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Isolation of Phenolic Compounds from Iceberg Lettuce and Impact on Enzymatic Browning

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ABSTRACT: Enzymatic browning is generally reported as the reaction between phenolic substances and enzymes. The quality of iceberg lettuce is directly linked to this discoloration. In particular, the color change of lettuce stems considerably reduces consumer acceptance and thus decreases sales revenue of iceberg lettuce. Ten phenolic compounds (caffeic acid, chlorogenic acid, phaseolic acid, chicoric acid, isochlorogenic acid, luteolin-7-*O*-glucuronide, quercetin-3-*O*-glucuronide, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-(6″-malonyl)-glucoside) were isolated from *Lactuca sativa* var. *capitata* by multilayer countercurrent chromatography (MLCCC) and preparative high-performance liquid chromatography (HPLC). In addition, syringin was identified for the first time in iceberg lettuce. This polyphenolic ingredient was previously not mentioned for the family of Cichorieae in general. The purity and identity of isolated compounds were confirmed by different NMR experiments, HPLC-DAD-MS, and HR-MS techniques. Furthermore, the relationship between discoloration of iceberg lettuce and enzymatic browning was thoroughly investigated. Unexpectedly, the total concentration of phenolic compounds and the activity of polyphenol oxidase were not directly related to the browning processes. Results of model incubation experiments of plant extract solutions led to the conclusion that in addition to the typical enzymatic browning induced by polyphenol oxidases, further mechanisms must be involved to explain total browning of lettuce.

KEYWORDS: *iceberg lettuce, Lactuca sativa var. capitata, multilayer countercurrent chromatography (MLCCC), enzymatic browning, phenolic compounds*

■ INTRODUCTION

Lettuces are the most popular vegetables in the world and are consumed in increasing amounts due to their potential beneficial health effects. Favorite species are butterhead, romaine, and, most importantly, iceberg lettuce. In 2010 the consumption per private household in Germany was 2.8 kg.[1] Crispy texture, fresh green color, and in particular the absence of browning are desirable properties and contribute to the acceptance of foods. Such properties are most relevant to vegetables such as lettuce; consumers associate them with freshness and wholesomeness.[2,3]

Browning of lettuce after minimal processing is one of the main causes of quality loss. The organoleptic and biochemical characteristics are significantly modified by the appearance of brown pigments, resulting in rejection by consumers. Iceberg lettuce tends to brown very rapidly specifically on the lettuce stem after harvesting, which complicates the storage of intact or processed heads.[4] To prevent these processes physical or chemical treatments are needed. It is a matter of common knowledge that the quality and shelf life of lettuce vary depending on cultivar, maturity, processing, and storage conditions.[5−7] Nevertheless, information on the molecular basis of tissue browning is limited.

After wounding of plant material, two biochemical key processes start. On the one hand, activation of the phenylpropanoid metabolism is induced by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), resulting in an accumulation of phenolic compounds.[8] On the other hand, wounding causes cellular disruption leading to exposure of phenolic substrates to polyphenol oxidase (PPO, EC 1.14.18.1). Generally, PPO catalyzes both hydroxylation of monophenols to *o*-diphenols and oxidation of colorless *o*-diphenols to *o*-quinones.[9] Then the *o*-quinones condense spontaneously with other *o*-quinones, polyphenols, and many other plant constituents such as proteins and carbohydrates to form high molecular weight polymers. These tannine-like compounds are the brown, red, or dark pigments in injured vegetable tissues.[10,11] PPO has a broad specificity toward different phenolic substrates.[10] Especially for iceberg lettuce, studies have shown that lettuce PPO can exclusively oxidize *o*-diphenols. On the contrary, no reactivity was found for monohydroxy and 1,2,3-trihydroxy substrates. PPOs isolated from photosynthetic or vascular tissues of lettuce indicated almost identical properties.[12] However, a comparison of PPO activities in the various parts of lettuce tissues including the stem has not been published.

Caffeoyl derivatives and flavonoids, especially quercetin and luteolin glycosides, represent typical polyphenolic compounds in iceberg lettuce. Among them, dicaffeoyl tartaric esters belong to the quantitatively major compounds.[13,14]

Many studies were conducted to gain insights into the mechanistic relationships between phenolic compounds and enzymatic catalyzed reactions in general. Caffeoyl derivatives led to a larger increase in enzymatic browning in comparison to flavonoids. Thus, they obviously play a significant role as substrates for polyphenol oxidases.[15] Furthermore, extensive investigations were carried out to describe the beneficial effects

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of physical or chemical treatments on the prevention of discoloration.[4,8]

However, for lettuce and also other kinds of fruits or vegetables, no precise relationship was reported for browning potential, PPO activity, and total or individual phenol accumulation or degradation.[16−18] Besides the chemistry of black tea fermentation, specific products of enzymatic browning were characterized very scarcely and, in addition, to the best of our knowledge, no study exists describing the relationship of PPOs and enzymatic browning in various tissues of the same plant.

On the basis of isolated phenols from iceberg lettuce, this work investigates the influence of specific phenolic compounds leading to colored structures during the browning processes on cut interfaces and bruised areas. With the present work, we correlate the intensive tissue browning particularly of the lettuce stem to changes in phenolic content.

