Colorimetric Assay of Alditols in Complex Biological Samples

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A colorimetric assay for the determination of alditols in complex extracts from biological sources, showing negligible interferences by other carbohydrates, has been worked out. The method involves mild oxidation of alditols by sodium periodate under acidic conditions, followed by reaction of the resulting formaldehyde with ammonia and acetylacetone (Hantzsch reaction) to yield a colored heterocycle amenable to spectrophotometric measurement. The assay is completed within a few minutes, and mannitol and other alditols can be detected down to the nanomolar range. The method is convenient in that it uses inexpensive chemicals of low potential hazard and requires only few minutes to complete. The method has been used for the determination of mannitol in aqueous extracts from olive leaves and fruits and has been validated by parallel determinations carried out by HPLC and enzymatic analysis with mannitol dehydrogenase.

Keywords: *Alditol determination; mannitol; Hantzsch reaction; olive*

INTRODUCTION

Alditols (sugar alcohols) constitute a group of compounds closely related to sugars and widely distributed in microorganisms, fungi, animals, and plants. In the plant kingdom, alditols with three to seven carbon atoms have been described in many different species ranging from algae to higher plants (Bieleski, 1982). Among these, hexitols are the most common, with mannitol being the most abundant sugar alcohol in nature. It has been found in over 100 different species of higher plants, in which it plays a role as translocatable assimilate together with sucrose. In addition, mannitol has been found to accumulate, in those plants capable of synthesizing it, in response to osmotic stress (Stoop et al., 1996).

A large number of chromatographic procedures have been developed to quantify mannitol in biological samples (Bieleski, 1982; Beck and Hopf, 1990). In particular, isocratic high-performance liquid chromatography (HPLC), with refractivity detection, developed for the analysis of carbohydrates, is commonly used for quantitation of alditols. This protocol, however, has the disadvantage of requiring previous fractionation of the sample, making it time-consuming and hence not suitable for batch measurement of alditols in biological materials. Mannitol can also be determined by enzymatic analysis (Horikoshi, 1984) using mannitol dehydrogenase (EC 1.1.1.67), which is now commercially available. This assay looks straightforward, but in practice has drawbacks resulting from the unfavorable equilibrium of the reaction and low affinity of the enzyme for mannitol ($K_m = 50$ mM), which requires the use of extreme pH, a large amount of enzyme, and addition of suitable reagents to trap the fructose produced by oxidation of mannitol.

Several chemical methods for the estimation of alditols have been developed. They are based on the use of glycol-splitting reagents, such as sodium periodate. An α-glycol group, which is composed of one primary and one secondary alcohol, is oxidized by sodium periodate, yielding formaldehyde. On the other hand, a secondary alcohol situated in the middle of a polyalcohol molecule is oxidized to formic acid. This property was exploited some 50 years ago by Cameron et al. (1948) to determine the mannitol content of seaweeds by estimation of the periodate consumed. Because the periodate oxidation of alditols is very rapid, errors due to oxidation of other substances present in the material were found to be negligible. Further developments of the chemical determination of mannitol, and other polyalcohols, involved the colorimetric determination of the formaldehyde formed upon oxidation with periodate. Thus, a method was developed for the determination of glycerol, and other polyalcohols, based on the reaction of formaldehyde with chromotropic acid (Burton, 1957). In our hands, however, this method has been found to be affected by interferences of other carbohydrates, which precludes its use in the determination of alditols in complex mixtures with sugars.

Another method for the estimation of formaldehyde is based on the Hantzsch reaction, i.e. the reaction of an aldehyde, an ammonia (or an amine), and a 1,3-dicarbonyl compound to yield a pyridine derivative (Sausins and Buburs, 1988). Thus, the reaction of formaldehyde with acetylacetone and ammonia results in the formation of a pyridine heterocycle with strong absorbance at 412 nm. This reaction has been exploited for the enzymatic determination of cholesterol by means of a test combination (Boehringer-Mannheim Biochemicals Catalog, 1995). The same strategy—periodate oxidation followed by Hantzsch reaction—has been used for the photometric detection of alditols after separation by ion-exchange chromatography (Samuelson and Strömberg, 1966) or HPLC (Honda et al., 1983). This post-column derivatization replaces the sometimes troublesome refractivity detection used for carbohydrate analysis. Under the conditions described in the latter report we found that the method was not specific for mannitol, with fructose giving as much as 50% of the colour developed by mannitol at the same concentration.

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Thus, assay conditions have been worked out to reduce the interference by carbohydrates normally present in complex biological extracts.

