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Study of the acetaldehyde induced polymerisation of flavan-3-ols by liquid chromatography–ion spray mass spectrometry

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Abstract

In some fruits, the loss of astringency has been attributed to the insolubilization of recombinated tannins, formed by reaction with acetaldehyde. This route has been investigated by on-line coupling of liquid chromatography with ion spray mass spectrometry (LC–ISP-MS). The results reported herein indicate that LC–ISP-MS can be successfully applied to tannin polymers and is very helpful in their identification. It also allows direct monitoring of the progress of polymerisation reactions. The mechanism of the acetaldehyde-induced polymerisation of flavan-3-ols has thus been demonstrated.

Keywords: Flavan-3-ols; Acetaldehyde; Catechin; Epicatechin

1. Introduction

Astringency is a young-wines feature which is usually correlated to tannin content [1–4]. This property tends to vanish in the course of wine ageing [5–8]. Until now, this evolution was suspected to result from an association between tannins and anthocyanins, either by direct condensation [7,9–11] or through acetaldehyde [9,10,12,13] produced by ethanol oxidation. Alternatively, the deastringency of persimmon fruits has been ascribed to a polymerisation reaction, involving epigallocatechin gallate residues and acetaldehyde [14]. In this case, the reaction leads to the formation of oligomers which precipitate given their hydrophobic nature. Similarly, the loss of astringency in aged wines may be due to competitive polymerisations through acetaldehyde, either between flavan-3-ol units or between flavan-3-ol and anthocyanin units. Whereas the latter reaction has been confirmed by mass spectrometry [12,15,16], acetaldehyde mediated catechin condensation has been considered only as a possible secondary pathway with regard to pigment-catechin condensation [17]. Structural hypotheses have been proposed for the products yielded by both reactions.

To investigate the acetaldehyde-induced polymerisation of catechin or epicatechin, the kinetics can be monitored by liquid chromatography on a reversed-phase column, allowing the separation of short oligomers (dimers, trimers). Nevertheless, the structural similarity of the reaction products should lead to similar retention times and possible co-elutions. In addition, as the basic chromophore remains the same in all oligomers, the UV–Vis detection does not

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allow them to be distinguished. Alternatively, mass detection should be a powerful tool in such applications. However, the conventional techniques like FAB-MS requiring the purification of compounds before mass analysis are difficult to apply to this particular case, given the rate of polymerisation and the instability of products thus formed. Consequently, the direct mass analysis after chromatographic elution is expected to be the best way to investigate the reaction. Among the spray techniques which can be interfaced with a liquid chromatographic system, the pneumatically assisted ion spray [18] has been selected because this particularly sensitive and soft ionization method appears to be the most suitable way to study thermolabile and polar compounds such as tannin derivatives.

2. Experimental

2.1. Chemicals

Deionised water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA) prior to use. Acetonitrile was of 'far UV grade' quality (BDH, Poole, UK). Formic acid of 'RP grade analytical reagent' quality (Prolabo, Fontenay S/Bois, France) was used as the mobile-phase additive. (+)-catechin and (−)-epicatechin were purchased from Sigma (St. Louis, MO, USA). Acetaldehyde was obtained from Merck (Darmstadt, Germany).

2.2. Reaction

An acidic solution was prepared with 17 μl of acetic acid and 50 μl of ethanol in 373 μl of water, yielding a pH value equal to 2.2. Six mg of flavan-3-ol (either catechin or epicatechin) were dissolved in the aqueous solution. The reaction was started by addition of acetaldehyde (60 μl) and then monitored by liquid chromatography coupled either with a diode-array detector (DAD) or with a mass-spectrometry (MS) detector.

2.3. HPLC–DAD analyses

HPLC–DAD analyses were performed by means of a Waters-Millipore (Millipore Corp., Milford, MA, USA) system including two pumps M510, a U6K manual injector, an automated gradient controller and a 990 diode-array detector. UV–Vis spectra were recorded from 250 to 600 nm and peak areas were measured at 280 nm. The column was a reversed-phase Lichrospher 100-RP18 (5 μm packing, 250×4 mm I.D.) protected with a guard column of the same material (Merck, Darmstadt, Germany). Catechin oligomers were eluted under the following conditions: 1 ml/min flow-rate; oven temperature 30°C; solvent A, water–formic acid (98:2, v/v); solvent B, acetonitrile–water–formic acid (80:18:2, v/v); 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% B in 10 min, followed by washing and reconditioning of the column.

