Introduction
During the development of a new HPLC method, there were unexpected results—all peaks, except for the injection peak, were negative (Figure 1). Even with advances in chromatography to eliminate problems, something unexpected is bound to happen. How would you go about finding out what’s wrong?

Although the web is full of HPLC troubleshooting guides, many of these are dozens of pages long.\textsuperscript{1,2,3} They can be helpful in teaching systematic approaches to troubleshooting, but when you need an answer fast, digging through these resources to find what you need can be slow and tedious.
An alternative is using a poster, such as the Thermo Scientific HPLC Troubleshooting Guide poster,\textsuperscript{4} where all troubleshooting suggestions are available at a glance and a useful suggestion can be found much faster.

An alternative for people with smart phones is the Thermo Scientific™ HPLC Troubleshooting Guide – an app for iPhone, Android, and Windows platforms. This mobile application incorporates all the information from the HPLC troubleshooting poster, and expands it to include electrochemical and nano LC solutions. In combination with solutions for charged aerosol detection, these additions make the troubleshooting app a comprehensive tool for various system setups. This mobile application is available for free at thermofisher.com/TroubleshootHPLC

**System**

- Thermo Scientific™ UltiMate™ 3000 x2 Dual System including:
  - SRD-3600 Solvent Rack with six degasser channels
  - DGP-3600RS Dual-Gradient Pump
  - WPS-3000TRS Thermostatted Wellplate Sampler
  - TCC-3000RS Thermostatted Column Compartment
  - DAD-3000RS Diode Array UV-Vis Detector with analytical flow cell

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS)

- All modules were connected using 0.005 in. (0.13 mm) i.d. Thermo Scientific™ Viper™ Fingertight Fitting System

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**Conditions**

<table>
<thead>
<tr>
<th>Column:</th>
<th>Thermo Scientific™ Acclaim™ 120, C18, 5 μm, 4.6 × 100 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard:</td>
<td>Caffeine, anthracene, and phenanthrene (0.05 mg/mL) in 70% ACN</td>
</tr>
<tr>
<td>Eluents:</td>
<td>A: 0.1% Formic acid in water</td>
</tr>
<tr>
<td></td>
<td>B: 0.1% Formic acid in acetonitrile</td>
</tr>
<tr>
<td>Gradient:</td>
<td>Time (min)  %B</td>
</tr>
<tr>
<td>0.0</td>
<td>60%</td>
</tr>
<tr>
<td>5.0</td>
<td>90%</td>
</tr>
<tr>
<td>5.5</td>
<td>90%</td>
</tr>
<tr>
<td>6.0</td>
<td>60%</td>
</tr>
<tr>
<td>10.0</td>
<td>60%</td>
</tr>
<tr>
<td>Flow:</td>
<td>1 mL/min</td>
</tr>
<tr>
<td>Column temp.:</td>
<td>30 °C</td>
</tr>
<tr>
<td>UV wavelength:</td>
<td>285 nm</td>
</tr>
<tr>
<td>UV bandwidth:</td>
<td>4 nm</td>
</tr>
</tbody>
</table>

**Figure 1. Negative peaks during method development experiments.**
Troubleshooting Case Study

A Few Clicks to Find the Probable Cause

Using the problem of negative peaks described above, the HPLC Troubleshooting Guide finds a solution as follows:

- From the app’s home screen, select “Start Troubleshooting” (Figure 2a).
- Because the issue is related to peaks, select “Peaks” (Figure 2b).
- Choose “Negative peaks” (Figure 2c).
- The app lists five “Possible cause/Solution” (Figure 2d), shown in Table 1.

Table 1. List of Possible Causes Generated by HPLC Troubleshooting Guide.

<table>
<thead>
<tr>
<th>Possible Causes</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Absorption/fluorescence of analyte is lower than mobile phase</td>
<td>1. Change UV/fluorescence detection wavelength(s) if possible. Use method without reference wavelength. Use Thermo Scientific’s DAD detector or another detector capable of handling low-level signals in the desired wavelength range.</td>
</tr>
<tr>
<td>2. Wrong polarization of analog output interface</td>
<td>2. Use mobile phase with less absorbance at reference wavelength. Use method without reference wavelength. Use Thermo Scientific’s DAD detector or another detector capable of handling low-level signals in the desired wavelength range.</td>
</tr>
<tr>
<td>3. Inappropriate reference wavelength setting (DAD)</td>
<td>3. Change UV/fluorescence detection wavelength(s) if possible. Use method without reference wavelength. Use Thermo Scientific’s DAD detector or another detector capable of handling low-level signals in the desired wavelength range.</td>
</tr>
<tr>
<td>4. Drainage spiking (Charged Aerosol Detection, CAD)</td>
<td>4. Drainage spiking (Charged Aerosol Detection, CAD)</td>
</tr>
<tr>
<td>5. The fluorescence of the substance is quenched by matrix or mobile phase (FLD)</td>
<td>5. The fluorescence of the substance is quenched by matrix or mobile phase (FLD)</td>
</tr>
</tbody>
</table>

