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Determination of organic acids in plant material

II. Determination of organic acids in plant extracts by anion exchange chromatography

Bestemmelse af organiske syrer i plantemateriale
II. Bestemmelse af organiske syrer i planteekstrakter ved anionbytningskromatografi

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Summary

A method for determination of organic acids in plant extracts is described. The method is based on anion exchange chromatography and comprises 2 steps.

First the acids are separated into 2 fractions by use of a short weak basic anion exchange column. The first fraction of acids was obtained by elution with formic acid and the second fraction by elution with ammonia.

The second step comprises a determination of the acids in each of the 2 fractions. The method gives almost a quantitative determination of quinic, shikimic, malic, succinic, citric, tartaric and oxalic acid. The recoveries were 89–101 per cent. For α-ketoglutaric acid the recovery was 66 per cent. An unknown compound seems to interfere with the dertermination of malonic acid.

Key words: Organic acids, anion exchange, chromatography, plants.

Resumé

I nærværende beretning er beskrevet en metode til bestemmelse af organiske syrer i planteekstrakter. Metoden er baseret på anionbytningskromatografi og omfatter 2 trin.

I første trin fjernes basiske og neutrale stoffer, og syrerne deles i 2 fraktioner. Planteekstrakten overføres til en kort kolonne (svag basisk anionbytter), hvor syrerne tilbageholdes. Efter udvaskning af basiske og neutrale stoffer med vand elueres første fraktion med myresyre og anden fraktion med en ammoniakopløsning.

Andet trin omfatter en bestemmelse af de organiske syrer i de 2 fraktioner. Syrerne adskilles på en 4 × 1000 mm stærkt basisk anionbytterkolonne ved eluering med natriumformiat og måles ved hjælp af et refraktometer.

Metoden giver tilnærmelsesvis en kvantitativ bestemmelse af quininsyre, shikiminsyre, æblesyre, ravsyre, citronsyre, vinsyre og oxalsyre. Genfindelse for disse syrer var fra 89–101%. For α-ketoglutarsyre var genfindelsen 66%. En uidentificeret forbindelse synes at inteferere med bestemmelsen af malonsyre, hvilket bevirker, at resultaterne for denne syre kan være behæftet med fejl.

Nøgleord: Organiske syrer, anionbytning, kromatografi, planter.

Introduction

Determination of organic acids in plant extracts has been carried out by paper chromatography, thin-layer chromatography, gas chromatography or liquid chromatography. For quantitative determination the most common methods are based on gas chromatography (*Clark,* 1969; *Philips & Jennings,* 1976) or liquid chromatography including partition chromatography (normal and reverse phase) (*Wager & Isherwood,* 1961; *Prior et al.,* 1973; *Rajakylä,* 1981; *Buslig et al.,* 1982) and anion exchange chromatography (*Palmer,* 1955; *Hulme & Wooltorton,* 1958; *Bengtsson & Samuelson,* 1969 and 1971; *Palmer & List,* 1973). In the paper a method is described based on the method developed by *Palmer* and *List* (1973), where sodium formate is used as eluent and the acids detected by a differential refractometer.

A disadvantage of the method is a peak overlap between citric and malic acid, no separation of succinic from tartaric acid and no separation of malic from malonic acid. The last may be a problem in examination of plant species which contain malonic acid e.g. the leguminous plants. The problems are not overcome by use of a formic acid gradient as eluent because this eluent gives peak overlap for some other acids (*Palmer,* 1955; *Hulme & Wooltorton,* 1958). However according to the elution pattern for a series of organic acids (*Palmer,* 1955; *Davies et al.,* 1965) it should be possible to separate the acids into 2 fractions using formic acid as eluent. One fraction (A) which contains succinic acid, malic acid, and some others and another fraction (B) which contains tartaric acid, citric acid, malonic acid and some others. Therefore it should be possible to separate all 5 acids in question by a method which combines elution with formic acid and sodium formate.

It is described in the following how the 2 elution systems can be combined without making the analytical procedure time-consuming. The analytical procedure comprises 2 steps. In the first step the sample is purified and the acids separated into 2 fractions. The second step comprises separation of the acids and quantitative determination of each.

