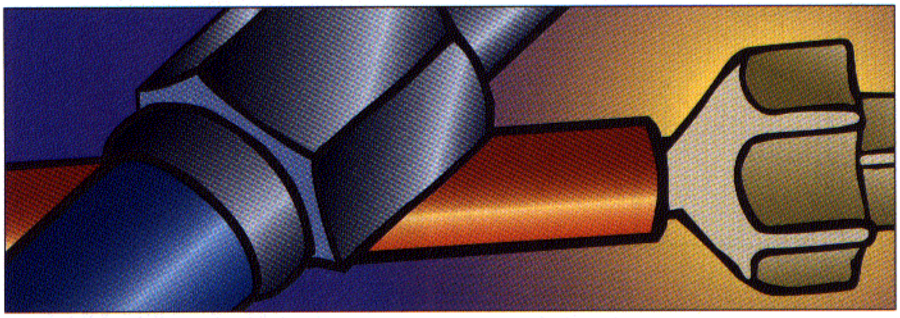
LC Troubleshooting



Gradient Background Peaks — A Case Study

Michael D. Nelson and John W. Dolan

When peaks appear in a blank gradient, isolating the problem source can be a challenge.

One problem chromatographers commonly encounter when performing gradient liquid chromatography (LC) runs is spurious peaks in blank baselines. Because these peaks occur when no sample is injected, the peaks originate from the solvents or system components. Recently we encountered excessively noisy baselines in one of our methods. The process we used to isolate the problem source serves as a good case study in isolating gradient contaminants.

THE METHOD

We performed the separation on a 150 mm × 4.6 mm, 5-µm d_p Inertsil ODS-3 column (MetaChem Technologies, Torrance, California) operated at a temperature of 30 °C and a flow rate of 1.5 mL/min. The LC system comprised two Shimadzu LC-10AD pumps (high-pressure mixing), a model SIL-10A autosampler, and a model SPD-10A variable-wavelength detector operated at a detection wavelength of 215 nm (all components from Shimadzu Scientific Instruments, Columbia, Maryland). Gradients of 0–100% B were run over 15 min, with a 5 min hold at 100%; a 10-min equilibration period was used between runs unless otherwise noted. For runs using buffer, the A solvent comprised 5:95 (v/v) acetonitrile–buffer and B was 80:20 (v/v) acetonitrile–buffer. A pH 7.0 10 mM phosphate buffer was prepared by blending 10 mM monobasic potassium phosphate and 10 mM dibasic potassium phosphate. In some experiments, water was substituted for buffer, as noted. We used HPLC-grade solvents for all experiments; HPLC-grade water was generated in-house using a Milli-Q water-purification system (Millipore, Bedford, Massachusetts).

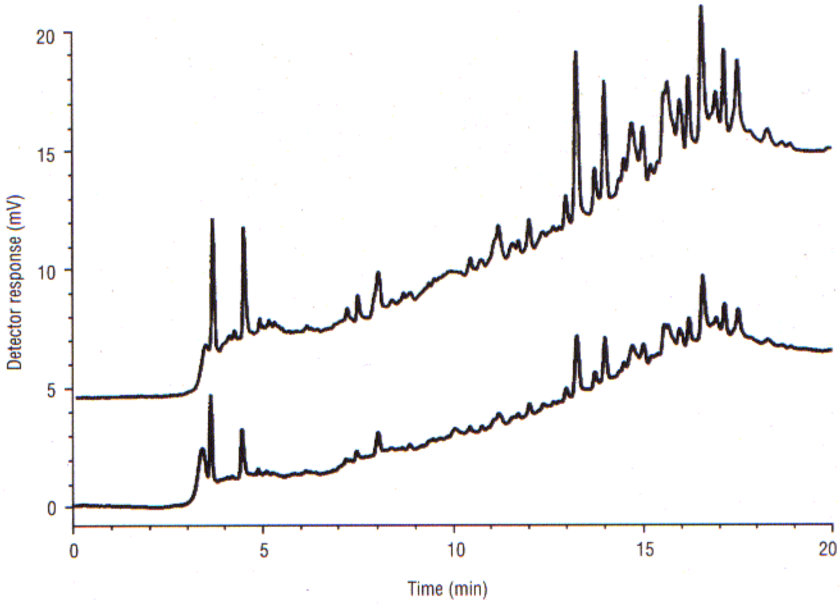


FIGURE 1: Extraneous peaks observed in a blank gradient. 10-min equilibration (lower) vs. 30-min equilibration (upper). See text for other conditions.

