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Indirect photometric detection of aliphatic acids separated by ion-exclusion chromatography using aromatic acidic eluents

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Abstract

The use of aromatic acids as eluents for the indirect photometric detection of aliphatic acids in ion-exclusion chromatography is described. A range of aromatic acids (benzoic, salicylic, phthalic, trimesic and pyromellitic acids) were investigated to obtain optimal conditions of separation and detection as well as to obtain symmetrical peaks. Phthalic acid was the best compromise among the possible eluents. A theoretical treatment predicts a linear response of peak height to solute concentration. The peak height should also be proportional to the solute dissociation constant and inversely proportional to the dissociation constant and concentration of aromatic acids used as modifiers. These predictions were confirmed experimentally. A linear response was obtained over range of concentrations 0.1–10 m*M* in 1 m*M* phthalic acid. The proposed method was demonstrated for use in the analysis of aliphatic acids in extracts of agricultural and Australian native plants.

Keywords: Detection, LC; Mobile phase composition; Aliphatic acids; Aromatic acids; Phthalic acid

1. Introduction

Ion-exclusion chromatography (IEC) with various detection techniques finds application in the analysis of weak and medium strength acids [1–3]. Detection techniques include direct UV absorbance [4,5], refractive index (RI) [6], potentiometric [7,8] and both suppressed [9] and non-suppressed conductivity [10–12]. Direct UV detection is commonly used in the analysis of aliphatic acids with sulfuric acid eluent at low wavelength (200–220 nm) [4,5], where detector response depends on the sum of the molar absorption of neutral and dissociated forms of acids. Detection limits in these cases are rather high because of the lack of a chromophore and the sample matrix may cause serious interference. Refractive index detection offers universal response but is characterized by high detection limits and thermal instability. The most commonly used IEC conductometric detector also lacks sensitivity due to poor dissociation of acids and is additionally depressed by the buffer.

Aliphatic acids can be separated by IEC with a water eluent. However, resolution is often poor because of fronting peaks caused by ionization of the acids. This effect is increased by hydrophobic absorption of undissociated forms of acids [10–14]. To overcome these problems, addition of various modifiers to the mobile phase is usually proposed. Such additions include (1) diluted strong acids [4,5], (2) ion paring reagents [9] or (3) a low concentration of

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polyalcohols or sugars (which increase the hydrophilicity of the cation-exchange resin) [10–12]. The actions mentioned above are suitable for bulk property detectors such as potentiometry [8,9], conductivity [11,12] and electrokinetry [13].

Indirect photometric detection (IPD) has been successfully used in ion chromatography [14,15] and capillary electrophoresis [16,17], where the eluting ion is the chromophore and is monitored by photometry. When a non-detectable ion passes the detection window, the increase or decrease in concentration of the chromophore in the analyte zone results in either an increase or decrease in the background signal [15,17]. Chromophores such as phthalate and salicylate [18], 2,6-anthraquinone disulfonate [19] and naphthalenedisulfonate [20], have thus been employed as mobile phases in ion chromatography. The use of IPD for ionic compounds in ion-exchange chromatography is based on the simplified mechanism of charge displacement [14,21]. The indirect response of ionic compounds in ion-interaction chromatography [22,23] have been developed for use in detection of ionic solutes [24]. In addition, reversed-phase liquid chromatography [25] based on the distribution of solutes in relation to the probe are governed by common interaction effects such as competition for the binding surface. However, to the best of our knowledge, indirect photometric detection of weak inorganic anions and organic acids separated by IEC has not been reported. Aromatic acids are frequently employed as eluents in ion chromatography and can be detected by both non-suppressed conductivity and indirect photometry due to their low conductance and high molar absorptivity in UV region [1]. Therefore, it was of interest to examine the possibility of the indirect photometric detection of acids by IEC using aromatic acidic eluents.

