Sensitive Electrochemical Detection Method of Melatonin in Food Supplements

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Melatonin (N-acetyl-5-methoxytryptamin) is a ubiquitous molecule widely distributed in nature. It is a hormone produced by the pineal gland, which plays a role in the body's sleep cycles and it is a powerful antioxidant. It is possible to find it in several foods (fruit, rice, corn) and as food supplement. Screen-printed carbon electrode (DRP-150) and graphene modified screen-printed electrode (DRP-110GPH) were tested as sensors for the detection of melatonin using electrochemical detection (differential pulse voltammetry), at different pH values (7.4, 7.0, and 6.4). Quantitative detection of melatonin was possible, with better results when graphene modified screen-printed electrodes were used. The pH influenced the position of the peak as well, with lowering the pH moves more in the right. Both electrodes have been tested on samples of food supplements containing melatonin, with good recovery degrees.

Keywords: melatonin, differential pulse voltammetry, screen-printed electrodes, graphene modified screen-printed electrode, food supplement

Melatonin (N-acetyl-5-methoxytryptamine) is a natural hormone principally produced in the mammalian pineal gland during the dark phase. With its hydrophilic and lipophilic character, melatonin crosses the blood – brain barrier and enters the cells [1]. It is commonly known as a sleep regulator but also along with its metabolites is shown to possess multiple functions, including antioxidation, immunomodulatory, and anti-inflammatory effects [1].

Melatonin (ML) has protecting properties against oxidative stress and has a powerful direct chain-breaking antioxidant activity [2]. There is evidence that melatonin stimulates a number of antioxidative enzymes including glutathione peroxidase, glutathione reductase, SOD and catalase [3]. Thus, melatonin acts as a direct scavenger of free radicals with the ability to detoxify both reactive oxygen and reactive nitrogen species, indirectly increasing the activity of the antioxidative defense systems [4,5]. In the last decade, the hydroxyl radical scavenging ability of melatonin was used as a rationality for testing its radio protective role and different clinical studies indicated that melatonin administration increases the efficacy of radiotherapy and decreases the toxicity during the treatment of human cancers [6].

It was found that melatonin is present in a lot of different types of foods and food supplements. The quantity of melatonin in the most common foods is variable: banana - 0.47 ng/g [7], apple - 0.05 ng/g [8], kiwifruit 0.02 ng/g [8], pineapple - 0.04 ng/g [8], strawberry - 0.01 ng/g [8], montmorency tart cherry - 13.46 ng/g [9], orange - 0.15 ng/g [10], mango - 0.70 ng/g [10], papaya - 0.24 ng/g [10], tomato - 8-16 pg/g [11,12], red wine - 17.07 ng/g [13,14], beer - 1.43 ng/g [13], bread crumb 3 - 1.57 ng/g [15], walnuts - 1.0 ng/g [15,16] , olive oil - 53-119 pg/mL [17], almonds - 39 ng/g [18], coffea canephora - 115.25 ± 6μg/g [19], beef - 2.1 ng/g [20], salmon - 3.7 ng/g [20].

Furthermore, melatonin supplements are not considered medicinal products but foods. They are consumed by a wide sector of the population [21-23]. These products have become very attractive because of their non-prescription status, quite low prices, and the opinion that natural products are safe. They are regulated by European food law (Regulation (EC) 178/2002 and Directive, 2002/46/EC) and considered as food [24]. It's been verified that ML supplements can be considered as efficient and safe in insomnia therapy and as an anti-oxidant [25,26].

Melatonin can be detected through several methods, such as radioimmunoassay (RIA) [13, 27-29], enzyme-linked immunosorbent assay (ELISA) [28,30-32] and high-performance liquid chromatography (HPLC) [19,33]. In order to increase the validity of results, determination of melatonin was performed successfully using mass spectrometry [34].

In the latest years melatonin has also been detected by using electrochemical detection [33-36]. Electrochemical methods offer advantages including acceptable sensitivity, low cost of instrument, wide linear concentration range, possibility of miniaturization.

