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Short Communication

Rapid detection and quantification of peroxidase activity in liquid chromatography

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ABSTRACT

A rapid and simple method for the detection and quantification of peroxidase activity is described. The method is based on the visible development of a brownish colour due the reaction of peroxidase in the presence of guaiacol and hydrogen peroxide. The main feature is the detection and quantification of a considerable number of samples at once, instead the spectrophotometric standard methods usually used for this purpose, especially in fractions obtained after liquid chromatography. The effect of substrate concentration and buffer composition on peroxidase determination by the proposed method was investigated.

INTRODUCTION

Detection of enzyme activity during the isolation and chromatographic purification of peroxidases usually relies on spectrophotometric determinations using cuvettes [1–10]. Although very convenient, such methods are laborious and time consuming for routine analysis, especially when the number of samples is large. An alternative and rapid procedure is described here, based on the two-fold serial dilution procedure commonly applied for haemagglutination in microtitre plates. Thus, qualitative and quantitative assays of peroxidase activity can be done directly with observation with the naked eye and automated expensive instruments are not required. Development of a brownish colour, due to the reaction of guaiacol and hydrogen peroxide with the peroxidases, indicates a positive reaction. The quantification is then expressed as the titre of activity, which is defined as the dilution factor of the fraction showing a capacity to develop the brownish colour in a given time. The test was shown

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to be reliable and reproducible in the range 0.5–100 μg/ml of protein and stable for at least 30 min at room temperature.

EXPERIMENTAL

Materials

Microtitre plates (96 wells, rigid U-bottom), microdiluter (Multi-Microdiluter handle) and pipette droppers were purchased from American Scientific Products (McGaw Park, IL, U.S.A.), Sephacryl S-200 superfine, CM Sepharose Cl-4B, bovine serum albumin and ribonuclease A from Pharmacia (Uppsala, Sweden), guaiacol, horseradish peroxidase (HRP) and other chemicals from Sigma (St. Louis, MO, U.S.A.) and hydrogen peroxide (20 volumes, 1.78 *M*) from a local drugstore.

Chromatography

Size-exclusion chromatography was performed on Sephacryl S-200 superfine with a column (94 × 1.6 cm I.D.) equilibrated with 0.1 *M* acetate buffer (pH 5.0) and a sample size of 6 ml of a protein mixture (horseradish peroxidase, bovine serum albumin and ribonuclease A, 2 mg each) at a flow-rate of 45 ml/h. Fractions of 2 ml were collected.

Ion-exchange chromatography was carried out on a CM Sepharose Cl-4B column (20 × 2.6 cm I.D.) with a bed volume of 50 ml. The equilibrium buffer was 0.02 *M* acetate buffer (pH 5.0) and the sample size was 1 ml of a protein mixture of 2 mg of horseradish peroxidase and 1 mg of lysozyme. After washing with two bed volumes of the equilibrium buffer, the molar concentration was increased linearly from 0.02 to 1.0 *M* in five bed volumes. The flow-rate was constant at 47 ml/h and the fraction size was 10 ml.

Qualitative detection of peroxidase activity

A 25-μl volume of 80 m*M* guaiacol solution [prepared in 0.1 *M* acetate buffer (pH 5.0)] were poured into the wells of a microtitre plate, then 25 μl from each chromatographic fraction and finally 25 μl of hydrogen peroxide solution (22.25 m*M*) were added to the wells so that the final volume per well was 75 μl. The plate was incubated at room temperature for 5 min and the development of colour was observed. Colourless wells indicated the absence of activity and, therefore, were considered to be negative. Blank controls (without enzyme) were processed with acetate buffer.

Quantification of peroxidase activity

On a separate plate containing 25 μl of 80 m*M* guiacol solution in each well, 25 μl from the peroxidase-positive fractions were poured into the first line of wells (A). Next, the two-fold serial dilution procedure was carried out with the aid of a 25-μl microdiluter (Multi-Microdiluter handle) from the first row of wells towards the last row at the end of the plate (H). This procedure allowed a consecurive two-fold dilution process with dilution factors from 1/2 to 1/256 to be obtained in a simple manner. Finally, 25 μl of hydrogen peroxide (22.25 m*M*) were added and the titre of activity was determined after incubation for 5 min at room temperature. The reciprocal of the highest dilution showing colour development was considered to be the activity titre. This number is usually plotted in $\log_2$ form to obtain a linear graph.

