Assessment of the Antioxidant Potential of Scotch Whiskeys by Electron Spin Resonance Spectroscopy: Relationship to Hydroxyl-Containing Aromatic Components

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Electron spin resonance (ESR) spectroscopy has been used to assess the antioxidant capacity of eight Scotch whiskeys by measuring the extent by which the original spirits, or pyridine solutions of their residues, reduced Fremy's radical or galvinoxyl radical. All whiskeys displayed antioxidant activity greater than that of a 0.2 mM solution of Trolox in the Fremy's assay and of a 0.1 mM solution of quercetin in the galvinoxyl assay. The relative antioxidant capacities determined according to the two assays were highly correlated and strongly related to the total phenol content as determined by using the Folin−Ciocalteu method. Activity was a consequence of maturation in oak casks with the "newmake" spirit showing no effect. Of 10 aromatic constituents analyzed, activity was most strongly correlated with ellagic acid and gallic acid in both assays. The reductive capacities of four major phenolics were determined, which, in summation, accounted for 31−53% of the total antioxidant activity of the whiskeys. There was no evidence for synergistic interaction between the phenols investigated.

Keywords: *Whiskey; phenolic; ESR spectroscopy; antioxidant; free radical*

INTRODUCTION

Scotch malt whiskeys contain complex mixtures of hydroxyl-containing, aromatic compounds (Figure 1), which originate from the wooden casks in which the maturation of the "newmake" spirit takes place. The profile of these aromatic constituents is influenced by several factors including the length of maturation, the species of oak from which the casks are made, the pretreatment of the cask by charring of the wood, prior usage of the cask for bourbon or sherry storage, and the number of times which the cask has been used for maturation (Clyne et al., 1993; Mosedale et al., 1995; Piggott et al., 1993; Singleton, 1995; Rous et al., 1983). Studies of naturally occurring phenolics in tea, coffee, and wine indicate that certain classes of compounds, such as catechins, gallates, cinnamic acids, and flavonoids, can exhibit potent antioxidant activity by acting as free radical scavengers through their hydrogen atom donor potential (Gardner et al., 1998; Rice-Evans et al., 1996). Likewise, polyphenols in whiskey, such as ellagic acid and gallic acid, have chemical structures that may confer significant antioxidant function. To assess the antioxidant activity in relation to production methods and the compositional profiles of nine phenolics and the furan derivative, 5-(hydroxymethyl)-2-furaldehyde (HMF), collectively referred to as "hydroxyl-containing aromatics", electron spin resonance (ESR) spectroscopy has been used to measure the radical scavenging abilities of eight whiskeys. ESR is a magnetic resonance technique that can detect and characterize species containing unpaired electrons such as paramagnetic transition metal ions and free radicals (Knowles et al., 1976). Two approaches have been adopted (Gardner et al., 1998): In the first, the water-soluble Fremy's radical (potassium nitrosodisulfonate) was reacted directly with whiskey. In the second, the water-insoluble galvinoxyl radical was reacted with whiskey residues that had been redissolved in pyridine. Trolox, a water-soluble vitamin E analogue, was used as an antioxidant standard for the Fremy's radical, and the flavonoid quercetin was the standard for the galvinoxyl radical experiments. The individual and combined antioxidant potentials of the four major aromatic constituents in the whiskeys were determined, and their contribution to the total antioxidant capacity of the whiskey residues was calculated.

MATERIALS AND METHODS

Whiskeys. Whiskeys, consisting of seven malts and one blend (Table 1), were supplied by the Scotch Whisky Research Institute (Edinburgh, U.K.).

HPLC Analysis. Whiskey aliquots (10 mL) were reduced to dryness using a Genevac centrifugal evaporator. The residues were redissolved by (a) addition of 0.5 mL of methanol/acetic acid solution (99:1) followed by vortex mixing (30 s) and sonication (10 min) and (b) addition of 0.5 mL of H_2O/acetic acid solution (99:1) followed by the same vortex mixing and sonication protocol. The solution was filtered and analyzed on a Hewlett-Packard 1090 LC equipped with a diode array detector and a Hichrom 5 μm ODS column. Gradient elution (Table 2) was performed using two solvents: solvent A [H_2O (89%), methanol (10%), acetic acid (1%)] and solvent B [methanol (90%), H_2O (9%), acetic acid (1%)]. The mobile phase flow was 1 mL min^{-1}. Signal detection was performed at 260, 280, and 320 nm.

