# Enantiomeric separation of D- and L-carnitine by integrating on-line derivatization with capillary zone electrophoresis

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## Abstract

A new capillary zone electrophoretic method has been developed for the enantiomeric separation and quantification of enantiomers of carnitine. D- and L-carnitine were derivatized with 9-fluorenylmethyl chloroformate in a flow system, working on-line with the capillary electrophoretic equipment. The separation was performed using a selective chiral buffer containing 2,6-dimethyl-β-cyclodextrin (heptakis). Triethanolamine was used as electroosmotic modifier and the separation was carried out in a uncoated capillary. Under the optimal conditions the resolution between D- and L-carnitine was 1.2 and the limits of detection for both isomers were about 5.0 $\mu M$. The proposed method was applied to the determination of D-carnitine in excess of L-carnitine in synthetic samples, and the results demonstrated that the maximal D-:L-carnitine ratio determined was $\approx$1:100.   © 1999 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Carnitine is a chiral biological substance found in different tissues of animals, plants and microorganisms. Its enantiomers show different biological activities. L-Carnitine is one of the most active substances as a mitochondrial fatty acid acyltransferase cofactor and it has interesting pharmacological and nutritional properties [1]. On the other hand, D-carnitine has been shown to have a considerable toxic influence on biochemical processes [2]; however, since 1960 DL-carnitine has been used as a drug for anorexia, dyspepsia, enterokinesis and other conditions [3]. So far, in the pharmaceutical formula-tions applied to overcome the L-carnitine deficiencies, the use of the pure L-enantiomer is recommended [1]. Consequently the separation of D- and L-carnitine is of great interest in the field of analytical chemistry, particularly in the pharmaceutical domain [4–6]. Several methods have been developed for the determination of these enantiomers, some of these methods have used the specific reaction catalysed by the enzyme carnitine acyltransferase (CAT) [7,8]. Simultaneous determination of D/L-carnitine has been performed by NMR measurements after formation of europium L-maleic acid–carnitine complex [6]. Liquid chromatography (LC) with pre-column derivatization using 9-anthroylnitrile, 9-anthryldiazomethane, 9-fluorenylmethyl chloroformate (FMOC-Cl) and [1-(9-fluorenyl)ethyl] chloroformate (FLEC) has been developed for the enantiomeric

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determinations of these compounds in pharmaceutical preparations [3,5,9].

The chemical and optical properties of the D- and L-carnitine do not allow its direct separation and detection by capillary electrophoresis (CE).These molecules do not show any UV–Vis or fluorescent signals. Interaction with a chiral agent such as cyclodextrin modifier, without previous derivatization reactions has not been reported yet. A few CE methodologies for the separation of these enantiomers have been described in the literature. Vogt et al. developed a method for separation of D/L-FMOC-carnitine using an electroosmotic flow modifier (tetrabutylammonium bromide) which presented a high background signal and low detection limits [2]; however, this result has been improved with the use of a modified capillary [1]. Another method has been developed using of chiral derivatization with FLEC, but the cost of the analysis was much increased [1,2].

This paper presents a new method for the determination of carnitine enantiomers by integrating the automatic derivatization with FMOC-Cl and CE separation. An uncoated capillary, 2,6 dimethyl-β-cyclodextrin as chiral additive and triethanolamine (TEA) as electroosmotic flow (EOF) modifier were used for the separation. The optimised method has been characterized and appropriately validated for the determination of these compounds.

## 2. Experimental

### 2.1. Instrumentation

A Beckman capillary electrophoresis instrument P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector operating at 204, 214 and 254 nm was used and a P/ACE software station was used to perform the data collection and controlling the operational variables of the system. A Gilson Minipuls-3 four-channel peristaltic pump, PTFE tubing of 0.5 mm I.D., a Rheodyne 5041 injection valve. The dimensions of the injection loop were 22 cm and the reactor was 330 cm. A laboratory-made programmable arm controlled by a microcomputer was also used as interface between the flow injection (FI) system and the CE equipment in order to automate the method [10].

### 2.2. Reagents and standards

Chemicals and solvents were HPLC or analytical grade from Merck (Darmstadt, Germany). Buffer solutions were prepared in 18 MΩ deionized water from a Millipore Milli-Q water purification system. D/L-Carnitine hydrochloride, D-carnitine hydrochloride, L-carnitine, FMOC-Cl, α-, β- and γ-cyclodextrin, 2,6-dimethyl-β-cyclodextrin and trimethyl-β-cyclodextrin were obtained from Sigma and sodium carbonate, sodium acetate, sodium dihydrogenphosphate, triethanolamine, acetone, hydrochloric acid, phosphoric acid and sodium hydroxide were obtained from Merck.

