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Identification and Quantification of Stilbenes in Fruits of Transgenic Tomato Plants (*Lycopersicon esculentum* Mill.) by Reversed Phase HPLC with Photodiode Array and Mass Spectrometry Detection

ISABELLA NICOLETTI,† ANTONELLA DE ROSSI,† GIOVANNA GIOVINAZZO,‡ AND DANILO CORRADINI*,†

Istituto di Metodologie Chimiche - CNR, Area della Ricerca di Roma 1, Via Salaria Km 29,300 Montelibretti, P.O. Box 10, 00016 Monterotondo Stazione (Rome), Italy, and Istituto di Scienze delle Produzioni Alimentari - CNR, Via Prov. Lecce-Monteroni, 73100 Lecce, Italy

Reversed-phase high-performance liquid chromatography (RP-HPLC) with photodiode array (PDA) and mass spectrometry (MS) detection was employed to study the accumulation of stilbenes and other naturally occurring polyphenol intermediates of flavonoid pathway in tomato fruits of plants genetically modified to synthesize resveratrol. The transgenic tomato fruits were obtained by overexpression of a grapevine gene encoding the enzyme stilbene synthase in tomato plants (*Lycopersicon esculentum* Mill.). Stilbenes and flavonoids, either glycosylated or free, were simultaneously identified by electrospray interface (ESI)-MS in negative ionization mode and were quantified by PDA detection at the wavelength corresponding to their maximum absorbance. The two detectors were coupled online with an HPLC system utilizing a narrow-bore C18 reversed-phase column, which was eluted by a multistep gradient of increasing concentration of acetonitrile in water containing 0.5% (v/v) formic acid. The results of these analysis revealed that the genetic modification of the tomato plants originated different levels of accumulation of four stilbenes (i.e., *trans*- and *cis*-piceid and *trans*- and *cis*-resveratrol) in their fruit depending on the stages of ripening. Either at immature or at mature stages of ripening the stilbenes were preferentially accumulated in the fruit peel as the glycosylated form. The highest amount of *trans*-piceid and *trans*-resveratrol were found in the peel of fruits harvested at mature stage of ripening. The variations in the levels of rutin, naringenin, and chlorogenic acid found in the samples extracted from the fruits of transgenic tomato plants, in comparison to that determined in the control lines, seemed to be related to the genetic transformation, whose effect on the flavonoid biosynthetic pathway needs to be elucidated by additional studies.

KEYWORDS: Transgenic tomato plants; *Lycopersicon esculentum* Mill.; stilbene; metabolic profiling; HPLC/PDA/MS

INTRODUCTION

Polyphenols are a large group of compounds containing multiple phenolic functionalities that are widely distributed in the plant kingdom and therefore form an integral part of the human diet. On the basis of their structure, polyphenols are divided in several classes, which include flavones, flavanols, flavonols, isoflavones, flavanones, chalcones, anthocyanidins, cinnamic acid derivatives, and stilbenes.

A significant role of polyphenols that has been widely investigated in recent years is their possible beneficial influence on human health. Many of the compounds comprising the above classes are potent antioxidants, and epidemiological studies have suggested a direct correlation between high polyphenols intake and reduced coronary heart disease mortality by suppressing the oxidation of low-density lipoprotein (*1*−*2*). Clinical studies have reported evidence that polyphenols may exhibit a great number of cell protective actions, such as modulation and induction of human cell receptors (*3*, *4*), enzymatic mechanisms of cell proliferation (*5*, *6*), and other beneficial effects against cancer (*7*, *8*), vasorelaxation (*9*), and allergy (*10*).

The increasing knowledge of polyphenol biosynthesis and the important function of these compounds in plants and human nutrition have made the biosynthetic pathways to these compounds excellent targets for metabolic engineering (*11*−*14*). Genetic engineering of plant secondary metabolism offers the opportunity to produce plants with improved nutritional char-

* To whom correspondence should be addressed. Phone: +39 06 90672254. Fax: +39 06 90672269. E-mail: danilo.corradini@imc.cnr.it.
† Istituto di Metodologie Chimiche.
‡ Istituto di Scienze delle Produzioni Alimentari.

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acteristics and as sources for compounds of pharmacological or nutritional value. One specific class of polyphenols that has significant potential for transgenic manipulation is the stilbenes, whose biosynthesis is controlled by the enzyme stilbene synthase (STS) (*15*). The expression of the gene encoding the STS enzyme has been obtained in several plants, including apple (*16*), papaya (*17*), lettuce (*18*), barley, and wheat (*19*).

In the development of food crops enriched with health-protective stilbene, an excellent candidate is tomato, one of the most important crops worldwide. In a recent study, transgenic tomato fruits were obtained by overexpression of a grapevine gene encoding the enzyme stilbene synthase in tomato plants (*Lycopersicon esculentum* Mill.) (*20*). The expression of the gene encoding the STS enzyme in tomato tissues caused the synthesis of new compounds, identified as *trans*-resveratrol and *trans*-piceid, and an increased total antioxidant capability (*20*). The establishment of a novel branch on flavonoid pathway through the introduction of stilbene synthase gene may induce a perturbation on the synthesis of other metabolites along the route, being the substrates for the enzyme stilbene synthase in common with chalcone synthase at the starting point of the pathway (*11*).

The aim of this paper was to develop a reliable and direct high-performance liquid chromatography (HPLC) method to study the accumulation of stilbenes and other naturally occurring polyphenol intermediates of flavonoid pathway in tomato genetically modified to synthesize resveratrol. The identification and quantification of polyphenols in plant extracts are generally carried out by reversed-phase HPLC with photodiode array (PDA) detection. Typically, the plant extracts are analyzed before and after acidic or enzymatic hydrolysis, and the level of the glycosilated polyphenols are estimated by subtracting the amount found in the unhydrolyzed sample from that determined after hydrolysis (*12*, *14*, *20*). The effectiveness of HPLC with mass spectrometric detection for analyzing polyphenols in plant extracts and food has been explored too (*21*−*23*).

The direct and simultaneous analysis of both free and glycosilated polyphenols in plant extracts was performed by reversed-phase HPLC using a narrow-bore column. To improve the identification of all analytes and to ensure the absence of interfering peaks in the sample extracted from the tomato fruits, mass spectrometric (MS) detection, with an electrospray interface (ESI), was included in addition to PDA detection. The synthesis of *trans*-resveratrol and *trans*-piceid and the accumulation of cinnamic acid derivatives (chlorogenic acid, ferulic acid, caffeic acid) of flavonols (quercetin, rutin) and flavanones (naringenin) were investigated in fruit peel and whole fruit at two stages of ripening (green and red), and the results were compared with their controls.

