Danish Research Service for Plant and Soil Science **Report no. 1707**
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Determination of organic acids in plant material

I. Extraction of organic acids from plants

Bestemmelse af organiske syrer i plantemateriale
I. Ekstraktion af organiske syrer fra planter

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Summary

The paper describes a method for extraction of organic acids, including oxalic acid from plants by adding a strongly acidic cation exchanger before extraction with water.

The method is based on addition of a relatively large amount of cation exchanger and homogenization of the sample and cation exchanger together.

The method was almost as effective as extraction with hot 0.5 M hydrochloric acid for determination of citric acid and oxalic acid in beet leaves, rye grass, green peas and string beans. Results obtained by use of 80 per cent ethanol were found to be considerably lower with exception of the results from determination of citric acid in beet leaves.

The recovery of citric acid and oxalic acid added to rye grass as calcium salts varied from 94 per cent to 102 per cent for citric acid and from 90 per cent to 97 per cent for oxalic acid.

The method has the advantage that the amount of chlorophyll extracted is very small which makes the purification of the extract very simple.

Key words: Organic acids, extraction, plants.

Resumé

I nærværende beretning er der beskrevet en ekstraktionsmetode, som gør det muligt at ekstrahere organiske syrer inklusive oxalsyre tilnærmelsesvis kvantitativt fra plantemateriale ved tilsætning af en stærkt sur kationbytter før ekstraktion med vand.

Metoden blev udarbejdet ved bestemmelse af citronsyre og oxalsyre i bederoeblade, rajgræs, grønne ærter og grønne bønner. Resultaterne blev sammenlignet med indholdet bestemt ved ekstraktion med 0,5 M saltsyre og en 80% ethanolopløsning. Endelig verificeredes metoden ved bestemmelse af indholdet af citronsyre og oxalsyre i rajgræs tilsat henholdsvis calciumcitrat og calciumoxalat, som må henregnes til de tungest opløselige salte af organiske syrer, der forekommer i planter.

Metoden er baseret på tilsætning af en forholdsvis stor mængde ionbytter til prøven og efterfølgende homogenisering af prøve og ionbytter. For at undgå anvendelse af store mængder ionbytter foretages der først en findeling af en større prøve (ca. 25 g) i frossen tilstand, hvorefter prøven (3 g) til ekstraktion afvejes. Der anvendes 2 g ionbytter pr. prøve.

Metoden fandtes at være næsten lige så effektiv som ekstraktion med en 0,5 M saltsyreopløsning. Ved ekstraktion med en 80% ethanolopløsning var resultaterne lavere med undtagelse af resultaterne for citronsyreindholdet i bederoeblade, hvor det fundne indhold var næsten ens for alle 3 metoder.

Ved tilsætning af henholdsvis calciumcitrat og calciumoxalat til rajgræs varierede den genfundne mængde citronsyre fra 94% til 102% og den genfundne mængde oxalsyre fra 90% til 97%.

I modsætning til metoder, hvor der anvendes en organisk opløsning som ekstraktionsmiddel, ekstraheres der med nærværende metode kun meget lidt klorofyl, hvilket forenkler den efterfølgende rensning af ekstrakten.

Nøgleord: Organiske syrer, ekstraktion, planter.

Introduction

In investigations of metabolic processes in plants it is often of interest to determine the content of organic acids because di- and tri-caboxylic acids take part in many metabolic processes. The content of organic acids is also of interest for estimating the quality of fruits, vegetables and other foods and feeds.

Analyses of plant material for organic acids comprises the following steps: Inactivation of the enzymes in the plant materials, extraction, purification of the extract and determination of the organic acids in the extract. Usually the enzymes are inactivated by boiling the plant tissue for a few minutes with the extraction solution.

Several extractants have been used including diluted solutions of sulfuric, hydrochloric or formic acids; water occasionally with addition of a strongly acidic cation exchanger; methanol; ethanol; and ether (*Schramm,* 1973; *Schramm et al.,* 1977). Occasionally the extraction is carried out by use of two different extractants e.g. a solution of ethanol combined with diluted hydrochloric acid or water with addition of a strongly acidic cation exchanger (*Fauconneau,* 1958; *Roux & Lesaint,* 1959; *Schramm et al.,* 1977). When solutions of sulfuric or hydrochloric acid are used the extraction is presumably quantitative because these extractants are able to dissolve such sparingly soluble salts as calcium oxalate. However, strong acids are difficult to remove from the extract and often interfere with the determination of the organic acids. 70 per cent to 80 per cent ethanol is the most common extractant used although salts of organic acids, especially some calcium salts, are sparingsly soluble in ethanol.