MATERIALS AND METHODS

Chemicals. Chemicals of the highest quality available were obtained from Roth (Karlsruhe, Germany) unless otherwise indicated. Methanol (HPLC grade), caffeic acid, chlorogenic acid, catechol, gallic acid, polyvinyl pyrrolidone (PVP, K15), and tyrosinase from mushroom (PPO, EC 1.14.18.1) were purchased from Sigma-Aldrich (Taufenkirchen, Germany). Methanol-d_4 (CD_3OD) and dimethyl-d_6 sulfoxide (DMSO-d_6) were obtained from Chemotrade (Leipzig, Germany). Folin−Ciocalteu's phenol reagent was ordered from Merck (Darmstadt, Germany).

Plant Materials. Lettuce and nashi pears were obtained from local wholesale markets in Germany. The lettuce heads were prepared for further processing by removing and discarding wrapper leaves and brown spots. For isolation of polyphenols, the material was freeze-dried and stored at 5 °C until use. For some experiments, the fresh lettuce head was separated into four tissue types: green leaves, yellow leaves, stem, and a mix of the entire head. Each lot was cut into squares (1 × 1 cm) using a sharp ceramic knife and mixed thoroughly. The material was separated in three fractions. One fraction of fresh-cut plant material was used for purification of polyphenol oxidase. The second fraction was stored into freezer bags, freeze-dried, and stored at −20 °C until used (hereinafter called "fresh sliced"). For browning, the last fraction was packed into Petri dishes and kept at room temperature. After 24 h, the brown material was treated just like the second fraction (hereinafter called "24-h-aged sliced").

Extraction of Plant Material. For isolation of polyphenols, 100 g of freeze-dried material was extracted with acetone/water (7:3 v/v, 1.5 L) at 5 °C for 24 h under argon atmosphere. The filtrates were concentrated under reduced pressure (<30 °C) until complete evaporation of acetone. The residual aqueous solution was successively extracted with diethyl ether (2 × 200 mL), ethyl acetate (2 × 200 mL), and *n*-butanol (2 × 200 mL). The ethyl acetate and *n*-butanol extracts were evaporated to dryness, and the residues were used for multilayer countercurrent chromatography (MLCCC).

For comparative studies on browning, 1 g of freeze-dried fresh sliced and of 24-h-aged sliced tissue types of iceberg lettuce were extracted with 10 mL acetone/water (7:3 v/v) at 5 °C under argon atmosphere. After 24 h, the samples were centrifuged at 4500 rpm for 10 min. A 1 mL aliquot of the clarified supernatant was dried under an argon stream. A 5 mL aliquot was concentrated under reduced pressure (<30 °C) until complete evaporation of acetone. The residue was extracted two times with 2 mL of diethyl ether. The combined diethyl ether phases were dried under argon stream.

Analytical HPLC-DAD-MS. A Jasco (Gross-Umstadt, Germany) quaternary gradient unit PU 2080, with degasser DG 2080-54, autosampler AS 2055, column oven (Jasco Jetstream II), and multiwavelength detector MD 2015 was used. Alternatively, the detector was coupled to an API 4000 triple-quadruple mass spectrometer (Applied Biosystems, Foster City, CA). Chromatographic separations were performed on stainless steel columns (Vydac CRT. 218TP54, 250 × 4.0 mm, RP 18, 5 μm, Hesperia, CA, USA) using a flow rate of 1.0 mL/min. The mobile phase consisted of water (solvent A) and MeOH/water (7:3, v/v, solvent B). To both solvents (A and B) was added 0.8 mL/L formic acid. Samples were analyzed using two different gradient systems. In gradient system 1, samples were injected at 10% B and held for 10 min, then the gradient was changed linearly to 65% B in 55 min and to 100% B in 5 min, and held at 100% B for 10 min. In gradient system 2, samples were injected at 50% B and held for 10 min. The gradient was changed linearly to 100% B in 60 min and held at 100% B for 10 min. The column temperature was always 25 °C. The effluent was monitored at 280 and 420 nm.

MS ionization was achieved using the Turbospray ionization source operated in positive and negative ion modes. Turbospray settings were as follows: curtain gas (N_2) at 40 psi, ion source gas 1 at 50 psi, ion source gas 2 at 60 psi, with source temperature at 500 °C and ion spray voltage at 4500 V and −4500 V, respectively. The total ion current (TIC) chromatogram with a scan range between 100 and 700 mass-to-charge units and mass spectral data on precursor ions were detected. Declustering potential, entrance potential, collision energy, and cell exit potential were optimized for each analyte.

MLCCC. The MLCCC system (Ito, Multilayer Separator-Extractor model, P. C. Inc., Potomac, MD, USA) was equipped with a Waters constant flow pump (model 6000 A), a Kontron spectrophotometer operating at 280 nm, and a sample injection valve with a 10 mL sample loop. Eluted liquids were collected in fractions of 8 mL with a fraction collector (LKB Ultrorac 7000). Chromatograms were recorded on a plotter (Servogor 200). The multilayer coil was prepared from a 1.6 mm inner diameter polytetrafluoroethylene (PTFE) tubing. The total capacity was 290 mL. The MLCCC was run at a revolution speed of 790 rpm and a flow rate of 2 mL/min in head-to-tail modus.

Samples of 1 g of dried extract were dissolved in a 1:1 mixture of light and heavy phase (10 mL) and injected into the system. Ethyl acetate extracts were separated by using water/ethyl acetate (1:1 v/v; solvent system A), and *n*-butanol extracts were isolated by using water/ethyl acetate/*n*-butanol (5:2:3 v/v; solvent system B).

