JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

New Insights into an Ancient Antibrowning Agent: Formation of Sulfophenolics in Sodium Hydrogen Sulfite-Treated Potato Extracts

Carlos-Eduardo Narváez-Cuenca,[†,‡] Tomas F.M. Kuijpers,[†] Jean-Paul Vincken,[†] Pieter de Waard,[§] and Harry Gruppen*[,†]

[†]Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700EV Wageningen, The Netherlands

[‡]Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia

[§]Wageningen NMR Centre, Wageningen University, Wageningen, The Netherlands

ABSTRACT: The effect of sodium hydrogen sulfite (*S*), used as antibrowning agent, on the phenolic profile of potato extracts was investigated. This extract was compared to one obtained in the presence of ascorbic acid (*A*). In the presence of *A*, two major compounds were obtained, 5-*O*-caffeoylquinic acid (5-CQA) and 4-*O*-caffeoyl quinic acid. With *S*, their 2′-sulfo-adducts were found instead, the structures of which were confirmed by nuclear magnetic resonance spectroscopy and mass spectrometry. Also, for minor caffeoyl derivatives and quercetin glycosides, the corresponding sulfo-adducts were observed. Feruloyl and sinapoyl derivatives were not chemically affected by the presence of *S*. Polyphenol oxidase (PPO) was thought to be responsible for the formation of the sulfo-adducts. This was confirmed by preparing 2′-sulfo-5-*O*-caffeoyl quinic acid in a model system using 5-CQA, sodium hydrogen sulfite, and PPO. This sulfo-adduct exhibited a small bathochromic shift (λ_{max} 329 nm) as compared to 5-CQA (λ_{max} 325 nm) and a strong hypochromic shift with an extinction coefficient of 9357 $\pm$ 395 M^{-1} cm^{-1} as compared to 18494 $\pm$ 196 M^{-1} cm^{-1}, respectively. The results suggest that whenever *S* is used as an antibrowning agent, the *O*-quinone formed with PPO reacts with *S* to produce sulfo-*O*-diphenol, which does not participate in browning reactions.

KEYWORDS: UHPLC-MS, *Solanum tuberosum*, sulfite, enzymatic browning, phenolic acids, sulfo-phenolics

INTRODUCTION

The prevention of enzymatic browning is a major concern during starch production and retrieval of other valuable components from potato. Phenolic compounds are considered to be the main precursors of the brown pigments. They constitute an abundant group of secondary metabolites in potato. Caffeic acid, 5-*O*-caffeoyl quinic acid (chlorogenic acid), its isomers, and rutin are representatives of hydroxycinamic acids (HCAs), HCA conjugates (HCAcs), and flavonols, commonly found in potato, respectively.[1−3] The content of phenolic compounds varies over a wide range depending on several factors, for example, variety. Chlorogenic acid and its isomers have been found in potato tubers to range from 31 to 1366 mg/100 g DW, caffeic acid from 1 to 19 mg/100 g DW, and rutin from 0 to 19 mg/100 g DW.[1−3]

The compounds mentioned can be oxidized by polyphenol oxidase (PPO) to produce *O*-quinones, which subsequently polymerize into brown-colored melanins.[4,5] This oxidation can be prevented by the addition of ascorbic acid or sulfites/hydrogen sulfites. Although the FDA prohibits the use of sulfites on fruits and vegetables for the fresh market, they are allowed in minimally processed potatoes.[6] Furthermore, they are commonly used in the potato starch industry, which, for example, in The Netherlands amounts to about 2.5 $\times$ 10^6 tons of starch potatoes annually.[7]

The antibrowning effect of ascorbic acid has been associated with its ability to reduce quinones to their precursor phenolics and with lowering the pH with a concomitant inhibition of PPO activity.[8] The sulfur-containing agents seem to control the browning reaction by irreversible inactivation of PPO[9] as well as by reacting with quinones to produce colorless compounds.[10] The latter mechanism has been proposed based on UV−vis data only, without structural elucidation or quantification of the end products.[10] Hence, information on the modification of the individual phenolic compounds is lacking to date.

Recently, we have reported a method for the identification of HCAs/HCAcs in potato by reverse-phase ultra high-performance liquid chromatography−diode array detection− mass spectrometry (RP-UHPLC-DAD-MS^n),[11] which is a useful tool for structural elucidation of a complex extract. In the present study, this method was employed to investigate how the addition of sodium hydrogen sulfite upon extraction of potato affects the composition of phenolics in the extract. Extraction in the presence of ascorbic acid was used as a reference to quantify the phenolic compounds in their unmodified state, as it inhibits enzymatic oxidation of phenolics that would otherwise occur.

MATERIALS AND METHODS

Chemicals. Caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, ascorbic acid, sodium hydrogen sulfite ($NaHSO_3$), and mushroom tyrosinase were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). *neo*-Chlorogenic acid (3-*O*-caffeoyl quinic acid) and *crypto*-chlorogenic acid (4-*O*-caffeoyl quinic acid) were from Phytolab (Vestenbergsgreuth, Germany). Rutin (quercetin-3-*O*-rutinoside) was from Merck (Darmstadt, Germany). UHPLC/MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands).

Received: April 15, 2011
Accepted: August 20, 2011
Revised: August 18, 2011
Published: August 20, 2011

ACS Publications © 2011 American Chemical Society

Water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). All other chemicals were from Merck.

Plant Material. Potato tubers (Nicola variety) were purchased from a local supermarket in Wageningen, The Netherlands. Tubers were washed under tap water and then processed further.

Extraction of Phenolic Compounds. Two hundred grams of fresh potato was diced (0.3−0.5 cm thickness) and immediately homogenized in a household blender with the addition of 200 mL of aqueous solutions of 20000 ppm ascorbic acid (extractant *A*) or 400 ppm $NaHSO_3$ (extractant *S*). Subsequently, the mixture was stirred for 10 min at 4 °C. Starch and fibers were allowed to settle for 20 min at 4 °C. After it was decanted, the solution was centrifuged (18000*g*; 20 min; 4 °C). The precipitated material and the pellet from centrifugation were collected, combined, and re-extracted with 100 mL of extractants *A* or *S*. When analyzed with RP-UHPLC-DAD-MS^n (see later), the fifth extraction yielded less than 1% of the 5-*O*-caffeoyl quinic acid and its derivatives, when compared to the summed up amount of the five extractions. Therefore, only the material from the first four extractions was combined. The extracts will be referred to as extracts *A* and *S*. The pH values of extracts *A* and *S* were 3.9 and 6.0, respectively. To remove proteins, the pH of extract *S* was adjusted to 4.0 by adding 100% acetic acid and left overnight at 4 °C. Extract *A* was kept overnight at 4 °C without the addition of acetic acid. The resulting materials were centrifuged (18000*g*; 20 min; 4 °C), and the supernatants were subsequently filtered through a 0.45 μm filter (Whatman, Scheicher & Schuell, Dassel, Germany). Aliquots (500 μL) of the extracts were ultrafiltrated using regenerated cellulose centrifugal filter units (Amicon ultra 0.5 mL, cutoff 10 kDa, Millipore) according to the instructions of the manufacturer. Filtrates were stored at −20 °C until further analysis. All extractions were done in triplicates.