Effect of substrate concentration and buffer composition on peroxidase assay

Following the previous method, different solutions of guaiacol (2.5, 5, 10, 20, 40, 80 and 160 m*M*) were used to measure the titre of a stock solution of horseradish peroxidase (*ca.* 15 μg/ml), using a constant final concentration of hydrogen peroxide (11.12 m*M*). In the same way, different concentrations of hydrogen peroxide (2.78, 5.56, 11.12, 22.25, 44.5, 89 and 890 m*M*) with a constant final concentration of guaiacol (40 m*M*) were tested. The buffer composition was also varied using different proportions of calcium and manganese chloride (0, 2.5, 5, 10, 25, 50, 75 and 100 m*M*), and the effects of sodium chloride, ammonium sulphate, methyl α-D-glucopyranoside, sodium sulphate (0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75 and 1.0 *M*), isopropanol, sodium dodecyl sulphate (SDS), Triton X-100 (0, 0.5, 1, 2, 4, 7.5 and 10%) and ethylene glycol (0, 2.5, 5, 10, 20, 40 and 50%) were also studied by mixing them with the hydrogen peroxide solution, valuating the effect on the titre determination of a stock solution of horseradish peroxidase.

Stability of horseradish peroxidase exposed to different chemicals used in liquid chromatography

The stability of HRP exposed to different chemicals which could be used during a chromatographic run was studied. One volume of a stock solution of HRP (15 μg/ml) was mixed with one volume of one of the following solutions: calcium chloride (20 m*M*), manganese chloride (200 m*M*); sodium chloride, sodium sulphate, methyl α-D-glucopyranoside, ammonium sulphate (2.0 *M*); isopropanol, SDS, Triton X-100 (20%); ethylene glycol (100%); and acetate (2.0 *M*), phosphate (0.6 m*M*) and citrate (1.0 *M*) buffers (pH 5.0). The mixture was incubated for 5 h at room temperature and then for 16 h at 4°C. The remaining activity was then measured by pouring 50 μl of the corresponding mixture in the empty first line of wells of the assay plate containing 25 μl of 80 m*M* guaiacol. After dilution using the 25-μl microdiluter, 25 μl of hydrogen peroxide solution (22.25 m*M*) were added and the titre was measured as indicated above.

RESULTS AND DISCUSSION

Figs. 1a and 2a, show the distribution of model proteins in size-exclusion chromatography on Sephacryl S-200 superfine and in ion-exchange chromatography on CM Sepharose Cl-4B, respectively.

Peroxidase activity was detected in fractions 35–60 in the size-exclusion experiment (Fig. 1b) and in fractions 2–27 in the ion-exchange chromatographic experiment (Fig. 2b). The higher intensity of the brownish colour also suggested higher peroxidase activity. Obviously this can be evaluated quantitatively using a microplate reader, but the method proposed here can be carried out without such apparatus. Fig. 3a shows the titre of the peroxidase-positive fractions (35–60) from the gel filtration run. The maximum activity corresponded to the HRP peak in Fig. 1a. The corresponding distribution pattern of peroxidase activity was obtained and plotted as the $\log_2$ (titre) against the fraction number (Fig. 3b) in order to obtain a linear plot.

Fig. 4a shows the titre of the peroxidase-positive fractions (2–27) from the ion-exchange chromatographic experiment, where two peaks could be detected. The first (fractions 2–10) corresponded to the unadsorbed material containing peroxidase,

