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Effect of sodium dodecyl sulfate as stationary phase on signal intensities of dansylamino acids in microcolumn liquid chromatography with on-column fluorimetric detection

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Abstract

The effects of stationary phase and mobile phase additive on fluorescence signal intensities of dansylamino acids were examined in microcolumn liquid chromatography. Sodium dodecyl sulfate (SDS), employed as the mobile phase additive, was dynamically introduced into an anion exchanger, and it worked as a stationary phase in the reversed-phase mode. Signal enhancement was achieved by on-column fluorimetric detection owing to the focusing and environmental effects of the SDS stationary phase.

1. Introduction

Armstrong et al. [1] reported the first known example of micellar enhanced fluorescence detection in liquid chromatography (LC). They demonstrated the separation of aromatic hydrocarbons with a micellar mobile phase, and the benefits of micellar enhanced fluorescence detection in LC were discussed. In spite of the promising features of micellar mobile phases, it seems that there have been few papers that appreciate fluorescence enhancement generated by micelles in LC.

The use of various micellar systems in spectrofluorimetric methods for the determination of dansyl (Dns)-amino acids, drugs and other compounds has been assessed [2–5]. It was shown that fluorescence enhancement due to micelles with factors varying from 8 to 20 was achieved for dansylamino acids in comparison with that in water alone, leading to an improvement in sensitivity [2].

On-column detection, defined as the case when analytes are detected in the presence of a stationary phase, provides increased sensitivity by a factor of $1 + k'$ compared with common detection in the absence of the stationary phase, where k' is the capacity factor of the analyte [6,7]. This is due to the focusing effect of the stationary phase. Takeuchi and Yeung [8] reported a signal enhancement due to both the focusing and environmental effects of the stationary phase in on-column fluorimetric detection in open-tubular capillary LC with an octylmethylsiloxane stationary phase. We [9] reported a signal enhancement of dansylamino acids by on-column fluorimetric detection using cyclodextrin-bonded stationary phases, in which fluorescence enhancement by inclusion complexation into cyclodextrin was demonstrated.

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It is frequently observed in LC that the pH, polarity and temperature of the mobile phase dramatically affect the position of the maximum emission wavelength and emission intensity [10]. Intensity changes of an order of magnitude and large wavelength shifts are sometimes found for molecules that can undergo strong solvent interactions [10]. Therefore, on-column fluorimetric detection is expected to accompany a dramatic effect in both the position of the maximum emission wavelength and emission intensity because analytes interact with the stationary phase when they are detected in the presence of the stationary phase.

It is expected that the on-column detection of dansylamino acids in the presence of micelles in the mobile phase can result in better sensitivity because the signal can be enhanced by both the micellar mobile phase and the focusing and environmental effects of the stationary phase.

This paper describes the effects of sodium dodecyl sulfate (SDS) as the stationary phase and mobile phase additive on the fluorescence intensity of dansylamino acids in LC.

2. Experimental

2.1. Apparatus

A microcolumn liquid chromatograph was assembled from an MF-2 microfeeder (Azumadenki Kogyo, Tokyo, Japan) equipped with a 0.5-ml MS-GAN 050 gas-tight syringe (Ito, Fuji, Japan) as a pump, an ML-522 microvalve injector with an injection volume of 0.11 μl (Jasco, Tokyo, Japan), a 150 × 0.35 mm I.D. microcolumn, an 820-FP fluorimetric detector (Jasco) with a laboratory-made flow cell and a Chromatopac C-R4AX data processor (Shimadzu, Kyoto, Japan). The empty and packed flow cells were prepared from fused-silica tubing of 0.32 mm I.D. (GL Science, Tokyo, Japan) as reported previously [11].

A U-330 bandpass filter (Jasco) was attached to the packed flow cell to prevent the second-order light from reaching the flow cell, and an L-39 cut-off filter (Jasco) was employed to prevent the scattered light from reaching the photomultiplier of the detector. Collection of scattered light for the empty flow cell was simply reduced by tilting the flow cell by ca. 30° [11]. The lengths of the flow cell tubes irradiated by the exciting light were 1.6 and 2.1 mm for the packed and empty flow cells, respectively. The wavelength of excitation was 335 nm. The time constant of the fluorimetric detector was kept at 3.5 s. Fluorescence spectra were also obtained with a model 820-FP fluorimetric detector.