Large-scale extraction was performed by processing 2 kg of fresh potato with 2 L of extractant *S* using identical conditions as with the 200 g fresh potato samples. After the filtration step (0.45 μm filter), the protocol was modified as follows. The supernatant obtained after precipitation of proteins (pH 4.0) and centrifugation was ultrafiltrated at 4 °C using a 2.5 L Amicon ultrafiltration cell (Millipore) with a regenerated cellulose membrane (cutoff, 10 kDa; Millipore). The system had a magnetic stirrer to minimize concentration polarization at the membrane and was pressurized (4 atm) with nitrogen. Low molecular weight polar compounds were removed from the ultrafiltrated liquid using solid-phase extraction with C18 35 mL/10 g Sep-Pak cartridges, according to the instructions of the manufacturer (Waters, Milford, MA). The methanolic fraction was evaporated under reduced pressure, and the remaining water was removed by freeze drying, yielding 768 mg of powder. A quantity of 200 mg of powder was suspended in MQ water to 5 mg/mL, stirred for 10 min, and centrifuged (12000*g*; 5 min, 4 °C). Subsequently, the resolubilized powder was fractionated by semipreparative RP-HPLC. Extraction for preparative purposes was performed once.

Semipreparative RP-HPLC. The resolubilized powder obtained from the large scale extraction from potato was fractionated by a Waters preparative HPLC system, using a semipreparative XTerra RP18 column (150 mm × 19 mm; particle size 5 μm; Waters) with a XTerra RP18 guard column (19 mm × 10 mm i.d.; particle size 5 μm; Waters). The solvents used were water/ACN/acetic acid (99:1:0.5, v/v/v) (eluent A) and ACN/acetic acid (100:0.5, v/v) (eluent B). Used was the following elution program: 0−5 min, 0% B; 5−35 min, 0−26% B; 35−37 min, 26−100% B; 37−42 min, 100% B; 42−44 min, 100−0% B; and 44−54 min, 0% B. Volumes of 10 mL of 5 mg/mL sample were injected. The flow rate was 12 mL/min. The eluate was monitored at 325 nm, and fractions (3.4 mL) were obtained during the time span of 15−25 min of each run. On the basis of RP-UHPLC-DAD-ESI-MS^n, two pools (I and II) were made. ACN was removed by evaporation under vacuum, and the remaining water was removed by freeze drying. Two hundred milligrams of powder yielded 4.3 and 20.4 mg of pools I and II, respectively. RP-UHPLC-DAD-ESI-MS^n analysis revealed that pool I was comprised of **22** (purity 45%, w/w), with impurities of tryptophan (45%, w/w) and **23** (10%, w/w). In pool II, **23** was the major compound, with a purity of approximately 71% w/w, with tryptophan (28%, w/w) and **22** (1%, w/w) as the main impurities.

PPO-Catalyzed Preparation of 2′-Sulfo-5-*O*-caffeoyl Quinic Acid in a Model System and Its Purification. To establish whether PPO is essential to the formation of sulfophenolics, 5-*O*-caffeoyl quinic acid and sodium hydrogen sulfite were incubated with and without commercial PPO. Only in the presence of PPO, 2′-sulfo-5-*O*-caffeoyl quinic acid was found as the major reaction product (data not shown). Five hundred milliliters of an aqueous solution of 5-*O*-caffeoyl quinic acid (1 mM) was fully converted after incubation with $NaHSO_3$ (2 mM) and mushroom tyrosinase (140 U/mL; PPO units according to supplier) at 20 °C for 2 h. The initial pH was adjusted to 6.5 by adding 0.1 M NaOH. The resulting material was purified by semipreparative RP-HPLC, similarly as described above. One hundred seventy-seven milligrams of 5-*O*-caffeoyl quinic acid yielded 77 mg of 2′-sulfo-5-*O*-caffeoyl quinic acid as the major reaction product, with a purity of 97% (after peak area integration at 325 nm), having identical retention times, UV, and MS^n data as **23**. An isomer of the major reaction product was the major impurity.

Determination of the Molar Extinction Coefficient. On the basis of stock solutions of 10 mg/mL of 5-*O*-caffeoyl quinic acid and 2′-sulfo-5-*O*-caffeoyl quinic acid (obtained by PPO-catalyzed preparation), dilution series in MQ water were made. The absorbances at 325 nm of these dilutions were measured against MQ water in a 1 mL quartz cuvette. The temperature of the solutions was maintained at 20 °C. The molar extinction coefficients (ε) were calculated using Abs = $\varepsilon \times 1 \times c$, in which Abs = absorbance at 325 nm, l = light path = 1 cm, and c = concentration (M). Furthermore, wavelength scans were made from 200 to 600 nm. Measurements were performed with six independently prepared replications.

RP-UHPLC-DAD-ESI-MS^n Analysis. Potato extracts, undiluted and 10× diluted, and reaction products synthesized in a model system with commercial PPO were analyzed using an Accela UHPLC system (Thermo Scientific, San Jose, CA) equipped with pump, autosampler, cooled at 7 °C, and a photodiode array detector (DAD), using a Hypersil gold RP column (150 mm × 2.1 mm i.d.; particle size 1.9 μm; Thermo Scientific) at 30 °C. The eluents used were water/ACN/acetic acid (99:1:0.2, v/v/v) (eluent A) and ACN/acetic acid (100:0.2, v/v) (eluent B). The elution program was 0−5 min, 0% B; 5−23 min, 0−60% B; 23−24 min, 60−100% B; 24−27 min, 100% B; 27−28 min, 100−0% B; and 28−35 min, 0% B. The flow rate was 400 μL/min. Sample volumes of 5 μL were injected. MS^n analysis was performed on a LTQ-XL (Thermo Scientific) using electrospray ionization (ESI). Detection was in the negative ion mode with a source voltage of 3.5 kV and an ion transfer tube temperature of 350 °C. The instrument was tuned using chlorogenic acid. A full-scan mass spectrum over a m/z range of 150−1500 was recorded. MS^2 spectra of extracts *A* and *S* were collected with a collision energy of 30% with the use of wideband activation, which ensures that both the parent ion and the subsequent water loss ion undergo fragmentation. The control of the instrument and data processing were done using Xcalibur 2.07 (Thermo Scientific). Annotation of HCAcs was done according to previous work.[11] Furthermore, retention times and spectroscopic data of 3-*O*-, 4-*O*-, and 5-*O*-caffeoyl quinic acid isomers, caffeic acid, and rutin were compared to standards. 5-*O*-Caffeoyl quinic acid was adopted as a standard for the quantification of caffeoyl quinic acid isomers. 2′-Sulfo-5-*O*-caffeoyl quinic acid, obtained with the commercial PPO, was used as the standard for the quantification of sulfo-caffeoyl quinic acid isomers. Other minor caffeoyl derivatives, different to (sulfo-) caffeoyl quinic acid isomers, were quantified using caffeic acid, with application of a MW correction factor. Ferulic acid and sinapic acid were used as standards for the quantification