MATERIALS AND METHODS

Chemicals and Standards. HPLC grade solvents and analytical-reagent grade formic acid were purchased from Carlo Erba (Milan, Italy). Deionized water was obtained by a Milli-Q unit (Millipore, Bedford, Ma). Chlorogenic acid, ferulic acid, caffeic acid, quercetin, and *trans*-resveratrol were purchased from Sigma (St. Louis, MO). Rutin, myricetin, naringenin, and kaempferol were obtained from Extrasynthese (Genay, France) and *trans*-piceid was supplied by Polyphenols Laboratories AS (Sandnes, Norway).

Plant Material. *Lycopersicon esculentum* Mill. (cv. Money Maker) plant tissues were utilized in all experiments. Details of the method used to produce transgenic tomatoes and their controls have been described by Giovinazzo et al. (*20*). Transgenic plants were maintained in vitro by somatic propagation. Rooted primary transformants were transferred to soil and were grown in a glasshouse at 28 °C with diurnal cycle of 16 h light and 8 h darkness.

Sample Preparation. Samples of tomato fruits both transgenic and wild type were obtained from three pairs of plants grown in a glasshouse. Three fruits were harvested from each plant at two different stages of ripening (immature and mature stages of ripening, referred to as green and red fruit). The outer layer of 2−3 mm thickness was separated from the fruit and was classified as peel. The fruit peel and the whole fruit were frozen in liquid nitrogen and were stored protected from light at −80 °C until sample extraction. Equal amounts of freeze-dried fruit tissues obtained from three individual fruits harvested from different plants (one for each fruit) were pooled to minimize sample variation. Amounts of 500 mg of the pooled samples were subjected to extraction with 5 mL of 70% (v/v) methanol−water mixture in a flask protected from light on a rotary shaker at room temperature for 1 h. The extracts were centrifuged at 13 000*g* for 10 min and the supernatant was dried under a stream of nitrogen and was stored at −80 °C until dissolution in 80% (v/v) methanol−water mixture just before analysis.

Liquid Chromatography and Electrospray Mass Spectrometry. Quantitative analysis was carried out using a Shimadzu LC-10A_{VP} HPLC system consisting of an SCL-10A_{VP} system controller, two LC-10AD_{VP} solvent delivery units, a SPD-M10A spectrophotometric diode array detector, a CTO-10AS_{VP} column oven, a DGU-14A online vacuum membrane degasser, and a Rheodyne (Cotati, CA) model 8125 semi-microinjection valve with a 5 μL sample loop. Data were processed using the Shimadzu Class VP 5.6 HPLC data system on a Pentium II 400 PC compatible computer. The compounds were separated using a Polaris C18A column (150 × 2.0 mm i.d, 5 μm; (Varian Inc., Lake Forest, CA) in conjunction with a C18 (30 × 2 mm, 5 μm) guard cartridge column. The column temperature was 30° ±1 °C. Separations were performed by a multistep gradient of increasing concentration of acetonitrile in water acidified with 0.5% (v/v) formic acid, at a flow rate of 0.2 mL/min, according to the following program: 8 min linear gradient from 10 to 18% (v/v) acetonitrile in water containing 0.5% (v/v) formic acid, followed by 2.0 min isocratic with 18% (v/v) acetonitrile, 5.0 min linear gradient from 18 to 25% (v/v) acetonitrile, 3.0 min linear gradient from 25 to 35% (v/v) acetonitrile, 10 min isocratic with 35% (v/v) acetonitrile, and 2.0 min increasing gradient segment to 60% (v/v) acetonitrile to elute any strongly retained components of natural samples. Then, the eluent composition was brought to the initial condition in 1 min, and the column equilibrates for 12 min before the next injection. UV−vis spectra were recorded in the 210−600 nm range, and the chromatograms were acquired at 280, 320, 306, and 370 nm.

The same column, at temperature of 30 ±1 °C, and gradient elution program described above were employed to confirm the identification of the selected analytes in real samples by HPLC-ESI-MS. The experiments were performed with a second Shimadzu HPLC instrument consisting of a high-performance liquid chromatograph/mass spectrometer model LCMS-2010 unit, comprising a SCL-10A*vp* system controller, two pumps model LC-10AD*vp* solvent delivery module, a SPD-M10Avp UV/vis photodiode array detector, and a single quadrupole mass analyzer model 2010 equipped with an electrospray (ESI) interface with nitrogen as the nebulizing and drying gas. Initial experiments were carried out with the ESI interface employed in both positive and negative ionization mode with different fragmentation voltages. Optimized conditions were determined by flow injection analysis (FIA) of standard solutions of the analytes at three different concentrations ranging from 0.1 to 50 mg/L. On the basis of the results of these experiments, MS acquisition was performed with the ESI interface in the negative ionization mode at the following conditions: nebulizing gas nitrogen at flow rate of 5.0 L/min; temperature of block heater, 200 °C; temperature of the curved desolvation line (CDL), 225 °C; probe voltage, −5 kV; CDL voltage, −54 V; Q-array voltages, −70, −70, −60 V; full scan *m*/*z* 100/500 were obtained every 0.5 s. System control and data processing were carried out by the Shimadzu LCMS solution software running on a Pentium IV personal computer (Gigabyte, Milan, Italy). Samples were introduced into the column by a Rheodyne model 8125 semi-microinjection valve with a 5 μL sample loop. The flow rate was 0.2 mL/min, and column effluent was first passed through the PDA detector before being directed to the quadrupole mass spectrometer with ESI interface.

