

Bioelectrochemical Determination of Citric Acid in Real Samples Using a Fully Automated Flow Injection Manifold

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An enzymic method for the determination of citric acid in fruits, juices and sport drinks is proposed. The method is based on the action of the enzymes citrate lyase, oxaloacetate decarboxylase and pyruvate oxidase, which convert citric acid into H₂O₂ with the latter being monitored amperometrically with a H₂O₂ probe. The enzymes pyruvate oxidase and oxaloacetate decarboxylase were immobilized. A multi-membrane system, consisting of a cellulose acetate membrane for the elimination of interferants, an enzymic membrane and a protective polycarbonate membrane were placed on a Pt electrode and used with a fully automated flow injection manifold. Several parameters were optimized, resulting in a readily constructed and reproducible biosensor. Interference from various compounds present in real samples was minimized. Calibration graphs were linear over the range 0.01–0.9 mM pyruvate, 0.015–0.6 mM oxaloacetate and 0.015–0.5 mM citrate. The throughput was 30 samples h⁻¹ with an RSD of 1.0% (*n* = 8); the mean relative error was 2.4% compared with a standard method. The recovery was 96–104%. A 8–10% loss of the initial activity of the sensor was observed after 100–120 injections.

Keywords: Amperometric citrate determination; multi-membrane biosensor; flow injection analysis; fruits, juices and sport drinks

Citric acid is present in numerous natural products and is a key tribasic acid involved in both plant and animal aerobic respiration. Several fresh fruits such as lemons and limes owe their sharp taste to the presence of the citrate anion.¹ Citric acid is also an additive in industry, mainly as a preservative and an acidulant. It is added to some dairy products to improve and protect both flavor and aroma. It has been used for radioactive Sr²⁺ from suspect milk after radiation fallout, and has been useful for chelating trace metals which can cause haze or deterioration of color and flavor.²

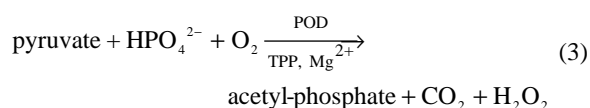
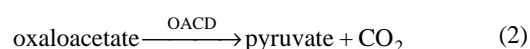
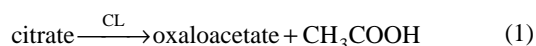
Several methods have been proposed for the determination of citric acid, based on ion-exchange chromatography,³ HPLC⁴ and isotachopheresis;⁵ these are time consuming procedures, and sample clean-up is required in order to separate citric acid from other co-existing tricarboxylic acids. Other approaches based on conductimetric⁶ or spectrophotometric methods⁷ suffer from selectivity as they are based on non-specific reactions with carboxylic acids.

The need for simple separation could be potentially solved, by using a high specificity enzyme such as citrate lyase⁸ (CL). Moellering and Gruber⁹ reported a method with soluble CL, lactate dehydrogenase and malate dehydrogenase (MDH), and monitored the decrease in the absorbance of the NADH at 340 nm. Although relatively slow and expensive in enzyme the approach works well, as a batch method, and is widely used in

routine off-line analysis. Since then several other approaches have been proposed; some make use of soluble CL and immobilized MDH with NADH¹⁰ detection, some use CL and oxaloacetate decarboxylase (OACD) in soluble¹¹ or immobilized¹² form in conjunction with polarography. The direct amperometric determination of citric acid has been proposed¹³ in connection with an ascorbate oxidase reactor to eliminate ascorbic acid interference. Gajovic *et al.*¹⁴ used CL, purified by recrystallisation and ultrafiltration and then entrapped in gelatin. This method needs recalibration every six runs probably owing to the gradual loss of the activity of the CL. The enzyme could be reactivated after 1 h incubation with adenosine 5'-triphosphate (ATP) and CH₃COOH. Measurements using a non-enzymic membrane were a proposed route to the elimination of interferent species.

CL is usually used in soluble form because of ready inactivation by complex formation with Mg²⁺ and Zn²⁺ and also the enol-form of oxaloacetate,¹⁵ itself the product of its action on citrate.

