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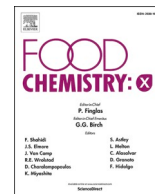
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Detection of nitrification inhibitor dicyandiamide: A direct electrochemical approach

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ABSTRACT

A single run approach for rapid detection of nitrification inhibitor, dicyandiamide (DCD) using electrogenerated chlorine assisted polymerization through azo bond, under acidic conditions and at a preanodized screen printed carbon electrode (SPCE*) is presented. The role of chloride containing support electrolyte in acidic medium along with oxygen functionalities/edge sites are found to be crucial for the successful oxidative polymerization and subsequent adsorption of oxidized products on the electrode surface. The SEM, cyclic voltammetry and X-ray photoelectron spectroscopy studies were used to characterize the polymer film formation. The system exhibited a linear range between 20 and 170 μM with a detection limit of 3 μM (S/N = 3). The method was successfully tested for the detection of DCD in dairy and water samples. Simultaneous detection of DCD in the presence of melamine has also been demonstrated.

1. Introduction

Adulteration with melamine and cyanuric acid in dairy products and pet foods to exaggerate nitrogen content to increase the apparent protein level has raised worldwide concern recently (Rovina & Siddiquee, 2016). The harmful nature of melamine, that can cause kidney failure and even death in cats and dogs, has prompted the necessity for routine examination of milk, dairy products and pet foods for melamine contamination leading to development of several analytical methods (Chen et al., 2015; Liao, Chen, Chang, & Zen, 2011a, 2011b; Liu et al., 2011; Rao et al., 2017; Tsai, Thiagarajan, & Chen, 2010). Similar to melamine, trace level detection of dicyandiamide (DCD) was reported in number of samples in New Zealand. DCD finds application in the synthesis of dicyandiamide-formaldehyde polymer/resin and serves as a precursor in melamine synthesis (Meng et al., 2014). This nitrogen rich molecule is also extensively used in the preparation of graphitic carbon nitrides (g-C₃N₄) that exhibit diverse catalytic properties (Goettmann,

Fischer, Antonietti, & Thomas, 2006; Jürgens et al., 2003; Thomas et al., 2008; Wang, Blechert, & Antonietti, 2012). DCD is one of the most widely used nitrification inhibitor in grazed pasture to decrease the leaching and denitrification from urea and ammonia based fertilizers, and to improve pasture productivity (Qiu, Sun, Gunatilake, She, & Mlsna, 2015). Cows grazing the pastures treated with DCD showed traces of the chemical in their milk samples (Zhang et al., 2015). Though the presence of DCD was limited and might not have caused a major health hazard, the US Food and Drug Administration has listed it as an issue of concern (Zou, Zhang, Feng, & Liang, 2014). The daily allowed limit of DCD in food samples is 1 mg/kg (Authority, 2004). This has warranted the development of analytical techniques for routine screening and quantification of DCD in dairy products.

Recent efforts resulted in the development of a few analytical methods to detect DCD in dairy products; HPLC-UV (Turowski & Deshmukh, 2004), HPLC-MS/MS (MacMahon, Begley, Diachenko, & Stromgren, 2012), molecularly imprinted polymer (MIP) technique

