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Short communication

Determination of *Citrus aurantium* protoalkaloids using HPLC with acidic potassium permanganate chemiluminescence detection

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ABSTRACT

Acidic potassium permanganate chemiluminescence was explored as a sensitive and selective mode of detection for phenolic phenethylamines (adrenergic amines) in consumer products containing *Citrus aurantium* extracts. Nine commercially available weight-loss products were analysed using rapid reversed-phase chromatography with a monolithic column (separation time of 4 min). The results were in good agreement with package labelling, with some notable exceptions. The products contained a wide concentration range of synephrine and total adrenergic amines, and the difference in consumer intake was even greater when the manufacturers' recommended daily consumption was considered. The quantity of the extract, often specified on the packaging as equivalent grams of dry *C. aurantium* fruit, was a poor indicator of the concentration of the active ingredients. Methionine, a thioether amino acid contained in some weight-loss products, was identified as a potential interferent for this mode of detection.

1. Introduction

The U.S. Food and Drug Administration's ban of ephedrine (Fig. 1) and related *Ephedra* protoalkaloids as ingredients in weight-loss products, due to a long association with strokes, heart attacks, and other adverse health effects, has led to a dramatic increase in the use of *Citrus aurantium* (Seville orange; Bitter orange) [1]. Extracts from the unripe fruit or peel of *C. aurantium* are rich in synephrine (oxedrine), and contain several other phenolic phenethylamines, including octopamine, tyramine, *N*-methyltyramine and hordenine (Fig. 1), that each posses adrenergic activity. There is a long history of *C. aurantium* consumption in herbal medicine, but its contemporary use may involve much higher daily intakes. Ingredients of weight-loss products can include highly concentrated extracts (up to 95% synephrine), pure individual adrenergic amines (octopamine, tyramine and/or hordenine), and herbs that contain high levels of caffeine. Concern has been expressed about the safety of these products [2].

As described in a recent review by Pellati and Benvenuti [3], many chromatographic and electrophoretic methods for the determination of synephrine (and to a lesser extent, octopamine and tyramine) have emerged, but relatively few have included quantitation of the five adrenergic amines detected in *C. aurantium* extracts. However, when examined, *N*-methyltyramine has been found at much higher concentrations than octopamine, tyramine or hordenine [4,5]. Previous chromatographic procedures have involved reversed-phase and ion-pairing separations using C_{18} columns with UV-absorbance [6–8] or fluorescence [9] detection; or separation on pentafluorophenylpropyl columns using mobile phases containing high levels of organic solvent, with UV-absorbance [4], mass spectrometric [10] or tandem mass spectrometric [5] detection.

Flow-injection analysis procedures for the determination of synephrine based on its enhancing effect on the chemiluminescent reactions of luminol with hexacyanoferrate(III) [11], and cerium(IV) with rhodamine B [12] have been reported, but these procedures are not sufficiently selective towards the target analyte in the presence of other phenolic compounds [13]. Acidic potassium permanganate can also be used for sensitive chemiluminescence detection of phenolic compounds [14,15] and has been coupled to chromatographic separations for the determination of *Papaver somniferum* (opium poppy) alkaloids in process liquors [16,17], neurotransmitter metabolites in urine [18], and synephrine in plant extracts and dietary supplements [19]. The key advantages of this mode of detection, compared to UV-absorbance and fluorescence, include the relatively high sensitivity and greater selectivity, which reduces the number of interfering compounds

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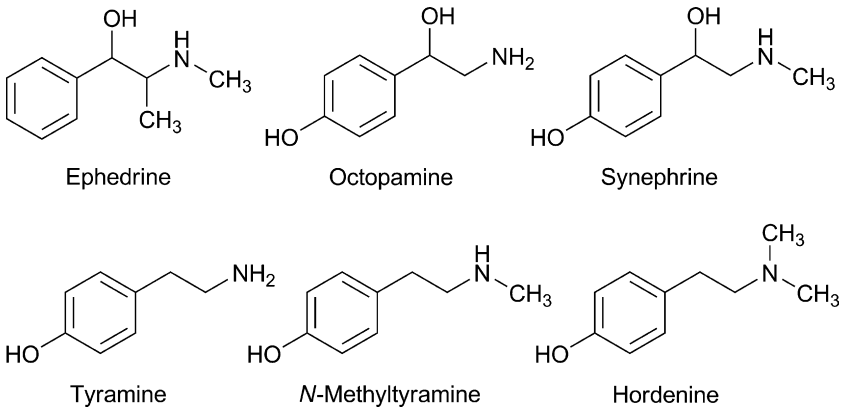

Fig. 1. Ephedrine (found in *Ephedra* species) and five phenolic phenethylamines found in *Citrus aurantium*.

that must be separated from the target analytes. However, the relationship between analyte structure and chemiluminescence intensity is yet to be fully elucidated [14]. Further investigation of potential interferences in complex sample matrices such as biological fluids and plant extracts is required to realise the full potential of chemiluminescence detection. In this study, we utilise monolithic column chromatography to explore acidic potassium permanganate chemiluminescence detection of the five bioactive phenethylamines found in weight-loss products containing *C. aurantium*.

