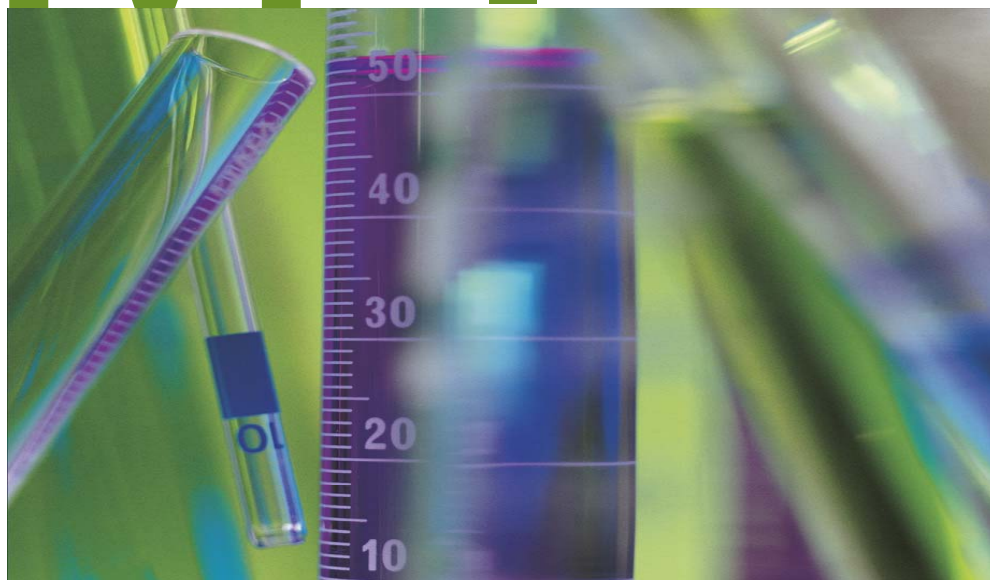


M

Analytical Method

Endotoxin analysis

ANALYTICAL METHOD 332



Applicability

This method is used for analyzing endotoxins

Standard(s) ¹

No standard.

Sampling system

Glass fibre filters with three-section cassettes or Button filter sampler

Recommended sampling volume and flow rate

Flow rate of 2L/min (time: 4 hours) at 10L/min (time: 1 hour)

Volume from 480 to 600 litres (depending on the sampling system)

Analysis

Spectrometer, LAL method

Minimum reported value (MRV)

0.4EU/m³ for 4 hours of sampling at 2L/min.

Range of application

Based on dilutions

Reability

Repeatability: 6.44%

Replicability: 1.96%

Analytical uncertainty (CVA)

15%.



Established in Québec since 1980, the Institut de recherche Robert-Sauvé en santé et en sécurité du travail (IRSST) is a scientific research organization known for the quality of its work and the expertise of its personnel.

OUR RESEARCH *is working for you !*

Mission

To contribute, through research, to the prevention of industrial accidents and occupational diseases as well as to the rehabilitation of affected workers.

To offer the laboratory services and expertise necessary for the activities of the public occupational health and safety prevention network.

To disseminate knowledge, and to act as scientific benchmark and expert.

Funded by the Commission de la santé et de la sécurité du travail, the IRSST has a board of directors made up of an equal number of employer and worker representatives.

To find out more

Visit our Web site for complete up-to-date information about the IRSST. All our publications can be downloaded at no charge.

www.irsst.qc.ca

To obtain the latest information on the research carried out or funded by the IRSST, subscribe to *Prévention au travail*, the free magazine published jointly by the IRSST and the CSST.

Subscription: 1-877-221-7046

Legal Deposit

Bibliothèque et Archives nationales
2009

ISBN: 978-2-89631-368-6 (PDF)

ISSN: 0820-8395

IRSST – Communications Division
505, De Maisonneuve Blvd West
Montréal (Québec)
H3A 3C2

Phone: 514 288-1551

Fax: 514 288-7636

sac.labo@irsst.qc.ca

www.irsst.qc.ca

© Institut de recherche Robert-Sauvé
en santé et en sécurité du travail,
2009



Analytical Method

Endotoxin analysis

 ANALYTICAL METHOD 332

Disclaimer

The IRSST makes no guarantee regarding the accuracy, reliability or completeness of the information contained in this document.

In no case shall the IRSST be held responsible for any physical or psychological injury or material damage resulting from the use of this information.