This paper describes IEC of aliphatic acids using aromatic acid eluents, with particular emphasis on detection using indirect photometric detection method. Addition of aromatic acids with different dissociation constants to the mobile phase decreases the dissociation of aliphatic acids and thus, influences retention times. Addition of a variety of aromatic acids to the mobile phase was investigated to determine conditions of optimal resolution and detection sensitivity for both separation and indirect photometric detection.

2. Experimental

2.1. Instrumentation

The ion chromatographic system consisted of a Waters (Milford, MA, USA) Model 510 pump, a Model 484 LC spectrophotometer and a U6K universal injector with a 100-μl sample loop. A Bio-Rad cation guard column and a HPX-87 organic acid column (300×7.8 mm I.D, Richmond, CA, USA) was used for separation of aliphatic acids. The column was placed in a Waters column heater. A UV detector was interfaced with a computer data processing system (Delta Software).

2.2. Chemicals and solutions

All reagents were of analytical grade and dissolved in Milli-Q water. Standard solutions of the aliphatic acids were prepared using analytical grade chemicals without further purification. Standards of the aliphatic acids tested were prepared daily from a 10 m*M* stock solution in Milli-Q water and diluted to the required concentrations before use. The mobile phase required for ion-exclusion chromatography was prepared by dissolution of an appropriate amount of aromatic acid solution in Milli-Q water. All eluents were filtered through a Millipore 0.45-μm membrane filter and degassed in an ultrasonic bath prior to use.

2.3. Procedure

The mobile phase was pumped through the column and UV detector at a flow-rate of 0.5 ml/min. The column temperature was maintained at 40°C and equilibrated for 30 min prior to use. The injected volumes of aliphatic acid solution ranged from 10 to 30 μl. The UV detector was operated at 250 or 300 nm with a sensitivity setting of 0.002 a.u.f.s.

3. Indirect detection model in IEC

Many eluents can be used in IEC for the separation of aliphatic acids [1–3] including water. The low resolution and poor peak shape which result when using water is due to poor retention caused by ionization of the aliphatic acids [3]. To improve peak

asymmetry, addition of strong acids (such as sulfuric acid) to the mobile phase decrease the degree of ionization of the aliphatic acids, and generally improve peak shape and resolution. Replacing sulfuric acid with aromatic acids, might reasonably be expected to improve both retention and detection.

When an aromatic acid, HB, is used as a eluent, the background absorbance, A_b, based on Lambert–Beert Law is given by;

$$A_b = l\varepsilon_{hb}[HB] + l\varepsilon_b[B^-] \quad (1)$$

where ε_{hb} and ε_b denotes the molar absorption coefficient of the free and dissociated acid and, l denotes the length of the optical path. It can be assumed that the molar absorption coefficients of the dissociated and undissociated form of acids are identical.

When the aliphatic acid to be quantified, HR, is weak and injected in small concentration, the concentration of the dissociated form of the aromatic acid is decreased according to the electroneutrality condition in the mobile phase, without changing its dissociation:

$$A_{max} = l\varepsilon_{hb}[HB] + l\varepsilon_b[B^-]_{max} \quad (2)$$

where subscript ''max'' denotes the peak maximum.

The maximum absorption peak, based on Eqs. (1) and (2) may be expressed as;

$$\Delta A = A_{max} - A_b = l\varepsilon_b([B^-]_{max} - [B^-]) \quad (3)$$

Under the above assumption we can write also:

$$[H^+] = [B^-] \quad (4)$$

and

$$[H^+] = [B^-]_{max} + [R^+] \quad (5)$$

The distribution coefficient of protons equal to ''1''. It can be assumed then that protons in Eq. (5) originate only from the aromatic acid and that their concentration is constant. From Eqs. (4) and (5) we can obtain:

$$[B^-]_{max} - [B^-] = -[R^-] \quad (6)$$

After substitution of Eq. (6) into Eq. (3),

$$\Delta A = -\varepsilon_h l[R^-] \quad (7)$$

The mass conversion equation of the aliphatic acid [3] can be then expressed as;

$$\frac{c_i v_i (V_s + V_m)}{V_r}\sqrt{\frac{N}{2\pi}} = [HR]_S V_s + ([HR + [R^-])V_m \quad (8)$$

where subscript ''S'' denotes the stationary phase, c_i and v_i are sample concentration and volume, respectively; V_m and V_s are dead and inner column volumes, respectively; V_r is the retention volume and N is the number equivalent to the theoretical plates.

