Carbohydrate Polymers **9** (1988) 277–285

Analysis of the Oligosaccharides from the Roots of *Arnica montana* L., *Artemisia absinthium* L., and *Artemisia dracuncula* L.

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(Received 12 February 1988; revised version received 14 April 1988; accepted 21 April 1988)

ABSTRACT

The oligosaccharides extracted from the roots of Arnica montana *L.,* Artemisia absinthium *L. and* Artemisia dracunculus *L. have been analysed by thin-layer and gel-permeation chromatography to assess their applicability as 'guide' substances for pharmacological activity. Differences observed in the oligosaccharide component composition of such extracts might be more related to the vegetative stage of the plants at time of harvest than to the species themselves. In addition to a series of non-reducing oligofructosides, a series of reducing inulin-type oligosaccharides was found at the initial stages of growth, whereas in later stages of growth only non-reducing oligofructosides were present. These differences have been related to different stages of biosynthetic activity within the plants.*

INTRODUCTION

Due to the growing interest in phytopharmaceuticals and their ever-widening use in therapy in recent years, emphasis has been placed on the importance of the standardisation both of the crude drugs themselves

Carbohydrate Polymers 0144-8617/88/$03.50 —

and of the preparations obtained from them (Sprecher, 1980; Menssen, 1981). This standardisation, necessary to ensure the reproducibility of the pharmacological action, is frequently based on the identification and the determination of the content of the active component, but in some cases is based on a convenient guide substance (Menssen, 1981).

Preparations derived from the roots or flowers of the plants under study are of interest due to their therapeutic properties (Schulte, 1963; Brünner, 1969; List & Hörhammer, 1972; Willuhn, 1981) which have been ascribed principally to the presence of: phenols, phenyl ethers, and phenolic esters which have antiseptic and antiphlogistic action; polyacetylenic compounds which have bacteriostatic and fungicidal action; sesquiterpenic substances which have antimythotic activity; and caffeic and chlorogenic acids and cynarin which have choleretic, chologogic and diuretic action. Whilst the overall qualitative and quantitative composition of each of these compounds is of considerable interest for the characterisation of such extracts, the identification and content of soluble carbohydrates or free amino acids are of considerable interest for the identication of potential 'guide' substances which could be used for characterisation (Sancin *et al.*, 1981; Lombard *et al.*, 1983; Rossetti *et al.*, 1984). As part of the study on the analysis of such 'guide' substances, the structure and composition of the soluble oligosaccharide components has been investigated (Lombard *et al.*, 1983) to assess the potential of these compounds as 'guide' substances.

Studies carried out previously on *Arnica montana* L. (Lombard *et al.*, 1981), *Artemisia absinthium* L. (Tourn & Lombard, 1973–74; Buffa *et al.*, 1980) and *Artemisia dracuncula* L. (Lombard *et al.*, 1974–75), have shown that a series of non-reducing oligofructosides with the structure $[\beta$-D-Fru*f*-$(2\rightarrow 1)]_n$-β-D-Fru*f*-$(2\leftrightarrow 1)$-α-D-Glc*p* where n = at least 3–6, are formed by the addition of successive D-fructofuranosyl residues to sucrose. In addition, depending upon time of harvesting, a homologous series of oligosaccharides of the inulin series, which lack the terminal α-D-glucopyranosyl residues (Lombard *et al.*, 1981), may also be present. The metabolic function of these D-fructosyl oligosaccharides is as acceptors or donors of D-fructosyl residues (Bacon, 1960).

Previous analysis of an extract from *Arnica montana* L. by thin-layer chromatography has allowed detection of oligosaccharides with a degree of polymerisation (DP) of 6–8, whereas gel-permeation chromatography has detected a DP of more than 10 (White *et al.*, 1985). An improved method based on both thin-layer and gel-permeation chromatography for the analysis of such extracts which readily resolves oligosaccharides of up to DP19 is now reported.

EXPERIMENTAL AND RESULTS

Samples

The roots of *Arnica montana* L. were collected during the initial (sprouting) stage of growth in June 1982 in Val Chisone, Piemonte, Italy at an altitude of 1600 m. Similarly the roots of *Artemisia dracunculus* L. were collected during their initial stage of growth in March 1984 in Pancalieri, Italy at an altitude of 250 m, whilst the roots of *Artemisia absinthium* L. were collected at the onset of the quiescence stage in September 1984 in Sestriere, Italy at an altitude of 1800 m. A hot 70% ethanol extract was prepared from the fresh roots within one day of collection in all cases in order to minimise any changes which might occur due to damage on collection. The crude carbohydrate residue was obtained on evaporation followed by lyophilisation. The oligosaccharides present in the extracts were analysed by means of thin-layer chromotography (TLC) and high-resolution gel-permeation chromatography.

