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Capillary electrophoresis as a metabolomic tool in antioxidant therapy studies

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Abstract

The development of an approach by which two CE methods operating with opposite polarities and orthogonal capillary electrophoretic separation modes (method 1: normal polarity cyclodextrin modified MEKC (CD-MEKC) and method 2: reversed polarity CZE) for the sequential application to urinary samples from a type I diabetes metabolomics investigation is discussed. During method development, problematic MEKC profile drift issues arising from the high glucose content of the diabetic animal urine samples required some electrolyte modifications involving the use of hexafluoroisopropanol (HFIP) to circumvent the drift. Data derived from both methods were subsequently subjected to alignment, normalization and multi-dimensional scaling (MDS) procedures. In such a way, classification of samples derived from control and diabetic animals receiving a placebo from those receiving an antioxidant nutraceutical, was successfully demonstrated. Such a strategy is a cost effective and comprehensive metabolomics tool useful for describing UV absorbing metabolite disease-related changes in nutra/pharma-ceutical studies.

Keywords: Diabetes; Antioxidants; Metabolic fingerprinting; Data alignment; Urine

1. Introduction

Metabolic fingerprinting is a complex matrix profiling strategy widely adopted by many researchers and which can be applied to a variety of sample matrices ranging from biofluids (e.g. tissue extracts, urine, plasma) to environmental samples (wastewater, soil or process plant effluent) and plant products (e.g. bark, root, leaf) [1]. The variety of metabolomic fingerprinting strategies has increased in recent years as a result of the technological developments with respect to both chromatographic and spectroscopic instrumentation capabilities. These developments have aided researchers when measuring the metabolome in overcoming major hurdles of chemical complexity and heterogeneity. The technology adopted in metabolomics studies must be capable of delivering efficient, reproducible and quantitative high resolution data on small sample volumes which are (ideally) subjected to minimal or no sample preparation steps. Example techniques have included nuclear magnetic resonance spectroscopy (NMR), high-pressure liquid chromatography–Mass spectrometry (HPLC–MS), gas chromatography mass spectrometry (GC–MS) and capillary electrophoresis hyphenated to either ultraviolet or mass spectrometry detection (CE-UV/MS). The latter and probably 'youngest' member of the metabolomics analytical toolbox—CE is proving a truly useful addition, especially given its relatively high throughput potential and low sample volume requirement. CE has the capability of enabling highly efficient and rapid separations of chemically diverse mixtures of analytes present in minute sample volumes, with minimum or indeed, no sample preparation—all factors of paramount importance in the quest for comprehensive and cost-effective metabolomics measurement tools.

CE is a strong candidate for system-level studies and is considered capable of 'tackling' the challenges surrounding generation of qualitative and quantitative data. Indeed, the applicability of CE to systems-based analytical data at the genomic, transcriptomic, proteomic, and metabolomic levels has been

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recently reviewed [2]. Nevertheless, CE applications in the metabolomics arena are still relatively scarce.

Another significant benefit of CE for metabolomics studies, which has been largely ignored in the published literature to date, includes the ability to provide a response for virtually all analytes in a sample (which is of course limited by the detection system) via employment of multiple separation modes on a single sample. For example, it is facile to combine data derived from a micellar electrokinetic chromatographic (MEKC) system separation with normal polarity [3], with a second and different CZE separation system employing reverse polarity method [4], to produce an extended representation of the sample. A second advantage is the ability to solve complex sample matrix effects through simple modifications of the electrolyte system. In this paper, for the first time employing CE-UV, we show how both of these advantages can be applied in a metabolomic fingerprinting study for a type I diabetes investigation.

The classical animal model for type I diabetes investigations is the streptozotocin (STZ) rat. STZ causes oxidative damage and a consequence of this is the death of pancreatic islet β cells. As type I diabetes progresses, markers of oxidative stress increase, and it is generally accepted that the majority of complications associated with type I diabetes, such as cardiovascular disease, nephropathy or cataracts, are related to these increases [5]. Recently we have been working to characterise the STZ rat model by developing suitable analytical methodology to profile the metabolites in biofluids and tissue extracts. The ultimate aim of the project will be to make available an acute model of short-term response for evaluation of *in vivo* activity of naturally derived antioxidant compounds. The antioxidant compounds we are particularly interested in are components suitable for use as dietary components.

In order to accurately follow the 'therapy' for a chronic disease such as type I diabetes, normalization of a targeted aspect of the metabolism must occur (without disruption of other metabolic pathway regulation). Furthermore, it is increasingly recognized that assessment of limited biomarker compounds to monitor therapy efficacy is fundamentally flawed and that more comprehensive snapshots of multiple metabolites must be taken. Such an approach is commonly employed in metabolomics investigations.

The aim of the work described herewith was to explore the utility of capillary electrophoresis as a facile and versatile metabolomics tool for rapid urinary UV-chromophore containing metabolite characterization of STZ rat model response to antioxidant treatment.

2. Experimental

The guidelines for metabolomics standards initiative (MSI) [6] have been followed for the experimental design description.

2.1. Animals and samples

Four groups of animals were employed for the assay and comprised of:

(a) a diabetic group receiving a placebo vehicle (200 mg triolein and 20 mg tween in 1 mL saline solution),
(b) a diabetic group receiving a placebo vehicle plus antioxidants,
(c) a non-diabetic group receiving a placebo vehicle substance,
(d) a non-diabetic group receiving a placebo vehicle plus antioxidants.

Rats (Sprague–Dawley strain from our animal quarters at San Pablo-CEU University) that received an intraperitoneal dose (50 mg/kg) of streptozotocin (STZ) and showed glucose levels in blood over 200 mg/dL after 4 days were classified as belonging to the diabetic group. Fourteen days post-STZ administration animals were sacrificed and both tissues and plasma stored at −80 °C for further determinations.

At 72, 64, 48, 40 and 24 h before sacrifice, rats received (by gavage) a dose of either an antioxidant mixture of vitamins C (264 mg) and E (30 mg) dispersed in 1 mL placebo vehicle or only the placebo vehicle. Throughout the experiments the animals were kept in appropriate conditions in the animal quarters of our University. The male rats, with 12 ± 2 weeks of age, were housed in groups of three in a room maintained at 22 ± 2 °C, 55 ± 10% humidity with 12 h light and dark cycles. The rats were fed with a standard diet (Harlan Global Diet 2014, Harlan Interfauna Iberica, Madrid, Spain) *ad libitum*. They also had free access to tap water. All studies were performed after achieving the necessary approval from the Ethical Committee of the University San Pablo CEU.

