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Ultrasensitive Nanosensor for Detection of Malic Acid in Tomato as Fruit Ripening Indicator

Anita Dalal[1,2] · J. S. Rana[2] · Ashok Kumar[1]

Abstract Malic acid is an important fruit ripening indicator. Fruit industry losses every year due to non-availability of rapid technology for early detection of ripening of fruits. Therefore, nanosensor was developed for detection of malic acid concentrations in tomato at early stage of ripening before transport to the market. The enzyme NADP-malate dehydrogenase (Malic enzyme) was covalently immobilized on to screen printed carboxylated-multiwall carbon nanotubes working electrode using EDC-NHS chemistry. The enzyme electrode was characterized using scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy. The immobilized enzyme/c-MWCNT electrode was used for amperometric determination of different concentrations of malic acid in tomato using differential pulse voltammogram (DPV) at scan rate of 100 mv/s. The limit of detection of malic acid was 0.01 mM. The nanosensor showed low K_m (0.12 mM), less response time (2 min), high sensitivity (0.01 mM) and better storage stability 180 days at 4 °C compared to earlier reported malate biosensor. The nanosensor was also validated at different stages of ripening of tomato using enzymatic method.

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Introduction

The emergence of nanotechnology offers great opportunities to improve the sensitivity, stability and anti-interference ability of the biosensing systems. In recent years, a number of novel nanomaterials have been used for fabrication of different biosensors. Performance of direct electron transfer between the enzyme and electrode is enhanced by carbon nanotubes (CNT) and metal nanoparticles (Sharma et al. 2009). Direct electrochemistry of enzyme has an important role in development of biosensors, biofuel cells and biomedical devices (Zhang et al. 2004; Prakash et al. 2009). CNT acts as an excellent transducer and is used in different electrochemical biosensor which promotes the electron transfer between immobilized enzyme electrode and substrate due to its extraordinary electron transport property (Iijima 1991; Nugent et al. 2001; Harris 2004; Arvinte et al. 2008). CNT improves the sensor performance because it posses high surface to volume ratio which help in higher enzyme loading and make an effective contact between deeply buried active sites of enzymes and the electrode (Tu et al. 2005; Doubnerova and Ryslava 2011; Hegde et al. 2011).Various biosensors have been reported for detection of water contamination, malic acid and glucose (Lin et al. 2004; Gautam et al. 2012).

Malic acid is found in many sour or tart-tasting unripe fruits. Malic acid has wide application in the food industries (Davis 1966; Kovacs and Djedjro 1994; Barden et al. (1997). L-malic acid is an important indicator for fruit maturity matrix (Kader 1999; Sotiropoulou and Chaniotakis 2003). The catalysis of malic enzyme generally proceeds in three steps: dehydrogenation of malate to produce oxaloacetate, decarboxylation of oxaloacetate to produce enol-pyruvate and then tautomerization of enol-pyruvate to produce pyruvate. The food industries take advantage of the fact that its natural products lend themselves for detection with biosensors. The ratio

of sugar to acid concentration provides a complete ripeness of fruits. There is a need to develop biosensor to determine malic acid which is a taste determining constituent of fruits and fruit juices. With this device, fruit growers, retailers and processors can monitor fruit quality in storage and marketing for sale.

Gajovic et al. (1997) used Clark type oxygen electrode sensor using immobilized malic enzyme for determination of L-malic acid in food samples. It has a linear detection from 1 μmol dm^{-3} to 0.9 mmol dm^{-3} with a response time of 1–5 min. Arif et al. (2002) developed amperometric biosensor incorporating malic enzyme for the measurement of L-malic acid in apple, potato and tomato horticultural samples and reported response time 6 min with 0.028–0.7 mM sensitivity. Lupu et al. (2004) developed malic acid biosensor based on screen printed electrode using polyethylenimine-glutaraldehyde cross linked membrane with NADP-dependent dehydrogenase enzyme. Arvinte et al. (2008) used amperometric techniques for exploring electrocatalytic property of single-walled carbon nanotubes (SWCNT) modified electrode for NADH detection with linearity from 0.2 to 1 mM (Arvinte et al. 2008). The chemical reaction of malic enzyme is as follows:

$$L\text{-Malate} + NADP^+ \Leftrightarrow Pyruvate + CO_2 + NADPH$$

In the present work, carboxylated multi-walled carbon nanotubes (c-MWCNT)-modified screen-printed electrode-based nanosensor was developed by immobilizing malic enzyme on working electrodes surface for detection of L-malic acid (L-malate) in fruits samples for testing maturity status of the fruits. The c-MWCNT increases the surface area for enzyme to bind and also acts as an electron transfer mediator, helping in enhancing the sensor response of enzyme electrode and thus increasing the sensitivity of the biosensor. The present enzyme-based biosensor can be used to detect malic acid concentrations in tomato as indicator of ripening.

Materials and Methods

Chemicals and Instruments

Malic enzyme (chicken liver), Malic Acid, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich, USA. Nicotinamide adenine dinucleotide phosphate (NADP), dimethylformamide (DMF) and other chemicals were procured from Sisco Research Laboratory, India. All electrochemical experiments were performed at room temperature (25 °C) using Potentiostat/Galvanostat (Model: FRA 2 μ AUTOLAB). Commercially available c-MWCNT electrode was obtained from Dropsens. Screen-printed electrode includes three-electrode configuration in which working (c-MWCNT), counter (carbon) and reference (silver) electrodes which are printed in close proximity. Fourier transform infrared spectroscopy (Model: Spectrum BXFTIR, Perkin Elmer) was performed at Delhi University and scanning electron microscopy (Model: FEI QUANTA 200F) was used at Department of Textile Technology, IIT Delhi.

Collection and Preparation of Fruit Samples

Four types of fresh tomatoes (unripened tomato, partially ripened tomato, ripened tomato and over ripened tomato) were obtained from local market. All tomato samples contain different concentrations of malic acid, which were tested using our fabricated nanosensor. Measurement of L-malic acid in tomato samples is indicator of fruit maturity/ripening. Fruit samples (tomato) 10 g each were washed with water dried and crushed in mixer, then squeezed with muslin cloth to collect juice. It was centrifuged at 6000×g for 10 min at 4 °C. Supernatant was collected and heated at 94 °C for 30 min to remove interferent from juices. The juices were used to detect the malic acid concentration in the samples using nanosensor.

Preparation of Enzyme Electrode

Screen printed c-MWCNT electrode was washed with Milli-Q water and dried. The working c-MWCNT electrode was treated with mixture of 0.2 M EDC and 0.2 M NHS (1:1, v/v) for 1 h. The electrode was washed with PBS (50 mM NaH$_2$PO$_4$, 50 mM Na$_2$HPO$_4$ and 0.9% NaCl) buffer, pH 7.4 and dried at room temperature. Malic enzyme (0.4 units/ml in PBS buffer, pH 7.4) 5 μl was immobilized on working electrode surface for 1 h at 25 °C. The enzyme electrode can be stored at 4 °C for further use. The surface of the fabricated nanosensor was characterized using FTIR and SEM.

