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A Simplified High-Performance Liquid Chromatography–Mass Spectrometry Method for Quantifying Soluble Sugars in Plant Leaves: Comparative Extraction Evaluation With Improved Sensitivity

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ABSTRACT

The development of rapid and reliable methods for soluble sugar analysis is essential for routine applications in plant physiology, ecology, and agriculture. Conventional techniques for sugar quantification often require dedicated detectors, derivatization steps, or lengthy sample preparation, limiting throughput and sensitivity. This study presents a fast, high-performance liquid chromatography–mass spectrometry method for the simultaneous determination of sucrose, glucose, and fructose in plant leaves. Chromatographic separation was achieved on a Rezex column with gradient elution, and detection was performed in negative electrospray ionization mode using selected ion recording. The method demonstrated excellent linearity ($R^2 \geq 0.99$), high precision (coefficient of variation $\leq$ 2%), and recoveries of 98%–102%. Limits of detection and quantification were 0.8 and 1.6 μg mL^{-1}, respectively, markedly improving sensitivity compared to conventional ultraviolet- or refractive index-based approaches. Several extraction protocols were evaluated, including water, methanol, and ethanol under thermal or ultrasonic conditions. Ultrasonic extraction with ultrapure water provided the highest efficiency, minimal matrix effects, and simplified sample preparation. The optimized workflow enables the preparation of multiple samples within a single day and overnight chromatographic analysis, offering a practical, cost-effective, and high-throughput solution for routine soluble sugar quantification in plant tissues.

1 | Introduction

Soluble sugars, including glucose, fructose, and sucrose, are central primary metabolites in plants, serving as sources of energy and carbon skeletons for growth and development [1]. Accurate quantification of soluble sugars is therefore essential for understanding plant physiology, carbon allocation, and responses to both abiotic and biotic stresses [2]. Traditional methods for sugar analysis include colorimetric assays, gas chromatography (GC), and high-performance liquid chromatography (HPLC) with sugar–dedicated detectors, i.e., evaporating light scattering detection (ELSD) and refractive index detection (RID) [3]. However, these methods either require specific detectors with limited sensitivity (ELSD and RID), lack specificity (colorimetry), or involve derivatization steps (GC), which are time–consuming and can introduce variability into the final data [4]. Moreover, these approaches often suffer from limited sensitivity (limit of detection [LOD] 0.5–1 mg mL^{-1}), long sample preparation times, and poor selectivity in complex matrices. Ultraviolet (UV)-based detection is widely used but is often inadequate for trace–level sugars [5]. Advanced techniques like HPLC coupled with mass spectrometry (MS) may offer higher sensitivity and selectivity,

enabling precise detection of individual sugars even at low concentrations.

This study presents an HPLC method employing a cost–effective single–quadrupole MS detector for quantifying soluble sugars in plant leaves. The effectiveness of common methods to extract soluble sugars was compared, i.e., with ice–cold ultrapure water or with mixtures of water with ethanol or methanol [6, 7]. Additionally, the effects of extraction temperature (80 ° C incubation) and ultrasonic treatment, as proposed by [8], were compared. The results indicate that ultrapure water combined with ultrasonic extraction provided the highest efficiency, outperforming extraction at 80 ° C. Moreover, optimization of the chromatographic conditions for sugar separation and quantification is presented. A Rezex column was employed to achieve effective separation of monosaccharides, and a post–column addition of 50% acetonitrile with 10 mM ammonium acetate was applied to enhance ionization in negative ion mode. Using this optimized protocol, researchers can prepare at least 20 samples for extraction and complete HPLC–MS runs overnight, obtaining results the following day. Plant material milled in liquid nitrogen and stored at –80 ° C is recommended, allowing the remaining material to be used for additional analyses.

2 | Materials and Methods

2.1 | Chemicals and Materials

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile, ammonium acetate, methanol, ethanol, and water were of LC–MS grade. Lettuce (*Lactuca sativa*) and barley (*Hordeum vulgare*) were cultivated in pots in controlled conditions.

2.2 | Instrumentation and Conditions

The method uses the following equipment: vortex (vortex Genie 2, Scientific Industries, NY, USA), centrifuge (Sigma 1–14K, Osterode am Harz, Germany), ultrasonic bath (VWR International, PA, USA), heat block with evaporation system (Dri–Block, TECHNE, UK), HPLC–MS (Arc HPLC Waters with UV detector, and qDa Waters mass detector, Waters, Milford, USA).

