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),...
avenanthamide B (2f) and avenanthamide 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 high 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 \((N-[3',4'-dimethoxycinnamoyl]-antranilic acid)\), 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; Darakshan and Pour 2015; Perrelli et al. 2018).

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 ultraviolet light (maximum in range of 315 – 365 nm) with \(\varepsilon\) 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

![structure of three main AVNs](image177x480 to 418x577)

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

<table>
<thead>
<tr>
<th>Name (Miyagawa et al. 1995)</th>
<th>Designation</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>(R_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N)-caffeoyl-5-hydroxyantrhancilic acid</td>
<td>AVN C (2c)</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>(N)-p-coumaroyl-5-hydroxyantrhancilic acid</td>
<td>AVN A (2p)</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>(N)-feruloyl-5-hydroxyantrhancilic acid</td>
<td>AVN B (2f)</td>
<td>OH</td>
<td>OCH(_3)</td>
<td>OH</td>
</tr>
</tbody>
</table>
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 \( (c = 100 \ \mu g.mL^{-1}) \) 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 2020 Kamil) were prepared by modifying the extraction procedure according to \( \text{(Maliarová et al. 2015)} \) 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: \( (A) \ 0.1 \ % \ HCOOH \ \text{in water}; \ (B) \ 0.1 \ % \ HCOOH \ \text{in ACN in volume ratio A : B = 77 : 23}; \) 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.

**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 \( \text{(Dolan 2014; Samanidou 2015; Şengül 2016; Barth 2018; Broeckhoven and Stoll 2022)} \). 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).

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**Table 1. Specifications of all three used columns.**

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

* Ethylene Bridged Hybrid.
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>
<thead>
<tr>
<th>Avenanthramide</th>
<th>Parameter</th>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Symmetry C18</td>
</tr>
<tr>
<td>AVN C</td>
<td>(t_R \pm SD) [min]</td>
<td>3.685 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>(k \pm SD)</td>
<td>2.802 ± 0.002</td>
</tr>
<tr>
<td>AVN A</td>
<td>(t_R \pm SD) [min]</td>
<td>5.899 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>(k \pm SD)</td>
<td>5.086 ± 0.003</td>
</tr>
<tr>
<td>AVN B</td>
<td>(t_R \pm SD) [min]</td>
<td>7.207 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>(k \pm SD)</td>
<td>6.435 ± 0.006</td>
</tr>
</tbody>
</table>

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.

![Chromatograms of AVNs standards on all columns](image-url)

**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.

<table>
<thead>
<tr>
<th>Column</th>
<th>AVN</th>
<th>$N \pm SD$</th>
<th>$R \pm SD$</th>
<th>$T \pm SD$</th>
<th>$HETP \pm SD$ [μm]</th>
<th>$h \pm SD$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symmetry C18</td>
<td>C</td>
<td>9475.954 ± 64.671</td>
<td>-</td>
<td>1.275 ± 0.008</td>
<td>10.553 ± 0.072</td>
<td>3.015 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>11612.732 ± 48.767</td>
<td>12.028 ± 0.009</td>
<td>1.1659 ± 0.036</td>
<td>8.611 ± 0.010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>11759.878 ± 61.077</td>
<td>5.411 ± 0.009</td>
<td>1.152 ± 0.044</td>
<td>8.504 ± 0.013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9277.356 ± 75.656</td>
<td>-</td>
<td>1.331 ± 0.004</td>
<td>10.779 ± 0.025</td>
<td></td>
</tr>
<tr>
<td>XBridge C18</td>
<td>A</td>
<td>10913.286 ± 94.849</td>
<td>10.574 ± 0.008</td>
<td>1.275 ± 0.007</td>
<td>9.164 ± 0.023</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>11175.433 ± 89.350</td>
<td>4.794 ± 0.008</td>
<td>1.254 ± 0.072</td>
<td>9.49 ± 0.087</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9591.997 ± 386.548</td>
<td>-</td>
<td>1.441 ± 0.004</td>
<td>10.442 ± 0.387</td>
<td></td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>A</td>
<td>12451.522 ± 423.197</td>
<td>10.779 ± 0.006</td>
<td>1.271 ± 0.274</td>
<td>8.039 ± 0.102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>13790.976 ± 426.995</td>
<td>5.324 ± 0.006</td>
<td>1.213 ± 0.225</td>
<td>7.258 ± 0.083</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Column</th>
<th>AVN C</th>
<th>AVN A</th>
<th>AVN B</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD [ng.mL$^{-1}$]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symmetry C18</td>
<td>91.4307</td>
<td>95.5642</td>
<td>91.4382</td>
</tr>
<tr>
<td>XBridge C18</td>
<td>76.3345</td>
<td>86.6991</td>
<td>68.4532</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>96.0841</td>
<td>80.6298</td>
<td>76.7470</td>
</tr>
<tr>
<td>LOQ [ng.mL$^{-1}$]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symmetry C18</td>
<td>304.7689</td>
<td>318.5472</td>
<td>304.7939</td>
</tr>
<tr>
<td>XBridge C18</td>
<td>254.4882</td>
<td>288.9970</td>
<td>228.1774</td>
</tr>
<tr>
<td>Cortecs C18</td>
<td>320.2802</td>
<td>268.7661</td>
<td>255.8233</td>
</tr>
</tbody>
</table>
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.

**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 \( R_k \) compared to columns with a 3.5 \( \mu \text{m} \) 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 \( \text{HETP} \) 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 \( \mu \text{m} \) (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.

**Acknowledgements**

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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