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Chocolate and cocoa: New sources of *trans*-resveratrol and *trans*-piceid

Christine Counet, Delphine Callemien, Sonia Collin *

Unité de Brasserie et des Industries Alimentaires, Université Catholique de Louvain, Croix du Sud, 2/Bte7, B-1348 Louvain-la-Neuve, Belgium

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Abstract

trans-Resveratrol and *trans*-piceid were found for the first time in dark chocolate (at least 0.4 ppm *trans*-resveratrol and 1 ppm *trans*-piceid) and cocoa liquor (at least 0.5 ppm *trans*-resveratrol and 1.2 ppm *trans*-piceid). Because these compounds are highly sensitive to light, a specific extraction procedure was required to recover them, involving delipidation with toluene and cyclohexane and ethanol/water (80/20, v/v) solid–liquid extraction at 60 °C before reverse-phase HPLC-MS/MS analysis (atmospheric pressure chemical ionization [APCI] in the positive mode). Thanks to an exceptionally high procyanidin content, chocolate products displayed higher antioxidant activity than much more concentrated commercial stilbene extracts.

Keywords: Resveratrol; Polyphenols; Stilbene; Procyanidin; Dark chocolate; Cocoa liquor

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin found in at least 72 species of plants distributed among 31 genera and 12 families (Jang et al., 1997). All of the families found to contain resveratrol belong to the spermatophytes division: *Vitaceae*, *Myrtaceae*, *Dipterocarpaceae*, *Cyperaceae*, *Gnetaceae*, *Leguminosae*, *Pinaceae*, *Moraceae*, *Fagaceae*, *Liliaceae* (Langcake & Pryce, 1976; Yoshiaki, Ke-Xu, Terashima, Yue-Hua, & Masatake, 2002). Resveratrol has most often been reported in nonedible plants: vine, eucalyptus, spruce, and the tropical deciduous tree *Bauhinia racemosa*, *Pterolobium Hexapetallum* (Cassady, Hanley, & Lamuela-Raventós, 2000; Soleas, Diamandis, & Golberg, 1997). Foods known to contain resveratrol are limited to grapes, wine, grape juice, cranberries, cranberry juice (0.278 ppm: Wang, Catana, Yang, Roderick, & van Breemen, 2002), peanuts, and peanut products (0.03–0.147 ppm in roasted peanuts: Sanders, McMichael, & Hendrix, 2000; Sobolev & Cole, 1999; 5.14 ± 2.85 ppm in boiled peanuts: Sobolev & Cole, 1999; and 0.27–0.75 ppm in peanut butter: Ibern-Gómez, Roig-Pérez, Lamuela-Raventós, & de la Torre-Boronat, 2000; Sobolev & Cole, 1999). Recently, Callemien, Counet, Cawet, and Collin (2003) detected *trans*-resveratrol, *trans*-piceid, and *cis*-piceid in hop, this suggesting that stilbenes might also be found in beer. Vastano et al. (2000) have shown that the roots of the weed *Polygonum cuspitadum* constitute one of the richest sources of resveratrol (2960–3770 ppm). High levels of resveratrol have also been detected in leaves of *Veratrum grandiflorum* and in roots and rhizomes of *Veratrum formosanum*. These last three plants have been extensively used in Japanese and Chinese folk medicine for their health properties (laxative, anti-arteriosclerosis, anti-cancer) (Soleas et al., 1997).

* Corresponding author. Tel.: + 32 10 47 29 13; fax: + 32 10 47 21 78.
E-mail address: collin@inbr.ucl.ac.be (S. Collin).

