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Research article

Grapevine tissues and phenology differentially affect soluble carbohydrates determination by capillary electrophoresis

Daniela Moreno, Federico Berli*, Rubén Bottini, Patricia N. Piccoli, María F. Silva

Instituto de Biología Agrícola de Mendoza, CONICET-Universidad Nacional de Cuyo, Facultad de Ciencias Agrarias, Almirante Brown 500, M5507 Chacras de Coria, Mendoza, Argentina

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ABSTRACT

Soluble carbohydrates distribution depends on plant physiology and, among other important factors, determines fruit yield and quality. In plant biology, the analysis of sugars is useful for many purposes, including metabolic studies. Capillary electrophoresis (CE) proved to be a powerful green separation technique with minimal sample preparation, even in complex plant tissues, that can provide high-resolution efficiency. Matrix effect refers to alterations in the analytical response caused by components of a sample other than the analyte of interest. Thus, the assessment and reduction of the matrix factor is fundamental for metabolic studies in different matrices. The present study evaluated the source and levels of matrix effects in the determination of most abundant sugars in grapevine tissues (mature and young leaves, berries and roots) at two phenological growth stages. Sucrose was the sugar that showed the least matrix effects, while fructose was the most affected analyte. Based on plant tissues, young leaves presented the smaller matrix effects, irrespectively of the phenology. These changes may be attributed to considerable differences at chemical composition of grapevine tissues with plant development. Therefore, matrix effect should be an important concern for plant metabolomics.

1. Introduction

The allocation of carbohydrates in different plant tissues determines the biomass accumulation, and hence crop yield and fruit quality (Calenge et al., 2006; Godt and Roitsch, 2006; Jammer et al., 2015; Roitsch, 1999; Zhu et al., 2007). Also, defines bud fertility since inflorescence induction and development depends on sugars availability (Bennett et al., 2005). The carbohydrates are produced in photosynthetic tissues, and are either stored or transported to different sinks. In grapevine, as in other higher plants, sucrose is the predominant metabolite for carbon transportation, and the partition of sugars is determined by the relative sink strength (Avigad, 1982; Lecourieux et al., 2014; Zapata et al., 2004). Hence, growing leaves, fruits, roots, and other storage organs compete for the photoassimilates (Albacete et al., 2011; Biemelt and Sonnewald, 2006; Xiang et al., 2011; Yang et al., 2004). Considering that the quality of wine is correlated with grape berry metabolic profile, an adequate sugar accumulation in the fruits is desired not only for ethanol production via fermentation, but also for the biosynthesis of compounds related to flavor (Hornsey, 2007). Sugars, especially glucose and fructose, are responsible for the sweet taste of grape juice and wine, and indirectly for ethanol and glycerol wine levels (Hufnagel and Hofmann, 2008).

Carbohydrate analysis is required for a variety of purposes such as food and beverage analysis, but also for the evaluation of plant physiology and metabolic studies. Gas chromatography coupled to flame ionization detection or mass spectrometry are common techniques for sugar analysis; however, multi-step derivatization is required for the sugars to become compatible with these chromatographic modes (Moreno et al., 2011; Murcia et al., 2016). Thus, several analytical approaches have been proposed for their direct determination in plant matrices. Commonly used techniques for underivatized carbohydrates are high performance liquid chromatography (HPLC) with pulsed amperometric detection, refractive index detector or evaporative light scattering detector (Carballo et al., 2014; Cataldi et al., 2000; Eyéghé-Bickong et al., 2012; Ma et al., 2014; Rocklin and Pohl, 1983; Soga et al., 1992). Nevertheless, HPLC is not optimally suited for routine analysis and sample

Abbreviations: BGE, background electrolyte; CE, capillary electrophoresis; HPLC, high performance liquid chromatography.

* Corresponding author.
E-mail addresses: dmoreno@fca.uncu.edu.ar (D. Moreno), fberli@fca.uncu.edu.ar (F. Berli), rbottini@fca.uncu.edu.ar (R. Bottini), ppiccoli@fca.uncu.edu.ar (P.N. Piccoli), msilva@fca.uncu.edu.ar (M.F. Silva).

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pre-treatment is necessary to remove interfering compounds present in complex plant matrices (Oliver et al., 2014; Weitzhandler et al., 1996). Besides, chromatographic methods involve long analysis times, mainly due to lengthy column re-equilibration following the analysis of samples with complex matrices.

