Determination of Low Molecular Weight Dicarboxylic Acids in Root Exudates by Gas Chromatography

Anna M. Szmigielska,*,† Ken C. J. Van Rees,† Grzegorz Cieslinski,† Pan Ming Huang,† and Doug R. Knott‡

Departments of Soil Science and Crop Science, Agriculture Building, University of Saskatchewan, 51 Campus Drive, Saskatoon, Saskatchewan S7N 5A8, Canada

Many high-pressure liquid chromatographic methods used for the determination of low molecular weight organic acids in root exudates lack specificity and cannot completely separate mixtures of mono-, poly-, and hydroxycarboxylic acids simultaneously. Hence, a new gas chromatographic (GC) method that is specific for the identification and quantitation of low molecular weight dicarboxylic acids as methyl esters was developed. Samples collected from root exudates of selected durum wheat and flax cultivars grown in sterile hydroponic solutions were extracted and concentrated by anion exchange membranes. Samples were then subjected to methylation in acidified methanol at elevated temperatures and injected into a GC. The five acids investigated in this study—oxalic, malonic, succinic, fumaric, and maleic—required 7 min for elution from a GC column and were quantified using methylmalonic acid as an internal standard. Good recovery and reproducibility as well as a low detection limit of ca. 1 ppm acid in the membrane eluate make this method very suitable for dicarboxylic acid determination in root exudates.

Keywords: *Dicarboxylic acids; root exudates; durum wheat; flax*

INTRODUCTION

Low molecular weight organic acids secreted by plant roots play an important role in a variety of processes at the soil–root interface. It is generally accepted that root exudates, besides microbial activity, soil pH, and ionic concentration changes, may modify the flow of nutrients in the rhizosphere (Mench et al., 1988). Secretion of low molecular weight organic acids contributes to the acidification of the rhizosphere and possibly the formation of soluble metal complexes (Mench and Martin, 1991). Mono-, di-, tri-, and hydroxycarboxylic acids have been reported to be present in root exudates (Foy et al., 1990; Petersen and Bottger, 1991; Mench and Martin, 1991).

Sensitive and specific quantitative analysis of low molecular weight organic acids in root exudates has been a difficult analytical problem due to the similarity in chromatographic behavior of the closely related compounds. Different methods have been tried for the separation of biologically important low molecular weight carboxylic acids from a variety of materials. Recently, several new high-performance liquid chromatographic (HPLC) procedures have been reported such as ion exclusion using a strong cation exchange resin (Peterson and Bottger, 1991; McFeeters, 1993; Foy et al., 1990), ion exchange using a weak anion exchange resin (Lieholm et al., 1992), and reversed phase with or without sample derivatization (Miwa, 1985). Most of these methods lack specificity and are not able to separate a mixture of mono-, poly-, and hydroxycarboxylic acids in a single chromatographic run (Blake et al., 1987).

Instead of trying to separate and identify all of the low molecular weight organic acids in root exudates simultaneously, we investigated a gas chromatographic (GC) approach based on the differences in the chemical structure of the acids. Trimethylsilylation (Akhavan and Wrolstad, 1980; Morvai and Molnar-Perl, 1990) and methylation (Supelco, 1990) have been used most frequently for the derivatization of nonvolatile organic acids. Dicarboxylic acids easily form methyl esters, while the hydroxy groups in hydroxy acids do not methylate under these conditions, rendering methyl esters of hydroxy acids unsuitable for GC analysis (Horning et al., 1967). This difference allowed an investigation of dicarboxylic acids without interference from hydroxy acids by GC. Volatile low molecular weight monocarboxylic acids, normally analyzed by GC without derivatization, elute with the solvent front when methylated.

The objective of this study, therefore, was to develop a specific and accurate GC method for the identification and quantification of low molecular weight dicarboxylic acids excreted by roots of durum wheat and flax plants grown in sterile hydroponic solutions.

