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COMPARATIVE STUDY ON EXTRACTION METHODS OF PECTIN FROM BY-PRODUCTS OF JUICED CARROTS

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Abstract

Carrot juice is a product of interest in food industry. Pomace, a by-product of the carrot juice industry, contains a significant amount of pectin. This natural polymer is rich in galacturonic acid and is used as food additive due to its gelling, thickening and stabilizing properties. The objective of this study was to compare different extraction methods of carrot pectin. Sodium citrate buffer, pH 5.0 and carrot pulp were used for pectin extraction. Methods used to obtain pectin required temperature, microwaves, ultrasounds and Celluclast 1.5L enzyme treatment. The extraction yield of each method was determined. The obtained pectin was characterized by the content of galacturonic acid, the degree of esterification, the degree of emulsification and cytotoxicity in a fibroblast cell line. The enzymatically extracted carrot pectin contained at least 65% galacturonic acid and had a high degree of esterification and emulsification. In vitro cytotoxicity tests have demonstrated the biocompatibility of pectin extracts. The results of this study have showed that valuable carrot pectin with high content of galacturonic acid and good biocompatibility can be obtained from carrot pomace. The enzymatic extraction method could be further studied for various industrial applications, and the obtained carrot pectin could be useful especially in food supplements.

Key words*: cytotoxicity, emulsion, esterification, galacturonic acid, pectin.*

INTRODUCTION

Food industry produces several by-products during fruits and vegetables processing, especially for natural juices manufacture, which can be valorised. The generated pomace is a valuable by-product that contains pectin, besides other components of interest. Pectin is a group of natural polysaccharides, rich in D-galacturonic acid units linked by α-1,4 glycosidic bonds, present in the cell walls of the superior plants. The galacturonic acid from pectin chain is largely esterified with methoxy and acetyl groups (Harholt et al., 2010). Due to its gelling, thickening and stabilizing properties, pectin is intensively used in the food industry, especially sweets, as an additive. It is a very safe food additive with no consumption limit. It is also used in the medical, cosmetic and pharmaceutical industries (May, 1990). There are no indications on its genotoxicity and it has no side effects or allergenic potential (Mortensen et al., 2017). At the beginning of the 20th century, the German apple juice producers used the remaining residue to obtain pectin, which was sold as a gelling agent. At present, apple pomace and citrus peels are the main sources for obtaining commercial pectin (Dranca & Oroian, 2018). 85.5% of the market pectin is from citrus peels, 14% from apple pomace and 0.5% from sugar beet pulp (Cirimina et al., 2016). New sources of pectin have been studied, such as tomatoes (Grassino et al., 2016), carrots (Jafaria et al., 2017), watermelon (Petkowicz et al., 2017), banana peels (Happi Emaga et al., 2008), passion fruit peels (Liew et al., 2014; Vasco-Correa et al., 2017), dragon fruit peels (Tongkham et al., 2017), grape pomace (Minjares-Fuentes et al., 2014) etc.

Both the extraction method and the source influence the quantity and the properties of the obtained pectin. The temperature and the buffer pH are parameters of great importance, which can vary during pectin extraction process. Pasandide et al. (2017) extracted a high esterified pectin from an aqueous solution of *Citrus medica* peel, at different temperatures and periods of extraction, with a maximum yield of 21.85%. Liew et al. (2014) obtained a high esterified pectin from passion fruit peels, varying the pH of citrate buffer and the maximum yield

corresponded to pH 2. Commercial pectin was also extracted from citrus peels or apple pomace using hot dilute acid at pH 2 (May, 1990).

Currently, environment-friendly methods based on microwaves, ultrasounds and enzymes are used in pectin extraction. Tongkham et al. (2017) obtained pectin from an aqueous solution of dragon fruit peels using microwaves at different powers and the maximum yield was 23.11%. Minjares-Fuentes et al. (2014) prepared a high esterified pectin with a yield of 29.4% from grape pomace in citric acid using ultrasounds. A maximum output of 19% pectin was obtained from apple pomace using a treatment with Celluclast 1.5L enzyme (Wikiera, 2015).

