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Research Article

Evaluation of hybrid hydrophilic interaction chromatography stationary phases for ultra-HPLC in analysis of polar pteridines

Retention characteristics of ultra-HPLC (UHPLC) hybrid stationary phases (bridged ethyl hybrid (BEH) and BEH Amide) were studied in hydrophilic interaction chromatography system in the group of polar basic pteridines (neopterin, biopterin, dihydroneopterin and dihydrobiopterin). The effect of mobile phase composition, buffer type, pH and concentration on retention of pteridines were examined in detail under UHPLC-fluorescence detection and UHPLC-MS conditions. The selectivity, retention properties and column performance were examined. BEH HILIC did not provide sufficient retention and selectivity for the separation of four pteridines under any tested conditions. BEH Amide provided strong retention for all pteridines especially at high pH values such as 9.8. However, at pH 9.8 the selectivity of separation for the pairs neopterin-dihydroneopterin and biopterin-dihydrobiopterin substantially decreased and resulted in very long analysis time. The best separation of four pteridine derivatives was obtained in the pH range 4.8–7.8 within a reasonable analysis time up to 8 min for UHPLC-fluorescence detection using higher concentrations of ammonium acetate buffer and up to 4 min for UHPLC-MS using lower concentrations of ammonium acetate.

Keywords: BEH Amide / Biopterin / Hydrophilic interaction chromatography / Neopterin / Ultra-HPLC
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1 Introduction

Hydrophilic interaction chromatography (HILIC) has recently become a popular separation mode in modern bio-analysis, as many biologically active compounds possess polar structure and therefore are difficult to be analyzed in other separation modes. HILIC has become an alternative of conventional RP-HPLC or normal-phase HPLC (NP-HPLC) as it is more convenient for the analysis of small polar molecules being weakly retained or eluted with dead volume in conventional RP-HPLC systems. A lack of retention for highly polar compounds is to a large extent caused by their solvation (polar functional groups enter dipolar bonds with the solvent). This process prevents nonpolar stationary phase to bind solvated compound and therefore it is eluted in void volume. Such property can be observed at charged functional groups or groups capable of entering strong dipolar hydrogen bonds [1].

The term HILIC was first utilized by Alpert [2] in 1990 for the description of chromatographic technique where the analytes interact with hydrophilic stationary phase and the elution is performed by relatively hydrophobic binary mobile phase containing water as a strongly eluting solvent. Since that time the explanation of HILIC mechanism does not seem to be perfectly clear. Suggested mechanism involves partitioning between hydrophobic mobile phase and a layer of mobile phase enriched with water being partially immobilized on the stationary phase. Hydrogen bonding is supposed to play an important role in this process [2–4]. Some authors referred also ionic interactions and a contribution of hydrophobic retention to various degrees, dependent on the particular conditions employed [5]. The HILIC mechanism is therefore believed to be complex, which was discussed in detail in the review article by Hemström and Irgum [1].

Under the HILIC conditions, stationary phase is of polar character, including plain silica, hydroxyl-ethyl-, amino groups, zwitterions and many other polar groups bound to silica, polymer or hybrid material [1]. Mobile phase is composed of high percentage of an organic solvent (typically ACN >70%) and it is complemented by a small percentage of water/volatile buffer part (at least 2.5% vol).

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Abbreviations: **BEH,** bridged ethyl hybrid; **BH2,** 7,8-dihydrobiopterin; **BIO,** biopterin; **CV,** cone voltage; **FD,** fluorescence detection; **HILIC,** hydrophilic interaction chromatography; **NEO,** neopterin; **NH2,** 7,8-dihydroneopterin; **NP,** normal phase; **SST,** system suitability test; **UHPLC,** ultra-HPLC

Elution of polar analytes is enabled by increasing polarity of mobile phase, thus the content of water.

The main advantages of HILIC approach were summarized by Hemström and Irgum [1] and McCalley [6] as follows: (i) good retention of polar compounds, (ii) the enhancement of sensitivity of MS due to high organic content in mobile phase, (iii) reasonable peak shapes obtained for bases, (iv) direct injection of extracts eluted from RP solid phase extraction columns and (v) higher flow-rates applicable due to high organic content of typical mobile phases. Another advantage (vi) of HILIC includes possibility to replace easily NP chromatography due to bad reproducibility of NP, toxic organic solvents used, poor dissolution of polar compounds in NP mobile phases and great difficulties when connection of NP chromatography with MS detection was required. On top of this, mobile phase preparation is less complicated since the need of total control over the solvent water is not necessary.