Differential Pulse Voltammetry (DPV) is often used to make electrochemical measurements. It has also been used in this research, being useful to study the redox properties of extremely small amounts of chemicals. Other electrochemical researches have studied melatonin using cyclic voltammetry [35-39] and square wave voltammetry [37].

Recently it was shown that the graphene-based electrodes can provide excellent capability in ultra-sensitive electrochemical detection of single nucleotide polymorphisms of DNA [40] and early detection of leukemia [41,42] using DPV.

The aim of this study is to develop and optimize rapid DPV method (testing different electrodes, at different pH values) to detect melatonin from food supplements. We evaluated the analytical performance of the different carbon-based electrodes for quantification of melatonin.

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All the authors had equal contribution at this original article

Experimental part

Material and methods

Chemicals

ML was purchased from Sigma Chemical (St. Louis, MO, USA), it is light-sensitive [43] and temperature-sensitive, for this reason till the first solubilisation it was kept in ice bath and covered with aluminum foils.

All reagents used were of analytical grade. Sodium hydrogen phosphate, sodium dihydrogen phosphate and citric acid were purchased from Poch (Sowinskiego, Poland). Phosphate buffer (PB) was prepared by dissolving appropriate amounts of sodium hydrogen phosphate 0.1M and sodium dihydrogen phosphate 0.1M according to the Merck method. Citrate-phosphate buffer (CPB) was obtained by dissolving appropriate amount of citric acid 0.1M and sodium dihydrogen phosphate 0.2M. Different buffers were prepared at *p*H 8.0, 7.4, 7.0, 6.4, 5.8 with PB, 4.6, 3.8, 3.0 with CPB.

Apparatus

All electrochemical measurements were performed keeping ML in ice-bath, with an Autolab potentiostat / galvanostat 302N (Eco Chemie, Utrecht, The Netherlands) coupled to a PC running NOVA 1.8 Software (Metrohm Autolab B.V.), and using a single-compartment three electrode cell by DropSens.

Electrodes type

All measurements were carried out using a screen printed three-electrode configuration. Two types of electrodes were tested: a screen printed carbon electrode (DRP-150) and a graphene modified screen printed electrode (DRP-110GPH). They were purchased from DropSens (www.dropsens.com) Oviedo, Spain.

The electrochemical cell of DRP-150 consists of a working electrode (carbon, 4 mm diameter); counter electrode (platinum); reference electrode (silver).

The electrochemical cell of DRP-110GPH consists of a working electrode(s): GPH / Carbon; Counter electrode: Carbon; Reference electrode: Silver. DRP- 110GPH are screen-printed carbon electrodes modified with graphene (CVD Graphene is suspended and the electrodes are modified by drop-casting). It was used a graphene nanopowder 8 nm flakes, according with data provided by the manufacturer company. No reduced graphene or reduced graphene oxide are used in reference 110GPH.

Electrochemical procedure

The measurement were conducted with the sensors immersed in 10 mL of PB or CPB at different *p*H. ML was examined using differential pulse voltammetry (DPV) in a potential range from -0.5 V to 1.0 V, scan rate of 10 mVs^{-1}, pulse time of 0.05 s, step potential of 5 mV and 15 s of equilibration time.

Sample preparation

Melatonin capsules 3 mg - additional ingredients soybean oil, glycerol, beeswax, soybean lecithin - (Melatonina, Bio-Synergie Activ S.R.L., France) were purchased from a local pharmacy. To avoid analyte degradation, the extraction of ML was done under dark conditions [36,43]. The liquid of the capsules was diluted with 3 mL of methanol according to the method of Gomez, et al. [43], then vortexed for 40 s and sonicated in an ultrasound bath for 10 min. The resulting extract was filtered in Nylon syringe filters 0.2µm (Nordic Invest S.R.L., Cluj-Napoca, Romania). On the day of the experiment, solutions were introduced in phosphate buffer and then analyzed.