Results and Discussion

Construction of Enzyme Electrode

The fabrication of malic acid nanosensor based on NADP-specific malate dehydrogenase (malic enzyme) immobilized on working c-MWCNT electrode (0.12 cm^2) is summarized in (Fig. 1). Malic enzyme was immobilized covalently onto c-MWCNT electrode using EDC-NHS coupling through amide bond formation between the free and unbound –COOH groups of c-MWCNT and –NH$_2$ groups of enzyme. Unbound enzyme on c-MWCNT (working electrode surface) was removed by 3–4 times washing with PBS buffer, pH 7.4. Due to covalent coupling of the enzyme on working electrode, the enzyme does not leach out during the repeated washing of the electrode confirm the attachment of enzyme on electrode surface.
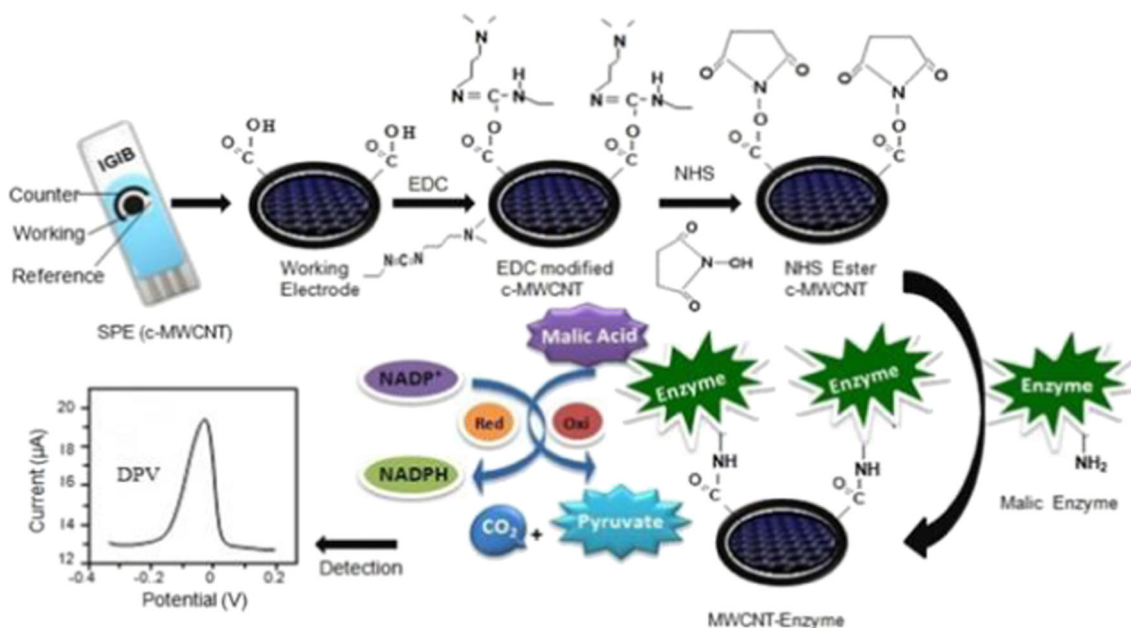
C-MWCNT: Carboxylated multiwalled carbon nanotubes(screen printed electrode), EDC: 1-Ethyl-3-(3-dimethyl amino propyl) Carbodiimide, NHS: N-Hydroxysucinimide, Malic enzyme, Substrate: Malic acid and NADP, Product:Pyruvate and CO_2, Oxi : Oxidation, Red: Reduction, DPV: Differential pulse voltammetry

Fig. 1 Schematic representation for fabrication of nanosensor for detection of malic acid in tomato fruit samples using covalent immobilization of enzyme on c-MWCNT through EDC-NHS chemistry

Differential Pulse Voltammetry Study

The screen-printed immobilized enzyme (malic enzyme/c-MWCNT) electrode was connected to Autolab potentiostat through wire connector. The nanosensor response was measured using differential pulse voltammogram (DPV) at potential range −0.6 to −0.2 V in the presence of different concentrations of malic acid. Figure 2a shows DPV of the enzyme/c-MWCNT in PBS, pH 7.4 with different concentrations of malic acid (0.03 to 0.2 mM). The maximum response was observed at −0.34 V and hence subsequent studies were carried out at this applied potential. The DPV was measured in a microcell containing fixed 1.0 µl NADP (4 mM) and 1.0 µl of different concentrations of malic acid in 48 µl of PBS, pH 7.4 on enzyme/c-MWCNT electrode. DPV peaks were increased with increasing concentrations of malic acid due to increased oxidation of malic acid. Malic enzyme catalyzes a reversible oxidative decarboxylation of L-malate to give carbon dioxide and pyruvate with the concomitant reduction of NADP to NADPH. The c-MWCNT increases the surface area for binding to the enzyme and also acts as an electron transfer mediator for enhancing sensor response of the enzyme electrode causing an increase in the sensitivity of the sensor. The response time of the nanosensor was 2 min and sensitivity was 0.01 mM malic acid which is lower than the earlier reported sensor (Lupu et al. 2004).

