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Sweat chloride quantification using capillary electrophoresis

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Abstract

Background: Cystic fibrosis (CF) is the less rare and severe genetic disease among the European population. Biochemical diagnosis of CF is based on the demonstration of increased chloride concentration in sweat samples, obtained during the sweat test (ST). WynSep developed a capillary electrophoresis with contactless conductivity detection (CE-C4D) able to measure sweat chloride with a low sample volume. We evaluated the clinical feasibility of this device in a cohort of patients suspected of CF, in comparison with the common coulometric method (ChloroChek chloridometer).

Methods: We determined sweat chloride concentration of 65 samples from patients referred to our institution to undergo a sweat test. Each sample was submitted to coulometric method first, then WynSep-CE, with or without internal standard (IS) subject to sufficient volume sample.

Results: A total of 53 samples were analysed by both coulometric and WynSep-CE (using IS) methods. The method validation showed comparable analytical performances for both methods ; no false positive or false negative was recorded. The two methods showed a high correlation ($r = 0.993$, $p < 0.001$) and a close agreement was demonstrated by two different statistical tests (Bland-Altman and Passing-Bablok).

Conclusions: WynSep-CE is an accurate, fast, easy-to-use and an appropriate method for CF diagnosis.

Keywords: sweat test, cystic fibrosis, capillary electrophoresis, chloride

1. Introduction

Cystic fibrosis (CF) is the less rare and severe genetic disease among European and North American populations, with an estimated incidence of 1/4700 newborns in France [1]. This autosomal recessive disorder is characterized by an impairment of exocrine glands, primarily in the lungs and the pancreas with respiratory consequences as the major causes of morbidity and mortality. This disease is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein which is involved in chloride and bicarbonate transport. CFTR mutations lead to impaired electrolyte transport resulting in an increased sweat chloride concentration [2].

Despite the availability of genetic testing, CF diagnosis is still based on the determination of increased chloride concentration in sweat samples [3, 4]. Sweat samples are collected by the sweat test (ST), a general term referring to the quantitative analysis of sweat chloride. Many methods are available to measure sweat chloride but it is recommended to use a direct measure of chloride like titration, potentiometry or coulometric quantitative tests [5]. In our institute (IFB, Purpan Hospital, Toulouse), the coulometric method (ChloroChek chloridometer) is used.

Capillary electrophoresis (CE) is able to directly measure chloride. CE is already known as a high performance separation technique, quick and a low-sample volume consumer/user, for/with different matrixes. Recently, Kuban et al. presented a novel approach to CF diagnosis, in which sweat chloride, sodium and potassium were simultaneously analysed by double opposite end injection (DOEI) - CE with C4D detection [6]. This method has been shown as a useful tool for analysis of sweat chloride. However, this approach has not yet been assessed in cohorts of patients

suspected of CF. Recently, the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) method has been proposed as a reference method (JCTLM database) for sweat chloride quantification [7].

Here, we aimed at evaluating the clinical feasibility of WynSep CE-C4D for CF diagnosis.

2. Materials and methods

2.1. Subjects

This study was carried out on subjects referred to our laboratory IFB, CHU Purpan for a ST between May and August 2017. During this period, a total of 65 sweat samples were processed for the diagnosis ($n = 48$) or for the initiation or follow-up of a new treatment (Lumacaftor/ivacaftor) for CF ($n=17$). This is a drug used only for adults and children > 12 years carrying the most common mutation (homozygous F508del mutation). The mean age of all subjects was 9.8 ± 10.7 years; median 5 (ranging from 0.1 to 58) years. There was no prior selection of patients for inclusion in the study, either on clinical or genotypic ground.

2.2. Sample collection

Sweat samples were obtained during the ST. This procedure involved sweat stimulation, sweat collection and quantitative measurement of chloride. All patients underwent ST in the same way, according to the ST guidelines [5]. Firstly, the sweat induction was carried out by pilocarpine iontophoresis, the gold standard, with Webster Sweat Inducer®. Then sweat was collected during 25 min with Macroduct® collector (Wescor system, Elitech, France), including a spiral plastic tube of around

85 μL total capacity [8]. Sweat produced was captured in the plastic tube and stained by a small quantity of blue water-soluble dye. This staining did not interfere with the assay and allowed to assess the volume assessment of obtained sweat. A minimal volume of 15 μL is required according to guidelines [8]. Finally, sweat was transferred to a microtube for transport (15 min) and storage at 4°C. Biological sample collection was registered under the number DC 2008-463 from the Toulouse Hospital biobank (CRB-TBR).

