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**Method Validation for the Determination of D-psicose in Raisins Using Ultrasound-assisted Extraction
Coupled with Ionic Chromatography**
Fahmi Maulana Zulkarnaen, Dr. Widiastuti Setyaningsih, S.T.P., M.Sc. ; Prof. Miguel Palma Lovillo
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In ion chromatography, the stationary phases are solid macromolecules with a cross-linked space structure that do not dissolve in water or other solvents. (Paull & Michalski, 2019)

There are also amphoteric ion exchangers, which can swap either anions or cations depending on the pH of the solution, and bipolar/zwitterionic ion exchangers, which can swap both types of ions. Two of the most important things that affect the separation quality are the types of stationary phases in the analytical column and the types of eluents (concentration, pH, flow rate, and addition of organic solvents). This made the chromatographic system easier to use. (Paull & Michalski, 2019)

2.8.2 Pulsed Amperometric Detection

The majority of carbohydrates are electrochemically active. Therefore their detection using electrochemistry is theoretically achievable. In the 1980s, Dennis Johnson and colleagues developed cyclic, pulsed amperometric techniques that enabled the detection of carbohydrates in acidic or alkaline solutions with reasonable sensitivity and specificity. In pulsed amperometry, the working electrode is elevated to an oxidizing potential (voltage) to oxidize its surface and remove any adsorbed oxidation products from the preceding detection cycle. After that, the working electrode is moved to a reducing potential, converting the gold oxide on its surface to gold (Hardy, 1989).

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The working electrode's potential stepped to a suitable value for oxidizing carbohydrate aldehyde or hydroxyl groups (usually around 0.05V relative to a silver/silver chloride reference electrode potential). After a brief delay (to allow the cell's charging current to dissipate), the cell's current is sampled for a defined time, and the charge is measured as the time-dependent variable. The overall cycle duration for this triple-pulse sequence is approximately 1 second, which provides sufficient temporal resolution for conventional HPLC analysis times (flow rate of 1ml min^{-1}, peak widths in the tens of seconds to minutes) (Hardy, 1989).

2.9 Validation Method

The analysis technique must be verified if the standard (compendial) method alters the acceptance criteria, if there are changes in methodological aspects such as reagents, instruments, chromatographic settings, and sample preparation and if non-standard (non-compendial) methods were developed as a result of laboratory needs (FDA, 2015).

Before undertaking the validation study, the approach and procedure of the analysis must be clarified. This is achieved through the creation of procedures based on a scientific basis and optimization research. Validation data must be generated in accordance with a Good Manufacturing Practice (GMP) implementation protocol by describing the

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methodology and all validation parameters; the equipment must have been qualified/calibrated (FDA, 2015).

In accordance with the ICH guidelines (Table 2.1), the validation procedures for evaluating HPLC-DAD include linearity, selectivity, accuracy (recovery), precision, the limit of detection, and the limit of quantitation (Monti et al., 2017). Examining the chromatogram to ensure there are no distracting peaks is a test for selectivity. Five radiant concentrations and three iterations were used to calculate linearity. The LOD and LOQ values are derived from the respective calibration curves.

Analyzing six samples, accuracy (recovery) and precision (reported as percent RSD) were examined. There are both intra-day (three repetitions on the same day) and daily (three repetitions on three different days) tests undertaken. Finally, measurement error is determined using validation data provided during each step of the analytical technique. Consideration is given to four sources of uncertainty when determining measurement uncertainty: matrix (recovery), standard preparation, sample preparation, and calibration curve (Alyassin et al., 2020)

Table 2.1 Parameters required in method validation (ICH,2005)

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES Quantitation limit		ASSAY - Dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision	-	+	-	+
- Repeatability	-	+ (1)	-	+ (1)

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- Intermediate Precision				
Spescificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

(1) Incases where reproducibility has been performed, intermediate precision is not needed
(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure
(3) Maybe needed in some cases

If there is a change in the conditions between the working conditions and the conditions when the validation of the previous method or a deviation from the standard method, the validation method is performed on the new method of analysis or its development. Benefits of method validation include reviewing the findings of analytical methods, assuring analysis procedures, ensuring accuracy and repeatability of analytical procedure results, and lowering the risk of abnormalities (Ravichandran et al., 2010).

2.9.1 Linearity

The correlation coefficient is a line-based indicator that indicates the proportional relationship between the analytical response and the observed concentration. The linear regression equation is determined by plotting the concentration of the standard solution against the peak area of each component, where a and b are calculated. A valid

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method of analysis has a correlation coefficient greater than 0.999. (Ravichandran et al., 2010)

$$Y = a + bX$$

$$a = \frac{\Sigma y - b\Sigma x}{n}$$

$$b = \frac{n\Sigma xy - \Sigma x\Sigma y}{n\Sigma x^2 - (\Sigma x)^2}$$

For testing the linearity of the relationship between concentration (X) and area (Y), the correlation coefficient (R^2) is utilized, which is determined using the formula: (Ravichandran et al., 2010)

$$R^2 = \left(\frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{\{n(\Sigma x^2) - (\Sigma x)^2\}\{(n\Sigma y^2) - (\Sigma y)^2\}}}\right)^2$$