Urine was collected during 24 h before sacrifice with animals housed in metabolic cages. Tubes with 3 M HCl (50 μL control samples and 100 μL diabetic samples) were kept for 12 h at room temperature and pooled with the second 12 h fraction. Finally urine samples were aliquoted in small tubes and frozen at −80 °C until the analysis by CE.

As a means by which the increased glomerular filtration rate characteristic of type I diabetes [7] could be accounted for, urine from control animals was firstly diluted 1:10 (v/v) with water and after that both samples were treated equally.

Filtered urine was added to BGE (1) (prepared as explained below) in a ratio 90:10 (urine: BGE (1), v/v) and injected directly onto the CE capillary. All samples were initially run with positive polarity and then with negative polarity, using the same sample vials.

2.2. Chemicals

Sodium tetraborate decahydrate and sodium dodecyl sulphate (SDS), methanol and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma (St. Louis, MO, USA), sulphated β-cyclodextrin (analytical grade, Sβ_CD) from Aldrich (Steinheim, Germany), sodium hydroxide from Panreac (Barcelona, Spain) and hydrochloric acid from Merck (Darmstadt, Germany). Reverse osmosed deionised water (Milli-Q Synthesis from Millipore, Bedford, MA, USA) was used for standard solution and electrolyte preparations. Standards used for biomarker identification were obtained from Sigma, except allantoin, AMPc, 2-OH-butyric acid, 3-OH-butyric acid, glutaric acid,

guanine, *n*-methylhistidine and serine from Fluka (Buchs, Swiss), creatine and dihydroxyacetone from Aldrich, tyrosine from Merck (Darmstadt, Germany), sodium oxalate from Panreac (Barcelona, Spain) and acetic acid from Prolabo (Fontenay, France).

2.3. Instrumentation

CE experiments were carried out on a P/ACE MDQ system (Beckman System, Palo Alto, CA, USA) equipped with diode array UV-absorbance detection (190–600 nm), a temperature-controlled (liquid cooled) capillary compartment and an autosampler. Electrophoretic data were acquired and analysed with 32 Karat software (P/ACE MDQ instrument).

CD-MEKC separations achieved with normal polarity (using BGE (1)) were performed in a 60 cm (total, length), 50 cm (effective length) × 75 μm (internal diameter) fused silica capillary (Composite Metal Services, Hallow, Worcester, UK). The conditioning procedure for a fresh capillary comprised of a 15 min pressure assisted flush (20 psi) with 0.1 M NaOH, followed by a 10 min deionised water flush (at 20 psi) and then a BGE (1) flush for 10 min (20 psi). Between analyses, the flushing procedure was a 5 min rinse with 0.1 M HCl (20 psi) and a 5 min rinse with deionised water (20 psi) followed by a 5 min rinse (20 psi) with BGE (1). BGE (1) comprised 25 mM sodium tetraborate decahydrate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin. The pH was adjusted to pH 9.50 (apparent) with 2 M NaOH (after addition of SDS and cyclodextrin). Buffer solutions were filtered through a 0.2 μm filter before use. During the optimization, either methanol (2.0, 5.0, 10.0%, v/v/v) or 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (1.5–2.5%, v/v in 0.25% increments, 2.5–5.0%, v/v in 0.5% increments and 10%, v/v) was added. The final composition employed was 2.25% (v/v) HFIP. The separation capillaries were maintained at 20 °C, with a 20 kV applied voltage (the current observed was 80 μA) and 10 s hydrodynamic injection. The UV-signal for all analyses was recorded at 200 nm with a 4 Hz data collection frequency.

CZE separations achieved with reversed polarity were performed using a 57 cm (total length), 50 cm (effective length) × 50 μm (internal diameter) polyacrylamide (PAG)-coated capillary (Beckman Coulter, Buckinghamshire, England). On first use, the conditioning procedure comprised a 1 min pressure assisted flush of 0.1 M HCl (20 psi) then a 10 min pressure flush of BGE (2) (20 psi), followed by a 10 min electrokinetic flush of BGE (2) (0.5 kV/cm) and finally a 10 min pressure flush with BGE (2) (20 psi). Between analyses the rinsing procedure proceeded under pressure for 4 min with BGE (2) (20 psi). All experiments were performed at 20 °C using a separation potential of −25 kV. Samples were injected at the cathode, with 0.5 psi (3447 Pa) pressure applied for 20 s. Resolved sample components were detected at the anode. BGE (2) was prepared with 0.2 M phosphoric acid, adjusted to pH 6.10 with NaOH, and 10% (v/v) methanol was then added (the current observed under these conditions was 100 μA). Data were collected at a frequency of 4 Hz and 200 nm wavelength.

2.4. Data alignment

Pairwise alignments of the electropherograms were performed using a modification of previously reported procedure [3]. In brief, each CE dataset was exported as an ASCII file and treated as a UV absorbance-time series, comprising (*N*) 5000 data points. In order to correct for base line noise and drift, each electropherogram was first convoluted with a simple ramp function, with absorbances recorded at time-point *n* (A_n) transformed as

$$A'_n = \Sigma k\, A_{n+k}$$

with *k* varied from −2 to 2, and *n* from 3 to $N-2$. The resulting first derivatives of the CE profiles were then mapped as ASCII character strings, with $-0.0002 < A'_i < 0.0002$ coded as '*N*', $A'_i < -0.0002$ as '*M*', and $A'_i > -0.0002$ as '*P*'. All sequences of the form $P_5N_iM_5$, with $0<i<7$, were then re-coded so as to flag the crowns of peaks as '*L*'; the sequence P_5NNM_5, for example, being re-coded as P_5LLM_5.

