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Determination of Bitter Orange alkaloids in dietary supplements standard reference materials by liquid chromatography with ultraviolet absorbance and fluorescence detection

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Abstract

Four adrenergic amines [synephrine, octopamine, tyramine, and *n*-methyltyramine] were determined in a variety of Bitter Orange containing dietary supplements. Two extraction techniques were evaluated in detail: Soxhlet extraction and sonication extraction. A liquid chromatographic separation using a reversed-phase C_{18} stationary phase and the ion-pairing reagent sodium dodecyl sulfate was developed to separate the Bitter Orange alkaloids. Ultraviolet absorbance detection at 220 nm and fluorescence detection with excitation at 273 nm and emission at 304 nm were used for the alkaloid detection. The method described was used for the assignment of the levels of the predominant alkaloids in three candidate standard reference materials containing Bitter Orange.

Keywords: *Citrus aurantium*; Bitter Orange alkaloids; Synephrine; Standard reference material; Fluorescence detection

1. Introduction

The terms Bitter Orange, sour orange, and Seville orange are commonly used to describe the citrus species *Citrus aurantium* and its fruits. Different *C. aurantium* varieties exist; the variety *C. aurantium* subspecies *amara* is an evergreen tree native to southern Vietnam. The botanical is also widely cultivated throughout the Mediterranean region. Various parts of the citrus tree are used in traditional herbal medicine to treat a range of health problems [1]. For example, Asian herbal medicine uses the entire unripe and dried fruit as an aid to digestion, whereas the leaves are used in South America and Mexico as a sedative. The essential oils of the fruits are used in liquors and in perfume.

Recently dietary supplements formulated with Bitter Orange have been marketed as an appetite suppressant to support weight loss. To a large extent, these products have replaced ephedra (*Ephedra sinica*) containing dietary supplements, which were banned by the Food and Drug Administration after association with strokes, heart attacks, and other severe adverse health effects [2,3]. These serious side effects have been attributed to certain adrenergic amines (i.e., ephedrine, norephedrine, pseudoephedrine) in ephedra [4,5]. The unripe fruits of *C. aurantium* contain several different adrenergic amines which differ in the number and position of hydroxyl substituents and include synephrine, octopamine, tyramine, *n*-methyltyramine, and hordenine (for structures see Fig. 1) [6]. Synephrine is the primary alkaloid found in the immature fruits, whereas the other alkaloids are present at significantly lower levels. Of these alkaloids, synephrine and octopamine exhibit the greatest activity. All of the Bitter Orange alkaloids raise the metabolic rate and the rate of oxidation of fat; however, synephrine and octopamine selectively activate the β-3 adrenoreceptors and appear to inhibit cAMP production [7–9]. Such receptors may be responsible for the thermogenic effects that influence the oxidation of fat. Reduced food intake, body slimming, and an alteration of electrocardiogram (ECG) parameters were observed after an intake of Bitter Orange extracts [10]. Recently increased concern about potential health risks associated with Bitter Orange-containing dietary supplements have been expressed, including elevated blood pressure and adverse cardiac effects [1]. The Bitter Orange fruits also contain high levels of flavonoids [11].

Several analytical approaches for the determination of synephrine in plant material, plant extract, juice, and dietary

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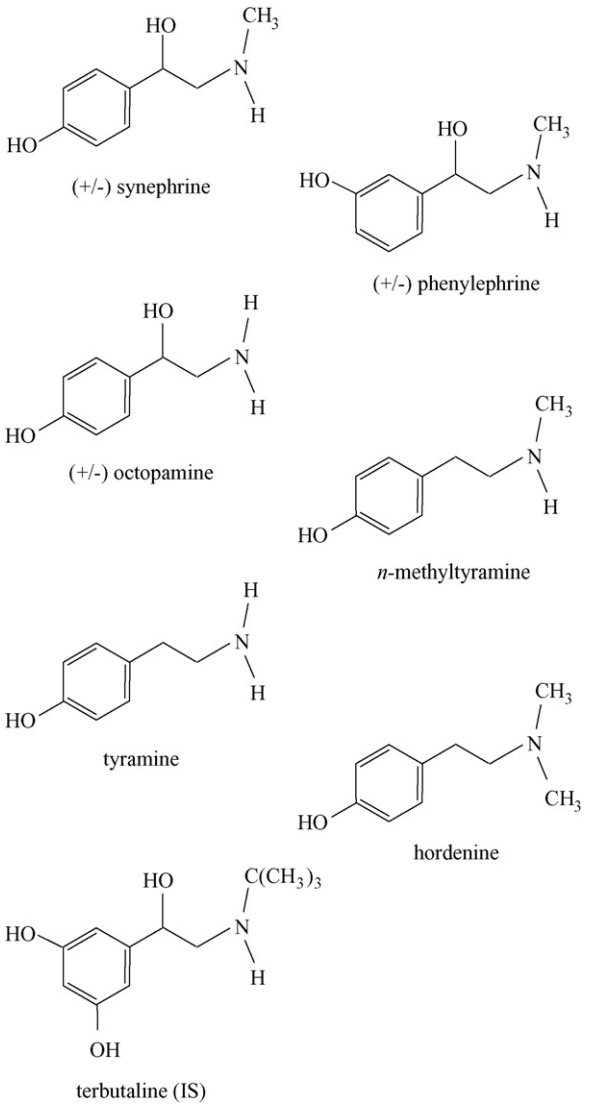

