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Microchip capillary electrophoresis with amperometric detection for rapid separation and detection of seleno amino acids

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Abstract

This article describes an effective microchip capillary-electrophoresis protocol for rapid and effective measurements of food-related seleno amino acids, including Se-methionine (Se-Met), Se-ethionine (Se-Eth), Se-methyl cysteine (Se-Cys), utilizing *o*-phtaldialdeyde/2-mercaptoethanol (OPA/2-ME) derivatization. Relevant parameters of the chip separation and amperometric detection are examined and optimized using a response surface methodology (RSM). Under optimum conditions, the analytes could be separated and detected in a 30 mM borate buffer (pH 9.3, with 28 mM sodium dodecul sulfate) within 300 s using a separation voltage of 2000 V and a detection voltage of +0.9 V. Linear calibration plots are observed for micromolar concentrations of the Se-amino acid compounds. The negligible sample volumes used in the microchip procedure obviates surface fouling common to amperometric measurements of selenoamino-acid compounds. The new microchip protocol offers great promise for a wide range of food applications requiring fast measurements and negligible sample consumption.

Keywords: Microchip; Capillary electrophoresis; Amperometric detection; Amino acids; Selenoamino acids

1. Introduction

Selenium is present in the biosphere in various chemical forms as the products of enzymatic reactions leading to selenoamino acid synthesis [1]. Selenium in the living body forms covalent Se-C bonds, with selenocysteine (Se-Cys) and selenomethionine (Se-Met) being the predominant forms in selenium-containing proteins. Selenoproteins, a Se-containing protein with genetically encoded selenocysteine, are found to be responsible for certain biological functions in mammals [2,3]. Other important Se-containing proteins, in addition to genetically encoded Se-Cys, are found in organisms through nonspecific incorporation of selenoamino acids. For example, Se-Met is randomly incorporated into methionine-containing proteins in the place of methionine [4] and is retained in tissue proteins to a greater extent then Se-Cys and inorganic selenium species [5]. The metabolism and bioavailability and antioxidant role of selenium and seleno-compounds have also been well documented [6,7]. The metabolic behavior, nutritional bioavailability and the toxicity of selenium are dependent on its chemical forms and concentrations. Therefore, there is an increasing recognition that the knowledge of selenium speciation in tissues and functional proteins is important for the accurate assessment of selenium status along with the demand for the determination of selenoamino acids in foodstuffs [8].

Various analytical techniques have been used so far in the separation and detection of Se-Cys including gas

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chromatography coupled atomic emission spectroscopy [9] or to mass spectrometry [10], high-performance liquid chromatography (HPLC) with reversed-phased columns [11] or ion exchange columns [12]. Similar techniques used in the separation of Se-Met have been reviewed recently [13]. An approach for screening and resolving selenium-containing plant proteins was recently developed based on the combination of sample preparation and multi-dimensional liquid chromatography coupled to inductively coupled plasma mass spectrometry (ICP-MS) [14]. Identification of Se-Cys and Se-Met in protein hydrolysates by HPLC after pre-column derivatization using *o*-phthaldialdehyde (OPA) reagent (in the presence of 2-mercaptoethanol, 2-ME) has also been reported [15]. Other methodological advances for determination of selenium-containing compounds have been reviewed recently, including speciation analysis in yeast and in biological compounds [16–23]. There has been an increasing interest in application of capillary electrophoresis (CE) for the separation of both inorganic and organic selenium compounds [24].

Microfluidic devices, particularly, microchip CE, have gained popular interest in the past decade owing to their speed, versatility, performance, negligible reagent/sample consumption and waste generation, and integration of various analytical steps [25,26]. Analytical advantages and methodological advances of such devices have been reviewed recently [27], along with recent developments in clinical [28] and point-of-care testing [29]. Of the detection schemes available, electrochemical detection is particularly suitable as it allows path length-independent measurements with excellent sensitivity and selectivity, compatibility with miniaturization capability, portability, and low-power requirements [30–31].

