Prediction of Polyphenol Oxidase Activity in Model Solutions Containing Various Combinations of Chlorogenic Acid, (−)-Epicatechin, O_2, CO_2, Temperature, and pH by Multiway Data Analysis

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Controlled or modified packaging (CA/MA) of lettuce is simulated in model solutions containing lettuce polyphenol oxidase (PPO), chlorogenic acid (CG), and/or (−)-epicatechin (EPI) under various combinations of CO_2, O_2, N_2, temperature, and pH conditions. Enzymatic browning is measured by PPO activity, color formation, substrate consumption, and development of products. Results were analyzed by multiway data analysis. CO_2 minimized the chemical polymerization and reduced PPO activity slightly. CG was a better substrate for PPO than EPI. CG was, in particular, involved in the primary enzymatic catalyzed oxidation, whereas EPI was important for the subsequent chemical polymerization in mixtures of CG and EPI. A very descriptive five-parameter multiplicative model was obtained from the PPO data by the multivariate method PARAFAC. This model describes the importance of substrate, atmospheric composition, pH, and temperature for PPO activity. Low pH (3.0) reduced PPO activity strongly (89%), whereas low temperature (5 °C) had a weaker effect on PPO activity (40% reduction of activity). The prediction of PPO activity (O_2 consumption) by kinetic UV−vis spectra correlated well ($r^2 = 0.87$) with traditional polarographic PPO assay.

Keywords: *Lettuce; Lactuca sativa; PPO; enzymatic browning; atmosphere; packaging; CA; MA; HPLC; polarographic assay; UV−vis spectra; multiway data analysis; PLS; PARAFAC*

INTRODUCTION

Undesirable browning of processed and stored fruits and vegetables is a well-described problem (Watada et al., 1990; Sapers, 1993; Martinez and Whitaker, 1995). In shredded lettuce, enzymatic browning is difficult to avoid or minimize because heating is not realistic, exclusion of O_2 develops off flavor (Heimdal et al., 1995), and the addition of browning inhibitors is undesirable. Polyphenol oxidase (PPO) is responsible for enzymatic browning in several fruits and vegetables (Martinez and Whitaker, 1995). Enzymatic browning is a very complex reaction, because it includes several substrates for PPO and nonenzymatic polymerization of, e.g. chlorogenic acid and (−)-epicatechin. In order to minimize the browning problem, it is necessary to fully know and understand the enzymatic reaction in detail for each vegetable food product. From experiments with intact or partly processed vegetables, it is difficult to make conclusions because of the complexity; therefore, the enzymatic reaction has been studied in model solutions (Janovitz-Klapp et al., 1990; Richard-Forget et al., 1992; Goupy et al., 1995). The mechanism of enzymatic browning has been investigated primarily for apple PPO (Richard-Forget et al., 1992; Goupy et al., 1995), grape PPO (Cheynier and Ricardo da Silva, 1991; Cheynier and Moutounet, 1992; Guyot et al., 1995), and commercially prepared tyrosinase (Oszmianski and Lee, 1990, 1991), but has never been examined in model solutions containing lettuce PPO. Furthermore, the influence of atmospheric composition, comparable to controlled or modified packaging (CA/MA) conditions for fruits and vegetables, on PPO activity and formation of brown compounds in model solution has never been reported.

The purpose of this study is to examine in further detail the enzymatic browning process in CA/MA packaged lettuce using a model system. Three experimental designs were necessary to obtain simultanous informations on (1) PPO activity (O_2 consumption), (2) formation of colored compounds (kinetic UV−vis spectra), and (3) substrate consumption and formation of reaction intermediates or products (HPLC). All experiments were performed using purified lettuce PPO, major substrates occuring in lettuce, singly and in mixtures, and various combinations of O_2, CO_2, N_2, temperature, and pH conditions.

MATERIALS AND METHODS

Materials. Five heads of fresh iceberg lettuce (*Lactuca sativa* L. cv. Saladin Iceball) were purchased from a commercial grower near Copenhagen, Denmark. Lettuce heads were trimmed, and leaves were torn into small pieces and immediately frozen in liquid nitrogen, lyophilized, ground into fine powder, and then stored at −20 °C in airtight bags until further use.

Ascorbic acid and ammonium sulfate were from Riedel-de Haën (Seelze, Germany). Potassium chloride, sodium dithionite, and L-cysteine hydrochloride monohydrate were from Merck (Darmstadt, Germany). Acetonitrile was HPLC grade quality (far-UV) from Lab-Scan (Dublin, Ireland), and all other chemicals were of reagent grade from Sigma (St. Louis, MO).

Gas mixtures of O_2, CO_2, and N_2 with certificate (±2% relative, Alphagaz) from the producer (Hede Nielsen, Horsens, Denmark) contained the following O_2/CO_2 ratios: 80/20, 80/10, 80/0, 20/20, 20/10, 10/20, 10/10, 10/0, 5/20, 5/10, 5/0, 0/20, and 0/10. Atmospheric air and N_2 were also used in the experiments (15 combinations).

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S0021-8561(96)00975-2 CCC: $14.00

Table 1. Design of Three Experiments Measuring the Activity of PPO as either O_2 Consumption, Color Production, or Substrate Consumption/Product Formation under the Stated Combinations of Substrate, O_2, CO_2, pH, and Temperature

factor	O_2 consumption (PPO)	UV−vis spectra (color)	HPLC (products)
substrate[a]	CG, EPI, MIX	CG, EPI, MIX	CG, EPI, MIX
O_2 (%)	0, 5, 10, 20, 80	0, 5, 10, 20, 80	0, 20, 80
CO_2 (%)	0, 10, 20	0, 10, 20	0, 20
pH	3.0, 4.5, 6.0	6.0	6.0
temp (°C)	5, 20, 30	30	30
time	initial rate	every 120 s for 1 h	0, 1, 5, 10, 30 min
combinations[b]	405	45	15

[a] Abbreviations: CG, chlorogenic acid; EPI, (−)-epicatechin; MIX, equimolar mixture of CG and EPI. [b] All combinations are performed except O_2/CO_2 0/20 in the HPLC experiment.

