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TRACE ANALYSIS OF ALDEHYDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRECOLUMN FLUORIGENIC LABELING WITH 5,5-DIMETHYL-1,3-CYCLOHEXANEDIONE

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SUMMARY

Dimedone (5,5-dimethyl-1,3-cyclohexanedione) is a highly specific and extremely sensitive reagent for the determination of aldehydes. We have adapted this reagent to the high-performance liquid chromatographic analysis of these compounds by precolumn derivatization. The reaction and chromatographic conditions were optimized. Using fluorimetric detection, the standard deviation at the 28 pmol level was about $\pm 2\%$ for most aldehydes. The detection limit was found to be about 30 fmol per injected aldehyde, but could be decreased by a factor of 100 by a single extraction step. Intercalibration with the standard 2,4-dinitrophenylhydrazine technique gave good agreement. Applications to various environmental and industrial samples are illustrated.

INTRODUCTION

It is well documented that low-molecular-weight aldehydes are environmentally hazardous substances. In complex materials such as tobacco smoke, foods, air and water pollution samples and physiological fluids (*e.g.*, urine and plasma) aldehydes are usually present in only trace amounts; however, they are very important because of their pronounced reactivity and toxicity and their known (or suspected) carcinogenic effects.

Numerous photometric and chromatographic techniques can be found in the literature for the determination of these compounds[1], but gas and liquid chromatographic techniques offer the greatest sensitivity and specificity. The simplest (and perhaps most powerful) chromatographic method for the analysis of trace organics, especially for aqueous samples, is one that permits the direct injection of samples with little or no pretreatment. In this respect, reversed-phase high-performance liquid chromatography (HPLC) is particularly noteworthy. Using this form of chromato-

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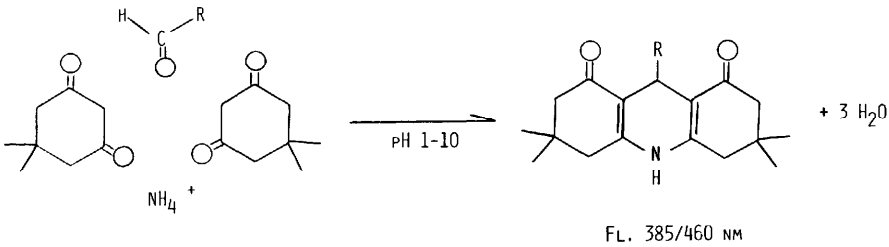

Fig. 1. Overall reaction of dimedone with aldehydes and ammonium, after Sawicki and Sawicki[1].

graphy, large volumes of aqueous samples can be injected onto the column with insignificant loss of resolution[2].

Precolumn derivatization has been shown to be a powerful technique for selectively enhancing the detectability of compounds in HPLC analysis[2]. The combination of this technique with reversed-phase HPLC has greatly simplified the trace analysis of some classes of compounds in aqueous samples. In the case of carbonyl compounds, four HPLC precolumn derivatization reagents have successfully been employed: 2,4-dinitrophenylhydrazine[3], 5-dimethylaminonaphthalene-1-sulphonylhydrazine[3], *p*-nitrobenzylhydroxylamine[4] and acetylacetone or Nash reagent[5]. The latter reagent is distinguished from the others in that it does not fluoresce nor produce fluorescent by-products, while, at the same time, it selectively condenses with aldehydes to produce highly fluorescent compounds (known as lutidine derivatives). This is advantageous because the presence of unreacted reagent does not interfere with the chromatographic separation of the derivatives. Therefore, an aliquot of the reaction mixture can be directly injected, without further purification, onto an HPLC column[5].

Dimedone (5,5-dimethyl-1,3-cyclohexanedione) reacts analogously to acetylacetone, Fig. 1, however the fluorescence quantum yields of the dimedone products are about 100 times higher than those of the lutidine derivatives[6]. The reaction mechanism has recently been elucidated[7]. This report presents our findings regarding the potential of dimedone as a precolumn fluorimetric reagent for the trace analysis of aldehydes.

