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DETERMINATION OF THE TANNING CAPACITY OF TANNIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The complexation of several tannic acids (purified tannins) with soluble and insoluble proteins has been studied by high-performance liquid chromatography. Tannins are analysed in both the straight phase and the reversed-phase mode. Bovine serum albumin (BSA) and beer proteins were chromatographed in the size exclusion mode. Soluble proteins seem to complex preferentially with the higher molecular weight tannin polygalloyl glucose components, whereas insoluble standardized hide powder binds preferentially to lower molecular weight tannin components. The tanning capacity of different tannic acids can be expressed in milligrams of BSA bonded per milligram of tannin under standard conditions.

INTRODUCTION

Tannins are isolated by extraction of leaves and gall nuts from different plant varieties. They can be divided into hydrolysable[1] and condensed tannins[2]. The latter are chiefly used in adhesives and resins[3]. In the group of hydrolysable tannins, the gallotannins are the most important. There are four commercial varieties, which differ in their vegetable origins. The Oriental or Chinese tannin (from *Rhus semialata*), the Sumac tannin *(R. coriaria)* and the Aleppo tannin *(Quercus infectoria)* are polygalloyl glucose mixtures. The Tara tannin *(Caesalpinia spinosa)* is a polygalloyl quinic acid. In each of these natural products galloyl groups are bonded to the central polyol nucleus by a hydrolysable ester bond. In a new formulation of tannic acids, glycosidic bonds have also been proposed[4]. Besides the galloyl glucose components there are in general also small amounts of free gallic, digallic and trigallic acids present in tannin extracts[5,6]. These can be determined by high-performance liquid chromatography (HPLC)[7,8].

The hydrolysable tannins find widespread application because of their antioxidation properties and their ability to form soluble and insoluble complexes with proteins[9,10]. They are used in the leather, food and pharmaceutical industries and in breweries. This requires good quality control. There are a number of testing procedures for tannins. The resin and gum test check the limpidity of water and ethanol solutions. Thin-layer chromatography (TLC) and HPLC are used to determine the

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non-bonded galloyl groups. Organoleptic tests are also common. We have devised HPLC methods to determine the tannic acid content of commercial samples[8]. This content of polygalloyl glucose or polygalloyl quinic acid is, however, not exactly identical with the so-called tanning power of a sample. To determine the tanning capacity (protein precipitating or binding power) the "hide powder assay" is an accepted method prescribed by the American Leather Chemists Association[11–13]. However, this method also determines non-tannins in a tanning extract and therefore gives purity percentages that do not always reflect the real tanning power. This has already been stated by Beasly *et al.*[14]. Other methods of studying tannin–protein complexation have been reported. Van Buren and Robinson studied the complexation with dinitrophenyl derivatized gelatin [10]. Hagerman and Butler reported complexation studies with ^{125}I-labelled BSA[15].

In the present paper a comparison is made between complexation of tannins with underivatized insoluble (hide powder) and soluble proteins (BSA and beer proteins) using HPLC. An attempt is made to express tanning power by a number which allows tannic acids to be compared.

EXPERIMENTAL

The chromatographic system consisted of a Varian 5020 LC pump, a Varian 9176 recorder, a Varian LC-50 UV detector and a 7000 p.s.i. 10-μl Valco sample loop injector. The columns were Lichroma tubes with Valco fittings. For straight phase chromatography a 25 × 0.46 cm I.D. column was packed with 5-μm ROSiL (a spherical silica gel from Alltech-RSL) or 5-μm RSiL (an irregular silica gel from Alltech-RSL). A gradient was run from solvent A (hexane) to solvent B methanol–tetrahydrofuran (3:1) containing 0.25% of citric acid in 30 min. For the reversed-phase mode a 15 × 0.46 cm I.D. column was packed with demineralized 5-μm ROSiL-C_{18}-D (a spherical octadecylated silica gel from Alltech-RSL). A gradient was run from 10% methanol to 100% methanol in water in 30 min; the mobile phase contained 0.5% phosphoric acid. The N-acetylaminopropyl stationary phase[16,17] was synthesized at our laboratory by reaction of N-acetylaminopropyltriethoxysilane with 10-μm RSiL. The dried phase is suspended in carbon tetrachloride and pumped into the column at a pressure of 400 bar with a Haskel air-driven fluid pump. Isooctane was used as the pressurizing liquid. The solvents hexane, methanol and tetrahydrofuran were HPLC-grade from Burdick & Jackson. Tannins were received from Gechem-Omnichem (Wetteren, Belgium). BSA (fraction V-powder) was from Sigma. Beer proteins were isolated from tannin–protein complexes obtained from tannin-stabilized and chillproofed beer. The sludge of precipitated tannin–protein complex is dried by lyophilisation and decomposed in methanol (24 h at room temperature) in which tannins dissolve and proteins are insoluble. The remaining solid material is stirred in water (24 h) to dissolve beer protein. The filtrate of this suspension is freeze-dried, giving pale yellow fluffy proteinic material.