Electrochemical detection on microchip CE for the determination of amino acids has previously been employed using either derivatization schemes [32,33] resulting in generation of electroactive compounds or direct oxidation [34–35]. The notable derivatization schemes demonstrated on microchip CE separation followed by amperometric detection are OPA [32] and naphthalene-2,3-dicarboxyaldehyde (NDA) [33]. Among direct detection schemes – without involving a derivatization steps – amperometric detection of amino acids at carbon-nanotube/Cu composite electrode in alkaline medium [34], MEKC separation and direct oxidation of aromatic amino acids [35], and pulsed amperometric detection [36] have been demonstrated. Recently, Graß et al. have presented an analytical method for the electrophoretic on-line preconcentration and separation of Se-Met, Se-Eth, and Se-Cys on a PMMA microchip coupled with conductivity detection [37]. Although good sensitivities were observed using conductivity detection, this is mainly due to the isotachophoresis (ITP) preconcentration step involved. However, in addition to the lack of specificity, such a detection scheme suffers from impurities present in the buffer system. Therefore, in the present study we have investigated an alternative approach based on derivatization of selenoamino acids with OPA (in the presence of 2-ME) to impart specificity and the subsequent separation and detection of derivatized selenoamino acids at an end-column amperometric detector. Optimizations of relevant parameters, such as the OPA and 2-ME concentrations, and factors affecting the separation (including the pH of the run buffer and sodium dodecyl sulfate concentration) have been optimized using a response surface methodology (RSM). The RSM optimization route estimates the reciprocal interaction between factors affecting the variability of the system. Such optimization, characterization and attractive performance of the new microchip protocol for detecting selenoamino acids are described in the following sections.

2. Experimental

2.1. Chemicals and reagents

Se-Methionine (Se-Met), Se-ethionine (Se-Eth), Se-methyl cysteine (Se-Cys), methionine (Met), 2-mercaptoethanol (2-ME), *o*-phtaldialdehyde (OPA), sodium tetraborate 10-hydrate, sodium dodecyl sulfate (SDS) and ethanol were obtained from Sigma (St. Louis, MO, USA). All chemicals were used without any further purification. Several borate buffers (at different concentrations and pH) were prepared daily in deionized water. The desired pH was obtained by titrating the buffer with 1 M NaOH as necessary. The optimal 'run buffer' consisted of 30 mM borate (pH 9.2, with 31 mM SDS). Stock solutions (10 mM of Se-Met and Se-Eth, and 4 mM for Se-Cys) of the seleno amino acids were prepared by dissolving the required amount in water; working standard solutions were obtained by diluting corresponding stock solutions in electrophoresis buffer.

2.2. Derivatization step

The derivatizing reagent was prepared dissolving 25 mg of OPA in a mixture of 500 mL of ethanol and 18 mL of 2-ME and finally making the volume up to 2 mL with 30 mM borate buffer, resulting in a final concentration of 75 mM OPA derivatizing solution.

The OPA derivatization reaction was carried in a 1.5 mL centrifuge vial by adding 30 μL of derivatizing (OPA and 2-ME) solution (with a final concentration of 2.25 mM OPA) and required amounts of seleno amino acids and the making the final volume up to 1 mL with the 'run buffer'. The mixture was allowed to react for 60 s and subsequently the reaction mixture was transferred into 'sample reservoir' prior to injection. The optimum reaction time was found to be 60 s and is in agreement with our earlier report using analogous measurements [32].

2.3. Apparatus

The simple-cross glass microchip (88 mm × 16 mm) was procured from Micralyne (Model MC-BF4-001, Edmonton,

Canada) and placed in a Plexiglas holder, accommodating the separation microchip and the end-column amperometric detector and the exact details were described elsewhere [31]. The simple-cross microchip consisted of a four-way injection cross with a 74-mm long separation channel and a 5-mm long injection channel. The original waste reservoir was cut off, leaving the channel outlet at the end of the chip, thus facilitating the end column amperometric detection [31]. The channels were 50 μm wide, 20 μm deep. Short pipette tips were inserted into the three (run buffer, sample and unused) reservoirs on the glass chip for solution contact between the channel on the chip and the corresponding reservoir on the chip holder. The amperometric detector, placed in the detection reservoir, consisted of an Ag/AgCl wire reference, a platinum wire counter, and the thick-film carbon screen-printed working electrode. The rectangular shaped screen-printed electrode was placed 50-μm away from the channel outlet using a thin layer spacer and the distance was controlled by a plastic screw [31]. Platinum wires, inserted into the individual reservoirs, served as contacts to the high-voltage power supply. A laboratory-made power supply, with an adjustable voltage range between 0 and 4000 V was used for injections and separations.