2.9.2 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The detection limit (LOD) is the lowest concentration of chemicals in a sample that can be detected and elicits a measurable reaction from the instrument. In contrast, the limit of quantitation (LOQ) refers to the least amount of chemicals in a sample that still passes the stringent and precise standards. LOD and LOQ values are generated statistically using a calibration curve-derived linear regression equation. The standard deviation and equation are then determined (Ravichandran et al., 2010)

$$S_{y/x} = \sqrt{\frac{\Sigma(Y - Yi)}{n - 2}}$$

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$$S_b = \frac{S_{y/x}}{\sqrt{\sum_i (x_i - \bar{x})^2}}$$

$$S_a = S_{y/x} \sqrt{\frac{\sum_i {x_i}^2}{n \sum_i (x_i - \bar{x})^2}}$$

$$LOD = \frac{3\,SD}{b}$$

$$LOQ = \frac{10\,SD}{b}$$

Note :
$S_{y/x}$ = Standard Deviation at the origin
S_b = Standard deviation of slope
S_a = Standard deviation of intercept
Y = The area detected at each concentration
Yi = The theoretical area of each concentration, calculated form the linear regression equation
n = Number of replicate sample injections
b = slope regression equation of the calibration curve

2.9.3 Accuracy

Accuracy is a parameter that reflects the degree to which the analysis findings correspond to the actual concentration of the analyte. Accuracy is reported as the number of analytes recovered as a percentage of the sample. Accuracy is achieved by comparing the reference's results to the fundamental reference material. The International Conference on Harmonization (ICH) suggests gathering data from nine determinations of levels with three distinct concentrations to demonstrate precision. Good percent recovery falls within the range of 95 to 105 percent for the active substance level of ≥ 0.1% w/w (AOAC, 2016)

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$$Recovery\ precentage\ = \frac{C1\ -\ C3}{C2} \times 100\%$$

Note :
C1 = concentration of compounds in the sample and standard
C2 = concentration of compounds in the sample
C3 = concentration of the actual compound added to the inside the sample

2.9.4 Precision

A precision test or equality test is used to assess the degree of similarity between the outcomes of the study in order to determine the random error analysis. Repeatability and reproducibility are two forms of precision tests (Ravichandran et al., 2010).

Repeatability test is a test for the correctness of the method whether it can be performed repeatedly by the analyst under the same conditions and in a little period of time. To provide a measure of equality under normal conditions, repeatability is determined using identical samples from the same batch. Comparatively, reproducibility is the resemblance of techniques carried out under different settings, locations, equipment, reagents, solvents, or analysts, with identical samples from the same batch. Reproducibility can also be achieved in the same laboratory by employing distinct instruments, reagents, solvents, or analysts. Meanwhile Intermediate precision describes variability within a laboratory, such as differences

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in days, analysts, and equipment. (Ravichandran et al., 2010).

Six or more replicates of a mixture of materials with a homogeneous matrix are used to conduct the repeatability precision test. If the approach yields a permitted proportion of relative standard deviation (RSD), stringent requirements are provided (Ravichandran et al., 2010).

2.9.5 Selectivity

The selectivity is determined by comparing the retention times of the standard solution, the sample solution, and the sample solution-standard mixture. So that the method can be stated selectively, the research must demonstrate that the standard and sample solutions and the mixing of sample solutions with the standard peak area occur when the retention time (Rt) is relatively the same (Ravichandran et al., 2010)

2.10 Hypothesis

1. D-fructose content will stable enough to be submited to the mid-high extraction temperature during the extraction under an hour using UAE because of the extraction condition does not accommodate Maillard reaction on the sugar compound.

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2. D-psicose contents in raisins may be degraded during high temperature treatment with long extraction time, but merely not degraded when its applied for a short extraction time (0-30 min.).
3. The extraction and chromatographic methods meet the validations parameters (precision, accuracy, linearity, selectivity, LOD, and LOQ).
4. Levels of D-psicose in regular food samples, specifically raisin samples, can be determined using the UAE-HPLC-PAD method with reliable results.

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CHAPTER 3
MATERIALS AND METHOD

3.1 Chemical and Reagents

From Sigma Aldrich Chemical Co., analytical grade standards of D-psicose, D-fructose, and lactose were purchased (St. Louis, MO, USA). In order to filter the ultrapure water that was necessary for this experiment, the Milli-Q system was used (Millipore Corp., Bedford, MA). Sigma Aldrich Chemical Company was also responsible for the distribution of reagents of analytical grade, such as anhydrous sodium acetate and sodium hydroxide (St. Louis, MO, USA).

3.2 Research Time and Place

The research to developed and validated a determination method for D-psicose in raisins was done in the Analytical Chemistry Laboratory, Departamento de Química Analítica, Universidad de Cádiz, Spain from February 7th until June 28th, 2022.

3.3 Sample Treatment

3.3.1 Raisins collection

Four commercial raisin samples comprising three different varieties were acquired at a local market for the method validation. The varieties include two Thompson seedless raisins, cultivated in Spain and Chile; a Muscat raisin from Spain; and a Sultana raisin from Turkey. The four samples were mixed and ground in a food processor (Thermomix

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TM31, Vorwerk International Strecker & Co., Wollerau, Switzerland) for 30 s, using the highest speed of the food processor's settings. Henceforth, a typical joint sample was used for method development. To test the method's applicability, those four samples were analyzed individually. Two additional samples from California (Sun-Maid Growers of California, Fresno, CA, USA) and Indonesia (PT. Trillions Naturalindo Multiboga, Bandung, Indonesia) were also included in the analysis. The ground samples were stored in a dry place at room temperature.

