Determination of neotame in non-alcoholic beverage by capillary zone electrophoresis

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Abstract

BACKGROUND: Neotame (NEO) is a new artificial sweetener approved as a food additive in many countries. The present method for the determination of NEO in various foodstuffs is high-performance liquid chromatography (HPLC). There are no reports on the determination of NEO in foods by capillary zone electrophoresis (CZE). Therefore a simple and rapid method to determine NEO is desired for quality control.

RESULTS: A CZE method combined with solid phase extraction was developed for the determination of NEO in non-alcoholic beverage. The optimum separation conditions obtained were 20 mmol L⁻¹ sodium borate buffer, pH 8, 25 kV applied voltage, 5 s hydrodynamic injection at 30 mbar and ultraviolet detection at 191 nm. The calibration curve showed good linearity ($R^2 = 1.000$) in the range 0.5–100 mg L⁻¹, and the limit of detection was 0.118 µg mL⁻¹. The method was successfully applied to the determination of NEO in two kinds of beverage with migration time less than 5 min, relative standard deviation ($n = 3$) less than 2% and recoveries ranging from 90 to 95%.

CONCLUSION: The proposed CZE method has the advantages of shorter analysis time and lower cost compared with HPLC, indicating that it may be a good alternative to the HPLC method.

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Keywords: artificial sweeteners; neotame; solid phase extraction; capillary zone electrophoresis

INTRODUCTION

Neotame, a dipeptide methyl ester derivative chemically related to aspartame, is a new artificial sweetener already approved as a food additive by the US Food and Drug Administration. Neotame has a sweetness factor that is approximately 7000–13 000 times greater than that of sucrose and approximately 30–60 times greater than that of sucrose, depending upon the particular food application. Its chemical structure is $N$-[3-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine-1-methyl ester (Fig. 1). The safety of neotame has been investigated and the results indicate that neotame is not carcinogenic, genotoxic, teratogenic or associated with any reproductive toxicity.2 Owing to its low cost, high-intensity sweetness, security, good stability and solubility, neotame has wide potential application as a second-generation dipeptide sweetener and can be used as a substitute for e.g. acesulfame-K, aspartame, cyclamate and saccharin in a number of countries, including China, Russia, Japan, Australia and New Zealand.

The main method used for the determination of neotame in various foodstuffs is high-performance liquid chromatography (HPLC). Among different HPLC detection methods, ultraviolet (UV) detection is the most widely used in routine testing. Owing to the high-intensity sweetness of neotame, the level added in foods is usually very low. Thus HPLC coupled with mass spectrometry (HPLC/MS) and HPLC with evaporative light-scattering detection (HPLC-ELSD) have been employed. However, compared with UV detection, these methods are more expensive.

Capillary electrophoresis (CE) is an analytical separation technique that generally offers shorter analysis time and is relatively economic, as the buffer salts and solvents required cost much less than those needed for HPLC. Owing to these advantages, CE is playing an increasingly important role in the fast growing field of sweetener analysis. Several papers have demonstrated the utility of CE for the analysis of many artificial sweeteners. At present, there are no reports on the determination of neotame in foods by CE. Therefore a simple and rapid method to determine neotame is highly desired for quality control.

In this study a simple capillary zone electrophoresis (CZE) method with UV detection was developed for the determination of neotame in non-alcoholic beverage. Compared with the national standard method (HPLC) for the determination of neotame in foods, the proposed CZE method has the advantages of shorter analytical time and lower cost and can be used as an alternative method for neotame analysis.

MATERIALS AND METHODS

Chemicals and reagents

HPLC-grade formic acid was purchased from Yongda Chemical Research Center (Tianjin, China). Other reagents used for the preparation of samples and running buffer were of analytical grade.

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Methanol, phosphoric acid and triethylamine were obtained from Kemel Chemical Co. (Tianjin, China). Boric acid was purchased from Bodi Chemical Co. (Tianjin, China). Sodium tetraborate was purchased from Guangcheng Chemical Co. (Tianjin, China). The water used in all studies was ultrapure water (18.25 MΩ cm) obtained from an ultrapure purification system (Yongjiieda, Hangzhou, China). The nitrogen used for evaporation of solvents was of 99.9% purity and supplied by Feiyuan Comprehensive Service Technology (Yantai, China). Agela Cleanert C18-SPE column cartridges (6 mL, 2000 mg) were purchased from Agela (Tianjin, China). All cartridges were conditioned with 10 mL of methanol followed by 15 mL of ultrapure water before use. Neotame (NEO) of >98% purity was purchased from Sanhe Biological Technology Co. (Weifang, China). The four sweeteners acesulfame-K (ACS-K), aspartame (ASP), cyclamate (CYC) and saccharin (SAC) were purchased from Xinghua Food Additive Co. (Zhengzhou, China). Beverage samples, i.e. cola drink and yoghurt, were purchased from local supermarkets (Yantai, China) and stored at 4 °C.

