

Article

The Effect of Different Extraction Conditions on the Physicochemical Properties of Novel High Methoxyl Pectin-like Polysaccharides from Green Bell Pepper (GBP)

Onome Obodo-Ovie, Mohammad Alyassin, Alan M. Smith  and Gordon A. Morris * 

School of Applied Sciences, University of Huddersfield, Huddersfield HD1 3DH, UK; onome.obodo-ovie@hud.ac.uk (O.O.-O.); m.alyassin2@hud.ac.uk (M.A.); a.m.smith@hud.ac.uk (A.M.S.)

* Correspondence: g.morris@hud.ac.uk; Tel.: +44-(0)-1484-256842

Abstract: Green peppers are massively produced all over the world; however, substantial quantities of peppers are wasted. Functional polysaccharides can be produced from pepper waste. A conventional acid extraction method was used to obtain pectin-like materials from green bell pepper (GBP). A 2³ experimental design (two-level factorials with three factors: temperature, pH, and time) was used to study the relationship between the extraction conditions and the measured physicochemical properties. The extracted polysaccharides were further analysed regarding their physicochemical and functional properties. The yields were in the range of (11.6–20.7%) and the highest yield value was extracted at pH 1. The polysaccharides were classified as “pectin-like”, as the galacturonic acid content was lower than 65%. Glucose and galactose were the major neutral sugars, and their relative amounts were dependent on the extraction conditions. The degree of esterification (DE) of the pectin-like extracts was greater than 50% and they were therefore classified as high methoxyl regardless of the extraction conditions. Also, important levels of phenolic materials (32.3–52.9 mg GAE/g) and proteins (1.5–5.4%) were present in the extract and their amounts varied depending on the extraction conditions. The green bell pepper polysaccharides demonstrated antioxidant and emulsifying activities and could also be used adequately to stabilise oil/water emulsion systems. This finding shows that green bell pepper could be used as an alternative source of antioxidants and an emulsifier/stabilising agent, and furthermore, the extraction conditions could be fine-tuned to produce polysaccharides with the desired quality depending on their application.

Keywords: pectin-like polysaccharide extraction; bell pepper polysaccharide; functional properties; polysaccharide characterisation; bioactive compound



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1. Introduction

Pectins and pectin-like polysaccharides are a very large family of complex heteropolysaccharides, which are usually found in large amounts in plants (primary cell walls, grass, middle lamella, and fibre cells in wood tissues). They play an important role in cell growth and differentiation, also contributing to the rigidity of plant tissues [1]. The primary cell wall consists of large amounts of pectin and very small amounts of cellulose, while the secondary cell wall usually contains very little or no pectin in some plants [2]. Pectic polysaccharides are made of several structural elements; the important ones are homogalacturonan (HG) and type I rhamnogalacturonan (RG-I) regions, often described in simplified terms as the “smooth” and “hairy” regions, respectively. The HG region is composed of (1→4) linked α-D-GalpA residues that can be partially methylated at C-6 [3] and possibly partially acetyl-esterified at O-2 and/or O-3 [4]. The degree of methylation (DM) and the degree of acetylation (Dac) are defined as the number of moles of methanol or acetic acid per 100 moles of GalA. The degree of methylation (DM) is used to classify the structure of pectin. Pectins with values of DM (>50%) are referred to as high methoxyl pectin (HMP) while pectins with values (<50%) are considered as low methoxyl pectin (LMP) [5,6]. Pectins

extracted from novel sources tend to have high DM values, although this will depend on the extraction conditions [6], plant species [7], season [8], geographical origin [9], genetic variety [10], and ripening stage [11].

Generally, pectin and pectin-like polysaccharides are complex in nature; to gather information and understand these complex polysaccharide structures and functionality, it is necessary to characterise these polysaccharides. This process is usually carried out in different stages. The first step involves the hydrolysis of the polysaccharide under different extraction conditions from the plant tissue. Historically, pectin has been extracted from plant sources (e.g., apple pomace and citrus peel) using conventional mineral acid (nitric, sulphuric, hydrochloric, or acetic) extraction under controlled conditions (temperature, time, and pH) [12,13]. In pectins with significant amounts of RG-I regions, harsher conditions may result in the loss of arabinose, in particular. After extraction, the viscous solution is centrifuged, filtered, and followed by precipitation with alcohol (methanol or ethanol), and finally the precipitated wet polysaccharide is then washed with acetone and dried in an oven [12]. Generally, to obtain pectin with a high yield and quality, the temperature and pH are adjusted to be in the range of (60–90 °C) and (1–3), respectively; however, as pectin can be sensitive to temperature, uncontrolled temperature can lead to the production of pectin with variable quality and yield [12]. Pectin extracted using conventional methods (hot acid extraction) is usually rich in galacturonic acid residues. Moreover, monitoring the extraction time, pH, type of acid, organic solvent, raw materials/water ratio, and temperature leads to increases in pectin yield with high quality [14].

Pectin and pectin-like polysaccharides have been isolated from different sources, such as beet, citrus, apple, and okra, and it has been found that they have surface activity and have the potential to stabilise oil-in-water emulsions [15,16]. Pectin-like polysaccharides have a potential to be used in the food industry as functional components, such as thickeners, viscosity enhancers, gel forming agents, stabilisers, texture modifiers, and emulsifiers [15–19].

Peppers, including sweet pepper (bell pepper), hot pepper, wax pepper, cayenne pepper, and jalapeño pepper, belong to the night shade Solanaceae family [20]. Pepper (*Capsicum annuum*, L) is cultivated and consumed in cuisines around the world for its nutritional value and flavour [20]. Peppers have been reported to contain dietary fibre (that includes pectin) and phenolic compounds [21].

Pectin has also reportedly shown some health benefits and bioactivity [22–25]. Free radicals are produced in the human body through the oxidation of the different compounds found in the body. Excess amounts of these free radicals lead to diseases such as cancer, cardiovascular disease, and aging of living tissues [26,27]. Synthetic or natural sources, such as plants, vegetables, and fruits with antioxidant capacity, can be used to reduce the excess free radicals that are produced in the human body, thereby protecting the human body from diseases. The antioxidant properties of these fruits and their extracted pectins are associated with the amount of extracted phenolic compounds and therefore will be related to the pectin structure [26,28], as phenolic compounds tend to be associated with arabinose groups, and hence the extraction conditions [29–32].

