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Application Note

Microbial Testing of Medical Cannabis Using Membrane Filtration

Ensure safety of your cannabis products according to Ph. Eur. 2.6.12, 2.6.13 and 2.6.31 test methods

Many cannabis products in the market have been found to be contaminated with microorganisms (including pathogenic bacteria, yeasts and molds) during production.[1] These contaminants are usually introduced during cannabis cultivation and storage. Possible contamination of cannabis products with microbial pathogens must be addressed to ensure consumer safety. Cannabis quality assurance and quality control teams play a vital role in ensuring product safety, consistency and compliance.[2] Microbial testing of cannabis involves two steps. While the total count tests for aerobic bacteria, yeasts and molds quantify a broad spectrum of microorganisms, the second test should be performed to detect specific pathogens in cannabis products.

The European Pharmacopoeia (Ph. Eur.) chapters 2.6.12, 2.6.13 and 2.6.31 give quality control departments guidance on how to ensure the safety of customers who consume medical cannabis products. The Total Aerobic Microbial Count (TAMC) and the Total Combined Yeast and Mold Count (TYMC) are described in 2.6.12. Determination of specific microorganisms listed in 2.6.13 and especially in chapter 2.6.31 should be used for herbal medicinal products for oral use and may be applied to medical cannabis products.[3,4,5]

Regarding the acceptance criteria for microbiological quality, the limits in Ph. Eur. chapters 5.1.8 and 5.1.4 apply.[6,7] The acceptance criteria and limits depend on the product. Readers are assumed to be familiar enough with both chapters.

To recover microorganisms present in the product, pour plate technique or membrane filtration can be used.[8] Both techniques have their advantages and disadvantages. While the filtration method can come with clogging of the membrane filter's pores if solid particles accumulate in the herbal extract, the pour plate technique requires handling skills because microorganisms will be killed if the temperature of the medium is too high when being poured, and air bubbles in the medium can lead to false-positive or false-negative results. The advantages of membrane filtration, in particular the method's ability to flush away inhibitors to avoid false-negative results, but also its lower likelihood of cross contamination than with an open system, the scalability of filtration volumes, the possibility to use ready-to-use agar plates and a reduced workload for lab technician give enough arguments to challenge the traditional recovery method.

The Milliflex Oasis® system for microbiological examination of aqueous fluids is composed of the Milliflex Oasis® pump, the Milliflex Oasis® single-use filtration units and the Milliflex Oasis® single-use media plates. For those experiments that require enrichment steps in liquid media, we recommend the Milliflex Oasis® head for the EZ-Fit® filtration unit which allows the use of EZ-Fit® filtration units with the Milliflex Oasis® filtration pump. The EZ-Fit® filtration unit offers the possibility to transfer the membrane filter into liquid media.

The scope of this application note study was to evaluate the effectiveness of the Milliflex Oasis® filtration system (**Part 1** using Milliflex Oasis® funnels, **Part 2** using EZ-Fit® funnels in combination with the Milliflex Oasis® filtration pump) for testing medicinal cannabis (please check for extracts the Ph. Eur. monograph <0765> "Herbal Drug Extracts" and for hemps the Ph. Eur. monograph <1433> "Herbal Drugs").

In **part 1** we determined the microbial load of several medical cannabis samples and in **part 2** we evaluated the suitability of the membrane filtration method for testing according to European Pharmacopoeia. As the matrix, 3 different hemp samples, and 3 different cannabis extracts (as defined above) were tested. All data in this study were determined by the BAV INSTITUT GmbH, A Tentamus Company, in Germany.[9]

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Materials

Cannabis Samples	THC (Tetrahydrocannabinol)	CBD (Cannabidiol)
Hemps		
Sample 1	27%	<1%
Sample 2	9.5%	12.5%
Sample 3	22%	<1%
Extracts		
Sample 4	25 mg/mL	1 mg/mL
Sample 5	50 mg/mL	50 mg/mL
Sample 6	12.5 mg/mL	12.5 mg/mL

Table 1: Approximate THC and CBD levels in the tested hemps and extracts.

*Please note in some regions, the term "hemp" has different regulatory definitions based on THC content. In North America and some LATAM regions for example, the regulatory threshold distinguishing hemp from Marijuana is greater than 0.3% THC. Please check your local regional requirements when referencing hemp products and associated terminology.

Table 1 lists the samples (1 to 6) with different levels of cannabinoids and terpenoids. Different samples were chosen to test the usage of rinsing solutions with neutralizers to avoid false negative results, to check for the absence of visible oil residues on the filtration funnel and to measure their filterability.