RESULTS AND DISCUSSION

Hide powder assay[11]

This is an accepted method for the determination of the percentage of tannins

in a tanning extract. The tannin purity is expressed as the amount of tannin compounds adsorbed on the standardized hide. Fractions that are not complexed are regarded as impurities. In practice, 50 mg of tannin is dissolved in 50 ml of water and stirred with 900 mg of hide powder for 20 min. The tannin–hide complex is filtered off. The evaporated filtrate is weighed and defined as the percentage impurity in the tannin. To evaluate this procedure we were interested in partial complexation, which would allow better comparison of the tannic acids.

To obtain only partial complexation, 50 mg of tannin was dissolved in 50 ml of water and only 350 mg of hide powder were added. After stirring and filtration, the filtrate was analysed by reversed-phase HPLC. For four different tannins the amount of complexed material is given in Table I. These values are calculated by comparing the sum of all peak integrations from the reversed-phase chromatograms of the solutions before and after the addition of hide powder.

TABLE I

BONDED TANNIN AFTER PARTIAL COMPLEXATION WITH HIDE POWDER

Tannin	*Tannin complexed (%)*	*Hide powder:tannin weight ratio*
Sumac	40	7.4
Chinese	46	7.1
Aleppo	72	7.1
Tara	92	7.0

The filtrate in each of these tests was freeze-dried, dissolved in methanol–tetrahydrofuran and analysed on a straight phase column. Fig. 1a and b shows the chromatograms of Chinese tannin and of such a filtrate, respectively. The successive peaks in the chromatogram are considered as the successive polygalloyl glucose components, following Beasly *et al.*[14].

Table II gives the percentage peak shares of the different polygalloyl glucoses in the Chinese tannin before and after partial complexation with the hide powder. The average number of galloyl groups bonded per glucose unit can be calculated from this table (see Table III).

After partial complexation the average composition is shifted to the higher molecular weight components. This means that the lower molecular weight components are preferentially bonded by the hide powder. That this would be the case is also evident from Table I, which lists the highest bonded figure for the lower molecular weight Tara tannin.

A possible explanation is that a smaller steric volume allows better penetration into the cavities of the insoluble hide. Smaller molecules and also the gallic acids (gallic, di- and trigallic acid) are however generally not considered as real tannins, in contrast to the higher molecular weight components[2]. The choice of hide powder for the determination of the tanning capacity can therefore be criticised.

TABLE II

PEAK SHARES (%) OF THE POLYGALLOYL GLUCOSES BEFORE (B) AND AFTER (A) COMPLEXATION WITH HIDE POWDER

Galloyl glucose	*Tetra*		*Penta*		*Hexa*		*Hepta*		*Octa*		*Nona*		*Deca*		*Undeca*		*Dodeca*	
	B	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*
Sumac			1.7	0	5.9	2.9	14.5	14.5	22.2	25.4	22.4	26.1	21	18.3	8.5	9.3	3.8	3.6
Chinese			4.4	0.2	10.4	5.9	18.1	18.8	21.5	24.5	19.3	22.3	13.8	15.5	8.2	8.8	4.3	4.1
Aleppo	13	2.6	31.5	27.9	31.7	38.5	15.9	21.2	6.2	8.1	1.7	2.5						

TABLE III

AVERAGE COMPOSITION EXPRESSED AS THE NUMBER OF GALLOYL GROUPS PER GLUCOSE UNIT

Tannin	*Number of galloyl groups per glucose unit*	
	Before complexation	*After complexation*
Sumac	8.7	8.9
Chinese	8.4	8.7
Aleppo	5.8	6.1

Bovine serum albumin

Since hide powder is unsatisfactory for establishing tanning capacity, an alternative is needed. Obviously a well-defined protein is indicated. Beer protein is not in this category, because it is a complex mixture of variable composition. We chose bovine serum albumin (BSA), which is commercially available and which is, in principle, monodisperse and not a mixture.

