Enzyme Assays for the Phenolic Content of Natural Juices

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The properties of a fungal laccase have been exploited to develop two enzyme assays for the phenolic content of natural beverages: first, by monitoring the fall in oxygen tension after addition of enzyme to a diluted juice sample in an end-point batch assay; second, by monitoring the current due to reduction of products after injection of sample into a flow injection analysis (FIA) system which incorporates the immobilized enzyme. Using apple juices, a linear ($R > 0.9$) correlation was observable between these enzyme assays and the Folin–Ciocalteu wet chemical assay for total phenol content. Using purified substrates, a study has been made of those phenolic constituents measured in the two enzyme assays. Oxidative treatment of juices with laccase was shown by HPLC to specifically remove these phenolics. These enzyme assays may prove helpful to predict the stability of natural beverages.

Keywords: *Enzyme assay; phenolics; apple juice; laccase; flow injection analysis (FIA)*

INTRODUCTION

Naturally occurring phenolics and their oxidation products are important constituents of natural beverages, contributing to color and taste. The use of enzymes, in particular those involved in the breakdown of carbohydrates and proteins, has long been established in the extraction and stabilization of juices (Pilnik, 1982). For apple (Lea, 1984) and grape juices (Singleton, 1987), excessive oxidation of phenolics has almost always been considered detrimental to the organoleptic quality of the product. Thus, measures are often taken to exclude oxygen during processing and reductants may be added to the final product. Recently, a controlled oxidation early in processing has been advocated for the production of wines (Müller-Späth, 1989) and apple juices. The rationale would be to remove by polymerization, precipitation, and filtration the phenolic components that may cause problems later, particularly during storage. Oxidation in the absence of a catalyst is very slow, the effectiveness of this treatment being greatly enhanced by addition of a fungal laccase to supplement the polyphenol oxidase activity naturally present (Cantarelli, 1986; Maier et al., 1990).

Thus, a measure of the phenolic content of a natural beverage is necessary both for an adequate organoleptic description and as an indicator of stability on storage. However, obtaining such a measure is complicated by the diverse chemical nature of the phenolic components. The main constituents can be divided into the phenolic carboxylic acids, esters, and aldehydes (e.g., chlorogenic acid, coniferaldehyde), the flavonoids (e.g., catechins, flavonols, flavonones, and anthocyanogens), and condensed phenolic polymers (the tannins). Many of the phenolics, in particular the flavonols, are present as glycosides.

Quantification of individual components may be achieved by the normal methods of separative analytical chemistry, including thin-layer, gas, and high-pressure liquid chromatography [for reviews see Singleton (1988) and Hardin and Stutte (1984)]. However, such methods are time-consuming, with no single set of conditions being available for the separation of all components. In addition, considerable effort is required to obtain standards, many of which must be purified from natural sources.

Wet chemical methods are available for estimations of the total phenolic content and for particular fractions. Of these, the two most common are the vanillin–sulfuric acid reagent for condensable flavanoids and the Folin-Ciocalteu assay for total phenolics. The latter is widely used, although it is known that this strong oxidizing agent will react with compounds other than the target phenols and that interfering reductants must be removed prior to the assay. The Folin–Ciocalteu assay has been automated in a continuous flow procedure (Slinkard and Singleton, 1977), a technique that has largely been superseded by flow injection analysis (FIA).

Various enzymatic assays and biosensors have been reported for phenolic compounds. The enzymes used were a fungal phenol hydroxylase [Fletcher et al., 1977 (a); Neujahr, 1982 (b)], tyrosinase from mushrooms [Hall et al., 1988 (c); Kulys and Schmidt, 1990 (d); Pacàkova et al., 1984 (e); Zachariah and Mottola, 1989 (f)], or polyphenol oxidase from higher plants [Macholan, 1987 (g); Sidewell and Rechnitz, 1986 (h); Uchiyama et al., 1988 (i)]. Different methods have been used for quantification: amperometrically using an oxygen electrode (b, e, h, i); amperometrically by reduction of the reaction products, either directly at a carbon electrode (c) or at a chemically modified electrode (d); and potentiometrically (a). To our knowledge, no practical application of these methods has gained acceptance to date.

