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ARTICLE

Qualitative and Quantitative Sugar Profiling in Olive Fruits, Leaves, and Stems by Gas Chromatography−Tandem Mass Spectrometry (GC-MS/MS) after Ultrasound-Assisted Leaching

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Qualitative and quantitative profiling of sugars in vegetal materials from *Olea europaea* cultivars is here reported. Vegetal tissues from olive fruits, leaves, and stems have been characterized by determination of 22 compounds belonging to monosaccharides, disaccharides, trisaccharides, sugar carboxylic acids and alcohols, cyclic polyols, and derived compounds. Sugar isolation was carried out by leaching into a 2:1 dichloromethane/methanol extraction solution under ultrasonic assistance. Multivariate optimization made possible complete isolation of the target fraction in 10 min with an efficiency similar to that provided by a conventional protocol based on 24 h maceration of the vegetal samples. An aliquot of the extract was dried and reconstituted for silylation prior to GC-MS/MS analysis for selective and sensitive identification/quantitation of sugars. Monitoring the target product ions generated after isolation of the precursor ions for each analyte increases the selectivity of the method. The proposed approach is of particular interest for characterization of the sugar fraction in *O. europaea*, which is of great relevance because of the role of sugars in the metabolism of lipids, proteins, and antioxidants.

KEYWORDS: Carbohydrates; *Olea europaea*; GC-MS/MS; ultrasound-assisted extraction

INTRODUCTION

The economic and social importance of *Olea europaea* in the Mediterranean basin is well-known. Apart from the olive oil industry, there are other industries derived from this cultivar such as cosmetics or biodiesel industries. At present, most of the studies about *O. europaea* are focused on olive oil and fruit composition, but other raw materials, such as leaves and stems, are not so frequently characterized. These materials are at present of great interest because of their potential as agricultural resources for the isolation of natural products such as phenols (*1*), polyhydroxylated compounds (*2*), or terpenic acids (*3*), among others.

Carbohydrates are photosynthesized in green plants initially as monosaccharides, which are then transformed into disaccharides, trisaccharides, and sugar alcohols. The primary function of sugars is as energy source for metabolic changes or as molecular precursors in the biosynthesis of lipids, proteins, antioxidants, and polysaccharides. Thus, olive ripening is characterized by a series of transformations in which sugars are especially involved. Patumi et al. (*4*) have reported a positive relationship between the oil content and the concentration of these precursors involved in lipid biosynthesis during maturation of olive fruit. A similar study suggested that the oil content of olive fruit depends on the metabolic activity of the given olive variety to convert substrates into oil (*5*). Another example corresponds to the processing of table olives, during which sugars act as carbon source for microorganisms to release secondary metabolites responsible for positive taste and distinctive flavor.

Sugars are the main soluble components in olive tissues of leaves and fruits, playing a key role in the cell-wall structure. Nonstructural carbohydrates, among other solutes, act as osmoregulators and osmoprotectors of the tolerance response to abiotic stresses. As an example, water deficiency, saline water, or soils with high salt content generate complex plant responses at the molecular level evidenced by biosynthesis, transport, and accumulation of osmolytes. For this reason, it is worth elucidating the conversion, storage, and transport patterns ascribed to soluble sugar compounds including alditols (*myo*-inositol, mannitol, sorbitol, dulcitol, galactinol, etc.) and saccharides (glucose, galactose, fructose, sucrose, raffinose, stachyose, etc.). For these purposes, qualitative and quantitative methods capable of monitoring a representative sugars profile under external stimuli are presently demanded.

Most methods for sugars analysis from plant extracts are based on chromatographic separation techniques. The main limitation of LC for sugars analysis is lower resolution at high-performance level as compared to GC and, thus, long gradients or stationary phase innovations are required (*6*, *7*). On the contrary, the resolution of GC is superior, although a derivatization step is required to convert sugars into volatile compounds such as acetyl trimethylsylil (TMS) ethers or oxime-TMS ethers. Detection is usually

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Table 1. Parameters of the GC-MS/MS Method[a]

