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Analysis of avenanthramides in oats with Ultra High Performance Liquid Chromatography

Bachelor's thesis in food science

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Abstract

Avenanthramides have been shown to have beneficial properties such as strong antioxidant activity and anti-inflammatory effects in humans, and are a key defence molecule for oats. The goal of this project was to create and validate a method for quantification of avenanthramides in oats that would take less than 20 minutes. A new extraction method and new liquid chromatography method were tested, as well as the introduction of an internal standard to improve quantification. We found that the internal standard selected was not stable during extraction and that it was necessary to use an external standard curve. We found that the liquid chromatography method was reproducible for standard compounds and that we could get separation of the three main avenathramides in oats for a total run time of 15 minutes. The extraction component of the method requires further work to improve recovery and stability before this method can be used for quantification of avenanthramides in oats.

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Goal

The goal of this project was to develop a validated method to quantify avenanthramides from various oat products by using High Performance Liquid Chromatography (HPLC)/Ultra Performance Liquid Chromatography (UPLC), with the target of an analysis time of less than 20 minutes. The goal is also to learn the function of HPLC/UPLC, test different types of extraction methods and how to validate an analytical method.

Background

Oats are considered a healthy food because of their high β-glucan content which can reduce blood cholesterol and reduce risk of cardiovascular disease. Oats also contain a class of polyphenols called avenanthramides. Avenanthramides have beneficial properties such as strong antioxidant activity, anti-inflammatory -and anti-proliferative effects. Avenanthramides may also give protection against skin irritation due to its anti-itching effects [1]

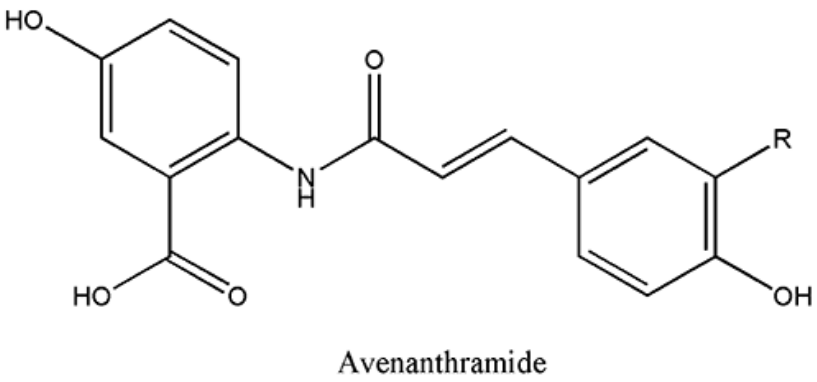

Figure 1 The basic structure of avenathramides. In the main avenanthramides tested in this work, R can be H, OCH3 or OH (see Table 1)

Avenanthramides function as anti-pathogenic compounds in oats, and concentrations of avenathrmaides in oats will increase when the oat plant is attacked, especially by fungal pathogens. [1]

There are more than 20 unique avenanthramides but most research has focused on the most

abundant avenanthramides. The most common avenanthramides are Av a, Av b and Av c, distinguished by a different substitution on the ferullic acid part of the avenathramide molecule. [2] The R in Fig.1 equals a different side chain depending on the avenanthramide and of these avenanthramides, c has the highest antioxidant activity. [3]

Table 1 – types of sidechains found on the most abundant avenanthramides in oats.

a	R=H
b	R=OCH_3
c	R=OH

Avenanthramides are exclusively found in oats and among all avenanthramides in oats, one third of them is found to be avenanthramide c. [4] The total concentration range of avenanthramides in oats is 3-289 mg/kg oats [5], showing that not all oat varieties are equal, and that it is important to measure avenanthramides to find varieties that contain the highest concentrations. The concentration of avenanthramides is associated with freshness, rancidity and bitterness in the oats. Common methods for processing oats for food use such as autoclaving and drum drying decreases its avenanthramide content. Some processing decreases certain types of avenanthramides. Avenanthramide a, decreases when steaming and flaking but these processes do not affect b and c [1].

As interest in avenanthramides as health-promoting compounds increases, it is important to re-evaluate and improve existing analytical methods. Current methods are generally based around HPLC analysis and external standard calibration curve-based quantification. We aimed to see if we could use UPLC and internal standard-based quantification to improve both speed and accuracy of the analysis.

