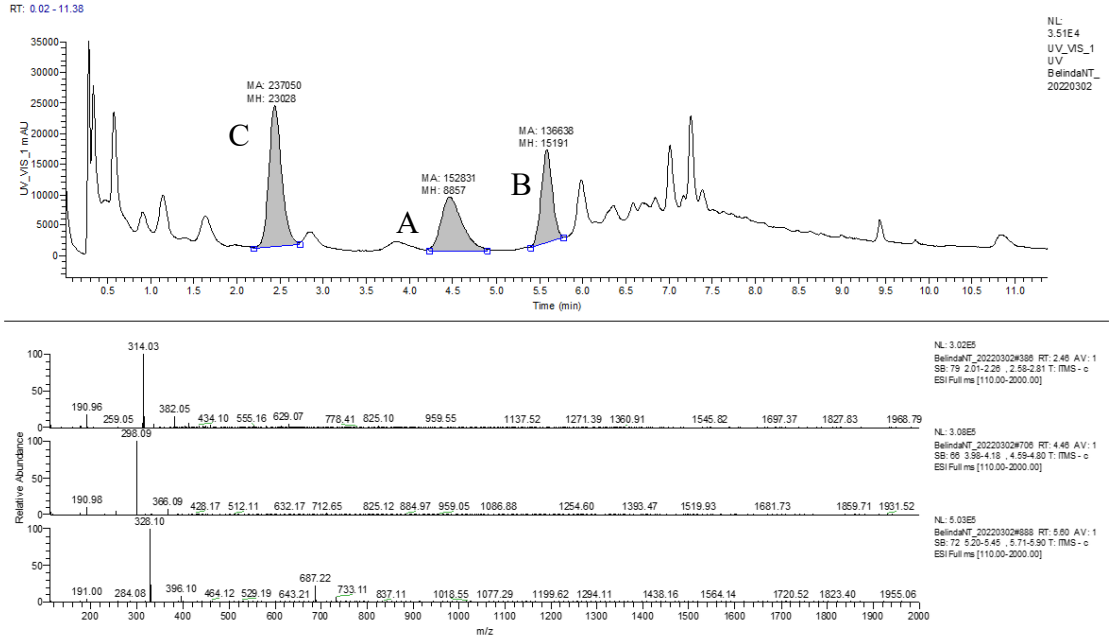


Figure 8 - LC-UV chromatogram of avenanthramides extracted from 0.1 g reference oats and dissolved in 100 µl methanol. Avenanthramides A, B and C are labelled, with the corresponding mass spectra below. UV was detected at 340 nm.

To investigate the impact of long-term storage and heat treatment on avenanthramide levels, avenanthramides were also extracted from oat kernels that were de-hulled, heat treated to 100ºC and stored in room temperature for 1 year. Similar to the fresh oats, avenanthramide extract from 0.1 g oat flour was evaporated and resuspended in 100 µl methanol prior to analysis on LC-UV/MS. Figure 9 shows the LC-UV chromatogram of the resulting extract. Interestingly, three peaks (in grey) can also be seen in the chromatogram with very similar retention times and m/z to those of the external standard mix previously described, although the area of the peaks of interest is lower than those of the fresh sample. The peak areas of avenanthramide A, B and C was 67%, 73% and 84% that of the non-heat treated oats respectively. While some of the avenanthramides may have been used up or degraded in the storage and heating process, the fact that there are still avenanthramides present in the resulting oat extracts suggest that the avenanthramides in the oat kernel were, to a certain extent, heat stable.

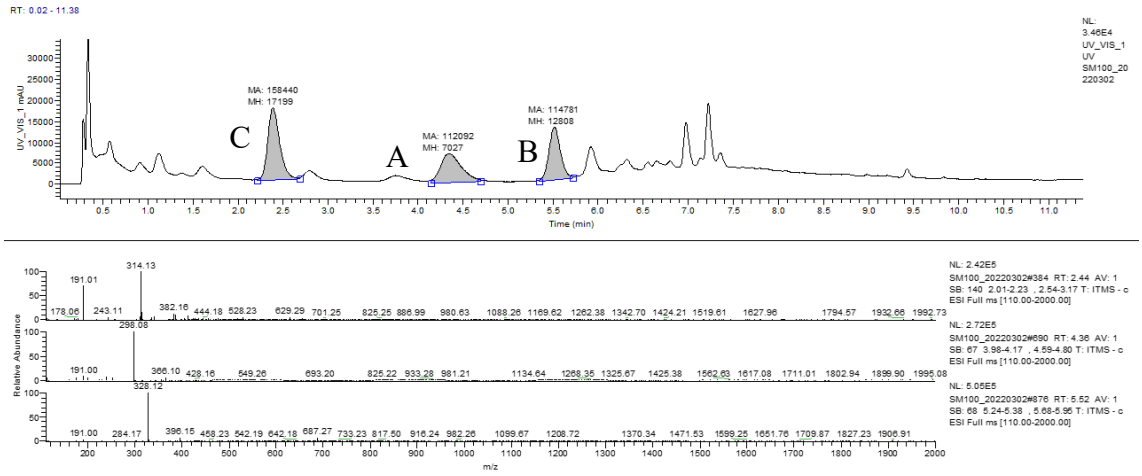


Figure 9 - LC-UV chromatogram of avenanthramides extracted from 0.1 g oats and dissolved in 100 µl methanol. The oats were dehulled, heat-treated to 100 ºC and stored in room temperature for 1 year. Avenanthramides A, B and C are labelled, with the corresponding mass spectra below. UV was detected at 340 nm.

