Nonchromatographic Cyclodextrin Assays: Evaluation of Sensitivity, Specificity, and Conversion Mixture Applications

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Methods based on various clathrate-forming molecules, sugar analyses in combination with glucoamylase treatment, or catalytic properties of cyclodextrins were evaluated as cyclodextrin assays. Optimum conditions for the assays were determined and, when necessary, appropriate modifications were made. The tests were made by using pure cyclodextrin standards, supplemented standards, and conversion mixtures obtained by the action of cyclodextrin glycosyltransferase on starch. The optimized methods varied considerably with respect to measurement range, relative sensitivity toward α-, β-, and γ-cyclodextrin, and susceptibility to interferences. On the basis of data from these experiments, typical applications of individual methods or their combinations are suggested. The specificity differences of assays based on methylorange, bromocresol green, and phenolphthalein were found especially useful in the analysis of individual CDs out of their mixtures. An example of the usage of this method combination is given.

Cyclodextrins (CD), the cyclic oligosaccharides of six, seven, or eight glucose units, are used increasingly to improve quality, stability, and functional properties of pharmaceuticals, foods, and agricultural products (Saenger, 1980; Frömming, 1981; Szejtli, 1981). Along with this development the need for CD analyses is growing and evaluation of proper methodology has become actual. Many of the direct CD assays are based on the measurement of clathrate-forming molecules, such as light-absorbing or fluorescent compounds, or enzyme substrates (Cramer et al., 1967; Lane and Pirt, 1973; Kondo et al., 1976a,b; Vikmon, 1981; Bender, 1981; Landert et al., 1981; Peterfi and Seres, 1981; Laakso et al., 1984; Kato and Horikoshi, 1984). These methods are rapid and easy to perform but may be interfered with by other components in the assay mixture. Even a change in ionic strength or temperature may disturb the facile equilibrium of complexation. Chromatographic CD assays have also been used (Beadle, 1969; Takeo and Kondo, 1970; Nakamura and Horikoshi 1976; Zsadon et al., 1978; Zsadon et al., 1979). They are often time-consuming and so less suitable to large series of samples. The HPLC applications (Hokse, 1980; Brunt, 1982; Koizumi et al., 1983, 1984) enable however the analysis of up to 10 samples/day and when used for simple mixtures or in combination with prepurification steps are well suited for simultaneous quantitation of α-, β-, and γ-subforms. Methods based on quantitation of CDs via reducing sugars present after a ring-saving glucoamylase treatment and total hydrolysis of the sample have been cited in the literature (Matzuzawa et al., 1975; Kobayashi et al., 1975; Nakamura and Horikoshi 1977).

Current literature is however deficient of data enabling comparison of various CD assays or selection of a method or their combinations to be used in, e.g., starch digests. Such information would also serve standardization purposes in a field ranging from scientific work to control of quality of various CD-containing products. In the present study we tested various nonchromatographic CD assays. It appeared that many of the published methods were in a preliminary state of development and required considerable modification and that the choice of proper method for different sample types is a key to successful CD analyses.

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METHODS

Spectrophotometric Methods. The name of the assay refers to the tracer molecule.

Phenolphthalein. Reagents: stock phenolphthalein solution, 3.75 mM, in 94% ethanol; phenolphthalein working solution, 0.4 mM; above stock solution is diluted 1:10 with destilled water just prior to use; Na_2CO_3 solution, 1.0 M, in water; CD standards in destilled water; α-CD, 0.5 mM; β-CD, 0.5 mM; γ-CD, 2.5 mM. Procedure: Into a test tube containing 50–500 μL of a CD sample are added 200 μL of the phenolphthalein working solution and 250 μL of Na_2CO_3 solution just prior to measurement. The final volume of 2.5 mL is made up with water. In the control assay, the sample is replaced by an equal volume of water. The calibration curves are made by using 100–500 μL of the CD standards in place of the samples. The reactants are mixed, and the color intensity is measured at 550 nm at 25 °C [modified from Vikmon (1981)].

