CASE STUDY

Troubleshooting Unwanted Peaks for HPLC: A Case Study Using Formic Acid

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Introduction

The adoption of a systematic approach to HPLC troubleshooting will help to definitively address the root cause of unexpected results caused by the observation of impurities during the implementation of reversed phase HPLC methods. Impurity peaks can potentially co-elute with relevant peaks of interest, causing a misinterpretation of the data. Altered results include percent relative standard deviation (%RSD), peak shape, as well as false quantitation values. A fast and simple way to identify the source of potential impurity peaks is by injecting a mobile phase blank. While commercial LC software can be used to remove peaks that appear in the mobile phase blank injection from sample injections to improve data accuracy (e.g., background subtraction), the best practice is to identify the source of impurity peaks and minimize, or eliminate, them to improve quantitation. Here, we present a case study of an application where the source of impurities was determined using basic troubleshooting techniques.

Application Overview

An application was run to analyze an Acid, Base, Neutral (ABN) mixture of 7 analytes using the Kinetex™ 2.6 µm C18 column. The conditions were as follows:

LC-UV Conditions

Column:	Kinetex 2.6 µm C18
Dimensions:	50 x 2.1 mm
Part No.:	00B-4462-AN
Mobile Phase:	A: 0.1 % Formic Acid in Water B: 0.1 % Formic Acid in Acetonitrile
Gradient:	see table below
Flow Rate:	0.4 mL/min
Injection Volume:	0.2 µL
Temperature:	30 °C
Detection:	UV @ 254 nm
LC System:	Waters® ACQUITY® iClass UHPLC

Time (min)	%B
0	5
0.5	5
5.5	95
6.5	95
7	5
10	5

Analytes:
1. Uracil (25 µg/mL in Methanol)
2. Pindolol (125 µg/mL in Methanol)
3. Chlorpheniramine (125 µg/mL in Methanol)
4. Nortriptyline (125 µg/mL in Methanol)
5. 3-Methyl, 4-Nitrobenzoic acid (125 µg/mL in Methanol)
6. 2-Hydroxy, 5-Methylbenzaldehyde (125 µg/mL in Methanol)
7. Hexanophenone (125 µg/mL in Methanol)

The chromatographic separation is shown in **Figure 1a**. Upon closer inspection, smaller impurity peaks were present as noted by the red boxes in the zoomed view in **Figure 1b**. Since this was a mixture prepared using standards of high purity for the seven analytes of interest, the presence of these additional peaks was not expected. This immediately brought into question the accuracy of the data that was gathered. A troubleshooting approach was implemented to determine the source of these impurities.

Key Concepts:

- A structured troubleshooting approach is best for identifying the source of extra peaks in a chromatogram.
- Formic Acid was identified as the source for the impurity peaks.
- “LC-MS Pure” does not mean LC-UV purity is guaranteed.

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Troubleshooting Approach

There were a few basic procedures that were performed as part of an initial troubleshooting approach. First, the column was cleaned to ensure no contaminant remained in the column. This was done by reverse flushing the column using ½ of the normal flow rate followed by forward flushing using stronger solvents (95 % Acetonitrile / 5 % Water; then 50:50 Acetonitrile / Tetrahydrofuran then 100 % Tetrahydrofuran) to remove the presence of any "sticky" compounds observed from the column. This procedure did not eliminate or reduce the presence of the observed impurity peaks. Next, the autosampler was flushed to ensure it was not the source for these contaminants. Many times, the solvent lines will be flushed for contaminants during troubleshooting but flushing the autosampler is overlooked. This was done by removing the column from the system and replacing it with a union. A vial filled with Isopropanol was placed in the autosampler tray, and a short method about a minute long is cued with at least 10 injections of the max loop volume to flush out anything that could be in the autosampler loop and port. This method operated at a constant flow of Acetonitrile / Water (50:50, v/v) at about 0.5 mL/min. The column was installed, and the analysis was repeated. Again, the impurity peaks appeared in the results.

For HPLC using a UV/VIS detector, some users perform caustic washes of their glassware. This effectively removes anything that was previously in the glassware, but it can lead to caustic soap residue remaining and being introduced in the next preparation of mobile phase and put into the system causing contamination and impurity detection. The glassware being used was thoroughly cleaned (brush cleaned with water and Liquinox® to remove grease and oils from glassware, followed by three rinses with HPLC grade water that was obtained from a Sartorius® arium® Comfort II water purification system and then finally 100 % Acetonitrile and left to air dry) to prevent any residues from being introduced and the analysis repeated. The same pattern of impurity peaks appeared in the resulting chromatogram. We had now ruled out the column and the injector as potential sources of these impurity peaks.

