

Effect of Enzyme Treatments and Drying Temperatures on Methylpyrazine Content in Cocoa (*Theobroma Cacao* L.) Powder Extract

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ABSTRACT: The effects of combining enzyme treatments and heating protocols on pyrazine formation in cocoa powder extracts have been studied. Five commercial enzyme preparations containing protease and carbohydrase activities were initially assessed for their ability to release amino acids and reducing sugars, both of which are substrates for Maillard reactions. The enzyme preparation Flavourzyme was subsequently selected for further study given its ability to liberate both types of substrate. Cocoa powder solutions were treated with 2 doses of Flavourzyme and processed at 2 drying temperatures. The combination of the higher dose of the enzyme (12% w/w enzyme/substrate) and the higher drying temperature (150 °C) resulted in a synergistic increase in the concentration of methyl- and dimethylpyrazines. A statistically significant increase in the concentration of tetramethylpyrazine occurred in samples treated at 150 °C that was independent of the enzyme dose assayed. No significant changes in trimethylpyrazine content were detected in treated compared to untreated samples.

Keywords: cocoa powder extract, enzyme treatment, flavor, Maillard reactions, pyrazines

Introduction

The Maillard reaction is of importance to the food industry because of its implication in food aroma, taste, and color, particularly in traditional processes such as the roasting of coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals, and the cooking of meat (Reineccius 1990; Mottram 1994; Friedman 1996). Cocoa powder is widely used as an ingredient in the food industry. The substances that have the greatest impact on the flavor of cocoa derivatives are N- and O-containing heterocyclic compounds generated during bean roasting.

Cocoa manufacture involves fermentation, drying, cleaning, roasting, and milling. After harvesting, cocoa pods are carefully cut open and the beans and pulp are removed. Microbial activities and the action of endogenous enzymes during fermentation induce chemical changes in the pulp surrounding the beans and within the beans themselves, leading to the accumulation of substrates for the Maillard reaction, mainly amino acids, peptides, and reducing sugars (Reineccius and others 1972; Yusep and others 2002). The seeds are subsequently dried and dispatched to processors as the raw material for the production of cocoa mass, cocoa powder, and cocoa butter. The first stage of their processing includes roasting, changing their color and flavor, followed by shell removal. Once roasted and shelled, the resulting “nib” is ground into a paste, which is also known as cocoa liquor. The liquor is pressed to remove a percentage of the cocoa butter, leaving behind a cake. The cocoa cake is ground into a fine powder that is mainly used in the baking and

confectionery industry (Bixler and Morgan 1999). Processing can be adjusted to produce cocoa powders of different composition and different fat content.

Flavor formation in cocoa is known to be influenced by thermal treatment, during which various modifications occur, including the Maillard reaction. More than 500 volatile compounds have been identified in roasted cocoa beans (Nijssen and others 1996). These compounds can be divided into more than 20 different chemical groups, among which pyrazines, aldehydes, and esters are the most representative (Gill and others 1984). Ninety-five pyrazines are implicated in cocoa aroma, the concentrations of which vary depending on the duration and temperature of the thermal treatment (Hashim and Chaveron 1994). Hashim and others (1999) reported that the main pyrazines produced during drying treatment are trimethyl- and tetramethylpyrazine. Pyrazines are used as an index of the roasting process because of their pronounced formation during roasting (Hashim and Chaveron 1994; Jinap and others 1998) and their importance to cocoa flavor.

A number of approaches have been considered for increasing the content of Maillard reaction substrates in order to improve aroma after roasting: the activation of remaining key proteases (Misnawi and others 2002); the addition of exogenous proteases (Voigt and others 1994a, 1994b; Brito and others 2004); direct addition of Maillard substrates (Bruto and others 2002). The objective of this work was to increase the availability of Maillard aroma substrates by the addition of enzymatic preparations containing proteolytic and glycosidic activities and to examine the effects of the subsequent drying conditions on the stimulation of methylpyrazine formation in cocoa powder extracts.

Materials and Methods

Cocoa powder samples

The raw material used was commercial cocoa bean powder of 10% fat content produced by Natraceutical S.A.