We have investigated the use of a fungal laccase to measure the phenolic content of natural beverages,

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concentrating on apple juices. In particular, we have studied the significance of the signal obtained using two amperometric detection systems: first, with an oxygen electrode to monitor oxygen consumption, in a batch assay using the soluble enzyme; second, with reductive detection of the reaction products at a glassy carbon electrode, in an FIA system using the immobilized enzyme.

MATERIALS AND METHODS

All chemicals were of the highest purity available from Fluka, Aldrich, or Merck. Phosphate buffers were prepared by mixing their sodium salts and acetate buffers by adjusting the pH of a carboxylic acid solution with 4 M sodium hydroxide, followed by volume adjustment to the final acid concentration as given.

Laccase of *Trametes versicolor* [activity = 40 international units (IU) mL^{-1}] was a kind gift from Dr. Matti Leisola of Cultor, Finland (lot 90401178). The partially purified extracellular fraction was sterile filtered on arrival and stored at 4 °C. One IU corresponds to the quantity of enzyme producing 1 μmol min^{-1} tetramethoxyazobis(methylenequinone) under the following conditions: To 800 μL of 50 mM sodium acetate, pH 5.0 (buffer A) at 25 °C, was added 100 μL of 0.5 mM syringaldazine (in ethanol; final concentration, 50 μM) followed by 100 μL of sample. The initial reaction rate was quantified spectrophotometrically (Shimadzu UV-240) at 530 nm (ϵ_{530} = 65 000 M^{-1} cm^{-1}) (Leonowicz and Grywnowicz, 1981). Protein concentrations were determined by the Coomassie blue binding method according to instructions supplied with reagent from Bio-Rad.

Immobilization on Epoxy-Activated Acrylic Beads. One milliliter of the laccase solution was exchanged into the coupling buffer, 1 M potassium phosphate, pH 7.5, by passage through Sephadex G-25 (PD-10 column, Pharmacia) in a final volume of 3 mL. The beads (0.25 g of Biosynth VA, Riedel-de-Haën) were incubated with 1.5 mL of enzyme solution for 24 h at 4 °C. Coupling yields were calculated by the fall in soluble enzyme activity of the supernatant. After coupling, the beads were washed with 0.1 M potassium phosphate, pH 7.5, and finally with buffer A and stored at 4 °C in the same buffer.

For use in the FIA system, beads were packed into teflon tubing (diameter, 1.5 mm; length, 30–50 mm) and retained behind porous polyethylene frits (30-μm Porex). The FIA hardware and setup was that described previously (Fawer et al., 1991). Two detectors were used: first, an electrochemical wall jet type (Metrohm 656, Metrohm, Switzerland), which incorporates an Ag/AgCl/3 M KCl reference electrode with a gold counter electrode and a glassy carbon working electrode held at 0.0 V for the reduction of oxidation products; second, an oxygen flow-through electrode (prototype kindly lent by Ingold AG, Urdorf, Switzerland) of the Clark type. Running buffer was buffer A, with flow rates in the range 1.0–1.3 mL min^{-1}. The injection volume for reductive measurements was 100 μL and for oxygen consumption measurements 600 μL. Either one of the outputs from the two detectors was fed over a chart recorder to the microprocessor, and for each peak a printout of height and area was obtained.

Cyclic voltammograms from 0.0 to 1.0 V were recorded with an electrochemical analyzer BAS 100A (Bioanalytical Systems, West Lafayette, IN) in buffer A at a scan rate of 20 mV s^{-1}. The glassy carbon working electrode was polished before each run with alumina powder and a Q-tip and sonicated in 1 M HCl for 60 s, and 10 initial sweeps in 3 mL of buffer A at 100 mV s^{-1} scan rate were carried out to stabilize the electrochemical signal. A 20-μL aliquot of substrate was added (dissolved in dimethylformamide when necessary) to give a final concentration of 670 μM and the voltammogram recorded.

