

AVALIAÇÃO DA SIMILARIDADE ESPECTRAL DE LINHAGENS DE *Penicillium citrinum* ISOLADAS DE UMA INDÚSTRIA FARMACÊUTICA POR ESPECTROSCOPIA DE INFRAVERMELHO POR TRANSFORMADA DE FOURIER

EVALUATION OF THE SPECTRAL SIMILARITY OF *Penicillium citrinum* STRAINS ISOLATED FROM A PHARMACEUTICAL FACILITY BY FOURIER-TRANSFORM INFRARED SPECTROSCOPY

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RESUMO

As indústrias farmacêuticas devem cumprir os requisitos de boas práticas de fabricação e manter baixos níveis de contaminação. A contaminação fúngica nestes locais é de grande preocupação pois os fungos podem se dispersar facilmente e contaminar diversas áreas. Neste estudo, 25 linhagens de *Penicillium citrinum* isoladas de diferentes áreas dentro de uma indústria farmacêutica, foram tipificadas utilizando a técnica de FTIR com IR Biotyper®. Todos os isolados foram cultivados de forma padronizada em SDA a $22,5 \pm 2,5$ °C por 5 dias. Foi gerado um dendrograma que agrupou as linhagens por diferenças de espectro. Como parâmetros foram considerados: local de isolamento, lote do produto e lote de placa de monitoramento ambiental. Quatro clusters foram identificados, representados por linhagens que apresentam alta similaridade espectral/bioquímica. Não houve relação entre os perfis espectrais e os lotes de produtos, nem local de isolamento. Porém, houve associação entre as linhagens do cluster 4 com os lotes das placas de monitoramento ambiental, sendo a maior prevalência de isolados provenientes do lote II. O valor de corte no dendrograma foi calculado automaticamente utilizando o software OPUS v.7.5. O gráfico *scatter plot* também permitiu a visualização dos 4 clusters e corroborou com os resultados obtidos no dendrograma. A técnica de FTIR foi capaz de diferenciar linhagens fúngicas rapidamente, sendo uma ferramenta aplicável no rastreamento de fontes de contaminações em indústrias e se mostrou eficaz para tipificação de fungos filamentosos.

Palavras-Chave: FTIR. indústria farmacêutica. *Penicillium citrinum*.

ABSTRACT

Pharmaceutical companies must comply with good manufacturing practice requirements and maintain low levels of contamination. Fungal contamination in these facilities is a major concern because fungi can spread easily and contaminate different areas. In this study 25 *Penicillium citrinum* strains isolated from different areas within a pharmaceutical facility were identified using FTIR spectroscopy with the IR Biotyper®. All isolates were cultured under standardized conditions on SDA at 22.5 ± 2.5 °C for 5 days. A dendrogram was generated that grouped the strains based on spectral differences. The following parameters were considered: isolation site, product batch, and environmental monitoring plate batch. Four clusters were identified, represented by strains exhibiting high spectral/biochemical similarity. There was no relationship between the spectral profiles and the product lots, nor the isolation site. However, there was an association between the strains in cluster 4 and the environmental monitoring plate batches, with the highest prevalence of isolates from batch II. The cutoff value in the dendrogram was calculated automatically using OPUS v.7.5 software. The scatter plot also allowed visualization of the 4 clusters and corroborated the results obtained in the dendrogram. The FTIR technique was able to rapidly differentiate fungal strains, making it a useful tool for tracing sources of contamination in industrial settings, and proved effective for the typing of filamentous fungi.

Keywords: FTIR. pharmaceutical industry. *Penicillium citrinum*.

1 INTRODUCTION

Pharmaceutical companies must comply with Good Manufacturing Practice (GMP) requirements to reduce the risk of contamination during production processes. To ensure product quality in pharmaceutical industries, particularly in areas that perform aseptic production, maintaining low levels of microbiological contamination is of great importance (Valadão et al., 2023). GMP are the part of Quality Management that ensures products are consistently manufactured and controlled in accordance with the quality standards appropriate for their intended use. GMPs relate to both production and quality control. One of the basic requirements of GMP is that all manufacturing processes must demonstrate the ability to produce drugs of the required quality and in compliance with their specifications. (Brasil, 2022).

Fungal contamination in environments, both within and outside clean areas, presents a problem of extreme importance (Tyski et al., 2025), as it is one of the most challenging microorganisms among the various types of microorganisms that cause microbial contamination (Sandle, 2021).

