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Effect of Mash Maceration on the Polyphenolic Content and Visual Quality Attributes of Cloudy Apple Juice

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The effects of enzymatic mash treatments on yield, turbidity, color, and polyphenolic content of cloudy apple juice were studied. Using HPLC-ESI-MS, cryptochlorogenic acid was identified in cv. Brettacher cloudy apple juice for the first time. Commercial pectolytic enzyme preparations with different levels of secondary protease activity were tested under both oxidative and nonoxidative conditions. Without the addition of ascorbic acid, oxidation substantially decreased chlorogenic acid, epicatechin, and procyanidin B2 contents due to enzymatic browning. The content of chlorogenic acid as the major polyphenolic compound was also influenced by the composition of pectolytic enzyme preparations because the presence of secondary protease activity resulted in a rise of chlorogenic acid. The latter effect was probably due to the inhibited protein−polyphenol interactions, which prevented binding of polyphenolic compounds to the matrix, thus increasing their antioxidative potential. The results obtained clearly demonstrate the advantage of the nonoxidative mash maceration for the production of cloud-stable apple juice with a high polyphenolic content, particularly in a premature processing campaign.

KEYWORDS: Cloudy apple juice; cryptochlorogenic acid; mash maceration; proteolytic enzymes; polyphenolic antioxidants; HPLC-MS; cloud stability

INTRODUCTION

Epidemiological studies have shown an inverse correlation of increased fruit and vegetable consumption with a reduced risk of chronic diseases including cardiovascular diseases (*1*, *2*). Fruits, vegetables, and their juices contain a broad spectrum of natural antioxidants that inhibit human low-density lipoprotein oxidation and could account for the beneficial health effect (*3*, *4*). Apples and apple juices have been shown to possess both in vitro and in vivo antioxidant activities that are attributed to their polyphenolic compounds (*5*−*10*).

The major polyphenolic compounds of apples are not equally distributed within the fruit (*11*, *12*). Whereas flavonol glycosides and anthocyanins are almost exclusively found in the skin, the main flavanols, catechins and proanthocyanidins, are mostly located in the skin, but lower levels are also present in the flesh and core, including the seeds. Chlorogenic acid, the predominant hydroxycinnamate, is mainly present in the core, whereas lower levels are found in the flesh and skin. Dihydrochalcones, phloridzin and phloretin xyloglucoside, are mainly located in the core, and lower levels are present in the skin and flesh. The concentrations of polyphenols are strongly dependent on the apple cultivar (*13*, *14*). However, because clear apple juice technology is primarily aimed at polyphenol reduction by juice fining, the process of juice production itself appears to have a greater influence on the polyphenolic contents of the juices than the variation of cultivars (*15*, *16*).

Apple mash maceration with pectolytic enzyme preparations is widely used in the juice industry to improve the juice yield and to facilitate the pressing operation. The effect of enzymation on the polyphenolic content of apple juice has been studied only under oxidative conditions of mash treatment according to the common production practice of clear apple juice concentrate (*17*−*19*). However, in the case of cloudy apple juice, the addition of ascorbic acid to the mash has been recommended for both cloud stability enhancement and prevention of oxidative browning, especially for the production of extremely light colored juices, for example, for the Japanese market (*20*). Moreover, inhibitory effects of oxidized polyphenols on the activity of pectolytic enzyme preparations have been reported (*21*).

Recently, apple pomace liquefaction with pectolytic and cellulolytic enzymes has been shown to increase the polyphenolic content, especially dihydrochalcones and quercetin glycosides, of the "extraction" juices as compared with the corresponding "premium" juices, which were produced by

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Table 1. Variants of Mash Maceration and Corresponding Juice Yields

variant code	enzyme preparation	pectolytic activity[a]	proteolytic activity (units/g)	dosage (mg/kg)	juice yield[b] (%)
C1[c]	control (without				78.3
C2[d]	enzymation)				77.5
A1	Rohavin CXL	160	91	60	85.8
A2		160	91	60	82.9
B1	Rohavin VR-X	80	10061	240	83.4
B2		80	10061	240	80.9

[a] Enzyme activity units according to product specification. [b] All are calculated to a standard apple juice strength of 11.2 °Brix. [c] 1 is a variant without ascorbic acid addition. [d] 2 is a variant with ascorbic acid addition.

conventional mash treatment with pectolytic enzymes (*22*). However, the use of cellulolytic enzymes for fruit juice production is still prohibited by law in the European Union. On the other hand, secondary protease activities of commercial pectolytic enzyme preparations (*23*), besides their major activities (polygalacturonases, pectin esterases and pectin lyases), are widely used in fruit juice technology. However, their potential contribution to the release of polyphenols has not yet been assessed.