Effect of Substrate Concentrations on Sensor (k_m)

To study the effect of substrate concentrations on sensor, the concentrations of malic acid were varied from 0.03 to 2.0 mM in PBS, pH 7.4 (Fig. 2a). A hyperbolic relationship was found between malic acid concentrations versus current (Fig. 2b). The initial rate of the reaction increases as substrate concentration increases. However, as substrate concentration gets higher, the enzyme gets saturated with substrate and rate of reaction reaches at maximum rate. The Lineweaver-Burk plot is linear (inset Fig. 2c) and follows the linear equation ($y = mx + c$) where, y is intercept corresponds to $1/I_{max}$ and x intercept of graph represents $−1/K_m$. A small value of K_m indicates that the enzyme requires only a small amount of substrate for maximum activity. Hence, maximum current was reached at relatively low substrate concentration. K_m value for malic acid as calculated from Lineweaver-Burk plot was found 0.12 mM which is lower than the 0.6 mM reported earlier (Siebert et al. 1979). The I_{max} was found 64.5 µA. The standard calibration curve of the nanosensor at different concentrations of malic acid showed that the sensor response was linear from 0 to 0.25 mM malic acid which is lower than the earlier reported 0.01 to 0.4 mM (Gajovic et al. 1997), 0.1 to 1.0 mM (Jayapraksha and Sakariah 1998) and 0.028 to 0.7 mM (Arif et al. 2002).
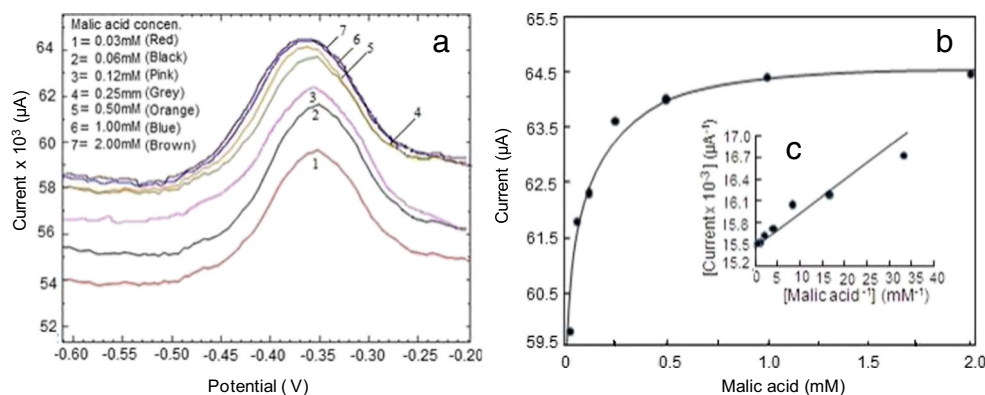
Fig. 2 **a** DPV response of enzyme/c-MWCNT electrode at different concentrations of malic acid in PBS, pH 7.4. **b** Measurement of DPV at variable malic acid concentrations on immobilized malic enzyme/c-MWCNT electrode. *Inset* **c** Lineweaver-Burk plot for determination of K_m of nanosensor based on NADP-malate dehydrogenase immobilized on c-MWCNT electrode

Effect of pH

The effect of pH on electrochemical response of malic enzyme electrode-based sensor was studied at pH 5.0 to 9.0 (data not shown). The maximum current response was obtained between pH 7.0 and 8.0 (optimum pH). At pH below 7.0 and above 8.0, the response of nanosensor decreased sharply. Therefore, 50 mM PBS, pH 7.4 was used throughout the experiment. The optimum pH of the present sensor was similar to that of previously reported amperometric malate sensor based on Clark-type oxygen electrode (Gajovic et al. 1997), polymer film (Gooding et al. 2000) aminopropyl glass beads (Esti et al. 2004), carbon ink and polymeric membrane (Maines et al. 2000) but slight higher than 2,7-dinitro-9-fluorenone film (pH 6.5) (Lupu et al. 2004).