HILIC has been used in analysis of various polar analytes including acidic [7, 8], basic [9, 10] and also neutral compounds [11]. Special interest is devoted to basic compounds, which might still cause considerable problems in RP chromatography due to interactions with ionized silanol groups and to overloading effects, where peak shape for charged bases deteriorates rapidly with increasing sample mass. This problem was experimentally studied by McCalley [6].

Pteridine molecules – neopterin (NEO), biopterin (BIO), 7,8-dihydroneopterin (NH2) and 7,8-dihydrobiopterin (BH2) – chosen for the purposes of this study belong to the group of polar basic compounds, Fig. 1. As an example, NEO contains two ionisable groups, therefore two pK values were determined: $pK_B = 2.23$ (basic-NH_2) and $pK_A = 7.89$ (acidic-OH) [12].

NEO is released by macrophages and is an immunologic marker for the activation of the cell-mediated immune system. Interferon-γ (secreted by T-lymphocytes) and tumor necrosis factor-α are the key cytokines, which lead to this immunologically triggered increase in NEO levels [13]. Thus, interferon-γ is probably the most important activator of pteridine synthesis and release; therefore, NEO is useful for the monitoring of cell-mediated (Th1-type) immune activation. Increased production and release of NEO and NH2 accompanies immune activation of macrophages both *in vitro* and *in vivo* [13].

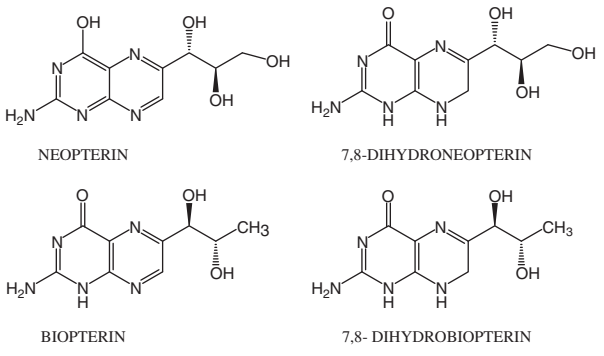

Figure 1. Structures of pteridines under study.

BIO and dihydrobiopterin are the oxidative products of tetrahydrobiopterin (BH4). Defects in BIO synthesis or regeneration might cause impairments in the biosynthesis of monoamine neurotransmitters. Autistic children were found to have significantly higher urinary BIO compared with control children [14]. NEO has been utilized as a marker of immune system activation and in inflammatory conditions. NEO is elevated among others in infections [15], cardiovascular disease [16], autoimmune diseases such as rheumatoid arthritis [17], systemic lupus [18] and atopic asthma [19], malignant diseases, immunomodulatory treatment monitoring, psychiatric disorders and sleep-disordered breathing [20]. NEO concentrations were also found to correlate with cognitive decline in Alzheimer's disease patients [21].

Pteridines have been analyzed only by the means of HPLC with fluorescence detection (FD) so far. Typically the separation was accomplished in RP chromatography mode [22–28] often employing 100% aqueous mobile phases containing phosphate buffer. Such mobile phases influence negatively RP column stability and efficiency. Aqueous mobile phase without any organic modifier might induce inadequate phase wetting and expulsion of eluent from pore space, which affects negatively separation efficiency on C18 stationary phase and leads to non-preproducible retention times [29]. In case of long time use such mobile phases can even lead to ''hydrophobic collapse of stationary phase'' [30]. Moreover, MS detection sensitivity might be significantly reduced with low concentration or no organic solvents in mobile phase. The elution order of pteridines was NEO first followed by BIO, which is in correspondence with polarity of molecules (see structures in Fig. 1) and their retention in RP system [22, 24, 27]. Dihydro- and tetrahydroderivatives were eluted before oxidized pterin forms [24, 26]. However, some newly developed methods are still highly time-consuming allowing NEO to be eluted in about 20 min and BIO in about 24 min [22]. The selectivity of FD is not sufficient in some cases of analysis of biological samples, as the interferences from urine matrix might often be observed [22]. In some articles the chromatogram to demonstrate method selectivity is not shown [25, 28]. Any more selective detection approaches including, *e.g.* MS were not applied; neither various separation modes employing, *e.g.* HILIC, which is highly convenient for the connection with MS detection.

The aim of this work was to evaluate retention properties of hybrid HILIC ultra-HPLC (UHPLC) stationary phases under conditions for FD and MS detection. The effects of mobile phase composition, buffer concentration, buffer pH and the effect of the type of volatile additives to the retention of polar pteridines and their separation were studied as there is a need for more studies on different types of polar stationary phases in order to gain better understanding of HILIC separation.

