



Key factors in the choice and development of a cleaning strategy

Part II

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This article discusses the removal of bacterial endotoxins in a washing process and the need for a continuous monitoring activity according to common cleaning validation practices. Results of washing tests performed with different load materials following an effective cleaning strategy to remove microbiological soil are presented. In addition, this paper compares two different online assay methods: conductivity analysis and Total Organic Carbon analysis.

Keywords: Cleaning, Microbiological Soil, Bacterial Endotoxins, Conductivity Analysis, Total Organic Carbon Analysis.

Introduction

The purpose of a washing treatment consists in removing the soil present on the load. Some aspects of soil removal, strictly of a chemical type, were previously discussed [1]. However, a new cleaning requirement is becoming increasingly widespread: treating a different soil, which is not chemical but microbiological. A very frequent requirement is indeed to remove bacterial endotoxins.

Once the washing process has been validated, the chemical and/or biological soil has been removed, and its absence on the load has been demonstrated, the process must remain in a continuous “monitoring mode”. Periodic checks or online monitoring of the rinse water contribute to:

- Increasing the safety margin that makes a validated washing process, reliable and always reproducible in its results;
- Protecting the end user of the product from an unexpected result that does not conform to expectations.

Removal of bacterial endotoxins

The U.S. FDA Cleaning validation guide states that “...control of the bioburden through adequate cleaning and storage of equipment is important to ensure that subsequent sterilization or sanitization procedures achieve the necessary assurance of sterility” [2]. Failure to remove/destroy endotoxins is certainly a limitation of steam sterilization; therefore, the “endotoxin content” of a generic load, intended for subsequent sterilization, is often monitored and controlled during its handling, production and washing.

Endotoxin removal can be investigated during the validation cycles of the cleaning process. In fact, three endotoxin log reductions, which is a standard requirement for the depyrogenation process, might be the additional target of a washing procedure. Obviously, the required reduction would not be achieved due to the high temperatures but due to the mechanical removal effect dictated by the cleaning medium, such as water, steam or aqueous solution with the addition of detergents.

Case study

The test, performed at the Research and Development department of Fedegari, removed bacterial endotoxins from different materials by a washing cycle and constituted a preliminary assessment of the effectiveness of this process in terms of endotoxin load reduction. The goal of this study was to verify the 3 log reduction of the inoculated endotoxin load on the surface of coupons measuring 100 mm x 100 mm.

The use of coupons in the initial setup of a washing process is suggested by PDA Technical Report No. 29: Points to consider for Cleaning Validation [3]. Stainless steel, 316 L, polypropylene and tempered glass coupons were investigated. The choice of materials to be inoculated was of a practical nature: materials usually found in pharmaceutical practices.

TEST PROCEDURES

The materials were depyrogenated through a thermal treatment performed at 250°C. Due to the high temperatures, it was not possible to expose the polypropylene coupons to the same process. This way, the plastic coupons were disinfected with a common biocidal agent representing a worst case in this study.

Every coupon was inoculated with a predefined quantity of endotoxins (Endotoxin Indicator ENDOSAFE, Charles River - Source: Escherichia coli 055:B5) and the inoculum was dried at 40°C by means of a laboratory heater. The drying time depended on the treated material. Appropriate supports for the coupons were studied to ensure that the washing medium reached the inoculated coupon surface (see Figure 1).

In addition, an appropriate cleaning equipment, composed of rotating arms with nozzles, was designed and built. Since the effectiveness of the process in terms of the intended goal was not known, a standard cycle already applied at a



Figure 1 - *Stainless steel coupons placed on special supports inside the washing machine*

customer facility was used. This cycle was mainly composed by various washing phases with water recirculation and a washing phase with water disposal, injection and simultaneous drain of water.

The washing machine located in the Research and Development laboratory of Fedegari is supplied with deionized water originating from a reverse osmosis plant. In order to remove from the results any endotoxin content due to the contribution of the water entering the system, an analysis of the water was carried out.