Capillary electrophoresis (CE) is a powerful separation technique that can provide high-resolution efficiency with minimal sample preparation. Due to the low cost per analysis, robustness and versatility, CE is becoming a standard tool for the analysis of agricultural interest compounds, even if complex matrices are involved (Landers, 2007). Nevertheless, since carbohydrates lack both a charge and a strong UV chromophore, most of CE approaches involve the complexity of derivatization, a time-consuming step (Guttman, 1997; Guttman et al., 1996; Honda et al., 1989, 1991; Suzuki et al., 2003). Alternatively, CE methods for the analysis of underivatized carbohydrates have been developed. These methods include the use of high alkaline electrolyte to ionize the carbohydrates and they are suitable for indirect UV detection (Cabálková et al., 2004; Jiang et al., 2015; Klockow et al., 1994; Rizelio et al., 2012; Soga and Serwe, 2000; Vorndran et al., 1992). The key advantages of CE over HPLC are that undesirable sample components can be easily and quickly flushed out after analysis. Moreover, a new capillary is much more affordable than a new HPLC column.

It has to be pointed out that matrix effect can be a serious problem in CE analysis of sugars, affecting quantitative analysis and method reproducibility (Piñero et al., 2011). Significant differences in the analytical signals are obtained between standard solutions and doped-samples as the result of chemical and/or physical interactions of sugars and matrix components with the capillary wall (Piñero et al., 2011). Little is known about matrix effect in the determination of soluble carbohydrates by CE with indirect detection. Indeed, there are no evidences concerning its relation with different plant tissues and/or development stages. Thus, the main purpose of the present work is to evaluate the source and levels of matrix effects in the determination of soluble sugars in different plant matrices. The optimized methodology was successfully applied for the determination of glucose, fructose and sucrose in young and mature leaves, berries and roots of a grapevine at the onset of ripening (pre-veraison) and veraison.

2. Material and methods

2.1. Plant material and sugar extraction

One-year-old grapevines of a selected clone of *Vitis vinifera* L. cv. Malbec were cultivated in plastic pots under field conditions (33°0′ S, 68°52′ W, 940 m asl). The vines were shoot-thinned to one shoot per vine, and one cluster was left at flowering. Samples of different tissues were taken at two stages of growth and development, the pre-veraison (stage 35) and veraison (stage 36) (Coombe, 1995). Young leaves (fifth fully expanded leaf from the shoot apex), mature leaves (third leaf from the base of the shoot), berries, and roots were sampled (n = 5).

The procedure for sugar extraction was performed following previous studies (Moreno et al., 2011). Briefly, the extraction was done homogenizing 100 mg of tissue samples with 5 mL of 80:20 (v/v) ethanol:water using a disperser (Ultra-Turrax, T 10 basic; IKA, Staufen, Germany). Then, the mixture was left for 90 min at 70 °C, centrifuged for 10 min at 15,000 *g*, and supernatants were collected.

2.2. Standards and solutions

Glucose and fructose, both ≥95% (Sigma-Aldrich, Milwaukee, WI, USA), and sucrose ≥97.0% (Fluka, Buchs, Switzerland), were used as standards for soluble sugars. Standard solutions containing the three sugars at concentrations between 80 and 500 mg L^{-1} were prepared.

The background electrolyte (BGE) solutions were based on Sugar Analysis Chemical Kit, Beverages, version 20.01.2014 (Lumex Ltd, St Petersburg, Russia; www.lumex.ru). In addition, methanol and acetonitrile (HPLC grade purity, JT Baker, Deventer, Holland) were used. Hexadecyltrimethylammonium bromide (CTAB), hydrochloric acid, potassium sorbate, sodium hydroxide were purchased from Sigma-Aldrich. Solutions were prepared using ultrapure water (18.3 Mm) from a Milli-Q system (Millipore, Paris, France), and stored in amber-colored glass bottles at 4 °C.

