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Determination of 25 Low Molecular Weight Carbohydrates in Tobacco by High Performance Ion Chromatography*

by

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SUMMARY

A High Performance Ion Chromatographic method for the qualitative and quantitative determination of low molecular weight carbohydrates in tobacco leaf is described. The carbohydrates were separated by running isocratic or gradient elutions on columns packed with amino-modified polystyrene divinylbenzene phases, using 0.1 N NaOH as eluent. The compounds were detected by a Pulsed Amperometric Detector, after post-column addition of 1 N NaOH solution. Twenty-two and twenty-four carbohydrates out of twenty-five were separated by isocratic and gradient elution, respectively. Some mono and disaccharides were identified in tobacco samples by their retention times and some quantitative determinations of glucose, fructose, inositol and saccharose were carried out using different varieties of tobacco.

ZUSAMMENFASSUNG

Eine Methode zur qualitativen und quantitativen Bestimmung von niedermolekularen Kohlehydraten in Tabakblättern durch (Hochleistungs-)Ionenchromatographie wird beschrieben. Die Trennung der Kohlenwasserstoffe erfolgte durch isokratische oder Gradientenelution mittels Säulenchromatographie. Die Säulen waren mit Polystyrendivinylphasen, die mit Aminogruppen modifiziert wurden, gefüllt, wobei 0,1 N NaOH als Elutionsmittel diente. Die Detektion der getrennten Verbindungen erfolgte amperometrisch nach Zugabe einer 1N NaOH-Lösung. 22 und 24 Kohlehydrate von insgesamt 25 wurden isokratisch beziehungsweise mittels Gradientenelution getrennt. Einige Mono- und Disaccharide wurden durch ihre Retentionszeit aus den Tabakproben identifiziert und einige quantitative Bestimmungen von Glukose, Fruktose, Inositol und Saccharose verschiedener Tabakvarietäten wurden durchgeführt.

RESUME

Ce travail décrit une méthode de chromatographie ionique haute performance utilisée pour l'analyse qualitative et quantitative des hydrates de carbone de faible poids moléculaire contenus dans les feuilles de tabac. Les hydrates de carbone ont été séparés au moyen d'élutions isocratiques et par gradient sur des colonnes garnies de phases au divinylbenzène polystyrène amino-modifiées en utilisant NaOH à 0,1 N comme éluant.

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Un détecteur ampérométrique pulsé a servi à mettre les composés en évidence après addition post-colonne d'une solution 1 N de NaOH. Vingt-deux et vingt-quatre hydrates de carbone sur vingt-cinq ont été respectivement séparés par élution isocratique et par gradient. On a identifié quelques mono et disaccharides dans les échantillons de tabac à partir de leurs durées de rétention; le glucose, le fructose, l'inositol et le saccharose ont été dosés dans différentes variétés de tabac.

INTRODUCTION

Carbohydrates make up a large portion of the leaf of many tobacco varieties, both in the free or in the combined state, and are of great interest because of their relationship to leaf quality and smoke flavour. Many papers have been published concerning the analysis of low molecular weight carbohydrates in tobacco leaf and in cigarette filler (1).
Some authors determined the total reducing sugars with colorimetric methods, others described gas chromatographic procedures using the trimethylsilyl derivatives or estimate the individual compounds by paper and thin-layer chromatography (2-6).
Many authors use the high-performance liquid chromatography for rapid characterization of tobacco sugars (7-16). The purpose of this work is to find the best conditions for detecting and determining as large a number as possible of both reducing and non reducing low molecular weight carbohydrates present in standard mixtures and to apply such conditions to the analyses of tobacco leaf and cigarette filler by the aid of the high-performance ion chromatography with pulsed amperometric detection.

