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Ceria nanocubic-ultrasonication assisted dispersive liquid–liquid microextraction coupled with matrix assisted laser desorption/ionization mass spectrometry for pathogenic bacteria analysis

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ABSTRACT

A new ceria (CeO_2) nanocubic modified surfactant is used as the basis of a novel nano-based microextraction technique for highly sensitive detection of pathogenic bacteria (*Pseudomonas aeruginosa* and *Staphylococcus aureus*). The technique uses ultrasound enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) with and without ceria (CeO_2) followed by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). In order to achieve high separation efficiency, we investigated the influential parameters, including extraction time of ultrasonication, type and volume of the extraction solvent and surfactant. Among various surfactants, the cationic surfactants can selectively offer better extraction efficiency on bacteria analysis than that of the anionic surfactants due to the negative charges of bacteria cell membranes. Extractions of the bacteria lysate from aqueous samples via UESA-DLLME-MALDI-MS were successfully achieved by using cetyltrimethyl ammonium bromide (CTAB, 10.0 μL, 1.0×10^{-3} M) as surfactants in chlorobenzene (10.0 μL) and chloroform (10.0 μL) as the optimal extracting solvent for *P. aeruginosa* and *S. aureus*, respectively. Ceria nanocubic was synthesized, and functionalized with CTAB (CeO_2@CTAB) and then characterized using transmission electron microscopy (TEM) and optical spectroscopy (UV and FTIR). CeO_2@CTAB demonstrates high extraction efficiency, improve peaks ionization, and enhance resolution. The prime reasons for these improvements are due to the large surface area of nanoparticles, and its absorption that coincides with the wavelength of MALDI laser (337 nm, N_2 laser). CeO_2@CTAB-based microextraction offers lowest detectable concentrations tenfold lower than that of without nanoceria. The present approach has been successfully applied to detect pathogenic bacteria at low concentrations of 10^4–10^5 cfu/mL (without ceria) and at 10^3–10^4 cfu/mL (with ceria) from bacteria suspensions. Finally, the current approach was applied for analyzing the pathogenic bacteria in biological samples (blood and serum). Ceria assist surfactant (CeO_2@CTAB) liquid–liquid microextraction (LLME) offers better extraction efficiency than that of using the surfactant in LLME alone.

1. Introduction

Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a soft ionization technique which allows sensitive analysis of biomolecules [1,2]. Today, MALDI-MS has been considered as a routine technique as it can be applied to analyze bacteria [3–8]. MALDI-MS is a rapid technique for microorganism analysis [3–8] based on their biomarker peaks.

Analysis of bacteria in biological samples is usually a challenge task because of their diversity, complexity, and low concentration. Detection of bacterial infection at the initial stage is tremendously important for clinical treatment. In contrast, MALDI-MS analysis from biological analysis typically suffers from intense interferences or suppression effect due to the presence of complicated and huge amount of biological biomolecules. Therefore, it is necessary to preconcentrate the environmental/biological samples prior to MALDI-MS analysis. Preconcentration techniques such as liquid–liquid extraction (LLE) or solid phase extraction (SPE) are necessary to analyze trace amounts of analytes [9–11].

Recent trend in analytical techniques is to reduce the solvent amount or chemicals that were used in the preconcentration steps. It is also necessary to miniaturize the solvents in order to decrease the analysis time [11-13]. The first attempt of liquid phase microextraction (LPME) was the single drop microextraction (SDME). SDME is a rapid, simple, and inexpensive technique [14,15]. For high extraction

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efficiency, a method called dispersive liquid–liquid microextraction (DLLME) [16] was proposed based on a dispersive solvent such as methanol, propanol, or butanol. Further, the ultrasound enhanced surfactant assisted dispersive liquid–liquid microextraction method (UESA-DLLME) was proposed [17–21]. It has been developed to avoid the use of disperser solvent such as methanol, propanol [17–21]. The UESA-DLLME could accelerate the extraction efficiency based on the use of ultrasonication and it also offers many analytical merits, such as relatively low cost, eco-friendly, easy handling, no toxic effects, and it can provide satisfactory results with the use of the least volume of the solvent. Surfactants are amphiphilic organic compounds which contain both hydrophobic and hydrophilic moieties, so it is soluble in both organic and aqueous medium. Surfactants can reduce the interfacial tension between the organic and water layers resulting in increase the contact areas and to improve the extraction efficiency. Nanoparticles (NPs) prepared in organic layers have been extensively applied in order to increase the mass transfer of target analytes. NPs miniaturize the extraction solvents, improve detection sensitivity and increase the extraction efficiency [22–24]. Potential applications of nanoparticles in sample preparation are discussed in [25]. Comprehensive discussions for various microextraction methods have been reviewed in [26–30]. Cerium oxide nanoparticles or ceria (CeO_2) were applied in biomedical applications due to their nice biocompatibility [31]. Ceria (CeO_2) nanocubic exhibits high positive zeta potentials, thus it can assist protein adsorptions [32]. The electrostatic interactions are the driving forces for the protein adsorption and cellular uptake of the ceria nanoparticles [32].

The main aim of the present study is to develop a sensitive, and rapid microextraction technique based on UESA-DLLME with and without nanoceria (CeO_2) coupled with MALDI-TOF-MS for pathogenic bacteria analysis (*Pseudomonas aeruginosa* and *Staphylococcus aureus*). The CeO_2 nanocubic modified surfactant was prepared, characterized and applied for bacteria microextraction. For the first time, we applied CTAB modified CeO_2 (CeO_2@CTAB) nanoparticles dispersed into organic solvents as liquid microdroplets for the detection of target bacteria from their suspensions. Ceria was selected because it has excellent ability to adsorb bacteria biomolecules, such as proteins based on the high positive zeta potential [32]. UESA-DLLME with and without ceria were applied to extract both bacteria (*P. aeruginosa* and *S. aureus*) lysates from aqueous medium. Ceria (CeO_2) could increase the affinity toward protein adsorption; decrease the lowest detectable concentration for more than tenfolds, and improving the peaks resolution. Three different biological samples were used to check the applicability for the current approach. The results revealed that the ceria-surfactant ultrasonic assist liquid–liquid microextraction is a superior technique to extract the bacteria lysate from aqueous and blood suspensions over than the approach which only applies the surfactants for extraction.

2. Materials and methods

Tetraoctylammounium bromide (TOAB), sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB) were purchased from Sigma-Aldrich (USA). Dichloromethane was purchased from ECHO Co. (Taiwan). Chloroform was purchased from J.T. Baker (USA). Chlorobenzene and sinapinic acid were purchased from Alfa Aesar (Great British). Ultrapure water was obtained from a Milli-Q Plus water purification system (18.2 MΩ, Millipore, Bedford, MA, USA) was used for all experiments.

