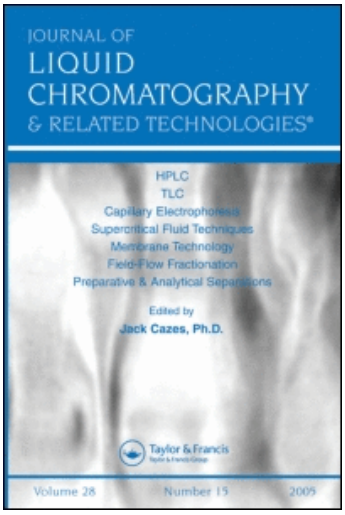

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SEPARATION OF HIGH MOLECULAR WEIGHT SAPONINS OF *ARCHIDENDRON ELLIPTICUM* BY HYDROPHILIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

Saponins of high molecular weight (Mr 2,000-2,100) from several species in the plant family Leguminosae were separated at both analytical and preparative scale using wide pore aminopropyl HPLC with acetonitrile-water mixtures, and gradient wide-pore butyl reversed-phase HPLC. The basis for the aminopropyl separation appears to be hydrophilic interaction chromatography, and the pore size of the media appears to play a key role in the separation.

INTRODUCTION

This paper reports the application of hydrophilic interaction chromatography methodology to the successful isolation of the cytotoxic elliptosides A-J from *Archidendron ellipticum* (Bl.) Nielsen (Leguminosae), at

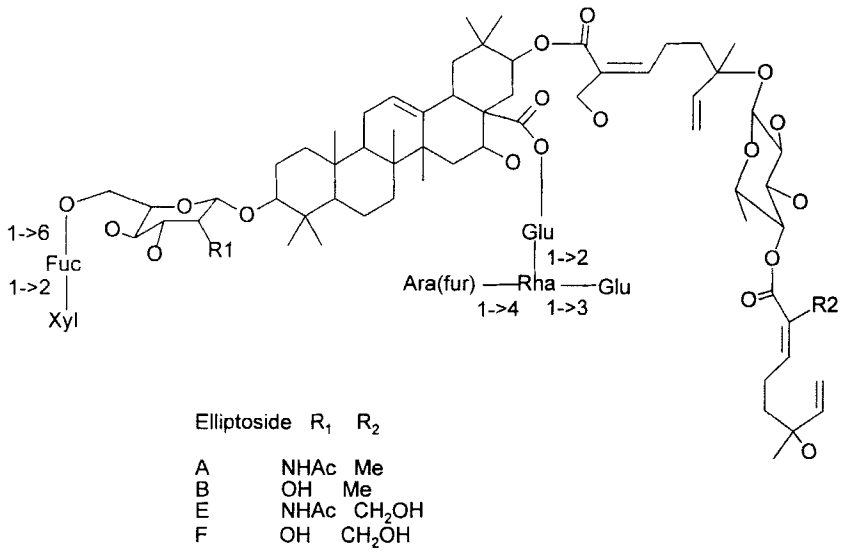

Figure 1. Structures of Elliptosides A,B, E, and F.

both the analytical and preparative scale. These closely related structures are ester glycosides of acacic acid with eight sugar residues and two monoterpene esters (Figure 1). Details of the structure elucidation and biological properties of elliptosides A-J are presented elsewhere.[1] We have found other saponin mixtures from the related trees *Archidendron jiringa* and *Cathormion altissimum* to contain similar cytotoxic saponins and have also separated these on an analytical scale. This technique has also been successfully applied in our laboratory, to the separation of saponins from other non-legume sources (data not shown).

RESULTS

The crude organic extract of *A. ellipticum* was partitioned between $CHCl_3$, MeOH, and water to give a cytotoxic aqueous fraction. This fraction was further permeated through Sephadex LH-20 in MeOH to give an early-eluting (i.e., higher molecular weight) cytotoxic fraction, composed of a complex mixture of saponins, as judged by ^{1}H-NMR. Later fractions had reduced or no cytotoxic activity. A variety of conventional separation methods were applied to the bioactive mixture in an effort to purify the saponins. Separations on silica gel, C_{18}, diol, cyano, PRP-1, and PRP-3 chromatography media were

evaluated by TLC and/or HPLC without obtaining adequate resolution. Numerous countercurrent systems were scouted by partition TLC on silica gel and several trial runs were made on a Sanki CPC apparatus. These methods were not effective, and, in addition, the use of centrifugal countercurrent methods led to significant loss of bioactivity in the recovered fractions.