The microcolumn was prepared from fused-silica tubing of 0.35 mm I.D. (GL Science) as reported previously [12], and 5-μm TSKgel IC-Anion SW anion exchanger (Tosoh, Tokyo, Japan) or 5-μm Develosil ODS-5 octadecyl-bonded silica gel (Nomura Chemical, Seto, Japan) was employed as the packing. The flow-rate of the eluent was 2.8 μl min^{-1}. Experiments were carried out at room temperature (ca. 25–30°C).

SDS was introduced dynamically into the anion exchanger by passing aqueous acetonitrile solution (50%, v/v) containing 40–100 m*M* SDS for ca. 1 h at 2.8 μl min^{-1}, followed by conditioning with the mobile phase employed.

2.2. Reagents

Guaranteed reagent-grade solvents and reagents were obtained from Nacalai Tesque (Kyoto, Japan), unless indicated otherwise. These reagents were employed as received. Dansyl derivatives of L-amino acids were purchased from Sigma (St. Louis, MO, USA). Distilled water was of HPLC grade (Nacalai Tesque). Eluents were prepared from the distilled water, acetonitrile, ammonium acetate and SDS.

3. Results and discussion

3.1. Fluorescence enhancement of dansylamino acids by SDS

Fluorescence enhancement was measured by using the 820-FP fluorimetric detector with a flow cell for conventional LC. Table 1 compares the wavelengths that give maximum fluorescence

Table 1
Fluorescence enhancement of Dns-L-amino acids on addition of SDS

Dns-L amino acid	λ_{max}(nm)		Enhancement factor
	Without SDS	With SDS	
αAB[a]	539	531	2.44
Ala	539	533	2.16
Arg[b]	539	527	3.88
Asn	541	533	2.05
Asp	541	539	1.08
Cysteic acid	539	539	1.11
Gln	539	533	2.08
Glu	540	540	1.08
Ile	539	529	3.22
Leu	539	529	3.12
Met	541	532	2.65
NVal	538	529	3.01
Phe	541	531	3.51
Pro	542	533	3.46
Ser	543	535	2.11
Thr[c]	539	535	2.03
Trp[d]	540	531	3.45
Val	539	531	2.65

Analyte solution: 40 μ*M* Dns-L-amino acid dissolved in 40 m*M* ammonium acetate containing 0.8% (v/v) acetonitrile with (pH 6.90) or without 100 m*M* SDS (pH 6.93). Excitation wavelength, 335 nm.
[a] Dansyl-L-α-aminobutyric acid.
[b] α-Dansyl-L-arginine.
[c] N-Dansyl-L-threonine.
[d] N^{α}-Dansyl-L-tryptophan.

intensity and the enhancement factors, defined as the ratio of the fluorescence intensity observed in the presence of SDS to that without SDS. The analyte solution prepared was 40 μ*M* Dns-L-amino acids dissolved in 40 m*M* ammonium acetate containing 0.8% acetonitrile with or without 100 m*M* SDS. The pH of the matrix solution was ca. 6.9. It was found that the largest enhancement factor was achieved for Dns-L-Arg, i.e., 3.88. Enhancement factors between 2 and 3.5 were observed for neutral and basic amino acids, whereas almost no enhancement was observed for acidic amino acids. This may be because SDS is anionic so that the acidic amino acids undergo electrostatic repulsion from the SDS micelle. In addition, the wavelength giving the maximum fluorescence intensity was slightly shifted to shorter wavelengths on addition of SDS. The larger the signal enhancement, the larger was the wavelength shift observed. The largest wavelength shift was observed for Dns-L-Arg, i.e., 12 nm.