Lastly, mobile phase preparation can lead to the introduction of impurities if not prepared and stored correctly. Using large filtering apparatuses to filter all mobile phase before use can help to remove these impurities from being introduced into the HPLC. Premade mobile phases may also be available for purchase; these can help to prevent the introduction of impurities brought about by user error during preparation of mobile phases in the lab. There are also additives like Sodium Azide that can help reduce the formation of bacteria in aqueous buffers, and their subsequent introduction into the HPLC. After attempting the previous troubleshooting techniques, a mobile phase blank was injected, and the results showed the exact same impurity profile as observed in the sample injection (**Figure 1c**). This confirmed that the impurities were coming from the mobile phase. The Formic Acid that was used for this analysis was the Fisher Chemical™ Formic Acid, 99.0+ %, Optima™ LC-MS Grade in a 50 mL bottle. HPLC water was obtained from a Sartorius arium Comfort II water purification system and Acetonitrile was obtained from ChemPure Brand Chemicals®.

Figure 1. ABN7 Analysis Using Fisher Chemical Formic Acid, 99.0+ %, Optima LC-MS Grade.

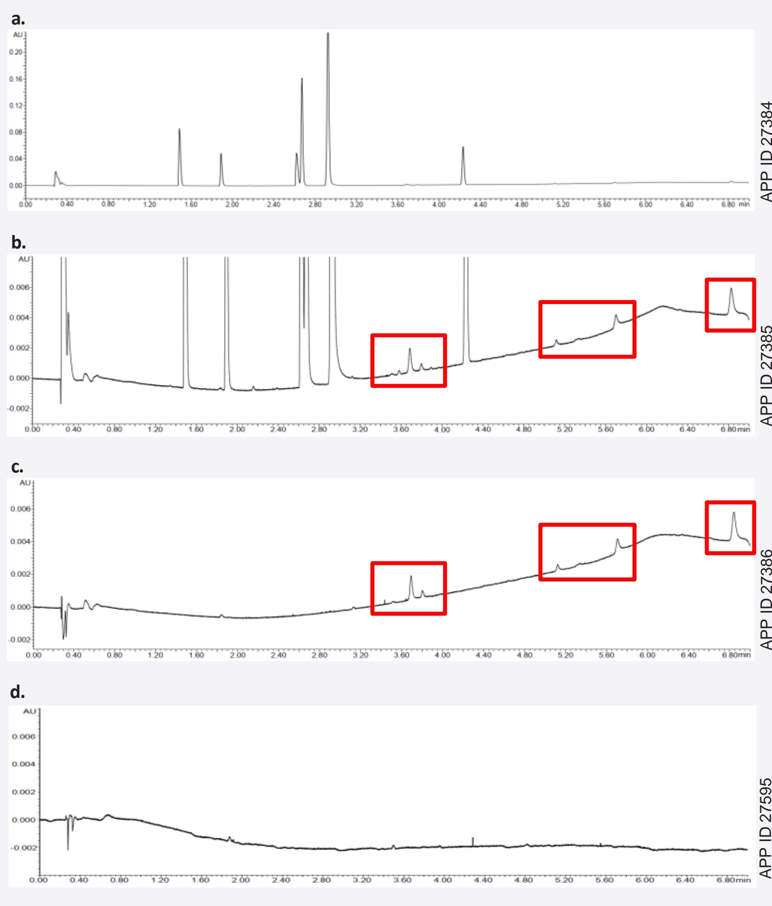
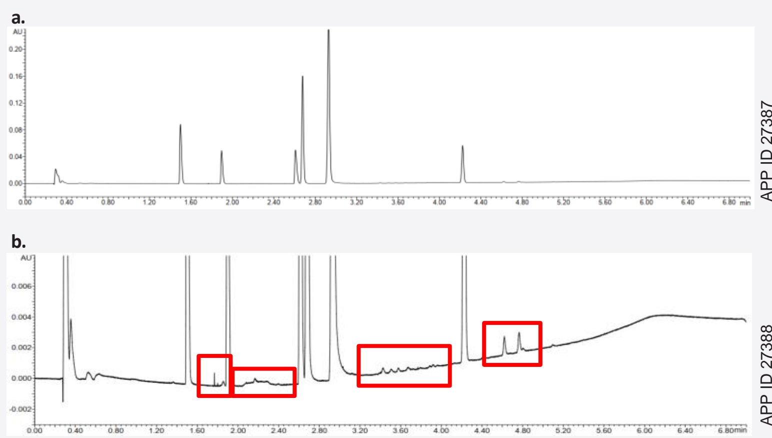


Figure 2. ABN7 Analysis Using Waters® Formic Acid.