Experimental

HPLC

High-performance-liquid-chromatography (HPLC), is an analytical instrument for separation, purification and detection of products. This instrument is useful in areas of industries such as pharmaceutical, biotechnology, environmental monitoring and applied research. A typical HPLC according to fig. 2 consists of solvents which leads to a mixing chamber and together creates the mobile phase, a pump which transfer the mobile phase through the system. An injector injects a liquid sample into the flow path together with the mobile phase and pushes it through a column packed with a stationary phase, which then separates different compounds via their different interaction with the stationary phase. The flow of mobile phase and compounds then leaves the column and passes through a detector which will detect the sample depending on changes of chemical compositions and result into a chromatograph containing peaks. Ideally each peak should represent a unique substance. [6]

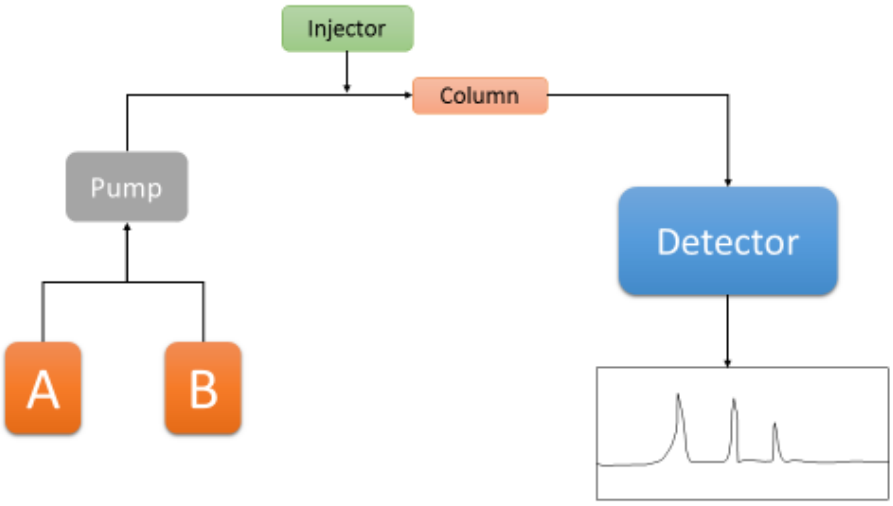

Figure 2 Typical HPLC system

http://www.chemguide.co.uk/analysis/chromatography/hplc.html

Varieties of chromatography methods

There are a lot of different techniques used within the HPLC that are developed depending on the analyte/analytes of interest. These techniques considers factors like polarity and size of analytes. There are different types of chromatography methods such as normal-phase, reversed-phase, size-exclusion (SEC) and ion-exchange (IC) chromatography.

Normal-phase chromatography

This method is based on a polar stationary phase and a mobile phase with low polarity and is used to separate polar analytes. The polar analytes interacts with the stationary phase and this causes a delay in the elution; the analytes with the highest polarity are most delayed and the compounds that are not as polar interact more with the mobile phase and elute faster than the polar analytes. This results in a separation of analytes mainly depending on the relative polarity of the stationary and mobile phases.

Reversed-phase chromatography

This method is similar to the normal-phase chromatography but as its name implies, it is reversed. The stationary phase is non-polar and the mobile phase consists of an aqueous-organic material instead. This makes it more efficient to separate non-polar analytes and elutes polar analytes faster. [6]

Size-exclusion chromatography

Also known as gel filtration, and acts more of a purification method before the finished product. This method separates particles depending on their size and is mainly focused on separate large molecules like proteins and polymers, from small particles. The gel in this method acts as a stationary phase and contains small pores. These pores traps small particles and delays their retention times whilst larger molecules ignores the pores and elutes much faster. The smaller the molecule the longer retention time it requires. [7]

UPLC

Ultra-performance liquid chromatography (UPLC) shown in fig.3 is the same as HPLC, but based around a system that can generate and withstand sustained back-pressure of around 1000 bar, compared to 300-400 bar for conventional HPLC. Using sub-2 µm column particles for better interaction between column and analytes in the mobile phase. UPLC is able to use higher velocities in the mobile phase as well because the instrumentations is able to operate in higher pressures. Decreasing the column particle size results in improved resolution, increased peak height, which leads to better sensitivity, and shorter columns can be used without loss of resolution, meaning that analyses can either have higher resolution or be faster. [8]

]

Figure 3. Acquity UPLC system similar to that used in this project.

