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MASS SPECTROMETRY OF FLAVONOID TRISACCHARIDES

THE STRUCTURE OF XANTHORHAMNIN, ALATERNIN AND CATHARTICIN

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Abstract—A general mass spectrometric method is described for the structure elucidation of flavonoid oligosaccharides. Information is obtained from the mass spectra of the perdeuteriomethylated compounds and their hydrolysis products, using GC–MS coupling.

The major part of the structures of the flavonoid troisides, xanthorhamnin, alaternin and cathartic in has been established by this method.

IN THE classical method for structure elucidation of flavonoid oligosides the structure of the aglycone and the sugar part of the molecule are determined separately after hydrolysis.[1] The aglycone structure is relatively easy to establish by use of chromatographic comparison with reference samples and physicochemical methods such as electron spectra, NMR and MS.[1] It is much more difficult to identify the sugar sequence (by partial hydrolysis), the attachment position of the oligosaccharide to the aglycone and the position of the interglycosidic linkages (usually accomplished by hydrolysis of the permethylated compounds and identification of the hydrolysis products). It is therefore not surprising that only a few structures of flavonoid oligosides containing more than two sugars are completely known.

It has previously been shown from the mass spectra of 13 perdeuteriomethylated flavonoid disacchardies of known structures that fragment ions could be recognized in these spectra allowing unequivocal identification of the aglycone type, its substituents, the sequence of sugars and the position of the interglycosidic linkage.[2]

In this paper we report that in the case of flavonoid triosides the mass spectra of their perdeuteriomethylated derivatives alone do not reveal all such structural information. However, mass spectrometric analysis of the perdeuteriomethylated flavonoid triosides before hydrolysis and of their constituent parts after hydrolysis provides a promising new technique for nearly complete structure elucidation of this class of compounds.

RESULTS AND DISCUSSION

Four flavonol trisacchardides all of which contain one galactose and two rhamnose

