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Short communication
METROLOGICAL CHARACTERISTICS IN URONIC ACID DETERMINATION FOR QUALITY CONTROL OF OLIGOGALACTURONIDE MIXTURES

Comunicación corta
Características metrológicas en la determinación de ácidos urónicos para el control de la calidad en mezclas de oligogalacturónidos

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ABSTRACT. Uronic acid content determination for the quality control of oligogalacturonides is extremely important, considering that they are directly related to their biological activity. The aim of this research was to analyze the metrological characteristics of Blumenkrantz and Asboe-Hansen spectrophotometric method, for uronic acid determination in oligogalacturonide mixtures, by using m-hydroxydiphenyl as chromophore. Linearity, precision and limits of detection and quantification were among the metrological characteristics analyzed. Blumenkrantz and Asboe-Hansen method resulted to be adjusted to a linear model within a concentration range of 10-80 µg mL^{-1}. Regarding such model, the correlation coefficient value is r= 0,996 whereas the determination coefficient is R^2= 0,992, indicating a very good correlation between uronic acid concentration and its absorbance. The precision of this method was determined through two procedures, which did not surpass the established value as a variability limit of 5 %. Detection and quantitation limits were in the order of 1,6 ± 0,4 µg mL^{-1} and 5 ± 1 µg mL^{-1}, respectively. Uronic acid concentration was recorded in the order of 890 µg mL^{-1}.

Key words: pectic acid, uronic acids, quality control, oligogalacturonides

RESUMEN. La determinación del contenido de ácidos urónicos en el control de la calidad de los oligogalacturónidos es sumamente importante, teniendo en cuenta que ellos están directamente relacionados con la actividad biológica que estos presentan. El objetivo de este trabajo fue realizar el análisis de las características metrológicas de la técnica espectrofotométrica de Blumenkrantz y Asboe-Hansen, en la determinación de ácidos urónicos en mezclas de oligogalacturónidos, empleando como cromóforo el m-hidroxidifenilo. Dentro de las características metrológicas analizadas se encontraron la linealidad, la precisión y los límites de detección y cuantificación. Como resultado el método de Blumenkrantz y Asboe-Hansen se ajustó a un modelo lineal en el rango de concentración de 10-80 µg mL^{-1}. Para el modelo ajustado, el valor del coeficiente de correlación es r= 0,996 y el coeficiente de determinación es R^2= 0,992, lo cual indicó muy buena correlación entre la concentración de ácidos urónicos y su absorbancia. La precisión del método se determinó por dos procedimientos, los cuales no sobrepasaron el valor establecido como límite de variabilidad del 5 %. Los límites de detección y cuantificación estuvieron en el orden de 1,6 ± 0,4 µg mL^{-1} y 5 ± 1 µg mL^{-1}, respectivamente. La concentración de ácidos urónicos se encontró en el orden de los 890 µg mL^{-1}.

Palabras clave: ácido péctico, ácidos urónicos, control de la calidad, oligogalacturónidos

INTRODUCTION

The oligogalacturonides are composed of linear oligomers of D-galacturonic acid linked by the (1-4) α type, which can be obtained by acid or enzymatic hydrolysis of pectic acid (1). The oligogalacturonides have demonstrated diverse biological activity in

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plants, among which is the of morphogenetic process promotion in plants (2) and the plant growth stimulation (3), among others. This biological activity is determined by its polymerization degree, the plant species and the concentration at which they are applied. It has also been studied use in sequestering of heavy metals (4). In this sense, the use of oligogalacturonides in reducing toxicity of Cu^{2+} ion in tomato seedlings have been studied, producing changes in absorption pattern of this metal (5).

The National Institute of Agricultural Sciences (INCA) has a patented technology nationally to obtain oligogalacturonide mixtures. The quality control of these mixtures includes determining the content of uronic acid, neutral sugars and reducing sugars present therein. Within these named characteristics, the content of uronic acids is one of the most important because it is closely related to the biological activity that these oligosaccharides have in plants. Such biological activity may be based on the ability of oligogalacturonides form a molecular trellis with Ca^{2+} ions named egg box (6). This arrangement is possible because of the many negative charges presented by oligogalacturonide, from the structural point of view and that depends on their polymerization degree.

