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Solid phase extraction/cyclodextrin-modified micellar electrokinetic chromatography for the analysis of melatonin and related indole compounds in plants

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ABSTRACT

The identification of melatonin in plants has inspired new investigations to understand its biological function. In this work, a robust, reliable, low-cost, quick and simple method based on solid phase extraction followed by micellar electrokinetic chromatography for the extraction, preconcentration and simultaneous determination of melatonin, tryptophan, serotonin and indole-3-acetic acid in plant material is proposed. Extraction of indole compounds from plant tissues was enhanced by ultrasound and solid phase extraction, which was carried out with C_8 SPE cartridges. The use of a dual pseudostationary phase system, involving a mixture of SDS anionic micelles and β-cyclodextrin, enabled to reach adequate selectivity. A BGE of 10 mM sodium tetraborate (pH 9.2), containing 20 mM β-CD, 20 mM SDS, and 10% (*v*/*v*) of acetonitrile, allowed baseline separation in less than 10 min. The proposed methodology provided limits of detection (LODs) down to low ppb levels. Under the optimal conditions, a successful application on Arabidopsis tissue, green, and linden tea leaves confirmed the validity of this method for food analysis and as a tool to contribute to the elucidation of the biological role of melatonin in plants.

1. Introduction

Melatonin (MT, N-acetyl-5-methoxytryptamine) is a ubiquitous molecule showing multiple mechanisms of action and functions in practically every living organism. It can be found in evolutionarily distant organisms: bacteria, mono- and multicellular algae, fungi, higher plants, invertebrates and vertebrates [1]. In addition, it is currently known that in some beverages, yeast can produce melatonin isomers [2,3]. MT has a spectrum of important properties and plays several crucial physiological roles, its antioxidant activity against a variety of toxic oxygen and nitrogen species has been well documented [4]. It also plays a key role in the circadian and seasonal rhythms and can act in animals as an anti-inflammatory and immunomodulator, showing antitumoral and anticancerigenic functions [5]. Very recently, it has been proposed the use of melatonin for the treatment of Ebola virus infection [6].

In plants, indoleamines regulate a variety of physiological functions during the growth, morphogenesis and stress-induced responses. Plant's melatonin has been evaluated in relation to several physiological aspects, including its role as circadian regulator, cytoprotector, growth promoter, organogenic agent, and stress protector against biotic and abiotic stimuli [7–10]. Experimental evidence indicates that MT biosynthesis pathway is similar in different organisms. Further studies using radioisotope tracer techniques indicated that, in higher plants, tryptophan (Trp) is the common precursor for melatonin (MT), serotonin (5-HT), and indole-3-acetic acid (IAA) [1,11].

Considering its low concentrations in plant tissues, various methods for melatonin extraction, purification, and determination have been employed. Chemical complexity of plant's extract can interfere with MT determinations, giving false positive results if methods from vertebrate's MT research are directly adopted, for example, due to coelution in LC or cross-reactivity with antibodies of immunological methods like RIA or ELISA [11,12]. Several analytical techniques have been developed. In the case of GC-MS, derivatization of MT using silanizing agents or pentafluoropropionic anhydride to form trimethylsilyl or pentafluoropropionyl melatonin; respectively, prior to analysis is necessary [13]. HPLC is a widely used technique for the analysis of melatonin, isocratic elution with a reversed phased is the most commonly used. In this case, different detectors such as fluorescence (FI) [14–17], electrochemical (ECD) [18,19], and UV [20–22] have been used. With the development of MS interface technology,

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high performance liquid chromatography coupled to mass spectrometry (HPLC–MS) has increased in popularity and has proved to be a powerful tool for complex sample analysis [2,3,23–25].

Recently, capillary electrophoresis (CE) has been applied in MT separation and determination [26–29]. High efficiency, minimal sample volume, low cost, and relatively short analysis time make CE a promising separation technique. Measurements have been carried out using mainly ECD, UV and Fl detection [30]. Separation of neutral compounds in CE is possible using micellar electrokinetic chromatography (MEKC) due to the presence of the pseudostationary phase (PS) in the BGE [31]. Various kinds of compounds have been employed as the PS. Sodium dodecyl sulfate (SDS), an anionic surfactant, is one of the most popular choice; however, cationic and macromolecular surfactants as well as cyclodextrins (CD) have also been used. The addition of a CD to the BGE alters the apparent retention factor of the analytes by introducing an additional equilibrium (the complex-formation) to the system [32]. Therefore, by addition of a CD to the BGE, the apparent distribution coefficient $K_{D,app}$ of the analyte between the micellar pseudophase and the aqueous phase is reduced by increasing the fraction of analyte in the non-micellar phase resulting in a significant decrease in the apparent retention factor $k_{BGE,app}$ [33,34]. Moreover, the CD can form an inclusion complex with the SDS monomer and hence the micellization of SDS molecules is affected resulting in an increase of the apparent critical micelle concentration (CMC_{app}) of SDS, which is another reason for the significant decrease of $k_{BGE,app}$ upon addition of CD to the BGE [35–38]. At the moment, to our knowledge, there are no reports dealing with the simultaneous determination of MT, Trp, 5-HT and IAA taking advantages of the separation abilities of dual pseudostationary phases in CE. Indeed, the applications of capillary electrophoresis to the analysis of melatonin in plants have been weakly exploited.