Methods

This project can be separated into two parts. Developing the method used for UPLC and the extraction of avenanthramides to validate the method.

Both HPLC and UPLC was used for this project to develop the best method for avenanthramides in oats. HPLC were used in the beginning of the method creation to understand the basics while UPLC was used later to apply and improve the method by using higher flow rates that were possible with HPLC.

Method with acidic mobile phase

HPLC was used to determine a method suited for extracting avenanthramides from oats. The mobile phase consisted of two solutions, A and B. A consisted of 0.1% formic acid in milliQ-water and B consisted of 0.1% formic acid in acetonitrile. These solvents were measured up to 1L to be able to make a lot of tests on the HPLC. Later on a new mobile phase A was created consisting of 5% acetonitrile and 0.1% formic acid in milliQ-water.

The columns used were mainly C18 which was the suited for these types of molecules. Then length, size of particle and particle with a solid core were tested for an optimized elution. In the end a short HPLC column: 2.1cmx50mm SB-C18 column (1.8 µm particle size, by Sigma) was used.

A gradient was created by applying different concentration set ups of the mobile phase during the elution of the samples. Individual avenanthramide standard solutions were made by diluting avenanthramide standards ’A’, ‘B’ and ‘C’ and then a mix of those three was made. 2 internal standards were made, 2-Phenyl-2’,4’,6’-trihydroxyacetophenon and Gallacetophenone, by measuring 10mg of each substance and then dilute those with 100ml methanol.

Avenanthramide extract from oats part 1

Following the avenanthramide sample preparation from [3] with some small alterations produce the avenanthramide extract such as using oat bran instead of oat flour. 1.00g oat bran was measured in a vial. 10ml 80% aqueous ethanol was added and then the mixture was vertical shaken for ~15min and then the supernatant was transferred to another vial. This process was repeated three times. The supernatant was evaporated at 40°C under with a flow of N_2 to speed up the process. The residue was dissolved in 20ml methanol and vortexed to

solubilize the dried extract. The resuspended supernatant was centrifuged 5min at 4000g to separate any solid particles from the liquid extract. Then the supernatant was transferred to a chromatography vial for analysis.

Two different methods were applied to this product to see the difference in the HPLC. Method 1 was to take 5ml avenanthramide extract and add the internal standard 2 - Phenyl-2', 4', 6'- trihydroxyacetophenone. This solution was then tested in the HPLC to determine the concentration of the avenanthramides. evaporate this amount and then re-suspend it in 200µl methanol to give the avenanthramides a higher concentration within the solution. The second method was similar to the first but avenanthramide extract was used instead of 5ml and then the solution was re-suspended in 100µl instead of 200µl.

To improve peak resolution and peak shape in the chromatogram a method of switching solution for the internal standard was applied. Three new solutions were made by combining different percentages of mobile phases A and B together. Ideally the solution used to dissolve the sample should be similar to the starting solvent conditions for the HPLC, though this is not always possible due to solubility issues for the compound of interest.

Table 2. Different combinations of A and B for a solution for the internal standard.

Solution	% A	%B
1	87	13
2	70	30
3	50	50

100µl internal standard was measured into three small vials with an insert tube. The content was evaporated in N_2 and with a temperature of 40°C. The residues of the evaporated liquids were dissolved in 100µl of the following % in table 2. The first vial contained 87µl of mobile phase A and 13µl of mobile phase B then the second vial contained 70µl mobile phase A and 30µl buffer B and the last vial contained 50µl buffer A and 50µl buffer B. These vials where vortexed before going into the HPLC for testing.

Method using ammonium acetate buffered mobile phase

The final method gradient was composed with a new mobile phase to get the best peak shape. The following table shows the gradient used for this method. The mobile phase was divided into two parts; A and B. A contains 99% H_2O and 1% 3,5M Ammonium acetate in 6.0pH and B contains 80% Acetonitrile, 19% H_2O and 1% 3,5M Ammonium acetate in 6.0pH

An Ascentis Express C18 Supleco (10cmx2.1mm, 2µm) analytical column was used for this method.