Reconstituted apple, pear, and grape juices and white and red wines were obtained from retail outlets. Fresh apple juice and juices oxidized by addition of fungal laccase prior to ultrafiltration, bottling, and pasteurization were prepared at the Institute of Oenology in Geisenheim as described (Maier et al., 1990). In this test series a control (no added laccase) and laccase-treated samples, with different methods of fining, were compared as follows: sample C1, control without fining; C2, control fined with gelatin (15 g hL^{-1}) and approximately 30% Kieselsol; C3, control fined with PVPP (100 g hL^{-1}); C4, control fined with bentonite (50 g hL^{-1}); L1, laccase treated without fining; L2, laccase treated with gelatin/Kieselsol fining; L3, laccase treated with PVPP fining; L4, laccase treated with bentonite fining.

The Folin–Ciocalteu assay for the determination of the total concentration of phenolic compounds in apple juices was carried out essentially as described (Tanner and Brunner, 1987) with the following modification to reduce the assay volume to 10 mL: To 8.4 mL of H_2O, add 0.1 mL of sample followed by 0.5 mL of Folin–Ciocalteu reagent (Merck), mix, and wait for 3–6 min; add 1 mL of saturated sodium carbonate (approximately 200 g of Na_2CO_3 in 1000 mL of H_2O).

For the determination of the enzyme-catalyzed oxygen consumption [laccase oxygen demand (LOD)], soluble laccase in a batch assay with an electrochemical processor, Model 2000, and oxygen electrode (Rank Brothers LTD, Cambridge, Great Britain) was used. The sample, standards, or juices suitably diluted in aerated 50 mM sodium acetate, pH 5.0, volume 3 mL, were added to the reaction chamber (thermostated at 25 °C). The chamber lid was replaced, and the fall in oxygen tension was recorded after addition of 20 μL (0.5 IU) of laccase through the entry channel of the closed chamber. The end point was reached after 5–15 min (at lower phenolic concentrations the end point is reached more quickly).

Calibration curves for enzymatic and Folin–Ciocalteu assays were constructed using chlorogenic acid as standard, phenolic content being expressed as chlorogenic acid equivalents in mg L^{-1}. For all assays the mean and coefficient of variation (cv) of a least three determinations were calculated.

High-pressure liquid chromatography (HPLC) separation of "neutral" and "acidic" polyphenolic fractions was carried out with minor modifications of the method described (Jaworski and Lee, 1987) in detail for grape phenolics. Chromabond C_{18} cartridges, 500 mg (Machery-Nagel), were preconditioned with 5 mL of methanol and 10 mL of H_2O (neutral cartridge) or 5 mL of methanol and 10 mL of 0.5% (v/v) acetic acid (acid cartridge). Apple juice samples were adjusted to pH 6.0 with 4 M NaOH, and a volume of 2–5 mL was applied to the neutral cartridge. The eluate and washings (5 mL H_2O, 2 mL of 0.5% acetic acid) were adjusted to pH 2.0–2.5 with 1 M H_2SO_4 before being applied to the acidic cartridge, which was itself washed with 5 mL of 0.5% acetic acid. Both cartridges were eluted with 2.5 mL of methanol and the eluates reduced to near dryness under reduced pressure. Prior to HPLC, the samples were taken up in 50% (v/v) aqueous methanol and filtered (0.45 μm). An HPLC system from Merck was used which incorporated a diode array detector. Forty-microliter samples were separated over a reversed phase (LiChrospher 100 RP-18, 5 μm) column with a discontinuous gradient from 10% (v/v) methanol in 0.5% (v/v) aqueous acetic acid to 100% methanol over 50 min. Phenolic compounds were identified by comparing their spectra and retention time with standards and by the standard addition method. Recoveries of spiked samples were between 80 and 100%. Chlorogenic acid, caffeic acid, coumaric acid, (+)-catechin, (−)-epicatechin, phloridzin, and phloretin were obtained from Roth (Karlsruhe). Procyanidin B2 was a gift from Dr. A. Lea (RSSL, Reading, U.K.). *p*-Coumarylquinic acid was quantitated as coumaric acid and the phloretin glycoside as phloretin.