Product Name	Cat. No.
Milliflex Oasis® filtration pump	**MMSYSTMM2**
Milliflex Oasis® filtration head	**MMHEADMM2**
Milliflex Oasis® filtration head for use with EZ-Fit® filtration units	**MMHEADEZ1**
Milliflex Oasis® 100 mL funnel, 0.45 µm, MCE membrane	**MMHAWG124**
EZ-Fit® filtration unit without absorbent pad	**EFHAW10MS**
Milliflex Oasis® Culture Media Plate TSA	**MMSMCTS48**
Milliflex Oasis® Culture Media Plate SDA	**MMSMCSD48**
NaCl peptone + LTH 1000 mL	**1464810006**
NaCl peptone + LTH 90 mL	**1465220006**
Tryptic Soy Broth Ready-to-use Bottles 100 mL	**1463570006**
Mossel Broth 100 mL	**1464630010**
Violet Red Bile Dextrose Agar Settle plate	**1460000020**
MacConkey Broth 100 mL	**1464610010**
MacConkey agar	**1460220020**
Cetrimide agar	**1460480020**
Mannitol Salt agar	**1460230020**
Rappaport Vassiliadis Medium Tubes 10 mL	**1461810020**
Xylose Lysine Deoxycholate (XLD) Agar - LI EP + USP 30 mL	**1460730020**

Table 2: Pump, filtration head and media used in this application study

Microorganism	WDCM	Cat. No. Vitroids™
Staphylococcus aureus ATCC© 6538	WDCM 00034	**VT000342-10EA**
Pseudomonas paraeruginosa ATCC© 9027	WDCM 00026	**VT000262-10EA**
Bacillus spizizenii ATCC© 6633	WDCM 00003	**VT000032-10EA**
Candida albicans ATCC© 10231	WDCM 00054	**VT000542-10EA**
Aspergillus brasiliensis ATCC© 16404	WDCM 00053	**VT000532-10EA**
Escherichia coli ATCC© 8739	WDCM 00012	**VT000122-10EA**
Salmonella enterica subsp. *enterica* NCTC 6017	WDCM 00029	**VT000292-10EA**

Table 3: Microorganism strains used in this study (**ATCC**© = American Type Culture Collection **www.atcc.org**; **NCTC** = National Collection of Type Cultures **www.culturecollections.org.uk**; **WDCM** = World Data Centre for Microorganisms a global microbial resources data centre, to improve access to microbial data).

Method

Pre-trials and final preparation of the test samples

Cannabis plants have long been known to contain cannabinoids with antimicrobial properties.[6] The high levels of cannabinoids and terpenoids in samples requires the use of rinsing solutions with neutralizers to avoid false negative results.

Different dilutions of the samples were tested for their filterability. The samples should also leave no visible oil residues on the filtration funnel.

Testing was performed on samples with different levels of contained tetrahydrocannabinol (THC) and cannabidiol (CBD), see **Table 1** above.

Preliminary tests show, best rinsing results were achieved when using sodium chloride peptone broth with added lecithin, histidine and Tween® 80 (LTH neutralizer solution).

The initial procedure for both part 1 and part 2 was as follows:

- 10 g of dried herbal material or cannabis extract were mixed with 90 mL of NaCl peptone + LTH solution to prepare a 1:10 dilution. Any further dilutions needed were prepared from this initial dilution.
- For the hemp, stomacher bags with filters were used for homogenization to minimize solid particles contained in the herbal extract to be carried over into the final filtration liquid.

Part 1 Evaluation of the microbial load of the examined medical cannabis samples according to EP 2.6.12

For the determination of TAMC and TYMC, Milliflex Oasis® 100 mL filtration funnels in combination with the Milliflex Oasis® culture media plates TSA (Trypticase Soy Agar) and SDA (Sabouraud-Dextrose-Agar), respectively, were used.

Part 2 Suitability of the membrane filtration method for microbial testing according to European Pharmacopoeia's guidance