The proposed method is based on a sequence of reactions involving thiamine pyrophosphate (TPP) and the enzymes CL, OACD and pyruvate oxidase (POD), according to the following scheme:



The final product H₂O₂ was monitored amperometrically by means of a Pt electrode, mounted on a wall-jet flow-through cell, polarised at +0.65 V *versus* an Ag–AgCl reference electrode. The multi-membrane biosensor gave an interferant-free system, with good analytical characteristics in terms of accuracy, reproducibility and operational simplicity, which is a key advantage of biosensors over conventional enzymic assays. Additionally, the system was combined with a flow injection (FI) manifold, fully automated by means of resident reported software.¹⁶

Experimental

Apparatus

This work was carried out using an in-house fully automated FI manifold. Electrochemical experiments were run using a computer-controlled potentiostat (Eco Chemie/Autolab, Utrecht, The Netherlands). Solutions were pumped through the manifold using a four-channel peristaltic pump (Gilson, Villiers

le Bel, France) and sample injections made with a pneumatically actuated injection valve (Rheodyne, Cotati, CA, USA). A resident program, synchronized with the acquisition software, ensured full control of pump and valves, eliminating manual manipulation; a detailed description of the program is given elsewhere.¹⁶ A three-electrode flow-through detector (Metrohm 656, Herisau, Switzerland) was used for the amperometric monitoring. This consists of a wall-jet thermostated cell (volume < 1 μ l) with a Pt (diameter 1.6 mm, Bioanalytical Systems, W. Lafayette, IN, USA) working electrode, a built-in Au auxiliary electrode and an Ag–AgCl reference electrode. A schematic diagram of the system is shown in Fig. 1(A).

Chemicals

POD (EC 1.2.3.3, from *Pediococcus* sp., 43 U mg^{-1}), OACD (EC 4.1.1.3, from *Pseudomonas* sp., 265 U mg^{-1}), CL (EC 4.1.3.6, from *Enterobacter aerogenes*, 0.26 U mg^{-1}) all in lyophilized form and cellulose acetate (approximately 40% acetyl), were obtained from Sigma (St. Louis, MO, USA). 3-Morpholinopropanesulfonic acid (MOPS) and poly(vinyl acetate) (PVA, M_r 167000 Da) were supplied by Aldrich (Gollingham, Germany). The enzymic test kit for citric acid determinations was purchased from Boehringer (Mannheim, Germany). All other (analytical-reagent grade) chemicals were purchased from Sigma.

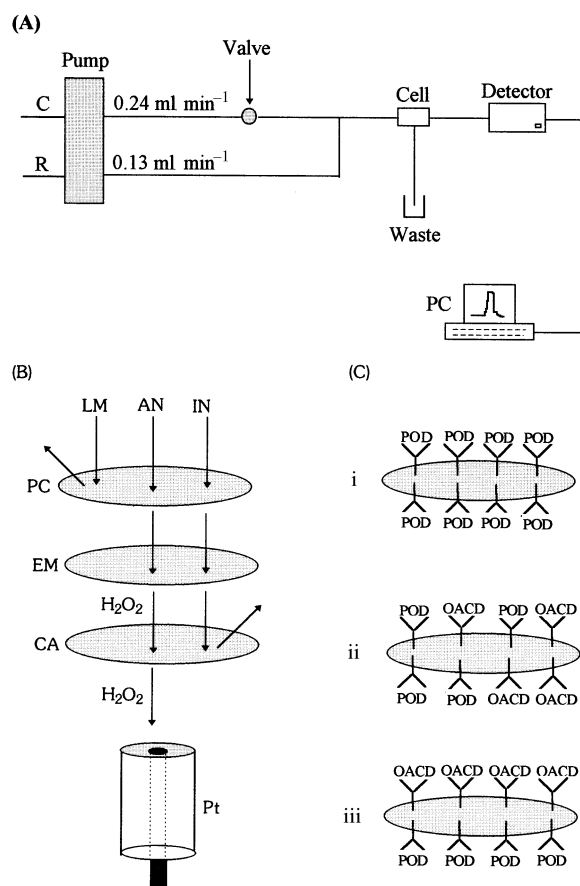


Fig. 1 (A) Schematic representation of FI manifold employed for citric acid determinations: C, carrier; R, TPP reagent. (B) Assembly of the biosensor; LM, large molecules; AN, analyte; IN, interferants; Pt, platinum electrode. CA, cellulose acetate; EM, enzymic membrane; PC, polycarbonate membrane. (C) i, asymmetric application; ii, random application; and iii, symmetric application.