Therefore, the main objective of the present study was to investigate the effect of mash maceration on the polyphenolic content of cloudy apple juice. Commercial pectolytic enzyme preparations with different levels of secondary protease activity were tested under both oxidative and nonoxidative conditions. The effects of mash maceration and the addition of ascorbic acid on yield, turbidity, cloud stability, and color properties were also studied to evaluate the potential application of enzyme preparations in cloudy apple juice production.

MATERIALS AND METHODS

Chemicals. Sources of reference compounds: flavonol glycosides (quercetin 3-*O*-galactoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, and isorhamnetin 3-*O*-glucoside), catechin, epicatechin, caffeic acid, *p*-coumaric acid, quercetin, and phloretin (Roth, Karlsruhe, Germany); chlorogenic acid (Sigma, St. Louis, MO); procyanidins B1 and B2 (Extrasynthese, Lyon, France); phloridzin (Fluka, Buchs, Switzerland); quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside (Plantech, Reading, U.K.). All reagents and solvents used were obtained from Merck (Darmstadt, Germany) and were of HPLC or analytical grade.

Apple Juice Sample Preparation. Apples (*Malus domestica* L. cv. Brettacher) were obtained from the Experimental Station of Hohenheim University at the early beginning of the harvest season 2003 and were immediately processed into cloudy apple juices. Batches of washed apples of ~10 kg each were ground in a Bucher grating mill (model Central 2, Niederweningen, Switzerland). To inhibit oxidative browning after grinding, ascorbic acid (1 g/kg) was immediately added to the mash (pH 3.2 ± 0.1). The mash with or without enzyme addition was left to stand for 1 h before pressing in a rack and cloth press (Wahler, Stuttgart, Germany). To remove part of the coarse pulp particles, the juice was centrifuged in a chamber separator (SA1-02-024, Westfalia, Oelde, Germany). For preservation, the juice was pasteurized (90 °C, 30 s) in a tubular heat exchanger (Ruland, Neustadt, Germany), hot bottled, sealed under steam injection, and cooled to ambient temperature.

Enzymatic mash maceration was carried out under periodic stirring at 20 ± 3 °C for 1 h. Commercial pectolytic enzyme preparations were added at equal pectolytic activities (150 PA/kg of mash) according to the specifications of the enzyme producer (AB Enzymes, Darmstadt, Germany). The secondary protease activity of the enzyme preparations was determined by casein digestion (*24*). All variants of enzymatic maceration are shown in **Table 1**.

Characterization of Polyphenolic Compounds by HPLC-MS. All samples were prepared for analysis according to the extraction procedure described in ref *25*, after the juices had been adjusted to pH 1.5. HPLC-MS analyses were performed using an Agilent (Waldbronn, Germany) HPLC series 1100 equipped with ChemStation software, a model G1322A degasser, a model G1312A binary gradient pump, a model G1313A autosampler, a model G1316A column oven, and a model G1315A diode array detector. The column used was an Aqua 5 μm C_{18} (250 × 4.6 mm i.d.) from Phenomenex (Torrance, CA), and a security guard C_{18} ODS (4 × 3.0 mm i.d.). The column was operated at a temperature of 25 °C. The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and acetonitrile (50:50, v/v; eluent B). The gradient program was as follows: 10−55% B (50 min), 55−100% B (10 min), 100−10% B (5 min). The injection volume was 10 μL. Simultaneous monitoring was performed at 280 nm (catechins, proanthocyanidins, and dihydrochalcones), 320 nm (hydroxycinnamic acids), and 370 nm (flavonols) at a flow rate of 1 mL/min. Spectra were recorded from 200 to 600 nm.