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The amount of resveratrol in grape products depends largely on the vine cultivar, the geographic origin (soil, weather), the intensity of fungal infection (especially *Botrytis cinerea*), and the enological practices used (skin maceration time, storage, enzymatic hydrolysis). Resveratrol synthesis is induced in grapes by stress, injury, infection, and UV irradiation (Adrian, Jeandet, Douillet-Breuil, Tesson, & Bessis, 2000; Cantos, Espín, & Tomás-Barberán, 2001; Cantos, García-Viguera, de Pascual-Teresa, & Tomás-Barberán, 2000; Chu, O'Dwyer, & Zeece, 1998; Darias-Martin, Rodriguez, Diaz, & Lamuela-Raventós, 2000; Douillet-Breuil, Jeandet, Adrian, & Bessis, 1999; Gilly, Mara, Oded, & Zohar, 2001; Jeandet, Bessis, Sbaghi, & Meunier, 1995; Lamikanra, Grimm, Rodin, & Inyang, 1996; Mattivi, Reniero, & Korhammer, 1995; Okuda & Yokotsuka, 1996; Rodriguez-Delgado, Gonzalez, Pérez-Trujillo, & Garcia-Montelongo, 2002; Romero-Pérez, Lamuela-Raventós, Buxaderas, & de la Torre-Boronat, 1996; Soleas et al., 1997; Trela & Waterhouse, 1996; Wang et al., 2002). Jeandet, Bessis, and Gautheron (1991) found that, in grapes, resveratrol is synthesized mainly by the skins (5–7 ppm). Lower amounts are found in grape seeds (1 ppm) and grape pulp (less than 0.1 ppm). According to Romero-Pérez, Ibern-Gómez, Lamuela-Raventós, and de la Torre-Boronat (1999), the *trans*-resveratrol content of red grape juices ranges from less than 0.010 to 1.090 ppm and the *cis*-resveratrol content from less than 0.003 to 0.23 ppm. On the other hand, these authors (Romero-Pérez et al., 1999) report only 0.05 ppm *trans*-resveratrol and no *cis*-resveratrol in white grape juices, owing to little contact with the skins during maceration. In red wines, from 1 to 11 ppm (mean: 3.15 ppm) *trans*-resveratrol and from 0.54 to 6.3 ppm (mean: 1.84 ppm) *cis*-resveratrol can be measured (Adrian et al., 2000; Goldberg et al., 1995; Lamuela-Raventós, Romero-Pérez, Waterhouse, & de la Torre-Boronat, 1995; McMurtrey, Minn, Pobanz, & Schultz, 1994; Ribeiro de Lima et al., 1999; Rodriguez-Delgado et al., 2002). Expectedly, only 0.1–2 ppm *trans*-isomer and 0.06 ppm *cis*-isomer are found in white wines, although maceration with skins can significantly increase the level (Cantos et al., 2000; Lamuela-Raventós & Waterhouse, 1993; Romero-Pérez, Lamuela-Raventós, Buxaderas, et al., 1996; Romero-Pérez, Lamuela-Raventós, Waterhouse, & de la Torre-Boronat, 1996). For rosé wines, intermediate levels are reported, i.e., from 0.05 to 1.19 ppm (Cantos et al., 2000; Romero-Pérez, Lamuela-Raventós, Waterhouse, et al., 1996). Wines with high quantities of the *trans*-isomer also contain more *cis*-resveratrol (Goldberg et al., 1995).

Nowadays, resveratrol is of prime interest because of its antioxidant properties (Blond, Denis, & Bezard, 1995; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999) and other physiological effects, including anti-platelet (Bertelli et al., 1995), anti-inflammatory (Martinez & Moreno, 2000; Subbaramaiah et al., 1998), estrogenic (Gehm, McAndrews, Chien, & Jamesson, 1997), cardioprotective (Hung, Chen, Huang, Lee, & Su, 2000), anti-tumor (Carbo, Costelli, Baccino, Lopez-Soriano, & Argiles, 1999; Jang et al., 1997; Nielsen, Ruth, & Vang, 2000; Schneider et al., 2000), and anti-viral (Docherty et al., 1999) action. Resveratrol is also suspected of being one of the compounds present in wines responsible for the "French Paradox": the observation that moderate drinking of red wine over a long period of time appears to prevent coronary heart disease (Frankel, Waterhouse, & Kinsella, 1993; Maxwell, Cruickjshank, & Thorpe, 1994; Siemann & Creasy, 1992).

The aim of the present work was to investigate the potential presence of resveratrol in chocolate and cocoa. Extracts were further compared with commercial polyphenolic extracts as regards their stilbene, procyanidin, flavanol, and phenolic acid contents. The data were finally related to antioxidant activities measured by means of the AAPH-induced oxidation assay (Liégeois, Lermusieau, & Collin, 2000).

2. Materials and methods

2.1. Materials

Dark chocolate and cocoa liquor from Ivory Coast were supplied by Belcolade (Puratos Group, Belgium). Red grape skin, white grape skin, white grape seed, and red wine extracts were supplied by the Société Française de Distilleries (France) and red grape seed *Polygonum cuspitadum* 20% resveratrol extracts by Naturex (France).