The endotoxin content was evaluated by applying a “Bacterial Endotoxin Test – BET”, as described in the various international Pharmacopoeias, harmonized in this regard [4-5]. This test is capable of identifying or quantifying endotoxins originating from gram-negative bacteria by using Limulus Polyphemus amoebocyte lysate.

There are three techniques for this type of test:

- gel clot;
- turbidimetric;
- chromogenic.

The European Pharmacopoeia [5] indicates the following analysis methods:

- Method A. Gel-clot method: limit test
- Method B. Gel-clot method: semi-quantitative test
- Method C. Turbidimetric kinetic method
- Method D. Chromogenic kinetic method
- Method E. Chromogenic end-point method
- Method F. Turbidimetric end-point method

The technique applied was the chromogenic kinetic one (method D of the European Pharmacopoeia).

The instrument used (see Figure 2), Endosafe PTS (Charles River), is a portable cartridge spectrophotometer, which measures the endotoxin content as a function of the time required to develop a given quantity of color. The higher is the endotoxin content; the lower is the response time.



Figure 2 – Endosafe PTS instrument, manufactured by Charles River, used for the evaluation of the endotoxin load of the samples being considered.

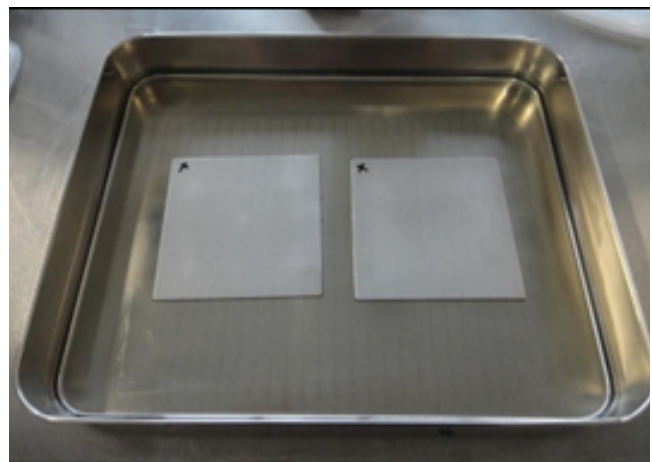


Figure 3 - Coupons immersed in water for injection; recovery of endotoxin content.

At the end of the washing treatment, the samples were extracted from the machine, placed in a non-pyrogenic container and immersed in a defined volume of water for injection (see Figure 3).

After approximately 60 minutes of immersion and continuous manual agitation, a series of dilutions of the solution was performed. It allowed the instrument to operate in its correct reading range.

The same procedure was applied for positive controls: coupons of various materials that were inoculated and not subjected to the washing process.

Comparing the positive control with the after treatment coupon results revealed a logarithmic reduction of approximately 3 log for the different materials tested. See Table 1 for more details.

Managing the unexpected: online control

Even for cleaning processes established and validated according to common Cleaning Validation practices, there are online controls that can help to “manage the unexpected”. These procedures allow continuous monitoring of the parameters indicating the performance of the

1	Samples	Endotoxin load	Logarithmic reductions
1a	Positive control: 316L stainless steel coupon	968 EU/ml	3.01
1b	316L stainless steel coupon after treatment	0.952 EU/ml	
2	Samples	Endotoxin load	Logarithmic reductions
2a	Positive control: tempered glass coupon	1001 EU/ml	3.02
2b	Tempered glass coupon after treatment	0.965 EU/ml	
3	Samples	Endotoxin load	Logarithmic reductions
3a	Positive control: polypropylene coupon	897 EU/ml	3.32
3b	Polypropylene coupon after treatment	0.434 EU/ml	

Table 1 - Endotoxin load of the coupons before and after treatment and corresponding logarithmic reduction.

process and are useful to identify any anomaly in the treatment.