In addition to the fact that objects, surfaces, and equipment contaminated with fungi are difficult to disinfect and eradicate (Tyski et al., 2025), fungal contamination can change the characteristics of pharmaceutical products due to the metabolism of compounds and/or the production of substances that may alter their composition or stability. Another relevant factor is that, due to spore production, filamentous fungi can disperse, potentially spreading and contaminating multiple areas (Moreira et al., 2022; Sandle, 2021).

Among the main microorganisms that contaminate clean areas in pharmaceutical industries, the genus *Penicillium* stands out (Vijayakumar et al., 2012). Species belonging to this genus are widely distributed across various environments, with *Penicillium citrinum* being one of the most common species (Egbuta et al., 2017). They produce mycotoxins, which are metabolic products of filamentous fungi. Species of the genus *Penicillium* can produce various toxins, such as citrinin, ochratoxin A, islanditoxin, penitrem, and rubratoxin, among others (Egbuta et al., 2017; Sandle, 2021). In addition, *P. citrinum* can produce citrinin, a nephrotoxic mycotoxin that may also be embryotoxic, teratogenic, and genotoxic (Mossini; Kemmelmeier, 2008). For this reason, pharmaceutical products, such as certain types of raw materials, can pose a serious risk when contaminated with mycotoxins (Sandle, 2021).

Effective environmental monitoring programs are the primary tools for ensuring that aseptic production areas remain within acceptable contamination levels (Costa et al., 2022). According to Annex 1 of the European Medicines Agency (EMA), microorganisms isolated

from environmental monitoring in areas classified as Grade A and B must be identified to the species level. The identification of microorganisms detected in Grade C and D areas should also be considered when action limits or alert levels are exceeded in environmental monitoring (EMA, 2022). When specified limits are exceeded, it is necessary to accurately identify and characterize microorganisms in order to investigate the microbiological contamination of a product or process (EMA, 2019). In addition, the identification of microorganisms from pharmaceutical production environments is necessary to reduce productivity losses, quality deviations, and batch rejections (Moreira et al., 2022). Several methodologies can be used for microbiological identification in the pharmaceutical industry, ranging from biochemical techniques to genomic methodologies, among these, MALDI-TOF MS (matrix-assisted laser desorption ionization–time-of-flight mass spectrometry), 16S rRNA gene sequencing for bacteria (Costa et al., 2024) and for the identification of fungi, the sequencing of the internal transcribed spacer (ITS) regions and the D1/D2 domains of ribosomal deoxyribonucleic acid (rDNA) (Moreira et al., 2022). In addition, typing methodologies can aid in the tracking of microbial lineages during investigations of deviations and process failures, proving useful for determining both the source of contamination and its persistence in production (De Miranda et al., 2023). Traditional microbiological assays, although efficient, may have limitations. To address these shortcomings, the Brazilian Pharmacopoeia encourages the use of alternative methods, once validated, to improve test quality and achieve faster results, enabling more assertive corrective actions. Fourier-transform infrared spectroscopy (FTIR) is described by the Brazilian Pharmacopoeia as a quick and simple alternative method that can be used to aid in the identification of microorganisms and is based on the vibration of molecules caused by radiation (Brasil, 2024).

The FTIR technique, performed using the IR Biotyper®, is a rapid and cost-effective method (Wang-Wang et al., 2022) that generates a spectrum enabling the identification of microorganisms in up to 3 hours (De Souza et al., 2025).

The objective of this study was to characterize the *P. citrinum* strains isolated from a pharmaceutical facility using FT-IR spectroscopy, in order to determine whether the isolates share the same spectral profile (indicating possible clonality) or have similar profiles, thereby allowing to infer persistence or a common source of contamination and, thus, contribute to the investigation of the contamination origins.

2 METHODOLOGY

2.1 TYPIFICATION BY FTIR ANALYSIS

Twenty-five strains isolated from a pharmaceutical company throughout the year 2025, collected from different areas within the company were previously identified as *Penicillium citrinum* by the MALDI Biotyper® (Bruker Daltonics, Bremen, Germany) and were cultured on sabouraud dextrose agar (SDA) at 22.5 ± 2.5 °C for 5 days. After growth, a small portion of a pure colony was removed using a 1 µL loop, transferred to a microtube containing 50 µL of 70% ethanol and homogenized, followed by the addition of 50 µL of distilled water and homogenized again.

For each strain, three 15-µL aliquots were dispensed into a 96-well silicone microplate. Test standards (IRTS1 and IRTS2) were added in duplicate. Once the plate was completely dry, it was loaded into the IR Biotyper® spectrometer (Bruker Optics-Daltonics, Bremen, Germany).

A dendrogram was generated by the IR Biotyper® software using raw data that grouped the strains based on spectral differences. The cutoff value for the dendrogram was calculated automatically using OPUS v.7.5 software (Bruker Optics-Daltonics GmbH, Bremen, Germany).

