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Development of a novel amide-silica stationary phase for the reversed-phase HPLC separation of different classes of phytohormones

Hayriye Aral [a], Tarık Aral [a,*], Berrin Ziyadanoğulları [b], Recep Ziyadanoğulları [b]

[a] University of Batman, Faculty of Science and Art, Department of Chemistry, Batman, Turkey
[b] University of Dicle, Faculty of Science, Department of Chemistry, Diyarbakır, Turkey

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ABSTRACT

A novel amide-bonded silica stationary phase was prepared starting from *N*-Boc-phenylalanine, cyclohexylamine and spherical silica gel (4 μm, 60 Å). The amide ligand was synthesised with high yield. The resulting amide bonded stationary phase was characterised by SEM, IR and elemental analysis. The resulting selector bearing a polar amide group is used for the reversed-phase chromatography separation of different classes of thirteen phytohormones (plant hormones). The chromatographic behaviours of these analytes on the amide-silica stationary phase were compared with those of RP-C18 column under same conditions. The effects of different separation conditions, such as mobile phase, pH value, flow rate and temperature, on the separation and retention behaviours of the 13 phytohormones in this system were studied. The optimum separation was achieved using reversed-phase HPLC gradient elution with an aqueous mobile phase containing pH=6.85 potassium phosphate buffer (20 mM) and acetonitrile with a 22 °C column temperature. Under these experimental conditions, the 12 phytohormones could be separated and detected at 230 or 270 nm within 26 min.

1. Introduction

High-performance liquid chromatography is very important for analysing a wide range of chemical species. The performance of this technique is strongly related to the properties of the stationary phase used [1–3]. Thus, chromatographers attach special importance to the design of efficient stationary phases for HPLC. The development of new silica-based stationary phases for HPLC remains a challenge for chromatographers. Silicas modified with C8 and C18 alkyl chains are the most widely used stationary phases for reversed-phase HPLC.

A recent trend in RPLC involves the use of stationary phases containing polar groups in addition of the non-polar RP ligands for separations of alkaline and acidic polar compounds [4–10].

Moderately polar stationary phases with chemically bonded cyano-, diol-, cyclodextrin-, polyethylene glycol and alkyls with embedded amide, carbamate or other functionalities were originally intended mainly for reversed-phase applications in water-rich mobile phases [11–14]. Subsequently, polar stationary phases have exhibited novel properties for polar analytes [9]. Recent investigations describing stationary phases containing embedded polar groups have shown the superior performance of these new phases over conventional C8 and C18 phases for alkaline analytes [15,16]. The polar groups, particularly amide, were originally chosen for their ability to interact with alkaline analytes [8]. In addition, the amide phases have shown enhanced selectivity towards low-molecular-weight acids [11]. The stationary phases containing embedded polar groups show different selectivities and are also less retentive, requiring a mobile phase with a lower concentration of organic solvent [12].

Low concentrations of phytohormones exert numerous important plant physiological responses at different stages of plant development, such as cell division, enlargement and differentiation; organ formation; seed dormancy and germination; and leaf and organ senescence and abscission [17]. Since the discovery of the first phytohormone, auxin, in 1926, scientists have identified several types of phytohormones, mainly including auxins, cytokinins, abscisic acid, gibberellins and ethylene [18]. Chlorinated phenoxy acids with auxin-like activity are also known as herbicides (Scheme 1: 2,4-D, 2,4-DP, 2,4-DB, 2,4,5-T and 4-PA). Herbicides can move in agricultural ecosystems, polluting surface and ground waters due to their solubility in water [19,20].

HPLC is the most widely used technique for the analysis of these polar phytohormones [21,22]. Liquid chromatography combined with mass spectrometry (LC-MS) has also been proposed for

Abbreviations: HPLC, high-performance liquid chromatography; RPLC, reversed-phase liquid chromatography; NPLC, normal-phase liquid chromatography; HILIC, hydrophilic interaction chromatography; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; H, hexane; EtOAc, ethyl acetate; TFA, trifluoroacetic acid; CPTMS, 3-chloropropyltrimethoxysilane; Boc, *tert*-butoxycarbonyl; SP, Stationary phase; CP-Si, 3-chloropropylated silica; ODS, octadecylsilyl; Pr, Propyl

* Corresponding author. Tel.: 90 488 2172205.
E-mail address: tarik.aral@batman.edu.tr (T. Aral).

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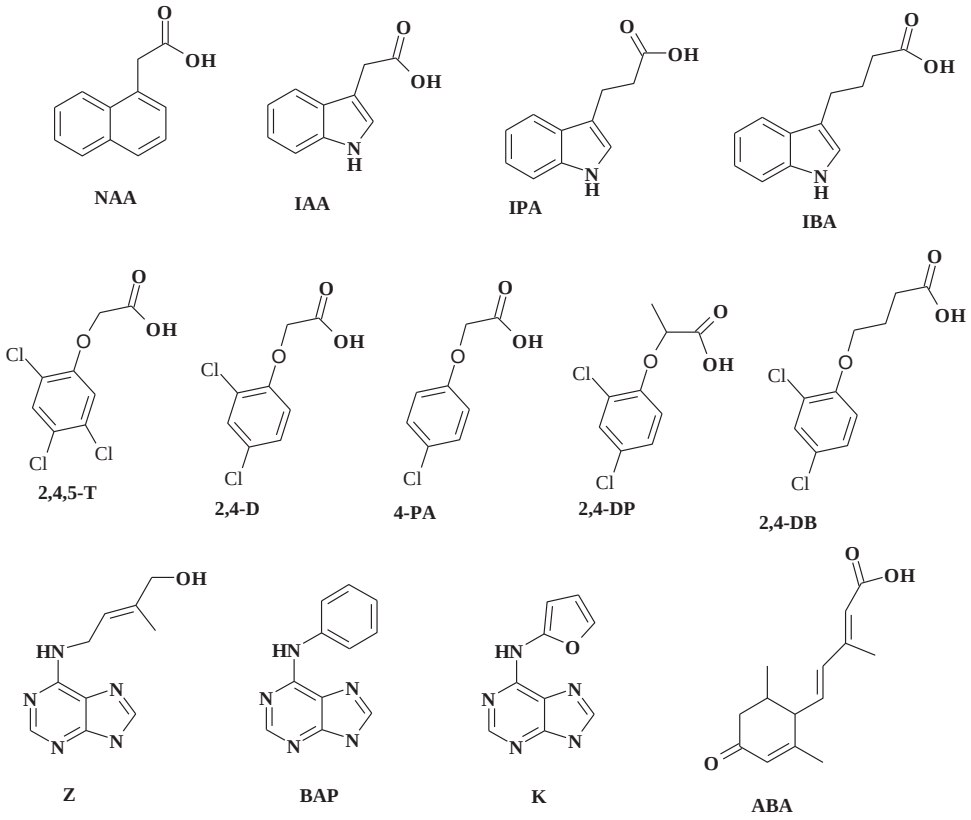