In the pure ion-exclusion mechanism;

$$[HR]_S = [HR] \quad (9)$$

In the absence of specific interaction, the neutral molecules are partitioned between two phases in a water eluent. The dissociation constant of the aliphatic acid is given by;

$$K_a = \frac{[R^-][H^+]}{[HR]} \quad (10)$$

After rearrangement, it is possible to obtain from Eqs. (7)–(10);

$$\Delta A = -\frac{\varepsilon_h l c_i v_i K_a (V_s + V_m)}{[H^+]V_r(V_s + V_m) + V_m V_r K_a}\sqrt{\frac{N}{2\pi}} \quad (11)$$

The dissociation constant of the aromatic acid used as the eluent can be expressed to similarly Eq. (10). After column equilibration, its mass balance can be presented in the form:

$$c_b = [HB] + [B^-] \quad (12)$$

where c_b represents the buffer concentration.

From Eqs. (10) and (11), the concentration of protons in the mobile phase can be estimated assuming no modification by the solute. Finally the absorbance change is described as;

$$\Delta A = -\frac{2\varepsilon_b l c_i v_i K_a (V_s + V_m)}{V_r(V_s + V_m)\left(\sqrt{K_b^2 + 4K_b c_b} - K_b\right) + 2V_m V_r K_a} \times\sqrt{\frac{N}{2\pi}} \quad (13)$$

The change in absorbance (ΔA) should be directly proportional to the amount of injected aliphatic acid. That is, a linear response between peak height and solute concentration is predicted. Peak height should be also proportional to the solute dissociation con-

stant and inversely proportional to the dissociation constant and the concentration of aromatic acid used as mobile phase modifier. Thus, it should be possible to couple indirect photometric detection with IEC using aromatic acid eluents. Finally it should be emphasised that aromatic acids influence retention of the aliphatic acids through competition for the hydrophobic adsorption sites on the surface of the stationary phase.

4. Results and discussion

A series of test aromatic acid eluents (benzoic, salicylic, phthalic, trimesic and pyromellitic acids) were used to examine the theoretical predictions from the proposed indirect photometric detection method. Aromatic acids with different dissociation constants (pK_a value ranged from 1.9 to 4.2) and high molar absorption coefficients [26] were used in order to obtain high detection sensitivity as illustrated in Eq. (13). Aliphatic acids, including oxalic, maleic, citric, tartaric, malic, formic and acetic, were selected as the solutes with different pK_a [27].

Fig. 1a and Fig. 1b show the IEC separation of the aliphatic acids using water and 2 m*M* sulfuric acid eluents, and detection by direct UV at 200 nm. Poor resolution and fronting peaks obtained with a water eluent were due to the large degree of ionization of the aliphatic acids and the corresponding hydrophobic adsorption effect [10–13]. In contrast, addition of sulfuric acid to the mobile phase, significantly improved resolution and peak shape. However, the change in absorbance (ΔA) was relatively small for most of the tested aliphatic acids.

A series of IEC separations of the test acids using a 2 m*M* aromatic acid eluent and indirect photometric detection can be seen in Fig. 2a–e. This