Pairwise alignments of the electropherogram character strings were performed using the dynamic programming algorithm of Needleman and Wunsch [8] as implemented in the SEQSEE package [9]. Self-matches between the characters '*M*' and '*P*' were each scored 50, and the self-matches between the characters '*N*' and '*L*' were scored as 0 and 100, respectively. Mismatches of '*L*' against any other character were scored 0, those involving '*M*' against '*P*' were scored −50, and those involving '*M*' against '*N*' or '*P*' against '*N*' were penalised by scores of −10. No penalty was incurred for any gap creation, but gap extension was penalised by a score of −5 per character position. All data alignment programs were operated *via* a standard PC.

2.5. Data treatment

After alignment and baseline correction, data were normalised. Afterwards, signals were subtracted point-by-point in sample pairs and results added to obtain a value corresponding to the total difference among signals between every pair of profiles. Differences found in each method (CD-MEKC and CZE) for the same pair of samples were added, with the numerical result giving an indication of how 'different' the profiles were, i.e. a low number indicated a higher degree of profile similarity than a high number. Using these numerical differences a matrix was constructed and subjected to multidimensional scaling (MDS).

3. Results and discussion

The rationale in this study for choosing urinary biofluids was that as the final aim of the study was to evaluate potential antioxidant nutraceuticals for human type I diabetic individuals, and the semi-invasive sampling advantage afforded by urine collection was obviously preferable to plasma/blood/tissue sampling. The animal model is an acute and short-term treatment model which was employed for these studies such that the metabolites varying in response to oxidative stress could be monitored (rather than inevitable diabetes related glucose

changes). Our aim was to exploit the cost-effective CE methodolody to provide a novel (rapid, simple and cost effective) means for following the metabolite changes of diabetic animals undergoing antioxidant treatment versus their corresponding controls. Our evaluations were based on the resolution and UV-detection of urinary metabolites differentially expressed in control, diseased and antioxidant-treated animals. As in many metabolite measurement studies, detection sensitivity was a primary crucial requirement for this work however, a secondary and important necessity was that the methodologies employed should exhibit wide selectivity (i.e. provision of identifiable signals from as many diverse metabolites (in terms of physicochemical properties and molecular structure) as possible). In these investigations, the opportunity to measure each sample with 'orthogonal' methods (which in this case were a CD-MEKC method operating under normal polarity conditions and a CZE method operating with reversed polarity) afforded the possibility to obtain a more holistic or 'comprehensive' metabolomic profile (at least for the UV absorbing components) for each sample.

3.1. Method optimization

The application of the CD-MEKC methodology, previously developed [3,4] and described above, to urine samples derived from control and diabetic rats under the normal polarity electrophoretic separation conditions clearly indicated how peaks corresponding to the same compounds were shifted (with respect to migration time) between the classes (Fig. 1). This variable peak migration issue rendered robust and correct automatic peak profile alignment for metabolite comparisons problematic. It should be noted that the peak shifting issues did not present themselves to the same extent with the CZE method and this was not surprising as this methodology utilized coated capillaries.

A study of potential matrix components responsible for such a peak shifting effect showed that glucose, with a medium value of 689 mg/dL (and obviously present in diabetic animal samples as compared with 177 mg/dL in controls), may be the cause.

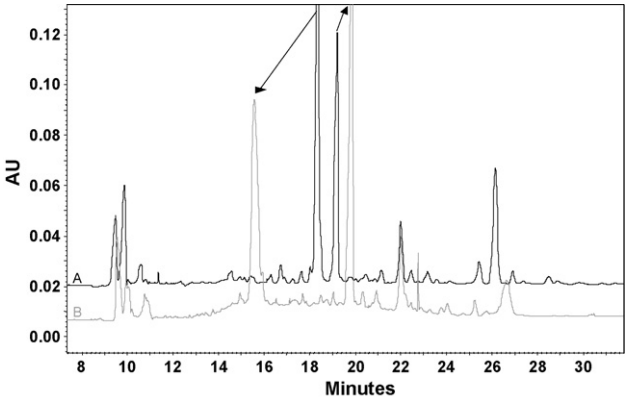

Fig. 1. Comparison of electropherogram profiles for two rat urines (A control and B diabetic) obtained using the normal polarity CD-MEKC method and demonstrating the peak shifting issues observed. CD-MEKC method conditions: BGE: 25 mM sodium tetraborate decahydrate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin, pH 9.50. Detection at 200 nm. For other conditions, see text.

This hypothesis was proven when, after adding a comparatively 'equal' amount of glucose as found in a diabetic urine, to a control urine sample, the profiles were once again 'alignable' and the peak shifting effects had disappeared. However, the addition of glucose to each sample was not a desirable or feasible option, not only in terms of lengthening sample preparation time but also because diabetic animals have variable amounts of glucose (ranging from 398 to 1013 mg/dL in this experiment). Furthermore, an important and compelling argument against the addition of glucose to samples pre-measurement was that as a frequent outcome of the antioxidant treatment should be a decrease in glucose levels, such an adjustment would be implausible. Instead, an alternative solution was investigated wherein the inherent advantages of rapid BGE screening capability for method development with CE were explored. Here, a range of BGE (1) compositions using various modifier additives were electrophoresed with diabetic and control samples with the hypothesis that a modifier introduced into the electrolyte might 'compete' with the glucose for retention sites on either the cyclodextrin or the micelles. In an attempt to test this premise, an initial experiment explored a methanol modifier in proportions up to 10% (v/v) (higher rates produced frequent high current instrumental 'cut-outs') but this modifier showed no improvement in the peak-shifting problem. Next, a solvent with a higher hydrogen bonding 'ability' was employed, namely hexafluoroisopropanol (HFIP). This compound is both water soluble and transparent in UV, but its employment as a modifier in CE electrolytes (at levels up to 30%, v/v) has been to date limited to the system described by Bossi and Righetti [11] for peptide analysis. These authors reported how HFIP acts mostly as a conductivity quencher in the BGE for that system. The actual mechanism for the HFIP contribution to the separation selectivity at this low concentration in the buffer employed is to date unknown, but this application could provide a clue for further studies and applications.

To test the utility of the HFIP, screening experiments with BGE (1) containing HFIP ranging at concentrations of 0.5–5% (0.25% increments) (v/v) were performed. The observations here were that the HFIP clearly had a marked effect in that the profile similarity was increasing. The HFIP 2.25% (v/v) was shown to be the optimum concentration, providing the maximum similarity (evaluated by overlaying the profiles). Fig. 2 shows an example electropherogram of a urine sample derived from control and diabetic animals using these modified BGE (1) conditions (and before any data alignment process). The effect of HFIP, when comparing Figs. 1 and 2 is clear.

