Reverse iontophoretic extraction of metabolites from living plants and their identification by ion-chromatography coupled to high resolution mass spectrometry.

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ABSTRACT

Introduction - The identification and characterisation of cellular metabolites has now become an important strategy to obtain insight into functional plant biology. However, the extraction of metabolites for identification and analysis is challenging and, at the present time, usually requires destruction of the plant.

Objective – To detect different plant metabolites in living plants with no pre-treatment using the combination of iontophoresis and ion-chromatography with mass spectrometry detection.

Methodology – In this work, the simple and non-destructive method of reverse iontophoresis has been used to extract *in situ* multiple plant metabolites from intact *Ocimum basilicum* leaves. Subsequently, the analysis of these metabolites has been performed with ion chromatography coupled directly to high resolution mass spectrometric detection (IC-MS).

Results – The application of reverse iontophoresis to living plant samples has avoided the need for complex pre-treatments. With this approach, no less than 24 compounds, including organic acids and sugars as well as ATP were successfully detected.

Conclusion- The research demonstrates that it is feasible to monitor, therefore, a number of important plant metabolites using a simple, relatively fast and non-destructive approach.

KEYWORDS

Ocimum basilicum, reverse iontophoresis, ion chromatography, mass spectrometry, plant metabolite identification

INTRODUCTION

Plants possess an enormously rich and diverse number of metabolites – measured in the many 1000s - including sugars, hormones, organic acids and bases, polyols, and amino acids, with multiple functions in manifold vital processes (Rivasseau et al., 2006). The identification and characterisation of cellular metabolites has now become an important strategy to obtain insight into functional plant biology (Sato et al., 2004). For example, the determination of organic acids provides information on biochemical pathways affected by stress or disease (Rivasseau et al., 2006). Adenosine triphosphate (ATP) is intimately involved in the energy balance and maintenance of cellular homeostasis and is considered an important signalling molecule that responds to stress (e.g., caused by trauma such as a wound) in plants (Kim et al., 2006). Equally, the intracellular concentrations of certain compounds can reveal phenotypes of specific proteins involved in metabolic regulation (Fiehn et al., 2000). However, the detailed profile of plant metabolites is complicated and limited by the physical barriers presented by the cuticles and epicuticular waxes that protect the organism (Bjarnholt et al., 2014). As a result, the extraction of metabolites for identification and analysis is challenging and, at the present time, usually requires destruction of the plant. Furthermore, once an aggressive extraction procedure has been implemented, the subsequent determination of the levels of the very many chemicals produced can be a daunting task (Stitt and Fernie, 2003).

It follows that the development of a method that is capable of simultaneously analysing the key compounds involved in plant homeostasis (as well as response to stress, etc.) is an important objective (Roessner et al., 2001). While a number of powerful techniques, including HPLC-MS (Li et al., 2011; Pan et al., 2010), gas chromatography-MS (Fiehn et al., 2000; Major et al., 2006; Werner et al., 2008) and electrophoresis (Rivasseau et al., 2006; Sato et al., 2004), are clearly able to detect and quantify samples containing a large number of different entities, preparation of the material for analysis typically requires destructive procedures such as plant tissue pulverization in liquid nitrogen and use of non-aqueous solvents. Such pre-treatments, prior to analysis, have been associated with artificial alteration of the biochemical status of the plant under study, rendering the results difficult, if not impossible, to interpret unambiguously (Bartels and Svatos, 2015). Logically, therefore, analytical methods requiring little or no sample pretreatment are preferred, as are procedures to acquire the samples *in situ* from living plants. Although it has been possible to chemically stain with exogenously applied compounds (and subsequently visualize microscopically) some metabolites in intact leaves (Sanchez-Perez et al., 2009), concerns remain about the 'collateral' effects of the materials used. Elsewhere, the determination of plant metabolites resulting from oxidative stress in intact leaves has been reported (Sun et al., 2014, Xu et al., 2009, 2010; Zheng et al., 2015), but the analytical methods used in this case were unable to provide multiple simultaneous determinations. More promisingly, the rapid evolution of mass spectrometric imaging for visualisation of plant metabolite distribution has significant potential; however, for the moment, the presence of cuticular waxes on leaf surfaces is an interference problem in need of resolution (Bjarnholt et al., 2014).