Scheme 1. Chemical structures of the phytohormone compounds separated by a new amide-silica column.

the determination of auxins [23,24] and abscisic acid [25,26] by RP-C18 columns. However, auxins, cytokinins, and abscisic acid have very different plant physiological functions and very different chemical properties. Thus, developing stationary phases and separation methods for the HPLC separation of different classes of phytohormones are extremely challenging. Based on our literature review, there are limited papers dealing with the separation of different classes of phytohormones [27–30]. In the last several years, Zhen Maa and co-workers reported the separation of four groups of phytohormones by LC-MS using an RP-C18 column. The authors indicated that this paper was the first to achieve the separation of four groups of phytohormones. In this study, eight phytohormones were separated in 50 min of analysis time [31]. In many other similar studies, RPLC was used for the separation and determination of plant hormones. In our wide review of the literature, we could not find any paper describing the separation of different classes of twelve phytohormones by HPLC.

Therefore, we describe herein the development of a new amide stationary phase and used as an HPLC column for the separation of 13 phytohormones, including indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-butyric acid (IBA), abscisic acid (ABA), zeatin (Z), 6-benzylaminopurine (BAP), kinetin (K), 1-naphthaleneacetic acid (NAA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxpropionic acid (2,4-DP), 2,4-dichlorophenoxybutiryc acid (2,4-DB), 4-chlorophenoxyacetic acid (4-PA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Scheme 1). The preparation of the new phase involves the synthesis of an amide ligand and the surface modification of spherical silica particles (4 μm, 60 Å) via the attachment of this ligand to the silica surface. The effects of different separation conditions, such as the mobile phase, pH, flow rate and temperature, on the separation and retention behaviours of 13 plant hormones in this system were studied and 12 phytohormones were separated. One of the best efficient commercial available ODS column ACE C18 was also used for reversed phase separation of thirteen phytohormones in two different pH and the results obtained by two columns are compared. The optimum separation conditions for the two columns are given in Figs. 1 and 2.

2. Experimental

2.1. Reagents and materials

Spherical silica gel (SuperSpher Si 60, 4 μm, 60 Å) was purchased from Merck. Silica gel 60 (Merck, 0.040–0.063 mm) and silica gel/TLC cards (F254), used for column chromatography and TLC, respectively, were purchased from Merck. ACE 5C18 column (250–4.6 mm, 5 μm, 100 Å, carbon load 15.5%, surface area 300 m^2/g) was used for comparisons. The solvents used in HPLC were of HPLC grade and purchased from Merck. All the selected analytes and all other reagents and solvents were purchased from Sigma-Aldrich or Merck. All reagent used in the synthesis were reagent grade unless otherwise specified. Melting points were determined by an Electrothermal 9300 apparatus with open capillaries. Infrared spectra were recorded on a Mattson 1000 FT-IR spectrometer. Elemental analyses were performed with a Thermo Scientific FLASH 2000 instrument. Deionised water was purified using a Millipore Milli-Q water system. Scanning electron microscopy (SEM) images were obtained with a JEOL/JSM-6510 LV instrument. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker DPX-400 high-performance digital FT-NMR spectrometer. The chemical shifts (d) and coupling constants (J) are expressed in parts per million (ppm) and Hertz, respectively.