Solutions

Working (MOPS 70 mM, pH 7.6) and immobilization (MOPS 50 mM, pH 7.35) buffer solutions were filtered through a 1.0 μ m pore diameter microporous polycarbonate (PC) membrane (Millipore, Bedford, MA, USA), prior to use. Stock solutions of pyruvic (50 mM) and oxaloacetic acid (50 mM) were prepared by dissolving 27.5 and 33.1 mg of sodium pyruvate and oxaloacetic acid in 5.0 ml of 0.1 and 0.5 M HCl, respectively; this prevented their polymerization or decarboxylation. Both solutions were stored at 4 °C and prepared weekly, owing to their instability.¹⁷ A stock solution of TPP (20 mM, pH 6.2) was prepared by dissolving 181.4 mg of TPP in 20 ml of the working buffer. Enzyme solutions were prepared in the immobilization buffer, otherwise working buffer solution was used throughout.

Membranes

Polymeric nylon 66 membranes, thickness 120 μ m, *viz.*, Biodyne A (amphoteric, 50% amino, 50% carboxyl, 0.2 μ m porosity), Biodyne B (positively charged with pore surfaces populated by a high density of quaternary ammonium groups, 0.45 μ m porosity), Biodyne C (negatively charged, contains 100% carboxyl, 0.45 μ m porosity) and Immunodyne ABC (pre-activated, 0.2 μ m porosity) were a kind gift from Pall Filtration (Milan, Italy). HA (mixed ester cellulose, 0.45 μ m porosity) membranes were purchased from Millipore. PC membranes (thickness 10 μ m, 0.03 and 0.05 μ m porosity) and dialysis tubing (12000 M_r cut-off) were supplied by Nuclepore (Cambridge, MA, USA) and Sigma, respectively. For casting cellulose acetate membranes a wet film applicator (5 in, 1–8 mils, URAI, Milan, Italy) was used.

Preparation of the Enzymic Membranes

POD was immobilized by ionic and/or covalent bonding onto nylon 66 membranes, following the spot wetting method:¹⁸ A 10 μ l (10 U) aliquot of the POD solution was symmetrically pipetted onto each side of the dry membrane (diameter 6 mm) and left to air-dry for 5 min. Alternatively, physical entrapment of POD was carried out by passing 50 μ l (10 U) of POD solution through an HA membrane (diameter 6 mm) while slight suction was applied.¹⁹ In both cases, unattached protein was removed by washing the membranes (3 \times 10 min) with the immobilization buffer.

POD and OACD were co-immobilized asymmetrically onto Biodyne B and Immunodyne ABC membranes, according to a procedure reported in the literature:²⁰ A 10 μ l (10 U) aliquot of the POD solution was applied onto the rough side of the dry membrane (1 \times 1 cm^2) and allowed to react for 2–3 min. Then, 6 μ l (15 U) of the OACD solution were applied onto the smooth side of the membrane and allowed again to react for 15 min. The membranes were washed and stored in the immobilization buffer at +4 °C.

Assembly of the Sensor

The cellulose acetate membrane (20 μ m thick, 100 Da cut-off) was first placed on the Pt surface to eliminate interference from electroactive species.²¹ This membrane was prepared in our laboratory by dissolving 3.996 g of cellulose acetate and 4 mg of PVA in a mixture of 60 ml of acetone and 40 ml of cyclohexanone (after a minor modification of the procedure reported by Palleschi *et al.*²¹). Then the membrane bearing the enzyme(s) was superimposed with an outer PC membrane in order to prevent microbial attack and also leaching of enzyme. With the oxaloacetic biosensor, the rough surface of the membrane was directed towards the cellulose acetate; POD membranes could be placed with any orientation. All the

membranes were tightly fitted over the electrode with the aid of an O-ring [Fig. 1(B)].

PC membranes of different porosity (0.03 and 0.05 μm) and a dialysis membrane (modified with 1% Na_2CO_3 and 10 mM Trizma-1 mM EDTA²²) were alternatively used as protective membranes. After preliminary evaluation of the effect of these membranes on the pyruvate biosensor, in terms of response times, sensitivity, linearity and operational stability, a porosity of 0.03 μm was finally selected.

Preparation of Sample Solutions

A 5 g amount of fruit sample was homogenized with a blender, diluted in 100 ml of the working buffer and finally filtered through a 1.0 μm PC membrane. Juice and sport drink samples were directly diluted in 10 ml of working buffer, while carbonated samples were sonicated (1 min, 100 W) prior to dilution.

