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Short communication

An improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates

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Abstract

Reversed-phase column liquid chromatography (RPLC) was used for the separation and quantification of 10 low-molecular-mass organic acids (malic, malonic, lactic, acetic, maleic, citric, *cis*-aconitic, succinic, fumaric, and *trans*-aconitic) in plant root exudates. A mobile phase of 93% 25 m*M* KH_2PO_4 at pH 2.5 and 7% methanol at a flow-rate of 1 ml min^{-1} resolved all 10 acids in 15 min on a C_{18} column. Experiments demonstrated a significant ($P<0.05$) effect of sample pH on detector response, with peak heights being significantly lower at pH 6.0 compared with pH 2.5, but peak area showed no significant difference. At pH 8.0 and above, both peak height and area differed significantly from injections made at pH 2.5. Limits of detection (LOD) for the 10 acids ranged from 0.05 to 24 μ*M*. Finally, the improved method was applied for the analysis of root exudates from soil cultured field pea, *Banksia attenuata*, white lupin, and chickpea.

Keywords: Root exudates; Rhizosphere extracts; Organic acids; Carboxylic acids

1. Introduction:

Soils in Western Australia (WA) are amongst the most heavily leached and nutrient impoverished in the world [1,2]. Plants of the Proteaceae family have evolved cluster roots [3] to enhance the mobility and availability of such scarce nutrients. Proteaceae and crop species such as white lupin and chickpea exude large amounts of root exudates as part of their mechanism(s) for nutrient acquisition. The biodiversity of the Proteaceae in WA is testimony to the success of these specific root adaptations. For the purpose of mobilising sparingly soluble nutrients, exudation involves the release of low-molecular-weight organic acids from the cluster roots into the rhizosphere [4]. The range of organic acids exuded by these plants varies, but citric, malic, malonic, aconitic, and fumaric acids are frequently found in plant root exudates [5,6]. This root-induced chemical change in the soil is of both ecophysiological and agricultural significance. Consequently, the analysis of organic acids becomes of importance when understanding the ecophysiology of such plant species in their adaptation mechanisms. With respect to crop species, a greater understanding of their nutrient acquisition mechanisms may result in more efficient fertiliser applications and use.

In order to collect the root exudates, plants are grown in modified Hoagland's nutrient solution

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(hydroponics) or soil, either in glasshouse or the field. Hydroponic culture of Australian native species involves low levels of nutrients and thus the solutions have low ionic strength. However, soil extracts contain large amounts of soluble salts and dissolved organic matter, and hence usually have considerably higher ionic strength. As such, sample matrices and organic acid concentrations are quite different. Another method of exudate collection is the placement of filter paper segments onto live root tissue for a known period of time, then the subsequent extraction of organic acids from the filter paper. This method of collection typically results in very low sample volumes (<100 μl). There is no sample pre-treatment prior to RPLC analysis except for acidification, then 0.2 μm filtration followed by direct injection. Our current method of analysis is a modification of the methods described in Refs [7] and [8], using a 100% aqueous mobile phase. However, a major problem with this method was the co-elution of *cis*-aconitic and fumaric acids, two common acids in the root exudates of Australian native plants [5,9]. One of the most common methods of organic acid analyses is by liquid chromatography, whether it be ion chromatography [10–12] or reversed-phase chromatography [7,13,14]. The sample matrices in which organic acids are analysed vary greatly, from soil solution and extracts [7,15], plant root exudates [9], plant extracts [16,17], fruit [18] and, recently, honey [19].

This paper presents the improvement of an existing RPLC method for the analysis and quantification of organic acids in soil collected plant root exudates. Analysis of root exudates from field pea, *Banksia attenuata*, white lupin and chickpea are presented using the improved method.

2. Experimental

2.1. Instrumentation

All RPLC analyses were conducted with a Waters (Milford, MA, USA) 600E dual head pump, 717 plus autosampler and a 996 photo-diode array (PDA) detector. Separation was performed on an Alltima C_{18} column (250 mm×4.6 mm, I.D.) with 5 μm particle size (Alltech Associates, Deerfield, IL, USA). A μBondapak (Waters) C_{18} guard column was used in-line prior to the analytical column.

