Low Molecular Weight Dicarboxylic Acids in Rhizosphere Soil of Durum Wheat

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A gas chromatographic (GC) method was developed for the determination of water extractable and HCl/MeOH extractable low molecular weight dicarboxylic acids in rhizosphere soils of durum wheat (*Triticum turgidum* var. Durum L.). Rhizosphere soils were collected after 2 weeks of plant growth by first removing the bulk soil from the root system and then by washing off the rhizosphere soil that adhered to the root surface with water. After shaking of the rhizosphere/water mixtures, dicarboxylic acids were concentrated on anion exchange membranes. Rhizosphere soils were then freeze-dried and the samples shaken with 0.5 M HCl in MeOH to remove any remaining dicarboxylic acids adsorbed to the soil particles after the water extraction. The efficiency of HCl/MeOH extraction from three different soils ranged from 85.9 to 106.3% for most acids; however, the recovery of oxalic acid varied with soil type. Oxalic, fumaric, and succinic acids were found in both the water and HCl/MeOH extracts of rhizosphere soils of durum wheat, while malonic acid was present only in the HCl/MeOH extracts. Dicarboxylic acids detected by GC in the water extracts were further identified by ion exclusion and ion exchange liquid chromatography. Washing soil from roots of durum wheat provided an efficient method for the collection of rhizosphere soil. Extraction of these soils with water and HCl/MeOH provided estimates of soluble and non-water-soluble low molecular weight dicarboxylic acids, respectively, using GC. Determination of these acids will aid in further understanding of their role in rhizosphere processes.

Keywords: *Rhizosphere; dicarboxylic acids; durum wheat*

INTRODUCTION

A wide variety of organic compounds is released by plant roots into the rhizosphere. Of the substances identified in root exudates, low molecular weight organic acids have received much attention due to their role in processes at the root−soil interface such as metal chelation, solubilization of nutrients, and acidification of the rhizosphere (Mench and Martin, 1991; Basu et al., 1994; Petersen and Bottger, 1991). Most studies on root exudation have been carried out in solution cultures; however, it has been shown that the nature and quantities of the exudates recovered from hydroponic cultures are different from those produced by plants growing in soil (Barber and Martin, 1976). Although solution culture studies permit determination of water-soluble exudates released by roots, this medium does not account for the complex interactions in rhizosphere soil that can render root exudates non-water-soluble. Therefore, to understand fully the processes at the root−soil interface, it is important to study organic acid composition in rhizosphere soils rather than in solution cultures.

Ion exclusion high-pressure liquid chromatography (HPLC) has been most frequently reported for the determination of low molecular weight organic acids collected from solution cultures or extracted from soils (Basu et al., 1994; Petersen and Bottger, 1991; Fox and Comerford, 1990; Hue et al., 1986; Mench and Martin, 1991). However, UV detection at 210 nm commonly used to monitor carboxylic acids with HPLC systems lacks sensitivity because the carboxyl group is only weakly chromophoric (Miwa, 1985). Also, because of interference from both organic and inorganic species capable of absorbing UV light and coelution of peaks, specificity is often a problem (Blake et al., 1987; Accorsi and Blo, 1991). Although gas chromatography (GC) requires sample derivatization, GC can provide better resolution and specificity than HPLC for low molecular weight organic acid separation (De Bruijn et al., 1984). We have recently proposed a GC method for the determination of low molecular weight dicarboxylic acids exuded from plant roots in hydroponic cultures (Szmigielska et al., 1995). This method was found to be accurate, precise, and specific.

The objective of this study, therefore, was to develop an extraction procedure for water-soluble and non-water-soluble low molecular weight dicarboxylic acids from rhizosphere soil of durum wheat (*Triticum turgidum* var. Durum L.) for the determination of the acids as methyl esters by GC.

MATERIALS AND METHODS

Pot Experiment. Surface soil samples were collected from three major soil zones in Saskatchewan, Canada, air-dried, and ground to pass a 2 mm sieve. Soil A was an Orthic Black Chernozem (Yorkton association) with a silty loam texture, soil B was classified as an Orthic Dark Brown Chernozem (Sutherland association) with a sandy loam texture, and soil C was an Orthic Gray Luvisol (Waitville association) having a sandy loam texture. Selected soil properties are listed in Table 1.