Storage Trial 1

In the first storage trial, samples were stored for 0, 5 and 72 hours in the above described conditions prior to analysis of PV, lipase activity, and hexanal detection. Figure 10 shows the PV of each storage condition at the three given timepoints, where it can be seen that the oat flour samples stored in water-methanol mixtures, including those that were avenanthramide enriched, were much lower compared to oat flour that was stored dry, indicating a lower level of lipid hydroperoxides at time of PV measurement.

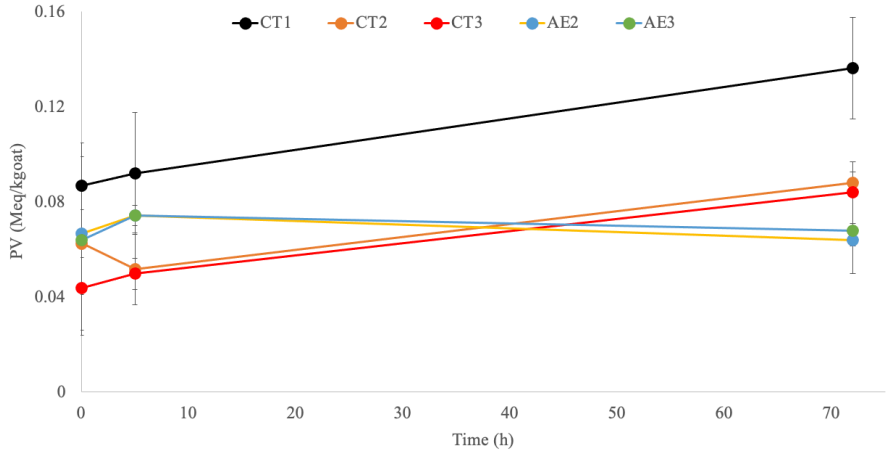


Figure 10 - PV analysis from the first storage trial. PV values are shown for 0, 5 and 72 hours after storage in each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM $CuSO_4$ (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM $CuSO_4$ (AE3)

Water is known to accelerate oxidation processes by plasticising the matrix of the sample, thereby promoting access to oxidation-inducing reactants and catalysts (Karel, 1980). One would therefore expect storage conditions where oat flour is mixed with water and methanol to have a higher level of lipid oxidation compared to oat flour that is stored dry. Since the extracted oat lipids were only stored at -20ºC and not -80ºC for a week prior to analysis, it is possible that, for the samples stored in water-methanol mixtures, lipid oxidation had already occurred and reached the end-stages by the time of analysis, resulting in lower peroxide values than the oat flour that was stored dry. This was later rectified in the second storage trial, where samples were stored at -80ºC or on dry ice whenever possible to prevent lipid oxidation from occurring outside the duration of the storage trial. The second storage trial was also shortened from 0, 5 and 72 hours to 0, 2 and 24 hours in hopes of better visualising the differences in lipid peroxide levels.

Figure 11 shows the lipase activity of each storage conditions at the three given timepoints in the first storage trial. It can be seen that that the oat flour samples stored in water-methanol mixtures, regardless of pro-oxidant concentration, were considerably lower compared to oat flour that was stored dry (approximately 22-23 U/mg oat). Lipase activity was completely lost in oat flour that was stored with avenanthramide enrichment (0 U/mg oat), and while oat flour without added avenanthramide enrichment still had some detectable lipase activity (approximately 2 U/mg oat) at hour 0, it was completely lost after hour 5.

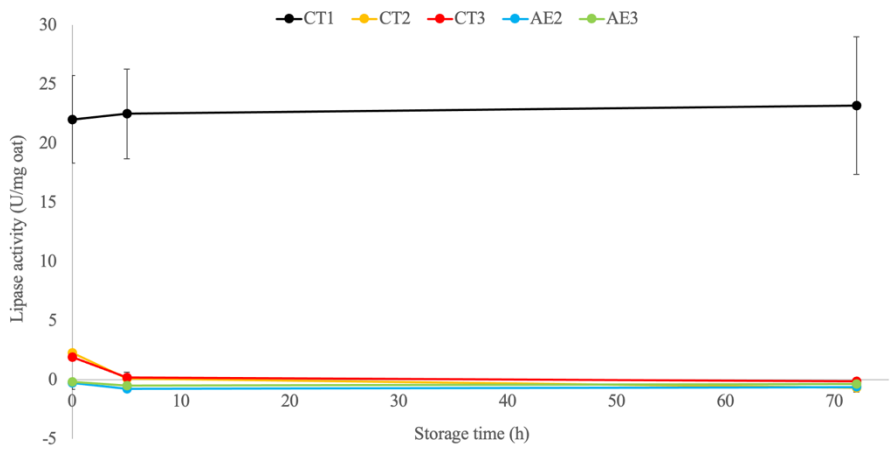


Figure 11 – Lipase activity from the first storage trial. Lipase activities are shown for 0, 5 and 72 hours after storage in each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol

One possible reason for the decrease in lipase activity is methanol inhibition. With the exception of the dry storage control (CT1), all other storage conditions involved mixing 0.15 g of oats with 200µl 75% v/v methanol. While not the case for all lipases (Samtambrogio *et al.*, 2013), methanol has been reported in literature to inhibit lipase activity (Lotti *et al.*, 2015). Alcohols such as methanol can result in the unfolding of the enzyme's tertiary structure, and can also act as competitive inhibitors (Lotti *et al.*, 2015). That being said, a small amount of lipase activity was still detected in oat flour without avenanthramide enrichment, suggesting that avenanthramide enrichment may have an added inhibitory effect on lipase activity. In order to better visualise the effect of avenanthramide enrichment, methanol concentration of the water-methanol mixture added to the oat flour was lowered from 75% to 10% in the second storage trial in order to minimise the inhibitory effect of methanol on lipase activity.

