2025 PDA Pharmaceutical Microbiology Conference |Oct 27 - Oct 29, 2025 | Washington, DC

FROM BENCH TO REALITY: THE ROLE OF NATURAL CONTAMINATIONS IN LER INVESTIGATIONS

by Alessandro Pauletto, Christian Faderl, Luca Di Bello, Gregory Devulder and Kevin L. Williams, bioMerieux

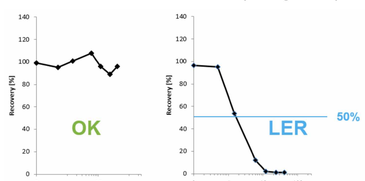
INTRODUCTION

There is ongoing debate regarding the most appropriate type of endotoxin to use in Low Endotoxin Recovery (LER) Hold Time Studies. PDA Technical Report 82 (TR82) recommends Reference Standard Endotoxin (RSE) and Control Standard Endotoxin (CSE) as the primary choices. Natural Occurring Endotoxins (NOEs) may be included as supportive data. However, their relevance remains controversial due to concerns that they may not accurately represent the type of endotoxin contamination likely to be encountered in actual drug products. In this study, we investigated the use of unprocessed water samples collected from Water for Injection (WFI) systems as a natural source of endotoxin. These samples, without undergoing any purification, were used to spike a formulation matrix relevant to biopharmaceutical products. The goal was to evaluate their behavior and reliability in LER studies.

CONCLUSION: This study, conducted by directly inoculating samples with naturally contaminated water, did not show relevant differences compared to results obtained using purified endotoxins (RSE and CSE). Therefore, there is no clear reason IF these COULD REPLACE the STANDARIZED worst-case model based on RSE.

BACKGROUND

Low Endotoxin Recovery (LER) refers to the phenomenon where endotoxins exhibit a reduced or undetectable response in the BET (Bacterial Endotoxin Test) after being exposed to certain conditions or matrices, such as proteins, buffers, or other pharmaceutical formulations. This reduced detection does not indicate the absence of endotoxins but rather a masking effect, where the endotoxins are not effectively recognized by the assay.



According to PDA Technical Report 82, hold time studies should be conducted by spiking the undiluted sample with Control Standard Endotoxins (CSE) or Reference Standard Endotoxins (RSE). The use of Naturally Occurring Endotoxins (NOE) is acceptable, but only as supplementary studies[1]). This guideline emerged after extensive debate on the use of NOE, ultimately favoring endotoxins that are more standardized in the manufacturing process to ensure more reproducible studies.

Claims have been made that the phenomenon only or primarily affects purified endotoxin including RSE and CSE[3]). Others have shown that some NOEs are prone to LER and some are less or much less affected [2]).

This study reinforces and expands previous studies using a more 'natural' NOE that was present in some pharmaceutical water samples and tap water. Rather than harvesting and growing the NOEs, this study innoculated samples directly with waters naturally contaminated by endotoxins.

Samples contaminated by the waters are a simple matrix based on 10 mM citrate + 0.05% Tween 20 at pH 6.2

The water samples used for spiking came from two different sources of drinking water (tap water) and two different sources from the WFI system. These are taken after the softener and before the RO (reverse osmosis) system. Two types of spiking has been proposed:

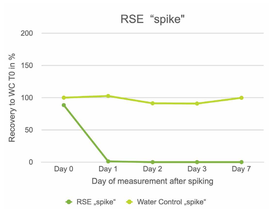
- **"Real"**
- **"Spike"**

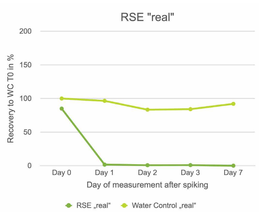
In **"Spike"** mode, spiking is performed as currently reported in many different LER hold time studies. This means the source of endotoxin has been directly added into the sample at a spiking level at around 10 EU/ml without adding more than 10% of the total volume as reported by PDA TR 82. In the **"Real"** mode, the individual water sample is used for the preparation of the defined matrix. The stock solutions of citrate and Tween 20 are added to the contaminated water for this study. This mimics the possible contamination process in drug manufacturing sites.

References:
1) PDA TR 82 Low Endotoxin Recovery (2019)
2) Low Endotoxin Recovery—Masking of Naturally Occurring Endotoxin, Johannes Reich et all – Int Journal of Mol. Sciences 2019 Feb;20:838
3) It is time to reconsider the use of naturally-occurring endotoxins in endotoxin recovery studies: Part 2 of a BioPhorum harmonized endotoxin recovery study. Jay Bolden et all – Biologicals 87 (2024) 101779

DATA

Real vs Spike with RSE:





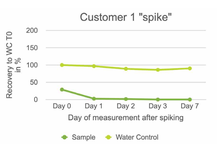
This table presents results of the matrix spiked by RSE at a theoretical value of 10 EU/ml in 2 different conditions: real and spike mode. All the samples are tested diluted 1:100 in BET water and tested by rFC ENDOZYME® II (bioMèrieux SA). There is no difference in results between spike and real mode. RSE shown a clear LER issue starting from the time point 1 (day 1)

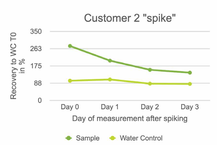
Real vs Spike with Drinking water samples

This table presents results of the matrix spiked by two different drinking water (tap water) from 2 different areas. One from Italy and another one form Germany. The theoretical spike level was around 10 EU/ml in both samples. All the samples are tested diluted 1:100 in BET water and tested by rFC ENDOZYME® II (bioMèrieux SA). There is no difference in results between spike and real mode. If compared with RSE the LER appears to be much quicker having <50% in the recovery already at the timepoint 0 (day 0). No relevant differences between both samples.

RESULTS

Real vs Spike with Water samples (WFI system)





The results from the tests conducted on the matrix contaminated with water samples derived from WFI systems show differences between the two sources.

In the case of Customer 1, the behavior is clearly similar to what was previously observed with the two drinking water samples, and especially with the RSE. The speed at which the LER phenomenon occurs is essentially the same as that seen in the sample contaminated with RSE.

In contrast, the behavior observed for Customer 2 is notably different. First, at time zero, the detected value is significantly higher than expected. Nevertheless, a marked reduction in endotoxin content is observed when compared to the initial value recorded at time zero for the same sample. No results are available at the 7-day time point due to contamination that occurred in the sample at that interval.

CONCLUSION

Traditionally, studies on Naturally Occurring Endotoxins (NOE) have followed a defined methodology:

- Culturing a bacterial isolate
- Harvesting the biomass
- Filtering out cellular debris before inoculating LER sample solutions for hold time studies, as required for BLA submissions.

In contrast, this study employed a more natural form of NOE, identified directly in pharmaceutical-grade and tap water samples. By avoiding any purification process, the native composition and state of the endotoxins were preserved. The findings confirm that Low Endotoxin Recovery (LER) is not limited to purified endotoxins such as Reference Standard Endotoxin (RSE), but also affects non-purified, naturally occurring endotoxins. This supports the continued use of RSE as the gold standard for LER assessment in hold time studies, due to its well-defined characterization and its ability to ensure robust and reliable quality control in pharmaceutical manufacturing.