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ORGANIC ACIDS ASSESMENTS IN MEDICINAL PLANTS BY CAPILLARY ELECTROPHORESIS

Georgiana TRUICĂ (BADEA),[a] Eugenia Dumitra TEODOR [a,*] and Gabriel Lucian RADU [a,b]

[a] National Institute for Biological Sciences, Centre of Bioanalysis, 296 Spl. Independentei, Bucharest 060031, Roumania
[b] University Politehnica Bucharest, Faculty of Applied Chemistry and Materials Science, 1-7 Polizu Str., Bucharest 011061, Roumania

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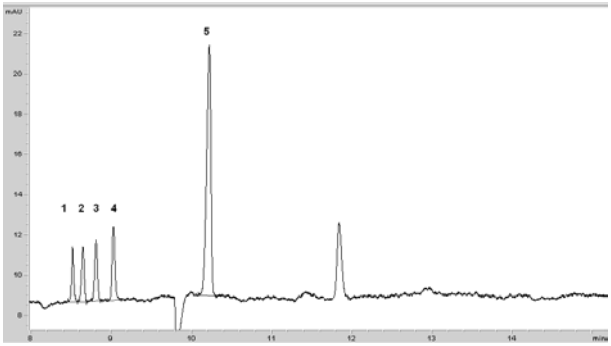
The content in short-chain organic acids of medicinal herbs is important for their taste, flavour and therapeutic effects. Short-chain organic acids from three types of medicinal plants were analyzed, chamomile *(Matricaria recutita*, Asteraceae), linden (lime, *Tilia platyphyllos*, Tiliaceae) and mint (menthe, *Mentha piperita*, Lamiaceae) as infusion and decoction. A diode array-capillary electrophoresis method has been partially validated that permits the direct measurement of 5 short-chain organic acids, respectively succinic, malic, citric, tartaric and lactic acid, in medicinal plants extracts (teas). The method is simple, rapid, reliable and low consumption of resources in comparison with chromatographic methods; it could be applied on other natural products and extracts (coffee, honey, fruits, juices and wines) and could be developed on a wide series of short-chain organic acids.



INTRODUCTION

Generally, plants contain considerable quantities of organic substances with a diversity of metabolites which total more than 200,000 products. The most important plant metabolites, present at concentrations ranging between 20-100 $\mu molg^{-1}$ in raw materials, are polysaccharides, polyols, amino acids, and organic acids. [1-2] Organic acids are involved as intermediate or end products in different fundamental pathways in plant metabolism and catabolism; for example the citrate, succinate, malate, fumarate, and acetate in the acetyl coenzyme A form, play an important role in the Krebs cycle which is the central energy yielding cycle of the cell.[3] Some of these short-chain organic acids serve as precursor for a variety of products, such as acetate or formate, others, such as malate, are involved in respiration and photosynthesis processes or in detoxification (oxalate and citrate).[4-5] Organic acids are responsible for the taste, the flavour, the microbial stability, and the product consistence of plant derived beverages and are used in food preservation because of their effects on bacteria.[6-7]

* Corresponding author: eu_teodor@yahoo.com

The chemical study of organic acids, as part of metabolomics analysis, provides biochemical information on cellular functioning and on pathways affected by stress or disease. Qualitative and quantitative determination of a large number of organic acids metabolites provides an overall view of the biochemical status of the cell.[1]

At the present time, several methods have been developed for identifying and quantifying organic acids in grape juices, wines, coffee, medicinal plants, so much individually, as non-enzymatic spectrophotometric and enzymatic methods or as a group of them simultaneously, as chromatographic and electrophoretic methods.[6,8-9] In recent years, chromatographic techniques have been replaced by capillary electrophoresis (CE) due to its good resolution, automation, simplicity, high speed, low consumption of chemicals and reduced sample preparation. Nevertheless, the methods used to determine simultaneously the short-chain organic acids such as tartaric, malic, succinic, lactic and citric acids from natural extracts (fruits, coffee, medicinal herbs, wines) are not very numerous.[6-8,10-20] Although the indirect UV detection was the most common used detection mode in capillary zone electrophoresis (CZE) for the determination of organic acids in these samples, direct UV detection seems to be more suitable due to the stability of the baseline.[6,10-12]

In this work a simple, reliable and rapid CE method was partially validated and applied for quantification of 5 short-chain organic acids (succinic, citric, malic, tartaric and lactic) in three types of medicinal plants, chamomile *(Matricaria recutita*, Asteraceae), linden (lime, *Tilia platyphyllos*, Tiliaceae) and mint (menthe, *Mentha piperita*, Lamiaceae). Generally, medicinal plants were evaluated only for the content in phenolic acids but short-chain organic acids also play important roles in plants metabolism and in their therapeutic power.