Figure 12 shows the levels of hexanal that was detected in each oat sample. As priority was placed on PV analysis and lipase activity determination, only one sample was taken for aldehyde derivatisation and analysis from each storage condition, as opposed to triplicates. Surprisingly, hexanal was only detected in oat samples with avenanthramide enrichment (AE2 and AE3), with levels increasing over time. Oat samples with avenanthramide enrichment and added pro-oxidant (AE3) had higher levels than samples without pro-oxidant (AE2). However, it is worth noting that the levels of hexanal detected were out of range of the standard curve used for quantification (below 1 µg/ml), and since the data for hexanal detection was not analysed in detail until after the second storage trial had already been conducted, this was not rectified until after both storage trials were completed. Hence, only the relative peak areas for hexanal detection are shown.

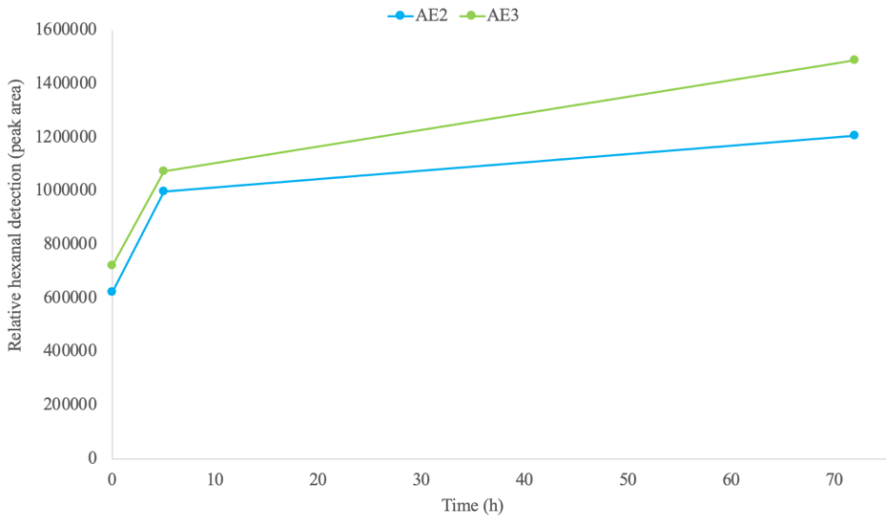


Figure 12 – Relative hexanal detection from the first storage trial. Hexanal levels are shown for 0, 5 and 72 hours after storage in each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM CuSO4 (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM CuSO4 (AE3). Hexanal was only detected in AE2 and AE3.

Storage Trial 2

Based on the findings of the first storage trial, a second storage trial was designed with earlier time points (0, 2 and 24 hours as opposed to 0, 5 and 72 hours) in order to better visualise the differences in PV. With the exception of the dry storage (CT1), the methanol concentration water-methanol mixture mixed with the oat flour (300µl with 0.15 g oat) was reduced from 75% to 10% v/v. This was done by concentrating the avenanthramide extracts by five fold, such that avenanthramides from the same quantity of starting material could be added to the oat flour with less methanol.

Figure 13 shows the PV of oat flour in each storage condition in the second storage trial. The biggest difference in PV between avenanthramide enriched (AE2, AE3) and non-avenanthramide enriched (CT2, CT3) samples can be seen after 2 hours, regardless of whether or not pro-oxidants were added to the system. CT2 and CT3 had a PV of 0.18 and 0.20, and AE2 and AE3 had a PV of 0.12 and 0.11 respectively. The PV of of CT2 and CT3 decreases after 24 hours, while that of AE2 and AE3 increases. An ANOVA test was conducted to further analyse the PV of each storage condition (with the exception of CT1) after 0, 2 and 24 hours (Table 6), and it was found that avenanthramide enrichment had a statistically significant effect

on PV compared to the other treatments after 2 and 24 hours ($p < 0.001$). The effect of avenanthramide enrichment was strongest at the 2 hour timepoint ($F = 64.5708$) as opposed to the 24 hour timepoint ($F = 29.07$). CT1 was not included in the statistical analysis as the storage conditions were drastically different from the rest of the storage conditions (stored dry).

