Evaluation of Ion Exclusion Chromatography as a Method for Determining the Total Carbon Dioxide (TCO_2) Concentration in Equine Plasma

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Abstract: Ion chromatography (IC), utilizing an ion exclusion column coupled to a conductivity detector (CD) *via* a post column reactor, had been evaluated for quantifying the Total Carbon dioxide (TCO_2) level in equine plasma. The concentrations obtained from the analysis of the ASE linearity set correlated with the results obtained when the measurement was done using a Beckman EL-*ISE*® instrument (R^2 = 0.998). Equine blood was analyzed using both instruments and the averaged absolute difference for 30 samples was 0.4 mM. The IC method was considered suitable for use to determine the compliance of 36 mM available CO_2 per liter of plasma concentration threshold stated in the Malayan Racing Association's (MRA) Regulations.

Keywords: Ion exclusion chromatography, total carbon dioxide (TCO_2), horse racing.

1. INTRODUCTION

TCO_2 comprises dissolved CO_2, bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and carbonic acid (H_2CO_3); and "Milkshaking" is the administration of approximately 500 g of sodium bicarbonate in 2 L of water, to a racehorse *via* a nasogastric tube 3 to 5 hours prior to the race [1, 2]. It is considered to have a performance enhancing effect by buffering the naturally occurring build-up of lactic acid produced during vigorous exercise, thus delaying the onset of fatigue and boosting the endurance of the treated horse [3]. Administration of the bicarbonate salts was shown [2, 4] to increase the plasma TCO_2 level, with several population surveys [5 - 7] being conducted to establish the normal range for thoroughbred racehorses, which provided the basis for the introduction of the international threshold of 36 mM. It was statistically determined that the possibility of a horse having a TCO_2 concentration of this threshold is less than 1 in 13,400; and, with a measurement uncertainty of 1.2 mM included, this probability changes to less than 1 in 641,000 [8]. Since the initial population surveys for plasma TCO_2 were done using the Beckman SYNCHRON EL-*ISE*® electrolyte system, it became the "gold" standard for this test.

The EL-*ISE*® employs an indirect approach for measuring the TCO_2. Upon the introduction of an acid, all forms of carbon dioxide in the sample matrix are assumed to be converted to their gaseous carbon dioxide form, which then diffuses through a silicone membrane and lowers the pH of a bicarbonate solution surrounding an electrode [9, 10] The EL-*ISE*® then calculates the TCO_2 based upon a two point calibration of 0 mM and 30 mM, under the basis that the rate of pH change is directly proportional to the sum of the all TCO_2 components. The chemical reaction is depicted in Scheme **1**. As the required TCO_2 working level is out of the calibration range, international Racing Laboratories had adopted the use of ASE linearity set (Australian Scientific Enterprise Pty Ltd, New South Wales, Australia) for the construction of a calibration curve, in a bid to improve the accuracy of the analysis. The plasma TCO_2 level is subsequently determined from the resulting calibration curve using linear regression analysis.

$$CO_2 + H_2CO_3 + HCO_3^- + CO_3^{2-} + RNHCOO^- + H^+ \rightarrow CO_2\,(g) + H_2O$$

Scheme 1. Chemical reaction for the analysis of TCO_2 using the Beckman SYNCHRON EL-*ISE*® Electrolyte System.

Since December 2008, the EL-*ISE*® instrument was declared obsolete by the manufacturer, resulting in the cessation of spare part supply and service required to keep the system operational. Consequently, Racing Laboratories have had to consider replacing this instrument with an alternative system. Several different ways of analyzing TCO_2 are available from a variety of vendors. For example, the Olympus system which uses an enzymatic method [11, 12] for measuring bicarbonate or the Kodak Dry Chemistry Analyzer which utilizes potentiometric analysis had been used for analyzing equine plasma samples [7], though, there have had been reports of organic acids interfering with its analysis [13, 14], similar to the EL-*ISE*® [8]. As with the EL-*ISE*®, these instruments utilize a principle of indirect measurement by converting the available TCO_2 into other forms, with the results determined from external calibration. This is a disadvantage, in comparison to the direct measurement approaches based on internal calibration generally adopted by Racing Laboratories to quantify other Prohibited Substances.

Instrumentations capable of separation and direct detection of TCO_2 are available, for example, ion

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chromatography (IC) [15, 16] and capillary electrophoresis (CE) [17] had both been shown to be potentially suitable for this purpose. In particular, IC, a variant of liquid chromatography (LC), is an attractive option as it can be done using a wide variety of LC pumps and auto-samplers, which are readily available and relatively inexpensive.