Methylorange. Reagents: methylorange, 0.30 mM, in 0.1 M malate buffer, pH 6.8; reagent dissolved by sonication; CD standards in water; α-, β-, and γ-CD, 0.5 mM each. Procedure: An appropriate dilution of a CD sample (100–600 μL) is pipetted into a test tube containing 1.0 mL of the methylorange solution. The mixture is made up to 1.5 mL with water and mixed and the absorbance measured at 543 nm at 25 °C. In controls and in assays of the standards, 500 μL of water or 100–500 μL of each of the 0.5 mM CD solutions is added in place of the sample (Landert et al., 1981).

p-Nitrophenolate. Reagents: p-nitrophenol (PNP), 0.1 mM, in 100 mM Na_2CO_3 buffer, pH 10.0; CD standards, 2.5 mM for α-, β-, and γ-CDs, in water. Procedure: CD samples (100–500 μL) are added to 0.5 mL of the PNP solution, and the final volume is brought to 1.5 mL with water. After mixing, the color intensity is measured at 425 nm (α-form) or at 400 nm (γ-form). The blank and the standards are prepared by adding, in place of sample, 1.0 mL of water or 0.1–0.5 mL of one of the standard solutions (Cramer et al., 1967).

Iodine. Reagents: Tris-HCl buffer, 30 mM, pH 7.2; iodine, 50 mM, in dimethyl sulfoxide (DMSO); CD standards in the above Tris-HCl buffer, 5 and 10 mM with respect to α- and β-CD. Procedure: Into 20-mL glass tubes are added 5 mL of the Tris-HCl buffer, 10–20 μL of the iodine solution, and either 10–100 μL of a CD sample or 0.05–1.0 μmol of CD standard. The mixture is incubated at 30 °C for 30 min, and the absorbances of iodine control (no CD added) and the clathrates formed are measured against the Tris-HCl buffer at 290 nm (Bender, 1981).

0021-8561/88/1436-0083$01.50/0 © 1988 American Chemical Society

Bromocresol Green. Reagents: sodium citrate buffer, 0.2 M, pH 4.2; bromocresol green (BCG), 5 mM in 20% (v/v) ethanol; CD standards, 0.5 mM in water. Procedure: Appropriate dilutions of CD samples (50–500 μL) are pipetted into test tubes containing 2.0 mL of the buffer. Of the indicator solution 50 μL is added, and the tubes are mixed by inversion. The absorbances are measured at 630 nm at 25 °C against a blank containing 500 μL of water in place of the CD sample (Kato and Horikoshi, 1984). Preferably cuvettes with 5-mm path length are used.

Fluorometric Methods. *2-p-Toluidinylnaphthalene-6-sulfonate (TNS).* Reagents: TNS from Sigma Chemical Co., 1 mM, in 0.08 M sodium acetate buffer, pH 5.3; CD standards in the same buffer, 0.5 mM for α-, β-, and γ-CD. Procedure: Appropriate dilutions of CD samples (50–1500 μL) are mixed with 1.5 mL of the TNS solution and made up to 3.0 mL with the acetate buffer. After mixing, CDs are quantitated at 25 °C by using excitation and emission wavelengths of 366 and 460 nm, respectively. The blank contains 1.5 mL of the acetate buffer instead of the TNS solution. Normally, a linear β-CD standard is obtained by using 50–500 μL of the corresponding standard solution. To ensure that the unknown samples fall within the linear measurement range, several sample volumes should be assayed (Kondo et al., 1976a,b).

1-Anilinonaphthalene-8-sulfonate (ANS). Reagents: ANS from Sigma, 2.3 mM, in sodium acetate buffer, pH 5.3; CD standards in the above acetate buffer, 2.5 mM for α-, β-, and γ-CD. Procedure: An appropriate dilution of CD samples (50–1500 μL) is mixed with 1.5 mL of the ANS solution and made up to final volume of 3.0 mL with the acetate buffer. After mixing, CDs are quantitated at 25 °C by using exitation and emission wavelengths of 410 and 510 nm, respectively. The ANS solution is replaced by 1.5 mL of the acetate buffer in the blank. Linear calibration curves are obtained by using 50–500 μL of the β-CD standard (Kondo et al., 1976a,b).