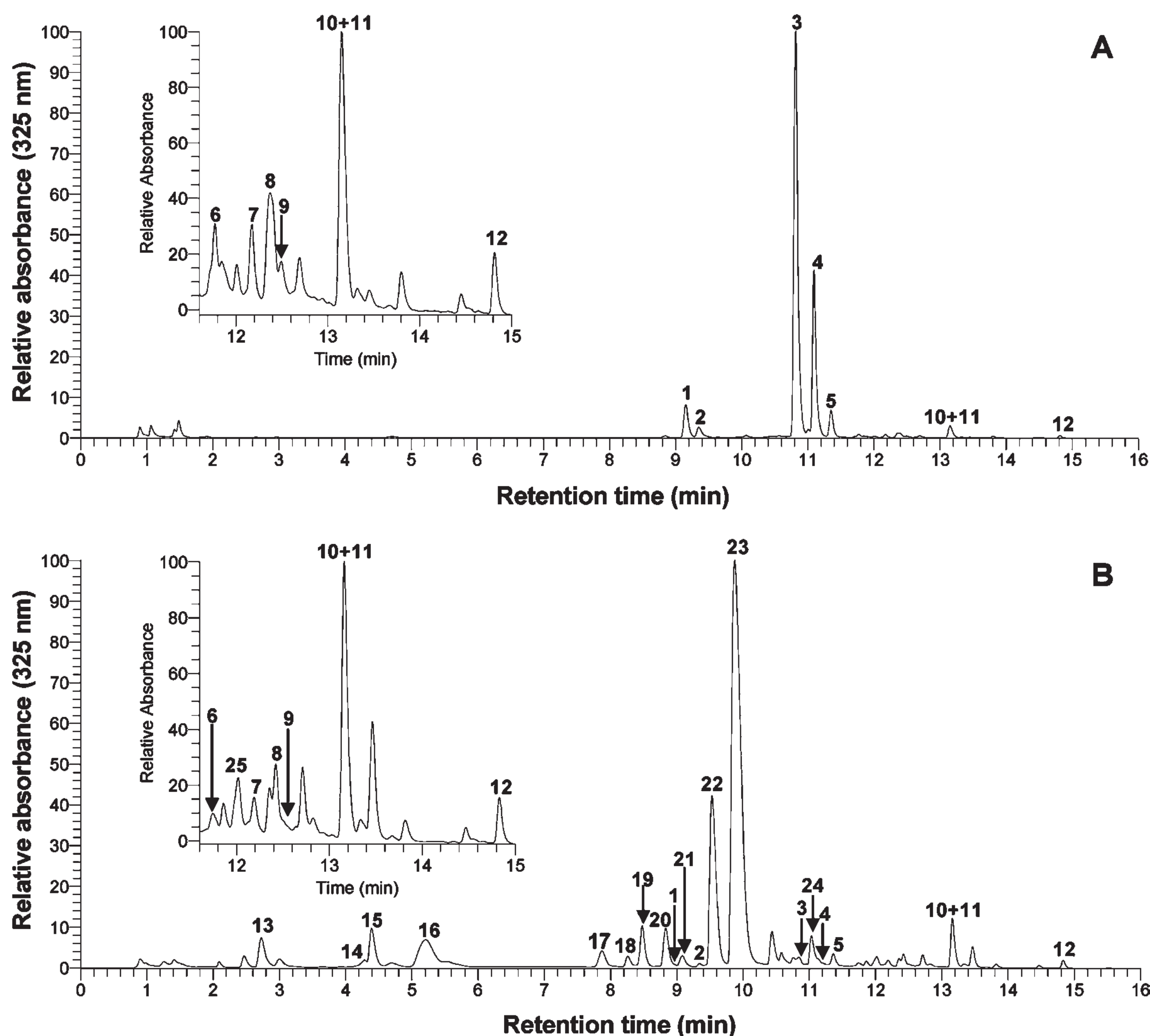

Figure 1. HPLC chromatogram recorded at 325 nm of the potato extract prepared with the addition of (A) 20000 ppm ascorbic acid or (B) 400 ppm $NaHSO_3$. The inserts are a zoom between 11.6 and 15.0 min.

of ferulic acid- and sinapic acid-containing compounds, respectively, with the use of MW correction factors ($MW_{HCAc}/MW_{external\ standard}$), assuming that the response of the HCAcs is determined by the HCA moiety. All HCAs/HCAcs were quantified at 325 nm. Quercetin glycosides were quantified based on calibration curves with rutin at 360 nm, and MW correction factors were used when necessary. In all cases, calibration curves were done at concentrations ranging from 0.05 to 30 μg/mL. Calibration curves with tryptophan (1−30 μg/mL) were carried out at 280 nm to calculate its content in pools I and II from the large scale potato extraction.

Nuclear Magnetic Resonance (NMR) Spectroscopy. Samples were dissolved in 0.35 mL of D_2O (99.9 atom %, Aldrich), and approximately 1 μL of acetone was added to each sample as internal standard. NMR spectra were recorded at a probe temperature of 300 K on a Bruker Avance-III-600 spectrometer, equipped with a cryo-probe located at Biqualys (Wageningen, The Netherlands). ^{1}H chemical shifts were expressed in ppm relative to internal acetone at 2.220 ppm. ^{13}C chemical shifts were expressed in ppm relative to internal acetone at 30.89 ppm. One- and two-dimensional correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence (HMQC) spectra were acquired using standard pulse sequences delivered by Bruker. For the ^{1}H COSY and ^{1}H TOCSY spectra, 400 experiments of four scans and 400 experiments of eight scans were recorded, resulting in measuring times of 1 and 2 h, respectively. The mixing time for the TOCSY spectra was 100 ms. For the [^{1}H,^{13}C]-HMBC and [^{1}H,^{13}C]-HMQC spectra, 1024 experiments of 16 scans and 512 experiments of four scans, were recorded, resulting in measuring times of 8 and 1.2 h, respectively.

Statistical Analysis. Data are reported as the mean with their standard deviation. Quantities of phenolics obtained in extracts *A* and *S* were compared by means of the Student's *t* test ($P < 0.05$).

■ RESULTS

Altered Composition of Potato Phenolics upon Use of $NaHSO_3$. When ascorbic acid or $NaHSO_3$ was used in the potato extract preparation, no visual browning was observed. In contrast, the omission of either ascorbic acid or $NaHSO_3$ led to rapid browning of the suspension (no further data shown). Interestingly, different chromatographic profiles were observed when the extracts were obtained in the presence of ascorbic acid (Figure 1A) or $NaHSO_3$ (Figure 1B).

Ascorbic Acid as an Antibrowning Agent. The chromatogram was dominated by HCAcs with 5-*O*-caffeoyl quinic acid, **3**, as the

Table 1. Retention Times, MS, and UV Data of HCAs/HCAcs and Flavonols in Potato

no.	RT (min)	MS (m/z)	MS[2a] (m/z)	UV λ_{max} (nm)	tentative identification
1	9.15	353	**191**, 179 (45), 173 (3), 161 (1), 135 (8)	223, 240 sh, 300 sh, 324	3-*O*-caffeoyl quinic acid[b]
2	9.35	249	**249**, 207 (7), 161 (<1) 135 (23)	223, 293 sh, 317	caffeoyl putrescine
3	10.82	353	**191**, 179 (3), 173 (<1), 161 (<1), 135 (<1)	223, 240 sh, 305 sh, 325	5-*O*-caffeoyl quinic acid[b]
4	11.10	353	191 (21), 179 (59), **173**, 135 (8)	223, 240 sh, 305 sh, 326	4-*O*-caffeoyl quinic acid[b]
5	11.35	179	179 (86), **135**, 161 (<1)	224, 305 sh, 323	caffeic acid[b]
6	11.77	353	**191**, 179 (4), 173 (<1), 161 (<1), 135 (1)	229, 240 sh, 305 sh, 325	caffeoyl quinic acid isomer
7	12.18	445	**385**, 223 (5), 205 (4), 179 (4)	225, 300 sh, 330	sinapoyl hexoside
8	12.37	367	193 (9), **191**, 173 (37), 134 (<1)	214, 301 sh, 324	5-*O*-feruloyl quinic acid
9	12.50	625	505 (9), 463 (11), 445 (31), 301 (49), **300**, 271 (22), 255 (11), 179 (3), 151 (4) MS[3] 300 (28), **271**, 255 (46), 179 (2), 151 (4)	260, 300 sh, 353	quercetin-3-*O*-diglycoside
10	13.15	309	**193**, 178 (<1), 149 (<1), 134 (<1), 115 (<1)	225, 300 sh, 326	feruloyl malate[c]
11	13.22	609	**301**, 300 (44), 271 (9), 255 (6), 179 (3) MS[3] 301 (74), **179**, 151 (92)	259, 300 sh, 351	rutin[b,c]
12	14.82	429	385 (25), **249**, 223 (<1), 205(44), 179 (21)	224, 311	sinapoyl conjugate