Fifty seeds of durum wheat cv. Kyle were placed in 15 cm plastic pots filled with 1.5 kg of soil and replicated three times. Seeds were germinated and plants were grown in a growth chamber at day/night temperatures of 25/18 °C with a 16 h light period of photon flux density 220 μmol m^{-2} s^{-1}. Plants were watered every other day to 65% of field capacity. Both bulk and rhizosphere soils were collected after 2 weeks of plant growth for analysis of low molecular weight dicarboxylic acids, as described below.

Sample Preparation. Plant roots were removed from the pots, and any soil loosely adhering to the roots was gently shaken off back into the pot. The soil in the pot was mixed,

0021-8561/96/1444-1036$12.00/0

Table 1. Selected Characteristics of Soils Used for the Pot Study

soil	pH	organic C (g/kg)	CEC ($cmol^+/kg$)	exchangeable Ca (g/kg)	exchangeable Mg (g/kg)
A	7.8	39	30.2	5.24	0.42
B	7.3	30	27.5	2.84	0.78
C	6.4	5	12.2	1.32	0.16

and a sample was taken which represented the bulk soil. Soil adhering to the root was washed off with approximately 500 mL of distilled water, and this soil represented the rhizosphere soil. Bulk soil samples (10 g) were extracted with 100 mL of distilled water, while rhizosphere soil samples were extracted with the water (i.e., 500 mL) used for the collection of the rhizosphere soil from the roots. The soils were shaken on a mechanical shaker for 8 h and the extracts were then centrifuged at 8000*g* for 15 min and filtered. Supernatants were transferred to plastic bottles along with one anion exchange membrane (7 cm^2) in bicarbonate form (Bio-Rad Laboratories, Richmond, CA), and the samples were shaken again for 8 h on a mechanical shaker. The membranes were then transferred to small vials containing 5 mL of 0.5 M HCl, and the dicarboxylic acids were eluted by shaking on a mechanical shaker for 8 h. For methylation, 1 mL of the membrane eluate was mixed with 0.1 mL of 100 μg mL^{-1} of internal standard (methylmalonic acid) solution in a small vial and the sample was acidified with 15 drops of 50% H_2SO_4. Next, 3 mL of MeOH was added and the vial heated at 60 °C for 30 min. After cooling, 3 mL of water and 0.5 mL of chloroform were added and the vial was shaken vigorously. The lower chloroform layer was analyzed by GC and represented the water extractable low molecular weight dicarboxylic acids.

The wet soil residues obtained after water extraction and centrifugation were freeze-dried and weighed to determine the dry weight of the rhizosphere soil collected from each pot. Next, 10 g of each rhizosphere soil was mixed with 10 mL of 0.5 M HCl in MeOH and shaken for 1 h on a mechanical shaker. The freeze-dried bulk soil samples were also extracted with 0.5 M HCl in MeOH in a 1:1 ratio. The extracts were centrifuged at 10000*g* for 10 min and filtered, and the volume of the extract was measured. One milliliter of the HCl/MeOH extract was then subjected to methylation as described above. However, after the sample had cooled and 3 mL of water had been added, the sample was filtered and the filter washed with an additional 4 mL of water before 0.5 mL of chloroform was mixed in the sample. This procedure helped to remove precipitates that otherwise made chloroform−water layer separation difficult. Since HCl/MeOH extraction followed water extraction, it can be assumed that extraction with HCl/MeOH released the dicarboxylic acids adsorbed to soil particles after the water extraction.

The data for individual dicarboxylic acids and total amounts of dicarboxylic acids were analyzed separately by a one-way ANOVA for water and HCl/MeOH extractions, and the statistical differences were determined by a Duncan's multiple range test.

Recovery of HCl/MeOH Extraction. To determine the efficiency of the HCl/MeOH extraction, samples of bulk soil were prepared for analysis using the same procedure as described above; i.e., 10 g of bulk soil was shaken with 100 mL of water on a mechanical shaker for 8 h and centrifuged, and the supernatant was discarded. The wet soil residue was spiked with 100 μg of each acid. Next, the "spiked" soil was freeze-dried and extracted with 0.5 M HCl in MeOH in a 1:1 soil/solution ratio by shaking for 1 h on a mechanical shaker. Blank samples (the wet soil that was not spiked) were analyzed together with spiked samples.

