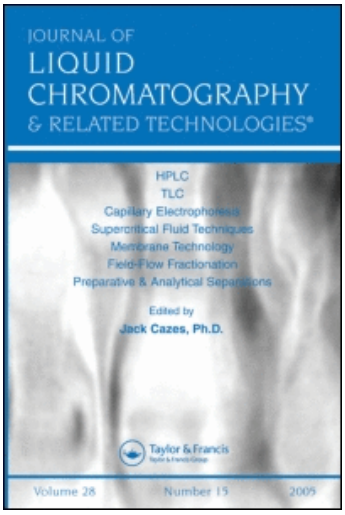

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Pulsed Electrochemical Detection of Aryl- and Alkylglycosides Following Reversed-Phase Liquid Chromatography

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ABSTRACT

Pulsed electrochemical detection (PED) coupled to reversed-phase high performance liquid chromatography is applied to the detection of aryl- and alkylglycosides. PED is used to detect these compounds sensitively without the need of derivatization. Detection limits using an optimized waveform are typically 80–40 and 400–200 nM for aryl- and alkylglycosides, respectively. For arylglycosides, PED proved generally to be comparable to absorbance detection at 254 nm (300–5 nM) and 204 nm (40–20 nM). The advantages of PED over UV detection are highlighted for alkylglycosides, for which optical detection at 204 nm produced no analytical signal. This work is applied to the determination of a

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commercial biological surfactant known as Elugent™, which is used to solubilize cell membrane proteins.

Key Words: Pulsed electrochemical detection; HPLC; Glycosides; Surfactants; Carbohydrates.

INTRODUCTION

Surfactants (e.g., aryl- and alkylglycosides) are used in a variety of chemical industries, including cosmetics, pesticides, pharmaceuticals, and plastics. Alkylglycosides, in particular, find application in the manufacture of biopharmaceuticals, and thus, it is extremely important to develop analytical procedures for the determination of trace amounts of these surfactants. In general, emphasis on trace quantities in foods, drugs, water supplies, and the like necessitates the need for selective and sensitive separation and detection systems.[1,2] Mixtures of aryl- and alkyl-substituted carbohydrates are amenable to separation using reversed-phase chromatography, but their detection at low levels is not always trivial. The detection of aryl-containing glycosides often relies on UV absorption,[3] but alkylglycosides[4] have little or no chromophores, fluorophores, and/or constant applied (dc) potential electrophores.

The simple, sensitive, and direct detection of numerous polar aliphatic compounds has been achieved with pulsed electrochemical detection (PED).[5–8] This technique exploits the electrocatalytic activity of noble metal electrode surfaces to oxidize various polar functional groups. In PED, multi-step potential–time waveforms at Au and Pt electrodes actualize amperometric/coulometric detection, while maintaining uniform and reproducible electrode activity. The use of PED following HPLC for the detection of carbohydrates has been reviewed.[9–11]

Typically, PED has been used to determine unsubstituted carbohydrates, such as glucose, in a variety of matrices. More recently, HPLC–PED has been applied to the determination of glucuronide metabolites of morphine in complex matrices.[12] This study showed that compounds consisting of a conjugated glycosidic moiety (not just simple or unsubstituted sugars) are amenable to detection by PED. Another technique that has emerged in the literature for determining glycosylated compounds is evaporative light scattering detection (ELSD). However, ELSD lacks the selectivity achieved by electrochemical detection, relying only on the chromatographic separation for specificity.[13] Furthermore, ELSD is limited to mobile-phase buffers that are volatile, and its linear range is markedly smaller than its dynamic range.[13] Finally, ELSD for glycosylated compounds is typically two orders of magnitude less sensitive than PED.[14,15]

Here, the application of PED to substituted carbohydrates is studied in detail. Model aryl- and alkylglycosides (e.g., alkylglucosides) are detected at a Au working electrode under alkaline conditions created by post-column addition of NaOH following reversed-phase liquid chromatography. Electrochemical characterization via cyclic voltammetry and pulsed voltammetry (PV)[16] is the basis of optimization of the PED waveform, and mechanistic information is used to improve the choice of the chromatographic solvent system. In addition to determining analytical figures of merit for model compounds, PED and UV detection modes will be compared. A chromatographic assay applicable to non-ionic detergent formulations, which are widely used in both biochemistry and biology enabling the isolation of and study of integral membrane proteins[17,18], will underscore the unique advantages of PED for the determination of glycosylated moieties with no optical activity.