[a] Bold numbers represent a relative abundance of 100%. In parentheses, the relative abundance is indicated. Values underlined are those that were diagnostic for the classification of compounds containing HCAs. In the case of quercetin glycosides, MS[3] of the 100% ion from MS[2] was included to provide extra information. [b] Similar retention times, MS and UV data as compared to authentic standards. [c]Coelution with other compounds. Peaks were resolved when the gradient was modified as follows: 0−5 min, 0% B; 5−23 min, 50% B; 23−24 min, 50−100% B; 24−27 min, 100% B; 27−28 min, 100−0% B; 28−35 min, 0% B at 40 °C, performed with eluents containing 0.1 instead of 0.2% HAc (Retention times of **10** and **11** were 12.98 and 13.20 min, respectively).

most abundant phenolic compound, followed by 4-*O*-caffeoyl quinic acid, **4**. From 12 identified compounds, 10 were HCA-containing compounds (caffeic acid, ferulic acid, and sinapic acid derivatives), including caffeic acid in the free form, and two were quercetin glycosides. The retention times, spectroscopic data, and mass spectrometric data of **1**−**12** are given in Table 1. The spectroscopic and mass spectrometric data of the 10 HCAs/HCAcs were in agreement with previous work.[11] Moreover, the retention times of 3-*O*-, 4-*O*-, and 5-*O*-caffeoyl quinic acid isomers and that of caffeic acid matched with those of authentic standards.

Two compounds were annotated as quercetin glycosides: quercetin 3-*O*-diglucoside (**9**) and rutin (**11**). After MS fragmentation, both compounds yielded the predominant ions m/z 300 ($[M - 2H - 324]^{-\bullet}$) and 301 ($[M - H - 324]^{-}$), which originated from homolytic and heterolytic cleavage of the glycosidic bond, respectively.[3,12] Retention times and spectroscopic data of rutin were in accordance with those of the authentic compound. The C-3 substitution of quercetin with glucose in compound **9** has been previously described in potato.[3] Furthermore, the presence of the ions m/z 505 and 445 during MS fragmentation was diagnostic for quercetin-*O*-dihexosides substituted at the C-3 position.[12]

$NaHSO_3$ as an Antibrowning Agent. Compounds that were identified in extract *A* (**1**−**12**) were found in extract *S* as well, although in different relative quantities. Particularly 5-*O*-caffeoyl quinic acid and 4-*O*-caffeoyl quinic acid (Figure 1A,B) were much lower in extract *S* than in extract *A*. Furthermore, about 18 new peaks were observed in extract *S*, of which 13 were tentatively identified (retention times, spectroscopic, and mass spectrometric data in Table 2). Compound **23** was the most abundant, followed by **22**.

The mass of the parent ions of the new compounds (**13**−**25**) revealed an increase of 80 amu (Table 2) when compared to the caffeoyl- and quercetin-containing compounds labeled **1**−**12** (Table 1), referred to in the text as precursor compounds. Eight isomers (**13**, **15**−**18**, **20**, **22**, and **23**) with MW of 434 were found, which represented the MW of caffeoyl quinic acid isomers plus 80 amu. Similarly, MS analysis revealed that the MW of **19** matched with that of caffeoyl putrescine plus 80 amu, **14** and **21** with a MW of caffeic acid plus 80 amu, **24** with a MW of quercetin-3-*O*-diglucoside plus 80 amu, and **25** with a MW of rutin plus 80 amu. The extra 80 amu indicated that a SO_3 substituent is present in the molecules.

MS Fragmentation and NMR Spectroscopy of the Two Main SO_3H-Caffeoyl Quinic Acids. Table 2 shows that **22** and **23** yielded, although in very low abundance, the ions m/z 353, 191, 179, 161, and 135, which are diagnostic for caffeoyl quinic acid isomers (**1**, **3**, **4**, and **6**; Table 1). Moreover, MS fragmentation data (Table 2) revealed ions that indicate a SO_3H moiety linked to the caffeic acid moiety. The ions m/z 259 (SO_3H caffeic acid), m/z 241 (dehydrated SO_3H caffeic acid), and m/z 215 (decarboxylated SO_3H caffeic acid) were used as diagnostic tool for the linkage of the SO_3H group to the caffeic acid moiety and not to the quinic acid moiety. Other ions found for **22** and **23**, but not for caffeoyl quinic acid isomers, were as follows: m/z 415 $[M - H_2O - H]^-$, 387 $[M - H_2O - CO - H]^-$, and 301 $[M - C_5H_6O_3 - H_2O - H]^-$. Interestingly, the ion m/z 259 was the most abundant for **23,** whereas for **22** the most abundant ion was m/z 301.

NMR spectroscopy was used to establish the position of the sulfite group on the caffeic acid moiety and the position of the ester linkage between caffeic acid and quinic acid. Interpretation of the 2D NMR spectra resulted in full assignment of both ^1H and ^{13}C spectra for **22** and **23** (Table 3). In the caffeoyl ring, the H-2′ was missing when compared to 4-*O*- and 5-*O*-caffeoyl quinic acid, indicating that the SO_3H group must be attached to position C-2′. The carbon chemical shift of 125 ppm for C-2′, assigned by a cross-peak in the HMBC between H-6′ and C-2′, was in accordance with a SO_3H group linked at position 2. In the HMBC of each compound, a cross-peak was assigned between a proton of quinic acid and C-9′ of caffeic acid, indicating the linkage position of quinic acid. For **22**, this was position 4. When compared to unsubstituted quinic acid, the downfield shift of 2.7 ppm for C-4 and upfield shifts of 2.4 and 2.0 ppm for C-3 and C-5, respectively, confirmed the linkage at position C-4.[13] For **23**, a cross-peak between H-5 of quinic acid and C-9′ of