Note that the content of the documents is protected by Canadian intellectual property legislation.

Analytical or calibration methods are the methods developed or chosen by the IRSST to carry out its different mandates. They may require the use of hazardous materials, operations or equipment. These methods do not mention all of the safety problems related to their use. It is the user's responsibility to establish the appropriate health and safety practices. Use of the data included in these methods is at the user's own risk: the IRSST is not responsible in any way for any errors or damage that may result from such use and application. The hyperlinks that appear in this document were validated at the time of publication.

Technical person in charge of the method

*Geneviève Marchand Ph.D., microbiologist,
Laboratory Services and Expertise Department, IRSST*

Approval

*Geneviève Marchand, M.Sc. chemist,
Marie-Claude Barrette, M.Sc. chemist, QA coordinator,
Jacques Lesage, M.Sc. chemist, director.
Laboratory Services and Expertise Department, IRSST*

Authorization for publication

*Marie Larue, M.Sc., president-chief executive officer,
Executive office, IRSST*



This publication is available free
of charge on the Web site.

IN CONFORMITY WITH THE IRSST'S POLICIES

The results of the research work published
in this document have been peer-reviewed.

TABLE OF CONTENTS

Preamble	1
1. Principle of the method	2
2. Interferences	2
3. Material	2
4. Reagents.....	3
5. Sampling.....	3
5.1 Relative exposure limit	3
6. Analytical protocol.....	4
6.1 Treatment of glassware and glass fibre filters	4
6.2 Sampling equipment	4
6.3 Endotoxin extraction	4
6.4 Programming the spectrometer	4
6.5 Loading of the plate	5
6.6 Analysis of samples	6
6.7 Acceptability of the results	6
7. Performance parameters.....	6
7.1 Limit of detection and limit of quantification	6
7.2 Reliability	7
7.3 Accuracy	7
7.4 Stability of samples	7
7.5 Desorption/recovery	8
8. Quality control.....	8
9. Calculations.....	9
10. OHS	9
11. References.....	9
12. Bibliography.....	9

Preamble

The goal of the [Act respecting occupational health and safety](#) in Québec is to eliminate, at the source, dangers to the health, safety and physical well-being of workers. Permissible exposure values (PEVs) for chemical substances have been established in Schedule I of the [Regulation respecting occupational health and safety](#) (RROHS). Section 44 of this regulation entitled “Methods” specifies that:

“... These dusts, gases, fumes, vapours and mists found in the workplace environment shall be sampled and analyzed to obtain an accuracy equivalent to that obtained by applying the [methods](#) described in the Sampling Guide for Air Contaminants published by the Institut de recherche Robert-Sauvé en santé et en sécurité du travail du Québec, as it reads at the time that it is applied.”

To achieve these objectives, analytical methods for quantifying the workers' degree of exposure are developed and written to establish appropriate means of control. In order to help health and safety professionals in workplaces, the IRSST publishes, periodically revises, and disseminates the [Sampling Guide for Air Contaminants in the Workplace](#), and the Laboratory Services and Expertise Department publishes contaminant analytical methods.

- ✓ *Act respecting occupational health and safety*. R.S.Q., chapter S-2.1. Éditeur officiel du Québec, (August 1, 2007).
http://www2.publicationsduquebec.gouv.qc.ca/dynamicSearch/telecharge.php?type=2&file=%2F%2FS_2_1%2FS2_1_A.htm
- ✓ *Regulation respecting occupational health and safety*. S-2.1, r.19.01, O.C. 885-2001. Éditeur officiel du Québec (July 25, 2007).
http://www2.publicationsduquebec.gouv.qc.ca/dynamicSearch/telecharge.php?type=2&file=%2F%2FS_2_1%2FS2_1R19_01_A.htm
- ✓ *Sampling Guide for Air Contaminants in the Workplace*. Operations Division, IRSST, T-15 Guide technique, Montréal, Québec, (March 2005) <http://www.irsst.qc.ca/files/documents/PubIRSST/T-15.pdf>
- ✓ NIOSH, National Institute for Occupational Safety and Health.
- ✓ ISO Guide 30, Terms and definitions used in connection with reference materials, 2nd edition, 1992.
- ✓ ISO, International vocabulary of basic and general terms in metrology (VIM), 2nd edition, 1993.
- ✓ American Industrial Hygiene Association (AIHA), organization that accredits the IRSST laboratory in the field of workplace chemical contaminant analysis and microbiological environmental analysis.