Conditions of elution and MS: The samples were run on HPLC–MS with Rezex RPM Pb^{2+} column (300 × 7.8 mm, 8 µm, Phenomenex, Torrance, CA, USA) with gradient elution (A: 10% acetonitrile in water, B: 30% acetonitrile in water), starting from 100% A, from 10 min to 25 min reaching 50% A and in 30 min back 100% A. Flow rate 0.6 mL min^{-1}. The column oven was adjusted to 65 ° C, and the UV detector was set to 190 nm. Before reaching the mass detector, HPLC column effluent was mixed via an isocratic solvent pump manager (ISM, Waters) with a solvent consisting of 50% acetonitrile (in water) with 10 mM ammonium acetate to improve ionization of the samples without affecting separation. Moreover, the addition of ammonium acetate enhances signal stability and reproducibility and lowers background in electrospray ionization (ESI) [9, 10]. A mass detector with ESI was used in negative mode with a cone voltage of 15 V, probe temperature of 600 ° C (temperature recommended by the manufacturer of Waters qDa mass spectrometer), and capillary voltage of 0.8 kV. The full mass scans were collected in a range of *m/z* 150–450. HPLC–MS was run on EMPOWER 3.6 software (Waters Corporation, Milford, MA, USA). Peak areas, mass–to–charge ratios, and key fragmentation ions were analyzed with ACD Labs software (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

2.3 | Sugar Extraction

The plant material was snap–frozen on dry ice, transported to the laboratory, ground in liquid nitrogen, and stored in –80 ° C freezers. 100 mg of the plant material was weighed into 2 mL Eppendorf tubes to which 1 mL of ice–cold extractant (water or 60 % methanol or 60 % ethanol) was added. Samples were vortexed for 10 s and incubated at 80 ° C or in an ultrasonic bath for 20 min to extract soluble sugars. After incubation, samples were centrifuged (12 000 × g, 3 min) and the extraction was repeated. Supernatants were combined. Ethanol/methanol was evaporated under a stream of N_2 in a heat block at 40 ° C. Water was added to adjust the volume; in this way, soluble sugars extracted with methanol or ethanol were dissolved in pure water before analysis, with no traces of other solvents. Samples were then filtered through a 0.2 µm polytetrafluoroethylene (PTFE) filter into HPLC vials with inserts (VWR, Cat no. 548–0425) and analyzed in HPLC–MS in conditions described above.

2.4 | Optimized Final Method

One hundred grams of plant material ground in liquid nitrogen was weighed into 2 mL Eppendorf tubes, and 1 mL of ice–cold water was added. Samples were vortexed for 10 s and incubated in an ultrasonic bath for 20 min to extract soluble sugars. After incubation, samples were centrifuged (12 000 × g, 3 min) and the extraction was repeated. Supernatants were combined. Samples were then filtered through a 0.2 µm PTFE filter into HPLC vials with inserts (VWR, Cat no. 548–0425) and analyzed in HPLC–MS with Rezex RPM Pb^{2+} column (300 x 7.8 mm, 8 µm, Phenomenex, Torrance, CA, USA) with gradient elution (A: 10% acetonitrile in water, B: 30% acetonitrile in water), starting from 100% A, from 10 min to 25 min reaching 50% A and in 30 min back 100% A. Flow rate 0.6 mL min^{-1}. The column oven was adjusted to 65 ° C. Before reaching the mass detector, HPLC column effluent was mixed via an ISM (Waters) with a solvent consisting of 50% acetonitrile (in water) with 10 mM ammonium acetate. A mass detector with ESI was used in negative mode with a cone voltage of 15 V, probe temperature of 600 ° C, and capillary voltage of 0.8 kV. The MS detector was operated in selected ion recording (SIR) mode, monitoring *m/z* 179 ($[M–H]^-$) for glucose and fructose and *m/z* 341 ($[M–H]^-$) for sucrose.

3 | Results and Discussion

3.1 | Comparison of Extraction Techniques

Extraction efficiency was compared using an ultrasonic bath and a heat block (80 ° C) with three solvents: ultrapure water, methanol, or ethanol. The ultrasonic bath was more effective

TABLE 1 | Comparison of soluble sugar extraction methods conducted on plant leaves of lettuce and barley, in mg g^{-1} FW (fresh weight). Standard deviation from four replicates in brackets. The results of the most effective extraction are marked in italic.

