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AN OXIDIZED TARTARIC ACID RESIDUE AS A NEW BRIDGE POTENTIALLY COMPETING WITH ACETALDEHYDE IN FLAVAN-3-OL CONDENSATION

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Key Word Index—Oxidation; iron; tartaric acid; tannin; catechin; acetaldehyde; wine-making.

Abstract—Iron-induced oxidation of (+)-catechin in a wine-like solution yielded typical products, including colourless and yellow compounds. Structural characterization of the major colourless one indicated that it is a dimer, in which flavanol units are linked through a carboxy-methine bridge. Unlike what might be expected, the structure showed that oxidation does not affect catechin directly, but tartaric acid, to give glyoxylic acid, which then reacts with two flavanol units. From a mechanistic point of view, the reaction resembles, once glyoxylic acid is formed, that of acetaldehyde-induced condensation well documented in enology. Thus, an ion series (up to trimer) was detected by mass spectrometry in the model solution. On the other hand, yellow products formed from colourless ones, especially from that isolated, suggests that a second pathway contributing to browning competes with polycondensation reactions. © 1997 Elsevier Science Ltd

INTRODUCTION

Browning causes major damage to food quality, mostly affecting fruit-based products and beverages in the course of their processing. It is an oxidative process involving, on one hand, fruit polyphenols and, on the other, molecular oxygen. The direct reaction between the two components is not easy, depending largely on physical and chemical conditions of the medium, and usually requires co-factors. One of the fastest processes leading to browning is under polyphenol oxidase (PPO) [1] control. By converting dihydroxyphenols to highly reactive quinones [2, 3], this catecholase activity is the starting point of non-enzymatic chain reactions, leading to brown pigments as end-products. Generally, enzymatic reactions occur during technological operations, like bruising, peeling or crushing. Copper and iron are the co-factors of oxygenases [4, 5] but, in the same way, such metallic ions can catalyse autoxidation by complexing molecular oxygen, thus yielding activated oxygen forms able to react with polyphenols [6].

Both type of oxidations (enzymatic and catalytic) take place in wine processing. The first one is very specific of the early stage of wine-making and occurs in grape musts [7], whereas the other, autoxidation, prevails in the later stages of wine-making and, further, in ageing of wines [8]. In addition, the polyphenols involved in enzymatic oxidation are restricted to grape PPO substrates (the major being caffeoyltartaric acid), whereas all polyphenols may be susceptible to autoxidation, depending on their own oxidation–reduction potential against that of oxygen. Nevertheless, oxidative-browning was shown to be especially related to flavanol content [9, 10].

Numerous studies deal with (+)-catechin oxidation, a flavanol which is widely distributed in fruits. Enzymatic reactions [11–14], as well as chemical reactions [15] and autoxidation [16], have been studied but few data are reported on the structural elucidation of the oxidation products thus obtained. However, dimers resulting from (+)-catechin oxidation by grape PPO were recently characterised [17]. The colourless products proved to be dehydrodicatechins of the B-type having a C-C or C-O interflavanic linkage, previously identified by Young and co-workers [15], whereas one of the yellow compounds corresponded with dehydrodicatechin A, also produced by peroxidase-catalysed oxidation [12]. Another product had a novel quinone methide structure.

In our previous paper [18], autoxidation of (+)-catechin was carried out in wine-like model solutions and the products compared with those yielded enzymatically. Evidence was found that specific products were formed by an iron-catalysed process. The present

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work concerns the characterization of the major one, which has been achieved by means of mass spectrometry and two-dimensional NMR spectroscopy.