Table 2. Retention Times, MS, and UV Data of Sulfo-HCAs/Sulfo-HCAcs/Sulfo-flavonols in Potato

no.	RT (min)	MS (m/z)	MS[2a] (m/z)	UV λ_{max} (nm)	tentative identification
13	2.73	433	433 (1), 415 (<1), 387 (3), 353 (80), 301 (2), *259* (13), *241* (44), *215* (3), **191**, 179 (6), 161 (34), 135 (1)	228, 246, 305 sh, 327	*O*-sulfate-caffeoyl quinic acid or sulfo-caffeoyl quinic acid
14	4.29	259	***259***, *241*(<1), *215* (13), 179 (47), 135 (13)	230, 250 sh, 289, 323	sulfo-caffeic acid isomer 1
15	4.39	433	433 (2), 415 (3), 387 (9), 353 (<1), 301 (2), ***259***, *241* (12), *215* (38), 179 (1), *161* (9), *135* (3)	227, 245, 305 sh, 327	sulfo-caffeoyl quinic acid isomer 1
16	5.21	433	433 (3), 415 (3), 387 (18), 353 (<1), 301 (2), *259* (36), ***241***, *215* (2), 179 (1), 161 (1), 135 (<1)	229, 250, 296, 324	sulfo-caffeoyl quinic acid isomer 2
17	7.87	433	433 (1), 415 (<1), 387 (9), 353 (<1), **301**, *259* (3), *241* (6), *215* (<1), 179 (<1), 161 (<1), 135 (<1)	229, 245, 295, 324	sulfo-caffeoyl quinic acid isomer 3
18	8.26	433	433 (1), 415 (2), 387 (3), 353 (<1), **301**, *259* (4), *241* (3), *215* (2), 179 (<1), 161 (2), 135 (1)	229, 291 sh, 313	sulfo-caffeoyl quinic acid isomer 4
19	8.48	329	329 (98), **249**, *241*(80), *215* (6), 161 (34), 135 (4)	229, 291, 321	sulfo-caffeoyl putrescine
20	8.83	433	433 (6), 415 (9), 387 (31), 353 (3), 301 (9), ***259***, *241* (29), *215* (37), 191 (12), 179 (3), 161 (34), 135 (3)	228, 280 sh, 315	sulfo-caffeoyl quinic acid isomer 5
21	9.08	259	*259* (6), *241* (<1), ***215***, 179 (1), 135 (2)	228, 281, 327	sulfo-caffeic acid isomer 2
22	9.53	433	433 (<1), 415 (<1), 387 (2), 353 (<1), **301**, *259* (<1), *241* (2), *215* (1), 161 (1)	228, 240 sh, 305 sh, 329	2′-sulfo-4-*O*-caffeoyl quinic acid
23	9.88	433	433 (9), 415 (9), 387 (38), 353 (<1), 301 (11), ***259***, *241* (37), *215* (45), 191 (<1), 179 (2), 161 (47), 135 (3)	224, 240 sh, 305 sh, 329	2′-sulfo-5-*O*-caffeoyl quinic acid
24	11.04	705	543 (4), 525 (1), ***381***, 301 (47), 271 (1)	229, 291 sh, 318[b]	sulfo-quercetin-3-*O*-diglucoside
25	11.96	689	***381***, 301 (53), 271 (1)	229, 259, 312[b]	sulfo-rutin

[a] Bold numbers represent a relative abundance of 100%. In parentheses, the relative abundance is indicated. Values underlined are those that were diagnostic for the precursor compounds. Values in italic are those that were diagnostic for the assignment of SO_3 attached to the aromatic ring of caffeic acid or quercetin. [b] UV maxima data agree with neither sulfo-quercetin nor with quercetin *O*-sulfate, (*25*, *26*) probably due to co-elution.

Table 3. ^{1}H and ^{13}C NMR Data of 22 and 23

	22				23			
atom no.	^{1}H shift	multiplicity	J coupling	^{13}C shift	^{1}H shift	multiplicity	J coupling	^{13}C shift
1				76.00				75.63
2	2.27	m		37.49	2.22	m		37.12
	2.11	m			2.10	m		
3	4.38	m		68.34	4.26	m		70.01
4	4.938	dd	9.1; 2.8	78.18	3.900	dd	8.5; 2.5	72.11
5	4.36	m		64.98	5.299	m		71.49
6	2.26	m		40.80	2.26	m		37.34
	2.12	m			2.14	m		
7				178.50				177.82
1′				125.24				125.11
2′				125.80				125.88
3′				143.21				143.14
4′				147.66				147.63
5′	7.027	d	8.4	118.35	6.974	d	8.4	118.21
6′	7.266	d	8.4	120.95	7.159	d	8.4	120.85
7′	8.493	d	15.7	144.66	8.436	d	15.7	144.45
8′	6.437	d	15.7	118.52	6.283	d	15.7	118.40
9′				168.90				168.77

caffeic acid indicated C-5 as linkage position. This was also confirmed by a cross-peak in the HMBC between H-5 and C-1, which was not present in case of linkage at position C-4, and by a downfield shift of 4.5 ppm for C-5 and upfield shifts of 3.4 and 3.6 ppm for C-4 and C-6, respectively, as compared to unsubstituted quinic acid.[13] Therefore, **22** was identified as 2′-SO_3H-4-*O*-caffeoyl quinic acid and compound **23** as 2′-SO_3H-5-*O*-caffeoyl quinic acid.

Identification of Minor SO_3H-Phenolics Based on MS Fragmentation. Table 2 shows that the six minor SO_3H-caffeoyl quinic acid isomers yielded ions similar to those that were found for the two major SO_3H-caffeoyl quinic acid isomers (**22** and **23**),

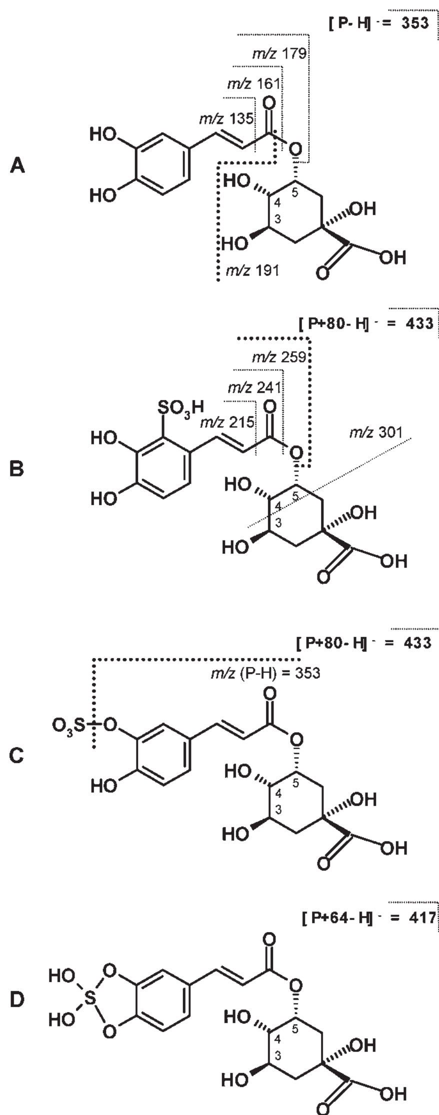

Figure 2. Distinctive MS fragmentation pattern of (A) 5-*O*-caffeoyl quinic acid (**3**), (B) 2′-sulfo-5-*O*-caffeoyl quinic acid (**23**), (C) 5-*O*-caffeoyl quinic acid sulfate, and (D) 5-*O*-caffeoyl quinic acid-double linked-*O*-sulfate. Part C was based on published results for 3-*O*- and 4-*O*-caffeoyl quinic acid-*O*-sulfate.[19] Part D was based on results for adducts between quercetin and sulfite.[21] In part C, the hydroxyl group to which SO_3 is attached is arbitrary. P denotes the molecular mass of the precursor compound, 354 amu. In each case, the thickest dotted line represents the most abundant fragment in MS^2.