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Enzyme assays

Flavourzyme 500L, Protamex, Fungamyl, NS-44045, and Celluclast enzyme preparations were obtained from Novozymes (Bagsvaerd, Denmark). To test the ability of the 5 enzyme preparations to release Maillard reaction substrates, 5 g of cocoa powder was dissolved in 25 mL of 0.1 M sodium phosphate buffered to pH 6.5 for Flavourzyme 500L and Protamex treatments, and buffered to pH 5 for Fungamyl, NS44045, and Celluclast. Three doses were tested for each enzyme preparation: 0.25%, 2.5%, and 25% w/w (enzyme/substrate) for NS-44045, Celluclast, and Fungamyl; 1.2%, 2.4%, and 12% w/w for Flavourzyme; and 0.2%, 0.4%, and 2% w/w for Protamex. All samples were incubated at 50 °C. After 24 h samples were heated at 100 °C for 10 min for enzyme inactivation and centrifuged at 4000 rpm, and the supernatants were assayed to determine amino acid and reducing sugar contents. A sample of untreated cocoa powder was included as a control.

To test the ability of Flavourzyme to increase pyrazine content, 2.4% or 12% w/w (enzyme/substrate) of the commercial preparation was added to suspensions of 40 g of cocoa powder in 200 mL of distilled water. The reaction mixtures were stirred in 500-mL glass vessels at 200 rpm and incubated at 50 °C for 8 h. Mixtures were subsequently heated at 100 °C for 10 min to achieve enzyme inactivation and extracted with 1 volume ethanol for 1.5 h with continuous stirring at 50 °C in a reactor connected to a refrigerator. The extract was then filtered and concentrated to 20 mL using a vacuum rotary concentrator to eliminate the ethanol. The concentrates were then dried in an oven at 120 °C or 150 °C for 1 h. The powdered extracts were stored in hermetically closed containers and kept at 4 °C until chromatographic analysis. Six different extracts were analyzed: four treated with Flavourzyme at doses of 2.4% and 12% w/w and drying temperatures of 120 °C or 150 °C, and two without Flavourzyme dried at the same temperatures.

Amino acid and reducing sugar determinations

Amino acid content was determined according to the method of Adler-Nissen (1979), based on a selective reaction between the amino groups and the reagent OPA (O-phthalaldehyde, Sigma-Aldrich Co., St. Louis, Mo, U.S.A.) in the presence of dithiothreitol (Sigma-Aldrich Co.).

The Nelson-Somogyi method was used to determine reducing sugar content (Somogyi 1952).

Pyrazine determination

For pyrazine analysis 0.8 g of sample and 0.2 g of NaCl were dissolved with 1 mL of distilled water in a 7-mL septum-sealed glass vial and analyzed by headspace solid-phase microextraction sampling. The vial was incubated in a water bath at 60 °C for 30 min for equilibration. A 65 μ m polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco, Belafonte, Pa., U.S.A.), previously conditioned following the supplier's instructions, was placed in the headspace of the vial for 15 min. Samples were analyzed in a HP5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) fitted with a flame ionization detector and an HP Innovax capillary column (length 30 m; internal diameter 0.25 mm; film thickness 0.25 μ m) (Agilent Technologies, Waldbronn, Germany). The injection block and detector temperatures were kept constant at 220 °C and 300 °C, respectively. The GC oven temperature was programmed as follows: 60 °C (3 min) to 150 °C at 2.0 °C/min and to 240 °C at 20 °C/min and then kept at 240 °C for 5 min. Compounds were identified by comparing retention times with those of standard compounds (Sigma-Aldrich Co.) and using an Agilent 5973N mass spectrometer detector coupled to an Agilent 6890 gas chromatograph under the same chromatographic conditions. For cali-

bration and quantitative analysis, relative response coefficients for methylpyrazines were determined by adding standard amounts (4 concentrations, in duplicates) of pure compounds (Sigma-Aldrich Co.) and 5 μ L of 0.1% (w/v) 2-octanol as an internal standard to nonroasted cocoa bean powder samples.

Statistical analyses

The statistical significance of the effects of the factors and the interactions between them was determined by analysis of the variance (ANOVA). Analysis of means was carried out by comparison of least significant difference (LSD) intervals at the 95% confidence level.

Results and Discussion

Effects of enzyme treatments on concentrations of Maillard substrates

The abilities of the 5 enzymatic preparations to release Maillard reaction substrates in cocoa powder were studied. Celluclast,