PPO Extraction and Purification Procedures. Extraction and purification procedures used in this study were modifications of the procedures described by Janovitz-Klapp et al. (1989) for apple PPO. Lyophilized (20.0 g) lettuce powder was homogenized with 500 mL of 0.1 M cold phosphate buffer at pH 7.0 containing 0.5% Triton X100 and 15 mM ascorbic acid for 1 min using a Warring blender and held for 15 min at 5 °C. The homogenate was centrifuged in a Sorvall (DuPont, Newtown, CT) RC5B refrigerated centrifuge (25000*g*, 40 min, 4 °C), and the clear supernatant (crude extract) was brought to 30−80% saturation with $(NH_4)_2SO_4$. The precipitate was separated by centrifugation (25000*g*, 20 min, 4 °C), dissolved in 0.05 M phosphate buffer at pH 6.5 containing 0.4 M $(NH_4)_2SO_4$ and 0.4 M KCl, and dialyzed overnight at 5 °C against the same buffer. After centrifugation (25000*g*, 20 min, 4 °C), the supernatant was applied onto a Phenyl Sepharose CL-4B column (Pharmacia, Uppsala, Sweden; 9 cm × 2.5 cm, 40 mL bed volume) equilibrated with the same buffer at a flow rate of 1 mL/min. Proteins were eluted with 100 mL of 0.05 M phosphate buffer (pH 6.5) containing 0.2 M $(NH_4)_2SO_4$ and 0.2 M KCl (1 mL/min), 100 mL of 0.01 M phosphate buffer (pH 6.5), and finally water. The absorbance at 280 nm (Perkin Elmer, Ueberlingen, Germany; Lambda 7 spectrophotometer) and the PPO activity (O_2 consumption) were determined for each 5 mL fraction.

PPO and Protein Assays. Polarographic polyphenol oxidase assay was carried out as described by Heimdal et al. (1994). All assays were performed in duplicate. Standard PPO assay was determined at 30 °C and pH 5.5 using chlorogenic acid (CG) (C3878, Sigma) as substrate. PPO activity in model solutions saturated with one of the 15 gas mixtures was expressed as nanomoles of O_2 consumed per second (= nkat), under the assumption that water contained 7.4, 8.8, or 12.3 mg of O_2/L at respectively 30, 20, or 5 °C, with a salinity (due to the buffer) of 0.1, 2.5, or 5.6 g/L (Hitchman, 1978) at respectively pH 3.0, 4.5, or 6.0. Experimental designs of model solutions are given in Table 1. The initial O_2 concentration of the gas mixture (80% O_2) or of atmospheric air (21% O_2) and sodium dithionite (0% O_2) was used to calibrate the Clark electrode (Rank Brothers, Bottisham, U.K.).

Protein was determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

HPLC Measurements. The model solution containing 4.775 mL of 0.001 M acetate buffer (pH 6.0, 30 °C) saturated with one of the five gas mixtures (O_2/CO_2 0/0, 20/0, 20/20, 80/0, or 80/20) and either 0.1 mL of 0.04 M CG, 0.04 M (−)-epicatechin (EPI) (E1753, Sigma), or an equimolar mixture of CG and EPI, resulting in 0.04 M of each substrate (MIX), and 25 μL of purified PPO reacted in a Clark electrode chamber (Rank Brothers) with continuous gas flow in the chamber (Table 1). For each time tested (0, 1, 5, 10, and 30 min), 0.2 mL was withdrawn from the chamber with a 0.250 mL syringe (Hamilton, Reno, NV) and immediately mixed with an equal volume of stopping solution containing 2 mM NaF, 2 mM cysteine, and 0.2 mM vanillic acid (internal standard). The samples were stored at 5 °C until analysis (same day).

Substrate consumption and product development were analyzed by reverse phase high-performance liquid chromatography (HPLC) using a series 1050 HPLC (Hewlett Packard), ODS Hypersil C18 column (5 μm, 250 mm × 4 mm, Hewlett Packard), flow rate 1 mL/min, detection at 280 and 390 nm with a 1040M series II diode array detector (Hewlett Packard), and a gradient method according to Richard-Forget et al. (1992). The solvents were (A) Millipore (Bedford, MA) water at pH 3.0 adjusted with 1 M phosphoric acid and (B) acetonitrile. The gradient profile was 0−5 min, 90% A; 5−12 min, 90−50% A; 12−14 min, 50% A; and 14−16 min, 50−90% A. The amount of each compound is expressed as peak area relative to peak area of internal standard. Spectra from diode array detection are measured in milliabsorbance units (mAU).

Color Measurements. Color development in the model solution was followed for 1 h by scanning with a Lambda 19 UV−vis/near-IR spectrometer (Perkin Elmer) from 220 to 500 nm (1 nm intervals) every second minute, resulting in 30 spectra. Measurements were made on closed quartz cuvettes containing 3.555 mL of 0.1 M acetate buffer (pH 6.0, 30 °C) saturated with one of the 15 gas mixtures, 25 μL of either 0.01 M CG, 0.01 M EPI, or an equimolar mixture of EPI and CG, resulting in 0.01 M of each substrate (MIX), and 20 μL of purified PPO (Table 1 for details).

