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Extraction of artemisinin and artemisinic acid from *Artemisia annua* L. using supercritical carbon dioxide

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Abstract

Artemisinin (an antimalaric compound) and its major precursor artemisinic acid, isolated as the active principles of the medicinal plant *Artemisia annua* L., were extracted by supercritical fluid extraction (SFE) and analyzed by supercritical fluid chromatography (SFC) using a capillary column, coupled with a flame ionization detector (FID). With optimized operating conditions, artemisinin and artemisinic acid were quantitatively extracted at a flow-rate of 2 ml min^{-1} in less than 20 min. The supercritical fluid was composed of carbon dioxide and 3% methanol with temperature and pressure fixed at 50°C and 15 MPa, respectively. From the kinetic curves, it appears that the extraction of artemisinin is not limited by the diffusion of the analyte from the plant into the extraction fluid but rather by the elution process.

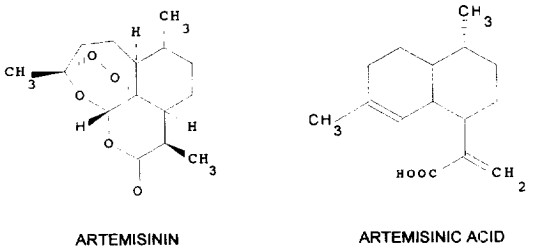
These conditions avoided degradation of the analyte and gave clean extracts ready to be analyzed by SFC. The SFE–SFC–FID method was successfully applied to six samples of *A. annua* containing various concentrations of artemisinin and artemisinic acid. Results were compared with two conventional liquid solvent extraction processes. © 1997 Elsevier Science B.V.

Keywords: Artemisinin; Artemisinic acid

1. Introduction

Malaria is a major disease in many countries, since, according to an estimation of the World Health Organisation (WHO), approximately 300 to 500 million people contract malaria yearly and almost 2 million die annually [1]. Controlling malaria is now becoming very problematic in view of the developing resistance of *Plasmodium falciparum* to chloroquine, mefloquine and other commonly used antimalarial drugs [2]. Therefore, it is vital to investigate new antimalarial compounds.

Artemisinin is a promising drug against chloroquine-resistant strains of *P. falciparum* and in the treatment of cerebral malaria [3–5]. This compound is an endoperoxide sesquiterpene lactone (Fig. 1) found in the aerial parts of the plant *Artemisia annua* L. (Asteraceae), a plant which has been used for many centuries in traditional Chinese medicine for the treatment of fever and malaria. Although the total



ARTEMISININ ARTEMISINIC ACID

Fig. 1. Structures of artemisinin and artemisinic acid.

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synthesis of artemisinin has been achieved [6], it is not competitive in price with the natural product. Concentrations of artemisinin obtained from cultivated *A. annua* vary in the range of 0.01 to around 1% of plant dry weight [4,5,7,8], according to various factors such as the plant's origin, its stage of development and the cultivation conditions. This is why it is important in a plant development project to have analytical methods at one's disposal which will allow the quantitative determination of artemisinin and possibly of its precursors.

A number of such analytical methods exist, such as high-performance liquid chromatography (HPLC) coupled with ultraviolet detection (UV) [9] and electrochemical detection (EC) [10], thin layer chromatography (TLC) [11], gas chromatography (GC) [12,13] and enzyme-immunoassay [14]. However, because artemisinin is thermolabile and does not possess a chromophore, it cannot be determined easily. Indeed, GC methods measuring artemisinin indirectly by detecting its degradation products and LC methods, equipped with a UV detector, need a pre-derivatisation step. It is possible to analyze artemisinin directly by HPLC equipped with an electrochemical detector. Nevertheless, electrochemical detection in reductive mode is not easily carried out for routine analyses because it requires the elimination of oxygen from the system. Recently, we have developed capillary and packed column SFC methods, coupled with a flame ionisation detector (FID) and an evaporative light scattering detector (ELSD) respectively, to analyse artemisinin and artemisinic acid directly without degradation [15].