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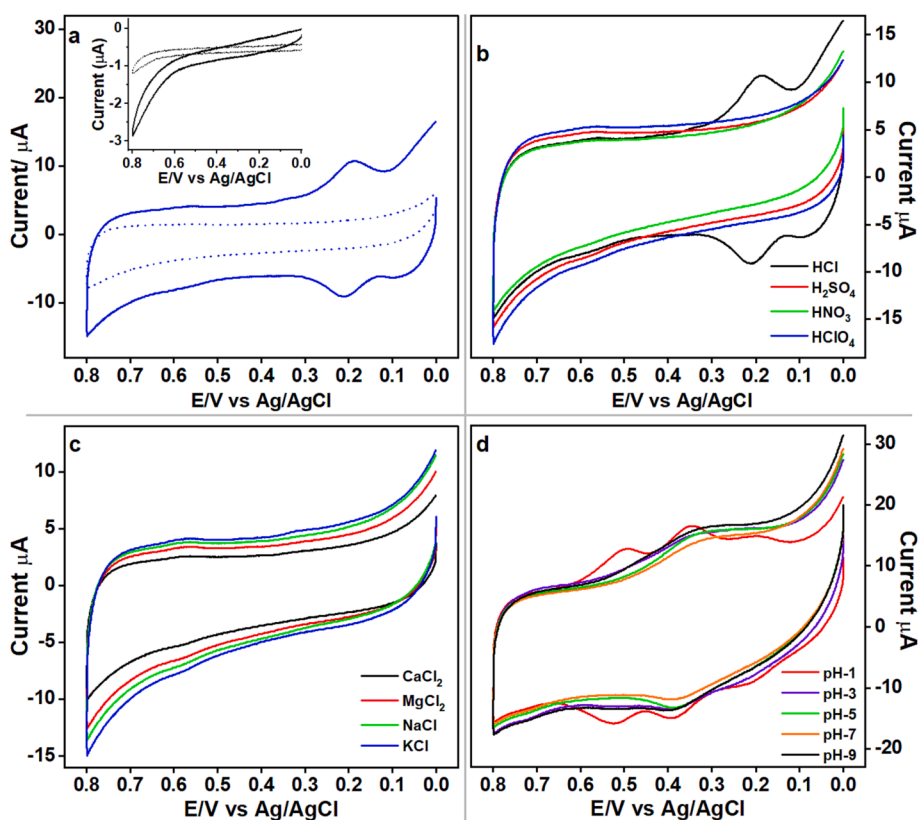


Fig. 1. (a) Reblank cyclic voltammograms (CVs) in 0.1 M pH 6.5 PB before (dot) and after (solid) oxidation of DCD on SPCE*. The reblank CV (inset Fig. 1a) of SPCE before (dot) and after (solid) oxidation of DCD in 0.1 M pH 6.5 PB. (b) Reblank cyclic voltammograms (CVs), obtained after oxidation of DCD in various acid medium, in 0.1 M pH 6.5 PB; (c) Reblank CVs, obtained after oxidation of DCD in different chloride containing supporting electrolytes, in 0.1 M pH 6.5 PB. (d) Reblank CVs obtained in 0.1 M HCl, after performing DCD oxidation in chloride containing supporting electrolyte at different pH values (1–9).

(Wang, Liu, Wei, Yao, & Gong, 2015; Wang, Liu, Yao, & Zhu, 2018), Point-Scan Raman Hyperspectral Imaging Technology (Yang et al., 2022), Surface-Enhanced Raman Spectroscopy (SERS) (Muhammad et al., 2020), ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) (Feng, Ping-Ping, Na-Si, Hong-Bin, & Xiao-Gang, 2013), ion exclusion chromatography (IEC-UV) (Mei-Lan, Guang-Wen, Kun, Xue-Ling, & Ming-Li, 2013), DART ionization source coupled with quadrupole time-of-flight tandem mass spectrometry (DART/Q-TOF MS/MS) (Zhang et al., 2015) and hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry (HILIC-MS/MS) (Inoue, Sakamoto, Min, & Todoroki, 2014). However, there is no direct electrochemical method available for the detection of DCD. Electrochemical method in combination with screen-printed carbon electrode (SPCE) can offer a cost-effective solution for rapid on-field analysis (Chiu, Yang, Liu, & Zen, 2009; Thiyagarajan, Chang, Senthilkumar, & Zen, 2014). Activated carbon electrodes have gained attention due to their porous structure, edge/defects sites and surface oxygen functional groups with enhanced adsorption and electrochemical behavior (Chiu, Wei, & Zen, 2011; Cumba et al., 2016; Prasad, Chen, Ay, & Zen, 2007; Prasad, Muthuraman, & Zen, 2008; Senthilkumar, Sithini, Thiyagarajan, Baskar, & Zen, 2015; Su, Tai, & Zen, 2013; Thirupathi, Thiyagarajan, Gopinathan, & Zen, 2016). We present hereby a simple and sensitive single-run electrochemical approach for DCD detection in dairy products. Unlike melamine, the present system exhibited a clear dependency on both chlorides containing supporting electrolyte and acidic pH. In addition, the role of oxygen functionalities and edge site were found to be crucial for efficient oxidation and its subsequent adsorption on the electrode surface. This proposed electrochemical method has been successfully employed for the determination of DCD in milk, infant formulas and water samples.

