# Diluting and shooting yourself in the foot: Complications with sample-to-sample variations in signal suppression

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BACKGROUND: Dilute and shoot (D&S) methods are widely used in clinical and bioanalytical practice. D&S takes less time, is less labor intensive, and costs less than any other sample preparation technique. It also requires little to no time for method development. Increasingly more sensitive LC-MS/MS systems make this strategy ever more appealing: the more sensitive the system, the more the sample can be diluted, thus minimizing matrix effects. Even when analytes elute in a signal suppression region, compensation by stable label internal standard is often deemed adequate to address the issue. Problems arise, however, when IS does not coelute exactly with the analyte of interest. It no longer compensates for matrix effects, which are not always eliminated even by high sample dilutions.

OBJECTIVE: Using our opioid method remodeling as an example, we wish to re-emphasize the importance of investigating matrix effects early on in the method validation process and on a large number of specimens in order to determine whether D&S sample preparation is adequate for the developed LC-MS/MS method or whether more extensive sample cleanup is necessary.

RESULTS: The decision was made to transfer our existing 8-opioid urine confirmation assay to a more sensitive LC-MS/MS platform (AB Sciex Triple Quad 5500$^{TM}$) and to expand it by four additional opioid normetabolites and two co-formulated opioids. To offset the increased cost of additional analyte and internal standard reference materials, D&S sample preparation was tested to replace the SPE procedure in the existing method. Initial experiments showed that as high as 20-fold dilution of neat patient specimens could be used with sufficient analytical sensitivity. During method comparison with an existing assay performed on Waters Acquity® TQD, significant discrepancies were found between oxycodone (OC) concentrations in SPE- vs. D&S-processed specimens. Figure 1 shows a method comparison plot for OC. At first, the poor OC correlation was attributed to known problems with OC quantitation in the existing method. However, a signal suppression experiment, performed on 32 patient specimens by co-infusing an internal standard mixture while injecting processed specimens, revealed a different picture. Even at 20-fold specimen dilution, several suppression regions were present, correlating somewhat with urine color and concentration. OC was especially vulnerable to matrix effects (see Table 1). In a number of specimens, the OC peak was found partially in a signal suppression region (see Figure 2.) Because OC IS (OC-d6) is separated significantly from OC and elutes outside of the signal suppression region, it did not compensate well for the suppression of OC signal. Using D&S, OC was underestimated as much as 45% compared to the existing method with SPE sample preparation.

Consequently, the decision was made to implement a modified SPE sample preparation in the new expanded opioid method. During the validation of the final method, the signal suppression experiment was performed on 56 patient specimens. No signal suppression was found at the retention times of any of the 14 opioid analytes. Figure 3 shows a significantly improved OC concentration correlation between the existing and the newly developed method.