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Other Formic Acid Sources

In order to determine if the Formic Acid was the source of these impurity peaks, alternative sources for Formic Acid were obtained and used to make up the mobile phase. The preparation of mobile phase A requires mixing Formic Acid with water. Both of these components could have introduced the contaminants that resulted in the impurity peaks. Several different sources of water (Milli-Q®, Barnstead®, and Sartorius®) were used to prepare the mobile phase. The same impurity profile was observed when the mobile phase blank was injected with each of the sources of water, but not when a water sample was run (**Figure 1d**).

Therefore, it was concluded that the source of the impurity peaks observed was most likely the Formic Acid. Another source of Formic Acid was used to prepare the mobile phase to determine if the impurity peaks persisted. The Waters® Formic Acid (purchased in two 1 mL ampules) was used in preparing the mobile phases and the ABN7 sample was analyzed under the same conditions. **Figure 2a** shows the same peak profile as seen in the previous analysis. Upon closer inspection, impurity peaks did appear in this analysis as well, but with a much different profile noted by the red boxes in the zoomed view in **Figure 2b**.

It was intriguing to find such different impurity profiles between two sources of Formic Acid. To further investigate these differences, other sources of Formic Acid were used to prepare the mobile phases and run under the exact same conditions. Reagent grade Formic Acid in a 100 mL bottle was purchased from Sigma-Aldrich® and the results can be seen in **Figure 3a** and **3b**. The impurity peaks, noted by the red boxes in the zoomed view in **Figure 3b**, have some peaks located with similar retention times as observed with the previous two sources of Formic Acid, but the chromatographic profile overall is different. RICCA® ProteoSpec® LC-MS Grade Formic Acid in 1 mL ampules was also used to prepare the mobile phase, and the analysis was repeated. As can be seen in **Figure 4a**, the use of this Formic Acid did not affect the separation of ABN7 since the peak profile is the same as the previous injections. As observed with the other sources of Formic Acid, there were impurity peaks (**Figure 4b**) present, but at much lower levels than observed with the other sources. The retention times of these impurity peaks were very close to that of the Sigma-Aldrich Formic Acid.

There was also the possibility of the introduction of impurities from an outside source because the mobile phases were all prepared in the lab by hand. A Supelco® premixed 0.1 % Formic Acid in Water and 0.1 % Formic Acid in Acetonitrile were used to rule out human error in mobile phase preparation. As seen with all the hand-mixed mobile phases, the peak profile for ABN7 was as expected (**Figure 5a**). Interestingly, there were impurity peaks in this run as well (**Figure 5b**). The profile of the impurity peaks was different than any of the chromatograms obtained using hand-mixed mobile phases. Since the profile was different, it was not possible to rule out impurities from outside sources during mobile phase preparation.

Figure 3. ABN7 Analysis Using Sigma-Aldrich Reagent Grade Formic Acid.

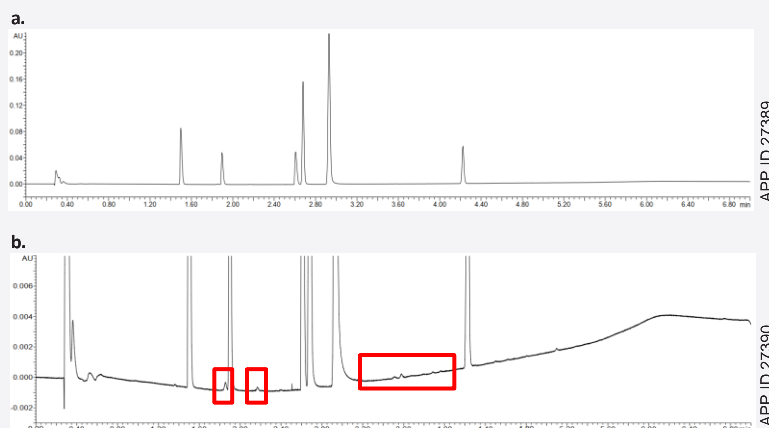

Figure 4. ABN7 Analysis Using RICCA ProteoSpec LC-MS Grade Formic Acid.