Thin-layer chromatography

The extracted oligosaccharides were fractionated on Kieselgel G (E. Merck, Darmstadt, FRG) thin-layer plates (20 × 20 cm) using chloroform–acetic acid–water (3:3·5:1) (De Stefanis & Poute, 1968; Buffa *et al.*, 1980) as solvent (three ascents at 28°C); 10 μl, corresponding to 20 mg of fresh material, were spotted on each plate. Reducing sugars were detected by the spray reagent triphenyltetrazolium chloride (Stahl, 1967), whereas non-reducing sugars were detected by the spray reagent diphenylamine aniline phosphate (Damonte *et al.*, 1971). The extracts from *Arnica montana* L. and *Artemisia dracunculus* L. were reactive to both reagents, whereas the extract from *Artemisia absinthium* L., apart from glucose and fructose, was reactive to diphenylamine aniline phosphate only (Fig. 1).

High-resolution gel-permeation chromatography

A water-jacketed column (140 × 2·8 cm id), maintained at 60°C to eliminate hydration effects relating to the gel (Heyraud & Rinaudo, 1972; John *et al.*, 1969) using a thermostatted circulatory bath (Grant FH15) was packed with Bio-Gel P2-400 mesh (Bio-Rad Laboratories, Watford, UK; particle size less than 37 μm). The packed column was continuously pumped at a constant flow rate (0·7 ml min^{-1}) with sodium

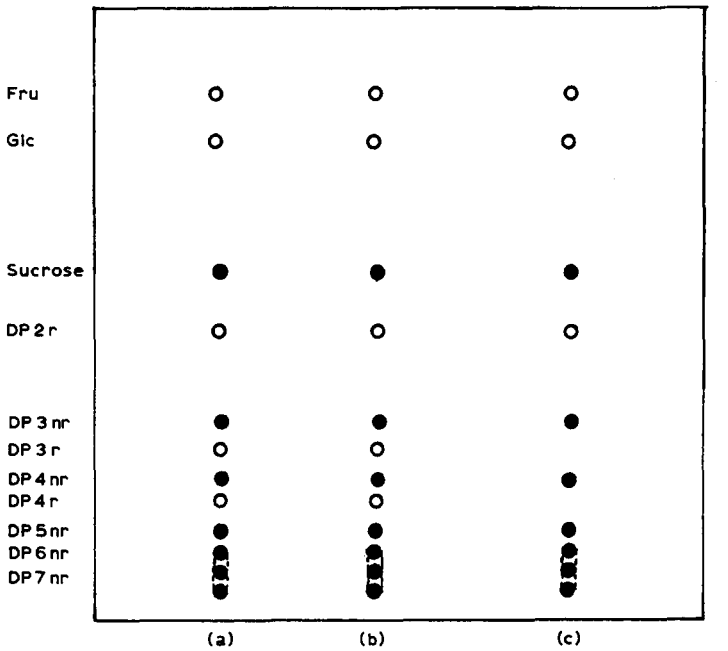

Fig. 1. Thin-layer chromatographic analysis of the extracts from (a) *Arnica montana* L., (b) *Artemisia dracunculus* L. and (c) *Artemisia absinthium* L. Spots marked ● reactive to diphenylamine-aniline phosphate (non-reducing sugars), whilst spots marked ○ reactive to triphenyltetrazolium chloride (reducing sugars).

chloride eluent (0·1 M) to prevent non-specific adsorption phenomena (Kennedy, 1972; White & Kennedy, 1979) using a piston pump (Metering Pumps Ltd, Series II, London). The column eluate was continuously monitored using an automated L-cysteine–sulphuric acid assay (Barker *et al.*, 1968; White & Kennedy, 1981) incorporating Technicon AA1 modules.

Samples of *Arnica montana* L. and *Artemisia absinthium* L. were analysed by preparing a solution (30 mg ml^{-1}) in water and loading an aliquot (100 μl) of the clear solution remaining after centrifugation onto the column. The extract from *Artemisia dracunculus* L. was analysed similarly with the centrifugation step omitted since it was totally soluble. For all extracts a series of chromatographic peaks corresponding to individual oligosaccharides up to DP19 were obtained, together with additional material of higher molecular weight (Fig. 2). This material has been divided into an intermediate molecular weight fraction (IMW) having a molecular weight range corresponding to materials of DP 17–25, and a high molecular weight fraction (HMW) having a molecular

