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# α-Lipoic acid in dietary supplements: Development and comparison of HPLC-CEAD and HPLC-ESI-MS methods

Arjumand I. Durrani [a], Heidi Schwartz [a], Walther Schmid [b], Gerhard Sontag [a,*]

[a] *Institute of Analytical and Food Chemistry, University of Vienna, Währinger Straße 38, A-1090 Vienna, Austria*
[b] *Institute of Organic Chemistry, University of Vienna, Währinger Straße 38, A-1090 Vienna, Austria*

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**Abstract**

α-Lipoic acid is an antioxidant used both in the prevention and treatment of various oxidative stress related diseases. It is an important constituent of some dietary supplements and can also be found in plant and animal sources. A rapid method for the determination of α-lipoic acid in dietary supplements based on high performance liquid chromatography coupled with a coulometric electrode array detector (CEAD) and an electrospray ionization mass spectrometer (ESI-MS) was developed. First, α-lipoic acid was extracted with methanol by sonication, chromatographic separation was then achieved by isocratic elution [acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid): 350/65/585, v/v/v] using an ACE 3-C-18 column at a flow rate of 0.45 ml/min. α-Lipoic acid was detected by means of a CEAD at +300, +400, +450, +500, +550, +600, +650, and +700 mV against palladium reference electrodes. For ESI-MS detection (negative mode), the composition of the mobile phase was changed to 0.1% acetic acid in water/acetonitrile 55:45, v/v applying a flow rate of 0.2 ml/min. The presented methods were utilized to determine the α-lipoic acid content in six dietary supplements. The results of both detection modes were in good correlation.
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*Keywords:* Lipoic acid; Reversed-phase high performance liquid chromatography; Coulometric electrode array detection; Electrospray ionization mass spectrometry; Dietary supplements

## 1. Introduction

α-Lipoic acid, 1,2-dithiolane-3-pentanoic acid, is an universal antioxidant present in prokaryotic and eukaryotic cells. In the human body, it is linked to lysine residues and acts as a cofactor in different multi-enzyme complexes [1]. α-Lipoic acid functions both in membranes and in aqueous phases and plays an important role in the synergism of antioxidants by directly recycling vitamin C, glutathione and coenzyme Q10 and indirectly recycling vitamin E [2]. Due to its unique properties, it has also been administered in the treatment of various oxidative stress related diseases such as alcoholic liver disease, heavy metal poisoning, mushroom poisoning, diabetes, glaucoma, ischemia/reperfusion injury of heart and heart mitochondria, chagas disease and neurodegenerative disorder [3]. The results of animal models suggest that α-lipoic acid also possesses anti-obesity effects [4].

Various analytical methods have been developed for the quantitative determination of α-lipoic acid in biological material, drugs and food samples. A review of chromatographic methods was published by Kataoka [5]. Gas chromatography with flame ionization detection [6], flame photometric detection [7] and mass spectrometry [8–10] are among the most frequently applied techniques. However, the necessity of different tedious derivatization steps is a considerable drawback. High performance liquid chromatography with different detection modes such as ultraviolet [11], fluorescence [12,13], electrochemical [14,15] and electrospray ionization mass spectrometry [16,17] requires, with the exception of fluorescence detection, no derivatization step [5]. Howard and McCormick [18] published a reversed-phase HPLC-UV method to separate α-lipoic acid and some of its analogues but the detection limits were high due to the lack of a strong chromophore. HPLC with fluorescence detection, based upon fluorogenic labelling reagent, proved to be suitable for the determination of α-lipoic acid [12,13] in human plasma and urine but required laborious sample preparation procedures. HPLC with electrochemical detection usually offers a sensitive quantification of organic compounds. Handelman et al. [19]

* Corresponding author. Tel.: +43 1 4277 52303; fax: +43 1 4277 9523.
*E-mail address:* gerhard.sontag@univie.ac.at (G. Sontag).