IC columns can be based upon reverse phase (using ion pairing or ion suppression), size exclusion, ion exclusion and ion exchange modes of separation. For this project we opted for an ion exclusion type column, commonly used for the analysis of organic acids. Separation in this mode is obtained by means of the Donnan membrane exclusion mechanism whereby strongly ionized substances are not retained and weakly ionizing substances, such as carbonic acid, in the presence of an acidic eluent, are able to penetrate into the pores of the stationary phase. Separation is thus achieved by partitioning between the external and internal liquid layer of the resin particles [18, 19] (Scheme **2**).

$$V_R = V_0 + K_d V_i$$

where V_R is the retention volume, V_0 is the interstitial volume (i.e. the volume of eluent flowing between the particles of stationary phase, V_i is the internal volume of eluent (i.e. the volume of occluded liquid inside the pores of the stationary phase), and K_d is distribution coefficient for the solute between the interstitial eluent and occluded liquid. For the above equation, fully ionized species are attributed a K_d value of 0, while neutral species, a value of 1.

Scheme 2. Equation representing theory of ion exclusion.

Whilst the eluent from the IC can be coupled to a wide variety of detectors, the cheapest and therefore most practical options were either ultraviolet (UV) or conductivity detector (CD). As carbonic acid does not possess a chromophore, an UV absorbing eluent would be required for its analysis *via* indirect measurement. When evaluated, conductivity detection was deemed to a superior option, with a lower running cost. The CD, combined with a post column reaction (PCR) using sodium hydroxide, avoided the need for expensive suppressors, was selected as a simple and responsive way of measuring TCO_2.

However, one disadvantage of IC, compared to conventional indirect analyzer machines, e.g. EL-*ISE*®, is the throughput factor, as longer time is required for chromatographic separation. Due to time constraint imposed upon us during pre-race analysis situations, we had developed two methods, screening and confirmatory, for the quantitation of TCO_2. The screening method involved the use of only the guard column for faster analysis, as opposed to the confirmatory method, utilizing both the guard and analytical column for a more thorough analysis with better separation.

In this paper, we present the possibility of using ion chromatography to detect TCO_2 *via* internal calibration quantitatively. This provides an alternate and independent quantitative method of analysis for the components that constitute TCO_2.

2. EXPERIMENTAL

2.1. Materials/Reagents

ASE Linearity Set Bicarbonate (batch 9020) with the concentration of the various calibrants at 26.93, 30.07, 32.96, 36.06, and 39.08 mM, and Certified Reference Material (CRM) with a TCO_2 concentration of 36.32 ± 0.28 mM (batch 9004) were obtained from Australia Scientific Pty. Ltd., National Measurement Institute (NMI). Sulfuric acid, for eluent preparation and sodium acetate, which was used as an internal standard (IS), were purchased from Merck (Singapore). HPLC grade Acetonitrile (ACN) (Tedia), and sodium hydroxide (NaOH) (QRëC) were obtained from Aik Moh Pants & Chemicals Pte. Ltd. (Singapore). Water was obtained by purifying tap water with a Millipore Milli-Q Advantage A10 water purification system. Universal Diluent, Universal Reference Reagent, Acid Reagent, and Alkaline Buffer used for the Beckman SYNCHRON EL-*ISE*® Electrolyte System were purchased from Beckman Coulter Singapore Pte. Ltd. Equine plasma samples were being acquired from the anonymous racehorses kept at the Singapore Turf Club.

2.2. Preparation of Plasma Sample

Blood tubes obtained from were centrifuged at 2500 *g* for 4 mins with Hettich Zentrifugen Universal 32 R centrifuge. The upper plasma layer was being subjected to analysis.

2.3. Instrumentation

2.3.1. EL-ISE® Analyses

EL-*ISE*® analyses were performed on Beckman SYNCHRON EL-*ISE*® Electrolyte System.