Results and discussions

Experimental results with sensor DRP-150

To better understand the behavior of melatonin with the electrode DRP-150 it has been decided to make a first analysis at high concentrations and then reduce it until finding the limit of detection for ML. The analysis at high concentration, conducted in a *p*H range 8-3.0, revealed that with acidic *p*H, the peak of melatonin is not well defined anymore, but it starts to split in two new peaks (fig. 1).

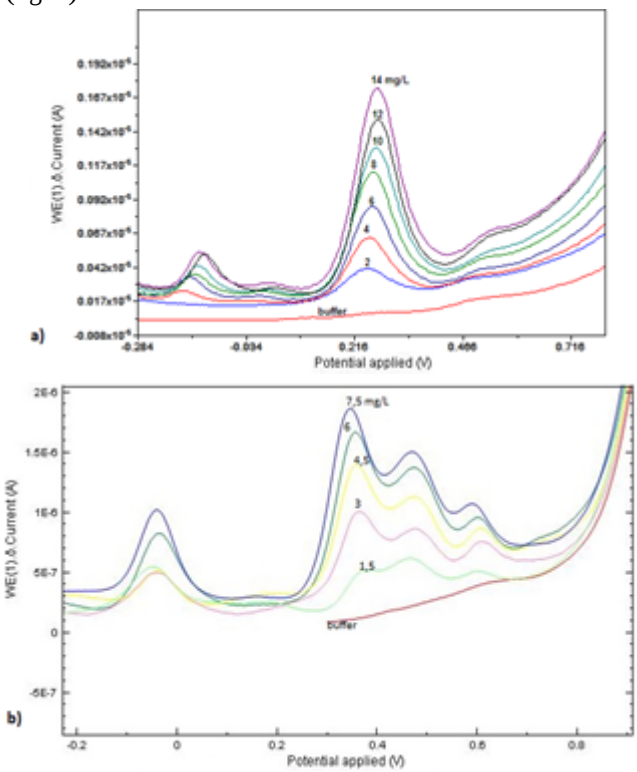

Fig. 1. Comparison of peaks between *p*H 7.0 (a) and *p*H 3.0 (b), at similar concentrations of melatonin

For this reason it has been decided to focus and study the behavior of low concentration of ML in *p*H of 6.4, 7.0 and 7.4. The same *p*H has then been used to study the behavior of ML with the second electrode, DRP-110GPH.

Furthermore, with the increasing of the concentration the slope changes considerably (fig. 2). It has been decided to study and work on the first dependence where the method seems to be more sensitive (bigger slope). The calibration lines for lower concentration of melatonin, at different *p*H values, are indicated in figure 3.

As we mentioned before, at *p*H 7.4, 7.0, and 6.4 were done experiments for both low and high concentrations. The dependencies (fig. 4) show high peaks (they are better defined), but our attention was to study the first slopes. The peaks are well defined and easy to measure (fig. 4

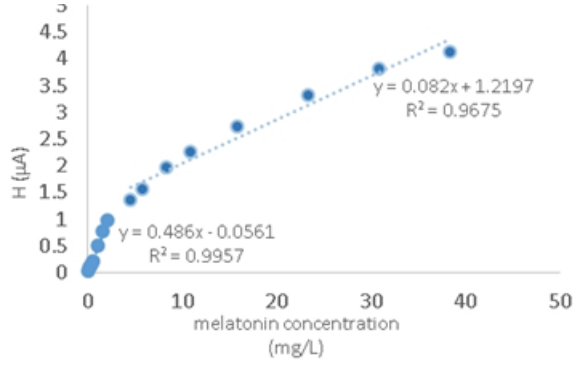

Fig. 2. Calibration curve with two liner domains, where slopes change depending on the concentration of melatonin (in range from 0.125 mg/L to 38 mg/L of ML)

