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**ÁREA DE CONCENTRAÇÃO**

**Desenvolvimento de Processos Químicos - ACDPQ**

# DESENVOLVIMENTO DE PROCESSO ENZIMÁTICO PARA REDUÇÃO DE SEDIMENTOS EM EXTRATOS DE CAFÉ SOLÚVEL

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Tese de Doutorado apresentada à Faculdade de Engenharia Química como parte dos requisitos exigidos para a obtenção do título de Doutor em Engenharia Química.

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**SUMÁRIO**

## NOMENCLATURA

| | |
|---|---|
| AcGal | Ácido galacturônico |
| Ara | Arabinose |
| $CO_2$ | Dióxido de Carbono |
| Da | Daltons |
| Gal | Galactose |
| Gli | Glicose |
| GPC | Cromatografia de Permeação em Gel |
| $H_2SO_4$ | Ácido Sulfúrico |
| HCl | Ácido Clorídrico |
| HPAEC | Cromatografia de Troca Aniônica |
| HPLC | Cromatografia Líquida de Alta Eficiência |
| Log M | Logaritmo da Massa Molecular |
| Man | Manose |
| PAD | Detector de Amperometria Pulsada |
| Ram | Ramnose |
| RI | Índice de Refração |
| TFA | Ácido trifluoracético |
| UV | Ultra-violeta |
| Xil | Xilose |

## RESUMO

Polissacarídeos são os principais constituintes do café verde, torrado e solúvel, sendo principalmente galactomananos e arabinogalactanos. Um aspecto importante em relação a estes polissacarídeos é a insolubilidade, umas das possíveis razões para a formação de sedimentos na produção de café solúvel, reduzindo o rendimento do processo. Em face disto, o objetivo deste trabalho foi investigar a redução de sedimentos em extrato de café pela ação de enzimas. Diversas preparações comerciais de pectinases foram selecionadas e suas diferentes atividades determinadas. Rohapect B1L apresentou a maior atividade de mananase. Adicionalmente, a maioria das preparações estudadas apresentou outras atividades enzimáticas, tais como mananase, endoglucanase, xilanase, além da atividade atestada pelos fabricantes. Frações solúveis e insolúveis do extrato de café e sedimento foram preparadas e suas composições determinadas. Os principais componentes do extrato de café e sua fração insolúvel foram carboidratos e proteínas, enquanto que a fração insolúvel do sedimento apresentou também uma fração significativa de lignina. Pela análise dos carboidratos, galactose, arabinose e manose foram os principais monossacarídeos obtidos após hidrólise ácida, confirmando a presença de galactomananos e arabinogalactanos. Galactomanano com baixo grau de ramificação foi o principal polissacarídeo encontrado nas frações insolúveis. Biopectinase CCM, Rohapect B1L, Pectinase 444L e Galactomananase ACH foram as preparações enzimáticas mais eficientes na redução de sedimento do extrato de café, sendo Rohapect B1L e Galactomananase ACH as mais viáveis, cujas concentrações ótimas foram 0,3 e 0,1 $mg_{proteína}/g_{substrato}$, respectivamente. A massa molecular média dos components do extrato de café foi 18 kDa, a qual decresceu 6,6% após hidrólise com Rohapect B1L. O principal açúcar liberado por esta enzima foi a manose, sugerindo sua ação sobre as cadeias de mananos do café, devido a alta atividade de mananase. Os extratos hidrolisados pelas preparações enzimáticas Rohapect B1L e Galactomananase ACH foram avaliados sensorialmente. Provadores treinados verificaram uma pequena a moderada diferença entre os extratos hidrolisados e o controle; entretanto, o tratamento enzimático não alterou a aceitação das amostras por parte dos consumidores. Os resultados mostraram boas perspectivas para a utilização de enzimas no processo de redução de sedimentos em extratos de café solúvel.



**Palavras-chave:** *extratos de café; sedimentos; hidrólise enzimática; polissacarídeos.*

**ABSTRACT**

Polysaccharides are the main components of green, roasted and soluble coffee, and they are mainly galactomannans and arabinogalactans. An important aspect about these polysaccharides is the insolubility, possibly one of the reasons for the sediments formation in the production of instant coffee, reducing the yield of the process. Thus, the aim of this work was to investigate the reduction of sediments in coffee extract by the action of enzymes. Several commercial preparations of pectinases were selected and their different activities were determined. Rohapect B1L presented the highest mannanase activity. Most of the commercial pectinases assayed presented other enzyme activities, like mannanase, endoglucanase, xylanase, besides the activity stated by the manufacturers. Soluble and insoluble fractions from coffee extract and sediment were prepared and their chemical composition determined. The main components of extract and its insoluble fraction were carbohydrates and proteins, while the insoluble fraction of sediment presented also a significant amount of lignin. Based on the carbohydrate analysis, galactose, arabinose and mannose were the main monosaccharides obtained after acid hydrolysis, confirming the presence of galactomannans and arabinogalactans. Galactomannan with low degree of branching was found to be the main polysaccharide of the insoluble fractions. Biopectinase CCM, Rohapect B1L, Pectinase 444L and Galactomannanase ACH were found to be the most effective enzyme preparations on the sediment reduction of coffee extract, being Rohapect B1L and Galactomannanase ACH the more viable ones, which optimum concentrations were 0.3 and 0.1 $mg_{protein}/g_{substrate}$, respectively. The average molecular weight of the coffee extract components was 18 kDa, which decreased 6.6% after hydrolysis with Rohapect B1L. The main sugar released by this enzyme preparation was mannose, suggesting its action on coffee mannans, due to the high activity of mannanase. The extracts hydrolyzed by Rohapect B1L and Galactomannanase ACH were evaluated for the sensory quality. Trained panelists indicated a small to moderate difference between the hydrolyzed extracts and the control; however, the enzymatic treatment did not alter the consumers' acceptance of the coffee extract. The results showed good perspectives for the use of enzymes in reducing sediments in coffee extracts.

**Keywords:** *coffee extracts; sediments; enzymatic hydrolysis; polysaccharides.*

# INTRODUÇÃO

O café é uma das bebidas mais consumidas no mundo como resultado de suas características sensoriais e seu efeito estimulante. Devido a este elevado consumo, a cadeia produtiva do café está entre os dez maiores setores da economia brasileira.

Em termos de composição química, os polissacarídeos constituem 50% da massa seca do café verde, sendo também importantes constituintes do café torrado e solúvel. Os principais polissacarídeos presentes no café são os arabinogalactanos, os galactomananos e a celulose.

Durante a etapa de extração de sólidos do café torrado, materiais insolúveis também são extraídos, formando sedimentos. Como conseqüência, ocorre a redução do rendimento do processo e uma etapa adicional para a retirada desses materiais é necessária. Além disso, nesta etapa adicional, geralmente constituída por uma operação de centrifugação, ocorre a perda de características importantes à qualidade da bebida, uma vez que óleos essenciais e aromas podem ser carreados do extrato.

Enzimas têm sido empregadas em diversas aplicações, incluindo a degradação de parede celular de plantas. Desta forma, a utilização de enzimas que atuam sobre os materiais insolúveis presentes em extratos de café é uma alternativa para a redução de sedimentos, o que refletiria em um maior rendimento do processo, além de facilitar e/ou eliminar algumas etapas no processamento do café solúvel.

Em face disto, esta Tese de Doutorado Direto teve por objetivo desenvolver um processo enzimático para a redução de sedimentos em extratos de café solúvel.

O trabalho foi distribuído em sete capítulos, sendo o CAPÍTULO I constituído pela revisão bibliográfica, que teve por objetivo investigar o estado da arte do tema desenvolvido.

Primeiramente, diversas preparações comerciais de enzimas foram selecionadas a fim de investigar quais poderiam atuar sobre os sedimentos do café. Diferentes atividades celulolíticas, hemicelulolíticas e pectinolíticas presentes em cada complexo foram determinadas, uma vez que alguns produtos denominados como pectinases continham alta atividade de mananase, podendo ser também empregadas no processo em questão. Por

outro lado, este mesmo estudo possibilitou a ampliação do objetivo original desta etapa do trabalho, uma vez que a caracterização das atividades das diferentes preparações enzimáticas estudadas elucidou a possibilidade de aplicação desses biocatalisadores em outros tipos de hidrólise enzimática de interesse comercial (CAPÍTULO II).

No CAPÍTULO III o extrato de café e o sedimento foram separados em suas frações solúveis e insolúveis, as quais foram caracterizadas quanto à composição. A redução de sedimentos pela ação de diferentes preparações comerciais de enzimas foi avaliada.

Uma vez determinadas as melhores preparações enzimáticas que atuam no processo de redução de sedimento, foram avaliados a massa molecular, o perfil de monossacarídeos e a qualidade sensorial dos extratos hidrolisados, comparando-os ao extrato não hidrolisado (CAPÍTULO IV).

No CAPÍTULO V uma análise econômica preliminar foi realizada visando a utilização de enzimas no processo industrial de produção de café solúvel.

Finalmente, nos CAPÍTULOS VI e VII são apresentadas as conclusões e as sugestões para trabalhos futuros, respectivamente.

# OBJETIVOS

## Objetivo geral

- ✓ Desenvolver um processo enzimático para a redução de sedimentos em extratos de café.

## Objetivos específicos

- ✓ Determinar a composição química das frações solúveis e insolúveis do extrato e sedimento;

- ✓ Selecionar preparações comerciais de enzimas que possam atuar na redução de sedimentos e determinar suas atividades;

- ✓ Avaliar o efeito de cada preparação enzimática na redução do sedimento e definir os melhores produtos e condições para o tratamento enzimático;

- ✓ Determinar a massa molecular dos componentes do extrato antes e após tratamento enzimático;

- ✓ Determinar o perfil de monossacarídeos do extrato antes e após hidrólise enzimática;

- ✓ Avaliar sensorialmente os extratos hidrolisados pelas melhores preparações de enzimas, comparando-os ao extrato não hidrolisado;

- ✓ Realizar uma análise econômica preliminar a fim de investigar a viabilidade da utilização de enzimas no processo de produção de café solúvel.

# *CAPÍTULO I*

# REVISÃO BIBLIOGRÁFICA

## 1. CAFÉ

### 1.1. Origem e expansão cafeeira no Brasil: importância sócio-econômica

O café é originário das regiões montanhosas das províncias de Kaffa-Lima, Sidamos e Harar na Etiópia (antiga Abissínia), no continente africano. Foi introduzido na Arábia no século quinze e a partir do século seguinte a técnica de infusão do café torrado se estabeleceu em muitas partes do mundo islâmico. Da Arábia o café foi levado para o Ceilão. O seu cultivo na Índia teve início por volta do ano de 1600 e posteriormente, em 1696, em Java, onde se constituiu as primeiras lavouras extensivas. A primeira casa de café inglesa data de 1650 e foi estabelecida em Oxford. Em 1706 foram ofertadas ao Jardim Botânico de Amsterdã algumas mudas de café procedentes de Java, sendo que em 1714 essas mudas foram levadas para as Américas, primeiramente à Guiana Holandesa (Suriname) e anos mais tarde à Ilha Martinica, de onde se estendeu aos outros países da América do Sul e América Central (SMITH, 1985; TEIXEIRA *et al*., 1974). Segundo os autores, o café foi introduzido no Brasil no ano de 1727 em Belém do Pará. Do Pará, o café foi para o Ceará, Bahia e Rio de Janeiro, expandindo-se então pela Serra do Mar e alcançando o Vale do Paraíba em São Paulo. Em 1830 o Brasil já era o principal produtor mundial de café. Mais tarde o café chegou ao Oeste, Nordeste e Alta Paulista, à Alta Sorocabana e também ao estado do Paraná.

O café é um dos mais importantes produtos da balança comercial brasileira, sendo responsável pela entrada de divisas da ordem de US$ 2,0 bilhões em 2004, US$ 2,9 bilhões em 2005, US$ 3,3 bilhões em 2006 e US$ 3,9 bilhões em 2007 (MAPA, 2008).

Atualmente o café é cultivado na América Latina, África, Ásia e Oceania. Os estados brasileiros notoriamente produtores de café são Minas Gerais, Espírito Santo, São Paulo, Paraná, Bahia e Rondônia (ABIC, 2008; CACIQUE, 2007; COFFEE BREAK, 2007).

Em 2007 o Brasil produziu cerca de 34 milhões de sacas de café, respondendo por aproximadamente 29% da produção mundial, seguido pelo Vietnã e Colômbia, com 14 e 11%, respectivamente. Assim, o Brasil é o maior produtor mundial de café, sendo que quase a totalidade (80%) dos cafeeiros plantados no Brasil é da espécie *Coffea arabica* (ABIC, 2008; ICO, 2008).

De acordo com a Associação Brasileira da Indústria de Café (ABIC, 2008), em 2007 foram exportados 28 milhões de sacas de café. Destas, 24,8 milhões de sacas correspondem ao café verde, 3,1 milhões ao café solúvel e 109 mil sacas correspondem ao café torrado. Isto demonstra a pequena quantidade de café industrializado exportado pelo Brasil e o enorme potencial para aumentar essa industrialização, o que geraria mais receita para a nação brasileira.

O café solúvel tem sido vendido nos Estados Unidos desde 1909, porém a produção em escala comercial teve seu início durante a Segunda Guerra Mundial, crescendo significativamente desde 1955 (SIVETZ & DESROSIER, 1979; SMITH, 1985). A qualidade do café solúvel depende não somente da matéria-prima empregada, mas também dos cuidados dispensados a ela (desde seu plantio e colheita) e do processamento industrial, que varia de acordo com a indústria e exigência do mercado.

Os principais países importadores do café solúvel brasileiro são: Estados Unidos, Rússia, Alemanha, Reino Unido e Japão (ABIC, 2008; ABICS, 2007).

A tendência dos dias atuais é a utilização de produtos de preparo mais simples e mais rápido, como é o caso do café solúvel, cujo consumo tem apresentado taxas surpreendentes de crescimento. Entre 1994 e 2004 o consumo mundial de café solúvel teve um crescimento de 16%, o qual deve continuar aumentando tanto pelas razões já mencionadas como também pela expansão do consumo de café em mercados emergentes, onde o café solúvel é freqüentemente a porta de entrada em substituição ao chá, como já ocorreu no passado em países como Japão, Rússia e Reino Unido. Mesmo no Brasil,

tradicional reduto do café torrado e moído, o consumo doméstico do solúvel vem aumentando à expressiva taxa de 8% ao ano (ABICS, 2007).

## 1.2. A planta do café

O cafeeiro é uma planta arbustiva pertencente ao grupo das plantas Fanerógamas, classe *Angiosperma*, subclasse *Dicotiledônea*, ordem *Rubiales*, família *Rubiaceae* e gênero *Coffea*. Duas são as espécies do gênero *Coffea* mais difundidas no mundo: *Coffea arabica* (café arábica) e *Coffea canephora* (café robusta). Dentre as espécies do gênero, *Coffea arabica* tem se destacado durante toda a história, responsável por cerca de 80% da produção mundial de café, produzindo café da mais fina qualidade. O café robusta é considerado de pior qualidade pela bebida que proporciona, mas apresenta alta produtividade e maior porcentagem de sólidos solúveis que o arábica, sendo utilizado principalmente na formação de ligas na indústria de café solúvel. Outras duas espécies, *Coffea liberica* e *Coffea dewevrei*, podem ser encontradas, mas possuem menor importância comercial. No Brasil é conhecido também o café conilon, variedade do robusta e correspondente a espécie *Coffea canephora var. Kouillou* (SIVETZ & DESROSIER, 1979; SMITH, 1985; TAGLIARI, 2003; TEIXEIRA *et al*., 1974).

O cafeeiro se desenvolve melhor em clima quente e úmido, típico das regiões tropicais, podendo alcançar naturalmente 10 metros, mas é mantido entre 2 e 3 metros para facilitar a colheita dos frutos (SMITH 1985; TAGLIARI, 2003).

O fruto maduro do café é comumente denominado “cereja”. A Figura 1 apresenta um corte longitudinal do fruto com as diferentes camadas de tecido, as quais são: casca, chamada de exocarpo ou epicarpo; polpa ou mesocarpo, que constitui uma zona facilmente desintegrável do fruto; pergaminho ou endocarpo, que é uma camada dura de fibras que envolve cada uma das sementes; espermoderma ou película prateada, que recobre o grão; e o grão (amêndoa), composto de endosperma e embrião. O endosperma, além de água, contém proteína, óleo, açúcar, dextrina, pentosanas, celulose, ácido clorogênico, substâncias minerais, etc. O embrião é pequeno, se localiza na base do endosperma e tem folhas cotiledonares cordiformes e justapostas. O exocarpo, mesocarpo e endocarpo formam o pericarpo do fruto, e a película prateada mais o grão formam a semente. Uma

"cereja" do café pode conter um ou dois grãos e raramente possui mais que esta quantidade, a qual é determinada pela fecundação dos dois óvulos (JARDINE, 1991; PRODOLLIET *et al*., 1995b; TEIXEIRA *et al*., 1974)

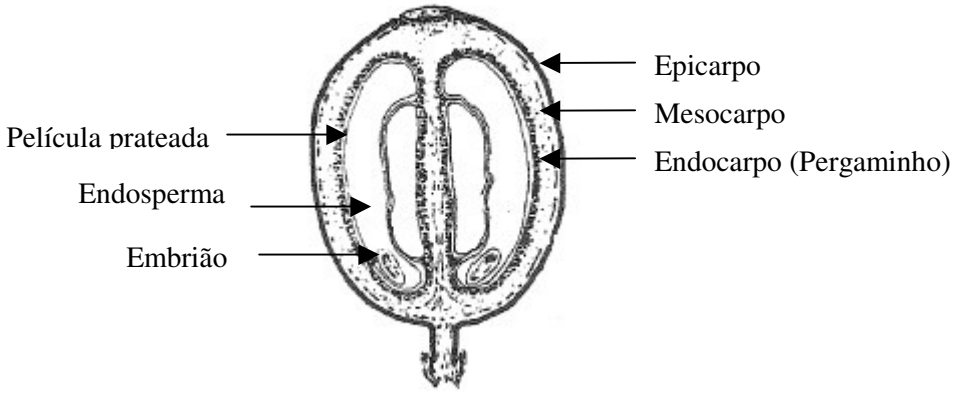


**Figura 1. Corte longitudinal do fruto do café.**

### 1.3. Processamento do fruto do café

O café é um produto agrícola que requer um cuidado especial em seu preparo a fim de que sejam preservadas as suas características. Existem dois métodos distintos para o processamento do fruto do café: processo por via úmida e processo por via seca. Pela via úmida são obtidos os cafés despolpados ou lavados, os quais possuem a semente envolta no pergaminho somente. Este processamento resulta numa bebida menos encorpada e mais ácida devido à fermentação nos tanques de lavagem. O processo por via seca é geralmente mais econômico e praticado em áreas de clima quente e seco, obtendo-se o café integral ou em coco, que são os grãos com todos os seus constituintes. Esta técnica consiste em se proceder a secagem das cerejas após a colheita, feita naturalmente por irradiação solar direta ou utilizando-se secadores artificiais. Praticamente todo o café produzido no Brasil é processado por via seca (JARDINE, 1991; SMITH, 1985; TAGLIARI, 2003).

## 1.4. Produção de café solúvel

A essência da manufatura do café solúvel consiste em produzir um extrato de café pela torrefação, moagem e extração dos grãos torrados, com a posterior remoção da água com o mínimo prejuízo à qualidade da bebida, sendo então envasado para o mercado consumidor. A Figura 2 apresenta o fluxograma da produção de café solúvel.

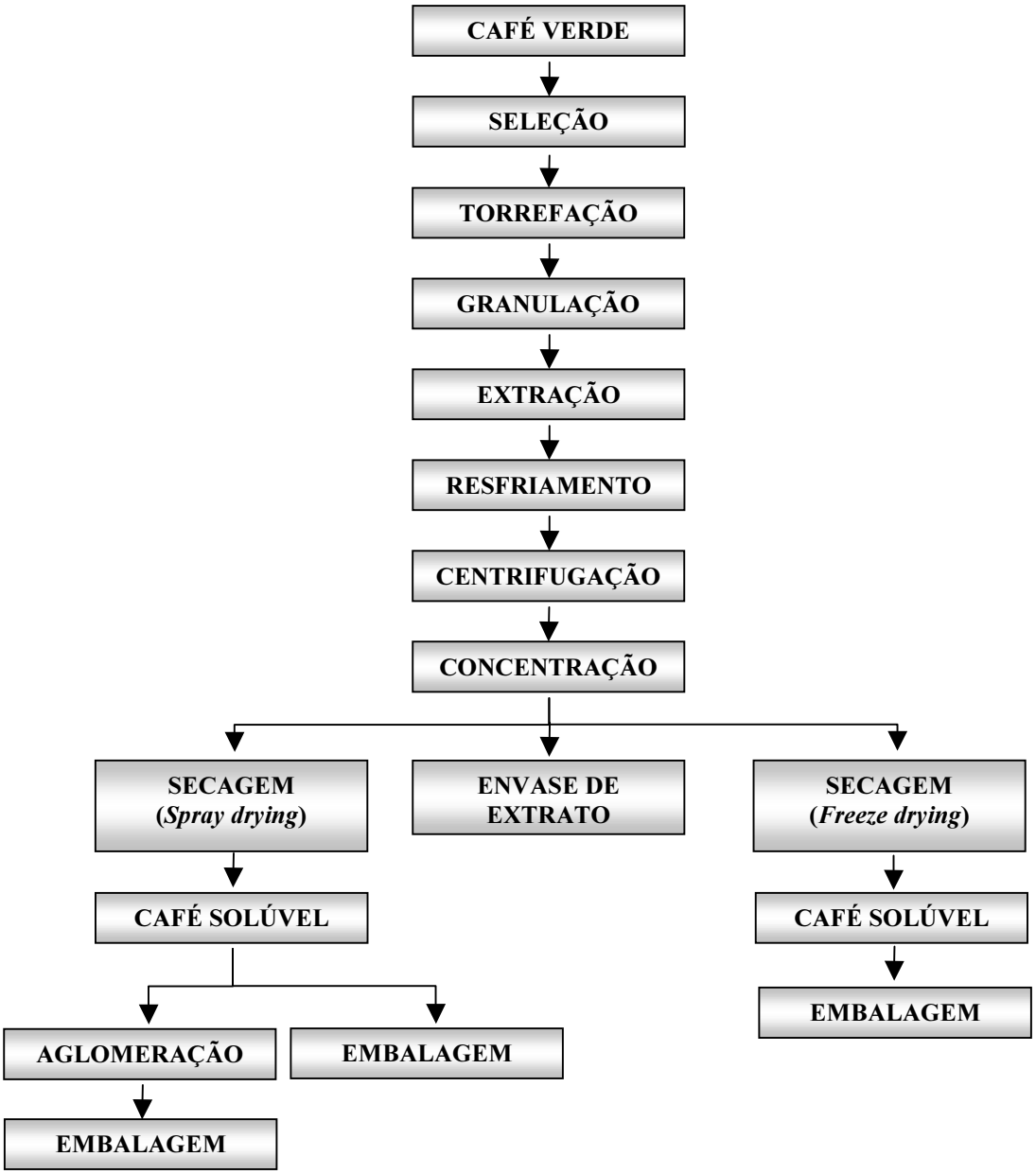


**Figura 2. Fluxograma da produção de café solúvel.**

Primeiramente, os grãos de café verde passam por uma seleção para a retirada de contaminantes como pedras, corpos metálicos, grãos defeituosos, folhas e outros. Os grãos beneficiados passam pelo processo de torrefação, responsável pelo desenvolvimento do aroma, cor e sabor característicos do café. Este processo consiste na aplicação de calor aos grãos, os quais devem ser mantidos em movimento até atingir a coloração desejada, quando são rapidamente resfriados por uma corrente de ar. Uma mistura (ou *blend*) de cafés pode ser feita antes ou após a torrefação, mas geralmente os *blends* são feitos com os cafés verdes. A composição desta mistura vai depender do mercado consumidor, preço e qualidade desejados. Um café com um baixo grau de torra possui maior acidez, menor corpo e possivelmente menor aroma. Com o aumento do grau de torra a acidez diminui, o corpo da bebida aumenta e o aroma aumenta até o ponto de torná-lo amargo. Durante o processo de torra os grãos passam por dois estágios de transformação. No primeiro estágio, cerca de 80% do tempo total, os grãos passam de verde a um marrom claro, e no segundo estágio ocorre uma reação de pirólise, com um inchaço dos grãos, cujos produtos são dióxido de carbono, aldeídos, cetonas, éteres, ácido acético, metanol, entre outros. Na torrefação, os grãos de café sofrem algumas transformações: escurecimento devido a reações de *Maillard* e caramelização; perda de peso por vaporização da água e pirólise; aumento de volume devido à liberação de $CO_2$ pela pirólise; diminuição do pH de 6 para 5 em função da transformação de carboidratos a ácidos orgânicos; redução de ácido clorogênico; degradação parcial de amidos e dextrinas e formação de compostos aromáticos importantes. O café torrado é então granulado, ou seja, quebrado em partículas menores a fim de obter maior eficiência na extração. Geralmente, partículas muito finas impedem a passagem do licor de café nas colunas de extração, não podendo também ser demasiadamente grandes, o que causa uma queda no rendimento da extração. Posteriormente à granulação, os grãos de café torrado são percolados com água em altas temperaturas e sob pressão, em extratores denominados percoladores ou colunas de extração. Estas colunas são carregadas com café torrado e moído e a extração se processa em contra corrente, ou seja, o extrato obtido na primeira coluna alimenta a segunda, a terceira e assim sucessivamente. Esta forma de extração em contra corrente, com temperaturas entre 100 e 190°C e sob altas pressões, permite o alto rendimento de extração e a obtenção de uma bebida de ótima qualidade. Ao final do processo o extrato se encontra a uma temperatura de 95 a 105°C e um teor de sólidos solúveis de 10 a 25°Brix. A

temperatura do extrato deve então ser reduzida rapidamente para evitar a volatilização dos compostos aromáticos, não influenciando assim na qualidade da bebida. A extração progressiva de sólidos solúveis causa também a retirada de material insolúvel do café torrado, que é o sedimento presente no extrato de café. A não solubilização deste material reflete diretamente no rendimento do processo. A etapa de centrifugação não era realizada até 1960, porém, atualmente a remoção destas partículas é uma etapa comum e essencial no processamento do extrato. A retirada de sólidos insolúveis na etapa de centrifugação também pode evitar problemas operacionais, como por exemplo, garantir que não haja entupimento nos bicos dos secadores. Entretanto, durante a centrifugação alguns compostos importantes, tais como óleo de café e aromas, podem ser retirados do extrato, acarretando perda na qualidade da bebida. Assim, alternativas que tornem toda ou parte destas partículas solúveis refletiriam diretamente em um maior rendimento do processo, além de facilitar ou eliminar etapas de processamento necessárias para a retirada deste material. A próxima etapa consiste na concentração do extrato pela remoção de água, cujo processo pode ser conduzido por duas formas: *spray* ou *freeze drying*. No primeiro processo o extrato é aquecido e evaporado sob vácuo a 60°C. No processo chamado *freeze drying* parte da água é congelada e os cristais de gelo formados são separados por prensagem; o extrato obtido é considerado de melhor qualidade, porém este processo é mais sofisticado e de maior custo. O extrato concentrado possui um teor de sólidos em torno de 40°Brix. Da mesma forma que a concentração, o processo de secagem também pode ser realizado de duas formas: *spray drying* ou *freeze drying*. No processo por *spray drying* o extrato é desidratado por atomização em co-corrente com ar superaquecido numa torre de secagem. O extrato concentrado, ao atingir o bico de pulverização da torre, é transformado em gotículas esféricas de menos de 1,0 mm de diâmetro, e em contato com o ar aquecido a temperatura acima de 300°C, sua água é evaporada quase instantaneamente, resultando em microesferas de sólidos solúveis de café com baixa densidade aparente. No processo por *freeze drying* o extrato é congelado e triturado, e as partículas congeladas são liofilizadas, que nada mais é que a sublimação da água sob vácuo, resultando numa bebida de qualidade superior e aroma mais intenso. Após a secagem o café solúvel pode então passar pela etapa de aglomeração, para a formação de grânulos, sendo finalmente envasado, de forma a prevenir a absorção de umidade, que pode levar a solidificação, como também acelerar a deteriorização. O extrato líquido de café pode também ser envasado e comercializado

para a produção de outras bebidas a base de café (IGUAÇU, 2007; JARDINE, 1991; SIVETZ & DESROSIER, 1979; SMITH, 1985).