All data were acquired and processed with Millennium$^{32®}$ chromatography software (Waters) with PDA acquisition from 190 to 400 nm. PDA output at 210 nm was used for the quantification of organic acids. Positive identification of organic acids was accomplished by comparing standard retention time and PDA peak spectral analyses with the unknowns. Spiking of samples with standard organic acid ensured there was no effect of sample matrix on spectral characteristics or peak response of the acids of interest (data not shown).

The mobile phase consisted of 25 m*M* KH_2PO_4 adjusted to pH 2.5 with concentrated *ortho*-phosphoric acid, and methanol at a flow-rate of 1 ml min^{-1}. The system was equilibrated with 30 column volumes at each new mobile phase composition prior to four injections of a mixed organic acid solution. Separation was achieved at ambient temperature of 25±2 °C. For the analysis of root exudate samples, a gradient elution was employed every 5th sample, using 60% methanol to fully flush the column of hydrophobic compounds from previous injections.

Limit of detection (LOD) was defined as a ratio of 3 for signal to noise (S/N), and all values reported for LOD are based on peak area.

Statistical analysis was performed with Genstat 5.0 software.

2.2. Chemical

Organic acid standards were obtained from ICN Biomedicals (Aurora, Ohio, USA), of analytical quality, in the free acid form, and were used as supplied. Methanol, KH_2PO_4 and acetonitrile were of HPLC grade (HiPerSolv) from Merck (Darmstadt, Germany) and used as purchased. For pH adjustment of mobile phase and solutions, H_3PO_4, H_2SO_4 and NaOH used were of analytical grade, purchased from Merck. All aqueous eluents were prepared with Milli-Q water (Millipore, Bedford, MA, USA) and were vacuum degassed and filtered to 0.2 μm prior to use with Pall Gelman (Ann Arbor, MI, USA) GH Polypro membrane filters.

3. Results and discussion

3.1. Effect of organic modifier addition

The use of 100% 25 m*M* KH_2PO_4 at pH 2.5 offered good separation of the organic acids with the exception of the co-elution of *cis*-aconitic and fumaric acids (Fig. 1A), both of which are frequently present in root exudate samples [5,9].

Methanol and acetonitrile were tested over the range 0–10% in the aqueous mobile phase, however acetonitrile was determined to be unsuitable. Increasing the percentage of methanol in the mobile phase decreased the retention factor for all organic acids as expected and resulted in *cis*-aconitic and succinic acids actually changing elution order.

A composition of 93% 25 m*M* KH_2PO_4 at pH 2.5 and 7% methanol was chosen as the mobile phase for future analysis, and used for further method development as this mobile phase offered separation of all interested organic acids (Fig. 1B).

3.2. Effect of sample pH on detector response

The pH of mixed organic acid solutions, initially prepared in 25 m*M* KH_2PO_4 at pH 2.5, were adjusted to pH values of 3.0, 3.5, 4.0, 6.0 and 8.0, and four 50 μl injections were made of each to investigate the effects of sample pH on detector