The screen-printed electrodes were printed with a semi-automatic printer (Model TF 100, MPM, Franklin, MA, USA). The Acheson ink (Electrodag 440B, catalog No. 49AB90, Acheson Colloids, Ontario, Canada) was used for printing electrode strips. Details of the printing process and exact dimensions of the carbon screen-printed electrode detector were described elsewhere [31].

2.4. End-column amperometric detection

Each electropherograms was recorded with a time resolution of 0.1 s using an applied potential of +0.9 V. Sample injections were performed after the stabilization of baseline. All experiments were performed at room temperature.

2.5. Electrophoresis procedure

The channels were treated before use by rinsing with deionized water for 10 min, with 0.1 M NaOH for 10 min, and deionized water for additional 10 min. The electrophoresis 'run buffer' (for separating seleno compounds) was a 30 mM borate buffer (pH 9.2) with SDS 31 mM. The 'run buffer', the 'unused' reservoirs and the detection reservoir were filled with electrophoresis run buffer solution, while the 'sample' reservoir with the reaction mixture of seleno amino acids and the derivatizing agent. The injection was affected by applying +1500 V between the sample reservoir and the grounded detection reservoir for 3 s. This drove the sample 'plug' into the separation channel through the intersection. Separations were performed by switching the high voltage contacts and applying a potential of +2000 V to the 'run buffer' reservoir with the detection reservoir grounded and all other reservoirs floating.

2.6. Safety considerations

The high voltage power supply and associated open electrical connections should be handled with extreme care to avoid electrical shock. 2-Mercaptoethanol and other chemical are toxic/irritant and should be handled with care. Skin and eye contact, and accidental inhalation or ingestion should be avoided.

3. Results and discussion

The aim of this study is to demonstrate the use and advantages of a microchip capillary electrophoresis with an amperometric detector for rapid simultaneous measurements of seleno amino acids. OPA derivatization was previously carried out for the determination of amino acids [32] but not in connection with selenoamino acids. In this study, selenoamino acids have been derivatized with OPA/2-ME mixture. Fig. 1 displays a typical electropherogram for a sample mixture containing three common seleno amino acids under optimum conditions. The electropherogram was obtained at the screen-printed carbon detector using submillimolar concentrations of MET (b), Se-MET (c), Se-ETH (d), Se-CYS (e). The five peaks are well resolved with favorable signal-to-noise characteristics, with the entire assay requiring less than 300 s. Analogous measurements with

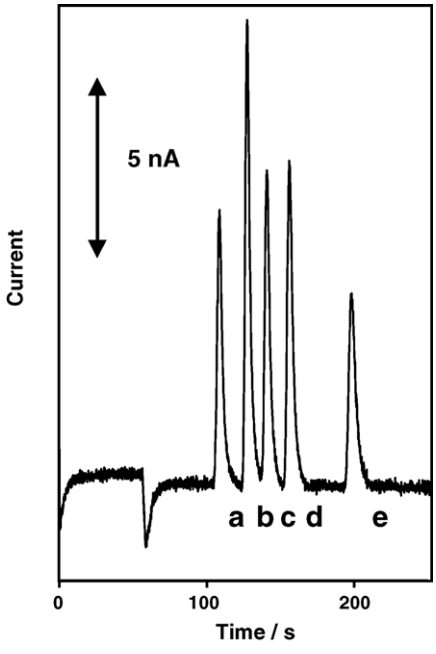

Fig. 1. Electropherograms for mixtures containing 200 μM of Met (b); Se-Met (c); Se-Eth (d); and Se-Cys (e) detected at the screen-printed carbon electrode. Peak a corresponds to the unreacted derivatizing agent. Operation conditions: separation voltage, 2000 V; injection voltage, 1500 V; injection time, 3 s; running buffer, 30 mM borate (pH 9.2) containing 31 mM SDS; detection potential, +0.9 V (vs. Ag/AgCl wire). The first peak (a) corresponds to the system peak generated from OPA/2-ME response.

HPLC required significantly longer (>40 min) periods [15]. A method for the determination of selenoamino acids by coupling of isotachophoresis/capillary zone electrophoresis on a PMMA microchip was unable to separate Se-Met and Se-Eth [37].