RESULTS AND DISCUSSION

Autoxidation of (+)-catechin was investigated in wine-like solution containing the highest iron concentration (20 mg l^{-1}) tested in our previous work [18]. The solution was maintained at 39° (instead of room temperature) in order to accelerate the autoxidation process. After two days, the HPLC trace resembled that obtained at room temperature after one month. Thus, both coloured and colourless products were detected in the heated solution as observed in our first experiments. Also, the formation of yellow pigments appeared to be related to the disappearance of some of the colourless compounds. Hence, we focused our attention on the major product which behaved like a precursor. After isolation by HPLC, it was analysed by electrospray mass spectrometry, in the negative ion mode. The main peak was located at *m/z* 635 (i.e. 636 M_r), along with minor ones (corresponding to yellow products), all detected at *m/z* 617. Since the mass difference between the major peak and a (+)-catechin dimer exceeds 58 units, the product was assumed to be a dimer with an additional residue. In order to identify the latter, experimental conditions were changed one by one, except for ferrous ions, as they were known to catalyse its formation. Thus, ethanol was replaced by two co-solvents (methanol or acetonitrile). In other solutions, various organic acids, such as malic, acetic or formic acids were used instead of potassium hydrogen tartrate. After 24 h, the solutions in which ethanol had been replaced gave the product expected, whereas the other one gave HPLC traces resembling those obtained without iron or by enzymatic oxidation [18]. Consequently, evidence was provided that formation of the isolated intermediate required both ferrous ions and tartaric acid. Hence, tartaric acid oxidation was suspected to occur under the conditions used, yielding oxidized fragments which further react with (+)-catechin. Our assumption was supported by the work of Genevois and Ribéreau-Gayon [19], in which they established the formation of ferritartrate (Fe^{3+}) when tartaric acid is in large excess against ferrous ions (100 to 1 molar ratio). According to their mechanistic hypothesis, ferric ions induce a series of oxidation reactions, giving intermediate species, such as dioxymaleic and dioxytartaric acids. Depending on the conditions, these acids can in turn decarboxylate, yielding glycolic and glyoxylic aldehydes, respectively. On the other hand, the high reactivity of aldehydes towards flavonoids is well known [20]. In the field of enology, the coupling reaction between anthocyanin and tannin mediated by acetaldehyde had been the subject of numerous studies [21–24]. Recent results obtained in our laboratory [25] demonstrated the mechanism of acetaldehyde-induced (+)-catechin polycondensation, by LC-MS monitoring of the reaction. Thus, (+)-catechin was shown to react with the protonated form of acetaldehyde to yield a novel intermediate ethanol adduct. By losing a water molecule, the latter suffered a second nucleophilic attack from another catechin unit, yielding an ethyl-linked dimer which was detected at *m/z*, along with larger oligomers. Other results on wine oxidation [26] supported the view that iron catalyses acetaldehyde combination with phenolic compounds. Thus, considering that a similar mechanism could occur in our solution and given our mass result, glyoxylic acid was postulated to be the aldehyde involved in catechin condensation. Though it was not mentioned among tartaric acid oxidation products, this hypothesis appeared quite plausible since glyoxylic acid has an additional degree of oxidation compared with glyoxylic aldehyde. To check this hypothesis, glyoxylic aldehyde and glyoxylic acid were individually put in reaction with (+)-catechin and the kinetics followed by HPLC separation and UV-visible detection. Traces of both solutions showed a product having the same R_t as that expected. However, no other specific compound was produced in the reaction carried out with the aldehyde, whereas both colourless and yellow products characteristic of the iron-catalysed reaction were detected in the solution containing the acid reagent. LC-mass spectrometric monitoring of the solutions confirmed the above results. When the reaction was conducted with the aldehyde, the mass expected (*m/z* 635) appeared only in trace amounts together with an ion *m/z* 619, corresponding with two (+)-catechin units linked by an aldehyde-methine bridge. When the reaction was conducted with glyoxylic acid, the product with the expected mass was found, along with that of yellow compounds. From mechanistic considerations, the reaction with glyoxylic acid was investigated in the same way as done with acetaldehyde and (+)-catechin [25]. The kinetics monitored by LC-mass spectrometry after 2 h of reaction allowed the detection of both oligomers and intermediate ions, as presented in the mass spectrum (Fig. 1). This demonstrated the polycondensation mechanism of glyoxylic acid with (+)-catechin, analogous to that of acetaldehyde. The ion series detected at *m/z* 363.2, 635.2, 709.2 and 981.2 could be attributed to the same intermediate types, namely the glyoxylic adduct of catechin, dimer, glyoxylic adduct of dimer and trimer, respectively. In addition to each *m/z* value there were several ions peaks, suggesting that they could be regio- and/or stereo isomers, since on one hand, the C-6 and C-8 carbons are both reactive sites on each flavanol unit and, on the other the carbon involved in the interflavanic junction may have the *R*- or *S*-configuration. Yellow compounds appeared as minor products under the conditions used above, whereas they were more abundant in less acidic and concentrated solutions such as the wine-like solution. Their detection at *m/z* 617, i.e. 18 mu less than that of

