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ANALYSIS OF MIXTURES OF α- AND β-CYCLODEXTRINS USING FLUORESCENT DYES

HITOSHI KONDO, HIROSHI NAKATANI, AND KEITARO HIROMI

Laboratory of Enzyme Chemistry, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)

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ABSTRACT

The fluorescence intensity of 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS) is markedly increased by the addition of cyclodextrins. Mixtures of α- and β-cyclodextrins can be analysed by utilizing the difference in the sensitivity of fluorescent dyes for the two cyclodextrins. One method utilizes the fluorescence increment of TNS and the fluorescence decrement of *o*-anisaldehyde produced on binding with cyclodextrins. An alternative method relates the increase in TNS fluorescence to the total amount of cyclodextrins, which is determined from the reducing power after complete hydrolysis by Taka-amylase A (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3).

INTRODUCTION

Cyclodextrins (α, β, and γ)* are cyclic oligosaccharides, respectively containing 6, 7, or 8 (1→4)-linked α-D-glucopyranose residues. Their inner diameters[1–3] are 6, 7.5–8, and 9.5–10 Å, respectively. The cyclodextrins are produced from starch by *Bacillus macerans* amylase[4,5], and because of their ability to form inclusion complexes with a variety of organic and inorganic compounds[4–8], they have been utilized for medicines, perfumes, and agricultural chemicals[9].

The three cyclodextrins are formed simultaneously from starch, and the reaction mixture also contains non-cyclic oligosaccharides. Therefore, methods for the quantitative analysis of mixtures of cyclodextrins are required in connection with the production and application of these saccharides.

Several methods have been proposed for the analysis of mixtures of cyclodextrins[10–16]. G.l.c., which is one of the most direct and accurate methods[13] for α- and β-cyclodextrins, requires prior esterification. The n.m.r. method[12] can be used directly, but requires 10–30% solutions of cyclodextrins in methyl sulphoxide. Gravimetry of cyclodextrins complexed with certain organic solvents (tetrachloroethane or bromobenzene) has been proposed[14], as has an enzymic method[16] using Taka-amylase A and glucoamylase. None of these methods is easily applicable to dilute, aqueous solutions of mixtures of cyclodextrins.

*Cyclohexa-, hepta-, and octa-amyloses.

Recently, we found that cyclodextrins caused a large increase in the fluorescence of the dye 2-*p*-toluidinylphthalene-6-sulphonate (TNS), and that this effect depends largely on the ring size of the cyclodextrin[17]. This phenomenon has been utilized in developing a new method for the analysis of mixtures of cyclodextrins.

EXPERIMENTAL

Materials. — α-Cyclodextrin was recrystallized from 60% aqueous 1-propanol and then from distilled water[18], and β-cyclodextrin was recrystallized from distilled water[18].

Potato amylose (Sigma Chemical Co, lot No. MOR4819, $\overline{\text{d.p.}}_n$ 600) and soluble starch (Nakarai Chemical Co. Ltd., lot No. M3F7484) were used without further purification.

TNS was recrystallized twice from distilled water before use[19], and the purity was monitored by t.l.c. on Kieselgel 60 F_{254} (Merck) using butan-2-ol saturated with 3% aqueous ammonia[19]. Stock solutions of TNS were freshly prepared with 0.08M sodium acetate buffer (pH 5.3), and concentrations were determined spectrophotometrically[20] by using the molecular absorptivity at 366 nm (ε 4,300).

The magnesium salt of 1-anilinonaphthalene-8-sulphonate (ANS) was determined spectrophotometrically[21] by using the molecular absorptivity at 350 nm (ε 4,950).

o-Anisaldehyde, L-kynurenine, *p*-phenylenediamine, salicylic acid, and anthranilic acid were purchased from Nakarai Chemical Co. Ltd. and used without further purification. 4-Benzamido-4′-aminostilbene-2,2′-disulphonate (MBAS) was a generous gift from Professor K. Yagi (Nagoya University).

Glucoamylase from *Rhizopus niveus* (Seikagaku Kogyo Co. Ltd., lot No. 4S17, pure grade) was used without further purification. The enzyme concentration was determined spectrophotometrically[22] at 280 nm, assuming $E^{1cm}_{1\%} = 14.2$ and a mol. wt. of 58,000.

