Carlos-Eduardo Narváez-Cuenca Hydroxycinnamoyl conjugates in potato tubers - Diversity and reactivity upon processing 2013

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Hydroxycinnamoyl conjugates in potato tubers

Diversity and reactivity upon processing

Carlos-Eduardo Narváez-Cuenca

Thesis

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To Gabriela, Samuel and Valentina

ABSTRACT: Despite the large amount of reports on the content of the hydroxycinnamic acid (HCA) caffeic acid and its conjugates (HCAcs; chlorogenic acid (ChA, 5-*O*-caffeoyl quinic acid), *crypto*-ChA (4-*O*-caffeoyl quinic acid) and *neo*-ChA (3-*O*-caffeoyl quinic acid)), other HCAcs/dihydroxicinnamic acid conjugates (DHCAcs) are rarely described in potato tubers. Furthermore, scarce information has been provided on the fate of phenolic acids when no anti-browning agents, or when the anti-browning agents ascorbic acid and $NaHSO_3$, are used during the preparation of potato juice. This thesis presents a method for the analysis of HCAs/HCAcs/DHCAcs based on RP-UHPLC-DAD-(H)ESI-MS^n. This analytical method revealed a total of 78 compounds tentatively identified as HCAs/HCAcs/DHCAcs when several Colombian potato cultivars and a single Dutch cultivar were analysed. The contribution of the less documented HCAcs/DHCAcs ranged from 7.1 to 20.1 % (w/w) of the total HCAs/HCAcs/DHCAcs in whole tuber. We provide for the first time molecular evidence on the formation of sulfonic acid derivatives when potato extracts are prepared in the presence of $NaHSO_3$. In that scenario, no ChA isomers were found but their sulfonic acid conjugates, with 2'-sulfo-ChA being found as the most abundant, followed by 2'-sulfo-*crypto*-ChA. Based on model experiments it was found that $NaHSO_3$ has a dual anti-browning effect: it reacts with ChA quinone, generated upon oxidation catalysed by polyphenol oxidase (PPO), to produce sulfo-ChA, and it inactivates PPO in a time-dependant way. When no anti-browning agent was used during the preparation of potato juice, the majority of the free ChA present in potato tubers was found to be associated with proteins. These results might explain why potato proteins, used as food ingredient, can turn brown in applications, when the protein is not isolated from potato tubers employing appropriate processing methodology.

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Chapter 1

General introduction

Background

Industrial isolation of potato starch produces large amounts of potato juice as by-product. Typically, proteins are precipitated from this potato juice by heat or acid treatment and used as animal feed. In recent years there has been an increased interest in the use of mild techniques to obtain proteins with good techno-functional properties, which makes them suitable for the food industry (*1*). Taking into account the European potato starch production, 140,000 tonnes of food-grade potato proteins are foreseen annually (*2*). The control of enzymatic browning during potato processing is a key factor in obtaining high quality food ingredients. For this, anti-browning agents, such as sodium hydrogen sulphite, are added during processing, in order to inhibit enzymes which convert phenolics into larger brown-coloured complexes. For understanding these browning reactions, it is important to have techniques for quantitative analysis of phenolic compounds, as well as methodologies to monitor their fate during processing.

Studies about phenolic compounds in potato tubers have been focused on the major representatives: Chlorogenic acid (5-*O*-caffeoyl quinic acid, ChA), *crypto*-ChA (4-*O*-caffeoyl quinic acid), *neo*-ChA (3-*O*-caffeoyl quinic acid), caffeic acid, and anthocyanin-linked hydroxycinnamic acids (HCAs). On the contrary, information on phenolic compounds other than these has been rarely provided. Information about the quantitative changes of the aforementioned compounds during processing is available in the literature. Nevertheless, while information on the concentrations of ChA, its isomers, and caffeic acid, in e.g. water or methanolic extracts of potato tubers has been provided, no information on their qualitative fate (tracking their structural changes upon reaction) and quantitative fate (tracking their concentrations, in all potential fractions, including insoluble materials, from the whole potato tuber) during enzymatic browning of potato, or upon use of anti-browning agents, is available.

The research described in this PhD thesis deals with the identification and quantification of HCAs, their conjugates (HCAcs) and dihydrohydroxycinnamic acid conjugates (DHCAcs) in potato tuber cultivars, with special emphasis on those phenolic compounds less commonly described. The qualitative and quantitative fate of the HCAs/HCAcs/DHCAcs in the presence (and absence) of anti-browning agents (ascorbic acid and sodium hydrogen sulfite) is described as well.

Potato tubers

Potato, original to the Andean region in South America, is to date one of the most important crops in the world. According to the FAO, 324 million tons of potatoes were produced worldwide in 2010. This production placed this crop as number five worldwide after sugar

cane (1,685 million tons), maize (844 million tons), rice (672 million tons) and wheat (651 million tons) (*3*). In The Netherlands there are more than 110 potato cultivars (*4*), with a production in 2010 of 6.8 million tons (*5*). These numbers contrast with the 43 cultivars reported in South American countries, such as Colombia, with a production in 2010 of 2.1 million tons (*5, 6*).

Some compositional attributes of potato tubers are shown in **Table 1**. On a dry weight basis, potato tubers are mainly composed of starch, with also considerable amounts of proteins present (*7, 8*). The ascorbic acid content of potato tubers (*8, 9*) is comparable to that of some wild fruits, nuts and leafy vegetables (*10*). Glycoalkaloids are also abundant in potato (*11*), with α-chaconine and α-solanine as well-known representatives (*12*). Because of their toxicity to humans, it has been advised not to consume potato tubers exceeding a content of 20 mg α-chaconine + α-solanine/100 g fresh weight (*13*). In recent years, it has been shown that glycoalkaloids might have beneficial effects related to e.g. their anti-cancer and anti-inflammatory properties (*13*). Furthermore, certain potato cultivars can contribute substantially to the recommended daily intake of minerals, e.g. up to 72% potassium, up to 41% iron, and up to 31% selenium (*14*).

Table 1. Summary of some nutritional components of potato tubers.

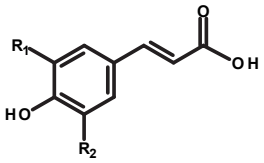
Parameter	Unit	Amount	References
Dry matter	g/100 g FW	16-34	(*7, 8, 15*)
Starch	g/100 g DW	60-73	(*8*)
Protein	g/100 g DW	4-14	(*7, 8*)
Ascorbic acid	mg/100 g DW	40-182	(*8, 9*)
Glycoalkaloids[a]	mg/100 g DW	18-550	(*11*)
Phenolic compounds			
by Folin-Ciocalteu	mg GA/100 g DW[b] DW	92-304	(*9, 16*)
	mg ChA/100 g DW[c]	279-440	(*8*)
by HPLC	mg/100 g DW[d]	27-527	(*16-20*)

[a]α-Solanine plus α-chaconine. [b]GA: expressed as gallic acid. [c]ChA: expressed as chlorogenic acid. [d]Includes hydroxycinnamic acids, hydroxycinnamic acid conjugates (including anthocyanin-linked HCAs), dihydrohydroxycinammic acid conjugates, hydroxybenzoic acids and flavonols. All compounds were quantified by HPLC, except the anthocyanin-linked HCAs. This last group of compounds was determined by the pH differential method, and expressed as petanin (petunidin-3-*O*-*p*-coumaroyl-rutinosyl-5-*O*-glucoside). FW: Fresh weight; DW: Dry weight.

Compared to other vegetables, potato tubers contain high levels of phenolic compounds (*21*). Phenolic compounds are involved in potato adaptation to abiotic and biotic stresses (*22, 23*). Phenolic compounds in potato are of particular interest for human consumption, as they have health-promoting effects, and they can negatively influence the sensory quality of potato during processing. Furthermore, they can negatively affect the nutritional and technological properties of other components, such as proteins (*24*).

HCAs, HCAcs and DHCAcs in potato tubers

Phenolic compounds in potato tubers are represented mainly by two groups: Phenolic acids and flavonoids. HCAs, HCAcs, DHCAcs and hydroxybenzoic acids are the representatives of phenolic acids in potato tubers. The chemical structure of HCAs is shown in **Figure 1**. HCAs are represented by *p*-coumaric acid (**1**), caffeic acid (**2**), ferulic acid (**3**), hydroxyferulic acid (**4**), and sinapic acid (**5**), each with a characteristic set of hydroxy and/or methoxy substituents on the aromatic ring. Caffeic acid is the only unconjugated HCA that is reported in potato tubers, with ferulic acid being reported sometimes in trace amounts (*16-19*).



HCAs	Substitutions
(1) *p*-Coumaric acid	$R_1 = R_2 = H$
(2) Caffeic acid	$R_1 = OH$, $R_2 = H$
(3) Ferulic acid	$R_1 = OCH_3$, $R_2 = H$
(4) Hydroxyferulic acid	$R_1 = OCH_3$, $R_2 = OH$
(5) Sinapic acid	$R_1 = R_2 = OCH_3$

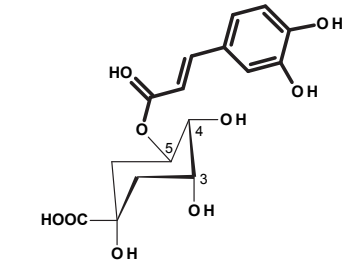
Figure 1. Chemical structure of hydroxycinnamic acids (HCAs).

HCAs can be conjugated to other molecules by their carboxylic acid group to yield HCAcs. For instance, HCAs can be linked to (poly) hydroxyl molecules or to (poly) amino molecules to produce ester or amide conjugates. In potato, the most abundant phenolic acid is chlorogenic acid (*24*) (5-*O*-caffeoyl quinic acid; ChA; (1*S*,3*R*,4*R*,5*R*)-5-{[(2*Z*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,3,4-trihydroxycyclohexane-carboxylic acid (*25*); **6** in **Figure 2**), an HCAc, in which caffeic acid is esterified with the poly-hydroxyl molecule quinic acid. Other HCAcs commonly reported in potato are two isomers of ChA: *crypto*-ChA (**7** in **Figure 2**) and *neo*-ChA (**8** in **Figure 2**) (*16-18*).

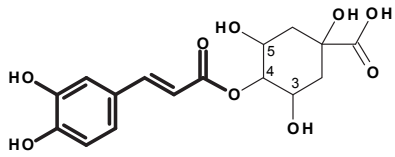
Anthocyanin-linked HCAs can be considered as a subclass of flavonoids. Anthocyanins acylated with *p*-coumaric acid, caffeic acid, and ferulic acid are commonly described in red-fleshed/skinned potatoes (*18, 26, 27*). Therefore, we classified them as HCA-(poly) hydroxyl conjugates. Other compounds of the type HCA-(poly) hydroxyl conjugates, less documented in potato are: Caffeoyl hexose, 1-*O*-caffeoyl quinic acid, 3-*O*-caffeoyl-5-*O*-feruloyl quinic acid, 5-*O*-feruloyl quinic acid, caffeoyl methyl quinate and 4,5-di-*O*-caffeoyl quinic acid (*19*). Regarding the HCAs linked to polyamino molecules, the conjugates caffeoyl putrescine (**9** in **Figure 2**) (*17*), caffeoyl spermine containing compound and ferulic acid amide have been reported in potato tubers (*19*).

DHCAcs differ from the HCAcs by the lack of unsaturation in the aliphatic chain of the HCAs. In potato, DHCAs are not reported in an unconjugated form, but as amides of dihydrocaffeic acid linked to spermine or spermidine to yield DHCAcs. Parr *et al*. (*28*) reported for the first time the presence of four dihydrocaffeoyl polyamines: N^1,N^{14}-

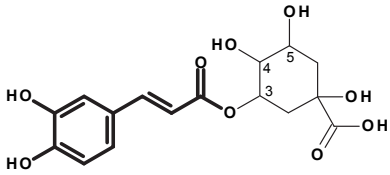
bis(dihydrocaffeoyl) spermine (Kukoamine A, **10** in **Figure 3**), N^1,N^{10}-bis (dihydrocaffeoyl) spermidine (**11** in **Figure 3**), N^1,N^5,N^{14}-tris(dihydrocaffeoyl) spermine, and N^1,N^5,N^{10}-tris(dihydrocaffeoyl) spermidine. In a more recent work, N^1,N^5,N^{10},N^{14}-tetra(dihydrocaffeoyl) spermine was found in potato tubers (*19*). Different to the *N*-substitution numbering used by Parr *et al*. (*28*) and by Shakya and Navarre (*19*), we have adopted the new numbering rules according to Bienz *et al*. for polyamines (*29*).



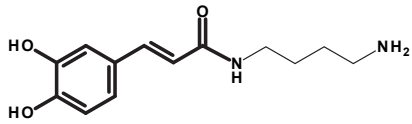
(6) 5-*O*-Caffeoyl quinic acid (Chlorogenic acid)



(7) 4-*O*-Caffeoyl quinic acid (*Crypto*-chlorogenic acid)

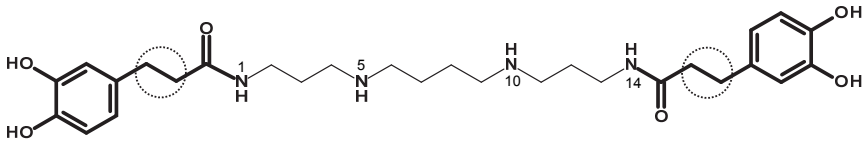


(8) 3-*O*-Caffeoyl quinic acid (*Neo*-chlorogenic acid)

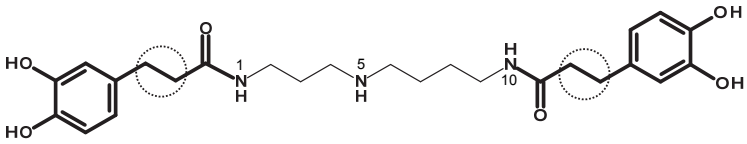


(9) Caffeoyl putrescine

Figure 2. Chemical structure of the most commonly reported hydroxycinnamic acid conjugates in potato tubers.



(10) N^1,N^{14}-bis(dihydrocaffeoyl) spermine (known as kukoamine A)



(11) N^1,N^{10}-bis(dihydrocaffeoyl) spermidine

Figure 3. Chemical structure of representative dihydrohydroxycinnamic acid conjugates in potato tubers. Dotted circles emphasize the absence of the double bond in the aliphatic chain of the dihydrocaffeoyl moiety, compared to a caffeoyl moiety. Numbering of the *N*-substitutions is according to Bienz *et al*. (*29*).

Within the group of phenolic acids, HCAs and HCAcs are present in higher amounts than DHCAcs and hydroxybenzoic acids (*19, 20*). In **Table 2**, the minimum and

maximum concentrations (mg/100 g DW potato) of each compound do not necessarily coincide with the others, as a variety that is the richest in one compound is not necessarily the richest in the others. The total amount of the most commonly described HCAs/HCAcs, i.e. ChA, *crypto*-ChA, *neo*-ChA and caffeic acid, ranges from 27 to 363 mg/100 g DW. If to the four mentioned compounds the total amount of anthocyanin-linked HCAs is added, then the range is from 27 to 527 mg/100 g DW. Flavonoids in potato are characterized by flavonols (mainly rutin and kaempferol-3-*O*-rutinoside), accounting for up to 44 mg flavonols/100 g DW. The contents of non-anthocyanin HCAs/HCAcs in potato are 3 to 21 times higher in peel than in flesh (*30*). Geographic origin, nutritional conditions of soils and storage conditions of harvested tubers might affect the contents of HCAs/HCAcs up to two orders of magnitude (*26, 31, 32*). Furthermore, the contents of HCAs/HCAcs can be increased, remain unchanged or decrease to even undetectable levels during home-processing (*16, 30*). In addition, isomerization of ChA into *neo*-ChA has been described during industrial processing of potato tubers (*20*).

Table 2. Phenolic compounds of potato tubers (mg/100 g DW).

Parameter	Amount	References
Phenolic acids		
HCAs/HCAcs[a]		
Chlorogenic acid	17-322	(*16-19*)
Crypto-chlorogenic acid	1-56	(*16-19*)
Neo-chlorogenic acid	Trace-44	(*16-19*)
Caffeic acid	Trace-48	(*16-19*)
Anthocyanin-linked HCAs[b]	Up to 293	(*18*)
Other HCAcs	Up to16[c]	(*17, 19*)
DHCAcs[d]	Up to 36[c]	(*19*)
Hydroxybenzoic acids	Up to 2[c]	(*20*)
Flavonoids		
Flavonols		
Rutin	Trace-19	(*17, 18*)
Kaempferol-3-*O*-rutinoside	Trace-43	(*17, 18*)

The reported intervals do not include compositional outliers. [a]HCAs: Hydroxycinnamic acids; HCAcs: Hydroxycinnamic acid conjugates. [b]Present in red/purple cultivars, determined by the pH differential method, and expressed as petanin (petunidin-3-*O*-*p*-coumaroyl-rutinosyl-5-*O*-glucoside). [c]Few varieties or limited number of compounds analysed. [d]DHCAcs: Dihydrohydroxycinnamic acid conjugates. DW: Dry weight.

HCAs/HCAcs/DHCAcs analysis

Analysis of HCAs/HCAcs/DHCAcs in potato has been performed with several techniques. While the Folin-Ciocalteu (F-C) reagent provides a rough estimation of the total phenolic content (*33*), other techniques, such as capillary electrophoresis, gas chromatography and

reversed phase (RP) - high performance liquid chromatography (HPLC), provide information on individual compounds in potato tubers (*20, 34, 35*).

Quantification by the F-C reagent is based on a redox reaction between phenolic compounds and the F-C reagent in alkaline conditions (*36*). The blue reaction product is measured spectrophotometrically with the advantage of being fast and relatively inexpensive. This method, nevertheless, tends to overestimate the actual concentration of phenolic compounds in extracts, due to the presence of other constituents, such as ascorbic acid, tryptophan and proteins, that can reduce the F-C reagent as well (*36*). From **Table 1** one could conclude that the F-C method gives a total content of phenolic compounds similar to that of the sum of individual phenolics. However, it is a coincidence that the interval reported for total phenolics, by F-C with ChA as standard (*8*), is similar to that of total phenolics by adding the contents of each compound from HPLC data (*16-20*), as they are collected from different studies. Phenolic compounds have different response factors with the F-C reagent (*37*). If analysis of total phenolic content in potato has to be performed by the F-C method, calibration lines with ChA as standard would be the best choice (as done in reference (*8*)), as ChA is the most abundant phenolic compound in potato.

Capillary electrophoresis in combination with UV detection has been used in the analysis of HCAs/HCAcs in potato tubers (*34*). Although it is considered a fast technique, analyses have been limited only to the most commonly reported HCAs/HCAcs (*34*). Furthermore, gas chromatography coupled to a mass spectrometer has been used for the quantification of ChA in potato tubers (*35*). This technique is time-consuming due to the derivatization of ChA prior to its analysis. Identification of ChA, its isomers *crypto*-ChA and *neo*-ChA, and caffeic acid in potato tubers has been performed by RP-HPLC coupled to a diode array detector (DAD) (*20*). Comparison of both retention times from RP-HPLC and UV spectral data allows the identification of those compounds. Quantification of the mentioned compounds can be performed by the external standard method with caffeic acid and ChA as standards.

The challenge in the identification of HCAcs/DHCAcs in potato tubers for which no commercial standards are available has been overcome by the use of RP-HPLC with an in-line tandem mass spectrometer (MS^n) (*19, 26, 28*). In recent years, RP-ultra (U)HPLC-MS^n has been introduced. Although it has been used for the analysis of other agricultural raw materials it has not been employed for potato (*38*). The effluent from the RP-(U)HPLC is directed into a tandem mass spectrometer, where the compounds are ionized. Phenolic compounds are generally ionized by electrospray ionization (ESI), which is preferred over atmospheric pressure chemical ionization (APCI). The latter is preferred for non-polar metabolites, such as carotenoids (*39*). Ionization can be performed in the negative mode to produce negative ions or in the positive mode to produce positive ions (*39, 40*).

In certain cases, mass spectrometry allows the screening of a specific group of phenolic compounds in complex mixtures, as has been shown by the screening of

prenylated isoflavonoids from soya and licorice (*41*). In that study, a guideline enabling the identification of the type of prenylation was formulated, based on diagnostic neutral losses and positive ions characteristic to the prenyl group. In the case of ChA, *neo*-ChA and *crypto*-ChA, it has been shown that the fragmentation of the parent ion, $[M-H]^-$ = 353, produces the daughters *m/z* 179, 161 and 135, characteristic of [caffeic acid-H]$^-$, [caffeic acid-H_2O-H]$^-$ and [caffeic acid-CO_2-H]$^-$, respectively. It also yields the daughters *m/z* 191 and 173, characteristic of [quinic acid-H]$^-$ and [quinic acid-H_2O-H]$^-$, respectively (*42*). Ions corresponding to neutral losses of CO_2 have been found for *p*-coumaric and other HCA containing compounds (*43*). Besides the ions produced by neutral losses of H_2O and CO_2, ions produced by neutral losses of CH_3 have been reported in the case of sinapic acid and ferulic acid containing compounds (*43*). Furthermore, it has been found that fragmentation of the parent ions of dihydrocaffeoyl spermine and dihydrocaffeoyl spermidine conjugates yields ions corresponding to neutral losses of 122 and 164 a.m.u. (*19*). HCAs/HCAcs have maxima of absorption at 310-330 nm and at 285-305 nm (*44, 45*). Maxima of absorption at 280 nm and at 320 nm have been reported for DHCAcs (*28, 46*). HCAs/HCAcs/DHCAcs have, therefore, characteristic mass spectrometric and spectral features. Nevertheless, a general strategy for the identification of compounds as HCAs/HCAcs/DHCAcs has not yet been reported.

Potato phenolics and human health

It is known that ChA and also the less documented HCAcs/DHCAcs have health-promoting effects, like anti-hypertensive and anti-cancer activity (*47-51*). When two potato cultivars were assessed for their composition of HCAs/HCAcs/DHCAcs, it was found that some of the less documented HCAcs/DHCAcs were quantifiable (*19*). Contribution of those less documented HCAcs/DHCAcs might play an important role, with respect to total intake of phenolic compounds and their individual health-promoting effects.

A serving portion of 200 g potato (FW) would provide 11-145 mg (35-424 µmol) of caffeic acid + ChA + *crypto*-ChA + *neo*-ChA, assuming a water content of 80% (w/w) (**Table 2**) (*16-19*). It has been found that after human ingestion of instant coffee the whole pool of HCAcs is metabolised and that the main products in plasma are (dihydro) caffeic acid, (dihydro) ferulic acid, and their sulfated and glucuronated adducts (*52*). In the study mentioned, a 200 mL serving of instant coffee provided 412 µmol of HCAcs, which included (di) caffeoyl quinic acid isomers, feruloyl quinic acid isomers and *p*-coumaroyl quinic acid isomers. Given the health-promoting effects related to HCAcs after moderate coffee consumption (*53*), the contribution of potato to the daily HCAs/HCAcs intake seems of interest, especially for non-coffee drinkers.

Reactivity of HCA(c)s during enzymatic browning

Polyphenol oxidases (PPOs) have been associated with enzymatic browning during fruit and vegetable processing (*54*). The enzymes tyrosinase and catecholase are often referred to as PPOs (**Figure 4**). Tyrosinase (also called cresolase, EC 1.14.18.1) catalyzes the hydroxylation of monophenolic compounds into *o*-diphenolic compounds and further oxidation to quinones, which are highly reactive (*55*). Catecholase (EC 1.10.3.1) only catalyzes the oxidation of *o*-diphenolic compounds to quinones (*56*). Some authors also include the enzyme laccase within the group of PPOs (*57*). Different to tyrosinase, laccase (EC 1.10.3.2) can oxidise ferulic acid to a phenoxy radical species (*57*). Potato PPO is able to oxidise ChA, caffeic acid and L-tyrosine, but not ferulic acid (*58, 59*). Therefore, it has tyrosinase activity, but not laccase activity. PPO extracted from potato has optimum activity at pH 6-7 and loses, irreversibly, most activity at pH values below 4 (*58, 59*), retaining less than 20% of activity after 48 h of incubation at pH 4. In the research described in this thesis, PPO refers to the tyrosinase activity in potato tubers, adopted from the references (*58, 59*).

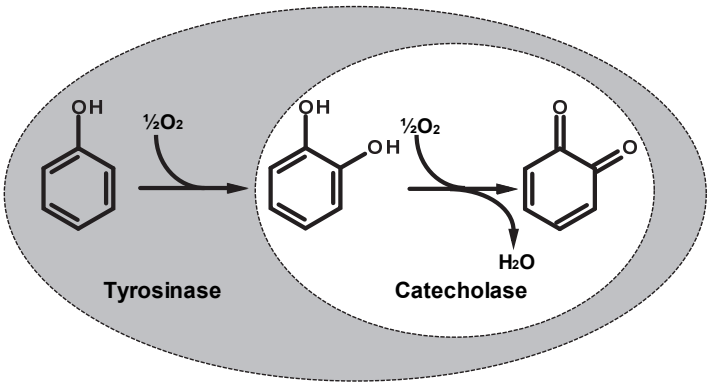


Figure 4. Typical reactions for tyrosinase and catecholase.

Peroxidase (EC 1.11.1.7, POD) oxidises phenolic compounds using H_2O_2 as an electron acceptor. Phenolic compounds upon POD oxidation yield phenoxy radical products that can react further, and participate in e.g. the biosynthesis of lignin (*60*). The role of POD in enzymatic browning during fruit and vegetable processing is controversial, given the low amounts of H_2O_2 expected in plant tissues and the higher affinity of PPO towards phenolic compounds commonly present in plant tissues than that of POD (*61*). In spite of this, it has been suggested that POD might play an important role in the presence of PPO (*61*).

Figure 5 shows a general overview of possible products formed upon reaction of ChA, based on model experiments with low molecular weight compounds (*48, 62-67*). From model systems it is known that ChA (**12** in **Figure 5**) is oxidised by PPO to produce ChA quinone (ChAq, **13** in **Figure 5**). Once the ChAq is produced, it can react further with a second molecule of ChA to produce several isomers of ChA dimers (ChA_2) (**14** in **Figure 5**) (*62, 63*). The chemical structures of dimeric oxidation products of caffeic acid have been elucidated by NMR and UV-vis spectroscopy and FAB-MS (*64*). Based on these structures, oxidation products of ChA, which include dimers of the naphthalene- (**15** in **Figure 6**), benzofuran- (**16** in **Figure 6**) and benzodioxane-type (**17** in **Figure 6**), have been proposed by others (*62, 63*). These isomeric compounds (**15**-**17**) were presented as one, as compound **14,** in **Figure 5**.

PPO-induced reactions of ChA in the presence of the *N*-protected amino acids (AA) cysteine, lysine, tryptophan, histidine, or tyrosine yielded, besides the homo-dimers of ChA (ChA_2), hetero-dimers (ChA-AA), trimers (ChA_2-AA), and tetramers (AA-ChA_2-AA) (*62, 63*). ChAq (**13** in **Figure 5**) reacts with an AA (**18** in **Figure 5**) to produce hetero-dimers of ChA-AA (**19** in **Figure 5**). This hetero-dimer might be a substrate of PPO and produce ChAq-AA (**20** in **Figure 5**), which in turn reacts with a second molecule of AA to yield trimers of AA-ChA-AA (**21** in **Figure 5**).

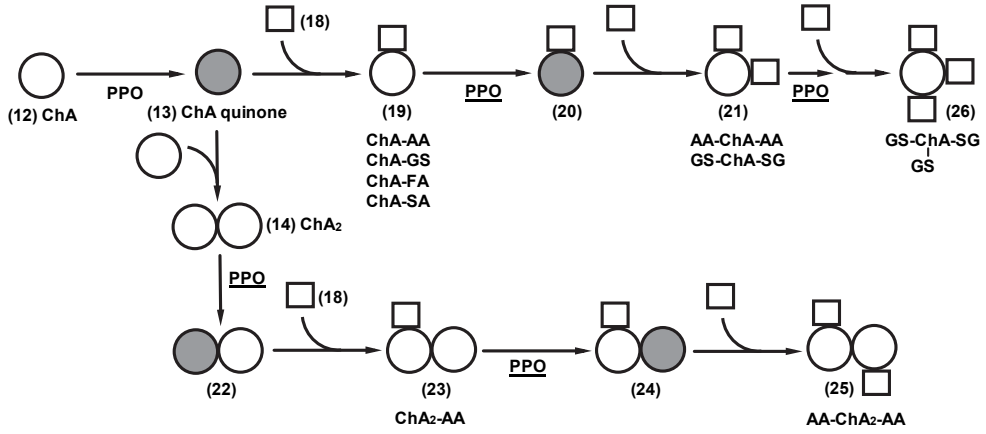


Figure 5. Schematic representation of the oxidation of chlorogenic acid (ChA,○) by polyphenol oxidase (PPO) and further reactions with several low molecular weight compounds: ChA, ferulic acid (FA), sinapic acid (SA), amino acids (AAs: *N*-protected-cysteine, *N*-protected-tryptophan, *N*-protected-histidine, *N*-protected-tyrosine) or glutathione (GSH). ChA quinone (**13**, ●) and some ChA_2-AA (**23**) are colored compounds. Figure based on model systems (*48, 62-65*). **18** (□) can represent an AA, GSH, FA or SA. **14** represents the three isomers that are depicted in **Figure 6** (**15**-**17**). PPO: The PPO-catalyzed reactions can be replaced by non-enzymatic redox reactions in which ChA quinone is converted to ChA.

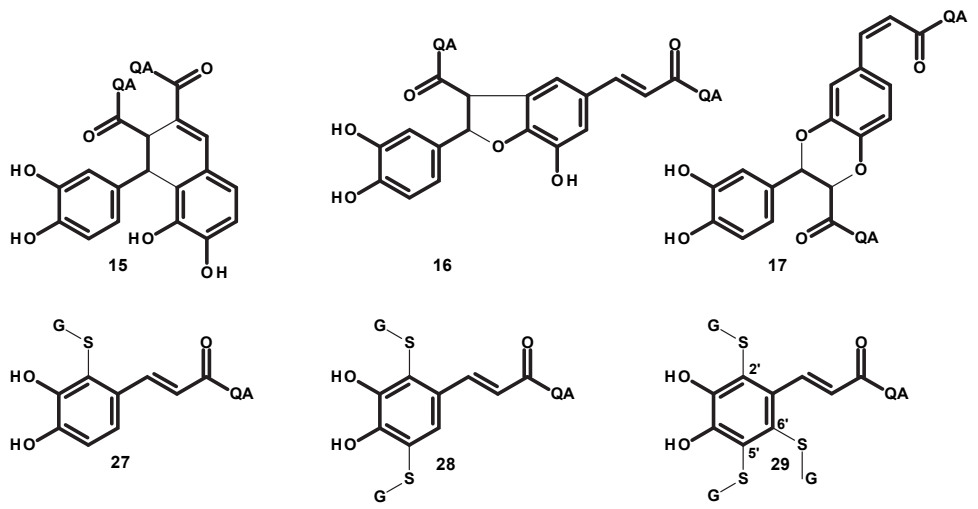


Figure 6. Proposed structures of chlorogenic acid (ChA) dimers, naphthalene- (**15**), benzofuran- (**16**) and benzodioxane- (**17**) type, based on reported oxidation products of caffeic acid (*64*). 2'-*S*-glutathionyl-ChA (**27**); 2',5'-*S*,*S*-diglutathionyl-ChA (**28**); and 2',5',6'-*S*,*S*,*S*-triglutathionyl-ChA (**29**). QA=quinic acid. GS= glutathione.

It has been proposed that production of trimers (ChA_2-AA) and tetramers (AA-ChA_2-AA) first involves dimerization of ChA into ChA_2, and subsequently the reaction with AAs. Once ChA_2 is formed (**14** in **Figure 5**), it can be oxidised again to produce a ChA_2 quinone (ChA_2q, **22** in **Figure 5**), which in turn reacts with an amino acid to produce the ChA_2-AA trimer (**23** in **Figure 5**) (*62, 63*). Likewise, ChA_2-AA might be oxidised to produce a reactive quinone (**24** in **Figure 5**), followed by a reaction with another amino acid to produce the AA-ChA_2-AA tetramer (**25** in **Figure 5**). Hetero reaction products between ChA and up to three molecules of the tri-peptide glutathione (**19**, **21** and **26** in **Figure 5**) were obtained by PPO catalysis and characterised by mass spectrometry and NMR (*48*). In those experiments the main reaction product was 2'-*S*-glutathionyl-ChA (**27** in **Figure 6**), followed by 2',5'-*S*,*S*-diglutathionyl-ChA (**28** in **Figure 6**), and by 2',5',6'-*S*,*S*,*S*-triglutathionyl-ChA (**29** in **Figure 6**). Compounds **27** and **28** were poor substrates of PPO (*48*). In addition to the PPO-catalyzed oxidation of phenolics, it has been proposed that quinones generated in the first steps of enzymatic browning, e.g. ChAq, can oxidise other phenolics. In that case, ChAq is reduced to ChA, which in turn can be oxidised again by PPO. Therefore, compounds **20**, **22**, **24** and **26** might be produced by either PPO-oxidation or by non-enzymatic redox reactions (**Figure 5**, represented as PPO) (*68*). It remains to be established whether further oxidation of homo- or hetero-dimers predominantly proceeds by direct action of PPO, or by non-enzymatic redox reactions.

To our knowledge, PPO-catalyzed oxidation products comprising two or more HCAcs have not been described. It has been shown though, that caffeic acid oxidised with POD/H_2O_2 reacts with ferulic acid and sinapic acid to yield the hetero-dimers (caffeic-ferulic acid and caffeic-sinapic acid) (*65*). Therefore, it might be hypothesised that the ChAq reacts with ferulic acid or sinapic acid, to yield hetero-dimers ChA-ferulic acid and ChA-sinapic acid (**19** in **Figure 5**).

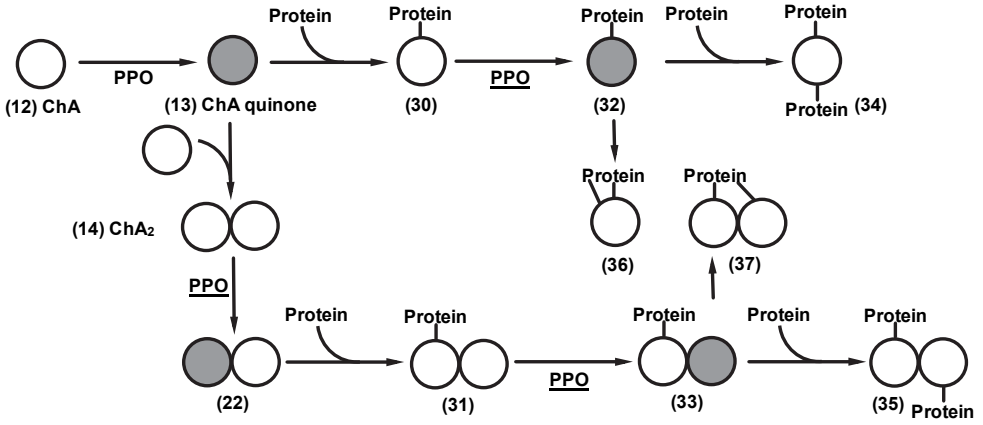


Figure 7. Schematic representation of the oxidation of chlorogenic acid (ChA, ○) by polyphenoloxidase (PPO) and further reactions with proteins. Figure based on model systems (*66, 67*). PPO: Those PPO-catalyzed reactions can be replaced by non-enzymatic redox reactions in which ChA quinone (●) is converted to ChA.

Oxidised ChA can covalently bind to proteins as shown in model experiments (*66, 67*). In such modifications, two, three or four units of ChA were found covalently attached to monomeric proteins (no large modification in MW of proteins). The final products also included cross-linked proteins (large increase in MW of proteins), presumably containing ChA.

The mechanism behind the covalent reaction between ChA and proteins is not completely clear. Based on what was described above in terms of ChA-AA oxidation products, it has been proposed that both the ChAq (**13** in **Figure 7**) and the ChA_2q (**22** in **Figure 7**) react with proteins to produce adducts ChA-protein (**30** in **Figure 7**) and ChA_2-protein (**31** in **Figure 7**), respectively (*63, 66*). The cross-linking of proteins has been explained as the result of further oxidation of ChA-protein (**30** in **Figure 7**) or ChA_2-protein (**31** in **Figure 7**), to produce ChAq-protein (**32** in **Figure 7**) or ChA_2q-protein (**33** in **Figure 7**), respectively (*63, 66*). Next, the quinoidal structures react with a second protein unit to produce protein-ChA-protein (**34** in **Figure 7**) or protein-ChA_2-protein (**35** in **Figure 7**). Given the expected presence of more than one surface exposed reactive amino acid in a protein unit, intra-molecular cross-linking leading to structures such as **36** and **37**

might be expected as well. PPO or non-enzymatic redox reactions might be involved in the production of **22**, **32** and **33**. As a result of the modification of proteins by ChA, techno-functional parameters (such as solubility and foam stability) and nutritional parameters (such as digestibility) were negatively affected (*66, 67*). The color of the proteins was also negatively affected. The actual effects of these modifications were dependent on the type of protein studied (*66, 67*).

With the presence of multiple phenolics (*19*), free amino acids (*69, 70*), glutathione (*71*) and proteins (*72*) in potato tubers, one might expect that enzymatic browning, during e.g. potato starch production, might be more complex than what has been shown in the model experiments described above. Given this complexity, only few studies have addressed the identification and quantification of oxidation products related to enzymatic browning during fruit or vegetable processing. The complexity of oxidation products and the challenge of their analysis has been underlined when studying low molecular weight enzymatic browning reaction products of cider apple juice (*73*). Nevertheless, no studies on potato tubers have been performed on this regard.

Inhibition of enzymatic browning

To control enzymatic browning during fruit and vegetable processing several techniques can be used, which include e.g. heat treatments and chemical additives (*74*). Chemical additives used as anti-browning agents are ascorbic acid, citric acid and sulphur-containing compounds, e.g. sulfites, glutathione and cysteine.

The anti-browning action of ascorbic acid has been attributed **(i)** to its capacity to reduce quinones to the original phenolic compounds, and to a lesser extension **(ii)** to irreversibly inactivate PPO by reducing the Cu^{2+} ions in the active site of the enzyme (*75*) and **(iii)** to a reduction of the pH of the matrix and, thereby, making PPO less active (*76*). The last property is shared by citric acid (*76*), a compound that is also considered to inhibit PPO by chelation of the Cu^{2+} ions present in PPO (*77*).

Sodium dioxide (SO_2) and sodium or potassium salts of sulfite (SO_3^{2-}), hydrogen sulfite (HSO_3^-) and metabisulfite ($S_2O_5^{2-}$) are effective anti-browning agents (*78-81*). The anti-browning effects of these compounds have been attributed, as described above for ascorbic acid, **(i)** to their capacity to reduce the quinones to the original phenolic compounds and **(ii)** to irreversible inactivation of PPO (*82*). Different to ascorbic acid, the anti-browning effect of sulfites and related compounds has also been attributed **(iii)** to their capacity to react with quinones and inhibit further reactions (*83*). The latter property was based on observations with UV-vis spectroscopy. No actual compounds have been identified. When these sulfur-containing inhibitors are dissolved in water the species HSO_3^- is present at pH values normally found in fruits and vegetables. This molecular species is

considered to be responsible for the inhibition of browning (*82*). At pH 6.0, e.g., approximately 95% (mol/mol) is represented by HSO_3^- (*84, 85*).

The FDA prohibits the use of SO_2 and other sources of HSO_3^- in fruits and vegetables to be served as raw or presented as fresh to the public (*86*), due to their adverse pulmonary reactions (*87*). These anti-browning agents are, nevertheless, still allowed in minimally processed potatoes, as well as in potato starch production (*86, 88*). Other sulfur-containing compounds have been shown to have promising anti-browning effects during fruit and vegetable processing, as is the case with the amino acid cysteine and the tri-peptide glutathione (*89*). Cysteine acts as an anti-browning agent by reacting with quinones, e.g. ChA quinone, to produce mainly the 2'-*S*-cysteinyl-ChA adduct (*62, 90, 91*). This adduct does not undergo further transformation and also behaves as a competitive inhibitor of PPO. Glutathione has also been found to form adducts with ChA quinone, as described previously, to produce mono-, di- and tri-*S*-glutathionyl-ChA adducts (*48*) (**27**, **28** and **29** in **Figure 6**).