3.3.2 Ultrasound-assisted Extraction

Extraction with UAE utilizes the application of Bandelin Sonopuls HD 4400 Ultrasonic Probe (Bandelin electronic, Berlin, Germany) with the aid of Frigiterm TFT-30 water bath circuit cooler (J.P. Selecta, Barcelona, Spain). It was proposed to prepare the sample solution before injection into the ion chromatography system. The sample to the solvent ratio we used for the whole extraction in this study was 1 gr to 20 mL of filtered water. Also, for specific ultrasound power and cycle, an intermediate condition of UAE was choosen; therefore, both ultrasound power and cycle were set to 100 W and 0.5/s. The ultrasonic parameter differences for each study we conducted were in the time and temperature settings. The fructose stability study applied a constant extraction temperature at 50°C with various extraction times (5,10,15,20, and 25 min). In contrast, we applied a constant

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extraction time at 10 min with various extraction temperatures (10, 25, 40, 55, 70, and 85 °C) for the psicose stability study. Also, for the method validation, we applied a constant extraction temperature of 25 °C and a constant extraction time of 10 min.

3.4 D-psicose Content Measurement

To determine the content of D-psicose in raisins matrices, chromatographic analyses were conducted using high-performance anion-exchange chromatography (HPAE) equipment (Metrohm 930 Compact I.C. Flex, Gallen, Switzerland). The detecting system incorporated a pulsed amperometric detector (PAD) with a gold working electrode. Using an aqueous mobile phase containing 300 mM sodium hydroxide and 1 mM sodium acetate, elution was performed at a flow rate of 0.5 mL min^{-1}. Separation of the components using a Metrosep Carb 2-150/4.0 column (Metrohm). The chromatographic analysis was documented using MagIC net®, version 3.1 (Metrohm) software. Before the chromatographic analysis, all samples were diluted 1:25 with HPLC grade water and filtered through a 0.45 µm nylon filter to ensure that the concentrations of the chemicals fell within the linear ranges of the calibration curves. The volume of the injection was fixed to 5 µL. Identifying the resulted peaks was done by comparing the retention period of the peaks to that of reference chemicals

3.5 Method Validation

Based on the recommendations of ISO 17025 and ICH Guideline Q2 (R1), the innovative method for the simultaneous separation of D-psicose

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compound in raisins matrices was verified ((ISO), 2005; ICH). The method's detection and qualification limitations, linearity range, and precisions were determined. Using the standard reference for the eight examined analytes, a series of solutions with concentrations ranging from 1 to 50 mg L^{-1} was created. After calculating the regression analysis, the linearity within the examined range was tested to demonstrate that the test results generated by the method are proportionate to the analyte concentration. On the basis of the slope and standard error regression data, the limits of detection (LOD) and quantification (LOQ) were estimated. Repeatability (intraday) and intermediate precision (interday) were utilized to represent the developed method's precisions. Six separate analyses of the same samples performed on the same day were utilized to determine the repeatability's value. Comparatively, three independent analyses were conducted on three consecutive days to assess the intermediate precision. Precision was expressed using coefficients of variation (CVs) of the retention time and peak area.

3.5.1 Selectivity

A mixture solution of 10 ppm for each three different standard (D-fructose, D-psicose, and lactose) solution was injected into the HPAEC system at a volume of 5 µL under specific conditions. Three repetitions are performed, the resolution indicates the separation of analytes from one another. Selectivity is determined by applying a formula to get the Rs resolution :

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$$Rs = 2\,\frac{(t_{r2} - t_{r1})}{(W_1 + W_2)}$$

Note :
R = Resolution
t_{r1} = retention time first peak
t_{r2} = retention time second peak
W_1 = base width of the first peak
W_2 = base width of the second peak

The purpose of determining the resolution value is to ensure the

separation of the eluted components close together and to ensure the efficiency of the system separation, the resolution will be met if the value of R > 1.5 (Emons, 2013)

3.5.2 Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ).

5 µL of a D-psicose solution in a filtered aquadest solvent with concentrations of 1, 2.5, 5, 7.5, and 10 mg/L was subsequently injected into an HPLC system under specified circumstances. The noted area is then converted into a standard curve. Each concentration repetition was performed three times.

The measurement data used to create the standard curve are then examined using linear regression A standard curve is produced for each tested substance. The linear correlation coefficient (R) must exceed 0.99 (Emons, 2013). The linearity of the outcomes demonstrates the analytical technique that yields outcomes exactly proportional to its concentration. In addition, the linear regression

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equation y = bx + a is utilized to calculate the detection limit (LOD) and the quantitation limit (LOQ).

LOD is defined as the lowest analyte concentration that can be detected, whereas LOQ is the lowest sample concentration that can be quantified with an acceptable level of accuracy and precision (Bernal et al., 2013). The detection limit (LOD) and quantitation limit (LOQ) of each chemical are determined statistically using a linear regression equation derived from the standard curve (Emons, 2013).

3.5.3 Accuracy

This determination method's accuracy is calculated using the usual addition approach (standard addition method). Accuracy test is performed by adding a D-psicose solution into the sample of extracted raisins solution in which level of D-psicose in the sample has known before.

Accuracy test done with 9 determinations consisting of 3 levels of 50, 100, 150% of D-psicose fortification based on the past literature about D-psicose content in raisins (Oshima et al., 2006). The solution from each determination is subsequently filtered using a 0.45 m filter prior to injection into the chosen HPEAC system. On the other hand, the same sample was likewise made without any additions. Comparing the amount of measured analytes for each

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standard addition to the theoretical analytic content yields the percent recovery (Emons, 2013).

The calculation formula for recovery

Percent Recovery (%) = ((B-A)/C) × 100
Note :
A= analyte levels before standard addition
B = analyte levels after standard addition
C = standard levels added

Good percent recovery is indicated by a range between 95 and 105 percent for the active ingredient content >0.1 percent b/b (AOAC, 2016)

3.5.4 Precision

The intraday precision of the raisins sample test solution was determined using fortification of 5 mg/L D-psicose standard solution, and then nine determinations were performed on the same day. The obtained area was recorded, and the RSD of each concentration was calculated.