Oxygraphy. Reagents: linoleic acid (>99%, from Sigma), 10 mM, in 0.2 M sodium borate buffer (pH 9.0) containing 10% (v/v) ethanol; Soybean lipoxygenase-1 (L-1) (EC 1.11.13.11) stock solution, 1 mg/mL, in the above borate buffer; lyophilized enzyme (Sigma), 140–180 U/mg of protein; CD standards, 5 mM for the α-, β-, and γ-subforms, in 0.2 M sodium borate buffer, pH 9.0. Procedure: The assay mixture contains, in 1.0 mL of 0.2 M sodium borate buffer (pH 9.0), 0.13–0.40 μmol of linoleate and 0.02–0.06 U of the enzyme. The samples and the standards are injected in 20–60-μL portions into the reaction mixture while the concentration of dissolved O_2 is continuously recorded polarographically. The change in the slope of the reaction velocity curve is used as a measure of CD added (Laakso et al., 1984).

Hydrolysis of Dimethylphenyl Acetate. Reagents: 3,5-Dimethylphenyl acetate (DMPA, not available commercially) was synthetized according to a previously described procedure (Chattaway, 1931). After this reaction, a small aliquot of diethyl ether was added to collect the ester. The ethereal solution was dried overnight over anhydrous Na_2SO_4 and the ether removed under N_2 stream at 40 °C (yield 80–90%). The identity of the liquid products was ascertained with ^1H NMR spectroscopy. The synthesis of 3,5-dinitrophenol acetate (yellowish solid) proceeded similarly. Procedure: The assay mixture consists of 1.25 mL of 0.6 M Na_2CO_3 buffer, pH 10.2, 0.1–1.0 mL (0.4–40 mM) of either α-CD standard or unknown sample, and water to give a final volume of 2.5 mL. The reaction temperature is 25 °C. Then, 10 μL of 0.03 M DMPA (in acetonitrile) is applied onto the cone-shaped groove of the stopper. The hydrolysis begins upon stoppering and quick inversions of the cuvette (within 2 s). The hydrolysis rate is followed at 240 nm as the slope of the reaction velocity curve and used as the measure of α-CD present (Lane and Pirt, 1973).

Methods Based on Glucose Analysis. *Glucoamylase–Anthrone–H_2SO_4 (Anthrone Method).* Reagents: glucoamylase (amyloglucosidase, EC 3.2.1.3) from a *Rhizopus* genus mold, 11 800 U/g solid (Sigma), used as 5.5 or 6.5 mg/mL solutions in water; 3,5-dinitrosalicylate (DNS), 10 g/L; anthrone–H_2SO_4, 20 mg of anthrone in 100 mL of 80% sulfuric acid; glucose standards, 1 and 0.2 mg/mL in water. Procedure: Total carbohydrate content in CD samples is adjusted to about 1% (w/w) by diluting with water, and the pH is adjusted to 5.5 with 0.2 M HCl. This sample and a water blank are supplemented with 0.1 mL of the glucoamylase (5.5 mg/mL) and incubated for 5 h at 40 °C with constant shaking. Reducing sugars in the digests are quantitated by the DNS method (Summer and Somers, 1944) and the 1.0 mg/mL glucose standard. For the determination of total carbohydrates less than 0.2% (w/w) of a sample, 0.4 mL of water, and 4 mL of the acidic anthrone are added. After careful mixing, the incubation is continued in boiling water for 12 min and the solution is cooled rapidly in cold water and, after mixing, measured at 630 nm against a blank containing 0.5 mL of water in place of the sample. Standards are prepared by using 0.05–0.5 mL of the glucose standard (0.2 mg/mL). The difference between total and reducing sugars is used as the measure of CDs present in the samples (Jermyn, 1975; Morris, 1948).

Glucoamylase-takadiastase. Reagents: glucoamylase as described above; α-amylase (EC 3.2.1.1) from *Aspergillus oryzae*, 240 U/mg of protein (type X-A from Sigma), diluted in water to contain 32.5 mg/mL; glucose standard, 1.0 mG, 1.0 mg/mL, in water. Procedure: the sample is divided into two 1.0-mL portions. One of the duplicates is treated with the glucoamylase as described above and the other under identical conditions with 0.1 mL of the glucoamylase (6.5 mg/mL) plus 0.2 mL of the α-amylase solution. Reducing sugars formed in the two mixtures are quantitated by the DNS method as above, and their difference is used as the measure of CDs.