Aim and outline of the thesis

As stated above, few attempts on the identification and quantification of phenolic acids other than ChA, *crypto*-ChA, *neo*-ChA, caffeic acid, and anthocyanin-linked HCAs have been done in tubers among different potato cultivars. Furthermore, little is known about the mechanism of action of sodium hydrogen sulfite as anti-browning agent. Finally, no information on the qualitative and quantitative fate of phenolic compounds during enzymatic browning is available. Therefore, this thesis is aimed at studying the HCAs/HCAcs/DHCAcs composition of potato tubers and their behaviour during potato tuber processing, following the several steps of potato starch production, with and without the use of anti-browning agents.

In **chapter 2**, a RP-UHPLC-DAD-ESI-MS^n method for the identification and quantification of HCAs/HCAcs/DHCAcs in potato tubers is described. The convenience of using only few available HCAs/HCAcs standards for the quantification of the whole set of HCAs/HCAcs/DHCAcs is also described in this chapter. The qualitative and quantitative variability of HCAs/HCAcs/DHCAcs within several Colombian potato cultivars is studied in **chapter 3**. The effect of ascorbic acid and $NaHSO_3$ on the composition of HCAs/HCAcs/DHCAcs in potato juice, while following an extraction method as the one involved in potato starch production is described in **chapter 4**. In **chapter 5** the inhibitory effect of ascorbic acid, $NaHSO_3$ and other sulfur-containing compounds on the activity of PPO in model experiments is shown. **Chapter 6** describes the quantitative fate of ChA during enzymatic browning, when no anti-browning agents are used during potato juice preparation. In **chapter 7**, a general discussion is presented and implications of the findings are elaborated.

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Chapter 2

Identification and quantification of (dihydro) hydroxycinnamic acids and their conjugates in potato by UHPLC-DAD-ESI-MS^n

ABSTRACT: Hydroxycinnamic acids (HCAs), their conjugates (HCAcs) and dihydrohydroxycinamic acid conjugates (DHCAcs) were identified and quantified in potato tuber extracts by UHPLC-DAD-ESI-MS^n. The HCAcs and DHCAcs identification took place by screening for product ions and neutral losses in combination with UV spectra. Thirty nine HCAs/HCAcs/DHCAcs were detected, including 17 previously reported in potato. HCAs were found unconjugated, linked to hydroxyl-containing compounds including hexose, quinic acid and malic acid, to amino-containing compounds, such as putrescine and octopamine, and to unknown compounds. DHCAs were present linked to spermine, spermidine and to still unidentified compounds. Chlorogenic acid was the most abundant compound (25.43±0.49 mg/100 g DW) followed by *crypto*-chlorogenic acid (7.31±0.38 mg/100 g DW), a non-hydrolyzable sinapic acid conjugate (2.80±0.06 mg/100 g DW) and *neo*-chlorogenic acid (2.41±0.10 mg/100 g DW), in total accounting for 83% (w/w) of the total concentration of HCAs/DHCAs-containing compounds. Quantifications of HCAs released after alkaline hydrolysis matched well with the quantification of the unhydrolyzed molecules. The UHPLC-DAD-ESI-MS^n method showed a larger diversity of HCAcs and DHCAcs in potato than described before.

Based on: 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 Chemistry* **2012**, *130*, 730-738.

Introduction

Plants accumulate a large variety of secondary metabolites, which includes phenolic compounds, such as hydroxycinnamic acids (HCAs), their conjugates (HCAcs) and dihydrohydroxycinnamoyl conjugates (DHCAcs). HCAs/HCAcs/DHCAcs are related to resistance of plants to stress (*1, 2*). These compounds also have technological importance, e.g. they can participate in browning reactions, and they can affect the applicability of proteins by binding to them (*3*). Furthermore, HCAs/HCAcs/DHCAs seem to have promising health benefits in humans, including their anti-hypertensive activity and prevention of atherosclerosis, type 2 diabetes, Alzheimer's disease, and cancer (*4, 5*). In addition, there is evidence of the inhibitory effect of HCAcs against HIV (*6*) and of their antimicrobial activity (*7*).

HCAs/HCAcs/DHCAcs are the most abundant secondary metabolites found in potato (*Solanum tuberosum*), with chlorogenic acid (5-*O*-caffeoylquinic acid) as the major representative. Isomers, like *crypto*-chlorogenic acid (4-*O*-caffeoylquinic acid) and *neo*-chlorogenic acid (3-*O*-caffeoylquinic acid), as well as caffeic acid are frequently reported in potato (*8, 9*). Although identification and quantification of these HCAs/HCAcs in potato is well reported, the large number of other HCAcs and DHCAcs is less documented (*9, 10*). Because of the potential implications of HCAs/HCAcs/DHCAcs in health and in the food industry, it is important to have a suitable method to reveal and quantify the complete array of these compounds present in potato. Recently, ultra high performance liquid chromatography (UHPLC) has been shown to offer unprecedented resolution in the separation of phytochemicals. Besides UV-vis data analysis, MS^n detection provides new opportunities for rapid identification of different phytochemicals (*11, 12*). Therefore, in this study, UHPLC-DAD-ESI-MS^n was employed to identify and quantify HCAs/HCAcs/DHCAcs in aqueous methanolic potato tuber extracts, with emphasis on those less documented compounds.

Materials and Methods

CHEMICALS.

p-Coumaric acid, caffeic acid, ferulic acid, sinapic acid, and chlorogenic acid were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). UHPLC/MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was obtained by a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were from Merck (Darmstadt, Germany).

PLANT MATERIAL.

Potato tubers (Victoria variety) were purchased from a local supermarket in Wageningen, The Netherlands. Samples were washed with tap water and the surface was allowed to dry at room temperature (3 h). Whole tubers were manually sliced (0.3 mm thickness) and freeze-dried. Freeze-dried material was milled into a fine powder with a DCFH 48 vegetable grinder, with a sieve size of 0.5 mm (Wilten Woltil, de Meern, The Netherlands) and stored in sealed plastic containers at -20 ºC until analysis.

EXTRACTION OF HCAS/HCACS/DHCACS.

Freeze-dried samples (2 g) were extracted using 10 mL of water/methanol (50/50, v/v) containing 0.5% (v/v) acetic acid. The mixture was stirred at 500 rpm for 15 min at 4 °C and then centrifuged (15,000 *g*; 15 min; 4 °C). This procedure was repeated four times. As the compounds investigated have absorption maxima ranging from 310 to 330 nm, the effectiveness of repetitive extractions was tested by measuring the absorbance at 320 nm. The fourth extraction represented less than 1% of the compounds that were extracted. The four supernatants were combined, filtered through a 0.45 µm filter (Schleicher & Schuell, Dassel, Germany), and stored at -20 °C until further analysis. This sample is further referred to as crude extract. Extractions were done in triplicates.

ALKALINE HYDROLYSIS OF PHENOLIC COMPOUNDS.

The crude extract, as such, was subjected to alkaline hydrolysis. Several concentrations of NaOH were tested: 0.0, 0.010, 0.025, 0.050, 0.075, 0.10, 0.25, 0.50 and 1.0 M. Crude extract (4 mL) was stirred with 4 mL of 0.0-2.0 M NaOH overnight at 20 °C; in all cases ascorbic acid and EDTA were added as antioxidants to final concentrations of 0.052 and 0.010 M, respectively. Afterwards, the mixture was acidified to pH 1-2 by adding 37% (w/v) HCl, and the volume was adjusted to 10 mL by adding the extraction solvent. The hydrolysates were analyzed by UHPLC-ESI-MS^n as described below. To verify the stability of the HCAs under alkaline conditions the recovery of *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid was tested with authentic compounds at concentrations similar to those expected in the extract.

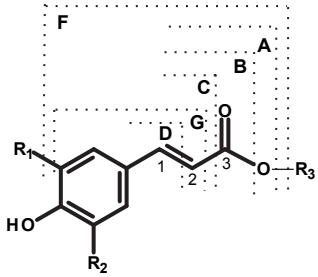
UHPLC-DAD-ESI-MS^N ANALYSIS.

Samples, with and without alkaline hydrolysis, undiluted and 10x diluted, were analyzed in a Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler and a photo-diode array detector (DAD) using a Hypersyl gold RP column (Thermo Scientific; 150 mm x 2.1 mm i.d.; particle size 1.9 µm) at 30 °C. The eluents used were water/ACN/acetic acid (99:1:0.1, v/v/v) (eluent A) and ACN/acetic acid (100:0.1, v/v) (eluent B). The elution program (elution condition I) was 0-5 min, 0% B; 5-23 min, 0 to 60% B; 23-24 min, 60 to 100% B; 24-27 min, 100% B; 27-28 min, 100 to 0%

B; 28-35 min, 0% B. The flow rate was 400 µL/min; injection volume was 5 µL. MS^n analysis was performed on a Thermo Scientific LTQ-XL using electrospray ionization (ESI) and detection 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 auto-tuned to optimize the ionization process and sensitivity using chlorogenic acid. A full-scan mass spectrum over a range of *m*/*z* values of 150-1500 was recorded. The control of the instrument and data processing were done using Xcalibur 2.07 (Thermo Scientific). Identification was done based on the molecular mass of the parent ion, MS^n and UV-vis spectra data. In a second set of runs (elution condition II) the elution program and temperature of the column were optimized to resolve feruloyl malate from sinapoyl malate, and ferulic acid from sinapic acid, which co-eluted using elution condition I. The elution condition II was 0-5 min, 0% B; 5-23 min, 0 to 50% B; 23-24 min, 50 to 100% B; 24-27 min, 100% B; 27-28 min, 100 to 0% B; 28-35 min, 0% B at 40 °C. Calibration curves were run at 320 nm for the external standards 5-*O*-caffeoylquinic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid at concentrations ranging from 0.05 to 5 µg/mL. 5-*O*-caffeoylquinic acid was adopted as an external standard for the quantification of the chlorogenic acid isomers. Concentrations of compounds containing caffeic acid, different to chlorogenic acid isomers, were calculated by using caffeic acid. The concentrations of *p*-coumaric, ferulic and sinapic acid containing compounds were calculated by using *p*-coumaric, ferulic and sinapic acid, respectively. Furthermore, assuming that the response of the HCAcs is mainly determined by the HCA moiety, molecular weight (MW) correction factors were used, $MW_{HCAc}/MW_{external\ standard}$. Dihydrocaffeic acid-containing compounds were calculated by using calibration curves with caffeic acid, including the MW correction factor. Limits of detection (LOD) and quantification (LOQ) were determined as three and ten times the standard deviation of the noise, respectively.

STRATEGY USED FOR THE IDENTIFICATION OF HCACS/DHCACS.

Figure 1 shows the fragmentation pattern in negative mode of HCAcs and DHCAcs. When compounds containing HCAs are fragmented in negative ion mode, the ions $[HCA\text{-}H]^-$, $[HCA\text{-}H_2O\text{-}H]^-$ and/or $[HCA\text{-}CO_2\text{-}H]^-$ are diagnostic. When OCH_3 groups are present, as in ferulic acid and sinapic acid, the ions $[HCA\text{-}CH_3\text{-}H]^-$ and $[HCA\text{-}CO_2\text{-}CH_3\text{-}H]^-$ are expected (*9, 11-18*). For some HCA-containing compounds, none of the five ions described above are produced, which is the case for e.g. 4-*O*- and 5-*O*-feruloyl quinic acid (*19*), and glycosylated flavonoids containing HCAs (*12, 20*). In that case, neutral losses corresponding to HCA or HCA-hexose are screened for (**Figure 1**). Besides HCAcs, DHCAcs are also present, whose negative ions are rarely reported (*16*). Compounds containing dihydrocaffeic acid are mainly identified by the neutral losses of 164 ($-C_9H_8O_3$) and 122 ($-C_7H_6O_2$), and those containing dihydroferulic acid by the neutral loss of 178 ($-C_9H_7O_3CH_3$) (*9, 14*).



HCA (Yes Δ^2)	Substitutions
p-Coumaric acid	R_1=R_2=H
Caffeic acid	R_1=OH, R_2=H
Ferulic acid	R_1=OMe, R_2=H
5-OH-ferulic acid	R_1=OMe, R_2=OH
Sinapic acid	R_1=R_2=OMe

DHCA (No Δ^2)	Substitutions
Dihydro-*p*-coumaric	R_1=R_2=H
Dihydrocaffeic acid	R_1=OH, R_2=H
Dihydroferulic acid	R_1=OMe, R_2=H
Dihydro-5-OH-ferulic	R_1=OMe, R_2=OH
Dihydrosinapic acid	R_1=R_2=OMe

HCA	§Negative ions					§Neutral losses		
	A^-	B^-	C^-	F^-	G^-	-B	-D	-B-
p-Coumaric acid	163*	145*	119*	N/A	N/A	146	-	308
Caffeic acid	179*	161*	135*	N/A	N/A	162*	-	324
Ferulic acid	193*	175*	149*	178*	134*	176*	-	338
5-OH-ferulic acid	209	191	165	194	150	192	-	354
Sinapic acid	223*	205 *	179*	208*	164*	206*	-	368
DHCA	A^-	B^-	C^-	F^-	G^-	-B	-D	-B-
Dihydro-*p*-coumaric acid	165	147	121	N/A	N/A	148	106	-
Dihydrocaffeic acid	181	163*	137	N/A	N/A	164*	122*	-
Dihydroferulic acid	195	177*	151	180	136	178*	136*	-
5-OH-dihydroferulic acid	211	193	167	196	152	194	152	-
Dihydrosinapic acid	225*	207*	181*	210	166	208	166	-

Figure 1. MS fragmentation in negative mode of the HCAcs/DHCAcs compounds found in potato. §Negative ions A: $[(D)HCA-H]^-$, B: $[(D)HCA-H_2O-H]^-$, C: $[(D)HCA-CO_2-H]^-$, F: $[(D)HCA-CH_3-H]^-$, G: $[(D)HCA-CH_3-CO_2-H]^-$ §Neutral losses -B: $-C_9H_4O_2R_1R_2$ in HCA and $-C_9H_6O_2R_1R_2$ in DHCA, -D: $-C_7H_4OR_1R_2$, -B-hex: $-C_9H_4O_2R_1R_2$-hexose in glycosylated flavonols. Values marked with * represent those belonging to HCAcs/DHCAcs in potato, found in this study. R_3 can represent an H (in HCAs as such), or a hydroxyl-, or an amino-compound. N/A: not applicable. MS data collected from literature (*9, 11, 12, 14-18, 20*).

The negative ions and neutral losses listed in **Figure 1** were screened in MS^2 and MS^3. Subsequently, the MW was calculated from the parent ion $[M-H]^-$, and the chemical identification was done. Furthermore, UV spectra were used to verify the annotations of compounds as HCAcs/DHCAcs (*10, 21*). Compounds such as quercetin-3-*O*-glucosyl-rutinoside and rutin (quercetin-3-*O*-rutinoside), which have been reported in potato (*9*), give the negative ion *m/z* 179 and neutral losses of 162 and 308 that can be misinterpreted as corresponding to HCA-containing compounds. Therefore, when the screening reveals the

presence of the mentioned negative ions and/or neutral losses, the UV absorption should be checked. If a maximum UV absorption at 343-350 nm is observed, then the compounds are annotated as flavonol glycosides (*22*). When HCAs are also present in these glycosylated flavonols, then a hypsochromic shift (310-336 nm) should be observed (*22*).

STATISTICAL ANALYSIS.

Data are reported as the mean with their standard deviation. Calculated quantities of HCAs that should be released from the crude extract were compared with the quantities of the HCAs obtained after alkaline hydrolysis of the crude extract by means of the Student's t-test ($P<0.05$).

Results and Discussion

IDENTIFICATION OF HCAS/HCACS/DHCACS IN THE CRUDE EXTRACT.

UV detection at 320 nm of the non-hydrolyzed crude extract revealed approximately 45 peaks (**Figure 2**). When screening for the negative ions and neutral losses listed in **Figure 1**, in total 39 compounds were annotated as HCA/DHCA-containing compounds (**Table 1**). Of these, 17 have been previously reported in potato. From the 22 compounds left, six have been reported in other botanical sources, two tentatively found for the first time in the plant kingdom, and 14 tentatively annotated as HCAs/DHCAs attached to unidentified compounds.

As presented in **Figure 3**, HCAs were found: unconjugated, linked to hydroxyl-containing compounds (hexose, quinic acid and malic acid), and linked to amino-containing compounds (spermine, putrescine and octopamine). DHCAs were found attached to amino compounds (spermine and spermidine). Furthermore, HCAs/DHCAs were also found attached to compounds, the exact structure of which could not be identified. All HCAs/HCAcs had maxima of absorption at 310-330 nm and some of them had shoulders at 285-305 nm, which agrees with data reported for HCAs/HCAcs (*11, 21*). All DHCAcs had maxima of absorption at 278-282 nm, with sometimes an additional signal at 310-320 nm, which is characteristic of dihydrocaffeoyl-spermine/spermidine conjugates (*10, 23*).

UNCONJUGATED HCAS.

Besides caffeic acid (**11**) and *p*-coumaric acid (**22**), also a caffeic acid methoxy compound ($[M-H]^-$ = 193, **A1**) was found in the crude extract, which did not coelute with the authentic ferulic acid standard. Probably, this compound was produced from caffeic acid and the methanol in the extractant during the analysis procedure. This compound is described later.

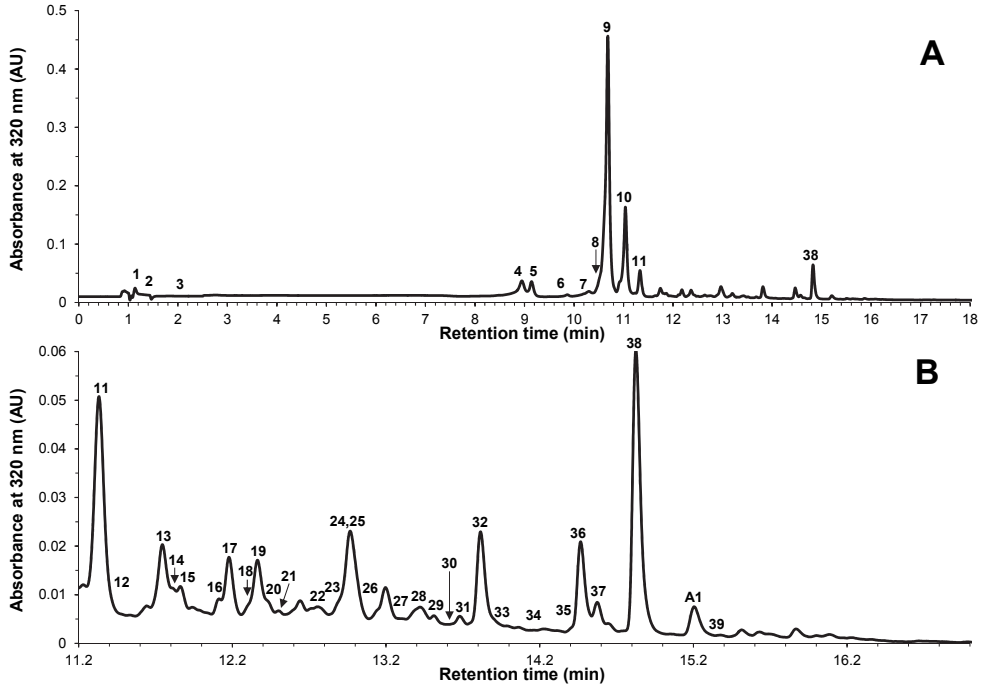


Figure 2. UHPLC-DAD profile of the non-hydrolyzed, undiluted methanolic extract of potato tuber recorded at 320 nm. (**A**) From 0.0 to 18.0 min. (**B**) Zoom from 11.2 to 17.0 min. Assignment of the peaks is given in **Table 1**.

HCAS LINKED TO HYDROXYL CONTAINING COMPOUNDS.

HCAs linked to hexose. Two caffeic acid hexoside-isomers were found (compounds **2** and **6**), of which only the one eluting close to the void volume (compound **2**) has been previously described in potato (*9*). Besides, two more HCA-hexosides were found for the first time in potato: sinapic acid hexoside (**17**) and *p*-coumaric acid hexoside (**21**). Several isomers of HCAs linked to hexose have been found in other plant sources (*11, 18*).

HCAs linked to quinic acid. Seven HCAs esterified to quinic acid were found, including one caffeoyl quinic acid isomer (compound **13**) reported for the first time in potato, which might represent the *cis* form of one of the caffeoyl quinic acid positional isomers. All seven compounds produced after fragmentation the ions 191 and 173, which corresponded to the [quinic acid-H]$^-$ and [quinic acid-H_2O-H]$^-$ ions, respectively. The annotation of the isomers 1-*O*-, 3-*O*-, 4-*O*- and 5-*O*-caffeoyl quinic acids (compounds **1**, **4**, **10** and **9**, respectively) was done according to Clifford, Knight and Kuhnert (*24*), all of them *trans* isomers. Based on the presence of the ion *m/z* 191 as base peak after fragmentation of the parent ion, compound **18** was identified as 5-*O*-feruloyl quinic acid (*19*).

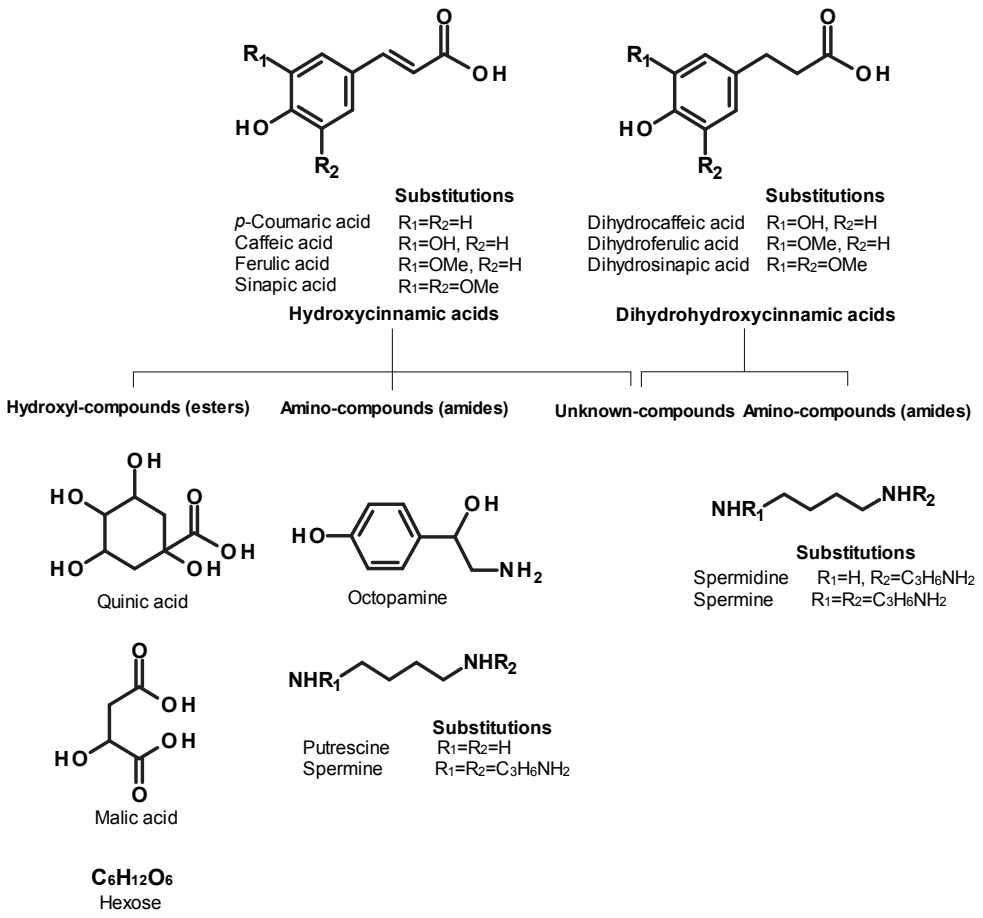


Figure 3. Chemical structures of the HCAs, DHCAs and attached moieties found in potato tubers.

HCAs linked to malic acid. HCAs esterified to malic acid have not been reported in potato yet. We found feruloyl malate (**24**) and sinapoyl malate (**25**), two compounds that are proposed to be involved in defense response of plants after fungal infection (*1*). These two compounds were resolved when the elution condition II was used with RT of 12.43 and 12.56 min, respectively. Esters of HCAs with malic acid have been previously reported in plants such as radish (*25*) and pak choi (*Brassica campestris*) (*12*). Our MS^n data matched with those reported in these studies.

Table 1. Retention time, UV, MS^n, and concentration of the HCAs/HCAcs/DHCAcs found in potato.

No	RT (min)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)[a]	UV λmax (nm)	Identification	Concentration (mg±SD/100 g DW)[b]
Unconjugated HCA						
11	11.32	179	179 (3), **135**, 161 (<1)	224, 305sh, 323	Caffeic acid[c,h] (*9*)	1.16±0.08 (2.5)
22	12.76	163	**163**, 145 (<1), 119 (14)	226, 300sh, 310	*p*-Coumaric acid[c,h] (*26*)	0.04±0.00 (0.1)
HCA-hydroxyl containing compounds						
	HCA-hexose					
21	12.50	325	**163**, 145 (8), 119 (41)	223, 285sh, 314	*p*-Coumaric acid-hexose (*11*)	0.07±0.03 (0.2)
2	1.16	401[g]	**341**, MS^3: **179**, 161 (20)	-[d]	Caffeic acid hexose isomer 1[c] (*9*)	NQ[e]
6	9.86	341	**179**, 161 (5), 135 (95)	223, 290, 310sh	Caffeic acid hexose isomer 2 (*11*)	0.14±0.01 (0.3)
17	12.18	445[g]	**385,** 223 (2), 205 (<1), 179 (6) MS^3: **223**, 205 (86), 179 (4)	225, 300sh, 330	Sinapic acid hexose (*18*)	0.59±0.01 (1.3)
	HCA-quinic acid					
1	1.02	353	**191** [M-H-162]$^-$, 179 (5), 173 (2), 161 (<1), 135 (<1)	-[d]	1-*O*-Caffeoyl quinic acid[c] (*9*)	NQ
4	8.95	353	**191** [M-H-162]$^-$, 179 (46), 173 (3), 161 (<1), 135 (8)	223, 240sh, 300sh, 324	3-*O*-Caffeoyl quinic acid[c] (*9*)	2.41±0.10 (5.3)
9	10.68	353	**191** [M-162]$^-$, 179 (5), 173 (<1), 161 (<1), 135 (<1)	223, 240sh, 305sh, 325	5-*O*-Caffeoyl quinic acid[c,h] (*9*)	25.43±0.49 (55.6)
10	11.03	353	191 [M-H-162]$^-$ (15), 179 (52), **173**, 135 (5)	223, 240sh, 305sh, 326	4-*O*-Caffeoyl quinic acid[c] (*9*)	7.31±0.38 (16.0)
13	11.74	353	**191** [M-H-162]$^-$, 179 (5), 173 (5), 135 (1)	224, 240sh, 300sh, 320	Caffeoyl quinic acid isomer	0.59±0.05 (1.3)
23	12.88	367	367 (47), 191 (30), **179**, 173 (5), 161 (11), 135 (37)	223, 305 sh, 324	Caffeoyl methyl quinate[c] (*9*)	BLOQ[f]
18	12.31	367	193 (10), **191** [M-H-176]$^-$, 173 (40), 134 (<1)	214, 295sh, 320	5-*O*-Feruloyl quinic acid[c] (*9*)	0.04±0.00 (0.1)
	HCA-malic acid					
24	12.97	309	**193**, 149 (<1), 133 [M-H-176]$^-$ (<1), 115 (<1), MS^3: 193 (<1), 178 (63), 175 (<1), **149**, 134 (77)	224, 300sh, 326	Feruloyl malate (*12*)	0.15±0.00 (0.3)

Table 1. (Continued)

No	RT (min)	[M-H]⁻ (*m/z*)	MS² (*m/z*)[a]	UV λmax (nm)	Identification	Concentration (mg±SD/100 g DW)[b]
25	12.97	339	**223**, 133 [M-H-206]⁻ (<1), MS³: 223 (64), **208**, 179 (65), 164 (61)	223, 323	Sinapoyl malate (*12*)	0.05±0.02 (0.1)
HCA-amino compounds (spermine, putrescine and octopamine)						
3	1.92	611	593 (42), 482 (97), 328 (65), **306**, 288 (6), 272 (42), 254 (14), 179 (3)	-[d]	Caffeoyl spermine conjugate[c] (*9*)	NQ
5	9.15	249	**249**, 207 (20), 161 (<1) 135 (70)	223, 293sh, 318	Caffeoyl putrescine[c,i] (*9*)	1.16±0.01 (2.5)
32	13.82	328	**310,** 135 (<1), MS³: **310**, 295 (19), 175 (<1), 149 (<1), 135 (10)	224, 293sh, 318	Feruloyl octopamine[c,i] (*27*)	0.70±0.01 (1.5)
DHCA-amino compounds (spermine and spermidine)						
8	10.52	529	**529**, 407 [M-H-122]⁻ (24), 365 [M-H-164]⁻ (51), 285 [M-H-122-122]⁻ (<1), 163 (<1)	223, 282, 318	N^1,N^{14}-bis (dihydrocaffeoyl) spermine[c,i] (*9, 10*)	1.10±0.20 (2.4)
14	11.80	472	**472**, 350 [M-H-122]⁻ (37), 308 [M-H-164]⁻ (63), 228 [M-H-122-122]⁻ (1), 186 [M-H-164-122]⁻ (<1), 163 (2)	223, 282, 320sh	N^1,N^{10}-bis (dihydrocaffeoyl) spermidine[c,i] (*9, 10*)	0.10±0.01 (0.2)
26	13.05	693	693 (28), 571 [M-H-122]⁻ (17), **529** [M-H-164]⁻, 449 [M-H-122-122]⁻ (1), 407 [M-H-164-122]⁻ (4), 365 [M-H-164-164]⁻ (2), MS³: **529**, 407 (35), 365 (63), 285 [M-H-164-122-122]⁻ (<1), 243 [M-H-164-164-122]⁻ (<1), 163 (1)	224, 282, 319sh	N^1,N^5,N^{14}-tris (dihydrocaffeoyl) spermine[c] (*9, 10*)	BLOQ
31	13.68	798	**636** [M-H-162]⁻, 472 [M-H-162-164]⁻ (<1) MS³: 514 [M-H-162-122]⁻ (17), **472** [M-H-162-164]⁻, 350 [M-H-162-164-122]⁻ (7), 308 [M-H-162-164-164]⁻ (4)	225, 282, 320sh	N^1,N^5,N^{10}-tris(dihydrocaffeoyl) spermidine hexoside[c] (*10*)	0.01±0.00 (<0.1)

Table 1. (continued)

No	RT (min)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)[a]	UV λmax (nm)	Identification	Concentration (mg±SD/100 g DW)[b]
35	14.40	812	**650** [M-H-162]$^-$, 486 [M-H-162-164]$^-$ (<1) MS^3: 650 [M-H-162]$^-$ (26), 528 [M-H-162-122]$^-$ (17), 486 [M-H-162-164]$^-$ (74), **472** [M-H-162-178]$^-$, 350 [M-H-162-164-136]$^-$ (4), 308 [M-H-162-178-164]$^-$ (6)	225, 278, 314sh	bis (dihydrocaffeoyl)-(dihydroferuloyl) spermidine hexoside	BLOQ
36	14.48	636	636 (22), 514 [M-H-122]$^-$ (16), **472** [M-H-164]$^-$, 350 [M-H-164-122]$^-$ (4), 308 [M-H-164-164]$^-$ (3), MS^3: **472**, 350 (26), 308 (44), 228 [M-H-164-122-122]$^-$ (<1), 186 [M-H-164-164-122]$^-$ (<1), 163 (1)	215, 278, 314	N^1,N^5,N^{10}-tris (dihydrocaffeoyl) spermidine[c,i] (*9, 10*)	0.38±0.02 (0.8)
39	15.27	650	650 (33), 528 [M-H-122]$^-$ (17), 486 [M-H-164]$^-$ (86), **472** [M-H-178]$^-$, 364 [M-H-164-122]$^-$ (<1), 350 [M-H-164-136]$^-$ (5), 308 [M-H-178-164]$^-$ (6)	225, 278, 309sh	bis (dihydrocaffeoyl)-(dihydroferuloyl) spermidine	BLOQ
HCA/DHCA-unknown compounds						
16	12.11	370	**190** [M-H-162]$^-$, 179 (4), 147 (4)	224, 300sh, 325	Caffeic acid amide	0.02±0.01 (0.1)
20	12.43	653	**473**, 429 (37), 267 (13), 249 (11), MS^3: **429**, 267 (26), 249 (15), 179 (5), 135 (2)	224, 278, 325	Caffeic acid conjugate	0.02±0.02 (<0.1)
19	12.36	443	425 (18), 411 (15), **267** [M-H-176]$^-$, 249 (14), 193 (8), 175 (<1), 134 (<1)	224, 300sh, 322	Ferulic acid conjugate	0.62±0.05 (1.4)
34	14.16	435	**417**, 273 (8), 255 (17), 237 (10), 193 (14)	224, 300sh, 325	Ferulic acid conjugate	BLOQ
12	11.48	475	**343**, 223 (4), 181 (60)	223, 295sh, 316	Sinapic acid conjugate	BLOQ

Table 1. (continued)

No	RT (min)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)[a]	UV λmax (nm)	Identification	Concentration (mg±SD/100 g DW)[b]
15	11.86	351	333 (26), 267 (22), **249**, 223 (<1), 205 (3)	223, 300sh, 322	Sinapic acid conjugate	0.32±0.02 (0.7)
27	13.26	403	359 (11), **223**, 205 (<1), 179 (32)	224, 270, 324	Sinapic acid conjugate	BLOQ
28	13.42	445	**283**, 249 (2), 223 (<1), 205 (<1), 179 (<1)	224, 271, 300sh, 324	Sinapic acid conjugate	0.30±0.02 (0.7)
30	13.58	403	359 (6), **223**, 179 (31)	224, 280, 315	Sinapic acid conjugate	BLOQ
37	14.58	429	385 (53), **249**, 223 (<1), 205(47), 179 (35)	225, 311	Sinapic acid conjugate[i]	0.21±0.07 (0.5)
38	14.83	429	385 (25), **249**, 223 (<1), 205(44), 179 (21)	224, 311	Sinapic acid conjugate[i]	2.80±0.06 (6.1)
7	10.29	417	**417**, 285 (28), 267(6), 241(14), 223 (2), 193 (2), 179 (8), 163 (11)	224,283, 311	HCA conjugate	BLOQ
29	13.52	449	**269**, 251 (4), 225 (2), 209(3), 207 (3)	225, 278, 310	Dihydrosinapic acid conjugate	BLOQ
33	13.93	405	**225**, 207 (4), 181 (9)	223, 278, 317	Dihydrosinapic acid conjugate	BLOQ

[a]Bold numbers represent a relative abundance of 100%. In brackets the relative abundance. In some cases MS^3 of the 100% ion from MS^2 was included to provide extra information. Values underlined were diagnostic for the classification of compounds as HCAs/HCAcs/DHCAcs (see also **Fig. 1**). In squared brackets are the ions which show neutral losses diagnostic for HCAcs/DHCAcs.
[b]Average of three independent extractions. Between brackets the percentage (w/w).
[c]Reported in potato.
[d]Coelution with other unidentified compounds.
[e]Not quantified.
[f]Below limit of quantification. Limits of quantification were 0.02 ng/injection for chlorogenic acid, *p*-coumaric acid, caffeic acid, and ferulic acid, and 0.03 ng/injection for sinapic acid. Limits of detection were 0.01 ng/injection for chlorogenic acid, *p*-coumaric acid and caffeic acid, and 0.02 ng/injection for ferulic and sinapic acid. In all cases R^2 values were 0.9999.
[g]Parent ions for compound **2** and **17** are supposed to be an adduct of acetic acid [M+60-H]$^-$.
[h]Retention time, UV and MS^n data equal to those of commercial compounds.
[i]Compounds that were not hydrolysable under the alkaline conditions.

HCAS LINKED TO AMINO COMPOUNDS.

We identified compound **32** as feruloyl octopamine. This compound had absorption maxima at 224, 293sh and 318 nm. After MS^2 fragmentation, a very abundant signal at *m/z* 310 [M-H_2O-H]$^-$ was observed. Further fragmentation (MS^3) yielded other ions, including *m/z* 175 and 149, which could be related to the ions [ferulic acid-H_2O-H]$^-$ and [ferulic acid-CO_2-H]$^-$, respectively (**Table 1**). Fragments found in MS^n were similar to those previously reported in potato for a feruloyl amide compound, with a parent ion of *m/z* 310 (*9*). Previously, feruloyl octopamine was found in potato cell cultures. Its identification was based on the abundant negative ions 328 and 310 in MS, but without further description of the fragmentation (*27*). The absence of the negative fragment [HCA-H]$^-$, the abundant peak

$[M-H_2O-H]^-$, and the λmax data were in accordance to results for *p*-coumaroyl octopamine and *p*-coumaroyl noradrenaline found in tomato (*28*).

DHCAS LINKED TO AMINO COMPOUNDS.

We identified seven DHCAcs of which five were linked to spermidine and two to spermine. Different to the *N*-substitution numbering used by Parr *et al*. (*10*) and by Shakya and Navarre (*9*), we have adopted the new numbering rules according to Bienz *et al*. for polyamines (*29*). Compounds **39** and **35** were tentatively identified as bis-(dihydrocaffeoyl)-(dihydroferuloyl) spermidine and its hexoside, respectively. To our knowledge these are reported for the first time in plants. The earlier elution of **36** than **39**, as well as the earlier elution of **31** than **35** agreed with the more lipophilic character of dihydroferulic acid in comparison to dihydrocaffeic acid. Compounds **8**, **14** and **26** were annotated as N^1,N^{14}-bis (dihydrocaffeoyl) spermine, N^1,N^{10}-bis (dihydrocaffeoyl) spermidine and N^1,N^5,N^{14}-tris (dihydrocaffeoyl) spermine, respectively, according to a previous study in which the *N*-substitutions were defined (*10*).

COMPOUNDS TENTATIVELY ANNOTATED AS HCACS/DHCACS.

We tentatively identified two compounds as caffeoyl conjugates (compounds **16** and **20,** the first one as a caffeoyl amide), two as feruloyl conjugates (compounds **19** and **34**) and seven more as sinapoyl conjugates (compounds **12**, **15**, **27**, **28**, **30**, **37** and **38**). One more (compound **7**) was classified as an HCAc, which had UV maxima characteristic for HCAcs, but negative ions corresponding to HCAs. Compounds **29** and **33** were tentatively identified as dihydrosinapoyl conjugates.

Although compounds such as feruloyl putrescine (*10*), 4,5-di-*O*-caffeoyl quinic acid, and N^1,N^5,N^{10},N^{14}-tetra(dihydrocaffeoyl) spermine (*9*) have been previously reported in potato, they were not found in the current work. This might be related to intervarietal differences, which are known to exist in potato (*9, 10*).

QUANTIFICATION OF HCACS/DHCACS.

The total amount of HCAs/HCAcs found in potato was 44.14±0.95 mg/100 g DW, whereas the total amount of DHCAcs was 1.58±0.14 mg/100 g DW (**Table 1**). From all HCAs/HCAcs/DHCAs, chlorogenic acid (**9**) was the most abundant one (25.43±0.49 mg/100 g DW), representing 55.6% (w/w) of the total HCAs/HCAcs/DHCAs, followed by *crypto*-chlorogenic acid (**10**, 16.0%, w/w), a sinapic acid conjugate (**38**, 6.1%, w/w), *neo*-chlorogenic acid (**4**, 5.3%, w/w), and caffeoyl putrescine (**5**, 2.5%, w/w), caffeic acid (**11**, 2.5%, w/w) and N^1,N^{14}-bis(dihydrocaffeoyl) spermine (**8**, 2.4%, w/w). Compounds containing caffeic acid were the largest group in both diversity and quantity of the HCAs/HCAcs/DHCAcs (83.6% of the total amount, w/w), followed by sinapic acid conjugates (9.3%, w/w) and ferulic acid conjugates (3.3%, w/w). Dihydrocaffeic acid-

containing compounds represented only 3.5% (w/w) of the total amount of HCAs/HCAcs/DHCAcs, and compounds containing *p*-coumaric acid even less (0.2%, w/w). Dihydroferulic acid- and dihydrosinapic acid-containing compounds were found in trace amounts. The contents of chlorogenic, *neo*-chlorogenic, *crypto*-chlorogenic and caffeic acids were within the range reported for several native Andean potato varieties (*30*), and lower than those in other potato varieties (*9, 26*). The quantity of caffeoyl putrescine was lower than that reported previously (*9*). The content of N^1,N^{14}-bis(dihydrocaffeoyl) spermine matched with that in one study (*10*), while being lower than the values found in another study (*9*).