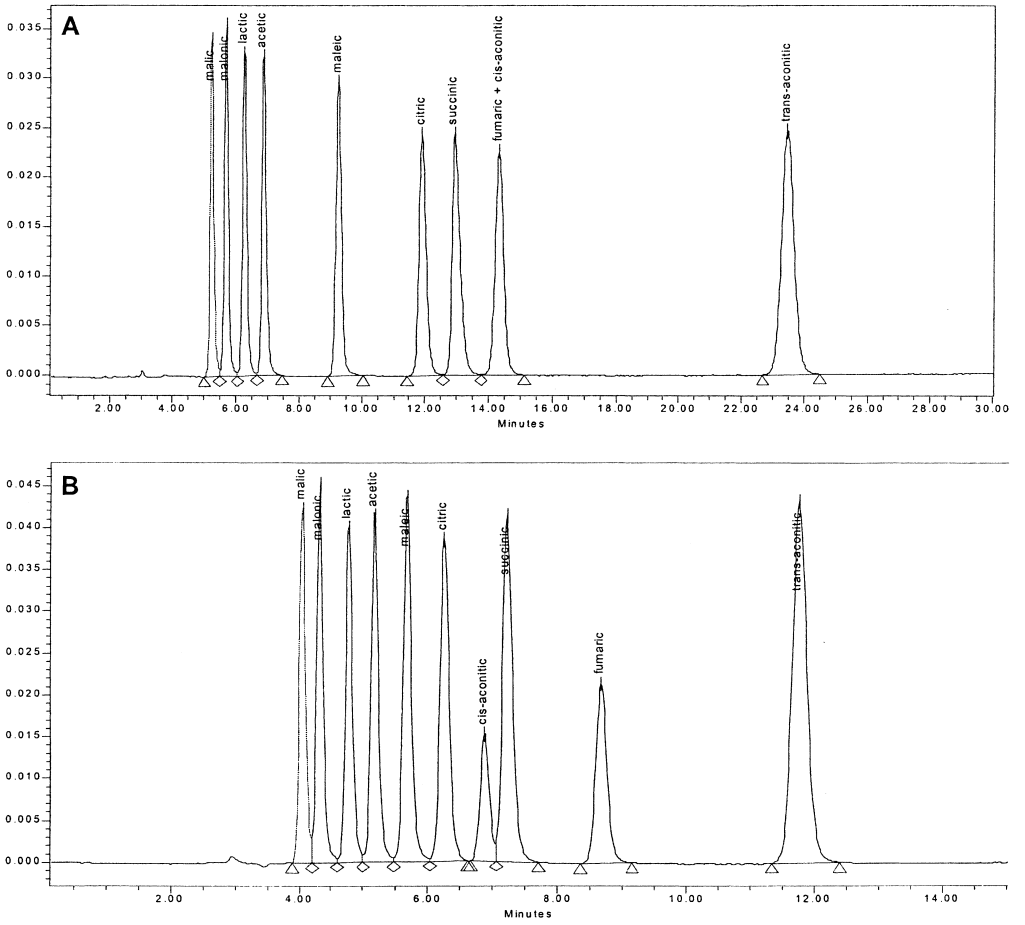

Fig. 1. Separation of organic acid standards by RP-high-performance liquid chromatography (HPLC) with (A) 100% 25 m*M* KH_2PO_4 pH 2.5 and (B) 93% 25 m*M* KH_2PO_4 pH 2.5 with 7% methanol (B), 1 ml min^{-1}; PDA detection at 210 nm.

response. The average of the four injections at pH 2.5 was used as the reference.

Over the pH range 2.5–4.0, neither peak height nor area were significantly ($P>0.05$) different than at pH 2.5 for all organic acids. Peak height was most sensitive to solution pH, significantly ($P>0.05$) decreasing at pH 6.0, whereas peak area demonstrated no significant decrease. At pH 8.0, both peak area and height were significantly reduced ($P<0.05$) for all organic acids analysed. Thus all samples and standards need to be acidified to pH≤4.0 for accurate quantification, with peak area being the optimum peak descriptor to use. The effects seen here are probably due to ionisation of the organic acids. As pH increases, less organic acids are fully ionised and bandspreading seems to occur, as area remains relatively constant, yet height significantly decreases over the pH 2.5 to 6.0 range.

3.3. Detection limits

Solutions were prepared to give a S/N of 3 at injection volumes of 5, 10, 20, and 50 μl. When comparing repeatability of peak area over four injections, 20 and 50 μl gave the lowest relative standard deviations (%RSD) of 2–13% and 3–6%, respectively.

