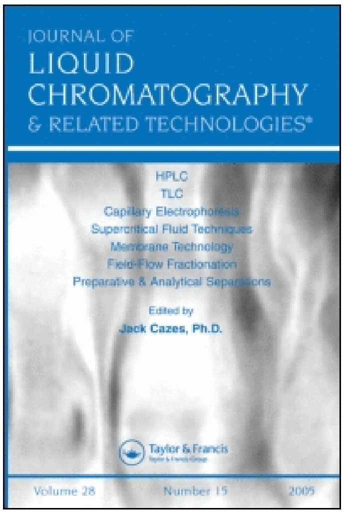

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NON-SUPPRESSED CONDUCTIVITY DETECTION OF ORGANIC ACIDS IN PLANT TISSUE EXTRACTS BY ION-EXCLUSION CHROMATOGRAPHY WITH AROMATIC ACID ELUENTS

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ABSTRACT

Aromatic acids were evaluated as eluents for ion exclusion chromatography of organic acids with non-suppressed conductivity detection. The separation of organic acids, performed on an Aminex HPX-87H ion-exclusion column, using a variety of aromatic carboxylic acid eluents, is described in detail. Phthalic acid was found to be a suitable eluent for the separation of the organic acid tested, giving reasonable resolution and detection sensitivity. When 1 mM phthalic acid was used as eluent, an excellent separation of eight acids was achieved in 20 min. Calibration plots for the test acids were linear in the range 10^{-2} M to 10^{-5} M with detection limits of the order of 10^{-6} M. Compared to eluent containing sulfuric acid, the proposed method permits a more sensitive detection for the organic acids tested due to the lower background conductance of the eluent used. The proposed method was used for the determination of organic acids in plant tissue extracts with direct injection of sample extract.

INTRODUCTION

Ion-exclusion chromatography (IEC) has been used widely for the separation of inorganic and organic weak acids, where the separation of weak acids is performed on a column packed with a high capacity sulfonated-styrene-divinylbenzene (PS-DVB) exchange resin in the H^+ form with an eluent containing strong acid or weak acid.[1-3] Acidic eluents reduce the dissociation of organic acids so that retention of organic acids on the resin matrix is increased. Consequently, the retention behavior of organic acids in ICE is dependent on their first dissociation constant (pKa_1) and their hydrophobicity.[1-3] Various strong and weak acids have been used as eluents for the separation of organic acids by IEC coupled with different detection methods.[2] Strong acids such as sulfuric acid[4] and aliphatic sulfonic acids[5] are commonly employed as eluents, and weak acids, including phosphoric,[6] perfluorobutric,[7] succinic, and benzoic acids,[8] have also been used as eluents in IEC of organic acids.

Detection methods applicable to non-suppressed IEC of organic acids involve UV absorbance (200-220 nm) or refractive index, and conductivity. In the case of UV absorbance or refractive index detection, strong acids such as sulfuric acid are used as eluents, but these detection methods are rather insensitive because some organic acids absorb only weakly at the low end of the UV spectral region.[1,2] Hydrochloric acid is often employed as an eluent with conductivity detection, after the eluent is passed through an additional suppression device to reduce background conductance.[9]

However, the precipitation of silver chloride tends to plug the column and increase the backpressure. Hence, various acids with low conductance have been used as eluents coupled with conductivity detection. For example, aliphatic sulfonic acids can be employed as an eluent for separation of carboxylic acids coupled with conductivity detection because of the relatively low background conductance without suppression.[5]

Tanaka et al.[8] compared the use of various weak acids as eluents for separation of aliphatic carboxylic acids by IEC, and found benzoic or succinic acids to be suitable eluents with highly sensitive conductivity detection and high resolution for aliphatic carboxylic acid. Regrettably, among the aromatic carboxylic acids, only benzoic acid was examined, and organic acids with a similar dissociation constant can not be separated well.(e.g., oxalic acid and maleic acid). More recently, Tanaka et al.[10,11] And, Morris et al.[12] suggested that addition of polyalcohols and sugars to mobile phase provide a reasonable resolution and highly sensitive detection of carboxylic acids.

Aromatic carboxylic acids are usually employed as eluents for the separation of anions in ion chromatography,[1,2] and there are few reports of them being employed as eluents in IEC coupled with non-suppressed conductivity detection. Therefore, it is of interest to test a variety of aromatic acidic eluents coupled with non-suppressed conductivity for the analysis of organic acids separated by IEC.

The experimental conditions affecting the retention and conductivity detection sensitivity for the test acids were examined in detail to determine the optimum conditions to achieve a reasonable resolution and high detection sensitivity. Finally, the proposed method was demonstrated by the determination of organic acids in plant tissue extracts.

