

Marianne de Lima Barboza

Extração e caracterização de pectinas naturais por método químico,
infravermelho e HPLC-ELSD

Recife
2011

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infravermelho e HPLC-ELSD

Tese apresentada ao Programa de Pós-Graduação em Nutrição, do Centro de Ciências da Saúde, da Universidade Federal de Pernambuco, para obtenção do Grau de Doutor em Nutrição, área de concentração - Ciência dos Alimentos.

Orientador Prof. Dr. José Almiro da Paixão

Recife
2011

Barboza, Marianne de Lima

Extração e caracterização de pectinas por
método químico, infravermelho e HPLC - ELSD /
Marianne de Lima Barboza. – Recife: O Autor, 2011.
1369 folhas: il., fig.; 30 cm.

Orientador: José Almiro da Paixão

Tese (doutorado) – Universidade Federal de
Pernambuco. CCS. Nutrição, 2011.

Inclui bibliografia e anexos.

1. Pectinas. 2. Infravermelho. 3.
Caracterização estrutural. 4. ELSD. 5. Coluna
Híplex. I. Paixão, José Almiro da. II. Título.

664.25

CDD (20.ed.)

UFPE

CCS2011-190

Título: Extração e caracterização de pectinas naturais por método químico, infravermelho e HPLC-ELSD.

Doutoranda: Marianne de Lima Barboza

Tese aprovada em: 22 de agosto de 2011

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Dedico este trabalho aos que amo:

Meus pais, Marlene e Edilton (in memorian)

Meu esposo, Lamartine e meu filho Vinícius

Meus Irmãos, César e Mariângela

Meus sobrinhos, Pedro Lucas e Paulo Gustavo

Meu cunhado Paulo Gustavo

Agradecimentos

A todos que me ajudaram nesta etapa da minha vida, em especial:

À **Deus**, pela sua presença em todas os momentos e por me conceder a vida.

À **minha família**, sem a qual não teria chegado até aqui e conquistado meus objetivos, sempre me apoiando e incentivando em todas as minhas decisões, com carinho amor e muita paciência.

Ao meu orientador, **Professor José Almiro da Paixão**, um exemplo de pesquisador. Obrigada por todo incentivo, dedicação, ensinamentos!

Ao corpo técnico do Laboratório de Experimentação e Análise de Alimentos - LEAAL, **Alexandre, Arthur, Camilo, Vivado, Moisés e Solange**, pela amizade e apoio.

À Professora **Samara Andrade**, pelas análises estatísticas e disponibilidade para me ajudar.

À minha amiga, **Roberta de Albuquerque Bento**, parceira que mesmo a distância sempre se mostrou presente. Muito obrigada!

À equipe da Central Analítica do Deptº de Química Fundamental da UFPE, especialmente a **Eliete e Conceição**, pelas análises por espectroscopia de infravermelho.

À **Rafael Padilha** do Laboratório de Imunopatologia Keizo Asami – LIKA pelas liofilizações das amostras de pectinas.

À **Prof. Angela Vieira**, do Dept. de Zootecnia, pelas análises de FDN e FDA.

À **Clayton Azevedo** pelo auxílio nas análises estatísticas e **Emmanuela Paiva**, pela colaboração junto ao Software LC solutions.

À Professora **Tânia Lúcia M. Stamford**, pela força e incentivo ao longo dessa jornada.

À secretaria da Pós-graduação, em especial a **Neci e Cecília Arruda**, pela ajuda nos momentos que precisei.

À **Facepe**, pelo projeto financiado pelo edital 10/2008, APQ-1302-4.05/08, que permitiu a Implantação da Central de Caracterização Química de Alimentos, Medicamentos e Suplementos Nutricionais, no Departamento de Nutrição, UFPE.

À **Capes**, pela concessão da bolsa de Doutorado.

Para tudo há um tempo determinado, sim, há um tempo para todo o assunto debaixo dos céus: tempo para nascer e tempo para morrer; tempo para plantar e tempo para desarraigar o que se plantou; tempo para matar e tempo para curar; tempo para derrocar e tempo para construir; tempo para chorar e tempo para rir; tempo para lamentar e tempo para saltitar; tempo para lançar fora pedras e tempo para reunir pedras; tempo para abraçar e tempo para manter-se longe dos abraços; tempo para procurar e tempo para dar por perdido; tempo para guardar e tempo para lançar fora; tempo para rasgar e tempo para costurar; tempo para ficar quieto e tempo para falar; tempo para amar e tempo para odiar; tempo para guerra e tempo para paz.

Eclesiastes 3:1-8

RESUMO

Este trabalho consiste numa abordagem metodológica de caracterização visando a viabilização de fontes naturais de pectina. Utilizou-se o mesocarpo da melancia (e demais frutos tropicais) como fonte de pectinas para demonstrar a caracterização estrutural por distintos métodos analíticos, considerando desde a etapa de extração até a caracterização estrutural. Foi avaliado o potencial do ácido cítrico e do HCl, em diferentes forças, em temperaturas distintas sobre o rendimento de extração (%) e o grau de metoxilação GM (%). Verificou-se também a influência da cadeia carbônica do álcool durante a precipitação de pectinas isoladas. Os métodos de caracterização estrutural de pectinas (padrões e extraídas de diversas frutas) foram o titulométrico, infravermelho e HPLC acoplado a detecção pelo espalhamento de luz (ELSD). A determinação do GM por infravermelho (IV) foi obtida a partir dos valores de absorbância das bandas 1750 cm^{-1} e 1650 cm^{-1} . Para determinação por HPLC foram avaliadas as fases estacionárias (*ODS-2* e *Hiplex*) e fases móveis diferenciadas para otimizar a separação e detecção. As melhores condições foram ajustadas ao uso da fase estacionária *Hiplex* em fase móvel H_2SO_4 50 mM. As condições de desesterificação dos padrões comerciais de pectinas foram ajustadas variando a força do NaOH, de 0,1 a 3,6 M e tempo de reação (5 min – 360 min), exploradas em ensaios multifatoriais. Obteve-se maior rendimento de extração de pectinas (3,38%) e maior GM (76,21%) utilizando o ácido cítrico, enquanto que o HCl produziu efeito desmetoxilante. A fase móvel H_2SO_4 50mM associada a coluna *Hiplex fast acid* mostraram-se eficazes na separação e detecção do metanol e ácido acético da pectina por *HPLC-ELSD*. A otimização do procedimento de desesterificação foi ajustada pela variável força molar minimizando a desmetoxilação entre 1,5-2,0 M ($p \leq 0,02$) requerendo em tempo de reação de 5-60 min. As condições ideais para tratar padrões comerciais quando asseguradas estatisticamente procedeu-se a curva extraída ($N=7$) para estudos de regressão e assim repassada para quantificação dos parâmetros estruturais em amostras de pectinas de frutas. As avaliações foram procedidas pelo método de padronização interna e externa que exibiram R^2 superior a 0,92. Enquanto para as amostras de pectinas de frutas tropicais o R^2 em torno de 0,75, independente do sistema de quantificação.

Palavras-Chave: pectinas; frutas; extração; caracterização estrutural; infravermelho; ELSD; coluna *Hiplex*.

ABSTRACT

This paper investigates the feasibility of using the region mesocarpic of fruits tropical as a source of natural pectin carrying to alternative methodologies involving since extraction procedure until analysis structural characterization by different analytical methods. From the watermelon mesocarpic was evaluated the potential of citric acid and HCl at different strengths, some one temperatures on the extraction yield (%) and the degree of methoxyl DM (%). It was also evaluated the influence of the carbon chain of alcohol during the precipitation of pectins. The methods of structural characterization of pectins (from various standards and fruits) were the titration, infrared and HPLC coupled to detection by evaporative light scattering (ELSD). The determination of DM by infrared (IR) was obtained from the absorbance values of the bands 1750 cm^{-1} and 1650 cm^{-1} . For HPLC determination were evaluated stationary phases (ODS-2 and Hiplex) and mobile phases in order to optimize the separation and detection. The best conditions were adjusted to the use of stationary phase Hiplex in H_2SO_4 50mM mobile phase. The conditions of desesterification of pectin standards were adjusted by varying the strength of NaOH, 0.1 to 3.6 M and time for reaction (5 min - 360 min), exploiting multifactorial assay. Considering extraction procedure obtained higher yield (3.38%) and DM (76.21%) using citric acid instead inorganic acid; while the HCl produced desmethoxylation effect. The mobile phase H_2SO_4 50mM coupled Hiplex fast acid column were effective in the separation and detection of methanol and acetic acid derived desesterification of standard pectins. Optimization of the desesterification procedure was adjusted by varying force molar maximizing DM when used between 1.5 and 2.0 M ($p \leq 0.02$) while was required time for reaction between 5-60 min. Thus the ideal conditions were guaranteed to standard of pectin extracted and next step was constructed curve calibration extracted in order to quantify of standards and samples of pectin fruits. The quantification systems evaluated were the method of internal standardization and external both exhibited R^2 greatest than 0.92. As for samples of tropical fruit pectins, the R^2 around 0.75, regardless of the system of measurement.

Keywords: pectins; fruit; extraction; structural characterization; infrared; ELSD; Hiplex column.

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1. Introdução

As pectinas pertencem ao grupo de polissacarídeos estruturais sendo utilizados como ingredientes na indústria de alimentos como agente de retenção de sabor e *flavor*, como hidrocolóide na função de dispersante e estabilizante de diversas emulsões e na produção de geléias (Lima et al., 2010).

As principais fontes para a produção comercial de pectinas são os resíduos da indústria de suco. O albedo, por exemplo, mesocarpo dos frutos cítricos, é considerado um resíduo agrícola que ao ser lançado no meio ambiente contribui para o acúmulo de lixo e desperdício como parte não comestível. Desta forma, pesquisas estão dando enfoque a estas e outras novas matrizes, uma vez que este processo torna-se sustentável, em virtude da grande quantidade de resíduos gerados industrialmente (FAO, 2010).

As pectinas extraídas de diferentes matérias-primas podem apresentar características diversas relacionadas à massa molar, grau de esterificação, teores de açúcares neutros, distribuição dos grupos carboxil-metoxilados e às propriedades de aplicação. Esta diferenciação depende principalmente das condições de extração. As condições de temperatura, força e natureza do ácido na extração e força da base na desesterificação, geralmente conduz a degradação da cadeia pectica. Em contraposição, um máximo de rendimento, comumente conseguido utilizando-se ácidos inorgânicos, muitas vezes são incompatíveis do ponto de vista toxicológico e compromete a segurança de uso como ingrediente alimentar. Em geral, o ponto de maior rendimento não corresponde à pectina com melhor comportamento reológico, ainda que está associado à estrutura da cadeia pectica (Willats et al., 2006).

A caracterização da pectina e posterior classificação em pectina de alta metoxilação (esterificação com metanol > 50%) e de baixa (esterificação com metanol <

50%) é feita com a finalidade de uma orientação tecnológica, uma vez que o comportamento funcional deste hidrocolóide depende majoritariamente desta característica (Kurita, et al., 2008). A caracterização de pectinas auxilia por exemplo na projeção de *blends* de geleias de frutas, o que permite uma maior versatilidade de produtos disponíveis no mercado com características sensoriais e nutricionais preservadas (Lima et al., 2010).

As técnicas de caracterização de pectinas variam de métodos químicos e biológicos (titulométrico, colorimétrico e enzimáticos) a instrumentais (espectroscopia de infravermelho, cromatografia líquida de alta eficiência e ressonância magnética), variando com isto também o grau de detectabilidade e sensibilidade e o tempo requerido para cada análise (Kerr & Wicker, 2000; Manrique & Lajolo, 2002; Levigne, et al., 2002). Os métodos tradicionais vêm sendo substituído pelas novas metodologias, visando rapidez, maior escala de detecção, e procedimentos não destrutivos da molécula de pectina, uma vez que devido a complexidade deste carboidrato, outros componentes como açúcares neutros e proteínas decorrentes da parede celular podem dificultar a caracterização deste carboidrato por ensaio titulométrico.

Considerando a importância da pectina e sua caracterização para a indústria de alimentos, principalmente para a elaboração de geléia, este trabalho prioriza o enfoque ao comportamento da pectina bem como de outros constituintes químicos dos frutos e outras matérias-primas. A abordagem também destina-se a suficiente caracterização buscando a viabilização da região do mesocarpo (resíduo agrícola) e de frutos integrais como fonte de pectinas naturais, desde a extração até a caracterização estrutural em confronto com procedimentos estabelecidos na literatura, e propondo alternativas e adequação metodológicas.

2. Objetivos

2.1 Geral

Otimizar a metodologia para extração e caracterização de pectinas, identificar e quantificar os componentes estruturais, com vistas a suscitar a metodologia mais adequada.

2.2 Específicos

- Avaliar o efeito da extração com ácido - orgânico e inorgânico, sobre o isolamento e caracterização das pectinas;
- Verificar a influência da natureza do álcool no isolamento de pectinas;
- Estimar parâmetros estruturais de pectinas de diferentes frutos por infravermelho e HPLC com detecção por ELSD;
- Avaliar o desempenho das fases estacionárias sobre a detectabilidade do ELSD acoplado ao HPLC;
- Otimizar o procedimento de desesterificação de pectinas, das variáveis força do NaOH (M) e tempo de reação (min);
- Avaliar o critério quantitativo entre os métodos titulométrico x infravermelho x HPLC-ELSD, através de ensaios estatísticos aplicada em padrões e amostras de pectinas isoladas de frutos.

3. Revisão de Literatura – Artigo 1

Artigo 1. Jellies quality: from the fruit's chemistry to the technological process.

Submetido à Revista Innovative Food Science and emerging Technologies.

ISSN: 1466-8564

Fator de Impacto (2010): 2.825

Artigo 1. Jellies quality: from the fruit's chemistry to the technological process.

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Abstract

This article contains a brief discussion about chemical constituents found in fruits that favoring gelation in order to obtain by products (jelly, jams and blends) with superior technological, nutritional and sensory properties. In this scope yet contribute the authors suggesting previous chemical characterization such as pectin content, degree of methoxylation, size of gel fragments and pectic substances have been shown able to improve new formulations and technological updating process from native fruits, exploiting unknown properties. In addition, the cooking system, mainly involving binomial effects of time versus temperature, interferes with the fine chemical structure of this complex polysaccharide. Chemical, spectral and microscopic characterization of pectin is roughly adjustable parameters from technological process to particular innovations in these by products

Keywords: pectin, fruit jelly, quality control, cooking system, characterization fruits

1. Introduction

Fruits waste consists on an old problem for population and aiming to avoid it, required simultaneously to develop preservation techniques to prolong the shelf life of products and ensure the full use of fruits and waste of raw materials involving quality control from primary to tertiary segments. Post-harvest losses may reach thirty percent or more of agricultural production depending on the fruit morphology and production system (Chitarra & Chitarra, 2005).

The main causes involved with losses include: insufficient qualified human resources, use of inappropriate technologies from planting to storage; carelessness in handling and packaging of fruits during transport coupled with long distances; pests attack and diseases, and impaired infrastructure to meet the special needs of the agricultural sector, such as consistent cooling from harvest to marketing.

There are countless products made from fruit, such as: jellies, juices, jams, paste sweet and compotes, canned fruit, pulp, nectar, candied fruits and others. The pattern of biochemical and oxidative losses are distinguished from losses related to fresh fruit in relation to the technological processes (Odriozola-Serrano, Soliva-Fortuny, Hernández-Jover, & Martín-Belloso, 2009). The lack of analytical methodology for measuring losses generates controversy about the relative importance of ingredient and nutrient losses and subsequent chemical modification; however, the rational use of these raw materials and agricultural by-products is nowadays highlighted in the equilibrated agroecologic industry as a target involving the best use of appropriate technology (Zhang, Wang, Yu, & Wu, 2011).

Population of the twenty-first century, computerized and demanding, associate their dietary choices with health notably by restricting the menu to products made from

whole fruits, natural and with technological value compatible with preconceived attributes for raw material (Koh, Jiang, Kasapis, & Foo, 2010). However, this information is provided insufficient and inconsistent, generally in the field of processing of fruit-based products for reasons that are discussed throughout this article, aiming to reach the academic productive medium. The goal is guiding both in some aspects and properties that provide better discriminating force among fruit-based products, involving chemical aspects and process technology.

In the case of jellies, which have pectin as main gelling ingredient, a hetero-functional carbohydrate with functional properties include some beneficial effects to health. In some cases, during jellies processing, pectin and other natural components of fruits undergo changes that depending on the magnitude may interfere with particularly nutritional, pharmacological, technological and sensory values (Lima, Paiva, Andrade, & Paixão, 2010).

Among the proven benefits of pectin in the literature, we can emphasize the increased levels (viable counts) of beneficial bacteria in the intestine, and use as micro-encapsulator of probiotics for the health and well-being of the organism, in other side, the prebiotic potential effect (oligogalacturonides), fiber content and general cardiovascular benefits by lowering LDL cholesterol and hypoglycemic effects (Anderson et al., 2009; Parkar et al., 2010).

Due to these nutritional features of pectin, its consumption has been increased during the last years. World consumption of pectin was 6,000 tons/year for low methoxyl (LM) and 8,000 tons/year for high-methoxyl (HM) in 1987. Prices (in 1987) ranged from 7.92 to 8.80 US \$/kg for LM and 11.00 to 13.20 US \$/kg for HM, moving

US\$ 22 million/year. The commercial value per kg citrus pectin HM reaching approximately US\$ 63.00 valorating about 400% (<http://www.quimibras.com.br>).

The review aims to contribute to the characterization of functional ingredients usually employed to obtain jellies, involving since the chemical components of the fruit (from pectin to other ingredients) until some choice pointed out technological process, emphasizing the influence of these parameters on the gelation mechanism that carried out highest valoration of final product. The approach will be highlighted in sub-topics: history of the jellies manufacture, fruit chemistry and technological process, demonstrating relevance and updates regarding the fruit's characterization and gelation mechanisms, suggesting reliable parameters for overall quality of products based-fruits.

2. History of jellies manufacture

The origin of fruits' jellies and other preserves such as candied fruits, compotes and candy bars, are credited to the Mesopotamians (tenth century), who elaborated these products to pharmaceutical and medicinal purposes, while the Arabs were pioneers in introducing sugar both in the pharmacopoeia as in the food formulations (Algranti, 2005a).

The art of making jams and jellies dates back to the sixteenth century, where the physician, astrologer and practitioner of alchemy Michel de Nostre-Dame, known as Nostradamus (1503-1566), when better stratify his knowledge on plant alchemy, including the medicinal potential effect of fruit jellies. His experiments were published in "*Traité des Fardements et des Confeitures*". The material milestone of Nostradamus was a primer involving twelve published issues containing thirty recipes among jams, jellies and other musts. This material originally instructed not only Dietary and Medicinal qualities of products but also the importance of hygiene containers,

packaging with good sealing. Such details could sensibly modify the overall appearance and taste of preserved products as well as a way to consume them (Algranti, 2002).

The popularization of jellies consumption retrieve to medieval people, particularly to Nostradamus. The formulations were accessible to more remedied classes and still have sugar as alternative, which was considered as a noble and rare product at the time available alternatively using honey or must (Algranti, 2005a).

From XVII century, the medicinal character of jams and jellies has spread worldwide these products, which increase the variability of this product by the use of exotic fruits as well, but the basic fomulation, where they are preserved at high levels of solutes (honey and must). Fewer modification about the technological process were registered between the XVI and XVIII centuries (Algranti, 2005b). After this period, the adoption of cane sugar as a substitute for honey, and consequently the spread of canning and confectionery industries given out rough revolution in this milestone technological process. The process employed from XVIII century given rough indication of the need to clarify sugar in many formulations recommended and cane sugar became the essential and innovative ingredient used for making candy and preserved fruits (Algranti, 2005a). When well established in order quantitative and qualitative to substitute honey for sugar industrial other characteristic pointed out as problematic was sugars (mono e dissacharides) met directy fruits. Therefore the compotes developed by Nostradamus, which could be also used honey, sugar or juice, ideally shown similar properties. Unlikely the published formulations dating from the fifteenth century, which used exclusively, honey (Algranti, 2002).

Anonymous author (1788) published in Portugal the book “*Arte nova e curiosa para conserveiros, confeiteiros e copeiros e mais pessoas que se ocupam em fazer*

doces e conservas com frutas”. Among other topics, health information was confirmed by Nostradamus, but the focus of this publication has not been on the nature and digestibility of foods, being showed the technical aspects on the art of making jellies, feeding practices, preparation, equipment and industrial or kitchen tools. In this treatise, most formulations dispensed the use of honey, but sugar was liquefied sufficiently in order to achieve consistency similar to that of honey (Algranti, 2005a).

By comparing the stage of formulation employed in the XVIII century with the work published by Nostradamus, it is clear that the main difference is the time required for preparation of jams. The preserved products in accordance with the treatise of the sixteenth century, could take about thirty days to be completed, retain the same preserved product while in the eighteenth century would take only ten days. This reduction was attributed to the quality of the newly edulcorant refined sugar and purest (Algranti, 2005b).