	Ultrasonic bath			Heat block, 80°c		
	Ultrapure water	60% Methanol	60% Ethanol	Ultrapure water	60% Methanol	60% Ethanol
Lettuce						
sucrose	*3.41*(0.01)	3.27(0.02)	3.25(0.03)	3.40(0.01)	2.99(0.02)	3.00(0.10)
glucose	*2.94*(0.03)	1.58(0.03)	1.54(0.04)	2.65(0.02)	1.83(0.05)	1.73(0.06)
fructose	*2.61*(0.02)	1.61(0.02)	1.51(0.03)	2.41(0.04)	1.43(0.05)	1.34(0.06)
Barley						
sucrose	*2.50* (0.05)	2.28(0.05)	2.05(0.03)	2.10(0.05)	1.97(0.05)	1.78(0.07)
glucose	*2.04*(0.04)	1.07(0.04)	1.00(0.02)	2.01(0.04)	1.00(0.05)	0.93(0.05)
fructose	*1.91*(0.03)	1.12(0.03)	0.99(0.03)	1.42(0.06)	0.93(0.05)	0.84(0.05)

than the heat block, and ultrapure water was more effective than methanol or ethanol (see Table 1). Moreover, extraction with ultrapure water in an ultrasonic bath was faster, due to two reasons: a) no need for cooling down after incubation at 80 ° C, b) no need for evaporation of methanol or ethanol from the supernatant.

3.2 | Method Development and Optimization

A Rezex column was selected for sugar analysis due to its high selectivity for monosaccharides and oligosaccharides, providing sharp, well–resolved peaks. Moreover, this column functions even in 100% water conditions. Gradient flow with acetonitrile separated sugars better, providing narrower peaks (see Figure S1). The flow rate range of 0.4–1 mL min^{-1} and column temperature range of 25–65 ° C were tested, and a flow rate of 0.6 mL min^{-1} at 65 ° C was found optimal (see Figure S2 for the effect of temperature).

Although soluble sugars can be quantified using a UV detector, as shown by [3] for honey samples using a UV detector operating at 190 nm, we also tried this option. However, the quality of chromatograms showed poor quality (see Figure S3), and reliable quantification was not possible at sugar concentrations below 0.5 mg mL^{-1}. Therefore, quantification of soluble sugars using an MS detector is proposed. Both positive and negative ESI modes were evaluated for the analytes. Negative ESI was preferred, as it produced approximately threefold higher signal intensity and simpler spectra with fewer detected *m/z* signals (see Figure S4). To achieve the highest sensitivity, the MS detector was operated in SIR mode, monitoring *m/z* 179 ($[M–H]^-$) for glucose and fructose and *m/z* 341 ($[M–H]^-$) for sucrose. Post–column addition of ammonium acetate may promote the formation of acetate adducts ($[M + CH_3COO]^-$), resulting in additional ions at *m/z* 239 for glucose and fructose and *m/z* 401 for sucrose. Chromatographic runs using 50% acetonitrile with ammonium acetate were compared to runs using 50% acetonitrile without ammonium acetate. As signal intensity was lower in the absence of ammonium acetate, the use of 50% acetonitrile with ammonium acetate is proposed for the quantification of soluble sugars.

Reducing sugars can undergo interconversion between their α and β anomeric forms (mutarotation) in aqueous solution, and under chromatographic conditions where the rate of interconversion is comparable to or slower than the elution time, this phenomenon can lead to multiple or broadened peaks for a single sugar compound [11]. In the proposed method, the absence of separate α– and β–anomer peaks is attributable to the Rezex column. Its ligand–exchange mechanism, combined with rapid mutarotation at 65 ° C, effectively averages the sugar forms during elution. Furthermore, detection by SIR monitoring (*m/z* 341 and 179) does not distinguish anomers, resulting in a single, sharp peak for each sugar.

Representative chromatograms and MS scans are shown in Figure 1, and key fragment ions of the sugars are provided in Figures S5–S7.