2.3. *Sample preparation*

The samples were submitted to both coulometric titration and, provided the sample volume was sufficient, CE analysis. Prior to CE analysis, the samples were made anonymous, diluted 1:1000 and an internal standard (IS) was added or not.

2.4. *Sample analysis*

2.4.1. *Coulometry ChloroChek Chloridometer (Elitech)*

The Wescor ChloroChek Chloridometer test system, model 3400 (EliTech), was used to measure the chloride in human sweat using the principle of coulometric titration. This assay required at least 10 μL of sweat and provided a digital readout in mmol/L [9]. Chloride levels measured by coulometric titration were taken as reference.

2.4.2. *Capillary Electrophoresis with Contactless Conductivity Detection (CE-C4D) (Wyn-CETM from WynSep)*

Analysis was performed by a Wyn-CE instrument (WynSep, Labège, France, equipped with a C4D detector (eDAQ, Australia). The separation voltage was - 25 kV, resulting in an electrophoretic current of - 8 μA . The separation capillaries used were neutral coated-silica capillary (50 μm ID, Microsolv, USA) with 35 cm total length and 23 cm effective cathodic length. Samples were injected by hydrodynamic injection

under 50 mbar during 5 seconds. A flushing step for 1 minute with the background electrolyte (BGE), was carried out between two successive runs. Analysis time was about 2 minutes (flushing time + separation time). This assay required only 2 μL . In all cases, measured responses correspond to the relative peak area (RPA) ratio of analyte normalized to sulfate as internal standard. Corrected peak areas were used for all calculations.

Chemicals and reagents

All chemicals were from reagent grade, and ultra-pure deionised water was used for background electrolyte (BGE) provided by WynSep, standard solutions, controls and sample dilutions. A 10 mmol/L stock solution of chloride and sulfate anions was purchased from Analab (France). Standard solutions were prepared by diluting the stock solution to the required concentrations for calibration.

Calibration standards, internal standard and quality control (QC) preparation

For calibration, five standard solutions (29, 43, 57, 100 and 200 $\mu\text{mol/L}$) containing an internal standard (IS) were prepared. Sulfate was added as IS at 100 $\mu\text{mol/L}$ per samples. To validate this new method, internal quality controls (IQCs) at three chloride concentrations (low level: 19 ± 2 mmol/L, medium: 43 ± 3 mmol/L, and high: 88 ± 7 mmol/L) provided by Elitech and external quality assessment (EQA), by Asqualab, were used, with IS diluted 1:1000.

2.5. Data analysis

Data are expressed as means \pm SD, and analysed using MedCalc statistical software program (Mariakerke, Belgium). A p value < 0.05 was considered to be statistically significant. Data from the different study groups were compared by a one-way and the repeated measures ANOVA. Comparisons between the groups were performed

using analyses of covariance followed by Bonferroni tests. Pearson r test was used to assess the relationship between both methods. Bland-Altman plots [10] and Passing-Bablok regression [11] were used to assess agreement between the two methods.

3. Results and discussion

3.1. Patient population

The concentration of sweat chloride, determined by coulometry, allowed us to distinguish three groups of patients, according to the new consensus guideline [8] : (i) *control group* (n = 44) with chloride < 30 mmol/L; (ii) *borderline group* (n = 3) with chloride between 30 and 59 mmol/L ; and (iii) *CF group* (n = 18) with chloride > 60 mmol/L. Subjects' characteristics are summarized in Table 1. We observed a positive correlation between sweat chloride value and the age of patients ($r = 0.39$, $p < 0.001$) as already described [12]. The concentration of electrolytes in the sweat varies through the life, with a transient increase during the first 24 hours after birth, followed by a rapid decline and an age-dependent increase [13]. For newborns, the ST was performed at one month.