Measurement of Total Phenols. The total phenol content of the whiskeys was measured by using the Folin−Ciocalteu

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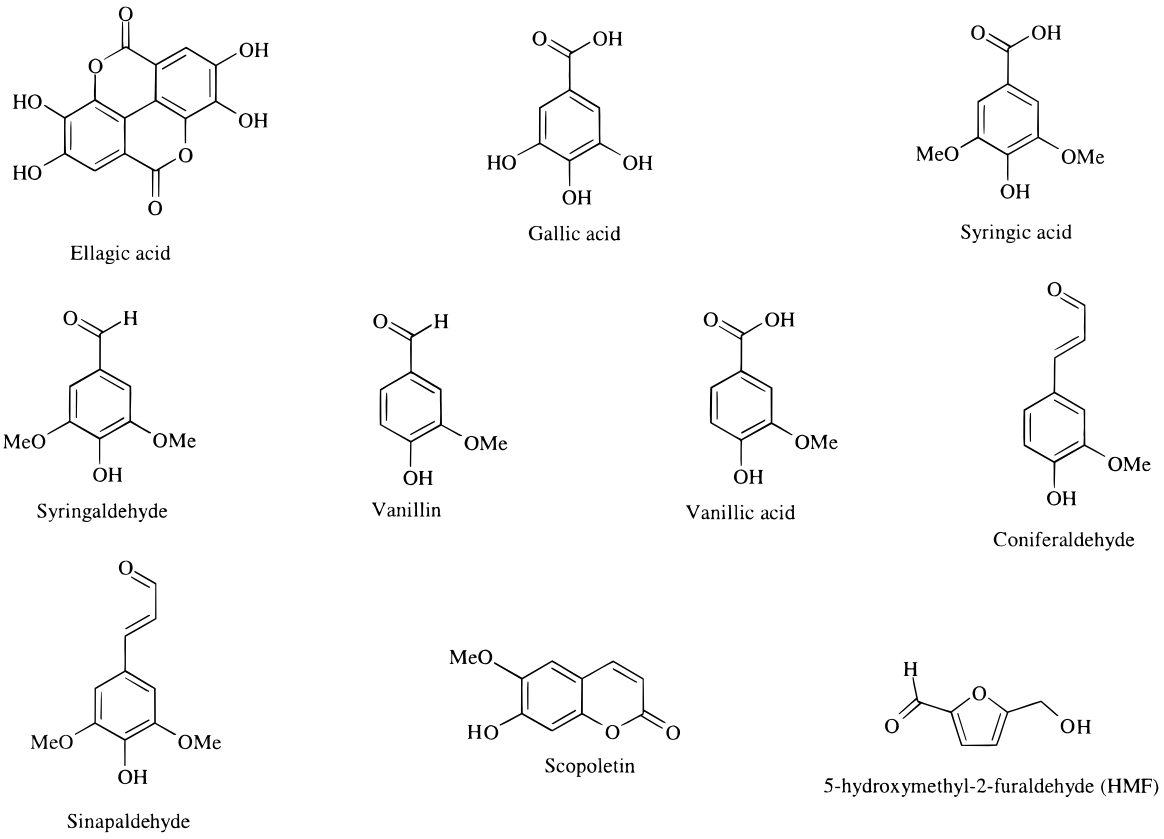

Figure 1. Structures of hydroxyl-containing aromatic compounds commonly found in Scotch whiskeys.

Table 1. Details of Whiskeys Used in This Work

whiskey	age (years)	classification	description
1	12	Island malt	sweet, fruity, some peat character
2	10	Highland malt	sweet, some sherry, little peat character
3	*a*	Speyside malt	balanced sweetness and fruit with some peat
4	12	Island malt	moderate peat character
5	10	Island malt	strong peat character
6	12	deluxe blended	high malt content, sweet, balanced, hint of peat
7	18	Speyside malt	sweet, fruity, some sherry character
8	10	Speyside malt	sweet, fruity, some sherry character

[a] Not specified.

