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Original Article

Introduction of the yeast strain *Cryptococcus uzbekistanensis* as a melatonin producer: Evaluation of the effect of different growth media on melatonin production

Ali Nosrati*

School of Allied Medicine, Tehran University of Medical Science (TUMS), Tehran, Iran

Abstract. Melatonin (N-Acetyl-5-Methoxytryptamine) is a neuro-hormone produced in the pineal gland. It is found in animals, plants and fungi. Its biological properties contribute to the circadian rhythm, reproductive physiology and antioxidant activity. Melatonin is known to help reduce the effects of the jet lag by cultivating the necessary re-set of the body's circadian rhythm. Natural production of melatonin in humans depends on the amount of light as well as age; in fact, natural melatonin production declines with age. The present study was conducted to evaluate melatonin expression in yeast species. One hundred and thirty yeast species were screened and analyzed for melatonin production. It was found that *Cryptococcus uzbekistanensis* produced 46 ppm of melatonin in the optimized condition. The Box-Behnken methodology was used to identify significant factors in terms of the carbon source, temperature, growth incubation, and tryptophan concentration for optimization of melatonin production. The Response Surface Methodology (RSM) was applied to optimize the levels of these factors. In the presence of 3 ppm of tryptophan in the growth media, a growth incubation time of 72 hours, glucose as carbohydrate, and a temperature of 308 Kelvin, *Cryptococcus uzbekistanensis* produced the maximum concentration of melatonin. This is among few instances that a microorganism is found to produce melatonin and thus the results can be utilized for industrial production of melatonin.

Keywords: Melatonin, *C. uzbekistanensis*, Media engineering, Experimental design method, Box-Behnken, RSM.

Introduction

Melatonin is a major neuro-hormone secreted during the dark hours of the night by the human pineal gland. Melatonin synthesis is inhibited when the retina detects light. First melatonin production in humans goes back to age 3–6 month. The maximum production of melatonin occurs at the age of 1 to 5 years [1]. Melatonin secretion by the pineal gland progressively declines by age. A significant reduction of circulating melatonin is also observed in numerous disorders including neurological conditions such as Alzheimer's disease and metabolic disorders particularly type 2 diabetes [2], which results in sleep problems. Melatonin has an effective role in preventing jet lag symptoms and therefore it could be used for treatment of the travelers experiencing the jet lag [3].

Melatonin also has anti-inflammatory and anti-oxidant effects. A study recently assessed the effects of melatonin on the COVID 19 patients and found that anti-inflammatory effects of melatonin could help the patients [4]. Using microorganisms to produce drugs and hormones, biotechnology can help ease the symptoms of human diseases.

Melatonin is found in some yeast species. According to a study, melatonin is a bioactive compound present in wine since it is synthesized by *Saccharomyces uvarum* and some strains of *Saccharomyces cerevisiae* during alcoholic fermentation. Rodriguez Naranjo et al. found melatonin in wine and introduced *Saccharomyces* strains as the source [5].

Another study found melatonin not only in saccharomyces strains but also in non-saccharomyces strains of wine yeasts [6]. Moreover, *Torulaspora delbrueckii*, *Hanseniaspora uvarum*, *Starmeralla bacillaris* and *Metschnikowia pulcherrima* also produce melatonin [7]. Microorganisms such as fungi could be used for expression and production of melatonin. Among fungi, yeasts are the best choices since they are safe; single-cell organisms; moreover, considering the aforementioned studies, there is strong evidences of the presence and production of melatonin in yeasts. The aim of the present study was to find yeasts that produce melatonin. One hundred and thirty species of yeasts from the collection of Environmental Biotechnology Laboratory of University of Tehran were

* Corresponding author: Ali Nosrati, MS (a-nosrati@student.tums.ac.ir)

screened to investigate melatonin production.

Materials and Methods

Collection of soil samples and isolation

In this study, 130 yeast isolates were collected from different areas of Iran and analyzed for isolation of potent yeasts.

Identification and phylogenic analysis

For identification of the selected yeast, PCR amplification of the ITS DNA was performed. The yeast was grown overnight in potato dextrose broth and the cells were harvested for DNA extraction using an extraction kit. Primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify ITS and the obtained fragment was then sequenced and compared with the GenBank data-base. Phylogenic analysis of the yeast was also performed using the MEGA software.