Effect of Interferrent

The effect of interferent on the response of enzyme electrode was measured in the presence of interfering substances. A number of compounds inhibit malic enzyme activity by competing with L-malic acid for enzyme binding sites. Some important inhibitors such as ADP, ascorbic acid, succinic acid, acetyl-CoA, glyoxylate, NADH, glutamic acid and citric acid were used in PBS, pH 7.4 in the presence of 1 mM malic acid and 4 mM NADP (Table 1). The response of the enzyme electrode at each interferent was tested and no significant changes were observed, suggesting no inhibition in the enzyme activity whereas, succinic acid (0.5 mM) acts as inhibitor in tomato (Goodenough et al. 1985). ADP acts as an inhibitor up to the concentration of 1.5 mM which is lower than 2.0 mM reported earlier (Cannata et al. 1979).

Stability of Nanosensor

The storage stability and reusability of enzyme/c-MWCNT electrode was studied at every 30 days on storage in PBS buffer at 4 °C over a period of 6 months The current response of the nanosensor maintained 82% of the initial current response even after regular 100 uses over a period of 6 months, which is better than earlier reported malate sensors 8 days (Gajovic et al. 1997), 5 days (Esti et al. 2004) and 6 months at −20 °C (Lupu et al. 2004). The results suggest that enzyme/c-MWCNT screen-printed electrode has good stability.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of c-MWCNT shows a peak at 2358 cm^{-1} associated with O–H stretch from strongly hydrogen bonded –COOH group. Increased strength of signal at 1166 cm^{-1}may be associated with C–O stretching in same functionalities. Peak at 1566 cm^{-1} can be associated with the stretching of carbon nanotubes backbone (Fig. 3(A)). An FTIR spectrum of immobilized enzyme with peaks at 3026 cm^{-1},1636 cm^{-1} and 1188 cm^{-1} corresponds to C-H stretching; N-H bending and C-N stretching, respectively (Fig. 3(B)). Medium intensity peaks of N-H occurred at 3497 cm^{-1}. The successful covalent

Table 1 Effect of interferent on response of Enzyme/c-MWCNT electrode based malate nanosensor

Interferent (Conc.)	Relative peak current (%)
Control (NADP + Malic acid)	100
ADP (1.5 mM)	98
Ascorbic acid (0.25 mM)	96
Succinic acid (0.5 mM)	100
Acetyl-CoA (0.5 mM)	100
Glyoxalate (2.0 mM)	100
NADH (0.3 mM)	100
Glutamic acid (0.5 mM)	108
Citric acid (0.5 mM)	101

ADP adenosine diphosphate, *NADH* nicotinamide adenine dinucleotide reduced, *CoA* coenzyme A

Fig. 3 FTIR spectra for *A* c-MWCNT electrode and *B* enzyme/c-MWCNT electrode at frequency 400–4000 cm^{-1}
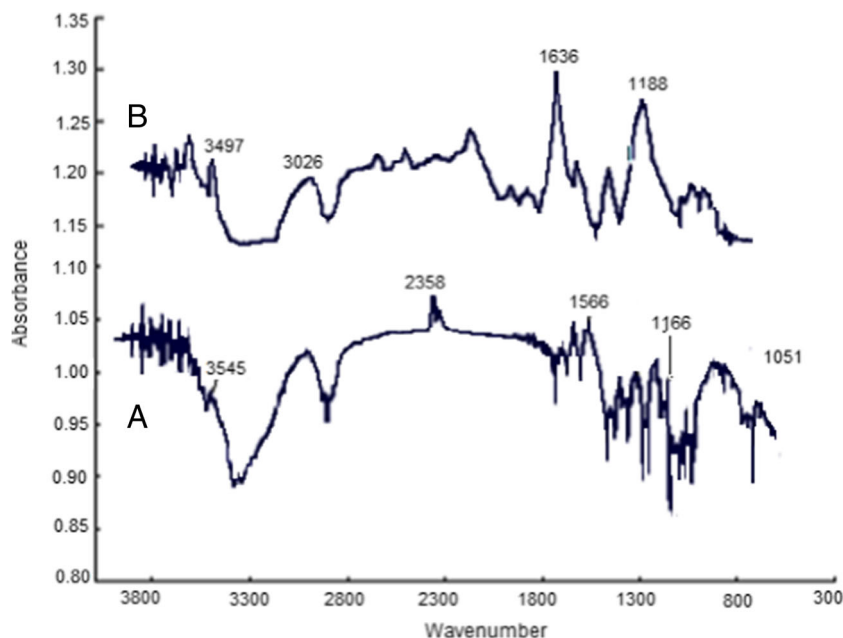
immobilization of enzyme onto c-MWCNT electrode was indicated by the appearance of the IR absorption of the amide I at 1636 cm^{-1} (Kong and Yu 2007).

