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HPAEC–PAD analysis of oligogalacturonic acids in strawberry juice

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Abstract

High performance anion-exchange chromatography (HPAEC), coupled with pulsed amperometric detection (PAD), was used to identify and quantify oligogalacturonic acids (OGAs) in strawberry juice. This method allowed good separation and detection of OGAs with degree of polymerization (DP) < 20 units, and has proved to be a useful tool for the study of the chemical composition of pectin breakdown products. However, the lack of availability of OGA standards above 3 DP units limited the quantification for these compounds. Treatment of strawberry juice with seven commercial pectolytic enzymes was investigated. Glucuronic acid occurred in strawberry only in small amounts (12 ± 0.5 mg/l) and its concentration was not affected by the enzymatic treatment. Polygalacturonic acid can be hydrolyzed in several ways, yielding different sets of oligomeric degradation products depending on the hydrolysis conditions. The use of Rohament® MAX enzyme, for 2 h at 45°C, produced juice with by far the highest concentration of OGAs. Moreover, treatment with Grindamyl Pectinase® LM was also extended to 4 and 6 h to measure the time course of pectinase activity and verify the stability of OGA. This enzyme generated high levels of pectin breakdown products proportionately with time (monitored up to 6 h) and increased the total level of OGAs with DP $\geqslant 4$ units from 78 to 771 mg/l.

Keywords: Pectin; Oligogalacturonic acids, OGAs; Pectolytic enzymes; HPAEC–PAD; Fruit juice

1. Introduction

Pectic substances, polysaccharides of high molecular weight located in the cell walls, are involved in the changes of firmness in fruits and vegetables during ripening (Hwang, Pyun, & Kokini, 1993). The processing of fruits and vegetables modifies the pectin composition of food and derived beverages. In particular, the pectic enzymes, which degrade the pectic substances, play an important role in the food industry mainly because of their ability to increase the extraction yield in the preparation of fruit juices (Whitaker, 1984). In fact, during juice manufacturing, high amounts of pectin are released into the juice and lead to problems during filtration and clarification (Voragen & Pilnik, 1989). Moreover, the stabilization of cloudy orange juice has been attributed to the depolymerization of pectic substances into soluble low molecular weight pectates (Baker & Bruemmer, 1972). The production of oligogalacturonic acids (OGAs) during the pectolytic activity is also of great interest from a regulatory point of view. In fact, a high level of OGAs is indicative of addition of peel and pulpwash in enzymatically treated orange juice (Farnell, 1995). The addition of these products to pure orange juice is not allowed in the UE.

There is an increasing interest in the metabolic benefit of fiber such as pectin for human health. Evidence suggests that dietary pectin may reduce the levels of serum total cholesterol and decrease low density lipoprotein cholesterol (Behall & Reiser, 1986). Strawberry is a soft fruit containing high levels of pectin in which the monomer units consist mainly of D-galacturonic acid linked with different neutral sugars (Legentil, Guichard, Piffaut, & Haluk, 1995). However, information on OGA composition of soft fruit and juice is still limited. Both the neutral and acidic sugars are useful compounds for monitoring the pectolytic degradation but specific analytical methods are required (De Vries, Den Uijl, Voragen, Rombouts, & Pilnik, 1983). The recent use of high

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performance anion-exchange chromatography (HPAEC) has allowed the separation of OGAs; in addition, pulsed amperometric detection (PAD) provides excellent selectivity and sensitivity (Hotchkiss & Hicks, 1993).

In the present work a HPAEC–PAD analytical method was used to study the composition of OGAs of strawberry juices, in order to evaluate the effects of pectolytic enzyme treatment on a breakdown of pectin. This work focused on strawberries because of the wide public acceptance and economic interest of this fruit.

2. Materials and methods

2.1. Pectolytic enzymes

Seven food grade commercial pectolytic enzymes were evaluated for processing the strawberry fruits: Pectinex® BE 3-L (Novo Nordisk Ferment Ltd, Dittingen, Switzerland), Rohapect® MB, Rohapect® B1L, Rohament® MAX (Röhm, Darmstad, Germany), Grindamyl Pectinase® LM, Grindamyl Pectinase® LX, and Pectinex® 3XL (Danisco, Brabrand, Denmark). Enzymes were used at the average dosage recommended by the manufacturers, resulting in a range of 3–30 g/100 kg mash.