Jellies and jams were restricted to the noble until the eighteenth century because they were considered superfluous for most of population did not include neither fruits in their diet. The popularization of these products is assigned to technological advances arising from scientific research in the nineteenth century. For example, the Appert method of conservation by heat sterilization released in 1810, the discovery of the solidification process of beet sugar, pasteurization and different packaging systems allowed the production of various canned and especially the jellies, with sensory characteristics closest to natural and integral products (Algranti, 2005b).

However, nowadays great interesting in demanding single standardization, involving heterogenous process that include exhotic aroma and flavor components, and so one factors. These parameters are typically found only in craft production with

commercial quality and certain uniformity to ensure certification with acceptable properties, reflecting itself the modern food chemistry about fruit and technological process. On the otherside, questions has suggested burned the great variability intrinsic in some steps involved obeyed standard since the choice of fruit that driving force until to technological processing.

3. Chemical composition of fruit

3.1 Structural carbohydrates and cell wall

The plant cell wall is a dynamic structure, whose shape, composition and properties are constantly changed throughout cell life in response to growth, differentiation, environment and cell activities. In general, the primary and secondary cell walls and middle lamella function as plant tissue support. The middle lamella fills the spaces between the primary walls and adjacent cells, consisting mainly of pectin, pectate and hemicellulose (Paiva, Lima, & Paixão, 2009).

The primary cell wall can be classified as: [1] type I, composed mainly of cellulose, xyloglucans, pectin and extensin, overall present in dicotyledons and some monocotyledons (non-commelinoids) and [2] type II, comprising mainly cellulose, glucoarabinoxilanas and phenolic compounds, with low amount of pectin, found in the Poaceae and most monocotyledons (Paiva et al., 2009).

The middle lamella is composed of pectin molecules attached via cross-links with the HG subsequently deposited in opposite pectin chains (Vincken et al., 2003). The structural polymers can be grouped as cellulose (β -D-1,4 glucan), pectin substances (galacturonans and ramnogalacturonans; arabinans; galactans and arabinogalactans I), hemicelluloses (xylans, including arabinoxylans, and 4-O-metilglicoronoxilans, glicomanans and galactoglicomans, xiloglicans and glycans β -D-

1,3 and β -D-1,4), other polysaccharides (arabinogalactans II and glicoromanans) and glycoproteins.

Cellulose is the most abundant polysaccharide of plant cell wall, is about 15 to 30% dry matter of the primary cell wall. This molecule provides strength to plant tissues. It is a neutral homo-polysaccharide formed by straight chains of anhydrous D-glucose linked by β -type 1,4, nutritionally known as endo- β -glucan (prebiotic agent found in cereals, grains and fruits) (Carpita & McCann, 2000).

The hemicellulose is comprised mainly of xyloglucans and contributes about 15% of the primary cell wall constituents. In general, xyloglucans are linked to microfibrils of cellulose, pectin and lignin by hydrogen bonds forming cross-links that stabilize the cell wall. Its neutral sugars skeleton is composed of bonds β -(1 \rightarrow 4) and the glucose residues can be replaced by xylose residues via α -(1 \rightarrow 6) bonds (Paiva et al., 2009).

The major monosaccharides that comprise the hemicelluloses of land plants are D-xylose, D-galactose, D-glucose, L-arabinose, D-mannose, D-glucuronic acid and acid 4-O-methylglucuronic. The major hemicelluloses are xyloglucans and glucuronoarabinoxylans, constituents of primary cell walls, in addition to mannans, glucomannans and galactoglucomannans (Carpita & McCann, 2000).

3.2 Pectic substances

Pectin was also discovered in 1790, when Nicolas-Louis Vauquelin found a soluble substance in fruits juice (Braverman, 1976). Later, in 1824, the French scientist Braconnot was the first to characterize it as fruits compound with gelling properties in acidic media and suggest the name of pectin, from the Greek $\pi\eta\chi\tau\omicron\varsigma$, which means

thick (Braconnot, 1825). In 1848, Fremy reported the existence of a pectic precursor insoluble in water, subsequently named protopectin (Joslyn & Deuel, 1963). Kelhofer in 1908, investigating the turbidity in pear fermented, concluded that the sediment observed consisted of a complex of proteins, pectins and oxidized phenolic compounds (Johnson, Donnelly, & Johnson, 1968). Smolenski, in 1923, was the pioneer to suggest that pectin would be a complex polymer, similar to the starch structure, with variable esterifications along its chain (Owens, Lotzkar, Schultz, & Maclay, 1946). Later, X-ray analysis of this molecule, performed by Meyer and Marck in 1930, proved that the comparison with the cellulose would be more appropriate. Thus, it was established the need for comparative studies on the composition, type and distribution of pectic compounds in plants, due to different extraction methods and analysis employed (discussed from 1790 to the present) (Baker, 1948).

Only in 1944, the committee from the American Chemical Society established the pectin substances as all materials containing galacturonic acid in their composition. Aiming to facilitate the understanding of these complex substances were classified as: protopectin, pectic acid, pectin acid and pectins (Jayani, Saxena, & Gupta, 2005).

Protopectins consist of the natural form of pectin, when combined with cellulose, hemicellulose and lignin, forming and crosslinking the primary and secondary cell walls. They are insoluble in water, and when heated (cooking system) in the presence of dilute acids form the pectin or pectic acids of different molecular sizes and composition; pectic acids consist of polygalacturonic acid chains entirely free from methyl ester groups and its salts are neutral pectates with low solubility acids in water; pectin acid is used to designate polygalacturonic acids containing a small proportion of methyl ester, poor defined in the literature for inaccuracy of methodologies in determining with accuracy of methyl ester groups (Jayani et al., 2005). Trading

standards of galacturonic acid are mixed with pectinic acid, when in fact, all standard commercial of galacturonic acid analyzed by Fourier transform infrared spectroscopy (FT-IR) have sufficient esterification with methanol, providing degree of methoxylation (DM) usually highest to obtained by titration.

Pectins are usually soluble in water, while when varying degrees of methyl ester groups and degree of neutralization capable of forming gels with sugars (Jayani et al., 2005). Modelling pectins are composed of three distinct regions: a linear fraction, known as homogalacturonans (HG) and branched fractions, the rhamnogalacturonans I (RG I) and rhamnogalacturonans II (RG II) found in different biological materials (Willats, Knox & Mikkelsen, 2006). Recently was credited that the main chain of pectic polysaccharides would consist of HG domains with single bonds called smooth regions and RG domains with branches containing various sugars, called hairy regions (Kjoniksen, Hiorth, & Nystrom, 2005). It has been proposed an alternative structure that considers the HG as a long branched RG I chain (Vincken et al., 2003; Willats et al., 2006).

RG I consists of alternating galacturonic acid units with rhamnose units [$\rightarrow 4\text{-}\alpha\text{-D-GalA-(1} \rightarrow 2\text{)-}\alpha\text{-L-Rham-(1} \rightarrow$]; it is a marked region, specifically where there is units of rhamnose, which produce loss of linearity, causing slight wrinkle in the pectin chain due to its hydrophobic effect. Rhamnose residues carry other sugars that alter the polarity, in addition to polygalacturonic, especially arabinans and galactans through C-4 linkage. Other sugars associated with this chain currently affect the structure of dispersant and gelling properties in accordance with media (acid and non-acid). Therefore leading to an incipient knowledge in this area linking the importance of pectin structure in the different technological processing involving sugars or other molecules of food colloidal systems (Willats et al., 2006).

The RG II portion is a complex pectic polysaccharide of low molecular weight, consisting of galacturonic acid, rhamnose, galactose and some rare sugars as apiose, acero acid, 3-deoxy-D-waste-heptulosaric acid (DHA) 2-keto-3-deoxy-D-mano-octulosonic acid (KDO), glucuronic acid (GlcA) (Lootens et al., 2003; Gulfi, Arrigoni, & Amado, 2007).

The HG are formed by repeated units of galacturonic acids, linked via galacturonic connections α -D-(1.4), thus forming a long polygalacturonic chain. Rhamnose units can be found occasionally in HG (Willats et al., 2006).

The carboxyl groups of galacturonic acid monomers in the HG portion may be esterified with methanol or acetic acid, where the percentage of esterified groups, treated as a DM and acetylation degree (DA), respectively (Mesbahi, Jamalian, & Farahnaky, 2005; Fishman, Chau, Hoagland, & Hotchkiss, 2006; Yapo, Robert, Etienne, & Wathelet, 2007). The carboxyl group esterified by methanol can reach up to 14% of the equivalent methoxyl, which mean about 80% DM, so called high methoxyl (HM), while low methoxyl (LM) has 7% of equivalent methoxyl, DM below to 50% picturing from Pomeranz & Meloan (2000).

DM is most commonly determined by titration (McCready, 1970). However, due to the susceptibility to chemical and physical bench agents, one should take into account the assumption of instrumental methods, without analyte derivation, such as NMR-proton proton (Kerr & Wicker, 2002) and HPLC (ODS-FR) (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002). FT-IR spectra show two bands of reliable pectins: 1750cm⁻¹ (carboxylic group esterified by methanol) and 1650cm⁻¹ carboxylate group, and less significant at 1400cm⁻¹ (Chatjigakis et al., 1998; Manrique & Lajolo, 2002). Through equations for relative bands sums, where $DM = [A_{1750cm^{-1}} / (A_{1650cm^{-1}} +$

A1750cm⁻¹)]. These main bands allowed characterize DM and establish a quantitative relation both methods viewed in Figure 1.

The pectin extraction can be accomplished by aqueous media mainly involving acid, basic and with chelating agents. The molecule also can be extracted by chelating agents such as calcium, ammonium oxalate and EDTA, which may not reach the fraction bounded to the primary cell wall (Manrique & Lajolo, 2002).

Usually, pectins are extracted with acidified water with phosphoric, acetic, sulfuric, nitric and hydrochloric acids, pH ranging from 1 to 3 at a temperature between 80-100°C. Several studies have reported that factors such as temperature, pH, exposure time and type of acid may influence not only the pectin yield, but also the chemical structure of the final product (Liu, Shi, & Langrish, 2006). Extended heating periods in the pectin extraction at temperatures above 90°C can cause hydrolysis and pectin degradation, resulting in a low-molar mass molecule (Mesbahi et al., 2005).

The Mc Cready's proposal for pectin extraction recommends the use of HCl to adjust the solution pH to 2.2 and later heating at 100°C for 30 minutes. The isolation of pectins extracted through this methodology occurs by using cloth cheese fabric (McCready, 1970). Adjustments in the methodology were recently published and suggest that solution acidification with citric acid at pH 2.2 and pectin isolation by filtration with polyester fabric (characterized mesh) and scanning electron microscopy (SEM), which ensure pectin fragments submitted to lyophilization for further analysis by FT-IR (Lima et al., 2010).

However, other recent publications recommend the use of hydrochloric acid (Kalapathy & Proctor, 2001; Iglesias & Lozano, 2004) and nitric acid as extractant agents (Liu et al., 2006). The extraction with these acids has some disadvantages, such

as environmental pollution and corrosion. Moreover, residues obtained after pectin extraction are not suitable for animal and human feeding (Ralet & Thibault, 1994).

Recent researches have focused on identifying the ideal and quantitative conditions of extraction with organic acids such as citric acid, to obtain a high quality pectin, benefiting the food industry from the economic, environmental and nutritional view points (Lima et al., 2010). However, other researchers continue comparing the pectin extraction with citric acid and hydrochloric acid. The best yield of extracted pectin from apple peel was obtained with 1% citric acid solution, showing 7.8% pectin yield. However, the pectin obtained in these extraction conditions showed 33.44% DM and 0.68% DA (Virk & Sogi, 2004). These data provide the suitable procedure presented by Lima and colleagues (2010).

Other researchers extracted and characterized pectin from apple pomace and optimized the process for laboratory routine. Through mathematical modeling determined that the best yield (17.82 % dry weight basis) was obtained with citric acid at 6.2% (w/v) at 100°C for 153 minutes and the pectin obtained showed 68.84% DM. Marcon (2004) obtained similar results, with 16.8% yield (w/w) when extracted pectin with 5% (w/v) citric acid at 100°C for 80 minutos (Canteri-Shemin, Fertoni, Wasczynski & Wosiacki, 2005).

Given the fruit's chemical composition, morphology and its compatibility with the jellies manufacturing, it is worth recognize that there are technical means to correct the fruit potential and design functional blends and improve the technological process of gelation, usually employed: adding mesocarp (orange and passion fruits), addition of commercial pectins and, ratio of fruits producing blends with different functional and

gelling properties (since fruit's pectins being well characterized in content, sugar content and DM) before processing.

These factors lead to technological difficulties in standardizing the process, deserving a quantitative optimization step for reliable parameters, which will be discussed in a later chapter (see 4.3), involving the characterization of fruits, ingredients and technological process.

3.3 Organic acids and nutritional components of fruits

These compounds are found dissolved in the plant cell cytoplasm in free form or combined with salts, cations, glycosides esters and other. They are also found in the roots sap moving to the stem, fruits and leaves differing in maturation stage. Give mainly the characteristic sour flavor of the fruit and also serve as a energy source in plant cells (Ito, 1971).

Citric acid is the main endocarp component of all fruits, in higher concentration in citrus fruits. In general, the exocarp has lower acid concentration than the mesocarp. The main acids found in the exocarp are oxalic, malic, malonic, and small amounts of citric acid (30-50% of anions). Unlike L-quinic acid, tartaric acid, benzoic acid and succine were found in the exocarp, mesocarp and endocarp of many citric fruits (Ito, 1971).

In the jellies industrialization, the acid media is essential to extract the pectin from its native form (protopectin) during cooking, encouraging the attraction between the chains of galacturonic in gelation media, and also give flavor to the final product; enabling or not the pectin depolymerization depending on the technological process. Regarding the pH favorable to gelation, many doubts raise in the literature, ranging from 2.2 to 3.5 at the risk of compromising the gel stability and with the

depolymerization it can generate retention levels of aroma and taste very different from those expected depending on the fruit chosen or combination thereof (blend), associated with main parameters of technicological processes (Fügel, Carle, & Schieber, 2005).

Minerals provide electrolytes required by the human body and also play a vital role in biochemical reactions and fruit morphology. Many of these elements are associated with enzyme systems and therefore are extremely important for the plants metabolism. Phosphorus, for example, is an element of nucleic acids and phosphoproteins, it is found in abundance in the orange juice (17.0 to 21.7 mg/100g). While potassium, calcium and magnesium occur in fruits in combination with organic acids such as citric, malic and oxalic sometimes unavailable in the fresh fruit. Magnesium is found in orange juice (7.3 to 15.3 mg/100g) but no trace of this mineral in this was detected in limes. Potassium can be found in orange juice (179-320mg/100g), tangerine (155-178mg/100g) and acid lime (140mg/100g), and markedly in banana (396 mg/100g) and avocado (599 mg/100g). The high levels of this mineral are associated with the high total acidity in fruits. Copper is known to have a destructive effect on ascorbic acid, since it catalyses its oxidation. Copper is found in tangerines at 0.7 to 0.9 mg/100g and acid lime (0.16 mg/100g). Calcium is present in abundance in acid lime (44mg/100g) and is also found associated with the pectic substances present in the form of calcium pectate in the middle lamella (Ito, 1971).

Minerals found in fruits are the first to ensure the possibility of gelation, which associated with pectin and other physicochemical parameters allow a rationality in the gelation process. Calcium and other divalent cations play a crucial role for the formation of lattice net in systems with LM pectin, known by the egg-box model (Grosso, Bobbio, & Airolidi, 2000). More recently was revealed a gelation characterized

by proton NMR involving LM pectins adding CaCl_2 (25mg Ca/g pectin) in the range of pH 6.0 which culminated in a innovative characterization as minimum pectin content that guarantee jelly process (1% in the LM and 0.1% in the HM) although this study highlighted these parameters using commercial standards (Kerr & Wicker, 2002).

A major contribution of fruits as food for humans is, doubtless, providing vitamins, especially vitamin C and A. Ingestion of 30-60 mg of these vitamins and provitaminic carotenoids is considered bellow of requirements for adults. Oranges generally contain 40-70 mg of vitamin C/100g juice, while tangerines and lemon provide 20-50 mg/100 suco (Stahl & Sies, 2005).

The instability of vitamin C is higher in the juice and losses are differentiated by processing mediated by enzymes such as ascorbate oxidase, fenolases, cytochrome oxidase and peroxidase, which oxidizes the ascorbic acid to dehydroascorbic and glucuronic. The presence of phenolics, flavonoids and acids may inhibit enzymatic oxidations, while copper and ion salts catalyze the enzymatic and non-enzymatic oxidation (Deutsch, 2000; Cardoso, Tomazini, Stringheta, Ribeiro, & Pinheiro-Sant'Ana, 2011).

Overall, the B complex vitamins are found mainly in the exocarp and mesocarp of fruits. In orange juice there is contains nicotinic acid (429 $\mu\text{g}/100\text{g}$), pyridoxine (57 $\mu\text{g}/100\text{g}$) and thiamine (40-100 $\mu\text{g}/100\text{g}$), grapes however, have enhanced levels of riboflavin (20-100 $\mu\text{g}/100\text{g}$), pantothenic acid (290 $\mu\text{g}/100\text{g}$) and biotin (0.4 to 3.0 $\mu\text{g}/100\text{g}$) (Ito, 1971).

In the guidance for simple jellies and blends prepared from whole fruit, it adds further value to the final product; it is expected to be transferred to jam high amounts of vitamins and minerals (retention level), which are concentrated in the mesocarp region,

endocarp and seeds. Blends intend to combine nutritional and sensory characteristics of two or more fruits providing pleasant taste and other sensory properties, thus gaining competitively international market.

4. Way of obtaining jellies and blends

4.1 Definition and commercial standard

In the United States, the Food and Drug Administration (FDA) published in December 2005 referencee the identity and standard for jellies in the Regulation Code (21 CFR 150,140). It considers this regulation that jellies and preserve as synonymous, but distinguish between jellies. Jelly and preserve are submmited cooking process and are prepared from fruits, therefore fitness this legislation approach. On this regulation, there are four types of designation for similar products: jam, preserves, jelly, marmalade. The jams and preserves are technically identical, with the following difference, jam fruit is crushed in paste; while in the preserve it remains fruits in its original way, whole or chunks. The jelly is a product obtained from the clarified juice and from fruit extracts. In order the better success of production well-gelled when finishing clearness and brightness and resemble flavor original. Jellies that include fruit pieces or strips in their whole gums are called alternatively marmalade, of citrus and non citrus fruits (<http://www.fda.gov>).

In addition, Brazilian regulamentation on identity of same products, defines jelly like as product obtained by cooking the whole fruits or in pieces, pulp or juice fruit with sugar and concentrated until to a gelatinous consistency, so that when removed from the container, it remains in semi-solid state respecting the properly combination of these ingredients, both in quality and order of addition during processing. (Cardoso et al., 2011). Yet determines brasilian rules that the jam should be transparent without

pieces of fruits, must exhibit elasticity to the touch, returning to its original form after a slight digital pressure. Color and odor must come from the own fruit. The flavor should be sweet, semi-acid, according to the fruit and/or blends (www.anvisa.gov.br).. Similarly European and American laws in order to improve qualify these products has specified yet cannot be colored and flavored artificially, being tolerated only the addition of natural acid and commercial pectin in order to compensate any deficiency in formulation (ISNN 1677-7042).

According to suitable and legislation, jellies are being contemplated in the category of products from fruits, whole or part, with or without seed, obtained by drying dehydration, lamination, fermentation, concentration, freezing, other technological processes considered safe for food production. They may be presented with or without a liquid medium, added sugar, salt, spice and other ingredient, since it does not mischaracterize the product. They may therefore have names by using terms that fall sensory properties, followed by expressions relative to ingredients characterizing the product as well as expressions emphasizing their functionality and way obtaining process, mainly driving american regulamentation.

This requirement of the competitive marketly in the XXI century fails mainly by analytical and sensory methodologies, which has attracted update in the scientific literature of newly products and process technological. The official physicochemical characterization recommended by the brasilian legislation of jellies are: total soluble solids, water-insoluble solids, pH, acidity total, acidity in organic acids, reducing sugar into glucose and non-reducing in sucrose equivalent. Microbiological standards for jelly, mashed fruits, paste or mass sweets and those similar follow the resolution of technical board establishing tolerances values for yeasts and molds at 10^{-4} viable cells/g (www.anvisa.gov.br/alimentos/legis/especifica).

4.2 Ways of processing jelly technology

Fruits for making jellies should be in their uniform ripeness, when they have better flavor, color, aroma and maximum content of soluble sugars. In the early stage of maturation, while failing proper sensory qualities (color and flavor), may also develop a brown color before and during cooking (Smith, 2003), because phenolase activity and Maillard reaction, simultaneously. Thus, it is recommended the addition of unripe fruits to supply protopectins in reasonable proportion with ripe fruits, usually from 20:80 to 30:70, depending of chemical nature of fruits.