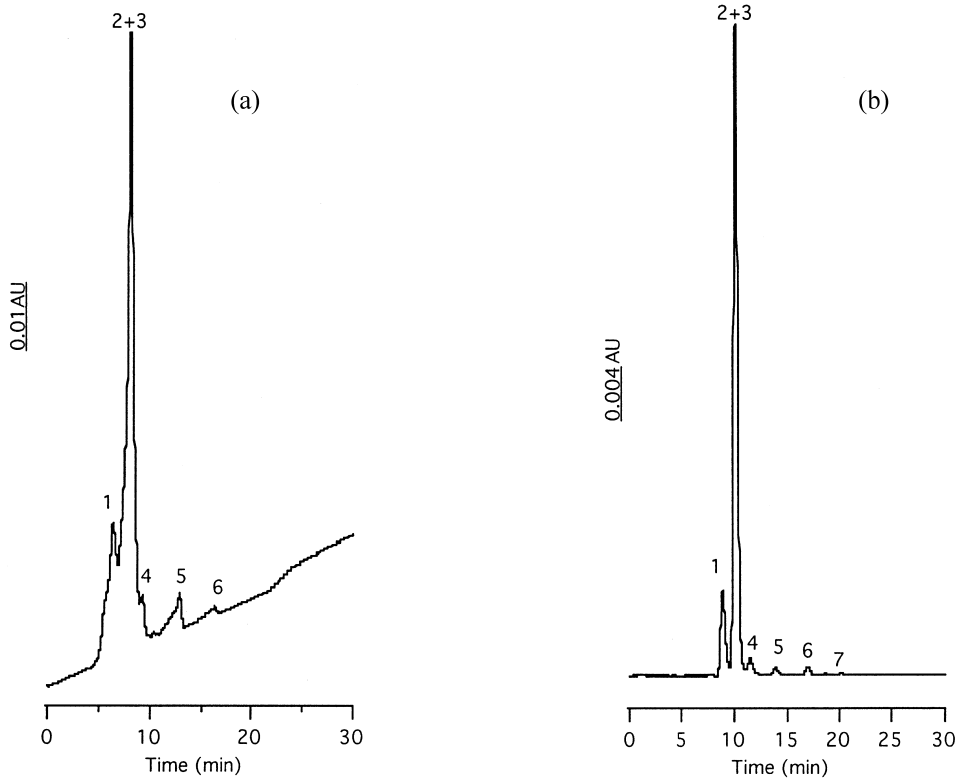

Fig. 1. (a) Ion-exclusion chromatography of aliphatic acids with a water eluent. (b) Ion-exclusion chromatography of aliphatic acids eluted by a 2 m*M* sulfuric acidic eluent. 1=oxalic, 2=maleic, 3=citric, 4=malic, 5=tartaric, 6=formic, 7=acetic acid. Conditions: UV detection at 210 nm; flow-rate: 0.5 ml/min; injected volume: 20 μl.