2.2. Sample preparation

Strawberry fruits (5.2°Brix and pH = 3.7) were purchased from local markets and the juice extraction process carried out in the laboratory as previously described (Versari, Barbanti, Biesenbruch, Farnell, & Galassi, 1998). Aliquots (50 g) of strawberry mash were poured into 250-ml glass conical flasks and pasteurized at 85°C for 2 min before pectolytic enzyme was added (treated samples). An enzyme/juice reaction time of 2 h at 45°C was used to simulate commercial processing practices. Treatment with Grindamyl Pectinase® LM was also extended to 4 and 6 h to measure the time course of pectinase activity and verify the stability of OGAs. Untreated juice was kept at 45°C and used as control (blank). After 2, 4 and 6 h, the samples were pasteurized, centrifuged at 15 000 rpm for 15 min (Ultracentrifuge MSE Europa 24 M, Kontron Instruments, UK), hence the supernatant was diluted to a solids content of 0.5°Brix. The non-polar compounds were removed from the supernatant by solid phase extraction (Sep-Pak-RPC18, Millipore, Watford, UK) as previously reported (Biesenbruch & Farnell, 1994). The juice was then filtered through 0.22-μm cellulose–acetate membrane (Sigma F-1039, Sigma Chemical Co., Dorset, UK) and analyzed by HPAEC–PAD.

2.3. HPLC analyses

Analysis of underivatized OGAs was carried out as previously described (Biesenbruch & Farnell, 1994) using a Dionex Ion Chromatograph 2010i system (Dionex Corporation, Sunnyvale, CA, USA) consisting of eluent degas module, autosampler, autoinjector (25 μl), advanced gradient pump and pulsed amperometric detector with gold electrode. A three-step PAD setting was used with the following time intervals (ms) and potentials (V): t_1: $300/E_1 = +0.05$ (detection); t_2: $120/E_2 = +0.6$ (cleaning); t_3: $60/E_3 = -0.6$ (regeneration). The column was a Dionex CarboPac PA-100 coupled to a guard column of the same material (250×4 and 50×4 mm, respectively). Analyses were done at room temperature using sodium hydroxide 0.1 M as eluent A, and sodium acetate 1 M–sodium hydroxide 0.1 M as eluent B. Solvents were HPLC grade (Sigma). The following elution gradient was used [time (min)/A (%)]: $t_1 = 0/99$; $t_2 = 5/99$; $t_3 = 35.4/0$; $t_4 = 43/0$; $t_5 = 48.4/99$; $t_6 = 52/99$.

2.4. Standards

Neutral sugars (rhamnose, glucose, fructose, sucrose, maltose, glucose trimer) and acidic sugars (D-glucuronic, D-galacturonic monohydrate, digalacturonic and trigalacturonic acid) were purchased from Sigma, and used as external standards for peaks identification and quantification. Standard solutions were prepared in the range of 1–5 mg/l and used for the calibration curves. Peak identification was based on the retention time. Moreover, a low methoxylated citrus pectin (Sigma) was enzymatically hydrolyzed with Pectinex® 3XL (Danisco) in order to use the breakdown products as natural standards for the higher OGA peaks identification. Commercial standards above 3 DP units were not available because of their chemical instability.

2.5. Statistical analysis

Data sets were analyzed by a linear fitting model using Statistica 5.0 software (StatSoft®, Tulsa, OK, USA). While the analytical variation limits the conclusion that can be drawn for treatment effects, the quantitative estimates are still useful for evaluating trends and providing a perspective on the amounts of OGA present in strawberry juices.