Statistical Analysis. SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC) software and the GLM (general linear model) procedure were used for analysis of O_2 consumption data (PPO activity) and HPLC data. A five-way (PPO activity) or three-way (HPLC and UV−vis data) analysis of variance was conducted with substrate, O_2 concentration, CO_2 concentration, temperature, and pH as factors (Table 1).

Multiway Data Analysis. PPO activity (O_2 consumption) was predicted from UV−vis spectra using multiway PLS1 (partial least-squares regression with one *y* variable) or N-PLS1 (multilinear PLS1, where the X matrices are at least three-linear) (Bro, 1996; Bro and Heimdal, 1996). The influence of CO_2 was predicted from UV−vis spectra using N-PLS1 (Bro, 1996; Bro and Heimdal, 1996). Substrate consumption and product development (HPLC) were predicted from UV−vis spectra using N-PLS2 (multilinear PLS with more than one *y* variable) (Bro, 1996; Bro and Heimdal, 1996). The influence of substrate, O_2, CO_2, temperature, and pH on O_2 consumption (PPO activity) was examined by five-way PARAFAC (parallel factor analysis) (Smilde, 1992; Bro and Heimdal, 1996). Both PLS and PARAFAC are available from the Internet in MATLAB code.

RESULTS AND DISCUSSION

In model solutions where the buffer is aerated with "0%" O_2, the O_2 concentration is actually approximately 1%, because of the O_2 content in the substrate and enzyme solutions.

PPO Purification and Activity. Purification results for lettuce PPO are shown in Table 2. The yield was 4%, and the purification factor was 2.0. Both the ammonium sulfate fractionation and the Phenyl Sepharose column resulted in very low yields and purification factor. PPO activity (37%) was found in a PPO peak with high protein concentration (fractions 58−64, Table 2) but was not used in this study. A purification factor of 13 was obtained for PPO from basil leaves using 30% $(NH_4)_2SO_4$, followed by Phenyl Sepharose column purification (Baritaux et al., 1991). Although the purification of lettuce was rather low, separation was obtained, and it was decided to use the pooled fractions 44−57 for further experiments. A high purification grade has been found to cause conformational changes of the enzyme, resulting in nonlinear kinetic curves (Lerner and Mayer, 1976).

The O_2 consumption is directly related to the PPO activity because O_2 is a substrate, and it has been checked that no other O_2-consuming enzymes are present, no interference with its determination by the Clark electrode is known, and O_2 is not produced during the reaction. To the contrary, the CG consumption as

Table 2. Results Obtained during Purification of Lettuce PPO from 20 g of Lyophilized Leaves (Cultivar Saladin)

step	total volume (mL)	total protein (mg)	total activity (nkat)	specific activity (nkat/mg)	yield (%)	purification factor
crude extract	400	960	187440	195	100	1.00
ammonium sulfate						
30%	420	416	78725	189	42	0.97
80%	17	228	49275	216	26	1.1
dialysis	21	290	52922	183	28	0.94
Phenyl Sepharose						
fractions 44−57	58	19	7314	385	4	2.0
fractions 58−64	27	20	4245	216	2	1.1

Table 3. Result of Classical Statistical Five-Way Analysis of Variance (GLM)[a]

	PPO activity, nkat/mL				
	0%	5%	10%	20%	80%
O_2	3.8e	13.5d	28.8c	58.6b	109a
CO_2	43.6a		43.5a	41.1a	

Substrate		
CG	EPI	CG + EPI
52.5a	30.0b	45.7a

pH		
3.0	4.5	6.0
6.3c	55.2b	66.6a

Temperature (°C)		
5	20	30
32.5b	46.0a	49.6a

[a] The results are means of PPO activity (nkat/mL determined as O_2 consumption) in model solutions containing chlorogenic acid (CG) and/or (−)-epicatechin (EPI) and purified lettuce polyphenol oxidase (PPO) in acetate buffer saturated with various combinations of O_2, CO_2, and N_2 (Table 1, 405 combinations). Values with the same letters within a row are not significantly different ($p < 0.05$).

determined by spectrophotometry as a measure of PPO activity is hampered by products absorbing at the same wavelength, and further CG can be reformed via the quinone (Martinez and Whitaker, 1995). Therefore, PPO activity determined by the various methods was compared to PPO activity (O_2 consumption).

PPO Activity (O_2 Consumption). In Table 3, means of determining PPO activity (nkat/mL) as a function of O_2 consumption and statistical significances, derived from classical statistical five-way analysis of variance (GLM), concerning the examined parameters are presented. The simple five-way multiplicative PARAFAC model, which describes the influence of various parameters on PPO activity, is presented in eq 1. The relative influences of the five parameters on PPO

$$PPO_{ijklm} = (O_2)_i(CO_2)_j sub_k pH_l temp_m \qquad (1)$$

activity, given as percent reduction of activity obtained from the two data analyses (GLM and PARAFAC), are almost identical (Table 4), which indicates that both models are usable. Low O_2 and pH levels resulted in greater reduction of PPO activity than substrate (EPI), low temperature, or high CO_2 level (Table 4).

