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Metabolomic approach to the nutraceutical effect of rosemary extract plus ω-3 PUFAs in diabetic children with capillary electrophoresis

Claudia Balderas[a,1], Alma Villaseñor[a,1], Antonia García[a], F. Javier Rupérez[a], Elena Ibañez[b], Javier Señorans[c], Julio Guerrero-Fernández[d], Isabel González-Casado[d], Ricardo Gracia-Bouthelier[d], Coral Barbas[a,*]

[a] CEMBIO (Center for Metabolomics and Bioanalysis) Facultad de Farmacia, Universidad San Pablo-CEU, Campus Montepríncipe, Boadilla del Monte, 28668 Madrid, Spain
[b] Instituto de Fermentaciones Industriales, CSIC, Madrid, Spain
[c] Sección Departamental de Ciencias de la Alimentación, Universidad Autónoma de Madrid, Madrid, Spain
[d] Servicio de Endocrinología Infantil, Hospital La Paz, Madrid, Spain

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ABSTRACT

Type 1 diabetes mellitus is a major endocrine disorder, affecting approximately 5% of the world's population. It not only leads to hyperglycaemia but also causes many complications, and numerous studies have demonstrated that oxidative stress contributes to these complications. As a new strategy to improve the oxidative damage in diabetes, interest has grown in the usage of natural antioxidants, even more in the long term. Among them, *Rosmarinus officinalis* (rosemary) has been widely accepted as one of the species with the highest antioxidant activity. In addition, ω-3 polyunsaturated fatty acids were efficient in delaying and decreasing cardiovascular risk factors associated with diabetes.

Type 1 diabetic children and the corresponding controls were enrolled in the assay. The aim was evaluating the effect of a special additive containing rosemary extract, vitamin E and PUFAs added to their standard diet through the meat. In the analytical point of view, a metabolomic approach with CE-UV was used to detect possible differences in urine of diabetic children as compared to controls. After the application of the appropriate multivariate statistical tools, clear differences could be observed between treated and non-treated diabetic children and some of the metabolites associated could be identified. This was specially challenging as most of the clinical biochemical parameters measured by target analysis showed no differences between the groups.

1. Introduction

Serious long-term complications of diabetes include cardiovascular diseases, chronic renal failure, retinal damage [1,2] and most of them have been related to oxidative stress [3,4]. Moreover, they appear even under a good glycemic control [5].

In terms of diabetic's lipidic profile, it has been demonstrated that under insulin therapy it can became normal although some lipoproteic changes may remain thus perpetuating the cardiovascular risks [6].

Therefore, to be able to reduce the long-term effects of both, oxidative stress and lipoproteic change, an antioxidant therapy combined with a diet rich in ω-3 fatty acids has been suggested, that might act modulating different processes associated to diabetes disease [7].

As for the antioxidant therapy, numerous clinical trials have tried to demonstrate the benefits of a diet supplemented, for instance, with α-tocopherol, ascorbic acid or β-carotene in type 1 diabetes mellitus patients (reviewed in [8]). Nevertheless, no conclusive results were obtained in these studies in terms of the therapeutic effect of the diet in the treatment of long-term complications in type 1 diabetes.

In a previous paper we set up the scientific bases of new functional meat products with both a balanced ω-6/ω-3 ratio and a synergic combination of antioxidants such as supercritical rosemary extracts and vitamin E [9] and proved that they showed a lipidic profile closer to fresh salmon than other meat products and an antioxidant activity similar to fruit functional beverages. Thus, these meat products could be good candidates to test the efficacy of a combined diet containing antioxidants and ω-3 PUFAs.

What seems clear is that the metabolic control of glucose and lipids plays a significant role in the prevention of the long-term complications of type 1 diabetes [10] but more information is required to decipher the real effects of an antioxidant therapy as

* Corresponding author. Tel.: +34 91 3724711; fax: +34 91 3724712.
E-mail address: cbarbas@ceu.es (C. Barbas).
[1] Equal work in the manuscript.

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a way to prevent or retard the appearance of symptoms related to oxidative stress in diabetic patients.