EXPERIMENTAL

Reagents

The twenty-five carbohydrates (Merck, grade for biochemistry) reported in Table 1 were used without further purification to prepare the standard solutions, using distilled water, purified in an Elgastat (ELGASTAT UHR system, by Elga) system (1).
These solutions were kept at 4 °C and were found to be stable for at least 15 days. Solutions of 0.1 N and 1.0 N NaOH (Merck or Fluka reagent grade) were used. All eluent solutions were prepared by bubbling helium through the water to prevent carbon dioxide contamination, and were kept under a blanket of helium during elution.

APPARATUS AND PROCEDURES

A Dionex Bio-LC chromatograph equipped with:

- a gradient pump module, operating up to 4000 p.s.i.;
- a Dionex eluent degas module with eluent container set;
- a pulsed amperometric detector (PAD-2) containing a working gold electrode;
- a Dionex sample injection valve with a 50 µl sample loop;
- a Dionex DOP-1 post column pumping system;
- On-guard RP filters;
- HPIC-AS6 column with amino modified polystyrene-divinylbenzene phase (250 x 4 mm, 10 µm particle size bed) protected by a guard column HPIC-AG6 (70 x 4 mm);
- HPIC-AS6A column (150 x 4 mm) with amino modified polystyrene-divinylbenzene phase, 5 µm particle size bed, protected by a guard column HPIC-AG6A (50 x 4 mm);
- a Varian 1200 recorder.

OPERATING CONDITIONS

Temperature: room-temperature in the range 18 - 23 °C

PAD operating parameters: E_1 = 0.10 V
E_2 = 0.60 V
E_3 = -0.80 V
$T_1 = T_2 = T_3$ = 300 msec

Output range: 10 µA; chart speed: 1.0 cm/min

For isocratic elutions:
Flow rate: 0.6 ml/min
Eluent: NaOH 0.1 N

For gradient elutions:
Flow rate: 1.0 ml/min;
Gradient program:

Time (min)	Water (%)	NaOH (0.2N) (%)
0	100	0
5	100	0
8	85	15
15	85	15
45	10	90
50	10	90
51	100	0

Post column reagent: NaOH 1 N at flow rate of 1 ml/min.

Table 1.
Chromatographic data of 25 carbohydrates in the HPIC system.

Peak Numbers	Sugars	[a]t_R (min)	[b]t_R	[c]RRT (min)
1	Inositol	1.71	0.10	0.19
2	Xylitol	1.89	0.22	0.21
3	Sorbitol	2.23	0.44	0.26
4	Mannitol	2.45	0.59	0.30
5	Trehalose	2.62	0.70	0.33
6	Fucose	2.62	0.70	0.58
7	2-Deoxy-D-Ribose	2.89	0.87	0.69
8	2-Deoxy-D-Galactose	3.04	0.97	0.77
9	Rhamnose	3.20	1.07	1.00
10	2-Deoxy-D-Glucose	3.56	1.30	1.13
11	Arabinose	3.56	1.30	1.15
12	Galactose	3.98	1.58	1.29
13	Lyxose	4.23	1.73	1.39
14	Glucose	4.23	1.73	1.44
15	Xylose	4.30	1.78	1.51
16	Fructose	4.63	1.99	1.55
17	Allose	4.99	2.23	1.64
18	Ribose	5.17	2.34	1.74
19	Melibiose	5.41	2.50	1.74
20	Sucrose	6.77	3.38	1.96
21	Lactose	7.21	3.66	2.32
22	Cellobiose	9.67	5.26	2.72
23	Gentiobiose	10.18	5.59	2.80
24	Raffinose	11.60	6.51	2.89
25	Maltose	15.99	9.34	3.55

* [a]t_R = Retention times in isocratic elution
[b]$t'_R = (t_c - t_0)/t_0$, where t_c = retention times of carbohydrates and t_0 = retention time of eluent in isocratic elution
[c]RRT = retention times relative to rhamnose in gradient elution.