VALIDATION OF THE QUANTIFICATION OF HCAS/HCACS/DHCACS VIA ALKALINE HYDROLYSIS.

To verify the quantification of the compounds assigned as HCAs/HCAcs/DHCAcs that was done on the crude extract, the constituent phenolic acids were quantified after alkaline hydrolysis. Hydrolysis with NaOH has been used by others with the purpose of confirming the identity of HCA conjugates found in crude extracts (*12*). We used this procedure more quantitatively, and tested whether the concentration of the large array of HCAs/HCAcs/DHCAcs found in the crude extract could be accurately determined with the few commercially available standards. **Figure 4** shows the chromatograms recorded at 320 nm when different concentrations of NaOH were tested. At concentrations of 0.025-0.075 M NaOH isomerization of chlorogenic acid to *neo*-chlorogenic acid and *crypto*-chlorogenic acid was observed. When the concentration of alkali was increased from 0.050 to 0.10 M, a gradual hydrolysis of all isomers of chlorogenic acid was observed, up to 95% at 0.10 M NaOH. With these concentrations of alkali the hydrolysis of caffeic acid-containing compounds did not yield caffeic acid as expected, but **A1**. Hydrolysis with 0.25 M NaOH yielded a large peak of caffeic acid, and formation of **A1** was suppressed. **A1** might represent one of the following structures: ferulic acid, isoferulic acid, or the methyl ester of caffeic acid. Its parent ion of *m/z* 193 gave the fragment ions *m/z* 178 $[M\text{-}CH_3\text{-}H]^-$, 149 $[M\text{-}CO_2\text{-}H]^-$ and 134 $[M\text{-}CO_2\text{-}CH_3\text{-}H]^-$, which are characteristic for ferulic acid and isoferulic acid (*31*), but not for caffeic acid methyl ester. Nevertheless, **A1** did not correspond to ferulic acid, as it eluted differently from authentic ferulic acid. It is also unlikely that **A1** corresponds to isoferulic acid, which is known to be stable at 2 M NaOH (*32*). The disappearance of **A1** at higher alkali concentrations might argue for the compound being the methyl ester of caffeic acid (*33*).

The individual HCAs were quantified after hydrolysis with 0.25 M NaOH. At 0.25 M NaOH the recoveries, in percentage, of *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid were 98.5±3.8, 102.1±2.5, 97.2±1.9 and 100.3±0.9, respectively. Ferulic acid and sinapic acid content was determined using elution condition II with retention times of 13.87 and 14.05 min, respectively. Alkaline hydrolysis of the crude extract yielded caffeic

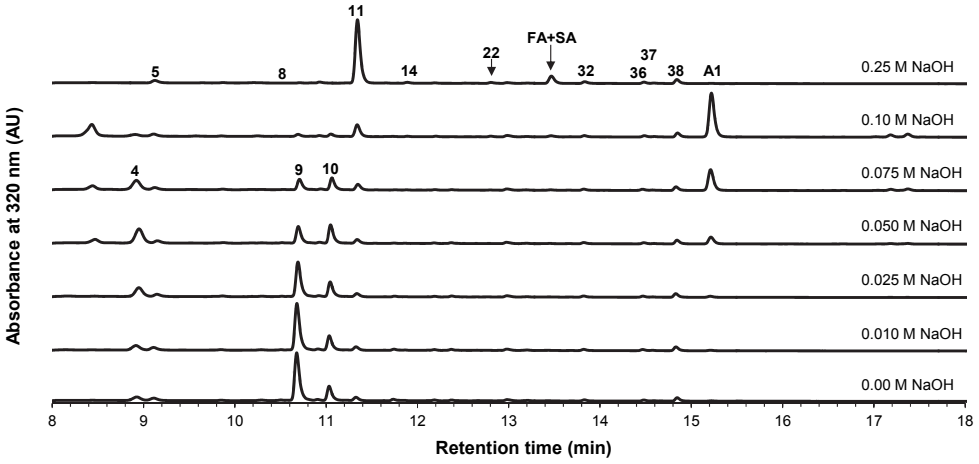


Figure 4. UHPLC-DAD profiles of the NaOH-hydrolyzed methanolic extract recorded at 320 nm. **4**, *neo*-chlorogenic acid; **5**, caffeoyl putrescine; **8**, N^1,N^{14}-bis (dihydrocaffeoyl) spermine; **9**, chlorogenic acid; **10**, *crypto*-chlorogenic acid; **11**, caffeic acid; **14**, N^1,N^{10}-bis (dihydrocaffeoyl) spermidine; **22**, *p*-coumaric acid; **32**, feruloyl octopamine; **36**, N^1,N^5,N^{10}-tris (dihydrocaffeoyl) spermidine; **37** and **38**, sinapic acid conjugates; **A1**, caffeic acid methyl ether; **FA**, ferulic acid; **SA**, sinapic acid.

acid as the most abundant HCA (21.82±0.89 mg/100 g DW), followed by sinapic acid (0.92±0.04 mg/100 g DW) and ferulic acid (0.60±0.06 mg/100 g DW), *p*-coumaric acid was less abundant (0.09±0.01 mg/100 g DW) (**Table 2**). Compounds N^1,N^{14}-bis (dihydrocaffeoyl) spermine (**8**), N^1,N^{10}-bis (dihydrocaffeoyl) spermidine (**14**) and N^1,N^5,N^{10}-tris (dihydrocaffeoyl) spermidine (**36**) were the only three DHCAcs that were present above the quantification level in the crude extract. These compounds were not hydrolysable under the alkaline conditions tested, which explains that no dihydrocaffeic acid was found in the hydrolyzates. Although dihydroferuloyl and dihydrosinapoyl conjugates were found in the crude extract, the respective DHCAs were not observed after alkaline hydrolysis, which might be related to the low concentration of the former compounds. Furthermore, the compounds caffeoyl putrescine (**5**), feruloyl octopamine (**32**), and two sinapic acid conjugates (**37** and **38**) were non-hydrolyzable under the conditions tested; their quantity represented approximately 11% (w/w) of the total amount of the HCAcs.

The calculated concentration of each HCA (from the concentration of HCA/ hydrolysable HCAcs reported in **Table 1**) was compared with the experimental concentration obtained after alkaline hydrolysis in **Table 2**. The calculated yield (HCA-found x 100/HCA-expected) was higher than 100% in all cases, with 119% for *p*-coumaric acid, 111% for caffeic acid, 154% for ferulic acid and 126% for sinapic acid. Considering that the quantification of individual HCAcs/DHCAcs in the crude extract was done by the use of a limited number of HCA standards, this is a good match.

Table 2. Hydroxycinnamic acids after alkaline hydrolysis of a methanolic potato extract.

HCAs[a]	Yield[b]	
	Expected (mg/100 g DW)	**Obtained (mg/100 g DW)**
p-Coumaric acid	0.08±0.01A	0.09±0.01A
Caffeic acid	19.60±0.46A	21.82±0.89A
Ferulic acid	0.39±0.01B	0.60±0.06A
Sinapic acid	0.73±0.01B	0.92±0.04A

[a]In all cases RT and spectroscopic data matched with those of the authentic compounds.
[b]Expected yield was obtained from the concentrations reported in **Table 1**. Different capital letters in the same row indicates significant difference according to the Student's t-test (P<0.05).

Conclusions

This paper provides a method that allows the simultaneous identification and quantification of 39 HCAs/HCAcs/DHCAcs. The large array of compounds found suggests that until now molecular diversity of polyphenols in potato has been underestimated. The use of a limited number of authentic compounds as external standards enabled accurate quantification of the large diversity of HCAcs/DHCAcs as judged from the quantification of the crude extract with and without alkaline hydrolysis.

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Chapter 3

Diversity of (dihydro) hydroxycinnamic acid conjugates in Colombian potato tubers

ABSTRACT: In potato tuber, caffeic acid (the predominant hydroxycinnamic acid (HCA)), its conjugates (HCAcs; i.e. chlorogenic acid (ChA), *crypto*-ChA, and *neo*-ChA), and anthocyanin-linked HCAs have been extensively described in the literature. In contrast, only little information is available on the occurrence of other HCAcs and didydrohydroxycinnamic acid conjugates (DHCAcs). Fifteen Colombian potato cultivars were screened for these less commonly described conjugates by reversed-phase ultrahigh performance liquid chromatography coupled to a diode array detector and a heated electrospray ionization mass spectrometer. A total of 62 HCAs/HCAcs/DHCAcs were found in extracts from peel and flesh. Among them, only twelve compounds were common to all cultivars in both peel and flesh. The less commonly described compounds accounted for 7.1-20.1% w/w of the total amount of HCAs/HCAcs/DHCAcs in whole tubers, highlighting their contribution to the total phenolic profile of potato tubers. Among all cultivars, the abundance (mg/100 g DW whole tuber) of *neo*-ChA (0.8 - 7.4) ranged in similar quantities as the less commonly reported feruloyl octopamine (1.2 - 5.2), 5-*O*-feruloyl quinic acid (0.1 - 7.5), *cis*-ChA (1.1 - 2.2), caffeoyl putrescine (0.6 - 2.5), sinapoyl hexose (0.1 - 1.8), N^1,N^{14}-bis-(dihydrocaffeoyl) spermine (0.2 - 1.7), N^1,N^{10}-bis-(dihydrocaffeoyl) spermidine (1.1 - 2.6), and N^1,N^5,N^{14}-tris-(dihydrocaffeoyl) spermine (trace - 11.1).

Based on: Narváez-Cuenca, C.-E.; Vincken, J.-P.; Zheng, Ch.; Gruppen, H. Diversity of (dihydro) hydroxycinnamic acid conjugates in Colombian potato tubers. *Submitted*.

Introduction

Potato is one of the most important crops in the world. It is considered a non-expensive source of high quality proteins, minerals, and antioxidants such as vitamin C and phenolic compounds (*1*). Phenolic compounds in potato tubers are mainly represented by hydroxycinnamic acids (HCAs), their conjugates (HCAcs), and dihydrohydroxycinnamic acid conjugates (DHCAcs). Among these, *trans*-5-*O*-caffeoyl quinic acid (chlorogenic acid, ChA) is the most abundant, with concentrations commonly ranging between 17 to 322 mg/100 g dry weight (DW) whole tuber, depending on the cultivar (*2, 3*). Besides ChA, its two isomers *trans*-4-*O*-caffeoyl quinic acid (*crypto*-ChA) and *trans*-3-*O*-caffeoyl quinic acid (*neo*-ChA), as well as *trans*-caffeic acid (CaA), are also commonly reported in potato tubers (*2, 3*) (**Figure 1**). Furthermore, anthocyanin-linked HCAs are frequently reported in red skin or flesh of potato tubers. They comprise glycosides of pelargonidin, cyanidin, peonidin, petunidin, and malvidin, containing *p*-coumaric acid, CaA or ferulic acid (*2, 4, 5*). Health-promoting effects have been attributed to ChA and CaA, such as inhibition of carcinogenesis (*6*), reduction of obesity (*7*), and hypotensive activity (*8*).

Other HCAcs/DHCAcs are less well documented than ChA, *crypto*-ChA, *neo*-ChA, CaA, and anthocyanin-linked HCAs (**Figure 1**). These compounds include HCAs/DHCAs linked to (poly) amines (*9, 10*), some of which are of particular interest because of their health-promoting activity. For example, kukoamine A (N^1,N^{14}-bis-(dihydrocaffeoyl) spermine, numbering of the *N*-substitutions according to (*11*)), has been reported to have hypotensive (*12*) and antitrypanosomal activity (*13*). Other nitrogen-containing HCAcs in potato tubers are known to be involved in plant resistance to stress factors; e.g. caffeoyl putrescine is a defense molecule against insects (*14*) and inhibits formation of lesions caused by ozone (*15*). Recently, 35 compounds, different to the most commonly reported, were visualized within a single cultivar, which represented 21% (w/w) of the total amount of HCAs/HCAcs/DHCAcs (*9*). It is, nevertheless, still unknown whether that diversity was characteristic of that specific cultivar or whether it is a common feature.

Colombia has 43 registered commercial potato cultivars, the most important of which rank as follows according to total cultivated area: Pastusa suprema > Diacol capiro > Parda pastusa > Criolla group (five cultivars) > Tuquerreña > Ica única ~ Ica puracé (**Table 1**) (*16*). Pastusa suprema and Parda pastusa are mainly used in domestic cooking, while Diacol capiro also known as “R-12 negra”, is used in the food industry (potato chips and French fries) as well (*16*). Different to the other cultivars, with red/purple peel and white/yellow flesh, the cultivars belonging to the Criolla group have an intense yellow color in both peel and flesh (*17*). The aim of the current work was to provide a

compositional blue print for the tubers of 15 Colombian potato cultivars (including the most cultivated ones), focused on the HCAs/HCAcs/DHCAcs in peel and flesh.

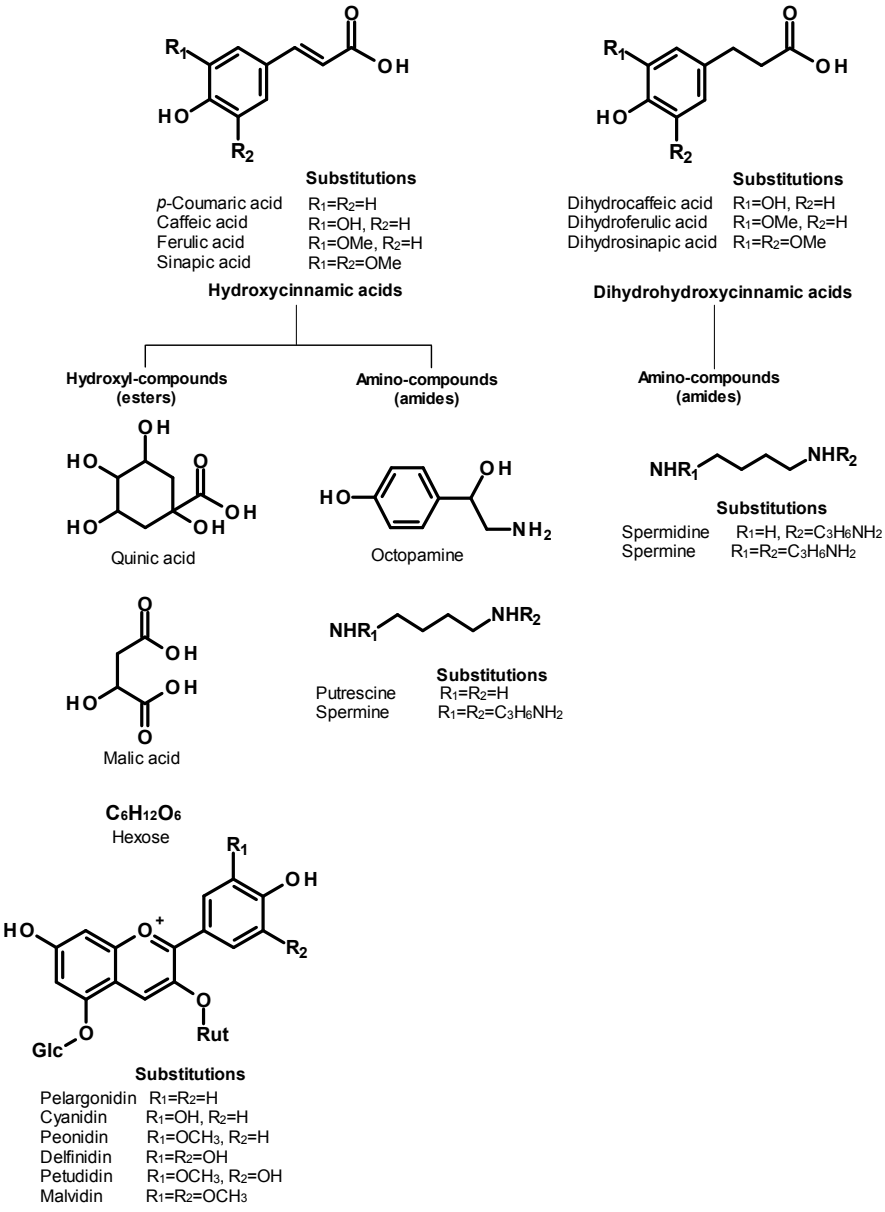


Figure 1. Chemical structures of hydroxycinnamic acids/(dihydro) hydroxycinnamic acid conjugates in potato tubers. Adapted from Narváez-Cuenca *et al*. (*9*). Glc: Glucose. Rut: Rutinose.

Materials and Methods

CHEMICALS.

p-Coumaric acid, caffeic acid (CaA), ferulic acid, sinapic acid and 5-*O*-caffeoyl quinic acid (chlorogenic acid, ChA), all *trans* isomers, were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 3-*O*-Caffeoyl quinic acid (*neo*-ChA) and 4-*O*-caffeoyl quinic acid (*crypto*-ChA), both *trans*-isomers, were purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). Malvidin cloride was purchased from Extrasynthese (Genay Cedex, France). Ultra high performance liquid chromatography (UHPLC)/MS grade 0.1% (v/v) acetic acid in acetonitrile (ACN) and UHPLC/MS grade 0.1% (v/v) acetic acid in water were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was obtained by a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were from Merck (Darmstadt, Germany).

PLANT MATERIAL.

Colombian potato tubers (15 cultivars) were produced in Centro Agropecuario Marengo, an experimental farm located at 2543 m above sea level, 14 km from Bogotá (Colombia). Samples were harvested in 2009. Potato tubers were washed with tap water and the surface was allowed to dry at room temperature (~3 h). Tubers were manually split into peel (0.1 - 0.2 mm outside layer) and flesh (peeled tubers) by using a stainless steel knife. Peel/flesh ratio (percentage, w/w) was calculated (**Table 1**). Flesh samples were manually sliced (0.3-0.5 mm thickness). Peel and flesh samples were freeze-dried. Afterwards, samples were shipped by commercial air freight to The Netherlands. There, freeze-dried material was milled into a fine powder with a DCFH 48 vegetable grinder, with a sieve size of 0.5 mm (Wilten Woltil, de Meern, The Netherlands) and stored in sealed plastic containers at -20 ºC until analysis. Relevant characteristics of the cultivars, i.e. proportion peel/flesh (%, w/w), color of peel and flesh, parents, pest resistance against *Phytopthora infestans*, and cultivated area in Colombia (%), are given in **Table 1**.

EXTRACTION OF PHENOLIC COMPOUNDS.

Freeze-dried, milled samples (50 mg) were extracted with 1 mL of methanol/water (50/50, v/v) containing 0.5% (v/v) acetic acid. The potato materials were extracted with continuous homogenization (1,000 rpm) during 15 min at 4 °C in a Thermomixer comfort shaker (Eppendorf AG, Hamburg, Germany). Next, mixtures were centrifuged (22,000 *g*; 15 min; 4 °C). Supernatant was collected and pellet was similarly re-extracted four times. The fifth extraction represented less than 1 % of the compounds that were extracted as measured at 325 nm. The five supernatants were combined, filtered through a 0.45 μm filter (Schleicher

Table 1. General characteristics of the Colombian cultivars analyzed[a].

Cultivar	Abbreviation	Peel/Flesh ratio[b]	Peel/Flesh color[c]	Parents	*P. infestans*[d]	Cultivated area (%)
Most cultivated						
Pastusa suprema	P. sup	18.0	sR/Y	[*S. stoloniferum* x CCC 81 yema de huevo (*S. phureja*)] × Parda pastusa	H	34
Diacol capiro	D. cap	14.8	P/C	CCC 751 (spp. *tuberosum*) × Tuquerreña (CCC 61 spp. *andigena*)	S	27
Parda pastusa	P. pas	23.6	dP/W	Quincha (spp. *andigena*) × Tocana colorada (spp. *andigena*)	M	20
Tuquerreña	Tuq	14.4	R/Y	Clone CCC 61	S	4
Ica única	I. uni	16.4	sP/Y	E-59-42 (*Neotuberosum*) × Bulk S. *andigena*	M	2
Criolla group						6
Criolla Colombia	C. Col	9.8	Y/Y	*S. phureja* (Yema de huevo Clone 1)	S	
Criolla latina	C. lat	11.6	Y/Y	Criolla Colombia × Amarilla de Perú (*S. goniocalyx*)	M	
Criolla paisa	C. pai	10.0	Y/Y	Amarilla de Perú (*S. goniocalyx*) × Criolla Colombia	M	
Criolla galeras	C. gal	10.8	Y/Y	Criolla Colombia × Amarilla de Perú (*S. goniocalyx*)	M	
Criolla guaneña	C. gua	12.4	Y/Y	Amarilla de Perú (*S. goniocalyx*) × Criolla Colombia	M	
Other cultivars						5
Punto azúl	P. azu	16.5	sP/W	(spp. *tuberosum* x spp. *andigena*) × Diacol Monserrate	H	
Esmeralda	Esm	18.0	R/Y	[Atzimba x CCC 81 yema de huevo (*S. phureja*)] × [D. Monserrate x I-1058]	M	
Roja nariño	R. nar	19.6	P/Y	87-502-41 [(spp. *tuberosum* × spp. *andigena*) × Parda pastusa] × [384329.1 × Waycha]	M	
Betina	Bet	15.9	sP/Y	UNC-Col 177 (*S. phureja*) × WI Bulk (*S. tuberosum*)	M	
Rubí	Rub	20.6	P/Y	83-209-2 [(spp. *tuberosum* × spp. *andigena*) × (spp. *tuberosum* × spp. *andigena*) x I-1058] × Parda pastusa	M	

[a]From Ñustez (*16*). ICA Puracé is within the most cultivated varieties (2% cultivated area), but was not analyzed in the current research.
[b]Percentage, w/w.
[c]dP, Dark purple; P, Purple; sP, slightly purple; R, Red; sR, Slightly red; Y, Yellow; C, Cream; W, White.
[d]Resistance against *Phytophtora infestans*. S, Susceptible; M, medium resistance; H, high resistance.

& Schuell, Dassel, Germany), flushed with nitrogen gas, and stored at -20 °C until further analysis. Extractions were done in triplicates. Peel samples were analysed undiluted, and diluted in a range from 1:2 to 1:20. Flesh samples were analysed undiluted, and concentrated in a range from 3:1 to 10:1 by using a Savant ISS-110 SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature under reduced pressure.

RP-UHPLC-DAD.

Samples were analysed in a Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler and a photo-diode array detector (DAD) using a Hypersyl gold RP column (Thermo Scientific; 150 mm x 2.1 mm i.d.; particle size 1.9 µm) at 30 °C. The eluents used were water/ACN/acetic acid (99:1:0.1, v/v/v) (eluent A) and ACN/acetic acid (100:0.1, v/v) (eluent B). The elution program (elution condition I) was 0-5 min, 0% B; 5-23 min, 0 to 60% B; 23-24 min, 60 to 100% B; 24-27 min, 100% B; 27-28 min, 100 to 0% B; 28-35 min, 0% B. The flow rate was 400 µL/min; injection volume was 5 µL. When using elution condition I there was co-elution of compounds, such as feruloyl malate and sinapoyl malate and of N^1,N^5,N^{14}-tris-(dihydrocaffeoyl) spermine and rutin. The mentioned compounds eluted separately when the elution program and temperature of the column were modified (elution condition II). The elution condition II was 0-5 min, 0% B; 5-23 min, 0 to 50% B; 23-24 min, 50 to 100% B; 24-27 min, 100% B; 27-28 min, 100 to 0% B; 28-35 min, 0% B at 40 °C. Anthocyanin-linked HCAs were better resolved with elution condition II. Therefore, this condition was used when performing their identification and quantification.

HEATED ELECTROSPRAY IONIZATION (HESI) MASS SPECTROMETRY (MS^N).

HESI-MS^n was aimed at identification of the less commonly described HCAcs/DHCAcs for which no commercial standards were available. The flow from the RP-UHPLC-DAD was directed to a HESI probe, which was coupled to a Thermo Scientific LTQ-XL. The instrument was auto-tuned for optimum ionization process and sensitivity with ChA. Detection was performed in negative mode, with a heater temperature of 230 °C, source voltage of 3.5 kV, ion transfer tube at 250 °C. Nitrogen was used as sheath and auxiliary gas, at flows of 25 arbitrary units and 10 arbitrary units, respectively. A full-scan mass spectrum over a range of *m*/*z* values of 150-1,500 was recorded. MS^n were performed with collision energy of 35%. Anthocyanin-linked HCAs were detected in the positive mode. The instrument was auto-tuned with malvidin cloride. Different from the settings described above, sheath and auxiliary gas flow was set at 40 and 30 arbitrary units and the temperature of the ion transfer tube was 350 °C. The control of the instrument and data processing were done using Xcalibur 2.1 (Thermo Scientific).

IDENTIFICATION AND QUANTIFICATION OF HCAS/HCACS/DHCACS.

ChA, *neo*-ChA, *crypto*-ChA, and CaA, all *trans* isomers, were identified by comparison of retention times and UV maxima against authentic standards. Identification of anthocyanin-linked HCAs was achieved by comparing parent ion and daughter ion data, in the positive ion mode, with literature data (*2, 5*). Identification of other HCAcs/DHCAcs was based on MS^n and UV maxima data as described previously (*9*). Briefly, the strategy for the identification involved three steps: (i) screening for characteristic MS^n daughter ions and neutral losses of HCAcs/DHCAcs, (ii) calculation of molecular weight according to the parent ion, and (iii) verification of assignment of a compound by UV maxima.

Quantification was based on the external standard method with seven data points for each standard. Calibration curves of HCAs/non-anthocyanin HCAcs/DHCAcs were made at 325 nm with ChA (0.05 to 20 µg/mL, $R^2 = 0.999$); *p*-coumaric acid (0.05 to 2 µg/mL, $R^2 = 0.998$); CaA (0.05 to 5 µg/mL, $R^2 = 1.000$); ferulic acid (0.05 to 5 µg/mL, $R^2 = 1.000$); and sinapic acid (0.05 to 2 µg/mL, $R^2 = 0.994$). Quantification of ChA, *neo*-ChA, and *crypto*-ChA was based on the calibration lines of ChA. Quantification of CaA was based on a calibration line with the authentic standard. Other CaA containing compounds, different to ChA, *neo*-ChA, and *crypto*-ChA, were quantified using CaA calibration. *p*-Coumaric, ferulic and sinapic acid containing compounds were quantified using *p*-coumaric, ferulic and sinapic acid calibration lines, respectively. Furthermore, assuming that the responses of the HCAcs are mainly determined by the HCA moiety, only molecular weight (MW) correction factors were used, $MW_{HCAc}/MW_{external\ standard}$. Concentrations of DHCAcs were calculated by using calibration curves of CaA, including the MW correction factor. Quantification of anthocyanin-linked HCAs found in red/purple peel potato samples was based on a calibration line with malvidin at 515 nm (0.2 to 3 µg/mL, $R^2 = 0.994$), with only correction factors accounting for the difference in molecular weight $MW_{anthocyanin}/MW_{malvidin}$. Although the molar extinction coefficients of anthocyanidins are affected (generally not more than 3-fold) by the type of anthocyanidin, glycosylation and acylation, e.g. with HCAs (*18*), we assumed that all anthocyanin-linked HCAs had the same molar extinction coefficient as malvidin. Limits of quantification (LOQ) were determined as ten times the standard deviation of the noise. Concentration was expressed as milligrams per 100 gram tissue (peel or flesh) dry weight (DW). Mean (n=3) and standard deviation were calculated.

Results and Discussion

IDENTIFICATION OF HCAS/HCACS/DHCACS.

Analysis of the 15 Colombian potato cultivars revealed a large array of HCAcs and DHCAcs. As an example of differences among cultivars and between peel and flesh chromatograms recorded at 325 nm for peel and flesh of Pastusa suprema and Criolla Colombia are shown in **Figure 2 (A** and **B)**. The chromatograms revealed high diversity in number of compounds found and in their relative abundances, when comparing peel versus flesh within one cultivar, as well as when comparing peel or flesh between two cultivars. This variation in the chromatographic profiles was observed among all the cultivars studied. In **Figure 2C**, a representative chromatogram recorded at 515 nm for the peel of Tuquerreña, the richest in anthocyanin-linked HCAs, is shown.

When analyzing all 15 cultivars, a total of 62 compounds were annotated as HCAs/HCAcs/DHCAcs (**Table 2**) and grouped as unconjugated HCAs, HCAs linked to hydroxyl containing compounds (including anthocyanin-linked HCAs, **Table 3**), HCAs linked to amino compounds, DHCAs linked to amino compounds, compounds tentatively annotated as HCAcs, and compounds tentatively annotated as DHCAcs. Only twelve compounds were common to all cultivars in both peel and flesh, with MS and UV data matching those of previous work (*9*). These compounds included the commonly reported ChA (**9**), *neo*-ChA (**1**), *crypto*-ChA (**10**) and CaA (**12**). The presence of the other 50 varied widely; e.g., compound **46** was found in all flesh samples and in seven of the peel samples, while compound **48** was found in thirteen peel samples and in nine flesh samples. Other compounds were rarely found, e.g. **31** was found only in the peel of Tuquerreña. *p*-Coumaric acid and ferulic acid were not found in the cultivars studied, although they were reported by others in potato tubers (*5, 9*). Within the 50 compounds that were not common to all cultivars a number of 25 were found previously in potato tubers (*2, 4, 5, 9, 19, 20*). Here, we focused on the annotation of the 25 compounds not described previously in potato tubers (seven HCA-hydroxyl containing compounds, fifteen tentatively annotated as HCAcs, and three tentatively annotated as DHCAcs). Anthocyanin-linked HCAs previously found in potato were also described.

Seven compounds were annotated as HCAs linked to hydroxyl containing compounds. Compound **14**, with shorter retention time than authentic ferulic acid and with parent ion *m/z* 355 [ferulic acid+162 a.m.u.-H]$^-$, was annotated as a feruloyl hexose. Compound **24** with parent ion *m/z* 279, gave the daughter ions *m/z* 163 and 119, characteristic to *p*-coumaric acid, as well as *m/z* 133, characteristic to malic acid. Therefore, **24** was annotated as *p*-coumaroyl malate (*21*). Compound **27**, with parent ion *m/z* 367, yielded daughter ions characteristic of *trans*-5-*O*-feruloyl quinic acid (**22**) (*22*). Based on MS^n information and the later elution of **27** than **22**, the former was annotated as *cis*-5-*O*-

feruloyl quinic acid. Compound **31**, with parent ion *m/z* 627, gave the daughter ions *m/z* 353, 191, 179, 173 and 135. Hence, it was annotated as a caffeoyl quinic acid conjugate.

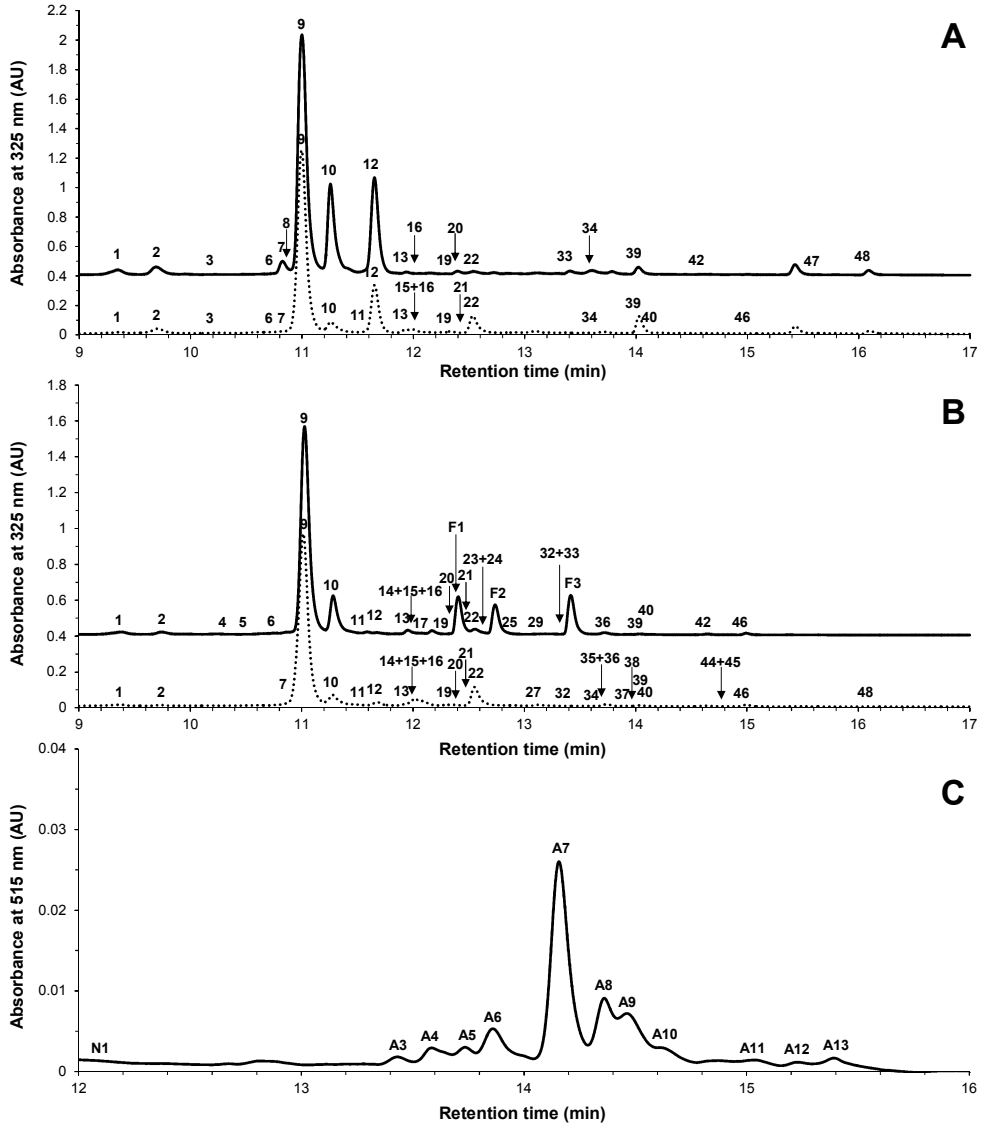


Figure 2. UHPLC profile at 325 nm (elution condition I), of aqueous methanolic extracts from peel (undiluted extracts) (**A**) and flesh (1.5 mL extracts concentrated to 0.2 mL) (**B**) of Pastusa suprema (solid line) and Criolla Colombia (dotted line) cultivars. F1: Quercetin rutinosyl glucoside; F2: Quercetin di-glucoside; F3: Rutin. UHPLC profile at 515 nm (elution condition II), from the peel of Tuquerreña, the richest in anthocyanin-linked HCAs sample (**C**). N1: Pelargonidin-3-*O*-rutinoside.

Table 2. Peak identification of compounds based on mass spectrometry and UV data.

No	RT (min)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)[a]	UV λmax (nm)	Identification	Occurrence[b]	
						Peel	Flesh
Unconjugated HCAs							
12	11.69	179	135, 179, 161	226, 305sh[c], 323	*trans*-Caffeic acid[de]	15	15
HCAs linked to hydroxyl containing compounds							
	HCAs linked to quinic acid						
1	9.38	353	*191*, 179, 135, *173*	225, 300sh, 324	*trans*-*Neo*-chlorogenic acid[de]	15	15
9	11.03	353	*191*, 179, *173*, 135	225, 305sh, 325	*trans*-Chlorogenic acid[de]	15	15
10	11.28	353	*173*, 179, *191*, 135	225, 305sh, 326	*trans*-*Crypto*-chlorogenic acid[de]	15	15
13	11.94	353	*191*, 179, 173, 135, 161	225, 303sh, 316	*cis*-Chlorogenic acid[e]	15	15
22	12.56	367	*191*, 173, 193	225, 305sh, 325	*trans*-5-*O*-feruloyl quinic acid[e]	15	15
27	13.11	367	*191*, 173, 193, 149, 134	225, 269, 313	*cis*-5-*O*-feruloyl quinic acid	2	11
31	13.36	627	353, 554, *191*, 453, 309, 179, 173, 291, 135	226, 295, 312sh	Caffeoyl quinic acid conjugate	1	0
41	14.23	515	353, 179, 161, *191*	226, 303sh, 320	3,5-di-*O*-caffeoyl quinic acid[f]	3	1
	HCAs linked to hexose						
4	10.27	341	135, 179, 161	224, 289, 316	Caffeoyl hexose[e]	0	10
14	12.00	355	193, 175, 134, 149	225, 302sh, 327	Feruloyl hexose[g]	0	10
19	12.33	385	205, 223, 179	225, 324	Sinapoyl hexose[e]	15	15
25	12.72	325	163, *161*, 119	225, 265, 320	*p*-Coumaroyl hexose[e]	0	5
	HCAs linked to malic acid						
24	12.69	279	163, 119, *133*	225, 301sh, 313	*p*-Coumaroyl malate[h]	3	7
28	13.16	309	193, 178, *133*, 149	226, 303sh, 325	Feruloyl malate[e]	8	13
29	13.16	339	223, 208, 179, *133*	225, 313	Sinapoyl malate[e]	7	10
HCAs linked to amino compounds							
2	9.75	249	249, 135, 207, 179, 161	225, 291sh[b], 319	Caffeoyl putrescine[e]	15	15
39	14.04	328	310, 234, 175, 149	227, 295sh, 319	Feruloyl octopamine[e]	15	15
48	16.10	312	312, 297, 178, 148	226, 291, 318	Feruloyl tyramine[i]	13	9

Table 2. (Continued)

No	RT (min)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)[a]	UV λmax (nm)	Identification	Occurrence[b]	
						Peel	Flesh
DHCAs linked to amino compounds							
8	10.85	529	529, *365*, *407*	225, 282, 319	N^1,N^{14}-bis(dihydrocaffeoyl) spermine[e]	15	15
16	12.06	472	472, *308*, *350*, 163	225, 282, 324	N^1,N^{10}-bis-(dihydrocaffeoyl) spermidine[e]	15	15
33	13.41	693	693, *529*, *571*, *449*, *407*, *365*, 163	227, 283, 312sh	N^1,N^5,N^{14}-tris-(dihydrocaffeoyl) spermine[e]	15	15
42	14.64	636	636, *472*, *514*, *308*	226, 284, 312	N^1,N^5,N^{10}-tris(dihydrocaffeoyl) spermidine[e]	10	8
47	15.58	857	857, *693*, *735*	226, 302sh, 325	N^1,N^5,N^{10},N^{14}-tetra(dihydrocaffeoyl) spermine[j]	7	2
Compounds tentatively annotated as HCAcs							
5	10.52	417	417, 285, 163, 179, 223	224, 293, 318sh[b]	HCA conjugate[e]	0	7
6	10.77	349	267, 249, 175, 193	225, 286, 313sh	Feruloyl conjugate	10	14
7	10.83	291	291, 171, 145, 119	225, 296, 305sh	Coumaroyl conjugate	9	7
11	11.56	349	249, 267, 205, 223	225, 289, 321	Sinapoyl conjugate	10	13
15	12.04	443	267, 193, 249, 175	225, 287, 322	Feruloyl conjugate	5	12
17	12.15	413	267, 249, 163, 119, 145	225, 300sh, 313	Coumaroyl conjugate	5	7
18	12.24	387	207, 163, 119	225, 285, 319	Coumaroyl conjugate	0	5
20	12.38	449	269, 287, 179, 225, 163	226, 286, 328	HCA conjugate	10	13
21	12.54	443	267, 249, 193, 175	225, 283, 321	Feruloyl conjugate	13	13
23	12.65	505	293, 445, 423, 173, 149	226, 303sh, 320	Feruloyl conjugate	0	2
30	13.19	391	331, 179, 161, 149, 193	225, 290sh, 326	HCA conjugate	3	8
32	13.40	403	223, 179, 359, 161, 135	225, 268, 321sh	HCA conjugate	0	14
34	13.60	445	283, 223, 179, 205	225, 283, 321	Sinapoyl conjugate[e]	12	6
35	13.65	462	307, 179, 161, 135	225, 295sh, 321	Caffeoyl conjugate	0	8

Table 2. (Continued)

No	RT (min)	[M-H]⁻ (*m/z*)	MS² (*m/z*)[a]	UV λmax (nm)	Identification	Occurrence[b]	
						Peel	Flesh
37	13.86	447	403, 367, 175, 149	225, 266, 319sh	Feruloyl conjugate	0	6
43	14.67	429	249, 205, 179, 223	225, 273, 320	Sinapoyl conjugate[e]	2	6
44	14.78	431	371, 145, 181, 209, 149, 119	226, 296, 318	HCA conjugate	5	9
45	14.81	397	397, 153, 165, 225, 163, 135	225, 261, 301	HCA conjugate	0	8
46	14.99	429	249, 205, 179, 385	225, 310	Sinapoyl conjugate[e]	7	15
Compounds tentatively annotated as DHCAcs							
3	10.24	351	215, 195, 177, 173, 151	224, 290, 319	Dihydroferuloyl conjugate	12	0
26	12.90	441	397, 359, 225, 207, 181	225, 302sh, 323	Dihydrosinapoyl conjugate	0	5
36	13.69	449	269, 225, 207, 251	225, 270, 320	Dihydrosinapoyl conjugate[e]	3	12
38	13.99	567	225, 181, 207, 179, 161	225, 319	Dihydrosinapoyl caffeoyl conjugate	0	2
40	14.12	405	225, 181, 207, 179, 161	225, 273, 320	Dihydrosinapoyl caffeoyl conjugate[e]	5	14

[a]Daugther ions in MS^2 are listed in order of intensity, first value is the base peak. Underlined data are diagnostic for the classification of compound as HCAcs/DHCAs. Data in italic are those ions which show neutral losses diagnostic for HCAcs/DHAcs.
[b]Number of cultivars where each compound is found.
[c]sh: shoulder.
[d]Retention times and UV maxima, parent ion and MS^2 data equal to those of authentic standards.
[e]Previously reported in potato tubers (*9*).
[f]Previously reported in other sources (*23*), but not in potato tubers.
[g]Previously reported in other sources (*24*), but not in potato tubers.
[h]Reported in other sources (*21*), but not in potato tubers.
[i]Previously reported in potato tubers (*20*).
[j]Previously reported in potato tubers (*19*).