## 2. COMPOSIÇÃO DO CAFÉ

### 2.1. Café verde

A composição química do café verde depende da espécie e da variedade em questão, e também de outros fatores como solo, clima, regime de chuvas, altitude do terreno, práticas da agricultura, grau de maturação e condições de armazenamento (COFFEE BREAK, 2007; SMITH, 1985). Segundo OOSTERVELD *et al*. (2003a), o café verde é composto basicamente por carboidratos, lipídeos e proteínas, como apresentado na Tabela 1. Dentre os açúcares presentes, manose representa 44%, galactose 23%, arabinose 12%, glicose 15%, ramnose 1% e xilose 2%. O restante consiste em ácidos clorogênicos, minerais, lignina, aminoácidos, ácidos alifáticos, trigonelina e cafeína (CLIFFORD, 1975).

**Tabela 1. Composição dos grãos de café arábica verde e torrado (% m/m)**

| | **Café verde** | **Café torrado** |
|---|---|---|
| Carboidratos | 54,8 | 48,1 |
| *Ramnose* | *1* | *1* |
| *Arabinose* | *12* | *6* |
| *Xilose* | *2* | *0* |
| *Manose* | *43* | *51* |
| *Galactose* | *23* | *21* |
| *Glicose* | *15* | *16* |
| Ácido urônico | *4* | *4* |
| Lipídeos | 11,3 | 15,9 |
| Proteínas | 5,8 | 6,8 |
| Total | 71,9 | 70,8 |

Fonte: OOSTERVELD *et al*. (2003a)

Assim, 80% dos carboidratos do café verde estão presentes como galactomananos e arabinogalactanos. Os galactomananos consistem em uma cadeia principal de manoses unidas por ligações glicosídicas 1→4 e ramificadas com unidades de galactose no C-6, com variado grau de ramificação, enquanto que os arabinogalactanos consistem em uma cadeia principal de galactoses unidas por ligações 1→3 e ramificadas no C-6 com cadeias laterais contendo resíduos de arabinose e/ou galactose (BRADBURY & HALLIDAY, 1990; FISCHER *et al*., 2001; NAVARINI *et al*., 1999; NUNES *et al*., 2006; OOSTERVELD *et al*., 2003a). Os arabinogalactanos estão, geralmente, ligados covalentemente a proteínas (NAVARINI *et al*., 1999; REDGWELL *et al*., 2002a; REDGWELL *et al*., 2005; REDGWELL & FISCHER, 2006). As Figuras 3 e 4 apresentam as estruturas de um galactomanano e um arabinogalactano, respectivamente.

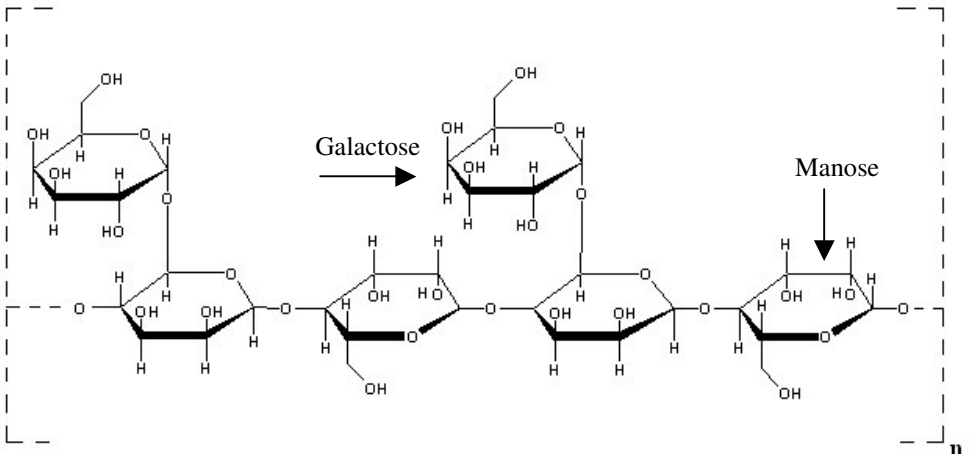


**Figura 3. Estrutura de um Galactomanano.**

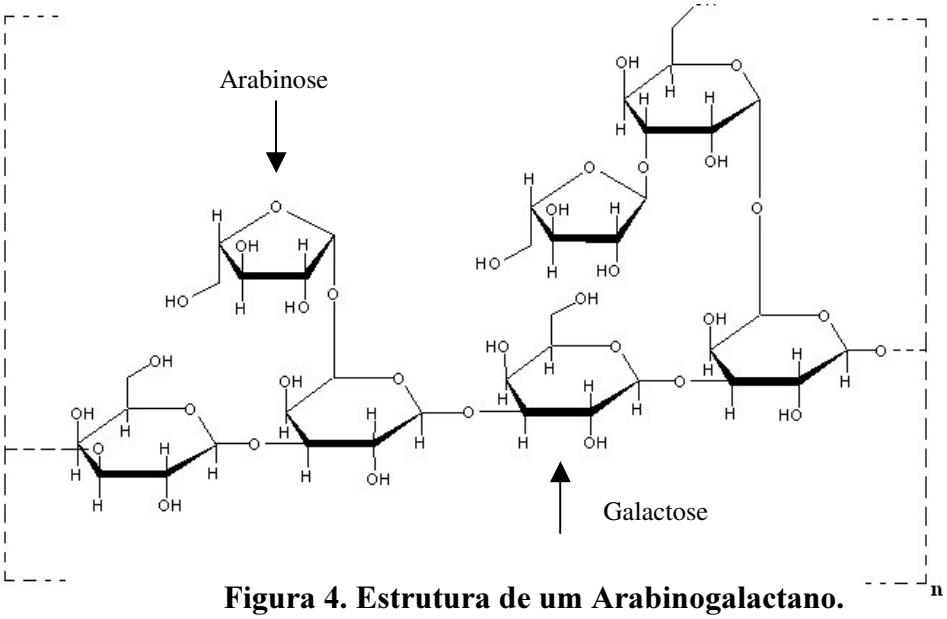


**Figura 4. Estrutura de um Arabinogalactano.**

Polissacarídeos, em geral, são heterogêneos considerando suas massas moleculares e grau de ramificação, o qual está relacionado às suas propriedades físico-químicas (NUNES & COIMBRA, 2001; OOSTERVELD *et al*., 2003a).

Os mananos são encontrados em endospermas de sementes de espécies de monocotiledôneas (por exemplo, *Phoenix dactylifera* e *Phytelephas macrocarpa*) e dicotiledôneas (*Coffea arabica*). Quanto menos ramificados, maior a indicação de que a função biológica está relacionada com a dureza e proteção do embrião. Por outro lado, quanto maior o grau de ramificação, mais solúvel o polissacarídeo. Abaixo de 10% de ramificação com galactose, os mananos (considerados lineares) tornam-se insolúveis e precipitam rapidamente em solução aquosa. Os mananos lineares, portanto, apresentam alto grau de interatividade intermolecular, formando cristais na parede celular, conferindo a dureza e reduzindo a solubilidade dos mesmos (BUCKERIDGE *et al*., 2000). REDGWELL & FISCHER (2006) também relatam que a solubilidade dos galactomananos do café aumenta com o grau de ramificação.

FISCHER *et al*. (2001) analisaram os polissacarídeos dos grãos de café arábica e robusta. Os autores verificaram diferenças na solubilidade, particularmente em relação aos arabinogalactanos, sendo os polissacarídeos do café robusta mais facilmente extraíveis que o grão de café arábica. Quantidades de arabinogalactanos e galactomananos mais solúveis e mais ramificados foram encontrados no café robusta. Segundo os autores, a principal dificuldade na caracterização de mananos ou galactomananos é a alta proporção de polímeros insolúveis. Esta dificuldade de solubilização indica uma associação entre arabinogalactanos, galactomananos e celulose, que pode ser causada por ligações não covalentes entre diferentes polissacarídeos, mas também pela possibilidade de haver ligações covalentes e, portanto, a presença de uma nova classe de polissacarídeos ainda não determinada.

## 2.2. Café torrado

A torrefação é uma etapa essencial na produção de café, pois há consideráveis mudanças na composição do café verde, ou seja, alguns componentes são degradados e

outros interagem para formar produtos complexos. O café é, provavelmente, um dos alimentos que mais se altera durante o processamento, e isto é refletido também em suas características sensoriais (TRUGO, 1985).

O processo de torra induz a uma mudança na quantidade e estrutura dos polissacarídeos em relação ao café verde (NUNES & COIMBRA, 2001; NUNES & COIMBRA, 2002a, 2002b; OOSTERVELD *et al*., 2003a, 2003b; REDGWELL *et al*., 2002b). Segundo OOSTERVELD *et al*. (2003a), a torrefação resulta numa perda de 8% da massa seca do grão de café. Como descrito na Tabela 1, o grão de café torrado é composto em média por 48% de carboidratos, 16% de lipídeos e 7% de proteínas. A diminuição do teor de carboidratos se deve à conversão de parte dos açúcares a produtos de reações de *Maillard* e reações de pirólise. Os lipídeos estão em maior quantidade provavelmente como resultado da degradação dos carboidratos durante a torra. As quantidades relativas de arabinose, xilose e galactose diminuem com a torra, enquanto que as quantidades relativas de manose e glicose aumentam devido à queda dos outros açúcares. Arabinose, ácido galacturônico e galactose são bastante sensíveis à degradação durante a torra, enquanto que manose e glicose são menos sensíveis.

Os galactomananos e arabinogalactanos presentes no café torrado possuem menos ramificações do que aqueles presentes no café verde, ou seja, o grau de ramificação decresce durante a torra, podendo resultar num decréscimo na solubilidade desses polissacarídeos. Entretanto, os polissacarídeos do café torrado possuem menor massa molecular que os polissacarídeos do café verde, aumentando assim a extrabilidade dos arabinogalactanos, pectinas e galactomananos (NUNES & COIMBRA, 2001; OOSTERVELD *et al*., 2003a, 2003b; REDGWELL *et al*., 2002b).

### 2.3. Extrato de café

Os polissacarídeos solúveis em água extraídos do café torrado são os principais componentes do extrato de café. Eles são importantes na retenção de compostos voláteis e contribuem para a viscosidade e a característica sensorial conhecida como “corpo” da bebida (DÍAZ-RUBIO & SAURA-CALIXTO, 2007; THALER, 1979).

Alguns estudos têm examinado a estrutura química dos polissacarídeos obtidos após extração do café torrado com água quente. Nestes trabalhos polissacarídeos puderam ser extraídos, porém a maior parte foi encontrada nos resíduos, indicando que a maioria dos polissacarídeos está firmemente associada à parede celular. OOSTERVELD *et al*. (2003b) realizaram extrações aquosas de café torrado a 90 e 170°C, por 60 e 20 minutos respectivamente, e as composições dos extratos estão descritas na Tabela 2.

**Tabela 2. Composição do extrato aquoso do café arábica após extração a 90°C (60 min) e 170°C (20 min)**

| Condições de Extração | % Carboidratos | | | | | | | Açúcares (% m/m) | Rendimento (% m/m) |
|---|---|---|---|---|---|---|---|---|---|
| | Ram | Ara | Xil | Man | Gal | Gli | AcGal | | |
| 90°C – baixo grau de torra | 2 | 13 | 0 | 39 | 38 | 1 | 7 | 51 | 9 |
| 90°C – alto grau de torra | 1 | 8 | 0 | 51 | 33 | 2 | 5 | 51 | 11 |
| 170°C - baixo grau de torra | 0 | 8 | 0 | 16 | 67 | 0 | 8 | 65 | 8 |
| 170°C – alto grau de torra | 0 | 5 | 0 | 40 | 48 | 1 | 7 | 56 | 7 |

Ram: ramnose; Ara: arabinose; Xil: xilose; Man: manose; Gal: galactose; Gli: glicose; Acgal: Ácido galacturônico.
Fonte: OOSTERVELD *et al*. (2003b)

Segundo os autores, a extração dos grãos de café torrado a 90°C a um baixo grau de torra produziu um extrato de polissacarídeos contendo manose (39%), galactose (38%), arabinose (13%) e ácido galacturônico (7%). A razão manose:galactose foi aproximadamente 1. A composição obtida indica a presença de galactomananos, arabinogalactanos e pectinas. Com o aumento do grau de torra, há uma queda na quantidade relativa de arabinose, galactose e ácido galacturônico, porém a quantidade relativa de manose aumentou, indicando que a extrabilidade dos galactomananos aumenta com o grau de torra. Na extração a 170°C a um baixo grau de torra, os autores obtiveram um extrato com uma maior quantidade de galactose do que o extrato obtido a 90°C, indicando que

era constituído principalmente de arabinogalactanos. Com o aumento do grau de torra, as quantidades relativas de galactose e arabinose diminuem e o teor de manose aumenta, indicando novamente que quanto maior o grau de torra, maior a extrabilidade dos galactomananos. Portanto, uma maior proporção de arabinogalactanos foi extraída a 170°C, enquanto que a quantidade de galactomananos foi menor comparada com a extração a 90°C. Aparentemente, uma maior temperatura de extração (170°C) em menor tempo (20 min) resultou em maiores níveis de arabinogalactanos e menores níveis de galactomananos. GNIECHWITZ *et al*. (2007) também relatam que a extrabilidade dos polissacarídeos do café torrado depende não somente do tipo de café como também do grau de torra.

LELOUP & LIARDON (1993) caracterizaram os carboidratos de extratos de café verde e torrado, sendo a extração realizada com água destilada em duas condições: 95°C-1h e 180°C-15 min. Os autores concluíram que a temperatura de torra e extração afetam quantitativa e qualitativamente a composição do extrato. A distribuição da massa molecular foi investigada por cromatografia de exclusão, e verificou-se que as massas moleculares dos carboidratos dos extratos variaram de 200 a 1.000.000 Da. Na extração realizada a 180ºC, a faixa de massa molecular decresceu de 200-200.000 (café verde) a 200-50.000 Da (café torrado).

Como descrito no item 1.4, sedimentos são observados em extratos de café devido à extração de moléculas insolúveis em água. Segundo BRADBURY (2001), arabinogalactanos se mantêm dissolvidos, porém, algumas moléculas de mananos lineares são menos solúveis e podem precipitar, levando a formação de sedimentos no processo de fabricação de café solúvel. O autor afirma que esta precipitação ocorre devido à associação de mananos lineares a fim de formar regiões cristalinas.

## 2.4. Café solúvel

Os polissacarídeos presentes no extrato de café contribuem para as características do café solúvel a ser produzido. A determinação dos perfis de carboidratos livres e totais em café solúvel permite detectar adulterações no produto, tais como, inclusão de cascas de café e pergaminho, cereais, malte, amido, maltodextrinas, xarope de glicose e açúcar

caramelizado. A principal técnica utilizada para a determinação de açúcares em café solúvel é a Cromatografia de Troca Aniônica com Detecção por Amperometria Pulsada (HPAEC-PAD). Altos níveis de manitol livre e xilose total indicam a presença de cascas de café ou pergaminho, enquanto que cafés contendo cereais ou açúcares caramelizados mostram grandes quantidades de glicose total. Frutose livre, glicose livre e sacarose também são bons indícios de adulteração. Amostras caracterizadas por baixas quantidades de carboidratos livres e altos níveis de galactose e manose totais são típicas de cafés solúveis puros (BERGER *et al*., 1991; PRODOLLIET *et al*., 1995a, 1995c).

## 3. ENZIMAS

Uma enzima é uma proteína que é sintetizada numa célula viva e catalisa ou acelera uma reação termodinamicamente possível. Sendo uma proteína, uma enzima perde suas propriedades catalíticas se submetida a agentes como calor, ácidos ou bases fortes, solventes orgânicos ou outro material que desnature a proteína (CONN & STUMPF, 1980). Graças a sua estrutura complexa, as enzimas podem apresentar um alto grau de especificidade, propriedade ausente nos catalisadores inorgânicos e, portanto, sua presença permite a seleção das reações que poderão ocorrer, ainda que milhares de compostos diferentes estejam presentes. Para que ocorra a catálise, os substratos devem ligar-se à molécula da enzima em uma região específica de sua superfície, chamada sítio ativo (BORZANI *et al*., 2001).

Enzimas são classificadas de acordo com as reações que catalisam, possuindo um número (*EC number*) de acordo com a nomenclatura da União Internacional de Bioquímica e Biologia Molecular (COPELAND, 2000). Unidades de atividade enzimática são geralmente expressas pela quantidade de substrato consumido ou produto formado por minuto (Unidade Internacional) ou por segundo (katal), no Sistema Internacional. A atividade específica é usualmente expressa em unidades ou katal por mg de proteína (FOX, 1991).

Basicamente, as enzimas podem hidrolisar os substratos de duas formas. Exoenzimas removem unidades dos terminais das cadeias poliméricas, enquanto que endoenzimas hidrolisam as ligações internas em qualquer ponto ao longo da cadeia, o que

pode ser visualizado na Figura 5. Endoenzimas podem alterar a viscosidade de soluções poliméricas drasticamente, pois está diretamente relacionada ao tamanho do polímero (MATHEWSON, 1998).

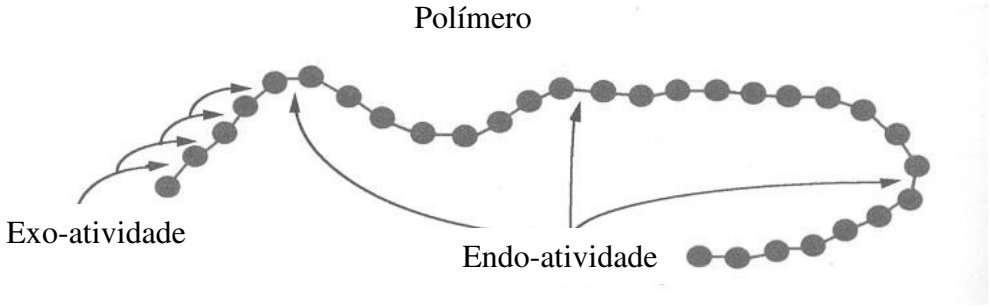


**Figura 5. Modos de ação de exo e endo-enzimas.**

### 3.1. Carboidrases

Carboidrases são enzimas que catalisam a hidrólise de ligações glicosídicas de carboidratos como amido, celulose, pectina, hemicelulose e outros polissacarídeos (DIXON & WEBB, 1979).

- ✓ *Celulase*

Celulose é o biopolímero mais abundante e renovável da Terra. Celulases são enzimas que hidrolisam as ligações β-(1→4) em celulose e seus derivados. A hidrólise da celulose ocorre pelo sinergismo de três diferentes enzimas: endoglucanases (EC 3.2.1.4), que hidrolisam a celulose a glico-oligossacarídeos, celobioidrolases (EC 3.2.1.91), as quais liberam celobiose a partir de celulose e β-glucosidases (EC 3.2.1.21), que convertem a celobiose a monômeros de glicose (CAO & TAN, 2002; JØRGENSEN *et al.*, 2003; JUHÁSZ *et al*., 2005; de VRIES & VISSER, 2001). Celulases podem ser obtidas de fungos como *Aspergillus, Trichoderma, Penicillium* e de bactérias. Este grupo de enzimas é utilizado comercialmente na degradação de materiais de plantas, detergentes, silagem, aditivos para alimentação animal e em aditivos para preparações digestivas (GERHARTZ, 1990).

- ✓ *Hemicelulases*

Hemicelulases são enzimas envolvidas na degradação de hemiceluloses, importantes componentes da parede celular de plantas. Hemiceluloses são polissacarídeos heterogêneos, lineares ou ramificados, compostos de xilose, arabinose, manose, glicose, galactose e outros açúcares. Estes polímeros podem também estar associados a moléculas de celulose e lignina. Devido à complexa estrutura das hemiceluloses, diferentes enzimas são necessárias para sua degradação ou modificação, tais como β-xilanases (EC 3.2.1.8), que quebram as ligações internas da cadeia de xilanos a xilo-oligossacarídeos; β-xilosidases (EC 3.2.1.37), que hidrolisam xilo-oligossacarídeos para produzir xilose; β-mananases (EC 3.2.1.78), que clivam as ligações internas de galacto(gluco)mananos a mano-oligossacarídeos; β-manosidases (EC 3.2.1.25), que hidrolisam mano-oligossacarídeos, liberando manose. Outras hemicelulases incluem α-L-arabinofuranosidase (EC 3.2.1.55), endo-arabinase (EC 3.2.1.99), α-galactosidase (EC 3.2.1.22), endogalactanase (EC 3.2.1.89), α-glucuronidase (EC 3.2.1.139), etc. Hemicelulases possuem diferentes aplicações, dentre elas, alimentação animal, hidrólise de biomassas e na indústria de papel e celulose (HEIDORNE *et al.*, 2006; JØRGENSEN *et al*., 2003; JUHÁSZ *et al*., 2005; OLSSON *et al*., 2003; SHALLOM & SHOHAM, 2003; SINGH *et al*., 2003; de VRIES & VISSER, 2001).

- ✓ *Pectinases*

Enzimas pectinolíticas ou pectinases é um heterogêneo grupo de enzimas que hidrolisam as substâncias pécticas, presentes principalmente em plantas. As substâncias pécticas consistem em uma cadeia formada por unidades de ácido galacturônico contendo unidades de ramnose e cadeias laterais de galactose e arabinose. Alguns dos grupos carboxil podem estar acetilados ou metilados. Pectinases podem ser classificadas em três grupos: *protopectinases*, as quais solubilizam protopectina formando uma pectina solúvel; *esterases*, que catalisam a desesterificação de pectinas pela remoção de ésteres, formando ácido péctico; e *depolimerases*, que catalisam a hidrólise da ligação glicosídica 1→ 4 de ácido galacturônico de substâncias pécticas. Este grupo de enzimas é empregado principalmente no processamento de frutas e vegetais (ALKORTA *et al*., 1998; BAI *et al*., 2004; de VRIES & VISSER, 2001; JAYANI *et al.*, 2005; KASHYAP *et al*., 2001;

MUTENDA *et al*., 2002; NIKOLIĆ & MOJOVIC 2007; PÉREZ *et al*., 2000; RIDLEY *et al*., 2001; SINGH *et al*., 1999).

### 3.2. Aplicações industriais de enzimas

As enzimas, em variadas formulações, são utilizadas em diferentes aplicações industriais. O mercado mundial de enzimas vem crescendo desde 1960, e este aumento tem refletido no aumento do número de enzimas disponíveis para uso industrial, diminuindo relativamente os custos de suas aplicações (CHAPLIN & BUCKE, 1990). O mercado global de enzimas é estimado em US$ 2,3 bilhões de dólares por ano, sendo que o Brasil representa 2% deste mercado (MUSSATTO *et al*., 2007).

A Tabela 3 apresenta algumas referências de trabalhos que relatam a aplicação de enzimas na redução da viscosidade e sedimentos de extratos de café. Entretanto, os trabalhos encontrados sobre a redução de sedimentos são patentes depositadas no Japão.

**Tabela 3. Aplicações de enzimas no tratamento de café**

| Enzimas | Aplicação | Referência |
|---|---|---|
| Galactomananases | Redução da viscosidade em extratos de café | EHLERS (1980) |
| Complexo SP-249 (pectinases, celulases, hemicelulases) | Redução da viscosidade em extratos de café | JARDINE (1991) |
| Mananase de *Sclerotium rolfsii* | Hidrólise de mananos para a redução da viscosidade | SACHSLEHNER *et al.* (2000) |
| Galactomananase | Preparação de bebida de café com a utilização de enzimas para a prevenção de precipitação | KASHIWAI *et al*. (2003); KASHIWAI & KITAJIMA (2003); OKADA *et al*. (2003) |

# 4. ANÁLISE DE CARBOIDRATOS

## 4.1. Composição de mono e polissacarídeos

De acordo com GUIGNARD *et al*. (2005), inúmeras dificuldades podem ser encontradas durante as análises cromatográficas de carboidratos, especialmente devido à alta diversidade e a grande variedade de grupos funcionais (hidroxil, amino, acetamino, fosfato).