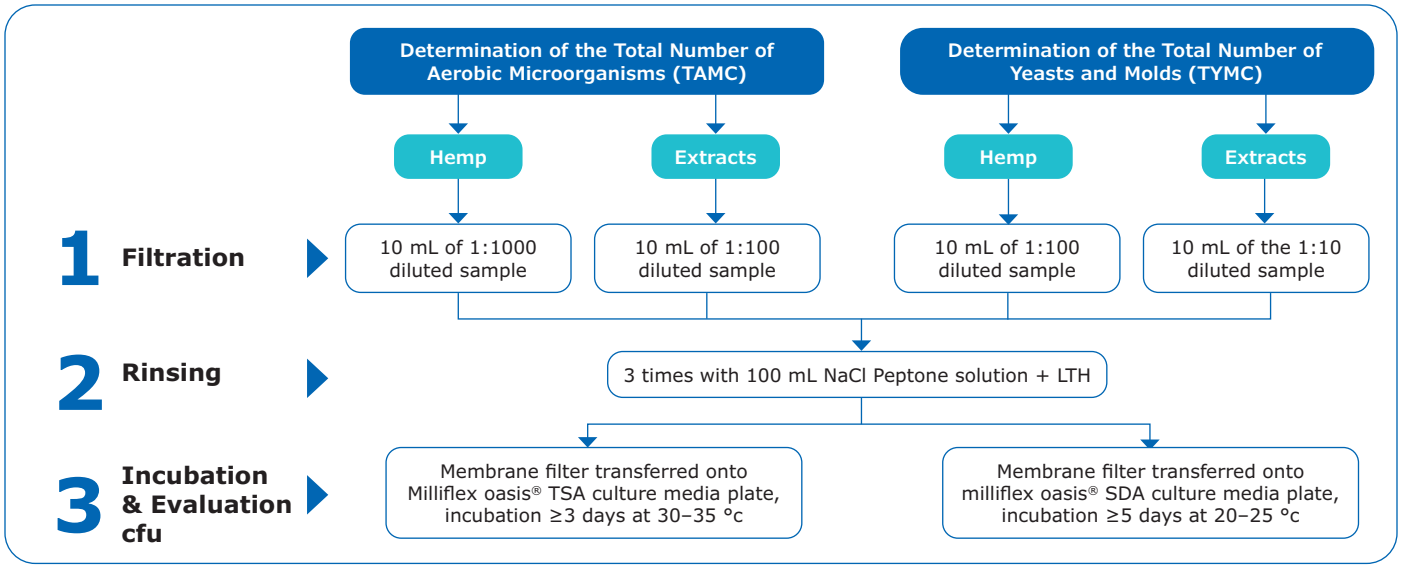

Image 1: Procedure of TAMC and TYMC determination

To determine if the filtration method can detect the pathogens listed in EP 2.6.13 and 2.6.31 in the presence of sample material, inocula of the reference microorganisms were added to the rinsing solution used for the final rinsing step.

The suitability of the test method is necessary to compare recovery rates of inoculated samples that contain hemp or extract with inoculated references that do not contain hemp or extract. In all cases, the inoculum was added into the rinsing solution used for the third and final rinsing step. The different test strains used are listed in **table 1**.

The test methods for TAMC and TYMC determination are described in Part 1 (see above).

The various test procedures to qualify the pathogens for which Ph. Eur. requires tests using selective media are described below. To incubate the membrane filter in liquid media, EZ-Fit® filtration units were used (as opposed to the Milliflex Oasis® filtration funnels used for part 1, but in combination with the Milliflex Oasis® filtration pump), because the EZ-Fit® filtration unit offers the possibility to manually transfer the membrane filter, which is not sealed at the base of the device.

