# Stilbenic Profile of Cocoa Liquors from Different Origins Determined by RP-HPLC-APCI(+)-MS/MS. Detection of a New Resveratrol Hexoside

Vesna Jerkovic, Meike Bröhan, Elise Monnart, Fanny Nguyen, Sabrina Nizet, and Sonia Collin*

Unité de Brasserie et des Industries Alimentaires, Earth and Life Institute, Faculté d'Ingénierie Biologique, Agronomique et Environnementale, Université Catholique de Louvain, Croix du Sud, 2 bte 7, B-1348 Louvain-la-Neuve, Belgium

*trans*-Resveratrol and *trans*-piceid were recently discovered in chocolate. In the present work, both were quantified by RP-HPLC-APCI(+)-MS/MS in 22 cocoa liquors from 11 different countries. A very large range of concentrations was observed for *trans*-piceid. The most concentrated sample (Arriba 06) reached 0.4 and 2.6 mg/kg of *trans*-resveratrol and *trans*-piceid, respectively, but in other cultivars stilbene levels were five times lower. Neither *cis*-resveratrol nor *cis*-piceid was found in cocoa liquors. An unknown compound eluting 0.5 min before *trans*-piceid and present at concentrations up to 0.8 mg/kg of *trans*-piceid equivalents in cocoa liquors was tentatively identified by HRMS as a *trans*-piceid-like hexoside.

KEYWORDS: Resveratrol; piceid; resveratrol galactoside; stilbene; polyphenol; cocoa; chocolate

## INTRODUCTION

Cocoa is increasingly recognized for its health benefits (*1−4*). It is a source of various polyphenolic compounds, including hydroxybenzoic acids (gallic/syringic/protocatechic/vanillic acids), hydroxycinnamic acids and analogues (caffeic/ferulic/*p*-coumaric/phloretic acids, clovamide, dideoxyclovamide), flavonols (quercetin), flavones (luteolin, apigenin), flavanones (naringenin), and flavan-3-ols ((+)-catechin, (−)-epicatechin, oligomers, and polymers/procyanidins) (*5,6*). Catechin and epicatechin have been found at concentrations of 150−1580 mg/kg in chocolate and 2530−3170 mg/kg in cocoa liquor (*4, 7−9*). Procyanidins (from monomers to hexamers) have been reported at concentrations from 2200 to 13230 mg/kg in various cocoa liquors (*6,8*). Many phenols are found as glycosides in cocoa, mainly glucoside, galactoside, and arabinoside (*10, 11*).

Besides hydroxyphenolics and flavonoids, two stilbenes were recently identified in a cocoa liquor from the Ivory Coast and in dark chocolate (*12*). *trans*-Resveratrol is known to exhibit interesting antiinflammatory, anticancer, cardioprotective, and estrogenic activities (*13, 14*). It has been reported in grapes (0.13−47.6 mg/kg) (*15−18*), wine (0−18.03 mg/L) (*15, 16*), grape juice (0.35 mg/kg) (*16*), cranberry juice (0.24 mg/kg) (*16*), peanuts (3.7 mg/kg) (*19*), strawberries and cranberries (3.57 and 19.29 mg/kg, respectively) (*20*), sorghum (0.5 mg/kg) (*21*), hop (0.05−2.28 mg/kg) (*22−25*), and beer (5 µg/L) (*26*). In cocoa liquor, 0.5 mg/kg of *trans*-resveratrol and up to 1.2 mg/kg of its glucoside *trans*-piceid have been determined (*12*). More recently, Hurst et al. (*27*) reported much higher levels in some cocoa-derived products: from 0.09 to 1.85 mg/kg and from 0.35 to 7.14 mg/kg for *trans*-resveratrol and *trans*-piceid, respectively. Their highest values were obtained for cocoa powder.

The aim of the present work was to determine the range of stilbene concentrations in cocoa liquors from different origins (Asia, South-America, and Africa). To the stilbene extraction method initially developed in our laboratory for hop analysis was added an Oasis cartridge purification step to avoid contamination of the samples by melanoidins (*22, 26*). Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with APCI(+) tandem mass spectrometry (MS/MS) was used for quantification.

## MATERIALS AND METHODS

**Materials.** Twenty-two cocoa liquors from two successive harvests of cocoa beans (2006 and 2007) were supplied by Belcolade (Puratos Group, Belgium) and investigated just after delivery. All fermented and dried beans imported from the Ivory Coast, New Guinea, Java, Madagascar, Arriba, Tobago, Ghana, Venezuela, Costa Rica, Dominican Republic, or Peru were roasted in the same way (150 °C, 30 min) by the supplier.

**Chemicals.** Ethanol (97%) was obtained from Belgaco (Gent, Belgium). Acetonitrile (99.99%) and cyclohexane (99.96%) were supplied by Fisher Scientific (U.K.). Formic acid (99%) was obtained from Aldrich (Germany). Methanol (99.9%) was supplied by Romil (Cambridge, U.K.). Ethyl acetate (97%) came from by Fisher Scientific (U.K.). *trans*-Resveratrol (99%), *trans*-piceid (97%), acetobromo-α-D-galactose (93%), dichloromethane (99.8%), and potassium hydroxide were supplied by Sigma-Aldrich (Bornem, Belgium). Acetic acid (100%) was obtained from Merck (Darmstadt, Germany). Aqueous solutions were made with Milli-Q (Millipore, Bedford, MA) water (resistance = 18 mΩ).