Scanning Electron Microscopy (SEM)

Morphological studies are the key features that provide the surface information of the modified electrode at different stages. SEM was employed to investigate the structure of c-MWCNT and immobilization of malic enzyme onto c-MWCNT electrode (Ruhal et al. 2012).

The SEM image of c-MWCNT electrode (Fig. 4a) revealed the uniform, homogenous and cable like morphology of the nanostructure of c-MWCNT. After immobilization of enzymes on c-MWCNT electrode, the globular clouded structure indicates the immobilization of enzymes onto the surface of c-MWCNT working electrode (Fig. 4b). Thus, change in the

surface morphology of the electrode after immobilization is the evidence of immobilization of enzyme on working electrode surface of the nanosensor.

Measurement of Malic Acid in Fruit Samples

Malic acid level in tomato fruit samples were determined using present nanosensor in a similar manner as described above under optimal conditions except that malic acid was replaced by fresh tomato fruit juice samples. Fig. 5a shows CV (cyclic voltammetry) of enzyme/c-MWCNT in PBS, pH 7.4 with different stages of tomato from un-ripened tomato to over-ripened tomato at the potential range from −0.1 to +0.4 V. The maximum response was observed at 0.21 V and hence subsequent studies were carried out at this potential. CV was measured in a microcell containing 1.0 μl NADP (4 mM in PBS, pH 7.4) and 49 μl tomato juices on enzyme/c-