2.1. MALDI-TOF MS analysis

MALDI-TOF-MS spectra were obtained from Microflex (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (N_2, wavelength 337 nm). The spectra were recorded in positive and linear mode using an acceleration voltage of 20 kV and 10 ns extraction delay time. Sinapinic acid was used as a matrix for all experiments. Sample preparation procedures were discussed in the extraction procedures.

2.2. Ultrasonic instrument

The aqueous or real sample suspensions were ultrasonicated using an ultrasonication machine (LC30H, Sunway Scientific Corporation, Taiwan).

2.3. Characterization of ceria (CeO_2) nanocubic modified surfactant

The ultraviolet–visible (UV–vis) spectrum of CeO_2 nanoparticles was recorded using a double beam UV–vis spectrophotometer (Perkin Elmer 100, Germany) in the range of 200–700 nm. The size of the CeO_2 nanoparticles was further confirmed with the transmission electron microscopy (TEM, Philips CM 200, Netherlands) at an accelerating voltage of 200 kV. The samples were prepared for TEM by depositing 10.0 μL of an aqueous solution of the CeO_2 nanoparticles on a copper grid, and then it was dried under vacuum overnight. The sample was analyzed to confirm the particle size/morphology.

3. Experimental section

3.1. Preparation of ceria (CeO_2) nanocubic

The ceria CeO_2 were synthesized and then modified with the suitable surfactants as described below:

1. *Synthesis of ceria (CeO_2) nanocubic:* Ceria (CeO_2) nanoparticle was synthesized through a hydrothermal treatment. Ce $(NO_3)_3 \cdot 6H_2O$ (1.0 g) was dissolved in 10.0 mL of deionized water. A solution of NaOH (10%) was added rapidly with stirring to the previous solution. A light yellow precipitate of amorphous CeO_2 was observed. After about 1 h of stirring, all slurry was then transferred into a 50.0 mL of Teflon autoclave tube, and diluted with deionized water up to 80% of the total volume. The autoclave tube was heated at temperatures 200 °C for 24 h. After cooling, the precipitate was filtrated and washed several times with deionized water and then dried at 60 °C as shown in Fig. 1A. Some of the physical parameters such as temperature and time are cited from literature [33].
2. *Surface capping of ceria* (CeO_2) *with cetyltrimethyl ammonium bromide* (*CTAB*): About 0.5 g of ceria (CeO_2) was dispersed in organic solvent ($CHCl_3$, C_6H_5Cl, individually) that contain cetyltrimethyl ammonium bromide surfactant (CTAB, 0.5 g). The solution was subjected to strong stirring for 3 h. The synthesized nanoparticles were separated by ultracentrifugation (18 kg, 20 min), and then washed several time to remove all unassociated surfactant. The prepared CeO_2@CTAB was characterized using FTIR, TEM and UV spectroscopy.

3.2. Preparation of CeO_2@CTAB in organic solvent

About 0.5 g of CeO_2@CTAB was dissolved in 15 mL of organic solvents (chlorobenzene, CB and chloroform, $CHCl_3$), individually. The suspension solutions were used directly for the extraction procedures.

3.3. Bacteria cultivation

Both bacteria were cultivated using the conventional method as reported in Refs. [34,35]. *S. aureus* (BCRC 10451) and *P. aeruginosa*