In order to establish the best detection conditions for the seleno amino acids, their behavior at screen printed carbon electrodes was investigated. The selection of the detection potential relies on the construction of hydrodynamic voltammograms (HDV). Fig. 2 depicts typical HDV for the oxidation of (A) Se-Met, (B) Se-Eth. The curves were developed pointwise by making 100 mV changes in the applied potential over the range of +0.4 to +1.20 V, while using a separation voltage of 2000 V. A sigmoidal response and the half-wave potentials observed for both the seleno amino compounds reflect the detection of the corresponding isoindole reaction product. The different voltammetric profiles and sensitivities obtained for Se-Met and Se-Eth are related to the chemical structure of the seleno amino acid residue of the isoindole products. The oxidation starts around +0.5 V (A, B) with a rapid increase in peak currents and leveling off around +0.9 (A, B). A higher baseline current with an increase in peak-to-peak noise was observed at potentials higher than +0.9 V. Hence, a detection potential of +0.9 V was used for all subsequent work as it

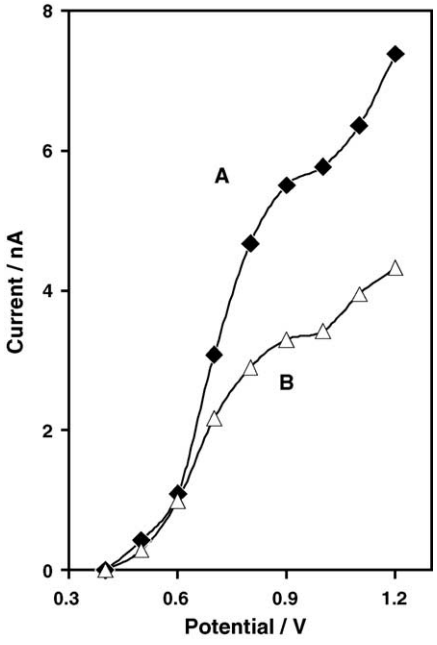

Fig. 2. Hydrodynamic voltammograms for 200 μM of Se-Met (A) and Se-Eth (B) at the screen-printed carbon electrode. Other conditions, as in Fig. 1.