In order to accurately follow the 'therapy' for a chronic disease such as type 1 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. Metabolic fingerprinting is a complex matrix profiling strategy widely adopted by many researchers and which can be applied to a variety of sample matrices [11,12]. Metabol(n)omics has gained great prominence in diabetes research within the last few years and has already been applied to understand the metabolism in a range of animal models and, more recently, attempts have been done to process complex metabolic data sets from clinical studies. A recent review summarizes the technologies currently being used in metabol(n)omics, as well as the studies reported related to diabetes [13].

The latter member of the metabolomics analytical toolbox—CE is proving a truly useful addition, especially given its high-throughput potential and low sample volume requirement. Another significant benefit of CE for metabolomics studies includes the ability to measure all type of analytes in a sample (which is only limited by the detection system) *via* employment of multiple separation mechanism modes on a single sample [14–19].

The aim of this study was checking the capabilities of CE-UV to detect differences in urine of diabetic children as compared to controls and, in such case, studying the possible effect of a change in the diet of diabetic children, including designed meat products with an ω-6/ω-3 ratio lower than 4 and a combination of natural antioxidants (supercritical rosemary extracts and vitamin E) at controlled dosage for 1 year.

2. Materials and methods

2.1. Chemicals

Sodium tetraborate decahydrate (STD), β-cyclodextrin sulphated (analytical grade, Sβ-CD) and methanol were purchased from Sigma–Aldrich (Steinheim, Germany), sodium dodecyl sulphate (SDS) and sodium hydroxide from Panreac Química S.A.U. (Barcelona, Spain), and hydrochloric acid from Fluka (Buchs, Switzerland). Standards used for peak identification were obtained from Sigma, except 2-OH-butyric acid, 3-OH-butyric acid, glutaric acid, guanine, L-pyroglutamic acid, DL-serine, 2,3-dihydroxybenzoic acid from Fluka, L-tyrosine from Merck (Darmstadt, Germany), sodium oxalate from Panreac. Reverse osmosed deionised water (Milli-Q Synthesis from Millipore, Bedford, MA, USA) was used for standard solution and electrolyte preparations.

2.2. Study design

33 type 1 diabetic children and 16 control ranging from 6 to 11 years old for boys and girls and including 27 of boys and 22 girls were enrolled in the assay. All parents signed informed-consent forms approved by the ethics committee of La Paz Hospital. This was an exploratory double blind randomized study.

The aim was evaluating the effect of a special extract containing 0.02% rosemary extract, 0.001% vitamin E and 0.3% PUFAs on T1DM young children when it was added to meat products (100 g per day, 3 days a week) as part of their regular diet. The functional meat product was supplied by Grupo Frial S.A. (Tres Cantos, Spain).

Children were divided into three groups: diabetic children with and without the extract and control children with the extract. All of them received the same meat products prepared weekly by a local industry (Grupo Frial S.A.). The diet was administered during 12 months and in each visit the adhesion was assessed. Mean adhesion to diet was around 80% and nobody was under 60%.

2.3. Urine samples

Punctual urine samples were collected at time zero, when the study started and after 12 months. Samples were kept at −80 °C until the analysis. The day of analysis the urine was defrosted and aliquoted for each CE method, vortexed and for CZE method 8 μl of urine were diluted with 32 μl of deionised water, for CD-MEKC method 10 μl urine were mixed with 10 μl BGE1 and 80 μl deionised water, and injected directly into the CE apparatus. All samples were initially run with negative polarity and then with positive polarity, using a separated aliquot for each method.

2.4. Instrumentation

CE experiments were carried out on a P/ACE MDQ system (Beckman Coulter, Fullerton, 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 Karats software (P/ACE MDQ instrument). Separations achieved with normal polarity were performed in a fused silica capillary (75 μm internal diameter), 60 cm total, 50 cm effective length (Beckman Coulter). New capillaries were conditioned for 5 min at 25 °C with water, followed by 0.1 M NaOH for 15 min, 8 min with deionised water and finally BGE(1) for 10 min. Before each analysis, the capillaries were washed with 0.1 M HCl and deionised water for 5 min, and then 5 min with the run buffer. The running buffer (BGE1) comprised 25 mM STD, 75 mM SDS and 1.43% (p/v) β-cyclodextrin sulphated. The pH was adjusted to pH 9.50 with 2 M NaOH. The capillaries were maintained at 20 °C, with 20 kV applied voltage and 10 s hydrodynamic injection (0.5 psi).