GC Conditions. Quantitative determination of low molecular weight dicarboxylic acids was performed on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with an HP FFAP capillary column (30 m × 0.53 mm i.d., film thickness 1.0 μm). The injector, column, and flame ionization detector temperatures were 200, 125, and 200 °C, respectively. Helium was used as a carrier gas at a flow rate of 7 mL min^{-1} and as a makeup gas at 23 mL min^{-1}. Two microliters of sample was injected at an attenuation setting of 3, and the chromatograms were recorded and peaks integrated using a Hewlett-Packard Model 3396 Series II integrator. The concentration of each acid in the standard mixtures was varied within the approximate range of concentrations found in the samples. Standards for the low molecular weight dicarboxylic acids (oxalic, malonic, succinic, fumaric, maleic, and methylmalonic used as an internal standard for the GC analysis) were obtained from Sigma Chemical Co. (St. Louis, MO).

Ion Exclusion HPLC. The HPLC system (Waters Associates) was equipped with an ion exclusion Shodex Ionpack KC-811 column (30 cm × 8 mm i.d.) maintained at a temperature of 40 °C. An aqueous solution of phosphoric acid (0.1%) was used as a mobile phase at a flow rate of 1 mL min^{-1}. Fifty microliters of standards and the water-extracted/membrane-concentrated samples were injected directly into the HPLC, and the peaks were detected by a UV detector set at 210 nm. Because of the large differences in the UV absorption coefficients of the dicarboxylic acids, concentrations of the acids in the standard mixture were adjusted so that all of the acids eluted with comparable peak sizes (5, 0.5, 50, 50, and 1 μg mL^{-1} for oxalic, maleic, malonic, succinic, and fumaric acid, respectively). The chromatograms were processed by a Baseline 810 chromatography work station.

Ion Exchange Liquid Chromatography. A Dionex Series 2110i ion chromatograph equipped with an HPIC-AS3 separator column, an Ionpac-AG3 guard column, and anion micromembrane suppressor was used. The regenerant for the suppressor was 12.5 mM H_2SO_4 at a flow rate of 3.4 mL min^{-1}. The mobile phase consisted of 2.8 mM $NaHCO_3$ and 2.2 mM Na_2CO_3 and was pumped at a flow rate of 3 mL min^{-1}. The injection loop was 100 μL. Standard mixture consisted of succinic, malonic, maleic, and oxalic acids at a concentration of 10 μg mL^{-1} for each acid. Peaks were detected by a conductivity detector at 3 μS and chromatograms recorded by a Dionex 4270 integrator.

The excessive amount of chloride ions present in the water-extracted/membrane-concentrated samples was removed with cation exchange membranes prior to ion chromatographic analysis as described below. The membranes (BDH, VWR Scientific of Canada Ltd., Toronto, ON) were prepared by washing five times with 0.5 M HCl and five times with 0.5 M $AgNO_3$. A cation exchange membrane (25 cm^2) was placed in a vial containing 4 mL of the sample and shaken for 15 min on a mechanical shaker. The chloride ions were precipitated through formation of insoluble AgCl. After the membrane was removed, the sample was filtered and injected into the ion chromatograph.

RESULTS AND DISCUSSION

Method Evaluation. Collection of the rhizosphere soil achieved by washing off the soil adhering to the roots with water rather than by mechanical removal of the soil particles from the roots (Fox and Comerford, 1990; Clemensson-Lindell and Persson, 1992) was found to be very easy and practical. Visual examination of roots after washing showed that little or no roots were broken because roots did not desiccate and therefore did not become brittle. It is possible that a small portion of roots or root hairs was mixed with the rhizosphere soil and resulted in some release of organic acids from the roots. However, contamination from washing soil from roots should be minimal compared to brushing off the soil particles from roots. A somewhat large volume of water was needed for an efficient removal of the soil from the roots; therefore, the water extract had to be enriched to allow for the acids' detection and quantitation. The method of sample concentration using anion exchange membranes, previously described for the determination of low molecular weight dicarboxylic acids in hydroponic growth solutions (Szmigielska et al., 1995), was successfully applied to the water extracts of rhizosphere soils. Through this procedure, an extract volume of ca. 500 mL was reduced to 5 mL, yielding