Materials and methods

A strongly acidic cation exchanger Merck I in hydrogen form was used for purification of the plant extract, column diameter 6 mm, resin height 50 mm. Before use the resin was washed several times with water. A weak basic anion exchanger, Amberlite CG 4B type II 200–400 mesh was used for further purification and separation of the acids into 2 fractions. The resin was equilibrated with 1 M formic acid and the finer particles were removed by allowing the coarser particles to settle and decanting the cloudy supernatant fluid. The resin was stored in a refrigerator until use. A column 10 × 120 mm with exactly 25 mm resin was employed. The resin in the column was equilibrated with 0.25 M ammonia by shaking it 3 times with 10 ml each time. Then the resin was washed with water until neutrality.

The separation of the acids was performed on a strong basic anion exchange resin Amines A 25, particle size 17.5 ± 2 μ, column height 1000 mm, bore 4 mm. The resin was converted to formate form and packed in wet condition. The column (glass) was equiped with a water jacket. The eluent was prepared by adjusting 1 M sodium formate to pH 7.3 using 1 M sodium hydroxide or 1 M formic acid. The eluent was heated to 70°C and evacuated for about half a minute. Standard solutions of organic acids were prepared in water. All chemicals used were of analytical reagent grade.

The plant materials used were rye grass, cocksfoot, timothy, meadow fescue, white clover, red clover and beet leaves. The plant tissue was frozen to –20°C immediately after harvest and extracted as described by *Kyllingsbæk* (1984).

Apparatus

Metering pump Constametric III Laboratory Data Control, Division of Milton Roy Co.

Differential refractometer Model 1107 Laboratory Data Control.

Recorder Perkin-Elmer Model 56, full scale steps of 1, 2, 5, 10, 20 and 50 mv.

Thermostate Hetotherm type 01 PF 623.

Six-way valve fitted with a 250 μl sample loop.

All tubes and fittings must withstand a pressure of at least 25–30 atm.

Flow rate: 0.4 ml/min.; Column temperature: 55°C; Recorder, full scale step: 10 mv; Chart speed: 5 mm/min.

Analytical procedure

The plant extracts (usually 25 ml) were purified by passing through the cation exchange column and subsequently through the weak basic anion exchange column where the acids were retained at the top of the column. 15 ml of water was added to remove neutral and basic substances. The acids were then separated into 2 fractions by elution with 27 ±0.5 ml of a 1 M formic acid which gave the first fraction (A). The second fraction was achieved by elution with 30 ml of a 0.25 M ammonia. To speed up the elution the ammonia was added in amounts of 10 ml and the resin suspended by shaking the column after addition of the first and the second amount.

The 2 fractions were evaporated to a volume of 2–3 ml at 30°C in a waterbath. For promotion of evaporation a stream of air was blown to the liquid surface. After evaporation a few ml of water was added and pH adjusted to 8.4 with sodium hydroxide and finally the volume made to 10 ml. In order to protect the separation column from contamination the sample was filtered through a membrane filter (0.45 μm). The samples were transferred to the column via a sample loop of known volume, 0.25 ml. A flow diagram is shown in Fig. 1.

Peak areas for a standard were used as base for calculation of the content of acids in unknown samples.

Thin-layer chromatography

For identification of organic acids a thin-layer chromatography method by *Chan et al.* (1971) was used. The eluted fraction corresponding to the peak which should be identified was treated with a cation exchanger in hydrogen form in order to convert the sodium formate to formic acid.

