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Application of a solid-phase microextraction-gas chromatography-mass spectrometry/metal oxide sensor system for detection of antibiotic susceptibility in urinary tract infection-causing *Escherichia coli* – a proof of principle study

Keywords

Volatile organic compounds; gas chromatography-mass spectrometry; metal-oxide sensor; antibiotic resistance; urinary tract infection.

Abstract

Purpose: Antibiotic resistance is widespread throughout the world and represents a serious health concern globally. There is therefore an urgent need for the development of novel tools for rapidly distinguishing antibiotic resistant bacteria from susceptible strains. Previous work has demonstrated that differences in antimicrobial susceptibility can be reflected in differences in the profile of volatile organic compounds (VOCs) produced by dissimilar strains. The aim of this study was to investigate the effect of the presence of cephalosporin antibiotics on the VOC profile of extended spectrum beta-lactamase (ESBL) and non-ESBL producing strains of *Escherichia coli*

Material and methods: In the study, VOCs from strains of *Escherichia coli* positive and negative for the most commonly encountered ESBL, CTX-M in the presence of cephalosporin antibiotics were assessed using solid-phase microextraction (SMPE) coupled with a combined gas chromatography-mass spectrometry/metal oxide sensor (GC-MS/MOS) system.

Results: Our proof-of-concept study allowed for distinguishing CTX-M positive and negative bacteria in 2 h after the addition of antibiotics. One MOS signal (RT: 22.6) showed a

statistically significant three-way interaction ($p = 0.033$) in addition to significant two-way interactions for culture and additive ($p = 0.046$) plus time and additive ($p = 0.020$). There were also significant effects observed for time ($p = 0.009$), culture ($p = 0.030$) and additive ($p = 0.028$). No such effects were observed in the MS data. *Conclusions:* The results of our study showed the potential of VOC analysis using SPME combined with a GC-MS/MOS system for the early detection of CTX-M-producing, antibiotic-resistant *E. coli*, responsible for UTIs. This proof of concept work involves bacterial cultures with the addition of antibiotics and future work would see the approach extended to urine samples of patients.

1. Introduction

Antibiotic resistance is widespread throughout the world and represents a serious health concern. The infections associated with antibiotic-resistant bacteria are increasing, in 2004 it was estimated that approximately 70 % of all infections were caused by bacteria resistant to at least one antibiotic [1]. Urinary tract infections (UTIs) are one of the most common bacterial pathologies with 150 million cases annually. UTIs are caused by a variety of bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* [2].

UTIs have tended to be treated with broad-spectrum antibiotics, fuelling antibiotic resistance in *Enterobacteriaceae* and others. The increasing prevalence of antibiotic resistance has led to the development of second, third and fourth generation antibiotics. However, bacteria are often able to develop resistance to new medications.

One significant concern is the resistance of *Enterobacteriaceae* to later generations of cephalosporins due to the acquisition of plasmids encoding extended-spectrum β -lactamases (ESBLs) [3]. The treatment options for ESBL producing bacteria are limited as they confer resistance to many available antibiotics [4]. The most common of the ESBL variants is the CTX-M group of enzymes capable of hydrolyzing third-generation cephalosporins such as cefotaxime. A recent study showed that the prevalence of UTIs caused by CTX-M producing cefotaxime-resistant *E. coli* reached 91 % in the American population [5], making the identification of ESBLs and CTX-M producing bacteria of crucial importance.

Despite the improvements in diagnostic technologies, the gold standard in the detection of antibiotic-resistant bacteria is still based on traditional microbiology testing. Antibiotic susceptibility tests are usually performed on either liquid or solid media and rely on changes in optical density or the presence of inhibition zones [6]. However, these tests usually take

between 24 to 48 hours and there have been several scientific attempts to reduce the time for diagnosis.

The application of molecular techniques based on polymerase chain reaction (PCR) and spectrographic methods has been evaluated for more rapid antibiotic susceptibility testing [7,8]. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF-MS) is able to reduce the time of analysis to 3 h and is used routinely for species identification, however, it is not an universal identifier of resistance [9]. However, these methods are rarely applied in clinical practice due to the high associated costs.