MATERIALS AND METHODS

Analytical-grade reagents (Aldrich, Sydney, Australia) were used in all cases. Standard solutions of the organic acids tested were prepared with deionized water obtained from a Milli-Q system (Millipore, Bedford, MA). Aqueous eluents containing aromatic acids were prepared as 0.1-5 mM solutions by dissolving the aromatic acid in deionised water.

The chromatographic system consisted of a Waters (Milford, MA, USA) model 510 HPLC, Model 710B intelligent sample processor and a Model 431 conductivity detector, and a Bio-Rad cation guard column and an Aminex HPX-87 organic acid column (300x7.8 mm I.D, Richmond, CA, USA).

The column was placed in a Waters column heater and maintained at 40°C. Data collection was done with chromatography software (Dapa, Perth, Australia) installed on a computer.

The eluent was passed though a Millipore 0.45 -µm membrane filter and degassed in an ultrasonic bath before use. The column was equilibrated for 60 min before use. 20 µL of solute solution was injected into the chromatographic system. Lyophilized tissues were pulverized using a Braun Mikro-Dimemator II to yield 1-2 µm particles.

Water-soluble solutes in tissue powder were extracted twice with 5 mL water (0.1 g /5 mL) in a water bath at 50°C for 60 min. The extract solution was filtered using a Millipore 0.45 -µm membrane before injecting to the chromatographic system.

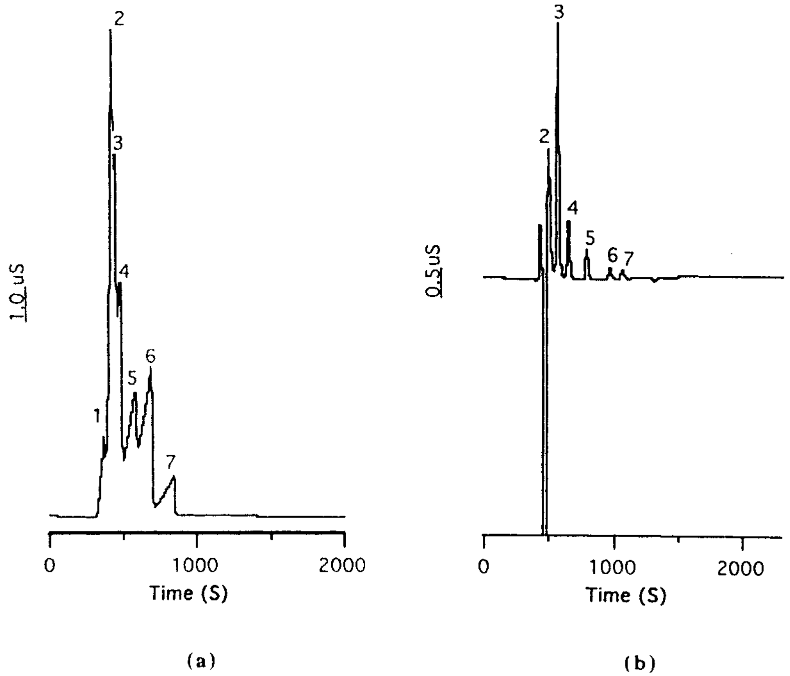

Figure 1. (a) Ion-exclusion chromatography of organic acids with water as eluent. (b) Ion-exclusion chromatography of organic acids with a 2mM sulfuric acid as eluent. (1) oxalic, (2) maleic , (3) citric, (4) malic, (5) tartaric, (6)formic, (7)acetic acid. Condition: flow rate: 0.5 mL/min; injected volume: 20 μL.

RESULTS AND DISCUSSION

Effect of Eluent on the Retention and Detection of Organic Acids

Addition of aromatic acids with different dissociation constants to mobile phase decreases the dissociation of the organic acids tested, and increases their retention times. In addition, the detection sensitivity also depends on the eluent containing the aromatic acids because the concentration of aromatic acid in the eluent influences the background conductance. Hence, it would be possible to use them as eluents in IEC for separation and detection of the organic acids tested. In the preliminary stages, benzoic, phthalic, trimesic, and pyromellitic acids were considered as eluents. All these species have a low conductance, and produce low background conductance.[1,2] Water and sulfuric