The main challenge in CF diagnosis is to obtain a sufficient sweat volume sample. It has been reported that the sweat rate depends on ethnic origin, weight and postmenstrual age at time of testing [13]. Other studies showed an association between a low sweat rate and decreased sweat chloride [14]. Hence, the Cystic Fibrosis Foundation (CFF) has recommended a minimum collected sample of 15 μ L [3]. Yet, one study showed no correlation between sweat rate and chloride ion concentration [15].

3.2. Separation

Figure 1 shows a representative analysis of sweat chloride from a CF patient, a non-CF patient and the standard solution. Within 1 minute, the peak of chloride separated well from the IS (sulfate). Under these analytical conditions, no other anions were found. The profile was similar to those described with other CE devices [7, 16].

3.3. Analytical validation

The analytical parameters of WynSep method were assessed and are summarised in Table 2. The calibration curve ($y = 0.0057x - 0.0047$) showed a linear relationship with a regression coefficient ($r^2 = 0.9997$) with coefficients of variation (CVs) of the slope and intercept of the calibration curve were 1.8 and 2.2 %, respectively (n=5). The limit of detection (LOD), determined using signal-to-noise ratio of 3, was 2 $\mu\text{mol/L}$ with CV at 2.4 % (n=10), which is 5000-fold lower than for the coulometric method (Table 2). Within-run and between-run precisions were very satisfactory with CVs < 5 % and < 6 %, respectively, except at low chloride levels (Table 2) and similar to those assessed with ChloroChek chloridometer [13]. All the standard deviations for the two methods were in accordance to the manufacturer device. Also, during our study four EQAs were analysed and the results obtained were excellent (Table 2). The allowable limit of performance for sweat chloride is not described on Westgard's database but is 15 % on Asqualab results.

These results demonstrate that WynSep-CE display analytical performances as good as those of the coulometric method.

3.4. Comparison of Sweat chloride in non-CF and CF patients

Analysis of different samples with chloride concentrations spanning a broad range of disease states allowed us to compare the two techniques. We compared the chloride

concentration determined by coulometry titration and WynSep-CE method with and without IS. Because of insufficient sample volume, 63 samples were analysed by WynSep CE without IS and 53 samples with IS. The comparison of samples analysed by WynSep CE without IS vs. coulometry showed a significant difference while after adding IS no difference was found. As shown in Table 3 the slope (1.02) and the correlation ($r = 0.993$) were significantly improved after addition of IS. A strong positive correlation ($y = 1.02x + 0.69$) between the two methods was observed with Pearson correlation coefficient at 0.993 and a confidence interval not statistically different from 1, meaning no bias between the two methods. With CE analysis, the CF or non-CF patient groups were unchanged. During this study, we detected only 3 patients with inconclusive diagnosis but only one could be analysed by CE. No false positive or false negative values were obtained. All affected patients were identified.

A Bland and Altman plot of the difference in chloride concentration between both methods versus their averages is depicted in figure 2A. There was a complete agreement in the results except for two pairs, out the 95th percentile. These values differed by less than 10 mmol/L as requested in the agreement of methods [17]. This might be due to the slightly condensate or evaporation in the stored tube. Agreement between the two methods was also assessed by Passing-Bablok regression. Figure 2B shows 95% confidence intervals for intercept and slope include value zero and one, respectively, meaning that there were no constant and proportional differences between two methods. Taken together, these results show no significant analytical differences between these two methods.

In recent years, the intra- as well as intersubject variability in sweat chloride values in patients with CF has been investigated. The majority of this variability (56 %) is determined by the specific CFTR genotype. Other causes of variability include:

variation over time (14 %), environmental factors (13 %), residual factors such as test variability (10 %) and unique individual factors (7 %) [18]. Some of these factors may have influenced the data reported here, as all our patients had the same genotype.

Apparently, the absolute value of chloride may not provide a 100% proof diagnostic method. Other ions (Na^+ , K^+) alone have very little diagnostic value. However, the measurement of Cl^- , Na^+ and K^+ in sweat could improve the diagnosis of CF and reduce the number of false positive or negative results [7, 16]. In the study of Faria et al [19], the quality of the sweat test is based on the proportion of sweat sodium and chloride. Recently, several studies [20] have confirmed an analytical variation that may impact the clinical interpretation. Changes in diagnostic conclusion were seen in 28 % of subjects, the most frequent being changing from indeterminate to “CF unlikely” range. In the present study, we observed no such changes.