Stock standard solutions (20 m$M$) of D-, L- and D/L-carnitine in water were prepared. Working standard solutions were obtained by appropriate dilution of the stock standard solutions and all standard solutions and buffers were prepared fresh daily. The pH of the separation buffer was adjusted with phosphoric acid and was filtered through a 0.45-μm membrane filter. The buffers used for the derivatization of carnitine were 100 m$M$ dipotassium hydrogenphosphate buffer at pH 8.0, 50 m$M$ sodium carbonate buffer at pH 10.4 and 50 m$M$ sodium acetate buffer at pH 4.2. The derivative reagent was 30 m$M$ FMOC-Cl prepared in acetone.

### 2.3. Operating conditions

A positive power supply of 16–18 kV was used with a constant current mode of operation (16 μA). Capillaries from Beckman of effective length of 57 cm×50 μm I.D. were used and the temperature was 30°C. The injection protocol was a washing step for 1 min with water and preconditioning step of 5 min by running the buffer followed by a hydrodynamic injection for between 3 and 6 s. The composition of the selected buffer was 20 m$M$ NaH$_2$PO$_4$, 50 m$M$ 2,6 dimethyl-β-cyclodextrin, 24 m$M$ triethanolamine at pH 4.3 and the detection was carried out at 200, 214 and 254 nm (the quantification was performed at 200 nm).

### 2.4. FI manifold

The derivatization reaction was carried out on a FI system coupled with the CE equipment via a pro-
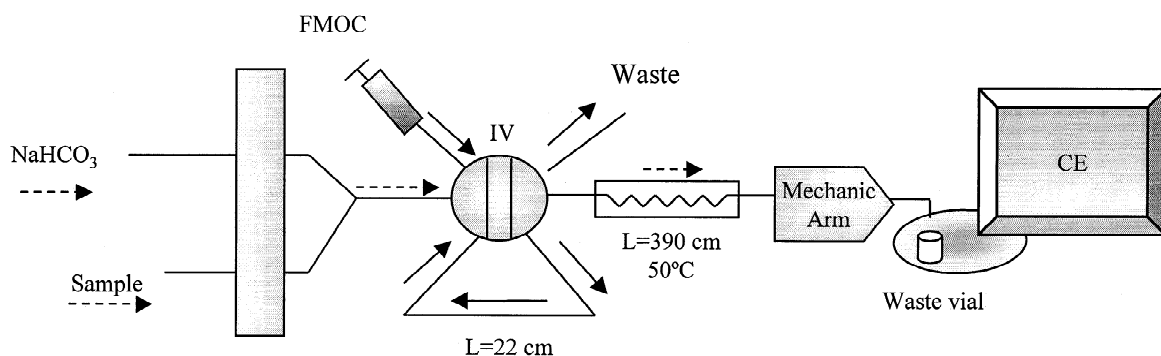
Fig. 1. FI manifold used for the derivatization of the carnitine enantiomers and their introduction into the CE system.

grammable arm [10,11]. The arrangement is shown in Fig. 1. The FMOC-Cl was introduced directly into the loop of the injection valve (IV) when load position was selected, while the sample was introduced by 2 min into the system and it was mixed with carbonate buffer. Then, valve was switched to the injection position allowing the mixing of sample–carbonate buffer and FMOC-Cl solution for 10 s. In this position the flow was stopped for 45 min in the reactor loop (390 cm), which was introduced into the thermostatic bath at 50°C. Finally, the reaction mixture was introduced via the mechanic arm into the CE system.

## 3. Results and discussion

Before the application, both the reaction used for the derivatization and the conditions for the CE separation were optimised.

