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LIQUID CHROMATOGRAPHY WITH VOLTAMMETRIC DETECTION FOR QUANTITATION OF PHENOLIC ACIDS

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SUMMARY

High-performance liquid chromatography with voltammetric detection was used to separate and quantitate a mixture of ten phenolic acids. Resolution from both the column and detector was necessary in order to achieve baseline separation of the mixture. The voltammetric detector yielded two-fold better precision than for amperometric detection of the same compounds, due to an inherent discrimination against electrode passivation. In addition, for all of the compounds studied, a significant improvement in precision was obtained when the difference in response at two separate potentials, was used for quantitation.

The best behavior was observed for 3,4-dihydroxycinnamic acid which exhibited a detection limit of 20 pmol. The detection limits for the other compounds were within a ten-fold range of this value.

INTRODUCTION

Within the past decade the popularity of electrochemical detectors has increased rapidly due to their high sensitivity and their increased selectivity within the voltage domain. However, a major limitation exists for quantitative analysis of species that are not chromatographically separable and/or where electroactive impurities are convoluted within the analytical peak. While it is possible to optimize the chromatographic conditions for a given sample, the same set of chromatographic conditions are often unacceptable for a different sample type.

Recently this problem has been addressed by using voltammetric detection where the potential domain was used successfully to resolve an overlapped chromatogram[1]. In this study, we present the results for various phenolic acids and their derivatives to further demonstrate the utility of voltammetric detectors.

Phenolic compounds are widely distributed in plants and animals. Phenolic acids are known for their hormonal activity in plant growth and their stimulatory or inhibitory activity (depending on the concentration) in biological tissue[2]. Various chromatographic procedures have been developed for identification of these compounds, including gas chromatography[3] and high-performance liquid chromato-

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graphy (HPLC)[4,5]. In contrast, quantitative analysis of these compounds has not been developed due to poor chromatographic separation and the absence of a selective detector. In most cases only an estimate of their concentration is reported[3–6].

Most phenolic compounds are electroactive and can be oxidized at a relatively low potential[7,8]. Those compounds with oxidation potentials 100–200 mV apart can be resolved by a voltammetric detector even if they are not separated by the chromatographic column.

EXPERIMENTAL SECTION

HPLC apparatus

Chromatography was carried out with a Model 8800 gradient liquid chromatographic system (DuPont Analytical Instrument Division, Wilmington, DE, U.S.A.). Sample was injected via a Micromeritics Model 725 automatic injector equipped with a 20-μl sample loop (Micromeritics, Norcross, GA, U.S.A.). Separations were achieved on a 250 × 4.6 mm I.D. DuPont Zorbax ODS reversed-phase column (5 μm porous support particles) or a 300 × 4.6 mm I.D. μBondapak C_{18} reversed-phase column (10 μm porous support particles, Waters Assoc.). The column effluent was monitored simultaneously with two detectors in series. The first detector was a Milton Roy variable-wavelength Spectromonitor D with a wavelength range of 190 to 700 nm (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). In addition to monitoring the chromatograms for any non-electroactive components which might be present, this detector provided a means to assess any band broadening caused by the second detector. The second detector was a coulostatic electrochemical detector equipped with a glassy carbon electrode. Technical details of this instrument have been reported elsewhere[9]. With this instrument, it is possible to scan the applied potential at a rate of 3 V/s, while recording up to 15 channels of chromatographic data, each corresponding to a different applied potential. This is analogous to scanning the wavelength and recording spectra with a diode array detector.

A microVAX (Digital Equipment) computer with one megabyte of memory was used to control the measurement process and record the data. The large memory was necessary in order to store the vast quantity of data generated by multiple-channel chromatograms.

Chemicals and reagents

Phenolic compounds were purchased from Aldrich, and from Sigma (4,8-dihydroxyquinoline-2-carboxylic acid and 1,3,5-trihydroxybenzol). All reagents were analytical reagent grade.

The mobile phase was methanol–acetate buffer (10:90, v/v), from which dissolved oxygen was removed by saturation with nitrogen. The acetate buffer was prepared by adjusting a 0.036 *M* ammonium acetate solution to pH 4.0 with 3 *M* acetic acid.