A cromatografia gasosa pode ser utilizada na análise de carboidratos, porém, devido a sua alta polaridade, hidrofobicidade e baixa volatilidade, estes compostos devem ser convertidos a acetatos antes da análise (FOX, 1999; GUIGNARD *et al*., 2005; LEIN *et al*., 2002; OLSON *et al*., 1988; RUMPEL & DIGNAC, 2006). Por estas razões, a cromatografia líquida de alta eficiência (HPLC) é geralmente preferida. Métodos de análise de carboidratos por cromatografia líquida têm empregado colunas tendo como suporte sílica modificada com grupos amino ou colunas tendo como suporte polímero com metal trocador catiônico, utilizando-se detector de índice de refração (RI) ou ultravioleta (UV). Estes métodos analíticos requerem atenção quanto à solubilidade, concentração da amostra, e no caso de colunas de troca catiônica, também requerem aquecimento. Além disso, estes métodos de detecção são sensíveis ao eluente e aos componentes da amostra, exigindo o uso de gradiente e a limpeza da amostra antes da injeção. Assim, foi desenvolvida uma técnica cromatográfica chamada Cromatografia de Troca Aniônica (High Performance Anion Exchange Chromatography - HPAEC) acoplada a um Detector de Amperometria Pulsada (Pulsed Amperometric Detector - PAD) (DIONEX, 2000).

A cromatografia aniônica com detecção por amperometria pulsada é uma ferramenta poderosa na análise de carboidratos, permitindo a quantificação sem a etapa de derivatização, com alta resolução e sensibilidade. As maiores vantagens da HPAEC são: método simples, sensível (alta resolução), alta velocidade da análise, mínima limpeza e preparação da amostra (CATALDI *et al*., 1998; CATALDI *et al*., 1999; DIONEX, 2000; LEE, 1996; PRODOLLIET *et al*., 1995a).

*Hidrólise ácida de polissacarídeos*

A composição de polissacarídeos é geralmente determinada medindo-se os monossacarídeos liberados após hidrólise ácida em altas temperaturas. Os principais ácidos utilizados são: ácido clorídrico (HCL), ácido trifluoracético (TFA) e ácido sulfúrico ($H_2SO_4$) (BORCH & KIRCHMAN, 1997; CHOW *et al*., 2005; HILZ *et al*., 2005; JOHANSSON *et al*., 2006; MAYWORM *et al*., 2000; OLSON *et al*., 1988).

Segundo PULS (1993), a hidrólise ácida é geralmente uma relação entre a completa solubilização e possível destruição, e a hidrólise incompleta dos polissacarídeos. Segundo o autor, após hidrólise com TFA e especialmente com HCl, alguma fração pode não ser hidrolisada, enquanto que, com $H_2SO_4$, a hidrólise dos açúcares neutros é completa, porém algumas unidades de monômeros podem degradar.

## 4.2. Determinação da massa molecular

Uma das técnicas para a determinação da massa molecular de polímeros é a Cromatografia de Permeação em Gel (GPC - Gel Permeation Chromatography). Esta técnica tem como princípio a separação de moléculas de acordo com seu tamanho em solução. Basicamente, qualquer molécula solúvel pode ser separada por GPC, desde moléculas de massas moleculares inferiores a 100 até moléculas de milhões de massa molecular (ALTGET & SEGAL, 1971).

A separação geralmente ocorre em colunas preenchidas com um gel ou algum outro material poroso, cujo tamanho do poro determina a faixa de massas moleculares através da qual a separação ocorre. Moléculas pequenas podem se difundir entre os poros, sendo, portanto, retardadas, apresentando um tempo maior de eluição. Já as moléculas maiores são excluídas, pois não conseguem penetrar nos poros, sendo eluídas mais rapidamente. Outras moléculas de tamanho intermediário podem penetrar em alguns poros maiores. O mecanismo de separação pode ser observado na Figura 6 (ALTGET & SEGAL, 1971; GRIGOLON, 2001).

.

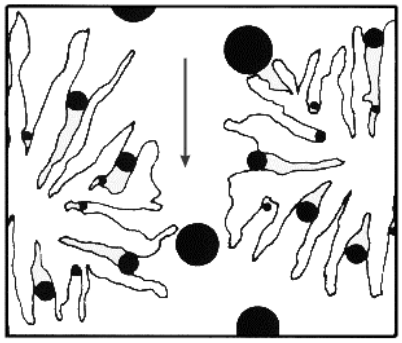

**Figura 6. Mecanismo de separação da Cromatografia de Permeação em Gel.**

Na Cromatografia de Permeação em Gel, diferentes tipos de calibrações podem ser realizados, de acordo com os detectores disponíveis (VISCOTEK, 2006):

- ✓ *Calibração convencional*

A coluna é calibrada utilizando-se somente um detector de concentração, tipicamene RI (índice de refração) ou UV (ultravioleta). São necessários padrões de diferentes massas moleculares, cujos logaritmos (Log M) em função do volume (ou tempo) de retenção fornecem a curva de calibração do sistema. Através da calibração convencional é possível obter a distribuição da massa molecular e também as massas moleculares relativas aos padrões e não valores absolutos.

- ✓ *Calibração universal*

A coluna é calibrada empregando-se um detector de concentração e um detector viscosimétrico.

- ✓ *Detecção tripla (triple detection)*

É uma técnica absoluta a qual emprega detectores de concentração, viscosimétrico e de espalhamento de luz. Com apenas uma injeção é possível obter as seguintes informações: distribuição da massa molecular, massas moleculares absolutas, viscosidade intrínseca, raio de giro, raio hidrodinâmico, incremento do índice de refração, e pode-se também obter informações de ramificação, agregação, estrutura e conformação.

## 5. ANÁLISE SENSORIAL

Historicamente, o interesse pela análise sensorial teve início nos anos 40 e foi desenvolvida principalmente nas décadas de 60 e 70. Esta ciência foi desenvolvida principalmente para alimentos, mas foi transferida com sucesso para outros produtos como cosméticos, automóveis, tecidos (CAYOT, 2007). A análise sensorial é feita através da utilização dos sentidos humanos: visão, gustação, olfato, audição e tato. As sensações que resultam da interação dos órgãos humanos com os produtos analisados, juntamente com os parâmetros químicos e microbiológicos, são utilizadas para determinar sua qualidade, aceitabilidade e em desenvolvimento de novos produtos (JELLINEK, 1985; KUTI *et al*., 2004; TEIXEIRA *et al*., 1987). Segundo FERIA-MORALES (2002), um painel sensorial bem treinado pode ser equivalente a um equipamento científico utilizado para medir parâmetros associados à qualidade do produto.

### 5.1. Métodos sensoriais

- ✓ *Métodos de Diferença ou Discriminativos*

O objetivo destes testes é determinar se há diferença sensorial entre duas ou mais amostras. Segue abaixo alguns exemplos (JELLINEK, 1985; MEILGAARD *et al*., 1999; STONE & SIDEL, 1993):

Teste triangular: utilizado quando se deseja saber se existe diferença sensorial entre dois produtos. O provador recebe três amostras codificadas, sendo duas idênticas e uma diferente, a qual deve ser selecionada pelo provador.

Teste duo-trio: utilizado quando o objetivo é determinar se há diferença sensorial entre duas amostras. O provador recebe uma amostra padrão e duas codificadas, tendo que avaliar qual é o próprio padrão dentre as amostras codificadas.

Teste de diferença do controle: este teste é utilizado quando se tem por objetivo (1) determinar se há diferença entre uma ou mais amostras e um controle e (2) estimar o tamanho de cada diferença. Geralmente, uma amostra é designada controle, referência ou

padrão, e todas as outras amostras são avaliadas em quanto cada uma difere do controle. Este teste é útil em situações nas quais pode haver diferença e o tamanho desta diferença afeta a decisão em relação ao objetivo do teste. Uma amostra controle juntamente com as amostras testes são apresentadas aos provadores, os quais devem avaliar, em uma escala, o grau de diferença entre cada amostra e o controle. É comum introduzir um padrão codificado às amostras a serem comparadas com o padrão declarado.

Podem ser feitos também testes de diferença em relação a um dado atributo.

- ✓ *Métodos Descritivos*

Todos os métodos sensoriais descritivos envolvem a detecção (discriminação) e a descrição dos aspectos sensoriais qualitativos e quantitativos de um produto. Os provadores, bem treinados, devem ser capazes de detectar e descrever os atributos sensoriais da amostra. Estes aspectos qualitativos definem o produto e incluem propriedades de aparência, aroma, sabor e textura. Os provadores devem também diferenciar e determinar a intensidade (quantidade) de cada aspecto (COSTELL, 2002; MEILGAARD *et al*., 1999). De acordo com MUNOZ *et al*. (1992), as principais vantagens deste método são a ausência da subjetividade na avaliação e a qualidade dos dados obtidos; enquanto que as principais desvantagens são o tempo e o custo necessários para treinamento dos provadores, e o tempo necessário para realização do teste e análise dos dados.

- ✓ *Testes Afetivos*

Os testes afetivos determinam a preferência ou aceitabilidade de um produto perante os consumidores. Estes testes têm aumentado a cada ano devido às seguintes razões: manutenção ou otimização de um produto, desenvolvimento de novos produtos, revisão de certa categoria de produtos e suporte a reclamações (MEILGAARD *et al*., 1999; STONE & SIDEL, 1993).

Em princípio, os métodos mais adequados de avaliação sensorial são aqueles nos quais é possível medir a magnitude da variabilidade entre um produto e um padrão previamente definido (escalas de intensidade ou método de diferença do controle), enquanto os métodos afetivos não são apropriados para a avaliação de rotina de qualidade

de produtos (COSTELL, 2002). Na prática, a seleção do método a ser utilizado depende dos objetivos e das características do produto a ser avaliado.

Em todo processamento do café faz-se o uso de profissionais altamente experientes para definir padrões de qualidade da bebida em questão. No que se refere à avaliação sensorial do café, a dependência constante de provadores treinados pode trazer alguns riscos para a continuidade e reprodutibilidade dos resultados. Porém, o uso de avançadas técnicas sensoriais, embasadas em tratamento estatístico adequado, pode ser uma excelente ferramenta nas indústrias de café, bem como nos demais segmentos da indústria de alimentos e bebidas (MENDES, 2005).

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# *CAPÍTULO II*

## AVALIAÇÃO DAS ATIVIDADES DE NOVE PREPARAÇÕES COMERCIAIS DE PECTINASES

Este capítulo é referente ao artigo "*Activities evaluation of nine commercial preparations of pectinases*", o qual será submetido à publicação.

Pectinases são enzimas que hidrolisam as substâncias pécticas e são empregadas essencialmente no processamento de frutas e vegetais. Neste trabalho, diferentes atividades celulolíticas, hemicelulolíticas e pectinolíticas foram determinadas em nove preparações comerciais de pectinases. Os ensaios foram realizados em tampão citrato 50 mM, pH 5,0 e 50°C. A maioria das preparações enzimáticas estudadas apresentou, além de poligalacturonase (atividade nominal), diversas atividades, incluindo endoglucanase, xilanase e mananase. Rohapect B1L foi o produto que apresentou a maior atividade de mananase. Portanto, além da hidrólise de pectina, essas preparações comerciais de pectinases poderiam ser empregadas para outros propósitos, como por exemplo, tratamento de extratos de café e hidrólise de biomassas. Pela análise de cluster foi possível detectar similaridades entre os diferentes produtos estudados

# ACTIVITIES EVALUATION OF NINE COMMERCIAL PREPARATIONS OF PECTINASES

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## Abstract

Pectinases are a heterogeneous group of enzymes that hydrolyze pectic substances, with activities of polygalacturonase, pectinlyase and pectimethylesterase. However, commercial pectinases preparations usually have other enzymatic activities. Nine commercial pectinases were assayed for cellulolytic, hemicellulolytic and pectinolytic activities in 50 mM citrate buffer, pH 5.0 at 50°C. Most of the preparations studied, which are indicated mainly for fruit processing by the manufacturers, contained, besides polygalacturonase, enzyme activities of endoglucanase, galactanase, xylanase and mannanase. By the cluster analysis it was possible to detect some similarities between the commercial products. Therefore, the enzymatic activity profiles obtained for the commercial pectinases suggested that, besides the hydrolysis of pectin in fruits, the preparations studied can potentially be employed for several other purposes, including biomass hydrolysis, animal feed, coffee treatment, etc.

*Keywords*: pectinases, cellulases, hemicellulases, enzyme activity, commercial enzymes.

## 1. INTRODUCTION

Enzymes are commonly used in many industrial applications. The global market of industrial enzymes is estimated in US$ 2.3 billion per year, and Brazilian market represents 2% of it (MUSSATTO *et al.*, 2007). From this global market, 35% of enzyme demands are for food processing, 28% for detergent and 23% for beverages, while less than 14% for medical and analytical fields.

Pectinolytic enzymes, also known as pectinases, are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants, helping in cell wall extension and softening during the maturation and storage (JAYANI *et al.*, 2005). These enzymes are usually employed in fruit and vegetable processing. Commercial pectinolytic enzymes often come from fungal source, especially from *Aspergillus niger* (BLANCO *et al*., 1999; JAYANI *et al.*, 2005; KASHYAP *et al.*, 2001; MUTLU *et al.*, 1999), being the genus *Aspergillus* a group of filamentous fungi with a large number of species (de VRIES & VISSER, 2001). Pectinolytic activities include polygalacturonase, pectinlyase and pectimethylesterase. Besides these, the commercial pectinases usually contain other enzymes produced by the fungis, which are not removed from the extract, such as hemicellulases, cellulases, proteases and amylases (MUTLU *et al.*, 1999), increasing the range of industrial applications.

Pectinases, hemicellulases and cellulases are important enzymes that are sold in large volumes for industrial applications, like food, brewery, wine, animal feed, textile and laundry, pulp and paper and agriculture. Some of these applications are listed in Table 1, which summarizes previous reviews.

In this study a selection of commercial pectinases was assayed for different cellulolytic, hemicellulolytic and pectinolytic activities in order to determine their overall suitability for various processing applications, besides that ones described by the manufacturers. Commercial users of enzymes might consider this study valuable and allow them to make a more informed choice regarding to the type of enzyme to be used in different applications.

**Table 1. Applications of cellulases, hemicellulases and pectinases**

| Enzyme | Applications |
|---|---|
| *Cellulases, hemicellulases and pectinases (macerating enzymes)* | Improvement maceration and extraction of grapes; filtration and clarification of wines.<br>Improvement in pressing and extraction of juice from fruits and oil from olives. |
| *Cellulases and hemicellulases* | Improvement in the nutritional quality of animal feed.<br>Bio-mechanical pulping; modification of fiber properties; deinking of recycled fibers.<br>Production of oligosaccharides as functional food ingredients and low-calorie food constituents.<br>Biomass conversion. |
| *Hemicellulases* | Solubilization of lignocellulose material to serve as a renewable energy and carbon source.<br>Improvement of animal feed.<br>Bio-bleaching and bio-pulping in the pulp and paper industry.<br>Processing flour in the bakery industry. |
| *Pectinases* | Coffee and tea fermentation.<br>Improvement of pressing, extraction and clarification of fruit juices.<br>Debarking in the paper and pulp industry. |
| *Cellulase-free xylanolytic enzyme* | Bio-bleaching of pulps. |
| *Xylanase* | Improvements of the bread volume and quality of the dough.<br>Increasing the feed conversion efficiency of animal feed.<br>Clarifying juices.<br>Producing oligosaccharides used as functional food additives or alternative sweeteners with |

| | |
|---|---|
| | beneficial properties.<br>Bleaching and delignification of pulps.<br>Viscosity reduction (fuel and starch). |
| *Cellulase, preferably endoglucanase rich* | Production of high quality fabrics. |
| *Pectinase and β-glucosidase* | Alteration of the sensory properties of fruits and vegetables. |
| *β-Glucosidase* | Improvement in the aroma of wines. |
| *Xylanases and endoglucanases* | Separation and isolation of starch and gluten from wheat flour. |
| *α-Galactosidases* | Improvement the gelling capacity of galactomannans, which have applications in the food, cosmetic and pharmaceutical industries.<br>Reduction the concentration of raffinose and other oligosaccharides in soybean milk, cowpea meal, and sugar beet syrup. |
| *β-Glucanases and mannanases* | Food safety and preservation. |
| *Endo-mannanase* | Production of water-soluble dietary fibers to enrich the fiber content of foods. |
| *Xylanases, mannanases, β-xylosidase and α-L-arabinofuranosidase* | Bio-bleaching of pulps. |
| *β-Glucanase and xylanase* | Improvement in the feed digestion and absorption. |

Sources: BHAT, 2000; de VRIES & VISSER, 2001; JAYANI *et al*., 2005; KASHYAP *et al*., 2001; KIRK *et al*., 2002; POUTANEN, 1997; SHALLOM & SHOHAM, 2003.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Commercial enzyme preparations, including nine pectinases and one cellulase, were obtained from different suppliers (Table 2). The substrates and standards were purchased from Sigma and Fluka. All other reagents were analytical grade chemicals.

**Table 2. Manufacturer, source and protein content of commercial pectinases and cellulase**

| Enzyme preparation | Manufacturer | Source | Protein (mg/ml) |
|---|---|---|---|
| Novo Shape | Novozymes | *Aspergillus oryzae* | 28.2 ± 0.21 |
| Pectinex 3XL | Novozymes | *Aspergillus aculeatus, Aspergillus niger* | 17.7 ± 0.42 |
| Pectinex Ultra | Novozymes | *Aspergillus aculeatus* | 50.6 ± 0.85 |
| Biopectinase CCM | Kerry | *Aspergillus niger* | 40.6 ± 1.70 |
| Biopectinase Super 8x | Kerry | *Aspergillus niger* | 57.9 ± 1.46 |
| Pectinase 444L | Biocatalysts | *Aspergillus sp.* | 16.9 ± 0.04 |
| Rohapect B1L | AB Enzymes | *Aspergillus niger* | 37.9 ± 0.14 |
| Rohapect D5L | AB Enzymes | *Aspergillus niger* | 12.9 ± 0.33 |
| Rohapect 10L | AB Enzymes | *Aspergillus* | 67.6 ± 2.62 |
| Econase CE * | AB Enzymes | *Trichoderma reesei* | 110 ± 4.24 |

* Cellulase

### 2.2. Protein Content

The protein content of the enzyme preparations was precipitated with 10% trichloroacetic acid and was determined according to LOWRY *et al*. (1951). Bovine Serum Albumine (BSA) was used as standard.

### 2.3. Activity assays

All the enzymes and substrates were prepared in 50 mM citrate buffer, pH 5.0 and the enzymatic activities were carried out at 50°C. The substrates and their concentrations, and the products measured are described in Table 3. Activities were expressed in nkat/$mg_{protein}$, where 1 nkat is defined as the amount of enzyme that catalyzes the conversion of 1 nmol of substrate to product in 1 second, under the conditions used in the assay. One nkat is equal to 0.06 IU. Cluster analysis was performed using the software Statistica 7.0, and the distances were measured as Euclidean distances.

**Table 3. Enzymatic activity assays: substrate nature, substrate concentration and product measured**

| Activity | Substrate | Substrate Concentration | Product measured | Reference |
|---|---|---|---|---|
| β-Glucosidase (EC 3.2.1.21) | *p*-nitrophenyl-β-D-glucopyranoside | 1 mM | *p*-nitrophenol | BAILEY & NEVALAINEN (1981) |
| α-galactosidase (EC 3.2.1.22) | *p*-nitrophenyl-α-D-galactopyranoside | 1 mM | *p*-nitrophenol | BAILEY & NEVALAINEN (1981); BAILEY & LINKO (1990) |
| β-mannosidase (EC 3.2.1.25) | *p*-nitrophenyl-β-D-mannopyranoside | 1 mM | *p*-nitrophenol | BAILEY & NEVALAINEN (1981) |
| α-arabinosidase (EC 3.2.1.55) | *p*-nitrophenyl-α-L-arabinofuranoside | 2 mM | *p*-nitrophenol | POUTANEN *et al*. (1987) |
| β-xylosidase (EC 3.2.1.37) | *p*-nitrophenyl-β-D-xylapyranoside | 5 mM | *p*-nitrophenol | BAILEY & NEVALAINEN (1981) |
| β-mannanase (EC 3.2.1.78) | Locust gum | 0.5% (w/v) | Reducing sugars | STÅLBRAND *et al*. (1993) |
| β-Xylanase (EC 3.2.1.8) | Xylan (4-*O*-methyl glucuronoxylan from *birchwood*) | 1% (w/v) | Reducing sugars | BAILEY *et al*. (1992) |
| β-Endoglucanase (EC 3.2.1.4) | HEC (hydroxy ethyl cellulose) | 1% (w/v) | Reducing sugars | BAILEY & NEVALAINEN (1981); IUPAC (1987) |
| Endo-β-galactanase (EC 3.2.1.89) | Lupin β-1,4-galactan | 0.5% (w/v) | Reducing sugars | LUONTERI *et al*. (2003) |
| Arabinase | Linear α-1,5 arabinan | 0.4% (w/v) | Reducing sugars | - |
| Polygalacturonase (EC 3.2.1.15) | Polygalacturonic acid | 0.4% (w/v) | Reducing sugars | BAILEY & PESSA (1990) |
| Pectinlyase (EC 4.2.2.10) | Pectin from citrus | 0.5% (w/v) | Unsaturated oligosaccharides | MANACHINI *et al*. (1988) |

## 3. RESULTS AND DISCUSSION

Enzymatic activities were assayed by using standard biochemical methods and were expressed in nkat per mg of protein. An important limitation of expressing the activities of enzymes is that the conditions of the assays are often not standardized between laboratories; therefore, although the term 'IU' or 'kat' is used, results are not comparable, unless the same assay conditions are used. Thus, enzymatic activity can vary from one laboratory to another. The determination of enzyme activity depends on many variables, including the assay method used, age and storage conditions of enzymes, temperature, pH, concentration of substrate, concentration of cofactors of the assay, leading to significant differences in enzyme activity. A better way to express the enzyme activity is to standardize them by the total protein content, as specific activity, giving a measurement of the purity of the enzyme.

The protein content of the commercial enzyme preparations was measured in order to allow the calculation of the specific activities and the protein results are present in Table 2. According to the manufacturers, all the commercial pectinases were obtained from *Aspergillus* and cellulase from *Trichoderma*. The protein content of each pectinase preparation ranged from 12.9 to 67.6 mg/ml, and was 110 mg/ml for cellulase (Econase CE).

Basically, enzymes can hydrolyze a polymeric substrate in two ways. Exo-enzymes remove units of one or more sugars from the ends of polymer chains; whereas endo-enzymes hydrolyze random bonds within the chains, thereby producing more ends for the exo-enzymes to act on (MATHEWSON, 1998; WARREN, 1996). Therefore, Tables 4 and 5 present the specific exo and endo-activities of the commercial enzyme preparations.

- ✓ *Exo-activities*

β-glucosidase activity was found mainly in Pectinase 444 L (48 nkat/$mg_{protein}$), and was also significant in Rohapect B1L and Novo Shape, which values were 19 and 15 nkat/$mg_{protein}$, respectively. All pectinases preparations presented very low or none activities of α-galactosidase and β-mannosidase. However, Pectinase 444L had the highest β-mannosidase activity amongst all other enzymes. Pectinex 3XL had the highest

α-arabinosidase activity (114 nkat/$mg_{protein}$), and this activity was also found in Pectinase 444L, Biopectinase CCM and Biopectinase Super 8x. Low levels of β-xylosidase activity were found in all enzyme preparations studied, but the highest value was found in Pectinase 444L (7 nkat/$mg_{protein}$). In general, the enzymes had low exo-activities and the product that had more distinct activities was Pectinase 444L.

- ✓ *Endo-activities*

Comparing Tables 4 and 5, it was verified that the activities found in the commercial pectinase preparations were mainly endo-activities. Polygalacturonase was the main activity of most of them; the exceptions were to Novo Shape, Pectinase 444L and Rohapect 10L. Polygalacturonase, which catalyses the hydrolysis of α-1,4-glycosidic linkages in polygalacturonan, is usually the main component of commercial pectinolytic enzymes (MOHAMED *et al*., 2003).

According to Table 5, Pectinex 3XL had a high polygalacturonase activity (10116 nkat/$mg_{protein}$), followed by galactanase (566 nkat/$mg_{protein}$) and xylanase (204 nkat/$mg_{protein}$). According to the manufacturer, Pectinex 3XL is commercialized for the degradation of pectin in fruits. Polygalacturonase, galactanase and xylanase were also the main activities found in Biopectinase CCM and Biopectinase Super 8x, which are used in fruit processing. Despite to the presence of other activities besides polygalacturonase, Pectinex 3XL, Biopectinase CCM and Super 8x can also be used for the degradation of galactans and xylans from different plant sources.

Pectinex Ultra had a high activity of polygalacturonase (4271 nkat/$mg_{protein}$), and also showed significant levels of galactanase and mannanase activities (499 and 195 nkat/$mg_{protein}$, respectively). SUUTARINEN *et al*. (2003) found polygalacturonase, pectin methylesterase, β-glucanase, and endoglucanase activities in this commercial product, which primary use is in fruit processing, for optimize juice yield and press capacity. Due to the galactanase and mannanase activities, this enzyme preparation can be employed on the hydrolysis of galactans, arabinogalactans and mannans.

Novo Shape and Rohapect 10L had little or no activities concerning to the endo-activities assayed. According to the manufacturer, the main activity of Novo Shape is pectin esterase, which was not assayed in this work.

