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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

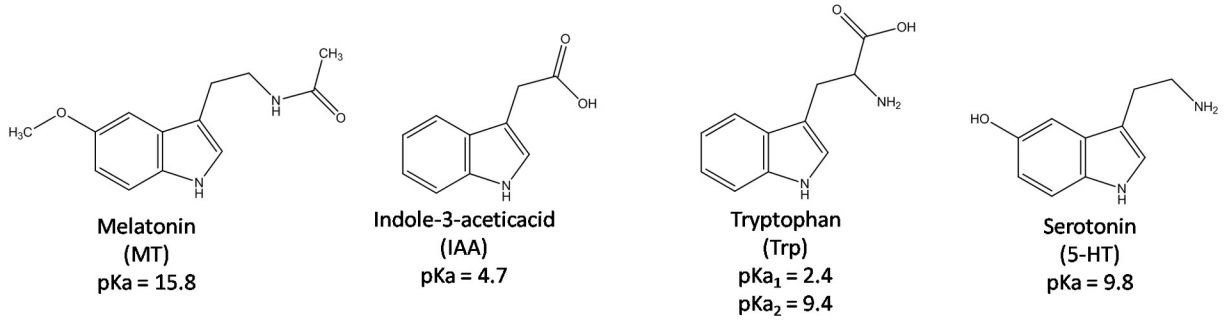

Fig. 1. Molecular structures and pKa values of the target analytes.

enhance the resolution of the analytes under study. Various amounts of organic modifier (5, 10, and 15%) were added into the buffer containing SDS. All buffers provided baseline-separation of L-Trp and IAA; however, MT and 5-HT coeluted. From all the possible combinations, the best results were obtained for the following conditions: sodium tetraborate 10 mM, pH = 9.50 containing 20 mM SDS, 10% ACN (*v*/*v*) as shown in Fig. 2. Thus, a 20 mM sodium dihydrogenphosphate–sodium tetraborate buffer solution, pH 9.5, was used as BGE for further study.

3.1.2. Cyclodextrin modified capillary electrophoresis (CD-CE)

Cyclodextrins have the ability to form selective host–guest inclusion complexes with solutes. So with the attempt to establish the simultaneous determination of MT, L-Trp, IAA; and 5-HT, the chromatographic mode CD-CE was considered. Hence, β-CD and γ-CD individually, as well as, a dual CD system were studied as running buffer modifiers. The concentrations were varied between 10 and 30 mM. Additionally, the effect of organic solvents such as MEOH, ACN and IPA (range 5–15% *v*/*v*) was also tested. The addition of γ-CD resulted in very poor peak shapes; the cavity size may be too big to form stable inclusion complexes with the indolamines under study. The combination of β-CD with γ-CD did not lead to satisfactory results in terms of baseline separation and/or adequate peak shapes. Although the analytes could be separated with a background electrolyte containing 10 mM sodium tetraborate, 20 mM β-CD with and without the addition of organic solvents including MEOH, ACN and IPA, MT peak shape was not satisfactory for the analysis of this indolamine in real samples.

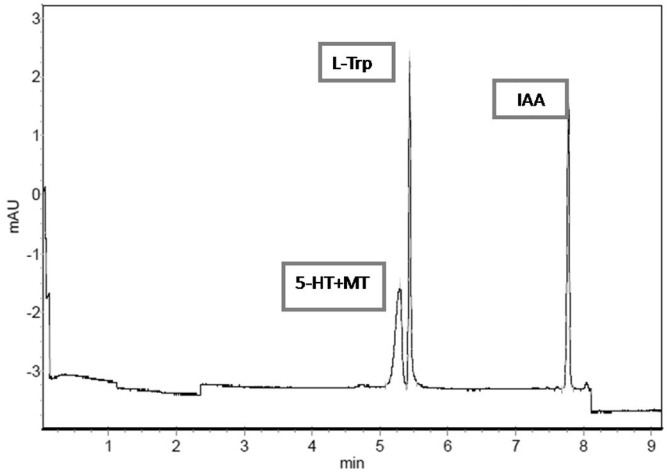

Fig. 2. Electropherogram of a standard mixture (5 mg L^{-1}) of melatonin, tryptophan, serotonin and indole-3-acetic acid by MEKC. Conditions: BGE: sodium tetraborate 10 mM, pH = 9.50 containing 20 mM SDS, 10% ACN (*v*/*v*), capillary: 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD; hydrodynamic injection 30 mbar, 3 s; 20 kV constant voltage; temperature was 25 °C, detection by UV absorbance at 220 nm.

