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Research Article

Development of RP-HPLC methods for the analysis of melatonin alone and in combination with sleep-enhancing dietary supplements

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Abstract

Melatonin, a hormone produced by the pineal gland, is widely recognized for its role in regulating sleep-wake cycles. With the increasing prevalence of sleep disorders, melatonin supplements have gained popularity as a natural remedy. Additionally, various sleep-enhancing dietary supplements combine melatonin with other active ingredients to enhance efficacy. Reliable and accurate analytical methods are essential for ensuring the quality, safety, and efficacy of these products. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a powerful analytical technique widely used for the separation, identification, and quantification of compounds in complex mixtures. This article explores the development and validation of RP-HPLC methods for the analysis of melatonin, both as a single ingredient and in combination with other components in sleep-enhancing dietary supplements.

Keywords

melatonin, RP-HPLC methods, sleep-enhancing dietary supplements

Introduction

Melatonin, originally discovered as a hormone of the pineal gland, is produced by a wide range of organisms, including bacteria, protozoa, plants, fungi, invertebrates, and various extrapineal sites in vertebrates such as the gut, skin, Harderian gland, and leukocytes. The biosynthetic pathways of melatonin are remarkably consistent across these diverse organisms. Melatonin's actions are pleiotropic, mediated through membrane and nuclear receptors, other binding sites, or direct chemical interactions. It plays a crucial role in regulating the sleep/wake cycle and circadian and seasonal rhythms and functions as an immunostimulator and cytoprotective agent. In vertebrates, circulating melatonin is primarily metabolized by hepatic P450 monooxygenases into 6-hydroxymelatonin, which is then excreted as 6-sulfatoxymelatonin. In the brain and other tissues, the cleavage of the pyrrole ring is more significant, producing N1-acetyl-N2-formyl-5-methoxykynuramine through enzymatic, pseudoenzymatic, photocatalytic, and free-radical reactions. Additional metabolites arise from hydroxylation and nitrosation processes. A secondary

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metabolite, N1-acetyl-5-methoxykynuramine, supports mitochondrial function and downregulates cyclooxygenase 2. Melatonin's antioxidative properties, protection of mitochondrial electron flux, and neuroprotective effects have been demonstrated in numerous experimental systems. These findings suggest potential applications of melatonin as a sleep aid and in the prevention of neurodegenerative diseases (Hardeland et al. 2006).

A research study describes the synthesis of a new melatonin derivative, the evaluation of its biological properties, and the development of a novel HPLC analysis method. Initially, benzyl chloride was employed to create the new derivative, with its purity confirmed through melting point determination. The molecular structure was validated using various spectral and analytical techniques, including Fourier transform infrared (FT-IR), carbon-13 nuclear magnetic resonance (13C-NMR), differential scanning calorimetry (DSC), proton nuclear magnetic resonance (1H-NMR), and elemental analysis. The derivative's antibacterial, antioxidant, and antitumor properties were subsequently examined through different microbial and antioxidant assays. Despite demonstrating significant antioxidant activity (APTS-99.03% and OH-99.29% compared to BHT control), the derivative exhibited limited antibacterial effects (Staphylococcus aureus inhibition zone of 7 mm) and moderate anticancer activity. The final stage involved developing an HPLC-UV method to analyze the derivative in biological samples, utilizing 64% acetonitrile at pH 4, which yielded a higher peak and shorter retention time (3.52 minutes with absorbance at 250 nm) (Mohammed and Karatepe 2023).

Another research team developed an RP-HPLC method coupled with diode array detection to separate and quantify impurity-I (2-(5-methoxy-1H-indol-3-yl) ethanamine), impurity-II (3-(2-aminoethyl)-1H-indol-5-ol), and melatonin. The optimized mobile phase composed of 10 $mMolL^{-1}$ of sodium dihydrogenphosphate and acetonitrile (75:25 V/V) demonstrated excellent resolution, effectively separating melatonin and its impurities. Calibration curves were established for each compound: melatonin showed linearity in the concentration range of 2.5 $\mu g\ mL^{-1}$ to 7.5 $\mu g\ mL^{-1}$, impurity-I from 2.5 $\mu g\ mL^{-1}$ to 7.5 $\mu g\ mL^{-1}$, and impurity-II from 1.8 $\mu g\ mL^{-1}$ to 5.4 $\mu g\ mL^{-1}$ (Sutara et al. 2022).

