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Uncovering the Quality Deficiencies with Potentially Harmful Effects in Substandard and Falsified PDE-5 Inhibitors Seized by Belgian Controlling Agencies

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Abstract: Illicit PDE-5 inhibitors are frequently encountered by regulatory agencies. Self-medicating with substandard and falsified (SF) PDE-5 inhibitors could be dangerous as they are likely taken without any medical supervision and might be of poor quality which could result in adverse reactions. In order to provide an overview of the quality deficiencies present in recently seized illicit PDE-5 samples that may pose health risks, we set out to identify the products' different chemical and/or biological risks. Our results indicate that 38% of the samples harbored a chemical risk including the significant exceedance of the maximum recommended dosage, a large heterogeneity in API content between the different tablets in the same package or blister and the presence of only 40% of the claimed dosage. Moreover, our results also demonstrate that 16 of the 32 samples were not compliant with the internationally set microbiological quality standards. Startlingly, two samples were severely contaminated with potentially pathogenic bacteria, which could result in a gastrointestinal illness upon oral intake.

Keywords: mass spectrometry; microbiology; PDE-5 inhibitors; substandard drugs

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1. Introduction

The global trade in illicit pharmaceuticals still remains a profitable crime area, despite the national efforts made by regulatory agencies and joint international actions, including the yearly Pangea operations [1]. In 2022, Operation Pangea XV, encompassing 94 Interpol member countries representing every continent, resulted in seizures of illicit medical products with an estimated value of around USD 11 million [2]. Moreover, incidents involving pharmaceutical crime increased by almost 65% over the last five years, impacting at least 142 countries and threatening the health and safety of patients and unaware consumers around the world [3]. Therefore, in order to come to a global uniform definition of the problem, the World Health Organization (WHO) has adopted the term "substandard and falsified (SF) medical products" to represent three mutually exclusive classes, namely substandard medical products, unregistered or unlicensed medical products and falsified medical products [4]. Substandard medicinal products are also called "out of specification" products, implying that these products are manufactured by regular companies; however, due to quality deficiencies, these items should be discontinued and destroyed. Fraudulent practices, including theft, is the main reason why such products can enter the regular market [1,4]. Unregistered or unlicensed medicines refer to products that are not approved for marketing by the national medicine regulatory authority of the market they are sold in.

Finally, falsified products are products that deliberately or fraudulently misrepresent their identity, composition or origin. Major issues with these SF medicines are the presence of only inactive ingredients, wrong ingredients or improper dosages [4].

For several decades, the falsifications of blockbuster drugs have been encountered on a worldwide scale but with regional differences in the products targeted by criminals and criminal organizations. Examples of these are the high occurrence of lifesaving medicines like antibiotics and antimalarial products in African and Asian countries [1,4–11]. Meanwhile, in Western countries, although there is prevalence of substandard and falsified lifesaving medicines, they are encountered less frequently. The focus in the west is directed toward lifestyle medicines like sexual performance enhancers, sport performance enhancers and weight-loss enhancers [12]. Based on the scientific literature, the major health risks associated with the use of these SF medicinal products are supplemented by the fact that they are not produced in accordance with the good manufacturing practices (GMP). This may result in them containing only inactive ingredients, wrong ingredients, an improper amount of an active pharmaceutical ingredient (API) or potentially toxic impurities and might also be contaminated with potentially pathogenic microorganisms [1,4–9,13–28]. Nevertheless, rogue online pharmacies still promote the use of unregistered or unlicensed medicinal products by distinguishing their product from "traditional" falsified products and their associated bad reputation. They claim that the product is being produced in genuine pharmaceutical companies and thus create the illusion of a safe but cheaper product.

In Europe, sexual-performance-enhancing SF medicinal products have been quite popular since the last decade, as illustrated by the many entries in the Know-X database that has been put in place by the European Directorate for the Quality of Medicines and HealthCare (EDQM) [29]. Likely, these products are purchased as a cheaper, easily accessible alternative to genuine products, and there is no requirement for a doctor's prescription for their purchase and thus no coincidental potential embarrassment [12]. In order to assess the online claims of these rogue online pharmacies and their potential threat to public health, we set out to perform a mapping of the potentially dangerous chemicals and biological risks, encountered in 32 real-life substandard or falsified PDE-5 inhibitors. These samples were intercepted by regulatory agencies in the period 2021–2022 and include mainly unlicensed and unregistered medicines or falsifications thereof.

2. Materials and Methods

Acetonitrile and formic acid, both MS-grade, were purchased from Biosolve (Valkenswaard, The Netherlands). Water was obtained using a milliQ-Gradient A10 system (Millipore, Billerica, MA, USA). Reference standards utilized for quantification purposes of sildenafil (sildenafil citrate, purity 98.7%), tadalafil (99.6% purity) and dapoxetine (dapoxetine hydrochloride, 99.4% purity) originated from Pfizer (New York, NY, USA), Dr. Ehrenstorfer™ (Augsburg, Germany) and Merck (Darmstadt, Germany), respectively. Chloroform (for gas chromatography), benzene, carbon tetrachloride (CCl4) (for spectroscopy) and ethylbenzene (for gas chromatography) were purchased from Merck (Darmstadt, Germany), and toluene (pesticide residues) and cyclohexane from VWR prolabo (VWR International, Fontenay-sous-Bois, France) were used as reference standards for analysis for residual solvents. Dimethyl Sulfoxide (DMSO), used as solvent for the samples, was purchased from Merck. HPLC-grade 2-propanol, acetone and dichloromethane and pesti-S-grade ethylacetate, used to quantify the detected residual solvents, were also purchased from Biosolve (Valkenswaard, The Netherlands).

Sabouraud dextrose agar with neutralizers (lecithin, tween and histidine) especially used for fungal organisms, trypto-casein-soy agar with neutralizers (lecithin, tween, histidine and thiosulfate), violet red bile glucose agar and buffered sodium chloride peptone with neutralizers (lecithin, tween, histidine and thiosulfate) were purchased from Merck (Darmstadt, Germany). EZ-Accu Shot™ pellets of the bacterial reference strains of *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* subp. *aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Salmonella enterica* subsp. *enterica* serotype Abony (NCTC 6017)

and *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633) and fungal strains of *Candida albicans* (ATCC 26790) and *Aspergillus brasiliensis* (ATCC 16404), purchased from Microbiologics (Saint Cloud, MA, USA), were used as positive controls to ensure proper growth conditions. Sterile analytical filter units 0.45 µm Nalgene™, used for the membrane filtration during the sterility test, were purchased from Thermo-Fisher Scientific (Rochester, NY, USA).

All samples were screened for the presence of synthetic drugs or medicines by both GC-MS and LC-MS2, according to the methodology described by Vanhee et al., 2018 [30]. Briefly, 30 mg of the ground tablet mixture was solubilized in 10 mL of methanol, sonicated for 15 min, and the solution was filtered through a 0.2 µm polytetrafluoroethylene (PTFE) filter prior to analysis by GC-MS and LC-MS2. Alternatively, when dealing with gel-like formulations, these samples were also solubilized in dichloromethane (1/10 dilution) prior to GC-MS injections. The GC-MS analyses were performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass detector. Full automation was achieved using Agilent MassHunter data acquisition and MassHunter qualitative analysis version 10.0 e (Santa Clara, CA, USA). Injections were made in pulsed splitless mode, with an injection port temperature of 250 °C. The solvents were separated in an Agilent Column DB-5MS (40 m × 0.25 mm × 0.25 µm film thickness) + 10 m EZ-guard which was purchased from Agilent technologies (Santa Clara, CA, USA). The column oven temperature was initially set to 80 °C for 2 min and then raised at a rate of 15 °C/min to 280 °C and held for 17 min, followed by a raise of 10 °C/min to 310 °C and held for 20 min. The total run time was 55 min. High-purity helium was used as the carrier gas with flow rate 1 mL/min. The MS was operated in electron ionization with electron energy of 70 eV. Data were acquired in full-scan mode with m/z ranging from 43 to 500. The MS data were analyzed by MassHunter, and spectra were compared to different libraries, including NIST20 mass spectral library and the Cayman spectral library. A 0.30 Da precursor tolerance for MS spectra was allowed, and at least a matching score of 85% was required to be considered a possible hit. The LC-MS analyses were performed on a Dionex UltiMate 3000 Rapid Separation LC (RSLC) system (Thermo Scientific, Sunnyvale, CA, USA) coupled to an amaZon™ speed ETD mass spectrometer (Bruker Daltonics, Bremen, Germany). The instrument system was calibrated using the manufacturer's calibration mixture, and the mass accuracy was determined to be <0.1 Da during the period of analysis. A sample volume of 1 µL was injected onto the system. The chromatographic separation was performed at 45 °C on an Acquity™ UPLC BEH C18 Column (150 × 2.1 mm, 1.7 µm particle size) (Waters, Milford, MA, USA) with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A general LC method, suitable for rapid screening of suspected samples was used. A linear gradient from 1% B to 99% B was accomplished in 9 min, followed by an isocratic elution for 2 min and 2 min at 1% B. The flow rate was 0.5 mL/min. The mass spectrometer was operated in alternating positive electrospray ionization (ESI+) and negative electrospray ionization (ESI-) mode, with respective spray voltage of 4.5 kV (ESI+) or 3.5 kV (ESI-) and end plate voltage 500 V. MS spectra were obtained within a mass range of 100–1000 m/z, and the smart parameter setting (SPS) was set to 475 m/z. For the MS2 precursor selection, the most intense ion was isolated (including singly charged ions) above the absolute intensity of 2500 and 5% relative intensity threshold. CID (collision-induced dissociation) was performed using helium as collision gas. The LC and MS data were analyzed by Compass Data Analysis 4.2 (Bruker Daltonics, Bremen, Germany), and the LC-MS/MS spectra were compared to different libraries, including the homemade library, enclosing a total of about 5000 MS and MS2 spectra. A 0.30 Da precursor tolerance for MS spectra and MS2 spectra was allowed.