3.2. Effect of concentration of acetonitrile and SDS

The enhancement factor was strongly affected by the acetonitrile concentration. Fig. 1 shows the effect of acetonitrile concentration on the signal intensity and the signal enhancement of Dns-L-Phe. The analyte was dissolved in aqueous acetonitrile containing 40 m*M* ammonium acetate (pH 6.9–7.5). In the absence of SDS, the signal intensity increased with increasing acetonitrile concentration. This phenomenon probably results because water quenches the fluorescence of dansylamino acids [13]. On the other hand, when SDS micelles are present in the solution, both the SDS micelles and the solvent effect due to acetonitrile contribute to the fluorescence

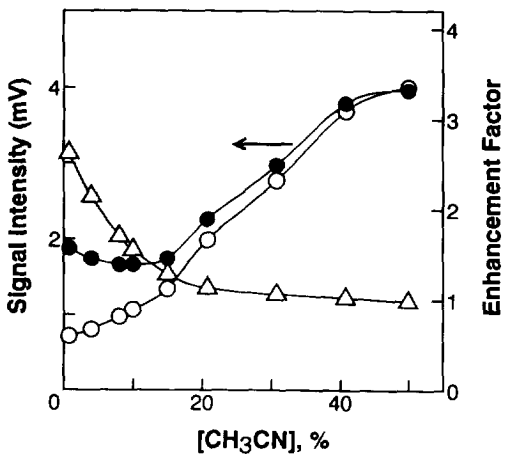

Fig. 1. Effect of acetonitrile concentration on signal intensity and signal enhancement. Analyte solutions, 40 μM Dns-L-Phe dissolved in aqueous acetonitrile in 40 mM ammonium acetate with or without 40 mM SDS (pH 6.9–7.5); excitation wavelength, 335 nm; ● and ○ refer to the signal intensity observed with or without 100 mM SDS, respectively, and △ refers to the signal enhancement.

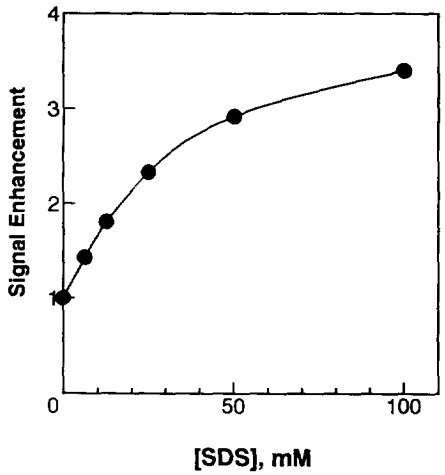

Fig. 2. Signal enhancement as a function of SDS concentration. Solutions, 40 μM Dns-L-Phe dissolved in 0.8% (v/v) acetonitrile containing 40 mM ammonium acetate and SDS as indicated (pH 6.90–6.93); excitation wavelength, 335 nm.

enhancement of dansylamino acids. With increasing acetonitrile concentration, the contribution of the solvent effect due to acetonitrile increases, whereas the signal enhancement due to SDS decreases at higher acetonitrile concentrations. The signal enhancement factor, therefore, decreases with increasing acetonitrile concentration and reaches almost unity at ca. 20% (v/v). In addition, the critical micelle concentration of SDS in water is 8.3 mM, whereas that in 20% acetonitrile solution was estimated to be ca. 30 mM from the measurement of its electrical conductivity.

The signal intensity is expected to increase with increasing SDS concentration. Fig. 2 illustrates the enhancement factor of Dns-L-Phe as a function of SDS concentration at an acetonitrile concentration of 0.8% (v/v). It is found that the enhancement factor increases with increasing SDS concentration. Lower enhancement factors were observed at higher acetonitrile concentrations. These results indicate that only a small signal enhancement of dansylamino acids can be expected when SDS is employed as the mobile phase additive.

3.3. On-column fluorimetric detection of dansylamino acids

In this work, "on-column detection" indicates the case when the analytes are detected in the presence of a stationary phase. On the other hand, when the analytes are detected in the absence of a stationary phase, the term "post-column detection" is utilized. Fig. 3 demonstrates the on-column and post-column fluorimetric detection of Dns-L-amino acids using an anion-exchange column dynamically modified with SDS, in which 0.11 μl of the sample solution containing 0.2 mM of each analyte is injected and the excitation wavelength is 335 nm. The noise levels observed for both detection methods were nearly the same, and better sensitivity was achieved with the on-column detection. Mass detection limits of the analytes at a signal-to-noise ratio of 3 achieved by on-column detection under the conditions in Fig. 3 were 60–130 fmol, which were 5–40 times lower than those achieved by the post-column detection. In addition, SDS micelles are not present in the mobile phase under the conditions in Fig. 3. Laser-based fluorimetric detection will further improve the detection limits.