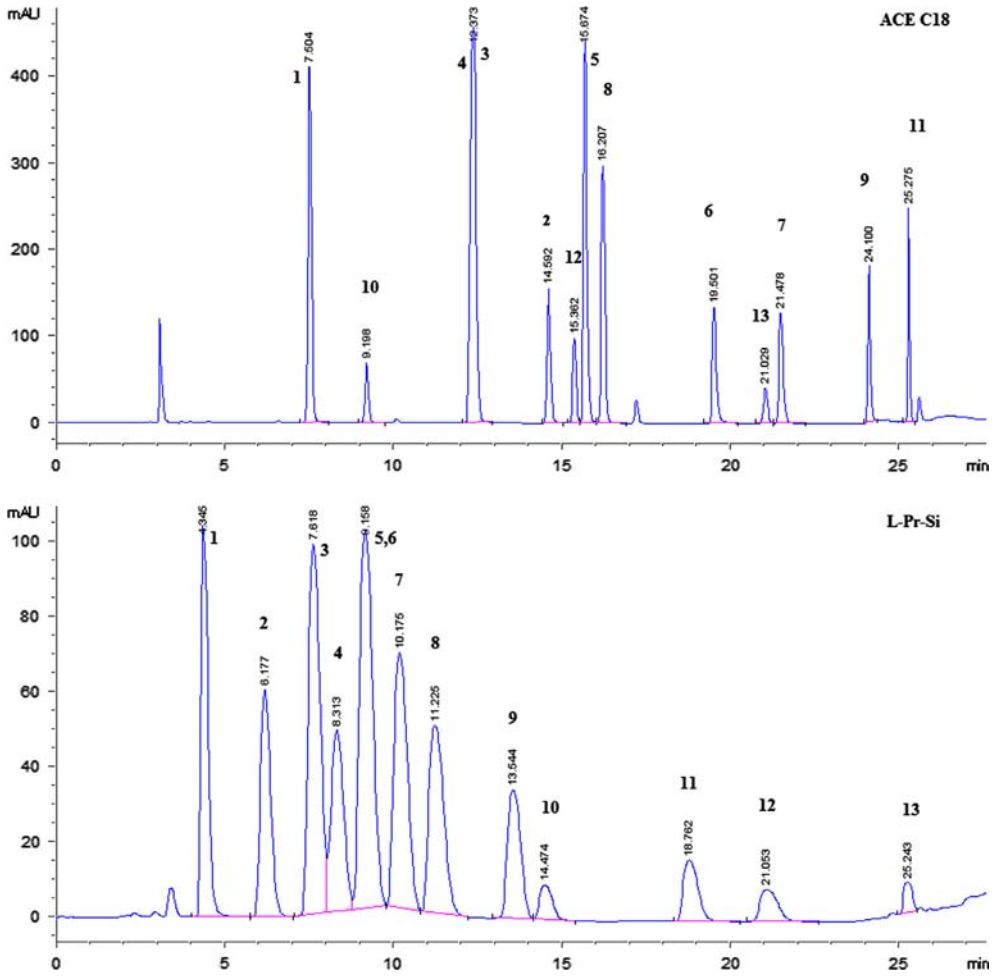


Fig. 1. Phytohormone separation on **L-Pr-Si** and **ACE C18** columns under the optimised conditions at pH=6.85. Gradient elution: ACN in phosphate buffer (pH: 6.85, 20 mM), 0–20 min; 10–25%, 20–30 min; 25–90%. Flow rate: 0–13 min; 0.9 mL/min, 13–30 dk; 1.2 mL/min. Temp.: 22 °C. Injection volume: 5 μL. Detection: 230 nm. Analytes: 1: IAA, 2: 4-PA, 3. IPA, 4: ABA, 5: IBA, 6: 2,4-D, 7: 2,4-DP, 8: NAA, 9: 2,4,5-T, 10: Z, 11: 2,4-DB, 12: K, 13: BAP.

2.2. HPLC conditions

The chromatographic equipment consisted of an Agilent 1260 HPLC system, including a quaternary pump, degasser, autosampler, DAD detector and thermostated column compartment (Agilent, Waldbronn, Germany). The column (250–4.6 mm, stainless steel) was packed with modified silica gel using the slurry packing technique under high pressure. Standard solutions of each analyte (1 mg mL^{-1}) were prepared in acetonitrile, whereas kinetin (*K*) and *N*-benzylaminopurine (BAP) were prepared in a 30:70 acetonitrile–0.1 N NaOH mixture. All solutions were stored at −25 °C. Further dilutions were performed with water to obtain a final concentration of 100 ppm per analytes (total concentration of 1300 ppm). The detection wavelengths used were 230 and 275 nm. The chromatographic conditions differing from the conditions given in this chapter are described in the figures.

2.3. Synthesis

2.3.1. *N*-Boc-amide (**1**)

Dicyclohexylcarbodiimine (DCC, 1.71 g, 8.3 mmol) dissolved in DCM (15 mL) was added to a stirred solution of *N*-Boc-phenylalanine (2 g, 7.55 mmol) and cyclohexylamine (820 mg, 8.3 mmol) in dichloromethane (15 mL) at 0 °C over 1 h. After stirring overnight at room temperature, the mixture was filtered and evaporated. The residue was purified by column chromatography on silica gel (H:EtOAc=4:1) to afford *N*-boc-amide (**1**) as a white solid. Yield: 3.11 g, 90%. Mp: 134–135. ^{1}H NMR ($CDCl_3$): δ (ppm); 0.90–1.95 (m, 19H), 2.91–2.99 (m, 1H), 3.05–3.15 (m, 1H), 3.65–3.76 (m, 1H), 4.18–4.27 (m, 1H), 5.18 (bs, 1H), 5.57 (bs, 1H), 7.21–7.33 (m, 5H). ^{13}C NMR ($CDCl_3$): δ (ppm); 26.64, 25.41, 32.71, 38.96, 48.08, 56.73, 79.11, 126.90, 128.64, 129.38, 136.95, 155.35, 169.90. IR ν (cm^{-1}); 3341, 3060, 3020, 29.60, 2930, 2853, 1690, 1649, 15.38, 1521, 1449, 1386, 1291, 1264, 1166, 743, 699.

2.3.2. *Deprotection of N-boc group (**2**)*

TFA/AcOH (1:1) (1.2 mL) was added to a stirred solution of *N*-boc-amide (**1**) (1 g, 2.89 mmol) in dichloromethane (5 mL) at 0 °C. After stirring overnight at room temperature, the solvent was evaporated and 1 N NaOH (5 mL) was added to the residue. The mixture was extracted with DCM (3 × 15 mL), dried over Na_2SO_4, filtered and evaporated under vacuum to afford a pure amide (**2**) a white solid. Mp: 217 (decomposed.). Yield: 710 mg, 99%. ^{1}H NMR ($CDCl_3$): δ (ppm); 1.1–2.2 (m, 12H), 2.90–2.95 (m, 1H), 3.18–3.23 (m, 1H), 3.75–3.81 (m, 1H), 4.16–4.19 (m, 1H), 6.07 (s, 1H), 7.19–7.33 (m, 5H). ^{13}C NMR ($CDCl_3$): δ (ppm); 24.94, 25.77, 28.99, 37.78, 51.29, 57.44, 127.28, 128.66, 129.44, 134.97, 173.17. IR ν (cm^{-1}); 3259, 3235, 3100, 3028, 2936, 2854, 1701, 1625, 1569, 1442, 1338, 1348, 1241, 1192, 1111,764, 696.