A number of studies have shown that volatile organic compounds (VOCs), carbon-based molecules which are volatile at ambient temperature, can be useful for distinguishing infections caused by antibiotic-resistant bacteria [10–12]. The hypothesis is that the ability of bacteria to show resistance to antibiotics results in metabolic changes, and this consequently affects the VOCs produced. Direct mass spectrometry methods such as Proton Transfer Reaction - Mass Spectrometry (PTR-MS) allowed the difference between susceptible and resistant strains of *Mycobacterium smegmatis* to be observed within 6 h [13]. However, PTR-MS does not allow for the identification of the metabolites responsible for enabling differentiation.

Gas chromatography-mass spectrometry is still the most commonly used method for the detection of bacterial VOCs. A pilot study comparing cephalexin and ciprofloxacin susceptibility in twenty-two UTI-causing bacteria using automated thermal desorption (ATD) with GC-MS showed clear differences between susceptible and resistant strains [14]. Another study [15] showed that the analysis of VOCs in the headspace above bacterial cultures allowed resistant and susceptible bacteria to be distinguished based on the abundance of six VOCs after an incubation time of 6h with an overall accuracy of 85.7 %.

MOS are sensors consisting of metal oxides e.g. tin dioxide, zinc oxide or tungsten oxide that are typically operated at elevated temperatures and exhibit reversible changes in electrical resistance when exposed to a range of gases and VOCs. MOS exhibit high sensitivity to a range of VOCs but the selectivity can be poor. They also tend to be sensitive to changes in environmental conditions such as temperature and humidity. Typically the selectivity is improved by the use of complex oxides [16], nanostructured materials or catalytic dopants. However, interfacing the MOS with a GC and using as a detector negates some of the poor selectivity and stability issues but takes advantage of the high sensitivity to a range of VOCs. Wiesner et al. [10] reported that metal oxide sensors (MOS) had potential use in the faster detection of antibiotic susceptibility. In previous work the combined GC-MS/MOS system showed the potential to detect VOCs in several matrices, thus combining the high sensitivity of MOS with the identification of specific VOCs by the mass spectrometer [17]. The analysis of headspace above *E. coli* cultures showed more peaks detected with the MOS than the MS detector [17]. In addition the MOS sensor was more sensitive than the MS detector when used for the detection of a mixture of 29 standard VOCs. Of these 29 compounds, the MOS exhibited better sensitivity to 17 when compared to the MS, including to typical bacterial VOCs such as butan-1-ol, butan-2-one and 1*H*-indole.

The aim of this study was to evaluate the changes in culture headspace VOC profiles when different generations of cephalosporin antibiotics were added into the CTX-M producing bacteria. This was assessed using solid-phase microextraction (SMPE) combined with a GC-MS/MOS system. This proof-of-concept study will hopefully lead to further development of rapid, point-of-care devices for the early detection of ESBL producing bacteria.

2. Material and Methods

2.1. Bacterial cultures

One CTX-M positive strain of *E. coli* isolated from primary care [18] and one ESBL negative laboratory strain were each inoculated from a single colony into 250 mL of Luria-Bertani broth (LB) and incubated overnight at 37 °C with shaking. The following day, the *E. coli* culture was used to inoculate 3 sets of 3 mL of fresh LB broth. To one set was added 20 µg/mL of cefotaxime (CTX), to the second, 40 µg/mL cefepime (CFP) and the third left free of antibiotic. Incubation was carried out for 2 and 4 h, respectively. The optical density of duplicate broths at each time point was measured at 550 nm and dilutions performed to keep the measurement in the optimum range for the spectrophotometer. Plate counts were performed on LB agar beforehand to allow calculation of cell counts at these densities. Two biological replicates of each strain were performed on different days from fresh primary agar culture each time, giving a total number of six replicates of each condition.

2.2. SPME

After the incubation with antibiotics, the SPME fibre with CAR/PDMS coating was inserted for 1 h into the headspace above the cultures heated to 55 °C. The higher temperature stopped further growth of the bacteria and was optimal for the transfer of VOCs into the headspace. After the heating stage, the fibres were retracted into the transport holders, secured with additional septa and transported to the GC-MS laboratory on ice.

2.3. GC-MS/MOS analysis

The fibres were introduced into a gas chromatography injector port with 0.75 mm ID splitless glass (PerkinElmer, Inc., Maryland, USA), set to a splitless mode and a temperature of 240 °C. The thermal desorption of VOCs was carried out for 10 min to avoid carryover (data not presented).