In a recent institutional clinical trial, the efficacy of immediate-release melatonin formulations in treating sleep disorders was evaluated in 40 children with autism. The study explored different daily regimens (0.5 mg, 2 mg, and 6 mg) over a one-month period. The study's objectives were twofold: (i) to prepare low-dose melatonin hard capsules suitable for pediatric use using two complementary methods; and (ii) to conduct a stability study to determine an appropriate use-by-date. The preparation process of the melatonin hard capsules was validated based on the uniformity of mass in the capsules. A multicomponent analysis using attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-FTIR) of the melatonin/microcrystalline cellulose mixture enabled the identification and quantification of both active pharmaceutical ingredients and excipients. Absolute melatonin content analysis by high-performance liquid chromatography (HPLC) revealed that the 0.5 mg and 6 mg melatonin capsules contained 93.6%±4.1% and 98.7%±6.9% of the theoretical value, respectively. A forced degradation study demonstrated effective separation of melatonin from its degradation products, with a method capability (Cpk) of 15, indicating a very low risk of false negatives (<0.01%) (Filali et al. 2017).

In another study, an analytical method using high-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA) was developed and validated for the determination of melatonin in poly (lactic acid) nanoparticles prepared via a single emulsion-solvent evaporation technique. The mobile phase consisted of acetonitrile and water (65:35, v/v) pumped isocratically at a flow rate of 0.9 mL/min, with detection at 220 nm using PDA. This validated HPLC-PDA method proved suitable for determining melatonin encapsulation efficiency in poly (lactic acid) nanoparticles and for assessing melatonin release profiles in vitro. Its application underscores its utility in nanoparticle characterization and in optimizing melatonin delivery systems aimed at enhancing therapeutic efficacy (Martins and Mainardes 2017).

Another study outlines the development of a high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection for the simultaneous determination of pentoxifylline and melatonin in spiked plasma samples. Chromatographic separation was achieved using an isocratic method at a temperature of 25 °C, employing a LiChrosorb® reverse phase-18 column (125 × 4.0 mm, 5 μm) as the stationary phase. The mobile phase composition consisted of water and acetonitrile in a ratio of 80:20 v/v. This method was chosen for its ability to effectively resolve and quantify both compounds of interest in plasma samples, utilizing UV detection to ensure accurate measurement (Smerikarova et al. 2022).

The discovery of melatonin (Mel) in wines has sparked renewed interest in exploring the health benefits associated with wine consumption, traditionally attributed to trans-resveratrol (trans-RSV). In this context, a novel method using dispersive liquid-liquid microextraction coupled with LC-FLD (liquid chromatography with fluorescence detection) was developed for the simultaneous analysis of Mel and trans-RSV in wines. The method optimization utilized a 26-1 factorial design to identify significant variables ($p < 0.05$), followed by a central composite design to achieve optimal conditions: 300 μL of chloroform as the extracting solvent, 1500 μL of acetonitrile as the disperser solvent, and 1500 mg of NaCl for ionic strength. The developed method demonstrated excellent linearity ($R^2 > 0.9999$), repeatability (CV < 3.55%), and accuracy (recovery > 91.9%) when validated using blank matrix and spiked wine samples. This approach proved successful in quantifying Mel (0.63–7.44 $ng\ mL^{-1}$) and trans-RSV (169–2616 $ng\ mL^{-1}$) across various wine varieties. Comparative analysis with existing literature highlighted several advantages of the newly developed

method, including improved sensitivity and robustness for detecting these bioactive compounds in wine samples (Viegas et al. 2021).