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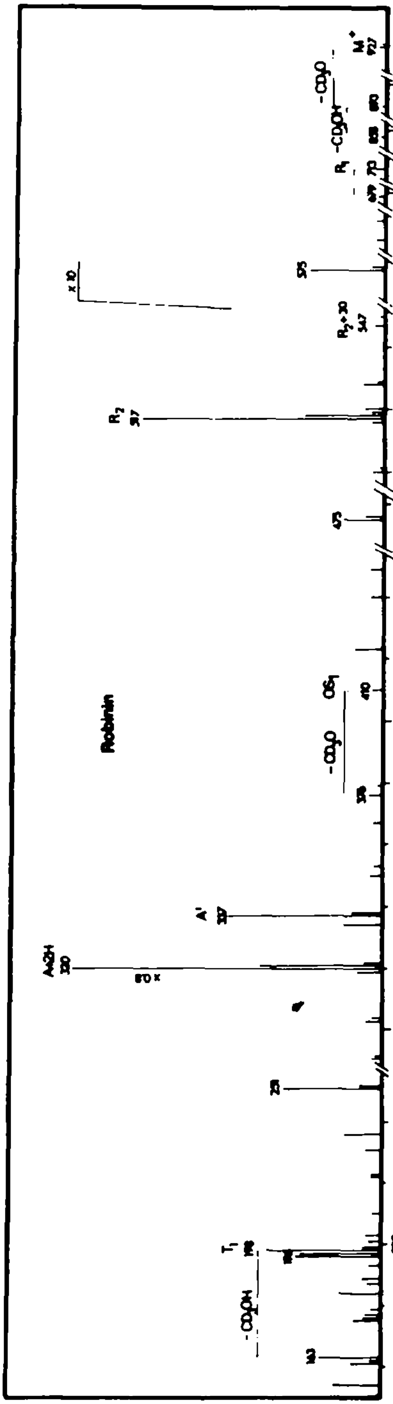
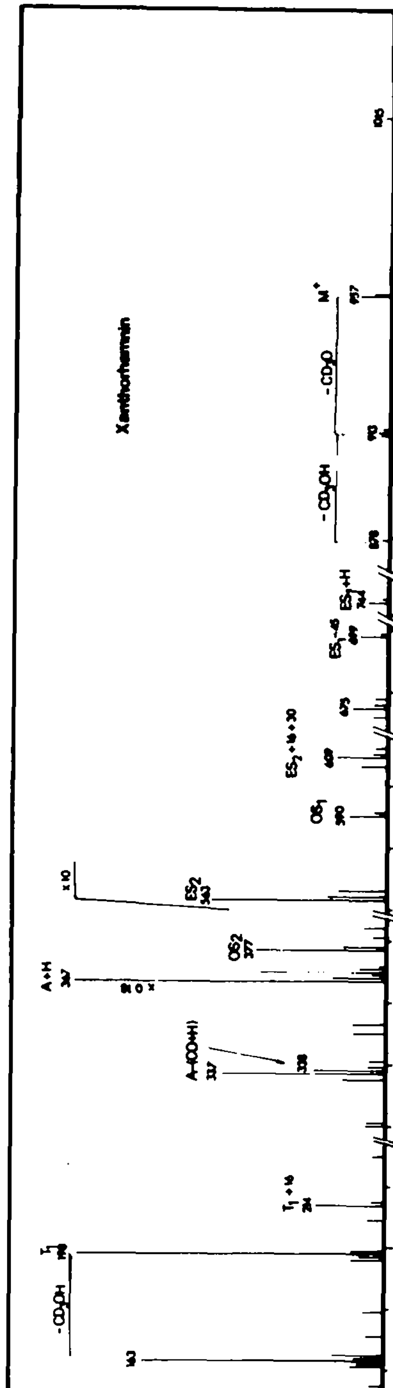
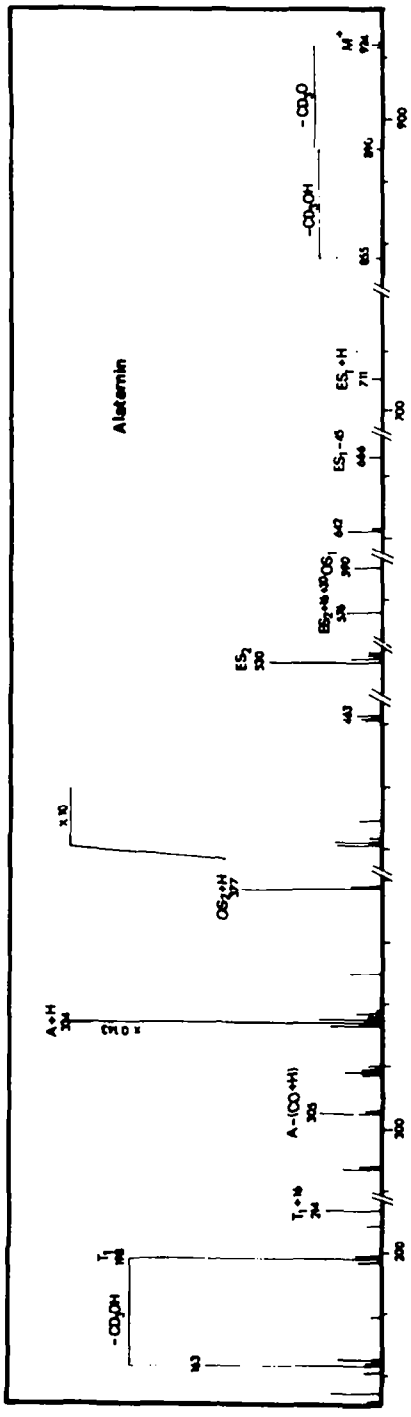

Robinin


Xanthorhamnin


Alatamin
Catharticin

residues have been studied. The partial structures of compounds I–IV known before the beginning of this work are given in Table 1. The mass spectra of these compounds after perdeuteriomethylation are reproduced in Figs 1–4. Peaks below *m/e* 150 are omitted. As described before[2] spectra of the permethylated compounds have been recorded to help the interpretation of the fragmentation pattern.