Avenanthramide extract from oats part 2

In order to optimise the extraction, milled rolled oats and milled oat bran were tested. 1g of both milled rolled oats and milled oat bran were each measured in triplicate into glass test tubes. 5ml of 80% aqueous ethanol was added to these vials and then vortexed for complete interaction with liquid and solid phase. Then the vials were sonicated for 15min at room temperature and later on the vials were placed into a vertical shaker for 30min. The samples were centrifuged and then the supernatant was poured into new vials. This process was repeated five times. The extracts were evaporated and re-suspended in 200 µl mobile phase starting conditions in an Eppendorf tube. The re-suspended samples were centrifuged again and the following supernatant was placed into a liquid chromatography vial.

Validation of the method

Tests were made to validate the gradient method including:

- Test the effect of starting solvent
- Test the limit of detection and quantification
- Test of the extraction method

To test the starting solvent effect the standard solution was altered in different compositions of methanol and mobile phase A.

A standard solution containing the wanted avenanthramides and the internal standard were evaporated in three different vials. The first vial where filled with 100% methanol, the second was filled with 75%methanol and 25% mobile phase A and the last vial was filled with 50% methanol and 50% mobile phase A. UPLC determined the best composition of methanol and mobile phase A by a comparison of the peaks showing in the chromatogram which was the best composition.

Then a standard curve was made to determine the limit of detection and quantification of the analytes in the solutions. Six different concentrations of the avenanthramide standard solution were made for the standard curve: 50 µg/ml, 25 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1 µg/ml. To get the best curve, every concentration were made in triplicates. The peak area of the avenathramides and internal standard was determined by integration using the UPLC

software. When organizing the standard curve the mean value of each avenanthramides peak area were plotted against the concentration.

Reproducibility

To test reproducibility of the method, various products containing oats were examined by applying the extraction method and UPLC gradient. Each oat product was milled and the samples divided into three large batches containing 11-12 different samples with and extra control sample to detect error. Each sample was analysed in triplicate. The peak area of every analyte was compared between the triplicates and to the standard curve to determine the concentration of each avenanthramide in the different oat products.
To get reproducible results, a coefficient of variation was calculated by comparing the peak areas for every replicate of each sample. See appendix.

Results

This gradient method was made together with the supervisor and was used in the UPLC to get the best peaks.

Table 3: The final gradient composition of A and B

Time (min)	Flowrate (ml/min)	A%	B%
0	0,5	80	20
2	0,5	80	20
4	0,5	55	45
5	0,5	0	100
7	0,5	0	100
8	0,5	80	20
12	0,5	80	20

This chromatogram show the peaks of a typical oat product.