The use of pectin on a larger scale requires new methods of extraction and valorisation of different sources, in order to obtain products with convenient properties. Carrot (*Daucus carota*) is a root plant, consumed worldwide in both fresh and cooked state for its high content in minerals and carbohydrates. During carrot juice preparation, an important amount of pomace is obtained, with a high content of valuable compounds including carotenoids, dietary fibres and pectin (Sharma et al., 2012). Jafaria et al. (2017) extracted the carrot pomace pectin in citric acid.

The purpose of this study was to compare the enzymatic methods of pectin extraction with the chemical methods using carrot pulp in citrate buffer as a source. The physical and chemical characterization of pectin was performed in order to obtain a product with improved properties for applications in the food and cosmetics industry.

MATERIALS AND METHODS

Materials

Carrots originating from Romania were bought from the supermarket, washed, cut and dried at 50°C, for 24 h. Then, the material was powdered using a grinder and stored in food bags, at -20°C until analysis.

Enzymatic extraction method

The powdered carrot was incubated in citrate buffer, pH 5 containing 88 U Celluclast 1.5L, in two different weight ratios of 1: 60 (variant a) and 1: 15 (variant b), 50°C, for 20 h (Sabater et al., 2018; Wikiera et al., 2015). The enzyme activity was stopped by boiling the mixture at 100°C, for 10 min. Controls (variants a1, b1) were prepared in the same conditions without adding the enzyme. The extracts were filtered through Whatman paper to remove the vegetal residues.

Thermal extraction method

Carrot powder (0.5 g) was incubated in 15 ml citrate buffer, pH 5, at 120°C, for 2 h (Pasandide et al., 2017). After cooling at room temperature, the solution was filtered through Whatman paper (variant c) and further processed for analysis.

Microwaves-assisted extraction method

In the case of microwaves-assisted extraction, a weight ratio of 1: 100 between the carrot powder and the citrate buffer was used. The homogenized solution was exposed to microwaves, at a power of 560 W, for 160 s (Tongkham et al., 2017). After cooling, the solution was filtered through Whatman paper and further analysed (variant d).

Ultrasonic extraction method

A mixture of carrot powder and citrate buffer pH 5, in a weight ratio of 1: 30 was exposed to ultrasounds treatment, at 10 kHz frequency, at 60°C, for 40 min (Minjares-Fuentes et al., 2014). The vegetal residues were removed by filtration and further processed (variant e).

Purification method

Pectin was purified by precipitation of filtrate with ethanol. In the first step, 2 volumes of ethanol were added over the obtained filtrate and incubated at 4°C, overnight. Then, it was centrifuged at 7000 g, for 30 min. The supernatant was discarded and the precipitate was washed with 10 ml absolute ethanol and separated by centrifugation at 7000 g, for 30 min. Pectin extract was dried at 50°C until constant mass was reached.

All pectin extracts were prepared in two separate experiments, in triplicate.

Yield determination

For each extraction method, the yield was calculated using the following equation:

% yield = (final weight/initial weight) x 100 where the final weight was the amount of dried pectin and the initial weight was the dried amount of raw material.

Determination of galacturonic acid

Determination of galacturonic acid content was performed by orcinol method (Moldovan et al., 2008). Briefly, over 1 ml of pectin solution, 3 ml orcinol reagent (orcinol mixed with FeCl3 and concentrated HCl) were added. The mixture was incubated at 100°C, for 40 min with gentle shaking. After cooling, the absorbance of each processed sample was read at 665 nm using a UV-VIS spectrophotometer. A standard curve was built using dilutions of 0.15 mM galacturonic acid solution. Determinations were performed in triplicate.

Determination of esterification degree

Determination of the esterification degree (DE) was performed by titrimetry (Liew et al., 2014). A pectin solution of 1 mg/ml concentration was titrated with NaOH, in the presence of 2 drops of phenolphthalein, until the colour turned pink (initial NaOH volume). The mixture was left at room temperature, for 2 h to de-esterize galacturonic acid. HCl was used to neutralize excess NaOH until the solution became colour-less. After that, 2-3 drops of phenolphthalein was added again and the obtained mixture was titrated with NaOH until pink (final NaOH volume).

$$\%DE = \left[\frac{\text{Final NaOH(ml)}}{\text{Final NaOH (ml)} + \text{Initial NaOH(ml)}}\right] \times 100$$

Determination of emulsification degree

Determination of the emulsification degree was performed by vortexing a mixture of 0.5 mg/ml pectin solution and sunflower oil containing 0.02% sodium azide, in equal parts, at maximum speed (Jafaria et al., 2017). Then, the emulsion was centrifuged at 4000 g, for 5 min. The emulsification degree was calculated using the following equation:

$$\%\text{Emulsification} = \left(\frac{\text{Emulsion volume}}{\text{System volume}}\right) \times 100$$

where emulsion volume was the volume of emulsion phase and the system volume was the volume of total system.

