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High-performance liquid chromatography of alditols with indirect photometric detection

Anne-Marie Dona*, Jean-François Verchère

Unité de Recherche Associée 500 du C.N.R.S., Université de Rouen, Faculté des Sciences, 76821 Mont-Saint-Aignan, France

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Abstract

Alditols were determined in aqueous solution using a novel indirect photometric technique as a post-column detection method. It allowed the use of the common UV detector for these transparent analytes. The method is based on the decrease of absorbance ($\lambda = 347$ nm) due to the competitive complexation of molybdate by the chloranilate ion (coloured complex) and an alditol (colourless complex). The experimental conditions were defined in a preliminary study which related the sensitivity for each analyte to the formation constant of its molybdate complex. D-Glucose, which forms a weak complex, does not interfere. The method was applied to the HPLC determination of mixtures of alditols with separation on a calcium column. The calibration curves are linear in the concentration range 0–100 $mg \cdot l^{-1}$. It demonstrates that indirect photometric detection may be used for the selective determination of specified analytes, in contrast with its usual application as a universal detection technique.

1. Introduction

Alditols are polyols obtained by reduction of the corresponding aldoses and ketoses. Their general formula is $HOCH_2-(CHOH)_{n-2}-CH_2OH$. Important industrial alditols are those with $n = 5$ or 6, namely xylitol, D-mannitol and D-glucitol that are used as additives and anti-crystallising agents in the food industry, especially in "sugar-free" products [1]. Mixtures of D-mannitol and D-glucitol (known as sorbitol) are manufactured by hydrolysis of sucrose to D-glucose and D-fructose followed by subsequent hydrogenation.

The determination of alditols in foodstuffs containing natural aldoses is requested for control of food quality. Mixtures of carbohydrates in aqueous solutions are easily separated by liquid chromatography [2] using anion-exchange resins in OH form or cation-exchange resins in Ca^{2+} [3,4], Pb^{2+} or Ag^{+} [5] form. Grafted amino or amino-propyl columns [6] may also be used. Such separations are often performed in borate buffer, since borate ions form complexes of different stabilities, depending on the configuration of the alditols [7]. In every case, the detection of carbohydrates presents a real problem, since classical techniques like refractive index or UV absorbance measurements below 200 nm are of low selectivity [8].

Conductimetric detection has been employed for chromatography in borate medium, because the borate complexes of alditols are strongly

* Corresponding author.

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acidic [9,10]. Pulsed amperometric detection [11] is a general method for all carbohydrates which requires an expensive apparatus. Whereas UV–visible photometric detection is not suitable for the direct determination of carbohydrates, it may be used after derivatization [8]. An interesting post-column derivatization method is based on the fast formation of a ternary Cu^{2+}–NH_3–carbohydrate complex [12]. In this work, we attempted to approach the problem of photometric detection in the indirect way [13,14] by provoking the "bleaching" of a coloured complex in response to the presence of an alditol. Such a process involves competitive complexation of a metal ion by an auxiliary ligand (forming an absorbing compound) and by an alditol forming a stronger, non-absorbing, complex.

Molybdenum (VI) was chosen as the complex-forming metal because the MoO_4^{2-} molybdate ion forms stable, colourless anionic complexes with carbohydrates L [15–19] in acidic solution. These species are dinuclear, (Mo:L = 2) and their structures have been determined by NMR studies [20–22] and crystal structure determinations [23,24]. Their stabilities are strongly dependent on the relative configurations of the hydroxyl groups of the ligand [22,25–27], alditols forming more stable complexes than aldoses [28,29].

Chloranilate (the ion of 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone, H_2C) was selected as the complex-forming auxiliary ligand, because this delocalized ion (Fig. 1a and 1b) possesses high molar absorbances in the UV and the visible spectrum, a high chemical stability, and an appreciable solubility in water. These properties are associated with interesting analytical potentialities that have received numerous applications [30], including indirect spectrophotometry [31]. The chloranilate ion C^{2-} is known to form a highly coloured 1:1 complex with molybdenum (VI) [32–34] (Fig. 1c). Finally, in the pH range 4–5, the molybdate–chloranilate complex was weaker than molybdate–alditol complexes (Fig. 1d), making the ligand-exchange process thermodynamically favorable.