Actually, senescence fruits show pectic substances partially decomposed into pectic acids, thus do not show the complete gelation capacity depending on the activity of desesterified (pectin esterase EC 3.1.1.11) and depolymerizing (the hydrolases, pectin hydrolase and polygalacturonases EC 3.2.1.11, EC 3.2.1.67 and EC 3.2.1.82. While lyase: pectin lyase and pectate lyase EC 4.2.2.2 and EC 4.2.2.10) enzymes are more exharcebated in unripe fruits (Uenojo & Pastore, 2007). The activity of pectin esterase EC 3.1.1.11 has been demonstrated intense as mature advanced, being known as enzyme associate to softening of fruit.

The raw material standardization (fruits and ingredients) is essential to obtain a good quality jelly, especially when seeds exocarp and mesocarp are added. These criteria generate information and determine tolerances about the type, shape, color, maturation degree, defects, presence of extraneous and toxic material and others (Smith, 2003).

The main selection may be the color desired in the final product, with the purpose for preparation of jellies, blends of red, purple or yellow fruits. Recently, it was proposed a brief classification, which considered the fresh fruit color: being white or

beige (sums of apple, pear, banana), orange (sums of guava, mango, nectarines, passion fruit, orange, papaya, peach, tangerine), blue/black (sums of blackberry, black grape), red/purple (sums of cherries, pomegranate, raspberry, red grape, strawberry and watermelon), pink (fig) and light green (melon, lime, kiwi) (Pennington & Fisher, 2009). This classification helps to guiding the preparation of jellies from fruits which theoretically would give more uniform products regarding coloring aspects.

Fruits can also be classified according to their ability to form gels, determined by titration (McCready, 1970) and FT-IR (Manrique & Lajolo, 2002) during last twenty years. Lima and colleagues (2010) have been addition mainly the aspect as pH, content and nature of pectins (HM and LM in tropical and temperate fruits) and so on. In this studied fruits were grouped according to their ability to gelation (group I - yield good jelly, group II - do not yield good jelly, fiber-rich chemical composition, and Group III - do not yield good jelly, starch-rich chemical composition), which score and set the conditions for jellies formulations and complement this guidance regarding the fruit color. From this strategy there is advantageous to produce blends using combinations of fruits of different groups (group I x group II and group I x group III), in order to compensate for the failure of some parameters.

In this early step is essential to adding water in amounts sufficient for all pectin in the fruit (protopectin plus pectin) is being extracted completely by lowheating. In some cases, the amount of water over the fruit should be optimized. Excess watering can produce pectin dilution, require more cooking time, reduce the gelation power by the molecule hydrolysis and promote losses of vitamin C, anthocyanins and other soluble substances (Smith, 2003). The literature does not provide precise data on the appropriate fruit:water ratio, depending on practical observations for each technological process, based on morphology and chemical criteria of the fruit. In the case of tropical

fruits values as fiber content and total solids (pectin, sugars and relation Ca^{2+} :solids) can be choiced the best optimization procedure.

The super cooking should be avoided because it can produce a cloudy juice difficult to filter, reduce the gelation power of pectins, cause losses in aroma and flavor of fruit and produce Maillard reaction by-products. This operation average time varies from 5 to 10 minutes for grapes; 20 to 25 minutes for sliced apples; 30 to 35 minutes for guavas and oranges, most common pectin source (Barrett, 2007).

When necessary it can be added to the diatomaceous earth liquor (celite) in an amount to be tested. The cooking causes the organic matter precipitation, which should be removed by the filtration step and consequently the liquor should be more clear, if the material is not appropriate it should be included a spin, combined directly with filtration (Baker, Berry, Hui, & Barrett, 2005).

4.3 Formulation strategies to improve jellies technology

The best result is obtained when the quality raw materials and flow chart are adjusted in order to achieve the low cooking time and fewer process steps (Diaz, Anthon, & Barrett, 2007).

The formulation depends on the presence and nature of pectins present in the fruit itself, and when it is absent or at insufficient condition, it requires the pectin addition: commercial forms of HM and LM; citrus fruits mesocarp; properties combined to generate blends of fruits (Lima et al., 2010). Furthermore, it is necessary acid media obtained by adding organic acids, usually citric acid, and sugar in amounts favorable to the gelation mechanism and according to formulation (Baker et al., 2005). The preparation of jam follows the flowchart has been illustrated in Figure 2.

The heterogeneous composition and divergence of ideal conditions with a focus on the defects of pectin gels compared to fruits which the presence of starch, fiber and its interaction with A_w (water activity) observed in practice from commercial products lead an interactive discussion regarding the pattern established for jellies (jelly) in this review, it can be addressed to the discussion from Figure 3, guiding new trends in relation usual regulations.

In vacuum cooking system where pectins can be added in parallel with the other ingredients (discussed later) is recommended the prior homogenization of pectin and sugar at 1:5 ratio (pectin:sugar). The sucrose present in the formulation tends to reduce the pectin solubility, which may encourage its precipitation and lumps formation (Barrett, Somogyi, & Ramaswamy, 2005). In an excess situation it almost always leads to crystallization and budget deficit, there was low aggregation of gel fragments producing exsudates.

When it comes to cooking in open pan, it is recommended the pectin addition in the final phase, before the acid addition, to minimize the degradation of pectic substances (Diaz et al., 2007).

Sugar acts favoring precipitation and pectin dehydration, narrowing the formation of three-dimensional network that provides gel stability, according to the pectin nature. It also acts as a chemical preservative, since it delays the growth of microorganisms, improves the appearance, flavor and final yield.

The sucrose (most common) used in this formulations, in quantities sufficient to obtain a jelly with 64 to 71° brix, 65° has been considered grade ideal in order to obtain a firm gel without evidence of crystallization. Alternatively, it can be used artificial sweeteners, and fruit-based or glucose-based syrups to serve differentiated

products (diet and reduced-calorie). However, care must be taken in relation to hysteresis and syneresis (Baker et al., 2005) both produce exsudate.

The power of pectin gelling in the fruits liquor is rough directly subject to the DM that can be empirically assessed by mixing 1:1 juice fruit:alcohol (95° Gay Lussac). The precipitated material obtained is also called "Insoluble Residue in Alcohol" and depending on the gelatinous mass consistency (precipitate), assessing jelly force of the liquor as lowest, medium and highest. Lima and colleagues (2010) have contributed to characterize the gel fragment size measured by Scanning Electronic Microscopic, showing that gel firmness was strongly dependent on the DM include assessment of size and diameter of the fragment retained in mesh-defined polyester in diverse fruits. This evaluation has guiding sugar amount to be added to the jellies formulation showing a better way of adjustable parameters before processing. If the juice is too rich in pectin, amount (1:1) juice:sucrose; if medium, 1:0.75; and if lowest, it is used ratio 1:0.5 (Leme Jr, 1968) therefore used as tool for gel quality. On the other side, acid components and heating can induce the generation of invert sugar, a mix now of sucrose, glucose and fructose have a higher solubility than sucrose in its pure form, aiming carry over a solid concentration above 68.8% without produce crystallization. The amount of invert sugar obtained during cooking is high, however short cooking is desirable. Thus, the addition of glucose and fructose syrup (powder), mainly in the vacuum cooking system (lowest reversal rate) is being desirable, thus obtaining a bright feature product (Diaz et al., 2007), sufficient prevention of syneresis and hysteresis with repercussion in calorie value and sweetness (Baker et al., 2005). However, synthetic sweeteners may be employed in the jelly formulation, such as sorbitol, mannitol, acesulfame K, aspartame, isomaltose, saccharin, sucralose, neohesperidina dihydrochalcone, neohesperidine, acesulfame, maltitol, lactitol, xylitol and erythritol. Care

must be taken once specific defects reported for some sweeteners emerging interaction with A_w and others ingredients.

To obtain a stronger gelation, the pH of fruits should be adjustable between 2.2 and 3.5. The natural pH of most fruits is generally outside this range, being necessary to correct with the addition of acidulants such as, citric, tartaric and malic acids, so that not to compromise the chemical structure of pectin, nor affect flavor, aroma and texture (Lima et al., 2010).

The most used acidulant is citric acid due to its high water solubility, chelating action on certain metals (copper and iron), scavenger or synergistic effect when associate to some compounds antioxidant, while the tartaric acid is not recommended for grape and apple jellies, as these fruits have naturally high levels of tartaric acid, which induces the crystallization of potassium tartrate in making jellies (Barrett, 2007).

The satisfactory gel situation (with a minimum flow, exsudates and defects) obtained under atmospheric pressure is recommended that the modern acid (even considering the use of organic acids) should be added to the end of processing, prior packaging to minimize the impact on the gels defects as syneresis.

The pectin contained in fruits or added, when subjected to heat in acid media can deploy the pectic polymers, yielding significant changes in gelling properties affecting functional, sensory and technological properties. The ideal systems should respecting the order because expected interaction of binomial dependent reactions, combining time and temperature (Acosta, Viquez, & Cubero, 2008).

In vacuum-processed jellies the acidification occurs in a single stage, since the operation temperature is always constant and below 60°C, with the advantage of operating in the partial absence of O_2 , which minimizes undesirable oxidative reactions,

little wide-broadcast system due to low availability of commercial models adjustable to this way of manufacturing (Fügel et al., 2005).

The gel state is considered as close to the solid state, which taking into consideration its organization, allows keeping its shape and resisting small changes. It consists of a solid three-dimensional network that holds a liquid phase among the mesh. Gel is a system in which pectin exists in the intermediate state between solution and precipitate. The addition of any ingredient that reduces pectin solubility will favor gel formation (Willats et al., 2006). Sugar nature complexed to galacturonic chain and double helix segments were registered as main factors that affect jelly properties borned pectin molecule. Chemically, the pectin colloidal solution has hydrated micelles and negative charges due to COO-groups, which cause repulsion between the micelles forcing the molecule to adopt a linear configuration (Baker et al., 2005).

The gelation property is undoubtedly the most debated feature, especially because polysaccharide gelling is a critical point in the formation of desirable texture to many foods and technological applications of different sizes and shapes of these biopolymers taking off functional properties and so one parameters involved in food formulations.

In order to transition from sol to gel is required to occur approximation of micelles, which happens only by reducing the negative field around these structures. For such, acids are used, which release H^+ ions forming the protonation of carboxylate groups leaving micelles closer. Usually this process takes place with the pH reduction to values between 2.2 and 3.5 for pectin (Lima et al., 2010).

With the protonation of carboxylic groups, micelles are at a shorter distance; however, it is still not enough to establish gel formation due to the presence of water

molecules around the micelles. At this gelling stage, free sugar has an important role, it favoring the approach of micelles by divergent mechanisms in the literature: 1 - some researchers believe that the sugar acts as a dehydrating agent (oldest view); 2 - while other suggest that the soluble solids, pectin and water interact via hydrogen bonds with sugars, proteins and acid components (Speiser & Eddy, 1946), 3 - and still others suggest that the sucrose function is controlling the water activity in the final product by osmotic dehydration (Baker et al., 2005). These mechanism likely sufficient itself for explanation interactions involved on jelly properties of pectin.

Other factors also vital to the sol-gel transition include: pectin characteristics such molecular weight, DM, degree of amidation, presence of acetyl esters at O-2 and O-3 in galacturonans chain, rhamnose and galactose and ester groups distribution along galacturonic chain temperature, divalent ions concentration and others.

Pectin chains adjustable and juxtaposed through hydrogen bonds and hydrophobic associations form the combination areas of molecules (Figure 3). Sucrose function is causing a dipole orientation, which will attract each other causing gel stiffening through hydrogen bonds. Ideally at 69% sucrose levels, satisfactory gel is formed, under excess conditions (above 71%) it induces the formation of crystals network, while at levels below 64%, fragile gel with exsudate will occur simultaneous Aw higher (Baker et al., 2005).

The pectin gel is formed by heating and depending on the cooking system its solidification occurs during cooling. The temperature at which the gel is formed is called gelation temperature, which is strongly influenced by pectin's DM (the greater, the higher gelation temperature). When DM is > 70% on cooling it gelled in the range 70-76°C when the processing occurs at temperatures above 100°C. While the DM \cong

60% gelled during the cooling temperature of 45-60°C, usually employed in vacuum cooking systems (Fügel & Schieber, 2005). This temperature setting is indicative suitable of sol-gel transition. Other interferences are discussed in the literature, for example, sugar beet pectin, the acetyl functions greatly reduce the gelling power, 2.6% acetylation almost completely reduces this property. The strongly acetylated pectic acid can show aptitude for gelation in acidic and sweet media for example pectin beet has 40% DA (degree of acetylation). The gelation function hampered by low amounts of acetyl groups (<1%) becomes possible when a strong acetylation recreates hydrophobic surfaces of acetyl groups (Morris, Castile, Smith, Adams, & Harding, 2010).

The pectin's desesterification pattern influences notoriously in the gelation power in a HM pectin with the use of three treatments: two enzymatic ones by pectinmethylesterase from native citrus and fungal *Aspergillus niger* compared to alkali treatment. Fungal PME produce chains derived from homogalacturonans (HG) with scattered arrangement of methyl esters along the chain. In contrast, plant PME usually have a block pattern action, producing long strands of non-esterified HG. The alkali treatment produces a random distribution of methyl esters. The effect of different patterns were shown by gel compression test (texture analyzer) of lemon pectin with added calcium. Two pectin gels with DM (41 and 43%), but with different distribution of esterified groups (obtained by plant and fungal PME), were produced and compressed under similar conditions. Gels with pectin hydrolyzed by plant PME demonstrated faulty results due to breaks, though not visible, which ran vertically through the gel. In contrast, pectin gels hydrolysed by fungal PME deformed with more plastic pattern (Willats et al., 2006)

The commercial LM pectins have variable esterification between 10 and 50% and form gels in the presence of divalent cations (Grosso et al., 2000; Cardoso,

Coimbra, & Lopes da Silva, 2003; Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006), favoring the gelling of fruits with different composition to the requirements of good gelation or even leading to diet jellies production. Gel is sufficient stabilized by ionic interactions between the pectin molecules and divalent cations (Figure 3), such as calcium or magnesium (Evageliou, Morris, & Richardson, 2000). Some researchers also suggest the involvement of calcium chelates intermolecular, leading also to the approximation of molecules. LM pectin gels cover a wide range of soluble solids (10-70%) and pH (ranging from 2.8 to 6.0) favoring the gelation process, which demonstrates its versatility in technological applications. In this case, gelation requires the presence of calcium (minimum 0.025%) which is bonding to carboxylate ions free of adjacent pectin molecules (Kerr & Wicker, 2002). Although a complex and interactive mechanism, being qualified usually as egg-box model, where calcium is the “egg” and the “box” limits of pectin chains (Grosso et al., 2000), depending the nature of pectin structure associating other components light modification can be assumed.

It is necessary two chains of 14 deesterified pectins units, symmetrically aligned, parallel and opposite in which the calcium ion forms chelate with the carboxylate ions. The calcium concentration in solution increases progressively in the longer bond sections (or junction zones). Gel is consolidated from a three-dimensional network with junction zones, thermo-reversible, softness and more elastic than those obtained with HM pectins (Kerr & Wicker, 2002; Willats et al., 2006). The gel strength is improved when the calcium level increases until a good consistency is reached. This gel tends to be elastic and more resistant. When there are excessive bonds among molecules, causing contraction and convergence between polysaccharide molecules, forming a brittle gel, more turbid and prone to syneresis, similar to the gels of gelatin and starch (El-Nawawi & Heikel, 1997), out of ideal situations. The low activity of methyl ester

groups of pectin and no accommodation of this waste in junction areas, result in distortions in the gel matrix requiring balance between Ca^{+2} forces and undesirable groups, such as $\text{GA} > 1\%$ to reverse the situation.

LM pectins may be obtained from an HM, where they are partially deesterified in the presence of ammonia and alcohol or in homogeneous system using aqueous concentrated ammonia. The amide groups show spontaneously within gel junction zones and, indeed, they seem to help with tolerance and reactivity of LM pectins (Racape, Thibault, Reitsma, & Pilnik, 1989).

Pectins with these property claim applications in products with low sugar content ($<50\%$), low-calorie, confectionery, and other applications involving sugar and oil restriction. The thermal reversibility of LM gels allows its use for glazing, fillings and toppings for bakery products and confetti, which undergo sterilization and pasteurization processes (Iglesias & Lozano, 2004).

The cooking evaporates water and concentrates solutes, maintaining and favouring gelation, but several volatile and thermolabile components responsible for quality attributes are lost at this stage (Barrett et al., 2005). The literature cites some unusual methods to concentrate juice to get jelly, such as freezer dried concentration where part of water is frozen and then crystals centrifuged (5.000 rpm) and concentration by reverse osmosis with cellulose acetate membranes, significantly reducing the cooking time in order to minimize oxidative, nutritional and sensory losses. The goal in this case is obtaining jellies with characteristics of greater retention of flavor, color and vitamins; however, most industries uses conventional processes as the open pan (atmospheric pressure) and vacuum cooking (ball-type pan), according to the pectin nature and/or way of processing chosen (Diaz et al., 2007).

The minimum time required for cooking ranges from 8 to 12 minutes (Baker et al., 2005), according to the cooking system, by extending causes demethoxylation associated additive of pectin hydrolysis and other non-pectic polysaccharides. In contrast, the heating under insufficient time period can generate low sucrose inversion, incomplete sugar absorption by the fruit, therefore speeding up the osmotic processes during processing and storage, compromising the nutritional and sensory quality of the jelly (Barret, 2007).

In vacuum cooking, the mixture of all ingredients is previously made in a pan and then transported to the hub. The concentration temperature is made at 50-60°C. When finished cooking, the jelly can be heated in the same hub at 85-90°C, or can be downloaded and heated in another pan before filling (reaching maximum 12 minutes). Pectins degradation, both natural and commercial, is minimal in this case requiring 50 to 10% less pectin compared to the open pan process and changing the shape and order of addition of pectins and acids components. In this case, is lighter in color and taste closer to fresh fruit, allowing the preparation of controlled batches (Khondkar, Tester, Hudson, Karkalas, & Morrow, 2007). The determination of the end point can be determined by various methods, the being the evaluation of boiling temperature and refractive index at 20°C the most rapid and accurate procedure, although vary according to process.

For example, in the open pan cooking system, the gelation point is reached generally 5-6°C above the water's ebullition point 105 to 106°C at sea level (decreasing 1°C per each 250 meters altitude. This value should be taken at the thermal center of pan and is equivalent to 65 to 68% of soluble solids; such method is widespread in the industry. There is an empirical test for determining the end cooking point for homemade

products, which consists of draining the material, presenting in the form of slides, suggests the end of the cooking process (Diaz et al., 2007).

In order to fill of vacuum-concentrated jellies, it becomes necessary to raise the mass temperature at 85°C followed by a cooling considered as "rapid pasteurization", aiming the microbiological quality of the final product, minimal color and flavor changes. Jellies processed under atmospheric pressure must be cooled at 85°C, simulating a slow pasteurization, minimizing the risk of breaking glass, darkening, giving even the partial interruption of the sucrose inversion and pectin hydrolysis (Root & Barrett, 2005). Filling temperature should be between 5 to 10°C, below the aforementioned recommended for the cooking system, minimizing the negative impact for each cooking system. Filling by hot fill system coupled to the technological process should be more appropriate than the thermal effect assuring in terms of destruction and/or inhibition of pathogenic microorganisms and enzyme inhibition (Acosta et al., 2008). Therefore it has been used as flash pasteurization safety processing (temperature of 90 °C by shorter time).

Glass packaging is the most widely used to pack jellies, since this material is easier to sterilization than coupled to the hotfill system, providing excellent results better product stability (Fügel & Schieber, 2005).

Glasses package tend to be overcome in the coming decades by polyvinyl chloride (PVC) or polypropylene (PP) for its practicality in joining qualities of transparency and reduction of O₂ and light migration (Fügel & Schieber, 2005).

The jelly labeling should follow the current resolution of each country. Jellies containing additives must bring in their labeling the indication of additives added as well as the class obeying regulamentation and legislation.

Shelf life of a food is defined as the time at which it can be stored under certain conditions of temperature, relative humidity, light and others, suffering minor changes acceptable to the manufacturer and following the protocol recommended by the current legislation simulating those product's use conditions by the consumer in order to verify the relevant changes by analyzing the parameters established by current legislation.