**Table 4. Exo-activities (nkat/mg$_{protein}$) of commercial pectinases and cellulase preparations**

| Enzyme preparation | β-glucosidase | α-galactosidase | β-mannosidase | α-arabinosidase | β-xylosidase |
|---|---|---|---|---|---|
| Pectinex 3XL | 13 | n.d. | 2 | 114 | 3 |
| Pectinex Ultra | 1 | n.d. | n.d. | 24 | 1 |
| Biop. CCM | 7 | 2 | 2 | 36 | 4 |
| Novo Shape | 15 | n.d. | n.d. | n.d. | n.d. |
| Biop. Super 8x | 7 | 2 | 2 | 47 | 3 |
| Pectinase 444L | 48 | 2 | 7 | 71 | 7 |
| Rohapect 10L | n.d. | n.d. | n.d. | n.d. | n.d. |
| Rohapect D5L | 11 | n.d. | n.d. | 6 | 3 |
| Rohapect B1L | 19 | n.d. | 2 | 7 | 3 |
| Econase CE * | 4 | n.d. | n.d. | 5 | 4 |

* Cellulase
n.d.: not detected

**Table 5. Endo-activities (nkat/mg$_{protein}$) of commercial pectinases and cellulase preparations**

| Enzyme preparation | Endoglucanase | β-galactanase | β-mannanase | Arabinase | β-Xylanase | Poly-galacturonase | Pectinlyase |
|---|---|---|---|---|---|---|---|
| Pectinex 3XL | 22 | 566 | 21 | 10 | 204 | 10116 | 5 |
| Pectinex Ultra | 39 | 499 | 195 | 8 | 65 | 4271 | 2 |
| Biop. CCM | 28 | 285 | 58 | 4 | 167 | 2979 | 3 |
| Novo Shape | n.d. | 4 | n.d. | n.d. | 3 | n.d. | 9 |
| Biop. Super 8x | 30 | 359 | 33 | 3 | 160 | 2457 | 2 |
| Pectinase 444L | 105 | 226 | 144 | 7 | 387 | 262 | 16 |
| Rohapect 10L | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Rohapect D5L | 43 | 12 | 10 | n.d. | 160 | 79992 | 1 |
| Rohapect B1L | 92 | 5 | 1280 | 2 | 190 | 9277 | n.d. |
| Econase CE * | 111 | n.d. | 15 | n.d. | 197 | 76 | n.d. |

* Cellulase
n.d.: not detected

Rohapect D5L was the preparation that presented the highest polygalacturonase activity (79992 nkat/$mg_{protein}$), but also had xylanase activity, which was 160 nkat/$mg_{protein}$.

According to Table 5, Pectinase 444L was found to have all the activities assayed, mainly, xylanase (387 nkat/$mg_{protein}$), polygalacturonase (262 nkat/$mg_{protein}$), galactanase (226 nkat/$mg_{protein}$), mannanase (144 nkat/$mg_{protein}$) and endoglucanase (105 nkat/$mg_{protein}$). According to the manufacturer, Pectinase 444L is commonly used to hydrolyze cell wall of fruits, increasing the extraction and solubility.

Polygalacturonase activity of Rohapect B1L was 9277 nkat/$mg_{protein}$. This product presented high level of mannanase activity (1280 nkat/$mg_{protein}$) and also xylanase activity (190 nkat/$mg_{protein}$). As described by the manufacturer, Rohapect B1L is a pectinase preparation with galactomannanase and other hemicellulolytic side activities, and is able to hydrolyze pectin, galactomannan and other hemicelluloses. Due to the presence of mannanase activity, this enzyme can be use in coffee processing, decreasing the viscosity and avoiding the formation of sediment (DELGADO *et al*., 2008).

Low amounts of arabinase and pectinlyase activities were detected in all studied products.

Cluster analysis is a powerful tool of multivariate statistics, used to divide the data in classes or clusters. The objects in the same cluster are similar to each other and different from objects located in other clusters (LEE *et al*., 2008; SAMOILENKO & OSEI-BRYSON, 2007). To better identify the similarities between the commercial products studied, cluster analysis was conducted for the main endo-activities assayed and the dendograms obtained are shown in Figure 1. For the endoglucanase activity (Figure 1(a)), the products were divided in two big clusters. Analyzing the sub-clusters, Biopectinase CCM was similar to Biopectinase Super 8x, and they formed another sub-cluster with Pectinex 3XL, which had no similarity to Pectinase 444L and Rohapect B1L. Galactanase activity was found mainly in Pectinex 3XL, Pectinex Ultra, Biop. CCM, Pectinase 444L and Biop. Super 8x, which were the products that formed a big cluster in Figure 1(b), composed by three sub-clusters (Pectinex 3XL - Ultra; Biop. CCM - Pectinase 444L; and Biopectinase Super 8x). Concerning to the mannanase activity, Rohapect B1L was found to have no similarity to the other products, as shown in Figure 1(c); and Pectinex Ultra was in the same sub-group that Pectinase 444L. For the xylanase activity (Figure 1(d)), Pectinase

3XL was similar to Rohapect B1L; and Biop. Super 8x was similar to Biop. CCM and Rohapect D5L. All these products together with Pectinex Ultra formed a big cluster, whereas Pectinase 444L was found to have no similarity to the other products. Concerning to the polygalacturonase activity, Rohapect D5L formed one group on the dendogram of Figure 1(e) and Pectinex 3XL was found to be similar to Rohapect B1L, while Biop. CCM was similar to Biop. Super 8x.

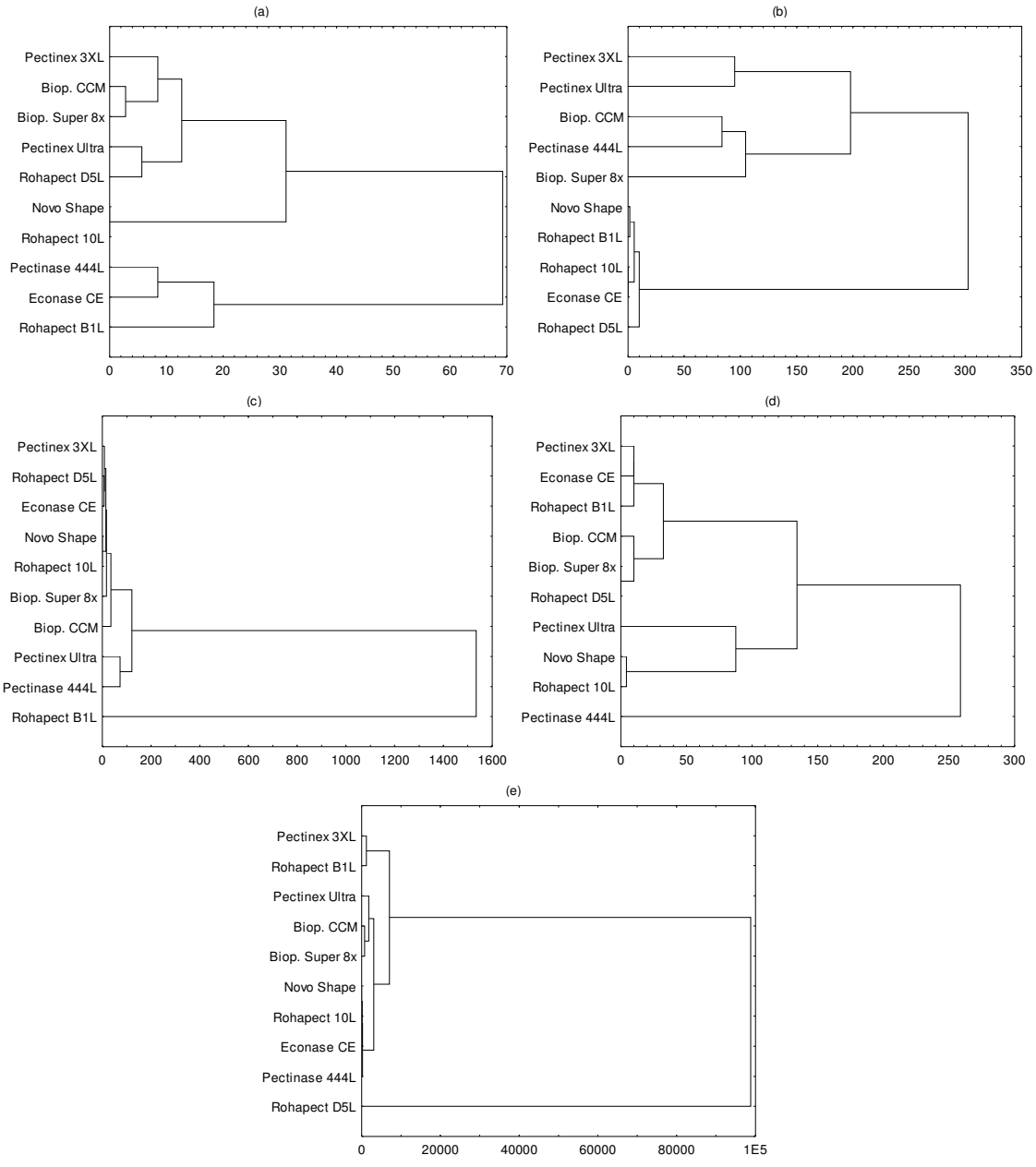


**Figure 1. Dendograms obtained by cluster analysis for the endo-activities: (a) endoglucanase; (b) galactanase; (c) mannanase; (d) xylanase; (e) polygalacturonase.**

Econase CE is a cellulase from *Trichoderma reesei* that is used for hydrolysis of biomass. Its activities were determined in order to compare this product with the commercial pectinases, and the results are also present in Tables 4 and 5. According to the results, the main specific activities of Econase CE were endoglucanase (111 nkat/$mg_{protein}$) and xylanase (197 nkat/$mg_{protein}$). By the cluster analysis, Econase CE was found to be similar to Pectinase 444 L and Rohapect B1L, concerning to the endoglucanase activity, as shown in Figure 1(a). For the xylanase activity, Econase CE was similar to Pectinex 3XL (Figure 1(c)); Pectinase 444L had approximately the double of the xylanase activity of Econase CE.

Due to the presence of endoglucanase, β-glucosidase, β-xylosidase, β-xylanase, β-mannanase and other activities, Pectinex 3XL, Pectinex Ultra, Biopectinase CCM, Biopectinase Super 8x, Pectinase 444L and Rohapect B1L can hydrolyze not only pectin, but also other cell wall polysaccharides and can be used for biomass hydrolysis (OHGREN *et al*., 2007; SAHA & COTTA, 2007). Nowadays, the use of renewable sources is increasing as an alternative to petroleum-derived fuels production, using cellulases and hemicellulases to release sugars. According to GRAY *et al*. (2006), the hemicellulases facilitate cellulose hydrolysis by exposing the cellulose fibres, thus making them more accessible. The efficient degradation of cell wall polysaccharides requires cooperative or synergistic interactions between the enzymes responsible for cleaving the different linkages (de VRIES & VISSER, 2001).

These results imply that most of these commercial enzyme preparations would have more applications than the ones suggested by the manufacturers.

## 4. CONCLUSIONS

Nine commercial pectinase preparations were assayed for cellulolytic, hemicellulolytic and pectinolytic activities. Besides the activity stated by the manufacturers, these products were found to have other activities, including β-galactanase, β-mannanase, β-xylanase and endoglucanase. The activities of one cellulase from *Trichoderma reesei* were also determined for comparison and cluster analysis was

performed in order to detect similarities between the commercial products studied. According to the enzymatic profile obtained in this work, besides their use in fruit and vegetable processing, Pectinex 3XL, Pectinex Ultra, Biopectinase CCM, Biopectinase Super 8x, Pectinase 444L and Rohapect B1L can also be employed in other applications, like biomass hydrolysis, coffee sediment treatment, pulp industry, animal feed, increasing the offer of enzymes.

## 5. ACKNOWLEDGEMENTS

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# *CAPÍTULO III*

## SEDIMENTOS EM EXTRATOS DE CAFÉ: COMPOSIÇÃO E CONTROLE PELA HIDRÓLISE ENZIMÁTICA

Este capítulo é referente ao artigo "*Sediments in coffee extracts: Composition and control by enzymatic hydrolysis*", o qual foi publicado no periódico *Food Chemistry*, Vol. 110, p. 168-176 (ANEXO 1).

Neste trabalho o extrato de café e seu respectivo sedimento foram isolados em suas frações solúveis e insolúveis, cujas composições químicas foram determinadas. Pela análise dos carboidratos, galactomanano foi o principal polissacarídeo encontrado nas frações insolúveis, as quais foram hidrolisadas por diversas preparações comerciais de enzimas, entre elas, celulase, protease, pectinases e galactomananase. Pectinase 444L foi o produto que proporcionou a maior liberação de açúcares, principalmente manose e galactose. O efeito destas preparações enzimáticas na redução de sedimentos em extratos de café foi então investigado. Biopectinase CCM, Rohapect B1L, Pectinase 444L e Galactomananase ACH foram as preparações mais eficientes na redução do sedimento, sendo Rohapect B1L e Galactomananase ACH as mais viáveis, cujas concentrações ótimas foram 0,3 e 0,1 $mg_{proteína}/g_{substrato}$, respectivamente.

# SEDIMENTS IN COFFEE EXTRACTS: COMPOSITION AND CONTROL BY ENZYMATIC HYDROLYSIS

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## Abstract

The water insolubility of some coffee extract components is one of the major limitations in the production of instant coffee. In this work, fractions from coffee extracts and sediments were prepared, and their chemical composition determined. Based on the carbohydrate analysis, galactomannan was found to be the main polysaccharide component of the insoluble fractions and probably responsible for sediment formation. The suitability of twelve commercial enzymes for the hydrolysis of the insoluble fractions was investigated. Pectinase 444L was the most effective enzyme in releasing sugars, mainly mannose and galactose, from these substrates. Biopectinase CCM, Rohapect B1L, Pectinase 444L and Galactomannanase ACH were found to be the most effective enzymes in reducing the sediment of coffee extracts. The highest sediment reduction was obtained using Rohapect B1L and Galactomannanase ACH, at enzyme concentrations of 0.3 and 0.1 $mg_{protein}/g_{substrate}$, respectively.

*Keywords:* coffee polysaccharides, galactomannans, instant coffee, enzymatic hydrolysis, coffee composition, sediment.

## 1. INTRODUCTION

Coffee is one of the world's most widely consumed beverages. The chemical composition of the coffee cell wall has not been studied in detail, since it is difficult to dissolve, extract and digest (KASAI *et al*., 2006). Polysaccharides comprise nearly 50% of the green coffee bean weight (FISCHER *et al*., 2001; NUNES & COIMBRA, 2001; NUNES *et al*., 2006), and those found in the coffee cell wall are mainly galactomannan, arabinogalactan and cellulose (FISCHER *et al*., 2001; OOSTERVELD *et al*., 2003a; REDGWELL *et al*., 2002a). Arabinogalactans consist of a main chain of 1→3 linked galactose branched at C-6, with side chains containing arabinose and galactose. Galactomannans consist of a main chain of 1→ 4 linked mannan with galactose unit side chains linked at C-6, and different degrees of branching (BRADBURY & HALLIDAY, 1990; NAVARINI *et al*., 1999; NUNES *et al*., 2006). The structure of the polysaccharides of industrialized coffee products depends on the degree of roasting (NUNES & COIMBRA, 2002; OOSTERVELD *et al*., 2003b; REDGWELL *et al*., 2002a).

The main obstacle for characterizing the coffee cell wall is the high proportion of insoluble polymers (BRADBURY & ATKINS, 1997; FISCHER *et al*., 2001; REDGWELL *et al*., 2002b). The solubility increases with increasing degree of branching and decreasing molecular weight (NUNES & COIMBRA, 2001). Arabinogalactans dissolve better than linear mannans, which can easily precipitate, and one of the reasons for this non-dissolution is an association of linear mannans to form crystalline regions (BRADBURY& ATKINS, 1997). This could be the reason for the formation of sediment during the manufacture of instant coffee. According to FISCHER *et al*. (2001) the difficulty in dissolving the cell wall polysaccharides indicates an intimate association between some of the arabinogalactan, galactomannan and cellulose molecules.

Proteins are another important component of coffee extracts. The roasting process causes degradation of the proteins into smaller products (NUNES & COIMBRA, 2001). In espresso coffee, the protein content was shown to be correlated with the foam volume (NUNES *et al*., 1997), and the protein is usually covalently linked to arabinogalactans (FISCHER *et al*., 2001; NAVARINI *et al*., 1999; REDGWELL *et al*., 2002b; REDGWELL *et al*., 2005). Information on coffee lipids is very limited, but it has been speculated that

the poor quality of coffee is also due to the hydrolysis of triacylglycerols (TAGs) with the release of free fatty acids, which, in turn, are oxidized (JHAM *et al*., 2001; NIKOLOVA-DAMYANOVA *et al*., 1998; SEGALL *et al*., 2005). The main classes of lipids present in green coffee are triacylglycerols (75%) and terpene esters (14%) (JHAM *et al*., 2001; NIKOLOVA-DAMYANOVA *et al*., 1998). There are still no reports available on the lignin content of coffee, but the lignin is found closely associated with the cellulose and hemicellulosic polysaccharides (de VRIES & VISSER, 2001; DÓKA *et al*., 2004; JUHÁSZ *et al*., 2005).

In Brazil, coffee extracts are processed into instant coffee or concentrated extract for exportation. However, during storage and commercial circulation, sediment is sometimes observed in the extracts, which is considered to be a quality defect and limits the utilization of the product.

These days, enzymes are commonly used in many industrial applications, including the degradation of plant cell walls. Cellulases, hemicellulases and pectinases are industrially important enzymes that are sold in large amounts for many applications. Different authors have investigated the use of enzymes to hydrolyze coffee polysaccharides. NUNES *et al*. (2006) isolated the galactomannans from light and dark roasted coffee infusions, and hydrolyzed them with endo-mannanase, decreasing the molecular weight of these polysaccharides. Mannanase can also be used to reduce the viscosity of the extract in the production of instant coffee, improving the effectiveness of the concentration process and reducing drying costs (SACHSLEHNER *et al*., 2000). These authors hydrolyzed the coffee mannan with free and immobilized mannanase from *Sclerotium rofsii*.

The aim of this work was to determine the composition and study the enzymatic hydrolysis of coffee fractions using different commercial enzyme preparations, and then apply the enzymatic treatment to the whole extract in order to reduce the sediment formed during coffee processing.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Coffee extract containing sediment was supplied by Cia Iguaçu de Café Solúvel (Cornelio Procópio, Paraná, Brazil). Enzyme preparations were obtained from different sources, and are described in Table 1. Monosaccharide standards were purchased from Sigma and Fluka, and all other reagents and solvents were of the highest purity.

**Table 1. Source, major activity and protein content of the commercial enzyme preparations**

| Enzyme | Source | Major activity[a] | Protein content (mg/ml) |
|---|---|---|---|
| Econase CE | AB Enzymes | Cellulase | 110.0 ± 4.24 |
| Protease GC 106 | Genencor | Protease | 86.0 ± 1.41 |
| Novo Shape | Novozymes | Pectinase | 28.2 ± 0.21 |
| Pectinex 3XL | Novozymes | Pectinase | 17.7 ± 0.42 |
| Pectinex Ultra | Novozymes | Pectinase | 50.6 ± 0.85 |
| Biopectinase CCM | Biofincon | Pectinase | 40.6 ± 1.70 |
| Biopectinase Super 8x | Quest | Pectinase | 57.9 ± 1.46 |
| Pectinase 444L | Biocatalysts | Pectinase | 16.9 ± 0.04 |
| Rohapect B1L | AB Enzymes | Pectinase | 37.9 ± 0.14 |
| Rohapect D5L | AB Enzymes | Pectinase | 12.9 ± 0.33 |
| Rohapect 10L | AB Enzymes | Pectinase | 67.6 ± 2.62 |
| Galactomannanase ACH | Sumizyme | Galactomannanase | 0.3 ±0.01[b] |

[a] According to the manufacturer
[b] mg/mg (enzyme powder)

## 2.2. Preparation of the coffee and sediment fractions

In the process used by the Cia Iguaçu, green coffee beans were roasted and ground, and the ground coffee then percolated by hot water under high pressure to extract the solids. The extract obtained was stored in tanks at 4°C, where the sediment formed. The whole extract (containing sediment) and the sediment alone were the samples used in the present work, being fractionated according to Figure 1. All the fractions were freeze dried.

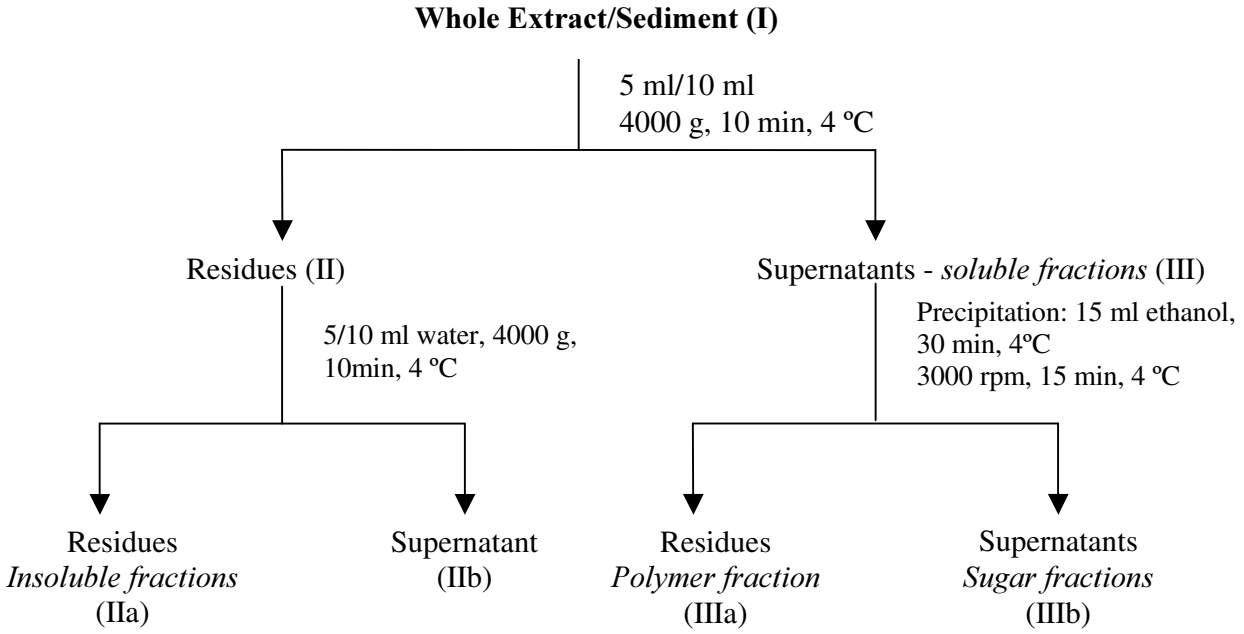


**Figure 1. Scheme for preparation of the coffee and sediment fractions.**

## 2.3. Chemical analysis

*Neutral and acidic sugars* were analyzed according to the Saeman hydrolysis (SELVENDRAN *et al*., 1979). In this method, 10 mg of sample were first added to 0.5 ml of 72% $H_2SO_4$ and maintained at room temperature for 3 hours. After this pre-treatment, distilled water was added to the mixture to dilute the $H_2SO_4$ to 1M, and incubated at 95°C for 2 hours. The monosaccharides were analyzed by High Performance Anion Exchange Chromatography (HPAEC) using a Dionex DX 500 system (Dionex Corp. Sunnyvale CA),

equipped with a GP40 gradient pump, ED40 electrochemical detector and AS 3500 autosampler. The analytical column was a CarboPac PA1 (250 x 4 mm) and the guard column was a CarboPac PA1 (25 x 3 mm). All determinations were carried out at 30°C, using a flow rate of 1 ml/min and a gradient with pure water, NaOH and NaOAc. *Reducing sugars* were determined using the dinitrosalicylic acid (DNS) method (BERNFELD, 1955; SUMNER, 1924). *Total protein content* was estimated by the Kjeldahl nitrogen method, and a factor of 6.25 was used to convert nitrogen into protein (BERNARD, 1992; BUCKEE, 1994). *Ash* was analyzed by incineration in a programmable muffler oven from room temperature to 550°C (4 hours at peak temperature). *The insoluble lignin content* was estimated according to BROWNING (1967). *The lipid content* was analyzed according to PARTANEN *et al*. (2005), using a Maran 23 MHz proton NMR spectrometer. The *soluble protein* concentration of the enzymes was determined according to the Lowry assay against a standard curve of bovine serum albumin (LOWRY *et al*., 1951), after precipitation of the protein from the samples using trichloroacetic acid. All the analyses were carried out in duplicate. The data were analyzed by ANOVA followed by Tukey's test, at a significance level of 0.05. Statistical analyses were performed using the software STATISTICA 7.0.

### 2.4. Enzymatic hydrolysis of insoluble fractions of the extract and sediment

Three-milliliter aliquots of insoluble fraction preparations, IIa (1% w/v in 50 mM citrate buffer, pH 5.0) were treated with 100 µl of different enzyme solutions (10 $mg_{protein}/g_{substrate}$) at 45°C for 20 hours. The reaction was stopped by heating the mixture in a boiling water bath for 10 minutes. The sugars released were determined by HPAEC. Control treatments (with no enzyme) were made in parallel to the enzymatic ones.

### 2.5. Enzymatic hydrolysis of the whole extract to reduce the sediment

To investigate the effect of the enzymatic treatment on the sediment contents, 4 ml of the whole extract was incubated with 100 µl of enzyme solution (initially 5mg $_{protein}/g_{substrate}$). The solutions were then cooled to 0°C, maintained at this temperature for 5 minutes and then centrifuged at 3000 rpm, 4°C for 10 minutes. The supernatant was

discarded and the sediment dried over night at 105°C. Before centrifugation, a small aliquot (25 µl) was collected for the reducing sugar analysis by the DNS method. The temperatures studied ranged from 35 to 55°C. Control treatments were made under similar conditions except that no enzyme was added. The sediment content was calculated according to the following equation:

$$SEDIMENT(\%) = \frac{\text{dried weight of residue}}{\text{dried weight of initial substrate}} \times 100$$

## 3. RESULTS AND DISCUSSION

### 3.1. Chemical composition of the extract and sediment fractions

The chemical compositions of the whole extract and the insoluble fractions isolated from the coffee extract and sediment are shown in Table 2. The whole extract (I) contained 43.4% of carbohydrate, the insoluble fraction of the extract 55.9% and the insoluble fraction of the sediment 12.8%. Thus the carbohydrate concentration was approximately 10% higher in the insoluble fraction of the extract than in the whole extract, and approximately 30% smaller in the insoluble fraction of the sediment. Concerning the whole extract, similar results have been described in the literature on roasted coffee (OOSTERVELD *et al*., 2003a; REDGWELL *et al*., 2002a).