Separations achieved with reverse polarity were performed using a capillary (Beckman Coulter) coated with polyacrylamide 60 cm of total length, 50 cm effective length and 50 μm internal diameter. On first use, the capillaries were conditioned by a pressure flush of 0.1 M HCl (2 min) and BGE (10 min). Between runs, the capillaries were flushed under pressure with deionised water (2 min) and BGE (5 min). All experiments were performed at 25 °C using a separation potential of −20 kV. Samples were injected at the cathode, with 0.5 psi of pressure applied for 25 s. Resolved sample components were detected at the anode. Electrophoretic buffer (pH 6.10) was prepared with 0.2 M ortho-phosphoric acid, adjusted to pH with NaOH saturated, and 10% (v/v) methanol HPLC grade was added. (The current observed under these conditions was −86 to −90 μA.) Data were collected at a frequency of 4 Hz. Buffer solutions were filtered through a 0.2 μm nylon filter before use.

2.5. Data alignment and treatment

Baseline correction and multialignments of the electropherograms were performed with an in-house program developed in Matlab® 7.0. For baseline correction the profiles were divided in p zones, in each zone the minimum value was accounted and all the minima were linearly interpolated. Multialignment was performed using the Correlation Optimized Warping (COW) method previously described [20]. For normalization, two approaches were tested: (1) the division of all signals by the creatinine peak (creatinine is often used to correct urine dilutions effects due to its

Table 1
Clinical parameters measured in children by La Paz Hospital at time zero.

Parameter		Glu** mg/dL	HbA1c** %	Total-Chol mg/dL	Chol-HDL mg/dL	TAG mg/dL	U. A.* mg/dL	TP* mg/dL	Creat mg/dL	mAlb mg/dL	Vit-E mg/dL
Patient											
Healthy (n = 16)	Mean	81.1	5.2	179.4	64.4	56.3	3.7	7.2	0.7	4.3	1035.8
	STD	6.2	0.3	34.4	13.3	19.1	0.7	0.4	0.1	3.0	220.8
	RSD (%)	7.7	4.8	19.2	20.6	33.9	18.0	5.6	10.8	69.5	21.3
Diabetic (n = 34)	Mean	172.9	7.6	172.0	63.1	50.0	3.1	6.9	0.7	4.0	1029.7
	STD	80.1	0.9	35.0	13.8	11.0	0.6	0.4	0.1	3.0	221.2
	RSD (%)	46.3	12.0	20.4	21.9	21.9	19.7	5.4	10.1	75.4	21.5

Glu, glucose; HbA1c, glycosylated haemoglobin; Total-Chol, total cholesterol; Chol-HDL, cholesterol HDL; TAG, triglycerides; U.A., uric acid; TP, total proteins; Creat, creatinine; mAlb, microalbumin; Vit. E, vitamin E.
* P value < 0.05.
** P value < 0.01.

excretion is almost constant during the day [21]). (2) Probabilistic quotient normalization. This method is based on the calculation of a most probable dilution factor by looking at the distribution of the quotients of the amplitudes of a test spectrum by those of a reference spectrum. It is demonstrated to be more robust and more accurate than other methods commonly employed [22]. Subsequently data were analysed with SIMCA P+ 12.0.1 (Umetrics, Sweden).

3. Results and discussion

Capillary electrophoresis provides a comprehensive snapshot of multiple metabolites in biological samples especially in urine because all analytes are already dissolved and most of them are easily separated due to its charge; despite its detection system, CE gives a general metabolic response. With different CE modes we can obtain a complete profile of a wide set of compounds charged and neutral using both polarities producing an extended representation [14,17–19,21,23,24]. The two methods operated with opposite polarities and orthogonal capillary electrophoretic separation modes. Method 1: normal polarity cyclodextrin modified micellar electrokinetic chromatography (CD-MEKC), mostly for cations and neutral compounds, and method 2: reversed polarity capillary zone electrophoresis (CZE), mainly for anions. The methodology already proved to be successful with urine from control and diabetic rats [14], but in humans with different diet, physiological, environment and genetic variation are still a challenge.