MATERIALS AND METHODS

Instrumentation. GC analysis of methylated dicarboxylic acids was performed on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a flame ionization detector. A capillary column (30 × 0.53 mm i.d.) coated with HP FFAP cross-linked stationary phase (film thickness 1.0 μm) was used. The injector, column, and detector temperatures were 200, 125, and 200 °C, respectively. Helium at a flow rate of 7 mL/min was used as the carrier gas. Two microliters of sample was injected at a sensitivity setting of 8×10^{-10} afs, and the chromatograms were integrated by a Hewlett-Packard Model 3396 Series II integrator.

Materials. Standards for the low molecular weight dicarboxylic acids (oxalic, malonic, succinic, fumaric, maleic, and methylmalonic) were obtained from Sigma Chemical Co. (St. Louis, MO), while methanol and chloroform used for sample preparation were BDH Assured reagents. Methylmalonic acid, not found in root exudates of durum wheat and flax plants, was used as an internal standard (IS) and was added to both standard mixtures and samples before derivatization. Anion exchange membrane strips (1 × 7 cm) used for acid extraction/concentration were cut from a Bio-Rex ion exchange membrane

† Department of Soil Science.
‡ Department of Crop Science.

0021-8561/95/1443-0956$09.00/0

sheet composed of AG 1-X8 exchange resin beads permanently enmeshed in a poly(tetrafluorethylene) membrane (Bio-Rad Laboratories, Richmond, CA). The AG 1-X8 resin is a strong anion exchanger with quaternary ammonium functional groups attached to the styrene divinylbenzene copolymer lattice. As recommended by the manufacturer, new membranes were washed several times in methanol to remove any tightly bound organic molecules. The membranes were used in the bicarbonate form due to the ease of exchange for other ions. New and used membranes were cleaned by washing five times with 0.5 M HCl and regenerated to the bicarbonate form by washing five times with 0.5 M $NaHCO_3$ (pH adjusted to 8.5 with 0.5 g of NaOH/L). Regenerated membranes were stored in deionized water.

Plant Material. Low molecular weight dicarboxylic acids were determined in root exudates of durum wheat (*Triticum turgidum* var. *durum* L.) cv. Sceptre and flax (*Linum usitatissiumum* L.) cv. Somme. Plants were grown in sterile hydroponic cultures using the following seed preparation and germination procedure.

Seed Surface Sterilization and Germination. Seeds were surface sterilized by rinsing in a 10% solution of calcium hypochlorite for 30 min. Seeds were then washed with sterile water and transferred (under sterile conditions) into Petri dishes filled with solid agar for germination. After 3 days of seed germination in the incubator at 25 °C, uncontaminated seedlings were selected and transferred into sterile plastic Mangenta containers (20 seedlings per container). Plants were supplied with a small amount (7 mL) of standard hydroponic nutrient solution (Zhang et al., 1991). Seedlings remained inside the containers (closed with transparent plastic lids equipped with filters of 0.45 μm pore diameter) for 14 days.

Plant Growth. Containers with plants were placed in a controlled environment phytotron (day/night temperature, 25/21 °C; light period, 16/8 h; photon flux density, 220 μmol m^{-2} s^{-1}). Five milliliters of nutrient solution was added to each container after 3 and 7 days from the time of seedling transfer. Plant cultures contaminated with microorganisms were eliminated. The hydroponic solution was collected from uncontaminated cultures at 14 days and used for the GC analysis of low molecular weight organic acids.

Acid Extraction and Concentration from Root Exudates. Hydroponic growth solutions were first centrifuged at 10000*g* for 10 min (Sorvall RC-5B refrigerated superspeed centrifuge, DuPont Instruments), and the supernatant was filtered through a 0.45 μm Millipore filter. Solutions were transferred to plastic bottles, anion exchange membranes added, and the bottles shaken overnight on a mechanical shaker. The membranes were then transferred to 5 mL of 0.5 M HCl and eluted by shaking on a mechanical shaker overnight (Schoenau and Huang, 1991).