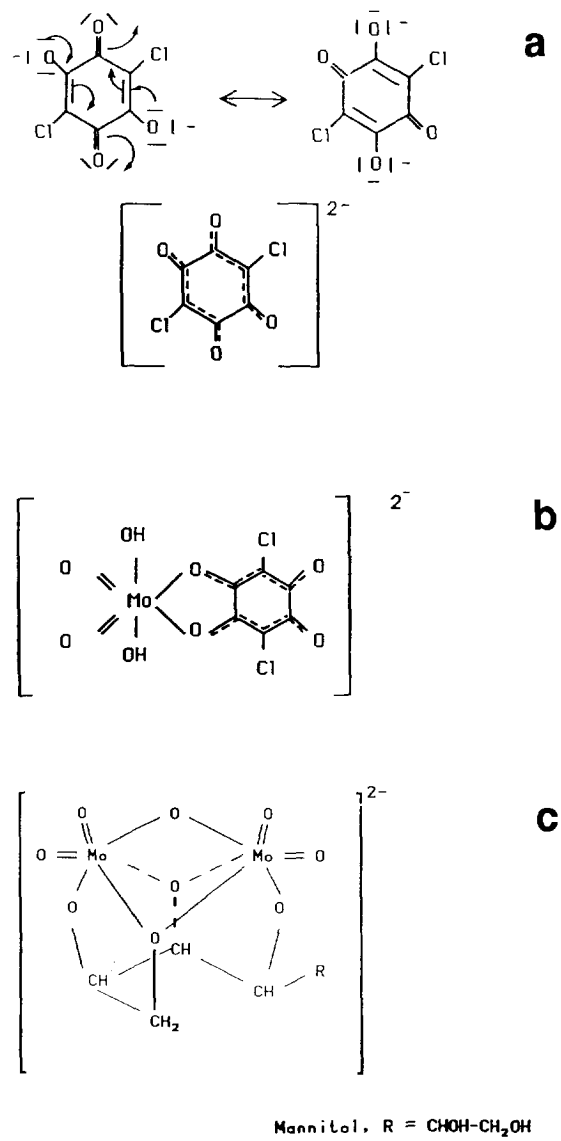

Fig. 1. Structures of (a) the chloranilate ion, (b) the (1,1,2) molybdate–chloranilate complex and (c) the (2,1,2) dimolybdate–mannitol complex. R = CHOH–CH_2OH.

2. Theory: principle of the indirect photometric detection of alditols

The analytical reaction is based on the competitive complexation of molybdate by chlorani-

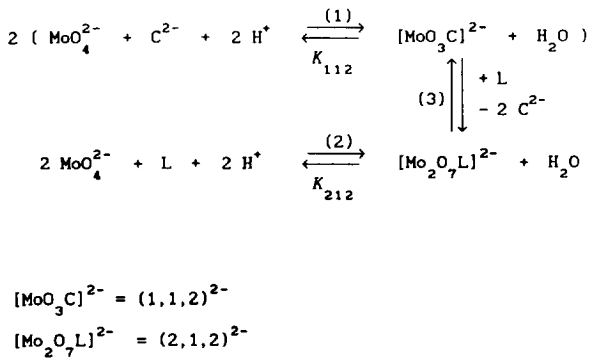

Fig. 2. Scheme of the competitive complexation of the molybdate ion by the chloranilate ion (C^{2-}, reaction 1) and an alditol (L, reaction 2). The overall analytical reaction is the ligand-exchange process of reaction 3.

late and an alditol as shown in Fig. 2. If the concentrations and pH are adjusted so that the alditol complex is more stable than the sacrificial chloranilate complex, the overall reaction is completely shifted to the formation of the alditol complex [reaction (3) in Fig. 2]. The optimal conditions of formation of the molybdate–chloranilate complex were determined in the following way. In order to use cells with optical path length $l = 1$ mm, the concentration of chloranilate was fixed at $5 \cdot 10^{-4}$ M. The ratio of concentrations $[MoO_4^{2-}]/[C^{2-}] = 2$ was chosen in order to increase the initial proportion of the sacrificial complex. A comparison of the curves of variation of absorbance versus pH for the molybdate–chloranilate complex, before and after partial decomposition by a model alditol (D-mannitol was used), showed that the maximum decrease of absorbance (at $\lambda = 347$ nm) occurred near pH 4.7. As this value is close to the pK of acetic acid, we eventually decided to use a 1:1 acetate buffer of pH 4.60.

