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# A new microwave-assisted extraction method for melatonin determination in rice grains

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ABSTRACT

A new microwave-assisted extraction (MAE) method has been developed for the extraction of melatonin from rice grains. The stability of melatonin under MAE conditions was studied in order to define the working range. The studied analytical conditions for the MAE were temperature (125–175 °C), microwave power (500–1000 W), time (5–15 min), solvent (10–90% EtOAc in MeOH), and ratio of solvent to sample (10:1–20:1). Extraction variables were optimized by Response Surface Methodology (RSM). Extraction temperature was found to have a highly significant effect on the response value ($p < 0.0001$) and the solvent and quadratic of time also had significant effects ($p < 0.1$). The optimized MAE conditions were as follows: extraction temperature 195 °C, microwave power 1000 W, extraction time 20 min, solvent 100% MeOH, and ratio of solvent to sample 10:1. The developed method showed high precision (in terms of CV: 4.97% for repeatability and 4.34% for intermediate precision). Finally, the new method was applied to real samples in order to investigate the presence of melatonin in a wide variety of rice grains.



## 1. Introduction

Melatonin is an indolamine (N-acetyl-5-methoxytriptamine) that occurs naturally in several vegetables and related foods and this compound has known biological activity (Cao et al., 2006; Paredes et al., 2009). Melatonin is involved in the regulation of circadian rhythm and the alleviation of sleep disorders, such as insomnia due to jet lag and shift work, as it plays a major role in the synchronization of the sleep/wake cycle (Buscemi et al., 2004). This compound also has potent anti-oxidation properties and anti-inflammatory effects (Reiter et al., 2005). It has also been demonstrated that melatonin mitigates neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Wang, 2009), and also acts as an anticancer agent (Anisimov et al., 2006).

Efficient analytical methods for the determination of melatonin, together with optimized extraction protocols, should help to confirm the presence of melatonin in food. Melatonin can be detected by several methods, such as immunological techniques, Radioimmunoassay (RIA) and Enzyme-Immunoassay (EIA) (De la Puerta et al., 2007); chromatographic techniques: GC–MS (Gonzalez-Gomez et al., 2009), HPLC–MS/MS (Hernández and Arnao, 2008; Rodriguez-Naranjo et al., 2011), HPLC–FD (Garcia-Parrilla et al., 2009), HPLC–ECD (Reiter et al., 2005); and chemiluminescent techniques (Garcia-Parrilla et al., 2009).

Chromatographic methods have been the most widely used separation techniques in this area in recent years. HPLC techniques are more economical and time efficient when derivatization of the sample is not required prior to analysis. Most of the HPLC methods reviewed have used reverse phase columns (e.g. $RP_{18}$ or $RP_8$) for melatonin separation and fluorescence detectors (FD) were found to be sensitive and versatile to quantify melatonin in food samples and also gave low limits of detection and quantification (Garcia-Parrilla et al., 2009).

Sample pre-treatments are required before chromatographic measurement for the determination of melatonin in solid samples.

*Abbreviations:* ANOVA, Analysis Of Variance; AOAC, Association of Official Agricultural Chemists; CV, coefficient of variance; EACEA, Education, Audiovisual and Culture Executive Agency; EIA, Enzyme-Immunoassay; EtOAc, ethyl acetate; FAO, Food and Agriculture Organization; FD, fluorescence detectors; GC–MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; HPLC–ECD, high performance liquid chromatography–electrochemical detection; HPLC–FD, high performance liquid chromatography–fluorescence detection; HPLC–MS/MS, high performance liquid chromatography–mass spectrometry/mass spectrometry; ICH, International Conference on Harmonisation; LOD, limit of detection; LOQ, limit of quantification; LSD, Least Significant Difference; MAE, microwave-assisted extraction; MeOH, methanol; RIA, Radioimmunoassay; RSM, Response Surface Methodology.

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Several procedures for the extraction of melatonin from vegetable and food samples have been reported, and these include ultrasound-assisted extraction (Cao et al., 2006), liquid–liquid extraction (Reiter et al., 2005) and solid phase extraction (Garcia-Parrilla et al., 2009). Methods based on microwave-assisted extraction (MAE) for melatonin were not found in a review of the literature. Numerous methods that involve the use of MAE instead of other extraction methods have already been published and, in many cases, these shorten extraction times for different kinds of compounds in vegetables and related foods (Barbero et al., 2006; Liazid et al., 2011; Rostagno et al., 2007).

In MAE, the rapid generation of heat and pressure forces compounds from within the matrix and produces good quality extracts with better target compound recovery (Hemwimon et al., 2007). The special heating mechanism of microwaves and the fact that different chemical substances absorb microwave radiation to different extents make MAE an efficient method for extraction and, more importantly, it makes the selective extraction of target compounds possible.

The efficiency of the MAE process depends on time, temperature, solid–liquid ratio and the type and composition of solvent used (Grigonis et al., 2005). The selection of appropriate solvent(s) influences the extraction yield during the MAE process, as the solvent acts as a conduit for energy coupling, mass transfer and exerting pressure on the matrix (Eskilsson and Björklund, 2000). Therefore, a chemometric approach based on the use of an experimental design was employed to evaluate the variables that affect MAE (Bas and Boyaci, 2007). This enables the overall number of experiments to be reduced and allows the possible interaction effects between the variables to be considered.