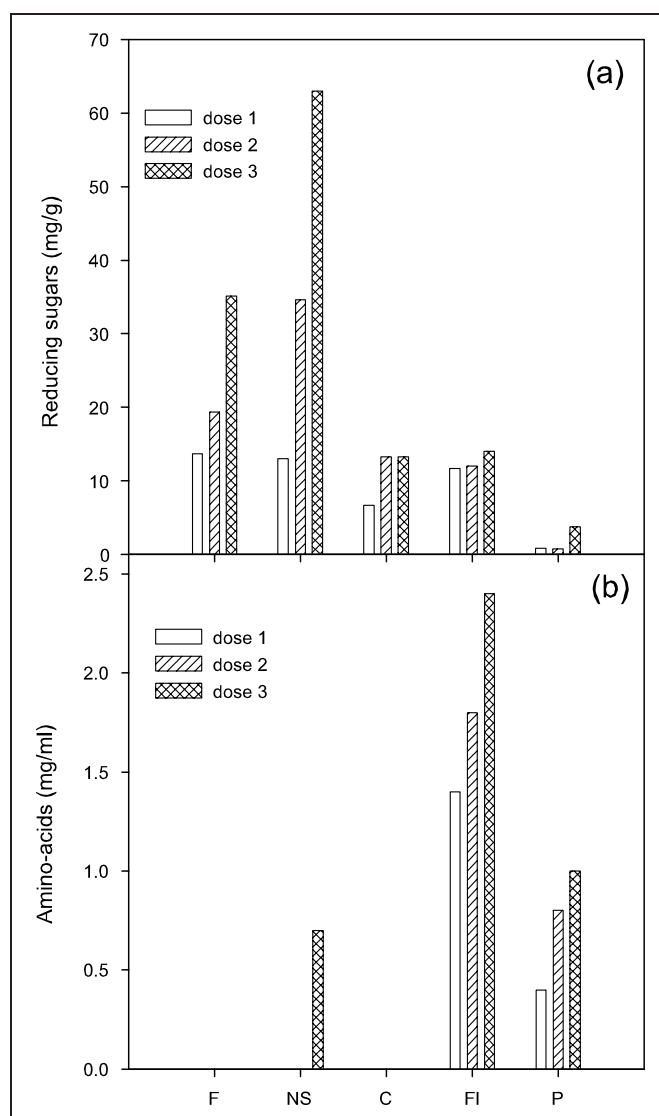


Figure 1 – (a) Reducing sugar and (b) amino acid contents of cocoa powder treated with different enzymes. Doses 1, 2, and 3 were, respectively, 0.25%, 2.5%, and 25% w/w (enzyme/substrate) for F (Fungamyl), NS (NS44045), and C (Celluclast); 1.2%, 2.4%, and 12% w/w for FI (Flavourzyme); 0.2%, 0.4%, and 2% w/w for P (Protamex).

Table 1 – Pyrazine concentrations in cocoa powder treated with Flavourzyme and subsequent heating treatment

Enzyme dose (% w/w)	Heating temperature (°C)	Pyrazines (mg/L)					
		2-MP ^a	2,3-DiMP	2,5-DiMP	2,6-DiMP	2,3,5-TriMP	2,3,5,6-TetraMP
0	120	1.753	1.254	1.643	6.841	2.251	2.204
		2.610	1.660	2.553	10.84	3.530	2.218
0	150	2.605	2.021	2.922	10.87	1.657	1.758
		2.236	1.580	2.851	10.46	3.325	1.307
2.4	120	2.852	1.078	0.857	10.26	0.808	2.402
		3.594	1.614	1.183	18.33	1.076	1.106
2.4	150	5.162	3.520	2.400	17.55	1.319	4.305
		6.673	3.116	3.131	26.93	2.735	3.389
12	120	9.306	2.028	2.219	25.33	1.370	3.242
		7.436	2.825	2.324	31.82	2.040	3.153
12	150	19.88	9.676	10.62	62.79	3.709	4.579
		17.56	8.192	9.790	67.65	3.578	5.028
Untreated cocoa powder		2.263	1.822	0.836	3.198	1.605	0.908
		1.709	1.534	0.631	2.497	1.204	0.594

^aMP: methylpyrazine.

Fungamyl, and NS-44045 are fungal multienzyme complexes enriched in cellulase, α -amylase, and carbohydrase activities, respectively. Protamex is a bacterial protease complex and Flavourzyme is a fungal preparation containing protease and aminopeptidase activities. Figure 1 shows the release of reducing sugars (A) and amino acids (B) from a buffered solution of cocoa powder incubated for 24 h with 3 different doses of each enzyme. NS-44045 and Fungamyl were the most effective preparations with respect to reducing sugar release, followed by Celluclast and Flavourzyme. Protamex treatment barely increased the reducing-sugar content of cocoa powder. As regards amino acid release, the greatest increases were achieved by Flavourzyme at the 3 doses assayed. To a lesser extent, increases were detected in Protamex-treated samples, and NS44045 also released amino acids at the highest dose tested. Fungamyl and Celluclast failed to increase amino acid content in cocoa powder.