Table 2. Recovery of Low Molecular Weight Dicarboxylic Acids from HCl/MeOH Extraction of Soils As Determined by GC after Sample Methylation

	amt found in unspiked samples (μg)			amt found in spiked samples (μg)			% recovery ± SD		
acid[a]	soil A	soil B	soil C	soil A	soil B	soil C	soil A	soil B	soil C
oxalic	61.3	35.6	91.9	89.7	70.8	196.2	28.4 ± 4.2	35.2 ± 2.3	104.3 ± 6.7
malonic	0	0	0	85.9	90	98.9	85.9 ± 1.2	90.0 ± 5.5	98.9 ± 3.7
fumaric	2.1	2.0	1.7	96.9	96.5	95.3	94.8 ± 1.8	94.5 ± 1.7	93.6 ± 2.6
succinic	0	0	0	93.6	91.6	96.0	93.6 ± 3.0	91.6 ± 4.2	96.0 ± 4.8
maleic	0	0	0	96.2	97.2	106.3	96.2 ± 2.7	97.2 ± 1.8	106.3 ± 2.7

[a] 100 μg of each acid added to 10 g of soil (n = 4).

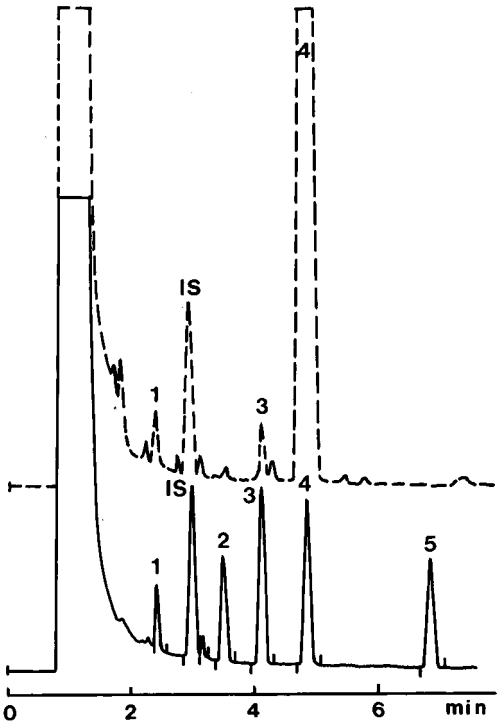

Figure 1. Gas chromatograms of standard mixture of low molecular weight dicarboxylic acids (solid line) and water extract of rhizosphere soil of durum wheat cv. Kyle grown in soil B (dashed line). Peaks: 1, oxalic; 2, malonic; 3, fumaric; 4, succinic; 5, maleic; IS, internal standard.

concentrations of acids in the extracts within the quantitation range (Figure 1). The efficiency of the acid concentration on the anion exchange membranes was high for most of the acids and ranged from 91 to 103%. It was lower for fumaric acid, i.e. 45%, probably due to instability of the double bond leading to a possible decomposition of fumaric acid.

The determination of acids remaining on soil particles after water extraction, however, was made with a more efficient extractant such as HCl/MeOH that had been previously reported for oxalic acid extraction from mycorrhizal fungi (Cromack et al., 1979). The HCl/MeOH was found to be quite effective in extracting malonic, fumaric, succinic, and maleic acids from the three soils with recovery values in the range of 85.9−106.3% as determined by GC after sample methylation (Table 2). Recovery of oxalic acid from HCl/MeOH extraction, however, ranged from 28.4 to 104.3% and varied with soil type. It has been reported that the determination of oxalic acid in soils (Lilieholm et al., 1992) and in other materials such as crop plants (Huang and Tanudjaja, 1992; Holloway et al., 1989) can be difficult due to the fact that oxalic acid is usually present in soluble and insoluble forms. This difficulty has been overcome in analysis of crop plants by use of water and 1 M HCl solution for the extraction of soluble and total oxalate, respectively, with very good recoveries (Huang and Tanudjaja, 1992; Holloway et al., 1989). In soil analysis, however, Lilieholm et al. (1992) reported that extraction with 1 M HCl yielded recoveries in the range of 16−104% and that the variability in recovery was linked to the amount of $CaCO_3$ present in the soil as well as to the cation exchange capacity. A similar relationship was found in this study for HCl/MeOH extraction of oxalic acid from soils; soils of high exchangeable Ca^{2+} (Table 1) resulted in lower oxalic acid recoveries (Table 2).

