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Biometric Metabolic Identification Using Fourier-Transform Infrared Spectroscopy

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Abstract: The rate of new antibiotic discoveries has recently slowed to a crawl. Antibiotic discovery has shifted back to phenotypic screening, but identifying mechanisms of action (MOA) remains a big challenge. Consequently, there is an urgent need for metabolic fingerprinting techniques like Fourier-Transform Infrared (FTIR) spectroscopy that can both deduce MOAs and screen complete cells at high throughput. To uncover the metabolic fingerprint caused by fifteen antibiotics on the metabolism of Escherichia coli, a bioassay based on high-throughput whole-cell FTIR spectroscopy was created. Spectra were swiftly obtained in the high-throughput mode after cells were exposed for a short period of time to a concentration four times greater than the minimum inhibitory concentration. Partially least squares discriminant and principal component analyses followed optimization of the preprocessing steps. Using either analysis algorithm, the biochemical fingerprints acquired using FTIR spectroscopy were highly specific enough to distinguish between several antibiotics across three separate cultures. These fingerprints aligned with the known modes of action (MOA) of all the antibiotics that were studied. This includes instances of antibiotics that target cell wall production, DNA, RNA, and proteins.

Keywords: Fourier-Transform infrared (FTIR), spectroscopy; high-throughput screening; mechanism-of-action (MOA); metabolic fingerprinting; multivariate analysis

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Introduction

Antibiotics have been a boon to contemporary medicine ever since their discovery, but progress has stalled since the heyday of discovery, when the majority of classes were presented [1]. The genomics era gave rise to target-based screening, however hits were often unsuccessful in penetrating their cellular targets, and no new drugs were developed as a result of these initiatives. Since phenotypic screening can target poorly understood

metabolic pathways and compounds that are effective against entire cells are more likely to become candidate molecules, it has largely replaced traditional antibiotic discovery strategies [3, 4]. It takes a lot of work later on in the discovery phase to figure out the candidate compounds' Mechanism-Of-Action (MOA), which these tests don't show. This leads to more compound rediscoveries but less insight into the pharmaceutical target and lower odds of discovering compounds with novel biological and/or chemical characteristics. Another area that phenotypic screening misses is the chemical grey matter, which consists of compounds that can modulate phenotypes to some extent but don't have the power to kill cells or inhibit growth. This group of compounds can be a gold mine for medicinal chemists looking to optimize leads [5].

In an effort to better understand how medicines and other bioactive small molecules work, a new field of study called chemoproteomics has emerged at the crossroads of medicinal chemistry, biochemistry, and cell biology. Quantitative mass spectrometry has recently become an integral part of many research strategies that integrate phenotypic screening with target identification to characterize drug-induced changes in cellular protein expression and post-translational modifications across the proteome [6]. This review summarises the experimental approaches taken by researchers in the field of chemical proteomics, talks about some recent successful applications, and identifies drug development domains that stand to benefit greatly from assays based on chemical proteomics that use native endogenous proteins.

Drug discovery and development remains a costly and inefficient process, even with significant investments in 'Omics' technologies. Although the importance of signaling networks in both health and illness has been better understood, target-based drug discovery remains the pharmaceutical industry's prevailing paradigm [7]. Proteins are the most common kind of therapeutic targets, hence methods based on proteomics, which allow for the study of many different proteins in a physiological setting, have a lot of potential. To be effective, however, these methods must be refined into practice so that they can supplement more conventional techniques to drug development. Therapeutic efficacy tests typically evaluate the biochemical activity of the isolated, pure target protein. Rather than using the full-length native proteins, recombinant enzymes or protein fragments are often utilized. Isolated recombinant proteins or protein fragments may not accurately portray the target's conformation and activity in a physiological setting due to factors such as improper folding, post-translational changes, or the absence of regulatory domains and interacting proteins. Because of this, results from these types of tests might not always indicate how well a medicine or chemical would work in in vivo or cell-based experimental settings. To analyze natural proteins in cell fractions or extracts, chemoproteomics uses conditions fine-tuned to maintain protein integrity, folding, post-translational modifications [8], and interactions with regulatory proteins. There are two main categories into which these techniques fall: (i) approaches based on global proteomics, which aim to characterize the cellular response to the small molecule at the cellular level (e.g., by measuring protein expression levels or defined post-translational modifications); and (ii) approaches based on targeted chemoproteomics, which use chemical probes designed to capture protein targets or entire sub-proteomes, for example, activity- or affinity-based target profiling. In this study, we will go over the basics of global proteomics and its recent uses in small molecule profiling, before moving on to chemoproteomics methods based on affinity and activity. Our goal is to show how these approaches can supplement or even replace the more traditional recombinant protein assays used in drug discovery [9].