segment	compound	ion preparation type	RT	MW	quantifier	excitation storage level	excitation amplitude	qualifier
3	D-(−)-arabinose	SRM	19.69, 19.75, 20.33	150	217	76.5	60	129, 143, 147
3, 4	L-rhamnose	SRM	19.96, 21.19	182	204	71.8	50	143, 147, 163
4	xylitol	SRM	21.39	152	319	90.2	60	129, 147, 243
4	1,6-anhydro-β-D-glucose	SRM	21.45	162	333	117.5	60	143, 171, 333
4, 5	D-(+)-xylose	SRM	21.56, 22.50	150	204	71.8	50	129, 143, 147
4	L-(−)-arabitol	SRM	21.65	152	217	76.5	60	129, 147, 149
4	adonitol	SRM	21.73	152	319	90.2	60	129, 147, 243
5	D-(+)-mannose	SRM	22.85	180	204	71	50	149, 151, 163
5, 6	D-(−)-fructose	SRM	23.14, 23.29, 24.79	180	437	120	60	229, 257, 345
5, 6	D-(−)-galactose	SRM	24.10, 24.98	180	435	120	60	218, 305, 331
6, 8	D-(+)-galacturonic acid	SRM	24.26, 26.83	212	217	76.5	60	129, 143, 152
6, 8	D-(+)-glucose	SRM	24.83, 26.75	180	204	71.8	50	143, 152, 163
7	D-mannitol	SRM	25.21	182	346	120	80	183,255,183
7	sedoheptulose	SRM	25.32	210	375	120	90	155,183, 255
7	D-(+)-chiro-inositol	SRM	25.85	180	319	90.2	80	147,255, 317
8	D-glucuronic acid	SRM	26.10	194	305	107.6	80	143,163, 217
9	*myo*-inositol	SRM	27.77	180	508	120	70	117, 265, 343, 417
9	*N*-acetyl-D-glucosamine	SRM	27.97, 28.07	221	173	60.9	40	116, 131, 158
11	D-(+)-sucrose	SRM	35.15	342	362	96	70	155, 183, 271
11	D-(+)-lactose	SRM	35.15, 36.70	360	204	71.6	50	149, 155, 163
12	galactinol	MS-MS	39.19	378	204	71.6	50	133, 152, 163
13	D-(+)-raffinose	SRM	43.69	594	361	120	80	169, 243, 271
13	maltotriose	SRM	45.48	504	361	120	70	169, 243, 271
13	D-(+)-melazitose (I.S.)	SRM	44.90	522	361	120	80	155, 169, 243, 271

[a] Segment, ion preparation type, retention time, molecular weight, quantifier and qualifier ions, excitation storage level, and excitation amplitude for each analyte.

performed with universal systems such as flame ionization detectors or mass spectrometers (*8*−*12*).

Isolation of sugars from plants has been usually performed by conventional time-consuming protocols, which have not been properly optimized. Some of these methods are based on agitation for long periods with aqueous or alcoholic solutions (*6*), and they do not ensure quantitative extraction of sugars (*7*, *13*). Sugars profile in olive tissues has been scarcely reported, with determination limited to a few analytes. Thus, Cataldi et al. (*6*) reported the determination of eight sugars in olive leaves (namely, *myo*-inositol, galactinol, mannitol, galactose, glucose, fructose, sucrose, raffinose, and stachyose). Another method reported by Marsilio et al. (*14*) was focused on predominant sugars such as *myo*-inositol, mannitol, galactose, glucose, fructose, and sucrose in olive fruits. The aim of this study was to develop a method for profiling analysis of 22 free sugars present in three different olive tree materials: fruits, leaves, and small stems of *O. europaea* cultivars characteristic of Andalucía (Spain). The method involves ultrasonic assistance for isolation of sugars from vegetal samples with subsequent silylation prior to GC-MS/MS analysis.

MATERIALS AND METHODS

Samples. Olive fruits, leaves, and stems were sampled from three different *O. europaea* varieties (Picual, Manzanilla, and Hojiblanca), in September 2009. For representativeness, the samples were taken from four different trees of each variety. The samples were dried at 40 °C for 24 h, milled with a mechanical mixer, and kept at −20 °C until use.

Reagents. Chromatographic grade dichloromethane, methanol, and ethanol were purchased from Scharlau (Barcelona, Spain). Deionized water (18 MΩ·cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA) was used to prepare the water/ethanol sugar standards. Carbohydrate standards D-(−)-arabinose, L-rhamnose, xylitol, 1,6-anhydro-β-D-glucose, D-(+)-xylose, L-(−)-arabitol, adonitol, D-(+)-mannose, D-(−)-fructose, D-(−)-galactose, D-(+)-galacturonic acid, D-(+)-glucose, D-mannitol, sedoheptulose, D-sorbitol, D-(+)-*chiro*-inositol, D-glucuronic acid, *myo*-inositol, *N*-acetyl-D-glucosamine, D-(+)-sucrose, D-(+)-lactose, galactinol, maltotriose, D-(+)-raffinose, stachyose, and D-(+)-melazitose (the last used as internal standard) were purchased from Sigma-Aldrich (St. Louis, MO). Derivatization reagents, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), and pyridine, were also from Sigma.

