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ESTIMATION OF THE DISTRIBUTION OF MOLECULAR WEIGHT FOR AMYLOSE BY THE LOW-ANGLE LASER-LIGHT-SCATTERING TECHNIQUE COMBINED WITH HIGH-PERFORMANCE GEL CHROMATOGRAPHY

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ABSTRACT

The weight-average molecular weights and distributions of molecular weight for various amyloses were estimated by high-performance gel chromatography using a low-angle laser-light-scattering photometer and a differential refractometer as detectors. The weight-average molecular weights of potato, sweet potato, tapioca, kuzu, and lily amyloses were 1,030,000 (d.p. 6,360), 880,000 (d.p. 5,430), 1,080,000 (d.p. 6,680), 518,000 (d.p. 3,220), 812,000 (d.p. 5,010), and 734,000 (d.p. 4,350), respectively. Amylose molecules are suggested to be less expanded than pullulan molecules, from the relationship between molecular weight and retention time in gel-permeation chromatography.

INTRODUCTION

Amylose is generally regarded as a long (1→4)-α-D-glucan with some molecules slightly branched through α-(1→6) linkages[1–3]. The molecular weight and the degree of branching are probably characteristic of a species[4]. Amylose is a minor component of most starch species, but it endows some functional properties on the starch. We have suggested[5,6] that the chain length or molecular weight of amylose is related to the tendency to retrograde. An understanding of the various physical and functional properties of amylose requires elucidation of its molecular structure. Average molecular weights of amylose have been measured by various methods[7–10], but the distribution of molecular weights has not been examined widely. Generally, the distribution has been estimated by fractional precipitation using suitable mixtures of solvents and non-solvents[10], but sharp fractionation is

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difficult to achieve and the procedures are tedious and time-consuming. We now report on the application of high-performance gel chromatography, using a low-angle laser-light-scattering photometer and a differential refractometer with flow-through cells as detectors, to this problem. By this technique, which has been applied to proteins[11,12] and a cellulose derivative[13], the polymer is fractionated according to its hydrodynamically effective volume. The concentration (c, by weight) of the eluate is given by the refractometer, and a value proportional to c × (molecular weight) is given by the photometer. Thus, the molecular weight is calculated from the ratio of the responses of the photometer and the refractometer. The details of this technique have been reported elsewhere[14].

EXPERIMENTAL

Materials. — Potato, sweet potato, and lily starches were prepared as described elsewhere[6]. Kuzu starch was a commercial product of Hirohachido Shoten Co. (Kagoshima) and its properties have been described[5]. Tapioca starch was an imported commercial product.

Amylose specimens were fractionated from various kinds of starch by the method of Lansky *et al.*[15], but heating in a boiling water bath was performed under nitrogen to avoid degradation. The fractionated amylose was purified by repeated crystallisations from 1-butanol-saturated water until it showed sufficient purity (an iodine-binding capacity of ≥20 g/100 g)[16]. The required number of recrystallisations depended on the kind of starch[16]; thus, potato, kuzu, tapioca, lily, and sweet-potato amyloses were recrystallised 4, 5, 6, 6, and 9 times, respectively. The lily amylose was the same specimen as that described elsewhere[6].

Methods. — The number-average degree of polymerisation ($\overline{\text{d.p.}}_n$) was determined by a modification of the Park–Johnson method[4]. Iodine-binding capacity (g/100 g) was measured by amperometric titration[17].

In high-performance gel chromatography, the relationship between the molecular weight (weight-average molecular weight) of a polymer and the output of the photometer at zero-scattering angle with a solution is given by[18] Eq. *1*.

$$\frac{(\mathrm{dn/dc})^2\, k'\mathrm{c}}{(\mathrm{LS})} = \frac{1}{\mathrm{M}} + 2\mathrm{Bc}, \qquad (1)$$

where dn/dc, c, (LS), M, and B are the refractive index increment of the polymer, the concentration of the solute, the output of the photometer, the molecular weight of the polymer, and the second virial coefficient, respectively, and k' is an instrumental constant that can be determined by measurements on standard polymers of known molecular weights. The output of the refractometer (RI) is proportional to the difference in refractive index between the solution and the solvent. Thus, c in Eq. *1* is proportional to (RI) × $(\mathrm{dn/dc})^{-1}$. When the concentration is sufficiently low, the second term in Eq. *1* can be ignored and Eq. *2* applies.