Simultaneously, the separation conditions with the reverse polarity CZE mode were optimized regarding capillary length, potential and injection time to provide maximum sensitivity without compromising resolution. Final conditions are described under the experimental section and corresponding electropherograms displayed in Fig. 3.

3.2. Identification

Inherent in most metabolomics strategies for biofluid profiling is the common 'reliance' on multivariate analyses as a means

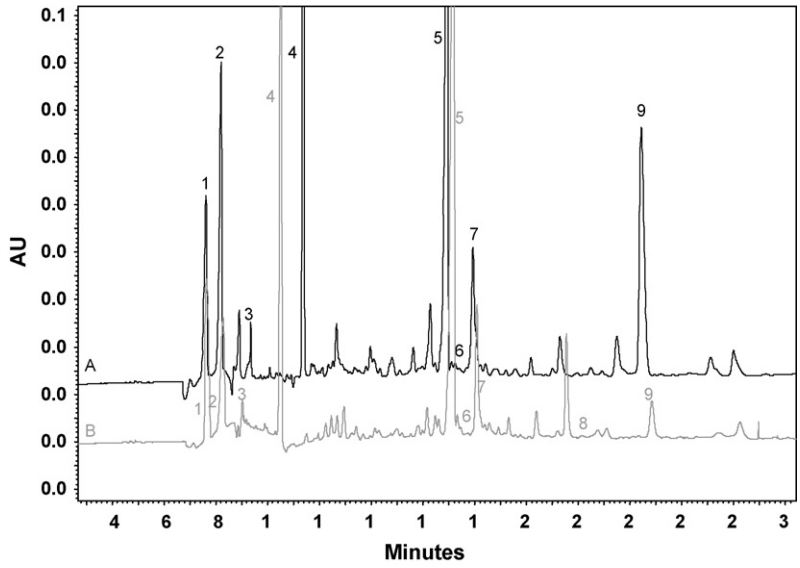

Fig. 2. Comparison of electropherogram profiles for two rat urines (A control and B diabetic) obtained using the normal polarity CD-MEKC method with a 2.25% HFIP BGE modifier to correct for glucose causing peak migration time shifting. Detection at 200 nm. Other conditions identical to Fig. 1. Peak identification: (A) control rat urine:1-urea/2-creatinine/3-uracil/4-allantoin/5-hippuric/6-phenyllactic/7-uric/9-benzoic acids. (B) diabetic rat urine: 1-urea/2-creatinine/3-uracil/4-allantoin/5-hippuric/6-phenyllactic/7-uric/8-3-OH-butyric/9-benzoic acids.

to directly compare large numbers of samples with complex profiles. This of course circumvents the necessity to assign identities to a multitude of signals. Nevertheless, once differences between patterns have been established, the obvious focus is identification of as many peaks in the profile as possible in order to obtain biochemical insights into the animal's evolution under treatment. As previously inferred, this is possibly the weakest point in CE when coupled with a UV detector, because identification must be done via a trial by error assay with pure standards of compounds expected in the profile. The assignment is performed by comparison of migration times and spectra and by spiking the sample with the pure standards. The spiking technique, due to the high separation efficiency in CE provides a good approach, while UV spectra comparison is not always possible because of the inherent lack of characteristic UV chromophoric detail. In the work presented here, 54 standards were tested selected on the basis of both previous experiences from the research group and those compounds known to be present in urine with concen-

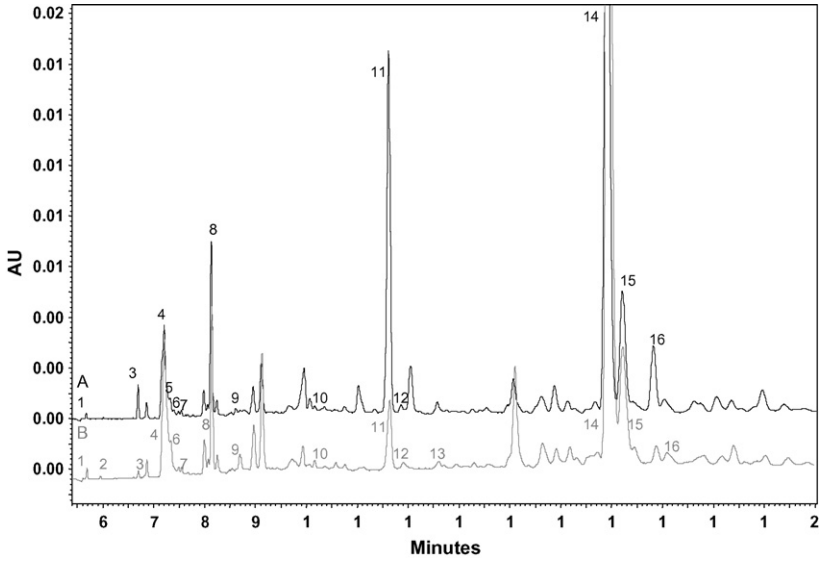

Fig. 3. Comparison of electropherogram profiles for two rat urines (A control and B diabetic) obtained using the reversed polarity CZE method. BGE: 0.2 M phosphoric acid, adjusted to pH 6.10 with NaOH, and 10% (v/v) methanol added. Detection at 200 nm. For other conditions see text. Peak identification: (A) control rat urine: 1-oxalic/3-fumaric/4-ketoglutaric/5-succinic/6-malic/7-isocitric/8-citric/9-acetic/10-acetoacetic/11-benzoic/12-2-ketocaproic/14-hippuric/15-uric/16-p-OH-phenyllactic acids. (B) diabetic rat urine: 1-oxalic/2-formic/3-fumaric/4-ketoglutaric/6-malic/7-isocitric/8-citric/9-acetic/10-acetoacetic/11-benzoic/12-2-ketocaproic/13-3-OH-butyric/14-hippuric/15-uric/16-p-OH-phenyllactic acids.

tration higher than 1 μM. The compounds identified to date for this work are shown in Figs. 2 and 3 for control and diabetic rat urine samples.