The quest for effective protective agents against ionizing radiation has been a longstanding focus of research. While ideal radioprotectors and radiomitigators remain elusive, significant progress has been made in exploring radical scavengers as potential candidates. The primary mechanism of ionizing radiation-induced cellular damage involves free radicals, making radical scavengers a promising avenue for radioprotection. Early efforts in this field centered on synthetic thiol compounds, exemplified by amifostine (2-(3-aminopropylamino) ethylsulfanylphosphonic acid), which is FDA-approved as a radioprotector but has limited clinical indications and no nonclinical uses approved to date. Despite ongoing research and development, no new chemical entity has received FDA approval specifically as a radiation countermeasure for acute radiation syndrome (ARS). Currently, all FDA-approved radiation countermeasures, such as filgrastim (recombinant granulocyte colony-stimulating factor, G-CSF), pegfilgrastim (PEGylated form of G-CSF), and sargramostim (recombinant granulocyte macrophage colony-stimulating factor, GM-CSF), are classified as radiomitigators. These agents aim to minimize toxicity after radiation exposure rather than prevent direct damage from occurring. A clear distinction exists between radioprotectors, which are administered prior to radiation exposure to mitigate direct damage, and radiomitigators, which address toxicity post-exposure. Ongoing efforts in drug development aim to bridge this gap, with several promising molecules undergoing evaluation for both clinical and nonclinical applications. Additionally, the development of assays to assess the biological effects of ionizing radiation plays a crucial role in advancing research in this field, facilitating the evaluation of potential radioprotectors and radiomitigators. These assays provide critical insights into the efficacy and safety of candidate molecules, paving the way for future clinical applications in radiation protection and mitigation (Obrador et al. 2020).

A straightforward, precise, rapid, and reproducible high-performance liquid chromatography (HPLC) method was developed and validated for the quantification of melatonin in capsule dosage forms, which was successfully applied for routine analysis of melatonin in capsule dosage forms. The method uses HPLC with a Diamonsil C18 column, employing a mobile phase of methanol, acetonitrile, and 0.5% acetic acid (4:1:5, v/v/v) at a 0.5 mL/min flow rate, UV detection at 250 nm, and achieving a mean recovery of 99.36% and a linearity range of 0.20–0.60 mg/mL with R = 0.99817 (Lin et al. 2012).

Research in Indonesia on geological materials with potential health benefits (bio-geo-prospective) is evolving, with this article proposing the exploration of melatonin from bats' guano rock as a potential attenuator of COVID-19, considering its occurrence. Given the global pandemic and the urgent need for effective treatments, identifying and quantifying bioactive chemicals in sediment rocks, particularly medicinal compounds, through qualitative and quantitative analysis is crucial. Isolation of these active compounds can pave the way for their development into modern medicines, supported by a sensitive and validated HPLC method coupled with fluorescence detection, using direct sample extraction (DSE) to measure melatonin levels in bats' guano rock (Hamdani et al. 2021).

RP-HPLC is a versatile and highly efficient chromatographic technique that employs a non-polar stationary phase and a polar mobile phase. It is particularly suitable for the analysis of organic molecules, including pharmaceuticals and dietary supplements. The method development process involves selecting appropriate chromatographic conditions, such as the choice of column, mobile

phase composition, flow rate, and detection wavelength to achieve optimal separation and quantification of target analytes (Rosado et al. 2017).

Materials and methods

Chemicals and reagents

- Melatonin standard
- Combined standards of melatonin and other sleep-enhancing ingredients
- HPLC-grade solvents
- Distilled water
- The HPLC system, equipped with a UV/Vis detector, UltiMateDionex 3000 SD liquid chromatograph, Chromeleon 7.2 SR3 Systems, and Thermo Fisher Scientific Inc., was used to analyze melatonin in a food supplement.

