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Food/beverage

# Pulsed amperometric detection waveforms for carbohydrate determinations

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## Goal

Discuss the waveforms that Thermo Fisher Scientific supports for HPAE-PAD carbohydrate determinations

## Introduction

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established technique for carbohydrate determinations. For review of the basics of HPAE-PAD and its application to a wide range of carbohydrates please see Thermo Scientific™ Technical Note 70671 and/or the author's recent book chapter.[1,2] This technical note updates Technical Note 21 (TN21) that introduced Waveform A, a 4-potential waveform for carbohydrate detection. Since the publication of TN21, this waveform, henceforth referred to as the 4-potential waveform, has been established as the nearly universally used waveform for carbohydrate determinations. There have been HPAE-PAD improvements that require waveform changes, and they will be addressed in this technical note. For example, there are now faster HPAE separations that required waveform changes. There is also a second type of reference electrode available, which impacts the waveform. This technical note will describe the waveforms used to accommodate those changes.

## Basics of electrochemical detection of carbohydrates

At high pH, carbohydrates are electrocatalytically oxidized at the surface of the gold electrode by application of a positive potential. The current generated is proportional to the carbohydrate concentration, and therefore carbohydrates can be detected and quantified. If only a single potential is applied to the electrode, oxidation products gradually poison the electrode surface. This electrode surface poisoning causes a loss of analyte signal. To prevent signal loss, the electrode surface is cleaned by a series of potentials that are applied for fixed time periods after the detection potential. A series of potentials applied for defined time periods is referred to as a waveform. Repeated application of a waveform is the basis of pulsed amperometry. The potentials of a waveform are designated $E_1$, $E_2$, $E_3$, etc., where $E_1$ is the detection potential. The remaining potentials clean and restore the electrode for subsequent detection. Potentials are maintained for time periods $t_1$, $t_2$, $t_3$, etc. The first time period ($t_1$) is subdivided into $t_{del}$ and $t_{det}$. The delay period, $t_{del}$, is the time that is allowed for the charging current (produced when changing potentials) to decay (dissipate) so that only current from analyte oxidation is measured during the detection period, $t_{det}$ (Figure 1).

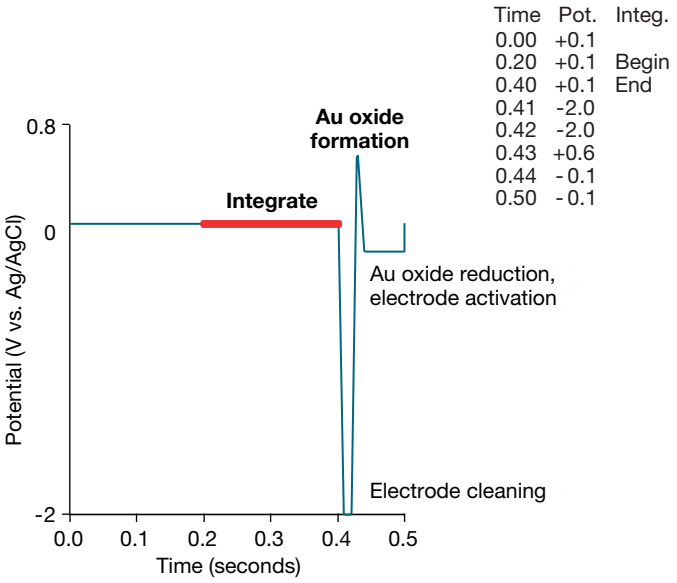


**Figure 1. Automated 4-potential waveform for carbohydrate detection**

Optimal values for all waveform parameters are determined by systematic variation of one parameter while holding the other parameters constant. An excellent discussion of the optimization of pulsed amperometry waveforms was published by LaCourse and Johnson.[3]

This technical note discusses waveforms Thermo Fisher Scientific supports for carbohydrate analysis. The discussion of each waveform will focus on when and how it should be used.