The HPLC system described above was connected in series with a Bruker (Bremen, Germany) Esquire 3000+ ion trap mass spectrometer fitted with an electrospray ionization source. Data acquisition and processing were performed using Esquire Control software. Negative ion mass spectra (in the range m/z 50−1500) of the column eluate were recorded. Nitrogen was used as the dry gas at a flow rate of 12.0 L/min and at a pressure of 70.0 psi. The nebulizer temperature was set at 365 °C. Collision-induced dissociation spectra were obtained with a fragmentation amplitude of 1.0 V, using helium as the collision gas (1.1×10^{-5} mbar).

Determination of Total and Protein Nitrogen Content. Lyophilized juice samples were analyzed for their nitrogen content using the FP-528 Determinator (Leco, Moenchengladbach, Germany). Protein nitrogen contents were obtained from juice samples dialyzed against distilled water for 48 h using dialysis membranes with a 14 kDa cutoff (type 36/32, Roth, Karlsruhe, Germany). Crude protein was calculated by multiplication of the protein nitrogen content by 6.25.

Color Measurement. Color properties (L^*, a^*, b^*) of juice sera, obtained after ultracentrifugation at 50000*g* for 1 h (20 °C), were determined with a Lambda 20 spectrophotometer equipped with an integration sphere RSA-PE-20 and WinCol software (all from Perkin-Elmer, Dreieich, Germany). The samples were filled in a 1 cm cell and L^*, a^*, b^* values were determined using Illuminant D65 and a 10° observer angle. Chroma (C^*) and hue angle ($h°$) were calculated from CIELAB a^* and b^* coordinates:

$$C^* = (a^{*2} + b^{*2})^{1/2}; \qquad h° = \arctan(b^*/a^*) + 180°$$

Browning indices (A_{420}) were also recorded.

Turbidity Measurement. The turbidities of juices were measured nephelometrically with an LPT 5 two-beam photometer (Dr. Lange, Duesseldorf, Germany) using 5 cm round cuvettes at color correction mode. Turbidity was expressed in nephelometric turbidity units (NTU). The resistance to clarification (cloud stability) was deduced from the relative turbidity (T%):

$$T\% = (T_c/T_o) \times 100$$

where T_o and T_c are the juice turbidities before and after centrifugation at 4200*g* for 15 min at 20 °C, respectively.

RESULTS AND DISCUSSION

The chromatograms of all the juices studied, as exemplified in **Figures 1** and **3**, showed polyphenolic profiles quite similar to those previously reported for the same apple cultivar (*13*). However, in the present study cryptochlorogenic acid (**Figure 1**) was identified by mass spectrometric detection for the first time. As recently demonstrated with coffee bean (*26*) and dried plum (*27*) extracts, the fragmentation patterns of caffeoylquinic acid isomers in negative mode ESI-MS proved to be reliable diagnostic tools. From **Figure 2** it becomes evident that

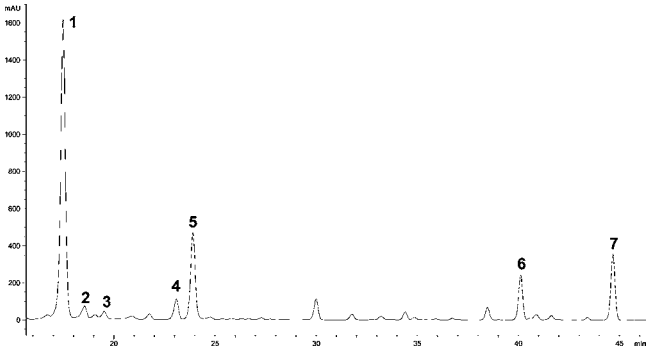

Figure 1. HPLC separation (280 nm) of polyphenolic compounds extracted from cloudy apple juice (variant C2). Peak assignment: (1) chlorogenic acid; (2) cryptochlorogenic acid; (3) procyanidin B2; (4) epicatechin; (5) *p*-coumaroylquinic acid; (6) phloretin xyloglucoside; (7) phloridzin.