For the 20 μl injection, the %RSD for fumaric acid was 13%, which may in part be due to a small yet broad alteration to the baseline where fumaric acid elutes, when a blank solution of mobile phase buffer alone is injected. Excluding fumaric acid, the %RSD range for a 20 μl injection is 2–10%. A 20 μl injection was selected as the injection volume for the determination of the LOD values presented in Table 1, as this is a common injection volume used in our analyses of filter paper collected root exudates with limited sample volume. However, a 50 μl injection would offer lower %RSD with respect to peak area and would be the injection volume of choice where sample volume is not limiting.

The LOD values reported here are similar to recent reports in the literature for HPLC analysis of organic acids [15,16]. The values have been calculated from standards prepared in clean solvents with no other analytes present; hence, one would assume that detection limits would increase somewhat when analysing real sample such as plant root exudates.

Table 1
Limit of detection (LOD) for organic acids analysed with the % relative standard deviation (%RSD), with conditions as described for Fig. 1B

Organic acid	Limit of detection (μ*M*)
Malic	7.0
Malonic	8.0
Lactic	13.0
Acetic	24.0
Maleic	0.05
Citric	5.0
Cis-aconitic	0.1
Succinic	15.0
Fumaric	0.06
Trans-aconitic	0.1

The extreme differences in detection limits between acids such as maleic (0.05 μ*M*), fumaric (0.06 μ*M*), and aconitic acids (0.1 μ*M*) with the rest (5–24 μ*M*) can be mostly attributed to the differences in molar absorptivity of the organic acids. The saturated organic acids have far greater molar absorptivity and thus lower detection limits.

Work is currently under way in our laboratory to quantify the LOD in a variety of root exudate samples, such as those from chickpea, field pea, lupin, selected Australian native species (*Banksia*, *Hakea*, *Grevillia*), wheat, and canola amongst others.

3.4. Calibration curves

Calibration curves for all 10 organic acids were determined at three different concentrations. Curves were obtained by plotting the mass of organic acid injected versus peak area. Linear regressions were generated across at least two orders of magnitude for all organic acids, with correlation coefficients (r^2) greater than 0.99. Calibration ranges for the acids were; malic: 7 μ*M*–2 m*M*; malonic: 8 μ*M*–1 m*M*; lactic: 13 μ*M*–1 m*M*; acetic: 24 μ*M*–4 m*M*; maleic: 0.05 μ*M*–9 μ*M*; citric: 5 μ*M*–0.7 m*M*; *cis*-aconitic: 0.10 μ*M*–13 μ*M*; succinic: 15 μ*M*–2 m*M*; fumaric: 0.06 μ*M*–60 μ*M*; *trans*-aconitic: 0.10 μ*M*–50 μ*M*.

3.5. Applications

The improved method offers the complete separation of *cis*-aconitic and fumaric acids, both of which were present in all analysed samples. Fig. 2

