

# **A proposed step-by-step optimization of HPLC method with pulsed amperometric detection for carbohydrates and amino acids by gold working electrode and palladium reference electrode**

## **Abstract**

High performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) by gold working electrode and anion-exchange separation in alkaline mobile phase was reported to be utilized for analyses of carbohydrates and amino acids without derivatization. But there were many different types of potential-time waveforms. Most of potentials were applied with respect to the glass-enveloped wet Ag/AgCl reference electrode that was liable to be alkali-corroded. We have developed a stable, sensitive, and reproducible waveform of potential-time parameters with respect to the solid-state palladium reference electrode that was acid- and alkali-resistant. The waveform we developed in this study was simply three-waved. No alumina polishing, chemical modification, or parts replacing was necessary for gold working electrode or palladium reference electrode. Regarding the performance of the reference electrode in HPLC-PAD system with the strongly alkaline mobile phase, the solid-state palladium was much better than the glass-enveloped wet Ag/AgCl.

## **1. Introduction**

For HPLC determination of carbohydrates, the refractive index detector can be used for high concentration of carbohydrates in the injected sample, the evaporative light scattering detector should be used for medium concentration of carbohydrates. The pulsed amperometric detector with gold working electrode has to be used if the concentrations of carbohydrates are very low. Some reporters said HPLC with pulsed amperometric detection by gold working electrode could also be utilized for analyses of amino acids without derivatization [1-6].

For the reference electrode of electrochemical detector in the popular HPLC market, there were the solid-state palladium and the glass-enveloped wet Ag/AgCl. The former was acid- and alkali-resistant, but the latter was only acid-resistant, not alkali-resistant. The strongly alkaline mobile phase could poison the glass-enveloped wet Ag/AgCl reference electrode. So we chose the solid-state palladium as the reference electrode with respect to gold working electrode [7].

We have developed a stable, sensitive, and reproducible efficient waveform of potential-time parameters by gold working electrode and palladium reference electrode for the pulsed amperometric detection of carbohydrates. The hydroxyl groups of monosaccharides in the strongly alkaline mobile phase would become anions, so it was rational to use the polymer-based anion-exchange column for the separation of

carbohydrates in HPLC-PAD.

## **2. Experimental**

This method has been developed with ESA 582 pump, ESA 5020 mobile-phase cell, ESA 542 autosampler, ESA Coulochem-3 pulsed electrochemical detector and its control-related thermal organizer, ESA 5040 analytical cell, and CarboHite 250x4mm analytical column. ESA 582 pump was connected with 2 kits of PEEK dampers. The applied potential of ESA 5020 mobile-phase cell was set by ESA GuardStat controller. ESA 542 autosampler was factory-equipped with Rheodyne 9740 PEEK injection valve. ESA Coulochem-3 pulsed electrochemical detector was operated in the pulsed mode and equipped with the thermal organizer that CarboHite column and ESA 5040 cell were placed in. ESA 5040 analytical cell was the amperometric cell equipped with gold working electrode and palladium reference electrode. All PEEK fittings, tubings, and unions were from Upchurch Scientific, Inc. The isocratic configuration of HPLC-PAD system for carbohydrates was shown in figure 1.

The alkaline mobile phase was prepared in PP 2L beaker, and filtered through VacuCap-60 filtering cap with Supor 0.1um membrane, and collected into PP 2L mobile-phase bottle. We did not use any glass labware in contact with the alkaline mobile phase. The mobile phase for separation of glucose, sorbitol and mannitol was 100mM sodium hydroxide. The mobile phase for separation of glucosamine-1-phosphate was 120mM sodium acetate and 50mM sodium hydroxide. All standards of glucose, sorbitol, mannitol, glycine, and glucosamine-1-phosphate were from Sigma. The analytical grade of sodium hydroxide and sodium acetate were from Merck.

We did not regenerate the analytical column before every injection of analysis. We did not use helium or nitrogen gas to do anything with the mobile phase. The mobile phase was not recycled. We did not polish or chemically modify the gold working electrode at all. The potential-time waveform, the temperature of analytical column and analytical cell, and the flow rate of mobile phase were discussed afterwards.