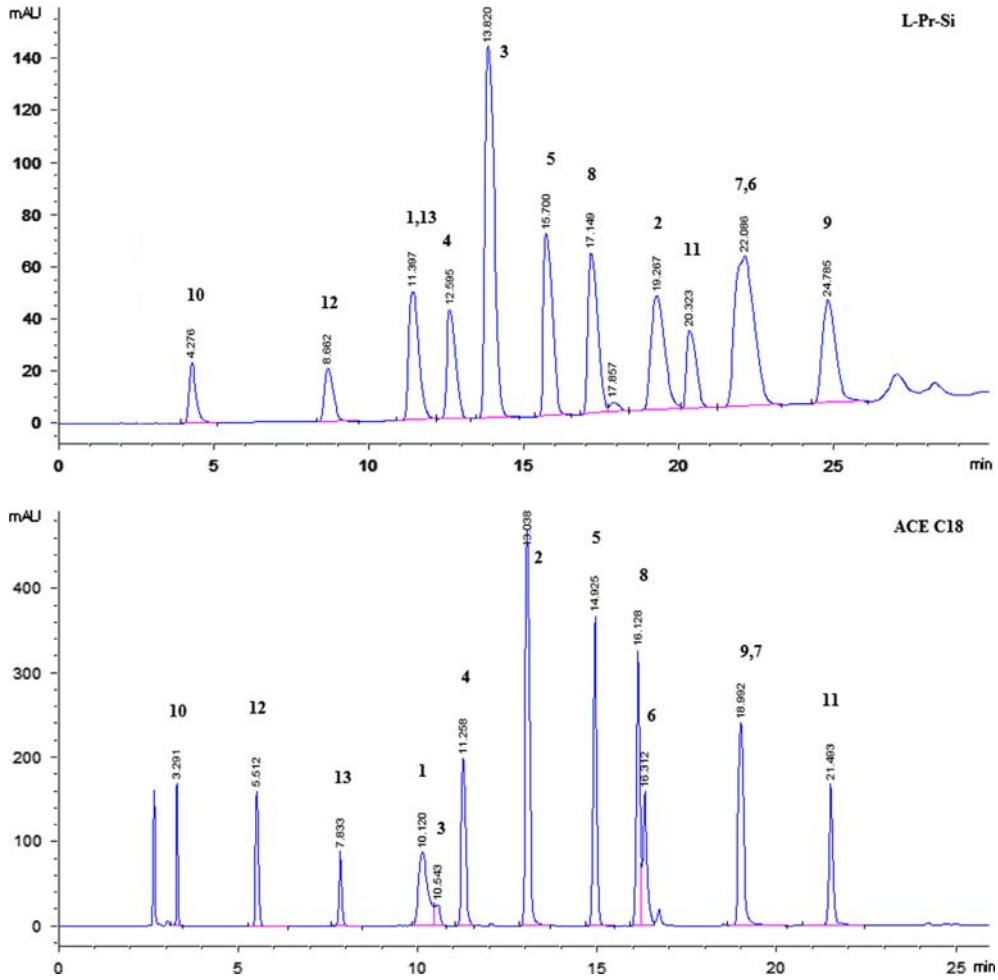


Fig. 2. Phytohormone separation on **L-Pr-Si** and **ACE C18** columns under the low pH (3.25) conditions. Gradient: ACN in phosphate buffer (10 mM); 20–80% from 0 to 30 min. Temp.: 22 °C, flow rate, 1 mL/min, injection volume: 5 μL. Detection: 230 nm. Analytes: 1: IAA, 2: 4-PA, 3. IPA, 4: ABA, 5: IBA, 6: 2,4-D, 7: 2,4-DP, 8: NAA, 9: 2,4,5-T, 10: Z, 11: 2,4-DB, 12: K, 13: BAP.

2.3.3. 3-Chloropropylsilica gel (**3**)

HPLC-quality spherical silica gel (4 μm, 60 Å) (4 g) was suspended with toluene (60 mL) and 3-chloropropyltrimethoxysilane (CPTMS, 2 g, 10 mmol) was added to the mixture. The mixture was stirred and refluxed for 4 days. The toluene was evaporated, and the chloropropylated silica was washed with chloroform by Soxhlet extraction overnight and dried under vacuum for 8 h to afford white powders. Elemental analysis: C 5.32, H 1.09 (1.11 mmol of loaded chloropropylsilyl/g CP-Si (**3**) based on C).

2.3.4. Amide-bonded stationary phase (**4**)

3-Chloropropylsilica gel (**3**) (4 g) was suspended with toluene (60 mL), and amide (**2**) (1.5 g, 6 mmol) was added to the mixture. The mixture was stirred and refluxed for 4 days. The toluene was evaporated, and the amide modified silica was washed with chloroform by Soxhlet extraction for 24 h and dried under vacuum for 7 h to obtain a white powder. Elemental analysis: C 21.45, H 3.24, N 2.46 (0.88 mmol of loaded amide/g SP (**4**) based on N. This means that 1.11 mmol of amid ligand was bonded to the each gram of CP-Si (**3**)). On the other hand, the chloroform was evaporated and the residue was dried to obtain 370 mg of unbounded ligand. 282 mg (1.15 mmol) of amide ligand was bonded to the each gram of 3 based on the account of the amount of the ligand recovered).