ANALYSES OF TOBACCO

The tobacco leaves or the cigarette filler were ground and amounts of 100 mg of tobacco powder were weighed. The samples were shaken twice with 50 ml of distilled and purified water and both extracts were combined and filtered. The solution was then injected through an On-Guard RP filter into the sample injection valve of the chromatograph using a 50 µl sample loop. Samples were analyzed not later than eight hours after the extraction, because the solutions of carbohydrates extracted from tobacco were unstable.

RESULTS

The chromatographic data of 25 carbohydrates are given in Table 1. The retention times and the values of t'_R were obtained under isocratic conditions. The retention times under gradient conditions are relative to rhamnose. Twenty-two out of twenty-five carbohydrates were separated by isocratic elution and twenty-four out of twenty-five by gradient elution. For detection and identification a pulsed amperometric detector has been used. The chromatograms are shown respectively in Figures 1 and 2. Several tobacco leaf samples, obtained from different varieties, were analyzed. Typical chromatograms of some main varieties are shown in Figures 3-6. Some mono and disaccharides were identified from their retention times; the most important were inositol, glucose, fructose, sucrose and galactose, the latter only in samples of Burley.

Some quantitative determinations of glucose, fructose, inositol and sucrose were carried out for samples of oriental tobaccos, bright and Kentucky. The range of concentrations are given in Table 2.

In order to check the range of linearity of the detector response a linear calibration graph was obtained for each of the four carbohydrates by plotting peak areas *vs* concentrations (range 1-100 µg/ml) with correlation coefficients in the range 0.99939 - 0.99996. The linearity of the responses was confirmed using rhamnose as internal standard. Linear calibration graphs were obtained by plotting the ratio A_c/A_r *vs* concentrations,

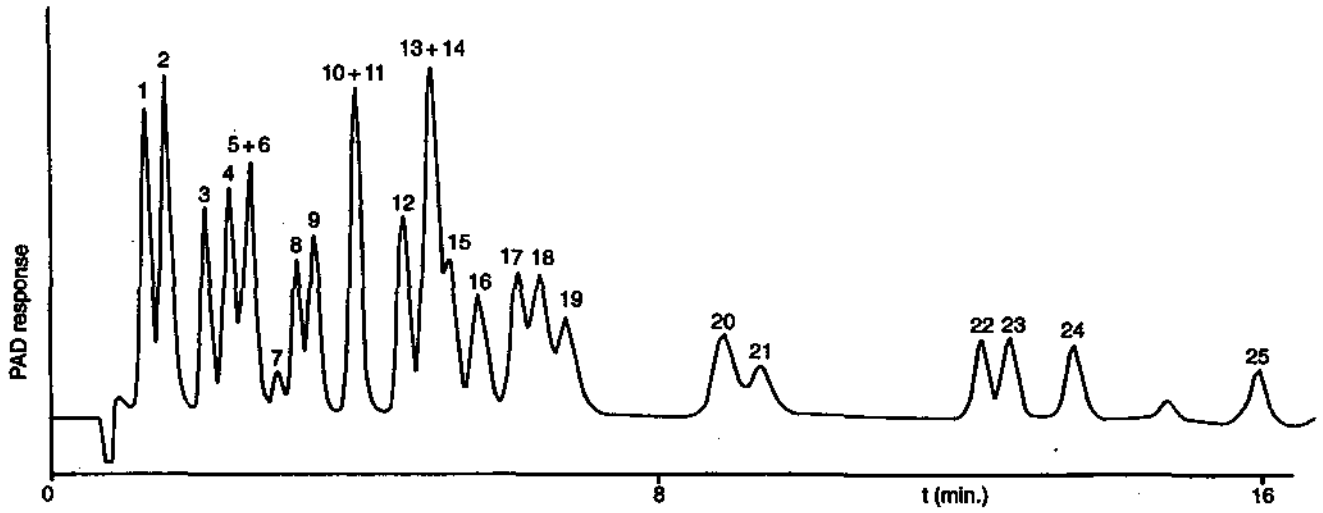

Figure 1.
Chromatogram of a standard mixture of carbohydrates separated by isocratic elution. The numbers of the peaks correspond to the compounds reported in Table 1.