The evaluation of the uronic acid content in the form of galacturonic acid may be performed by spectrophotometric evaluation. For this, the best-known spectrophotometric methods in this determination are those used as chromophores to m-hydroxydiphenyl (7, 8, 9, 10), 3,5-dimethylphenol (11, 12) and the carbazole (13, 14) . More recently it has been employed high performance liquid chromatography resolution (15, 16) and the zonal capillary electrophoresis, as quantization method with galacturonic acid with UV detection (17).

Generally, the method using by the m-hydroxydiphenyl as chromophore (7) is the most quoted of all and it is characterized by quantitative, sensitive and specific reasons why it was selected as a technique for quality control of oligogalacturonide mixtures. This method is based on the appearance of a pink chromogen when uronic acid is heated to 100 °C in a mixture of sulfuric acid/sodium tetraborate and subsequently treated with m-hydroxydiphenyl (Figure 1).

The aim of this study was the evaluation of some metrological characteristics in the determination technique of uronic acid using as chromophore the m-hydroxydiphenyl and thus to contribute to its further validation.

MATERIALS AND METHODS

Oligogalacturonide mixture was obtained by enzymatic hydrolysis (18), with the use of polygalacturonic acid (SIGMA) and Pectinase pectinolytic prepared derived from *Aspergillus aculeatus* fungus from Novozymes A/S Corporation. The determination of uronic acids was performed according to Blumenkrantz and Asboe proposed by Hansen-method (7) using as galacturonic acid pattern in the concentration range of 10, 20, 40, 60 and 80 ug mL^{-1}. Readings were taken at 520 nm in a UV visible Genesys 6 of Thermo Electron Corporation. The metrological characteristics analyzed were: linearity, accuracy and detection limits and quantification method (19, 20). Linearity (n=3) was determined by the method of least squares with the evaluation of the slope, intercept, correlation coefficients and determination, as well as the lack of adjustment existence, analysis graph waste and the coefficient of variation of response factor (CVf); the latter was determined from the following sequence of equations.

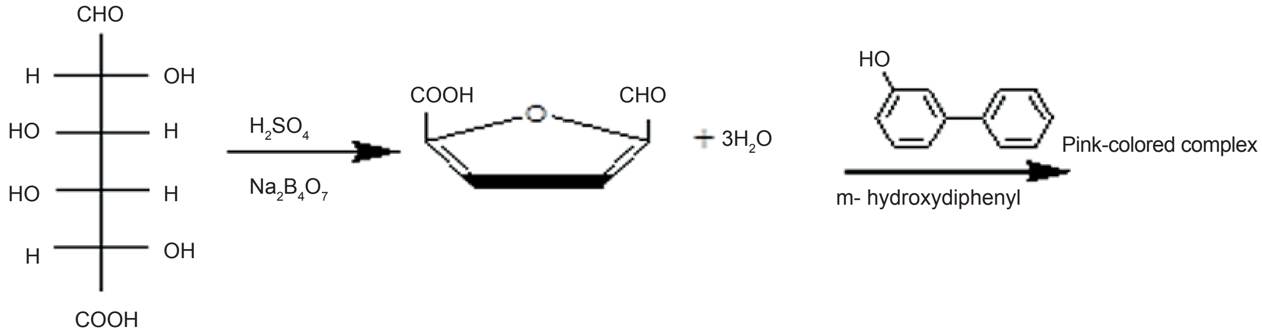


Figure 1. Schematic representation of the pink–colored complex formation between the galacturonic acid and the m-hydroxydiphenyl

$$f_i = \frac{y_i}{x_i} \qquad f = \frac{\sum f_i}{n}$$

$$SD_f = \sqrt{\frac{\sum (f_i - f)^2}{n-1}} \qquad CVf = \frac{SD_f}{\overline{f}} * 100$$

where:

f is the response factor, y_i and x_i are the absorbance and galacturonic acid concentration, respectively, SD_f is the standard deviation of the response factor, *CVf* is the coefficient of variation of the response factor and n the number of replicas