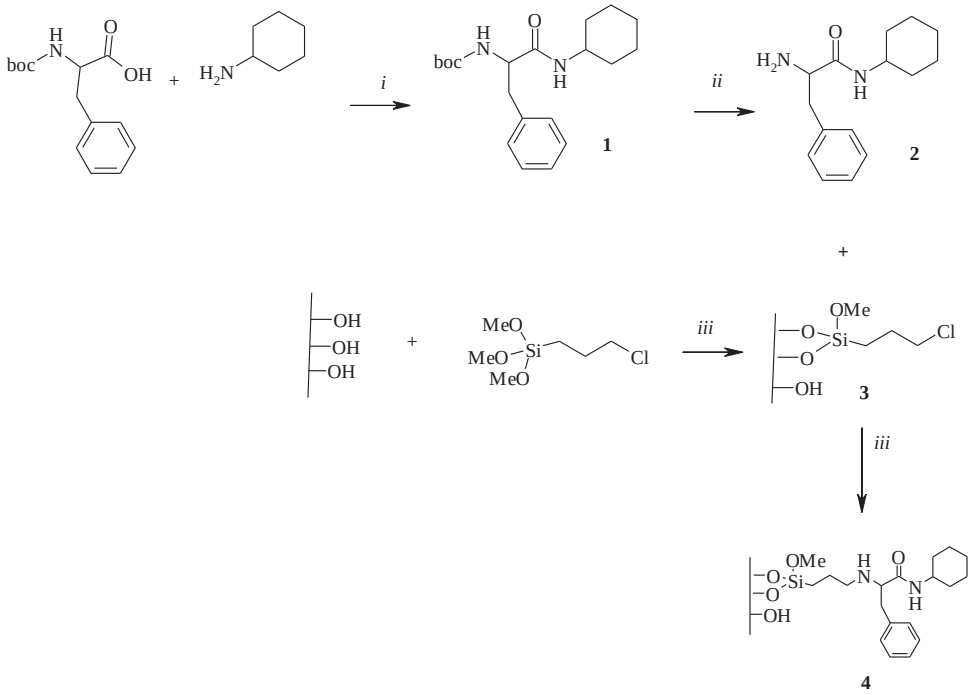
3. Results and discussions

3.1. Syntheses

The preparation of the amide-functionalised silica is outlined in Scheme 2. This stationary phase was chosen because its structure bears both amine and amide polar groups, which can interact well with alkaline and acidic analytes. The stationary phase also includes aliphatic and aromatic groups and can show selectivity towards polar analytes bearing aromatic rings and aliphatic chains. For the synthesis of the stationary phase, the amide ligand was first synthesised in two steps (Scheme 2). By the reaction of *N*-boc-phenylalanine with cyclohexylamine, *N*-boc-amide (**1**) was obtained with 90% yield. The deprotection of **1** gave amide ligand (**2**) in quantitative yield. 3-Chloropropyltrimethoxysilane (CPTMS) was attached to the HPLC-quality spherical silica gel (4 μm, 60 Å) to obtain 3-chloropropylsilica gel (**3**). After 3 was washed by Soxhlet extraction using 150 mL of chloroform overnight and dried under vacuum, compound **2** was covalently bonded to **3** to obtain the amide-silica stationary phase (**4**).

3.2. Characterisation of the stationary phase

The prepared novel amide-silica stationary phase was characterised by SEM, FT-IR and elemental analysis. The elemental analysis



Scheme 2. Reagents and conditions. (i) DCC, 0 °C-rt, 24 h; (ii) TFA/AcOH (1:1, v-v), DCM; (iii) toluene, reflux, 4 days.

Table 1
Elemental analysis results of silica, chloropropylated silica and ligand bonded silica.

Silica materials	% C	% H	% N
Silica (Si)	0	0.03	0
CP-Si (**3**)	5.32	1.09	0
Li-Pr-Si (**4**)	21.45	3.24	2.46

results and selected IR absorbance were given in the Experimental section. Table 1 shows the elemental analysis of blank silica (Si), chloropropylated silica (**CP-Si**, **3**) and ligand-bonded silica (**L-Pr-Si**, **4**). According to these data, all the CPTMS was replaced with the amide ligand. The resulting bonded stationary phase consists of 0.88 mmol of the amide ligand on each gram of stationary phase.

The IR spectrum of **Si** showed a strong silanol (SiOH) band at 3484 cm^{-1}. This band was absent (or very weak) in the spectrum of **CP-Si**. The bands at 1636 and 963 cm^{-1} were also very weak, and new weak bands appeared at 2990–2980 cm^{-1}, which were assigned to C–H asymmetric and symmetric stretching for CH_2. Thus, CP-Si (**3**) has higher coverage on the surface. L-Pr-Si (**4**) has stronger bands at 2970–2980 and many new bands between 1610–1450 and 900–600 cm^{-1}. Therefore, the FT-IR spectra obtained from the solid samples confirmed the success of the reactions on the surface of the silica.

The morphology, shape and size of the silica material particles were characterised by high-resolution scanning electron microscopy. The comparison of SEM spectra of blank silica gel and the amide-bonded silica showed a narrow particle size distribution with well-defined spherical particles. Thus, a new amide stationary phase was synthesised from the spherical silica gel, and elemental analysis, FT-IR and SEM measurements confirmed the successful derivatisation.

3.3. HPLC evaluations

The chromatographic evaluations were performed with a column packed with the amide-silica phase using a standard test mixture composed of thirteen phytohormones: indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-butyric acid (IBA), abscisic acid (ABA), zeatin (Z), 6-benzylaminopurine (BAP), kinetin (*K*), 1-naphthaleneacetic acid (NAA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxpropionic acid (2,4-DP), 2,4-dichlorophenoxybutiryc acid (2,4-DB), 4-chlorophenoxyacetic acid (4-PA), and 2,4-dichlorophenoxyacetic acid (2,4-D) (Scheme 1). The effects of the separation conditions, such as mobile phase, pH, flow rate and temperature, on the separation and retention behaviours of 13 phytohormones in this system were studied, and 12 phytohormones were separated. Different gradient elution programs were tested to identify the optimum separation conditions for the 13 phytohormones. The best separation was achieved using a gradient elution with an aqueous mobile phase containing 20 mM potassium phosphate buffer, pH = 6.85, and acetonitrile with a 22 °C column temperature. Under these experimental conditions, the 12 phytohormones could be separated and detected at 230 and/or 270 nm within 26 min. The chromatographic behaviours of the prepared stationary phase for these analytes were compared with those of a reversed phase C18 column in two different gradient conditions and mobile phase pH (6.85 and 3.25) as shown in Figs. 1 and 2 respectively.

It can be observed in the chromatograms that both L-Pr-Si and C18 columns exhibited similar and high selectivity in both pH. While the number of separated analytes and analysis times were similar, the elution orders of analytes on the two columns are different, especially comparing the different classes of analytes. In generaly, elution order of the analytes within a class is very similar and more hydrophobic analyte is more retained on both columns. But in different class, more hydrophilic analyte class which is less retained on C18 is more retained on L-Pr-Si. For example, while chlorinated acids were more retained on C18, alkaline analytes BAP, Z and K were more retained on L-Pr-Si. This case shows that not only hydrophobic interaction, but also polar interactions are responsible for the retention behaviour of these analytes on L-Pr-Si.

3.3.1. Influence of pH on the separation process

The mobile phase pH can have very strong impact on retention and selectivity in reversed-phase chromatography by influencing solute ionisation in the mobile phase. The effect of the mobile

