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Evaluation of chromatographic performance of three C18 columns for avenanthramides separation

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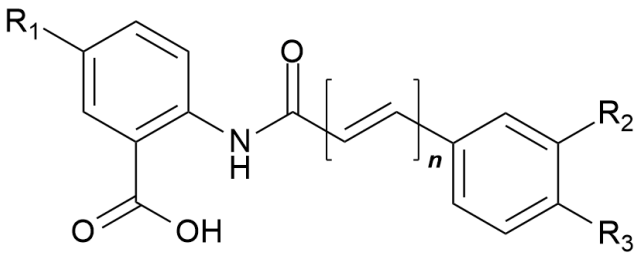
Abstract

Phenolic amides contained in oats (Avenanthramides; AVNs) are biologically active substances with strong antioxidant activity. In this paper, we evaluated efficiency of three C18 chromatographic columns (Symmetry, XBridge, Cortecs) with different particle technology and particle sizes for the separation of three major avenanthramides (AVN A, AVN B, AVN C). We compared columns in terms of retention times, retention factors of AVNs and in terms of other parameters such as number of theoretical plates, height equivalent to a theoretical plate, reduced plate height, resolution and in terms of peak symmetry, respectively. Limits of detection and limits of quantification of AVNs on all columns were calculated. Retention results of AVNs on individual columns showed a significant reduction in retention times of AVNs on solid core column with a particle size 2.7 µm compared to columns with particle size 3.5 µm. Within columns with 3.5 µm particles, separation on Symmetry C18 column appeared to be more efficient than on the XBridge C18 column. In general, results achieved on Cortecs C18 column can be considered as the best in terms of both separation efficiency and retention times.

Introduction

Avenanthramides (AVNs) are exceptional low molecular weight polyphenol amides produced in oats as phytoalexins (secondary metabolites) (Wise 2013; Ishihara *et al.* 2014; Tripathi *et al.* 2018). AVNs were first described in 1989 by Collins as a group of hydroxycinnamoylanthranilate alkaloids (Chen *et al.* 2007). AVNs are derivatives of hydroxycinnamic acid and anthranilic acid coupled by an amide bond. It has been described approximately 40 AVNs consisting of these two acids so far. Among the other minor AVNs in oats, which are generally not included in the quantitative determinations, there are three major AVNs, the most important (Fig. 1): Avenanthramide A (2p),

avenanthramide B (2f) and avenanthramide C (2c) (Dokuycu *et al.* 2003; Ishihara *et al.* 2014; Pridal *et al.* 2018; Tripathi *et al.* 2018; Li *et al.* 2019; Hernandez-Hernandez *et al.* 2021). They are present in higher concentrations mainly in oat grains - bran and outer layers of the kernel but may also be present in other parts of the plant (Boz 2015; Kulichová *et al.* 2018). AVNs are relatively stable compounds in UV light and in acidic and neutral pH. In alkaline pH (7-12), AVN B slightly decompose, AVN C decompose to a greater extent and above pH 12 AVN C is completely decomposed, but AVN A is stable in this pH range and thus the stability depends on the structure (Dimberg *et al.* 2001). In addition to natural AVNs, there are also their synthetic structural analogues. An example is the drug called Tranilast, chemically (*N*-[3′,4′-dimethoxycinnamoyl]-anthranilic acid), firstly described by Koda *et al.* in 1976. Tranilast has been licensed in Japan and South Korea since 1982. It is mainly used in the treatment of bronchial asthma and atypical dermatitis (Koda *et al.* 1976; Darakhshan and Pour 2015; Perrelli *et al.* 2018).



Name (Miyagawa *et al.* 1995**)**	**Designation**		**R_1**	**R_2**	**R_3**
N-caffeoyl-5-hydroxyanthranilic acid	AVN **C**	2*c*	OH	OH	OH
N-*p*-coumaroyl-5-hydroxyanthranilic acid	AVN **A**	2*p*	OH	H	OH
N-feruloyl-5-hydroxyanthranilic acid	AVN **B**	2*f*	OH	OCH_3	OH

Fig. 1. Structure of three main AVNs, where n = 1 (structure made in *ChemDraw Professional 16.0 software*).