$$\frac{(LS)}{(RI)} = k\left(\frac{dn}{dc}\right) M, \tag{2}$$

where k is a constant. Thus, (LS)/(RI) is proportional to the molecular weight. If a polymer of known molecular weight (Ms) and with the same (dn/dc) as the specimen is used as a standard, the molecular weight of the specimen, Ma, is given by Eq. *3*.

$$Ma = \frac{R_a}{R_s} Ms, \tag{3}$$

where R = (LS)/(RI), and subscripts a and s denote the specimen and standard, respectively. Then, Ma is calculated from Ms and the outputs of both the photometer and the refractometer for the specimen and the standard polymer measured under the same conditions.

Equation *1* is confined to the scattering intensity at zero-scattering angle (θ), and the practical difficulty of measurement at $\theta = 0°$ is overcome by extrapolation of the data at several angles (for example, 30–130°) in a Zimm plot[19]. The low-angle laser-light-scattering photometer used in this system measures the scattering intensity at $\theta = 5°$, where the angular dependence can be ignored generally. Therefore, it makes tedious Zimm-plotting unnecessary and is usable as a monitoring device with a flow-through cell. Prior to the application of this technique to amylose, a series of standard pullulan specimens was examined in order to ascertain whether this simplification was possible. The error at $\theta = 5°$ was found to be negligibly small.

The fractionated specimens of pullulan, which are manufactured by the Hayashibara Biochemical Institute and have specified, narrow distributions of molecular weight, were used as standards[20,21]. Pullulan is a linear α-D-glucan with (1→4) and (1→6) linkages, and is assumed to have the same (dn/dc) as amylose; fraction P-400 (mol. wt., 348,000) was used as a standard, because it gave suitable intensities with the photometer and the refractometer operated under the same conditions as those for the measurement of amylose.

The instrumental assembly is illustrated in Fig. 1. Fractionation was performed at 35° on three sequentially linked columns of TSK-GEL (spherical particles of cross-linked, hydrophilic vinyl polymer[20]), namely, C_1 [G3000PW, suggested range of molecular weight for fractionation of poly(ethylene glycol), 0–5 × 10^4], C_2 (G4000PW, 2 × 10^3–3 × 10^5), and C_3 (G6000PW, 4 × 10^4–8 × 10^6). The eluate was monitored by using a flow-through cell (30 μL) in a Toyo Soda LS-8 photometer (633 nm of He–Ne laser, 5-mW output, ~5° scattering angle), and then a smaller cell (10 μL) in a Toyo Soda RI-8 differential refractometer. The outputs of the detectors were recorded with a dual pen recorder (chart speed, 20 cm/h). The operation ranges of the photometer and refractometer were set at 16.

The elution solvent was 50mM sodium phosphate buffer (pH 6.1) containing

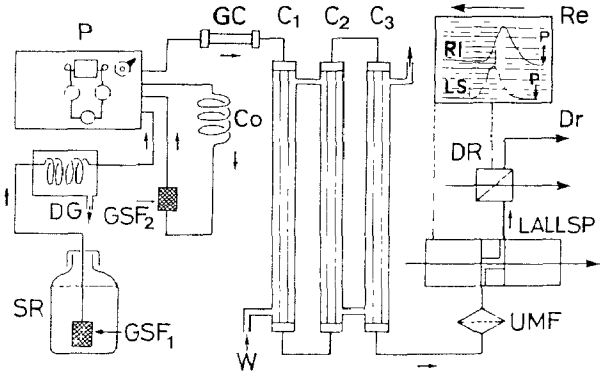

Fig. 1. Outline of instrumentation (Toyo Soda Co., unless stated otherwise): SR, solvent reservoir (3.5 L); GSF_1, sintered stainless-steel filter (Umetani Seiki Co., Model SYF); DG, degasser (Elma Optical Works, Model ERC-3310); P, high-speed liquid chromatograph (Model HLC-803D) with a 500-μL sample loop; Co, helical stainless-steel tube (0.2ø × 6 m); GSF_2, sintered stainless-steel filters (Umetani Seiki Co., Model SLF), GC, guard-column (Model TSK GSWP, 7.5 × 100 mm); C_1, C_2, and C_3, packed columns (each 7.5 × 600 mm) of TSK-GEL G3000PW, G4000PW, and G6000PW, respectively; W, circulating water, UMF, ultramembrane filter (two Millipore filters, Type LS-WP 01300, pore size 1 μm, in series); LALLSP, low-angle laser-light-scattering photometer (Model LS-8); DR, differential refractometer (Model RI-8); Re, double pen recorder; P, pen position; Dr, drain; RI and LS, differential refractometer and low-angle laser-light-scattering photometer curves, respectively