3. Results and discussion

3.1. HPAEC–PAD analysis

The elution profiles of the standard compounds analyzed by HPAEC–PAD (Fig. 1) show a satisfactory resolution for both the peaks of neutral and acidic sugars (Fig. 1A) and also for the peaks of breakdown products of low methoxylated citrus pectin (Fig. 1B). Peaks for which standards were available were numbered (1–10), while the other peaks were lettered (a–e)

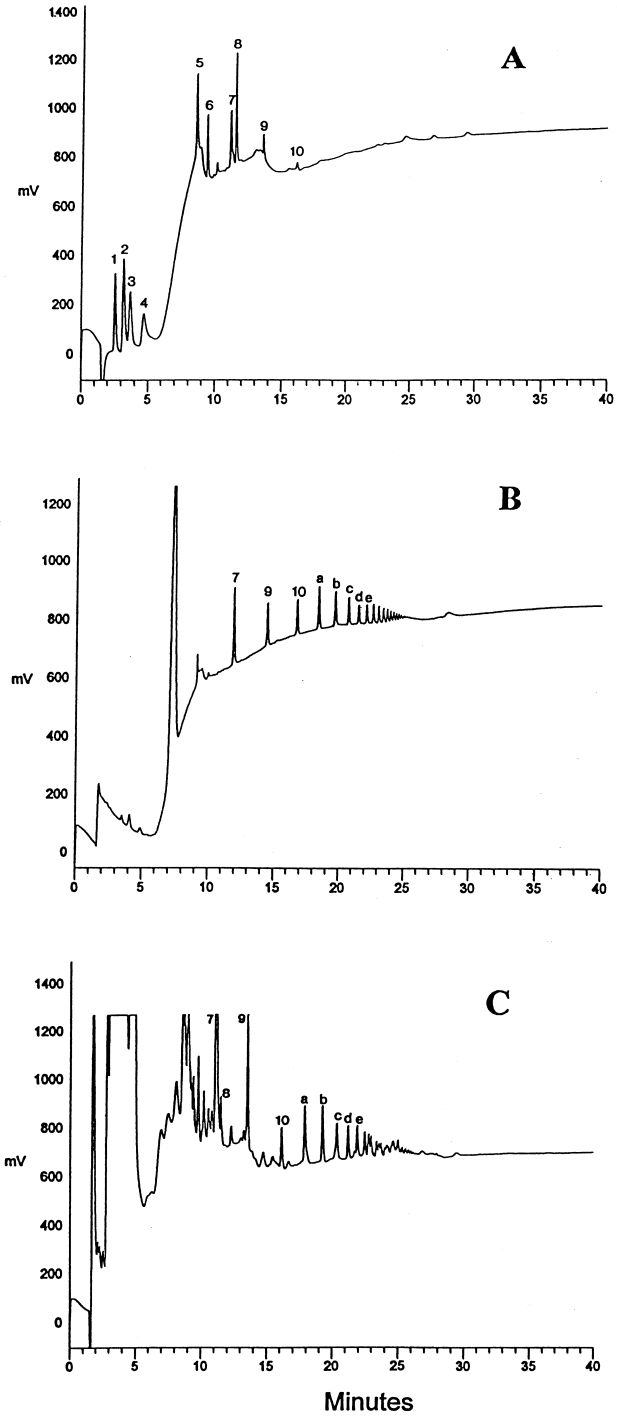

Fig. 1. HPAEC–PAD profiles of: (A) neutral and acidic sugar standards; (B) low methoxilated citrus pectin breakdown products; (C) strawberry juice treated with Pectinase® LM (45°C for 4 h). Peak identification: (1) rhamnose; (2) glucose; (3) fructose; (4) sucrose; (5) maltose; (6) glucose trimer; (7) D-glucuronic; (8) D-galacturonic acid; (9) digalacturonic; (10) trigalacturonic acid; (a) tetragalacturonic acid; (b) pentagalacturonic acid; (c) esagalacturonic acid; (d) eptagalacturonic acid; and (e) octagalacturonic acid.