The accuracy was determined in terms of repeatability, maintaining the same measurement procedure, the same operator and measuring system and the same operating conditions, with equal replicas in a short period of time (21). The precision was expressed by the expert and determined as the ratio between the standard deviation and the mean value of the response factor, expressed in percent. Precision was evaluated in two ways, by determining the CV (from the analysis of three concentration levels galacturonic acid: 10, 40 and 80 ug mL^{-1}) and by analyzing six samples of oligogalacturonide under repeatability. For oligogalacturonide mixture analysis of these dilutions were performed at 1000 ug mL^{-1} in deionized water. The detection limits (LOD) and quantitation (LOQ) were determined by Long and Winefordner formulas (22).

$$LOD...y..LOQ = \frac{K.SD_B}{m \pm t_a SD_m}$$

where:

K=3 for LOD and 10 for LOQ

SD_B = standard deviation of the absorbance of the blank

m is the average of the slope of the calibration curves

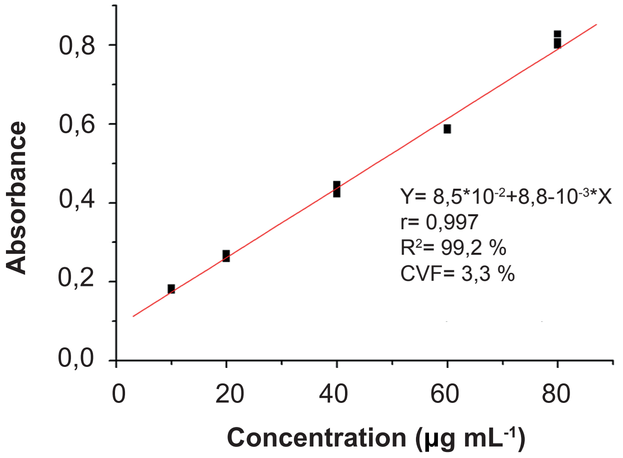
t_a=t Student distribution for a=0,01

SD_m=standard deviation of the slopes of the calibration curves

The limits were calculated from analysis of seven white.

RESULTS AND DISCUSSION

Linearity analysis of the uronic acids determination technique was performed through the calibration curve in the concentration range 10-80 ug mL^{-1} (Figure 2). The regression analysis showed that a linear model described by the linear equation was adjusted $y=0,09+8,8.10^{-3x}$ for the specified range of concentrations. For the adjusted model, the value of the correlation coefficient is r = 0,996172, while the coefficient of determination is R_2 = 0,992, indicating an excellent correlation between the concentration and the absorbance. The significance analysis showed that the slope error probability is less than 0,05 (P=0.0000), which indicated that this is significantly different from zero. Moreover, the significance analysis showed that intercept the probability for error, determined from the t Student test is less than 0,05 (P=0,0000), indicating the intercept (0, 09) it must be taken into account in the analysis. The variation coefficient of response factor (CVf) in the range of concentrations tested was 3,3 %, which is less than 5 % variability known as limit value, which is considered suitable (23).



Galacturonic acid is used as pattern and m-hydroxybiphenyl as chromophore (n = 3)

Figure 2 . Calibration curve used in the determination of uronic acids by Blumenkrantz and Asboe-Hansen method (7)

The unadjusted test checks whether the selected model (linear model) is adequate to describe the observed data or if it should be used a more complex model. The probability (P = 0,0012) associated with *F* value of the lack of adjustment was less than 0,05, so there is a lack of adjustment to the statistically significant linear model for a confidence level of 95 % (Table). Although it is known that the linearity analysis in a method of spectrophotometric determination is made from the search for the linear equation that describes in a specified range of concentrations, the use of statistical software *STATGRAPHICS PLUS 5.1* (24) verifies, from alternative curvilinear models, which is the best mathematical model to describe the system.