2. Materials and methods

2.1. FIA and HPLC conditions

The flow-injection analysis (FIA) instrumentation was configured as previously described [19]. Each standard solution (70 μL) was injected into a water carrier stream that merged with the acidic potassium permanganate reagent stream at a T-fitting prior to entering a coiled transparent flow-cell, positioned against a photomultiplier tube within a light-tight housing. The solution flow rate was 3 mL/min per line. In the HPLC experiments, the column eluate (instead of the FIA carrier stream) was merged with the acidic potassium permanganate reagent stream within the chemiluminescence detector. The adrenergic amines were separated using a monolithic column (Chromolith SpeedROD RP-18e, 50 mm × 4.6 mm i.d., with 5 mm × 4.6 mm i.d. guard) under isocratic conditions (5 min: 98% deionised water adjusted to pH 2.15 with trifluoroacetic acid; 2% methanol). The column was flushed after each run, by increasing the methanol to 80% over 1 min and maintaining that concentration for 12 min, and was then re-equilibrated at the separation conditions. The flow-rate through the column was 1 mL/min and the injection volume was 10 μL.

2.2. Other instrumentation

^{1}H NMR was performed with a JEOL EX 270 MHz FT-NMR spectrometer, with samples dissolved in $CDCl_3$. Peaks were recorded as: chemical shift δ (ppm) (integral, multiplicity (s = singlet, d = doublet, m = multiplet), coupling constant J (Hz), assignment). Melting point was obtained using a heated block (SMP3, Stuart Scientific, UK). A 6210 MSDTOF mass spectrometer (Agilent Technologies, Australia) was used with the following conditions: drying gas: nitrogen (7 mL/min, 350 °C); nebuliser gas: nitrogen (16 psi); capillary voltage: 4.0 kV; vaporizer temperature: 350 °C; cone voltage: 60 V.

2.3. Reagents, standards and samples

The chemiluminescence reagent contained 1×10^{-3} M potassium permanganate (Chem-Supply, Australia) and 1% (w/v) sodium polyphosphate (Sigma–Aldrich, Australia) in deionised water and was adjusted to pH 2.5 with sulfuric acid (Merck, Australia). Synephrine, octopamine and tyramine were purchased from Sigma–Aldrich. Hordenine was synthesised using a modified Eschweiler Clark *N*-methylation reaction [20]: tyramine (1.01 g, 7.4 mmol) in methanol (50 mL) and formaldehyde (36% solution; 5.45 mL) was heated to reflux for 90 min, then cooled and stirred overnight. Sodium borohydride (1 g, 26.4 mmol) was added slowly over 30 min and stirred for a further 90 min. The solvent was removed *in vacuo*. The solid was then dissolved in water (50 mL) and extracted with chloroform (4 × 40 mL). The organic extracts were combined and dried with anhydrous sodium sulfate, filtered, and the solvent was removed. The crude product was recrystallised with 1:1 ethyl acetate:petroleum spirits to afford hordenine (615.8 mg, 51% yield; m.p. 117.3–118.1 °C, Lit. 117–118 °C [21]; MS: m/z 166.12559 $[M+H]^+$, calc. 166.12264. ^{1}H NMR (270 MHz, $CDCl_3$): δ 2.30 (6H, s, $N(CH_3)_2$), 2.55 (2H, m, $ArCH_2CH_2N$), 2.69 (2H, m, $ArCH_2CH_2N$), 6.62 (2H, d, J = 8.40, Ar), 6.99 (2H, d, J = 8.40, Ar). *N*-Methyltyramine was supplied by CSIRO Animal Health Laboratories, Australia.

The weight-loss products were supplied by or purchased from manufacturers in the USA and Australia. Tablets were ground with mortar and pestle. The powders from capsules were removed from their outer coating. A previous comparison of the effect of various solvents on the extraction of *C. aurantium* protoalkaloids from dietary supplements (using sonication or stirring with a magnetic bar) [4] revealed that water is more efficient than 0.1 M HCl, methanol or a mixture of 37% HCl and methanol (0.8:99.2, v/v). In a related study [9] it was shown that aqueous HCl (0.37% mass fraction) extracted greater quantities of the amines than methanol or acidified methanol (using sonication), but neutral aqueous solutions were not examined. In this study, samples were dissolved in a known volume of water (50–250 mL) by stirring for 45 min with a magnetic stir bar. The sample solutions were diluted with deionised water (if required) and filtered with Acrodisc PSF syringe filters (Pall Australia).

3. Results and discussion

3.1. Method development

N-Methyltyramine had not previously been detected with acidic potassium permanganate, and therefore the reaction was initially