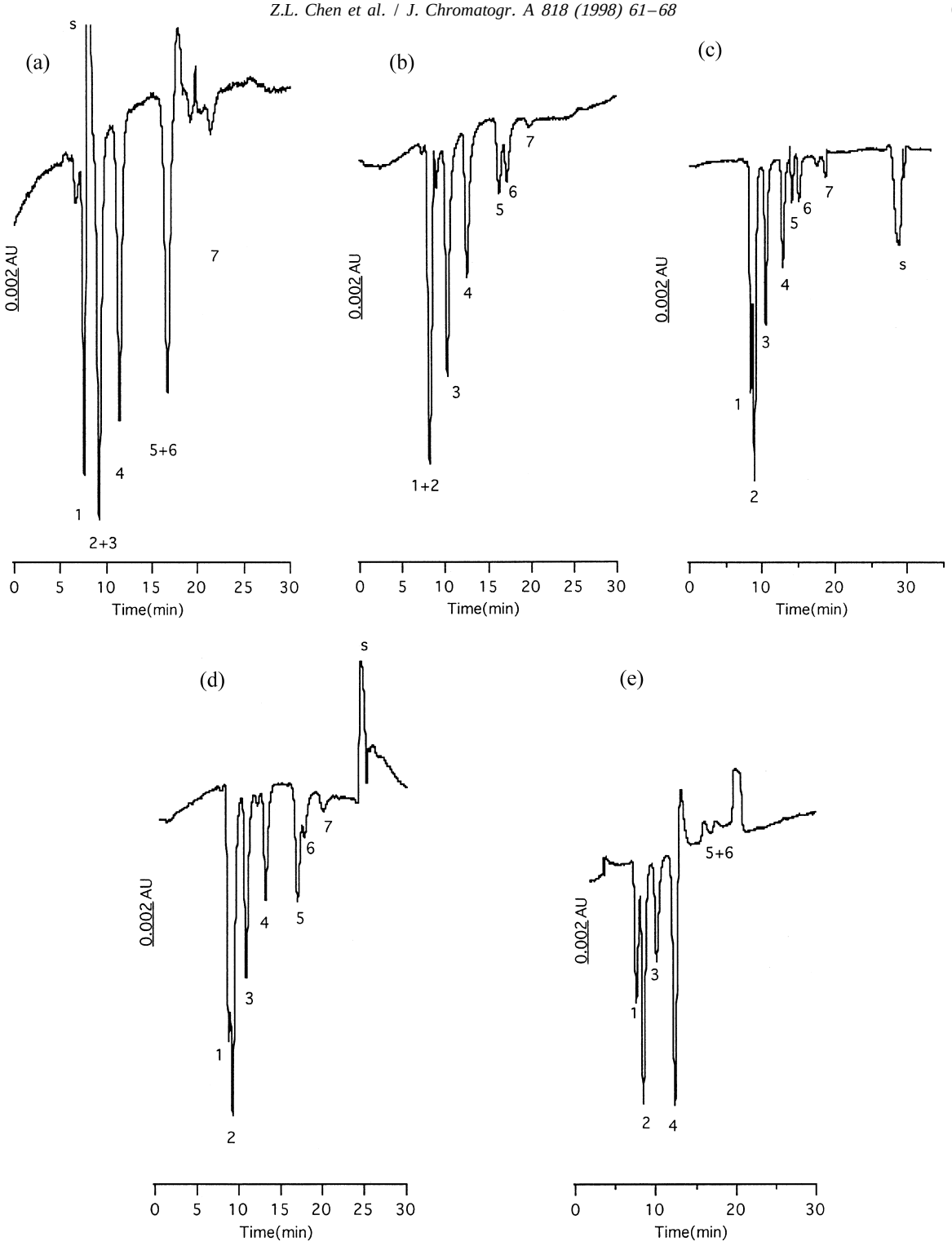

Fig. 2. Ion-exclusion chromatography of aliphatic acids by eluted with aromatic acid eluents. (a) Benzoic (250 nm), (b) salicylic (254 nm), (c) phthalic (300 nm), (d) trimesic (300 nm), (e) pyromellitic acid (300 nm). Other conditions as in Fig. 1.

preliminary study shows that the aromatic acidic eluents are applicable to the IEC separation of the aliphatic acids. Compared with a water eluent, resolution and peak shape were greatly improved by using aromatic acids due to an increase in hydrophobic adsorption of aliphatic acids and decreasing degree of ionization of the aliphatic acids, leading to improved peak asymmetry [3,13]. Negative peaks were observed and ΔA for the aliphatic acids increased in comparison to that obtained using sulfuric acid as the eluent. Chromatograms obtained from the eluents (a) benzoic, (b) salicylic (c) phthalic, (d) trimesic and (e) pyromellitic acid, indicated that ΔA decreased with decreasing pK_a of the aromatic acids. ΔA for the tested aliphatic acids using aromatic acid eluents were of the order: benzoic>trimesic≈phthalic≈salicylic>pyromellitic acid. In addition, peak height for the aliphatic acids increased with increasing dissociation constants as shown in Fig. 2. These results are in agreement with the theoretical calculation e.g., Eq. (13) and predicts that peak height should be proportional to the solute dissociation constant and inversely proportional to the dissociation constant of the aromatic acid used as a mobile phase modifier. Best resolution was obtained when phthalic acid was employed as an eluent. Some aliphatic acids can not be easily separated using other aromatic acid eluents. e.g., maleic and citric acid are not well resolved in a benzoic acid eluent; oxalic and maleic acid are not well resolved in a salicylic acid eluent; and tartaric and formic acid tend to co-elute when using a pyomellitic acid eluent. System peaks were observed in 13 ml of phthalic acid eluent, and in 5 ml of pyromellitic acid eluent and may be a result of disturbance of column equilibria caused by the injection of the sample [15]. For both chromatographic separation and detection, phthalic acid was the most suitable for IEC of aliphatic acids with indirect photometric detection.