0.02% of sodium azide, the flow rate was 0.513 mL/min, and 0.5 mL of the solution described below was injected.

The dry, powdered specimen (5–10 mg) was suspended in aqueous 50% ethanol (100 μL) and dissolved by the addition of M sodium hydroxide (500 μL). The solution was diluted with water (to ~8 mL) and neutralised with M hydrochloric acid (500 μL), 0.5M sodium phosphate buffer (pH 6.1, 1 mL) containing 0.2% of sodium azide was added, and the volume was made up to 10 mL. The solution was filtered through a Millipore FA type filter (1.0 μm), and stored at 45° ($\leq$1 h) before h.p.l.c.

RESULTS AND DISCUSSION

Because it is possible to measure the scattering intensity at the low angle of θ = 5° with a dilute solution and a low-angle laser-light-scattering photometer, no corrections for the scattering intensity are required generally[11,12,14]. Therefore, the photometer is suitable for monitoring molecular weight during chromatography. Standard specimens of pullulan were used in order to determine that this simplification is possible for long, unbranched polysaccharides. The weight-average molecular weights of these pullulan specimens (P-5, P-10, P-50, P-100, P-200, P-400, and P-800), determined using the low-angle laser-light-scattering photometer, with bovine serum albumin and hen ovalbumin as standards, were 5,300 (5,300), 10,400 (12,000), 45,500 (46,700), 100,000 (95,800), 187,000 (194,000), 348,000 (338,000), and 747,000 (758,000), respectively. These values agreed well with those (shown in

brackets) obtained using a sedimentation equilibrium method[21], indicating that corrections for angular and concentration dependences are unnecessary when measuring dilute solutions. This finding is consistent with the fact that pullulan is a flexible random-coil polymer in aqueous solution, as suggested by viscosity, sedimentation, and light-scattering measurements[21,22]. The error of measurement at $\theta = 5°$ was assumed to be negligible for amylose, which has also been suggested to be a flexible coil in aqueous solutions[8,23–25]. The assumption was reasonable because, as noted below, amylose is a less expanded molecule than pullulan.

We have reported[4] that the number-average molecular weights of amylose specimens from several origins are in the range 10^5–10^6, and similar values have been reported by other investigators, using various methods[7–10]. Probably, a reserve polysaccharide such as amylose has a wide distribution of molecular weight. Therefore, gel columns were tested for the fractionation of an unbranched polysaccharide having a wide distribution of molecular weight by using a mixture of several standard pullulan specimens (Fig. 2), and three TSK-GEL columns (G3000PW, G4000PW, and G6000PW) in sequence were found to be suitable. The pullulan specimens were well separated into the four components, and the intensity ratios of the photometer and the refractometer decreased with the progress of the elution.

There was an apparent time-lag between the positions of elution detected by the photometer and the refractometer due to positional differences of the pens of the recorder and the two monitoring devices in the flow-line. This lag could be corrected for by using a standard specimen with no distribution of molecular weight. With hen ovalbumin as the standard, the tracing of the photometer was 1.95 min ahead of that of the refractometer.

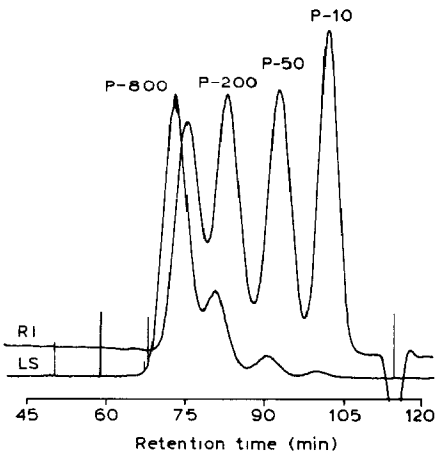

Fig. 2. H.p.l.c. elution chart of mixed pullulan specimens; the retention time is correct for the differential refractometer trace (RI), which is 1.95 min behind the low-angle laser-light-scattering trace (LS).