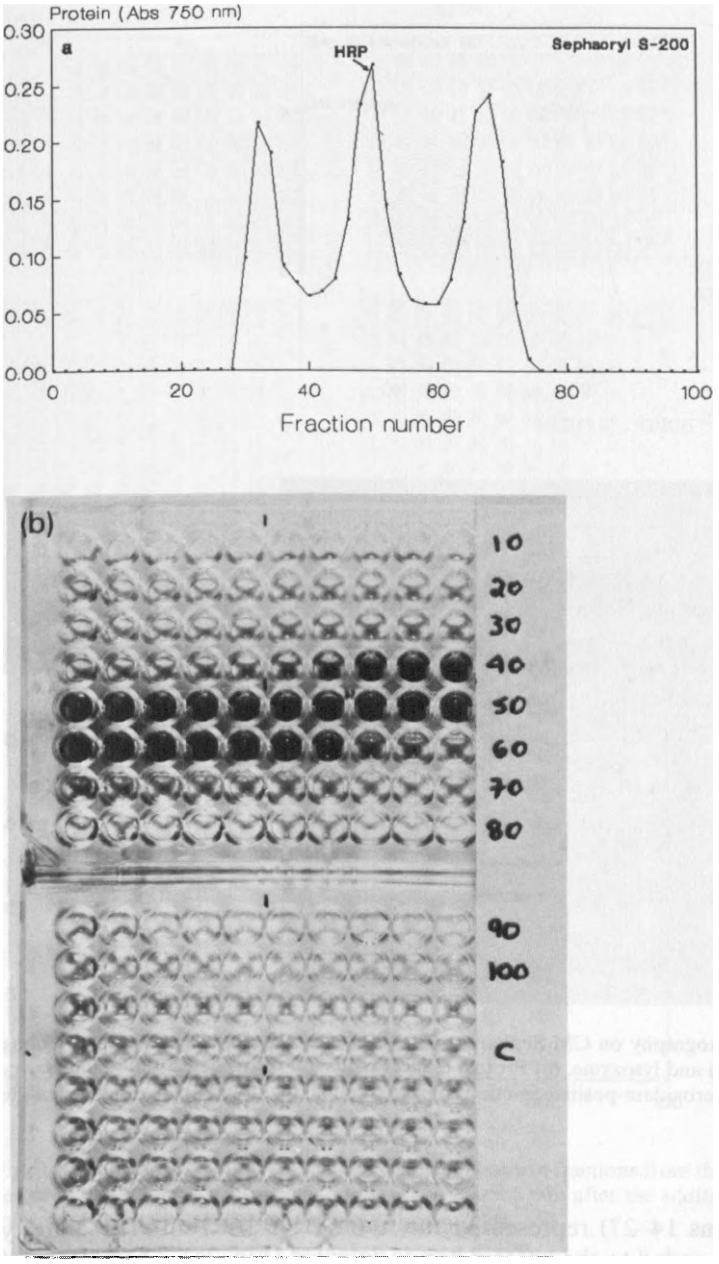

Fig. 1. Size-exclusion chromatography on Sephacryl S-200 of a model protein mixture containing bovine albumin (MW = 65 000), horseradish peroxidase (HRP) (MW = 40 000), and ribonuclease A (MW = 13 700). (a) Protein elution pattern. Protein detection by the Lowry method [11]. (b) Detection of peroxidase-positive fractions. Photograph taken 2 min after the addition of the hydrogen peroxide solution.