The amounts of sildenafil and tadalafil were determined based on a previously validated methodology described in Sacré et al., 2011 [31]. Briefly, all analyses were performed on a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA) including a binary solvent manager, sampler manager-flow through needle, column heater and photo-diode array (PDA) detector connected to Waters Empower 3.7.0 data station. The chromatographic separation was performed at 45 °C on an Acquity™ UPLC BEH Shield RP18 Column

(100 × 2.1 mm, 1.7 µm particle size) (Waters, Milford, MA, USA) with a mobile phase consisting of 10 mM ammonium formate, pH 3.5 (A) and acetonitrile (B). The gradient started with 25% B to 35% B in 2.5 min, followed by an increase to 45% B the next minute and a final increase to 70% B at 3.8 min. Next, an isocratic elution took place for 0.7 min, and the next 0.5 min was utilized to return to the initial conditions. These conditions were also utilized to quantify the amount of dapoxetine present in one sample. A quantification was performed on 2 separate units (tablet or liquid bag) and a mixture of different units (minimum 3 units present in the mixture).

For the analysis of residual solvents, the samples were injected on a GC-MS system using a 7890B headspace sampler (Agilent Technologies, Palo Alto, CA, USA). The analyses were performed on an Agilent 7890B gas chromatograph coupled to an Agilent 7000C Quadrupole MS/MS EI system (Santa Clara, CA, USA). Full automation was achieved using Agilent MassHunter data acquisition and MassHunter qualitative analysis version 10.0. After incubation of the sample (1 mL in a 10 mL headspace vial) at 85 °C for 10 min, during which it was shaken, 1 mL of the vapor phase was injected into the GC/MS system in a split-injection mode (split ratio 16.2:1). The temperatures of the headspace loop, the transfer line and the EPC volatile interface were 95, 105 and 160 °C, respectively. The solvents were separated on a Phenomenex 624 capillary column (60 m × 0.32 mm; 1.8 µm film thickness) (Phenomenex, Torrance, CA, USA). The oven temperature was programmed from 40 °C (held for 20 min) to 240 °C at 10 °C/min; 240 °C was held for 20 min. The total run time was 60 min. The temperatures of the injection port, the ion source, the quadrupole and the interface were set at 160, 230, 150 and 280 °C, respectively. For the identification of the solvents present in the samples, the mass spectrometer was operated in full-scan mode while for quantification, the mass spectrometer was operated in SIM mode [15]. Starting from the stock solutions, dilutions were prepared in dimethyl sulfoxide (DMSO), a solvent also used successfully previously for the analysis of residual solvents in falsified PDE-5 inhibitors [15]. The solutions were brought in vials and automatically sealed. Dimethyl sulfoxide was used as blank. For the analysis of tablets, the tablets were broken in two before addition of 1 mL of DMSO. Samples were prepared in duplicate and also injected in duplicate. A one-point calibration was performed in the linear range, established for the encountered residual solvents.

Bioburden testing aims to count aerobic microorganisms that are possibly present in pharmaceutical preparations. These microorganisms are grown to visible colonies, and it is assumed that a colony is formed from one colony-forming unit (CFU). Bioburden testing was performed based on Ph. Eur. using the membrane filtration method [29]. Briefly, the sterile ground sample is solubilized in buffered peptone with neutralizers, applied to a membrane filter, washed at least two times with phosphate buffer saline (PBS) solution and subsequently placed on top of either Sabouraud dextrose agar with neutralizers or trypto-casein-soy agar with neutralizers to determine the total yeast and mold count (TYMC) and the total aerobic microbial count (TAMC). Additionally, the filter was also incubated on violet red bile glucose agar, a medium that promotes the growth of bile-tolerant bacteria and enterobacteria. Negative controls were performed on culture media with buffered peptone solution, used to solubilize the samples, PBS solution (used to rinse the samples on the membrane) and the membrane filters. Positive controls, EZ-Accu Shot™ pellets (Microbiologics, Saint Cloud, MA, USA) of the different reference strains, were also used to demonstrate that the proper material and growth conditions were used.

The bacteria detected in the positive samples were isolated on tryptone-casein-soy agar and subsequently analyzed via matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) by means of the MALDI Biotyper® as described previously in Janvier et al. [27]. The data generated were processed using the MALDI Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany). Only hits with log(score) values equal or higher than 2.00 were considered as a high-confidence identification at species level. In case of no hits with a sufficient log(score), hits with log(score) values between 1.70 and 2.00 were reported on genus level (in case of *Bacillus* spp.). The fungi encountered in

the samples were also isolated on Sabouraud medium prior to fungal material extraction and subsequent analysis by MALDI-TOF [28]. Also, here, hits with log(score) values equal or higher than 2.00 were considered as a high-confidence identification at the species level.

In case of a high-confidence identification as *Bacillus cereus s.l.*, a little of biomass was transferred to grow on Columbia blood agar (Oxoid, Thermofisher Diagnostics, Erembodegem, Belgium) for 24 h at 30 °C to ensure that the cells were in their vegetative state. Afterward, a colony was sub-cultured overnight in BHI broth (BIO-RAD, Temse, Belgium) at 30 °C. Before DNA extraction, 2 mL of the brain heart infusion (BHI) culture was resuspended in 400 µL of a 25 ng/µL lysozyme (Merck, Overijse, Belgium) in TE buffer (pH 8, Invitrogen, ThermoFisher, Rochester, NY, USA) solution and incubated for 30 min at 37 °C to facilitate the degradation of its cell wall. The DNA was extracted using the Maxwell RSC Cultured Cells kit (Promega, Leiden, The Netherlands) following the manufacturer's instruction. The concentration of the DNA extract was estimated using a Nanodrop 1000 device (ThermoFisher, Rochester, NY, USA) and stored at −20 °C before being sent to Eurofins Genomics GmbH for DNA sequencing on the Illumina NovaSeq platform (NovaSeq 6000 S4 PE150 XP, Illumina, San Diego, CA, USA). The raw fastq sequencing reads were trimmed using Trimmomatic v.0.38 before de novo assembly using SPAdes v.3.15.4 with the isolate option enabled. The parameter cov-cutoff was set to 10.0, and –k was set to Auto [32,33]. The species of the isolates was determined using fastANI v.1.33 with default parameters and genomes of 13 reference strains of the *B. cereus sensu lato* clade [34]. The highest average nucleotide identity (ANI) score was used for species determination; a minimal ANI of 95% is required [35]. The presence of virulence genes was determined using BTyper3 v.3.3.4; plasmid detection was performed using PlasmidFinder [35,36].

The suspected *Bacillus anthracis* strain, present in sample 14, was additionally tested by real-time PCR targeting *pagA* and *capC* genes [37]. PCR amplification was conducted in a 25 µL volume containing premixed PCR reagents (Roche, Basel, Switzerland), forward and reverse primers at 0.4 µM/each and double-dye probes at a 0.2 µM concentration. Amplification was performed on a LightCycler® 480 System (Roche, Basel, Switzerland) using the following condition: 15 min at 95 °C (1 cycle), followed by 20 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C (45 cycles). The phenotypical analysis was performed by testing for penicillin susceptibility, spreading the encountered strain on Polymyxin B—Lysozyme—EDTA—Thallous acetate Agar (PLET) medium and assessing susceptibility to *B. anthracis* phage preparations (gamma phage). Susceptibility testing for penicillin was performed according to CLSI M45 guidelines for potential bacterial agents of bioterrorism [38]. PLET agar plates were prepared by following instructions as described previously [39]. Briefly, heated Heart Infusion Agar solution was supplemented with polymyxine (30 UI/mL) (Oxoid, Hampshire, UK), lysozyme (40 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA), EDTA (300 µg/mL) (Thermo Fisher Scientific, Inc., UK), thaliumacetate (40 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and sheep blood (5%) (Oxoid, Thermo Fisher Scientific, Inc., Hampshire, UK) and poured in plates. *B. anthracis* will form small gray colonies while the growth of other bacteria will be suppressed or limited. Next, phage inoculation was conducted in blood agar plate by incubating 1 drop of the phage (in-house production) on inoculated plates. After one-night incubation at 37 °C, assessment of the growth of the tested strain was performed in parallel with that of the control strain.

3. Results

3.1. Physical Characteristics of the Samples

Thirty-two samples were seized by the Belgian Federal Agency for Medicine and Health Products (FAMHP) and subsequently sent to our Official Medicines Control Laboratory (OMCL) where pictures were taken upon arrival and the information available on the packaging or blister was recorded (see Table 1).