**Figure 1.** Oxycodone method comparison: existing method (SPE) vs. new method (D&S).

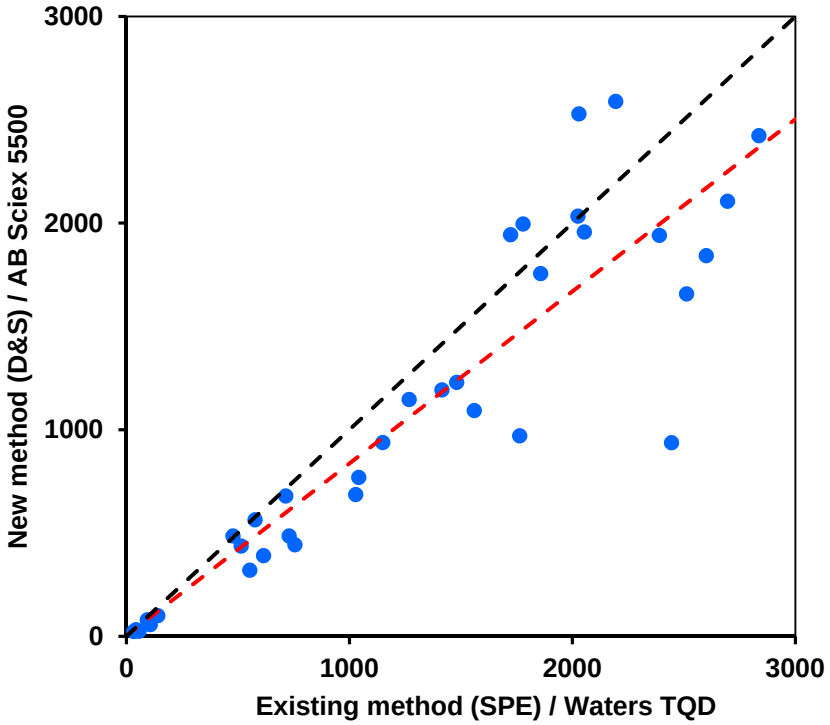



**Table 1.** Comparison of concentrations of SPE- vs. D&S-processed patient urine specimens used in the signal suppression experiment. Only OC-positive specimens are listed.

| Sample ID | Urine appearance | SPE (ng/mL) | D&S (ng/mL) | %Bias D&S vs SPE |
|---|---|---|---|---|
| SP 02 | very light yellow | 130 | 154 | 19% |
| SP 03 | very light yellow | 41 | 42 | 3% |
| SP 11 | very light yellow | 1487 | 1759 | 18% |
| SP 19 | very light yellow | 188 | 224 | 19% |
| SP 01 | light yellow | 2009 | 2794 | 39% |
| SP 04 | light yellow | 1182 | 2369 | 100% |
| SP 07 | light yellow | 1803 | 2377 | 32% |
| SP 09 | light yellow | 2242 | 2333 | 4% |
| SP 30 | light yellow | 253 | 204 | -19% |
| SP 31 | light yellow | 143 | 140 | -2% |
| SP 13 | medium yellow | 374 | 428 | 15% |
| SP 18 | medium yellow | 2272 | 1931 | -15% |
| SP 20 | medium yellow | 1023 | 1129 | 10% |
| SP 21 | medium yellow | 2226 | 2344 | 5% |
| SP 25 | medium yellow | 2594 | 2803 | 8% |
| SP 06 | orange | 3752 | 2055 | -45% |
| SP 17 | dark yellow | 1232 | 748 | -39% |
| SP 29 | murky dark brown | 2348 | 1547 | -34% |