The efficiency of ion exchange extraction was compared with the efficiency of solvent extraction using ether and ethyl acetate from neutral and acidified aqueous solutions.

Sample Methylation for GC. One milliliter of the membrane eluate was transferred to a small vial for methylation, 0.1 mL of 100 ppm internal standard (IS) solution was added, and the sample was acidified with 15 drops of 50% H_2SO_4. Three milliliters of MeOH was then added and the vial heated at 50 °C for 30 min. Once the sample had cooled, 3 mL of water and 0.3 mL of chloroform were added, and the vial was shaken vigorously. After layer separation, 2 μL of the lower chloroform layer was injected onto the GC column.

One milliliter of the standard mixture with 0.1 mL of 100 ppm internal standard solution added was derivatized according to the same procedure as that for the samples.

Recovery. Recovery of the extraction/concentration and derivatization procedure was evaluated in hydroponic solutions collected from the durum wheat and flax growth cultures. Hydroponic solutions were first analyzed to determine the initial organic acid concentration. Five milliliters of a standard mixture containing 10 ppm of each acid was added to 25 mL of durum wheat and 15 mL of flax hydroponic solution. Volumes of 25 and 15 mL represented average volumes collected from typical hydroponic growth cultures of durum wheat and flax, respectively. Acids were extracted and concentrated on the anion exchange membranes, and 1 mL of the membrane eluate was subjected to GC analysis as described above.

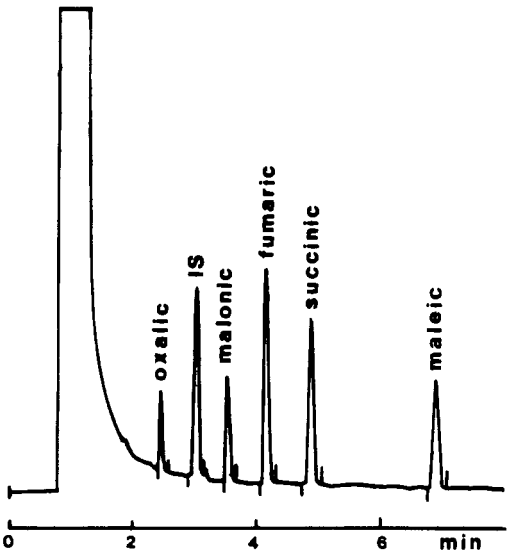

Figure 1. Gas chromatogram of methylated standard mixture of dicarboxylic acids. Two microliters of solution containing 10 ppm of each acid was injected at 8×10^{-10} afs; HP FFAP capillary column (30 m x 0.53 mm i.d.) at 125 °C; helium flow rate, 7 mL min^{-1}; IS, internal standard.

RESULTS AND DISCUSSION

GC Separation of Dimethyl Esters. Methyl esters of low molecular weight dicarboxylic acids from standard mixtures were well resolved on a high-polarity GC packing (Figure 1). Methylmalonic acid, not found in root exudates, was a suitable internal standard because its peak eluted close to the peaks of other acids without overlapping. Since methylmalonic acid behaved like other dicarboxylic acids during methylation, reproducibility of GC responses was improved. The five acids investigated in this study, i.e., oxalic, malonic, succinic, fumaric, and maleic acid, required 7 min for elution, making the GC monitoring of the dicarboxylic acid content in hydroponic solutions very fast and efficient. Acid concentrations as low as 1 ppm in membrane eluate were easily detected. Methylation of standard mixtures yielded clean chromatograms with no artifacts formed (Figure 1).

Sample Extraction and Concentration. Unconcentrated samples from hydroponic growth solutions showed no detectable peaks; hence, samples had to be concentrated. Concentration of the sample by removal of water through evaporation, however, was found to be unpractical due to the loss of oxalic acid. When samples were allowed to evaporate at room temperature, the amount of oxalic acid was reduced by ca. 50%; drying in the oven at 60 °C or in a freeze-drier resulted in the total loss of oxalic acid. It is known that a considerable loss of oxalic acid occurs through sublimation (*Merck Index*, 1976).