If an excess of tannin is used, the affinity of BSA for the different tannins can be evaluated. With an excess of protein, the affinity of the tannic acids for BSA can be established.

Excess tannin. To obtain partial complexation with BSA, 50 mg of tannin is dissolved in 5 ml of 0.01 *M* phosphate–citrate buffer (pH 4.3; about the pH of beer), added to a solution of 5 mg of BSA in 5 ml of buffer and stirred for 30 min at room temperature. The milky suspension is filtered on a paper filter (Schleicher and Schull, F.R.G., diameter 7 cm, number 600) under atmospheric pressure. The remaining tannin in the filtrate is determined by reversed-phase HPLC. The amounts complexed are listed in Table IV.

TABLE IV

HPLC ANALYSIS OF THE FILTRATE AFTER PARTIAL COMPLEXATION WITH BSA

Tannin	*Tannin : BSA weight ratio*	*Tannin complexed (%)*
Chinese	8.9	56.5
Sumac	10.1	48.3
Aleppo	9.2	35.8
Tara	9.7	37.2

The tannins with lower molecular weight (Aleppo and Tara) are bonded least by BSA. The filtrate of the Sumac–BSA complex was freeze-dried and analysed by straight phase HPLC. Fig. 2a and b shows the chromatograms of the original and partially complexed Sumac, respectively. The components with low molecular weight do not form insoluble complexes with BSA, in contrast to hide powder.

TABLE V

PEAK SHARES (%) OF THE POLYGALLOYL GLUCOSES BEFORE (B) AND AFTER (A) COMPLEXATION WITH BSA

Galloyl glucose	*Tetra*		*Penta*		*Hexa*		*Hepta*		*Octa*		*Nona*		*Deca*		*Undeca*		*Dodeca*	
	B	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*
Sumac			1.7	1.1	5.8	5.1	14.6	16.4	23.4	26.3	24.0	25.2	16.8	14.6	9.3	6.4	4.4	–
Chinese			3.8	2.3	9.2	8.1	16.3	18.1	20.8	23.4	19.7	20.4	14.9	13.0	9.5	7.1	5.8	–
Aleppo	13.5	12.4	31.4	33.9	29.8	30.8	15.8	14.9	6.8	5.7	2.7	2.4						

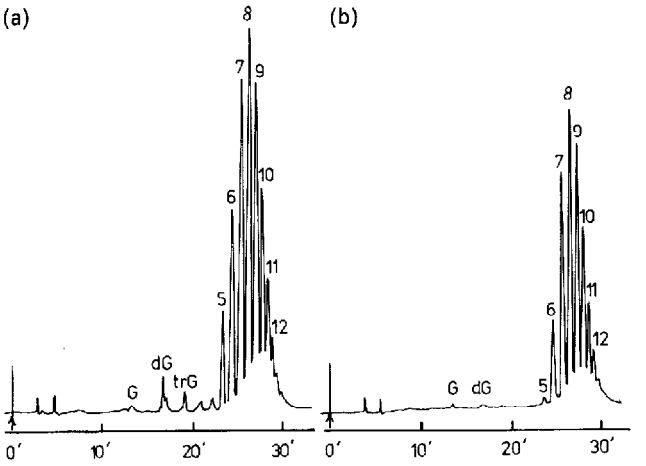

Fig. 1. (a) Straight phase analysis of Chinese tannin. Peaks: G = gallic acid; dG = digallic acid; trG = trigallic acid; 5–12 = penta- to dodecagalloyl glucose. Column, 25 × 0.46 cm I.D. filled with extra demineralized 5 μm ROSiL; mobile phase, A = hexane, B = methanol–tetrahydrofuran (3:1) with 0.25% citric acid, programmed from 80% A–20 % B, to 50% A–50% B at 15 min, to 35% A–65% B at 30 min; flow-rate, 1 ml/min; recorder speed, 0.5 cm/min; detection wavelength, 280 nm. (b) Straight phase analysis of Chinese tannin after partial complexation with hide powder.