### 3.1. Optimisation of derivatization reaction

The enantiomeric separation of D- and L-carnitine without previous derivatization was not possible due to their very similar molecular structures, which did not allow the interactions with chiral agents such as cyclodextrins. Derivatization was necessary in order to introduce a polar group which allowed the chiral interaction and the direct UV detection of the adducts. D- and L-carnitine were derivatized with a

non-chiral reagent, FMOC-Cl, according to the following reaction:
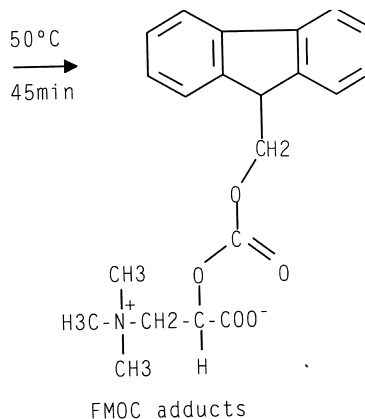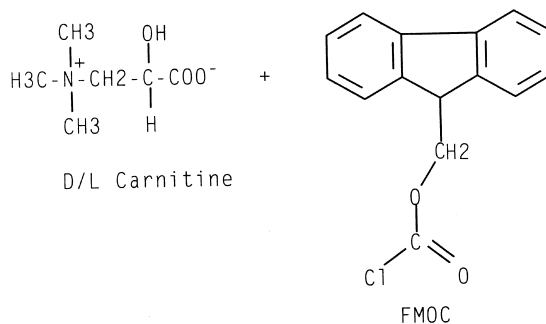


This reaction was normally carried out at pH 10.4 and acetic acid buffer (pH 4.2) was added to the reaction mixture 45 min later, to stop the derivatization reaction.

The reaction time and the concentration of FMOC-

Cl were evaluated and optimised. The reaction time was varied between 0 and 90 min under the following conditions: 10 m$M$ of D- and L-carnitine (racemic mixture) and 100 m$M$ of FMOC-Cl at 50°C (the response signals were obtained by CE). The results showed that the sensitivity was improved by using a time of 45 min. Longer reaction times did not give better results. The concentration of the FMOC-Cl reagent must be high in order to achieve the complete reaction of the analytes. Different concentrations were tested and 30 m$M$ of FMOC-Cl was sufficient to complete the reaction, at 50°C for 60 min.

The acetic acid–acetate buffer at pH 4.2 was necessary to stop the derivatization reaction; however, it was suppressed when the on-line derivatization system was used because the mixture between the FMOC-carnitine adducts and the acidic CE separation buffer in the injection vial of the CE equipment (Fig. 1) produced the same effect. In order to improve the life of the pump tubes, the FMOC (prepared in acetone) was introduced directly to the IV using a PTFE syringe. Finally, the time necessary for the on-line derivatization reaction and the direct introduction into the CE system was 48 min: 2 min for the sample introduction, 45 min for the derivatization reaction and 40 s for elution into the autosampler.

### 3.2. Optimisation of the CE separation

CE using a background electrolyte containing a cyclodextrin as chiral agent is the most useful operating mode for enantiomer separations [12,13]. In this case, different cyclodextrin were tested, using NaH$_2$PO$_4$ at different concentrations as the background electrolyte and triethanolamine as modifier of the electroosmotic flow [12,14,15]. The type of the chiral additive was the first parameter to be evaluated; different cyclodextrins were added to the separation buffer. The results demonstrated that when a natural cyclodextrin ($\alpha$, $\beta$ and $\gamma$) was assayed at different concentrations (between 5 and 20 m$M$) separation of D- and L-carnitine was not achieved. Trimethyl- and dimethyl-$\beta$-cyclodextrin were then tested; no resolution of the racemates was observed using trimethyl-$\beta$-cyclodextrin, while with dimethyl-$\beta$-cyclodextrin, a resolution of 1.2 was achieved at a concentration was 50 m$M$. The results are shown in Fig. 2. The role of TEA in the run buffer was to reduce the electroosmotic flow and to allow a better interaction between cyclodextrin and FMOC-carnitine molecules. The optimal concentration of TEA was 24 m$M$. In order to improve the separation efficiency, the concentration of electrolyte in the run buffer was tested between 10 and 60 m$M$. Increasing the concentration of NaH$_2$PO$_4$ produced
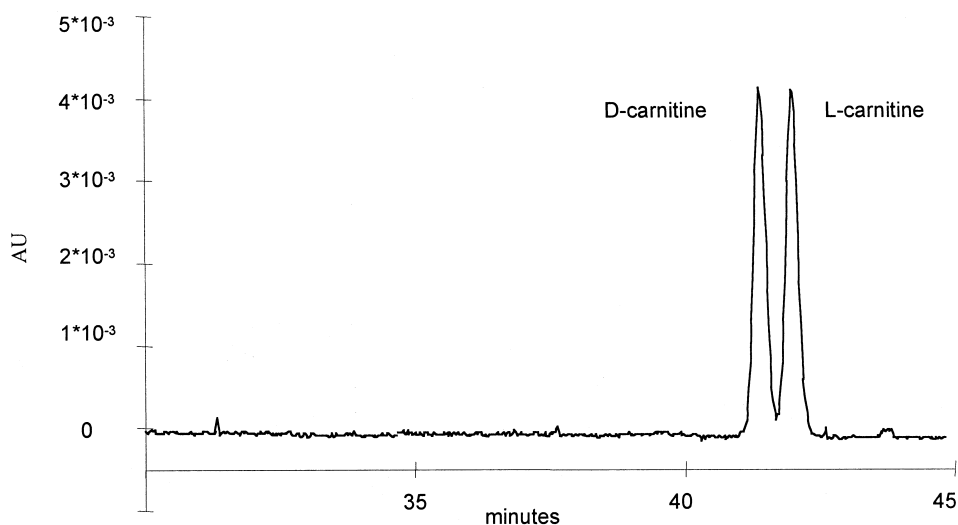