Several clinical and biochemical parameters (glucose, glycosylated haemoglobin (HbA1c), total cholesterol, HDL-cholesterol, total proteins, triglycerides, creatinine, uric acid, vitamin E and microalbumin) were measured in these children at the beginning of the study and no differences were found except for glucose and HbA1, which were higher in diabetics while uric acid and total proteins were significantly lower (Table 1). Therefore the first challenge was to test the capability of our methodology to discriminate between the two groups, considering that none of these parameters were detected.

Fingerprints were obtained with a relatively inexpensive tool such as CE-UV, without any previous treatment other than filtration. After that, data pre-treatment of electrophoretic profiles (baseline correction, alignment, scaling and normalization) was applied. That step proved to be critical to obtain satisfactory results as CE profiles usually are affected by some type of shift, and different natural degrees of dilution in urine can alter the comparison. The fingerprints obtained with two polarities after alignment are shown in Fig. 1.

Normalization is a pre-processing method which accounts for different dilutions of samples by scaling the spectra to the same virtual overall concentration. Best results, considering sample clustering and quality of the models, were obtained with the probabilistic quotient normalization and that was the procedure used throughout the work.

Finally chemometric tools such as PCA (principal components analysis), PLS-DA (partial least squares discriminant analysis) and OPLS-DA (orthogonal partial least squares discriminant analysis) were applied. Regarding multivariate data analysis, a satisfactory result for pattern recognition was obtained with a supervised analysis such as OPLS-DA using Pareto scaling (most commonly used for metabolomic research), as can be observed in Fig. 2, after deleting from the group several strong outliers, according to the Hotellings T^2 range plot (SIMCA Umetrics).

Fingerprinting ignores the assignment problem presented by the multitude of signals and, instead, uses multivariate analysis to compare sets of profiles and hence the samples from which the profiles were derived. Nevertheless, once differences between patterns have been established, the obvious interest is the identification of as many peaks in the profile as possible in order to obtain

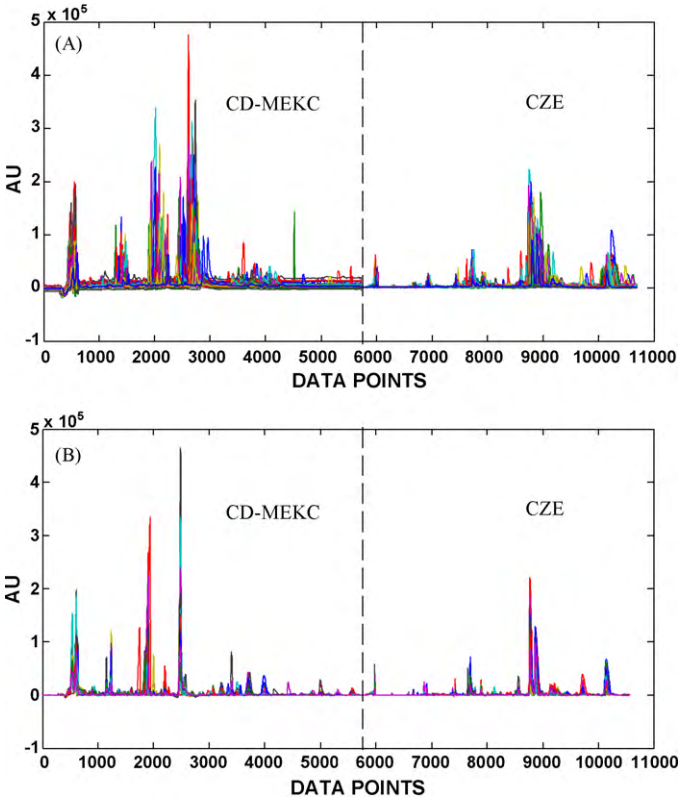

Fig. 1. (A) Raw data with the results coming from both polarities added up (first half normal polarity, second half second polarity) and (B) 98 profiles (CE-UV) after alignment and scaling with COW algorithm after optimizing the slack.