Preparative HPLC-UV. A Besta HD 2-200 pump (Wilhelmsfeld, Germany) was used at a flow rate of 8 mL/min. Elution of materials was monitored by an UV detector (Jasco UV-2075, Gross-Umstadt, Germany). Chromatographic separations were performed on a stainless steel column (Vydac CRT. 218TP1022, 250 × 23 mm, RP 18, 10 μm). The mobile phases used were solvents A and B, identical to the analytical HPLC-DAD system. An isocratic method was chosen according to the characteristics of the target structures. From the individual chromatographic fractions, solvents were removed under reduced pressure. After addition of water, solutions of polyphenols were freeze-dried.

Accurate Mass Determination (HR-MS). The high-resolution positive and negative ion ESI mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), a radio frequency (RF)-only hexapole ion guide, and an external electrospray ion source (APollo; Agilent, off-axis spray). Nitrogen was used as the drying gas at 150 °C. The samples were dissolved in methanol, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL/h. The data were acquired with 256K data points and zero filled to 1024K by averaging 32 scans.

Magnetic Resonance Spectroscopy (NMR). NMR experiments were performed on a Varian Unity Inova 500 instrument (Darmstadt, Germany) operating at 500 MHz for ^1H and at 200 MHz for ^{13}C, respectively. Samples were dissolved in either methanol/d_4 or dimethyl-d_6 sulfoxide, and all spectra were run at 27 °C. Chemical shifts are given relative to external Me_4Si and were referenced to internal CD_3OD (^1H, δ 3.310; ^{13}C, δ 49.000) and DMSO-d_6 (^1H, δ 2.500; ^{13}C, δ 39.520), respectively.

Incubation of Lettuce Extracts. Dried extracts (20 mg acetone/water, diethyl ether, ethylacetat, *n*-butanol, and water extract) were dissolved in phosphate buffer (0.2 M, pH 6.5). By diluting, an

appropriate intensity was adjusted. After the addition of 3 U of PPO solution (isolated from nashi, mushroom, or lettuce) and adjustment to 200 μL with buffer, the samples were incubated at 37 °C for 120 min. The absorbance of these solutions was measured at 420 nm every 1 min with a Tecan Infinite M200 Microplate reader including i-control software (Männedorf, Switzerland). After 90 min, the incubations reached a plateau. The corresponding absorbance change was plotted into graphs.

Purification of Lettuce PPO. PPO activities were determined in extracts prepared using a method of Fukumoto et al.,[19] with some modifications. Fresh lettuce samples (fraction 1) were cut in 1 × 1 cm squares using a ceramic knife. A 12.5 g sample was homogenized in a semimicro blender (Ultra-Turrax, IKA, Staufen, Germany) with 25 mL of chilled 0.05 M phosphate buffer (pH 6.2) and 1.88 g of PVP. The mixture was blended for 60 s, filtered through four layers of Schleicher & Schuell papers No. 905[1], and centrifuged at 4500 rpm for 30 min at 4 °C. The supernatant was filtered through Schleicher & Schuell papers No. 589[1], and the clarified filtrate was kept for a maximum of 5 h on ice until assayed.

Protein Content. Protein content was measured using the method of Bradford[20] with bovine serum albumin (BSA from Roth, Karlsruhe, Germany) at several concentrations (0.005–0.05 mg/mL) as standard. A 500 μL aliquot of extracts or phosphate buffer (0.05 M, pH 6.2) was added to half microcuvettes. After addition of 500 μL of Bradford reagent the Parafilm-covered cuvette was inverted three times, and the absorbance was recorded at 595 nm in a Jasco V-530 UV–vis spectrometer (Gross-Umstadt, Germany).

Assay of PPO Activity. PPO activity was assayed as reported by Fukumoto et al.:[19] A 50 μL aliquot of extract was added to a cuvette containing 2.95 mL of 10 mM catechol solution (in 0.05 M phosphate buffer, pH 6.2). The Parafilm-covered cuvette was inverted three times, and the absorbance was recorded at 420 nm every 2 s for 120 s. Slopes for the linear portions of each resultant curve were calculated for the first 30 s of the recording. One unit of PPO activity was defined as a 0.001 absorbance unit change per minute at 420 nm. PPO activity is expressed as units per milligram protein. All data were the mean of three samples. Mean values ± standard deviations were plotted.

Spectrophotometric Analysis. For the determination of the optical density 1 g of freeze-dried 24-h-aged sliced lettuce was extracted with methanol/water (7:3 v/v). The extracts were measured at 420 nm with a Jasco V-530 UV–vis spectrometer. Mean values of three samples ± standard deviations were reported in the data.

Total Phenolic Determination. The total phenolic content was determined according to the Folin–Ciocalteu method. Freeze-dried material (1.5 g) was extracted with 30 mL of acetone/water (7:3 v/v) under sonication for 15 min and for 24 h at 5 °C under argon atmosphere. To 125 μL of filtered lettuce extract were added 500 μL of deionized water and 125 μL of the Folin–Ciocalteu's phenol reagent. The mixture was kept for 6 min. Then 1250 μL of 20% aqueous sodium carbonate solution and 1000 μL of water were added to a final volume of 3 mL at room temperature. After exactly 90 min, the absorption was measured at 760 nm against water as a blank using a Jasco V-530 UV–vis spectrometer. The amount of total phenolics was expressed as gallic acid equivalents (GAE, mg gallic acid/g dried sample) referenced to the calibration curve of gallic acid. The calibration curve ranged from 10 to 200 μg/mL (R^2 = 0.9982). All data were mean values of triplicate analyses with standard deviations.

