

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 5
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JUAN PABLO CADENA SARMIENTO

Asunto: Radicación: 11-130827- -5
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 10293
Folios: 5

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2010/026787				
Fecha de Presentación Internacional	10/03/2010				

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios	7 a 26	04/Octubre/2011
Reivindicaciones:	Folios	46 a 49	27/Octubre/2011
Dibujos:	Folios	33 a 35	04/Octubre/2011
Prioridades:	US	61/159,292	11/Marzo/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Se aceptan para estudio todas las 20 reivindicaciones presentadas (Fls. 46 a 49) porque las tasas pagadas cubren en su totalidad lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única.

Título de la solicitud: "Proceso de fabricación de producto de cacao" (Fl. 1).

El problema planteado en esta solicitud consiste en la necesidad de producir un producto de cacao con deseables propiedades organolépticas.

Para resolver este problema técnico, la solicitud en estudio proporciona un proceso de tratamiento de un producto de cacao con proteasas y carbohidrasas a ciertas condiciones de tiempo y temperatura que permitan darle al producto final un color deseable.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP) A23G 1/02, 1/32.

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1, 2, 8, 11, 15 y 17 no son claras porque las expresiones "*al menos*", "*a un tiempo y a una temperatura suficiente para incrementar*" y "*combinaciones de las mismas*" son imprecisas, por lo cual no permiten distinguir la invención del estado de la técnica. Se sugiere sustituirlas por términos precisos o un rango concreto.

Las reivindicaciones 1, 8, 5, 13 y 15 no son claras porque las expresiones "*a un tiempo y a una temperatura suficiente para incrementar*" y "*valor L menor a 19*" son resultados a alcanzar, lo que no está permitido, no define al producto y/o método porque la invención no queda definida por el resultado a alcanzar, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere remplazarla por las características esenciales estructurales que contribuyen a la obtención de ese efecto.

Las reivindicaciones 1, 2, 8, 11, 15 y 17 no son concisas porque las definiciones de proteasa y carbohidrasa, son géneros funcionales, se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos que han sido sintetizados ó probados, es decir, que se encuentran debidamente sustentados.

Las reivindicaciones 5, 13 y 15 no son claras porque la expresión el valor L no es conocido en el campo técnico de la invención. Se sugiere definir el término dentro del capítulo reivindicatorio.

Las reivindicaciones 18 y 20 no son claras porque hacen referencia a un producto, pero presenta características técnicas de un método al indicar la forma de obtención. Al respecto no debe olvidarse que las reivindicaciones de producto y de procedimiento son diferentes y, por ello, deben redactarse de manera independiente en el pliego reivindicatorio. Sobre las mismas, el Tribunal Andino de Justicia ha dicho lo siguiente:

"El objeto de la invención de producto está constituido por un cuerpo cierto destinado a llenar una necesidad industrial que el estado de la técnica aún no ha satisfecho. En cambio, el objeto de la invención de procedimiento se encuentra conformado por "una serie o sucesión de operaciones que inciden sobre una materia (solida, liquida o gaseosa) que se encamina a la obtención de una cosa o un resultado"¹.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad invocada, es decir, el 11 de marzo de 2009.

¹ Otero Lastres, José Manuel. Ob. Cit. Proceso 129alPa2007.

Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Item	Documento	Título	Fecha de Publicación
D1	US 5888562	"Enzymatic treatment of cocoa"	30/Marzo/1999
D2	Art 1 ²	"Effect of polyphenol oxidase (PPO) and air treatments on total phenol and tannin content of cocoa nibs"	Abril/2002
D3	Art 2 ³	"Use of a proteolytic enzyme in Cocoa (<i>Theobroma cacao</i> L.)"	Agosto/2004
D4	Art 3 ⁴	"Effect of enzyme treatments and drying temperatures on methylpyrazine content in cocoa (<i>Theobroma cacao</i> L.) powder extract."	2006

El documento D1⁵ divulga el tratamiento enzimático con proteasas y carbohidrasas sobre cocoa (Col. 3, líneas 5 a 9, Col. 4, líneas 6 a 23, Col. 4, líneas 55 a 60, Col. 5, líneas 18 a 23, Col. 14, reivindicación 15).

El documento D2 divulga el tratamiento enzimático con polifenol oxidasa sobre cocoa (Pág. 47, Col. 1).

El documento D3 divulga el tratamiento enzimático con proteasas y carbohidrasas sobre cocoa sin ajuste de pH (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).

El documento D4 divulga el tratamiento enzimático con proteasas y carbohidrasas sobre cocoa (Pág. 622, Col. 1, Párr. 1).

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 18 D486)

La reivindicación 1 consiste en "*Un proceso para producir un producto tratado de cacao, el proceso comprendiendo las etapas de...*" (Fl. 46).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D4
Proceso para producir un producto tratado de cacao, el proceso comprendiendo las etapas de:	Tratamiento de licor o trozos de cocoa (Col. 2, líneas 28 a 37).	Tratamiento de cocoa en polvo con enzimas (Pág. 622, Col. 1, Párr. 1).
Proporcionar un producto de cacao	Se tiene el cocoa en líquido o en sólido (Col. 2, líneas 28 a 37).	Se toma cocoa en polvo y se disuelve en agua (Pág. 622, Col. 1, Párr. 1).
Añadir una mezcla de enzimas con proteasa	Se adiciona una proteasa (Col. 2, líneas 28 a 37).	Se adicionan proteasas (Pág. 622, Col. 1, Párr. 1, Fig. 1).
y carbohidrasa en presencia de agua	Se emplea también una invertasa o sacarasa que produce glucosa y fructosa a partir de sacarosa (Col. 3, líneas 5 a 9).	Se adicionan carbohidrasas (Pág. 622, Col. 1, Párr. 1, Fig. 1).
Tratar el producto de cacao durante un tiempo y temperatura	Se deja un tiempo suficiente para tener aminoácidos libres (Col. 2, líneas 28 a 37).	Se mantiene a una temperatura de 50°C y durante 8 horas (Pág. 622, Col. 1, Párr. 2).

² de Brito et al. Ciência e tecnologia de alimentos, Campinas. Vol. 22 (1). 2002. Págs. 45 a 48.

³ de Brito et al. Brazilian archives of biology an technology. Vol. 47 (4). 2004. Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3.

⁴ Moulay et al. Journal of food science. Vol. 71 (9). Págs. 621 a 625.

⁵[http://worldwide.espacenet.com/publicationDetails/biblio?](http://worldwide.espacenet.com/publicationDetails/biblio?DB=worldwide.espacenet.com&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=19990330&CC=US&NR=5888562A&KC=A)

DB=worldwide.espacenet.com&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=19990330&CC=US&NR=5888562A&KC=A

De acuerdo con la tabla anterior, las características esenciales del método de la reivindicación 1 habían sido divulgadas previamente en los documentos D1 o D4, por lo tanto, la materia reivindicada no es nueva.

