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The use of chitosan modified with glutaraldehyde and glyoxal as chromatographic support for the separation of flavonoids from *Aleurites moluccana* leaves

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This paper describes the preparation of chitosan modified with dialdehydes, glutaraldehyde (CH-Glu) and glyoxal (CH-Gly) and its application in the isolation of the flavonoids swertisin and 2″-*O*-rhamnosylswertisin from *A. mollucana*. The additional non-polar alkyl groups increase the hydrophobicity of the sorbent. The results show that the separation was mediated by hydrophobic interaction (CH-Glu), as well as hydrogen bonding, between phenolic OH or rhamnosil residues from the flavonoids, and the free amine groups (CH-Gly).

1. Introduction

Various types of modified chitosan have been used as a good chromatographic support for the separation and purification of protein, enzymes, dyes, flavonoids, aromatic hydrocarbons, etc. (Wang et al. 2002; Saito et al. 2002; Girardi et al. 2003; Tangpasuthadol et al. 2003). Recently, a novel crosslinked chitosan phase, with different degrees of cross-linking, was synthesized as the stationary phase for liquid chromatography in the separation of polycyclic aromatic hydrocarbons (Saito et al. 2002). Hydrophobic interaction chromatography has emerged as a useful chromatographic method for separation of enzymes and proteins. The reaction of chitosan (free NH_2) with glutaraldehyde and glyoxal (aldehyde ends) produces intramolecular crosslinking (Schiff's base reaction) and can make chitosan much more hydrophobic. Glutaraldehyde modified chitosan has been evaluated as a matrix for hydrophobic interaction chromatography, and the results show that this adsorbent can be used to separate enzymes (glucose oxidase, pectinase and alkaline phosphatase) (Agarwal and Gupta 1995; Kucera 2004).

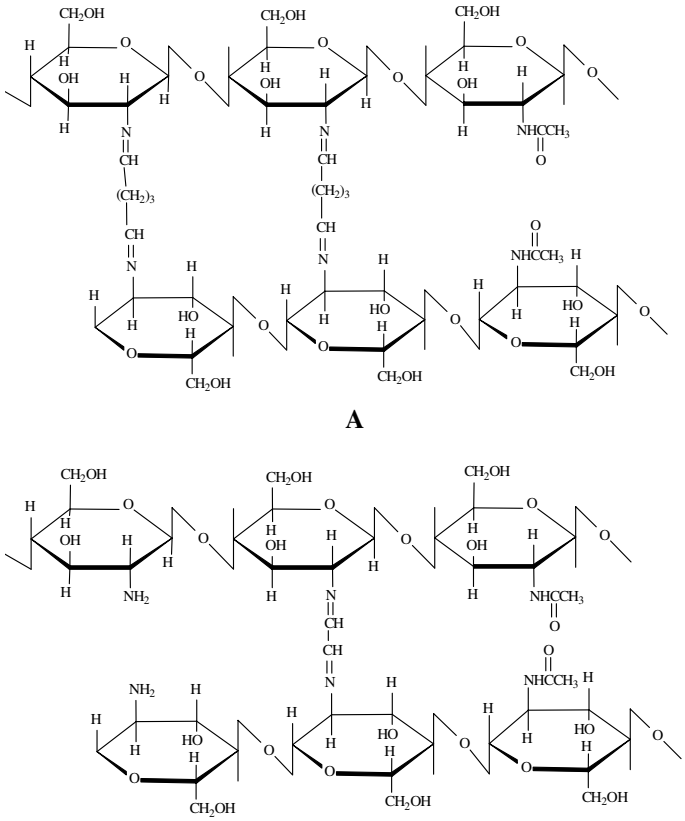
Aleurites moluccana (L.) Willd. has been extensively used in folk medicine for treatment of headache, fevers, diarrhea and hypercholesterolemia (Duke 1991). Pharmacological evaluation of this plant has demonstrated pronounced antinociceptive and hypolipidaemic properties. Chemically, we have isolated hydrocarbons, sterols, flavonoids and triterpenes, which contribute to the biological effects of this plant (Meyre-Silva et al. 1998, 1999; Pedrosa et al. 2002). This present study evaluates glyoxal- and glutaraldehyde-modified chitosan as an adsorbent for the separation of flavonoids, swertisin and 2″-*O*-rhamnosylswertisin from *A. moluccana*.

2. Investigations, results and discussion

Glutaraldehyde and glyoxal crosslinking occur through a Schiff's base reaction with an imine function. The degree of crosslinking (CD) of the chitosan, after the reaction with dialdehyde, was 100% for glutaraldehyde and 22% for glyoxal (determined by potentiometric titration). The IR spectra of CH-Glu and CH-Gly (not shown) present two absorption peaks at 2930 cm^{-1} (ν_{C-H} of CH_2 group) and 1400 cm^{-1} (δ_{C-H} of CH_2 group). The spectrum of modified chitosan exhibits strong absorbance at 1634 cm^{-1} which represents $\nu_{C=N}$ (C=N Schiff base) (Knaul et al. 1999; Brugnerotto 2001).

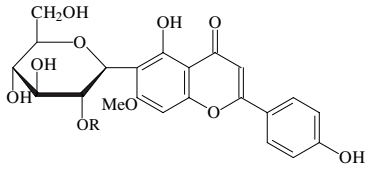
The present work explores the behaviour of chitosan modified with dialdehydes as an adsorbent for the separation of bioctive flavonoids from *A. moluccana,* with the aim of extending this to the separation of flavonoids (glycosylated an aglicam) from medicinal plants. The crosslinked chitosan films, membranes and powder are used in the separation of proteins, enzymes and aromatic hydrocarbons (Agarwal and Gupta 1995; Saito 2002; Kucera 2004).