Investigations concerning the efficiency of different methods are few. *Fauconneau* (1958) and *Roux* and *Lesaint* (1959) found that extraction with 80 per cent ethanol was insufficient for a quantitative extraction of some organic acids. *Fauconneau* (1958) made an additional extraction with a diluted hydrochloric acid to obtain a quantitative extraction of citric acid whereas *Roux* and *Lesaint* (1959) used water with a strongly acidic cation exchanger. On the contrary *Schramm et al.* (1977) found that extraction with 80 per cent ethanol was sufficient. Judging from the solubility of salts of different organic acids in water and ethanol, water should be a better extractant than ethanol. If a strongly acidic cation exchanger is used in addition to water even a sparingly soluble salt as calcium oxalate should be dissolved although it takes place very slowly. The above mentioned must also apply to salts of organic acids in plants. Therefore it should be possible to carry out a quantitative extraction of organic acids from plant tissue by water in presence of a strongly acidic cation exchanger. However, the prerequisite to obtain a quantitative extraction in a reasonably short time is that the ratio of cation exchanger to plant tissue is high and that the suspension is homogeneous.

An attempt has been made to improve the extraction method using water in presence of a strongly acidic cation exchanger for extraction of organic acids from different plant materials.

The method was compared to methods using the most common extractants 0.5 M hydrochloric acid and 80 per cent ethanol.

The effectiveness of the method was further elucidated by its capability of recovering citrate and oxalate added to rye grass as their calcium salts.

Materials and methods

Plant material

The plant materials used were ryegrass, beet leaves, green peas and string beans. The plant tissue was frozen immediately after harvest and stored at ÷20°C.

25 g of the frozen plant material was divided into fine particles using a mincer (Moulinex Moulinette) at 10.000 r.p.m. in 30 seconds. Then samples of 3 g were weighed directly into 25 ml centrifuge tubes. Performing the operations in a cold store (5°C) and cooling the tools in a freezer before use makes it possible to keep the plant tissue constantly frozen.

Extraction

Extraction with water and a strongly acidic cation exchanger was performed by addition of 10 ml of water to the frozen sample and immediately placing the centrifuge tubes in a boiling water bath for 10 minutes. The suspension was cooled, 2 g of a strongly acidic cation exchanger (Merck I) was added and then the mixture was homogenized by an Ultra Turax TP 18 for 2–3 minutes. The suspension was stored in a refrigerator at 5°C overnight and then heated in boiling water for 10 minutes. After cooling the suspension was centrifuged at 6000 × g and the residue extracted twice with 7–8 ml of water each time. Then the collected extract was diluted to 25 ml with water.

Extraction with alcohol was performed by addition of 5 ml of 96 per cent ethanol to the frozen sample and homogenizing it as described before. Then 10 ml of 80 per cent ethanol v/v was added and the suspension stirred frequently for 15 minutes. After centrifugation the residue was extracted twice for 15 minutes with 15 ml of 80 per cent ethanol, the second time in a water bath at 50°C. The extract collected (45 ml) was evaporated to near dryness at 50°C and the residue extracted five times with 4–5 ml of water each time. After filtration the extracts were collected and diluted to 25 ml with water.

Extraction with hydrochloric acid was performed by addition of 10 ml of 0.5 M hydrochloric acid to the frozen sample and placing the centrifuge tubes in boiling water for 10 minutes. After cooling the sample was homogenized as previously described. The sample was stored in a refrigerator at 5°C overnight. Next day the suspension was centrifuged and the residue extracted twice with water, 7–8 ml each time. After filtration the extract collected was diluted to 25 ml with water.

Analyses

Determination of oxalic acid and citric acid. All the extracts were purified by precipitation with tungstate phosphoric acid before determination of the acids (*Anonymous*, 1967). The oxalic acid in the extracts was determined by titration with potassium permanganate (*Anonymous*, 1967) and citric acid by use of a method of *Taylor* (1953).

Results

With respect to the method using water with addition of a strongly acidic cation exchanger (cation exchangerwater extraction) it was found that the effectiveness was increased by the following steps: a) Heating the plant tissue in boiling water before addition of the cation exchanger. b) Increasing the amount of the cation exchanger to about 2 g per 3 g fresh plant tissue. c) Homogenization of the plant tissue together with the cation exchanger. d) Storing the suspension overnight. e) Heating the suspension in boiling water before the final extraction.