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and Han et al. [20] used a dual Hg–Au electrode system with pulsed amperometric detection to determine α-lipoic acid and dihydrolipoic acid at nanomolar levels but the electrodes lost sensitivity after 30–50 injections and had to be reconditioned. Teichert and Preiss [15] measured α-lipoic acid in human plasma using amperometric detection at the high oxidation potential of +1.1 V. However, the method suffered from an extreme loss in sensitivity due to poisoning of the electrode. Later the same authors overcame this drawback by applying pulsed amperometric detection [21]. High performance liquid chromatography with dual electrode coulometric detection on porous carbon electrodes was then used to determine α-lipoic acid in biological samples, but a very high working electrode potential (+850 mV) was necessary [14]. Recently, Chen et al. [16] and Trivedi et al. [17] developed a relatively simple and sensitive analytical method for the quantitative determination of α-lipoic acid in human plasma using LC coupled with electrospray ionization single quadrupole mass spectrometry in the negative selected ion monitoring (SIM) mode.

Very few analytical data are available on the quantitative determination of α-lipoic acid in dietary supplements. Sitton et al. [22] published a method for the quantitative determination of α-lipoic acid in a dietary supplement by capillary electrophoresis using UV detection at 208 nm. The main drawback was the adsorption of α-lipoic acid at the capillary wall after a few injections so that the capillary needed to be washed for further use. A rapid high performance liquid chromatographic method was developed and validated for the determination of α-lipoic acid in pharmaceutical dosage forms using UV detection at 332 nm [6]. The limit of detection and limit of quantification were high, 4.4 and 16.8 μg/ml, respectively, due to the low absorption coefficient of pure α-lipoic acid ($\varepsilon = 150\,\mathrm{L\,mol^{-1}\,cm^{-1}}$) at 332 nm.

The aim of the present work was to develop a rapid and reliable HPLC-CEAD and ESI-MS method for the determination of α-lipoic acid in commercially available supplements. Six different dietary supplements were analyzed and the data were compared.

## 2. Materials and methods

### 2.1. Reagents and chemicals

α-Lipoic acid [DL-α-lipoic acid; purity ≥99.0%] and bisphenol A (99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate (≥99.0%, p.a.), *ortho*-phosphoric acid (85%), HPLC-gradient grade acetonitrile and methanol were delivered from Merck (Merck Company, Darmstadt, Germany). Deionized water, prepared using a Barnstead Easypur LF (Dubuque, Iowa, USA) water purification system, was used in all experiments.

### 2.2. Preparation of solutions

#### 2.2.1. Stock solutions

A stock solution of α-lipoic acid was prepared by dissolving 10.0 mg of α-LA in 100 ml of methanol. Calibration solutions of six different concentrations between 0.005 and 1 μg/ml were made by diluting the stock solution with water:acetonitrile (70:30, v/v). A stock solution containing 1 mg bisphenol A/ml in methanol (internal standard) was diluted with water:acetonitrile (70:30, v/v) to 2 μg/ml. All the solutions were stored in tightly sealed amber volumetric flasks at 4 °C.

#### 2.2.2. Mobile phase

*Mobile phase I*: Acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid): 350/65/585, v/v/v were mixed, filtered through a 0.45 μm Sartolon polyamide filter (Sartorius AG, Goettingen, Germany) and degassed by sonication for 20 min prior to use.

*Mobile phase II*: 0.1% acetic acid in water and acetonitrile (55:45, v/v).

### 2.3. Samples and sample preparation

Six different dietary supplements (capsules/tablets/sachet) were purchased from local pharmacies as well as from the US market. Ten tablets were weighed, finely ground and the average weight of one tablet was determined. Likewise, the content of 10 capsules was pooled and the average weight of one capsule was taken. Three aliquots (50 mg) of each homogenized supplement and of the sachet were dissolved in 8.0 ml of methanol and sonicated for 30 min at room temperature. The mixtures were then transferred into 25 ml volumetric flasks and the volume was made up to the mark with distilled water. Two milliliters of the extracts were pipetted into Eppendorf tubes and centrifuged for 10 min at 10,000 rpm. The supernatants were further diluted with water:acetonitrile (70:30, v/v). Five hundred microliters of these solutions were then transferred to autosampler vials containing 450 μl water:acetonitrile (70:30, v/v) and 50 μl of bisphenol A solution (2 μg/ml).