**Specific Extraction Procedure for Stilbenes.** This method has been first developed in our laboratory (*22, 26*) to analyze stilbenes in hop pellets. All extraction steps have been done with protection against day light, in

*To whom correspondence should be addressed (e-mail, sonia. collin@uclouvain.be; fax, +32-10-47 21 78).
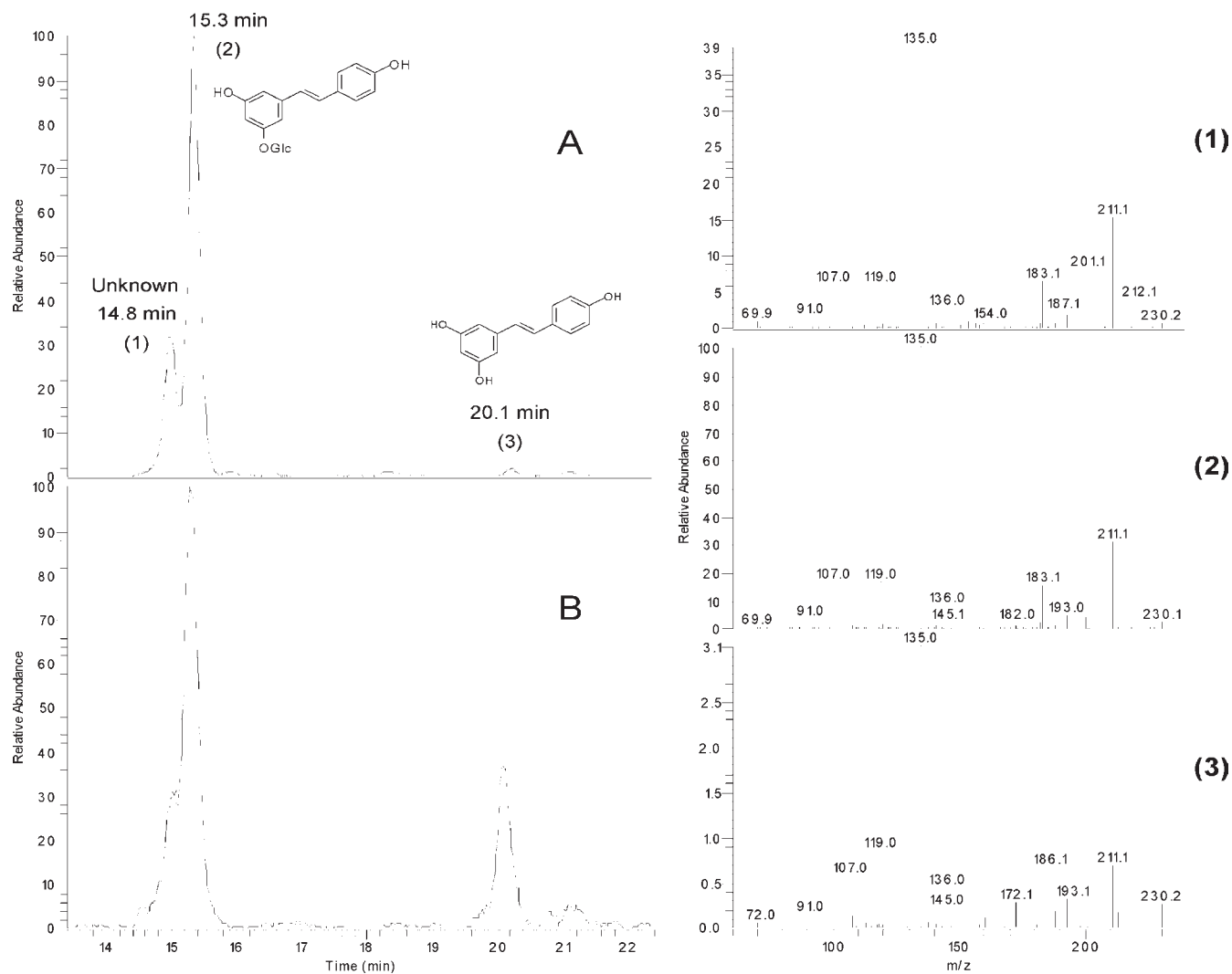
**Figure 1.** RP-HPLC-APCI(+)-MS/MS data of (**A**) Peru 07 and (**B**) Peru 06 cocoa liquor extracts. MS/MS chromatogram ($m/z$ = 229) and experimental mass spectra for the unknown (1), *trans*-piceid (2), and *trans*-resveratrol (3).

duplicate. An Oasis cartridge purification step was added to avoid sugar and melanoidin contamination of the mass spectrometer.

*Lipid Removal.* Cocoa liquor (5 g) was reduced to powder and introduced into a centrifugal vial. By successive 10 min extractions, lipids were removed with $3 \times 50$ mL cyclohexane at room temperature under gentle stirring. At the end of each step, the sample was centrifuged for 10 min at 2500$g$. At the last step, cocoa powder was dried under vacuum to get rid of residual solvent.

*Stilbene Extraction.* Defatted cocoa liquor was extracted three times with 40 mL ethanol−water (80:20 v/v), each time for 10 min under gentle stirring at 60 °C. After each extraction, the sample was centrifuged for 10 min at 2500$g$ and the supernatant collected. The combined supernatants were concentrated by rotary evaporation (35 °C) under vacuum to obtain ~20 mL of extract and then filtrated to remove residual particles.

*Solid-Phase Extraction Purification Step.* The 500 mg Oasis cartridge (Waters) was preconditioned with methanol and 1.5 M formic acid. Approximately 20 mL of stilbene extract was loaded on the cartridge, and sugar and melanoidin were removed with 15 mL of 1.5 M formic acid followed by 15 mL of water containing 5% methanol and 2% formic acid. Stilbenes were then eluted with 24 mL of ethyl acetate. The eluate was concentrated by rotary evaporation (35 °C) to dryness. The residue was solubilized in 1 mL of a 50:50 (v/v) mixture of ethanol−water and filtered before analysis.