As far back as 1927, pectin has been used as an emulsifying agent by several food industries, and more recently, as an emulsion stabilizing agent to prolong the life span of food and pharmaceutical products [33]. The structure of pectin usually affects their physiochemical properties, which are linked to their source, genetic variation, growing conditions, and extraction procedures [7,10,34]. This, therefore, is one of the main reasons why novel pectin sources and optimisable extraction conditions are of industrial interest. Thereby, different polysaccharides that serve different applications needs could be produced by fine-tuning the extraction conditions to produce pectin (or pectin-like) polysaccharides with the desired characteristics.

Therefore, the aim of this study is to use an experimental design to produce and characterise pectin-like polysaccharides from green bell pepper (GBP) using different

conditions of the conventional acid extraction method to understand the relationship between their physicochemical and functional properties.

2. Materials and Methods

2.1. Raw Materials and Chemicals

Green bell peppers were purchased (September 2020) from a local supermarket (Sainsbury's, Huddersfield, UK); they were then chopped into smaller pieces (approximately 1 cm³), weighed, and dried in an oven at 45 °C for 48 h prior to the polysaccharide extraction. Ethanol (96% *w/w*), hydrochloric acid 37%, acetone ≥99.9%, and concentrated sulphuric acid were used as supplied. 1-diphenyl-2-picrylhydrazyl (DPPH), sulfamic acid 99.3%, sodium azide ≥99.5%, Bradford reagent, bovine serum albumin (BSA) 99%, sodium hydroxide, sodium tetraborate (borax) 99.0%, phenol, trifluoroacetic acid, potassium sulfamate ≥99.0%, *m*-hydroxydiphenyl, standards for glucose, galactose, arabinose, mannose, xylose, fucose, galacturonic acid, glucuronic acid, galactosamine, glucosamine, and rhamnose were purchased from Sigma-Aldrich (Gillingham, UK).

2.2. Experimental Design

Thermal acid extraction was carried out on bell pepper using various parameters (pH, time, and temperature) adapted from previous extraction methods for melon and sugar beet pectin [25,32], which are typical of conventional acid extraction conditions used in pectin isolation [12]. Minitab version 19 was used to create a full factorial design (two-level factorials with three factors) (Table 1) to study the relationship between the extraction conditions (pH, time, and temperature) and the measured polysaccharide properties, which included yield, galacturonic acid content, protein content, DM, intrinsic viscosity, and emulsifying and antioxidant properties.

Table 1. Full factorial design for the extraction procedure using Minitab 19.

Sample	pH	Time (h)	Temperature (°C)
1	1	2	60
2	1	2	80
3	1	4	60
4	1	4	80
5	3	2	60
6	3	4	60
7	3	2	80
8	3	4	80

2.3. Polysaccharide Extraction Process

A dried sample of green bell pepper was soaked in ethanol for 1 h followed by acetone for 24 h to remove soluble phenolics and low-molecular-weight material to obtain an alcohol-insoluble residue. The alcohol-insoluble residue (AIR) was placed in an oven at 45 °C for 4 h to dry. The dried AIR was then crushed into a powder. A total of 5 g of AIR was suspended in 100 mL of hydrochloric acid (pH 1 or pH 3) under different extraction conditions and heated on a hot plate with magnetic stirring. After extraction, the viscous solution was filtered using a muslin cloth. The filtrate was centrifuged at 4200 RPM for 10 min. The supernatant was then neutralised to pH 6.5 with 2 M NaOH, and the resultant solution ~30 mL was concentrated to approximately 2 mL using a rotary evaporator (BUCHI labortechnik CH-9230 R-11 Rotavapor, Flawil, Switzerland) at 50 °C. The polysaccharides in the solution were precipitated using absolute ethanol (4 volumes) and left for 1 h, and then filtered; the precipitate was washed with acetone and then filtered

again. The resultant precipitate (wet polysaccharide) was freeze-dried for 48 h (Christ Alpha 2–4 LSC basic). The extraction yield was calculated using Equation (1).

$$\text{Extraction yield of polysaccharide (g/100 g)} = \frac{\text{Dried polysaccharide}}{\text{dried powder weight}} \times 100\% \quad (1)$$

2.4. Determination of Neutral Sugar and Galacturonic Contents

High-performance anion exchange chromatography coupled with a pulsed amperometry detector (HPAEC-PAD) was used to analyse the monosaccharide constituents of GBP polysaccharides. Three milligrams (3.0 mg) of the polysaccharide extract was added to a pressure tube containing trifluoroacetic acid (TFA, 2 mL of 2 M). The pressure tube was sealed and heated at 100 °C for 4 h; the sample was left to cool at room temperature and then dried under a stream of nitrogen at 60 °C to remove the TFA using Zymark TurboVap (Hopkinton, MA, USA). Deionised water (3 mL) was added to the dried sample, which was mixed thoroughly and then filtered using a syringe filter (0.45 µm) prior the HPAEC-PAD analysis. The instrument used was the Dionex ICS-3000 HPAEC-PAD system (Dionex Corporation, Sunnyvale, CA, USA) that consisted of an Auto Sampler (AS-AP), ICS-3000-DP (Dual Pump), and an ICS-3000 DC (Detector/Chromatography) compartment with an electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. A Dionex CarboPac™ PA-20 analytical column (150 mm × 3 mm I.D.) and a Dionex CarboPac™ PA-20 guard column (50 mm × 3 mm I.D.) (Dionex Corporation, Sunnyvale, CA, USA) were used. The used mobile phase was NaOH (10 mM) prepared using Milli-Q (18.2 MΩ cm), and a NaOH (200 mM) solution was used for the column regeneration. The flow rate used was 0.3 mL/min with an injection volume of 20 µL. The sulfamic colorimetric assay (sulfamic *m*-hydroxyphenyl) [35] was used to determine the galacturonic content of the polysaccharide extracts [33]. In brief, polysaccharide sample solutions (1 mg/mL) were prepared along with several standards using galacturonic acid (20, 40, 60, 80, and 100 ppm). A total of 500 µL of samples and standard solutions were transferred into a 15 mL glass tube with a screw cap and 40 µL of a potassium sulfamate solution (4 M and pH 1.6) was added and mixed. Concentrated sulphuric acid containing sodium tetraborate (2.4 mL of 75 mM) was added and mixed vigorously with a vortex mixer. The solution was transferred into a water bath at 100 °C for 20 min. Prior to heating, the glass tube was secured with the screw cap to prevent the contamination of the samples from condensation reaction. Thereafter, the heated mixture was cooled in an ice bath for about 10 min. A total of 80 µL of 0.15% *m*-hydroxyphenyl in 0.5% *w/v* sodium hydroxide was added to the cooled solution and mixed using a vortex mixer. The solution was then allowed to stand for a few minutes until a pink colour developed. The absorbance of the pink solution was then measured at 525 nm using a UV-Vis spectrophotometer (Agilent Technologies, Cheshire, UK Cary 60 UV-Vis). The absorbance values of the galacturonic acid standards were used to plot a calibration curve, which was used to determine the galacturonic content of the polysaccharide sample.