For the determination of the intermediate precision in the D-psicose test solution, 5 mg/L analytes were used, and each determination was performed on the first, second, and third days on a sequence. The acquired area was recorded, and RSD and the standard deviation of the average concentration were calculated from each concentration (Ravichandran et al., 2010)

3.5.5 Real Sample Applications

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The real sample applications conducted by extracting several different commercially available raisins with validated extraction and ion chromatographic methods. For each varieties of commercial sample, 6 extraction and analysis were run to evaluate the average value of D-psicose levels in each raisin sample.

3.6 Statistical Analysis

Minitab Statistical Software (Minitab, LLC., USA) was utilized for the stability study of D-fructose and D-psicose to run a one-way ANOVA ($p=0.05$) subsequently with a least significant difference (LSD, $p = 0.05$) test was conducted to determine if there were significant differences between the means. Microsoft Office's Analysis ToolPak was used to evaluate the linear regression of the experimental outcomes of the D-psicose calibration curve for further calculation of LOD and LOQ.

CHAPTER 4 : RESULTS AND DISCUSSION

4.1 Fructose and Psicose Stability

The purpose of this investigation into the stability of fructose and psicose is to ensure that the UAE used in this determination method did not significantly ($p<0.05$) impact the analyte content while it was being analyzed. The results from the study on the stability of fructose showed that increasing the ultrasound period the level of fructose remains stable for up to 25 minutes of extraction time, at a temperature that was considered to be mid-high for

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extraction (50 °C). Sustained ultrasonic waves provide adequate heat accumulation, which may lead to decomposing the fructose content that is dissolving in the solvent. This is because the extraction system absorbs ultrasound energy.

As seen in Figure 4.1, the results of this specific experiment showed no statistically significant difference in the mean peak area responses between any of the groups of observed periods. Based on these findings, it can be concluded that fructose is robust enough to withstand temperatures higher than the mid-high range for the entire 25-minute extraction period.

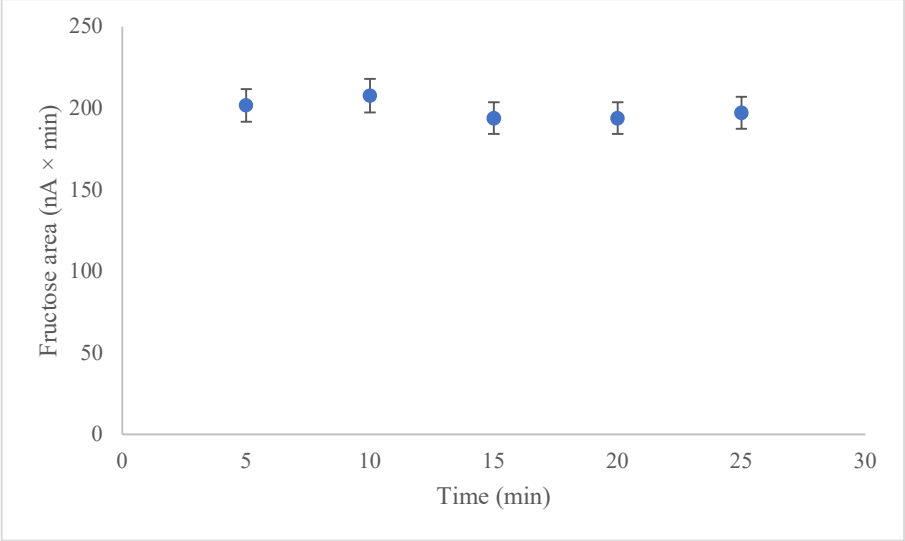


Figure 4 1. Fructose peak area response versus different extraction times

Nonetheless, during the course of our process of collecting data, we discovered an unstable behavior of fructose peak area response in the 5 minute group, a result of fructose level was much lower than the other results using the same condition, as depicted in Appendix 1. Therefore, 10 minutes of extraction time was selected as the parameter that would remain constant

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throughout the planned method validation. The findings of this study are consistent with those of earlier research on the behavior of fructose during heating at various temperatures and for varying amounts of time (Woo et al., 2011). In addition, this 10-minute extraction period was the same characteristics as the research of psicose in various food matrices, which was another approach that was offered to determine the amount of psicose contained in raisins (Oshima et al., 2006).

In contrast, for the study on the stability of psicose, the observation of different extraction temperatures with a constant extraction time (10 minutes) in raisin matrices also resulted in a stable amount of psicose being present in any of the groups of temperatures that were observed, as can be seen in Figure 4.2. Based on these findings, it can be deduced that extractions of psicose can be carried out at any temperature between 10-85 °C without the need to be concerned about large changes in the amount of psicose brought about by the extraction process itself.

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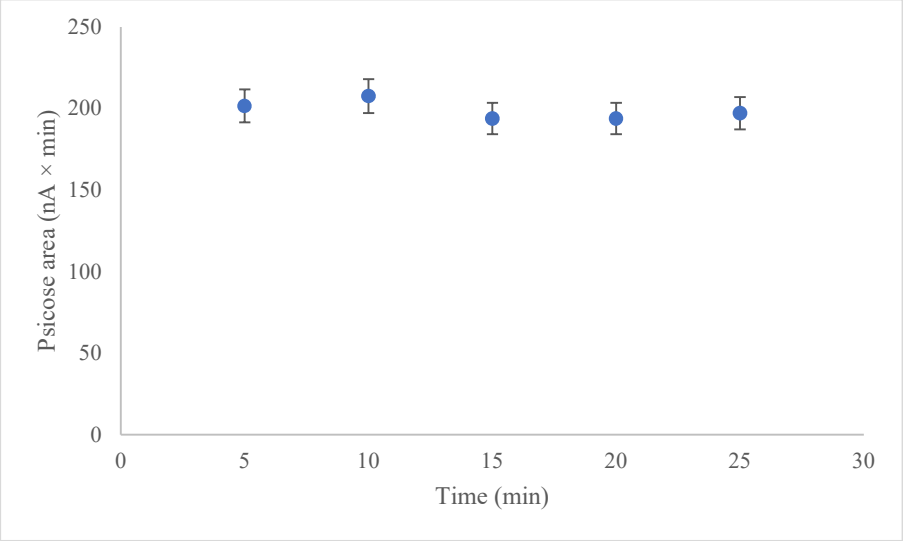