2.2. Chemicals

Acetone (99.9%), (−)-epicatechin, (+)-catechin, *trans*-resveratrol, rutin, vanillic acid, ferulic acid, and syringic acid were from Sigma–Aldrich (Bornem, Belgium). Methanol (99.9%) and dichloromethane (99.9%) were purchased from Romil (Cambridge, UK). Acetic acid (99.8%) was from Acros (Geel, Belgium); Milli-Q (Millipore, Bedford, MA, USA) double-distilled water (resistance = 18 mΩ) was used.

2.3. Specific extraction procedure for stilbenes

2.3.1. General

This method has been developed in our laboratory by Callemien, Jerkovic, Rozenberg, and Collin (2004) to analyze stilbenes of hop pellets. All extraction steps were done with protection against day light, in duplicate.

2.3.2. Removal of lipids

Dark chocolate (10 g), or cocoa liquor from Ivory Coast (5 g) was reduced to powder and introduced into a centrifugal vial. In successive 10 min steps, lipids were removed at room temperature with gentle stirring, first with 50 ml toluene (3 times) and then with 50 ml cyclohexane (3 times). At the end of each step, the sample was centrifuged for 10 min at 3000*g*. At the last step, cocoa products were also dried under vacuum to get rid of residual solvent.

2.3.3. Stilbenes extraction

Delipidated dark chocolate, or cocoa liquor, was extracted three times with 40 ml ethanol/water (80/20, v/v); each time for 10 min with gentle stirring at 60 °C. After the extraction, the sample was centrifuged for 10 min, at 3000*g* and the supernatant collected. After filtration to remove residual particles, the combined supernatants were concentrated by rotary evaporation (35 °C) to dryness. The residue was solubilized in 2 ml of 50:50 (v/v) mixture of ethanol/water.

2.4. RP-HPLC-APCI-MS/MS analysis of stilbenes

Analyses were performed on a C18 Prevail column (150 × 2.1 mm, 2 μm) (Alltech, Deerfield, IL, USA) eluted with a linear gradient from A (water containing 1% acetonitrile and 0.1% formic acid) to B (acetonitrile). Gradient elution was 95–55% A, 0–23 min; 55–0%. A 23–30; 30–40 min isocratic, at a flow rate of 200 μl/ min. 10 μl samples were injected into the column kept at 30 °C. A SpectraSystem equipped with an AS3000 autosampler and a P4000 quaternary pump was used. The system was controlled with the Xcalibur software version 1.2 (Finnigan Mat). Mass spectra were acquired using a LCQ mass spectrometer equipped with an APCI source (Finnigan Mat). The following APCI inlet conditions were applied: vaporization temperature, 470 °C; capillary voltage, 3 V; capillary temperature, 175 °C; sheath gas, 40 psi; auxiliary gas, 7 psi and discharge current 5 μA. Collision-induced dissociation spectra were recorded at 37% relative collision energy.

2.5. NP-HPLC polyphenol extract analysis

For preliminary studies, the chocolate extracts were prepared according to Counet and Collin (2003) and Counet, Rosoux, Ouwerx, and Collin (2004).

2.6. NP-HPLC-UV analysis for commercial extracts and preliminary studies on chocolate

A SpectraSystem (Finnigan Mat, San Jose, CA, USA) equipped with a SCM degasser, an AS3000 autosampler, a P4000 quaternary pump and a diode array detector UV6000LP was used. The system was controlled with the Xcalibur software version 1.2 (Finnigan Mat). Polyphenols were separated on a Phenomenex 5-μm normal-phase Luna silica column, 250 × 4.6 mm i.d. (Bester, Holland) at 25 °C. Separations were carried out at a flow rate of 1 ml/min with a linear gradient from A (dichloromethane) to B (methanol) and a constant 4% level of C (acetic acid and water, 1:1 v/v). Gradient elution was 14–28% B, 0–30 min; 28–50% B, 30–60 min; 50–86% B, 60–65 min; 65–70 min isocratic. A 10 mg polyphenol sample of extract was diluted in 1 ml methanol before injection with the 20 μl Rheodyne loop (Berkeley, USA). The standard-addition method which involves adding increasing concentrations of each compound to the extract, was used to confirm quantification. The UV detector was set at 270 nm for gallic and syringic acids and rutin, at 280 nm for procyanidin monomers to hexamers and vanillic acid, at 306 nm for resveratrol; at 320 nm for caffeic, *p*-coumaric and ferulic acids, and at 370 nm for ellagic acid, myricetin and quercetin.