AVNs as natural substances, in addition to their high antioxidant activity, which has been demonstrated *in vitro* and *in vivo*, are characterized by many other potential effects such as anti-irritant and anti-inflammatory. AVNs as strong antioxidants are able to prevent development of diseases caused by oxidative stress (e.g., cancer) (Sur *et al.* 2008; Meydani 2009; Gani *et al.* 2012; Turrini *et al.* 2019). Many various methods for extraction and determination of AVNs have been proposed (Gangopadhyay *et al.* 2015). Methanol or ethanol is most often used as the extraction agent (Pridal *et al.* 2018). The conditions under which the three main AVNs are extracted to the maximum extent (extraction with 70 % methanol at 55 °C) have been described by Maliarová *et al.* (2015). These authors used Response Surface Methodology (RSM) to determine the optimal parameters for the extraction of AVNs from oat. A relatively large number of methods have been proposed for the quantification of AVNs in oats. Reverse-phased high performance liquid chromatography (RP-HPLC) coupled with UV detection is frequently used because of fact that AVNs absorb the ultra-violet light (maximum in range of 315 – 365 nm) with ε in range of 23,000 – 28,000 $dm^3.mol^{-1}.cm^{-1}$. However, the disadvantage of UV detection is the limit of quantification (100 – 400 $ng.mL^{-1}$). Based on this, several methods have been developed using HPLC-MS (using negative or positive ionization mode) or partially selective but sensitive electrochemical detection to determine AVNs in some types of oats (Jastrebova *et al.* 2006; Xie *et al.* 2017; Kulichová *et al.* 2018; de Bruijn *et al.* 2019). None of published papers do not refer to performance evaluation of different RP C18 stationary phases for three main AVNs separation (e.g., in terms of separation efficiency).

The aim of this paper was to evaluate the chromatographic performance of three different endcapped C18 columns including classic silica substrate, hybrid substrate and solid core column as

well for three main AVNs analysis in oat samples and calculate chromatographic parameters.

Experimental

Chemicals, reagents, and samples

All standards of AVNs (A, B, C) were obtained from Sigma-Aldrich (Darmstadt, Germany). Formic acid (HCOOH), acetonitrile (ACN) and methanol (HPLC grade) were obtained from Centralchem, s. r. o. (Bratislava, Slovakia). Ultrapure water was prepared using Simplicity UV device. All grinded oat samples were obtained from National Agricultural and Food Centre, Research Institute of Plant Production (Piešťany, Slovakia).

Preparation of standards and oat samples

Standard stock solutions (c = 100 μg.mL^{-1}) of AVNs were prepared in acidic mixture of 0.1 % HCOOH in methanol and 0.1 % HCOOH in water (70 : 30, v/v) and then diluted to desired concentrations. Grinded oat samples (2014 Avenuda, 2020 Avenuda and 2014 Kamil, 2020 Kamil) were prepared by modifying the extraction procedure according to (Maliarová *et al.* 2015) as follows: 0.6 g of oats was weighed into a 15 mL tube and 3 mL of acidic mixture 0.1 % HCOOH in methanol:0.1 % HCOOH in water (70 : 30, v/v) was added. Extraction of analytes was performed at 55 °C for 15 min in ultrasonic bath. After extraction step, the samples were centrifuged at 13,000 rpm for 15 min. Supernatants were stored at -15 °C until HPLC analysis. Supernatants were filtered through a 0.45 μm nylon filter before HPLC analysis and then injected to HPLC system without dilution.

HPLC system and HPLC analysis

Waters HPLC system consisted of 2695 separation module, 2998 photodiode array detector and Empower 3 software (Waters, Milford, MA, USA). Separations were performed on three equally long reversed-phase endcapped columns: Symmetry C18 100 × 4.6; 3.5 μm (Waters, Milford, MA, USA), XBridge C18 100 × 4.6; 3.5 μm (Waters, Milford, MA, USA) and Cortecs C18 100 × 4.6; 2.7μm (Waters, Milford, MA, USA). Detailed specifications of used columns are given in Table 1.

All separations were performed according to previously optimized isocratic method. All chromatographic parameters were calculated from data obtained using this isocratic method: composition of the mobile phase was: (A) 0.1 % HCOOH in water: (B) 0.1 % HCOOH in ACN in volume ratio A : B = 77 : 23; temperature of column was set at 40 °C; flow rate was 1 mL.min^{-1}; injection volume was 10 μL and detection of analytes was carried out with PDA scan in range of 210 – 400 nm.

