

**RETENTION BEHAVIOUR
OF *N*-CBZ-*D*-PHENYLALANINE AND *D*-TRYPTOPHAN:
EFFECT OF IONIC LIQUID AS MOBILE-PHASE
MODIFIER**

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SUMMARY

Studies have been performed to investigate the effect of an ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate, as mobile-phase modifier on the retention behaviour of *N*-carbobenzyloxy-*D*-phenylalanine and *D*-tryptophan on a reversed-phase silica-based column. Retention of these amino acids was determined with water–acetonitrile mobile phases and with mobile phases containing 2, 5, or 10 mmol L⁻¹ ionic liquid. The effect of the amount of ionic liquid modifier on retention was also studied, and interpreted on the basis of intermolecular interactions. The ionic liquid showed promise as an additive in high-performance liquid chromatography.

INTRODUCTION

Ionic liquids (IL) are a new class of solvent with unique properties which have been widely used in numerous disciplines of modern chemistry. Increased interest in IL is reflected by articles in the scientific literature [1,2]. This suggests they might also lead to exclusive and interesting opportunities in separation science in general and chromatography in particular. A quantity of scientific papers which grows progressively from year to year implies IL have enormous advantages in high-performance liquid chromatography (HPLC) [3–8]. It has already been proved that IL can be used as mobile-phase additives in reversed-phase chromatography when mixed with other solvents of low viscosity. Eluent strength in liquid chromatography is adjusted by changing the composition of the mobile phase. Numerous experiments have been performed with water, methanol, acetonitrile, and their mixtures as mobile phases. According to several authors [9] IL have a dual nature. They have unique properties, for example a strong coulombic field around them, which promotes strong orientation and in-

duction interactions. The solvent properties of the IL make them possible candidates for mobile phases in HPLC. They are polar solvents whose miscibility with water is very dependent on their structure. Bare silica has been proved to be useful with IL either in cation-exchange mode [10] or when dynamically modified materials are used [11,12]. The chemical nature of the IL leads to the conclusion that when they are used as mobile-phase additives in HPLC they occur in solution in the mobile phase and also become coated on reversed-phase silica-based columns. Some authors note that adsorption of IL cations on the surface of silica depends on the presence of accessible silanol groups [4,11]. Ternary phase diagrams have been prepared for the acetonitrile–water–1-butyl-3-methylimidazolium system [13]. It was found that acetonitrile was the best organic solvent for work with this IL. These investigations showed that as mobile-phase additives IL could play a multiplicity of roles, for example blocking residual silanol groups, modifying the stationary phase, or acting as ion-pairing agents. One should note that the effects of IL on separation mechanisms are, unfortunately, not yet clearly understood. This, and successful examples of the use of IL for the chromatographic separations, is powerful stimulus for further studies on this topic.

In this work the effect of 1-butyl-3-methylimidazolium tetrafluoroborate as mobile-phase modifier on the retention behaviour of the *N*-carbobenzyloxy-*D*-phenylalanine and *D*-tryptophan was investigated. This IL might not be the best; it was selected because it is a good representative IL which can be used as a typical example of an IL forming a liquid system with water.

EXPERIMENTAL

Material and Reagents

Acetonitrile (HPLC grade) and potassium nitrite (KNO₂) were from Sigma (St Louis, MO, USA). The amino acids *N*-carbobenzyloxy-*D*-phenylalanine ((*N*-CBZ-*D*)-phenylalanine) and *D*-tryptophan, were Sigma–Aldrich products. 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIm][BF₄]; 99.99%) was obtained from C-tri (Namyang, Korea).

Distilled water was deionized with a Millipore (Belford, MA, USA) water-purification system.