2.3.2. IC Analyses

IC analyses were performed on Metrohm 882 Compact IC plus system coupled to Metrohm 863 Compact Autosampler, and Metrohm 800 Dosino. The loop volume was 10 µl. The screening analyses were performed with Phenomenex Rezex RFQ Fast Fruit H+ guard column, 50 mm x 7.8 mm, 8 mm. The confirmatory analyses were performed with Phenomenex Rezex RFQ Fast Fruit H+, 50 mm x 7.8 mm, 8 mm and Phenomenex Rezex RFQ Fast Fruit H+ analytical column, 100 mm x 7.8 mm, 8 mm. The mobile phase was 0.5 mM H_2SO_4 with 5% ACN, flow rate 1.2 ml/min, column oven temperature was 40°C; and the post column reaction solvent was 3 mM NaOH, flow rate 1.2 ml/min delivered using Agilent 1200 series quaternary pump. The program used was Metrohm MagIC Net version 1.1, build 35. The integration of the negative polarity trace used a smoothing of 30 and a sensitivity of 50, with a minimum height of 0.01 mS/cm. The calculations were performed using Microsoft® Office Excel® 2007.

2.4. Analysis

2.4.1. IC Screening Analysis

Approximately 0.05 g of sodium acetate (OAc) was dissolved in 100 ml of water, and used as IS_a. Linearity was established *via* a 9:1 dilution of ASE Linearity Set Bicarbonate solutions (batch 9020) with IS_a. All pipetting work was performed with Thermo Scientific 100-1000 µl electronic Finnpipette, utilizing reverse pipetting mode, with both the rate of aspiration and pipetting set at 1. Each set consisted of 1.35, 1.49, 1.64, 1.80, 1.95 mM. Both the plasma samples and CRM were diluted in the same manner; with the diluted CRM used as a quality check (QC), with a back-calculated tolerance of 36.32 ± 1 mM to compensate for dilution error. Ratio of HCO_3 peak height (corresponding

to TCO_2) over OAc peak height was plotted against the nominal value of the standards. Calibration curves were generated with a linear least-square fit with a weighing of 1 using the 5 different diluted ASE solutions.

2.4.2. IC Confirmation Analysis

Approximately 0.5 g of sodium acetate was dissolved in 100 ml of water, and used as IS_b. Linearity was established *via* a 1:1 dilution of ASE Linearity Set Bicarbonate solutions (batch 9020) with IS. All pipetting work was performed with Thermo Scientific 100-1000 µl electronic Finnpipette, utilizing reverse pipetting mode, with both the rate of aspiration and pipetting set at 1. Each set consisted of 13.48, 14.93, 16.44, 17.99, 19.47 mM. Both the plasma samples and CRM were diluted in the same manner; with the diluted CRM used as a quality check (QC), with a back-calculated tolerance of 36.32 ± 1 mM to compensate for dilution error. Ratio of HCO_3 peak height over OAc peak height was plotted against the nominal value of the standards. Calibration curves were generated with a linear least-square fit with a weighing of 1 using the 5 different diluted ASE solutions.

2.4.3. EL-ISE® Analysis

The 2 point calibrants used for unlocking the machine were dilute 0.5mM sulfuric acid (0 mM TCO_2) and ASE 30 (approximately 30 mM TCO_2) (from ASE Linerity Set Bicarbonate), instead of the SYNCHRON EL-*ISE*® Calibrators Levels 1 and 2. Calibration curves were generated with the subsequent analysis of the ASE Linearity Set Bicarbonate solutions (batch 9020) without dilution, and plotted with a linear least-square fit with a weighing of 1. The centrifuged plasma samples were analyzed without further dilution.

3. RESULTS AND DISCUSSION

3.1. Setting Up of IC Method

The Phenomenex Rezex RFQ Fast Fruit H+ guard and analytical columns separate compounds based on ion exclusion principles, with the degree of ionization of the solute being the main influencing factor for its retention. 0.5mM sulfuric acid with 5% (v/v) ACN was chosen to be the eluent for the IC work. Within the column, the presence of strong sulfuric acid is expected to suppress the ionization of the weak carbonic acid, thus rendering it a neutral species, with an attributed K_d value of approximately 1. Assuming the sole presence of ion exclusion chromatographic influence, substances with similar K_d values are expected to elute out at similar retention times. This would potentially include organic compounds such as propionic and benzoic acid [19]. However, it is believed that their presence is rather insignificant, as CO_2 is expected to be liberated in the presence of an acid, and greatly impacting the results. Additionally, ACN was added to the eluent to induce separation of the organic compounds from the carbonic acid peak; and aid in the removal of certain organic components to increase the lifespan of the column.