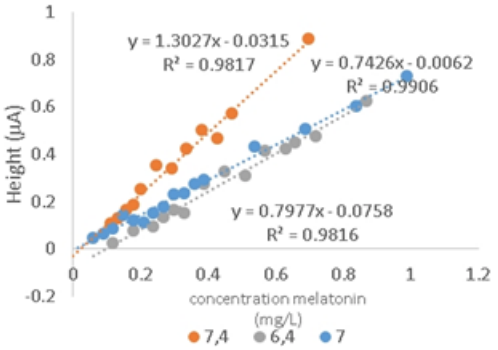

Fig. 3. First slopes of every *p*H

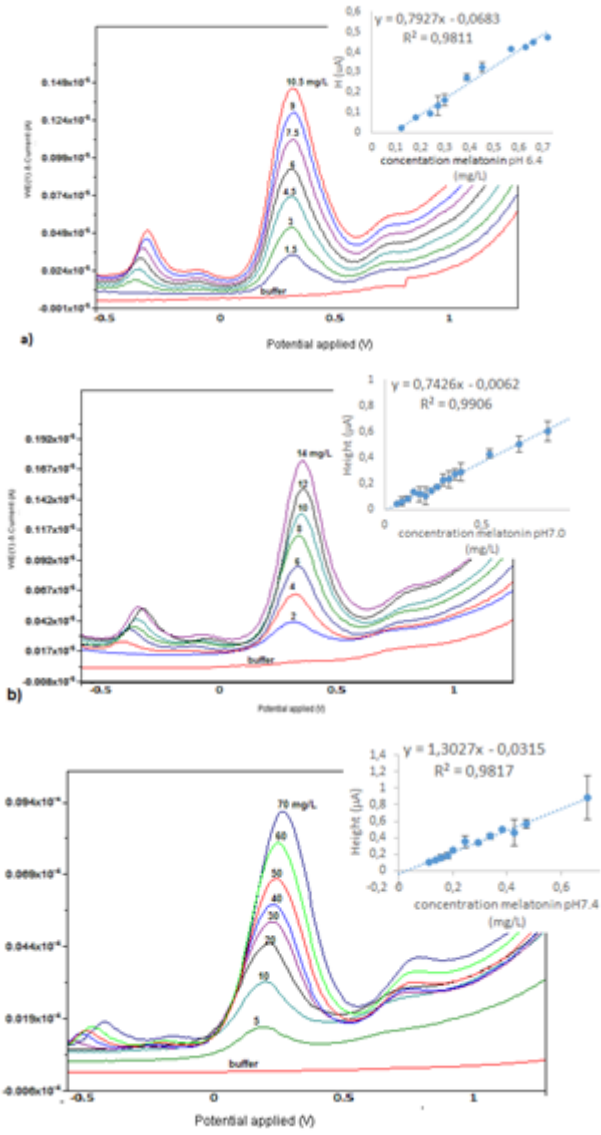

Fig. 4. DPV's measurements of melatonin solutions for *p*H 6.4 (a), 7.0 (b), and 7.4 (c), using DRP-150 sensor

a,b,c) at low concentration, but is not always easy to find or measure the peak because it could have a non-regular basis or have an asymmetric form.

Influence of pH

*p*H 7.4 results to be the best pH to analyze ML with the electrode DRP-150, due to the better sensitivity - slope of the calibration line was 1.3027 vs. 0.7426 at *p*H=7.0 and 0.7977 at *p*H=6.4 (fig. 5).

The *p*H influenced the position of the peak as well, with lowering the pH moves more in the right (fig. 6). The peak position for *p*H 6.4 was at 0.379 (± 0,066) V, for *p*H 7.0 was at 0.341 (±0.013) and for *p*H 7.4 was at 0.268 (±0.014) V.