Freeze-drying of the wet soil residues obtained after water extraction resulted in no substantial loss of the dicarboxylic acids (Table 2). Drying of the soil samples increased the efficiency of HCl/MeOH extraction and subsequently the methylation as compared to wet soil samples. Recoveries of acids extracted from moist soil samples were 12.2, 55.9, 94.3, 93.7, and 78.3% for oxalic, malonic, fumaric, succinic, and maleic acid, respectively; extraction of wet soil samples resulted in recoveries of 0, 39.7, 67.0, 76.3, and 56.2% for oxalic, malonic, fumaric, succinic, and maleic acid, respectively.

Because dicarboxylic acid recoveries from HCl/MeOH extraction were high (except for oxalic acid) (Table 2), apparently, the water and HCl/MeOH extractions applied in a sequence removed most or all of the labile and potentially labile low molecular weight dicarboxylic acids, respectively, from the soils as determined by additions of dicarboxylic acid standards.

To avoid changes in organic acid composition, soils were processed immediately after collection and all extracts were stored in the freezer. This procedure reduced the possibility of microbial activity during sample preparation; however, the effects of possible microbial activity on organic acid content in rhizosphere soil during the extraction procedure were not examined in this study.

Dicarboxylic Acid Identification. Very good GC separation was obtained for methyl esters of low molecular weight dicarboxylic acids on an HP FFAP capillary column (10 pg of each acid injected) (Figure1). Peaks were well resolved and therefore easy to identify on the basis of retention times. Of the five dicarboxylic acids investigated in this study, oxalic, succinic, and fumaric acids were found in both the water and HCl/MeOH extracts of rhizosphere soils collected from 2-week-old durum wheat plants, while malonic acid was detected only in the HCl/MeOH extracts (Tables 3 and 4). Maleic acid, however, was not found in either extract.

Selected water extracts of the rhizosphere soils that had been concentrated on the anion exchange membranes were injected into an ion exclusion HPLC without any further sample modification. Despite some problems such as peak coelution, low sensitivity of UV detection, and interference from other UV-absorbing components in the samples (Blake et al., 1987; Accorsi and Blo, 1991), ion exclusion HPLC allowed for positive identification of succinic and fumaric acids (Figure 2).

Table 3. Concentration of Water Extractable Low Molecular Weight Dicarboxylic Acids (Micrograms per 10 g of Dry Soil) in Rhizosphere and Bulk Soil of Durum Wheat Cv. Kyle Grown in Three Different Soils As Determined by GC after Sample Methylation[a]

	soil A		soil B		soil C	
acid	rhizosphere	bulk	rhizosphere	bulk	rhizosphere	bulk
oxalic	nd[b]	nd	6.57[a]	nd	5.86[a]	nd
malonic	nd	nd	nd	nd	nd	nd
fumaric	nd	nd	2.29[b]	nd	0.45[a]	nd
succinic	0.52[a]	nd	385.19[c]	nd	99.51[b]	nd
total	**0.52**[a]	**nd**	**394.05**[c]	**nd**	**105.82**[b]	**nd**

[a] Means within the same row having the same superscript letter are not significantly different ($p \leq 0.05$). [b] Not detectable.