**Combinatorial Synthesis of Stilbene Galactosides** was achieved according to the methodology described in the literature for stilbene glucosides (*28*). Under red light, 5 mg of *trans*-resveratrol, 9 mg of acetobromo-α-ᴅ-galactose, and 2.45 mg of potassium hydroxide were dissolved in

180 $\mu$L of ethanol. The reaction medium was stirred for 1 week at room temperature. Then, 500 $\mu$L of ethanol was added, and the mixture was filtered before analysis by RP-HPLC-MS/MS.

**RP-HPLC-APCI(+)-MS/MS Analysis of Stilbenes.** Quantifications were performed on a 150 mm × 2.1 mm, 2 $\mu$m C18 Prevail column (Grace, Deerfield, IL) eluted with a linear gradient from water (containing 1% acetonitrile and 0.1% formic acid) to acetonitrile. Gradient elution was as follows: from 95% water to 55% in 23 min, 55% to 0% in 7 min, and isocratic for 10 min at a flow rate of 200 $\mu$L/min. Ten microliters of the sample was injected onto the column. 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 in positive mode were applied: vaporization temperature, 470 °C; capillary voltage, 30 V; capillary temperature, 175 °C; sheath gas, 40 psi; auxiliary gas, 7 psi; discharge current, 5 $\mu$A. After the first monitoring on the $m/z$ 229, collision-induced dissociation spectra were recorded at 37% relative collision energy.

**RP-HPLC-APCI(+)-HRMS Analysis of Stilbenes.** RP-HPLC was performed on an Accela system (Thermo Fisher), as described above. HRMS analyses were carried out using an LTQ-Orbitrap-XL (Thermo Fisher) mass spectrometer equipped with an APCI source. The system was controlled with the Xcalibur software version 2.0.7. The same APCI inlet conditions were applied as described above. For MS/MS (monitoring on $m/z$ 229), collision-induced dissociation spectra were recorded at 35% relative collision energy. LC-HRMS applied on the unknown in the

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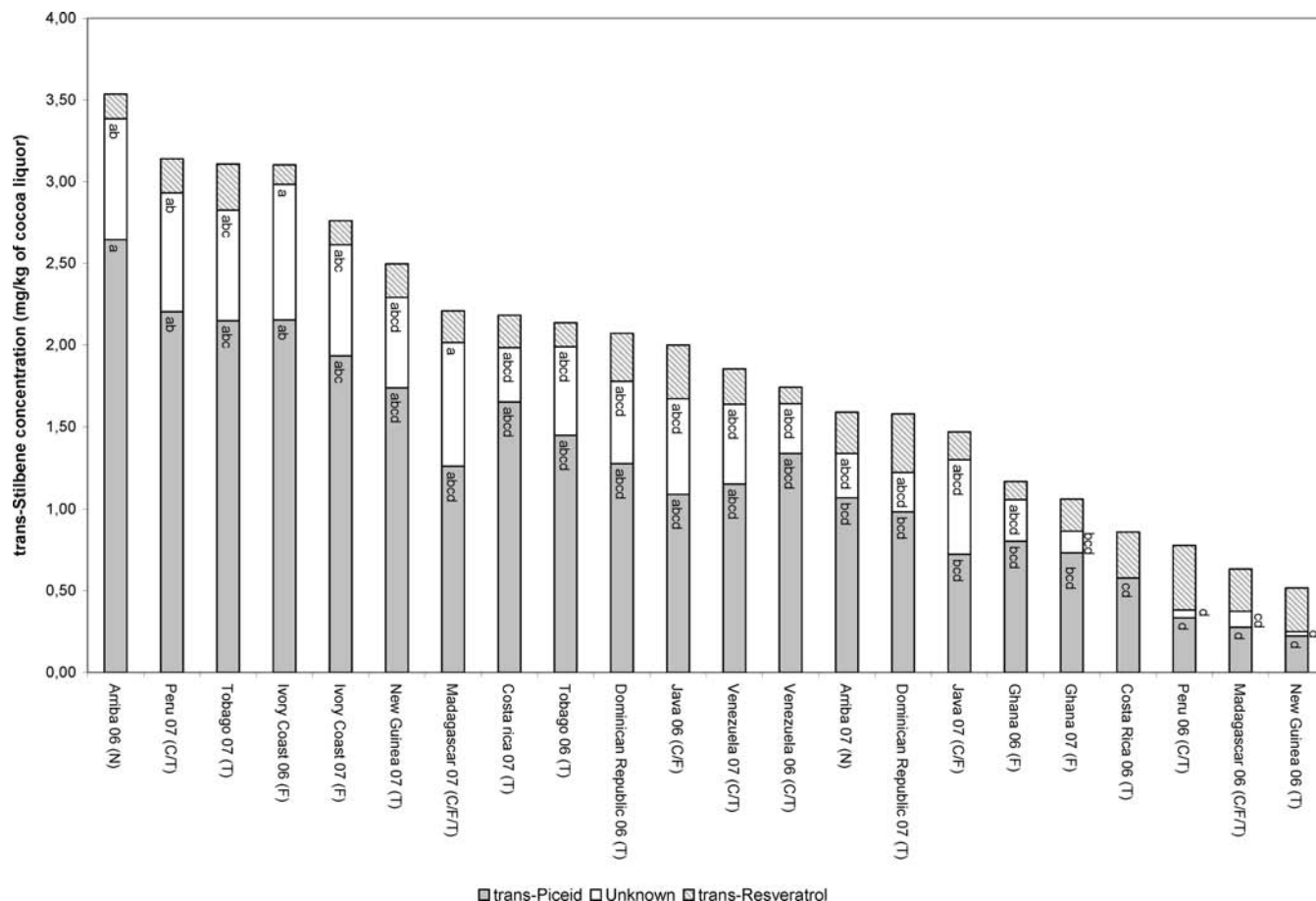*J. Agric. Food Chem.,* Vol. 58, No. 11, 2010 **7069**