Cell cytotoxicity tests

Cells from mouse fibroblast NCTC clone L929 cell line were seeded in 96-well culture plates and incubated in 5% CO_2 humid atmosphere, at 37°C, for 24 h. For experiments, the culture medium was replaced with fresh medium containing pectin extracts in the concentration range of 50-1000 µg/ml and the plates were incubated in standard conditions for 24 h and 48 h. Cell viability was determined at the end of incubation period by MTT assay, as previously described (Scudiero et al., 1988; Quentin-Leclercq et al., 1992). MTT assay consists in the reduction of tetrazolium bromide salt by mitochondrial dehydrogenases present in metabolically active cells. Briefly, the culture medium was replaced with MTT solution, followed by incubation at 37ºC, for 3 h. The formed formazan crystals were dissolved in isopropanol by gentle shaking and the absorbance of the coloured solution was read at 570 nm using a microplate reader. Untreated cells served as negative control, considered as 100% viable cells, while cells treated with 0.03% H_2O_2 represented the positive control. Determinations were performed in triplicate.

Cell morphology observations of the cultures treated with pectin extracts for 48 h were performed using an optic microscope equipped with a digital camera.

RESULTS AND DISCUSSIONS

In this study, pectin was extracted from carrot pulp in sodium citrate buffer, pH 5.0 using the enzymatic, thermal, microwave- and ultrasound-assisted methods.

Extraction of pectin and yield

The purified pectin extract was dried to obtain a yellowish or white powder (Figure 1).

The yield of pectin extraction from carrot in citrate buffer, pH 5, varied between 7-20%, according to each treatment procedure (Figure 2). Thus, the highest value was 20%, in the case of microwave extraction of pectin from carrot pulp. In the case of ultrasonic extraction, the yield was almost 3 times lower than that of microwave-assisted extraction of pectin. Enzymatic extraction of pectin in variant b had a yield of 17%, which was with 23.52% higher than the yield obtained in enzymatic treatment variant a.

Figure 1. Pectin extracts obtained from carrot pulp by enzymatic (a), thermal (c) and microwave-assisted (d) methods

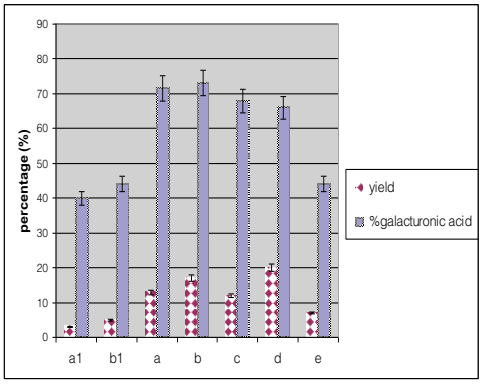

Figure 2. Extraction yield and galacturonic acid content of carrot pectin obtained by enzymatic (1: 60) (a) with its control (a1), enzymatic (1: 15) (b) with its control (b1), thermal (c), microwave-assisted (d) and ultrasonic (e) extraction methods

Pectin extracts obtained in the same conditions, but enzyme-free (a1 and b1 controls) had a very small yield of 3% and 5%, respectively, indicating that Celluclast was efficient in pectin extraction.

Celluclast 1.5L is a cocktail of enzymes with xylanolytic and cellulolytic activity. This enzymatic cocktail helps to release the pectin by destroying the cellulosic wall of the plant cell (Wikiera et al., 2015). Still, the yield of enzymatic extraction variant b was with 15% lower than the yield of microwave-assisted extraction. Thermal extraction of pectin had a yield of 12%. Similar studies indicated a yield of 23.1% in the case of microwave-assisted extraction of pectin from dragon fruit peels, in citrate buffer pH 2, for 10 min, at a power of 600 W (Tongkham et al., 2017).