Table 1. Overview of the information available on the blister or package and description of the appearance of the different samples. Abbreviations: Mfr. = manufacturer; mfg. lic. no. = manufacturer's license number; N = not present; n.a. = not available; Y = present.

n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored
1	100 mg sildenafil	Y	Y	Y	1 October 2024	Quinoline yellow, brilliant blue and titanium dioxide	green	"KGR 100" and logo "ap" on other side	N
2	50 mg sildenafil	Y	Y	Y	1 July 2023	Quinoline yellow, brilliant blue and titanium dioxide	green	"KGR 50" and logo "ap" on other side	N
3	100 mg sildenafil 50 mg tadalafil	Y	N	N	1 July 2024	Red oxide iron, indigo caramine, Ponceau 4R and titanium dioxide	red	n.a.	Y
4	100 mg sildenafil 60 mg dapoxetine	Y	Y	N	1 September 2023	n.a.	green	n.a.	N

Table 1. *Cont.*

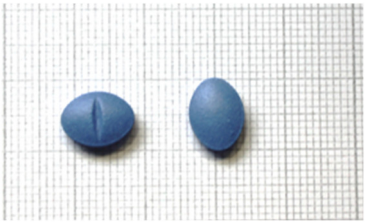
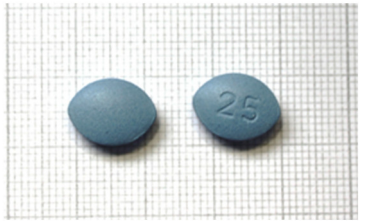
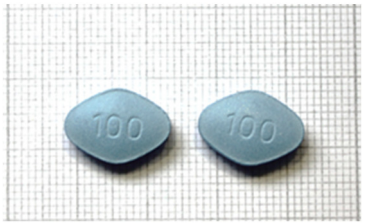
n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored	
5	50 mg sildenafil	Y	Y	Y	1 April 2024	Titanium dioxide and lake indigo carmine	blue	n.a.	Y	
6	200 mg sildenafil citrate	Y	Y	N	1 February 2023	n.a.	yellow	"200" on one side	N	
7	25 mg sildenafil	Y	N	N	1 March 2023	Indigo carmine and titanium dioxide	blue	"25" on one side	N	
8	100 mg sildenafil	Y	Y	N	1 April 2024	n.a.	blue	"100" on both sides	N	

Table 1. *Cont.*

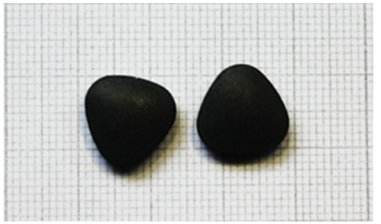
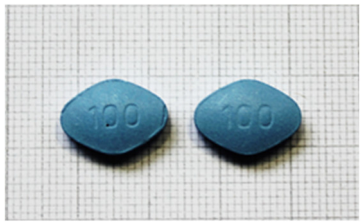
n°	Information on the Outer Packaging or Blister						Appearance of the Tablet		
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored	
9	200 mg sildenafil	Y	Y	Y	1 March 2024	Black oxide or iron	black	n.a.	N	
10	100 mg sildenafil	Y	Y	N	1 September 2024	n.a.	purple	"100" on one side and "F" on the other side	N	
11	100 mg sildenafil	Y	Y	Y	1 April 2024	Indigo carmine	blue	"100" on both sides	N	
12	100 mg sildenafil	Y	Y	N	1 October 2023	Brilliant blue and indigo carmine	blue	"100" on one side	N	

Table 1. *Cont.*

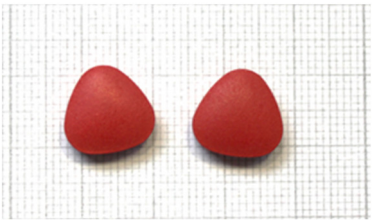
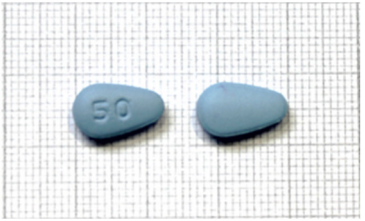
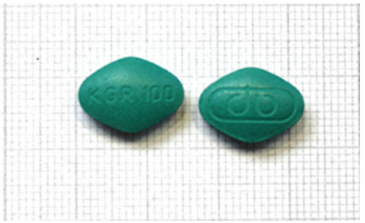
n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored	
13	150 mg sildenafil	Y	Y	Y	1 April 2024	Titanium dioxide, iron oxide red, lake of indigo carmine and lake of Ponceau 4R	red	n.a.	N	
14	120 mg sildenafil	Y	N	N	1 January 2024	n.a.	red	"120" on one side	N	
15	50 mg sildenafil	Y	N	N	1 December 2023	n.a.	blue	"50" on one side	N	
16	100 mg sildenafil	Y	Y	Y	1 December 2024	Quinoline yellow, brilliant blue and titanium dioxide	green	"KGR 100" and logo "ap" on other side	N	

Table 1. *Cont.*

n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored
17	100 mg sildenafil	Y	Y	Y	1 July 2023	Lake of sunset yellow, lake of quinoline yellow and erythrosine	coloring depends on the flavor	"KGR 100" and logo "ap" on other side	N
18	100 mg sildenafil	Y	Y	N	1 September 2023	n.a.	blue	"100" on both sides	N
19	200 mg sildenafil	Y	Y	y	1 April 2024	Black oxide or iron	black	n.a.	N
20	100 mg sildenafil	Y	Y	Y	1 June 2024	Quinoline yellow, sunset yellow, Ponceau 4R and brilliant blue	/	/	/

Table 1. *Cont.*

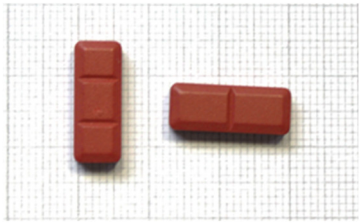
n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored	
21	100 mg sildenafil	Y	Y	N	1 January 2024	n.a.	/	/	/	
22	100 mg sildenafil 20 mg tadalafil	Y	N	N	1 September 2024	Red oxide iron, indigo caramine, Ponceau 4R and titanium dioxide	red	n.a.	Y	
23	100 mg sildenafil	Y	Y	N	1 January 2025	Indigo caramine and titanium dioxide	blue	“100” on one side	N	
24	100 mg sildenafil	Y	Y	N	1 November 2023	Indigo carmine	blue	“100” on one side	N	

Table 1. *Cont.*

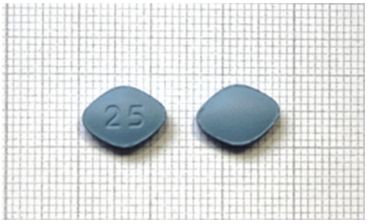
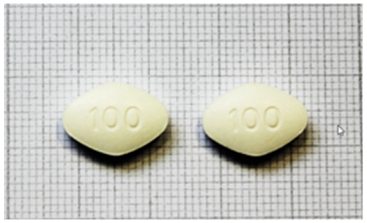
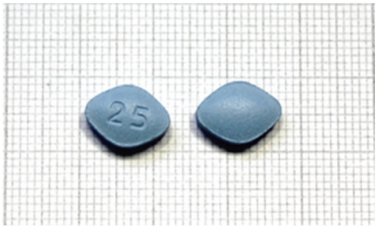
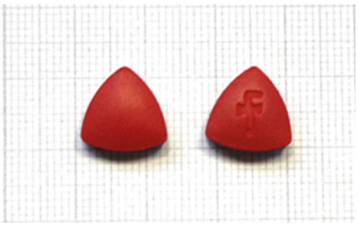
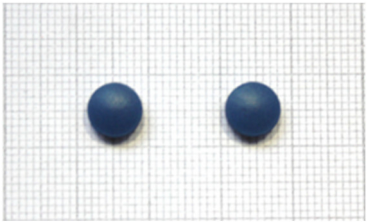
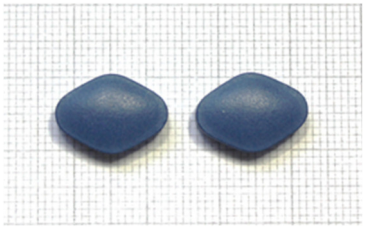
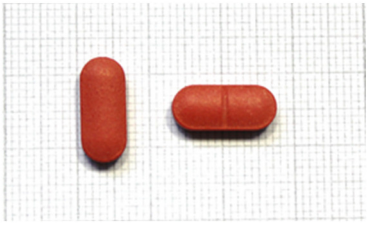
n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored	
25	25 mg sildenafil	Y	N	N	1 April 2024	Indigo carmine	blue	“25” on one side	N	
26	100 mg sildenafil	Y	Y	Y	1 August 2024	n.a.	white	“100” on both sides	N	
27	25 mg sildenafil	Y	Y	Y	1 October 2024	Indigo carmine	blue	“25” on one side	N	
28	not clear	Y	Y	Y	1 May 2023	n.a.	red	“F” on one side	N	