TABLE 1

Flavonoid trisaccharides, a- and b-series[a]	aglycone	position of sugar attachment	sugars[b]	interglycosidic linkage	literature
Robinin I	kaempferol	3, 7	rha, rha-gal	1 → 6	3
Xanthorhamnin II	7-O-methylquercetin	3	rha rha gal	?	4
Alaternin III	7-O-methylkaempferol	?	rha rha gal	?	5
Catharticin IV	7-O-methylkaempferol	3?	rha rha gal	?	6

[a] a-series: natural compounds; b-series: compounds after perdeuteriomethylation.
[b] rha = rhamnose: gal = galactose.

All 4 perdeuteriomethylated flavonoid trisaccharides (Ib–IVb) show molecular ion (M^+) peaks. The main fragments which have been identified are given in Table 2 and explained below (Fig 5).

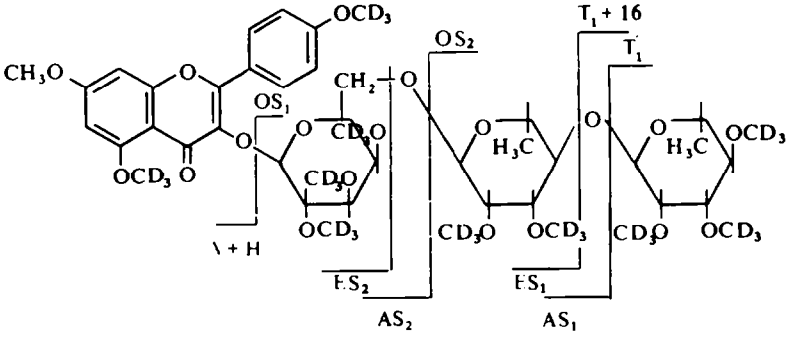


FIG 5

Cleavage at each side of the glycosidic oxygen of the terminal sugar leads to the strong fragments T_1 and $T_1 + 16$. The former successively loses 2 molecules of CD_3OH as indicated by metastable ions. Retention of charge on the aglycone containing part of the molecule leads to the sequence ions ES_1 or AS_1. ES_1 peaks

are accompanied by H-transfer. The same type of fragmentation occurs between the second and third sugar giving rise to the sequence ions ES_2 or AS_2 and the disaccharide ion OS_2.

A peak 45 m.u. below ES_1 is explained by the loss of (CH_3CHO + H°) from the second rhamnose moiety.

Ions 30 m.u. higher than AS_2 can be represented by the structure shown in Fig 6.

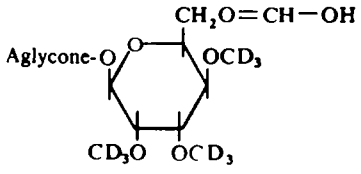


FIG 6

The lower part of the spectra is dominated by ions due to the usual fragmentation pathways of permethylated sugars.[7]

The perdeuteriomethylated diglycosidic trisaccharide robinin (Ib) under electron impact loses first the rhamnosyl residue of the disaccharide as indicated by fragment R_1 (Fig 1). Loss of the rhamnosyl residue on C-7 of the aglycone follows and leads to R_2. Finally, the galactosyl fragment is lost to give the aglycone peak at *m/e* 320 (A + 2H).