EXPERIMENTAL

Apparatus

Varian Model 5020 and Beckman Model 324 gradient HPLC systems were used in this study. The chromatographs were equipped with Valco (Model CV-6UHpa-N60) sampling valves with 20- or 200-μl sample loops. The columns used were Altex Ultrasphere ODS (octadecyl silane, 5 μm, 250 $\times$ 4.6 mm) and in-house packed Nucleosil ODS (5 μm, 200 $\times$ 4.6 mm).

The HPLC fluorescence detectors used were a Kratos Model FS 970 (excitation: 385 nm; emission: >440 nm, cut-off filter) and a Perkin-Elmer Model 650 S-LC (excitation: 385 nm; emission: 460 nm). An Aminco-Bowman spectrophotofluorometer was used for spectral determinations and for some optimization tests of the reagent composition.

Chemicals

Reagent grade chemicals and double distilled deionized water were exclusively employed in this study. Organic solvents used in the HPLC mobile phase, derivatization procedures and standard solutions were distilled-in-glass HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain; J. T. Baker, Philipsburgh, NJ, U.S.A.). Dimedone was obtained from Fluka (Buchs, Switzerland). Individual and mixed aldehyde standards (E. Merck, Darmstadt, G.F.R.) were dissolved in isopropanol–water (1:1) to give 1 m*M* solutions.

Derivatization procedure

The reagent is prepared as follows: 60 g ammonium acetate and 2.1 g dimedone (in isopropanol, 0.12 g ml^{-1}) are made up to 100 ml in water. This solution is stable for several weeks at room temperature when stored in an amber glass bottle. The solution is allowed to age one day prior to use. To eliminate aldehyde contamination, the reagent is heated at 100°C for 30 min in a sealed vessel, cooled to room temperature and extracted with two 20-ml portions of carbonyl-free dichloromethane[8]. Alternatively, the reacted reagent is eluted through a C_{18} Seppak (Waters Associates) at a rate of 3–4 ml min^{-1}; the Seppak is preconditioned in 2 ml methanol and 5 ml water. To 2 ml of aqueous sample (or aldehyde standard) are added 1 ml of extracted reagent and 0.25 ml of 9 *M* H_2SO_4 (made carbonyl-free by refluxing for 2 h). The reaction tube is sealed and placed in a bath of boiling water for 20 min. The reaction is stopped by immersion of the tube in an ice-bath. An aliquot (5–200 μl) of the reaction mixture is injected directly onto the HPLC column. The derivatives are stable for at least 8 h if stored in the dark at 0°C. Alternatively, the derivatives can be quantitatively extracted from the aqueous reaction mixture with one 5-min extraction with 0.6 ml dichloromethane. The derivatives are stable indefinitely in the organic phase. A C_{18} Seppak can also be used to enrich the derivatives.

HPLC procedures

All runs were made at ambient temperature and at flow-rates of 1.0 ml min^{-1}. For gradient runs, the weaker mobile phase (A) was water and the stronger mobile phase (B) was acetonitrile. The gradient typically used was: 50% to 55% B in 4 min; 55% to 80% B in 3 min; isocratic at 80% for 7 min; 80% to 100% B in 1 min; isocratic at 100% B for 4 min; 100% to 50% B in 2 min.

Alternatively, C_1–C_7 aldehydes can be adequately separated in a reasonable analysis time (*ca.* 20 min) by isocratic elution using 60% B. It is recommended that a simple column switching technique be used to eliminate strongly retained components[2,9].

RESULTS AND DISCUSSION

Chromatographic conditions

Several organic solvents were tested for their effect on resolution and selectivity of dimedone derivatives in reversed-phase HPLC. The greatest selectivity was obtained with acetonitrile. The separation of propanal and benzaldehyde was somewhat difficult with methanol and tetrahydrofuran, but was readily achieved with acetonitrile, Fig. 2.

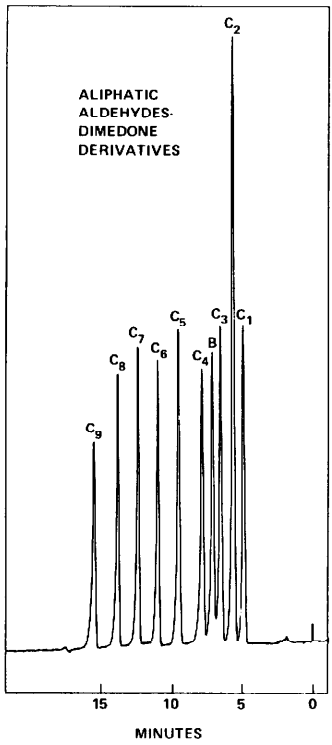

Fig. 2. Reversed-phase separation of straight-chain aliphatic aldehydes and benzaldehyde (B); derivatization and chromatographic conditions as in the text. Each peak represents 20 pmol (40 pmol for C_2 and C_3).