In an alditol-free solution, the formation of the (1,1,2) molybdate–chloranilate complex may be written:

$$MoO_4^{2-} + C^{2-} + 2H^+ \rightleftarrows (1,1,2)^{2-} \qquad (1)$$

In the presence of an alditol L, equilibrium (1) is shifted backwards, in response to the formation of the (2,1,2) molybdate–alditol complex:

$$2MoO_4^{2-} + L + 2H^+ \rightleftarrows (2,1,2)^{2-} + 3H_2O \qquad (2)$$

The equilibrium constant for reaction (2) is represented by K_{212} and defines the formation constant [22] of the complex.

According to overall reaction (3) in Fig. 2, when an alditol was added to a solution of the molybdate–chloranilate complex, a decrease of absorbance at 347 nm and an increase of absorbance at 318 nm were simultaneously observed (Fig. 3). The variations were due to the dissociation of the (1,1,2) molybdate–chlorani-

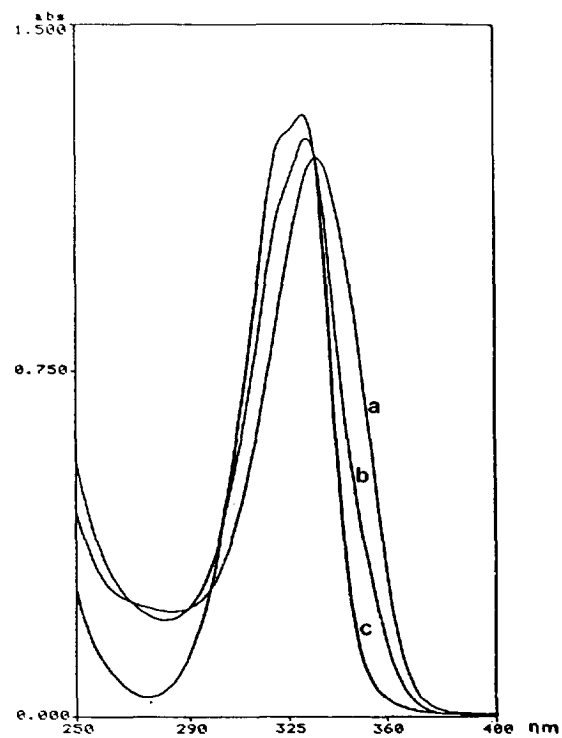

Fig. 3. UV Spectra of (a) the initial molybdate–chloranilate complex, (c) the final mixture of free chloranilate and alditol complex and (b) an intermediate stage with ca. 50% reaction at pH 4.60 (acetate buffer). Optical path length $l = 1$ mm. Analytical concentration of chloranilic acid, $c_T = 5 \cdot 10^{-4}$ M, and of disodium molybdate, $c_{Mo} = 1 \cdot 10^{-3}$ M.

late complex (Fig. 3, a) to form the colourless molybdate–alditol complex and free chloranilate ion (Fig. 3, c). When the reaction was not complete, an intermediate spectrum was obtained (Fig. 3, b). The observation of the isosbestic point at $\lambda = 335$ nm showed that no other species were involved in the analytical reaction.

3. Experimental

3.1. Reagents

All chemicals were of analytical-reagent grade and were used without further purification. A stock solution of molybdate–chloranilate complex in acetate buffer (pH = 4.60) was prepared by mixing in the following order: sodium hydroxide (0.1 *M*), chloranilic acid (Fluka, puriss, $5 \cdot 10^{-4}$ *M*), acetic acid (0.2 *M*), and sodium molybdate (10^{-3} *M*). It was finally diluted to the required concentration with purified (Millipore) water. This solution could be kept for one month at room temperature if protected from light.

For HPLC, the stock solution of the sacrificial complex was prepared as described above, but the concentration of chloranilic acid ($2.5 \cdot 10^{-4}$ *M*) and sodium molybdate ($5 \cdot 10^{-4}$ *M*) were modified in order to ensure the reading of a peak intensity close to 1 with the absorbance detector.

For discontinuous indirect photometric titration, the introduction of analytes into 100 cm^3 of the above solution was made from aliquots of solutions of alditols (typically 10–50 $g \cdot l^{-1}$), using a Gilson micropipette ($V_{max} = 2$ cm^3). Interfering substances were added in solid form. For *HPLC*, the solutions of alditols (1 $g \cdot l^{-1}$) were prepared by weighing and dilution in purified water.

