A Comparative Study of Stationary Phase for Separation of Biflavonoids from *Rheedia gardneriana* Using Column Chromatography

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This paper describes a comparative study by using different chromatographic supports (silica gel, chitin and chitosan) to separate biflavonoids from *Rheedia gardneriana* by column chromatography. The results indicated that chitin can be used as alternative method, but the yield of the compounds is lower than when silica gel is employed. In contrast, chitosan is not a good chromatographic support for the separation of the biflavonoids under the same experimental conditions.

Introduction

Biflavonoids form an important class of natural products, which exert different pharmacological activities (Alcaraz and Jimenez, 1988; Pathak *et al.*, 1991). We have recently isolated some phytoconstituents present in the *Rheedia gardneriana* Plant et Triana leaves, a Brazilian medicinal plant, by column chromatography using silica gel as stationary phase (Luzzi *et al.*, 1997). Such compounds (see Fig. 1) were identified as volkensiflavone (**1**), GB-2a (**2**), fukugetin (morelloflavone) (**3**) and fukugeside (**4**), which were the main active components of the ethyl acetate fraction, showing marked analgesic effects in mice (Luzzi *et al.*, 1997). Since their molecular structures are very similar, the chromatographic separation using silica gel as a stationary phase proved to be very laborious. Such observation led us to determine other possible chromatographic supports, which could be used as alternative method for this purpose.

For this reason, we have attempted to determine whether chitin or chitosan can be used as a stationary phase to isolate the biflavonoids of *R. gardneriana*.

Chitin, which is perhaps the second most important natural polysaccharide, is the straight homopolymer composed of β (1,4)-linked GlcNAc units with a three-dimensional α-helical configuration stabilized by intramolecular hydrogen bonding (Kas, 1997).

Muzzarelli and co-workers used chitin as a chromatographic support and adsorbent in column chromatography (CC) for collection of metals ions from organic and aqueous solution (Muzzarelli and Tubertini, 1969). Bloch and co-workers used chitin for the purification of wheat germ aglutinin using affinity chromatography (Bloch and Burger, 1974). Chitin has been used in thin layer chromatography (TLC) for separation of amino acid peptide saccharides, phenols and carboxylic acids (Nahlik *et al.*, 1985).

Chitosan, or β (1,4)2-amino-2-deoxy-D-glucose, is a hydrophilic biopolymer obtained industrially by hydrolyzing the aminoacetyl group of chitin, which is the main component of the shell of crab, shrimp and krill, by alkaline treatment (Kas, 1997).

Because the amount of NH_2 free (60–100%), the chitosan is a useful support for CC in separa-

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tion of the inorganic ions and purification of the protein (trypsin) (Shi *et al.*, 1996). Chitosan has also been used in TLC for separation of amino acid, peptides, saccharides, phenols and carboxylic acids, nucleic acid, nucleotide and nucleosides (Nahlik *et al.*, 1985; Nagasawa *et al.*, 1970).

In this study we have compared the efficacy of chitin, chitosan and silica gel to separate the biflavonoids present in an ethyl acetate fraction of *R. gardneriana*.

Material and Methods

Preparation of extract

The leaves of *R. gardneriana* Plant et Triana were collected in a Blumenau city, in September 1997. The plant was identified by Prof. Marcos Sobral (Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre). The vouchers were deposited in Dr. Roberto Miguel Klein Herbariun (Departamento de Ciências Naturais, FURB, Blumenau) under numbers 534 to 540.

Air dried leaves (545 g) were powdered and macerated with 95% methanol (8 l) at room temperature for approximately 10 days. After solvent removal under reduced pressure, the extract was successively partitioned with solvents of increasing polarity namely, hexane, chloroform, ethyl acetate (22 g) and butanol (700mL each) in order to obtain the respective fractions. Only the ethyl acetate fraction was studied further.

Preparation of chromatographic support

Chitin flakes (85% N-acetylation) were obtained in NIQFAR laboratories according to the literature method from shrimp shells captured Atlantic south cost (Bagio *et al.*, 1989). The material was ground and sieved and fractions with sizes of between 43–100 μm were used for preparation of the chromatographic column.

Chitosan (76% N-desacetylation) was obtained through basic hydrolysis of chitin according to previously described literature (Bagio *et al.*, 1989).The material was ground and sieved and fractions with size between 43–65 μm were used for the preparation of the chromatographic column.