**Figure 2.** Concentration (mg/kg of cocoa liquor) of *trans*-piceid, *trans*-resveratrol, and the unknown (in *trans*-piceid equivalents) in different varieties of cocoa liquor. Key: C, *Criollo*; F, *Forastero*; T, *Trinitario*; N, *Nacional*. Assay in duplicate. For *trans*-piceid or the unknown, all samples that do not share a common letter are significantly different (*p* < 0.05) according to the Tukey test (for *trans*-resveratrol all samples were found in the same group).

Tobago 07 extract confirmed an elemental formula of $C_{20}H_{23}O_8$ (experimental mass of *m*/*z* 391.13932, theoretical mass of *m*/*z* 391.13874, delta = 0.57 ppm, well in the variation range of the apparatus) with a major fragmentation into *m*/*z* 229.08630 (theoretical mass of protonated *trans*-resveratrol ($C_{14}H_{13}O_3$) = 229.08592, delta = 1.66 ppm). As expected, the MS/MS applied on *m*/*z* 229 gave an intense ion at *m*/*z* 135.04388.

**NP-HPLC-APCI(+)-MS/MS Analysis of Stilbenes.** Separations were carried out on a 250 mm × 2.1 mm, 2 *μ*m Alltima Silica column (Grace, Deerfield, IL) at a flow rate of 200 *μ*L/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 14−50% B, 0−30 min; 50−56% B, 30−35 min; 56−85% B, 35−40 min; 40−50 min isocratic. Ten microliters of the sample was injected in the column. The mass spectrometry conditions were the same as for RP analyses.

**Statistical Analyses.** The statistical analysis system (SAS Institute, Inc., Cary, NC) was used. Significant differences among samples were determined by analysis of variance (ANOVA), and the multiple comparisons of means were performed with the Tukey test.
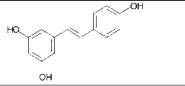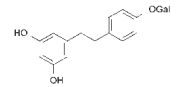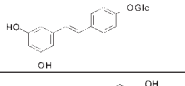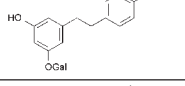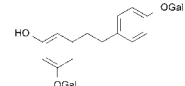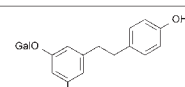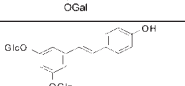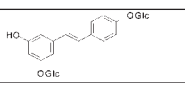
## RESULTS AND DISCUSSION
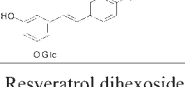
**Optimization of Stilbene Extraction.** The method was initially developed to analyze resveratrol in hop (*22*). According to the literature (*29*), preliminary removal of lipids and other hydrophobic constituents is required for recovering high amounts of polyphenols. Ether is often chosen for preliminary cleaning (*30*). Unfortunately, because of the S1-type solubility of resveratrol (>3.3% soluble in both water and ether) (*22*, *29*, *31*), solvents more hydrophobic than diethyl ether were required here for precleaning. A three-step solid/liquid precleaning with cyclohexane proved sufficient for removing lipids from cocoa liquor. Stilbenes

were then extracted from the defatted cocoa liquor with ethanol−water (80:20) at 60 °C as proposed by Callemien et al. (*22*). The presence of Maillard colored compounds in the ethanol−water extract, however, made it necessary to perform an additional purification step on an Oasis HLB cartridge (*26*). Elution with ethyl acetate, often used for stilbenoid extraction (*16*, *31−33*), enabled us to recover 80% *trans*-resveratrol and 60% *trans*-piceid (determined by standard addition in cocoa liquor). These recovery values were used for further quantifications.

**Stilbenoids in Cocoa Liquors.** The optimized procedure was applied to 22 cocoa liquors of different origins (Africa, America, and Asia; 11 countries). Indicative data on cocoa varieties (fine grades, *Criollo*, *Trinitario*, and *Nacional*; bulk-basic grade, *Forastero*) were given by the producers. RP-HPLC-APCI(+)-MS/ MS enabled us to easily separate *trans*-piceid (RT = 15.3 min in **Figure 1A**) and *trans*-resveratrol (RT = 20.1 min in **Figure 1A**) in all varieties. As expected (*28*), fragmentation of the monoglucoside was characterized by loss of the sugar, leading in all cases to an intense aglycon M + 1 ion. Therefore, both *trans*-stilbenes were measured just by selecting *m*/*z* 229. As in hop, grape juices, wine, and cocoa-containing products (*23*, *27*, *34*, *35*), *trans*-piceid emerged as the major form (**Figure 1A**; its lower recovery is balanced on the chromatogram by the two times higher sensitivity of the mass spectrometer for piceid than for its aglycon). Only three cocoa liquors (Peru 06, Madagascar 06, and New Guinea 06) exhibited a distinct profile with a higher peak for *trans*-resveratrol (**Figure 1B**).