Las reivindicaciones dependientes 2 a 4 y 7 no mencionan alguna característica que haga nuevo el método de la reivindicación 1, por lo cual se considera que no son nuevas, ya que D1 divulga carboxipeptidasas, aminoproteasas, endoproteasas, celulasas y glucanasas para el tratamiento con concentraciones entre 0.01 y 5%, con condiciones de hidrólisis de 10 a 60°C y durante 20 minutos a 2 horas, donde el producto final es tostado (Col. 3, líneas 5 a 9, Col. 4, líneas 6 a 23, Col. 4, líneas 55 a 60, Col. 5, líneas 18 a 23, Col. 14, reivindicación 15).

La reivindicación 18 consiste en “*Un producto alimenticio que comprende: un producto de cacao tratado enzimáticamente por una mezcla de enzimas...*” (Fl. 48).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D4
Producto alimenticio que comprende un producto de cacao tratado enzimáticamente	El producto de cocoa proviene de un tratamiento con proteasas (Pág. 621, Col. 1, Párr. 2 a Col. 2 Párr. 2)
El producto tratado de cacao se incorpora en un producto comestible seleccionado de productos lácteos, ingredientes culinarios, productos de horneado, dulces o confitería y cualquier combinación de los mismos.	Cocoa en polvo es distribuido después del tratamiento a la industria de confitería y horneado (Pág. 621, Col. 1, Párr. 2)

De acuerdo con la tabla anterior, las características esenciales del producto de la reivindicación 18 habían sido divulgadas previamente en el documento D4, por lo tanto, la materia reivindicada no es nueva.

Las reivindicaciones dependientes 19 y 20 no mencionan alguna característica que haga nuevo el método de la reivindicación 1, por lo cual se considera que no son nuevas, ya que D4 divulga el empleo de cocoa en diversos producto con contenido de grasa lo que conllevaría a su uso en relleno, recubrimientos y siendo implícito para la persona versada en la materia que existe la opción de que el pH para el tratamiento enzimático no sea ajustado³ (Pág. 624, Col. 2).

Nivel inventivo (Art 18 D486)

La reivindicación 6 consiste en “*El proceso de la reivindicación 1, en donde la mezcla de la enzima comprende además una polifenol oxidasa*” (Fl.46).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
La mezcla de la enzima comprende además una polifenol oxidasa	No se menciona	Se emplea una polifenol oxidasa para mejorar las condiciones organolépticas de un producto de cocoa (Pág. 47, Col. 1).

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 6, porque divulgan métodos enzimáticos para tratar productos de cacao.

La diferencia entre la invención, definida en la reivindicación 6 y el método del documento D1 consiste en que D1 no menciona la polifenol oxidasa.

La diferencia entre la invención, definida en la reivindicación 6 y el método del documento D2 consiste en que en D2 no se menciona las proteasas y carbohidrasas.

El efecto que logra el incluir la polifenol oxidasa es que permite mejorar las condiciones organolépticas del producto final de cacao de forma más eficiente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así “¿cómo modificar el método de D1 para que se mejoren las condiciones organolépticas de un producto final de cacao de forma más eficiente?”.

Sin embargo, la enzima polifenol oxidasa, ya está divulgada en el documento D2 que se refiere a un estudio sobre el tratamiento de cocoa con polifenol oxidasa, el cual es un producto del cacao (Pág. 47, Col. 1).

En consecuencia, una persona normalmente versada en la materia incluiría la polifenol oxidasa de D2 en el procedimiento de la anterioridad D1, esperando con certeza lograr el método, como la de la reivindicación 6 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

La reivindicación 8 consiste en “*Un proceso para producir un producto tratado de cacao, el proceso comprendiendo las etapas de...*” (Fl. 47).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D3
Proceso para producir un producto tratado de cacao, el proceso comprendiendo las etapas de:	Tratamiento de licor o trozos de cocoa (Col. 2, líneas 28 a 37).	Estudio del tratamiento de cocoa con una enzima (Pág. 554).
Proporcionar un producto de cacao	Se tiene la cocoa en líquido o en sólido (Col. 2, líneas 28 a 37).	Se tiene el frijol de cocoa fermentado y esterilizado (Pág. 554, Col. 1, Párr. 3).
Añadir una mezcla de enzimas con proteasa	Se adiciona una proteasa (Col. 2, líneas 28 a 37).	Se adiciona una proteasa (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).
y carbohidrasa en presencia de agua	Se emplea también una invertasa o sacarasa que produce glucosa y fructosa a partir de sacarosa (Col. 3, líneas 5 a 9).	No se menciona
Tratar el producto de cacao durante un tiempo y temperatura	Se deja un tiempo suficiente para tener aminoácidos libres (Col. 2, líneas 28 a 37).	Se estudia temperaturas alrededor de 50°C y de 1 a 6 horas (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).
Sin ajustar el pH	No se menciona	Durante el estudio no se ajusta el pH siendo así implícito que este tipo de material proporciona el pH adecuado para una adecuada actividad enzimática (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).

Los documentos D1 y D3 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 8, porque divulgan métodos enzimáticos para tratar productos de cacao.

La diferencia entre la invención, definida en la reivindicación 8 y el método del documento D1 consiste en que D1 ajusta el pH.

La diferencia entre la invención, definida en la reivindicación 8 y el método del documento D3 consiste en que en D3 no se menciona las carbohidrasas.

El efecto que logra el no ajustar el pH es que permite evitar la etapa de adicionar un buffer para mejorar las condiciones organolépticas del producto final de cacao de forma más eficiente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así “¿cómo modificar el método de D1 para que se mejoren las condiciones organolépticas de un producto final de cacao de forma más eficiente?”.

Sin embargo, una proteasa que no requiere buffer, ya están divulgada en el documento D3 que se refiere a un estudio sobre el tratamiento de cocoa con una proteasa teniendo en cuenta la concentración y temperatura únicamente (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).

En consecuencia, una persona normalmente versada en la materia incluiría la proteasa sin buffer para ajustar pH de D3 en el procedimiento de la anterioridad D1, esperando con certeza lograr el método, como la de la reivindicación 8 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

Puesto que las reivindicaciones dependientes 9 a 12 y 14 no mencionan características adicionales que hagan inventiva la solicitud, se considera que tales reivindicaciones tampoco tienen nivel inventivo, ya que D1 divulga carboxipeptidasas, aminoproteasas, endoproteasas, celulasas y glucanasas para el tratamiento con concentraciones entre 0.01 y 5%, con condiciones de hidrólisis de 10 a 60°C y durante 20 minutos a 2 horas, pH entre 3 y 8, donde el producto final es tostado y siendo implícito para la persona versada en la materia el empleo de polifenol oxidasa² (Col. 3, líneas 5 a 9, Col. 4, líneas 6 a 23, Col. 4, líneas 55 a 60, Col. 5, líneas 18 a 23, Col. 14, reivindicación 15).

