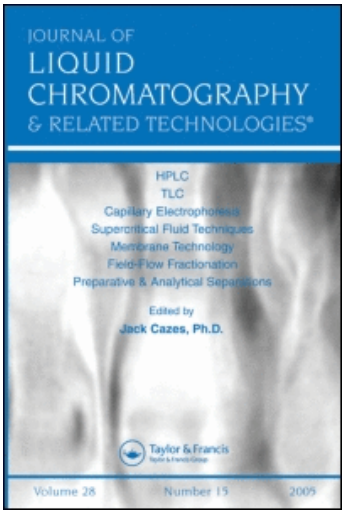

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SEPARATION OF ORGANIC ACIDS IN PLANT TISSUE BY HPLC WITH A TWIN PHASE, ION EXCHANGE AND REVERSE PHASE, COLUMN

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ABSTRACT

No single isocratic chromatographic technique allows the complete separation of common organic aliphatic and alicyclic acids of plants. In order to obtain a better isocratic separation with a single HPLC method we combined in one chromatography assay the Ion Exchange and Reverse Phase technics in building a twin phase column. The first attempts are promising. This double chromatography based on the polarity of molecule (Reverse Phase) and on its acidic characteristics (Ion Exchange) has the advantages of both methods and allows good separations of the acids.

INTRODUCTION

The number of chromatographic techniques used reflects the difficulty of natural organic acid separation. The most common methods are Gas Liquid (1,2,3,4,5) and Liquid-Liquid Chromatogra-

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phy. High performance Liquid Chromatography (HPLC) has recently been uses in three forms : Reverse Phase (6,7,8,9,10,11), Ion Exchange (12,13,14,15,16,17,18,19,20) and Counter Ion Chromatography (21). No single method allows the complete separation of common organic aliphatic and alicyclic acids present in plants and consequently each problem needs a specific solution. Chromatographic columns with specific characteristics (12,13) are available commercially but they often involve expensive and delicate procedures, particularly due to the high cost of columns and the need of a gradient apparatus.

In order to obtain a good isocratic separation of organic acids with a single HPLC method, we combined in one chromatography assay, the Ion Exchange and Reverse Phase techniques in building a twin phase column. For this purpose we took advantage of two main properties of the organic acid molecules :

- their polarity (brought out by the Reverse Phase)
- their acid dissociation constant (brought out by the Ion Exchange).

MATERIALS

Choice of supports

The reverse phase support, Lichrosorb RP 18 (Merck) particle size 10 µ, was chosen because of the silica-bonded octadecyl groups (C_{18}) which are the best support for the retention of polar organic acids. The ion exchange support, Lichrosorb AN (Merck) particle size 10 µ, was chosen because of the silica-bonded ammonium quaternary groups which are strongly anionic.

Column characteristics

Supports	RP 18	Ion Exchange
Length	320 mm	80 mm
Diameter	4 mm	4 mm
Particle size	10 µ	10 µ

Choice of eluents

We used as mobile phases aqueous phosphate buffers at different pH and concentrations.

Chromatographic equipment

The chromatographic system included the following equipment :

. a rotary solvent distribution valve (Reodyne 50-03)

. an injector valve (Teflon rotary valve Reodyne 50)

. a high pressure pump (ORLITA D MP-AE 10-4)

. an UV detector (PHILIPS Pye Unicam LC), 210 nm

. a pulse damper (TOUZART et MATIGNON)

. a flow rate and pressure control unit (TOUZART et MATIGNON)

. a recorder (KIPP and ZONEN BD 41)

. recorder-computer (SHIMADZU)

. column packing equipment (TOUZART et MATIGNON)

. a temperature regulator (JULABO).

METHODS

The column was first partially filled with the Reverse Phase support and then totally filled with the Ion Exchange support. The following solvents were used :

- for Reverse Phase : a mixture of ethanol (8 ml) and bromoform (15 ml), with similar densities of the support and solvent (d = 2.1)
- for Ion Exchange : the phosphate buffer later used as the chromatographic mobile phase.

The suspension of each support was homogenized and degassed by ultrasonics during several minutes and then poured into the filling apparatus precolumn TOUZART et MATIGNON (23 ml capacity). The suspension was pushed from the precolumn to the chromatographic column using a 450 bars filling pressure, with ethanol for the Reverse Phase, and phosphate buffer for the Ion Exchange part. The required time to built a twin phase column was about 3 hours.

The functional direction was the same as the filling direction; thus the column was used first in the Ion Exchange part and second in the Reverse Phase part.

After preparation the column was stabilized with the chromatographic mobile phase and tested for its efficiency before final attatchment to the chromatograph.

RESULTS AND DISCUSSION

Our chromatographic studies were directed toward the separation of organic acids (quinic, shikimic, tartaric, malic, fumaric, succinic, citric and oxalic) which contribute to the leaf tissue acidity of trees. HPLC chromatography performed either by Ion Exchange or with Reverse Phase did not give good results. The table gives representative results. Using the twin phase column, we

TABLE : Average values of the capacity coefficients of acids in increasing order for a 30 cm length Lichrosorb AN 10 µ diameter column (k'_{AN}) and a 15 cm length Lichrosorb RP_{18} 5 µ diameter column ($k'_{RP_{18}}$).