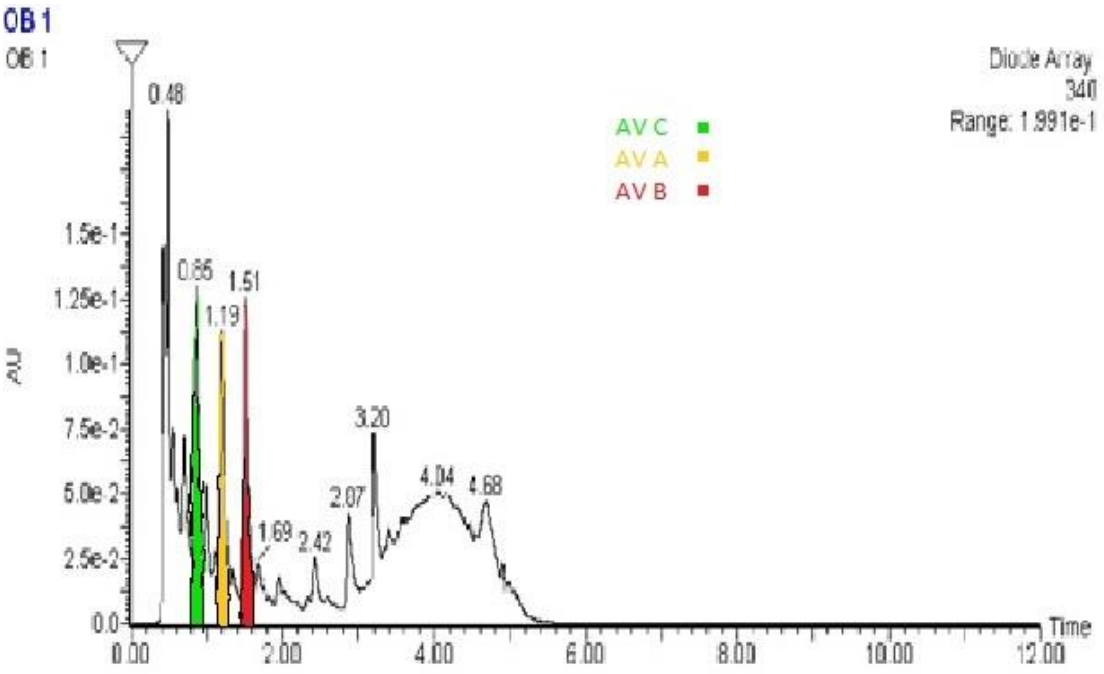

Figure 4. A typical chromatogram of an oat product which shows the avenanthramide peaks and some other peaks not related to the avenanthramides

This chromatogram shows the peaks of standard solution 25 µg/ml in two types of wavelengths to detect both the avenanthramides and the internal standard. The avenanthramides is shown with a diode array between 339-341nm and the internal standard is shown in the interval of 279-281nm.