Finding the exact mechanism of action of antibiotics has been just as difficult as discovering them [10]. An area where phenotypic screening is currently lacking is understanding how antibiotics work; here is where metabolomics could be a game-changer. Given the urgent need for novel antibiotics, the capacity to quickly infer MOA and, if feasible, the biomolecular target of antibiotics, is of utmost importance [11]. The growing availability of natural product libraries and the relative simplicity of synthesizing bioactive compounds have made the screening of hundreds of thousands of compounds a feasible drug discovery program throughput at now.

Macromolecular synthesis assays, the gold standard for studying the action mechanism, are infamously slow, prone to error, and defined by poor resolution and throughput [12]. Another limited choice is biochemical techniques like affinity chromatography, which can identify the particular biomolecule that a target molecule binds to [13]. Unfortunately, it's not unlike fishing in that it requires a little molecule with a strong affinity and a protein receptor that is abundantly available [13]. Another big problem with traditional mechanism of

action assays is that they require an excessively large dose of the drug being tested.

More frequently than not, genome-wide transcriptional or translational profiles show indirect stress responses rather than the precise chain of events that causes the inhibitory effect [14], but these profiles have recently been utilized to uncover the target of candidate molecules. Placing the metabolome at the base of the Omics cascade allows for its application in identifying drug-induced inhibition because it represents the substrates and products of different metabolic enzymes. Therefore, the initial metabolomics investigations into antibiotic action mechanisms focused on identifying the target(s) by observing changes in metabolite concentrations caused by a single molecule [15]. Metaboomics has been utilized more and more to construct thorough multi-parametric profiles of the MOA as our understanding of it has evolved from targets to networks [16]. Beyond non-metabolic targets, these profiles characterize drug-induced effects on a genome-scale [16]. Metabolic profiling has the potential benefit of taking into account the antibiotic impact of a drug through its underlying MOA as well as its on-target and off-target effects. Consequently, metabolic profiles were the primary focus of metabolomics investigations on MOA. By comparing the metabolic profiles of two isogenic strains of Staphylococcus aureus that are susceptible to and resistant to methicillin, researchers have been able to identify metabolic changes that are unique to the three antibiotics' actions on the three main biosynthetic pathways: cell wall, DNA, and protein biosynthesis [17]. Predicting epistatic drug interactions has also made use of metabolic profiling of drug exposure in conjunction with chemogenomic and metabolic profiles of single-deletion strains. Finding nonantibiotic chemicals that, when mixed, exert antimicrobial action allows for the rational creation of medication combinations. Dereplication and guided fractionation of new natural compounds with antibacterial characteristics also involve metabolic profiling.