Fig. 1. Structures of the natural Bitter Orange alkaloids found in *Citrus aurantium* (terbutaline and phenylephrine are reagents that were evaluated for use as internal standards).

supplements have been published. Typically, methods utilize liquid chromatography (LC) with ultraviolet absorbance detection (UV) [6,12,13], fluorescence detection (FL) [14], or mass spectrometry (MS) [15–18]. Ganzera et al. [6] determined ephedrine and Bitter Orange alkaloids in plant materials and dietary supplements by ion-pair chromatography on a reversed-phase (RP) C_{18} column. The method employed a complex quaternary solvent mixture consisting of 3 mmol/L aqueous sodium dodecyl sulfate (SDS) pH 4, 0.1% phosphoric acid containing 3 mmol/L SDS pH 2, and a mixture of acetonitrile and methanol. Alkaloids were extracted by sonication with hydrochloric acid at ambient temperature. Pellati et al. [12] used a Lichrospher RP-C_{18} column and an isocratic mixture of citric acid and sodium phosphate at pH 3 to separate synephrine, octopamine, and tyramine from fruits, extracts, and herbal products. The alkaloids were extracted with water at ambient temperature. Analyte retention was quite limited and peak tailing was observed for synephrine and tyramine. Niemann and Gay [14] described the separation of ephedrine alkaloids and synephrine from dietary supplements by employing LC column switching with two strong cation exchange (SCX) columns with UV absorbance and FL detection. The first SCX column was used as an on-line sample cleanup to simplify the sample preparation. Gay et al. [16] and Sander et al. [18] described the separation of ephedrine alkaloids and synephrine from a variety of samples with RP phenyl columns and MS detection. These methods lack the capability to resolve the Bitter Orange alkaloids with sufficient selectivity and/or sensitivity to permit the determination of both the major and minor Bitter Orange alkaloids.

This report describes a new LC/UV/FL method that uses the ion-pair reagent SDS to separate the Bitter Orange alkaloids on an inert RP C_{18} column. The separation was monitored with UV absorbance detection at 220 nm and with fluorescence detection with excitation at 273 nm and emission at 304 nm. Three candidate Standard Reference Materials (SRMs) were investigated: SRM 3258 Bitter Orange (Fruit), SRM 3259 Bitter Orange extract, and SRM 3260 Bitter Orange-containing solid oral dosage form. Two extraction approaches were evaluated, and levels of synephrine and three minor alkaloids reported for the candidate SRMs. Data obtained using these methods will be combined with LC/MS results and results reported by collaborating laboratories to assign certified values on these materials. This effort is part of an ongoing collaboration between the National Institute of Standards and Technology (NIST), the National Institutes of Health, Office of Dietary Supplements (NIH/ODS), and the Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) to develop dietary supplement SRMs [19].

2. Experimental

Certain commercial equipment, instruments, or material are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

2.1. Reagents

HPLC-grade acetonitrile, methanol, and water were used without further purification. The Bitter Orange reference standards [synephrine, octopamine HCl, tyramine, *n*-methyltyramine, and hordenine sulfate] as well as the possible internal standard phenylephrine HCl were purchased from ChromaDex (Santa Ana, CA, USA). The internal standard terbutaline hemisulfate salt was obtained from Sigma (St. Louis, MO, USA). Ammonium hydroxide, hydrochloric acid, and glacial acetic acid were analytical grade. Diatomaceous earth (Hydromatrix) for the pressurized fluid extraction (PFE) was obtained from Isco (Lincoln, NE, USA). Sodium dodecyl sulfate from Polyscience Inc. (Warrington, PA, USA) was used for mobile phase preparation.