Concentration of acids from hydroponic solutions by extraction with organic solvents yielded low recoveries due to the poor solubilities of the low molecular weight organic acids in nonpolar solvents (*Merck Index*, 1976). Ethyl acetate and ether extraction, however, had been reported for isolating aromatic acids with very good recoveries (Horning et al., 1966). Recoveries from ether or ethyl acetate extraction of dicarboxylic acids in this study were comparable and were generally very low, i.e.,

ca. 1, 9, 28, and 3% for oxalic, malonic, succinic, and maleic acid, respectively. Acidification of the aqueous phase before extraction with concentrated HCl (pH reduced to 1) increased the recovery of organic acids to ca. 3, 26, 37, and 35% for oxalic, malonic, succinic, and maleic acid, respectively. Thus, solvent extraction of low molecular weight organic acids with either ether or ethyl acetate from neutral and acidified solutions was not effective and left most of the acids in the aqueous phase. Of the five acids investigated here, fumaric acid is more soluble in nonpolar organic solvents than in water. Thus, its recovery from solvent extraction was high, i.e., ca. 67 and 81% from neutral and acidified solutions, respectively.

Ion exchange procedures had been recommended for the isolation of organic acids from a variety of materials such as biological fluids (Sims et al., 1981), hydroponic growth solutions (Petersen and Bottger, 1991), and food products (Akhavan et al., 1980). The advantage of an ion exchange procedure over a solvent extraction is that all of the acids are adsorbed and recovered with an eluant from the ion exchanger (Horning et al., 1967). Solvent extractions are considered to be simple and rapid; however, as discussed above, the degree of recovery of low molecular dicarboxylic acids was low and varied with the chemical nature of the acid. Commonly, ion exchange procedures are performed using chromatographic columns. To simplify acid isolation using an ion exchange method, ion exchange membranes were used instead of columns. The advantage of ion exchange membranes over ion exchange resins in the bead form is that they are very easy to use and many samples can be prepared simultaneously (Schoenau and Huang, 1991), while resins require columns and large volumes of solvents. To concentrate a sample after elution from a column, the solvent has to be removed through evaporation. In the case of the membranes, the volume of the eluting solvent can be adjusted so that a desirable degree of sample concentration can be achieved. Thus, solvent evaporation is not necessary. The membrane size (7 cm^2) was selected on the basis of the ion exchange capacity of 0.064 mequiv/cm^2 of membrane surface and the expected organic acid and other inorganic anion concentration in the hydroponic solutions. Addition of two or three membranes did not improve the efficiency of the acid extraction from the hydroponic solutions. One elution with 5 mL of 0.5 M HCl removed all of the organic acids from the membrane as no organic acids were found in the eluate from a second or third elution of the membranes. Acid extraction/concentration of hydroponic growth solutions using the anion exchange membranes yielded chromatograms free of impurities (Figure 2). Thus, sample extraction and derivatization allowed selective isolation of acids and effectively removed interfering substances.

Oxalic, malonic, succinic, and fumaric acids were identified on the basis of retention times in the hydroponic cultures of wheat and flax plants. Maleic acid was not found in the samples. It should be noted that the retention time reproducibility was very good as represented by coefficients of variation (CV) of <0.5% (n = 6). The peaks were well resolved and therefore easy to quantify.