Compound **41** had a parent ion of *m/z* 515, with daughter ions *m/z* 353 (base peak), 179, 161 and 191. Fragmentation of *m/z* 353 in MS^3 gave the ion *m/z* 191 as base peak and the ion *m/z* 179 (45%). The absence of the ion *m/z* 335 in MS^2, together with base peak *m/z* 191 and the abundant *m/z* 179 during MS^3 fragmentation (*23*) allowed us to annotate compound **41** as 3,5-di-*O*-caffeoyl quinic acid. We did not find the isomer 4,5-di-*O*-caffeoyl quinic acid, previously found in potato tubers (*19*). Two anthocyanins had not been described in potato tubers. Based on parent ion and daughter ions, **A1** was tentatively annotated as cyanidin-3-*O*-caffeoyl-rutinosyl-5-*O*-glucoside. **A13**, giving parent ion and daughter ions as **A8**, was annotated as peonidin-3-*O*-*p*-coumaroyl-rutinosyl-5-*O*-glucoside isomer.

Fifteen compounds, not reported in potato tubers previously, were tentatively annotated as HCAcs based on negative ions, neutral losses and UV maxima characteristic to HCAs, but their full annotation was not achieved. In addition, three compounds, not reported previously in potato tubers, were tentatively annotated as DHCAcs. Because of the documented cross-talk between HCAs/DHCAs and amines (*25*), we screened for the presence of amines as being part of those 18 compounds. Nevertheless, parent ion (*m/z* [HCA+amine-H]$^-$ or *m/z* [DHCA+amine-H]$^-$) screening did not reveal the presence of the amines putrescine, spermidine, spermine, agmatin, tyramine, serotonin, tryptamine, octopamine, noradrenaline, dopamine and anthranilate.

Table 3. Peak identification of anthocyanin-linked HCAs in peel extracts from Colombian potato cultivars different to the Criolla group, based on mass spectrometric data.

No	RT (min)	M^+ (*m/z*)	MS^2 (*m/z*)[a]	Identification	Occurrence[b]
A1	13.00	919	757, 287, 449	Cyan-3-*O*-CaA-rut-5-*O*-glc	6
A2	13.20	949	317, 787, 479	Pet-3-*O*-CaA-rut-5-*O*-glc[cd]	2
A3	13.42	903	741, 271, 433	Pel-3-*O*-CaA-rut-5-*O*-glc[c]	7
A4	13.65	933	771, 301, 463	Peo-3-*O*-CaA-rut-5-*O*-glc[e]	9
A5	13.74	903	741, 287, 449, *757*	Cyan-3-*O*-*p*-CouA-rut-5-*O*-glc[d]	10
A6	13.86	933	317, 771, 479, *787*	Pet-3-*O*-*p*-CouA-rut-5-*O*-glc[cde]	6
A7	14.16	887	725, 271, 433, *741*	Pel-3-*O*-*p*-CouA-rut-5-*O*-glc[ce]	10
A8	14.36	917	755, 301, 463, *771*	Peo-3-*O*-*p*-CouA-rut-5-*O*-glc[cde]	10
A9	14.46	917	755, 271, 433, *741*	Pel-3-*O*-FerA-rut-5-*O*-glc[ce]	9
A10	14.61	947	785, 301, 463, *771*	Peo-3-*O*-FerA-rut-5-*O*-glc[ce]	10
A11	15.05	725	271, 433, *579*	Pel-3-*O*-*p*-CouA-rut[c]	5
A12	15.20	887	725, 271, 433, *741*	Pel-3-*O*-*p*-CouA-rut-5-*O*-glc isomer[c]	6
A13	15.34	917	755, 301, 463, *771*	Peo-3-*O*-*p*-CouA-rut-5-*O*-glc isomer	5
A14	15.40	755	271, 433, *579*	Pel-3-*O*-FerA-rut[c]	3

RT obtained using elution condition II. Data in italic are those ions which show neutral losses diagnostic for HCAcs.

Cyan: Cyanidin; Pet: Petunidin; Pel: Pelargonidin; Peo: Peonidin; CaA: caffeic acid; *p*-CouA: *p*-coumaric acid; FerA: ferulic acid; rut: rutinose; glc: glucose

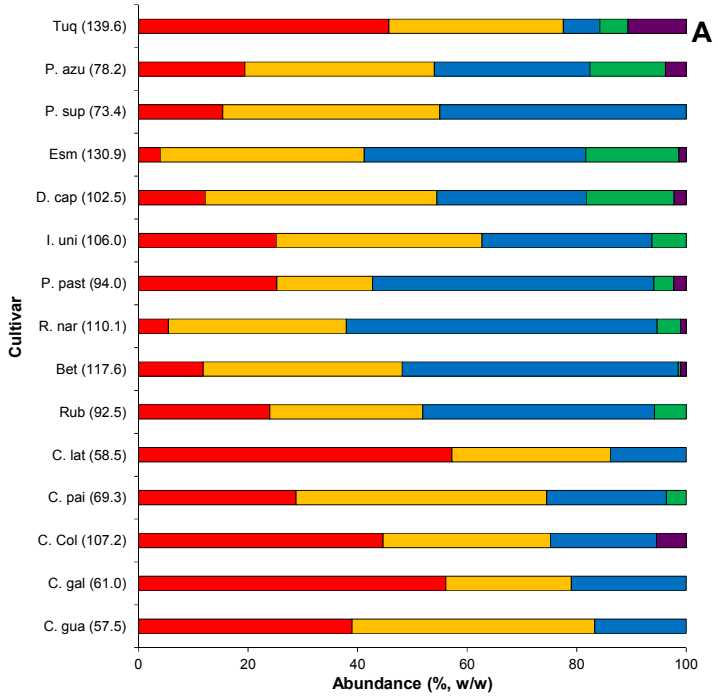
[a]Daugther ions in MS^2 are listed in order of intensity, first value is the base peak.

[b]Number of cultivars where each compound is found.

[c]Previously reported in potato tubers (*5*).

[d]Previously reported in potato tubers (*2*).

[e]Previously reported in potato tubers (*4*).


A
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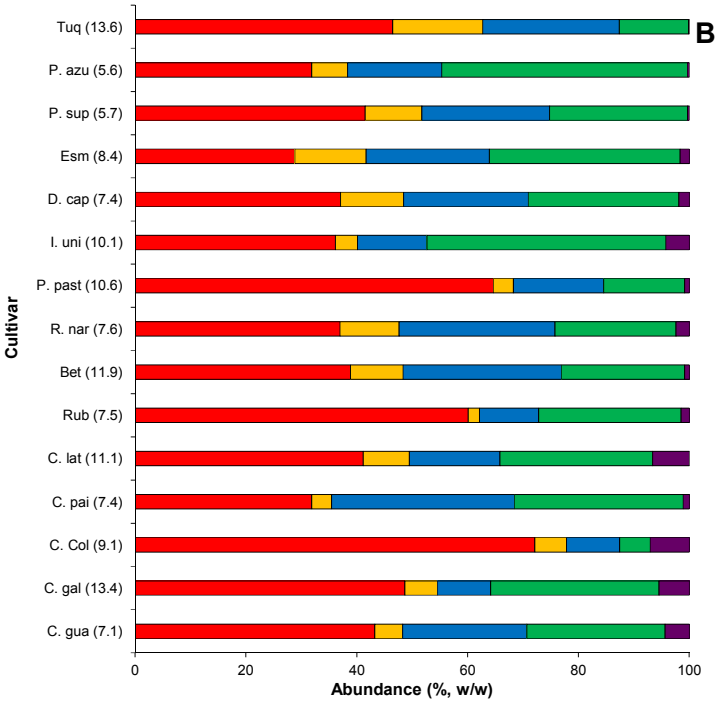
P. azu (78.2)
P. sup (73.4)
Esm (130.9)
D. cap (102.5)
I. uni (106.0)
P. past (94.0)
R. nar (110.1)
Bet (117.6)
Rub (92.5)
C. lat (58.5)
C. pai (69.3)
C. Col (107.2)
C. gal (61.0)
C. gua (57.5)
Cultivar
0
20
40
60
80
100
Abundance (%, w/w)


B
Tuq (13.6)
P. azu (5.6)
P. sup (5.7)
Esm (8.4)
D. cap (7.4)
I. uni (10.1)
P. past (10.6)
R. nar (7.6)
Bet (11.9)
Rub (7.5)
C. lat (11.1)
C. pai (7.4)
C. Col (9.1)
C. gal (13.4)
C. gua (7.1)
Cultivar
0
20
40
60
80
100
Abundance (%, w/w)

A total of 14 anthocyanins was found acylated with HCAs when screening the purple/red peel extracts of the ten potato cultivars at 515 nm (**Figure 2C**, **Table 3**), different to those of the Criolla group (**Table 1**). Only four anthocyanins were common to the ten purple/red peel cultivars: cyanidin-3-*O*-*p*-coumaroyl-rutinosyl-5-*O*-glucoside (**A5**), pelargonidin-3-*O*-coumaroyl-rutinosyl-5-*O*-glucoside (**A7**), peonidin-3-*O*-*p*-coumaroyl-rutinosyl-5-*O*-glucoside (**A8**), and peonidin-3-*O*-feruloyl-rutinosyl-5-*O*-glucoside (**A10**). Commonly, annotation of anthocyanin-linked HCAs is based on molecular weight, intensity of ions, *m/z* value of daughter ions related to neutral losses of sugar units, and UV-vis spectra (*26*). As shown in **Table 3**, fragmentation of anthocyanin-linked HCAs gave ions, corresponding to neutral losses of HCAs (146 a.m.u., *p*-coumaric acid; 162 a.m.u., caffeic acid; and 176 a.m.u, ferulic acid) in the positive mode, although in low intensity (1 to 3%). In principle, these neutral losses of 146, 162, and 176 a.m.u. might also correspond to rhamnosyl, glucosyl (or galactosyl), or galacturonyl residues, respectively. To our knowledge, no anthocyanins with *O*-3 linked trisaccharide side chains have been reported in potato, and therefore the *O*-3 substitution was annotated as a rutinosyl side chain, end-capped with one of the HCAs.

OTHER PHENOLIC COMPOUNDS.

Other non HCA-acylated phenolic compounds were found. As shown in **Figure 2**, three non-acylated flavonols were found, mainly in the flesh samples: Quercetin rutinosyl glucoside (**F1**), quercetin di-glucoside (**F2**) and rutin (**F3**). Their parent and daughter ions matched those in the literature (*3*). Besides the 14 HCA-acylated anthocyanins, two unacylated anthocyanins were found: pelargonidin-3-*O*-rutinoside (**N1**) and cyanidin-3-*O*-rutinoside (**N2**). Their presence was cultivar-dependent.

QUANTIFICATION OF HCA/HCACS/DHCACS.

Less commonly described HCAcs/DHCAcs. Scarcely described HCAcs/DHCAcs were divided into the categories: HCA-hydroxyl containing compounds, HCA-amines, DHCA-amines, tentatively annotated HCAcs and tentatively annotated DHCAcs. Their relative abundances in peel and flesh are shown in **Figure 3** and their absolute contents in whole tubers are shown in **Figure 4**. High intervarietal differences in these compounds can be observed. Except for the second category, in which the range was higher in peel than in flesh, the ranges obtained for the quantities of compounds of the other categories overlapped in peel and flesh.

Figure 3. Relative distribution of the less commonly described HCAcs/DHCAcs in peel (**A**) and flesh (**B**) of Colombian potato tubers. HCA-hydroxyl containing compounds, ■; HCA-amines, ■; DHCA-amines, ■; tentatively annotated as HCAcs, ■; tentatively annotated as DHCAcs, ■. Abbreviations of cultivars are as in **Table 1**. In brackets the total amount in mg/100 g DW.

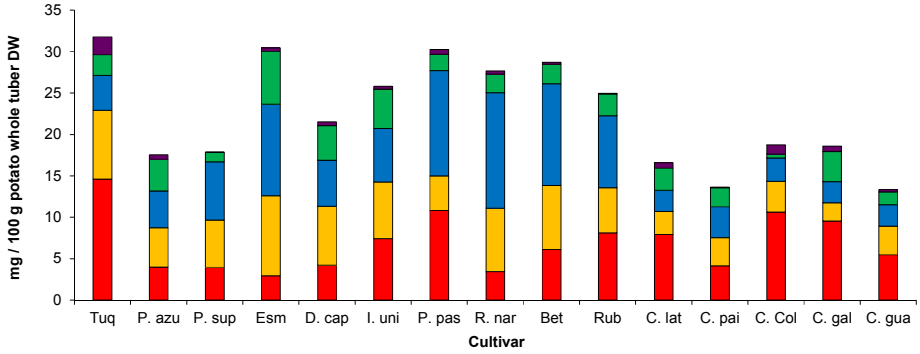


Figure 4. Contents of the less commonly described HCAcs/DHCAcs in whole tubers. HCA-hydroxyl containing compounds, ■; HCA-amines, ■; DHCA-amines, ■; tentatively annotated as HCAcs, ■; tentatively annotated as DHCAcs, ■. Abbreviations of cultivars are as in **Table 1**.

Within the groups of HCA-hydroxyl containing compounds and HCA-amines, compounds that have been related to plant disease resistance were identified (*25*). Interestingly, no correlation was found between *Phytophtora infestans* resistance and quantities of feruloyl malate (**28**), sinapoyl malate (**29**), caffeoyl putrescine (**2**), feruloyl octopamine (**39**), feruloyl tyramine (**48**) in peel, flesh, or whole tuber. This supports the idea that besides the preformed defense compounds (non-pathogen challenged), compounds induced after host-pathogen interaction (not tested here) play an important role in pest resistance (*27*).

Regarding the whole tuber contents of the two DHCA-amines with health-promoting effects it was found that N^1,N^{10}-bis-(dihydrocaffeoyl) spermidine contents ranged from 1.1±0.2 mg/100 DW to 2.6±0.4 mg/100 g DW, while N^1,N^{14}-bis-(dihydrocaffeoyl) spermine content ranged from 0.2±0.1 mg/100 g DW to 1.7±0.5 mg/100 g DW, values that fit well with those of others (*9, 10, 19*).

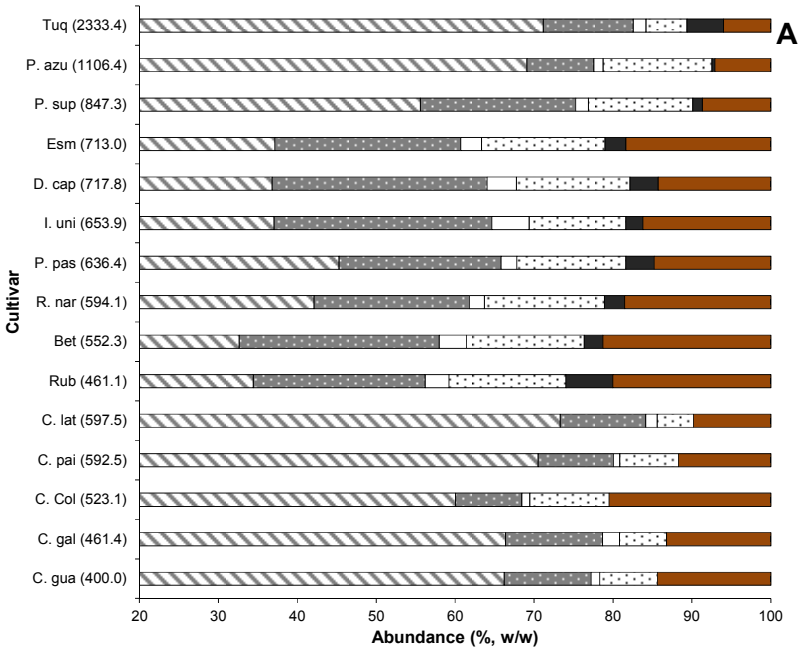
Most commonly described HCAcs/DHCAcs. Figure 5 shows the relative proportions of ChA, *crypto*-ChA, *neo*-ChA, CaA, and HCAs linked to anthocyanins. The contribution of the less commonly described HCAcs/DHCAcs, shown in the previous section, was reported as a single group in **Figure 5**. Interestingly, the group of the less commonly described HCAcs/DHCAcs, being much more diverse in representatives than the group of ChA/*crypto*-ChA/*neo*-ChA/CaA/HCA-anthocyanins, accounted for 6.0-21.3% (w/w) in peel and for 8.0-20.1% (w/w) in flesh, indicative of their important contribution to the total. The total content of HCAs/HCAcs/DHCAcs was 7 to 28 times higher in peel than in flesh. In peel there was a 5.6-fold variation in the total content of HCAs/HCAcs/DHCAcs, with the lowest value for Criolla guaneña (400.0±50.8 mg/100g DW) and the highest for Tuquerreña (2233.4±131.3 mg/100g DW). The variation in flesh was within a 3.5-fold

range, with the lowest value for Diacol capiro (37.7±1.5 mg/100g DW) and the highest for Tuquerreña (133.6±11.8 mg/100g DW). The higher amounts of total HCAs/HCAcs/DHCAcs in peel than in flesh is in agreement with previous data on ChA, its isomers and CaA contents (*5, 28*). Peel is expected to be richer in phenolics than flesh, partially due to exposure to light during handling and storage of whole potato tubers (*29*), and also because the peel forms the first line of defense, in which these compounds play an important role (*30*).

Also, high variation was observed in the concentrations of the most commonly described HCAs/HCAcs. In general, in peel samples of all fifteen cultivars the relative abundance followed the order ChA (32.6 – 71.2%, w/w) > *crypto*-ChA (8.4 – 27.6%, w/w) > CaA (4.6 – 15.6%, w/w) > *neo*-ChA (0.8 - 4.7%, w/w) ~ anthocyanin-linked HCAs (0.0 – 5.9%, w/w). This contrasted with the lower contribution of CaA to the flesh composition, with the order ChA (48.9 - 76.1%, w/w) > *crypto*-ChA (7.2 - 24.0%, w/w) > CaA (1.4 – 4.1%, w/w) ~ *neo*-ChA (0.7 – 3.9%, w/w) (**Figure 5**).

Unacylated anthocyanins comprised only between 0.0 to 4.1% (w/w) of the total amount of anthocyanins, underlining the prevalence of anthocyanin-linked HCAs in potato tubers. The peel of the Tuquerreña cultivar had the highest content of anthocyanin-linked HCAs (108.7±7.5 mg/100 g DW), while the peel of Punto azúl had the lowest content (4.4±0.3 mg/100 g DW). In Tuquerreña peel, pelargonidin-3-*O*-coumaroyl-rutinosyl-5-*O*-glucoside (**A7**, 51% w/w) was the main anthocyanin, followed by both pelargonidin-3-*O*-feruloyl-rutinosyl-5-*O*-glucoside (**A9**, 14% w/w) and peonidin-3-*O*-*p*-coumaroyl-rutinosyl-5-*O*-glucoside (**A8**, 13% w/w).

Absolute amounts of ChA, *neo*-ChA, *crypto*-ChA, CaA, anthocyanin-linked HCAs, and other HCAcs/DHCAs were calculated in whole tubers (**Figure 6**) based on the peel/flesh ratio (**Table 1**) and the quantity of each compound in peel and flesh (**Figure 5**). Absolute amounts in whole tubers of ChA+*neo*-ChA+*crypto*-ChA ranged from 66.5±6.4 mg/100 g DW to 384.0±24.0 mg/100 g DW. CaA in whole tubers ranged from 4.4±0.7 mg/100 g DW to 26.0±2.2 mg/100 g. Variation of both ChA isomers and CaA among Colombian potato cultivars is lower than that observed for potatoes grown in Canada (*31*), in the USA (*3*) and in most of those from Peru (*2*).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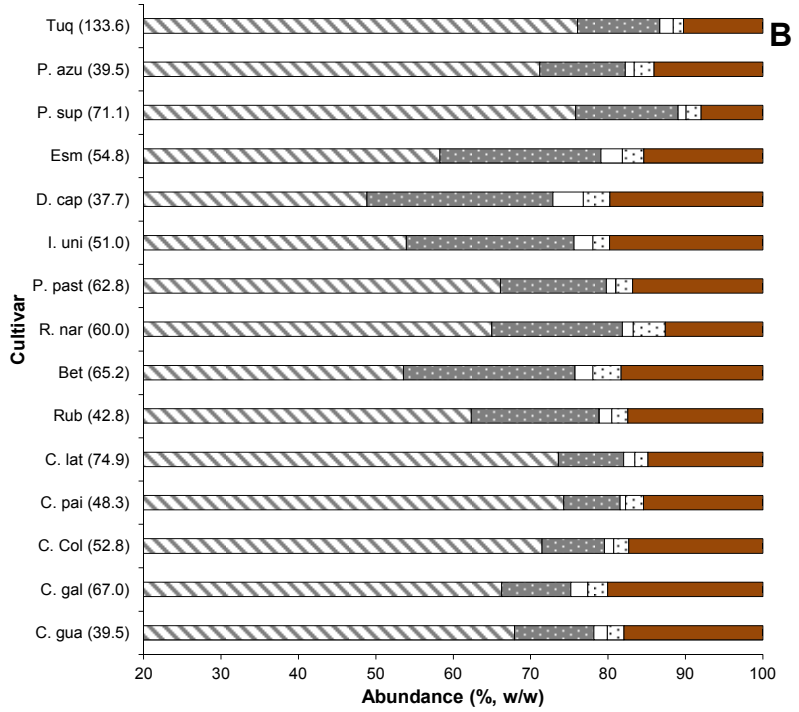
P. sup (847.3)
Esm (713.0)
D. cap (717.8)
I. uni (653.9)
P. pas (636.4)
R. nar (594.1)
Bet (552.3)
Rub (461.1)
C. lat (597.5)
C. pai (592.5)
C. Col (523.1)
C. gal (461.4)
C. gua (400.0)
Cultivar
20
30
40
50
60
70
80
90
100
Abundance (%, w/w)


B
Tuq (133.6)
P. azu (39.5)
P. sup (71.1)
Esm (54.8)
D. cap (37.7)
I. uni (51.0)
P. past (62.8)
R. nar (60.0)
Bet (65.2)
Rub (42.8)
C. lat (74.9)
C. pai (48.3)
C. Col (52.8)
C. gal (67.0)
C. gua (39.5)
Cultivar
20
30
40
50
60
70
80
90
100
Abundance (%, w/w)

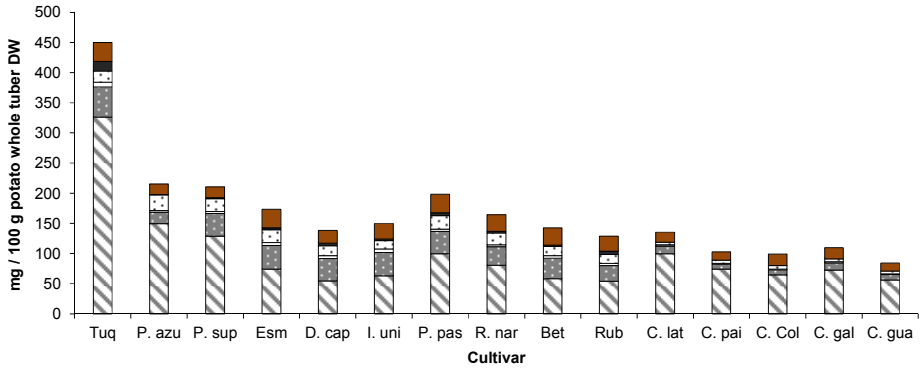


Figure 6. Contents of various constituents in the whole tuber of 15 Colombian potato cultivars. Chlorogenic acid (ChA), ; *crypto*-ChA, ; *neo*-ChA, ;caffeic acid, ; anthocyanin-linked HCAs, ; less commonly described (dihydro) hydroxycinnamic acid conjugates, . Abbreviations of cultivars are as in **Table 1**.

Conclusions

In total, 62 compounds were tentatively annotated as HCA/HCAcs/DHCAcs. With only twelve compounds being common to all cultivars in both peel and flesh, the remaining 50 compounds accounted for a large diversity among cultivars. The less commonly described compounds in whole tubers accounted for 7.1-20.1% w/w of the total amount of HCAs/HCAcs/DHCAcs, highlighting their contribution to the phenolic profile of potato tubers.

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Figure 5. Relative distribution of various constituents in the peel (**A**) and flesh (**B**) of Colombian potato tubers. Chlorogenic acid (ChA), ; *crypto*-ChA , *neo*-ChA, ; caffeic acid, ; anthocyanin-linked HCAs, ; less commonly described (dihydro) hydroxycinnamic acid conjugates, . Values in brackets are total amount in mg/100 g DW. Abbreviations of cultivars are as in **Table 1**.

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Chapter 4

New insights into an ancient anti-browning agent: formation of sulfo-phenolics in sodium hydrogen sulfite treated potato extracts

ABSTRACT: The effect of sodium hydrogen sulfite (*S*), used as anti-browning 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*-caffeoyl quinic acid (ChA) 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 ChA, sodium hydrogen sulfite and PPO. This sulfo-adduct exhibited a small bathochromic shift (λmax 329 nm) compared to ChA (λmax 325 nm), and a strong hypochromic shift with an extinction coefficient of 9,357±395 $M^{-1}cm^{-1}$ compared to 18,494±196 $M^{-1}cm^{-1}$, respectively. The results suggest that whenever *S* is used as anti-browning agent, the *o*-quinone formed with PPO reacts with *S* to produce sulfo-*o*-diphenol, which does not participate in browning reactions.

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Introduction

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, ChA), 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, e.g. variety. ChA and its isomers have been found in potato tubers to commonly range from 23 to 350 mg/100 g DW, caffeic acid from trace to 48 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 2.5×10^6 tons of starch potatoes annually (*7*).

The anti-browning effect of ascorbic acid has been associated to its ability to reduce quinones to their precursor phenolics and by 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, by reduction of the quinones to the original phenolic compounds (*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/DHCAcs in potato by reverse-phase ultrahigh 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 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 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 (ChA), ascorbic acid, sodium hydrogen sulfite ($NaHSO_3$), and mushroom tyrosinase were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). *Neo*-ChA (3-*O*-caffeoyl quinic acid) and *crypto*-ChA (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). Water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were from Merck (Darmstadt, Germany).

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 20,000 ppm ascorbic acid (extractant *A*) or 400 ppm $NaHSO_3$ (extractant *S*). Subsequently, the mixture was stirred for 10 min at 4 ºC. Starch and fibers were allowed to settle for 20 min at 4 ºC. After it was decanted, the solution was centrifuged (18,000 x g; 20 min; 4 ºC). The precipitated material and the pellet from centrifugation were collected, combined, and re-extracted with 100 mL 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 ºC. Extract *A* was kept overnight at 4 ºC without addition of acetic acid. The resulting materials were centrifuged (18,000 x g; 20 min; 4 ºC), 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, cut-off 10 kDa, Millipore) according to the instructions of the manufacturer. Filtrates were stored at -20 ºC 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 ºC using a 2.5 L Amicon ultrafiltration cell (Millipore) with a regenerated cellulose membrane (cut-off 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, USA). The methanolic fraction was evaporated under reduced pressure, and the remaining water was removed by freeze-drying, yielding 768 mg powder. A quantity of 200 mg of powder was suspended in MQ water to 5 mg/mL, stirred for 10 min, and centrifuged (12,000 x g; 5 min, 4 ºC). Subsequently, the resolubilised 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 × 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). The following elution program was used: 0-5 min, 0% B; 5-35 min, 0 to 26% B; 35-37 min, 26 to 100% B; 37-42 min, 100% B; 42-44 min, 100 to 0% B; 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 comprised **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 sulfo-phenolics, 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 mL of an aqueous solution of 5-*O*-caffeoyl quinic acid (1 mM) were fully converted after incubation with $NaHSO_3$ (2 mM) and

mushroom tyrosinase (140 units/mL; PPO units according to supplier) at 20 °C during 2 h. The initial pH was adjusted to 6.5 by adding 0.1 M NaOH. The resulting material was purified by semi-preparative 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 time, 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 cuvet. Temperature of the solutions was maintained at 20 ºC. The molar extinction coefficients (ε) were calculated using Abs = ε * 1 * c, in which Abs = absorbance at 325 nm, l = light path = 1 cm, 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 10x diluted, and reaction products synthesized in a model system with commercial PPO were analyzed using an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler, cooled at 7 °C, and a photo-diode array detector (DAD), using a Hypersil gold RP column (150 mm x 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 to 60% B; 23-24 min, 60 to 100% B; 24-27 min, 100% B; 27-28 min, 100 to 0% B; 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 auto-tuned using ChA. 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 standard for the quantification of caffeoyl quinic acid isomers. 2'-Sulfo-5-*O*-caffeoyl quinic

acid, obtained with the commercial PPO, was used as 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 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 to 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 D_2O (99.9 atom%, Aldrich) and approximately 1 μL 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 h 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 h 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 anti-browning agent. The chromatogram was dominated by HCAcs with 5-*O*-caffeoyl quinic acid (**3**) as the 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 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*-, 5-*O*-caffeoyl quinic acid isomers and that of caffeic acid matched with those of authentic standards.

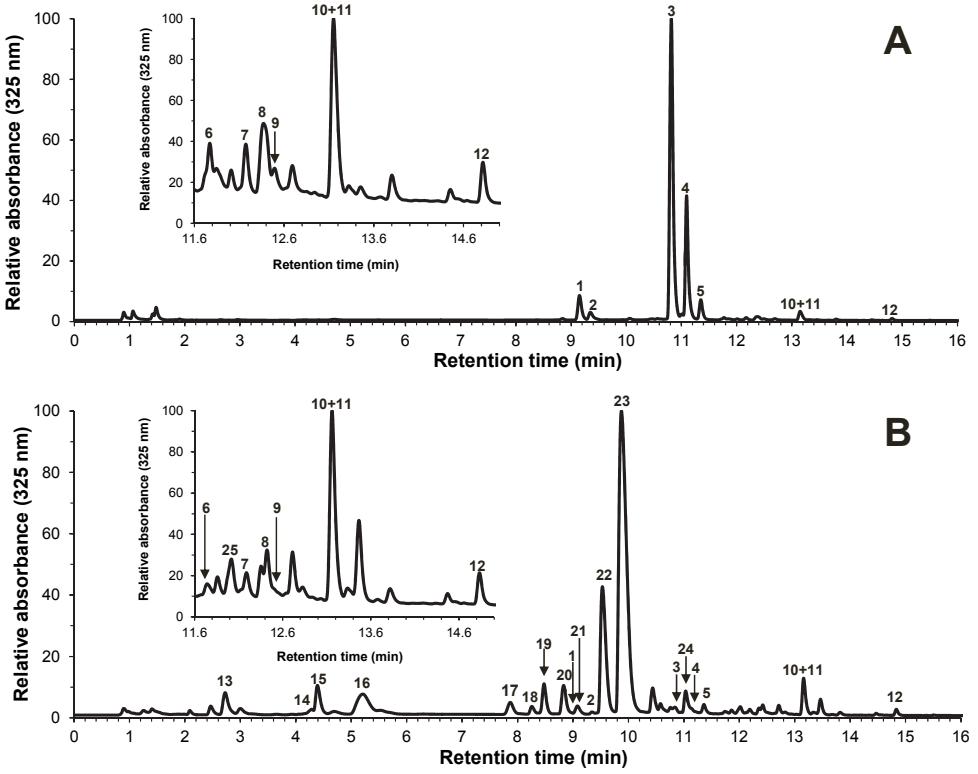

Figure 1. UHPLC chromatogram recorded at 325 nm of the potato extract prepared with the addition of (**A**) 20,000 ppm ascorbic acid or (**B**) 400 ppm $NaHSO_3$. The inserts are a zoom between 11.6 and 15.0 min.

Table 1. Retention times, MS, and UV data of HCAs/HCAcs and flavonols in potato.

#	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, 240sh, 300sh, 324	3-*O*-caffeoyl quinic acid[b]
2	9.35	249	**249**, 207 (7), 161 (<1) 135 (23)	223, 293sh, 317	Caffeoyl putrescine
3	10.82	353	**191**, 179 (3), 173 (<1), 161 (<1), 135 (<1)	223, 240sh, 305sh, 325	5-*O*-caffeoyl quinic acid[b]
4	11.10	353	191 (21), 179 (59), **173**, 135 (8)	223, 240sh, 305sh, 326	4-*O*-caffeoyl quinic acid[b]
5	11.35	179	179 (86), **135**, 161 (<1)	224, 305sh, 323	Caffeic acid[b]
6	11.77	353	**191**, 179 (4), 173 (<1), 161 (<1), 135 (1)	229, 240sh, 305sh, 325	Caffeoyl quinic acid isomer
7	12.18	445	**385,** 223 (5), 205 (4), 179 (4)	225, 300sh, 330	Sinapoyl hexoside
8	12.37	367	193 (9), **191**, 173 (37), 134 (<1)	214, 301sh, 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, 300sh, 353	Quercetin-3-*O*-diglycoside
10	13.15	309	**193**, 178 (<1), 149 (<1), 134 (<1), 115 (<1)	225, 300sh, 326	Feruloyl malate[c]
11	13.22	609	**301**, 300 (44), 271 (9), 255 (6), 179 (3) MS^3 (74), **179**, 151 (92)	259, 300sh, 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 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, 0 to 50% B; 23-24 min, 50 to 100% B; 24-27 min, 100% B; 27-28 min, 100 to 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).

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 time 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*).

Table 2. Retention times, MS, and UV data of sulfo-HCAs/sulfo-HCAcs/sulfo-flavonols in potato.

#	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, 305sh, 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, 250sh, 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, 305sh, 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, 291sh, 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, 280sh, 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, 240sh, 305sh, 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, 240sh, 305sh, 329	2'-Sulfo-5-*O*-caffeoyl quinic acid
24	11.04	705	543 (4), 525 (1), ***381***, 301 (47), 271 (1)	229, 291sh, 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 brackets, 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 does agree with neither sulfo-quercetin nor with quercetin *O*-sulfate (*13, 14*), probably due to co-elution.

$NaHSO_3$ as anti-browning 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 a.m.u. (**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 a.m.u. Similarly, MS analysis revealed that the MW of **19** matched with that of caffeoyl putrescine plus 80 a.m.u., **14** and **21** with a MW of caffeic acid plus 80 a.m.u., **24** with a MW of quercetin-3-*O*-diglucoside plus 80 a.m.u., and **25** with a MW of rutin plus 80 a.m.u. The extra 80 a.m.u. 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: *m/z* 415 $[M\text{-}H_2O\text{-}H]^-$, 387 $[M\text{-}H_2O\text{-}CO\text{-}H]^-$ and 301 $[M\text{-}C_5H_6O_3\text{-}H_2O\text{-}H]^-$. 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 crosspeak 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 crosspeak 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 (*15*). For **23** a crosspeak between H-5 of quinic acid and C-9' of caffeic acid indicated C-5 as linkage position. This was also confirmed by a crosspeak 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, compared to unsubstituted quinic acid (*15*). 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.

Table 3. ^{1}H and ^{13}C-NMR data of **22** and **23**.

atom no	22				23			
	^{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

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**), 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 (*16*), 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 (*17, 18*).

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 (*19*), 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 SULFO-CHA AND QUANTIFICATION OF PHENOLICS AND SULFO-PHENOLICS IN POTATO EXTRACTS.

The presence of the SO_3H moiety in 5-*O*-caffeoyl quinic acid caused a small bathocromic (λmax shift from 325 to 329 nm) and a large hypochromic shift (**Figure 2**). The hypochromic shift effect was reflected in the extinction coefficient of 2'-sulfo-5-*O*-caffeoyl quinic acid (9,357±395 $M^{-1}cm^{-1}$), which was half that of 5-*O*-caffeoyl quinic acid (18,494±196 $M^{-1}cm^{-1}$). The extinction coefficient of 5-*O*-caffeoyl quinic acid was comparable to that reported in the literature (18,130±242 $M^{-1}cm^{-1}$) (*20*).

Quantification of the (modified) HCAs/HCAcs/flavonols obtained in the presence of *S* revealed that most chlorogenic acid isomers were modified (less than 1%, w/w, 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

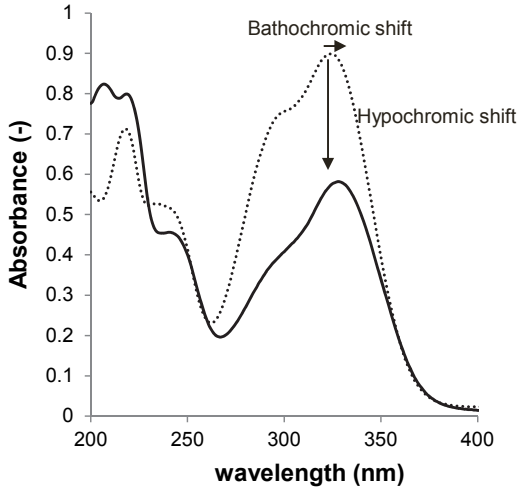

Figure 2. UV spectra of 0.05 mM 2'-sulfo-5-*O*-caffeoyl quinic acid (solid line) and 0.05 mM 5-*O*-caffeoyl quinic acid (dotted line).

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 ChA quinone, by which ChA and ChA-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 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 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 a.m.u. extra 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 a.m.u. extra (*21*). Interestingly, the MS^2 fragmentation pattern of those sulfated adducts differs from those of **22** and **23** (**Figure 3**). This suggests that in that study the sulphur-containing groups are differently attached to the aromatic ring 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 $[M\text{-}SO_3\text{-}H]^-$ with *m/z* 353 as base peak after MS^2 (*21*), 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 (*22*). 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 (*23*), but these have only 64 a.m.u. extra, instead of 80.