Standard samples were prepared by dissolving the appropriate amount of phenolic compounds in the mobile phase. The grape juice samples were diluted ten-fold prior to injection. Samples were filtered through Sep-Pak C_{18} cartridges (Waters Assoc.) before injection into the chromatograph.

RESULTS AND DISCUSSION

Table I lists the relative peak area as a function of applied potential for a series of phenolic compounds. These data were obtained from single component injections of each analyte. The detector was operated in an identical manner for all samples; a series of 50 mV steps was applied, and the response was recorded at each potential. In this manner, several parallel data channels were obtained for each chromatographic run. For any given compound, the chromatographic peak was integrated over the same time interval for each channel. Each value reported is the average of four identical samples.

The values listed for each compound in Table I represent points along the hydrodynamic voltammogram. Therefore, the table can be used to select the appropriate voltage for quantitation of each compound in addition to showing the relative responses. The table also indicates the degree of voltammetric resolution which can be attained between the various compounds.

The behavior observed in Table I for 4-hydroxybenzoic acid is unusual. The response initially increases rapidly to a plateau, then decreases severely before returning to a significant level, and eventually going off-scale as the solvent begins to oxidize. No satisfactory explanation for this behavior can be given at this time; however, it can not be attributed to instrumental artifact because the compound exhibits similar behavior when subjected to cyclic voltammetry.

Fig. 1 is a three dimensional chromatogram of a mixture of phenolic acids. The electrochemical response (y-axis) was plotted *versus* voltage (z-axis) and time (x-axis). Each horizontal trace represents a single channel separated by 50 mV from the adjacent channels, all of which were collected simultaneously from a single chromatographic injection. The point density in the figure has been reduced four-fold from that of the acquired data, in order to avoid overlap between the lines which run parallel to the z-axis. Although the column resolution is quite good for this complex mixture, compounds b, c, and d, and also f and g, are not completely resolved chromatographically. Voltammetric resolution of a pair of overlapped peaks is possible whenever their oxidation potentials differ by at least 120 mV/n; where n is the number of electrons transferred. For example, peaks c and d in the figure are resolved in the voltage domain, and peaks b and c are partially resolved, while no voltammetric resolution can be achieved for peaks f and g. Peak g actually represents two compounds which are partially voltammetrically resolved as evidenced by the shoulder which appears on the trailing edge of the peak at higher potentials. Compounds i and j are resolved in both the voltage domain and by the column under the current chromatographic conditions.

The peaks for compounds f, g and h in the figure are very broad. Comparison between the UV and voltammetric outputs indicated that the unusual peak shape was due to poor oxidation characteristics, rather than chromatographic behavior. With amperometric detection, the response for these compounds was found to be erratic and decreased rapidly indicating electrode passivation by the oxidation products.

The peak integrity of each component was determined using the peak ratio method[10]. Peak areas for the phenolic compounds were obtained at two different potentials; one corresponding to the mass transfer limited region (maximum re-

TABLE I

RELATIVE RESPONSES *VERSUS* VOLTAGE

Responses in percent, relative to 3,4-dihydroxybenzoic acid at 0.9 V.

Compound	*Voltage (V)*									
	0.60	*0.65*	*0.70*	*0.75*	*0.80*	*0.85*	*0.90*	*0.95*	*1.00*	*1.05*
2-Hydroxybenzoic acid	0.00	0.00	0.00	0.00	0.69	2.39	6.49	12.74	36.98	44.60
4-Hydroxybenzoic acid	3.86	15.47	53.38	23.59	0.84	7.57	5.64	9.67	42.55	–
4-Hydroxy-3-methoxybenzoic acid	0.00	0.00	0.00	0.00	0.70	8.51	10.87	11.60	10.05	35.57
2,3-Dihydroxybenzoic acid	34.04	34.16	34.23	69.70	77.75	80.84	87.07	81.62	81.54	80.31
2,6-Dihydroxybenzoic acid	0.00	0.00	0.00	0.00	0.00	0.00	1.35	2.20	3.98	14.93
3,5-Dihydroxybenzoic acid	0.00	0.00	0.00	0.00	0.00	5.92	8.92	7.67	20.54	66.92
3,4-Dihydroxybenzoic acid	33.76	24.37	49.90	65.37	76.59	85.14	100.00	71.18	60.34	49.36
3-Hydroxyphenylacetic acid	0.00	0.00	0.00	0.00	0.00	0.24	0.37	1.92	4.83	16.55
3,4-Dihydroxyphenylacetic acid	6.38	13.69	35.74	49.51	52.80	84.33	81.27	69.09	65.37	63.00
4,8-Dihydroxyquinolin-2-carboxylic acid	0.00	0.00	0.00	0.00	2.52	26.30	23.75	34.62	38.14	33.34
1,3,5-Trihydroxybenzol	0.00	0.00	0.00	0.00	0.00	2.63	5.02	6.96	18.56	40.61
3,4-Dihydroxycinnamic acid	32.00	38.92	62.00	75.00	80.50	85.06	76.20	68.10	65.31	53.20