Liew et al. (2016) extracted the pectin from the passion fruit by the enzymatic method using Celluclast and obtained a maximum yield of 9.17%. Wikiera et al. (2015) obtained apple pectin with a maximum yield of 19%. Jahari et al. (2017) obtained pectin from carrot pomace using a temperature of 50- 90°C for 30-150 min, at a pH ranging between 0.5-2.5. By varying temperature, time and pH parameters, yields ranged between 5-15.2%. The maximum yield was obtained in the case of pH 1.3, 90°C for 79.8 minutes.

Galacturonic acid content

The method of pectin extraction from carrot pulp influences its galacturonic acid content. According to FAO, pectin used in the food industry have at least 65% galacturonic acid content, as an indicator of its purity (May, 1990). In this study, pectin obtained by enzymatic extraction with Celluclast had a high content of galacturonic acid (Figure 2), ranging from 71.5% (variant a) and 73% (variant b). The percentage of galacturonic acid in thermal extracted pectin was 68% and in microwaves extracted pectin was 66%. The pectin extracted by ultrasound had a lower percentage of galacturonic acid (44%).

By enzymatic extraction, using Celluclast, we obtained a pectin with a 73% galacturonic acid content. Jafaria et al. (2012) showed that pectin obtained from carrot pomace, under acidic conditions, at high temperature contained 75.5% galacturonic acid. In turn, pectin obtained from passion fruit by enzymatic extraction using an

extract from *G. klebahii* had 85.4% galacturonic acid content (Vasco-Correa et al., 2017).

Esterification degree

The esterification degree represents the percentage of galacturonic acid carboxyl groups esterified with methoxy (in most cases) or acetyl groups.

The pectin extracted by different extraction methods in this study had a high degree of esterification. It was higher than 70% for all treatment procedures, as presented in Figure 3. The highest esterification degree of pectin extracted from carrot was 87%. A product with an esterification degree of 81% was obtained by thermal extraction. The microwave-assisted extraction resulted in a product with 77% esterification degree, while ultrasound-extracted pectin had a degree of esterification of 75%.

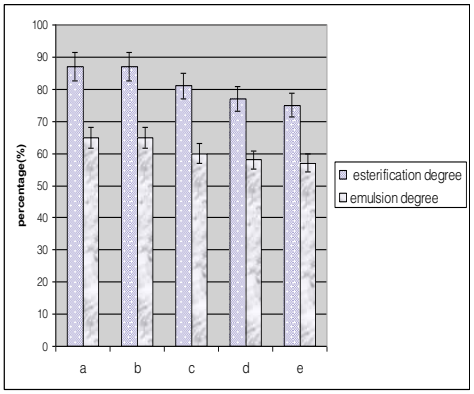

Figure 3. The esterification and emulsion degree of carrot pulp pectin obtained by enzymatic (1: 60) (a), enzymatic (1: 15) (b), thermal (c), microwave-assisted (d) and ultrasonic (e) extraction methods

A similar result was obtained by Liew et al. (2016) for enzymatically extracted passion fruit pectin, presenting 86.96% esterification degree.

Pectin esterification degree is >50% for high methylated pectin or <50% for low methylated pectin, respectively. The esterification degree affects pectin properties (Liew et al., 2014). The high methylated pectin can form gels at low pH. Low esterified pectin forms gels in the presence of Ca^{2+} (Venzon et al., 2015).

Venzon et al. (2015) attempted to decrease the pectin esterification degree by treatment with NaOH for 1 h, at 55°C. Lowering the esterification degree with a few percent, the pectin solution viscosity was reduced.

Emulsion degree

Pectin can be used as an emulsifier in the food industry and its quality depends on the degree of emulsification. In this study, enzymatically obtained carrot pectin had the highest degree of emulsification (65%) (Figure 3). The pectin obtained by ultrasounds treatment had a lower emulsification degree (57%). Thermally obtained pectin had the emulsification degree of 60%, while that of microwaves-extracted pectin was 58%. A similar degree of emulsification of 60.3% was reported for pectin obtained from carrot pomace (Jafaria et al., 2017).