A few preliminary tests showed that the derivatives can also be readily separated using the normal phase mode. A column packed with porous silica (Nucleosil, 7 μm, 200 $\times$ 4.6 mm) was used with a mobile phase of hexane–isopropanol (99:1) and a flow-rate of 1 ml min^{-1}. The normal phase mode was useful when the derivatives were extracted from the aqueous reaction mixture into dichloromethane (see Experimental).

Fluorescence wavelengths

The maximum excitation and emission wavelengths for thirteen aldehyde derivatives are given in Table I. The wavelengths are very similar for most aldehydes suggesting that R group has little effect on the electronic environment of the actual fluorophore. Only the benzaldehyde derivative has significantly different wavelengths, perhaps due to resonance between the phenyl group and the fluorophore.

pH of the reaction mixture

Sawicki and Carnes[6] used pH $\approx$ 6.5 in their dimedone reaction mixture, while Compton and Purdy[7] reported that the reaction of dimedone with aldehydes is optimal at pH 2–3. Because of this difference, we decided to examine in some detail the effect of pH on the reactivity of dimedone.

The reaction mixture consisted of 20 g ammonium acetate, 0.7 g dimedone (in

TABLE I

FLUORESCENCE RESPONSES AND MAXIMUM EXCITATION/EMISSION WAVELENGTHS OF DIMEDONE DERIVATIVES

Derivative of:	*Wavelengths* (nm)*	*Responses relative to formaldehyde*
Formaldehyde	393/462	1.0
Acetaldehyde	383/455	1.1
Propanal	387/455	0.3
n-Butanal	386/456	1.0
Isobutanal	392/459	<0.1
n-Pentanal	385/458	0.9
n-Hexanal	385/457	0.9
n-Heptanal	384/457	0.8
n-Octanal	385/458	0.8
n-Nonanal	384/457	0.7
Benzaldehyde	380/448	0.9
Acrolein	386/460	<0.1
Crotanaldehyde	392/450	<0.1

* Measured in aqueous reaction mixture, diluted 1:1 in acetonitrile.

isopropanol, 0.12 g ml^{-1}) and various amounts of 9 *M* H_2SO_4 or 10 *M* NaOH in a final volume of 100 ml. After reaction at 100°C for 20 min, the fluorescence intensities at the different pH values were measured; the results for formaldehyde and propanal are depicted in Fig. 3. For formaldehyde the optimal pH range is 4–8, while for propanal (and other aldehydes) the optimal pH range is 2–5. We have chosen a pH of 4.5 as a compromise.

The reason for the two pH dependencies is probably related to the finding that the fluorophore is formed via two reaction pathways[7], each pathway having a different pH optimum. Apparently, different aldehydes (formaldehyde *versus* other aldehydes) react at different rates along these pathways.

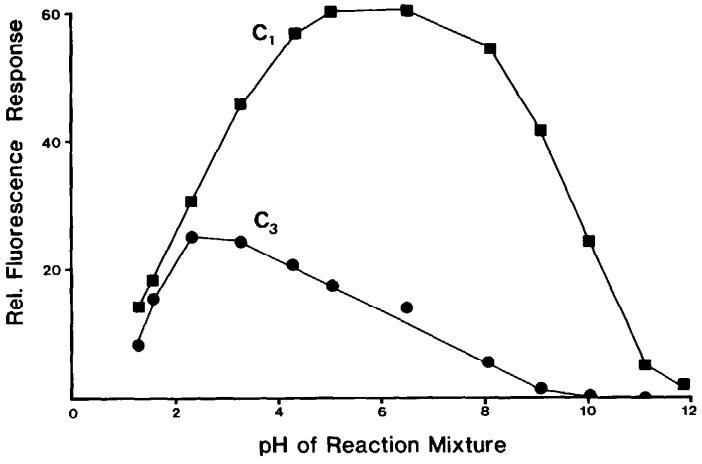

Fig. 3. Effect of pH on the reaction of dimedone with aldehydes; reaction conditions as in the text.