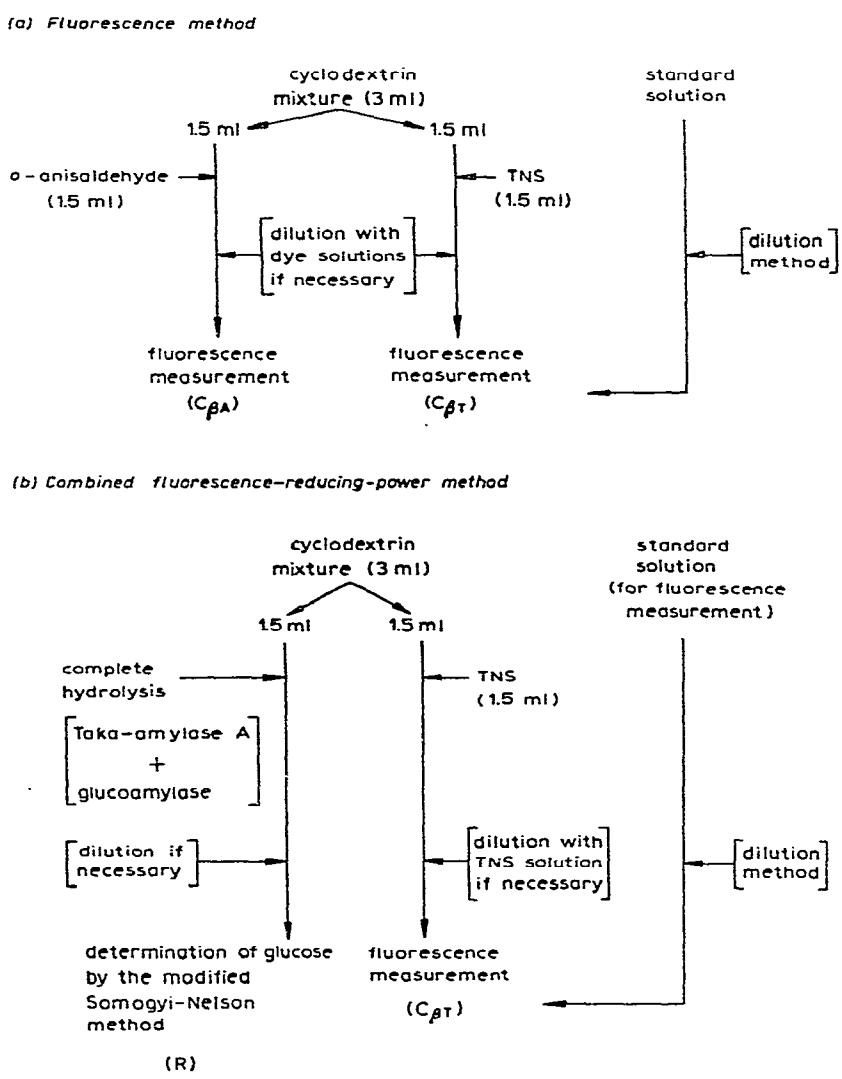
Crystalline Taka-amylase A was prepared[23] from "Taka-diastase Sankyo" by a modified procedure[24], and its concentration was determined spectrophotometrically[21] at 280 nm, assuming $E^{1cm}_{1\%} = 22.1$ and a mol. wt.[26] of 51,000.

Other chemicals were of reagent grade.

Methods. — Fluorescence measurements of dyes were performed in the following systems at 25° with a Hitachi MPF-2A Spectrofluorimeter: TNS (2.57×10^{-5}–5.27×10^{-4}M), ANS (1.15×10^{-3}M), MBAS (1.90×10^{-4}M), and salicylic acid (10^{-3}M), 0.08M sodium acetate buffer (pH 5.3); L-kynurenine (8.50×10^{-5}M), 0.45M Tris-HCl buffer (pH 8.2); anthranilic acid (10^{-3}M), 0.1M acetate buffer (pH 1.3); *p*-phenylenediamine (10^{-3}M), 0.1M acetate buffer (pH 2.5); and *o*-anisaldehyde (5.80–6.30×10^{-4}M), 0.1M acetate buffer (pH 3.8). The excitation wavelengths were fixed at 366 nm for TNS, 360 nm for ANS and L-kynurenine, 354 nm for *o*-anisaldehyde, 342 nm for MBAS, and 292 nm for *p*-phenylenediamine. The emission wavelengths were fixed at 510 nm for ANS, 460 nm for TNS, and 425 nm for

o-anisaldehyde. Fluorescence titrations of ANS, TNS, and *o*-anisaldehyde with cyclodextrins were made at constant concentrations of each dye by the successive-dilution method[17]. The initial sample was used as a standard to correct for instrumental drift.