Apparatus and Instruments. Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium alloy probe (12.70 mm diameter), which was immersed into a laboratory-made stainless steel container filled with water. The container possesses eight compartments to place test tubes equidistant from the probe tip, which is located in the center of the container. A mechanical electrical stirrer (Selecta, Barcelona, Spain) was used for conventional extraction by agitation. A Varian CP-3800 gas chromatograph (Walnut Creek, CA) equipped with a split/splitless SPI/1079 programmable temperature injector and coupled to a Saturn 2200 ion trap mass spectrometer (Sunnyvalley, TX) was used for analysis of the target compounds. This instrument was equipped with a FactorFour fused-silica capillary column (VF-5 ms, 30 m × 0.25 mm, 0.25 μm) provided by Varian.

Proposed Extraction Procedure. One-tenth of a gram of milled sample was placed in a test tube with 6.5 mL of extractant (2:1 dichloromethane/methanol). The tube was immersed into a water bath (a cylindrical stainless steel container) at room temperature for a 10 min preset time under ultrasonic irradiation (duty cycle = 0.5 s, output amplitude = 60% of the converter, applied power = 450 W with the probe placed 1 cm from the bottom of the container). The extracts were subsequently centrifuged at 13.6*g* (3500 rpm) for 2 min, filtered through 0.45 μm Millipore nylon membrane, and concentrated to dryness under vacuum at 30 °C. The dried extracts were reconstituted in 1 mL of pyridine. Aliquots of 50 μL of extract were derivatized to obtain the trimethylsilyl derivatives using 100 μL of BSTFA containing 2% TMCS for 1 h at room temperature immediately before GC-MS analysis.

Reference Extraction Procedure. One-tenth of a gram of milled leaves and 6.5 mL of extractant (2:1 dichloromethane/methanol) were placed in a beaker and subjected to stirring at room temperature for 24 h. The extracts were treated as in the proposed protocol.

GC-MS/MS Separation−Detection. After derivatization, 1 μL of the analytical sample was injected into the chromatograph. The injector temperature was fixed at 280 °C, and the injection was in the split/splitless mode. The splitter was opened (2:1) for 0.5 min, closed for 3.5 min, and then opened at 100:1 split ratio for 10 min. Helium was circulated at a constant flow rate of 1.3 mL/min as carrier gas. The oven temperature program was as follows: initial temperature = 65 °C (held for 2 min), increased at 6 °C/min to 300 °C (held for 30 min). The total analysis time