As the eluent had a high conductance which would affect anion detection, PCR with 3 mM NaOH solution was employed to enhance the signal through indirect conductivity detection. Due to the fact that acid-base reaction is a very fast process, and the presence of a large void volume before the detector, a mixing module was not included in the setup. The efficiency of the mixing was indicated by a relatively stable baseline upon analysis of blanks and plasma samples. This way of enhancing the signal of the carbonic acid peak was deemed to be more cost effective than the use of expensive suppressors, which required periodic replacement. Furthermore, when the ASE linearity set and plasma samples were analyzed with the packed bed post-column suppressor that was supplied within the Metrohm 863 IC system (using 0.1M LiCl as regenerant), significant tailing of the TCO_2 peak was observed, rendering difficulty in integration (results not shown).

Prior to the analysis of the samples, several compounds were screened as potential IS candidates. Of which, sodium acetate was finally chosen as no interfering peak was observed in the same elution region in both the undiluted plasma and blank plasma (n =30) diluted with water; and that the addition of it to the plasma and the ASE Linearity Set Bicarbonate solutions did not affect the carbonate results significantly. A series of serial dilution of the plasma sample with IS_b was performed with a good linear correlation of 0.999 achieved. Results are shown in Table **1**.

Table 1. Analysis of Serially Diluted Plasma with IS_b

Volume of Plasma Added (µl)	Volume of IS Added (µl)	Back-Calculated Reading (mM)
0	1000	0
100	900	3
200	800	5.9
300	700	8.9
400	600	11.8
500	500	14.7
600	400	17.8
700	300	20.6
800	200	23.7
900	100	26.7
1000	0	29.6
	R^2	0.999

TCO_2 peak (labeled as HCO_3) was observed at about 2.1 minutes for the screening analyses, and about 4.9 mins for the confirmatory analyses, while the IS peak (labeled as OAc) was observed at about 1.6 min and about 3.9 min respectively. Example chromatogram of the screening method is shown in Fig. (**1**), while example chromatogram of the confirmatory method is shown in Fig. (**2**). The average (n = 6) R^2 for the ASE linearity set for the screening calibration curve, generated over several days for the screening analysis, was 0.994 (%RSD = 0.3%). The average (n = 6) RSQ for the ASE linearity set for the confirmatory calibration curve, generated over several days for the confirmatory analysis, was 0.995 (%RSD = 0.3%). Results are shown in Table **2**.

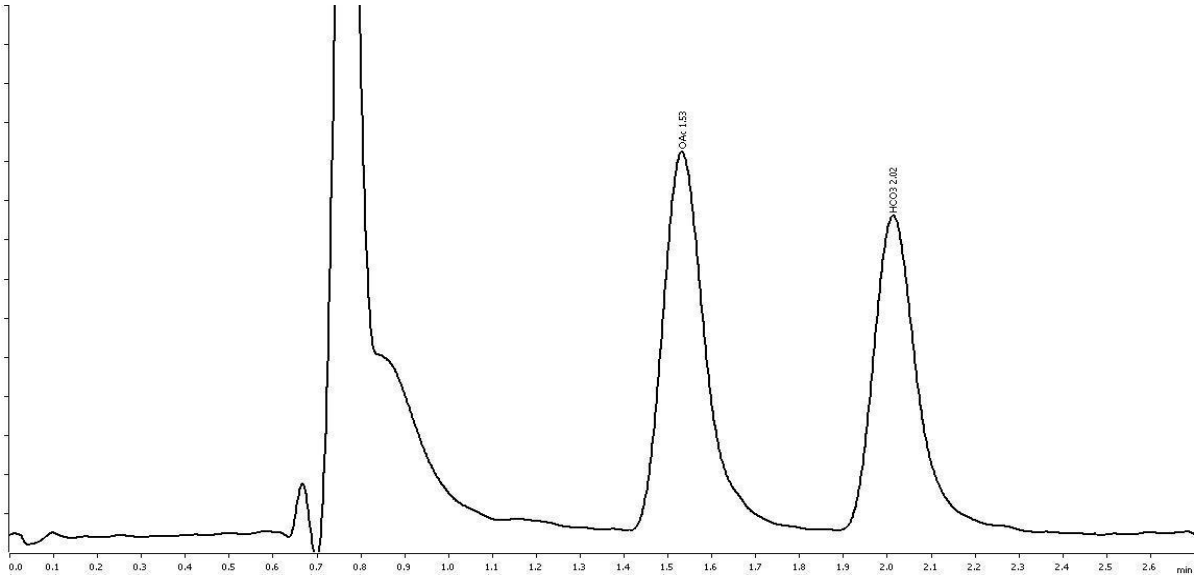

Fig. (1). Example chromatogram of screening analysis.