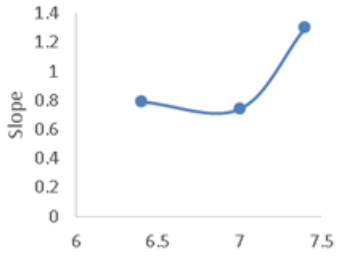

Fig. 5. Correlation between the slope of calibration lines and *p*H, for DVP detection of melatonin using DRP-150 sensor

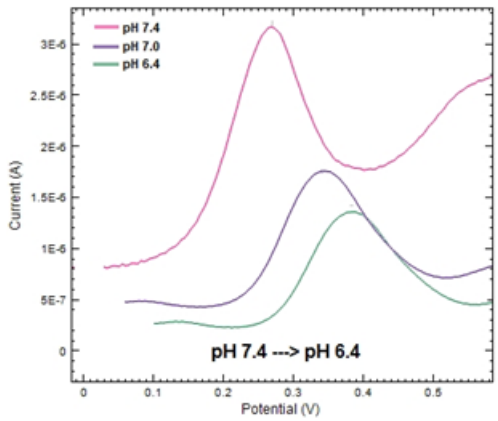

Fig. 6. Movement of 10mg/L of ML peaks depending on the *p*H

Real samples analysis

In order to evaluate the analytical applicability of the proposed method, it was also applied for the determination of melatonin in food supplements containing melatonin as active compound. The calibration line, obtained with the standard solutions, has been used to calculate the experimental concentration of melatonin in real samples, then it has been calculated the recovery degree.

In the first part of the analysis it has been added a standard solution of melatonin and, once a good calibration line was obtained, it was continued with the solution containing the real sample. The recovery degree shows the correlation between the real concentration of the sample (as it is indicated on the package of the food supplement) and the experimental concentration. Test on real samples have been conducted at *p*H 6.4, *p*H 7.0, and *p*H 7.4. All of them showed a good recovery degree.

At *p*H 6.4 the recovery degree was (109.94 ± 6.37) %, at *p*H 7.0 was (103.13 ± 8.14) %, and at *p*H 7.4 was (107.42 ± 10.30) %.

Experimental results with sensor DRP-110GPH

With this sensor are being studied just low concentrations of melatonin at *p*H 6.4, 7.0, and 7.4. The peaks with this sensor are not well-defined (fig. 7), but it is possible to measure them in each case.

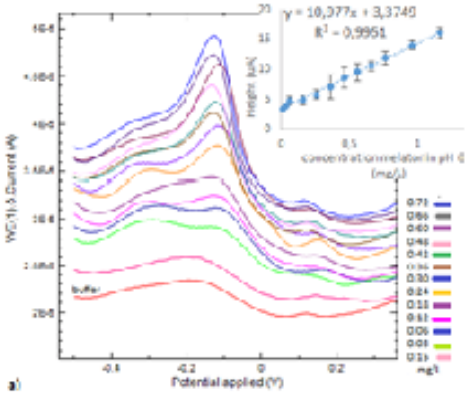

Fig. 7. DPV's peaks for different concentration of melatonin, for *p*H 6.4 (a)using DRP110GPH sensor

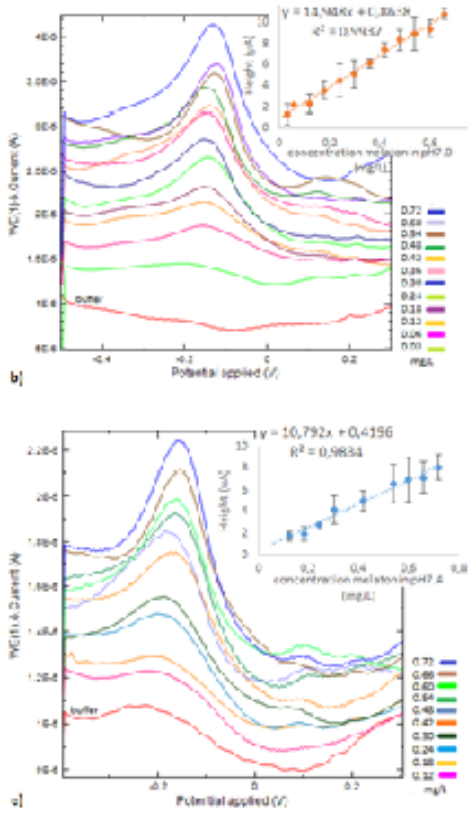

Fig. 7. DPV's peaks for different concentration of melatonin, for *p*H 7.0 (b), and 7.4 (c), using DRP110GPH sensor

Also for this sensor the slopes changed depending on the concentration. It's been studied the first slope for all the *p*H (fig. 8).