The main changes occurring during processing and its control have been discussed in previous sections, thus integrating the concept of overall quality for jellies, it has considered that the functionality can be compromised by biochemical changes since fruit's harvest to storage and marketing of final product. In this sense, it has been to provide guidance, regarding aggregate values related to the desirable characteristics in a product using concept overall quality assessed through the possible biochemical, nutritional and microbiological indicators described below:

- Anthocyanins retention degree: unstable in acid medium and susceptible to changes in color especially during exposure to oxygen and storage, adding the effect of time and processing temperature;
- Vitamins and pro-vitamin: those liposoluble can be concentrated on the processing of jellies, however, the thermal impact of the cooking system at atmospheric pressure causes the molecules interaction with oxygen that are strong triggers of changes in α and β -carotene, β -cryptoxanthin and xanthophylls. Those of B-complex, by its ionic nature may undergo chemical changes, modifying the pattern of hydro and liposoluble vitamins found in fruit. In this sense, the maximum retention (ideal between 90-99%) should be taken as indicators of good quality of technological process, allowing indicate that degree below cutoff should reorientate processing technology;

- Sugars: there is a slight increase of reducing sugars and reduction of non-reducing sugars during storage due to the acid hydrolysis. Sugar additions after processing can generate intensification of Maillard products, while the unstability favouring the crystallization and/or syneresis;
- Aroma: loss of aromatic compounds, accentuated in the conventional filling systems, associated with inadequate thermal processing may favor the appearance of extraneous smells almost always due to contamination before and during the thermal process. It is worth noting organoleptic properties statistically significant for this component of overall quality, involving the concept related to aroma and flavors respecting the fruit's chemistry peculiarity.
- Flavor: Hansson and colleagues studied the flavor release in pectin gels. The gels were produced with different pectin (1.5-2%) and syrup (34% sucrose, 24% glucose, 22% fructose and 20% water) contents. As pectin concentration increased, it was observed rough increase in gel strength. In this studies aromatics compounds (isopenthyl acetate, ethyl hexanoate, cis-3-hexenil acetate) were significantly higher in the air/gel partition coefficient in the weak gels compared to the rigid ones. Observe yet that mono and disaccharides exhibited a salting-out effect, which increased volatility of aromatic compounds. When the sucrose interacted with water, increasing aromatic compounds retention diminishing free water (exhibiting low exsudates (Naknean & Meenune, 2010)).
- Microorganisms (yeasts and bacteria): Due to the acidic characteristic of fruit jellies associated with intermediate $A_w \approx 0.8$ and high osmotic pressure, some one products are often contaminated by yeasts (usually, maximum $10^3/\text{g}$). Sporulating species (*Chrysosporium inops* and *C. xerophilus*) can withstand from heat processing, mainly under vacuum cooking system (Leong, Pettersson, Rice, Hocking, &

Schnürer, 2011). Appropriate indicators should be oriented according to the fruit's nature, morphology and the technological processes, considering since the ingredients characterization (and raw material) to the filling and packaging steps of processing system.

- Water activity: whereas this parameter is not considered by Brazilians and american laws, it has suggested that as a product of intermediate water activity (0.75-0.85), it is a stronger quality indicator evidenced by the practice market observing in some commercial jellies even disregarding the process, exudates' level above ideal contrast of definition. In the particular formulation involving a blend, it has observed frequently hysteresis and synerese, considering visual evidence of defects tracking out extremely acidic medium, exhotic fruits, low-solid content jellies and combinations without some criteria before formulation.

5. Updating remarks

With the aid of analytical tools for structural characterization of pectins now discussed in this issue, it can be categorized some considerations in order to clearance contribution about pectins characterization related to jelly technological processes. In a academic sense has the study ranking atributes of the production chain to concept of overall quality in products, particularly on the chemistry of fruits until for making jellies and correlates:

- In addition to conventional parameters has been shown a previous caracterization of pectin fraction (DM, DA, average size of gel fragments and pectin content) exhibiting rough correlations with structural parameters of gels pectins from liquor fruits, and also helps to guide the previous characterization

of raw materials and ingredients in order to ascertain suitable formulation, improving sensory and nutritional quality;

- Other physical and chemical parameters (cellulose, soluble and insoluble fiber, divalent cation, vitamins and minerals) known in fruits allows to orientate technological and nutritional adequacy concerning to significant improving in value, involving cycles and critical hazards since primary production;
- The controversial role of the sugar or sugars in jellies formulation and its interaction with pectin and other organic acids, naturally involved in the taste and flavor chemistry, as well as related as critical hazards from the fruit post harvest until jellies production, allow to improve the technological process;
- The chemical and micro-structural ordination of sugars (usually mono- and disaccharides) in relation to the natural pectin chains, and integrated to the technological process, respecting compatibility with the cooking system it was showed rationalize the technological process through optimal formulation (choosing essentially range critical factors).
- A sequence of new technological descriptors (chemical, nutritional, sensory and microbiological attributes) aiming the compatibility in newly formulations is suggested, especially for evaluations of formulas involving the exotic and native fruit alone and involving some mixture (blends), yet poorly characterized as to jelly force parameters.

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Captions of figures

Figure 1. FT-IR spectra: (a) pectin extracted from orange mesocarp (*Rutaceae* sp) DM=69.0%; (b) pectin (alkaline media) extracted from passion fruit mesocarp (*Passiflora edulis*) DM=14.0% (c) pectins standard (Fluka) DM=72.0%, (d) galacturonic acid standard DM=10.8%.

Figure 2. Processing of fruit jelly related to thermal treatment

Figure 3. Junction zones formation in gels containing pectin (a) HM and (b) LM.

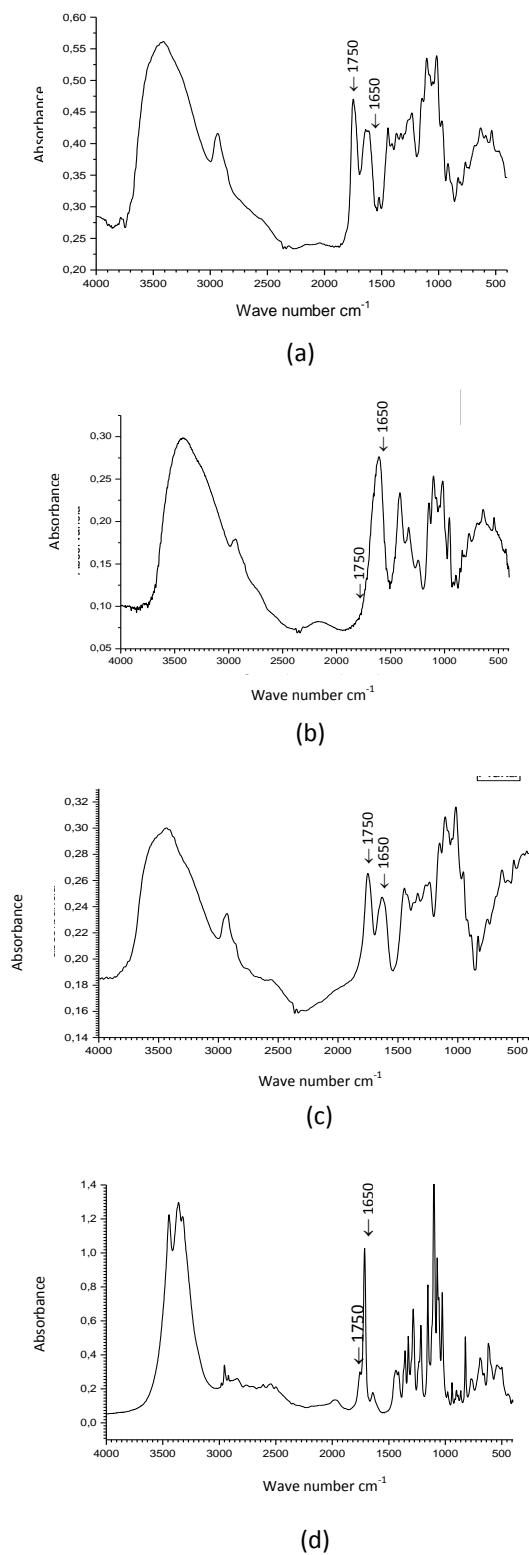


Figure 1. FT-IR spectra: (a) pectin extracted from orange mesocarp (*Rutaceae* sp) DM=69.0%; (b) pectin (alkaline media) extracted from passion fruit mesocarp (*Passiflora edulis*) DM=14.0%; (c) pectins standard Fluka DM=72.0%; (d) galacturonic acid standard DM=10.5%.

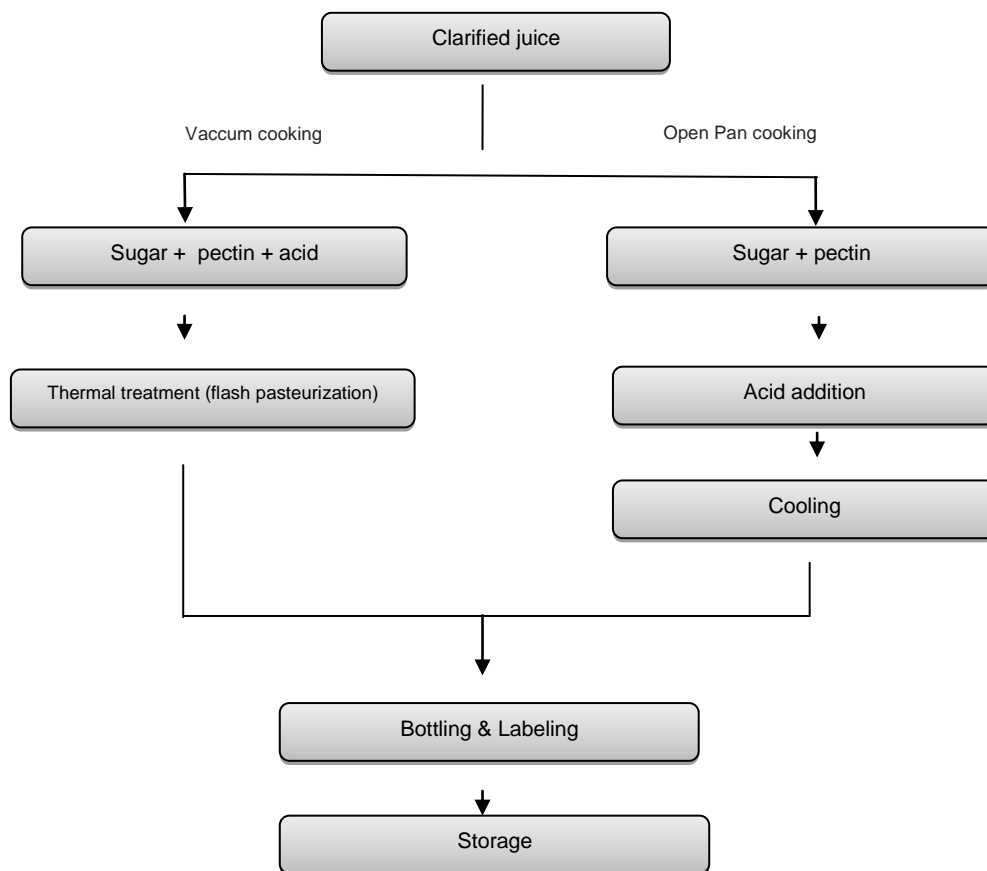


Figure 2. Flow processing of fruit jelly related to thermal treatment

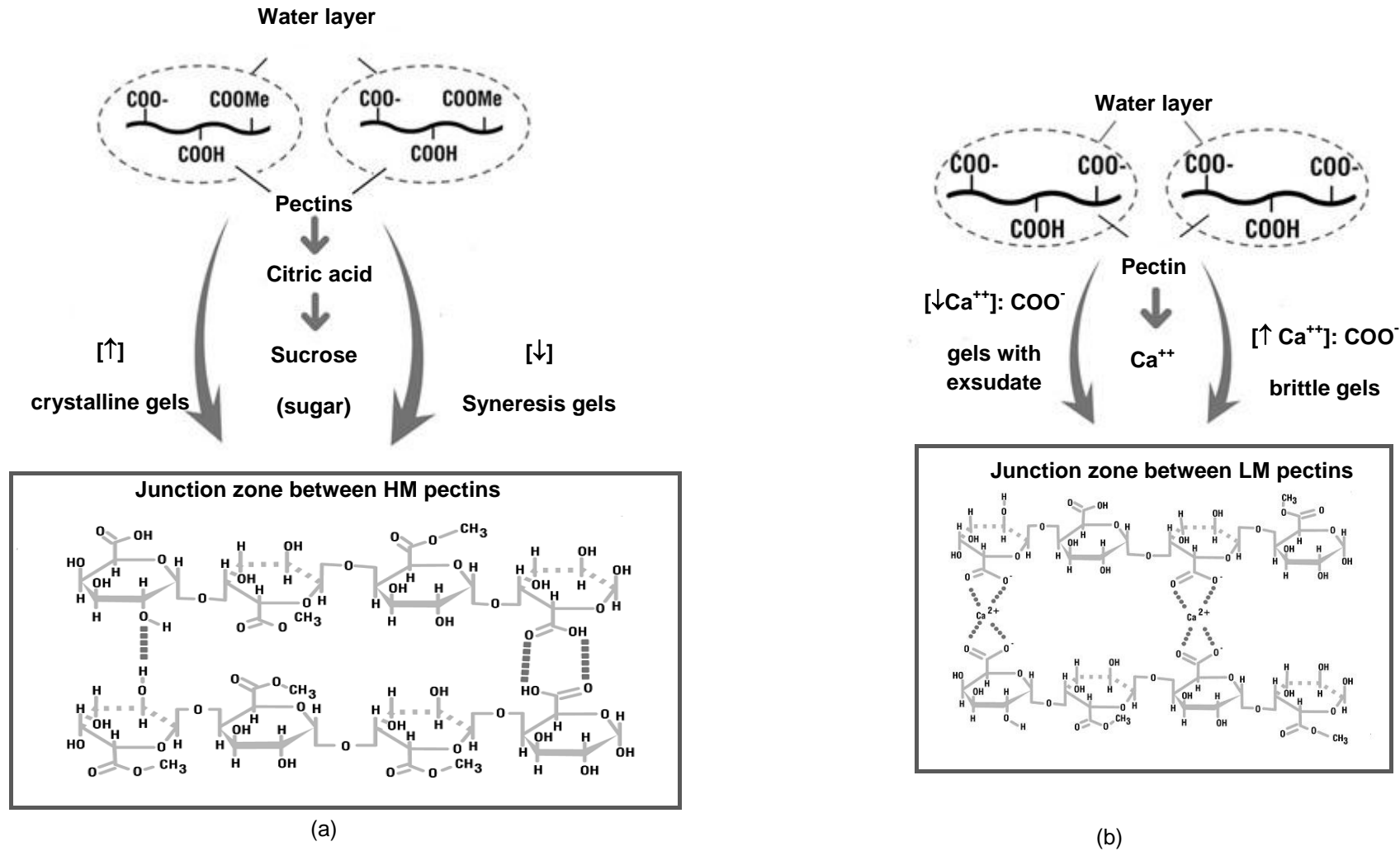


Figure 3. Junction zones formation in gels containing pectin (a) HM and (b) LM

4. Material e Métodos

4.1 Materiais

4.1.1 Amostras de frutos

Todas as frutas foram selecionadas e adquiridas respeitando estado de maturação em função de caracteres sensoriais, a partir de análises visuais.

Foram utilizados frutos inteiros: maçã (*Malus silvestres*), morango (*Fragaria vesca*), Kiwi (*Actinidia deliciosa*), banana (*Musa paradisiaca*), mamão (*Carica papaya*), acerola (*West Indian cherry*) e goiaba (*Psidium guava*). A partir de laranja (*Rutacea sp*), maracujá (*Passiflora edulis*) e melancia (*Citrullus vulgaris Schrad*) foi removido manualmente a região mesocárpica do fruto. A introdução dos materiais investigados no procedimento de extração de pectina foi respeitado um plano amostral e diluição em relação ao teor de água. A melancia foi utilizada para investigar o potencial deste resíduo como fonte de pectinas.

4.1.2 Padrões de pectinas

Foram utilizados padrões de ácidos galacturônicos GM=10,59% (Fluka, cód.48280) e GM=1,52% (Sigma, cód.48280). Padrões de pectinas de alto GM: 68% (CP Kelco, Genu tipo 105), 72% (Sigma, cód.P8471) e 90% (Sigma, cód.P9135), 85% (Fluka, cód P9561); e padrão de pectina de baixo GM: 30% (CP Kelco, cód. Genu LM102AS).

Todos os reagentes utilizados na análise por cromatografia líquida foram de grau HPLC, e os demais, de grau analítico.

4.1.3 Equipamentos

Para determinação do GM (%) dos padrões e das amostras de pectinas utilizou-se o espectrômetro de infravermelho Bruker IFS66.

A análise por HPLC procedeu em Cromatógrafo líquido (LC-20AD-Shimadzu) acoplado a degaseificador (DGU-20A₃) e CBM-20A. A detecção do HPLC ocorreu por *evaporative light scattering detector* (ELDS-LTII Shimadzu), utilizando gás nitrogênio grau analítico 5.0 (White Martins).

4.1.4 Fases estacionárias e fases móveis

Para a adequabilidade de detecção pelo novo sistema (ELSD) foram experimentadas as seguintes fases estacionárias: Hipler H Acid 100x7.7mm, (8µm), empacotada com *styrenodivinilbenzene with lead sulfonate (copolymers)* (Varian, PL1170-2823); Waters Spherisorb ODS-2 150x3.2mm (5µm) (54904); e Waters Spherisorb S5 ODS2 100x2mm (5µm) microbore column (PSS831922). As fases móveis testadas foram H₂O Mili-Q e ácido sulfúrico 5mM, 50 mM e 100mM.

4.2 Métodos

4.2.1 Procedimento para extração de pectinas (estudo piloto)

Inicialmente foi realizado uma otimização de extração de pectinas, a partir de mesocarpo de melancia. Tomou-se 100 g de mesocarpo a partir de 1000 g picados em cubos, aos quais foram adicionados 300-400 ml de água deionizada e a mistura foi triturada em multiprocessador. Posteriormente, o pH foi ajustado a 2,2, com ácido cítrico 10%. A mistura foi aquecida a 100°C e mantida por 30 minutos. Após o resfriamento, foi realizada uma filtração utilizando bomba à vácuo e funil de Buchner, com auxílio de um retentor. No líquido extraído foi adicionado álcool *quantum satis* para

precipitar exaustivamente a pectina. Foram testados nesta etapa diferentes tipos de álcool, a fim de se avaliar o comprimento da cadeia carbônica sobre a estrutura da pectina. Os álcoois avaliados foram: metanol, etanol e isopropanol (95%). O precipitado foi lavado com o respectivo álcool 99% e posteriormente com acetona 99%. O precipitado final foi deixado em temperatura ambiente por 24h para evaporar resíduos de álcool e em seguida submetido à liofilização para fins de preparação da pastilha de KBr.

4.2.2 Ensaio de otimização (efeito da natureza do ácido e da temperatura da reação)

Neste ensaio foi avaliado o efeito da natureza do ácido (orgânico *versus* inorgânico) e da temperatura sobre a extração de pectinas e GM (%). Foram utilizados dois planejamentos fatoriais 2^2 , com um ponto central, tendo como variável independente ácido cítrico (%) x temperatura (°C) (1º planejamento) e HCl (M) x temperatura (°C) (2º planejamento); e dependentes: rendimento (%) e grau de metoxilação (%) (tabela 1). Cada planejamento consistiu em 7 ensaios, com três repetições no ponto central (tabela 2). A partir destes resultados, foram selecionadas as condições de extratibilidade de pectinas nos demais frutos.

Tabela 1. Avaliação do tipo e da força do ácido na extração de pectinas. Níveis decodificados das variáveis independentes.

Desenho fatorial			
Experimento I			
	Código	Ácido cítrico (%)	Temperatura (°C)
	-1	5	50
	0	10	75
	1	15	100
Experimento II			

Código	HCl (M)	Temperatura (°C)
-1	0.025	50
0	0.06	75
1	0.10	100

Tabela 2. Combinações experimentais no perfil de extração da pectina.

Ensaio	Condição de ensaio (ácido cítrico)	Condição de ensaio (ácido clorídrico)
1	5% x 50°C	0.025M x 50°C
2	15% x 50°C	0.10M x 50°C
3	5% x 100°C	0.025M x 100°C
4	15% x 100°C	0.10M x 100°C
5	10% x 75°C	0.06M x 75°C
6	10% x 75°C	0.06M x 75°C
7	10% x 75°C	0.06M x 75°C

4.2.3 Determinação do grau de metoxilação

Padrões comerciais de pectinas, amostras de pectinas liofilizadas e KBr foram dessecadas à vácuo por 24h. As amostras foram homogeneizadas com KBr (90:10 sal-amostra), e com auxílio de compressor foi obtida a pastilha, a qual foi encaminhada ao espectrômetro de infravermelho. O espectro FT-IR foi coletado em absorbância na faixa de 400-4000 cm^{-1} , na resolução de 4 cm^{-1} (em duplicatas).

Após análise por espectroscopia de infravermelho das amostras isoladas, foram tomadas as bandas 1650 e 1750 cm^{-1} dos espectros, no que através da equação: $[A_{1750} \text{ cm}^{-1} / (A_{1750} \text{ cm}^{-1} + A_{1650} \text{ cm}^{-1})]$ quantificou-se o GM das amostras.