Some methods are very operator-dependent and time consuming with a high variability observed in the results [21] emphasizing the need for experienced technical operators [17, 21].

The present work has some limitations. First, almost all the patients included in this study had sweat chloride values well above the diagnostic cut-off of 60 mmol/L. We do not know whether variability is similar in patients with sweat chloride values around this cut-off would have resulted in group changes remains to be determined.. Second, after coulometric analysis, all the samples were stored at + 4 °C until CE analysis, and no simultaneously evaluated by CE. The storage at 4 °C during several months is possible as mentioned by the Asqualab procedure. However, this had no impact on the correlations.

Finally, the WynSep CE device offers a strong financial advantage, it is not dedicated to only sweat chloride assay and can be used for measurement of a variety of analytes [22].

4. Conclusion

WynSep CE is an accurate, fast, easy-to-use method and an appropriate technique for CF diagnosis.

Acknowledgements

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Abbreviations :

CE : capillary electrophoresis ; CF : cystic fibrosis ; CFTR : cystic fibrosis
transmembrane conductance regulator ; CV : coefficient of variation ; IS internal
standard ; LOD : limit of detection ; ST : sweat test ;

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Legends

Table 1 : Clinical features of the subjects

(based on the sweat chloride concentration determined by the coulometric test, individuals were classified into three groups)

Table 2 : Repetability and reproducibility of the two methods

Table 3 : Relationship between chloride concentrations as determined by WynSep CE (without or with IS) and ChloroChek chloridometer . Paired sweat levels. * comparison with ChloroChek chloridometer

Figure 1 : Electropherograms of chloride analysis for CF diagnosis.

A : Standard solution ; B : Control (non-CF) patient and C : CF pathologic samples.

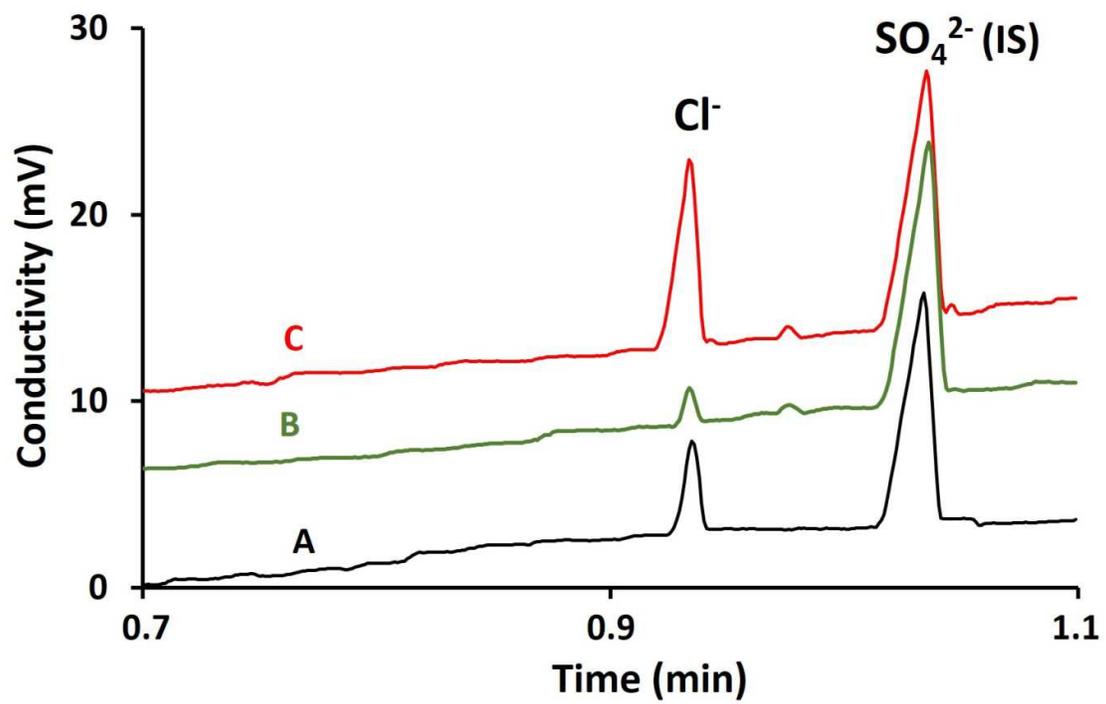
Separations carried out on a Wyn-CE system (WynSep). CE conditions were as follows: neutral coated 50 μm DI capillary 35-cm total length and 23-cm effective cathodic length; background electrolyte (BGE) : CF-Buffer from WynSep ; voltage - 25 kV; C4D detection; hydrodynamic injection under 50 mbars during 5 s.

