Anal. Chem. **2000,** *72,* 3902−3907

Determination of Sugar Compounds in Olive Plant Extracts by Anion-Exchange Chromatography with Pulsed Amperometric Detection

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We describe a chromatographic method that uses isocratic elution and pulsed amperometric detection to determine soluble carbohydrates in plant tissues. Such a method provides a rapid and convenient means to obtain a complete profile of the sugar components of leaves and roots from olive (*Olea europaea* L. cv. Coratina) plants. A simple purification of plant extracts using pure water was developed, which is far less time-consuming and retains a high level of accuracy. Excellent separation of *myo*-inositol, galactinol, mannitol, galactose, glucose, fructose, sucrose, raffinose, and stachyose was achieved with an anion-exchange column and 12 mM NaOH spiked with 1 mM barium acetate as an eluent. At a flow rate of 1.0 mL/min, the time of analysis was less than 25 min, and repeatability of the method on the order of 2.2% as RSD or better for retention times and lower than 5.2% for peak areas. Recoveries approximated 100% (range 97.2−104.5%), and the method provided good precision with a coefficient of variation which ranged between 0.9 and 3.3%. Among identified carbohydrates extracted from leaves and roots of olive plants, glucose and mannitol were major compounds. Their molar ratio was estimated to be 1.2 ± 0.1 and 2.2 ± 0.3 for olive leaves and roots, respectively. The occurrence of soluble galactinol in plant tissues was also validated.

Green plants produce carbohydrates through the process of photosynthesis. These compounds are used either as energy sources for vegetative growth and development or as precursors in the biosynthesis of a wide range of molecules, including lipids, proteins, and polysaccharides.[1] Selected plant species appear to have evolved the biochemical processes and physiological conditions necessary for withstanding unfavorable environmental variables, including drought, flooding, salinity, irradiance, and both high and low temperatures.[2,3] Nonstructural carbohydrates, among other solutes, are involved as osmoregulators and osmoprotectants in tolerance to abiotic stresses.[4,5,6] Plant molecular responses to water deficiency as well as to high-salt soil−water contents are complex as evidenced by osmolyte biosynthesis, transport, accumulation, and cellular protection.[7,8] In this respect, there is a growing interest in examining the bioconversion, storage, and transport patterns associated with soluble sugar compounds including alditols (*myo*-inositol, mannitol, sorbitol, dulcitol, galactinol, etc.) and saccharides (glucose, galactose, fructose, sucrose, raffinose, stachyose, etc.).[9,10,11] Olive plants (*Olea europaea* L.) are particularly resistant to water deficiency, and a wide range of sugar compounds, such as glucose, mannitol, sucrose, and members of the raffinose family (stachyose, verbascose, etc.), seem to be involved in the basic mechanisms developed by these trees to overcome adverse environmental conditions.[4,12] Yet, a detailed knowledge of their physiological role in plants can be successfully investigated only if a sensitive, reliable, and rapid analytical method is available.

Chromatographic techniques are usually applied for this type of task.[13,14] Numerous procedures based on high-performance liquid chromatography (HPLC) have been described. A major problem with carbohydrate detection in HPLC is that these compounds lack chromophores and fluorophores, thus eliminating the use of ultraviolet and fluorescence detectors.[13] Other detectors include differential refractive index (RI) and evaporative light scattering, both of which suffer from poor sensitivity, poor specificity of detection, and considerable drift.[15] Nowadays,

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10.1021/ac000266o CCC: $19.00
Published on Web 07/20/2000

carbohydrate analysis can take advantage of the much greater selectivity and sensitivity of anion-exchange chromatography (AEC) in conjunction with pulsed amperometric detection (PAD).[15−21] The anion-exchange columns are made with anion-exchange pellicular resins especially designed for efficient separations of carbohydrates, including alditols, without any derivatization.[16,21] Alkaline mobile phases (pH 12−13) are usually employed to form the required sugar anion that interacts with the positively charged stationary phase. As the contamination of carbonate ion interferes with a reliable chromatographic analysis of carbohydrates, separation of complex mixtures necessitates carbonate-free alkaline eluents. Indeed, carbonate ions tend to gradually occupy the active sites of the column, thereby progressively decreasing the retention of sugar molecules, especially when dilute alkaline eluents are employed. In a previous paper,[22] it has been demonstrated that alkaline solutions (e.g., <20 mM NaOH) spiked with millimolar amounts of barium acetate could be used as eluents for the separation of mono- and disaccharides without using column regeneration between chromatographic runs. Apparently, the addition of barium leads to an efficient abatement of carbonate ions through the precipitation of $BaCO_3$, thus allowing very good reliability of chromatographic data.