The disaccharide ion OS at *m/e* 410 which loses CD_3O giving rise to a peak at *m/e* 376 indicates a 1 → 6 linked bioside since it is formed with loss of hydrogen.[2]

A strong peak at *m/e* 197 not found in linear oligosides is indicative of a diglycoside.[2]

Aglycone structures and sugar attachment position. Aglycone masses are recognized as strong A + H peaks in the mass spectra of the perdeuteriomethylated glycosides. The relative intensity of these peaks gives some clue to the nature of the aglycone.[2] Its very high intensity in compounds Ib–IIIb suggests flavonol aglycones while this seems questionable for perdeuteriomethylated catharticin, IVb. However, comparison of the mass spectra of kaempferol and the aglycone of catharticin (obtained by hydrolysis of the underivatized glycoside) prove that the latter is a kaempferol monomethylated in the A-ring thus substantiating the results obtained by spectral analysis.[6]

Inspection of the A + H column in Table 2 indicates the following substituents (except sugars) of the flavonol ring:*

Robinin	2 OCD_3, 1 OH
Xanthorhamnin	3 OCD_3, 1 OCH_3
Alaternin	2 OCD_3, 1 OCH_3
Catharticin	mixture of 2 OCD_3, 1 OCH_3 and 3 OCD_3

The substitution pattern of the aglycone is derived from the mass spectra of the perdeuteriomethylated flavonoids after hydrolysis which leaves the attachment point of the sugars as the only free OH group.

* Calculation for xanthorhamnin: 238 m.u. (flavonol) + 30 m.u. (OCH_3) + 3 × 33 m.u. (OCD_3) gives *m/e* 367 for A + H after perdeuteriomethylation.

TABLE 2

Flavonoid (perdeuteriomethylated)	M^+	ES_1^*	AS_1	ES_2	AS_2	A + H	OS_1^*	OS_2^*	T_1
Xanthorhamnin, IIb	957	744[a]	—	563	—	367	590	377	198
Alaternin, IIIb	924	711[a]	—	530	—	334	590	377	198
Catharticin, IVb	924	—	726	530	546	334	590	377	198
	927		729	533	549	337			
Robinin, Ib	927					320[a]			

[a] Accompanied by H-transfer

Though flavonols undergo little fragmentation and show weak or no peaks due to retro-Diels–Alder cleavage,[8,9] information regarding the substitution of the A- and B-ring is obtained by fragments *a* and *b*, respectively[8] (Fig. 7).

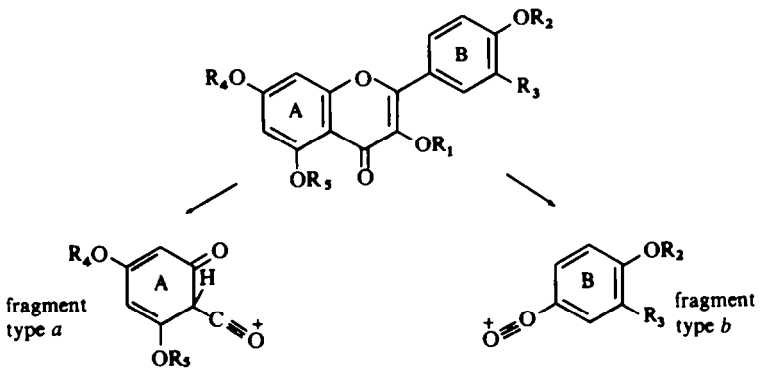


FIG 7

Weak but significant *a*-type fragments are found in three of the four aglycone derivatives. Their values as given in Table 3 indicate that no free OH groups are present; therefore the trisaccharides of xanthorhamnin, alaternin and catharticin have not been attached to the A-ring. The same conclusion for the B-ring can be drawn from the values of the *b*-type fragments of the robinin, xanthorhamnin and alaternin aglycone derivatives. Since the A- and B-rings of xanthorhamnin and alaternin after perdeuteriomethylation and hydrolysis contain no free OH group the sugars must have been attached through the 3-OH group in these compounds. In catharticin, however, the presence of a free OH group in the *b*-type fragment (as indicated by its *m/e* value in Table 3) provides direct evidence for attachment of the sugar residue to the B-ring.