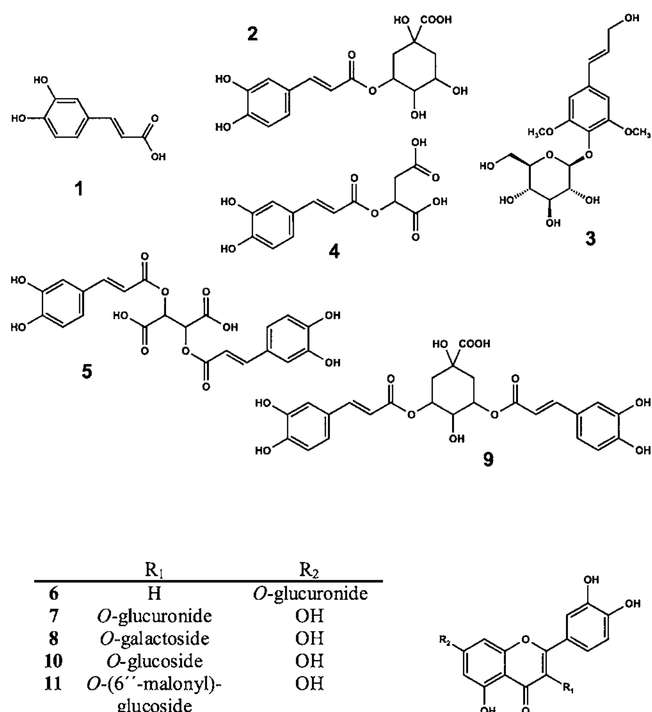
RESULTS AND DISCUSSION

Isolation and Elucidation of Phenolic Compounds. The extracts from iceberg lettuce (*Lactuca sativa* var. *capitata*) were screened for phenolic compounds by analytical HPLC-DAD. The qualitative determination of caffeic acid, chlorogenic acid, quercetin-3-*O*-galactoside, and quercetin-3-*O*-glucoside was carried out by comparison of retention time and UV–vis spectra of the eluting peak with authentic standards.

Basically, the ethyl acetate and *n*-butanol extracts revealed the same target structures. Therefore, both extracts were used for isolation of further phenolic substances.

The first step of purification was separation by MLCCC. Solvent system A was used for ethyl acetate extract and solvent system B for *n*-butanol extracts. Afterward, fractions containing the phenolic compounds were separated by preparative HPLC to isolate pure substances. The final structural evidence was achieved by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) measurements, as well as heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and homonuclear correlation spectroscopy (H,H–COSY) techniques.

The following structures were verified (Figure 1): caffeic acid **1** (λ_{max} = 324 nm), chlorogenic acid **2** (λ_{max} = 326 nm; 5-caffeoylquinic acid); syringin **3** (λ_{max} = 271 nm); phaseolic acid **4** (λ_{max} = 329 nm, 2-caffeoylmalic acid); chicoric acid **5** (λ_{max} = 328 nm; 2,3-dicaffeoyl-L-tartaric acid); luteolin-7-*O*-glucuronide **6** (λ_{max} = 350 nm); quercetin-3-*O*-glucuronide **7** (λ_{max} = 350 nm); quercetin-3-*O*-galactoside **8** (λ_{max} = 350 nm); isochlorogenic acid **9** (λ_{max} = 328 nm; 3,5-dicaffeoylquinic acid); quercetin-3-*O*-glucoside **10** (λ_{max} = 350 nm); and quercetin-3-*O*-(6″-malonyl)-glucoside **11** (λ_{max} = 350 nm) . All compounds besides **3** have been isolated from iceberg lettuce before. NMR data of **7**, **9**, and **11** were in line with those of Ferreres et al.[21] NMR data of **4**, **5**, and **6** were in agreement with the literature.[15]



	R_1	R_2
6	H	*O*-glucuronide
7	*O*-glucuronide	OH
8	*O*-galactoside	OH
10	*O*-glucoside	OH
11	*O*-(6′′-malonyl)-glucoside	OH

Figure 1. Phenolic compounds isolated from iceberg lettuce: **1**, caffeic acid; **2**, chlorogenic acid; **3**, syringin; **4**, phaseolic acid; **5**, chicoric acid; **6**, luteolin-7-*O*-glucuronide; **7**, quercetin-3-*O*-glucuronide; **8**, quercetin-3-*O*-galactoside; **9**, isochlorogenic acid; **10**, quercetin-3-*O*-glucoside; **11**, quercetin-3-*O*-(6″-malonyl)-glucoside.