Whatever the analytical method used, an extraction procedure of the plant material is required. Liquid solvent extraction with toluene, hexane or petroleum ether is the most currently applied technique [16], with extraction times which can vary from a few minutes to several hours. Furthermore, these procedures use a large amount of potentially hazardous solvents which have to be eliminated before analysis. Therefore, in view of its properties already described in the literature [17–19], supercritical fluid extraction (SFE) with carbon dioxide is an interesting alternative to conventional liquid solvent extraction methods, especially in the case of plant material [20–22]. In this study, we applied SFE to the extraction of artemisinin and its major precursor artemisinic acid in *A. annua*. After an optimisation of the experimental parameters, we compared the results obtained with those of conventional liquid solvent extraction processes. Analytical determinations of both compounds were carried out by supercritical fluid chromatography coupled to a flame ionisation detector.

2. Materials and methods

2.1. Materials

For supercritical fluid chromatography and extraction, 99.99% purity CO_2 (Polygaz, Geneva, Switzerland) contained in a cylinder with an eductor tube, was used. A charcoal packed column and a molecular sieve packed column were incorporated in front of the pump to prevent possible contamination by hydrocarbons.

HPLC-grade methanol, acetonitrile and ethanol were purchased from Maechler AG (Basel, Switzerland), toluene (puriss p.a.) was purchased from Fluka SA (Buchs, Switzerland). Crystalline artemisinin was obtained from Sigma (St. Louis, USA) and dodecanol was from Merck (Darmstadt, Germany). Artemisinic acid was kindly provided by Dr. N. Acton (Walter Reed Army Institute of Research, Washington DC, USA). Authentic air-dried plant material was kindly provided by Dr. N. Delabays (Station Fédérale de Recherche Agronomique, Conthey, Switzerland).

2.2. Supercritical fluid extraction

The air-dried plant material was thoroughly ground in a domestic mixer. 100 mg of this material was introduced in a 1-ml (14-mm×10-mm I.D.) JASCO (Jasco International, Tokyo, Japan) extraction cell. The temperature was controlled by a column oven (Jasco CO-965) adjustable to ±1°C betwen 30°C and 80°C. The CO_2 and the modifier were pumped by two HPLC pumps operated in constant flow mode (JASCO PU-980). The total flow-rate was set between 0.5 ml min^{-1} and 3 ml min^{-1} (expressed as the sum of liquid CO_2 and modifier). The pressure in the system was regulated from 15 MPa to 30 MPa at ±0.1 MPa through a

variable restrictor (Jasco 880-01 Back Pressure Regulator). The latter was heated at 40°C to avoid dry ice formation and the sample was collected by bubbling the CO_2 through 5 ml of methanol placed in a 15-ml conical centrifuge tube.

The extract was evaporated to dryness under a nitrogen flow at 40°C and the dry residue was redissolved in 500 μl of acetonitrile containing 1 mg ml^{-1} dodecanol as chromatographic internal standard. This solution was filtered through a 0.22-μm membrane filter before SFC analysis.

2.3. Liquid–solid extraction

The air-dried plant material was thoroughly ground in a domestic mixer. 100 mg of this material was introduced into a 15-ml centrifuge tube with 5 ml of toluene. This mixture was then either sonicated with an ultrasonic probe (Branson sonifier 250, Heat systems–ultrasonics, New-York, USA) or vigorously mixed with a dynamic mixer (Polytron, Luzern, Switzerland). The extraction time in both cases was 5 min. The mixture was then centrifuged for 10 min at 1000 *g* (Hermle Z380, Gosheim, Germany) and filtered through a 0.45-μm PTFE membrane (Titan syringe filter, Scientific resources, Eatontown, USA). The residue was taken in 1 ml of toluene and the procedure was repeated. The organic phases were collected and evaporated to dryness under a nitrogen flow at 40°C and the dry residue was redissolved in 500 μl of acetonitrile containing 1 mg ml^{-1} dodecanol as chromatographic internal standard. This solution was filtered through a 0.22-μm membrane filter before SFC analysis.