The use of a C_4 wide-pore separation[2] using a MeOH-H_2O-HOAc gradient partially resolved the saponin mixture; however, NMR and mass spectral analysis made it clear that further separation would be required to obtain pure compounds. Wide-pore aminopropyl media resolved the mixture into six major peaks. Retention times for the peaks increased with increasing content of CH_3CN in the mobile phase, indicating that the separation did not reflect reverse-phase chromatography. When analyzed by C-4 HPLC, each of the peaks also proved to be a mixture. The sequential application of amino and C-4 HPLC, however, permitted complete separation of elliptosides A-H in pure form (Figures 2 and 3). Wide-pore phenyl separation of a third peak from the aminopropyl chromatography yielded two further saponins, elliptosides I and J. The compounds isolated by this method had consistent ^1H- and ^{13}C-NMR spectra, as well as a single major pseudomolecular ion by FABMS and, thus, were judged to be pure.

The effect of pore size on the aminopropyl separation was studied in three ways:

1) Chromatography columns having aminopropyl bonded phase chemistry, but different nominal pore size, were used to characterize the Sephadex LH-20 eluate from *A. ellipticum*. As shown in Figure 4, a 60Å column (Rainin Dynamax, 8μ irregular, 4.6 x 250 mm) did not give a useful chromatographic separation, while a column with 120Å pores (Waters Carbohydrate column, 4.6 x 300 mm) gave some resolution, and a wide pore 300Å column (YMC Amino, 4.6 x 250 mm) gave a superior separation of the same material. Further trial separations using 120Å, 5μ spherical aminopropyl and polyamine media (YMC) gave poor resolution, confirming the necessity for wide pore media and eliminating particle size and shape as major factors affecting the separation.

2) The organic and aqueous extracts of several other plants which also exhibited cytotoxicity similar to that from *A. ellipticum* were processed by the same partition and Sephadex LH-20 chromatography steps described above. The earliest Sephadex LH-20 peak was then analyzed using an analytical YMC 300Å 5 μ aminopropyl HPLC column.