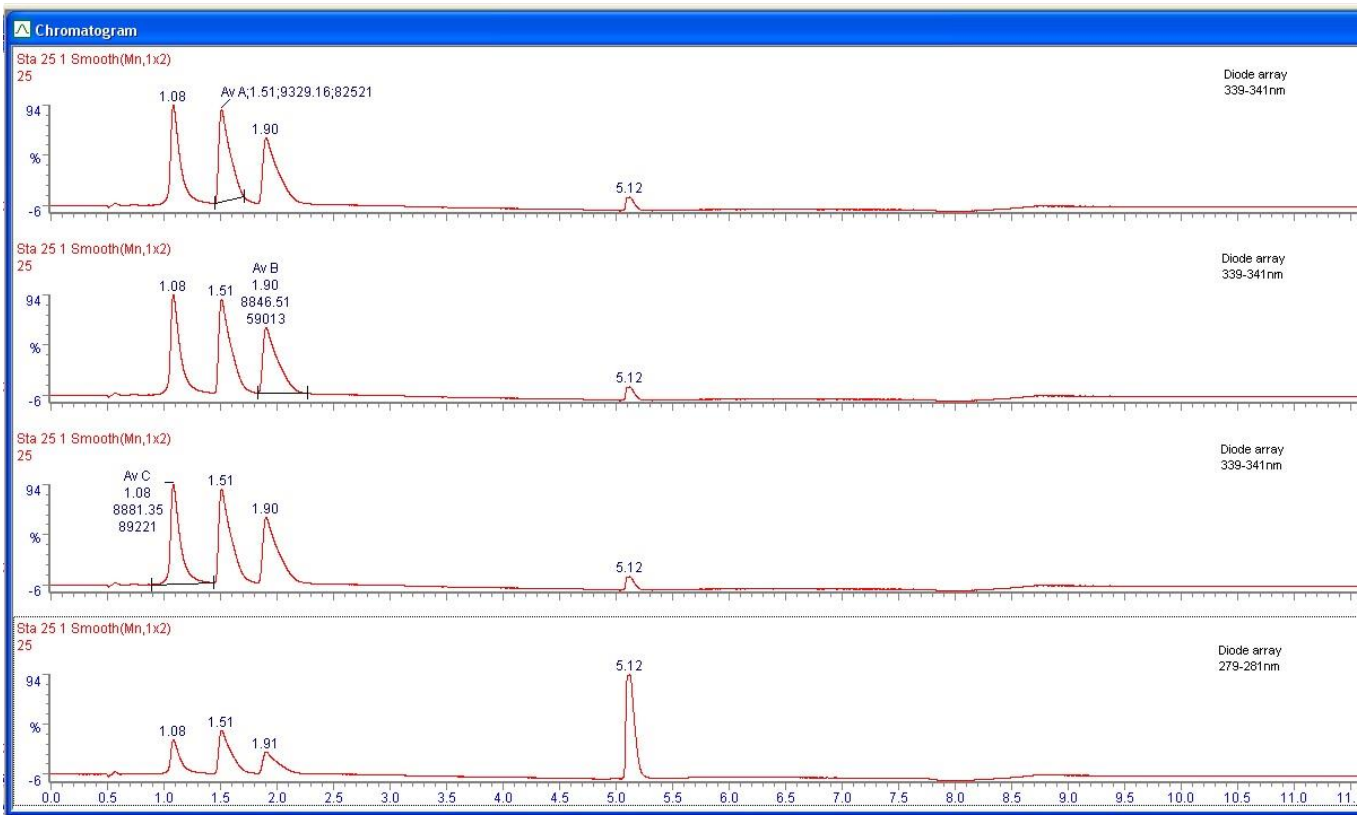

Figure 6. Chromatogram of a standard solution containing 25µg/ml of avenanthramide standards and internal standard in mobile phase

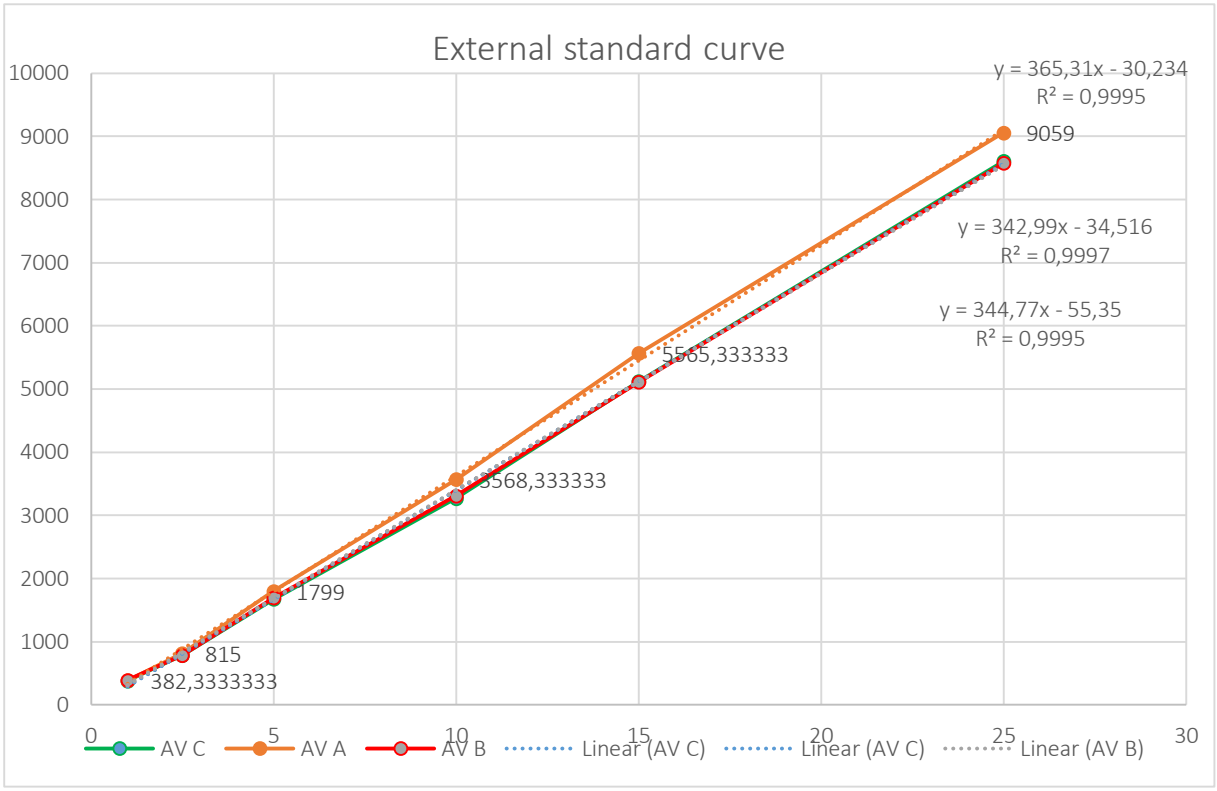

Figure 7. An external standard curve with a linear trend line.

This curve was used to get a linear concentration curve for standard solutions of lower concentrations as this goes from 30µg/ml to 1µg/ml.

These samples were reproducible which resulted in concentration read from the external standard curve.