**Apparatus**

CZE analyses were carried out on a LUMEX CAPEL 105 Capillary Electrophoresis System (LUMEX Ltd, St Petersburg, Russia) equipped with a UV detector and Chrom&Spec Version 1.5x software for chromatography. A 60 cm (50.5 cm effective length), 75 µm i.d. fused silica capillary (Yongnian Ruifeng Chromatographic Devices Limited Company, Hebei, China) was used. A PH5500 dual-channel pH/ion meter (Crison, Singapore) was used for pH adjustment. The pressure-blowing concentrator used for evaporation of the solvent was purchased from Hengao Technology Development Co. (Tianjin, China).

**Standard and sample preparation**

A stock standard solution of NEO (1 mg mL⁻¹) was prepared by dissolving 0.01 g of NEO in ultrapure water in a 10 mL volumetric flask. A series of calibration solutions (0.5, 1, 5, 10, 50 and 100 µg mL⁻¹) was prepared by diluting the stock standard solution with ultrapure water. All samples were pretreated by solid phase extraction according to the national standard method (HPLC) for the determination of NEO in foods. A 10 mL sample (cola drink samples were completely degassed by ultrasonication before use) was extracted with 30 mL of a mixed solution of formic acid/triethylamine/ultrapure water (4:125:5000 v/v/v) in an ultrasonic bath for 15 min and made up to a volume of 50 mL. After centrifugation of the sample at 1250 g for 10 min, 20 mL of the supernatant was loaded into an Agela Cleanert C18-SPE cartridge. The cartridge was then rinsed with 10 mL of the mixed extraction solution. NEO was eluted with 10 mL of methanol at a flow rate of approximately 1–2 mL min⁻¹. The eluant was evaporated to dryness in a stream of nitrogen at 40 °C, reconstituted with 1 mL of ultrapure water and then homogenised thoroughly in a vortex mixer for 2 min. All solutions were stored at 4 °C and filtered through 0.45 µm Millipore filters before analysis.

**CZE conditions**

Each new capillary was rinsed sequentially with ultrapure water for 10 min, 0.5 mol L⁻¹ NaOH for 40 min, ultrapure water for 10 min and running buffer for 30 min. At the beginning of each day the capillary was conditioned with ultrapure water for 2 min, 0.2 mol L⁻¹ NaOH for 10 min, ultrapure water for 5 min and running buffer for 10 min. The capillary was conditioned only with running buffer for 2 min between consecutive injections. Pressure injection at the anodic side in hydrodynamic mode was set at 30 mbar for 5 s. Electrophoresis was carried out by applying a voltage of 25 kV to the capillary, with the cathode at the detector end. All solutions were filtered through 0.45 µm Millipore membrane filters before injection.

**RESULTS AND DISCUSSION**

**Optimisation of CE method**

The UV absorption spectrum of NEO aqueous solution from 190 to 400 nm was measured. As seen in Fig. 2, the maximum absorption of NEO occurred at a wavelength of 191 nm, so 191 nm was used as the detection wavelength in order to get maximum sensitivity. Both borate and phosphate were tested as the background electrolyte. The results showed that the borate buffer gave a better peak shape and shorter migration time than the phosphate buffer, so the borate buffer was selected for subsequent CZE analysis.

**Influence of buffer concentration**

Borate buffer at 10, 20, 30, 40 and 50 mmol L⁻¹ was investigated. With increasing buffer concentration, the analysis time lengthened. As shown in Fig. 3, with increasing buffer concentration, the migration time lengthened and the peak shape deteriorated.

**Results and discussion**

Figure 1. Structure of neotame.

![Structure of neotame](image1)

Figure 2. UV absorption spectrum of neotame from 190 to 400 nm.

![UV absorption spectrum of neotame](image2)
and the sensitivity improved slightly. In order to obtain a shorter migration time as well as a better signal-to-noise ratio, a buffer concentration of 20 mmol L\(^{-1}\) was selected.

### Influence of buffer pH

To verify the effects of running buffer pH on migration behaviour, experiments were performed using 20 mmol L\(^{-1}\) sodium borate running buffer at pH 7, 8, 9 and 10. At pH 7, NEO could not separate from the electro-osmotic flow, while at pH 10 it produced an asymmetric leading peak. The analysis time at pH 9 was longer than that at pH 8. Thus pH 8 proved to be ideal for the subsequent analysis.