Table 1. Repeatability and Reproducibility of Retention Times of Polyphenols

	repeatability					reproducibility			
	time			area		time		area	
compound	min	SD	RSD%	SD	RSD%	SD	RSD%	SD	RSD%
chlorogenic acid	6.879	0.038	0.56	11 283	0.87	0.026	0.38	15 430	1.19
caffeic acid	8.372	0.061	0.73	42 712	1.93	0.035	0.42	25 770	1.16
ferulic acid	13.118	0.069	0.52	48 825	2.05	0.056	0.42	34 305	1.43
trans-piceid	13.837	0.073	0.53	27 237	1.99	0.052	0.38	18 537	1.35
rutin	14.479	0.049	0.34	14 206	1.77	0.044	0.31	13 510	1.68
myricetin	18.704	0.047	0.25	25 006	1.91	0.040	0.22	70 238	5.02
trans-resveratrol	19.707	0.029	0.15	57 520	1.69	0.024	0.12	38 357	1.12
quercetin	20.980	0.024	0.11	40 108	2.52	0.022	0.11	55 003	3.78
naringenin	22.110	0.031	0.14	22 285	2.04	0.025	0.11	12 752	1.16
kaempferol	23.562	0.050	0.21	41 822	3.15	0.044	0.19	60 004	4.28

Identification and Quantification of Polyphenols. The identification of individual analytes was performed on the basis of their retention times and both spectroscopic and mass spectrometric spectra. Stock solutions of each standard compound were prepared by dissolving weighted amounts of each standard in 80% (v/v) methanol−water mixture. These solutions were individually injected into the HPLC column and were eluted with the above gradient elution method determining their chromatographic retention times and collecting both UV and MS spectra by the PDA and MS detector, respectively. The stock solutions were diluted with the 80% (v/v) methanol−water mixture to obtaining six working solutions of each compound covering the following concentration ranges: chlorogenic acid, 0.31−500 mg/L; caffeic acid, 0.15−15 mg/L; ferulic acid, 0.22−45 mg/L; *trans*-piceid, 0.3−600 mg/L; rutin, 0.25−200 mg/L; myricetin, 0.1−50 mg/L; *trans*-resveratrol, 0.44−35 mg/L; quercetin, 0.35−35 mg/L; naringenin, 0.44−350 mg/L; kaempferol, 0.5−50 mg/L. Quantification of individual compounds was performed by the external standard method using a six-point regression curve of the UV absorption data collected at the following wavelength of maximum absorbance of each analyte: chlorogenic acid, caffeic acid, and ferulic acid, 320 nm; *trans*-resveratrol and *trans*-piceid, 306 nm; rutin, myricetin, quercetin, and kaempferol, 370 nm; naringenin, 280 nm. The *cis*-resveratrol and *cis*-piceid peaks were identified by comparing their retention time and UV spectra with the new peak appearing after UV irradiation of *trans*-resveratrol and *trans*-piceid standard solution, respectively. As *cis*-resveratrol and *cis*-piceid are not commercially available, calibration curves for these compounds were obtained at 285 nm (maximum absorption for these compounds) with the same solutions used for the *trans*-isomer, after exposure of the solution to UV light at 366 nm for 30 min, which is the time necessary to determine the conversion of at least 90% of the *trans*- to *cis*-isomer. The quantity of this compound was ascertained on the basis of the decrease in the *trans*-isomer following UV irradiation (*24*). The concentrations of the standard solution previously reported for *trans*-resveratrol were obviously corrected for the percentage of conversion obtained (*25*). As it is known from the literature (*26*−*28*), the UV spectra of the resveratrol glucosides are very close to that of resveratrol aglycons, therefore, the quantification of *trans*- and *cis*-piceid was based on the assumption of identical molar extinction coefficient of the *trans*- and *cis*-resveratrol at 306 and 285 nm, respectively. Peak purity was checked to exclude any contribution from interfering peaks.

RESULTS AND DISCUSSION

Optimization of Chromatographic Separation. The analytical strategy employed in this study was focused on the combined use of reversed-phase HPLC coupled online with both a photodiode array detector and a mass spectrometer equipped with an electrospray ion source (ESI-MS) to obtain resolution, identification, and quantification of the polyphenols molecules, characterizing the flavonoid pathway in tomato fruits (**Table 1**). Initial phases of the investigation were focused on the optimization of the chromatographic conditions required to obtain the selective resolutions of these compounds by RP-HPLC using a Polaris C-18A column. To ensure appropriate resolution of the compounds of interest and the absence of interfering peaks in samples extracted from tomato fruits, the experiments were performed subjecting to chromatography standard mixtures of the selected compounds and real samples obtained as described in the Materials and Methods.

In RP-HPLC-ESI-MS, the chemical composition of the mobile phase plays an important role both on the chromatographic separation (*29*, *30*) and on spray stability and signal sensitivity (*31*, *32*). In addition, sample detection by ESI-MS restricts the use of buffers and other mobile-phase modifiers to volatile compounds. Thus, several factors such as gradient shape, flow rate, type, and concentration of the acidic additive of the hydro-organic mobile phase were optimized before testing the utility of online ESI-MS to confirm peak identity.

Acidic mobile phases were considered to suppress the ionization of carboxylic compounds and favorite their hydrophobic interaction with the C-18 stationary phase. The two volatile acidic mobile phase additives formic acid and acetic acid were evaluated for this purpose, testing their capability in resolving the secondary metabolites extracted from tomato fruits, both wild type and transgenic, in combination with either methanol or acetonitrile as the organic modifier employed for increasing the eluotropic strength of the mobile phase during gradient elution.

The optimum conditions for chromatographic resolution and sample detection by ESI-MS were obtained with 0.5% (v/v) formic acid and acetonitrile as the aqueous and organic components of the mobile phase, respectively, using the multisegments elution gradient program described in the Materials and Methods. The chromatogram displayed in **Figure 1** shows the separation of a test mixture of the selected analytes, obtained under the optimized chromatographic conditions. The analytes were completely resolved in less than 26 min with highly repeatable retention times, as reported in **Table 1**.

Identification and Quantification of the Analytes. The identification of the selected compounds in the mixture of the secondary metabolites extracted from transgenic and wild-type tomato fruits were preliminarily performed on the basis of their retention times and UV spectra acquired in the wavelength range comprised between 210 and 600 nm with the photodiode array (PDA) detector. The *cis*-resveratrol and *cis*-piceid were identified by comparison of the retention times and UV spectra of the additional peaks observed after UV irradiation at 366 nm of *trans*-resveratrol and *trans*-piceid, respectively (see Materials and Methods). **Figure 2** shows the separation of a mixture of *trans*-piceid and *trans*-resveratrol before (**A**) and after (**B**) the exposure of the solution to UV light at 366 nm