The concentration ranges of cyclodextrins employed were 3.91×10^{-5}–1.0×10^{-2}M for α-cyclodextrin, and 1.56×10^{-5}–1.0×10^{-2}M for β-cyclodextrin. At higher concentrations, the mixtures of cyclodextrins to be analysed were appropriately diluted with dye solutions (TNS and *o*-anisaldehyde) to the concentration range



Scheme 1.

where the standard curve for fluorescence intensity–cyclodextrin concentration is linear (Figs. 2 and 3).

The total amount of cyclodextrins was determined by measuring the reducing power after complete hydrolysis effected by the simultaneous action of Taka-amylase A (4.04×10^{-6}M) and glucoamylase (9.55×10^{-7}M) at 30° for 30 h. The reducing power was measured by the modified Somogyi–Nelson[27] and phenol–sulphuric acid methods[28].

The procedures for the analysis of mixtures of α- and β-cyclodextrins are given in Scheme 1.

RESULTS

Cramer *et al.*[7] have shown that ANS forms inclusion complexes with cyclodextrins, accompanied by a pronounced increase in the fluorescence intensity. We have found that TNS not only has a much higher fluorescence intensity than ANS, but also a larger difference in binding affinities towards α- and β-cyclodextrins[17]. Furthermore, of the fluorescent dyes examined, TNS and *o*-anisaldehyde were the most useful for analysing mixtures of α- and β-cyclodextrins, since the fluorescence intensities of these dyes are markedly, but differently, changed by α- and β-cyclodextrins. A typical example of titrations is shown in Fig. 1 for TNS– and *o*-anisaldehyde–β-cyclodextrin systems. The fluorescence emission of TNS is very weak, but

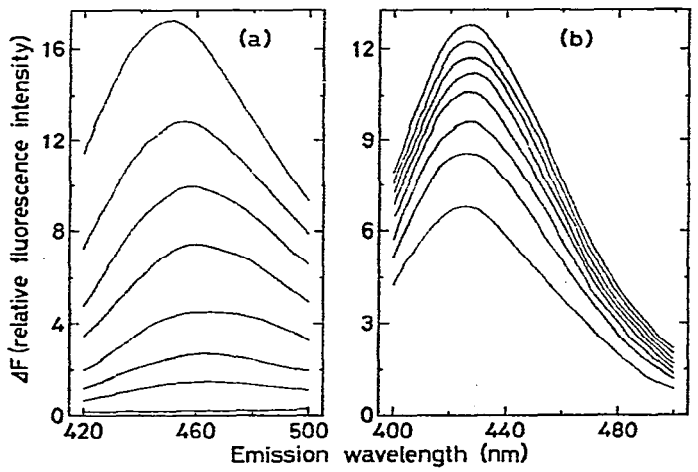


Fig. 1. Fluorescence spectra of TNS– and *o*-anisaldehyde–β-cyclodextrin systems. (*a*) TNS concentration: 4.36×10^{-4}M. β-Cyclodextrin concentrations from top to bottom: 7.85, 3.93, 1.96, 0.98, 0.49, 0.25, 0.12, and 0mM. The lowest curve is the fluorescence spectrum of TNS (λ_{ex} 366 nm). The buffer was 0.08M sodium acetate (pH 5.3). (*b*) *o*-Anisaldehyde concentration: 6.16×10^{-4}M. β-Cyclodextrin concentrations from top to bottom: 0, 0.88, 1.32, 1.98, 2.96, 4.45, 6.67, and 10.0mM. The highest curve is the fluorescence spectrum of *o*-anisaldehyde (λ_{ex} 354 nm). The buffer was 0.1M acetate (pH 3.8).

