Short Communication

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Hydrolysis of chlorogenic acid in apple juice using a *p*-coumaryl esterase of *Rhizoctonia solani*

Mareike Siebert,* Ralf Günter Berger and Franziska Pfeiffer

Abstract

BACKGROUND: Apple juice is rich in polyphenolic compounds, especially in chlorogenic acid. A sour and bitter taste has been attributed to the compound. Chlorogenic acid in coffee powder was quickly hydrolysed by a *p*-coumaryl esterase of *Rhizoctonia solani* (Rs*p*CAE) at its optimal pH of 6.0. It was unknown, however, if Rs*p*CAE would also degrade chlorogenic acid under the strongly acidic conditions (pH 3.3) present in apple juice.

RESULTS: Treatment of apple juice with Rs*p*CAE led to a chlorogenic acid degradation from $53.38 \pm 0.94\,mg\,L^{-1}$ to $21.02 \pm 1.47\,mg\,L^{-1}$. Simultaneously, the caffeic acid content increased from $6.72 \pm 0.69\,mg\,L^{-1}$ to $19.33 \pm 1.86\,mg/L^{-1}$. The aroma profile of the enzymatically treated sample and a control sample differed in only one volatile. Vitispirane had a higher flavour dilution factor in the treated juice. Sensory analysis showed no significant difference in the taste profile ($p < 0.05$).

CONCLUSION: These results demonstrated a high stability and substrate specificity of Rs*p*CAE. An increase in caffeic acid and a concurrent decrease in chlorogenic acid concentration may exert a beneficial effect on human health.
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Keywords: chlorogenic acid; apple juice; *Rhizoctonia solani*; *p*-coumaryl esterase

INTRODUCTION

Apple juice belongs to the most popular fruit juices with a *per capita* consumption of 7.6 L in Germany in 2017.[1] It is rich in polyphenolic compounds, mainly chlorogenic acids, which are isomer esters of caffeic and quinic acids.[2] Chlorogenic acids are also present in high amounts in coffee, where their effect on human health is controversially discussed. Though chlorogenic acids contribute to the antioxidative potential of coffee and are related to risk mitigation of several diseases,[3,4] they are also supposed to trigger stomach irritation for sensitive people after consumption.[5,6] Furthermore, a sour and bitter taste has been attributed to chlorogenic acid.[7,8] In a previous study, 5-*O*-chlorogenic acid (5-CQA), the most abundant isomer in coffee, was enzymatically hydrolysed to improve the digestibility without altering the aroma and taste profile of the resultant beverage. At the same time, an increase in caffeic acid concentration was obtained.[9] It is suggested that caffeic acid contributes more than chlorogenic acid to the beneficial health effects.[10,11] For chlorogenic acid hydrolysis, a *p*-coumaryl esterase of the basidiomycete *Rhizoctonia solani* (Rs*p*CAE) with an optimum of pH 6.0, which matches the natural pH of a coffee brew, was used at the temperature optimum of the enzyme (30 °C).[9,12]

This study aimed at transferring the method to the mitigation of 5-CQA in apple juice, to potentially achieve similar beneficial health effects. Since the pH optimum of Rs*p*CAE is 6.0, the enzyme stability at the comparatively low pH value of apple juice (pH 3.3) was of particular interest. In addition, the enzymatic treatment was performed at room temperature, and the reaction time was minimized to facilitate the technical application. The specificity of the esterase used was highly relevant due to the presence of a variety of fruit esters in apple juice, which are known to contribute to its characteristic aroma profile. To determine whether the treatment of apple juice with Rs*p*CAE altered its aroma and taste, aroma dilution analyses and sensory studies were performed. The increased insight into the stability and specificity of Rs*p*CAE provided by this study will be relevant with respect to further applications of Rs*p*CAE in the food industry.

MATERIAL AND METHODS

Chemicals

All chemicals were purchased from Carl Roth (Karlsruhe, Germany), except caffeic acid (Fluka, Buchs, Switzerland) and formic acid (Sigma-Aldrich, Steinheim, Germany). For chemical analysis, ultra-pure water (TKA-GenPure, Labor- und Analysen-Technik GmbH, Garbsen, Germany) and rectified solvents (GC grade) were

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used. Commercial clear apple juice (Schlossmarke; Kelterei Wilkening GmbH, Bad Münder, Germany) was obtained from a local supermarket.

Enzymatic treatment of apple juice

For enzymatic treatment of the apple juice, a heterologously produced and purified *p*-coumaroyl esterase from *Rhizoctonia solani* (Rs*p*CAE) was used.[12] The enzyme activity was determined as described by Siebert *et al.(2018)*.[9] The filtered apple juice (syringe filter Chromafil® RC-45/25; Macherey-Nagel, Düren, Germany) was incubated with an Rs*p*CAE solution (40 mU per millilitre juice, room temperature, 30 min). Afterwards, the enzymatic reaction was stopped by doubling the volume with acetonitrile. Control samples were prepared with water instead of enzyme solution. Determination of chlorogenic acid concentration was performed in triplicate.