EXPERIMENTAL

Reagents and Solutions

All solutions were made with water that was purified using a reverse osmosis system coupled to multi-tank/ultraviolet ultrafiltration stations (US Filter/IONPURE, Lowell, MA). All solvents were HPLC grade. All solvents were filtered with a vacuum filtration apparatus (Fisher) utilizing a 0.2-μm PTFE membrane filter (Alltech Associates, Inc., Deerfield, IL). Post-column reagent, 300 mM NaOH, was prepared from 50% (w/w) NaOH (VWR Scientific Products Corp., West Chester, PA). Phenyl-β-D-galactoside, phenyl-β-D-glucoside, and all alkylglucosides were obtained from Sigma Chemical Company (St. Louis, MO). D-Amygdalin and helicin were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All glycoside solutions were prepared in purified water except the alkylglucosides, which were made in a solution of ACN/water (52/48, v/v). If not used immediately, standard solutions were stored in a refrigerator set at 4°C. The real sample, Elugent™ (Calbiochem-Novabiochem Corporation, San Diego, CA), is a biological detergent containing a mixture of several alkylglucosides, and it was prepared by diluting the appropriate amount to volume with 100% MeOH and further diluting this solution in water.

Instrumentation

Cyclic voltammetric (CV) data was collected at a gold, 3.0-mm diameter rotating disk electrode (RDE) on an Epsilon Electrochemistry System (Bioanalytical Systems, Inc., West Lafayette, IN) using the Epsilon software

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on a Dell Dimension 2100 desktop computer. PV experiments were carried out on a model AFRDE4 Bi-Potentiostat (Pine Instrument Company, Grove City, PA). Pulsed voltammetric waveforms were generated with ASYST scientific software (Asyst Software Technologies, Rochester, NY) on a 286/16 MHz IBM compatible computer interfaced via a DAS-20 AD/DA expansion board (Keithley Data Acquisition, Taunton, MA). The auxiliary electrode was a platinum wire, and the reference electrode was a Ag/AgCl electrode (model 13-620-45; Fisher Scientific). The electrochemical cell (ca. 125 mL) for all voltammetric experiments only was constructed of Pyrex™ glass with two side arms separated from the cell body with fine glass frits.

The chromatographic system consists of an Advanced Gradient Pump (Model GP-40) equipped with an Eluent Degas Module (Dionex Corporation, Sunnyvale, CA). An acetonitrile (ACN, Fisher Scientific, Pittsburgh, PA)/water (80/20, v/v) solution filled one reservoir of the chromatography apparatus, while another reservoir contained solely purified water. The flow rate was set at 1.0 mL min^{-1}. The injection valve (model 9010; Rheodyne, Inc., Rohnert Park, CA) was fitted with a 20-μL injection loop. All separations were performed on a reversed-phase (C18; 250 × 4.6 mm, 5 μm) Econosphere column (Alltech Associates) with guard column (C18; 7.5 × 4.6 mm, 5 μm; Econosphere; Alltech). UV absorbance detection at either 254 or 204 nm was performed using a Model VDM-2 variable wavelength detector (Dionex). Post-column addition of NaOH, necessary to promote PED-activity, was accomplished by a reagent delivery module (RDM; Dionex). The post-column apparatus was placed between the UV detector and the electrochemical detector. The NaOH reagent at 0.6 mL min^{-1} was added to the eluent via a mixing-tee, followed by a 250 μL, knitted, Teflon™ tubing reaction coil[19] to achieve a final solution concentration of ca. 0.1 M NaOH. PED was accomplished using a pulsed electrochemical detector (Dionex) employing a quad-potential waveform[20] optimized for glycoside detection. The cell consisted of a Au working electrode (1.0 mm diameter), a Ag/AgCl reference electrode (model 42442; Dionex), and a stainless steel body serving as the auxiliary electrode. Data acquisition and instrument control was accomplished using Dionex PeakNet software, version 4.30, on a 33 MHz Eclipse PC.