RESULTS AND DISCUSSION

After more attempts, the optimum results for the simultaneous quantification of succinic, malic, citric, tartaric and lactic acids were obtained using 0.5 M H_3PO_4 and 0.5 mM cetyltrimethylammonium bromide (CTAB, pH=6.25) as background electrolyte (BGE) and water as the sample matrix.[12] After we establish the optimal conditions for the separation, the selectivity, linearity, accuracy, precision, limit of detection and limit of quantification were tested. Under the described experimental conditions the values of the migration times were: 8.579 for succinic acid, 8.712 for malic acid, 8.877 for tartaric acid, 9.097 for citric acid and 10.340 for lactic acid.

The linearity of the method was determined by injecting a series of organic acids standards (six calibration points) with the concentration range between 25 and 250 μgmL^{-1} for malic, tartaric, citric and succinic acids and between 60.45 and 604.50 μgmL^{-1} for lactic acid. Analyses were performed in triplicate and the calibration curves were obtained by plotting the peak area *vs*. concentration (μgmL^{-1}). The important parameters of calibration curves, *i.e.*, slope (a), intercept (b) and correlation coefficient are presented in Table1. The sensitivity of the method, expressed as values of detection and quantification limits calculated as 3xS/N, respectively 10xS/N, is also shown in Table 1.

Table 1

The most important parameters for the calibration curves obtained by capillary electrophoresis

Organic acid	***y*= a*x* +b**	**R^2**	**Concentration range μgmL^{-1}**	**LOD (μgmL^{-1})**	**LOQ (μgmL^{-1})**
Succinic	y=0.1334x - 0.0326	0.9994	25-250	0.97	3.25
Malic	y=0.1354x + 0.2101	0.9995	25-250	3.10	10.33
Tartaric	y=0.1533x - 0.0835	0.9986	25-250	2.18	7.25
Citric	y=0.1963x - 0.1242	0.9995	25-250	2.53	8.43
Lactic	y=0.0756x + 3.392	0.9954	60.45-604.5	89.73	298.82

a – Slope; b – Intercept; R^2 – coefficient of correlation; (LOD) Limits of detection and (LOQ) limits of quantification (S – signal, N – noise).