**Table 2. Chemical composition of the whole extract and insoluble fractions % (w/w)**

| Fraction | Total Sugars | Free sugars | Protein | Lipids | Ash | Insoluble Lignin |
|---|---|---|---|---|---|---|
| Whole extract | 43.4±1.34[a] | 4.7±0.14[a] | 19.2±0.28[a] | ~ 0.0 | 10.5±0.69[a] | 3.8±0.32[a] |
| Insoluble fraction of extract | 55.9±1.93[b] | 3.4±0.14[b] | 15.2±0.57[b] | ~ 0.0 | 6.2±0.31[b] | 5.2±0.35[a] |
| Insoluble fraction of sediment | 12.8±0.08[c] | 0.6±0.03[c] | 30.5±1.41[c] | 12.2±0.5 | 3.4±0.14[c] | 27.6±2.26[b] |

Different letters in the same column are significantly different (P< 0.05) according to Tukey's test.

The protein content of the whole extract was found to be higher, 19%, than the value reported by OOSTERVELD *et al*. (2003a), which was only 6.8%, but was closer to the value reported by FRANCA *et al*. (2005), which was 14.9%. As the protein content was calculated from the total nitrogen content of the samples, it may have been overestimated due to the presence of other nitrogen containing substances (caffeine, trigonelline, free amines and amino acids). In addition to polysaccharides and proteins, the whole extract also contained ash (10.5%) and insoluble lignin (3.8%). Lipids were not found. The insoluble fraction of the extract showed higher contents of carbohydrate (54.7%) and insoluble lignin (5.2%), and lower contents of protein (15.2%) and ash (6.2%), when compared to the whole extract; whereas the insoluble fraction of the sediment had relatively lower contents of sugar (12.9%) and ash (3.4%) and higher contents of protein (30.5%), lipid (12.2%) and insoluble lignin (27.6%). Since the insoluble lignin content was higher in the insoluble fraction of the sediment than in the other fractions, this leads to believe that the lignin may be bound to the cellulose, hemicellulose and protein, forming a large amount of insoluble particles. Besides polysaccharides, proteins, lipids, ash and insoluble lignin, the material could also contain soluble lignin, chlorogenic acids, aliphatic acids, humic acids and melanoidins (CHARLES-BERNARD *et al*., 2005; SMITH, 1985).

The monosaccharide profiles of the coffee fractions are shown in Table 3, and were composed mainly of galactose, arabinose and mannose, building blocks of the galactomannans and arabinogalactans.

The predominant sugars in the whole extract (I) were galactose and mannose (44.1 and 33.2%, respectively), followed by arabinose (10.5%). The ratios of galactose:mannose and arabinose:galactose were, respectively, 1.3 and 0.24. OOSTERVELD *et al*. (2003a) examined the chemical structure of the polysaccharide fractions obtained after the hot water extraction of roasted coffee. After an extraction with water at 90°C, the authors found 49% of mannose, 33% of galactose and 9% of arabinose. After extraction at 170°C, the sugar profile was 32% of mannose, 53% of galactose, and 8% of arabinose; the ratios galactose:mannose and arabinose:galactose were 1.7 and 0.15, respectively, values close to the present results. Acidic sugars were also found in the whole extract (methylglucuronic acid, galacturonic acid and glucuronic acid), representing 7% of the total carbohydrate content. According to REDGWELL *et al*. (2002a, 2002b), rhamnose and galacturonic acid

are components of pectin or rhamnogalacturonan, that can be present in the coffee bean cell wall, while glucuronic acid can exist as terminal residues on the side chains of arabinogalactans.

Concerning the insoluble fractions (IIa), it was found that the main polysaccharide was a galactomannan. The ratios of galactose:mannose were 0.41 and 0.37 for the extract and sediment insoluble fractions, respectively. Therefore, the galactomannan present in these fractions is less branched than that found in the whole extract. The low solubility of these fractions may result from the low degree of branching (BUCKERIDGE *et al*., 2000; OOSTERVELD *et al*., 2003a). The polymer fractions (IIIa) were both primarily composed of galactose units, indicating the presence of arabinogalactans and galactans. In the sugar fractions (IIIb), the main sugar was mannose, followed by galactose and arabinose. The ratio of mannose:galactose was approximately 1, suggesting the high solubility of these fractions.

**Table 3. Monosaccharide composition of the coffee fractions after acid hydrolysis (% w/w)**

| Sample | Rha | Ara | Gal | Glu | Xyl | Man | Fru | MeGlcA | GalA | GlcA | Total Carbohydrate (mg/100 mg solids) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Extract** | | | | | | | | | | | |
| Whole | 1.0±0.07[a] | 10.5±0.18[a] | 44.1±0.33[a] | 3.2±0.01[a] | 0.4±0.03[a] | 33.2±0.10[a] | 1.2±0.07[a] | 0.6±0.02[a] | 5.2±0.02[a] | 0.6±0.02[a] | 43.4±1.34[a] |
| Insoluble fraction | 0.6±0.04[b] | 6.1±0.05[b] | 25.3±0.24[b] | 2.1±0.01[b] | 0.2±0.02[b] | 61.3±0.10[b] | 0.7±0.00[b] | 0.5±0.02[a] | 3.0±0.18[b] | 0.5±0.02[b] | 55.9±1.93[b] |
| Polymer fraction | 0.8±0.02[c] | 5.4±0.10[c] | 60.4±0.35[c] | 1.8±0.01[c] | 0.3±0.01[a] | 22.0±0.08[c] | 0.3±0.00[c] | 0.7±0.42[a] | 7.9±0.30[c] | 0.4±0.00[c] | 64.6±0.37[c] |
| Sugar fraction | 1.6±0.05[d] | 20.0±0.08[d] | 33.5±0.19[d] | 6.2±0.02[d] | 0.6±0.02[c] | 36.1±0.05[d] | 2.0±0.14[d] | Na | Na | Na | 24.9±0.70[d] |
| **Sediment** | | | | | | | | | | | |
| Insoluble fraction | 1.3±0.04[e] | 8.2±0.00[e] | 21.5±0.14[e] | 4.3±0.07[e] | 0.3±0.01[a] | 57.4±0.08[e] | 0.7±0.03[b] | 2.0±0.01[b] | 2.5±0.02[b] | 2.0±0.01[d] | 12.8±0.08[e] |
| Polymer fraction | 1.1±0.01[a] | 6.5±0.06[f] | 65.2±0.03[f] | 2.0±0.01[b,c] | 0.3±0.03[a] | 24.6±0.01[f] | 0.3±0.00[c] | Na | Na | Na | 57.1±1.55[b] |
| Sugar fraction | 1.7±0.01[d] | 20.9±0.01[g] | 33.0±0.07[d] | 6.2±0.11[d] | 0.7±0.01[d] | 35.3±0.09[g] | 2.2±0.03[e] | Na | Na | Na | 23.4±0.43[d] |

Values expressed on dry bases. Na, not analyzed.
Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Fru, fructose, MeGlcA, Methylglucuronic acid; GalA, Galacturonic acid; GlcA, Glucuronic acid.
Different letters in the same column are significantly different ($P < 0.05$) according to Tukey's test.

### 3.2. Enzymatic hydrolysis of the insoluble fractions

Insoluble fractions (IIa), previously isolated from the coffee extract and sediment, were treated with twelve commercial enzyme preparations containing cellulases, hemicellulases, pectinases and protease. The protein content of the enzymes was also investigated in order to standardize suitable concentrations for them in the coffee treatments (Table 1). These enzyme preparations are complex mixtures of various different hydrolytic enzymes, but the major activity according to the manufacture was also given.

Tables 4 and 5 show the monosaccharide compositions of the products resulted after enzymatic hydrolysis of the insoluble fractions of the extract and sediment, respectively. The content of each sugar released after acid hydrolysis of these fractions was also included, for comparison. The free sugar contents of the non-hydrolyzed (control treatments) insoluble fractions were also determined, in order to compare with the values obtained after the enzymatic treatments.

**Table 4. Monosaccharide composition of the insoluble fraction of the extract after enzymatic hydrolysis (mg in 100 mg of dried substrate)**

| Enzyme | Rha | Ara | Gal | Glu | Xyl | Man | Fru | Total |
|---|---|---|---|---|---|---|---|---|
| Control treatment [a] | 0.04 | 1.24 | 0.79 | 0.17 | 0.04 | 0.77 | 0.29 | 3.35 |
| Total sugars (acid hydrolysis) [b] | 0.31 | 3.30 | 13.67 | 1.16 | 0.09 | 33.12 | 0.35 | 52.00 |
| Econase CE | 0.04 | 1.65 | 1.19 | 0.58 | 0.17 | 1.45 | 0.31 | 5.38 |
| Protease GC 106 | 0.04 | 1.55 | 2.69 | 0.57 | 0.06 | 6.72 | 0.39 | 12.02 |
| Pectinex 3XL | 0.04 | 2.17 | 9.30 | 0.12 | 0.04 | 26.35 | 0.72 | 38.75 |
| Pectinex Ultra | 0.05 | 1.96 | 8.68 | 0.71 | 0.04 | 9.30 | 0.53 | 21.28 |
| Biopectinase CCM | 0.04 | 2.07 | 8.06 | 0.90 | 0.04 | 25.21 | 0.74 | 37.07 |
| Biop.Super 8x | 0.04 | 2.07 | 6.92 | 0.82 | 0.04 | 28.93 | 0.73 | 39.56 |
| Pectinase 444L | 0.04 | 2.17 | 9.63 | 1.32 | 0.04 | 30.35 | 0.85 | 44.40 |
| Rohapect B1L | 0.04 | 1.76 | 2.89 | 1.24 | 0.04 | 25.94 | 0.71 | 32.62 |
| Rohapect D5L | 0.04 | 1.71 | 1.50 | 0.64 | 0.08 | 1.27 | 0.32 | 5.56 |

Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Fru, fructose.
[a] Free sugars of the control treatment (no enzyme).
[b] Total sugars obtained after acid hydrolysis of the insoluble fraction of the extract.

**Table 5. Monosaccharide composition of the insoluble fraction of the sediment after enzymatic hydrolysis (mg in 100 mg of dried substrate)**

| Enzyme | Rha | Ara | Gal | Glu | Xyl | Man | Fru | Total |
|---|---|---|---|---|---|---|---|---|
| Control treatment [a] | 0.04 | 0.24 | 0.15 | 0.04 | 0.04 | 0.13 | 0.05 | 0.69 |
| Total sugars (acid hydrolysis) [b] | 0.17 | 1.05 | 2.77 | 0.54 | 0.03 | 7.35 | 0.09 | 12.01 |
| Econase CE | 0.04 | 0.42 | 0.30 | 0.30 | 0.04 | 0.42 | 0.06 | 1.59 |
| Protease GC 106 | 0.04 | 0.33 | 0.69 | 0.24 | 0.04 | 2.69 | 0.07 | 4.10 |
| Pectinex 3XL | 0.04 | 0.59 | 1.76 | 0.04 | 0.04 | 6.30 | 0.13 | 8.89 |
| Pectinex Ultra | 0.04 | 0.49 | 1.65 | 0.31 | 0.04 | 2.48 | 0.11 | 5.13 |
| Biopectinase CCM | 0.04 | 0.51 | 1.55 | 0.37 | 0.04 | 6.30 | 0.15 | 8.96 |
| Biop. Super 8x | 0.04 | 0.51 | 1.40 | 0.29 | 0.04 | 6.20 | 0.13 | 8.61 |
| Pectinase 444L | 0.07 | 0.53 | 1.64 | 1.31 | 0.04 | 6.47 | 0.23 | 10.29 |
| Rohapect B1L | 0.04 | 0.44 | 0.62 | 0.73 | 0.04 | 6.72 | 0.15 | 8.74 |
| Rohapect D5L | 0.04 | 0.42 | 0.39 | 0.34. | 0.04 | 0.40 | 0.07 | 1.72 |

Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Fru, fructose.
[a] Free sugars of the control treatment (no enzyme).
[b] Total sugars obtained after acid hydrolysis of the insoluble fraction of the sediment.

As shown in Table 4, the control of the insoluble fraction of the extract (without the addition of enzyme) was found to have 3.35% of free sugars, composed mainly of arabinose, followed by galactose and mannose. After treatment with Econase, the free sugar content increased to 5.33%, glucose being the main sugar released by this enzyme. Econase is a preparation of cellulases obtained from *Trichoderma reesei*, containing several endoglucanases and exoglucanases and various hemicellulases (SUUTARINEN *et al*., 2003). Hydrolysis with Protease GC106 produced mainly mannose, followed by galactose and glucose, which were certainly released due to the presence of other hydrolytic activities apart from the protease activity. Galactose and mannose were found to be the main sugars released by hydrolysis with Pectinex 3XL, Pectinex Ultra, Biopectinase CCM, Biopectinase Super 8x, Pectinase 444 and Rohapect B1L. It was evident that these pectinase preparations were mixtures of various enzymes, which hydrolyzed mannans, galactans and other carbohydrates. Of all the pectinases tested, Rohapect D5L, which is used in fruit juice processing, achieved the lowest yield of released sugars. After hydrolysis with Pectinase

444L, the mannose content increased significantly (42 times) and 80% of the total carbohydrates were present as free sugars, indicating the hydrolysis of mannans.

When the insoluble fraction of the coffee sediment (Table 5) was treated with the commercial enzymes, similar results to those obtained with the insoluble fraction of the extract were found, although lower amounts of reducing sugars were observed. As shown in Table 3, both the extract and sediment insoluble fractions were composed of the same carbohydrates, but the extract fraction had a higher carbohydrate concentration than the sediment fraction.

Two other enzyme complexes (Novo Shape and Rohapect 10L) were also tested, but apparently had no effect on the carbohydrate composition of the substrates (data not shown).

The glucose and fructose contents were higher after enzymatic hydrolysis than after acid hydrolysis. Acid hydrolysis probably degraded part of these sugars, but if the acid hydrolysis conditions had been less drastic, complete hydrolysis of the polysaccharides might not have been possible. According to PULS (1993), after TFA and HCl hydrolysis, part of the sample may still not have been hydrolyzed, whilst with $H_2SO_4$, the hydrolysis of the neutral sugars is complete but some of the monomer units may degrade.

Therefore it was concluded from the results that Pectinase 444L was the most efficient enzyme in releasing sugars from insoluble coffee materials.

### 3.3. Enzymatic hydrolysis of the whole extract to reduce the sediment

As it is important to reduce the amount of sediment during the production of instant coffee, the efficiency of the different enzymes on the coffee extract was studied. The contents of the main components found in the whole extract can be seen in Table 2. The performance of the enzymes in the hydrolysis of the coffee extract can depend on factors such as substrate concentration, enzyme type and concentration, and process conditions such as pH, temperature, mixing rate and reaction time. The pH of the industrialized coffee extract is around 5.0, which was maintained, since this is the optimum pH of the enzymes

studied and due to the difficulty of adjusting the pH during the industrial manufacture of instant coffee.

The first set of experiments was conducted in order to determine which enzymes most efficiently reduced the amount of sediment in the whole extract, employing an enzyme concentration of 5 mg $_{protein}$/g $_{substrate}$ at 45ºC for 2 hours. The effects of the enzymes on the sediment and on the reducing sugars are shown in Figure 2.

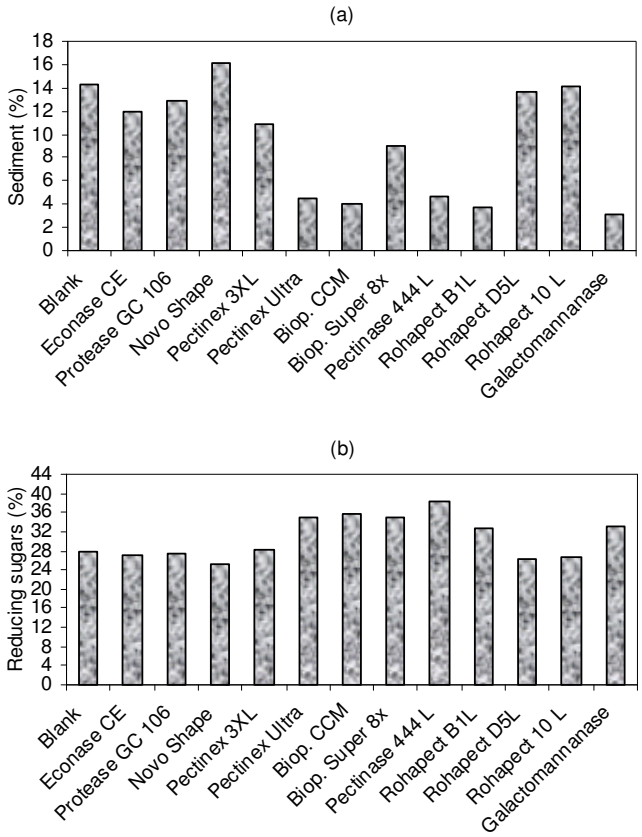


**Figure 2. Screening of commercial enzymes for sediment reduction. The effect of enzymatic hydrolysis on: (a) the amount of sediment and (b) the release of reducing sugars.**

The amount of sediment obtained in the non-enzymatically treated coffee was 14% at 45°C. Treatment with Econase, Protease, Novo Shape, Pectinex 3XL, Rohapect 5DL and Rohapect 10L had no effect on the amount of sediment. Hydrolysis with Pectinex Ultra, Biopectinase CCM, Pectinase 444 L, Rohapect B1L and Galactomannanase ACH resulted

in approximately the same efficiency (≈ 4% of the amount of sediment). Figures 2 (a) and (b) show that the profiles of the amount of sediment and of the sugars released were not related, i.e., Pectinase 444 L was shown to release the highest concentration of sugars.

Equivalent conversions can be achieved with lower concentrations of enzymes during longer treatments; therefore the influence of enzyme concentration on the hydrolysis was evaluated. Hydrolysis experiments with the four best enzyme preparations (Biopectinase CCM, Pectinase 444L, Rohapect B1L and Galactomannanase ACH) were performed at 50ºC and the results can be seen in Figures 3 (a) to (d), respectively. Hydrolysis with Biopectinase CCM was carried out in a concentration range from 1-4 $mg_{protein}/g_{substrate}$, as shown in Figure 3 (a). Increasing enzyme concentrations resulted in decreasing amounts of sediment. However, when 4 $mg_{protein}/g_{substrate}$ was used, the final sediment content was 5% (w/w), and so this enzyme was not considered to be economically viable for an industrial process, since quite a large amount of enzyme would be required for a long period of time. Similar hydrolysis performances were obtained by Pectinase 444L, as shown in Figure 3 (b), the maximum rate being achieved with an enzyme concentration of 4 $mg_{protein}/g_{substrate}$, which represents 16% (w/w) of enzyme per quantity of dried substrate, a value considered too high for a commercial application. However, when Rohapect B1L was employed (Figure 3 (c)), it was observed that after 40 min of incubation, a significantly lower amount of enzyme (0.3 $mg_{protein}/g_{substrate}$), which represents 0.9% (w/w) of enzyme per amount of substrate, resulted in a decreased amount of sediment (approximately 3.5%). Further increases in the enzyme concentration did not result in a better effect. Galactomannanase ACH treatment (Figure 3 (d)) resulted in a high and fast sediment reduction. The lowest enzyme concentration to obtain the minimum sediment, 3.5% after one hour, was 0.1 $mg_{protein}/g_{substrate}$, which represents 0.03% (w/w) of enzyme per amount of substrate. Above this, the enzyme concentration did not appear to enhance the hydrolysis yield; although using higher concentrations of enzyme the same yield was achieved in a shorter period of time. Therefore, the present results indicated that, potentially, Rohapect B1L and Galactomannanase ACH could be employed for sediment reduction in coffee processing.

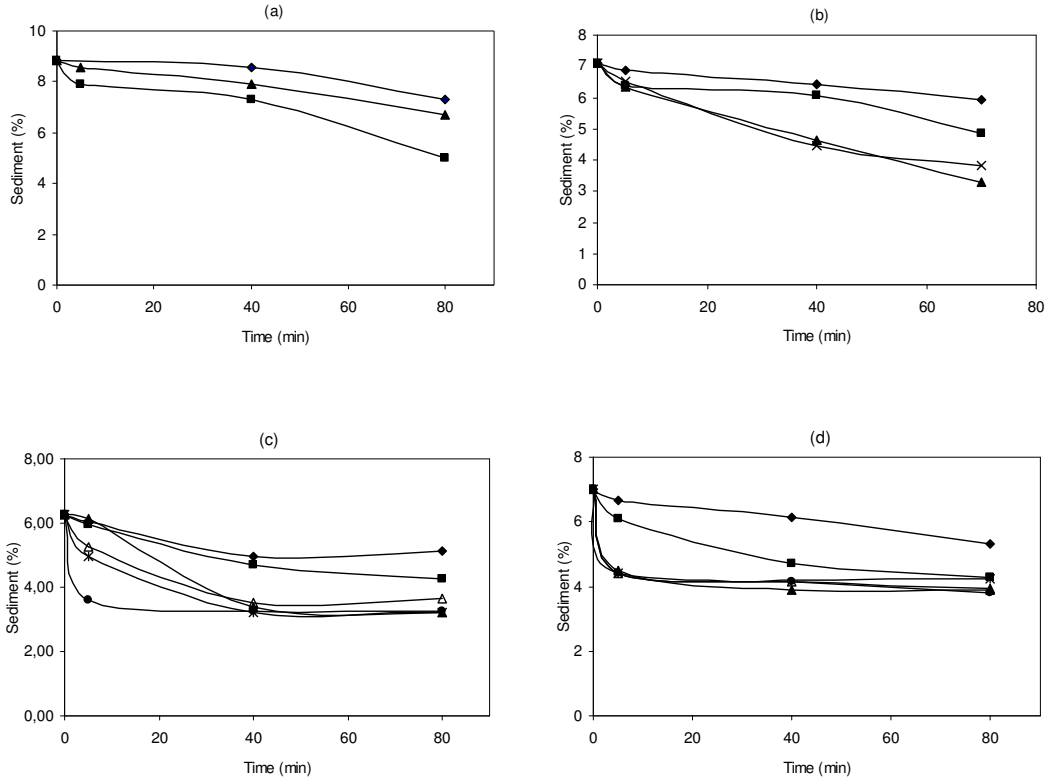


**Figure 3. Time course curves of the enzymatic hydrolysis of coffee extracts at 50ºC. Effect of the enzyme concentrations on the amounts of sediment.**

(a) Biopectinase CCM (-◆-) 1.0 $mg_{protein}/g_{substrate}$, (-▲-) 2.0 $mg_{protein}/g_{substrate}$, (-■-) 4.0 $mg_{protein}/g_{substrate}$
(b) Pectinase 444L (-◆-) 0.5 $mg_{protein}/g_{substrate}$, (-■-)1.0 $mg_{protein}/g_{substrate}$, (-▲-) 2.0 $mg_{protein}/g_{substrate}$, (-×-) 4.0 $mg_{protein}/g_{substrate}$
(c) Rohapect B1L (-◆-) 0.05 $mg_{protein}/g_{substrate}$, (-■-) 0.1 $mg_{protein}/g_{substrate}$, (-▲-) 0.3 $mg_{protein}/g_{substrate}$, (-Δ-) 0.5 $mg_{protein}/g_{substrate}$, (-×-) 1.0 $mg_{protein}/g_{substrate}$, (-●-) 2.0 $mg_{protein}/g_{substrate}$
(d) Galactomannanase ACH - (-◆-) 0.05 $mg_{protein}/g_{substrate}$, (-■-) 0.1 $mg_{protein}/g_{substrate}$, (-▲-) 0.5 $mg_{protein}/g_{substrate}$, (-Δ-) 1.0 $mg_{protein}/g_{substrate}$, (-×-) 2.0 $mg_{protein}/g_{substrate}$, (-●-) 4.0 $mg_{protein}/g_{substrate}$.

The effect of temperature on the dissolution of the sediment was also studied, since during the manufacture of coffee the sediment is mostly observed at low temperatures. The kinetic curves for the release of reducing sugars and the amount of sediment are shown in Figures 4 and 5 for the enzymes Rohapect B1L and Galactomannanase ACH, respectively, at five different temperatures (35, 40, 45, 50 and 55°C).

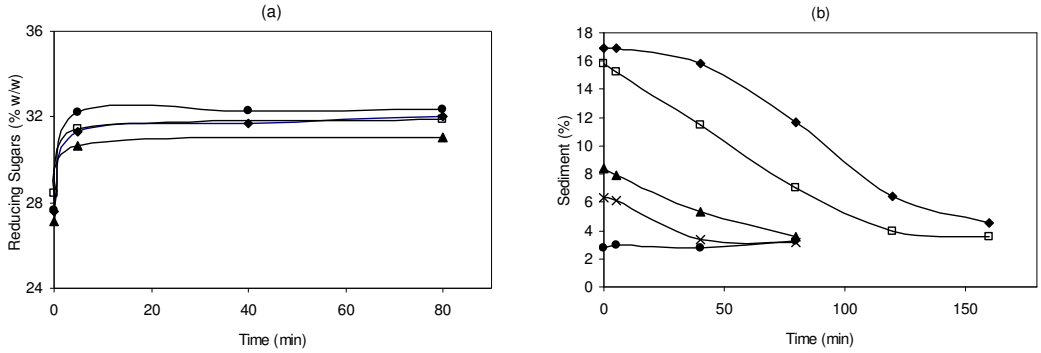


**Figure 4. Time course curves of the enzymatic hydrolysis of coffee extracts with Rohapect B1L. The effect of temperature on: (a) the release of reducing sugars and (b) the amount of sediment.**

(-◆-) 35°C, (- -) 40°C, (-▲-) 45°C, (-×-) 50°C, (-●-) 55°C

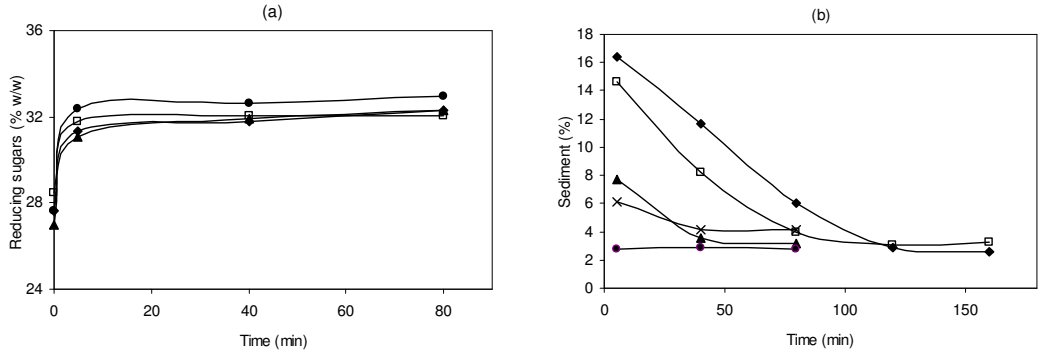


**Figure 5. Time course curves of the enzymatic hydrolysis of coffee extracts with Galactomannanase ACH. The effect of temperature on: (a) the release of reducing sugars and (b) the amount of sediment.**

(-◆-) 35°C, (- -) 40°C, (-▲-) 45°C, (-×-) 50°C, (-●-) 55°C

Hydrolysis started quickly, as can be deduced from the reducing sugars profiles. The concentration of reducing sugars increased during the first 5 minutes and maintained constant until the end of the reaction, independent of temperature. However, while the formation of total reducing sugars ceased after 5 min, the decrease in sediment showed different kinetics, depending on which temperature was used for hydrolysis. The fact that the reduction in sediment persisted while the release of reducing sugars had finished, indicates that the commercial preparations used in this work contained a mixture of

enzymes with a wide spectrum of different activities, such as high activities of endo-enzymes, for example, endo-mannanase, and other activities.