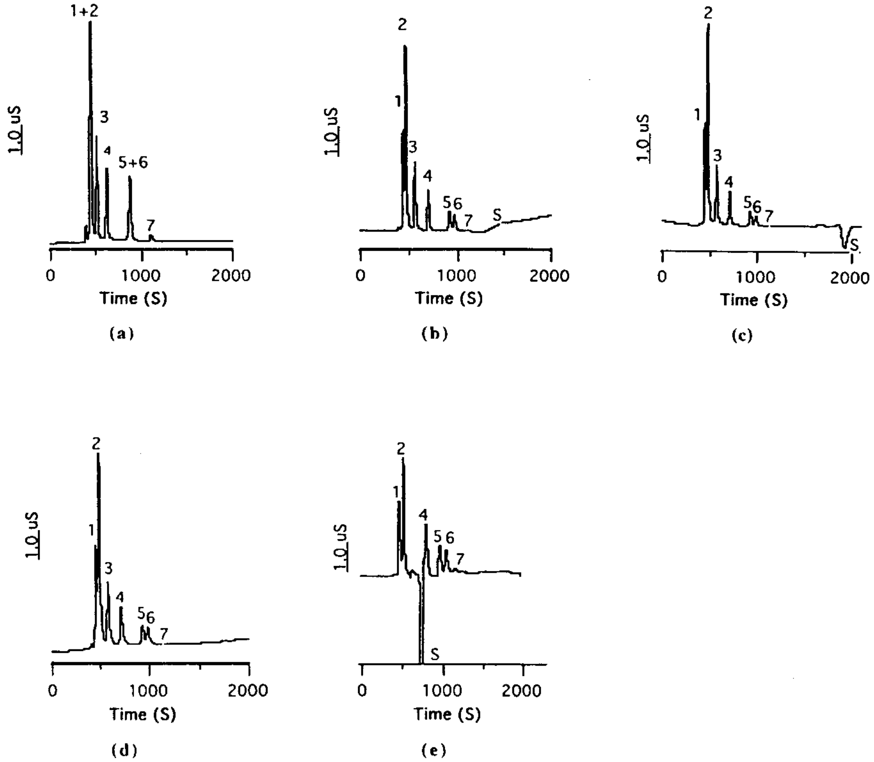

Figure 2. Ion-exclusion chromatography of aliphatic acids by elution with aromatic acidic eluents. (a) benzoic, (b) salicylic, (c)phthalic, (d)trimesic, (e) pyromellitic acid. Other conditions same as in Fig.1.

acid also were tested as eluent for comparison with the aromatic acidic eluents used. The elution behavior of the organic acids, including oxalic, maleic, citric, tartaric, malic, succinic, formic, and acetic acid, were investigated by using eluents containing 2 mM aromatic acids to characterize the column selectivity.

Figurel (a) and (b) show the IEC separation of the test acids using water and a 2 mM sulfuric acid as eluents, and detection by non-suppressed conductivity detection. It is clear that poor resolution and fronting peak were obtained by using water eluent resulting in a great degree of ionization of the test acid and hydrophobic adsorption effect.[3]

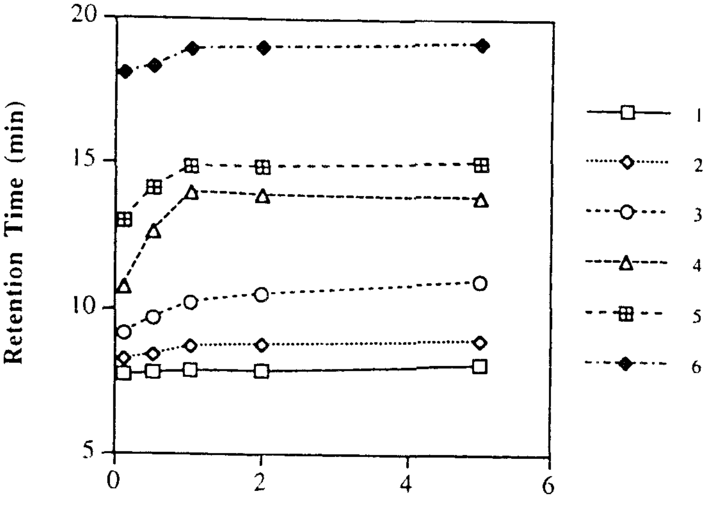

Figure 3. The effect of the concentration of the phthalic acid in mobile phase on the retention time of organic acids. Other conditions same as in Fig.1.