From Eq. (13), it was expected that not only would ΔA depend on the dissociation constant of both solute and aromatic acid used as a modifier, but would also depend on its concentration in the eluent. Fig. 3 presents the relationship between ΔA and eluent concentration. ΔA decreased with increasing concentration of phthalic acid in the range 0.1–5 m*M* as expected in Eq. (13). Retention times of aliphatic acids were only slightly changed under these conditions. Optimal separation of aliphatic acids and detection using indirect photometric detection was obtained with 1 m*M* phthalic acid eluent as shown in Fig. 4. The symmetry of peaks was due to phthalic acid buffering the aliphatic acid and strongly absorbing onto the resin, resulting in an increase in resin

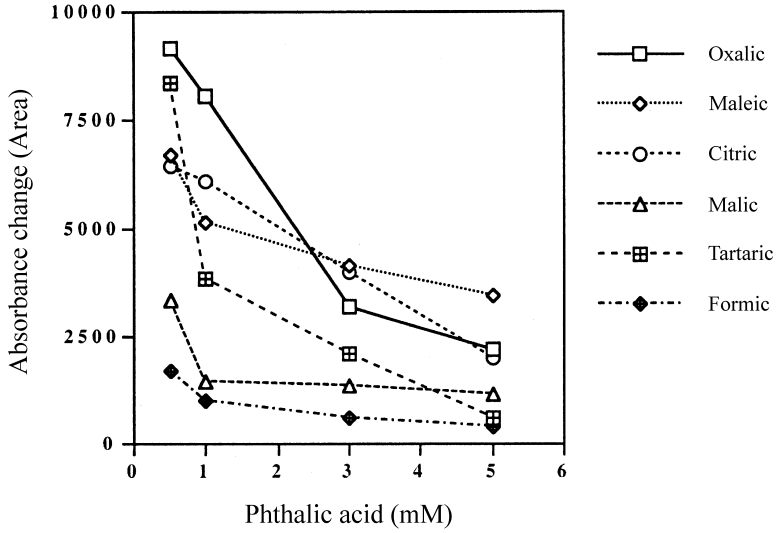

Fig. 3. The effect of concentration on the detection sensitivity for the test acids. Other conditions as in Fig. 1.

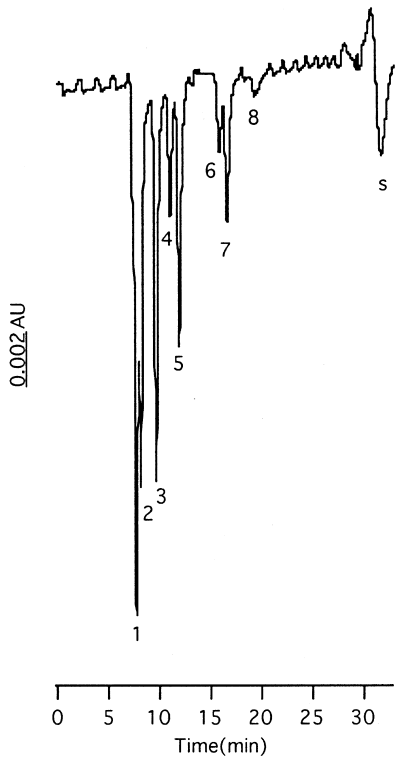

Fig. 4. Ion-exclusion chromatography of aliphatic acids using 1 m*M* phthalic acid in the mobile phase. 1=oxalic (1 m*M*), 2=maleic (1 m*M*), 3=citric (1 m*M*), 4=*tert*.-aconitic (1 m*M*), 5=malic (1 m*M*), 6=tartaric (1 m*M*), 7=formic (2 m*M*), 8=acetic acid (2 m*M*). Injected volume: 30 μl. Other conditions as in Fig. 1.