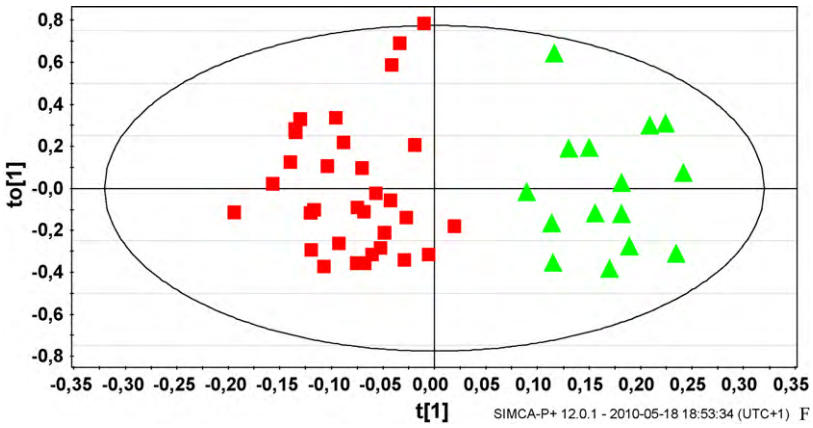

Fig. 2. OPLS-DA data derived using the total profile added of CD-MEKC (normal polarity) and CZE (reverse polarity) methods at zero months. Identification: ▲, healthy; ■, diabetic.

biochemical insight into the pathology condition. This is possibly the weakest point in CE with UV detection, because identification can only be made *via* trial and error, using 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. Fig. 3 displays 26 metabolites using CD-MEKC method (normal polarity) from a total of 71 compounds tested; some of the identified standards gave analytical signal in the same migration time and increased the same peak into the profile. Fig. 4 shows an example of an electropherogram of a urine sample in which 30 compounds were identified by CZE method (reverse polarity) out of 76 standards tested.

After that, the corresponding loading plots were employed to identify those compounds that contributed most to the sample grouping. Among the compounds identified so far, we noticed increased nitrites, citrate, phenyllactate, glutamate, creatinine and urea in diabetic children and lower quantities of glutarate, guanidine, phospho-L-serine, benzoate, urate, and glycerate (Table 2). In addition, there were 14 significant metabolites still unknown and for that reason they have not been included in the table.

In a previous study [25] with Proton Nuclear Magnetic Resonance (^{1}H NMR), profiles of urine from children and adolescents with type 1 diabetes showed highly significant differences for citrate, alanine, lactate, and hippurate, which were all higher in diabetic children than in the unaffected individuals. According to these authors, the consistently higher citrate, alanine, and hippurate might reflect the increased glomerular filtration rate characteristic of type 1 diabetes [26] and/or a modification of the transport mechanisms at the tubular level. The latter may be related either to altered cellular function or to the presence of high glucose concentrations in the tubular lumen. It should be also taken into account

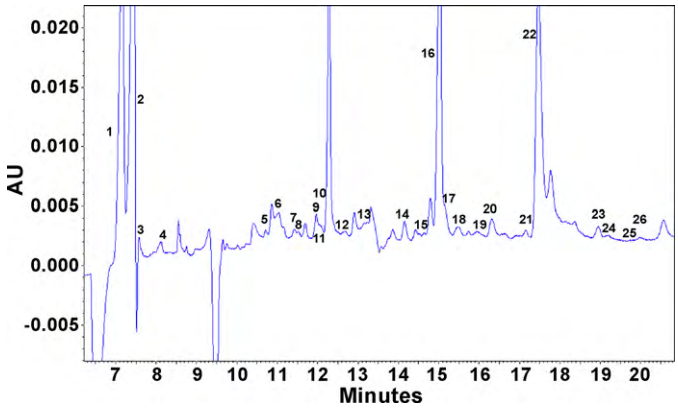

Fig. 3. Electropherogram profile for urinary sample obtained using the normal polarity CD-MEKC method (for conditions see text). Peak identification: 1, urea; 2, creatinine; 3, guanidinoacetate acid; 4, ornithine; 5, histidine; 6, tyrosine, 7, metionine; 8, serine; 9, phenylalanine; 10, 5-methyluridine; 11, 2,5-dihydroxybenzoate/glutamine; 12, tryptophan; 13, asparagine; 14, phenaceturate; 15, uridine; 16, hippurate; 17, β-phenyllactate; 18, guanosine; 19, p-hydroxyphenyllactate; 20, quinaldate; 21, inosine; 22, urate; 23, pyroglutamate; 24, β-3,4-dihydroxyphenylalanine; 25, isovanillate; 26, 3-nitro-L-tyrosine.