In contrast, addition of dilute sulfuric acid to the mobile phase greatly improved the resolution and peak shape by reducing ionization of the test acids and hydrophobic effect, but the detection sensitivity was relatively low for most of the test acids due to the increase in the background conductance.[8] Fig.2 (a) to (e) show the chromatograms obtained by using various aromatic acid eluents. It indicates that all aromatic acids tested can be used for the elution of the organic acids tested with a symmetric peak and reasonable detection sensitivity. However, it was observed that oxalic and malic acids, tartaric and formic acids can not be separated well using benzoic acid eluent. With other aromatic acid eluents, good separation of the test acids and detection sensitivity was obtained. The detection sensitivities decreased in the order of benzoic> phthalic = trimetric = salicylic > pyomellitic acid. Clearly, the conductivity sensitivity increased as *pKa* of the aromatic acids in eluent increased. In addition, a system peak was observed due to the aromatic acid in the eluent adsorbing strongly on the PS-DVB resins.[1] In all cases, for both separation and detection, phthalic acid was found the best eluent. The chromatogram shown in Fig.2 (c) was obtained by using a 2 mM phthalic acid eluent with conductivity detection of the acids tested. Resolution and peak shapes were significantly improved

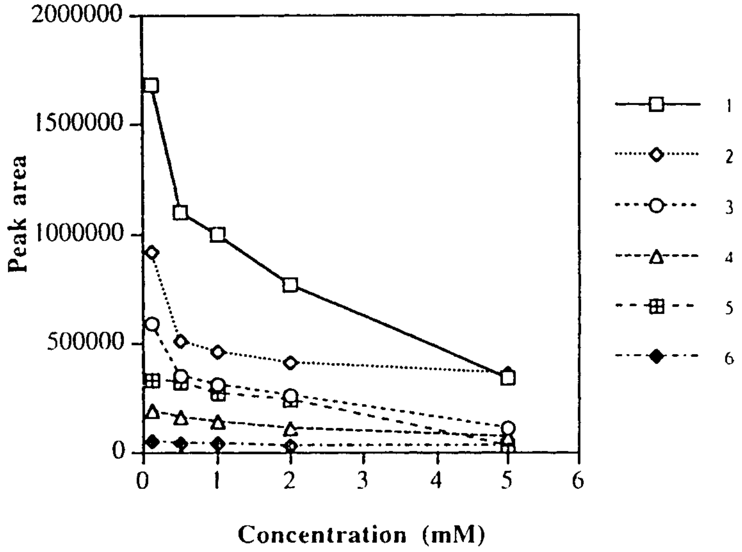

Figure 4. The effect of the concentration the phthalic acid in mobile phase on the detection sensitivities for the test acids. Other conditions same as in Fig.1.

using phthalic acid eluent. This is the result of decreased ionization of the test acids when using weak acid eluent.[3] In addition, the detection sensitivities for the test acids by using phthalic acid eluent are higher than those obtained with sulfuric acid eluent due to its the background conductance.[2] Thus, phthalic acid was employed as an eluent in the following section.

Effect of Phthalic Acid Concentration

Tanaka et al.[8] reported that use of benzoic acid eluent in IEC improved the retention of hydrophobic carboxylic acid. In our case, the test acids were hydrophilic, so it was expected that the concentration of phthalic acid would have little affect on their retention, but would significantly influence the detection sensitivity due to background conductance varying with the concentration of phthalic acid in eluent. Such effects were examined by using 0.1-5 mM phthalic acidic eluent to find the optimum conditions for both separation and detection. Figures 3 and 4 show that the retention of the test acids increased as the concentration of phthalic acid in eluent increased from 0.1 to 1 mM, but the retention for oxalic and maleic acids changed little.

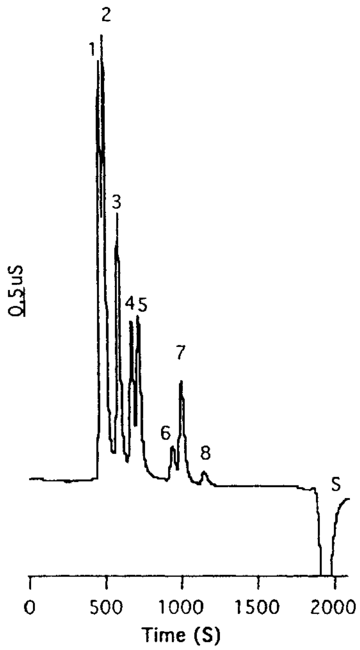

Figure 5. Ion-exclusion chromatography of organic acids using 1 mM phthalic acid as eluent. (1) oxalic (1 mM), (2) maleic (0.5 mM), (3) citric (1 mM), (4) T-aconitic (0.5mM), (5) malic (1 mM), (6) tartaric (1 mM), (7) formic (1 mM), (8) acetic acid (2 mM). Injected volume: 30 μL. Other condtions same as in Fig. 1.