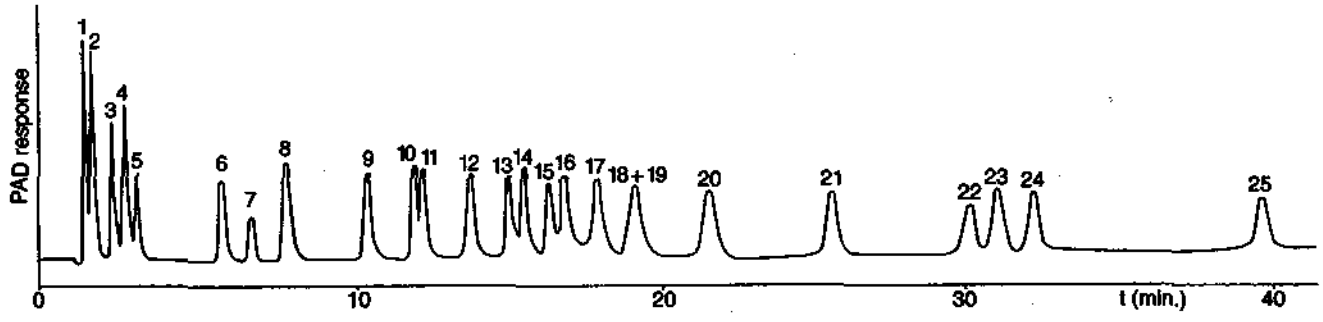

Figure 2.
Chromatogram of a standard mixture of carbohydrates separated by gradient elution. The numbers of the peaks correspond to the compounds reported in Table 1.

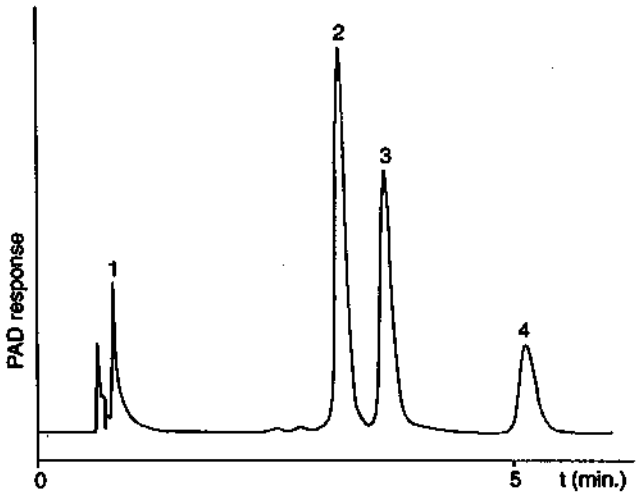

Figure 3.
Bright - Isocratic elution. Meter scale: 10 µA.

1	:	Inositol	2	:	Glucose
3	:	Fructose	4	:	Sucrose

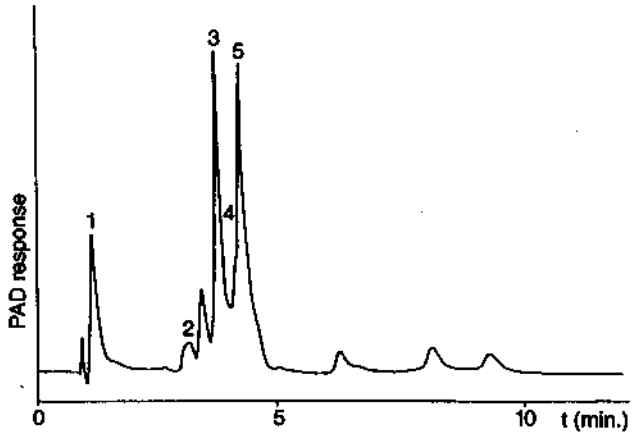

Figure 4.
Xanty - Isocratic elution. Meter scale: 10 µA.