2.4. Capillary SFC analysis

SFC analyses were performed on a Carlo-Erba SFC 3000 equipped with FID detection (Carlo Erba Instruments, Milano, Italy). Injection was performed in pneumatic actuation, the injection volume was 200 nl and the injection time was 1 s. A 20-m×0.1-mm I.D capillary column of DB-WAX (polyethylene glycol phase), film thickness 0.1 μm, (J and W Scientific, Folsom, USA) was used. The following CO_2 density program was carried out: initial density 0.25 g ml^{-1} hold for 7 min, increased at 0.04 g ml^{-1} min^{-1} to 0.75 g ml^{-1}, with a final hold for 7 min. The oven temperature was set at 100°C. The restriction was a tappered fused-silica restrictor (laboratory-made) with a flow-rate of 10 ml min^{-1} (gas state) at 0.25 g ml^{-1}. The detector temperature was 300°C.

3. Results and discussion

3.1. Optimization of the experimental conditions

Supercritical fluid extraction of plant secondary metabolites, with pure or modified carbon dioxide preceding an analytical determination, is now recognised as a very powerful technique [20–22]. However, several parameters have to be optimized in order to extract, quantitatively, the analytes of interest in a short period of time. Among them, the pressure and the temperature of the fluid, the nature and the concentration of the modifier, the flow-rate and the extraction time are generally considered as the most important factors [19]. The optimization of the method can be carried out step-by-step or by using an experimental design. However, because the extraction is a dynamic process, it can be useful in determining the extraction kinetic curves. Furthermore, the method development should be conducted on real material because the behaviour of native and spiked analytes, in a complex matrix such as a plant, is not necessarily the same.

Sesquiterpene lactones, such as artemisinin, are slightly polar compounds which can be extracted by supercritical fluids. Santonine [23], parthenolide [24,25] and costunolide [25] were extracted from plant material by supercritical fluid extraction with carbon dioxide. Previous experiments showed that artemisinin could also be extracted from *A. annua* with carbon dioxide [26,27] and that a small addition of methanol was sufficient to achieve a rapid and quantitative extraction, whatever the pressure and the temperature used. Furthermore, an experimental design (factorial design 2^3) [28] was carried out with the three factors of pressure, temperature and amount of methanol. Inferior and superior levels were 15 and 30 Mpa, 40 and 80°C, 1 and 10% for pressure, temperature and methanol concentration, respectively. Results led us to confirm that only the latter parameter had a significant influence on the ex-

traction yield. Thus, artemisinin being a thermolabile compound, the temperature was fixed at 50°C and the pressure at 15 MPa.

As described by Hawthorne [19], the extraction process can be divided into three parts: (1) partitioning the analytes into the bulk supercritical fluid, (2) sweeping the analytes out of the cell and (3) collecting the analytes from the depressurized CO_2. The shape of the extraction kinetic curves can help the analyst to determine the limiting step. These curves are obtained by plotting the cumulative amount extracted versus the extraction time.

Since the modifier concentration is the most significant parameter, its nature was also tested. For this purpose, extraction kinetic curves were plotted for methanol, ethanol, methanol–water (50/50, v/v) and toluene. These solvents were selected on the basis of preliminary tests and of their solvating power for artemisinin. Extraction kinetic curves were determined using various concentrations of modifier, by collecting fractions after 2.5, 5, 10, 15, 20 and 30 min. Methanol, ethanol and toluene gave similar results, as shown in Fig. 2, with a quantitative extraction obtained in less than 15 min with 3% of modifier in CO_2. However, toluene presents the major drawback of possessing a high boiling point, thus inducing longer evaporation times. Only methanol–water gave unsuccessful results with extraction kinetic curves which rose more slowly for an extended period of time. This result can be explained by the