3.3. Validation

Although in most metabolomics studies present in the scientific literature the analytical methodologies employed are not generally employed for accurate metabolite quantification, the potential of the system presented here for quantification was explored. In order to do this, several compounds were selected from each method which had the following characteristics:

1. a range of chemical structures (and associated diverse physicochemical properties),
2. existed in urine over a wide range of concentration levels and
3. migration times across the total migration time window (for each method).

Compounds fitting these selection criteria were, for the normal polarity CD-MEKC method: urea, creatinine, allantoin, hippuric acid and uric acid; and for the reversed polarity CZE method: oxalic, citric, benzoic and hippuric acids.

Additionally, the method was validated separately for urines obtained from control and diabetic animals, in order to detect any possible influence of glucose in the signal. Tables 1 and 2 display this data (for the normal polarity CD-MEKC and reversed polarity CZE methods). In the first attempt, samples were prepared separately for each method and the BGE (1) was only added to samples for the normal polarity CD-MEKC method. A simultaneous decrease in hippurate parallel with an increase in benzoate (data not shown) occurred in the reversed polarity CZE method during pre-validation assays and disappeared when the samples were buffered; therefore, that step was included in the sample treatment. In summary the methods were individually proven to be suitable for their intended purpose. They can be considered as linear, accurate and precise with all data falling within the normal acceptance criteria for CE.

3.4. Data alignment and treatment

Once the glucose-causing peak shifting issue had been successfully overcome, initial attempts to use the electropherograms directly for comparisons proved unsuccessful. This failure to discriminate between control and diabetic animal samples was precluded by a minor, but nonetheless significant, peak migration shifting, occurring randomly across electropherograms derived from both control and diabetic urines (and so obviously not attributable to specific urinary components). It is important to note also that while this effect was displayed by both methods, in relative terms the effect was minor for the CZE reversed polarity method (which was unsurprising as this method employed coated capillaries). After performing baseline correction and data alignment procedures however, the situation was dramatically improved. While the pairwise alignment procedure can be tedious, it does permit the attainment of a high degree of profile alignment even for complex electropherograms containing a plethora of major and minor peaks.

Another pre-processing procedure which was deemed necessary before metabolite comparisons could be performed was concentration normalization. Urine samples derived in studies such as these all have different volumes and dilutions and so normalization in this manner is usually necessary. For normalization here, two approaches were tested: (1) division of all signals by the creatinine peak area (which is facile because, as explained later, this compound appears at the initial part of the profile for the positive polarity separation. (2) Expression of each raw data point as the initial value minus the minimum value of signal in the profile divided by the range (difference between the maximum and the minimum values of signal in the profile).

Both approaches provided equivalent results when normalizing the control samples, however, when one control and one diabetic sample profile were compared, results were different, which could be misleading in the process of making conclusions regarding relative increases or decreases of specific compounds between different samples. This effect was previously pointed out by Craig et al. [12] with electronically generated NMR data. The selection of the normalizing procedure was therefore based on prior sample knowledge, as kidney pathology is frequent in diabetes and can even lead to renal failure [13] with the consequent alteration in creatinine excretion. Therefore, normalization and not creatinine correction was chosen. The differences between the profiles, firstly overlaid as they are produced (i.e. no pre-processing) are shown in Fig. 4A and after alignment and normalization for two controls are shown in Fig. 4B. There can be observed that, when subtracting both profiles point by point, the resulting differences will be minimal. Fig. 5 contains the non-treated (A) and treated (B) profiles of a control and a diabetic animal, with peaks clearly overlaid, but noticeable differences in the corresponding heights, that will drive to a bigger value in the MDS matrix.

The final procedure adopted incorporated after the pairwise alignment a normalization step to the 0–1 range followed by multi-dimensional scaling (MDS) analysis, which was a relatively facile and rapid procedure once the pairwise distances and corresponding matrix had been calculated. MDS also permitted a combination of the two matrices corresponding to each separation mode which provided a more holistic snapshot of information about the behaviour of large range of metabolites from a single sample. In general, the goal of a MDS analysis is to detect meaningful underlying dimensions that allow the researcher to explain observed similarities or dissimilarities (distances) between the investigated objects. Essentially, MDS attempts to arrange "objects" in a space with a particular number of dimensions (two-dimensional in this case) so as to reproduce the observed distances. As a result, we can "explain" the distances in terms of underlying dimensions; in our case, we could explain the distances in terms of the control/diabetic state and placebo/antioxidant treatment. MDS is not so much an exact procedure as rather a way to "rearrange" objects in an efficient manner, so as to arrive at a configuration that best approximates the observed distances. It uses a function minimization algorithm that evaluates different configurations with the goal of

Table 1
Validation data for selected compounds with the normal polarity CD-MEKC method (CU: control urine; DU: diabetic rat urine)

Standards			Urea	Creatinine	Allantoin	Hippuric	Uric
	Range (mM)		37.40–112.19	0.45–1.35	1.50–4.49	0.60–1.81	0.10–0.30
Linearity	Standards	Intercept ± C.L.	32895.4 ± 20379.2	40010.6 ± 41619.7	259342.8 ± 48352.6	253571.8 ± 78727.6	35071.6 ± 21360.5
		Slope ± C.L.	3027.9 ± 184.9	599474.0 ± 31436.8	427502.6 ± 10972.3	1486551.1 ± 44236.2	2175920.6 ± 72430.9
		r	0.993	0.995	0.999	0.998	0.998
Accuracy	Standards	Recovery (%)	99.5	99.7	100.0	100.1	100.1
		RSD (%)	8.0	6.6	2.2	2.4	2.7
	CU sample	Recovery (%)	100.1	99.3	107.3	103.9	103.8
		RSD (%)	3.8	2.8	5.1	3.6	2.8
	DU sample	Recovery (%)	100.7	101.8	102.7	102.1	100.8
		RSD (%)	1.3	2.8	4.4	4.5	4.3
Standards, precision (C)	Intra-assay	*n*	10	10	10	10	10
		RSD (%)	1.7	2.1	3.0	3.1	1.7
	Intermediate	*n*	20	20	20	20	20
		RSD (%)	2.0	2.2	2.9	2.8	1.3
Control sample, precision for concentrations	Intra-assay	*n*	10	10	10	10	10
		RSD (%)	4.1	2.0	4.3	1.5	2.8
	Intermediate	*n*	20	20	20	20	20
		RSD (%)	3.4	1.5	4.5	3.4	2.6
Diabetic sample, precision for concentrations	Intra-assay	*n*	10	10	10	10	10
		RSD (%)	1.6	2.0	2.4	3.0	3.0
	Intermediate	*n*	20	20	20	20	20
		RSD (%)	2.2	2.2	1.9	2.6	4.5
Standards, precision for migration time	Intra-assay	*n*	10	10	10	10	10
		RSD (%)	1.3	1.9	1.3	1.5	1.6
	Intermediate	*n*	20	20	20	20	20
		RSD (%)	1.1	1.5	1.6	2.0	2.1
Control sample, precision for migration time	Intra-assay	*n*	10	10	10	10	10
		RSD (%)	0.7	0.8	1.4	1.4	1.3
	Intermediate	*n*	20	20	20	20	20
		RSD (%)	0.7	0.7	1.5	1.4	1.5
Diabetic sample, precision for migration time	Intra-assay	*n*	10	10	10	10	10
		RSD (%)	1.0	0.9	1.6	2.3	2.4
	Intermediate	*n*	20	20	20	20	20
		RSD (%)	1.2	1.0	2.5	2,7	3.3