Fig. 2. Electropherogram obtained by using of 2,6 dimethyl-$\beta$-cyclodextrin as chiral additive (see experimental conditions in text).

an increase in the migration time, but no advantage was observed in the separation of analytes. The concentration finally used was 20 m*M*.

One of the most important parameter in CE is the pH of the run buffer because the EOF velocity and the dissociation of FMOC-carnitine compound depend on this parameter. Phosphoric acid was used to fix the pH in the buffer solution between 3.0 and 5.5. The results demonstrated that the maximum resolution of 1.2 was obtained by using the buffer at pH 4.3. In Fig. 3A the dramatic influence of pH in the

separation is evident; even small changes in the pH produced important variation on the resolution of the enantiomers.

Temperature played an important role because it influenced the viscosity and pH of the run buffer, and the stability of the inclusion complex of L- and D-carnitine with the cyclodextrin. The results (Fig. 3B) showed that resolution increased with low temperatures, but the migration times were considerably increased (60 min).

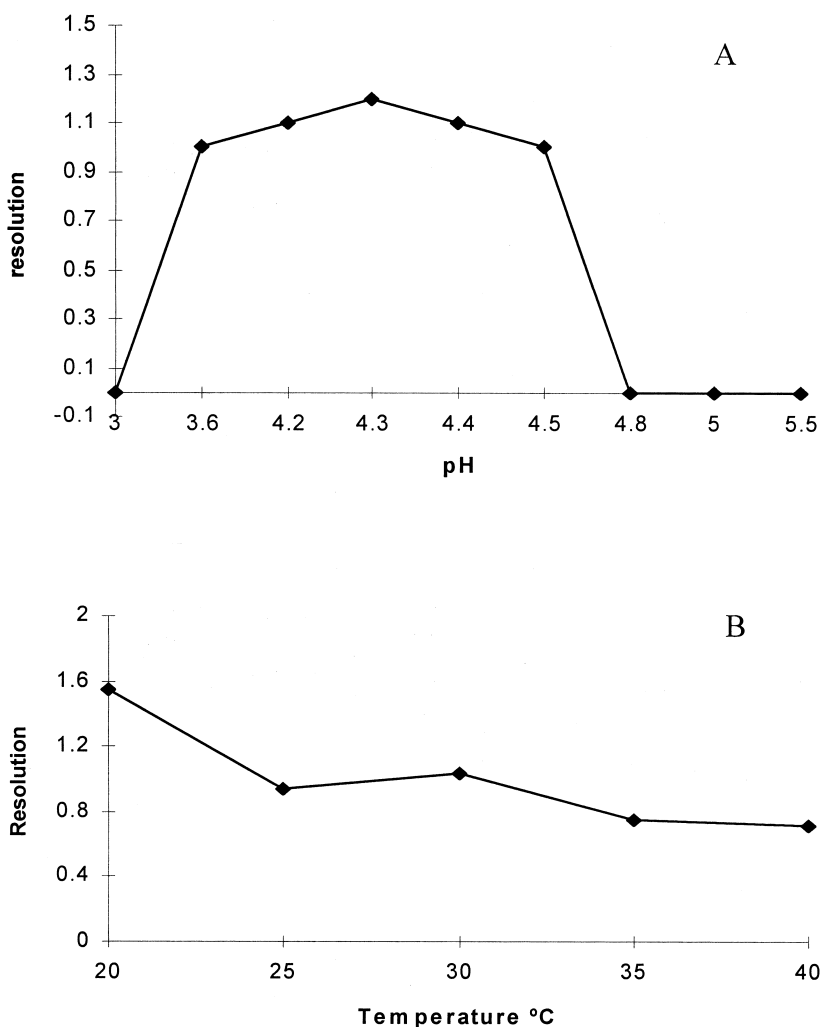The enantiomeric separation was tested at different