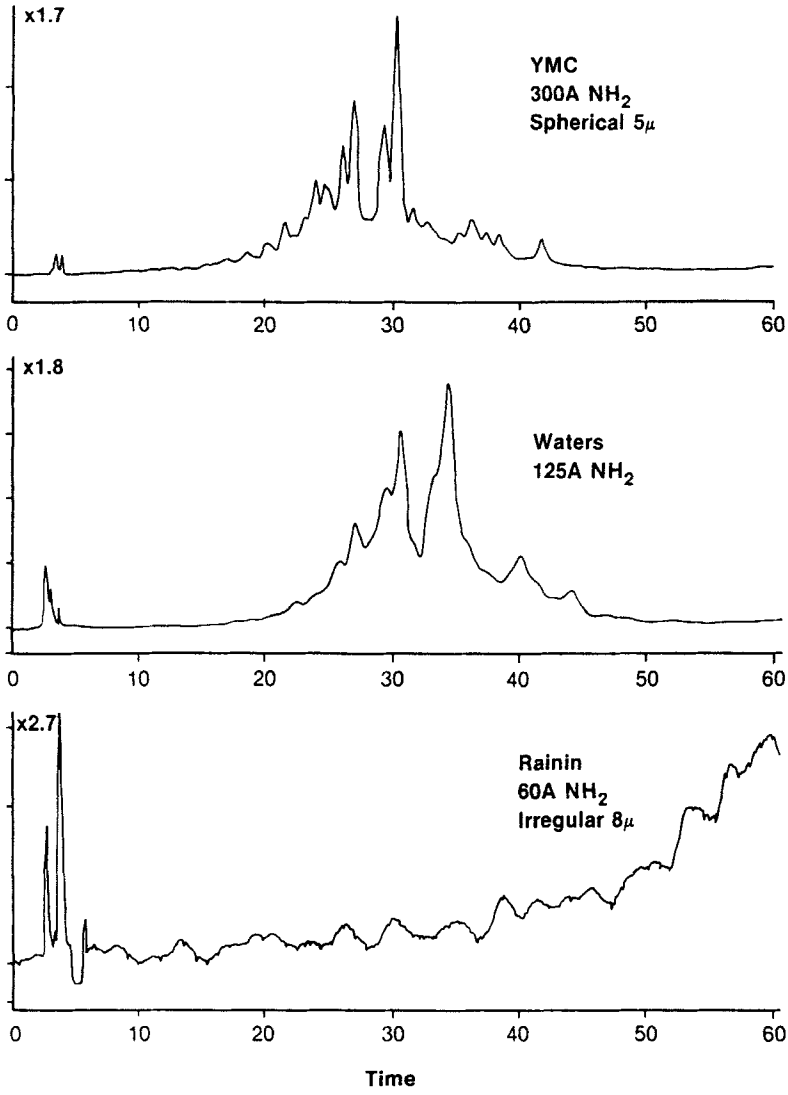

Figure 2. Comparison of the separation of crude *A. ellipticum* saponin mixture on a) YMC 300Å 5 μ spherical particle analytical column; b) Waters 125Å "Carbohydrate" column c) Rainin 60 Å aminopropyl 8 μ irregular particle column. All columns were 250 mm in length, flow 1 mL/min., 80% CH_3CN, detection at 230 nm.

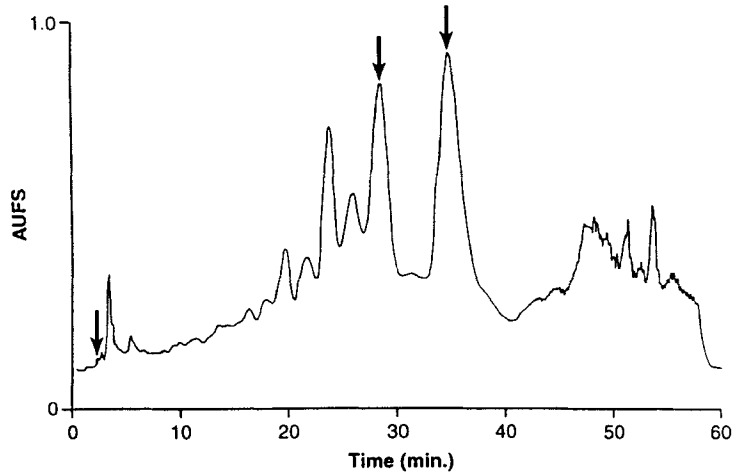

Figure 3. Preparative separation of crude saponin mixture from *A. ellipticum* on YMC 20mm x 250 mm 300 Å 5 μ spherical aminopropyl column, 80% CH_3CN, 15 mL/min, detection 238 nm. Load 100 mg. Arrows indicate elliptoside A-D and E-H containing fractions respectively.

Fractions from the plants *Cathormion altissimum* (Hook.f.) Hutch.& Dandy and *Archidendron jiringa* (Jack) I.Nielsen gave a similar high-resolution separation of saponin peaks (Figure 5). Thus, this method could be applied to the separation of saponin mixtures from several plants in the family Leguminosae.

3) Construction of computer models of the elliptosides showed that the maximum dimensions of the molecules should be no greater than 100Å. While a compound of this size would not usually be considered large enough to demand 300Å pores for efficient mass transfer, the effect of hydration of the saponin may increase the effective size of the molecule.

In addition, the functionalization and hydration of the aminopropyl silica media may decrease the effective pore size of the particle. Alternatively, the saponins might form mixed micelles of a large enough size to require larger pore media, but in that case separation of the distinct components would not appear probable.