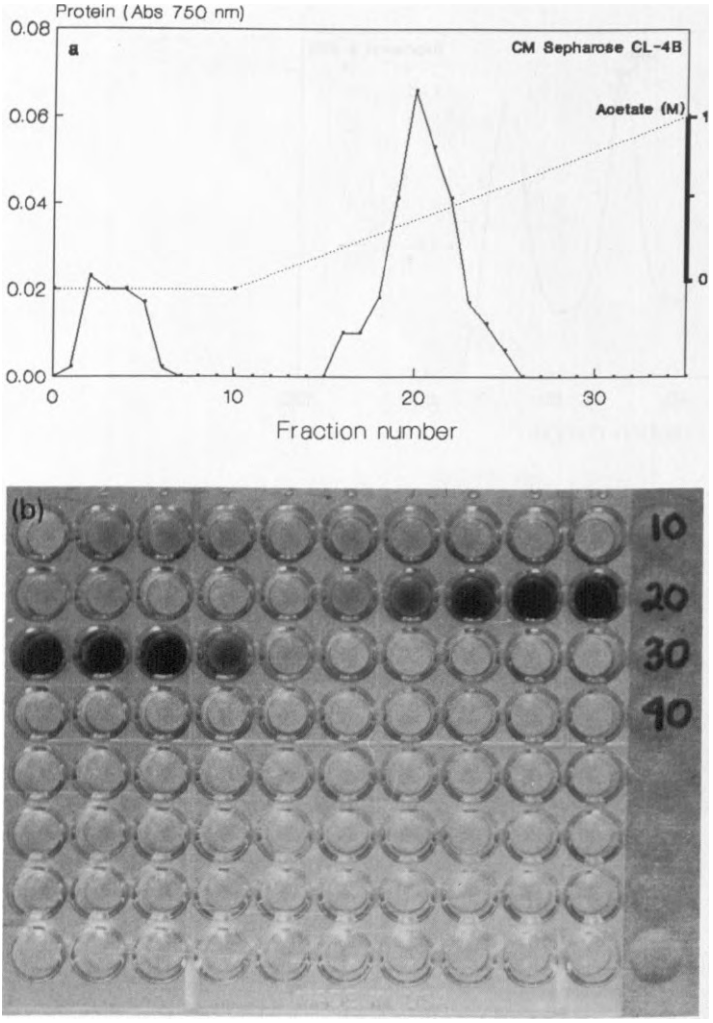

Fig. 2. Ion-exchange chromatography on CM Sepharose CL-4B of a model protein mixture containing horseradish peroxidase (HRP) and lysozyme. (a) Protein elution pattern. Protein detection by the Lowry method [11]. (b) Detection of peroxidase-positive fractions. Photograph taken 1 min after the addition of the hydrogen peroxide solution.

and the second (fractions 14–27) represented the main HRP fraction. The activity distribution also corresponded to the protein bands detected (Fig. 2a). The pattern of elution of the peroxidase activity is shown in Fig. 4b.

The titre of a stock solution of HRP (*ca.* 15 μg/ml) is dependent on guaiacol concentration, being maximum at 40 m*M*. The effect of different concentrations of hydrogen peroxide, on the other hand, is two-fold; it increases colour development up to 22.25 m*M*, above which there is a notable inhibitory effect, reflected in a reduced titre.