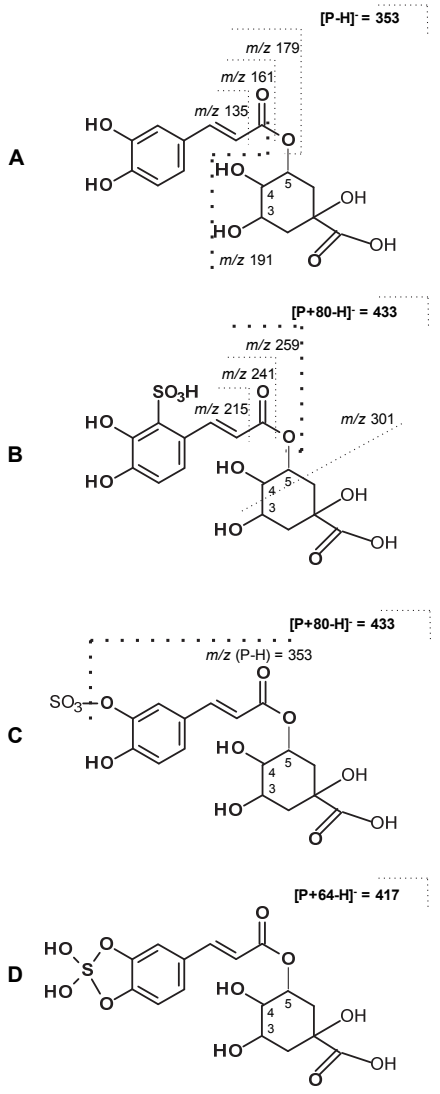

Figure 3. Distinctive MS fragmentation pattern of (**A**) 5-*O*-caffeoyl quinic acid (**3**) (**B**) 2'-sulfo-5-*O*-caffeoyl quinic acid (**23**) (**C**) 5-*O*-caffeoyl quinic acid sulfate, and (**D**) 5-*O*-caffeoyl quinic acid-double linked-*O*-sulfate. Part **C** was based on published results for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

Following the MS fragmentation data, as presented in **Figure 3, 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]$^-$ (*24*), 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 and, 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 (*17*). 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 (*17*). 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 (*18*). 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 (*17, 18, 25*). The importance of the double activation is evident if compared to the reactivity of dihydrocaffeic acid *o*-quinone (*18*). 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.

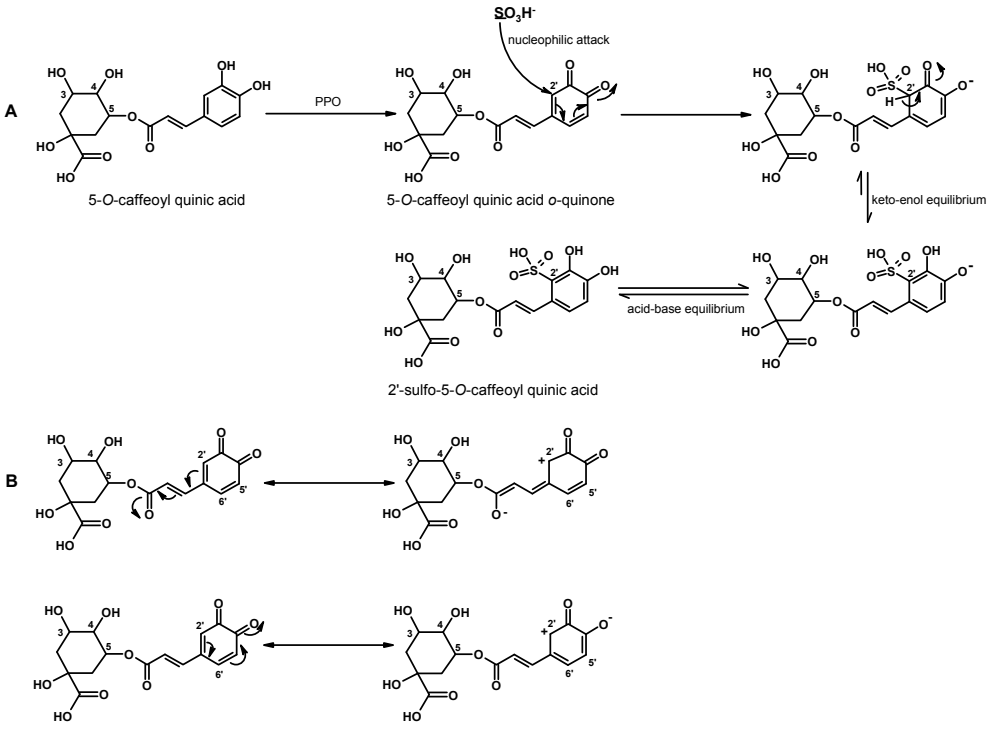

Figure 4. (A) Reaction between 5-*O*-caffeoyl quinic acid and HSO_3^-, after enzymatic activation of the aromatic ring to produce 2'-sulfo-5-*O*-caffeoyl quinic acid. (**B**) Double activation of the C-2' position of caffeic acid in 5-*O*-caffeoyl quinic acid.

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 (*26*). 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 (*25*), and for adducts of catechin and glutathione (*18*). 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 and; therefore, a nucleophilic attack is more probable at this position (*18*). Hence, we postulate that **24** and **25** correspond to 5'-sulfo-quercetin-3-*O*-diglucoside and 5'-sulfo-rutin, respectively.

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Chapter 5

Inhibition of enzymatic browning of chlorogenic acid by sulfur-containing compounds

ABSTRACT: The anti-browning activity of sodium hydrogen sulfite ($NaHSO_3$) was compared to that of other sulfur-containing compounds. Inhibition of enzymatic browning was investigated using a model browning system consisting of mushroom tyrosinase and chlorogenic acid (ChA). Development of brown color (spectral analysis), oxygen consumption, and reaction product formation (RP-UHPLC-DAD-MS) were monitored in time. It was found that the compounds showing anti-browning activity either prevented browning by forming colorless addition products with *o*-quinones of ChA ($NaHSO_3$, cysteine, glutathione) or by inhibiting the enzymatic activity of tyrosinase ($NaHSO_3$, dithiothreitol). $NaHSO_3$ was different from the other sulfur-containing compounds investigated, as it showed a dual inhibitory effect on browning. Initial browning was prevented by trapping the *o*-quinones formed in colorless addition products (sulfo-ChA), while at the same time tyrosinase activity was inhibited in a time-dependent way, as was shown by pre-incubation experiments of tyrosinase with $NaHSO_3$. Furthermore, it was demonstrated that sulfo-ChA and cysteinyl-ChA were not inhibitors of mushroom tyrosinase.

Based on: Kuijpers, T.F.M.; Narváez-Cuenca, C.-E.; Vincken, J.-P.; Verloop, A.J.W.; Van Berkel, W.J.H.; Gruppen, H. Inhibition of enzymatic browning of chlorogenic acid by sulfur-containing compounds. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 3507-3514.

Introduction

Enzymatic browning is a major quality problem in the processing of fruits and vegetables. The enzymes responsible for enzymatic browning are polyphenol oxidases (PPOs), which, depending on their source, can either catalyze the *o*-hydroxylation and subsequent oxidation of a wide range of phenolic substrates (tyrosinase, cresolase or monophenolase activity), or only the oxidation of *o*-diphenols (catecholase or diphenolase activity) (*1, 2*). The oxidation of *o*-diphenols results in *o*-quinones, which in turn react with each other and other compounds present, resulting in dark colored pigments (*3*). Additives widely used to inhibit enzymatic browning include sulfites and ascorbic acid (*2, 4*).

Different sulfites used as food additives are sodium sulfite (Na_2SO_3), sodium metabisulfite ($Na_2S_2O_5$), and sodium hydrogen sulfite ($NaHSO_3$, also referred to as sodium bisulfite). When dissolved, these salts yield a mixture of SO_3^{2-} and HSO_3^- (*5*). Three possible mechanisms for browning inhibition by sulfite have been suggested: (i) irreversible inhibition of PPO (*6*), (ii) reduction of *o*-quinones, thereby reversing the enzymatic reaction (*4*), and (iii) formation of addition products between sulfite and *o*-quinones, preventing them to react further into brown pigments (*7*).

Using sulfites in food products is controversial because of related health risks (*8*). An alternative anti-browning agent is ascorbic acid. The mechanism by which ascorbic acid inhibits browning is well known: *o*-quinones formed by PPO are reduced to their precursors, *o*-diphenols, which subsequently can be oxidized again (*9*). This redox cycling continues until all ascorbic acid is consumed, after which the brown colored pigments are still formed. Therefore, ascorbic acid can delay browning, but it does not inhibit enzymatic activity. Hence, much effort has been put in finding natural sulfite alternatives. Many PPO inhibitors have been identified and often their mode of inhibition has been determined (*10*). Surprisingly, the mechanism by which sulfite inhibits browning is still unclear, despite its common use for a long time.

Recently, it was found that, when extracting phenolic compounds from potato in the presence of sulfite, the expected phenolic compounds, mainly chlorogenic acid, were absent in the extracted material (*11*). Sulfonated derivatives were found instead. It was proposed that PPO-catalyzed *o*-quinone formation was a prerequisite for the formation of these sulfophenolics, and that formation of these compounds might be responsible for inhibition of browning.

In the present study, the effect of sulfite on PPO-catalyzed browning was further investigated. In contrast to the previous research (*11*), a model browning system was used, consisting of chlorogenic acid (**Figure 1**) and a commercially available mushroom PPO (tyrosinase; EC 1.14.18.1). The mechanism of action of $NaHSO_3$ is compared to that of other sulfur-containing compounds (**Figure 1**). Cysteine and glutathione (GSH) were

selected, as these compounds are known to form adducts with *o*-quinones (*12-15*). Besides formation of adducts with *o*-quinones, the anti-browning activity of cysteine has also been proposed to be due to irreversible inhibition of PPO (*16*). Dithiothreitol (DTT) was chosen because it was reported to be an inhibitor of tyrosinase-catalyzed browning. However, different explanations have been given: DTT was found to form addition products with *o*-quinones, it was found to reversibly inhibit tyrosinase, and it was found to reduce the copper in the active site of tyrosinase (*17, 18*). It should be noted that the results for the sulfur-containing compounds mentioned above were obtained in different studies, under a variety of conditions. For example, type of substrate, substrate concentration, inhibitor concentration, and enzyme source varied over the different studies.

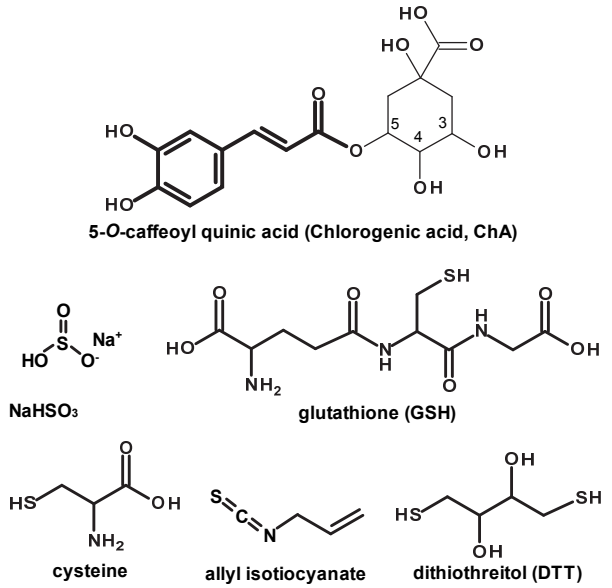

Figure 1. Structures of substrate and different sulfur-containing compounds used in this study.

Here, we compared the anti-browning properties of the different sulfur-containing compounds under similar conditions. To investigate whether other natural sulfur-containing compounds might have the potential to function as a sulfite alternative, allyl isothiocyanate was selected. Isothiocyanates result from the hydrolysis and subsequent rearrangements of their precursors, glucosinolates, which widely occur in for instance the Brassicaceae plant family (*19*).

Materials and Methods

CHEMICALS.
Mushroom tyrosinase, chlorogenic acid (5-*O*-caffeoylquinic acid, ChA), sodium hydrogen sulphite ($NaHSO_3$), L-ascorbic acid, L-cysteine, L-glutathione (GSH), dithiothreitol (DTT) and allyl isothiocyanate were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultra-high-performance liquid chromatography/mass spectrometry (UHPLC/MS) grade acetonitrile (ACN) was obtained from Biosolve BV (Valkenswaard, The Netherlands). Water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA).

PURIFICATION OF MUSHROOM TYROSINASE.
Commercial mushroom tyrosinase was purified by a single gel filtration step (*20*). A HiLoad 26/60 Superdex 200 column connected to an Akta Explorer system (GE Healthcare, Uppsala, Sweden) was used. A total of 50 mg of the commercial enzyme [dissolved in 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer pH 6.8] was loaded and eluted with 50 mM HEPES buffer at pH 6.8 at 4 mL/min. Fractions (5 mL) were collected, and activity was assayed by a spectrophotometric assay: 50 μL of each fraction was combined with 100 μL 0.8 mM tyrosine in a 96-well plate, and absorbance at 520 nm was monitored in time. Active fractions were pooled and stored at -20 °C until use. Tyrosinase activity was expressed in units, according to the suppliers definition (1 unit increases the A_{280} by 0.001 min^{-1} with L-tyrosine as substrate, at pH 6.5 and 25 °C).

INCUBATION OF CHA AND TYROSINASE WITH BROWNING INHIBITORS.
ChA (0.1 mM), sulfur-containing compounds (0.2 mM) or ascorbic acid (0.2 mM), and tyrosinase (7 units/mL) were mixed in 10 mM HEPES buffer pH 6.5. Samples were incubated at room temperature for 1 h, centrifuged (10,000 × g; 5 min; room temperature), and analyzed by RP-UHPLC. For spectrophotometric analysis, the same reaction mixtures were incubated in quartz cuvettes in a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Wavelength scans were made from 200 to 600 nm. A scan was made every min, for a total of 60 min.

RP-UHPLC ANALYSIS.
Samples were analysed on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler and a diode array detector (DAD). Samples (5 μL) were injected onto a Hypersil Gold column (2.1 × 150 mm, particle size 1.9 μm; Thermo Scientific). Water acidified with 0.1% (v/v) acetic acid, eluent A, and ACN acidified with 0.1% (v/v) acetic acid, eluent B, were used as eluents. The flow rate was 400

μL/min, and the column oven temperature was controlled at 30 °C. The DAD was set to measure the range 200-600 nm. The following elution profile was used: 0-1 min, isocratic on 0% (v/v) B; 1-6 min, linear gradient from 0% to 35% (v/v) B; 6-7 min, linear gradient from 35% to 100% (v/v) B; 7-9 min, isocratic on 100% (v/v) B; 9-10 min, linear gradient from 100% to 0% (v/v) B; and 10-12 min, isocratic on 0% (v/v) B.

ELECTROSPRAY IONIZATION-MASS SPECTROMETRY (ESI-MS).

Mass spectrometric data were obtained by analyzing samples on a LTQ-XL (Thermo Scientific) equipped with an ESI probe coupled to the RP-UHPLC system. Nitrogen was used as sheath gas and auxiliary gas. Data were collected over the *m/z* range 150-1,500. Data-dependent MS^n analysis was performed with a normalized collision energy of 35%. The MS^n fragmentation was performed on the most intense product ion in the MS^{n-1} spectrum. Most settings were optimized via automatic tuning using 'Tune Plus' (Xcalibur 2.07, Thermo Scientific). The system was auto-tuned with ChA in negative ionization (NI) mode. The transfer tube temperature was 350 °C, and the source voltage 3.5 kV. Data acquisition and reprocessing were done with Xcalibur 2.07 (Thermo Scientific).

PREPARATIVE HPLC PURIFICATION OF SULFOCHA.

Samples of ChA (1 mM) incubated with $NaHSO_3$ (2 mM) and tyrosinase (70 units/mL) were fractionated on a preparative HPLC system (Waters, Milford, MA, USA), using a semi-preparative Hypersil Gold column (20 × 150 mm, particle size 5 μm; Thermo Scientific) with a Hypersil guard column (20 × 20 mm, particle size 5 μm; Thermo Scientific). Water acidified with 0.2% (v/v) acetic acid (eluent A) and ACN acidified with 0.2% (v/v) acetic acid (eluent B) were used as eluents. The flow rate was 30 mL/min. The following elution profile was used: 0-2 min, isocratic on 2% (v/v) B; 2-17 min, linear gradient from 2 to10% (v/v) B; 17-20 min, linear gradient from 10 to100% (v/v) B; 20-25 min, isocratic on 100% (v/v) B; 25-27 min, linear gradient from 100 to 2% (v/v) B; and 27-37 min, isocratic on 2% (v/v) B. Fractions (14 mL) were collected and pooled based on UV response at 320 nm. The major reaction product was found to be 2'-SO_3H-ChA (*11*), with a purity of 94% (based on peak integration at 320 nm), which was in accordance with the MS base peak chromatogram.

PREPARATIVE PURIFICATION OF CYSTEINYL-CHA USING FLASH CHROMATOGRAPHY.

Samples of ChA (1 mM) incubated with cysteine (2 mM) and tyrosinase (70 units/mL) were fractionated on a flash chromatography system (Grace, Deerfield, IL, USA) using a Reveleris C18 flash cartridge (Grace). Water acidified with 0.1% (v/v) acetic acid (eluent A) and ACN acidified with 0.1% (v/v) acetic acid (eluent B) were used as eluents. The cartridge was equilibrated with 5 column volumes of B, followed by 5 column volumes of A. The following elution profile was used: 0-10 min, linear gradient from 0 to 40% (v/v) B;

10-14 min, linear gradient from 40 to100% (v/v) B; and 14-16 min, isocratic on 100% (v/v) B. Fractions (25 mL) were collected and pooled based on UV response at 320 nm. The major reaction product was found to be 2'-*S*-cysteinyl-ChA (*12*), with a purity of 95% (on the basis of peak integration at 320 nm), which was in accordance with the MS base peak chromatogram.

OXYGEN CONSUMPTION MEASUREMENTS.

Oxygen consumption of tyrosinase was measured using an Oxytherm System (Hansatech, Kings Lynn, UK). Incubations with ChA (0.5 mM) and tyrosinase (35 U/mL) with or without sulfur-containing compound (1 mM) or ascorbic acid (1 mM) were done in a total volume of 1 mL 10 mM HEPES buffer at pH 6.5 and 25 °C. Data acquisition and analysis were performed using Oxygraph Plus software (Hansatech).

In case of pre-incubation experiments, tyrosinase (35 units/mL) and sulfur-containing compounds (1 mM) were incubated for different times (specified in the text) at 25 °C, after which concentrated ChA (50 mM) was added to reach a final concentration of 0.5 mM.

Results and Discussion

***IN VITRO* BROWNING OF CHA BY TYROSINASE.**

To investigate the effect of different sulfur-containing compounds on ChA conversion by tyrosinase, the reaction products of the *in vitro* browning of ChA by tyrosinase were analyzed. The formation of reaction products was monitored by both RP-UHPLC-DAD-MS analysis and recording absorption spectra of the reaction mixture at different time intervals (**Figure 2**). Results were compared to those obtained in the presence of ascorbic acid, which was used as a reference anti-browning agent.

After incubation the absorption spectra of a mixture of ChA and tyrosinase (**Figure 2A**) showed a clear decrease in absorbance at the typical maximum for ChA, 326 nm. New maxima at 250 nm and around 400 nm appeared, the latter being responsible for the brown color observed after the reaction. The corresponding RP-UHPLC-DAD trace (**Figure 2F**) showed that different reaction products were formed. Based on the rise of the baseline, it seemed that a multitude of compounds eluted between 4.5 and 6.5 min, with three clear peaks standing out (peaks **1, 2** and **3**). Peak **1** was identified as ChA, both based on MS analysis (**Table 1**) and by comparing to RP-UHPLC analysis of the authentic standard. MS analysis of peak **2** revealed an *m/z* of 351, corresponding to a mass of 352, which might represent the mass of the *o*-quinone of ChA, an expected reaction product of ChA and tyrosinase (*21*). MS^2 analysis yielded *m/z* 215 as the most abundant product ion, which could not be explained. Two less abundant product ions were *m/z* 177 and *m/z* 133,

which might correspond to the fragments *m/z* 179 and *m/z* 135, respectively, found in MS^2 of ChA, taking into account the loss of two protons upon oxidation of ChA. Peak **3** had *m/z* 705, and MS^2 resulted in a predominant fragment with *m/z* 513, the MS^3 fragmentation of which resulted in a major product ion of *m/z* 339. Dimers of ChA, resulting from oxidation by tyrosinase, showing the same MS fragmentation have been described before (*22*), and were also found in oxidized apple extracts (*23*). Based on these data, compounds **2** and **3** (**Table 1**) were tentatively identified as the *o*-quinone and dimer of ChA, respectively.

Table 1. Identification of reaction products of ChA, $NaHSO_3$, cysteine, GSH and tyrosinase.

No	RT (min)	$[M-H]^-$ (*m/z*)	MS^n fragments (% relative abundance) (*m/z*)	UV λ_{max} (nm)	Tentative identification
1	5.11	353	MS^2 [353]: 191 (100), 179 (4), 135 (1)	326	5-*O*-caffeoylquinic acid (ChA)
2	4.75	351	MS^2 [351]: 215 (100), 177 (15), 195 (8), 133 (6), 307 (6), 173 (5), 123 (5), 261 (2), 155 (2), 149 (2), 191 (1)	251, 300	ChA quinone
3	5.61	705	MS^2 [705]: 513 (100) MS^3 [705 → 513]: 339 (100), 321 (10), 496 (7), 295 (2)	323	ChA dimer
4	4.45	433	MS^2 [433]: 259 (100), 415 (47), 215 (38), 241 (23), 387 (17), 161 (16) 301 (7)	240, 328	2'-sulfo-5-*O*-caffeoylquinic acid (2'-SO_3H-ChA)
5	1.77	433	MS^2 [433]: 353 (100), 191 (30), 241 (24), 161 (13), 415 (7), 271 (4), 179 (1) MS^3 [433 → 353]: 191 (100), 179 (6), 135 (1)	244, 302	Sulfo-caffeoylquinic acid isomer
6	3.16	433	MS^2 [433]: 241 (100), 415 (93), 387 (11), 259 (9), 213 (7), 416 (4), 433 (3), 242 (3), 161 (1), 133 (1) MS^3 [433 → 241]: 213 (100), 241 (56), 161 (5), 133 (3)	296, 324	Sulfo-caffeoylquinic acid isomer
7	4.09	433	MS^2 [433]: 259 (100), 415 (37), 215 (33), 241 (20), 387 (18), 161 (12), 301 (11)	315	Sulfo-caffeoylquinic acid isomer
8	4.49	472	MS^2 [472]: 385 (100), 193 (18), 191 (17), 236 (12), 280 (3), 351 (2), 298 (2), 167 (2) MS^3 [472 → 385]: 191 (100), 193 (89), 211 (1)	250, 326	2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid
9	4.57	658	MS^2 [658]: 385 (100), 466 (55), 272 (24), 193 (13), 529 (12), 191 (11), 448 (4), 379 (4), 254 (4), 337 (2), 211 (2), 340 (1), 306 (1), 293 (1), 210 (1) MS^3 [658 → 385]: 191 (100), 193 (94), 211 (1)	252, 325	2'-*S*-glutathionyl-5-*O*-caffeoylquinic acid

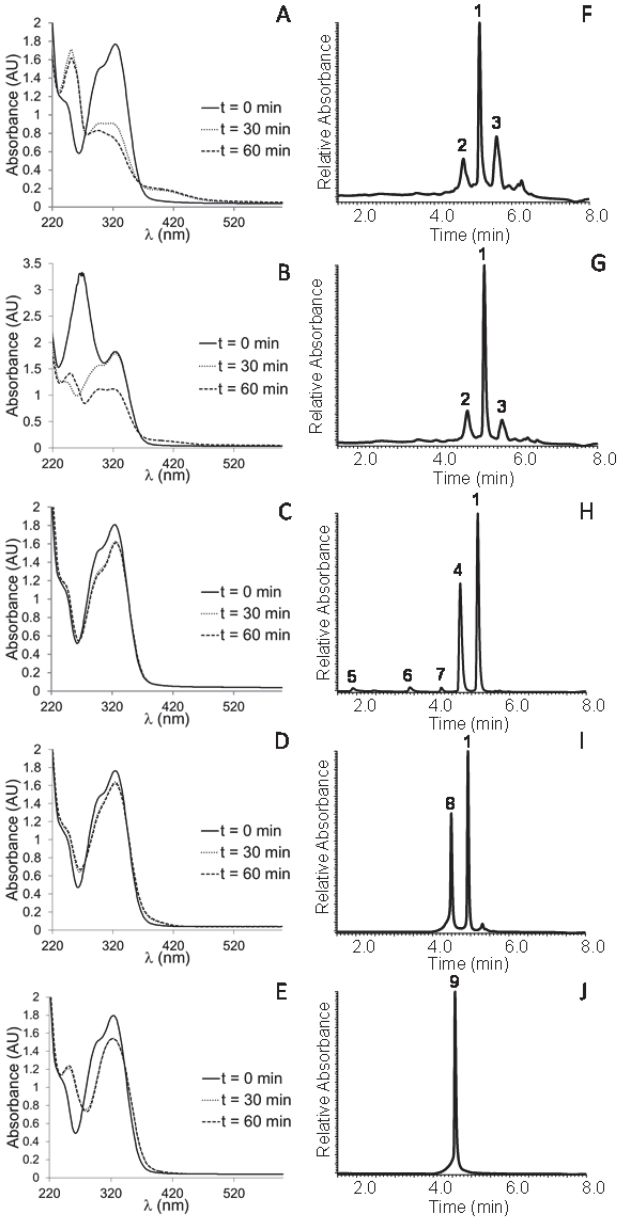

Figure 2. Absorption spectra of ChA (0.1 mM) incubated with tyrosinase (7 units/mL) with and without potential browning inhibitors (**A** to **E**) at different incubation times and RP-UHPLC-DAD traces (320 nm) of the reaction mixtures after incubation (**F** to **J**): (**A** and **F**), ChA with tyrosinase alone; (**B** and **G**), ChA with tyrosinase and ascorbic acid (0.2 mM); (**C** and **H**), ChA with tyrosinase and $NaHSO_3$ (0.2 mM); (**D** and **I**), ChA with tyrosinase and cysteine (0.2 mM); and (**E** and **J**), ChA with tyrosinase and GSH (0.2 mM).

The absorption spectra of ChA and tyrosinase incubated in the presence of ascorbic acid (**Figure 2B**) showed that ascorbic acid delays enzymatic browning. At time zero, besides the typical 326 nm maximum for ChA, also a maximum at 265 nm was observed, caused by the ascorbic acid present. After 30 min of incubation, this maximum disappeared, whereas the ChA maximum remained. After 60 min of incubation, the spectrum was similar to the spectrum of ChA incubated with tyrosinase after 30 and 60 min: a decrease in absorbance at 326 nm, and new maxima formed at 250 nm and around 400 nm. The RP-UHPLC-DAD trace (**Figure 2G**) was similar to that of ChA incubated with tyrosinase alone (**Figure 2F**). The same compounds were present, only the ratio between them differing. These results confirmed that ascorbic acid delays browning.

EFFECT OF DIFFERENT SULFUR-CONTAINING COMPOUNDS ON CHA CONVERSION BY TYROSINASE.

To investigate the influence of different sulfur-containing compounds on tyrosinase-catalyzed browning of ChA, combinations of ChA, tyrosinase, and $NaHSO_3$, cysteine, GSH, DTT, or allyl isothiocyanate were incubated. Formation of reaction products was monitored as described above.

The absorption spectra of ChA did not change substantially upon incubation with tyrosinase and $NaHSO_3$ (**Figure 2C**). After 30 and 60 min a small decrease in absorption at 326 nm was observed, with no new maxima formed. RP-UHPLC-DAD analysis resulted in five peaks (**Figure 2F**). Besides ChA (compound **1**), four reaction products were present, which were identified by MS analysis (**Table 1**). Comparing the product ions found for compounds **4-7** with published MS fragmentation data for ChA (*24-26*), the fragments diagnostic for ChA were observed, only with an increase of 80 a.m.u.. The product ions with *m/z* 259, 241 and 215 corresponded to product ions with *m/z* 179, 161 and 135, respectively. According to the fragmentation pattern of ChA, these three product ions all contained the phenolic ring of the caffeic acid moiety of ChA, indicating that the addition of HSO_3^- occurred on this phenolic ring. Compounds **4-7** were tentatively identified as different sulfo-caffeoyl quinic acid isomers, with the main reaction product being 2'-sulfo-5-*O*-caffeoylquinic acid (2'-SO_3H-ChA) (*11*).

For the incubation of ChA with tyrosinase and cysteine, the absorption spectra also did not change substantially (**Figure 2D**). After incubation, a small decrease in absorption at 326 nm and a small increase in absorption at 260 nm were observed. RP-UHPLC-DAD analysis revealed two major peaks (**Figure 2I**). Besides ChA, a new peak was observed (compound **8**). MS analysis of compound **8** revealed a mass of 473, equal to the combined masses of ChA (354) and cysteine (121), minus 2 for the two hydrogen atoms lost due to the formation of a covalent bond between cysteine and ChA. Upon fragmentation of compound **8**, a product ion with *m/z* 385 was predominantly formed. The occurrence of this product can be explained by the loss of part of the cysteine moiety, with the sulfur of

cysteine remaining bound to ChA. MS^3 analysis of this fragment resulted in fragments with *m/z* 191 and 193, which could be explained as the quinic acid and dehydrated caffeoyl moiety with sulfur attached, respectively. Similar fragmentation has been observed for addition products of ChA and *N*-acetyl-cysteine (*22*). An addition product of cysteine and ChA has been identified as 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid (*12*). Based on these data, compound **8** was tentatively identified as 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid.

When incubated with tyrosinase and GSH, the absorption spectra of ChA showed a small decrease in absorption at 326 nm and a bathochromic shift for the minimum observed at 260 nm before incubation to 275 nm after incubation (**Figure 2E**). RP-UHPLC-DAD analysis revealed total depletion of ChA with formation of a single reaction product, compound **9** (**Figure 2J**). MS analysis of compound **9** resulted in a mass of 659, corresponding to a covalent addition product of ChA and GSH (354 + 307 – 2). MS^2 and MS^3 analysis resulted in the same fragments as described above for the fragmentation of 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid. On the basis of this and previous data (*15, 27*), compound **9** was tentatively identified as 2'-*S*-glutathionyl-5-*O*-caffeoylquinic acid.

Incubation of ChA with tyrosinase and DTT resulted in total inhibition of ChA conversion: The absorption spectra after 30 and 60 min were the same as at 0 min, and RP-UHPLC analysis showed that only ChA was present after incubation (See panels **A** and **C** of **Figure S1** of the **Supporting Information**). Complete inhibition of tyrosinase by DTT was found before (*18*), while also adduct formation between *o*-quinones and DTT was observed (*17*). Interestingly, our results did not indicate such adduct formation. Possibly, adduct formation is concentration dependent: at relative high concentrations of DTT tyrosinase is strongly inhibited, while at lower concentrations tyrosinase remains (partly) active, making *o*-quinones available for addition of DTT.

Allyl isothiocyanate seemed to have little influence on ChA conversion, both absorption spectra and UHPLC-DAD trace (See panels **B** and **D** of **Figure S1** of the **Supporting Information**) were similar to the ones obtained with the control experiment of only ChA and tyrosinase (Panels **A** and **F** of **Figure 2**).

INFLUENCE OF SULFUR-CONTAINING COMPOUNDS ON BROWN COLOR FORMATION AND OXYGEN CONSUMPTION.

The rate of color formation and oxygen consumption during incubations of ChA with tyrosinase and different potential anti-browning agents was determined by monitoring the absorbance at 400 nm and the oxygen consumption in time (**Figure 3**). It can be seen that there is not always a correlation between these parameters. While incubations with cysteine and GSH showed oxygen consumption rates comparable to the control experiment of only ChA and tyrosinase, they showed much less color development. The reduced color formation corresponded to the formation of 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid and 2'-*S*-glutathionyl-5-*O*-caffeoylquinic acid, respectively (Panels **I** and **J** of **Figure 2**). Ascorbic

acid showed a higher oxygen consumption compared to the control experiment, while the onset of color formation in the presence of ascorbic acid was delayed. This confirmed the mechanism of browning inhibition by ascorbic acid, as described before. Incubation with allyl isothiocyanate resulted in both slightly reduced oxygen consumption and slightly reduced brown coloration. DTT seemed to prevent browning by inhibiting tyrosinase activity: both oxygen consumption and brown color formation were totally inhibited at the concentration used. This corresponds well with the absorption spectra and UHPLC-MS analysis of the incubation of ChA with tyrosinase and DTT (See panels A and C of **Figure S1** of the **Supporting Information**), where no conversion of ChA was observed. When ChA was incubated with tyrosinase and $NaHSO_3$, it was observed that oxygen consumption leveled off during the experiment, to a plateau value of around 75% of the starting concentration of oxygen. This indicated that tyrosinase activity was somehow lost during the course of the incubation. In the presence of $NaHSO_3$, no color development was observed, consistent with the spectra in **Figure 2C**.

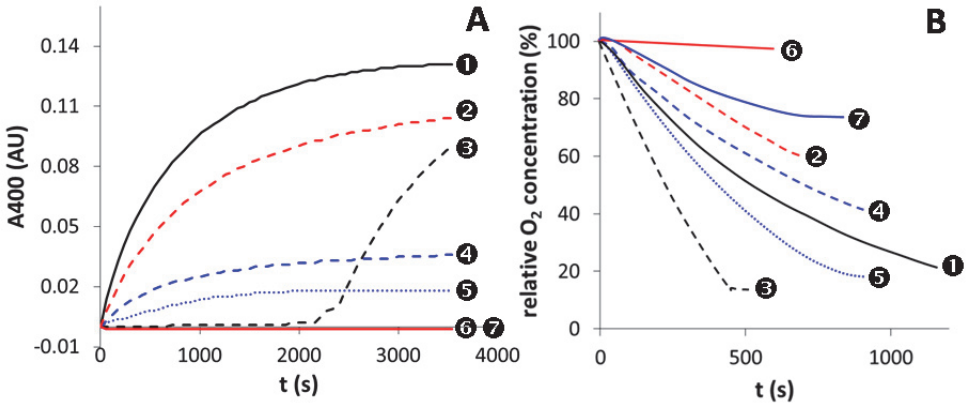

Figure 3. Development of brown color measured by (**A**) monitoring absorbance at 400 nm and (**B**) monitoring of oxygen consumption in time during incubations of ChA (A, 0.1 mM; B, 0.5 mM) with tyrosinase (A, 7 units/mL; B, 35 units/mL), in the presence of different sulfur-containing compounds and ascorbic acid (A, 0.2 mM; B, 1 mM). ❶, control; ❷, allyl isothiocyanate; ❸, ascorbic acid; ❹, cysteine; ❺, GSH; ❻, DTT; ❼, $NaHSO_3$.

In contrast to incubations of ChA and tyrosinase with $NaHSO_3$, those with cysteine and GSH resulted in some browning. Comparing the UHPLC-DAD absorption spectra of the different addition products and ChA, it was found that 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid and 2'-*S*-glutathionyl-5-*O*-caffeoylquinic acid had a higher absorbance around 400 nm compared to ChA, while that for 2'-SO_3H-ChA was comparable to that of ChA (**Figure 4**). Although the absorbance at 400 nm of 2'-*S*-glutathionyl-5-*O*-caffeoylquinic acid is higher than that of 2'-*S*-cysteinyl-5-O-caffeoylquinic acid, for the

total reaction mixtures with these compounds one observes the opposite (**Figure 3A**). This suggests that the absorbance of the cysteine addition product only partially explains the observed browning. A possible explanation might be that some of the *o*-quinones formed reacted further into brown pigments prior to reaction with cysteine.

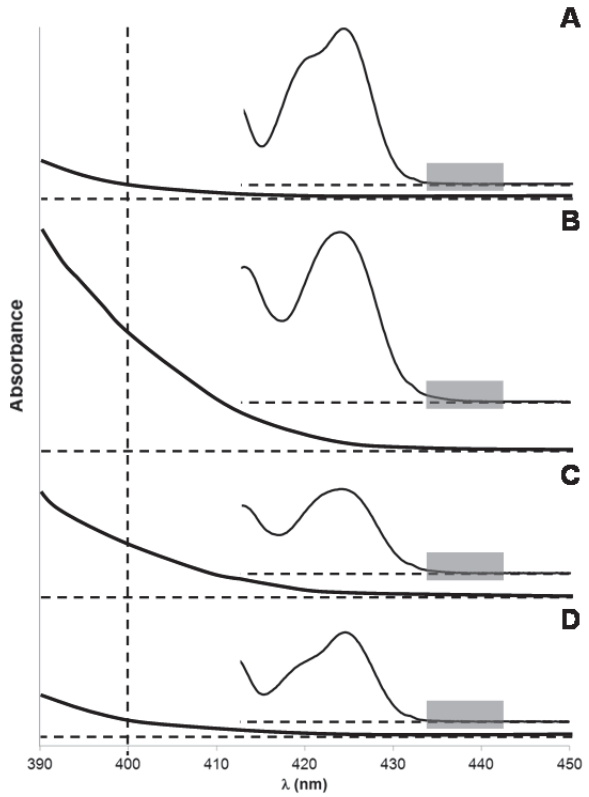

Figure 4. UHPLC-DAD absorption spectra of (**A**) ChA, (**B**) 2'-*S*-glutathionyl-5-*O*-caffeoylquinic acid, (**C**) 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid, and (**D**) 2'-SO_3H-5-*O*-caffeoylquinic acid. Absorption spectra were derived from peaks **1**, **9**, **8** and **4** (**Figure 2**), respectively. Insets show spectra of 250-500 nm, and the grey windows indicate the wavelength range (390-450 nm) of the zoom.

REDUCTION OF TYROSINASE ACTIVITY DURING FORMATION OF SULFOCHA.

$NaHSO_3$ showed a distinctly different effect compared to the other inhibitors investigated: no brown color formation was observed and oxygen consumption leveled off during incubation with ChA and tyrosinase. This observation was investigated further by monitoring the amount of ChA converted by tyrosinase in time with or without $NaHSO_3$ using RP-UHPLC (See **Figure S2** of the **Supporting Information**). It was found that, with $NaHSO_3$ present, not all ChA was converted, consistent with the observation that less

oxygen was consumed when ChA was incubated in combination with $NaHSO_3$ and tyrosinase, compared to an incubation of ChA and tyrosinase alone. The addition of a second dose of tyrosinase restored oxygen consumption and ChA conversion (**Figure 5A**). Markakis and Embs (*28*) found a similar effect of $NaHSO_3$ when following the activity of mushroom tyrosinase in a reaction mixture containing tyrosine and $NaHSO_3$.

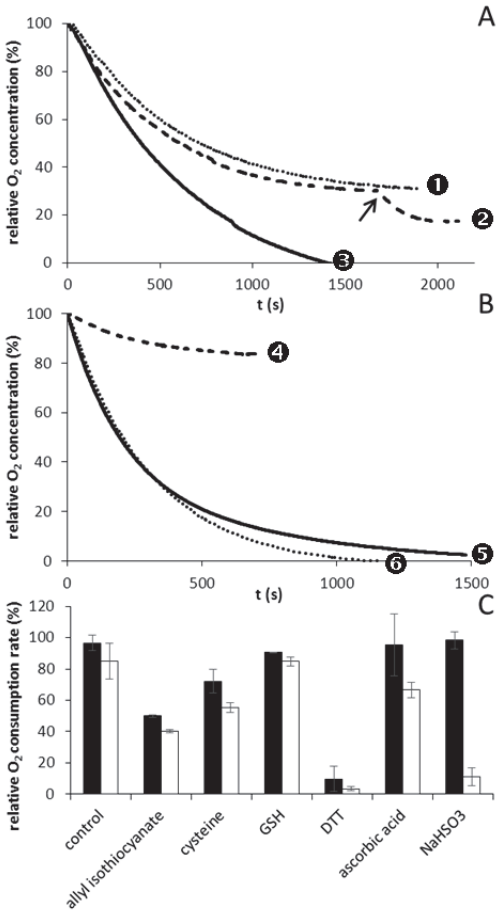

Figure 5. Oxygen consumption in time upon (**A**) incubation of ChA (0.5 mM) and tyrosinase (35 units/mL) with and without $NaHSO_3$ (1 mM), (**B**) incubation of ChA (0.5 mM) with 2'-SO_3H-ChA (0.5 mM) and ChA (0.5 mM) with tyrosinase pre-incubated with $NaHSO_3$ (1 mM, 1 h, 25 °C), and (**C**) relative O_2 consumption rate obtained when incubating ChA (0.5 mM) with different potential browning inhibitors (1 mM) and tyrosinase (35 units/mL), which was either pre-incubated with the potential browning inhibitor (15 min, 25 °C; white bars) or not pre-incubated (black bars). ❶, ChA + $NaHSO_3$ + tyrosinase (single addition); ❷, ChA + $NaHSO_3$ + tyrosinase (double addition); ❸, ChA + tyrosinase; ❹, ChA + pre-incubated tyrosinase with $NaHSO_3$; ❺, ChA + tyrosinase; ❻, ChA + 2'-SO_3H-ChA + tyrosinase. The arrow indicates the second tyrosinase addition.