RESULTS

Calibration curves in the linear range ($R > 0.98$) for the three enzyme assays are shown in Figure 1. Using the immobilized enzyme in the FIA system, the reductive response (FIA-RR) was the most sensitive (detection limit approximately 50 nM, cv = 1.5%, n = 8, 1.0 μM chlorogenic acid), enabling injection of juice samples at high dilution (typically 0.1–2.5%). This method gave reproducible results for juice samples, with a typical cv = 2% and linear working range of 0.05–50 μM (0.02–

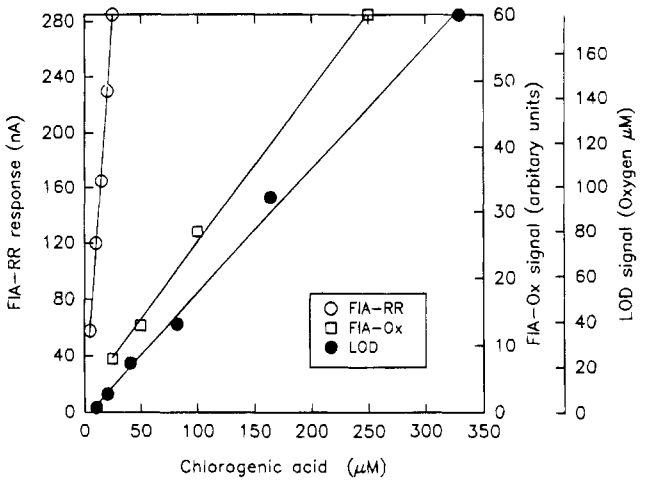

Figure 1. Calibration curves for three enzyme assays using chlorogenic acid as standard.

20 mg L^{-1}) chlorogenic acid. With this nonequilibrium assay, absolute values are dependent on the activity of the enzyme column and the state of the electrode, so that daily calibration is required. Electrode fouling necessitated daily cleaning of the electrode surface. A preliminary study (Cliffe et al., 1992) had shown that loss of enzyme activity, approximately 1% reduction in peak height every 20 injections, determined the lifetime of the laccase column. Of the three immobilization matrices compared previously, controlled pore glass, activated cellulose membranes, and epoxy activated acrylic beads, best results in terms of sensitivity and peak stability were obtained with the latter matrix, which was thus chosen for the current study (Fawer, 1992).

Monitoring the transient fall in oxygen levels (FIA-Ox) gave a markedly less sensitive assay (detection limit of 20 μM chlorogenic acid). Nevertheless, with standard solutions, reproducible (typical cv = 2–3%) results were obtained with many phenolic compounds. However, the low sensitivity necessitated injection of juice samples at 5–20% dilution, where a considerable zero signal was observed. To overcome this, a batch assay was developed, measuring the total oxygen demand (LOD) after addition of soluble laccase to a previously diluted juice sample. While similar slopes of the calibration curves are observed for the FIA-Ox and LOD assays, the detection limit in the latter is reduced to 10 μM chlorogenic acid and little zero signal was observed with suitably diluted juices. Thus, accurate quantification of juice samples was achieved with a typical cv = 2–6% and linear working range of 10–350 μM (5–125 mg L^{-1}) chlorogenic acid.