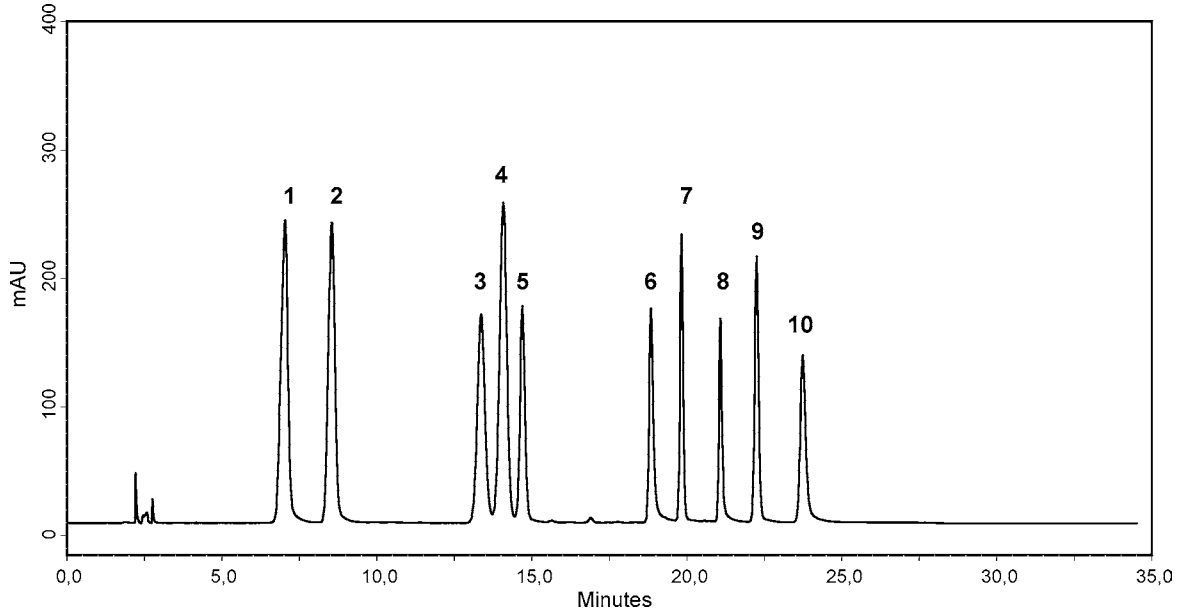

Figure 1. Separation of a test mixture comprising stilbenes, flavonols, flavanones, and cinnamic acid derivatives. Column, Polaris C18A (150 × 2.0 mm I.D, 5 μm) with C18 (30 × 2 mm, 5 μm) guard cartridge, eluted by the multistep gradient reported in the Materials and Methods; detection by PDA at 306 nm, temperature 30 ±1 °C. Identification of peaks: 1, chlorogenic acid; 2, caffeic acid; 3, ferulic acid; 4, *trans*-piceid; 5, rutin; 6, myricetin; 7, *trans*-resveratrol; 8, quercetin; 9, naringenin; 10, kaempferol.

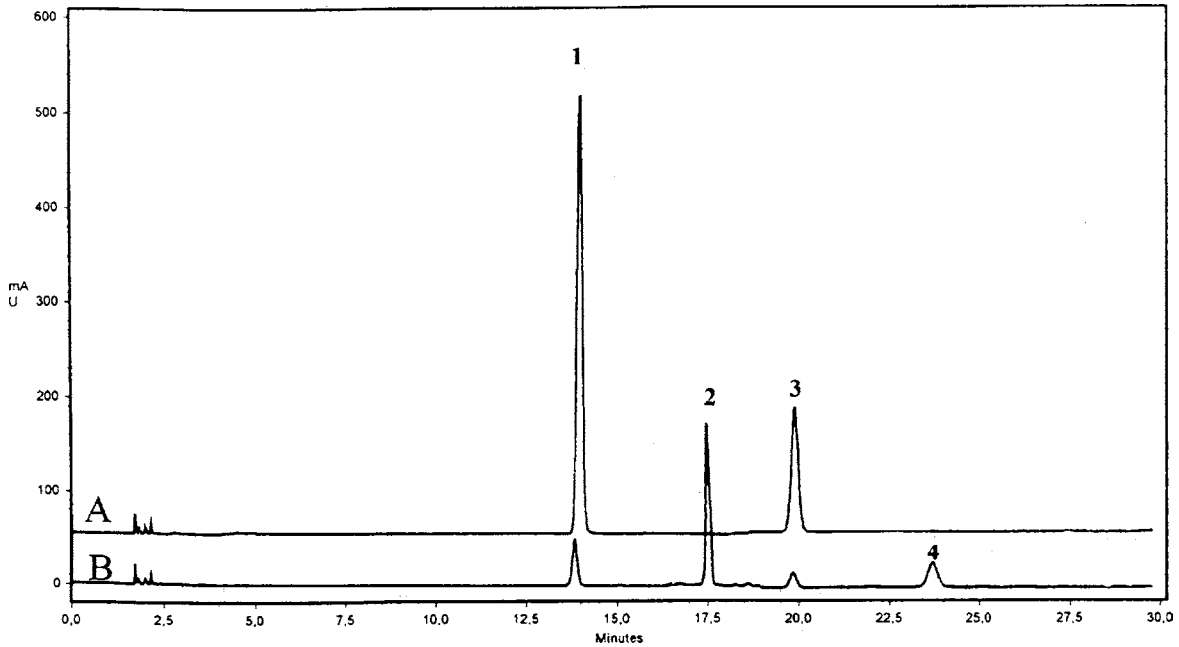

Figure 2. Separation of a mixture of *trans*-piceid and *trans*-resveratrol before (**A**) and after (**B**) the exposure of the solution to UV light at 366 nm for 30 min. Column and experimental condition as in **Figure 1**. Identification of peaks: 1, *trans*-piceid; 2, *cis*-piceid; 3, *trans*-resveratrol; 4, *cis*-resveratrol.

for 30 min. Both chromatograms were detected at 306 nm. The additional peaks detected after UV irradiation of the standard solution were identified as *cis*-piceid (peak 2) and *cis*-resveratrol (peak 4) by comparing their UV and mass spectrum to those of *trans*-piceid and *trans*-resveratrol, respectively. These additional peaks are drastically smaller than those of *trans*-resveratrol and *trans*-piceid because of the molar adsorptivity of the *cis*-isomers at 306 nm, which is about 3.4 times lower than that of the *trans*-forms (*24*).

For all analytes, peak identification was confirmed by carrying out ESI-MS detection in the single-ion monitoring (SIM) mode in conjunction with PDA detection, which resulted in the appearance of signals at *m*/*z* values corresponding to the main ion of the selected analytes in correspondence to the peaks of the PDA chromatogram. Stilbenes and phenolic compounds were detected by ESI-MS in the negative ionization mode, which resulted in higher sensitivity and lower background noise than in positive mode, at the conditions reported in the Materials and Methods that were optimized by flow injection analysis of standard solutions of the analytes at concentrations ranging between 0.1 and 50 mg/L.