A scatter plot was also generated using the IR Biotyper® software to visualize the relationship between the isolates based on spectral similarity. Each point on the graph represents the spectra of the isolates, where proximity between points indicates greater spectral/biochemical similarity, while greater distance indicates greater divergence between the analyzed profiles.

3 RESULTS

The characterization of the 25 *P. citrinum* strains using FT-IR analysis allowed for the identification of four groups, referred to as clusters, as shown in the dendrogram (Figure 1) generated by the IR Biotyper®. Each cluster, numerated from 1 to 4, consists of strains that exhibit high spectral and biochemical similarity, suggesting a possible clonal relationship among them.

The dendrogram analyzed the following parameters from a pharmaceutical company: isolation site (10 different sites, represented by the letters A through J in the dendrogram), product batch (14 product batches, represented by P1 through P14 in the dendrogram), and

environmental monitoring plate batch (6 batches of environmental monitoring plates, numbered I through VI). Three batches could not be identified and were designated as NI (not identified).

According to this analysis, there was no correlation between the spectral profiles obtained and the product batches or the isolation site. On the other hand, an association was observed between the strains in cluster 4 and the environmental monitoring plate batches.

In clusters 1 and 2, a higher prevalence of isolates from environmental monitoring plates in batch V was observed. Clusters 3 and 4 had the highest number of isolates, with cluster 4 showing the highest prevalence of isolates from environmental monitoring plates in batch II.

Analysis of cluster 4 also shows that isolates with high spectral similarity (with possible clonal relationship among themselves) were collected from different isolation sites and isolated from different products, although most were from the same batch of environmental monitoring plates.

The scatter plot (Figure 2) showed clusters equal to those observed in the dendrogram; thus, it allowed for the observation of the same clusters obtained in the dendrogram, but through a different form of visualization.

In Figure 2, Cluster 1 is represented by the blue circle, Cluster 2 by the red circle, Cluster 3 by the yellow circle, and Cluster 4 by the black circle.