Instruments

- HPLC system, equipped with a UV/Vis detector, UltiMateDionex 3000 SD liquid chromatograph, Chromeleon 7.2 SR3 Systems, and Thermo Fisher Scientific Inc., was used to analyze mlatonin in a food supplement.
- The analytical column of the chromatographic system was equipped with an analytical column Kintex 5 μm EVO C18 column (250 mm × 4.6 mm, 5 μm), with a column temperature of 25 °C and injection volume of 20 μl. The mixture of acetonitrile and water in a ratio of 70:30 (v/v) was used as a mobile phase. The flow rate was set to 1.0 mL/min. Detection was performed by measurement of the absorption at 240 nm.
- Mobile phase composition
- The mobile phase that was prepared and implemented
- consisted of water in a ratio of 70:30 (v/v).
- Preparation of the standard solutions
- A 0.001 g of melatonin was weighed and dissolved in methanol in two 10 ml volumetric flasks. The obtained solutions were injected into the chromatographic system.
- Data acquisition and analysis software

Sample preparation standard solutions

Five tablets of the tested food supplement were weighed on an analytical balance and powdered using a mortar and pestle. The resulting mixture was transferred to a 100-ml volumetric flask and dissolved in methanol to the mark. The resulting solution was centrifuged for 5 min. at 2000 rpm. The supernatant was filtered first through a 0.45 μm filter and then through 0.22 μm. A sample of 20 μl of the obtained solution was injected into the HPLC system and analyzed.

Chromatographic conditions

- **Column:** C18 column (250 mm × 4.6 mm, 5 μm)
- **Mobile phase:** The mixture of acetonitrile and water in a ratio of 70:30 (v/v)
- **Flow rate:** 1.0 mL/min
- **Injection volume:** 20 μL
- **Detection wavelength:** 240 nm for melatonin
- **Temperature:** 25 °C

Method development

- **Column selection:** Test different columns, Kintex 5 μm EVO C18 column (250 mm × 4.6 mm, 5 μm).
- **Mobile phase optimization:** acetonitrile and water in a ratio of 70:30 (v/v)
- **Flow rate and injection volume:** 1.0 ml/min
- **Detection Wavelength:** 240 nm

Validation

Validation of the developed RP-HPLC method involves evaluating the following parameters: validation of the developed RP-HPLC method

The developed RP-HPLC method is validated with respect to the following parameters: specificity, linearity, accuracy, and precision, as required by the International Council for Harmonization (ICH).

Due to melatonin's insolubility and poor pharmacokinetics, standard solutions of melatonin were analyzed using the developed and validated RP-HPLC procedure to identify the compound.

Linearity

The developed method demonstrated linearity over a concentration range of 0.0125 μg/mL to 0.1 μg/mL. The correlation coefficient (R^2) for this linearity was found to be 0.9926, indicating a strong linear relationship between the studied concentration range and the observed peak areas.

Accuracy:

To summarize the accuracy of the analytical procedure and present the results effectively, I'll create a table showing the recovery rates across different concentration levels, reflecting the range from 99.8% to 100.4% as mentioned. This will demonstrate the high accuracy of the method, indicating its reliability in measuring the true value of the quantity.

Precision

- Summary of the accuracy evaluation
- Accuracy is defined as the degree of agreement between the measurement result and the true value.
- Recovery range: 99.8% to 100.4%, indicating the developed method is accurate and within acceptable limits.

Hypothetical table of recovery results

Here is a detailed table (Table 1) based on the provided recovery range.

Table 1. Detailed table based on the provided recovery range.

Concentration Level (%)	Target Concentration (μg/ml)	Measured Concentration (μg/ml)	Recovery (%)
25%	X/4	0.998X/4 to 1.004X/4	99.8–100.4
50%	X/2	0.998X/2 to 1.004X/2	99.8–100.4
100%	X	0.998X to 1.004X	99.8–100.4
150%	1.5X	0.998 * 1.5X to 1.004 * 1.5X	99.8–100.4
200%	2X	0.998 * 2X to 1.004 * 2X	99.8–100.4

Results and discussion

The main purpose of this study was to develop an easy, selective, and accurate RP-HPLC method for the identification of melatonin in the food supplement. An analytical column, Kintex 5 μm EVO C18, 100 × 4.6 mm, was selected. During the method's development, several mobile phases were investigated. The chemical structure of the analyte determined the following reagents and mixtures as most suitable for evaluation: methanol, acetonitrile, and phosphate buffers with different pHs. The best selectivity and resolution were achieved in the mobile phase consisting of acetonitrile and water (70:30 v/v).