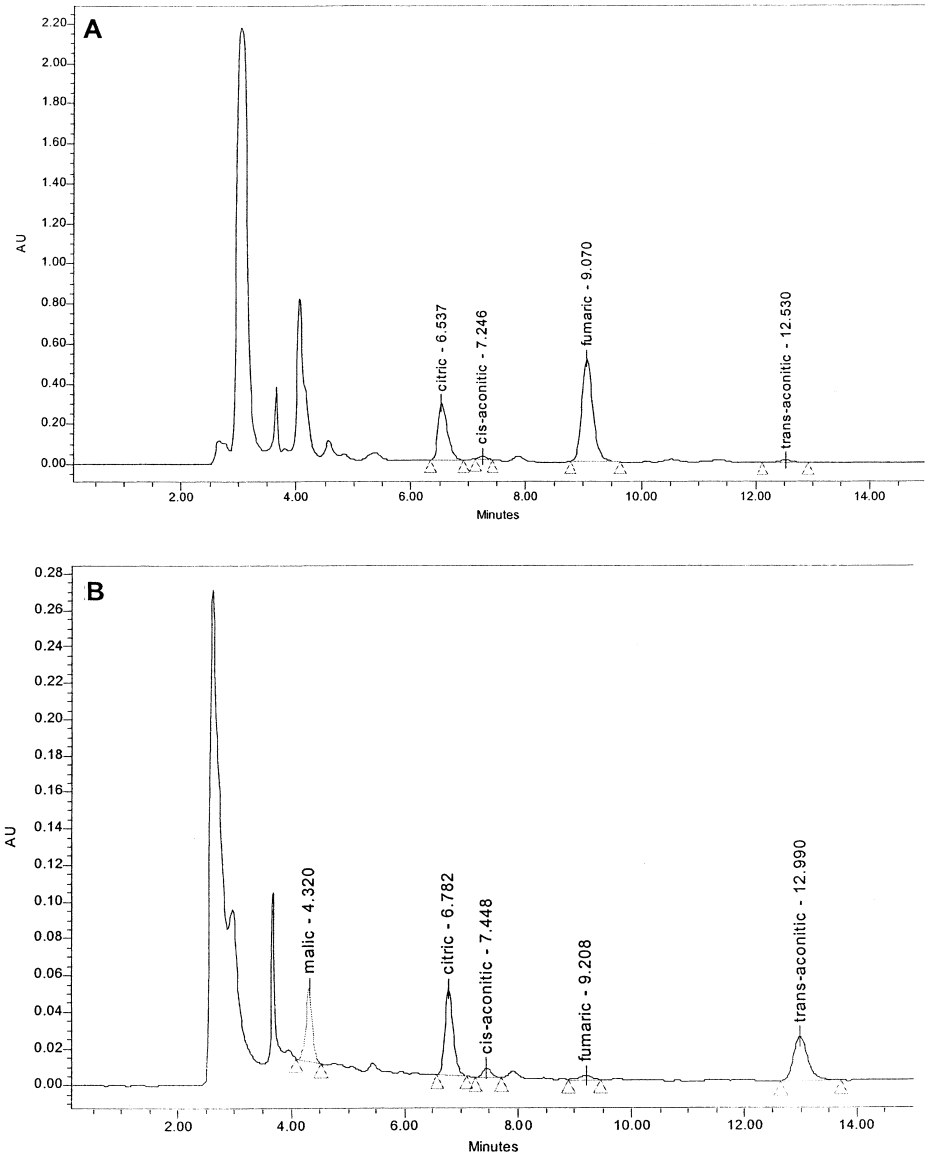

Fig. 2. (A) Chromatogram of root exudate sample from soil grown field pea. (B) Chromatogram of root exudate sample from soil grown *Banksia attenuata*. (C) Chromatogram of root exudate from soil grown white lupin. (D) Chromatogram of root exudate from soil grown chickpea.

contains chromatograms of field pea, *Banksia attenuata*, white lupin and chickpea root exudate (rhizosphere) extracts analysed using this procedure. The concentrations of the saturated organic acids (i.e. fumaric and aconitic) were up to two orders of magnitude lower than the unsaturated acids (i.e. malic, malonic, and citric) in the analysed samples.

Twelve white lupin (*Lupinus albus)* extracts analysed all contained malic (172 to 387 μM) and citric (250 to 887 μM) acids and these two acids were present in the highest concentration and thus perhaps ecophysiologically the most important. The concentrations of fumaric (1.5 to 3.5 μM), *cis*-aconitic (not detected (nd) to 0.45 μM) and *trans*-aconitic (nd to