Fig. 3. (A) Influence of pH on the resolution of FMOC-derivatives of D- and L-carnitine. (B) Influence of the temperature on the resolution. The experimental conditions were 20 m*M* NaH$_2$PO$_4$, 24 m*M* triethanolamine and 50 m*M* 2,6 dimethyl-β-cyclodextrin and the separation was carried out at 18 kV.

voltages; the best results were obtained at 18 kV, which generated a current of between 14 and 16 $\mu$A. At this voltage, the reproducibility of the migration times was poor, and hence the constant current modality was used, resulting in an improvement of the reproducibility of the migration times (a relative standard deviation of $\pm 2.4\%$ was obtained for D-carnitine). Finally, the operational mode used for CE separation was the constant current mode at 15.5 $\mu$A.

These results agree with those previously obtained by Vogh et al. with reference to the limit of detection (LOD) and linearity, but in this case, the time of separation was increased and the resolution was lower than that obtained by using FLEC as a chiral derivatization reagent [2]. However, considering the high cost of this reagent, it represents a considerable advantage. On the other hand, the proposed method allowed the separation in an uncoated capillary, with TEA as EOF modifier, which represent an improvement in the analysis of carnitine enantiomers, because generally modified capillaries have a shorter lifetime than uncoated capillaries, which also increases the cost of analysis.

### 3.3. Analytical applications

No interference produced by the reagents used was found in the electrophoretic region of interest. Linearity was observed between 0.01 and 10 m$M$ of each enantiomer and the LOD and limit of quantification (LOQ) were calculated as the blank value plus three and ten times its standard deviation, respectively. The results are shown in Table 1. The reproducibility of the migration time was about 2.3%.

The purpose of the present method was the determination of D-carnitine in the presence of excess of L-carnitine because the presence of the D-isomer is

not recommended in the pharmaceutical production of the L-carnitine for the treatment of L-carnitine deficiencies [1]. In order to demonstrate the applicability of the proposed method, the determination of D-and L-carnitine in synthetic samples containing different enantiomeric relationships was assayed. The resolution of the different enantiomeric ratios under optimal conditions of separation was determined and the results are shown in Table 2 which demonstrates that the separation of enantiomers was possible up to an enantiomeric ratio of $\approx$1:100 of D/L-carnitine. When this ratio was >1:100, no resolution was observed. An electropherogram obtained for this molar ratio of D- and L-carnitine mixture under the optimal condition of separation is shown in Fig. 4, where the resolution obtained was 1.0. The results for the quantitative analysis of D-carnitine in presence of excess of L-carnitine in synthetic samples are shown in Table 3. The concentrations found were close to the real concentrations of D-carnitine, and the application of the statistical *t*-test demonstrated the concordance between both results (as Table 4 shows). This confirms the validation of the proposed method.

Table 2
Resolution obtained for different D-/L-carnitine concentration ratios

| Molar ratio D-carnitine:L-carnitine (m$M$) | Resolution |
|---|---|
| 0.03:0.3 | 1.03 |
| 0.03:0.6 | 1.01 |
| 0.03:0.9 | 0.98 |
| 0.03:2.1 | 1.01 |
| 0.03:3.0 | 1.02 |
| 0.03:6.0 | – |

Table 1
Figures of merit of the proposed method for the determination of D- and L-carnitine[a]

| Analyte | $y = a \pm bx$ | | $S_{y/x}$ | $r$ | $R^2$ (%) | RSD (%) | LOD ($\mu M$) | LOQ ($\mu M$) |
|---|---|---|---|---|---|---|---|---|
| | $a$ | $b$ | | | | | | |
| D-Carnitine | $-0.032 \pm 7.3 \cdot 10^{-3}$ | $0.258 \pm 2.6 \cdot 10^{-3}$ | $9.5 \cdot 10^{-3}$ | 0.999 | 99.98 | 2.35 | 5.0 | 15.0 |
| L-Carnitine | $-0.025 \pm 4.8 \cdot 10^{-2}$ | $0.254 \pm 1.7 \cdot 10^{-2}$ | $6.3 \cdot 10^{-2}$ | 0.995 | 99.11 | 2.40 | 5.0 | 15.0 |