Chloride concentrations : 96 mmol/L for pathologic sample, 13 mmol/L for Healthy sample, and 43 mmol/L for standard solution. Internal Standard : sulfate at 100 μM .

Figure 2 : Comparison between data obtained by CE and reference coulometric method by *Bland-Altman test* (A : the central line show the mean differences between methods, upper and lower lines correspond to ± 1.96 times the standard deviation) and *Passing-Bablok regression* (B : the dotted lines indicate the envelope of $\pm 95\%$ confidence limits about the regression, shown in a thick black line).

CI, confidence interval. Concentrations are expressed in mmol/L.

Figure 1



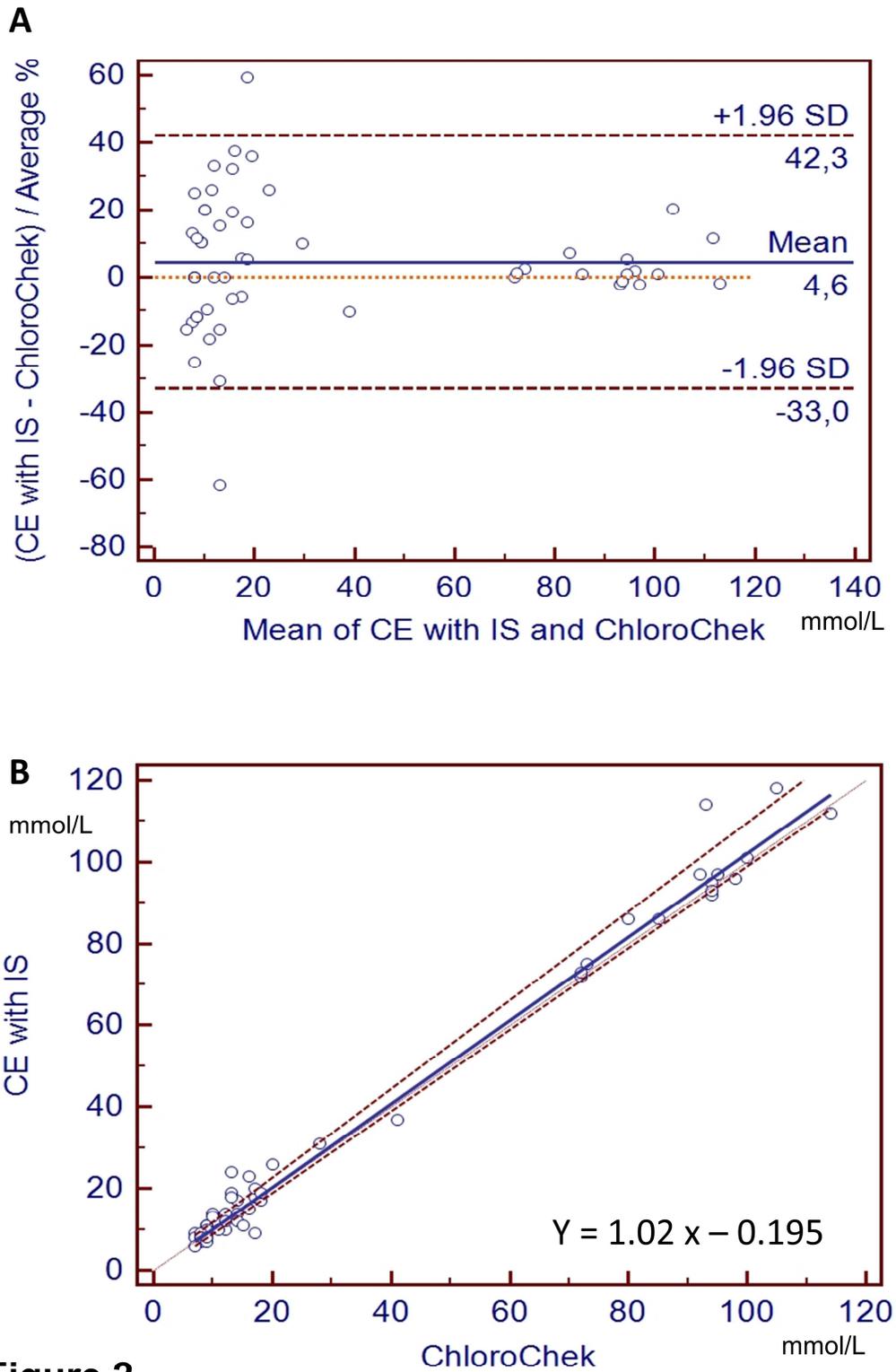


Figure 2

Table 1 : Clinical features of subjects

	Controls < 30 mmol/L	Borderline 30 – 59 mmol/L	CF > 60 mmol/L
N	44	3	18
Chloride (mmol/L) (ChloroChek Chloridometer)	12.3 ± 5.03	40.3 ± 2.08	90.6 ± 13.72
Age (years)	6 ± 7.4	29 ± 25.2	15.8 ± 8.8
Sex ratio M/F	21/23	2/1	11/7
Associated symptomatology	7	1	0
- gastroenteric	27	2	18
- lung disease	10	0	0
- other			
F508del mutation	-	-	18

Table 2 : Repetability and reproducibility of the two methods

	Assay controls (mmol/L)	ChloroChek Chloridometer	WynSep CE
Sweat sample volume		20 µL	2 µL
LOD		10 mmol/L	2 µmol/L
Within-run (Cl, mmol/L ; n ; CV %)			