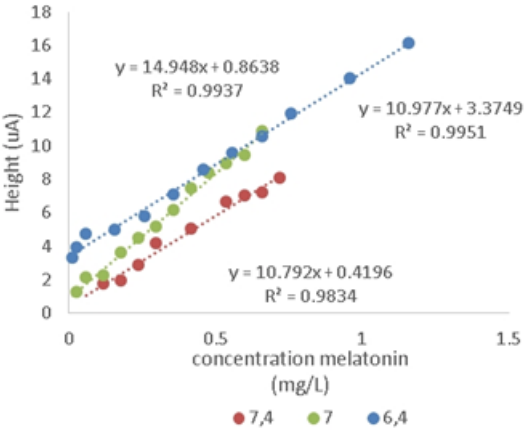

Fig. 8. Calibration lines for melatonin detection, for *p*H 6.4, 7.0, and 7.4

Influence of pH

*p*H 7.0 results to be the best pH to analyze ML with the electrode DRP-110GPH, with a slope of 14.948 vs. 10.792 at *p*H 7.4 and 10.977 at *p*H 6.4 (fig. 9).

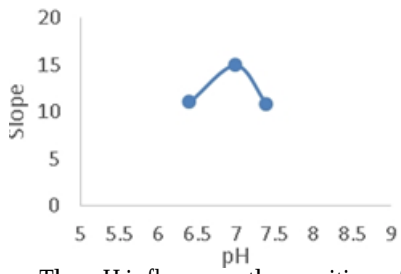

Fig. 9. Correlation between the slopes of calibration line and *p*H, using DPV measurements with DRP-110GPH

The *p*H influences the position of the peak as well, with lowering the *p*H it moves more in the right (fig. 10). The peak position at *p*H 6.4 is at -0.104 (±0.048) V, *p*H 7.0 at -0.144 (±0.029) and *p*H 7.4 at -0.160 (±0.018) V.

Real samples analysis

The calibration line has been used to calculate the experimental concentration of melatonin in real samples, then it has been calculated the recovery degree. The same methods for the analysis with the other sensors were applied here. Test on real samples have been conducted at *p*H 7.0 and *p*H 7.4. Both showed an excellent recovery degree. At *p*H 7.0 the recovery degree is (97.78±7.20)%; at *p*H 7.4 is (101.03±8.45)%.

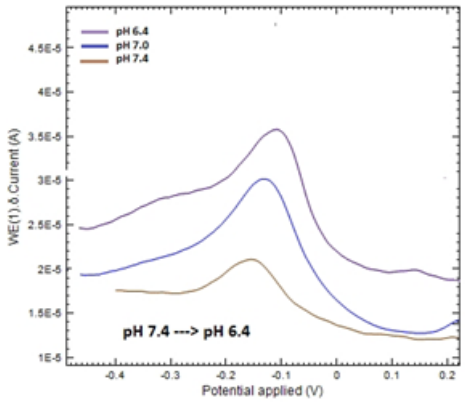

Fig. 10. Movement of peaks of DPV measurements with DRP-110GPH (for 0.66 mg/L of ML), depending on *p*H

Comparison between DRP-150 and DRP-110GPH

The experimental curves showed low background currents related to capacitive effects appearing when screen printed electrodes were immersed in buffer solutions [44]. The background currents were similar for both DRP-150 and DRP-110GPH electrodes.

The detection with DRP-150 shows two peaks (fig. 11a); instead with DRP-110GPH the detection shows one definite peak (fig. 11b). The height is really obvious compared to the data obtained using the other sensor.