Table 2 reports the *m*/*z* values of the major ions observed for each analyte at optimized values of applied voltages at the three-stage high-frequency lens system (Q-array), which is located in the area of the MS detector where pressure is relatively high immediately after ions have been introduced into

Table 2. Ions Observed in Negative ESI-MS for Standard Polyphenols[a]

compound	MW	main ions observed
chlorogenic acid	354.31	353
caffeic acid	180.16	179 (135)
ferulic acid	194.18	193
rutin	610.51	609
trans-piceid	390.38	389 (227)
trans-resveratrol	228.22	227
quercetin	302.24	301
naringenin	272.25	271
kaempferol	286.24	285

[a] MW, molecular weight. Ions of lower abundance are reported in parentheses.

vacuum and ion fragmentation may occur. It is observed that with applied voltages of −70, −70, and −60 V at Q-array 1, Q-array 2, and Q-array 3, respectively, ion fragmentation was limited and almost all analytes produced mass spectra with the base peak corresponding to the deprotonated species $[M - H]^-$, which is referred to as the molecular ion.

Quantification of the identified analytes was performed by HPLC-PDA detection using the external standard method with six-point calibration graphs, which were constructed by plotting concentration of the standard samples in mg/L as a function of peak area, detected at the wavelength corresponding to their maximum absorbance. Linear least-squares regression analysis was employed to calculate slope, intercept, and correlation coefficient of the calibration graphs that are reported in **Table 3**. This table also reports for each analyte the limit of detection (LOD), determined from the amount of analytes required to give a signal-to-noise ratio of 3, and the limit of quantification, defined as the lowest concentration giving a signal-to-noise ratio of 10.

The repeatability and precision of the method was assessed by analyzing 10 repeated times standard mixtures (30 mg/L each analyte) during the same day and over a period of a week. In the latter case, the measured values, representing the means of five determinations per day and per analyte, were used for the evaluation of the overall between-day precision for the method. Data are reported in **Table 1**, and the accuracy was assessed through quintuplicate analysis of samples containing known amounts of the detected analytes, which resulted in relative standard deviation (RSD) values lower than 5.0%.

Occurrence of Polyphenols in Transgenic Tomato Fruits. The developed RP-HPLC method, employing electrospray ionization (ESI) mass spectrometry (MS) to identify and PDA detection to quantify stilbenes and other occurring polyphenols in plant extracts, offers the advantage of allowing the direct and simultaneous analysis of resveratrol and its glycosilated forms, which in a previously proposed method (*20*) were separately analyzed before and after enzymatic hydrolysis of samples, as currently reported in the literature (*12*−*14*). In addition, the employment of a narrow-bore column allows working with samples of small size and operating at the low mobile phase flow rate requested by the ESI interface, which has recently proven to be a powerful tool for the identification and characterization of stilbenes and flavonoids by online mass spectrometry detection (*23*, *28*). The method was employed to examine the accumulation of stilbenes in fruits of tomato genetically modified to synthesize stilbenes. The amounts of cinnamic acid derivatives (chlorogenic acid, ferulic acid, caffeic acid), flavonols (quercetin, rutin), and flavanones (naringenin) present in the fruits of wild-type tomato were determined to monitor any alteration in the levels of these metabolites as a consequence of introducing a new branch along the flavonoid pathway. The analysis was carried out on samples extracted from the peel, and whole fruit was harvested at immature and mature stages of ripening, and the results were compared with those determined in their wild-type counterpart.

The separation of the components of the sample extracted from the peel of transgenic red tomato fruits obtained by HPLC-ESI-MS in negative full-scan mode is depicted by the total ion current (TIC) chromatogram reported in **Figure 3**. The identification of the peaks detected under full-scan conditions was obtained analyzing the extracted-ion chromatograms of the ion current at *m*/*z* values corresponding to the $[M - H]^-$ ions of the individual investigated compounds (see **Table 2**).

Acquiring the extracted-ion chromatogram of the ion current at *m*/*z* 389, corresponding to the $[M - H]^-$ ion, evidenced the presence of two peaks at retention times of 14.2 and 17.6 min, attributable to *trans*- and *cis*-piceid, the monoglycosylated form of resveratrol (MW 390). The identity of these peaks was further investigated by acquiring the extracted-ion chromatogram of the ion current at *m*/*z* 227, corresponding to the $[M - H - 162]^-$ ion resulting from the loss of a 162 mass fragment, equivalent to the hexose group of the glycosylated *trans*- and *cis*-resveratrol. These signals appeared in correspondence to the peaks acquired by SIM detection mode of the ion current at *m*/*z* 389, confirming their attribution to *trans*- and *cis*-piceid, respectively, whose elution order was deduced by comparing their retention times with those of the authentic standard *trans*-piceid, before and after UV irradiation at 366 nm, respectively.

The extracted ion chromatogram of the ion current at *m*/*z* 227 showed the presence of another peak at retention time of 19.8 min, which was attributed to *trans*-resveratrol (MW 228.22). The SIM detection mode allowed the attribution of the peaks at retention times of 6.9, 8.4, 14.5, 22.11, and 23.6

Table 3. Calibration Data, Wavelength of Detection, and Limits of Quantification (LOQ) and Detection (LOD) for the Selected Polyphenols

compound	λ (nm)	linear range (mg/L)	regression equation[a]	correlation coefficient	LOQ (μg/L)	LOD (μg/L)
chlorogenic acid	320	0.31–500	$y = 0.39x + 0.50$	0.9992	196	70
caffeic acid	320	0.15–15	$y = 10.03x - 0.22$	0.9999	80	27
ferulic acid	320	0.22–45	$y = 9.10x - 0.36$	0.9996	110	40
trans-piceid	285	0.3–600	$y = 0.54x + 0.64$	0.9998	105	45
rutin	370	0.25–200	$y = 3.02x - 0.32$	0.9974	165	55
cis-piceid	285	0.1–1.86	$y = 1.88x + 0.14$	0.9996	105	45
myricetin	370	0.5–50	$y = 0.59x - 0.88$	0.9973	420	180
trans-resveratrol	306	0.44–35	$y = 1.35x - 0.34$	0.9928	25	10
quercetin	370	0.35–35	$y = 6.64x - 0.84$	0.9995	230	80
naringenin	280	0.44–350	$y = 4.10x - 0.98$	0.9977	145	50
kaempferol	370	0.5–50	$y = 0.60x - 0.81$	0.9986	410	175

[a] y expresses the detection response (peak area in arbitrary units) and x the concentration for polyphenols (in mg/L).