2. Materials and methods

2.1. Reagents and chemicals

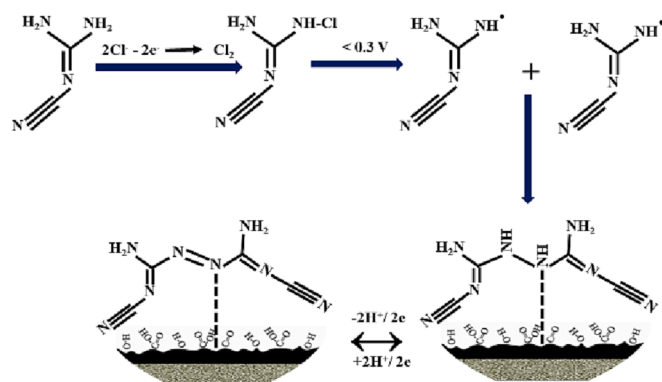
Dicyandiamide (Alfa Aesar, England) and all other chemicals were of ACS-certified reagent grade and used without further purification. 0.1 M (pH 7.4) phosphate buffer solution (PB) and all other solutions were prepared with Millipore (Billerica, MA) water. Milk powder (Anchor, Fonterra, New Zealand) and milk (Fresh delight, Taiwan) samples were purchased from a local shop. All real samples were treated by dissolving 0.1 g of dry milk powder/milk and 0.058 g of NaCl (Sigma, USA) in 10 ml of 0.1 M HCl. After 1 min of vortex shaking and ultrasonication for 5 min, the solution mixture was centrifuged at 4000 rpm for 20 min and the supernatant was filtered through a 0.25 µm polytetrafluoroethylene (PTFE) membrane filter (Advantec, Japan). The stock sample solutions were appropriately diluted for real sample analysis.

2.2. Apparatus and electrodes

Electrochemical measurements were performed with CHI832a electrochemical workstation (CH Instruments, Austin, TX) and Modulab potentiostat (Solartron Analytical, UK) in a three-electrode cell with Ag/AgCl reference electrode, platinum auxiliary electrode and preanodized SPCE (SPCE*) as working electrode. The SPCE electrode (working area of 0.196 cm²) was purchased from Zensor R&D (Taichung, Taiwan). X-ray photoelectron spectroscopy (XPS) measurements carried out by Omicron DAR 400 (Germany), using an Al K α X-ray source (1486.6 eV) with 0.1 eV resolution. The pressure inside the analyzer was maintained at about 10⁻¹⁰ Torr.

2.3. Procedure

Prior to electrochemical analysis, the bare SPCE was cleaned electrochemically in 0.1 M (pH 7.4) PB by cycling between -1.0 and 1.2 V



Scheme 1. Schematic representation of DCD oxidation and dimerization/polymerization assisted by electrogenerated chlorine.

for 20 segments. For the electrode activation, the electrode was pre-anodized electrochemically at an applied potential of 2.0 V vs. Ag/AgCl for 300 s in 0.1 M PB (pH 7.4) (designated as SPCE*). We found that a minimum anodization potential of 1.8 V vs Ag/AgCl was necessary to observe the redox peaks associated with the polymerized DCD. While 2 V vs Ag/AgCl was found to be optimal, higher anodization voltage (2.2 V vs Ag/AgCl) resulted in a higher background current (capacitive current) due to the oxide layer formation and defect/edge sites, which may not be suitable for the sensor applications. Electrochemical oxidation of dicyandiamide (DCD) was carried out at SPCE/SPCE* with 1 mM solution of DCD in 0.1 M HCl, unless mentioned, at a potential of 1.5 V vs.