## **3. Results and discussion**

### **3.1 Requirements of HPLC-PAD system**

Due to the strong alkalinity of mobile phase, the deliberate selection of components of HPLC-PAD system must be taken into account for alkali-resistance. Glass labware was not alkali-resistant. PP labware was good for alkali-resistance. For the mobile phase reservoir, we found PP bottle had a better alkali-resistance than HDPE bottle did. Furthermore, PP bottle was thick enough to be used as the vacuum bottle to collect the membrane-filtered mobile phase by vacuum suction.

The on-line vacuum degasser was not used here, because we were not sure if it was alkali-resistant or not. Two kits of PEEK dampers were utilized in series right after pump to significantly reduce the pressure noise from pump. The washing solvent of pump should be pure water without any organic solvent.

ESA 5020 mobile-phase cell was coulometric and utilized right before autosampler to reduce the background current of mobile phase, and to facilitate the baseline of ESA 5040 analytical cell to stabilize. The mobile-phase cell applied with a higher potential than the detection potential of analytes could electrochemically pre-treat the mobile phase before it carried the injected sample and entered into the analytical column and analytical cell.

Regarding the sample injection valve of autosampler, the PEEK injection valve with Tefzel rotor seal should be used instead of the stainless-steel injection valve with Vespel rotor seal. According to Rheodyne informations, PEEK and Tefzel were alkali-resistant up to pH 14, but Vespel was only to pH 10. The washing solvent of autosampler should be pure water without any organic solvent.

ESA 5040 amperometric cell was equipped with gold working electrode, palladium reference electrode, and 316SS counter electrode.

ESA thermal organizer was utilized to install and heat both CarboHite column and ESA 5040 cell. The heating temperature could be controlled directly by ESA Coulochem-3 pulsed electrochemical detector.

### 3.2 Develop and optimize the potential-time waveform

At the beginning of this study, we had tried those potential-time parameters according to some published journals and application notes. Unfortunately, either the background baseline was not stable, or the detection response did not meet our expectation. The reason should be that we used the solid-state palladium, instead of the glass-enveloped wet Ag/AgCl, as the reference electrode of amperometric detector. So we had to develop a pulsed waveform of potential-time parameters for the electrochemical detection of carbohydrates by gold working electrode and palladium reference electrode. So afterwards, all potentials mentioned in this study were applied with respect to the solid-state palladium reference electrode.

We were trying to find those optimal parameters for E1, E2, E3, E4, T1, T2, T3, T4, and AD. Primarily, we thought, after the analytical column was completely equilibrated with the alkaline mobile phase, every entry of a correct parameter should result in a stable background current. After every entry of a new parameter, we observed the background current for about one minute. If a new parameter resulted in an unstable background current, we thought this new parameter was on the wrong way. As long as the background current was stable in about one minute after every new parameter's entry, we immediately injected 10 $\mu$ L of glucose standard solution 1 $\mu$ g/mL into HPLC and repeated three times for the verification of reproducibility.