Procedure

The carrier (70 mM MOPS of pH 7.6 containing 3.22 mM MgCl_2 and 1.61 mM KH_2PO_4) and TPP [2.64 mM, pH 6.2 (2.64 ml of 20 mM TPP in 20 ml of water)] streams were continuously pumped at flow rates of 0.23 and 0.14 ml min^{-1} , respectively, towards the probe until a stable baseline current was reached (1–2 nA, within 10–15 min). Standard or sample solutions of citric acid [40 μl (0.4 U) CL + $x \mu\text{l}$ sample + (960 – x) μl carrier] were introduced as short pulses of 120 μl via the loop injection valve. For the oxaloacetate or pyruvate biosensor, the composition of the standards was [$x \mu\text{l}$ standard + (5000 – x) μl carrier]. The peak height of the current response was taken as a measure of the analyte concentration.

Results and Discussion

Enzyme Membranes

Commencing from the first enzymic step [eqn. (3)], POD was immobilized onto several commercially available membranes. The wide range of the chemical and physical properties of the membranes led us to find the most efficient matrix for the construction of the pyruvate biosensor. As well as the membrane spot wetting immobilization, membrane immersion¹⁸ was tested using a 0.32 mg per 2 ml (10 U POD) enzymic solution; the latter gave poor immobilization efficiencies. Enzyme loading was tested separately for POD and OACD and defined the enzyme loading necessary to obtain diffusional limitation of response, *i.e.*, the response maximum.¹⁴ Saturation study of the membrane was made using the Immunodyne ABC membrane; this is claimed to have the greatest binding capacity. The response to pyruvate with different enzyme loading solutions (2–12 U POD) was studied (Fig. 2). Experiments were then performed, applying the useful enzyme content (10 U POD) onto different membranes. The relative apparent measurable efficiencies, which reflect the biocatalytic efficiency of the immobilized enzymes, are shown in Table 1. Biodyne B and Immunodyne ABC membranes showed the best results and so further work was carried out using these membranes.

By keeping the amount of POD at the optimum value, further membranes were prepared by varying the amount of the applied OACD from 5 to 20 U. The responses of oxaloacetate biosensors with different enzyme loadings are shown in Fig. 2. Asymmetric co-immobilization of POD and OACD gave greater current signals than those with random application of the same amount of the enzymes [Fig. 1(C)].

Probe Lifetime

POD is a relatively unstable enzyme probably owing to the gradual loss of its cofactor flavin adenine dinucleotide (FAD),

during both the immobilization procedure and the measurements. The addition of several compounds such as FAD, TPP, MgCl_2 , glycerol and $(\text{NH}_4)_2\text{SO}_4$ to the immobilization or storage buffer did not improve either the immobilization efficiency or the long-term stability. Indeed, this led to a more complicated storage of the membranes and greater cost. OACD is a more stable enzyme; therefore, lifetime experiments were carried out with the pyruvate biosensor (Table 2). The proposed approach is more economical than other attempts, with similar¹³ or slightly better lifetimes²³ where the preparation time of the enzymic membranes and the amount of the enzymes are significantly greater (> 30 h preparation time and 10–26-fold amounts of the enzyme).

Concentration of Activators and Cofactors

It has been reported²⁴ that the activity of POD is affected by the presence of inorganic phosphate, divalent cations such as Mg^{2+} , Ca^{2+} , Mn^{2+} or Co^{2+} and cofactors such as TPP and FAD. This optimization was carried out with the probe assembled as a pyruvate biosensor, using 0.5 mM pyruvate solution as a sample.

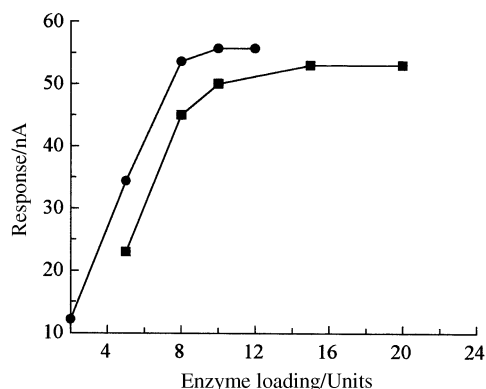


Fig. 2 Enzyme loading test using the Immunodyne ABC membrane: ● POD (2–12 U); ■, POD (10 U) + OACD (5–20 U); 0.5 mM substrate. Parameters: 70 mM MOPS; pH 7.5; 1 mM MgCl_2 ; 2 mM TPP; 1 mM KH_2PO_4 .