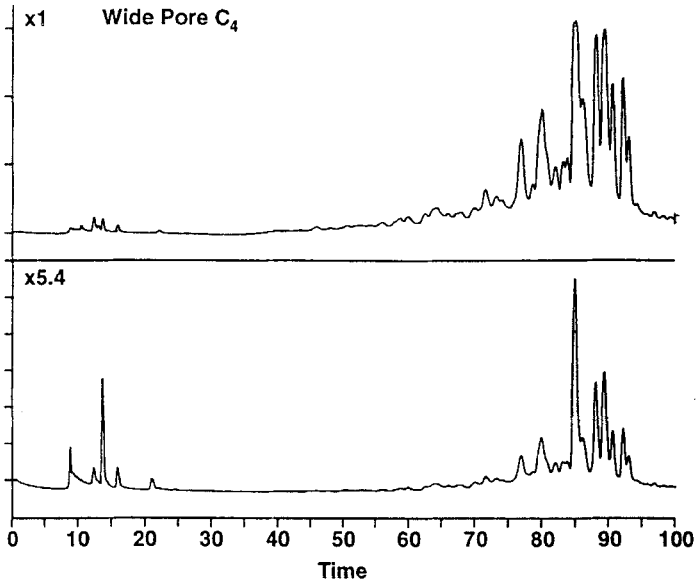

Figure 4. Preparative separation of partially purified elliptosides A-D on Rainin C_4 300 Å 5 μ spherical column, gradient from 50% MeOH (40 mM HOAc) to 100% MeOH (40 mM HOAc). Load 25 mg.

DISCUSSION

The majority of previously characterized saponins have been of molecular weight <1500 daltons (i.e., containing one to four sugar moieties). Saponins containing larger numbers of sugars have generally been difficult to purify due to the complexity of such mixtures. In the present case, monoterpene esters attached to different parts of the molecule further complicated the task. While simple saponins have been successfully purified by conventional chemical techniques such as adsorption and partition chromatography, as well as by countercurrent chromatography,[3] more complex mixtures of high molecular weight saponins have historically been difficult to purify to homogeneity.

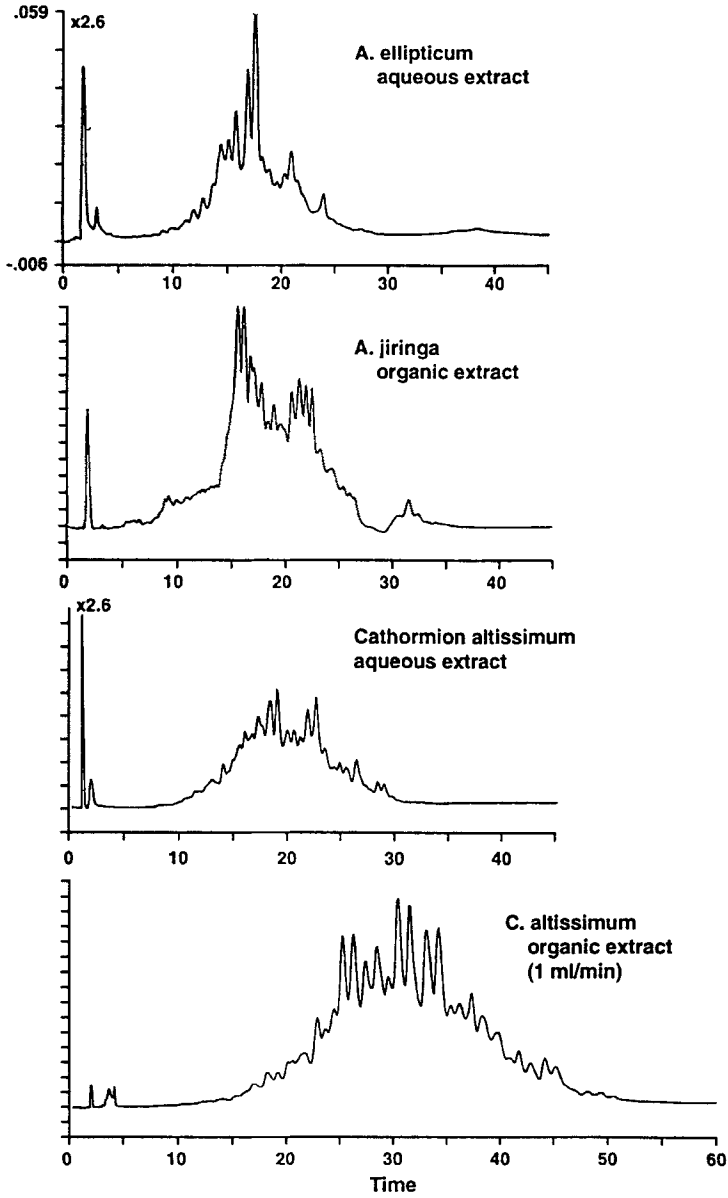

Figure 5. Analytical separation of saponin fractions from various plant samples on aminopropyl bonded phase media. 2 mL/min 80% CH_3CN except for d) 1 mL/min.