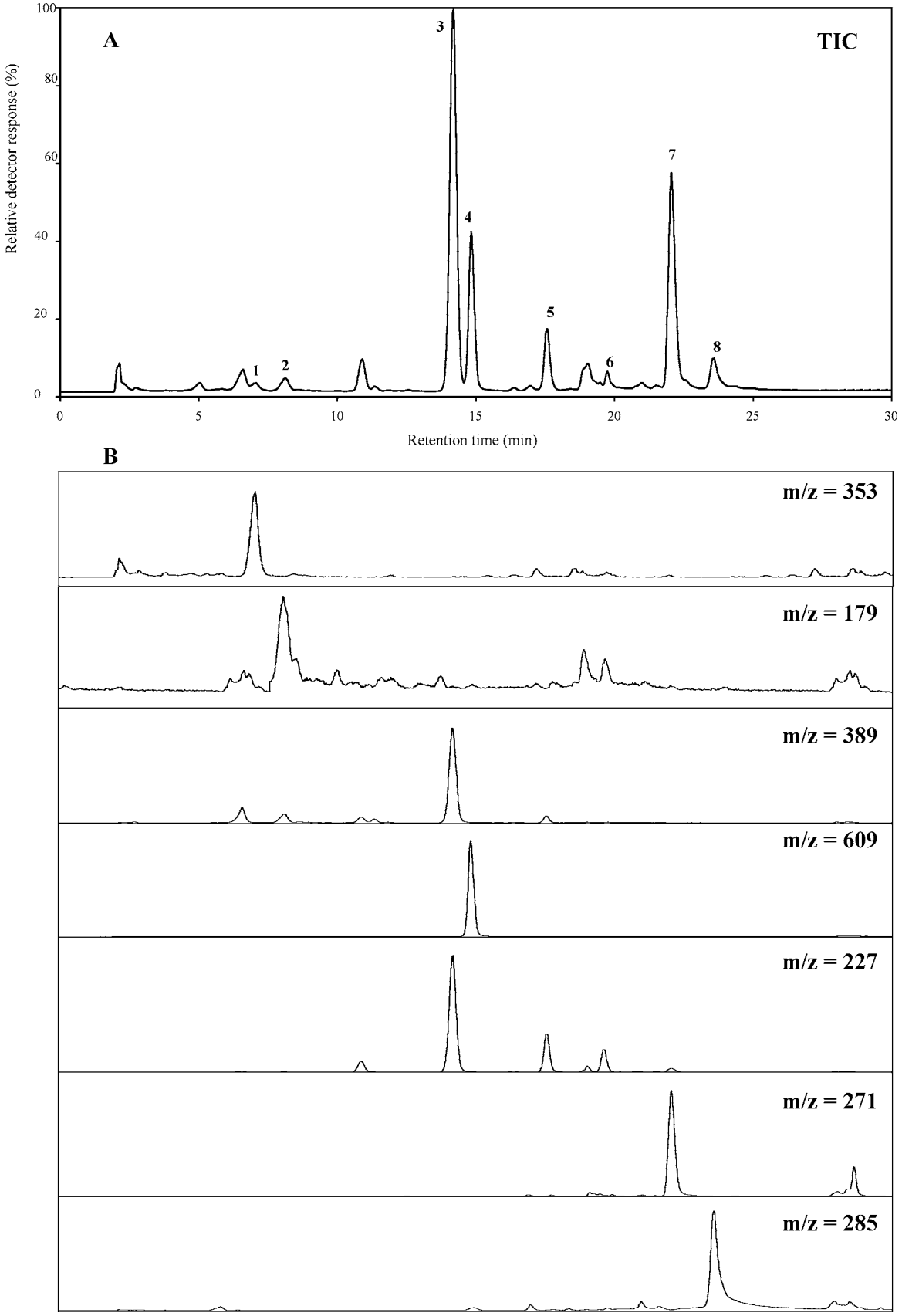

Figure 3. HPLC-ESI-MS of the sample extracted from the peel of transgenic red tomato fruits: (**A**) total ion current chromatogram (TIC) acquired in negative full-scan mode; (**B**) ion chromatograms extracted at *m*/*z* values corresponding to the $[M - H]^-$ ion of each identified polyphenol. Column and elution gradient program as in **Figure 1**; ESI-MS conditions as in Materials and Methods. Identification of peaks: 1, chlorogenic acid; 2, caffeic acid; 3, *trans*-piceid; 4, rutin; 5, *cis*-piceid; 6, *trans*-resveratrol; 7, naringenin; 8, kaempferol.

min as chlorogenic acid, caffeic acid, rutin, naringenin, and kaempferol, respectively. Peak retention times and UV−vis spectra acquired in the wavelength range comprised between 210 and 600 nm with the photodiode array (PDA) detector were

Table 4. Quantification of Polyphenols in Wild-Type and Transgenic Tomato Fruits

	green fruit				red fruit			
	whole		peel		whole		peel	
compound	wild type	transgenic	wild type	transgenic	wild type	transgenic	wild type	transgenic
chlorogenic acid	35.39 ± 0.87	22.31 ± 0.62	1.67 ± 0.04	10.41 ± 0.57	19.71 ± 0.65	2.74 ± 0.57	nd	0.82 ± 0.01
caffeic acid	nd	nd	nd	nd	0.17 ± 0.01	nd	0.26 ± 0.01	0.58 ± 0.02
ferulic acid	1.16 ± 0.01	0.21 ± 0.02	nd	nd	0.19 ± 0.01	nd	1.21 ± 0.01	0.91 ± 0.01
rutin	11.43 ± 0.19	3.55 ± 0.09	30.0 ± 1.65	26.24 ± 1.43	4.36 ± 0.12	3.27 ± 0.10	80.3 ± 2.86	39.13 ± 0.93
quercetin	0.23 ± 0.01	1.05 ± 0.04	nd	nd	nd	nd	nd	nd
trans-piceid	nd	21.52 ± 0.15	nd	54.25 ± 1.21	nd	21.16 ± 2.57	nd	126.58 ± 3.46
cis-piceid	nd	2.26 ± 0.03	nd	nd	nd	0.52 ± 0.01	nd	nd
trans-resveratrol	nd	0.79 ± 0.01	nd	nd	nd	15.82 ± 0.58	nd	48.48 ± 0.99
cis-resveratrol	nd	0.42 ± 0.02	nd	2.52 ± 0.02	nd	nd	nd	nd
naringenin	1.04 ± 0.03	0.38 ± 0.01	nd	nd	23.92 ± 2.34	8.42 ± 0.26	31.68 ± 0.38	13.2 ± 1.25

also employed to further confirm the peak identity attributed by HPLC-ESI-MS.