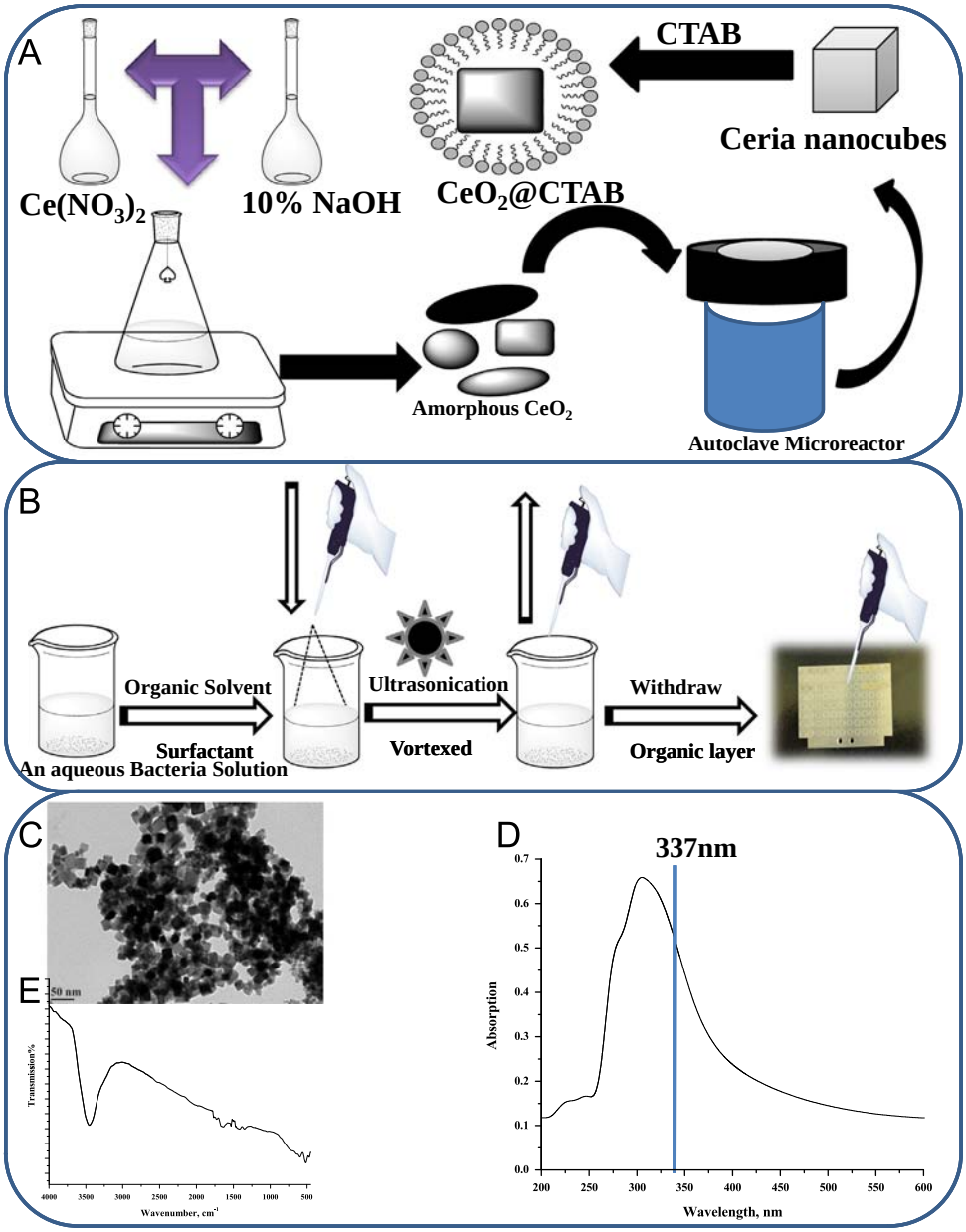

Fig. 1. (A) Schematic presentation of CeO_2 nanocubic preparation, (B) Schematic procedure of ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME), characterization of CeO_2@CTAB nanopaticles using (C) TEM micrograph, (D) UV spectrum, vertical line represent absorption of nanoparticles at the wavelength of N_2 laser (MALDI).

(BCRC 10303) standard cultures were purchased from Bioresource Collection and Research Center (BCRC, Hsin-Chu, Taiwan). The bacterial cultures are generally stored in powdered solid-phase (lyophilized) and were recovered by aseptically adding 0.3–0.5 mL of appropriate liquid medium into the vials with a sterile pipette then mixed thoroughly by pipetting up and down. Then 0.1–0.2 mL of the resuspended culture was streaked directly onto an agar medium plate and incubated at 37 °C. The bacteria were cultured repeatedly into fresh medium every two days for a week (sub-culturing) before using it for the experiments. Sub-culture after growth was suspended in sterilized deionized water for all experiments.

3.4. Extraction procedure

A sample suspensions (1.0 mL) containing different colony forming of bacteria were drawn into eppendorf tube (1.5 mL). Different surfactants TOAB, CTAB and SDS (1.0×10^{-3} M) were injected rapidly into the sample suspension. A cloudy suspension was observed due to the dispersion of the immiscible extraction solvent in the aqueous sample. In order to enhance the transfer of the bacteria lysate, we used ultrasonication at different time 1, 3, 5, 10, 15, 20, and 25 min. After ultrasonication, bacteria suspensions were vortexed and suspensions were leaved at ambient temperature prior to setting and extraction. The bottom organic phase was extracted with a micropipette as shown in Fig. 1B.

3.5. CeO2@CTAB assisted ultrasonication liquid–liquid microextraction

The prepared CeO_2@CTAB (0.5 g, 15 mL) with different organic solvents (CB, $CHCl_3$) were used directly. A sample suspensions (1.0 mL) containing different colony forming of bacteria were drawn into eppendorf tube (1.5 mL). Different volumes (10–20 μL) of CeO_2@CTAB

(0.5 g, 15 mL) were injected rapidly into the sample suspension. A cloudy solution was observed due to the dispersion of the immiscible extraction solvent in the aqueous sample. The bacteria were transferred from aqueous to organic layer that enhanced by ultrasonication for 10 min. After ultrasonication, bacteria suspensions were vortexed and incubated at ambient temperature prior to setting and extraction. The bottom organic phase was extracted with a pipette as shown in Fig. 1B.

3.6. Preparation of MALDI spotting

About 10.0 μL of extracting drop (with and without CeO_2) was mixed with sinapinic acid (10.0 μL, 50 mM). About 1.0 μL of the mixture was spotted onto the MALDI-MS plate and were leaved for air drying at room temperature before MALDI-MS analysis (Fig. 1B).

For control experiment, bacteria suspensions (10.0 μL) were mixed with sinapinic acid (10.0 μL, 50 mM). About 1.0 μL of the mixture was spotted onto the MALDI-MS plate and was leaved for air drying at room temperature before MALDI analysis.

3.7. Bacteria counting using standard plate counting

In order to calculate the bacteria colony forming units (cfu/mL), we used standard plate counting protocol as described here. Series of diluted bacteria suspensions were used (1–4 dilutions). Briefly, using aseptic technique, the initial dilution is made by transferring 1 μL of bacteria (*S. aureus* and *P. aeuginosa*) sample to 99 μL sterile water (this is a 1/100 or 10^{-2} dilution). The 10^{-2} dilution is then shaken by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and break up any clumps. Immediately after the 10^{-2} dilution has been shaken, uncap it and aseptically transfer 1 μL to second 99 μL sterile water. This second blank represents a 10^{-4} dilution of the original sample. Repeat the process to produce other dilutions. About 10 μL of the different dilution were spotted on agar plate, and then melted agar was poured with shaking to spread the bacteria on the full plate. At the end of the incubation period (24 h, 37 °C), select all of the petri plates containing suitable colonies (between 30 and 300 colonies). A plate having 30–300 colonies was chosen because this range is considered statistically significant. Count the colonies on each plate (it is more accurate to use more than one plate and take the average). Then, calculate the number of bacteria (cfu) per milliliter form the following equation:

$$\text{The number of cfu/mL} = \text{The number of colonies (30–300 plate)} \times \text{The dilution factor of the plate counted} \quad (1)$$

3.8. Blood sample preparations

Blood samples from two different sources (mouse or sheep) were spiked with two pathogenic bacteria (S. *aureus*, 6.1×10^3 cfu/mL and *P. aeruginosa*, 1.5×10^4 cfu/mL). The extraction procedure was the same as those of the above described procedures.

4. Result and discussion

4.1. Characterization of nanoceria (CeO_2) modified surfactant (CTAB)

Ceria (cerium dioxide, CeO_2) nanocubic has been synthesized via oxidation of $Ce(NO_3)_3 \cdot 6H_2O$ in basic medium according to Eq. 2. From the TEM images in Fig. 1C, the nanoparticles exhibit a clear cubic character (Fig. 1C) with size of almost about 20 nm. The ultraviolet (UV–Vis) spectrum of CeO_2 displays a broad and strong absorption centered at 314 nm (Fig. 1D). Ceria nanocubic displays also absorption at 337 nm that match with the laser wavelength (nitrogen, N_2) of MALDI. This absorption offers enhancement of the MALDI signals. FTIR spectrum of CeO_2@CTAB is plotted in Fig. 1E. Spectrum (Fig. 1E) shows peaks at 3500 cm^{-1} corresponding to O–H stretching. Bands around 610 cm^{-1} may be due to Ce–O stretching vibration:

$$2Ce(NO_3)_3 + 6OH^- + 0.5O_{2(g)} \rightarrow 2CeO_{2(s)} + 6NO_3^- + 3H_2O \quad (2)$$

Generally, biological samples have trace number of bacteria cells or their lysates. Thus, preconcentration approach is necessary. In routine analysis, liquid–liquid extraction (LLE) is widely applied as a sample preparation technique, not only for cleanup, or enrichment but also to improve the signals of low sample content.