2.3. Capillary electrophoresis (CE)

Analyses of underivatized carbohydrates in different grapevine extracts were performed with reverse polarity and dynamic coating using a Capel 105M (Lumex Ltd, St Petersburg, Russia) system equipped with an UV detector and a 0–25 kV high-voltage power supply. Data were collected on a PC configured with Elforun software version 3.2.2. Capillary columns were bare fused-silica capillaries 55 cm effective length with 75 μm ID and 375 μm OD (MOLEX Incorporated, Polymicro Technologies, Phoenix, AZ, USA). Sample supernatants and standard solutions were filtered through 0.45 μm membrane pore size and centrifuged at 10,000 *g* for 5 min, just before being introduced into the capillary by pressure injection at 30 mbar for 5 s. Electropherograms were performed at 254 nm with Indirect UV detection, and all operations were carried out at 20 °C. Then, sugars were determined according to the protocol from Sugar Analysis Chemical Kit with modifications.

2.4. Evaluation of matrix effect

A matrix effect is defined as a change in the analytical signal caused by anything else in the sample other than analyte. The most effective way to evaluate matrix effect affecting trueness and precision of the analytical method is to use the standard addition technique (Stüber and Reemtsma, 2004). Standard addition is especially appropriate when the sample composition is unknown or complex and affects the analytical signal. If small volume of concentrated standard is added, the concentration of the matrix will not be significantly changed (Ćirić et al., 2012).

The matrix effect during the development of the analytical method was examined by comparing the analytical response (peak areas) of an analyte in spiked grapevine tissue extracts with the response of the same analyte present in pure solvent at several concentration levels.

The relative matrix effect was calculated following equation (1), where X1 and X2 are the slopes of each analyte calibration curves in the samples and in pure solvent, respectively (Gomez et al., 2013).

$$\textit{Matrix effect}\ (\%) = 100 - [(X1\ /X2) \times 100] \quad (1)$$

3. Results and discussion

3.1. CE separation

The Lumex protocol for sugar analysis (Sugar Analysis Chemical Kit, Beverages, version 20.01.2014, Lumex Ltd, St Petersburg, Russia) was developed for alcoholic drinks and we found that reproducibility for such approach was not adequate for our plant extracts. Therefore, organic modifiers such as acetonitrile and methanol (2–20% v/v) were used to enhance the reproducibility and resolution. Additionally, we tested the experimental conditions for the

effective separation of the analytes. The following parameters were consecutively optimized: sample conditioning, pH, BGE composition and concentration, organic modifiers, sample and capillary temperatures, separation voltage and injection mode. The optimization of the experimental conditions was accomplished by the traditional method of one-at-a-time, being the analytes separated in less than 7.50 min. A BGE of 7 mM potassium sorbate, 0.50 mM CTAB, 50 mM NaOH and 10% (v/v) methanol, pH = 12.80 found to be optimal. Indirect detection was performed at 254 nm and other electrophoretic parameters were as follows: capillary: 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD; hydrodynamic injection: 30 mbar, 5 s; −20 kV constant voltage and 20 °C temperature. Fig. 1 shows characteristics CE electropherograms for 500 mg L^{-1} of sugars standards (glucose, fructose and sucrose) added to pure solvent and in the extracts of the different tissues matrices at veraison.

3.2. Matrix effect

Usually the matrix effect is observed when a significant difference in response is obtained between standards prepared in solvent and those prepared in the matrix extract. This effect can be positive, leading to an increase in the electrophoretic signal or negative, when there is a decrease of this signal. These changes are the result of adsorption of analytes and matrix components in the capillary wall or other instrument components. The origin and mechanism of matrix effects are not fully understood. Consequently, after selecting the proper electrophoretic approach for each sample, the effect of the matrix was assessed by comparing the signal of the analytes in pure solvent to the signal in the different sample matrices. As can be seen in Fig. 1, we found that the different matrices caused changes in the baseline of electropherograms as well as peak areas of the analytes. Nonetheless, no differences at migration times of carbohydrates were detected for all matrices.

For each compound (fructose, glucose and sucrose), calibration curves were obtained in pure solvent and in the extracts of the matrices (leaves, berries, and roots) in the concentration range from 10 to 500 mg L^{-1}. We found differences on intersect as well as slopes of all analytical curves (solvent × matrix). As an example, Fig. 2 shows the linear regressions for fructose peak areas at different concentrations, added to pure solvent and different tissues at pre-veraison and veraison. Similar results were observed for the other carbohydrates studied. In all cases the coefficients of determination were above 0.90. The difference in the slopes was attributed to a proportional systematic error, caused by matrix components (De Sousa et al., 2012).