Analysis of VOCs was performed using a single quadrupole Perkin Elmer GC-MS Clarus 500 instrument (PerkinElmer, Inc., Maryland, USA). The separation of VOCs was undertaken using a Zebron-624 column, 60 m × 0.25 mm × 1.40 μm, (Phenomenex, Torrance, USA). The carrier gas was 99.9995 % pure helium (AirProducts, Crewe, UK) set at 22.8 psi, 31.5 cm s⁻¹, with a column flow of 3.1 ml min⁻¹ at 40 °C dropping to 1.6 ml min⁻¹ at 240 °C was used throughout this work. The GC output was split using an S-Swafer (PerkinElmer, Inc., Maryland, USA) equally between the MS and MOS detectors.

The oven temperature program was set as follows: 40 °C held for 2 min, and increased to 240 °C at a rate of 10 °C/min and maintained at the final temperature for 8 min, giving a total run time of 30 min. Mass spectra were obtained by electron ionization acquired in full scan mode with a scan range of m/z 35–450 used for data acquisition. The operating conditions for the MS were as follows: electron ionization mode at an energy of 70 eV; transfer line and ion source temperatures were 280 °C and 180 °C, respectively. Total ion chromatograms (TIC) were analyzed with the Turbomass software (PerkinElmer, Inc., Maryland, USA) and compounds are expressed as the area under the curve. Moreover, the chromatograms were analyzed using the free software Automated Mass Spectral Deconvolution and Identification System (AMDIS). The deconvoluted peaks were tentatively identified, where possible, by comparison of the mass spectra with the NIST/EPA/NIH Mass Spectral Library (version 2.2, 2014). Only the components with a match factor > 70 % were listed by name, while other compounds were named as “unknown”. For all the peaks, retention indexes were calculated according to d’Acampora Zellner et al. [19].

All MS responses were subject to a manual search and in order to directly compare (synchronise) the MS (peak area) and MOS (resistance change) data the MOS output was converted to $\Delta R/\Delta T$ and was aligned with the GC-MS chromatogram by comparing the largest

peaks. This was carried out in order to tentatively identify the peaks detected by the MOS and used in the statistical models.

2.4. MOS preparation

MOS are highly sensitive detectors, in which the detection is typically via a reversible change in their electrical resistance [20]. In n-type materials such as tin oxide/zinc oxide the interaction with reducing VOCs causes a decrease in the electrical resistance. By displaying the negative only $\Delta R/\Delta T$ of the MOS sensor this produces a trace which aligns well with a typical total ion chromatogram produced by the MS detector.

The preparation of the sensor coating and test chamber have been described in detail elsewhere [17]. The sensor substrate was coated with a thick film metal oxide paste containing 50 % zinc oxide nanopowder and 50 % tin oxide (IV) nanopowder, both with a particle size < 100 nm purchased from Sigma Aldrich (Saint Louis, MO, USA).

The sensor was mounted on a polytetrafluoroethylene base and placed in an aluminium chamber of 5 cm³ volume. The aluminium chamber was mounted above the GC oven and the GC column was interfaced with the chamber and positioned perpendicular to the sensor, approx. 5 mm from the sensor surface. The sensor operating temperature was 450 °C. The flow of helium from a GC column was dropping from 3.1 mL per min at 40 °C to 1.6 mL per min at 240 °C. The sensor chamber was purged with synthetic air (287478-L-C, BOC Ltd, Guildford, UK) at a constant flow of 180 mL per min. This is because the sensor requires oxygen to be present in order to work effectively.

2.5. Data analysis

Multivariable linear discriminant analysis was used to identify VOCs which would discriminate between sensitive and resistant strains and additives at both 2 and 4 h. The

experimental study comprised three factors: incubation time (2 h, 4 h), additive (CFP, CTX, LB) and culture (sensitive, resistant) with three replicates of each, giving $2 \times 2 \times 3 = 12$ -factor combinations ($N = 36$ in total). As such MOS and MS output data would be amenable to analysis using an analysis of variance for a $2 \times 2 \times 3$ fully independent design.

Comparison of the peak areas of individual VOCs of CTX-M negative and CTX-M producing strains, at 2 and 4 h, with different antibiotics was performed at each retention time using an analysis of variance (ANOVA) for a $2 \times 2 \times 3$ independent design.