The objective of the present research work was to devise an anion-exchange chromatographic method with pulsed amperometric detection for the determination of sugars and alditols in plant tissues. Our effort was to combine the advantages of highly sensitive electrochemical detection at a gold working electrode with the advantage of efficient separations carried out with dilute alkaline eluents liberated from carbonate. Interestingly, it will be demonstrated that limited sample preparation and high sensitivity of sugars and alditols make our proposal an ideal means for the determination of soluble carbohydrates in plant extracts, with a total analysis time of less than 25 min under isocratic elution. Since an authentic standard was not available, galactinol in olive extracts was identified from running a sample of watermelon cotyledon. It is anticipated that the present proposal will allow elucidation of the role of sugar compounds in plants under abiotic stress conditions, including drought, salinity, UV radiation, and temperature.

MATERIALS AND METHODS

Chemicals. Sodium hydroxide, 50% w/w solution in water (d = 1.515 g/mL), $Ba(CH_3COO)_2$ 99%, and D-galactose 97% were purchased from Aldrich (St. Louis, MO); *myo*-inositol, D-mannitol, D-glucose 99.5%, D-fructose 99%, sucrose 99+%, D-raffinose pentahydrate >99%, stachyose >98%, D-2-deoxyribose >99%, and D-3-*O*-methylglucopyranose >98% were from Sigma Chemical Co. (Steinheim, Germany). All chemicals were used as received. Membrane-filtered (0.45 μm) and degassed Mill-Q water (Millipore, Bedford, MA) was used to prepare alkaline eluents from a carbonate-free 50% (w/w) NaOH mother solution. The exact concentration of hydroxide ions in the mobile phase was determined by titration against a standard solution of hydrochloric acid. Stock solutions of sugars and alditols were prepared in pure water and stabilized with 0.1% sodium azide to prevent bacterial growth. Just before use, standard solutions were prepared from the stock solutions by dilution to the desired concentration.

HPAEC-PAD System. All experiments were performed using a metal-free programmable gradient pump (Dionex, Sunnyvale, CA), model GP40, a Dionex pulsed amperometric detector (model ED40), and a metal-free rotary injection valve (Rheodyne, Cotati, CA) with a 10-μL injection loop. The thin-layer-type amperometric cell contained a 1.0-mm-diameter gold working electrode and an Ag|AgCl reference electrode with the titanium cell body serving as the counter electrode. Two anion-exchange columns in separate experiments were employed: (i) a Dionex CarboPac PA1 column (250 mm × 4 mm i.d.) plus a guard column (50 mm × 4 mm i.d.) and (ii) a CarboPac MA1 column (250 mm × 4 mm i.d., Dionex) with a guard column (50 mm × 4 mm i.d.). Pulsed amperometric detection was carried out with the following pulse settings: (i) E_{OX} = +800 mV (t_{OX} = 180 ms), E_{DET} = +250 mV (t_{DEL} = 240 ms, t_{INT} = 200 ms), and E_{RED} = −250 mV (t_{RED} = 360 ms) when CarboPac PA1 column was used; and (ii) E_{OX} = +650 mV (t_{OX} = 190 ms), E_{DET} = +50 mV (t_{DEL} = 150 ms, t_{INT} = 300 ms), and E_{RED} = −150 mV (t_{RED} = 340 ms) with CarboPac MA1 column. The response time was 1 s. Data acquisition was completed using a Chrom-Card for Windows from CE Instruments (ThermoQuest, Milan, Italy). The temperature of the columns was kept at 22 ± 1 °C using a homemade water jacket coupled with a circulating water bath model WK4DS from Colora (Colora, Messtechnik GmbH, Germany).