Matrix effect was observed for all the plant tissues under study, and interestingly, those effect were also different for each analyte and developmental stage (Table 1). No signal enhancement, but response reductions within the range 19–57% due to matrix interference was observed except for young leaves at pre-veraison stage. Sucrose was the sugar showing the least matrix effects, being fructose the analyte most affected. Concerning the different tissues, young leaves presented the smaller matrix effects, irrespectively of phenology. Consequently, quantification was carried out following the standard addition method.

These changes are the result of chemical and/or physical interactions of sugars and matrix components with the capillary wall. Differences may be to the fact that the chemical composition of leaves, fruits and roots, as in all grapevine tissues, varied considerably with plant development (Vivin et al., 2003). For example,

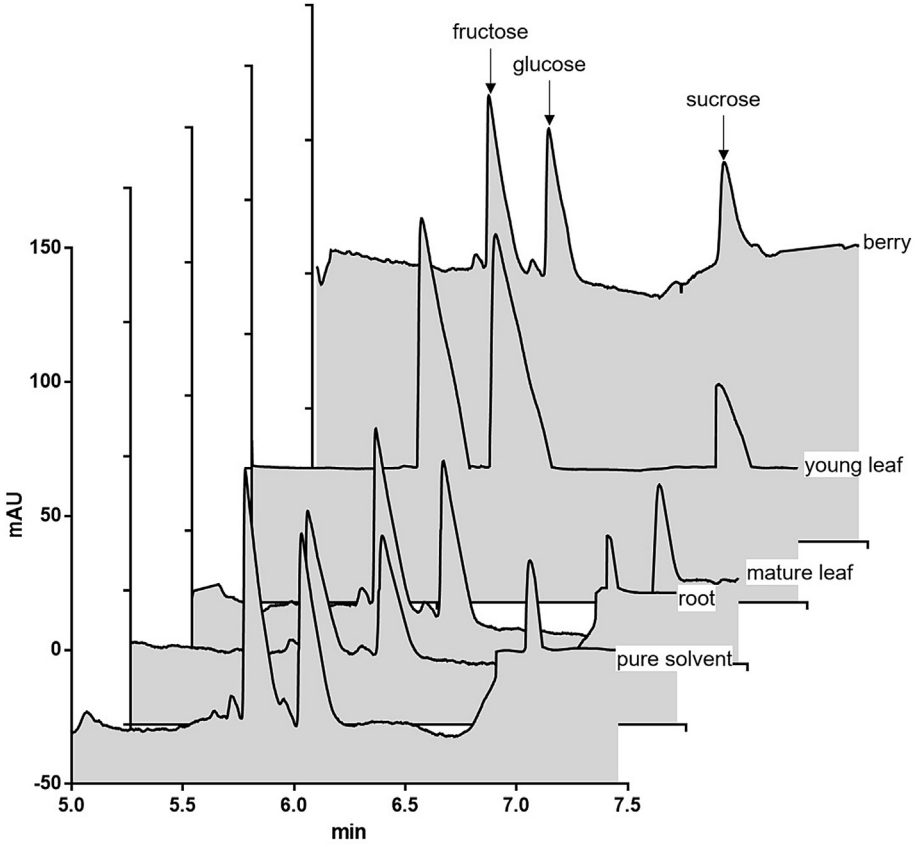

Fig. 1. Characteristic CE electropherograms for 500 mg L^{-1} of sugars standards (glucose, fructose and sucrose) added to pure solvent and in the extracts of the matrices at veraison (mature leaf, young leaf, berries and roots).