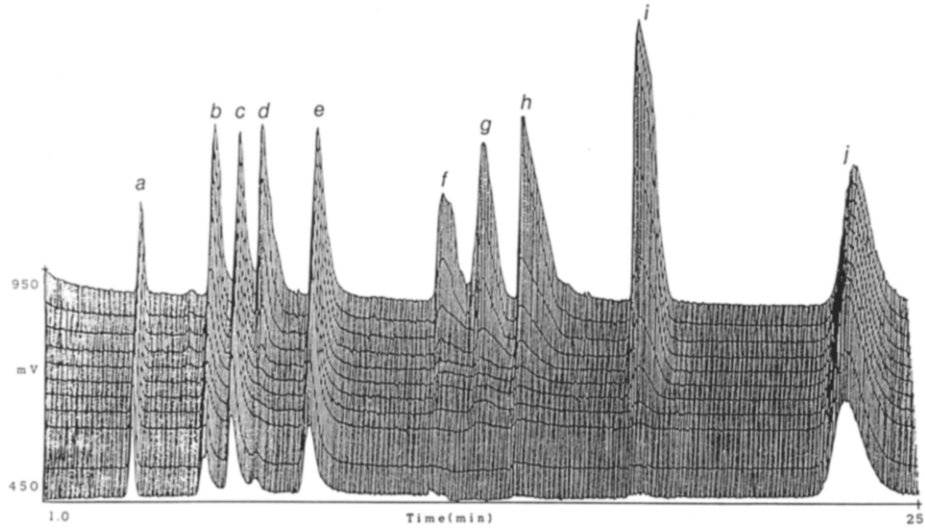

Fig. 1. Three dimensional chromatogram of phenolic acid mixture. Response *versus* voltage and time. a = 6 ng of ascorbic acid, b = 6 ng of trihydroxybenzoic acid, c = 6 ng of 2,3-dihydroxybenzoic acid, d = 6 ng of 3,5-dihydroxybenzoic acid, e = 4 ng of 3,4-dihydroxybenzoic acid, f = 10 ng of 2-hydroxybenzoic acid, g = 8 ng of 4,8-dihydroxyquinoline-2-carboxylic acid and 4 ng of 4-hydroxybenzoic acid, h = 12 ng of 2,6-dihydroxybenzoic acid, i = 15 ng of 4-hydroxy-3-methoxybenzoic acid, and j = 6 ng of 3,4-dihydroxycinnamic acid.

sponse) and the other at the lower Tafel region (slight response). Table II lists the characteristic peak area ratio for each compound. For most of the compounds the relative standard deviation (R.S.D.) of the ratio was less than 5% for five repetitive measurements; however, for 3,4-dihydroxybenzoic acid the coefficient of variation was 7%. In addition to testing for peak integrity, the peak–area ratio can also be used to identify an unknown phenolic acid. The retention time can be used to obtain