Table 2. HPLC Gradient Elution Protocol Used in Hydroxyl-Containing Aromatic Analysis

time (min)	solvent A (%)	solvent B (%)
0.00	100	0
30.00	60	40
40.00	0	100
45.00	0	100
50.00	100	0

method (Singleton et al., 1965), and the results are expressed as gallic acid equivalents.

Reduction of Fremy's Radical by Whiskeys. An aqueous solution of radical (1 mM, 3 mL) was mixed with an equal volume of whiskey, Trolox solution standard [0.2 mM in aqueous ethanol (40% v/v)], or aqueous ethanol (40% v/v) control. The reaction mixture was transferred to a quartz solution cell and the ESR spectrum (Figure 2a) obtained after 20 min, by which time the reaction had gone to completion. The amount of radical remaining was determined by double integration of the low-field peak of the spectral triplet. The experimental procedure was replicated three times, and the mean and standard deviation were obtained.

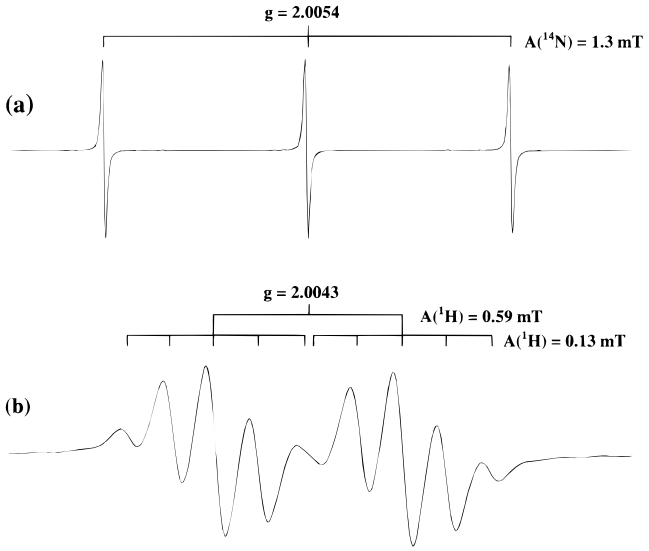

Figure 2. ESR spectra of **(**a**)** Fremy's radical and **(**b**)** galvinoxyl radical with *g* values and hyperfine coupling constants (*A* values).

Reduction of Galvinoxyl Radical by Whiskey Residues. Extracts of whiskey residues were prepared by evaporation of 30 mL of whiskey to dryness, using a Genevac centrifugal evaporator, and then redissolution to the original volume in pyridine. A solution of galvinoxyl in pyridine (0.5 mM, 3 mL) was combined with an equal volume of extract, quercetin standard (0.1 mM in pyridine), or pyridine (control), and the ESR spectrum was obtained (Figure 2b) after 5 min, by which time the reaction had gone to completion. The amount of radical remaining was determined by double integration over the complete spectrum. The experimental procedure for each sample was replicated three times.

Table 3. Compositional Analysis by HPLC of Hydroxyl-Containing Aromatics in Whiskey and Wine Samples (Expressed as Parts per Million)