2 Materials and methods

2.1 Chemicals and reagents

Working standards of NEO, BIO, NH2 and BH2 were used for the purpose of this study. All compounds were obtained from Sigma Aldrich (Prague, Czech Republic). Ammonium acetate, acetic acid, ammonia, all of them LC-MS, were purchased by Sigma Aldrich as was HPLC gradient grade ACN and LC-MS grade ACN. HPLC grade water was obtained with a Milli-Q reverse osmosis Millipore system (Bedford, MA, USA) and met the requirements of the European Pharmacopoeia.

2.2 Chromatography

The Acquity UHPLC system (Waters, Prague, Czech Republic) was used for the purposes of this study. The system consisted of an ACQ-binary solvent manager, an ACQ-sample manager and an ACQ column thermostat, where the analytical column was kept at 30°C. All injected solutions were stored in the auto-sampler at 4°C. The partial loop with needle overfill mode was set up to inject 1 or 2 μL (which was the maximum to be injected because of overloading effect). ACN was used as a strong wash, and 20% ACN in water was used as a weak wash solvent. Fluorescence spectrometry and MS detection was used. ACQ FLR detector was set up at 353 nm for excitation wavelength and at 438 nm for emission wavelength. An MS/MS triple quadrupole system Quattro Micro (Micromass, Manchester, GB) was equipped with a Multi-Mode Ionisation Source (ESCI). Ion source in ESI positive mode was set up as follows: capillary voltage: 3000 V, ion source temperature: 130°C, extractor: 2.0 V and RF lens: 0.2 V. The desolvation gas was nitrogen at a flow of 400 L/h and a temperature of 375°C. Cone voltage (CV) was set up individually for each analyte. Nitrogen was also used as a cone gas (50 L/h) to prevent contamination of the sample cone. Triple quadrupole was set up to the selected reaction monitoring experiment. Argon was used as the collision gas, and collision energy (CE) was optimised for each analyte individually. NEO was monitored at selected reaction monitoring transition: $254.1 > 206.2$, CE = 15 V, CV = 25 V; BIO: $238.1 > 178.1$, CE = 20 V, CV = 30 V; BH2: $240.1 > 166.2$, CE = 10 V, CV = 15 V; NH2 $256.1 > 165.2$, CE = 25 V, CV = 25 V. MassLynx 4.1 software was used for data MS control and data gathering. QuanLynx software was used for data processing and integration.

Tested stationary phases included Acquity UHPLC stationary phases based on BEH hybrid sorbent:

Acquity UPLC BEH HILIC (100 × 2.1 mm, 1.7 μm, pH 1–8)

Acquity UPLC BEH Amide I (100 × 2.1 mm, 1.7 μm, pH 2–11)

Acquity UPLC BEH Amide II (100 × 2.1 mm, 1.7 μm, pH 2–11)

BEH Amide I represents stationary phase belonging to Acquity columns phase I, while BEH Amide II represents Acquity columns phase II. Phase II columns should offer improved performance (due to improved A and C term of Van Deemter plot) and reduced back-pressure [31]. The mobile phase tested for the retention of pteridines always contained ACN (50–90%) and volatile component being acetic acid, ammonium acetate buffer, water or formic acid at different concentrations and various values of pH. Mobile phase flow-rate was 0.4 mL/min for both MS andFD.

2.3 Preparation of standard solutions and samples

Reference standard solution of pteridines was prepared by dissolution of NEO and BIO in the mixture of water/ACN (50:50) and for reduced forms NH2 and BH2 by dissolution in pure water. Stock solutions were further diluted by the mixture of water/ACN (50:50) for optimization purposes and by appropriate mobile phase for system suitability test (SST) experiments.

Stock solution of pteridines had to be kept in dark and cool ambient (4°C), without the access of oxygen. Stock solutions were prepared fresh every day.

2.4 Evaluation of retention properties and SST

During this study the effect of the ratio of organic and water content of mobile phase together with the effect of additive type, pH and concentration on retention of pteridines was investigated. ACN always formed organic part of mobile phase. Water and volatile additives easily miscible with ACN at high concentration were included in the study: acetic acid, formic acid, ammonium acetate, ammonia and ammonium formate. The effect of pH variations was tested within stability range of stationary phase (2–11 for BEH Amide 1–8 for BEH) and with the regard to the ability of tested volatile additives to maintain certain pH (acidic pH – formic and acetic acid, basic pH – ammonia). There is a difference in buffer concentration used in MS applications and common detection approaches including UV, FD and others. MS detection requires much lower concentration of volatile additives (10 mM and less) as significant signal suppression occurs with higher concentrations compared with conventional detection approaches, which typically employ 10–100 mM buffers.