O_2 consumption in model solutions containing purified lettuce PPO, CG, and/or EPI decreased significantly with declining initial O_2 concentration (Figure 1 and Table 3), which was expected because O_2 is one of the substrates for the enzymatic reaction. Carbon dioxide in the model solution had no influence on the PPO activity using classical statistical analysis (Figure 1 and Table 3). Results from multiway data analysis like PARAFAC confirm a systematic, nonlinear reduction of

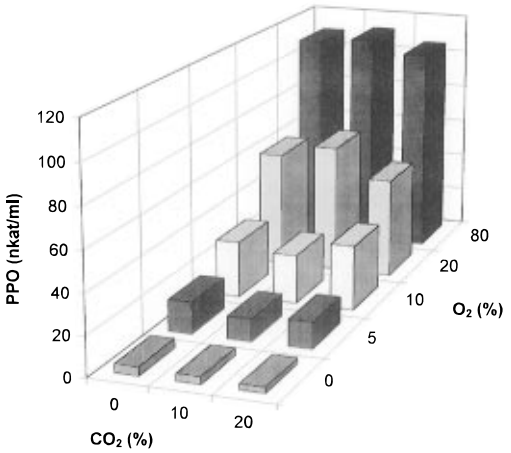

Figure 1. Influence of O_2 and CO_2 on polyphenol oxidase (PPO) activity (O_2 consumption) in model solutions containing substrate (chlorogenic acid (CG) and/or (−)-epicatechin (EPI)) and purified lettuce PPO in acetate buffer (pH, 3.0, 4.5, or 6.0 at 5, 20, or 30 °C).

PPO activity when CO_2 was included in the gas mixture (Bro and Heimdal, 1996), which is in accordance to Siriphanich and Kader (1985), who found weak inhibition of spectrophotometric PPO activity in lettuce segments stored in air + 15% CO_2. The inhibitory effect of CO_2 might be competitive because of structural similarity between O═O and O═C═O. Whether the size of CO_2 fits into the active center in PPO is unknown.

Multiway methods are appropriate for the data modeled here, resulting in more robust and interpretable results compared to classical methods. It is thus obvious that important effects are not necessarily revealed by classical statistical analysis.

CG is a better substrate than EPI for this enzyme (Table 3), which is in accordance with Heimdal et al. (1994). A mixture with an equal amount of the two substrates gave an insignificant lower O_2 consumption (Table 3) compared to CG alone. Goupy et al. (1995) found that CG quinones could oxidize EPI in a coupled reaction, leading to regeneration of CG.

In model solutions with CG, EPI, or MIX at pH 3.0, no or very low O_2 consumption was obtained (Table 3). At pH 4.5 and 6.0, both close to the optimum pH for the enzyme (pH 5.5, Heimdal et al., 1994), high O_2 consumption is obtained. PPO activity is reduced by 91% at pH 3.0 compared to that at pH 6.0. The same activity reduction by pH of lettuce PPO is reported by Fujita et al. (1991).

We found lower O_2 consumption at 5 °C compared to that at 20 and 30 °C (Table 3). PPO activity is reduced by 40% at 5 °C compared to that at 30 °C (Table 4), which is the optimum temperature for the enzyme (Heimdal et al., 1994). Higher activity reduction at low temperature (5 °C) is found for PPO in vascular (50%) and photosynthetic (58%) lettuce tissues (Heimdal et al., 1994).

Table 4. Maximum Influence of the Five Examined Parameters as Percent Reduction of PPO Activity (nkat/mL) Determined as O_2 Consumption (Table 1, 405 combinations)[a]

	O_2 (%)	CO_2 (%)	substrate	pH	temp (°C)
parameters	0, 5, 10, 20, 80	0, 10, 20	CG, EPI, MIX	3.0, 4.5, 6.0	5, 20, 30
reduction of activity (%)					
GLM	97	6	43	91	34
PARAFAC	96	8	45	89	40

[a] The estimates are results from classical five-way statistical analysis (GLM) and multiway data analysis (PARAFAC) obtained by the following equation: reduction of activity = (maximum − minimum) × 100/maximum. Abbreviations: CG, chlorogenic acid; EPI, (−)-epicatechin; MIX, equimolar mixture of CG and EPI; GLM, general linear model; PARAFAC, parallel factor analysis; PPO, polyphenol oxidase.