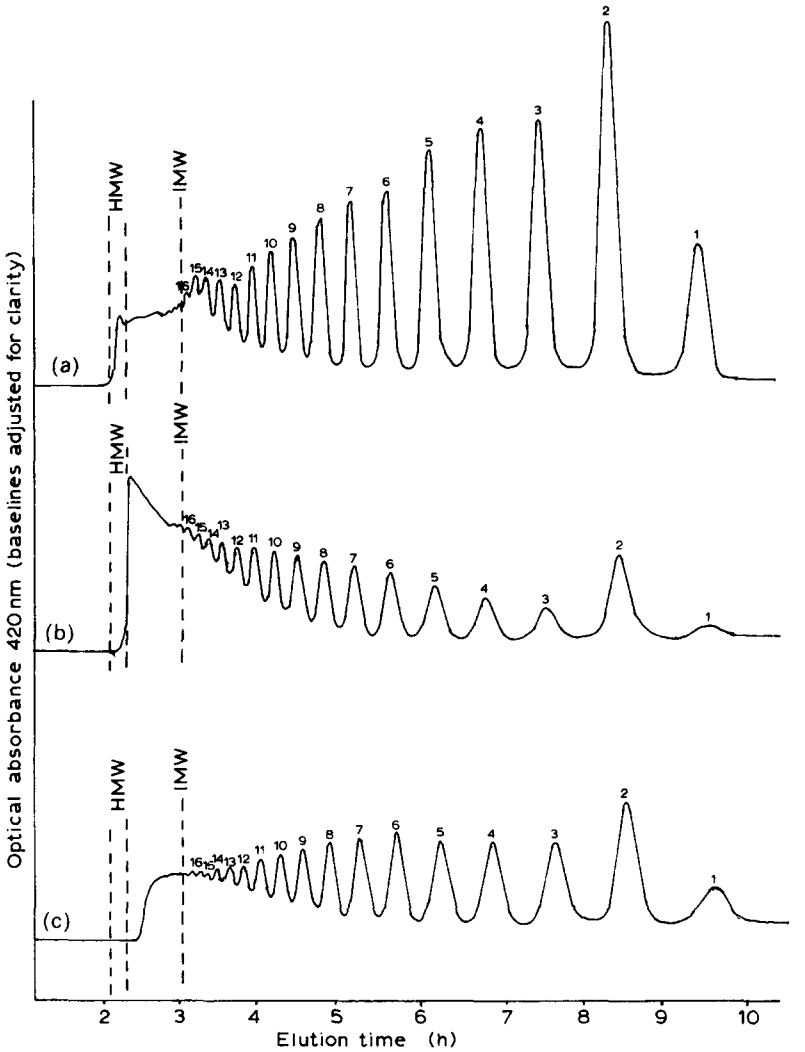

Fig. 2. Gel-permeation chromatograms of the 70% ethanol extracts from the roots of (a) *Arnica montana* L., (b) *Artemisia absinthium* L. and (c) *Artemisia dracunculus* L.

weight in excess of 4000 Daltons representing material wtih DP greater than 25. Although material of up to DP19 is observable, it is not possible to quantitate it with any degree of accuracy and it is therefore included in the IMW fraction. The extracts from *Arnica montana* L. and *Artemisia dracunculus* L. contained either very little or no HMW fraction, whereas the extract from *Artemisia absinthium* L. contained a HMW fraction and a large IMW fraction.

Using the assumption that the relative area under each chromatographic peak is proportional to the actual amount of each oligosaccharide present (which implies that the response of the L-cysteine–sulphuric acid assay for a given weight of an oligosaccharide is constant, irrespective of the DP of the oligosaccharide), the percentage composition (by weight) of each oligosaccharide in the 70% extracts was calculated (Table 1).

TABLE 1

Oligosaccharide Component Composition of the 70% Ethanol Extracts from the Roots of *Arnica montana* L., *Artemisia absinthium* L. and *Artemisia dracunculus* L.

	Composition as a percentage of total		
	Arnica montana *L.*	Artemisia absinthium *L.*	Artemisia dracunculus *L.*
DP			
1	5·9	0·5	3·7
2	19·0	6·1	11·5
3	10·9	2·2	7·5
4	9·3	2·6	7·4
5	7·2	3·3	7·5
6	6·0	3·6	7·2
7	5·3	3·8	6·6
8	4·7	4·0	6·0
9	3·8	4·1	5·0
10	3·5	4·4	4·7
11	2·8	4·3	4·2
12	2·6	4·4	3·9
13	2·4	4·2	3·4
14	2·4	3·9	3·0
15	2·3	3·6	2·7
16	1·7	3·5	2·3
IMW	9·3	38·6	13·5
HMW	0·9	3·1	0·0

DISCUSSION

The use of thin-layer chromatography to determine the oligosaccharide component composition of complicated mixtures has several serious disadvantages. Firstly, separation above DP6–8 is not possible and therefore no quantitative assessment can be complete on the basis of this method alone. Secondly, quantitation of the separated oligosaccharides

is normally very difficult and achievable only via scanning densitometric techniques. On the other hand, the use of gel-permeation chromatography is a useful and well-documented technique widely used for studying the oligosaccharide component composition of hydrolysates of starch and other polysaccharides. It has the advantages over thin-layer chromatography of not only being fully quantitative but also providing information on the complete range of oligosaccharides present. However, gel-permeation chromatography has the disadvantage in that separation of very closely related homologous series of oligosaccharides is not achievable, in contrast to thin-layer chromatography, since separation takes place on a molecular exclusion basis only.