La reivindicación 15 consiste en “*Un proceso para producir un producto tratado de cacao, el proceso comprendiendo las etapas de...*” (Fl. 48).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D4	D3
Proceso para producir un producto tratado de cacao, el proceso comprendiendo las etapas de:	Tratamiento de cocoa en polvo con enzimas (Pág. 622, Col. 1, Párr. 1, Fig. 1).	Estudio del tratamiento de cocoa con una enzima (Pág. 554).
Añadir una mezcla de enzimas con proteasa	Se adicionan proteasas (Pág. 622, Col. 1, Párr. 1, Fig. 1).	Se adiciona una proteasa (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).
y carbohidrasa a un producto de cacao en presencia de agua	Se adicionan carbohidrasas (Pág. 622, Col. 1, Párr. 1, Fig. 1).	No se menciona
Tratar el producto de cacao durante un tiempo y temperatura	Se estudia una temperatura de 50°C y durante 8 horas (Pág. 622, Col. 1, Párr. 2).	Se estudian temperaturas alrededor de 50°C y de 1 a 6 horas (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).
Sin ajustar el pH	No se menciona	Durante el estudio no se ajusta el pH siendo así implícito que este tipo de material proporciona el pH adecuado para una adecuada actividad enzimática (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).
Producir un polvo tratado de cacao	El producto del proceso es en polvo (Pág. 621, Col. 1, Párr. 2).	No se menciona

Los documentos D4 y D3 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 15, porque divulgan métodos enzimáticos para tratar productos de cacao.

La diferencia entre la invención, definida en la reivindicación 15 y el método del documento D4 consiste en que D4 ajusta el pH.

La diferencia entre la invención, definida en la reivindicación 15 y el método del documento D3 consiste en que en D3 no se menciona carbohidrasas.

El efecto que logra el no ajustar el pH es que permite evitar la etapa de adicionar un buffer para mejorar las condiciones organolépticas del producto final de cacao de forma más eficiente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así “¿cómo modificar el método de D4 para que se mejoren las condiciones organolépticas de un producto final de cacao de forma más eficiente?”.

Sin embargo, una proteasa que no requiere buffer, ya están divulgada en el documento D3 que se refiere a un estudio sobre el tratamiento de cocoa con una proteasa teniendo en cuenta la concentración y temperatura únicamente (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).

En consecuencia, una persona normalmente versada en la materia incluiría la proteasa sin buffer para ajustar pH de D3 en el procedimiento de la anterioridad D4, esperando con certeza lograr el método, como la de la reivindicación 15 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

Puesto que las reivindicaciones dependientes 16 y 17 no mencionan características adicionales que hagan inventiva la solicitud, se considera que tales reivindicaciones tampoco tienen nivel inventivo, ya que D4 divulga enzimas como celulasas, amilasas, aminopeptidasa y complejo de proteasas (Pág. 622, Col. 1, Párr. 1, Fig. 1, Pág. 623, Col. 1, Párr. 1) y D3 divulga que se estudian temperaturas alrededor de 50°C y de 1 a 6 horas (Pág. 554, Col. 1, Párr. 4 a Col. 2, Párr. 3).

7. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 5888562	1 a 4 y 7	Novedad/ Nivel inventivo
		6, 8 a 12 y 14	Nivel inventivo
D2	Art 1	6	Nivel inventivo
D3	Art 2	8 a 12 y 14 a 17	Nivel inventivo
D4	Art 3	1, 18 a 20	Novedad/Nivel inventivo
		15 a 17	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-06-18

Elaboró: Jorge Eduardo Cortazar Gomez
Revisó: Leonardo Rojas Vega

EFFECT OF POLYPHENOL OXIDASE (PPO) AND AIR TREATMENTS ON TOTAL PHENOL AND TANNIN CONTENT OF COCOA NIBS¹

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SUMMARY

Cocoa flavour is greatly influenced by polyphenols. These compounds undergo a series of transformations during cocoa processing leading to the characteristic cocoa flavour. The use of exogenous polyphenol oxidase (PPO) proved to be useful to reduce polyphenol content in cocoa nibs. The effect of a PPO associated or not with air over total phenol and tannin content was evaluated. Cocoa nibs were autoclaved and treated with a PPO or water in the absence or presence of an air flow for 0.5, 1, 2 and 3 hours. Total phenol content was reduced in PPO or water treatments, but when associated with air there was an increase in phenol content. Tannin content was reduced only by the treatment with water and air.

Keywords: cocoa; processing; PPO; enzyme; air; *Theobroma cacao*.

RESUMO

EFEITO DOS TRATAMENTOS COM POLIFENOL OXIDASE (PPO) E AR SOBRE O TEOR DE FENÓIS TOTAIS E TANINOS EM NIBS DE CACAU. O sabor do cacau é fortemente influenciado pelos polifenóis. Esses compostos sofrem uma série de transformações durante o processamento do cacau dando origem ao sabor característico do cacau. O uso de polifenol oxidase (PPO) exógena mostrou ser útil na redução do teor de polifenóis em nibs de cacau. O efeito de uma PPO associada ou não com ar sobre o teor de fenóis totais e taninos foi avaliado. Nibs de cacau foram autoclavados e tratados com PPO ou água na presença ou não de um fluxo de ar por 0,5; 1; 2 e 3 horas. O teor de fenóis totais foi reduzido nos tratamentos com PPO ou água, mas quando associados com ar houve um aumento desses teores. O teor de taninos foi reduzido apenas pelo tratamento com água e ar.

Palavras-chave: cacau; processamento; PPO; enzima; ar; *Theobroma cacao*.

1 – INTRODUCTION

During cocoa fermentation the phenolic compounds originally compacted into vacuoles of specific cells diffuse through the cotyledon [7]. The phenols are oxidized and the resulting compounds associate reversibly with proteins by hydrogen bonds or, irreversibly, by condensation with reactive groups of amino acids, peptides, proteins and polysaccharides. These reactions are important to the development of cocoa flavour [1, 8]. The total phenols in cocoa get reduced during fermentation to 30% of the initial value and the (-)-epicatechin, principal substrate of cocoa polyphenol oxidase (PPO), is reduced by 90%, with a proportional increase in catechin content [2, 10, 15]. Polyphenol reduction during drying was attributed to enzymatic browning followed by non-enzymatic browning from quinone polymerization. The PPO would not have appropriate conditions to react during fermentation, but during drying with the increase in pH and high O₂ uptake, the conditions will be appropriate to phenolic oxidation [4]. However, the reduction of the phenolic compounds could not be explained only by enzymatic oxidation since, in the fifth day of fermentation, the PPO activity was reduced from 5 to 13% of the beginning with a complete or strong inactivation [6, 12, 14]. Information about other changes in phenolic content during further cocoa processing is rare [16].

Today there is a great interest in cocoa polyphenols not only because of their contribution to flavour but also

for their antioxidant activity and possible beneficial health effects [17]. Total phenol content in cocoa varies from 12 to 18% of defatted and dry weight. The principal compounds are (+)-catechin, (-)-epicatechin and 60% of proanthocyanidins, of which half are dimers. Other compounds identified are quercetin, quercetrin and *p*-coumaric, caffeic and chlorogenic acids [10, 13].