### 3.2.1 Finding the optimal E1 potential

We used a procedure of quasi-DC mode to find the optimal E1 potential of the pulsed waveform for the detection of monosaccharides. Those parameters of ESA Coulochem-3 pulsed electrochemical detector were set as follows.  $E1 = E2 = E3 = E4 = X$  mV (milli-volt),  $T1 = 1000$  mS (milli-second),  $T2 = T3 = T4 = 2$  mS, AD (acquisition delay) = 2 mS, and R (detection range) = 100 nCFS. The potential of ESA 5020 mobile-phase cell was set  $E1 + 100$  mV. The 10  $\mu$ L of glucose standard solution 1  $\mu$ g/mL was injected into HPLC. We found there was a lower detection response in every successor injection than the predecessor injection. Like many reporters said [1-3], this suggested that glucose oxide could adsorb onto the surface of gold working electrode and then lowered its electrochemical capability. So after every injection of analysis was completed, we manually set  $E2 = +1000$  mV and  $E3 = -1000$  mV for about 10 seconds to clean and activate the surface of gold working electrode, and then set both E2 and E3 back to X mV waiting for baseline equilibrium and next injection. The temporary setting of  $E2 = +1000$  mV and  $E3 = -1000$  mV for about 10 seconds and waiting for about 20 minutes could get a stable baseline and a reproducible next-analysis. The X mV was tested from 0 mV up to +400 mV by an increment of 50 mV, we found +200 mV had the optimal signal-to-noise ratio for detection response. So we chose +200 mV as E1 potential, i.e., the detection potential of the pulsed potentials waveform. Regarding the E2 potential, we logically speculated it should be a negative potential, since E1 was positive. Regarding the E3 potential, it should be positive, since E2 was negative. Regarding the E4 potential, it should be not necessary, since E2 and E3 were already applied with the negative and positive potentials in order to clean and activate the surface of gold working electrode, and as long as the magnitudes of E2 and E3 potentials were enough capable of achieving the cleaning and activating task. At this moment, we used a three-wave form (+ - +) of pulsed amperometric potentials, and set  $E1 = +200$  mV,  $E2 = -1000$  mV,  $E3 = +1000$  mV,  $T1 = 1000$  mS,  $T2 = T3 = 100$  mS, AD = 500 mS, and the detection range R = 100 nCFS. The potential of ESA 5020 mobile-phase cell was set +300 mV.

### 3.2.2 Finding the optimal E2 potential

E2 potential was modified from -1000 mV up to -300 mV by an increment of 100 mV. We found the higher the E2 potential was, the lower the background current was. (P.S. -300 mV was higher than -1000 mV.) But the detection response did not differ significantly. In the potential range of from -1000 mV up to -300 mV for E2 entry, we found -600 mV was the optimal potential for detection response and baseline stability. So we chose -600 mV as E2 potential.

### 3.2.3 Finding the optimal E3 potential

E3 potential was modified from +1000mV down to +300mV by a deduction of 100mV. We found the lower the E3 potential was, the higher the background current was, and the lower the detection response was. So we returned E3 potential from +300mV up to +1300mV by an increment of 100mV. We found the higher the E3 potential was, the lower the background current was, and the higher the detection response was. Furthermore, the detection response was enhanced significantly when E3 potential was set above +1000mV. In the potential range of from +300mV up to +1300mV for E3 entry, we found +1200mV was the optimal potential for detection response and baseline stability. So we chose +1200mV as E3 potential.

### 3.2.4 Finding the optimal T2 and T3 times

Both T2 and T3 times were modified from 100mS down to 50mS, 20mS, and 10mS. This modification did not alter the baseline stability, but slightly enhanced the detection response. This fact suggested that both T2 and T3 times were not necessary to be long, as both E2 and E3 potentials were qualified for the cleaning and restoring of the surface of gold working electrode. So we chose 10mS as both T2 and T3 times.

### 3.2.5 Finding the optimal AD time

AD time was modified from 500mS down to 100mS by a deduction of 100mS. According to the operation manual of ESA Coulochem-3 pulsed electrochemical detector, the acquisition delay (AD) time was set to skip the charging current that was caused by the capacitor effect of gold working electrode applied with the pulsed potentials. We found the lower the AD time was, the higher the background current was, and the significantly higher the detection response was. But the background current was very unstable and rising fast, while AD time was set 200mS or 100mS. In the time range of from 500mS down to 100mS for AD entry, we found 300mS was the optimal time for detection response and baseline stability. So we chose 300mS as AD time.

### 3.2.6 Finding the optimal T1 time

T1 time was modified from 1000mS down to 500mS by a deduction of 100mS. We found the lower the T1 time was, the lower and the more stable the background current was, and the lower the detection response was. In the time range of from 1000mS down to 500mS for T1 entry, we found 800mS had the optimal signal-to-noise ratio for detection response and baseline stability. So we chose 800mS as T1 time.

Finally, there was the same result, when we injected sorbitol and mannitol standard

solution instead of glucose standard solution. We also injected glucosamine-1-phosphate standard solution, but with the mobile phase consisting of 120mM sodium acetate and 50mM sodium hydroxide. Those results also met our expectation.