### 2.4. Equipment and chromatographic conditions

#### 2.4.1. HPLC-CEAD and HPLC-UV

The HPLC system consisted of a Merck Hitachi pump L-6200, a Basic Marathon autosampler (Spark, Holland B.V., Emmen, The Netherlands), and an ACE 3-C-18 (150 mm × 3.0 mm, particle size 3 μm) column (Advanced Chromatography Technologies, Aberdeen, Scotland).

A coulometric electrode array detector was controlled by Coul Array Win/Software (ESA, Chelmsford, USA). The potentials of the electrodes were set at +300, +400, +450, +500, +550, +600, +650, and +700 mV against palladium reference electrodes.

A Merck Hitachi L-4250 UV/Vis detector was used together with D-6000 Interface (Merck Hitachi). The wavelength was set at 215 nm. Data were obtained using chromatography data station software, HPLC manager version 2.

Twenty microliters of the standard solutions or the samples were injected into the HPLC system. Chromatographic separations were achieved isocratically using mobile phase I at a flow rate of 0.45 ml/min. By plotting the peak height versus the potential of the working electrodes the current/voltage curves for α-lipoic acid and bisphenol A (Fig. 1) were obtained.

Table 1
Validation parameters (RSD: relative standard deviation; LOD: limit of detection)

| Chromatographic method | Repeatability (% RSD) | | LOD (μg/ml) | Linear regression data | | Regression coefficient ($R^2$) |
|---|---|---|---|---|---|---|
| | Intra-day ($n = 5$) | Inter-day ($n = 15$) | | Regression equation | Observed linear range (μg/ml) | |
| HPLC-CEAD | 2.56 | 2.73 | 0.005 | $y = 3.21x + 0.0073$ | 0.01–1 | 0.9997 |
| HPLC-ESI-MS | 3.03 | 4.23 | 0.003 | $y = 10.4x + 0.038$ | 0.005–1 | 0.9999 |
| HPLC-UV | 2.61 | 2.85 | 0.025 | $y = 1.34x + 0.0002$ | 0.025–1 | 0.9989 |

*2.4.2. HPLC-ESI-MS*

The HPLC 1100 series system (Agilent Technology, Palo Alto, CA, USA) consisted of a G1312A binary pump, a G1322A mobile phase vacuum degassing unit, a G1313A autosampler, an ACE 3-C-18 (150 mm × 2.1 mm, particle size 3 μm) column (Advanced Chromatography Technologies, Aberdeen, Scotland) and an ion trap mass spectrometric (MS) detector (HCT plus, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Hystar 3.1, software for chromatography and hyphenated techniques, was used to acquire the data. Data were then integrated using the software, Data analysis™ version 3.3 (Bruker Daltonics). Isocratic elution was carried out with mobile phase II at a flow rate of 0.2 ml/min. Electrospray ionization of the column effluents was performed with nitrogen as nebulizing gas at 10 l/min, 40 psi nebulizing pressure, and 300 °C drying gas temperature. The capillary voltage was set at 4.5 kV.

Data were collected in the multiple reaction monitoring mode [MRM] by fragmenting the deprotonated molecular ion [*m/z* 205] without isolating it and by successively isolating the fragment ion *m/z* 171 $[\alpha\text{-LA-H}_2\text{S-H}]^-$ and by isolating *m/z* 227 $[\text{BPA-H}]^-$. During the run time, the divert valve was set to waste from 0 to 3.5 min and to the source from 3.5 to 7.5 min to avoid contamination from the matrix of supplements.

## 2.5. Validation of the methods

Standard calibration curves for α-lipoic acid with HPLC-UV, HPLC-CEAD and HPLC-ESI-MS were generated (Table 1) on five successive days using the analyte/I.S. peak area ratios versus the nominal concentrations of the analytes and the relative standard deviation of the slope was calculated. The limit of detection (LOD) was determined at a signal-to-noise ratio of 3. The intra-day repeatability was checked by injecting α-lipoic acid standard solution (0.19 μg/ml) five times on one day. The inter-day repeatability was tested by injecting the same solution three times a day successively for 5 days. Stability of α-lipoic acid in the standard solutions was checked both at 4 °C and at room temperature.