up to octagalacturonic acid (Table 1). The analytical conditions used in this study allowed the determination of only the acidic sugars (OGAs). This was because the excess amounts of neutral sugars in strawberry samples overloaded the detector. The results (Fig. 1B) show that citrus pectin breakdown products with DP <20 units were well resolved in less than 25 min. Moreover, the elution order of OGAs increased with increasing degree of polymerization and there was a good correlation ($r=0.97$) between the DP and the retention time (Rt) values of the OGAs. The linearity of the calibration curve, expressed as the coefficient of determination (r^2), was studied over five concentration levels in the range of 1–5 mg/l. The results (Table 1) indicated that good linearity was obtained for OGA standards ($r^2 \geqslant 0.99$). The limit of detection (LOD), calculated according to Miller and Miller (1993), showed lower values for glucuronic acid (0.1 mg/l) when compared with those of galacturonic acids (0.6–0.9 mg/l) (Table 1). Due to unavailability of commercial standards, the OGA above 3 DP units (peaks a–e) were quantified with reference to the trigalacturonic acid standard. Unfortunately, PAD does not give a predictable response factor for each oligosaccharide on weight or molar basis (Hotchkiss & Hicks, 1993), hence the concentration of OGA above 3 DP units had to be considered only as indicative (probably underestimated). Finally, to minimise baseline drift in the chromatogram the system noise (blank) should be recorded and the use of 'blank subtraction' techniques is recommended.

3.2. Characterization of strawberry OGA profile

The influence of pectolytic enzymes on the concentration of OGA in strawberry juices is shown in Table 2. The concentration (mg/l) of each individual OGA at 2, 4 and 6 h of reaction time, was compared to the control. Glucuronic acid occurred in strawberry only in small amounts (12 ± 0.5 mg/l) and its concentration was not affected by the enzymatic treatment (data not shown). Juices treated with Pectinex® BE 3-L and Pectinex® 3XL enzymes, respectively, contained only galacturonic and digalacturonic acids as breakdown products of pectin. In comparison, the other enzymes were able to produce additional breakdown products resulting in a complex OGA profile. In particular, Rohament® MAX yielded juice with by far the highest total concentration of OGA, followed by Grindamyl Pectinase® LM (Table 2). This finding can be explained by taking into consideration that the 'main' and the 'side' activities of the pectolytic enzymes could affect the result of enzymatic degradation (Will & Dietrich, 1994). According to Ceci and Lozano (1998) we formulated the hypothesis that the commercial pectinases used in this study contained a mixture of enzymatic activities, including polygalacturonases, pectin lyase and pectin esterase. Each enzymatic activity could vary considerably, and the complete breakdown of pectin in fruit juice can only be ensured if the different types

Table 1
Retention time (Rt), calibration curve parameters ($y = a + bx$) and limit of detection (LOD) values determined by HPAEC–PAD for the neutral and acidic sugar standards

Compound	Peak code	Calibration curve[a]				
		Rt (min)	r^2 ($n = 5$)	$y = a + bx$		LOD (mg/l)
				(a)	(b)	
Rhamnose	1	2.5	0.99	−281 731	1 311 164	0.4
Glucose	2	3.2	0.99	138 972	1 366 099	0.7
Fructose	3	3.7	0.98	−2704	140 087	0.8
Sucrose	4	4.7	0.93	−131 928	495 187	0.2
Maltose	5	8.6	0.99	482 114	830 863	0.3
Glucose trimer	6	9.3	0.99	24 608	634 300	0.3
Galacturonic acid	7	11.1	0.99	10 034	504 329	0.8
Glucuronic acid	8	11.5	0.99	134 078	642 707	0.1
Digalacturonic acid	9	13.9	0.99	−19 300	120 040	0.6
Trigalacturonic acid	10	16.4	0.99	1277	67 550	0.9
Tetragalacturonic acid	a	18.4				
Pentagalacturonic acid	b	19.7				
Esagalacturonic acid	c	20.7				
Eptagalacturonic acid	d	21.5				
Octagalacturonic acid	e	22.1				

r^2, Determination coefficient of calibration curve; LOD, limit of detection = [(3×standard error of calibration curve)/slope].
[a] Calibration curve: y (signal, high performance anion-exchange chromatography [HPAEC] area) = a (intercept, signal) + b (slope, signal mg/l) x (concentration, mg/l).