## The 4-potential waveform

This waveform was first described in a publication by Rocklin et al.[4] and in TN21. It differed from the other waveforms used at the time in that it used reductive cleaning rather than oxidative cleaning for the gold working electrode. Figure 1 shows a schematic of the waveform and the individual potentials and time periods. This waveform dramatically improved long-term peak area reproducibility. When positive cleaning potentials are used there is a gradual decrease in carbohydrate peak areas over time due to working electrode wear (recession below the plastic housing). The 4-potential waveform significantly minimizes electrode wear. This is demonstrated by comparing Figures 2 and 3. Figure 2 shows a two-week repetitive analysis of monosaccharide standards (100 pmol each) using a new working electrode and a waveform that uses a positive potential for electrode cleaning. This waveform, the recommended waveform before introduction of the 4-potential waveform, will be discussed later in this technical note. Figure 3 shows the same analysis using the 4-potential waveform. While there is a gradual decrease in peak area response using the waveform with a positive cleaning potential, response is constant using the 4-potential waveform. The 4-potential waveform provides the best reproducibility of absolute electrochemical response.

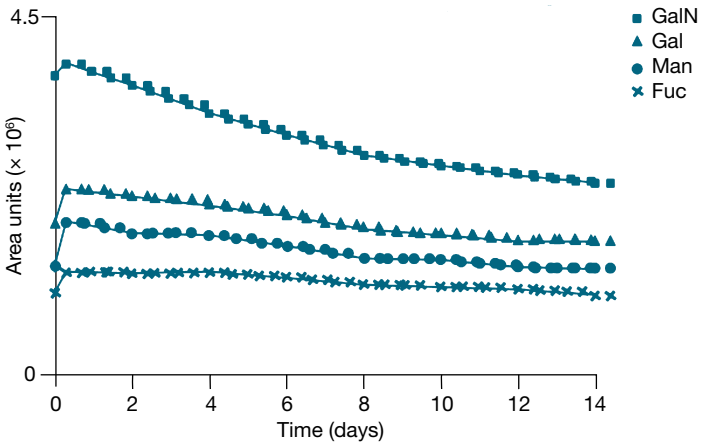


**Figure 2. Monosaccharide response over two weeks—oxidative cleaning**

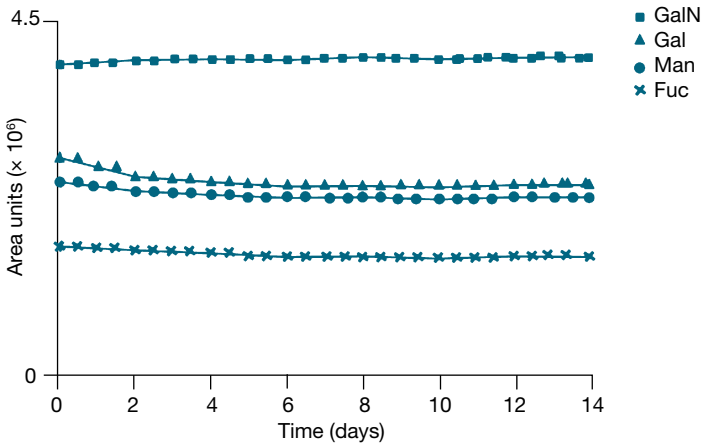


**Figure 3. Monosaccharide response over two weeks—reductive cleaning**

A review of Figure 1 shows that the detection potential, $E_1$, is 0.1 V. This potential is maintained for 400 ms. The first 200 ms are $t_{del}$ and the second 200 ms are $t_{det}$. The working electrode is cleaned for 10 ms at -2.0 V ($E_2$). This is followed by a quick excursion to 0.6 V ($E_3$). The latter potential were found to be necessary to initiate electrocatalysis to maintain an active working electrode surface. These two steps, reductive cleaning and transient gold oxide formation, require only 40 ms. The final potential of this waveform, $E_4$, is -0.1 V, and it is required to reduce the small amount of gold oxide formed in $E_3$. This potential is maintained for 60 ms and therefore this waveform requires a total of 500 ms. For more details on the theory of cleaning at negative potentials see the publication by Jensen and Johnson.[5]

The greatest benefit of using the 4-potential waveform is consistent long term peak area response. This is beneficial when comparing two or more systems that are analyzing monosaccharides. Because the 4-potential waveform requires only 500 ms, data can be collected at 2 Hz. This is twice the data collection rate of previously used waveforms. Because the 4-potential waveform allows twice as many data points per peak, sharp, early eluting peaks (e.g., fucose) are detected with greater reproducibility. A further advantage of the 4-potential waveform is that it is less subject to interference from electroactive amino acids than previously used waveforms.