2.7. NP-HPLC-ESI-MS-SIM analysis

MS analyses were carried out using a LCQ Duo (Finnigan Mat) multipole mass spectrometer equipped with an ESI interface. The negative ion mode with a 4.5 kV source voltage, a 225 °C capillary temperature and a shear gas (N_2) of 50 arb (arbitrary units) were selected for the polyphenol analysis. MS analysis was tuned for each phenolic compound. Capillary voltage used for resveratrol was −20 kV. Only 1:10 of the HPLC flow was directed to the ESI interface of the mass analyser. Data were collected on a computer (Xcalibur software) using multiple selective ion monitoring (SIM) mode.

2.8. Antioxidant assay

Reduction power of some extracts was measured by a method proposed by Liégeois et al. (2000). The oxidation of linoleic acid was induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) in an aqueous dispersion in the absence or presence of antioxidant. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. A Shimadzu UV–visible 240 spectrophotometer (Antwerp, Belgium) equipped with an automatic sample positionner allowed analysis of six samples every minute. In all cases, the measurements were run in duplicate against the buffer and compared with a separate AAPH-free control to check for any spontaneous oxidation. All appropriate dilutions of extracts were prepared in Milli Q double distilled water.

3. Results and discussion

A polyphenol extract prepared from chocolate has recently been investigated in our laboratory (Counet & Collin, 2003). From the acetone–water–acetic acid mixture (70:28:2, v/v) a major chocolate procyanidin fraction (8536 mg/100 g dry extract) was recovered containing compounds ranging in size from P1 (catechin and epicatechin) to P10 (decamer). The extract also contained quercetin (25 mg/100 g dry extract) and ferulic acid (24 mg/100 g dry extract).

Unexpectedly, despite the absence of protection against light during analysis, it appeared that a small peak (UV-detection at 306 nm) eluting very early in normal-phase HPLC (Fig. 1, retention time = 5.4 min, just after caffeine and theobromine) might be resveratrol (ion 227.2 at 5.4 min in NP-HPLC-MS-ESI-SIM).

In order to confirm this hypothesis, a specific procedure, more adapted to stilbenes (darkness, delipidation with a non-ether solvent) was applied to cocoa products. This procedure was recently optimized in our laboratory by Callemien et al. (2004) for the analysis of hop-pellet stilbenes. As depicted in Fig. 2(c), *trans*-resveratrol (A) but also its glucoside, *trans*-piceid (B), were detected in the dark chocolate extract (analysis done in duplicate). Fig. 3 shows the *trans*-resveratrol (A) and *trans*-piceid (B) mass spectra obtained. An Ivory Coast cocoa liquor

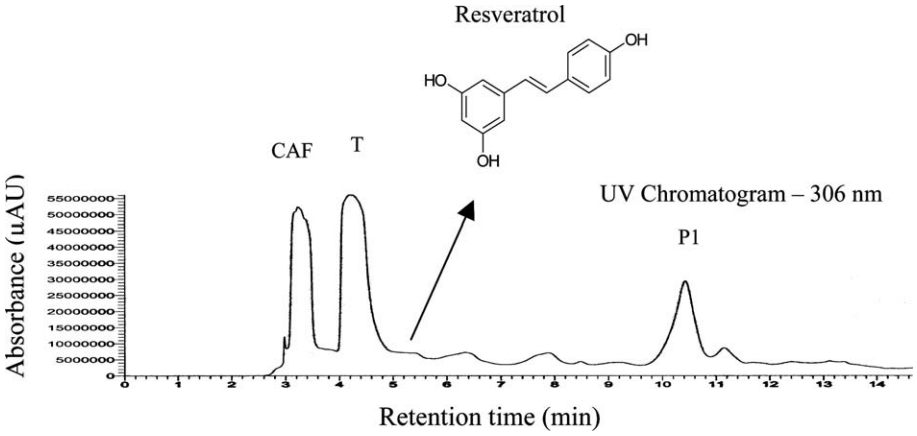

Fig. 1. NP-HPLC-UV (306 nm) chromatogram of the dark chocolate. CAF, caffeine; T, theobromine, P1, procyanidin monomers.