Sample	AV C (µg/g)	AV A (µg/g)	AV B (µg/g)
Gyllenhammar havregryn	22,3	16,7	21,5
Semper glutenfria havregryn	23,0	14,0	19,2
Kungsörnen havrekli	18,2	11,5	18,5
Axa havegryn	28,0	26,1	27,0
Havrevälling	24,0	16,0	22,0
Ekologiska havregryn	40,1	32,1	33,0

Table 4. Quantified avenanthramides in various oat samples.

We found that after the first batch there was a problem with peak height and reproducibility. We troubleshooted the UPLC method using standards, and found that these were reproducible. We then troubleshooted the extraction method using standards and found that there were high losses of both the avenanthramides and internal standard (Tables 5-10).

Table 5. Peak area values from both avenanthramides and internal standards of an evaporator with nitrogen gas.

Evaporator	Sample	AV C	AV A	AV B	IS
Control	1	2317	2397	3172	1344
	2	2608	2513	3306	1530
	3	2332	2472	3147	1516
Kväve 30°C	1	748	856	776	372
	2	953	1007	1216	423
	3	918	972	1337	261
Kväve 40°C	1	815	933	883	437
	2	604	677	736	319
	3	820	972	894	324

Table 6. Mean values of the peak areas from table 5.

AVC	AVA	AVB	IS
2419	2460,667	3208,333	1463,333
873	945	1109,667	352
746,3333	860,6667	837,6667	360

Table 7. The losses of avenanthramide content and internal standard content in evaporation with nitrogen gas.

Evaporators	AV C %	AV A %	AV B %	IS %
kväve 30°C	64 %	62 %	65 %	76 %
kväve 40°C	69 %	65 %	74 %	75 %

Table 8. Peak area values from both abenanthramides and internal standard for a vacuum evaporator.

Evaporators	Sample	AV C	AV A	AV B	IS
control	1	1843	1862	1742	1724
	2	1791	2056	1710	1546
	3	2290	2461	2937	1558
vacuum	1	990	993	1266	614
	2	671	767	610	553
	3	791	905	813	619

Table 9. Mean values of the peak areas from table 8.

AV C	AV A	AV B	IS
1974,667	2126,333	2129,666667	1609,333
817,3333	888,3333	896,3333333	595,3333

Table 10. The losses of avenanthramide content and internal standard content in an vacuum evaporator

Evaporator	% AV C	% AV A	% AV B	% IS
Vacuum 30°C	59 %	58 %	58 %	63 %

Discussion

This project can be divided into several parts which include the testing of a gradient method in HPLC/UPLC, validation of this method and then an extraction method of various oat samples was made. The testing of a gradient methods was more of an instructive part of the project to learn more about how HPLC and extractions works. Which was very useful because it let me improve my skill with various software concerning HPLC and it also improved my troubleshooting of chromatographs including problems such tailing, wide peaks and fused peaks. This is affected by many things such as polarity, pressure, flowrates, different mobile phases and the gradient.

A gradient was used to elute the avenanthramides one at a time to form good peaks in the chromatogram. The goal was to get the avenanthramides to elute fast and one at the time to have the best separation of these molecules. By using a gradient the %A and %B in the mobile phase could vary to elute the mixtures both fast and slow.

One of the concentration gradient used for the analytical method is shown in Table 5. Where the focus lies on the % of B because it interacts more with the stationary phase and lets the injected sample elute through the column. The avenanthramides elute between 3-5min, the internal standard shortly after, followed by a washout phase to remove all compounds injected

from the column, followed by a re-equilibration phase so that the column is in the same state for each sample when it is injected.

Table 11. One of the concentration gradient.

Time(min)	%A	%B
0	87	13
7	70	30
8	0	100
10	0	100
11	87	13
18	87	13

A lot of gradients were applied to various solutions to get the final gradient method. Firstly separate avenanthramides were tested individually and then together to see how they were located on the chromatogram in contrast to each other.

The final gradient was made in a UPLC because it can handle a higher pressure value which means it tolerates a higher flowrate of the mobile phase that can elute the avenanthramides faster.