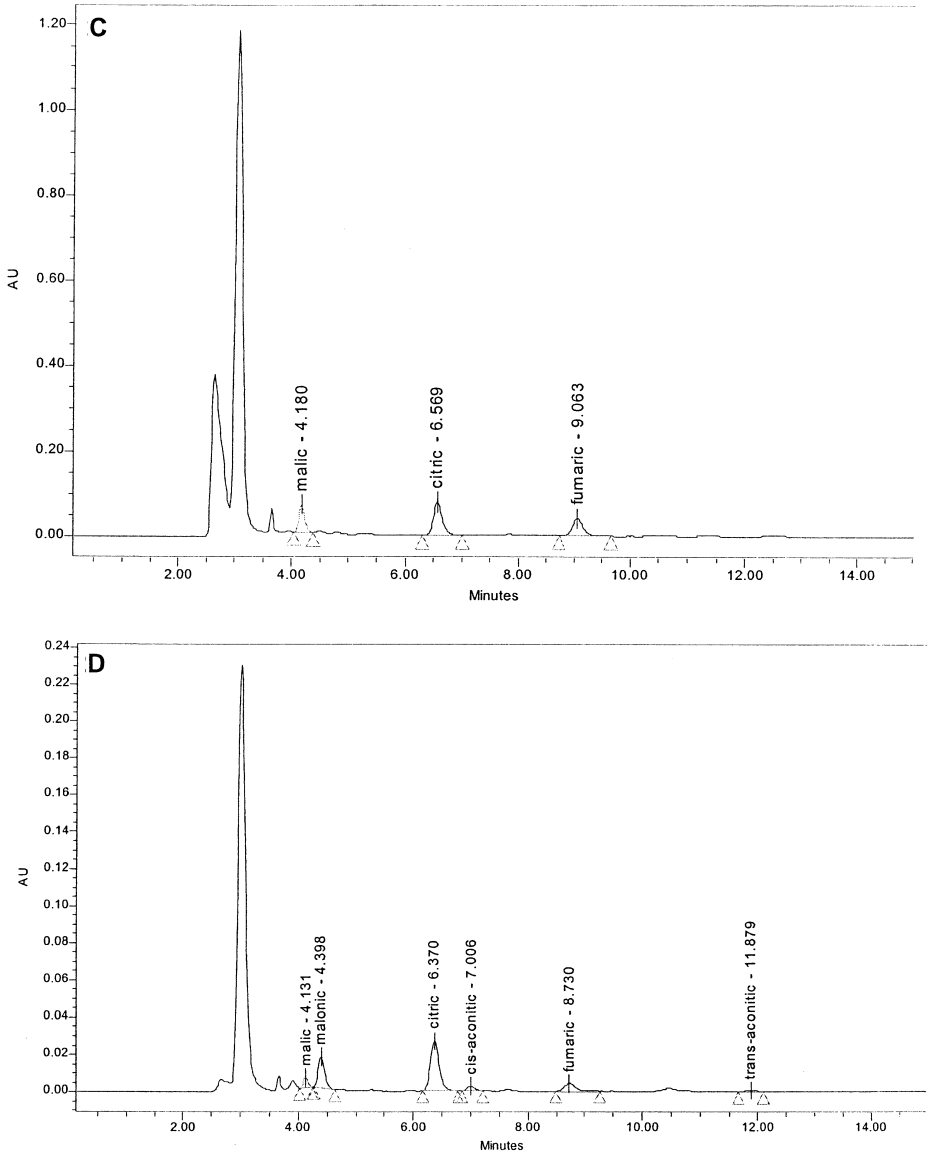

Fig. 2. (*continued*)

1.0 μ*M*) acids were significantly lower, but were found in 100%, 83% and 92% of all extracts, respectively.

Chickpea (*Cicer arietinum)* was the only species that contained malonic acid, and it was the major organic acid released by this species (230 to 955 μ*M*), with citric acid (95 to 567 μ*M*) also of major importance and the concentration range of malic acid an order of magnitude lower (10 to 68 μ*M*). The concentration of fumaric (0.43 to 1.9 μ*M*), *cis*-aconitic (0.06 to 0.94 μ*M*) and *trans*-aconitic acids (0.14 to 1.3 μ*M*) were an order of magnitude lower again in 12 rhizosphere extracts of chickpea analysed. All six organic acids were detected in each of the 12 extracts.

For the 12 field pea (*Pisum sativum)* rhizosphere extracts, citric acid (67 to 552 μ*M*) and malic acid (nd to 175 μ*M*) were essentially the only acids

released by the roots, as the relative proportions of the other acids present on a concentration basis, fumaric (0.60 to 4.7 μ*M*), *cis*-aconitic (0.09 to 1.2 μ*M*) and *trans*-aconitic (nd to 1.0 μ*M*), would seem insignificant. Although the concentration ranges may be quite different, the three acids were present in all 12 extracts, suggesting that each organic acid plays some role in the nutrient acquisition process.