An extract of supplement ‘a’ (Table 2) was prepared and diluted (1:2, v/v) with water/acetonitrile (70:30, v/v) and stored at 4 °C. Nine hundred and fifty microliters of this solution and 50 μl of bisphenol A solution (2 μg/ml) were mixed in the autosampler vial and injected three times each day to check the instrument performance.

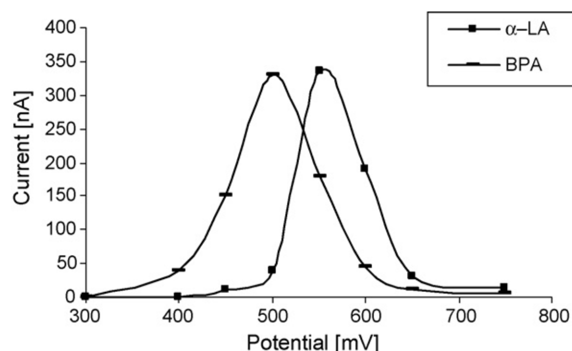


Fig. 1. Current/voltage curves of α-lipoic acid (α-LA) and bisphenol A (BPA).

## 2.6. Quantitative determination of α-lipoic acid

*2.6.1. Calibration curve*

A calibration curve was established by plotting the peak area ratios versus the concentrations of α-lipoic acid in the standard solutions. The content of α-lipoic acid in the supplements was determined by multiplying the obtained concentration with the appropriate dilution factor, correcting by the weighed portion and considering the recovery.

*2.6.2. Standard addition*

Supplement ‘f’ was selected for the standard addition method as it contained a lower amount of α-lipoic acid than other supplements. Fifty milligrams of supplement (in triplicate) were treated as in Section 2.3 and the α-lipoic acid content was determined with the external calibration function. Fifty milligrams aliquots of the supplement ‘f’ were then spiked with 50, 100, 150, 200% of the experimentally calculated α-lipoic acid content (each in triplicate). The unspiked aliquots and each of the spiked samples were then treated as described above (Section 2.3) and analyzed by HPLC-CEAD and HPLC-ESI-MS. The analyte concentration in the supplement was determined by plotting the peak area ratios

Table 2
Content of α-lipoic acid in dietary supplements

| Supplement | Claimed (mg/g) | Found (mg/g) | | |
|---|---|---|---|---|
| | | HPLC-CEAD | HPLC-MS | HPLC-UV |
| a | 34.9 | 33.6 | 33.9 | |
| b | 0.7 | 0.4 | 0.5 | 0.4 |
| c | 1.3 | 0.9 | 0.9 | |
| d | 20.6 | 12.1 | 13.2 | 12.1 |
| e | 13.1 | 9.9 | 10.5 | |
| f | 1.0 | 0.4 | 0.4 | |

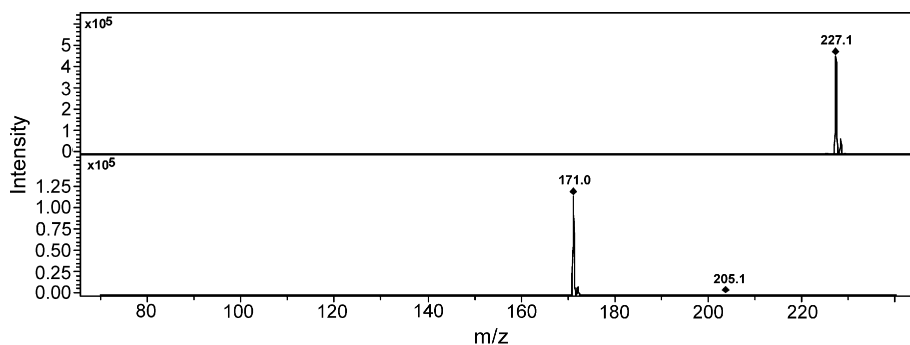


Fig. 2. Mass spectrum of α-LA fragment ion at *m/z* 171 ([α-LA-$H_2$S-H]$^-$) and for internal standard BPA at *m/z* 227 ([BPA-H]$^-$ using MRM mode.

versus the added concentrations of the standard compound, linear regression and division of the *y*-intersection (*d*) by the slope of the regression line $y = kx + d$.