Ag/AgCl for 300 s. This modified electrode was designated as SPCE*-DCD. Further, the SWV method was employed for the selective identification of DCD with optimized parameters of amplitude = 25 mV, frequency = 15 Hz and step = 5 mV. All experiments were performed at ambient temperature (25 °C). Sample preparation method for real sample analysis: Step 1: Measure 0.1 g of milk powder (For whole milk, 0.1 ml was taken) into test tubes (50 ml). Mix 100 mM of NaCl with 1 mM of DCD in 10 ml of 0.1 M HCl. Step 2: The mixture was subjected to vortex shaking for 5 min and ultrasonication for 5 min. Then, the solution was heated at 60 °C until completely dissolved. Step 3: Then the mixture was centrifuged at 4000 rpm for 20 min and subsequently the supernatant was filtered through a 0.25 μm polytetrafluoroethylene membrane filter. The sample solutions were suitably diluted for further analysis.

3. Results and discussion

3.1. Electrochemical behavior

The electrochemical behavior of dicyandiamide was studied by carrying out the oxidation at a potential of 1.5 V (vs. Ag/AgCl) in 0.1 M HCl for 300 s followed by reblank in 0.1 M PB, pH-6.5 solution. As shown in Fig. 1a, in the absence of DCD (dotted), no peak response was noticed at bare SPCE (inset) and SPCE* (dotted) during the reblank. However, in the presence of DCD, a well-defined reversible redox peak with a $E_{1/2} \sim 0.206\text{ V}$ and $\Delta E_p \sim 0.02\text{ V}$ was observed at SPCE* (solid). The oxidation performed at unactivated SPCE electrode (inset) did not show any redox peak during the reblank performed in PB (solid). The