3.3 | Method Validation

Method specificity was assessed using plant leaf extracts containing endogenous sugars. Spiked extracts, prepared by adding known amounts of the target sugars to the leaf matrix, showed clear analyte peaks at the expected retention times with no additional signals, confirming that the method can reliably detect and quantify the sugars in the presence of the natural matrix (see Figure 1D for a mixture of standards, Figure 1E for plant extract with no added sugars, thus only with endogenous ones). The method exhibited linearity with correlation coefficients (R^2) ≥ 0.99 over the range of 0.0005–1 mg mL^{-1} for sucrose and 0.001–1 mg mL^{-1} for other sugars (Table 2). Accuracy was evaluated by spiking plant leaf extracts with known amounts of sugars at three concentrations. Recoveries ranged from 98%–102% for fructose and glucose, and for sucrose, 99%–101% (calculated for both plant species), demonstrating that the method provides reliable quantification of the analytes in the presence of the natural matrix. Precision was evaluated at three concentrations (0.01, 0.1, and 1 mg mL^{-1}), with intra- and inter-day coefficient of variation (CV%) of 2.2% (fructose and glucose) and 1.7% (sucrose). LOD and limit of quantitation (LOQ) were determined with the signal– to–noise ratio for each of the standards based on peak area with the MS detector. LOD was set at 3:1 and LOQ at 10:1 signal–to–noise, respectively (see Table 2). Matrix effects were evaluated by comparing analyte responses in neat solution (in the case of the proposed method, sugars in water) versus spiked matrix (formed by the addition of soluble sugars in 0, 0.1, 0.5,