In Table 1, the relative oxygen demands (measured using FIA-Ox and FIA-RR) are listed for a series of naturally occurring and synthetic compounds. The substrate specificity of laccase appears to be limited primarily by the redox potential of the substrate, which, when above approximately 650 mV, renders the substrate resistant to attack. Thus, laccase is able to directly oxidize many (including the main component chlorogenic acid) but not all of the phenolics present in an apple juice—phloridzin being noticeably resistant. In addition to direct enzymatic oxidation, some substances may be oxidized by reactive reaction products or intermediates. In test systems over a period of days, the presence of mediators results in the oxidation of substrates of higher oxidation potential (Bourbonnais and Paice, 1987); however, given the time scale of the enzyme assays, approximately 5 s for FIA and 10 min

Table 1. Enzyme Assays for Phenolic Standards Using Flow Injection Analysis

substance	E_{Ox}[a] (mV)	FIA response: FIA-Ox relative signal[b]	FIA response: FIA-RR (nA μM^{-1})
hydroquinone	306	1.0	6.3
caffeic acid	333	1.0	11.7
4-methylcatechol	350	1.1	20.7
gallic acid	350	1.3	<0.5
dopamine	354	1.1	14.5
chlorogenic acid	356	1.0	11.5
syringaldazine	364	0.8	1.3
(−)-catechin	380	0.3	1.2
(±)-catechin	383	0.4	0.6
(+)-catechin	400	0.4	0.5
catechol	422	1.1	20.2
syringic acid	550	1.8	0.6
syringaldehyde	638	0.9	0.8
phloridzin	786	0	0
p-coumaric acid	793	0	0

[a] From cyclic voltammetry experiments according to procedure described under Materials and Methods. [b] Comparison of molar response.

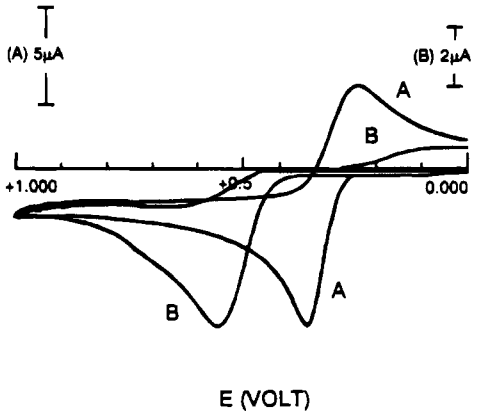

Figure 2. Cyclic voltammograms of chlorogenic acid (A, 5-μA scale) and syringic acid (B, 2-μA scale).

for the batch assay, we consider it unlikely that this effect plays a role here. Of more significance may be the role chlorogenic acid is proposed to play in mediating oxidation in apple juices (Lea, 1984), where this mediation may speed up oxidation of compounds that only undergo slow direct oxidation.

Also from Table 1 it can be seen that not all substrates detectable by oxygen consumption gave a reductive response. A reductive response is expected only when the oxidation is reversible at the applied potential of 0.0 V. Cyclic voltammetry, Figure 2, confirmed that only those compounds showing at least semireversible oxidation in the range 0.0–1.0 V gave a reductive response. Thus, chlorogenic acid (almost fully reversible) shows a much greater reductive response than syringic acid (almost totally irreversible).

The correlation observed between the two enzyme methods and the total phenol content as determined by the Folin–Ciocalteu method is shown in Figure 3 for 13 apple juices. It can be seen that the LOD assay gives values of approximately 70% the value given by the Folin–Ciocalteu method, while the reductive response lies considerably lower. While the absolute quantities are significantly different, there is clearly a correlation between the values obtained by the three methods. If we consider the juice to have n phenolic components, $P_1, P_2, \ldots, P_n$, then the true value for the total phenolic content is given by $\Sigma[P_n]$. Any one assay for the total phenolic content will give a value of $\Sigma w_n[P_n] + I_{np}$, where w_n represents the *weighting* or sensitivity of a particular