Yeast isolation

A collection of 130 yeast strains was available from previous work of yeast isolation. Soil samples were collected from various regions of Iran and enriched and cultured in YPG containing 5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose with 100 mg/L added chloramphenicol and 500 mg/L Rose Bengal to isolate indigenous yeasts. The obtained isolates were purified on YPG agar and stored at 4˚C.

Sample preparation and evaluation of melatonin production

In the next step 1×10^6 cell/mL of each yeast was cultured in a 50-mL flask containing 100 mL Czapek Dox broth (30 g/L sucrose, 3 g/L sodium nitrate, 1 g/L dipotassium phosphate, 0.5 g/L magnesium sulfate, 0.5 g/L potassium chloride and 0.01 g/L ferrous sulfate) supplemented with 0.59 g/L standard tryptophan (Trp) (Rodriguez-Naranjo et al. 2012). The flasks were shaken at 120 rpm at 28˚C. After 24 hours, 1 mL of the culture medium was taken for melatonin production examination. It should be noted that all of the flasks and samples were protected from light during incubation since melatonin is a light-sensitive compound. Melatonin production was assayed using the HPLC method (Hamase et al. 2000).

Selection of spectrophotometric positive species

Saccharomyces *cerevisiae* is a melatonin producer. Our method for spectrophotometric detection of positive species was to culture Saccharomyces *cerevisiae* in Czapek Dox broth medium in the presence of 1% of tryptophan by shaking at 120 rpm for two days. Five milliliters of growth medium was then separated and centrifuged for 5 minutes at 400rpm.Next, 15ml of dichloromethane was injected as solvent to separate melatonin after shaking the mixture. Then, 3mL was separated from the top phase in which melatonin existed, and analyzed using a spectrophotometer with ϰ=285nm, and its absorption was 0.0713. Using this method, all 130 yeast isolates were cultured. The species with the highest absorption and melatonin production were selected.

TABLE 1

Code	Variables	Levels		
1	Carbone source	Indole	Glucose	Starch
2	Tryptophan concentration(ppm)	1	2	3
3	Growth time (hour)	24	48	72
4	Temperature(K)	301	304.5	308

HPLC method

A high performance liquid chromatography system with a UV detector was used for the determination of melatonin (Shimadzu, Japan). The HPLC unit consisted of a pump (LC-10ADVP), injector (7725a), fluorescence detector (254 nm), integrator (SCL10AVP) and analytical column (CLC-ODS (25 cm) PN: 228-17873-92. The eluent included 70% acetonitrile and 30% H2O with a flow rate of 1 ml/min (Hamase et al. 2000).

Optimization of culture condition

Various factors were evaluated for determining the ideal condition for melatonin production including the carbon source, four different concentrations of tryptophan, temperature and growth incubation. The aforementioned factors went subjected to Response Surface Methodology (RSM).

Results

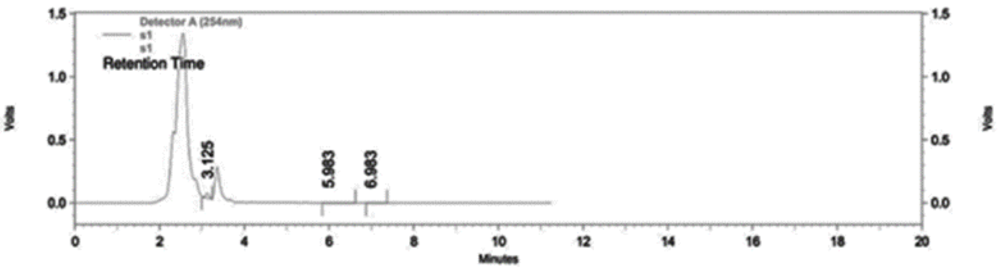
HPLC positive species

Cryptococcus *uzbekistanesis* (absorption: 2.634), Cryptococcus *aerius* (absorption: 2.609) and Cryptococcus *adelinsis* (absorption: 1.118) were spectrophotometry positive species analyzed using the HPLC to check their melatonin production. The HPLC results showed that; C. *uzbekistanesis* was a melatonin-producer yeast. Considering the absorption of melatonin solute using HPLC, the following equation was obtained for melatonin concentration: Y=4851.5x+15013; where Y is optical density and X is melatonin solution (ppm). The amount of melatonin that was produced was 45ppm.