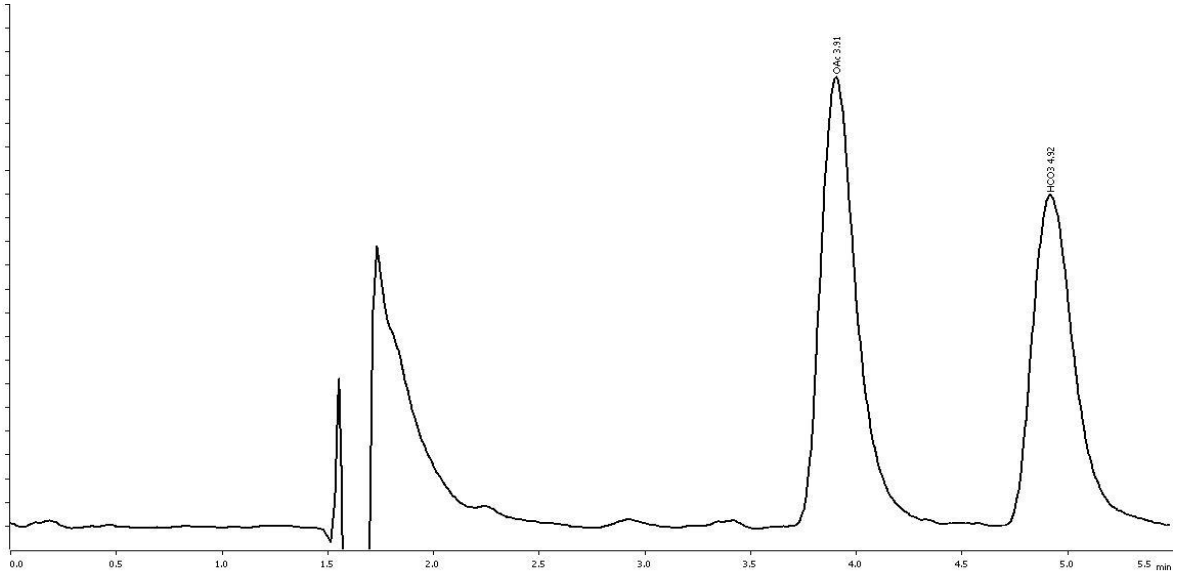

Fig. (2). Example chromatogram of confirmatory analysis.

Table 2. R^2 of Calibration Curves from 6 Different Days of Calibration Data on IC Using ASE Linearity Set

	Screening Analyses	Confirmatory Analyses
	0.995	0.997
	0.990	0.991
	0.993	0.993
	0.991	0.996
	0.998	0.997
	0.997	0.998
Average	0.994	0.995
%RSD	0.3	0.3

For the screening method, due to the limited capacity of the guard column, it was deemed necessary to dilute the plasma to minimize saturation of the guard column, and a 10-fold dilution was found to be effective in reproducing decent sensitivity; while a 2-fold dilution was performed for the confirmatory method. In the event of a decrease in peak resolution, the column may be back-flushed with approximately 10% ACN in water to regenerate the column. Generally, it was found that a guard column was able to perform approximately 200 analyses before the need for back-flushing. In order to achieve consistent results, it is recommended to replace the guard column after approximately 500 analyses.

3.2. Comparison of EL-ISE® Versus Screening IC Method

As the EL-*ISE*® system was the 'gold' system for equine TCO_2 analysis; some analyses were performed to compare

Table 3. Analysis of ASE Linearity Solutions on EL-ISE® and IC Confirmatory Analysis

	1st Set		2nd Set		3rd Set		4th Set		5th Set	
	EL-ISE®	IC	EL-ISE®	IC	EL-ISE®	IC	EL-ISE®	IC	EL-ISE®	IC
ASE 27	26.8	27.1	26.8	27.2	26.7	27.1	26.9	27.1	26.9	27.2
ASE 30	30.2	30.3	30.1	30.1	30.2	30.3	30.2	30.3	30.2	30.4
ASE 33	32.7	32.9	32.7	33.2	32.6	33.1	32.7	33.1	32.7	33.2
ASE 36	35.8	36.2	35.8	36.3	35.8	36.2	35.8	36.3	35.8	36.3
ASE 39	39.1	38.8	39.2	38.8	39.3	39	39.1	39	39.2	39.1
R^2		0.999		0.997		0.998		0.999		0.999
Average R^2	0.998									
RSD	< 0.001									