2. Experimental

2.1. Instrumentation and conditions

Separations were carried out using a Capel™ 105M (Lumex, St Petersburg, Russia) equipped with an UV detector and a 0–25 kV high-voltage power supply. The data were collected on a PC configured with Elforun software version 3.2.2. The capillary columns used for separation were bare fused-silica capillaries 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD from MTC MicroSolv Technology Corporation (Eatontow, USA). The capillary tube was conditioned daily prior to its use by flushing with water (2 min), 0.10 mol L^{-1} NaOH for 3 min, followed by water for another 2 min and, finally, with the running buffer for 4 min. The separation voltage was 20 kV and the capillary temperature was 25 °C. Samples were injected by hydrodynamic injection at 30 mbar for 3 s. Electropherograms were recorded at 220 nm. Between runs, the capillary was flushed with water (2 min), 0.10 mol L^{-1} NaOH (2 min), water (2 min) and fresh buffer (2 min). The capillary tube was rinsed with 0.10 mol L^{-1} NaOH for 10 min, then with water for 10 min, every day after use.

2.2. Reagents and solutions

Melatonin, serotonin, L-tryptophan, indole-3-acetic acid, isopropyl alcohol (IPA), methanol (MEOH), β cyclodextrin (β CD) and γ-cyclodextrin (γ-CD) were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was from Merck (Buenos Aires, Argentina), sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) from Mallinckrodt (St. Louis, MO), and sodium dodecyl sulfate was supplied by Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan). Boric acid was purchased from JT Baker (Xalostoc, Mexico).

Stock standard solutions were prepared by dissolution of each compound (50 mg L^{-1}) in MEOH/water (0.01% *v*/*v*) and were diluted appropriately with pure water prior to use. The running buffer was sodium tetraborate or borate buffer containing multiple buffer additives, SDS, ACN, MEOH, IPA, β-CD or γ-CD, at the desired concentrations. The water used in all studies was ultra-high-quality water obtained from a Barnstead Easy pure RF compact ultrapure water system. All solutions were degassed by ultrasonication (200 W, 15 °C; Cleanson 1106, Buenos Aires, Argentina) for 10 min. The running electrolytes and samples were filtered through 0.22 μm PTFE syringe filters (Osmonics®). Strata C_8 (500 mg/6 mL), Strata C_{18} (200 mg/6 mL), and Strata-X (200 mg/6 mL) were purchased from Phenomenex® (Torrance, CA, USA).

Limits of detection and quantification were estimated from peak areas, where signal to noise ratios of 3 and 10; respectively, were used.

2.3. Sample treatment and SPE procedure

The extracts were prepared from leaves (0.60 g) of lyophilized *Arabidopsis thaliana* tissue or commercial tea leaves (*Camellia sinensis* and *Tilia cordata*). Vegetal tissues were transferred to 15 mL glass tubes. After that, 8 mL of 50% (*v*/*v*) methanol–water were added to each sample and then, tubes were vortexed during 30 s. Ultrasonication was employed to assist and accelerate the extraction of the analytes in an ultrasonic bath (200 W, 15 °C) filled with cold water for 10 min. The supernatant was decanted and centrifuged for 5 min at 3500 rpm (1852.2 g). The resulting extract was filtered through a 0.22 μm PTFE syringe filter.

The solid phase extraction/preconcentration step was carried out as follows: C_8 cartridges were preconditioned with 1 mL of MeOH and 1 mL of ultrapure water. After the conditioning step, an aliquot of 6 mL of plant extract was loaded into the cartridge. The retained analytes were eluted with 2 mL of MeOH. This eluate was evaporated to dryness under a gentle stream of nitrogen (to prevent analyte degradation), and the residue was reconstituted in 300 μL water: methanol (50:50 *v*/*v*). Then, this extract was filtered through a 0.22 μm PTFE syringe filter before injecting into the CE system.

3. Results and discussion

The analytes were selected taking into account the natural products sharing MT biosynthesis in plants. In higher plants, tryptophan is the common precursor for melatonin, serotonin, and indole-3-acetic acid. Fig. 1 shows the structures and pKa values of the target analytes. Considering that MT is neutral in a wide range of pH, capillary zone electrophoresis mode is not suitable for the separation of the analytes. Thus, a CE chromatographic separation mode is mandatory.

3.1. Separation optimization

The effects of several experimental parameters upon the separation parameters have been thoroughly evaluated and optimized. The optimization of the experimental conditions has been accomplished by the traditional method of one-at-a-time.

3.1.1. Micellar electrokinetic chromatography

The separation mechanism in MEKC is an interesting interaction between chromatography and electrophoresis. The development of a MEKC approach involves optimizing the surfactant, organic solvents and buffer types and concentrations. Organic modifiers are very important to improve separation in many systems because they can change the partition coefficient of the analytes.

The following parameters were consecutively optimized: buffer pH, buffer concentration, surfactant concentration, and organic modifier type and concentration. Therefore, sodium borate as well as sodium tetraborate solutions (5–10 mM) at different pH values: 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5 were tested. The SDS concentration was varied from 10 to 50 mM. Methanol, acetonitrile, isopropyl alcohol, as well as different ACN/MEOH mixtures were evaluated as organic modifiers to