Iontophoresis (Glikfeld et al., 1988, 1989) is a technique that has been used to increase the transport of primarily ionised compounds across biological barriers, such as the skin (Leboulanger et al, 2004) and leaves (Gonzalez-Sanchez et al., 2015), and represents a putative,

non-destructive way to extract plant metabolites. The approach uses a small electric current to promote the transport of compounds by electromigration and/or electroosmosis. Generally speaking, this approach has been targeted at increasing drug delivery across human skin (Delgado-Charro and Guy, 2014; Kalia et al., 2004), although the symmetry of the method has led to its application in "reverse" mode to non-invasively extract, for monitoring purposes, analytes such as glucose, urea and lithium (Delgado-Charro and Guy, 2003; Leboulanger et al., 2004; Rao et al., 1993, 1995; Wascotte et al., 2008). Most recently, reverse iontophoresis was employed for the first time to extract salicylate from the leaves of an *Ocimum basilicum* plant *in situ* (Gonzalez-Sanchez et al., 2015); the metabolite was subsequently analysed by cyclic voltammetry on a multi-walled, carbon nanotube, screen-printed electrode. Because the specificity of the technique originates in the analytical approach employed, it follows that reverse iontophoresis is able to extract many other important plant metabolites in a process that is easy, relatively fast and non-destructive.

In this paper, therefore, the ability of reverse iontophoresis to extract multiple endogenous metabolites from the intact leaves of a living *Ocimum basilicum* plant and their subsequent quantification with ion chromatography – mass spectrometry (IC-MS) is demonstrated. IC-MS has been chosen as the analytical tool for its powerful ability to separate, detect, and identify biological compounds (De Vos et al., 2012) and its wide use in the analysis of metabolites in crude aqueous-alcohol plant extracts (De Vos et al., 2012; Li et al., 2011; Pan et al., 2010).

EXPERIMENTAL

Materials and reagents

Pyruvate, fumarate, 2-isopropylmalic acid, phosphoenolpyruvate, sedoheptulose-7-phosphate, glyceric acid, uric acid, ribose-5-phosphate, mucic acid, ATP, quinic acid, 2-oxoglutatarate, citramalic acid, fructose-6-phosphate, oxalic acid, fructose-1-6-biphosphate, lactate, methylglutaric acid, gluconic acid, glucose-1-phosphate, succinate, glucose-6-phosphate, malate, citrate, potassium chloride, potassium monohydrogen phosphate and potassium hydroxide were purchased at their highest available purity from Sigma-Aldrich (UK) and were used as received. Stock solutions of the standards (10 μg/ml) were prepared using deionized, sterile-filtered water ((resistivity ≥ 18.2 MΩ·cm at 25°C) (Millipore, Watford, UK)). A phosphate buffer solution (100 mM, pH = 7.0) was used as the as the supporting electrolyte for iontophoretic extraction. This solution was prepared using mixtures of 100 mM solutions of Na_2HPO_4 and NaH_2PO_4 to obtain the desired pH. pH was measured with a pH213 Microprocessor pH meter (Hanna instruments). *Ocimum basilicum* (basil) plants were purchased at a local supermarket and were used within 5 days. As a key goal of the study was to demonstrate that the method can be applied to any type of plant, no attempt was made to control either the growth conditions or the age of plants. The choice of basil followed on from our previous work (Gonzalez-Sanchez et al., 2015) but was otherwise quite arbitrary – the plant is readily available in good health and the size and texture of its leaves are suitable for the iontophoresis experiments undertaken.