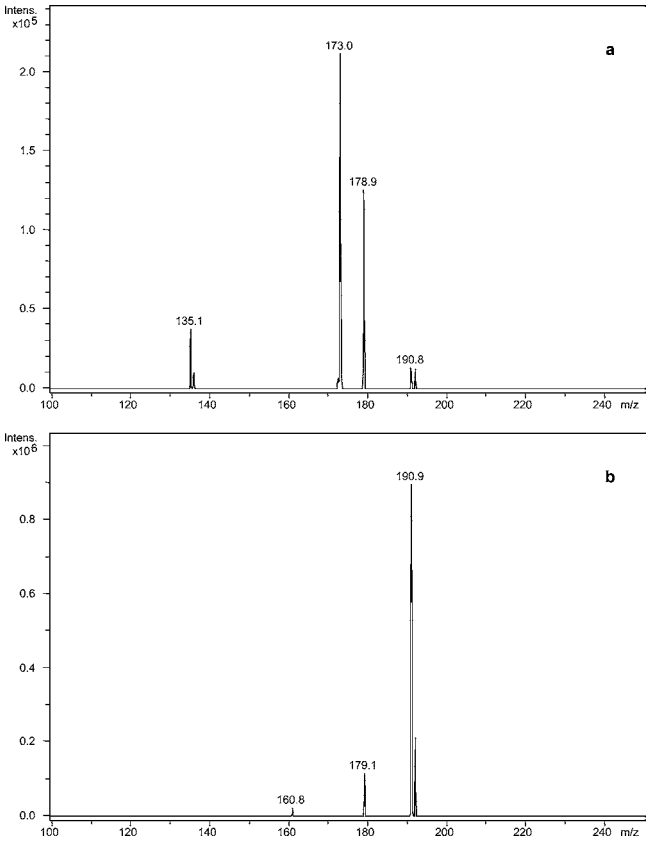

Figure 2. MS^2 spectra of the caffeoylquinic acid isomers (pseudomolecular ion of *m*/*z* 353 $[M - H]^-$) extracted from cloudy apple juice (variant C2): (**a**) cryptochlorogenic acid; (**b**) chlorogenic acid.

4-caffeoylquinic acid (cryptochlorogenic acid) can easily be identified by its MS^2 base peak at *m*/*z* 173. In contrast, 5-caffeoylquinic acid (chlorogenic acid) produced a base peak at *m*/*z* 191 in the MS^2 experiment. The presence of isorhamnetin 3-glucoside (**Figure 3**) in apple juices derived from cv. Brettacher apples, which has so far been considered a marker for pear fruits and juices (*28*−*30*), could be confirmed.

The contents of polyphenolic compounds of the juices are shown in **Table 2**. The main polyphenolic classes were hydroxycinnamates and dihydrochalcones. Flavanols were also found in appreciable amounts, whereas flavonol glycoside contents were considerably lower. The latter result is in good agreement with the recent findings that the majority of the flavonol glycosides are retained in the pomace, which is

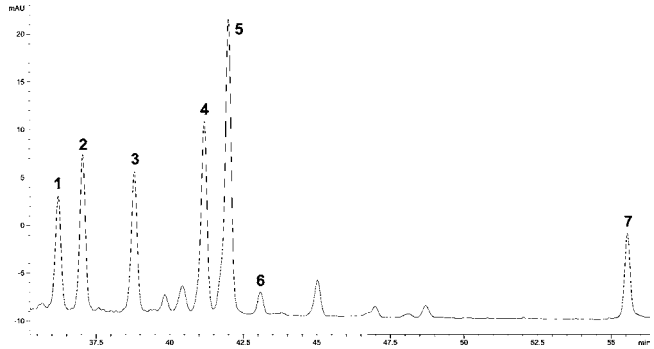

Figure 3. HPLC separation (370 nm) of flavonol glycosides extracted from cloudy apple juice (variant C2). Peak assignment: (1) quercetin 3-galactoside; (2) quercetin 3-glucoside; (3) quercetin 3-xyloside; (4) quercetin 3-arabinoside; (5) quercetin 3-rhamnoside; (6) isorhamnetin 3-glucoside; (7) quercetin (aglycone).