Fig. 4 SEM image of **a** c-MWCNT electrode and **b** after immobilization of malic enzyme onto c-MWCNT electrode
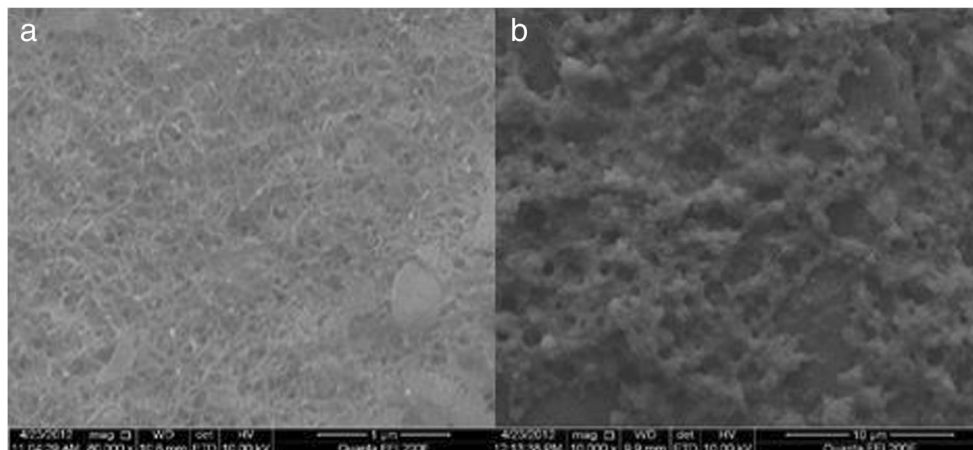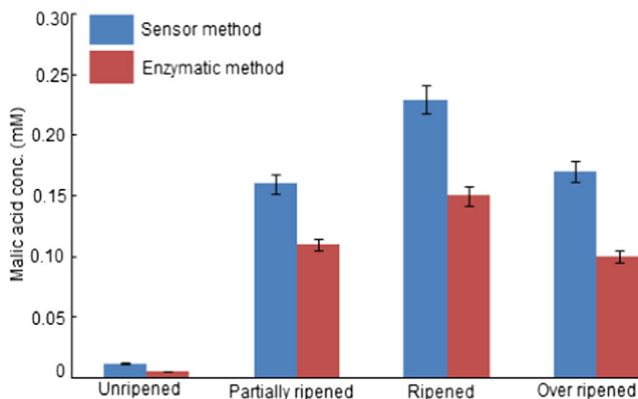
Fig. 5 **a** CV of malic acid at different stages of ripening of tomato. **b** DPV of malic acid at different stages of ripening of tomato
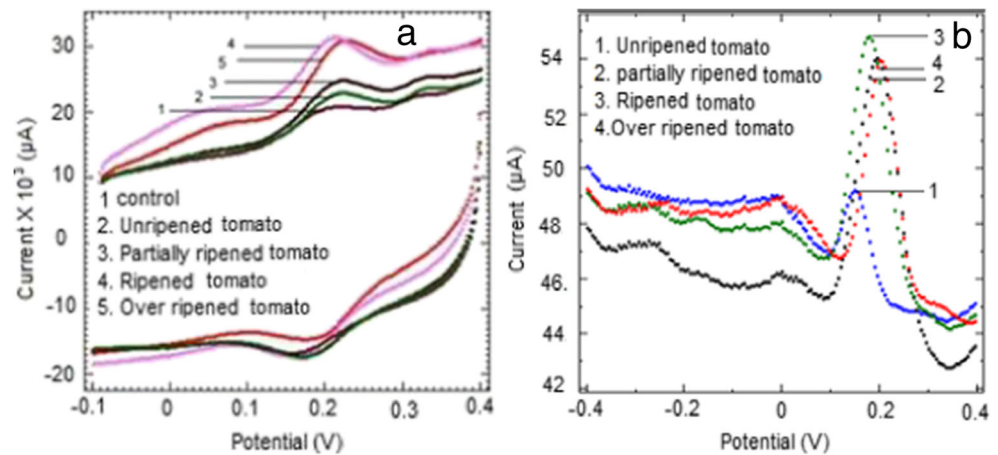
MWCNT electrode. CV peaks increased with un-ripened tomato to ripened tomato but peaks decreased in over-ripened tomato. Similarly, Fig. 5b shows DPV of enzyme/c-MWCNT in PBS, pH 7.4 with different stages of tomato from unripened to over-ripened tomato at the potential range from −0.4 to +0.4 V. The maximum response was observed at 0.19 V and hence subsequent studies were carried out at this potential. From CV and DPV, it was concluded that malic acid concentration increased from un-ripened stage of tomato to ripened stage but it decreased in over ripened stage.

Validation of Malic Acid Nanosensor

Validation of the sensor was performed at different stages of ripening in tomato fruits using biosensor and spectrophotometric enzymatic methods (Suye et al. 1992). Variation in malic acid concentrations at different stages of ripening shows nanosensor and enzymatic method are comparable and more significant than the enzymatic method (Fig. 6). Malic acid nanosensor response time was 2 min and lower limit of detection was found 0.01 mM. In over ripened stage, increased respiration might be responsible for the decline in the concentration of malic acid. Since malic acid concentration is the fruit

maturity (ripening) indicator, it can be used as a viable tool for detection of maturity of fruits.

Conclusions

Nanosensor was developed for detection of malic acid concentrations in tomato as fruit ripening indicator and also validated with enzymatic (spectrophotometric) method. The present nanosensor takes only 2 min response time. It helps fruit industries in transport, storage and to prevent from spoiling.

Compliance with Ethical Standards

Conflict of Interest Anita Dalal declares that she has no conflict of interest. J. S. Rana declares that he has no conflict of interest. Ashok Kumar declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

Fig. 6 Validation of the nanosensor with enzymatic method. Malic acid concentration was measured at different stages of ripening in tomato fruits using nanosensor and enzymatic (spectrophotometric) method

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