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Short Communication

Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography

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ABSTRACT

The classical method of procyanidin thiolysis was modified so as to render it suitable for the identification of small amounts (< 0.1 mg) of compound. High-performance liquid chromatographic monitoring of the degradation kinetics gives access to the intermediate products. The use of sulphur dioxide instead of acetic acid as the hydrolysing agent allows a faster reaction, thus minimizing structural modifications, and prevents alterations due to procyanidin oxidation.

INTRODUCTION

Proanthocyanidins are dimeric or oligomeric compounds which release anthocyanidins on heating in a strongly acidic medium. Most of them are composed of catechin or gallocatechin units, possibly esterified with gallic acid, linked by C-4–C-8′ bonds. The major methods of identification are fast atom bombardment mass spectrometry (FAB-MS) [1,2] and ^{1}H [3–5] and ^{13}C [6–8] NMR spectrometry.

Nevertheless, degradation by thiolysis [9] is essential to confirm the structure of these compounds [10,11]. This consists in heating proanthocyanidins in the presence of acetic acid and phenylmethanethiol at 100°C for 4–24 h; the "lower" units are released by hydrolysis whereas the "upper" units give benzylthioethers which can be reduced with hydrogen using Raney nickel (RaNi) as a catalyst. By this method, Morimoto *et*

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al. [12] identified a series of homogeneous C-4–C-8 linked oligomers with up to six epicatechin units.

The usual procedures require more than 1 mg of each compound in order to collect the reaction products, generally by thin-layer chromatography (TLC) [7,13] or liquid column chromatography [12], and identify them. Moreover, prolonged heating requires the use of an oxygen-free medium and may result in significant epimerization, even under these moderately acidic conditions [14].

The purpose of this work was to develop a sensitive thiolysis and HPLC method suitable for the identification of procyanidins available in small amounts (<0.1 mg) and minimizing their alteration.

EXPERIMENTAL

Chemicals

Phenylmethanethiol and gallic acid were purchased from Fluka (Buchs, Switzerland). An ethanol suspension of Raney nickel (Merck, Darmstadt, Germany) was obtained by rapidly wiping the commercial preparation on filter-paper prior to introducing it into ethanol (100 mg/ml). Ultrasound treatment was applied before each pipetting.

Sulphurous acid solution (2%, w/w) was obtained by dissolving gaseous sulphur dioxide in water.

Instrumentation

The HPLC apparatus was a Millipore–Waters (Milford, MA, U.S.A.) system including a Model 710B autoinjector, a Model 720 system controller and two Model M510 pumps connected to a Model 3100 variable-wavelength UV detector (Milton Roy) set at 280 nm with 1 a.u.f.s. sensitivity unless specified otherwise. The column was a reversed-phase Spherisorb ODS-2 (5 μm particle size) (Knauer, Berlin, Germany) (250 × 4 mm I.D.) protected with a guard column packed with the same material. The elution conditions were as follows: solvent A, 2.5% acetic acid; solvent B, acetonitrile–solvent A (80:20, v/v); linear gradient from 5 to 50% B in 35 min, followed by washing and re-equilibrating the column. The oven temperature was 30°C.

Kinetics of thiolysis

A series of 20-μl volumes of pure proanthocyanidin solutions (1 mg/ml in ethanol) were introduced into five glass bulbs together with 20 μl of phenylmethanethiol (5% in ethanol) and 2 μl of sulphurous acid (2%). After sealing, reactions were carried out at 60°C for 15, 30, 45, 60 and 90 min.

The kinetics were monitored by HPLC. An elution gradient was necessary because of the magnitude of the polarities of the components between the flavanols and their thioether derivatives.

Desulphurization of the thioethers

The thioethers eluted from the column were collected and evaporated to dryness under vacuum at 30°C in a rotary evaporator. After addition of 50 μl of ethanol, the solution was transferred into a 4-ml vial together with 50 μl of Raney nickel ethanolic suspension and 5 μl of gallic acid (10 mg/ml in ethanol).