Table 1. Specifications of all three used columns.

Column	Dimensions [mm]	Particle size [μm]	Particle substrate	Particle technology	Surface area [m^2.g^{-1}]	Pore size [Å]	Carbon load [%]
Symmetry C18	4.6 × 100	3.5	Silica	-	335	100	19
XBridge C18	4.6 × 100	3.5	Hybrid	BEH*	185	130	18
Cortecs C18	4.6 × 100	2.7	Silica	Solid Core	100	90	6.6

* Ethylene Bridged Hybrid.

Calculation of chromatographic parameters

The retention factor (k), height equivalent to a theoretical plate ($HETP$), reduced plate height (h), limits of detection (LOD) and limits of quantification (LOQ) were calculated manually according to a literature (Dolan 2014; Samanidou 2015; Şengül 2016; Barth 2018; Broeckhoven and Stoll 2022). Other three parameters: resolution (R), tailing factor (T) and number of theoretical plates (N) were calculated using Empower 3 System Suitability software (Waters, Milford, MA, USA).

Results and Discussion

Retention times and retention factors of AVNs

The retention times of the individual AVNs and their retention factors were monitored at three concentration levels (1; 5; 10 µg.mL^{-1}).

Table 2 gives an overview of the averages (five injections for each concentration level) of retention times and retention factors of AVNs on the different columns.

Table 2. Overview of AVNs retention times (t_R) and retention factors (k) on the different columns. Retention times of AVNs were obtained from average of five injections at each concentration level.

Avenanthramide	Parameter	Column		
		Symmetry C18	XBridge C18	Cortecs C18
AVN C	$t_R \pm$ SD [min]	3.685 ± 0.002	2.623 ± 0.003	2.003 ± 0.002
	$k \pm$ SD	2.802 ± 0.002	1.664 ± 0.003	1.349 ± 0.002
AVN A	$t_R \pm$ SD [min]	5.899 ± 0.003	4.006 ± 0.004	3.031 ± 0.003
	$k \pm$ SD	5.086 ± 0.003	3.068 ± 0.004	2.554 ± 0.003
AVN B	$t_R \pm$ SD [min]	7.207 ± 0.005	4.807 ± 0.005	3.649 ± 0.004
	$k \pm$ SD	6.435 ± 0.006	3.882 ± 0.005	3.279 ± 0.005

AVN C eluted first because it contains only OH groups as substituents (Fig. 1), which generally reduce the lipophilic character of the molecule and therefore AVN C was less retained on nonpolar stationary phase. AVN A, which has one hydroxy group in position R_2 (Fig. 1) replaced by a hydrogen atom, eluted second and AVN B, which has a methoxy group attached at this position (making AVN B less polar) eluted last. The elution order of AVNs was the same on all used columns (Fig. 2). When comparing individual columns, a decreasing trend of AVNs retention times as well as their retention factors can be observed. The most significant reduction in AVNs retention times (and thus analysis times) was observed for the Cortecs C18 column (Fig. 2). It is important to note here that the Cortecs C18 have a particle size of 2.7 µm while the other columns used have 3.5 µm.