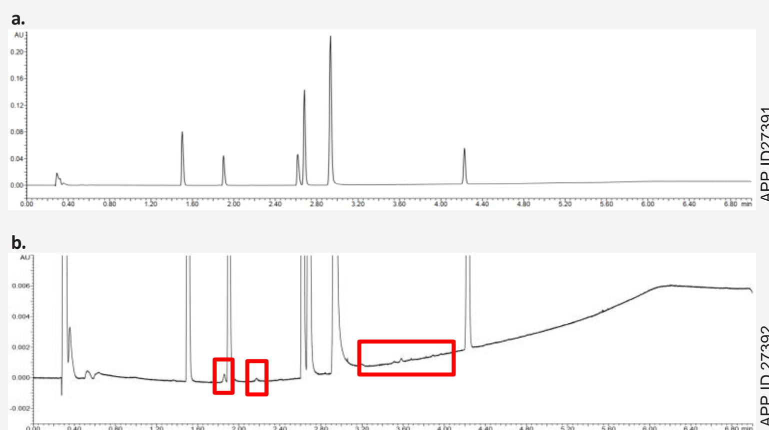

Figure 5. ABN7 Analysis Using Supleco Premade Mobile Phases.

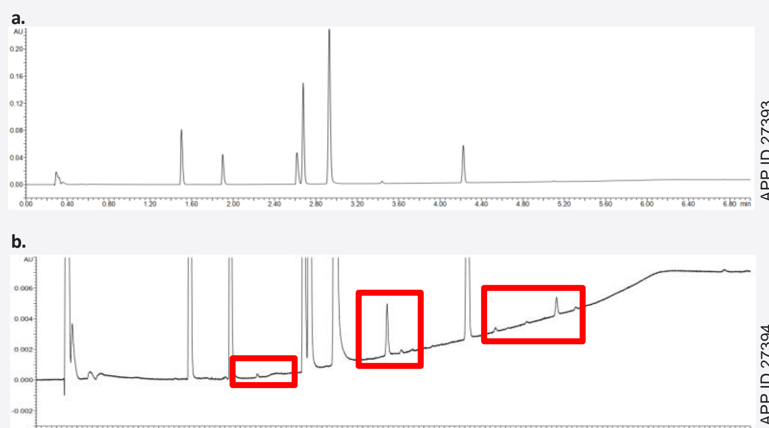

Table 1. Impurity Percentage of Total Absorbance and Highest Absorbance Impurity for Each Formic Acid Source.

Formic Acid Source	Impurity Percentage of Total Absorbance (%)	Highest Impurity Absorbance Percentage of Total Impurities (%)	Retention Time (min) of Highest Absorbance Impurity
Fisher Chemical™	0.81	32.35	6.825
Waters	0.70	28.14	4.766
Sigma-Aldrich	0.19	42.98	1.856
RICCA ProteoSpec	0.26	35.48	1.86
Supelco Premade	0.92	59.28	3.439

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Conclusion

Unexpected peaks of unknown origin would be of concern to any HPLC or UHPLC user. Impurities are a common cause for these peaks and could very easily cause issues in data analysis and interpretation. There are several troubleshooting approaches that could be taken in order to find the source. Here we showcased a study that had impurity peaks originating in the Formic Acid used for mobile phase preparation. It is clear that each source of Formic Acid, including the premade mobile phases, has a unique impurity peak profile with the main impurity in each source being different except between the Formic Acids from Sigma-Aldrich® and RICCA® ProteoSpec® (**Table 1, Figure 6**). Another point to consider is that "LC-MS pure" does not mean "LC-UV pure." When faced with the presence of unexpected or unknown peaks in the chromatogram, one can identify potential sources and determine if they are sample-related or otherwise and troubleshoot accordingly.