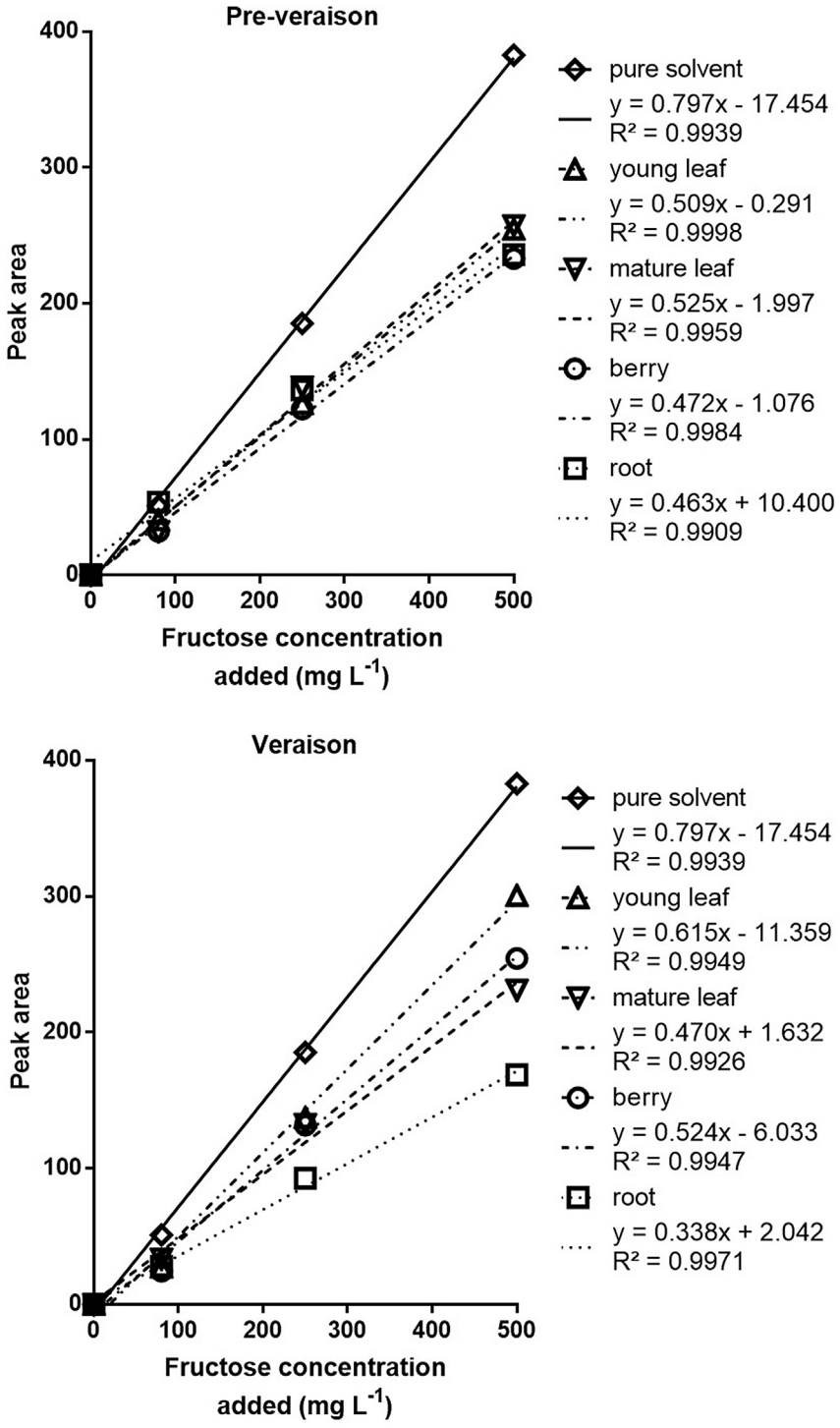

Fig. 2. Linear regressions for fructose peak areas at different concentrations, added to pure solvent and different tissues matrices (mature leaf, young leaf, berries and roots) at pre-veraison and veraison.

leaf and berry proteins decrease during maturation, while phenolic compounds increase (Vivin et al., 2003). Also, organic acids and lipids rises with ripening in leaves, while declines in fruits. Interestingly, the chemical nature of the sugar also influences matrix effect, and it could be attributable to the fact that sucrose (the least affected) is a non-reducing disaccharide without free aldehyde and ketone groups. In berries, the concentration of sugars increase dramatically at veraison, partly diluting fractions of others compounds as well as organic acids (Ruffner, 1982), and at harvest, carbon reach up to 50% of berry dry weight (Ollat and Gaudillere, 2000; Vivin et al., 2003). Soluble sugars represents the major proportion of this carbon, in addition to structural carbohydrates, proteins and amino acids (Coombe, 1992; Dai et al., 2010; Vivin et al., 2003).