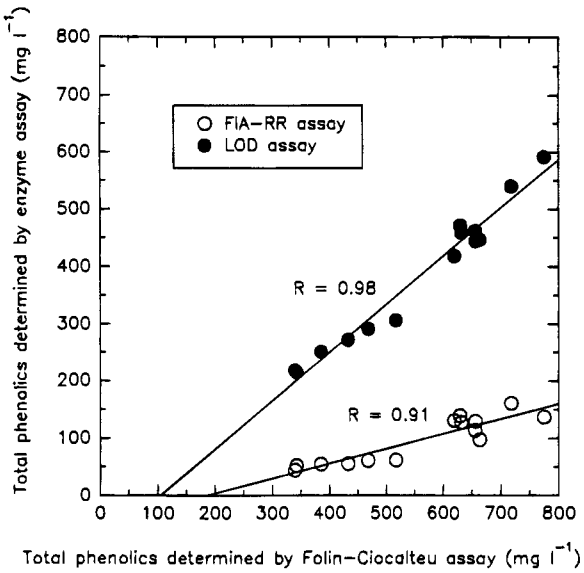

Figure 3. Comparison between enzyme and chemical assays for the total phenolic content of apple juices.

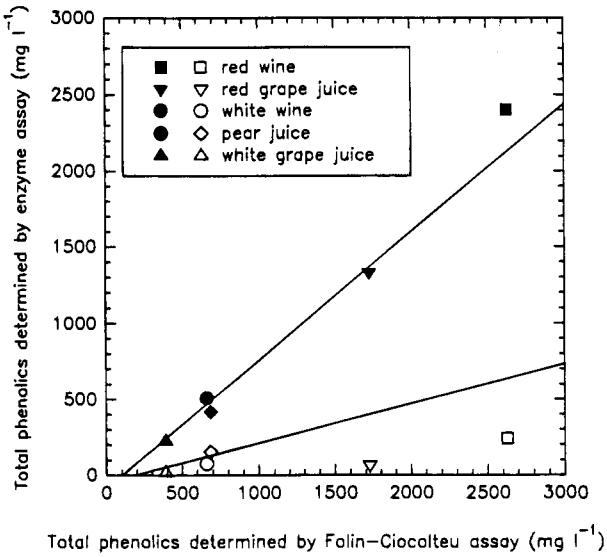

Figure 4. Comparison between enzyme and chemical assays for the total phenolic content of different juices: (solid symbols) LOD assay; (open symbols) FIA-RR assay.

phenolic in the assay and I_{np} represents signal due to *nonphenolic* components such as sugars, nucleic acids, and proteins. Determination of weighting factors for all phenolics in each of these assays was not feasible during this study; however, we can reach some conclusions, in particular with regard to the enzyme assays, in the light of the present results. Since laccase will not oxidize all phenolics, w_n will equal zero for a small number of phenolic components in the LOD assay and the value of w_n for positive constituents will vary with the stoichiometry of the oxidation reaction. By comparison, with the FIA-RR assay, w_n will equal zero for a larger subset of phenolics and the values of w_n will vary over a wider range. Thus, we would expect the phenolic content as measured by the enzyme assays, and in particular with the FIA-RR assay, to vary with the relative quantities of different phenolic constituents.

To test this hypothesis, we have measured the phenolic content of a limited number of non-apple-derived juices. If we take the regression lines for the LOD and FIA-RR assays from Figure 3 and then compare these with values obtained from other beverages as depicted in Figure 4, we can see that the correlation between the three assays remains surprisingly similar. However, there is more variation. In particular, in comparison to the Folin–Ciocalteu values, the FIA-RR values for grape juices and wines lie under that obtained for apple juices, while the LOD values vary in a similar fashion.

In Table 2 the analysis data are given for a series of four apple juices that were prepared after the must had been exhaustively oxidized in the presence (L1–L4) or absence (C1–C4) of added laccase. The total phenolic content as measured by Folin–Ciocalteu fell by ca. 50% after laccase treatment, while the response in the LOD assay fell by over 90% and the RR fell by over 97%.