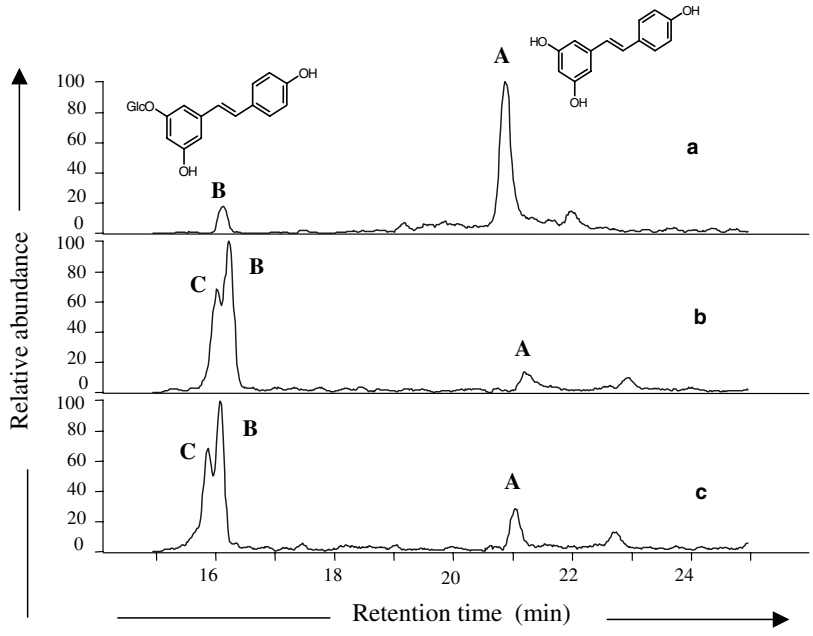

Fig. 2. RP-HPLC-APCI(+)-MS/MS chromatogram (a) of a 5 ppm standard solution of *trans*-piceid (retention time = 16.04) and *trans*-resveratrol (retention time = 20.87), (b) of the cocoa liquor extract and (c) of the dark chocolate extract. A, *trans*-resveratrol; B, *trans*-piceid; C, unknown.

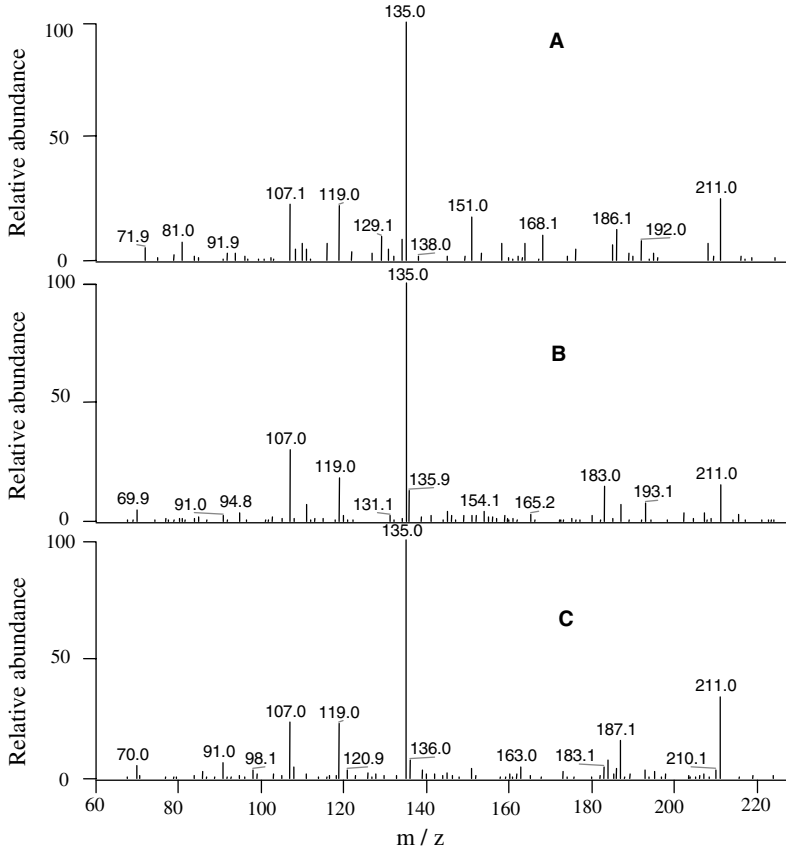

Fig. 3. RP-HPLC-APCI(+)-MS/MS spectra of: (A) *trans*-resveratrol (collision with the pseudomolecular ion of *trans*-resveratrol: $[M + H]^+ = 229.2$), (B) *trans*-piceid, and (C) the unknown in the dark chocolate extract (c in Fig. 2). Note that, during the mass spectrometry, *trans*-piceid is quickly degraded into sugar and *trans*-resveratrol (similar MS/MS spectra for *trans*-piceid and *trans*-resveratrol).

extract analyzed in duplicate by the same specific procedure yielded similar data (Figs. 2(b) and 4). In both cases (Figs. 3 and 4), a third mass spectrum (C) similar to *trans*-resveratrol, was detected at the retention time of 15.9 min (possibly another glycoside of resveratrol).