These coefficients were obtained with a potassium orthophosphate buffer as eluent (concentration 0.1 M, pH = 2.5)

ACIDS	SHIKIMIC	QUINIC	TARTARIC	MALIC	SUCCINIC	CITRIC	OXALIC	FUMARIC
k'_{AN}	0.50 ⟷	0.54	1.50 ⟷	1.62	2.08	5.5	11.7 ⟷	27

ACIDS	OXALIC	TARTARIC	QUINIC	MALIC	SHIKIMIC	CITRIC	FUMARIC	SUCCINIC
$k'_{RP_{18}}$	0.10 ⟷	0.28 ⟷	0.33	0.60 ⟷	0.75	2.20 ⟷	2.26 ⟶	2.33

⟷ Non separated acids : common part > 1/2 the height of the smaller peak

⟷ Partially separated acids : common part < 1/2 the height of the smaller peak

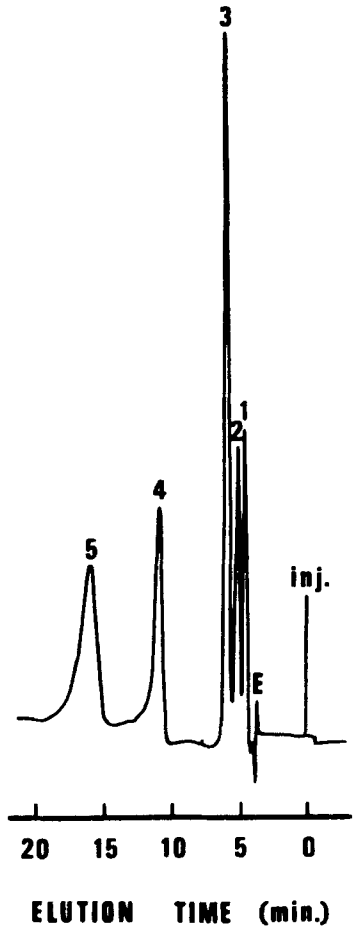

FIG. 1

Acids	Concentration µg/ml	k'
1. Quinic	225	0.26
2. Tartaric	200	0.39
3. Shikimic + Malic	11 + 700	0.98
4. Succinic + Citric	200 + 200	1.98
5. Fumaric	10.5	3.36

Sensitivity : S = 0.08 A.U.F.S.
Temperature : 20°C
Flow rate : 1.45 ml/minute
Pressure : 81 bars
Buffer : (KH_2PO_4-H_3PO_4) c = 0.33 M ; pH = 2.5
PEAKS : E = Eluent

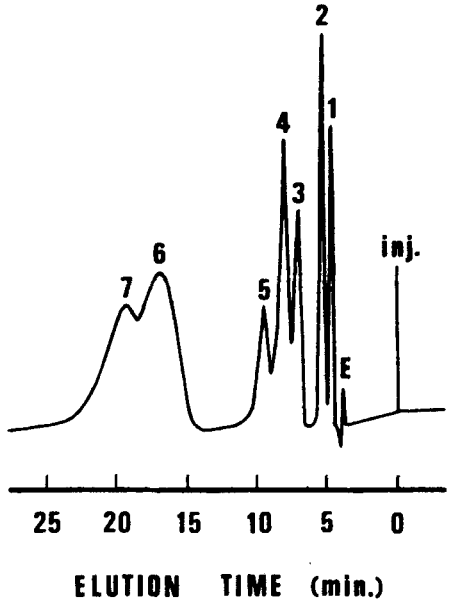

FIG. 2

Acids	Concentration µg/ml	k'
1. Quinic	300	0.25
2. Shikimic	11	0.43
3. Malic	300	0.86
4. Tartaric	300	1.14
5. Succinic	300	1.54
6. Oxalic + Fumaric	300	≈ 3.5
7. Citric	300	≈ 4.5

Sensitivity : S = 0.08 A.U.F.S.
Temperature : 20°C
Flow rate : d = 1.29 ml/minute
Pressure : 71 bars
Buffer : (KH_2PO_4) c = 0.2 M ; pH = 4.5

PEAKS : E = Eluent

expected to obtain both the advantages of the two kinds of chromatography :

- a good separation of quinic and shikimic acids brought by the Reverse Phase
- an increase by the Anion Exchange support of the capacity coefficients (k') of oxalic, and perhaps tartaric, acids observed with Reverse Phase, in order to displace their chromatographic peaks, respectively, away from those of quinic and shikimic acids.