Chromatography

300 mg of the ethyl acetate fraction, which contained the biflavonoids, was chromatographed on a column chromatography (CC) (2.0 × 30 cm) using 6 g of chitin eluted with $CHCl_3$:MeOH gradient and fractions of 5 ml were collected. After being monitored by thin layer chromatography (TLC) eluted with $CHCl_3$:MeOH 70:30 v/v, the fractions which showed positive reaction with $FeCl_3$ were combined and rechromatographed as in the previous case.

Similarly, 300 mg of the ethyl acetate fraction were chromatographed over silica gel (9 g) and chitosan (6 g).

The purity of all isolated substances was examined by TLC precoated with a 0.25 mm layer of silica gel 60 HF_{254} from Merck and eluted with $CHCl_3$:MeOH 85:15 v/v. The compounds were detected by spraying with a $FeCl_3$ (2% in ethanol) solution or visualized under UV light (254 nm). The compounds were identified by direct comparison with authentic samples.

Results

We have selected chitin and chitosan for investigation, since some authors have described the efficiency of these natural polymers for chromatographic separation of organic compounds (Rózylo *et al.*, 1988, 1989; Rodrigues *et al.*, 1998).

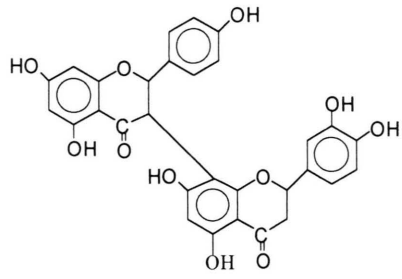
Chitin is a natural biopolymer with physical and chemical similarity to cellulose, which is often used in chromatography. Chitin is an odorless, solid substance. It is insoluble in water and resistant to acid, bases and organic solvents.

Since a great variety of the functional groups exist on the chitin surface (OH-groups, free and acetylated amino groups) chitin can be used for the separation of many types of compounds (Rózylo *et al.*, 1988).

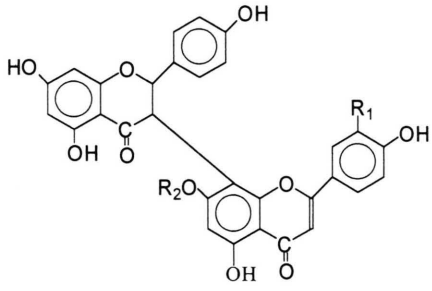
The structures of isolated compounds of *R. gardneriana* using chitin or silica gel as chromatographic support are shown in Figure 1.

The order of elution of biflavonoids in chitin was the same with silica gel, i.e., volkensiflavone, GB-2a, fukugetin and fukugeside, respectively. The amount of the isolated substances is shown in the Table I.

The retention of biflavonoids on the surface of chitin seems to occur due interaction by hydrogen



(2) GB-2a



(1) volkensiflavone $R_1 = R_2 = H$
(3) fukugetin R_1=OH, R_2 = H
(4) fukugeside R_1 = OH, R_2 = ï Dgluc

Fig. 1. Molecular structures of biflavonoids isolated in this study.

bonding of OH phenolics with a variety of functional groups on surface (OH-groups, free or acetylated amino groups) (Rózylo *et al.*, 1988, 1989). Similar behavior occurs in isoflavonoids separation when polyamide is used as chromatographic support (Heftmann, 1992).

Another interesting observation is the low yield of pure compounds when chitin was used as support . This can be attributed to the strong interaction between free amino groups of chitin (15%) and OH-groups of the phenolic compounds, resulting in retention of a large amount of compounds in the column. In an attempt to confirm this hypothesis, we have substituted chitin with its derivative chitosan as a chromatographic support.

However, the presence of many free amino groups on the surface of chitosan (75%), which acts as an electron donor and strongly reacts with OH ions present in biflavonoids, resulted in a complete retention of the compounds in column.

These results indicated that chitin can be suitable as chromatograpic support for separation of biflavonoids present in *R. gardneriana*, although in lower quantities when compared with silica gel, Table I. On the other hand, chitosan was not efficient to separate these substances under the same experimental conditions.

Table I. Efficiency of different support studied in the separation (300 mg of ethyl acetate fraction) of biflavonoids of *R. gardneriana* (in mg).

Support	Volkensiflavone	Fukugeside	GB-2a	Fukugetin
Chitosan	0	0	0	0
Chitin	15	126	20	14
Silica Gel [a]	45	150	11.2	25

[a] From reference Luzzi *et al.* (1997).

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