3.2. Apparatus

After waiting for 5 min for the equilibration of temperature, absorbance measurements were performed at $\lambda = 347$ nm and 318 nm on a Kontron Uvikon 860 spectrophotometer equipped with quartz cells (path length $l = 1$ mm). The temperature was fixed at 25.0 ± 0.3°C by a water bath and a pump. The pH values were measured with a Hanna pH meter and a combined glass electrode.

The HPLC apparatus is schematically represented in Fig. 4 and included two pumps (Perkin Elmer, series 10). The carrier (water) is transported by the first pump P1 and the molybdate–chloranilate complex reagent by the second pump P2. Absorbance measurements were performed with a Spectra-Physics UV detector (Spectra 100) equipped with a fixed 6-mm path length cell, and operated at 347 nm. The separations of alditols were realised on a Phenomenex column (Rezex Cal., monosaccharide) containing an ion-exchange resin in Ca^{2+} form. This column was heated to 85°C in a Prolabo oven (Stabitherm). The reaction coil (Teflon) was 230 cm long and the volume of the injection loop was 50 μl. The samples were injected into the chromatographic system with a 100-μl syringe (Hamilton).

For the comparison with refractometric detection, we used the same column fitted with a Varian RI-4 refractometer, with minimal changes in the chromatographic conditions, except for the injection loop (100 μl).

All chromatograms were recorded and treated on a microcomputer, using laboratory-made software. The flow rates were 0.5 cm^3/min for pump P1 and 0.4 cm^3/min for pump P2. The absorbance before injection had a value close to 1 that was compensated to zero before injecting the sample.

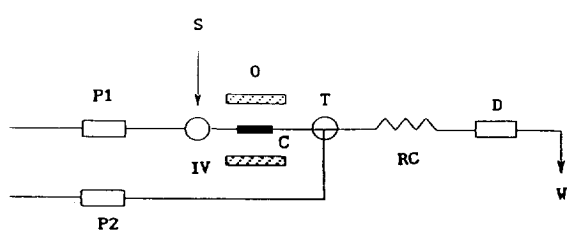

Fig. 4. Schematic diagram of the manifold for HPLC experiments. P_1 = pump for carrier (water), P_2 = pump for the molybdate–chloranilate complex reagent, IV = injection valve (sample volume, 50 μl), S = sample, C = analytical column, O = oven (*t*, 85°C), T = mixing tee, RC = reaction coil, D = UV detector, W = waste.

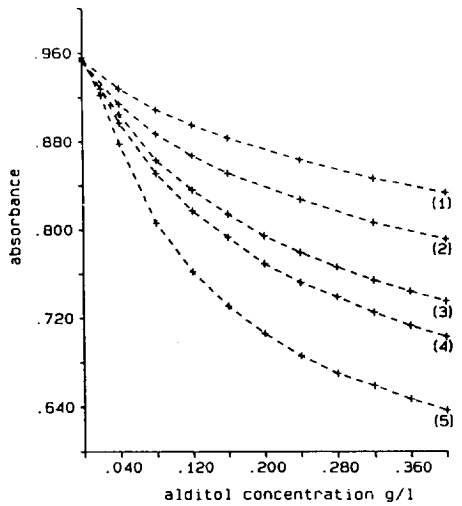

Fig. 5. Variations of the absorbance ($\lambda = 347$ nm) of the molybdate–chloranilate reagent with addition of alditols (1) xylitol, (2) D-arabinitol, (3) D-glucitol, (4) D-mannitol and (5) galactitol at pH 4.60 (acetate buffer). Optical path length $l = 1$ mm. Analytical concentration of chloranilic acid, $c_T = 5 \cdot 10^{-4}$ M, and of disodium molybdate, $c_{Mo} = 1 \cdot 10^{-3}$ M.

4. Results and discussion

4.1. Validation of the indirect photometric procedure

Determination of alditols at pH 4.60

The method was first applied to the determination of alditols by discontinuous indirect photometric titration. When aliquots of a solution of alditol are added to a buffered solution (pH = 4.60) of the molybdate–chloranilate complex, the dissociation of the sacrificial complex due to the formation of the alditol complex, according to reaction (3) in Fig. 2, induces a decrease of absorbance at 347 nm and an increase at 318 nm that were monitored. Fig. 5 represents the variations of absorbance at 347 nm versus the concentration of five analytes: galactitol, D-mannitol, D-glucitol, D-arabinitol and xylitol. The reproducibility of the absorbance measurements was better than 1%. These plots define calibration curves for the determination of each alditol in aqueous solution. A noteworthy feature of the method is that each analyte responds in a specific way, depending on the formation constant of its molybdate complex. The relative positions of the curves are related to the stabilities of the complexes (Table 1) defined by their formation constants [22]. For aldoses in the same concentration range, horizontal response plots were obtained, indicating that they would not interfere in the determination of alditols. This agrees with the low values of the formation constants of the molybdate complexes of aldoses [28,29].