Whereas the fractionation range of a chromatographic gel support under study can be predetermined by the degree of cross-linking within the gel matrix, defining the so-called exclusion limit, the resolution of such gels is largely dependent upon the particle size of such gels. The use of small particle-size gels ensures tighter packing within a column, thereby increasing the number of theoretical plates and hence improving resolution. On this basis the use of a smaller particle-size gel of the same exclusion limit should enhance the resolution on any chromatograph obtained.

In a previous study (White *et al.*, 1985) the 70% ethanol extract from the roots of *Arnica montana* L. was analysed for oligosaccharide component composition using a column of Bio-Gel P2 200–400 mesh (i.e. particle size 75–37 μm) with resolution of individual oligosaccharides up to DP10 being possible. This sample has been rerun for comparison in this paper and it is immediately obvious that resolution of up to DP19 is now achieved, although accurate quantitation is not possible above DP16.

There are considerable differences in the oligosaccharide component composition for all extracts examined, although the extract from *Arnica montana* L. does show some similarity to the extract from *Artemisia dracunculus* L. in that each contains relatively high proportions of lower molecular weight oligosaccharides (up to DP7) and considerably more IMW and HMW material than that from the extract of *Artemisia absinthium* L. (Table 1). This is significant, since TLC clearly showed (see Fig. 1) that the extracts from the roots of *Arnica montana* L. and *Artemisia dracunculus* L. contained two series of oligosaccharides, namely a reducing series of inulin-type oligosaccharides which were reactive to triphenyltetrazolium chloride, and a non-reducing series of oligofructosides, reactive to diphenylamine aniline phosphate, to which α-D-glucose was glycosidically (2 ↔ 1) bound at the reducing end of the

fructo-oligosaccharide. The extract from *Artemisia absinthium* L. was only reactive towards diphenylamine aniline phosphate, demonstrating that this extract contained non-reducing oligofructosides only.

The differences in oligosaccharide component composition in all extracts and the presence of both reducing and non-reducing oligofructosides in the extracts of *Arnica montana* L. and *Artemisia dracunculus* L. and only non-reducing oligofructosides in *Artemisia absinthium* L. is almost certainly related to biosynthetic activity within each plant.

The roots from *Arnica montana* L. and *Artemisia dracunculus* L. were collected in June and March respectively at the initial stage of growth (sprouting stage) for which maximal biosynthetic activity is to be expected. The similarity between the two samples is also shown in their oligosaccharide component composition (see Table 1). Whilst the actual values differ for the two sample types, the same basic distribution in oligosaccharide components, namely a low (4–6%) level of DP1, a high (11–19%) level of DP2, then a gradual decrease in the content of individual oligosaccharides from DP3 to DP16, is seen. The level of intermediate molecular weight (IMW) material is slightly lower than that of DP2 (i.e. 9–13%), with a small (0–1%) level of high molecular weight material. The presence of inulin-type reducing oligosaccharides (as indicated by thin-layer chromatography) in both these extracts would strongly suggest that the oligofructosides are acting as D-glucopyranosyl residue donors (Bacon, 1960) or storage material which can generate the more metabolically-active sucrose in this early stage of development.

On the other hand, the roots from *Artemisia absinthium* L. were collected in September after flowering and at the onset of the quiescence stage, at which biosynthetic activity would have either stopped or slowed down considerably. This is borne out by a different oligosaccharide component composition (see Table 1), in that levels of IMW (DP16–25) are very high and the level of HMW is also raised, whilst the distribution in the range DP3 to 16 follows a gradual increase from 2·2% (DP3) to 4·4% (DP10) and then a decrease to 3·5% (DP16). Since this extract contained only non-reducing oligofructosides it would appear that all D-glucopyranosyl residue transfer activity or storage of material which can produce sucrose has ceased.

In conclusion it has been shown that the vegetative stage of the plants affects the use of thin-layer and gel-permeation chromatography analysis of the oligosaccharides extracted from the roots. Further studies into the optimal concentration of pharmacologically-active ingredients within the roots of such plants related to oligosaccharide composition need to be undertaken. Such a study would be of use in determining the optimal

time of harvest for such plants to ensure the desired pharmacological properties. The diversity of the oligosaccharide composition reported herein would appear to have some promise in the development of 'guide' substances for such purposes.

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