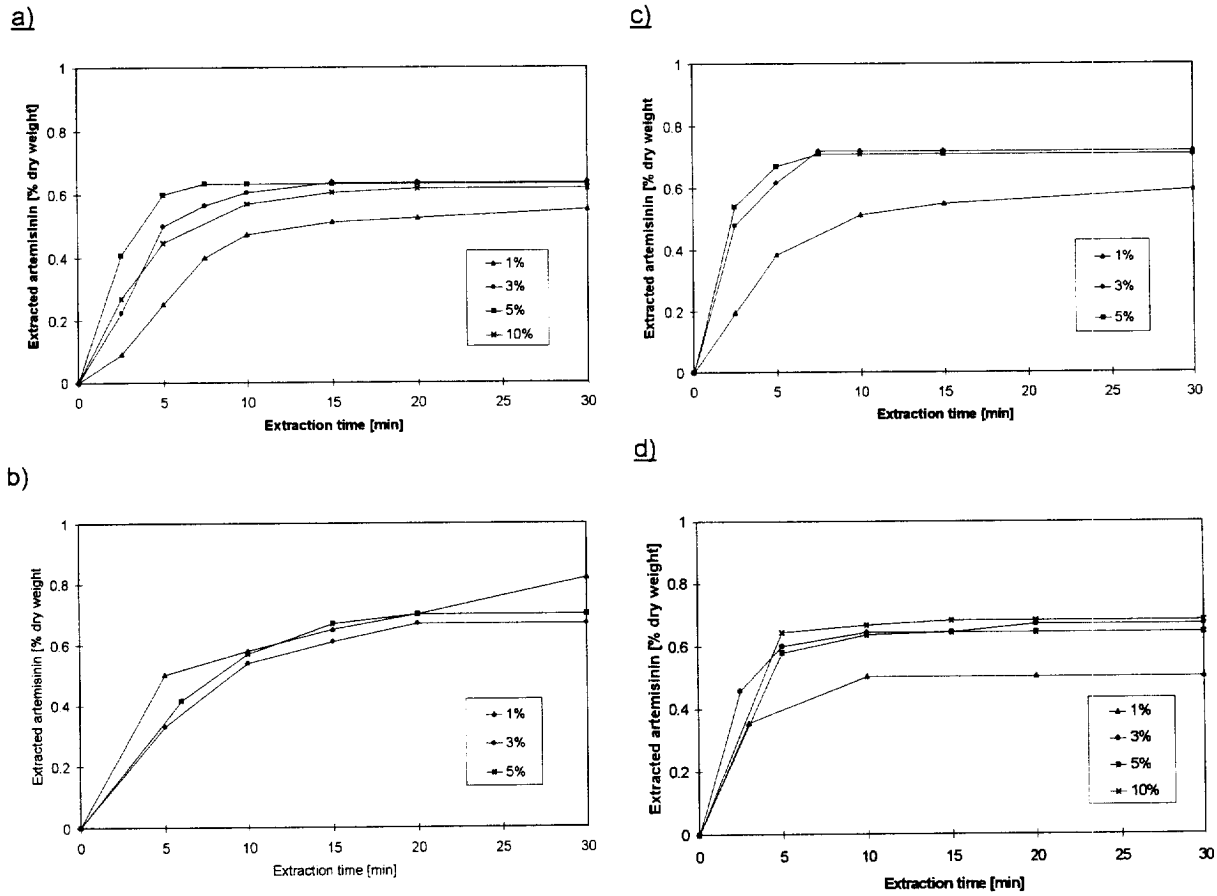

Fig. 2. Extraction kinetic curves for artemisinin from *A. annua* by SFE (15 MPa, 50°C, flow-rate 1 ml min^{-1} with different modifiers: (a) methanol, (b) methanol–water, (c) ethanol, (d) toluene. Each measure was carried out in duplicate.

low solvating power of water for artemisinin (0.46 $g\,l^{-1}$ at 37°C [1]). Finally, extraction with pure CO_2 was also tested. In these conditions, artemisinin was extracted, but with a slower kinetic profile, and some adsorption problems into capillaries (from the restrictor to the collecting solvent) occurred. Therefore, it was impossible to obtain quantitative and reproducible results.

The influence of the flow-rate was also determined and, as shown in Fig. 3, this parameter was important. A quantitative extraction of artemisinin was obtained in less than 10 min with a flow-rate of CO_2 (liquid state) set at 2 ml min^{-1}.

The method for recovering artemisinin from the supercritical fluid was not optimized because artemisinin is not volatile. In all experiments, the analyte was trapped in a few milliliters of methanol at ambient temperature. Nevertheless, we checked that no entrainment of artemisinin occurred in the gas flow from the collection solvent.

Finally, the optimized extraction conditions of artemisinin from *A. annua* were the following: pressure=15 MPa, temperature 50°C, methanol (or ethanol) concentration=3%, flow-rate=2 ml min^{-1}, collection=bubbling in methanol, extraction time=20 min.