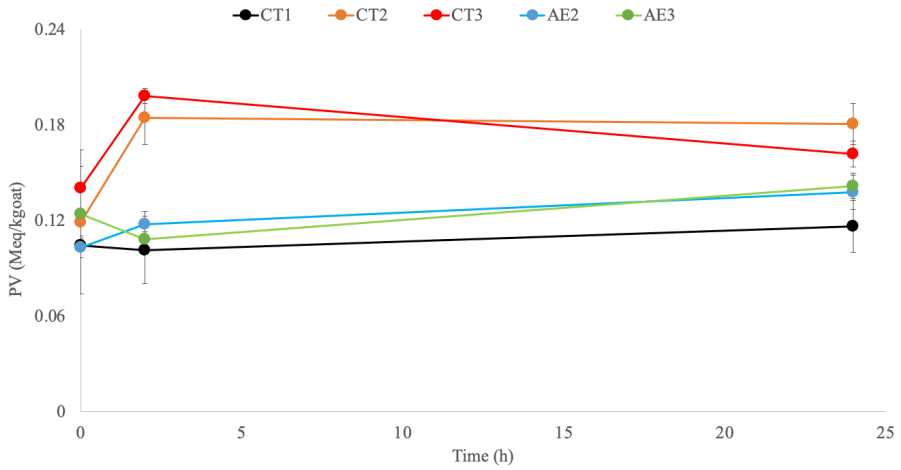


Figure 13 - PV analysis from the second storage trial. PV values are shown for 0, 2 and 24 hours after storage in each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM $CuSO_4$ (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM $CuSO_4$ (AE3)

Table 6 - ANOVA test comparing PV after 0, 2 and 24 hours of the oat flour from each storage condition (with the exception of CT1). Avenanthramide enrichment was found to have a significant effect on PV after 2 and 24 hours.

ANOVA - Initial PV

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	7.91e−4	1	7.91e−4	0.934	0.362
Pro-oxidant Concentration (mM)	0.00136	1	0.00136	1.609	0.240
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	6.05e−8	1	6.05e−8	7.14e−5	0.993
Residuals	0.00677	8	8.47e−4		

ANOVA - PV after 2 hours

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	0.01846	1	0.0185	64.5708	<.001
Pro-oxidant Concentration (mM)	1.21e−5	1	1.21e−5	0.0425	0.842
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	3.90e−4	1	3.90e−4	1.3632	0.277
Residuals	0.00229	8	2.86e−4		

ANOVA - PV after 24 hours

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	0.00298	1	0.00298	29.07	<.001
Pro-oxidant Concentration (mM)	1.63e−4	1	1.63e−4	1.59	0.243
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	3.90e−4	1	3.90e−4	3.80	0.087
Residuals	8.20e−4	8	1.03e−4		

Figure 14 shows the lipase activity of each storage conditions at the three given timepoints in the second storage trial. Compared to the lipase activity observed in the first storage trial, it is clear that lowering the concentration of methanol in the storage trials decreased lipase inhibition. There is also a considerably larger difference between the lipase activity of oat samples stored with and without avenanthramide enrichment, and samples with avenanthramide enrichment (AE2, AE3) were observed to have a substantially lower lipase activity compared to samples without (CT2, CT3). An ANOVA test was also conducted to further analyse the initial lipase activity of each storage condition (again, with the exception of CT1) at hour 0, 2 and 24 (Table 7), and it was found that, in addition to its effect on PV, avenanthramide enrichment also had a statistically significant effect on lipase activity after 0, 2 and 24 hours of storage ($p < 0.001$).

Unexpectedly, pro-oxidant concentration was also found to have a significant effect on lipase activity at after 0 and 2 hours of storage ($p = 0.02$), but no significant effect was found after 24 hours. Interactions between pro-oxidant concentration and avenanthramide enrichment was also found to be statistically significant after 2 ($p = 0.014$) and 24 ($p = 0.004$) hours. F-values for avenanthramide enrichment are much greater than pro-oxidant concentration, suggesting that avenanthramide enrichment has a much stronger effect on lipase activity. The effect of avenanthramide enrichment on lipase activity was further investigated in additional experiments by looking at the lipid composition of the resulting oat flour samples.

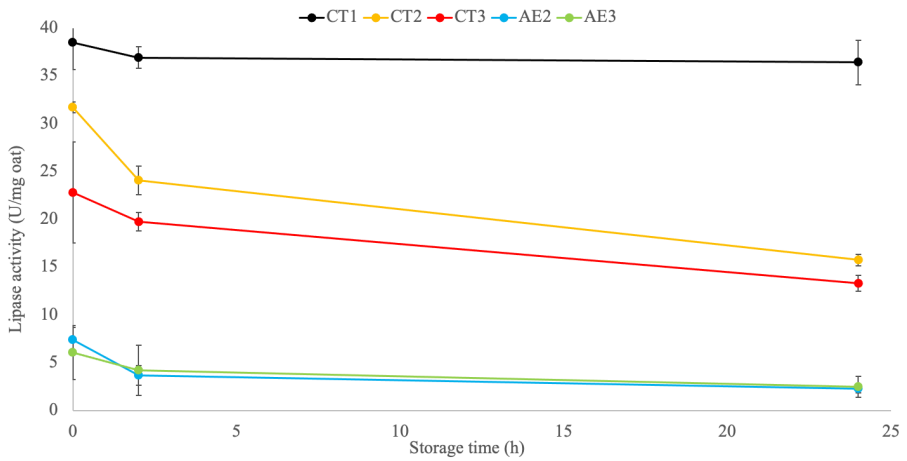


Figure 14 - Lipase activity from the second storage trial. Lipase activities are shown for 0, 2 and 24 hours after storage in each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM $CuSO_4$ (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM $CuSO_4$ (AE3).