Optimization by response surface methodology

The results indicated that the indigenous yeast *Cryptococcus uzbekistanensis* was able to produce melatonin in the fermentation broth. A statistical experiment design was used to engineer production media for different carbon sources, temperatures, growth incubation times, and tryptophan concentrations. Response surface methodology (RSM) was applied to determine and optimize the effective factors in melatonin production.

Optimization of process parameters was carried out using the Box-Behnken design with the parameters found, including time, carbon source, tryptophan concentration



Detector A (254nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	3.125	204501	98.927	35914	99.603
2	5.983	1149	0.556	56	0.155
3	6.983	1070	0.518	87	0.241
Totals		206720	100.000	36057	100.000

Fig. 1 Melatonin production by C. uzbekistanesis

and temperature. **Table 3** presents the design table.

Interpretation of the results collected by regression analysis of the plots of **Tables 4, 5, 6, 7, 8 and 9** using the Box-Behnken tool showed, the following results:

Indole

Melatonin (ppm) = 10.8 - 8.5 tryptophan + 1.676 time + 8.06 tryptophan*tryptophan - 0.02175 time*time + 0.698 tryptophan*time.

Glucose

Melatonin (ppm) = -22.1 + 17.0 tryptophan + 2.530 time + 8.06 tryptophan*tryptophan - 0.02175 time*time + 0.698 tryptophan*time.

Starch

Melatonin (ppm) = 7.6 + 2.5 tryptophan + 2.041 time + 8.06 tryptophan*tryptophan - 0.02175 time*time + 0.698 tryptophan*time.

ANOVA of the quadratic regression model demonstrated that tryptophan concentration and growth incubation time were highly significant factors ($p \leq 0.005$) (**Table 4,5, and 6)**. The goodness of fit of the model was checked using the determination coefficient (R^2). The R^2 value was 0.9667and the adjusted R^2 value was 95.64. It was in reasonable agreement with the predicted R^2 (90.82). Graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between test variables in order to deduce the optimum conditions. The interaction effects and optimal levels of the variables were determined by plotting three-dimensional (3D) response surface curves [8, 9] (**Table 7, 8, and 9**). The response surface curves, which represent the interactions between variables, showed that a tryptophan concentration of 3 ppm, growth incubation time of 72 hours, glucose as the carbon source, and a temperature 308 k yielded the maximum melatonin concentration. The shape of the response surface curves showed a strong positive interaction between these tested variables.

Morphological identification

The gene sequence similarity search in the EzTaxon database showed that the sampled yeast belonged to **Cryptococcus** *Uzbekistanis* with 100 % similarity. The isolate was deposited in University of Tehran Microorganisms Collection and its sequence was registered in the GenBank under accession number KX347452.

Discussion

About 94% long-haul travelers experience jet-lag due to changes in the circadian rhythm. The body's internal clock is controlled by the release of a hormone called melatonin. This pineal gland Hormone is regulated by light. The levels of melatonin are highest in children and diminish with age. [10] The functional repertoire of melatonin makes it a medical 'wonder drug'.

Naturally, it is prescribed for people with sleep problems like insomnia or disrupted circadian rhythms. It is also used to treat problems related to the central nervous system like depression or to prevent damages in neurons [11]. Melatonin is also administered as a drug to ameliorate diseases like Parkinson's or Alzheimer's disease due to its ability to reduce oxidative stress through its free radical scavenging effect as well as indirect enhancement of the antioxidant defense systems. Several experiments were performed using Melatonin to probe its oncostatic effect on different cancer cells, and so its use as a drug is extended to other disorders like hypertension, diabetes, and urinary incontinence.

Furthermore, melatonin shows regenerative properties in tissues like the bone, muscle, and cartilage as well as the ability to sustain a successful *in vitro* embryo development in some animals because it promotes the development of blastocysts and increases the rates of embryo implantation, pregnancy, and postnatal survival of offspring. [12]

In Europe, around 1,700,000 people die solely due to

TABLE 2
Response surface methodology experiments.