1	:	Inositol	2	:	Arabinose
3	:	Glucose	4	:	Xylose
5	:	Fructose			

were A_c is the peak area of each carbohydrate and A_r is the peak area of rhamnose.
The correlation coefficients were in the range 0.99984 - 0.99999.

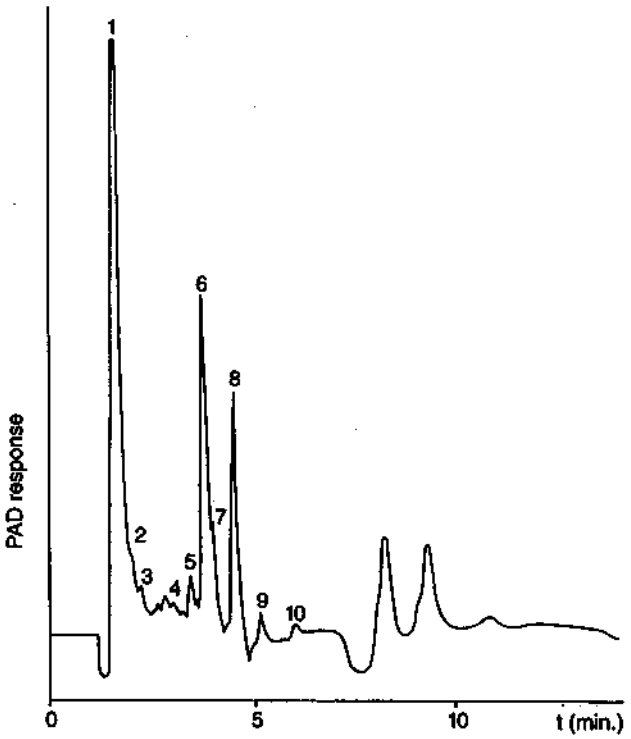

Figure 5.
Kentucky - Isocratic elution. Meter scale: 3µA.

1	:	Inositol	2	:	Sorbitol
3	:	Mannitol	4	:	Rhamnose
5	:	Arabinose	6	:	Glucose
7	:	Xylose	8	:	Fructose
9	:	Melibiose	10	:	Sucrose

DISCUSSION AND CONCLUSIONS

Carbohydrates behave as weak acids with a pKa in the range 12 - 14. They are present in the anionic form in solutions with a pH > 12 and can be retained and separated on anion exchange columns. Carbohydrates are also easily oxidized on the surface of noble metal electrodes such as gold (17-22).
The pulsed amperometric detector uses a multistep potential wave form of three different voltages, each applied for a few hundreds milliseconds, resulting in alternating anodic and cathodic polarizations. The first positive voltage allows, through an oxidating reaction, the amperometric detection of the $-CH_2OH$ functions of carbohydrates; the second higher positive potential cleans electrochemically the electrode surface, while the third negative voltage reduces gold oxide back to gold.