Mass spectrometry (MS) is another method that has shown promise; it is particularly useful when coupled with chromatographic separation techniques and can detect a broader variety of metabolites with greater sensitivity. Annotation, or the process of identifying metabolites from these peaks, remains the bottleneck in MS-based metabolomics, although adequate throughput can be achieved with an untargeted approach, allowing thousands of ion peaks to be detected from individual samples [18]. Actually, the necessary throughput to methodically determine the MOA of relatively large collections of antibiotics can be achieved using untargeted metabolomics [19]. Nevertheless, eliminating the chromatographic phase is the current solution to achieve the 10-100x throughput improvement needed for large-scale studies [20]. This tradeoff forces researchers to choose between coverage and separation. So, a chemical library used in industry has its MOA predicted utilizing high-throughput untargeted metabolomics with flow injection electrospray, however at the expense of resolution. Alternative analytical methods may be more appropriate for the quick processing of many samples with little preparation and modification, falling under the "throughput" rather than "resolution" category; however, their output is intrinsically less meaningful. One well-established metabolic fingerprinting method is Fourier-Transform Infrared (FTIR) spectroscopy, which is reagent-and label-free, requires little sample handling, and has a high throughput [21].

Using Fourier-transform infrared spectroscopy, we characterized stomach cell infections caused by various strains of Helicobacter pylori.

About half of the global population is infected with Helicobacter pylori, a Gram-negative bacterium that colonizes the apical surface of human gastric epithelial cells and the mucous layers. Geographic location, age, socioeconomic position, living environment, and occupation are some of the factors that influence the prevalence of H. pylori. The frequency in underdeveloped nations often surpasses 80%, whereas in the West it is between 20% and 50% and falling. Because it thrives in acidic conditions, Helicobacter pylori is the most abundant bacterium in a "healthy" stomach. H. pylori may be nearly nonexistent in atrophic stomachs since it has less of a chance of survival compared to other bacteria. While the majority of people infected with H. pylori do not have any symptoms at all, about 10% get gastric or duodenal peptic ulcer disease (PUD), 1-3% get gastric cancer (GC), and a few others get mucosa-associated lymphoid tissue lymphoma [22]. Environmental variables, host genetics, and virulence characteristics of H. pylori strains are among the many causes of stomach diseases. Some examples of environmental influences are dietary intake, smoking habits, alcohol use, and the use of certain medications, such as anti-inflammatory and antibiotics [9]. Infection outcomes can be impacted by host characteristics such as polymorphisms in inflammatory response genes (e.g., IL-1 and IL-10), HLA haplotype, and Toll-like receptor

2 and 4 gene types. These factors can either intensify or dampen the inflammatory response linked to the infection, leading to pathology-related negative outcomes [23]. Various virulence factors of Helicobacter pylori also regulate the bacterial-gastric epithelial cell and immune system interaction. The severity of gastric disease is influenced by several virulence factors of Helicobacter pylori, the most important of which are vacuolating cytotoxin A (VacA) and cytotoxin associated gene A (CagA). These factors, among others, reduce cellular matrix adhesion, break intercellular junctions, and induce cell motility. Although not all strains of Helicobacter pylori express CagA, the most dangerous ones do, and those that do tend to cause the worst gastrointestinal problems and even more cases of gastric cancer [24]. The 120-145 kDa cytotoxin CagA is introduced into the host cell through a type IV secretion system. This triggers a cascade of reactions, including but not limited to: cellular morphology disruption (by encouraging the formation of gastric epithelium cell pedestals), cytoskeleton changes, cell polarity alteration, cell proliferation stimulation, and gastric epithelium cell IL-8 secretion.

An auto-transport of a secreted toxin measuring 88 kDa is caused by a 140 kDa precursor that is encoded by the VacA gene. The toxin has the potential to persist in cells' plasma membranes as anion-selective channels formed by huge oligomers with a "snowflake" structure. In vitro, internalized VacA induces significant "vacuolization" [25], which is defined as a cluster of giant vesicles with endosome characteristics. When host cells absorb VacA, it can trigger a cascade of events that include a lowered membrane potential, malfunction in the mitochondria, cell death, increased activity of mitogen-activated protein kinases, and a decrease in T cell activity. All known strains of Helicobacter pylori possess the VacA gene, which encodes a protein; the amount of this protein is genotype dependent. Each gene is categorized according to the amount of variability found at its signal sequence (alleles s1 and s2), mid-region (alleles m1 and m2), and intermediate-regions (alleles i1, i2, and i3). In comparison to less dangerous strains, those with the VacA s1/m1 genotype are more likely to cause gastric mucosal inflammation, gastric atrophy, and cancer. Type I H. pylori strains are typically involved in the induction of gastroduodenal disease because they are more likely to express the CagA+ and VacA s1/m1 genotypes, which are associated with increased gastric mucosal inflammatory cell infiltration and gastric epithelial injury compared to strains without these genotypes. In addition, it appears that antibiotic treatment is not as effective against these latter strains [26].