The dark chocolate extract proved to contain 2 ppm *trans*-resveratrol and 5 ppm *trans*-piceid, whilst the cocoa liquor extract reached 1.3 ppm *trans*-resveratrol and 3 ppm *trans*-piceid. Taking into account the concentration factor applied through extraction (2 ml extract/10 g dark chocolate or 5 g cocoa liquor), the initial dark chocolate contained at least 0.4 ppm *trans*-resveratrol and 1 ppm *trans*-piceid. In the same way, it was determined that at least 0.5 ppm *trans*-resveratrol and 1.2 ppm *trans*-piceid were present in the cocoa liquor (recovery factor of 100% applied).

Commercial polyphenol extracts were analyzed for comparison. The resveratrol concentration was, as expected, much higher in the *Polygonum cuspitadum* extract (Fig. 5), commercially claimed to be a "resveratrol" concentrate (20%). Also to be emphasized is the high level found in an extract mimicking the composition of red wine (337 mg/100 g dry extract). Both red grape-skin extracts and the white grape-skin sample were found to contain from 60 to 75 mg/100 g dry extract. No resveratrol was found in olive extract.

Grape-seed extracts displayed the highest amount of procyanidins (Fig. 6(b)). Up to 573 ppm gallic acid, 59 ppm ellagic acid, and 50 ppm vanillic acid were also found in the grape-skin extracts. *Polygonum cuspitadum* extract (20% resveratrol) contained a relatively high amount of ferulic acid (155 ppm). Flavonols were also present in the commercial red wine extract (295 ppm quercetin and 181 ppm myricetin). Grape-skin extracts contained flavonols at concentrations ranging from 99 to 190 mg/100 g dry extract (up to 138 mg/100 g of dry extract for rutin and 152 mg/100 g dry extract for quercetin).

As depicted in Table 1, a similar distribution of procyanidin oligomers was found in all commercial extracts. Probably also because of a better recovery factor (Counet & Collin, 2003) P1 appeared in all cases much more concentrated than P2, P3,...,P6. Monomers and dimers were found to account for up to 71% of the total procyanidin fraction.

As shown in Fig. 6(a), the antioxidant activity measured by means of the AAPH procedure recently proposed by Liégeois et al. (2000), was found to be

