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Haemolytic micromethod for rapid estimation of toxic alfalfa saponin

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Abstract

On a plate covered with a thin layer of blood suspension with gelatine 10 microliters of alfalfa juice or alfalfa meal extract is spotted. As a result of saponin diffusion and their reaction with blood a haemolytic ring appears, the diameter of which is proportional to the concentration of toxic saponins. Selected by this method low-saponin individual alfalfa plants proved non toxic for fishes and did not inhibit the growth of *Trichoderma viride*.

INTRODUCTION

The varieties of alfalfa obtained by breeding universally grown as valuable fodder plants with high protein content contain considerable quantities of toxic compounds, glycosides of pentacyclic triterpenoides.

These compounds called saponins cause bloat in ruminants, inhibit the growth of animals and decrease egg-laying of hens (Birk 1969). As regards chemical composition, the saponins of alfalfa may be divided into glycosides of soyasapogenols and those of medicagenic acid. Particularly toxic are the glycosides of medicagenic acid. They are characterized among other features by strong fungistatic properties and haemolytic activity.

From the agricultural point of view the breeding of alfalfa varieties with a low-saponin content is an important problem. For this purpose rapid methods of plant testing are necessary. Literature gives several methods of saponin determination in alfalfa but these methods are not always convenient in breeding work. The gravimetric method of Van Atta et al. (1961) is very laborious and requires many laboratory devices as well as a considerable amount of plant material.

The biological methods which utilise the toxic properties of medicagenic acid glycosides (Zimmer et al. 1967; Jones and Elliott

1969) are much simpler but they also require at least one gramme of plant material.

The proposed microhaemolytic method is simple, not very labourconsuming, small quantities of material suffice and may be applied for testing individual plants in a relatively early stage of growth.

MATERIAL AND METHOD

Saponin extracts

a) *Plant juice.* Several drops of alfalfa juice are squeezed out, by means of a small press, the kind used when sugar is analysed in beet juice, from about 10 alfalfa leaves into small test tubes. If dilution is necessary (saponin concentration $> 0{,}2\%$) more than 20 µl of juice, is strictly measured with a micropipette, and a definite quantity of isotonic NaCl (0.9%) solution, coloured with aniline blue of 1 mg/ml concentration is added and mixed.

b) *Extraction of alfalfa meal.* Finely ground dried alfalfa (100 mg) is put into 2-ml ampoules and 1 ml of the isotonic NaCl solution is added.

The sealed ampoules are placed in a water bath and boiled during two hours. After centrifugation and decantation some crystals of aniline blue are added to the extract until an intense blue colour of the solution is obtained.

In case of too low-saponin content in the alfalfa meal ($< 0.02\%$) 100-200 µl of extract can be dried at 70°C and the dry residue dissolved in a strictly measured quantity of water coloured with aniline blue.

Standard solutions

a) *For the determination of saponins in alfalfa juice.* About 0.5 kg of freshly cut clover are crushed by a grinding machine and the juice is filtered through a G-3 filter under reduced pressure. This juice is used for preparation of standard solutions of alfalfa saponins of 0.02-0.2% concentrations. The standard saponins have been isolated according to Gestetner et al. (1970) from cultivated alfalfa varieties.

b) *For determination of saponins in alfalfa meal.* 20 g of dried and ground clover is extracted with 200 ml of isotonic NaCl solution on a boiling water bath during two hours. The filtered extract coloured with aniline blue is used for preparing standard saponin solutions.

Preparation of blood conserved with sodium citrate (Borkowski 1959)

10 ml of aqueous 3.65% sodium citrate are put into a sterilized Erlen-

mayer flask and 90 ml of ox blood taken directly from the artery are added and mixed gently. Blood prepared in that way may be kept in a refrigerator for one week.

Preparation of plates covered with blood suspension

4.5 g of gelatine is placed in an Erlenmayer flask and 75 ml of isotonic (0.9%) NaCl solution is added. After standing for 30 min. at room temperature the mixture is heated on a water bath at about 80°C until complete dissolution of the gelatine. The whole is cooled to 30°C, and 20 ml of ox blood conserved with sodium citrate is added with careful mixing. Very clean glass plates 10×20 cm are covered with a 0.25 mm layer of blood suspension by means of a spreader used for thin-layer chromatography. The plates are stored in horizontal position for about half an hour for coagulation.

Testing of plants for saponin content. The bottom of a petri plate is covered with a thin layer of blood suspension in gelatine. After coagulation of the suspension 10 µl of juice from alfalfa leaves is introduced and the covered dishes are left to stand overnight. The sice of the appearing haemolytic circle gives evidence of the saponin content. A lack of the ring shows that the concentration of saponins in the juice does not surpass 0.02%.