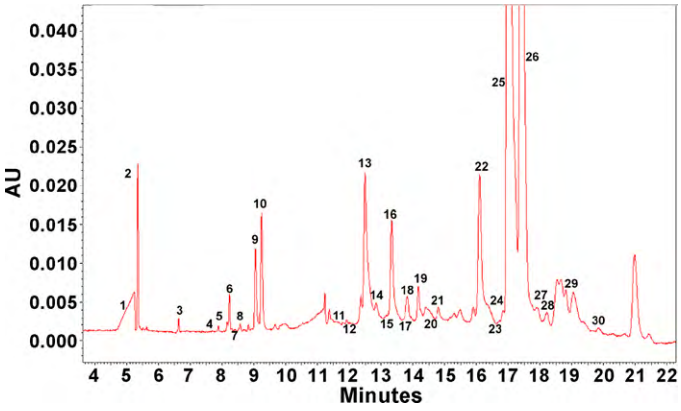

Fig. 4. Electropherogram profile for urinary sample obtained using the reverse polarity CZE method (for conditions see text). Peak identification: 1, chloride; 2, nitrate; 3, oxalate; 4, fumarate; 5, trans-aconitate; 6, 2-ketoglutarate; 7, malate/succinate; 8, isocitrate; 9, glutarate; 10, citrate; 11, acetoacetate; 12, lactate; 13, O-phospho-L-serine; 14, glycerate; 15, 2-hydroxybutyrate; 16, benzoate; 17, 3-hydroxybutyrate; 18, pyroglutamate; 19, 2,5-dihydroxybenzoate/glutamine; 20, 2,3-dihydroxybenzoate; 21, aspartate; 22, β-phenyllactate; 23, quinoline-2-carboxylate; 24, glutamate; 25, hippurate; 26, urate; 27, p-hydroxyphenyllactate; 28, homovanillate; 29, vanillylmandelate/2-aminoadipate; 30, glucuronate.

Table 2
Discriminant metabolites found using OPLS-DA model for type 1 diabetic disease in children before the treatment as compared to controls.

Metabolites	Diabetics
Nitrite	↑
Citrate	↑
Glutarate	↓
Phenyllactate	↑
Glutamate	↑
Creatinine	↑
Guanidinoacetate	↓
Phospho-L-serine	↓
Benzoate	↓
Urea	↑
Urate	↓
Glycerate	↓

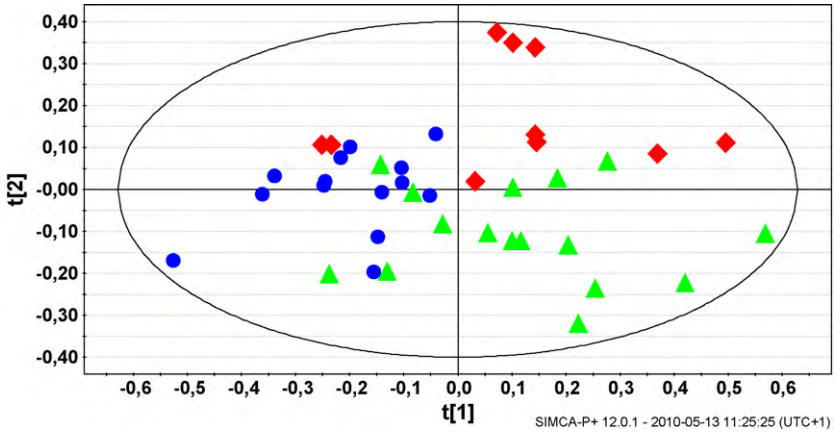

Fig. 5. PLS-DA data derived using the total profile added of CD-MEKC (normal polarity) and CZE (reverse polarity) methods at 12 months. Identification: ▲, healthy children with extract (H); ♦, diabetic children treated with extract (TD) and ●, diabetic children non-treated with the extract (NTD).

that activity of ATP-citrate lyase, a cytosolic enzyme, is enhanced by insulin; therefore accumulation of citrate may occur in absence of insulin and/or insulin resistance.