Preparation of Conversion Mixtures. Potato starch hydrolysates were prepared by cyclodextrin glycosyltransferases (CGTase, 2.4.1.19) from alcalophilic *Bacillus sp.* (ATCC 21783). The enzyme (1 mg/mL of an ammonium sulfate concentrate) was added in 0.2 M glycine–NaOH buffer (pH 8.5) containing 10 mM $CaCl_2$ and 1–15% (w/v) potato starch. The suspension was liquified by vigorous shaking at 90 °C for 30 min (prehydrolysis). The temperature was lowered to 60 °C, 0.40% (w/w) fresh CGTase was added in some of the samples, and the incubation was continued for an additional 21 h. According to HPLC (Zsadon et at., 1979), the product mixture consisted of 5.4, 72.2, and 22.4 mol % of α-, β-, and γ-CD, respectively. Modifications in the reactions were as follows: sample 1, starch 15%, prehydrolyzed with 0.01% CGTase and the incubation continued with 0.40% CGTase; sample 2, starch 5%, treated with the CGTase as above; sample 3, starch 1%, treated with the CGTase as above; sample 4, starch 15%, prehydrolyzed with 0.01% CGTase; sample 5, starch 5%, prehydrolyzed with 0.01% CGTase; sample 6, starch 1%, prehydrolyzed with 0.01% CGTase.

RESULTS AND DISCUSSION

The methods were tested for their usability as CD assays in pure and supplemented CD standard solutions (Tables I and II) and in conversion mixtures consisting of acyclic

Table I. Measurement Ranges and Relative Sensitivities of the CD Assays

method	upper limit of linear assay, mM			rel senstivity (β-CD = 100)	
	α-CD	β-CD	γ-CD	α-CD	γ-CD
phenolphthalein	5.0	0.05	0.4	1	15
methylorange	0.15	0.25	0.25	214	86
p-nitrophenol	1.5	2.0	2.0	232	32
iodine	0.1	2.0		5000	
bromocresol green			0.1	10	10000
2-*p*-toluidinylnaphthalene-6-sulfonate	0.25	0.15	0.1	3	165
1-anilinonaphthalene-8-sulfonate	1.5	1.0	0.4	9	271
oxygraphy	0.5	0.3	0.4	60	57
hydrolysis of dimethylphenyl acetate	1.0	1.0		63	
glucoamylase–anthrone–H_2SO_4	6.4/ 0.7[a]	5.5/ 0.6[a]	4.8/ 0.5[a]	86[b]	114[b]
glucoamylase-takadiastase	6.4	5.5	4.8	86[b]	114[b]

[a] The former refers to the DNS assay and the latter to the anthrone–sulfuric acid test. [b] Theoretical values based on the number of glucose units in CD.

dextrins and a mixture of CD subforms (Table III). In light of these data none of the tested methods seem to be of general applicability in CD analytics; depending on the method used, significantly different results were obtained in the conversion mixtures due to differences in specificities of the methods for the CD subforms and in their susceptibility to interferences. When sample treatments, such as heating during the glucoamylase catalysis, are required, changes in the equilibrium composition may occur and be reflected in the recorded values. For these reasons guidelines for the choice of proper method according to the type of sample should be available. These and other aspects concerning the application of the CD assays are considered below in more detail.

The method based on complexation of phenolphthalein is easy to carry out, sensitive, and relatively specific to β-CD (Cramer, 1952). The original procedure (Vikmon, 1981) required minor modifications to work reproducibly: The assay pH (Landert et al., 1981; Lane and Pirt, 1973) is stabilized by 100 mM Na_2CO_3 instead of the previously described 4 mM solutions, the color intensity is measured either immediately after mixing of the reactants or at timed intervals due to spontaneous bleaching of the color, and the linear assay range is set up to 0.05 mM β-CD. Deviation from linearity above this concentration reflects incomplete complexation. This phenolphthalein assay is suited for the determination of β-CD in complex mixtures containing, e.g. ethanol, protein, and acyclic dextrins, bivalent cations, and even reducing agents (Table II). Instead, the detection limit of β-CD is high enough to exceed the interference threshold of many sample constituents.