As depicted in **Figure 2**, concentrations ranged in the 22 investigated cocoa liquors from 0.1 to 0.5 mg/kg *trans*-resveratrol

**Table 1.** Structures, Retention Times (RT, min) on C18 and Silica Columns, and Relative Retention Times (rRT = 1 for *trans*-Piceid) of Stilbene Glucosides and Galactosides, Potentially Candidates for Being the Unknown (rRT C18 = 0.97; rRT Silica =1.35) in Cocoa Liquor.[a]

| Structure | Name | *m/z* [M +H]$^+$ | RT C18(rRT) | RT Silica(rRT) |
|---|---|---|---|---|
| Resveratrol | | | | |
| | *trans*-Resveratrol or *trans*-3,4',5-Trihydroxystilbene | 229 | 20.1(1.31) | 4.0(0.37) |
| Resveratrol monohexosides | | | | |
| | *trans*-Resveratroloside-like galactoside or *trans*-3,4',5-Trihydroxystilbene-4'-*O*-β-D-galactopyranoside | 391 | 13.5(0.88) | 14.9(1.37) |
| | *trans*-Resveratroloside or *trans*-3,4',5-Trihydroxystilbene-4'-*O*-β-D- glucopyranoside | 391 | 13.9(0.91) | 12.5(1.15) |
| | *trans*-Piceid-like galactoside or *trans*-3,4',5-Trihydroxystilbene-3-*O*-β-D-galactopyranoside | 391 | 14.8(0.97) | 11.7(1.07) |
| | *trans*-Piceid or *trans*-3,4',5-Trihydroxystilbene-3-*O*-β-D-glucopyranoside | 391 | 15.3(1.00) | 10.9(1.00) |
| Resveratrol dihexosides | | | | |
| | *trans*-3,4',5-Trihydroxystilbene-3,4'-*O*-β-D-digalactopyranoside | 553 | 9.9(0.65) | 24.9(2.28) |
| | *trans*-3,4',5-Trihydroxystilbene-3,5-*O*-β-D-digalactopyranoside | 553 | 10.6(0.69) | 21.4(1.96) |
| | *trans*-3,4',5-Trihydroxystilbene-3,5-*O*-β-D-diglucopyranoside | 553 | 11.6(0.76) | 19.1(1.75) |
| | *trans*-3,4',5-Trihydroxystilbene-3,4'-*O*-β-D-diglucopyranoside | 553 | 10.9(0.71) | 20.6(1.89) |

[a] Chromatograms given in Figure 5. For all compounds, the APCI(+)-MS/MS spectrum obtained after selecting *m/z* = 229 was similar to that of the unknown.

(one statistical group according to Tukey test) and from 0.2 to 2.6 mg/kg *trans*-piceid (three statistical groups according to Tukey test). Fine-cocoa liquors, to which shorter fermentations are usually applied, are known to contain higher levels of total procyanidins (*6*). Stilbenes did not systematically follow this rule. The fine Arriba/Nacional sample did exhibit the highest level, but fine samples from Java and Madagascar showed levels at the bottom of the range.

Such large variations between samples have been previously observed in grapes and hop (*18*, *23−25*). As stilbenes are phytoalexins, growing area, climate conditions, interactions with pathogens, and many other factors can modulate their concentrations. Counet et al. (*12*) found values in the same range, with 0.5 mg/kg *trans*-resveratrol and 1 mg/kg *trans*-piceid in their cocoa liquor from the Ivory Coast. On the other hand, this range is lower than the values reported by Hurst et al. (*27*) for baking chocolate products (i.e. from 3.81 to 4.20 mg/kg for *trans*-piceid). The use of a different preextraction procedure (acidic hydrolysis which could release bound fractions) and of UV detection (less selective than MS) may partially explain these differences. Moreover, as polyphenols are known to be degraded through fermentation (*6*, *36*), we can suspect that samples issued from less fermented cocoa beans (often used in the United States) were investigated.

**Identification of the Unknown Compound Eluting 0.5 min before *trans*-Piceid in Cocoa Liquors.** Worth stressing is the presence of another peak (peak 1 in **Figure 1A**), closely related to piceid, in the cocoa liquor MS/MS chromatograms (*m/z* 229). The identical MS/MS spectra for both peaks (after selecting either *m/z* 229 or *m/z* 391) suggest a glycoside structure. In *trans*-piceid equivalents, from 0 to 0.8 mg/kg of the unknown compound were quantified in the 22 investigated cocoa liquors (**Figure 2**).

A library of stilbene glucosides previously obtained by combinatorial chemistry (*28*) enabled us to rule out *trans*-resveratroloside and two *trans*-stilbene diglucosides (3,4′,5-trihydroxystilbene-3,4′-*O*-β-D-diglucopyranoside and 3,4′,5-trihydroxystilbene-3,5-*O*-β-D-diglucopyranoside) (**Table 1**), all exhibiting similar MS/MS (*m/z* 229) spectra but eluting at retention times different from that of the unknown compound. Methoxylated and tetrasubstituted analogues (*28*) also proved not to be potential candidates, because of their very distinctive mass spectra.

As galactoside phenols are known in cocoa, resveratrol galactosides were synthesized in the present work. Two monogalactosides and two digalactosides were characterized (RP-HPLC elution data given in **Figure 3** and **Table 1**). As previously emphasized for *trans*-piceid, the first fragmentation of the monogalactoside was the loss of the sugar, leading to an MS/MS spectrum (*m/z* 229) very similar to that of resveratrol and piceid (**Figure 4A**, **B**). The MS/MS spectra recorded after selecting the total molecular weight (*m/z* 391) also proved similar for glucosides and galactosides (**Figure 4C**, **D**).