3.1.3. Cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC)

In view of the above mentioned results for MECK and CD-CE, it was decided to evaluate a dual-pseudostationary phase provided by neutral micelles and cyclodextrin. As analytes are separated based on their differential partitioning between the micelles and CD, the relative concentrations were investigated to optimize selectivity. Several other BGE modifiers, including IPA, MEOH and ACN, as well as key parameters for CE separation (pH value, separation voltage, injection mode and volume, temperature, and wavelength detection), were investigated. The dual-PSP composition allowing the best compromise between separation, selectivity, efficiency and analysis time was 20 mM SDS and 20 mM β-CD. Table 1 shows the experimental variables tested as well as the optimized parameters for the CD-MEKC approach. Optimal values for standard aqueous solutions prepared as stated in the Experimental section. Fig. 3 shows the electropherogram of a standard mixture of indoleamines at the optimized conditions. As can be seen, excellent separation was achieved for the simultaneous determination of target analytes with the proposed CD-MEKC methodology.

3.2. Solid phase extraction optimization

As has been stated above, the determination of melatonin and its biosynthesis-related indole compounds in plant tissues represents a highly analytical challenging task considering their low concentration as well as the complex matrix nature. A solid phase extraction step was tested with the aim to adjust the concentration of the analytes of interest to yield reasonable detector responses on one hand, and to reach a proper sample clean up, on the other.

To evaluate the capability of the sorbents for the selective preconcentration of indolamines from plant extracts, different parameters such as sorbent chemical nature, pH, and elution conditions were optimized. A factor by factor method was used for the optimization of the parameters affecting the extraction efficiency. The optimization studies focused on obtaining both high preconcentration factors (CFs) and overall analyte recoveries. The recoveries were optimized by monitoring the peak area of the indolamines analyzed under the optimized CD-MEKC conditions. Three kind of bonded silica sorbents: C_8 (56 μm particle size), C_{18} (55 μm), and Strata-X (28–34 μm) were tested, being the C_8 sorbent (500 mg) the most efficient.

The volume of sample was evaluated within the interval between 1 and 10 mL. The best results in terms of preconcentration factor were obtained for a 6 mL sample volume. Remaining this volume constant, the other SPE factors were tested. For column conditioning, different solvent systems were considered: acetonitrile, methanol, and water between 1 and 4 mL of each one. The best results were obtained with 1 mL of methanol followed by 1 mL of water. Then, volumes ranging from 500 μL to 2 mL of acetonitrile, water, methanol, and acidified methanol (2% (*v*/*v*) formic acid) were tested for the elution step. It was observed that 2 mL MeOH was sufficient to reach quantitative

Table 1
Experimental variables optimized for separation.

Variable		Range	Optimal value
BGE	pH	8.0–10.5	9.2
	Buffer agent	Sodium tetraborate/boric acid	Sodium tetraborate
		5–20 mM	10 mM
	SDS	10–50 mM	20 mM
	Cyclodextrin	β-CD/γ-CD	β-CD
		10–30 mM	20 mM
	Organic solvent	MeOH–ACN–IPA	ACN
		5–15% (*v*/*v*)	10% (*v*/*v*)
CE conditions	Voltage	15–25 kV	20 kV
	Hydrodynamic	2–7 s	3 s
	Injection	10–30 mbar	30 mbar
	Capillary temperature	15–30 °C	25 °C
	Detection wavelength	200–300 nm	220 nm

recoveries. The obtained 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*) mixture. Then this solution was filtered through a 0.22 μm PTFE syringe filter before injecting into the CE system.