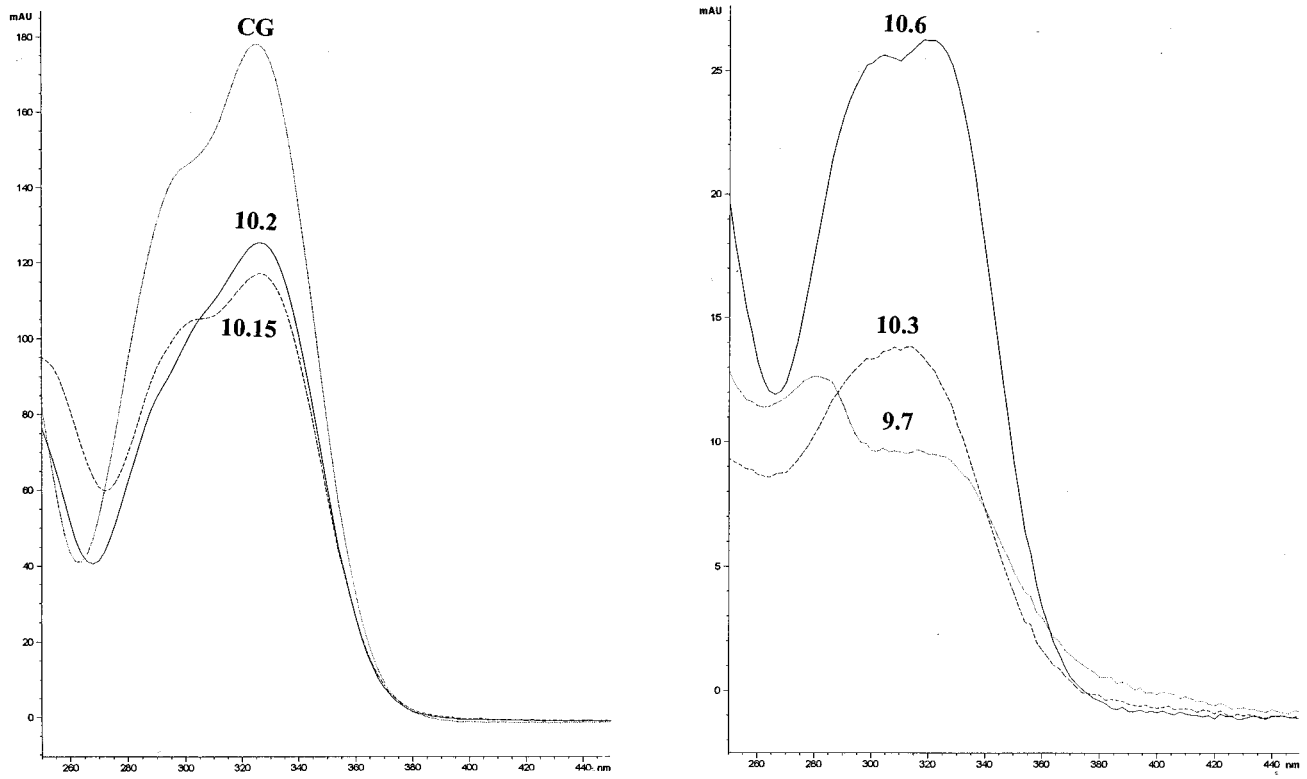

Figure 2. Diode array spectra of chlorogenic acid (CG) and five major products (named by retention time) derived from HPLC measurements after 60 min of reaction in model solutions containing CG, lettuce polyphenol oxidase (PPO), and acetate buffer (pH 6.0, 30 °C) saturated with 80% O_2, 0% CO_2, and 20% N_2. Absorbance is expressed as milliabsorbance units (mAu). For further details, see HPLC Measurements under Materials and Methods.

The results of the classical and multiway data analyses lead to the conclusion that a low pH or O_2 level is more efficient in reducing lettuce PPO activity (O_2 consumption) than low temperature or high CO_2 level.

HPLC (Formation of Intermediates in the PPO-Catalyzed Reaction). In order to follow the course of the PPO reaction and the formation of intermediates in the polymerization reactions, samples were withdrawn from the assay mixture at 0, 1, 5, 10, and 30 min and subjected to HPLC analysis using diode array to obtain spectra of every compound.

Retention times (t_R) for CG, vanillic acid (internal standard, IS), and EPI are 9.0, 9.9, and 10.1 min, respectively, when detecting at 280 nm. At 390 nm, EPI products but not EPI, CG but not CG products, and MIX products absorb the light. CG products have very low absorption in the visual area (380−780 nm), resulting in low development of color from that substrate (Figure 2). Many compounds develop during the time course when PPO acts on diphenolic substrates, but only the first developed and greatest peaks from the HPLC chromatograms enter the following description.

Chlorogenic Acid (CG). Lettuce PPO in model solution with CG and a buffer (pH 6.0) aerated with 100% N_2 (0/0) is not able to oxidize the substrate in spite of the low O_2 content (~1%) from substrate and enzyme solutions (data not shown). CG concentration is decreasing during the time in model solutions containing PPO, CG, and buffer aerated with O_2 (20% or 80%, data not shown). No significant differences in substrate consumption are observed in this small number of samples when varying O_2 and CO_2 concentrations in the model solutions, possibly because the quinone and CG have the same t_R. Two of the products formed during PPO's action on CG had t_R = 9.7 and 10.3 min (Figure 2). The compound with t_R = 9.7 min is probably 2,5-di-*S*-cysteinyl-CG, due to addition of the quinone scavenger, cysteine, to the stopping solution. This is judged by the similarity to the spectrum of 2,5-di-*S*-cysteinyl-caffeic acid identified by Cilliers and Singleton (1990). The compound with t_R = 10.3 min is probably a 2-*S*-cysteinyl-CG because of the similarity to the spectrum identified by Cilliers and Singleton (1990) and Richard et al. (1991). The relative peak areas of these two compounds (t_R = 9.7 and 10.3 min) were rather constant over time, indicating that these compounds originate from quinones. Another product with t_R = 10.6 min (Figure 2) is probably a dimer of CG because of the similarity to the spectrum of caffeic acid dimer identified by Cilliers and Singleton (1991). This compound (t_R = 10.6 min) is the first developed after PPO addition; therefore, the possiblity for it being a trimer is very low.

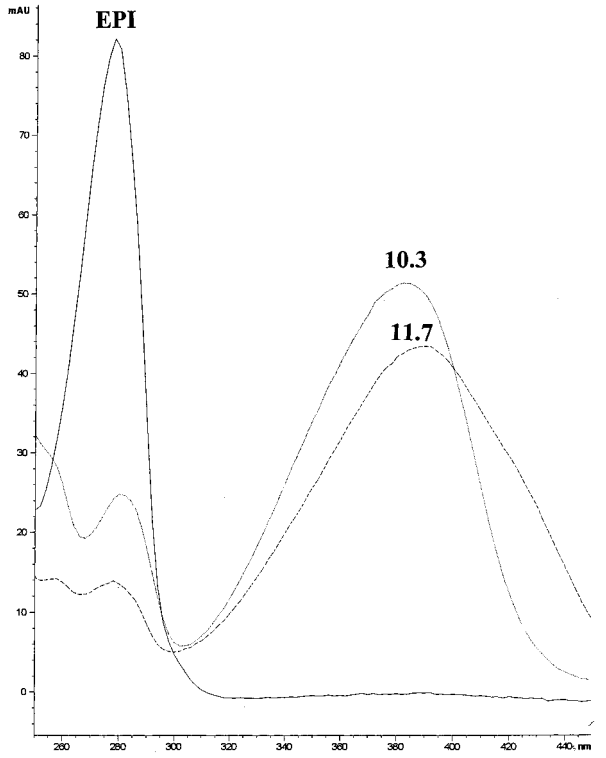

Figure 3. Diode array spectra of (−)-epicatechin (EPI) and two major products (named by retention time) derived from HPLC measurements after 60 min of reaction in model solutions containing EPI, lettuce polyphenol oxidase (PPO), and acetate buffer (pH 6.0, 30 °C) saturated with 80% O_2, 0% CO_2, and 20% N_2. Absorbance is expressed as milliabsorbance units (mAu). For further details, see HPLC Measurements under Materials and Methods.