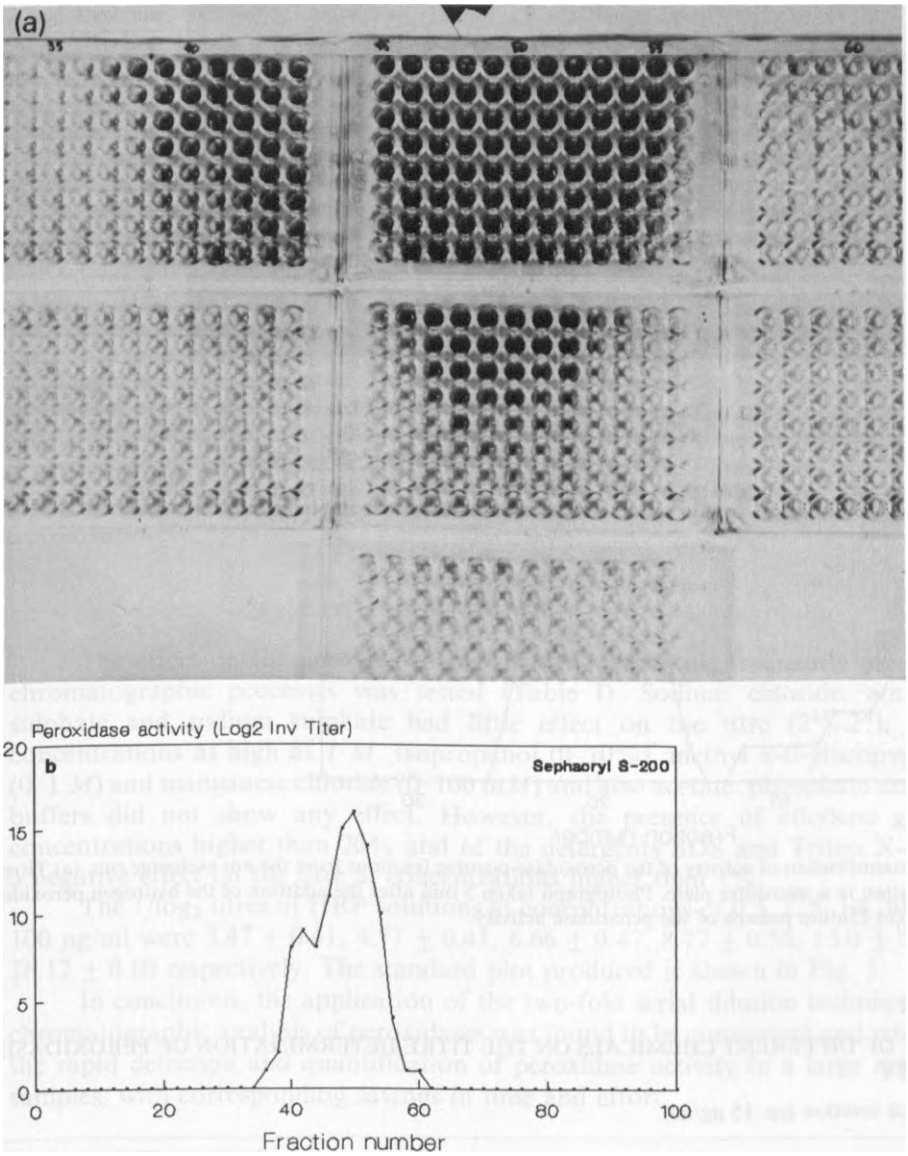

Fig. 3. Quantification of activity of the peroxidase-positive fractions from the size-exclusion run. (a) Titre determination in a microtitre plate. Photograph taken 5 min after the addition of the hydrogen peroxide solution. (b) Elution pattern of the peroxidase activity.

The positive role of calcium in peroxidase activity has been reported previously [12,13]. Therefore, the presence of 25 m*M* of calcium in the reaction mixture induced an increase in the peroxidase titre (2^{10}–2^{12}); however, at higher concentrations the titre was reduced. Interestingly, preincubation of HRP with calcium diminished the requirement for this cation for maximum activity (10 m*M*).

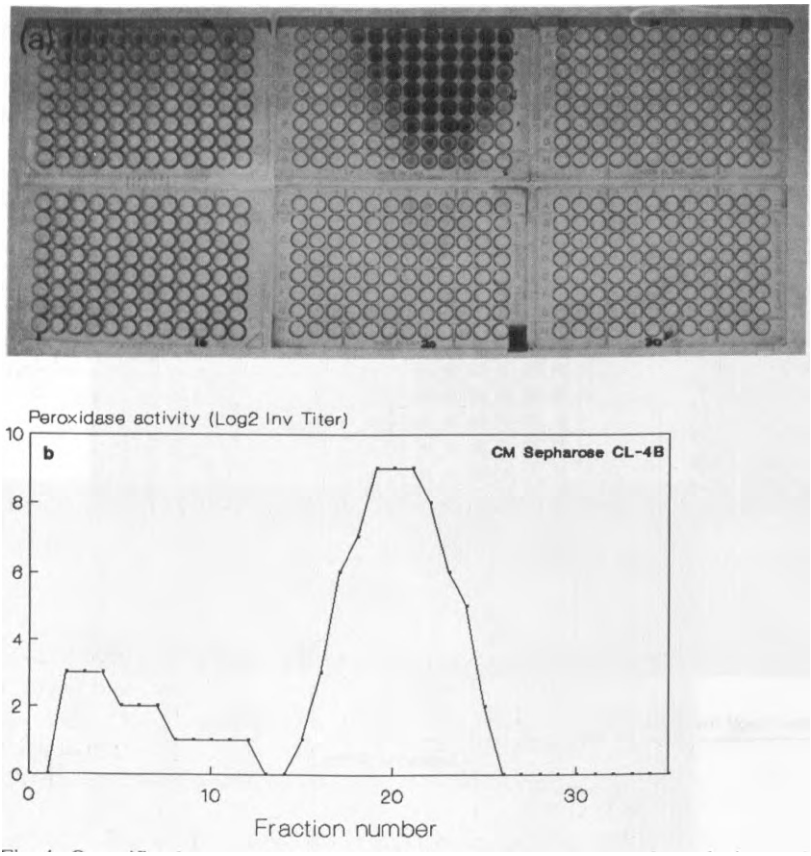

Fig. 4. Quantification of activity of the peroxidase-positive fractions from the ion-exchange run. (a) Titre determination in a microtitre plate. Photograph taken 5 min after the addition of the hydrogen peroxide solution. (b) Elution pattern of the peroxidase activity.