Table 1. *Cont.*

n°	Information on the Outer Packaging or Blister					Appearance of the Tablet			
	Quantity	Lot Number	Mfr.	Mfg. Lic. No.	Expiration Date	Colorants	Tablet Color	Inscription on the Tablet	Scored	
29	25 mg sildenafil	Y	N	N	1 September 2024	Indigo carmine	blue	n.a.	N	
30	100 mg sildenafil	Y	N	N	1 September 2024	Indigo carmine	blue	n.a.	N	
31	50 mg sildenafil	Y	N	N	1 May 2024	Not mentioned on the blister	blue	"50" on one side	N	
32	100 mg sildenafil	Y	Y	Y	1 August 2024	Ponceau 4R, sunset yellow, titanium dioxide (marked in French)	red	n.a.	Y	

All samples mentioned the API(s) that should be present in the product, the batch number and the expiration date on the packaging except one sample (sample 28) that did not clearly declare the amount of APIs it contained. Five samples (samples 6, 9, 13, 14, 19) mentioned the presence of sildenafil in a dosage that exceeded 100 mg, which is the highest recommended sildenafil dose for treating erectile dysfunction, in accordance with the outcome of the clinical trials. Moreover, two samples (sample 3 and 22) disclosed the presence of both sildenafil and tadalafil, a mixture that does not exist as a genuine medicine in Europe or the USA. Sample 22 claimed the presence of 50 mg tadalafil, which exceeds the highest recommended dose by a factor of 2.5. Additionally, sample 4 also disclosed the presence of a mixture of sildenafil 100 mg and dapoxetine 60 mg, a cocktail that is also not legally available in Belgium [40].

From those 32 samples, 22 samples mentioned that they were produced in India, 1 mentioned to be produced in Germany, and 9 samples did not reveal any information on the manufacturer. Less than half of the samples (14/32) mentioned a manufacturer license number. Moreover, the majority of the blisters or packaging also stated which colorants were used, occasionally with spelling mistakes (e.g., indigo caramine instead of the correct indigo carmine). The most popular colorants used, according to the declared information, were indigo carmine (mentioned 13 times) and titanium dioxide (mentioned 10 times). The other colorants, including brilliant blue, sunset yellow, quinolone yellow, Ponceau 4R, red oxide iron, black oxide iron and erythrosine, were mentioned less than six times (see Table 1). Almost half of the tablets (14/30) were blue, while six tablets were red, four had a green color (same "brand"), two samples had tablets that were black, one contained yellow tablets, one consisted of purple tablets, and one sample contained white tablets (see Table 2). Sample 17 contained tablets in four different colors, depending on the flavor.

3.2. Identification and Quantification of the Detected APIs

Based on the scientific literature, one of the major health risks associated with the use of SF medicinal products is the fact that they might contain an improper amount of the active pharmaceutical ingredient (API) or the wrong ingredients [5–14,16–22,26]. A serious incident with counterfeit PDE-5 inhibitors occurred in Singapore in 2008, where 150 non-diabetic patients suffered from drug-induced hypoglycemia due to four brands of sexual-enhancement drugs that were contaminated with glyburide [13]. Moreover, a study performed by Pullirsch et al. demonstrated that 92% (24/26) of their illegal PDE-5 inhibitor samples did not contain the labeled amount of the PDE-5 inhibitor, taking into account an acceptance criterion of ±10% of the labeled amount [26]. Additionally, 21 samples (81%) were underdosed, whereas 3 samples (12%) were about two-fold overdosed. Interestingly, 14 of the 26 samples (54%) also contained trace amounts of a second PDE-5 inhibitor. Therefore, we set out to screen for the presence of APIs, including non-PDE-5 inhibitors, and quantify the APIs encountered. This screening was performed as described by Vanhee et al., 2018 [30], and the encountered APIs are listed in Table 2. All samples contained the declared API while no other APIs could be detected in the samples. Next, the amount of sildenafil, tadalafil and dapoxetine was determined. A quantification was performed on two separate units (tablet, capsule or liquid bag) and a mixture of different units, as is routinely done by our OMCL laboratory for this type of sample.

From the results mentioned in Table 2, it can be concluded that 10 out of the 31 samples (sample 28 did not declare the amount of sildenafil present) contained an amount of the API that corresponded to a 95–105% concentration interval, taking into account the measurement uncertainty (see Figure 1). Additionally, 11 samples contained at least 90% of the declared dosage. In contrast to the study performed in 2014 by Pullirsch and colleagues [26], our results demonstrated that 68% of the analyzed samples did contain the labeled amount of the PDE-5 inhibitor taking into account an acceptance criterion of ±10% of the labeled amount. Moreover, eight of the remnant samples contained at least 80% of the declared dosage, encompassing 94% of the samples. Two samples, samples 3 and 21, contained less than 40% of the declared dosage of at least one API, representing 6% of the

samples. Additionally, some of these samples (6/32) displayed a larger variation (>5%) for the amount of the API quantified in the different tablets belonging to the same sample; however, this variation never exceeded 20%.

Table 2. Summary of the APIs detected in the samples and the amount present in the sample. The measurement uncertainty values (MUs), marked with an asterisk, demonstrated large variations (>5% RSD) between the different tablets. The MU is expressed as confidence interval using the standard deviation of the generated quantification results. Abbreviations: n.a. = not available.

n°	Quantity API Declared	API Detected	API Quantified (mg)	% Declared Dose	% MU
1	100 mg sildenafil	sildenafil	93.2	93.2	1.6
2	50 mg sildenafil	sildenafil	90.5	90.5	3.8
3	100 mg sildenafil	sildenafil	86.0	86.0	1.5
	50 mg tadalafil	tadalafil	19.6	39.1	0.5
4	100 mg sildenafil	sildenafil	89.11	94.4	4.7
	60 mg dapoxetine	dapoxetine	50.4	83.9	3.6
5	50 mg sildenafil	sildenafil	46.3	92.7	2.2
6	200 mg sildenafil citrate	sildenafil	186.5	93.3	0.5
7	25 mg sildenafil	sildenafil	23.3	93.0	0.4
8	100 mg sildenafil	sildenafil	96.3	96.3	1.0
9	200 mg sildenafil	sildenafil	195.5	97.8	0.5
10	100 mg sildenafil	sildenafil	78.9	78.9	9.5 *
11	100 mg sildenafil	sildenafil	87.6	87.6	1.1
12	100 mg sildenafil	sildenafil	78.3	78.3	1.5
13	150 mg sildenafil	sildenafil	143.4	87.6	1.8
14	120 mg sildenafil	sildenafil	105.3	87.8	6.5 *
15	50 mg sildenafil	sildenafil	45.1	90.2	1.8
16	100 mg sildenafil	sildenafil	17.9	17.9	3.9
17	100 mg sildenafil	sildenafil	83.0	83.0	1.4
18	100 mg sildenafil	sildenafil	91.4	91.4	0.3
19	200 mg sildenafil	sildenafil	174.4	87.2	4.4 *
20	100 mg sildenafil	sildenafil	93.1	93.1	0.6
21	100 mg sildenafil	sildenafil	34.9	34.9	0.6
22	100 mg sildenafil	sildenafil	89.4	89.4	2.9
	20 mg tadalafil	tadalafil	19.8	99.0	1.2
23	100 mg sildenafil	sildenafil	89.1	89.1	6.8 *
24	100 mg sildenafil	sildenafil	93.8	93.8	0.4
25	25 mg sildenafil	sildenafil	21.4	85.5	7.0
26	100 mg sildenafil	sildenafil	100.9	100.9	1.3
27	25 mg sildenafil	sildenafil	18.7	74.8	11.1
28	n.a.	sildenafil	97.4	n.a.	8.7 *
29	25 mg sildenafil	sildenafil	19.6	78.4	8.7
30	100 mg sildenafil	sildenafil	91.1	91.1	4.0
31	50 mg sildenafil	sildenafil	39.6	79.2	15.8 *
32	100 mg sildenafil	sildenafil	97.4	97.4	0.4

3.3. Screening and Quantification of Residual Solvents

Organic solvents are often used during the synthesis of APIs and excipients or during the preparation of drug products either to enhance the yield, increase the solubility or aid crystallization. In some cases, the presence or the amount of these residual solvents in the finished product has proven to be toxic and even had lethal outcomes [41,42].

For some time, pharmacopoeias [43–45] have adopted the guideline proposed by the International Committee for Harmonization [46]. The guideline divides the residual solvents into three classes. Class 1 consists of solvents that should be avoided in pharmaceutical preparations due to their high toxicity, class 2 are solvents that should be limited, and class 3 consists of solvents with relatively low toxicity. Following the European Pharmacopoeia, most of the class 1 solvents (e.g., benzene, carbon tetrachloride, etc.) are limited to very low concentrations ranging from 2 to 8 ppm. For the class 2 solvents (e.g., chloroform, methanol, dichloromethane, etc.), the limits vary between 50 and 4500 ppm, while the class 3 solvents (e.g., acetic acid, ethanol, isopropanol, etc.) are limited to 5000 ppm.