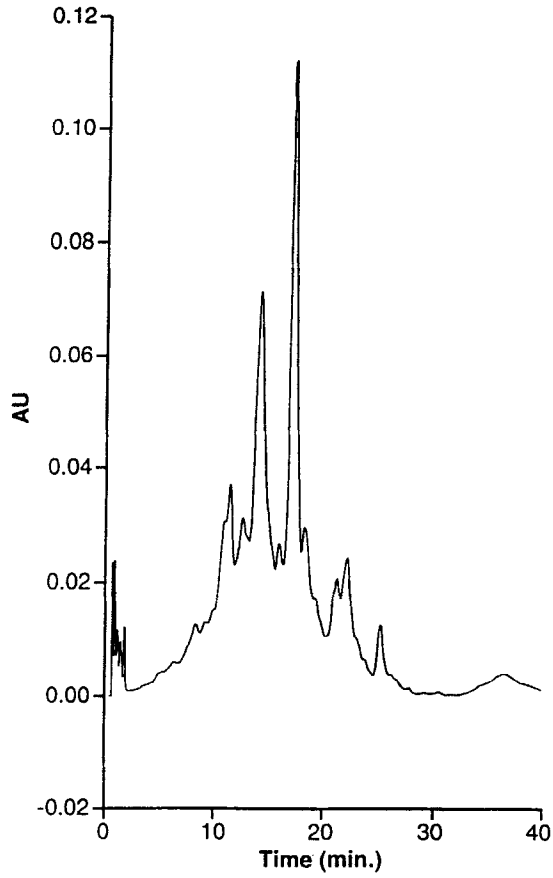

Figure 6. Analytical separation of crude saponin mixture from *A. ellipticum* on YMC 4.6 mm x 250 mm 300 Å 5 μ spherical polymeric amino column, CH_3CN-H_2O gradient, 2 mL/min, detection 230 nm. Load 100 μg.

A recent advance in large saponin purification methodology is the use of wide-pore butyl (C_4) HPLC,[2] where high molecular weight (Mr1,800) saponins from *Quillaja saponaria*, useful as immune adjuvants,[4] were purified from a complex mixture. The use of wide-pore (330Å) HPLC media was not specifically identified as being crucial to this separation. The well-established technique of separating simple sugars and oligosaccharides by the use of so-called "carbohydrate" HPLC columns (aminopropyl phase-bonded silica gel) eluted with mixtures of acetonitrile and water has been well-known since 1975.[5,6] A partition mechanism has been proposed for the separation,[7,8,9]

involving formation of a water-rich layer in the stationary phase. The most polar analytes are proposed to associate preferentially with the water-rich layer. The recent formulation of the concept of hydrophilic interaction chromatography (HILIC[10]) has been made to generalize this method of separation. While the elliptosides contain eight sugars, the basis for their separation on aminopropyl media would appear not to depend on the specific sugar moieties, since elliptosides A and B, which are not separated by this system, have different internal sugars (Glc vs. GlcNAc), while elliptosides A and E, which have identical sugar composition but a different monoterpene, are separated. The most polar saponins are eluted after the less polar saponins, the opposite elution order from that expected in reverse-phase chromatography. HILIC appears to be the basis for this separation.

"Carbohydrate" separation technology or HILIC has rarely been applied to the separation of glycosides of any type, including saponins. The only examples found in the literature were the analytical separation of ginseng saponins on an amino column of unspecified pore size,[11] and separation of neutral steroidal *Dioscorea* saponins.[12]

The aminopropyl column performance rapidly deteriorated after several days of preparative use. Replacement of the guard column returned the system to its initial performance. The use of a vacuum flash chromatography step using CH_3CN-H_2O on flash chromatography grade aminopropyl media prior to HPLC did not appear to improve column lifetime, and resulted in poor mass recoveries.

Short column lifetime is a recognized limitation of aminopropyl HPLC media.[13] Switching to a "polymeric" amino bonded phase column has yielded useful separations with substantially increased column lifetime (Figure 6). The wide pore amino column separation of high molecular weight saponins was successfully applied to the resolution of saponin mixtures from diverse plants in the family Leguminosae. It may also prove useful for separation of complex saponin mixtures from other families.