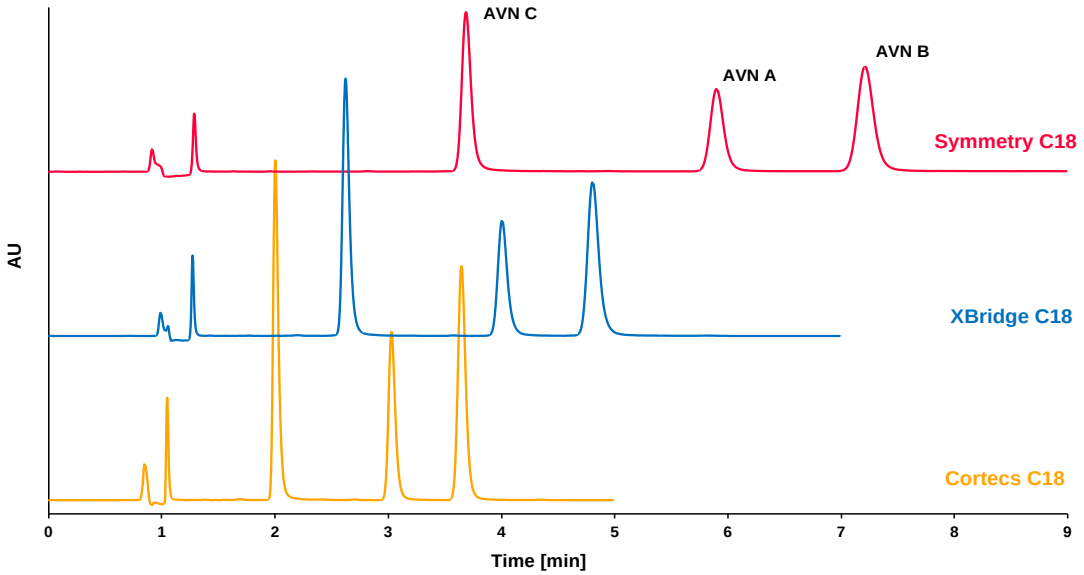


Fig. 2. Chromatograms of AVNs standards on all columns: Symmetry C18 100 × 4.6; 3.5 µm (red); XBridge C18 100×4.6; 3.5 µm (blue); Cortecs C18 100 × 4.6; 2.7 µm (yellow); the elution order was the same on all columns: 1. peak: AVN C, 2. peak: AVN A and 3. peak: AVN B.

However, this is not the only reason for the reduction of AVNs retention times, the second reason for reduction of AVNs retention times may be a significantly lower carbon load in the case of Cortecs C18 column, which is consistent with the assertion that with decreasing carbon load are analyzes shortened. In comparison of the Symmetry C18 and Cortecs C18, retention times of AVNs were reduced by approximately 47.88 % on average, while in comparison of columns with same particle size but different surface area (Symmetry C18 – XBridge C18) were retention times of AVNs reduced by approximately 31.40 % on average. This reduction in retention times in comparison of columns with the same particle size (Symmetry C18 and XBridge C18) is due to their different surface area (Table 1). In general, higher surface area provides the greater retention of analytes (Lakka and Kuppan 2019).

Chromatographic column performance and system suitability of three C18 columns

As in the previous case, all chromatographic column performance parameters were calculated from five injections of three different concentrations of AVNs. An overview of the calculated values of individual parameters on different columns is given in Table 3.

Table 3. Overview of calculated column performance parameters. The calculation was performed from five injections of three different concentrations of AVNs. Individual parameter values are listed with the standard deviation.

Column	AVN	Parameter				
		N **± SD**	***R*** **± SD**	***T*** **± SD**	***HETP*** **± SD [µm]**	***h*** **± SD**
Symmetry C18	C	9475.954 ± 64.671	-	1.275 ± 0.008	10.553 ± 0.072	3.015 ± 0.021
	A	11612.732 ± 48.767	12.028 ± 0.024	1.1659 ± 0.009	8.611 ± 0.036	2.460 ± 0.010
	B	11759.878 ± 61.077	5.411 ± 0.014	1.152 ± 0.014	8.504 ± 0.044	2.429 ± 0.013
XBridge C18	C	9277.356 ± 75.656	-	1.331 ± 0.004	10.779 ± 0.088	3.080 ± 0.025
	A	10913.286 ± 94.849	10.574 ± 0.044	1.275 ± 0.008	9.164 ± 0.079	2.618 ± 0.023
	B	11175.433 ± 89.350	4.794 ± 0.017	1.254 ± 0.010	8.949 ± 0.072	2.557 ± 0.020
Cortecs C18	C	9591.997 ± 386.548	-	1.441 ± 0.010	10.442 ± 0.438	3.867 ± 0.162
	A	12451.522 ± 423.197	10.779 ± 0.209	1.271 ± 0.006	8.039 ± 0.274	2.978 ± 0.102
	B	13790.976 ± 426.995	5.324 ± 0.087	1.213 ± 0.005	7.258 ± 0.225	2.688 ± 0.083