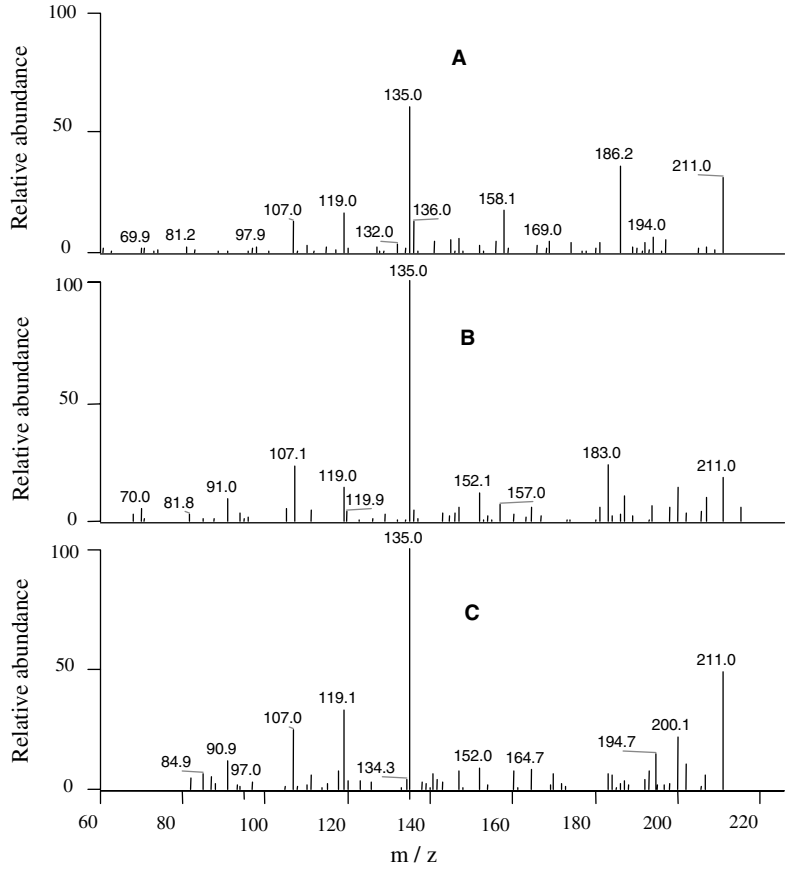

Fig. 4. RP-HPLC-APCI(+)-MS/MS spectra of: (A) *trans*-resveratrol (collision with the pseudomolecular ion of *trans*-resveratrol: $[M + H]^+ = 229.2$), (B) *trans*-piceid, and (C) the unknown in the cocoa liquor extract (b in Fig. 2).

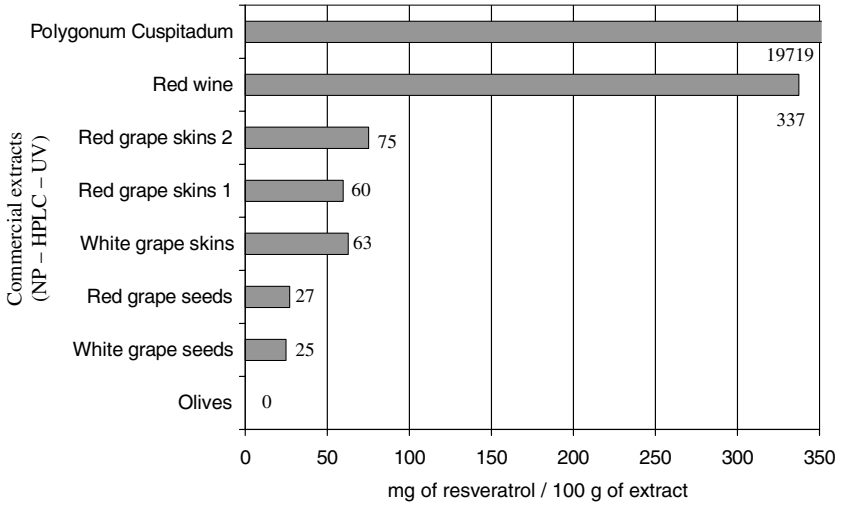

Fig. 5. Concentration of resveratrol (NP-HPLC-UV-306 nm) in commercial extracts (mg/100 g of dry extract).

mainly linked to this procyanidin fraction. The *Polygonum cuspitadum* extract showed a very short inhibition time, despite its exceptionally high resveratrol concentration. On the other hand, grape-seed extracts emerged as the best antioxidants (inhibition time up to 110 min/ppm). In our AAPH assay, polymeric procyanidins appeared better antioxidants than a single phenol such as resveratrol. This could be explained by a higher degree of radical delocalisation in polymerised flavanoids (Fig. 7). Fundamental studies are, however, still needed to understand what does exactly explain the exceptional antioxidant activity of higher procyanidins.

