by repeating the experiment at least once.

A total of 34 decamer primers (series OPA, OPB etc.) from Operon Tech, CA, USA were screened using three representative genomic DNA samples of black pepper. Of these, 24 primers that yielded consistent and clear band patterns were selected for the final analysis of the 22 accessions used in the present study. The 24 selected 10-mer primers generated 372 amplification products; the total number of markers per primer ranged from 4 (OPV05) to 21 (OPF09). The range of polymorphic markers per primer was 3 (OPV05) to 21 (OPF09), with a mean of 15.3 polymorphic bands per primer.

Cultivar-specific bands were obtained for all the released varieties except Panniyur-3 (Table 1). Panniyur-1 and Panniyur-2 generated specific bands with OPA 08 (Figure 1 *a* and *b*). OPA10 was efficient in differentiating three varieties, viz. Panniyur-4, Panniyur-5 and Panchami. Among the land races, cultivar-specific bands were observed for Cheriakaniakadan, Malligeswara and Karimunda. Panniyur-1 is the most popular pepper variety grown in India, while land races Cheriakaniakadan and Karimunda are extensively used for pepper improvement work. The utility of the specific RAPD markers can be increased by sequencing their termini and designing longer primers (SCARS) for specific amplification.

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Determination of oxalate in foodstuffs with arylamine glass-bound oxalate oxidase and peroxidase

In plants, oxalic acid is derived from oxidative breakdown of carbohydrate and protein. Edible plant tissues contain considerable amount of soluble oxalate, some of which forms an insoluble salt with calcium and thus makes the calcium unavailable in the diet. Patients with calcium oxalate urolithiasis are advised by clinicians to avoid an oxalate-rich diet, as high consumption of oxalate leads to a high level of oxalate in plasma, which might deposit in the kidney as insoluble calcium oxalate to form urinary stones[1]. Hence the measurement of oxalate in foodstuffs is very important. Different methods have been used to measure oxalate in foodstuffs such as permanganate titrimetry[2], colorimetry[3], capillary electrophoresis[4], spectrophotometry[5], GLC[3], HPLC[6], isotachophoresis[7] and ion chromatography[8]. Of these methods, the first two are non-specific and less accurate, while the rest require expensive apparatus and trained personnel to operate.

Although an enzymatic colorimetric method employing oxalate oxidase and peroxidase is simple, sensitive, and specific and does not require costly equipment[9], it becomes expensive for a large number of samples, due to the requirement of bulk quantity of enzyme. Immobilization of enzyme on insoluble support provides its reuse and thus reduces the cost of procedure. Recently, we have reported the immobilization of barley oxalate oxidase onto alkylamine glass beads through glutaraldehyde coupling, for determination of oxalate in a few foodstuffs[10]. However, the immobilization of an enzyme on alkylamine glass through glutaraldehyde coupling has the disadvantage of extensive self-polymerization nature of glutaraldehyde and protein crosslinking, and hence the support needs to be washed well prior to enzyme addition[11]. Further, the glutaraldehyde coupling involves Schiff's base formation, which has a drawback of reversibility of the reaction at low pH[12]. These problems were overcome by immobilizing oxalate oxidase and peroxidase onto arylamine glass beads through diazotization[13,14]. The present report describes the use of arylamine glass-bound enzymes for determination of oxalate in various foodstuffs.

Zirconia-coated arylamine glass beads (pore diameter 55 nm) (Corning Glass Works, New York); horseradish peroxidase (RZ = 1.1), oxalic acid, 4-aminophenazone (Sigma Chemicals Co, USA), DEAE-Sephacel and Sephadex G-200 (Pharmacia LKB, Sweden) were used. All other chemicals were of analytical reagent grade. The various foodstuffs analysed in the present study were purchased from a local market.