Sensitivity and limits of detection

The sensitivity (S) was defined as the slope of the initial linear part of the plot of absorbance versus the concentration of alditol, and was calculated at 347 and 318 nm for each alditol (Table 1). For all alditols, the ratio S_{347}/S_{318} had

Table 1
Formation constants of the molybdate complexes (log K_{212}), sensitivities (S) and limits of detection (LOD) for the alditols

	Galactitol	D-Mannitol	D-Glucitol	D-Arabinitol	Xylitol
log K^a_{212}	17.30	16.87	16.75	16.34	16.00
S_{347} ($l \cdot g^{-1}$)	1.88	1.28	1.14	0.83	0.56
S_{318} ($l \cdot g^{-1}$)	1.19	0.76	0.71	0.53	0.34
S_{347}/S_{318}	1.58	1.68	1.61	1.57	1.65
$LOD_{347} \cdot 10^2$ ($g \cdot l^{-1}$)	1.05	1.6	1.75	2.4	3.6
$LOD_{318} \cdot 10^2$ ($g \cdot l^{-1}$)	1.7	2.6	2.8	3.8	5.9

[a] K_{212} is the equilibrium constant [29] for the formation of the (2,1,2) molybdate–alditol–proton complex. The reagent is a solution of the chloranilate ($c_T = 5 \cdot 10^{-4}$ M)–molybdate ($c_{Mo} = 1 \cdot 10^{-3}$ M) complex at pH 4.60 (acetate buffer). The sensitivity is defined (see text) by the ratio of the decrease of absorbance at 347 nm or the increase of absorbance at 318 nm, using 1-mm cells, versus the concentration of alditol. The limit of detection was calculated from the standard deviation for absorbance, $3\sigma = 0.02$.

a constant value close to 1.6, showing excellent agreement between the measurements at both wavelengths.

Under our standard conditions, the initial absorbance at 347 nm of the molybdate–chloranilate complex was 0.95 + 0.02 for $l = 1$ mm, from ten replicate determinations. Hence, the limit of detection (LOD) was defined as LOD = 0.02/S. LOD values for the five alditols are given in Table 1. The higher sensitivity was found for galactitol. The lower sensitivity was that for xylitol (almost 3 times lower than for galactitol) that forms the weakest complex. At 347 nm, $1.05 \cdot 10^{-2}$ g·l^{-1} of galactitol can be detected ($1.7\ 10^{-2}$ g·l^{-1} at 318 nm). The mean ratio LOD_{347}/LOD_{318} is close to 0.6.

Interferences

Because the determination of alditols is generally required for biological samples and food control, the possible interferences of common inorganic anions: sulphate, nitrate, chloride and phosphate ions ($c = 10$ g·l^{-1}) were examined at $\lambda = 347$ nm (Table 2). The highest interference is due to the phosphate ion which decreases the absorbance by 20%. On the other hand, sulphate, nitrate and chloride ions interfere weakly, as the decrease of absorbance is at most equal to 2%.

Organic acids and hydroxy acids like oxalic [26,35,36], tartaric [37] and citric [38] acids, that are known to form stable molybdate complexes, interfered significantly. The strongest interference is due to oxalic acid that dissociates the molybdate–chloranilate complex more readily than any alditol. Since the final objective of this work was to apply the indirect photometric method to the post-column detection of alditols after high-performance liquid chromatography on an ion-exchange resin in Ca^{2+} form, such interferences were not considered a serious drawback, because these anions would react with Ca(II) ions sequestered in the analytical column and therefore must be removed prior to analysis with an anion-exchange resin treatment. Thus, inorganic or organic anions are not expected to be present in the chromatographic samples.