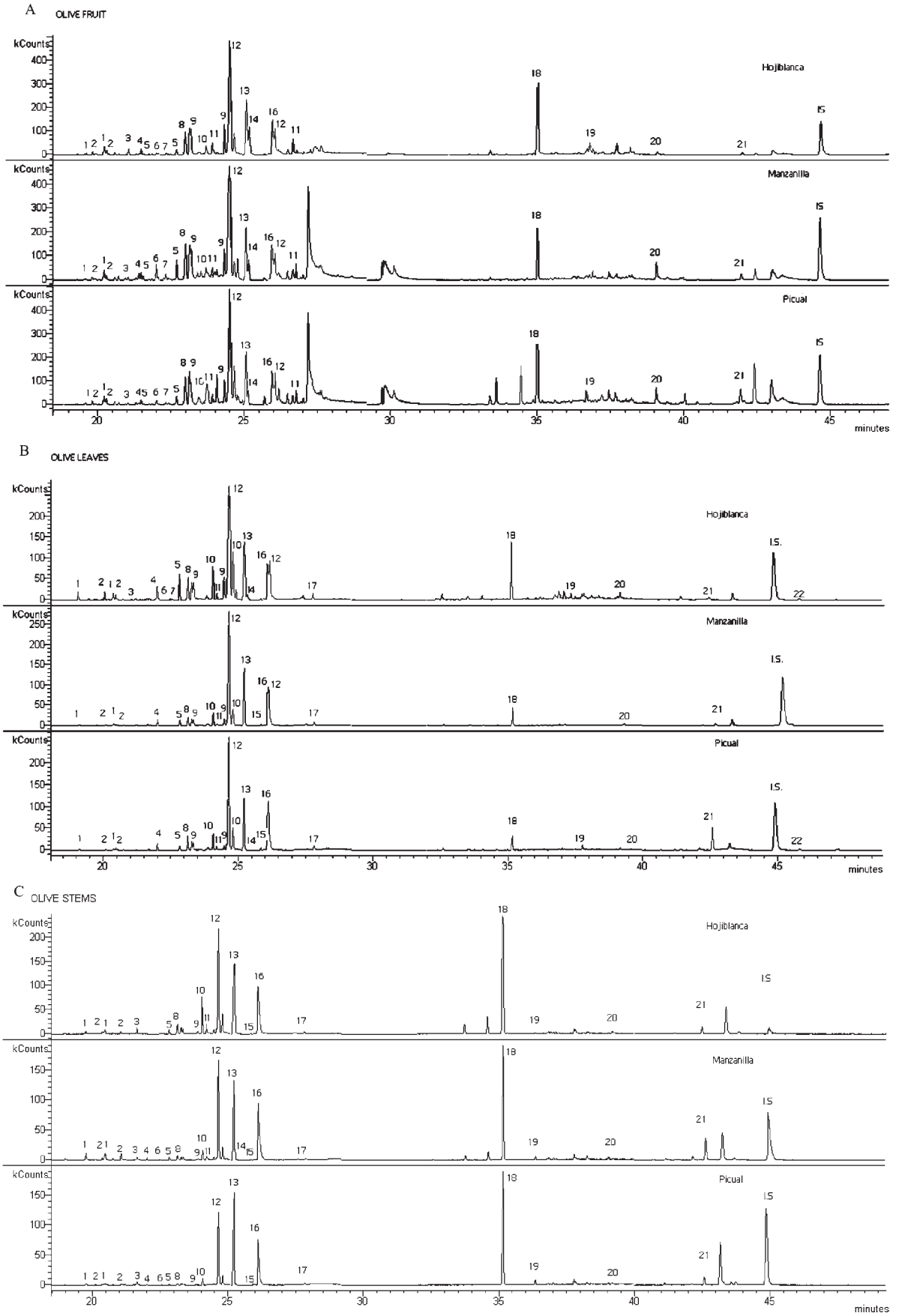

Figure 1. Chromatograms of the extracts from different vegetal tissues sampled from three olive cultivars. 1, D-(−) arabinose; 2, L-rhamanose; 3, xylitol; 4, 1,6-anhydro-β-D-glucose; 5, D-(+)-xylose; 6, LL-(−)-arabitol; 7, adonitol; 8, D-(+)-mannose; 9, D-(−)-fructose; 10, D-(−)-galactose; 11, D-(+)-galacturonic acid; 12, D-(+)-glucose; 13, D-mannitol; 14, sedoheptulose; 15, D-(+)-*chiro*-inositol; 16, D-glucuronic acid; 17, *myo*-inositol; 18, D-(+)-sucrose; 19, D-(+)-lactose; 20, galactinol; 21, D-(+)-raffinose; 22, maltotriose; I.S., internal standard.

Table 2. Calibration Equation, Regression Coefficient, and Detection and Quantification Limits (LOD and LOQ) for Each Analyte by GC-MS/MS

compound	calibration eq[a]	r^2	LOD (μg/mL)	LOQ (μg/mL)
monosaccharides				
pentoses				
D-(−)-arabinose	$y = 0.0085x - 0.0043$	0.997	0.076	0.250
D-(+)-xylose	$y = 0.0132x - 0.0046$	0.998	0.076	0.250
hexoses				
D-(+)-glucose	$y = 0.0033x - 0.0015$	0.997	0.015	0.050
D-(+)-mannose	$y = 0.0006x - 0.0008$	0.997	0.303	1.000
D-(−)-galactose	$y = 0.001x - 0.0002$	0.997	0.076	0.250
ketoses				
D-(−)-fructose	$y = 0.0003x - 0.0007$	0.995	0.030	0.100
sedoheptulose	$y = 0.0002x - 0.0005$	0.990	0.152	0.500
anhydrosaccharides				
1,6-anhydro-β-D-glucose	$y = 0.0017x - 0.0008$	0.999	0.030	0.100
disaccharides				
D-(+)-sucrose	$y = 0.009x + 0.0281$	0.990	0.015	0.050
D-(+)-lactose	$y = 0.0035x + 0.0053$	0.999	0.076	0.250
trisaccharides				
D-(+)-raffinose	$y = 0.0346x - 0.0478$	0.992	0.076	0.250
maltotriose	$y = 0.0798x + 0.114$	0.992	0.076	0.250
methylpentoses				
L-rhamnose	$y = 0.0012x - 0.0025$	0.994	0.076	0.250
sugar carboxylic acids				
D-(+)-galacturonic acid	$y = 0.0073x - 0.0096$	0.998	0.076	0.250
D-glucuronic acid	$y = 0.0040x - 0.0060$	0.998	0.076	0.250
sugar alcohols				
xylitol	$y = 0.0109x + 0.0073$	0.997	0.076	0.250
L-(−)-arabitol	$y = 0.0086x + 0.0042$	0.998	0.076	0.250
adonitol (ribitol)	$y = 0.0078x + 0.0149$	0.991	0.076	0.250
D-mannitol	$y = 0.0009x - 0.0013$	0.996	0.076	0.250
cyclic polyols				
D-(+)-*chiro*-inositol	$y = 0.0003x + 0.0003$	0.994	1.515	5.000
myo-inositol	$y = 0.0005x + 0.0004$	0.992	0.076	0.250
galactinol	$y = 0.0008x + 0.0057$	0.987	0.076	0.250

[a] y = sugar area; x = sugar concentration.

was 76 min with 5 min of extra time to reestablish and equilibrate the initial conditions. The ion trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current = 80 μA; transfer line, ion trap, and manifold temperatures = 280, 200, and 50 °C, respectively. A filament multiplier delay of 6 min was established to prevent instrument damage. The MS/MS step was carried out by collision-induced dissociation (CID) in nonresonant excitation mode.