2.4. MS apparatus

Measurements were performed on a Sciex API I Plus simple quadrupole mass spectrometer with mass range of 2400 u, equipped with an ion-spray ion source (Sciex, Thornhill, Ont., Canada). The mass spectrometer was operated in the negative-ion mode. The ion spray voltage was selected at −4 kV and orifice voltage at −60 V. For direct injection, the solution was introduced into the electrospray source at a constant flow-rate of 10 μl/min with a medical syringe infusion pump (Harvard Apparatus, Model 22, Southnatick, USA) in combination with a 100-μl syringe.

2.5. LC/MS analyses

HPLC separations were carried out on a narrow-bore reversed-phase column with an ABI 140B solvent delivery system (Applied Biosystems, Weiterstadt, Germany). The column was connected with the ES interface via a fused-silica capillary (length 100 cm, 100 μm I.D.). The reaction mixture was injected with a rotary valve (Rheodyne Model 8125) fitted with a 20-μl sample loop. The separation was achieved on a Superspher 100-RP18 (3 μm packing, 125×2 mm I.D., Merck, Darmstadt, Germany) column by using a two-step linear gradient at a flow-rate of 200 μl/min. The 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 absorbance at 280 nm was monitored by an ABI 785A programmable absorbance detector. The flow was split so that 50 μl/min went to the electrospray source. Mass data were acquired in two ways. The first one, referred to as the scan mode, consisted of scanning over a given mass range (namely, between m/z 280 and 1300) using a 0.15-u step size. The other one, referred to as the multiple-ion mode, allowed us to make miniscans over a narrow mass range centered about specified values. In this study, centre masses were selected at m/z 315, 635, 945, 1255, 1575 and 1869 and the width to be scanned around them was either 5 or 60 u. The latter width was chosen so as to detect both the molecules with a given polymerisation degree (from the monomer to the hexamer) and the intermediate ethanol adducts leading to the next polymerisation step.

3. Results and discussion

Acetaldehyde-induced polymerisation was carried out separately on the two epimers, (+)-catechin and (−)-epicatechin, using 50 as the aldehyde to flavan-3-ol molar ratio. The reaction was studied with pH values ranging from 2.2 to 4 and monitored by on-line LC–UV photodiode-array detection. The UV traces recorded at 280 nm after 30 min revealed the presence of three majors peaks in addition to that of catechin, whereas four new ones were detected with epicatechin. Besides, polymerisation kinetics slowed down for both epimers when the pH increased, in agreement with the literature data [17,19].

After appropriate conditions for monitoring the reaction were determined, LC–ISP-MS analyses were optimized using the catechin monomer. Despite the acidity of the HPLC solvents, the mass response was better in the negative-ion mode than in the positive-ion mode. Thus, the set of experiments were run in the negative-ion mode, allowing the detection of $[M-H]^-$ ions.

The reaction was carried out at pH 2.2 and initiated a few minutes prior to the first LC–MS analysis, so that polymerisation could be followed over successive injections. Besides, getting an idea of the reaction progress versus time (which amounts to detecting the highest mass present at each stage), a large range of masses (between 280 to 1300) was scanned. The total ion current (TIC) recorded after 45 min indicated the formation of mainly dimers and trimers eluting in order of increasing molecular mass, along with the monomer ion peak (Fig. 1). Their m/z values detected at 605 and 921, respectively, showed that the constitutive units are linked by ethyl bridges, confirming the role of acetaldehyde in the polymerisation mechanism. Moreover, ethanol adducts on monomer, dimer and trimer species were also detected at m/z 333, 649 and 965, as shown on the mass chromatograms presented in Fig. 2. The detection, for the first time, of these intermediates in dimers, trimers and tetramers formation supports the mechanism previously postulated by Timberlake and Bridle [9]. This process, presented in Fig. 3, starts with the protonation of acetaldehyde in the acidic medium. This limiting stage (suggested by the pH effect) yields a carbocation, which then suffers a nucleophilic attack by the flavan unit (C-6 or C-8 of the A ring). The ethanol adduct thus formed gives a new carbocation by loosing a water molecule. The corresponding species is, in turn, attacked by a second flavan unit to yield a dimer, and this process continues.