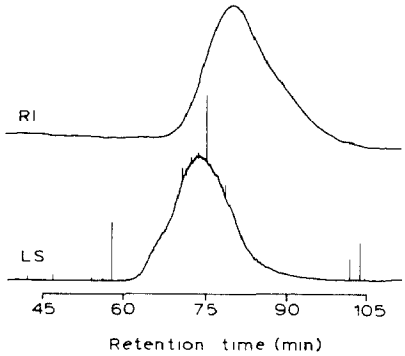

Fig. 3. H.p.l.c. elution charts of kuzu amylose monitored by a low-angle laser-light-scattering photometer (LS) and a differential refractometer (RI). The retention time is correct for the RI curve, but is 1.95 min behind the LS curve.

Fig. 3 shows the h.p.l.c.-elution chart for kuzu amylose. The weight-average molecular weights of amylose samples can be calculated from the ratios of the peak areas under the photometer and the refractometer curves, according to Eq. *3*. The concentrations of solutions injected were <0.1% and were diluted more than 10-fold in the eluate, and thus it was assumed that the term involving the second virial coefficient in Eq. *1* could be ignored. Three independent determinations of molecular weights of kuzu amylose gave values of 522,000, 525,000, and 514,000. Usually, the values agreed to within ±5%.

The possible association of the molecules in solutions stored at 45° was examined because of the high tendency for retrogradation. No increase but a decrease in molecular weight was observed, which was irregular in extent and was independent of the amylose specimens, *e.g.*, for potato amylose, the molecular weight decreased from 1,030,000 to 975,000 and 720,000 after 3 and 16 h, respectively. Storage of the solution at ~100° for 10 min did not cause any significant changes in molecular weight. Therefore, the oxygen-sensitive bond suggested by Baum and Gilbert[26] appears to have no relation to the degradation. Precautions were taken to avoid contamination[27] by amylases (*e.g.*, from saliva) during the preparation of solutions, which were usually analysed within 1 h.

Fig. 4 shows the h.p.l.c.-elution curves (corrected for the lag) of various amylose specimens. The eluate was divided into 10 subfractions of equal weight, and the weight-average degrees of polymerisation ($\overline{d.p.}_w$) for these fractions are listed in Table I. The higher and the lower limits of the molecular weights are hard to determine accurately from the elution patterns in Fig. 4. Therefore, the range of apparent d.p. distribution is tentatively expressed as the $\overline{d.p.}_w$ of the subfractions having the lowest and highest molecular weights. The exact distribution of molecular weight will be much broader. The properties of individual amyloses are summarised in Table II.