The present experiments showed that the initial amount of sediment decreased from 16 to 3.5% when the temperature increased from 35 to 55°C. With an increase in extract temperature, the amount of sediment decreases, but according to the manufacturer becomes insoluble again on cooling. In the present work, the coffee extract was treated at five different temperatures, but the best temperature should be determined by the manufacturer.

## 4. CONCLUSIONS

This study showed that both the whole extract and its insoluble faction were composed mainly of carbohydrates and proteins, while the insoluble fraction of the sediment, besides these compounds, contained considerable amounts of insoluble lignin and lipids.

Analyzing the sugar compositions of the coffee fractions, it was observed that all of them consisted mainly of galactose, mannose and arabinose, which are the building blocks of galactomannans and arabinogalactans. The galactomannan present in the insoluble fractions had a lower galactose:mannose ratio than that found in the whole extract, which may explain the lower solubility of the insoluble fractions.

The insoluble fractions of the extract and sediment were treated with commercial enzymes. After analysis of the sugar composition, it was shown that the main sugars released were arabinose, galactose and mannose.

Four enzymes, Biopectinase CCM, Pectinase 444L, Rohapect B1L and Galactomannanase ACH, were considered efficient in treating the sediment, but of these only Rohapect B1L and Galactomannanase ACH were economically viable for an industrial application. The lowest enzyme concentration required to reduce the sediment to approximately 3.5% was 0.3 and 0.1 mg protein/g substrate, for Rohapect B1L and Galactomanannase ACH, respectively.

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# *CAPÍTULO IV*

## HIDRÓLISE ENZIMÁTICA DE EXTRATO DE CAFÉ: DETERMINAÇÃO DA MASSA MOLECULAR, PERFIL DE MONOSSACARÍDEOS E AVALIAÇÃO SENSORIAL

Este capítulo é referente ao artigo "*Enzymatic hydrolysis of coffee extract: Determination of molecular weight, monosaccharides profile and sensory evaluation*", o qual será submetido à publicação ao periódico *Food Chemistry*.

No capítulo anterior, foram determinadas as mais eficientes e viáveis preparações enzimáticas que atuam na redução de sedimento em extratos de café, as quais foram Rohapect B1L e Galactomananase ACH. Neste trabalho o extrato de café foi hidrolisado por estas preparações enzimáticas e a massa molecular, o perfil de monossacarídeos e a avaliação sensorial foram investigados a fim de comparar os extratos hidrolisados e o extrato não hidrolisado. A massa molecular média dos componentes do extrato controle foi 18 kDa, a qual decresceu 6,6% após o tratamento enzimático com Rohapect B1L. O principal açúcar liberado por este produto foi manose, sugerindo sua ação sobre as cadeias de mananos do café, devido a alta atividade de β-mananase. Nas análises sensoriais, provadores treinados verificaram uma pequena a moderada diferença entre o extrato controle e os extratos hidrolisados; entretanto, o tratamento enzimático não alterou a aceitação das amostras por parte dos consumidores.

# ENZYMATIC HYDROLYSIS OF COFFEE EXTRACT: DETERMINATION OF MOLECULAR WEIGHT, MONOSACCHARIDES PROFILE AND SENSORY EVALUATION

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**Abstract**

Coffee extracts were hydrolyzed by two commercial enzyme preparations (Rohapect B1L and Galactomannanase ACH), and molecular weight measurement, monosaccharides profile and sensory evaluation were performed for comparison of treated and non treated extracts. The weight-average molecular weight of the non treated extract components was 18 kDa, which decreased 6.6% after hydrolysis with Rohapect B1L, while the polidispersity increased from 1.48 to 1.63. The main sugar released by this enzyme preparation was mannose, suggesting its action on mannans chains, due to the high activity of β-mannanase. In the sensory evaluation, trained panelists verified a small to moderate difference between the extracts treated by both enzymes and the control; however, the enzymatic treatment did not alter the acceptance of the coffee extract, as shown in the acceptance test done by coffee consumers.

*Keywords:* coffee extract, enzymatic hydrolysis, molecular weight, monosaccharides, sensory evaluation.

## 1. INTRODUCTION

Coffee is one of the world's most widely consumed beverages. Polysaccharides are the main constituents of green, roasted and instant coffee, and they are mainly galactomannans, arabinogalactans and cellulose (BRADBURY, 2001; FISCHER *et al*., 2001; NUNES & COIMBRA, 2001; NUNES *et al*., 2006; OOSTERVELD *et al*., 2003; REDGWELL *et al*., 2002). The insolubility of galactomannans and arabinogalactans may lead to the formation of sediments during the instant coffee manufacturing, which is considered to be a quality defect and limits the utilization of the product.

Different authors have investigated the use of enzymes to hydrolyze coffee polysaccharides. NUNES *et al*. (2006) isolated the galactomannans from light and dark roasted coffee infusions and hydrolyzed them with endo-mannanase, decreasing the molecular weight of these polysaccharides. Mannanase can also be used to reduce the viscosity of coffee extract, improving the effectiveness of the concentration process and reducing drying costs in the production of instant coffee (JARDINE, 1991; SACHSLEHNER *et al*., 2000).

In recent work done by Delgado and co-authors, coffee extracts were hydrolyzed by several commercial enzyme preparations in order to reduce sediments. The best preparations were selected to obtain the minimum costs and maximum yields, which were reached with Rohapect B1L and Galactomannanase ACH (DELGADO *et al*., 2008). Thus, the aim of this work was to hydrolyze coffee extract by these enzyme preparations and perform the molecular weight measurement, monosaccharides profile and sensory evaluation before and after treatment, comparing the treated extracts to the non treated one.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Coffee extract (20% of solids) was supplied by Cia Iguaçu de Café Solúvel (Cornélio Procópio, Paraná, Brazil). Rohapect B1L was supplied by ABEnzymes and Galactomannanase ACH by Sumizyme. Substrates and monosaccharide standards were purchased from Sigma and Fluka. Polyethylene oxide standard was purchased from Polymer Standard and Dextran from American Polymer. All other reagents and solvents were analytical grade chemicals.

### 2.2. Activity assays

Enzymes and substrates were prepared in 50 mM citrate buffer, pH 5.0 and the enzymatic activities were carried out at 50°C. Activities were expressed in nkat/$mg_{protein}$, where 1 nkat is defined as the amount of enzyme that catalyses the conversion of 1 nmol of substrate to product in 1 second, under the conditions of the assay. One nkat is equal to 0.06 IU. The soluble protein concentration of the enzymes was determined according to the Lowry assay (LOWRY *et al*., 1951), after precipitation of the proteins from the samples using trichloroacetic acid. Enzymatic activities were determined according to the following assays:

*β-Glucosidase (EC 3.2.1.21), α-galactosidase (EC 3.2.1.22), β-mannosidase (EC 3.2.1.25), α-arabinosidase (EC 3.2.1.55) and β-xylosidase (EC 3.2.1.37)* activities were determined by measuring the amount of *p*-nitrophenol released from the respective *p*-nitrophenyl-glycosides: 1 mM *p*-nitrophenyl-β-D-glucopyranoside, 1 mM *p*-nitrophenyl-α-D-galactopyranoside, 1 mM *p*-nitrophenyl-β-D-mannopyranoside**,** 2 mM *p*-nitrophenyl-α-L-arabinofuranoside, 5 mM *p*-nitrophenyl-β-D-xylapyranoside (BAILEY & NEVALAINEN, 1981; BAILEY & LINKO, 1990; POUTANEN *et al*., 1987).

*β-mannanase (EC 3.2.1.78)* activity was determined according to STÅLBRAND *et al*. (1993), using 0.5% locust gum as substrate.

*β-Xylanase (EC 3.2.1.8)* activity was determined using 1% xylan (4-*O*-methyl glucuronoxylan from *birchwood*), according to BAILEY *et al*. (1992).

*β-Endoglucanase (EC 3.2.1.4)* activity was done according to BAILEY & NEVALAINEN (1981) and IUPAC (1987), using 1% HEC (hydroxyethyl cellulose) as substrate.

*Endo-β-galactanase (EC 3.2.1.89)* activity was determined according to LUONTERI *et al*. (2003). The substrate used was 0.5% Lupin β-1,4-galactan.

*Arabinase* activity was done using a linear α-1,5 arabinan (97% purity) as substrate in a concentration of 0.4%.

*Polygalacturonase (EC 3.2.1.15)* activity was determined modifying the method described by BAILEY & PESSA (1990), using 0.4% polygalacturonic acid as substrate.

*Pectinlyase (EC 4.2.2.10)* activity was determined according to MANACHINI *et al*. (1988), using pectin from citrus as substrate.

**2.3. Enzymatic hydrolysis of coffee extract**

Enzymatic hydrolysis of coffee extract was carried out at 40°C for 160 minutes and the enzyme concentration was 0.3 and 0.1 $mg_{protein}/g_{substrate}$, for Rohapect B1L and Galactomannanase ACH, respectively. These conditions were determined in work done by Delgado and co-authors (DELGADO *et al*., 2008). To 250 ml of extract, 6.3 ml of enzyme solution was added. Control treatments were made under similar conditions except that no enzyme was added.

**2.4. Molecular weight measurement**

The molecular weight of coffee extracts components, before and after enzymatic treatment, was determined by Gel Permeation Chromatography (GPC), using a Viscotek Chromatography model TDA 302, equipped with three detectors (Refractive Index - RI, Differential Viscosimeter – DP and Right Angle Light Scattering – RALS). An Ultrahydrogel Guard Column (40 x 6.0 mm) followed by two Ultrahydrogel Linear Columns (300 x 7.8 mm, Waters) were used in series, the mobile phase was 0.1 M $NaNO_3$, the flow rate was 0.8 ml/min and the column temperature was 40°C. Polyethylene oxide standard was used to calibrate the equipment and dextran standard to check the calibration. Before injection (200 µl), the samples were filtered through a 0.2 µm membrane filter. Data were obtained from the Software OmniSec 4.1 (Viscotek).

**2.5. Monosaccharide analysis**

The monosaccharides were determined by High Performance Anion Exchange Chromatography (HPAEC), using a Dionex DX 300 system (Dionex Corp. Sunnyvale CA), equipped with a pulsed amperometric detector. The analytical column was CarboPac PA1 (250 x 4 mm) and the guard column was CarboPac PA1 (25 x 3 mm). All determinations were carried out at 30°C, the mobile phase was 1 mM NaOH and the flow rate was 1 ml/min. All the standards and samples were filtered through a 0.2 µm membrane filter before injection. For the analysis of total carbohydrates, the coffee extract was submitted to Saeman hydrolysis (SELVENDRAN *et al*., 1979) and the monosaccharides determined.

**2.6. Sensory analysis**

Two sensorial tests were performed on the coffee extracts before and after enzymatic treatment (MEILGAARD *et al*., 1988):

*Difference test*

Difference-from-control test was used to compare the extracts treated by enzyme preparations and the non-treated one, called as control sample. This test was carried out by 14 trained panelists comprised of staff of Food Technology Institute of Campinas (ITAL), using a 7-point scale, which ranged from '1- no difference' to '7- extreme difference' from the control. Samples were diluted with water (1:5) and served to the panelists at 70°C in internally black cups, in booths illuminated with red light. Each panelist received the control sample and three samples coded with a randomized three digit number (amongst them the control). The data were analyzed by ANOVA, and the difference between control and samples' means were analyzed by Dunnett's test at a significance level ($p< 0.05$).

*Affective test*

The acceptance test was carried out to evaluate the acceptability of the coffee extract before and after enzymatic hydrolysis. Thirty consuming assessors recruited amongst the students and staff of Food Technology Institute of Campinas (ITAL), without restriction on age, social class and gender, were instructed to determine how much they liked or disliked the coffee extracts, using a 9-point hedonic scale, which ranged from '1- disliked extremely' to '9- liked extremely'. Samples were diluted with water (1:5) and served to the panelists at 70°C in white plastic cups, in booths illuminated with fluorescent lights. Each sample was coded with a randomized three digit number and the panelists were instructed to sweet all the samples by the same way. The data were analyzed by ANOVA followed by Tukey's test at a significance level ($p<0.05$).

## 3. RESULTS AND DISCUSSION

### 3.1. Enzyme activity

Rohapect B1L and Galactomannanase ACH were assayed for different cellulolytic, hemicellulolytic and pectinolytic activities, and the results are present in Table 1. Polygalacturonase was the main activity of Rohapect B1L (9277 nkat/$mg_{protein}$), followed by β-mannanase (1280 nkat/$mg_{protein}$). According to the manufacturer, this product is a pectinase with high activities of hemicellulases. As expected, the main activity of Galactomannanase ACH was β-mannanase, which was 2540 nkat/$mg_{protein}$; this preparation was also found to have polygalacturonase activity (244 nkat/$mg_{protein}$). Both products presented low levels of β-xylanase and β-endoglucanase activities. Therefore, due to the high activity of β-mannanase, both enzyme preparations can be use in coffee treatment, however, a synergism between the different activites must occur, improving the hydrolysis efficiency.

**Table 1: Enzymatic activities of Rohapect B1L and Galactomannanase ACH (nkat/$mg_{protein}$*)**

| Activity | Rohapect B1L | Galactomannanase ACH |
|---|---|---|
| β-Glucosidase | 19 | 3 |
| α-Galactosidase | n.d. | n.d. |
| β-Mannosidase | 2 | n.d. |
| α-Arabinosidase | 7 | n.d. |
| β-Xylosidase | 3 | 2 |
| β-Endoglucanase | 92 | 24 |
| β-Galactanase | 5 | 7 |
| β-Mannanase | 1280 | 2540 |
| Arabinase | 2 | n.d. |
| β-Xylanase | 190 | 87 |
| Polygalacturonase | 9277 | 244 |
| Pectinlyase | n.d. | n.d. |

* Protein content: Rohapect B1L (37.9 mg/ml) and Galactomannanase ACH (0.3 mg/mg)
n.d.: not detected

### 3.2. Molecular weight

Gel Permeation Chromatography (GPC) is the most widely used technique to determine molecular weights of natural and synthetic polymers and has been used on characterization of polysaccharides, separating the molecules according to their size in solution (ALTGET & SEGAL, 1971; CHENG *et al*., 2002; FREITAS *et al*., 2005; POPOVICI & SCHOENMAKERS, 2005). A triple detector system permits the determination of molecular weights, without employing the conventional calibration curve.

Weight-average (Mw) and number-average (Mn) molecular weights, polydispersity (Mw/Mn) and hydrodynamic radius (Rh) of coffee extract components were determined before and after enzymatic treatment and the results are described in Table 2. The chromatographic profiles obtained from refractive index and viscosimeter detectors are present in Figure 1.

**Table 2: Weight-average (Mw) and number-average (Mn) molecular weights, polydispersity (Mw/Mn) and hydrodynamic radius (Rh) of coffee extract components**

| Sample/Treatment | Mw (kDa) | Mn (kDa) | Mw/Mn | Rh (nm) |
|---|---|---|---|---|
| Control | 18.10 | 12.22 | 1.48 | 2.13 |
| Rohapect B1L | 16.91 | 10.37 | 1.63 | 1.95 |

The weight-average molecular weight of the control extract components was approximately 18 kDa, which was higher than the values reported in the literature for coffee polysaccharides. NAVARINI *et al*. (1999) fractionated the polysaccharides extracted from roasted coffee and obtained three fractions with the following weight-average molecular weight (Mw): 10.9, 8.3 and 6.8 kDa, respectively. REDGWELL *et al*. (2002) found for galactomannans and arabinogalactans of roasted coffee a weight-average molecular weight around 10 kDa. In the work reported by LELOUP & LIARDON (1993), the molecular weight of polysaccharides extracted from green and roasted coffee ranged from 0.2 to 1000 kDa, influenced by the extraction conditions.

As shown in Table 2, after enzymatic hydrolysis with Rohapect B1L, the weight-average molecular weight decreased 6.6%; the number-average molecular weight and the hydrodynamic radius also decreased. The polydispersity increased from 1.48 to 1.63 after the enzymatic treatment, indicating that part of the long polymer chains might have been hydrolyzed and shorter chains were generated, what can also be observed in Figure 1 (a). According to the response of viscosimeter detector, it seems that the viscosity of the coffee extract decreased after hydrolysis with Rohapect B1L (Figure 1(b)).

Therefore, the enzymatic treatment of coffee extract leaded to a decrease of molecular weights of coffee extract components, resulted by the hydrolysis of the glycoside chains of polysaccharides.

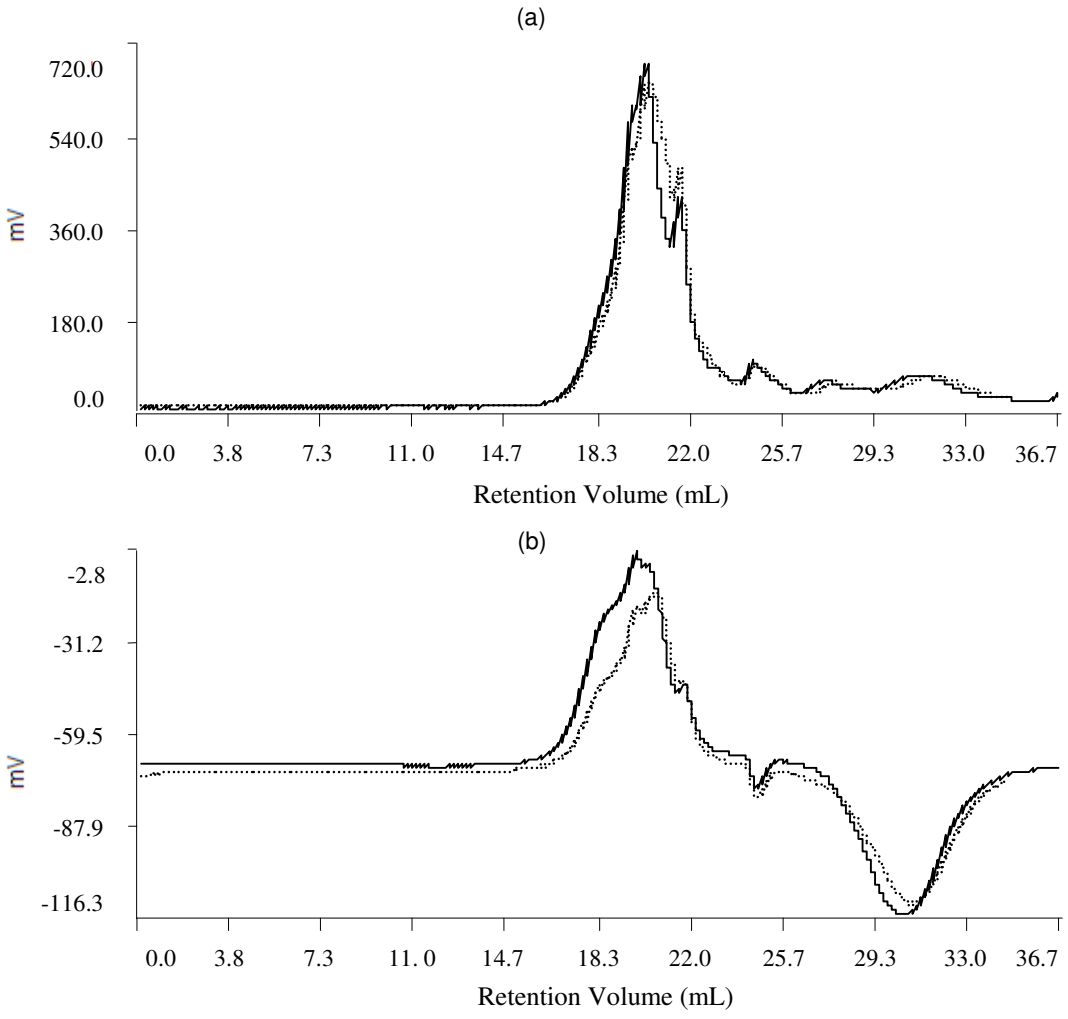


**Figure 1. Chromatographic profiles of coffee extract components obtained from (a) Refractive Index and (b) Viscosimeter detectors**

——— *Control extract* ····· *Extract hydrolyzed by Rohapect B1L*

### 3.3. Monosaccharides profile

The profiles of total and free sugars before and after enzymatic treatment of the coffee extract were determined and the results are shown in Table 3. The total carbohydrates of coffee extract were composed mainly by galactose (19.14%), mannose (14.41%) and arabinose (4.56%), indicating the presence of galactomannans and arabinogalactans. Galactose, mannose and arabinose were the predominant free monosaccharides of the control extract. The hydrolysis of coffee extract with Rohapect B1L resulted in a release of arabinose, fructose and mainly mannose, suggesting the cleavage of mannans by the enzyme preparation. It can be observed that the release of sugars was low, avoiding the formation of syrup and maintaing the quality of the product.

**Table 3: Monosaccharide profiles of coffee extract – total sugars and free sugars before and after hydrolysis with Rohapect B1L (% dry bases)**

| Monosaccharide | Total sugars * | Free sugars - control extract | Free sugars - extract hydrolyzed by Rohapect B1L |
|---|---|---|---|
| Arabinose | 4.56 | 0.55 | 0.61 |
| Galactose | 19.14 | 0.45 | 0.45 |
| Glucose | 1.39 | 0.14 | 0.19 |
| Xylose | 0.17 | - | - |
| Mannose | 14.41 | 0.37 | 0.72 |
| Fructose | 0.52 | 0.26 | 0.42 |

* After acid hyrolysis

Limits of acceptability of three carbohydrates in instant coffee have been defined by AFCA-SOLE (the European Soluble Coffee Manufacturer's Association) to assure the highest quality of the beverage. Table 4 describes the maximum levels of total glucose, total xylose and free fructose allowed to be present in instant coffee. Higher levels of these carbohydrates might indicate adulterations. By HPAEC-PAD, it is possible to detect these adulterations in instant coffee, which can include cereals, caramels, maltodextrin, glucose syrup, starch and coffee husks (BERGER *et al*., 1991). According to PRODOLLIET *et al*.

(1995), high levels of free mannitol and total xylose indicate the presence of coffee husks or parchments; while high level of total glucose indicates the presence of cereals or caramelized sugars. Free fructose, free glucose and sucrose are also good indicatives of adulteration. Samples with high levels of total galactose and mannose indicate a pure instant coffee.

**Table 4: Tolerable control limits for carbohydrates in instant coffee**

| Carbohydrate | Maximum Content in Pure Coffee (%) | Control Limit (%) |
|---|---|---|
| Total Glucose | 2.1 | 2.6 |
| Total Xylose | 0.4 | 0.6 |
| Free Fructose | 0.6 | 1.0 |

Source: FAO (2007)

As shown in Table 3, the total glucose and xylose contents of the control coffee extract were 1.39 and 0.17%, respectively. The level of free fructose was 0.26%. All these values were below to the tolerable limits described in Table 4, indicating that the coffee used in the present work was not adulterated; therefore, it is possible to produce a high quality instant coffee from this product. Concerning to the free sugars, the main monosaccharides of the control extract were arabinose (31%), galactose (25%) and mannose (21%), indicating again the good quality of the extract. After hydrolysis of the coffee extract with Rohapect B1L, the concentration of free fructose increased to 0.42%, which is still below of the limit established in Table 4 (1.0%).

Therefore, the enzymatic treatment did not affect the quality of the coffee extract, concerning to the monosaccharides composition.

### 3.4. Sensory evaluation

Coffee beverage has some distinctive sensorial properties, which should not be changed by enzymatic treatment, since they determine the consumers' acceptance of the product. Therefore, sensory evaluation of coffee extracts is extremely important in addition

to chemical parameters. According to STONE & SIDEL (1993) and KUTI *et al*. (2004), sensory evaluation is the identification, measurement, analysis and interpretation of the characteristics of foods and materials as they are perceived through the senses of sight, smell, taste, touch and hearing. Two sensorial analyses were conducted on the coffee extract to study possible effects of the enzymatic treatment on its sensory quality.

Table 5 summarizes the results of the difference-from-control test, which is a technique used to determine whether there is difference between one or more samples and a control, and to estimate the size of such difference (MEILGAARD *et al*., 1988; CARR *et al*., 2006). According to the results, the extracts treated by the enzyme preparations were statistically different from the control extract at a significance level ($p<0.05$). The mean values for treatments with Rohapect B1L and Galactomannanase ACH were, respectively, 3.57 and 3.07, which differences were considered small to moderate, according to the scale used. Some panelists had commented that the treated samples were more acid and bitterer then the control one.

**Table 5: Average values attributed by trained panelists for coffee extracts – *Difference-from-control test***

| Sample/Treatment | Mean values |
|---|---|
| Control | $1.29 \pm 0.47^{a}$ |
| Rohapect B1L | $3.57 \pm 1.22^{b}$ |
| Galactomannanase ACH | $3.07 \pm 1.54^{b}$ |

Different letters in the same column are significantly different ($p< 0.05$) according to Dunnett's test.

By an acceptance test, it is possible to determine if the difference perceived by the trained panelists is meaningful to consumers. Then, this test was performed to determine how well the consumers liked or disliked the samples and the results are summarized in Table 6. It can be observed that the control and hydrolyzed samples had close acceptances and consumers liked them slightly, according to the hedonic scale. The percentage of acceptance was approximately 66% for all samples.