Quantification of chlorogenic acid and caffeic acid

Quantification of 5-CQA and caffeic acid was performed using high-performance liquid chromatography (HPLC) equipped with an ultraviolet–visible light detector. The HPLC method was as described by Siebert *et al.* (2018).[9] Standards solutions of 5, 10, 25, 50, 75, 100, 250, 500 μg mL^{-1} (5-CQA) and 4, 10, 20, 40, 60, 100, 175, 250 μg mL^{-1} (caffeic acid) were used for quantification *via* linear regression.

Analysis of key aroma compounds

Stir bar sorptive extraction was applied to isolate the volatile compounds. A stir bar, coated with polydimethylsiloxane (10 mm × 0.5 mm; Gerstel, Mülheim, Germany), was combined with the sample in a headspace vial. Enzymatically treated samples were prepared by adding 40 mU enzyme solution per millilitre juice to 5 mL apple juice. For the control samples, water was used instead of enzyme solution. After incubation (room temperature, 1 h, 120 rpm, Telemodul 40S; H + P Variomag, Daytona Beach, FL, USA), the stir bar was rinsed with deionized water, dried, and stored in a closed vial at 4 °C. Sodium chloride (100 g L^{-1}) and a new stir bar were added to the samples. The second extraction was performed as just described. The stir bars of the first and second extractions were analysed together in a glass tube of the TDS3 thermal desorption system (Gerstel).[13] An Agilent 6890 N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with mass spectrometry–olfactometry and thermodesorption as injection technique was used to identify the key aroma compounds. Flavour dilution (FD)-factors were determined by means of aroma dilution analyses. The samples were diluted after thermodesorption within the gas chromatography system by increasing the carrier gas flow (helium, 44.4 mL min^{-1}) in the injection system (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512).[14] Two capillaries with different polarity were used: VF-5ms (30 m, 0.25 mm ID, film thickness 0.25 μm; Agilent J&W GC Columns, Santa Clara, CA, USA) and HP-INNOWax (30 m, 0.25 mm ID, film thickness 0.25 μm; Agilent J&W GC Columns). Initial temperature of the thermodesorption system was 20 °C. The temperature, after raising at a rate of 60 °C min^{-1} to 150 °C, was held for 2 min in the splitless mode for desorption. Subsequently, the volatiles were refocused in the cold injection system (CIS4; Gerstel) at −10 °C. The cold injection system was heated at 12 °C s^{-1} to 230 °C and held for 2 min in the solvent vent mode to transfer the volatiles onto the column. The oven program was as follows: injection at 40 °C (held for 5 min) and heating at 8 °C min^{-1} to the final temperature of 230 °C (held for 10 min). The flow of the helium carrier gas was 1.3 mL min^{-1}. The effluent was split 1:2 between a mass spectrometer and an olfactory detection port (ODP3; Gerstel) at the end of the column. The temperatures of the mass spectrometer and olfactory detection port were 250 °C and 230 °C respectively. To determine the retention indices, a homologous series of *n*-alkanes (C8–C25) was injected.[15]

Sensory analysis

For sensory analysis, the apple juice was treated with Rs*p*CAE solution (40 mU mL^{-1} juice) for 30 min at room temperature. Samples were boiled for 1 min to terminate the reaction. All of the judges (five male and six female) were experienced panellists. The intensity of eight attributes describing the taste (bitter, sour, sweet, fruity, apple-like, peach-like, metallic, musty) were ranked on a linear scale from 0 (not perceived) to 10 (strongly perceived). Untreated apple juice was offered as a reference sample to define the centre of the linear scale. Tasting was performed in triplicate. Two-tailed Student's *t*-tests ($p < 0.05$) were calculated to highlight differences between the samples.

RESULTS

Enzymatic mitigation of 5-CQA in apple juice

The enzymatic treatment of apple juice decreased the 5-CQA concentration by 61% from 53.38 ± 0.94 mg L^{-1} to 21.02 ± 1.47 mg L^{-1} under the conditions mentioned earlier. At the same time, the caffeic acid concentration increased from 6.72 ± 0.69 mg L^{-1} to 19.33 ± 1.86 mg L^{-1} (Fig. 1).