### 3.3 Effect of temperature of analytical column

The heating temperature onto analytical column would improve the separation efficiency of carbohydrates, due to the better mass transfer of analytes among the packing gels of analytical column. The heating temperature of ESA thermal organizer was set 35°C because of taking account of the temperature limits of ESA 5040 cell and CarboHite column, since they were installed in the same one thermal organizer.

### 3.4 Effect of temperature of analytical cell

We found the heating temperature onto analytical cell would significantly enhance the detection response of carbohydrates in the pulsed amperometric detection. The heating temperature of ESA thermal organizer was set 35°C because of taking account of the temperature limit of ESA 5040 cell.

### 3.5 Effect of flow rate of mobile phase

The lower flow rate of mobile phase would result in the higher peak height of carbohydrates in the pulsed amperometric detection, due to the longer period of the electrochemical reaction of carbohydrates at the surface of gold working electrode.

However, the lower the flow rate of mobile phase was, the broader the peak width was, when the flow rate of mobile phase was set below 0.8mL/min. So the compromised flow rate was set 0.8mL/min.

### 3.6 Effect of pH value of mobile phase

For the separation of sorbitol and mannitol epimers, the mobile phase consisting of 100mM sodium hydroxide would result in good retention and resolution, while the mobile phase consisting of 20mM sodium hydroxide resulted in poor retention and resolution. We did not use any higher concentration of alkaline solution than the concentration of mobile phase to regenerate the CarboHite analytical column before every injection of analysis. According to the Merck Index, the pKa values of sorbitol and mannitol were near 13.5 and 13.6 respectively. So we thought 20mM sodium hydroxide was too weak to completely ionize sorbitol and mannitol. We speculated the mobile phase consisting of 200~500mM sodium hydroxide might provide better retention and resolution for the separation of sorbitol and mannitol epimers. The CarboHite analytical column could resist the alkaline mobile

phase with pH up to 14. Any mobile phase consisting of sodium hydroxide above 1000mM or with pH > 14 should not be used for CarboHite analytical column.

### 3.7 Detection response of acetic acid

We had injected the acetic acid solution (50% V/V in pure water) into HPLC-PAD with the mobile phase consisting of 100mM sodium hydroxide, and found no detection response. This fact suggested the carboxylic acid group in alkaline solution could not proceed the electrochemical reaction in the pulsed amperometric detection of gold working electrode. This fact also suggested the reason why sodium acetate could be used as a component of the alkaline mobile phase in HPLC-PAD.

### 3.8 Detection response of amino acid glycine

We had injected the amino acid glycine (10 $\mu$ L of 5 $\mu$ g/mL H<sub>2</sub>O) into HPLC-PAD with the mobile phase consisting of 100mM sodium hydroxide, and found the significant detection response. The obvious difference of molecular structure between glycine and acetic acid was that glycine had an amino group. So, the difference of detection response between glycine and acetic acid suggested the amino group of amino acid could be the group that led amino acid to proceed the electrochemical reaction in HPLC-PAD with gold working electrode and alkaline mobile phase.

If this hypothesis was true, those amino acids, peptides, and their adducts including the reduced and oxidized glutathiones, chloro-tyrosine, nitro-tyrosine, etc., that were not easily detected by the constant-potential electrochemical detection of the carbon-graphite or glassy-carbon working electrode, could probably be analyzed by utilizing HPLC-PAD system with gold working electrode, palladium reference electrode, and the appropriate combination of analytical column and mobile phase.

### 3.9 Re-checking the PAD detection of carbohydrates in neutral and acidic mobile phase

At the beginning of this study, we were wondering why many reporters still used a glass-enveloped wet Ag/AgCl as the reference electrode in alkaline mobile phase for the pulsed amperometric detection of carbohydrates and amino acids, since that glass was very liable to be corroded by alkaline solution was a common fact. Some reporters said that carbohydrates had to be detected in alkaline mobile phase, if HPLC-PAD was utilized. But they all used a glass-enveloped wet Ag/AgCl reference electrode in their HPLC-PAD systems [1-6].