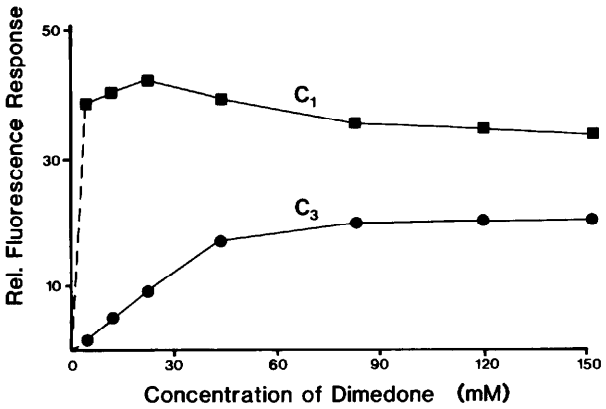

Fig. 4. Effect of dimedone concentration on the reaction of dimedone with aldehydes; see text for reaction conditions.

Concentration of dimedone

The concentration of dimedone in the normal reaction mixture (see Experimental) was varied from 3.6 to 152 m*M*. The aldehyde concentration was maintained at 40 μM. After reaction at 100°C for 20 min the fluorescence intensities were measured. Fig. 4 depicts the results for formaldehyde and propanal. Variations in dimedone concentration had little effect on the reaction with formaldehyde, however a minimum dimedone concentration of about 45 m*M* is needed for quantitative derivatization of other aldehydes.

Concentration of ammonium acetate

The concentration of ammonium acetate in the normal reaction mixture was varied from 0.3 to 9.7 *M*. The pH was 6.5 and the dimedone concentration was 71

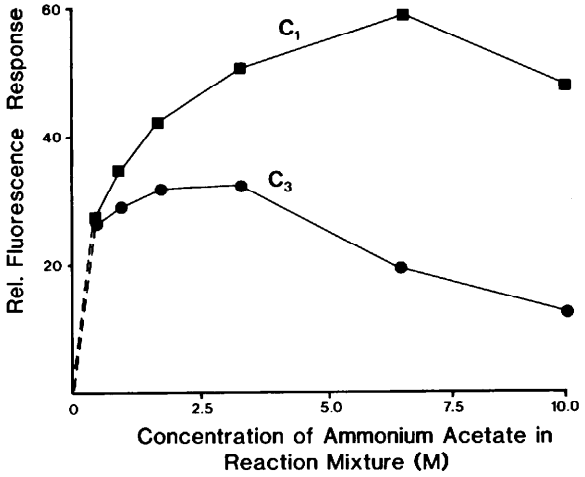

Fig. 5. Effect of ammonium acetate concentration on the reaction of dimedone with aldehydes; reaction conditions as in the text.

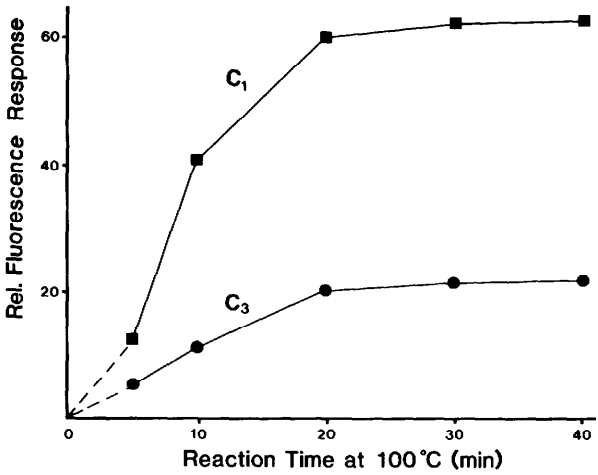

Fig. 6. Effect of reaction time at 100°C on the production of fluorescent dimedone derivatives of aldehydes; reaction conditions as in the text.

m*M*. The fluorescence intensities were measured after a 20-min reaction at 100°C. The results for formaldehyde and propanal are shown in Fig. 5. The optimal ammonium acetate concentration for formaldehyde is 3.0–9.5 *M*, while for the other aldehydes the optimum is 0.8–3.2 *M*. As a compromise, an ammonium acetate concentration of 2.6 *M* was chosen.