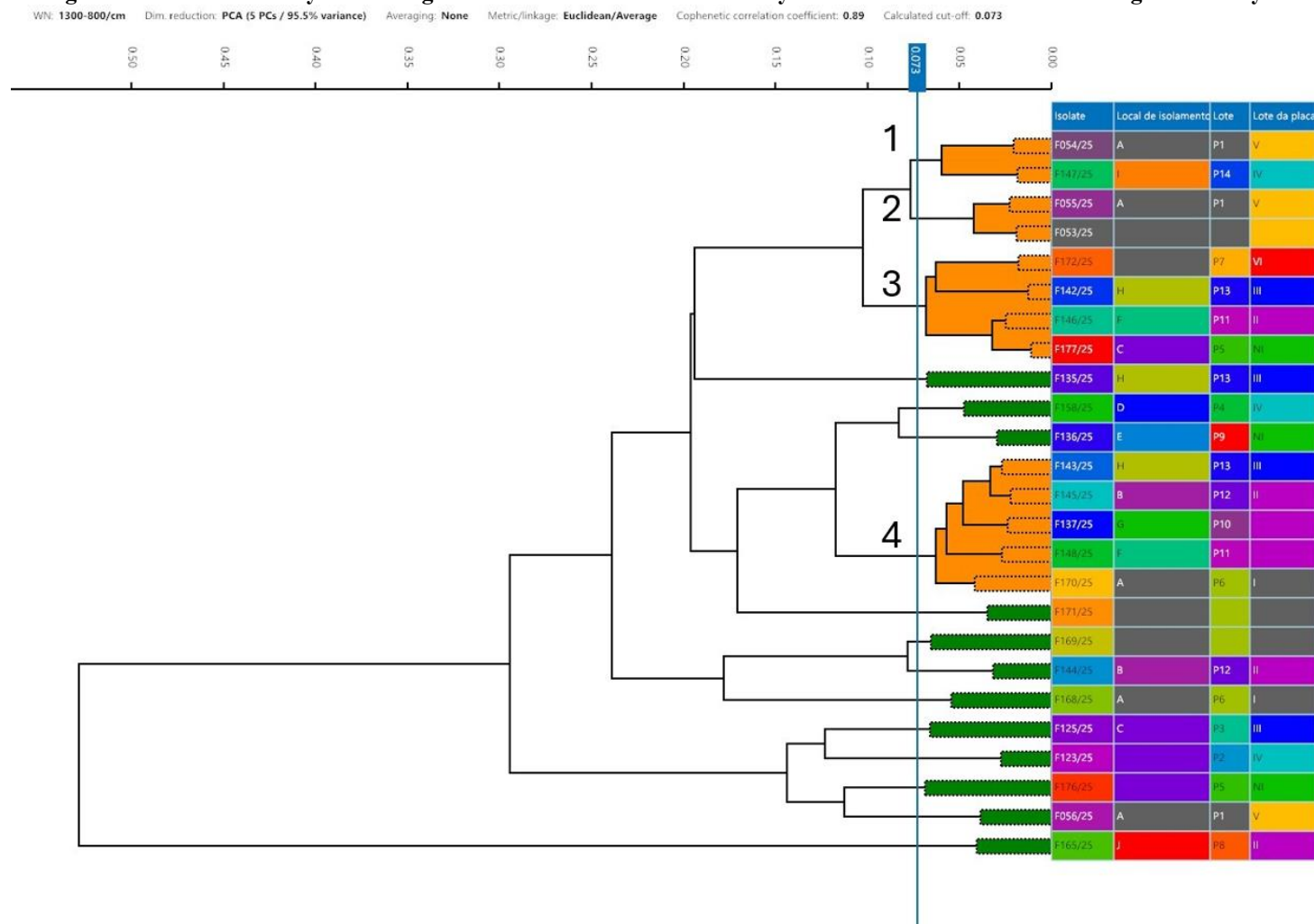
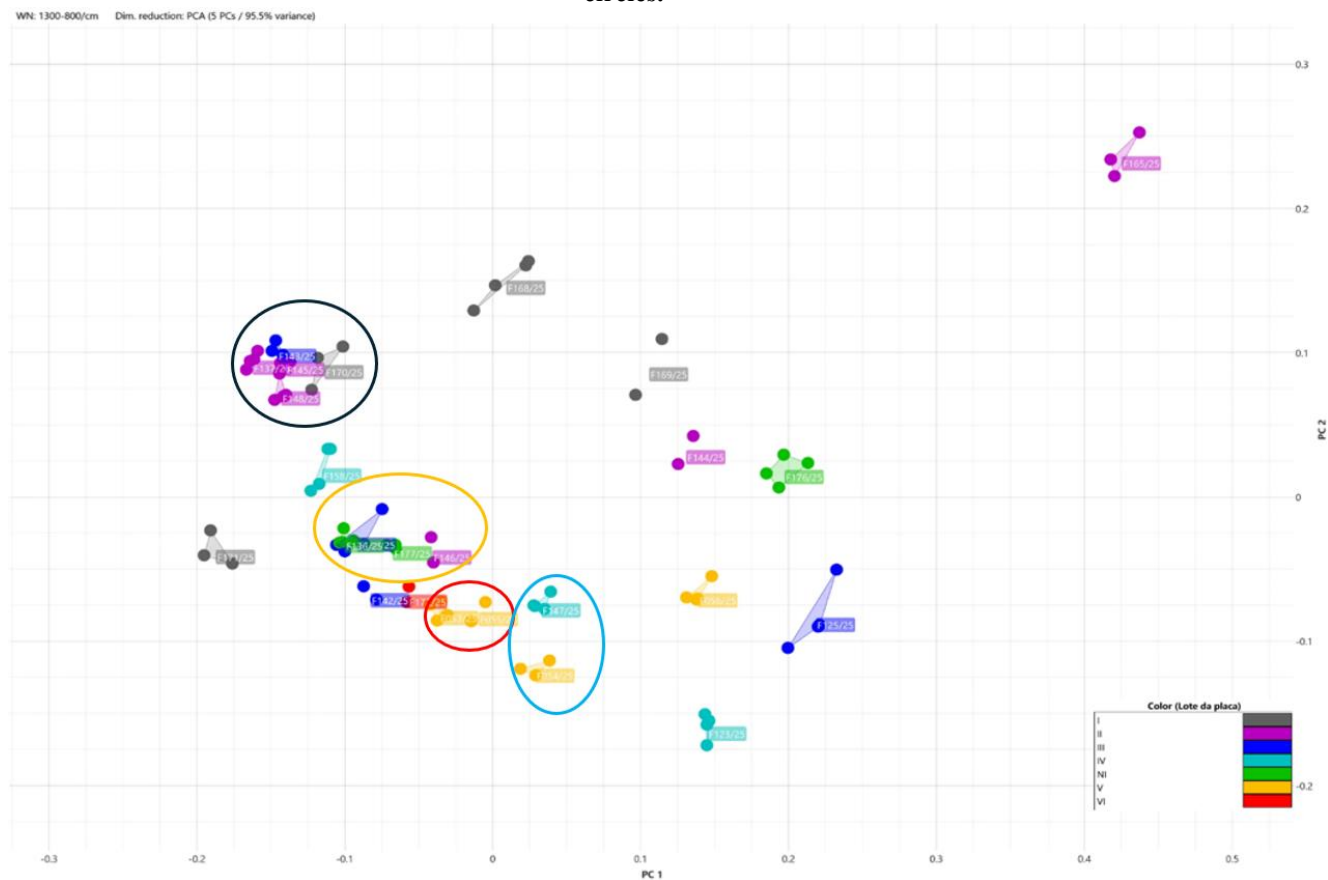
Figure 1. Dendrogram of the FT-IR analysis showing four distinct clusters of the twenty-five *Penicillium citrinum* strains generated by the IR Biotyper®.