The *trans*-resveratroloside-like galactoside revealed slightly more polar than the unknown on the reversed phase. Both digalactosides
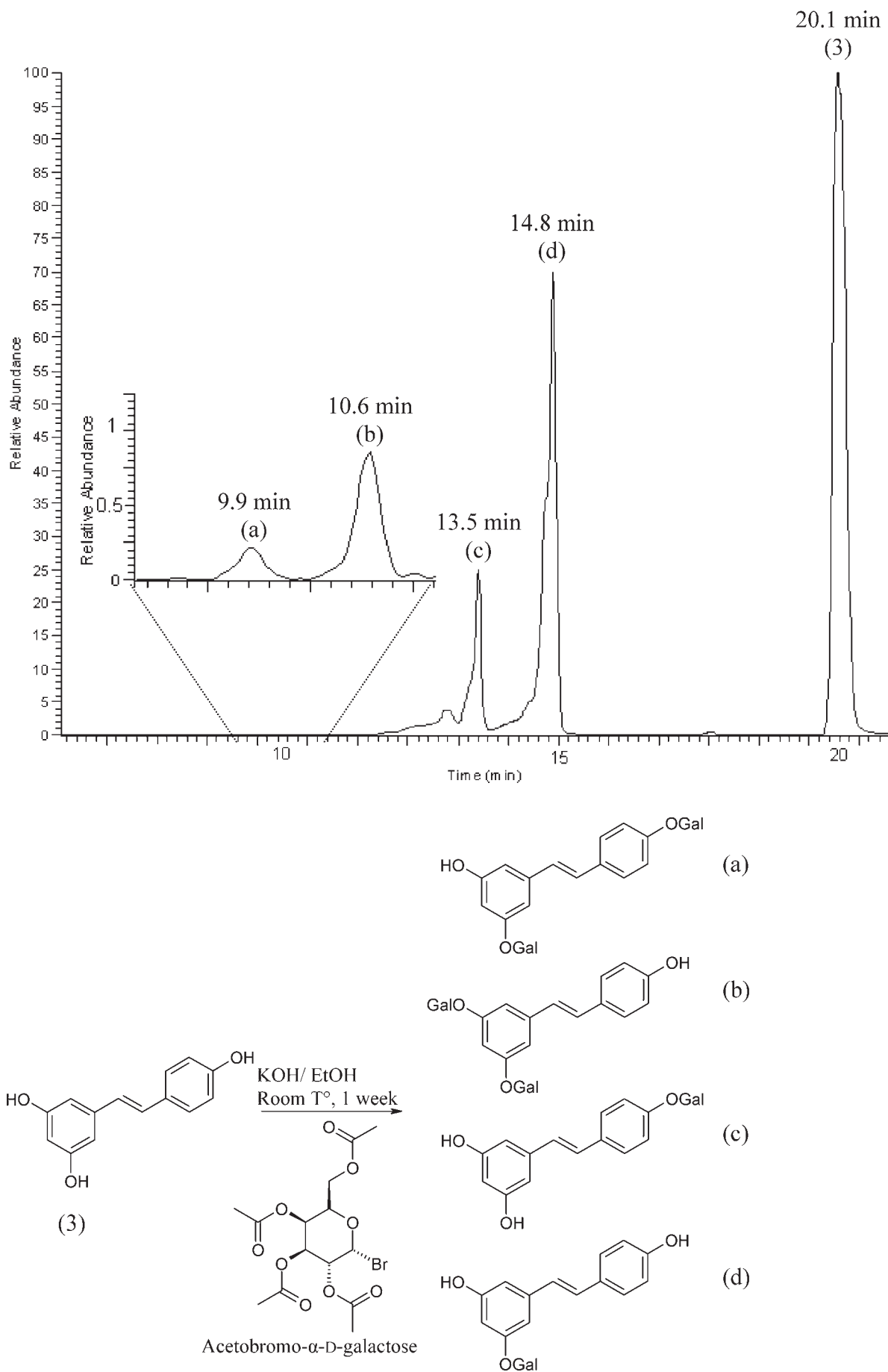
**Figure 3.** Synthesis scheme and RP-HPLC-APCI(+)-MS/MS chromatograms of synthesized galactosides (a, b, c, d) issued from *trans*-resveratrol (3).