O grau de metoxilação dos padrões e amostras de pectinas foi determinado por método titulométrico (N=5) de acordo com a proposta de McCready (1970), modificada para a quantidade inicial de padrão para facilitar a visualização da reação.

4.2.4 Determinação do GM por HPLC-ELSD (*Evaporative light scattering detector*).

Na primeira fase, os experimentos foram produzidos para estabelecer as condições cromatográficas, variando o ganho do detector de ELSD de 1 a 12, combinado a intervalos crescentes de temperaturas de nebulização do detector (de 20 a 60° C). Nesta fase foram utilizados padrões de pectina de alta metoxilação (superior a 90% GM) e de ácido galacturônico (abaixo de 1% GM), desesterificados. A confirmação dos picos ocorreu por injeção de padrões desesterificados e por injeção de mistura de padrões (metanol + ácido acético + isopropanol) em quantidades equimolares. As fases estacionárias (spherisorb ODS-2 x hiplex) foram avaliadas. A repetibilidade da extração foi avaliada nas condições NaOH 1M nos tempos de 5, 30, 60 e 120 min.

Na segunda fase, visando adequar as condições de desesterificação dos padrões e amostras de pectinas, foi utilizado um planejamento fatorial de decomposição central rotativo (DCCR), onde as variáveis independentes foram planejadas variando a força de NaOH (0.5 M a 2 M) e tempo (5 a 120 min) (tabela 3). As variáveis decodificadas resultaram em um total de 11 experimentos (tabela 4). Após ajuste do procedimento de saponificação, foi aplicado o método otimizado para os padrões e amostras.

Tabela 3. Ensaio de desesterificação de pectinas. Níveis decodificados das variáveis independentes

Código	Tempo de reação (min)	Força do NaOH (M)
---------------	------------------------------	--------------------------

-1,41	5	0,5
-1,00	21,71	0,74
0,00	62,5	1,25
1,00	103,28	1,78
1,41	120	2

Tabela 4. Experimentos de desesterificação de pectinas.

Ensaio	Tempo de reação (min)	Força do NaOH (M)
1	21,71	0,74
2	103,28	0,74
3	21,71	1,78
4	103,28	1,78
5	5	1,25
6	120	1,25
7	62,5	0,5
8	62,5	2
9	62,5	1,25
10	62,5	1,25
11	62,5	1,25

4.2.5 Otimização e adequabilidade da saponificação das amostras de pectinas

Aproximadamente de 5 a 10 mg de pectinas foram suspensas em 10mM de CuSO_4 . 0.5-1.0 mL de NaOH [-0.5-1.0–2.0 ∞ -] (M) foi adicionado para proceder a saponificação a 4°C . O tempo requerido para a saponificação foi experimentado de modo combinado [5x30x60x120] (min) de acordo com ensaio fatorial DCCR. Após a saponificação procedeu-se a centrifugação por 10 min a 8000g, em condições

refrigeradas. O sobrenadante foi neutralizado e adicionou-se o isopropanol como padrão interno. Foi utilizada a alíquota de 20 µl para injeção no HPLC-ELSD. A eluição ocorreu no modo isocrático, no fluxo de 0,6ml/min

4.2.6 Curvas de calibração (padrões x estudo de exatidão em amostras)

O GM dos padrões e das amostras de pectinas obtidas das frutas foram avaliadas por titulometria (McCready, 1970). Foi utilizado o sistema de equação para bandas de infravermelho (Manrique & Lajolo, 2002) e por HPLC-ELSD respostas em área integrada em 7 distintos padrões de pectinas, nas quais procedeu-se a saponificação/desesterificação, conforme proposta apresentada nesta investigação para compor as quantificações imprescindíveis no estudo de pectinas naturais. Assim foram construídas três curvas de calibração relacionando os resultados em distintas amostras e padrões: titulometria x FT-IR; titulometria x HPLC e FT-IR x HPLC através do programa Origin 8.0.

4.3 Análises estatísticas

Os resultados foram analisados através de análise de variância (ANOVA), e comparados pelo teste de Tukey ao nível de 5% de significância por comparações múltiplas e avaliação de contrastes.

O ensaio de otimização da extração e da desesterificação das pectinas, teve os resultados obtidos pelo planejamento fatorial, que foram avaliadas através da metodologia de superfície de resposta (MSR), com eixo central de resposta, e aplicado o teste de ortogonalidade por Anova e contrastes (programa R).

5. Resultados

A seguir serão apresentados os artigos originais referentes às observações dos ensaios laboratoriais de investigação das condições de extração e caracterização das pectinas por método titulométrico, Espectroscopia de infravermelho e por HPLC com detecção por ELSD. Os dois artigos foram submetidos a revistas internacionais, os quais serão apresentados em ordem cronológica de elaboração em suas versões originais.

Artigo 2. Watermelon (*Citrullus vulgaris* Schrad) mesocarp as a source of pectin: a spectral and chemical analysis.

Submetido à Revista Food Hydrocolloids **ISSN:** 0268-005X

Fator de Impacto (2010): 2,659

Artigo 3. HPLC-ELSD coupled Hipler columns as analytical tool for screening degree of acetylation and methoxylation of pectins from tropical fruits.

Submetido à Journal Chromatograph A **ISSN:** 0021-9673

Fator de Impacto (2010): 4,194

Artigo 2. Watermelon (*Citrullus vulgaris* Schrad) mesocarp as a source of pectin: a spectral and chemical analysis.

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Abstract

Watermelon is the most produced fruit in the world and it also has a mesocarp:epicarp plus endocarp ratio of (1:1) compared to citric fruits. With a view to preserve the chemical-functional characteristics, the aim of this study was to determine the chemical and spectral characteristics of pectin extracted from watermelon mesocarp, highlighting the influence of the kind of acid used for extraction by swelling and the alcohol carbon chain used. Two experiments were drawn up to evaluate the influence of the concentration of the type of acid (organic and inorganic) and the temperature. With citric acid (15%), there was a tendency for the degree of methoxylation to decrease slightly, although the maximum yield was observed. Under other conditions there was a limiting factor producing low yields and maximum values of DM, associated with the lowest temperature and the citric acid. With the inorganic acid, the conditions that

allowed maximum extractability did not coincide with the maximum DM, suggesting a combined effect of the kind and the strength of the acid registered in both experiments. The appropriate alcohol for precipitating the pectin was ethanol, although isopropanol furnished a higher yield, and methanol performed best in terms of retaining methoxyls, ensuring a maximum retention in a chemical and natural source environment. The variables recommended in terms of DM and yield associated with chemical components of the cell wall would seem to be appropriate for characterization of pectins in different foodstuffs and agricultural waste.

Key-words: watermelon, pectin, mesocarp, extraction, characterization, methoxylation

1. Introduction

In the agricultural production process, the generation of waste, generally 40% of primary products, especially in tropical countries, has worsened environmental pollution, and clean technologies are needed to improve the process of using the whole of the fruit (Oreopoulou & Russ, 2007).

Of all fruits produced in the world, watermelon is produced in the largest quantities (97 million tonnes), followed by bananas (86 million tonnes), grapes (67 million tonnes), apples (66 million tonnes) and oranges (65 million tonnes). In Brazil, watermelon is the fourth most produced fruit (2.1 million tonnes) behind oranges (18.7 million tonnes), bananas (7.1 million tonnes) and pineapple (2.7 million tonnes) (FAO, 2010).

Fruit wastage can be as high as 65-70% of the total weight, varying somewhat according to morphology. The endocarp (the edible part) accounts for 65% of the total weight, the epicarp and the mesocarp (which are waste materials) make up 35% of the

weight of watermelon, which is different from the proportion in the malic and citric fruits normally used to obtain commercial pectins.

Agricultural waste has been studied as a natural source of pectins, oligogalacturoides and oligosaccharides, nucleotides, and other bioactive compounds and functional ingredients (Kalapathy & Proctor, 2001; Emaga, Robert, Ronkart, Wathelet, & Paquot, 2008; Koubala et al., 2008; Vriesman, Teófilo, Petckowicz, 2011a) can provide added value, such as flavonoids, carotenoids, and phytoesters, compounds which may or may not help to link the components of the cell wall (Min et al., 2011).

The mesocarp of watermelon (*Citrullus vulgaris* Scharad) currently has the potential to be used to manufacture dressings, deserts, marmalades, and jelly, but although functional, it is not widely accepted as a foodstuff. Huor et al. (1980) have reported that it is possible to use the non-edible parts of watermelon as a source of pectin, although the best extraction conditions have not been investigated and characterized by way of accuracy studies.

Pectin extraction can be accomplished using an aqueous medium, mainly containing acid, base and chelating agents. The molecule can also be extracted by chelating agents such as calcium, ammonium oxalate and EDTA, which may not reach the part attached to the primary cell wall (Manrique & Lajolo, 2002). Full extraction has been registered and it is recommended that quantum satis alcohol be used to ensure selective extraction (Lima, Paiva, Andrade, & Paixão, 2010). Ethanol 99% in fact has been found to fully precipitate gel fragments, which can then be submitted to lyophilization, once residual alcohol has evaporated and other slight modifications carried out. McCready (1970) recommended a pectin extraction method using HCl followed by drying by heating with a significant effect on the functionality of pectin and the chemical environment.

Canteri-Schemin et al. (2005) have used comparative studies of various organic (citric, malic and tartaric) and inorganic acids (phosphoric, hydrochloric, nitric and sulfuric) to assess the potential of these means of extracting pectin from apple pomace. Extraction was carried out under reflux at 97° C for 30 min, adjusted to pH 2.5 and precipitated in 95% ethanol. The citric acid was shown to be more efficient at extracting pectin from apples, with higher figures than those for other acids, although, they did not measure methoxyls and other parameters that provide a functional chemical environment, such as acetyls and other structural parameters involved in technological applications, such as dispersion and stability of emulsion in foods, and preservation of flavor and color (Kurita, Fujiwara, & Yamazaki, 2008).

Pectins have also been extracted using water acidified with phosphoric, oxalic, acetic, sulfuric, nitric and hydrochloric acids, with pH ranging from 1 to 3 at a temperature of between 80-100°C (Garna et al., 2007; Koubala et al., 2008; Yapo, 2009). Several studies have reported the main factors to be temperature, pH, and type of acid and these may influence not only the pectin yield, but also the chemical fine structure (Liu, Shi, & Langrish, 2006). It is, however, still a considerable challenge for appropriate methodologies to adjust the structure-function relation. Extended heating periods (usually of over 60 min), used for pectin extraction at temperatures of above 90°C produce hydrolysis and result in a low-mass molecule (Mesbahi, Jamalain, & Farahnaki, 2005). Structural analysis should be monitored by comparing the effect on the degree of methoxylation (DM) using infrared (Manrique & Lajolo, 2002; Lima et al., 2010; Gan, Manaf, & Latiff, 2010), since it is a nondestructive method, though other methods, such as HPLC (Quemener, Marot, Mouillet, Riz, & Diris, 2000; Levigne, Thomas, Ralet, Quemener, & Thibault, 2002) and RMN (Joye & Luzio, 2000; Popov

et al., 2011; Zhang, Wang, Yu, & Wu, 2011; Vriesman, Amboni, & Petkowicz, 2011a; Ele-Ekouna, Pau-Roblot, Courtois, & Courtois, 2011), are more appropriate.

The most reliable proposal for pectin extraction recommends the use of HCl to adjust the solution pH to 2.2 and later heating to 100°C (boiling point) on a hot plate for 30 minutes. A cheese cloth has been recommended for the isolation of pectins in this way (McCready, 1970). Fine adjustments have recently been made to the method and it is suggested that acidification with citric acid at pH 2.2 and isolation by way of filtration through polyester fabric (mesh defined, aperture size 1072.35µm), measurement using scanning electron microscopy (SEM) may provide pectin gel fragments (4478.8 µm) that can be submitted to FT-IR analysis (Lima et al., 2010).

The main aim of this study was to evaluate the non-edible portion (the mesocarp) of watermelon as a source of pectin, drawing attention to its chemical and spectral characteristics in view of the kind of acid used for extraction and the kind alcohol used, in order to achieve full precipitation of pectin.

2. Materials and Methods

2.1 Materials

2.1.1 Fruit samples

Watermelon (*Citrullus vulgaris* Schrad) mesocarp was used. The fruits were selected on the grounds of the firmness of the peel, external appearance, weight and stage of ripeness.

2.2.2 Pectin standards

The standards used for galacturonic acids were DM=10.59% (Fluka, Code 48280) and DM=1.52% (Sigma, Code 48280). The standard for high DM pectins were:

68% (CP Kelco, Genu Type 105), 72% (Sigma, Code P8471) and 90% (Sigma, Code P9135), 85% (Fluka, Code P9561); and the standard for low DM pectins: 30% (CP Kelco, Code Genu LM102AS).

2.2 Methods

2.2.1 The procedure for extraction of pectins (developed by Lima et al., 2010)

A sample of 100 g of watermelon mesocarp diced into cubes was taken from 1000g. Subsequently \cong 400 mL of deionized water was added and the mixture ground up in a blender. The pH was then adjusted to 2.2, with 10% citric acid and other concentrations. The same procedure was followed for the other acids and other concentrations. The mixture was heated to boiling point for 30 minutes (fixed) or alternatively exposed to different temperatures followed an experiment design. After cooling, the mixture was filtered using a vacuum pump and a Buchner funnel, with the aid of a rubber seal. Alcohol was added *quantum satis* to the resulting liquid extract to fully precipitate the pectin. At this stage, different types of alcohol were tested, in order to assess the length of the carbon chain on the pectin structure. The alcohols tested were: methanol, ethanol, and isopropanol (95%). The precipitate was washed with one of these alcohols (99%) and then with 99% acetone. The final precipitate was left at room temperature for 24h to evaporate of the alcohol residues and was then lyophilized. After homogenize and prepare was submit to KBr pellets.

2.2.2 The degree of methoxylation and the pectin yield

Commercial pectin standards, samples of lyophilized pectins and KBr were desiccated in a vacuum for 24h. The samples were homogenized with KBr (9:1 salt-sample), with the aid of a compressor to obtain the pellet, which was sent to the infra-

red spectrometer. The FT-IR spectrum was collected at an absorbance in the range of 400-4000 cm^{-1} , and at a resolution of 4 cm^{-1} (in duplicate).

Infrared spectroscopy was used to analyze the pectin samples in isolation, taking the 1650 and 1750 cm^{-1} bands of the spectrum, at which, using the equation $[A_{1750} \text{ cm}^{-1} / (A_{1750} \text{ cm}^{-1} + A_{1650} \text{ cm}^{-1})]$ enabled the DM of the samples to be quantified. The pectin yield (%) was determined after weighing the residues that had been dried by way of lyophilized.

The DM of the pectin standards was determined using the titrimetric method (N=5) as proposed by McCready (1970), modified to the initial quantity of the standard so as to make it easier to view the reaction. The curve was plotted with the aid of the Origin 8.0 computer program. The IR spectra of the commercial standards and samples were taken in triplicate. The regression was plotted and adjusted using minimum squares and the linear correlation coefficient for the calibration equation was 0.9766, where $DM = (Y - 0.05822) / 0.0615$, taking the data for standards (N=7) by titrimetry and FT-IR.

2.2.3 Optimization Assay (the effect of the kind of acid and the reaction temperature)

This assay assessed the effect of the kind of acid (organic or inorganic) and the temperature on the extraction of pectins and the DM. Two 2^2 factorial sampling plans were used with a central point, taking citric acid as the independent variable (%) x temperature ($^{\circ}\text{C}$) (1st planning) and HCl (N) x temperature ($^{\circ}\text{C}$) (2nd planning); and the dependent variables were yield (%) and DM (%). Each planning consisted of seven assays, whose conditions (codified and de-codified levels) can be found in Table 1.

2.2.4 Statistical Analysis

The resulting data for yield (%) and DM (%) were subjected to ANOVA, and compared using the Duncan test with a 5% level of significance.

With a view to assaying the extractability and characterization of the pectins, the results obtained using factorial planning were evaluated using the response surface methodology, showing the contrasts and the interaction observed, using the Statistic 6.1 computer program.

3. Results and Discussion

3.1 Optimization assay and effect of kind acid and alcohol in order to precipitate

Table 2 shows the results and contrast values obtained with the planning using citric acid as the extracting agent. The influence of the effects on the dependent variables can be seen, with the yield being positively (Figure 1) influenced in the case of both independent variables (acid strength and temperature), while the DM was impacted negatively only by the strength of the acid to both assays.

Temperature and acid strength positive influenced the yield and the higher of these was obtained from higher temperature favored by interaction (Table 2).

The strength of the citric acid had a negative impact on the DM (76-72%) (Figure 2), which could be explained by the slight demethoxylation of the pectin under acidic conditions. While temperature was the variable that had the greatest impact on the DM viewed in Figure 3. The interaction also was rough strength acid x temperature.

The second design experiments and the contrast values can be found in Table 3, which shows that the increase in temperature and inorganic acid strength led to a higher yield (1.52) (Figure 4), half that found for the main effect of citric acid. The DM varied from 40 to 60%, suggesting that it conformed to the surface response of the contours

(Figure 5) in relation to the same parameter for the condition of organic acid (citric acid).

Under inorganic acidic conditions, a significant reduction in DM and yield was observed, showing that this condition has favored both demethoxylation and depolymeration reactions in the pectin structure.

Table 4 shows the alcohols effect (of the different size of the carbon chain) on extraction and the chemical characterization of pectin. The isopropanol was found to be the most efficient agent for precipitating pectins, since the length of the alcohol chain seemed to contribute to the yield. While methanol tended to conserve the DM, dissolving with the methoxyl surrounding more efficiently, thereby providing a chemical environment for the preservation of technological properties associated with protection against demethoxylation. Although the aim was to maintain higher values of DM, food safety and processing costs need to be borne in mind. For toxicological questions, ethanol was selected, as it is widely used in industry for this purpose and provides small differences when measuring the potential of new sources.

The function x structure relation guides the technological applications of pectin, although the diversity of applications, gelification (pH 2.0 – 3.5), as a dispersant (pH 1.0 – 10.0) and a stabilizer of emulsions (pH 2.0 – 9.0), require different methods and a different focus for determination of relation structural bioactivity from distinct methodologies (Funami et al., 2011).

Pectin in its structured form in the plant is found in the middle lamella (Lima et al. 2010) and is intrinsically associated with the primary and the secondary cell wall, which controls the heterogeneity of materials in nature (Yoshioka, Hayama, Tatsuki & Nakamura, 2011; Ortiz, Graell & Lara, 2011).

Recent articles that have produced results for extraction and purification conditions at pH 1.0; 3.0; 6.0 and 8.0 have reported co-extracted substances that naturally affect the integrity of the pectin molecule (Morris, Ralet, Bonnin, Thibault, & Harding, 2010). These substances of variable composition and molecular weight are mostly derivatives of structural non-pectic, amylaceous and proteinaceous polysaccharides (Houben, Jolie, Fraeye, Loey, & Hendrickx, 2011; Jaramillo, Roberts, & Coupland, 2011). Most studies that lead to this characterization take as their starting point the procedure for extraction of the portion that is insoluble in water or alcohol (80%) (kurita, Fujiwara, & Ymazaki, 2008). Although some authors (McCready, 1970; Lima et al., 2010) recommended 95% ethanol, this approach treats the intrinsic heterogeneity of the plants and agricultural waste in a different manner, carrying structural changes.

The demethoxylation reaction leads to depolymerization that results in uronic acids, sugars and other kinds of acid. The conditions for evaluating polymers of varying chain length have still not been clearly established. This leads to erroneous interpretations of the function-structure relation of pectin and the polymers resulting from its hydrolysis or decomposed under any conditions (Ngouémazon et al., 2011).

The most recent work that confirms this discussion suggests that, in pectin extracted from the epicarp of citrus fruits, treatment with citric acid favors the preservation of the DM, by inhibiting the action of the pectin methyl esterase and the same study also found an increase in viscosity in solutions containing more than 1M, even at temperatures of 50-65°C, conditions under which citric pectin was characterized using two distinct portions that varied from 2000–70 Kda. The molecule cleavage profile tended in the direction of producing more molecules in the range of 500 – 70 Kda. The most incongruous issue is that this assay was carried out at pH 5.0-8.0, 65°C.

2 h (Vriesman et al., 2011a). Others recent authors using methodology for molecular weight registered from range similar for pectin extracted and submitted in others conditions (Vriesman et al., 2011b).

In fruit, pH and exposure time associated with the strength of the acid used for extraction are certainly conducive to depolymerization reactions. The presence of starch and fibers did not affect pectin extraction (Lima et al., 2010) when characterized using IR, and, no practical difficulties were encountered throughout the extraction and isolation stages, probably because the pH was kept at optimum levels and the most suitable concentration of citric acid (10%) was used for this new protocol.