The overall levels of impurities from the different sources of formic acid tested are summarized in detail in **Table 2**, with the corresponding chromatograms shows in **Figure 6**. Impurity peaks are identified with "*" in the chromatograms in **Figure 6**. From the data summarized in **Table 2**, a couple of conclusions can be drawn:

1. Formic Acid obtained from RICCA and Sigma-Aldrich gave the lowest overall levels of chromatographic impurities.
2. The Supelco® pre-made Formic Acid in water and Formic Acid in Acetonitrile yielded similar overall levels of observed impurities as several other sources (Waters®, Fisher Chemical™).
3. Impurity profiles for the various sources of Formic Acid were dramatically different, suggesting different manufacturing and/or purification processes are used.
4. One cannot assume that all reagents, in this case Formic Acid, are equivalent from an impurity profile point of view.
5. Finally, it is important to test each reagent to ensure that impurities which may be present do not interfere with the chromatographic analysis.

Figure 6. Stacked Chromatograms of Impurity Peak Profiles. "*" Indicates Impurity Peaks.

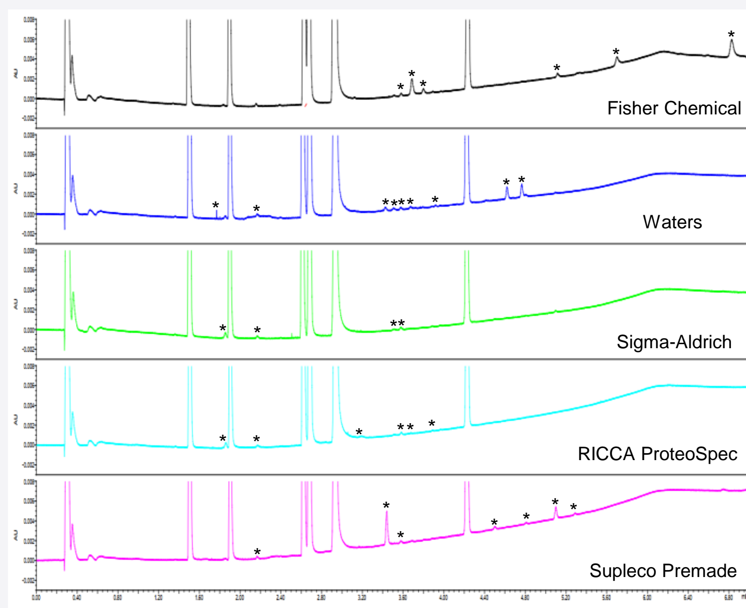

Table 2. Peak Profiles and Absorbance for All Formic Acid Sources.

Peak No.	Analyte	Retention Time (min)	Peak Height				
			Fisher Chemical	Waters	Sigma-Aldrich	RICCA	Supelco
1	Pidolol	1.49	86283	88658	86046	80553	81271
2	Impurity	1.86	-	295	539	551	-
3	Chlorpehiramine	1.9	48754	49368	48971	44438	44542
4	Impurity	2.17	-	455	273	210	237
5	Nortriptyline	2.62	49365	50156	49842	46471	46750
6	3-Methyl, 4-Nitrobenzoic Acid	2.68	161967	160483	156666	143209	149351
7	2-Hydroxy, 5-Methylbenzaldehyde	2.93	256593	250329	246498	223699	236065
8	Impurity	3.19	-	-	-	266	-
9	Impurity	3.43	-	363	-	-	3371
10	Impurity	3.51	-	266	152	140	-
11	Impurity	3.58	296	278	290	259	245
12	Impurity	3.67	1643	248	-	-	-
13	Impurity	3.8	499	-	-	127	-
14	Impurity	3.9	-	212	-	-	-
15	Hexanophenone	4.23	57463	55468	57133	53675	55116
16	Impurity	4.5	-	-	-	-	309
17	Impurity	4.62	-	1216	-	-	-
18	Impurity	4.77	-	1305	-	-	152
19	Impurity	5.1	405	-	-	-	1179
20	Impurity	5.3	-	-	-	-	194
21	Impurity	5.69	792	-	-	-	-
22	Impurity	6.83	1738	-	-	-	-
		Total Impurities	**5373**	**4638**	**1254**	**1553**	**5687**

Key Takeaways:

- Impurities are a common cause for unexpected peaks and could very easily cause issues in data analysis and interpretation.
- Impurity profiles and levels were observed to be different for each Formic Acid source.
- The main impurity in each source is different except for Sigma-Aldrich and RICCA ProteoSpec.

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