The measure of retention was expressed as capacity factor for two chosen representative analytes NEO and BIO. The experiments were performed under UHPLC-FD conditions (50 and 100 mM buffers) and under UHPLC-MS conditions (1 mM buffers and lower concentrations of formic and acetic acids).

SST was performed under the optimized chromatographic conditions for separation of four pteridines in order to verify chromatographic performance. A number of theoretical plates, peak asymmetry, resolution of individual compounds and repeatability of reference standard solution

injection were established in UHPLC-FD measurements. Only repeatability of reference standard solution injection was established in UHPLC-MS measurements. Details for determination and limits of individual parameters are given in Pharmacopoeias [32, 33].

3 Results and discussion

Stationary phases of two different polarities were selected for the retention and separation of four structurally similar polar basic pteridines. All the columns are based on hybrid support – BEH enabling to work in extended pH range (1–8) and (2–11) when ligand is attached. The particles are formed by organic and inorganic material [34]. Column dimensions were the same as well as the particle size: 2.1 × 100 mm, 1.7 μm.

On plain silica stationary phases the water shielding its surface will still not prevent the dissociation of free silanols and the cation exchange properties. In fact, the presence of water will promote formation of free silanols and also make the dissociation more facile. The negatively charged surface will attract cationic solutes; moreover, it will also cause a decreased retention of negatively charged polar compounds due to electrostatic repulsion forces of the surface. Addition of electrolyte (preferentially a buffer) is therefore obligatory in order to control the mixed mode separations induced by dissociated silanols, in particular with basic solutes [1]. When using salt buffers, the high organic content of the mobile phase forces the buffer salt to migrate into this polar layer. With increasing salt concentration, a larger hydrophilicity of the polar layer results, thus encouraging a stronger retention of solutes [35].

Similar interactions occur on underivatized BEH particles. However, due to the ethylene bridged groups embedded within the silica matrix, nearly one-third of the surface silanols are removed – see Fig. 1 in [36]; therefore retention due to interactions with these silanols is reduced. The surface of BEH particle is more alkaline in nature compared with traditional silica, where residual silanols tend to be rather acidic. Therefore, BEH particle differs in surface pK_a and charge state of the residual surface silanols, which can impact retentivity [34, 36].

The amide group is less reactive and lacks basic properties demonstrated, *e.g.* by amine group. Retention on amide stationary phases should be thus less sensitive to eluent pH and less prone to irreversible chemisorption [1]. The presence of amide group might enable both partitioning and hydrogen bonding separation mechanism as well.

3.1 Evaluation of UHPLC stationary phases – retention

3.1.1 Elution order

The retention and separation of pteridines in a conventional RP-HPLC system might be complicated due to their polarity and basic character. Typical approaches developed so far employed mostly aqueous mobile phases (91–100%) using C18 RP for separation [22, 28]. Using HILIC chromatography, there is a possibility to adjust the retention and separation of pteridines without the need of application of mostly aqueous mobile phases incompatible with sensitive MS detection and long-time use of C18 stationary phase. Under RP-HPLC conditions pteridines elute in following order: NH2, NEO, BH2, BIO [22, 24, 26, 27]. Under HILIC conditions the elution order was observed to be inversed as it was previously described in many HILIC applications.

This phenomenon was confirmed on BEH Amide stationary phase under all tested conditions. Elution order of pteridines could not be well evaluated on BEH HILIC, as complete separation of pairs NEO-NH2 and BIO-BH2 was not achieved at any tested chromatographic conditions, unless the peak shape of reduced forms of NH2 and BH2 was unacceptably wide or broadening.

3.1.2 Mobile phase composition – ACN content

During this study the influence of the ratio of organic and water content of mobile phase together with the influence of additive type, pH and concentration on retention of pteridines was studied. ACN always formed organic part of mobile phase. The measure of retention was expressed as capacity factor for two chosen analytes NEO and BIO under UHPLC-FD conditions and under UHPLC-MS conditions.

The strongest impact on retention of NEO and BIO was induced by changing water/organic part ratio. Even using pure water without any additive provided quite a decent retention ($k > 1.5$) at ACN contents higher than 85% on BEH HILIC, while on BEH Amide already at the concentration higher than 70%. Under HILIC conditions, the higher percentage of ACN was applied, the higher retention of pteridines was reached, thus k value increased up to 50 enabling elution of NEO at retention times about 35 min using 5% of water component on BEH Amide. BEH HILIC provided significantly lower retention for pteridines compared with both BEH Amide phases. These results were in agreement with Guo *et al.* [35], who also published results showing higher retention of basic compounds on amide stationary phase (TSK gel Amide-80) compared with plain silica and other stationary phases.