Instrumentation

Iontophoretic extraction: Iontophoretic experiments were controlled by a computer-interfaced potentiostat (μAUTOLAB Type III, ECO-chemie, NL). The vertical, glass iontophoresis cell (Figure 1) comprises lower and upper compartments, with the latter having two electrode chambers separated by an intervening space. The basil leaf was interposed horizontally, with the abaxial side facing up, between the upper and lower parts of the cell (contact area: 1.6 cm^2). The anode and cathode compartments were filled with 1.0 mL of phosphate buffered saline (pH 7.0). The lower chamber was charged with 7.0 mL of the same buffer. A constant current of 0.5 mA (with the voltage limited to 10V) was imposed, for periods of 1, 2, 4 and 8 hours, between the Ag/AgCl cathode and the silver wire anode, which were introduced into the electrode chambers prior to the initiation of the experiment. The electrical potential gradient promotes the transport of both ionized and polar, uncharged plant metabolites from the leaf by electromigration and electroosmosis, respectively. Charged compounds are extracted, of course, into the electrode chamber of opposite polarity, while electroosmotic flow carries net-neutral compounds in the direction of ions that have a charge opposite to that of the leaf surface (Kalia et al., 2004). Following iontophoresis, aliquots were taken from upper compartments and the extracted compounds were analyzed using ion-chromatography with mass spectrometric detection (IC-MS).

Ion chromatography with mass spectrometry detection: Compounds from the plant extracts were identified using a Thermo Scientific ICS-5000+ ion chromatography system coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Scientific, San Jose, CA). The ICS-5000+ HPLC system incorporated an electrolytic anion generator (KOH) which was programmed to produce an OH^- gradient over 37 minutes to facilitate ion chromatography. An inline electrolytic suppressor removed the OH^- ions and cations from the post-column eluent prior to MS analysis (Thermo Scientific Dionex AERS 500). A 10 μL partial loop injection was used for all analyses and the chromatographic separation was performed using a Thermo Scientific Dionex IonPac AS11-HC 2 × 250 mm, 4 μm particle size column with a Dionex Ionpac AG11-HC 4 μm 2x50 guard column inline. The IC flow rate was 0.250 mL/min. The total run time was 37 minutes and the hydroxide ion gradient comprised as follows: 0 mins, 0 mM; 1 min, 0 mM; 15 mins, 60 mM; 25 mins, 100 mM; 30 mins, 100 mM; 30.1 mins, 0 mM; 37 mins, 0 mM. Analysis was performed in negative ion mode using a scan range from 80-900 m/z and resolution set to 70,000 (arbitary units). The tune file source parameters were set as follows: Sheath gas flow 60 (arbitary units); Aux gas flow 20 (arbitary units); Spray voltage 3.6 V; Capillary temperature 320 °C; S-lens RF value 70 V; Heater temperature 450 °C. AGC target was set to 1e6 ions and the Max IT value was 250 ms. The column temperature was kept at 30°C throughout the experiment. Full scan data were acquired in continuum mode. Peak retention times were identified from the injection of authentic standards and metabolite identification was performed using a combination of accurate mass analysis (<2 ppm) and retention time using Thermo Scientific Quanbrowser software (Thermofisher Scientific, Hemmel, UK).

RESULTS AND DISCUSSION

The method developed comprised the extraction of metabolites from living *Ocimum basilicum* (basil) and their detection using IC-MS. A schematic diagram of the iontophoresis diffusion cell is shown in Figure 1 (Glikfeld et al., 1988; Glikfeld et al., 1989). The leaf separated the upper compartment, which has two chambers that provide electrode access, from the lower compartment. The effective, exposed area of the leaf was 1.6 cm^2. When the two halves of the cell are aligned correctly with the leaf in place, the electrode chambers were electrically and physically isolated from one another and the current path traversed the leaf itself in both 'in' and 'out' directions.

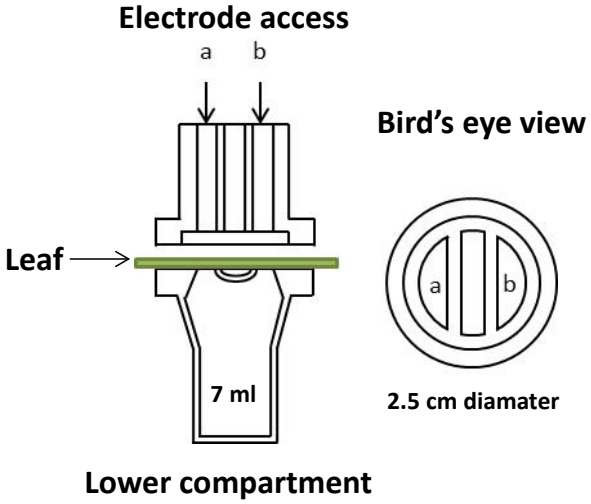


Figure 1. *Iontophoresis diffusion cell. The leaf is situated between the upper and the lower compartments of the cell. A bird's eye view looking down on the top of the cell is shown on the right of the figure.*