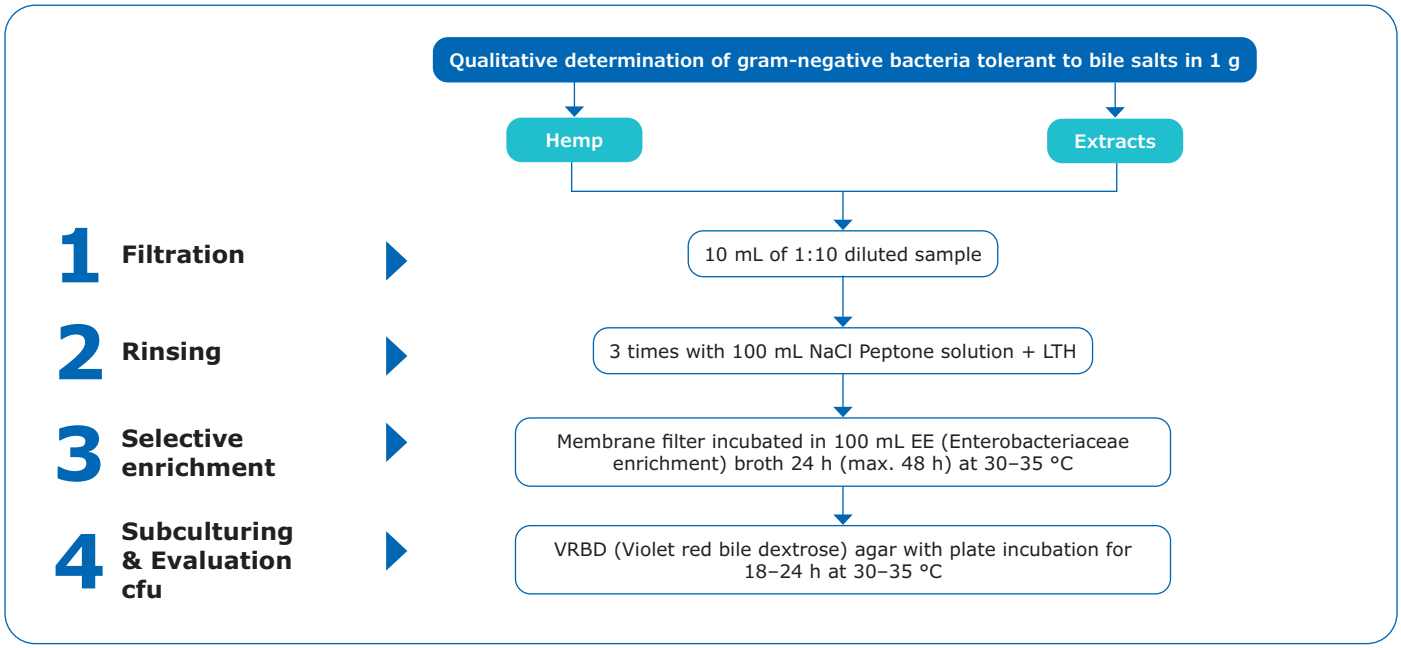

Image 2: Procedure of the qualitative determination of gram-negative bacteria tolerant to bile salts in 1 g