Table 7 - ANOVA test comparing lipase activity at 0, 2 and 24 hours of the oat flour for each storage condition (with the exception of CT1). Avenanthramide enrichment and pro-oxidant concentration was found to have a significant effect on initial lipase activity, as well as lipase activity after 2 hours of storage.

ANOVA - Initial Lipase Activity

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	1261.5	1	1261.55	133.98	<.001
Pro-oxidant Concentration (mM)	79.3	1	79.27	8.42	0.020
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	42.9	1	42.92	4.56	0.065
Residuals	75.3	8	9.42		

ANOVA - Lipase Activity after 2 Hours

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	860.16	1	860.16	579.81	<.001
Pro-oxidant Concentration (mM)	9.62	1	9.62	6.48	0.038
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	15.52	1	15.52	10.46	0.014
Residuals	10.38	7	1.48		

ANOVA - Lipase Activity after 24 Hours

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	416.39	1	416.387	823.86	<.001
Pro-oxidant Concentration (mM)	1.72	1	1.724	3.41	0.102
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	8.41	1	8.407	16.63	0.004
Residuals	4.04	8	0.505		

Figure 15 shows the levels of hexanal that was detected in each oat sample in the second storage trial. It can be seen that the hexanal levels in the second storage trial were inconsistent with the first storage trial. As a result of the findings of the first storage trial, a control was prepared with just avenanthramide extract dissolved in methanol and water for derivatisation with DNPH and aldehyde analysis as well. Hexanal was also detected in the avenanthramide control (66% of that detected in CT1 at time point 0). Since only one sample was taken for aldehyde derivatisation and analysis from each storage condition, no meaningful conclusions can be drawn from this alone. Additional experiments were therefore conducted to gain further insight to avenanthramide enrichment on hexanal levels.

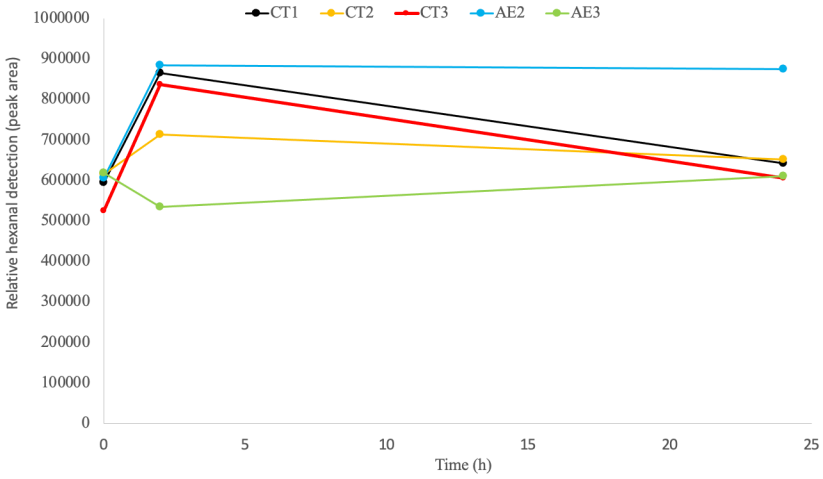


Figure 15 - Relative hexanal detection from the second storage trial. Hexanal levels are shown for 0, 2 and 24 hours after storage for each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM CuSO4 (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM CuSO4 (AE3).

Supplementary Experiments

To further investigate the effect of avenanthramide enrichment on hexanal levels and lipid composition in oat samples, an additional storage trial was conducted with the exact same conditions as storage trial 2. Samples were taken at 0 and 24 hours for analysis of aldehydes to determine hexanal levels, as well as lipid extraction and subsequent analysis with HPTLC to determine lipid composition.

In order to concentrate the samples for analysis, the quantity of oat flour used for analysis was increased to 200 mg from 150 mg, and the derivatised aldehydes were resuspended in 200 µl acetonitrile instead of 300 µl. However, despite the efforts to concentrate the samples, levels of hexanal detected were still out of range of the standard curve used for quantification (below 1 µg/ml). Hence, only the relative peak areas for hexanal detection are shown. Hexanal levels after 0 and 24 hours of storage are shown in Figure 16, and the same findings are also presented in a box plot (Figure 17) to better visualise the effects of avenanthramide enrichment and pro-oxidant concentrations. Avenanthramide enrichment was observed to have a statistically significant effect on hexanal levels after 24 hours of storage (Table 8), and the difference is most prominent in oat samples without added pro-oxidant ($CuSO_4$). One of the oat samples that was stored with avenanthramide extract and pro-oxidant (AE3) taken at 0 hours had

considerably higher hexanal levels than expected (Figure 16 and 17), which is possibly due to biological variance or human error. Hexanal levels in the additional trial (Figure 16) are slightly lower than those in the second storage trial (Figure 15), despite having the same storage conditions, perhaps due to human error and slight differences in handling between batches of milled oat flour.