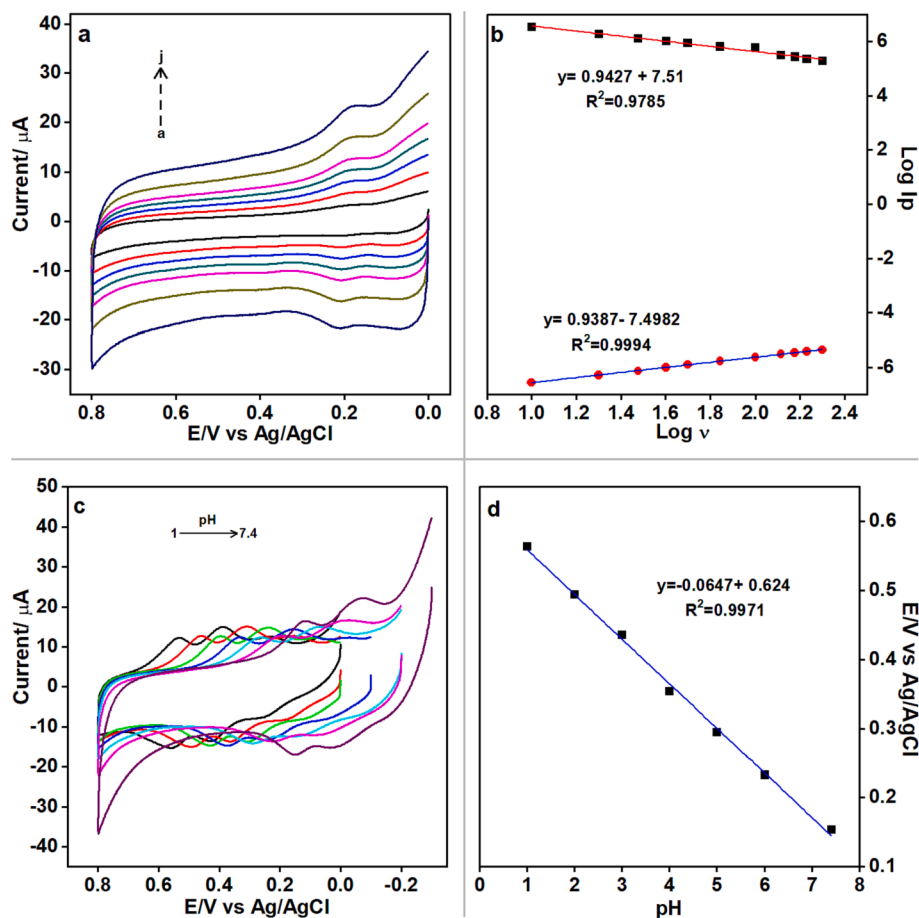


Fig. 2. (a) Cyclic voltammograms (CVs) of SPCE*-DCD electrode at various scan rate in 0.1 M pH 6.5 PB (a–j: 10, 20, 30, 40, 50, 70, 100, 120, 150, 170 and 200 mV/s). (b) Plot of log scan rate vs log peak current. (c) CV response of SPCE*-DCD modified electrode at various pH in 0.1 M HCl/PB (pH 1–6) and PB (pH-7.4); (d) Plot of pH vs potential.

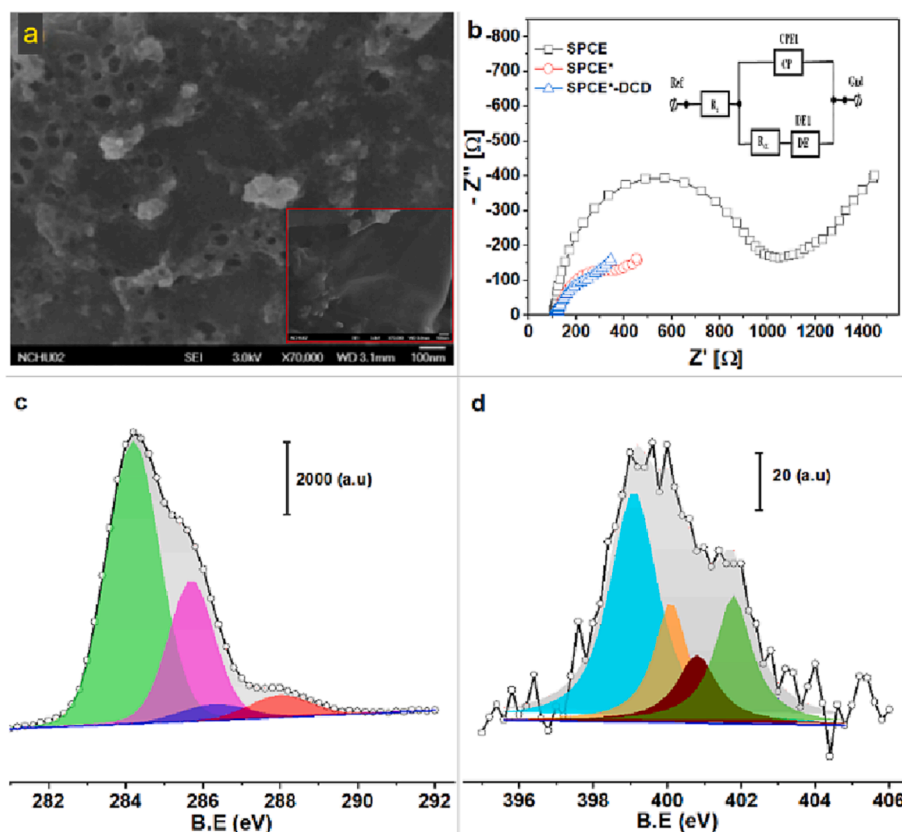


Fig. 3. (a) SEM image of SPCE*-DCD; Inset SEM image of bare SPCE (scale bar 100 nm). (b) EIS spectra of SPCE, SPCE* and SPCE*-DCD electrode obtained at 0.23 V in 0.1 M KCl containing 5 mM Ferricyanide. (c and d) The deconvoluted high resolution C1s and N1s XPS spectra of SPCE* and SPCE*-DCD electrodes.