The Table gives the amount of swertisin (**1**) and 2″-*O*-rhamnosylswertisin (**2**) obtained with CH-glu. When CH-glu was used as an adsorbent, there was an increase in the amount of compounds isolated, compared with chitin, fully *N*-acetylated chitin and silica gel. The crosslinking reaction of chitosan with glutaraldehyde eliminates the free NH_2 groups (CD 100%), reducing the hydrogen bond interaction between the adsorbent and phenolic OH from the flavonoids. Crosslinking with glutaraldehyde can also increase hydrophobicity (additional groups $=CH(CH_2)_3HC=$) to the adsorbent, resulting in an increase in the amount of compounds isolated. Similar results were previously reported by (Morch et al. 2004), particularly for swertisin. On the other hand, crosslinking can



CH-glu (A) and CH-gly (B)

Table: Efficiency of different supports studied in the separation of flavonoids of *A. moluccana* (150) mg from ethyl acetate extract



1 R = H
2 R = Rhamnosyl

Support	Swertisin (1)		2″-*O*-Rhamnosylswertisin (2)	
	mg	%	mg	%
Chitin[a]	11.2	8.0	10.9	7.2
Chitin-100[a]	12.8	9.5	15.8	10.5
Silica gel[a]	3.0	2.3	9.2	6.1
CH-Bz[b]	31.5	21.0	36.0	24.0
CH-glu	17.5 ± 0.7[c]	11.7 ± 0.9	17.0 ± 0.2	11.3 ± 0.5
CH-gly	34.0 ± 1.5	22.7 ± 1.7	6.20 ± 0.4	4.1 ± 0.6

[a] Morsch et al. (2002)
[b] Girardi et al. (2003)
[c] means for three experiments ± s.d.

reduce the space occupied and increase the rigidity of the polymer chain, decreasing its interaction with the phenolic compound (especially 2″-*O*-rhamnosylswertisin), and reducing the efficiency of the adsorbent. Another interesting result was found when CG-gly was used as an adsorbent. The amount of isolated swertisin was higher when compared with the other adsorbents.

On the other hand, CH-gly contains high amounts of free NH_2 groups (CD 22%) that can form a strong hydrogen bond with OH groups of phenolic or rhamnosil residues, resulting in the retention of **2** in the column. The adsorbent presents similar characteristics to chitosan, therefore a large amount of compound is retained in the column. Similar results were reported when chitosan was used to separate biflavonoids from *Rheedia gardneriana* (Rodrigues et al. 2000).

The results from this study show that the adsorbents used here appear to be useful matrices for the separation of phenolic compounds. They are inexpensive and easy to prepare, compared with other adsorbents generally used in chromatography. In addition, CH-glu and CH-gly have efficiency similar to that of other adsorbents derived from chitosan.

3. Experimental

3.1. Plant material

A. moluccana was collected in Tijucas, in the south of Brazil, and a voucher specimen was deposited at the Barbosa Rodrigues Herbarium, Itajaí, under number VC Filho 001. A methanolic extract was obtained after maceration with methanol at room temperature for 10 days (600 g dried leaves). This extract was concentrated and then successively partitioned with hexane, dichloromethane, and ethyl acetate, to give the respective fractions. The ethyl acetate fraction, rich in flavonoids, was dried at room temperature (1.2 g) and selected for further studies.

3.2. Preparation of stationary phase

Chitosan (76% *N*-desacetylation) was obtained through basic hydrolysis of chitin, according to methods previously described in the literature (Rinaudo et al. 1993). The material was ground and sieved, and fractions of 43–80 μm were used for the preparation of the modified chitosan and the chromatographic column.

Chitosan (5.0 g) was suspended in 250 ml of glurataraldehyde aqueous solution (15% v/v) or 250 ml of glyoxal aqueous solution (15% v/v) and continuously stirred for 24 h. Chitosan modified with di-aldehydes was

washed extensively with distilled water, and then with ethanolamine (0.1 M, pH 7.0). This treatment was carried out to quench all the aldehyde groups of the modified polymer. Afterwards, the imine groups were reduced with cyanoborohydride for 12 h. Finally the glutaraldehyde modified chitosan (CH-glu) (**A**) and glyoxal-modified chitosan (CH-gly) (**B**) were washed extensively with distilled water, filtered and dried at 60 °C.
The material obtained was characterized by IR, and potentiometric titration (Brugnerotto et al. 2001). The IR spectra were obtained in KBr disks on a Bomem MB-100 IR-FT spectrophotometer. The potentiometric titration was carried out using an ORION model A920 pH meter.

3.3. Chromatography

The ethyl acetate fraction (150 mg), containing the biflavonoids, was chromatographed on a chromatographic column (CC) (2.0×30 cm) using 3 g of CH-Glu and CH-Gly, eluted with $CHCl_3$: MeOH gradient, and fractions of 5 ml were collected. After monitoring by TLC eluted with $CHCl_3$: MeOH 70 : 30 v/v, the fractions which showed a positive reaction with $FeCl_3$ were combined.
The purity of all the isolated substances was examined by TLC, pre-coated with a 0.25 mm layer of Merck silica gel 60 HF_{254} and eluted with $CHCl_3$: MeOH 85 : 15 v/v. The compounds were detected by spraying with $FeCl_3$ (2% in ethanol) solution or visualization under UV light (254 nm). The compounds were identified by direct comparison with authentic samples.

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