The fact that a second addition of tyrosinase was necessary to convert all ChA in the presence of $NaHSO_3$ indicated that tyrosinase was somehow inhibited in the course of the reaction. Two explanations for the observed enzyme inhibition might be: (i) $NaHSO_3$ inhibited tyrosinase directly in a rather slow, time-dependent manner or (ii) the 2'-SO_3H-ChA formed inhibited the enzyme. To investigate these possible scenarios of inhibition, 2'-SO_3H-ChA was purified from a reaction mixture in order to test its influence on tyrosinase activity.

The addition of 0.5 mM 2'-SO_3H-ChA to an incubation of ChA with tyrosinase did not influence the rate of oxygen consumption (**Figure 5B**), showing that 2'-SO_3H-ChA was not a tyrosinase inhibitor. This is in contrast with addition products of cysteine and ChA, which were found to be competitive inhibitors for PPO from apple (*13*). Based on the observations with apple PPO, 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid was purified from a reaction mixture containing ChA, cysteine and mushroom tyrosinase. Addition of 0.5 mM purified 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid to an incubation of ChA and tyrosinase did not result in tyrosinase inhibition (results not shown). Thus, 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid does not inhibit mushroom tyrosinase, similar to 2'-SO_3H-ChA. The fact that 2'-*S*-cysteinyl-5-*O*-caffeoylquinic acid was found before to inhibit apple PPO (*13*) might be explained by the different origin of the two enzymes.

To investigate whether $NaHSO_3$ inhibits the enzymatic activity of tyrosinase in time, tyrosinase was pre-incubated with $NaHSO_3$ (1 h). Oxygen consumption measurements with this pre-incubated tyrosinase showed a decreased activity compared to its control without prior pre-incubation with $NaHSO_3$ (**Figure 5B**). This experiment indicated that scenario (i) is most likely. To establish the time dependency of tyrosinase inhibition by $NaHSO_3$, tyrosinase was pre-incubated with $NaHSO_3$ for different times, after which a concentrated substrate solution was added. The initial reaction rate was determined by measuring the oxygen consumption rate (See F**igure S3** of the **Supporintg Information**). It was found that the reaction rate rapidly decreased with an increasing pre-incubation time: already after 1 min, approximately 50% of activity was lost. After 15 min of pre-incubation, activity decreased further to approximately 10% of the initial activity. Sayavedra-Soto and Montgomery (*6*) found that pre-incubation of pear PPO with sulfite resulted in irreversible inhibition of the enzyme: indications for modification of the protein structure were found, although the nature of the modifications was not established.

The effects of pre-incubation of tyrosinase with the other sulfur-containing compounds tested in this study were also investigated. Oxygen consumption with and without pre-incubation of tyrosinase was determined and expressed relative to a control experiment using untreated tyrosinase. Pre-incubation of tyrosinase with other sulfur-containing compounds had little effect on tyrosinase activity (**Figure 5C**).

$NaHSO_3$ HAS A DUAL INHIBITORY EFFECT ON TYROSINASE-CATALYZED BROWNING.

In conclusion, our results show that different sulfur-containing compounds can inhibit *in vitro* browning of ChA by mushroom tyrosinase in two different ways: by inhibition of enzymatic activity ($NaHSO_3$, DTT) or by formation of colorless adducts with enzymatically formed *o*-quinones ($NaHSO_3$, cysteine, GSH). A schematic representation of enzymatic browning of ChA and possible inhibitory routes is shown in **Figure 6**. It is evident that $NaHSO_3$ has a unique position within the group of sulfur-containing compounds investigated: it has a dual inhibitory effect on tyrosinase-catalyzed browning of ChA. Initially, the formation of brown pigments is inhibited by formation of sulfo-ChA, while at the same time tyrosinase is inhibited in a time-dependent way. The exact mechanism of the time-dependent inhibition of tyrosinase by $NaHSO_3$ remains unclear. It is possibly due to covalent interactions between $NaHSO_3$ and tyrosinase. This will be subject of further investigation.

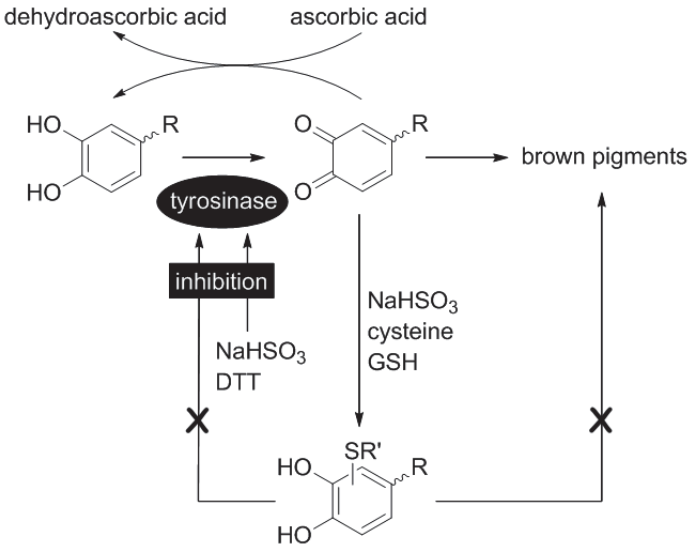

Figure 6. Schematic representation of the action of tyrosinase on ChA, together with possible mechanisms of inhibition of ChA browning.

Acknowledgements

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acronym SO2SAY, coordinated by TTZ Bremerhaven. This work was partly supported by COLCIENCIAS and Universidad Nacional de Colombia by providing a fellowship for Carlos-Eduardo Narváez-Cuenca.

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Supporting information

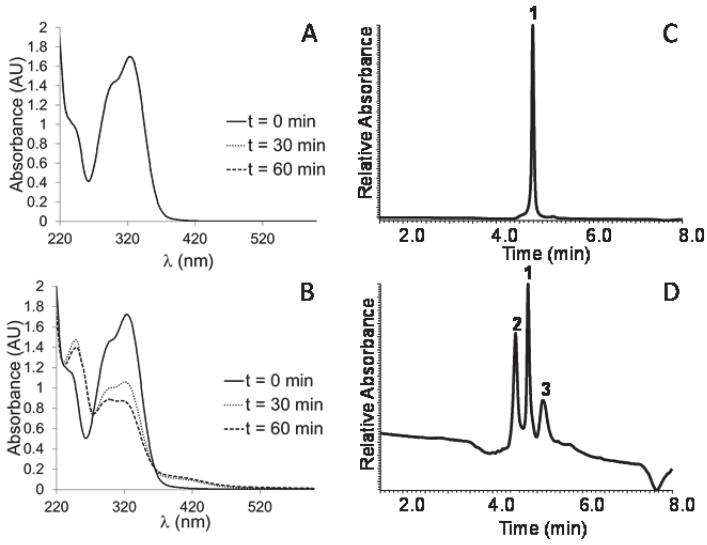

Figure S1. Absorption spectra of ChA (0.1 mM) incubated with tyrosinase (7 units/mL) with different sulfur-containing compounds (**A** and **B**) at different incubation times and RP-UHPLC-DAD traces (320 nm) of the reaction mixtures after incubation (**C** and **D**). (**A** and **C**) ChA with tyrosinase and DTT (0.2 mM); (**B** and **D**) ChA with tyrosinase and allyl isothiocyanate (0.2 mM).

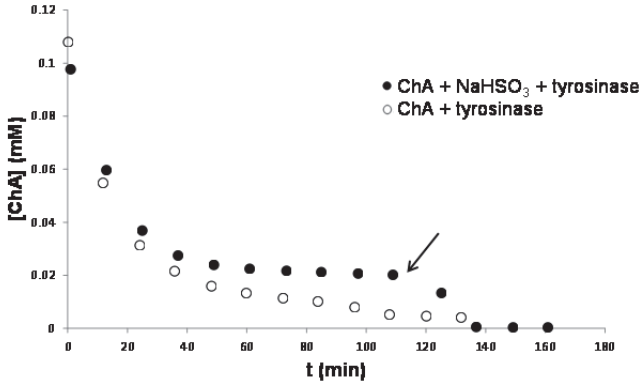

Figure S2. Decrease in concentration of ChA in time when incubated with tyrosinase with or without $NaHSO_3$, determined by RP-UHPLC-DAD analysis. The arrow indicates the second addition of tyrosinase.

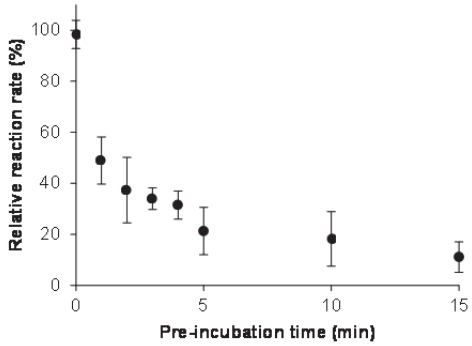

Figure S3. Relative oxygen consumption rate of incubations of ChA (0.5 mM) with tyrosinase (35 units/mL) pre-incubated with $NaHSO_3$ (1 mM) for different times. Data points represent duplicate measurements, error bars indicate standard deviations.

Chapter 6

Quantitative fate of chlorogenic acid during enzymatic browning of potato juice

ABSTRACT: The quantitative fate of chlorogenic acid (ChA) during enzymatic browning of potato juice was investigated. Potato juice was prepared in water without the use of any anti-browning agent (*OX* treatment). As a control, a potato juice was prepared in the presence of $NaHSO_3$ (*S* control). To study the composition of phenolic compounds in potato in their native states, also a potato extract was made with 50% (v/v) methanol containing 0.5% (v/v) acetic acid (*MeOH* control). Water-soluble low molecular weight fractions (LMWFs), and high molecular weight fractions (HMWFs), from *S* and *OX* extracts were obtained by ultrafiltration and dialysis, respectively. Pellets obtained after the *OX* treatment and the *S* and *MeOH* controls were also analysed for ChA content. While in the *S*-LMWF all ChA was converted to sulfonic acid adducts, no free ChA was found in the *OX*-LMWF, indicating its high reactivity upon enzymatic browning. Analysis of protein in the HMWFs showed a higher content of reacted ChA in *OX* (49.8±7.1 mg ChA/100 g potato DW) than in *S* (14.4±1.5 mg ChA/100 g potato DW), as evidenced by quinic acid release upon alkaline hydrolysis. The presence of quinic acid in *S*-HMWF was unexpected, but a mass balance incorporating the ChA content of LMWF, HMWF and pellet for the treatment and the two controls suggested that ChA might have been attached to polymeric material, soluble in the aqueous environment of *S*, but not in that of *MeOH*. Size exclusion chromatography, combined with proteolysis, revealed that ChA reacted with patatin and protease inhibitors to produce brown soluble complexes.

Based on: Narváez-Cuenca, C.-E.; Vincken, J.-P; Gruppen, H. Quantitative fate of chlorogenic acid during enzymatic browning of potato juice. Submitted.

Introduction

Enzymatic browning is associated with the oxidation of phenolics, such as hydroxycinnamic acids (HCAs) and their conjugates (HCAcs), to yield quinones, by the action of polyphenoloxidase (PPO) or peroxidase/H_2O_2 (*1-7*). Chlorogenic acid (ChA) and its isomers, *neo*-ChA and *crypto*-ChA, are the most abundant HCAcs in potato, their amounts commonly ranging from 23 to 350 mg/100 g dry weight, depending on the cultivar (*8-10*). ChA consists of a caffeic acid esterified to the C-5 OH group of a quinic acid moiety. Dimers and, to a lesser extent, trimers of HCAs/HCAcs as well as conjugates comprising up to two HCAs/HCAcs and up to two amino acid moieties have been reported as low molecular weight oxidation products. Some of these had absorption in the visible light spectrum (*1, 3-7*). High molecular weight conjugates of HCAs/HCAcs and proteins have been reported as well, such as between ChA and proteins, sometimes leading to cross-linking of proteins (*5, 6*). When soy proteins were modified by oxidized caffeic acid or oxidized ChA, an increase in the absorption from 250 to 500 nm was observed compared to unmodified proteins, with the absorption at 400-500 nm being indicative of brown color (*7*). To our knowledge, only a few attempts have been made towards the identification of oxidation products upon enzymatic browning of fruits or vegetables, e.g. the study of low molecular weight oxidation products in cider apple juice (*11*). In the work cited, several isomers of dimers of ChA, as well as ChA-catechin and ChA-procyanidin B2 conjugates, were found as oxidation products.

So far, studies on enzymatic browning have not focused on the fate of major phenolics in complex mixtures of proteins and phenolics during fruit or vegetable processing. Neither information on possible changes in the chemical identity of phenolics, nor information on their quantities, including their presence in insoluble fractions, is available. Hence, it is not known to what extent the oxidized phenolics react with proteins, with free amino acids, with other phenolics, or with themselves. The aim of the present research was to follow the quantitative fate of ChA when potato tubers were homogenized with water, without the use of any anti-browning agent. In this respect, the occurrence of ChA in a modified form after processing was studied. Low molecular weight oxidation products as well as high molecular weight oxidation products were investigated in the oxidized potato extract. A potato tuber extract prepared in the presence of sodium hydrogen sulfite, known to inhibit browning by formation of sulfo-*o*-diphenolics and by direct inhibition of PPO (*12, 13*), served as a control. To assess the parental composition of phenolic compounds in non-oxidized potato, without formation of sulfonic acid derivatives, an aqueous methanolic extract was prepared as well (*14*).

Materials and Methods

CHEMICALS.

Chlorogenic acid (ChA), ferulic acid, sinapic acid, tyrosine, quinic acid, 37% (v/v) HCl, $NaHSO_3$, α-amylase (from *Bacillus licheniformis*) and amyloglucosidase (from *Rhizopus* sp.) were purchased from Sigma-Aldrich (Steinheim, Germany). Proteinase K (number 92905, 825 units/mg) was from Fluka (Steinheim, Germany). UHPLC/MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were from Merck (Darmstadt, Germany).

PLANT MATERIAL.

Three batches of potato tubers (Nicola cultivar) were purchased in January 2012 from local supermarkets in Wageningen, The Netherlands. Before further processing potato tubers were washed under tap water and dried with paper tissue. Part of the potato tubers was freeze-dried.

PREPARATION OF BROWN POTATO EXTRACT AND FRACTIONATION.

To study the reaction products, potato extract was prepared in water (*OX* extract). The procedure, schematically, is shown in **Figure 1**. Fresh potato tubers (200 g) were diced (0.5-1 cm thick) and immediately homogenized in a household blender with 200 mL of water. Subsequently, the mixture was stirred for 10 min at 4 ºC. Next, starch and fibers were left to settle for 10 min at 4 ºC. After decanting, the solution was centrifuged (37,000 g; 30 min; 4 ºC). The pellet was extracted three times with 100 mL of water. The four supernatants were combined and subsequently filtered through a 0.45 μm filter (Whatman, Schleicher & Schuell, Dassel, Germany). The filtrate (pH 6.0) represented the *OX* extract, which was immediately processed. Part of the *OX* extract (20 mL) was processed to obtain a low molecular weight fraction (LMWF), while the remainder was used to obtain a high molecular weight fraction (HMWF). The remaining pellet after 4 extractions and centrifugations, rich in starch, was freeze dried, ground with a mortar and pestle, and kept at -20 °C until further analysis.

Preparation of LMWF involved the adjustment of the pH of the 20 mL-aliquot to 3.5 by adding glacial acetic acid. The aliquot was left overnight at 4 ºC. The resulting material was centrifuged (9,000 g; 30 min; 4 ºC) and the supernatant was filtered through a 0.45 μm filter (Whatman, Schleicher & Schuell). Remaining soluble proteins were retained by ultrafiltration using regenerated cellulose centrifugal filter units (Millipore, Amicon ultracel YM-10, 15 mL, cut-off 10 kDa, Bedford, MA, USA). The filtrate was denoted LMWF. Afterwards, 10 mL of the LMWF were applied onto an activated Sep-Pak cartridge

(C18, 6 mL/1 g, Waters, Milford, MA, USA). Next, the cartridge was washed with 5 mL of MQ water and the compounds retained were eluted with 5 mL of methanol. The methanolic fraction was concentrated using a Savant ISS-110 SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature under reduced pressure. The final volume was adjusted to 500 µL with 50% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid. The concentrated LMWF as well as the LMWF before Sep-Pak were flushed with N_2 and kept at -80 ºC until further analysis.

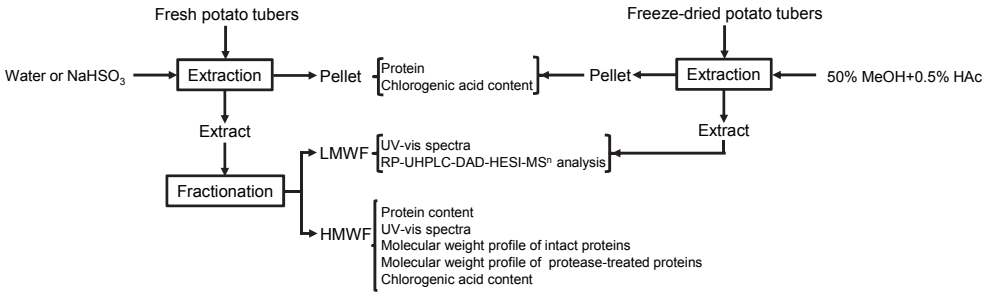

Figure 1. General scheme for the preparation and analysis of the potato extracts.

Preparation of HMWF involved removal of phenolics and other low molecular weight compounds by dialysis. Since most of the proteins in potato tubers (98% w/w) have molecular masses higher than 20 kDa (*15, 16*), extract *OX* was extensively dialyzed against water at 4 °C in cellulose ester dialysis tubes with a cut-off of 12 - 14 kDa (Medicell International, London, UK). The dialyzed *OX* extract was freeze-dried, denoted HMWF, and stored at -20 °C until further analysis.

PREPARATION OF CONTROL POTATO EXTRACTS AND FRACTIONS.

As a control, a potato extract was prepared in the presence of $NaHSO_3$ (*S* extract) and the LMWF, HMWF and remaining pellet were obtained (**Figure 1**). The extract *S* was obtained as described above for extract *OX*. The first extraction was done with 200 mL 400 mg/L $NaHSO_3$ solution and the subsequent extractions with 100 mL 200 mg/L $NaHSO_3$ solution. Different to *OX*-HMWF, to avoid any further reaction between phenolics and proteins, the *S*-HMWF extract was first dialyzed against 200 mg $NaHSO_3$/L until no further change in the conductivity of the retentate was observed. Subsequently, it was extensively dialysed against water. The pellet after the fourth extraction was processed as described for the *OX* extract.

To assess the composition of phenolics without producing sulfo-*o*-diphenolics (*12*), an extract from 2 g of freeze-dried material, of the same potato tuber batch, was obtained in 50% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid (extract *MeOH*)

as described elsewhere (*14*) (**Figure 1**). This extraction at acidic pH was aimed to precipitate proteins (*17*), inhibit PPO activity and, therefore, inhibit enzymatic oxidation of phenolics. This extraction is reported to yield full recovery of ChA from potato (*18*) and is considered to be indicative for the total amount of native phenolics present in LMWF. No HMWF was prepared, because during this acidic extraction proteins were precipitated. The pellet remaining after extraction was processed as described for the *OX* extract.

Extractions *OX*, *S*, and *MeOH* were performed on three independent batches of potato tubers, and their data were reported as averages with standard deviation.

ANALYSIS OF LMWF.

UV-Vis spectra. LMWF was diluted 5 times with water and the UV-vis spectra were recorded.

RP-UHPLC-DAD-HESI-MS^n analysis. Tyrosine, (sulfo) HCAs, (sulfo) HCAcs, and HCAs/HCAcs oxidation products were analysed by reversed phase-ultra high performance liquid chromatography (RP-UHPLC) with diode array detection (DAD) and an in-line heated electrospray ionization (HESI) mass spectrometer (MS^n). The same system was used to quantify un-bound ChA in the *OX*-HMWF and *S*-HMWF. Conditions for the UHPLC were according to previous work (*14*), except that the flow rate was 300 µL/min and the injected sample volumes were 2 µL. The eluate of the column was directed to a HESI probe, which in turn was coupled to an LTQ-Velos (Thermo Scientific, San Jose, CA, USA). HESI-MS^n was performed in negative mode in a full-scan mass spectrum mode over a *m*/*z* range of 150-1,500 with tune files for tyrosine during the first 3 min and for ChA for the rest of the run. Other settings were: Source voltage: 3.5 kV; ion transfer tube temperature: 250 °C; heater source: 230 °C. MS^2 spectra were collected with a collision energy of 35%, with the use of wideband activation. The control of the instrument and data processing were done using Xcalibur 2.1 (Thermo Scientific). Annotation of (sulfo-) HCAs / (sulfo-) HCAcs was done according to previous work (*12, 14*).

Quantification of (sulfo-) ChA isomers was based on calibration curves for ChA (0.1 to 5 µg/mL, R^2 = 1.000, five data points). Concentrations of sulfo-ChA isomers were expressed as ChA equivalents after correction of the difference between the molar extinction coefficients of ChA and sulfo-ChA (*12*) by the formula $X = [(Y_{325nm} - a) / b]*(\varepsilon_{ChA} / \varepsilon_{sulfo\text{-}ChA})$; where X = µg ChA/mL; Y_{325nm} = absorbance at 325 nm of the sulfo-ChA isomers; a = intercept of the calibration curve of ChA; b = slope of the calibration curve of ChA; ε_{ChA} = extinction coefficient of ChA (18,494±196 $M^{-1}cm^{-1}$); $\varepsilon_{sulfo\text{-}ChA}$ = extinction coefficient of sulfo-ChA (9,357±395 $M^{-1}cm^{-1}$). Other caffeic acid conjugates were quantified by means of caffeic acid calibration curves after correction of the difference in molecular weight by the formula $X = [(Y_{325nm} - a) / b]*(MW_{caffeic\ acid\ conjugate} / MW_{caffeic\ acid})$; where X = µg caffeic acid conjugate/mL; Y_{325nm} = absorbance at 325 nm of the caffeic acid

conjugate; *a* = intercept of the calibration curve of caffeic acid; *b* = slope of the calibration curve of caffeic acid; $MW_{caffeic\ acid\ conjugate}$ = molecular weight of the caffeic acid conjugate; $MW_{caffeic\ acid}$ = molecular weight of caffeic acid. Ferulic acid and synapic acid were adopted as standards for quantification of ferulic acid and sinapic acid conjugates, respectively. The latter concentrations were calculated likewise as for caffeic acid conjugates. Calibration lines for caffeic, ferulic and sinapic acids consisted of five data points at concentrations ranging from 0.05 to 2 µg/mL (R^2 = 0.999, in all cases). Limits of quantification were determined as ten times the standard deviation of the noise.

Quantification of tyrosine was based on selective reaction monitoring (SRM) during HESI-MS^n. UHPLC conditions were as described above in this section, but HESI-MS^n settings were different. HESI-MS^n analysis during the first three min of the run was performed using SRM mode, for which the parent ion of tyrosine (*m/z* 180) was fragmented to produce the ion *m/z* 163 as base peak. The ion *m/z* 163 was used for quantification of tyrosine. Calibration curves were performed with tyrosine (1 to 8 µg/mL, R^2 = 0.994, five data points). Repeatability was tested by analysing a solution of 5 µg/mL tyrosine evenly distributed within the sequence of sample analysis (five data points, coefficient of variation = 0.9%).

ANALYSIS OF HMWF AND PELLET.

Protein content. Protein contents of HMWFs and pellets were measured by the Dumas method (conversion factor 6.25 (*15*)) using a Flash EA 111 NC analyser (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

UV-vis spectra. HMWFs were prepared in 50 mM sodium phosphate buffer, containing 40 mM NaCl, at pH 7.0 in a ratio 3 mg powder to 1 mL buffer. After centrifugation (18,000 g, 10 min, 20 °C), samples were diluted with buffer to obtain 0.05 mg protein/mL buffer. Subsequently, UV-Vis spectra were recorded.

Molecular weight profile of unhydrolyzed proteins. Size exclusion chromatography (SEC) of the HMWFs of *S* and *OX* was performed on an ÄKTAmicro system (GE Healthcare, Uppsala, Sweden). A Superose 12 HR 10/30 column (GE Healthcare) was equilibrated and ran using 50 mM Tris/HCl buffer (pH 8.0), containing 150 mM NaCl at a flow rate of 500 µL/min at room temperature. A 100 µL-sample (5 mg/mL unhydrolyzed proteins at pH 7.5) was injected onto the column. The eluate was monitored at 280, 320, and 400 nm. Void volume of the column was access with blue dextran (2,000 kDa, void volume). Apparent distribution of molecular masses was estimated with ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa). The included volume was estimated with glutathione.

Enzymatic digestion of proteins and effect on molecular weight profile. Hydrolysis of the proteins present in the HMWFs of *S* and *OX* was performed by Proteinase K using a pH-stat set-up. For this, 8 mL HMWF solution (5 mg/mL) were preheated to 40 °C in the temperature controlled pH-stat. Subsequently, the pH was adjusted to pH 7.5 with 0.5 M NaOH. Next, 500 µL of a 10 mg/mL proteinase K solution was added. The pH was kept constant by the pH-stat (pH 7.5) during 24 h of incubation with Proteinase K. The obtained hydrolysate was analysed with SEC, without any further dilution, as described above.

Estimation of "reacted" chlorogenic acid. Determination was based on the release of quinic acid by alkaline hydrolysis (*19*). HMWFs from *OX*, *S* and *MeOH* (25 mg) were suspended in water and clarified by adjusting the pH to 8 with 0.5 M NaOH. Afterwards, the volume was adjusted to 1 mL using water. Next, 1 mL of 1 M NaOH was added. Mixtures were vortexed and then hydrolysed by incubation at 100 °C during 1 h (*19*). After cooling to room temperature 37% (v/v) HCl was added to the hydrolysates to reach a pH of 3-4. Acidified hydrolysates were centrifuged (9,000 g; 5 min; 4 °C). The supernatant was kept and the pellet was washed twice with 0.5 mL 0.1% (v/v) HCl. Supernatants were combined and final volume was adjusted to 5 mL with water. Aliquots (500 µL) were ultrafiltrated using regenerated cellulose centrifugal filter units (Amicon ultra 0.5 mL, cut-off 10 kDa, Millipore). Blanks, without alkaline treatment, were run to estimate the amount of free quinic acid and free (sulfo-) ChA present in HMWF extract. Hydrolysates and blanks were stored at -20 °C until further analysis.

The pellets from *OX*, *S* and *MeOH* (100 mg) were each suspended in 20 mM sodium acetate buffer pH 5.2 (1 mL), followed by the addition of 50 µL of 1 mg/mL α-amylase. The suspension was incubated at 80 °C for 1 h and cooled to room temperature. Next, 50 µL of 1 mg/mL α-amylase and 50 µL of 1 mg/mL amyloglucosidase were added to the suspension, which was incubated at 30 °C during 12 h. Two cycles of enzymatic treatment were performed. The de-starched suspension was freeze-dried and analyzed for ChA content. Enzyme-treated pellets were analyzed before and after alkaline hydrolysis for their content of free quinic acid and free (sulfo) ChA.

An Accela UHPLC system (Thermo Scientific) equipped with a pump, an autosampler cooled at 7 °C, and a Hypercarb column (100 mm x 2.1 mm i.d.; particle size 3 µm; Thermo Scientific) at 30 °C was used to determine the quinic acid released. The eluents used were 100% water (eluent A), 100% ACN (eluent B), and 15% (v/v) aqueous formic acid (eluent C). The elution program in terms of A/B/C composition was as follows: 0-5 min, 100/0/0%; 5-6 min, 100/0/0%→0/25/75%; 6-8 min, 0/25/75%; 8-9 min, 0/25/75→50/50/0%; 9-11 min, 50/50/0%; 11-12 min, 50/50/0%→100/0/0%, and 12-16 min, 100/0/0%. The flow rate was 400 µL/min. Sample volumes of 5 µL were injected. The eluate was directed to a splitter (1:9), with the lowest flow going into the HESI, which was coupled to an LTQ-Velos Pro (Thermo Scientific). Settings for the HESI-MS^n were as

described above, except that the system was auto-tuned with quinic acid. Quantification of quinic acid was performed by SRM analysis by monitoring the fragment *m/z* 85 produced from the fragmentation of the parent ion of quinic acid (*m/z* 191). The concentration of ChA in hydrolyzed HMWF was calculated by means of calibration curves that were performed by hydrolysis of authentic ChA (0.04-20 µg ChA/mL, R^2 = 0.999, ten data points). Free quinic acid content in the blanks was calculated by means of calibration curves with authentic quinic acid (0.02-10 µg quinic acid/mL, R^2 = 0.999, ten data points). Free (sulfo-) ChA was analysed in the blanks as described above. Repeatability was tested with a solution of 5 µg/mL alkali-treated ChA, which was analysed evenly distributed within the sequence of sample analysis (6 data points, coefficient of variation = 2.0%).

STATISTICAL ANALYSIS.

Data were reported as the means with their standard deviation. Quantities of phenolics in *MeOH* extract, *S*-LMWF, and *OX*-LMWF were analysed using a one-way ANOVA as a completely randomized design with three replications. Means of phenolics in *MeOH* extract, *S*-LMWF, and *OX*-LMWF were compared by Tukey's test ($P<0.05$). Data from *S*-HMWF and *OX*-HMWF were compared by the Student's t-test ($P<0.05$). Means of ChA in the mass balance were compared by Tukey's test ($P<0.05$).

Results and Discussion

COLOR OF THE POTATO EXTRACTS.

When potato extract was prepared in water (*OX* extract), a reddish coloration was observed in the low molecular weight fraction (LMWF), while a brown color was observed in the high molecular weight fraction (HMWF) (Inserts in **Figure 2**). In contrast, colorless LMWF and HMWF were obtained when the extraction was performed in the presence of $NaHSO_3$ (*S* extract) (Inserts in **Figure 2**). The UV-vis spectra of *S*-LMWF showed absorption maxima at 273 and 324 nm (**Figure 2A**). The first maximum corresponded to the free aromatic amino acids tyrosine and tryptophan, reported to be present in potato (*18*). The second maximum corresponded to sulfo-chlorogenic acid (sulfo-ChA) isomers that are expected to be present in *S* (*12*). In the *OX*-LMWF there was an increase in the absorption in the visible region with a maximum at 469-480 nm (**Figure 2A**) when compared to *S*-LMWF. The absence of a maximum of absorption at 324 nm (as expected for ChA and its isomers) in *OX*-LMWF indicated a depletion of the ChA population.

The UV-vis spectrum of the *S*-HMWF (**Figure 2B**) showed that it had a maximum of absorption at 276 nm, indicative of the presence of tyrosine and tryptophan, present in proteins in significant quantities (*20*). Shoulders observed in *S*-HMWF at 320 nm and at 400 nm indicated the presence of oxidized phenolics (*7*). *OX*-HMWF showed similar

maxima/shoulders of absorption as those observed in *S*-HMWF, but with higher values. Furthermore, *OX*-HMWF showed absorption at 450-650 nm, indicative of browning, while absorption for *S*-HMWF in that region of the spectrum was negligible (**Figure 2B**). These results, therefore, suggested that both low and high molecular weight oxidation products were generated during enzymatic browning of potato extracts.

PHENOLICS IN LOW MOLECULAR WEIGHT FRACTIONS.

Composition of phenolics in MeOH and low molecular weight fraction of *S*. The *MeOH* extract contained ChA isomers, caffeic acid and caffeoyl putrescine (**Table 1**, **Figure 3**). In contrast, in *S*-LMWF, ChA isomers and caffeoyl putrescine were found as their sulfonic acid derivatives (**Table 1**, **Figure 3**). Tyrosine, together with ferulic and sinapic acid containing compounds, were constituents of both extracts (**Table 1**, **Figure 3**). Tyrosine (**1**), ChA (**4**), and caffeic acid (**6**) were identified by the combination of their retention times, UV spectra and MS^n data. These data coincided with those of the authentic standards. MS^n and UV data of other compounds, including the sulfonic acid derivatives, matched those found in previous work (*12, 14*).

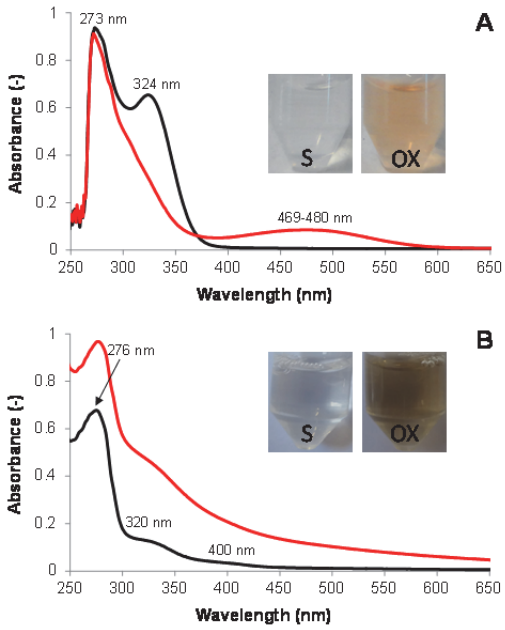

Figure 2. (A) Low molecular weight fraction (LMWF) of potato extracts prepared in the presence of $NaHSO_3$ (black line, *S*) and in water (red line, *OX*), both 5× diluted. Inserts show pictures of undiluted *S*-LMWF and *OX*-LMWF. **(B)** High molecular weight fraction (HMWF) of potato extracts prepared in the presence of $NaHSO_3$ (black line, *S*) and in water (red line, *OX*), both 0.5 mg protein/mL. Inserts show pictures of *S*-HMWF and *OX*-HMWF, both 2.0 mg protein/mL.

Table 1. Retention times, MS, and UV-Vis data of HCAs/HCAcs, sulfonic acid conjugates, and oxidation products in the low molecular weight fraction of potato extracts.

No	RT (min)	MS (*m/z*)	MS^2 (*m/z*)[a]	UV-Vis λmax	Tentative identification
1	2.43	180	163, 119, 136, 93	226, 275	Tyrosine
2	10.36	353	191, 179*, 135*, 173, 161*	225, 240sh, 300sh, 324	3-*O*-Caffeoyl quinic acid
3	10.60	249	249, 135*, 207, 179*, 161*	225, 294sh, 317	Caffeoyl putrescine
4	11.86	353	191, 179*, 161*, 135*, 173	226, 240sh, 305sh, 326	5-*O*-Caffeoyl quinic acid
5	12.08	353	173, 179*, 191, 135*, 161*	226, 240sh, 305sh, 326	4-*O*-Caffeoyl quinic acid
6	12.69	179	135*, 179*, 161*	225, 305sh, 323	Caffeic acid
7	13.42	367	191, 173, 193, 178, 134	225, 300sh, 325	5-*O*-Feruloyl quinic acid
8	14.06	309	193, 178, 149, 133, 115, 134	225, 305sh, 326	Feruloyl malate
9	14.91	328	310, 295, 135, 149, 175	226, 294sh, 320	Feruloyl octopamine
10	15.77	429	249, 205, 385, 179, 223	225, 311	Sinapic acid conjugate
11	3.61	433	191, 353*, *241*, 161*, *259*, 179*, 135*	225, 246, 295, 327sh	Sulfo-caffeoyl quinic acid isomer
12	5.76	433	*259*, *215*, 161*, *241*, 415, 387, 433, 179*, 301, 191, 135*, 353*	225, 240, 305sh, 327	Sulfo-caffeoyl quinic acid isomer
13	6.64	433	*241*, *259*, 387, 415, 433, 301, 191, 161*, 179*, 353*	225, 250, 295, 323	Sulfo-caffeoyl quinic acid isomer
14	7.35	329	329, 249, 161*, 135*, *215*	224, 294sh, 320	Sulfo-caffeoyl putrescine isomer
15	9.01	433	301, 387, 433, *241*, *259*, 415, 191, 161*, *215*, 179*, 353*	225, 245sh, 295sh, 324	Sulfo-caffeoyl quinic acid isomer
16	9.44	433	301, *259*, *241*, 387, 415, 161*, 135*, 191, 433	225, 289sh, 310	Sulfo-caffeoyl quinic acid isomer
17	9.80	329	329, 249, *241*, 161*, *215*, 135*	225, 295sh, 322	Sulfo-caffeoyl putrescine isomer
18	9.93	433	*259*, 161*, *241*, 387, *215*, 415, 191, 301, 353*, 179*, 135* , 433	225, 280sh, 315	Sulfo-caffeoyl quinic acid isomer
19	10.63	433	301, 387*, *241*, 161, 387	225, 240sh, 305sh, 329	2'-Sulfo-4-*O*-caffeoyl quinic acid
20	10.95	433	*259*, 161*, *241*, 387, *215*, 415, 135*, 179*, 191, 433, 301, 353*	225, 240sh, 305sh, 329	2'-Sulfo-5-*O*-caffeoyl quinic acid
21	2.41[b]	192	148, 120	475	Dopachrome
22	10.48	461	167, 353*, 191, 152	230, 250sh, 286, 460sh	Caffeoyl quinic acid oxidation product
23	10.89	192	148	225, 316	Oxidation product
24	10.94	396	178, 222 (NL 174)[c], 173, 191	230, 294, 513	Caffeoyl quinic acid oxidation product
25	12.68	489	429, 191, 179*	-	Caffeoyl quinic acid oxidation product

Table 1. Continued

No	RT (min)	MS (*m/z*)	MS^2 (*m/z*)[a]	UV-Vis λmax	Tentative identification
26	13.92	561	501, 479, 369 (NL 192), 516, 191, 179*	-	Caffeoyl quinic acid oxidation product
27	14.40	523	313, 331 (NL 192), 287, 269, 191, 173	230, 240sh, 309, 420sh	Caffeoyl quinic acid oxidation product
28	15.06	523	331 (NL 192), 287, 313, 269, 191, 173	231, 280, 320sh, 420sh	Caffeoyl quinic acid oxidation product

[a]Ions are written in order of intensity, the first one is the base peak in MS^2.
[b]Coelution with tyrosine.
[c]NL: neutral loss. Underlined values are related to a neutral loss (NL) of quinic acid or to ions expected for quinic acid.
*Fragments related to the presence of chlorogenic acid or caffeic acid. In italic those ions diagnostic of SO_3 attached to the aromatic ring of caffeic acid.

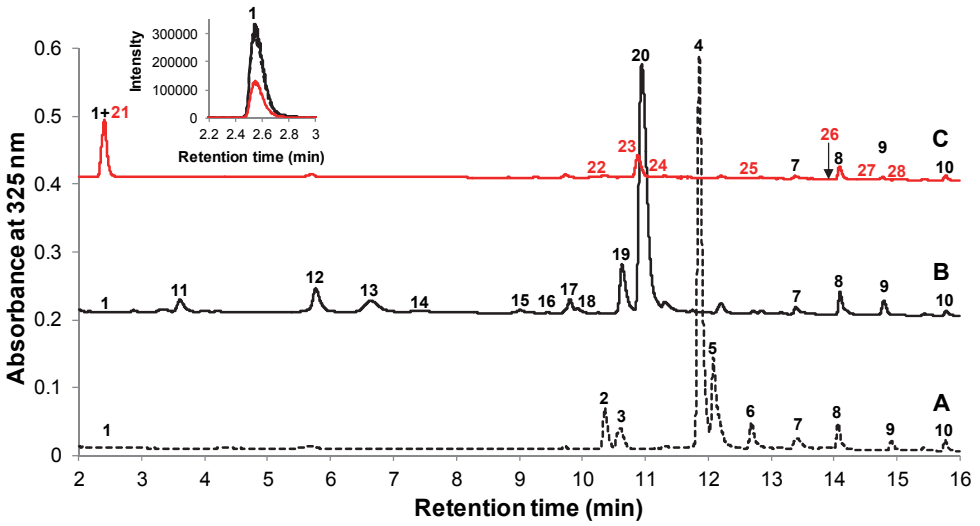

Figure 3. UHPLC profile recorded at 325 nm of the low molecular weight fraction of undiluted extracts prepared in 50% (v/v) methanol plus 0.5% (v/v) acetic acid (black dotted line, **A**), $NaHSO_3$ (black solid line, **B**) and water (red solid line, **C**). Compounds **1**-**28** correspond to those in **Table 1**. Insert shows the UHPLC-HESI-MS-SRM chromatograms from 2.2 to 3 min of the ion *m/z* 163.