Indicators of precision in calibration curves of galacturonic acid, determining the limits of detection and quantification

Sample	Concentration (μg mL^{-1})	Mean absorbance	DE	CV	LOD	LOQ
Galacturonic acid	10	0,182	0,003	1,77 %	1,6 ± 0,4 μg mL^{-1}	5 ± 1 μg mL^{-1}
	_40	0,434	0,012	2,76 %		
	_80	0,808	0,013	1,64 %		
Mixture oligogalacturonide	1000	Concentration (μg mL^{1})				
			29	3,3 %		

SD: standard deviation CV: coefficient of variation LOD: limit of detection LOQ: limit of quantification

The adjusted model that best described the system was square root with a R$_2$ = 99,41 %; however, the linear model only differ from this in 0,17 %, consistent with the lack of adjustment found in the variance analysis. Moreover, the residual plot showed that the results show a random distribution, confirming that the model chosen is the right (Figure 3).

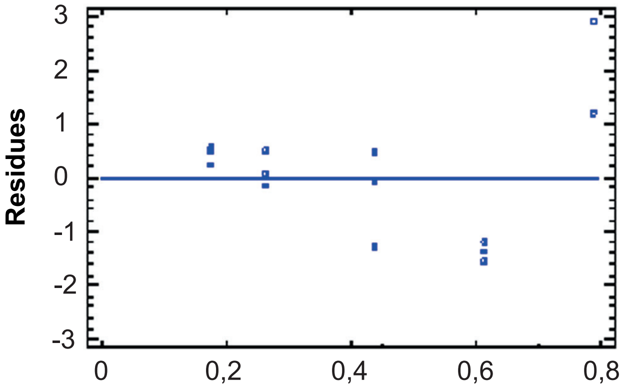


Figure 3. Graph of residues corresponding to the calibration curves used in the determination of uronic acids

The linearity evaluation in a spectrophotometric method is based on the property of substances to absorb light and must comply with the Lambert-Beer law, which states that the absorbed light is directly proportional to the concentration of the substance (25). Therefore, it can say that this relationship was described from the linear equation, y =0,09+8,8.10^{-3}x, whose correlation coefficients and determination demonstrated the proportionality between concentration and absorbance, fulfilling and with the Lambert-Beer.

The accuracy of the method was evaluated in terms of repeatability. Variation coefficient determining at three different concentration levels showed that this ranges from 1,64 to 2,76 %, which is below the limit value set as variability (5 %), so the method it is considered appropriate (Table).

Determining of the detection and quantitation limit in the calibration curve (Table) showed the lowest level of uronic acids which can be detected by this technique is 1,6 ± 0,4 µg mL^{-1}. In turn, the lowest concentration which can be determined accurately is 5 ± 1 µg mL^{-1}. The values of detection and calculated quantization limits indicate that the method of determination of uronic acids is suitable, considering that the concentration of uronic acids present in samples of oligogalacturonide mixtures is in the order of 890 ug mL^{-1}, being these major components, representing approximately 89 % of the samples. This concentration is in the order of the values in the literature on the commercial pectic acid characterization (SIGMA) of citric origin (26), wherein the concentration of uronic acid accounted for 87 % of the sample. Determining the detection limit allowed to refer that the linear response method had a range to 1,6 to 80 ug mL^{-1}. This result was found in the order reported by other authors (27) when performing the spectrophotometric determination of the uronic acids in green tea samples where the linear response of the method is between 1 and 100 ug mL^{-1}.

CONCLUSIONS

In general and as a conclusion, taking into account all metrological characteristics discussed above, it can be said that the determination technique of uronic acids, according to that proposed by Blumenkrantz and Asboe-Hansen (7) method, can be used as technical quality control in oligogalacturonide mixtures, since it meets the criteria of linearity, accuracy and detection and quantification limits of suitable for this purpose.

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