Table II. Effect of Various Supplements to CD Standards on the Reproducibility of Various CD Assays

method	ratio of α-CD (β-CD) concentrations[a] in the presence of						
	starch syrup (15%)	albumin (1%)	ethanol (20%)	$(NH_4)_2SO_4$ (20%)	ascorbic acid (1%)	Mg^{2+} (5 mM)	starch (1%)
phenolphthalein	2 (1.0)	2 (1.0)	1.0 (1.0)	2 (2)	2 (1.4)	2 (1.4)	1.7
methylorange	1.0 (1.0)	1.2 (1.3)	0.9 (1.0)	0.8 (0.8)	0.9 (0.9)	0.6 (0.2)	
iodine	0.0 (0.0)	0.5 (0.0)	1.0 (1.0)	1.0 (0.7)	0.5 (0.0)	1.2 (0.0)	1.6
2-*p*-toluidinylnaphthalene-6-sulfonate	2 (2)	2 (2)	0.3 (1.0)	0.5 (0.8)	0.8 (1.0)	2 (1.3)	2
1-anilinonaphthalene-8-sulfonate	2 (2)	2 (2)	0.4 (1.2)	0.3 (0.9)	0.7 (1.0)	2 (1.0)	2
oxygraphy	1.0 (1.1)	0.8 (0.8)	1.1 (0.4)	(1.5)	2 (2)	2 (2)	1.0
glucoamylase-takadiastase	2 (2)	1.1 (0.7)	0.3 (1.1)	0.0 (0.0)	0.3 (0.8)	1.0 (0.8)	1.0

[a] Ratios of the observed analysis value to the calculated CD concentration.

Table III. Comparison of CD Assays in the Analysis of Conversion Mixtures Prepared by Hydrolyzing Various Amounts of Starch by CGTase from Alkalophilic *Bacillus sp.*

method	CD concentration,[a] mM					
	starch (15%)		starch (5%)		starch (1%)	
	I	II[b]	I	II[b]	I	II[b]
phenolphthalein	43.0 (42.0)	13.5 (16.0)	18.5 (17.0)	10.7 (10.8)	3.5 (4.0)	5.7 (6.8)
methylorange	55.0 (55.0)	16.0	24.0 (19.0)	12.6	1.7 (1.5)	6.7
p-nitrophenol	45.0		28.0		2.3	
iodine	700 (650)	80	200 (350)	50	250 (100)	20
bromocresol green	6.8	6.7	3.2	3.2	0.7	0.8
2-*p*-toluidinylnaphthalene-6-sulfonate	38.0 (36.0)	40.0 (44.0)	12.0 (11.0)	19.0 (20.0)	1.3 (1.5)	5.8 (6.5)
1-anilinonaphthalene-8-sulfonate	61.0 (64.0)	52.0 (55.0)	17.0 (18.0)	27.0 (32.0)	2.7 (3.3)	9.3 (11.0)
oxygraphy	31.0		9.8		0.8	
glucoamylase–anthrone–H_2SO_4	30.0	12.9	10.0	10.6	1.3	3.6
glucoamylase-takadiastase	31.0 (29.0)	14.1	11.1 (11.0)	11.1	2.1 (1.3)	3.4

[a] Based on β-CD standard or internal β-CD standardization (in parentheses). The relative amounts of CD subforms are shown in Methods. [b] Only the prehydrolysis was performed.

The phenolphthalein method is suited for starch digests since the dye poorly complexes with high molecular weight dextrins as tested by separate size fractionations on Biogel P6.

The complexation of methylorange to CDs is characterized by a change of color at neutral pH, and therefore it has been used for the assays of CGTase activities (Landert et al., 1981). However, the dye is equally applicable to assays of CD concentrations. Then, $CaCl_2$ required by the enzyme can be omitted. The analyses are slightly weighted to β-CD (Table I), and by doubling the concentration of methylorange from that of the original paper (Landert et al., 1981) an about twofold assay range and sensitivity was obtained. The color is stable for several hours, evidently contributing to the high reproducibility of the analyses. The absorbance changes at 546 nm are however quite small within the linear measurement range (maximally 0.06 for β-CD and 0.140 for α-CD). It has been proposed that the changes would be larger at a lower pH region (Szejtli et al., 1978). Methylorange offers a relatively interference-free CD analysis. It may tend to form complexes with proteins (Table II) and so overestimate CD concentrations as suggested also by the slightly above-mean values in the enzyme digests. On the other hand, standardization with β-CD also leads to overestimations in the case of methods weighted to α-CD.