Table 2. Contents of Polyphenolic Compounds (Milligrams per Liter)[a] of Cloudy Apple Juices Produced by Oxidative and Nonoxidative Mash Maceration

	oxidative			nonoxidative		
compound	C1	A1	B1	C2	A2	B2
chlorogenic acid	89.2x	63.7y	78.5x	176.7z	153.0w	173.8z
cryptochlorogenic acid[b]	4.7x	4.7x	4.3x	7.5y	7.3y	7.1y
p-coumaroylquinic acid[c]	18.3x	17.0x	17.2x	24.5y	20.1x	20.3x
procyanidin B2	2.3x	2.0x	2.0x	17.3y	15.0y	15.3y
epicatechin	3.5x	3.7x	3.3x	26.7y	24.3y	27.0y
phloretin xyloglucoside[d]	13.6x	12.6x	12.5x	14.2x	14.0x	14.1x
phloridzin	20.3x	18.8x	21.5xz	25.4y	21.6xz	23.3yz
quercetin 3-galactoside	0.7x	0.7x	0.9x	0.9x	0.8x	0.9x
quercetin 3-glucoside	1.1x	1.1x	1.2x	1.4x	1.4x	1.4x
quercetin 3-xyloside	0.6x	0.6x	0.7x	0.9x	0.9x	0.9x
quercetin 3-rhamnoside	2.7x	2.9x	3.0x	4.0y	3.7y	4.0y
isorhamnetin 3-glucoside	1.8x	1.8x	1.8x	1.8x	1.9x	1.8x
total	158.8	129.6	146.9	301.3	264.0	289.9

[a] All values are calculated to a standard apple juice strength of 11.2 °Brix. Sample preparation and HPLC determination were performed in triplicate. Values within a row with the same letters are not statistically different at the 5% significance level (Scheffe's test). [b] Calculated as chlorogenic acid. [c] Calculated as *p*-coumaric acid. [d] Calculated as phloridzin.

conceivable because these compounds are almost exclusively located in the skins (*31*).

As expected, the contents of chlorogenic acid, epicatechin, and procyanidin B2 decreased substantially due to enzymatic browning when the mash maceration was performed without ascorbic acid addition. These compounds are well recognized as substrates of apple polyphenol oxidase and/or are involved in nonenzymatic coupled oxidation mechanisms (*32*). Phloretin xyloglucoside and phloridzin were oxidized to a lesser extent.

The contents of chlorogenic acid as the major polyphenolic compound, representing 50−70% of the total polyphenols detected by our HPLC system, were also affected by the enzymatic treatment. They were significantly lower in the case of enzyme A treated samples, as compared to the enzyme B and control samples. These effects could be ascribed to the different levels of the secondary protease activity of the enzyme preparations used (**Table 1**), because the apple mash was treated with both preparations at an equivalent level of their major pectolytic activity. Consequently, consistent with the results of Beveridge and Weintraub (*33*), higher protein nitrogen and crude protein contents, respectively, were found for the enzyme-treated samples (**Table 3**). It should be noted that the total nitrogen content of the enzyme preparations (data not shown), at the

Table 3. Total and Protein Nitrogen and Crude Protein Contents (Milligrams per Liter)[a] of Cloudy Apple Juices Produced by Nonoxidative Mash Maceration

variant	total N	protein N	crude protein
C2	154.4	2.9	18.1
A2	162.0	13.8	86.3
B2	276.5	17.3	108.1

[a] All values are calculated to a standard apple juice strength of 11.2 °Brix. Data are the mean values of two replicate determinations.

Table 4. Color Properties and Browning Index (A_{420}) of Sera of Cloudy Apple Juices Produced by Oxidative and Nonoxidative Mash Maceration[a]

variant	L^*	a^*	b^*	C^*	$h°$	A_{420}
C1	92.9	–2.6	25.1	25.2	95.9	0.375
A1	92.8	–3.4	27.5	27.7	97.0	0.424
B1	92.8	–3.3	27.8	28.0	96.8	0.428
C2	96.5	–0.9	4.6	4.7	101.1	0.097
A2	96.5	–0.9	4.2	4.3	102.1	0.092
B2	96.2	–0.9	4.9	5.0	100.4	0.102

[a] Data are the mean values of two replicate determinations.

dosages added to the mash, was <2% of the total nitrogen content of the corresponding juices. Moreover, Scheibe et al. (*34*) observed that the pressing operation significantly reduced the pectinase levels of pressed juices as compared to enzyme-treated apple mashes. Therefore, the increased protein content is assumed to be caused by a synergistic action of pectolytic and proteolytic activities enhancing the degradation of matrix constituents. In contrast, the higher polyphenolic content of the enzyme B treated samples as compared to enzyme A may probably be attributed to the secondary protease activity, inhibiting the protein−polyphenol interactions by hydrolyzing the released proteins. The latter assumption is supported by the increased total nitrogen content of the enzyme B treated sample (**Table 3**).