Table 2
Validation data for selected compounds with the reversed polarity CZE method (CU: control urine; DU: diabetic rat urine)

Standards			Oxalic	Citric	Benzoic	Hippuric
	Range (mM)		0.04–0.12	1.20–3.60	0.05–0.15	0.25–0.75
Linearity	Standards	Intercept ± C.L.	30.10 ± 75.45	586.54 ± 75.46	604.30 ± 468.6	6775.9 ± 6896.6
		Slope ± C.L.	24543.7 ± 900.24	13369.1 ± 2558.0	401119.4 ± 3139.2	665808.8 ± 9265.6
		r	0.999	0.999	0.999	0.999
Accuracy	Standards	Recovery (%)	100.1	100.0	100.0	100.0
		R.S.D. (%)	1.1	0.3	0.6	1.1
	Sample CU	Recovery (%)	99.8	100.7	98.1	96.2
		R.S.D. (%)	1.3	2.2	1.5	2.9
	Sample DU	Recovery (%)	102.3	103.9	107.4	98.0
		R.S.D. (%)	1.6	2.0	5.1	2.1
Standards, precision concentrations	Intra-assay	*n*	10	10	10	10
		R.S.D. (%)	3.8	1.8	2.7	1.3
	Intermediate	*n*	20	20	20	20
		R.S.D. (%)	3.4	2.0	2.6	1.2
Control sample, precision concentrations	Intra-assay	*n*	10	10	10	10
		R.S.D. (%)	4.1	3.1	3.0	3.5
	Intermediate	*n*	20	20	20	20
		R.S.D. (%)	3.9	3.6	3.1	3.7
Diabetic sample, precision concentrations	Intra-assay	*n*	10	10	10	10
		R.S.D. (%)	2.6	2.9	7.1	1.1
	Intermediate	*n*	20	20	20	20
		R.S.D. (%)	5.0	5.6	6.7	5.1
Standards, precision migration time	Intra-assay	*n*	10	10	10	10
		R.S.D. (%)	0.5	0.5	0.4	0.4
	Intermediate	*n*	20	20	20	20
		R.S.D. (%)	0.5	0.5	0.4	0.4
Control sample, precision migration time	Intra-assay	*n*	10	10	10	10
		R.S.D. (%)	0.5	0.5	0.4	0.4
	Intermediate	*n*	20	20	20	20
		R.S.D. (%)	0.4	0.4	0.3	0.3
Diabetic sample	Intra-assay	*n*	10	10	10	10
		R.S.D. (%)	0.3	0.4	0.3	0.3
Precision migration time	Intermediate	*n*	20	20	20	20
		R.S.D. (%)	0.5	0.5	0.3	0.3

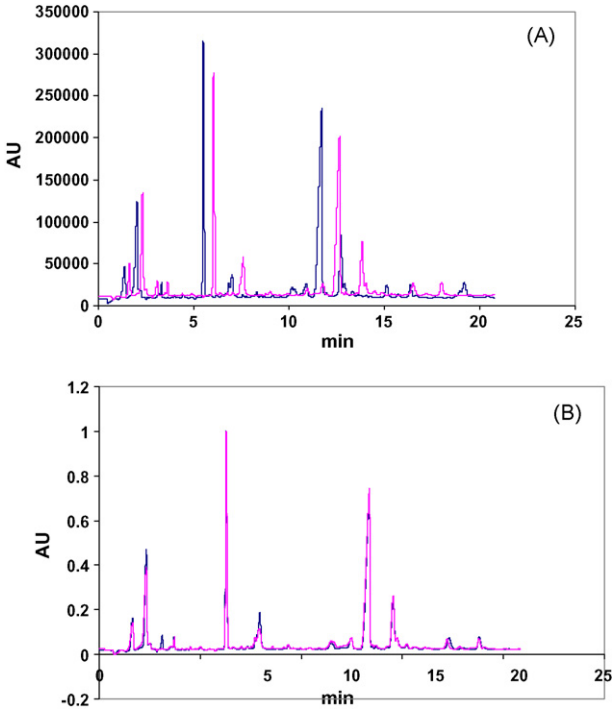

Fig. 4. Comparison of electropherogram profiles for control rat urines obtained using the normal polarity CD-MEKC method (with HFIP modifier—conditions as for Fig. 2) and demonstrating how profile is before (A) and after (B) the alignment.