RESULTS AND DISCUSSION

Optimization of the Determination Step. The experimental chromatographic variables were optimized, resulting in the operating conditions described under Materials and Methods. Optimization of the chromatographic step was carried out with standard solutions using the mass spectrometer in full-scan mode. Melazitose was selected as internal standard (I.S.) because of its physical and chemical characteristics being similar to those observed for the derivatized analytes. This compound is a nonreducing trisaccharide produced in sap from many plants, and it was not found in the samples. Melazitose eluted at 44.9 min, which is close to other analytes such as raffinose (43.59 min) or maltotriose (45.48 min), but with no interference in chromatographic resolution. Samples were spiked with 60 μg from the melazitose stock solution to inject into the gas chromatograph a final amount of 20 ng. The chromatographic method was optimized with the carbohydrates multistandard by studying the temperature program to obtain complete separation in the shortest time, which was accomplished in 76 min. Identification of the target compounds was carried out by comparison with the retention time and mass spectra provided by the standards. The optimization of the ion trap mass spectrometer conditions was carried out in full-scan mode looking for the ions of the target compounds. This analysis enables selection of the precursor ions for each compound to optimize MS/MS analysis, as shown in **Table 1**. The next step was the selection of the collision energy to favor the formation of the target product ions with quantification purposes. The fastest way to determine this parameter is the automatic method development (AMD) ion preparation mode, which is able to determine the collision energy for one precursor ion per segment in a single run. Both nonresonant and resonant CIDs were tested to determine the best conditions for each compound. Nonresonant waveform was selected for all analytes. The use of MS^2 in single-reaction monitoring (SRM) as ion preparation mode is a function of the number of precursor ions present in a segment. In the case of one precursor ion, regardless of the number of compounds determined in that segment, MS^2 should be used. On the other hand, in the case of two or more precursor ions per segment, the use of SRM is mandatory. **Figure 1** illustrates the chromatograms provided by analysis of the different *O. europaea* vegetal tissues obtained after MS/MS analysis. **Table 1** shows the ion preparation mode used in each segment. The mass spectra of saccharides with the pyrano-ring (5 C) are mainly characterized by the m/z 204 fragment ion. On the other hand, the spectra of the furano-ring saccharides (4 C) are mainly characterized by the m/z 217 ion. In fact, these two fragments are discriminant as qualifier ions to identify monosaccharides as TMS derivatives in complex extracts. As can be seen in **Table 1**, most monosaccharides (pentoses and hexoses) presented two GC peaks, which were ascribed to the α- and β-configurations of the hydroxyl group in the pyrano- and furano-rings. Both isomers were also present in vegetal samples and were summed to report one value for

each compound. The presence of open-ring compounds was additionally detected for some sugar standards such as arabinose and fructose together with their cyclic isomers. Ketols and aldols exist as an equilibrium mixture with their cyclic isomers. However, the proportion of open-ring forms was negligible and, therefore, was not taken into account for quantification. Disaccharides and trisaccharides were directly identified as TMS derivatives by the m/z 361 ion (together with m/z 204) (*15*).

Analytical Characterization of the GC-MS/MS Method. Calibration plots were run by using the standard peak/internal standard peak ratio as a function of standard concentration. Calibration was performed with multistandard solutions at 10 concentration levels between 0.1 and 150 μg/mL, which were analyzed in triplicate by using the derivatization protocol described under Materials and Methods. Stock solutions containing 150 μg/mL of each reference compound were prepared and diluted to 10 appropriate concentrations, and 1 μL of each solution was injected to run the calibration curves. The calibration equations and regression coefficients are shown in **Table 2**. The lowest limits of detection (LLOD), expressed as the mass of analyte which gives a signal that is 3σ above the mean blank signal (where σ is the standard deviation of the blank signal), ranged between 0.02 and 1.52 μg/mL (or 0.04−8.41 pmol on-column). The lowest limits of quantification (LLOQ), expressed as the mass of analyte which gives a signal that is 10σ above the mean blank signal, ranged between 0.05 and 5.0 μg/mL (or 0.14−27.77 pmol on-column).