Figure 4 2. D-psicose peak area response versus extraction temperature

However, a particular extraction temperature of 25 ◦C was selected as our fixed extraction parameter for the projected method validation. This was done in part because of the unstable behavior of peak area response in the group of 10 ◦C (Appendix 2). Additionally, selecting an extraction temperature that is not too far from room temperature will save more time and energy than selecting a higher temperature for the extraction itself when it comes to preparing the extraction environment for further D-psicose determination in raisin matrices. With this decision, our study results agreed with the earlier UAE parameters for psicose determination in various food matrices (Oshima et al., 2006) and the study results of psicose decreasing behavior affected by heating temperature in various processed foods. (Oshima et al., 2014).

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4.2 Method Validations

4.2.1 Selectivity

Selectivity is the ability of a method to dependably measure the target analyte in the presence of matrix interfaces. The absence of false-positive results was investigated by analyzing a solution of psicose standard mixed with two other standard compounds (fructose and lactose) that were eluted just before and after the psicose in the ion chromatography method. This was done in order to determine whether or not the solution contained any false-positive results.

As can be seen in Figure 4.3a, there are no interference peaks during the retention time (7.71 min) that is predicted for psicose. It is necessary to determine the resolution value before coming to the conclusion that the psicose peak is eluted as a single compound and that the psicose peak was unaffected by any other compounds. According to our findings (Appendix 3), the mean resolution value between fructose and psicose was greater than 1.5, which indicates that the fructose-psicose peaks are distinct from one another. On the other hand, the mean resolution value between psicose and lactose was less than 1.5, which indicates that the psicose-lactose peak is not fully separated (Emons, 2013).

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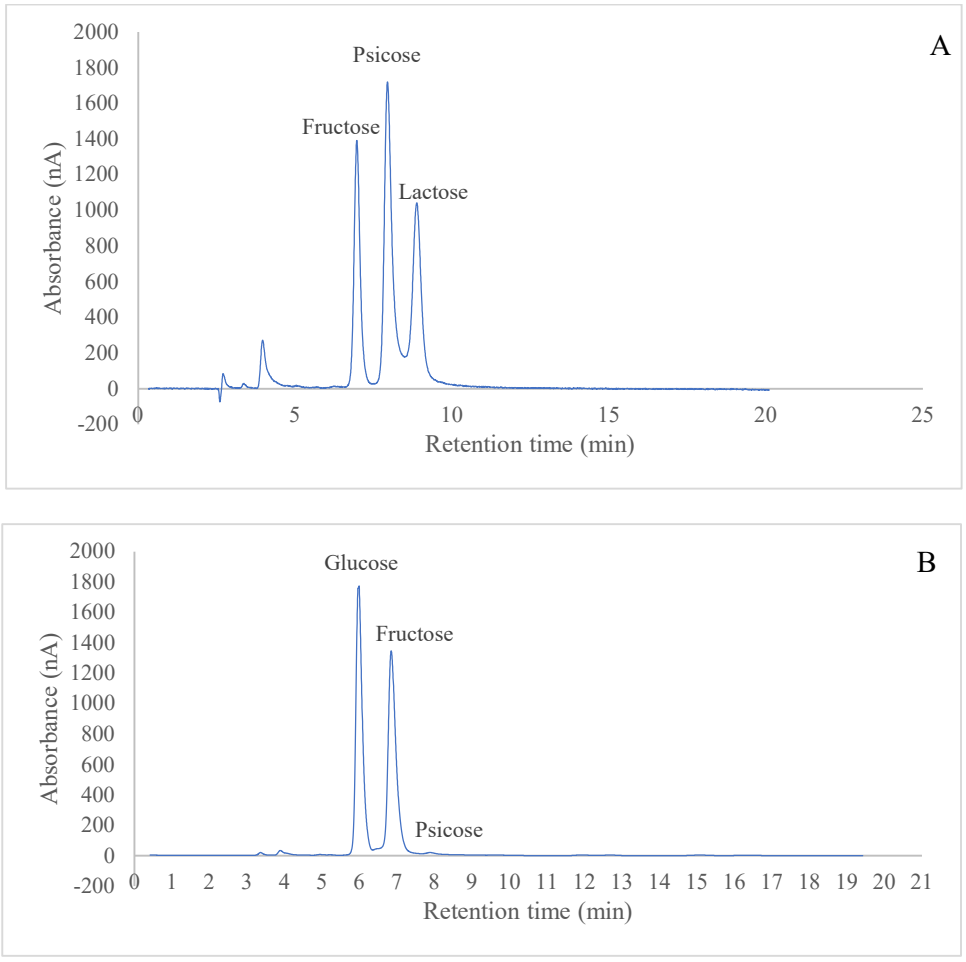


Figure 4 3. The representative chromatograms of the detected samples. (A) a mixture of 3 standard solutions (fructose-psicose-lactose); (B) Extracted raisins solution spiked with psicose standard

However, our observation (Figure 4.) in natural raisin matrices spiked with psicose standard demonstrated that lactose was not detected in raisin matrices; consequently, the problems regarding not fully separated peaks between psicose and lactose could be ruled out from this determination method. Figure 4.3 shows the results of our observation in natural raisin matrices spiked with psicose standard.