RESULTS AND DISCUSSION

Model Compounds

Figure 1 shows the molecular structures of the model compounds selected for this study, which include phenyl-β-D-glucoside, phenyl-β-D-galactoside, helicin, D-amygdalin, and a series of alkylglucosides. In general, the

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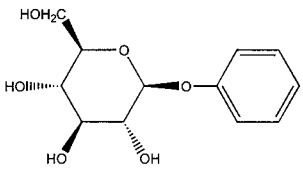
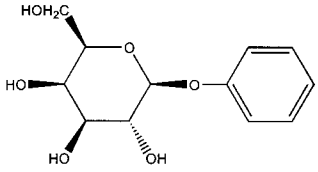


Phenyl-β-D-glucoside



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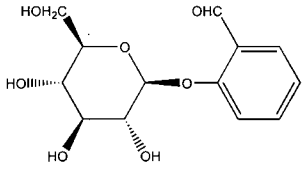
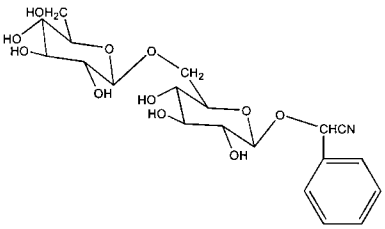
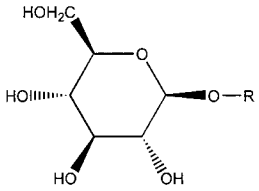


Helicin



D-amygdalin



Compound	R
Hexylglucopyranoside	C_6H_{13}
Heptylglucopyranoside	C_7H_{15}
Octylglucopyranoside	C_8H_{17}
Nonylglucopyranoside	C_9H_{19}

Figure 1. Molecular structures of aryl- and alkylglycosides.

aryl-containing glycosides serve as models for glycosylated flavonoids (e.g., ginsenosides), and the alkylglucosides represent a class of non-ionic surfactants. D-amygdalin, which is a constituent of a wide range of seeds (e.g., from bitter almonds, apples, apricots, peaches, plums, and cherries) and plant components, is a cyanogenic glycoside capable of releasing HCN, which can result in death upon ingestion by animals and humans.[21] In particular, the model compounds were chosen to exhibit varying degrees of conjugation (i.e., aryl- and alkylglycosides), which affects both UV-activity and lipophilicity and

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glycation. The extent of glycation ultimately determines relative PED-activity. Since the response mechanisms in PED are dominated by the surface properties of the electrodes, members of each chemical class of compounds produce virtually identical voltammetric responses. This fact leads to two conclusions: (i) model compounds can be used to represent a wide variety of analogues and (ii) PED requires a priori separation of complex mixtures to be effective.

Cyclic Voltammetry and Pulsed Voltammetry-Waveform Optimization

The current–potential response shown in Fig. 2 is for phenyl-β-D-galactoside (—) at a Au RDE in de-aerated 0.1 M NaOH (-----). The anodic signal for the oxidation of the hydroxyl groups (wave a) on the glycosidic group begins at ca. −200 mV and reaches a plateau at ca. +50 mV. The response is attenuated abruptly (>ca. +350 mV) with the onset of surface oxide formation (wave b). The anodic discharge of H_2O to produce O_2