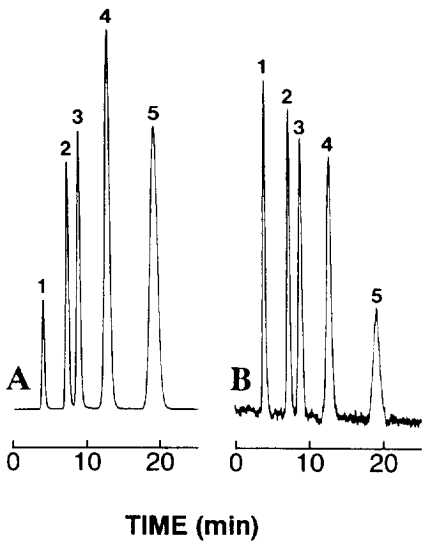

Fig. 3. (A) On-column and (B) post-column fluorimetric detection of dansylamino acids. Column, 150 × 0.35 mm I.D., packed with TSKgel IC-Anion SW; eluent, 15% (v/v) acetonitrile in 40 m*M* ammonium acetate and 5 m*M* SDS (pH 7.07); flow-rate, 2.8 μl min $^{-1}$; excitation wavelength, 335 nm; emission wavelength, 535 nm; sample, 0.2 m*M* each of dansyl derivatives of (1) L-Glu, (2) L-Ala, (3) L-Val, (4) L-Leu and (5) L-Phe; injection volume, 0.11 μl.

The retention time of the analytes was affected by the concentration of acetonitrile and SDS. When the eluent contained no SDS, the analytes could not be eluted in a reasonable time because SDS introduced into the anion exchanger were gradually eluted from the stationary phase, leading to exposure of bare ion-exchange sites.

Reproducibilities of the retention time, peak height and peak area for on-column detection were calculated for five successive measurements under the same conditions except for the SDS concentration (40 m*M*) as in Fig. 3. The relative standard deviations of the retention time, peak height and peak area were 0.3–0.5, 2.3–3.3 and 2.8–4.0%, respectively.

Takeuchi and Yeung [8] derived an equation that allows the estimation of the environmental effect of the stationary phase by measuring peak heights of analyte solutes as follows:

$$\frac{h_{oi}}{h_{pi}} = \frac{\alpha_o \varepsilon_o I_{om}}{\alpha_p \varepsilon_p I_p} \cdot \left(k_i' \cdot \frac{\varphi_{osi}}{\varphi_{pi}} + 1 \right) \quad (1)$$

where subscripts o, p, s, m and *i* denote on-column detection, post-column detection, the stationary phase, the mobile phase and the analyte component *i*, *h* is peak height, α is a constant, ε is the collection efficiency of fluorescence, *I* is the light intensity, k' is the capacity factor of the analyte and, φ is a factor accounting for the quantum efficiency, selectivity, absorptivity, energy transfer, etc.

The $\varphi_{osi}/\varphi_{pi}$ value represents the environmental effect of the stationary phase. When the value is larger than unity, signal enhancement due to the environmental effect of the stationary phase exists, whereas when it is equal to unity, the focusing effect only contributes to the signal enhancement. On the other hand, when $\varphi_{osi}/\varphi_{pi}$ is smaller than unity, the stationary phase quenches the fluorescence.

When analytes *i* and *j* are detected in a single chromatographic run, Eq. 1, is valid for the component *j*:

$$\frac{h_{oj}}{h_{pj}} = \frac{\alpha_o \varepsilon_o I_{om}}{\alpha_p \varepsilon_p I_p} \cdot \left(k_j' \cdot \frac{\varphi_{osj}}{\varphi_{pj}} + 1 \right) \quad (2)$$

The ratio of Eq. 1 to Eq. 2 is given by the following equation:

$$\left(\frac{h_{oi}}{h_{pi}}\right) \Big/ \left(\frac{h_{oj}}{h_{pj}}\right) = \left(k_i' \cdot \frac{\varphi_{osi}}{\varphi_{pi}} + 1 \right) \cdot \left(k_j' \cdot \frac{\varphi_{osj}}{\varphi_{pj}} + 1 \right) \quad (3)$$

When k_j' is zero or $\varphi_{osj}/\varphi_{pj}$ is known, $\varphi_{osi}/\varphi_{pi}$ is calculated from the peak-height ratios and the capacity factors of the analytes.