In general, for a 4.6 × 100 mm, 5 µm column, *N* is equal to 5,000 – 8,000 (Ravisankar *et al.* 2017). In our case, we used columns with same dimensions but with smaller particle sizes (3.5 or 2.7 µm). It follows that we should achieve higher *N* values on all three columns than the typical values given for a column with the same size but larger particles. All three AVNs had close *N* values. The *N* values of all three AVNs were highest on the Cortecs C18 column, which means the most efficient separation was on Cortecs C18 column. However, *N* values obtained on Cortecs C18 are accompanied by the highest SD. In the case of AVN C, the *N* values were very similar on all three columns. In case of two other AVNs, the differences in *N* values between the Cortecs C18 column and two other columns were more considerable. Peaks eluting later have higher number of theoretical plates, as confirmed by the data in Table 3. Another, more general parameter which can be used to evaluate the chromatographic column efficiency is the height equivalent to theoretical plate (*HETP*) (Fornstedt *et al.* 2015), *HETP* values for all analytes separated on individual columns are

shown in Table 3. Based on theory (Barth 2018), higher values of *HETP* means lower separation efficiency. It follows that *N* is directly proportional and *HETP* is indirectly proportional to the efficiency. Based on this, it could be said that the column efficiency expressed by the number of theoretical plates *N* is in our case consistent with the column efficiency expressed by the height equivalent to a theoretical plate *HETP*. While for the first eluting analyte (AVN C) *HETP* values were similar for all columns (as in previous discussed parameter). On the other hand, for the last eluting analyte (AVN B) the differences between *HETP* values were more considerable, especially for the Cortecs column which has smaller particle size (2.7 μm) and separation on this column was also most efficient in terms of this parameter.

Columns with different particle sizes were used in our work (two with a particle size 3.5 μm and one with a particle size 2.7 μm), we can use another parameter to compare column efficiency, namely reduced plate height (*h*). This parameter was designed to allow comparison of separation efficiency between columns with different particle sizes. Based on the literature, values between 2 and 3 indicate the optimal separation efficiency (Anderson 1995). The same principle as for *HETP* applies for *h*, the lower *h* values mean more efficient separation. As can be seen in Table 3, only two values deviate from this theoretically optimal range. The *h* values were expected to be the lowest for the column with a 2.7 μm particle size, but *h* values were similar or overlapped with *h* values for the columns with a 3.5 μm particle size. This phenomenon could be caused by other factors (for example the flow rate of the mobile phase). In the case of a request to reduce the values of this parameter, it would be possible to optimize method by changing chromatographic conditions (e.g., temperature or flow rate of a mobile phase).

The symmetry of the peaks is another indicator of efficiency that we have evaluated. Peaks having a tailing factor $T \leq 1.5$ are acceptable for a large number of applications. If the *T* value of any peak in the chromatogram is greater than 2 (Dolan 2012), which is no longer acceptable, it is necessary to adjust the separation conditions. The tailing factors of the AVNs did not exceed 1.5 on any of the columns used (Table 3). The best results in peak symmetry evaluation were obtained on a Symmetry C18 column. Slightly higher *T* values were observed on XBridge C18 and Cortecs C18 columns, but values were still acceptable. The highest *T* value (1.441) was observed on a Cortecs C18 column at the AVN C peak. Based on *T* values peaks of all three AVNs on all columns used showed slight tailing. As mentioned above, this effect can also be reduced by adjusting chromatographic conditions, either by changing the mobile phase or by adjusting a ratio of mobile phase components.

The last discussed parameter in this category is the resolution (*R*) for which different calculation methods can be used. However, many data acquisition systems use peak widths at 50 % peak heigh to calculate resolution, as it is easier and advantageous to use with tailing peaks (Dolan 2014; Ravisankar *et al.* 2017). Resolution between the individual analyte pairs was more than sufficient on all columns and exceeded 4,794 in all cases. The best *R* was obtained on a Symmetry C18 column, where, however, AVNs had the highest retention times. The result on Cortecs C18 column can be considered the best in terms of both separation efficiency and retention times.

Limits of detection (LOD) and limits of quantification (LOQ) of AVNs

LOD and LOQ values were calculated from the calibration lines (AVNs concentration range from 250 to 2,500 ng.mL^{-1}), with $R^2 \geq 0.9996$ in all cases. Calculated LOD and LOQ values for AVNs on all columns are shown in Table 4.

Table 4. Calculated LOD and LOQ values of AVNs on all columns.