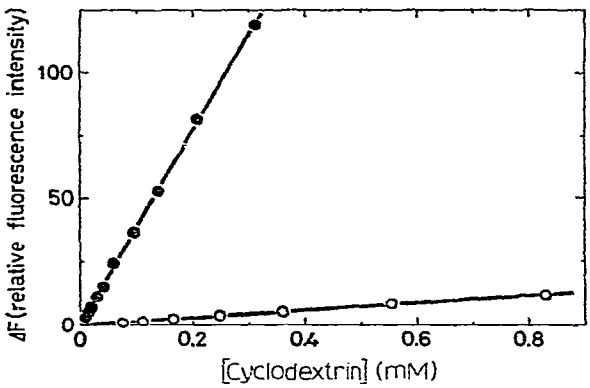


Fig. 2. Standard curves of fluorescence intensities of TNS–cyclodextrin systems. TNS concentration: 4.88×10^{-4}M; −○−, TNS–α-cyclodextrin system; −●−, TNS–β-cyclodextrin system (λ_{em} 460 nm). The other conditions are the same as those in Fig. 1.

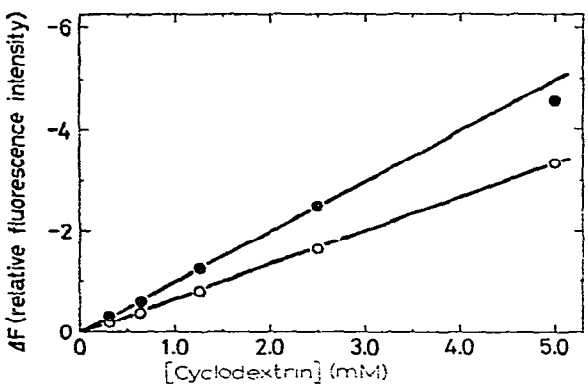


Fig. 3. Standard curves of fluorescence intensities of *o*-anisaldehyde–cyclodextrin systems. *o*-Anisaldehyde concentration: 5.77×10^{-4}M; −○−, *o*-anisaldehyde–α-cyclodextrin system; −●−, *o*-anisaldehyde–β-cyclodextrin system (λ_{em} 425 nm). The other conditions are the same as those in Fig. 1.

when added to cyclodextrin solutions its fluorescence intensity increases markedly. On the other hand, when *o*-anisaldehyde was added to cyclodextrin solutions, its fluorescence intensity decreased appreciably. Figs. 2 and 3 show the standard curves of change in fluorescence intensity of TNS and *o*-anisaldehyde on binding with α- and β-cyclodextrins, respectively. The difference in the relative slopes for TNS and *o*-anisaldehyde reflects the difference in sensitivities of the two dyes towards α- and β-cyclodextrins.

Based on these findings, the analysis of mixtures of α- and β-cyclodextrins was attempted by two procedures.

Fluorescence method using TNS and o-anisaldehyde.— So far as the additivity of fluorescence intensity of TNS-cyclodextrin systems holds, the equations *1* and *2* can be obtained, where F_T is the total, observed fluorescence-intensity for TNS-cyclodextrin systems, $F_{\alpha T}$ and $F_{\beta T}$ denote the contributions from the binding with α- and

$$F_T = F_{\alpha T}+F_{\beta T} = f_{\alpha T}\cdot C_\alpha + f_{\beta T}\cdot C_\beta = f_{\beta T}(T\cdot C_\alpha + C_\beta) \quad (1)$$

$$T = f_{\alpha T}/f_{\beta T} = 0.037 \quad (2)$$

β-cyclodextrins, respectively, C_α and C_β denote the α- and β-cyclodextrin concentrations to be determined, and $f_{\alpha T}$ and $f_{\beta T}$ denote proportionality coefficients of fluorescence intensities of TNS complexes with α- and β-cyclodextrins, which are determined from the standard curves (Fig. 2). For *o*-anisaldehyde–cyclodextrin systems, the amounts of fluorescence decrease ($F_{\alpha A}$ and $F_{\beta A}$) are proportional to α- and β-cyclodextrin concentrations as seen in Fig. 3. The observed fluorescence-decrease F_A is given by the sum, $F_{\alpha A}+F_{\beta A}$. Accordingly, for *o*-anisaldehyde, equations *3* and *4* hold.