Since the polymerisation mechanism was demonstrated with (+)-catechin, a comparison was done with (−)-epicatechin. Analogous products were detected but a clear difference could be noted in the

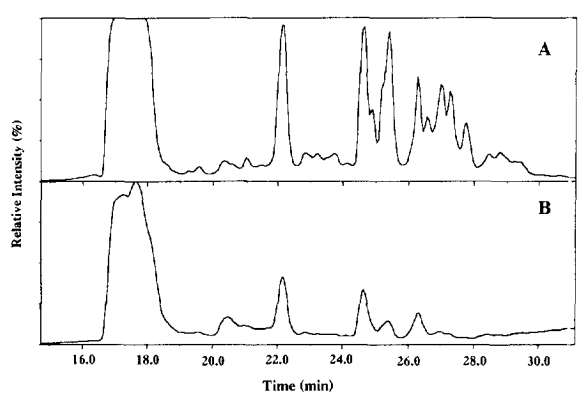

Fig. 1. LC–ISP-MS analysis of the reaction mixture obtained with catechin and acetaldehyde after 45 min. The UV trace at 280 nm (A) and TIC trace (B) are displayed.

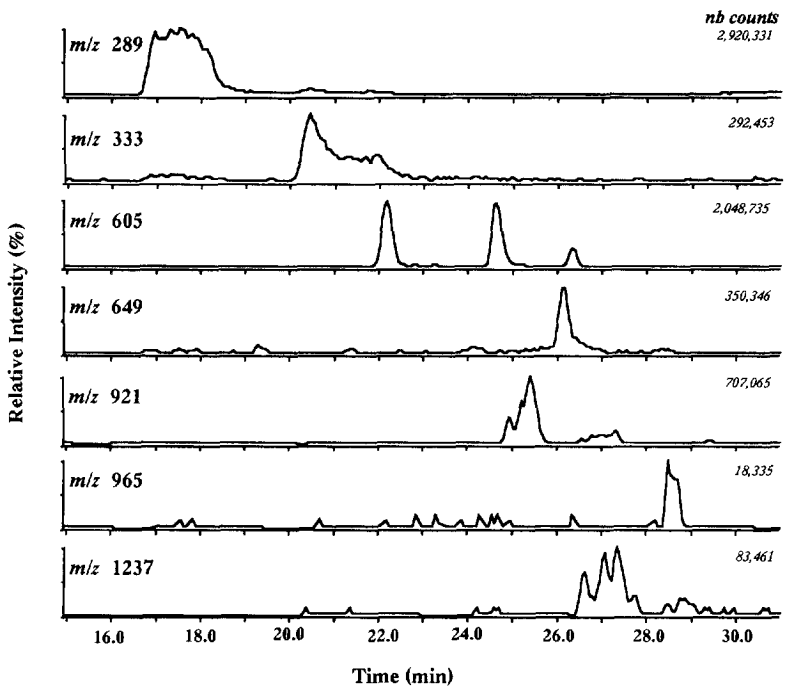

Fig. 2. Mass chromatograms extracted from the TIC trace displayed on Fig. 1. The m/z values correspond, going from the top to the bottom, to catechin, catechin–ethanol adduct, dimer, dimer–ethanol adduct, trimer, trimer–ethanol adduct and tetramer.