At first, we studied the effects of eluent pH, and ionic strength on the retention of each acid by a 40 cm length twin phase column (RP 18 and A.N. in the proportion 1/4). It appeared that the acids fell into two groups :

- those whose capacity coefficient k' were little influenced by pH and ionic strength ; they are quinic, shikimic, malic and succinic acids ;
- those whose capacity coefficient k' were more dependant on pH and ionic strength ; they are citric, fumaric, oxalic and tartaric acids.

Generally, k' decreases when ionic strength increases at constant pH, and k' increases with increasing pH at constant ionic strength. The pH effect is more noticeable for tartaric and citric acids, especially with low eluent concentrations (0.1 M). At pH = 2.5 and above oxalic acid is completely dissociated and consequently strongly retained by the Anion Exchange support. We obtained a very high k' coefficient for low eluent concentrations.

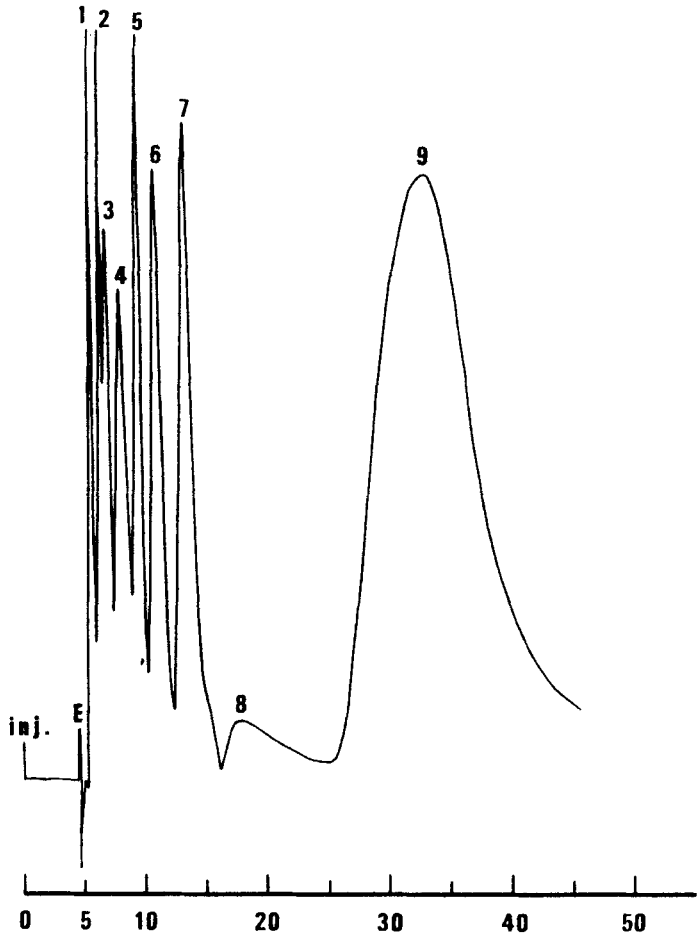

FIG. 3

Acids	Concentration µg/ml	k'
Quinic	250	0.21
Shikimic	7.5	0.38
Malic	250	0.50
Tartaric	250	0.75
Succinic	250	1.05
Malonic	250	1.37
Fumaric	5.0	1.91
Citric	250	3.01
Oxalic	250	6.22

Sensitivity : S = 0.04 A.U.F.S.
Temperature : 40°C
Flow rate : d = 0.99 ml/minute
Pressure : 41 bars
Buffer : (KH_2PO_4-H_3PO_4)
c = 0.2M, pH = 3.8

With this data in hand, we used the same column to analyse mixtures of acids using different experimental conditions. Our results show that flow rate, temperature, and sample mixture composition play a prominent part in the acids separations. The figures give representative examples of the separation obtained. Figure 3, for instance, shows the separation of 9 organic acids at a temperature of 40°C, which is a very promising result. It must be noted that although, an increase of temperature improves acid separation, good results were obtained at 25°C for more less complex cases (Fig. 1,2).

The choice of the proportion of the support in the column (Reverse Phase 3/4 - Anion Exchange 1/4) was not well suited to oxalic acid identification. Increasing the ratio of the Reverse Phase support would give better results. Another way improvement would be obtained with the use of very low pH ; such a pH suppresses an important dissociation of this acid, and consequently it would strongly lower its retention time. Unfortunately these low pH's are uncompatible with silicon supports. An Anion Exchange Chromatography at very low pH (pH < 2.5) has been used by others to identify oxalic acid (13,14). An ammonium styrene support type, which does not allow the use of high solvent pressure, was used.

CONCLUSION

The first attempts to separate aliphatic and alicyclic acids of tree foliar tissue on a twin phase column, using Reverse Phase and Ion Exchange supports, are promising. This double chromatogra-

phy, based on the polarity of the molecule (Reverse Phase) and on its acid characteristics (Ion Exchange), uses the advantages of both methods. The possibility of varying the proportions of the two supports in a single column allows the researcher a personalized column whose properties can be modulated complementarly by the eluent ionic strength, the eluent pH, and the temperature conditions.

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