that is, the ions m/z 259 (SO_3H caffeic acid), 241 (dehydrated SO_3H caffeic acid), and 215 (decarboxylated SO_3H caffeic acid). The differences observed in the relative abundances of the ions among the six molecules suggest a distinctive effect of the position of the ester linkage between caffeic acid and quinic acid,[14] as well as of the position of the SO_3H moiety. In the UV spectra, there was a small bathochromic shift (maximum shift 5 nm) for **13**, **15**, **19**, **21**, **22**, and **23**, if compared to the precursor compounds (those without the SO_3H group). On the contrary, there was a hypsochromic effect (maximum shift 10 nm) for **18** and **20**, if compared to precursor compounds. In literature, both bathochromic and hypsochromic spectral shifts have been reported upon nucleophilic attack of glutathione to caffeoyl-containing compounds.[15,16]

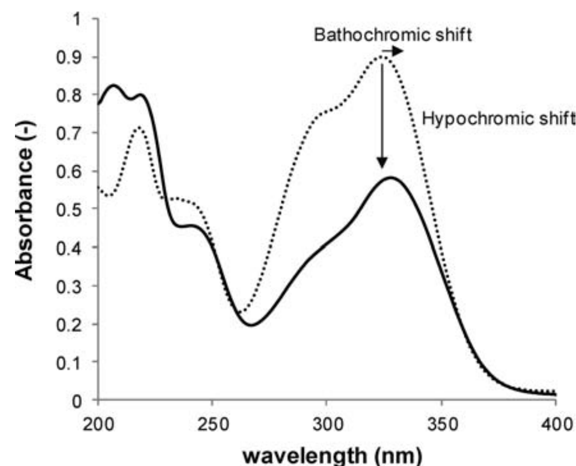

Figure 3. UV spectra of 0.05 mM 2′-sulfo-5-*O*-caffeoyl quinic acid (solid line) and 0.05 mM 5-*O*-caffeoyl quinic acid (dotted line).

In our study, the ion m/z 381, observed with both **24** and **25** (Table 2), demonstrated that the SO_3H was attached to the flavonoid skeleton and not to the saccharide moiety. The ions m/z 179 and 151, representing the A ring moiety after C ring cleavage of quercetin,[17] were observed in **9** and **11** (Table 1) as well as in **24** and **25** (Table 2). The lack of ions m/z 179 + 80 and 151 + 80 in MS^n spectra of **24** and **25** suggested that the SO_3H group should be linked to ring B.

Extinction Coefficient of Sulfochlorogenic Acid and Quantification of Phenolics and Sulfophenolics in Potato Extracts. The presence of the SO_3H moiety in 5-*O*-caffeoyl quinic acid caused a small bathocromic (λ_{max} shift from 325 to 329 nm) and a large hypochromic shift (Figure 3). The hypochromic shift effect was reflected in the extinction coefficient of 2′-sulfo-5-*O*-caffeoyl quinic acid (9357 ± 395 M^{-1} cm^{-1}), which was half that of 5-*O*-caffeoyl quinic acid (18494 ± 196 M^{-1} cm^{-1}). The extinction coefficient of 5-*O*-caffeoyl quinic acid was comparable to that reported in the literature (18130 ± 242 M^{-1} cm^{-1}).[18]

Quantification of the (modified) HCAs/HCAcs/flavonols obtained in the presence of *S* revealed that most chlorogenic acid isomers were modified (less than 1% of total chlorogenic acid isomers were found as such), accounting for 95% (w/w) of all phenolic compounds. The content of sulfo-chlorogenic acid isomers obtained in the presence of *S* was 113 ± 25 mg/100 g potato DW, which corresponds to 102 ± 20 mg total chlorogenic acid isomers/100 g potato DW, a quantity that is well within the range of total chlorogenic isomers found in potato.[1,2,11] Surprisingly, the total amount of chlorogenic acid isomers obtained in the presence of *A* was lower (60 ± 6 mg/100 g potato DW, $P < 0.05$) than that obtained in the presence of *S*. Although the amount of ascorbic acid was nonlimiting, as judged by UHPLC-DAD-ESI-MS^n (data not shown), it might be that ascorbic acid competes with proteins in reacting with chlorogenic acid quinone, by which chlorogenic acid and chlorogenic acid−protein complexes are formed, respectively. The latter are not analyzed by our method.

■ DISCUSSION

For the first time, molecular evidence of reaction products upon addition of $NaHSO_3$ as antibrowning agent against PPO during food processing is provided. Two new major compounds

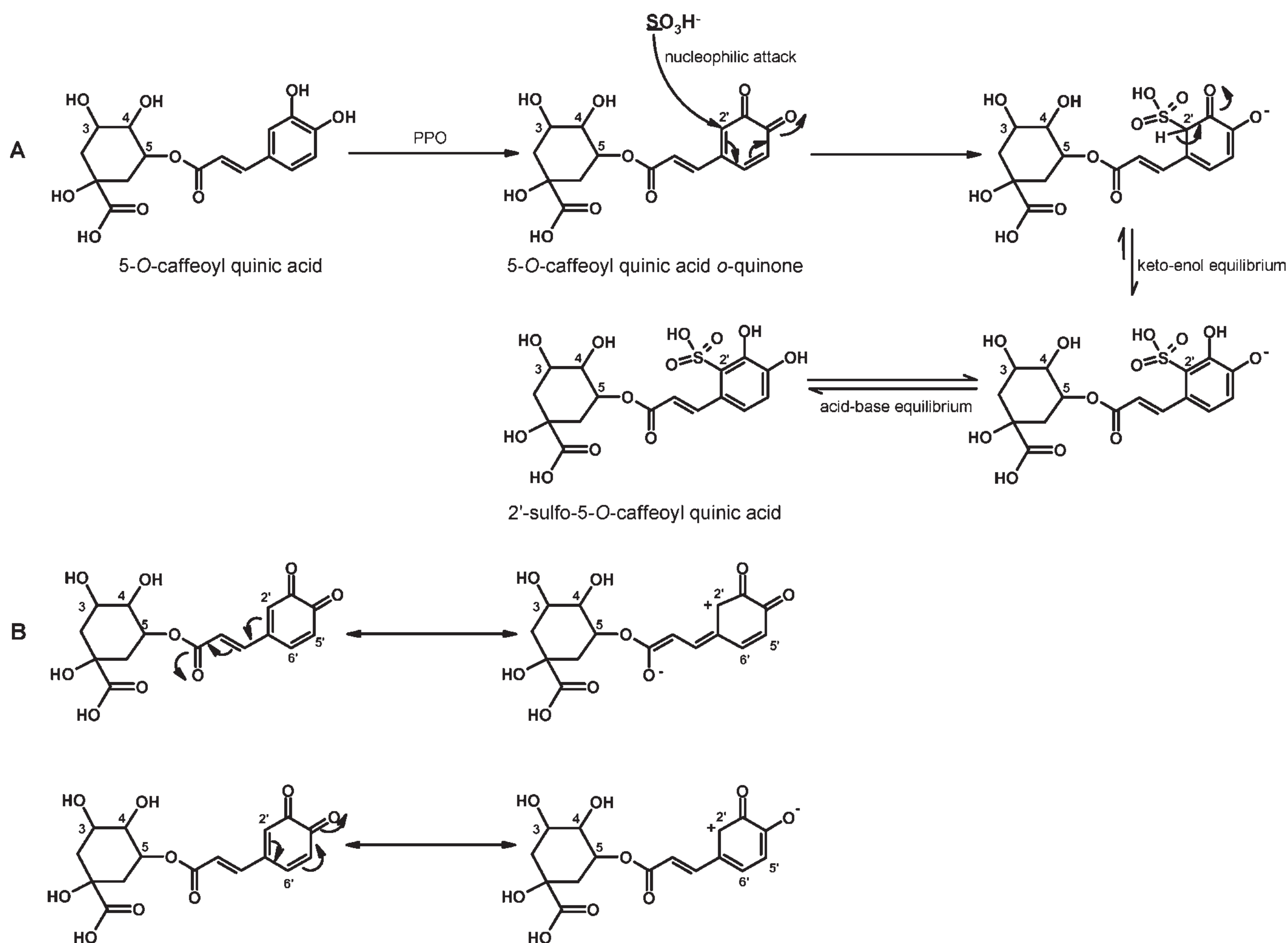

Figure 4. (A) Reaction between 5-*O*-caffeoyl quinic acid and HSO_3^-, after enzymatic activation of the aromatic ring to produce 2′-sulfo-5-*O*-caffeoyl quinic acid. (B) Double activation of the C-2′ position of caffeic acid in 5-*O*-caffeoyl quinic acid.