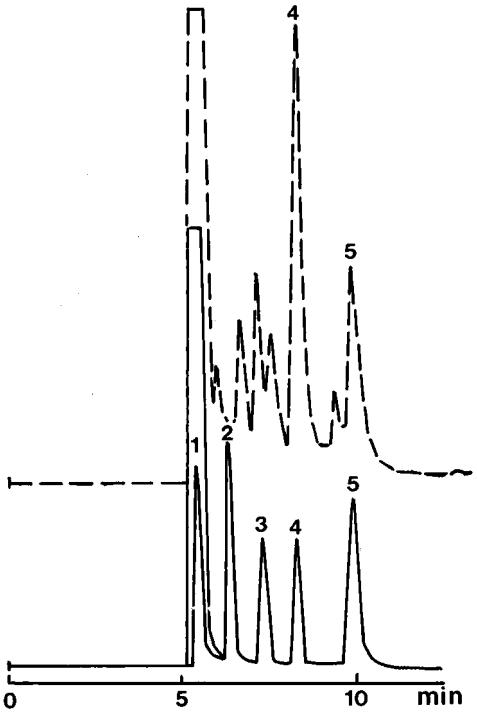

Figure 2. Ion exclusion liquid chromatograms of standard mixture of low molecular weight dicarboxylic acids (solid line) and water extract of rhizosphere soil of durum wheat cv. Kyle grown in soil B (dashed line). Peaks: 1, Cl^- and oxalic; 2, maleic; 3, malonic; 4, succinic; 5, fumaric.

As seen in a chromatogram of a standard mixture (Figure 2), low molecular weight dicarboxylic acids were well separated by the ion exclusion HPLC, except for oxalic acid which coeluted with the large peak in the dead volume of the column. Succinic acid was present in the samples at high concentrations (Figure 1); therefore, it was easily detected by the HPLC (Figure 2), even though its response to UV detection was somewhat low (log ϵ 2.0 at 208 nm). The presence of fumaric acid was also easily detected by the HPLC because of the high UV absorption coefficient (log ϵ 4.2 at 208 nm) of fumaric acid, yielding a large peak even at low concentrations (Figure 2). Malonic acid was found by GC only in HCl/MeOH extracts of rhizosphere soil from plants grown in soil B (Table 4). However, because HCl/MeOH extracts were not suitable for HPLC analysis, the identity of malonic acid was not confirmed. The remaining peaks in the ion exclusion chromatograms of water extracts of rhizosphere soils (Figure 2) were not investigated in this study.

Because the oxalic acid peak was obscured by the large peak of inorganic anions in the ion exclusion chromatograms (Figure 2), its presence was substantiated by ion exchange chromatography. Ion exchange chromatography with conductivity detection has been reported for the determination of soluble and insoluble oxalates both in soils and in other materials (Lilieholm et al., 1992; Huang and Tanudjaja, 1992; Holloway et al., 1989). When HCl was used for the extraction of insoluble oxalates, however, the high concentration of Cl^- ions masked the oxalic acid peak. To remove the excessive amount of Cl^- ions, Huang and Tanudjaja (1992) used cation exchange cartridges in the Ag^+ form before chromatographic analysis, while Lilieholm et al. (1992) diluted the extracts to bring the Cl^- concentration down. Cation exchange membranes in the Ag^+ form used in this study were simple to use and very efficient in the removal of Cl^- ions from the extracts. As seen in a chromatogram of a standard mixture (Figure 3), the oxalic acid peak was well separated from the peak representing succinic, malonic, and maleic acids, while fumaric acid did not elute from the column under these conditions. In sample chromatograms (Figure 3), due to the relatively high concentration of inorganic anions and succinic acid that eluted before oxalic acid, oxalic acid formed a peak on a shoulder of a large peak. However, because the peaks were sufficiently separated, ion chromatography allowed for the confirmation of the presence of oxalic acid in the extracts of rhizosphere soils.

Quantitative Determination. To illustrate the application of the developed method, bulk and rhizosphere soils from durum wheat cv. Kyle grown in three different soils were analyzed by GC for low molecular weight dicarboxylic acids. Concentrations of water extractable and HCl/MeOH extractable low molecular weight dicarboxylic acids are listed in Tables 3 and 4, respectively. No water extractable acids were found in the bulk soils (Table 3). When the bulk soils were extracted with HCl/MeOH, mainly oxalic acid and small quantities of fumaric acid were found (Table 4). Succinic acid was generally the most abundant among the water extractable acids from rhizosphere soils, followed by oxalic and fumaric acids (Table 3). In the HCl/MeOH extracts of rhizosphere soils, oxalic and succinic acids were found at the highest concentrations followed by fumaric and malonic acids (Table 4). Total amounts of