As mentioned above robinin must be considered to be a diglycoside because of the mass of its aglycone fragment. Since the *b*-type fragment of the robinin aglycone derivative contains no free OH group (Table 3), the sugars must be linked through the 3-OH group and an OH group in the A-ring.

TABLE 3

Aglycone[a] obtained from	*m/e* Values				Intensity[b]
	M^+	$M^+ - CO—H$	Type a fragment	Type b fragment	$M^+ - 1$
Robinin	320	291	—	138	10
Xanthorhamnin	367	338	184	171	12
Alaternin	334	305	184	138	10
Catharticin[c]	334	305	185	121	110

[a] Aglycone after hydrolysis of the perdeuteriomethylated flavonoid glycoside.
[b] Percent intensity relative to M^+.
[c] Additional peaks at *m/e* 337, 308 and 188 show "Catharticin" to be a mixture of the kaempferol- and 7-O-Methylkaempferol-glycosides.

The aglycones obtained after hydrolysis of perdeuteriomethylated robinin, xanthorhamnin and alaternin give mass spectra which as a group are different from that of the catharticin aglycone prepared in the same way. The latter containing a OCD_3 group in 3-position is distinguished by two features (Table 3): the presence of an intense $M^+ - 1$ peak to which the structure shown in Fig 8 can be ascribed, and the transfer of D instead of H in the formation of the *a*-type fragment (in agreement with this interpretation the *a*-type fragment is observed at *m/e* 181 when CH_3I is used for derivatization).

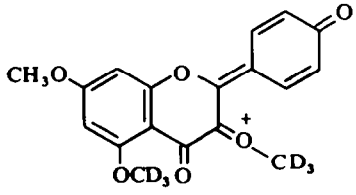


FIG 8

TABLE 4[a]

Flavonoid (perdeuteriomethylated)	Terminal sugar			Sugar 2		Sugar 3	
	$T_1 + 16$	T_1	$M^+ - S_1$	$S_1 - S_2$	$OS_2 - T_1$	$S_2 - A$	$OS_1 - OS_2$
Xanthorhamnin IIb	214	198	213	181	179	196	213
Alaternin IIIb	214	198	213	181	179	196	213
Catharticin IVb	214	198	198	180	179	196	213
		T_1	$M^+ - R_1$	$R_1 - R_2$	$OS_1 - T_1$	$R_2 - A$	
Robinin, Ib		198	214	196	212	197	

[a] Values in m.u. S stands for ES or AS

TABLE 5

Flavonoid (perdeuterio-methylated)		RT_{glu}[a]	L	H	h	F_1	f	E	D	proposed structure	blocked[b] positions
	sugar 1	0·48	—	—	—	167 (168)	107 (108)	134 (134)	120 (121)	1,5-di-O-acetyl-2,3,4-tri-O-methyl-rhamnitol	1,5
Xanthorhamnin IIb	sugar 2	0·98	—	—	—	167 (168)	107 (108)	—	120 (121)	1,4,5-tri-O-acetyl-2,3-di-O-methyl-rhamnitol	1,4,5
	sugar 3	2·83	239 (239)	192 (192)	132 (132)	167 (168)	108 (108)	—	120 (121)	1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-galactitol	1,5,6
	sugar 1	0·48	—	—	—	167 (168)	107 (108)	134 (134)	120 (121)	1,5-di-O-acetyl-2,3,4-tri-O-methyl-rhamnitol	1,5
Alaternin IIIb	sugar 2	0·95	239 (240)	—	—	—	—	134 (134)	120 (121)	1,3,5-tri-O-acetyl-2,4-di-O-methyl-rhamnitol	1,3,5
	sugar 3	2·89	239 (239)	192 (192)	132 (132)	167 (168)	107 (108)	—	120 (121)	1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-galactitol	1,5,6
	sugar 1	0·48	—	—	—	167 (168)	107 (108)	134 (134)	120 (121)	1,5-di-O-acetyl-2,3,4-tri-O-methyl-rhamnitol	1,5
Catharticin IVb	sugar 2	0·95	—	—	—	167 (168)	107 (108)	—	120 (121)	1,4,5-tri-O-acetyl-2,3-O-methyl-rhamnitol	1,4,5
	sugar 3	2·83	239 (239)	192 (192)	132 (132)	167 (168)	107 (108)	—	120 (121)	1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-galactitol	1,5,6

[a] Retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol.