Table 2
Interferences of neutral biomolecules and inorganic ions

Interferent	$c = 4$ g·l^{-1}	$c = 10$ g·l^{-1}
D-Glucose	0.8	2.1
D-Xylose	1.4	2.9
D-Ribose	6.2	–
D-Mannose	6.3	–
Urea	–	0.5
Sulphate	–	1.4
Nitrate	–	1.8
Chloride	–	2.0
Phosphate	12.5	20.1

Reported values indicate the decrease (in %) of the absorbance ($\lambda = 347$ nm) of a solution of the chloranilate ($c_T = 5 \cdot 10^{-4}$ M)–molybdate ($c_{Mo} = 1 \cdot 10^{-3}$ M) complex at pH 4.60 (acetate buffer) in the presence of the specified concentration of interferent (optical path length 1 mm).

Consequently, the interferences of neutral biomolecules were examined in more detail, as such compounds would not be removed by the preliminary exchange-resin treatment. Urea interferes less than inorganic anions. Four aldoses were examined: D-glucose, D-xylose, D-ribose and D-mannose (Table 2). At a concentration of 4.0 g·l^{-1}, D-glucose and D-xylose interfere weakly, as they decrease the absorbance by 1 to 2%, but D-ribose and D-mannose decrease the absorbance by more than 6%. It agrees with the relative order of stability of their molybdate complexes: D-mannose > D-ribose > D-xylose > D-glucose [28,29].

However, since the concentrations of aldoses (4.0 g·l^{-1}) used in the interference experiments were considerably higher than the concentrations of alditols (0.01–0.40 g·l^{-1}), the alditols may be determined in this concentration range without interference of aldoses, even with D-mannose and D-ribose in moderate amounts. This result is in agreement with a report describing the polarimetric determination of D-mannitol and D-glucitol [39] in the form of their molybdate complexes, that possess enhanced optical rotation [15]. It was found that D-glucose did not interfere, as would be expected because of the weakness of its molybdate complex [29].

The proposed indirect photometric method is fast and simple. However, because each alditol decreases the absorbance of the reagent with a

specific sensitivity, the method is not suitable for the determination of mixtures of alditols.

4.2. High-performance liquid chromatography of alditols

Mixtures of alditols were analyzed by HPLC, using a cation-exchange resin in Ca^{2+} form, heated at 85°C. We examined the applicability of indirect photometry as a post-column detection method. In principle, the effluent is mixed with a stream of the sacrificial complex and the reaction takes place in the reaction coil. Finally, the absorbance is measured by the UV detector. The elution of each alditol would then give rise to a negative peak at $\lambda = 347$ nm. Advantages would be the use of a very common detector and a partial selectivity, since only compounds that can complex molybdate would be detected.

Retention times and relative peak heights

The same five alditols were studied: galactitol, D-mannitol, D-glucitol, D-arabinitol and xylitol, and were injected as pure samples for standardization or in binary mixtures. Measurements made in triplicate showed that the retention times had similar values for pure alditols or in mixtures. The mean results are summarized in Table 3, together with the Fischer projections of the alditols. Most couples of alditols are eluted with time intervals higher than 30 s and are well separated except xylitol/D-glucitol and D-mannitol/D-arabinitol that give only one peak.

Table 3
Retention times and Fischer formulas of the five alditols

Alditol	Formula	Retention time[a]
Galactitol	$HOCH_2$ ⊥⊥⊥ CH_2OH	24 min 45 s
D-Mannitol	$HOCH_2$ ⊥⊥⊥ CH_2OH	21 min 35 s
D-Glucitol	$HOCH_2$ ⊥⊥⊥ CH_2OH	26 min 00 s
D-Arabinitol	$HOCH_2$ ⊥⊥ CH_2OH	22 min 00 s
Xylitol	$HOCH_2$ ⊥⊥ CH_2OH	25 min 30 s

[a] Accuracy ± 15 s. Conditions as in Experimental Section.

Table 4
(h) for various alditols

Alditol	h
Galactitol	0.269
D-Mannitol	0.210
D-Glucitol	0.147
D-Arabinitol	0.082
Xylitol	0.048

Relative peak height h is given in absorbance units at $\lambda = 347$ nm. Conditions as in Experimental Section. Injection: 50 μl of aqueous solution of alditol ($c = 1.00\ g \cdot l^{-1}$).

The peak heights obtained for the injection of solutions of alditols ($c = 1.00\ g \cdot l^{-1}$) are compared in Table 4. The values obtained in four independent determinations did not differ by more than 1%. The response order is similar to that found in the preliminary study and agrees with the relative stabilities of the corresponding molybdate complexes. It means that a specific calibration curve must be drawn for every alditol present in the chromatographic mixture.