Gallic acid, or another polyphenolic compound, is essential to limit irreversible adsorption of the compound of interest on the catalyst. The vial was vented with hydrogen, tightly stoppered and shaken several times for 1 h. The suspension was then filtered through a Millex HV4 filter (Millipore) and injected into the previously described chromatographic system, with the detector set at a higher sensitivity (0.2 a.u.f.s.).

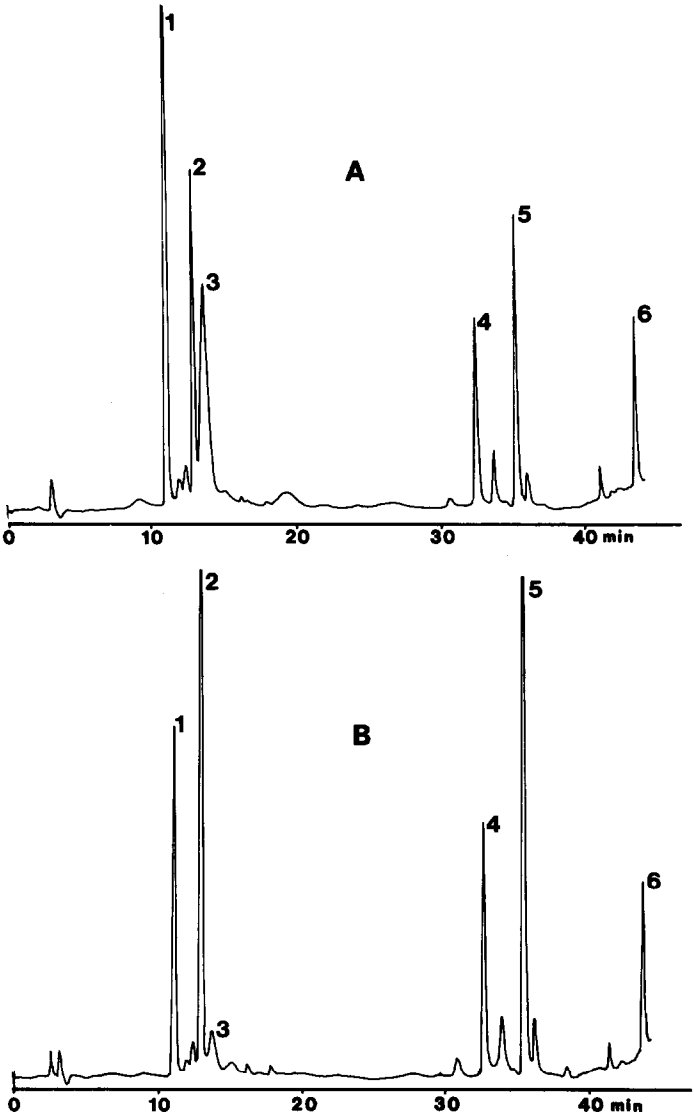

Fig. 1. HPLC of the compounds resulting from partial thiolysis of a procyanidin trimer after heating at 60°C for (A) 15 or (B) 30 min. Peaks: 1 = procyanidin B1; 2 = catechin; 3 = procyanidin trimer, to be identified; 4 = 4′-benzylthioprocyanidin B2; 5 = 4-benzylthioepicatechin; 6 = phenylmethanethiol.

RESULTS AND DISCUSSION

Fig. 1 shows two steps in the thiolysis of an unknown procyanidin originating from grape seeds which behaved like a trimer, according to TLC and FAB-MS analysis. The reaction of this compound with phenylmethanethiol in presence of sulphurous acid gave four products.

Examination of the two chromatograms obtained after thiolysis for 15 min (Fig. 1A) and 30 min (Fig. 1B) allowed us to distinguish between intermediate (peaks 1 and