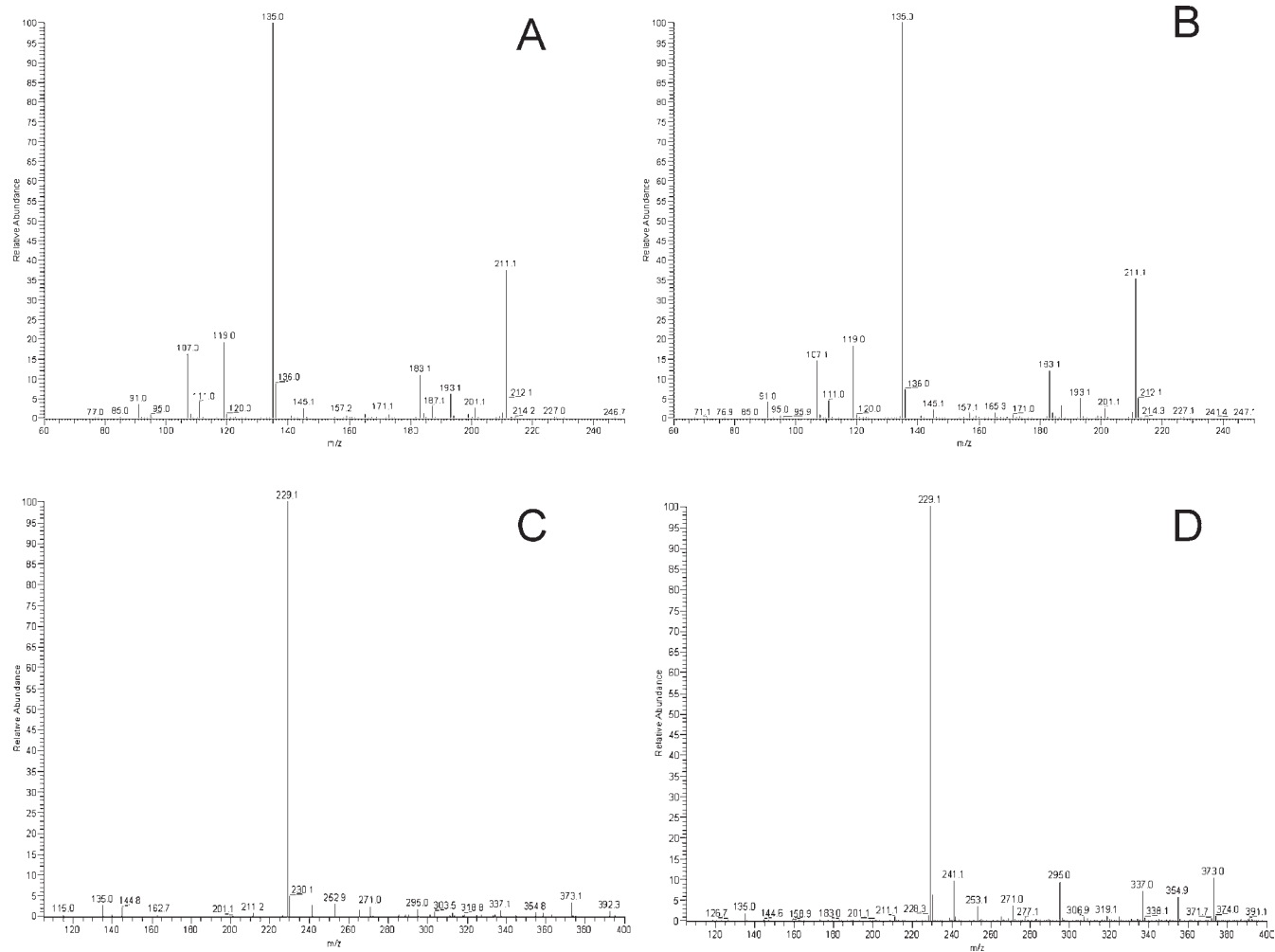
**Figure 4.** APCI($+$)-MS/MS spectra (*m/z* 229 for **A** and **B**, *m/z* 391 for **C** and **D**) of *trans*-piceid (**A**, **C**) and *trans*-piceid-like galactoside (**B**, **D**).
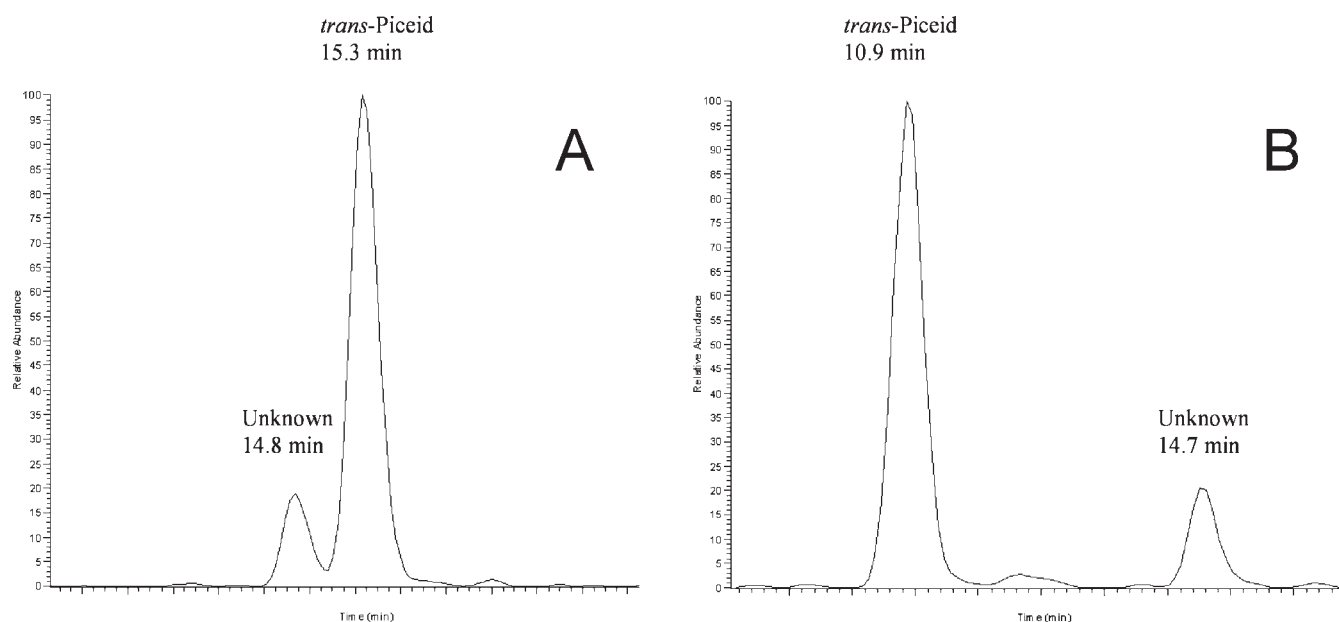