Since the control extract had close acceptance to the treated ones, it seems that some changes in sensorial properties can occur in the next steps of instant coffee manufacturing, such as concentration and drying process.

**Table 6: Average values attributed by panelists for coffee extracts – *Acceptance test***

| Sample/Treatment | Mean values |
|---|---|
| Control | $5.8 \pm 2.0^{a}$ |
| Rohapect B1L | $5.7 \pm 2.1^{a}$ |
| Galactomannanase ACH | $5.7 \pm 1.9^{a}$ |

Same letters are not significantly different ($p < 0.05$) according to Tukey's test.

Despite of the trained panelists had verified a small to moderate difference between the treated extracts and the control, the consumers' acceptance for these samples was very close. Therefore, the results of the overall sensory evaluation showed that the utilization of enzymes in coffee treatment resulted in an acceptable coffee beverage in terms of sensorial properties.

## 4. CONCLUSIONS

Coffee extract was hydrolyzed by two commercial enzyme preparations, Rohapect B1L and Galactomannanase ACH, which β-mannanase activities were 1280 and 2540 $nkat/mg_{protein}$, respectively. The control and enzyme treated extracts were submitted to molecular weight measurement, monosaccharides profile determination and sensory evaluation.

The weight average molecular weight of the control extract components was approximately 18 kDa, which decreased 6.6% after hydrolysis with Rohapect B1L. The polidispersity increased from 1.48 to 1.63 with the treatment, indicating the hydrolysis of long chains and the generation of shorter ones.

The total and free sugars presented in coffee extract were mainly galactose, mannose and arabinose. After hydrolysis with Rohapect B1L, mannose was the main sugar

released, suggesting that this enzyme preparation acted on the hydrolysis of mannans of coffee extract. The levels of total glucose, total xylose and free fructose indicated absence of adulterations before and after enzyme treatment.

Concerning to the sensory evaluation, difference-from-control test showed that the panelists verified a small to moderate difference between treated extracts and the control. However, by the acceptance test, done by coffee consumers, it was verified that the enzymatic treatment did not alter the acceptability of the coffee extract.

Therefore, Rohapect B1L is well suited for the treatment of coffee extract. Mannans chains were hydrolyzed and the chemical and sensorial qualities were maintained.

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# *CAPÍTULO V*

# ANÁLISE ECONÔMICA PRELIMINAR

## AUMENTO DO RENDIMENTO NA PRODUÇÃO DE CAFÉ SOLÚVEL PELO PROCESSO ENZIMÁTICO: ANÁLISE ECONÔMICA PRELIMINAR

Visando uma possível aplicação de enzimas no processamento industrial de café solúvel, faz-se necessário uma avaliação preliminar entre o custo das enzimas, o aumento do rendimento do processo e o custo do investimento. Considerou-se o investimento necessário para a inserção de uma nova etapa na planta em operação e também não foram considerados os custos de utilidades.

Como apresentado em capítulos anteriores, a preparação enzimática Rohapect B1L proporcionou um ótimo rendimento na redução de sedimento sem alteração da qualidade química e sensorial do extrato de café. Assim, esta enzima foi utilizada no estudo da viabilidade econômica. A seguir, seguem informações necessárias para os cálculos:

- ✓ *Preço do café verde*
  - US$ 112,00/saca (preço médio de 2007 de acordo com ABIC, 2008).
- ✓ *Preço de venda do café solúvel*
  - US$ 5,00/kg
- ✓ *Concentração da enzima - Rohapect B1L*
  - Concentração de enzima no tratamento enzimático = 0,3 $mg_{proteína}/g_{sólidos}$
  - Teor de proteína da enzima = 37,9 $mg_{proteína}$/ml

Portanto, a concentração de enzima necessária para o tratamento enzimático é 0,79% (m/m – enzima/sólidos).

- ✓ *Preço da enzima*
  - US$ 26,00/ kg
- ✓ *Reator*
  - Aço inoxidável - 1500 litros
  - US$ 14.000,00

De acordo com os resultados obtidos no Capítulo III, a 45°C o teor de sedimento do extrato de café foi aproximadamente 8,5%, o qual decresceu até 3,5% após hidrólise enzimática com Rohapect B1L. A Tabela 1 apresenta o rendimento da produção de café solúvel para os processos convencional e enzimático, partindo-se de 100 kg de café verde. Observa-se a obtenção de 37,5 kg de café solúvel pelo processo convencional, enquanto que pelo processo enzimático este valor é de 39,6 kg.

**Tabela 1. Rendimento da produção de café solúvel - processos convencional e enzimático**

| | **Convencional (kg)** | **Enzimático (kg)** |
|---|---|---|
| Café verde | 100,0 | 100,0 |
| Extrato de café | 41,0 | 41,0 |
| Sedimento | 3,5 | 1,4 |
| Café solúvel | 37,5 | 39,6 |

A análise econômica preliminar da utilização de enzimas no processo de produção de café solúvel foi realizada para uma base de cálculo de 100 kg/h de café verde. Os custos, receitas e lucro anuais obtidos para os processos convencional e enzimático estão descritos na Tabela 2. De acordo com os resultados apresentados, verifica-se que o lucro obtido pelo processo enzimático é maior que o lucro obtido pelo processo convencional, sugerindo assim a viabilidade da aplicação de enzimas no processo estudado.

**Tabela 2. Custos, receitas e lucro bruto anuais da produção de café solúvel - processos convencional e enzimático*.**

| | Convencional (US$/ano) | Enzimático (US$/ano) |
|---|---|---|
| Custo - café verde | 1.400.000,00 | 1.400.000,00 |
| Custo - enzima | - | 64.350,00 |
| Receita - café solúvel | 1.406.812,50 | 1.483.687,50 |
| Lucro bruto | 6.812,50 | 19.337,50 |

* Base de cálculo: 100 kg/h café verde. 7500 horas/ano.

Para a implantação desta etapa deve-se considerar o investimento inicial, um reator tanque agitado de aço inoxidável cotado à US$ 14.000,00. Estimou-se o tempo de retorno do investimento pelo método *payback* (CÉSAR, 2005). De acordo com RODRIGUES (2007), esta é uma medida comumente utilizada como uma avaliação rápida da rentabilidade, não considerando o valor do dinheiro no tempo. Para um processamento de 100 kg/h de café verde, o prazo de retorno do investimento (*payback*) é de aproximadamente 1 ano, considerando-se o somente lucro adicional proporcionado pelo processo enzimático.

Portanto, a análise econômica preliminar revelou a potencialidade de aplicação de enzimas no processamento de café.

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# *CAPÍTULO VI*

## CONCLUSÕES

A fim de selecionar enzimas que poderiam atuar na redução de sedimentos em extratos de café, diferentes atividades presentes em preparações comerciais de pectinases foram determinadas. Observou-se que estas preparações comerciais são compostas basicamente por endo-enzimas, hidrolisando as ligações internas dos respectivos substratos. Rohapect B1L foi o produto que apresentou a maior atividade de mananase, podendo ser potencialmente empregado na hidrólise de galactomananos. Esta etapa nos possibilitou também sugerir diferentes aplicações para as preparações enzimáticas estudadas, devido a atividades de endoglucanase, galactanase, mananase e xilanase, além da atividade atestada pelos fabricantes.

O extrato de café e sedimento foram separados em suas frações solúveis e insolúveis, as quais foram caracterizadas quanto à composição. Os principais constituintes do extrato de café e sua fração insolúvel foram carboidratos e proteínas. A fração insolúvel do sedimento apresentou também uma fração significativa de lignina. Sugerimos uma associação entre lignina, proteínas e carboidratos, ocasionando a não solubilização deste material.

Pela análise dos carboidratos do extrato e suas frações, galactose, arabinose e manose foram os principais monossacarídeos obtidos após hidrólise ácida, confirmando assim que os polissacarídeos do café são essencialmente galactomananos e arabinogalactanos. Considerando as frações insolúveis, o principal polissacarídeo encontrado foi um galactomanano com baixo grau de ramificação, e portanto, pouco ou não solúvel.

Pectinase 444L, Biopectinase CCM, Rohapect B1L e Galactomananase ACH foram as preparações enzimáticas que apresentaram maior eficiência na redução do sedimento, porém Rohapect B1L e Galactomananase ACH foram as mais viáveis, cujas

concentrações ótimas foram 0,3 e 0,1 $mg_{proteína}/g_{substrato}$, respectivamente.

A massa molecular dos componentes do extrato não hidrolisado foi 18kDa, a qual decresceu 6.6% após hidrólise com Rohapect B1L.

Manose foi o principal açúcar liberado pelo complexo Rohapect B1L, confirmando assim a ação desta enzima sobre as cadeias de mananos presentes no extrato de café. Podemos concluir também que a hidrólise enzimática não alterou os índices estabelecidos para alguns carboidratos em café solúvel, o que poderia sugerir adulterações no extrato estudado.

Nas análises sensoriais provadores treinados verificaram uma pequena a moderada diferença entre o extrato controle e os extratos hidrolisados pelas preparações Rohapect B1L e Galactomananase ACH; entretanto, o tratamento enzimático não alterou a aceitação do extrato por parte dos consumidores.

Na avaliação econômica preliminar, verificou-se um aumento no lucro bruto quando empregado o processo enzimático, sugerindo assim a viabilidade da aplicação de enzimas na produção de café solúvel.

Assim, os resultados mostraram boas perspectivas para a utilização de enzimas no processo de redução de sedimentos em extratos de café.

# *CAPÍTULO VII*

## SUGESTÕES PARA TRABALHOS FUTUROS

- ✓ Investigar a imobilização da enzima, visando sua reutilização e minimização dos custos.

- ✓ Verificar a eficiência da mistura Rohapect B1L/Galactomananase ACH na redução de sedimento.

- ✓ Avaliar o processo em escala piloto.

- ✓ Investigar a utilização de complexos contendo tanases/esterases na redução de sedimento.

- ✓ Verificar a interferência das enzimas nos compostos voláteis do café.

# ANEXO 1

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Analytical Methods

# Sediments in coffee extracts: Composition and control by enzymatic hydrolysis

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**Abstract**

The water-insolubility of some coffee extract components is one of the major limitations in the production of instant coffee. In this work, fractions from coffee extracts and sediments were prepared, and their chemical composition determined. Based on the carbohydrate analysis, galactomannan was found to be the main polysaccharide component of the insoluble fractions and probably responsible for sediment formation. The suitability of twelve commercial enzymes for the hydrolysis of the insoluble fractions was investigated. Pectinase 444L was the most effective enzyme in releasing sugars, mainly mannose and galactose, from these substrates. Biopectinase CCM, Rohapect B1L, Pectinase 444L and Galactomannanase ACH were found to be the most effective enzymes for reducing the sediment of coffee extracts. The highest sediment reduction was obtained using Rohapect B1L and Galactomannanase ACH, at enzyme concentrations of 0.3 and 0.1 mg protein/g substrate, respectively.


*Keywords*: Coffee polysaccharides; Galactomannans; Instant coffee; Enzymatic hydrolysis; Coffee composition; Sediment

## 1. Introduction

Coffee is one of the world's most widely consumed beverages. The chemical composition of the coffee cell wall has not been studied in detail, since it is difficult to dissolve, extract and digest (Kasai, Konishi, Iwai, & Maeda, 2006). Polysaccharides comprise nearly 50% of the green coffee bean weight (Fischer, Reimann, Trovato, & Redgwell, 2001; Nunes & Coimbra, 2001; Nunes, Reis, Domingues, & Coimbra, 2006), and those found in the coffee cell wall are mainly galactomannan, arabinogalactan and cellulose (Fischer et al., 2001; Oosterveld, Harmsen, Voragen, & Schols, 2003; Redgwell, Trovato, Curti, & Fischer, 2002). Arabinogalactans consist of a main chain of 1→3 linked galactose branched at C-6, with side chains containing arabinose and galactose. Galactomannans consist of a main chain of 1→4 linked mannan with galactose unit side chains linked at C-6, and different degrees of branching (Bradbury & Halliday, 1990; Navarini et al., 1999; Nunes et al., 2006). The structures of the polysaccharides of industrialized coffee products depend on the degree of roasting (Nunes & Coimbra, 2002; Oosterveld, Voragen, & Schols, 2003; Redgwell, Trovato, et al., 2002).

The main obstacle to characterizing the coffee cell wall is the high proportion of insoluble polymers (Bradbury & Atkins, 1997; Fischer et al., 2001; Redgwell, Curti, Fischer, Nicolas, & Fay, 2002). The solubility increases with increasing degree of branching and decreasing molecular weight (Nunes & Coimbra, 2001). Arabinogalactans dissolve better than do linear mannans, which can easily precipitate, and one of the reasons for this non-dissolution is an association of linear mannans to form crystalline regions (Bradbury & Atkins, 1997). This could be the reason for the formation of sediment during the manufacture of instant coffee. According to Fischer et al. (2001), the

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difficulty in dissolving the cell wall polysaccharides indicates an intimate association between some of the arabinogalactan, galactomannan and cellulose molecules.

Proteins are another important component of coffee extracts. The roasting process causes degradation of the proteins into smaller products (Nunes & Coimbra, 2001). In espresso coffee, the protein content was shown to be correlated with the foam volume (Nunes, Coimbra, Duarte, & Delgadillo, 1997), and the protein is usually covalently linked to arabinogalactans (Fischer et al., 2001; Redgwell, Curti, et al., 2002; Navarini et al., 1999; Redgwell, Schmitt, Beaulieu, & Curti, 2005). Information on coffee lipids is very limited, but it has been speculated that poor quality of coffee is also due to the hydrolysis of triacylglycerols (TAGs) with the release of free fatty acids, which, in turn, are oxidized (Jham, Velikova, Muller, Nikolova-Damyanova, & Cecon, 2001; Nikolova-Damyanova, Velikova, & Jham, 1998; Segall, Artz, Raslan, Jham, & Takahashi, 2005). The main classes of lipids present in green coffee are triacylglycerols (75%) and terpene esters (14%) (Jham et al., 2001; Nikolova-Damyanova et al., 1998). There are still no reports available on the lignin content of coffee, but the lignin is found closely associated with the cellulose and hemicellulosic polysaccharides (de Vries & Visser, 2001; Dóka, Bicanic, & Bunzel, 2004; Juhász, Szengyel, Réczey, Siika-aho, & Viikari, 2005).

In Brazil, coffee extracts are processed into instant coffee or concentrated extract for exportation. However, during storage and commercial circulation, sediment is sometimes observed in the extracts, which is considered to be a quality defect and limits the utilization of the product.

These days, enzymes are commonly used in many industrial applications, including the degradation of plant cell walls. Cellulases, hemicellulases and pectinases are industrially important enzymes that are sold in large amounts for many applications. Different authors have investigated the use of enzymes to hydrolyze coffee polysaccharides. Nunes et al. (2006) isolated the galactomannans from light and dark roasted coffee infusions, and hydrolyzed them with endo-mannanase, decreasing the molecular weight of these polysaccharides. Mannanase can also be used to reduce the viscosity of the extract in the production of instant coffee, improving the effectiveness of the concentration process and reducing drying costs (Sachslehner, Foidl, Foidl, Gübitz, & Haltrich, 2000). These authors hydrolyzed the coffee mannan with free and immobilized mannanase from *Sclerotium rofsii*.

The aim of this work was to determine the composition and study the enzymatic hydrolysis of coffee fractions using different commercial enzyme preparations, and then to apply the enzymatic treatment to the whole extract in order to reduce the sediment formed during coffee processing.

## 2. Materials and methods

### 2.1. Materials

Coffee extract containing sediment was supplied by Cia Iguaçu de Café Solúvel (Cornelio Procópio, Paraná, Brazil). Enzyme preparations were obtained from different sources, and are described in Table 1. Monosaccharide standards were purchased from Sigma and Fluka, and all other reagents and solvents were of the highest purity.

### 2.2. Preparation of the coffee and sediment fractions

In the process used by the Cia Iguaçu, green coffee beans were roasted and ground, and the ground coffee then percolated by hot water under high pressure to extract the solids. The extract obtained was stored in tanks at 4 °C, where the sediment formed. The whole extract (containing sediment) and the sediment alone were the samples used in the present work, being fractionated according to Fig. 1. All the fractions were freeze-dried.

### 2.3. Chemical analysis

*Neutral and acidic sugars* were analyzed according to the Saeman hydrolysis (Selvendran, March, & Ring, 1979). In this method, 10 mg of sample were first added to 0.5 ml of

Table 1
Source, major activity and protein content of the commercial enzyme preparations

| Enzyme | Source | Major activity[a] | Protein content (mg/ml) |
|---|---|---|---|
| Econase CE | AB enzymes | Cellulase | 110 ± 4.24 |
| Protease GC 106 | Genencor | Protease | 86.0 ± 1.41 |
| Novo Shape | Novozymes | Pectinase | 28.2 ± 0.21 |
| Pectinex 3XL | Novozymes | Pectinase | 17.7 ± 0.42 |
| Pectinex Ultra | Novozymes | Pectinase | 50.6 ± 0.85 |
| Biop. CCM | Biofincon | Pectinase | 40.6 ± 1.70 |
| Biop. Super 8x | Quest | Pectinase | 57.9 ± 1.46 |
| Pectinase 444L | Biocatalysts | Pectinase | 16.9 ± 0.04 |
| Rohapect B1L | AB enzymes | Pectinase | 37.9 ± 0.14 |
| Rohapect D5L | AB enzymes | Pectinase | 12.9 ± 0.33 |
| Rohapect 10L | AB enzymes | Pectinase | 67.6 ± 2.62 |
| Galactomannanase ACH | Sumizyme | Galactomannanase | 0.3 ± 0.01[b] |

[a] According to the manufacture.
[b] mg/mg (enzyme powder).

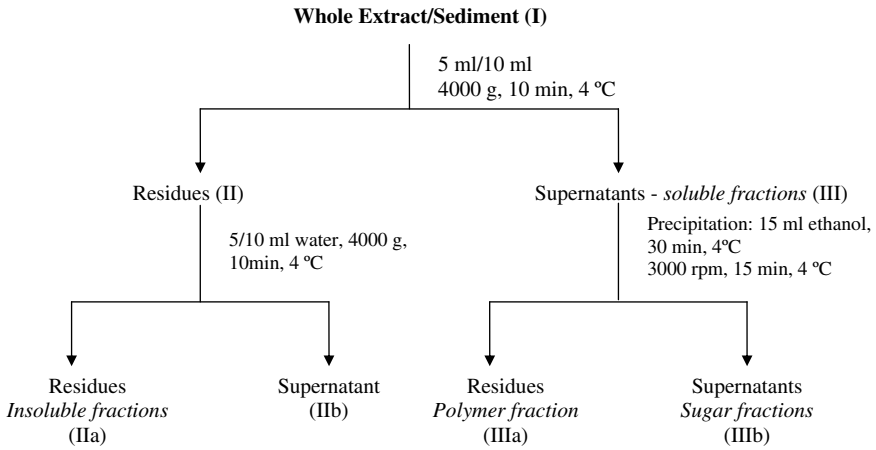


Fig. 1. Scheme for preparation of the coffee and sediment fractions.

72% $H_2SO_4$ and maintained at room temperature for 3 h. After this pre-treatment, distilled water was added to the mixture, to dilute the $H_2SO_4$ to 1 M, and incubated at 95 °C for 2 h. The monosaccharides were analyzed by high performance anion-exchange chromatography (HPAEC), using a Dionex DX 500 system (Dionex Corp. Sunnyvale CA), equipped with a GP40 gradient pump, ED40 electrochemical detector and AS 3500 autosampler. The analytical column was a CarboPac PA1 (250 × 4 mm) and the guard column was a CarboPac PA1 (25 × 3 mm). All determinations were carried out at 30 °C, using a flow rate of 1 ml/min and a gradient with pure water, NaOH and NaOAc. *Reducing sugars* were determined using the dinitrosalicylic acid (DNS) method (Bernfeld, 1955; Sumner, 1924). *Total protein content* was estimated by the Kjeldahl nitrogen method, and a factor of 6.25 was used to convert nitrogen into protein (Bernard, 1992; Buckee, 1994). *Ash* was analyzed by incineration in a programmable muffler oven from room temperature to 550 °C (4 h at peak temperature). *The insoluble lignin content* was estimated according to Browning (1967). *The lipid content* was analyzed according to Partanen, Hakala, Sjövall, Kallio, and Forssell (2005), using a Maran 23 MHz proton NMR spectrometer. The *soluble protein* concentration of the enzymes was determined according to the Lowry assay against a standard curve of bovine serum albumin (Lowry, Rosebrough, Farr, & Randall, 1951), after precipitation of the protein from the samples using trichloroacetic acid. All the analyses were carried out in duplicate. The data were analyzed by ANOVA followed by Tukey's test, at a significance level of 0.05. Statistical analyses were performed using the software STATISTICA 7.0.

### 2.4. Enzymatic hydrolysis of insoluble fractions of the extract and sediment

Three-millilitre aliquots of insoluble fraction preparations, IIa (1% w/v in 50 mM citrate buffer, pH 5.0) were treated with 100 µl of different enzyme solutions (10 mg protein/g substrate) at 45 °C for 20 h. The reaction was stopped by heating the mixture in a boiling water bath for 10 min. The sugars released were determined by HPAEC. Control treatments (with no enzyme) were done in parallel to the enzymatic ones.

### 2.5. Enzymatic hydrolysis of the whole extract to reduce the sediment

To investigate the effect of the enzymatic treatment on the sediment contents, 4 ml of the whole extract were incubated with 100 µl of enzyme solution (initially 5 mg protein/g substrate). The solutions were then cooled to 0 °C, maintained at this temperature for 5 min and then centrifuged at 3000 rpm, 4 °C for 10 min. The supernatant was discarded and the sediment dried overnight at 105 °C. Before centrifugation, a small aliquot (25 µl) was collected for the reducing sugar analysis by the DNS method. The temperatures studied ranged from 35 to 55 °C. Control treatments were made under similar conditions except that no enzyme was added. The sediment content was calculated according to the following equation:

$$\text{SEDIMENT}(\%) = \frac{\text{dried weight of residue}}{\text{dried weight of initial substrate}} \times 100.$$

## 3. Results and discussion

### 3.1. Chemical composition of the extract and sediment fractions

The chemical compositions of the whole extract and of the insoluble fractions isolated from the coffee extract and sediment are shown in Table 2. The whole extract (I) contained 43.9% of carbohydrate, the insoluble fraction of the extract 54.7% and the insoluble fraction of the sediment 12.9%. Thus the carbohydrate concentration was approximately 10% higher in the insoluble fraction of the extract than in the whole extract, and approximately 30% smaller in the insoluble fraction of the sediment. Concerning the whole extract, similar results have been described in

Table 2
Chemical composition of the whole extract and insoluble fractions % (w/w)

| Fraction | Total sugars | Free sugars | Protein | Lipids | Ash | Insoluble lignin |
|---|---|---|---|---|---|---|
| Whole extract | $43.4 \pm 1.34^{a}$ | $4.7 \pm 0.14^{a}$ | $19.2 \pm 0.28^{a}$ | ~0.0 | $10.5 \pm 0.69^{a}$ | $3.8 \pm 0.32^{a}$ |
| Insoluble fraction of extract | $55.9 \pm 1.93^{b}$ | $3.4 \pm 0.14^{b}$ | $15.2 \pm 0.57^{b}$ | ~0.0 | $6.2 \pm 0.31^{b}$ | $5.2 \pm 0.35^{a}$ |
| Insoluble fraction of sediment | $12.8 \pm 0.08^{c}$ | $0.6 \pm 0.03^{c}$ | $30.5 \pm 1.41^{c}$ | $12.2 \pm 0.5$ | $3.4 \pm 0.14^{c}$ | $27.6 \pm 2.26^{b}$ |

Different letters in the same column are significantly different ($P < 0.05$) according to Tukey's test.

the literature on roasted coffee (Oosterveld, Harmsen, et al., 2003; Redgwell, Trovato, et al., 2002).

The protein content of the whole extract was found to be higher, 19%, than the value reported by Oosterveld, Harmsen, et al. (2003), which was only 6.8%, but was closer to the value reported by Franca, Mendonça, and Oliveira (2005), which was 14.9%. As the protein content was calculated from the total nitrogen content of the samples, it may have been overestimated due to the presence of other nitrogen-containing substances (caffeine, trigonelline, free amines and amino acids). In addition to polysaccharides and proteins, the whole extract also contained ash (10.5%) and insoluble lignin (3.8%). Lipids were not found.

The insoluble fraction of the extract showed higher contents of carbohydrate (54.7%) and insoluble lignin (5.2%), and lower contents of protein (15.2%) and ash (6.2%), when compared to the whole extract, whereas the insoluble fraction of the sediment had relatively lower contents of sugar (12.9%) and ash (3.4%) and higher contents of protein (30.5%), lipid (12.2%) and insoluble lignin (27.6%). Since the insoluble lignin content was higher in the insoluble fraction of the sediment than in the other fractions, this leads us to believe that the lignin may be bound to the cellulose, hemicellulose and protein, forming a large amount of insoluble particles. Besides polysaccharides, proteins, lipids, ash and insoluble lignin, the material could also contain soluble lignin, chlorogenic acids, aliphatic acids, humic acids and melanoidins (Charles-Bernard, Kraehenbuehl, Rytz, & Roberts, 2005; Smith, 1985).

The monosaccharide profiles of the coffee fractions are shown in Table 3, and were composed mainly of galactose, arabinose and mannose, building blocks of the galactomannans and arabinogalactans.

The predominant sugars in the whole extract (I) were galactose and mannose (44.1% and 33.1%, respectively), followed by arabinose (10.6%). The ratios of galactose:mannose and arabinose:galactose were, respectively, 1.3 and 0.24. Oosterveld, Harmsen, et al. (2003) examined the chemical structure of the polysaccharide fractions obtained after the hot water extraction of roasted coffee. After an extraction with water at 90 °C, the authors found 49% of mannose, 33% of galactose and 9% of arabinose. After extraction at 170 °C, the sugar profile was 32% of mannose, 53% of galactose, and 8% of arabinose; the ratios galactose:mannose and arabinose:galactose were 1.7 and 0.15, respectively, values close to the present results. Acidic sugars were also found in the whole extract (methylglucuronic acid, galacturonic acid and glucuronic acid), representing 7% of the total carbohydrate content. According to Redgwell, Trovato, et al. (2002) and Redgwell, Curti, et al. (2002), rhamnose and galacturonic acid are components of pectin or rhamnogalacturonan, that can be present in the coffee bean cell wall, while glucuronic acid can exist as terminal residues on the side chains of arabinogalactans.