Table 1 Relative efficiencies of various polymeric membranes used as supports for the immobilization of POD. 0.3 mM pyruvate; 10 U POD

Membrane	Current/nA	RE (%) [*]
Biodyne A	29.3	68
Biodyne B	42.8	100
Biodyne C	27.3	64
Immunodyne ABC	40.7	95
Millipore HA	22.1	52

^{*} Relative efficiency with respect to the most active membrane.

Table 2 Lifetime of the pyruvate biosensor. Immunodyne ABC membrane; 10 U POD; 0.2 mM pyruvate^{*}

Day	Current/nA	Activity (%)
1	26.0	100
2	23.4	90
3	20.7	80
4	19.0	73
5	9.2	35
6	5.5	21
7	1.0	4

^{*} Continuous use: 100–200 injections (8–10% loss of the initial activity). Use after storage: minimum 15 injections per day.

TPP was initially included in the carrier stream, but it was found to be unstable in this environment (decay of signal output), and so was pumped separately [Fig. 1(A)] achieving, furthermore, better mixing with the sample solution. In the absence of TPP no response was observed while for concentrations higher than 0.8 mM TPP the response became constant; subsequent work used 1 mM TPP. Several cations were examined as activators of POD. In addition to sensitivity, the criterion for the final selection was the stability of the sensor. Since Ca^{2+} and Co^{2+} precipitate with phosphate they must be avoided; Ca^{2+} also competed with the binding site for Mg^{2+} , vital for CL activity. A 2 mM MgCl_2 concentration was found to be optimum for the performance of the biosensor. Inorganic phosphate, as KH_2PO_4 , was tested in the concentration range 0–2 mM and a concentration of 1 mM was selected; the graph was similar to that seen with Mg^{2+} . FAD in the carrier stream (up to 0.5 mM) had no effect on the current output; it is likely that the enzyme preparation contains sufficient FAD for the catalytic reaction and no further FAD was needed.

Using POD–OACD membranes and 0.15 mM oxaloacetate, the effect of TPP, MgCl_2 and KH_2PO_4 was re-examined and similar optima were observed. The effect of Mn was further investigated and it was found to increase the activity of the system (approximately 6–8%) when a concentration of 0.1 mM MnCl_2 was added to the carrier stream. At higher concentrations a progressive decrease of the signal was observed, accompanied by the formation of $\text{Mn}_3(\text{PO}_4)_2$; hence, subsequent work was carried out in the absence of MnCl_2 .

Working Conditions

The pH of the working buffer was also investigated in several buffering systems, such as MOPS, glycylglycine and Trizma-HCl, covering the pH range 7–8. The last gave lower responses, probably owing to its reaction with Mg^{2+} and oxaloacetate ions. Using POD–OACD membranes, sequential injections of 0.3 mM pyruvate and 0.3 mM oxaloacetate, at different pH values of 70 mM MOPS, were performed. The optimum pH for both the pyruvate and oxaloacetate biosensors is 7.5 as shown in Fig. 3. The numbers that appear on the graph were calculated as $(S_1/S_2) \times 100$ (where S_1 and S_2 represent the signals obtained for standards of 0.3 mM oxaloacetate and pyruvate, respectively) and reflect the efficiency of the conversion of oxaloacetate to pyruvate.

The efficiencies of the conversion were also investigated at different flow rates (reaction times), following the procedure given above. Flow rate profiles are shown in Fig. 4. An overall flow rate of 0.37 ml min^{-1} was finally selected, which reconciles fairly high peaks and satisfactory sample throughput (30 h^{-1}). A sample volume of $120 \mu\text{l}$ was used as it prevented

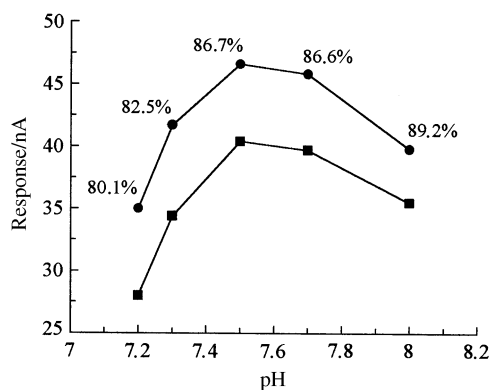


Fig. 3 pH profile of the pyruvate (●) and oxaloacetate (■) biosensors, using the Immunodyne ABC membranes, 0.3 mM substrate; Parameters: 70 mM MOPS; 1 mM KH_2PO_4 ; 2 mM MgCl_2 ; 1 mM TPP.