TABLE I

EFFECT OF DIFFERENT CHEMICALS ON THE TITRE DETERMINATION OF PEROXIDASE ACTIVITY

HRP stock solution (*ca.* 15 μg/ml).

Chemical final concentrations	Activity [$\log_2$ (inverse titre)]
Calcium chloride 0, 2.5, 5, 10, 25, 50, 75, 100 m*M*	10, 10, 10, 10, 12, 11, 11, 10
Sodium chloride 0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 10, 10, 9, 9, 8
Ammonium sulphate 0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 9, 9, 9, 9, 8
Sodium sulphate 0, 0.05, 0.1, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 10, 10, 10, 10
Isopropanol 0, 1, 2, 4, 5, 7.5, 10%	10, 10, 10, 10, 10, 10, 10
Methyl α-D-glucopyranoside 0, 0.05, 0.1, 0.3, 0.5, 0.75, 1 *M*	10, 10, 10, 10, 10, 10, 10
Manganese chloride 0, 2.5, 5, 25, 50, 75, 100 m*M*	10, 10, 10, 10, 10, 10, 10
Ethylene glycol 0, 2.5, 5, 10, 20, 40, 50%	10, 10, 10, 10, 10, 7, 5
SDS 0, 0.5, 1, 2, 4, 7.5, 10%	10, 10, 8, 7, 7, 6, 6
Triton X-100 0, 0.5, 1, 2, 4, 7.5, 10%	10, 9, 7, 6, 6, 5, 5

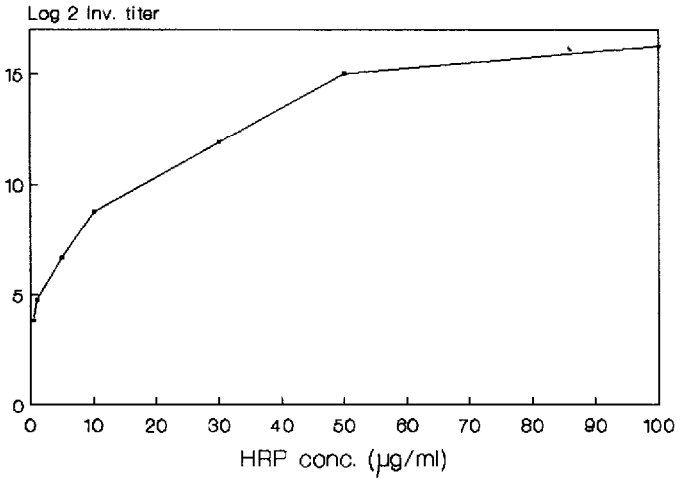

Fig. 5. HRP standard plot. Activity expressed as $\log_2$ (inverse of the titre of HRP solution of different concentration).

The effect on the peroxidase titre of some chemicals frequently used during chromatographic processes was tested (Table I). Sodium chloride, ammonium sulphate and sodium sulphate had little effect on the titre (2^{10}–2^8), even at concentrations as high as 1 *M*. Isopropanol (0–10%), methyl α-D-glucopyranoside (0–1 *M*) and manganese chloride (0–100 m*M*) and also acetate, phosphate and citrate buffers did not show any effect. However, the presence of ethylene glycol at concentrations higher than 20% and of the detergents SDS and Triton X-100 had a negative effect on the titre at concentrations higher than 1%.

The $1/\log_2$ titres of HRP solutions at concentrations of 0.5, 1, 5, 10, 30, 50 and 100 µg/ml were 3.47 ± 0.41, 4.77 ± 0.41, 6.66 ± 0.47, 8.77 ± 0.56, 15.0 ± 0.47 and 16.12 ± 0.60 respectively. The standard plot produced is shown in Fig. 5.

In conclusion, the application of the two-fold serial dilution technique in the chromatographic analysis of peroxidases was found to be convenient and reliable for the rapid detection and quantification of peroxidase activity in a large number of samples, with corresponding savings in time and effort.

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