Figure 2 represents a typical HPLC-DAD chromatogram of an acetone/water extract from the fresh sliced iceberg lettuce. Phenolic compounds eluted after 10 min. The acetone/water extract contained all of the isolated compounds. The

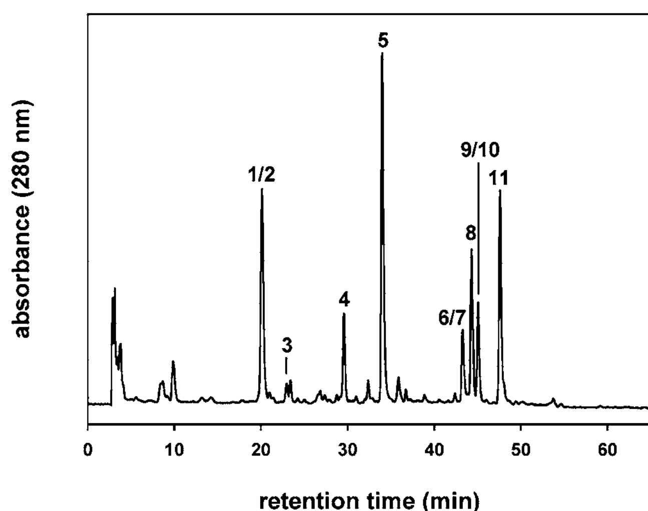


Figure 2. HPLC-DAD chromatogram of acetone/water extract from fresh iceberg lettuce. Phenolic compounds eluted after 10 min; retention times are given in parentheses: **1**, caffeic acid (20.2 min); **2**, chlorogenic acid (21.1 min); **3**, syringin (23.0 min); **4**, phaseolic acid (29.6 min); **5**, chicoric acid (34.0 min); **6**, luteolin-7-*O*-glucuronide (43.2 min); **7**, quercetin-3-*O*-glucuronide (44.2 min); **8**, quercetin-3-*O*-galactoside (44.4 min); **9**, isochlorogenic acid (45.0 min); **10**, quercetin-3-*O*-glucoside (45.2 min); **11**, quercetin-3-*O*-(6″-malonyl)-glucoside (47.5 min).

concentrations ranged from 2 to 20 μg/g fresh weight (FW). **4** (3.0 ± 0.7 μg/g FW), **6** (5.1 ± 0.7 μg/g FW), and **9** (6.8 ± 2.2 μg/g FW) were present in small amounts. Higher concentrations were quantified for **2** (13.8 ± 1.7 μg/g FW) and **7** (11.1 ± 1.2 μg/g FW). **5** and **11** were the main components, with concentrations ranging between 15.2 and 23.3 μg/g FW for **5** and between 11.6 and 24.9 μg/g FW for **11**. Large variations in polyphenolic content (about 2 magnitude orders) have already been reported, which may be linked to the different extraction methods used.[22−24] Nevertheless, our findings are in agreement with Degl'Innoocenti et al. and Ribas-Agusti et al.[24,25]

Compound **3** was isolated as a white crystalline substance with concentrations ranging between 6.6 and 12.8 μg/g FW and analyzed by MS and NMR. LC-ESI-MS delivered for signal **3** a pseudomolecular ion of m/z 395.1 [M + Na$^+$]. This finding was confirmed by high-resolution mass spectrometry (found, m/z 395.13112 [M + Na$^+$]; calcd, m/z 395.13125 [M + Na$^+$] for $C_{17}H_{24}O_9Na$). Thin layer chromatography indicated the presence of a glucose moiety after acid hydrolysis. NMR spectral data of **3** are given in Table 1. The results of NMR spectroscopy verified the glucose moiety with ^{1}H signals at δ_H 3.03−3.58 (for H-1′ to H-6′) and an anomeric proton at δ_H 4.90 (1H, d, ^3J = 6.3 Hz) for H-1′. In the ^{13}C NMR data the ketalic carbon signal at δ_C 102.6 (C-1′) and the signals derived from the glucose moiety between δ_C 60.9 (C-6′) and δ_C 77.2 (C-5′) were confirmed. Coupling constant and chemical shift verified a *β*-*O*-glucopyranoside, linked to a phenylpropanoid skeleton. The presence of sinapyl alcohol was evident from ^{1}H NMR: δ_H 6.46 (1H, d, ^3J = 15.8 Hz, H-7), 6.33 (1H, dt, ^3J = 15.8 Hz, ^3J = 5.0 Hz, H-8), and 4.10 (2H, d, ^3J = 5.0 Hz, H-9). The coupling constant of ^3J = 15.8 Hz was attributed to one pair of trans protons, which are characteristic for cinnamic acid derivatives. A meta-substituted aromatic ring system was verified by signals at δ_H 6.72 (2H, s, H-3 and H-5) and two *O*-methyl groups at δ_H 3.77 (6H, s, 2- and 6-OCH_3). The signal

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (200 MHz) Spectroscopic Data of Syringin (in DMSO-d_6)[a]

syringin

C/H	δ^1H [ppm]	δ^{13}C [ppm]
1	-	133.9
2,6	-	152.7
3,5	6.72 (s, 2H)	104.5
4	-	132.6
7	6.46 (d, 1H, ^3J = 15,8 Hz)	128.4
8	6.33 (dt, 1H, ^3J = 15.8 Hz, ^3J = 5.0 Hz)	130.2
9	4.10 (d, 2H, ^3J = 5.0 Hz)	61.4
2,6-OCH_3	3.77 (s, 6H)	56.4
1'	4.90 (d, 1H, ^3J = 6.3 Hz)	102.6
2'	3.20 (m, 1H)	74.2
3'	3.20 (m, 1H)	76.5
4'	3.13 (m, 1H)	69.8
5'	3.03 (m, 1H)	77.2
6'	3.41 (dd, 1H, ^2J = 11.2 Hz, ^3J = 5.0 Hz)	60.9
	3.58 (dd, 1H, ^2J = 11.2 Hz, ^3J = 1.4 Hz)	