2.5. Degree of Methyl Esterification (DM) of GBP Polysaccharides

The titrimetric method reported by Sayah et al. (2016) [36] was used, with few modifications, to estimate the degree of methyl esterification of the galacturonic acid unit present in the sample. A total of 5 mg of the sample was soaked in 3 mL of ethanol and then dissolved in deionised water (25 mL) in a small beaker, and the solution was stirred until the samples were completely dissolved, which was followed by adding a few drops of phenolphthalein. Two drops of sodium chloride solution (0.1 M) were added to make the end point more visible [37]. The solution was titrated with 0.01 M sodium hydroxide to neutralise the free galacturonic acid and the volume of NaOH used was recorded as (V_1). A total of 25 mL of 0.25 M NaOH was added to the sample solution and the resulting pink solution was left for 30 min; then, 25 mL of hydrochloric acid was added to the solution and the pink colour of the solution disappeared. The solution was then back-titrated with

0.1 M NaOH. Once the pink colour reappeared, the titration was stopped, and the volume was recorded as V_2 . The degree of methyl esterification was estimated using Equation (2).

$$\text{DE \%} = \frac{V_2}{V_1 + V_2} \times 100 \quad (2)$$

where V_1 = volume of NaOH used in the first titration and V_2 = volume of NaOH used in the second titration.

2.6. Determination of the Protein and Phenolic Contents of GBP Polysaccharides

The Bradford assay was used to determine the total protein content of the polysaccharide sample using bovine serum albumin (BSA) as a standard [38]. The Folin–Ciocalteu method was used to determine the total phenolic content as gallic acid equivalents (GAE, mg/g) using gallic acid as a standard [39].

2.7. Degree of Acetylation of GBP Polysaccharides

The McComb and McCready method [40] was used to measure the acetyl content of the polysaccharide sample using D-glucose pentaacetate as a standard. Using the values of the galacturonic acid (GalA) content and the acetyl content, the degree of acetylation was estimated using Equation (3).

$$\text{DA \%} = \frac{176 \times \text{acetyl content} \left(\frac{\%w}{w} \right)}{43 \times \text{GalA content} \left(\frac{\%w}{w} \right)} \times 100 \quad (3)$$

where 176 and 43 g/mol are the molecular weights of galacturonic acid and the acetyl groups, respectively.

2.8. DPPH Radical Scavenging Activities of GBP Polysaccharides

The antioxidant activities of the GBP extracts were investigated using the DPPH radical scavenging method, as reported by Yanhong, L. et al. [41], with slight modifications. A total of 5 mg of polysaccharide samples was dissolved in 5 mL of deionised water. To this mixture, 2 mL of DPPH solution (0.1 mM) in ethanol (95%) was added and the solution was mixed thoroughly and left to stand for 30 min at room temperature. A blank was also prepared using 2 mL of DPPH solution. UV-Vis spectroscopy was used to measure the absorbance of the mixture at 517 nm and the scavenging activity was measured (Equation (4)).

$$\text{DPPH scavenging activities \%} = \left[\frac{(\text{blank absorbance} - \text{sample absorbance})}{\text{blank absorbance}} \right] \times 100 \quad (4)$$

2.9. Emulsifying Properties of GBP Polysaccharides

In summary, 5 mL of polysaccharide sample solutions (5 mg/ mL) was prepared in deionised water. To this mixture, 0.2 mg of sodium azide was added, as a preservative, and mixed thoroughly. The solution was transferred to a 50 mL centrifuge tube followed by the addition of 5 mL of sunflower oil [42]. The mixture was treated with a high-speed homogeniser (IKA T 18 basic, Ultra-Turax, Staufen, Germany) for approximately 3 min and an ultrasound processor (Model UP 100H, Hielscher Ultrasonics, Teltow, Germany) for 45 s. The emulsifying activity (EA) was calculated using Equation (5) [43]:

$$\text{EA \%} = \frac{VE}{VT} \times 100 \quad (5)$$

where VE represents the volume of the emulsion level and VT represents the total volume of the complex mixture.

The same procedure for the preparation of the emulsion was used to determine the emulsion stability. The emulsion produced was stored at different temperatures (room

temperature $\sim 20\text{ }^{\circ}\text{C}$ and $4\text{ }^{\circ}\text{C}$) for several days (1, 5, 10, 20, and 30 days). The emulsion stability (ES) was calculated using Equation (6) [43]:

$$\text{ES \%} = \frac{VR}{VI} \times 100 \quad (6)$$

where VI = initial emulsion volume and VR = remaining emulsion volume.

2.10. Measurement of Intrinsic Viscosity and Molecular Weight of GBP Polysaccharides

Viscosity measurements provide information about the molecular weight and conformation (shape). An Ostwald Ubbelohde glass capillary viscometer (Sigma-Aldrich, Gillingham, UK) was used to measure the intrinsic viscosity of the GBP polysaccharide solution. A total of 25 mg of sample was dissolved in 25 mL of 0.1 M sodium chloride [36,44,45]. The solution was transferred into a 50 mL centrifuge tube and then centrifuged for 10 min, followed by filtration using a $0.45\text{ }\mu\text{m}$ filter membrane. A total of 15 mL of the sample solution was pipetted into an Ostwald capillary viscometer and then placed in a water bath at $25 \pm 0.1\text{ }^{\circ}\text{C}$. The time taken for the solution to run from the upper level of the viscometer to the lower level was measured (flow time). These values were used to calculate the intrinsic viscosity of the polysaccharide. The experiment was performed in triplicate. The intrinsic viscosity can be estimated at one concentration by applying the Solomon and Ciută approximation (Equation (9)) [46–50]. The intrinsic viscosity of the polymer can be approximated using this method, as shown by Morris (2001) and Kravtchenko and Pilnik (1990), with an error of $\sim 1\%$ when using a single low concentration ($<5\text{ mg/mL}$) measurement [49,51]. This modified method and the commonly used Huggins and Kraemer plots are expected to agree and produce the same values (see, e.g., [49–51]), which is beneficial when samples are not available in large quantities. The intrinsic viscosity (Equation (9)) and the Mark–Houwink equation (Equation (10)) were used to estimate the average molecular weight of the polysaccharides [52]. As the chemical compositions of the extracted polysaccharides were similar in terms of composition and relatively low in galacturonic acid, it was decided that the same Mark–Houwink equation could be used for each sample.