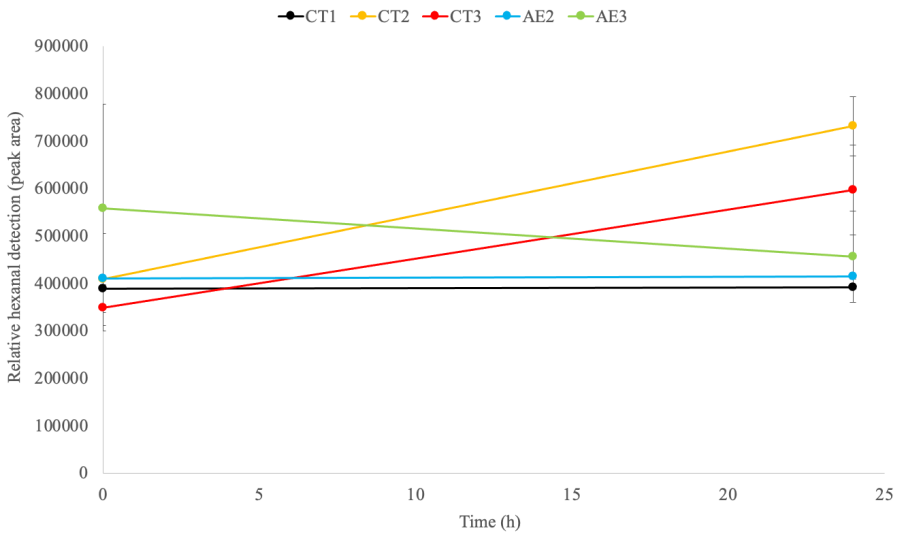


Figure 16 - Relative hexanal detection from additional storage trial. Hexanal levels are shown for 0, 24 hours after storage for each storage condition. Milled oat flour was stored dry (CT1), with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM CuSO4 (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM CuSO4 (AE3).

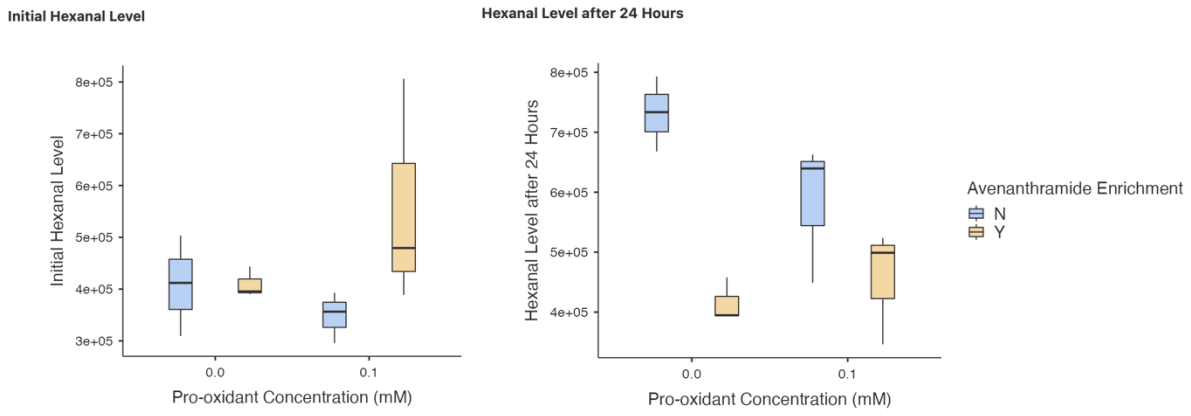


Figure 17 - Relative hexanal detection from additional storage trial, presented in the form of box plots for hexanal levels of storage conditions CT2, CT3, AE2 and AE3 after 0 and 24 hours. Milled oat flour was stored with a water-methanol mixture (CT2), with a water-methanol mixture containing 0.1 mM CuSO4 (CT3), with a water-methanol based avenanthramide extract (AE2), and finally with a water-methanol based avenanthramide extract containing 0.1 mM CuSO4 (AE3).

Table 8 - ANOVA test comparing relative hexanal levels at 0 and 24 hours of the oat flour for each storage condition (with the exception of CT1). Avenanthramide enrichment was found to have a significant effect on hexanal levels after 24 hours.

ANOVA - Initial Hexanal Level

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	3.35e+10	1	3.35e+10	2.198	0.176
Pro-oxidant Concentration (mM)	5.86e +9	1	5.86e +9	0.385	0.552
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	3.25e+10	1	3.25e+10	2.134	0.182
Residuals	1.22e+11	8	1.52e+10		

ANOVA - Hexanal Level after 24 Hours

	Sum of Squares	df	Mean Square	F	p
Avenanthramide Enrichment	1.48e+11	1	1.48e+11	20.87	0.002
Pro-oxidant Concentration (mM)	8.54e +9	1	8.54e +9	1.21	0.304
Avenanthramide Enrichment ✻ Pro-oxidant Concentration (mM)	2.68e+10	1	2.68e+10	3.78	0.088
Residuals	5.66e+10	8	7.07e +9		

In addition to aldehyde analysis, the lipid composition was also determined at 0 and 24 hours after storage for oat samples that were stored in dry storage (CT1), with a water-methanol mixture (CT2), and with a water-methanol based avenanthramide extract (AE2). Since lipase inhibition can already be clearly observed in samples without added pro-oxidant ($CuSO_4$) in the second storage trial, oat samples stored in accelerated conditions with $CuSO_4$ (CT3 and AE3) were not included in lipid composition analysis. Milled oat flour samples were collected for lipid extraction, which was then analysed with HPTLC. A mixture of heptane, diethyl ether and acetic acid at a ratio of 70:30:1 was used for the separation of neutral lipids (NL). TLC plates for NL separation is shown in Figure 18.