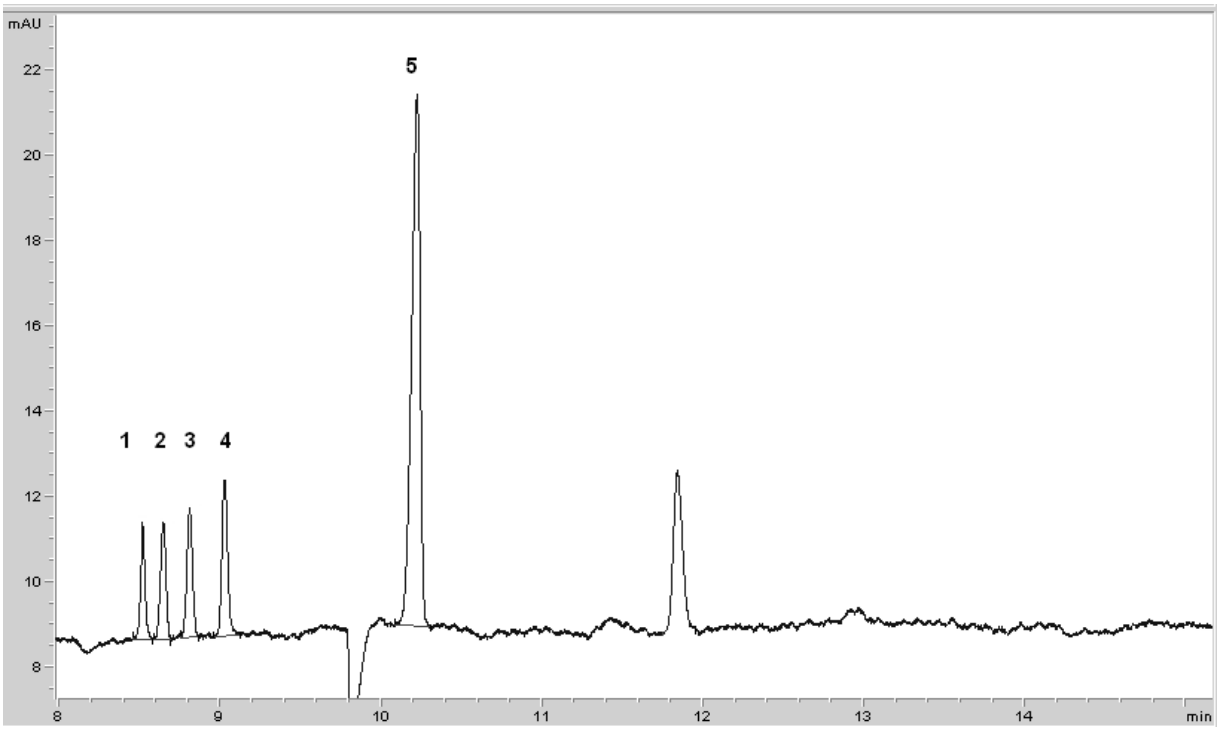


Fig. 1 – Electropherogram corresponding to a mixture of 5 organic acids at 50 μgmL^{-1} concentration: 1 – succinic acid, 2 – malic acid, 3 – tartaric acid, 4 – citric acid, and 5 – lactic acid.

Table 2

Results obtained in the repeatability and inter-assay precision studies

Organic acid	**Repeatability RSD % (n=6)**		**Inter-assay precision RSD% (n=4x6)**	
	Migration time	Peak area	Migration time	Peak area
Succinic	2.79	5.32	5.30	4.57
Malic	2.84	3.14	5.42	2.56
Tartaric	2.89	2.61	5.56	3.46
Citric	2.99	4.69	5.55	5.53
Lactic	3.88	0.96	6.69	5.36

Electropherogram corresponding to a mixture of 5 organic acids at 50 μgmL^{-1} concentration (from standard solutions of calibration curve) is presented in Fig. 1.

Because the measurements were performed in a single laboratory we approached only repeatability and intermediate precision (for precision study of the method). Repeatability was tested comparing 6 consecutive injections of a standard solution of concentration 50 μgmL^{-1} of each organic acid under the same conditions, the same analyst on the same day. Intermediate precision was demonstrated by injecting 6 times a mixture solution of concentration 50 μgmL^{-1} of each organic acid, for a period of 4 days. The results are presented in Table 3 as relative standard deviation percentage (RSD %). The obtained RSD values (below 6%) show an excellent repeatability and precision of the proposed method.

For accuracy estimation were used all 6 types of samples (3 plants and 2 modes of extraction) spiked with a concentration of 25 μgmL^{-1} for malic, succinic, tartaric and citric acid and with a concentration of 90.68 μgmL^{-1} for lactic acid, and were made three consecutive injections. The obtained results expressed as recovery percentage are given in Table 3 and are in the range of 75-134%, showing that in the domain of concentrations studied, the sampling and the method itself does not significantly affect the results.

Table 3

Results expressed as recovery obtained for accuracy studies of organic acids in tea samples

Organic acid	Recovery % – Infusion	Recovery % – Decoction	Recovery domain* %
Succinic	104-114	92-105	92-114
Malic	88-100	101-103	88-103.2
Tartaric	100-116	101-134	99-134
Citric	98-123	94-101	94-123
Lactic	81-96	74-91	74-96

* n=3 consecutive injections for each sample

Table 4

Concentration of organic acids (mgL^{-1}) in various tea samples

Samples	Organic acid content* mgL^{-1}				
	Succinic	Malic	Tartaric	Citric	Lactic
Chamomile – infusion	nd	20.45 ±0.08	nd	17.34 ±0.22	nd
Chamomile – decoction	9.98 ±0.10	43.25 ±0.06	nd	31.36 ±0.16	nd
Linden – infusion	nd	18.20 ±0.09	nd	nd	nd
Linden – decoction	nd	34.30 ±0.11	nd	7.30 ±0.09	nd
Mint – infusion	5.77 ±0.06	87.64 ±0.11	19.02 ±0.08	76.43 ±0.06	nd
Mint – decoction	9.74 ±0.02	111.53 ±0.12	24.76 ±0.10	104.49 ±0.16	nd

* Average of 3 measurements
nd – not detected or under detection limit

In conclusion, the values obtained from the recovery studies can be considered adequate for the levels of analytes and the characteristics of the method.

The final results obtained from the analysis of tea samples, infusion and decoction, are presented in Table 4 and in Fig. 2 are shown the electropherograms for an undiluted tea samples and for the same sample, spiked with a known concentration of each analyzed organic acid.

Organic acids from tea samples were identified comparing the migration times (t_M) with those obtained for standard organic acids. As could be observed from Table 4, four short-chain organic acids from five were permanently identified in tea samples, except for lactic acid which is under the detection limit or absent in all 6 extracts. Tartaric acid is significantly present only in mint extracts and succinic acid is present in mint and chamomile but in low concentrations. Malic acid is always present in significant levels, between 18.3 and 111.5 mgL^{-1}, in all the analysed extracts (especially in mint). Citric acid was identified in chamomile and mint tea (17.34-104.49 mgL^{-1}) and only in low concentrations in linden tea. It should be noted that all the tea samples prepared as decoction offered higher concentrations in organic acids compared to samples prepared by infusion.