Treatment based on the combination of numerous antibiotics is already failing in several world regions, such Western Europe, where it fails in more than 20% of patients largely owing to antibiotic resistance. Unfortunately, there is currently no vaccination available for this bacterial infection. Hence, more research into the intricate relationships between H. pylori traits, host traits, and environment is required to tackle this major health concern. This knowledge could be enhanced by developing a technique that allows for the sensitive, high-throughput, and easy examination of how different strains of H. pylori affect host cell metabolism in various environments. The mid-infrared area of Fourier transform infrared (FTIR) spectra, which represents the fundamental vibrational modes of important biological macromolecules such proteins, carbohydrates, lipids, and nucleic acids, could be a useful tool in this regard [27]. Additionally, FTIR spectroscopy may detect the complete molecular composition of cells in a label-free, high-throughput (using, for example, 96-well microplates), simple (minimum sample preparation), inexpensive (no chemicals needed), and extremely sensitive manner.

As a result, Fourier transform infrared spectroscopy (FTIR) has found many uses in biology, including the assessment of cell growth, nutrient consumption (e.g., glucose and glycerol), metabolic by-products (e.g., ethanol and acetate) production and consumption, and the production of plasmids and recombinant proteins, among many others [27]. Bacterial typing and subtyping, cell cycle event detection and mechanism discrimination (between apoptosis and necrosis), and study and diagnosis of many diseases (including cancer) have all been made possible by the great sensitivity of FTIR spectroscopy.

Gastric adenocarcinoma (AGS) cell lines were examined in an earlier study using a high-throughput 96-well microplate FTIR spectroscopy equipment. The cells were cultured with H. pylori strains that exhibited two distinct genotypes, CagA+ and Vac-s2. In that study, three absorbance ratios were used to distinguish between the two bacterial genotypes and AGS cells that were treated with the bacteria. The current study assessed an intermediate genotype of H. pylori with more variability compared to its predecessors, the CagA−/VacA−s1 genotype. The molecular fingerprint of the bacteria incubated with AGS cells was also linked to the stomach

disorders such as non-ulcer dyspepsia (NUD) [28], PUD, or GC in the patients from whom the germs were recovered. To differentiate between AGS cells incubated with H. pylori and cells not incubated, to determine the H. pylori genotype in relation to CagA and VacA, and to identify the gastric pathology from which the bacteria were isolated, a significantly higher number of absorbance ratios—fourteen instead of three—were examined. Our goal is to help find spectral [29] biomarkers that can identify H. pylori infections and distinguish between different strains of the bacteria, particularly those linked to serious stomach diseases, and to spread awareness of a new way to learn about the complicated process of H. pylori infection.

Half of the world's population has Helicobacter pylori infections, which can cause a variety of gastrointestinal problems including ulcers and cancer throughout the course of a person's lifetime. In this study, H. pylori strains with different genotypes regarding the virulent factors vacuolating cytotoxin A and cytotoxin associated gene A were incubated with adenocarcinoma gastric cells, and Fourier-transform infrared spectra were obtained from these cells to aid in the search for biomarkers of bacterial infection. In order to determine if the test assumptions were satisfied, various statistical inference methods were used to assess the defined absorbance ratios. From the gastric cells, we were able to determine a variety of absorbance ratios that allowed us to distinguish between three things: (i) the type of infection; (ii) the genotype of the bacteria; and (iii) the gastric disease that the patients had. By providing a foundation for a new diagnostic approach that is sensitive, specific, and quick to diagnose infection and bacterial pathogenicity, these biomarkers have the potential to speed up our understanding of the intricate infection process [30].