Kalapathy & Proctor (2001) investigated the effect of the concentration of inorganic acid (HCl, varying from 0.05-0.3M) on soya bean husks, and found that the pectin yield was lower at both of the highest strength. The recommended level for the highest pectin yield was 0.05 M along with precipitation using 95% ethanol at pH 3.5. Medium pH lower than 2 favored the precipitation of other non-pectic compounds. The study showed that, at high concentrations of inorganic acid, there was a reduction in the coalescence of the gel associated with the defragmentation of pectin into shorter chains of oligogalacturonides, which also affects the viscosity.

Other publications recommend the use of hydrochloric acid (Kalapathy & Proctor, 2001; Iglesias & Lozano, 2004) and nitric acid as extraction agents (Liu et al., 2006). The use of these acids for extraction has some disadvantages, such as environmental pollution and corrosion. Moreover, residues obtained after pectin extraction is not very suitable for animal and human food (Ralet & Thibault, 1994) because it is difficult to minimize toxic acid residues ensure safe processing.

More complete investigation requires advanced methods for characterizing the structure of pectin, and, when the influence of the extraction conditions has been

assessed, the chemical nature of the material and the technology applied should also be taken into consideration. Vriesmann et al. (2011a) have proposed a methodology that associates the discussion of data from ^{13}C NMR x HPSEC, which characterized the composition of monosaccharides and others carbohydrates in pectin from cocoa wastage using the first technique and also found a polymer with a molecular mass of 1681 Kda eluted when using the refraction index (and another of smaller mass), while the scattering detection method revealed only one compound.

Recent studies have focused on identifying the ideal and quantitative conditions for extraction using organic acids, such as citric acid, as a way of obtaining high quality pectin, which is of great benefit to the food industry from the an economic, environmental and nutritional point of view (Lima et al., 2010).

Technological and nutritional safety in food and pharmaceutical applications are desirable in order to provide subsequent segmental applications that are in greater compliance with international laws for production of ingredients and supplementary applications.

4. Conclusion

The factorial design assay in comparative conditions evaluates the effect of the kind and the strength for interaction acid x alcohol in terms of yield and the DM show a tendency for the inorganic acid be more aggressive than the organic one.

Watermelon is a good source of pectin for industrial purposes as it has 2 to 3 times the amount in the mesocarp region and this represents 40-45 % of the non-edible portion of the fruit.

The organic (citric) acid performed better than the inorganic acid (HCl) in terms of preserving the DM, pectin yield and size of gel fragment, used as a model to estimate the structural parameters of the pectin extracted from the mesocarp of tropical fruits and wastage.

An association between demethoxylation x depolymerization was indirectly observed under particular conditions in assays which had a low yield and DM was comparable to the other treatments.

It can also be concluded that, in the case of the influence of alcohol the length of the carbon chain affected yield extraction and the kind of alcohol choice should be considered safety and nutritional of alcohols.

5. Acknowledgement

The authors are grateful to Capes, Facepe and The Federal University of Pernambuco for financial and human resource support.

6. References

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Table 1. Decoded levels of independent variables.

Factorial Design		
Factorial Experiment I		
Coding	Citric Acid (%)	Temperature (°C)
-1	5	50
0	10	75
1	15	100
Factorial Experiment II		
Coding	Hydrochloric Acid (M)	Temperature (°C)
-1	0.025	50
0	0.06	75
1	0.10	100

Table 2. Contrast values obtained from factorial assay I.

Assay	Conditions of assay	Yield (%)	DM (%)
	(citric acid)		
1	5% x 50°C	0	0
2	15% x 50°C	0	0
3	5% x 100°C	0.043	76.12
4	15% x 100°C	3.389	72.10
5	10% x 75°C	0.120	76.21
6	10% x 75°C	0.126	76.09
7	10% x 75°C	0.122	76.15
Factor			
(1) Citric Acid		1.67	-2.01
(2) Temperature		1.72	74.11
1 x 2		1.67	-2.01

Table 3. Contrast values obtained from factorial assay II.

Assay	Conditions of Assay (HCl)	Yield (%)	DM (%)
1	0.025M x 50°C	0	0
2	0.10M x 50°C	0	0
3	0.025M x 100°C	0.046	63.2
4	0.10M x 100°C	1.520	41.8
5	0.06M x 75°C	0.103	52.6
6	0.06M x 75°C	0.112	52.5
7	0.06M x 75°C	0.110	52.6
Factor			
(1) HCl		0.74	-10.70
(2) Temperature		0.78	52.50
1 x 2		0.74	-10.70

Table 4. Effect of the size of the carbon chain of alcohol to precipitate pectins.

	Yield (%)	DM (%)
Methanol	3.1 ^a	86 ^a
Ethanol	3.2 ^a	72 ^b
2-propanol	4.01 ^b	73 ^b

Equal columns letters do not differ statistically by 5%.

Captions of figures

Figure 1. Surface response for variable yield (%) exploiting temperature *versus* strength citric acid.

Figure 2. Infrared spectrum of pectin extracted from the mesocarp of watermelon (DM = 72%).

Figure 3. Surface response for variable degree of methoxyl exploiting temperature *versus* strength citric acid.

Figure 4. Surface response for variable yield (%)exploiting temperature *versus* strength HCl.

Figure 5. Surface response for variable degree of methoxyl exploiting temperature *versus* strength HCl.

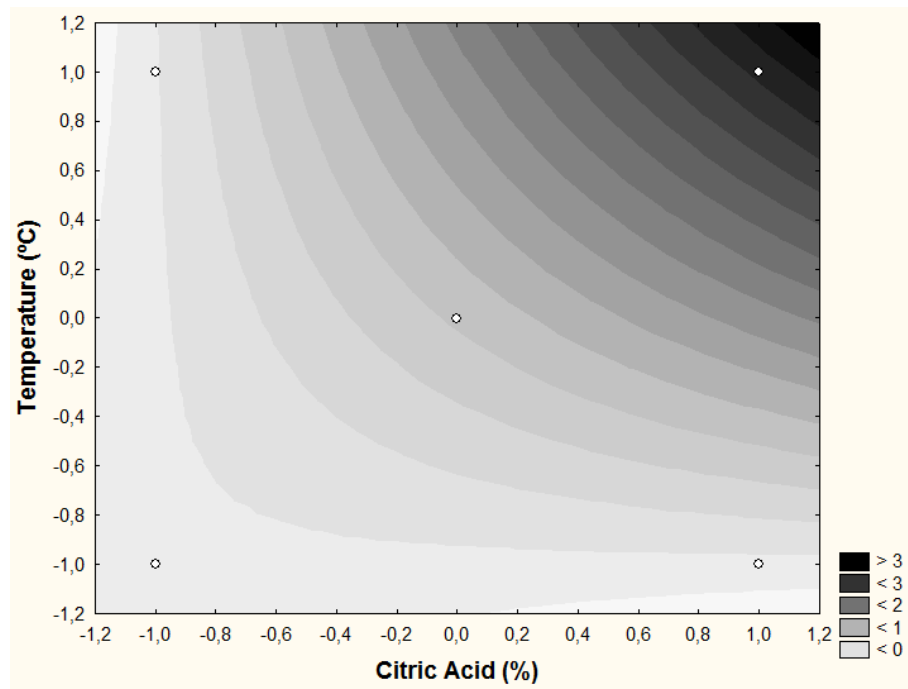


Figure 1. Surface response for variable yield (%) exploiting temperature *versus* strength citric acid.

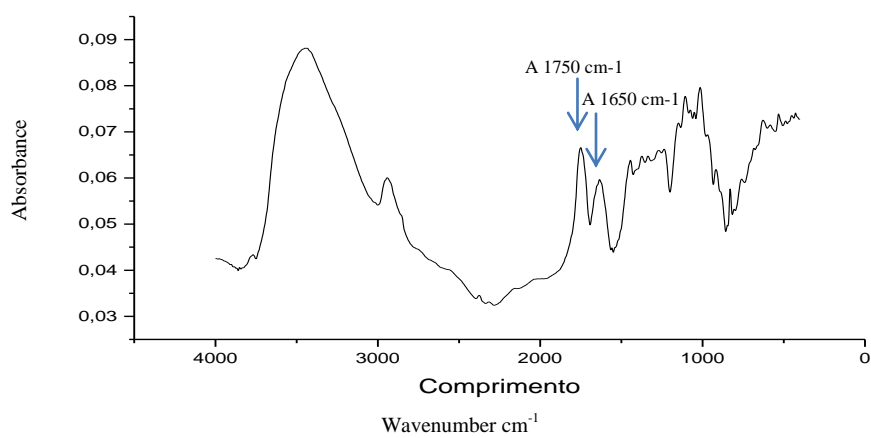


Figure 2. Infrared spectrum of pectin extracted from the mesocarp of watermelon (DM = 72%).

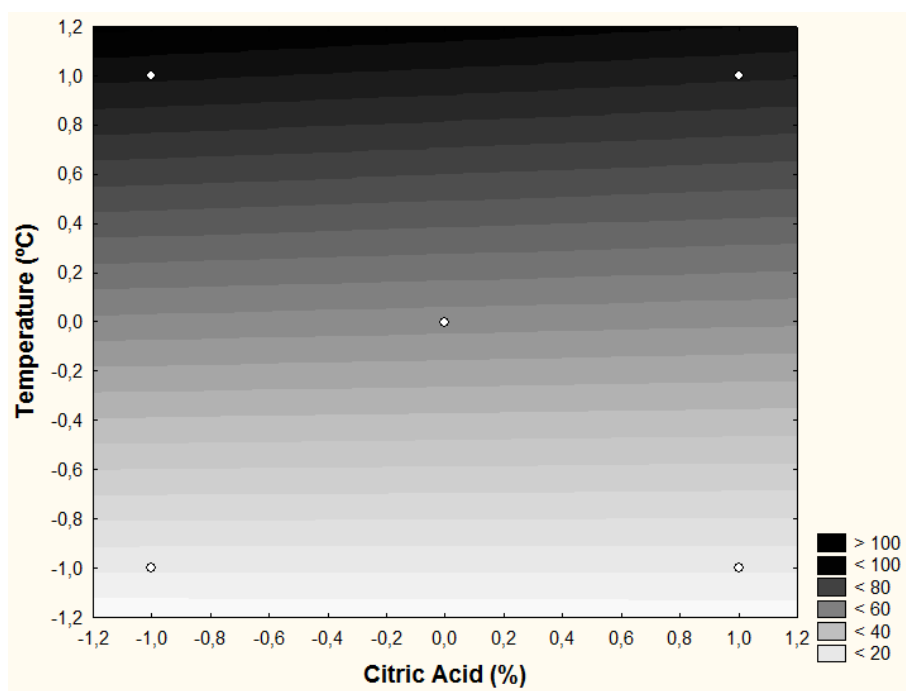


Figure 3. Surface response for variable degree of methoxyl exploiting temperature *versus* strength citric acid.

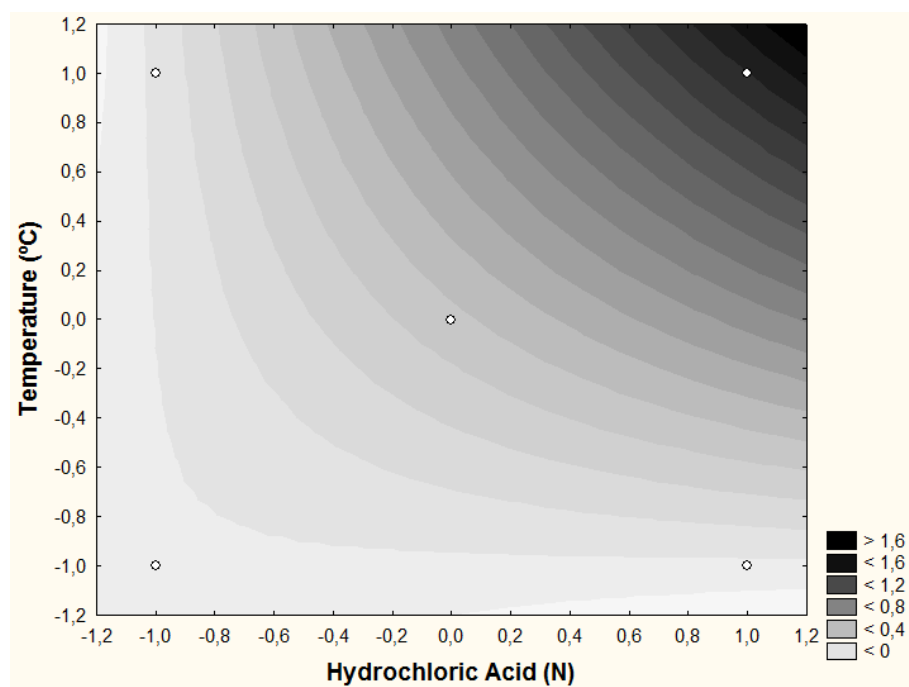


Figure 4. Surface response for variable yield (%) exploiting temperature *versus* strength HCl.

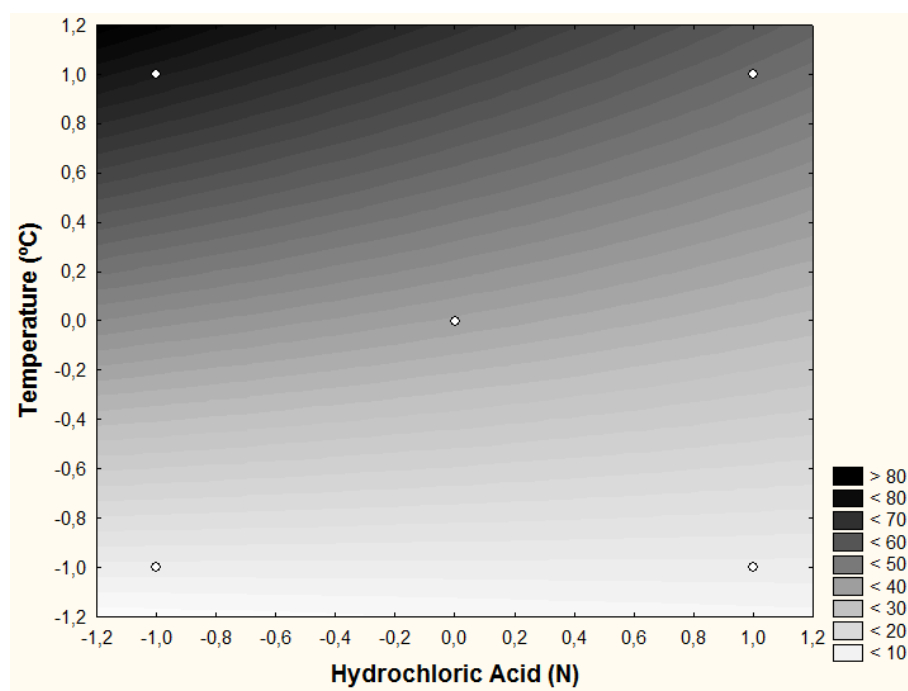


Figure 5. Surface response for DM exploiting temperature *versus* strength HCl.

Artigo 3. HPLC-ELSD coupled Hiplex fast column as analytical tool for screening degree of acetylation and methoxylation of pectins from tropical fruits.

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Abstract

Pectin is found in the cell wall and middle lamella of plant tissue linked by covalent chemical bonds to proteins, neutral sugars, ferulic acid and variable linkages to the methoxyl (degree of methoxyl - DM) and acetyl (degree of acetylation - DA). Usually analytical methods employed for characterization of DM and DA for pectins have generated conflicting data, mainly involving extraction methods can act in a destructive way, defragmenting the native structure throughout pectic heteropolysaccharides. In fact, non-destructive techniques appear to be rough candidates a useful tool and precise

characterization of pectic substances. This work aiming develop a suitable separation and roughness detection by HPLC-ELSD identified and quantified by different procedure through extracted curve. In the first phase of research, it was optimized the conditions of detectability and the interaction of the stationary phases x mobile phase x ELSD detector. Different materials were evaluated (H Hiplex fast acid and Spherisorb ODS-2), combined mobile phases using water and sulfuric acid. In sulfuric acid HPLC grade was registered better results when varied from 5 to 100 mM. Conditions which adjusted detect ability parameters, such fogging temperature and gain were determined in order to improve analytical signal by newer ELSD detector. Strengths of NaOH at 1.78 M was the best condition that exhibited maximum of esterification of the samples when compared standards of pectins, associated to minor significant parameter evaluated time for reaction 60 minutes (lowest deviation standard and maximum yield). The results of the linear regression after comparison between the methods: titrimetric x infrared; titrimetric x HPLC-ELSD and infrared x HPLC-ELSD showed a correlation > 0.91. Samples of pectin extracted from fruits showed correlated > 0.75 through internal and external standard regression by instrumental techniques.

Key words: pectin, characterization, HPLC, ELSD, infrared, fruits.

1. Introduction

Pectin is a polysaccharide complex containing in the main chain galacturonic acid units, where main L-rhamnose are inserted and attached through glycosidic linkages with neutral sugars in content variables, carrying differences in the secondary structure. Beside the carboxylic groups are partially esterified with methoxyl, acetyl and amide groups [1]. This structural orientation aiming to classify compounds found naturally, differ vegetable material and still guiding the technological application [2].

Recent studies characterized the pectin extracted at pH 1-3.5, and obtained by ^{13}C -NMR 65-80% of carbohydrates, and 3-6% of proteins, nonavailable biologically, and other fractions less important as fenolic and ferulic, probably burning from primary and secondary cell wall [3,4]. The content of galacturonic acid is also variable, from 20 to 80% while the methodological doubtless emerge from derivation chemical carried out interferences medium burning conflicting nature of this determinations, implicate unbiased functional and technological properties [3,5,6,7]. The main determinations that guiding this properties yet is uncertain, while emphasis has been ascertained to degree of acetyl (DA), degree of methoxyl (DM) by conferring properties of surface chemical stabilized by weak forces and hydrophobic [8]. In the molecule of pectin these parameters are useful guides on the evaluation of properties of gelling (DM) and other functional properties [2] as dispersant, encapsulating and stabilizer, requiring chemically environment for different applications such food, drugs, cosmetics, cleaning products and others.

Despite the low molecular weight, methoxyl and acetyl groups associated with the other structural parameters confer diverse properties, such as hydrophobic linkage (galacturonic acid chain) and electrostatic interaction established in gelling capacity

(neutral and polar sugars) and dispersing of pectin (ratios of amides and non-polar and neutral sugars), among minority others sugars such as galactose, arabinose and fucose [1].

While the usual methods for these determinations being the titration, colorimetry with or without enzymatic reactions, the vulnerability and the interactions with components by crossing reactions generate conflicting data in the literature in order to clearance of structural properties. Non-destructive methods are generally more reliable, employing less aggressive extraction techniques associated with detection without any structural changes such as infrared [2,9,10], NMR [3,5,6,7,11] and more recently by HPLC using RP-IR [12,13]. Satisfactory results were achieved through simplified methodologies which respect the instability of chemical groups, before a curve typically building with uncertain standards regarding the need the extraction of this groups by de-esterification. Few records have registered in the literature about methods to ensure the purity standards of pectins, Houben and collaborators [14] propose four classes of ratios: GalA/Fuc+Rham+Gal+Xyl; 2-Rham/GalA; Ara+Gal/Rham; and Man/Xyl.

By the 1970's some results were obtained by GC and spectrophotometry, and from 1990 until now the predominant infrared and other non-destructive methods [15]. In the last decade the business valuation was more than 4 times for the HM pectin, deserving studies of accuracy of methods as an additional way to confirm values relationship functional properties to meet prior to the formulation of jelly, blend and other applications as coatings, films, and as agents dispersion. The chemical structure and function relationship, despite being widely investigated since 2000, is far from being understood because of the heterogeneity associated with naturally occurring molecule and other interactions with the environment in which the extraction is achieved mainly by cross-links between proteins and carbohydrates [4,16].

The environmental chemical variables that favor the de-methoxylation and the de-acetylation still interact strongly with the source by modifying the fine structure of pectin [2]. The industry requires modifications intentioned that occasionally favors any technological process. The unknown nature of these factors associated with the instability of analytes lead the influence of time for reaction associated with molar force means basic or acid in varying conditions of temperature, thus compromising the structure and yielding different heteropolymers that has been recently associate in prevent cancer and anti-inflammatory [3].

Levigne and collaborators [13] presented a study to determine DM and DA, using the stationary phase C-18 250x4 mm (5 μ m) and mobile phase 4 mM sulfuric acid using isopropanol as internal standard. Others attempts to get these determinations are demonstrated by HPSEC, however in this analytical tool has been directed more roughly oligogalacturonides of different molecular weight. Recent studies have demonstrated de-polymerization of pectin (approximately 2000 kDa) treated with acid generating fragments below 500 kDa, associated with de-methoxylation, even using citric acid as acidifying agent [6,17].

The objective of this study was to optimize the analytical tool for determining DM and DA by HPLC-ELSD, and prove impact of structure of pectin by suitability tool, and estimate the accuracy of the methods based on information obtained from standard analytical and samples of pectin extracted from fruits.

2. Material and method

2.1 Material

2.1.1 Sampling of fruits

All fruits were selected and acquired respecting maturation stage in sensory properties (color, firmness and others) .