3.3. Analytical figures of merit

In order to determine the repeatability of the methodology, replicate injections of a standard mixture solution under the selected optimum conditions were carried out. In all cases, the RSD was better than 2.28% for the migration time and 3.42% for the peak area (n = 6). The reproducibility (between-day precision) was also evaluated over 3 days by performing six injections each day. The %RSDs on the basis of migration time and peak area were better than 2.30 and 3.28%, respectively.

In order to determine the matrix effect over each analyte response, calibration curves from a spiked matrix and spiked pure solvent samples were created. Matrix to solvent slope ratios for each of the studied analytes were calculated. All the analytes showed a minimum matrix effect. The later demonstrated the efficiency of the proposed SPE approach; all slope ratios were close to one (1.00 ± 0.02). Thus external calibration was chosen.

Calibration curves for the determination of the four target compounds were constructed under the optimal conditions. Six concentrations were used for each calibration curve within the range: 15–5000 ng g^{-1} for serotonin and 15–5000 ng g^{-1} for melatonin, tryptophan, and indole-3-acetic. Standard solutions were subjected to the whole SPE followed by CD-MECK process. The corrected peak area and concentration of each analyte were subjected to regression analysis to obtain the calibration equations and correlation coefficients. The results showed that, within the tested concentration range, there was an excellent correlation between the corrected peak area and the concentration of each analyte. Correlation coefficients of area ratio equations were >0.99 for all target compounds. All of them showed a linear range from the LOQ to 5000 ng g^{-1} at least. The limits of detection (LODs) and quantification (LOQs) were evaluated on the basis of signal-to-noise ratios (S/N) of 3 and 10, respectively. Table 2 shows calibration equations, limits of detection, limits of quantification as well as theoretical plates and enhancement factors for the indole compounds under study.

In order to determine the accuracy of this method, a 6 g of lyophilized vegetal tissue (*A. thaliana* tissue, green and linden tea tissue) was collected and divided into 10 portions of 0.60 g each. The proposed method was applied and the average concentrations determined for each compound were taken as a base value. The amounts of target species were within the following ranges: 0–160 ng g^{-1} for 5-HT, 380–550 ng $g^{-1}$for MT, 460–1000 ng g^{-1} for L-Trp and 0–770 ng g^{-1} for IAA. Then, tree levels of known quantities (50, 100 and 500 ng g^{-1}) of the analytes were added to the samples, and the indole compounds were determined following the recommended procedure (n = 3). The recovery behaviors were satisfactory; leading to recoveries higher than 87% and lower than 108% for all the target analytes under study.

Finally, Table 3 lists the main analytical features obtained in the selected related works found in the literature involving the determination of MT and its precursors involving separation techniques. The LODs obtained and the total analysis times in this work were very competitive with the previous reported.

3.4. Analysis of samples

The method was successfully applied for the simultaneous analysis of melatonin, serotonin, tryptophan, and indole-3-acetic in *A. thaliana* leaves, *C. sinensis* (green tea) and *T. cordata* (linden tea) samples. Fig. 4 shows an electropherogram of the assayed samples. The obtained values are shown in Table 4 and are in agreement with previous works [23,39–41]. Indole compounds were detected and quantified, indicating the valuable potential application of the present method for food and vegetal analysis.

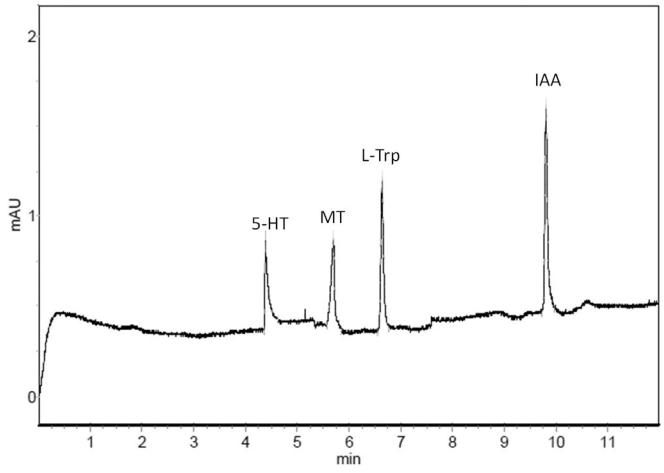

Fig. 3. Electropherogram of a standard mixture (1 mg L^{-1}) of melatonin, tryptophan, serotonin and indole-3-acetic acid by CD-MEKC. Conditions: capillary: 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD; hydrodynamic injection 30 mbar, 3 s; 20 kV constant voltage; temperature was 25 °C, detection by UV absorbance at 220 nm.