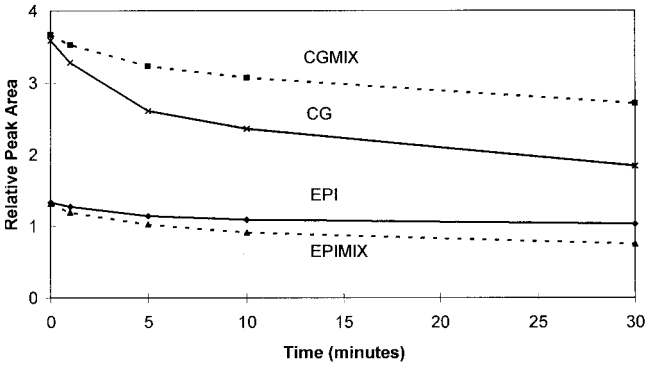

Figure 4. Influence of the substrates chlorogenic acid (CG), (−)-epicatechin (EPI), or a mixture of CG and EPI (CGMIX and EPIMIX) on the time-dependent consumption of CG and/or EPI (measured as HPLC peak area (280 nm) relative to internal standard (vanillic acid)) by the polyphenol oxidase (PPO). For further details, see HPLC Measurements under Materials and Methods.

Compounds with t_R = 10.15 and 10.2 min are less polar than CG, but their identities are unknown (Figure 2). To obtain further information about the reaction path when CG is substrate for PPO, multiway data analysis of UV−vis spectra (Table 1) using N-PLS2 (Bro, 1996) was used to predict substrate consumption and product development data (otherwise obtained by HPLC analysis). The overall shape of these UV−vis spectra could be analyzed and dissolved into the most important contributing spectra (Bro and Heimdal, 1996). They turned out to be of almost the same shape as the spectrum of the possible dimer (t_R = 10.6 min) and as the spectra of CG or the unknown products with t_R = 10.15 and 10.2 min from the HPLC analysis (Figure 2). The decrease in UV absorbance at 325 nm is not an exact measure of the CG consumption, as is assumed by Tono et al. (1987). Our results indicate that contribution from a dimeric compound to spectral changes in absorbance in model solutions containing CG and PPO is of importance. In addition, the results indicate that substrate consumption obtained by HPLC analysis can be predicted from kinetic UV−vis spectra in model systems (Bro and Heimdal, 1996). The time-consuming and expensive HPLC diode array analysis can thus be substituted by simple kinetic UV−vis spectrometry in combination with multiway data analysis without missing the most important information related to formation of products and consumption of substrate (Bro and Heimdal, 1996).

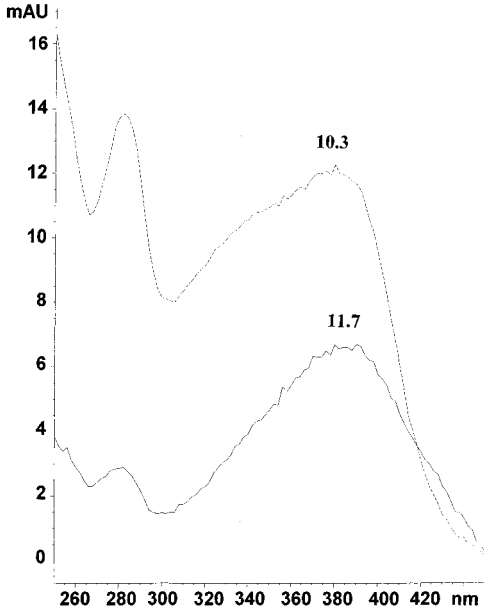

Figure 5. Diode array spectra of major products (named by retention time) derived from HPLC measurements after 60 min of reaction in model solutions containing chlorogenic acid (CG), (−)-epicatechin (EPI), lettuce polyphenol oxidase (PPO), and acetate buffer (pH 6.0, 30 °C) saturated with 80% O_2, 0% CO_2, and 20% N_2. Absorbance is expressed as milliabsorbance units (mAu). For further details, see HPLC Measurements under Materials and Methods.

(−)-Epicatechin (EPI). In model solutions containing EPI, PPO, and buffer aerated with each of the five gas mixtures, the EPI concentration decreases during reaction with PPO. The rate of substrate consumption is lower in solutions with 20% CO_2 compared to that in solutions containing 0% CO_2 but the same in O_2 concentrations (data not shown). In solutions with very low O_2 concentration (0/0), the slightest substrate consumption is observed (data not shown).

Many colored products are produced when EPI is substrate for the enzyme. The two major compounds with t_R = 10.3 and 11.7 min, respectively, show high absorbance in the visual area (Figure 3). The latter (t_R = 11.7) is probably a dimer, as judged by the similarity to the spectrum of a dehydrodicatechin A found in model solutions containing catechin and grape PPO (Guyot et al., 1995) at pH 6. The former (t_R = 10.3) has approximately the same polarity as EPI (t_R = 10.1) and might be a quinone-scavenged cysteinyl derivative. A compound with this spectrum was not found in model solutions without addition of cysteine to the stopping solution (Guyot et al., 1995).