In contrast, the conductivity sensitivity greatly decreased as the concentration of phthalic acid in the eluent increased. This is attributed to increasing background conductance[2] Hence, for both separation and detection, a 1 mM phthalic eluent seems to be the best suitable eluent for this system.

Calibration Plots, Reproducibility, and Detection Limits

Figure 5 is a typical chromatogram obtained from using a 1 mM phthalic acid eluent, and demonstrates eight organic acids separated by IEC and detected using non-suppressed conductivity. The test acids were eluted in order: oxalic , maleic, citric, malic, trans-aconitic, tartaric, formic, and acetic acids. The retention times were 7.9, 8.4, 10.0, 11.6, 12.3, 16.2, 16.8, and 19.5 min, respectively. Good resolution, symmetrical peaks, and sensitive detection were obtained with these conditions. This demonstrates that addition of

Table 1

Analytical Characteristics for the Test Organic Acids in Ion-Exclusions Chromatography with Conductivity Detection*

Acids	Retention	Calibration Curve	r^2	RSD(%) (n=5)	DL Con. (μM)	DL UV (μM)[13]
Oxalic	7.90	$Y=2.73\times10^5x-2.94\times10^4$	1.00	2.26	1	—
Maleic	8.43	$Y=5.83\times10^5-1.84\times10^3$	0.999	1.82	1	—
Citric	9.96	$Y=1.80\times10^5x-1.37\times10^3$	1.00	1.11	2	20
T-aconitic	11.6	$Y=5.83\times10^5x-1.84\times10^3$	1.00	1.87	3	—
Malic	12.3	$Y=1.27\times10^5x-4.12\times10^3$	1.00	1.24	5	40
Tartaric	16.2	$Y=3.27\times10^4x-1.14\times10^3$	1.00	2.05	4	—
Formic	16.8	$Y=4.88\times10^4x-1.62\times10^3$	0.999	1.92	5	110
Acetic	19.5	$Y=4.64\times10^3x+4.53\times10^2$	0.995	1.19	8	150

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* Conditions as Fig. 5. DL-Detection Limits (Single/Noise (S/N)=3); Con. - Conductivity.

phthalic acid to the mobile phase decreased the dissociation of the test acids, and hence increased the retention. In addition, the low background conductance of the phthalic acid eluent enhanced the detection sensitivity. The calibration plots of the test acid were obtained by plotting peak area *vs* the concentration of the test acid, and was found linear in the range of 0.01 to 10 mM. The correlation coefficients (r^2) were in the range of 0.995 to 1.00. The detection limits (S/N=3) were in the order of 1.0 μM and the reproducibility (relative standard derivation, n=5) from injecting a 1 mM test acids solution ranged between 1.0-2.5%.

The analytical characteristics for the proposed method, together with UV detection (210 nm) of organic acids separated by IEC with sulfuric acid eluent obtained from reference,[13] are listed in Table 1. It can be seen that the detection limits obtained with phthalic acid eluent are lower than those achieved with sulfuric acid eluent.

Analysis of Plant Extracts

The proposed method was used to determine the organic acids in various plant tissue extracts. The extracts were filtered by passing through a Millipore 0.45 -μm membrane. The extracts were diluted tenfold before injection. Good resolution for the organic acids of interest was obtained with the exception of oxalic acid. The organic acids in plant tissue extracts are listed in Table 2.

Table 2

Analysis of Organic Acids in Plant Tissue Extracts

Sample	Maleic Acid (mM)	Citric Acid (mM)	Malic Acid (mM)	Tartartic Acid (mM)	Formic Acid (mM)
Pea extracts	0.85	0.99	1.13	---	0.21
Faba extract	---	0.91	0.34	0.24	0.36

The result shows that the proposed method is useful for the determination of organic acids in plant tissue extracts with direct injection. However, the problem is co-elution of oxalic acid with inorganic anions such as Cl^- and NO_3^{-}[14]. Extraction procedure to eliminate anion interference is required for determination of oxalic acid in plant extracts.

CONCLUSION

The findings of this research have demonstrated that aromatic acids such as phthalic acid can be used as eluent in IEC for the separation of organic acids detected by non-suppressed conductivity. The results showed that good resolution and sensitivity detection with a simple analytical procedure was achieved with the separation of the organic acids in 20 min. Compared to UV detection with sulfuric acid as eluent, lower detection limits for the test acids were obtained by non-suppressed conductivity detector. Work is in progress to improve column selectivity for organic acids of interest by using various weak acids to separate more organic acids in plant extracts.

ACKNOWLEDGMENTS

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