Furthermore, all the terminology used in this method is described in work instruction “I-G-014” of the document management system associated with the IRSST's quality system.

1. PRINCIPLE OF THE METHOD

Drawing a known volume of air through a 37-mm glass fibre filter to collect the endotoxins. Extracting the filter in an aqueous solution, which will subsequently be treated in the ultrasonic bath and by agitation.

The analytical method quantifies the toxigenic level of endotoxins contained in the extraction solution.

The determination is done by kinetic chromogenic analysis using a spectrophotometer at a wavelength of 405 nm.

2. INTERFERENCES

This method's sensitivity varies in relation to:

- ✓ Products encountered that can interact with the enzymatic reaction.
- ✓ Products encountered that modify the dispersion of endotoxins.
- ✓ The presence of serum proteases that can produce false positives.
- ✓ Coloration or turbidity of a product that can interfere with the LAL analysis.

Different products can interfere with the LAL analysis. The presence of interference must be verified for each new product used, and for each group of samples analyzed.

It is essential to demonstrate with each group of samples that there is no interference. The majority of the interferences are dependent on the concentration and can be countered by dilution with LRW water ("LAL reagent water").

3. MATERIAL

3.1 Sampling

- ✓ Plastic cassette in three sections or Button filter sampler
- ✓ Support for glass filter
- ✓ Glass fibre filter, 37mm (Pall type A/E # 61652)
- ✓ Cellulose strip (Millipore # AP4003705)
- ✓ High volume pump

3.2 Laboratory equipment

- ✓ 96-well spectrophotometry microplate reader
- ✓ "Softmax Pro" software
- ✓ Multi-tube vortex
- ✓ Ultrasonic bath
- ✓ Precision micropipettes
- ✓ Repetition pipette or multi-pipette
- ✓ Certified endotoxin-free tips
- ✓ Certified endotoxin-free glass tubes

- ✓ Certified endotoxin-free 96-well microplates
- ✓ Certified endotoxin-free 2 mL tubes
- ✓ Polypropylene 50 mL centrifuge tube
- ✓ Incubator ($37^{\circ}\text{C} \pm 2^{\circ}\text{C}$)
- ✓ Pasteur oven
- ✓ Dessicator
- ✓ Centrifuge

4. REAGENTS

- ✓ Sterile water for irrigation, USP (Baxter)
- ✓ Tween 20
- ✓ LAL reagent water
- ✓ CSE, i.e., “Control Standard Endotoxin” (10ng/vial 50 EU/mL or 2 EU/mL)
- ✓ Pyrochrome LAL (A.C.C.)
- ✓ Interference control buffer

5. SAMPLING

Sampling is done using glass fibre filters that can be mounted either in three-section cassettes or in a Button filter sampler. The sampling rate is 2L/min when three-section cassettes are used, 4L/min for personal sampling, and 10L/min for environmental sampling when the Button filter sampler is used. The sampling time varies from 1 to 4 hours.

5.1 Relative exposure limit

5.1.1 For endotoxin analysis, the relative exposure limit value is used. This limit is based on a comparison with a baseline level, which is often the outdoor air. When respiratory problems are recognized, an exposure limit of 10X the baseline level is recommended, while a limit of 30X the baseline level is considered to be acceptable when no problem is noted.

5.1.2 Always perform a control, meaning a negative control.

For more details about the preparation of the sampling material, the calibration, and the strategy used, refer to the IRSST Sampling Guide¹.

6. ANALYTICAL PROTOCOL

6.1 Treatment of glassware and glass fibre filters

Glassware not certified as endotoxin-free that is used for endotoxin analysis, as well as the glass fibre filters and filter supports must be treated in the oven at 180°C for at least 4 hours or at 210°C for 60 minutes.

6.2 Sampling equipment

Mount the cassettes in a sterile location so as to reduce the risks of contamination. Minimize contact with the filter and the inside of the cassette.

6.2.1 Mounting of filters in a plastic cassette

1. Start by inserting the glass fibre support and then the glass fibre filter.
2. Press the cassette and ensure that it is tightly sealed.
3. Install a cellulose strip to seal the cassette.
4. Identify the cassette with a label.

6.2.2 Mounting of filters in a Button filter sampler

1. Start by inserting the glass fibre support and then the glass fibre filter.
2. Identify it with a label.

6.3 Endotoxin extraction

Before any equipment is used, make sure that a quality control has been done and that the endotoxin level is below 0.05 EU/mL.