When the retention time for an analyte is plotted against the percentage of ACN in the eluent, a linear relationship would provide evidence for a partitioning mechanism, augmented by the observation that at extreme ACN concentration a deviation is evident toward excessive retention. When the data are visualized as log k against the linear and logarithmic ratio of water (the eluting member) of the eluent, curvatures are evident in most of the plots. However, in general the data adhere better to a log–log plot [1]. Such plots are presented in Fig 2 showing quite a good linearity in log–log plots for both analytes NEO and BIO on all tested BEH stationary phases. The type of additive in

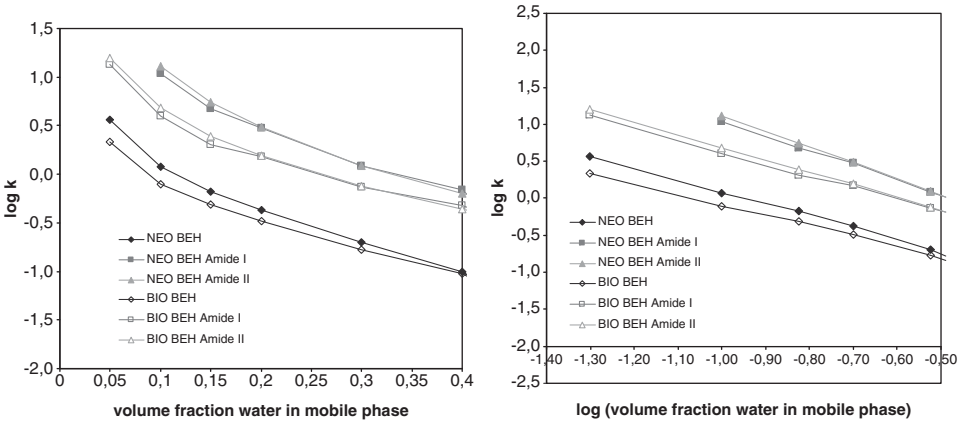

Figure 2. Plots of log *k versus* volume fraction water in mobile phase (left) and log *k versus* log volume fraction water in mobile phase (right) for NEO and BIO on three various HILIC stationary phases with 0.1% acetic acid as mobile phase.

mobile phase had only slight effect on correlation coefficients expressed as r^2. Presented data represent 0.1% acetic acid as an additive. As a result, the log–log plot also demonstrates lower retention for BEH HILIC and higher for BEH Amide. Using acetic acid, BEH Amide of phase I demonstrates slightly stronger retention properties.

3.1.3 Mobile phase composition – the type, concentration and pH of additive

Most of inorganic salts typically used in RP-HPLC are not suitable for HILIC due to poor solubility in the mobile phase containing high percentage of ACN. Excluding salts incompatible with MS detection only ammonium acetate, ammonium formate and ammonium bicarbonate are convenient for HILIC-MS methods. Various types of additives were compared in this study. The additives were examined at low concentrations for UHPLC-MS applications (1 mM for buffers, 0.001–0.1% for acids and ammonia) and at higher concentrations for UHPLC-FD applications (50 and 100 mM). The effect of pH variations was tested within stability range of stationary phase (2–11 for BEH Amide phases, 1–8 for BEH) and with regard to the ability of tested volatile additives to maintain certain pH (acidic pH–formic and acetic acid, basic pH–ammonia).

At UHPLC-FD conditions with higher concentrations of buffer the strongest retention was achieved with 100 mM ammonium acetate buffer, pH 9.8, on tested BEH columns. The decrease in concentration to 50 or 10 mM decreased the retention. There was about two times difference between 100 and 10 mM ammonium acetate buffer ($k = 21$ decreased to 12 on BEH HILIC, while $k = 26$ decreased to 13 on BEH Amide). There was only a slight difference in the effect of pH on retention within the pH range 4.8–8.8. Lower pH buffers (3.8) together with diluted formic and acetic acid provided more significantly lower retention.

At UHPLC-MS conditions substantially lower concentrations of additives must be used in order to prevent signal suppression. With decreasing buffer concentration the retention of analytes decreased as well. Similarly, BEH HILIC stationary phase demonstrating the lowest retention capability provided almost no retention for BIO at UHPLC-MS conditions – $k > 1.5$ only when 95% of ACN was present in mobile phase. At such mobile phase composition maximum k value was 2.4 for BIO and 8.8 for NEO. Contrary to UHPLC-FD conditions, where higher buffer concentration was used, the highest retention was not achieved with ammonium acetate, pH 9.8, on BEH HILIC. Lower pH additives including ammonium formate, formic and acetic acid provided stronger retention. This phenomenon might correspond to cation exchange activity of residual silanols, which are not shielded by any polar substituent on the surface of BEH particle. Increased buffer concentration allows partition mechanism to prevail, while at low buffer concentration cation exchange process predominates (positively charged amine group of pteridines at low pH).