A different study with Doppler ultrasonography [27] found urinary nitrite and nitrate concentrations significantly increased in children with diabetes compared with control subjects. In particular, prepubertal children with diabetes had significantly higher $NO_2^- + NO_3^-$ serum levels than prepubertal healthy children. The authors concluded that this study demonstrates that in children with diabetes, chronic hyperglycaemia may act through a mechanism that involves increased NO production and/or action and contributes to generating intrarenal hemodynamic abnormalities [28].

Urines obtained after 12-month treatment were submitted to the same procedure and PLS-DA results are shown in Fig. 5. Quality parameters of the model showed a good result for the explained variance ($R^2 = 0.64$), but a poor one for the predicted variance ($Q^2 = -0.16$).

Comparing the three groups, results showed that there is a significant change in metabolism due to the diet. In Fig. 5 it can be appreciated that latent variable 1 seems to be related to the diet while variable 2 is more related to the pathology. For analysing metabolites playing a more significant role, we compared in OPLS-DA each two groups making three different comparisons that are showed in Fig. 6 for the scores plot. In addition we obtained the loadings S-plot where each variable is represented by its contribution in the group (covariance) and its confidence (correlation). The interval of covariance selected was ±0.15 and those variables which resulted significant are summarized in Table 3. In addition, Jack-knife intervals were obtained to check homogeneities, also included in the table.

Guanidinoacetate and creatinine are metabolites related to catabolism of aminoacids (glycine) that is known to be increased in diabetics due to impairment of insulin. Although treatment has not decreased this catabolism, the accumulation of guanidinoac-