StdOrder Run Order	Run Order	P Ttype	Blocks	Tryptophan (ppm	Time (hour)	Temperature (K)	Carbon Source	Melatonin (ppm)
12	1	2	1	2	72	308	indole	146
40	2	2	1	2	72	301	starch	175
21	3	2	1	3	48	301	glucose	295
37	4	2	1	1	48	308	starch	98
29	5	0	1	2	48	304/5	glucose	179
28	6	0	1	2	48	304/5	glucose	183
30	7	0	1	2	48	304/5	glucose	181
2	8	2	1	3	24	304/5	indole	145
25	9	2	1	2	72	301	glucose	204
11	10	2	1	2	24	308	indole	105
5	11	2	1	1	48	301	indole	74
26	12	2	1	2	24	308	glucose	127
18	13	2	1	1	72	304/5	glucose	115
10	14	2	1	2	72	301	indole	129
3	15	2	1	1	72	304/5	indole	90
24	16	2	1	2	24	301	glucose	110
16	17	2	1	1	24	304/5	glucose	86
7	18	2	1	1	48	308	indole	73
1	19	2	1	1	24	304/5	indole	30
41	20	2	1	2	24	308	starch	100
44	21	0	1	2	48	304/5	starch	137
32	22	2	1	3	24	304/5	starch	162
35	23	2	1	1	48	301	starch	95
34	24	2	1	3	72	304/5	starch	285
13	25	0	1	2	48	304/5	indole	102
20	26	2	1	1	48	301	glucose	107
33	27	2	1	1	72	304/5	starch	100
42	28	2	1	2	72	308	starch	180
22	29	2	1	1	48	308	glucose	110
45	30	0	1	2	48	304/5	starch	165
36	31	2	1	3	48	301	starch	250
9	32	2	1	2	24	301	indole	98
8	33	2	1	3	48	308	indole	183
19	34	2	1	3	72	304/5	glucose	315
14	35	0	1	2	48	304/5	indole	107
23	36	2	1	3	48	308	glucose	292
27	37	2	1	2	72	308	glucose	220
4	38	2	1	3	72	304/5	indole	202
39	39	2	1	2	24	301	starch	146
6	40	2	1	3	48	301	indole	195
38	41	2	1	3	48	308	starch	215
17	42	2	1	3	24	304/5	glucose	178
43	43	0	1	2	48	304/5	starch	179
15	44	0	1	2	48	304/5	indole	136
31	45	2	1	1	24	304/5	starch	73

have indicated the high potential of melatonin as an anti-cancer drug against breast cancer. [13, 14, 15] since it is a natural antioxidant hormone produced in the human body, it can be administered to the elderly cancer patients who have lower levels of melatonin.

The use of melatonin and other natural hormones is

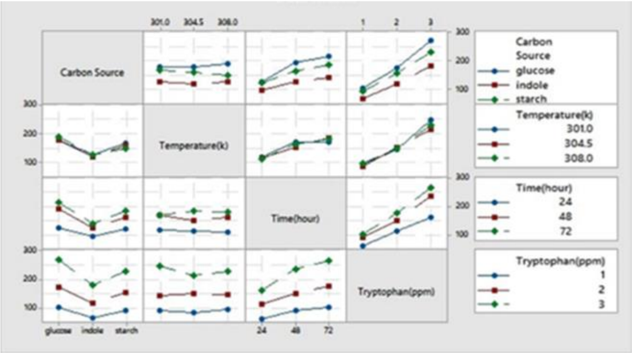


Fig. 2 Interaction plot for melatonin (ppm). Data means.

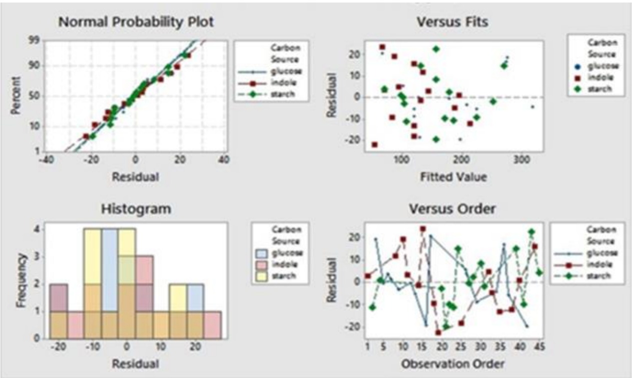


Fig. 3 Residual plot for melatonin (ppm).