Ten-day-old seedling plants of grain sorghum hybrid (CSH-5) were raised in the laboratory and their leaves were collected as described by Pundir and Nath[15]. The extraction and purification of oxalate oxidase from sorghum leaves were carried out at 4°C (ref. 16). The leaves were homogenized in cold distilled water in 1 : 3 ratio (w/v) in a chilled mortar with pestle and the homogenate was centrifuged at 15,000 × *g* for 30 min. The yellowish-brown supernatant was collected and treated as crude enzyme. The crude enzyme was purified by 0–80% $(NH_4)_2SO_4$ precipitation, ion exchange chromato-

graphy on DEAE-Sephacel column (2.5 cm × 29 cm) using a linear gradient of KCl (0.6 M) in 0.02 M potassium phosphate buffer, pH 6.7 for elution of enzyme followed by 0–80% $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-200 column (1.5 cm × 45 cm) using 0.02 M potassium phosphate buffer, pH 7.2 for elution. The active fractions of the last step were pooled and passed through Amicon centriflo to concentrate the enzyme. The purified enzyme gave a single band in PAGE using Coomassie blue as protein strain with 12.24% per cent yield and a specific activity of 0.85 U/mg. One unit of the enzyme is defined as the amount of enzyme which generates 1 µmol of H_2O_2 per min under the standard assay conditions.

The purified oxalate oxidase was immobilized on arylamine glass beads through diazotization, described by Pundir *et al*[13]. Hundred mg of the arylamine glass beads was taken into a 10 ml conical flask kept on ice bath. One ml 2 N HCl and 25 mg solid $NaNO_2$ were added to the glass beads. The diazotization reaction was allowed to proceed for about 30 min in the same ice bath. The diazotized beads were washed several times with 0.1 M sodium phosphate buffer, pH 7.0 to remove the excess of acid, until pH of the washing discard was 7.0. One ml of purified enzyme (0.37 U) was added and the beads were kept at 4°C for 48 h with occasional stirring. The unbound enzyme was decanted and tested for activity and protein. The glass beads were washed with reaction buffer until no activity was detected in the washing.

Commercially available horseradish peroxidase was dissolved in 0.1 M sodium phosphate buffer (pH 7.0; 4.0 mg/ml) and immobilized on arylamine glass beads through diazo-coupling as described in the case of oxalate oxidase.

The reaction mixture containing 1.8 ml of 50 mM sodium succinate buffer (pH 5.5), 0.1 ml of 10 mM $CuSO_4$ and 100 mg of arylamine glass beads bound to sorghum oxalate oxidase and 100 mg glass beads bound to horseradish peroxidase in a 25 ml conical flask wrapped with black paper were preincubated at 45°C for 2 min. The reaction was started by adding 0.1 ml oxalate solution containing 0.2 to 20 µmol of oxalic acid. After incubation at 45°C for 5 min, 1.0 ml colour reagent was added to the flask and kept at room temperature (30 ± 2°C) for 10 min to develop the colour. The reaction mixture was transferred carefully into the cuvette with the help of an Eppendorf pipette. A_{520} was read in Spectronic-20. A curve was plotted between varying oxalate concentrations and A_{520}.

The colour reagent consisted of 50 mg 4-aminophenazone and 100 mg solid phenol per 100 ml 400 mM sodium phosphate buffer, pH 7.0 and was stored in an amber-coloured bottle at 4°C and prepared fresh after one week.

Fifty g fresh leaf/stem/fruit samples was crushed separately and 2 g of these extracts was homogenized in 5 ml distilled water in pestle and mortar. The homogenate was centrifuged at 5000 rpm for 15 min and both pellet and supernatant were collected. The supernatant was used for determination of soluble oxalate. The pellet was mixed with 2 ml 1 N HCl, kept at 100°C in a boiling water bath for 1 h, diluted to 4 ml with distilled water and used for determination of insoluble oxalate analysis. In the case of chocolate, 50 mg of chocolate bar (brand: 5-Star) was mixed in 10 ml distilled water and centrifuged at 3000 rpm for 10 min. The supernatant was collected and used for oxalate assay. Cold drinks were used as such.

Determination of oxalate in foodstuffs was carried out in the same manner as described for preparation of the standard curve for oxalate, except that the oxalate solution was replaced by the pre-treated food sample/soft drink. To determine insoluble oxalate, 400 mM sodium succinate buffer, pH 5.5 was used as a reaction buffer to resist any fall in its pH due to the highly acidic nature of the sample. Control was prepared for each sample by adding the food extract into the assay, which did not contain immobilized enzyme. The oxalate concentration in the sample was calculated from the standard curve between A_{520} and oxalate concentration.

To reuse the immobilized enzymes, the arylamine glass beads bound to oxalate oxidase and peroxidase were washed with reaction buffer 3–4 times, prior to their use in the next assay. During each washing, the buffer was carefully withdrawn by an Eppendorff pipette, avoiding removal of the glass beads. The beads were stored in distilled water at 4°C, when not in use.