[a] *a*, Intercept; *b*, slope; $S_{y/x}$, standard deviation of residuals; $R^2$, curve fitting level (%) obtained by analysis of variance (ANOVA) for validation of the model; RSD (%), reproducibility of injections.
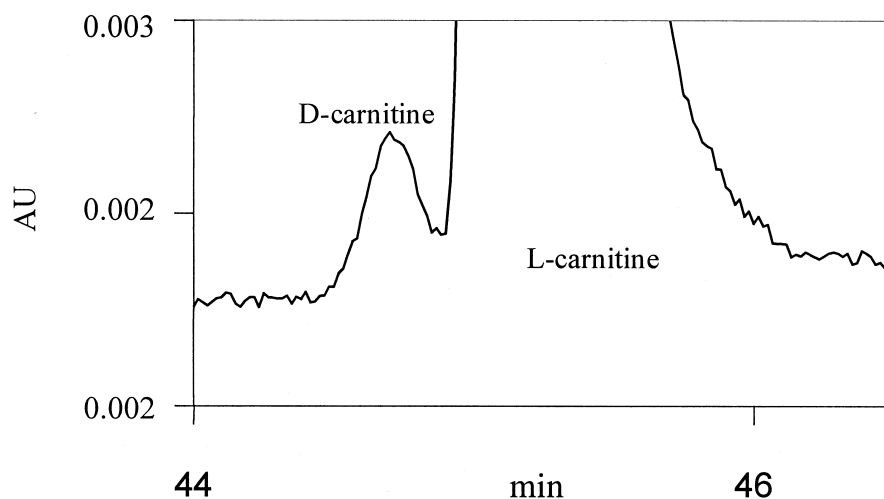
Fig. 4. Electrophoretic separation of a synthetic sample containing a D-:L-carnitine ratio of 1:100, obtained under optimal conditions of separation.

Table 3
Quantitative determination of D-carnitine in presence of L-carnitine (external calibration has been used for the calibration)

| Real concentration | | Found concentration | | % Error for D-carnitine |
|---|---|---|---|---|
| L-carnitine ($\mu M$) | D-carnitine ($\mu M$) | L-carnitine ($\mu M$) | D-carnitine ($\mu M$) | |
| 300 | 30 | 300±2 | 30±2.5 | 0 |
| 600 | 30 | 601±3 | 28±2.3 | 6 |
| 900 | 30 | 899±3 | 30±2.9 | 0 |
| 2100 | 30 | 2100±6 | 27±2.4 | 10 |
| 3000 | 30 | 3002±5 | 25±3.1 | 16 |
| 6000 | 30 | 6003±6 | [a] | – |

[a] No resolution.

Table 4
Statistical analysis of the results obtained for the determination of D-carnitine in excess of L-carnitine

| Sample statistic | | |
|---|---|---|
| | $n$ | 5 |
| | Average | 28 |
| | Variance | 4.5 |
| | Standard deviation | 2.12 |
| | Median | 28 |
| Confidence interval for mean | 95% | 25.3–30.6 |
| | | 4 Degree of freedom |

Null hypothesis, $H_0$: $\bar{x} = 30$
Alternative hypothesis, $H_1$: $\bar{x} \neq 30$
$t$ critical ($n=5$, $\alpha=0.05$, two tails)$=2.78$
$t$ experimental$=2.10$

## 4. Conclusions

The new method proposed here integrates a flow injection system for the automatic derivatization of carnitine on-line, coupled with the CE equipment in which the derivatized sample product is analysed. The FI system allowed a continuous derivatization of the D/L-carnitine, while the CE system allowed the enantiomeric separation in a large concentration range with a precision and sensitivity which is highly satisfactory. The time for both on-line derivatization and CE analysis was 90 min, however, both methodologies worked synchronically by using the mechanic arm, so during the electrophoretic analysis of one sample the derivatization of the next sample was being performed, resulting in a reduction of the overall time. Separation of carnitine enantiomers has been previously described by CE with a modified capillary; however, the proposed method allowed the separation in a uncoated capillary with TEA as EOF modifier with sufficient resolution. The use of a chiral additive (2,6-dimethyl-β-cyclodextrin) allowed the enantiomeric separation of D- and L-carnitine up to a 1:100 D-:L-ratio.

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