Figure 2A is a photograph of an actual experiment with the Ag anode and Ag/AgCl cathode, between which a current of 0.5 mA was applied, in place. The iontophoretic current causes a variety of plant metabolites to be extracted from the leaf into the electrode chambers: negatively-charged compounds are attracted to the anode, positive moieties to the cathode, by electromigration, as shown in Figure 2B (Delgado-Charro and Guy, 2003). Neutral, polar chemicals can also be extracted by the mechanism of electroosmosis (Glikfeld et al., 1989), the dominant direction of which depends upon the net charge of the leaf itself (see below).

Samples of the anodal and cathodal solutions were taken as a function of time and the iontophoretically-extracted metabolites were analyzed by IC-MS. In total, no less than 24 compounds were identified in this way, including organic acids, sugars and the nucleotide ATP (Figure 3). These molecules play important roles in plant metabolism including DNA glycolysis, the Calvin cycle, and other key pathways; their detection and quantification is important, therefore, in the study of plant metabolism. Mass spectrometric data for the identified compounds are shown in Table 1 and, by way of an example, the IC-MS chromatograms and mass spectra for three important compounds (glucose-6-phosphate, citrate and malate) are presented in Figure 1S (Supplementary Material).

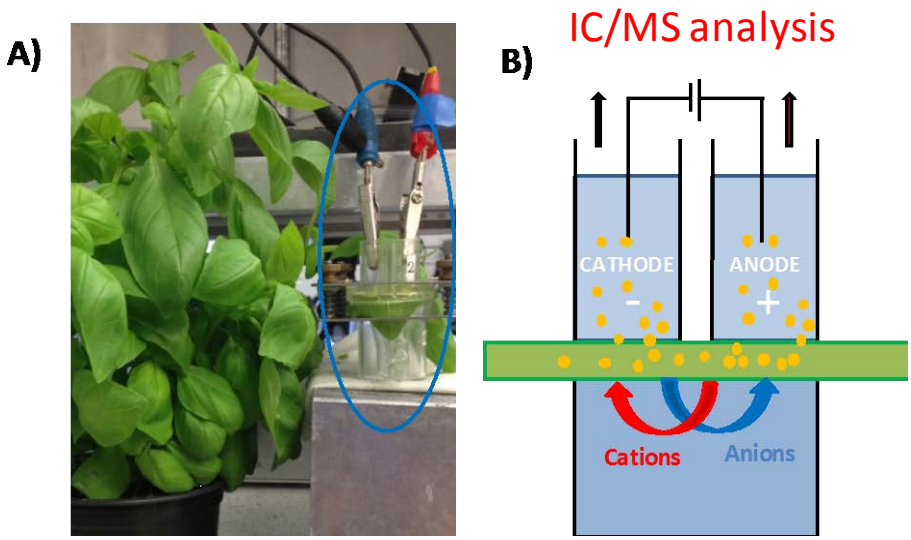


Figure 2. *(A) Photograph of the experimental set-up used for the iontophoretic extraction of plant metabolites from an Ocimum basilicum leaf. (B) Schematic illustration of the extraction of anions and cations by reverse iontophoresis.*