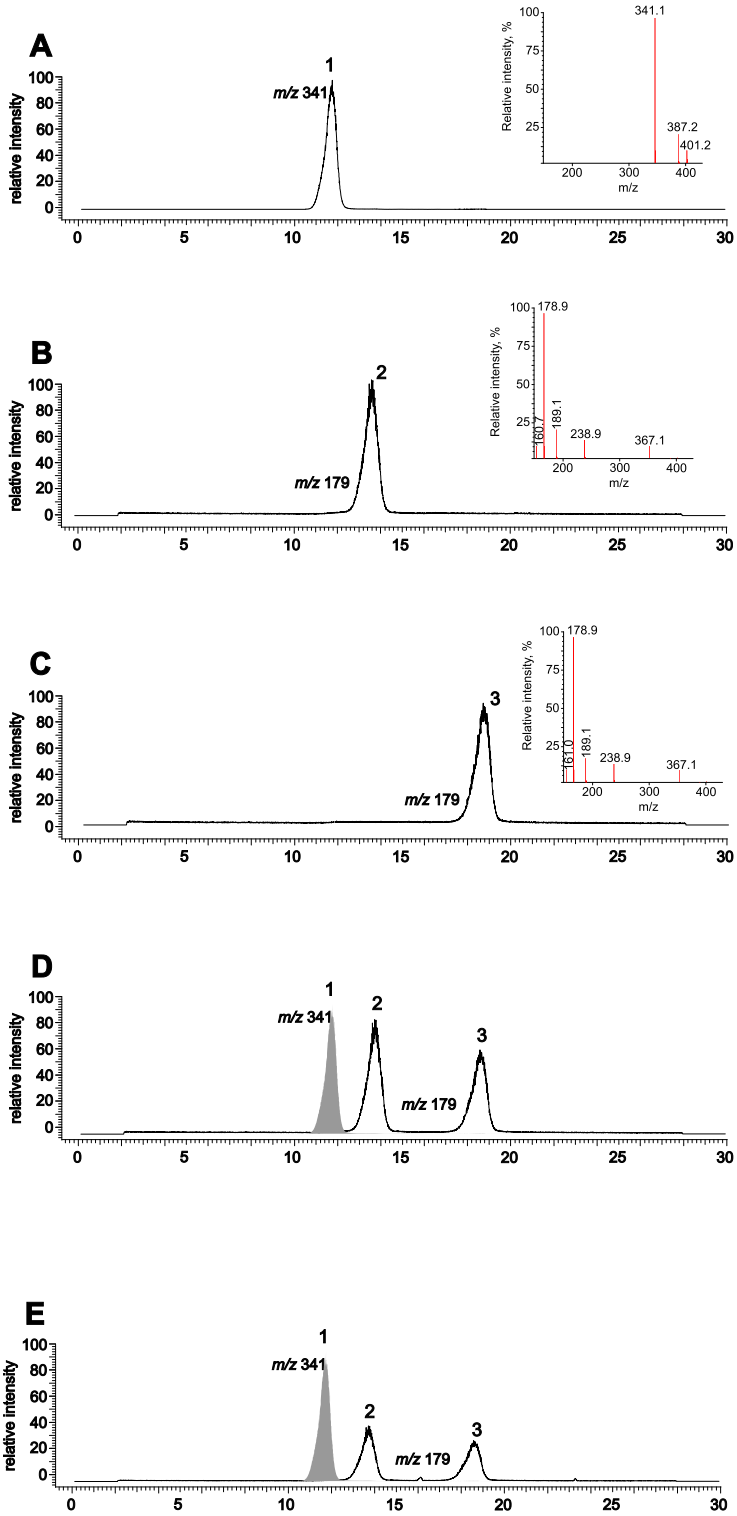

FIGURE 1 | Selected ion chromatograms (SIR) and mass spectra of soluble sugar standards and plant leaf samples. (A) SIR chromatogram of sucrose with corresponding mass spectrum, (B) SIR chromatogram of glucose with corresponding mass spectrum, (C) SIR chromatogram of fructose with corresponding mass spectrum, (D) overlaid SIR chromatograms of soluble sugar standards (1 mg mL^{-1} each), and (E) overlaid SIR chromatograms of soluble sugars extracted from lettuce leaves using water and ultrasonic bath. concentrations in lettuce: 3.4, 2.9, and 2.6 mg g^{-1} FW (fresh weight), respectively. Peak assignments: 1 – sucrose, 2 – glucose, 3 – fructose.

TABLE 2 | Limits of detection and quantitation, and linearity for high-performance liquid chromatography–mass spectrometry (HPLC–MS) detection.

Compound	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Linearity (R^2)
Sucrose	0.25	0.50	0.9987
Glucose	0.75	1.50	0.9966
Fructose	0.80	1.60	0.9977

and 1 mg mL^{-1} into 100 mg of plant material, both plant species separately). Matrix effect was calculated as the peak area in the spiked matrix divided by the peak area in the neat solution (water) multiplied by 100. The matrix effect % was 97.5% for fructose and glucose and 98% for sucrose. Minimal matrix effects were observed, likely due to the use of water as the extraction solvent, which selectively extracts the target sugars while limiting co–extraction of interfering compounds. The stability of sugars in plant leaf extracts was evaluated under short–term (room temperature, 24 h), autosampler (12 h), and long–term (−20 °C, 30 days) conditions. Measured concentration remained within ±5% of the initial values, indicating that the analytes are stable under routine sample handling and storage conditions.

3.4 | Comparison With Other Methods

Traditional approaches for soluble sugar analysis, including colorimetric assays, GC, and HPLC with RID or ELSD, have been widely used but present several limitations [3]. Colorimetric assays lack specificity and are prone to interference from other plant metabolites [12], while GC requires derivatization steps that are time–consuming and can introduce variability [13]. HPLC with RID or ELSD avoids derivatization but suffers from limited sensitivity and poor performance at low analyte concentrations (<0.5 mg mL^{-1}), and these detectors are often less robust in complex plant matrices [14]. UV-based detection, although convenient, generally provides insufficient sensitivity for trace–level sugars and often produces broad or poorly resolved peaks [15]. In contrast, coupling HPLC with MS, as implemented in the current study, significantly improves both sensitivity and selectivity. Using SIR mode at m/z 179 and 341, sugars can be quantified unambiguously even at sub-mg mL^{-1} concentrations, with minimal matrix interference. Furthermore, the combination of a Rezex column with post-column addition of ammonium acetate ensures sharp, well–resolved peaks, effectively averaging α- and β-anomers, which are often partially resolved in traditional chromatographic approaches [11]. Compared with previous reports, the optimized method provides faster sample preparation, reduced solvent use, and the ability to process multiple samples overnight, while achieving lower limits of detection and higher reproducibility. Overall, the presented HPLC–MS approach demonstrates clear advantages over classical colorimetric, GC, and non–MS HPLC methods, offering a practical and robust solution for routine quantification of plant soluble sugars.

4 | Conclusions

In conclusion, this study demonstrates that fast HPLC–MS provides an efficient and reliable approach for the simultaneous detection of sucrose, glucose, and fructose in plant leaves. Among the tested protocols, extraction with ultrapure water assisted by an ultrasonic bath proved to be the most effective for recovering soluble sugars. The use of a mass detector further enhanced sensitivity compared with conventional methods. The optimized HPLC–MS/SIR method allows direct, high-sensitivity quantification of soluble sugars, minimizing sample preparation and achieving lower limits of detection than commonly reported methods. Together, these optimized extraction and detection strategies offer a practical and time–saving solution for routine sugar analysis in ecological and agricultural research.

Author Contributions

Sylwia Adamczyk: data curation, formal analysis, investigation, visualization, and writing – original draft. **Bartosz Adamczyk**: conceptualization, funding acquisition, methodology, supervision, validation, and writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: sscp70219-sup-0001-SuppMat.pdf.