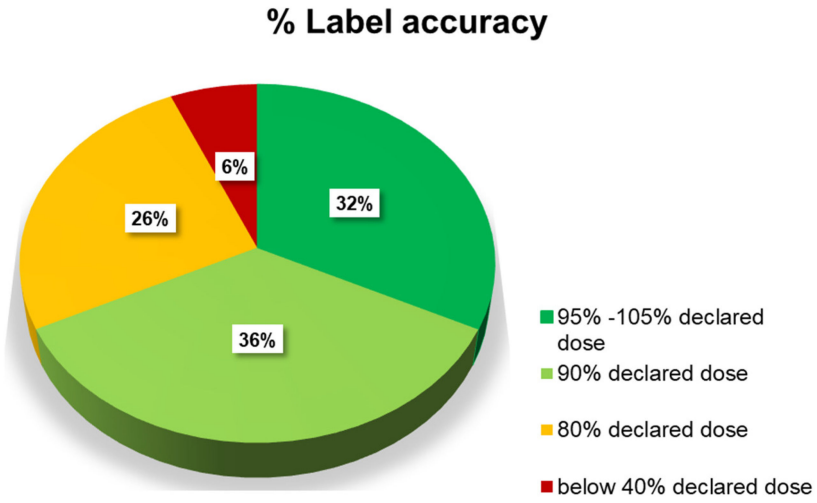


Figure 1. Results of the quantitative analysis of the 32 samples for label accuracy expressed as % deviation of the claimed concentration.

A previous study demonstrated that 3 out of 44 illegal PDE-5 inhibitors were not compliant as two of them contained the class 1 solvent carbon tetrachloride and another sample contained the class 2 solvent chloroform at a level that exceeded their respective tolerated limits [15]. Moreover, often, contaminations with class 3 solvents, not present in the analyzed genuine samples, were also encountered. However, the class 3 solvents did not exceed the threshold limit of 5000 ppm. Additionally, the amount of residual solvents was assessed based on the methodology described by Deconinck et al., 2013 [15]. Our results, summarized in Figure 2, demonstrate that from these 32 samples, 3 samples contained a class 2 solvent, dichloromethane, and a total of 17 samples contained also a class 3 solvent, including acetone and/or 2-propanol. Interestingly, these encountered solvents were also often encountered in the illegal samples analyzed in 2013 but not present in the genuine medicines, indicating that similar synthesis or possible reaction solvents are still being used in the falsified medicine business. However, in this study, the amount of the residual solvents present in the samples did not exceed the acceptable limit as is depicted in Figure 2.

3.4. Bioburden Determination and Identification of the Microorganisms

The microbiological content in non-sterile products has to be controlled to a level that is consistent with patient safety; therefore, microbial enumeration tests are performed to check if the production occurred under acceptable hygienic conditions. Whenever pharmacopoeial limits are exceeded, adverse effects on patient health cannot be excluded. Moreover, according to the US and European pharmacopoeias, the significance of recovered microorganisms must be evaluated and the absence of specific pathogens demonstrated, depending on the route of administration [43,45]. A previous study on the microbial quality of falsified PDE-5 inhibitors demonstrated that 23% (12/52) of the samples were not compliant for the total aerobic microbial count (TAMC). These samples were mainly contaminated with bacteria from the genus *Bacillus* [26]. *Bacillus* ssp. can form resistant endospores which can germinate upon the encounter of more favorable conditions. The genus *Bacillus* contains multiple clades, including the *Bacillus cereus sensu lato* clade, comprising a number of illustrious human pathogens, e.g., *Bacillus cereus sensu stricto*, an organism often associated with foodborne illnesses [47–49]. Luckily, none of the identified *Bacillus* spp. were part of the *Bacillus cereus s.l.* clade. Nevertheless, these 12 samples that did not comply with the pharmacopoeial limits could still be dangerous since many *Bacillus* spp. are known to be opportunistic pathogens [50]. Another study, conducted in 2019 on falsified antimicrobiologicals, demonstrated that 35% of the samples (6/17) were not compliant for the total yeast and mold count (TYMC) and were mainly contaminated with

Penicillium rubens, a fungus that is very common in subtropical regions [28]. Although this species has rarely been linked to human mycoses, this mold can produce mycotoxins that can cause liver and kidney intoxication [51].

Figure 2. Mean amount of residual solvent encountered in a sample. The error bars represent the measurement uncertainty (n = 4), and the red dashed line illustrates the maximum concentration tolerated. The uncertainty of the measurement is expressed as confidence interval using the standard deviation of the generated quantification results.

Therefore, we initially set out to check the TAMC and the TYMC as described by Tie et al., 2019 [28]. According to the USP and EP, the acceptance criteria for non-aqueous preparations for oral use are 10^3 colony-forming units (CFU)/g in the TAMC test and 10^2 CFU/g in the TYMC test, corresponding to a maximum acceptable count of, respectively,

2000 CFU/g and 200 CFU/g [26,43,45]. Moreover, we also checked for the occurrence of bile-tolerant Gram-negative bacteria (e.g., *Escherichia coli*, *Salmonella* ssp. and other enterobacteria). The results of the microbial contamination and the bacteria and fungi identified (solely those fungi where the TYMC was exceeded) are summarized in Table 3. It can be concluded that 16 samples (50%) exceeded either the limits set for the TAMC and/or the limits set for the TYMC. Indeed, 13 samples (41%) exceeded the TAMC, and 5 samples (16%) exceeded the limits for the TYMC.

Table 3. Microbial load and identified bacterial species present in the samples and the fungi present in those samples that exceeded the pharmacopoeial limits. Abbreviations: n.a. = not applicable.

n°		Microbial Load		Identification of Microorganism
1	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Sutcliffiella cohnii* and *Bacillus thermoamylovorans* (biofilm)
2	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
3	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
4	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Bacillus mojavensis*
5	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
6	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Bacillus cereus sensu lato*
7	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Bacillus pumilus*
8	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
9	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
10	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Bacillus* spp.
11	TAMC TYMC VRBG	compliant compliant no growth	≈20 CFU/g - -	*Micrococcus luteus*
12	TAMC TYMC VRBG	not compliant compliant no growth	biofilm ≈20 CFU/g -	*Bacillus* spp.

Table 3. *Cont.*

n°		Microbial Load		Identification of Microorganism
13	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Bacillus* spp. (biofilm) and *Corynebacterium* ssp.
14	TAMC TYMC VRBG	not compliant not compliant growth of fungi and molds	biofilm >200 CFU/g ≈110 CFU/g	Bacteria: *Bacillus cereus sensu lato* and *Paenibacillus amylolyticus* Fungi: *Alternaria triticimaculans*, *Penicillium citrinum*, *Penicillium rubens*, *Aspergillus versicolor*, *Aspergillus fumigatus* and *Penicillium chrysogenum*
15	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
16	TAMC TYMC VRBG	compliant compliant no growth	≈20 CFU/g ≈20 CFU/g -	*Staphylococcus hominis*
17	TAMC TYMC VRBG	not compliant compliant no growth	biofilm - -	*Bacillus* spp. and *Kocuria rhizophila*
18	TAMC TYMC VRBG	compliant compliant no growth	≈20 CFU/g ≈60 CFU/g -	n.a.
19	TAMC TYMC VRBG	compliant compliant no growth	≈20 CFU/g ≈20 CFU/g -	*Bacillus beringensis*
20	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
21	TAMC TYMC VRBG	compliant compliant no growth	- - -	n.a.
22	TAMC TYMC VRBG	compliant compliant no growth	≈20 CFU/g ≈20 CFU/g -	*Staphylococcus hominis*
23	TAMC TYMC VRBG	compliant compliant no growth	≈20 CFU/g - -	*Priestia megaterium* and *Micrococcus luteus*
24	TAMC TYMC VRBG	not compliant not compliant no growth	biofilm >200 CFU/g -	Bacteria: *Bacillus* spp., *Priestia megaterium* and *Micrococcus luteus* Fungi: *Aspergillus flavus*
25	TAMC TYMC VRBG	compliant not compliant growth of molds	≈100 CFU/g >200 CFU/g ≈20 CFU/g	Bacteria: *Peribacillus simplex* and *Bevundimonas vesicularis* Fungi: *Aspergillus fumigatus*
26	TAMC TYMC VRBG	not compliant compliant no growth	biofilm ≈100 CFU/g -	*Bacillus licheniformis*
27	TAMC TYMC VRBG	not compliant compliant no growth	Biofilm ≈20 CFu/g -	*Alkalihalobacillus* spp. (biofilm) and *Corynebacterium liphiloflavum*

Table 3. *Cont.*

n°		Microbial Load		Identification of Microorganism
28	TAMC TYMC VRBG	not compliant compliant growth of molds	biofilm ≈140 CFU/g ≈40 CFU/g	*Alkalihalobacillus halodurans* (biofilm) and *Micrococcus luteus*
29	TAMC TYMC VRBG	compliant compliant no growth	≈40 CFU/g ≈20 CFU/g -	n.a.
30	TAMC TYMC VRBG	compliant compliant no growth	≈300 CFU/g - -	*Corynebacterium* spp.
31	TAMC TYMC VRBG	compliant not compliant growth of molds	≈80 CFU/g >200 CFU/g ≈40 CFU/g	Bacteria: *Bacillus* spp. Fungi: *Penicillium frequentans* and *Talaromyces amestolkiae*
32	TAMC TYMC VRBG	compliant not compliant growth of molds	≈ 20 CFU/g >200 CFU/g ≈ 40 CFU/g	Bacteria: *Staphylococcus haemolyticus* and *Kocuria atrinae* Fungi: *Aspergillus fumigatus* *Aspergillus jensenii*

For all samples where the TAMC exceeded the limits, a biofilm was generated on the surface of the filter, and occasionally, also growth outside the outer borders of the filter was observed, which is typical for bacteria that possess motility traits, including Bacillus species (see Figure 3). This biofilm clearly surpasses the tolerated 100 CFU on the membrane as 50 mg of the product was analyzed per membrane. Next, also the ability of the encountered bacterial Gram-negative species and yeast or molds to grow on bile acids was assessed. Also, here, five samples displayed growth on the violet red bile glucose (VRBG) medium, and the encountered colonies displayed the typical phenotypical characteristics of yeasts and molds. No bacterial phenotypes were encountered on this medium, which indicates that no detectable viable Gram-negative enterobacteria or coliforms were present in the samples.