TABLE II

PEAK AREA RATIOS AND RETENTION TIMES

Compound	*V1 (V)**	*V2 (V)***	*Peak area ratio ± S.D.*	t_R *(min)*
1,3,5-Trihydroxybenzol	1.00	0.85	7.05 ± 0.31	5.21
2,3-Dihydroxybenzoic acid	0.85	0.60	2.37 ± 0.12	6.70
3,5-Dihydroxybenzoic acid	1.00	0.85	3.47 ± 0.02	7.25
3,4-Dihydroxybenzoic acid	0.85	0.65	3.49 ± 0.25	9.08
2-Hydroxybenzoic acid	1.05	0.85	18.66 ± 0.82	16.50
4-Hydroxybenzoic acid	1.00	0.85	5.38 ± 0.20	16.90
2,6-Dihydroxybenzoic acid	1.05	0.95	6.78 ± 0.32	17.50
3-Hydroxyphenylacetic acid	1.00	0.85	20.13 ± 0.53	18.12
4-Hydroxy-3-methoxybenzoic acid	1.05	0.85	4.18 ± 0.06	19.50
3,4-Dihydroxycinnamic acid	0.80	0.60	2.68 ± 0.07	22.00

* Mass transfer limited region (maximum response).
** Lower Tafel region (slight response).

a list of several possible candidates, and the peak–area ratio can be used to select the most likely possibility, or eliminate all of the candidates selected from the retention time alone. Of the compounds listed in Table II, only 3,4-dihydroxybenzoic acid and 3,5-dihydroxybenzoic acid have similar retention times *and* peak–area ratios which differ by less than their statistical variation.

Fig. 2 is a section of the chromatogram of the phenolic acids shown in Fig. 1. The upper trace corresponds to an applied potential of 900 mV. The large peak in the center represents 13 nmol of 3,5-dihydroxybenzoic acid and the smaller peak 0.47 nmol of 2,3-dihydroxybenzoic acid. The lower trace corresponds to 750 mV, at which 3,5-dihydroxybenzoic acid is not significantly oxidized. Therefore, the 2,3-dihydroxybenzoic acid can be quantitated at 750 mV without significant interference from the 3,5-dihydroxybenzoic acid. If the response at 750 mV is subtracted from that at 900 mV, one obtains the difference chromatogram shown in the center trace. This trace can be used to quantitate the 3,5-dihydroxybenzoic acid without significant interference from the 2,3-disubstituted acid. Although the column resolution for these compounds is about 0.64 (determined by UV absorption), the voltammetric detector provided complete resolution.

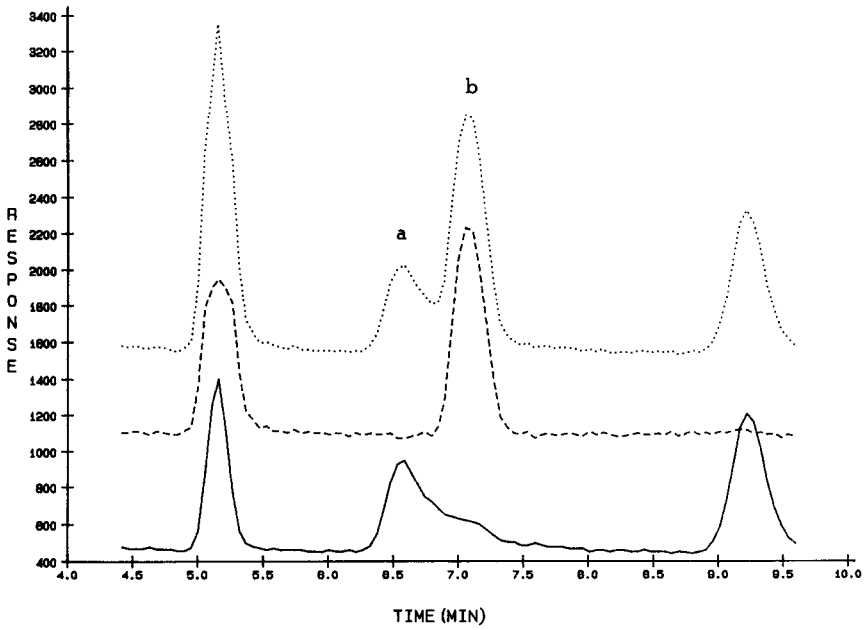

Fig. 2. Chromatograms which illustrate voltage resolution. Response at 900 mV potential (.....), response at 750 mV (———), difference chromatogram (upper trace – lower trace) (---------). a = 0.47 nmol of 2,3-dihydroxybenzoic acid, b = 13 nmol of 3,5-dihydroxybenzoic acid.