Concerning the insoluble fractions (IIa), it was found that the main polysaccharide was a galactomannan. The ratios of galactose:mannose were 0.41 and 0.37 for the extract and sediment insoluble fractions, respectively. Therefore, the galactomannan present in these fractions is less branched than that found in the whole extract. The low solubility of these fractions may result from the low degree of branching (Buckeridge, Tiné, Santos, & Lima, 2000; Oosterveld, Harmsen, et al., 2003). The polymer fractions (IIIa) were both primarily composed of galactose units, indicating the presence of arabinogalactans and galactans. In the sugar fractions (IIIb), the main sugar was mannose, followed by galactose and arabinose. The ratio of mannose:galactose was approximately 1, suggesting the high solubility of these fractions.

### 3.2. Enzymatic hydrolysis of the insoluble fractions

Insoluble fractions (IIa), previously isolated from the coffee extract and sediment, were treated with twelve commercial enzyme preparations containing cellulases, hemicellulases, pectinases and protease. The protein content of the enzymes was also investigated in order to standardize suitable concentrations for them in the coffee treatments (Table 1). These enzyme preparations are complex mixtures of various different hydrolytic enzymes, but the major activity according to the manufacture is also given.

Tables 4 and 5 show the monosaccharide compositions of the products resulting from enzymatic hydrolysis of the insoluble fractions of the extract and sediment, respectively. The content of each sugar released after acid hydrolysis of these fractions is also included, for comparison. The free sugar contents of the non-hydrolyzed (control treatments) insoluble fractions were also determined, in order to compare with the values obtained after the enzymatic treatments.

As shown in Table 4, the control of the insoluble fraction of the extract (without the addition of enzyme) was found to have 3.35% of free sugars, composed mainly of arabinose, followed by galactose and mannose. After treatment with Econase, the free sugar content increased to 5.33%, glucose being the main sugar released by this

Table 3
Monosaccharide composition of the coffee fractions after acid hydrolysis (% w/w)

| Sample | Rha | Ara | Gal | Glu | Xyl | Man | Fru | MeGlcA | GalA | GlcA | Total carbohydrate (mg/100 mg solids) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Extract* | | | | | | | | | | | |
| Whole | $1.0 \pm 0.07^{a}$ | $10.5 \pm 0.18^{a}$ | $44.1 \pm 0.33^{a}$ | $3.2 \pm 0.01^{a}$ | $0.4 \pm 0.03^{a}$ | $33.2 \pm 0.10^{a}$ | $1.2 \pm 0.07^{a}$ | $0.6 \pm 0.02^{a}$ | $5.2 \pm 0.02^{a}$ | $0.6 \pm 0.02^{a}$ | $43.4 \pm 1.34^{a}$ |
| Insoluble fraction | $0.6 \pm 0.04^{b}$ | $6.1 \pm 0.05^{b}$ | $25.3 \pm 0.24^{b}$ | $2.1 \pm 0.01^{b}$ | $0.2 \pm 0.02^{b}$ | $61.3 \pm 0.10^{b}$ | $0.7 \pm 0.00^{b}$ | $0.5 \pm 0.02^{a}$ | $3.0 \pm 0.18^{b}$ | $0.5 \pm 0.02^{b}$ | $55.9 \pm 1.93^{b}$ |
| Polymer fraction | $0.8 \pm 0.02^{c}$ | $5.4 \pm 0.10^{c}$ | $60.4 \pm 0.35^{c}$ | $1.8 \pm 0.01^{c}$ | $0.3 \pm 0.01^{a}$ | $22.0 \pm 0.08^{c}$ | $0.3 \pm 0.00^{c}$ | $0.7 \pm 0.42^{a}$ | $7.9 \pm 0.30^{c}$ | $0.4 \pm 0.00^{c}$ | $64.6 \pm 0.37^{c}$ |
| Sugar fraction | $1.6 \pm 0.05^{d}$ | $20.0 \pm 0.08^{d}$ | $33.5 \pm 0.19^{d}$ | $6.2 \pm 0.02^{d}$ | $0.6 \pm 0.02^{c}$ | $36.1 \pm 0.05^{d}$ | $2.0 \pm 0.14^{d}$ | Na | Na | Na | $24.9 \pm 0.70^{d}$ |
| *Sediment* | | | | | | | | | | | |
| Insoluble fraction | $1.3 \pm 0.04^{e}$ | $8.2 \pm 0.00^{e}$ | $21.5 \pm 0.14^{e}$ | $4.3 \pm 0.07^{e}$ | $0.3 \pm 0.01^{a}$ | $57.4 \pm 0.08^{e}$ | $0.7 \pm 0.03^{b}$ | $2.0 \pm 0.01^{b}$ | $2.5 \pm 0.02^{b}$ | $2.0 \pm 0.01^{d}$ | $12.8 \pm 0.08^{e}$ |
| Polymer fraction | $1.1 \pm 0.01^{a}$ | $6.5 \pm 0.06^{f}$ | $65.2 \pm 0.03^{f}$ | $2.0 \pm 0.01^{b,c}$ | $0.3 \pm 0.03^{a}$ | $24.6 \pm 0.01^{f}$ | $0.3 \pm 0.00^{c}$ | Na | Na | Na | $57.1 \pm 1.55^{b}$ |
| Sugar fraction | $1.7 \pm 0.01^{d}$ | $20.9 \pm 0.01^{g}$ | $33.0 \pm 0.07^{d}$ | $6.2 \pm 0.11^{d}$ | $0.7 \pm 0.01^{d}$ | $35.3 \pm 0.09^{g}$ | $2.2 \pm 0.03^{e}$ | Na | Na | Na | $23.4 \pm 0.43^{d}$ |

Values expressed on dry bases. Na, not analyzed.
Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Fru, fructose; MeGlcA, methylglucuronic acid; GalA, galacturonic acid; GlcA, glucuronic acid.
Different letters in the same column are significantly different ($P < 0.05$) according to Tukey's test.

enzyme. Econase is a preparation of cellulases obtained from *Trichoderma reesei*, containing several endoglucanases and exoglucanases and various hemicellulases (Suutarinen et al., 2003). Hydrolysis with Protease CG106 produced mainly mannose, followed by galactose and glucose, which were certainly released due to the presence of other hydrolytic activities apart from the protease activity. Galactose and mannose were found to be the main sugars released by hydrolysis with Pectinex 3XL, Pectinex Ultra, Biopectinase CCM, Biopectinase Super 8x, Pectinase 444 and Rohapect B1L. It was evident that these pectinase preparations were mixtures of various enzymes, which hydrolyzed mannans, galactans and other carbohydrates. Of all the pectinases tested, Rohapect D5L, which is used in fruit juice processing, achieved the lowest yield of released sugars. After hydrolysis with Pectinase 444L, the mannose content increased significantly (42 times) and 80% of the total carbohydrates was present as free sugars, indicating the hydrolysis of mannans.

When the insoluble fraction of the coffee sediment (Table 5) was treated with the commercial enzymes, similar results to those obtained with the insoluble fraction of the extract were found, although lower amounts of reducing sugars were observed. As shown in Table 3, both the extract and sediment insoluble fractions were composed of the same carbohydrates, but the extract fraction had a higher carbohydrate concentration than had the sediment fraction.

Two other enzyme complexes (Novo Shape and Rohapect 10L) were also tested, but apparently had no effect on the carbohydrate composition of the substrates (data not shown).

The glucose and fructose contents were higher after enzymatic hydrolysis than after acid hydrolysis. Acid hydrolysis probably degraded part of these sugars, but if the acid hydrolysis conditions had been less drastic, complete hydrolysis of the polysaccharides might not have been possible. According to Puls (1993), after TFA and HCl hydrolysis, part of the sample may still not have been hydrolyzed, whilst with $H_2SO_4$, the hydrolysis of the neutral sugars is complete but some of the monomer units may degrade.

Therefore it was concluded, from the results, that Pectinase 444L was the most efficient enzyme in releasing sugars from insoluble coffee materials.

### 3.3. Enzymatic hydrolysis of the whole extract to reduce the sediment

As it is important to reduce the amount of sediment during the production of instant coffee, the efficiencies of the different enzymes on the coffee extract were studied. The contents of the main components found in the whole extract can be seen in Table 2. The performance of the enzymes in the hydrolysis of the coffee extract can depend on factors, such as substrate concentration, enzyme type and concentration, and process conditions, such as pH,

Table 4
Monosaccharide composition of the insoluble fraction of the extract after enzymatic hydrolysis (mg in 100 mg of dried substrate)

| Enzyme | Rha | Ara | Gal | Glu | Xyl | Man | Fru | Total |
|---|---|---|---|---|---|---|---|---|
| Control treatment[a] | 0.04 | 1.24 | 0.79 | 0.17 | 0.04 | 0.77 | 0.29 | 3.35 |
| Total sugars (acid hydrolysis)[b] | 0.31 | 3.30 | 13.67 | 1.16 | 0.09 | 33.12 | 0.35 | 52.00 |
| Econase CE | 0.04 | 1.65 | 1.19 | 0.58 | 0.17 | 1.45 | 0.31 | 5.38 |
| Protaese GC 106 | 0.04 | 1.55 | 2.69 | 0.57 | 0.06 | 6.72 | 0.39 | 12.0 |
| Pectinex 3XL | 0.04 | 2.17 | 9.30 | 0.12 | 0.04 | 26.4 | 0.72 | 38.8 |
| Pectinex Ultra SP-L | 0.05 | 1.96 | 8.68 | 0.71 | 0.04 | 9.30 | 0.53 | 21.3 |
| Biopectinase CCM | 0.04 | 2.07 | 8.06 | 0.90 | 0.04 | 25.2 | 0.74 | 37.1 |
| Biopectinase Super 8x | 0.04 | 2.07 | 6.92 | 0.82 | 0.04 | 28.9 | 0.73 | 39.6 |
| Pectinase 444L | 0.04 | 2.17 | 9.63 | 1.32 | 0.04 | 30.4 | 0.85 | 44.4 |
| Rohapect B1L | 0.04 | 1.76 | 2.89 | 1.24 | 0.04 | 25.9 | 0.71 | 32.6 |
| Rohapect D5L | 0.04 | 1.71 | 1.50 | 0.64 | 0.08 | 1.27 | 0.32 | 5.56 |

Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Fru, fructose.
[a] Free sugars of the control treatment (no enzyme).
[b] Total sugars obtained after acid hydrolysis of the insoluble fraction of the extract.

Table 5
Monosaccharide composition of the insoluble fraction of the sediment after enzymatic hydrolysis (mg in 100 mg of dried substrate)

| Enzyme | Rha | Ara | Gal | Glu | Xyl | Man | Fru | Total |
|---|---|---|---|---|---|---|---|---|
| Control treatment[a] | 0.04 | 0.24 | 0.15 | 0.04 | 0.04 | 0.13 | 0.05 | 0.69 |
| Total sugars (acid hydrolysis)[b] | 0.17 | 1.05 | 2.77 | 0.54 | 0.03 | 7.35 | 0.09 | 12.01 |
| Econase CE | 0.04 | 0.42 | 0.30 | 0.30 | 0.04 | 0.42 | 0.06 | 1.59 |
| Protaese GC 106 | 0.04 | 0.33 | 0.69 | 0.24 | 0.04 | 2.69 | 0.07 | 4.10 |
| Pectinex 3XL | 0.04 | 0.59 | 1.76 | 0.04 | 0.04 | 6.30 | 0.13 | 8.89 |
| Pectinex Ultra SP-L | 0.04 | 0.49 | 1.65 | 0.31 | 0.04 | 2.48 | 0.11 | 5.13 |
| Biopectinase CCM | 0.04 | 0.51 | 1.55 | 0.37 | 0.04 | 6.30 | 0.15 | 8.96 |
| Biopectinase Super 8x | 0.04 | 0.51 | 1.40 | 0.29 | 0.04 | 6.20 | 0.13 | 8.61 |
| Pectinase 444L | 0.07 | 0.53 | 1.64 | 1.31 | 0.04 | 6.47 | 0.23 | 10.3 |
| Rohapect B1L | 0.04 | 0.44 | 0.62 | 0.73 | 0.04 | 6.72 | 0.15 | 8.74 |
| Rohapect D5L | 0.04 | 0.42 | 0.39 | 0.34. | 0.04 | 0.40 | 0.07 | 1.72 |

Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Fru, fructose.
[a] Free sugars of the control treatment (no enzyme).
[b] Total sugars obtained after acid hydrolysis of the insoluble fraction of the sediment.

temperature, mixing rate and reaction time. The pH of the industrialized coffee extract is around 5.0, which was maintained, since this is the optimum pH of the enzymes studied, and due to the difficulty of adjusting the pH during the industrial manufacture of instant coffee.

The first set of experiments was conducted in order to determine which enzymes most efficiently reduced the amount of sediment in the whole extract, employing an enzyme concentration of 5 mg protein/g substrate at 45 °C for 2 h. The effects of the enzymes on the sediment and on the reducing sugars are shown in Fig. 2.

The amount of sediment obtained in the non-enzymatically treated coffee was 14% at 45 °C. Treatments with Econase, Protease, Novo Shape, Pectinex 3XL, Rohapect 5DL and Rohapect 10L had no effect on the amount of sediment. Hydrolysis with Pectinex Ultra, Biopectinase CCM, Pectinase 444L, Rohapect B1L and Galactomannanase ACH resulted in approximately the same efficiency (≈4% of the amount of sediment). Fig. 2a and b shows that the profiles of the amount of sediment and of the sugars released were not related, i.e., Pectinase 444L was shown to release the highest concentration of sugars.

Equivalent conversions can be achieved with lower concentrations of enzymes during longer treatments; therefore the influence of enzyme concentration on the hydrolysis was evaluated. Hydrolysis experiments with the four best enzyme preparations (Biopectinase CCM, Pectinase 444L, Rohapect B1L and Galactomannanase ACH) were performed at 50 °C and the results can be seen in Fig. 3a–d, respectively. Hydrolysis with Biopectinase CCM was carried out in a concentration range from 1–4 mg protein/g substrate, as shown in Fig. 3a. Increasing enzyme concentrations resulted in decreasing amounts of sediment. However, when 4 mg protein/g substrate was used, the final sediment content was 5% (w/w), and so this enzyme was not considered to be economically viable for an industrial process, since quite a large amount of enzyme would be required for a long period of time. Similar hydrolysis performances were obtained by Pectinase 444L, as shown in Fig. 3b, the maximum rate being achieved with an enzyme concentration of 4 mg protein/g substrate, which represents 16% (w/w) of enzyme per quantity of dried substrate, a value considered too high for a commercial application. However, when Rohapect B1L was employed (Fig. 3c), it

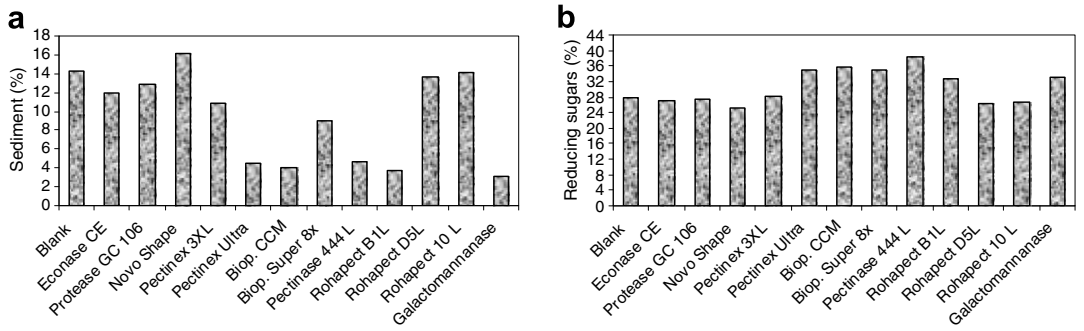


Fig. 2. Screening of commercial enzymes for sediment reduction. The effect of enzymatic hydrolysis on: (a) the amount of sediment and (b) the release of reducing sugars.

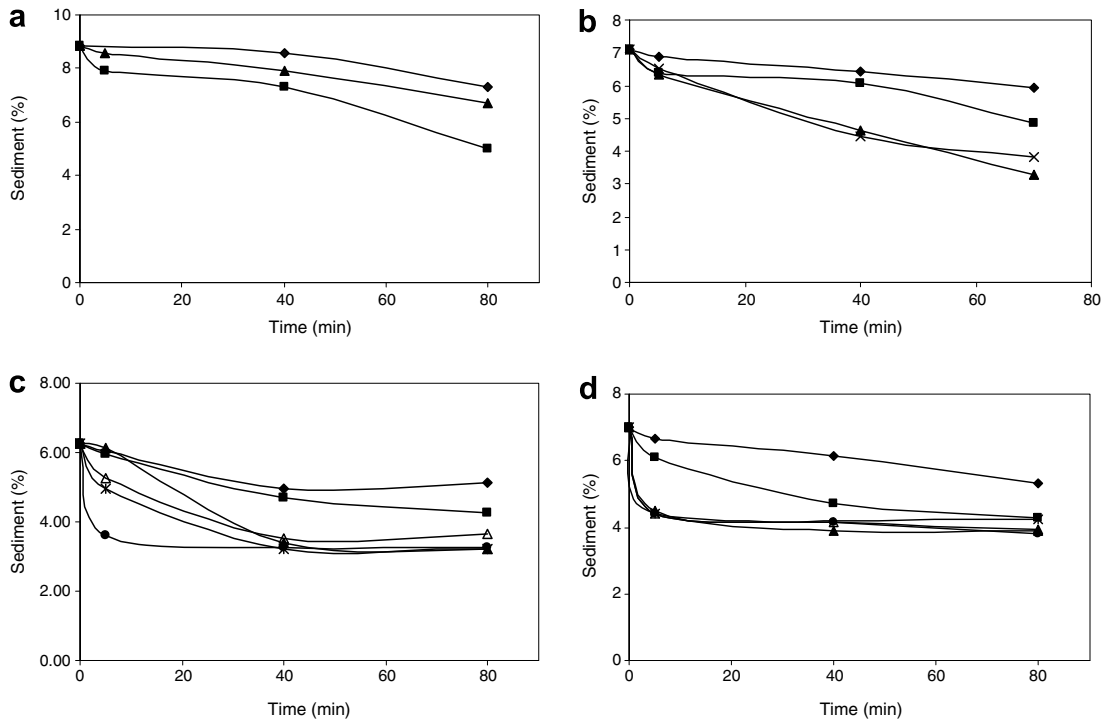


Fig. 3. Time course curves of the enzymatic hydrolysis of coffee extracts at 50 °C. Effect of the enzyme concentrations on the amounts of sediment. (a) Biopectinase CCM (-♦-) 1 mg prot/g substrate, (-▲-) 2 mg prot/g substrate, (-■-) 4 mg prot/g substrate; (b) Pectinase 444L (-♦-) 0.5 mg prot/g substrate, (-■-)1.0 mg prot/g substrate, (-▲-) 2.0 mg prot/g substate, (-×-) 4.0 mg prot/g substrate; (c) Rohapect B1L (-♦-) 0.05 mg prot/g substrate, (-■-) 0.1 mg prot/g substrate, (-▲-) 0.3 mg prot/g substrate, (-Δ-) 0.5 mg prot/g substrate, (-×-) 1.0 mg prot/g substrate, (-●-) 2.0 mg prot/g substrate; (d) Galactomannanase ACH – (-♦-) 0.05 mg prot/g substrate, (-■-) 0.1 mg prot/g substrate, (-▲-) 0.5 mg prot/g substrate, (-Δ-) 1.0 mg prot/g substrate, (-×-) 2.0 mg prot/g substrate, (-●-) 4.0 mg prot/g substrate.

was observed that, after 40 min of incubation, a significantly lower amount of enzyme (0.3 mg protein/g substrate), which represents 0.9% (w/w) of enzyme per amount of substrate, resulted in a decreased amount of sediment (approximately 3.5%). Further increases in the enzyme concentration did not result in a better effect. Galactomannanase ACH treatment (Fig. 3d) resulted in a high and fast sediment reduction. The lowest enzyme concentration to obtain the minimum sediment, 3.5% after 1 h, was 0.1 mg protein/g substrate, which represents 0.03% (w/w) of enzyme per amount of substrate. Above this, the enzyme concentration did not appear to enhance the hydrolysis yield; however, when using higher concentrations of enzyme, the same yield was achieved in a shorter period of time. Therefore, the present results indicated that, potentially, Rohapect B1L and Galactomannanase ACH could be employed for sediment reduction in coffee processing.

The effect of temperature on the dissolution of the sediment was also studied, since during the manufacture of coffee the sediment is mostly observed at low temperatures. The kinetic curves for the release of reducing sugars and the amount of sediment are shown in Figs. 4 and 5 for the enzymes Rohapect B1L and Galactomannanase ACH, respectively, at five different temperatures (35, 40, 45, 50 and 55 °C).

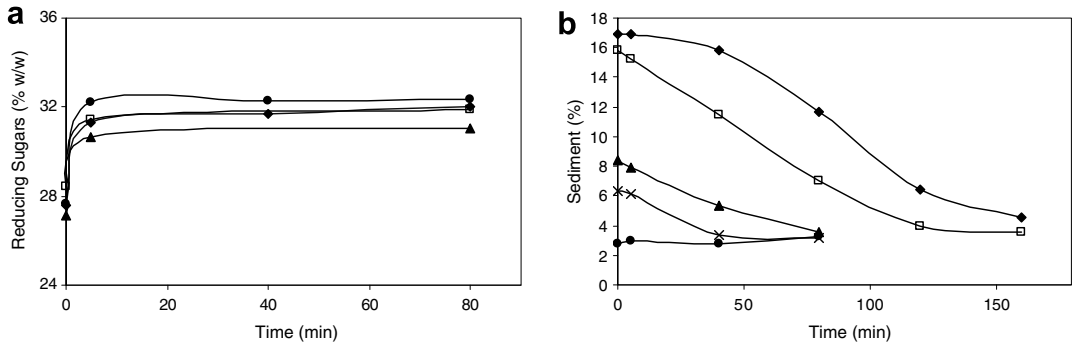


Fig. 4. Time course curves of the enzymatic hydrolysis of coffee extracts with Rohapect B1L. The effect of temperature on: (a) the release of reducing sugars and (b) the amount of sediment. (-♦-) 35 °C, (-□-) 40 °C, (-▲-) 45 °C, (-×-) 50 °C, (-●-) 55 °C.

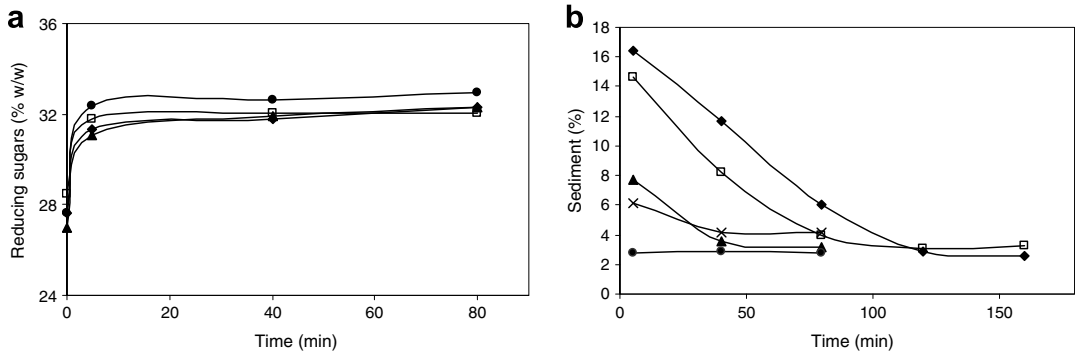


Fig. 5. Time course curves of the enzymatic hydrolysis of coffee extracts with Galactomannanase ACH. The effect of temperature on: (a) the release of reducing sugars and (b) the amount of sediment. (-♦-) 35 °C, (-□-) 40 °C, (-▲-) 45 °C, (-×-) 50 °C, (-●-) 55 °C.

Hydrolysis started quickly, as can be deduced from the reducing sugars profiles. The concentration of reducing sugars increased during the first 5 min and remained constant until the end of the reaction, independent of temperature. However, while the formation of total reducing sugars ceased after 5 min, the decrease in sediment showed different kinetics, depending on which temperature was used for hydrolysis. The fact that the reduction in sediment persisted while the release of reducing sugars had finished, indicates that the commercial preparations used in this work contained a mixture of enzymes with a wide spectrum of different activities, such as high activities of endo-enzymes, for example, endo-mannanase, and other activities.

Sensory evaluation of enzymatically treated coffee extracts has been studied and is the objective of another publication. Our studies showed that practically none or few differences were observed by the trained panellists and consumers (Delgado, 2008).

The present experiments showed that the initial amount of sediment decreased from 16% to 3.5% when the temperature increased from 35 to 55 °C. With an increase in extract temperature, the amount of sediment decreases, but according to the manufacturer becomes insoluble again on cooling. In the present work, the coffee extract was treated at five different temperatures, but the best temperature should be determined by the manufacturer.

## 4. Conclusions

This study showed that both the whole extract and its insoluble faction were composed mainly of carbohydrates and proteins, while the insoluble fraction of the sediment, besides these compounds, contained considerable amounts of insoluble lignin and lipids.

Analyzing the sugar compositions of the coffee fractions, it was observed that all of them consisted mainly of galactose, mannose and arabinose, which are the building blocks of galactomannans and arabinogalactans. The galactomannan present in the insoluble fractions had a lower galactose:mannose ratio than that found in the whole extract, which may explain the lower solubility of the insoluble fractions.

The insoluble fractions of the extract and sediment were treated with commercial enzymes. After analysis of the sugar composition, it was shown that the main sugars released were arabinose, galactose and mannose.

Four enzymes, Biopectinase CCM, Pectinase 444L, Rohapect B1L and Galactomannanase ACH, were considered efficient in treating the sediment but, of these, only Rohapect B1L and Galactomannanase ACH were economically viable for an industrial application. The lowest enzyme concentration required to reduce the sediment to approximately 3.5% was 0.3 and 0.1 mg protein/g substrate, for Rohapect B1L and Galactomanannase ACH, respectively.

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