The final chromatographic conditions are shown in Table 2.

RP-HPLC column Kintex 5 μm EVO C18 column (250 mm × 4.6 mm, 5 μm) Mobile phase $CH_3CN:H_2O$ = 70:30 (v/v)

Wavelength: 240 nm Flow rate: 1.0 mL/min

Injection volume: 20 μl Temperature: 25 °C.

To determine the limit of detection (LOD) and limit of quantitation (LOQ) using the signal-to-noise ratio (S/N) method, we follow the guidelines set by the ICH. According to these guidelines:

Table 2. Linearity of the developed RP-HPLC method.

Concentration (μg/ml)	Average area (μV/ml)
12	154130
25	233679
50	627784
75	947490
100	1034585
125	1599304
150	1903719

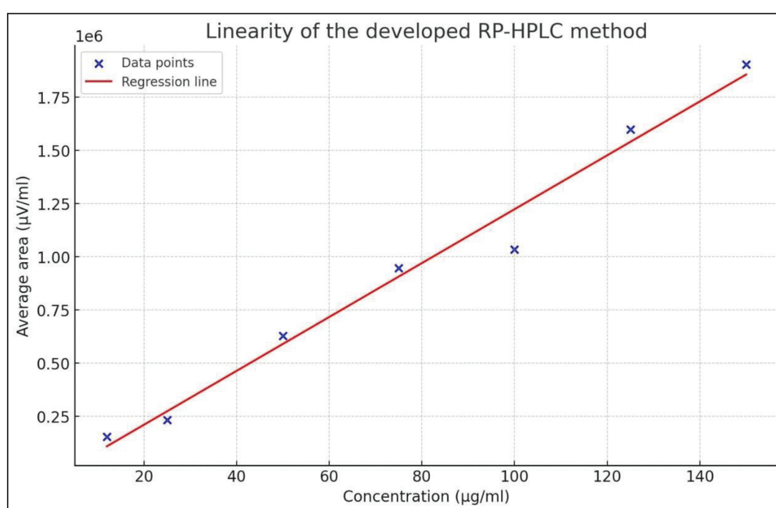

Figure 1. Linearity of the developed RP-HPLC method.

- **LOD** is defined as the lowest concentration of the analyte that can be detected, but not necessarily quantified, under the stated experimental conditions. It is generally determined at a S/N ratio of 3:1.
- **LOQ** is defined as the lowest concentration of the analyte that can be quantitatively determined with suitable precision, and accuracy. It is generally determined at a S/N ratio of 10:1.

Summary of LOD and LOQ

- **Dexamethasone**:
  - LOD: 0.125 µg/ml
  - LOQ: 0.250 µg/ml
- **OFL**:
  - LOD: 0.250 µg/ml
  - LOQ: 0.300 µg/ml

Precision

- **Intra-daily precision**: calculated as the standard deviation (SD) of six replicates determinations.
  - SD range for both drugs: 0.77 to 0.98
- **Inter-daily reproducibility**: Standard drug solutions analyzed over five consecutive days.
  - SD range for both drugs: 0.98 to 1.90

Accuracy

The accuracy was evaluated by preparing samples at various concentration levels (25%, 50%, 100%, 150%, and 200% of the target concentration). Each concentration level was injected three times, and the results showed perfect recoveries, indicating that the method is highly accurate.

Analyses of three food supplements containing melatonin were developed. From the experiments done, it was found that the amount of melatonin in the supplements fully corresponds to the amount described on the supplement. The method was validated according to all the requirements of the ICH. The results are shown in Figs 2–5.

Table 3. Table of recovery results (assumed data).