The distributions of molecular weights of amyloses appear to be unique to

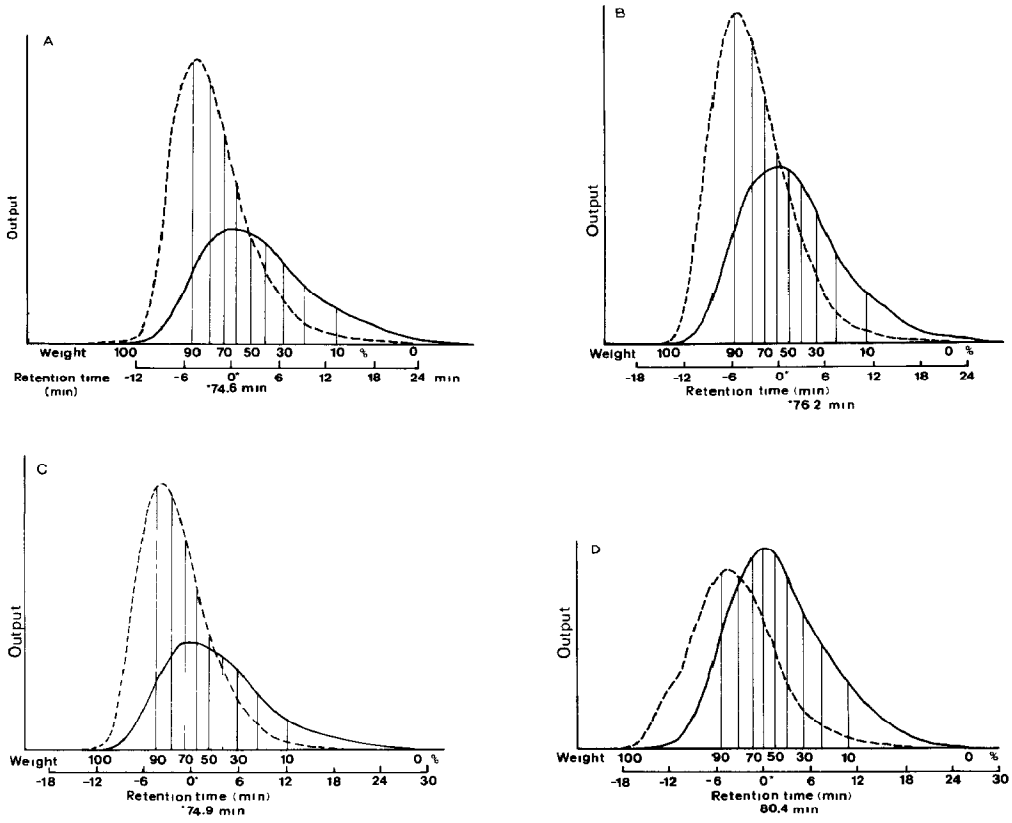

Fig. 4. H.p.l.c. elution curves [low-angle laser-light-scattering photometer (----) and differential refractometer (——)] of amylose specimens corrected for time lags. Amylose specimens: A, potato; B, sweet potato; C, tapioca; D, kuzu. The maximum elution position for the refractometer is shown as 0-time, and its real retention time is indicated thereunder.

TABLE I

WEIGHT-AVERAGE DEGREES OF POLYMERISATION OF AMYLOSE AND ITS SUBFRACTIONS

Subfraction	*Potato*	*Sweet potato*	*Tapioca*	*Kuzu*	*Lily*
0–10	840	840	580	480	360
10–20	1,300	1,070	1,390	770	650
20–30	1,830	1,710	2,220	970	1,110
30–40	2,710	2,360	3,090	1,460	1,700
40–50	3,430	2,790	3,580	1,890	2,280
50–60	4,750	3,850	5,790	2,230	3,360
60–70	6,140	5,610	6,890	2,930	4,600
70–80	8,710	6,440	8,840	3,710	6,920
80–90	12,500	10,200	12,100	5,220	9,830
90–100	21,800	19,100	22,400	12,300	18,900
Whole	6,360	5,430	6,680	3,220	5,010

TABLE II

SUMMARISED PROPERTIES OF AMYLOSE

Amylose	$\overline{D.p.}_n$	$\overline{D.p.}_w$	$\overline{D.p.}_w/\overline{d.p.}_n$	*D.p. (max.)*[a]	*Apparent d.p. distribution*[b]
Potato	4,920	6,360	1.29	6,200	840–21,800
Sweet potato	4,100	5,430	1.31	4,400	840–19,100
Tapioca	2,660	6,680	2.51	6,090	580–22,400
Kuzu	1,540	3,220	2.08	2,560	480–12,300
Lily	2,310	5,010	2.17	3,290	360–18,900

[a]D.p. at the maximum of the elution peak with RI. [b]See text.