Since Flavourzyme was able to release both types of Maillard substrate, this enzyme mixture was selected for further experiments. An incubation time of 8 h was chosen because no differences were observed in the amounts of reducing sugars and amino acids release at 8 and 24 h (data not shown).

Effect of Flavourzyme treatment and drying temperatures on the generation of pyrazines in cocoa powder extract

To study the effect on pyrazine content in cocoa powder treated with Flavourzyme, all possible combinations of the enzyme doses used (0%, 2.4%, and 12% w/w enzyme/substrate) and the 2 drying temperatures (120 °C and 150 °C) were assayed in duplicate. As a control untreated cocoa powder was included. The experimental design and the concentrations of pyrazines in the samples are shown in Table 1. The highest concentration of pyrazines was observed in the sample treated with 12% w/w Flavourzyme and dried at 150 °C.

To assess the relative contributions of enzyme dose and drying temperature on the increases in pyrazine content a multifactor ANOVA analysis was carried out. *P* values were calculated to test the statistical significance of the treatments (Table 2). The effects of enzyme dose and drying temperature, and the interaction between them, were statistically significant for all the pyrazine compounds assayed with the exception of triMP. Figure 2 shows the interaction plots between both factors for each pyrazine and its concentration in the untreated sample. The concentrations of 2-methylpyrazine (MP), 2,3-dimethylpyrazine (2,3-diMP), 2,5-dimethylpyrazine (2,5-diMP), and 2,6-dimethylpyrazine (2,6-diMP) showed synergistic increases for the combination of 12% w/w of enzyme and drying at 150 °C, which were significantly higher than any other treatment as-

sayed. The combination of 120 °C drying temperature and 12% w/w enzyme dose led to lesser but nevertheless significant increases in MP and 2,6-diMP content. The combination of 150 °C and 2.4% w/w led to significant increases in the concentrations of MP, 2,3-diMP, and 2,6-diMP, while the increase in the concentration of 2,3,5,6-tetramethylpyrazine (tetraMP) was not significantly different from that obtained with the 12% w/w enzyme dose at the same temperature but was greater than the concentration achieved in samples to which no enzyme was added. The concentrations of MP and 2,3-diMP in untreated cocoa powder were not significantly different from those for nonenzymically treated samples dried at 120 °C or 150 °C, thus showing the relevance of the enzyme treatment in increasing the content of these pyrazines.

Conclusions

It has recently been demonstrated that proteolytic enzyme treatment of cocoa beans improves cocoa flavor (Brito and others 2004). Hashim and Chaveron (1994), Jinap (1994), and Jinap and others (1998) previously reported that heating at more than 130 °C is an important parameter for the development of cocoa flavor. The combination of adding 12% w/w Flavourzyme to cocoa powder and subsequent drying at 150 °C to promote Maillard reactions could thus be expected to lead to synergistic increases in the contents of the main cocoa methylpyrazines. MP, 2,3-DiMP, 2,5-DiMP,

Table 2 – Results of ANOVA for the Flavourzyme-treated samples

Pyrazine	Effects	<i>P</i> value
2-MP ^a	A: Dose	0.0000
	B: Temp.	0.0003
	Interaction AB	0.0010
2,3-diMP	A: Dose	0.0001
	B: Temp.	0.0001
	Interaction AB	0.0006
2,5-diMP	A: Dose	0.0000
	B: Temp.	0.0000
	Interaction AB	0.0000
2,6-diMP	A: Dose	0.0001
	B: Temp.	0.0009
	Interaction AB	0.0031
2,3,5-triMP	A: Dose	0.1080
	B: Temp.	0.0938
	Interaction AB	0.1702
2,3,5,6-tetraMP	A: Dose	0.0027
	B: Temp.	0.0124
	Interaction AB	0.0158

^aMP: methylpyrazine.

2,6-DiMP, and tetraMP concentrations were approximately 7.7-, 5-, 3.5-, 6-, and 2.2-fold higher, respectively, than those detected in samples treated at 120 °C or 150 °C without enzyme addition. These pyrazines have been linked to sensorial attributes such as nutty, cocoa, chocolate, roasted nuts, coffee, green, caramel, peanut, and hazelnut (Sanagi and others 1997; Serra-Bonvehí 2005). The addition of enzyme preparations such as Flavourzyme, having the capacity to

release amino acids and reducing sugars, followed by heat treatment to promote Maillard reactions has been shown to provide a means of increasing the content of these aroma compounds in cocoa powder extracts. However, further experiments will be necessary to establish the impact of these treatments on the sensorial attributes of cocoa powder extract and their potential applications in confectionery, ice creams, and beverages.