whis-key	ellagic acid	gallic acid	HMF	syring-aldehyde	vanillin	syringic acid	vanillic acid	conifer-aldehyde	sinap-aldehyde	scopoletin	total
1	13.4 ± 0.5	6.27 ± 0.5	2.79 ± 0.2	2.88 ± 0.1	1.51 ± 0.07	1.44 ± 0.03	0.99 ± 0.08	0.42 ± 0.02	0.55 ± 0.04	0.49 ± 0.10	30.7 ± 0.7
2	15.6 ± 0.5	6.62 ± 0.5	1.22 ± 0.1	3.75 ± 0.1	1.95 ± 0.09	1.79 ± 0.04	1.17 ± 0.09	0.52 ± 0.02	0.47 ± 0.03	0.65 ± 0.14	33.7 ± 0.8
3	9.55 ± 0.3	4.48 ± 0.3	12.6 ± 1.0	2.19 ± 0.1	1.10 ± 0.05	1.02 ± 0.02	0.68 ± 0.05	0.49 ± 0.02	0.83 ± 0.05	0.41 ± 0.08	33.4 ± 1.1
4	10.3 ± 0.4	4.79 ± 0.4	6.42 ± 0.5	1.56 ± 0.1	0.87 ± 0.04	0.91 ± 0.02	0.64 ± 0.05	0.28 ± 0.01	0.25 ± 0.02	0.39 ± 0.08	26.4 ± 0.7
5	10.2 ± 0.4	4.04 ± 0.3	4.40 ± 0.3	4.69 ± 0.2	2.37 ± 0.11	1.79 ± 0.04	1.15 ± 0.09	0.76 ± 0.04	0.87 ± 0.06	0.64 ± 0.13	30.9 ± 0.6
6	9.26 ± 0.3	3.94 ± 0.3	6.98 ± 0.5	1.80 ± 0.1	0.85 ± 0.04	0.95 ± 0.02	0.65 ± 0.05	0.30 ± 0.01	0.34 ± 0.02	0.43 ± 0.09	25.5 ± 0.7
7	28.4 ± 1.0	13.0 ± 1.0	1.25 ± 0.1	6.01 ± 0.2	2.24 ± 0.10	2.22 ± 0.05	1.62 ± 0.12	0.85 ± 0.04	1.09 ± 0.07	0.46 ± 0.10	57.2 ± 1.4
8	36.0 ± 1.2	17.0 ± 1.3	5.43 ± 0.4	5.70 ± 0.2	3.41 ± 0.16	3.08 ± 0.07	2.10 ± 0.16	1.14 ± 0.05	0.16 ± 0.01	0.68 ± 0.14	74.8 ± 1.9
av	16.6	7.53	5.15	3.57	1.79	1.65	1.12	0.59	0.57	0.52	39.1

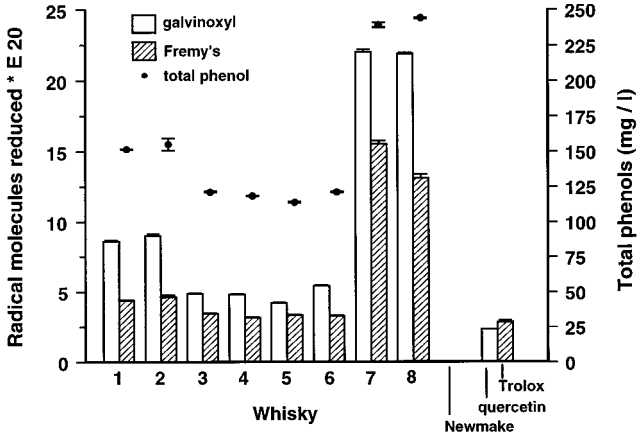

Figure 3. Reduction of Fremy's and galvinoxyl radicals by whiskey and whiskey extracts, respectively. Data are expressed as the number of radical molecules reduced per liter of whiskey or extract. Values for Trolox and quercetin standards are expressed as the number of radicals reduced per liter of a 0.2 and 0.1 mM solution, respectively. Newmake data are for galvinoxyl reduction and expressed in the same units as the whiskeys.

Table 4. Correlation between the Radical Scavenging Potential of the Eight Whiskeys and Their Hydroxyl-Containing Aromatic Composition

phenol	r^a (Fremy's assay)	r^a (galvinoxyl assay)
ellagic acid	0.938***	0.974***
gallic acid	0.932***	0.971***
HMF	−0.396 ns	−0.432 ns
syringaldehyde	0.827*	0.810*
vanillin	0.696 ns	0.733*
syringic acid	0.808*	0.853**
vanillic acid	0.862**	0.899**
coniferaldehyde	0.790*	0.791*
sinapaldehyde	0.198 ns	0.065 ns
scopoletin	0.247 ns	0.328 ns

[a] Levels of probability: *, **, ***, significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. ns, not significant.

Reduction of Galvinoxyl by Major Whiskey Hydroxyl-Containing Aromatics. Stock solutions of ellagic acid (0.05 mM), gallic acid (0.05 mM), syringic acid (0.1 mM), syringaldehyde (0.1 mM), HMF (1 mM), and galvinoxyl (0.5 mM) were prepared in pyridine. An aliquot (3 mL) of the hydroxyl-containing aromatic solution was reacted with an equal volume of galvinoxyl solution. The number of radical molecules reduced was calculated according to the same ESR protocol used with the whiskey residues. From the component concentrations in the individual whiskeys, the number of galvinoxyl molecules which each compound could be attributed to reducing in 1 L of whisky was obtained. To investigate synergistic effects, solutions were prepared containing the five compounds at their respective concentrations in the whiskeys. After reaction with galvinoxyl, the number of radicals that 1 L of the solutions could reduce was calculated and compared with the values obtained by summation of the reducing potential of the individual components. The percentage contributions to the reductive capacities of the eight whiskeys attributable to the five major components was also obtained.