The incorporation of p-nitrophenolate in CDs produces an about 15-nm blue shift in the yellow color of the uncomplexed dye (Cramer et al., 1967). When this charge is monitored at 425 nm, a relatively large assay range is obtained (Table I). The respective absorbance changes do not however exceed 0.11 and 0.06 for α- and β-CD, complicating the analysis of dilute CDsamples. With respect to specificity for different subforms and stability of the color, the method resembles that based on methylorange. However, the susceptibility of the two methods to interference may often be different due to the smaller molecular weight of *p*-nitrophenolate, and in this light special applications for the *p*-nitrophenolate-based method could be found.

The iodine method is a derivative of the CGTase assay of Bender (1981). The increase in absorbance is measured at 290 nm when molecular iodine is imbibed into the cavity of CD. In the present study iodine bound most selectively to α-CD while only marginal interaction with β-CD and none with γ-CD occurred (Table I). The high specificity and sensitivity for α-CD is counteracted by a narrow measuring range. The aqueous iodine reagent is prepared by using DMSO. As dilute solutions, iodine tends to be bleached spontaneously and so controlled incubation times are required. Due to the low measuring wavelength (290 nm) high background absorbances are expected in many instances. Tables II and III show that starch and its hydrolysates interfere with the assay; the former increases while the hydrolysates tend to decrease the intensity from those of pure α-CD solutions. High concentrations of protein, reducing agents, and bivalent cations are also typical sources of interference (Table II).

The original CD assay based on TNS was described for 2–7 mM concentrations (Kondo et al., 1976a). The measurements are then however significantly beyond the linear measurement range (Table I), and there appears an inflection point at about 1 mM CD concentration, reflecting a change in the complexation mechanism or fluorescence self-absorption of TNS. However, within the range indicated in Table I pure CD solutions can be assayed reproducibly down to 1 μM concentrations. In spite of sensitivity, the method is obviously of limited practical use since it is subjected to interferences both in the complexation step and in the fluorescence process (e.g., turbidity), or in both.

The fluorometric measurements based on ANS were carried out at an excitation wavelength of 410 nm instead of the reported 360 nm (Kondo et al., 1976a) since there is a minimum at this point. The assay ranges for each of the subforms are broader than with TNS (Table I). An inflection point (between 2 and 5 mM) is observed also with ANS. The ratio of emission intensities with the TNS and ANS methods is about 30. In contrast to earlier suggestions (Kondo et al., 1976a,b) the relative specificities of these fluorometric methods were found practically equal for the CD subforms. In spite of the extended measurement range and poorer sensitivity, the ANS method is equally prone to interferences as the TNS method (Table II) and requires preliminary removal of e.g. starch and protein.

The polarographic method (Laakso et al., 1984) determines all the CD forms with almost equal sensitivity (Table I). The assay range is up to 0.5 mM (β-CD). The relatively poor sensitivity is compensated for by the possibility to adjust and compromise the reaction conditions according to the needs with respect to analysis time and sensitivity. If, for example, the oxygenation rate is lowered by reducing the concentration of linoleate (tracer) and the enzyme, the sensitivity toward CD is correspondingly improved at the expense of prolonged analysis time. The method is rather unaffected by starch digests and protein. However, reducing agents and bivalent cations (Table II) either react with linoleic acid or interfere with the oxygenation mechanism itself. The below average values obtained in starch digests cannot be attributed to interference by acyclic oligosaccharides (Table III) or interconversion of the CD subforms but rather suggest spontaneous conversion of cyclic to acyclic dextrins under the conditions of the assay. The polarographic monitoring system does not require preliminary clarification steps, and therefore turbid samples are a particular application area (Laakso et al., 1984).