4. Conclusions

Plant neurobiology is an exciting and original topic recently presented; focusing on natural products whose biosynthesis is shared by animal and plant organisms, i.e., indoleamines and catecholamines. The methodology proposed herein takes advantage of the unique

Table 2
Figures of merit.

Analyte	Regression equation[a]	R^2	LOD (ng g^{-1})	LOQ (ng g^{-1})	N[b]	EF[c]
5-HT	y = 1.39x − 1.03	0.994	4.16	13	331,634	17
MT	y = 5.35x − 1.29	0.999	0.79	2.62	60,168	89
L-Trp	y = 7.45x − 0.47	0.998	0.72	2.41	228,017	94
IAA	y = 8.88x − 0.68	0.996	0.55	1.83	251,023	127

Conditions: BGE: sodium tetraborate 10 mM, pH = 9.2 containing 20 mM β-CD, 20 mM SDS, ACN 10% (*v*/*v*) capillary: 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD; hydrodynamic injection 30 mbar, 3 s; 20 kV constant voltage; temperature was 25 °C, detection by UV absorbance at 220 nm.

[a] Regression equation is y = *b*x + *a*.
[b] Number of theoretical plates.
[c] Enrichment factor.

Table 3
Selected analytical works for determination of MT and its precursors.

Analyte*	Sample	Separation technique	Detection	Analysis time (min)	LOD (ng g^{-1})	Coments	Ref
MT	–	MEKC	DAD	4	13	They also determined two MT metabolites	[13]
MT	Wine and plant tissues	CEC	DAD	10	0.01	c-MWNT inmobilized fused-silica capillary through covalent modification	[26]
5-HT, MT and L-Trp	Human serum	MEKC	UV	12	130, 30 and 21	Comparison of sweeping and stacking techniques for on-line preconcentration	[27]
5-HT, MT, L-Trp and IAA	Serum and urine	MEKC	LIF	16	2.01, 2.40, 1.40 and 0.96	They analyzed other seven indole compounds	[30]
5-HT, MT and L-Trp	Pharmaceutical preparation, biological fluids and pineal gland	MEKC	UV and FD	13	260, 370 and 160	They analyzed other tree indole compounds	[42]
MT	Pharmaceutical preparation	MEKC	DAD	8	500	They also proposed an spectrofluorimetry method for direct MT analysis	[43]
5-HT, MT and IAA	Plant tissues	HPLC	MS	10	0.1, 0.005 and 0.05	MT and IAA coelute. Analysis is not simultaneous	[44]
5-HT, MT, L-Trp and IAA	Plant tissues	CD-MEKC	UV	10	4.16, 0.79, 0.72 and 0.55	SPE step for clean-up and preconcentration	This work

properties of the combination of SPE with the Dual-PSP to achieve LODs in the low ppb levels with excellent separation features.

The present work proposes a novel SPE followed by CD-MEKC method for the simultaneous and sensitive determination of melatonin (the darkness molecule), serotonin (the happiness molecule), tryptophan (an essential amino acid) and indole-3-acetic acid (the most common, naturally-occurring, plant hormone of the auxin class). In comparison with other methodologies that used HPLC and CE, the present approach is more selective, simpler, cheaper, quicker and greener. The optimized modified CE methodology provides a robust and reliable approach for the analysis of important indole compounds in complex plant matrices.