$$\eta_{\text{rel}} = t/t_0 \quad (7)$$

$$\eta_{\text{sp}} = \eta_{\text{rel}} - 1 \quad (8)$$

$$[\eta] \approx \frac{\sqrt{2\eta_{\text{sp}} - 2(\ln \eta_{\text{rel}})}}{c} \quad (9)$$

The intrinsic viscosity is related to the molecular weight of the polymer through the Mark–Houwink equation (Equation (10)) [52]:

$$[\eta] = k' M_w^{\alpha} \quad (10)$$

where t is the flow time of a sample, t_0 is the flow time of the solvent, and k' and α are Mark–Houwink constants that depend on the temperature and the solvent and can therefore be used to estimate the average molecular weight from intrinsic viscosity measurements. The “ α ” exponent provides an indication that the solution conformation has been estimated to be in the range of 0.61–1.43 [36,50,53–56] for pectin, which is typical of a semi-flexible coil/rigid rod [57], where higher values of the exponent indicate more rigid polymers. The constants α and k' are usually found in the literature; however, they can be calculated by an experimental method by calculating the intrinsic viscosity of several polymers with widely different molecular weights. Then, $\log [\eta]$ and $\log M_w$ are used to plot a calibration curve, where the slope of the graph and intercept is represented by α and $\log k'$, respectively [52]. The k' and α values depend on the sample (solute), temperature, and solvent properties. The values of the Mark–Houwink constants k' and α were taken to be 9.55×10^{-2} and 0.73, respectively [53], as due to the low GalA content ($<50\%$), it was assumed that the polysaccharides would likely have a semi-flexible coil conformation ($\alpha = 0.6\text{--}0.8$) [47,57].

2.11. Statistical Analysis

The experimental data were analysed using Minitab 19 to study the relationship between the extraction conditions (pH, time, and temperature) and the measured polysaccharide properties, (yield, galacturonic acid content and protein content, and DM). The main effect plots were applied to visualise and show a graphical representation of the interaction between the extraction variables and responses. A one-way analysis of variance (ANOVA) showing grouping information using Tukey's method and a 95% confidence level was used to show significant difference between groups.

3. Results and Discussion

3.1. Yield and Physicochemical Properties of GBP Polysaccharides

The yield and chemical constituents of the polysaccharides extracted from green bell pepper are represented in Table 2. The highest polysaccharide yield of GBP (20.7%) was extracted at pH 1, while the lowest yield was extracted at pH 3 (Figure 1). This agrees with multiple reports in the literature that found that pectin polysaccharides from different plant sources with high yield are generally extracted under low pH (high acidic strength) [25,32,43,58–61]. The extraction yield of GBP was higher than the reported value for Japanese pepper fruit (8.4%) [62], sugar beet pulp (16.2%) [60], honeydew melon (7.9%) [25], green sweet pepper (8%) [20], and orange peel pectin (18.52%) [63], and therefore, is potentially a good source of polysaccharides, as large quantities are available as biowaste [64].

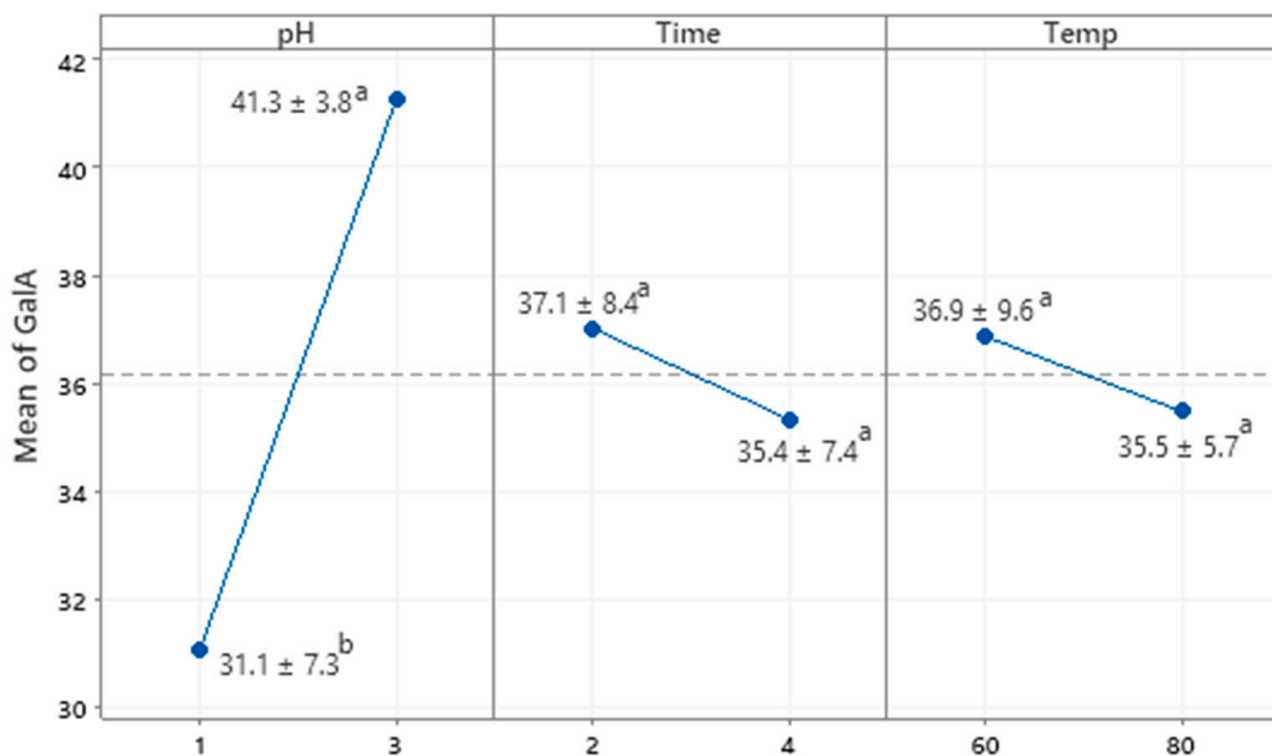


Figure 1. Main effect plots of pH, time (hours), and temperature (°C) for the galacturonic acid (GalA) content (%). Data in each panel show the mean $\pm$ one standard deviation, and different letters indicate a significant difference at 95% confidence limits. In this case, for example, there is a significant difference with the pH, but not with time and temperature.