6.3.1 Transfer the glass fibre filter to the polypropylene centrifuge tube.

6.3.2 Add 20 mL of irrigation water with 0.05% Tween 20 added.

6.3.3 Place the tube in the ultrasonic bath for 60 minutes, making sure that the temperature of the bath is not above 30°C.

6.3.4 Place the tube in the multi-tube vortex for 30 minutes and centrifuge for 10 minutes at 2000 RPM

6.3.5 Carry out the same step with a tube that does not contain a filter. This is the handling control ("check sample").

6.3.6 Carry out the analysis (sections 6.4 to 6.7).

The samples can be stored in certified endotoxin-free tubes and must be analyzed as soon as possible (i.e., within a period of 24 to 48 hours after sampling).

6.4 Programming the spectrometer

“SoftMax Pro” software is used to calculate the spike recovery percentage, and indicates whether the sample is valid. A template can be created and remain permanent. The following data must be included: calibration curve, sample solution, handling check solution, interference spikes, negative control, positive control. Each of the solutions must be analyzed in duplicate in two adjacent wells.

The reader uses the results of the calibration curve to calculate the concentrations and takes the dilutions into account.

It also stores the instrument reading conditions: (reading interval (15 sec.), durations of readings (1.5 hrs), wavelength (405nm), “Onset time” at 0.2, agitation (yes) once before the first reading).

6.4.1 Adjust the incubation temperature to 37°C.

6.4.2 Produce the template for the plate to be extracted according to the number of samples. Specify the dilutions carried out and the interference spikes.

6.4.3 Check whether the reading conditions are appropriate.

6.5 Loading of the plate

6.5.1 Agitate well with the vortex before taking an aliquot of the suspensions of the sample (endotoxins adhere easily to the walls).

6.5.2 Prepare the CSE (“Control standard endotoxin”) solution according to the manufacturer’s specifications. The CSE concentration is 50 EU/mL.

6.5.3 Prepare a calibration curve from 0.01 to 1 EU/mL, by performing 1:10 dilutions from the 50 EU/mL CSE solution. Use certified endotoxin-free tubes.

6.5.4 According to the previously recorded template, place 50 µL or 100 µL (depending on the manufacturer) of each of the solutions: **standard, sample suspension, negative control** (LRW water) **as well as the positive control** which is a replicate of the central point on the calibration curve.

6.5.5 Interference test

6.5.5.1 For each of the suspensions of sample replicates, perform a spike.

6.5.5.2 The addition is done by adding 10µL of the standard 1EU/mL to the well already containing the suspension of the sample. ATTENTION: The concentration of the spike must correspond to the concentration at the centre of the calibration curve.

- 6.5.6 Incubate the plate at 37°C for 10 minutes before adding the LAL.
- 6.5.7 Make sure that the spectrophotometer has reached its incubation temperature of 37°C.
- 6.5.8 Add 50 µL or 100 µL of LAL, based on the manufacturer's recommendations, starting with the wells with **the lowest concentrations**. Use the repetition pipette or a multi-pipette. This step must be performed very rapidly.
- 6.5.9 Place the plate in the reader, and remove the cover before taking the reading.

6.6 Analysis of samples

The reading data are recorded and interpreted by "Softmax Pro" software throughout the incubation period.

6.7 Acceptability of the results

In order to ensure validation of the results, different parameters must be checked.

- 6.7.1 The correlation coefficient (r) of the calibration curve must be greater than or equal to 0.995, and the coefficient of variation (c.v.) of the standards must be less than 4%.
- 6.7.2 The blanks (negative controls) of the plates must have a significantly longer "Onset Time" than that of the least concentrated standard on the curve.
- 6.7.3 The concentration obtained for the positive control in LRW water must be between 50% and 150% ($\pm 50\%$) of its expected value.
- 6.7.4 The concentration obtained for the spikes of the samples, controls and "checks" must be between 50% and 150% ($\pm 50\%$) of their expected value.
- 6.7.5 The endotoxin concentration of the "check" (laboratory control) must be below the least concentrated point (γ) on the calibration curve.

7. PERFORMANCE PARAMETERS

The scopes are specific to products from the Charles River Company. The data may vary with product suppliers.

7.1 Limit of detection and limit of quantification

- 7.1.1 **Linearity:** The linearity of the calibration curve was demonstrated for concentrations varying between 0.0015 and 50 EU/mL. This corresponds to concentrations from 0.063 to 2000 EU/m³ of air, respectively, for a sampled air volume of 480 L and a final extraction solution volume of 20 mL without any dilution of the extraction solution.