For all storage conditions, oat samples taken at 0 hours contained primarily triacylglycerols (TAG). After 24 hours, oat flour placed in dry storage (CT1) also consisted of primarily TAG, while oat flour placed with water-methanol mixtures (CT2) and avenanthramide extract (AE2) had detectable quantities of methyl-esters (orange), which is likely due to the methanol present in the samples during storage. While both CT2 and AE2 had detectable quantities of free fatty acids (FFA; green), it is worth noting that the bands for TAG are clearer for AE3 than CT2 (red), suggesting higher levels of TAG in AE2. AE2 also has detectable levels of diacylglycerols (DAG) that is not found in CT2 (yellow).

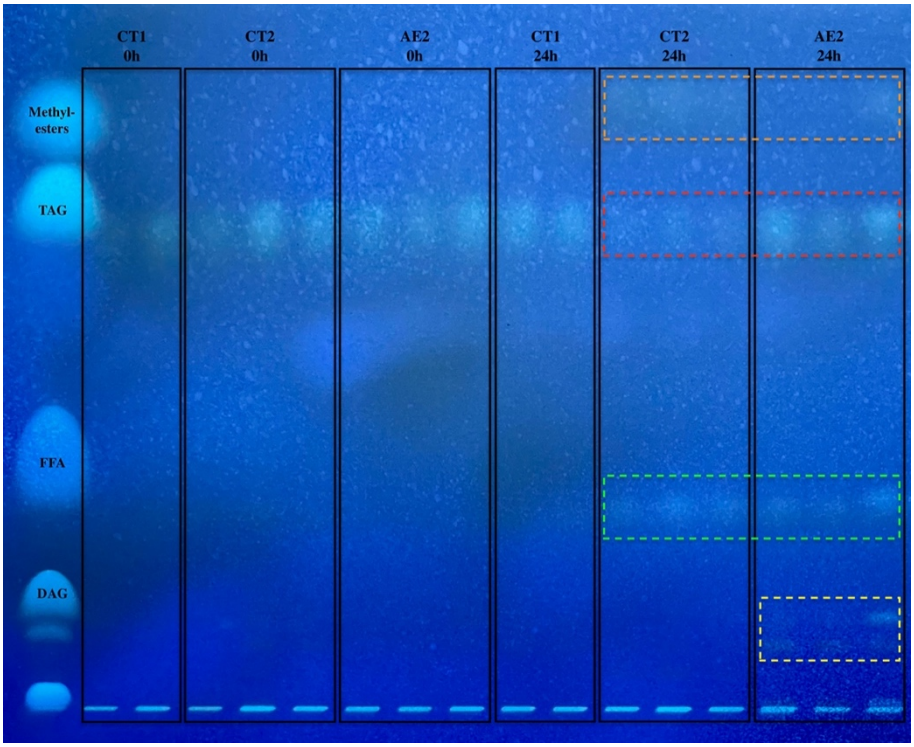


Figure 18 - HPTLC plate of oat flour samples. Neutral lipids (NL) were separated using a mixture of heptane, diethyl ether and acetic acid (70:30:1) as the mobile phase. Milled oat flour was stored with a water-methanol mixture (CT2), and with a water-methanol based avenanthramide extract (AE2).

Role of avenanthramide enrichment on rancidification in oats

Based on the results obtained from the second storage trial, it is possible that avenanthramide enrichment, at least within the specific way the storage trial was conducted, leads to a delay in lipid oxidation, and, as a result, rancidification in oats. Avenanthramide enrichment in the second storage trial was shown to result in a statistically significant decreases in peroxide values after 2 and 24 hours, as well as decreases in lipase activity at all three time points that were tested, with the largest differences observed after 2 hours for the PV measurements, and after 24 hours for the lipase activity determinations.

PV measures the level of peroxides in a solution. While samples that are high in PV can indicate a high level of lipid oxidation, it is important to note that, as summarised previously, peroxides are converted into other non-volatile and volatile endproducts as oxidation progresses, and it is typically expected for peroxide levels to reach a maximum before decreasing as secondary products are formed (see “Oat lipids and rancidification”). This phenomenon is reflected in the PV analysis of the second storage trial as well, where the PV of oat flour that is stored without

avenanthramide enrichment (CT2 and CT3) increases from 0 to 2 hours, and decreases from 2 to 24 hours. This was not observed in samples with avenanthramide enrichment (AE2 and AE3). It is therefore possible that, for samples without avenanthramide enrichment, PV reached a maximum between 2 and 24 hours, while, for samples with avenanthramide enrichment, the maximum PV has yet to be reached, indicating that avenanthramide enrichment has slowed the process of lipid oxidation in the oat flour. This is likely due to the antioxidant activity of avenanthramides (Fagerlund, 2009), and possibly due to inhibition of lipase activity as well.

Although there is not much published literature regarding avenanthramides and lipase activity, studies have demonstrated that phenolic compounds can have an inhibiting effect on pancreatic lipases (Buchholz and Melzig, 2015). Phenolic compounds are hypothesised to do so via nonspecific binding to the enzyme surface (Lotti *et al.*, 1996). As mentioned previously, lipase hydrolyses storage lipids into free fatty acids which not only results in hydrolytic rancidification, but are also much more susceptible to lipid oxidation (Mahne, 2018). The inhibitory effect on lipase activity by avenanthramide enrichment could therefore also play a role in delaying lipid oxidation in oats. In the second storage trial, addition of copper sulphate was also observed to result in a statistically significant decrease in lipase activity, corroborating other publications that have previously reported such effects of copper sulphate on lipase activity (Shastry and Raghavendra Rao, 1976).