Ultrasound enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) is a preconcentration technique. It is based on ternary component solvent systems (aqueous, surfactant, and organic extracting solvent). Before using surfactant assisted liquid–liquid microextraction, it is necessary to optimize the influential factors (e.g., types and volumes of extraction surfactants, and extraction time) in order to obtain the ideal extraction conditions.

Note that bacteria are ill-define microorganism and they are extremely complicated biological samples with many biomolecules/proteins to be analyzed using MALDI-MS. Thus, control experiments are required to evaluate the extraction method and to optimize various parameters that would influence on the microextraction process. Furthermore, MALDI profile of the bacteria depends on the culture age and incubation time.

4.2. Selection of the types of surfactant and organic solvent

Factors affecting microextraction efficiency such as types, volumes of extraction solvent, surfactant, and extraction time, were optimized. For simultaneous optimization of the type of surfactant and organic solvent, three different surfactants including tetraoctylammounium bromide (TOAB), sodium dodecyl sulfate (SDS), and cetyltrimethyl ammonium bromide (CTAB) were dissolved in three different organic solvents such as chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2) and chlorobenzene (CB) and were investigated individually. TOAB and CTAB are cationic surfactant, while SDS is an anionic surfactant.

The selection of surfactant is the paramount influential factor in order to achieve a satisfactory microextraction. Surfactants could accelerate dispersion and assist mass transfer of bacteria lysate from aqueous layer to extraction solvent (water-immiscible) under ultrasound irradiation. All selected surfactants should be soluble in the extraction solvent and miscible in water, then enabling the formation of fine droplets and enable mass transfer. Thus, it shows high extraction efficiency. Both cationic surfactants (TOAB and CTAB) in different extracting solvents ($CHCl_3$, CH_2Cl_2, CB) produced bacteria biomarker peaks that can identify the *P. aeruginosa* (Fig. 2A and B) and *S. aureus* (Fig. S2A and B). In other side, the anionic surfactant (SDS) does not produce any peaks for *P. aeruginosa* (Fig. S1A) or *S. aureus* (Fig. S1B). This might be due to the negative charge of cell membranes that are compatible with cationic charge (CTAB and TOAB) other than negative charge (SDS) [36]. Generally, the cell membrane consists of the lipid bilayer with embedded proteins. The cell membrane of pathogenic bacteria is acidic (negative charge) due to teichoic acid in Gram positive (*S. aureus*) and lipopolysaccharide in Gram negative (*P. aeruginosa*). They are more compatible with the cationic surfactants (CTAB or TOAB) due to electrostatic forces. Since SDS is an anionic surfactant, it is inconvenient with the acidity of the bacteria cell membranes as they carry the same type of charges. SDS also has higher hydrophilicity than CTAB and TOAB. The cationic surfactants could function as a hook to attract the bacteria cell membranes via electrostatic forces. The results

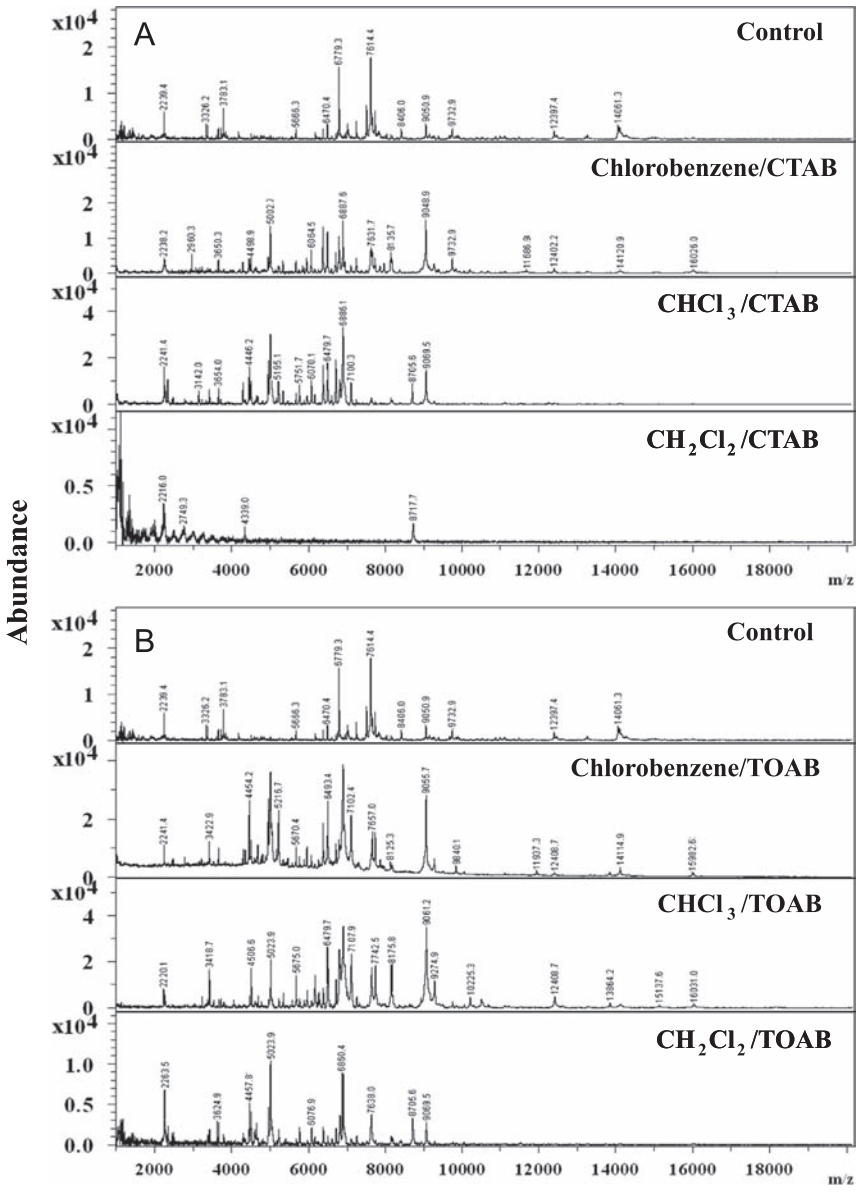

Fig. 2. Effect of surfactant ((A) CTAB and (B) TOAB) and organic solvent ($CHCl_3$, CB, CH_2Cl_2) types on the extraction efficiency of *Pseudomonas aeruginosa*. Extraction conditions: 20.0 μL organic solvent, 20.0 μL surfactant ((A) CTAB and (B) TOAB, with concentration = 1.0×10^{-3} M), bacteria concentration *P. aeruginosa* = 2.0×10^5 cfu/mL.

indicate that the cationic surfactant had the best extraction efficiency for the pathogenic bacteria. As both cationic surfactants (CTAB and TOAB) exhibit excellent extraction capability. We further select CTAB as it can be modified easily onto the nanoparticle surfaces. CTAB also has good ability to suppress matrices peaks during MALDI-MS analysis to improve the peak resolution [37].