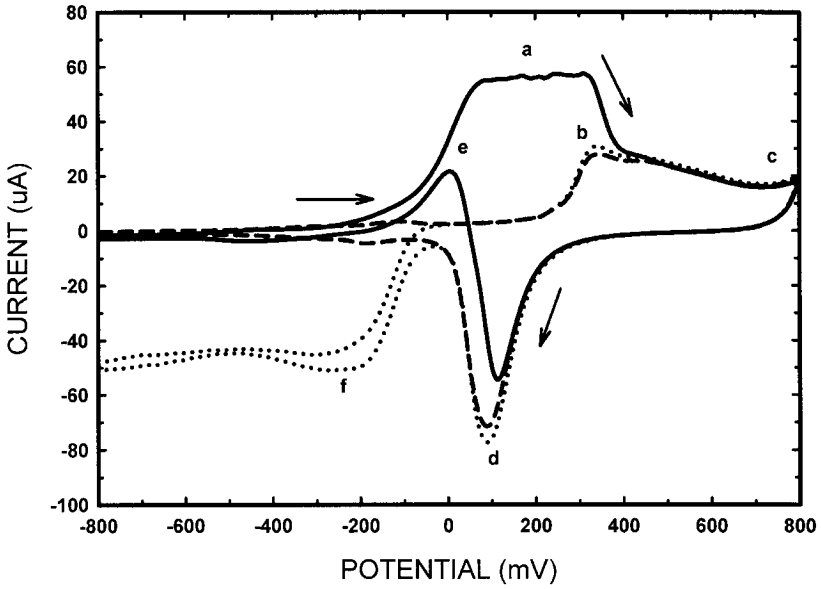

Figure 2. CV response for phenyl-β-D-galactoside at a Au RDE in 0.1 M NaOH. Conditions: 900 rpm rotation speed; 150 mV s^{-1} scan rate. Solutions: (· · · · · ·) aerated 0.1 M NaOH; (------) deaerated 0.1 M NaOH; (——) 0.3 mM phenyl-β-D-glucoside in de-aerated 0.1 M NaOH.

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(wave c, $>+800$ mV) defines the positive limit of the voltammetric scan. The cathodic peak signal for the subsequent negative scan corresponds to the reduction of the surface oxide (wave d, ca. $+300$ to -50 mV), and, as the oxide is removed from the electrode surface, the reactivity of the Au surface is restored and an anodic peak is observed for hydroxyl oxidation (wave e, ca. $+100$ to -200 mV). If dissolved O_2 is present in the solution, its reduction is observed as a cathodic wave in both the forward reverse potential scans. The potential region between dissolved O_2 reduction (wave f) and the onset of oxide formation (wave b) is typically denoted as the "oxide-free" window, where carbohydrate detection can be monitored with the least background interference. These observations are in agreement with oxide-free detection mechanisms for carbohydrates.[9–11]

Although cyclic voltammetry is useful to the investigation of electrochemical mechanisms, PV has been shown to be the definitive method for characterizing analyte response and selecting potential and time waveform parameters in PED.[16] Figure 3 displays the background-corrected current

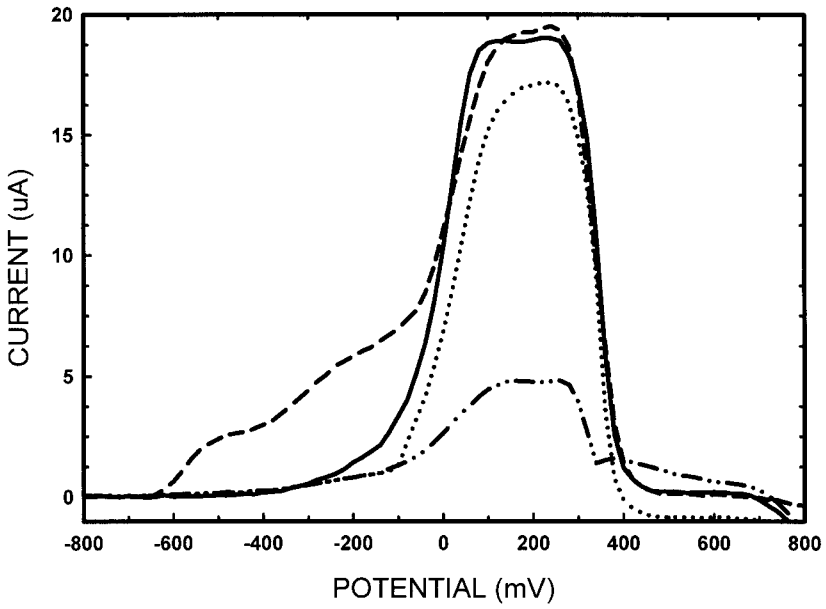

Figure 3. Background-corrected pulsed voltammetric responses as a function of E_{det} for arylglycosides at a Au RDE in 0.1 M NaOH. Conditions: 900 rpm rotation speed. Solutions (0.4 mM): (——) phenyl-β-D-galactoside; (· · · · · ·) phenyl-β-D-glucoside; (------) helicin; (— · —) D-amygdalin.

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to potential responses of (—) phenyl-β-D-galactoside, (· · · · · ·) phenyl-β-D-glucoside, (------) helicin, and (— · —· ·) D-amygdalin at a Au RDE in deaerated 0.1 M NaOH. Helicin shows an anodic wave beginning at ca. −600 mV and extending to ca. +400 mV due to the oxidation of the aldehyde group to the corresponding carboxylate group.[11] The response between ca. −300 and ca. +400 mV corresponds to the electrocatalytic oxidation of the hydroxyl groups of the carbohydrate moieties in all the compounds. As in the CV, the signal is attenuated beyond ca. +400 mV due to the formation of surface oxide inhibiting detection of carbohydrates. The optimized detection potential is determined to be +100 mV, based on maximizing the signal-to-noise ratio for all the model compounds and, hence, virtually all substituted glycosides. The overall waveform is based on a quad-potential pulsed waveform, which is used to achieve high sensitivity *and* long-term stability.[20] Table 1 lists the values of the optimized waveform used for all the studies in this paper.