THE PROBLEM

Figure 1 illustrates the problem we encountered. The lower trace shows a blank gradient (no injection) using the standard conditions stated above. Background peaks as large as 1 mV (1 × 10^{-3} absorbance units [AU]) are allowable in HPLC-grade water. The lower chromatogram of Figure 1 generates numerous peaks approximately twice this size. These peaks commonly originate from the A solvent through on-column concentration, in which nonpolar materials from the A solvent accumulate at the head of the column during equilibration and then are eluted as peaks by the gradient. One easy check for this problem is to extend the equilibration time between runs — the extended equilibration time increases the amount of material that collects at the head of the column and yields larger peaks in the gradient. The upper trace of Figure 1 shows the result of a 30 min equilibration between runs. The peaks roughly triple in size, confirming that the peaks originate in the A solvent. We rarely have problems with our HPLC-grade water, so we suspected that the phosphate buffer was the problem source. This problem also gave us an excuse to compare the backgrounds generated by different brands of buffer.

PHOSPHATES COMPARED

We purchased mono- and dibasic phosphate salts from four vendors and prepared buffers as described above. To facilitate comparison of background peaks, a 30-min equilibration period was used. Figure 2 shows the four blank chromatograms. We were surprised to see very similar chromatograms for all four brands. Each product generated unacceptably large background peaks. As a check, we used a water blank (bottom trace), in which phosphate was replaced by purified water. Several of the larger peaks persisted in the water, for example the peaks near 12, 13, 16, and 17 min. The fact that all the buffers had similar appearances and that the peaks were much larger than expected led us to suspect that the extra peaks originated from inadvertent contamination during buffer preparation.

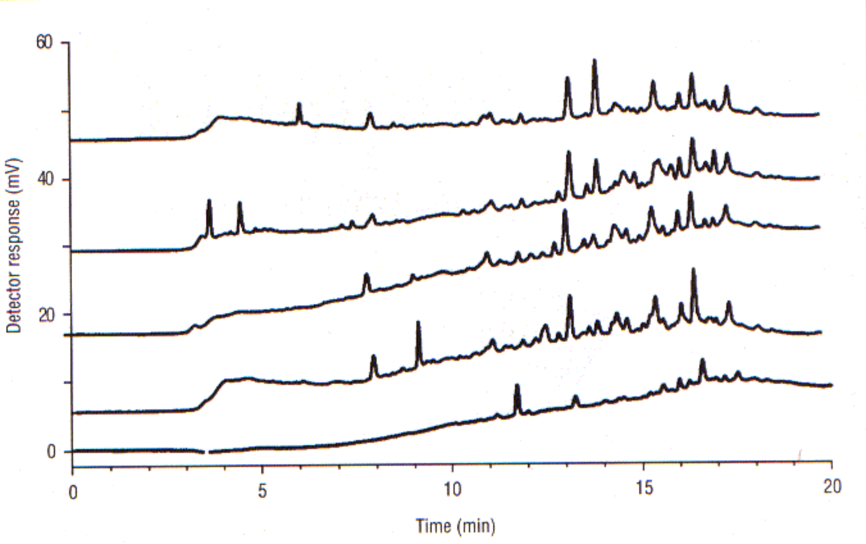


FIGURE 2: Blank gradients from four phosphate buffers (upper traces). Lowest chromatogram uses purified water instead of buffer. All runs included a 30-min equilibration.

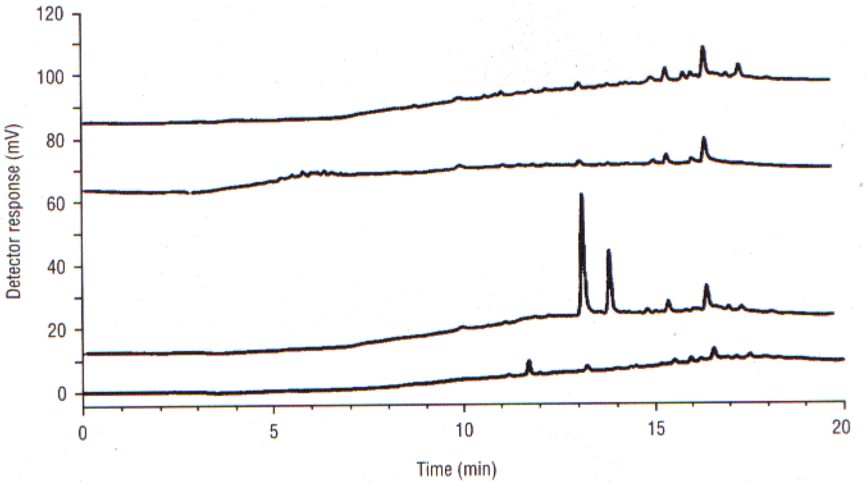


FIGURE 3: Chromatograms resulting from use of suspected contamination sources, including glassware contact (upper), solvent filtration (next to top), pH meter probe (next to bottom), degassing (bottom). See text for other conditions.