larger number of isomers formed from the latter. As observed in UV traces, the three and four peaks obtained from the reaction of catechin and epicatechin, respectively, were also present in the TIC profile, corresponding to the major dimers among the reaction products. This result was confirmed by using another mode of mass data acquisition referred to as the multiple-ion mode, which consists of scanning over narrow mass ranges between designated intervals. In this way, the sensitivity of the detection was increased so that eight dimers and nine (at least) trimers could be numbered for epicatechin on the three-dimensional map of the LC–ISP-MS analysis (Fig. 4). Since only four dimers can be theoretically formed [2 C6-C8 (*R* and *S*), 1 C6-C6, 1 C8-C8] our results suggested that the contamination of epicatechin by catechin (checked by the presence of a peak showing its retention time and m/z value) yielded co-dimers. This hypothesis is also supported by the fact that epicatechin, usually less contaminating given the direction of the epimerisation process, was not found in the three-dimensional map of catechin and the co-dimers scarcely detectable. With regard to the polymerisation kinetics, the UV traces of the reaction hardly changed during the early hours, whereas after 20 h all the peaks had disappeared and were replaced by a single unresolved hump at the end of the chromatogram.

As the separation became ineffective beyond a certain degree of polymerisation (tetra or pentamers), the reaction was infused through a syringe drive in order to improve detection sensitivity. In addition to the concentration increase thus obtained, the gain of the sensitivity noticeable in the spectrum (Fig. 5) may be attributed to the fact that all isomeric forms of one product appeared as a single peak. In this way, the highest value found at m/z 1869 corresponded to the mass of a hexamer and was detected along with the entire series presenting lower polymerisation degrees. Nevertheless, it is likely that at this stage of progress, larger oligomers were formed, but the only way to observe them would be as

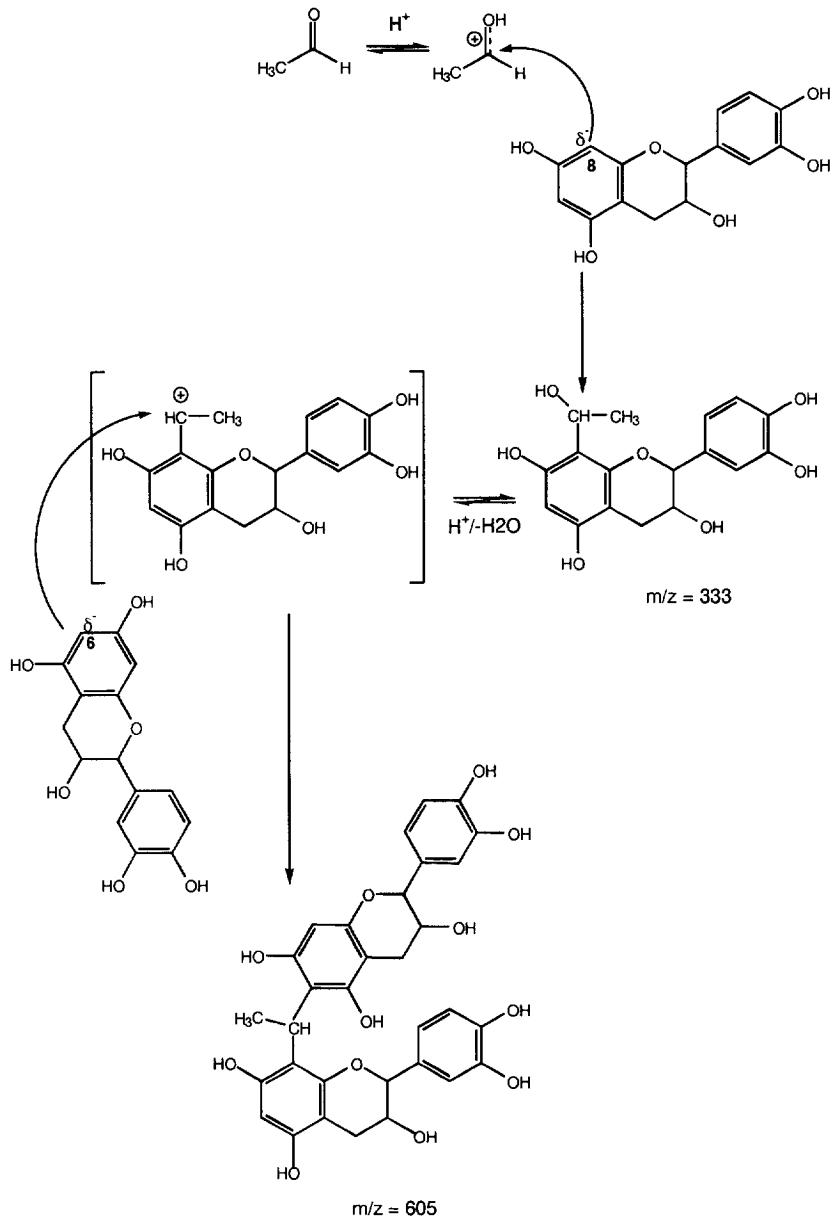

Fig. 3. Mechanism of the acetaldehyde-induced polymerisation of flavan-3-ols.

multi-charged ions, since, given the quadrupole limits (2400 u), the largest detectable mono-charged ion would be a heptamer.