SPCE*, given their edge/defect sites and oxygen functionalities that could improve the adsorption characteristics, acted as a suitable platform for polymer formation (Thiruppathi et al., 2016). Further, in order to explore optimal oxidation conditions, different acid containing supporting electrolyte solutions; HCl, H₂SO₄, HNO₃, HClO₄ (0.1 M) were chosen for the oxidation process (Fig. 1b). As can be observed from these experiments, electrochemical oxidation of DCD only performed in supporting electrolytes that contain chloride ions resulting in the appearance of redox wave upon reblank performed in PB. The role of electrogenerated Cl₂ in the oxidation of amine group for facile immobilization of adenine on electrode surface has been demonstrated (Senthilkumar et al., 2015). To further access any such role of Cl₂ in the oxidation of DCD, different Cl⁻ containing electrolytes such as KCl, MgCl₂, CaCl₂ and NaCl were employed during oxidation. However, no redox signal was observed under any of these conditions (Fig. 1c). The observation indicated a possible role of protonation that may be necessary for the oxidation process. To validate this, an electrolyte solution containing 0.1 M KCl with varying pH range between 1 and 9 were employed. Unlike melamine that could be oxidized under weakly acidic conditions (though polymerization was not substantial), the signal originating from the oxidized DCD product was observed at only pH 1.0 and oxidation performed under other conditions failed to produce any redox signal upon reblank (Fig. 1d). It was previously verified that sufficiently acidic pH is needed to stabilize the radical cation in order for the polymerization step to proceed (Baskar, Liao, Chang, & Zen, 2013; Mu & Kan, 1996) and thus a similar mechanism is expected in the case of DCD as well (Scheme 1). Finally, chloride-containing supporting electrolyte and a sufficiently acidic pH (pH-1.0) were found as necessary preconditions for DCD oxidation and polymerization process. Note that when acids other than HCl are employed to adjust the pH, roughly double concentration of chloride containing salt to that of the acid was found to work well.

Fig. 2 (a) depicts the influence of the scan rate on the CVs of DCD-SPCE* prepared using 1.0 mM DCD, at different scan rates ranging from 10 to 200 mVs⁻¹ in 0.1 M PBS (pH 7.4). The anodic peak current (I_{pa}) and cathodic peak current (I_{pc}) increased with increasing scan rates and a plot of log of scan rate vs current exhibited a linear increase with a slope of ~0.9 indicating an adsorption-controlled process at SPCE* (Fig. 2b) and verifies the surface confined redox behavior or the polymer formation. Fig. 2 (c) shows the CV of DCD-SPCE* at different pH values from 1.0 to 6.0 and at pH 7.4 in 0.1 M PBS. As can be seen, the peak potential shifted to negative potentials with the increasing pH. The peak potentials have shown a linear dependence on pH over the range from 1.0 to 7.4 with a slope value of 64 mV/pH (Fig. 2d) demonstrating a Nernstian behavior with equal number of electrons coupled with equal number of protons involved in the redox process. Though only one redox peak is expected for this azo system, the appearance of second redox peak could be due to different chemical nature of the azo moiety as the radical generated can also attack the nucleophile carbon. Further, the storage stability of DCD modified electrode was studied over a period of 5 days. Not much change in the current response of the redox peak was observed between day 1 and day 3, however on day 5 the current response decreased by half. The results indicate a reasonable storage stability of the DCD modified electrode.

Surface morphology of DCD-SPCE*

The electrode surface after oxidation of DCD was characterized by SEM (scanning electron microscopy) and XPS (X-ray photoelectron spectroscopy) techniques to confirm the polymeric film formation and to validate the proposed mechanism of polymer formation via azo type coupling through electrogenerated Cl₂ assisted amine oxidation. The SEM image, Fig. 3 (a), corresponds to DCD modified SPCE. The, small sponge like white structures, albeit scattered and fewer, could be seen on