There are some small disadvantages associated with the 4-potential waveform. Though the response is, in some cases, higher with the 4-potential waveform compared to earlier waveforms, the noise is also higher. Taken together, the minimum detection limits using the 4-potential waveform are usually not as low as those found with earlier waveforms, but this benefit is only transient as the gold working electrode loses gold with the oxidative cleaning of earlier waveforms. The 4-potential waveform has a greater sensitivity to dissolved oxygen and therefore higher backgrounds (16–22 nC) and higher noise. This may be apparent when using the Thermo Scientific™ CarboPac™ PA1 column, where the baseline dip, due to reduction of dissolved oxygen, is between glucosamine and mannose. The Thermo Scientific™ CarboPac™ PA10 column, places the baseline dip, due to dissolved oxygen, after the carbohydrate elution window.

When using the 4-potential waveform, there is occasionally a small dip observed after amino sugars (Figure 4). This dip is largest after glucosamine but is also observed after galactosamine and mannosamine. Dips are not observed after the acetylated amino sugars *N*-acetylglucosamine and *N*-acetylgalactosamine. Increasing E1 to 0.15 V minimizes this dip, though the long term (> 1 month) effect of this change on peak area reproducibility has not been measured.

| | | Peaks: | |
|---|---|---|---|
| Column: | CarboPac PA10 | 1. Fucose | 250 pmol |
| Eluent: | 18 mM sodium hydroxide | 2. 2-deoxy glucose | 250 |
| Flow rate: | 1.5 mL/min | 3. Galactosamine | 250 |
| Inj. volume: | 10uL | 4. Glucosamine | 250 |
| Detection: | Pulsed Amperometric Detection (PAD) | 5. Galactose | 250 |
| | | 6. Glucose | 250 |
| | | 7. Mannose | 250 |

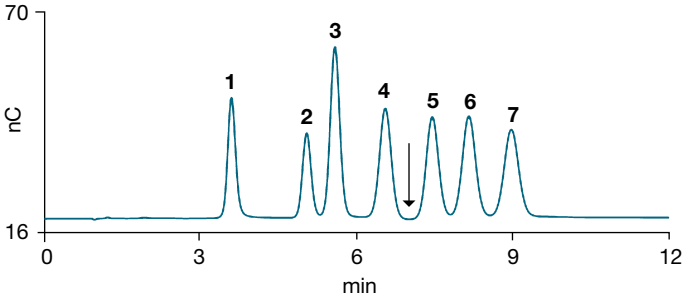


**Figure 4. Small dip occasionally observed after amino sugars using the 4-potential waveform**

The 4-potential waveform requires an ion chromatography system with a pump that has vacuum degas and a new working electrode. That working electrode can be a disposable gold working electrode. If a conventional gold working electrode is used, polish the working electrode before installing it in the electrochemical cell. The procedure for polishing the working electrode can be found in the Polishing Amperometry Cell Gold Working Electrodes manual.[6] The 4-potential waveform can be selected with the Instrument Method wizard in the Thermo Scientific™ Chromeleon™ chromatography workstation software. Under the EDet1 Options choose the Ag/AgCl reference electrode and then under the Waveform Selector pick the waveform labeled "Gold, Carbo, Quad". We also suggest unchecking the 3D_Amp box whenever using the this wizard. Make sure the pump degas is functioning. Do this using the Chromeleon chromatography workstation software. Click on the Instruments tab and then click on the Pump. Properties will be displayed on the right. Right click on the Properties field and select Expert mode. Then scroll down to find Degasser. It should show "On". If "Off", toggle it to "On". Immediately below Degasser is DegasserVacuum. That should show as "Ok". If not Ok, the connections may need to be tightened or the degasser pump replaced. On systems predating the Thermo Scientific™ Dionex™ ICS-3000 system, set the pump to degas for 30 s every 4 min. During the first day peak areas may increase as the working electrode surface is activated. This increase may be observed anytime the electrode is polished. Only polish new working electrodes and those believed to be fouled. Evidence of fouling is visible electrode discoloration or a decrease in peak area response that occurs without working electrode wear.