Table 4. Concentration of HCl/MeOH Extractable Low Molecular Weight Dicarboxylic Acids (Micrograms per 10 g of Dry Soil) in Bulk and Rhizosphere Soil of Durum Wheat Cv. Kyle Grown in Three Different Soils As Determined by GC after Sample Methylation[a]

	soil A		soil B		soil C	
acid	rhizosphere	bulk	rhizosphere	bulk	rhizosphere	bulk
oxalic	63.03[ab]	61.33[ab]	78.62[bc]	35.58[a]	111.61[c]	91.90[c]
malonic	nd[b]	nd	11.00[a]	nd	nd	nd
fumaric	3.65[ab]	2.08[ab]	3.43[ab]	1.98[ab]	4.44[b]	1.69[a]
succinic	6.85[a]	nd	82.29[c]	nd	34.66[b]	nd
total	**73.53**[ab]	**63.41**[ab]	**175.34**[d]	**37.56**[a]	**150.71**[c]	**93.59**[b]

[a] Means within the same row having the same superscript letter are not significantly different ($p \leq 0.05$). [b] Not detectable.

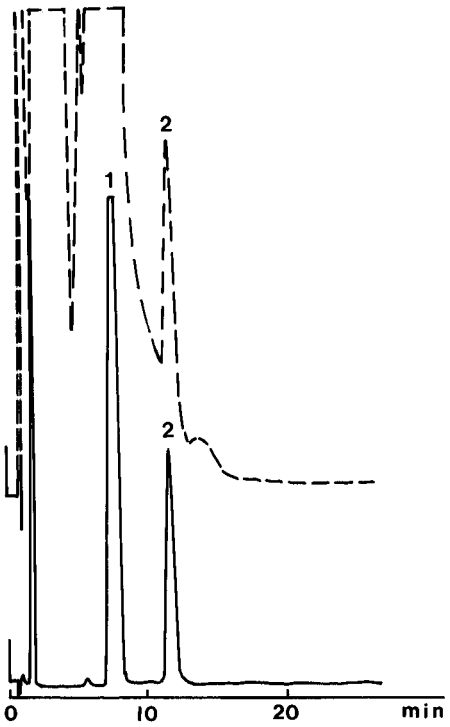

Figure 3. Ion exchange liquid chromatograms of standard mixture of low molecular weight dicarboxylic acids (solid line) and water extract of rhizosphere soil of durum wheat cv. Kyle grown in soil B after Ag^+ cation exchange membrane treatment (dashed line). Peaks: 1, succinic, malonic, and maleic; 2, oxalic.

water extractable acids were significantly different ($p \leq 0.05$) among the soils with the highest acid concentrations found in soil B, followed by soil C and soil A (Table 3). A similar trend was also observed for the total HCl/MeOH extractable acids; the highest total acid concentration was found in soil B and the lowest in soil A with significant differences ($p \leq 0.05$) among the three soils (Table 4). These results demonstrated a strong soil effect on the content of low molecular weight dicarboxylic acids in the rhizosphere soils. The factors leading to these differences were not investigated in this work and merit further study.

Conclusions. The GC method described here allows for the determination of water-soluble and non-water-soluble low molecular weight dicarboxylic acids in rhizosphere and bulk soils. An easy and practical method for collection of the rhizosphere soil was achieved by washing the soil particles adhering to the roots with water. The extract obtained after shaking of the rhizosphere soil with water can be efficiently concentrated on the anion exchange membranes. The high recoveries of dicarboxylic acids added to the soils using HCl/MeOH extraction indicate that HCl/MeOH can be used successfully for the determination of non-water-soluble low molecular weight dicarboxylic acids in soils. The developed methodology will be useful in further studies of the effects of various low molecular weight organic acids on rhizosphere processes.

ACKNOWLEDGMENT

We thank Dr. Doug Knott of the Crop Science Department for supplying the seeds for the pot experiment and Dr. Jeff Schoenau of the Soil Science Department for advice and expertise on the chemistry and use of ion exchange membranes. Contribution R779, Saskatchewan Centre for Soil Research, University of Saskatchewan, Saskatoon, SK, Canada.

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Received for review May 4, 1995. Revised manuscript received December 15, 1995. Accepted January 2, 1996.[⊗] Funding was provided by the Canadian Network of Toxicology Centres and the Strategic Grant (STR0134336; Soil Rhizosphere and Reduction of Cadmium Levels in Durum and Flax) from the Natural Sciences and Engineering Research Council of Canada.

JF950272Z

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1996.