Quantification of HCAs/HCAcs in the *MeOH* extract (**Table 2**) revealed that caffeic acid-containing compounds represented 94% (w/w) of the total content of HCAs/HCAcs, while ferulic acid- and sinapic acid-containing compounds accounted for 6% (w/w). In the *MeOH* extract a total amount of ChA isomers of 80.6±10.6 mg/100 g DW potato was found, which is comparable to literature data (*9, 18*). When the amounts of sulfo-ChA isomers were expressed as ChA, a total value of 77.6±2.3 mg/100 g potato DW (**Table 2**) was found in *S*-LMWF. The amount of tyrosine in the *S*-LMWF (55.2±7.9 mg/100 g potato DW) was markedly lower than that in the *MeOH*-LMWF (110.2±13.8 mg/100 g potato DW), which might be caused by the formation of sulfonic acid derivatives

of the *o*-diphenol 3,4-dihydroxyphenylalanine (DOPA), formed upon hydroxylation of tyrosine by PPO and the subsequent reaction of sulphite with DOPA quinone. Such a derivative would elute close to the void volume and would escape detection with the conditions applied.

Table 2. Quantification of compounds found in the low molecular weight fraction of potato extracted with 50% (v/v) methanol+0.5% (v/v) acetic acid (*MeOH*), with aqueous $NaHSO_3$ (*S*), and with water (*OX*).

No	Compound	mg/100 g DW[1]		
		MeOH	***S***	***OX***
1	Tyrosine	110.2±13.8[a]	55.2±7.9[b]	20.8±5.4[c]
Quinic acid containing compounds				
2	3-*O*-Caffeoyl quinic acid	6.6±1.2	ND[2]	ND
4	5-*O*-Caffeoyl quinic acid	57.2±6.9	ND	ND
5	4-*O*-Caffeoyl quinic acid	16.8±2.8	ND	ND
	Total caffeoyl quinic acid isomers	80.6±10.6	ND	ND
7	5-*O*-Feruloyl quinic acid	0.6±0.0[a]	0.2±0.0[b]	0.1±0.0[c]
Other hydroxycinnamic acids				
3	Caffeoyl putrescine	3.4±0.5	ND	ND
6	Caffeic acid	2.2±0.3	ND	ND
8	Feruloyl malate	2.5±0.3[a]	1.4±0.2[b]	0.8±0.1[c]
9	Feruloyl octopamine	1.0±0.2	<0.03[3]	<0.03[3]
10	Sinapic acid conjugate	1.4±0.3[a]	0.3±0.0[b]	0.2±0.0[c]
Sulfocaffeoyl quinic acid isomers				
11	Sulfocaffeoyl quinic acid isomer	-	2.8±0.2	-
12	Sulfocaffeoyl quinic acid isomer	-	5.5±0.4	-
13	Sulfocaffeoyl quinic acid isomer	-	5.4±0.3	-
15	Sulfocaffeoyl quinic acid isomer	-	0.7±0.0	-
16	Sulfocaffeoyl quinic acid isomer	-	0.1±0.1	-
18	Sulfocaffeoyl quinic acid isomer	-	1.1±0.1	-
19	2'-Sulfo-4-*O*-caffeoyl quinic acid	-	10.3±0.6	-
20	2'-Sulfo-5-*O*-caffeoyl quinic acid	-	51.7±3.0	-
Sulfo-caffeoyl quinic acid isomers expressed as chlorogenic acid		-	77.6±2.3	-
14, 17	Sulfo-caffeoyl putrescine isomers	-	NQ[4]	-

Values within the same row with different superscripts show significant differences (Tukey's test, $P < 0.05$).
[1]Data are expressed as the mean with standard deviation.
[2]ND: Not detected by MS.
[3]Below limit of quantification: 0.03 mg feruloyl octopamine/100 g potato DW.
[4]NQ: Not quantified due to lack of standards.

Composition of phenolics in low molecular weight fraction of *OX*. ChA isomers, caffeic acid and caffeoyl putrescine were not detected in *OX*-LMWF. At 325 nm the RP-UHPLC chromatogram of *OX*-LMWF (**Figure 3**) was dominated by compound **21**, co-eluting with tyrosine. When recording the chromatogram at 478 nm (chromatogram not shown) still a peak was observed, which could not be attributed to tyrosine. Compound **21**, having a maximum of absorption at 478 nm, was then considered to be responsible for the reddish

color observed in *OX*-LMWF (Insert in **Figure 2A**). MS analysis revealed that compound **21** had a molecular parent ion of *m/z* 192, which in MS^2 gave the fragments *m/z* 148 ([M-CO_2-H]$^-$) and *m/z* 120. This might be due to the neutral loss of CO from the quinoidal ring of the ion *m/z* 148 (**Table 1**). Based on the maximum absorption at 478 nm and MS^n data, compound **21** was tentatively annotated as dopachrome. To our knowledge, no mass fragmentation data have been reported for dopachrome. Compound **23**, with parent ion *m/z* 192 and UV maximum at 316 nm, was neither present in the *MeOH* extract nor in *S*-LMWF. Furthermore, **23** neither showed absorption in the visible spectrum, nor fragments characteristic to caffeic acid or quinic acid. Compound **23** was considered to be an oxidation product, but its exact identity remains unclear.

To screen for trace oxidation products of ChA, *OX*-LMWF was concentrated by Sep-Pak. Compounds **22**, **24**, **27** and **28** showed absorption between 420 to 515 nm. This indicates that they were co-responsible, although to a lesser extent than dopachrome, for the red color of *OX*-LWMF. It has been shown (*1, 4*) that during oxidation of ChA and caffeic acid the aromatic ring can sometimes be modified leading to a fragmentation pattern different to that characteristic of HCAcs. With this in mind, we screened not only for negative ions (*m/z* 179, 161, 135) and neutral losses (180 a.m.u., 162 a.m.u) of caffeic acid containing compounds (*14*), but also for negative ions (*m/z* 191, 173) and neutral losses (192 a.m.u, 174 a.m.u.) characteristic to quinic acid containing compounds. When applying this strategy, compounds **22** and **24-28** were found (**Table 2**, **Figure 3**) and, therefore, they are considered to be chlorogenic acid oxidation products.

Potato tubers contain amino acids, phenolics different to ChA, and glutathione (*21*) that can react with ChA quinone to produce hetero-adducts as has been shown in *in-vitro* systems (*4, 11, 13*). Nevertheless, parent ions (*m/z* ranging from 396 to 523) of compounds **22** and **24-28** did not match those of such hetero-dimers. It could be that unstable low molecular weight homo- or hetero-adducts of ChA were formed, which have reacted further. Compounds **22** and **24-28** were neither found in *MeOH* nor in *S*-LMWF, indicating the anti-browning effectiveness of these extractants. Neither ChA dimers (parent ion in negative mode *m/z* 705) nor hydrated-ChA dimers (*m/z* 723) were found in the brown potato extract, also not when those parent ions were screened in the selected ion monitoring (SIM) mode. ChA dimers have been found during *in vitro* experiments in which ChA was oxidized by fungal tyrosinase (*4*). ChA dimers (*m/z* 705) and hydrated-ChA dimers (*m/z* 723) have been found in oxidized cider apple juice (*4*).

ChA isomers, caffeic acid, caffeoyl putrescine and tyrosine reacted upon enzymatic browning of potato extract (**Figure 3**). A stronger decrease in the amounts of ChA and caffeic acid compared to tyrosine (**Table 2**) was found in the oxidized potato extract. This indicated a higher reactivity of PPO towards ChA and caffeic acid than towards tyrosine, consistent with the literature (*22*). Dopaquinone might react with proteins in a non-enzymatic way, leading to brown polymers known as eumelanin (*23*).

Furthermore, proteins containing surface-exposed tyrosine might also be oxidized by PPO and contribute to browning of proteins as suggested by others (*5*). The high molecular weight oxidation products of tyrosine were considered to be outside the scope of the current research, and not investigated further.

CHLOROGENIC ACID IN HIGH MOLECULAR WEIGHT FRACTION.

"Reacted" ChA content of high molecular weight fractions. Because no ChA isomers and only small amounts of oxidation products of ChA were present in *OX*-LMWF, we searched for "reacted" ChA in the *OX*-HMWF. We assumed that the oxidized caffeic acid was the reactive moiety of ChA that was present in HMWF with the quinic acid moiety still unmodified. The release of quinic acid by alkaline treatment of the HMWF (*19*) was used to assess the quantity of ChA occurring in HMWF. The "reacted" ChA content in *OX*-HMWF was higher (1.87±0.25%, w/w) than that in *S*-HMWF (0.68±0.17%, w/w). As expected, proteins were found to be the major constituents of *S*-HMWF (85.4±1.5%, w/w) and *OX*-HMWF (81.8±2.4%, w/w).

Effect of chlorogenic acid on molecular weight distribution and UV-vis absorption of proteins. The molecular weight distribution of unhydrolyzed *S*-HMWF obtained at 280 nm by size exclusion chromatography (SEC; **Figure 4A**) showed patatin isoforms (eluting as dimers of approximately 85 kDa) and protease inhibitors (approximately 20 kDa) as the most abundant fractions in *S*-HMWF, as judged from the obtained molecular masses, in agreement with previous reports (*15, 16*). Within the family of protease inhibitors, PSPI has been reported as the most abundant representative in potato juice (*24*). Some aspecific binding to the column material was observed as judged from the peak (280 nm) eluting after the included volume. Compared to *S*-HMWF, SEC of the unhydrolyzed *OX*-HMWF revealed an increase of the absorption at 280 nm in the void volume, patatin fraction and protease inhibitors fraction (**Figure 4B**), even though the difference in protein content in both solutions did not exceed 5% (w/w). Contrasting with the lack of absorption at 400 nm in the profile of *S*-HMWF, in *OX*-HMWF all protein fractions exhibited absorption at 400 nm. It is known that proteins modified with oxidized phenolics exhibit an increase in the absorption in the UV-vis range compared to un-modified proteins (*7*). Hence, the absorbance at 400 nm, together with the increase in absorbance at 280 nm in *OX*-HMWF, were assumed to be the result of the presence of oxidation products of ChA associated with proteins. SDS-PAGE (not shown) of *OX*-HMWF revealed a band that did not migrate into the gel, suggesting that the peak at the void volume observed for *OX*-HMWF was caused by, besides aggregation effects, cross-linking of proteins.

The SEC profiles at 400 nm and at 280 nm for the unhydrolyzed *OX*-HMWF coincided (**Figure 4B**). Furthermore, when analyzing *OX*-HMWF, before alkaline treatment, by RP-UHPLC-DAD-HESI-MS^n, no low molecular weight oxidation products of

ChA were found (chromatogram not shown). These results suggested two possible scenarios for attachment of ChA to proteins. (i) Oxidized ChA was covalently attached to proteins. (ii) Oligomeric ChA, formed upon oxidation, was non-covalently attached to the potato proteins, with similar affinity to patatin and PSPI, and therefore with a elution pattern coinciding with that of the proteins. The first scenario seems to be most likely.

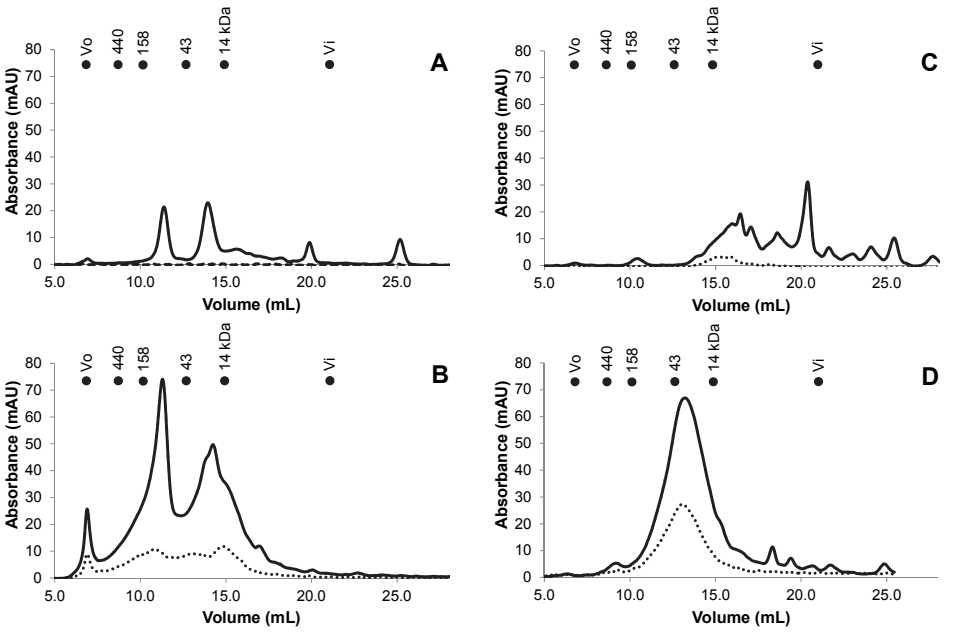

Figure 4. Molecular weight distribution of unhydrolyzed proteins from potato extracts prepared by using (**A**) sodium hydrogen sulfite (*S*) or (**B**) water, and of protease-treated proteins from potato extracts prepared by using (**C**) sodium hydrogen sulfite or (**D**) water. All profiles were monitored at 280 nm (solid lines) and at 400 nm (dotted lines).

Effect of protease treatment on molecular weight distribution of proteins. To further determine whether "reacted" ChA in *OX*-HMWF was covalently linked to proteins or present as oligomeric ChA, enzymatic hydrolysis of the proteins was performed. The elution profiles of the protease treated samples were evaluated at 280 and 400 nm on SEC, and compared with the *S*-HMWF profile of the unhydrolysed proteins. As expected, protease treatment of *S*-HMWF (**Figure 4C**) generated a wide distribution of products with lower molecular mass, e.g. as judged by the shift to higher elution volume in the absorption profile at 280 nm. The high amount of protease K required to achieve the hydrolysis of proteins in the *S*-HMWF might be the result of the presence of protease inhibitors as major components of potato protein preparations (*15*). After hydrolysis, a small peak at an elution volume between 14 to 17 mL was observed at 400 nm. This signal might be the result of

partial oxidation of aromatic amino acids in the proteins induced by the pH (7.5) and temperature (40 °C) applied during the protease treatment.

The moderate shift in elution volume after enzymatic treatment of the *OX*-HMWF (**Figure 4D**) was indicative of less extensive hydrolysis of this sample compared to *S*-HMWF. The difficulty to degrade the oxidized proteins suggested that modified ChA was strongly associated to proteins. After partial enzymatic hydrolysis, the chromatographic profiles at 280 and 400 nm coincided, suggesting that the oxidized ChA remained attached to the peptides produced. Considering that *in vitro* studies have shown that ChA is covalently attached to proteins, such as lysozyme and bovine serum albumin (*5, 6*), we speculated that oxidized ChA was attached to the potato proteins, similarly. Other studies with individual NH_2-blocked amino acids and oxidized ChA have shown that cysteine, lysine, tryptophan, histidine, and tyrosine are targets for covalent attachment of ChA (*4, 25*). Lysine, tryptophan, histidine, and tyrosine were indeed found to be present on the surface of patatin and PSPI (EMBL-EBI LION Bioscience AG, htpp://www.ebi.ac.uk/thornton-srv/databases). Therefore, one might speculate that oxidized ChA will be covalently attached to these solvent-exposed amino acid residues in potato proteins.

"REACTED" CHLOROGENIC ACID IN PELLETS.

The amounts of pellet that were obtained in the three extractions were 68.4±1.3 g pellet/100 g potato DW, 79.1±0.2 g pellet/100 g potato DW, and 68.6±1.0 g pellet/100 g potato DW, in *OX*, *MeOH* and *S*, respectively. As expected, the amount of pellet found in *MeOH* was higher, due to the lower solubility of e.g. proteins and polysaccharides in methanol than in aqueous environment (*OX*, *S*). The amounts of protein present in the pellet of *OX* and *S* were approximately similar with 2.69±0.15 g protein/100 g potato DW and 2.27±0.27 g protein/100 g potato DW, respectively. The amount of protein in the pellet of *MeOH* was 4.31±0.27 g protein/100 g potato DW, consistent with poor solubility of proteins in methanol.

The amount of (reacted) ChA was also analysed in the pellet fractions. No free ChA was present, as expected after the exhaustive extraction procedure. The amount of "reacted" ChA found in the *MeOH*-pellet (0.029±0.007 g "reacted" ChA/100 g pellet DW) was higher than that found in *S*-pellet (0.016±0.002 g "reacted" ChA/100 g pellet DW), but similar to that found in *OX*-pellet (0.031±0.005 g "reacted" ChA/100 g pellet DW). The presence of "reacted" ChA in the *MeOH*-pellet was surprising, as we considered oxidation of ChA by PPO unlikely during this control extraction. A possible explanation for this is that quinic acid might be part of other potato tuber constituents, such as the plant cell wall, although this has never been reported in the literature to our knowledge.

TRACKING CHLOROGENIC ACID DURING ENZYMATIC BROWNING.

To determine the quantitative fate of ChA during enzymatic browning a mass balance was made (**Figure 5**). Concentrations of sulfo-ChA isomers obtained in *S*-LMWF were expressed as ChA. In the *S* experiment, the LMWF accounted for 77.6±2.3 mg ChA/100 g potato DW, the HMWF accounted for 14.4±1.5 mg ChA/100 g potato DW, and the pellet for 10.5±1.1 mg ChA/100 g potato DW. Thus, a total amount of 102.5±2.4 mg ChA/100 g potato DW was found, which matched very well (less than 1% difference) with that obtained in *MeOH* (103.2±6.2 mg/100 g potato DW).

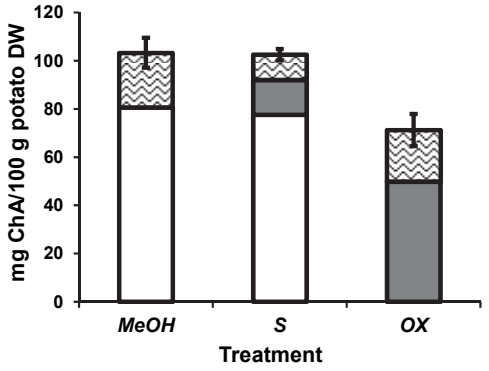

Figure 5. Total chlorogenic acid (ChA) in low molecular weight fraction (LWMF, white bars), high molecular weight fraction (HMWF, gray bars), and pellet (patterned bars). Error bars represent the standard deviation of the total amount.

No ChA was found in the *OX*-LMWF, but 49.8±7.1 mg ChA/100 g potato DW was found in *OX*-HMWF and 21.4±3.5 mg ChA/100 g potato DW in the pellet. The total amount of ChA in the *OX* treatment represented only 70% of that obtained in *S* treatment. Possibly, the quinic acid part of ChA participated in the later stages of the enzymatic browning reaction and participated in further reactions with proteins or was released as a low molecular weight oxidation product. It has been shown that oxidation of quinic acid produces compounds, such as citric acid, malic acid, and hydroquinone (*26, 27*), but no information on reactions in the presence of other components, such as proteins, is available.

Remarkably, the total amount of "reacted" ChA recovered (represented by the sum of the amounts of ChA in HMWF and pellet) was similar in the *MeOH* and *S* extraction (**Figure 5**), but the contribution of ChA to the pellets differed. We speculate that all "reacted" ChA is precipitated, together with proteins and polysaccharides, in the methanolic environment of *MeOH*, whereas in the aqueous environment of *S*, part of the "reacted" ChA remained soluble, and ended up in the HMWF. We do not have a good explanation for our observations that the pellets of *MeOH* and *OX* have similar amounts of "reacted" ChA, and

that the pellet of *OX* has a larger amount of "reacted" ChA than that of *S*, despite the fact that the aqueous extraction conditions of *OX* and *S* were similar. Perhaps the "reacted" ChA in *OX*-HMWF becomes part of the *OX*-pellet after further action of PPO, e.g. as a result of precipitation of proteins cross-linked by ChA. These ChA cross-linked proteins should be rich in ChA as the protein content of the *OX*- and *S*-pellet are similar. This might explain the observed discrepancy in amount of "reacted" ChA in pellet of *S* and *OX*. During further oxidation reactions the quinic acid moiety of ChA might participate, and can subsequently not be analysed as such anymore. This might explain why the amount of "reacted" ChA is not higher in the *OX*-pellet, and the similar amounts of "reacted" ChA in the pellets of *MeOH* and *OX* might actually be a coincidence. In conclusion, our results show that the majority of the free ChA present in potato tubers is associated with (high molecular weight) proteins upon processing of potato juice in the absence of anti-browning agents.

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Chapter 7

General discussion

The research described in this thesis was focused on the quantification of HCAs/HCAcs/DHCAcs in different potato tuber cultivars, with special emphasis on the less documented compounds, and on the behaviour of HCAs/HCAcs/DHCAcs during the preparation of potato juice. The use of UHPLC-DAD-ESI-MS^n as an analytical technique for the identification and quantification of HCAs/HCAcs/DHCAcs and their oxidation products was elaborated. Enzymatic browning, its inhibition by the anti-browning agent $NaHSO_3$, and the reaction of oxidized phenolics with proteins were also addressed.

HCAs/HCAcs/DHCAcs in potato tubers

In **chapter 2**, a method for the identification of HCAs/HCAcs/DHCAcs based on the screening of negative fragments and neutral losses characteristic to HCAcs/DHCAcs was described. This method allowed the tentative annotation of 39 compounds as HCAs/HCAcs/DHCAcs in a single cultivar, with only 17 of them reported previously in potato. In **chapter 3**, the screening method mentioned was applied to several Colombian potato cultivars and a total of 62 HCAs/HCAcs/DHCAcs were found. In the purple/red skinned Colombian potato cultivars a total of 14 HCA-acylated anthocyanins were found. When totalizing the number of compounds described in **chapters 2** and **3**, 78 compounds were tentatively identified as HCAcs/HCAcs/DHCAcs, with only 12 compounds being common to all varieties. A large number of HCAcs has been described in other plant sources, such as in pak choi cultivars (*Brassica campestris* L. ssp. *chinensis* var. *communis*) (*1*). DHCAcs have been found in other sources, e.g. in naranjilla fruits (*Solanum quitoense* Lam. Var. Puyo), where conjugates of dihydrocaffeic acid with either spermine or spermidine have been described (*2*).

Chromatographic conditions in which potato extracts were analysed in chapters **2** and **3** were identical. A closer look at the chromatograms in both chapters shows that retention times did not coincide, retention time of e.g. chlorogenic acid (ChA) in **chapter 2** was 10.68 min while in **chapter 3** it was 11.03 min. The UHPLC device used in each case was the same while the columns were from different batches, which might explain the observed differences in retention times. Nevertheless, this discrepancy when using different column batches did not affect the resolution, reproducibility and accuracy of the chromatographic technique. The large number of compounds different to the most documented ones (ChA, *crypto*-ChA, *neo*-ChA, caffeic acid, and anthocyanin-linked HCAs) that was found in our studies has not been described before in potato tubers. This outcome is, in our opinion, the result of higher resolution during chromatographic analyses obtained with UHPLC compared to HPLC, combined with our screening method, based on the information of parent molecular mass and fragmentation ions of separated metabolites. In recent years RP-UHPLC coupled to in-line tandem mass spectrometers has provided new

opportunities for the analysis of complex samples, including non-volatile plant secondary metabolites (*3, 4*).

Oxidation products of ChA

When we applied the screening method (**chapter 2**) for reaction products of ChA in the LMWF of brown potato juice, only two compounds were found (Compounds **25** and **26**, **chapter 6**) giving, after fragmentation, the ion *m/z* 179, characteristic of caffeic acid. Apparently, most of the ChA had reacted during the production of potato juice.

The screening method used for the identification of caffeic acid containing compounds in **chapters 2** and **3** included the search for the negative ions *m/z* 179, 161 and 135 and the neutral loss 162. These ions, nevertheless, were not always observed for the oxidation products of caffeic acid or ChA (*5, 6*). Typically, a variety of other masses were observed. The fact that oxidation products of caffeic acid or of ChA did not follow the typical fragmentation pattern expected for caffeic acid containing compounds became an analytical challenge that was addressed in **chapter 6**. We proposed in that chapter that the screening of oxidation products of ChA would involve, besides the screening of negative ions and neutral losses characteristic to the caffeic acid moiety of ChA containing compounds, the screening of neutral losses or ions characteristic to the quinic acid moiety. Therefore, with the objective to follow the qualitative fate of HCAcs during processing, the screening of negative ions and neutral losses characteristic to HCAs was complemented with the screening of negative ions and neutral losses of the quinic acid moiety.

When applying the strategy mentioned, new oxidation products of ChA were found in the oxidized potato extract (Compounds **22**, **24**, **27** and **28**, **chapter 6**). In addition, compound **23** in **chapter 6** was considered to be an oxidation product. Nevertheless, **23** did not have absorption in the VIS region and did not show ions or neutral losses characteristic to caffeic acid or to quinic acid.

Figure 1 shows a general strategy for the screening of oxidation products of ChA. The first level is to look for ions or neutral losses in MS^n characteristic to caffeic acid. Next, if those features are not found, a screening for ions or neutral losses characteristic to quinic acid should be done (second level). When caffeic acid or quinic acid moieties are found as part of a compound, that compound should be screened in a control extract, where browning has been inhibited. The absence of that particular compound in the control extract might indicate that that compound is an oxidation product and its tentative annotation can be done. If neither caffeic acid nor quinic acid moieties are detected by MS^n, but a peak with absorption in the UV or VIS spectra is detected, then that peak should be screened in the control extract (third level). The peak might be an oxidation product if it is absent in the

control extract. In the third level of screening, nevertheless, it is not possible to qualify the oxidation product as an oxidation product derived of ChA.

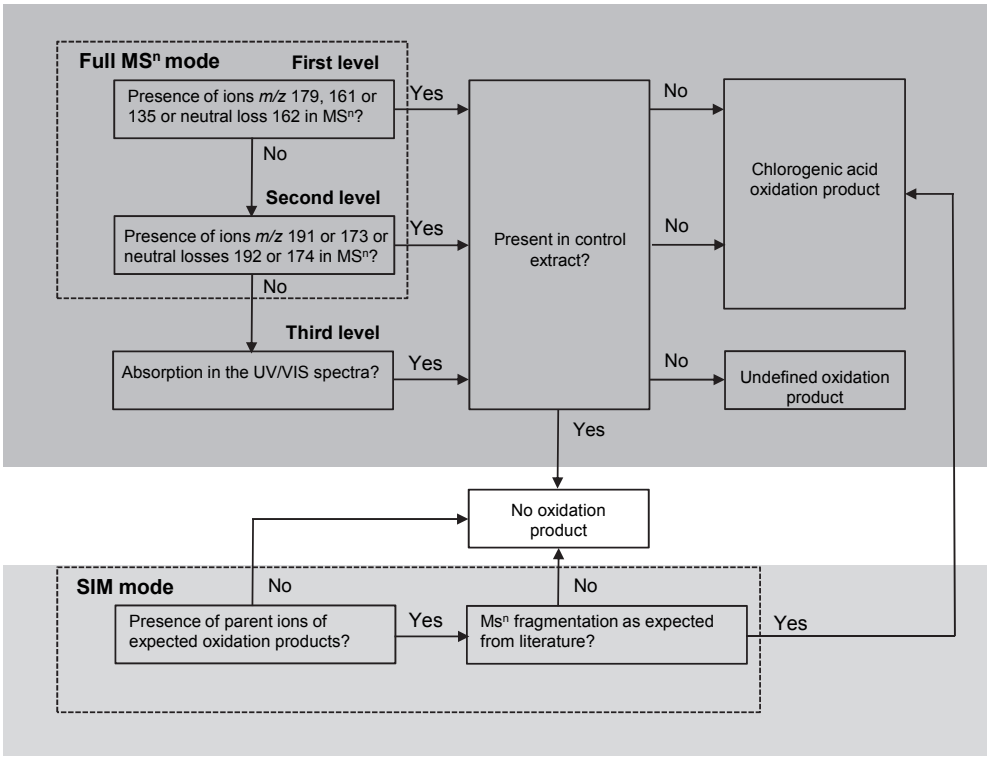

Figure 1. Strategy for the screening of oxidation products of chlorogenic acid in potato juice.

When co-elution of compounds occurs, MS detection in a full MS^n mode might be hampered (*7*). As a consequence, information on the possible presence of oxidation products might be missed. This situation was encountered in **Chapter 6**. In this case, an alternative strategy can be used, i.e. the selected ion monitoring (SIM) mode, to screen for specific parent ions of oxidation products. This strategy requires information on the phenolic composition of a non-oxidised control extract in the full MS^n mode. With oxidation pathways derived from model experiments, molecular masses of oxidation products can be predicted. Finally, if the parent and daughter ions measured match those calculated based on the literature, the compound is annotated as an oxidation product. In our potato extracts, this strategy was applied to oxidation products of tyrosine and ChA. Parent ions for the expected oxidation products of ChA were screened in the SIM mode and the fragmentation pattern was studied. Homo- and hetero-dimers oxidation products of ChA, i.e. ChA dimer (*m/z* 705), ChA-tryptophan (*m/z* 555) were screened. Nevertheless, the

corresponding parent ions were not found. When the SIM mode was used for oxidation products of tyrosine, DOPAchrome was found.

Quinic acid moiety in oxidised ChA

In **chapter 6**, the quinic acid released from the HMWFs and pellets, by alkaline treatment, was quantified and used as a strategy to calculate how much ChA was present in those fractions. When a mass balance of ChA was made, the total ChA content in the methanolic control (*MeOH*) was statistically equal to that in the $NaHSO_3$ control (*S*). Furthermore, the total amount of ChA found in the oxidized treatment (*OX*) represented 70% of that found in *S*. This observation suggested that in the *OX* treatment both moieties of ChA were oxidised. As suggested in **chapter 6**, ChA quinone and/or ChA dimer quinone reacted with proteins. Although quinic acid is not a substrate of PPO, it could be that ChA quinone, or other oxidizing agents generated during the enzymatic browning, oxidized the quinic acid moiety of the ChA attached to proteins.

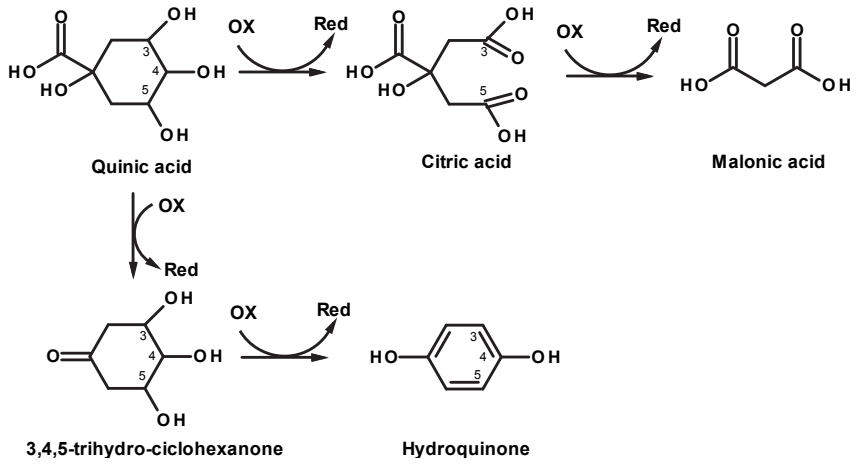

Figure 2. Oxidation of quinic acid. Figure based on references (*8*) and (*9*).

It has been shown that quinic acid can be oxidised by H_2O_2 to yield citric acid and malonic acid (*8*). This reaction involves the conversion of the C-3 and C-5 hydroxyl groups of quinic acid into aldehyde groups and then into carboxylic groups (**Figure 2**). Quinic acid can also be oxidised in the presence of NaOCl into 3,4,5-trihydrocyclo hexanone, which can be further oxidised to hydroquinone (*9*). If such reactions occur to the quinic acid moiety of the ChA-protein complexes, the oxidizing agents might be ChA quinone or ChA dimer (ChA_2) quinone. These reactions might eventually lead to cleavage of the ester

linkage of ChA. Consequently, oxidation products of quinic acid, instead of quinic acid itself, might be expected, and some of the modified quinic acid might be un-attached as citric acid, malonic acid and other unknown compounds. Furthermore, oxidation products of quinic acid might react with proteins, as a result of which either unknown compounds might be released after alkaline hydrolysis, or the (modified) quinic acid might not be released at all from the proteins after alkaline hydrolysis.

If the quinic acid moiety is oxidised and released from ChA during oxidation, one would expect homo- and hetero-dimers (or even trimers) as oxidation products of ChA, not only containing ChA, but also caffeic acid. On the other hand, if oxidised quinic acid is not released from ChA, it could participate in further cross-linking with low molecular weight compounds and with proteins. These possible scenarios make the identification and quantification of oxidation products of ChA difficult.

Anti-browning effect of sodium hydrogen sulfite

Starch production from potato requires the use of $NaHSO_3$ or related compounds to prevent enzymatic browning. In **chapter 4** we provided evidence, for the first time, on the formation of sulfonic acid derivatives of *o*-diphenolic compounds as a mechanism of browning inhibition. We showed that the use of $NaHSO_3$ during the preparation of potato juice generated sulfonic acid derivatives of the two most abundant *o*-diphenolics: ChA and *crypto*-ChA. 2'-Sulfo-5-*O*-caffeoyl quinic acid (2'-sulfo-ChA), followed in abundance by 2'-sulfo-4-*O*-caffeoyl quinic acid (2'-sulfo-*crypto*-ChA), were the two most abundant compounds found in potato extract upon $NaHSO_3$ treatment. Other *o*-diphenolics also followed the same reaction with $NaHSO_3$: caffeic acid, caffeoyl putrescine, *neo*-ChA, rutin and quercetin-3-*O*-diglucoside. In **chapter 5** we demonstrated that $NaHSO_3$ has a double action as an anti-browning agent. On the one hand it reacts with ChA quinone to produce 2'-sulfo-ChA, stopping the oxidation process, and on the other hand it inhibits PPO. As stated in **chapter 1**, it has been suggested that $NaHSO_3$ can reduce the *o*-quinones back to their *o*-diphenolics. Nevertheless, we did not perform experiments to prove this mechanism of action.

Because of the allergies related to the use of $NaHSO_3$ (*10*), a concentration of this compound as low as possible is advisable. At $NaHSO_3$ concentrations lower than that of phenolics, quenching of all quinones formed by $NaHSO_3$ is impossible. During the time required for $NaHSO_3$ to inactivate PPO, the surplus of quinones formed will rapidly react further, leading to brown color formation. It would, thus, be advantageous to complement the action of $NaHSO_3$ with other anti-browning agents to counteract the action of PPO during the time span that $NaHSO_3$ requires to inactivate PPO. Both ascorbic acid and citric acid might be used for this purpose, in a mixture with $NaHSO_3$. Ascorbic acid might reduce

the *o*-quinones back to their corresponding *o*-diphenolics at the initial stages of the reaction, providing the time required to inactivate PPO by $NaHSO_3$. The PPO-inhibitor properties of citric acid comprise its chelating (of the catalytic copper ions in PPO's active site) and acidulant properties. Both combinations, ascorbic acid/$NaHSO_3$ and citric acid/$NaHSO_3$, might reduce the pH of potato juice, if the buffering capacity of potato juice is overcome. At lower pH, away from the pH optimum of PPO, quinone formation will be severely reduced. It has been shown that potato proteins precipitate when modifying the pH of the medium from 7.0 to lower values (*11*). For example, only 40% of the proteins remained soluble when the pH of the medium was lowered to 5.0 by the addition of citric acid (*11*). As a consequence of the reduction in the solubility of proteins, a starch richer in protein and a potato juice with a lesser amount of proteins might be obtained. This indicates that there might not always be a simple solution for counteracting PPO action in the time span required for direct inactivation of PPO by $NaHSO_3$.

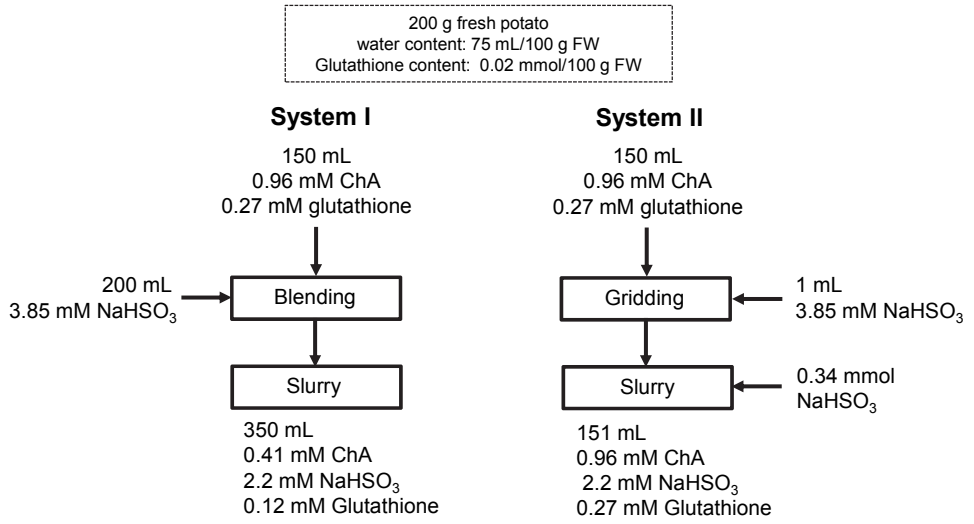

Figure 3. Molar mass balance of $NaHSO_3$ and glutathione when potato juice was prepared by blending 200 g fresh potato with 200 mL of 400 mg/mL $NaHSO_3$ (*system I*) and when it was prepared by blending 200 g fresh $NaHSO_3$-soaked potato slices and solid $NaHSO_3$ (35 mg) was added to the slurry (*system II*). Glutathione content was estimated according to reference (*12*). ChA concentration was calculated from values given in **chapter 4**.

When testing the effect of $NaHSO_3$ on the composition of *o*-diphenolic compounds in potato juice, 200 g of potato were blended with 200 mL of 400 ppm (3.85 mM) $NaHSO_3$ (*system I*). Different to the four extractions that were described in **chapter 4**, in *system I* only one extraction was performed. We also performed an extraction without addition of water (*system II*). In both *systems I* and *II*, potatoes from the Nicola variety were

tested. In *System II*, 200 g fresh potatoes were sliced, soaked in an aqueous solution of 400 mg/L $NaHSO_3$. Potato slices were gently shaken in a sieve to remove the excess of $NaHSO_3$ solution, and milled in a domestic type extractor (approximately 1 mL of the 400 mg/mL $NaHSO_3$ solution remained with the potato slices). After milling, 35 mg (0.34 mmol) of solid $NaHSO_3$ was added to the slurry. In both systems *I* and *II*, starch was sedimented for 20 min at 4 ºC and the supernatant was centrifuged (18,000 g; 20 min; 4 ºC). A molar mass balance for the main components in systems *I* and *II* is shown in **Figure 3**. After removing proteins, by acidic precipitation and ultrafiltration, the supernatants were analysed by RP-UHPLC (method as in **chapter 4**). Chromatographic profiles of phenolic compounds in *systems I* and *II* are shown in **Figure 4**.

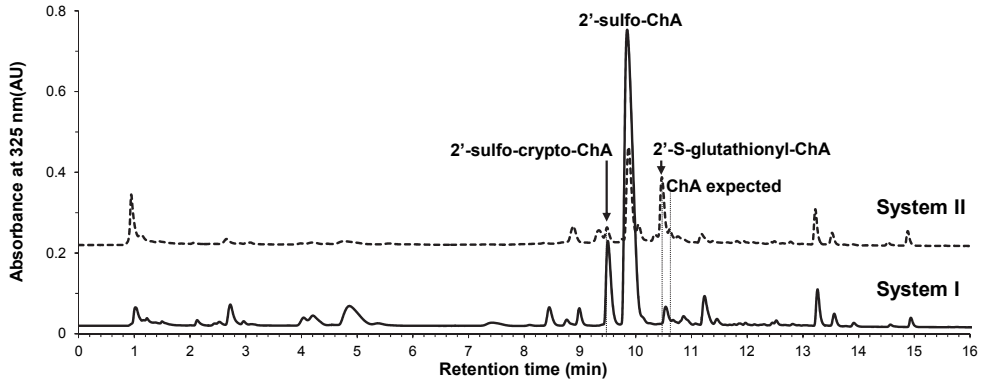

Figure 4. UHPLC chromatogram of potato juice prepared in the presence of $NaHSO_3$, with (*system I*) and without water (*system II*).