In fact, the doubly charged ion of a pentamer was found at 776 beside the singly-charged ion (1554) (Fig. 6), on the basis of their respective isotope peak spacing (approximately 0.5 u for the doubly-charged and 1.0 u for the mono-charged). Other peaks presumably corresponding to the calculated values of multi-charged ions were also present although spacing between their isotope peaks did not indicate so clearly what they really were.

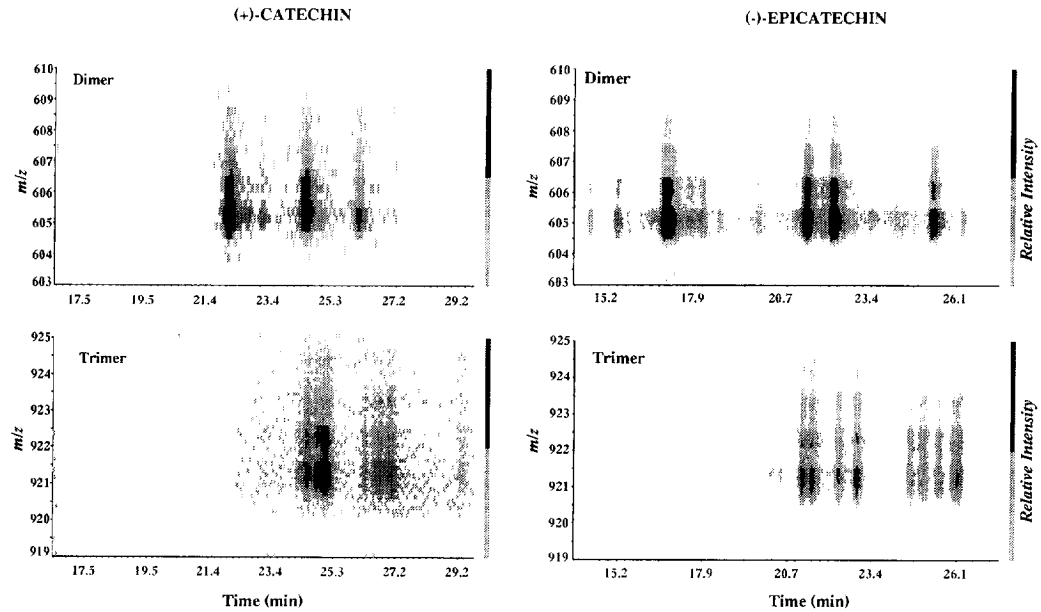

Fig. 4. Comparison of the LC–ISP-MS three-dimensional maps of dimers and trimers yielded by (+)-catechin and (−)-epicatechin polymerisation through acetaldehyde.