High-Performance Liquid Chromatography

HPLC was performed with a Waters (USA) liquid chromatograph comprising a pump, diode-array detector DAD, and interface. Compounds were separated on a 25.0 mm × 4.6 mm i.d. column packed with octadecyl-bonded silica, average particle-size 15 μm, in our laboratory. The mobile phase flow rate was 1 mL min⁻¹ and detection was at 254 nm. The isocratic mobile phases were 40, 50, 60, 70, 80, and 90% (v/v) acetonitrile in water (pure reversed-phase systems). Different amounts (2, 5, and 10 mmol L⁻¹) of the IL were also added to the mobile phase. Amino acids were prepared as 1 mg mL⁻¹ solutions in methanol. The solutions were stored at 277 K and the working standards were re-prepared every two days to avoid potential errors from decomposition of the compounds. The injection volume was always 5 μL. Retention factors, k , were calculated by use of the formula $k = (t_R - t_0)/t_0$, where t_R is the retention time of the analyte and t_0 is the retention time of an unretained peak (taken as the first deviation of the baseline after injection of 5 μL KNO₂). Three replicate injections were made to determine retention time, and average values were used to calculate retention factors. Results from the experiment were evaluated by the techniques of mathematical statistics. The relative error of a single measurement did not exceed 5%. All experiments were performed at 293 K.

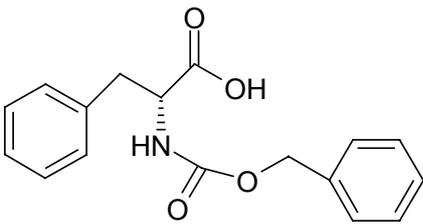
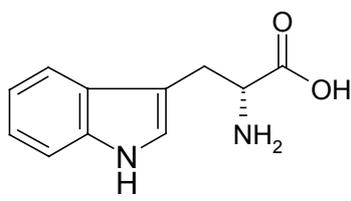
RESULTS AND DISCUSSION

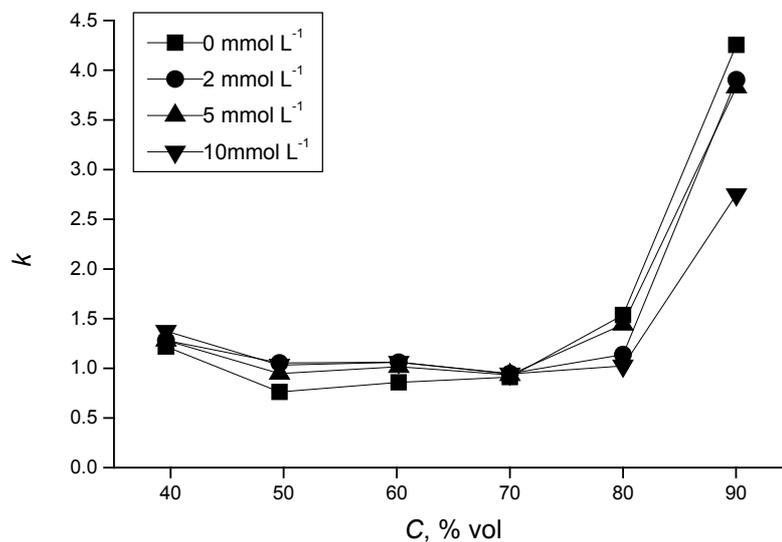
Chemically modified silica–aqueous acetonitrile mobile phases have been widely used in HPLC. As noted above, however, in addition to buffer salts a variety of additives, for example IL, are often needed for optimum performance. The retention factors of *N*-CBZ-*D*-phenylalanine and *D*-tryptophan were determined with water–acetonitrile mobile phases and with mobile phases modified by addition of 2, 5, and 10 mmol L⁻¹ [BMIm][BF₄]. The structures and molecular weights of the solutes are given in Table I.

Correlations of retention factors (k) with the concentration of the organic solvent (C) were of particular interest. Experiments with pure reversed-phase systems indicated that separation of these acids could not be achieved satisfactorily using acetonitrile alone as mobile-phase modifier. Retention factors of the amino acids were always very low and did not exceed 1.0. The parabolic nature of dependence $k = f(C)$ is most clearly apparent for IL-modified mobile phases, as shown in Figs 1 and 2.