	Column	**AVN C**	**AVN A**	**AVN B**
LOD [ng.mL^{-1}]	Symmetry C18	91.4307	95.5642	91.4382
	XBridge C18	76.3345	86.6991	68.4532
	Cortecs C18	96.0841	80.6298	76.7470
LOQ [ng.mL^{-1}]	Symmetry C18	304.7689	318.5472	304.7939
	XBridge C18	254.4482	288.9970	228.1774
	Cortecs C18	320.2802	268.7661	255.8233

In comparison of LOD and LOQ of individual AVNs, we can observe certain differences between columns. In general, LOD and LOQ values may appear relatively high. However, as mentioned in the theoretical part (Kulichová *et al.* 2018) as well as other authors (Jastrebova *et al.* 2006) AVNs have significantly higher LOD and LOQ when using UV (DAD) detection. LOD is in the range 30 – 140 ng.mL^{-1} and LOQ was in the range 100 – 400 ng.mL^{-1} using this type of detection. LOD and LOQ results for AVNs on all columns were in these intervals. Significantly improved LOD and LOQ should be achieved using HPLC-MS.

Analysis of oat samples

In the last part, we analysed real oat samples on all columns using the isocratic method described above. We randomly selected 2 different oat samples (out of more than 150 samples available in our laboratory) collected in 2014 and the same 2 oat samples collected in 2020.
In both samples from 2014, we successfully separated AVNs from other components with minimal interference on all three columns. Fig. 3 shows the chromatogram of a selected mixture of AVNs standards (blue solid line) and the chromatogram of oat sample – 2014 Avenuda (orange dotted line) analysed on XBridge C18 column. Interfering components can be seen close to AVN C and AVN B peaks, which could be eliminated by modifying the extraction technique or in case of determination of these components, it would be better to use gradient elution to separate not only the AVNs but also other components of oat samples such as phenolic acids. Overall, this isocratic method was primarily designed to evaluate the efficiency of three C18 columns with different particle technologies, for possibility of use the best column with gradient elution to provide simultaneous separation and quantification of AVNs and phenolic compounds in oat samples.
In the case of oat samples collected in 2020 (Avenuda and Kamil), there was slightly fewer interfering components using this isocratic method, but AVNs were also in much lower concentrations than in the case of oat samples from 2014.

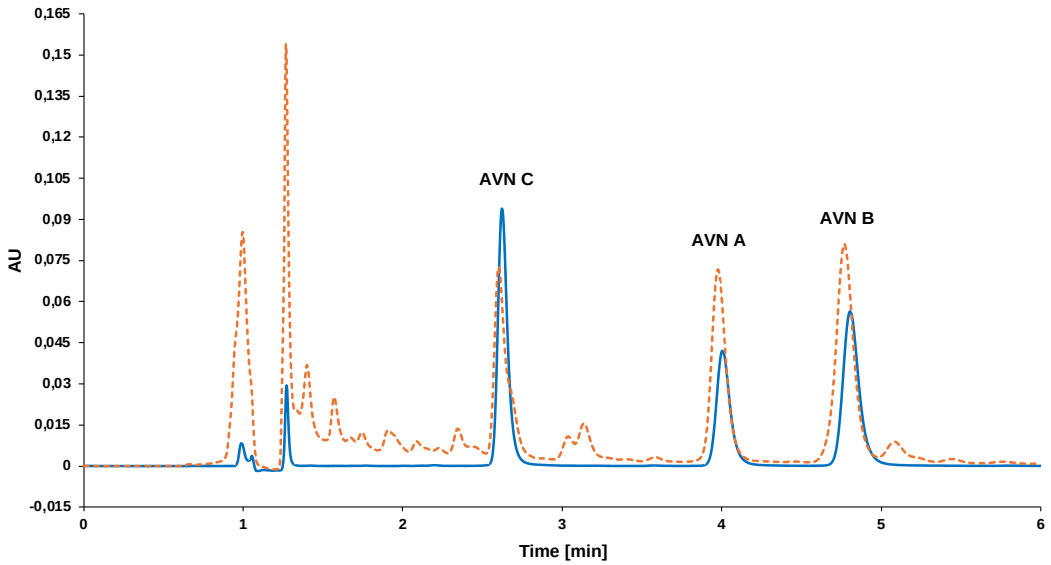


Fig. 3. Overlayed chromatograms of AVNs standard mixture (blue solid line) and 2014 Avenuda sample (orange dotted line) analyzed on XBridge C18 100 × 4.6; 3.5 µm.