To proceed efficient microextraction, the extraction solvent should have the following properties: (1) its density should be higher than water density; (2) it should have bio-affinity toward the pathogenic bacteria especially cell membrane that consists of the lipid bilayer with embedded proteins; (3) it should be available and volatile to assist drying after spotting on the MALDI target plates. Three organic solvents were tested in our study including dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$) and chlorobenzene (CB) based on the three requirements.

For organic layer, results reveal that chlorobenzene is the optimized organic solvent for *P. aeruginosa* (Fig. 2A and B); while chloroform is the optimized extracting solvent for *S. aureus* (Fig. S2A and B). The optimized solvent is the solvent that show high peaks number that matches with control spectrum.

4.3. Optimization the exposure time of ultrasonication

Generally, more analytes could be extracted into the organic solvent if the extraction phase is exposed to the sample solution for a longer time [38]. However, long equilibration times may result in the loss of analytes in the extraction process. Initially, the extraction solvent (water immiscible) at ambient temperature is added to the aqueous bacteria suspensions. When the temperature is increased via ultrasonic source, complete dissolution could takes place, and after a slowly cooling, a cloudy solution is formed. Finally, after vortex, the extraction-solvent droplet is collected at the bottom of the eppendorf tubes. Different intervals (1, 3, 5, 10, 15, 20 and 25 min) of ultrasonication processes were investigated. MALDI-MS spectra of *P. aeruginosa* (Fig. S3A) and *S. aureus* (Fig. S3B) show increase in bacteria biomarker peaks with the increase in ultrasonication time. Data reveal that average time 10 min is the

optimal extraction time for both pathogenic bacteria. Fig. S3 of both bacteria shows high number of biomarker peaks at short time (10 min) of *P. aeruginosa* (Fig. S3A) than *S. aureus* (Fig. S3B). The prime reason is due to Gram negative bacteria (*P. aeruginosa*) which is rich by lipopolysaccharide than Gram positive (*S. aureus*) that have thick peptidoglycan layers. The exposure time of both bacteria was fixed as 10 min for further experiments.

4.4. Effect of the surfactant and organic volume

Among various LLME techniques, the surfactant assisted LLME is a very popular liquid-phase microextraction technique. The reasons for the popularity of this technique are because it is inexpensive, simple equipment required, easy to operate, and is nearly solvent free. The extraction volumes of organic solvents (chlorobenzene and chloroform) were optimized from 10.0 to 40.0 μL. MALDI-MS spectra (Fig. S4) reveal that 10.0 μL was the optimal volume as the extracting solvent. In general, spot of MALDI plates require only a small fraction (1–5 μL). Fig. S4A of *P. aeruginosa* and Fig. S4B of *S. aureus* present 10.0 μL is sufficient for both bacteria in order to give a high number of peaks that match bacteria peaks of control (Fig. S4).

Volume of surfactant is paramount important in UESA-DLLME, especially when MALDI-MS is used. Surfactant can suppress molecules ionization [37], so optimization on the surfactant volume is necessary. Furthermore, high concentration of surfactant results in relatively high viscosity that causes reduction of bacteria extraction in the organic phase. Data revealed that 10.0 μL of surfactants (1.0×10^{-3} M) are the optimized volume for the microextraction process (Fig. 3A and B). Spectral evaluation of *P. aeruginosa* (Fig. 3A) and *S. aureus* (Fig. 3B) indicates that 10.0 μL of surfactant (CTAB, 1.0×10^{-3} M) is minimum volume that offer a significant peaks which were matched with standard spectrum.

4.5. Ceria nanostructure@CTAB based dispersive liquid–liquid microextraction

The CeO_2@CTAB-ultrasonic assisted liquid–liquid microextraction consist of two steps: (1) inject an appropriate volume (10.0–20.0 μL) of