In *systems I* and *II*, neither ChA nor *crypto*-ChA were found. Instead the sulfonic acid derivatives were found. Retention times, UV and MS^n data of 2'-sulfo-ChA and 2'-sulfo-*crypto*-ChA were as in **chapter 4**. In *system II*, surprisingly, a glutathionyl adduct was observed as well. Parent ion $[M-H]^-$ = 658 and base peak after fragmentation *m/z* 385 were consistent with this compound being a glutathionyl-caffeoyl quinic acid adduct. As described in **chapter 4**, the 2'-C position in ChA quinone is the most activated and, therefore, more prone to nucleophilic attack. Accordingly, the most abundant glutathionyl-caffeoyl quinic acid isomer in *system II* is thought to be 2'-*S*-glutathionyl-ChA (**Figures 4** and **5**). This product is also formed in minute amounts in *system I*.

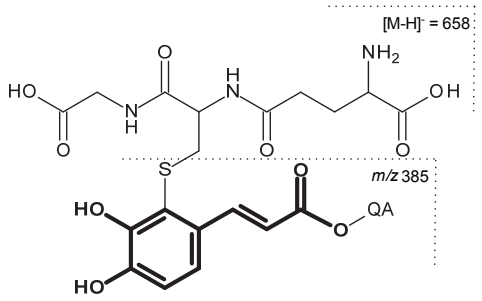

Figure 5. Fragmentation expected for 2'-*S*-glutathionyl-chlorogenic acid. QA=quinic acid.

Formation of glutathionyl adducts in *system II* might be the result of the limited amount of $NaHSO_3$ present in comparison to the production of ChA quinone during the first seconds of the extraction, at the beginning of the blending (**Figure 3**). In *system II*, potato slices were soaked in $NaHSO_3$ before blending. During the time span between blending and the addition and solubilisation of $NaHSO_3$ into the slurry, only a small amount of $NaHSO_3$ was present. We estimated that after soaking only 1 mL of 3.85 mM $NaHSO_3$ remained with the sliced potatoes. That would account for 0.03 mM $NaHSO_3$ in the slurry before adding the solid $NaHSO_3$, in contrast to the 0.96 mM ChA present in *system II*. Under those conditions, ChA quinone reacted with gluthatione. Once $NaHSO_3$ was added to the slurry, ChA quinone reacted with $NaHSO_3$. Because *system II* was more concentrated than *system I*, and assuming that 2'-*S*-glutathionyl-ChA has a similar molar extinction coefficient as 2'-sulfo-ChA, one would expect that the peak areas for ChA containing compounds would be higher in *system II* than in *system I*. When looking at the peak areas in both systems we found that the total area for ChA derivatives was much lower in *system II* than in *system I*. From **Figure 3** it can be observed that in the slurry of *system II*, glutathione (0.27 mM) was present in a lower molar amount than ChA (0.96 mM). Therefore, reaction of ChA quinone with proteins might occur, before addition of $NaHSO_3$ to the slurry. The reaction of ChA quinone with proteins might explain the lower peak area of the chromatogram at 325 nm observed in *system II* than in *system I*.

In *system I*, both $NaHSO_3$ and endogenous glutathione were present from the beginning of the extraction. As final products, mainly sulfonic acid derivatives of *o*-diphenolics were found, and only a minute amount of 2'-glutathionyl-ChA. The relatively higher abundance of sulfonic acid adducts in comparison to the glutathionyl adduct, in *system I*, is in accordance with the higher concentration of $NaHSO_3$ (2.2 mM) compared to glutathione (0.12 mM), and with the similar reactivity towards quinones of the two sulphur compounds (*13*) at the pH conditions employed (*14*).

Future prospects

The research carried out in this Ph.D. study revealed new information in terms of the HCAcs/DHCAcs present in potato tubers. HCAcs/DHCAcs had been underestimated for a number of representatives and, therefore, also the total quantity of HCAcs/DHCAcs had been understimated. While cultivars rich in HCAcs/DHCAcs might be desirable concerning their health-promoting effects, from a technological point of view those HCAcs/DHCAcs-rich cultivars might be detrimental to the techno-functional properties of other important potato constituents, such as proteins.

ChA is well-known because of its anti-oxidant properties (*15*). The antioxidant capacity of ChA has been attributed on the one hand to its chelating activity towards transition metals by the hydroxyl groups of the aromatic ring (*16*), and on the other hand to its radical-scavenging activity (*17, 18*). The radical-scavenging activity has been linked to the hydrogen-donating capacity of the hydroxyl groups of polyphenols, to their redox potential and to the stability of the phenoxy radicals (*18-20*). Minimally processed potatoes, such as pre-peeled potatoes or sliced potatoes, are dipped in anti-browning agents, such as $NaHSO_3$ (*21*). In countries, such as Colombia, the use of sulfiting agents is allowed in the food industry. For example, in pulp fruit preservation at refrigerator temperatures, sulfiting agents are commonly used. With the possible presence of *o*-diphenolic compounds in the treated fruit, the formation of sulfo-phenolic derivatives is likely to occur. It might be expected, therefore, that the electron withdrawing effect of the sulfonic acid moiety would affect the anti-oxidant properties of sulfo-phenolics as a result of the deactivation of the aromatic ring (*18, 19, 21*). The main concern on sulfiting agents has been focused on the adverse pulmonary reaction in people (*10*). With our finding of large quantities of sulfo-phenolics in processed potato, and the probability that sulfo-phenolics might also be formed upon processing of other fruits and vegetables, it might be worthwhile to consider their bioactivity, i.e. by investigating whether sulfo-ChA (and other sulfo-phenolics) can cause allergic reactions at the concentrations present in sulfite-treated plant materials.

Enzymatic browning during preparation of potato juice was found to consume all *o*-diphenolic compounds present, represented mainly by ChA. While low molecular weight oxidation products of ChA were found in trace amounts, the majority of the oxidised ChA was found associated to soluble proteins. Potato juice is composed of PPO, ChA, other phenolics (sinapic acid and ferulic acid containing compounds), amino acids, glutathione and proteins. In this complex natural setting, we propose that oxidised ChA is associated mainly to proteins after enzymatic browning. Whether ChA quinone or ChA_2 quinone reacted with proteins to produce both soluble and insoluble complexes is still unknown. Furthermore, whether ChA quinone and or ChA_2 quinone reacted with amino acids, or with other phenolics, and after re-oxidation (by PPO or by non-enzymatic reactions), reacted

with proteins, still remains to be answered. In this respect, MALDI-TOF MS might be a useful technique for the further elucidation of the mechanism behind the reaction between proteins and ChA. In literature it has been proposed that oxidised phenolics can also react with themselves or with other phenolics to produce (co) polymeric phenolics (*22*). Our results strongly suggested that the oxidation products of the main phenolic compound, ChA, were linked to protein fractions as main oxidation product, and not as polymeric ChA.

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Summary
Samenvatting
Resumen

SUMMARY: Hydroxycinamic acids (HCAs) and their conjugates (HCAcs) are the most abundant phenolic compounds in potato tubers. Dihydrohydroxycinnamic acid conjugates (DHCAcs) are less abundant in potato tuber. HCAs/HCAcs/DHCAcs are correlated with health-promoting effects and are involved in plant resistance against stress. In addition, they are involved in enzymatic browning during processing, which might be detrimental to color and functional properties of other components such as proteins. Most of the studies have been focused on the quantification of chlorogenic acid (ChA), the most abundant HCA-conjugate in potato tuber, its isomers *crypto*-ChA and *neo*-ChA, caffeic acid, and anthocyanin-linked HCAs, in whole tubers. Sometimes, these studies monitor the changes in the levels of the compounds mentioned during processing. Information about other HCAcs/DHCAcs is less documented. Furthermore, there are no systematic studies on the fate of ChA during enzymatic browning in potato juice. The aim of the current research was to study the HCAs/HCAcs/DHCAcs composition of potato tubers and their behaviour during the preparation of potato juice, prepared with and without the use of anti-browning agents, commonly used in the potato starch isolation process.

Chapter 1 provides a general introduction on the nutritional components of potato tubers, highlighting the composition of phenolic compounds. A background in terms of the chemical structure, relative abundance, analytical techniques and strategies for analysis is provided. The health-promoting effects of HCAs/HCAcs/DHCAcs are also discussed. Based on results from model experiments, the role of polyphenoloxidase (PPO) and its activity towards ChA and caffeic acid in enzymatic browning is elaborated.

In **chapter 2**, an analytical method was developed for the identification and quantification of HCAs/HCAcs/DHCAcs in a single cultivar. The method was based on reversed phase (RP) - ultra high performance liquid chromatography (UHPLC) - diode array detector (DAD) - electrospray ionization (ESI) - tandem mass spectrometry (MS^n). In contrast to other analytical methods (in which the assignment of compounds was primarily based on UV-vis spectroscopy of individual peaks in chromatograms), our analytical method started with the screening of compounds from MS data. The strategy of the identification was based on three steps: i) screening for negative ions and neutral losses characteristic to HCAs, ii) calculation of the molecular weight of the parent ion, and iii) verification of the annotation of compounds as HCAcs/DHCAcs by checking the UV spectra. A total of 39 HCAs/HCAcs/DHCAcs were found, with ChA as the most predominant representative. Only 17 compounds had been described in potato tubers previously. For quantification, the external standard method was used. Quantification of HCAs after alkaline hydrolysis of a crude potato extract indicated that there was good accuracy in the quantification of the large array of compounds by the use of just a few standards.

In **chapter 3**, the RP-UHPLC-DAD-heated ESI-MS^n was used to further study the composition of HCAs/HCAcs/DHCAcs in the most cultivated Colombian potato cultivars. High variation in terms of number of representatives as well as in their concentration was observed. The contribution of the less commonly reported HCAcs/DHCAcs ranged between 7.1 to 20.1 % (w/w) of the total HCAs/HCAcs/DHCAcs in whole tuber. More emphasis, therefore, should be put on the composition of less commonly reported compounds when compositional studies on potato tubers are performed. ChA, typically reported in potato cultivars, was the most abundant compound in all cultivars, in both peel and flesh. *Neo*-ChA, also commonly reported in potato tubers, ranged in concentrations (0.8 - 7.4 mg/100 g potato dried weight, DW), similar to other compounds less commonly reported in potato tubers, e.g. sinapoyl hexose (0.1 - 1.8 mg/100 g potato DW), feruloyl octopamine (1.2 - 5.2 mg/100 g potato DW), and Kukoamine A (0.2 - 1.7 mg/100 g potato DW).

With the analytical RP-UHPLC-DAD-(H)ESI-MS^n method developed for the analysis of HCAs/HCAcs/DHCAcs, the effect of the use of the anti-browning agents $NaHSO_3$ and ascorbic acid on the HCAs/HCAcs composition, while preparing potato juice, was tested (**chapter 4**). For years, it has been accepted that one of the possible mechanisms of action of $NaHSO_3$ as anti-browning agent is its capacity of react with *o*-quinones to form adducts. Nevertheless, no actual molecular information had been provided until now. In **chapter 4** we showed that with the use of ascorbic acid, a typical HCAs/HCAcs composition was obtained: ChA and *crypto*-ChA were the two most abundant phenolic compounds found in the potato juice prepared. In contrast, with the use of $NaHSO_3$, neither ChA nor *crypto*-ChA were found, but their sulfonic acid adducts: 2'sulfo-chA and 2'-sulfo-*crypto*-ChA. Their molecular identification was achieved by ESI-MS^n data from the mass spectrometer coupled *in-line* to the RP-UHPLC, and by nuclear magnetic resonance (NMR) spectroscopy of purified compounds. PPO was proven to be involved in the production of the sulfonic acid adducts. Results from quantification of (sulfo) ChA isomers suggested that, in the presence of ascorbic acid, ChA partially reacted with proteins. Good accuracy in the quantification of sulfonic acid derivatives was achieved by using PPO-synthetized 2'-sulfo-ChA as standard, as the molar extinction coefficient of 2'-sulfo-ChA (9,357±395 $M^{-1}cm^{-1}$) was lower than that of ChA (18,494±196 $M^{-1}cm^{-1}$).

In **chapter 5**, the anti-browning effect of ascorbic acid, $NaHSO_3$, and other sulphur-containing compounds (cysteine, glutathione and dithiothreitol), was further investigated based on model experiments. ChA, PPO, and the anti-browning agents mentioned were incubated under controlled conditions. $NaHSO_3$ had a double action as anti-browning agent: on the one hand, it yielded sulfo-ChA adducts, and on the other hand, it inactivated, in a time-dependent way, PPO. It was found that ascorbic acid reduced the *o*-quinones, generated by the PPO catalized oxidation of ChA, to the non-oxidized ChA. The main disadvantage of ascorbic acid is that once it is totally reacted, browning still

occurs. The anti-browning effect of cysteine and glutathione was related to their capacity to yield cysteinyl-ChA and glutathionyl-ChA adducts, respectively. In contrast, dithiothreitol did not yield adducts with ChA, but inactivated PPO. No inhibitory action towards PPO was observed for sulfo-ChA or cysteinyl-ChA.

In **chapter 6**, the molecular identity and quantity of reaction products of a brown potato juice was evaluated. For doing so, a potato juice without the use of any anti-browning agent (*OX*) was prepared. The juice was fractionated into a low molecular weight fraction (LMWF) and a high molecular weight fraction (HMWF). The pellet after extraction was also analysed. Control extracts with an aqueous acidic methanolic solvent (*MeOH*) and with an aqueous $NaHSO_3$ solution (*S*) were prepared. No ChA as such was found in the LMWF of the *OX* treatment. In that fraction, trace amounts of oxidation products of ChA were found. "Reacted" ChA was found mainly linked to the *OX*-HMWF (49.8±7.1 mg ChA/100 g potato DW). In the *OX*-pellet, 21.4±3.5 mg "reacted" ChA/100 g potato DW was found. A mass balance revealed that the total amount of ("reacted") ChA in the control *MeOH* (103.2±6.2 mg/100 g potato DW) was statistically equal to that in the control *S* (102.5±2.4 mg ChA/100 g potato DW). "Reacted" ChA in the *OX* treatment represented 70% of that found in the *S* control. Results from protease digestion of the proteins present in the *OX*-HMWF suggested that modified ChA was strongly associated with proteins. Whether this association is covalent in nature, whether oligomeric/polymeric ChA are non-covalently bound to proteins, awaits further proof.

In **chapter 7**, the use of RP-UHPLC-DAD-ESI-MS^n as an analytical technique for the identification of HCAs in crude (oxidized) potato extract was addressed. The identification and quantification of the quinic acid moiety in oxidized ChA is described as part of a decision tree in which a general strategy to the identification of oxidation products of ChA is provided. Screening in full MS^n and in selected ion monitoring (SIM) are part of the method. Finally, the use of $NaHSO_3$ and other anti-browning agents, implications and perspectives of the current research are provided. The use of combinations of $NaHSO_3$/ascorbic acid or $NaHSO_3$/citric acid might be more advantageous than the use of $NaHSO_3$ alone. The main advantage of such combinations might be that the action of PPO is immediately counteracted by reduction of quinones (ascorbic acid), or inhibited (acidulant effect of citric acid and ascorbic acid), while $NaHSO_3$ directly inactivates PPO in a slow fashion, but with a more durable effect.

SAMENVATTING: Hydroxykaneelzuren (hydroxycinnamic acids, HCAs) en hun conjugaten (HCAcs) zijn de meest voorkomende phenolische verbindingen in aardappelknollen. Dihydroxykaneelzuurconjugaten (DHCAcs) komen minder voor in aardappels. HCAs, HCAcs en DHCAcs worden geassocieerd met gezondheidsbevorderende effecten en zijn betrokken bij plantenafweer tegen stress. Daarnaast zijn ze betrokken bij enzymatische bruinkleuring tijdens verwerking, hetgeen negatieve gevolgen voor de kleur en de functionele eigenschappen van andere verbindingen, zoals eiwitten, kan hebben. De meeste onderzoeken zijn gericht op het kwantificeren van chlorogeenzuur (chlorogenic acid, ChA), het meest aanwezige HCAc in aardappels, en zijn isomeren *crypto*-ChA en *neo*-ChA, koffiezuur, en met anthocyaan verbonden HCAs. In sommigen van deze studies worden veranderingen in gehaltes van deze verbindingen gedurende bewerking gevolgd. Over andere HCAcs en DHCAcs is minder informatie beschikbaar. Bovendien zijn er geen systematische studies naar het lot van ChA tijdens enzymatische bruinkleuring van aardappelsap. Het doel van het huidige onderzoek was het bestuderen van de HCAs/HCAcs/DHCAcs compositie van aardappels en het gedrag van deze componenten tijdens het bereiden van aardappelsap met en zonder anti-bruinings middelen zoals gewoonlijk gebruikt in het aardappelzetmeel isolatieproces.

Hoofdstuk 1 geeft een algemene inleiding op de voedingskundige samenstelling van aardappels, met speciale aandacht voor de compositie van phenolische verbindingen. Er wordt achtergrondinformatie over de chemische structuur, relatieve hoeveelheid, analytische technieken en analysestrategieën gegeven. De gezondheidsbevorderende effecten van HCAs, HCAcs en DHCAcs worden ook besproken. Gebaseerd op resultaten van modelexperimenten wordt de rol van PPO en zijn activiteit op ChA en koffiezuur gedurende enzymatische bruining behandeld.

In **hoofdstuk 2** wordt een analytische methode voor de identificatie en kwantificatie van HCAs, HCAcs en DHCAcs in een enkele cultivar beschreven. De methode is gebaseerd op *reversed phase (RP) – ultra high performance liquid chromatography (UHPLC) – diode array detector (DAD) – electrospray ionization (ESI) – tandem mass spectrometry (MS^n)*. In tegenstelling tot andere analytische methoden (waar toekenning van componenten vooral gebaseerd wordt op UV-vis spectroscopie van individuele pieken in chromatogrammen) begon onze methode met het screenen voor verbindingen in MS data. De strategie voor identificatie was gebaseerd op drie stappen : i) screenen voor negatieve ionen en neutrale verliezen karakteristiek voor HCAs, ii) berekening van het molecuulgewicht van het *parent ion*, en iii) bevestigen van annotatie van verbindingen als HCAcs of DHCAcs door controle van de UV spectra. In totaal werden 39 HCAs, HCAcs en DHCAcs gevonden, met ChA als meest voorkomende verbinding. Slechts 17 verbindingen waren eerder in aardappels beschreven. Voor kwantificatie werd gebruik gemaakt een externe standaard. Kwantificatie van HCAs na alkalische hydrolyse

van een ruw aardappelextract gaf aan dat een grote variëteit aan verbindingen nauwkeurig gekwantificeerd kon worden door gebruik te maken van slechts enkele standaarden.

In **hoofdstuk 3** wordt gebruik gemaakt van RP-UHPLC-DAD-heated ESI-MS^n om de compositie van HCAs, HCAcs en DHCAcs in de meest geteelde Colombiaanse aardappel variëteiten in meer detail te bestuderen. Grote verschillen werden gezien in zowel het aantal aanwezige verbindingen als in hun concentraties. Het aandeel van minder vaak gerapporteerde HCAcs en DHCAcs varieerde tussen 7,1 en 20,1 % (w/w) van het totaal gehalte aan HCAs, HCAcs en DHCAcs in de hele aardappel. Daarom zou er meer nadruk moeten worden gelegd op het gehalte aan verbindingen die nu minder vaak gerapporteerd worden in studies naar de samenstelling van aardappels. In alle cultivars was ChA de verbinding die het meest voorkwam, zowel in de schil als het vruchtvlees. *Neo*-ChA, ook vaak gerapporteerd in aardappels, kwam voor in concentraties (0,8 - 7,4 mg/100 g aardappel drooggewicht, DW) vergelijkbaar met andere verbindingen die minder vaak gerapporteerd worden in aardappels, bijvoorbeeld sinapoly hexose (0,1 - 1,8 mg/100 g DW), feruloyl octopamine (1,2 - 5,2 mg/100 g DW) en kukoamine A (0,2 - 1,7 mg/100 g DW).

De analytische RP-UHPLC-DAD-(H)ESI-MS^n methode ontwikkeld voor de analyse van HCAs, HCAcs en DHCAcs werd gebruikt om het effect van de anti-bruiningsmiddelen $NaHSO_3$ en ascorbinezuur op de concentratie HCAs en HCAcs tijdens het maken van aardappelsap te onderzoeken (**hoofdstuk 4**). Al jaren wordt aangenomen dat één van de mogelijke mechanismen waarmee $NaHSO_3$ bruining voorkomt berust op de reactie van $NaHSO_3$ met *o*-chinonen, waarbij adducten gevormd worden. Desalniettemin is er tot op heden geen daadwerkelijk moleculair bewijs geleverd voor vorming van adducten. In hoofdstuk 4 tonen we aan dat door gebruik te maken van ascorbinezuur een typische HCAs/HCAcs samenstelling wordt verkregen: ChA en *crypto*-ChA waren de phenolische verbindingen die in de hoogste concentratie werden gevonden in het aardappelsap. Met gebruik van $NaHSO_3$ daarentegen, werd ChA, noch *crypto*-ChA gevonden, maar wel hun sulfonzuur adducten: 2'-sulfo-ChA en 2'-sulfo-*crypto*-ChA. Moleculaire identificatie van deze verbindingen werd verkregen door ESI-MS^n data van de *in lijn* aan de RP-UHPLC gekoppelde massa spectrometer, en door kernmagnetische resonantie (nuclear magnetic resonance, NMR) spectroscopie van gezuiverde verbindingen. Aangetoond werd dat PPO betrokken is bij de vorming van sulfonzuur adducten. Kwantificatie van (sulfo) ChA isomeren suggereerde dat in de aanwezigheid van ascorbinezuur ChA voor een deel reageerde met eiwitten. Accurate kwantificatie van sulfonzuur afgeleiden werd bereikt door gebruik te maken van PPO-gesynthetiseerd 2'-sulfo-ChA als standaard, omdat de molaire extinctie coëfficiënt van 2'-sulfo-ChA (9.357 ± 395 M^{-1} cm^{-1}) lager was dan die van ChA (18.494 ± 196 M^{-1} cm^{-1})

In **hoofdstuk 5** werden de anti-bruinings eigenschappen van ascorbinezuur, $NaHSO_3$ en andere zwavelhoudende verbindingen (cysteïne, glutathion en dithiothreitol)

onderzocht met behulp van model experimenten. ChA, PPO en de genoemde anti-bruiningsmiddelen werden geïncubeerd onder gecontroleerde condities. $NaHSO_3$ had een dubbele werking als anti-bruiningsmiddel: enerzijds zorgde het voor de vorming van sulfo-ChA adducten, anderzijds remde het PPO op een tijdsafhankelijke manier. Gevonden werd dat ascorbinezuur de *o*-chinonen, resulterend van PPO-gekatalyseerde oxidatie van ChA, reduceerde naar ChA. Het grootste nadeel van ascorbinezuur is dat bruining nog steeds optreedt, nadat alle ascorbinezuur is geoxideerd. Het anti-bruinings effect van cysteïne en glutathion berustte op het feit dat ze cysteinyl-ChA en glutathionyl-ChA konden vormen. In tegenstelling tot dithiothreitol, dat geen ChA adducten vormde maar PPO remde. Geen remmende werking op PPO werd geobserveerd voor sulfo-ChA of cysteinyl-ChA.

In **hoofdstuk 6** worden de reactieproducten in een bruin aardappelsap onderzocht. Om dit mogelijk te maken werd een aardappelsap gemaakt, zonder gebruik te maken van enige anti-bruiningsmiddelen (*OX*). Het extract werd gefractioneerd in een fractie met laag moleculair gewicht (LMWF) en in een fractie met hoog moleculair gewicht (HMWF). Het onoplosbare materiaal na extractie werd ook geanalyseerd. Controle extracten werden gemaakt met waterige, aangezuurde methanol (*MeOH*) en met een $NaHSO_3$ oplossing in water (*S*). Geen ChA als zodanig werd gevonden in LMWF van het *OX* monster. In deze fractie werden sporen van oxidatieproducten van ChA gevonden. ChA dat had gereageerd werd voornamelijk gevonden gebonden aan *OX*-HMWF (49,8 ± 7,1 mg ChA/100 g aardappel DW). In het onoplosbare materiaal van *OX* werd 21,4 ± 3,5 mg gereageerd ChA/100 g aardappel DW gevonden. Een massabalans onthulde dat de totale hoeveelheid (gereageerd) ChA in de controle *MeOH* (103,2 ± 6,2 mg/100 g aardappel DW) statistisch gelijk was aan de hoeveelheid in controle *S* (102,5 ± 2,4 mg/100 g aardappel DW). Gereageerd ChA in het *OX* monster vertegenwoordigde 70% van de hoeveelheid gevonden in de *S* controle. Resultaten van protease digestie van de eiwitten aanwezig in *OX*-HMWF suggereerden dat gemodificeerd ChA sterk gebonden was met eiwitten. Of deze binding covalent is, of dat oligo- of polymeer ChA non-covalent gebonden is aan eiwitten moet nog verder worden onderzocht.

In **hoofdstuk 7** wordt het gebruik van RP-UHPLC-DAD-ESI MS^n als een analytische techniek voor de identificatie van HCAs in ruw (geoxideerd) aardappelextract besproken. De identificatie en kwantificatie van de chinazuur groep in geoxideerd ChA wordt beschreven als onderdeel van een beslisboom, welke gebruikt kan worden als een algemene strategie voor het identificeren van oxidatieproducten van ChA. Screenen in volledige MS^n en in selectieve ion monitoring (SIM) zijn onderdeel van die methode. Tot slot worden het gebruik van $NaHSO_3$ en andere antibruiningsmiddelen en implicaties en perspectieven van het huidige onderzoek besproken. Het gebruik van combinaties van $NaHSO_3$ en ascorbinezuur of $NaHSO_3$ en citroenzuur zouden voordeliger kunnen zijn dan gebruik van $NaHSO_3$ alleen. Het grootste voordeel van zulke combinaties zou kunnen zijn dat de activiteit van PPO onmiddellijk wordt tenietgedaan door reductie van chinonen (in

het geval van ascorbinezuur) of dat PPO wordt geremd (het aanzurende effect van citroen- en ascorbinezuur), terwijl $NaHSO_3$ PPO remt op een relatief langzame manier, maar met een meer duurzaam effect.

RESUMEN: Los compuestos fenólicos más abundantes en papa son los ácidos hidroxicinámicos (HCAs) y sus compuestos conjugados (HCAcs), siendo los compuestos conjugados de ácidos dihydro-hidroxicinámicos (DHCAcs) menos abundantes. HCAs/HCAcs/DHCAcs están relacionados con efectos positivos en la salud y están involucrados en la resistencia de las plantas al estrés. Este tipo de compuestos están también involucrados en el pardeamiento enzimático que tiene lugar durante el procesamiento de papa en fábrica, el cual va en detrimento del color y de las propiedades funcionales de otros compuestos, como las proteínas. La mayoría de los estudios llevados a cabo hasta el momento se han enfocado en la cuantificación de ácido clorogénico (ChA) (HCAc más abundante en papa), sus isómeros *cripto*-ChA y *neo*-ChA, ácido caféico y HCAs que contienen antocianinas. En algunos casos, estos estudios registran los cambios producidos en los niveles de los compuestos anteriormente mencionados durante el procesamiento. A pesar de todos estos estudios, hay poca información disponible acerca de otros HCAcs/DHCAcs. Además, no hay estudios sistemáticos acerca del destino final del ChA durante el pardeamiento enzimático del jugo de papa, un subproducto de la industria de producción de almidón de papa. Por ello, el objetivo de esta investigación fue estudiar el contenido de la papa en HCAs/HCAcs/DHCAcs, así como su comportamiento durante la preparación del jugo de papa, preparado con y sin el uso de agentes anti-pardeamiento, los cuales son de uso común en la industria de obtención de almidón de papa.

El **Capítulo 1** brinda una introducción general de los componentes nutricionales de la papa, resaltando su contenido en compuestos fenólicos y efectos beneficiosos de los mismos (HCAs/HCAcs/DHCAcs) para la salud. En este mismo capítulo se proporciona también una descripción general de la estructura química, abundancia relativa, técnicas analíticas y estrategias de análisis de estos compuestos. Por último, se describe el rol de la polifenoloxidasa (PPO) y su actividad sobre el ChA y el ácido caféico en el pardeamiento enzimático en relación a los resultados obtenidos en estudios anteriores con sistemas modelo.

El **Capítulo 2** describe el desarrollo de un nuevo método analítico de cromatografía líquida de ultra alta eficacia (CLUAE) en fase inversa (RP) con detector de arreglo de diodos (DAD), acoplado a un detector de espectrometría de masas con ionización por electrospray (ESI-MS^n), para la identificación y cuantificación de HCAs/HCAcs/DHCAcs en un cultivar de papa. En contraste con otros métodos analíticos, en los que la asignación de los compuestos se basa principalmente en espectroscopía UV-vis de los picos cromatográficos, nuestro método analítico comienza con la búsqueda de compuestos con datos de masa proporcionados por el MS^n. La estrategia de identificación se basa en tres pasos: i) Búsqueda de iones negativos y pérdidas neutras característicos de HCAs, ii) cálculo del peso molecular con base en el ión molecular, y iii) verificación de la anotación de los compuestos como HCAcs/DHCAcs mediante estudio del espectro UV. En

total se encontraron 39 HCAs/HCAcs/DHCAcs, siendo el ChA el compuesto más abundante. Hasta el momento, sólo 17 compuestos habían sido descritos en papa. Para la cuantificación de los 39 HCAs/HCAcs/DHCAcs se empleó el método del estándar externo. La cuantificación de HCAs, tras la hidrólisis alcalina de un extracto de papa, mostró una buena exactitud en la cuantificación de una gran diversidad de compuestos mediante el uso de pocos estándares.

En el **Capítulo 3** la técnica RP-CLUAE-DAD-*heated* ESI-MS^n se empleó para estudiar la composición de HCAs/HCAcs/DHCAcs en los cultivares de papa más comercializados en Colombia. Se observó una alta variación tanto en el número de compuestos como en la concentración. La contribución de los compuestos HCAcs/DHCAcs, menos reportados en papa, varió entre el 7,1 y el 20,1% (w/w) en comparación con la concentración total de HCAs/HCAcs/DHCAcs. Por tanto, en futuros trabajos relacionados con el estudio de la composición nutricional de papa se debe poner más énfasis en la composición de los HCAcs/DHCAcs, reportados con menos frecuencia. El ChA, normalmente reportado en papa, fue el compuesto más abundante en todos los cultivares, tanto en la pulpa como en la corteza. El *neo*-ChA, también frecuentemente reportado en papa, varió en concentraciones (0,8 - 7,4 mg/100 g papa base seca, BS) similares a la de otros compuestos escasamente reportados, por ejemplo, sinapoil hexosa (0,1 - 1,8 mg/100 g papa BS), feruloil octopamina (1,2 - 5,2 mg/100 g papa BS) y Kukoamina A (0,2 - 1,7 mg/100 g papa BS).

Con el método analítico RP-CLUAE-DAD-(H)ESI-MS^n desarrollado para el análisis de HCAs/HCAcs/DHCAcs se estudió el efecto del empleo de los agentes anti-pardeamiento $NaHSO_3$ y ácido ascórbico sobre la composición de HCAs/HCAcs durante la preparación de jugo de papa (**Capítulo 4**). Durante muchos años se ha aceptado que uno de los posibles mecanismos de acción del $NaHSO_3$ como agente anti-pardeamiento es su capacidad de reaccionar con *o*-quinonas para formar aductos. Sin embargo, no se había proporcionado información molecular hasta ahora. En el **capítulo 4** mostramos que con el uso del ácido ascórbico se obtiene una composición típica de HCAs/HCAcs: los dos compuestos fenólicos más abundantes encontrados en el jugo de papa fueron ChA y *cripto*-ChA. En contraste, con el uso de $NaHSO_3$, no se encontró ni ChA ni *cripto*-ChA. En estas condiciones, se encontraron los derivados del ácido sulfónico: 2'-sulfo-ChA y 2'-sulfo-*cripto*-ChA. La identificación molecular de estos compuestos se hizo mediante ESI-MS^n acoplado en línea al RP-CLUAE y mediante resonancia magnética nuclear (RMN) de los compuestos purificados. Se probó que la PPO está involucrada en la producción de los derivados del ácido sulfónico. Los resultados de la cuantificación de los isómeros de (sulfo) ChA sugirieron que en presencia de ácido ascórbico el ChA reacciona parcialmente con proteínas. Debido a que el coeficiente de extinción molar del 2'-sulfo-ChA (9.357±395 $M^{-1}cm^{-1}$) fue menor que el de ChA (18.494±196 $M^{-1}cm^{-1}$), el empleo de 2'-sulfo-ChA,

sintetizado mediante PPO, como estándar, permitió la cuantificación exacta de los derivados del ácido sulfónico.

En el **Capítulo 5** se estudió, en experimentos modelo, el efecto anti-pardeamiento del ácido ascórbico, $NaHSO_3$ y de otros compuestos que contienen azufre (cisteína, glutatión y ditiotreitol). El ChA, PPO y los agentes anti-pardeamiento mencionados, fueron incubados bajo condiciones controladas. El $NaHSO_3$ mostró una doble acción anti-pardeamiento. Por un lado, éste generó aductos del tipo sulfo-ChA y, por otro lado, éste inactivó, de una manera dependiente del tiempo, la PPO. También se encontró que el ácido ascórbico reduce las quinonas, generadas por la oxidación del ChA por acción de la PPO, a la forma no oxidada de ChA. La principal desventaja del ácido ascórbico es que una vez éste es totalmente consumido, aún ocurre el pardeamiento. El efecto de los compuestos anti-pardeamiento cisteína y glutatión se relacionó con su capacidad de generar los aductos cisteinil-ChA y glutationil-ChA, respectivamente. En contraste, el ditiotreitol no generó aductos con el ChA. Sin embargo, este compuesto inactivó a la PPO. No se observó efecto inhibidor de sulfo-ChA ni de cisteinil-ChA sobre la actividad de PPO.

En el **Capítulo 6** se llevó a cabo la identificación molecular y cuantificación de los productos de reacción de un jugo pardeado de papa. Para esto, se preparó un jugo de papa sin el uso de ningún compuesto anti-pardeamiento (*OX*). El jugo fue fraccionado en una fracción de bajo peso molecular (FBPM) y en una fracción de alto peso molecular (FAPM). También se analizó el pellet al final de la extracción. Se prepararon extractos control con metanol acuoso acidulado (*MeOH*) y con una solución acuosa de $NaHSO_3$ (*S*). En la FBPM del tratamiento *OX* no se encontró ChA. En esa fracción se encontraron cantidades traza de productos de oxidación del ChA. El ChA "modificado" se encontró principalmente en la *OX*-FAPM (49,8±7,1 mg ChA/100 g papa BS). En el *OX*-pellet se encontraron 21,4±3,5 mg ChA "modificado"/100 g papa BS. El balance de masa reveló que la cantidad total de ChA ("modificado") en el control *MeOH* (103,2±6,2 mg/100 g papa BS) fue estadísticamente igual al encontrado en el control *S* (102,5±2,4 mg ChA/100 g papa BS). La cantidad de ChA "modificado" en el tratamiento *OX* representó un 70% (w/w) del encontrado en el control *S*. Los resultados de la digestión enzimática de las proteínas presentes en la *OX*-FAPM indicaron que el ChA "modificado" estaba fuertemente asociado a las proteínas. Futuras pruebas son necesarias para distinguir si esta asociación es de naturaleza covalente o si se trata de ChA oligomérico/polimérico asociado a proteínas de manera no covalente.

En el **Capítulo 7** se analizó el empleo de la técnica RP-CLUAE-DAD-(H)ESI-MS^n en el estudio de extractos (oxidados) de papa. La identificación y cuantificación del ácido quínico en el ChA oxidado se describe como parte de un diagrama de toma de decisiones, en el que se proporciona una estrategia general para la identificación de productos de oxidación de ChA. La búsqueda en el modo "full MS^n" y en el modo de monitoreo selectivo de iones (SIM) son también parte del método. Finalmente, se proporciona información sobre el uso, implicaciones y perspectivas de $NaHSO_3$ y de otros

agentes anti-pardeamiento. El empleo de mezclas $NaHSO_3$/ácido ascórbico o $NaHSO_3$/ácido cítrico podría tener mayores ventajas que el empleo de únicamente $NaHSO_3$. La ventaja principal de tales combinaciones estaría relacionada con la reducción de las quinonas (ácido ascórbico), generadas por acción de la PPO, y con la inhibición de la PPO (efecto acidulante del ácido cítrico y del ácido ascórbico), mientras que el $NaHSO_3$ inhibe de una manera lenta pero con efecto duradero la PPO.

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About the author

Curriculum Vitae

Carlos Eduardo Narváez Cuenca was born on December 28th 1972 in Iquira, Colombia. After graduating from secondary education (Colegio Misael Patrana Borrero, Teruel-Colombia) in 1988, he moved to Bogotá and started his study in 1989 in Food Engineering at Universidad Jorge Tadeo Lozano. In 1992 he started his study in Chemistry at Universidad Nacional de Colombia (UNal). He graduated from two separate studies, from Food Engineering in 1995 and from Chemistry in 1998. His thesis in Chemistry was performed in the field of soil chemistry, on the adsorption of water-soluble aluminium ions by composts and raw plant materials, with professor Carmen-Rosa Gómez-Laverde. He performed the second half of his Chemistry studies with a fellowship provided from UNal.

The first half of 1999 he worked as a lecturer at Universidad Central, Bogotá. Following this, he moved to Florencia (south of Colombia) to work as a lecturer at Universidad de la Amazonia from 1999 to 2004. In the meantime, from 2000 to 2002, he followed his Master studies in chemistry at UNal with a fellowship provided by the aforementioned university. His MSc thesis was on the study of the polyphenol oxidase, peroxidase and catalase activities during ripening and refrigerated storage of *Pourouma cecropiaefolia*, a fruit from the Amazon rainforest. He conducted his MSc thesis under the supervision of professor Luz-Patricia Restrepo-Sánchez.

In 2005, Carlos got a permanent position at Universidad Nacional de Colombia as an assistant professor at the Chemistry Department of analytical chemistry and food chemistry. In 2006 he visited the Laboratory of Food Chemistry at Wageningen University, The Netherlands, for a period of six months, where he studied the nature of a yellowish coloration observed in purified potato protein fractions. He was supervised by dr. Nathalie Creusot and by professor dr. ir Harry Gruppen. After that experience, Carlos was offered the opportunity to start his PhD under the supervision of prof. Gruppen. In 2009, while he was still employed by Universidad Nacional de Colombia, he started his PhD at Wageningen University in the Laboratory of Food Chemistry, as a guest employee, with a fellowship provided by Colciencias, a Colombian institution that supports research and technology in Colombia. From 2009 to 2013 he conducted his PhD research on the diversity and reactivity of phenolic acids in potato during processing as described in this thesis. After his defence he will resume his work at UNal in Bogotá.

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Overview of completed training activities

Discipline Specific Activities

- 3rd international advanced course on industrial proteins, Wageningen, The Netherlands, 2006
- 6th international advanced course on reaction kinetics in food science, Wageningen, The Netherlands, 2009
- XXV International conference in polyphenols, Montpellier, France, poster presentation, 2010
- 3rd international course advanced food analysis, Wageningen, The Netherlands, 2010
- Advanced statistics course design of experiments, Wageningen, The Netherlands, 2010
- Euro-Mediterranean symposium for fruit & vegetable processing, Avignon, France, oral presentation, 2011
- XXVI International conference in polyphenols. Florence, Italy, poster presentation, 2012

General Courses

- VLAG PhD week, Maastricht, The Netherlands, 2009
- English speaking and listening IV, Wageningen, The Netherlands, 2010
- Information literacy for PhD including EndNote introduction, Wageningen, The Netherlands, 2010
- Interdisciplinary research: Crucial knowledge and skills, Wageningen, The Netherlands, 2010
- Presentation skills, Wageningen, The Netherlands, 2011
- Scientific writing, Wageningen, The Netherlands, 2011
- Techniques for writing and presenting a scientific paper, Wageningen, The Netherlands, 2011

Optionals

- Preparation PhD research proposal, Wageningen, The Netherlands, 2009
- Food Chemistry PhD trip, Ghent, Belgium, 2009
- Food Chemistry PhD trip, Italy and Switzerland, 2010
- Food Chemistry PhD Trip, Singapore and Malaysia, 2012
- Member of the organization committee for the PhD Trip to Singapore and Malaysia, 2012
- Food Chemistry seminars, Wageningen, The Netherlands, 2009-2012
- Food Chemistry colloquia, Wageningen, The Netherlands, 2009-2012

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