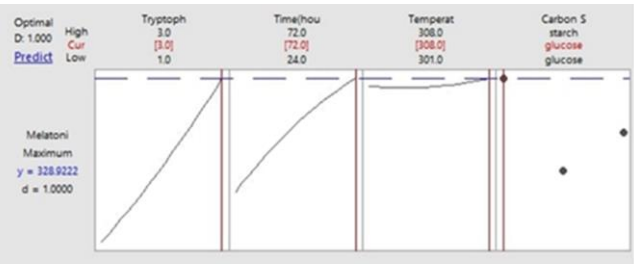


Fig. 4

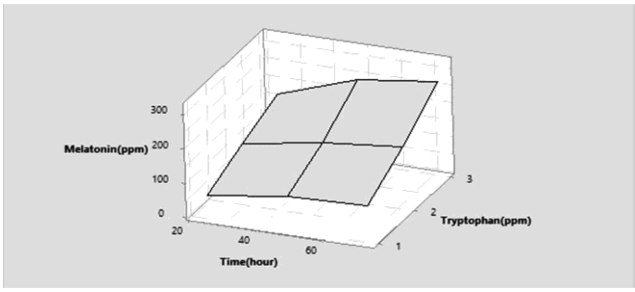


Fig. 5 Surface plot of melatonin (ppm) vs. tryptophan (ppm). Time (hour).

regulated in Europe and it can be only purchased as a medication with a prescription. The pharma grade of melatonin is expensive; hence, many people purchase cheap and crude forms. However, these unregulated cheap products contain impurities and are considered unhealthy

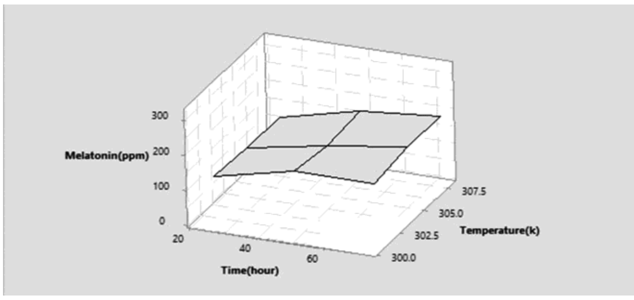


Fig. 6 Surface plot of melatonin (ppm) vs. temperature (k). Time (hour).

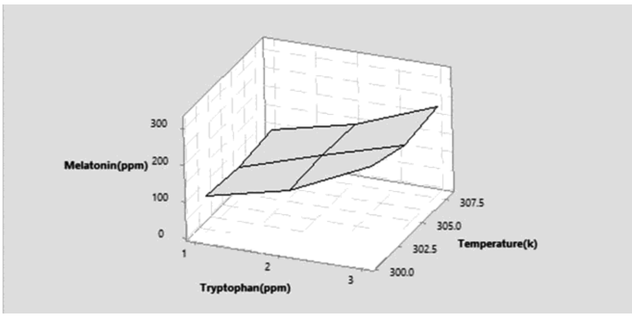


Fig. 7 Surface plot of melatonin (ppm) vs. temperature (k), tryptophan (ppm).

and toxic by European scientists. Many people import melatonin capsules from other countries like the UK as

OTC (Over-The- Counter) drugs. The German [16] and Dutch [17] markets have supported the regulated use of melatonin given the positive effects backed by scientific research.

In the U.S., people spent 32 billion USD on the sleep market in 2012 [18] to which melatonin is closely linked because of the stressful lifestyle and increased life expectancy. That huge amount of money persuaded pharma companies to work in melatonin production using chemical synthesis methods but they did not achieve a high level of purity, which could be dangerous for the patient since it can trigger some secondary reactions. [19] A wonder drug like melatonin with its multi-functional properties needs to be made available to the general population of Europe to curb cancer, autism, multiple sclerosis, depression, rare sleep-wake disorders, insomnia, heart disease, high blood pressure, and aging. The project described in the present study may facilitate attaining this goal. Chemically produced melatonin contains impurities that sometimes cause fatal secondary effects. Highly- pure melatonin is also available, but its price makes it unprofitable.

This paper reports a biologic source for producing melatonin. The results showed that C. *uzbekistanesis* as a biologic source could produce melatonin. Media engineering was used to improve melatonin production in the studied yeast species.

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Conflict of Interest

The authors declare no conflicts of interest.

References

1. Fauteck JD, Schmidt H, Lerchl A, Kurlemann G, Wittkowski W. Melatonin in epilepsy: first results of replacement therapy and first clinical results. Biologic Signals Recept 1999 Jan-Apr;8(1-2):105-101.

