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# Development of HPLC method for aspartame, L-aspartyl-D-phenylalanine methyl ester, and 5-benzyl-3,6-dioxo-2-piperazine acetic acid using relative molar sensitivity based on $^{1}$H-qNMR

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## Abstract

Aspartame is a widely used artificial sweetener. For quality control purposes, it is necessary to quantify the impurities L-aspartyl-D-phenylalanine methyl ester (L, D-APM) and 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP). However, the sale of L, D-APM standards has been temporarily suspended in Japan, leading to significant analytical challenges for the conventional HPLC method. Additionally, a method that can simultaneously quantify aspartame, L, D-APM, and DKP to improve analytical efficiency is required. In this study, we developed a method for the simultaneous analysis of aspartame, L, D-APM, and DKP using the relative molar sensitivity (RMS) based on $^{1}$H-quantitative nuclear magnetic resonance ($^{1}$H-qNMR) spectroscopy. Standard solutions prepared based on the accurate purity of each compound determined by $^{1}$H-qNMR were analyzed by HPLC, and the RMS values for aspartame were calculated from the slope ratio of the calibration curve. In method validation using samples spiked with aspartame, no statistically significant differences were observed between the RMS method and the conventional absolute calibration method, demonstrating excellent precision. The proposed method enables reliable simultaneous quantification without the use of challenging to obtain quantitative standards while maintaining compatibility with existing regulatory HPLC conditions and improving analytical efficiency. Furthermore, it could significantly improve the quality control of aspartame.

Keywords : aspartame, L-aspartyl-D-phenylalanine methyl ester, 5-benzyl-3,6-dioxo-2-piperazine acetic acid, relative molar sensitivity, HPLC

## I Introduction

Aspartame, the methyl ester of a dipeptide (L-aspartyl-L-phenylalanine methyl ester) comprising L-aspartic acid and L-phenylalanine (Fig. 1), was discovered in 1965.[1, 2)] It is a sweetener with a sweetness level of approximately 200 times that of sucrose, is known to have an energy value of 4 kcal/g and is characterized by a refreshing natural sweetness.[1-3)] Although aspartame has the physicochemical property of being unstable against heat,[3)] it is used in various applications from foods, such as soft drinks, dairy products, and confectionery, to more than 600 types of pharmaceutical products.[4, 5)] For safety evaluation of aspartame, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set an acceptable daily intake of 40 mg per kg of body weight per day (mg/kg bw/day). In addition, the U.S. Food and Drug Administration has set a daily intake limit of 50 mg/kg bw/day.[6)] The safety of the use of aspartame within these limits has been confirmed. In Japan, to ensure the quality and safety of aspartame, Japan's Specifications and Standards for Food Additives[7)] has established the following standards based on safety assessments: (1) the allowable aspartame content in food additive products (98.0-102.0%), (2) the maximum permissible levels of by-products originating from raw materials or generated during manufacturing [e.g., lead, arsenic, 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP), and L-aspartyl-D-phenylalanine methyl ester (L, D-APM)] (Fig.

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