Recovery. Recovery of the acids from the extraction/concentration and methylation procedure was examined by spiking hydroponic growth solutions obtained from durum wheat cv. Sceptre and flax cv. Somme with selected acids. As seen in Tables 1 and 2, very good

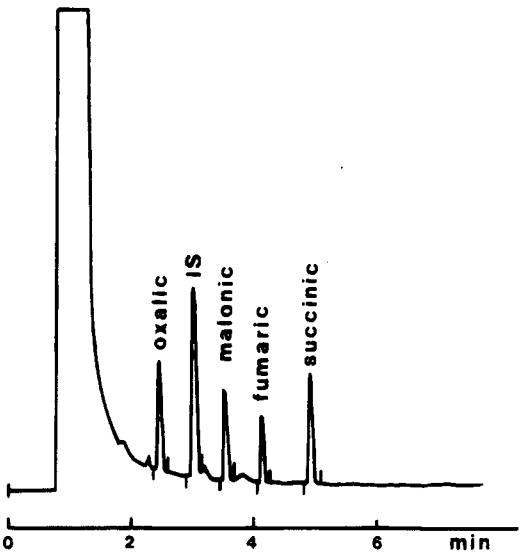

Figure 2. Gas chromatogram of methylated hydroponic solution from durum wheat cv. Sceptre growth culture after extraction/concentration on the anion exchange membrane. GC conditions were as in Figure 1.

Table 1. Recovery and Precision of Sample Extraction/Concentration and Methylation[a]

acid	amt in the unspiked samples (μg)	amt in the spiked samples (μg)	recovery (%)	CV (%)
oxalic	87.5	134.5	94.0	2.9
malonic	55.0	100.4	90.8	10.4
fumaric	37.5	68.6	62.2	13.4
succinic	202.5	245.2	85.4	3.0
maleic	0	55.6	111.2	16.6

[a] Fifty micrograms of each acid was added to 25 mL of hydroponic culture solution of durum wheat cv. Sceptre (n = 6).

Table 2. Recovery and Precision of Sample Extraction/Concentration and Methylation[a]

acid	amt in the unspiked samples (μg)	amt in the spiked samples (μg)	recovery (%)	CV (%)
oxalic	0	50.5	101.1	12.7
malonic	20.3	76.8	113.0	5.8
fumaric	10.5	55.5	90.0	17.2
succinic	6.8	56.7	99.8	9.9
maleic	0	57.5	115.1	18.7

[a] Fifty micrograms of each acid was added to 15 mL of hydroponic culture solution of flax cv. Somme (n = 6).

recovery and reproducibility were obtained for the saturated dicarboxylic acids, i.e., oxalic, malonic, and succinic acid. The unsaturated dicarboxylic acids, i.e., fumaric and maleic acid, showed some degree of instability. It has been reported that the methylation of the unsaturated dicarboxylic acids may be accompanied by undesirable side reactions as well as cis–trans isomerization (Mlejnek, 1972). The results of method evaluation (Tables 1 and 2) demonstrate that the method developed in this study is accurate and precise.

Sample Stability. Methyl esters of dicarboxylic acids were quite stable and thus the samples did not have to be injected immediately after derivatization. Derivatized samples were stored in the refrigerator. However, original as well as membrane extracted/concentrated hydroponic solutions were kept in the deep freezer to avoid any possible changes in the acid composition. Samples analyzed after 1 month of storage in the freezer did not show any signs of decomposition;

however, refrigerated samples showed a decrease in the acid content with time.

CONCLUSIONS

The developed GC method for the determination of low molecular weight dicarboxylic acids released by plant roots into sterile hydroponic cultures is precise and accurate. It should be emphasized that the high degree of accuracy was achieved due to the very good recovery of the acids from the extraction/concentration step performed with anion exchange membranes before sample methylation. Since the method is specific for dicarboxylic acids, it allowed for acid identification based on retention times. Because sterile conditions were used for plant growth, it was evident that the low molecular weight dicarboxylic acids determined according to this method were the acids secreted by the roots. The application of the developed method will be extended to study the excretion of low molecular weight dicarboxylic acids by plant roots into rhizosphere soil.