In addition, Fourier transform infrared spectroscopy (FTIR) is a metabolic fingerprinting method that may reliably and quickly identify significant metabolome changes, especially those caused by antibiotics and stress factors. Furthermore, clinical isolates with antibiotic resistance have been successfully identified using FTIR microscopy. Particularly encouraging about FTIRS is the fact that it combines the best features of both phenotypic screening assays, which are known for their high-throughput nature and their low-information nature, with the low-throughput/high-information metabolomic assays [31]. Although FTIRS does not produce exhaustive metabolite-level data, the abundance of biological information it provides enables a better evaluation of the biomolecular processes underpinning the antibiotic effect; this has proven to be appropriate in research focused on MOA. Research on FTIRS's capacity to identify antibiotic-specific fingerprints has actually been extensive.

Application of Fourier transform infrared microscopy to the study of antimicrobial agent efficacy

When it comes to potentially fatal illnesses in both humans and other animals, bacteria are high on the list of culprits. Even though antibiotics are among the most powerful medications against bacteria, there are mutant strains that are resistant to these drugs. In fact, there are already many strains that are resistant to every antibiotic that is on the market. To ensure effective future therapy, it is crucial to find novel anti-bacterial medications and to develop early evaluation procedures for the treatments' efficiency [32]. Also, using quick identification procedures and accurate ways to measure the efficacy of the treatment could drastically cut down on infectious disease-related deaths and expenses. The majority of the identification methods that are now on the market rely on nutritional (media composition, sugar assimilation, enzymatic testing, etc.) and physiological (morphology, growth temperature, etc.) traits. The problem is that these traditional exams take a long time and don't always provide accurate results Examples :

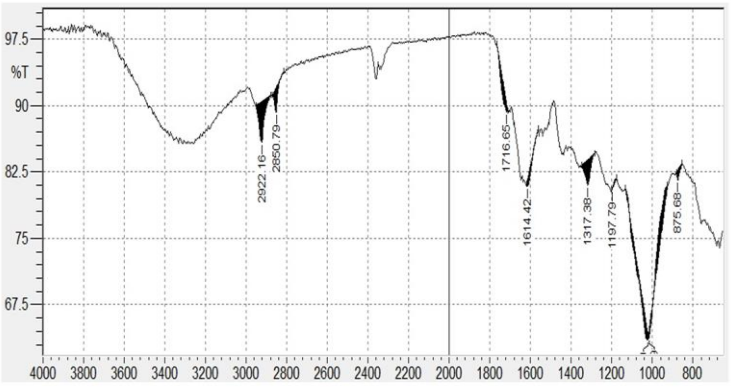

Table 1. FT-IR peak values of solid analysis of *Passiflora caerulea.*

No.	Peak (Wave number cm-1)	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area	Type of Intensity	Bond	Type of Vibration	Functional group assignment	Group frequency
1.	875.68	81.563	1.123	881.47	854.47	2.266	0.084	Strong	=C–H	Bending	Alkenes	650-1000
2.	1016.49	63.828	0.776	1020.34	925.83	12.733	0.091	Strong	C-F	Stretch	alkyl halides	1000-1400
3.	1024.20	63.550	0.936	1132.21	1020.34	16.466	0.352	Strong	C-F	Stretch	alkyl halides	1000-1400
4.	1197.79	80.489	0.124	1199.72	1176.58	2.079	0.004	Strong	C-F	Stretch	alkyl halides	1000-1400
5.	1317.38	81.051	2.838	1346.31	1284.59	5.107	0.417	Strong	C-F	Stretch	alkyl halides	1000-1400
6.	1614.42	80.833	0.751	1618.28	1562.34	4.241	0.112	Bending	N-H	Stretch	Amide	1550-1640
7.	1716.65	89.259	0.978	1764.87	1710.86	1.969	0.261	Strong	C=O	Stretch	Acid	1700-1725
8.	2850.79	89.239	2.861	2870.08	2814.14	2.088	0.208	Strong	C-H	Stretch	Alkane	2850-3000
9.	2922.16	85.898	4.577	2951.09	2879.72	3.716	0.655	Strong	C-H	Stretch	Alkane	2850-3000