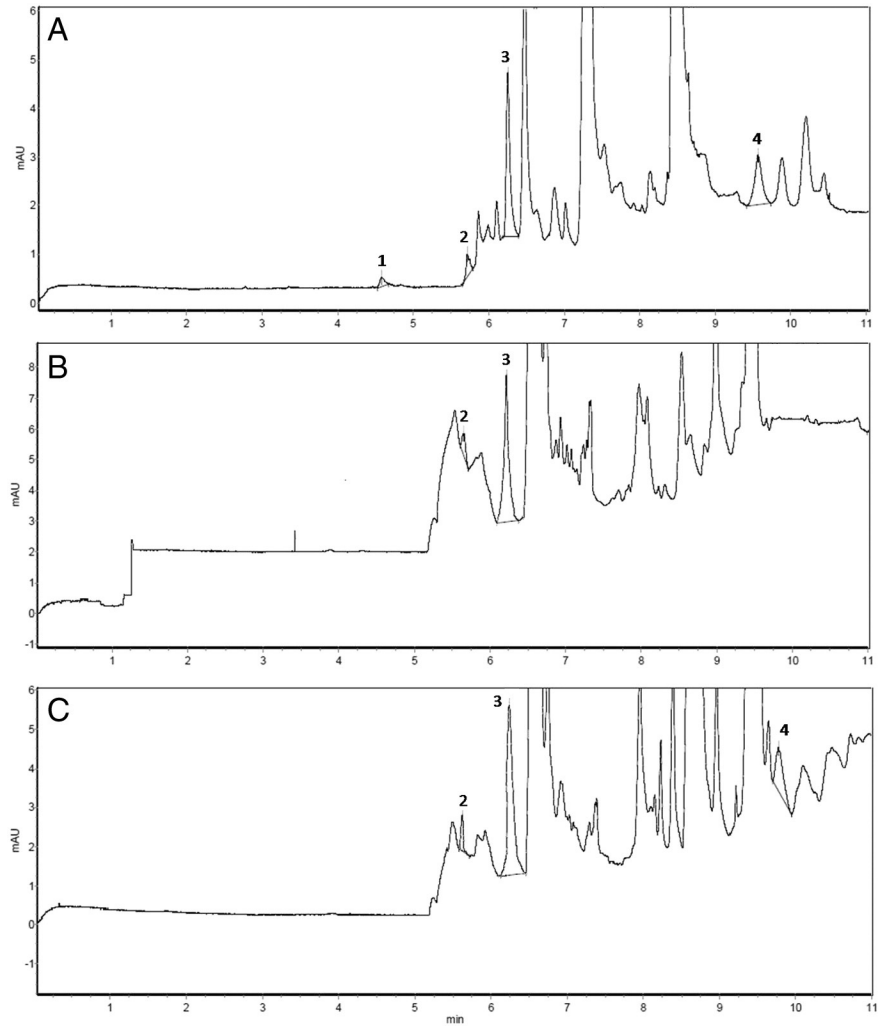

Fig. 4. Electropherograms of *Arabidopsis thaliana* (A), *Camellia sinensis* (B), and *Tilia cordata* (C) leaves by CD-MEKC. 1: serotonin, 2: melatonin, 3: tryptophan, and 4: indole-3-acetic acid. Conditions: capillary: 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD; hydrodynamic injection 30 mbar, 3 s; 20 kV constant voltage; temperature was 25 °C, detection by UV absorbance at 220 nm.

Table 4
Analysis of samples.

	5-HT ng g^{-1}	MT ng g^{-1}	L-Trp ng g^{-1}	IAA ng g^{-1}
Green tea	ND	386 ± 21	671 ± 20	ND
Linden tea	ND	410 ± 16	993 ± 9	543 ± 10
Arabidopsis	166 ± 2	548 ± 26	459 ± 15	765 ± 20

Values are means ± SD.
Conditions: BGE: sodium tetraborate 10 mM, pH = 9.2 containing 20 mM β-CD, 20 mM SDS, ACN 10% (v/v) capillary: 57 cm full length, 50 cm effective length, 75 μm ID and 375 μm OD; hydrodynamic injection 30 mbar, 3 s; 20 kV constant voltage; temperature was 25 °C, detection by UV absorbance at 220 nm.

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