LC–UV–PED

Aryl- and alkylglycosides are readily separated by a reversed-phase mechanism with a mixture of water and ACN as the mobile phase. Acetonitrile was chosen because of its compatibility with low-wavelength UV detection and it is not PED-active. Figure 4 shows a plot of capacity factor (k') vs. percent ACN in the mobile phase. A mobile phase of water/ACN, 90/10 v/v, allowed for the baseline resolution of the four model arylglycosides, and it was used for all chromatographic separations unless otherwise noted. Since none of the model compounds had ionizable groups, a mobile phase buffer was not needed. If a mobile phase buffer is needed, sodium acetate buffers are suggested for PED compatibility. Post-column addition of NaOH

Table 1. Optimized PED waveform parameters.

Potential (mV) vs. Ag/AgCl	Time (msec)	Integration interval
100	0	
100	200	Begin
100	400	End
−1,500	410	
−1,500	420	
600	430	
−100	440	
−100	500	

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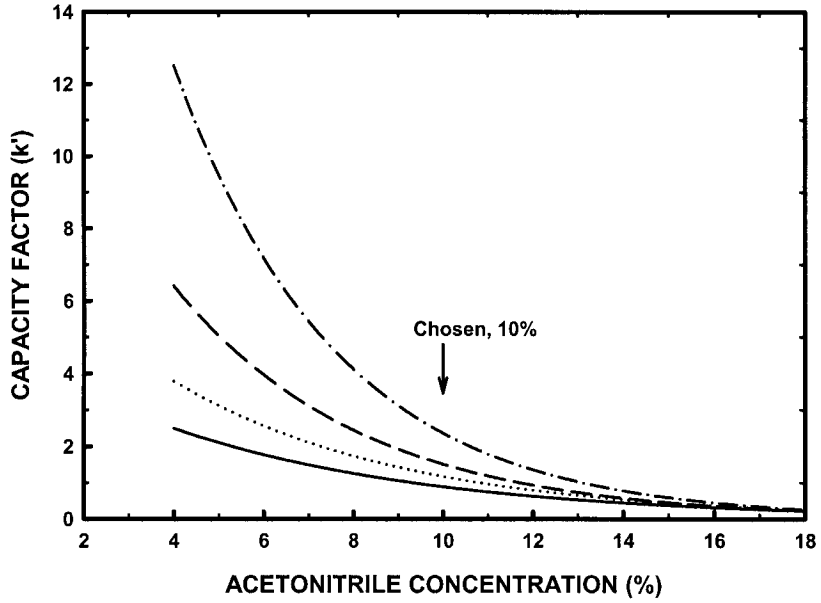

Figure 4. Capacity factor (k') plot of arylglycosides. Compounds: (——) phenyl-β-D-galactoside; (· · · · · ·) phenyl-β-D-glucoside; (------) helicin; (—·—·) D-amygdalin.

reagent (i) provides the supporting electrolyte for electrochemical detection, (ii) facilitates the highly alkaline conditions required for electrocatalytic detection of carbohydrates at noble metal electrodes, and, if used, (iii) ameliorates the effects of any mobile phase buffers or gradients.[22]

Figure 5 shows the chromatograms of the four model compounds using (A) UV detection at 254 nm and (B) PED. Baseline resolution of the four arylglycosides is achieved, including the closely related compounds phenyl-β-D-galactoside (peak 1) and phenyl-β-D-glucoside (peak 2). The chromatograms are normalized to have the same noise for comparison of the signals. Under these conditions, PED shows a fairly uniform response as compared to UV detection. Table 2 lists the analytical figures of merit for UV detection at 204 nm and 254 nm and PED using the optimized waveform. Regression analysis showed that both UV and PED were linear over the range of concentrations tested, which was at least 3 decades in all cases. Limits of detection (LOD) for UV at 254 nm ranged from 0.005 to 3 μM, while those for UV at 204 nm ranged from 0.02 to 0.04 μM. LODs for PED, calculated in the same manner, were found to have values between 0.04 and 0.08 μM. All LOD were found using peak height of the analytical signal. Repeatability,