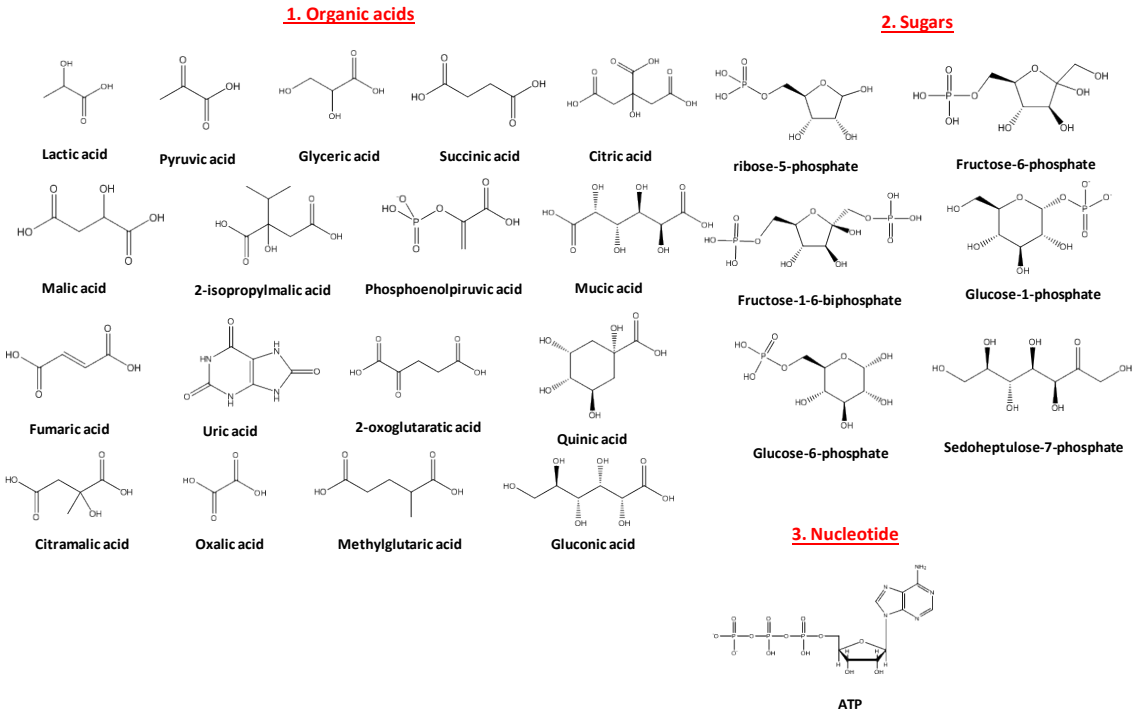


Figure 3. *Plant metabolites, and their chemical structures, extracted from Ocimum basilicum leaves by iontophoresis and identified by IC-MS.*