Table 2. Properties of the pectin-like polysaccharides extracted from GBP using acid extraction.

Extract	Yield (%)	Phenolic Content (mg GAE/g)	Protein (%)	DA (%)	DM (%)	Glc	Gal	Ara	Rha	GalA	[η] (mL/g)	M _w (kg/mol)
1	15.4 ± 3.1 ^a	40.2 ± 13.4 ^a	2.9 ± 1.0 ^{ab}	22.1 ± 2.3 ^a	88.5 ± 0.2 ^a	11.2 ± 1.0 ^{abc}	7.7 ± 0.9 ^{ab}	1.3 ± 0.4 ^a	1.2 ± 0.2 ^a	32.4 ± 2.0 ^{bc}	20.2 ± 1.9 ^{cd}	1533 ± 60 ^{cd}
2	18.5 ± 7.8 ^a	52.9 ± 7.7 ^a	2.3 ± 0.6 ^{ab}	24.3 ± 1.8 ^a	87.7 ± 5.0 ^a	8.6 ± 1.3 ^{bc}	7.9 ± 1.0 ^{ab}	1.1 ± 0.5 ^a	0.8 ± 0.1 ^a	30.7 ± 1.6 ^{bc}	36.4 ± 8.3 ^{ab}	3434 ± 453 ^{ab}
3	20.7 ± 1.8 ^a	48.6 ± 5.2 ^a	1.5 ± 0.1 ^b	23.3 ± 2.8 ^a	89.7 ± 1.1 ^a	9.1 ± 0.4 ^{bc}	4.4 ± 0.1 ^b	0.8 ± 0.1 ^b	0.3 ± 0.1 ^a	26.4 ± 2.2 ^c	30.8 ± 9.2 ^{abc}	2731 ± 522 ^{abc}
4	20.0 ± 1.7 ^a	47.8 ± 1.3 ^a	5.4 ± 1.1 ^a	19.5 ± 2.2 ^a	89.6 ± 1.8 ^a	7.0 ± 1.3 ^{bc}	7.6 ± 1.8 ^{ab}	1.2 ± 0.4 ^a	1.2 ± 0.4 ^a	34.3 ± 2.7 ^{abc}	41.4 ± 5.6 ^a	4096 ± 264 ^a
5	19.8 ± 2.8 ^a	48.1 ± 2.8 ^a	2.6 ± 0.4 ^{ab}	15.6 ± 3.0 ^a	88.9 ± 0.1 ^a	9.4 ± 2.4 ^{bc}	7.0 ± 0.9 ^{ab}	1.4 ± 0.5 ^a	n. d.	45.3 ± 8.0 ^a	19.0 ± 3.3 ^{cd}	1409 ± 128 ^{cd}
6	19.2 ± 1.7 ^a	48.3 ± 4.5 ^a	4.8 ± 1.6 ^{ab}	14.7 ± 1.7 ^a	90.5 ± 0.3 ^a	16.6 ± 3.9 ^a	9.7 ± 2.5 ^a	1.2 ± 0.8 ^a	n. d.	42.8 ± 4.5 ^{ab}	12.7 ± 2.0 ^d	812 ± 65 ^d
7	16.6 ± 4.8 ^a	52.0 ± 13.0 ^a	3.2 ± 1.1 ^{ab}	16.9 ± 2.8 ^a	89.7 ± 1.1 ^a	7.4 ± 2.1 ^c	5.8 ± 1.8 ^{ab}	1.5 ± 0.8 ^a	0.6 ± 0.1 ^a	43.0 ± 5.2 ^{ab}	20.2 ± 1.9 ^{cd}	1533 ± 60 ^{cd}
8	11.6 ± 0.6 ^a	32.3 ± 13.9 ^a	5.3 ± 0.8 ^a	16.6 ± 3.9 ^a	87.6 ± 1.8 ^a	11.3 ± 0.9 ^{abc}	5.1 ± 0.4 ^b	0.8 ± 0.6 ^b	2.1 ± 0.5 ^a	36.6 ± 5.4 ^{abc}	19.0 ± 0.2 ^{cd}	1409 ± 3 ^{cd}

All extractions were performed in triplicate and are presented as mean values ± 1 SD. GAE: gallic acid equivalent; DA: degree of acetylation; DM: degree of methyl esterification; Glc: glucose; Gal: galactose; Ara: arabinose; Rha: rhamnose; GalA: galacturonic acid; [η]: intrinsic viscosity. Means that do not share a superscript letter (a, b, c, or d) within a column are significantly different ($p < 0.05$), whilst means that share a letter are not significantly different. n. d. = not determined/detected. Mannose is not shown as, in all cases, the values were approximately 1%, and there were no significant differences between the samples.

Neutral sugar constituents were determined using HPAEC-PAD, while the GalA contents were analysed using the colorimetric method. The neutral sugar and uronic analyses of bell pepper polysaccharides revealed the presence of five different neutral monosaccharides (Glc, Ara, Rha, Gal, and Man) together with GalA (Table 2), which was the main constituent. Xylose and fucose were present, but they could not be quantified as they were below the limits of detection. However, the presence of GalA, Ara, Rha, and Gal suggested that the extracted polysaccharides consist of pectin-like materials. The arabinose content increased as the pH increased (decreased acidic strength). The arabinose content extracted at pH 3 was higher than that at pH 1, suggesting that, in a high acidic medium, the pectin side chain may be degraded, leading to a reduction in arabinose. Similar findings were reported for polysaccharides from sugar beet pulp [60] and apple pomace [65]. Unexpectedly, large amounts of glucose (16.6%) were observed in the extracts. This may be attributed to hemicellulose hydrolysis that was attached to pectin polysaccharides during the extraction process, consequently increasing the glucose content [65,66]. There is also the possibility that cellulose and/or starch were co-extracted [25]. Similarly, high amounts of glucose (14.64%) were reported for pectin extracted from another member of the Solanaceae family, tomato [67], and in citrus peels (25–49.2%) [68], although these samples were extracted and purified under different conditions.