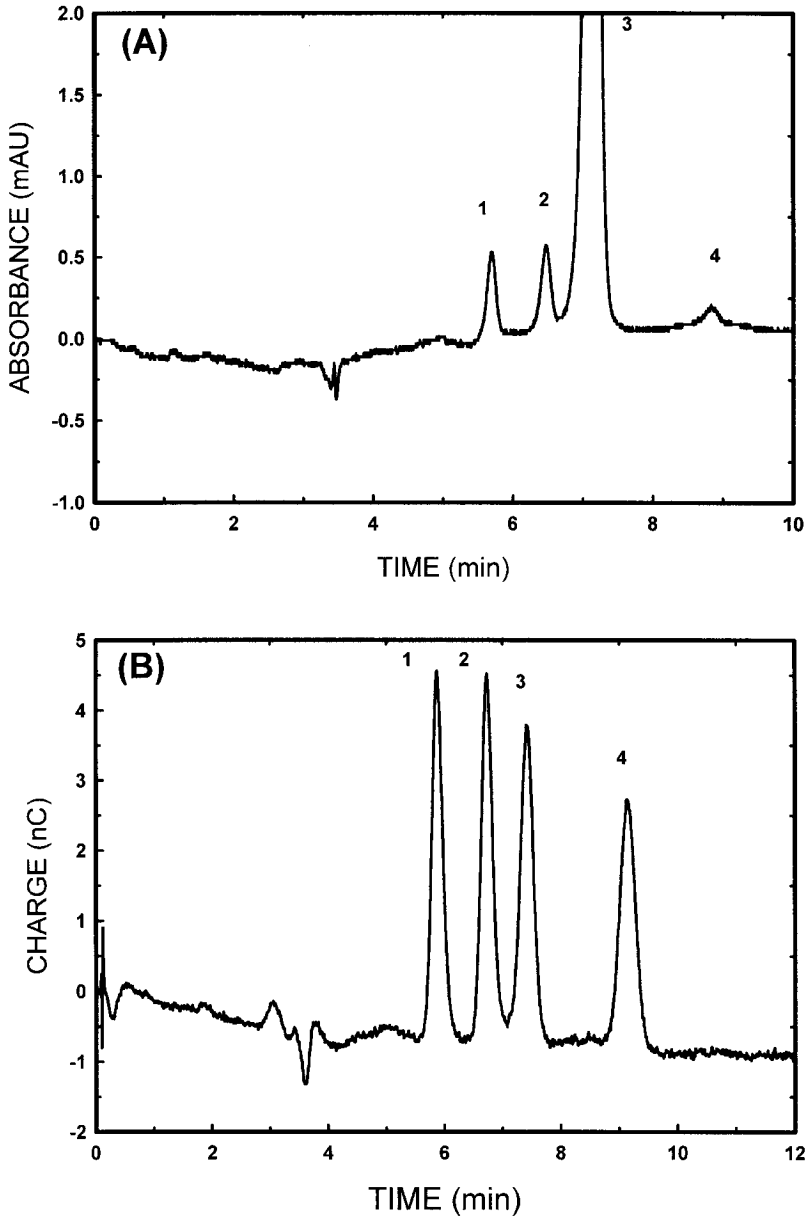

Figure 5. Chromatograms for a mixture of arylglycosides with detection by (A) UV at 254 nm and (B) PED. Peaks (1 ppm): (1) phenyl-β-D-galactoside; (2) phenyl-β-D-glucoside; (3) helicin; (4) D-amygdalin.

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Table 2. Quantitative parameters model compounds by UV detection and PED.