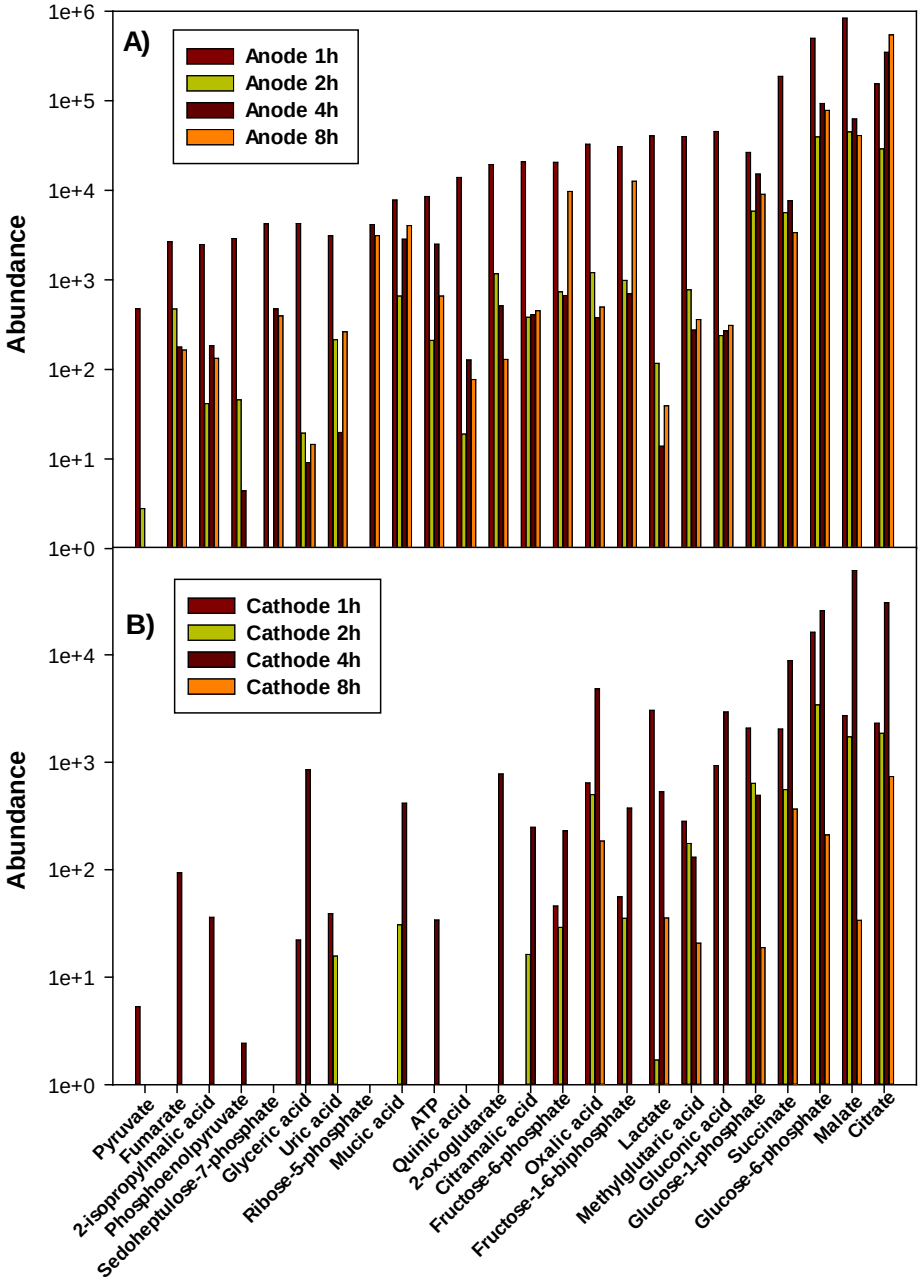
The relative abundance information on the plant metabolites extracted from *Ocimum basilicum* leaves by reverse iontophoresis together with the relevant pK_a value(s) and net charge at physiological pH, is presented in Table 2. For each compound, the maximum relative signal, and the time at which that maximum abundance was attained, are shown. Those substances which were clearly charged at pH 7 were extracted essentially exclusively to the electrode of opposite polarity as expected. For the acidic compounds, there is a consistent trend of high

abundance at the anode rather than cathode. In the case of sugars, they show less difference in abundance between the anode and cathode, although there are lower levels in the cathode samples. It is not surprising that these compounds would likely be negatively charged *in vivo* and hence migrate towards the anode; however, the fact that they are also present in the cathode sample suggests more than one mechanism of migration. Interestingly, uric acid, which is uncharged at neutral pH, was preferentially extracted to the anode. This suggests that the surface of the basil leaf supports a net positive charge; i.e., counterion flow is due to the movement of anions. Leaf surfaces that are positively charged have been reported before under certain circumstances, but it cannot be said that the observation is ubiquitous to all plants (Leach, 1984; Leach and Apple, 1984). Furthermore, the molecular origin of the positive charge on basil leaves remains, for now, unknown.

The time-dependent extraction of the plant metabolites to the anode and cathode over an 8-hour period is shown in Figure 4. Typically, the maximum signal at the anode was attained at 1 h, whereas at the cathode 4 h were usually required. The decrease in signal with further increase in the duration of iontophoresis may be the result of the depletion of local 'reservoirs' of certain compounds that are not necessarily present throughout the plant at the same level; such a phenomenon has been observed when reverse iontophoresis is applied to human skin *in vivo*, where the local tissue concentrations of certain amino acids, for example, are significantly higher than those in the blood (Sieg et al., 2009; Sylvestre et al., 2010). While compound degradation, or any number of redox reactions (due to the constant application of an electric current), may also be responsible, the fact that the plant continues to grow normally post-iontophoresis suggests that the extraction method is essentially non-destructive. Practically speaking, it would appear that 1 hour of iontophoresis is probably sufficient to permit the simultaneous extraction of a significant number of interesting plant metabolites.