MATERIALS AND METHODS

General

All solvents were HPLC grade. Gel permeation was carried out using glass columns packed with Sephadex LH-20 (Pharmacia) and eluted with

MeOH, and monitored with a variable wavelength monitor (ISCO V^4) tuned to 230 nm, or detuned to higher wavelengths at higher loadings. HPLC was carried out using a Waters model 600E gradient pumping system equipped with a model 990 diode array detector, or a Waters Delta Prep 3000 with a Waters model 481 variable wavelength detector. Columns used were Rainin Dynamax wide pore C_4 (250 mm, 5μ, 300 Å) and YMC aminopropyl (250 mm, 5μ spherical 300Å), polyamine (PBMN, 250 mm, 5 μ spherical 300Å), and phenyl (250 mm, 5 μ spherical 300Å) columns of various diameters, equipped with guard columns containing the same packing materials. A Waters "Carbohydrate" column (4.6 x 300 mm, 125Å), YMC narrow pore (120 Å) aminopropyl column, and Rainin Dynamax aminopropyl column (4.6 x 250 mm, 60Å, 8 μ irregular) were also used in characterizing the effect of pore size on the separation.

Extracts

Extracts used in the present work were obtained from the NCI natural products repository. The extracts were prepared as described below from plant materials collected by NCI contractors in South America, Africa, and Southeast Asia. Voucher herbarium specimens were deposited by the collectors.

Isolation of Saponins from *Archidendron ellipticum* Leaf Extract

Milled leaves of *Archidendron ellipticum* (824 gms) were percolated with a mixture of CH_2Cl_2-MeOH (1:1, v/v) to extract the organic soluble materials. The solvent was drained and a MeOH rinse was performed on the marc. The combined solvents were evaporated to dryness on a rotary evaporator at 35°C. This yielded 98 g of organic extract (11.8% w/w). 14.4 g of extract were dissolved and partitioned with a mixture of $CHCl_3$ (500 mL), MeOH (450 mL), and H_2O (200 mL). Two further 500 mL portions of $CHCl_3$ were used to wash the aqueous phase. The aqueous phase was evaporated to give 4.03 gms of cytotoxic polar material. This material was chromatographed on a 7 x 38 cm column of Sephadex LH-20 in MeOH, using uv detection at 238 nm to differentiate the eluted fractions. The earliest eluting peak was evaporated to give 949 mg of crude saponin mixture with increased cytotoxicity. This material was separated by wide-pore aminopropyl HPLC (YMC 20 x 250 mm, 5μ spherical, 300Å column) using 80% acetonitrile as eluant, followed by a linear gradient to 72% CH_3CN (see Figure 3). This resolved six major peaks, two of which were then separately chromatographed on wide- pore butyl HPLC (Rainin 21.4 x 250 mm 5μ spherical, 300Å column), using a gradient from 50% MeOH/H_2O, 40 mM HOAc to 100% MeOH, 40 mM HOAc (see Figure

4). In this fashion elliptosdes A-H were isolated in the following amounts: A-16 mg, B-5.1 mg, C -5.9 mg, D-3.4 mg, E -25 mg, F-11.8 mg, G- 14.7 mg, H -10.6 mg.

Isolation Using a Vacuum Flash Chromatography Step

The same sequence of separation was followed as above, however, a vacuum flash chromatography column step using aminopropyl media (YMC 120Å, 40-63μ, irregular) was performed before the aminopropyl HPLC in an effort to increase the HPLC column lifetime. The column was eluted with 70% CH_3CN, 50% CH_3CN and H_2O.

Purification of Aqueous Extract of *Archidendron Ellipticum* Leaves

The marc from the organic extraction above was extracted overnight with distilled water and the aqueous extract lyophilized to give an aqueous extract. Trituration of 314 mg of extract with MeOH yielded a methanol-soluble fraction which was permeated on Sephadex LH-20 in MeOH. The earliest peak from this chromatography (29 mg) was identical in cytotoxic activity, aminopropyl HPLC profile, and NMR spectrum to the corresponding fraction from the organic extract.

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