Figure 2. 2D FT-IR scatter plot of the twenty-five *Penicillium citrinum* strains generated by the IR Biotyper® showing the related isolates indicated by four colored circles.



4 DISCUSSION

The FTIR technique using the IR Biotyper[®], which was employed in this study for the screening of *P. citrinum*, allows for the typing of microorganisms in as little as 3 hours (De Souza et al., 2025), although the time required for identification depends on the technique used, such as sequencing (which takes longer) or MALDI-TOF (which is faster).

According to the analysis of the dendrogram and scatter plot, the strains were separated into 4 distinct clades, with the largest number of isolates present in clade 4. Similar studies, such as that by De Melo et al. (2025), have shown that for the yeast *Candida auris* (*C. auris*), the FTIR technique was also capable of distinguishing between different clades. According to the study, strains belonging to clade IV which were highly similar to one another collected from three different hospitals in Pernambuco, Brazil. Of these hospitals, two shared strains that were very closely related, which makes sense given that patients were transferred between these two hospitals, leading to the spread of *C. auris*. In the present study, clade 4 also groups strains that exhibit high spectral similarity and were found in different geographic areas; however, in this case, these strains originate from the same batch of plates used in environmental monitoring, this fact indicates that there was also a spread of very similar *P. citrinum* strains to other areas within the industry, suggesting that the possible source of contamination and the spread may have originated from batch II of the environmental monitoring plates.

Therefore, it can be inferred that there may have been issues during the transport, storage, or handling of the plates in this batch.

Although genotyping techniques were not performed in the present study to confirm and enhance the reliability of the results obtained by FTIR, the study by Melo et al. (De Melo et al., 2025) also included genotyping analyses (short tandem repeat-STR) because this approach offers greater discriminatory power, which is essential for tracing the sources of contamination, and these analyses corroborated the FT-IR results, demonstrating that they correspond to the results of the genotype-based technique.

There is a lack of studies in the literature addressing fungal typing using FTIR, however, for bacterial typing, this methodology has been described more consistently in studies, particularly those related to the investigation of hospital outbreaks to trace the source of contamination, as demonstrated in studies such as that by Martak et al. (2019), who evaluated the ability of FTIR to differentiate clones of Gram-negative bacilli (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Acinetobacter baumannii*) responsible for

hospital outbreaks. As reported by Martak et al., the IR Biotyper® was generally able to detect the spread of clones of *P. aeruginosa*, *K. pneumoniae*, *E. cloacae*, and *A. baumannii* in less than 3 hours. Another study also demonstrated the ability of FTIR to distinguish 1 isolates of *P. aeruginosa* and correctly group the strains associated with SARS-CoV-2 co-infections (De Souza et al., 2024), corroborating the findings mentioned above. These data suggest that FTIR can serve as a quick and effective tool for distinguishing strains and understanding the dynamics of microorganism propagation in the pharmaceutical industry.

With regard to the analysis of the scatter plot, a study demonstrated that it was possible to differentiate isolates of different *Staphylococcus aureus* serotypes using the scatter plot (Hong et al., 2022), which was similar to the results found in this study: according to the scatter plot, the strains with the highest spectral similarity were clustered together, while the most distinct ones appeared more scattered.

5 FINAL CONSIDERATIONS

Based on the results obtained in this study, it was concluded that the FTIR technique was able to rapidly differentiate fungal strains, demonstrating that it is a useful tool for tracing sources of contamination in industrial settings, and not only in the clinical setting for investigating hospital outbreaks. In addition to proving to be an effective technique for the typing of filamentous fungi, the analysis of the dendrogram, corroborated by the scatter plot, identified four clades of *P. citrinum*. Each clade contained strains that showed a high degree of similarity to one another, and the analysis of clade 4 was the most analytically significant, since it allowed us to infer that the strains belonging to this clade originated from the same batch of environmental monitoring plates, indicating that this was the likely source of the spread of *P. citrinum*.

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