**Figure 2.** Comparison of signal suppression experiment results for a patient urine specimen processed by SPE vs D&S.

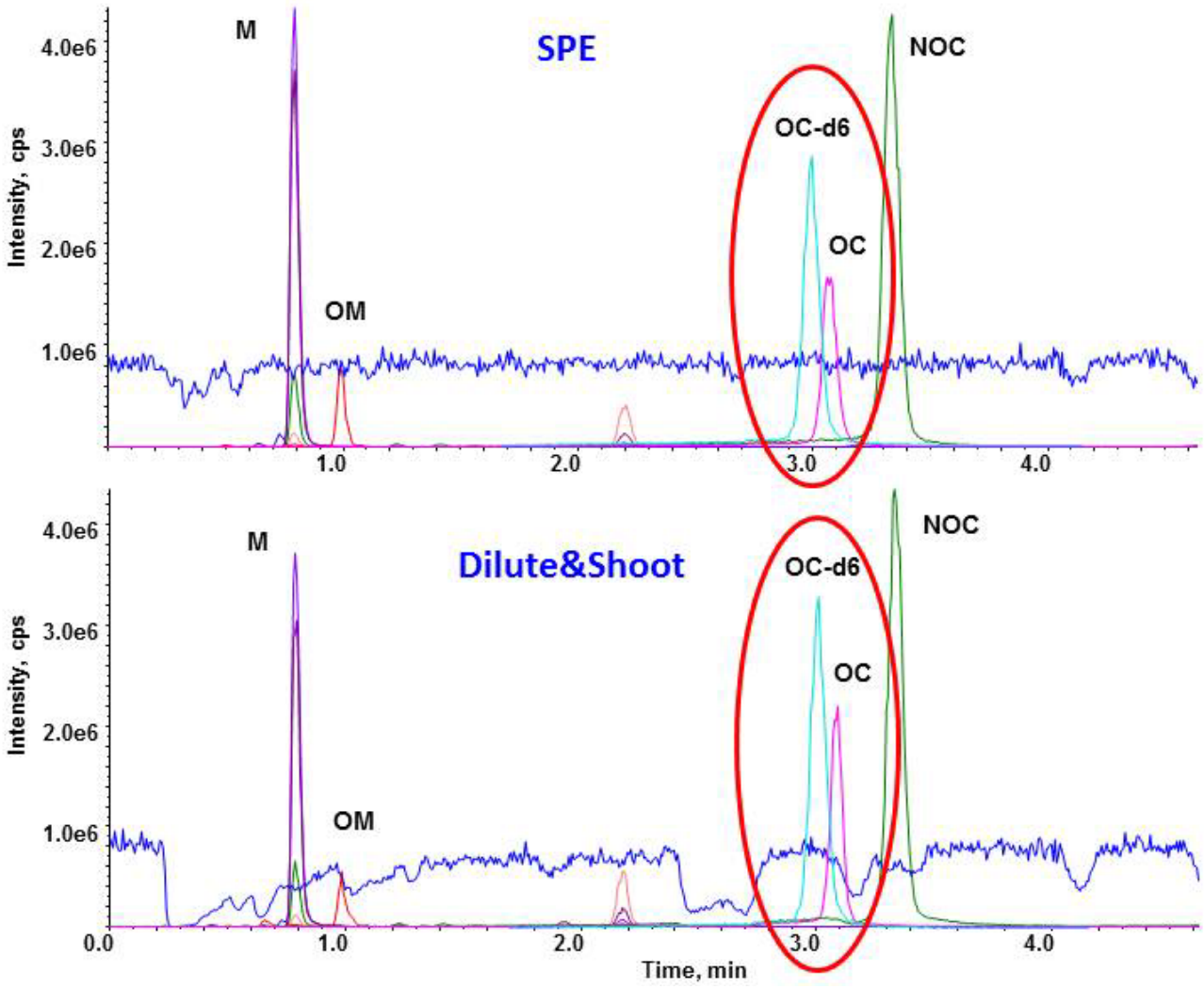
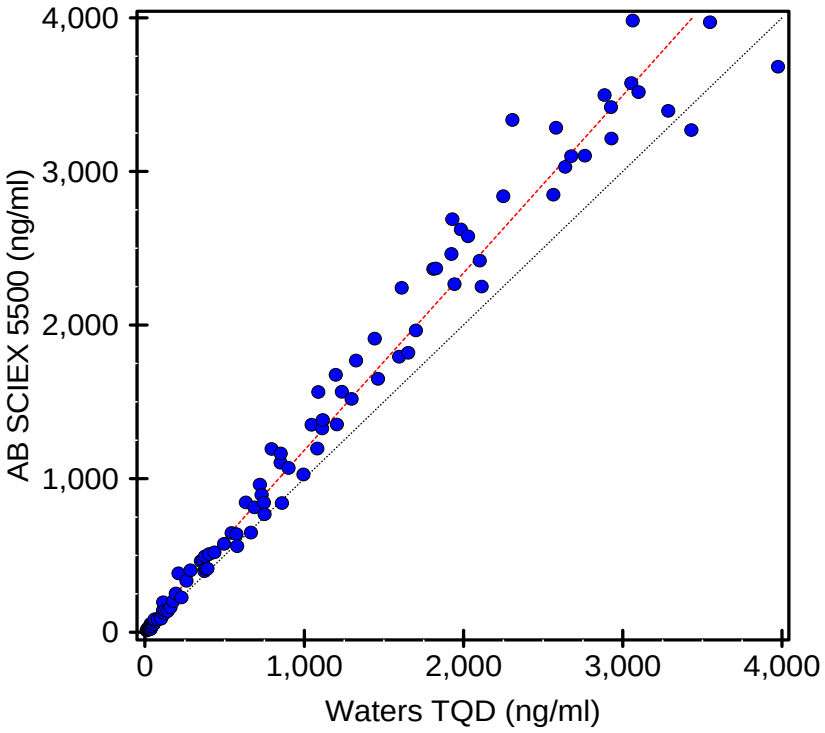


**Figure 3.** Oxycodone comparison method plot for existing method vs. final new method, both using an SPE sample preparation procedure.




CONCLUSION:

To ensure that the newly developed LC-MS/MS method is robust and provides quality data, we strongly advocate investigating matrix effects by performing the signal suppression experiment early on during method validation or even as part of the method development process and on a large number of specimens to determine if D&S sample preparation is adequate for the new method or whether more extensive sample cleanup is necessary.