Findings from the additional storage trial seem to in part corroborate the findings from the second storage trial. Avenanthramide extract was found to lead to a statistically significant decrease in hexanal levels detected 24 hours after storage, in accordance with the PV findings from the second storage trial suggesting a reduced rate of lipid oxidation. Analysis of lipid composition with HPTLC also showed higher levels of TAG and DAG in avenanthramide enriched oat samples compared to the control stored in water-methanol mixture. While presence of DAG can be an indication of higher levels of lipase activity, it is worth noting that further investigation is required to draw meaningful and quantitative conclusions regarding the relationship between avenanthramide enrichment and oat lipid composition.

Despite the promising results found in the second storage trial and supplementary experiments, it is worth noting that, as mentioned previously, lipid oxidation is highly complex and, depending on factors such as pH and concentration, extracts that are originally added with the intent of delaying oxidation have been found to exhibit prooxidant activity instead (Chedea *et*

al., 2010; Zhou and Elias, 2013). It is therefore possible that the apparent delay of lipid oxidation by avenanthramide enrichment are only specific to the particular conditions used in the second and later storage trials. While meaningful conclusions cannot be drawn due to lack of replicates, the detection of hexanal in avenanthramide enriched oat flour but not crude oat flour in the first storage trial highlights a potential for avenanthramide enrichment to induce lipid oxidation rather than prevent it. While avenanthramide enrichment was based on the same quantity of starting material for all storage trials, the avenanthramide extracts were further concentrated after the first storage trial, a process that could have changed the final concentration of avenanthramides added to the oat flour for storage. The concentration of other compounds extracted alongside the avenanthramides, such as polar lipids and other phenolic compounds, may have also been affected as a result of the concentration step. A better understanding of the composition of avenanthramide extracts used is therefore needed in order to gain more insight into the antioxidative/prooxidative activities of avenanthramide enrichment in oats and other food lipids.

Overall, the experimental findings from this study provide valuable insight into the potential of avenanthramide-rich oat extracts to prevent rancidification in oat products. Avenanthramide enrichment could be used on its own, or in conjunction with other rancidification-preventing measures such as heat-treatment to allow for a milder treatment process overall. Applications in lipid-based foods other than oats could also be a possibility, especially with foods that are highly prone to oxidation such as meat and fish, as demand for natural antioxidants have increased in recent years due to increasing awareness regarding the detrimental health concerns of synthetic antioxidants (Shah *et al.*, 2014). Findings from this study can also be used to inform agricultural decisions, particularly in cultivar selection and growth conditions to optimise avenanthramide levels in oats. The inhibitory effect of avenanthramide enrichment on lipase activity could also be of interest in the field of obesity research, as inhibition of human pancreatic lipase has been considered to prevent excess calorie intake and, subsequently, development of obesity (Lunagariya *et al.*, 2014).

Future Directions

To better understand and evaluate the potential of avenanthramide enrichment to prevent rancidity development of oats and other foods, it would be incredibly interesting to study in

depth the composition of the avenanthramide-rich oat extracts. The concentration of different avenanthramide congeners could be quantified, and studies could also be conducted to identify any other compounds that are also extracted along with the avenanthramides. This will not only allow for better controlled storage trials in future research, but also provide insight to other mechanisms taking place due to additional compounds present in the avenanthramide extracts used for enrichment. Storage trials including earlier timepoints (e.g. 0-10 hours) could also be helpful for mapping out the level of peroxides over time and providing more detailed information with regards to the lipid oxidation process. Simultaneously, oat flour could also be stored for a longer period of time in order to see clearer differences in hexanal detection. Finally, storage trials could also be conducted with other lipid-based foods or oil emulsions to study the potential of avenanthramide enrichment on delaying lipid oxidation in other foods as well.

Conclusion

In conclusion, this present study explores the role of avenanthramides with regards to lipid oxidation in oats. Avenanthramide-rich oat extracts were added to milled oat flour in accelerated storage trials, and rancidity markers such as peroxide value (PV), lipase activity and hexanal detection were followed over time to investigate the potential of avenanthramides as a means to slow rancidity development. After preliminary characterisations of the oats' lipid composition and avenanthramide content, three storage trials were conducted over the course of 72 hours for the first storage trial, and 24 hours for the subsequent storage trials. In the later storage trials, avenanthramide enrichment was found to result in statistically significant decreases in PV, lipase activity and hexanal levels after 24 hours compared to the controls. While further research will be needed to gain further insight into the exact mechanisms taking place, the results from this study demonstrate a potential for avenanthramide enrichment as a milder treatment alternative to be used either solely or in conjunction with the heat-inactivation of lipolytic enzymes in oat products. Other potential applications of avenanthramide enrichment include the possibilities of delaying oxidation in other lipid-based food products aside from oats, as well as informing agricultural decisions such as strain selection and growth conditions to optimise avenanthramide levels in oats. Overall, the findings of this study serve to provide an overview of avenanthramide enrichment as a means of delaying lipid oxidation and can be used to guide further research into how avenanthramide extracts can be used to optimise oxidative stability in oats and other lipid-based foods. Interestingly, in addition to delaying lipid oxidation, avenanthramides also seem to be effective lipase inhibitors, which is perhaps something that can be further investigated in later studies.

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