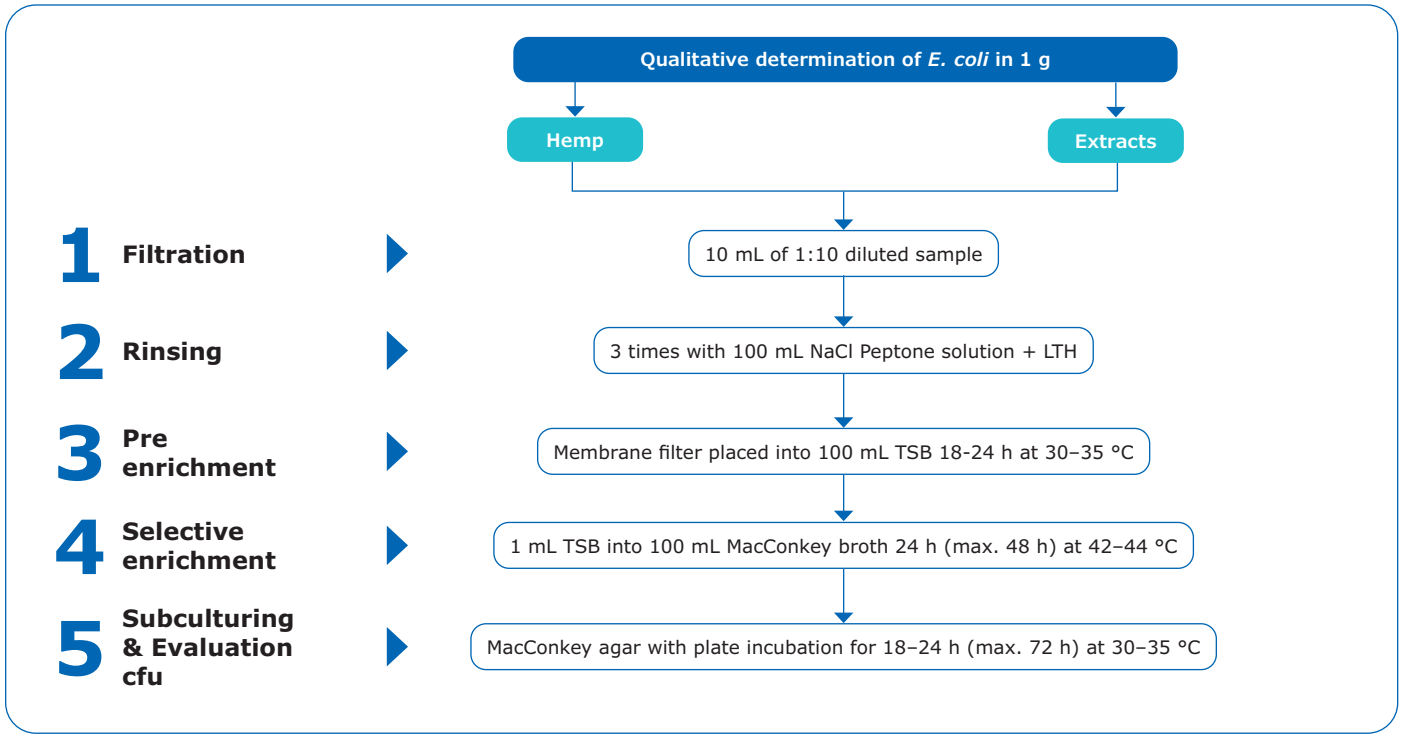

Image 3: Procedure of the qualitative determination of *Escherichia coli* in 1 g

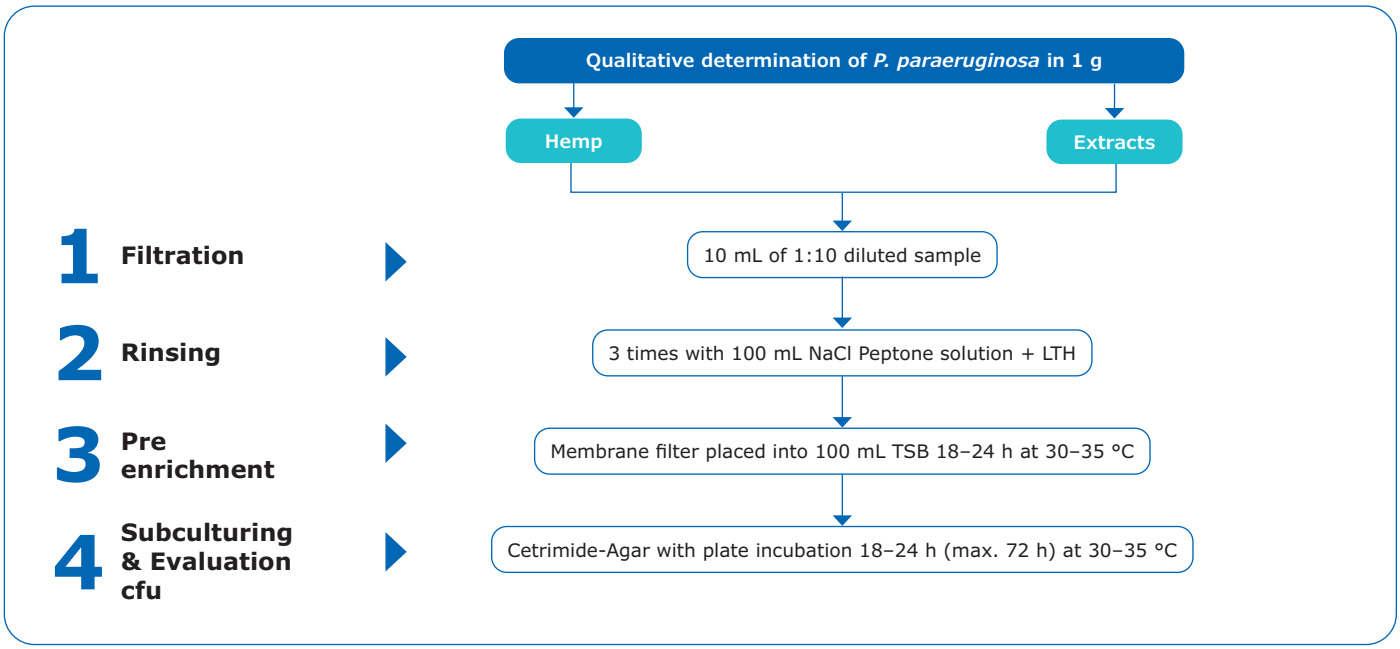

Image 4: Procedure of the qualitative determination of *Pseudomonas paraeruginosa* in 1 g