Figs. 4 and 5 illustrate the relationships between the peak height and the capacity factor of the analytes for post-column and on-column detection, respectively, where eluents with different acetronitrile concentrations are employed. With post-column detection, the peak height of the analytes decreases with increasing capacity factor, whereas the dependence of the peak height on the capacity factor is not obviously recognized in on-column detection. In addition, nearly the same levels of baseline noise were observed for both detection methods, indicating that the sensitivity is improved by on-column detection.

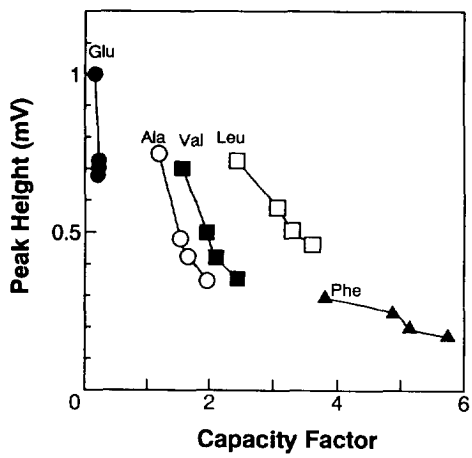

Fig. 4. Relationship between peak height and capacity factor in post-column fluorimetric detection. Mobile phase, 5–15% (v/v) acetonitrile in 40 m*M* ammonium acetate and 40 m*M* SDS (pH 6.98–7.07); other operating conditions as in Fig. 3B.

The ratio of the peak height achieved by on-column detection to that achieved by post-column detection is plotted as a function of the capacity factor in Fig. 6. It is found that the peak-height ratio increases with increasing capacity factor and the on-column detection

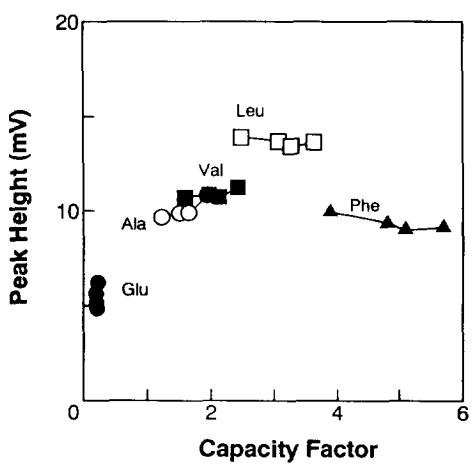

Fig. 5. Relationship between peak height and capacity factor in on-column fluorimetric detection. Mobile phase, 5–15% (v/v) acetonitrile in 40 m*M* ammonium acetate and 40 m*M* SDS (pH 6.98–7.07); other operating conditions as in Fig. 3A.

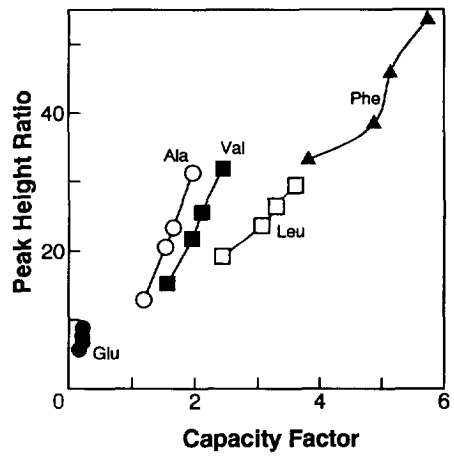

Fig. 6. Ratio of peak height observed in on-column detection to that in post-column detection as a function of capacity factor. Operating conditions as in Figs. 4 and 5.

improves the detection limits by a factor of 5.6–54 under the conditions in Fig. 6.

The concentration of SDS in the mobile phase also affected the retention time of the analytes. The capacity factor decreased slightly with increasing SDS concentration, e.g., the capacity factor of Dns-L-Phe was 5.44 for 5 m*M* SDS and 3.86 for 40 m*M* SDS when aqueous acetonitrile solution (15%, v/v) containing 40 m*M* ammonium acetate was used as the mobile phase. In addition, the peak heights achieved by on-column detection were almost constant, irrespective of the SDS concentration in the region 5–40 m*M*.