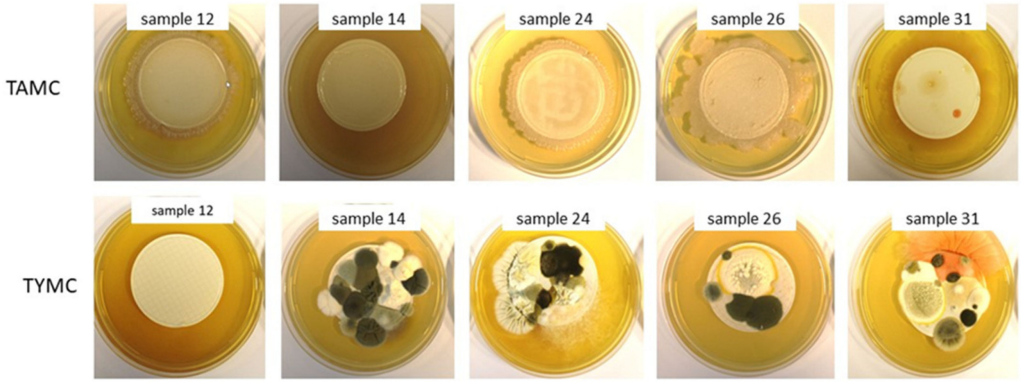


Figure 3. Examples of some heavily contaminated samples with biofilm formation and/or severe yeast and mold contamination. The amount of colony-forming units (CFU) are reported in the table. Abbreviations: TAMC = total aerobic microbial count; TYMC = total yeast and mold count.

Next, the identification of the bacterial biofilms was performed according to the methodology described by Janvier et al., 2018 [27]. Our results (see Table 3) demonstrate that the non-compliant samples were mainly contaminated with bacteria from the Bacillus genus, similar to the study of 2014 [26]. Except for samples 6 and 14, most of the *Bacillus* ssp. that were identified did not belong to the illustrious *Bacillus cereus sensu lato* clade.

Nevertheless, the remaining 11 samples that were contaminated with Bacillus ssp. exceeded the pharmacopoeial limits and thus could pose an increased risk to patients' health. Subsequently, whole-genome sequencing (WGS) was performed to determine the species present in samples 6 and 14. We found that the strain present in sample 6 corresponded to *Bacillus cereus sensu stricto* (ANI of 98.8% with a *Bacillus cereus* s.s. strain), containing enterotoxin genes (*nhe*, *hbl*, *cytK2* genes) but lacking the genes responsible for the production of the emetic toxin (see Supplementary Materials). The genetic profile of this strain indicated the presence of a potential pathogen that could result in gastrointestinal illness upon oral intake. The *Bacillus* strain present in sample 14 was very similar to *Bacillus anthracis* (ANI of 98.6% with the *B. anthracis* Ames strain); nevertheless, the core pathogenic factors (*pagA*, *lef* and *cya* genes) residing on the pXO1 and pXO2 plasmids (Ba virulence plasmids) were not detected, indicating that the encountered strain would not cause anthrax disease. These findings were also corroborated as the qPCR, targeting either the *pagA* or the *capC* gene, respectively, present on pXO1 and pXO2, resulted in no amplification while the positive control did. Moreover, this strain also did not show growth on a *B. anthracis*-specific growth medium, was resistant to penicillin and showed no susceptibility to the phage gamma, indicating that this *B. cereus s.l.* strain did not correspond to *B. anthracis*. However, this strain does contain the same enterotoxin genes as the abovementioned pathogenic *Bacillus cereus s.s.* strain, indicating that oral intake could still result in a potential gastrointestinal pathogenicity.

In addition to bacteria, several samples were also heavily contaminated with fungi. Several fungi were identified, including opportunistic pathogens (e.g., *Penicillium citrinum*, *Penicillium rubens*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus versicolor*) which have the ability to produce mycotoxins which may have nephrotoxic, genotoxic, teratogenic, carcinogenic and/or cytotoxic properties, and, as a consequence, these toxins may cause liver carcinomas and renal dysfunctions [51–56].

4. Discussion

PDE-5 inhibitors containing sildenafil or tadalafil are prescription medicines whose usage should be monitored by a medical doctor, taking into consideration the potential side effects and drug–drug interactions. Taking these medicines without medical supervision already holds a certain risk. Moreover, purchasing these medicines from an unreliable source represents a far greater danger. In this study, we analyzed 32 different substandard and falsified PDE-5 inhibitors, including unlicensed or unregistered versions. Our results, summarized in Table 4, demonstrate that 22 of 32 samples might pose a health risk. All 32 analyzed samples contained the API(s) mentioned on the label, and no other APIs were detected in the samples. Moreover, 21 samples, representing 66% of the sample set, contained at least 90% of the declared dosage, and 94% of the sample set contained at least 80% of the declared dose. However, two samples contained less than 40% of the claimed dosage, which could be dangerous as the desired effect might not occur and could tempt the patient in taking a second dose. Moreover, five samples exceeded the maximum recommended dosage up to 1.4 times, and five samples demonstrated a high level of heterogeneity in API content between different tablets in the same package or blister (RSD > 5%). Although 15 samples were compliant for the performed analysis, it cannot be excluded that they might pose additional chemical risks. This can be due to the possibility that they may contain higher than tolerated levels of API-related impurities, metals and metalloids or even have dissolution profiles with low equivalence, as often seen with substandard and falsified medicines [57,58] or substandard medicines [59]. The latter can then increase the temptation for the patient to consume a second dose, hence increasing the risks again.

Table 4. Overview of the chemical and biological risks present in each sample. [a] denotes the chemical risks other than the use of prescription medicine, which is a risk in itself.

Sample n°	Identified Chemical Risk [a]	Identified Biological Risk
1		Bacterial contamination
2		
3	Mixture of sildenafil and tadalafil, and amount of tadalafil found was less than 40% of the API mentioned on the package/blister	
4		Bacterial contamination
5		
6	Sildenafil found exceeds the recommended dosage (186.5 mg/unit)	Contamination with potential pathogenic bacteria
7		Bacterial contamination
8		
9	Sildenafil found exceeds the recommended dosage (195.5 mg/unit)	
10	Large variations (RSD > 5%) in API content between the different tablets in one package/blister	Bacterial contamination
11		
12		Bacterial contamination
13	Sildenafil found exceeds the recommended dosage (143.4 mg/unit)	Bacterial contamination
14	Large variations (RSD > 5%) in API content between the different tablets in one package/blister	Contamination with potential pathogenic bacteria and fungal contamination
15		
16	Amount less than 20% declared dosage	
17		Bacterial contamination
18		
19	Sildenafil found exceeds the recommended dosage (174.4 mg/unit) and large variations (RSD > 5%) in API content between the different tablets in one package/blister	
20		
21	Amount less than 40% declared dosage	
22		
23	Large variations (RSD > 5%) in API content between the different tablets in one package/blister	
24		Bacterial and fungal contamination
25		Fungal contamination
26		Bacterial contamination
27		Bacterial contamination

Table 4. *Cont.*

Sample n°	Identified Chemical Risk [a]	Identified Biological Risk
28	Large variations (RSD > 5%) in API content between the different tablets in one package/blister	Bacterial contamination
29		
30		
31	Large variations (RSD > 5%) in API content between the different tablets in one package/blister	Fungal contamination
32		Fungal contamination

Additionally to the chemical risks, we also identified the biological risk as a non-compliance to the microbiological quality standards set by the USP and European pharmacopoeia. This bioburden analysis demonstrated that 50% of the samples were severely contaminated by bacteria or fungi, indicating that improper hygienic standards were used during the production of these medicinal products. Startlingly, also two different potential pathogens were encountered in two different samples. Genetic analysis demonstrated that these pathogens belonged to the illustrious *Bacillus cereus sensu lato* clade, and both strains contained enterotoxin genes (*nhe*, *hbl*, *cytK2* genes). It stands to reason that an oral uptake of these samples could potentially result in gastrointestinal pathogenesis. In addition to bacteria, several samples were also heavily contaminated with opportunistic fungal pathogens which have the ability to produce mycotoxins which may have nephrotoxic, genotoxic, teratogenic, carcinogenic and/or cytotoxic properties.