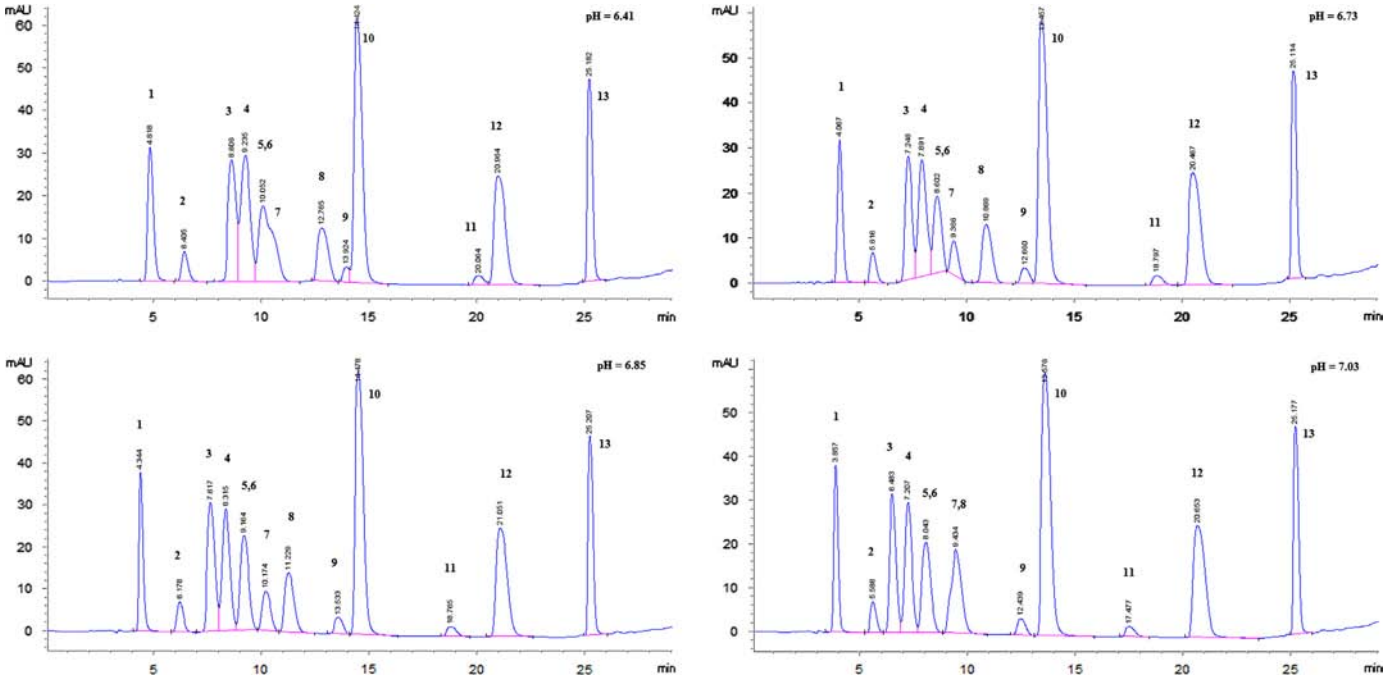


Fig. 3. Effect of buffer pH=6.41–7.03 on phytohormone separation. Gradient elution: ACN in phosphate buffer (20 mM), 0–20 min; 10–25%, 20–30 min; 25–90%. Flow rate: 0–13 min; 0.9 mL/min, 13–30 min; 1.2 mL/min. Temp.: 22 °C. Injection volume: 5 μL. Detection: 270 nm. Analytes: 1: IAA, 2: 4-PA, 3: IPA, 4: ABA, 5: IBA, 6: 2,4-D, 7: 2,4-DP, 8: NAA, 9: 2,4,5-T, 10: Z, 11: 2,4-DB, 12: K, 13: BAP.

phase pH on the RPLC separation was investigated in this study using buffer solutions with different pH. A 20 mM potassium dihydrogen phosphate/ potassium monohydrogen phosphate buffer was selected for pH 6–7 (6.42, 6.73, 6.85, and 7.03) (Fig. 4). A stock solution of 20 mM, pH=6.73 phosphate buffer was first prepared, and then the pH of the stock solution was adjusted to desired pH values (6.42, 6.85, 7.03). Phosphoric acid/potassium dihydrogen phosphate buffer was selected for a lower pH (pH=3.12, 10 mM) (Fig. 3). For pH 4.6, an acetic acid/ammonium acetate buffer (10 mM) was used (Fig. 4).

Comparing the retention times and separation efficiency as functions of acidity in Figs. 4 and 5, increasing the acidity of the mobile phase decreases the retention times of the alkaline analytes BAP, Z and K but increases the retention times of the acidic analytes. The chlorinated acids were especially well retained on the amide stationary phase with decreasing mobile phase pH (increasing acidity). All chlorinated acids were eluted the latest in pH 3.25 and were eluted quite early in pH 7.03. Furthermore, the separation efficiency of chlorinated acids decreased as the mobile phase pH decreased. Considering only the chlorinated acids, the most effective separation was observed at pH 4.6, corresponding to the ammonium acetate buffer. However, the separation efficiency of the alkaline analytes BAP, Z and K, known as cytokinins, does not change as the acidity increases, despite the decreasing elution times. The amide silica stationary phase shows great selectivity toward these alkaline analytes. Possibly because they contain both acidic and basic groups, the indole derivative acids (IAA, IPA, IBA) were eluted intermediate to chlorinated acids and cytokinins, depending on the pH. The pH value has a significant impact on the separation of the acidic and acidic–alkaline analyte mixtures used in this study.

The relationship between the polarity and the elution order of different classes of analytes is strongly dependent on the pH of the mobile phase. Although non-polar analytes within a class generally leave the column later, the comparison is more complex among different classes of analytes. For example, retention times of IAA, IBA and IPA, which are in the same class, is IBA > IPA > IAA. Similarly, retention order of cytokinins, which are alkaline analytes, is BAP > K > Z. In both cases, the three compounds contain same polar groups but different non-polar groups. Therefore, the more hydrophobic compound left column later; analytes that are more hydrophobic are difficult to dissolve in water and interact more strongly with a stationary phase containing both phenyl and cyclohexyl groups. However, compounds in different classes (containing different types and numbers of polar and non-polar groups) cannot be compared in a straightforward manner because the analytes interact with both the mobile and stationary phases. The acidic amide, alkaline amine group, aromatic and aliphatic rings in the stationary phase lead to a large number of interactions with different classes of analytes bearing different polar and non-polar groups. However, cytokinins, which have the most polar groups, were retained for longer in the pH 6–7 range. Although some chlorinated acids (2,4-D, 4-PA, 2,4-DP) were elated early in the pH 6–7 range, they eluted very late at pH 3.2. The retention time ordering of all other analytes varied with pH.

3.3.2. *Influence of the mobile phase on the separation process*

Even though different organic modifiers, such as alcohols, THF and ACN, have been used in mobile phase mixtures in RPLC or HILIC separations, better results have been reported for ACN [4]. In fact, the mobile phase applied in this separation mode usually contains water and ACN mixtures. Thus, all chromatographic evaluations were performed using gradient elution with acetonitrile and an aqueous mobile phase containing potassium phosphate or ammonium acetate buffer.