There were more organic acids detected in the rhizosphere extracts of *Banksia attenuata*, although considerably more extracts were analysed (77 samples). The major acids identified were malic (nd to 465 μ*M*) and citric (nd to 1710 μ*M*), present in 25% and 52%, respectively, of the 77 extracts analysed. Fumaric acid was detected in high proportion of extracts (85%), but again the concentration range for fumaric (nd to 1.5 μ*M*) was significantly lower than that for malic and citric. However, unlike the cropping species, the concentration range of *cis*-aconitic acid (nd to 6.5 μ*M*) and *trans*-aconitic acid (nd to 77 μ*M*), was considerably higher and they were detected in 58% and 91%, respectively. The detection of high concentrations of *trans*-aconitic acid is quite common for Australian native species in the Proteaceae [5,9,20]. Extra organic acids were also detected in *Banksia*, but again on a per concentration basis, were present in quite insignificant amounts. Maleic acid (nd to 0.14 μ*M*) was detected in 17% of the extracts, lactic acid (nd to 60 μ*M*) in 5%, and succinic acid (nd to 225 μ*M*) was found in only 4% of rhizosphere extracts from *Banksia*.

In general, malic and citric acids are the major organic acids present in the soil collected root exudates of field pea, *Banksia*, and white lupin. Malonic acid, exclusively found in chickpea exudates, was the dominant organic acid present for this species. Both fumaric and aconitic (*cis* and *trans* isomers) acids are also present but in much lower quantities, indicating the importance of malic, malonic and citric acids in rhizosphere chemistry and nutrient acquisition mechanisms. However, the high frequency of samples containing fumaric and aconitic acids in the root exudates of all four species may indicate they are also essential in the nutrient acquisition process of these species, or they are partaking a vital role in rhizosphere chemistry. It would be of interest to study the effectiveness of the unsaturated organic acids as opposed to the saturated organic acids in their nutrient acquisition efficiency. Of the four species investigated, only *Banksia* contained traces of maleic, succinic and lactic acids.

Another major benefit from the improved method described here is the PDA spectral purity of the more common acids in the separation, with the former method using 100% buffer, frequently resulting in peaks being spectrally impure due to co-elution of unknown compounds. With the new separation method, PDA peak purity of all acids significantly improved, resulting in greater accuracy and confidence in the result. Often with LC, co-elution can have quite adverse affects on sample analysis and any improvements in peak purity are a definite gain in research accuracy.

Finally, with the method described here, the degree of sample pre-treatment has been kept to an absolute minimum. This results in quicker and less costly analyses, thus more samples can be analysed in the same time period as opposed to samples requiring laborious and accuracy affecting pre-treatment, such as solid-phase extraction and liquid–liquid extraction.

4. Conclusions

With 7% methanol and 93% 25 m*M* KH_2PO_4 at pH 2.5 and a flow-rate of 1 ml min^{-1}, separation and resolution of malic, malonic, lactic, acetic, maleic, citric, *cis*-aconitic, succinic, fumaric and *trans*-aconitic acids was achieved using a C_{18} column. Analysis of the rhizosphere extracts of four plant species showed the major organic acids present were malic, malonic and citric acids, but not all species contained these three main acids, in fact chickpea was the only species to contain malonic acid in the root exudates. The improved method offers better peak resolution of fumaric and *cis*-aconitic acids, both of which were minor constituents in essentially all plant root exudate samples analysed. The resolution of the most common organic acids, would allow the application of this method to many other sample matrices, with the knowledge that all samples and standards need to be acidified to pH≤4. Concentrations of saturated organic acids can be detected at n*M* level whilst the unsaturated organic acids have low μ*M* detection limits. With the sample matrix

analysed here, the improved method offers vast improvement in photodiode array peak purity analyses, greater accuracy in the result with no sample pre-treatment, thus providing cheaper per sample analyses.

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