The material choiced was taken as whole fruit: apple (*Malus wild*), strawberry (*Fragaria vesca L.*), kiwi (*Actinidia deliciosa*), banana (*Musa paradisiaca*), papaya (*Carica papaya*), acerola (*West Indian cherry*) and guava (*Psidium guava*). From mesocarp it was extracted pectin from orange (*Rutacea sp*), passion fruit (*Passiflora edulis*) and watermelon (*Citrullus vulgaris Schrad*). The mesocarpic region of fruits was manually removed. In order to submit the extraction protocol of pectins was considered a sampling strategy and further dilution in relation to water content of material (edible and non edible),.

2.1.2 Standard of pectin and reagents

The commercial standards were galacturonic acid DM=10.59% (Fluka, 48280) and DM=1.52% (Sigma, 48280), standards of pectins high DM: 68% (CP Kelco, Genu 105), 72% (Sigma, P8471) and 90% (Sigma, P9135), 85% (Fluka, P9561); and standards of pectins low DM: 30% (CP Kelco, Genu LM102AS). Data were obtained from seven standards, using the technique of titration (N = 5), described by McCready [18] and infrared (N = 3), described by Manrique and Lajolo [9].

All reagents used in the analysis by liquid chromatography were HPLC grade, and the others were analytical grade.

2.1.3 Equipment

For determination of DM (%) was used the infrared spectrometer (Bruker IFS66). The HPLC analysis was in liquid chromatograph (LC-20AD, Shimadzu Proeminence LC solution) coupled to a degasser (DGU-20A3) and CBM-20A. The HPLC detection was by evaporative light scattering detector (Shimadzu ELDS-LTII) using nitrogen gas analytical grade 5.0 acquired by White Martins. Milli-Q integral was supplied water proper to assays of mobile phase.

2.1.4 Stationary phases and mobile phases

Suitability test for the new system of detection (ELSD) was tried the following stationary phases: Hipler H Acid 100x7.7mm (8 μ m), filled with styrenodivinilbenzene with lead sulfonate copolymers (Varian, PL1170-2823); Waters Spherisorb ODS-2 150x3.2mm (5 μ m) (cat. Number 54904); and Spherisorb S5 ODS2 100x2mm (5 μ m) microbore column (PSS831922). The mobile phases were tested: H₂O Milli-Q and sulphuric acid from 5mM.

2.2 Methods

2.2.1 Experimental

In the initial phase the experiments were carried out in order to establish the chromatographic conditions, varying the ELSD detector gain from 1 to 12, combined with increasing ranges for temperature detector fogging (from 20 to 70° C).

At this stage were used HM pectin standards (above 90% DM) and galacturonic acid (below 1% DM), de-esterified according to Levigne and collaborators [13]. The confirmation of the peaks occurred by injection of de-esterified standards and by injection of mixture (acetic acid:methanol:isopropanol) in quantities of 1:1:1 (v/v/v). The stationary phase Spherisorb ODS-2 versus hiplex fast acid were evaluated using water and H₂SO₄ as mobile phase. The repeatability of extraction procedure was evaluated under the conditions NaOH 1M in the times for reaction varying from 5-120 minutes.

In the second phase, aiming at improving the conditions of the de-esterified of standard and samples of pectin extracted, used a factorial design decomposition central rotary (DCCR), where the independent variable were designed varying the strength of NaOH (0.5 M to 2 M) and time for reaction (5 to 120 min). The decoded variables (Table 1) resulted in a total of 11 experiments (Table 2). After appropriateness of the procedure developed, the combination ascertained was applied to seven standards by different methods and compared results to infrared, which the samples of pectin fruits were evaluated. Calibration extracted curves were obtained by regression information using for correlation parameters titrimetric x infrared, infrared x HPLC and titrimetric x HPLC.

2.2.2 Extraction of pectin (whole fruit and mesocarp)

Initially were taken (100g) samples diced and shredded of mesocarp and whole fruit. Then was added Milli-Q water in proportions that varied from 1:4 to 1:8 w/v, according to the water content. The mixture was ground into multiprocessor in order to obtain a smooth paste. After dissolved it was adjusted the pH of the mixture to 2.2 using 10% citric acid and then heating until to the boiling point, maintained by 30 minutes,

according to the methodology proposed by Lima and others researchers [2]. After cooled to room temperature, the mixture was filtered through a polyester and the filtrate was added ethanol (95%) *quantum satis* to precipitate the pectin thoroughly, then was made successive washings with ethanol and acetone (99%). The recovered material (gels of different shapes and cohesiveness) was maintained at 20° C for 12 to 24 hours, before submit to lyophilization. After this procedure the material was prompt for different techniques.

2.2.3 Determination of DM by FT-IR

Standards of pectin, lyophilized samples and KBr were dried under vacuum (12 h) prior to analysis. The samples were mixed with KBr (9:1), and with the help of compressor (Bechman 00-25) it was obtained from the inserts. After analysis by FT-IR (4000-400 cm^{-1}) of the samples, we identified the bands 1650 and 1750 cm^{-1} spectra, which through the equation: $[A_{1750 \text{ cm}^{-1}} / (A_{1750 \text{ cm}^{-1}} + A_{1650 \text{ cm}^{-1}})]$ was quantified DM samples and standards [2]. Remaking the standard curve from infrared using standard in extreme points of methoxyl groups in order to calculate accuracy studies (infrared x HPLC-ELSD).

2.2.4 Optimization and suitability of the de-esterification of standard pectins

Approximately 5 to 10 mg of pectin of each standard, were suspended in 10 mM of CuSO_4 . 0.5-1.0 ml of NaOH 0.5 to 3.2 M was added to make the de-esterification at 4°C. The time required for complete desesterification was tested so combined (varying from 5 to 120 min) according to test factorial (Table 2). After this step proceeded to

centrifugation for 10 min at 8000g at 4° C. The supernatant was neutralized adding drops of HCl diluted then added isopropanol as internal standard. Always was injected 20 µl onto the columns hyphenated to HPLC-ELSD.

2.2.5 Determination of DM by HPLC-ELSD (evaporative light scattering detector)

The neutralized supernatant was injected into the equipment (UFLC LC20AD Prominence, Shimadzu) connected to column H Hipler Acid. The elution was isocratic mode, flow 0.6 mL/min using a mobile phase of 50 mM H₂SO₄, coupled to detection by ELSD (LT-II, Shimadzu).

2.2.6 Accuracy studies through calibration curves

The DM of standards (N=7) and samples obtained from the fruit pectins (N=12) were evaluated by titration [18]. Was used the system equation for the infrared bands [9] aiming correlation conventional methods to HPLC-ELSD. In according to research presented to make the essential measurements in this study. Were built three calibration curves relating the results in different samples and standards: FT-IR x titration; titrimetry x HPLC-ELSD; and infrared x HPLC-ELSD using the program Origin 8.0. For samples of pectins of fruits was evaluated fit linear using external an internal standard correlation analysis of infrared x HPLC-ELSD.

2.2.7 Statistical analysis

The results were analyzed by means of analysis of variance (ANOVA), and compared by Tukey test 5% level of significance for multiple comparisons and contrasts.

The test for optimization of de-esterification of standards pectins had the results obtained by factorial planning, which were evaluated by response surface methodology (RMS) with the central axis of response, and applied the test of orthogonality for analysis of variance (ANOVA) using to program (R).

3. Results and Discussion

3.1 Optimizing the ability for detection by ELSD

The initial records ELSD detector coupled to reverse phase ODS-2 columns are summarized in Table 3 and attached to the column hiplex presented in Table 4.

The stationary phases evaluated showed significant interaction with the detectability of acetyl and methoxyl desesterified from pectin standards. The column hiplex fast acid was superior to reversed-phase previously described by Levigne and collaborators [13] although the column evaluated in this study were of shorter length (150 and 100 mm, both ODS-II), thus was deserved the same manufacturer, packaging level and size particle for comparatives assays. Standard of pectin HM (90%) and LM (10%) may be demonstrated in figure 1. Favor can be assumed by detection of methoxyl and acetyl mainly when combined to sulfuric acid. The mobile phase water did not work properly, while the sulfuric acid yet required strength adjustment (5 mM to 100 mM) in relation to the Levigne and collaborators [13]. Although there was a favorable situation from 50 to 100 mM, the molar strength was inversely related to the

detectability, the mobile phase of 5 mM appeared similar to the condition of isocratic elution in water (low detection) while Levigne worked refraction index detector . The mobile phase water that generated better detectability for HM pectin was not chosen why the goal was carried to better suitability for the acetyl and methoxyl, observed previous assays. Almost unsuccessful until this moment quantifies acetyl by similar curve because only standards of medium and low DM were registered detection for this peak (3.2 min) (figure 1). Others samples of fruits and horticoles are being investigated in order to improve detection and quantification of acetyl.

Using isopropanol as internal standard, from the extracted curve protocols with extreme points, previous stepping offered low condition pectin LM which registered evident sign of acetyl, while the peak of isopropanol (7.5 min) and acetyl (3,5 min) were also confirmed by injection of standards (mixture 1:1:1). The peak isopropanol was adjusted from low molarity (25 μ M) described by Levigne and collaborators [13], from 1.7 μ L to 350 μ L (rough necessary to internal standard curve). Then set the methodology by increasing the volume of isopropanol for confirmatory purposes. This peak also was adjusted in the order of addition of it, because it was only possible measure it when added the internal standard after centrifugation step.

3.2 Optimization of conditions of desesterification of pectins

The strength of NaOH was the most expressive parameter showing maximum extraction between 1.5-2.0 M with combinations of time ranging from 5-60 min. The fact that time does not significantly influence was asked to select the values assigned to the variable time. In testing there was a tendency to reduce the strength of the extraction times above 60 min in a repeatability of standards. The significant differences by

multiple comparisons of averages of 5-30 min, 30-60 min and 5-60 min, indicated that time should not exceed 60 min, which was greater variability in weather extremes (figure 2 and table 5). Table 6 summarizes the effect of strength NaOH indicating that this parameter only worked on the modeling polynomial regression, although the quadratic model failed to explain the linear relationship that ensured the interpretation range from -1 to +1 in order to perform the best conditions. In this case -1 related to strength (0.5M) and +1 (1.78 M), was choose the positive value by the ration of orthogonal projection and a positive correlations.

A second experiment of conditions of desesterification was tested on standards and observed that above NaOH 2 M, probably has met cutoff sustentable hypothesis that unaffected natural structure of pectin. The HM and LM pectins were intensely demethoxylated in this conditions (above 2 M NaOH at any time. In this experience was preserved the integrity of the molecule with probably minimal effects on its structure, when used below 1.78 N for standards and samples of pectin extracted from tropical fruits (below 1.78 M was used in this procedure).

Calibration with commercial standards required certification that the standards being appropriate to analytical conditions. Due to the difficulty of finding commercial pectin with acetyl, this quantification can be accomplished by extraction of pectin samples that have high levels of acetyl, as highlighted by Levigne and collaborators [13]. Beets and pears are examples of matrices that contain high acetyl (10 to 40%). In the samples yielded lower values were suggested be associated with the limit of detection of the new analytical tool. Only with pectin DM 10.5% yielded quantitative acetyl (figure 1), and in some samples of pectin of fruits (figure 3 and 4). The extreme strength of NaOH evaluated were improperly able to desesterification extracting poorly or destroying firstly observed in HM and LM pectins. Below 0.5 M NaOH

showed insufficient to desesterification of samples, while levels NaOH 3.2 M strongly interacted with high time (from 120 to 325 min), producing intense and exhaustive demethoxylation (figure 5), notably observed in the HM pectin, yielding probably acid pectinic. However, this variation in response may also produce β -elimination any products were few investigated in the literature. In this experimental context only from 2.0 to 2.3 M in the time for reaction from 120 to 325 min yielded any methoxyl groups, however lowest than half the value found in the essay that allowed the hypothesis integrality reported in this discussion. Therefore time for reaction and strength NaOH associated to simultaneous effect on pH have corroborated to recent studies may occur of way variable to degradation by β -elimination associated mainly the loss of branching [2].

Recent studies have given profile for chemical hydrolysis and enzymatic hydrolysis associated with sugars and other chemical compounds making a prediction of chemical composition of pectin extracted under different experimental conditions [16]. When comparing this result with new system of measuring purity of pectins from ratio of individual sugars and non sugars can be validate and certify commercial standard through distinct protocol.

Another less drastic method to evaluate the chemical composition of pectin is by ^{13}C NMR, which is not destructive method [3]. Itself while need any procedure of extraction, this technique has provided reliable composition and trend pointed interactions with the extraction procedure. It should be noted that the ELSD detector for recording signals of compounds found in the structure of pectin, under inert atmosphere (350 kPa), consist of one powerless candidate of class of non-destructive detectors, whereas technical adjustments as outlined in this application ensuring maximization of the signs, especially for chemical components of character of conflictant.

3.3 Determination of DM (%) of standards and samples of pectins extracted of fruits

The construction of the standard curves allowed to estimate the significant correlations in three distinct methods by comparing the values of $R \geq 0.92$, indicating the suitability of the methods in question, when relationship titration x infrared x HPLC-ELSD.

The external and internal standardization can be recommended using the combination detection by ELSD x column Hipelex fast acid.

In the evaluation of samples with levels of difficulty by chemical composition, the optimization procedure has shown able to quantify and correlate satisfactorily. Excepted when making use of internal standard taking isopropanol peak which provided low-sensibility signal from the initial studies, offereying more resistance to a satisfactory quantitative result, ensured a correlation of 0.75. In this approach, considering the positive results of comparable measures with external standard, this is possibility in repeating the analysis to include a reliable quantification which ensuring a stronger correlation of quantitative procedure. The internal standard used in the proposed Levigne and collaborators [13] does not seem so essential to the systems studied considering quantification tool. Moreover, the equation described by same authors considering the amount of galacturonic, in ratio to standards and samples were taken for dispensable values due to nonspecific reaction crossover [13], in the quantification of any uronic. This makes the system unreliable requiring quantitative structural assessment, considering the demand for accuracy in studies with minimal impact on the fine structure of the pectin molecule, carrying with successful knowledge of pectin function and really crossing over reactions carried out by extraction procedure. To consider the quantification using internal standardization should be pointed out in

some security feature of the method, not observed for isopropanol, such as no interaction with the matrix during procedure of extraction, eluting between what is intended to quantify repetitive signals. Therefore the system of quantification by external standardization seems as reliable as the internal standardization, by considering the regression parameters IR x HPLC-ELSD (table 7). More advantageous likely favorable to external standard because shorter run,

The process of extraction of pectin in different materials, related to the strength of organic and inorganic agents extractors occurs in various acid pH of 1.5-7.0, combined with other parameters such as temperature and reaction time, are distancing themselves from the possibility of securing the native structure of pectin mainly by β -elimination reaction with or without de-methoxylation [10]. This can lead to molecules of varying size even in less destructive and faulting procedure which ensures the conflicting character of methodologies for polysaccharides [8].

The oligogalacturonides and oligosaccharides with size molecular, DM and DA variable need to be characterized and thus indirectly confirms any change structural of pectin fruits and other matrices [16]. Recent characterizations of this nature allowed associating an anti-inflammatory activity of rhamnogalacturonana extracted from sweet pepper (around 510 kDa) while galacturonana, arabinogalacturonana require more studies relation structure versus function. Any molecule with similar properties pectin and so one derived of medium pH effect believe be equally behavior to Gal-3 met in lectin functionally registered as inhibitors of various cancers [3].

4. Conclusion

The experimental conditions of gain, fogging temperature and interactions with the stationary phases were differentiated by ELSD detector, and favored by the

combination Hiplex column, which showed advantage in sensitivity and separation for acetyl and methoxyl.

Sulfuric acid mobile phase was adjusted to better the condition of detection of the compounds of interest (50 mM) and shown to be advantageous in relation to water by comparing results in both stationary phases (hiplex and ODS-II)..

The elution of the analytes respected the order of increasing molecular weight in the conditions agreed on two stationary phases, using the same standards extracted HM and LM, while pectins as well injected mixture of analytes as solvents;.

The strength of NaOH proved significant and growing up 1.78 M, evaluated in any combination of time for reaction (5-60 min), conditions which were set the procedure of desesterification of standards (extractable curve) and samples.

The intense demethoxylation was hit on the strength of NaOH over 3 M in combinations of time exceeding 120 min, while below 0.5 M NaOH phase separation has not occurred after precipitating and centrifugation step.

The systems of quantification evaluated by regression using comparative results titrimetric x infrared and infrared x HPLC-ELSD being alternatively external and internal standardization provided R superior 0.92; while in the samples 0.75, performing the accuracy in this new method.

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Table 1. Decoded levels of independent variables.

Coding	Time for reaction (min)	Strenght NaOH (M)
-1.41	5	0.5
-1.00	21.71	0.74
0.00	62.5	1.25
1.00	103.28	1.78
1.41	120	2

Table 2. Contrast values obtained from assay factorial

Assay	Time for reaction (min)	Strenght NaOH (M)
1	21.71	0.74
2	103.28	0.74
3	21.71	1.78
4	103.28	1.78
5	5	1.25
6	120	1.25
7	62.5	0.5
8	62.5	2
9	62.5	1.25
10	62.5	1.25
11	62.5	1.25

Table 3. Evaluating Spherisorb ODS-2 as stationary phase to HPLC-ELSD

	Mobile phase	Methanol rT	ELSD (mV)	Acetic acid rT	ELSD (mV)	(2-propanol) rT	ELSD (mV)
ODS-2 150x3.2mm (5µm)							
Pectin DM= 30%	H ₂ SO ₄ (5mM)	~ 2.5	250.0	nd	nd	nd	nd
Pectin DM= 90%	H ₂ SO ₄ (5mM)	~ 2.5	150.0	nd	nd	nd	nd
solvent mixture (Methanol + ethanol + 2-propanol)	H ₂ SO ₄ (5mM)	~ 2.5	250.0	nd	nd	nd	nd
ODS2 100x2mm (5µm)							
Pectin DM= 30%	H ₂ SO ₄ (5mM)	nd	nd	nd	nd	nd	nd
Pectin DM= 90%	H ₂ SO ₄ (5mM)	~ 1.5	150.0	nd	nd	nd	nd
solvent mixture (methanol + ethanol + 2-propanol)	H ₂ SO ₄ (5mM)	~ 1.5	250.0	nd	nd	nd	nd

nd: not detected

rT: time retention **Detection:** ELSD (mv)

Flow: 0.6mL /min **Temperature:** 40°C **Gain:** 1 **Pressure:** 350Kpa

Extraction conditions: in accordance with Levigne and collaborators [13].

Table 4. Evaluating Hiplex fast acid as stationary phase to HPLC-ELSD

	Mobile phase	Methanol	ELSD	Acetic acid	ELSD	(2-propanol)	ELSD
		rT	(mV)	rT	(mV)	rT	(mV)
Pectin DM= 10%	H ₂ O	2.5	6.0	nd	nd	nd	nd
	H ₂ SO ₄ 5mM	nd	nd	nd	nd	7.5	50.0
	H ₂ SO ₄ 50mM	2.5	75.0	3.2	10.0	7.5	25.0
	H ₂ SO ₄ 100mM	2.5	75.0	nd	nd	7.5	200.0
Pectin DM= 90%	H ₂ O	2.5	6.0	3.2	26.0	nd	nd
	H ₂ SO ₄ 5mM	2.5	35.0	3.2	20.0	7.5	5.0
	H ₂ SO ₄ 50mM	2.5	135.0	3.2	20.0	7.5	50.0
	H ₂ SO ₄ 100mM	2.5	200.0	3.2	nd	7.5	150.0

nd: not detected

rT: retention time **Detection:** ELSD

Flow: 0,6mL /min **Temperature:** 40°C **Gain:** 1 **Pressure:** 350Kpa

Extraction conditions: in accordance with Levigne and collaborates [13].

Table 5. Repeatability of standards of pectins HM and LM extraction conditions.

Times for reaction (Multiples comparative)	Standard Deviation	
	Pectin HM	Pectin LM
5-120 (min)	1.1×10^{-5}	0.8×10^{-5}
30-120 (min)	1.4×10^{-5}	1.1×10^{-5}
60-120 (min)	1.6×10^{-5}	1.1×10^{-5}
5-30 (min)	0.45×10^{-5}	0.9×10^{-5}
5-60 (min)	0.6×10^{-5}	0.9×10^{-5}
30-60 (min)	0.3×10^{-5}	0.30×10^{-5}

Table 6. Contrast analysis by source of variations through factorial assay.

		T value	P (> table) 95%
Pectin LM (below 1%)	Time for reaction (min)	0.282	0.2549
	NaOH strength (M)	3.685	0.0142
	Time x NaOH	0.182	0.8629
	Quadratic model $R^2 = 0.7894$		
Pectin HM (DM ~ 90%)	Time for reaction (min)	0.257	0.8076
	NaOH strength (M)	3.302	0.0214
	Time x NaOH	0.235	0.8237
	Quadratic model $R^2 = 0.7290$		

Table 7. Linear regression applied to commercial standar of pectin and samples of pectins from fruits.