3. Results

Although the addition of antibiotics to the media resulted in the eventual inhibition of bacterial growth as expected, all cultures began by showing a degree of turbidity in the first 60-90 min of incubation. After 150 min had elapsed, the CTX-M producing strain showed high turbidity in the antibiotic-free media and the presence of the third-generation cephalosporin, CTX. In the presence of the fourth-generation cephalosporin, CFP, cell lysis had occurred as expected given the high concentration used, giving low turbidity. In contrast, the CTX-M negative strain showed a reduction in turbidity with both antibiotics after 150 min.

Figure 1 and Table 1 show the comparison between the output MOS and MS detectors when analyzing the headspace above the same bacterial culture. Only the chromatographic peaks detected in at least half of the replicates were considered during the statistical analysis of the data. Using this method a total of 74 signals were detected using MOS, while MS allowed for the detection of 61 individual peaks. Fifty of these features were found to be common between the two detectors, meaning that the MS detected 11 additional peaks and the MOS 24 additional peaks..

Canonical Discriminant analysis of the MOS output (Figure 2) showed that discrimination between the effects of the three antibiotics (irrespective of the antibiotic susceptibility) after 2 h, could be achieved using a model based on two sensor signals (retention time (RT): 19.5 and 22.3) giving 66.7, 50.0 and 100 % accuracy for CFP, CTX and LB, respectively. Comparing the signals to the MS did not allow for the chemical identification of these compounds, because the NIST library match was too low, < 70 % match for the peak at 19.5 minutes, whereas the second signal (RT: 22.3 min) has no corresponding peak in the MS chromatogram.

Extending the time to 4 h allowed for distinguishing between the effects of the three antibiotics with 100 % accuracy based on a linear combination of nine MOS signals (RT: 13.05, 14.93, 16.3, 17.9, 18, 20.6, 20.85, 21.4, 21.6). The comparison of these MOS retention times to the corresponding MS output allowed for the tentative identification of 2-methylpropan-1-ol, propanoic acid, 3-methylbut-2-enal, butyl propanoate and heptan-2-one. Whereas, three MOS signals had corresponding MS peaks with a low NIST library match quality < 70 % (classified as unknowns), while one peak (RT: 21.6) had no corresponding MS peak.

The separation between CTX-M negative and positive bacteria (irrespective of the antibiotic added) allowed for the determination of CTX-M positivity with 100 % accuracy after 2 h. This was based on a statistical model consisting of seven MOS signals (RT: 13.05, 15.12, 15.4, 20.2, 20.85, 21.5, 23.25) with 100 % accuracy. Four compounds corresponding to the signals used in the statistical model were tentatively identified as 2-methylpropan-1-ol, 1-pentanol, 2-ethylhexan-1-ol and 1-phenylethanone, while one compound had a low match quality (unknown) and two had no corresponding MS peak.

Extending the time to 4 h allowed a statistical model based on a reduced number of VOCs (RT: 16.2, 19.2, 21.2, 26.5) to be developed. This model had a high accuracy of 88.9 % for

sensitive) and 100 % for resistant bacteria respectively. Three compounds were tentatively identified as hexanal, butyl 2-methylpropanoate, phenylmethanol, while one compound had no corresponding MS peak.

The same statistical analysis applied to the MS data (Figure 3) did not allow for the construction of a successful model after 2 h incubation. The models were not able to separate the different classes on the basis of either antibiotic or antibiotic susceptibility.

After 4 h incubation, the applied antibiotics (irrespective of the antibiotic susceptibility) could be separated with 100% accuracy by a statistical model based on five VOCs (RT: 12.44, 15.0, 18.3, 20.24, 20.57). The tentative identification of the compounds using the NIST database showed that the VOCs allowing for the separation between the antibiotics are: butan-2-one; propanoic acid; 2,5-dimethylpyrazine; 2-ethylhexan-1-ol and an unknown (tentatively identified as a pyrazine derivative).

The separation between CTX-M negative and positive bacteria with 88.9% accuracy was achieved using a statistical model based on five VOCs (RT: 15.15, 17.03, 17.38, 17.93, 19.98). The NIST library allowed for the tentative identification of two of these VOCs 1-pentanol and heptan-2-one, whereas one compound was unknown but tentatively classified as a xylene derivative (17.38), and the remaining two compounds were classified as unknowns.