### Influence of organic additives

Several organic solvents (100 mL L\(^{-1}\)), i.e. acetonitrile, acetone, methanol, ethanol, n-amyl alcohol and sodium dodecyl sulfate, were added separately to 20 mmol L\(^{-1}\) borate buffer at pH 8. However, their presence led to no obvious improvement in peak shape. On the contrary, the migration time increased. Therefore no organic modifier was selected.

### Influence of injection time

Considering the low concentration of NEO added to the samples, different hydrodynamic injection times of 1, 3, 5, 8 and 10 s at 30 mbar were further studied in order to obtain better sensitivity and peak shape. The results indicated that 5 s was a good choice.

From the above results the following CZE separation conditions were selected: 20 mmol L\(^{-1}\) sodium borate (pH 8), 5 s pressure injection at 30 mbar, 25 kV applied voltage at 25 °C, detection at 191 nm. The system suitability parameters migration time, tailing factor and theoretical plate number for NEO were 4.18 ± 0.01 min, 0.91 ± 0.02 and 881 752 ± 11 305 respectively. A typical electropherogram of NEO standard is shown in Fig. 3a. Under these conditions, good separation of NEO from the other four artificial sweeteners ACS-K, ASP, CYC and SAC was also achieved as shown in Fig. 3b. The reason for choosing these artificial sweeteners for separation is that they are commonly used as food additives together with NEO.

### Validation of CZE method

#### Linearity, limit of detection and limit of quantification of method

Calibration curves were obtained by plotting peak area (A) as a function of NEO concentration (C). The obtained correlation was \(A = 4.4422C + 0.3533\) \((R^2 = 1.000)\) and the linearity range was 0.5–100 µg mL\(^{-1}\). The limit of detection (LOD) and limit of quantification (LOQ) were determined as 3σ/s and 10σ/s respectively, where σ is the standard deviation of the blank responses and s is the slope of the calibration curve. The LOD and LOQ were 0.118 and 0.395 µg mL\(^{-1}\) respectively.

#### Precision and accuracy study

In order to investigate the precision of the proposed method, the intra-day and inter-day repeatability of migration time and peak area were determined under the selected experimental conditions. The intra-day repeatability was determined by replicate injections \((n = 6)\) of a 10 µg mL\(^{-1}\) solution of NEO in one day, while the inter-day repeatability was determined by performing injections for six consecutive days. In all cases the relative standard deviation (RSD) was lower than 2.2% for the migration time and less than 2.5% for the peak area.

The accuracy of the method was tested with fortified samples of two representative food products: cola drink and yoghurt. Before fortification, blank samples were analysed in order to determine the actual content of NEO in the samples. To determine the accuracy, each sample was divided into three subsamples spiked at different levels. Each subsample was analysed in triplicate according to the procedure described above. The results are presented in Table 1. Satisfactory recoveries (90.3–95.1%), with RSD below 2%, were obtained regardless of the type of sample matrix and the spiking level.

### Sample analysis

As we know, NEO is present in real samples at very low concentration. The complex matrix of food samples also makes NEO difficult to determine accurately. To overcome this limitation,
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**Figure 4.** Electropherograms of (a) yogurt sample, (b) yogurt sample spiked with 0.5 µg mL⁻¹ neotame, (c) cola drink sample and (d) cola drink sample spiked with 0.5 µg mL⁻¹ neotame. CZE conditions: same as in Fig. 3.

**Table 1.** Quantitation of neotame in beverage samples (n = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of NEO (µg mL⁻¹)</th>
<th>Added (µg mL⁻¹)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cola drink</td>
<td>1.00 ± 0.011</td>
<td>0.50</td>
<td>92.3</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>95.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00</td>
<td>93.4</td>
<td></td>
</tr>
<tr>
<td>Yoghurt</td>
<td>1.14 ± 0.018</td>
<td>0.50</td>
<td>90.3</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>90.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00</td>
<td>93.2</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean ± standard deviation of three measurements.

some preconcentration and purification approaches have been developed for NEO analysis.¹³

In this experiment, all samples were pretreated by solid phase extraction before analysis, and the optimised CE method was applied to analyse NEO in beverage samples. Typical electropherograms of the samples are shown in Fig. 4. The results satisfactorily demonstrated that this method was applicable for the quantification of NEO with high accuracy (Table 1).

**CONCLUSIONS**

A simple CZE method for the determination of NEO in beverage has been described. Compared with the national standard method (HPLC) for the determination of NEO in foods,²² the proposed CZE method has the advantages of shorter analysis time and lower cost, indicating that it maybe a good alternative to the HPLC method for NEO determination.

**ACKNOWLEDGEMENT**

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**REFERENCES**