CONCLUSIONS

The successful extraction and identification of several endogenous plant metabolites from intact leaves of *Ocimum basilicum* has been achieved by reverse iontophoresis coupled with IC- MS. The research demonstrates that it is feasible to monitor, therefore, a number of important plant metabolites using a simple, relatively fast and non-destructive approach. A key achievement of the technique is that it can be used *in situ* on the leaves of a living plant; in other words, the extraction of endogenous compounds is accomplished without separating the leaf from the plant, and without any potentially destructive and confounding pre-treatments of the leaf to obtain the metabolites. This non-invasive extraction, combined with sensitive IC-MS analysis, has permitted no less than 24 plant metabolites to be easily detected, including organic acids, sugars and the nucleotide ATP, and offers a new approach to probe and to better understand the complexity of plant metabolism.



F**igure 4**. *Time-dependent, reverse iontophoretic extraction of plant metabolites to the anode (A) and the cathode (B).*

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TABLES

Table 1. *Mass spectrometric data and analyte identification.*

Accepted Compound ID	m/z	Charge	Retention time (min)	Formula	Mass Error (ppm)	Chromatographic peak width (min)	Isotope Distribution	Maximum Abundance	Adducts	Isotope Similarity	Retention Time Error (mins)
pyruvate	87.00859	1	12.56	$C_3H_4O_3$	-1.97	0.41	100 - 2.54	244	M-H	98.54	-1.45
Fumarate	115.0036	1	12.18	$C_4H_4O_4$	-0.36	0.36	100 - 2.91	1516	M-H	97.61	-0.05
2-Isopropylmalic acid	175.061	1	13.15	$C_7H_{12}O_5$	-0.91	0.36	100 - 4.35	1529	M-H	95.56	1.22
Phosphoenolpyruvic acid	166.9749	1	18.34	$C_3H_5O_6P$	-1.38	0.17	100 - 2.14	1606	M-H	97.46	-0.04
Sedoheptulose-7-P	289.0336	1	13.86	$C_7H_{15}O_{10}P$	1.98	0.30	100 - 4.09	2115	M-H	94.08	1.05
Glyceric acid	105.0192	1	12.56	$C_3H_6O_4$	-1.30	0.46	100 - 2.91	2168	M-H	98.64	-1.06
Uric acid	167.0208	1	19.31	$C_5H_4N_4O_3$	1.28	0.55	100 - 5.11	2283	M-H	97.45	0.35
Ribose - 5 phosphate	275.0178	1	14.84	$C_5H_{11}O_8P$	-0.79	0.51	100 - 5.92 - 0.383	4155	M+FA-H	98.63	0.60
Mucic acid	209.0301	1	11.96	$C_6H_{10}O_8$	-0.74	1.39	100 - 7.44 - 0.439	5041	M-H	98.12	1.45
ATP	505.9889	1	24.20	$C_{10}H_{16}N_5O_{13}P_3$	0.89	0.83	100 - 11.6 - 2.42	5349	M-H	97.26	-0.06
Quinic acid	173.0452	1	7.52	$C_7H_{12}O_6$	-0.16	1.21	100 - 6.41 - 3.11	7145	M-H2O-H	97.09	0.96
2-Oxoglutarate	145.0141	1	13.55	$C_5H_6O_5$	-0.90	0.34	100 - 3.15	11504	M-H	96.55	0.05
Citramalic acid	147.0297	1	11.96	$C_5H_8O_5$	-1.35	0.77	100 - 4.28	12040	M-H	97.59	1.47
Frusctose-6-Phosphate	259.0223	1	19.13	$C_6H_{13}O_9P$	-0.65	0.94	100 - 2.83 - 2.62	13139	M-H	95.58	1.26
Oxalic acid	88.98795	1	14.02	$C_2H_2O_4$	-0.89	0.46	100 - 0.962 - 0.054	18144	M-H	97.91	1.07
Fructose-1,6-diphosphate	338.9898	1	19.13	$C_6H_{14}O_{12}P_2$	2.89	0.94	100 - 6.29 - 2	19428	M-H	98.47	-0.84
Lactate	89.02429	1	7.77	$C_3H_6O_3$	-0.98	1.78	100 - 3.25 - 0.421	20645	M-H, M+FA-H	99.60	-1.17
3-Methylglutaric acid	145.0503	1	11.96	$C_6H_{10}O_4$	-2.10	1.05	100 - 3.53	23220	M-H	96.06	0.62
Gluconic acid	195.0508	1	7.57	$C_6H_{12}O_7$	-0.91	0.66	100 - 6.55 - 1.32	23567	M-H	99.29	-0.94