Eluent solutions were prepared as described previously.[22] Briefly, pure water for the eluent preparation was degassed before use by flushing helium for about 20 min. Upon addition of the proper aliquot of labeled carbonate-free 50% (w/w) NaOH and the proper amount of $Ba(OAc)_2$, the eluent solution was kept in a plastic bottle and a Dionex eluent organizer (EO1) was used to saturate it with helium gas to minimize CO_2 adsorption. Before use, it is advisable to keep the eluent solution saturated overnight with an inert gas, thus allowing complete precipitation of the low amount of barium carbonate in the eluent reservoir.[20] After each daily working session, the analytical and guard columns were flushed for ∼30 min with 200 mM NaOH.

Extraction and Purification Procedures. Two-year-old olive (*Olea europaea* L. cv. Coratina) plants were grown outdoors in 18-L pots containing a medium consisting of a 3:1 mixture of field soil (73.2% sand, 13.3% silt, and 13.5% clay) and peat. The pots were arranged in rows in a North−South direction and placed on a raised structure approximately 10 cm from the ground. During plant growth, the soil/water ratio was maintained constant, that is, roughly 85%, integrating each evening the water lost through transpiration. Leaves, from the central portion of brunches, and roots were collected. Following harvest, tissues were immediately stored at −80 °C until lyophilization. The lyophilized tissues were ground and stored in airtight vials at room temperature. The ratio between fresh and dry weights of leaves and roots was estimated to be 2.25 and 2.55, respectively. Approximately 50 mg of leaves

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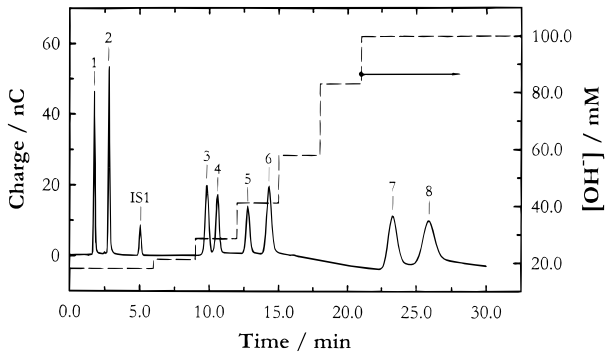

Figure 1. Separation and detection of a pool of carbohydrates by HPAEC-PAD under a gradient elution. Peak and concentration: (1) *myo*-inositol, 20 μm, (2) mannitol, 20 μM, (IS1) 2-deoxyribose, 20 μM, (3) galactose, 20 μM, (4) glucose, 20 μM, (5) sucrose, 20 μM, (6) fructose, 20 μM, (7) raffinose, 20 μM, and (8) stachyose, 20 μM. Column, CarboPac PA1 plus guard (Dionex) at a controlled temperature of 22 °C. Flow rate, 1.0 mL/min. Detection potential at the gold working electrode, E_{DET} = +0.20 V vs Ag|AgCl. A gradient elution comprising 200 mM NaOH + 1 mM $Ba(OAc)_2$ and 1 mM $Ba(OAc)_2$ in water is shown by the dashed line.

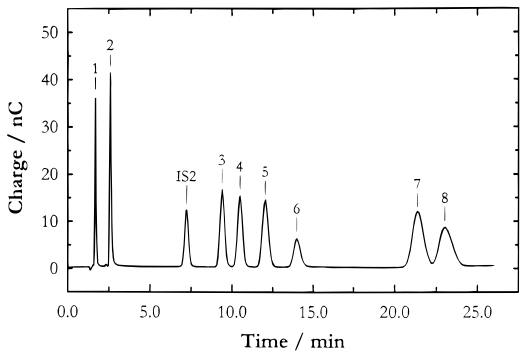

Figure 2. HPAEC-PAD isocratic separation of a mixture of carbohydrates. Peak and concentration: (1) *myo*-inositol, 8 μM, (2) mannitol, 20 μM, (IS2) 3-*O*-methylglupyranose, 40 μM, (3) galactose, 20 μM, (4) glucose, 20 μM, (5) sucrose, 20 μM, (6) fructose, 20 μM, (7) raffinose, 20 μM, and (8) stachyose, 20 μM. Eluents, 12 mM NaOH + 1 mM $Ba(OAc)_2$. E_{DET} = +0.25 V vs Ag|AgCl. Other conditions as in Figure 1.