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4.2.2 Linearity

The concentration range over which the method can be applied was evaluated with the help of five calibration levels produced in psicose standard solution: 1.0, 2.5, 5.0, 7.5, and 10 mg L^{-1}. This was done in order to determine the range of the method's applicability. As shown in Figure 4.4, a linear calibration curve for psicose was detected between the peak area and the analyte concentration of the five calibration levels. This may be seen as a correlation between the two variables. The regression coefficient on the calibration curve, denoted by R^2, was 0.9982 As a consequence, the validity of the linearity of the analyte responses across the investigated range was confirmed. The lowest concentration that can be detected is referred to as the LOD, while the lowest concentration that can be quantified is referred to as the LOQ. LOD and LOQ for the chromatographic method can be calculated, however it is more useful determining both parameters for the whole method, it means including the extraction step. Therefore, the lowest concentration of psicose that could be detected using the method that was attempted was 208.6 ng/kg, while the lowest concentration of psicose that could be measured was 695 ng/kg.

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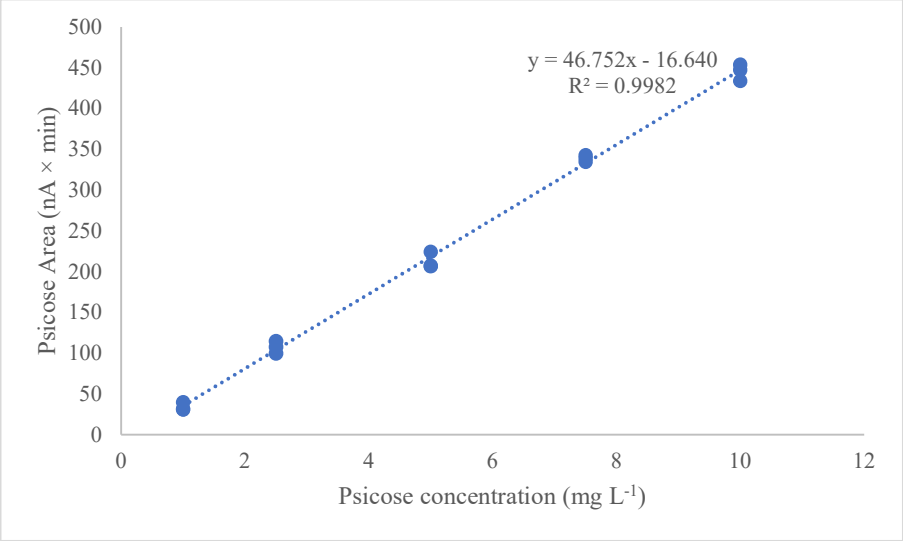


Figure 4 4. HPAEC-PAD calibration curve of psicose in solvent

4.2.3 Accuracy and precision

For the purpose of ensuring accuracy and precision, nine replicate samples of raisins that had been spiked with psicose standard solution at three different concentrations (2, 6, and 10 mg/kg) were examined. Recovery (acceptable range: 80–120 percent) and coefficient of variation (CV) (acceptable when below 10 percent) were used to evaluate a procedure's accuracy and precision, respectively. The acceptable range for recovery was 80–120%. According to Table 4.1, the mean recovery was 101.6 percent, 91.64 percent, and 89.78 percent, and the coefficient of variation (CV) intra-day was 4.80%, while the CV inter-day was 4.34% , respectively.

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Table 4 1 The average recovery and coefficient of variation (CV) results of psicose spiked at 2, 6, and 10 mg/kg (9 replicates)

Spiking levels	Spiking recovery replicates	Recovery (%)	Mean Recovery (%)	CV intra-day (%)	CV inter-day (%)
	Sp_R1	107.24			
Psicose 2 mg/kg	Sp_R2	112.47	101.06		
	Sp_R3	83.46			
	Sp_R4	96.03			
Psicose 6 mg/kg	Sp_R5	68.86	91.64	4.80	4.34
	Sp_R6	110.05			
	Sp_R7	94.54			
Psicose 10 mg/kg	Sp_R8	86.64	89.78		
	Sp_R9	88.18			

4.2.4 Real Sample Applications

After it was demonstrated that the suggested method for HPAEC-PAD with the UAE determination method for psicose in raisins had high precision and accuracy, it was put to use to extract a variety of raisins samples in order to evaluate the method's applicability. This was done in order to determine whether or not the method could be applied to other situations. The fructose stability and psicose stability tests were each carried out on four different samples of raisins, while the method validation tests were carried out on a single sample for each variety, including Thompson, Sultana, Malaga, and Bio. The applicability of the suggested determination method was evaluated with the use of two additional raisins sourced from Indonesia and the United States of America.

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According to the findings of the sample application, our method was successful in measuring one replication of Bio varieties raisins from Spain with a psicose concentration of 520 mg kg^{-1}. The proposed approach could not detect psicose in other raisin samples. However, this results was in agreement with the level range of psicose that previously reported (Oshima et al., 2006)

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CHAPTER 5 : CONCLUSION AND RECOMMENDATION

5.1 Conclusion

- Fructose was stable enough versus a mid-high extraction temperature for 0 –25 min extraction time using proposed UAE method.
- Psicose was stable enough versus a range of extraction temperatures from 10 – 85 °C) in UAE conditions
- The following UAE working conditions can be applied with no degradation of fructose nor of psicose in the UAE working conditions: 25° C extraction temperature, 10 min. extraction time.
- A linearity test, an accuracy test, a precision test, a limit of detection test, and a limit of quantification test were carried out in order to verify the method. The conclusions reached by the ICH were consistent with the findings that were presented. Resulting a LOD value at 208.6 ng kg^{-1} and LOQ value at 695 ng kg^{-1} .
- The only raisin showing psicose content was the one produced under organic conditions at the level of 520 mg kg^{-1}. These findings shown that the method is able to determine psicose content in raisins matrices at levels greater than the LOD of the suggested method of determination. The results was in agreement with the level of psicose reported in the past research reports.