Table I

Names, structures, and molecular weights of the amino acids

Name	Structure	Molecular weight
<i>N</i> -CBZ-D-Phenylalanine ((<i>R</i>)-2-(benzyloxycarbonylamino)-3-phenylpropanoic acid)		299
D-Tryptophan ((<i>R</i>)-2-amino-3-(1H-indol-3-yl)propanoic acid)		204

**Fig. 1**Effect of [BMIm][BF₄] concentration and amount of acetonitrile (C, %) on the retention factor (*k*) of D-tryptophan

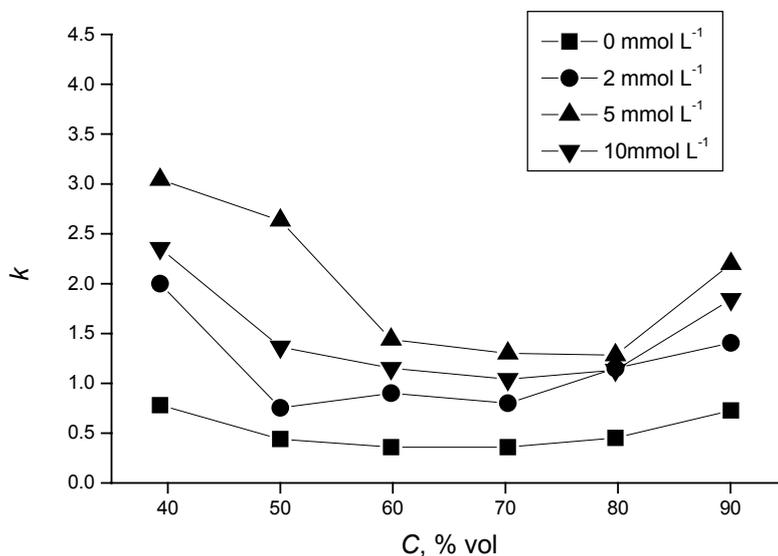


Fig. 2

Effect of [BMIm][BF₄] concentration and amount of acetonitrile (*C*, %) on the retention factor (*k*) of *N*-CBZ-*D*-phenylalanine.

First, with increasing concentration of acetonitrile retention factors decrease. When the concentration reaches 50–70% this trend is reversed. Similar relationships have been observed for a wide variety of substances [14–17]; in all these papers it is asserted that the increase in retention at high *C* values is because of polar interactions with the solid phase and the decrease in the concentration of water in the mobile phase. It is important to note here that after each experiment with a particular concentration of the IL, and before experiments with a different concentration of the IL, the column was flushed for at least 3 h to remove IL used previously and/or to fully equilibrate the column. Chromatograms obtained from pure solutions containing 1 mg mL⁻¹ *D*-tryptophan and *N*-CBZ-*D*-phenylalanine are shown in Figs 3 and 4, respectively.

The results revealed that peak height decreased slightly with increasing IL concentration in the range of 2–10 mmol L⁻¹. Efficiency and peak shape for chromatography of *D*-tryptophan were quite similar, irrespective of IL content (Fig. 3). Retention times were also identical (Figs 1 and 3). Thus addition of IL did not affect retention of *D*-tryptophan. Addition of the IL to the mobile phase did, however, affect the chromatographic behaviour of *N*-CBZ-*D*-phenylalanine. Retention of this compound changed as

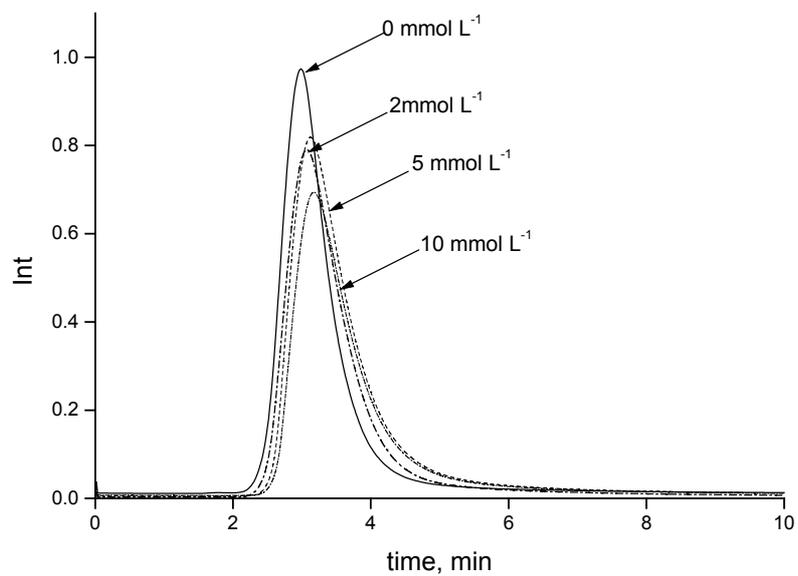


Fig. 3

Chromatograms obtained for D-tryptophan by use of water–acetonitrile, 50:50 (% v/v) containing different concentrations of [BMIm][BF₄] as mobile phase