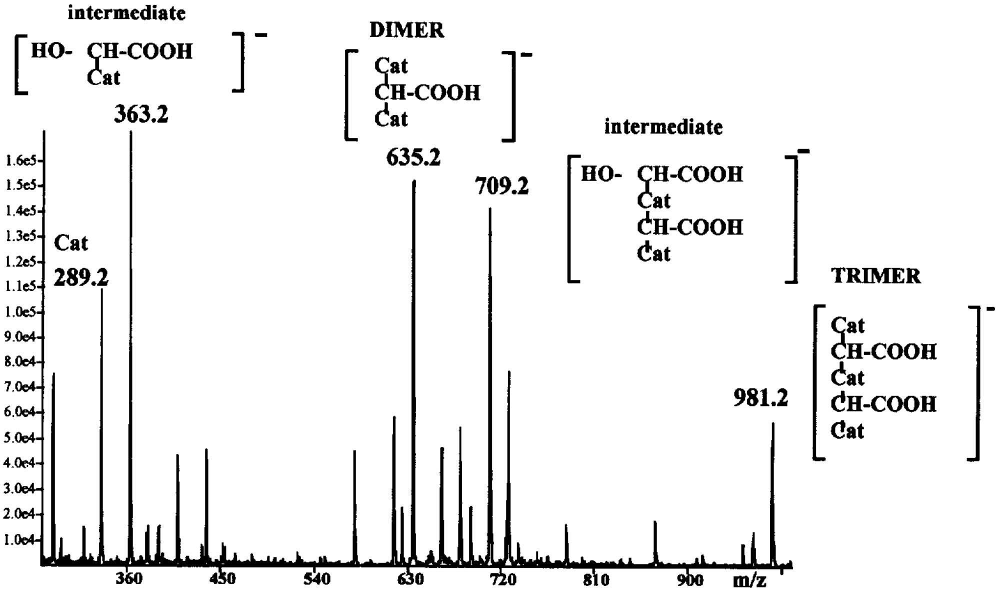

Fig. 1. LC-MS analysis of (+)-catechin/glyoxylic aldehyde solution after 2 h reaction. The mass spectrum corresponds with the entire TIC trace.

dimer products, suggested that they had lost a water molecule.

For full structural elucidation, the major colourless dimer was synthesized from glyoxylic acid and (+)-catechin. This reaction was prefered to that of tartaric acid, ferrous ions and (+)-catechin, as it is direct and gives a higher yield. ID (^{1}H, ^{13}C) and 2D (homo- and hetero-correlations) NMR experiments allowed us to assign protons and carbons (Table 1), corresponding with the structure shown on Fig. 2. Thus, the presence of two distinct proton systems confirmed the dimeric structure with an additional singlet at δ 5.67, attributed to the methine proton linking both catechin units. Then, the linkage position on each flavanol moiety was deduced unequivocally from HMBC experiments. Correlations from C-5 both to H-4 and to the proton of the hydroxyl group on C-5 indicated C-8 as the junction atom in each catechin unit. Given the apparent symmetry of the corresponding dimer, one would expect the non symmetrical regio-isomer, linked on one side to C-6 and the other to C-8. Nevertheless, the structure (Fig. 2) has no element of symmetry, neither plane (σ), nor axes (C_n and S_n), nor centre (i). Thus, all protons are non-interchangeable through any symmetry interactions (heterotopic protons) and therefore they are not chemical shift equivalent; the two flavanol systems are distinguishable. In addition, a more detailed examination of proton signals indicated a clear difference in coupling constants between the two heterocycle spin systems, suggesting that their confirmation equilibria could be different. NMR analysis performed in DMSO-d_6 at 30 and 70° compared with D_2O, allowed us to observe changes in both heterocycle proton shifts and coupling constants of each catechin moiety, indicating conformation modifications. These differences of benzopyrane conformation can result from intramolecular hydrogen binding between the carbonyl and a phenolic hydroxyl.