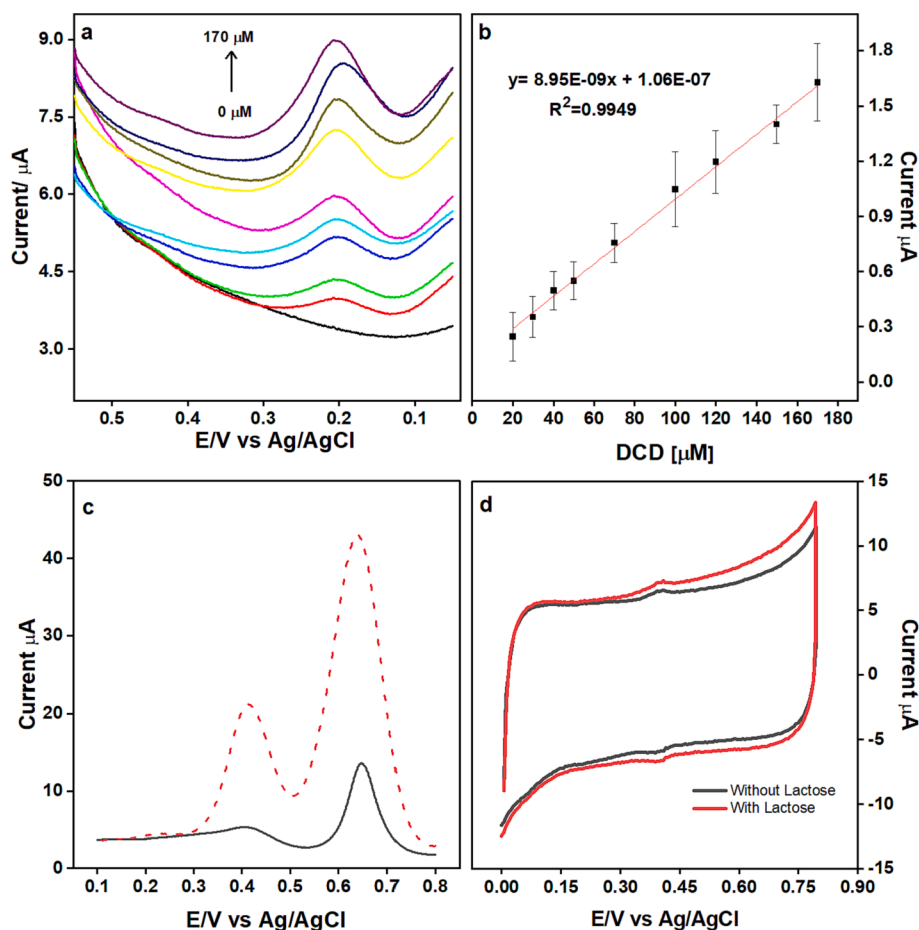


Fig. 4. (a) Reblank SWV response of SPCE*-DCD with increasing concentration of DCD in 0.1 M PB, pH 6.5. (b) Plot of concentration vs current response. (c) Reblank SWV response of SPCE*-DCD/Mel in 0.1 M HCl; 20 μM DCD and 20 μM Melamine (solid) and 150 μM DCD and 50 μM Melamine (dashed), respectively. (d) Reblank CVs obtained in 0.1 M HCl, after oxidation of 200 μM of DCD in the presence and absence of 5% lactose.

the surface indicating formation of polymeric film similar to that of polymelamine and presence of porous structures on the electrode surface can be attributed to the preanodization process. While SPCE shows a relatively smooth graphite structure (inset Fig. 3a).

To further analyze the electron transfer behavior of the DCD modified electrode, impedance measurement for the SPCE, SPCE* and SPCE*-DCD modified electrodes using 5 mM Ferricyanide redox probe in 0.1 M KCl electrolyte. DC Level = 0.23 V. Amplitude = 10 mV. Start frequency: 100 Hz. End frequency: 0.5 Hz. The R_{CT} values were found to be 0.074 k Ω , 8.45E-08 k Ω and 1.28E-08 k Ω for the SPCE, SPCE* and SPCE*-DCD modified electrodes, respectively. It shows that the modification of SPCE surface with DCD can result in improved electron transfer process at the electrode/electrolyte interface (Fig. 3b). Further, X-ray photoelectron spectroscopy (XPS) studies were performed to obtain details about the chemical state of components present on the surface. The deconvoluted spectra C1s spectra has peaks at 284.19 eV (C—C), 285.67 eV (C—OH), 286.29 eV (C—O) and 288.20 eV (O—C=O), respectively, confirming the formation of oxygen functionalities at the preanodized electrode surface (Fig. 3c). The fitted N1s spectra of DCD-SPCE*, Fig. 3 (d), exhibits peaks around 399.1 eV, 400.8 eV and 401.8 eV, which indicates the presence of imine, imide and quaternary nitrogen group, respectively. In addition, the peak around 400.1 eV can be assigned to the azo functional group (Kesavan & John, 2014). It is to be noted that DCD does not have any azo functionality as such. The SEM and XPS results thus indicate the polymerization of DCD on electrode surface through formation of azo bonds upon oxidation.