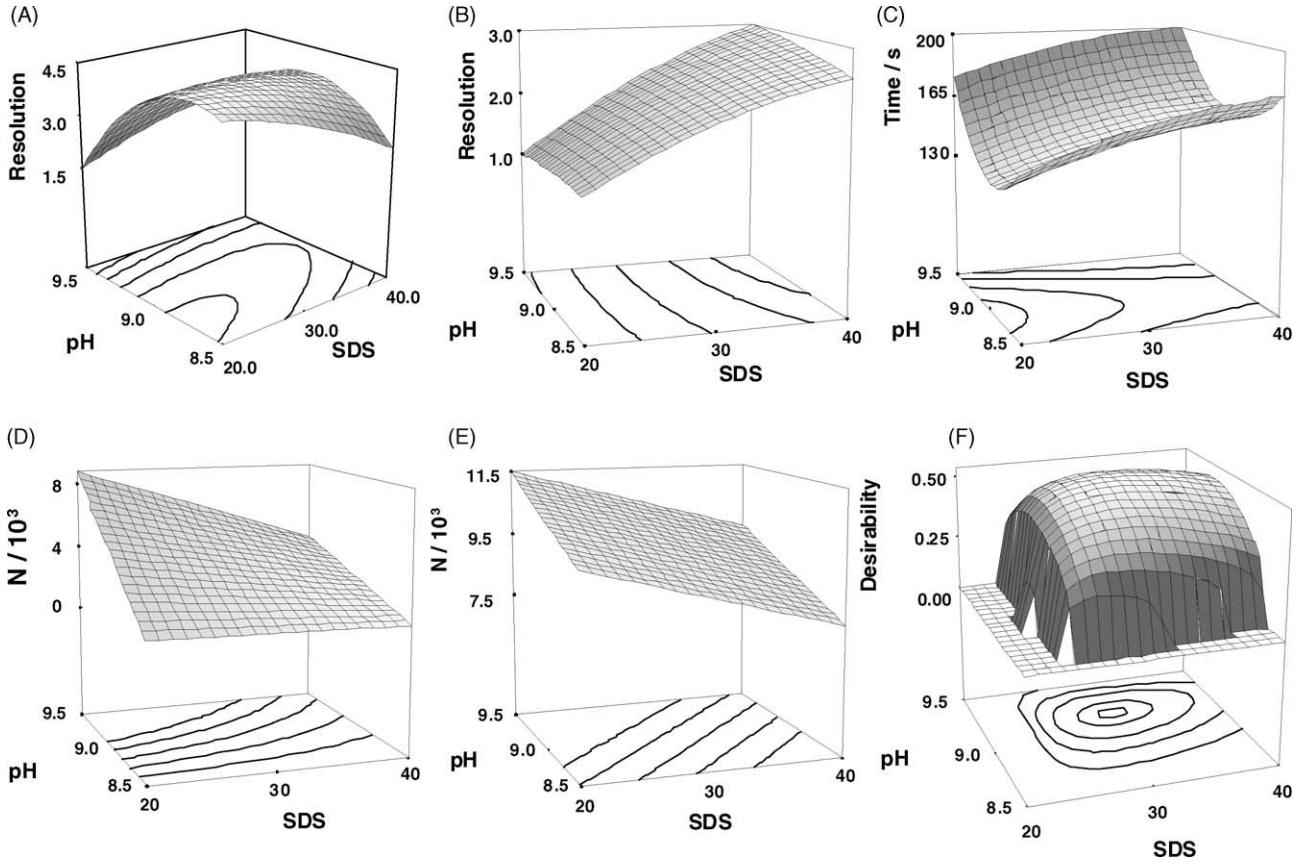

Fig. 3. Optimization of pH and SDS concentration using response surface methodology (RSM). Response surfaces for (A) resolution between OPA/2-ME and Met; (B) resolution between Met and Se-Met; (C) overall time of analysis; (D) theoretical plate number for Met; (E) theoretical plate number for Se-Met; and (F) desirability plot. Level of borate concentration is 30 mM.

provided optimum sensitivity and baseline noise characteristics.

The composition of the 'run buffer' and its pH are important parameters while obtaining efficient separations. While the pH of the run buffer is an important parameter since it determines the extent of ionization and mobility of each solute, the concentration of the borate run buffer determines the separation voltages employed (and hence the assay time). Furthermore, the concentration of the SDS is also an important factor in MEKC separations [38]. Therefore, an attempt has been made to optimize these conditions using a RSM to provide a map of response in the form of three-dimensional (3D) rendering. RSM provides better results compared to classical optimization performed using one variable at a time [39,40]. Using RSM it is possible to estimate reciprocal interaction between factors affecting the variability of the system, to check linearity or to reveal the presence of curvatures in the responses and, at the same time, to reduce at minimum the number of experiments to be performed in order to obtain optimum performance.

The RSM was obtained by a central 19 independent experiments, as required by a central composite design, with one central point repeated five times, 6 axial points, and 8 factorial points. Each point is the average of five running replicates. Six responses [theoretical plate numbers (N_{Met} and $N_{Se\text{-}Met}$), the resolution between OPA/2-ME system peak and Met ($R_{1–2}$) and Met and Se-Met ($R_{2–3}$) and finally, the overall analysis time (t)] were studied with the analysis of variance and results are plotted in the response surface map in Fig. 3A–E. The analysis of response surface maps shows the importance of each factors and their reciprocal interaction on the single responses.

The numerical optimization for several responses and factors was obtained combining all these six responses into one function, called desirability function [41] and plotted in Fig. 3F. The analysis of this map is useful to find out the maximum of desirability and optimal conditions for a run buffer composition from such a map are found to be 30 mM borate, pH 9.2 and 31 mM of SDS.

The preparation of the derivatising agent is a critical step as it not only influences the sensitivity of the response, but also the overall resolution. The amount of the derivatising agent should be enough to react completely with the analytes; however, excessive amount will result in a wider system peak (OPA/2-ME), thereby influencing the separation and resolution. Several mixtures of the derivatizing agent have been prepared following an experimental design with response surface methodology. With this technique, only 11 experiments have been necessary in order to obtain the information on the optimum concentrations of OPA and 2-ME and the resulting response surface map is shown in Fig. 4. The response surface map shows a curvilinear dependence between the resolution of the first two peaks ($R_{1–2}$) and the concentration of OPA and 2-ME. As expected, higher concentrations of derivatising agent worsen the resolution because it broadens the unreacted OPA/2-ME system peak. This is also true for low concentration of the derivatizing agent, probably due to decrease in the efficiency of the derivatization reaction. Optimal conditions were found at the maximum of the surface map for a derivatising reagent composed by 25 mg of OPA and 18 mL of 2ME and used in all the subsequent work.