Taken together, our results illustrate the different types of risks that might be associated with the use of substandard and falsified medicinal products, including unlicensed or unregistered medicines, and the potential threat they may pose to patient health. Moreover, they also demonstrate the need for continuing the vigilance of the international and national regulatory agencies and law enforcement agencies to safeguard this aspect of public health. This can be achieved by safeguarding the medicine supply and distribution chain and also by informing and warning the public of the possible risks of these types of falsified medicines, exemplified by real-life data.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/forensicsci3030031/s1, Table S1: ANI values for *Bacillus cereus sensu lato* species determination, Table S2: Determination of the species, toxin genes and plasmid of the two *Bacillus cereus sensu lato* isolates, Table S3: Metadata on the reference genomes used for species determination, Table S4: real-time PCR and phenotypical analysis.

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References

1. OECD; EUIPO. *Trade in Counterfeit Pharmaceutical Products, Illicit Trade*; OECD Publishing: Paris, France, 2020. [CrossRef]
2. INTERPOL. USD11 Million in Illicit Mediicnes Seized in Global INTERPOL Operation. Available online: https://www.interpol.int/en/News-and-Events/News/2022/USD-11-million-in-illicit-medicines-seized-in-global-INTERPOL-operation (accessed on 18 January 2023).
3. Pharmaceutical Security Institute. Available online: https://www.psi-inc.org/incident-trends (accessed on 18 January 2023).
4. World Health Organization. *WHO Global Surveillance and Monitoring System for Substandard and Falsified Medical Products*; World Health Organization: Geneva, Switzerland, 2017; Available online: https://apps.who.int/iris/handle/10665/326708 (accessed on 18 January 2023).
5. Fadeyi, I.; Lalani, M.; Mailk, N.; Van Wyk, A.; Kaur, H. Quality of the antibiotics—Amoxicillin and co-trimoxazole from Ghana, Nigeria, and the United Kingdom. *Am. J. Trop Med. Hyg.* **2015**, *92*, 87–94. [CrossRef] [PubMed]
6. Kaur, H.; Allan, E.L.; Mamadu, I.; Hall, Z.; Green, M.D.; Swamidos, I.; Dwivedi, P.; Culzoni, M.J.; Fernandez, F.M.; Garcia, G.; et al. Prevalence of substandard and falsified artemisinin-based combination antimalarial medicines on Bioko Island, Equatorial Guinea. *BMJ Glob. Health* **2017**, *2*, e000409. [CrossRef] [PubMed]
7. Frimpong, G.; Ofori-Kwakye, K.; Kuntworbe, N.; Buabeng, K.O.; Osei, Y.A.; Boakye-Gyasi, M.E.; Adi-Dako, O. Quality Assessment of Some Essential Children's Medicines Sold in Licensed Outlets in Ashanti Region, Ghana. *J. Trop Med.* **2018**, *2018*, 1494957. [CrossRef]
8. Abuye, H.; Abraham, W.; Kebede, S.; Tatiparthi, R.; Suleman, S. Physicochemical Quality Assessment of Antimalarial Medicines: Chloroquine Phosphate and Quinine Sulfate Tablets from Drug Retail Outlets of South-West Ethiopia. *Infect. Drug Resist.* **2020**, *13*, 691–701. [CrossRef] [PubMed]
9. Irungu, B.N.; Koech, L.C.; Ondicho, J.M.; Keter, L.K. Quality assessment of selected co-trimoxazole suspension brands marketed in Nairobi County, Kenya. *PLoS ONE* **2021**, *16*, e0257625. [CrossRef]
10. Chiumia, F.K.; Nyirongo, H.M.; Kampira, E.; Muula, A.S.; Khuluza, F. Burden of and factors associated with poor quality antibiotic, antimalarial, antihypertensive and antidiabetic medicines in Malawi. *PLoS ONE* **2022**, *17*, e0279637. [CrossRef]
11. Adigwe, O.P.; Onavbavba, G.; Wilson, D.O. Challenges Associated with Addressing Counterfeit Medicines in Nigeria: An Exploration of Pharmacists' Knowledge, Practices, and Perceptions. *Integr. Pharm. Res. Pract.* **2022**, *11*, 177–186. [CrossRef]
12. Deconinck, E.; Vanhee, C.; Keizers, P.; Guinot, P.; Mihailova, A.; Syversen, P.V.; Li-Ship, G.; Young, S.; Blazewicz, A.; Poplawska, M.; et al. The occurrence of non-anatomical therapeutic chemical-international nonproprietary name molecules in suspected illegal or illegally traded health products in Europe: A retrospective and prospective study. *Drug Test. Anal.* **2021**, *13*, 833–840. [CrossRef] [PubMed]
13. Kao, S.L.; Chan, C.L.; Tan, B.; Lim, C.C.; Dalan, R.; Gardner, D.; Pratt, E.; Lee, M.; Lee, K.O. An unusual outbreak of hypoglycemia. *N. Engl. J. Med.* **2009**, *360*, 734–736. [CrossRef]
14. Jackson, G.; Arver, S.; Banks, I.; Stecher, V.J. Counterfeit phosphodiesterase type 5 inhibitors pose significant safety risks. *Int. J. Clin. Pract.* **2010**, *64*, 497–504. [CrossRef]
15. Deconinck, E.; Canfyn, M.; Sacré, P.Y.; Courselle, P.; De Beer, J.O. Evaluation of the residual solvent content of counterfeit tablets and capsules. *J. Pharm. Biomed. Anal.* **2013**, *81–82*, 80–88. [CrossRef] [PubMed]
16. Veronin, M.A.; Nutan, M.T.; Dodla, U.K. Quantification of active pharmaceutical ingredient and impurities in sildenafil citrate obtained from the Internet. *Ther. Adv. Drug Saf.* **2014**, *5*, 180–189. [CrossRef] [PubMed]
17. Neves, D.B.D.J.; Caldas, E.D. GC-MS quantitative analysis of black market pharmaceutical products containing anabolic androgenic steroids seized by the Brazilian Federal Police. *Forensic. Sci. Int.* **2017**, *275*, 272–281. [CrossRef] [PubMed]
18. Weber, C.; Krug, O.; Kamber, M.; Thevis, M. Qualitative and Semiquantitative Analysis of Doping Products Seized at the Swiss Border. *Subst. Use Misuse.* **2017**, *52*, 742–753. [CrossRef]
19. Vanhee, C.; Janvier, S.; Moens, G.; Goscinny, S.; Courselle, P.; Deconinck, E. Identification of epidermal growth factor (EGF), in an unknown pharmaceutical preparation suspected to contain insulin like growth factor 1 (IGF-1). *Drug Test Anal.* **2017**, *9*, 831–837. [CrossRef]
20. Janvier, S.; Cheyns, K.; Canfyn, M.; Goscinny, S.; De Spiegeleer, B.; Vanhee, C.; Deconinck, E. Impurity profiling of the most frequently encountered falsified polypeptide drugs on the Belgian market. *Talanta* **2018**, *188*, 795–807. [CrossRef]
21. Zawilska, J.B.; Kuczyńska, K.; Kosmal, W.; Markiewicz, K.; Adamowicz, P. Carfentanil—From an animal anesthetic to a deadly illicit drug. *Forensic. Sci. Int.* **2021**, *320*, 110715. [CrossRef]
22. Chapman, B.P.; Lai, J.T.; Krotulski, A.J.; Fogarty, M.F.; Griswold, M.K.; Logan, B.K.; Babu, K.M. A Case of Unintentional Opioid (U-47700) Overdose in a Young Adult After Counterfeit Xanax Use. *Pediatr. Emerg. Care.* **2021**, *37*, e579–e580. [CrossRef]
23. Fabresse, N.; Gheddar, L.; Kintz, P.; Knapp, A.; Larabi, I.A.; Alvarez, J.C. Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders. *Forensic. Sci. Int.* **2021**, *322*, 110771. [CrossRef]