In view of these results, it appeared that artemisinin could be quantitatively extracted from *A. annua* in less than 20 min with a supercritical fluid (carbon dioxide modified with a small amount of methanol). The mild conditions used for this extraction avoided a possible degradation of artemisinin, which is a major advantage vis-à-vis conventional liquid extraction methods. It was necessary to add a small proportion of the modifier mainly for increasing solubility and therefore avoiding the adsorption of the analyte on the capillary wall and certainly not to enhance its diffusion through the plant material. Indeed, a methanol concentration higher than 3% in CO_2, did not significantly increase the extraction yield whatever the temperature and pressure chosen. Furthermore, the extraction curves showed no severe kinetic limitations for all the tested modifiers and a significant influence of the flow-rate. Finally, pure carbon dioxide was able to extract artemisinin from *A. annua* and water as modifier gave decreased extraction efficiency.

It has recently been reported [29,30] that artemisinin is localized on the foliar tissues of *A. annua*, and more particularly in the glandular trichomes allowing a rapid extraction with ether, acetonitrile or chloroform. Further investigations are

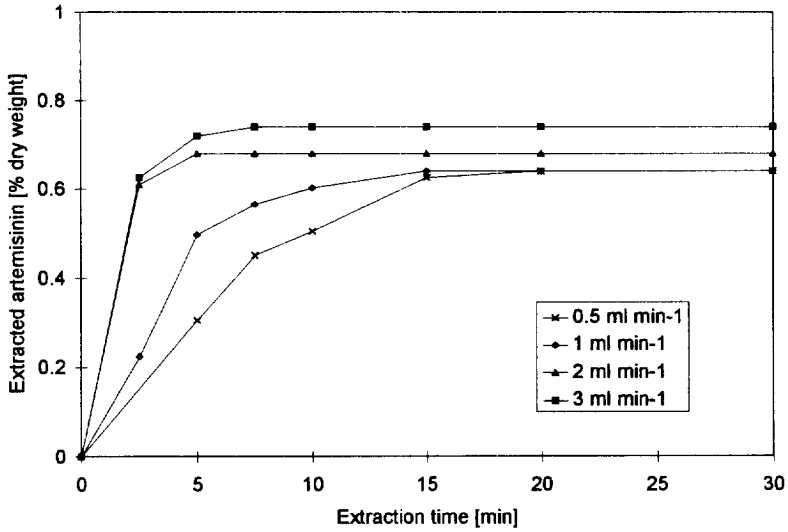

Fig. 3. Extraction kinetic curves for SFE of artemisin from *A. annua* (15 MPa, 50°C, 3% methanol added as modifier) with different flow-rates. Each measure was carried out in duplicate.

in progress in our laboratory to confirm that the artemisinin extraction is not limited by a diffusion mechanism.

3.2. Quantitative results and comparison with conventional extraction procedures

The determination of artemisinin and artemisinic acid, its major precursor, by SFC was validated previously [15] and a chromatogram obtained with standards and plant extracts is shown in Fig. 4.

Repeatability of the process was determined by extracting in replicate ($n=6$) a batch of *A. annua* containing 0.73% dry weight of artemisinin. The relative standard deviation (R.S.D.) of the SFE–SFC–FID method was 7.1% and 10.2% for artemisinin and artemisinic acid, respectively (Table 1). The inter-day variability of the process was 6.3% and 8.4% for artemisinin and artemisinic acid, respectively ($n=20$).