Table V lists the percentage peak shares of the different polygalloyl glucoses before and after complexation with BSA (calculated from the chromatograms). As for hide powder, the average compositions of the original and partial complexed Sumac can be calculated. These are shown in Table VI.

The average molecular weight is obviously smaller after complexation, which means that the larger galloylglucoses have the highest affinity for the soluble BSA.

Apparently there is an important difference between the complexation of tannin with insoluble hide and soluble BSA. The "non-tannin" residue found by the

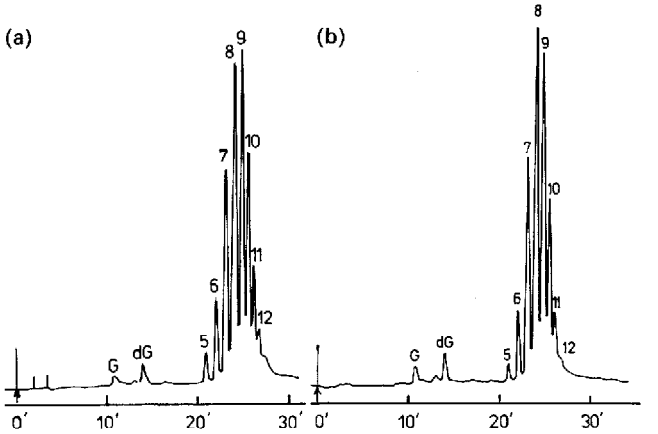

Fig. 2. (a) Straight phase analysis of Sumac tannin. Conditions as in Fig. 1a. (b) Straight phase analysis of Sumac tannin after partial complexation with BSA.

TABLE VI

AVERAGE COMPOSITION AS THE NUMBER OF GALLOYL GROUPS PER GLUCOSE UNIT BEFORE AND AFTER COMPLEXATION WITH BSA

Tannin	*Number of galloyl groups per glucose unit*	
	Before complexation	*After complexation*
Sumac	8.7	8.0
Chinese	8.4	7.7
Aleppo	5.8	5.7

hide powder method is in fact the largest polygalloyl glucoses, with the highest tanning capacity for soluble protein. The purity percentage or the tanning capacity of a tannin determined by the hide powder method, is therefore no guarantee of quality for its use as a complexer for soluble proteins (beer protein)!

Following Table IV, BSA has the strongest affinity for Chinese tannic acid.

Excess protein. To compare the affinities of the different commercial tannic acids for BSA, an excess of the protein is used and the following procedure was developed. A solution of 85 mg of BSA in 9 ml of buffer (pH 4.3, as above) is mixed with a solution of 10 mg of tannin in 1 ml of buffer. This suspension is stirred for 30 min followed by filtration through a filter paper as discussed before. A 1-ml volume of the clear solution was added to 1 ml of internal standard (salicylic acid, 0.30 mg/ml buffer, pH 4.3) and analysed on the acetylamide column. Other HPLC analyses of BSA have been reported[18,19] and could also be used. The gradual increase of column pressure is caused by the adsorption of tannins and soluble tannin–protein complexes. The normal pressure can be restored by rinsing the column with water (to prevent precipitation of salts in the detector cell and capillaries) followed by acetonitrile. Fig. 3 shows four different chromatograms of BSA (and internal standard). The amount of protein-bonded tannin can be calculated by subtracting the amount of BSA in the filtrate (from the calibration curve $Y = 0.0591X + 0.0015$; correlation coefficient $r = 0.9989$; the concentration of BSA in the calibration solutions varied between 10 and 40 mg per 10 ml of buffer, and the concentration of the internal standard, salicylic acid, was kept constant at 1.50 mg per 10 ml) from the original amount before complexation. The tanning capacity is expressed as milligrams of protein bonded per milligram of tannin. The results are presented in Table VII.