Conclusion

This paper offered efficiency comparison of three C18 chromatographic columns with different particle technology, to determine biologically important substances - three main avenanthramides. To simplify comparison, a pre-optimized isocratic method was used to evaluate efficiency of columns. In terms of retention times, a solid-core column with a 2.7 µm particle size (Cortecs C18) appears

to be the best, as there was a significant reduction in t_R compared to columns with a 3.5 μm particle size. Overall, the retention times of all three AVNs on each column decreased in the following order: Symmetry > XBridge > Cortecs. LOD and LOQ were similar on all columns but relatively high (which is standard for this type of detection) compared to LOD and LOQ, which can be achieved by a more sensitive HPLC-MS method. However, nowadays there are still many laboratories that do not have HPLC-MS or even UHPLC-MS available. Therefore, it is constantly necessary to look for a suitable stationary phase and optimize the HPLC-UV (HPLC-DAD) separation conditions. The results of columns efficiency evaluation show differences between columns. The most efficient separation of AVNs in terms of *N* and *HETP* parameters was achieved on a Cortecs C18 column, which predetermines it as the most suitable column (of three tested columns) for AVNs separation. If we focus on the comparison only between columns with the same particle size of 3.5 μm (Symmetry C18 *vs*. XBridge C18), in overall evaluation, separation was more efficient on the Symmetry C18 column, on which was achieved the best results in terms of peak symmetry (follows from the column name). On the other hand, it is important to note that all calculated efficiency parameters are either directly or indirectly dependent on ambient conditions such as flow and mobile phase composition, ratio of individual mobile phase components or column temperature.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Anderson DJ (1995) High-performance liquid chromatography (advances in packing materials). Anal. Chem. 67: 475-486.

Barth HG (2018) Chromatography Fundamentals, Part V: Theoretical Plates: Significance, Properties, and Uses. LC GC N. Am. 36: 830-835.

Boz H (2015) Phenolic Amides (Avenanthramides) in Oats - A review. Czech J. Food Sci. 33: 399-404.

Broeckhoven K, Stoll DR (2022) But Why Doesn't It Get Better? Kinetic Plots for Liquid Chromatography, Part II: Making and Interpreting the Plots. LC GC N. Am. 40: 58-62.

Chen C-YO, Milbury PE, Collins FW, Blumberg JB (2007) Avenanthramides are bioavailable and have antioxidant activity in humans after acute consumption of an enriched mixture from oats. J. Nutr. 137: 1375-1382.

Darakhshan S, Pour AB (2015) Tranilast: A review of its therapeutic applications. Pharmacol. Res. 91: 15-28.

de Bruijn WJC, van Dinteren S, Gruppen H, Vincken J-P (2019) Mass spectrometric characterization of avenanthramides and enhancing their production by germination of oat (*Avena sativa*). Food Chem. 277: 682-690.

Dimberg LH, Sunnerheim K, Sundberg B, Walsh K (2001) Stability of oat avenanthramides. Cereal Chem. 78: 278-281.

Dokuyucu T, Peterson DM, Akkaya A (2003) Contents of antioxidant compounds in Turkish oats: Simple phenolics and avenanthramide concentrations. Cereal Chem. 80: 542-543.

Dolan JW (2012) Troubleshooting basics, Part IV: Peak shape problems. LC GC N. Am. 30: 564-569.

Dolan JW (2014) Estimating resolution for marginally separated peaks. LC GC N. Am. 32: 718-725.

Fornstedt T, Forssén P, Westerlund D (2015) Basic HPLC Theory and Definitions: Retention, Thermodynamics, Selectivity, Zone Spreading, Kinetics, and Resolution. *In* Anderson JL, Berthod A, Estévez VP, Stalcup AM (Eds.), Analytical Separation Science, Wiley, New York, pp. 1-24.

Gangopadhyay N, Hossain MB, Rai DK, Brunton NB (2015) A review of extraction and analysis of bioactives in oat and barley and scope for use of novel food processing technologies. Molecules 20: 10884

Gani A, Wani SM, Masoodi FA, Hameed G (2012) Whole-grain cereal bioactive compounds and their health benefits: A Review. J. Food Process. Technol. 3: 1000146.