To determine the reliability of the method, solid oxalate (25 mg/100 ml) was added to the spinach extract and oxalate content was determined before and after addition of oxalate. The per cent recovery of added oxalate was calculated.

To work out the reproducibility of the method, the oxalate content was determined in three samples of spinach extract repeatedly on the same day (within day) and then in the same samples after their storage at – 20°C for one week (between batch). The within day and between batch coefficient of variation (CV) was calculated for determination in food sample.

An oxalate oxidase has been immobilized onto arylamine glass through diazocoupling with a conjugation yield of 9.2 mg/g and 34.1% retention of initial activity of free enzyme, confirming our earlier results[13]. This is a better conjugation yield than that for barley enzyme on the same support (6.63 mg)[17]. Peroxidase has also been immobilized onto arylamine glass with a conjugation yield of 18.0 mg/g and 60% retention of its initial specific activity, which is better than those for the same enzyme on alkylamine glass beads (Table 1)[14]. Arylamine glass-bound oxalate oxidase and peroxidase lost 20% of their initial activity during

Table 1. Immobilization of sorghum leaf oxalate oxidase and horseradish peroxidase on arylamine glass beads (pore diameter 55 nm)

Enzyme	Enzyme added to 200 mg glass beads (mg)	Enzyme units added to glass beads (µmol H_2O_2/min)	Enzyme coupled to 200 mg glass beads (mg)	Conjugation yield (mg/g)	Per cent retention of initial specific activity
Oxalate oxidase	2.48	0.37	1.84	9.2	34.1
Peroxidase	8.00	1.20	3.60	18	80

One enzyme unit for oxalate oxidase is defined as the amount of enzyme required to generate 1 µmol H_2O_2 per min under the standard assay conditions. One enzyme unit for peroxidase is defined as the amount of enzyme required to utilize 1 µmol H_2O_2 per min under the standard assay conditions.

their regular use for about 200 times over a period of 6 months, while free sorghum oxalate oxidase lost 50% of its initial activity after 15 days storage at 4°C in 0.02 M sodium phosphate buffer, pH 7.2.

The immobilized oxalate oxidase showed maximum activity at pH 5.5, when incubated at 45°C for 5 min and immobilized peroxidase had maximum activity at pH 7.5 for 25 min.

The arylamine glass-bound sorghum oxalate oxidase and horseradish peroxidase were employed to develop an enzymic colorimetric method for determination of oxalate in foodstuffs. In the method, H_2O_2 generated by immobilized oxalate oxidase from solubilized oxalate of foodstuffs is measured by a colour reaction consisting of 4-aminophenazone, phenol and peroxidase as a chromogenic system. The resulting quinonemine dye absorbs maximally at 520 nm. A linear relationship was obtained between oxalate concentration ranging from 4.5 mg/l to 108 mg/l and A_{520}. The minimum detection limit of the method is 4.5 mg/l. The analytical recovery of added oxalate (25 mg/100 ml) in spinach samples was 90.0 ± 3.0% (mean ± SD). The within and between batch coefficients of variation (CV) for determination of oxalate in spinach samples were < 3.5% and < 7.0% respectively. The method has the advantage that it allowed the reuse of the immobilized enzymes.

Table 2. Oxalate content in soft drinks as determined by using arylamine glass-bound oxalate oxidase and peroxidase

Drink	Oxalate content (mg/100 ml)
Plain orange juice	1.3
Mirinda (orange soft drink)	1.3
Coca cola	3.6
Limca	0.83
Beer (brand – Kingfisher)	11.8

The oxalate content in various foodstuffs as measured by the present method is given in Tables 2 and 3. The oxalate content in plain orange juice was 1.3 mg/100 ml, which is comparable to that reported by the enzyme electrode method, employing membrane-bound barley oxalate oxidase (1.4 mg/100 ml)[18]. The oxalate content in Mirinda, an orange soft drink was 1.3 mg/100 ml, which is higher than that reported for orange soft drink by enzyme electrode method (0.6 mg/100 ml)[18]. The oxalate content in beer (brand – Kingfisher) was as high as 11.8 mg/100 ml, confirming the earlier speculation of the excessive foaming of beer due to high oxalate content[19], but in contrast to the earlier report of Kasidas and Rose[20], who found practically no oxalate in beer.