Fig. 7 illustrates the fluorescence intensity and signal enhancement for Dns-L-Glu as a function of acetonitrile concentration. It is found that the signal enhancement is not significant and is almost independent of the acetonitrile concentration. This may be because Glu is an acidic amino acid, and its net negative charge makes it difficult to interact with the anionic detergent.

By assuming that $\varphi_{osi}/\varphi_{pi}$ for Dns-L-Glu is unity, $\varphi_{osi}/\varphi_{pi}$ values for other dansylamino acids can be calculated. Table 2 lists the $\varphi_{osi}/\varphi_{pi}$ values under different mobile phase conditions. The mobile phase contained SDS micelles under the conditions in Table 2, and the micelle concentration increased with decreasing acetonitrile

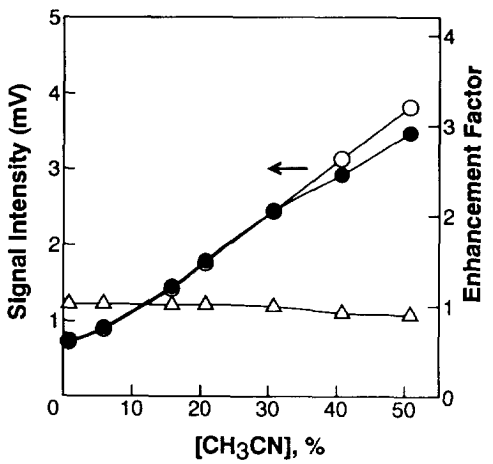

Fig. 7. Fluorescence intensity and signal enhancement for Dns-L-Glu as a function of acetonitrile concentration. Analyte solutions, 40 μM Dns-L-Phe dissolved in aqueous acetonitrile in 40 mM ammonium acetate with or without 40 mM SDS (pH 6.9–7.5); excitation wavelength, 335 nm; ● and ○ refer to the signal intensity observed with or without 40 mM SDS, respectively, and △ refers to the signal enhancement.

concentration. $\varphi_{osi}/\varphi_{pi}$ values smaller than unity were observed for Dns-L-Leu at lower acetonitrile concentrations, indicating that the φ value of Dns-L-Leu in the mobile phase is larger than that in the stationary phase. On the other hand, the largest $\varphi_{osi}/\varphi_{pi}$ values were observed at 10% acetonitrile for Dns-L-Ala and Dns-L-Val.

When octadecyldimethylsilyl-bonded silica (ODS) and an ammonium acetate solution of acetonitrile without SDS were used as the stationary and mobile phase, respectively, $\varphi_{osi}/\varphi_{pi}$ was 2.3–2.4 for dansyl derivatives of Ala, Val, Leu and Phe, indicating that ODS also enhances the fluorescence of the dansylamino acids [14]. Considering these results, there is no evidence supporting the existence of micellar or aggregates of SDS on the stationary phase. In other words, SDS micelles may be present only in the mobile phase even when the SDS concentration is higher than its critical micelle concentration. In addition, the length of alkyl group, the type of modifier and variations in charge on the micelle reagents may affect the fluorescence enhancement. These effects are being investigated.

4. Conclusions

On-column fluorimetric detection with the stationary phase dynamically modified with SDS improved the detection limits of dansylamino acids. The environmental effects of the stationary phase were estimated from the peak heights. Laser-based on-column fluorimetric detection will further improve the detection limits.

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Table 2
Environmental effect ($\varphi_{osi}/\varphi_{pi}$) of SDS stationary phase

Acetontrile (%)	$\varphi_{osi}/\varphi_{pi}$				
	Glu	Ala	Val	Leu	Phe
15	1	1.38	1.38	1.21	1.53
10	1	1.80	1.52	1.08	1.23
8	1	1.66	1.47	0.98	1.23
5	1	1.69	1.39	0.85	1.11

Operating conditions as in Fig. 4 except for the mobile phase: 5–15% acetonitrile containing 40 mM SDS and 40 mM ammonium acetate (pH 7.0–7.1).

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