and roots tissues from two-year-old olive plants was extracted at room temperature using deionized water with 17.8 MΩ×cm resistivity. Then the addition of an appropriate volume of D-3-*O*-methylglucopyranose (MeGlu) as the internal standard, to give a concentration of 40 μM, was accomplished. To facilitate the best contact between plant tissue and extraction solvent, the sample was shaken for 15 min, and finally, the suspension was centrifuged at 3000 rpm for 10 min. Prior to injection, the aqueous extracting phase was filtered through a single-use 0.22-μm nylon syringe filter (Aldrich) and passed on a cartridge OnGuard A (Dionex) to remove anion contaminants. Such a solution turned out to be colorless. The same solution was injected and the sample separation carried out, three times, to assess the repeatability of retention. As mannitol and glucose were present at relatively high levels, a sixteen-fold dilution was accomplished before injection.

RESULTS AND DISCUSSION

Optimization of the Elution Conditions. Eight main sugar compounds previously identified in extracts of olive plants by Flora and Madore[4] were pooled and analyzed by AEC-PAD. Method development was therefore carried out on standard solutions containing *myo*-inositol, mannitol, galactose, glucose, sucrose, fructose, raffinose, and stachyose. Various eluent combinations and elution modes were tested to enable their separation. Figure 1 shows the chromatographic plot recorded under a gradient elution at a flow rate of 1.0 mL/min. A standard mixture of the sugar compounds spiked with 2-deoxyribose (IS1) as an internal standard was resolved. Increasing concentration of the hydroxide ion was obtained by using two reservoirs containing (A) 200 mM NaOH + 1 mM $Ba(Ac)_2$, and (B) 1 mM $Ba(Ac)_2$ in water. Zero-time conditions were fixed at 9% A and 91% B. Then, the gradient elution followed the adjusted profile shown in Figure 1 until termination of the run at 35 min. This was followed by, at the least, a 25-min equilibration time with the zero-time eluent mixture prior to injection of the next sample. As the mobile phase is carbonate-free, there is no need to flush the column after each chromatographic run with a more concentrated sodium hydroxide eluent. Under these experimental conditions, a run time takes longer than 60 min for completion. Thus, a large number of separations in a daily session is precluded and not all the advantages of HPAEC-PAD can be fully exploited, including speed, limited sample treatment, selectivity, baseline stability, and sensitivity.

To separate the pool of carbohydrates in a reasonable time, the possibility of utilizing an isocratic elution was investigated. For such a purpose, it was necessary to define the best alkaline eluent solution. It is known that the anion-exchange chromatographic separation of isomer monosaccharides, such as galactose and glucose, both contained in olive tree leaves and roots, requires fairly low NaOH concentrations, (i.e., <20 mM).[20,22] As mentioned earlier, dilute alkaline eluents can be successfully employed upon addition of barium ion as $Ba(OAc)_2$. Baseline separation of the sugar compounds of interest was obtained with the following optimized elution conditions, 12 mM NaOH spiked with 1 mM $Ba(OAc)_2$ at a flow rate of 1.0 mL/min and a column temperature of 22 °C. All carbohydrates were eluted in less than 25 min (see Figure 2). A key advantage over conventional alkaline eluents (i.e., no addition of barium ions) is that no column regeneration and or equilibration is needed upon the chromatographic run.[20] Besides, the use of a NaOH gradient elution to separate sugar analytes is less advantageous than that of an isocratic one, as, during the run, changes in hydroxide concentration make the detector baseline unstable (see Figure 1). We wish also to mention that a constant retention behavior makes chromatographic peaks readily identifiable and leads to a better evaluation of peak areas (intensities). On this basis, isocratic elution is generally preferred because the run time is comparably shorter and the sample throughput can be increased quite significantly. All subsequent work was carried out under such experimental conditions.