The influence of the buffer concentration on retention was evaluated using 0, 5, 10, 20 and 40 mM of aqueous potassium phosphate buffer at the optimum pH, 6.85 (Fig. 5). The best separation is achieved by 20 mM buffer. Suitable separation could not be achieved when buffer-free (0 mM) aqueous ACN was used; a long analysis time, poorly resolved peaks and low selectivity were observed (data not shown). The chromatogram improved when using 5 mM buffer. The separation selectivity of acidic

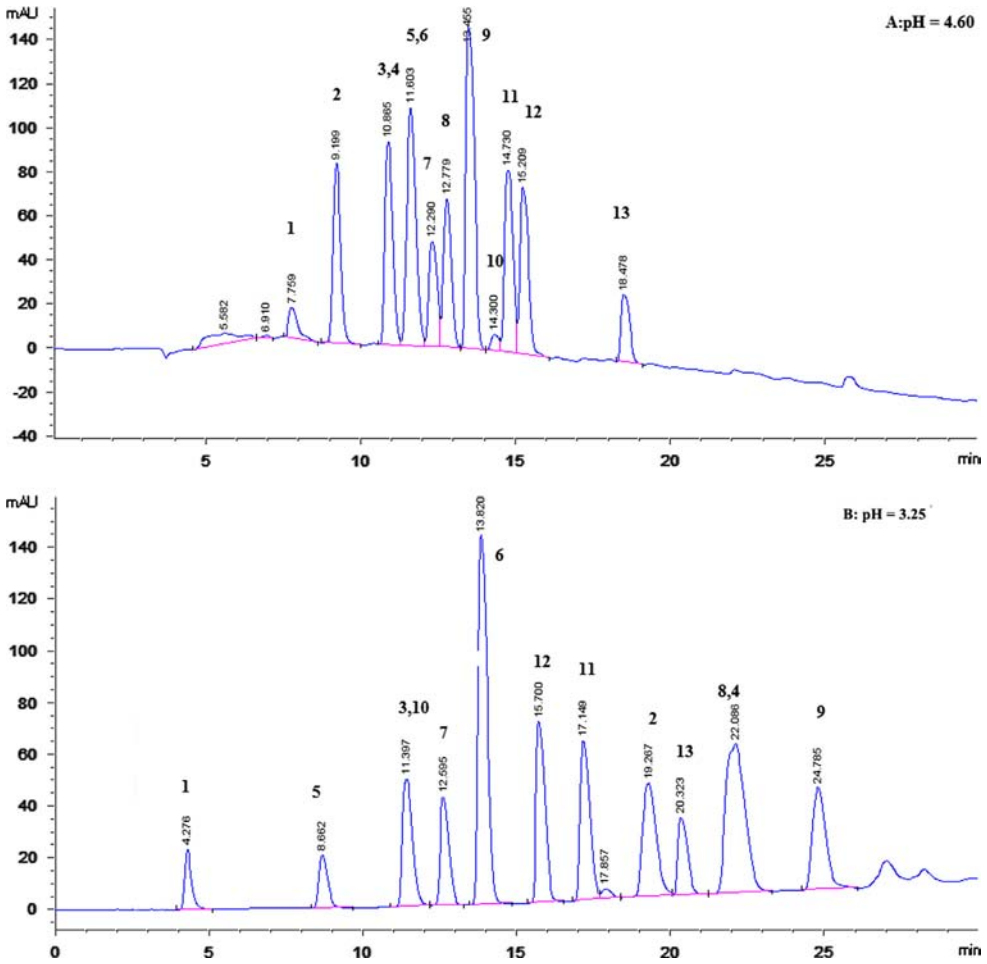


Fig. 4. Effect of pH=4.6 and 3.25 on phytohormone separation. Temp.: 22 °C, flow rate, 1 mL/min, Injection volume: 5 μL. Solvent. (A) Gradient: ACN in ammonium acetate buffer (10 mM); 10–25% from 0 to 20 min. (B) Gradient: ACN in phosphate buffer (10 mM); 20–80% from 0 to 30 min. Detection: 230 nm. Analytes: 1: Z, 2: 4-PA, 3: IAA, 4: 2,4-D, 5: K, 6: IPA, 7: ABA, 8: 2,4-DP, 9: 2,4,5-T, 10: BAP, 11: NAA, 12: IBA, 13: 2,4-DB.