Optimization of the Ultrasound-Assisted Extraction Protocol. Ultrasonic-assisted extraction was selected as sample preparation approach to isolate quantitatively the target sugar fraction. Four variables (extractant volume, duty cycle expressed in relative terms, ultrasonic irradiation amplitude, and extraction time) were optimized by a multivariate experimental design. Preliminary tests were carried out to select a suitable extraction solvent depending on the sample matrix. Several ethanol/water mixtures, according to the methods of Romani et al. (*7*) and Norikishi et al. (*16*), and a 2:1 dichloromethane/methanol mixture, previously used for isolation of carbohydrates from environmental samples (*15*), were assayed. The last mixture was found to be especially suited for this application due to the efficient isolation of intra- and extracellular sugars from vegetal material without distinction and, thus, was selected as leaching medium.

Optimization of the extraction step started with a screening study to elucidate the main factors influencing this step. The study was based on a half-fractional factorial design involving eight randomized runs including three center points. An independent optimization study was applied to each vegetal sample to evaluate the incidence of the matrix on the leaching process. The conclusions of this screening study were that niether the extractant volume nor the percentage of duty cycle of ultrasonic exposure was an influential factor for any type of sample in the ranges under study. However, the results showed better recoveries with the medium values of both parameters. Thus, the intermediate values tested for both variables, 6.5 mL and 0.5 s, respectively, were selected for subsequent experiments. On the other hand, both the ultrasonic radiation amplitude and extraction time were influential factors for isolation of the target sugars with a positive effect. Therefore, the next step was to check the influence of higher values of both variables by using a central composite design 2^2+stars involving 10 runs with 4 degrees of freedom and generating a surface response with 3 center experiments. In this case, neither the ultrasound radiation amplitude nor extraction time was an influential factor in the ranges monitored for olive leaves and small stems. As shown in the response surface in **Figure 2A**, the optimum values for both parameters were 50% ultrasound radiation amplitude and 10 min of sonication time. In the case of olive fruit (**Figure 2B**), the radiation amplitude was significant for the extraction of sugars; however, higher values of irradiation amplitude are not recommended by the ultrasonic probe manufacturer. The influence of the sonication time was not clear. For this reason, quantitative extraction of sugars was assessed by a kinetics study, testing different sonication times and setting the other parameters at their optimum values. The results obtained with this univariate study completing sugar isolation after 10 min of sonication, which was set as definitive value for all sample matrices.

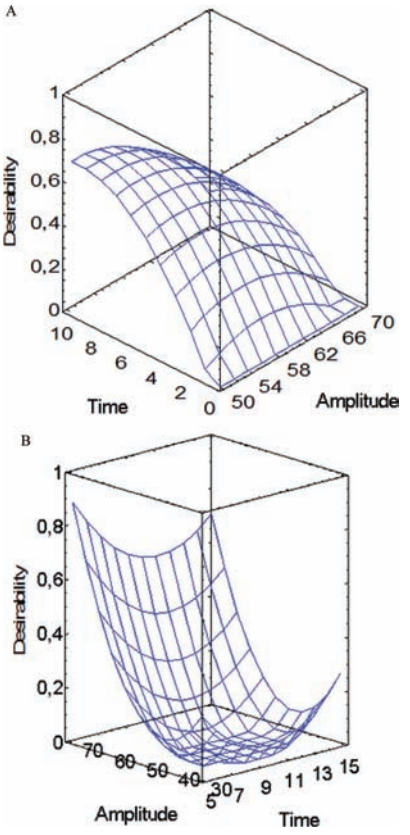

Figure 2. Experimental response generated in the study for optimization of carbohydrates leaching assisted by ultrasonic energy using 6.5 mL of 2:1 dichloromethane/methanol and 0.5 s dutty cycle: (**A**) leaves; (**B**) fruits.

Comparison of the Ultrasound-Assisted Extraction with a Reference Protocol. The proposed ultrasonic approach was validated by comparison of the leaching efficiency with a conventional reference protocol. The strategy selected was based on maceration of the sample by 24 h of agitation with the same extractant (2:1 dichloromethane/methanol). The proposed methodology was tested with the three different olive matrices: leaves, stems, and fruits. As a result, comparable efficiencies were obtained with both isolation protocols, which emphasizes the enhancement effect of ultrasound application as auxiliary energy to accelerate the extraction process. The percentages of sugars extracted with the reference method were 19.95, 14.16, and 28.74% of the total mass versus 22.93, 13.56, and 28.01% isolated with the proposed extraction procedure in leaves, fruits, and stems, respectively.