Assay Performances, Sample Extraction, and Recovery of Carbohydrates. The following compounds, *myo*-inositol, mannitol, galactose, glucose, sucrose, fructose, raffinose, and stachyose, were detected and quantified by AEC-PAD with MeGlu being used as an internal standard (IS). The choice of such an IS is based on two conditions: (i) it is absent from vegetable samples and (ii) it has a chromatographic peak which does not overlap those of other solutes. Experimental parameters including column

Table 1. Quantitative Parameters of Carbohydrates Present in Olive Plant Extracts and Determined by HPAEC-PAD[a]

analyte (A)	k' [b]	$(A_A/A_{IS}) = a + b \times [C]$[c] $a = t_{95} \times s_a$	$b = t_{95} \times s_b$ (μM^{-1})	r	RSD %[d] (μM)
myo-inositol	0.22	-0.1 ± 0.1	0.111 ± 0.005	0.9993	2.0 (8)
mannitol	0.88	0.1 ± 0.4	0.062 ± 0.007	0.9988	2.9 (20)
galactose	5.81	-0.3 ± 0.4	0.088 ± 0.007	0.9992	2.3 (20)
glucose	6.62	-0.2 ± 0.3	0.090 ± 0.005	0.9995	2.2 (20)
sucrose	7.72	0.2 ± 0.2	0.076 ± 0.007	0.9991	2.2 (20)
fructose	9.14	-0.1 ± 0.2	0.046 ± 0.003	0.9992	2.7 (20)
raffinose	14.5	0.0 ± 0.1	0.14 ± 0.01	0.9991	4.0 (2.5)
stachyose	15.7	0.0 ± 0.1	0.13 ± 0.01	0.9989	5.2 (2.5)

[a] Column, CarboPac PA1 with guard column; flow rate, 1 mL/min; mobile phase, 12 mM NaOH spiked with 1 mM $Ba(OAc)_2$; sample loop, 10 μL. [b] The capacity factor, k', was calculated according to $k' = (t_R - t_M)/t_M$, where t_R is the retention time and t_M is the column dead time, measured from the front disturbance in the chromatogram; dead time, 1.38 min. [c] A_A/A_{IS} represents the peak areas ratio between analyte and internal standard. The confidence limits for the slope and intercept were evaluated with t taken at the 95% confidence level. [d] Relative standard deviations of peak areas were calculated using three replicate injections.

capacity factor (k') and precision (RSD%) for the sugar compounds with AEC-PAD are listed in Table 1. The calibration graphs, based on the corrected peak area ratios of analyte/IS, were established for all sugar compounds up to 2−3 orders of magnitude above the limit of detection. The correlation coefficients (r) were better than 0.9988. Limits of detection evaluated with a signal-to-noise ratio of 3 were near the picomole level, which is typical for sugar compounds in PAD.[22] Under the optimized chromatographic conditions, the repeatability of retention was assessed by carrying out three replicate analyses of a sample mixture, and it was less than 2.2% as RSD. These data confirm the already-reported findings[20] that dilute alkaline eluents spiked with barium acetate provide a stable retention, as the contamination of carbonate ion has been efficiently removed.

Determination of carbohydrates in plant tissue encompasses first an extraction procedure followed by separation, identification, and quantification. Once the separation conditions were optimized, we attempted to simplify the sample pretreatment. Traditional methods for extracting sugar compounds in plant tissues are based on the use of ethanol−water (75:25, v/v)[4,9,12] or methanol, chloroform, and water (12:5:3, v/v) mixtures.[23,24] When the extraction of sugar compounds from crop tissues is carried out with polar organic solvents, many other substances are also extracted, including organic acids, pigments, lipids, and phenolic compounds. This makes the next steps of sample purification time-consuming and labor-intensive. Furthermore, the chromatographic results often prove difficult to interpret, as numerous peaks are present. As described in the Experimental Section, pure water was a very effective extractive solvent of sugars and alditols from vegetable samples (Figure 3). Compared with conventional methods, the present extraction procedure has the advantages of being less expensive and more rapid for extracting soluble carbohydrates. Additional details will be presented in the next section.

Recoveries were evaluated for each sugar constituent in both tissues by spiking the extraction solutions with pure carbohydrates at the level of 50−100% of the measured content and performing triplicate assays before and after each addition. On the basis of their high water solubility, sugars were recovered to a high degree; this recovery on average (Table 2) ranged from 97.2 ± 1.0% for stachyose to 104.5 ± 3.4% for glucose, and coefficients of variation ranged between 0.9 and 3.3%. There were no consistent differences in recovery from either leaf or root extracts.