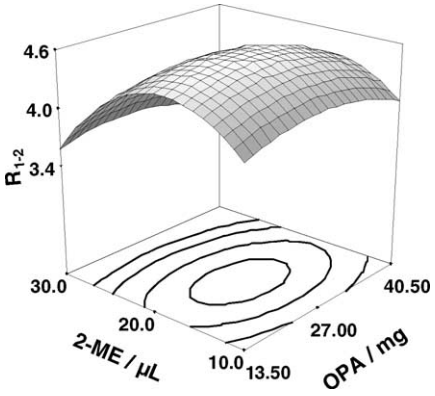

Fig. 4. Response surface plot for optimizing the OPA and 2-ME concentrations in order to maximize the resolution between the first peak of the derivatizing agent and the first isoindole compound.

The amperometric detection at the screen-printed electrode results in a well defined concentration dependence. An electropherograms for sample mixtures containing increasing levels of Se-Met and Se-Eth in 50 μM steps are shown in Fig. 5A–F. Defined peaks, proportional to the concentration of both analytes, are observed. The resulting calibration

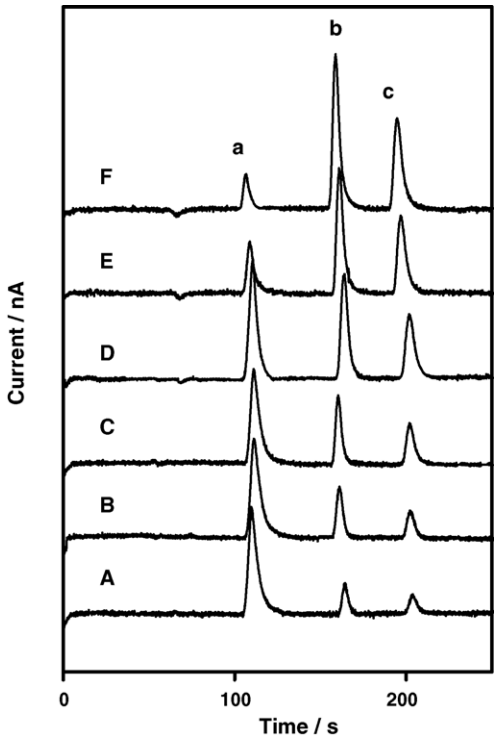

Fig. 5. Electropherogram for a mixture of Se-Met (b) and Se-Eth (c) with an increasing concentration from 50 to 300 μM of in increments of 50 μM (A–F). Peak (a) corresponds to the unreacted derivatizing agent. Detection potential, +0.9 V (vs. Ag/AgCl wire); other conditions, as in Fig. 1.

plot (not shown) is highly linear with correlation coefficients for Se-Met, Se-Eth and Se-Cys of 0.999, 0.997, and 0.994, respectively. The detection limits (based on a S/N ratio of 3) are found to be 15, 25 and 42 μM for Se-Met, Se-Eth, and Se-Cys, respectively.

No passivation problem was observed using the CE/electrochemical-detector microsystem for the detection of seleno amino compounds. A series of 10 repetitive injections of a mixture containing 200 μM of Se-Met (using the same detector strip) was performed for estimating the relative standard deviations (RSDs) of the peak current and migration time for all the analytes under optimum conditions. The RSDs of the peak current and migration time are 4.7 and 1.3%, respectively. Good precision reflects the negligible surface passivation due to the injection of extremely small sample volumes.

4. Conclusions

We have demonstrated the utility of CE microchips with screen-printed electrochemical detectors for the separation and detection of important seleno amino acids compounds. The fast separation and negligible waste production (compared, for example, to common liquid chromatographic protocols) is attractive for various practical applications. The optimization of various experimental parameters is carried out using response surface methodology.

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