hydrophilicity [3,13]. Similar results have been obtained using polyalcohol eluents [11,12]. Aliphatic acids were elueted in order of their increasing pK_a values and molecular dimensions [3]: oxalic (3.9 ml), maleic (4.1 ml), citric (4.9 ml), *trans*-aconitic (5.6 ml), malic (6.0 ml), tartaric (8.0 ml), formic (8.4 ml) and acetic (9.8 ml).

Peak area was linearly related to concentration over the range of 0.1–10 m*M* with correlation coefficients of 0.998 to 1.00. Detection limits for aliphatic acids, obtained at signal-to-noise ratio of 3, ranged from 10 to 70 μ*M*. The reproducibility of peak areas by repeated injection of a 1 m*M* standard ranged between 1.2–3.8%. The characteristics of the proposed method are listed in Table 1.

5. Analysis of plant extracts

The proposed method was used to determine the concentration of aliphatic acids in extracts of tissues of agricultural and Australian native plants. Lyophilised tissues were pulverised 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 extracts were filtered by passing through a Millipore 0.45-μm membrane and diluted tenfold prior to injection. Good resolution of the aliphatic acids was obtained with the exception of oxalic acid. The organic acids

Table 1
Analytical characteristics for the aliphatic acids separated by IEC using indirect UV detection

Acid	Retention V_r (ml)	Regression equation	r^2	R.S.D. (%) ($n=5$)	DL (μ*M*)
Oxalic	3.9	$y=1.61\cdot10^3x+3.01\cdot10^3$	0.998	1.8	10
Maleic	4.1	$y=2.48\cdot10^4x+6.94\cdot10^2$	0.998	2.8	20
Citric	4.9	$y=1.43\cdot10^3x-5.60\cdot10^2$	1.00	1.2	10
trans.-Aconitic	5.6	$y=8.72\cdot10^3x+2.1\cdot10^1$	0.999	1.9	30
Malic	6.0	$y=9.43\cdot10^3x+1.7\cdot10^1$	0.999	2.2	30
Tartaric	8.0	$y=2.05\cdot10^3x+3.45\cdot10^2$	0.998	2.3	40
Formic	8.4	$y=6.20\cdot10^2x-6.8$	0.999	1.9	50
Acetic	9.8	$y=2.57\cdot10^3x-2.15\cdot10^2$	0.998	3.8	70

Conditions as in Fig. 4. DL=Detection limit.

Table 2
Analysis of aliphatic acids in Australian plant tissue extracts by the proposed method

Sample	Maleic acid (m*M*)	Citric acid (m*M*)	Malic acid (m*M*)	Formic acid (m*M*)
Lupin leaves	20.7	3.5	23.6	3.4
Chickpea	4.2	4.6	15.7	1.5
Pea	8.5	9.9	11.3	2.1
Wheat	2.1	0.3	1.42	0.5
Clover root	2.1	0.3	–	0.8

Conditions as in Fig. 4. – (Malic acid) was not found in Clover root extract.

in various plant tissues are listed in Table 2. The problem of co-elution of oxalic acid with inorganic anions such as Cl^- and NO_3^- has been noted previously [28] and extraction procedures must be modified to eliminate such anion interference.

6. Conclusion

The presented study has shown that aromatic acids can be used as eluents for separation of aliphatic acids in ion-exclusion chromatography using indirect photometric detection. A proposed model has been shown to reliably predict experimental results. Resolution and peak shape are significantly improved in place of water or other eluents. Of the tested aromatic acids, the best results were obtained with a 1 m*M* phthalic acid, and the method was demonstrated for analysis of aliphatic acids in plant tissue samples.

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