However, we used a solid-state palladium reference electrode in our HPLC-PAD system. As the reference electrode, palladium was totally different from Ag/AgCl. Due to that we used palladium instead of Ag/AgCl for the reference electrode, we re-checked the pulsed

amperometric detection of sorbitol and mannitol in neutral and acidic mobile phase with the finally optimized potential-time parameters. We just removed the CarboHite analytical column from our HPLC-PAD system, and then used a union to connect both tubing ends. So, there was no problem for the elution of sorbitol or mannitol in neutral or acidic mobile phase. The flow rate of mobile phase was set 0.2mL/min. 10 $\mu$ L of 500 $\mu$ g/mL sorbitol and mannitol prepared in pure water were injected respectively. 10 $\mu$ L of pure water was injected prior to sorbitol and mannitol for the identification of the front peak caused by pure water. No peak could be recognized as sorbitol or mannitol.

#### 4. Conclusion

Carbohydrates could be separated by anion-exchange chromatography with alkaline mobile phase and detected by pulsed amperometric detection of gold working electrode applied with an appropriate potential-time waveform with respect to palladium reference electrode. The optimal potential-time profile for HPLC-PAD of carbohydrates was illustrated in figure 2. The HPLC-PAD chromatograms were very good and reproducible. The detection limits of sorbitol, mannitol, and glucose were below 50ng/mL with the injected volume 10 $\mu$ L.

This HPLC-PAD method might be used with the binary gradient elution of sodium hydroxide and sodium acetate for separation and detection of carbohydrates, if the binary gradient pumping system was utilized. Sodium hydroxide and/or sodium acetate as the components of mobile phase did not cause a high background current in HPLC-PAD.

The optimizing procedures for the potential-time parameters mentioned in this study might be utilized in the same idea for the development of the pulsed amperometric detection of amino acids without derivatization.

Regarding the performance of the reference electrode in HPLC-PAD system with the strongly alkaline mobile phase, the solid-state palladium was much better than the glass-enveloped wet Ag/AgCl.

#### References

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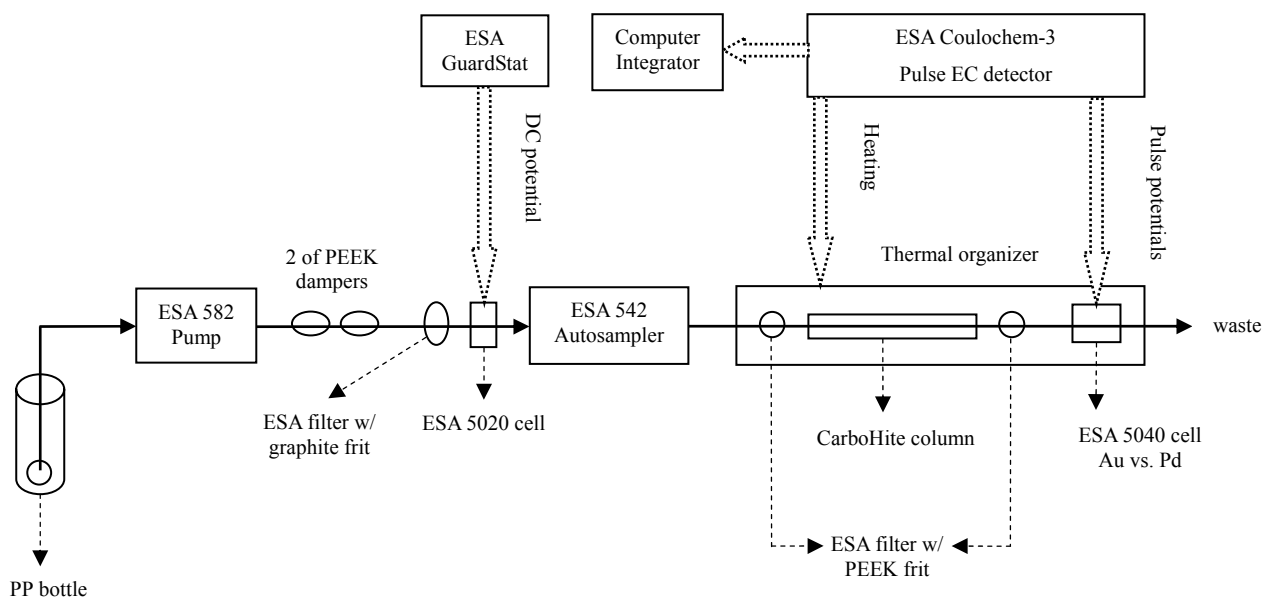


Figure 1. The isocratic configuration of HPLC-PAD system for carbohydrates.