Quantification System	F	p value	R²	a	b
Titrimetric x Infrared*	1421.52296	< 0.0001	0.9766	0.09668	0.00559
Titrimetric x HPLC-ELSD*					
External Standard	315.45909	< 0.0001	0.92215	281134.32544	10079.97022
Internal Standard	592.36463	< 0.0001	0.94563	0.09848	0.00371
Infrared x HPLC-ELSD*					
External Standard	376.58651	< 0.0001	0.91943	277212.11823	10299.1025
Internal Standard	633.73469	< 0.0001	0.94901	0.10034	0.0368
Infrared x HPLC-ELSD **					
External Standard	75.62989	< 0.0001	0.75667	-118,46183	2.55215
Internal Standard	73.21187	< 0.0001	0.75055	-128,71796	2.73054

*N=35 for commercial standard of pectin

**N=10 for pectin extracted of fruits

Captions of figures

Figure 1. Chromatogram standard of pectin DM=90% (Sigma Aldrich) and galacturônico acid DM=10.5% (Fluka).

Figure 2. Histogram of confidence level for pectin LM and HM (repeatability studies)

Figure 3. Chromatograms of pectin extracted from fruit mesocarp: (a) watermelon; (b) orange; (c) passion fruit.

Figure 4. Chromatograms of pectin extracted from fruit: (a) apple and (b) guava.

Figure 5. Chromatogram of pectin Hm subjected to extremes strenght NaOH (3M) and reaction time (240 min). Only showed isopropanol peak.

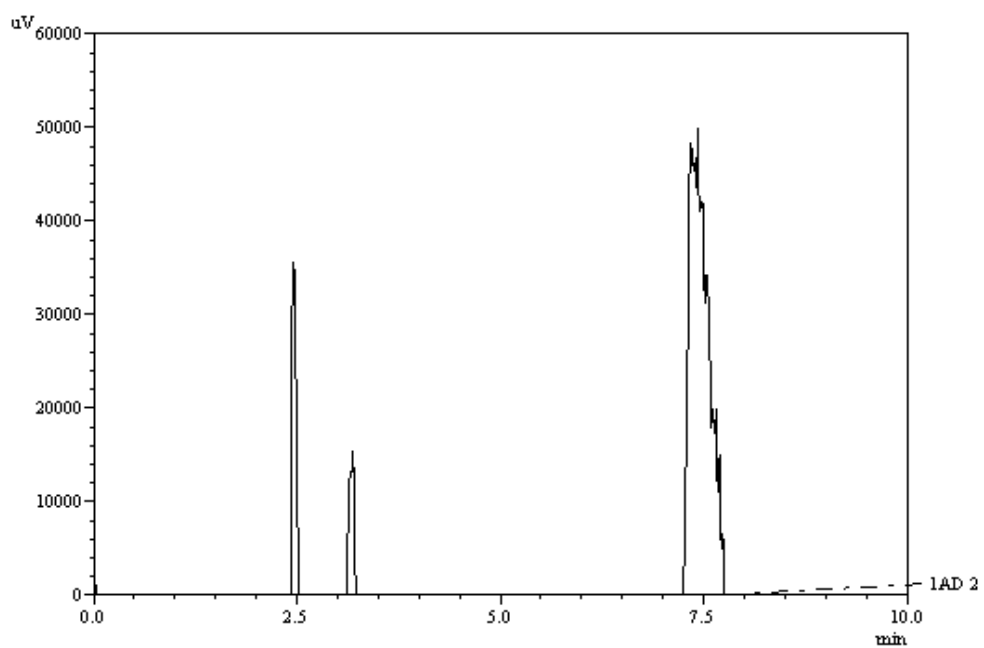
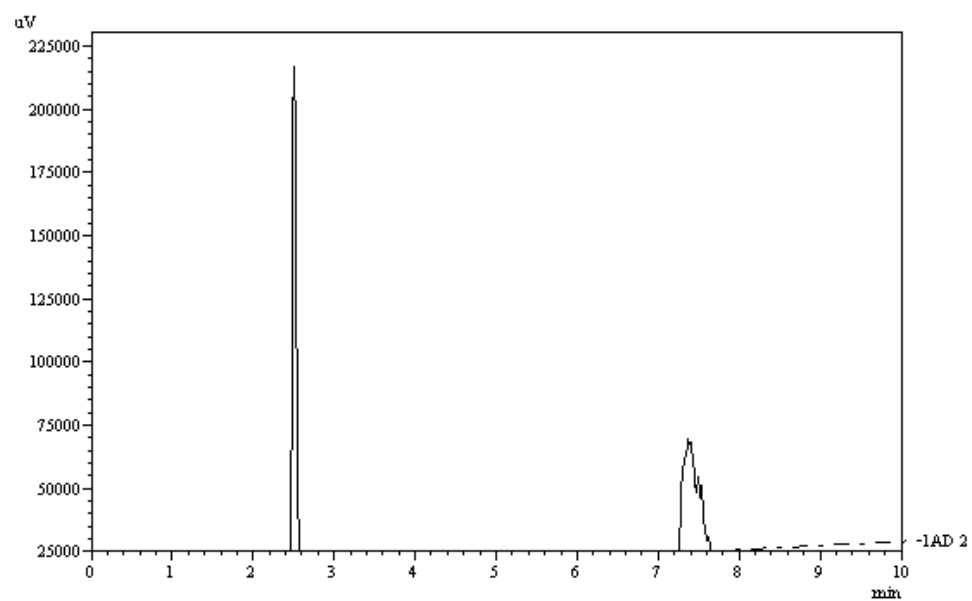
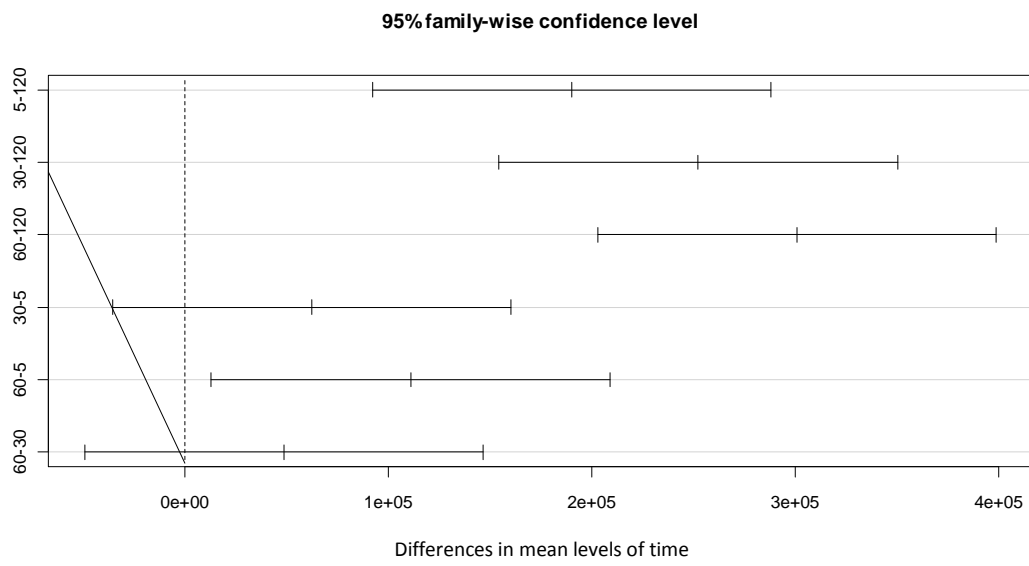


Figure 1. Chromatogram standard of pectin DM=90% (Sigma Aldrich) and galacturônico acid DM=10.5% (Fluka).

Pectin HM



Pectin LM

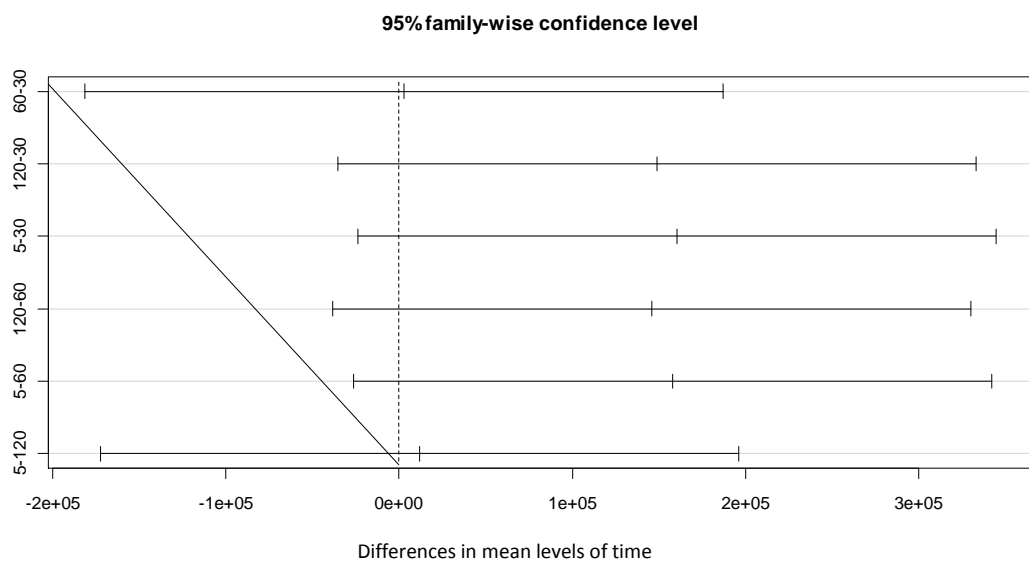


Figure 2. Histogram of confidence level for pectin LM and HM (repeatability studies)

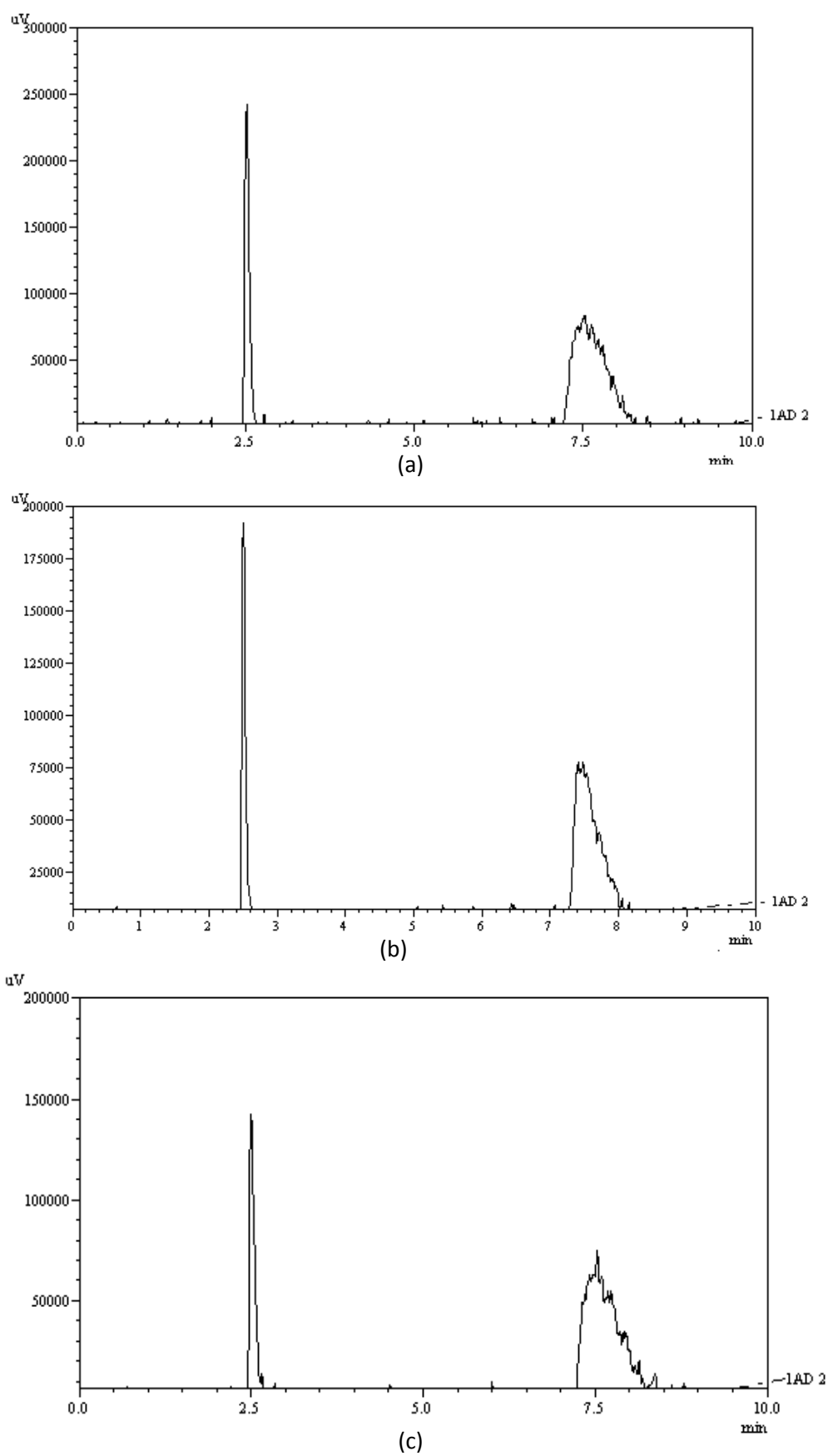


Figure 3. Chromatograms of pectin extracted from fruit mesocarp: (a) watermelon; (b) orange; (c) passion fruit.

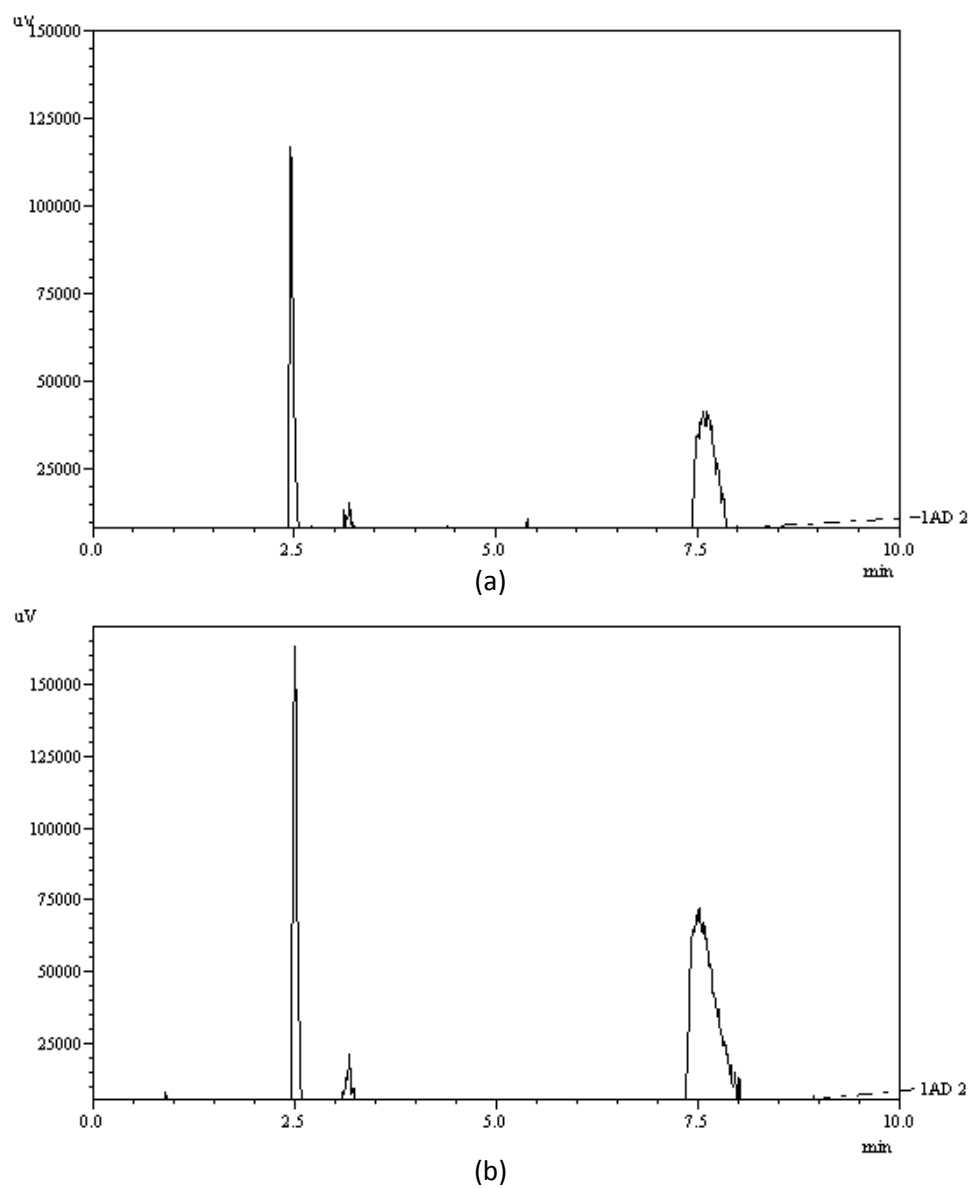


Figure 4. Chromatograms of pectin extracted from fruit: (a) apple and (b) guava.

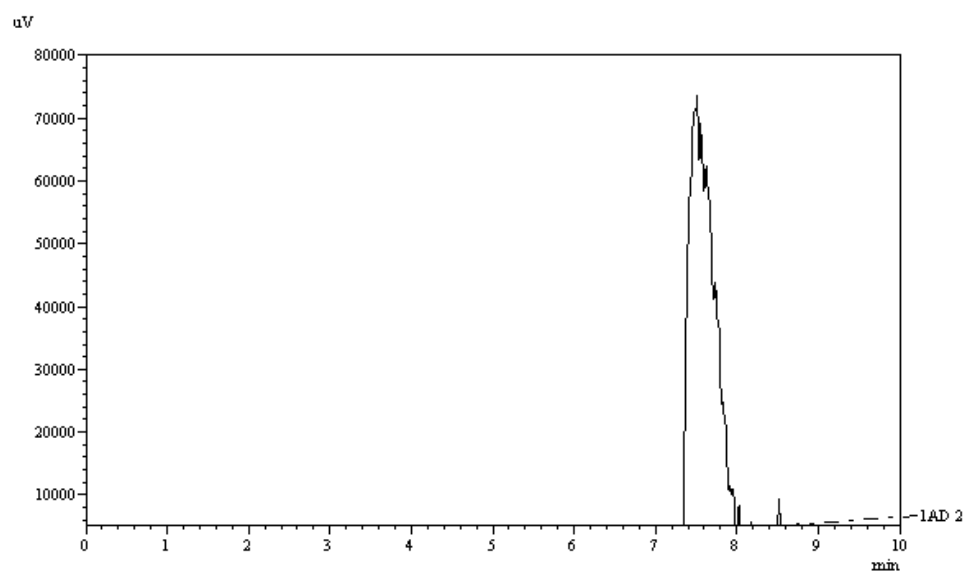


Figure 5. Chromatogram of pectin HM subjected to extremes strength NaOH (3M) and reaction time (240 min). Only showed isopropanol peak.

8. Conclusões

- O ácido orgânico (cítrico) mostrou ser mais eficiente na extração de pectinas do mesocarpo de melancia e demais frutos, exibindo maiores rendimentos e associado ao maior grau de metoxilação;
- Por questões toxicológicas, o etanol é o álcool mais apropriado para o isolamento de pectinas;
- A coluna Hipler fast acid e a fase móvel H_2SO_4 foram apropriadas para a análise da metoxilação de pectinas por HPLC-ELSD;
- A saponificação de pectinas com NaOH 1,78M no tempo de reação de 60 minutos mostrou-se apropriada para posterior análise por HPLC-ELSD;
- Os métodos analíticos apresentaram correlação de $R^2 > 0,91943$ para padrões, e $R^2 > 0,75055$ para pectinas extraídas de frutas.

9. Perspectivas

A caracterização de pectinas para a indústria farmacêutica e de alimentos é uma ferramenta tecnológica que auxilia na orientação de novas formulações com características apropriadas.

Novos enfoques metodológicos permitirão clarear relação estrutura x função de natureza polêmica nos últimos artigos encontrados.

O resultado levantado permite orientação tecnológica no tocante as multi-funções assumidas pelas moléculas de pectinas (e derivados).

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Anexos

ANEXO A – Documentação de encaminhamento do artigo ao periódico

Submission Confirmation

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Responder 

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ANEXO C – Documentação de encaminhamento do artigo ao periódico

Journal of Chromatography A: Submission Confirmation

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Title: HPLC-ELSD coupled Hiplax columns as analytical tool for screening degree of acetylation and methoxylation of pectins from tropical fruits.
Corresponding Author: Dr. Marianne Lima

Dear Dr. Lima,

This is to confirm that the above-mentioned manuscript has been received for consideration in Journal of Chromatography A.

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With kind regards
Journal Staff