Thus, the NMR data proved that the dicatechin-carboxy- methine isolated was the C-8/C-8 regio-isomer, in which each flavanol moiety probably is in a different conformational equilibrium. Moreover, assuming that the stereochemistry of (+)-catechin is maintained during the reaction process, the dimer isolated was enantiomerically pure, because the methine carbon is achiral.

In conclusion, structural elucidation of this dimer demonstrated a polycondensation mechanism through carboxy-methine bridges, arising themselves from iron-catalysed oxidation of tartaric acid. Although the latter oxidation process is already well documented in enology, glyoxylic acid is a new intermediate, which seems particularly reactive towards flavanols. Considering the relative amounts of tartrate and ferrous ions in grapes and wines, this reaction may be an important route in wine ageing processes, probably competing with acetaldehyde-induced polycondensation. Moreover, yellow products derived from colourless ones seem to be more stable and could contribute to the coloured pigments involved in browning processes.

EXPERIMENTAL

Standards. (+)-Catechin was purchased from Sigma, potassium hydrogen tartrate and ferrous sulphate were supplied by Prolabo (France) and glyoxylic aldehyde and glyoxylic acid by Aldrich.

Autoxidation in wine-like solution. (+)-Catechin was dissolved in aq. potassium hydrogen tartrate soln (20 mM, pH 3.7) containing EtOH (20%) and

Table 1. ^1H and ^{13}C chemical shifts (DMSO-d_6) of dimer synthesised from glyoxylic acid and (+)-catechin

	Proton	δ(500 MHz); *J*(Hz)	Carbon	δ(125 MHz)
C-ring	H-2C	3.98; $J_{2,3} = 8.3$	C-2C	81.2
	H-3C	3.66; *m*	C-3C	66.5
	H-4αC	2.74; $J_{4\alpha,\beta} = 16.1$; $J_{4\alpha,3} = 6.0$	C-4C	29.2
	H-4βC	2.22; $J_{4\beta,4\alpha} = 16.1$; $J_{4\beta,3} = 9.5$		
B-ring			C-1′B	130.7
	H-2′B	6.62; $J_{2',6'} = 1.9$	C-2′B	114.5
			C-3′B	144.6
			C-4′B	144.7
	H-5′B	6.61; $J_{5',6'} = 8.3$	C-5′B	114.7
	H-6′B	6.38; $J_{6',5'} = 8.3$; $J_{6',2'} = 1.9$	C-6′B	118.7
A-ring			C-4aA	99.5
			C-8aA	153.0
	H(OH-5A)	8.96; *s*	C-5A	154.0
	J-6A	5.85; *s*	C-6A	95.5
			C-7A	154.s
			C-8A	104.6
Bridge	H(C)-	5.67; *s*	C(H)	35.0
			C(OOH)	174.5
D-ring			C-4aD	99.2
			C-8aD	152.4
	H(OH-5D)	9.02 *s*	C-5D	154.2
	H-6D	5.93; *s*	C-6D	94.9
			C-7D	152.6
			C-8D	104.5
F-ring	H-2F	4.56; $J_{2,3} = 5.9$	C-2F	80.4
	H-3F	3.74; *m*	C-3F	66.0
	H-4αF	2.36; $J_{4\alpha,\beta} = 16.1$; $J_{4\alpha,3} = 6.1$	C-4F	26.3
	H-4βF	2.34; $J_{4\beta,4\alpha} = 16.1$; $J_{4\beta,3} = 5.0$		
E-ring			C-1′E	130.4
	H-2′E	6.58; $J_{2',6'} = 1.9$	C-2′E	113.7
			C-3′E	144.4
			C-4′E	144.3
	H-5′E	6.40; $J_{5',6'} = 8.3$	C-5′E	114.9
	H-6′E	6.13; $J_{6',5'} = 8.3$; $J_{6',2'} = 1.9$	C-6′E	117.0

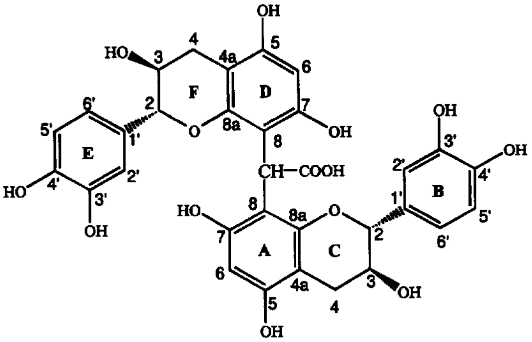

Fig. 2. Structure of the isolated colourless dimer.