The content in organic acids obtained by CE analysis was difficult to be compared with other data from literature because short-chain organic acids are poorly studied in plant extracts.

Among the identified compounds in our study, malic, fumaric and citric acids were previously described in *C. annuum* seeds.[21-22] Guimarães *et al*., 2013, has a recent study about Roman chamomile, *Chamaemelum nobile* L. (Asteraceae) which has been used for medicinal applications, mainly through oral dosage forms (decoctions and infusions). This herb and its infusion are a source of phenolic compounds (flavonoids such as flavonols and flavones, phenolic acids and derivatives) and organic acids (oxalic, quinic, malic, citric and fumaric acids) that showed antioxidant and antitumor activities, without hepatotoxicity.[23] From our knowledge, tartaric acid was not yet identified in medicinal plants.

EXPERIMENTAL

Chemicals and reagents

All the reagents were of analytical reagent grade (purity > 98%): DL- lactic acid from Fluka (Buchs, Switzerland), L-(+)-tartaric acid, citric acid, succinic acid, malic acid and the surfactant added to background electrolyte, cetyltrimethylammonium bromide (CTAB) from Sigma-Aldrich (Germany). Solvents and solutions were filtered on 0.2 μm membranes (Millipore, Bedford, MA, USA) and degassed before use. Phosphoric acid 85% was purchased from Merck (Germany), HPLC-grade water, 0.1N and 1N sodium hydroxide solutions were purchased from Agilent Technologies (USA).

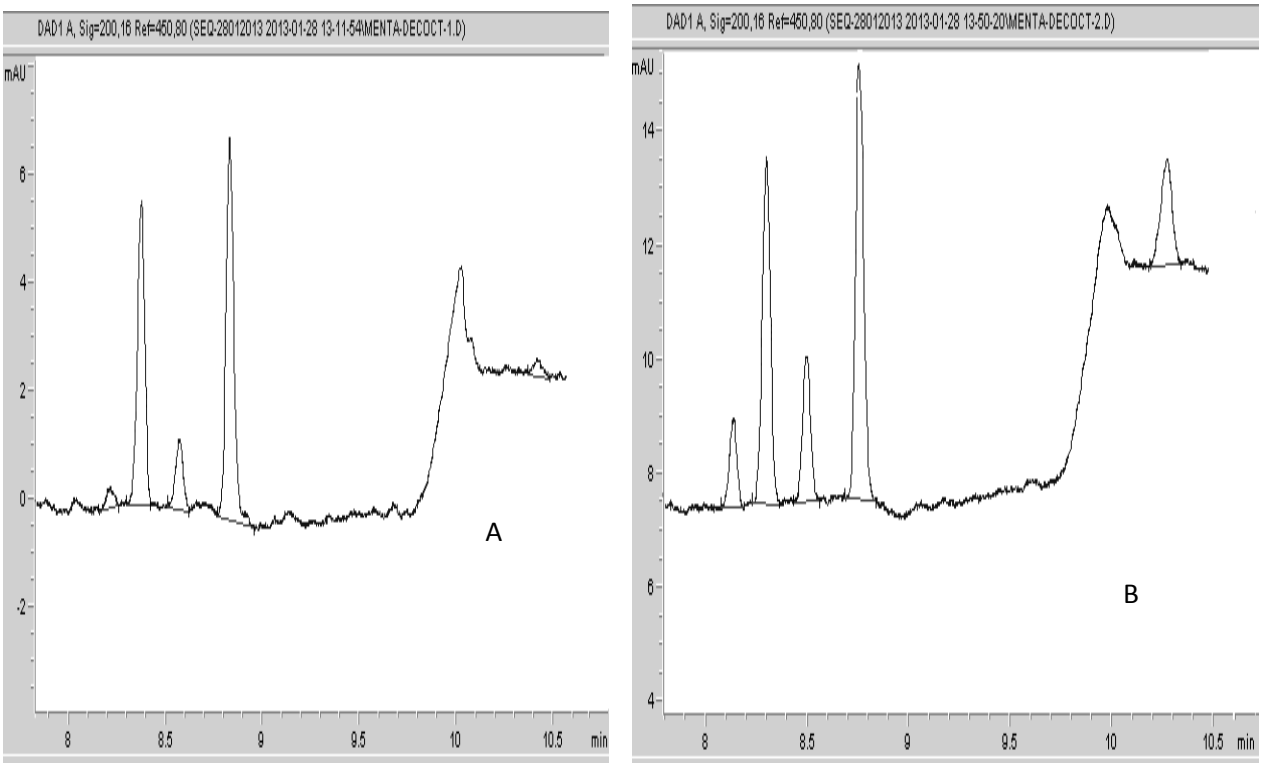


Fig. 2 – Electropherogram of a tea sample (mint, infusion) undiluted injected (A) and spiked with 25 μgmL^{-1} of malic, tartaric, succinic and citric acid, and respectively 90.68 μgmL^{-1} lactic acid (B).