Table 1

Real sample analysis recovery data obtained from dairy and water samples.

Sample	Dilution factor	Spiked (μM)	Found (μM)	Recovery (%)	RSD (%) (n = 3)
Milk powder	100	20	22.00	102.0	4.44
		50	45.50	95.5	3.92
		100	103.30	103.3	1.72
Milk	100	20	23.70	103.7	4.67
		50	49.80	99.8	1.20
		100	100.20	100.2	4.87
Tap water	100	20	18.70	98.7	3.19
		50	44.79	94.8	1.82
		100	98.60	98.6	1.18

3.3. Analytical performance

The overall electrochemical polymer formation is expected to provide good selectivity for the determination of DCD in dairy products. To further improve the detection sensitivity, squarewave voltammetry (SWV) technique was adopted. The calibration curve of DCD was obtained using the optimized SWV parameters (Fig. 4a) and a linear response between 20 and 170 μM , with a regression coefficient 0.996 and detection limit of 3.0 μM ($S/N = 3$) was obtained (Fig. 4b). The limit of quantification is 9.9 μM ($S/N = 10$). The applicability of the present method for testing of real samples was examined under the optimized

condition using the standard addition method. The dairy products were found to contain no DCD, thus a known amount of DCD was spiked in to the commercial milk powder, milk and tap water samples. The proposed method successfully detected DCD with good recovery values for all samples in the range of 94.8–103.7% and the results are summarized in Table 1. Further, in order to validate the selectivity of the present method, simultaneous detection of 20 and 150 μM of DCD was demonstrated in the presence of both 10 and 50 μM of melamine. The SWV results obtained in 0.1 M HCl showed a well separated peak for melamine and DCD, respectively (Fig. 4c). The results indicated that the presence of melamine does not influence the polymerization of DCD significantly and thus, the presence of either DCD/melamine or both could be detected in dairy products using a single method. Since lactose is present up to 5% in whole milk, we carried out the DCD (200 μM) oxidation in the presence of 5% lactose. On comparison, the reblank CVs of polymerized DCD obtained in the presence and absence of lactose did not show any significant difference (Fig. 4d).

4. Conclusions

We have successfully established a selective and sensitive single run electrochemical approach for the detection of DCD in various matrix. The presence of chloride containing electrolyte and sufficiently acidic pH are essential for DCD polymer formation on SPCE*. The good recovery values obtained in commercial milk powder, milk and tap water indicate the usefulness of this method. The proposed single run method is highly suitable for on-site and real time detection of DCD. Based on the experimental results, the anodization process plays a vital role for the oxidation and subsequent adsorption of the oxidized products. However, the increased background current affects the detection of low concentration. Exploring suitable electrochemical pretreatment conditions, including other electrolyte medium and optimizing the preanodization time may enable detection/quantification of even lower concentration.

CRedit authorship contribution statement

Thiyagarajan Natarajan: Conceptualization, Methodology, Supervision, Validation. **Manavalan Gopinathan:** Data curation, Formal analysis, Methodology, Investigation, Validation. **Murugan Thirupathi:** Formal analysis, Investigation. **Omotayo Adeniyi:** Formal analysis, Investigation. **Jen-Lin Chang:** Formal analysis, Investigation. **Jyh-Myng Zen:** Conceptualization, Project administration, Resources, Supervision. **Solomon Tesfalidet:** Investigation. **Jyri-Pekka Mikkola:** Project administration, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are all available in this article.

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