[a] δ, chemical shift; *J*, coupling constant; hydrogen/carbon assignments were verified by HMBC, HSQC, H,H−COSY, and APT measurements.

at δ_C 56.4 (2,6-OCH_3) confirmed the two *O*-methyl carbons. The chemical shift of C-1 and the HMBC signal between H-1′ and C-1 verified the link between glucose and the phenylpropanoid skeleton. On the basis of one-dimensional and two-dimensional NMR data, compound **3** was thus identified as the *β*-*O*-glucoside of sinapyl alcohol, that is, syringin. **3** was identified for the first time in lettuce, especially iceberg lettuce. It was isolated first from the bark of *Syringa vulgaris* and has been verified in many types of plants as an allelopathic secondary metabolite. Bioactive functions as a hypotensive and antioxidative substance were described.[26]

All of these phenolic structures have been associated with enzymatic browning. In particular, the caffeic acid derivatives were reported to play an important role. The latter were also identified in other fruits and vegetables that naturally have a high tendency to brownish discoloration.[11]

Browning of Lettuce Tissues. The quality of iceberg lettuce is most easily assessed in relation to the discoloration of the lettuce stem. The greater the discoloration of the lettuce stem, the older is the iceberg lettuce. In the present experiments the browning in different parts of the plant was studied and compared to PPO activity and phenolic content. These aimed to increase our understanding of why the lettuce stem leads to more intensive browning in relation to specific phenolic compounds.

The underlying experimental setup was as follows: the lettuce was divided into four parts (outer (green) leaves, inner (yellow) leaves, lettuce stem, and, for comparison, a mixture of the total head). These subsamples were crushed, stored at room temperature for 24 h, and then extracted with methanol/water. Figure 3A represents the determined absorptions at 420 nm. It clearly states that the lettuce stem led to the strongest browning, followed by the yellow inner leaves. The outer leaves showed the slightest discoloration. Due to the high proportion of green leaves, the mixed total sample had a lower intensity versus the stem sample. Obviously, increased browning activity

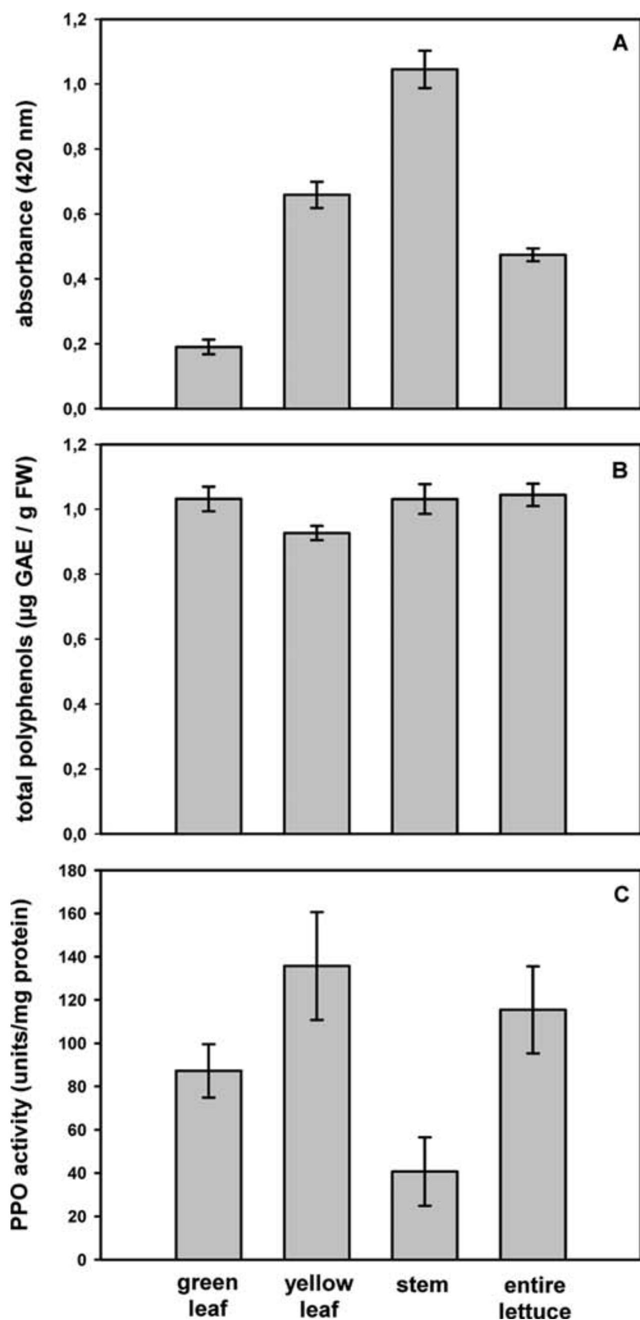


Figure 3. (A) Optical density in the process of enzymatic browning in different parts of lettuce. (B) Total phenolics expressed as microgram gallic acid equivalents (GAE) per gram fresh weight. (C) PPO activity in iceberg lettuce using catechol as substrate.

correlates to decreased chlorophyll content. However, all of the examined parts of the iceberg lettuce showed a browning reaction.