LITERATURE CITED

Akhavan, I.; Wrolstad, R. E.; Richardson, D. G. Relative effectiveness of ion exchange and lead acetate precipitation methods in isolating pear sugars and acids. *J. Chromatogr.* **1980**, *190*, 452–456.

Blake, J. D.; Clarke, M. L.; Richards, G. N. Determination of organic acids in sugar cane process juice by high performance liquid chromatography: improved resolution using dual Aminex HPX-87H cation-exchange columns equilibrated to different temperatures. *J. Chromatogr.* **1987**, *398*, 265–277.

Foy, C. D.; Lee, E. H.; Coradetti, C. C.; Taylor, G. J. Organic acids related to differential aluminium tolerance in wheat (*Triticum aestivum*) cultivars. In *Plant Nutrition-Physiology and Applications*; van Beusichem, M. L., Ed.; Kluwer Academic Publishers: Boston, 1990; pp 381–389.

Horning, M. G.; Knox, K. L.; Dalgliesh, C. E.; Horning, E. C. Gas-liquid chromatographic study and estimation of several urinary aromatic acids. *Anal. Biochem.* **1966**, *17*, 244–257.

Horning, M. G.; Boucher, E. A.; Moss, A. M. The study of urinary acids and related compounds by gas phase analytical methods. *J. Gas Chromatogr.* **1967**, *5*, 297–302.

Lilieholm, B. C.; Dudley, L. M.; Jurinak, J. J. Oxalate determination in soils using ion chromatography. *Soil Sci. Soc. Am. J.* **1992**, *56*, 324–326.

McFeeters, R. F. Single injection HPLC analysis of acids, sugars and alcohols in cucumber fermentations. *J. Agric. Food Chem.* **1993**, *41*, 1439–1443.

Mench, M.; Martin, E. Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L. and *Nicotiana rustica* L. *Plant Soil* **1991**, *132*, 187–196.

Mench, M.; Morel, J. L.; Guckert, A.; Guillet, B. Metal binding with root exudates of low molecular weight. *J. Soil Sci.* **1988**, *39*, 521–527.

Merck Index, 9th ed.; Merck & Co.: Rahway, NJ, 1976.

Miwa, H. Reversed-phase ion pair chromatography of some biologically important carboxylic acids as 2-nitrophenylhydrazides. *J. Chromatogr.* **1985**, *333*, 215–219.

Mlejnek, O. Methylation of dibasic acids with diazomethane in gas chromatography. *J. Chromatogr.* **1972**, *70*, 59–65.

Morvai, M.; Molnar-Perl, I. Simultaneous determination of organic acids and sugars in apples by gas-chromatography. *J. Chromatogr.* **1990**, *520*, 201–207.

Petersen, W.; Bottger, M. Contribution of organic acids to the acidification of the rhizosphere of maize seedlings. *Plant Soil* **1991**, *132*, 159–163.

Schoenau, J. J.; Huang, W. Z. Anion-exchange membrane, water and sodium bicarbonate extractions as soil tests for phosphorus. *Commun. Soil Sci. Plant Anal.* **1991**, *22*, 465–492.

Sims, P.; Truscott, R.; Halpern, B. Improved procedure for the anion exchange isolation of urinary organic acids. *J. Chromatogr.* **1981**, *222*, 337–344.

Supelco. Analyzing fatty acids by packed column gas chromatography. GC Bulletin 856, 1990.

Zhang, F.; Romheld, V.; Marschner, H. Release of zinc mobilizing root exudates in different plant species as affected by zinc nutritional status. *J. Plant Nutr.* **1991**, *14*, 675–686.

Received for review July 19, 1994. Revised manuscript received January 18, 1995. Accepted February 13, 1995.[⊗] Funding was provided by a Strategic Grant (STR0134336), "Soil Rhizosphere and Reduction of Cadmium Levels in Durum and Flax", from the Natural Sciences and Engineering Research Council of Canada and the Canadian Network of Toxicology Centres.

JF940402O

[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1995.