To obtain further information about the reaction path when EPI is substrate for PPO, multiway data analysis of UV−vis spectra (Table 1) using N-PLS2 (Bro, 1996) was used to predict substrate consumption and product

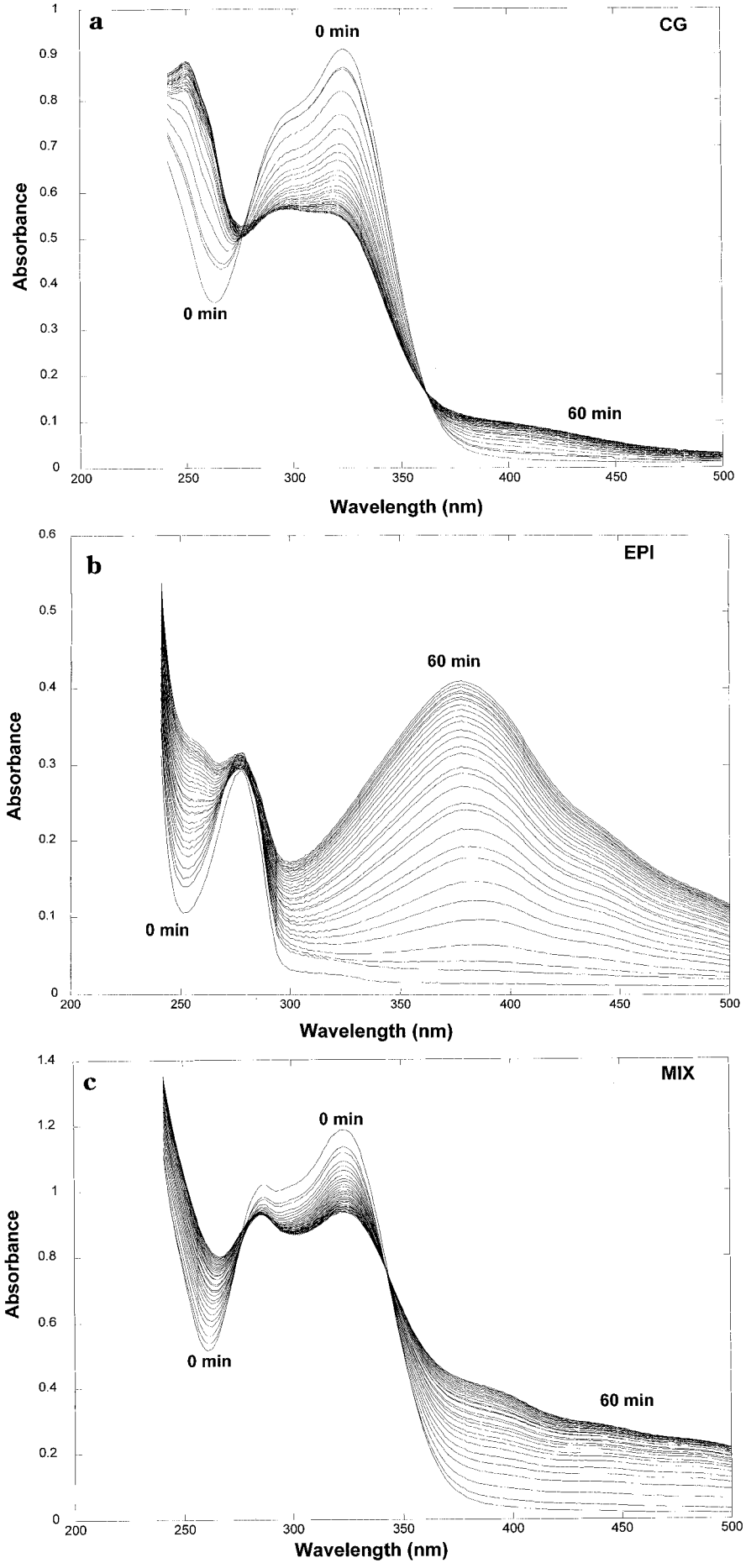

Figure 6. Time-dependent UV−vis spectra of model solutions containing acetate buffer (pH 6.0, 30 °C) aerated with atmospheric air, lettuce polyphenol oxidase (PPO), and (a) chlorogenic acid (CG), (b) (−)-epicatechin (EPI), or (c) an equimolar mixture of CG and EPI (MIX).

development data (otherwise obtained by HPLC analysis). The overall shape of the UV−vis spectrum was dissolved into two spectra of nearly the same shape as the major products from HPLC analysis (t_R = 10.3 and 11.7 min; Bro and Heimdal, 1996). Like in the CG experiments, the contribution from a dimeric compound to spectral changes in absorbance in the model solution containing EPI and PPO is important.

Chlorogenic Acid and (−)-Epicatechin (MIX). When both phenolic substrates are available for the enzyme, the CG consumption is lower and the EPI consumption is higher compared to the substrate consumption with one of the phenolic substrates alone (Figure 4). The same picture is seen for the five gas mixtures, except for gas 0/0, where very low and equal substrate consumption is seen (data not shown). The decrease in substrate concentration measured by HPLC is primarily a result of incorporation into a polymer (Cheynier et al., 1988). The results presented in Figure 4 confirm the theory by Cheynier et al. (1989) that CG is a primary substrate for PPO and CG-quinone is reduced back to CG, while EPI is oxidized to EPI-quinone. EPI-quinone polymerizes irreversibly with EPI and CG, resulting in colored products. Products from EPI and MIX oxidation with t_R = 11.7 min have very similar spectra and, therefore, probably are EPI dimers or trimers (Figure 5). Oxidations of CG, EPI, and MIX all gave a product with t_R = 10.3 min. The 10.3 peak in the MIX oxidation is, therefore, probably a mixture of two or more compounds, which explains the shape of the spectrum (Figure 5). In general, many compounds are developed during the reaction when MIX is oxidized by PPO, resulting in HPLC peaks that contain more than one compound.