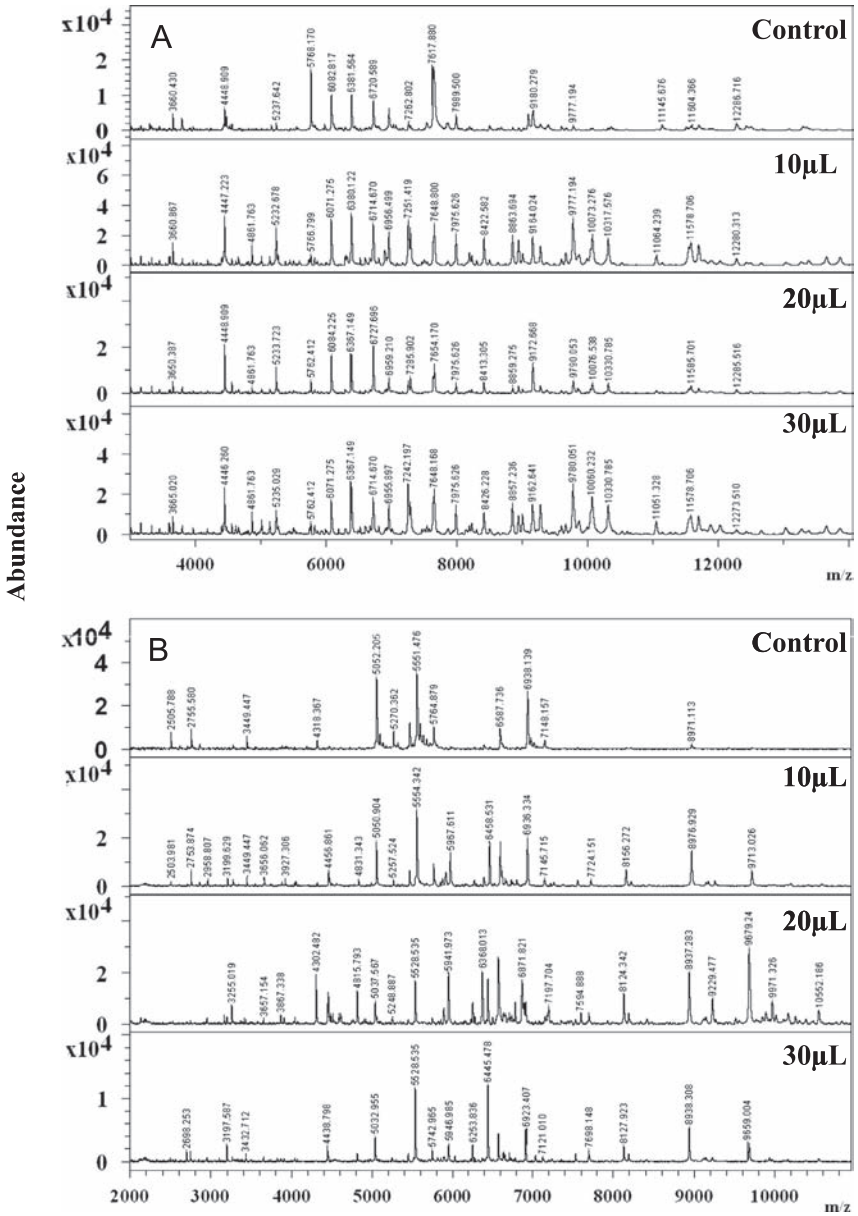

Fig. 3. Effect of surfactant volume on the extraction efficiency of (A) *P. aeruginosa* and (B) *Staphylococcus aureus*. Extraction conditions: 20.0 μL organic solvent, 10.0–40.0 μL surfactant (1.0×10^{-3} M), bacteria concentration of *S. aureus* = 3.5×10^6 cfu/mL and *P. aeruginosa* = 2.0×10^5 cfu/mL.

the extracting mixture which contains extracting solvent (chlorobenzene for *P. aeruginosa* and chloroform for *S. aureus*) and CeO_2@CTAB into 1.0 mL of aqueous solution containing bacteria. In this step, the extraction solvent was dispersed into the aqueous sample and a very fine microdroplets were formed that enriched by bacteria lipid bilayer embedded proteins. In order to increase the large surface area and contact area between the extraction solvent and the sample, ceria was added to the extracting solvent. (2) Subject eppendorf to ultrasounds about 10 min (Fig. 2). After vortexing, the organic phases that contain the bacteria extract onto the nanoparticle surface were sedimented and extracted using a micropipette. The extracted crude was mixed with organic matrix then was spotted into MALDI target plate for analysis.

Ceria (CeO_2) can increase the bioaffinities of the organic solvent via adsorption of the protein onto the nanoparticle surface, as it has high positive zeta potential [32]. Also, CeO_2 nanocubic can increase desorption/ionization due to large surface area. Ceria (CeO_2) nanoparticles have high positive zeta potential which can assist protein adsorptions [32]. The electrostatic interactions are the driving force for the protein adsorption and cellular uptake of the ceria nanoparticles [32]. Zeta potentials, electrostatic interactions and hydrogen bonding interactions are responsible for the extraction process. The competition between two phases (aqueous and organic) for the bacteria biomolecules is high than ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME). Furthermore, CeO_2@CTAB increases the contact area and improves the extraction efficiency

Nanoparticle could also play a significant role in the process of MALDI analysis; it can increase the surface area that enhance the desorption of proteins and to increase peak resolution by acting as the co-matrix with sinapinic acid (Fig. 4A and B) [39]. The UV spectrum (Fig. 1C) shows the absorption of CeO_2@CTAB at 337 nm that match with the wavelength of the MALDI-laser (N_2 laser, 337 nm). This absorption may be the prime reason why the MALDI signals were increased and numerous numbers of bacteria peaks could be detected [40]. MALDI spectrum of *P. aeruginosa* (Fig. 4A) exhibits improvement in bacteria biomolecules > 10 KDa. Gram positive bacteria (*S. aureus*) display low peak intensities in the control spectrum (Fig. 4B), while the CeO_2@CTAB shows highest improvement than *P. aeruginosa* (Fig. 4A). Although MALDI-MS