Reaction time at 100°C

Reaction times from 5 to 40 min were tested and the results for formaldehyde and propanal are shown in Fig. 6. A reaction time of 20 min is sufficient. Varying the dimedone concentration from 35 to 140 m*M* in the final reaction mixture only slightly affected the reaction rate for formaldehyde. However, the other aldehydes required a longer reaction period at lower dimedone concentrations (*e.g.*, $\approx$ 30 min at 35 m*M* dimedone).

Fluorescence responses

The fluorescence responses of twelve aldehydes, relative to formaldehyde, are listed in Table I. These responses were obtained using the derivatization procedure given in the Experimental section. As can be seen, the responses of most straight-chain aldehydes are very similar to that of formaldehyde. For comparison, the response factors of most aldehydes using the procedure of Sawicki and Carnes[6] were about 5–20 times lower than that obtained for formaldehyde. Therefore, depending on the derivatization procedure used, dimedone can be used either as a general reagent for aldehydes or as a fairly specific reagent for formaldehyde.

The low fluorescence responses for isobutanal, acrolein and crotonaldehyde, Table I, suggest that branching or unsaturation at the α position hinders the reaction. In the case of isobutanal, steric hindrance is the probable cause of its low reactivity.

Reaction yield

Gram quantities of dimedone derivatives of propanal and pentanal were prepared according to Sawicki and Carnes[6]. The reaction yield, using the derivatization procedure given in the Experimental section, was checked for these aldehydes. The concentration of aldehydes in the reaction mixture was 20 μM. The yields for propanal and pentanal were 37% and 89%, respectively. Since the fluorescence responses of most aldehydes are similar to that of pentanal, Table I, their reaction yields are presumably also similar to that of pentanal ($\approx 90\%$). This assumes that the fluorescence quantum yields of the dimedone fluorophores of the different aldehydes are similar. This assumption is probably correct for aliphatic aldehydes since excitation and emission wavelengths are nearly identical for C_1–C_9 derivatives, Table I.

Extraction of the fluorescent products

Hexane, 1,1,2-trichloro-1,2,2-trifluoroethane, butanol, ethyl acetate, chloroform, dichloromethane and C_{18} Seppaks were tested for their extraction efficiencies. The last three solvents and C_{18} Seppaks proved to be the most effective. For example, >97% of the derivatives could be extracted by one 5-min extraction with dichloromethane (see Experimental).

Since the fluorescent products can be preferentially extracted from the reaction solution, aldehyde contaminants in the reagent can be quantitatively removed by prereaction and extraction (see Experimental).

The fluorescent products were found to be significantly more stable in nonpolar solvents than in the aqueous reaction mixture, therefore extracted samples could be analyzed weeks after derivatization. In addition, the sensitivity of the method could easily be increased by a factor of 100 by extraction and concentration.

Precision, accuracy and detection limit

A series of eleven isocratic runs were performed with a ten-component aldehyde standard mixture (C_1–C_9 and benzaldehyde). Equal aliquots of this mixture were derivatized by the usual procedure (see Experimental) prior to each injection. Using peak areas, the average standard deviation (for C_3–C_9 and benzaldehyde) was $\pm 1.7\%$ at the 28 pmol level. The standard deviations for formaldehyde and acetaldehyde were $\pm 4.1\%$ and $\pm 3.6\%$, respectively. The higher standard deviations for these aldehydes were probably due to contamination.

The dimedone technique was intercalibrated with a 2,4-dinitrophenylhydrazine method[3]. Aliquots of automobile exhaust (collected in methanol–water, 1:4) were analyzed simultaneously with both HPLC procedures. The 2,4-dinitrophenylhydrazine method gave the following concentrations in the sampling solution: formaldehyde, 27.4 μM; acetaldehyde, 3.4 μM; benzaldehyde, 0.97 μM. For the dimedone method the corresponding values were: 23.0 μM; 4.5 μM; 0.99 μM. Therefore, the agreement between the methods is very good.