In order to test the precision of the detector for quantitative measurements, experiments were performed in which five measurements of 0.8 nmol of each sample, were made under identical conditions. The peak area was measured at two different potentials as in the previous experiment; however, in this case a peak–area-difference

TABLE III

PRECISION FOR VOLTAMMETRIC DIFFERENCE-PEAKS

Area values in percent, relative to 3,4-dihydroxybenzoic acid at 900 mV.

Compound	*V1 (V)*	*V2 (V)*	*Δ Peak area ± S.D.*	*R.S.D. (%)*
1,3,5-Trihydroxybenzol	1.00	0.85	15.93 ± 0.34	2.1
2,3-Dihydroxybenzoic acid	0.85	0.60	46.80 ± 0.13	0.3
3,5-Dihydroxybenzoic acid	1.00	0.85	14.62 ± 0.13	0.9
3,4-Dihydroxybenzoic acid	0.85	0.65	60.77 ± 0.26	0.4
2-Hydroxybenzoic acid	1.05	0.85	42.21 ± 0.62	1.5
4-Hydroxybenzoic acid	1.00	0.85	35.61 ± 0.38	0.1
2,6-Dihydroxybenzoic acid	1.05	0.85	14.93 ± 0.38	2.5
3-Hydroxyphenylacetic acid	1.00	0.85	4.59 ± 0.10	2.2
4-Hydroxy-3-methoxybenzoic acid	1.05	0.85	27.06 ± 0.34	1.3
3,4-Dihydroxycinnamic acid	0.85	0.60	42.42 ± 0.63	1.5

was obtained. The differences were used as a measure of response for each analyte. These results are shown in Table III, and the average peak areas for single potential measurements are shown in Table IV. For any given compound, the entries in both tables were obtained from the same set of data. In all cases the R.S.D. of the single-channel peak areas are considerably larger than those for difference peaks, even though the peak area is decreased in the latter case. This is a remarkable finding, since the precision of the voltammetric measurement is already two-fold better than for amperometric determinations (in our hands) of the same compounds (*e.g.*, for 2-hydroxybenzoic acid the R.S.D. was 3.0% and 5.6% for voltammetric and amperometric methods, respectively). The precise reason for this phenomenon is unknown; however, it is definitely associated with electrode ageing. For voltammetric, and more so amperometric measurements, the electrode response slowly decreases as oxidation products are deposited on the electrode surface. The problem is less severe for voltammetric measurements because the sawtooth waveform tends to either clean

TABLE IV

PRECISION FOR VOLTAMMETRIC SINGLE-PEAK MEASUREMENTS

Area value in percent, relative to 3,4-dihydroxybenzoic acid at 900 mV.

Compound	*Voltage (V)*	*Peak area ± S.D.*	*R.S.D. (%)*
1,3,5-Trihydroxybenzol	1.00	18.16 ± 0.50	2.8
2,3-Dihydroxybenzoic acid	0.85	80.84 ± 2.62	3.2
3,5-Dihydroxybenzoic acid	1.00	20.57 ± 1.13	5.5
3,4-Dihydroxybenzoic acid	0.85	85.14 ± 2.16	2.5
2-Hydroxybenzoic acid	1.05	44.60 ± 1.32	3.0
4-Hydroxybenzoic acid	1.00	43.75 ± 0.63	1.4
2,6-Dihydroxybenzoic acid	1.05	14.93 ± 0.42	2.8
3-Hydroxyphenylacetic acid	1.00	4.83 ± 0.21	4.3
4-Hydroxy-3-methoxybenzoic acid	1.05	35.57 ± 1.30	3.7
3,4-Dihydroxycinnamic acid	0.80	80.50 ± 1.96	2.4

the electrode, or reduce a large fraction of the oxidation products from the previous scan, before they become deposited (or polymerized) on the electrode surface. The voltammetric difference measurements apparently discriminate against the electrode ageing by removing a variable component of the oxidation current. The electrode ageing affects the oxidation current to the same extent for both channels involved in the difference-peak measurement. When the responses at the two different potentials are subtracted, the component of the analytical current affected by the electrode passivation is removed, leaving a second more stable component which is the value reported for the difference-peaks in Table II.