Table 1 shows the amount of oxalic acid extracted from beet leaves using different concentrations of hydrochloric acid as extractants with and without heating the suspensions in boiling water for 10 minutes. The results show that the amount of oxalic acid extracted is increased by increasing concentration of hydrochloric acid and by heating

Table 1. Oxalic acid in beet leaves found by extraction with various concentrations of hydrochloric acid.
Oxalsyreindhold i bederoeblade fundet ved ekstraktion med saltsyre i forskellig koncentration.

HCl M	Without heating	Heated 10 min. at 100°C
	mg per g dry matter:	
0.1	23.7	23.7
0.2	25.2	28.4
0.5	37.4	57.3
1.0	53.8	58.9

the suspension. When heating the suspension the amount of oxalic acid extracted is almost the same by use of a 0.5 M as a 1.0 M hydrochloric acid as extractant.

Table 2. Oxalic acid in different species of plants found by extraction with 3 different extractants.
Oxalsyreindhold i forskellige plantearter fundet ved ekstraktion med 3 forskellige ekstraktionsmidler.

Extractants	Plant species: beet leaves	rye-grass	green peas	string beans
	mg per g dry matter:			
0.5 M HCl	47	0.9	trace	2.4
Ion exchanger water	43	0.8	trace	2.1
80 per cent ethanol	6.3	trace	trace	trace

The results in Table 2 show the amount of oxalic acid and the results in Table 3 the amount of citric acid in different plant species extracted with 3 different extractants. From the results in Table 2 it can be seen that the amount of oxalic acid found was almost the same whether 0.5 M hydrocxhloric acid or cation exchanger-water were used as extractants whereas the amount found was considerably lower when using 80 per cent ethanol. From Table 3 it can be seen that the amount of citric acid found as the amount of oxalic acid is almost the same whether 0.5 M hydrochloric acid or cation exchanger-water were used as extractants. It is interesting to note that 80

Table 3. Citric acid in different species of plants found by extraction with 3 different extractants.
Citronsyreindhold i forskellige plantearter fundet ved ekstraktion med 3 forskellige ekstraktionsmidler.

Extractants	Plant species: beet leaves	rye-grass	green peas	string beans
	mg per g dry matter:			
0.5 M HCl	8.4	9.9	7.7	6.6
Ion exchanger water	8.1	9.0	6.9	5.7
80 per cent ethanol	8.5	3.6	4.6	4.9

per cent ethanol extracts almost the same amount from beet leaves as the 2 other extractants, whereas the amount extracted from grass, green pea and string bean was considerably lower with 80 per cent ethanol.

Table 4. Recovery of oxalic acid added to ryegrass as calcium oxalate.
Genfindelse af oxalsyre i rajgræs tilsat calciumoxalat.

Added mg per g dry matter	Per cent recovery
20	97
40	95
66	90
88	91

In Table 4 and Table 5 the results are shown from an examination of the recovery of oxalic acid and citric acid from plant tissue by use of cation exchanger-water extraction. The investigation was carried out by extraction of rye grass after addition of calcium oxalate or calcium citrate to the samples of frozen plant tissue. The recovery (Table 4) for oxalic acid was from 90 per cent to 97 per cent when calcium oxalate corresponding to 20–88 mg oxalic acid per g of plant dry matter was added.

It can be seen from Table 5 that the recovery for citric acid was from 94 per cent to 102 per cent when calcium citrate corresponding to 3–8 mg citric acid per g of plant dry matter was added.

Table 5. Recovery of citric acid added to ryegrass as calcium citrate.
Genfindelse af citronsyre i rajgræs tilsat calciumcitrat.

Added mg per g dry matter	Per cent recovery
3.2	94
8.1	102

Discussion

When water is used as extractant, addition of a strongly acidic cation exchanger releases the anions of the organic acids from their salts and at the same time denaturates and coagulates the proteins. However, the coagulated proteins settled on the particles of the cation exchanger and the plant material, which makes further extraction difficult (*Schramm,* 1973). Coagulation of the proteins by boiling the plant tissue before addition of the cation exchanger was found to reduce their harmful effect.

Disintegration of the frozen plant material made it possible to obtain a representative sample by taking only 3 g fresh weight which also reduced the amount of cation exchanger necessary to obtain the recommended ratio to the plant sample.