## 3. Results and discussion

### 3.1. HPLC-CEAD

Mobile phase I was found to be appropriate to eliminate the influence of the matrix compounds at the beginning of the chromatogram (Fig. 4A) and to separate the compounds present in the supplements in a short analysis time. Bisphenol A, though not chemically related with α-lipoic acid, was chosen as an internal standard for its similar chromatographic and electrochemical behaviour to check the stability of the HPLC-CEAD system. Due to the similarity of the current/voltage curves of α-lipoic acid and bisphenol A (Fig. 1) the evaluation was made in adjacent channels (+500 and +550 mV).

### 3.2. HPLC-ESI-MS

Direct infusion of a standard solution of pure α-lipoic acid into the ESI-MS apparatus revealed (Fig. 2) that the deprotonated molecular ion (*m/z* 205) was partially fragmented to *m/z* 171 [M-$H_2$S-H]$^-$. This fact was also observed by Chen et al. [16] but due to the limited possibilities with a single quadrupole instrument, the deprotonated molecular ion was favoured for quantitative determination of α-lipoic acid. One advantage of the ion trap instrument is the possibility to use the multiple reaction monitoring mode (MRM) for quantitative analysis. In this mode the deprotonated molecular ion (*m/z* 205) can first be fragmented without being isolated, then the fragment ion (*m/z* 171) can be isolated and the corresponding peak area can be used for quantitation. This approach offers both higher selectivity due to measurement of the fragment ion and higher sensitivity because both the original fragment ion (the one created in the source) and the fragment ion created from the deprotonated molecular ion in the trap contribute to the total ion current (Fig. 3).

### 3.3. Calibration, linearity, limit of detection and repeatability

The methods were compared concerning the characteristic analytical parameters for α-lipoic acid. The calibration curves were linear in the investigated concentration range. The limit of detection (S/N = 3) for the HPLC-ESI-MS method turned out to be slightly lower than for HPLC-CEAD. On the other hand, the intra- and inter-day repeatability of the electrochemical method was better than the mass spectrometric method (Table 1). Calibration solutions were stable for more than 1 week when stored at 4 °C and were unstable at room temperature (25 °C or more) after 2 days.

### 3.4. Determination of α-lipoic acid in supplements

#### 3.4.1. Extraction

α-Lipoic acid is soluble in both aqueous and non-aqueous phases. To avoid degradation of α-lipoic acid and to provide sufficient extraction efficiency, sonication of the sample was performed at room temperature in methanol. Different sonication times were tested and 30 min were found to be sufficient for a quantitative extraction.

#### 3.4.2. α-LA in dietary supplements

In both chromatographic methods, isocratic elution was carried out with different mobile phases and different column diameters to obtain a shorter run time for α-lipoic acid and bisphenol A. Representative chromatograms for α-lipoic acid in supplement ‘a’ are depicted in Fig. 4A and B. The relative standard deviation ($n = 3$) for the content of α-lipoic acid in supplement ‘a’ was lower then 10% for both methods.

Quantitative evaluation revealed that the experimentally calculated values matched the manufacturer’s claim only in case of

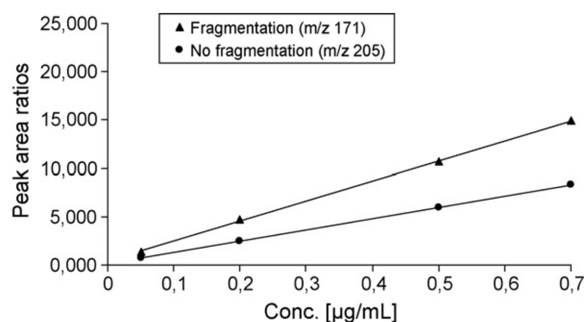


Fig. 3. Comparison of calibration curves obtained from two different modes in the negative ESI-MS system: (▲) complete fragmentation of α-LA molecular ion peak (*m/z* 171); (●) α-LA molecular ion peak (*m/z* 205).