The use of enzymes in cocoa nibs was applied with success to reduce polyphenol contents by about 25% [3, 19]. These authors reported a greater polyphenol reduction when nibs were autoclaved, which facilitates enzyme penetration into tissues and enzyme action. FERNÁNDEZ BARBERY [3] also demonstrated that astringency and bitterness were reduced by polyphenol oxidase action, which contributed to a better acceptance of the cocoa products. The use of Polyphenol oxidase in cocoa processing was also suggested, since these enzymes were involved in the formation of aldehydes from amino acids [8].

In this work we studied the effect of polyphenol oxidase and air effects on the total phenol and tannin content of cocoa nibs.

2 – MATERIAL AND METHODS

2.1 – Processing

Fermented and dried “Forastero” cocoa beans were supplied by INDECA S.A. (Embú-SP, Brasil). A cut-test

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was performed to assay bean characteristics after which they were broken into nibs (3 – 6mm) prior to treatment.

Polyphenol oxidase (E.C. 1.14.18.1) from mushroom (Sigma, St Louis, MI, USA) with 5,350U/mg was used at a final concentration of 200U/mL. To 80g of autoclaved cocoa nibs (121°C for 15min), 40mL of enzyme solution was added. The sample was submitted to a vacuum of 200mmHg in a desiccator for 1min to facilitate the penetration of the enzyme solution.

To evaluate the effect of air on enzyme action, the sample was placed in a glass column. The height of the packed sample was 10cm and the internal diameter was 3cm. An air flow of 250L/h was passed through the column. To verify the effect of air alone an experiment was done with water instead of the enzyme solution. Controlled experiments were conducted without air by placing the samples with enzyme or water into beakers.

The experiments were conducted at ambient temperature and aliquots were taken at 0, 0.5, 1, 2 and 3 hours of treatment. The samples were dried at 65°C, defatted with petroleum ether and stored at -18°C until the analysis.

2.2 – Total phenol analysis

Total phenols were determined by the Prussian Blue assay as follows. Fifty mg of defatted sample were extracted twice with 5mL of acetone:water (7:3, v/v) at 4°C, centrifuged for 10min at 4100xg and the supernatants were combined. An aliquot of 0.1mL was added to 50mL of distilled water followed by the addition of 3.0mL of 0.10 M $\text{FeNH}_4(\text{SO}_4)_2$ in 0.10 M HCl. After exactly 20 min, 0.008 M $\text{K}_3\text{Fe}(\text{CN})_6$ was added. The absorbancy was read after 20min at 720nm [11]. A standard curve was constructed using tannic acid and the results expressed as mg phenols/g sample.

2.3 – Tannins analysis

Fifty mg of defatted samples were extracted three times (3, 3 and 4mL) with methanol:water (1:1, v/v) and centrifuged for 5min at 4100g and the supernatants were combined. To an aliquot of 1mL, 2mL of BSA (bovine serum albumin, Type 5, 1mg/mL in 0.20 M CH_3COOH : 0.17 M NaCl buffer, pH 4.9) was added. The sample was immediately mixed and maintained at 4°C for 24 hours for tannin precipitation. After a centrifugation at 4100g for 15min, the supernatant was discarded and the precipitate dissolved in 4mL SDS:TEA (10g/L SDS and 50mL/L triethanolamine). Then, 1mL of 0.01 M FeCl_3 in 0.01 M HCl was added and mixed well. After 15 min the absorbancy was read at 510nm [5]. A standard curve was constructed with tannic acid and the results were expressed as mg tannic acid/g sample.

2.4 – Statistics

Each treatment was performed three times and each sample was analysed three times. The results were compared by analysis of variance using the software package Statistica version 5.0. Significant differences

among means were confirmed using the Tukey test for multiple comparisons at $P < 0.05$.

3 – RESULTS AND DISCUSSION

The cut test revealed a large proportion (25/100) of violet beans, which indicated under-fermentation. The total phenol content was 7.9mg/g and the tannin content 2.6mg/g. The results for total phenols and tannin are shown in *Tables 1* and *2*, respectively. Total phenol content in the sample treated with water and air had no significant change during the process, but tannin content presented a significant decrease. In the sample treated with water, the total phenol content was reduced at 0.5h and returned gradually to the initial value, but tannin content after an initial increase until 1 hour remained unchanged after that. For the sample treated with enzyme and air there was a large increase of total phenols at the beginning of the process until 0.5h followed by a decrease to the initial value. The same could be observed for tannin content. On the other hand, the enzyme treated sample without air had little decrease in total phenol content at the beginning of the process and this value remained unchanged for 3 hours. The tannin content was the same as that of the sample treated with enzyme and air. *Figure 1* shows the percentage contribution of tannin to total phenols. The samples treated with air had a lower tannin percentage, which indicated that air had a positive effect on tannin content reduction, especially in the sample treated with water and air.

TABLE 1. Total Phenol content (mg tannic acid/g sample) in cocoa nibs after treatment with PPO, air and water.

Time (h)	Sample			
	PPO + Air	PPO	H ₂ O + Air	H ₂ O
0	7.9 ± 0.4 a, A	7.9 ± 0.4 a, A	7.9 ± 0.4 a, A	7.9 ± 0.4 a, A
0.5	10.4 ± 0.7 b, A	6.8 ± 0.2 b, B	8.4 ± 0.6 a, C	5.9 ± 0.1 b, D
1	8.6 ± 0.4 a, A	6.4 ± 0.5 b, B	8.5 ± 0.9 a, A	6.4 ± 0.8 b, B
2	8.2 ± 0.1 a, A	6.3 ± 0.5 b, B	8.8 ± 0.9 a, A	6.4 ± 0.1 b, B
3	7.9 ± 0.2 a, A	6.5 ± 0.6 b, B	7.9 ± 0.6 a, A	7.3 ± 0.1 a, A

Values are mean ± S.D. Means within the same treatment followed by the same lower case letters are not significantly different at $p < 0.05$. Means within the same time followed by the same upper case capital letters are not significantly different at $p < 0.05$.

TABLE 2. Tannin content (mg tannic acid/g sample) in cocoa nibs after treatment with PPO, air and water.

Time (h)	Sample			
	PPO + Air	PPO	H ₂ O + Air	H ₂ O
0	2.6 ± 0.6 a,b, A	2.6 ± 0.6 a,b, A	2.6 ± 0.6 a,b, A	2.6 ± 0.6 a, A
0.5	3.5 ± 0.6 a, A	3.6 ± 0.4 a, A	2.9 ± 0.6 a, A	2.9 ± 0.5 a, A
1	3.1 ± 0.6 a, A	3.1 ± 0.2 a,b, A	3.1 ± 0.2 a, A	3.3 ± 0.2 a, A
2	2.7 ± 0.1 a, A	2.7 ± 0.1 b, A	2.1 ± 0.1 b, B	3.3 ± 0.2 a, C
3	2.2 ± 0.3 b, A	2.5 ± 0.3 b, A	1.7 ± 0.1 c, B	3.3 ± 0.1 a, C

Values are mean ± S.D. Means within the same treatment followed by the same lower case letters are not significantly different at $p < 0.05$. Means within the same time followed by the same upper case capital letters are not significantly different at $p < 0.05$.