Under UHPLC-MS conditions substantially stronger retention was observed on BEH Amide compared with BEH HILIC. Overall, capacity factor values were about ten times higher compared with BEH HILIC. The retention was decreased compared with UHPLC-FD using higher concentrations of additives, *e.g.* for pH 9.8 at 100 mM $k = 26$, at 50 mM $k = 20$, at 10 mM $k = 14$ and at 1 mM $k = 12$. The same phenomenon as on BEH HILIC was observed at low pH with low buffer concentration. The retention was increased with acidic and neutral pH additives, while ammonium acetate, pH 9.8, and ammonia solution induced a decrease in the retention of pteridines.

3.2 Evaluation of UHPLC stationary phases – selectivity for separation of pteridines

BEH HILIC stationary phase demonstrated lower retention properties and the insufficient selectivity, as only the separation of NEO and BIO was enabled under any of tested conditions, while the separation of BIO and BH2 reduced form as well as NEO and NH2 reduced form was impossible (neither at UHPLC-MS nor at UHPLC-FD conditions).

Examples of separation of pteridines on BEH Amide are given in Figs. 3 and 4. Separation of all four pteridines was

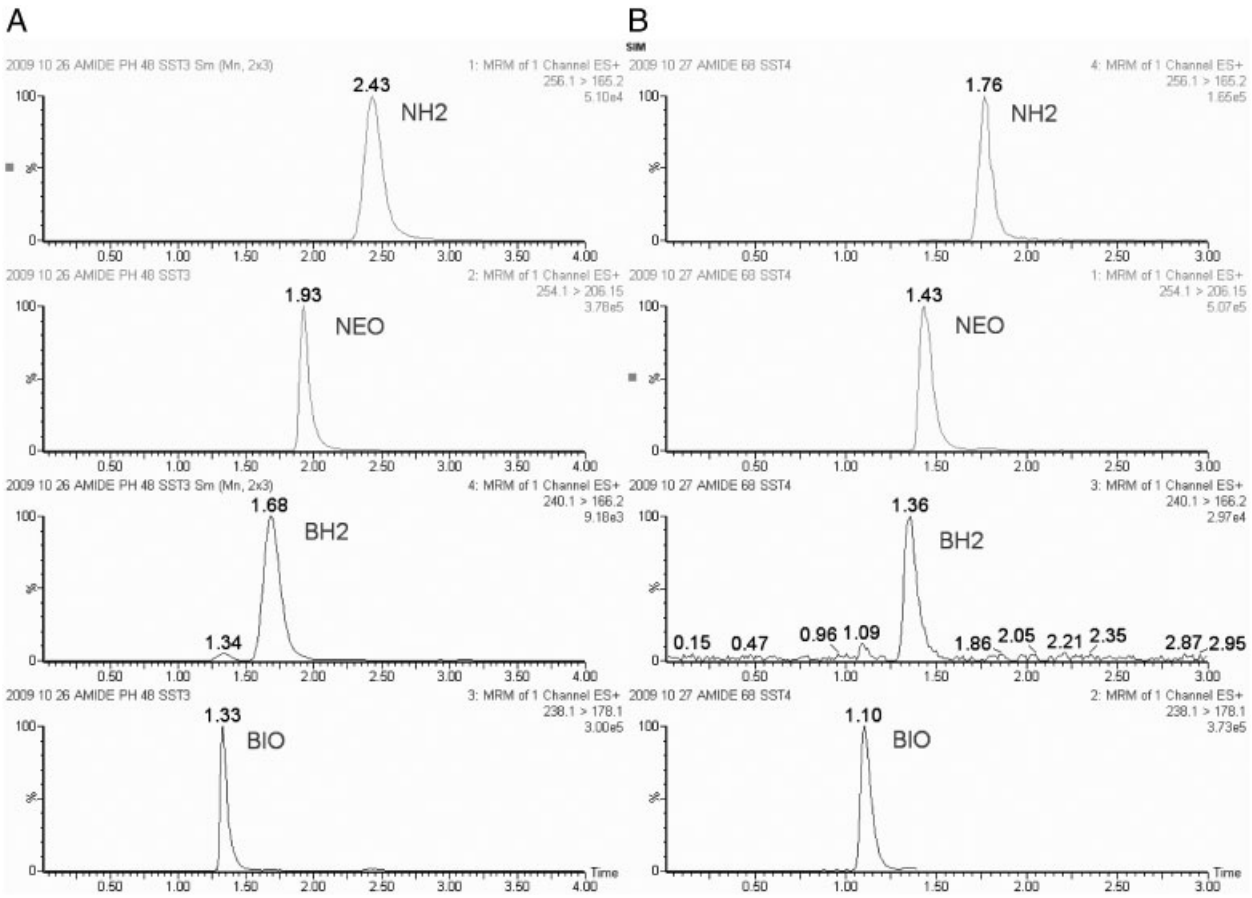

Figure 3. Chromatogram of separation of pteridines under UHPLC-MS conditions. Separation on BEH Amide II (A) ACN/1 mM ammonium acetate pH 4.8 (77:23) and (B) ACN/1 mM ammonium acetate pH 6.8 (72:28) at flow-rate 0.4 mL/min.