Because of their simplicity, speed, low cost, and sensitivity, spectroscopic methods hold considerable potential for the detection and identification of microorganisms. The quantitative and qualitative data about a sample can be uncovered by employing spectroscopic techniques. A distinct "finger print" can be extracted from the infrared spectra of each given chemical [33]. The combination of this with the extensive knowledge of spectrum bands derived from FTIR spectra of live cells makes FTIR spectroscopy a promising tool for the detection and identification of infectious diseases. Several types of microbes, cancer cells, and virus-infected cells were positively identified using Fourier transform infrared spectroscopy. Recently, it has also found application in testing how well antiviral medications work.

For thousands of years, people have turned to propolis (PE), a honeybee product made from resins extracted from specific plants and trees, for a variety of medicinal uses. It is believed that flavanoids, the most abundant and biologically active class of PE chemicals, are crucial to the bioactivities of the molecule. This substance exhibited remarkable antibacterial action, primarily against Gram positive bacteria, among its other bioactivities.

The Fourier transform infrared spectroscopy has been an invaluable tool for chemists in their quest to understand both organic and inorganic substances. This study looked at the possibility of using Fourier transform infrared microspectroscopy to assess the efficacy of antimicrobial treatment at an early stage. Caffeic acid phenethyl ester (CAPE) and ampicillin were tested for their effects on bacterial infection development in cell culture for this aim. Among the many antimicrobial properties of propolis, a natural honeybee product, CAPE stands out. Our findings reveal distinct and noteworthy spectrum indicators for effective CAPE treatment as early as 2 hours after treatment, however several of these biomarkers exhibited different patterns in Gram-negative bacteria compared to Gram-positive bacteria. The intensity of the bands at 682 and 1316 cm−1 drops in all the Gram (−) bacterial strains that were tested, while it spikes dramatically in all the Gram (+) bacterial strains. Conversely, as compared to the control group of untreated bacteria, ampicillin-treated Gram (+) and Gram (−) bacteria exhibited no spectral differences. Early evaluation of the efficacy of the antibacterial effect of CAPE and likely other utilized medications can be achieved with the use of FTIR spectroscopy.

Conclusion:

Antibiotic discovery has shifted back to phenotypic screening, but identifying mechanisms of action (MOA) remains a big challenge. Consequently, there is an urgent need for metabolic fingerprinting techniques like Fourier-Transform Infrared (FTIR) spectroscopy that can both deduce MOAs and screen complete cells at high throughput. To uncover the metabolic fingerprint caused by fifteen antibiotics on the metabolism of Escherichia coli, a bioassay based on high-throughput whole-cell FTIR spectroscopy was created. Spectra were swiftly obtained in the high-throughput mode after cells were exposed for a short period of time to a concentration four times greater than the minimum inhibitory concentration. Partially least squares discriminant and principal component analyses followed optimization of the preprocessing steps. Using either analysis algorithm, the biochemical fingerprints acquired using FTIR spectroscopy were highly specific enough to distinguish between several antibiotics across three separate cultures. These fingerprints aligned with the known modes of action (MOA) of all the antibiotics that were studied. This includes instances of antibiotics that target cell wall production, DNA, RNA, and proteins. There is promising future usage for Fourier transform infrared spectroscopy for high-throughput screening of antibiotic candidates and, maybe, for elucidating the mechanisms of action (MOA) of existing antibiotics, as it provides a comprehensive fingerprint of the impact of antibiotics on cellular metabolism.

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