The total oxalate content in spinach leaves was 0.32 mg/100 mg fresh wt., which is comparable to that reported by the enzyme electrode method (0.281 mg/100 mg fresh wt.)[18], but lower than that by titrimetric method (1.014 mg/100 mg of fresh wt.)[21], capillary electrophoretic method (0.774 mg/100 mg)[4] and also lower than that reported by Gopalan *et al.* (0.658 mg/100 mg)[22]. The total oxalate content in *Amaranthus* leaves was 2.0 mg/100 mg fresh wt. and 6.03 mg/100 mg dry wt., which is almost similar to those by titrimetric method (1.9 mg/100 mg fresh wt.)[21] and (6.0 mg/100 mg dry weight)[23]. The total oxalate content in the leaves of *Chenopodium album* was 1.74 mg/100 mg fresh wt., which is slightly higher than that measured by the titrimetric method (1.43 mg/100 mg fresh wt.)[21]. The soluble oxalate content of *Chenopodium* leaves was 0.5 mg/100 mg fresh wt., which is similar to an earlier report (0.5 mg/100 mg fresh wt.)[1]. In *Coriandrum sativum*, the total oxalate content was 0.29 mg/100 mg fresh wt., which is lower than that reported earlier (1.26 mg/100 mg fresh wt.)[21].

The total oxalate content of *Lycopersicon* fruit was 0.109 mg/100 mg fresh wt., which is much higher than that reported earlier (0.005 mg/100 mg)[1]. The seeds of *Phaseolus radiatus* had a total oxalate content of 0.095 mg/100 mg dry wt., which

Table 3. Oxalate content in various foodstuffs by arylamine glass-bound oxalate oxidase and peroxidase

	Oxalate content (mg)			
	Soluble		Insoluble	
Foodstuff	Per 100 mg fresh wt.	Per 100 mg dry wt.	Per 100 mg fresh wt.	Per 100 mg dry wt.
Spinacea oleracea (leaf), spinach	0.04	0.396	0.28	2.76
Amaranthus viridis (leaf), chulai	0.01	0.03	2.00	6.00
Brassica campestris (leaf), sarson ka sag	0.02	0.13	0.36	2.48
Chenopodium album (leaf), bathu	0.50	2.26	1.24	5.65
Brassica oleracea (leaf), cabbage	0.04	0.27	0.12	0.80
Medicago falcata (leaf)	0.01	0.084	0.66	5.50
Lagenaira vulgaris (fruit) apple gourd	0.015	0.183	0.06	0.68
Colocasia antiquorum (modified stem), arvi	0.016	0.069	0.08	0.33
Cucumis sativas (fruit), cucumber	0.03	0.66	N.D.	N.D.
Coriandrum sativum (leaf), coriander	0.03	0.204	0.26	1.77
Psidium gugava (fruit), guava	0.01	0.05	0.026	0.13
Daucus carota (modified stem), carrot	0.042	0.512	N.D.	N.D.
Cucurbita moschata (fruit), pumpkin	0.01	0.12	0.27	3.00
Lycopersicon esculentum (fruit), tomato	0.03	0.42	0.079	1.097
Pisum sativum (fruit), pea	0.03	0.12	0.12	0.47
Chocolate	N.D.	N.D.	0.06	0.063
Phaseolus munga (mung)	N.D.	N.D.	0.14	0.145
Phaseolus radiatus (urd)	N.D.	N.D.	0.09	0.095
Cicer arietinum (horsegram)	N.D.	N.D.	0.49	0.50
Lathyrus sativus (kesari dal)	N.D.	N.D.	0.163	0.17

N.D. = Not detected; Data are the mean of three replications.

is lower than that measured in *Phaseolus munga* (0.14 mg/100 mg). The oxalate content of seeds of *Cicer arietinum* was 0.49 mg/100 mg fresh wt., which is comparable to that reported by Gopalan *et al.* (0.417 mg/100 mg fresh wt.)[22]. The total oxalate content of seeds of kesari dal was 0.163 mg/100 mg fresh wt., which is comparable to that reported by Gopalan *et al.* (0.122 mg/100 mg fresh wt.)[22]. The total oxalate content of chocolate was 0.06 mg/100 mg fresh wt., which is comparable to that measured by oxalate oxidase electrode method (0.049 mg/100 mg)[18].

Our results have shown that *Chenopodium*, pumpkin, *Brassica campestris*, spinach and beer had high oxalate content. Hence the restricted consumption of these oxalate-rich foodstuffs can be recommended to urinary stone patients, to avoid the risk of deposition of calcium oxalate in their urinary tract.

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