The ageing effect described above can not be due simply to a reduction in the active electrode surface area. If it were, the current decrease would be proportional to the actual current at any potential, and the peak-difference technique would yield no better precision than for single-potential measurements. A possible explanation, which is consistent with all of the observed facts, is that the oxidation current for the phenolic acids is composed of two components. One which is affected by the electrode passivation, and another which is unaffected or affected to a much smaller degree. Furthermore, one must conclude that the oxidation associated with the latter component occurs at a higher potential than for the component of the current affected by passivation, and that the variable component represents oxidation of an intermediate generated by the high-voltage process. In that case, the stable current component would be observed only at the larger of the two potentials involved in the peak-difference measurement, while the variable component would be observed at both potentials. Therefore, when the difference in the two responses was calculated, the variable part of the oxidation current would be removed.

Other researchers[11] have also observed complex voltammetric behavior for phenolic acids. This is particularly true for the monohydroxy phenolic acids which are often irreversible due to competing chemical reactions that consume electrochemical intermediates. A thorough study of the oxidation mechanism will be necessary before a definitive explanation can be given for the improvement in precision observed for difference-peaks.

If two channels are to be used for quantitation, it is necessary for the response to be linear in both channels. Therefore, the response for several phenolic acids was evaluated over a 0.1–1 nanomole range. In all cases examined, the response was linear with a correlation coefficient of at least 0.99. The best behavior was observed for 3,4-dihydroxycinnamic acid which exhibited a slope of 28 800 area units/nmol, an intercept of -1550 area units, and standard error of estimate of 200 area units. This leads to a detection limit of 20 pmol at the 95% confidence level. The detection limits for the other compounds tested were within a ten-fold range of this value.

Fig. 3 is a three dimensional chromatogram representing the first 20 min of elution for a sample of Welch's grape juice which was diluted ten-fold, spiked with 3,4-dihydroxybenzoic acid, and injected directly on the column after an initial pass through a Sep-Pak C_{18} column. Some components eluted up to 90 min after injection; however, several phenolic acids are eluted within the time span of the figure. By inspection along the voltage axis, it can be seen that the large unknown peak which elutes between ascorbic acid and trihydroxybenzoic acid is actually composed of at least three components. Many of the unknown compounds which coelute with trihydroxybenzoic acid are also resolved in the voltage domain.

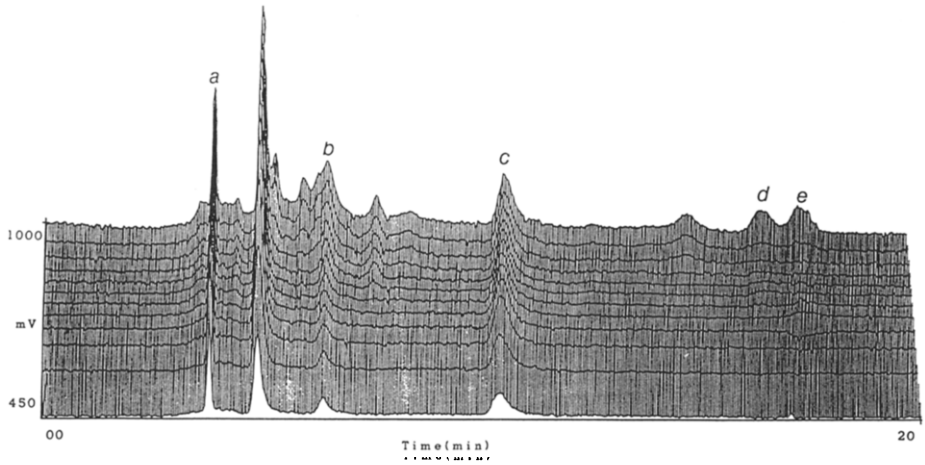

Fig. 3. Three dimensional chromatogram of first 20 min of elution for grape juice. a = Ascorbic acid, b = trihydroxybenzoic acid, c = 3,4-dihydroxybenzoic acid, d = 2-hydroxybenzoic acid, and e = 4-hydroxybenzoic acid.

CONCLUSIONS

The added resolution provided by voltammetric detection allows separation and quantitation of the phenolic acids at the sub-nmol level. Of the compounds investigated, only two (4-hydroxybenzoic acid and 4,8-dihydroxyquinoline-2-carboxylic acid) were not completely resolved by the column–detector pair in complex mixtures.

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