7.1.2 **Limit of detection:** The limit of detection of the endotoxin analysis method is 0.0023 EU/mL. It is defined as being the concentration equivalent to three times the standard deviation calculated from 20 measurements on a solution of LRW water.

7.1.3 **Lower limit of quantification:** The lower limit of the endotoxin analytical method is 0.0078 EU/mL. It is defined as being the concentration equivalent to ten times the standard deviation calculated from 20 measurements on a solution of LRW water.

7.2 Reliability

7.2.1 Replicability

Replicability was determined for four endotoxin concentrations. For each concentration, 12 fractions of the same sample were subjected to the analytical method. Replicability was calculated from two aliquots analyzed on the same day, by the same analyst, and on the same instrument. It corresponds to 1.96%.

7.2.2 Repeatability

Repeatability was determined for four endotoxin concentrations. For each concentration, two fractions of the same sample were subjected to the analytical method. Repeatability was calculated from two aliquots analyzed on three different days, with two different calibration curves, on the same instrument, and by three different analysts. It corresponds to 6.44%.

7.3 Accuracy

The accuracy of the analytical method is verified at each analytical sequence by using a positive control prepared by our laboratories. The concentration of this positive control is in the central part of the curve where the concentrations of the majority of the samples are found. The percentage difference obtained from the spikes carried out using 32 plates over the last 3 years was calculated as 43%.

7.4 Stability of samples

7.4.1 The non-extracted samples, stored at room temperature in a dry location, do not seem to show variation in their endotoxin content for approximately two weeks. However, it is preferable to analyze them as soon as possible.

7.4.2 The extracted samples, stored in a refrigerator, do not seem to show variation in the endotoxin content for approximately 48 hours.

7.5 Desorption/recovery

A desorption/recovery test was performed for four different endotoxin concentrations. For each concentration, twelve aliquots were added to glass fibre filters. After the aliquots were dried, the filters were subjected to the analytical method. All the analyses were carried out on the same day, by the same analyst, and on the same instrument. The results obtained from these tests are presented in Table 1.

Table 1: Desorption/recovery test (n=12)

Concentration (EU/mL)	0.1	0.25	0.625	1.25
Coefficient of des / rec (%)	105.18	94.19	96.51	112.22

Recovery average: 102%

8. QUALITY CONTROL

For each series of analyses, an extraction control (check) is done. This control is a sample without a filter that is subjected to exactly the same analytical process as the remainder of the samples. The analytical result for this suspension of sample must be below 0.05EU/mL.

For each analytical plate analyzed, a negative control (LRW water) and a positive control (the central point on the calibration curve, namely 0.1EU/mL) are performed. The negative control must have an endotoxin concentration below 0.05EU/mL. The positive control must have a concentration of approximately 50% of the target concentration.

For each suspension of sample, a spike is performed. This spike must be recovered at approximately 50% of the target value. This value is generally the concentration equivalent to the middle of the curve, namely 0.1EU/mL.

For all the duplicates of samples analyzed, the coefficient of variation between the two results obtained must not be greater than 10%. For the calibration curve, this variation between the duplicates must not exceed 4%.

9. CALCULATIONS

To obtain the results in EU/m³, the concentration obtained is multiplied by the dilution and the volume of extraction solution used, and is then divided by the volume of air (m³) sampled.

$$EU/m^3 = \frac{\text{concentration} \times \text{dilution} \times \text{extraction volume}}{\text{air volume sampled in L}} \times \frac{1000 \text{ L}}{m^3}$$

10. OHS

Not applicable

11. REFERENCES

- 1 *Sampling Guide for Air Contaminants in the Workplace*. Operations Division, IRSST, T-15 Guide technique, Montréal, Québec, (February 2005).
<http://www.irsst.qc.ca/files/documents/PubIRSST/T-15.pdf>
- 2 *Limulus Amebocyte Lysate, endosafe® endochrome-K™ “multi-test vial for endotoxin detection, users’ guide*, Charles River Endosafe, Charleston, South Carolina, USA.

12. BIBLIOGRAPHY

Munson, T.E., *Guideline for validation of the LAL test as an end-product endotoxin test for human and biological drug products, in Bacterial Endotoxins: Structure, Biomedical Significance, and Detection with the Limulus Amebocyte Lysate*, pages: 211-220, 1985.