$Fe(SO_4)_2$ ions (20 mg l^{-1} Fe). The soln was maintained at 39° and 25 μl aliquots taken each day for HPLC analyses.

Condensation of (+)-catechin with glyoxylic acid and glyoxylic aldehyde. Glyoxylic acid (50% soln) was added to aq. (+)-catechin soln containing EtOH (20%). Final concns were 4 and 22 mM for (+)-catechin and glyoxylic acid, respectively. The soln was maintained at 39° and the reaction kinetics monitored by HPLC after 24 h. The reaction with glyoxylic aldehyde was carried out so as to keep the same final concns mentioned above.

HPLC/DAD analyses. HPLC/DAD analyses were performed using a two-pump system, a manual injector, an automated gradient controller and a diode array detector (DAD). UV-visible spectra were recorded from 250 to 600 nm and peak areas measured at 280 nm. The column was a reverse-phase Lichrospher 100-RP18 (5 μm packing, 250 × 4 mm i.d.) protected with a guard column of the same material (Merck). Catechin oligomers were eluted under the following conditions: 1 ml min^{-1} flow rate; oven temperature

30°; solvent A, H_2O–HC_2H (49:1); solvent B, MeCN–H_2O–HCO_2H (4:9:1); elution with linear gradients from 5 to 30% B in 40 min, from 30 to 50% B in 20 min and from 50 to 80% in 10 min, followed by washing and reconditioning of the column.

LC/MS analyses. Measurements were performed on a quadrupole instrument with a mass range of 2400 mu, equipped with an Ion Spray ion source (Sciex, Canada). The MS was operated in the negative-ion mode. The ion spray voltage was −4000 V, the orifice voltage −60 V. HPLC sepns were carried out on a narrow-bore reverse-phase column (Superspher 100-RP18, 3 μm packing, 125 × 2 mm i.d., Merck). The column was connected to the electrospray source *via* a fused-silica capillary (length 100 cm, 100 μm i.d.). The reaction mixt. was injected with a rotary valve fitted with a 20 μl sample loop. Sepn was achieved using a two-step linear gradient at a flow rate of 200 μl min^{-1}. Elution was done with solvents A and B used in HPLC/DAD analyses and the conditions adapted as follows: linear gradients from 5 to 30% of solvent B in 20 min and from 30 to 50% in 10 min, followed by washing and reconditioning of the column. The *A* at 280 nm was monitored by a programmable *A* detector. The flow was split so that 50 μl min^{-1} went into the electrospray source.

Synthesis of dimer. (+)-Catechin (100 mg) was dissolved in EtOH (1 ml) and glyoxylic acid soln (9 ml of 0.2 M soln). After 2 h reaction at 35°C, the soln (20 μl) was analysed by LC-MS. In the rest of the soln, the reaction was slowed down by increasing the pH to 3.5 with NaOH soln (0.5 ml of 1M soln). The soln was subdivided (10 × 1 ml) kept at −80°, which were thawed prior to prep. sepn. The equipment used for purification was a high-press. prep. system including two gradient pumps, an injection pump and a UV detector set at 280 nm. The sepn was carried out on a reverse-phase column (Lichrospher 100-RP18, 5 μm packing, 125 × 22 mm i.d., Merck). Elution was done under following conditions: 10 ml min^{-1} flow rate; solvent A, H_2O–HOAc (99:1); solvent B, MeOH–solvent A (4:1); elution with linear gradients from 5 to 30% B in 20 min, from 30 to 100% B in 5 min, followed by washing and reconditioning of the column. Pure frs were collected, evapd and freeze-dried.

NMR. Spectra were recorded (^{1}H: 500 MHz ^{13}C: 125 MHz), using a 3 mm probe locked to the deuterium resonance of the solvent, DMSO-d_6. Chemical shifts are reported in δ referenced to the solvent.

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