our IC method against the EL-*ISE*®. Firstly, the analysis of the ASE linearity set as samples after linearity was achieved. Though the EL-*ISE*® system was supposedly able to analyze TCO_2 concentrations from 0 to 30 mM [7], it had difficulties analyzing samples spiked with the acetate IS. This was potentially due to the acetate ion acting as a buffer, thus inhibiting the proper release of CO_2 gas. This highlighted an important advantage of IC separation technique, with the capability to separate and quantify TCO_2 separately. The ASE linearity set was thus being analyzed undiluted on the EL-*ISE*® system, and the results compared to a 2-fold dilution using the screening IC method. An average correlation of $R^2 = 0.998$ (n = 5, RSD = < 0.001) was being achieved, indicating that the results from both analyses correlate well. Results are shown in Table **3**.

Subsequently, analyses on equine plasma samples were being carried out with 15 random equine plasma samples (previously stored in refrigerator at approximately 5ºC for 1 week) were analyzed on both the IC and Beckman EL-*ISE*® systems on two separate days continuously. Results are shown in Table **4**. The averaged intraday differences were -0.6 mM (RDS = -0.7 mM) and 0.2 mM (RSD = 3.6 mM) with an intraday average difference of -0.2 mM; while the averaged intraday absolute differences were 0.6 mM (RSD = 0.5 mM) and 0.7 mM (RSD = 0.5 mM) with an interday average difference of 0.7 mM. Reasons accounting for differences in the readings between the 2 respective instruments include the matrix effect, individual instrument performance, human error such as poor pipetting techniques, and inadequate mixing involved during the addition of IS for the IC technique. Despite the potential disadvantages incurred during the addition of IS, its presence enabled the constant monitoring of the system performance in return.

4. CONCLUSION

We found the use of IC to be suitable for our general equine TCO_2 screening to determine the compliance of TCO_2 threshold set by MRA, and is considered a suitable replacement of the EL-*ISE*® system. The advantages of IC are that it is able to separate and quantify the TCO_2 species from the matrix using direct analysis; and that it allows the presence of an IS to aid in the performance monitoring of the system, and for internal calibration, which is more analogous to other methods of quantitative analysis.

Table 4. Comparison Results Between EL-*ISE*® and IC Confirmatory Analysis

Day 1	EL-*ISE*® (mM)	IC (mM)	Difference (mM)	Abs. Ave. Difference (mM)
1	29.7	30.3	-0.6	0.6
2	29.5	30.4	-0.9	0.9
3	30	30.4	-0.4	0.4
4	28.7	29.9	-1.2	1.2
5	29	29.6	-0.6	0.6
6	29.2	29.7	-0.5	0.5
7	29	29.8	-0.8	0.8
8	28.7	28.5	0.2	0.2
9	28.2	29.4	-1.2	1.2
10	29.3	29.1	0.2	0.2
11	29.1	29.4	-0.3	0.3
12	27.8	28.7	-0.9	0.9
13	28.1	28.6	-0.5	0.5
14	27.4	28.4	-1	1
15	28.2	28.6	-0.4	0.4
AVE	28.8	29.4	-0.6	0.6
RSD			-0.7	0.5
Day 2	**EL-*ISE*® (mM)**	**IC (mM)**	**Difference (mM)**	**Abs. Ave. Difference (mM)**
1	27.1	26.8	0.3	0.3
2	26.3	25.7	0.6	0.6
3	26.4	25.9	0.5	0.5
4	26.5	25.9	0.6	0.6
5	27	27.8	-0.8	0.8
6	26.1	25.5	0.6	0.6
7	26.3	26.8	-0.5	0.5
8	28	26.2	1.8	1.8
9	29	28.4	0.6	0.6

(Table 4) contd…..

Day 2	EL-*ISE*® (mM)	IC (mM)	Difference (mM)	Abs. Ave. Difference (mM)
10	26.5	27	-0.5	0.5
11	25.9	25.5	0.4	0.4
12	26.2	27.2	-1	1
13	27.5	26.7	0.8	0.8
14	28	29.1	-1.1	1.1
15	25.8	24.7	1.1	1.1
AVE	26.8	26.6	0.2	0.7
RSD			3.6	0.5
			Absolute Difference (Average of Day 1+2)	0.7

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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