Several batches were made to test the reproducibility of the final gradient method for the UPLC. These batches contained various oat products such as rolled oats, oat bran, oat cereal and specific grown oats etc. Every batch were made in the same way so it was strange that the results varied so much. The first batch was the best since half of the samples were approved in the reproducible test, the results in the upcoming batches were a disappointment though since only one sample per batch was approved. This could have been affected by the composition of the mobile phase. When the final gradient method was made the mobile phase changed as well to an ionic solution with an acidic pH of 6.0 and in the first batch I used a buffered salt solution made by my supervisor but for the upcoming batches I try to make one of those myself and the pH went lower than 6.0 and it resulted in peaks eluting later which greatly affected the reproducibility. I created a new buffered salt solution which improved the results. This solution was used to make the mobile phase for the standard curve test shown in Fig.4. This indicated that the method is very sensitive to changes in the mobile phase, and when this was at the correct pH, the UPLC method was reproducible and detector response was linear.

Later on the method was working well but the extraction part was not, since the latter chromatographs mostly showed a loss of the internal standard but a loss of the avenanthramides as well. To test this the extraction method a regular standard solution was compared with an extracted standard solution that should have been equal; this was not the case, which determined that the evaporation process was at fault.

Every sample I produced for the batches were evaporated in 40°C with nitrogen gas, it worked in the first batch but became less effective the more samples I run through the UPLC so I had to try out other evaporation systems. First I tested if the temperature with nitrogen gas did any difference by testing the reproducibility in different temperatures. I tested both 30°C and 40°C with three samples each. Both of the temperatures did not give better results so another test had to be made. Next a vacuum evaporator was tested. I repeated the temperature test on this new evaporator and found out that the internal standard disappeared in both temperature but the avenanthramides stayed almost intact when evaporated in 30°C.

Some of my samples in the batches gave a really low value on the peak areas which could be related to that the extraction process was insufficient or that the sample did not contain a lot of avenanthramides. Some of my samples were cereals and cookies that contained oat which means that the oat is processed and this may have decreased the avenanthramide contents. In all of my samples, the cookies stood out the most because of its texture. Since cookies contains fat, the milled versions of these cookies became gooier than the dried oat products. This could have been prevented if the fat was removed before extracting the avenanthramides from these products, because avenanthramides are non-polar like fat molecules, which can make them difficult to separate during my extraction process.

Conclusion

The purpose of this project was to develop and validate a method for quantification of avenanthramides in oats which should be under 20min. The UPLC method is working with a gradient that mixes two different mobile phases to elute avenanthramides fast. The avenanthramides are vulnerable to heat and the quantification of avenanthramides decreased while enduring higher temperature. The internal standard used for this project is vulnerable to evaporation though it almost disappeared after the treatment of evaporation. To summarize

this project, the developed method was completed for UPLC and is reproducible but the extraction process part is not completed.

Next steps

Since the reproducibility of the UPLC method was working fine, I would focus more on the extraction method. I would analyse every part of the extraction method to identify why the errors were made in my attempts of the extraction process. Try new evaporators in different temperatures to see which evaporator and which temperature would be optimal for avenanthramides and the internal standard. Another option would be to look for another suitable internal standard with greater stability.

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Appendix

General calculation method of the CV% for Gyllenhammars havregryn:

	Replicate A				Replicate B				Replicate C				Correction factor
	Area	Area/IS	Amount (µ	%	Area	Area/IS	Amount (µ	%	Area	Area/IS	Amount (µ	%	
Avenanth	7849	0,673618	13,4	35,106	7977	0,727364	14,5	35,47225	7485	0,666162	13,3	36,38794	1
Avenanth	6408	0,549949	11,0	28,66088	7144	0,651409	13,0	31,76805	6051	0,538537	10,7	29,41663	1
Avenanth	8101	0,695245	13,9	36,23312	7367	0,671743	13,4	32,75969	7034	0,626023	12,5	34,19543	1
Total aver	**22358**	**1,918812**	**38,27672**	**100**	**22488**	**2,050515**	**41,0021**	**100**	**20570**	**1,830723**	**36,49402**	**100**	1
Internal st	11652				10967				11236				
Amount o	20 µg												
Weight of	1,003 g			NB: Dry weight									
Weight of	1,000 g												
Weight of	1,003 g												
Average results													
	Concentra	Std Dev	CV%										
Avenanth	13,8	0,689247	5,011343										
Avenanth	11,6	1,259902	10,8827										
Avenanth	13,3	0,710581	5,358787										
Total aver	38,6	2,270407	5,883264										