Preparation of stock solutions, **calibration standards**, **and quality control samples**

Stock solutions for each standard were prepared at a concentration of 1 $mgmL^{-1}$ in water and stored at +4°C. Working solutions were prepared daily by diluting the stock solutions in the concentration range between 25 and 250 μgmL^{-1} for malic, tartaric, succinic and citric acids, and between 60.5 and 604.5 μgmL^{-1} for lactic acid.

Three types of medicinal plants were analyzed, chamomile *(Matricaria recutita*, Asteraceae), linden (lime, *Tilia platyphyllos*, Tiliaceae) and mint (menthe, *Mentha piperita*, Lamiaceae). The samples of plants were obtained from different brands of medicinal teas available on the market.

The samples were prepared by extraction of organic acids from medicinal plants (infusion and decoction). For infusions, one tea bag (approx. 1g) of each plant category was minced in a mortar (homogenized), mixed with 200 mL hot distilled water (100° C) and let to infuse for 5 minutes; when the solution was cold it was filtered through a 0.2 µm Millipore filter and injected undiluted in the instrument. Samples preparation of tea decoction form: the same amount of sample, 1 g (1 tea bag) from each category of medicinal plants was added over boiling water and boiled for 5 minutes, and then was left to cool, filtered and injected undiluted into the instrument.

Capillary electrophoresis

Electrophoretic separation was carried out using an Agilent CE instrument with DAD detector and CE standard bare fused-silica capillary having 50 µm internal diameter and 72 cm effective length. Before use, the capillary was washed successively with basic solutions: 5 min with 1N NaOH, 5 min with 0.1 N NaOH followed by Ultra Pure Water 5 min and buffer 20 min.

After several attempts, the CE method selected by us belongs to reversed polarity CE category,[12] and the conditions are described below. The applied voltage was -10 kV and the best UV detection was performed at 200 nm (direct detection). Sample injection was performed using the hydrodynamic mode 35 mbar/5 sec while the capillary was maintained at constant temperature of 25°C. The analyte migration time was found by injecting dilutions of the stock solutions of each compound. Organic acids were analyzed with 0.5M H_3PO_4 and 0.5 mM CTAB (pH=6.25), filtered on 0.2 µm membranes (Millipore, Bedford, MA, USA) and degassed before use. The organic acids order of elution was succinic, malic, tartaric, citric and lactic, and the analysis time of 15 minutes. The capillary was flushed between runs with 0.1M NaOH for 3 min, H2O for 3 min and the background electrolyte for 5 min.

Validation

After we establish the optimal conditions for the separation, the selectivity, linearity, precision, accuracy (recovery), limit of detection and limit of quantification were tested.

CONCLUSIONS

The content in short-chain organic acids of medicinal herbs is important for their taste, flavour and therapeutic effects. Organic acids from three types of medicinal plants were analyzed,

chamomile *(Matricaria recutita*, Asteraceae), linden (lime, *Tilia platyphyllos*, Tiliaceae) and mint (menthe, *Mentha piperita*, Lamiaceae) as infusion and decoction. A DAD-capillary electrophoresis method has been partially validated that permits the direct measurement of 5 short-chain organic acids in medicinal herbs extracts (teas). The method is simple, rapid, reliable and low consumption of resources in comparison with chromatographic methods and could be applied on other natural products or extracts (coffee, honey, fruit juices and wines) and could be developed on a wide range of short-chain organic acids.

The organic acids of interest were identified in tea samples prepared as infusion and decoction, except for the lactic acid which is under the detection limit or absent. Tartaric acid was significantly present only in mint extracts and succinic acid was present in mint and chamomile but in low concentrations. Malic acid was always present in significant levels, between 18.3 and 111.5 mgL^{-1}, in all the analysed extracts (especially in mint). Citric acid was identified in chamomile and mint tea (17.34-104.49) and only in low concentrations in linden tea.

Usually, the plants (herbs) bioactivity could be explored in the medicine, food, and cosmetic industries.

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