The use of rinsing water samples can keep the process in a “state of control” during routine production and is prescribed in the guidelines for validation processes written by the U.S. FDA [6] in 2011: “...Ongoing assurance is gained during routine production that the process remains in a state of control”

Appropriate analytical methods can be applied to meet the previously described requirements: analysis of conductivity and of total organic carbon on the waste water of the process are a typical example.

ONLINE CONDUCTIVITY ANALYSIS METHOD

The most widespread method for online analysis of waste water quality in a washing system is the measurement of conductivity. Conductivity analysis is a non-specific method that allows to detect ionic substances dissociated in water samples but is unable to distinguish among different ions.

Conductivity analysis can be used to measure residues of API (Active Pharmaceutical Ingredients) or detergents, if they are of ionic nature. The conductivity value of the final rinse water is an additional requirement commonly included in the performance parameters of a cleaning process. E.g.: ‘If the readouts of the conductivity at the end of the rinse phase is higher than the preset value, the system must continue rinsing of all washing lines (repetition of maximum number of phases) until the required value is obtained’. URS of a Pharma company.

Application of online conductivity analysis on Fedegari steam washers

In washing machines, the conductivity sensor is integrated in the hydraulic system and connected to the process controller (see Figure 4).

Analysis of conductivity

- Measurement of water conductivity is performed during final rinsing. For this purpose, a conductivity meter is installed in the drain line.
- Rinses are repeated automatically until the conductivity reading is lower than a preset value or until the maximum preset number of rinses has been reached. If the conductivity set point is not reached within the set number of rinses, the process controller reports an alarm. In this manner, the operator can apply the procedures required to solve the anomaly in progress.
- The conductivity meter is fully managed by ‘Thema4’ process controller installed in Fedegari steam washers.



Figure 4 - Schematic view of the online application of conductivity sensors in Fedegari steam washers.

METHOD FOR ONLINE ANALYSIS OF TOTAL ORGANIC CARBON

Until recently, conductivity analysis was the only online method used to monitor the quality of the water of the last rinse. Recent technological developments related to the measurement of Total Organic Carbon (TOC) in water samples have allowed to integrate it in a washing system.

Why is this parameter monitored as well?

Most of the products involved in biotechnological processes are characterized by its organic nature; so simple conductivity analysis cannot detect them. A TOC analyzer is used to determine residues of detergents and API (Active Pharmaceutical Ingredients) composed of organic molecules when the identity of the analyte is not required.

TOC analysis can be applied to every sample containing organic carbon. The TOC analyzer oxidizes the organic carbon present in the molecule that constitutes the sample and measures the resulting carbon dioxide obtained from the oxidized organic molecule. The instrument is capable of converting organic compounds into inorganic compounds, which then can be analyzed by conductivity measurements.

TOC analyzers manufactured by various suppliers and based on different measurement methods are commercially available. However, the following characteristics are common to all TOC analysis devices, regardless of the supplier:

- the end result of the TOC measurement is derived from conductivity readings,
- the concentration of the carbon content derived from organic material present in the water is measured in parts per billion (ppb).

The TOC analysis is a highly sensitive method, but it is clearly nonspecific. In fact, the instrument is capable of detecting organic residues of products that have degraded during washing processes but is unable to identify the analyte in the sample.

Application of online analysis of the TOC sensor on Fedegari washing machines

This case study regards the online application of the conductivity and Total Organic Carbon sensors in Fedegari steam washers. In these machines, both the conductivity meter and the TOC analyzer are integrated in the hydraulic system and connected to the process controller (see Figure 5).



Figure 5 - Schematic view of the online application of conductivity and Total Organic Carbon sensors in Fedegari washing machines.