SUSPECTED SOURCES

Several buffer preparation steps have the potential for contaminating the solvents. These possibilities included glassware contact, microfiltration, pH adjustment, and degassing. We chose to simplify the system and use water instead of buffer for the isolation steps. During normal buffer preparation, the buffer was exposed to six separate pieces of glassware, so glassware was tested by swirling purified water in six 600-mL beakers. Filtered water was prepared by passing it through a 0.45-μm membrane filter three times. We checked the pH-adjustment step by placing a stir bar in a beaker of purified water and stirring it for 10 min with the pH meter probe immersed in the water. Degassing by helium sparging was used only for the degassing test. For each test, we avoided exposure to the other sources if possible (for example, only one batch of solvent was degassed, but all solvents required at least a minimum exposure to glassware).

> *As a first guess, analysts should assume that a single source is responsible for any given LC problem.*

Figure 3 shows chromatograms for the various water treatments (30-min equilibration). The degassed and filtered water are very similar and have the fewest contaminant peaks. Dirty glassware appears to be the source of most of the smaller peaks. We were surprised to see two large peaks at approximately 13 and 14 min that were present only when the solvent was exposed to the pH meter probe.

DIRTY GLASSWARE

Because the test above suggested that some of the contaminants originated from the glassware, we thoroughly investigated the dishwashing procedure. All glassware is washed by hand using a commercial laboratory dish soap, rinsed six times with tap water, then six times with deionized water. Most glassware is baked dry in a laboratory oven. We first suspected that the wrong soap dilution was used, and we were right — the typical sink full of dishes contained approximately 10% of the recommended detergent. Even this reduced detergent concentration appeared to leave unacceptable residues.

We found that rinsing the glassware an additional five times before use eliminated most of the spurious peaks. In addition, we changed the pH adjustment procedure so that aliquots of buffer were removed from the main batch to check the pH so that the pH meter probe was not placed in the bulk buffer. Figure 4 compares a phosphate gradient using the extra rinses and external pH adjustment (lower trace) with the original problem gradient (upper trace) of Figure 1.

pH METER

The previous test showed that the glassware and pH meter contributed to contamination. When extraclean glassware was used for the preparation of buffer and the pH meter probe was inserted in the solvent for 30 min, we obtained an unacceptable baseline, as illustrated in the upper trace of Figure 5. Compare this result to the lower trace in which the same starting buffer was adjusted using the external

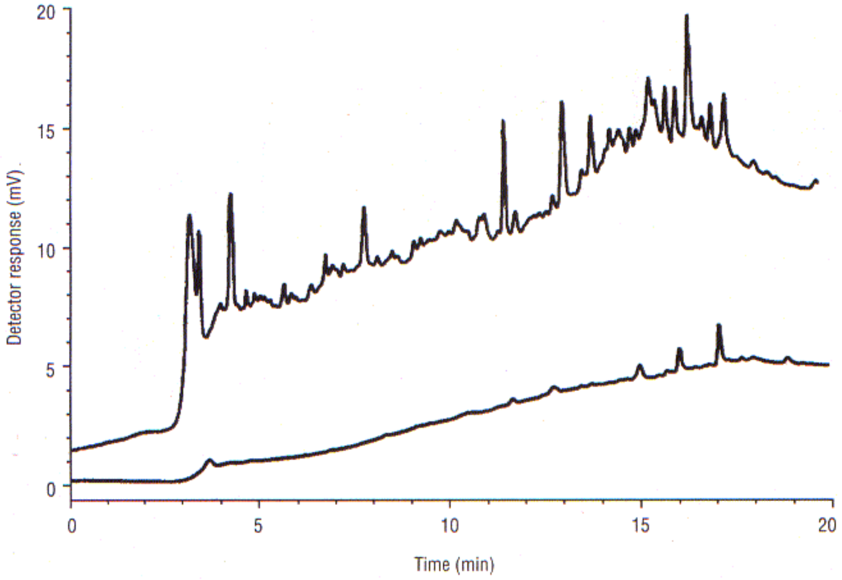


FIGURE 4: Comparison of chromatograms generated using contaminated buffer (top) and buffer prepared with extraclean glassware and no exposure to the pH meter probe (bottom).