Mode	Compound	LOD[a] (μM, pmol)	Linear range μC (AU for UV) = a (pmol) + b			% RSD (μM, n)
			a	b	R^2	
PED	Phenylgalactoside	0.07, 1.4	57,797	8,440	0.99934	2.97 (4.1, 6)
	Phenylglucoside	0.08, 1.6	60,147	7,139	0.99929	2.26 (4.0, 6)
	Helicin	0.06, 1.2	59,752	11,322	0.99909	2.16 (3.6, 6)
	D-Amygdalin	0.04, 0.8	53,362	7,457	0.99975	3.40 (2.2, 6)
	Hexylglucoside	0.2, 3.3	26,761	12,271	0.99952	4.81 (3.6, 12)
	Heptylglucoside	0.2, 3.8	27,721	6,414	0.99987	4.87 (3.4, 12)
	Octylglucoside	0.2, 4.9	19,936	6,510	0.99899	4.89 (3.6, 12)
	Nonylglucoside	0.4, 8.2	15,566	4,285	0.99926	2.57 (3.2, 12)
UV, 254 nm	Phenylgalactoside	0.09, 1.8	473	407	0.99925	2.06 (4.1, 6)
	Phenylglucoside	0.09, 1.8	493	331	0.99932	2.20 (4.0, 6)
	Helicin	0.005, 1.1	10,490	13,998	0.99931	0.44 (3.6, 6)
	D-Amygdalin	3, 0.6	131	−105	0.99910	0.94 (2.2, 6)
UV, 204 nm	Phenylgalactoside	0.03, 0.6	7,813	5,870	0.99926	3.48 (4.1, 6)
	Phenylglucoside	0.04, 0.8	60,147	7,139	0.99924	2.21 (4.0, 6)
	Helicin	0.02, 0.4	15,284	10,624	0.99933	2.65 (3.6, 6)
	D-Amygdalin	0.03, 0.7	5,005	3,950	0.99930	3.71 (2.2, 6)

[a]Limits of detection were determined at 3 times S/N ratio from concentrations within 10 times the LOD.

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or the percent relative standard deviation (%RSD), of repetitive injections at the limit of quantitation ($S/N = 10$) for the modes of detection, were found to be comparable—PED (2.2–3.0%) and UV (0.44–3.7%). The data in Table 2 suggests that the sensitivity of PED and UV detection at both 254 and 204 nm are essentially the same. If the absorptivity of a substituent increases relative to the carbohydrate composition of an analyte, the sensitivity for optical detection methods will be enhanced relative to PED. For instance, helicin ($\varepsilon_{max} = 10{,}094\,M^{-1}\,cm^{-1}$) is an order of magnitude more sensitive by UV (at 254 nm) then PED. Conversely, amygdalin (i.e., a substituted disaccharide) is similarly more sensitive by PED then by UV detection at 254 nm. Comparable sensitivity to PED for amygdalin can be achieved at 204 nm. However, the lack of specificity for low-wavelength UV detection often leads to interference from matrix components in real samples, and the use of gradient elution is compromised since baseline shifts become evident due to the significant absorbance of mobile phase components. If buffers are required for the separation, it should be noted that phosphate buffers, which are commonly used in reversed-phase separations due to their weak absorbance at low-wavelengths, are not compatible with PED,[23] and the PED-compatible acetate buffers absorb significantly below 240 nm. Hence, it is not always practical to use such low-wavelengths, with or without PED, on line.

Application to Alkylglucosides

For compounds lacking chromophores, such as alkylglucosides, UV detection becomes ineffective even at low-wavelengths. Figure 6(A) shows a chromatogram of *n*-hexyl-glucopyranoside (peak 2), *n*-heptyl-glucopyranoside (peak 3), *n*-octyl-glucopyranoside (peak 4), and *n*-nonyl-glucopyranoside (peak 5) using PED. Peak 1 was determined to be an impurity of *n*-pentyl-glucopyranoside in the alkylglucoside samples. Elution of the alkylglucosides required the organic modifier (i.e., ACN) in the mobile phase to be increased to 36%. Table 2 also lists the analytical figures of merit for the alkylglucosides. Regression analysis showed PED was linear over the range of concentrations tested, which was at least three decades in all cases. LODs and repeatabilities were found to be 0.2–0.4 μM and 2.6–4.9%, respectively. UV detection shows no response, even with UV detection at 204 nm (see inset of Fig. 6(A)).