peak broadening (dispersion coefficient 1.22–1.25) and also ensured high sensitivity. The sensitivity of the oxaloacetate biosensor also increased with the temperature, levelling off at a maximum value of 47°C . Above this temperature thermal inactivation dominates over the increase in the collision frequency, resulting in a decrease of the signal. All experiments were carried out at 30°C , where the stability of the biosensor was the same as at room temperature.

Amount of Citrate Lyase

Preliminary experiments were carried out with immobilized CL. The resulting probe showed poor reproducibility and a marked loss of activity with repeated injections of citric acid, eliminating the advantages from the immobilization of the other enzymes. Planta *et al.*¹⁰ reported a 50% loss of the initial activity after 15–20 sample injections. Magnesium complexes of the enolic form of oxaloacetate appear to be responsible for the inactivation of the enzyme.¹⁵ The effect of the CL on the response was investigated by varying the enzyme additions ($0\text{--}0.6 \text{ U ml}^{-1}$) to 0.15 mM citric acid standards. The saturation point is 0.4 U ml^{-1} . The same profile was also recorded when a standard solution of 0.4 mM citric acid was used. The Zn^{2+} –enol oxaloacetate complex is less inhibitory¹⁵ and so the effect of ZnCl_2 on the activity of CL was examined. Addition of ZnCl_2 to standard solutions up to 0.03 mM showed no effect on the response. In contrast, at concentrations up to 0.13 mM a decrease in the response (approximately 8–10%) was observed as Zn^{2+} is also an inhibitor of OACD.²⁵ At concentrations higher than 0.2 mM the formation of a $\text{Zn}_3(\text{PO}_4)_2$ precipitate was evident.

Interferences

Interference by metal ions, amino acids and other organic acids present in real samples was investigated by applying the method of mixed solutions in the presence of 0.1 mM citric acid. Interferants were added at concentrations much higher than those in the real samples after dilution. The effect on the relative response is shown in Table 3, where only for malic acid was a small increase in the signal observed, presumably owing to its structural similarity with oxaloacetic acid. Because of the cellulose acetate membrane there is no interference effect from ascorbic acid.

Application to Standards and Real Samples

Under the optimum conditions, a series of calibration graphs, $\text{current/nA} = f([\text{analyte}/\text{mM}])$, were constructed, applying the least-squares method. Using the Immunodyne ABC membrane

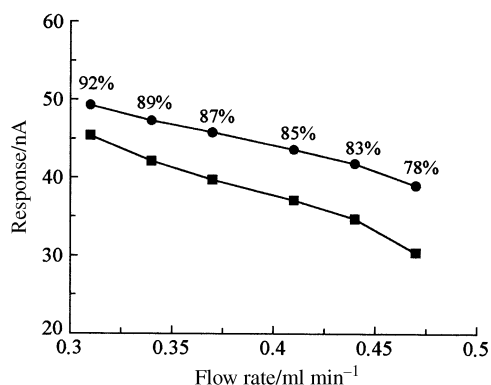


Fig. 4 Flow rate profile of the pyruvate (●) and oxaloacetate (■) biosensors, using the Immunodyne ABC membranes; 0.3 mM substrate; Parameters: 70 mM MOPS, pH 7.5; 1 mM KH_2PO_4 ; 2 mM MgCl_2 ; 1 mM TPP.

Table 3 Interference effect of various compounds on the assay of citric acid. The values in parentheses are the concentrations of the compounds in mM. All solutions contained 0.1 mM citric acid and were compared with the activity of plain 0.1 mM citric acid taken as 100%

Interferant	Relative activity (%)
None	100
Potassium (5)	101
Sodium (5)	100
Alanine (5)	98.5
Lysine (5)	98.5
Leucine (5)	99
Glutamic acid (5)	99.5
Lactic acid (2)	101.5
Adipic acid (2)	99.5
Tartaric acid (2)	103.5
Butyric acid (2)	101.5
Malic acid (2)	106
Ascorbic acid (1)	101
Acetic acid (2)	100
Oxalic acid (2)	100
Isocitric acid (2)	101