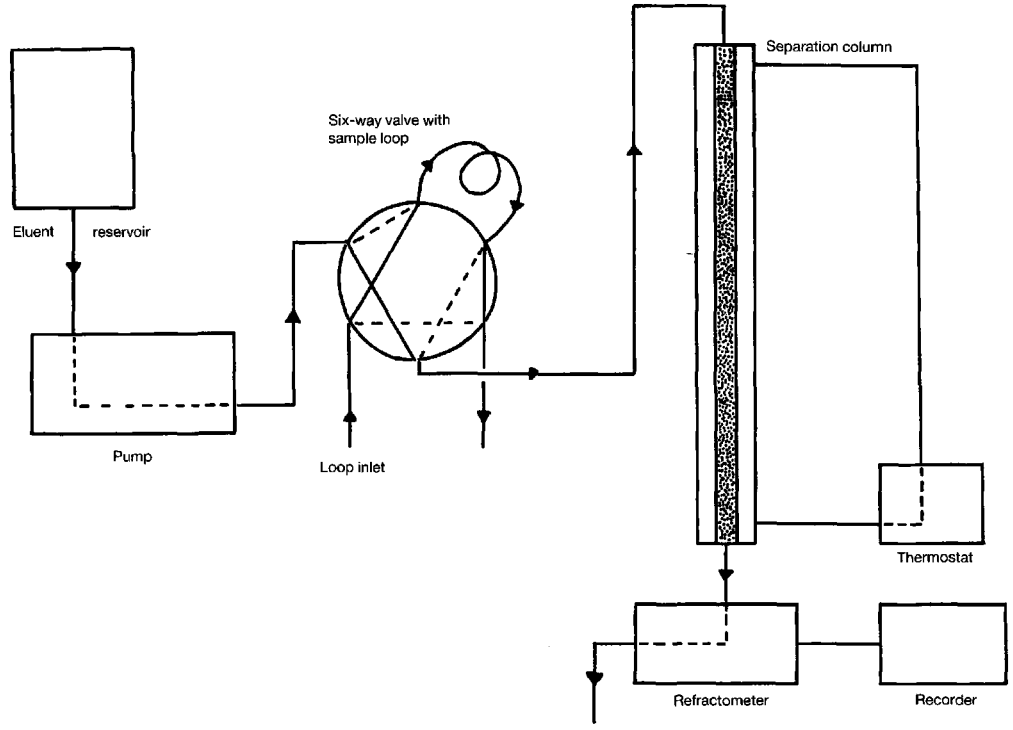

Fig. 1. Flow diagram for organic acid analyzer.
Flow diagram for analysator til bestemmelse of organiske syrer.

Then the solution was evaporated to a volume of about 100 μl which was applied to a cellulose coated plate (Merck). The plate was developed in the upper phases of the solvent system ethyl ether – formic acid – water in the volume ratio 20:5:3. The plate was dried over night at room temperature and then sprayed with bromophenol blue (0.04 per cent w/v with 0.05 per cent sodium acetate in 96 per cent ethanol). The acids appeared as yellow spots on a blue-green background.

Results

It was found in preliminary investigations that the separation of fumaric acid from α-ketoglutaric acid and of citric acid from malonic acid is influenced by the pH of the sample and the pH of the eluent. At pH 6.9 for both the sample and the eluent the separation of fumaric and α-ketoglutaric acid was better than of a pH of 8.4 for the sample and of 7.3 for the eluent. On the contrary pH of 8.4 and 7.3 for the sample and the eluent respectively gave a better separation of citric and malonic acid. The detector response for citric acid also seemed to be lower when the pH of the eluent was 6.9 than when the pH was 7.3.

Chromatograms from runs of standards corresponding to the 2 fractions A and B are shown in Fig. 2. It is seen that the separation of all the acids