**Figure 5.** Elution of *trans*-piceid and the unknown in the Tobago 07 cocoa liquor extract by RP- (**A**) and NP- (**B**) HPLC-APCI($+$)-MS/MS (*m/z* 229).

were also ruled out because of their much higher polarity. On the other hand, the galactoside analogue of *trans*-piceid (RT = 14.8 min) (**Table 1**) revealed to coeluate with our unknown in RP-HPLC. However, complementary analysis performed on a polar phase led us to discard the piceid-like galactoside as well (less polar than the unknown: RT on Silica = 11.7 min against 14.7 min for the unknown) (**Figure 5** and **Table 1**).
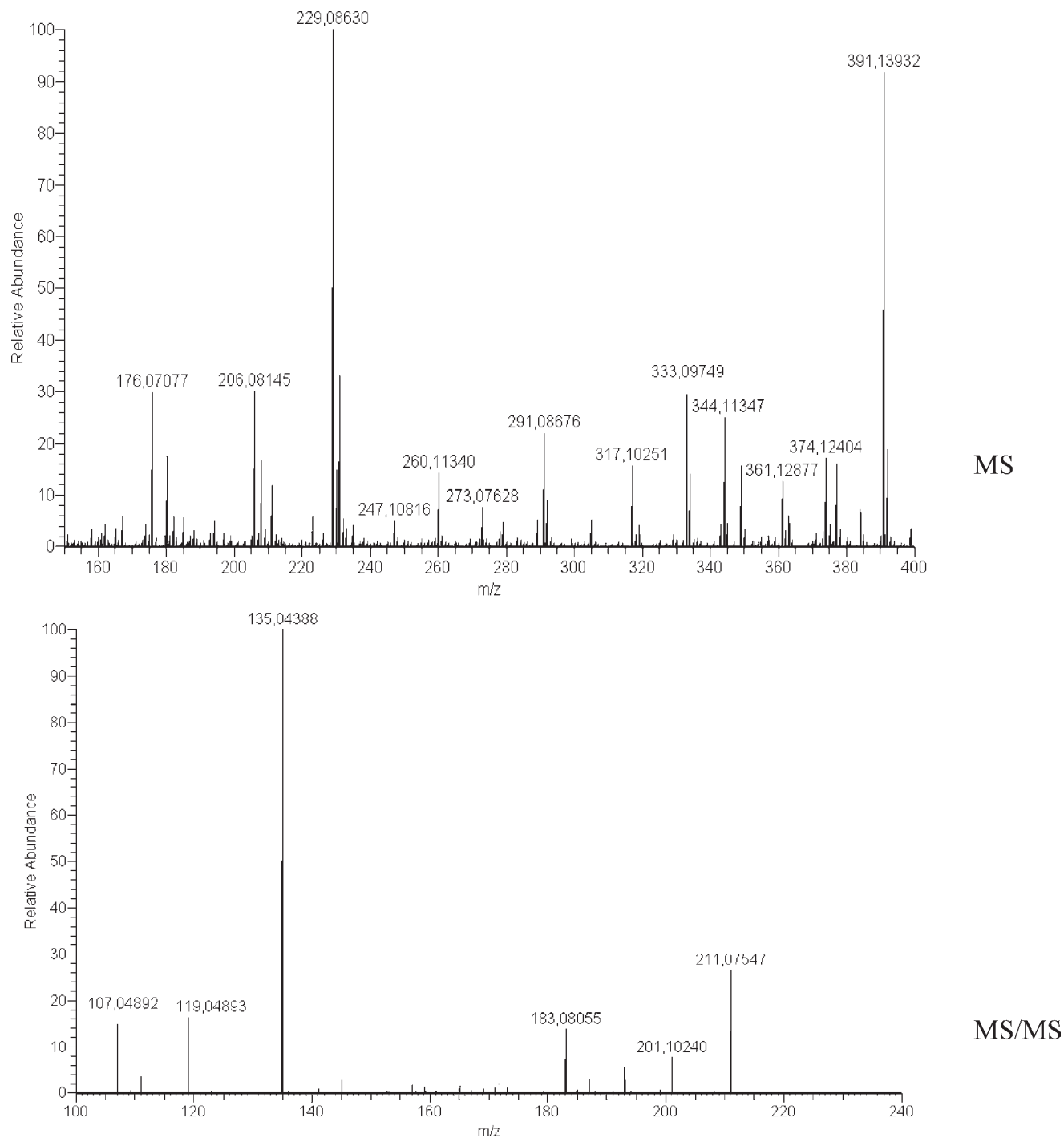
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*J. Agric. Food Chem.*, Vol. 58, No. 11, 2010    **7073**



**Figure 6.** HR-MS and -MS/MS data of the unknown in the Tobago 07 cocoa liquor extract.

LC-HRMS applied on the Tobago 07 extract confirmed an elemental formula of $C_{20}H_{23}O_8$ for the unknown compound (**Figure 6**, experimental mass of $m/z$ 391.13932), corresponding to a resveratrol hexoside structure. According to the HPLC retention times, a sugar slightly more polar than galactose is required. Mannose and fructose could be potential candidates. Unfortunately, the corresponding acetobromo reagents are not commercially available, requiring therefore another methodology to synthesize piceid-like mannoside and fructoside.

In conclusion, cocoa liquor is a significant source of stilbene hexosides, especially *trans*-piceid. Compared to the aglycons, stilbene glycosides are known for their higher resistance through the intestinal tract and for their better bioavailability (*5*). Therefore, both stilbene hexosides here investigated could be key elements for the nutritional properties of cocoa. More investigations are still needed to identify the sugar linked to resveratrol in the unknown compound.

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