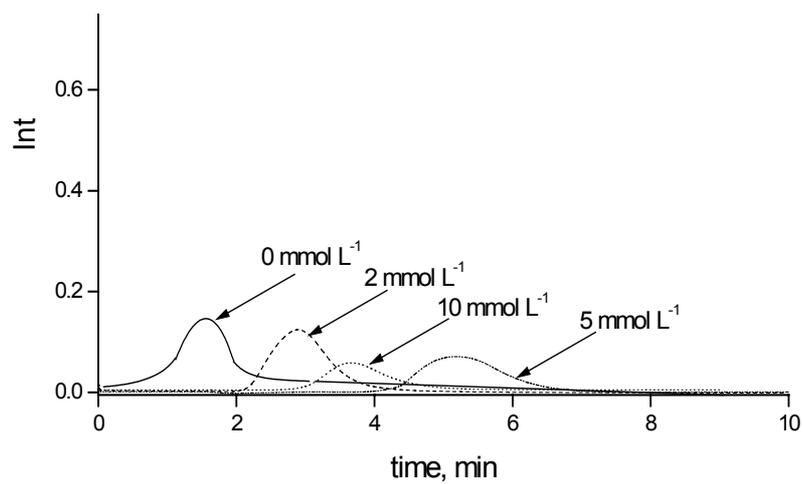
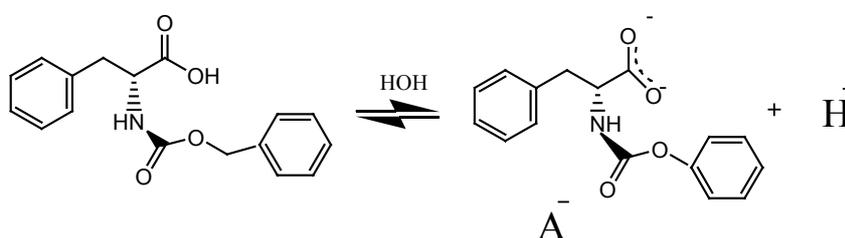


Fig. 4

Chromatograms obtained for *N*-CBZ-D-phenylalanine by use of water–acetonitrile, 50:50 (% v/v) containing different concentrations of [BMIm][BF₄] as mobile phase

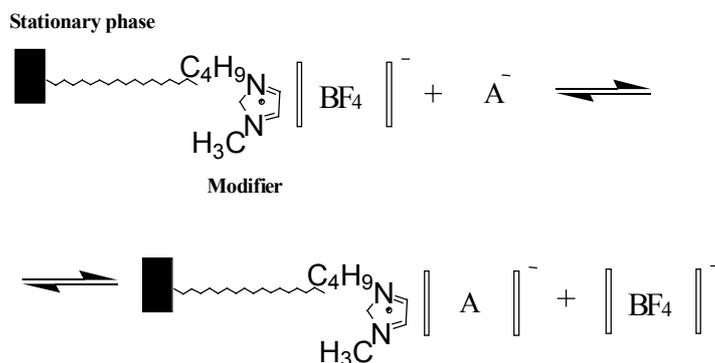
the IL content was increased from 0 (pure reversed-phase system) to 10 mmol L⁻¹ (Figs 2 and 4). This can be explained on the basis of intermolecular interactions between the solute and the solid and mobile phases. The special features of the chemical structure of this amino acid determines possible modes of interaction. The phenylalanine has an *N*-carbobenzyloxy group blocking the amino nitrogen. Because of this, the molecule acquires acidic properties and in the aqueous medium dissociates as shown below:



Thus in aqueous solution the *N*-CBZ-D-phenylalanine is an organic acid and occurs mainly as the anionic form (A⁻).