Our findings support the suggestion of Castaner et al. that the photosynthetic tissue also enables browning, although other natural pigments such as chlorophyll are masking the discoloration.[4]

Phenolic Distribution in Different Parts of Lettuce. Figure 3B clearly demonstrates that the total phenolic content assessed by Folin–Ciocalteu showed no significant difference in the various subsamples. Thus, differences in browning cannot be related to total phenolic content. However, with the Folin–Ciocalteu method, all of phenolic compounds are addressed, even those that are not substrates of PPO.

In addition to the substrate specificity, the enzyme activity of PPO plays a significant role. This was investigated in a catechol assay. This assay method, commonly used for the activity determination of most PPOs, is based on the oxidation of catechols to *o*-quinones. The activity was determined by following the reaction progress at 420 nm. It is reported that PPO is inactivated by freezing storage conditions.[18] Therefore, we used only fresh lettuce samples from a local wholesale market. The PPOs showed different activities in the various parts of lettuce. In Figure 3C the activities are presented in units per milligram protein. The highest enzyme activity was verified in the inner yellow leaves followed by the external green chlorophyll-rich leaves, whereas the lettuce stem showed the lowest enzyme activity in the catechol assay. The activity of the entire head was clearly influenced by the major green leaf fraction. Therefore, the PPO-browning activities were not positively correlated to the actual browning in the different parts of lettuce.

Browning Process of Different Extracts from Lettuce. The above observations of the enzymatic browning naturally occurring in the plant material were reviewed by a browning model in which specific enzymes were added to the five extracts for induction of color formation. The acetone/water extract contained the total of extractable material, whereas diethyl ether, ethyl acetate, *n*-butanol, and water extract represented subsets of increasing polarity. After 90 min at 24 °C, the color intensity was measured at 420 nm. To compare the effectiveness of lettuce PPO, additionally mushroom PPO and nashi pear PPO were used, to address a broad spectrum of substrates. By matter of preparation, the enzyme extracts of lettuce PPO and nashi pear PPO were enzyme mixtures. Only the mushroom PPO was a purchased purified standard.

Figure 4 exhibits that PPO isolated from iceberg lettuce had the strongest browning potential.

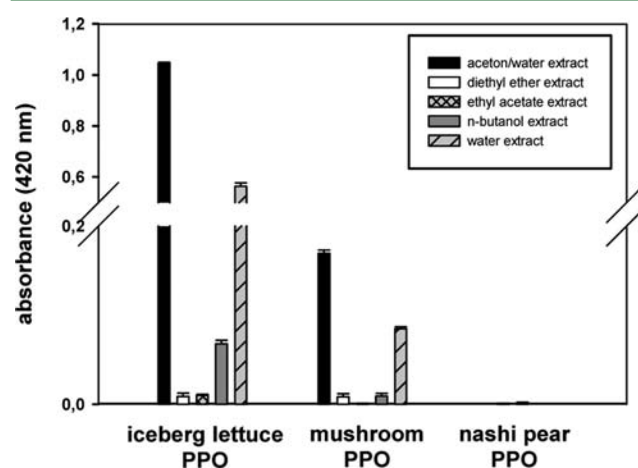


Figure 4. Effect of PPO from iceberg lettuce, mushroom, and nashi pear on browning in different extracts of iceberg lettuce.

The addition of mushroom PPO and nashi pear PPO to the extracts led to a smaller change in absorbance. The substrate specificity of the polyphenol oxidase is the responsible factor for the formation of *o*-quinones with PPOs of different sources having different affinities to specific phenolic compounds.[27,28] Obviously for mushroom PPO and nashi pear PPO, the

phenolic profile of the extracts was much less specific, in contrast to the freshly extracted lettuce PPO. Therefore, this lettuce enzyme extract was used for further experiments. The greatest change in absorption was achieved for the acetone/water extract.

Correlation of Lettuce Ingredients with the Enzymatic Browning. In addition to the browning in the tissue and in the total extracts, the influence of the conversion of individual phenolic compounds contained in the acetone/water extract was measured by HPLC-DAD-MS techniques. As reported in previous studies, the caffeoyl acid derivatives are the important substrates for browning.[12,27,29] Thus, in Figure 5 the phenolic

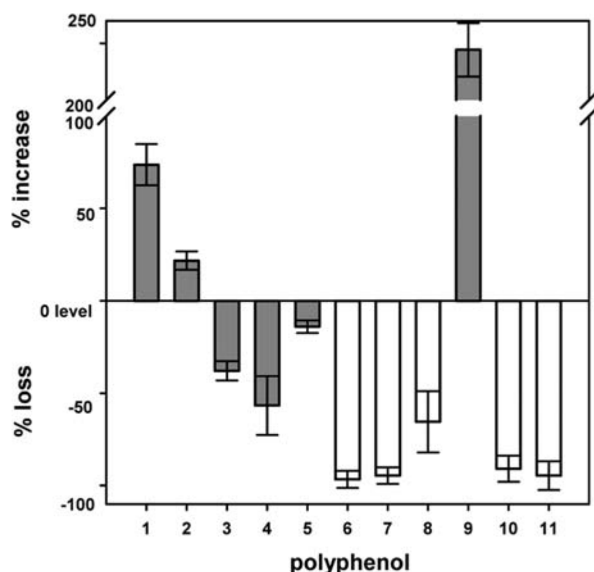


Figure 5. Percentage change of individual phenolic compounds in 24-h-aged sliced lettuce compared to fresh sliced lettuce (shaded bars, caffeic acid derivatives; white bars, flavonoids).

compounds were divided into two groups, the caffeoyl derivatives (shaded bars) and flavonoids (white bars). The comparison of the acetone/water extract of fresh sliced iceberg lettuce and of 24-h-aged sliced lettuce gave decreasing and increasing signal intensities.