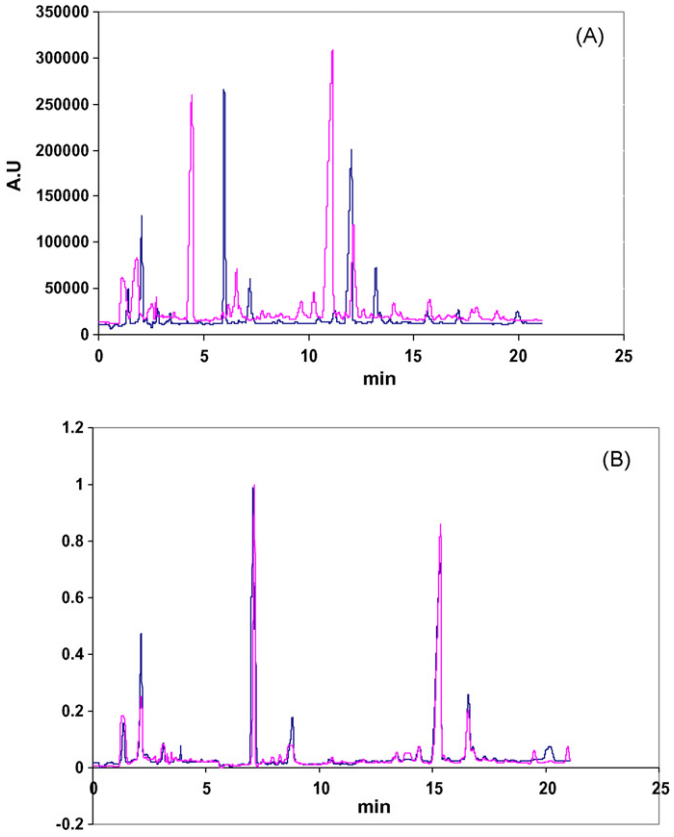

Fig. 5. Comparison of electropherogram profiles for control and diabetic rat urines obtained using the normal polarity CD-MEKC method (with HFIP modifier—conditions as for Fig. 2) and demonstrating how profile is before (A) and after (B) the alignment.

maximizing the goodness-of-fit (or minimizing "lack of fit") [10]. Statistical analyses were performed using Statistica 7.1 (Statsoft Inc.).

In Figs. 6–8 each dot represents an animal; labels for their identification have been kept so correlations could be established with their individual metabolic parameters if necessary.

Using this approach therefore, whereby data was aligned, normalised and subjected to MDS analyses, the results obtained for the normal polarity CD-MEKC method (corresponding mainly to cationic and neutral compounds), are shown in Fig. 6. Here samples are clearly classified in dimension 1 with respect to their status as control or diabetic (this of course not being the objective, as it is more sensible, cheaper and facile to measure glucose). Additionally, antioxidant treated animal data tends to occupy the positive region of dimension 2 in Fig. 6, but here, classification is less pronounced than the control versus diabetic status in dimension 1. Similarly, results obtained for the reverse polarity CZE method, corresponding mainly to anionic compounds, are shown in Fig. 7. Here, it can be observed that the classification criteria are quite different (which serves to support the complementarity of, and rationale for, the two methods). In fact, this analysis shows how the CZE data alone renders sample classification difficult, although diabetic animal data does tend to occupy the right upper region of Fig. 7 (dimension 2), with treated animals clustering in closer proximity to the centre of the classifying space. Finally Fig. 8 shows the results of applying MDS to the data from both methods combined. Here, it can be observed that once again, a spatial separation between control and diabetic samples is clear in dimension 1, however this is no longer the case for dimension 2 which fails to spatially separate the group of antioxidant treated diabetic animals (positive axis space) from antioxidant treated controls (negative axis space). Moreover, urine samples from antioxi-

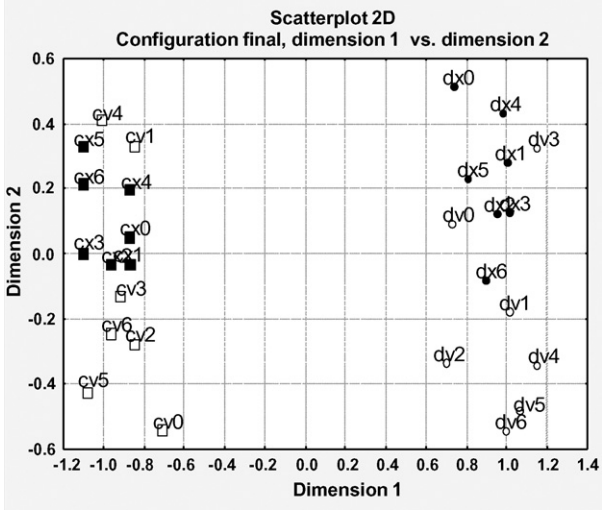

Fig. 6. MDS data derived using electropherograms obtained from the normal polarity CD-MEKC method (as in Fig. 4): (□) control (CV); (●) control treated with antioxidants (CX); (◯) diabetic (DV); (■) diabetic treated with antioxidants (DX). Numbers identified different animals in the group.

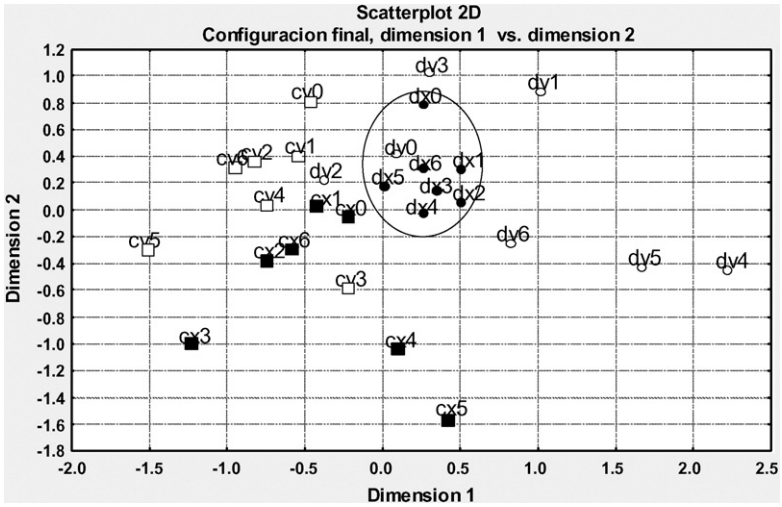

Fig. 7. MDS data derived using electropherograms obtained with the reversed polarity CZE method (conditions as in Fig. 3): (□) control; (●) control treated with antioxidants: (◯) diabetic; (■) diabetic treated with antioxidants.