TABLE VII

TANNING CAPACITIES OF FOUR DIFFERENT TANNINS

Tannin	*mg Bonded protein per mg tannin*	*Standard deviation (3 analyses) (%)*
Chinese	6.99	2.4
Sumac	6.14	1.1
Aleppo	6.83	1.2
Tara	4.77	4.1

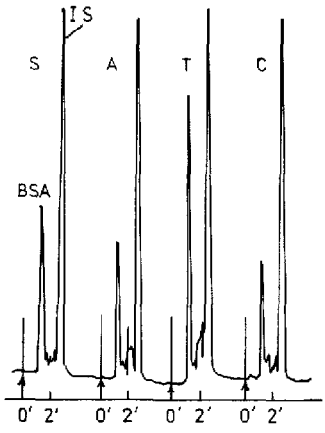

Fig. 3. Analysis of BSA, after partial complexation, in the steric exclusion mode. The surface area of BSA is compared with that of the internal standard, salicylic acid. Peaks: S = Sumac; A = Aleppo; T = Tara; C = Chinese tannin. Column, 15 × 0.46 cm I.D. filled with 10 μm irregular N-acetylaminopropyl silica gel; mobile phase, 0.01 *M* sodium dihydrogen phosphate, 0.01 *M* disodium hydrogen phosphate, with 2% sodium chloride (pH 6); flow-rate, 1 ml/min; recorder speed, 0.5 cm/min; detection wavelength, 280 nm; presaturation column, 5 × 0.46 cm I.D. filled with silica gel (Prep-RSiL d_p 12–40 μm).

The Chinese and Aleppo tannins have the highest tanning capacities: they are each able to bond about seven times their own weight of BSA. Tara, with an average molecular weight of *ca.* 800, has the lowest tanning power. Industrial experience shows that Tara tannin is not so suited for beer stabilization but is more valuable in other technical applications (inks and textiles). However, it is interesting that Aleppo has a large tanning power despite its lower molecular weight.

It has been stated that the tanning capacity is directly related to molecular weight[20], with an optimum[2] of *ca.* 3000. Haslam, however, reported[21] that β-D-pentagalloyl glucose with MW 940 would bind best with proteins and should have a tanning capacity similar to those of the Chinese and Sumac tannins (mean MW *ca.* 1500). Another view is that the tanning capacity will be improved if a number (minimum two) of specific groups in a molecule are sterically well separated and each of them is able to bond to proteins. The molecular weight is of minor importance in this case[2,20]. This fact is used to explain the high tanning capacity of some tannins despite their lower molecular weights. However, Table V shows that the larger polygalloyl components (deca-, undeca- and dodecagalloyl glucose) in the Chinese and Sumac tannins are preferentially complexed by BSA! Dodecagalloyl glucose is relatively most bonded by the protein.

In the case of Aleppo, the percentage of the pentagalloyl glucose is higher after complexation, which means that the higher molecular weight polygalloyl glucoses are more bonded by the soluble protein. In this case these are the octa- and nonagalloyl glucoses. These facts contradict the β-D-pentagalloyl glucose hypothesis! Whether the idea of Metche is true (2) that 3000 MW is the best cannot be decided by our experiments.

Beer proteins

An important application of tannins is as beer stabilizers. In aged beer, proteins combine with polyphenols to form complexes and these are responsible for the formation of chill haze[22]. Therefore purified tannins (tannic acid) are added to the finished beer in which soluble and insoluble complexes are formed. After a chill-proofing period the beer is filtered. The protein level in the beer is consequently decreased to an appropriate level, thus increasing the shelf life.

A similar procedure with beer proteins was followed as for the partial complexation of BSA: 5 mg of Sumac tannin are stirred with 10 mg of beer protein in 8 ml of buffer (pH 4.3). The suspension is stirred for 30 min and filtered through a filter paper, and the filtrate is lyophilized. The residue is dissolved in methanol–THF and analysed by HPLC on a straight phase column. Fig. 4a and b shows chromatograms of a Chinese tannin before and after partial complexation with beer proteins. No quantitative study was done because of the large differences in beer proteinic material, depending on the beer and the isolation procedure. Moreover, it is difficult to integrate accurately the beer protein patterns (see Fig. 5). However, the behaviour is similar to that observed with the BSA.