low	19 ± 2	19.7 ± 1.16 (10 ; 5.9 %)	19.3 ± 0.9 (11 ; 4.7 %)
medium	43 ± 3	42.6 ± 0.52 (10 ; 1.2 %)	43.4 ± 0.9 (11 ; 2.1 %)
high	88 ± 7	87.9 ± 1.97 (10 ; 2.2 %)	88.5 ± 1.0 (11 ; 1.1 %)
Between-run (Cl, mmol/L ; n ; CV %)			
low	19 ± 2	19.4 ± 1.41 (34 ; 7.3 %)	19.6 ± 1.1 (28 ; 5.6 %)
medium	43 ± 3	42.3 ± 1.43 (46 ; 3.4 %)	43.7 ± 1.7 (28 ; 3.9 %)
high	88 ± 7	86.2 ± 2.88 (32 ; 3.3 %)	88.9 ± 2.4 (28 ; 2.7 %)
Standard solution	100	97.1 ± 4.52 (19 ; 4.7 %)	97.5 ± 2.8 (11 ; 2.9 %)
Asqualab (EQA)		Chloride Biais (%) Z score	Chloride Biais (%) Z score
Asqualab 01	68.1	69 1.3 0.51	69.8 2.5 0.96
Asqualab 02	112	112 0 0	114.3 2.1 0.38
Asqualab 03	51.6	50 - 3.1 - 0.89	51.2 - 0.8 - 0.23
Asqualab 04	80	79 - 1.3 - 0.36	82.4 + 3 + 0.86

	Non-CF Cl < 59 mmol/L	CF Cl > 60 mmol/L	slope	intercept	r	p
ChloroChek Chloridometer (n = 65)	14.1 ± 5.03 (n = 47)	90.6 ± 13.72 (n = 18)				
WynSep CE without IS (n = 63)	19.2 ± 7.32 (n = 45) p < 0.001 *	106.1 ± 18.55 (n = 18) p = 0.003 *	1.13	3.62	0.988	< 0.001 *
WynSep CE with IS (n = 53)	14.0 ± 5.98 (n = 38) p = 0.28 *	93.8 ± 14.12 (n = 15) p = 0.24 *	1.02	0.69	0.993	< 0.001 *

Table 3 : Relationship between chloride concentrations as determined by WynSep CE vs ChloroChek chloridometer

(* Comparison with ChloroChek chloridometer)