When precautions were taken to minimize aldehyde contamination from glassware, reagent solution and air, the detection limit of the dimedone method was found to be about 30 fmol per injected aldehyde (C_1–C_9 and benzaldehyde) with a signal-to-noise ratio of about 3. This corresponds to an injection of 100 μl of a sample with 0.3-nM levels of aldehydes. The detection limit could be lowered by a factor of 100 by extraction with dichloromethane or C_{18} Seppaks and concentration (see Experi-

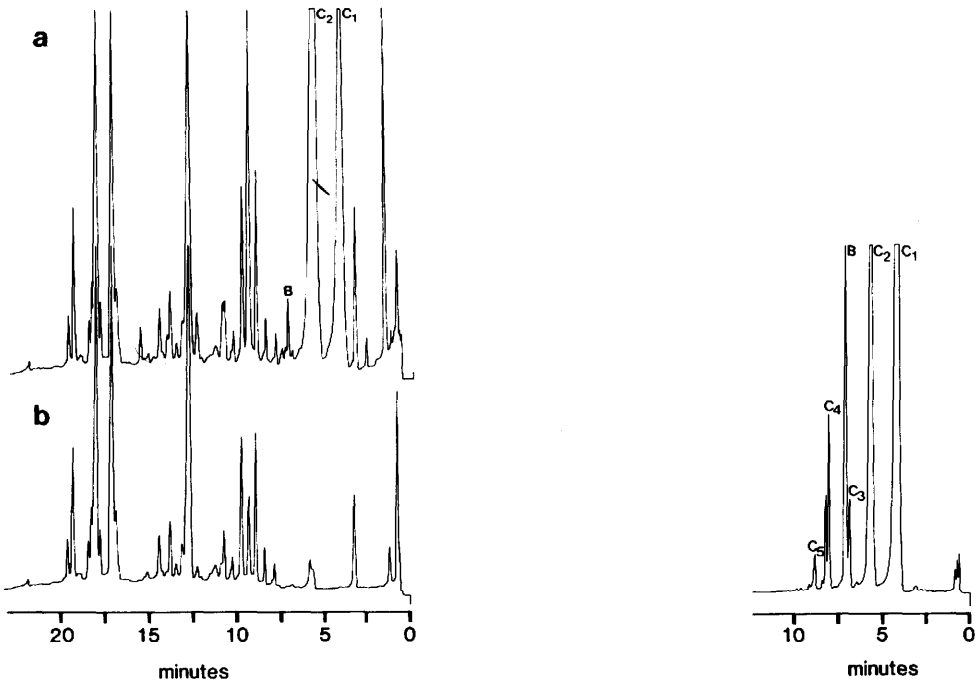

Fig. 7. Chromatograms corresponding to a direct injection of 4 μl of a derivatized perfume sample (a); (C_1 = formaldehyde; C_2 = acetaldehyde; B = benzaldehyde) and fluorescent compounds in an injection of an equivalent amount of underivatized perfume (b).

Fig. 8. Chromatogram of dimedone derivatized aldehydes in automobile exhaust, collected in methanol–water (1:4). Peaks: C_1 = formaldehyde; C_2 = acetaldehyde; C_3 = propanal; B = benzaldehyde; C_4 = butanal; C_5 = pentanal.

mental). Dimedone appears to be the most sensitive reagent presently available for HPLC analysis of aldehydes.

Additional comments

An analogous reagent, 1,3-cyclohexanedione[6], was also examined as part of this study. Preliminary tests showed that this reagent reacts more rapidly with aldehydes than dimedone, and that the derivatives are also easily separated by reversed-phase HPLC. However, the reagent itself was found to contain higher levels of contamination and the reagent solution was somewhat less stable than that for dimedone. If further tests show 1,3-cyclohexanedione to be a superior reagent, we will report the results in a future note.

Figs. 7 and 8 show various practical applications of the dimedone method. The method is simple, highly specific and extremely sensitive, therefore, it should be particularly useful for trace analysis of aldehydes, especially for aqueous samples with complex matrices. We are presently using this technique for study of aldehyde production and consumption in sea-water, where levels are typically in the nanomolar range.

ACKNOWLEDGEMENTS

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