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5.2 Recommendation

- In order to do further research, it may be necessary to use a longer separation column to provide clearer D-psicose peak to avoid the effect of D-fructose boardening peak. If so, the dilution step before the chromatographic analysis could be avoided, then decreasing the LOD by a factor of 25:1 at the least.

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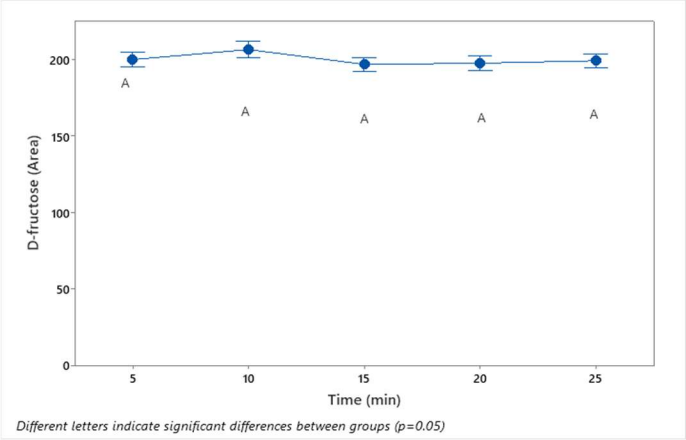
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Appendix 1. Fructose stability data collection (red signed data was an outlier)

Time (min)	Fructosa (Area)	Mean
5	201.706	190.1973
	200.361	
	168.525	
10	207.673	206.9337
	207.201	
	205.927	
15	193.892	197.0900
	195.579	
	201.799	
20	193.892	197.8543
	195.799	
	203.872	
25	197.160	199.4660
	198.930	
	202.308	



Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

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Time (min)	4	132,2	33,05	2,68	0,101
Error	9	111,1	12,35		
Total	13	243,3			

Appendix 2. Psicose stability data collection

Temperature	Psicose Area	Mean
	198.661	
	196.703	
	93.852	
10	104.185	197.682
	194.452	
	204.812	
	192.040	
25	206.068	199.343
	192.299	
	210.309	
	198.883	
40	200.782	200.568
	202.215	
	108.483	
	198.556	
55	186.410	195.727
	198.485	
	211.191	
	205.784	
70	196.138	202.900
	209.650	
	217.928	
	205.635	
85	206.064	209.819

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	5	435,8	87,16	1,88	0,159

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Error	15	697,0	46,47		
Total	20	1132,8			

Appendix 3. Resolution works data collection

Repetition	Fructose - Psicose	Psicose - Lactose
R1	1.5714	1.4308
R2	1.5714	1.4844
R3	1.4776	1.3333
Mean	**1.5401**	**1.4162**

Appendix 4. Psicose calibration curve

Samples	Mean Area
0.1005	3.70
0.2513	7.40
0.5025	16.71
0.7538	26.75
1.0050	34.27
2.5125	107.52
5.0250	212.83
7.5375	338.81
10.0500	444.95

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999664896
R Square	0.999329904
Adjusted R Square	0.999234176

	LOD	**LOD(ppm)**
	6.69	0.1489
LOQ	20.27	0.4512

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Standard Error	4.52342224
Observations	9

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	213601.5	213601.5	10439.27	2.27E-12
Residual	7	143.2294	20.46135		
Total	8	213744.7			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-5.935441246	2.027459	-2.92753	0.022102	-10.7296	-1.141263273	-10.72961922	-1.141263273
X Variable 1	44.93341161	0.439779	102.1727	2.27E-12	43.8935	45.97332353	43.89349969	45.97332353

Appendix 5. Psicose recovery works

Psicose Recovery in Real Samples (Standard added Before Extraction)

No	Sample	Area Value	Standard	Area Value	Spiked Sample	Area Value	%Recovery	Mean %Recovery
4	Raisins	N.D.	Psicose 2 ppm	23.783	S. Psicose 2	25.505	107.24%	101.06%
5		N.D.		30.435	S. Psicose 2	34.231	112.47%	
6		N.D.		29.034	S. Psicose 2	24.233	83.46%	
4		N.D.	Psicose 6 ppm	57.890	S. Psicose 6	55.589	96.03%	91.64%

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5		N.D.		68.163	S. Psicose 6	46.936	68.86%	
6		N.D.		50.513	S. Psicose 6	55.589	110.05%	
4		N.D.	Psicose 10 ppm	132.199	S. Psicose 10	124.977	94.54%	89.78%
5		N.D.		154.192	S. Psicose 10	133.590	86.64%	
6		N.D.		150.211	S. Psicose 10	132.451	88.18%	

N.D. = Not detected

Appendix 6. Psicose precision works

Repeatability

Repetition	Area Value	Mean	SD	RSD	CV
1	139.151	140.3647	6.7379	0.0480	4.80%
2	147.887				
3	149.195				
4	135.303				
5	144.394				
6	135.554				
7	133.331				
8	146.654				
9	131.813				

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Intermediate Precision

Day	Repetition	Area Value	Mean	SD	RSD	CV
1	1	139.151	143.4550	6.2214	0.0434	4.34%
	2	146.654				
	3	154.394				
2	1	144.776				
	2	148.470				
	3	141.313				
3	1	145.027				
	2	133.984				
	3	137.326				

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Appendix 8. GRASEQA Research Posters

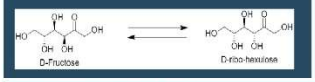
VALIDATION OF A RAPID ULTRASOUND-ASSISTED EXTRACTION COUPLED WITH ION CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF D-PSICOSE IN RAISIN MATRICES

Fahmi Maulana Zulkarnaen [1], Widiastuti Setyaningsih [1] and Miguel Palma [2]

1 Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Gadjah Mada University, Jalan Flora, Bulaksumur, 55281 Yogyakarta, Indonesia.
2 Department of Analytical Chemistry, Faculty of Sciences, IVAGRO, University of Cadiz, (CeIA3), Campus del Río San Pedro, 11510 Puerto Real, Cadiz, Spain.