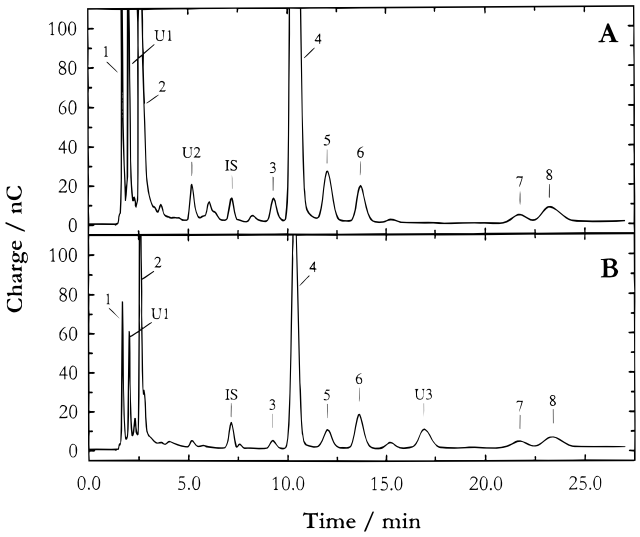

Figure 3. Separation of soluble carbohydrates in olive tree leaf (A) and root (B) extracts. For peak identification and chromatographic conditions, see the legend to Figure 2. Peaks U1, U2, and U3 represent three unknown electroactive compounds.

Sugar Compounds in Plant Extracts. Typical chromatograms of leaf and root extracts from *Olea europaea* are illustrated in parts A and B of Figure 3, respectively. Peak attributions were made by comparison with retention times of carbohydrate standards injected under the same experimental conditions. The number of the labeled peaks corresponds to the sugar compounds reported in Figure 2. Along with three unknown compounds (U1, U2, and U3), only a few minor peaks were not identified; it is most likely that these samples may contain other sugars as well as electroactive compounds. The following standard carbohydrates were injected without there being any correspondence found with the unknown peaks of Figure 3 (A and B): fucitol; arabitol; αα-, αβ-, and $\beta\beta$-trehaloses; fucose; rhamnose; arabinose; galactosamine; glucosamine; and glucuronic acid. Besides, the use of diluted alkaline eluents eliminated the possibility of interfering amino acids in the unknowns. As will be described later, however, the retention time of peak U1 probably corresponds to galactinol.

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Table 2. Recoveries (%) of Pure Sugar Compounds Added (mg L^{-1}) to Olive Tissue Samples ($n = 3$) at High and Low Concentration (Half of High Concentration)[a]

	leaves		roots		
sugar	low	high	low	high	(mean ± SD)
		%			
myo-inositol (8.0; 1.5)[b]	100.9	105.1	98.9	101.6	101.6 ± 2.6
mannitol (140; 32)	101.3	101.3	108.2	103.5	103.6 ± 3.2
galactose (3.0; 1.0)	99.7	101.1	99.0	98.7	99.6 ± 1.1
glucose (180; 60)	103.5	100.2	107.9	106.3	104.5 ± 3.4
sucrose (4.0; 10.0)	100.4	100.9	101.1	103.5	101.5 ± 1.4
fructose (11.0; 3.0)	101.6	102.1	100.8	103.3	101.9 ± 1.0
raffinose (1.0; 1.5)	98.7	98.0	100.0	98.3	98.8 ± 0.9
stachyose (9.0; 5.0)	96.7	98.5	97.3	96.3	97.2 ± 1.0

[a] Each sample was assayed three times before and after each addition, and the average of the results is presented. [b] In brackets are reported the high concentration of each constituent used to evaluate recovery in leaves and roots extracts, respectively.

Both tissue samples were extracted and purified in the same way, prior to carrying out the analytical separation. While previous methods, based on ligand-exchange chromatography with refractometric detection, are subjected to various interferences in the analysis of plant extracts, minimal sample workup was required under the present experimental conditions.[23,25] It should be mentioned that while Romani et al.[12] used two serial columns plus a guard one operating at 90 °C and eluted with water−acetonitrile 95:5 (v/v), here a single column ensured a good separation of soluble carbohydrates in about 25 min of chromatographic analysis.