Quantitation of Sugars in Olive Fruits, Leaves, and Stems. The overall method was applied to analyze the sugars profile composed by 22 analytes in olive fruits, leaves, and stems collected from 3 *O. europaea* varieties (see **Figure 1**). The results obtained are summarized in **Figure 3**; the latter represents the concentration of sugars depending on the olive tissue and cultivar variety. Mannitol and glucose, the primary photosynthetic products,

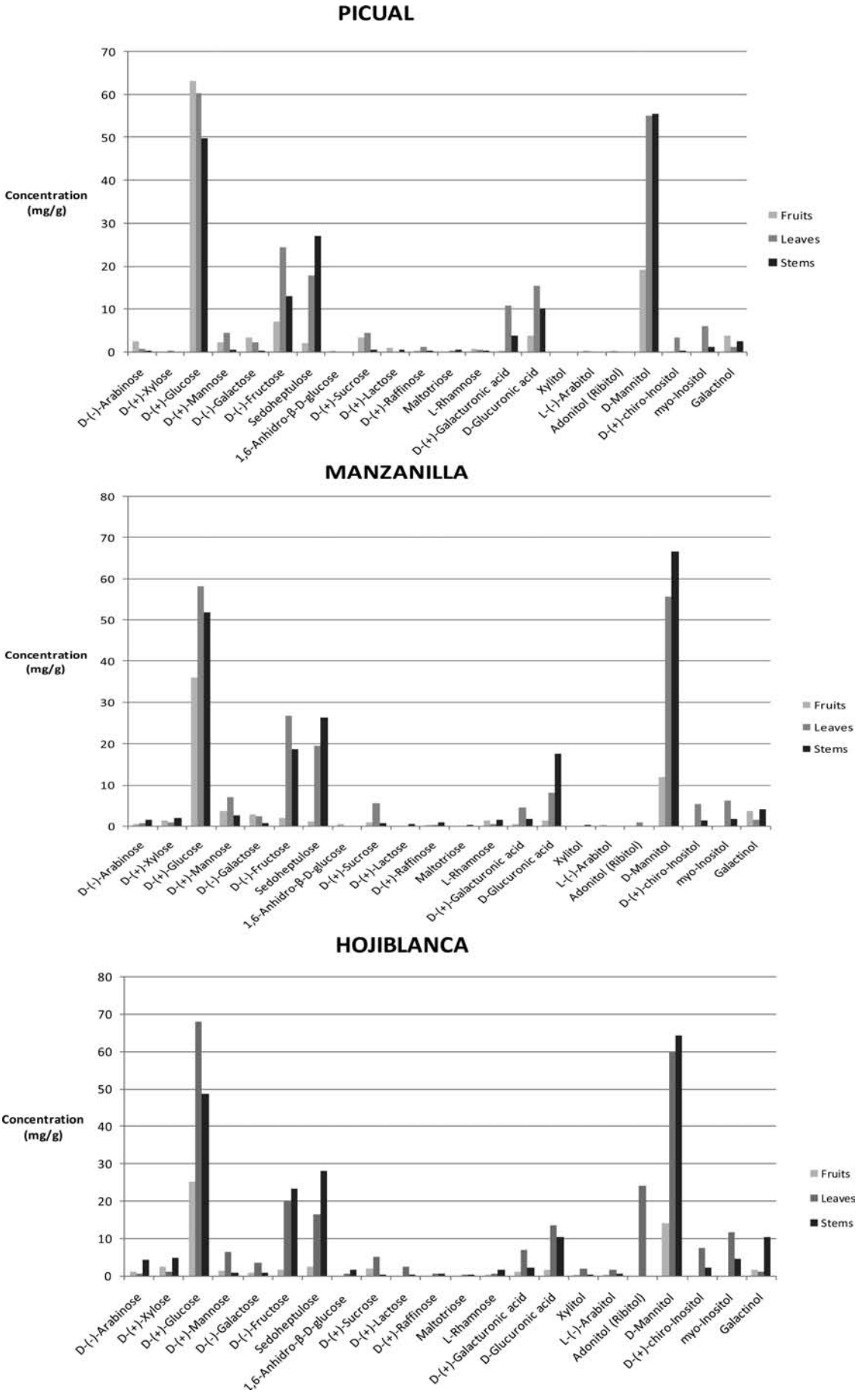

Figure 3. Continued

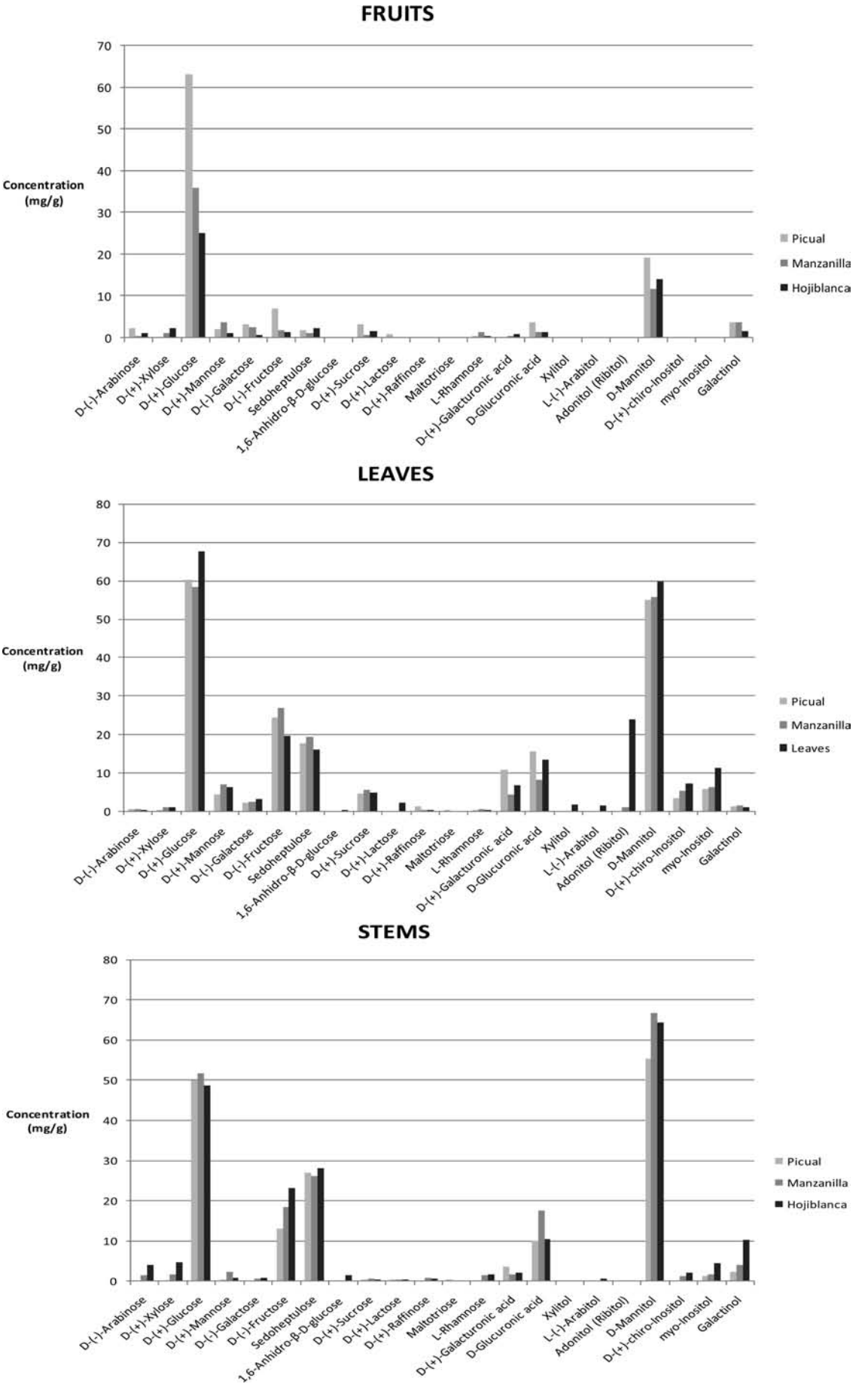

Figure 3. Concentration of carbohydrates found for each cultivar variety and depending on the vegetal tissue. Results are expressed as mg/g dry weight.

together with fructose and galactose, were the predominant sugars in fruits and leaves. In fact, these sugars represent >60% of the total soluble carbohydrates in these olive tissues. For small stems, the predominant sugars are sucrose, D-glucuronic acid, mannitol, and glucose. This is not surprising as mannitol and glucose represent the major transport sugars in olive trees and contribute significantly to osmotic adjustment. Complementarily, glucose was detected in lower amounts than mannitol, which is in agreement with previous results reported by Drossopoulos and Niavis (*17*) and Priestley (*18*). These findings do not agree with the results of Flora and Madore (*19*), who assigned a major role to glucose as compared with that of sucrose and mannitol. In fruits, glucose was the major sugar in all *O. europaea* varieties.

The remaining sugars in the samples, such as xylitol, 1,6-anhydro-β-D-glucose, arabitol, adonitol, *N*-acetyl-D-glucosamine, and lactose, are in very low concentration in all tissues for the varieties assayed. The capability of supporting prolonged water deficiency is well-known for *O. europaea* because the basic mechanisms of osmotic adjustment by accumulation of organic solutes, such as sugar compounds, in the cytoplasm have evolved properly. The activity of these substances is related to their ability to raise the osmotic potential of the cell, thus balancing the potential of an externally increased osmotic pressure.

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