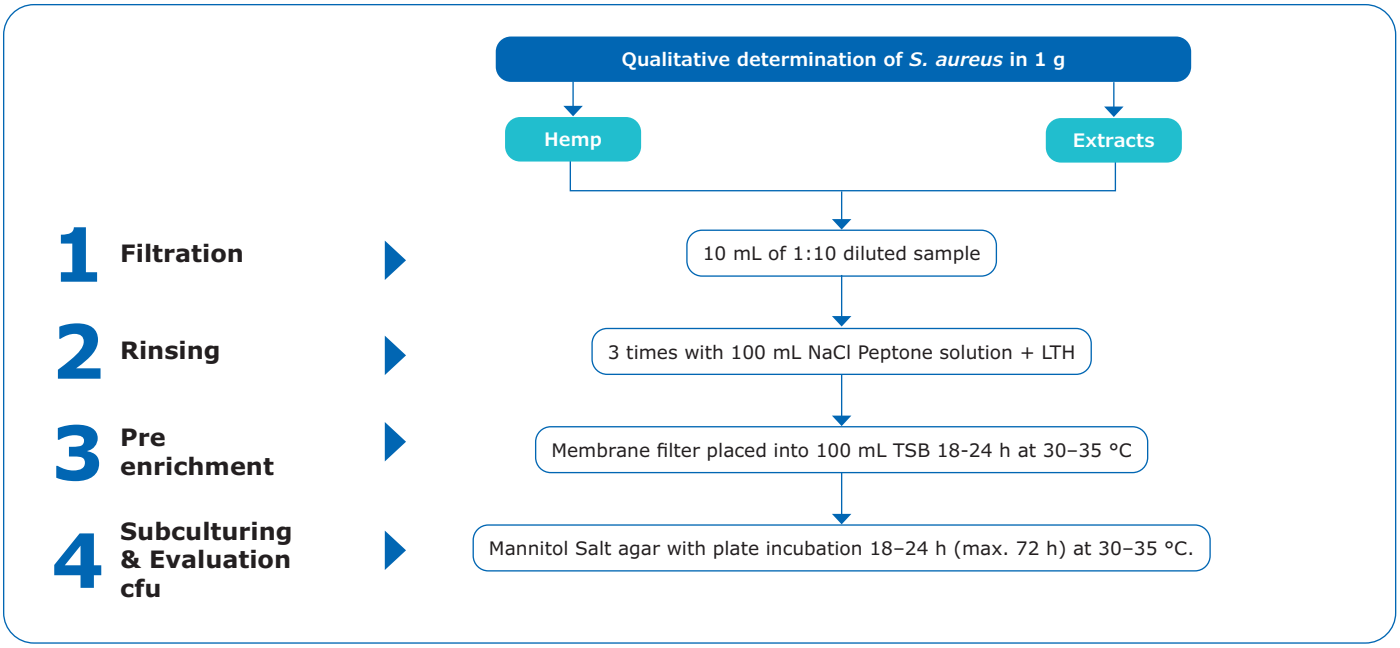

Image 5: Procedure of the qualitative determination of *Staphylococcus aureus* in 1 g

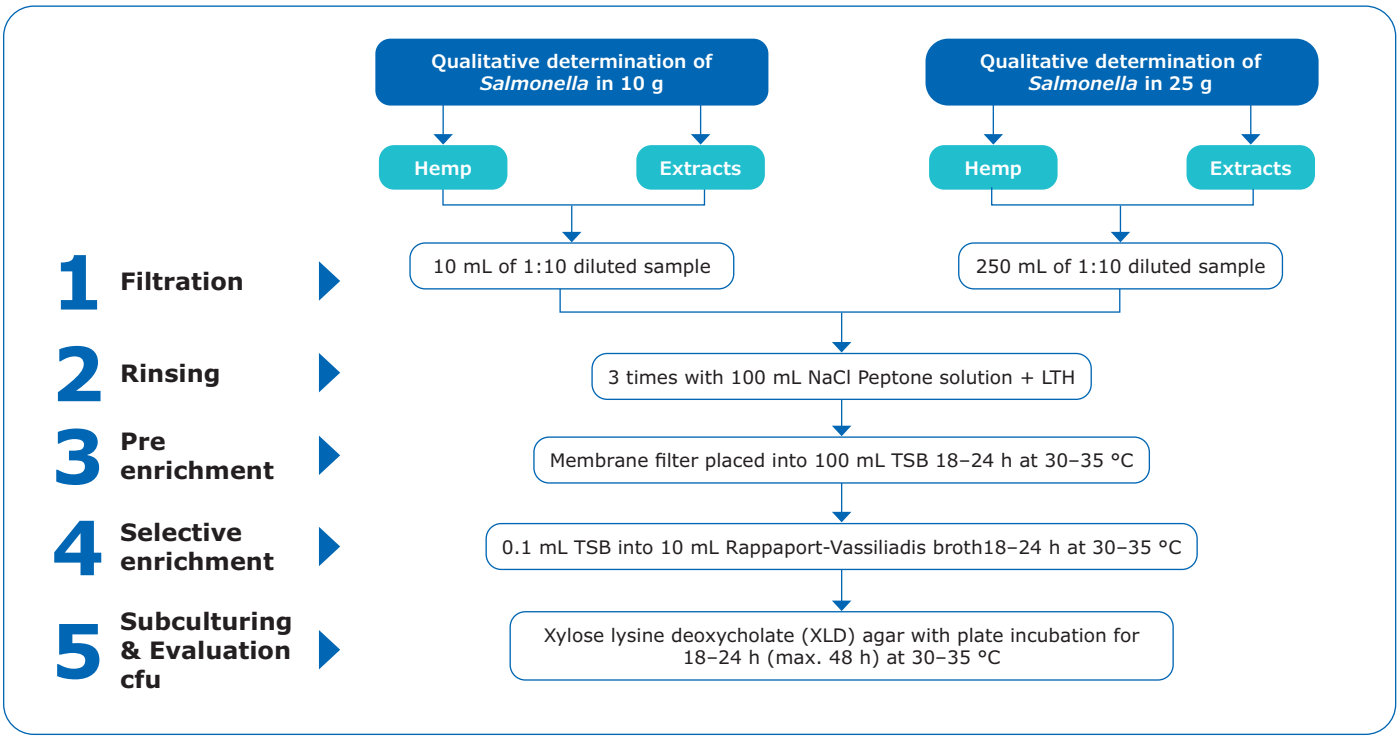

Image 6: Procedure of the qualitative determination of *Salmonella* in 10 g and 25 g

Results

Part 1 Determination of the microbial load of medical cannabis samples according to EP 2.6.12

The filtration volume of 10 mL required to determine the Total Aerobic Microbial Count (TAMC) and the Total Combined Yeast And Mold Count (TYMC) of the hemp and extract samples passed easily through the Milliflex Oasis® filtration funnels, so TAMC and TYMC determination could be performed successfully for all samples. **Table 4** summarizes the outcome of this test panel method.