Glucose 1 phosphate	241.0118	1	13.46	$C_6H_{13}O_9P$	-0.43	1.50	100 - 4.81 - 0.209	24748	M-H2O-H	96.19	0.34
Succinate	117.0193	1	12.15	$C_4H_6O_4$	-0.62	1.54	100 - 4.12 - 0.451	112613	M-H	99.17	-0.07
Glucose-6-Phosphate	259.0224	1	13.15	$C_6H_{13}O_9P$	-0.29	3.22	100 - 6.51 - 0.866	415245	M-H	98.35	-0.02
Malate	133.0141	1	12.18	$C_4H_6O_5$	-0.52	2.75	100 - 4.17 - 0.76	486629	M-H2O-H, M-H	99.28	-0.04
Citrate	191.0197	1	17.28	$C_6H_8O_7$	-0.37	1.14	100 - 6.64 - 1.13	538354	M-H	99.25	0.05

Table 2. *Plant metabolites extracted by iontophoresis from Ocimum basilicum leaves.*

Name	pK_a values	Charge at pH 7	Maximum relative signal (A)	Maximum relative signal (C)
Pyruvate	2.93*	-1	4.74×10^2 (1h)	5.32 (1h)
Fumarate	3.55*	-2	2.67×10^2 (1h)	93.70 (4h)
2-isopropylmalic acid	3.63*	-2	2.48×10^3 (1h)	36.02 (4h)
Phosphoenolpyruvate	0.76*	-3	2.89×10^3 (1h)	2.43 (4h)
Sedoheptulose-7-phosphate	1.49*	-2	4.23×10^3 (1h)	NF
Glyceric acid	3.42*	-1	4.25×10^3 (1h)	8.54×10^2 (4h)
Uric acid	7.61*	0	3.11×10^3 (1h)	39.08 (1h)
Ribose-5-phosphate	1.22*	-2	4.15×10^3 (4h)	NF
Mucic acid	2.83*	-2	7.78×10^3 (1h)	NF
ATP	0.90, 5.00*	-3	8.52×10^3 (1h)	34.07 (4h)
Quinic acid	3.46*	-1	1.39×10^4 (1h)	0.07 (1h)
2-oxoglutarate	2.66*	-2	1.94×10^4 (1h)	7.77×10^2 (4h)
Citramalic acid	3.35*	-2	2.08×10^4 (1h)	2.48×10^2 (4h)
Fructose-6-phosphate	0.97, 6.11 (Bhattacharyya & Rohrer, 2012b)	-2	9.69×10^3 (8h)	2.30×10^2 (4h)
Oxalic acid	1.36*	-2	3.27×10^4 (1h)	1.84×10^2 (8h)
Fructose-1-6-biphosphate	0.93*	-4	3.08×10^4 (1h)	3.75×10^2 (4h)
Lactate	3.78*	-1	4.06×10^4 (1h)	3.04×10^3 (1h)
Methylglutaric acid	3.91*	-2	3.99×10^4 (1h)	2.82×10^2 (1h)
Gluconic acid	3.39*	-1	4.53×10^4 (1h)	2.94×10^3 (4h)
Glucose-1-phosphate	1.10, 6.13(Bhattacharyya & Rohrer, 2012b)	-2	2.65×10^4 (1h)	2.08×10^3 (1h)
Succinate	3.55*	-2	1.87×10^5 (1h)	8.85×10^3 (4h)
Glucose-6-phosphate	0.94, 6.11(Bhattacharyya & Rohrer, 2012a)	-2	4.97×10^5 (1h)	3.42×10^3 (4h)
Malate	3.20*	-2	8.34×10^5 (1h)	6.09×10^4 (4h)
Citrate	2.87, 4.35, 5.69 (Bhattacharyya & Rohrer, 2012a)	-3	1.54×10^5 (1 h)	3.05×10^4 (4 h)

*pK_a values from http://www.hmbd.ca/metabolites

A = anode, C = cathode; NF = not found; values in parentheses indicate the period of extraction in hours.