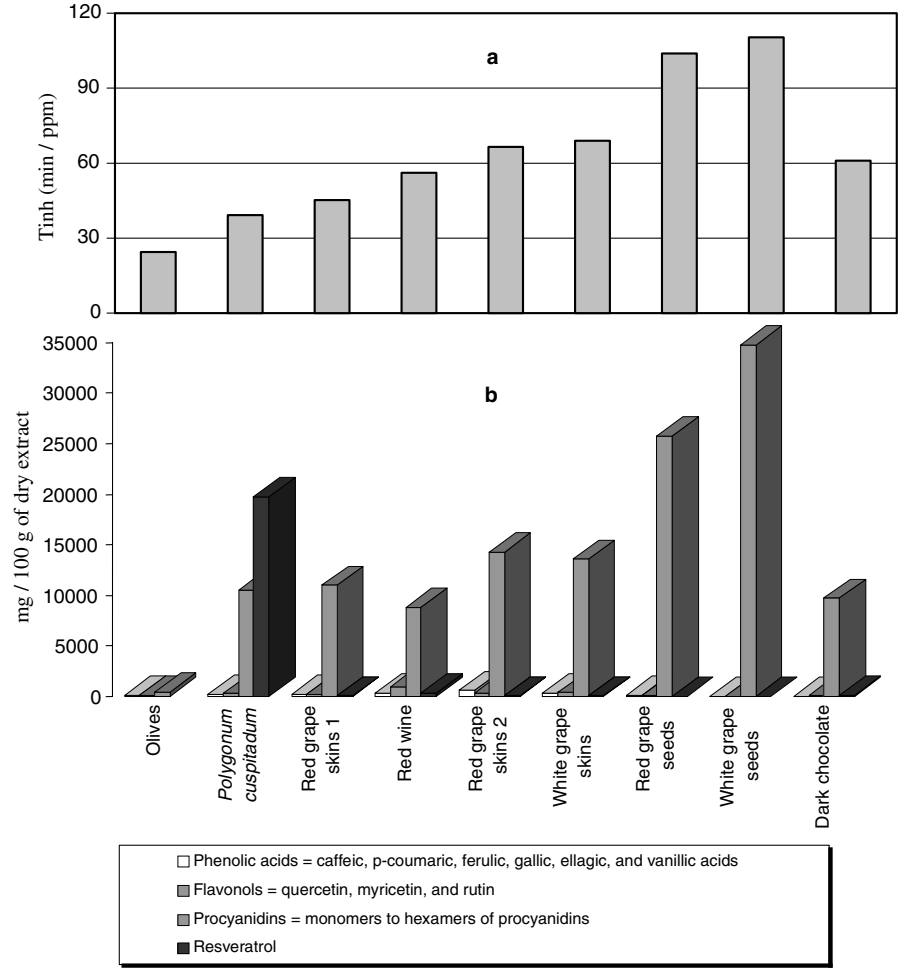

Fig. 6. Comparison between: (a) inhibition time (AAPH assay), and (b) procyanidin content of commercial and chocolate extracts.

Table 1
Procyanidins (HPLC-UV, mg/100 g of dry extract) in commercial and laboratory polyphenol extracts

	P1[a]	P2[a]	P3[a]	P4[a]	P5[a]	P6[a]	T[b]
Commercial extracts							
White grape seeds	9812	8276	7900	5401	2052	1323	34764
Red grape seeds	10583	6672	4087	2746	1182	472	25742
White grape skins	6212	3453	1536	1664	359	363	13587
Red grape skins 1	4578	4081	900	3757	413	523	14252
Red grape skins 2	5027	2665	1215	1498	401	198	11004
Red wine	2724	1750	1776	1664	473	425	8812
Polygonum cuspitadum	3859	515	3644	936	742	858	10554
Olives	0	0	0	0	0	0	0
Laboratory extract							
Dark chocolate	4298	1463	1170	784	504	317	8536

[a] P1–P6: monomeric to hexameric procyanidins.
[b] T, summation of P1–P6.

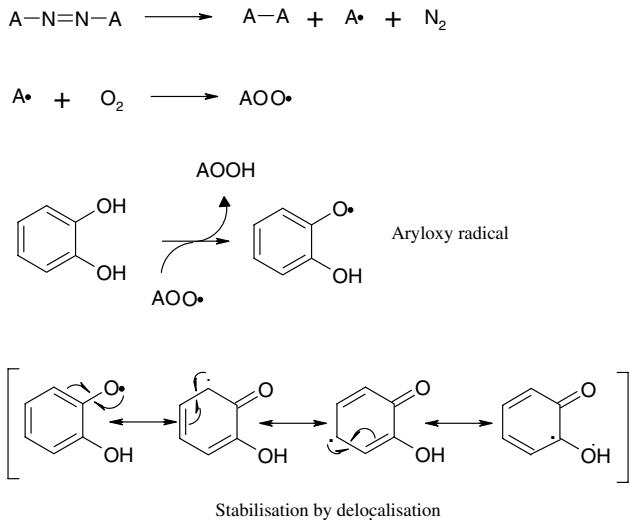

Fig. 7. AAPH generation of free radicals, scavenging activity of the catechol group and stabilisation of the aryloxy radical by delocalisation ($A = -C(CH_3)_2C(NH_2) = NH \cdot HCl$).

4. Conclusions

For the first time, *trans*-resveratrol and *trans*-piceid have been identified in dark chocolate and cocoa liquor extracts. Yet, the exceptional antioxidant activity of chocolate must be related more to its high procyanidin content than to the presence of stilbenes.

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