

## HPLC troubleshooting guide

A free app that will save time and frustration

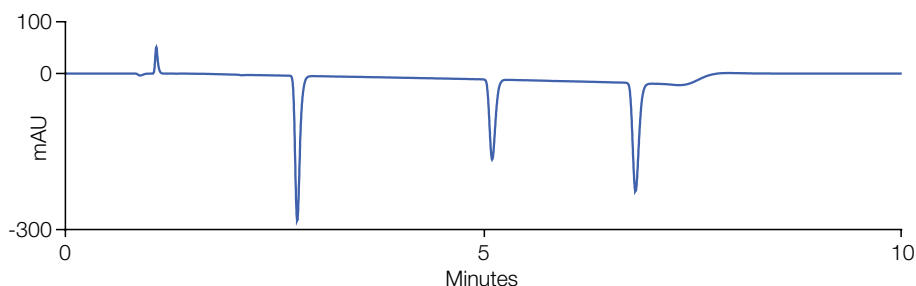


Thermo Scientific HPLC Troubleshooting Guide – an app for iPhone, Android, and Windows platforms.

### 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.<sup>1,2,3</sup> 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.



**Figure 1. Negative peaks during method development experiments.**

An alternative is using a poster, such as the Thermo Scientific HPLC Troubleshooting Guide poster,<sup>4</sup> 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](http://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

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

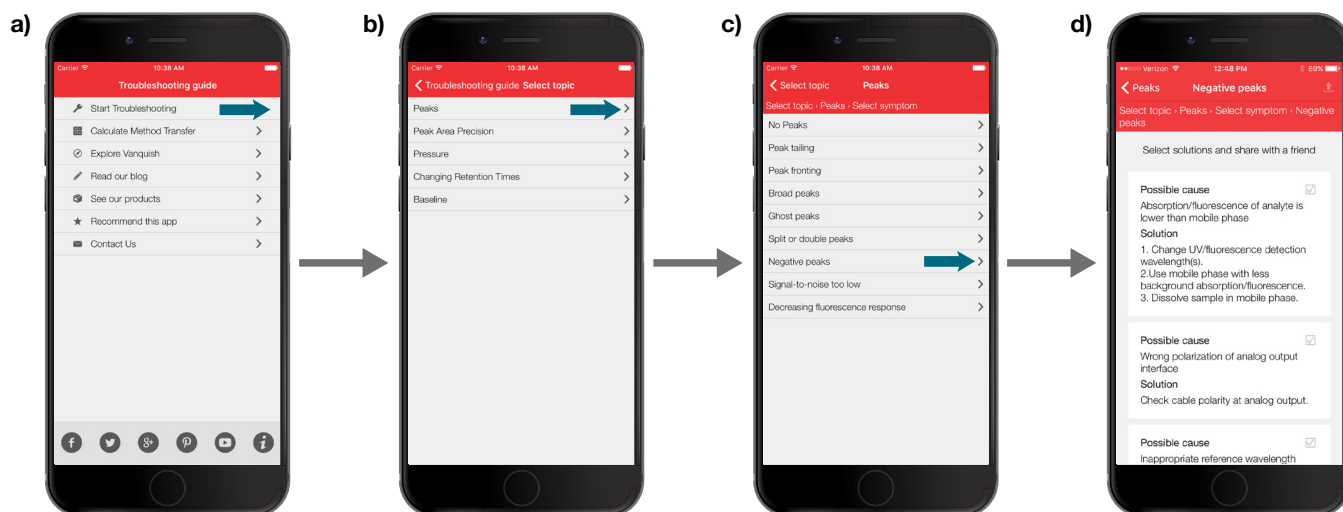


Figure 2. Generating probable cause using the Thermo Scientific HPLC Troubleshooting Guide.