As expected, stilbenes were not detected in the extracts of wild-type whole fruit and fruit peel, either at immature or at mature stages of ripening. The concentrations of stilbenes in the transgenic fruits as well as those of polyphenols in both transgenic and wild-type fruits were determined by the HPLC-PDA method described above. The results are reported in **Table 4** as mg/kg of fresh weight. *cis*-Piceid, which was detected in the peel of transgenic red tomato fruits by ESI-MS (see **Figure 3**), was not quantified in this sample because its amount was below the limit of quantification by PDA detection.

The results of these analysis revealed that the genetic modification of the tomato plants originated different levels of accumulation of four stilbenes (i.e., *trans*- and *cis*-piceid and *trans*- and *cis*-resveratrol) in their fruit depending on the stages of ripening and fruit samples (whole or peel). Both at immature and at mature stages of ripening, the stilbenes were preferentially accumulated in the fruit peel and the highest amount of *trans*-piceid and *trans*-resveratrol was found in the peel of fruits harvested at mature stage of ripening, being 126.58 and 48.48 mg per kg of fresh weight of fruit peel, respectively. The results indicate that the novel antioxidant molecules preferentially accumulate in the fruit peel and are stored in the glycosylated form, according to Giovinazzo et al. (*20*). In a recent paper reporting the results of a study on tomato genetically modified to synthesize stilbenes, Schijlen et al. (*33*) reported opposite conclusions regarding the relative amounts of aglycon and glycosilated resveratrol, without specifying which geometrical isomer (either *trans*- or *cis*-form) was detected in the fruit peel.

The quantification of polyphenols naturally occurring in tomato fruits revealed no appreciable differences in the concentrations of caffeic and ferulic acid in any sample extracted from peel and whole fruit of either transgenic or wild-type tomato plant. The concentration of these polyphenols was never greater than 1.21 mg per kg of fresh weight and in some samples was below the limit of quantification.

Significant accumulation of chlorogenic acid was determined in the whole fruit, especially at immature stage of ripening, which was 35.39 and 22.31 mg per kg of fresh weight in wild-type and transgenic fruit, respectively. These values were lower in red tomatos, where the content of chlorogenic acid was again lower in transgenic fruit (2.74 mg per kg of fresh weight) than in the control (19.71 mg per kg of fresh weight).

The most abundant flavonoids determined in the samples extracted from peel and whole fruit of control lines harvested at mature stage of ripening were the flavonol rutin and the flavanone naringenin. The levels of accumulation of these flavonoids appeared to be lowered in transgenic fruit peel and whole fruit as compared to the equivalent samples from the wild-type lines. The content of naringenin in peel and whole red transgenic fruit was 13.2 and 8.42 mg per kg of fresh weight, respectively, whereas in the controls the amount of naringenin was 31.68 and 23.92 mg per kg of fresh weight, respectively. The flavonol rutin, which is the glycosylated form of quercetin, lowered the levels of accumulation in transgenic fruit to 39.13 and 3.27 mg per kg fresh weight in the peel and whole fruit, respectively, from the content of 80.32 and 4.36 mg per kg fresh weight that was determined in the equivalent samples of wild-type plants. In both wild-type and in transgenic fruit, quercetin was below the limit of detection, with the exception of samples extracted from the whole green fruit where this flavonol was 1.16 and 0.21 mg per kg fresh weight in wild-type and transgenic plants, respectively.

In conclusion, the results of this work demonstrate that RP-HPLC utilizing a narrow-bore C18 columns and employing ESI-MS and PDA detection for peak identification and quantification, respectively, allows the unambiguous recognition and reliable quantification of *trans*- and *cis*-isomers of resveratrol and its glycosylated form in the fruits of tomato plants overexpressing a grape stilbene synthase gene. Our investigation indicates that the highest accumulation of stilbenes is located in the peel of fruits harvested at mature stages of ripening and that stilbenes are preferentially stored in their glycosylated form. The differences in the levels of rutin, naringenin, and chlorogenic acid found in the samples extracted from the fruits of transgenic tomato plants, in comparison to those determined in the control lines, seem to be related to the genetic transformation. On the other hand, the comparable levels of caffeic and ferulic acid determined in the fruits of both plants are indicative of a limited effect on the biosynthesis of these metabolites. Therefore, additional investigations need to be undertaken to elucidate the influence of the overexpression of the grape stilbene synthase gene on the flavonoid biosynthetic pathway.

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LITERATURE CITED

(1) Curin, Y.; Andriantsitohaina, R. Polyphenols as potential therapeutic agents against cardiovascular diseased. *Pharmacol. Rep.* **2005**, *57* (suppl.), 97−107.

(2) Meyer, A. S.; Heinonen, M.; Frankel, E. N. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chem.* **1998**, *61*, 71−75.

(3) Ashida, H.; Fukuda, I.; Yamashita, T.; Kanazawa, K. Flavones and flavonols at dietary levels inhibit a transformation of aryl hydrocarbon receptor induced by dioxin. *FEBS Lett.* **2000**, *476*, 213−217.

(4) An, J.; Tzagarakis-Foster, C.; Scharschmidt, T. C.; Lomri, N.; Leitman, D. C. Estrogen receptor β-selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J. Biol. Chem.* **2001**, *276*, 17808−17814.

(5) Frey, R. S.; Li, J.; Singletary, K. W. Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: involvement of Cdc2, $p21^{waf/cip1}$, $p27^{kip1}$, and Cdc2C expression. *Biochem. Pharmacol.* **2001**, *61*, 979−989.

(6) Kobuchi, H.; Roy, S.; Sen, C. K.; Nguyen, H. G.; Packer, L. Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. *Am. J. Physiol. Cell Physiol.* **1999**, *277*, C403−C411.

(7) Wadsworth, T. L.; Koop, D. R. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem. Pharmacol.* **1999**, *57*, 941−949.

(8) Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.