Results	Hemp (cfu)			Extracts (cfu)		
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
TAMC/g	<100	<100	<100	<10	<10	<10
TYMC/g	<10	<10	<10	<1	<1	<1

Table 4: Microbial load of the medical cannabis hemps and extracts (in cfu per gram of original sample)

Part 2 Suitability of the membrane filtration method for microbial testing according to European Pharmacopoeia (quantitative and qualitative tests)

For the tests to determine if the filtration method can detect the pathogens listed in EP 2.6.13 and 2.6.31 in the presence of sample material, inocula of the reference microorganisms were added to the rinsing solution used for the final rinsing step (**Table 3:** list of microorganisms used). Controls not containing any sample material were also inoculated and the results compared. If found to be suitable, the filtration test method can be applied to detect microbial contamination in hemp and extract samples according to the European Pharmacopoeia.

Table 5 summarizes the quantitative TAMC and TYMC test results for the hemps and **table 6** for the cannabis extracts on the media TSA and SDA, respectively.

European Pharmacopoeia 2.6.12 chapter 4-6 stipulates that the mean count of any of the test organisms must not differ by a factor greater than 2 from the value in absence of the product.

Results	Hemps (CFU)			Control (w/o sample; CFU)	CFU range for test to pass (factor 2)	Test result
	Sample 1	Sample 2	Sample 3			
TAMC (10 mL of 1:1000 dilution) – TSA 3 days at 30–35 °C						
P. paraeruginosa	24	20	19	28	14–56	Passed
S. aureus	20	21	31	22	11–44	Passed
B. spizizenii	35	36	29	39	20–78	Passed
C. albicans	26	31	32	28	14–56	Passed
A. brasiliensis	40	44	39	39	20–78	Passed
TYMC (10 mL of 1:100 dilution) – SDA 5 days at 20–25 °C						
C. albicans	29	30	30	29	15–58	Passed
A. brasiliensis	39	47	34	44	22–88	Passed

Table 5: TAMC and TYMC results of hemp samples using the membrane filtration method

Results	Extract (CFU)			Control (w/o sample; CFU)	CFU range for test to pass (factor 2)	Test result
	Sample 4	Sample 5	Sample 6			
TAMC (10 mL of 1:100 dilution) – TSA 3 days at 30–35 °C						
P. paraeruginosa	25	22	25	21	11 - 42	Passed
S. aureus	25	25	18	22	11 - 44	Passed
B. spizizenii	56	50	53	52	26 - 104	Passed
C. albicans	20	14	13	22	11 - 44	Passed
A. brasiliensis	49	50	54	55	28 - 110	Passed
TYMC (10 mL of 1:10 dilution) – SDA 5 days at 20–25 °C						
C. albicans	19	14	20	21	11 - 42	Passed
A. brasiliensis	45	42	42	48	24 - 96	Passed

Table 6: TAMC and TYMC results of cannabis extract samples using the membrane filtration method

Tables 5 and **6** show that the number of CFU for each reference microorganism in the presence of the product did not differ by a factor of more than 2 from the value obtained for the reference control solutions, which contained no sample. Therefore, the requirements for the determination of the total number of aerobic microorganisms and for the determination of the total number of yeasts and molds according to European Pharmacopoeia 2.6.12 were fulfilled for all the hemps (samples 1 to 3) and extracts (samples 4 to 6).

The results of the qualitative test to ascertain the suitability of the membrane filtration method to detect the specific pathogens stated in EP after subculturing on selective media, based on the specified original amount (in grams) of the three hemp samples, are presented in **table 7**.

Results	Hemps (microbial growth observed)			Control (w/o sample; growth)	Test result
	Sample 1	Sample 2	Sample 3		
Qualitative determination of gram-negative bacteria tolerant to bile salts in 1 g					
E. coli	Positive	Positive	Positive	Positive	Passed
P. paraeruginosa	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *E. coli* in 1 g					
E. coli	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *P. aeruginosa* in 1 g					
P. paraeruginosa	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *S. aureus* in 1 g					
S. aureus	Positive	Positive	Negative in 100 mL of TSB, Positive In 1000 mL In TSB	Positive	Passed
Qualitative determination of *Salmonella* in 10 g					Membrane clogged
Qualitative determination of *Salmonella* in 25 g					Membrane clogged
Qualitative determination of *Salmonella* in 1 g*					
Salmonella enterica* ssp. *enterica	Positive	Positive	Positive	Positive	Passed

Table 7: Test results to determine the suitability of the membrane filtration method to detect specified pathogens in the hemp samples 1 to 3 on selective media. *Test for *Salmonella* in 1 g of sample is not specified in the European Pharmacopoeia. 10 mL of the 1:10 diluted sample were filtered for all strains. For *Salmonella*, 100 mL and 250 mL were initially filtered.