## 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.

Possible Causes
1. Absorption/fluorescence of analyte is lower than mobile phase
2. Wrong polarization of analog output interface
3. Inappropriate reference wavelength setting (DAD)
4. Drainage spiking (Charged Aerosol Detection, CAD)
5. The fluorescence of the substance is quenched by matrix or mobile phase (FLD)

## 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.*

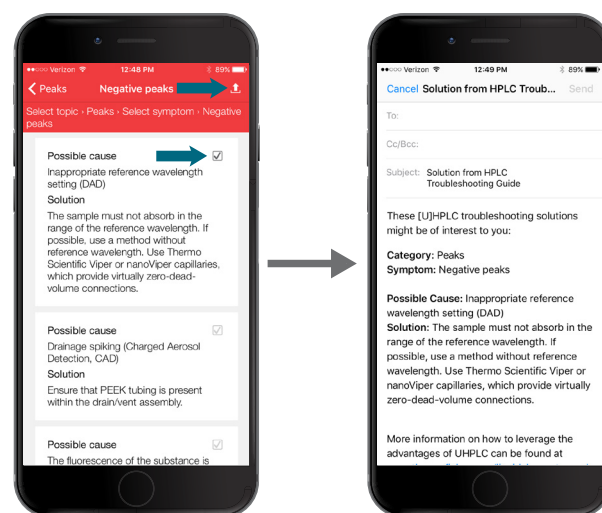


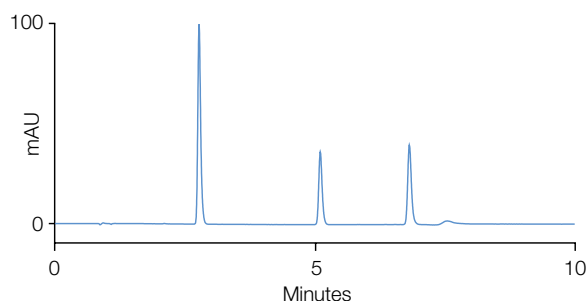
Figure 3. The app showing probable cause "Inappropriate reference wavelength setting (DAD)," with the solution. Also note the app has the ability to share the solution with a colleague and links if you want to learn more.

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.



**Figure 4. Obtaining the chromatogram without reference (Reference: off) displays the peaks in a normal way and removes side effects such as baseline drift and injection peak.**

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?

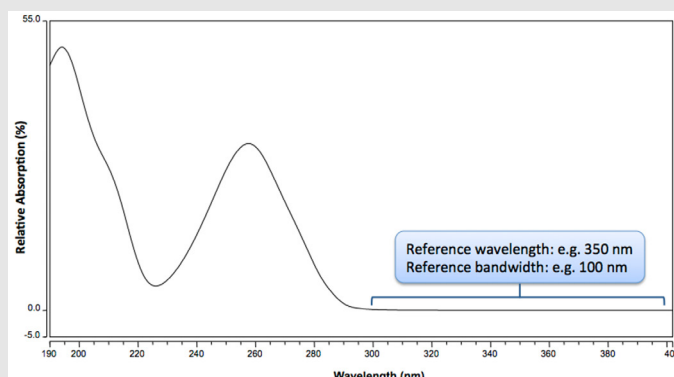
### How reference and detection wavelengths work

#### 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.



**Figure 5. Butylparabene spectrum with recommended reference wavelength settings.**

#### 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.<sup>5</sup>



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.

1. Successful HPLC Operation – A Troubleshooting Guide – Version 1.1, Thermo Electron Corporation, 2004. <https://assets.thermofisher.com/TFS-Assets/CMD/Product-Guides/TG-20421-HPLC-Troubleshooting-Guide-TG20421-EN.pdf>
2. HPLC Troubleshooting Guide, Bulletin 826E, Sigma-Aldrich, 2009.
3. HPLC Troubleshooting Guide, Phenomenex Inc., 2008.
4. Thermo Scientific HPLC Troubleshooting Guide Poster (XX70308), 2016.
5. Thermo Scientific UltiMate 3000 Series Diode Array Detectors Operating Instructions, Revision 1.4. <https://assets.thermofisher.com/TFS-Assets/CMD/manuals/MAN-LC-DAD-MWD-Operation-Sep13-DOC4820-8250-1-4.pdf>

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

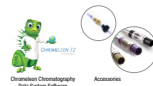


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For a copy of the troubleshooting poster, please request it from your Thermo Fisher Scientific sales representative.

Find out more at [thermofisher.com/liquidchromatography](http://thermofisher.com/liquidchromatography)

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