Currently, with regard to growing consumer demand for healthy nutrition, the main technological challenge is to optimize apple juice production with respect to increased bioactive compounds content while maintaining a high sensoric quality. In the case of cloudy apple juices, color and turbidity are decisive quality attributes. As shown in **Table 4**, the color properties changed with the oxidation, but not with the enzymatic treatment. The L^* values, representing the lightness, increased with the addition of ascorbic acid. Correspondingly, nonoxidized samples were characterized by lower browning indices. These results confirm that ascorbic acid addition definitely inhibits polyphenol oxidase-catalyzed oxidation and subsequent darkening of apple mash. Because immature apples were processed, a relatively large amount of ascorbic acid was used in our experiment. As reported earlier (*35*, *36*), unripe apples are highly susceptible to browning, which is attributable to lower contents of native ascorbic acid and higher polyphenol oxidase activity in immature fruits. The simultaneous decrease of the chroma value and a slight shift of the hue angle characterizes the color hue of the nonoxidized samples as yellowish green. Because the color preferences vary widely, from amber yellow in Germany to whitish green in Japan, an adjustment of ascorbic acid dosage with respect to the polyphenolic antioxidants content and the desired color properties is possible. It should also be noted that visual color perception is influenced by the light scattering of cloud particles, which is perceived as juice turbidity.

Table 5. Intensity and Stability of Turbidity of Cloudy Apple Juices Produced by Oxidative and Nonoxidative Mash Maceration[a]

variant	T_0 (NTU)	T_c (NTU)	T%
C1	17	13	76.5
A1	38	19	50.0
B1	42	22	52.4
C2	42	24	57.1
A2	489	250	51.1
B2	615	318	51.7

[a] Data are the mean values of two replicate determinations.

With respect to turbidity, the following quality requirements for cloudy apple juices have been established: $T_c \geq 250$ NTU and $T\% \geq 50\%$ (*37*). As shown in **Table 5**, all juices matched the reference value for the relative turbidity. However, only in the case of nonoxidative enzymatic mash treatment (A2 and B2) were cloudy apple juices meeting the minimal requirement of turbidity obtained. These results must be ascribed to the insufficient maturity of the apples, which were processed immediately after harvest just at the beginning of the season. When fully mature apples are used, the production of cloud-stable juices with an intense turbidity does not pose a problem, and turbidity parameters can even be improved after apple cold storage (*38*).

Therefore, nonoxidative enzymatic mash treatment could find a specific application in cloudy apple juice production, improving the turbidity-forming potential of apples, particularly when applied in premature dejuicing campaign. Interestingly, oxidative conditions obviously did not inhibit the maceration effect of the pectolytic preparations because the juice yields were mainly affected without enzymatic treatment (**Table 1**). However, mash oxidation appears to limit the transition of cloud-forming proteins released during maceration into the juice. This result is probably due to their interaction with oxidized polyphenols. The latter assumption is supported by the recent findings that apple procyanidins, especially those of a higher degree of polymerization, rapidly associate with the cell wall matrix (*39*).

The results of the present study clearly demonstrate the potential of nonoxidative enzymatic mash treatment for the production of cloud-stable apple juices having high polyphenolic contents, allowing improved juice yields. Moreover, optimization of the enzyme composition is shown to be worthwhile, because the secondary protease activity of the commercial pectolytic preparations is apparently correlated with the release of polyphenols, as shown for chlorogenic acid, the major polyphenolic compound in the juice. Therefore, studying the effects of different processing steps on the protein−polyphenol interactions may provide useful information to improve cloudy apple juice technology with respect to the release of polyphenolic compounds into the juice, which is a prerequisite for their in vivo antioxidant activity.

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