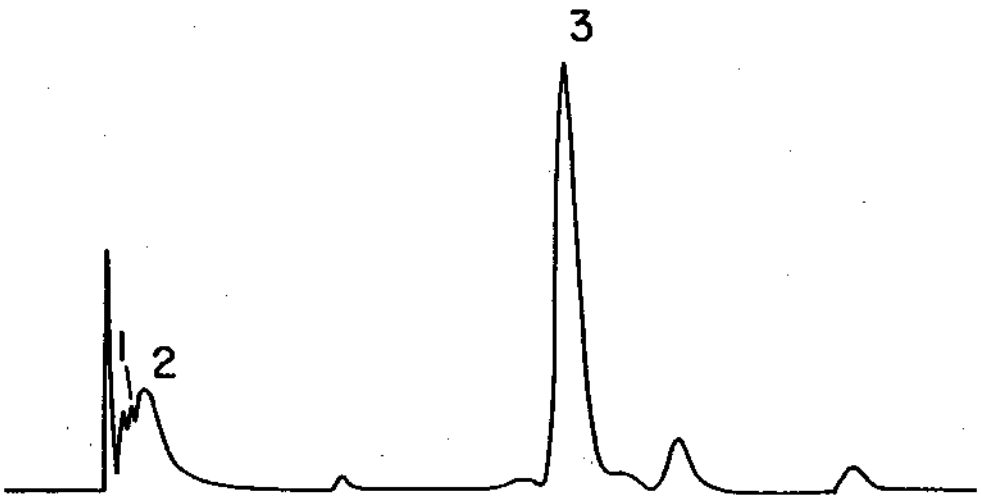

Figure 6.
Burley - Isocratic elution. Meter scale: 10μA.
1 : Inositol 2 : Xylitol 3 : Galactose

With such a detector only molecules having oxidizable functional groups can be detected and therefore only compounds with amino or sulfide groups can interfere, while carboxylic acids and most of the inorganic species do not. The molecules containing carbonyl functions are not oxidized at the operating conditions followed in the present work (23, 24).
Only some of them, such as formaldehyde and acetaldehyde, are detected when they are present in very high concentrations, 100 μg/ml and 200 μg/ml respectively, giving however only low signals.
Furthermore, the anion-exchange chromatography allows a good separation of a large number of carbohydrates, from monosaccharides to low molecular-weight polysaccharides, while non-ionic and basic compounds, even if oxidizable, are not retained and elute in the void volume without any interference. It is still possible to achieve high sensitivity: amounts in the range 0.02-1.00 μg/ml were easily detected, while the detection limits for the most important sugars present in tobacco samples were in the range 5-25 ppm (Table 3). In the gradient elutions the sensitivity is increased by adding a more concentrated solution of sodium hydroxide (1.0 N) after the analytical column, thereby keeping the pH in the detector cell relatively constant and achieving also a lower drift of the baseline. Carbohydrate degradation in high pH solutions was not observed: these reactions are slow on the time scale of chromatography employed.

Table 2.
Range of concentrations of some sugars detected in tobacco varieties.

Varieties	Number of Samples	Inositol (%)	Glucose (%)	Fructose (%)	Sucrose (%)
Oriental	38	0.69 ÷ 1.70	2.0 ÷ 9.5	2.4 ÷ 8.5	traces
Bright	84	0.72 ÷ 1.60	3.2 ÷ 17.0	4.9 ÷ 12.2	0.5 ÷ 6.0
Kentucky	21	1.20 ÷ 1.40	0.5 ÷ 3.0	1.3 ÷ 2.5	traces

Table 3.
Detection limits for some sugars in tobacco.

Peak Numbers	Sugars	S µg/ml	D.L. ppm
1	Inositol	0.02	5
2	Xylitol	0.02	
3	Sorbitol	0.04	
4	Mannitol	0.03	
5	Trehalose	0.07	
6	Fucose	0.06	
7	2-Deoxy-D-Ribose	1.00	
8	2-Deoxy-D-Galactose	0.05	
9	Rhamnose	0.05	
10	2-Deoxy-D-Glucose	0.06	
11	Arabinose	0.06	10
12	Galactose	0.05	10
13	Lyxose	0.06	
14	Glucose	0.05	10
15	Xylose	0.06	
16	Fructose	0.05	10
17	Allose	0.05	
18	Ribose	0.10	
19	Melibiose	0.10	
20	Sucrose	0.10	25
21	Lactose	0.10	
22	Cellobiose	0.15	
23	Gentiobiose	0.10	
24	Raffinose	0.10	
25	Maltose	0.15	

* S = sensitivity
D.L. = Detection limits

Anion-exchange liquid chromatography with pulsed amperometric detection proved to be an efficient technique for the separation, identification and quantitative determination of low molecular weight carbohydrates in tobacco leaves and in cigarette filler.
This system represents a method of choice, providing many advantages such as specificity, selectivity, sensitivity and reliability, which can be used when it is necessary to determine the amounts of the individual reducing and non reducing sugars.

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