The experimental study comprised three factors: incubation time (2 h, 4 h), additive (CFP, CTX, LB) and culture (sensitive, resistant) with three replicates of each, giving $2 \times 2 \times 3 = 12$ -factor combinations (N = 36 in total). The proof-of-concept study hypothesis would be for a three-way interaction i.e. the effect of additive (CFP, CTX, LB) would differentially vary with culture (sensitive, resistant) and this dependency would differ between 2 and 4 h. For MOS one signal (RT: 22.6) showed a statistically significant three-way interaction ($p = 0.033$), significant two-way interactions for culture and additive ($p = 0.046$), time and additive ($p = 0.020$), and with significant main effects for time ($p = 0.009$), culture ($p = 0.030$) and

additive ($p = 0.028$) as shown in Figure 4. No such effects were present in the MS data. Interestingly, a signal reported at 22.6 min on MOS has no corresponding peak in the MS, therefore the tentative identification using NIST was not possible.

4. Discussion

UTIs are often treated with inappropriate antibiotics, thus resulting in several complications, leading to higher medical costs and higher mortality [21]. It is of particular importance for the infections caused by CTX-M-producing bacteria, for which the risk of mortality is much higher if the appropriate treatment is applied too late [22]. Additionally, early identification of patients colonized by CTX-M producing strains can lead to improved infection control procedures and significant reduction in the spread of resistance. There is an urgent need for developing rapid and simple tools for identifying CTX-M producing bacteria.

There have been many advancements in the early detection of antibiotic resistance [1], but many of these technologies are sold as “only for research”, are still in development, or the further steps to incorporate them into clinical practice have not been adequately considered [23].

There are several promising tests based on the amplification of specific resistance genes which can give a result in 2.5 h [24,25]. Despite the high potential of these tests, the number of the resistance genes detected by such assays is limited and novel genes frequently emerge and can be involved in antibiotic resistance. Moreover, the presence of resistant genes does not always correspond to phenotypic resistance [26] leading to unanticipated therapeutic failure. Tests that rely on phenotype, i.e. the growth of bacteria irrespective of the specific genes, is potentially more promising.

Phenotype-based dual-enzyme trigger-enabled cascade technology (DETECT) assay showed promise to detect resistant UTI causing bacteria in 3 h from bacterial culture with 97.9, 94 and

89.4 % accuracy for ciprofloxacin, nitrofurantoin and trimethoprim/sulfamethoxazole respectively [27]. However, the main drawback of this assay is dependence on bacterial growth which can be the most limiting step. In our study, promising results could be obtained after 2 h of bacterial incubation with antibiotic. This time is shorter than that reported for other assays [9,14,27] and further reductions using our methodology are still feasible. In addition MOS are inexpensive and can be miniaturized and coupled with an appropriate statistical algorithm to produce a useful point-of-care device. This would be beneficial as transport and other logistic considerations cause delays in testing patient samples and reaching an appropriate diagnosis to enable effective treatment.

MOS have previously shown the potential for bacterial pathogen detection in various matrices [28–31]. This is the first time they have been combined with simultaneous MS detection and applied to the differentiation of bacteria by volatile profile. In this study, we showed that the higher sensitivity of the MOS allows for the detection of third generation cephalosporin resistant, CTX-M-producing *E. coli* in 2 h after the addition of antibiotics to the medium, which was not possible when using the MS alone.

MOS has some advantages over the MS detector. Firstly, the MOS does not detect siloxanes which are common contaminant of the chromatograms resulting from column bleed, septa bleed and degradation of SPME fibres based on PDMS [32]. Siloxanes are large molecules, which possess high volatility and are not oxidized by metal oxides [17]. It is an advantage of MOS over MS because siloxanes can interject false information into group comparison since despite often being method derived from GC columns/septa etc., are commonly used in cosmetics, personal care products and household items [33]. The detection of siloxanes would also reduce the ability of the sensor to recover and mask other, potentially more relevant analyte responses. In our study, all siloxanes were removed from the MS output as they do not contribute any significant information for antibiotic-resistance testing. Secondly, the

advantage of MOS is the very low price compared to the MS. E-nose technology using an array of MOS combined with pattern recognition software has found multiple applications to date [34–36].