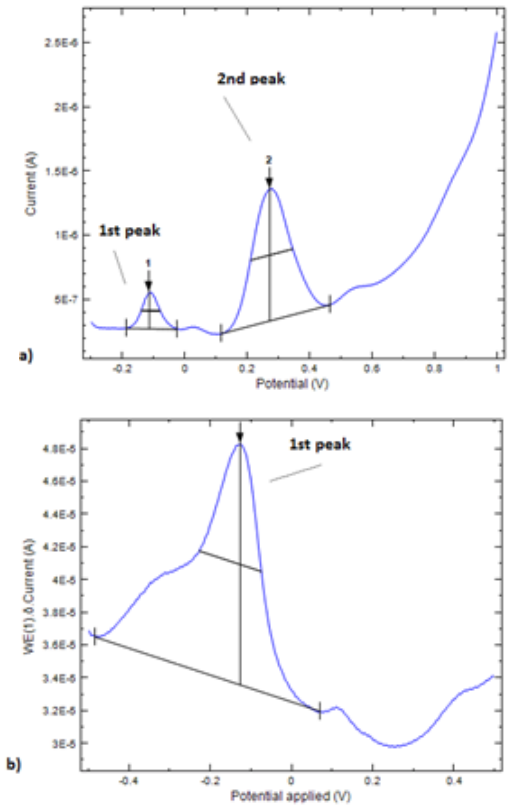

Fig. 11. Comparisons of the peaks position using the two sensors DRP-150 (a) and DRP-110GPH

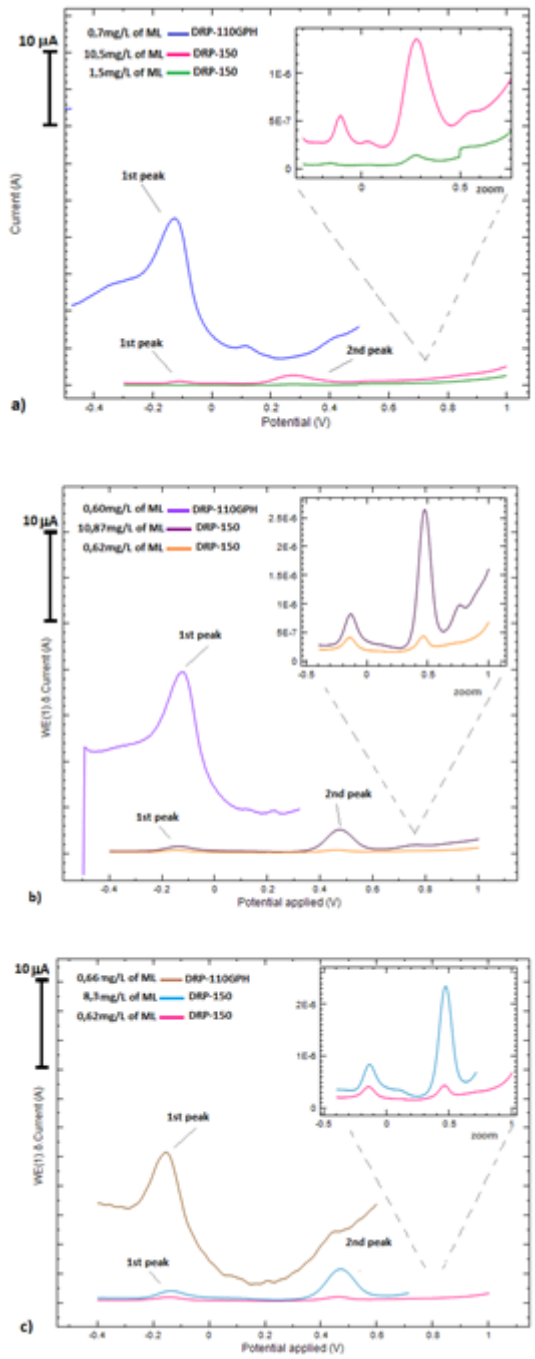

Fig. 12. Comparison of melatonin peaks with DRP-150 and DRP-110GPH at *p*H 6.4 (a), *p*H 7.0 (b) and *p*H 7.4 (c), zoom on the peaks of DRP-150

Table 1

THE DETECTION LIMITS FOUND FOR DETECTION OF MELATONIN USING DPV METHOD, DRP-110GPH AND DRP-150 SENSORS

pH	LOD (mg/L)		
	pH 7.4	pH 7.0	pH 6.4
DRP-110GPH	0.06	0.03	0.015
DRP-150	0.11	0.09	0.12

For DRP-150 the calibration line has been built on the second peak. It's possible to notice that the second one has a better increasing than the first one (fig. 4). In the case of DRP-110, instead, the calibration line has been built on the first, and only one, peak (fig. 7).