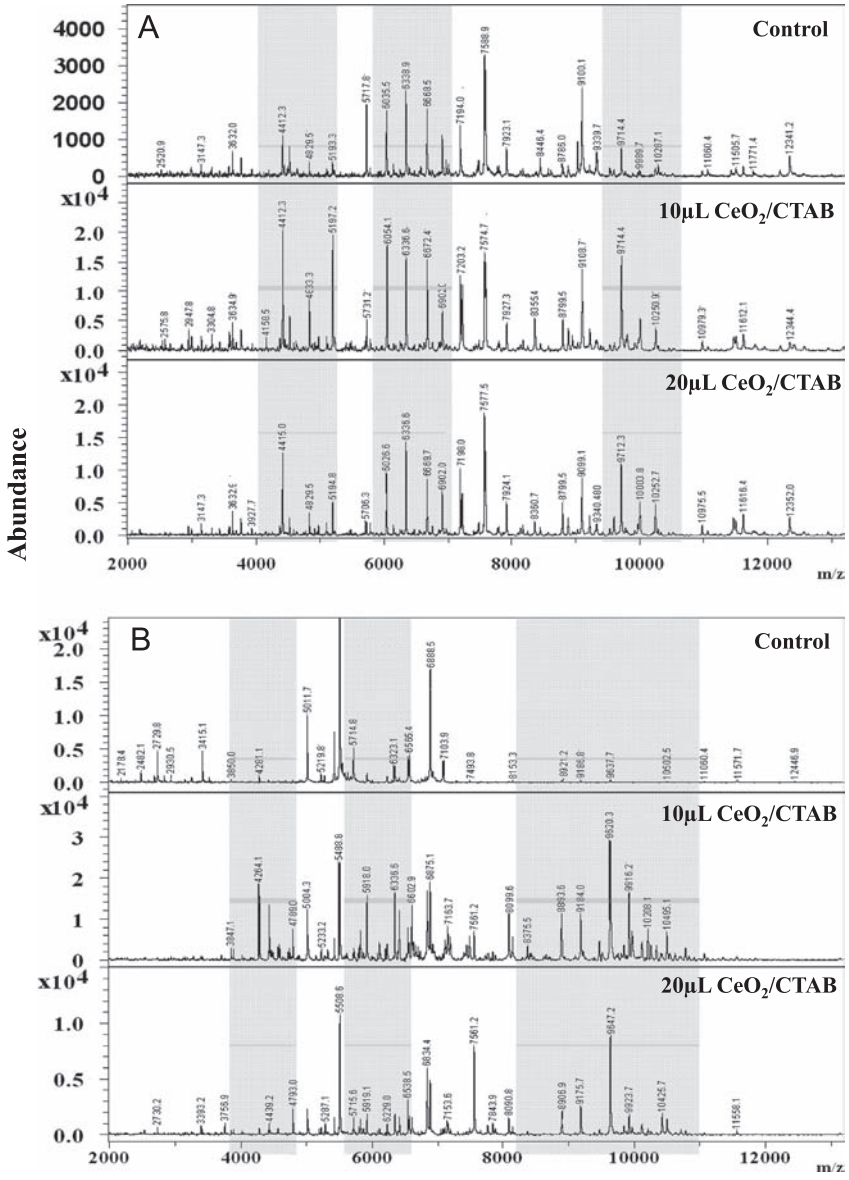

Fig. 4. CeO_2@CTAB-ultrasonic assisted liquid–liquid microextraction of (A) *P. aeruginosa* and (B) *S. aureus*. Extraction conditions: 10–20 μL CeO_2@CTAB, bacteria concentration of *S. aureus* = 3.5×10^5 cfu/mL, *P. aeruginosa* = 2.0×10^4 cfu/mL. White highlight (darker 35%) represents the bacteria enhancement.

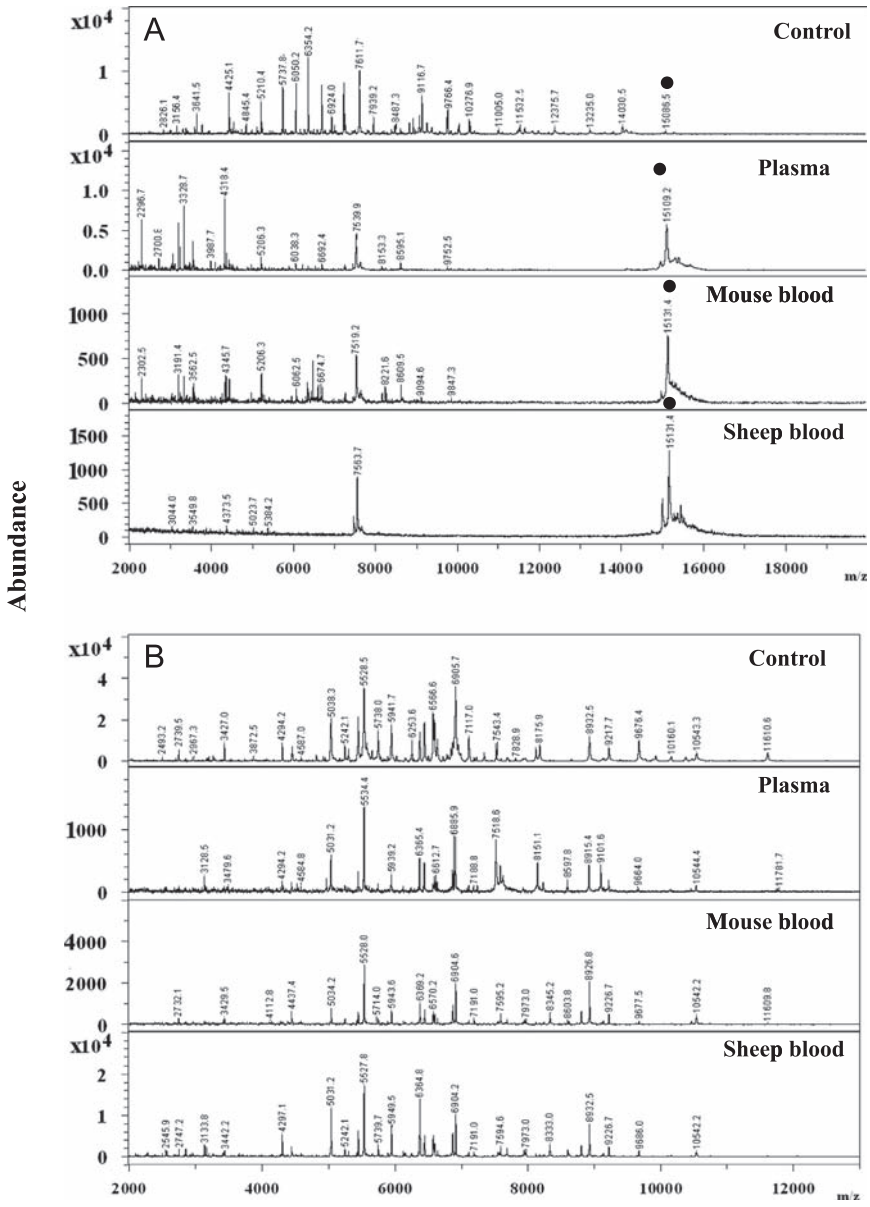

Fig. 5. CeO_2@CTAB assisted liquid–liquid microextraction of real sample analysis (plasma, mouse and sheep blood) using spike technique of (A) *S. aureus* = 6.1×10^3 cfu/mL and (B) *P. aeruginosa* = 1.5×10^4 cfu/mL.

results typically cannot be used to calculate the absolute extraction recoveries as it is a qualitative technique. However, MALDI data show high consistence of both bacteria *P. aeruginosa* (Fig. S5A) and *S. aureus* (Fig. S5B).

Comparing ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) in the presence and absence of CeO_2 nanoparticles, data reveals increase of microextraction sensitivity (Figs. S6 and S7). MALDI-MS spectra of *P. aeruginosa* without and with CeO_2 (Fig. S6A and B) reveal improvement in the lowest detectable concentration. Data indicate bacteria detection decrease from 3.8×10^4 to 1.5×10^4 cfu/mL. While *S. aureus* shows decrease of lowest detectable concentration from 1.5×10^5 (without CeO_2, Fig. S7A) to 6.1×10^4 cfu/mL (with CeO_2, Fig. S7B). CeO_2@CTAB reveals decrease of lowest detectable concentration more than tenfolds than using the surfactant alone as shown in Table 1.