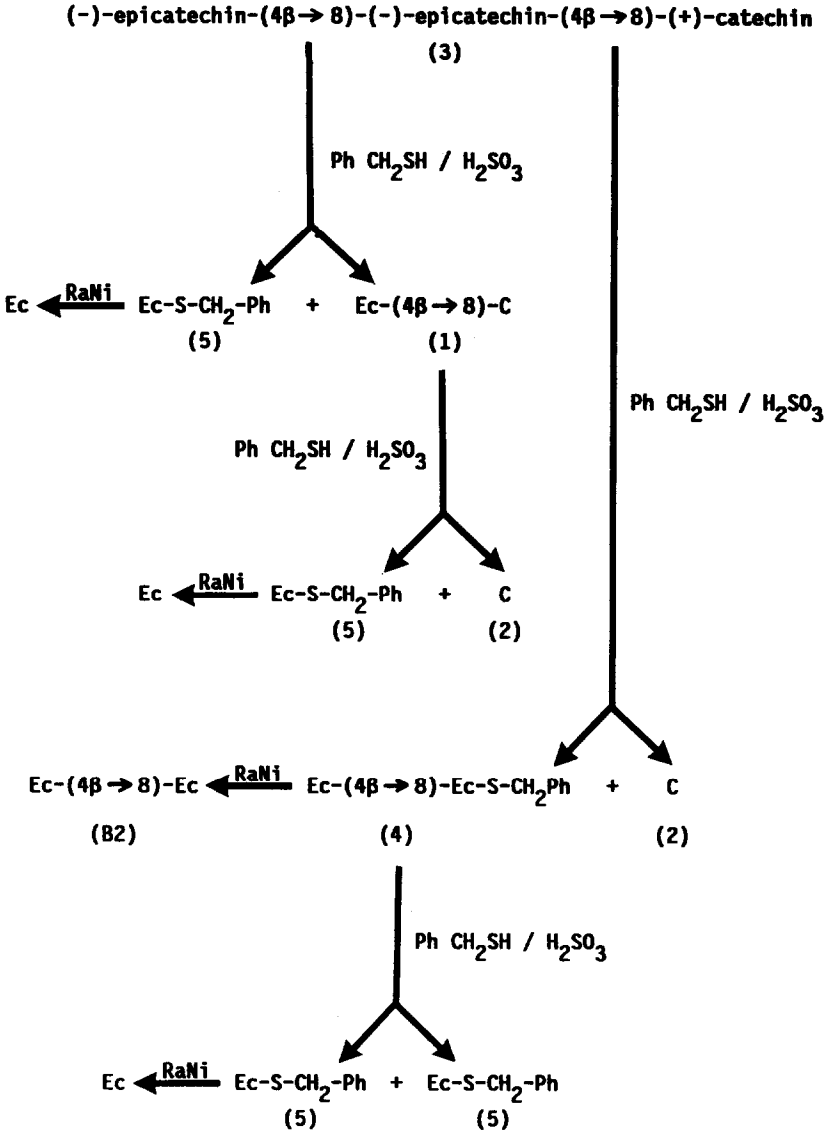

Fig. 2. Scheme of procyanidin trimer thiolysis. C = (+)-Catechin; Ec = (−)-epicatechin; $PhCH_2SH$ = phenylmethanethiol.

4) and end products (peaks 2 and 5). Peaks 1 and 2 were identified as procyanidin B1 [(−)-epicatechin-(4β→8)-(+)-catechin] and (+)-catechin, respectively, according to their retention times. Thus, the "lower" unit of the unknown trimer was (+)-catechin, the only free monomer released by the reaction, linked by a 4β→8 linkage to a (−)-epicatechin central unit. Collection and reduction by Raney nickel of compounds 4 and 5 led to identify them as sulphur derivatives of procyanidin B2 [(−)-epicatechin-(4β→8)-(−)-epicatechin; intermediate product 4] and (−)-epicatechin (final product 5), indicating that the upper part of the trimer was (−)-epicatechin-(4β→8)-(−)-epicatechin. From this information, summarized in Fig. 2, the unknown procyanidin trimer was confirmed as (−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(+)-catechin.

Owing to the systematic use of HPLC, the proposed method makes it possible to work with small amounts (< 0.1 mg) of proanthocyanidins (often difficult to obtain in a pure state) and of phenylmethanethiol, a very unpleasant reagent. Further, replacement of acetic acid with sulphurous acid protects procyanidins from oxidation and allows smooth heating conditions, hence minimizing alterations of the compounds.

This method is currently being applied successfully in our laboratory to identify procyanidin oligomers from grapes, including C-4–C-6 linked compounds and galloyl derivatives.

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