24. Cohen, P.A.; Travis, J.C.; Vanhee, C.; Ohana, D.; Venhuis, B.J. Nine prohibited stimulants found in sports and weight loss supplements: Deterenol, phenpromethamine (Vonedrine), oxilofrine, octodrine, beta-methylphenylethylamine (BMPEA), 1,3-dimethylamylamine (1,3-DMAA), 1,4-dimethylamylamine (1,4-DMAA), 1,3-dimethylbutylamine (1,3-DMBA) and higenamine. *Clin. Toxicol.* **2021**, *59*, 975–981. [CrossRef]
25. Wang, F.; Yu, S.; Liu, K.; Chen, F.E.; Song, Z.; Zhang, X.; Xu, X.; Sun, X. Acute intraocular inflammation caused by endotoxin after intravitreal injection of counterfeit bevacizumab in Shanghai, China. *Ophthalmology* **2013**, *120*, 355–361. [CrossRef] [PubMed]
26. Pullirsch, D.; Bellemare, J.; Hackl, A.; Trottier, Y.L.; Mayrhofer, A.; Schindl, H.; Taillon, C.; Gartner, C.; Hottowy, B.; Beck, G.; et al. Microbiological contamination in counterfeit and unapproved drugs. *BMC Pharmacol. Toxicol.* **2014**, *15*, 34. [CrossRef] [PubMed]
27. Janvier, S.; Wattijn, E.; Botteldoorn, N.; De Spiegeleer, B.; Deconinck, E.; Vanhee, C. Are injectable illegal polypeptide drugs safe? Case report demonstrating the presence of haemolytic *Bacillus* cereus in 2 illegal peptide drugs. *Drug Test. Anal.* **2018**, *10*, 791–795. [CrossRef]
28. Tie, Y.; Adams, E.; Deconinck, E.; Vanhee, C. Substandard and falsified antimicrobials: A potential biohazard in disguise? *Drug Test. Anal.* **2020**, *12*, 285–291. [CrossRef] [PubMed]
29. European Directorate for the Quality of Medicines and Health Care. Available online: https://www.edqm.eu/en/know-x (accessed on 18 January 2023).
30. Vanhee, C.; Tuenter, E.; Kamugisha, A.; Canfyn, M.; Moens, G.; Courselle, P.; Pieters, L.; Deconinck, E.; Exarchou, V. Identification and Quantification Methodology for the Analysis of Suspected Illegal Dietary Supplements: Reference Standard or no Reference Standard, that's the Question. *J. Forensic. Toxicol. Pharmacol.* **2018**, *7*, 1. [CrossRef]
31. Sacré, P.Y.; Deconinck, E.; Chiap, P.; Crommen, J.; Mansion, F.; Rozet, E.; Courselle, P.; De Beer, J.O. Development and validation of a ultra-high-performance liquid chromatography-UV method for the detection and quantification of erectile dysfunction drugs and some of their analogues found in counterfeit medicines. *J. Chromatogr. A* **2011**, *1218*, 6439–6447. [CrossRef]
32. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics* **2014**, *30*, 15. [CrossRef]
33. Prjibelski, A.; Antipov, D.; Meleshko, D.; Lapidus, A.; Korobeynikov, A. Using SPAdes De Novo Assembler. *Curr. Protoc. Bioinform.* **2020**, *70*, 1. [CrossRef]
34. Jain, C.; Rodriguez-R, L.M.; Phillippy, A.M.; Konstantinidis, K.T.; Aluru, S. High troughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **2018**, *9*, 5114. [CrossRef]
35. Carroll, L.M.; Cheng, R.A.; Kovac, J. No Assembly Required: Using BTyper3 to assess the congruency of a proposed taxonomic framework for the Bacillus cereus group with historical typing methods. *Front. Microbiol.* **2020**, *11*, 580691. [CrossRef]
36. Carattoli, A.; Zankari, E.; Garcia-Fernandez, A.; Larsen, M.V.; Lund, O.; Villa, L.; Aarestrup, F.M.; Hasman, H. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* **2014**, *58*, 7. [CrossRef] [PubMed]
37. Wattiau, P.; Klee, S.R.; Fretin, D.; Van Hessche, M.; Ménart, M.; Franz, T.; Chasseur, C.; Butaye, P.; Imberechts, H. Occurrence and genetic diversity of Bacillus anthracis strains isolated in an active wool-cleaning factory. *Appl. Environ. Microbiol.* **2008**, *74*, 4005–4011. [CrossRef] [PubMed]
38. Clinical and Laboratory Standards Institute Document M45. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Available online: https://clsi.org/standards/products/microbiology/documents/m45/ (accessed on 18 January 2023).
39. Knisely, R.F. Selective medium for Bacillus anthracis. *J. Bacteriol.* **1966**, *92*, 784–786. [CrossRef]
40. Belgian Centre for Pharmacotherapeutic Information. Available online: https://www.famhp.be/en/human_use/medicines/medicines/information_about_medicines/bcficbip (accessed on 18 January 2023).
41. Rentz, E.D.; Lewis, L.; Mujica, O.J.; Barr, D.B.; Schier, J.G.; Weerasekera, G.; Kuklenyik, P.; McGeehin, M.; Osterloh, J.; Wamsley, J.; et al. Outbreak of acute renal failure in Panama in 2006: A case-control study. *Bull. World Health Organ.* **2008**, *86*, 749–756. [CrossRef] [PubMed]
42. World Health Organization. Available online: https://www.who.int/news/item/05-10-2022-medical-product-alert-n-6-2022-substandard-(contaminated)-paediatric-medicines (accessed on 18 January 2023).
43. *United States Pharmacopoeia 42*; United States Pharmacopeial Convention, Inc.: Rockville, MD, USA, 2022.
44. *Japanese Pharmacopoeia*, 18th ed.; Society of Japanese Pharmacopoeia: Tokyo, Japan, 2022.
45. *European Pharmacopoeia 10.0*; Council of Europe: Strasbourg, France, 2022.
46. International Conference on Harmonisation (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Q3C: Impurities: Guidelines for Residual Solvents, Step 4. 2021. Available online: https://database.ich.org/sites/default/files/ICH_Q3C-R8_Guideline_Step4_2021_0422_1.pdf (accessed on 18 January 2023).
47. Patel, S.; Gupta, R.S. A phylogenomic and comparative genomic framework for resolving the polyphyly of the genus Bacillus: Proposal for six new genera of *Bacillus* species, *Peribacillus* gen. nov., *Cytobacillus* gen. nov., *Mesobacillus* gen. nov., *Neobacillus* gen. nov., *Metabacillus* gen. nov. and *Alkalihalobacillus* gen. nov. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 406–438. [CrossRef]
48. Cui, Y.; Märtlbauer, E.; Dietrich, R.; Luo, H.; Ding, S.; Zhu, K. Multifaceted toxin profile, an approach toward a better understanding of probiotic Bacillus cereus. *Crit. Rev. Toxicol.* **2019**, *49*, 342–356. [CrossRef]
49. Ehling-Schulz, M.; Lereclus, D.; Koehler, T.M. The *Bacillus cereus* Group: *Bacillus* Species with Pathogenic Potential. *Microbiol. Spectr.* **2019**, 7. [CrossRef]

50. Celandroni, F.; Salvetti, S.; Gueye, S.A.; Mazzantini, D.; Lupetti, A.; Senesi, S.; Ghelardi, E. Identification and Pathogenic Potential of Clinical Bacillus and Paenibacillus Isolates. *PLoS ONE.* **2016**, *11*, e0152831. [CrossRef]
51. Zhang, X.; Li, Y.; Wang, H.; Gu, X.; Zheng, X.; Wang, Y.; Diao, J.; Peng, Y.; Zhang, H. Reply to Comment on "Screening and Identification of Novel Ochratoxin A-Producing Fungi from Grapes". *Toxins* **2016**, *8*, 333"—In Reporting Ochratoxin A Production from Strains of Aspergillus, Penicillium and Talaromyces. *Toxins* **2017**, *9*, 66. [CrossRef]
52. Egbuta, M.A.; Mwanza, M.; Babalola, O.O. Health Risks Associated with Exposure to Filamentous Fungi. *Int. J. Environ. Res. Public Health* **2017**, *14*, 719. [CrossRef]
53. Hesse, S.E.; Luethy, P.M.; Beigel, J.H.; Zelazny, A.M. Penicillium citrinum: Opportunistic pathogen or idle bystander? A case analysis with demonstration of galactomannan cross-reactivity. *Med. Mycol. Case Rep.* **2017**, *17*, 8–10. [CrossRef] [PubMed]
54. Navale, V.; Vamkudoth, K.R.; Ajmera, S.; Dhuri, V. Aspergillus derived mycotoxins in food and the environment: Prevalence, detection, and toxicity. *Toxicol. Rep.* **2021**, *8*, 1008–1030. [CrossRef] [PubMed]
55. Ráduly, Z.; Szabó, L.; Madar, A.; Pócsi, I.; Csernoch, L. Toxicological and Medical Aspects of Aspergillus-Derived Mycotoxins Entering the Feed and Food Chain. *Front. Microbiol.* **2020**, *10*, 2908. [CrossRef] [PubMed]
56. Perrone, G.; Susca, A. Penicillium Species and Their Associated Mycotoxins. *Methods Mol. Biol.* **2017**, *1542*, 107–119. [CrossRef]
57. Deconinck, E.; Andriessens, S.; Bothy, J.L.; Courselle, P.; De Beer, J.O. Comparative dissolution study on counterfeit medicines of PDE-5 inhibitors. *J. Pharm. Anal.* **2014**, *4*, 250–257. [CrossRef]
58. Tie, Y.; van Loock, K.; Deconinck, E.; Adams, E. Evaluation of impurities and dissolution profiles of illegal antimicrobial drugs encountered in Belgium. *Drug Test. Anal.* **2020**, *12*, 53–66. [CrossRef]
59. Rahman, M.S.; Yoshida, N.; Tsuboi, H.; Maeda, E.; Ibarra, A.V.V.; Zin, T.; Akimoto, Y.; Tanimoto, T.; Kimura, K. Patient safety and public health concerns: Poor dissolution rate of pioglitazone tablets obtained from China, Myanmar and internet sites. *BMC Pharmacol. Toxicol.* **2021**, *22*, 12. [CrossRef]