As mentioned the MOS sensor was found to be more sensitive than the MS to a range of VOCs [17]. However, not all VOCs can be detected at low concentrations by the MOS. For example, nonane and other alkanes are difficult to detect by MOS due to the high number of carbons and the lack of oxygen species which impedes the catalytical breakdown [17]. Similar findings were reported by Kohl et al. [37], who compared the responses of MOS consisting of different metal oxides and their mixtures to alkanes, alcohols, aldehydes, ketones, acids and esters. ZnO/SnO₂ containing MOS were found to be very sensitive to aldehydes, alcohols and acids with a limit of detection approaching 1 vppb, but the MOS could not detect alkanes at the concentration range used in the study [37].

In this study the MOS did not detect sulfides (methylsulfanylmethane, (methyldisulfanyl)methane and (methyltrisulfanyl)methane), which were detected in the headspace above bacterial cultures by the MS detector. This highlights that although the MOS has enhanced sensitivity for many oxygenated compounds they have inherent selectivity which limits their sensitivity to alkanes and siloxane impurities and other classes of compounds such as sulfides. Although metal oxides have been utilised extensively for the detection of sulfides [38], many studies reported a degradation in performance possibly caused by poisoning of the catalytic metal oxides. There are several factors influencing the sensitivity of the MOS, including ambient temperature and humidity and surface reactions, surface modification and microstructure of the sensing layers [39]. Therefore, MOS can be optimized for better detection of sulfides if required. For example, gold particles were previously found to enhance the response to sulfides [40].

In our study, the main aim was to obtain the maximum number of peaks/signals, increasing the chance of finding markers associated with antibiotic susceptibility. A possible reason that the sensitivity to sulfides is reduced could be related to the use of nanomaterials in contrast to the previously reported study [36]. Even though it was suggested that using nanomaterials increased the overall sensitivity of the sensor [41,42], the excessive decrease of the particle size can cause the reduction in the structural stability and consequently changes in the surface and catalytic properties of the MOS [43,44].

In the models obtained via Canonical Discriminant analysis, the most common VOCs identified as being significant discriminators were 2-methylpropan-1-ol, propanoic acid, heptan-2-one and 1-pentanol. The presence of propanoic acid has previously been identified to indicate the presence of anaerobes [45]. Heptan-2-one is known to be produced during the decarboxylation of fatty acids. Heptan-2-one was previously reported as a distinguishing VOC between methicillin-resistant and -susceptible *Staphylococcus aureus* [11]. Similarly, 1-pentanol was previously reported to discriminate between groups of susceptible and resistant *E. coli* after incubation with gentamicin [45].

The main limitation of this study is that it was performed on isolated bacterial cultures and not on real urine samples from UTI patients, in which the concentration of bacteria could be much lower. Since the MOS is more sensitive than MS, the VOCs in the urine matrix could be detectable despite dilution. Furthermore, the normalization of urine samples to account for concentration differences should be considered when designing future studies. This will help to translate these findings into clinical practice. Moreover, the techniques described in this study could be extended to other bacteria causing UTIs, not only *E. coli*, and could identify additional resistance mechanisms in parallel. In addition the technique of VOC analysis using GC-MOS sensor instruments coupled with pattern recognition software or statistical

algorithms could be used to detect a variety of pathogenic infections of humans. These same techniques could be extended to other human diseases causing metabolic changes.

Future work on detecting antibiotic susceptibility of bacteria should focus on addressing the challenges of detection in clinical samples and extending the range of bacteria and antibiotics utilized. Once this has been established efforts to reduce the time of the assay should be the primary focus.

5. Conclusions

In conclusion, the results of our study showed the potential of the GC-MS/MOS system for early detection of ESBL-producing, antibiotic-resistant *E. coli*, responsible for UTIs. Our proof-of-concept study allowed for distinguishing susceptible and resistant bacteria, where the very common ESBL, CTX-M is the resistance mechanism involved, in 2 h after the addition of antibiotics, which has the potential to be further shortened in future studies. The results obtained, even though promising, were performed on small sample size and has to be confirmed in the large scale studies with a much larger number of samples to be analyzed. Moreover, the future studies with the application of real samples (urine) must be performed to confirm our findings.

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