A simple explanation of these transformations is very difficult since PPO promotes *o*-hydroxylation of mono-phenols and the oxidation of *o*-diphenols to *o*-quinones.

The o-quinones also take part in secondary reactions with compounds like anthocyanins and many other phenolic compounds, which reduce o-quinones to diphenols, or can condense with phenolic compounds [18]. A study of astringency and bitterness of flavan-3-ol monomers, dimers and trimers revealed that molecular size was the major factor influencing the sensory properties of these attributes. As molecular size increased, bitterness decreased and astringency increased [9]. The use of PPO without air also contributed to reduction of astringency and bitterness in cocoa nibs, and improved the acceptance of a chocolate formulated with treated cocoa [3]. Despite the changes observed in PPO treated samples we achieved a greater tannin reduction in the treatment with water and air.

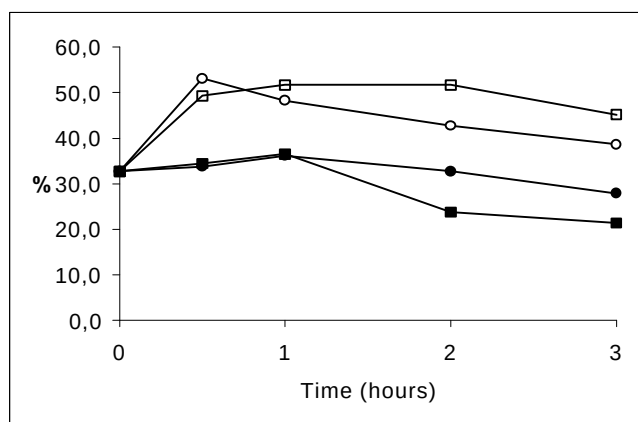


FIGURE 1. Tannin percentage in relation to total phenol in cocoa nibs treated with PPO + air (●), PPO (○), H₂O + air (■) and H₂O (□).

The polyphenol oxidase from mushrooms reduced the total phenol content in cocoa nibs by 20%, which was lower than that obtained by PPO of *Coriolus versicolor* for the same time of treatment [19] and of *Annona squamosa* after 6 hours of treatment [3], both presented a 25% reduction. Tannin content was not reduced by mushroom PPO, whereas it was reduced by PPO from other sources [3, 19]. This could be explained by different experimental conditions and/or different specificity of the enzyme studied. Cocoa beans are known to have different pH and polyphenol content, depending on their origin and fermenting practices.

4 – CONCLUSION

Despite the use of PPO, tannin content was significantly reduced by air application. These findings indicated that air processing of cocoa nibs could help in cocoa flavour improvement by changing the ratio between tannin and total phenol content and, consequently, the astringency and bitterness of insufficiently fermented beans. Further studies are needed to understand the specific transformations of phenolic compounds during air processing of cocoa.

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Use of a Proteolytic Enzyme in Cocoa (*Theobroma cacao* L.) Processing

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ABSTRACT

Protein hydrolysis using an exogenous protease on cocoa nibs was performed to verify the formation of precursors and the effect on cocoa flavour. An experimental design was used to check the influence of temperature (30 to 70 °C) and enzyme : substrate ratio [E/S] (97.5 to 1267.5 U g⁻¹ of protein). The % Degree of Hydrolysis (% DH) was affected mainly by [E/S] leading to a 4-fold increase (from 5 to 20 %) after 6 hours of treatment. During cocoa nibs roasting, there was a greater consumption of hydrolysis compounds in the sample treated with protease as compared to the control, indicating their participation in the Maillard reaction. An increased perception of chocolate flavour and bitter taste was observed in a product formulated with protease treated cocoa.

Key words: Cocoa, flavour, protein hydrolysis, processing

INTRODUCTION

Fermented and dried cocoa beans constitute the raw material used by the chocolate industry to produce various different products, which are largely appreciated for their characteristic flavour. During fermentation the pulp surrounding the seeds is metabolized by the microorganisms resulting in a temperature rise and a drop in pH, which are responsible for cotyledon death. During this some other substances such as ethanol, acetic and lactic acids and the formation of flavour precursors, basically peptides, free amino acids and reducing sugars (Reinnecius et al., 1972; Mohr et al., 1976) are also produced. These precursors participate in the Maillard reaction during cocoa roasting, leading to the characteristic chocolate flavour.

Proteolysis is very important for cocoa flavour development (Voigt et al., 1994a; 1994b). Amino acids and peptides are produced during fermentation by the combined action of an aspartic endoprotease and a carboxipeptidase. The main substrate for these enzymes is a globulin described as a cocoa vicilin. A specific cocoa aroma was obtained when this globulin was degraded by exogenous proteases and the resulting products were roasted in the presence of reducing sugars (Voigt et al., 1994a; 1994b). However, other cocoa proteins should be considered in flavour precursor formation (Lerceteau et al., 1999). During subsequent roasting, amino acid levels were reduced, but this reduction was not complete and there were different rates of reaction for the distinct amino acid groups (Seiki, 1973; Abeygunasekera and Jansz, 1989).

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The use of enzymes in cocoa nibs was proved to be successful in reducing polyphenol contents by nearly 25% (Yoshiyama and Ito, 1996; Fernández Barbery, 1999). These authors found a greater polyphenol reduction in autoclaved nibs, which facilitated enzyme penetration into tissues and action. Fernández Barbery (1999) also demonstrated that bitter taste and astringency were reduced by polyphenoloxidase action, which contributed to a better acceptance of the cocoa products.

The purpose of this work was to study the effect of using an exogenous proteolytic enzyme in previously fermented and dried cocoa nibs. Enzyme concentration and temperature of the process were evaluated in order to improve protein hydrolysis.

MATERIAL AND METHODS

Sample

Fermented cocoa beans of the Forastero variety collected from an IAC experimental station in Pariqueira Açu, São Paulo, Brazil were used. The tegument and germ tissues were removed and the cotyledons were broken into nibs. The material sieved through a 3.5 mesh screen but retained by an 8 mesh screen was autoclaved at 121°C for 15 min prior to enzyme treatment.

Enzymatic Treatment

A proteolytic enzyme (Flavourzyme MG, type A), with an activity of 1,000 Leucine Aminopeptidase

Units (LAPU) g⁻¹ was provided by Novo Nordisk A/S (Bagsvaerd, Denmark). The enzyme was dissolved in 90 mL of distilled water and placed in a beaker containing 150 g of cocoa nibs. The samples were then subjected to a 200 mm Hg atmosphere for 1 min. During the experiments samples were maintained in a water bath at the desired temperature and the nibs were mixed at 15 min intervals. At appropriate times the reaction was stopped by drying samples at 65°C for 30 min and then kept at -18°C until analysed.