Analysis of Total Organic Carbon (TOC)

- The conductivity of the rinse water is measured during final rinsing through a conductivity sensor (which is external to the TOC sensor) located on the drain line. The rinses are repeated automatically until the conductivity value is lower than a predetermined value included within the operating limits of the TOC analyzer.
- At the end of this phase, the system for monitoring Total Organic Carbon is activated and a controlled volume of water from the final rinse is conveyed to the TOC sensor, opening an isolation valve.
- The TOC sensor uses the oxidation induced by an ultraviolet lamp to determine the concentration of Total Organic Carbon that is contained in the sample of water from the final rinse. Integrated conductivity sensors provide real-time conductivity measurements before and after sample oxidation; the final value of the Total Organic Carbon derives from conductivity readings.
- Once sampling has ended, the measured TOC value is transmitted to the process controller of the washing machine. The controller can be programmed to repeat the washing phase until a predefined TOC value or a maximum number of rinses is reached. If the preset TOC value is not reached within the maximum number of rinses, then the Process Controller reports an alarm. In this manner, the operator can apply the procedure required to solve the anomaly in progress.
- As highlighted in the preceding application, the conductivity meter and the TOC sensor are fully managed by 'Thema4' process controller installed on Fedegari washing machines.

While the conductivity measurement is instantaneous, the introduction of an online TOC analysis entails a longer cycle time. The washing system is in fact in a standby state during sample analysis in order to assess the need to perform further rinsing.

Nevertheless, comparison between off-line and online measurements clearly shows that the online application of conductivity and TOC allows to eliminate manual sampling, the transport of samples to laboratories, the delay in laboratory time analysis, the "human variable" in the assessment of the results, and the risk of cross contamination. Online application allows continuous monitoring of process parameters in the reporting of any errors during a previously validated process.

A schematic summary of the advantages/disadvantages of the online application of conductivity and TOC is provided hereafter.

Advantages of the online conductivity and TOC analysis methods

- Faster analyses: elimination of manual sampling
- Reduced risk of cross contamination: elimination of manual sampling
- Reduced costs: no manual analysis in a laboratory
- Reduction of inactivity periods: waiting for laboratory analysis
- Analysis reliability : continuous monitoring of process parameters
- Real-time "alarm system"
- High sensitivity of the measurement

Disadvantages of the online conductivity and TOC analysis methods

- Nonspecific methods
- Only for the TOC analyzer: initial cost of instrument

FINAL CONSIDERATIONS

Removal of bacterial endotoxins - The washing tests performed at Fedegari R&D department have proven to be effective in removing not only chemical but also microbiological soil. A more intense washing process is expected to yield a reduction of endotoxin load distinctly higher

than 3 log. A supplementary test, starting from a “chemically dirty” load, might constitute an additional challenge in the removal of inoculated bacterial endotoxins.

Online analyses of Total Organic Carbon and conductivity are complementary for continuous monitoring of the washing process performances. The conductivity meter and the TOC sensor can be fully integrated in the hydraulic system of a washing machine and connected to the process controller, as in the case of Fedegari steam washers. The complete integration with the controller allows regulatory compliance to the requirements of the FDA 21 CFR part 11: the data related to each deviation from the standard required in terms of washing performance are recorded and stored in the system history. Through these applications, the process remains in a state of control during routine production as indicated by the FDA itself [6].

1.

References

1. M.L. Bernuzzi. Fattori chiave nella scelta/ sviluppo di una strategia di lavaggio; 1a parte. ASCCA News, 2014; 4: 6-9.
2. Guide to Inspections of Validation of Cleaning Practices; U.S. Food and Drug Administration: 1993. Available at: www.fda.gov/ICECI/Inspections/InspectionGuides/ucm074922.htm (accessed February 24, 2015)
3. Technical Report No. 29: Points to Consider for Cleaning Validation; Parenteral Drug Association: Bethesda, MD, 1998.
4. USP 37 General Chapter <85> BACTERIAL ENDOTOXINS TEST, 2014.
5. European Pharmacopoeia 8.0. Bacterial Endotoxins, 01/2014:20614.
6. Guidance for Industry. Process Validation: General Principles and Practice; U.S. Food and Drug Administration: [on-line] 2011. Available at: www.fda.gov/downloads/Drugs/Guidances/UCM070336.pdf (accessed February 24, 2015)