Comparison of volatile compounds of enzymatically treated and control apple juice

By means of aroma dilution analysis, 17 aroma active compounds with an FD-factor of ≥16 in at least one sample were determined (Table 1). Gas chromatography–olfactometry analysis resulted in only one compound with a different FD-factor. Substance 13 (vitispirane) showed a higher FD-factor in the enzymatically treated sample than in the control sample. However, overlaying the gas chromatographic total ion chromatograms of the enzymatically treated and control samples showed that both had a nearly identical profile (Fig. 2). Thus, the discrepancy is most likely due to the inherent fuzziness of sensory judgments.

Sensory comparison of enzymatically treated and control apple juice

Sensory evaluation was performed to evaluate the effect of enzymatic treatment on the taste profile. Enzymatically treated and control apple juice shared the same intensities for the attributes sour, peach-like, metallic, and musty. The enzymatically treated apple juice was perceived as slightly more bitter, sweet, fruity, and apple-like. None of the differences were statistically significant (Fig. 3). In addition, the panellists noticed neither an off-taste nor an off-odour.

DISCUSSION

With a pH optimum of 6.0, the activity of Rs*p*CAE decreases by lowering the pH value. In a range from pH 4.0 to 7.0, more than 50% activity is retained. However, the enzyme is unstable below pH 4.0.[12] The results in the present study showed that 5-CQA was successfully hydrolysed by Rs*p*CAE in apple juice, which had a

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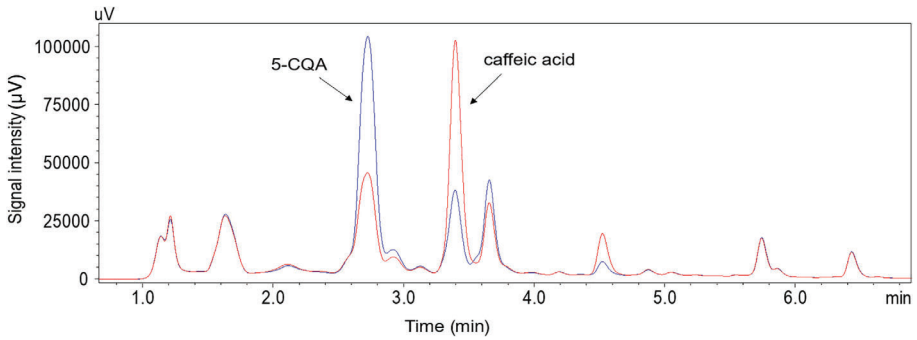

Figure 1. HPLC chromatograms of control (blue) and enzymatically treated (red) apple juice (λ = 323 nm). Decrease of 5-CQA went along with an increase of caffeic acid.

Table 1. Aroma compounds with flavour dilution (FD)-factors ≥16 of the enzymatically treated ('treated') and control apple juice ('control')

	Retention index[a]				FD-factor[d]				
	VF-5ms		HP-Innowax						
No.	Exp.[b]	Lit.[c]	Exp.[b]	Lit.[c]	Control	Treated	Odour[e]	Substance	Identification criteria[f]
1	<800	764	–	1244	16	16	Glue like	1-Pentanol	B[g]
2	<800	780	–	1008	16	16	Glue like	Methyl 2-methylbutanoate	B[g]
3	804	802	1033	1028	32	32	Flowery, glue like	Ethyl butanoate	A, B
4	851	842	1048	1056	128	128	Fruity, candy like	Ethyl 2-methylbutanoate	A, B
5	874	865	1367	1360	16	16	Grass, woody	1-Hexanol	A, B
6	983	975	1298	1317	64	64	Mushroom	1-Octen-3-one	A, B
7	989	988	1263	1261	32	32	Mushroom, musty	3-Octanone	A, B
8	1097	1096	1862	1862	64	64	Spicy, leather like	*o*-Guaiacol	A, B
11	1172	–	–	–	32	32	Woody	n.i.[h]	
13	1294	1272	1529	1515	4	16	Fresh mint like	Vitispirane	A, B
15	1381	1385	–	1596	16	16	Woody	Hexyl hexanoate	B[g]
16	1390	1390	1822	1819	512	512	Juicy, fruity	(*E*)-*β*-Damascenone	A, B
17	1428	1415	–	1805	64	64	fruity	*β*-Damascone	–[7]

[a] Retention indices determined on VF-5ms and HP-INNOWax column.[15]
[b] Experimentally determined retention indices.
[c] Retention indices from reference library.[16]
[d] Determined on VF-5ms column.
[e] Odour description perceived on VF-5ms column.
[f] All of the compounds have previously been identified in apple juice. Besides odour quality and retention index on VF-5ms column, the following criteria were used for identification: A, comparison of retention index on HP-INNOWax column with reference library; B, mass spectrum compared with commercial mass spectra database NIST 14.[16]
[g] Tentatively identified.
[h] n.i.: not identified.