MATERIALS AND METHODS

Chemicals. Alditols and carbohydrates used as standards, as well as mannitol dehydrogenase from *Actinobacillus* sp., were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Preparation of Carbohydrate Extracts. Mature leaves from olive (*Olea europaea*) and spinach (*Spinacia oleracea*) were thoroughly rinsed with water, blotted on tissue paper, and ground to a powder in liquid nitrogen. The resulting powder was lyophilized overnight and the lyophilate stored at −25 °C until used. Similarly, developing olive fruits were broken in liquid nitrogen, the endocarp was removed, and the pulp tissue was ground to a powder, lyophilized, and stored. To extract carbohydrates, the lyophilized powder (~50 mg) was placed in a capped 1.5-mL microfuge tube (Eppendorf type) and 1 mL of hot (80 °C) distilled water was added. The tube was heated at 80 °C for 30 min and then cooled and centrifuged at 12000*g* for 15 min twice. Plant extracts were deionized by passage through cationic and anionic resins (Redgwell, 1980). To monitor quantitative recovery of sugars, a suitable amount of radiolabeled glucose was added to the extract prior to deionization through ion-exchange resins.

Colorimetric Assay of Mannitol. The biological extract (or standard solution), containing from 5 to 150 nmol of mannitol in 0.1 mL of water was dispensed in a capped 1.5-mL microfuge tube, to which 0.5 mL of 0.5 M formate, pH 3.0, was added. To this solution was added 0.3 mL of 5 mM sodium periodate (reagent 1), and the tube was closed, vortexed, and left at room temperature for up to 15 s upon addition of the periodate solution. Then 0.3 mL of a solution consisting of 0.1 M acetylacetone, 2 M ammonium acetate, and 0.02 M sodium thiosulfate (reagent 2), was added. The tube was closed and heated in boiling water for 2 min and cooled under running tap water, and, after temperature equilibration, the absorbance at 412 nm was measured. The use of lid bridles, or alternatively screw-capped tubes, is necessary to avoid opening of the tubes during heating.

Chromatographic Analysis of Sugars. The sugar fraction was subsequently analyzed by HPLC on Supelcosil LC-NH_2 (Supelco, Bellefonte, PA) using water/acetonitrile (85:15) as the mobile phase with refractivity detection.

Enzymatic Analysis of Mannitol. Mannitol in olive extracts was determined by enzymatic analysis with mannitol dehydrogenase in the presence of 0.1 M glycine, pH 9.5, 5 mM NAD, and mannitol dehydrogenase (0.1 unit/mL). The reaction was started by addition of the enzyme and was carried out at 30 °C. Absorbance at 334 nm was monitored in a dual-wavelength spectrophotometer (Sigma ZFP-22).

RESULTS AND DISCUSSION

Oxidation Reaction. Alditols are rapidly oxidized by periodate under mild conditions, but more drastic conditions result in the oxidation of other carbohydrates, thus decreasing specificity of the alditol determination in sugar samples. Preliminary attempts to determine mannitol were performed by following the assay conditions described by Honda et al. (1983) for the postcolumn derivatization of alditols. Thus, 0.6 mL of standard solution, either in water or in borate buffer, pH 8.0, was mixed with 0.3 mL of 50 mM sodium periodate; after vortexing, the reaction mixture was allowed to stand at room temperature for 2 min, and then 0.3 mL of a solution consisting of 15% ammonium acetate, 2% acetylacetone, and 5% sodium thiosulfate was added. The mixture was heated at 100 °C for 2 min, which resulted in the formation of a yellow color the intensity of which, assessed by measuring absorbance at 412 nm, was linearly dependent on the concentration of mannitol in the range from 5 to 150 nmol. The molar absorption coefficient for mannitol at 412 nm was calculated to be 8500 M^{-1} cm^{-1} (results not shown). Under these conditions, however, some carbohydrates (sucrose, glucose, galactose, and fructose) were found to give positive reaction. Among carbohydrates, fructose was found to be particularly susceptible to oxidation by periodate, yielding as much as half the color developed by mannitol at the same concentration. Since fructose is normally present in extracts from biological tissues, the reaction conditions for the oxidation step were thoroughly investigated to reduce interference by this ketose. Thus, the effects of reaction temperature and time, concentration of sodium periodate, and pH on the response of fructose relative to mannitol were examined. Decreasing the reaction temperature to 0−5 °C did not result in any great reduction of the response of fructose, but shortening the oxidation time from 2 min to 15 s resulted in a 2-fold decrease in the response of fructose relative to mannitol.