To highlight the analytical utility of LC–PED, Elugent™ was assayed for alkylglucoside content. Elugent™ is a biological non-ionic detergent that is used to solubilize cell membrane proteins. The sample was diluted 1 : 100 mL Elugent : MeOH, and further diluted 1 : 100 mL in water. Figure 6(B) is a typical chromatogram of Elugent™ using PED. For this separation, the

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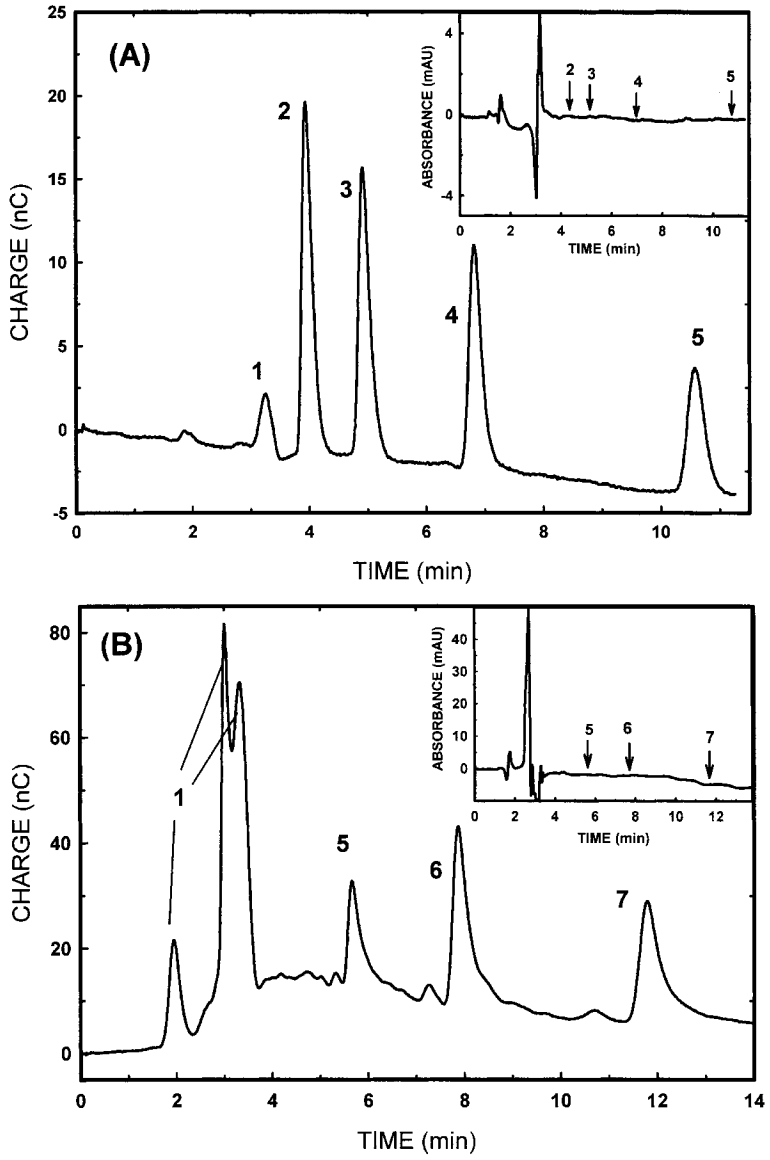

Figure 6. Separation and detection of (A) alkylglucoside standards and (B) Elugent™ using HPLC–PED with inset chromatograms of the same separations but with UV detection at 204 nm. Peaks in (A): (1) *n*-pentylglucoside; (2) *n*-hexylglucoside, 1 ppm; (3) *n*-heptylglucoside, 1 ppm; (4) *n*-octylglucoside, 1 ppm; and (5) *n*-nonylglucoside, 1 ppm. Peaks in (B): (5) *n*-nonylglucoside; (6) *n*-decylglucoside; and (7) *n*-undecylglucoside.

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amount of organic modifier in the mobile phase was increased to 45%. The sample was determined to contain a mixture *n*-nonylglucoside (peak 5), *n*-decylglucoside (peak 6), and *n*-undecylglucoside (peak 7) at concentrations of 4.54 ± 0.03%, 9.35 ± 0.02%, and 9.51 ± 0.11%, respectively. The listed concentrations are 5%, 9%, and 9%, respectively. Peaks 1 are unidentified components of the formulation. Again, UV detection at 204 nm offers no analytical utility for these compounds (inset of Figure 6(B)).

CONCLUSIONS

This study shows that PED, with post-column addition of NaOH, is compatible with reversed-phase chromatography, thus allowing for the separation and detection of a wide variety of moderately polar to nonpolar substituted carbohydrates. PED can be used to detect aryl- and alkyl glycosides, regardless of whether or not the compounds of interest contain a chromophore, often with equal or superior sensitivity than UV detection. This is a rugged and reliable technique, useful in determining virtually any substituted glycoside.

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