were identified as 2′-sulfo-5-*O*-caffeoyl quinic acid and 2′-sulfo-4-*O*-caffeoyl quinic acid, whereas several other minor compounds were tentatively assigned as positional isomers of sulfo-caffeoyl quinic acid, sulfo-caffeoyl putrescine, sulfo-caffeic acid isomers, and sulfo-quercetin-3-*O*-glycosides. Our results suggest that whenever sodium hydrogen sulfite is used as an antibrowning agent during fruit and vegetable processing, one can expect *O*-diphenolics to react into sulfo-*O*-diphenolics, which might change their nutritional and sensory properties.

Structural Elucidation of the SO_3H-Containing Phenolics. In this study, we have found a series of phenolics with a molecular mass of 80 amu extra as compared to the common compounds found in ascorbic acid-treated potato juice, which is diagnostic for the attachment of a sulfite group. With ^1H and ^{13}C NMR spectroscopy, it was possible to establish that the SO_3H moiety was attached to the C-2′ position on the caffeic acid moiety as well as the position of the ester linkage between caffeic acid moiety and quinic acid moiety. Nevertheless, it could not be established whether the SO_3H group is attached to the aromatic ring through the sulfur or oxygen atom. Others have reported sulfated adducts of caffeic acid, 3-*O*- and 4-*O*-caffeoyl quinic acid isomers in urine and plasma, also with 80 amu extra.[19] Interestingly, the MS^2 fragmentation pattern of those sulfated adducts differs from those of **22** and **23** (Figure 2). This suggests that in that study, the sulfur-containing groups are differently attached to the aromatic ring as compared to **22** and **23**. The MS^2 fragmentation of **22** and **23** yielded m/z 353 in very low abundance, whereas m/z 259, 241, and 215 were quite abundant. In contrast, 5-*O*-caffeoyl quinic acid-*O*-sulfate yielded the ion $[M - SO_3 - H]^-$ with m/z 353 as base peak after MS^2,[19] and further fragmentation followed that of the precursor compound. No m/z 415, 387, 301, 259, 241, and 215 ions were reported in MS^2, as in that study they were present in very low intensities.[20] Hence, it is hypothesized that **22** and **23** are sulfo-adducts, in which the sulfur atom is attached directly to the aromatic ring. This is also consistent with the proposed mechanism underlying the formation of **22** and **23** (see further). In wine model solutions, double-linked sulfate adducts have been reported,[21] but these have only 64 amu extra, instead of 80.

Following the MS fragmentation data, as presented in Figure 2, **14**−**18**, **20**, and **21** are, analogous to **22** and **23**, annotated as sulfo-phenolics, more specifically, **15**−**18** and **20** as sulfo-caffeoyl quinic acid isomers and **14** and **21** as sulfo-caffeic acid isomers. Isomer **13**, with parent ion of m/z 433, gave the ion m/z 353 with relatively high intensity (80%) after MS fragmentation, which indicated that **13** might be a sulfated compound. Nevertheless, the presence of ions m/z 259, 241, and 215 in lower intensity suggested that it can not be excluded that **13** is a

sulfo-compound. MS^2 fragmentation of **19**, identified as sulfo-caffeoyl putrescine, gave the ion m/z 249 [caffeoyl putrescine − H]$^-$ as base peak and m/z 241 [sulfocaffeic acid − H_2O − H]$^-$ in high abundance.

Similar to sulfo-caffeoyl adducts, two modified flavonols were found and assigned as sulfo-quercetin glycosides. Fragmentation of the two modified quercetin glycosides (**24** and **25**) yielded the ion m/z 381 [sulfoquercetin − H]$^-$ as base peak (Table 2). Its further fragmentation produced the aglycone m/z 301 [quercetin − H]$^-$. In contrast, MS fragmentation of a glycosylated sulfate-flavonol has been shown to result in the glycosylated flavonol ion [M − 80 − H]$^-$,[22] and its further fragmentation yielded the aglycone as base peak. It seems that the glycosidic linkage in quercetin glycoside sulfates is stronger than the bond attaching the sulfates, whereas in sulfo-quercetin glycosides this is the opposite.

Mechanism and Position of Sulfonation. With the commercial PPO, it was shown that PPO is involved in the reaction between 5-*O*-caffeoyl quinic acid and $NaHSO_3$ to produce the sulfo-derivative. PPO catalyzes the oxidation of the *O*-diphenol in 5-*O*-caffeoyl quinic acid to its respective *O*-quinone (Figure 4A), which is prone to nucleophilic attack by HSO_3^-. After this attack, the resulting ketone tautomerizes to the thermodynamically more stable enol form, which can exist in equilibrium with 2′-sulfo-5-*O*-caffeoyl quinic acid depending on the pH. This final compound is less reactive than the *O*-quinone; therefore, the browning process is stopped.

For caffeic acid-containing compounds, the addition might occur at positions C-2′, C-5′, and C-6′. As shown in Figure 4B, the C-2′ position is preferred due to the conjugative delocalization of an electron to the side chain carbonyl, as well as to an oxygen on the aromatic ring.[15] For the C-5′or C-6′ position, only a single activation by electron migration to the oxygens attached to the aromatic ring is possible.[15] On the other hand, from a steric perspective, the preferred substitution should follow the order C-5′ > C-6′ > C-2′, with C-2′ as the least accessible carbon.[16] We postulate that the double activation overrules the steric hindrance effect. Our data are in agreement with the PPO-catalyzed reaction between glutathione or cysteine and different caffeic acid derivatives, resulting in C-2′ reaction products as the most abundant ones.[15,16,23] The importance of the double activation is evident if compared to the reactivity of dihydrocaffeic acid *O*-quinone.[16] In the latter case, no conjugative delocalization of an electron to the side chain carbonyl is possible. Hence, the C-5′ position is the most reactive, followed by C-6′ and C-2′, with the steric hindrance effect playing a major role.

Reaction of Other Phenolics with $NaHSO_3$. The lack of reactivity of ferulic acid- and sinapic acid-containing compounds with $NaHSO_3$ supports that PPO is involved in the reaction, as ferulic acid can not be oxidized by PPO extracted from potato.[24] In the case of sinapic acid, no *O*-hydroxylation by PPO, and thus no further *O*-quinone formation, is possible.

The C-5′ preferred nucleophilic attack has been shown for adducts of epicatechin/catechin and cysteine[23] and for adducts of catechin and glutathione.[16] In these reactions, the initial enzymatic oxidation is crucial for the activation of the B ring. With no enzyme present, no adduct formation was observed. The C-5′ is the most electrophilic and the least sterically hindered position; therefore, a nucleophilic attack is more probable at this position.[16] Hence, we postulate that **24** and **25** correspond to 5′-sulfo-quercetin-3-*O*-diglucoside and 5′-sulfo-rutin, respectively.