their botanical species. The apparent d.p. distribution of potato amylose was from 840 to 21,800, which is a little wider than the values described by other investigators. Everett and Foster[28] reported a $\overline{d.p.}$ range of 930–13,600 by fractionation into seven subfractions and measurements by light scattering in dimethyl sulfoxide, and similar values have been reported by other investigators[25,29–31]. The d.p. distribution range observed for the elution curve (Fig. 4A) was wider (approximately 800–27,000). This range is fairly wide, but a bell-shaped elution peak was obtained with the refractometer (Fig. 4A) and the d.p. at the maximum agreed well with $\overline{d.p.}_w$; the low $\overline{d.p.}_w/\overline{d.p.}_n$ (Table II) indicated a relatively narrow distribution of molecular weight for this amylose. Sharp fractionation by gel-permeation chromatography and the determination of molecular weight in the flow-line of chromatography probably gave a wider distribution of molecular weight than those of earlier studies[25,29–31]. Sweet-potato amylose had molecular properties similar to those of potato amylose, but it was composed of slightly smaller molecules. Tapioca amylose showed an apparently wide distribution of molecular weight due to the presence of a small proportion of small molecules (10% $\overline{d.p.}$ 580) and quantities of large molecules (Table I). The d.p. at the maximum of the refractometer curve was close to the $\overline{d.p.}_w$, suggesting that such large molecules were abundant. Lily amylose contained the smallest molecular fraction among the specimens, but 50–100% of subfractions were composed of large molecules similar to those of sweet-potato amylose. Results of detailed analyses of this amylose will appear elsewhere[27]. Kuzu amylose gave the lowest $\overline{d.p.}_n$ and $\overline{d.p.}_w$, and the majority of its subfractions were composed of smaller molecules than other kinds. The triangular elution curve with RI (Fig. 4D) showed the unique distribution of molecular weight of this amylose.

The retention times of the amylose specimens were a little longer than those of pullulans having the same molecular weights (Fig. 5), suggesting that amylose has a slightly smaller hydrodynamic volume and a less expanded molecule. The molecular weights of amylose specimens were ~35% higher than those of the pullulan specimens at the same elution positions. Whereas amylose is mainly a (1→4)-α-D-glucan, pullulan is an α-D-glucan having two-thirds (1→4) linkages and one-third

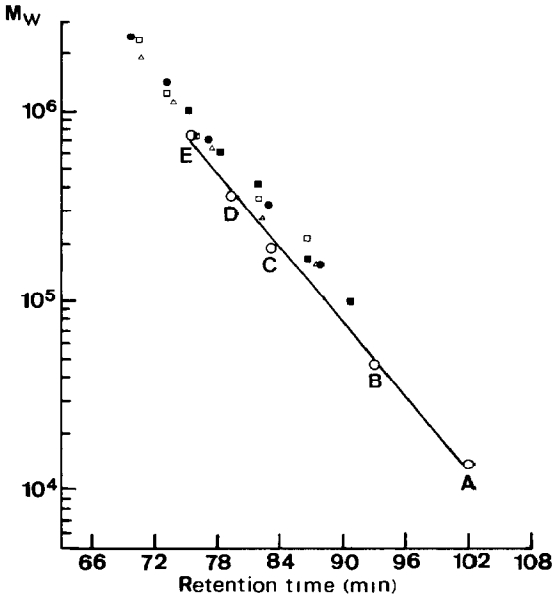

Fig. 5. Relationships between molecular weights and retention times for amylose and pullulan specimens. Amylose specimens [potato (●), sweet potato (△), tapioca (□), and kuzu (■)] are marked at the positions where 10, 25, 50, 75, and 90% (by weight) were eluted. Standard pullulan specimens were: A, P-10; B, P-50; C, P-200; D, P-400; and E, P-800.

(1→6) linkages. The lengths of the repeating units in the *C1* conformation of amylose[32] and linear dextran[33] are reported to be 4.25 and 5.70 Å, respectively. In addition, amylose samples are slightly branched[4], whereas pullulan is an unbranched molecule. These structural differences, in part, may explain[22] the smaller hydrodynamically effective volume of amylose.

The weight-average molecular weight of a polymer is usually determined by sedimentation equilibrium or light-scattering measurements. The technique described here does not require extreme purification of the solvent as do conventional light-scattering studies, because the light-scattering volume of the photometer is only 0.1 μL and the solvent is purified with ultramembrane filters (1 μm) fitted in the flow-line. In addition, the molecular weights of specimens fractionated by h.p.l.c. can be measured continuously by monitoring the eluate with the photometer together with a differential refractometer. By conventional gel-permeation chromatography, the molecular weight of an eluted specimen is often estimated indirectly from the retention time (or elution volume) of a standard specimen and is less accurate even when the same kind of standard polymer is used. The h.p.l.c. method using a low-angle laser-light-scattering photometer and a differential refractometer is a convenient and accurate technique for the determination of weight-average molecular weights and for the estimation of the distributions of molecular weight of water-soluble polysaccharides or polymers. It requires only a small amount of a specimen (at most 500 μg) and takes only 2–3 h.

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