The galacturonic acid content of GBP was in the range of 26.4–45.3% (Table 2), which was very low compared to the galacturonic acid (66.9%) extracted from Japanese pepper [62] and sugar beet pulp extracts (35.2–76.3%) [60]. With regard to the application of pectin in the pharmaceutical and food industries, the European Union and Food and Agricultural Organisation recommend that, in order to be classified as pectin, the GalA content needs to be >65% [69]. The GalA content in this work was below the 65% benchmark. Therefore, the polysaccharides extracted from the extract should be classified as pectin-like materials. From the main effect plot for galacturonic acid (Figure 1), it is clear that pH was the most important parameter that influenced the GalA content, since, as the pH increased (decreasing acidic strength), the content of GalA increased. In other words, pectin-like polysaccharides extracted at pH 3 are richer in galacturonic acid and consist of more pure pectin compared to pectin-like polysaccharides extracted at pH 1.

3.1.1. Neutral Sugar Ratios

The calculation of the different ratios of sugar constituents in GBP was used to reveal the extracted polysaccharide fingerprint, provide information, and determine the structure of pectin. Different sugar ratios were used to define the properties of the pectin structure. The sugar ratio of pectin backbone GalA to the different monosaccharide constituents (Rha, Ara, and Gal) (Table 3) was used to establish the linearity of the pectin structure, with high values indicating a more linear structure. In Table 3, the linearity of the extracted pectin varies with the extraction pH, and samples 5, 7, and 8 extracted under high pH conditions (low acidic medium) appear to be more linear compared to those extracted at a low pH (high acidic medium). The values in the present work (3.2–5.4) are in range with the values published for water-soluble pectin fractions from broccoli stem (3.1), carrot root (3.6), and tomato fruit (4.8) [67]. The second ratio of the pectic monosaccharide composition (Rha, Ara, Gal, and GalA) to Glc was used to indicate the pectin quality (Table 3). A large value indicates a “more pure” pectin. Samples 5, 7, and 8 extracted under a high pH (low acidic medium) have larger values compared to those extracted at a low pH (high acidic strength), and therefore, better quality of pectin. This also suggests that more pectin and/or less contaminants were extracted at a high pH (low acidic strength).

Table 3. Linearity and quality of pectin extracted from GBP polysaccharides.

Sample	Pectin Linearity ^a $\frac{\text{GalA}}{\text{Ara+Gal+Rha}}$	Pectin Quality ^b $\frac{\text{Rha+Ara+Gal+GalA}}{\text{Glc+Man}}$	$\frac{\text{Rha}}{\text{GalA}}$ ^c	$\frac{\text{Ara+Gal}}{\text{Rha}}$ ^d	%HG (GalA – Rha)	%RG-I (2Rha + Ara + Gal)	HG: RG-I
1	3.2	3.6	0.03	7.5	31.2	11.4	2.7
2	3.1	4.4	0.03	11.3	29.9	10.6	2.8
3	4.8	3.3	0.01	17.3	26.1	5.8	4.5
4	3.4	6.0	0.03	7.3	33.1	11.2	3.0
5	5.4	5.1	-	-	45.3	8.4	5.4
6	3.9	3.0	-	-	42.8	10.9	3.9
7	5.4	6.3	0.01	12.2	42.4	8.5	5.0
8	4.6	3.5	0.06	2.8	34.5	10.1	3.4

No rhamnose was detected in samples 5 and 6. ^a A larger value is indicative of more linear/less branched pectin.

^b A larger value is indicative of a “more pure” pectin extract. ^c A smaller value is indicative of more linear/less branched pectin. ^d A larger value is indicative of a larger average size of the branching side chains. Ratios were estimated and adapted from [25,67].

The third ratio $\frac{\text{Rha}}{\text{GalA}}$ was used to estimate the contribution of the RG-I region to the entire pectin structure. In Table 3, these values are very small and show little variation with the extraction conditions, apart from sample 8. These small values indicate that the pectin-like polysaccharide extracted from GBP has a linear structure, and consequently, less branched pectin. The values (0.01–0.06) in the present work are similar to the values (0.02–0.09) reported for broccoli, carrot, and tomato fruit [43]. The fourth ratio $\frac{\text{Ara+Gal}}{\text{Rha}}$ was used to establish the size and length of the RG-I branched region by comparing the neutral sugar side chain (Ara and Gal) with the rhamnose content of the entire pectin structure. High values indicate that the pectin side chain has longer branches. In the results below (Table 3), the pectin extracts 2, 3, and 7 show a high degree of branching, with values in the range of (2.8–17.3). Furthermore, similar results with heavy side-chain branching were also reported for broccoli and carrot [67] and honeydew melon [25].

The amounts of neutral sugars and GalA can further be used to calculate the percentage of the key pectin regions HG and RG-I (Table 3). We can see that all pectin-like polysaccharides extracted are richer in HG regions than those extracted from honeydew melon [25], although it is expected that this is, in part, due to the low amounts of rhamnose in all samples and particularly in samples 5 and 6, in which rhamnose was not detected. The quantity of HG regions is larger at a higher pH (pH 3), which agrees with the pectin linearity ratio. However, the quantity of RG-I is essentially the same at both pH 1 and pH 3.

3.1.2. Total Protein Content of GBP Polysaccharides

According to the Food and Agricultural Organisation (FAO), the protein content allowable to be classified as pectin should not be >15.6% [43]. In this study, the protein content varied in the range of 1.5–5.4% depending on the extraction conditions (Table 2). The highest amount of protein was extracted at pH 3, since, as the pH (low acidic medium), temperature, and time increased, the protein content increased. Similar findings were published for sugar beet pectin [32]. Pectin and protein have been co-extracted together from various plant sources by different researchers, for example, pectin extracted from sweet pepper using a simulated gastric medium was found to contain 5.8% protein [70], which is similar to the value (5.4%) reported in the present study. Pectin from another member of the Solanaceae family, eggplant, was found to contain 9.13% protein [39]. Proteins linked to pectin structures carry out important functions. During emulsion formation, this protein helps to bind and adsorb at the newly dispersed liquid (emulsion) layer, leading to a more stable emulsion [42].

3.1.3. Total Phenolic Content of GBP Polysaccharides

The total phenolic content measured in this work is presented in (Table 2). Slightly higher amounts of phenolics (32.3–52.9 mg/g) were co-extracted with pectin-like polysaccharides compared to 32.5 mg/g phenolic content extracted from red pepper [16], and sour orange peel (35.95 mg/g) [71]. The extraction conditions (pH, time, and temperature) had a minor effect on the phenolic content. As the pH increased (low acidic strength), the phenolic content increased slightly. The findings in the present study are in good agreement with the findings reported for sugar beet pulp pectin [70]. Also, as the extraction time increased, the phenolic content increased. Levigne et al. (2002) reported phenolic content values in the range of 2.6–16.7 mg/g and proposed that the arabinose content of sugar beet pectin was related to the phenolic content [32]. However, in the present work, there were no observed links and relationship with arabinose and phenolic content, possibly as the arabinose content was quite low.