It is possible to notice the peaks of DRP-110GPH are much higher than the peaks of DRP-150 (fig. 12 a,b,c). As concerns *p*H 6.4 the slope of the first peak of DRP-110GPH is approximately 14 times bigger than the slope of the second peak of DRP-150; for *p*H 7.0 the slope is approximately 20 times bigger and for 7.4 the slope is approximately 8 times bigger. This change of sensitivity could be explained by the electroactive surface area of the grapheme screen-printed electrodes which is much larger than the geometrical electrode surface area of the normal working electrodes due to their roughness [45].

The detection limits of melatonin, identified with DPV method and found with DRP-110GPH and DRP-150 are listed in table 1.

The obtained results are in line with others found in scientific literature where it has been observed that the oxidation of other electroactive compounds (dopamine) at the surface of modified electrode occurs at a potential less positive than that of an unmodified carbon paste electrode [46]. The detection limit of $5.6x10^{-8}$ M for dopamine was obtained using square wave voltammetry.

The processes at the graphene-based sensor surface are indicated in literature studies [45], showing that oxidation peaks were proportional with the scan rate, indicating a surface controlled process. These processes are in agreement with other data reported in the literature for melatonin [47]. It was observed that graphene is more suitable for electrochemical applications because it has a nanostructured configuration and it can improve the sensitivity of the sensing procedure by increasing the number of electroactive sites on the surface of working electrodes and enhancing the rate of electron transfer, according to other reported literature data as well [45].

Electrochemical methods offer the possibilities to use the techniques for different application in life sciences (medicine, pharmacy, food control, environmental monitoring) [48-55] due to their advantages including simplicity, good sensitivity, wide linear concentration range, low expenses of sensors and equipment's, suitability for real-time detection, possibility of miniaturization and use as remote devices [56].

The analysis of melatonin in foods presents some difficulties. First, the content of melatonin in some plants is in the microgram per gram range, but there are cases of plants when the amount of melatonin found is much lower. Also, due to the antioxidant properties of melatonin, it exists the possibility to react with other food biological active constituents, so careful handling of the sample is thus a prerequisite [57]. Because of the expected interferences from the food matrix and because the molecules of melatonin are light sensitive because of their oxidation, a prior step of purification is generally required, as well as other specific stability and interferences studies.

Conclusions

This work shows that nanomaterial as graphene modified screen-printed electrodes (DRP-110GPH) provides an easy and fast analytical tool for the detection of ML with proper reproducibility, good versatility and good sensitivity of the electrode process; the sensitivity for the determination of melatonin increases markedly. The result is that the use of the graphene electrode (DRP-110GPH) is significantly better than the carbon one (DRP-150).

The values of currents of the oxidation peak were found to increase when the concentrations of melatonin increased in the analytical system. The dependence between peak current and concentration of melatonin, allows peak current measurements to be used for quantitative applications.

Applicability of the sensor was studied by analyzing commercial melatonin formulations as food supplements. Although the amount of melatonin in the food supplement was indicated by the producer, recovery studies were

successfully performed. We could conclude that the proposed method has a high sensitivity and could be applied for the analysis of products containing melatonin.

Acknowledgements: Mihaela Badea and Laura Floroian were partially sustained in this work by fellowship funds of UTBV for international cooperation between mixed partnerships (Bursa Universitatii Transilvania, 2016). Angela Miccoli thanks to the Erasmus Plus program for the financial sustaining of her fellowship in UTBV. Preliminary results of this study were presented at the 6th International Conference on Food and Tourism BIOATLAS 2016 (May 27-28, 2016, Brasov, Romania).

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Manuscript received: 18.09.2017