The qualitative determination of gram-negative bacteria tolerant to bile salts, of *E. coli*, of *P. paraeruginosa* and of *S. aureus* was possible and thus the Pharmacopoeia requirements fulfilled.

For one product of the medicinal cannabis hemps (sample 3), no growth of the reference microorganism *S. aureus* could be observed for 1 g of the product after initial insertion of the membrane filter in 100 mL of TSB. This test was repeated but with the membrane filter placed into 1000 mL of TSB instead of 100 mL, upon which growth was observed after sub cultivation on the selective medium. Further tests with a change of the filtration protocol were not conducted, as incubation in 1000 mL in TSB led to successful detection.

For the qualitative determination of *Salmonella*, Ph. Eur. regulation requires filtration of 100 mL (equivalent to 10 g of sample) or 250 mL (equivalent to 25 g of sample). In both cases it was observed that herbal particles contained in the sample clogged the membrane filters, and the full volumes could not be filtered. To evaluate if *Salmonella* can be detected in principle, it was decided to filtrate only 10 mL (equivalent to 1 g of product). In this case, the requirement of *Salmonella* detection was fulfilled.

The cannabis extract results of the qualitative test to determine the suitability of the membrane filtration method to detect specific pathogens using the selective media are presented in **table 8**.

Results	Extracts (microbial growth observed)			Control (w/o sample; growth)	Test result
	Sample 4	Sample 5	Sample 6		
Qualitative determination of gram-negative bacteria tolerant to bile salts in 1 g					
E. coli	Positive	Positive	Positive	Positive	Passed
P. paraeruginosa	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *E. coli* in 1 g					
E. coli	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *P. aeruginosa* in 1 g					
P. paraeruginosa	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *S. aureus* in 1 g					
S. aureus	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *Salmonella* in 10 g					
Salmonella enterica* ssp. *enterica	Positive	Positive	Positive	Positive	Passed
Qualitative determination of *Salmonella* in 25 g					Membrane clogged

Table 8: Results to determine the suitability of the membrane filtration method to detect specified pathogens in the extract samples 4 to 6 on selective media.

The EP requirement to detect reference pathogens in specified amounts of the extract samples (4 to 6; see THC and CBD levels in **table 1**) on selective media was generally successful for all strains. Although the highest volume of 250 mL for the qualitative determination of *Salmonella* was not fully filterable due to membrane clogging, filtration succeeded with a lower filtration volume of 100 mL (equivalent of 10 g product). This proves that in principle *Salmonella* in the extract samples can be detected according to Ph. Eur. requirements.

Conclusion

Three different hemp and three different cannabis extract samples were used to evaluate the suitability of the membrane filtration method for microbial testing of medical cannabis (in both herbal and extract format) according to Ph. Eur. chapters 2.6.12, 2.6.13 and 2.6.31.

The objective was to find the right sample dilution and a suitable rinsing liquid to remove inhibitors from the sample that could inhibit the microorganism growth, increasing the risk of false negatives. Dilutions from 1:10 to 1:1,000, and using Sodium Chloride Peptone broth with added lecithin, histidine and Tween® 80 (LTH neutralizer solution) as the rinsing solution achieved the best results.

In this study, we determined the microbial load of the six medical cannabis samples and evaluated the suitability of the membrane filtration method for testing according to European Pharmacopoeia, which involved qualitative and quantitative tests using a panel of pathogens as stated in EP chapters 2.6.13 and 2.6.31.

The overall results showed that the Milliflex Oasis® filtration system, in combination with Milliflex Oasis® funnels and EZ-Fit® funnels, is suitable for microbiological testing according to Ph. Eur. of medical cannabis in both its herbal form and as an extract solution.

For the detection of *Salmonella*, the EP requirement of significantly higher volumes of 100 mL and 250 mL from the tested cannabis hemp clogged the membrane filter due to particles contained in the plant material, but the observation that *Salmonella* could be detected when 10 mL (equivalent of 1 g product) was filtered demonstrated that, in general, these pathogens can be detected via membrane filtration according to Ph. Eur.

For the extracts, membrane clogging was observed if 250 mL were filtered, but no negative filtration issues were observed for a volume of 100 mL. It should be noted that cannabis, as a part of the herbal product family, is well known to show marked batch-to-batch differences regarding filterability and required microbial testing conditions. Depending on the physical properties of products, the European Pharmacopoeia requires a suitable dilution medium and rinsing solution to be determined.

Literature

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9. BAV INSTITUT GmbH, A Tentamus Company, in Germany (Hanns-Martin-Schleyer-Str. 2577656 Offenburg)

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MK_AN14742EN Ver. 1.0
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09/2025