3.1.4. Degree of Acetylation (DA) of GBP Polysaccharides

The degree of acetylation of GBP polysaccharides was in the range of 14.7–24.3% (Table 2), which was similar to the data reported for sugar beet pulp pectin (3.1–29.2% [70] and 16–35% [32]) and much higher than the data published for citrus (1.4–1.6%) [72] and apple (5%). The extraction pH had a more significant effect on the DA than the time and temperature. The highest value of DA was obtained at pH 1. This implies that the DA content increased as the pH decreased (high acidic strength). However, the opposite was reported, with DA content increasing as the pH increased (low acidic strength) for sugar beet pulp pectin [60]. Increasing the temperature appeared to have an insignificant effect on DA. In the table below (Table 4), the DA of samples 1, 2, 3, and 4 were found to be high (19.5–24.3%). This implies that, during the extraction process, a large amount of acetic acid was released into the environment.

Table 4. Values of emulsifying activities and emulsion stability under different extraction parameters.

Sample	EA % at 22 °C	ES at 22 °C after 1 Day (%)	ES at 22 °C after 30 Days (%)	ES at 4 °C after 1 Day (%)	ES at 4 °C after 30 Days (%)
1	52.1 ± 0.1	96.2 ± 5.1	93.1 ± 1.3	98.0 ± 0.0	98.0 ± 0.0
2	52.1 ± 0.8	96.1 ± 3.1	93.1 ± 1.3	97.1 ± 1.3	97.0 ± 1.4
3	52.1 ± 0.7	97.5 ± 3.5	93.9 ± 0.2	97.0 ± 1.4	98.0 ± 0.0
4	51.6 ± 1.5	97.0 ± 4.2	94.0 ± 0.0	98.0 ± 0.1	98.0 ± 0.0
5	52.3 ± 0.4	95.0 ± 4.2	94.1 ± 0.1	97.0 ± 0.0	97.0 ± 1.4
6	54.1 ± 2.1	100 ± 0.0	94.0 ± 0.0	98.0 ± 0.0	98.0 ± 0.0
7	52.6 ± 0.0	100 ± 0.0	94.0 ± 0.0	97.0 ± 1.3	98.0 ± 0.0
8	52.6 ± 0.0	100 ± 0.0	94.0 ± 0.0	98.1 ± 0.1	98.0 ± 0.0

All extractions were performed in triplicate and are presented as mean values ± 1 SD. There are no significant differences between the mean values at a 95% confidence limit. EA: emulsifying activity and ES: emulsion stability.

3.1.5. Degree of Methyl Esterification (DM) of GBP Polysaccharides

In Table 2, the degree of esterification of pectin-like polysaccharides from green bell pepper using the titrimetric method is in the range of 87.6–90.5%. Therefore, the pectin-like polysaccharides extracted from GBP can be classified as high methoxy (HM). The DM values in this study are close to the results (over 80%) reported for Japanese pepper [62]. The temperature and time were the major parameters that affected the degree of methyl esterification, although only the time was at a significant level at 95% confidence limit. As the time increased from 2 to 4 h, the DM also increased. A harsher temperature decreased the DM, although not significantly ($p > 0.05$). A higher temperature helps to increase the de-methylation of the galacturonic acid unit and, consequently, decreases the

DM [73]. Similar results were reported for sugar beet [32], sugar beet pulp [60], and citrus peel [74]. The reverse trend, with a higher temperature increasing the degree of methyl esterification, was previously observed for grapefruit and citrus peel [36] and apple pomace and citrus peel [66]. It also appears that the pH has little or no effect on the degree of methyl esterification. In terms of pectin applications as a gelling agent, this is important, as pectin has different degrees of esterification gel via different mechanisms and, in the case of high methoxyl (HM) pectin from GBP, this means large amounts of sucrose would be required to promote gelation.

3.1.6. Intrinsic Viscosity of GBPP and the Molecular Weight of GBP Polysaccharides

The intrinsic viscosity of the pectin-like materials extracted from GBP were measured in 0.1 M of sodium chloride and calculated using (Equation (9)). The value of the intrinsic viscosity varies with the extraction conditions (12.7–41.4 mL/g) (Table 2). These values were lower when compared to the data published for Japanese pepper fruit (179 mL/g) under similar extraction conditions [62]. The molecular weight is in the range of 812–4096 kg/mol, although with the caveat that the conformation of the different polysaccharides is unknown. However, based on the compositional analysis, it is reasonable to assume that they will all be of similar conformation and that, as the galacturonic acid content is relatively low, they would be more likely to adopt a semi-flexible coil conformation [47,57]. The values obtained in this present study are larger than the molecular weights obtained for apple (473 kg/mol) [75], citrus peel (226.6 kg/mol) [36], and sugar beet pectins (70–355 kg/mol) [32]. From the main effects plot for the molecular weight (not shown), the time, pH, and temperature all affected the molecular weight. As the temperature increased, the molecular weight increased, and as the pH decreased (high acidic strength), the molecular weight also increased. It appears that harsh conditions (a high temperature and low pH) favour the extraction of high-molecular-weight polysaccharides from green bell pepper, while a low temperature and high pH favour the extraction of pectin with a low molecular weight. However, the opposite trend was reported for sugar beet pulp with harsher conditions (a high temperature and low pH), favouring the release of lower-molecular-weight pectin due to degradation [60].

3.2. Functional Properties of GBP Polysaccharides

3.2.1. Emulsifying Activity (EA) and Emulsion Stability (ES) of GBP Polysaccharides

The emulsifying activities (EA) and emulsion stability (ES) were calculated and are presented in Table 4. The emulsifying activities of the GBP extracts were in the range of (51.6–54.1%). These values are higher than the results published for sour orange peel (40.7%) [76], citrus peel (46.5%) [74], and sugar beet pulp (43.2 and 47.1%) [60]. The emulsion stabilities at 4 °C and 22 °C after 1 day were in the ranges of (97–98%) and (96–100%), respectively. However, after 30 days, the emulsion stabilities at 4 °C and 22 °C were 98% and 93.1%, respectively. From these observations, the emulsions are more stable at 4 °C than at 22 °C. Similar findings from previous studies were reported for citrus peel and sugar beet pulp [60,74]. It was also observed that the extraction pH, temperature, and time played a major role in the EA. As the pH, time, and temperature of extraction increased, the emulsifying activity of the pectin-like material increased. Protein and phenolic compounds co-extracted with many polysaccharides have been linked to emulsion activities [77–79]. In the present work, large amounts of phenolic compounds (32.3–52.9 mg/g) and proteins (1.5–5.4%) were reported, which could be responsible for the high emulsion activity. Moreover, acetyl groups have also been associated with emulsion stability [78]. Therefore, the combination of the acetyl content, phenolic compounds, and protein residues in GBP polysaccharides could be attributed to the high emulsion activity and stability of the GBP stabilised emulsions.