The reasons for improving signals of both bacteria are that CeO_2 have large surface area, participate in desorption/ionization process as it exhibit absorption at $\lambda = 337$ nm (Fig. 1D), and inherent multifunctional properties (zeta potential, electrostatic interactions and hydrogen bonding) of surfactant and nanoparticles (i.e. CeO_2@CTAB). To address the reasons why there are improvements in peaks number, we shall differentiate between ionization of intact cell (control spectrum) and ionization of bacteria after microextraction using UESA-DLLME. When laser radiation desorbs intact cell (unbroken cell) from MALDI plate, a few biomolecules can undergoes desorption/ionization. In contrast, the UESA-DLLME disturbs the whole cell (intact cell) via extraction the bacteria biomolecules, thus improve bacteria identification (Fig. 4). Because the thickness of peptidoglycan in Gram positive (*S. aureus*, Fig. 4B)

Table 1
Limit of detection of UESA-DLLME with and without nanoceria.

Techniques/bacteria (cfu/mL)	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
UESA-DLLME without CeO_2	3.8×10^4	1.5×10^5
UESA-DLLME with CeO_2	1.5×10^3	6.1×10^4

Table 2
Comparison between different techniques that used to extract and identify bacteria.

Technique	Species	Nanoparticles/organic layer	LOD (cfu/mL)	Ref.
SDME-MALDI	*Escherichia coli* and *Serratia marcescens*	Platinum (Pt) nanoparticles conjugated with ionic liquid drop	10^6	[14]
Affinity probe	*S. marcescens and E. coli*.	Immunoglobulin immobilized on platinum nanoparticles	10^5	[40]
UESA-DLLME	*P. aeruginosa* and *S. aureus*	CeO_2@CTAB	10^3–10^4	This work
SPME-GC–MS	VOC of *Streptococcus* (*Strep.*) *pneumonia*, *Haemophilus* (*H.*) *influenzae*, *Pseumdomonas* (*P.*) *aurginosa*, *S.* (*Staph.*) *aureus*, *Moraxella* (*M.*) *catarrhalis* and *Stenotrophomonas* (*S.*) *maltophilia*	Fibers (divinylbenzene/carboxenon polydimethylsiloxan)	ND	[41]
SPME	Classification of bacteria based on fatty acid methyl ester (FAME) profiles	Fibers	ND	[40]
SPME-IRMS	Volatile Compounds Produced during Fermentation by Lactic Acid Bacteria	Lactic acid bacteria	ND	[41]

is higher than Gram negative (*P. aeruginosa*, Fig. 4A), intact cell (control spectrum, Fig. 4) of the former bacteria shows low peaks number over than *P. aeruginosa*. Beside that *P. aeruginosa* (Gram negative) [41] is rich by lipopolysaccharide (endotoxin) than *S. aureus* (Gram positive) that can undergo mass transfer from aqueous to organic layers. Because of these significant improvements, the current approach is excellent to be applied for real (biological) sample analysis.

4.6. Analysis of bacteria in mouse and sheep blood samples

The presence of pathogenic bacteria (*P. aeruginosa* and *S. aureus*) in blood can lead to a serious health problem. It can cause septic shock because the recipients of blood transfusions are usually critically sick and have low immunity. Therefore, it is extremely necessary to monitor the presence of bacteria in the blood before recipient's transfusion. The major drawbacks of blood analysis using MALDI-MS, is not only interferences originated from blood albumin, blood cell, or salts, but also due to bacteria signals cause ionization suppression, isobaric submerge, mass shift due to salts and interferences association. Thus, identification of bacteria in blood has been major challenges for analytical chemists [4–8].

In order to test the applicability of the proposed method to real sample analysis, blood samples (plasma, mouse and sheep blood) were investigated using spiked protocol. UESA-DLLME without ceria nanoparticles for both biological samples produced low number of peaks which failed for identification (Fig. S8A and B). The prime reason may be due to large interference of small molecules in the blood samples which transfer smoothly than the large biomolecules which belong to bacteria cells. Furthermore, bacteria may be suffering from the ion suppression. In contract, when ceria (CeO_2) were added into the extraction system, all biological samples (plasma, mouse and sheep blood) produced a great numerous of peaks for both bacteria (*S. aureus* (Fig. 5A), *P. aeruginosa* (Fig. 5B)) and allow successful detection of pathogenic bacteria into real samples (Fig. 5). Peaks belong to blood background were marked by (•), while the rest belongs to bacteria peaks. UESA-DLLME conjugated CeO_2@CTAB approach improves peaks ionization because CeO_2 nanoparticles display absorption at 337 nm.

The major challenge of liquid–liquid microextraction is in selectivity. However, UESA demonstrates better selectivity than other microextraction protocols based on surfactant type. Data reveal that cationic surfactant can only serve as a disperser due to the acidity of cell membrane. In contrast, anionic surfactants are incompatible due to the same charge of the surfactant and bacteria cell membrane. Because of the complexity and diversity of bacteria, volatile organic compounds (VOCs) have been proposed to identify the bacteria using solid phase microextraction [42–45]. A comparison of the different microextraction techniques is tabulated in Table 2. Data (Table 2) indicate that ceria@surfactant assisted liquid–liquid microextraction is lower limit of detection, simple, eco-friendly and fast. Furthermore, the present technique gives the opportunity for more modification. Microextractions techniques such as LLE and LSE methods have been used to solve challenges such as low concentration, time, environmental concerns, and cost. They not only offer the ability to separate the target analytes from the sample solution, but also reduce, control or even eliminate the interferences originally present [42–45]. However, it is tedious as there are many parameters need to optimize before using DLLME. There is a need to optimize carefully influential factors (e.g., types and volumes of extraction and disperser solvents, extraction time, sample amount, pH, and salt addition). While, it is quite simple in UESA-DLLME [17–21].

5. Conclusions

We presented a sensitive method for the extraction/detection of pathogenic bacteria in blood samples using ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) with and without nanoceria coupled with MALDI-TOF-MS. The novel approach is an attractive and powerful technique to analyze the pathogenic bacteria in different biological samples. Ceria@surfactant assisted liquid–liquid microextraction introduces a great contribution to meet better microextraction objective, due to its simplicity, rapidity of operation, high resolution and low consumption of solvents/reagents. According to the structure of bacteria cell membrane, as a general rule, cationic surfactants are more effective in binding bacteria biomolecules than anionic surfactants. The main driving force for bacteria partitioning from the intact cell in aqueous phase into the CeO_2@CTAB-phases is hydrophobic interaction, while other forces such as zeta potential (electrostatic interactions), dipolar interaction and hydrogen bonding play secondary roles in lysate–CeO_2@CTAB interactions. CeO_2@CTAB combined UESA-DLLME method offers high microextraction efficiency, low limit of detection and eco-friendly.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.078.

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