dant treated diabetic animals cluster in a space approaching that of their respective untreated controls. This fact might be supportive of the hypothesis that a real metabolic improvement in the animal status has occurred after antioxidant treatment. Nevertheless, spatial separation is not absolute as two data-points representing diabetic non-treated animals cluster in the same group (but this kind of problem is probably unavoidable due to the high metabolic dispersion in any group of animals despite the rigorous control over variables). Many parameters related with biochemical and oxidative stress and/or general status of these animals have been measured in our laboratory (data to be published). After looking closely at the characteristics of the two diabetic animals in close proximity to the antioxidant treated group, it was evident that both of these animals fell outside the average parameters for the group. DV0 rat was in fact the diabetic animal with the lowest glucose value in the group (398 mg/dL vs. 689 mg/dL, mean value in the group). We have observed a trend wherein a decrease in glucose occurs with antioxidant treatment and probably for that reason, the animal with the lowest glucose in the diabetic group is closer to the treated animals. Moreover, DV2 rat was unusual with very high glucose and ketone bodies, which made the diabetes status unquestionable, but on the other hand this animal exhibited low triglycerides (66 mg/dL vs. 393 mg/dL, medium value in the group), lactic acid (1.93 mM vs. 2.75 mM medium value in the group), and an abnormal amount of lumbar adipose tissue (3.664 g vs. 0.878 g, medium value in the group). These parameters by themselves do not justify the change in the metabolic profile getting closer to the controls, but they could point to a metabolic adaptation related to the energetic metabolism, that can be detected with this methodology.

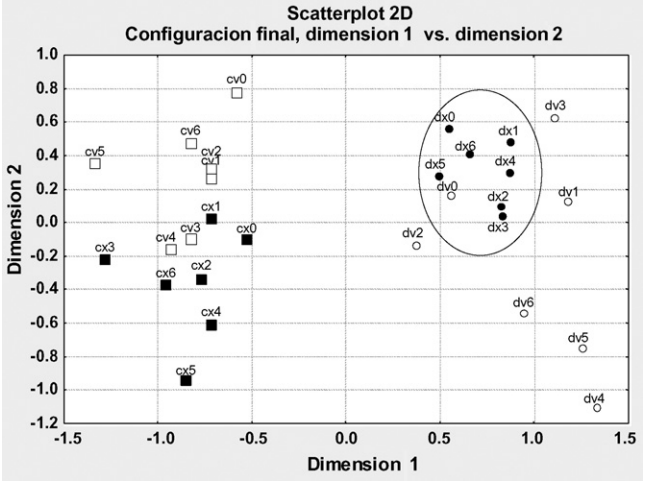

Fig. 8. MDS data derived using summed electropherograms from both the normal polarity (CD-MEKC) and reversed polarity (CZE) methods (conditions as in Figs. 4 and 3, respectively): (□) control; (●) control treated with antioxidants; (◯) diabetic; (■) diabetic treated with antioxidants.

Regarding the quality of the model, the distortion of the correlation of distances between points on the MDS map and the matrix input by the user is measured by a *stress* function. Stress may be caused either by insufficient dimensionality, or by random measurement error. The stress values for these matrices were around 0.09. As a general rule, values under 0.1 are excellent and values over 0.15 are unacceptable. Therefore, the quality of the model was considered acceptable.

4. Conclusions

Capillary electrophoresis with UV detection has been successfully employed for the first time, in a comprehensive metabolomic profiling approach. The strategy developed and validated combines two methods operating in opposite polarities and with coated and uncoated capillaries and exploiting the separation mechanisms from two CE separation modes—namely CD-MEKC (with normal polarity) and CZE (with reversed polarity). The approach has been applied to the analyses of urinary samples derived from control and STZ rats which were treated with either a placebo or antioxidant nutraceutical. Various hurdles were overcome during the method development process including the significant problem of electrophoretic pro-

file drifting for the CD-MEKC method which was attributable to glucose in the diabetic samples. This particular problem was overcome in a novel way by employing HFIP a BGE modifier. Once the methods were developed and validated, a data analysis strategy for classification was developed which comprised of alignment, normalization and a MDS procedure. These analytical and chemometric tools in combination allowed for a classification of controls and diabetic animals from antioxidant-treated animals. Furthermore, the results may be indicating that the antioxidant employed produces a positive response in that the diabetic animal metabolite profile exhibited signs of becoming more akin to that of a profile produced by a control animal.

Such a tool is both facile and cost effective for routine clinical screening laboratories, and should be considered as a truly practicable complement to the more widely employed NMR, LC–MS and GC–MS techniques in the analytical toolbox for metabolomics investigations.

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References

[1] W.B. Dunn, D.I. Ellis, Trends Anal. Chem. 24 (2005) 290–303.
[2] C.V. Suresh Babu, E.J. Song, Md. Sheikh, E. Babar, M.H. Wi, Y.S. Yoo, Electrophoresis 27 (2006) 97–110.
[3] C. Guillo, D. Barlow, D. Perrett, M. Hanna-Brown, J. Chromatogr. A 1027 (2004) 203–212.
[4] A. Garcia, C. Barbas, R. Aguilar, M. Castro, Clin. Chem. 44 (1998) 1905–1911.
[5] J.W. Baynes, S.R. Thorpe, Diabetes 48 (1999) 1–9.
[6] Metabolomics Standards Initiative CIMR: In vivo Context. MSI working groups available on http://msi-workgroups.sourceforge.net/.
[7] A. Körner, A.C. Eklöf, G. Celsi, A. Aperia, Diabetes 43 (1994) 629–633.
[8] S.B. Needleman, C.D. Wunsch, J. Mol. Biol. 48 (1970) 443–453.
[9] D.S. Wishart, R.F. Boyko, L. Willard, F.M. Richards, B.D. Sykes, Comput. Appl. Biosci. 10 (1994) 121–132.
[10] http://www.statsoft.com/textbook/stmulsca.html Accessed August 2007.
[11] A. Bossi, P.G. Righetti, J. Chromatogr. A 840 (1999) 117–129.
[12] A. Craig, O. Cloarec, E. Holmes, J.K. Nicholson, J.C. Lindon, Anal. Chem. 78 (2006) 2262–2267.
[13] M. Nair, Br. J. Nurs. 16 (2007) 184–188.