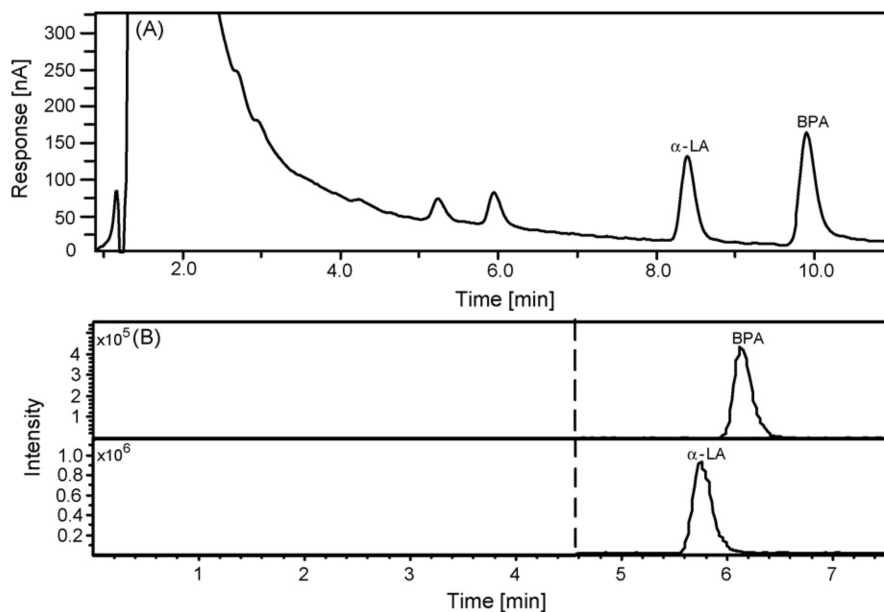


Fig. 4. Representative chromatograms of α-lipoic acid in supplement 'a' using high performance liquid chromatography with coulometric electrode array (A) and ESI-MS (B) detection. α-LA: α-lipoic acid fragment ion (*m*/*z* 171); BPA: bisphenol A (*m*/*z* 227).

supplement 'a' (Table 2). The contents of the supplements b, c, d and e determined by HPLC-CEAD and HPLC-ESI-MS showed a good correlation but were below the values claimed by the manufacturers. Additionally two supplements as representatives for a low and a high concentration (b and d) were investigated by HPLC-UV and corresponding results were obtained (Table 2). A wavelength of 215 nm was used because of better sensitivity than at 332 or 208 nm.

In order to check whether the matrix compounds influence the measured α-lipoic acid content or not, standard addition was performed. The recovery was determined by division of the slope of the regression lines, obtained using standard addition method, by the slope of the external calibration functions recorded in the same analysis run and multiplication by 100 [23] and was $96 \pm 2.1\%$ ($n = 3$). The data of the standard addition method confirm the results obtained by the external calibration method. The deviation between the claimed values and the data found could be due to improper handling during the production of the supplements or degradation of α-lipoic acid during storage.

## 4. Conclusion

Two rapid and reliable HPLC methods coupled with CEAD and ESI-MS detection were developed and validated for the quantitative determination of α-lipoic acid in dietary supplements. The sample preparation consists of sonication at room temperature and does not require solid-phase extraction and/or tedious derivatization steps. The CEAD mode using eight channels (working electrodes) set at different potentials has an advantage over amperometric, pulsed amperometric and dual coulometric electrode detection as α-lipoic acid can be detected at a comparable low potential. This is because, in coulometric electrode array detection, the optimal detection potential corresponds approximately to the half wave potential of the compound or to the potential at the peak maximum of the current/voltage curve (Fig. 1). Lower working electrode potentials are responsible for a decreased noise of the detector and a lower detection limit.

ESI-MS measurements applying the negative MRM mode offers, in the case of α-lipoic acid, the possibility of complete fragmentation of the $[M\text{-}H]^-$ ion into the fragment ion *m*/*z* 171 [24] which is responsible for the more sensitive measurement of this compound. The detection limit of α-lipoic acid in the HPLC-ESI-MS system is a bit lower than that of HPLC-CEAD. The highest detection limit was found for HPLC-UV (Table 1).

Rapid and simple sample preparation and a short run time make these methods highly useful for assaying large stocks of samples from different origin. In addition, the high sensitivity and selectivity of both methods could be of great importance in pharmacological studies where lower concentrations have to be determined.

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