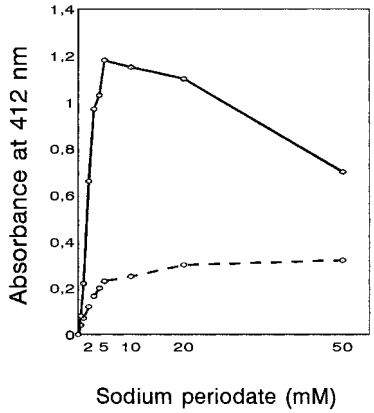

Figure 1. Effect of sodium periodate concentration on the color reaction yielded by mannitol (solid line) and fructose (dashed line). In both cases 100 nmol of analyte in 0.6 mL of water was mixed with 0.3 mL of sodium periodate solution of the stated concentration; after 2 min at room temperature, 0.3 mL of reagent 2 was added and the mixture heated at 100 °C for 2 min.

The concentration of sodium periodate was found to exert a differential effect on the oxidation of mannitol and fructose. As depicted in Figure 1, the color formation elicited by mannitol increased with the concentration of periodate anion up to 5 mM, whereas 50 mM periodate resulted in a decrease in the color formed, an effect likely to be due to bleaching of the dye generated through the Hantzsch reaction (see below). With fructose, on the other hand, the color formed increased with the concentration of sodium periodate, thus resulting in a higher interference by this carbohydrate. From these data the concentration of the sodium periodate solution (reagent 1) used in the oxidation reaction was fixed at 5 mM.

The pH of the oxidation reaction was found to be critical for the relative response of mannitol and fructose. As shown in Figure 2, the color developed by mannitol had a peak at pH 4.0, whereas that yielded by fructose increased with the pH from 2.0 to 7.0. The mannitol to fructose ratio of color development was found to peak at pH 2.5, but since the pK_a of formic acid is 3.7, and the reaction was strongly affected by pH, a pH of 3.0, which is within the buffering range of formate, was selected as the optimum.

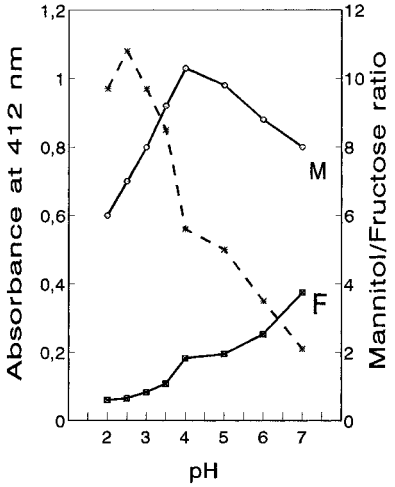

Figure 2. Effect of the pH of the oxidation reaction on the color developed by mannitol (M) and fructose (F). The mannitol to fructose ratio of color development is depicted by the dashed line. The reaction medium contained 100 nmol of analyte in 0.1 mL of water plus 0.5 mL of 0.1 M buffer of the stated pH (formate for pH 2−3.5, citrate for pH 4 and 5, and phosphate for pH 6 and 7).

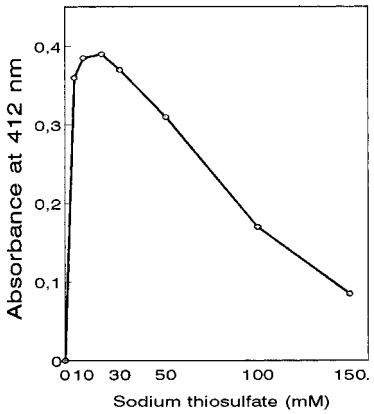

Figure 3. Effect of sodium thiosulfate on the color reaction elicited by formaldehyde, which formed upon periodate oxidation of 50 nmol of mannitol.

Color Reaction. As mentioned before, the Hantzsch reaction involves an aldehyde, an ammonia, and a 1,3-dicarbonyl compound as the reactants (Sausins and Duburs, 1988). Thus, formaldehyde produced upon oxidation with periodate anion was reacted with ammonium acetate and acetylacetone; the excess of periodate remaining from the oxidation reaction, which completely prevents the formation of color, was removed by including sodium thiosulfate in reaction mixture. The effect of these three components of the reagent solution on the formation of color was systematically investigated. (In all cases mannitol in 0.6 mL of 0.4 M formate, pH 3.0, was oxidized with 0.3 mL of 5 mM sodium periodate for 15 s at room temperature.)