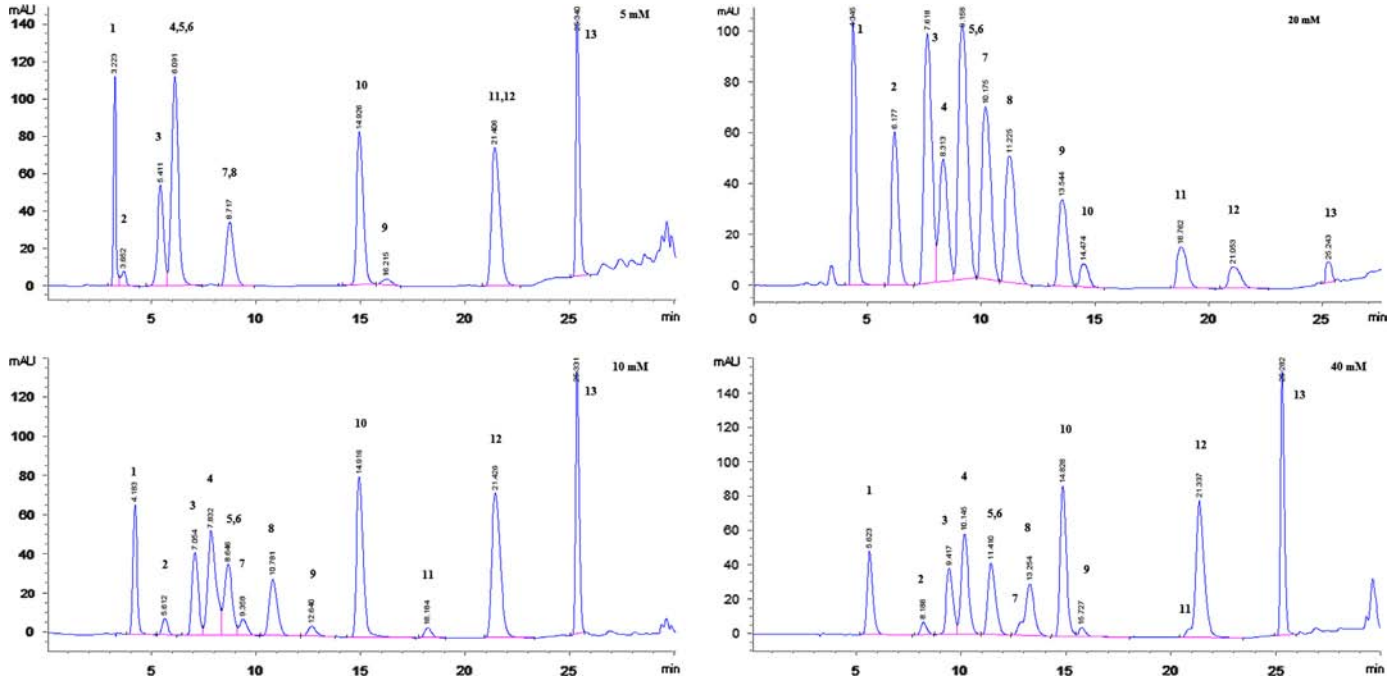


Fig. 5. Effect of the buffer concentration in the gradient elution on phytohormone separation. All other conditions are same as the optimum conditions (Fig. 1). Detection: 270 nm. Analytes: 1: IAA, 2: 4-PA, 3: IPA, 4: ABA, 5: IBA, 6: 2,4-D, 7: 2,4-DP, 8: NAA, 9: 2,4,5-T, 10: Z, 11: 2,4-DB, 12: K, 13: BAP.

analytes eluted between 0 and 10 min was increased by increasing the buffer concentration. Unlike the alkaline analytes BAP, Z and K, which were indifferent to the buffer concentration, the acidic analytes, especially chlorinated acids, were strongly affected by changes in the buffer concentration. With increasing buffer concentration, the elution of acidic analytes from the column slowed, as shown in Fig. 5. Thus, the best separation was obtained by 20 mM buffer. The higher concentration (40 mM) led to the merging of the peaks separated by the 5–20 mM buffer.

The influence of ACN content on retention was evaluated by varying its starting concentration in the gradient elution in the range of 5–90%, 10–90% and 15–90% v/v over 40 min and maintaining a constant phosphate buffer (pH: 6.85) concentration at 20 mM. Although only 11 analytes could be separated starting from 5% and 15% ACN, 12 analytes were separated starting from 10% ACN. Additionally, the analysis time was shortened by increasing the ACN content in the mobile phase, which is typical behaviour for reversed-phase columns. However, the elution order of the analytes according to their polarity is not completely the same on RPLC. The elution order of analytes is discussed above.

3.3.3. Influence of column temperature on the separation process

The column temperature is also an important parameter that affects the analyte retention. In most cases, an increase in column temperature reduces the analysis time because the exothermic enthalpy changes associated with the transfer of solutes from the mobile to stationary phases dominate the retention process in typical RP chromatographic systems [32]. This change in selectivity as a function of column temperature is dependent on the functional groups of both the analyte and the stationary phase as well as the composition of the mobile phase [33–35]. It is widely accepted that a mobile phase becomes more hydrophobic at high temperatures [36], reducing the retention of analytes in RPHPLC. Based on this theory, the opposite phenomenon should occur in HILIC: the retention of hydrophilic compounds should increase as a function of the temperature because the strength of the mobile phase decreases. Nevertheless, both increasing and decreasing retention have been documented in the literature as a consequence of increasing temperature. Thus, these findings prove that the nature of both the analyte and the stationary phase and their mutual interactions influence the overall retention [37].

In this study, the effect of temperature on the retention and separation of phytohormones was investigated by varying the column temperature from 10 to 30 °C under the optimum conditions using gradient elution. All analytes followed the typical behaviour for RPLC mode. The retention times of all analytes decreased with increasing temperature. However, the retention times change with increasing temperature, affecting the selectivity. As the temperature increased from 10 to 22 °C, the selectivity between ABA and IBA increased but that between 2,4-DP and NAA decreased. At 25 °C, 2,4-DP and NAA were elated at same time. Therefore, the best temperature was 22 °C.

3.3.4. Influence of flow rate on the separation process

To better separate the analytes eluted between 0 and 20 min of analysis, the influence of flow rate on the selectivity was studied with 0.7, 0.9 and 1 mL/min for the first 20 min. The flow rate between 20 and 30 min was constant at 1.2 mL/min. There was little difference between the three flow rates in terms of selectivity. However, the 0.9 mL/min flow rate was selected for between 0 and 20 min of analysis because of its superior peak shape compared to 0.7 mL/min.

3.4. Column stability tests

The stationary phase, L-Pr-Si, was tested in acidic condition. The chromatographic conditions for the test: mobile phase, ACN/20 mM, pH=3.20 aqueous potassium phosphate buffer (50/50); flow rate, 1 mL/min; temperature, 40 °C; detection, 280 nm; probes, kinetin (K) and naphthaleneacetic acid (NAA) as alkaline and acidic probes, respectively.

The column was continuously purged with the mobile phase at a rate of 1 mL/min, and periodically tested. The retention times of K and NAA were recorded. After about 1500 column volumes of mobile phase, there was essentially no change of retention for either alkaline or acidic probes. The column is very stable at pH=3.20 which is the lowest pH studied in this work.

4. Conclusion

A new stationary phase containing amide polar group embedded into the cycloalkyl chain was prepared by the modification of spherical silica gel. Initial chromatographic tests show that these phases can be used under reversed-phase conditions to separate phytohormones. The retentions of these analytes on the amide-silica stationary phase were compared with those of RP-C18 column. The amide-silica stationary phase displayed RPLC properties in the separation of phytohormones and may possess several retention mechanisms, such as partitioning, hydrogen-bonding and electrostatic interaction mechanisms. The effects of pH, buffer and acetonitrile concentration, temperature and flow rate on retention were investigated to better understand its chromatographic properties. This new type of polar embedded stationary phase could efficiently separate 12 plant hormones from different classes under gradient eluent conditions and can be very good alternative to commercial available columns for separation of acidic, alkaline and neutral compounds. Further investigations of this new stationary phase in other application fields are being undertaken in our laboratory.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.007.

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