The above methods based on chlathrate formation were all found sufficiently simple to carry out, and they require only standard laboratory equipments. The sensitivities vary considerably due to differences in the equilibrium constants, stoichiometry of complexation, and magnitude of change of the measured parameter. For example, acid–base indicators have, at a proper pH, different p*K*s in complexed and free states (e.g., phenolphthalein), and the respective change in the absorption wavelength is therefore normally large, i.e. the method is sensitive. If, instead, the electronic distribution of the tracer is affected by complexation, a relatively small spectral shift is observed and the sensitivity is lower but the color is more stable and less susceptible to changes in the reaction conditions.

The complexation methods show a good correlation with the HPLC analysis when used to properly supplement each others. Combined use of methods based on methylorange, phenolphthalein, and bromocresol green appears especially useful: Assays of conversion mixtures with bromocresol green give directly the concentration of γ-CD, and the value for β-CD is then obtained by reducing 0.15 times the concentration of γ-CD from the apparent result of the phenolphthalein method. According to Table I only about 1% of α-CD is determined by phenolphthalein and can be ignored. If the values of γ- and β-CD are both reduced from that given by the methylorange assay and this difference divided by a coefficient of 2.14 resulting from the

usage of β-CD standards in Table I, the concentration of α-CD is obtained. Corresponding combinations can be based on any of the complexation methods. However, the advantage of the given combination lies in the fact that it can be used without the aid of computers.

The measurement of CDs according to the rate of cleavage of phenyl acetates provides a simple kinetic assay. The method requires strict control of reaction temperature and timing even if carried out as a fixed-time assay. Base-catalytic "background" hydrolysis occurs and, in order to maintain it tolerable, substrate concentrations below 0.15 mM should be used. Under carefully controlled conditions the method works well but does not offer clear advantages with respect to sensitivity, relative specificity (Table I), or susceptibility to interferences (Tables II and III).

The assay of CDs by the two-stage sugar analysis is based on the inability of glucoamylase to cleave the cyclic dextrins, and, as such, the method is suited for starch digests. The modified Somogyi-Nelson (Hiromi et al., 1963) or phenol–sulfuric acid methods (Dudois et al., 1956) can be used in place of the DNS method for the determination of reducing sugars in the enzyme digests. The requirement for two independent sugar analyses complicates the overall assay: The measurement range of total sugars is about 10-fold below that of reducing sugars necessitating two different dilutions of the sample, and the less sensitive step determines the overall sensitivity. Due to the multistage nature of the assay, a broad spectrum of interfering factors is likely to exist. Strict precision is required in the analyses since CD concentration of the digests may be small in comparison to the overall carbohydrate content; a reliable minimum conversion percent in the CGTase digests was 5. Monosaccharides other than glucose are natural sources of errors. Recovery tests should precede each glucoamylase treatment since the cleavage of β-CD also occurs in proportion to the enzyme concentration. In this study the concentration of glucoamylase (0.5 mg/mL) was adjusted to allow 1–2% decomposition of β-CD while total conversion of the acyclic dextrins to glucose was achieved. As can be judged from the ring size, γ-CD is totally lost while α-CD remains unattacked (French, 1965). The linear measuring ranges of CDs in Table I are those of the respective sugar analyses of the method. Thus, the values are valid for pure CD solutions and/or for solutions where the total carbohydrate content is about 10 and 0.6 mg/mL for the DNS and anthrone–H_2SO_4 tests, respectively. The relative sensitivities of the method (Table I) were calculated on a molar basis from the molecular weights of CDs. The values for γ-CD can be calculated only in the case of pure standard solutions. Enzymic decomposition of γ-CD may also explain the slightly lower CD contents in comparison to the other methods in Table III.

The need for two separate analyses and the inconvenience encountered in the use of the anthrone–H_2SO_4 reagent led us to develop an enzymic modification where the sample is divided into two portions, one of which is treated with glucoamylase and the other with a mixture of glucoamylase and takadiastase. Glucose formed during these two parallel treatments is measured with DNS. The linear assay ranges in Table I are presented on the same basis as with the glucoamylase-anthrone method. In practical situations similar or slightly higher values are obtained by this two-enzyme method. This modification might have use in glucose analyses by enzyme electrodes.

ACKNOWLEDGMENT

This work was supported in part by the Biotekniikan ja Käymisteollisuuden Tutkimussäätiö and the Finnish Academy of Sciences.