ESR Analysis. Spectra were obtained at room temperature on a Bruker ECS 106 spectrometer, operating at ∼9.5 GHz (X-band) frequency and equipped with a cylindrical (TM_{110} mode) cavity. Instrument settings for the Fremy's radical experiments were microwave power = 2 mW and modulation amplitude = 0.01 mT, and for galvinoxyl radical experiments, setting were microwave power = 1 mW and modulation amplitude = 0.03 mT. Relative radical concentrations were calculated by double integration of the signal.

RESULTS AND DISCUSSION

Hydroxyl-Containing Aromatic Composition. During the production of Scotch whiskey, maturation of the "newmake" spirit in oak casks results in the extraction of lignin-derived, monomeric phenols (Singleton, 1995). HPLC analysis of the eight whiskeys (Table 3) showed that the content of phenolics and HMF ranged from 25 to 75 ppm (average = 39.1 ppm, standard deviation = 17.5). The variation in the component profile of the whiskeys is a consequence of the different methods of maturation adopted by the distilleries. The four most abundant compounds are ellagic acid, gallic acid, HMF, and syringaldehyde, which account for 29−50, 13−23, 2−38, and 6−15%, respectively, of the total hydroxyl-containing aromatic constituents analyzed in the individual whiskeys.

Free Radical Reducing Potential. The antioxidant capacities of the whole whiskeys were assessed by their abilities to reduce the water-soluble Fremy's radical. Direct reaction of the whiskeys with a solution of Fremy's radical indicated that 3.1×10^{20}−15.5×10^{20} molecules of radical could be reduced by 1 L of whiskey (Figure 3). The antioxidant potential of the whiskeys was therefore greater than that of a 0.2 mM solution of the vitamin E analogue, Trolox, which reduced 2.84×10^{20} molecules of radical/L. Reduction of galvinoxyl radical by whiskey residues, which had been redissolved in pyridine, again demonstrates significant activity (Figure 2), with 4.20×10^{20}−22.0×10^{20} molecules being reduced by the residue obtained from 1 L of whiskey. This compares with 2.33×10^{20} molecules of radical reduced by 1 L of a 0.1 mM solution of the flavonoid quercetin. In relation to tea extracts (Gardner et al., 1998), 1 L of strong black tea will reduce 81×10^{20} molecules of galvinoxyl, assuming that a mug (220 mL) of tea contains 2.2 g of dry extract (Balentine, 1992). No reduction of galvinoxyl was observed with the "newmake" spirit (Figure 2), which demonstrates that the antioxidant capacity of the whiskeys is a consequence of maturation of the spirit in oak casks. Regression analysis showed a significant correlation ($r = 0.983$)

Table 5. Calculation of the Number of Molecules of Radical Reduced by Whiskey Residues and Hydroxyl-Containing Aromatic Solutions in Pyridine

whiskey	total no. of radicals predicted to be reduced by the four major phenols[a] ($\times 10^{20}$)	actual no. of radicals reduced by a mixed phenol solution[b] ($\times 10^{20}$)	% predicted/actual[c]	no. of radicals reduced by whiskey residues ($\times 10^{20}$)	% reduction by whiskey residue accountable in terms of mixed phenol solution
1	2.95 ± 0.13	3.41 ± 0.03	87 ± 3.8	8.63 ± 0.07	40 ± 1.8
2	3.29 ± 0.14	3.74 ± 0.05	88 ± 3.9	9.03 ± 0.13	41 ± 1.9
3	2.11 ± 0.09	2.28 ± 0.03	92 ± 4.2	4.90 ± 0.05	47 ± 2.2
4	2.25 ± 0.10	2.30 ± 0.02	98 ± 4.4	4.83 ± 0.01	48 ± 2.1
5	2.11 ± 0.09	2.22 ± 0.01	95 ± 3.9	4.20 ± 0.03	53 ± 2.2
6	1.95 ± 0.08	1.92 ± 0.01	101 ± 4.3	5.44 ± 0.02	35 ± 1.5
7	6.17 ± 0.27	6.91 ± 0.05	89 ± 3.9	22.0 ± 0.18	31 ± 1.4
8	7.95 ± 0.35	8.18 ± 0.07	97 ± 4.3	21.9 ± 0.07	37 ± 1.7