An experiment with an enzyme/substrate ratio [E/S] of 682.5 and 50°C (central point) was done and samples were collected after 1, 2, 4 and 6 hours of reaction. Reaction times of 4 and 6 hours were chosen for further sensorial studies.

A central composite rotatable design (CCRD) (Barros Neto et al., 1996) with two variables was used to study the response pattern, degree of hydrolysis, and to establish a model for cocoa protein hydrolysis. The variables studied were temperature and enzyme/substrate ratio, each one at five levels. Table 1 shows the symbols and levels.

Results were evaluated by response surface methodology (RSM) and adequacy of the model was assessed by analysis of variance ($P < 0.10$) using the software STATISTICA (Statsoft, v. 5.0). In order to verify the model and to carry out a sensory evaluation, cocoa nibs were treated with enzyme or water (control) at a fixed condition and roasted at 150°C for 38 min (PRE 1Z sample roaster, Probat-Werke, Germany).

Table 1 - Variables and their levels for CCRD.

Variables	Symbol	Levels				
		- α	-1	0	+1	+ α
Temperature (°C)	X_1	30.0	35.9	50.0	64.1	70.0
[E/S] (U g ⁻¹)	X_2	97.5	269.1	682.5	1095.9	1267.5

Analyses

The amino groups (α -N) were extracted with a mixture of trichloroacetic acid/sodium acetate/acetic acid (0.11 M:0.22 M:0.33 M) (Murthy et al., 1997) and determined spectrophotometrically after reacting with o-phthalaldehyde (Church, 1985). The values were expressed as glycine content (mg g⁻¹). The absorbance of the resulting extract was also read at A_{275} to determine levels of amino acids and peptides with aromatic groups (Murthy et al., 1997). Total nitrogen was estimated

by a semi-micro Kjeldhal procedure (AOAC, 1997).

Degree of protein hydrolysis (DH) was established as the ratio between amino-terminal groups and total nitrogen and expressed on a percentage base (equation 1).

$$\% \text{ DH} = (\alpha\text{-N} / \text{total-N}) \times 100 \quad (\text{eq. 1})$$

Sensory Evaluation

The roasted nibs were milled in a cooled three-roll mill. The mass obtained was mixed with sugar and dairy cream (35% fat) in a proportion of 48.8:26.2:25 (w/w), respectively. The resulting product was cooled and cut into pieces (10 g weight) for sensory evaluation. A just right scale was used to evaluate the intensity of chocolate and bitter taste. The samples were presented to judges (N=30) who were asked to indicate the strength of the taste, in a 9-point structured scale ranging from “much too weak” to “much too strong” (Meilgaard et al., 1987).

RESULTS AND DISCUSSION

Fig. 1 shows the dynamics of protein hydrolysis at fixed conditions (central point). At the beginning of the process a 5 %DH was found. This value could be explained by the natural hydrolysis that happened during the fermentation. After 6 hours of treatment the DH experienced a 4-fold increase.

Table 2 shows the conditions and %DH after enzyme treatment for 4 and 6 hours. Data were analysed for main effects and interactions. The models expressed by equations 2 and 3 were generated, representing the %DH at 4 and 6 hours, respectively, as a function of the more significant variables ($P < 0.10$).

$$\%DH_{4 \text{ hours}} = 15.64 + 1.12X_2 - 1.45X_1X_2 \quad (\text{eq. 2})$$

$$\%DH_{6 \text{ hours}} = 19 + 1.04X_2 - 2.4X_2^2 \quad (\text{eq. 3})$$

The F -test showed that the model for A_{275} was not reliable since the calculated $F_{0.1}$ values were lower than the tabled values (data not shown). These results indicated that aromatic amino acids were not released during proteolysis or that A_{275} estimation was not a useful procedure to follow proteolysis in cocoa. Table 3 shows the analysis of variance for the %DH after 4 and 6 hours of treatment. The $F_{0.1}$ value for $\%DH_{4 \text{ hours}}$ was lower than the tabled one, and the $\%DH_{6 \text{ hours}}$ was greater than it, but not 3-5 times greater which would indicate a model with high predictive power (Barros Neto et al., 1999). Despite this fact, it was decided to evaluate the model for 6 hours of treatment and its effect on % DH and on sensory properties. In order to determine the most adequate conditions and analyze the process, the response surface was plotted using Eq. (3) (Fig. 2). The non-significant effect of temperature on %DH

could be explained by non-uniform heat distribution inside the nibs due to their size (5 mm) and distribution inside the beaker. As the temperature had no influence in %DH, it was decided to use the lower temperature (30 °C), which implied a lower energy cost. For [E/S] it was decided to use the central point value since the highest %DH was obtained near this point.

An additional experiment was performed at a fixed condition ($X_1 = -\alpha$, $X_2 = 0$) and a control sample with water instead of enzyme solution. The results are shown in Table 4. The % DH (18.1%) obtained in this latter experiment was lower than the values observed previously (20.0 %) and predicted (19 %) by the model, but it was higher than the control. The high level of %DH in the control could be explained by the action of endogenous enzymes (Hansen et al., 1998) that were brought to a suitable condition during the experiment. After roasting, the enzyme treated sample presented a greater consumption of hydrolysis products as compared to the control. These results indicated that the products formed during enzymatic hydrolysis were consumed at a higher extent during roasting, probably due to their participation in the Maillard reaction.

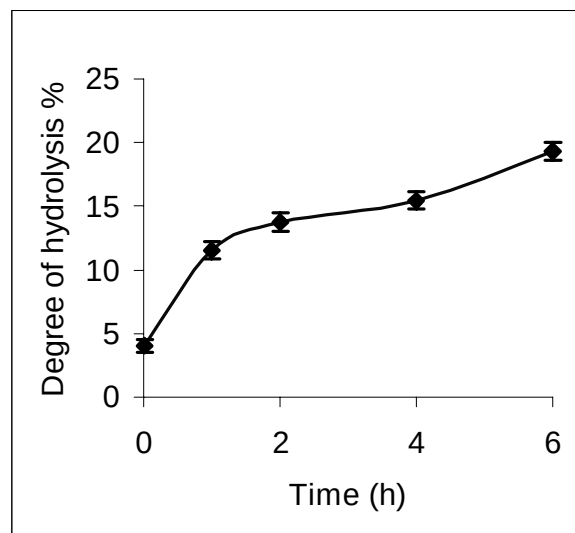


Figure 1 - Dynamics of protein hydrolysis with time. Test was performed at fixed conditions ([E/S]=682.5 and 50 °C). Values are mean of three replicates $\pm$ SD.

Fig. 3 (a and b) presents the results obtained in the sensory evaluation of a product formulated with enzymatic treated cocoa and the control. For

chocolate flavour and bitter taste, the treated sample presented a distribution more right-dislocated than the control, which indicated a stronger perception of the attributes. Such behaviour indicates that there are too many hydrolysis products available to form the flavour

components during roasting. The results in this work also emphasize the need for a good fermentation process, since during this process proteolysis occurred naturally.