The composition of nonstructural carbohydrates of *Olea europaea* is comparable in both leaves and roots samples, and relevant data are summarized in Table 3. In agreement with previous results,[5] the analysis of olive tissue extracts revealed that mannitol and glucose, the primary photosynthetic products along with fructose and sucrose, were the predominant sugar compounds in the investigated samples. These sugars represent more than 90% of the total soluble carbohydrates in olive tissues (see columns 3 and 5 in Table 3). This is not surprising, as mannitol and glucose represent the major transport sugars in olive trees and contribute significantly to osmotic adjustment. The relative amounts of the two detected sugars changed according to the type of olive tissue; based upon triplicate injections, the molar ratio of glucose to mannitol was estimated as 1.2 ± 0.1, and 2.2 ± 0.3 for olive leaves, and roots, respectively. The glucose/mannitol ratio in leaf tissues resulted in good agreement to 1.33, which is the value estimated from data of Romani et al.[12] Our average value is, however, slightly lower than that reported by Flora and Madore[4] of 1.9 ± 0.1, which was based on ^{14}C-labeled sugars upon liquid chromatographic separation, postcolumn fraction collection, and scintillation counting. Galactose, raffinose, and stachyose were present at relatively lower concentrations compared with those of glucose and mannitol in the extract syrups from leaves and roots of *Olea europaea*. Both these tissues possess the same pool of sugar compounds, thereby providing evidence for the existence of carbohydrate transporters in the olive plants. Apparently, this vegetal species is sufficiently capable of supporting prolonged

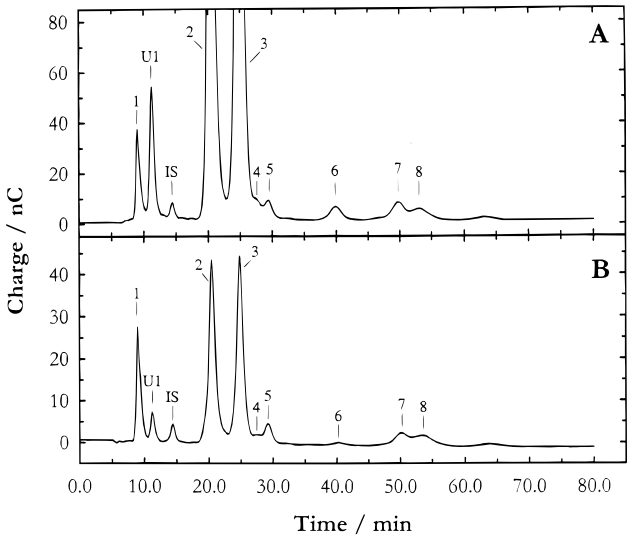

Figure 4. Chromatographic separations of extracts from olive leaves (A) and roots (B) by HPAEC with integrated PAD using 500 mM NaOH as the mobile phase at a flow rate of 0.4 mL/min. Peak identification: (1) *myo*-inositol, (2) mannitol, (3) glucose, (4) galactose, (5) fructose, (6) sucrose, (7) stachyose, (8) raffinose. Column, CarboPac MA1 plus guard (Dionex). Detection potential at the gold working electrode, $E_{DET} = +0.05$ V.

water deficiency probably because it has evolved the basic mechanisms of osmotic adjustment by accumulation of organic solutes, such as sugar compounds, in the cytoplasm. The activity of these substances is related to their ability to raise the osmotic potential of the cell, thus balancing the osmotic potential of an externally increased osmotic pressure.[7,8] We speculate that the present method will help to establish functions and roles of carbohydrates to dissect drought stress-signaling pathways, and to determine critical and rate-limiting cellular processes for water stress tolerance. Work is underway and will be reported elsewhere. We wish also to emphasize that the method for carbohydrate analysis in plant extracts proposed in the present work is much more convenient in terms of sensitivity, rapidity, and accuracy than previously proposed approaches based on radioactively labeled carbohydrates.[4]

To gain further information on the sugar composition, a chromatographic separation with a second anion-exchange column was carried out. Although the CarboPac PA1 column is well suited to the chromatography of mono- and linear oligosaccharides, it is less well suited to separate alditols. These compounds are weaker acids than their nonreduced counterparts and are therefore poorly retained on the above-mentioned anion-exchange column. However, it is possible to achieve a better separation of alditols using a CarboPac MA1 column. The resulting chromatograms are shown in parts A and B of Figure 4, leaf and root extracts, respectively. Samples were eluted with 500 mM NaOH at a flow rate of 0.4 mL/min. Although the same carbohydrates of Figure 3 were identified, a longer analysis time (>70 min) for each single run was needed. More importantly, poor separation occurred between glucose, galactose, and fructose (peaks 3, 4, and 5) as well as between raffinose and stachyose (peaks 7 and 8). Overall, the present results indicated that the separation of soluble carbohydrates in olive plants is better performed isocratically with a column having a relatively low anion-exchange capacity and dilute alkaline eluents upon addition of barium acetate.