DETERMINATION OF SAPONINS

Alfalfa juice (10 µl) or extract from alfalfa meal are introduced on a previously prepared glass plate covered with the blood suspension. The intervals between samples should be at least 1.5 cm. The same volumes of appropriate standards should be placed beside the samples. 50 samples can be placed on the 10×20 cm plate. The plate is placed in a camera. The camera consists of a frame made of plexiglass with dimensions 11×22 cm inside and 14×25 cm outside and thickness 6 mm. Two glass plates of dimension 14×25 cm serve as bottom and the cover of the camera. Measurement of the haemolytic ring diameter with the help of a photographic enlarger may be done after 20 hours at a×5-10 mangification (see Fig. 1). From the standards the curve is prepared (see Fig. 2) from which the saponin content of the samples is read.

TESTING AND SELECTION OF ALFALFA

One hundred individual alfalfa plants *(Medicago media* Pers) were taken for selection from each of 'Miechowska' and 'Piaskowa' varieties. Testing was performed in the third cutting of the third year of utiliza-

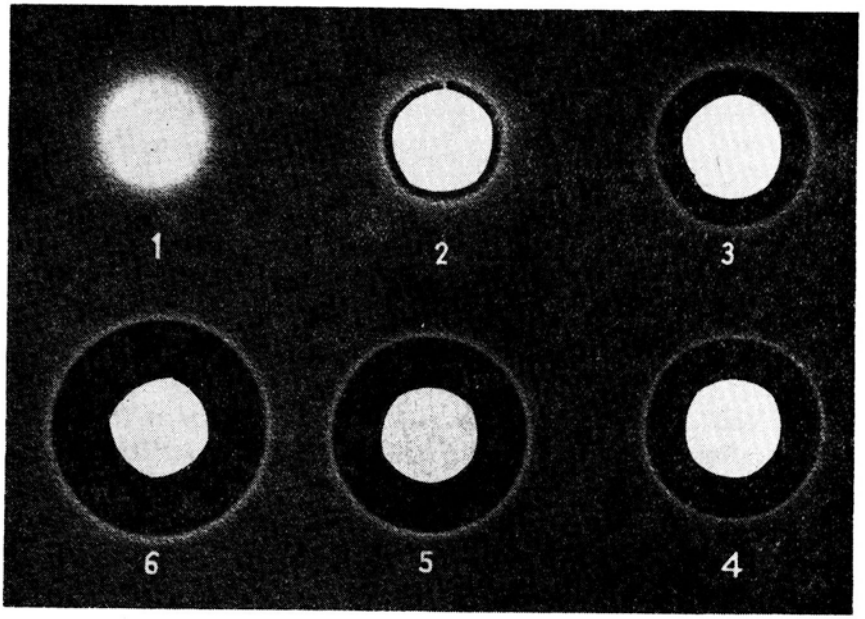

Fig. 1. Haemodiffusiogram (fotonegative) of medicagenic acid glycosides: 1, 2, 3, 4, 5 and 6 — 0, 0.05, 0.1, 0.15, 0.20 and 0.30%, respectively. Five times enlarged

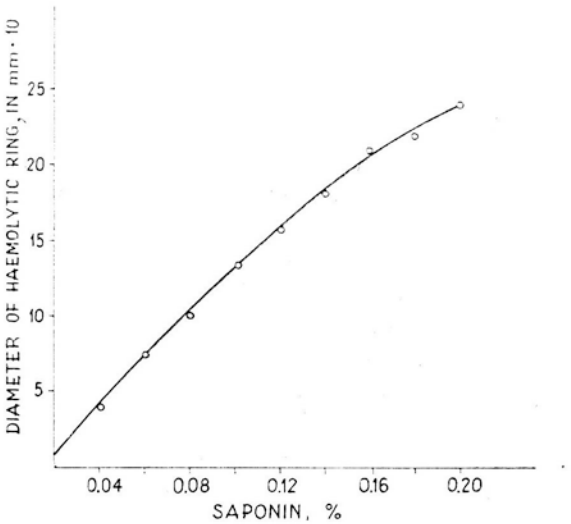

Fig. 2. Standard curve for the quantitative analysis of toxic saponins in alfalfa meal extracts

tion at the beginning of flowering. Immediately after testing the plants were cut, dried at 60°C, finely ground and analysed for saponin content.

The toxicity of alfalfa meal, obtained from the selected individual

plants of low saponin content was analysed by biological assays. The toxicity in regard to fishes was checked by the Jones and Elliott (1969) method only instead of *Pimephales promelas Libistes reticulatus* was used.