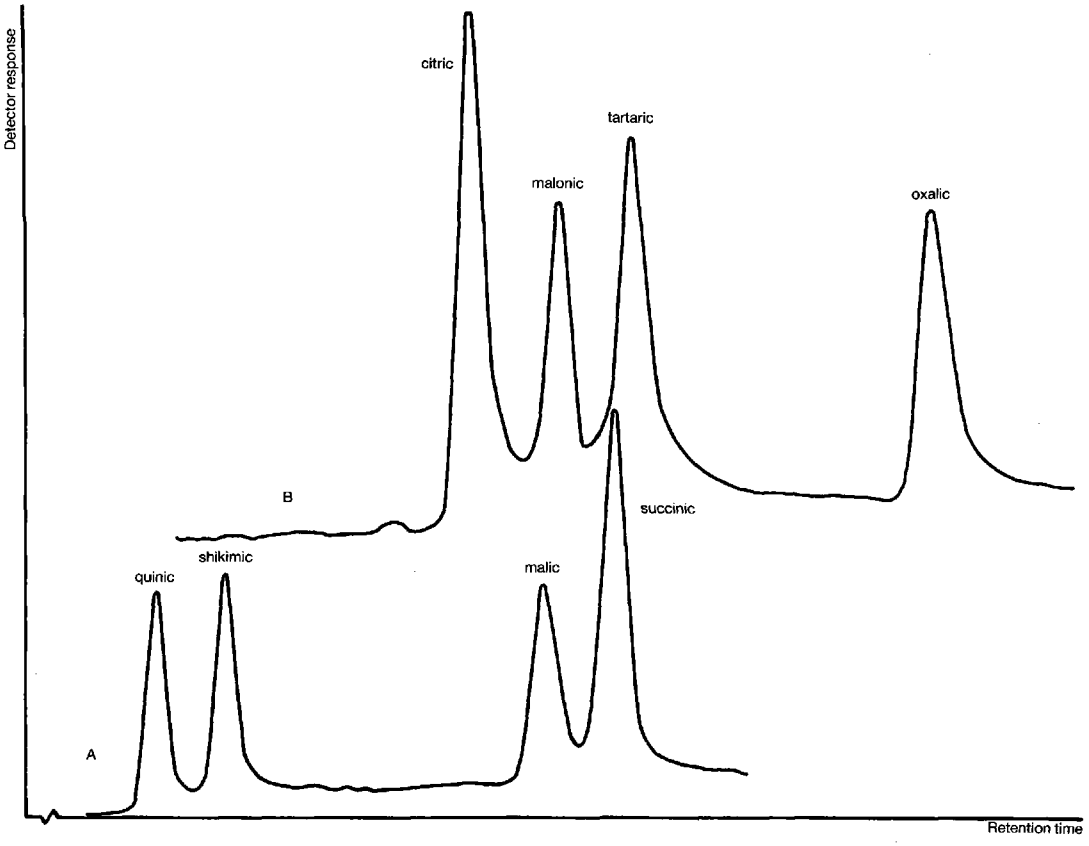

Fig. 2. Eluation pattern for some organic acids.
A) standard mixture corresponding to the first fraction
B) standard mixture corresponding to the second fraction
Elueringsmønster for nogle organiske syrer.
A) standardopløsning svarende til første fraktion
B) standardopløsning svarende til anden fraktion

Table 1. Coefficient of variation for results from determination of some organic acids in standard solutions.
Relative standardafvigelser for resultater fra bestemmelse af nogle organiske syrer i standardopløsninger.

No.	Acid	µg injected	Coefficient of variation a	b
1	quinic	48.1	2.6	2.5
2	shikimic	43.4	2.4	3.4
3	malic	83.8	1.3	3.7
4	succinic	147.6	1.1	1.0
5	citric	160.1	2.1	6.4
6	malonic	130.1	0.9	3.2
7	tartaric	187.6	2.0	2.9
8	oxalic	225.1	0.4	4.3
9	α-ketoglutaric	274.0	3.3	2.7

a: Based on 2 types of standard solutions containing acid 1, 2, 3, 4 and 5, 6, 7, 8, 9, respectively.
b: Based on standard solutions containing all the acids, but before analysis the acids were divided into 2 fractions containing acid 1, 2, 3, 4 and 5, 6, 7, 8, 9, respectively.
a: Baseret på 2 forskellige typer standardopløsninger indeholdende henholdsvis syrerne 1, 2, 3, 4 og 5, 6, 7, 8, 9.
b: Baseret på standardopløsninger indeholdende alle syrerne, men før analysering delt i 2 fraktioner indeholdende henholdsvis syrerne 1, 2, 3, 4 og 5, 6, 7, 8, 9.

from each other is quite good and that the retention time is almost the same for malic acid and malonic acid and for succinic acid and tartaric acid.

The precision of the analyses is illustrated in Table 1. The relative standard deviation calculated from results obtained by running 5 identical samples from each of the 2 standard solutions representing the acids in the 2 fractions A and B is shown in the table. The coefficient of variation when 5 identical, standard solutions containing all the acids are separated into 2 fractions each using the techniques described above is also shown. From the results it can be seen that for most of the acids separation into 2 fractions has led to an increase of the coefficient of variation.

Table 2. Recovery of some organic acids added to rye grass.
Genfindelse af forskellige organiske syrer tilsat rajgræs.

Acid	Added mg/g dry matter	% recovery
Quinic	4.4	92
Shikimic	4.0	91
Malic	7.7	98
Succinic	13.5	98
Citric	14.6	99
Malonic	11.9	95
Tartaric	17.2	101
Oxalic	20.6	89
α-ketoglutaric	25.1	66

Table 2 shows the recovery of different acids added to samples of frozen rye grass, followed by extraction of the samples as described by *Kyllingsbæk* (1984). From the table can be seen that with the exception of the result for α-ketoglutaric acid (recovery 66 per cent) the recoveries of the acids vary from 89–101 per cent.

Table 3 shows results from determination of organic acids in different plant species. The separation of these acids was almost as good as the separation of the acids in the standard samples but

Table 3. Levels of some organic acids in different plant species.
Indhold af nogle organiske syrer i forskellige plantearter.