INTRODUCTION

D-psicose (D-ribo-2-hexose or D-allulose) is an epimer of D-fructose at C3 [1]. With the rise in obesity, diabetes, and other sugar-related health issues, this sugar provides a low-caloria option for sucrose as table sugar. D-psicose provides 70% of sucrose's sweetness and 0.39 kcal g-1 to the body [2]. Small amounts can be recovered from Itea and wheat [3].



D-psicose is rarely formed in nature, although food processing can promote its formation. Time and temperature positively correlate with processed food D-psicose content, according to Oshima et al. Dried figs and kiwis have more D-psicose than raw. Raisins have more D-psicose (up to 38.7 mg 100 g-1) than other dried fruits [4]

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ANALYTICAL METHODS

UAE was conducted using a Bandelin Sonopuls HD 4400 Ultrasonic Probe and a Frigiterm TFT-30 water bath circuit cooler (J.P. Selecta, Barcelona, Spain). High-performance ani-on-exchange (HPAE) chromatography was used (Metrohm 930 Compact I.C. Flex, Gallen, Switzerland). Metrosep Carb 2-150/4.0 was used to separate the components (Metrohm AG, Gallen, Switzerland). MagIC net♀ (Metrohm AG, Switzerland) was used to manage the chromatographic system.



1 gr of a mixture of four grounded raisins sample was diluted with 20 mL HPLC grade water

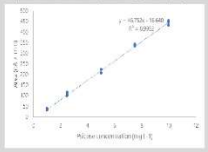
Extraction by UAE with 100 W UAE power, 0.5 s⁻¹ UAE cycle, 25 °C Contraction temperature and 10 min. extraction time

all samples were diluted 1:25 with HPLC grade water and filtered through a 0.45 µm nylon filter

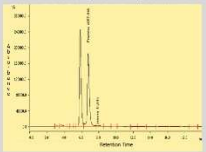
Injection to HPAEC-PAD with a constant injection volume (5 mL)

RESULTS

D-psicose Calibration curve (Linear range 1-10 mg L⁻¹)



Extracted raisins solution spiked with D-psicose standard

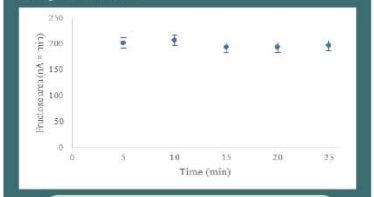


Analytical parameters for HPAEC-PAD (Linear range 1-10 mg L⁻¹)

Validation Parameters		Results
Linear Equation		y=46.752x-10.198
R^2		0.9982
LOD (mg kg⁻¹)		200.6
LOQ (mg kg⁻¹)		605.4
Recovery (%)	Psicose spiked 2 mg kg⁻¹	101.6
	Psicose spiked 6 mg kg⁻¹	91.64
	Psicose spiked 10 mg kg⁻¹	89.78
Intra-day, CV(%) n=9		4.80
Inter-day, CV (%) n=3 x 3		4.34

D-FRUCTOSE STABILITY

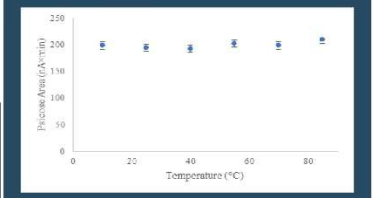
The stability results of D-psicose and its precursor (D-fructose) ensured that UAE as a sample preparation step in the detection procedure did not appreciably modify the analyte in the matrix. The ultrasound energy may allow psicose epimerization from degraded D-fructose after it is extracted from the sample. Therefore, D-fructose stability was checked in the regular solvent used for the UAE in the range from 5 to 25 min.



It was proved that D-fructose was stable unless until 25 min of extraction time at 50 °C

D-PSICOSE STABILITY

In the D-psicose stability investigation, various extraction temperatures were observed to find the UAE extraction temperature that does not produce D-psicose degradation. This observation was done at 10, 25, 40, 55, 70, and 85 °C for a constant extraction time (10 min.).



These studies show that D-psicose may be extracted at temperatures between 10-85 °C using UAE without degradation.

REAL SAMPLE APPLICATIONS

Our method measured one replicate of Bio raisins from Spain with D-psicose concentration of 833,0 ng kg-1. The proposed approach could not detect D-psicose in other raisin samples . Therefore, additional work should be done to be able to determine D-psicose in raisins. However, the current method shows a LOQ lower than the values reported for D-psicose in several food samples with a minimum of 22 mg kg-1 in cereals [4].

CONCLUSIONS

The method's linearity, accuracy, precision, detection limit, and quantification limit were evaluated. ICH's recommendations were followed. D-psicose was found only in one specific commercial raisin sample. These results showed that the methodology can detect and determine D-psicose in raisins matrices and requires only a fast sample preparation. This is the first study validating a method for the determination of D-psicose.

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