(9) Duffy, S. J.; Keaney, J. F.; Holbrook, M.; Gokce, N.; Swerdloff, P. L.; Frei, B.; Vita, J. A. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation* **2001**, *104*, 151−156.

(10) Yamada, K.; Shoji, K.; Mori, M.; Ueyama, T.; Matsuo, N.; Oka, S.; Nishiyama, K.; Sugano, M. Structure-activity relationship of polyphenols on inhibition of chemical mediator release from rat peritoneal exudates cells. *In Vitro Cell. Dev. Biol.* **1999**, *35*, 169−174.

(11) Dixon, R.; Steele, C. Flavonoids and isoflavonoids - a gold mine for metabolic engineering. *Trends Plant. Sci.* **1999**, *4*, 394−400.

(12) Verhoeyen, M. E.; Bovy, A.; Collins, G.; Muir, S.; Robinson, S.; de Vos, C. H. R.; Colliver, S. Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. *J. Exp. Bot.* **2002**, *53*, 2099−2106.

(13) Niggeweg, R.; Michael, A. J.; Martin, C. Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nat. Biotechnol.* **2004**, *22*, 746−754.

(14) Muir, S. R.; Collins, G. J.; Robinson, S.; Hughes, S.; Bovy, A.; de Vos, C. H. R.; van Tunen, A. J.; Verhoeyen, M. E. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. *Nat. Biotechnol.* **2001**, *19*, 470−474.

(15) Weisshaar, B.; Jenkins, G. I. Phenylpropanoid biosynthesis and its regulation. *Curr. Opin. Plant Biol.* **1998**, *1*, 251−257.

(16) Szankowski, I.; Briviba, K.; Fleschhut, J.; Schönherr, J.; Jacobsen, H.; Kiesecker, H. Tranformation of apple (*Malus domestica Borkh*) with the stilbene synthase gene from grapevine (*Vitis vinifera L.*) and a PGIP gene from kiwi (*Actinidia deliciosa*). *Plant Cell Rep.* **2003**, *22*, 141−149.

(17) Zhu, Y. J.; Agbayani, R.; Jackson, M. C.; Tang, C. S.; Moore, P. H. Expression of the grapevine stilbene synthase gene VST1 in papaia provides increased resistance against diseases caused by *Phytophthora palmivora*. *Planta* **2004**, *220*, 241−250.

(18) Liu, S.; Hu, Y.; Wang, X.; Zhong, J.; Lin, Z. High content of resveratrol in lettuce transformed with a stilbene synthase gene of *Parthenocissus henryana*. *J. Agric. Food Chem.* **2006**, *54*, 8082−8085.

(19) Leckband, G.; Lorz, H. Transformation and expression of a stilbene synthase gene of *Vitis vinifera L.* in barley and wheat for increasing fungal resistance. *Theor. Appl. Genet.* **1998**, *96*, 1004−1012.

(20) Giovinazzo, G.; D'Amico, L.; Paradiso, A.; Bollini, R.; Sparvoli, F.; De Gara, L. Antioxidant metabolite profiles in tomato fruit constitutively expressing the grapevine stilbene synthase gene. *Plant Biotechnol. J.* **2005**, *3*, 57−69.

(21) Huck, C. W.; Buchmeiser, M. R.; Bonn, G. K. Fast analysis of flavonoids in plant extracts by liquid chromatography-ultraviolet absorbnce detection on poly(carboxylic acid)-coated silica and ectrospray ionization tandem mass spectrometric detection. *J. Chromatogr., A* **2001**, *943*, 33−38.

(22) Stöggl, W. M.; Huck, C. W.; Bonn, G. K. Structural elucidation of catechin and epicatechin in sorrel leaf extracts using liquid chromatography coupled to diode array-, fluorescence-, and mass spectrometric detection. *J. Sep. Sci.* **2004**, *27*, 524−528.

(23) Dugo, P.; Lo Presti, M.; Öhman, M.; Fazio, A.; Dugo, G.; Mondello, L. Determination of flavonoids in citrus juices by micro-HPLC-ESI/MS. *J. Sep. Sci.* **2005**, *28*, 1149−1156.

(24) Trela, B. C.; Waterhouse, A. L. Resveratrol: isomeric molar absorptivities and stability. *J. Agric. Food Chem.* **1996**, *44*, 1253−1257.

(25) Goldberg, D. M.; Ng, E.; Karumanchiri, A.; Yan, J.; Diamandis, E. P.; Soleas, G. J. Assay of resveratrol glucosides and isomers in wine by direct-injection high-performance liquid chromatography. *J. Chromatogr., A* **1995**, *708*, 89−98.

(26) Mattivi, F.; Reniero, F.; Korhammer, S. Isolation, characterization, and evolution in red wine vinification of resveratrol monomers. *J. Agric. Food Chem.* **1995**, *43*, 1820−1823.

(27) Waterhouse, A. L.; Lamuela-Raventòs, R. M. The occurrence of piceid, a stilbene glucoside, in grape berries. *Phytochemistry* **1994**, *37*, 571−573.

(28) Careri, M.; Corradini, C.; Elviri, L.; Nicoletti, I.; Zagnoni, I. Direct HPLC analysis of quercetin and *trans*-resveratrol in red wine, grape, and winemaking byproducts. *J. Agric. Food Chem.* **2003**, *51*, 5226−5231.

(29) Corradini, D.; Kalghatgi, K.; Horváth, Cs. Effect of mobile phase additives on peptide retention in reversed phase chromatography with pellicular and totally porous sorbents. *J. Chromatogr., A* **1996**, *728*, 225−233.

(30) Corradini, D.; Cannarsa, G. A comparative study on the effect of hydrochloric, phosphoric and trifluoroacetic acid in the reversed phase chromatography of angiotensins and related peptides. *J. Liq. Chromatogr.* **1995**, *18*, 3919−3931.

(31) Mallet, C. R.; Lu, Z.; Mazzeo, J. R. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Commun. Mass Spectom.* **2004**, *18*, 49−58.

(32) Beaudry, F.; Vachon, P. Electrospray ionization suppression, a physical or a chemical phenomenon? *Biomed. Chromatogr.* **2006**, *20*, 200−205.

(33) Schijlen, E.; Ric de Vos, C. H.; Jonker, H.; van den Broeck, H.; Molthoff, J.; van Tunen, A.; Martens, S.; Bovy, A. Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruits. *Plant Biotechnol. J.* **2006**, *4*, 433−444.

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