$$F_A = F_{\alpha A}+F_{\beta A} = f_{\alpha A}\cdot C_\alpha + f_{\beta A}\cdot C_\beta = f_{\beta A}(A\cdot C_\alpha + C_\beta) \quad (3)$$

$$A = f_{\alpha A}/f_{\beta A} = 0.654 \quad (4)$$

When equations *1* and *3* are solved simultaneously, equations *5* and *6* are obtained,

$$C_\alpha = \frac{C_{\beta A}-C_{\beta T}}{A-T} = \frac{C_{\beta A}-C_{\beta T}}{0.617} \quad (5)$$

$$C_\beta = \frac{A\cdot C_{\beta T}-T\cdot C_{\beta A}}{A-T} = \frac{A\cdot C_{\beta T}-T\cdot C_{\beta A}}{0.617} \quad (6)$$

where $C_{\beta T}(=F_T/f_{\beta T})$ and $C_{\beta A}(=F_A/f_{\beta A})$ denote β-cyclodextrin concentrations corresponding to the observed fluorescence-intensities F_T and F_A, respectively, which are obtained from the standard curves (Figs. 2 and 3). The concentrations C_α and C_β can be obtained in so far as the values T and A are different from each other. In the present case, T was 0.037 and A was 0.654, which were determined from the standard curves (Figs. 2 and 3).

The procedure was then applied to solutions of mixtures of α- and β-cyclodextrins of known ratios, and the results shown in Tables I–III indicate that the method is accurate to ~10%, except for low, relative concentrations of α-cyclodextrin ($C_\alpha/C_\beta<0.5$). The greater error at low C_α/C_β ratios is due to the lower sensitivity of TNS fluorescence towards α-cyclodextrin.

Combined fluorescence–reducing-power method. — An alternative analytical procedure involves a combination of the change of TNS fluorescence-intensity with the measurement of the total amount of cyclodextrins. The latter value can be obtained from the reducing power (R) of the D-glucose formed on complete hydrolysis of cyclodextrins by Taka-amylase A and glucoamylase. Equation *7* relates the cyclo-

TABLE I

APPLICABILITY OF THE FLUORESCENCE METHOD FOR DETERMINING α- AND β-CYCLODEXTRINS (TOTAL CONCENTRATION: 2.5×10^{-3}M)

α-Cyclodextrin				*β-Cyclodextrin*			
Fraction	*Calc.* (10^{-3}M)	*Found*[a] (10^{-3}M)	*Error* (%)	*Fraction*	*Calc.* (10^{-3}M)	*Found*[a] (10^{-3}M)	*Error* (%)
0.71	1.818	1.69	−7.2	0.29	0.727	0.70	−4.1
0.56	1.364	1.30	−4.9	0.44	1.091	1.05	−3.6

[a]TNS concentration: 3.92×10^{-4}M. *o*-Anisaldehyde concentration: 5.77×10^{-4}M.

TABLE II

APPLICABILITY OF THE FLUORESCENCE METHOD FOR DETERMINING α- AND β-CYCLODEXTRINS (TOTAL CONCENTRATION: 1.42×10^{-3}M)

α-Cyclodextrin				*β-Cyclodextrin*			
Fraction	*Calc.* (10^{-4}M)	*Found*[a] (10^{-4}M)	*Error* (%)	*Fraction*	*Calc.* (10^{-4}M)	*Found*[a] (10^{-4}M)	*Error* (%)
0.10	1.420	1.64	+15.1	0.90	12.780	11.74	−8.1
0.30	4.209	3.90	−7.3	0.70	9.940	9.12	−8.2
0.40	5.680	5.10	−10.2	0.60	8.520	7.88	−7.5
0.50	7.100	6.43	−9.4	0.50	7.100	6.26	−11.8
0.60	8.520	7.13	−16.3	0.40	5.680	5.24	−7.8
0.70	9.940	8.23	−17.3	0.30	4.209	3.94	−6.4
0.90	12.780	11.66	−8.8	0.10	1.420	1.44	+1.1

[a]TNS concentration: 4.60×10^{-4}M. *o*-Anisaldehyde concentration: 5.77×10^{-4}M.