enabled preferably at lower and neutral pH. At high pH values such as 9.8 reduced forms BH2 and NH2 were no more well separated from NEO and BIO unless very high percentage of ACN (90%) was present in mobile phase using at least 50 mM concentration of ammonium acetate buffer. This however prolonged analysis time unacceptably, Fig. 4C.

3.2.1 Peak shape under various UHPLC conditions

Under various mobile phase compositions there was a great difference in peak shape. It was a limiting factor of applicability for some mobile phases, as very wide and non-symmetric peaks would not provide high efficiency repeatable results.

Generally, on BEH HILIC stationary phase a negative influence of acidic pH on peak shape was observed already at 50% of ACN in mobile phase. This phenomenon was observed for all acidic mobile phases including formic acid, acetic acid and ammonium acetate, pH 3.8. Eluting peaks were very wide especially for BH2 and NH2 demonstrating strong tailing with increasing concentration of ACN. No separation of oxidized and reduced forms of NEO and BIO was obtained. The peak shape improved significantly with increasing pH, unless the amount of ACN was increased up to 20% or more. Low selectivity and significant peak tailing probably induced by free silanol groups interacting with basic compounds did not permit the separation of oxidized and reduced form of NEO/NH2 and BIO/BH2.

Similar beavior was observed on BEH Amide stationary phase with one important difference – increased selectivity, which enabled the separation of oxidized and reduced forms. Similarly, all acidic modifiers including formic acid, acetic acid, ammonium formate and ammonium acetate until pH 3.8 exhibited very wide peaks for NH2 and BH2 often hardly baseline separated from their oxidized forms. With increasing pH the peak shapes were significantly improved and all four pteridines were nicely separated within pH region 4.8–7.8. Further pH increase induced change in selectivity; therefore, pH higher than 8.8 did not allow the separation of oxidized and reduced forms any more in spite of very nice peak shapes obtained either with ammonium acetate or with ammonia solution, unless very high concentration of ACN and high molarity buffer was used, which significantly prolonged analysis time, Fig. 4C.

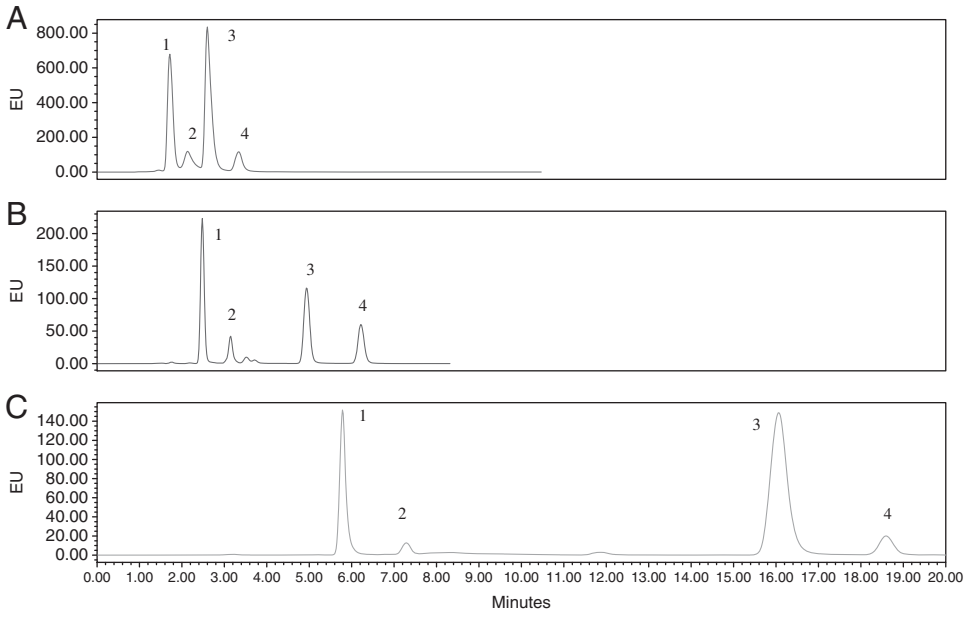

Figure 4. Chromatogram of separation of pteridines under UHPLC-FD conditions. Separation on BEH Amide II (A) ACN/50 mM ammonium acetate pH 3.8 (80:20), (B) ACN/50 mM ammonium acetate pH 6.8 (85:15) and (C) ACN/50 mM ammonium acetate, pH 9.8 (90:10). Elution order: BIO (1), BH2 (2), NEO (3), NH2 (4).