Apart from **1**, **2**, and **9** all phenolic compounds decreased. Obviously, they were oxidized by enzymatic processes, and, thus, may contribute to color formation.

On the contrary, the concentrations of **1**, **2**, and **9** increased. This was in line with reports that PAL activity increases during browning,[30,31] thus leading to de novo synthesis of phenolic compounds and other structures. Both caffeoyl derivatives and flavonoids must be involved in the color change of iceberg lettuce. However, this experiment cannot show the contribution of individual structures.

Investigation of Acetone/Water Extracts. The acetone/water extracts of fresh sliced, of 24-h-aged sliced, and of fresh sliced iceberg lettuce incubated for 24 h with lettuce PPO were analyzed by HPLC-DAD (Figure 6). For this chromatography, method 2 was used. Extracts of both the 24-h-aged sliced iceberg lettuce and the PPO-incubated sample showed an obvious increase in total color formation at 420 nm in comparison to the extract of the fresh sliced lettuce. However, only the HPLC-DAD chromatogram of the 24-h-aged sliced iceberg lettuce indicated discrete signals, in contrast to fresh

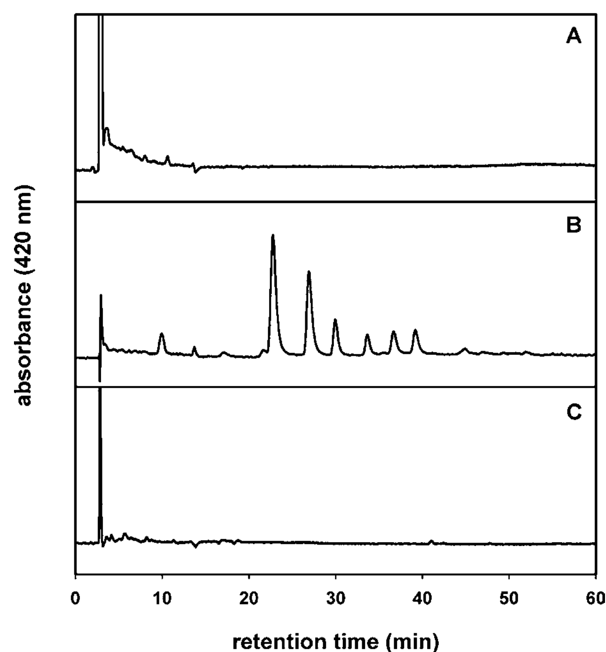


Figure 6. HPLC-DAD chromatogram of (A) acetone/water extract from fresh sliced iceberg lettuce at λ = 420 nm; (B) acetone/water extract from 24-h-aged sliced iceberg lettuce at λ = 420 nm, unknown colored structures eluted between 20 and 40 min; (C) acetone/water extract from fresh sliced iceberg lettuce incubated for 24 h with PPO.

sliced lettuce and PPO-incubated lettuce, where no signals were detected.

In an attempt to obviate the notion that with acetone/water possible browning adducts were discriminated, fresh iceberg lettuce shredded in phosphate buffer (pH 6.5) was supplemented with enzyme solution and incubated under continuous oxygen supply for 24 h. Identical to the incubated acetone/water extract (data not shown) a change in total browning was detected at 420 nm, but not colored structures were identified by HPLC-DAD.

This is in line with a previous report of the addition of lettuce PPO leading to a change in absorption, but no structures for resulting pigments were given. The authors assumed the compounds to be nonpolar.[30] This can be confirmed by the present investigation, as method 2 used a far more unpolar gradient profile. Obviously, PPO-induced browning is not related to the synthesis of the specific structures shown in Figure 6.

The following substances from *L. sativa* var. *capitata* were isolated and identified: caffeic acid **1**, chlorogenic acid **2**, syringin **3**, phaseolic acid **4**, chicoric acid **5**, luteolin-7-*O*-glucuronide **6**, quercetin-3-*O*-glucuronide **7**, quercetin-3-*O*-galactoside **8**, isochlorogenic acid **9**, quercetin-3-*O*-glucoside **10**, and quercetin-3-*O*-(6″-malonyl)-glucoside **11**. Within this context, compound **3** was verified for the first time in iceberg lettuce. In addition, we clarified the influence of phenolic compounds and of polyphenol oxidase activity on the enzymatic browning in iceberg lettuce. As a result, tissue browning after cutting and storage was not related to change in concentrations of phenolic compounds or to the specific activity of PPO.

It can be concluded that additional factors are involved in the process of browning in lettuce. Besides the enzymatic browning of phenolic compounds leading to unspecific browning other

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reactions such as polymerization of yet unknown educts makes the browning process of iceberg lettuce unclear. Thus, in follow-up studies, we intend to isolate the specific colored products indicated in the present study and characterize their impact on sensorial changes.

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Notes

The authors declare no competing financial interest.

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