HPLC analysis of samples C1 and L1 is given in Table 3. Laccase treatment resulted in almost complete removal of chlorogenic acid, caffeic acid, and (−)-catechin, while levels of *p*-coumarylquinic acid, phloretin glycoside, and phloridzin remained largely unaltered. These results are consistent with the data given in Table 1 and suggest that at least the latter three laccase-resistant phenolics are not oxidized indirectly in a mediated reaction. We observed a total phenolic fraction estimated by HPLC as 10–15% of the Folin–Ciocalteu values, which was in agreement with previously published data (Wrolstad et al., 1990). Recoveries of standards from spiked samples were consistently above 80%. While the absorption coefficients (measures of w_n for spectrophotometric detection) assumed for some compounds may have resulted in errors, it is also likely that, as yet, unknown phenolics remain undetected by the HPLC system used. Thus, we have no definitive means to determine I_{np}, the signal due to nonphenolic components of the juice. However, since enzymatic oxidation is considerably more specific than chemical oxidation, we would expect the value of I_{np} to be much smaller for the enzyme assays than for the Folin–Ciocalteu method.

Table 2. Effect of Laccase-Catalyzed Oxidation on Phenolic Content of Apple Juice As Measured by Enzyme or Chemical Assay

	enzyme assay		
juice sample	LOD (mg/L)	FIA-RR (mg/L)	chemical assay FC (mg/L)
C1	291	61	470
C2	252	55	385
C3	215	52	340
C4	272	56	430
L1	14	<1	190
L2	35	<1	210
L3	14	<1	160
L4	17	<1	174

Table 3. Effect of Laccase-Catalyzed Oxidation on Phenolic Content of Apple Juice As Measured by HPLC

	peak area (mg/L)	
phenolic compd	sample C1	sample L1
chlorogenic acid	27.3	2.4
(−)-epicatechin	9.0	1.0
caffeic acid	0.7	0.1
procyanidin B2	3.1	0.9
coumaric acid	1.2	1.0
phloridzin	7.1	5.0
phloretin glycoside	8.2	7.0
p-coumarylquinic acid	9.5	10.5
total	66.1	27.9

DISCUSSION

The enzyme assays (FIA-RR and LOD) gave absolute values for the total phenolic content of unoxidized apple juices that represented approximately 20% and 70%,

respectively, of the values obtained by the Folin–Ciocalteu method. This can be compared with a previously reported correlation between chlorogenic acid levels (approximately 8% total), measured by HPLC, and the total Folin–Ciocalteu phenolic content (Cilliers et al., 1990). While absolute values from the enzyme assays may vary with fruit and possibly cultivar, these assays provide accurate measures of the total phenol content liable to laccase-catalyzed oxidation. Initial results with juices aerated in the presence of laccase (Ritter et al., 1992) suggest that it is mainly these phenolics that may be critical to the stability of processed juices. Due to the instability of plant polyphenol oxidase, a possible narrower substrate range for the native enzyme, and the exclusion of oxygen during processing, many of these phenolics remain present in conventionally prepared juices. Thus, the enzyme assays may provide a measure of potential polyphenolic instability, particularly for products that do not contain antioxidants.

The LOD assay provides an accurate end-point measurement that is insensitive to small fluctuations in enzyme activity. Automation of the present LOD protocol would be possible in a flow system using a stopped flow procedure. The FIA-RR assay provides at present an automated, reproducible, and quick measurement. However, it is a nonequilibrium technique, and further work is required to determine to what extent the assay suffers from interference.

ABBREVIATIONS USED

FIA, flow injection analysis; FIA-Ox, flow injection analysis assay for total phenolics with detection of oxygen consumed; FIA-RR, flow injection analysis assay for total phenolics with amperometric detection of products formed (reductive response); LOD, batch assay for total phenolics measuring the laccase oxygen demand; HPLC, high-pressure liquid chromatography; cv, coefficient of variation; w_n, weighting or sensitivity of a particular phenolic; I_{np}, signal due to nonphenolic compounds.

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