[b] Positions originally blocked in the flavonoid and liberated by hydrolysis of its perdeuteriomethylated derivative followed by reduction.
Numbers in brackets give *m/e* values after reduction with $NaBD_4$ instead of $NaBH_4$.
Symbols for fragment species are taken from ref 11.

Another effect which can be ascribed to the 3-OCD_3 group present only in perdeuteriomethylated catharticin (IVb) is the weak relative intensity* of its A + H peak (Fig 4) as compared to the strong A + H peaks in the mass spectra of the other perdeuteriomethylated glycosides (Figs 1–3).

Sugar sequence and linkage. By comparing the mass differences shown in Table 4 with the calculated masses for perdeuteriomethylated rhamnose and galactose, the sequence rha-rha-gal-aglycone is clearly established in all 3 flavonoid triosides (IIb–IVb).

Unfortunately, unlike in the case of flavonoid biosides, the presence or absence of H-transfer accompanying the cleavage of an interglycosidic linkage does not give much information on the position of such linkage in triosides. Transformation of the monosaccharides obtained by hydrolysis of the perdeuteriomethylated flavonoid triosides into their alditol acetates and analysis of these by GC–MS coupling—a method introduced by the Stockholm group for the analysis of polysaccharides[10–13]—is a general method to obtain the position of the interglycosidic linkages.

This method indicates not only the sugar OH groups blocked in the oligosaccharide and liberated by hydrolysis and reduction but because of its combination with GC gives also some information on the stereochemistry of the sugars and thereby helps around a severe limitation of mass spectrometry.

GC indicates that alditol acetates are obtained in roughly 1:1:1 ratio after reduction and acetylation of the hydrolysis mixture of a perdeuteriomethylated flavonoid trioside.

Table 5 shows retention times and *m/e* values of fragments having structural significance. These data lead to the conclusion that the sugar sequences and interglycosidic linkages are

$rha\xrightarrow{1\to4}rha_p\xrightarrow{1\to6}gal$—	or	
$rha\xrightarrow{1\to5}rha_f\xrightarrow{1\to6}gal$—	for	xanthorhamnin†
$rha\xrightarrow{1\to3}rha\xrightarrow{1\to6}gal$—	for	alaternin
$rha\xrightarrow{1\to4}rha_p\xrightarrow{1\to6}gal$—	or	
$rha\xrightarrow{1\to5}rha_f\xrightarrow{1\to6}gal$	for	catharticin†

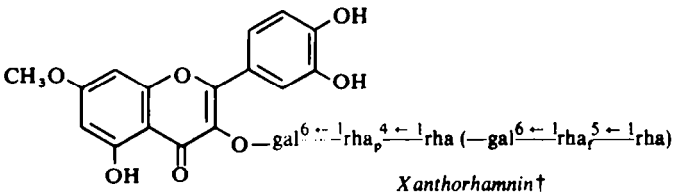
The ambiguity for xanthorhamnin and catharticin stems from the fact that a 4-linked hexopyranose and a 5-linked hexofuranose give the same partially methylated alditol acetate.