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Table 3. Sugar Content of Olive Tissues of *Olea Europaea*. Data Represent the Means of Five Measurements (±SD)

sugar	leaf sugar content, μmol (grams dry weight)$^{-1}$	percentage of sugar content	root sugar content, μmol (grams dry weight)$^{-1}$	percentage of sugar content
myo-inositol	14.3 ± 1.1	1.9%	2.5 ± 4.2	0.9%
mannitol	309 ± 17	41.0%	77.9 ± 6.4	29.0%
galactose	4.8 ± 0.3	0.6%	1.2 ± 0.1	0.4%
glucose	370 ± 26	49.2%	168 ± 16	62.6%
sucrose	21.8 ± 2.5	2.9%	8.5 ± 0.6	3.2%
fructose	20.7 ± 0.7	2.8%	6.5 ± 0.9	2.4%
raffinose	2.7 ± 0.1	0.4%	1.0 ± 0.05	0.4%
stachyose	9.7 ± 1.3	1.2%	2.7 ± 0.4	1.0%

Galactinol Peak Assignment. As mentioned above, we have been successful in the chromatographic separation of sugar compounds in method development experiments, but we were unable to assign which carbohydrate in plant extracts is present in the peak at 2.05 min (see Figure 3). It was initially hoped that the use of a different anion-exchange column might be a way to assign such a peak to galactinol (*O*-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol).[26] In plants containing relatively large amounts of raffinose oligosaccharides, galactinol occurs in significant concentrations.[27] In previous work[12] using HPLC-RI detection, the occurrence of galactinol in plant extracts of *Olea europaea* was ascertained. Because of the unavailability of galactinol as a standard compound, an attempt to discover the identity of peak 2 was made by running a sample of watermelon cotyledon. In Figure 5 two separations relevant to extracts from watermelon cotyledons and olive leaves, dashed and solid curves, respectively, are compared. The same conditions described for Figure 2 were employed. As the identity of galactinol (peak 2) might be still in doubt, it was unambiguously identified using the CarboPac MA1 column in which the same sample extracts were injected (not shown).

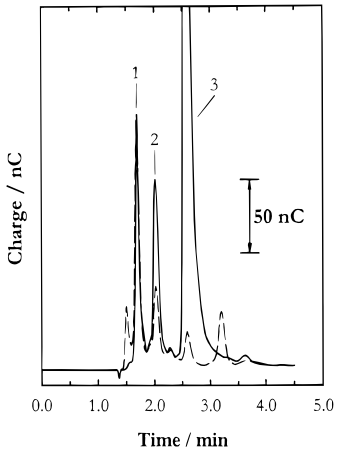

Figure 5. Chromatographic profiles of sample extracts from olive leaves (solid curve) and watermelon cotyledons (dashed curve). Peak: (1) *myo*-inositol, (2) galactinol, and (3) mannitol. Other conditions as in Figure 2.

CONCLUSIONS

The conditions developed in this study for carbohydrate analysis in extracts of vegetal tissues by HPAEC-PAD are a powerful improvement over previous methods in terms of sensitivity, simplicity, and efficiency. It is well suited to satisfy the demands for accurate and sensitive detection of alditols and mono-, di-, and trisaccharides with minimal sample preparation and cleanup. Although this method reported was developed and validated specifically for olive plants, it can be applied to tissue extracts of others vegetables. We believe that HPAEC-PAD is especially suitable for studies involving changes of concentration of soluble carbohydrates in plants subjected to environmental stress conditions, so work is currently under way along these directions.

ACKNOWLEDGMENT

This work is part of the Ph.D. Thesis of G.M. still in progress in our laboratory. The work was supported by the National Research Council of Italy (CNR, Rome) and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, Rome).

Received for review March 6, 2000. Accepted June 7, 2000.

AC000266O

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