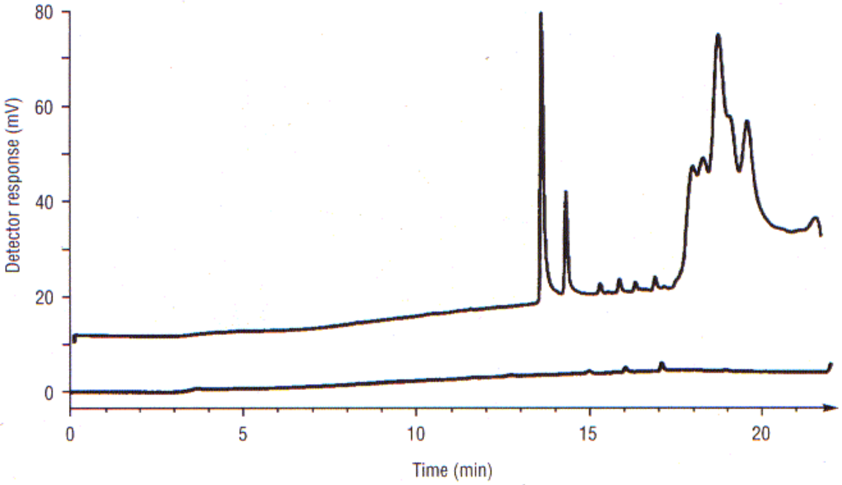


FIGURE 5: Chromatograms generated using buffer exposed for 30 min to the pH meter probe (top) and using buffer prepared with extraclean glassware and no exposure to the pH meter probe (bottom).

pH meter probe technique. The pH meter probe obviously is a major contamination source.

THE CULPRITS EXPOSED

As a final set of tests, we examined the contamination sources more closely. When unused (for example, between tests), the pH meter probe is kept immersed in a commercial pH 7 calibration buffer. We diluted this buffer 100-fold with purified water and used it instead of phosphate buffer. The lower trace of Figure 6 shows a blank gradient for this sample. The large peak at 4 min normally is not a problem; the other small peaks later in the run may or may not correlate with problem peaks in the normal gradient. In any event, no large peaks appear to contribute to the general problem.

Our second experiment used a 100-fold dilution of the saturated potassium chloride solution used to fill the pH meter probe. The upper trace of Figure 6 shows a gradient using upper trace of Figure 6 shows a gradient using this solution instead of buffer. The large peak near 14 min is one of the peaks that consistently appears in problem gradient runs (some retention time shifts are observed between days for this peak because of slightly different LC conditions). The potassium chloride appears to contribute significantly to the buffer contamination problem.

Finally, soapy dishwater was diluted 1:1 with purified water and 50 µL was injected into the system. Figure 7 compares the chromatogram for soapy water (upper trace) to a (dirty) blank phosphate gradient (lower trace). (Note that the upper trace is attenuated fivefold over the lower one.) Clearly several of the peaks in the contaminated phosphate correlate with peaks from the soapy water.