Hernandez-Hernandez O, Pereira-Caro G, Borges G, Crozier A, Olsson O (2021) Characterization and antioxidant activity of avenanthramides from selected oat lines developed by mutagenesis technique. Food Chem. 343: 128408.

Ishihara A, Kojima K, Fujita T, Yamamoto Y, Nakajima H (2014) New series of avenanthramides in oat seed. Biosci. Biotech. Bioch. 78: 1975-1983.

Jastrebova J, Skoglund M, Nilsson J, Dimberg LH (2006) Selective and sensitive LC-MS determination of avenanthramides in oats. Chromatographia 63: 419-423.

Koda A, Nagai H, Watanabe S, Yanagihara Y, Sakamoto K (1976) Inhibition of hypersensitivity reactions by a new drug, N-(3′,4′- dimethoxycinnamoyl) anthranilic acid (N-5′). J. Allergy Clin. Immunol. 57: 396-407.

Kulichová K, Sokol J, Maliarová M (2018) Štúdium avenantramidov ako významných biologicky aktívnych látok fenolového charakteru. Chem. Listy 112: 848-854.

Lakka NS, Kuppan C (2019) Principles of chromatography method development. *In* Boldura OM, Baltă C, Awwad N (Eds.), Biochemical Analysis Tools – Methods for Bio-Molecules Studies, pp. 1-20.

Li Z, Chen Y, Meesapyodsuk D, Qiu X (2019) The biosynthetic pathway of major avenanthramides in oat. Metabolites 9: 163.

Maliarová M, Mrázová V, Havrlentová M, Sokol J (2015) Optimization of parameters for extraction of avenanthramides from oat (*Avena sativa* L.) grain using response surface methodology (RSM). J. Braz. Chem. Soc. 26: 2369-2378.

Meydani M (2009) Potential health benefits of avenanthramides of oats. Nutr. Rev. 67: 731-735.

Miyagawa H, Ishihara A, Nishimoto T, Ueno T, Mayama S (1995) Induction of avenanthramides in oat leaves inoculated with crown rust fungus, *Puccinia coronata* f. sp. *avenae*. Biosci. Biotech. Bioch. 59: 2305-2306.

Perrelli A, Goitre L, Salzano AM, Moglia A, Scaloni A, Retta SF (2018) Role of antioxidants in the protection from aging-related diseases. Oxid. Med. Cell Longev. 2018: 6015351.

Pridal AA, Böttger W, Ross AB (2018) Analysis of avenanthramides in oat products and estimation of avenanthramide intake in humans. Food Chem. 253: 93-100.

Ravisankar P, Swathi V, Babu PS, Sulthana S Md, Gousepeer SK (2017) Current trends in performance of forced degradation studies and stability indicating studies of drugs. J. Pharm. Biol. Sci. 12: 17-36.

Samanidou VF (2015) Basic LC Method Development and Optimization. *In* Anderson JL, Berthod A, Estévez VP, Stalcup AM (Eds.), Analytical Separation Science, Wiley, New York, pp. 25-42.

Şengül Ü (2016) Comparing determination methods of detection and quantification limits for aflatoxin analysis in hazelnut. J. Food Drug Anal. 24: 56-62.

Sur R, Nigam A, Grote D, Liebel F, Southall MD (2008) Avenanthramides, polyphenols from oats, exhibit anti-inflammatory and anti-itch activity. Arch. Dermatol. Res. 300: 569-574.

Tripathi V, Singh A, Ashraf MT (2018) Avenanthramides of oats: Medicinal importance and future perspectives. Pharmacogn. Rev. 12: 66-71.

Turrini E, Maffei F, Milelli A, Calcabrini C, Fimognari C (2019) Overview of the anticancer profile of avenanthramides from oat. Int. J. Mol. Sci. 20: 4536.

Wise ML (2013) Avenanthramides: Chemistry and Biosynthesis. *In* Chu YF (Eds.), Oats Nutrition and Technology, Wiley, New York, pp. 195-226.

Xie Z, Mui T, Sintara M, Ou B, Johnson J, Chu YF, O'shea M, Kasturi P, Chen Y (2017) Rapid quantitation of avenanthramides in oat-containing products by high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC-TQMS). Food Chem. 224: 280-288.