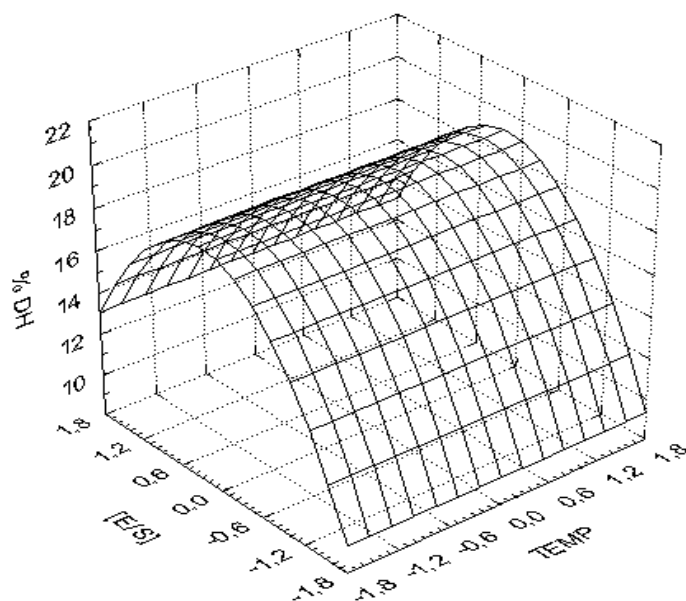


Figure 2 - Response surface diagram of % DH at 6 hours as function of [E/S] and temperature.

Table 2 - Conditions and results for percentage degree of protein hydrolysis (%DH) at 4 and 6 hours according to central composite rotatable design.

Treatment Nº	X_1	X_2	Responses (%DH)	
			4 h	6 h
1	+1	+1	16.29	18.61
2	-1	+1	18.06	18.65
3	+1	-1	16.02	16.84
4	-1	-1	11.97	15.04
5	$+\alpha$	0	14.74	15.92
6	$-\alpha$	0	14.49	20.04
7	0	$+\alpha$	18.00	14.56
8	0	$-\alpha$	16.17	12.44
9	0	0	15.88	18.65
10	0	0	15.82	19.36
11	0	0	14.65	19.66

Table 3 - Analysis of variance of %DH after 4 or 6 hours of treatment ^a.

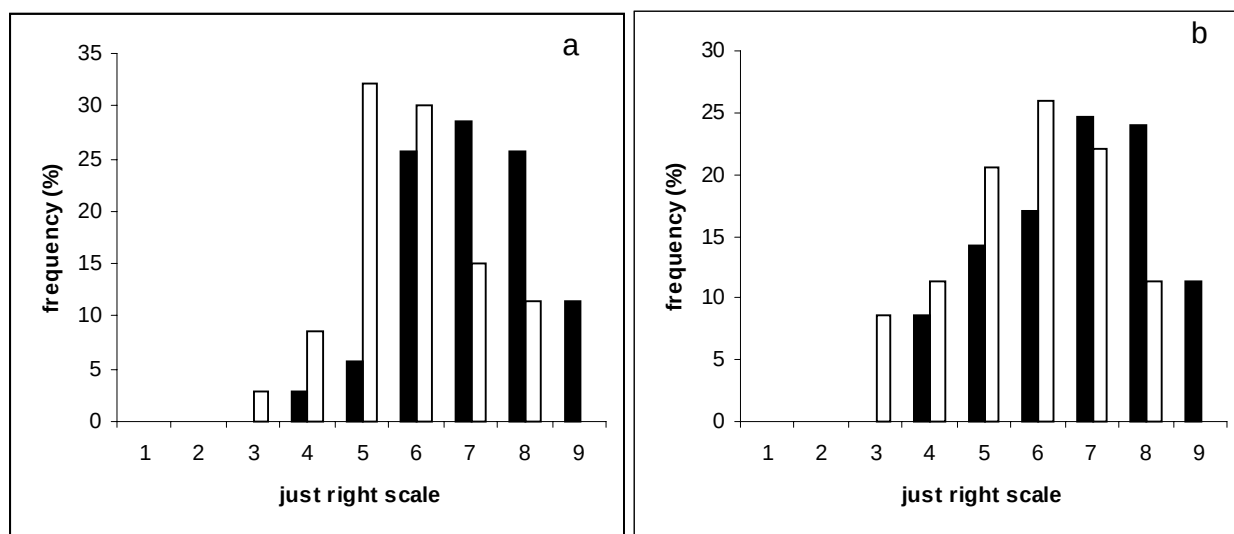
Source of variation	Sum of squares		Mean square		Degree of freedom		F-value	
	4 h	6 h	4 h	6 h	4 h	6 h	4 h	6 h
Regression	18.84	44.57	9.24	22.29	2	2	7.1	10.9
Residual	10.47	16.44	1.3	2.05	8	8		
Total	28.95	61.01			10	10		
Correlation coefficient	0.638	0.730						

^a $F_{0.90;2;8} = 9.37$ **Table 4** - Degree of hydrolysis as predicted by the model and for cocoa samples treated with water and enzyme for 6 hours, before and after roasting.

Sample	Degree of hydrolysis (%)
Observed ^a	20,0
Predicted by the model	19,0
Control ^b	17,4
Control roasted	13,7
Enzyme treated	18,1
Enzyme treated roasted	12,2

a - experiment 6, table 2

b - treated with water

**Figure 3** - Percentage of responses for chocolate flavour (a) and bitter taste (b) for cocoa products formulated with enzyme treated cocoa (■) and control (□) (1=much too weak, 5=just right, 9=much too strong).

CONCLUSION

The use of a proteolytic enzyme was useful in improving cocoa flavour precursors and affected the flavour perception in their products. The difficulty to establish a model could be suppressed

by changing the process in such a way to enhance the access of enzyme to substrate and a better heat transfer.

ACKNOWLEDGMENT

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RESUMO

Foi feita uma hidrólise da proteína dos nibs de cacau usando-se uma protease para verificar a formação de precursores e o efeito sobre o sabor do cacau. Um desenho experimental foi usado para verificar a influência da temperatura (30 a 70 °C) e razão enzima : substrato [E/S] (97,5 a 1267,5 U g⁻¹ de proteína). O grau de hidrólise % (%DH) foi afetado principalmente pela [E/S], tendo sofrido um aumento de 4 vezes (de 5 para 20 %) após 6 horas de tratamento. Durante a torração dos nibs houve um consumo maior dos compostos de hidrólise na amostra tratada com protease em comparação com o controle, indicando a participação desses compostos na reação de Maillard. Foi observado um aumento na percepção do sabor de chocolate e do gosto amargo em um produto formulado com o cacau tratado com a protease.