The formation of color from mannitol upon periodate oxidation was absolutely dependent on the presence of sodium thiosulfate in the reaction medium (Figure 3), and its optimal concentration in the reagent solution was found to be 20 mM, with higher concentrations resulting in a sharp decrease in the color formed, which agrees with results reported prevously (Honda et al., 1983). The effect of the concentrations of ammonium acetate and acetylacetone in the reagent solution was also examined; ammonium acetate was found to increase color formation up to 2 M concentration, whereas acetylacetone reached optimal concentration at 0.1 M (results not shown).

Table 1. Specificity of the Assay

analyte	rel response	analyte	rel response
mannitol	100	fructose	11
arabitol	90	galactose	4
erythritol	87	mannose	1
glycerol	75	glucose	0
sorbitol	73	*myo*-inositol	0
ribitol	56	ascorbic acid	0
mannitol 1-phosphate	38	sucrose	0
glycerol 3-phosphate	10	raffinose	0

The formation of color was completed in 1 min at 100 °C and bleaching began after 3 min at that temperature. Thus, 2 min in boiling water was chosen as the optimal condition for the color reaction. On the other hand, changing the pH of the reaction medium from 2.0 to 7.0 had only a minor effect in the development of color (results not shown). The color formed should be measured within 1 h, since it fades with a half-life of ~8 h.

Sensitivity and Specificity of the Assay. Under the conditions described under Material and Methods the assay was linear with mannitol concentration from 10 to 120 nmol, and the molar absorption coefficient was calculated to be 12 000 M^{-1} cm^{-1}, thus allowing the estimation of mannitol down to the nanomolar range.

A number of carbohydrates and polyalcohols have been tested for their response to the method. The results (Table 1) show that the assay is highly specific for alditols and, among those tested, mannitol gave the highest response. The difference in relative response between different alditols is likely to be due to differences in their stereochemistries, since the mechanism of periodate oxidation involves the formation of an intermediate cyclic complex of the glycol and periodate. Moreover, phosphorylation of a primary alcohol, as in mannitol 1-phosphate and glycerol 3-phosphate, resulted in a lower molar response. The absence of reaction by *myo*-inositol is not surprising because formaldehyde is formed upon oxidation of primary alcohols only, and these are absent in cyclic polyalcohols. Ascorbic acid, which also possesses the glycol structure consisting of a primary and a secondary alcohol, also yielded negative reaction to the assay.

Of all the carbohydrates tested, only fructose gave significant color development under the assay conditions, which precludes its application to extracts containing high amounts of this ketose.

Validation of the Method. Olive, like other members of the Oleaceae, is capable of forming and translocating mannitol (Bieleski, 1982). Aqueous extracts from olive leaves and fruits, after deionization (see Material and Methods), were submitted to mannitol determination by enzymatic analysis, HPLC, and the proposed colorimetric assay. As shown in Table 2 the colorimetric assay yielded values of mannitol content in full agreement with those obtained by the other two methods, and also in accordance with the available reported values of mannitol content in olive leaves (Drossopoulos and Niavis, 1988; Flora and Madore, 1993; Tattini et al., 1996). On the other hand, spinach, a species that does not form alditols, was used as a negative control and was found not to give any response when deionized leaf extracts were assayed for alditols by using the colorimetric assay, indicating that the method can be used in complex mixtures of carbohy-

Table 2. Validation of the Colorimetric Assay for Mannitol[a]

extract	mannitol content (mg/g of dry wt)		
	enzymatic analysis	HPLC	colorimetric assay
olive leaf	31.3 ± 1.9	32.1 ± 1.1	29.8 ± 0.4
olive fruit	50.0 ± 2.1	50.9 ± 0.9	51.6 ± 1.0
spinach leaf	not measured	0	0

[a] Results are average of three determinations (± standard deviation) carried out on deionized extracts.

drates from biological sources. In addition, the results obtained by using the colorimetric assay on crude extracts of olive leaves were equivalent to those shown in Table 2, which were obtained on deionized extracts. As a result, the colorimetric assay has been used in our laboratory for routine determination of mannitol content in crude, nonfractionated extracts from olive leaves and repeatedly validated by parallel determinations by both HPLC and enzymatic analysis.

Therefore, the proposed colorimetric assay has clear advantages over the other two alternative methods used here. First of all, it is highly specific for alditols (Table 1) and hence can be performed on crude, nonpurified extracts, thus resulting in considerable savings of labor and time. Moreover, the assay itself is completed within a few minutes. Second, it is convenient in using low-hazard and inexpensive reagents and requiring only easily available equipment. Finally, the method is sensitive and highly reproducible. All of these characteristics make this method suitable for batch measurements of alditols in biological tissues, as well as to monitor alditol formation in enzymatic reactions.

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