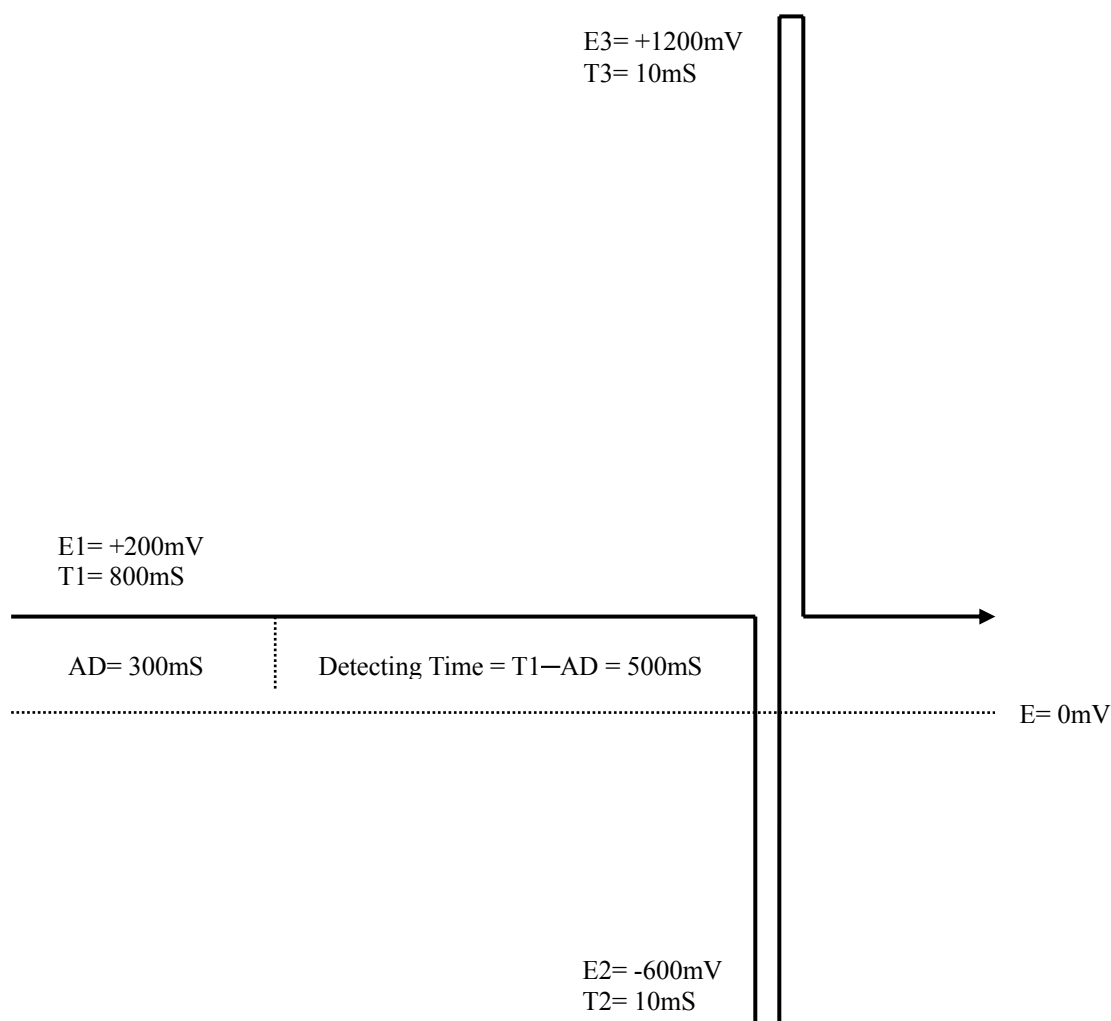


Figure 2. The optimal potential-time profile for HPLC-PAD of carbohydrates.