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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,

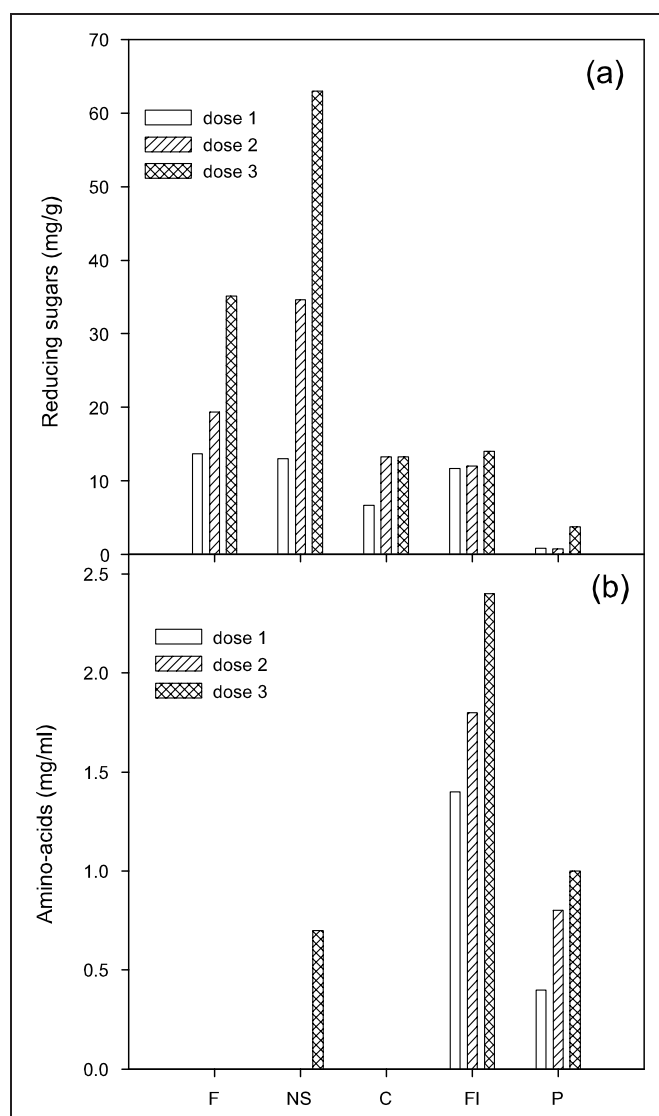


Figure 1 – (a) Reducing sugar and (b) amino acid contents of cocoa powder treated with different enzymes. Doses 1, 2, and 3 were, respectively, 0.25%, 2.5%, and 25% w/w (enzyme/substrate) for F (Fungamyl), NS (NS44045), and C (Celluclast); 1.2%, 2.4%, and 12% w/w for FI (Flavourzyme); 0.2%, 0.4%, and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

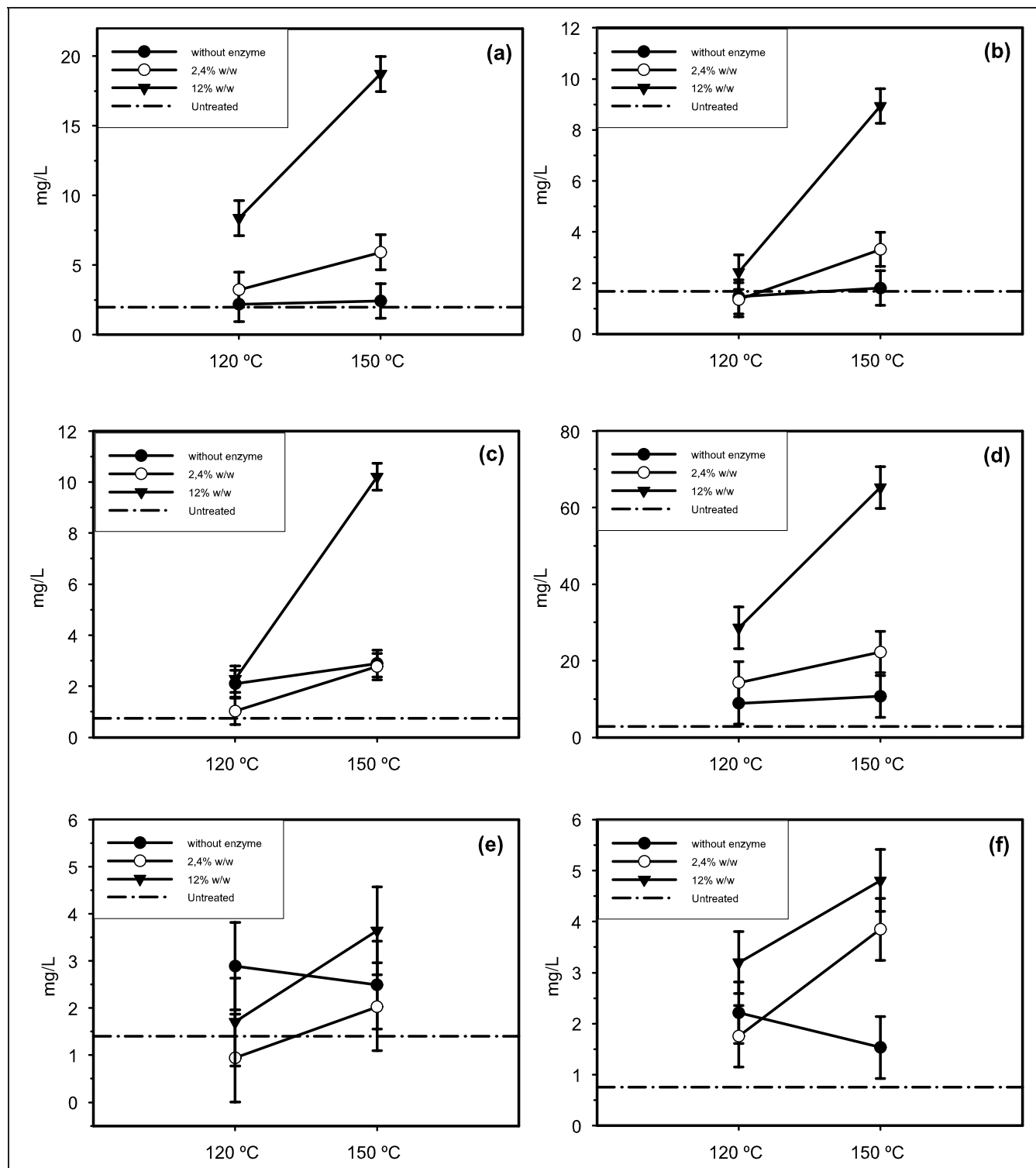


Figure 2—Concentrations of 2-methylpyrazine (a), 2,3-dimethylpyrazine (b), 2,5-dimethylpyrazine (c), 2,6-dimethylpyrazine (d), 2,3,5-trimethylpyrazine (e), and 2,3,5,6-tetramethylpyrazine (f) in cocoa powder extract treated with 0%, 2.4%, or 12% w/w Flavourzyme and dried at 120 °C or 150 °C. Error bars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

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