As the cation exchanger is disintegrated during the extraction the particle size is unimportant. By addition of the acidic cation exchanger the cations, including calcium, are exchanged with hydrogen ions. However, if this process is to finish in a reasonably short time it has to be accelerated. A possibility for this was to reduce the distance between the particles of the cation exchanger and the crystals of the salts. In agreement with this it was found that the efficiency of the extraction was increased by increasing the amount of cation exchanger and homogenize the suspension of plant tissue and cation exchanger. The effect of storing the suspension overnight also indicates that releasing the acids is not a momentary process.

Calcium oxalate is the most sparingly soluble salt of the common organic acids in plants and most published methods for determination of oxalic acid involve extraction with hot or cold hydrochloric acid (*Hodgkinson,* 1977). According to the results in Table 1 extraction with hot 0.5 or 1.0 M hydrochloric acid seems to give a quantitative extraction of oxalic acids from beet leaves which have a relatively high calcium and oxalic acid content. According to the amount of oxalic and citric acids found in different plant species (Table 2 and 3) cation exchanger-water seems to be nearly as effective as 0.5 M hydrochloric acid whereas the efficiency seems to be smaller for a 80 per cent ethanol especially for extraction of oxalic acid. Since organic acids in plants partly exist as salts of different cations which is known to be less soluble in ethanol than in water the above findings may be due to the inability of the 80 per cent ethanol to dissolve the salts of the acids especially the calcium salts.

It is interesting that 80 per cent ethanol seems to be considerably more effective for extraction of citric acid from beet leaves than from ryegrass althougt the content of citric acid in the 2 plant species seems to be almost the same according to results obtained using the 2 other extractants, Table 3.

These findings are probably linked to the oxalic acid/calcium ratio in the 2 plant species. In ryegrass the level of oxalic acid is low and equivalent to only about one fifth of the calcium level (*Dijkshoorn,* 1973) whereas the level of oxalic acid in beet leaves is very high and exceeds the level of calcium (*Egmond & Houba,* 1970). This difference with respect to the content of calcium and oxalic acids in the 2 plant species may be responsible for the different capability of 80 per cent ethanol for extraction of citric acid from ryegrass and beet leaves respectively. When beet leaves are extracted the whole amount of calcium is precipitated as calcium oxalate and no calcium citrate which is sparingly soluble in ethanol is formed. However, when ryegrass is extracted there is a possibility of formation of calcium citrate because the level of oxalic acid is only one fifth of the calcium level.

This may also contribute to explain the disagreement between results from investigations of

the efficiency of different extraction methods. In such investigations *Schramm et al.*, (1977) found that an alcohol extraction was satisfactory for extraction of citric acid whereas *Fauconneau* (1958) and *Roux* and *Lesaint* (1959) found it insufficient.

The recoveries of citric and oxalic acid added to rye grass as calcium citrate and calcium oxalate respectively indicate that with the present method it should be possible, to extract citric and oxalic acid almost quantitatively from most of the cultivated plant species. Since calcium citrate and especially calcium oxalate are the most sparingly soluble salts of organic acids normally present in plants it may be expected that the method should also be suitable for extraction of the other acids present.

By use of water as extractant it may be expected that the risk of reactions of the extractant with the acids should be very small. According to *Schramm* (1973) this might be a problem when using alcohol, particularly heated alcohol. Furthermore the use of water as extractant has the advantage, to the use of organic solvents, that the amount of chlorophyll extracted is very small. This means that the purification of the extract may be reduced to passing it through a cation exchanger for further deproteinization if necessary and then passing it through an anion exchanger. The use of an anion exchanger for the purification also makes it possible to concentrate and fractionate the acids which makes a determination of the acids by ion exchange chromatography more accurate (*Kyllingsbæk,* 1984).

Conclusion

Use of the described method makes it possible to extract organic acids including oxalic acid almost quantitatively by addition of a strongly acidic cation exchanger to the plant tissue and extract with water. The amount of oxalic and citric acid extracted from different plant species was almost equal to the amount extracted by 0.5 M hydrochloric acid. The amount of the 2 acids extracted by 80 per cent ethanol was lower. An exception was the amount of citric acid extracted from beet leaves which was equal for all 3 extractants.

An advantage of the present method is that the amount of chlorophyll extracted is very small which makes the purification of the extract easy and simple.

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Manuscript received 8 July 1983.