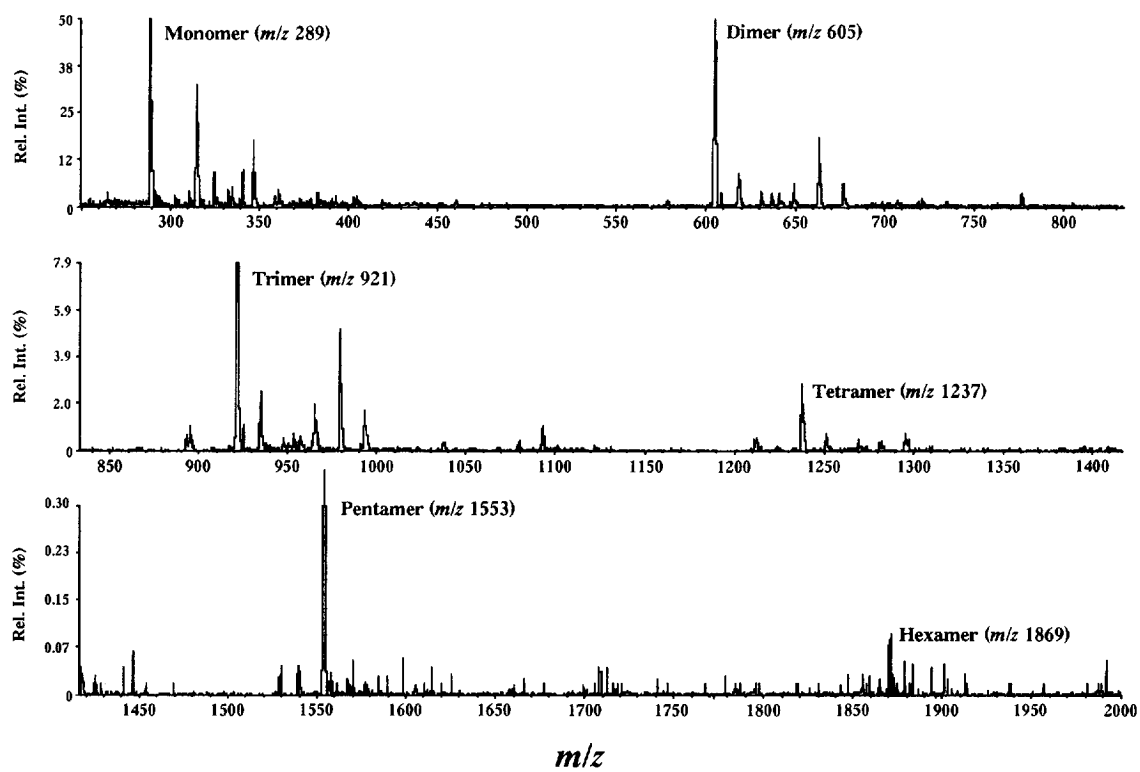

Fig. 5. Mass spectrum of the infused reaction mixture, showing the mono-charged ions from the monomer up to the hexamer.

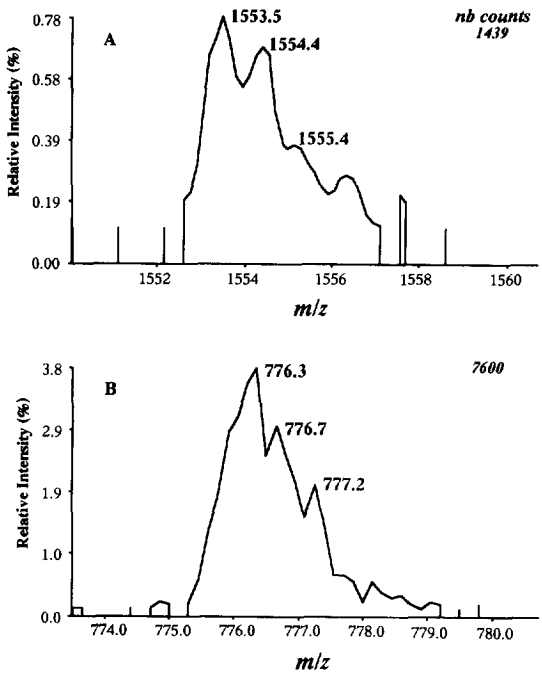

Fig. 6. Comparison of isotope peak spacing between the mono-charged ion (A) and the doubly-charged ion (B) of an epicatechin pentamer.

4. Conclusion

LC–ISP-MS proved to be a powerful tool to investigate a reaction like acetaldehyde-induced flavan-3-ol polymerisation, allowing the detection of oligomers up to the hexamer as mono-charged ions. In addition, the mass difference provided by the presence of an ethyl bridge between consecutive units rendered feasible the detection in the infusion mode, and so, to observe multi-charged ions. For the first time, the mechanism previously postulated by Timberlake and Bridle [9] has been demonstrated with the detection of intermediate species (ethanol adducts on oligomers) so that all other hypotheses have been ruled out.

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