Acid	White clover	Red clover	Beet leaves	Rye grass	Cocks-foot	Timothy	Meadow fescue
				mg/g dry matter			
Quinic	–	–	–	2.5	2.4	6.9	5.0
Shikimic	9.1	7.8	0.8	1.7	1.2	2.1	2.4
Malic	21.1	24.6	6.0	20.0	5.8	10.9	10.8
Citric	13.0	13.2	16.5	9.4	2.2	7.5	6.5
Malonic	–	(7.5)	–	(0.6)	(6.4)	(2.8)	(5.4)
Oxalic	4.4	3.8	54.1	2.5	3.4	1.8	1.5

the difference between replicates could in some cases be 10 per cent, especially when the content of the acid was low. For grasses an unknown peak was eluated just after shikimic acid.

The content of malonic acid found, especially in the grass samples was high compared to the amount generally found in grasses (*Dijkshoorn,* 1973). As a control of the identity the eluent fraction corresponding to the peaks representing malonic acid was collected for further examination by thin-layer chromatography. It was found that the spots on the thin-layer plate were considerably smaller than might be expected from the size of the peak on the chromatograms when compared with the results obtained for standard solutions of malonic acid. This was especially the case for samples of meadow fescue. In an attempt to remove the compound which seems to interfere with the malonic acid all the samples from grasses were purified by filtration through a charcoal filter which made them almost colourless. However, the purification did not reduce the area of the peak representing malonic acid nor the peak areas of the other acids present. The purification was found only to have a protective effect against contamination of the column. None of the acids were absorbed by the charcoal filter, which was established by a filtration of a standard solution through charcoal filters 5 times.

Discussion

Since the separation of some acids and the detector response was found to be influenced by the pH of both the sample and the eluent it is recommended to always adjust pH to a fixed value. In most cases pH 8.4 of the sample and pH 7.3 of the eluent was suitable. However, for samples where fumaric and α-ketoglutaric acid occur together a better separation for the 2 acids is obtained by adjusting pH of the eluent to 6.9

The preliminary separation of the acids into 2 fractions before the analysis makes the method more time consuming than the method of *Palmer* and *List* (1973), however a much better resolution is achieved. From Fig. 2 it can be seen that when the 2 fractions were analyzed together malic and malonic acid would be almost unresolved as would succinic and tartaric acids. The figures in Table 1 show that the separation may be carried out with a precision acceptable for most purposes. The enlargement of the coefficient of variation observed when the acids were separated into 2 fractions (see Table 1) is probably not only caused by the manipulations connected to the separation of the acids. It may to some extent be caused by a decrease in the efficiency of the separation column used for the quantitative determination of the acids. The decrease in the efficiency appeared as a peak tailing and a decrease in the resolution of the peaks.

According to the recoveries of different acids added to rye grass it seems to be possible to determine the most common organic acids in plant materials almost quantitatively by use of the method of *Kyllingsbæk* (1984) for extraction of the plant materials and the present method for determination of the acids in the extract.

The levels of organic acids found in different plant species (Table 3) are on the whole in agreement with the levels found by others (*Dijkshoorn,* 1973). However,an exception is the relatively large amount of malonic acid found in cocksfoot, timothy and meadow fescue.

The examination by thin-layer chromatography of the eluent fraction representing malonic acid indicated that another compound is eluated together with malonic acid. That means that without a separation of the acids into 2 fractions this compound, if present will also interfere with the determination of malic acid. The picture on the thin-layer plate only shows spots corresponding to malonic acid. The spots representing samples from grasses, especially samples from meadow fescue did not have a size and colour intensity that could be responsible for the corresponding peak areas found by anion exchange chromatography. That means that the results obtained for malonic acid may in some cases be influenced by interference from another compound. Charcoal has not been able to absorb the disturbing compound at pH 8.4 since filtration of the samples through a charcoal filter did not reduce the peak areas, but

only removed the colour substances from the samples. Further examinations are necessary for identification of the compound.

Conclusion

When using a method based on anion exchange chromatography and a formate solution as eluent for determination of organic acids in plant materials the separation of the acids is influenced by the pH of the sample and the pH of the eluent. Therefore it is recommended to adjust pH to a fixed level. In most cases pH 8.4 for the samples and pH 7.3 for the eluent is suitable.

A problem with unresolved peaks for the pairs malic and malonic acid and for succinic and tartaric acid was overcome by separation of the acids into 2 fractions followed by determination of the acids in each fraction.

By the described method it is possible to determine the acids quinic, shikimic, malic, succinic, citric, tartaric and oxalic acid almost quantitatively. The recoveries were 89–101 per cent. For α-ketoglutaric acid a recovery of 66 per cent was found. An unknown compound seems to interfere with the determination of malonic acid which to some extent makes the results obtained for this acid erroneous.

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