3.2.2. Antioxidant Properties of GBP Polysaccharides

A DPPH radical scavenging assay was used to estimate the antioxidant properties of GBP polysaccharides, and the results vary with the extraction conditions (Table 5). Upon being added to the sample, DPPH free radicals accept electrons (hydrogen) from the sample, thus reducing and forming a stable complex. Also, the purple colour of the solution changes to a yellow colour as the concentration of the free radicals of the DPPH decreases, making it a simple and inexpensive measurement of the antioxidant activities. From the results, the antioxidant activities of the pectin-like material extracted from GBP were in the range of 50.3–73.4%, indicating high antioxidant activity. However, the antioxidant results obtained in this study are lower than the result obtained for another member of the Solanaceae family, eggplant (94%) [43]. In the main effects plot for DPPH activities (Figure 2), the extraction pH and time were the major factors affecting the antioxidant activity. As the extraction pH decreased (high acidic strength), the antioxidant activities increased. Moreover, when the extraction time was reduced from 4 to 2 h, the antioxidant activities also increased. This could be due to the phenolic content (Table 5), as when the pH decreased, the phenolic content and antioxidant activity increased. The antioxidant properties of the GBP extracts could therefore be attributed to the degree of esterification, galacturonic content, and phenolic content [43,80].

Table 5. Values of the free radical scavenging (DPPH assay) effect under different extraction conditions.

Sample	Free Radical Scavenging Effect of DPPH (%)
1	72.8 ± 5.1
2	70.4 ± 4.3
3	62.8 ± 17.8
4	61.9 ± 8.1
5	67.0 ± 5.1
6	73.4 ± 3.9
7	50.3 ± 5.1
8	66.7 ± 2.8

All extractions were performed in triplicate and are presented as mean values ± 1 SD. There is no significant difference between the mean values at 95% confidence limit. DPPH: 1-diphenyl-2-picrylhydrazyl.

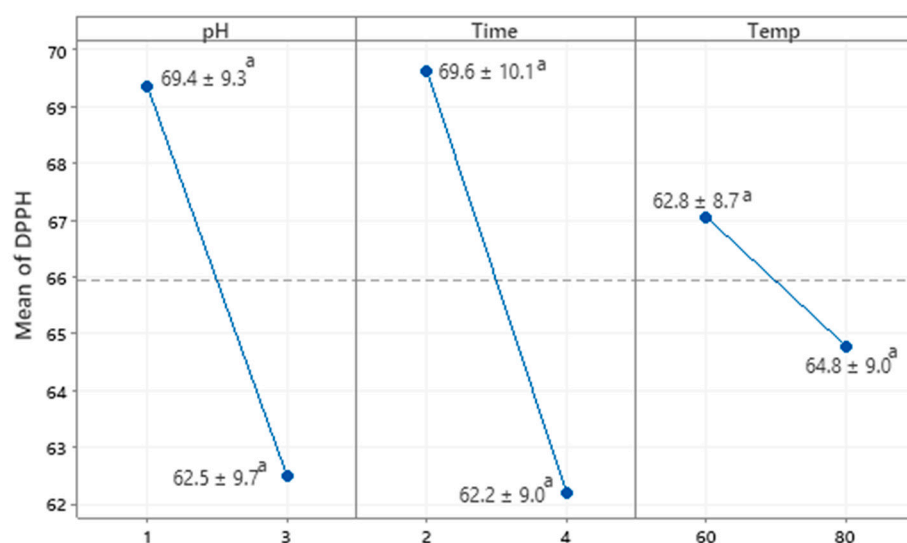


Figure 2. Main effects plot of the pH, time (hours), and temperature (°C) for the DPPH assay for the GBP extracts. Data in each panel show the mean ± one standard deviation, and the same letter indicates no significant difference at 95% confidence limits. DPPH: 1-diphenyl-2-picrylhydrazyl.

Although it might appear that, at first, there are only limited differences between the samples depending on their extraction conditions, which could be considered as a drawback in the application of GBP polysaccharides, it does also present the probability that polysaccharides extracted from GBP are unlikely to be greatly affected by unintended changes in the processing conditions during isolation, unlike pectins from citrus [12,13], honeydew melon [25,81], or sugar beet [32], for example.

4. Conclusions

Pectin-like materials and other polysaccharides were successfully extracted (pectin with a small amount of co-extracted starch and/or cellulose), purified, and characterised under various extraction conditions. The different extraction parameters (temperature, pH, and time) affected the extraction yield, phenolic content, protein content, degree of esterification, antioxidant properties, composition of pectin, degree of acetylation, emulsifying, and emulsion stability, making it possible to achieve pectin-like materials with the desirable quality and functionality. Therefore, this means that, in principle, each extraction yields a different pectin-like polysaccharide, and it is also expected that the “pectin quality”/amount of other co-extracted polysaccharides may have affected the measured properties. Based on the high degree of esterification values, the pectin-like polysaccharides extracted from GBP were classified as high methoxyl. Furthermore, the extracted polysaccharides showed good antioxidant properties and emulsifying activities and could also be used adequately to stabilise an oil/water emulsion system. However, the high amount of protein and phenolic compounds observed in the GBP extracts probably influenced the antioxidant and emulsion activities.

To corroborate the results observed in this study, a further investigation is needed to analyse the physicochemical and functional properties of GBP polysaccharides with regard to their extraction conditions. This can be achieved by choosing other conditions, for example, a wider range of temperatures or pH levels or it is also possible to study different cultivars [10], ripeness [11], or by comparison with peppers of different colours [9,10]. In addition, SEC-MALLS or sedimentation techniques would be very useful to measure the *absolute* molecular weight of the polysaccharides, as this will enable the estimation of conformational information and the number and length of side chains [81], as functional properties are related to polysaccharide structure/composition, physical properties (molecular weight), and conformation/shape in a solution [82].

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