Discussion of possible causes and solutions

Negative peaks can be caused by the detector or by how the detection signal is processed. Consequently, the different causes are specific to the types of detectors. In this example, different causes in combination with UV detection (variable wavelength, VWD) or diode array detection (DAD), analog data acquisition, fluorescence detection (FLD) and charged aerosol detection (CAD) are listed and demonstrate the unique diversity of the Troubleshooting Guide.

In this example, results were obtained digitally by DAD. Possible causes 2, 4 and 5 in Table 1 relate to analog data acquisition, CAD and FLD, therefore, do not apply. This leaves two other possible causes. Starting with cause 3: Inappropriate reference wavelength setting (DAD), the HPLC Troubleshooting Guide shows that the related solution for an inappropriate reference wavelength is (Figure 3):

The sample must not absorb in the range of the reference wavelength. If possible, use a method without reference wavelength.
For a UV-Vis DAD, you can select both a reference and a
detection wavelength. When used properly, the reference
signal corrects for changing conditions such as baseline
drift. If the reference wavelength is not chosen correctly
and absorbs in the range of your analyte, then you may
get a negative response. (See the sidebar Did You Know?
for more information.)

In this case study, the reference and detection
wavelengths for the analysis of butylparabene were
selected incorrectly.

- With a reference wavelength of 240 nm and a bandwidth
  of 100 nm, the reference absorption was taken from
  190 nm to 290 nm.
- With a detection wavelength of 285 nm and a
  bandwidth of 4 nm, detection absorption was taken
  from 283 nm to 287 nm.

The range of the reference indeed overlaps in the range
of analyte detection, making this the probable cause for
negative peaks. To verify that this is cause, the reference
wavelength can be switched to “off”. “Reference: off” is
actually the recommended operation of Thermo Scientific
Diode Array Detectors mainly to avoid implications with
incorrect reference settings. The resulting chromatogram
(Figure 4) shows that this change immediately solves the
issue of negative peaks.

Note that the positive injection peak and the baseline
drift are now gone. Both were caused by the absorption
of formic acid as part of the mobile phase, affecting
wavelengths below 260 nm. The injection peak was
positive as the sample diluent lacks formic acid and
therefore absorbed less than the mobile phase. With
the initial, improper reference wavelength settings,
the negative peak was inverted and shown as a
positive peak.

**Did you know?**

**How reference and detection wavelengths work**

**Reference wavelength**

The reference wavelength is intended to correct for
(subtract) absorption that can occur from changes in
conditions during analysis. Those changes can
sometimes be significant, such as if there is a reduction
in the lamp energy (lamp drift). The reference wavelength
selected therefore must be in a quiet area of the
spectrum where little to no absorption of the sample
analytes occurs. Because the signal from the reference
wavelength is subtracted from the signal received from
the detection wavelength, it has a direct impact on
results.

**Reference bandwidth**

Reference bandwidth serves to average several
photodiode signals above and below the reference
wavelength. A reference bandwidth should be selected
as wide as possible, for example 30–100 nm, but narrow
enough not to interfere with the absorption spectrum
of the analytes. Figure 5 below visualizes appropriate
reference settings for a butylparabene spectrum.

**UV-Vis Detection Wavelength**

In contrast to the reference wavelength, the UV-
Vis detection channel wavelength for recording the
analyte absorption should be set to the absorbance
maxima of the analytes of interest.\(^5\)
Conclusion

A solution is just a few taps away; select your problem and the HPLC Troubleshooting Guide – an app that will guide you to the answer. Comprehensive in its inclusion of specific technologies such as fluorescence detection, charged aerosol detection and nano LC, this powerful, free tool effectively diagnoses various potential issues with HPLC analysis. A Share function even lets you send the solution to your team. Simply check the troubleshooting solution of interest and select share, and enter the email address from your contacts list.

References


Download your free HPLC Troubleshooting Guide

The app is available for iPhone, Android, and Windows phones, free at thermofisher.com/TroubleshootHPLC or scan this quick response (QR) code to download the app:

For a copy of the troubleshooting poster, please request it from your Thermo Fisher Scientific sales representative.

Find out more at thermofisher.com/liquidchromatography

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