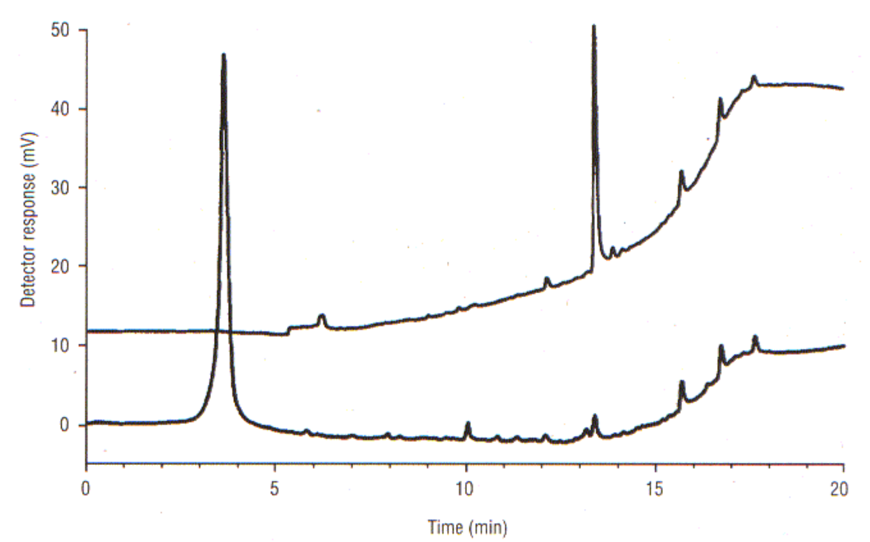


FIGURE 6: Chromatograms generated using mobile phase prepared from possible contamination sources, including the pH 7.0 calibration standard (lower) and the saturated potassium chloride used for filling the pH meter probe (upper). See text for other conditions.

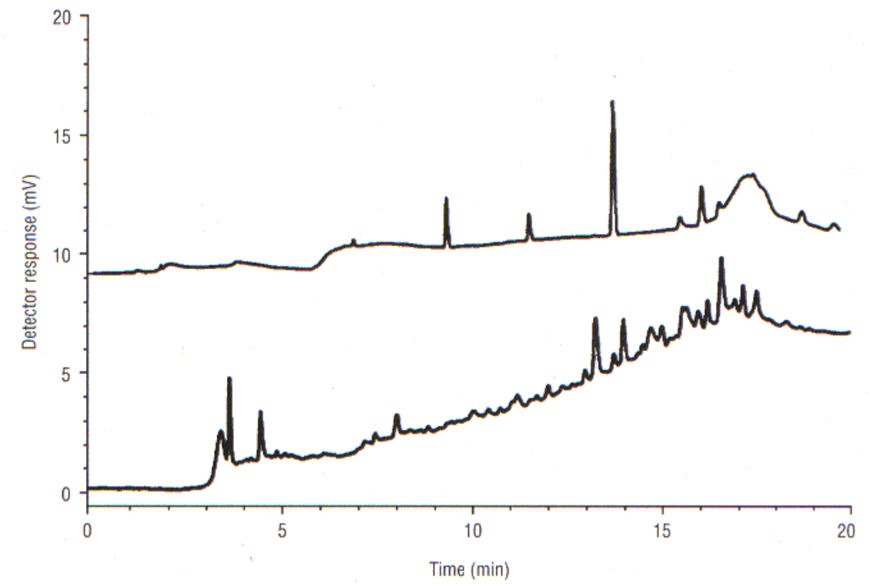


FIGURE 7: Chromatograms showing contaminants from dishwater (upper trace) and phosphate buffer (lower trace). Note that the upper trace is fivefold less

CONCLUSIONS

This case study shows one example of how analysts can use a stepwise technique for isolating the problem source. From the current study, we can draw the following conclusions:

- In our procedure, six tap water rinses followed by six deionized water rinses is insufficient to remove soap residues that create unwanted peaks in the present method. We adjusted the procedure to use two 10-fold rinses and now obtain satisfactory results.

> *Spurious peaks in blank baselines occurred when no sample was injected, so they originated from the solvents or system components.*

- The 0.45-µm filter used for mobile phases does not appear to contaminate the solvents.
- Degassing by vigorous helium sparging for 5–10 min does not appear to contaminate the solvents.
- Although the storage solution for the pH meter probe does contain potential contaminants, they do not appear to transfer to the mobile-phase buffer. The lack of contaminants is likely due to our practice of rinsing the probe thoroughly before each use.
- The saturated potassium chloride solution contained in the pH meter probe contributes significant contamination when the probe is immersed in the buffer during preparation. By checking the pH external to the bulk buffer (and then discarding the tested aliquot), we were able to determine buffer pH without contaminating the bulk buffer.
- The experiments described here used UV detection at 215 nm. Many of the problem peaks probably would not be of concern at higher wavelengths (for example, 250 nm).

As a first guess, analysts should assume that a single source is responsible for any given LC problem. Systematic problem isolation then can be used to find the problem source by changing one variable at a time until the problem is eliminated. Although the present problem was caused by two separate sources and we did not always change just one thing at a time during isolation, stepwise problem isolation led us quickly to the sources.

Michael D. Nelson is a chemist at LC Resources Inc.'s laboratory in McMinnville, Oregon.

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of LC•GC's editorial advisory board. Direct correspondence about this column to "LC Troubleshooting," LC•GC, 859 Willamette Street, Eugene,