Table 2
Effect of different commercial pectolytic enzymes on the concentration of oligogalaturonic acids (OGAs) in strawberry juices (treatment conditions: 45°C for 2 h)

Compound (mg/l)	Control	Pectinex® BE 3-L	Pectinex® 3XL	Pectinase® LX	Pectinase® LM	Rohament® MB	Rohament® B1L	Rohament® MAX
Galacturonic acid	8.0	43.3	43.6	19.6	75.5	19.7	15.3	49.5
Digalacturonic acid	128.1	284.3	259.3	131.4	190.4	169.3	155.5	142.3
Trigalacturonic acid	0.0	0.0	0.0	4.5	79.8	6.7	7.5	196.4
Tetragalacruronic acid	0.0	0.0	0.0	12.7	20.6	23.8	18.1	165.0
Pentagalacturonic acid	0.0	0.0	0.0	22.6	91.0	21.7	13.1	208.9
Esagalacturonic acid	0.0	0.0	0.0	44.4	58.5	23.1	17.4	88.1
Eptagalactuonic acid	0.0	0.0	0.0	11.2	63.7	11.3	5.1	72.5

of enzyme are present in the correct proportions. Unfortunately, any further information about the composition of the selected commercial pectinases was not available.

The release of OGA during the action of pectolytic enzymes is also of great interest. The chromatogram of strawberry juice treated with Pectinase® LM (Fig. 1C) shows the presence of OGA as a consequence of pectinase activities. The control, which contained only galacturonic acid (8 mg/l) and digalacturonic acid (128 mg/l), did not show changes in the amount of the individual compounds with time. On the other hand, treatment with Pectinase® LM increased the total OGA content of strawberry juices and also affected the levels of each individual OGA proportionately with time (Fig. 2). Galacturonic acid increased from 8 to 116 mg/l

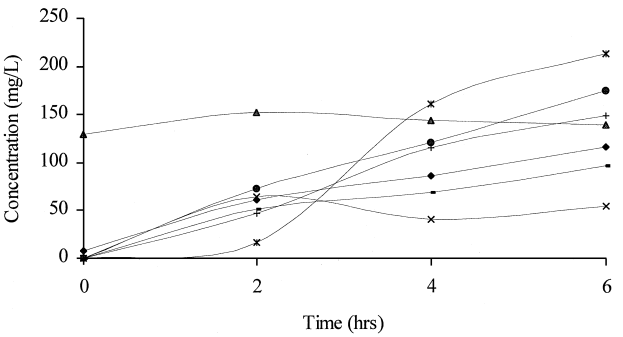

Fig. 2. Changes in oligogalacturonic acid (OGA) concentration with time for strawberry juice treated with Pectinase® LM (treatment conditions: 45°C for 2, 4 and 6 h). ◆, Galacturonic; ▲, digalacturonic; ×, trigalacturonic; ⋆, tetragalacturonic; ●, pentagalacturonic; +, esagalacturonic; −, eptagalacturonic acid.

(after 6 h), while the sum of OGA with DP $\geqslant 4$ units rose from 78 to 771 mg/l (after 6 h). The overall rate of production of OGA ranged between 15 and 39 mg/l/h, for epta- and tetragalacturonic acid, respectively. The continuous increase of OGA may indicate that the breakdown products of pectin are not suitable substrates for further pectinase activities. Finally, the concentration of digalacturonic acids remained constant with time (141 ± 7 mg/l), while trigalacturonic acid showed great variability with time.

4. Conclusions

HPAEC–PAD analysis provided a valuable technique to characterize the profile of OGAs and to monitor the pectolytic activity in strawberry juice. Breakdown of pectins in strawberry juice with commercial pectolytic enzymes resulted in the release of characteristic sets of pectin breakdown products, depending on the hydrolysis conditions. The composition of OGA varied in relation to the type of enzyme and the hydrolysis time. Hence, the separation of OGAs is a prerequisite to study the degradation of galacturonic acid polymers using pectolytic enzymes. Since galacturonic acid was always released by the selected pectinase enzymes, its detection provided the best marker for monitoring the enzymatic activitiy. Moreover, the HPAEC OGA profile could be used as 'fingerprint' in authenticity investigations. These results provide more insight into the problems concerning the clarification and filtration of juices, as well as providing data for nutritional and biochemical investigations.

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