CONCLUSION

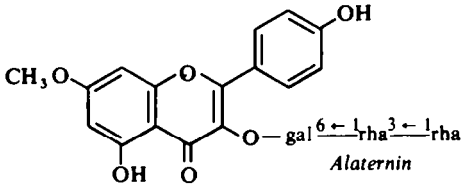
By using mass spectrometry and GC-MS coupling, the structures of the flavonoid triosides, xanthorhamnin, alaternin and catharticin have been established to be:

* Relative to the fragment at *m/e* 107 abundant in most perdeuteriomethylated sugars.[7]

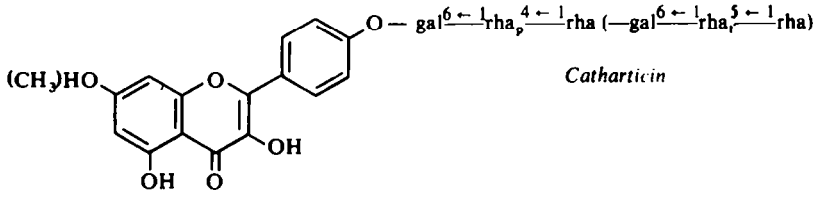
† rha_p: rhamnopyranose; rha_f: rhamnofuranose; gal_p: galactopyranose; gal_f: galactofuranose.



Xanthorhamnin†



Alaternin



*Cathartic*in

The configuration at C-1 and C-5 of the sugars remains unknown. Since robinin has been found to be a mixture of 4 isomers[15] differing by the ring size of the rhamnose in 7-position and its anomeric linkage, it is quite possible that similar isomerisms are present within the 3 flavonoid triosides discussed.

The present work shows that it is possible to establish the full structure of flavonoid triosides by a combination of mass spectrometric methods. The following approach is suggested:

(1) The flavonoid oligoside is perdeuteriomethylated and a mass spectrum recorded. One obtains the sugar sequence and some information on the aglycone type and substitution.

(2) The perdeuteriomethylated compound is hydrolyzed and a mass spectrum is taken of the precipitated aglycone. This provides the substitution pattern of the aglycone and the sugar attachment point.

(3) The sugars in the supernatant of the hydrolysis mixture are reduced with $NaBD_4$ and acetylated. After analysis by GC–MS one obtains the position of the interglycosidic linkages and—by virtue of GC—information on the sugar stereochemistry.

While in favorable cases the full structure of flavonoid oligosaccharides may be resolved by this method, some limitations should be pointed out.

(i) In the absence of sufficient standards, stereochemistry of the sugars remains obscure.

(ii) The sugar configuration at C-1 and C-5 is not resolved.

(iii) In special cases the exact interglycosidic linkage cannot be given. For instance, one cannot decide between the sequences †

$$\text{—gal}\xrightarrow{3\leftarrow 1}\text{gal}_f\xrightarrow{5\leftarrow 1}\text{rha}$$

$$\text{—gal}\xrightarrow{3\leftarrow 1}\text{gal}_p\xrightarrow{4\leftarrow 1}\text{rha}$$

$$\text{—gal}\xrightarrow{4\leftarrow 1}\text{gal}_p\xrightarrow{3\leftarrow 1}\text{rha}$$

because all three give the same mixture of alditol acetates.

(iv) The exact localisation of substituents in the aglycone may prove difficult (e.g., differentiation between a 5,7- and a 6,7-dihydroxy-flavonoid).

While (i) usually can be solved by thin layer or paper chromatography and (iv) by spectrophotometry, at present there seems to be no straightforward method to solve (ii) and (iii) if only small amounts of higher flavonoid oligosides are available.

EXPERIMENTAL

Perdeuteriomethylation and permethylation of the samples were carried out as described before.[2] Hydrolysis was achieved by heating for 1 hr at 100° in 1 N H_2SO_4.

After neutralisation with $BaCO_3$, preparation of the alditol acetates was achieved as described in the lit.[13]

Stationary phase for GC was 3% OV-225 on Gas–Chrom Q, 100–120 mesh. Running conditions were taken from the lit.[14]

The gas chromatograph (Varian 1540) was coupled via a Biemann separator to an AEI–MS 9.

Temp of the connecting units and the source was maintained at 200°.

All mass spectra were recorded on an AEI–MS 9 instrument using a direct insertion probe and operating at 70 eV. Temperature varied between 180 and 250°.

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