TABLE III

APPLICABILITY OF THE FLUORESCENCE METHOD FOR DETERMINING α- AND β-CYCLODEXTRINS (TOTAL CONCENTRATION: 5.00×10^{-4}M)

α-Cyclodextrin				*β-Cyclodextrin*			
Fraction	*Calc.* (10^{-4}M)	*Found*[a] (10^{-4}M)	*Error* (%)	*Fraction*	*Calc.* (10^{-4}M)	*Found*[a] (10^{-4}M)	*Error* (%)
0.10	0.500	—	—	0.90	4.500	4.48	−0.5
0.25	1.250	0.68	−45.8	0.75	3.750	3.92	+4.4
0.50	2.500	2.43	−3.0	0.50	2.500	2.73	+9.2
0.75	3.750	3.65	−2.8	0.25	1.250	1.38	+10.6
0.90	4.500	4.28	−5.0	0.10	0.500	0.50	−1.0

[a]TNS concentration: 5.27×10^{-4}M. *o*-Anisaldehyde concentration: 6.29×10^{-4}M.

dextrin concentrations and R, where R denotes the total amount of

$$6 \cdot C_\alpha + 7 \cdot C_\beta = R \tag{7}$$

cyclodextrins expressed in glucose equivalent. This imples that a mole each of α- and β-cyclodextrins contribute in the ratio 6:7 to the reducing power.

C_α and C_β can be obtained by solving equations *1* and *7*.

$$C_\alpha = \frac{R - 7 \cdot C_{\beta T}}{6 - 7 \cdot T} = \frac{R - 7 \cdot C_{\beta T}}{5.741} \tag{8}$$

$$C_\beta = \frac{6 \cdot C_{\beta T} - T \cdot R}{6 - 7 \cdot T} = \frac{6 \cdot C_{\beta T} - T \cdot R}{5.741} \tag{9}$$

The results calculated from equations *8* and *9* are given in Table IV (*cf.* Table III). This method improves the accuracy of determinations of α-cyclodextrin at lower C_α/C_β ratios.

TABLE IV

APPLICABILITY OF THE COMBINED FLUORESCENCE–REDUCING-POWER METHOD FOR DETERMINING α- AND β-CYCLODEXTRINS (TOTAL CONCENTRATION: 5.00×10^{-4}M)

α-Cyclodextrin				*β-Cyclodextrin*			
Fraction	*Calc.* (10^{-4}M)	*Found*[a] (10^{-4}M)	*Error* (%)	*Fraction*	*Calc.* (10^{-4}M)	*Found*[a] (10^{-4}M)	*Error* (%)
0.10	0.500	0.60	+19.5	0.90	4.500	4.48	−0.5
0.25	1.250	1.21	−3.0	0.75	3.750	3.83	+2.0
0.50	2.500	2.40	−4.1	0.50	2.500	2.59	+3.6
0.75	3.750	3.66	−2.4	0.25	1.250	1.34	+7.5
0.90	4.500	4.59	+2.0	0.10	0.500	0.49	−2.5

[a]TNS concentration: 5.27×10^{-4}M. The total concentration of D-glucose residues was determined by using Taka-amylase A and glucoamylase.

DISCUSSION

The foregoing analytical methods are much simpler and more convenient than the conventional methods[12–14,16]. For a C_α/C_β ratio of 1, the enzymic method of Kobayashi *et al.*[16] has a relative error of <5%, whereas that of the combined fluorescence–reducing-power method is $< \sim 4\%$ and that of the fluorescence method is $\sim 10\%$.

The limitations of these two methods are as follows. (1) They cannot be applied with accuracy at C_α/C_β ratios of <0.5 in the fluorescence method, and <0.25 in the combined fluorescence–reducing-power method. The reason is that both TNS and *o*-anisaldehyde have a lower affinity for α-cyclodextrin than for β-cyclodextrin.

(2) The upper limit of total cyclodextrin concentrations to be determined is 2×10^{-4}M for TNS and 4×10^{-3}M for *o*-anisaldehyde, as indicated by the linearity in the standard curves in Figs. 2 and 3. The lower limit of total cyclodextrin concentration is 5×10^{-5}M for TNS and 5×10^{-4}M for *o*-anisaldehyde. Therefore, the fluorescence method is applicable to total cyclodextrin concentrations higher than 5×10^{-4}M ($\sim$1.5 mg/3 ml), and the combined fluorescence–reducing-power method to concentrations higher than 5×10^{-5}M ($\sim$0.15 mg/3 ml).