Cytotoxicity tests

Cytotoxicity of pectin extracts was tested in NCTC fibroblast culture after 24 h and 48 h of cultivation, by MTT assay and cell morphology observations. The results are presented in Figure 4.

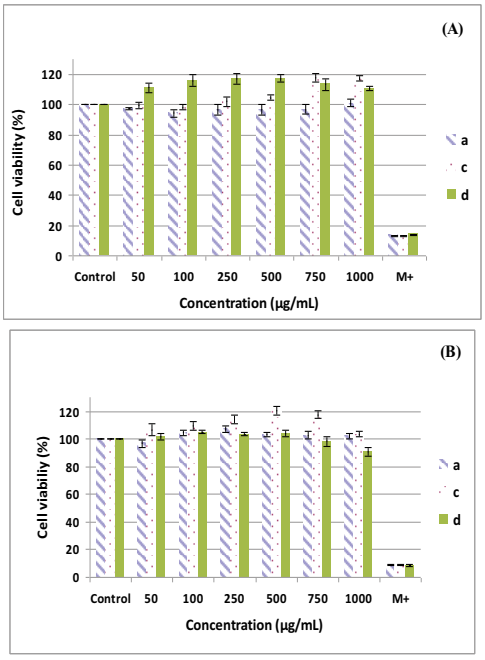

Figure 4. Cytotoxicity of carrot pectin extracted enzymatically (a), thermal (c) and microwave-assisted (d) and cultivated in NCTC fibroblast cell culture for 24 h (A) and 48 h (B)

The viability was high after 24 h of NCTC cells cultivation in the presence of pectin, surpassing 94% (Figure 4. A).
It was observed that cells cultivated with microwaves-extracted pectin with concentrations of 50-1000 μg/ml presented high values of viability, between 110.94-117.61%.
The viability of cells cultivated with thermally-extracted pectin increased in a concentration-dependent manner, with the highest value at 1000 μg/ml concentration. After 48 h of cultivation in the presence of pectin extracts, the cell viability was similar or higher than that of control cells, considered 100%, excepting microwaves-extracted pectin at 1000 μg/ml (Figure 4. B).
Thermally-extracted pectin maintained a high value of cell viability, ranging between 103.72% and 124.44%, for concentrations of 50-750 μg/ml.
Cell morphology of NCTC fibroblasts cultivated in the presence of pectin extracts was observed after 48 h of cultivation (Figure 5). No change was observed in the morphology of pectin-treated cells, compared to untreated cells. Fibroblast cells presented the characteristic spindle-shape phenotype. These observations confirmed the quantitative data obtained by MTT assay.

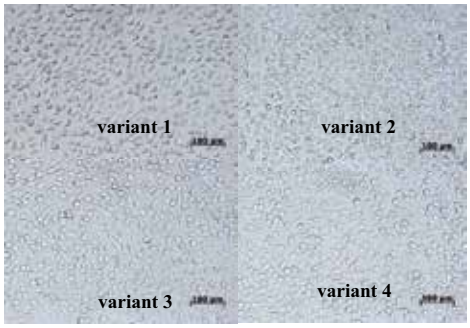

Figure 5. Morphology of NCTC cells cultivated with 250 μg/ml pectin extracted by enzymatic method (1), microwaves method (2) and thermal method (3), for 48 h. Control cells were cultivated in plastic culture plates (4)

The cytotoxicity tests demonstrated that all three types of carrot pectin were not cytotoxic. Moreover, thermally-extracted pectin stimulated the growth of NCTC cells.

CONCLUSIONS

All results demonstrated that high quality pectin extract could be obtained from carrot pulp by environmentally friendly methods based on enzymatic or microwaves treatment. They avoid strong acids or high temperature treatment for long periods of time. Each method of extraction influenced pectin extract characteristics. Thus, pectin extracts were highly esterified (>70%), had a high degree of emulsification (>50%) and over 65% galacturonic acid content. The best yield was obtained in the case of microwaves extraction, while the highest percentage of galacturonic acid was found in enzymatic-extracted pectin. The MTT assay results showed that thermally-, enzymatically- and microwaves-extracted pectin did not have cytotoxic effects in a fibroblast cell culture. In conclusion, the high quality pectin extracted from carrot pulp could be further studied for various industrial applications, especially in food supplements.

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