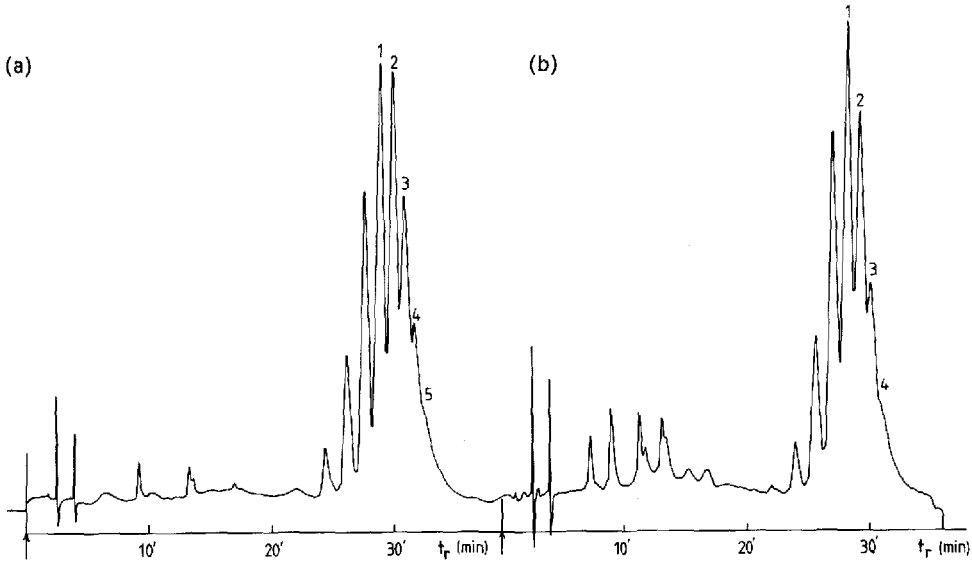

Fig. 4. (a) Analysis of Chinese tannin. Column, 25 × 0.46 cm I.D. with 5 μm RSiL (irregularly shaped silica gel from Alltech-RSL); mobile phase, A = hexane, B = methanol–tetrahydrofuran (2:1) with 0.25% citric acid; programmed from 80% A–20% B to 50% A–50% B at 15 min to 35% A–65% B at 30 min; flow-rate, 1 ml/min; recorder speed, 0.5 cm/min; detection wavelength, 280 nm. (b) Analysis of Chinese tannin after partial complexation with beer proteins. Conditions as in (a).

The higher molecular weight polygalloyl glucoses are preferentially combined with the beer protein (peak 5 has disappeared, peaks 3 and 4 are considerably decreased). Smaller molecules such as the gallic acids are found in larger amounts after complexation. Fig. 5 shows the chromatograms of beer proteins after complexation with four commercial tannins. Peak 1 (blank) is the original beer protein pattern

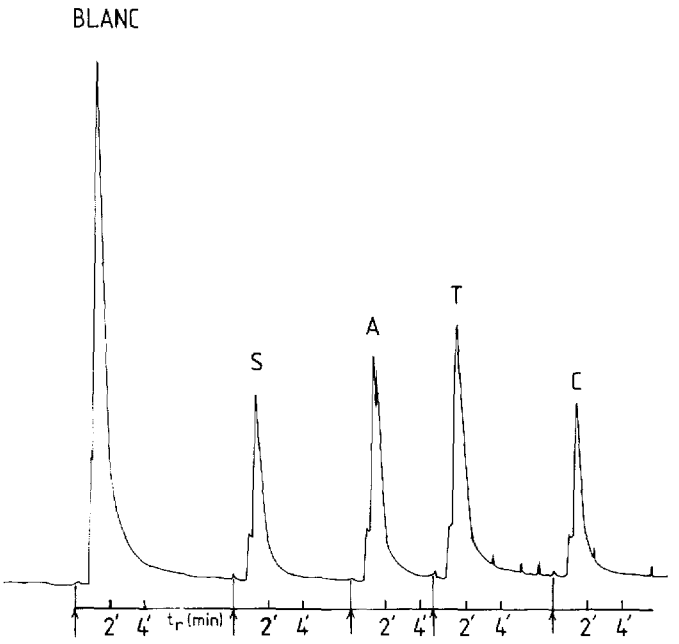

Fig. 5. Analysis of beer proteins before and after partial complexation. Peaks: B = blank; S = Sumac; A = Aleppo; T = Tara; C = Chinese tannin. Conditions as in Fig. 3.

before adding the tannins. The results are similar to those of the BSA experiments: Chinese and Sumac are very similar and have about the same tanning capacity, followed by Aleppo. Tara has again the lowest precipitating power.

In conclusion, it can be stated that the higher polygalloyl glucoses possess the largest precipitating power for beer protein, for BSA and probably for other soluble proteins as well. The optimal configuration of the tannin is of minor importance according to our experiments.

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