2. Hardeland R. Melatonin in aging and disease -multiple consequences of reduced secretion, options and limits of treatment. Aging Dis. 2012 Apr;3(2):194-225.

3. Herxheimer A, Petrie KJ. Melatonin for preventing and treating jet lag. Cochrane Database Syst Rev. 2001;(1):CD001520.

4. Acuña-Castroviejo D, Escames G, Figueira JC, de la Oliva P, Borobia AM, Acuña-Fernández C. Clinical trial to test the efficacy of melatonin in COVID-19. J Pineal Res 2020;00: e12683.

5. Rodriguez-Naranjo, M. I., Torija, M. J., Mas, A., Cantos-Villar, E., and GarciaParrilla, M. D. C (2012). Production of melatonin by Saccharomyces strains under growth and fermentation conditions. J. Pineal Res 53, 219–224. doi: 10.1111/j.1600-079X.2012. 00990.x

6. Fernandez-Cruz, E., González, B., Muñiz-Calvo, S. et al. Intracellular biosynthesis of melatonin and other indolic compounds in Saccharomyces and non-Saccharomyces wine yeasts. Eur Food Res Technol 245, 1553–1560 (2019).

7. Parra, Mª Ángeles & Gonzalez, Beatriz & Beltran, Gemma & Mas, Albert & Torija, Maria. (2019). Melatonin and glycolytic protein interactions are related to yeast fermentative capacity. Food Microbiol 87. 103398. 10.1016/j.fm.2019.103398.

8. Minitab 17 Statistical Software (2010). [Computer software]. State College, PA: Minitab, Inc. (www.minitab.com)

9. D. C. Montgomery, John Wiley & Sons, New York, NY, USA, 1991.

10. Pandi-Perumal, S. R., Trakht, I., Srinivasan, V., Spence, D. W., Maestroni, G. J., Zisapel, N., &Cardinali, D. P. (2008). Physiological effects of melatonin: role of melatonin receptors and signal Transduction pathways. Progr Neurobiol 85(3):335-353.

11. Polimeni, G., Esposito, E., Bevelacqua, V., Guarneri, C., & Cuzzocrea, S. (2014). Role of Melatonin supplementation in neurodegenerative disorders. Front Biosci (Landmark Ed), 19, 429-446.

12. Rivara, S., Pala, D., Bedini, A., & Spadoni, G. (2015). Therapeutic uses of melatonin and Melatonin derivatives: a patent review (2012-2014). Expert Opin Ther Pat 25(4), 425-441.

13. El Moneim, N. A. A., El Masry, H., Sorial, M. M., Hewala, T. I., Embaby, A., & Sheweita, S. A Molecular Case-Control Study on the Association of Melatonin Hormone and rs#10830963 Single Nucleotide Polymorphism in its Receptor MTNR1B Gene with Breast Cancer. Middle East J Cancer 2015;6(1), 11-20.

14. Hill SM, Belancio VP, Dauchy RT, Xiang S, Brimer S, Mao L, Hauch A, Lundberg PW, Summers W, Yuan L, Frasch T, Blask DE. Melatonin: an inhibitor of breast cancer. Endocr Relat Cancer. 2015 Jun;22(3):R183-204.

15. Hevia, D., González-Menéndez, P., Quiros-González, I., Miar, A., Rodríguez-García, A., Tan, D.X., & Sainz, R. M. Melatonin uptake through glucose transporters: a new target for melatonin inhibition of cancer. J Pineal Res 2015;58(2), 234-250.

16. Rivendell.eu. Rivendell News the Netherlands. [Online] Available at: http://www.rivendell.eu/internet/articles/articles.asp?pageid=276 [Accessed 29 Oct. 2015].

17. Public Assessment Report of the Medicines Evaluation Board in the Netherlands Melatonin TioFarma 1 mg, 3 mg and 5 mg tablets TioFarma b.v., the Netherlands Melatonin. 29 June 2012.

18. Mackey, M. Sleepless in America: A $32.4 Billion Business. [Online] The Fiscal Times. Available at: http://www.thefiscaltimes.com/Articles/2012/07/23/Sleepless-in-America-A-32-4-Billion-Business [Accessed 29 Oct. 2015].

19. Laforce, R., Rigozzi, K., Paganetti, M., Mossi, W., Guainazzi, P., & Calderari, G. Aspects of melatonin manufacturing and requirements for a reliable active component. Neurosignals 1999;8(1-2):143-146.