3.3 System suitability test

System suitability test was performed by ten times injecting of mixed pteridine solutions at optimum found chromatography conditions – details provided in Table 1. Testing of system suitability provided satisfactory results at all tested conditions – see Table 1. For UHPLC-FD all monitored parameters met required criteria for separation method [32, 33]. Separation at pH 3.8 demonstrated the lowest separation efficiency. The separation efficiency increased with increasing pH. Hence, the best results were observed at pH 9.8. When comparing efficiency of BEH Amide I and Amide II at pH 6.8, substantial decrease is observed in case of BEH Amide I.

4 Concluding remarks

HILIC approach using hybrid stationary phases in UHPLC system was found to be highly advantageous for the analysis of polar pteridines as the retention was enabled by simple volatile mobile phases without any content of inorganic buffers. Such approach is easily compatible with MS detection and allows high sensitivity and selectivity.

Retention characteristics of BEH HILIC and BEH Amide were studied in the group of polar basic pteridines (NEO, BIO, NH2 and BH2). BEH HILIC was not found to be convenient, as its selectivity did not enable complete separation of NEO-NH2 and BIO-BH2 pairs. On the other hand, BEH Amide provided both, sufficient retention and selectivity for separation of four pteridine derivatives. The effect of mobile phase composition, buffer type, pH and concentration on retention of pteridines were examined in detail under UHPLC-FD and UHPLC-MS conditions. The retention of pteridines in HILIC system was affected by all

Table 1. SST parameters for selected HILIC separation conditions

	BIO	BH2	NEO	NH2
BEH AMIDE II				
20% 50 mM AmAc, pH 3.8/ACN, $f = 0.4$ mL/min, FD				
t_R (%RSD)	0.08	0.07	0.05	0.05
A (%RSD)	1.66	0.28	5.21	1.05
Asymmetry	1.08	0.99	1.79	1.11
Resolution	–	2.24	2.68	3.00
Theoretical plate number	1512	1958	1978	4124
15% 50 mM AmAc, pH 6.8/ACN, $f = 0.4$ mL/min, FD				
t_R (%RSD)	0.12	0.27	0.13	0.26
A (%RSD)	0.59	0.31	1.14	1.00
Asymmetry	1.18	0.95	1.09	1.07
Resolution	–	6.60	11.78	5.80
Theoretical plate number	8297	15 934	8674	13 474
10% 50 mM AmAc, pH 9.8/ACN, $f = 0.4$ mL/min, FD				
t_R (%RSD)	0.09	0.02	0.02	0.02
A (%RSD)	1.40	0.99	1.85	0.79
Asymmetry	1.29	0.99	1.14	1.14
Resolution	–	7.31	18.76	4.45
Theoretical plate number	28 786	10 519	9017	16 931
23% 1 mM AmAc, pH 4.8/ACN, $f = 0.4$ mL/min, MS				
t_R (%RSD)	0.00	0.17	0.00	0.20
A (%RSD)	1.43	4.82	1.43	5.61
28% 1 mM AmAc, pH 6.8/ACN, $f = 0.4$ mL/min, MS				
t_R (%RSD)	0.00	0.37	0.21	0.17
A (%RSD)	3.00	3.58	1.98	3.84
BEH AMIDE I				
20% 50 mM AmAc, pH 6.8/ACN, $f = 0.4$ mL/min, FD				
t_R (%RSD)	0.15	0.23	0.14	0.21
A (%RSD)	0.69	2.73	2.87	1.52
Asymmetry	1.04	1.84	1.12	1.19
Resolution	–	2.11	4.00	2.95
Theoretical plate number	2150	1961	3446	4526

factors – the content of ACN being the most significant one, buffer pH – the best separation was obtained within pH range 4.8–7.8, and buffer concentration – the stronger the buffer concentration, the stronger was the retention at basic pH, while at acidic pH probably cation-exchange effect induced stronger retention of analytes with low buffer concentrations.

The difference between Acquity UHPLC column phase I and phase II was investigated using BEH Amide stationary phase. BEH Amide column phase I demonstrated slightly stronger retention for pteridine derivatives, substantially lower efficiency of separation process and 7–19% decrease in column back-pressure.

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