The fungistatic test was performed according to Zimmer et al. (1967) with 0.5 g of alfalfa meal per 100 ml of potatoe-dextrose-agar medium. The haemolytic index was determined by Borkowski's (1959) method.

RESULTS AND DISCUSSION

The following factors influence the repeatability of the results obtained by the haemolytic micromethod: the layer thickness and density of the diffusing medium, the concentration and quality of blood as well as the temperature and humidity of the atmosphere. The influence of these factors on the results may be reduced if the standard solutions of saponins are placed on each plate beside the analysed extracts.

An inadequate kind of gelatine may disqualify its usability in the described method. We have used successfully different kinds of home comestible gelatine; but the imported chemically pure gelatine proved unsuitable because it caused clotting of the blood.

The plates covered with blood suspension with the addition of gelatine should be optically homogeneous and the suspension ought to become compact enough before the samples are spotted, because then the solution can preserve the shape of the drops.

Saponin extracts and standard solutions must be coloured with aniline blue in order to make the haemolytic rings stand out distinctly in colour from the site of extract deposition. In case of testing alfalfa juice chlorophyll plays the role of pigment and aniline blue is not necessary.

The standard solutions used for analysis of the extract of alfalfa meal instead of the clover meal extract may be prepared by dissolving saponins in isotonic NaCl solution. These solutions can be kept in a refrigerator during several months. On the contary, the standard solutions for the analysis of alfalfa juice, because of their great viscosity, must be prepared by dissolving the saponins in the juice of some other plant, which does not contain any haemolytic saponins. Clover is the most adequate for this purpose. Such solutions are not stable however and can be kept in the refrigerator during two days only.

However, in breeding work, where the absolute content of saponins is not important only the forms of low-saponin content are searched the breeder need not prepare any standard curve, but the approximate content of saponins may be estimated by comparing the analysed indi-

vidual plants with the values obtained for a mean population. Thus, the proposed method may be applied both for semi-quantitative determinations and for an approximate evaluation of haemolytic plant saponins.

The smallest possible to determine quantity of saponins is 2 μg. The highest accuracy of the method ranges from 0.05% to 0.15% of saponins in the solution. Two persons can test 100 samples a day.

Applying the described method we have tested 100 alfalfa plants from each, of the varieties 'Miechowska' and 'Piaskowa'. As shown in the Table 1 both analysed varieties had a similar contents of haemolytic

Table 1

Saponin content and biological activity of selected low-saponin individual plants of two alfalfa varieties

Alfalfa var.	Toxic saponin content in %		Haemolytic index	Min. before immobilization of fishes	Inhibition of *Trichoderma viride* in %
	juice	meal			
'Miechowska'	0.80	0.51	60	32	34
M/56	0.15	0.26	21	225	18
M/20	0.11	0.19	18	227	11
M/84	0.13	0.13	12	not toxic	6
M/32	0.13	0.10	15	„ „	8
M/60	0.07	0.10	10	„ „	8
M/52	0.10	0.07	10	„ „	3
M/87	0.11	0.05	10	„ „	4
M/24	0.08	0.00	0	„ „	−2
'Piaskowa'	0.82	0.55	75	33	46
P/83	0.16	0.18	18	195	10
P/42	0.09	0.18	21	205	17
P/52	0.05	0.12	15	not toxic	6
P/13	0.10	0.00	0	„ „	−3
P/60	0.08	0.00	0	„ „	−2

saponins, both in the juice of leaves and in their meal. On the other hand, within the varieties a high variability was found between the individuals. Alfalfa meal obtained from plants with low saponin content had a low haemolytic index and their water extracts were in most cases nontoxic for fish and inhibited only in a slight degree the growth of *Trichoderma viride*. Among the individuals with a low-saponin content, three plants have been found with complete lack of haemolytic properties in the extracts and stimulating the growth of the test fungus.

The obtained results confirm the usefulness of the haemolytic micromethod for breeding purposes. It seems that the method may also be applied in het search for plants with high saponin content for pharmacological and idustrial purposes.

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Hemolityczna mikrometoda szybkiego oznaczania toksycznych saponin w lucernie

Streszczenie

Na płytki szklane pokryte cienką warstwą zawiesiny krwi z dodatkiem żelatyny nanosi się po 10 µl świeżego soku lucerny lub ekstraktu z mączki lucernianej. W wyniku dyfuzji saponin i ich reakcji z krwią powstaje pierścień hemolityczny, którego średnica jest proporcjonalna do stężenia saponin. Wyselekcjonowane za pomocą tej metody pojedyncze rośliny lucerny, wykazujące niski indeks hemolityczny, nie są toksyczne dla ryb i nie hamują wzrostu grzyba *Trichoderma viride*.