The objective of the study reported here was to develop an optimal and reliable analytical protocol for the extraction of melatonin using a microwave-assisted extraction from food samples, with the approach planned using experimental design. Rice (*Oryza sativa* L.) was found to be an appropriate sample for the extraction of melatonin as it is the second most widely grown cereal crop in the world and serves as a staple food for more than half of the world's population (FAO, 2011). The amount of melatonin in rice was previously found to be 1 $\mu g\ kg^{-1}$ of rice, which was the highest melatonin level found in twenty-four different edible plants investigated by Hattori (Hattori et al., 1995).

## 2. Materials and methods

### 2.1. Materials and chemicals

HPLC-grade methanol, acetic acid and ethyl acetate were purchased from Merck (Darmstadt, Germany). Melatonin standard, M-5250, was obtained from Sigma–Aldrich (St. Louis, MO, USA). Water was purified with a Milli-Q purification system (Millipore, Billerica, MA, USA).

### 2.2. Rice samples

A long grain variety of rice was obtained from a commercial market in Spain. A rice sample (20 g) was placed in a plastic cylinder and rice grains were milled with an Ultraturrax homogenizer (IKA® T25 Digital, Germany) for 10 min prior to extraction. The milling process was stopped every 1 min to avoid excessive heating of the sample. The fine grain was then homogenized by stirring and then stored in a closed, labelled bottle. The final extraction method was applied to 13 different rice products available in the market and these covered a variety of short, long, aromatic, exotic, integral and processed rice grains.

### 2.3. Extraction of melatonin

MAE experiments were performed in a Milestone Ethos 1600 (Sorisole, Italy) equipped with the vessels which are made of tetrafluoromethoxyl and lined with Teflon liners. Rice powder (2.5 g) was accurately weighed and placed into an extraction vessel. According to the experimental design, a set volume and type of solvent was added to the extraction vessel and the extraction was performed under different MAE conditions. After extraction, the vessels were allowed to cool down in an ice bath for 10 min and carefully opened in a fume cupboard. Solid material from the sample was separated from liquid, using a paper filter. The solid material was then washed using new solvent. The liquid must be dried under vacuum condition by a rotary evaporator, then re-dissolved with methanol into 2 mL and filtered using 0.45 μm cellulose filter (Millipore) prior to injection on the HPLC–FD system.

### 2.4. Determination of melatonin

Liquid chromatography analyses were carried out on an Alliance HPLC 2695 system, controlled by an Empower Pro 2002 data station (Waters, Milford, MA) and a fluorescence detector (Waters 474 Fluorescence Detector) was used for melatonin determination. A reverse phase RP-18 Lichrospher Column (LiChroCART 250 × 4 (5 μm)) from Merck was used.

A gradient elution program was used with two mobile phases: A (2% acetic acid and 5% methanol in water) and B (2% acetic acid and 88% methanol in water). The gradient applied was as follows: (time, solvent B): 0 min, 0%; 5 min, 35%; 12 min, 40%; 15 min, 40%; 20 min, 45%; 25 min, 50%. The flow rate was fixed at 0.5 mL/min. For the fluorescence detector, the fixed conditions were as follows: excitation and emission wavelength (nm) was set at 290/330, the sensitivity was set at gain 1000 and attenuation was fixed at 16. Injection volume was set to 10 μL.

### 2.5. Performance of the method

The analytical procedure for the chromatographic method for melatonin determination was carried out according to the recommendations of ICH Guideline Q2 (R1) and suggestions of ISO 17025 (ICH, 2006; ISO, 2005). Linearity, range, precision, detection and quantification limits of the method were evaluated.

Linearity was assessed in order to express the ability of the method to obtain test results that are directly proportional to the concentration of melatonin in two different ranges. Appropriate dilution from a stock solution of melatonin was carried out to give concentrations ranging from 0.75 to 15 $\mu g\ L^{-1}$ and 15 to 750 $\mu g\ L^{-1}$. Gnumeric 1.10.17 was used to generate the regression analysis. Calibration curves were obtained from this regression analysis and the quantification of melatonin in extracts was performed. The standard deviation ($\sigma$) obtained for the response and the slope ($a$) from the regression were then used to calculate the limit of detection (LOD) and limit of quantification (LOQ).

The precision of the method was evaluated by studying repeatability (intra-day) and intermediate precision (extra-day). Repeatability was assessed by ten independent injections of samples on the same day while intermediate precision was determined by five independent HPLC analyses on three consecutive days. Precision was expressed as Coefficient of Variance (CV) of retention time and peak height. The acceptable limit of CV was ±10% according to the AOAC manual for the Peer-Verified Methods program (AOAC, 1993). The CV values for both repeatability and intermediate precision were less than 3%, showing that the method has excellent precision. Analytical properties for the chromatographic method for the determination of melatonin are listed in Table 1.