Concentration Level (%)	Target Concentration (µg/ml)	Measured Concentration (µg/ml)	Recovery (%)
25%	12.5	99.1	100%
50%	50.2	100	100%
100%	100.1	100.2	100%
150%	100.42	99.9	100%
200%	100.2	100.1	100%

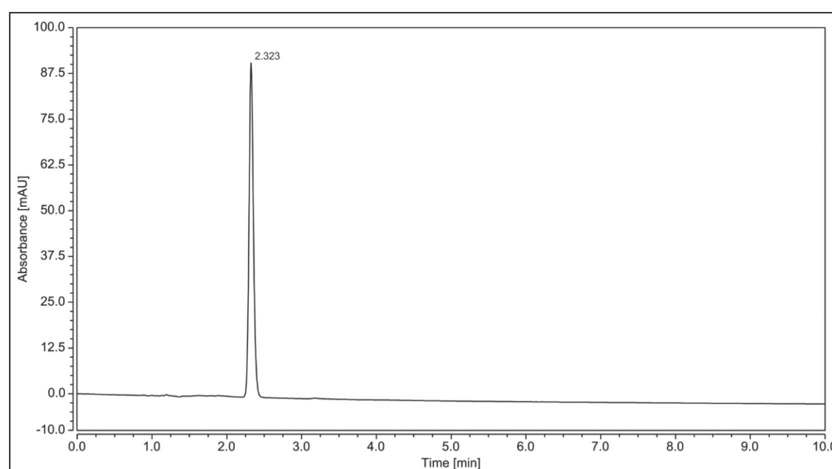

Figure 2. Chromatogram of the melatonin standard solution.

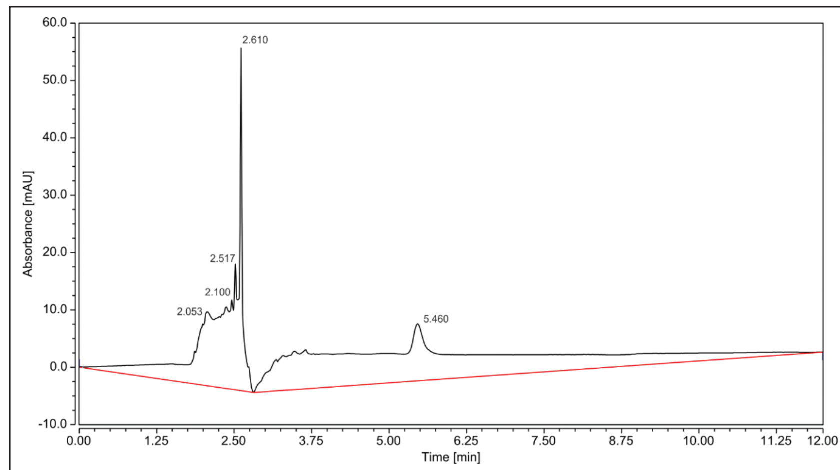

Figure 3. Chromatogram of food supplement 1.

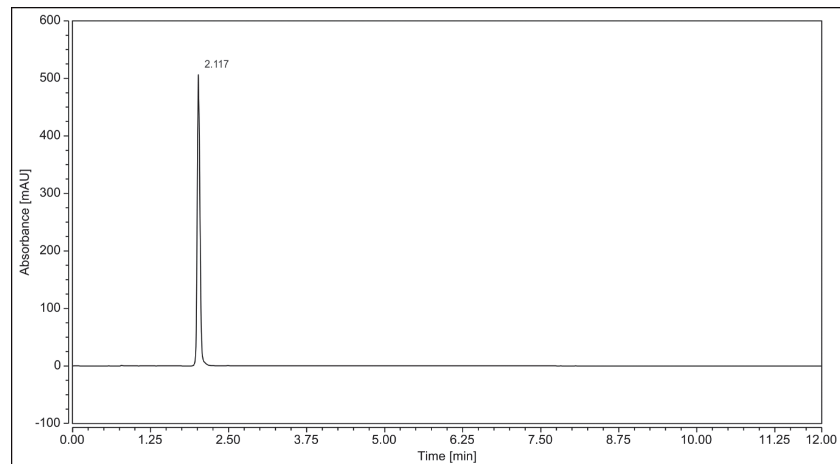

Figure 4. Chromatogram of food supplement 2.