pH of 3.3. Surprisingly, an incubation time of 30 min was enough to mitigate chlorogenic acid concentration by 61% but not long enough to inactivate the enzyme completely. Rs*p*CAE has a temperature optimum of 30 °C but possesses about 80% residual activity at 20 °C. Furthermore, it shows the highest stability at 20 °C.[12] Incubation at room temperature may compensate the stability loss caused by the low pH value and may be the main reason for the fast degradation even in an acidic beverage like apple juice. In addition, hydrolysis of 5-CQA led to a distinct increase in caffeic acid concentration. This is connected to enhanced antioxidative properties, and therefore related to a beneficial effect on human health.[17,18]

Rs*p*CAE is a *p*-coumaryl esterase, which hydrolyses ester linkages of hydroxycinnamic acids such as methyl caffeate or chlorogenic acid. To our knowledge, it has not been examined yet whether this enzyme will also hydrolyse other ester compounds common in food. Since apple juice is rich in short and medium-chain carboxylic acid esters, which contribute strongly to the popular aroma characteristics, it was investigated whether the treatment affected the aroma and taste profile. Seventeen key aroma compounds were determined in both samples. All aroma compounds identified have been described before as apple juice volatiles in the literature. No differences were seen between the volatile profiles of the samples in the total ion chromatogram of gas chromatography–mass spectrometry analysis (Fig. 2). An increase in caffeic acid has been described to enhance bitter and sour taste.[19] Contrary to expectations, the mitigation of 5-CQA did not result in a significant change of the taste profile. It is concluded that 5-CQA possesses acidic and bitter properties similar to its cleavage products, caffeic and quinic acids, and thus the decrease compensated the increase in caffeic acid. Since the perceived acidity is a decisive factor for consumer liking of apple juice, an unchanged sour taste is of great importance.[20]

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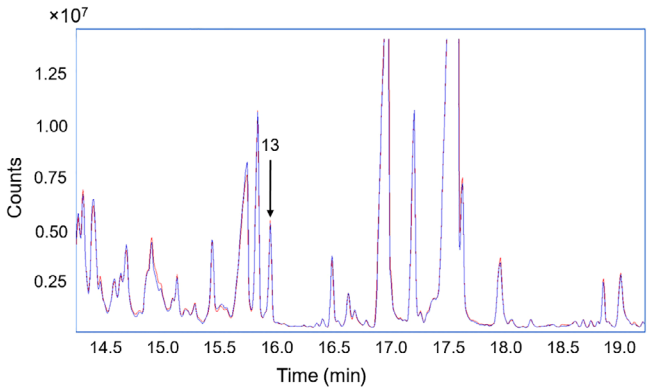

Figure 2. Total ion chromatogram of control (blue) and enzymatically treated (red) apple juice. Both samples showed the same profile of volatiles. There is no visible difference for substance 13 (vitispirane).

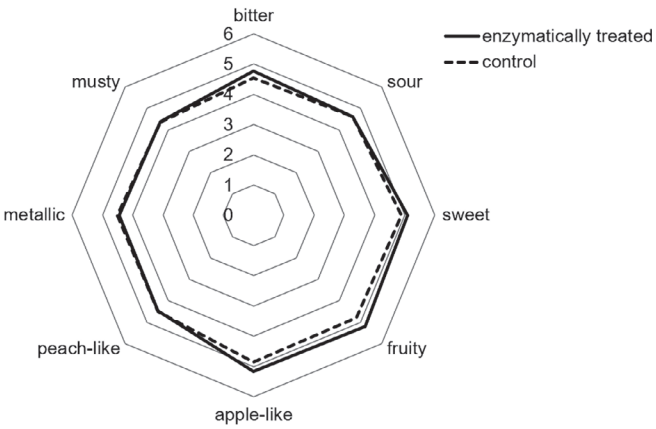

Figure 3. Perceived mean intensities of taste describing attributes of a control (dashed line) and an enzymatically treated (solid line) apple juice.

Carboxylic ester hydrolases, such as Rs*p*CAE, can be used to valorize agro-industrial side streams.[21,22] In the food sector, the application of esterases of this class was described for the treatment of coffee[17,23] and in baking[24] to improve taste and texture properties. The high stability and substrate specificity of Rs*p*CAE demonstrated in this study are promising for applying this enzyme in other fields in the food industry.

CONCLUSION

Hydrolysis of 5-CQA at a low pH value (3.3) and mild reaction conditions (short incubation time, room temperature) without the cleavage of other ester compounds showed the high stability and substrate specificity of Rs*p*CAE. The enzymatic treatment did not alter the taste profile substantially. These results are a promising basis for further application in the food industry. In addition, an increase in caffeic acid and a decrease in chlorogenic acid concentration may have a beneficial effect on human health. This will have to be proven by performing intervention studies.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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