These methods may be applied to mixtures of cyclodextrins in the presence of amylose or starch, since the contribution of these non-cyclic saccharides to TNS fluorescence can be removed by the action of glucoamylase. A preliminary experiment showed that amylose and starch (0.044%) initially added to β-cyclodextrin (0.1%), after being treated by glucomylase, produced a relative error of $<0.1\%$. Any error due to interaction between TNS and proteins could be avoided, in principle, by subtracting the fluorescence remaining after complete hydrolysis of cyclodextrins from the total intensity.

Although α- and β-cyclodextrins are always formed simultaneously and together with considerably smaller amounts of γ-cyclodextrin by the action of *Bacillus macerans* amylase[14] on starch, the above analytical methods are useful for the analysis of mixtures of α- and β-cyclodextrins.

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REFERENCES

1 K. Takeo and T. Kuge, *Agr. Biol. Chem.*, 33 (1969) 1174–1180.
2 K. Takeo and T. Kuge, *Agr. Biol. Chem.*, 34 (1970) 568–574.
3 K. Takeo and T. Kuge, *Agr. Biol. Chem.*, 34 (1970) 1787–1794.
4 D. French, *Adv. Carbohydr. Chem.*, 12 (1957) 189–260.
5 J. A. Thoma and L. Stewart, in R. L. Whistler and E. F. Paschall (Eds.), *Starch: Chemistry and Technology*, Academic Press, New York, 1965, Vol. 1, pp. 209–249.
6 W. Lautsch, W. Broser, W. Biederman, and H. Gnichtel, *J. Polym. Sci.*, 17 (1955) 479–510.
7 F. Cramer, W. Saenger, and H.-Ch. Spatz, *J. Am. Chem. Soc.*, 89 (1967) 14–20.
8 K. Takeo and T. Kuge, *Stärke*, 24 (1972) 331–336.
9 T. Kuge and K. Takeo, *Denpun Kagaku*, 21 (1974) 151–168.
10 J. N. J. J. Lammers, *Stärke*, 19 (1967) 70–73.
11 J. N. J. J. Lammers, *J. Chromatogr.*, 41 (1969) 462–466.
12 B. Casu, G. G. Gallo, M. Reggiani, and A. Vigevani, *Stärke*, 20 (1968) 387–391.
13 J. B. Beadle, *J. Chromatogr.*, 42 (1969) 201–206.
14 K. Takeo, Y. Kondo, and T. Kuge, *Kyoto Furitsu Daigaku Gakujutsu Hokoku, Nogaku*, 22 (1970) 106–110.
15 J. H. Carter and E. Y. C. Lee, *Anal. Biochem.*, 39 (1971) 521–542.
16 S. Kobayashi, K. Kainuma, and S. Suzuki, *Denpun Kagaku*, 21 (1974) 131–137.
17 H. Kondo, H. Nakatani, and K. Hiromi, *J. Biochem. (Tokyo)*, 79 (1976) 393–405.

18 D. FRENCH, M. L. LEVINE, J. H. PAZUR, AND E. NORBERG, *J. Am. Chem. Soc.*, 71 (1949) 353–356.
19 W. O. MCCLURE AND G. H. EDELMAN, *Biochemistry*, 5 (1966) 1908–1919.
20 W. O. MCCLURE AND G. H. EDELMAN, *Biochemistry*, 6 (1967) 559–566.
21 G. WEBER AND L. B. YOUNG, *J. Biol. Chem.*, 239 (1964) 1415–1423.
22 Y. TSUJISAKA, personal communication.
23 S. AKABORI, T. IKENAKA, AND B. HAGIHARA, *J. Biochem. (Tokyo)*, 41 (1954) 577–582.
24 Y. MATSUSHIMA, personal communication.
25 H. TODA AND S. AKABORI, *J. Biochem. (Tokyo)*, 53 (1963) 102–110.
26 K. NARITA, H. MURAKAMI, AND T. IKENAKA, *J. Biochem. (Tokyo)*, 59 (1966) 170–175.
27 K. HIROMI, Y. TAKASAKI, AND S. ONO, *Bull. Chem. Soc., Jap.* 36 (1963) 563–569.
28 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.