Color Formation. In model solutions containing atmospheric air, CG, and PPO, the spectrum changes over time as a result of substrate degradation and development of products (Figure 6a). No great absorbance is seen at 380 nm. The time-dependent spectra of EPI as substrate for PPO in atmospheric air results in high absorbance at 380 nm, as shown in Figure 6b. Time-dependent spectra for CG and EPI are in accordance with results obtained by Richard-Forget (1992). Time-dependent spectra obtained in model solutions containing atmospheric air, CG, EPI, and PPO are shown in Figure 6c. Model solutions influenced by other combinations of O_2, CO_2, and N_2 resulted in spectra with the same shape but different velocities (data not shown).

Results from color development observed by UV−vis spectrometry were analyzed by the multiway data analysis N-PLS separately for each substrate (Bro and Heimdal, 1996) in order to predict the influence of CO_2 and O_2 on color formation. However, the results were not very conclusive and will, therefore, not be described in detail. For the CG-incubated samples, no effect of CO_2 could be observed, while for the EPI-incubated samples, there was a distinguishable difference between the presence and absence of CO_2. This suggests that color formation by flavonoids is influenced by CO_2, while color formation by hydrocinnamics is not.

The facts that PPO activity (O_2 consumption) is slightly influenced by CO_2 (Table 4), that flavonoids primarily are involved in polymerization (Figure 4), and that color development is reduced by CO_2 only when flavonoids are available as substrates indicate that CO_2 diminishes the polymerization and slightly reduces PPO activity. The mechanism of CO_2 inhibition of polymerization is unknown. It could be speculated that it might be due to an electrophilic attack by CO_2 on the slightly negatively charged quinones, resulting in compounds

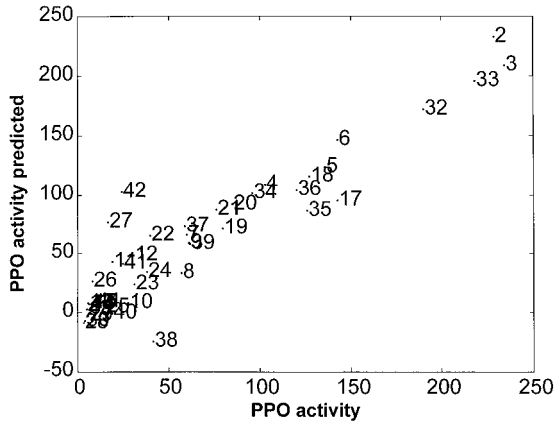

Figure 7. Polyphenol oxidase (PPO) activity predicted from kinetic UV−vis spectra (45 combinations, Table 1) versus PPO activity (O_2 consumption) using the multivariate N-PLS method.

which are unable to polymerize. The inhibition by CO_2 of lettuce PPO activity found by Siriphanich and Kader (1985) is probably due to a combination of inhibition of the enzymatic activity and the polymerization, because a spectrophotometric PPO assay was used.

Prediction of PPO Activity under Varying Conditions. We have showed that, even though we vary substrate type, pH, temperature, O_2 level, and CO_2 level, it is still possible to make a general calibration model for PPO activity. This implies that we can now take a sample with varying and unknown levels of these factors and estimate the PPO activity. In this case, we found that, due to the relatively few samples available, we had to separate the calibration models into three models according to "substrate" (CG, EPI, and MIX). The PPO activity (O_2 consumption) is the dependent variable to be predicted, and the independent variables from which the activity is predicted are the UV−vis absorbance spectra determined at various times after incubation. The dependent variables in this case are thus a "landscape of absorbances". In the work of Bro and Heimdal (1996), several methods were investigated for estimating the calibration models. The results using the N-PLS model will be shown here. In Figure 7 (r^2 = 0.87), the predictions of activity are shown. Classical spectrophotometric PPO assays using measurements from only one wavelength gave a poor estimation of PPO activity (r^2 = 0.64), probably because of the complexity of the color formation (Bro and Heimdal, 1996).

The result of the multivariate data analysis shows that it is possible to predict PPO activity even though the samples are of very different constitutions. When comparing standard univariate analysis with multivariate analysis, it is shown that a univariate approach will not be able to correctly predict the concentration when the sample constitutions differ as much as they do here (Bro and Heimdal, 1996).

Conclusion. Overall, the statistical analysis of the combination of PPO activity (O_2 consumption), color development, and formation of reaction products from this study of model solutions led to the conclusion that enzymatic browning of shredded lettuce might be minimized if it is stored in an atmosphere with high CO_2 (≥10%), low O_2 %, low pH, and low temperature. This is in accordance with the finding that the lowest enzymatic browning was observed in shredded lettuce stored in an atmosphere with low O_2 and high CO_2 concentrations quickly obtained in the MA packagings (Heimdal et al., 1995).

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Received for review December 30, 1996. Revised manuscript received April 1, 1997. Accepted April 1, 1997.[⊗] This research was supported by the Danish Ministry of Education as part of the FØTEK program.

JF960975W

[⊗] Abstract published in *Advance ACS Abstracts*, June 15, 1997.