Table 1
Matrix effect (%) on soluble carbohydrates for different grapevine tissues at two phenological stages by standard addition method. Values are means (n = 5).

Tissue	Phenological growth stage	Matrix effect (%)		
		Fructose	Glucose	Sucrose
young leaf	pre-veraison	36.139	26.217	0.172
mature leaf	pre-veraison	26.179	24.124	5.401
berry	pre-veraison	40.768	43.648	22.807
root	pre-veraison	41.884	33.672	44.892
young leaf	veraison	22.830	22.982	19.161
mature leaf	veraison	41.031	40.508	17.785
berry	veraison	34.270	35.067	30.444
root	veraison	57.489	50.278	28.965

Table 2
Soluble carbohydrates concentration (mg L^{-1}) for different grapevine tissues at two stages of growth and development. Values are means ± SEM (n = 5).

Tissue	Phenological growth stage	Fructose (mg L^{-1})	Glucose (mg L^{-1})	Sucrose (mg L^{-1})
young leaf	pre-veraison	550.10 ± 89.64	543.15 ± 149.29	794.41 ± 150.84
mature leaf	pre-veraison	754.50 ± 99.17	718.53 ± 115.63	78.97 ± 24.23
berry	pre-veraison	283.25 ± 12.67	461.70 ± 77.58	0.00 ± 0.00
root	pre-veraison	78.40 ± 14.82	123.62 ± 25.36	66.25 ± 8.54
young leaf	veraison	663.65 ± 52.94	1209.02 ± 84.53	982.43 ± 61.25
mature leaf	veraison	783.20 ± 126.55	1323.29 ± 62.83	235.79 ± 18.81
berry	veraison	7249.00 ± 520.28	11,178.80 ± 852.81	0.00 ± 0.00
root	veraison	431.68 ± 116.55	1121.35 ± 155.17	320.51 ± 30.74

3.3. Analytical performance and samples analysis

In order to estimate the intra-day repeatability, and intermediate precision, pure as well as spiked samples were analyzed under the selected optimal conditions: 5 blank samples, three replicate measurements at 80, 250 and 500 mg L^{-1} sugar concentration levels; were analyzed. In all cases repeatability was better than 6.48% for peak area. The same experiment was repeated on four other independent occasions with at least one week interval. The overall within-laboratory intermediate precision ranged from 1.60 to 10.70% for peak areas at the tested concentration levels.

Taking into account that a certified reference material of the studied matrices with an informed value for soluble carbohydrates does not exist, the trueness of the measurements was evaluated through recovery of additions of known amounts of the analytes to samples. For this purpose, a pool of samples for each plant tissue was used. The studies were satisfactory leading recoveries higher than 86.30% and lower than 106.60%. In summary, and taking into account the matrix complexity, the reported values for the method assessment parameters could be considered highly satisfactory.

Once the optimal conditions were established for the different matrices under study, the developed methodologies were applied to the analysis of berry, leaf and root samples at pre-veraison and veraison stages. Table 2 shows the endogenous sugar concentration found for each type of sample, at different phenological stages.

The glucose to fructose ratio in berries at the stages of pre-veraison and veraison was 1.63 and 1.54, respectively. During berry ripening, glucose/fructose change due to the action of an epimerase, being on the order of 1.5 at veraison, and then drops below 1 at full maturity (Ribéreau-Gayon et al., 2006). The sugars, synthesized in the leaves, migrate exclusively as sucrose through the phloem to the berries, where they are mostly hydrolyzed to monosaccharides.

4. Conclusions

Capillary zone electrophoresis with reversed polarity and dynamic coating proved to be a robust, low cost and versatile methodology for the analysis of underivatized carbohydrates in different grapevine extracts. Taking into account that matrix effect was observed for all the plant tissues under study, quantification was carried out following the standard addition method.

The results found in this study clearly demonstrate that the deep evaluation of matrix effect should be an integral and important issue of quantitative determination of carbohydrates at different plant tissues and/or developmental moments. These changes are the result of chemical and/or physical interactions of sugars and matrix components with the capillary wall. The chemical nature of the analytes as well as differences of matrix components of the plant tissues and phenology showed marked influence on matrix effects. Optimal conditions for the extraction, separation and quantitation should be carefully settled and exhaustively controlled for each sugar and each plant tissue.

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