AUTHOR INFORMATION

Corresponding Author

*Tel: +31-317-483211. Fax: +31-317-484893. E-mail: harry.gruppen@wur.nl.

Funding Sources

We are grateful to COLCIENCIAS and Universidad Nacional de Colombia for providing a fellowship to C.E.N.-C. supporting this work. Part of this work was supported by the Commission of the European Communities within the Seventh Framework Programme for research and technological development (FP7), Grant Agreement No. 226930, title “Replacement of sulphur dioxide (SO_2) in food keeping the SAme qualitY and shelf-life of the products”, acronym SO2SAY.

ACKNOWLEDGMENT

We thank Prof. Ricaurte Rodriguez-Angulo from Universidad Nacional de Colombia for his advice on the reactivity of *O*-quinones with HSO_3^- (Figure 4). Information about fragmentation of sulfated hydroxycinnamic acids by Dr. Angélique Stalmach, Prof. Alan Crozier (both University of Glasgow), and Prof. Gary Williamson (University of Leeds) is greatly appreciated.

REFERENCES

(1) Andre, C. M.; Oufir, M.; Guignard, C.; Hoffmann, L.; Hausman, J. F.; Evers, D.; Larondelle, Y. Antioxidant profiling of native Andean potato tubers (*Solanum tuberosum* L.) reveals cultivars with high levels of β-carotene, α-tocopherol, chlorogenic acid, and petanin. *J. Agric. Food Chem.* **2007**, *55*, 10839–10849.

(2) Shakya, R.; Navarre, D. A. Rapid screening of ascorbic acid, glycoalkaloids, and phenolics in potato using high-performance liquid chromatography. *J. Agric. Food Chem.* **2006**, *54*, 5253–5260.

(3) Tudela, J. A.; Cantos, E.; Espín, J. C.; Tomás-Barberán, F. A.; Gil, M. I. Induction of antioxidant flavonol biosynthesis in fresh-cut potatoes. Effect of domestic cooking. *J. Agric. Food Chem.* **2002**, *50*, 5925–5931.

(4) Gómez-López, V. M. Some biochemical properties of polyphenol oxidase from two varieties of avocado. *Food Chem.* **2002**, *77*, 163–169.

(5) Makris, D. P.; Rossiter, J. T. An investigation on structural aspects influencing product formation in enzymic and chemical oxidation of quercetin and related flavonols. *Food Chem.* **2002**, *77*, 177–185.

(6) Timbo, B.; Koehler, K. M.; Wolyniak, C.; Klontz, K. C. Sulfites—A food and drug administration review of recalls and reported adverse events. *J. Food Prot.* **2004**, *67*, 1806–1811.

(7) Brunt, K.; Drost, W. C. Design, construction, and testing of an automated NIR in-line analysis system for potatoes. Part I: Off-line NIR feasibility study for the characterization of potato composition. *Potato Res.* **2010**, *53*, 25–39.

(8) Guerrero-Beltrán, J. A.; Swanson, B. G.; Barbosa-Cánovas, G. V. Inhibition of polyphenoloxidase in mango puree with 4-hexylresorcinol, cysteine and ascorbic acid. *Lebensm.-Wiss. Technol.* **2005**, *38*, 625–630.

(9) Sayavedra-Soto, L. A.; Montgomery, M. W. Inhibition of polyphenoloxidase by sulfite. *J. Food Sci.* **1986**, *51*, 1531–1536.

(10) Ferrer, O. J.; Otwell, W. S.; Marshall, M. R. Effect of bisulfite on lobster shell phenoloxidase. *J. Food Sci.* **1989**, *54*, 478–480.

(11) Narváez-Cuenca, C. E.; Vincken, J.-P.; Gruppen, H. Identification and quantification of (dihydro) hydroxycinnamic acids and their conjugates in potato by UHPLC-DAD-ESI-MS^n. *Food Chem.* **2012**, *130*, 730–738.

(12) Ferreres, F.; Llorach, R.; Gil-Izquierdo, A. Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2004**, *39*, 312–321.

(13) Scholz-Böttcher, B. M.; Ernst, L.; Maier, H. G. New stereoisomers of quinic acid and their lactones. *Liebigs Ann. Chem.* **1991**, 1029–1036.

(14) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. Hierarchical scheme for LC-MS^n identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900–2911.

(15) Cheynier, V. F.; Trousdale, E. K.; Singleton, V. L.; Salgues, M. J.; Wylde, R. Characterization of 2-S-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986**, *34*, 217–221.

(16) Moridani, M. Y.; Scobie, H.; Jamshidzadeh, A.; Salehi, P.; O'Brien, P. J. Caffeic acid, chlorogenic acid, and dihydrocaffeic acid metabolism: Glutathione conjugate formation. *Drug Metab. Dispos.* **2001**, *29*, 1432–1439.

(17) Chen, M.; Song, F.; Guo, M.; Liu, Z.; Liu, S. Analysis of flavonoid constituents from leaves of Acanthopanax senticosus Harms by electrospray tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 264–271.

(18) Dao, L.; Friedman, M. Chlorogenic acid content of fresh and processed potatoes determined by ultraviolet spectrophotometry. *J. Agric. Food Chem.* **1992**, *40*, 2152–2156.

(19) Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. *Drug Metab. Dispos.* **2009**, *37*, 1749–1758.

(20) Stalmach, A.; Williamson, G.; Crozier, A. Personal communication, 2011.

(21) Nonier, M. F.; Vivas, N.; Gaulejac, N. V. d.; Absalon, C.; Vitry, C. Study by LC/ESI/MS^n and ESI/HR/MS of SO_2 interactions in flavanols-aldehydes nucleophilic reactions. *Food Chem.* **2010**, *122*, 488–494.

(22) Ferreres, F.; Fernandes, F.; Oliveira, J. M. A.; Valentão, P.; Pereira, J. A.; Andrade, P. B. Metabolic profiling and biological capacity of *Pieris brassicae* fed with kale (*Brassica oleracea* L. var. *acephala*). *Food Chem. Toxicol.* **2009**, *47*, 1209–1220.

(23) Richard, F. C.; Goupy, P. M.; Nicolas, J. J.; Lacombe, J. M.; Pavia, A. A. Cysteine as an inhibitor of enzymatic browning. 1. Isolation and characterization of addition compounds formed during oxidation of phenolics by apple polyphenol oxidase. *J. Agric. Food Chem.* **1991**, *39*, 841–847.

(24) Ni Eidhin, D.; Degn, P.; O'Beirne, D. Characterization of polyphenol oxidase from rooster potato (*Solanum tuberosum* cv. Rooster). *J. Food Biochem.* **2010**, *34*, 13–30.

(25) Balcerzak, M.; Kopacz, M.; Kosiorek, A.; Swiecicka, E.; Kus, S. Spectrophotometric studies of the interaction of noble metals with quercetin and quercetin-5′-sulfonic acid. *Anal. Sci.* **2004**, *20*, 1333–1337.

(26) Justino, G. C.; Santos, M. R.; Canário, S.; Borges, C.; Florêncio, M. H.; Mira, L. Plasma quercetin metabolites: Structure-antioxidant activity relationships. *Arch. Biochem. Biophys.* **2004**, *432*, 109–121.