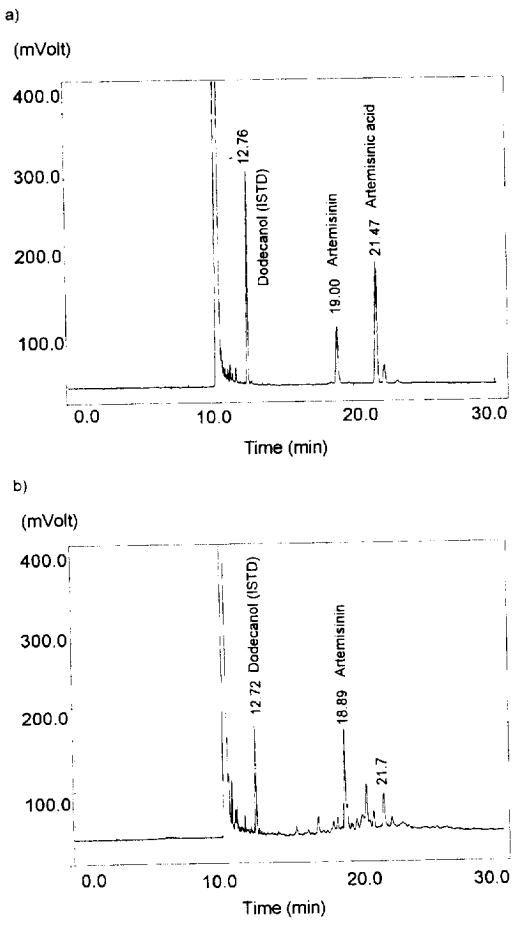

Fig. 4. Capillary SFC–FID chromatograms. (a) Standard solution of artemisinin and artemisinic acid at 1 mg ml^{-1}, (b) SFE extract of 100 mg *A. annua* (Chromatographic conditions: cf. Section 2.4).

The SFE method was compared with two standard liquid solvent extraction procedures. These two methods used toluene as solvent and only differed between them in the mixing process (cf. material and methods). In all three cases, analysis of the extracts was carried out by SFC–FID giving similar chromatograms. Repeatabilities of both liquid solvent processes ($n=6$) were determined on the same batch of *A. annua* used for determining the SFE repeatability, and showed similar R.S.D.s (Table 1).

Table 1 reports the averaged determinations of duplicate SFE and liquid solvent extractions. These results show that artemisinin and artemisinic acid can be quantitatively extracted from *A. annua* by a supercritical fluid extraction and that comparable concentrations of both compounds were obtained by SFE and classical extraction procedures. However, the supercritical extracts obtained were slightly yellow whereas toluene extracts were dark green. As already mentioned in the literature [25], SFE is more selective than standard liquid solvent extractions for this kind of compounds. This better selectivity could explain the higher artemisinin content observed in samples 4 and 5 (Table 1). Furthermore, SFE is less tedious and minimizes the risk of artemisinin degradation. From these results, we can also observe that different artemisinin and artemisinic acid concentrations were found in different batches of *A. annua* (from 0.1 to 1.5% of plant dry weight) as already reported in the literature [8].

4. Conclusion

Artemisinin found in the aerial parts of *A. annua* can be quantitatively extracted in less than 20 min with a supercritical fluid composed of carbon dioxide and 3% (v/v) methanol at pressure and temperature fixed at 15 MPa and 50°C, respectively. These mild operating conditions avoid a degradation of the analyte and allow clean plant extracts to be obtained.

Table 1
Quantitative results of the SFE and liquid solvent extraction methods of six different batches of *A. annua*

Sample	Liquid–solid		Sonication		Supercritical	
	Artemisinin	Artemisinic acid	Artemisinin	Artemisinic acid	Artemisinin	Artemisinic acid
1	0.10	1.61	0.10	1.61	0.13	1.43
2	0.85	0.09	1.02	0.08	0.96	0.07
3	0.27	0.10	0.30	0.12	0.31	0.13
4	0.51	0.59	0.50	0.55	0.69	0.64
5	0.05	0.25	0.05	0.17	0.13	0.17
6	0.78	0.17	0.73	0.14	0.73	0.19
Repeatability (n=6) on sample 6 R.S.D (%)	7.5%	4.0%	9.9%	15.4%	7.1%	10.2%

Extracts were analyzed by capillary SFC–FID; results are expressed in % of dry plant material.

Therefore, the SFC–FID determination of artemisinin and its major precursor artemisinic acid can be conducted directly on the extracts without an additional precleaning step.

From the kinetic curves, it appears that the extraction of artemisinin is not limited by the diffusion of the analyte from the plant into the extraction fluid but rather by the elution process. Actually, artemisinin is not strongly bounded in the plant material and can be easily extracted by SFE. This hypothesis is confirmed by the localization of this compound in the glandular trichomes of *A. annua*.

Finally, the SFE–SFC–FID analytical method offers good precision which allows the determination of artemisinin and artemisinic acid in plant extracts in the expected concentration range and shows comparable results with conventional liquid solvent extraction procedures. Furthermore, SFE gives a better selectivity for compounds of interest, is less tedious and can be easily coupled on-line with the SFC–FID method.

Acknowledgements

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