[a] The number of molecules reduced for each phenol was calculated from the reductive capacity of the phenol and the concentration of the phenol in the whiskey. The data represent the sum of the contributions from the individual phenols. [b] The number of molecules of radical reduced by a solution containing all four major phenols at the concentration with which they occur in the whiskeys. [c] The percent by which the sum of the reduction achieved by the individual phenols relates to the reduction achieved by a combined solution.

between the relative antioxidant activities of the whiskeys determined according to the two assays, despite the different chemical natures of the oxidizing radical species involved and the solvent systems used. The total phenol content, expressed as gallic acid equivalents, ranged from 114 to 211 mg L^{-1}. This content was strongly correlated to the antioxidant capacity as determined by the Fremy's assay ($r = 0.971$, $p < 0.0001$) and the galvinoxyl assay ($r = 0.998$, $p < 0.0001$).

Relationship between Antioxidant Activity and Hydroxyl-Containing Aromatic Profile. The Fremy's radical experiment showed a significant correlation between antioxidant activity and the presence of six phenolics (Table 4). The correlation was greatest for ellagic and gallic acids ($p < 0.001$), whereas no relationship was observed with HMF, the major component in whiskey 3. In the galvinoxyl assay, seven constituents correlated with the observed activity (Table 4) with ellagic and gallic acid, as in the Fremy's assay, being most significant ($p < 0.0001$). Again, HMF content was not related to the activity. Although these data establish a relationship between the concentration of particular compounds and the relative antioxidant capacities of the whiskeys, they do not, in themselves, provide definitive evidence of the ability of particular compounds to act as radical scavengers. The possibility that a nonreactive compound is derived, either from the same polymeric precursor in the oak cask as a reactive component or directly from a reactive component, could result, fortuitously, in an association between the relative antioxidant capacities and compositions of the whiskeys. To resolve this problem, the antioxidant capacity of the major constituents has been established. Using galvinoxyl as the oxidizing species, the number of molecules of radical reduced by one molecule of the compound was determined. Of the major constituents, ellagic acid and gallic acid had the highest activities with reductive capacities (stoichiometries) of 6.2 and 6.4 molecules of radical quenched per molecule of phenol, respectively. The high antioxidant activity of ellagic acid is of particular interest due to its reported bioavailability (Mandal et al., 1990). Syringaldehyde displayed little radical scavenging ability with a capacity of 0.07, whereas syringic acid had an approximately equimolar capacity of 1.1. HMF displayed no radical scavenging activity. This may be explained by the inability of any putative radical center formed on the hydroxyl oxygen to interact with the conjugated π-electron system of the furan ring, thereby making hydrogen abstraction energetically less favorable. From a knowledge of the concentration of the constituents in each whiskey and their individual reductive capacities, the number of radical molecules that each of the four major components can be attributed to quenching has been calculated and the combined total obtained (Table 5). These values have been compared with those obtained by reaction of the radical with pyridine solutions containing all four phenols simultaneously at the concentrations in which they occur in the whiskeys (Table 5). The antioxidant capacity, calculated by summation of the component parts, ranges from 87 to 101% of the values obtained from the combined phenol solutions. This indicates that no significant synergistic or antagonistic effects on the antioxidant capacity occur when the phenolics are oxidized together. Comparison of the number of molecules of radical reduced by pyridine solutions of the whiskey residues and combined solutions of ellagic acid, gallic acid, syringic acid, and syringaldehyde in the proportions found in the whiskeys (Table 5) indicates that these four phenols account for 31−53% of the total antioxidant capacity of the whiskey residues. This suggests that unidentified compounds, such as ellagitannins, flavonoids from the sherry casks, or oligomer breakdown products of lignin, also contribute to the total antioxidant activity of the whiskeys.

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