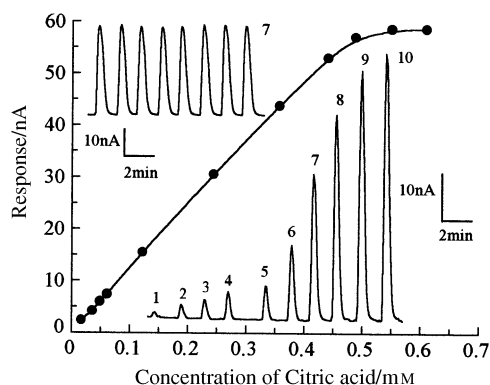


Fig. 5 Calibration graph of citric acid with all the parameters optimized (see text). FI traces top left, reproducibility of the system (0.24 mM citric acid, $n = 8$). Bottom right, calibration graph for citric acid. Peaks 2–10 correspond to concentrations within the linear range while peak 1 represents a concentration of 0.007 mM citric acid.

(5 U POD per side), a linear relationship was obtained between the response and the pyruvate concentration in the range 0.01–0.9 mM with a correlation coefficient, $r = 0.999$. Data fitted the equation $y = (-0.02 \pm 0.51) + (136.27 \pm 1.27)$ [pyruvate]. The detection limit was 5 μM pyruvate for a signal-to-noise ratio of 3 ($S/N = 3$).

By using Immunodyne ABC and Biodyne B membranes, two calibration graphs, linear over the concentration range 0.015–0.6 mM oxaloacetate, were plotted. The equations for the straight lines were $y = (0.18 \pm 0.43) + (131.10 \pm 1.53)$ [oxaloacetate], and $y = (0.303 \pm 1.07) + (106.77 \pm 3.71)$ [oxaloacetate], with correlation coefficients $r = 0.999$ and $r = 0.998$, respectively. The detection limits ($S/N = 3$) were 4 and 10 μM oxaloacetate, respectively. By applying these graphs, pyruvate and oxaloacetate were determined in standard solutions and the mean relative error was 1.8% and 2.1%, respectively.

Using the Immunodyne ABC membrane and citric acid standards, a calibration graph, linear over the range 0.015–0.5 mM, with a correlation coefficient $r = 0.999$, fitting the equation $y = (0.09 \pm 0.44) + (123.42 \pm 2.67)$ [citrate], was constructed. The detection limit ($S/N = 3$) was 4 μM citrate and the RSD of the method was calculated as 1.0% ($n = 8$, 0.24 mM). Results are shown in Fig. 5.

Table 4 Determination of citric acid in various real samples. The standard deviation of the mean ranges from 0.01 to 0.09 mM

Sample	Dilution ratio	Proposed method* / mM	Reference method† / mM	Relative error (%)
Lemonade (IVI)	10	2.24	2.26	-0.9
Ice-tea lemon	10	1.11	1.16	-4.3
Lemon juice	100	2.41	2.38	+1.3
Juice (Florina)	10	3.50	3.65	-4.1
Lucozade sport	10	3.00	3.05	-1.7
Orange juice	50	1.66	1.63	+1.8

* Average of three runs. † Boehringer–Mannheim test kit.

Table 5 Recovery of citric acid added to real samples

Sample	Added/ 10^{-4} M	Found/ 10^{-4} M	Recovery (%)
Lemonade (IVI)	0.60	0.58	96
Juice (Florina)	0.80	0.84	105
Lucozade sport	0.70	0.68	98
Ice-tea lemon	0.80	0.78	98
Lemon juice	0.60	0.62	103
Apple (5%)	0	0	—
Apple (5%)	17.11	17.70	104
Avocado (5%)	0	0	—
Avocado (5%)	17.11	16.61	97

The proposed method was applied to fruits, juices and sport drinks for the determination of citric acid. The results for various samples are summarized in Table 4. Each sample required a minimum dilution of 1 + 99, whereas for orange and lemon juices a dilution of 1 + 449 and 1 + 1999, respectively, was required. The results were compared with those obtained with the Boehringer test kit. The mean relative error was 2.4%. The accuracy of the method was also verified by recovery studies performed by adding standard citric acid solutions to samples. According to the literature, apple and avocado do not contain citric acid²⁶ and this was also verified with the proposed method. Recoveries of 96–105% were achieved, as shown in Table 5.

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