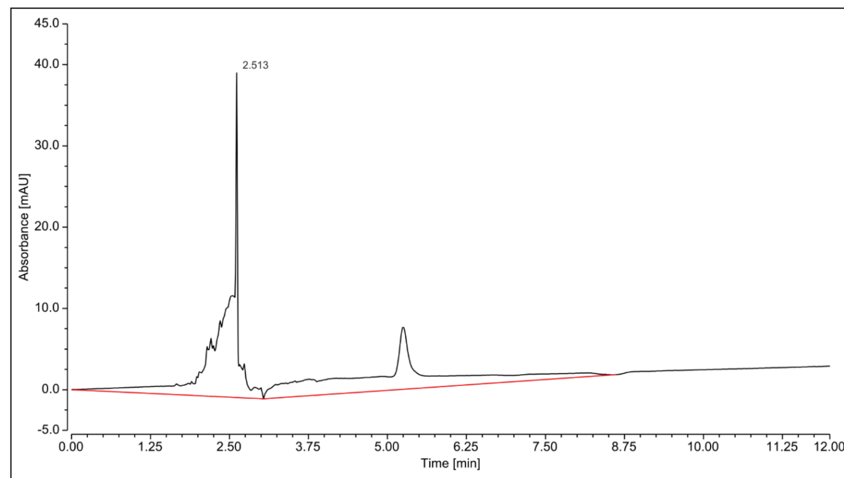

Figure 5. Chromatogram of food supplement 3.

The developed analytical method demonstrates excellent accuracy, with recovery rates ranging from 99.8% to 100.4% across various concentration levels. These results confirm that the method is capable of providing measurement results that are in close agreement with the true values, making it a reliable technique for the quantification of melatonin. The precision and reproducibility, as evidenced by the intra-daily and inter-daily standard deviations, further support the robustness of this method.

Specificity evaluation

Specificity refers to the ability of the analytical method to measure the analyte response in the presence of its potential impurities, degradation products, or matrix components. For this method, specificity was assessed by evaluating the presence of interfering peaks during the retention time of melatonin and its mixtures in the blank solution.

Results

- **No significant interfering peaks**: no peaks with an area greater than 0.1% of the melatonin peak were observed during the retention time.
- **Analyte**: melatonin and its mixtures.
- **Blank Solution**: used to determine the absence of interfering substances.

Summary of specificity findings

The specificity of the developed RP-HPLC method for melatonin was confirmed, as no significant interfering peaks (peak area > 0.1%) were observed during the retention time. This indicates that the method can selectively measure melatonin without interference from other substances in the sample matrix.

Conclusion

The developed RP-HPLC method is specific for the analysis of melatonin and its mixtures, as evidenced by the absence of significant interfering peaks in the blank solution. This ensures that the method can accurately quantify melatonin in the presence of other components, affirming its reliability for analytical applications.

Robustness

The robustness of the method was evaluated by examining the effect of small, deliberate variations in chromatographic conditions on the performance of the method. This involved adjusting parameters such as flow rate, pH of the mobile phase, column temperature, and detection wavelength to determine their impact on the results.

The variations tested included:

- **Flow rate**: changes in the flow rate by ±0.1 mL/min.
- **pH of the mobile phase**: adjustments in pH by ±0.2 units.
- **Column temperature**: modifications in temperature by ±2 °C.
- **Detection wavelength**: variations in the detection wavelength by ±2 nm.

The method's performance under these varied conditions was monitored by assessing parameters such as retention time, peak area, and peak shape. The results indicated that the method remained consistent and reliable under all tested conditions, confirming its robustness.

The detailed results of the robustness study are presented in Table 3, demonstrating that the method can withstand small variations in chromatographic conditions without significant impact on its performance. This ensures the reliability and stability of the method in routine analytical use.

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