Separation and determination of mixtures of galactitol and D-glucitol

The retention times of galactitol and D-glucitol differ by more than 1 min (Table 3) and these alditols are eluted as two peaks that slightly overlap (Fig. 6). However, the separation was reasonably complete, since we verified that the same peak intensities were found when the compounds were injected separately, or as mixtures. The calibration curves are linear in the 0–0.10 $g \cdot l^{-1}$ concentration range. Higher concentrations may be determined either by direct reading on the calibration curves, or after suitable dilution.

The sensitivities were defined as the slopes of the linear portion of the calibration curves and are given in Table 5. The limits of detection obtained with the UV detector are given in Table 6, together with those for the refractometric detector which was currently used in the laboratory. The limit of detection in the indirect photometric mode is 2 to 7 times smaller than with refractometric detection, although the volume of injection loop was doubled in the case of

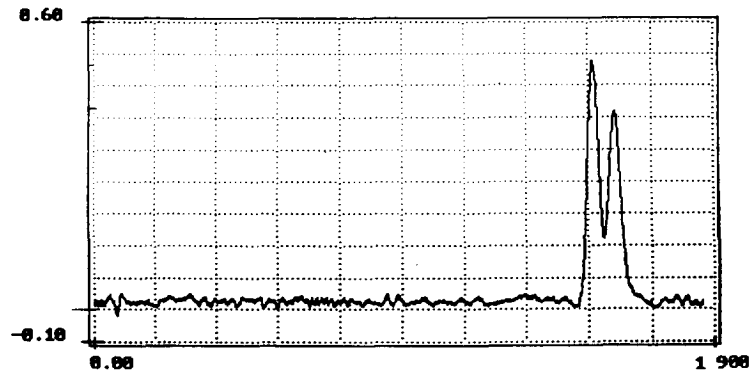

Fig. 6. Chromatogram of an equimolar mixture of galactitol and D-glucitol with indirect photometric detection (λ = 347 nm). The peak height is higher for galactitol (eluted first) because of the larger sensitivity. The signal input was reversed in order to show positive deviations. Vertical axis: arbitrary absorbance units. Horizontal axis: time (190 s/square).

Table 5
Sensitivities (S) for the chromatographic determination of galactitol and D-glucitol

	Galactitol	D-Glucitol
S ($l \cdot mol^{-1}$)	143.9	56.5
S ($l \cdot g^{-1}$)	0.79	0.31

Peak heights are in absorbance units. λ = 347 nm. Conditions as in Experimental Section.

refractometric detection. Moreover, the limit of detection is the same for galactitol and D-glucitol with refractometric detection, whereas the limit of detection is related to the stability of the alditol–molybdate complex in the case of photometric detection, which is particularly advantageous for galactitol.

Table 6
Comparison of the detection limits of refractometric and photometric detection in the HPLC separation of galactitol and D-glucitol

Mode of detection	Refractometric	Photometric[a]
Volume injected (μl)	100	50
Galactitol	$5 \cdot 10^{-4}$ M	$7 \cdot 10^{-5}$ M
D-Glucitol	$5 \cdot 10^{-4}$ M	$2.5 \cdot 10^{-4}$ M

[a] Indirect photometry at λ = 347 nm, according to the proposed method.

Interferences

Since the analytical column should not be supplied with samples containing ions that would react with calcium ions, no interference can arise from inorganic or organic anions that must be removed from the samples by a preliminary treatment with an anion-exchange resin. Among possible interfering sugars, we checked that the ubiquitous glucose did not interfere, first by injecting solutions ($c = 2$ g $\cdot l^{-1}$) of D-glucose alone, that did not produce a noticeable signal, and then by injecting mixtures of D-glucitol and D-glucose, that gave a single signal equal to that for D-glucitol alone. It demonstrates that the method is useful for food control, as alditols introduced as additives may be determined in the presence of natural sugars, without prior separation.

5. Conclusion

The present method employing a solution of the molybdate–chloranilate complex as the coloured reagent offers a new mode of indirect photometric detection for alditols in the presence of aldoses. The selectivity of the determination is due to the sensitivity of the ligand-exchange reaction to the configuration of the analyte. Its accuracy compares favourably with that of refractometry index detection.

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