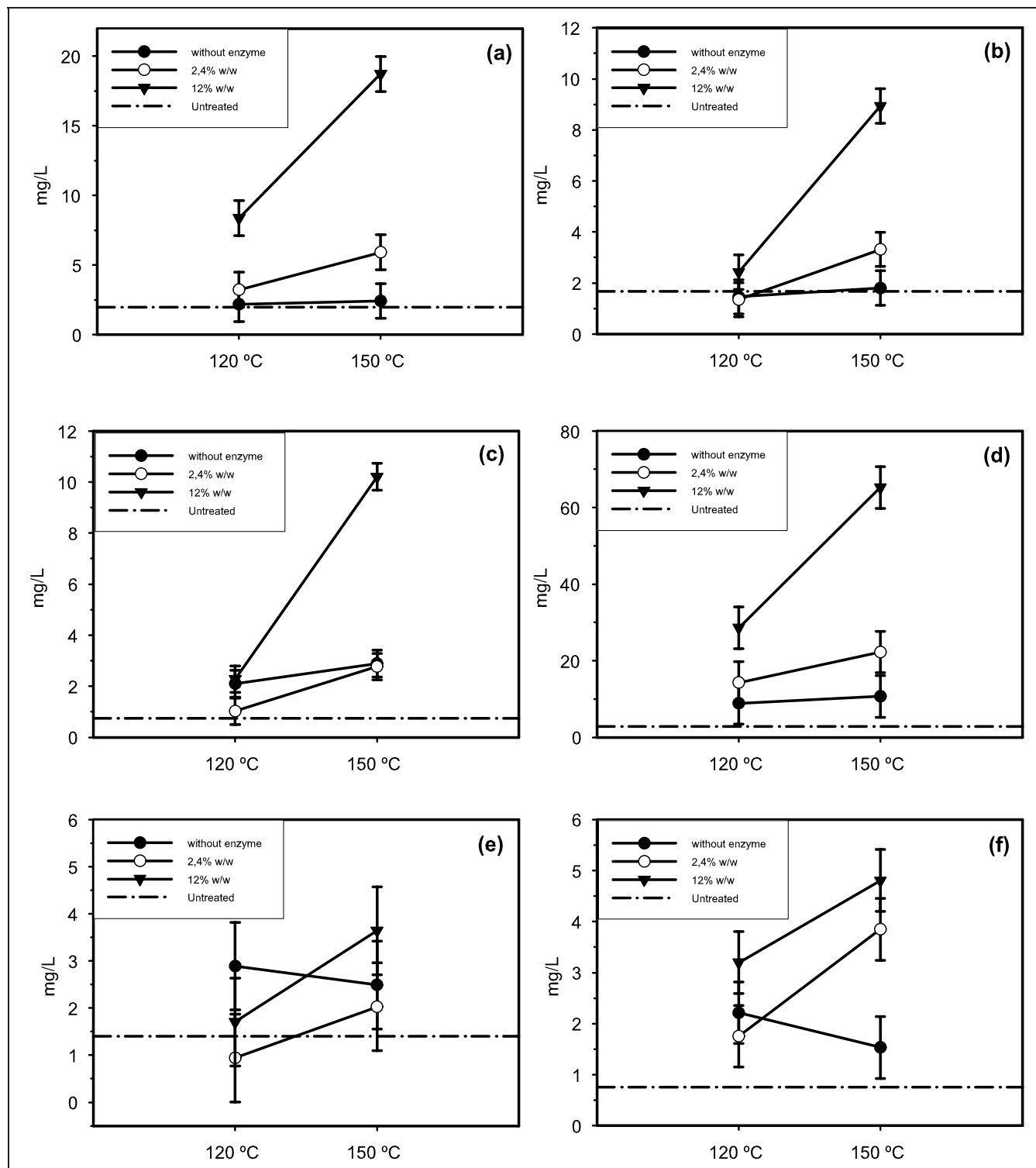


Figure 2—Concentrations of 2-methylpyrazine (a), 2,3-dimethylpyrazine (b), 2,5-dimethylpyrazine (c), 2,6-dimethylpyrazine (d), 2,3,5-trimethylpyrazine (e), and 2,3,5,6-tetramethylpyrazine (f) in cocoa powder extract treated with 0%, 2.4%, or 12% w/w Flavourzyme and dried at 120 °C or 150 °C. Error bars indicate the least significant difference intervals at the 95% confidence level.

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