Registry No. I_2, 7553-56-2; α-cyclodextrin, 10016-20-3; β-cyclodextrin, 7585-39-9; γ-cyclodextrin, 17465-86-0; phenolphthalein, 77-09-8; methyl orange, 547-58-0; p-nitrophenol, 100-02-7; bromocresol green, 76-60-8; 2-p-toluidinylnaphthalene-6-sulfonate, 7724-15-4; 1-anilinonaphthalene-8-sulfonate, 82-76-8; dimethylphenyl acetate, 28675-18-5; glucoamylase, 9032-08-0; anthrone, 90-44-8; takadiastase, 9001-19-8.

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Received for review December 12, 1986. Accepted August 25, 1987.

Aflatoxin Contamination in Dried Figs: Distribution and Association with Fluorescence

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The aflatoxin distribution was studied in a naturally contaminated batch of figs by analyzing more than 400 single figs and samples totalling over 90 kg. The situation closely resembles the pattern described for peanuts: only a very small number of figs were contaminated; the levels of contamination however were very high in individual fruits. The degree of contamination was estimated to be ca. 1 in 100. Evidence is presented in detail that bright greenish yellow (BGY) fluorescence under UV light (365 nm) is strongly correlated with the occurrence of aflatoxin contamination in a qualitative (but not in a quantitative) way. Removal of all BGY-fluorescent figs from a 56-kg sample effectively lowered the original contamination level from 22.6 to 0.3 ppb aflatoxin B_1. The sorting procedure is simple and fast and may easily be used to efficiently "clean" large batches of dried figs on an industrial scale before retail distribution. Microbiological investigations revealed the presence of aflatoxin-producing strains of *Aspergillus flavus* and *Aspergillus parasiticus*.

In 1985 the laboratory of a food retail chain reported that dried figs, imported from Turkey, were partly contaminated with aflatoxins. Various food-control laboratories in Switzerland subsequently increased their activities in analyzing figs for aflatoxin and reported the detection of tens of ppb's in samples (homogenates) of 2–5 kg. Single figs were found to contain up to 5 mg of aflatoxin B_1/kg (unpublished reports, Kantonales Laboratorium of Basle). On the basis of these findings, the Federal Office of Public Health set the legal limit for aflatoxins on figs to the same levels as it is for nuts; i.e. 1 ng/g of AFB_1 and 5 ng/g for the sum of toxins B_1, B_2, G_1, and G_2.

The occurrence of aflatoxin in figs was observed several years ago (*Food Chem. News*, 1974), and aflatoxin production during ripening was studied by Buchanan et al. (1975). Other carbohydrate-rich fruit, such as pineapple and cooked apricot, were recognized as good substrates for aflatoxin production by Morton et al. (1979). Figs had not been recognized as a high-risk commodity in Switzerland, and the distribution of aflatoxin contamination in figs does not seem to be known. The existing sampling plans (Campbell et al., 1986) apparently have been derived by extrapolating from the situation found in peanuts.

With contaminated figs of uniform origin, we studied the distribution of aflatoxin within the batch, the association of aflatoxin with toxin-producing *Aspergillus* species, and the association of bright greenish yellow (BGY) fluorescence with aflatoxin occurrence on individual figs.

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MATERIALS AND METHODS

Figs. The average weight of dried Turkish figs (1985 crop) used for this work was 22.3 g (CV = 17.2%; n = 189). This weight was used to calculate the number of figs in the analyzed samples.

Aflatoxin Standard Solution. Aflatoxins B_1, G_1, B_2, and G_2 were obtained from Sigma Chemical Co. A solution in toluene/acetonitrile (98:2), containing 0.5 mg/L of each toxin, was prepared and assayed according to AOAC Method 26.009 (Horwitz, 1980).

Detection of BGY Fluorescence. A Blak Ray long-wave ultraviolet lamp (Model B-100 A; 360 nm) was used. During the sorting and inspecting of figs, UV-protective spectacles, polyethylene gloves, and dust-protective facial masks were worn.

Color Slides. These were taken on Fujichrome 400 ASA with a Minolta XE-1 camera (lens 1:3.5, f = 100 mm).

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