The molecule of D-tryptophan is a weak nucleophile, because of the aromatic indolyl group, but is neutral overall and in aqueous medium forms a zwitterion. This molecule does not, therefore, form stable ions in aqueous solution and remains neutral. According to He et al. [4] IL form a layer (pseudo-stationary phase) on the surface of the modified silica gel. It has also been shown that IL cations can interact and compete with silanol groups on the silica surface of the alkylated mobile phase [4, 18–20]. The non-polar alkyl groups of the stationary phase can also interact with the alkyl groups on the heterocyclic rings of the cation. This phenomenon effectively shields the residual silanols, which improves peak shapes and affects the retention times of the solutes. The relationship between the concentration of IL as modifier and the thickness and the stability of this layer may be affected by other factors. Addition of IL as modifier to a mobile phase leads to competition between the IL cations and the polar groups of solutes for the polar silanol groups on the surface of the alkylated silica. The modifier thus also disables the alkyl groups of the stationary phase, which leads to a sharp decrease in the possibility of dispersion interactions between the solute and the alkyl groups of the stationary phase. If the concentrations of the IL are slightly increased, their cationic interactions with the silanol groups on the silica surface, because of specific interactions, or with the alkyl groups, because of hydrophobic and non-specific interactions, gradually strengthen, resulting in an increase in the carbon content

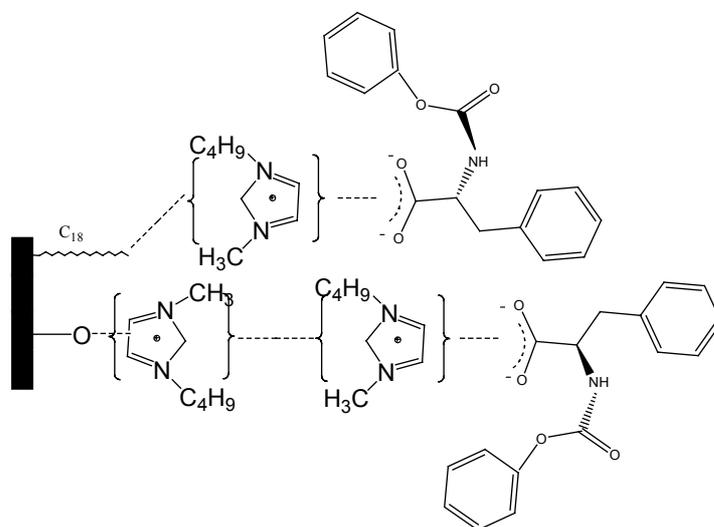
of the stationary phase. With a further increase in concentration of the IL modifier cations interact with the silanols groups by electrostatic interaction, producing a weak bilayer electronic structure, and interact with the alkyl groups, by hydrophobic and non-specific interactions. Dynamic modification of the adsorbent by the IL, and formation of the pseudo-stationary phase, is therefore possible. By dynamic modification is usually understood addition of different components to the mobile phase which interact uniformly with the components of the mobile phase and with the surface of the adsorbent, thus changing the properties of the chromatographic system. Interaction of the modifier with the adsorbent and solute often occurs by very complex mechanisms leading to a completely different mechanism of separation. Thus, the *N*-CBZ-D-phenylalanine, which occurs in solution as anion, can participate in specific ionic interactions with adsorbed cations of IL (pseudo-stationary phase). Such chromatographic behaviour can lead to an ion-exchange mechanism of retention. Formation of an anion exchanger on the surface of a reversed-phase adsorbent is illustrated below:



Adsorption of the lipophilic part of the IL cations by the alkyl chains of the adsorbent is more probable. This fixes the position of the hydrophilic cationic part of the modifier, leading to subsequent absorption of the new IL ions. This creates an electrostatic potential on the surface which results in additional retention of ionized analytes. The surface thus acquires ion-exchange properties and retention becomes dependent on the laws governing ion-exchange chromatography. Retention in ion exchange chromatography is usually limited by two processes:

- distribution of the sample between the aqueous mobile phase and organic solid phase, and
- formation of ion pairs

with the latter process prevailing. The distribution of the substance between the phases depends on the strength of electrostatic interactions of the charged ionized groups of solute with the charged groups of the pseudo-stationary phase. Retention in ion-exchange HPLC results from fairly complicated, and competing, equilibrium processes. Absorption of hydrophobic ions on the silica surface of the alkylated stationary phase is possible, because of hydrophobic interactions and because of displacement of the mobile phase from the polar medium. Retention of the analyte decreases with increasing ionic strength of the mobile phase and increases with increasing ion-exchange capacity of the adsorbent. Besides ion-ion interactions, secondary, non-ionic, interactions occur on the surface of the adsorbent, because of adsorption and formation of hydrogen bonds between the solute and the non-ionic part of the adsorbent, because of the limited solubility of the sample in the mobile phase. Polar hydrophilic groups on the entire surface of the adsorbent remain accessible for interactions with the solutes, and retention of the *N*-CBZ-D-phenylalanine anion occurs predominantly by an ion-exchange mechanism. A schematic diagram of probable interactions is given below:



The ionic strength of the mobile phase is the main property affecting retention of the anions, which, in turn, very strongly affects chromatographic behaviour. When the IL content of the mobile phase is relatively low, the adsorbed layer contains quite a small amount of IL molecules. With increasing amounts of IL in the mobile phase there is a rapid in-

crease in the amount of adsorbed IL. Our experiment indicated that for IL concentrations up to 5 mmol L^{-1} this leads to increased retention. Increasing the IL content of the mobile phase to 10 mmol L^{-1} leads to a decrease in the retention of *N*-CBZ-D-phenylalanine. This phenomenon can be explained on the basis of the assumption that for 10 mmol L^{-1} IL in the mobile phase, formation of adsorbed layer is complete so there will be no further increase of retention of the *N*-CBZ-D-phenylalanine and there will, concurrently, be a decrease in retention because of increased interactions of the *N*-CBZ-D-phenylalanine with IL in the mobile phase. Greater IL concentrations drive more ions into the water-rich mobile phase, which results in an increase in the volume and hydrophilicity of the IL layer, leading to an increase in its mobility. The increase in the contribution of specific interactions with the mobile phase and the increased mobility of the IL layer on the surface of the adsorbent results in a decrease of the retention of ionized solutes. It can be assumed that relatively high concentrations (10 mmol L^{-1}) of the modifier in the mobile phase lead to formation of a multilayer (bilayer or trilayer) on the surface of the stationary phase.

The chromatographic behaviour of D-tryptophan was substantially stable, however. A change in the mobile phase had almost no effect on the retention and shape of the peak. For this compound a change in the polarity and ionic strength of the mobile phase did not affect the retention time of the neutral (zwitterionic) molecule of D-tryptophan. This phenomenon has been shown to effect the contribution of specific solute–adsorbent interactions, leading to the conclusion that dipole–dipole and van der Waals intermolecular interaction with the stationary phase are more important than ionic interactions. A sharp increase in the retention of D-tryptophan was observed if the acetonitrile content of the mobile phase was increased to more than 80% (v/v), however. It seems probable that chromatographic retention of this amino acid is also determined by its solubility in water. Use of a mobile phase containing 90% (v/v) acetonitrile sharply increases the retention time of D-tryptophan because its solubility in the mobile phase was reduced. For D-tryptophan, therefore, the conventional mechanism of reversed-phase chromatography is most likely.

CONCLUSION

We have investigated the effect of the IL 1-butyl-3-methylimidazolium tetrafluoroborate as mobile-phase modifier on the retention behaviour

of *N*-carbobenzyloxy-*D*-phenylalanine and *D*-tryptophan. For mobile phases containing a relatively low concentration of the IL (from 2 to 5 mmol L⁻¹) increasing the concentration of the modifier resulted in increased retention of *N*-CBZ-*D*-phenylalanine, because formation of a layer of IL on the surface of the stationary phase results in an electrostatic potential, leading to additional retention of ionized analytes and, therefore, significant adsorption of the *N*-CBZ-*D*-phenylalanine. Under these conditions the chromatographic behaviour of *D*-tryptophan was fairly stable; i.e. changing the composition of the mobile phase hardly changed the retention of *D*-tryptophan. The occurrence of multilayer adsorption of the IL has a substantial effect on the retention of ionized analytes and realization of an ion-exchange mechanism of retention, in contrast with that for non-dissociated molecules, which is the ordinary mechanism of reversed-phase chromatography, leading to small differences in analyte retention. The contributions of the counter-ion to solute retention are currently under investigation. The role of IL is complex and further research is needed to quantitatively explain some of the phenomena. The performance of IL as additives in HPLC suggests they may have promising applications.

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