## 4-potential waveform for faster separations

The introduction of shorter column formats containing anion-exchange resins with smaller particle sizes has enabled faster separations with more efficient (sharper) peaks. This change demands a waveform that collects more data per second to obtain more data points for each analyte peak to deliver the best possible reproducibility. To meet this need, the 4-potential waveform was optimized to achieve a shorter overall cycle time and collect three data points per second rather than two (Figure 5). This waveform is the first choice for separations of five minutes or less. This waveform can be selected with the Instrument Method wizard in the Chromeleon chromatography workstation software as described above. Choose the Ag/AgCl reference electrode and then "Gold, Sialic Acids, 3Hz Quad" in the Waveform Selector.

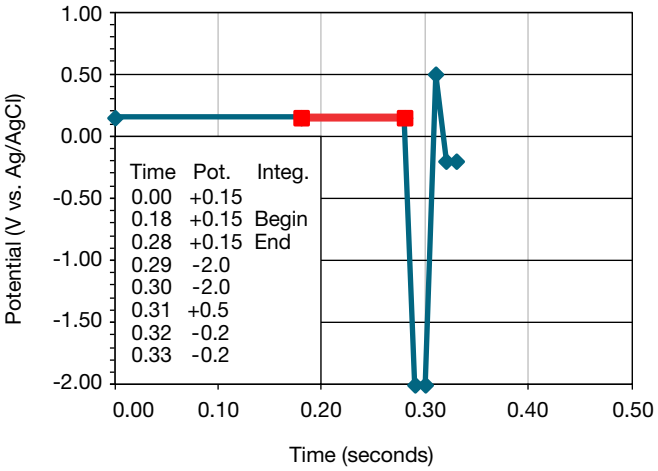


**Figure 5. 3 Hz 4-potential waveform**

## 4-potential waveform with a palladium hydrogen reference electrode

There is now a palladium hydrogen (PdH) reference electrode available that can used in place of the combination Ag/AgCl-pH reference electrode traditionally used for HPAE-PAD carbohydrate determinations. The PdH reference electrode is a solid-state electrode, unlike the aqueous Ag/AgCl reference electrode. The Ag/AgCl reference electrode should be changed every six months and requires calibration at start up. Solid-state reference electrodes are known to exhibit long lifetimes and provide a stable response. Thermo Fisher Scientific's Technical Note 73348 compares using a PdH reference electrode with a Ag/AgCl reference electrode.[7]

Because the PdH electrode has a different $E_0$ than the Ag/AgCl electrode, the applied potentials of the 4-potential waveform must be adjusted. Table 1 shows the potentials for the 4-potential waveform using the PdH reference electrode. Note that the set potentials must consider the temperature of the separation and the eluent's hydroxide concentration and Table 1 shows three waveforms to address four temperature/eluent concentration conditions. This waveform can be selected with the Instrument Method wizard in the Chromeleon chromatography workstation software as described above, but with the following differences. After selecting the PdH reference electrode, pick the Eluent type (Base), the concentration, and the detection temperature. Then using the Waveform Selector pick the waveform labeled "Gold, PdH RE, Carbo. Quad". The wizard will then build a 4-potential waveform to account for your eluent concentration and detection temperature.