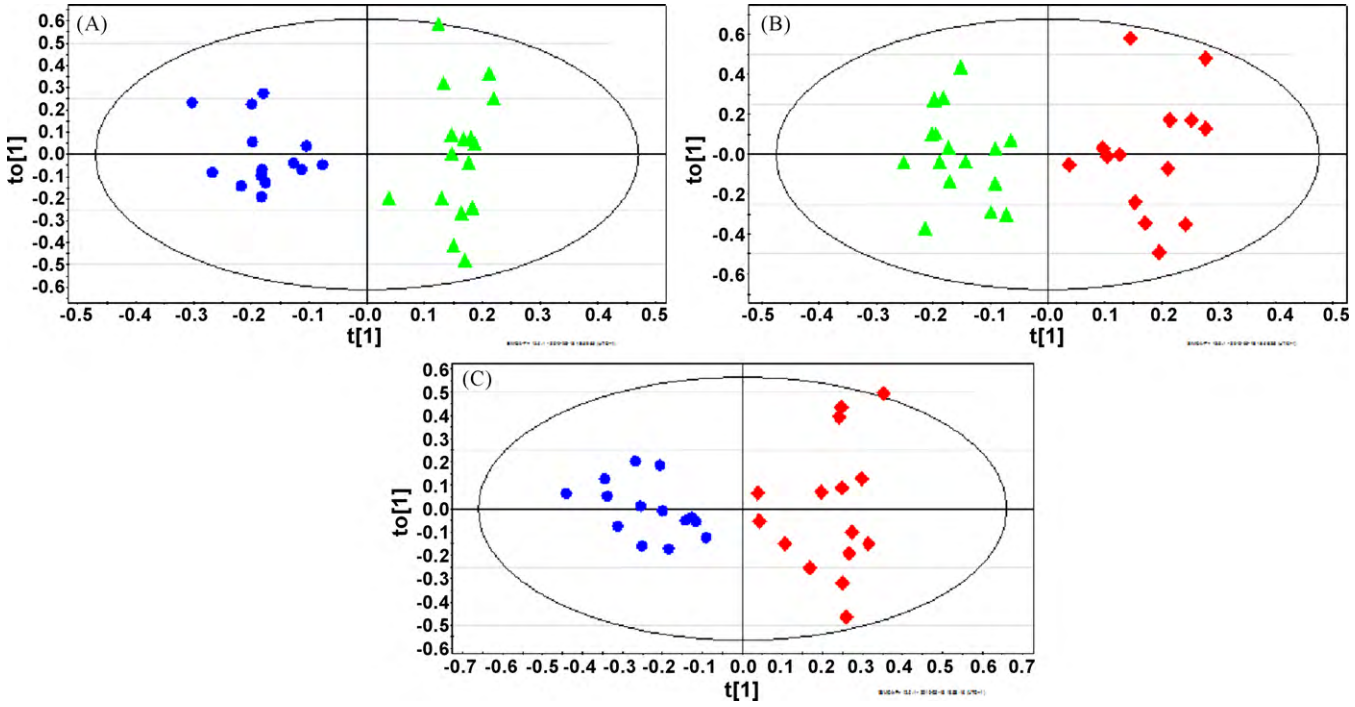

Fig. 6. Different comparisons of OPLS-DA data derived using the total profile obtained by adding CD-MEKC (normal polarity) and CZE (reverse polarity) methods at 12 months. (A) Healthy with extract vs. diabetic children without extract; (B) healthy vs. diabetic children both with extract; (C) diabetic children with and without extract. Identification: ▲, healthy children with extract; ♦, diabetic children with extract and ●, diabetic children without extract.

Table 3
Discriminant metabolites found in OPLS-DA model for type 1 diabetic disease in children after 12 months of receiving the extract. (i) Treated diabetic (TD) vs. healthy children (H); (ii) non-treated diabetic (NTD) vs. healthy children (H) and (iii) non-treated diabetic (NTD) vs. treated diabetic (TD). Homogeneity was expressed with letters: common letter(s) mean no evidence enough for significant differences, being *a* the letter corresponding to the lowest value.

Metabolites	Comparisons			Homogeneity		
	TD/H	NTD/H	NTD/TD	H	NTD	TD
Nitrite	↑	↓	↓	b	a	c
Citrate	↑	↑	↓	a	b	c
Ketoglutarate	↑	↑		a	a	b
Aminoadipate	↑	↓	↓	b	a	c
Phenyllactate	↑			a	ab	b
Glutamate	↑	↑		a	a	b
Creatinine	↑		↓	a	a	b
Guanidinocetate		↑	↑	a	b	a
Phospho-L-serine	↑		↓	a	a	b
Pyroglutamate	↑			a	ab	b
Benzoate		↓	↓	b	a	ab
Urea	↓	↑	↑	b	c	a
Urate		↓	↓	b	a	b
3-Hydroxybutyrate		↑		a	b	ab
p-Hydroxyphenyllactate	↓	↓	↓	c	a	b
Phenaceturate			↓	ab	a	b

11 significant metabolites were unknown and are not assigned in the table.

etate seen in diabetic children without diet was overcome and catabolism could proceed to the last step when they received the supplemented diet. Urea changes must be investigated more in detail: although the reduction in the increased urea excretion characteristic of diabetics should be considered positive, the level of controls was found to be higher than in treated diabetics, and maybe not all the ammonium was excreted as urea. Increase in aminoadipate should be treated with caution as well, because the level of this metabolite of tryptophan, lysine and OH-lysine catabolism when diabetes and diet were both present was higher than in controls. Urate has long been considered the main antioxidant of non-lipidic media, and reductions of it have been attributed to oxidative stress increase [29]. When children took the diet with adequate PUFAs and antioxidants they presented urate levels not different from healthy controls. In addition, 3-OH butyrate in treated diabetic was not different from controls, pointing at a better metabolic control (less ketogenesis). Insulin deficiency with glucagon excess leads to the release of ketone bodies (acetoacetate, 3-OH-butyrate) by the liver and excretion in the urine [30].

It is also noteworthy that some metabolites with aromatic ring, such as phenyllactate and p-OH-phenyllactate, phenaceturate and benzoate have been found to be significantly changed due to diabetes and/or treatment. Their detection is related to the analytical tools used (UV) but they have not been reported previously as altered in this condition, and probably deserve further studies.

Results presented demonstrated the usefulness of CE with UV detection for metabolomics. The use of this inexpensive and fast tool allowed following the evolution of the metabolic profile of diabetic children with a diet modified by the consumption of meat products enriched with antioxidants (supercritical rosemary extract plus vitamin E) plus ω-3 PUFAs compared to diabetic children with no enriched meat products and with healthy children. After the application of the appropriate statistical tools, clear differences could be observed between treated and non-treated diabetic children and some of the metabolites associated could be identified. More in depth analytical and biochemical interpretation of the results is needed to understand the real effect of the diet in the antioxidant status of the diabetics. Nevertheless, this is an encouraging starting point to apply other technologies with higher elucidation capabilities.

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