| **Eluent concentration** | 12 mM | 29 mM | 12 mM | 29 mM |
|---|---|---|---|---|
| **Detection temperature** | 20 °C | 20 °C | 30 °C | 30 °C |

| Waveform | | | | | |
|---|---|---|---|---|---|
| **Time (sec)** | **Voltage (V)** | | | | **Integration** |
| 0 | 0.96 | 0.98 | 0.98 | 1.01 | Off |
| 0.2 | 0.96 | 0.98 | 0.98 | 1.01 | On |
| 0.4 | 0.96 | 0.98 | 0.98 | 1.01 | Off |
| 0.41 | -1.14 | -1.12 | -1.12 | -1.09 | Off |
| 0.42 | -1.14 | -1.12 | -1.12 | -1.09 | Off |
| 0.43 | 1.46 | 1.48 | 1.48 | 1.51 | Off |
| 0.44 | 0.76 | 0.78 | 0.78 | 0.81 | Off |
| 0.5 | 0.76 | 0.78 | 0.78 | 0.81 | Off |

## 3-potential waveform

The 3-potential waveform (Figure 6, referred to as Waveform B in TN21) was the recommended waveform between 1993 and 1998. This waveform was developed to increase sensitivity, minimize the sensitivity to dissolved oxygen, and minimize baseline drift when separating oligosaccharides with sodium acetate gradients compared to the previously used waveforms. It provides the greatest carbohydrate sensitivity with a new working electrode, the least sensitivity to dissolved oxygen, and is equivalent to the 4-potential waveform in baseline drift using sodium acetate gradients. Because this waveform uses oxidative cleaning ($E_2$ = 0.75 V), there is working electrode wear and a gradual decrease in carbohydrate peak area over time (Figure 2) and as discussed earlier, the sensitivity benefit is transient. Even with this peak area decrease, quantitative carbohydrate analyses are possible by using internal standards and regularly spaced external standards.[8,9] This waveform cannot be used with disposable gold working electrodes. After less than ten injections the gold surface will be completely gone and thus there will be no peaks detected.

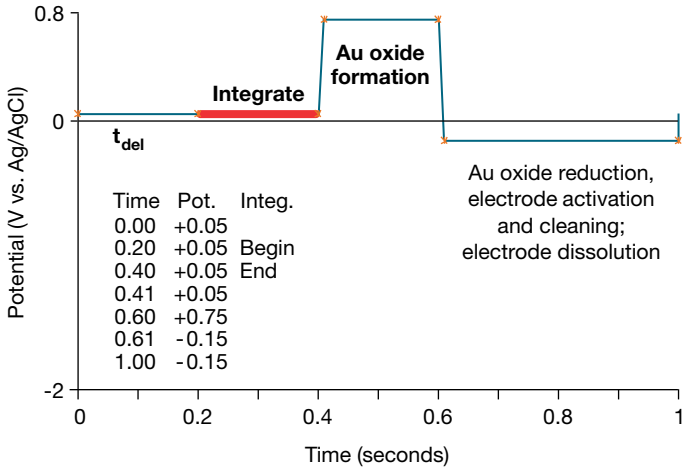


**Figure 6. 3-potential waveform for carbohydrate detection**

The 3-potential waveform is still in use because it is reported in some methods published by standards setting organizations. For example, it is used in the AOAC method for determining trans-galactooligosaccharides in food products.[10] There are also analysts that have this waveform in their standard operating procedures and some analysts that believe this waveform offers superior electrode cleaning for some samples. This waveform can be selected with the program wizard in the Chromeleon chromatography workstation software. It is labeled as "Gold, CWE RE, Carbo, Triple".

## Other considerations

Choosing the correct waveform is only one part of the process for successful HPAE-PAD carbohydrate determinations. The reader is encouraged to read the User's Guide to Electrochemical Detection and the Thermo Fisher Scientific technical notes that discuss proper eluent preparation, using disposable gold working electrodes, and using the PdH reference electrode for HPAE-PAD carbohydrate determinations.[7,11–13]

Carbohydrates are also detected using the waveform for AAA-Direct, a technique for separating and detecting amino acids. This waveform has been used for detecting aminoglycoside antibiotics as it demonstrated slightly more sensitivity than the 4-potential waveform.[14] If the amino acid waveform is chosen for aminoglycoside determinations, all the system and electrochemical recommendations for AAA-Direct must be followed.[15]

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