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Dietary Intake of Melatonin from Tropical Fruit Altered Urinary Excretion of 6-Sulfatoxymelatonin in Healthy Volunteers

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ABSTRACT: This study assessed the melatonin content of six tropical fruits and examined whether human consumption could contribute to dietary melatonin as measured by 6-sulfatoxymelatonin (aMT6-s, a marker of circulating melatonin in the body). Melatonin was extracted using methanol and analyzed by high-performance liquid chromatography. In a clinical crossover study, 30 healthy volunteers consumed selected fruits one at a time, with a 1week wash-out period between fruits, until completing all six fruits. Most fruits had moderate melatonin content. Significant increases in urine aMT6-s concentrations were seen after the consumption of pineapple (266%, $p = 0.004$), banana (180%, $p = 0.001$), and orange (47%, $p = 0.007$). The need to analyze melatonin both in fruit and as in vivo uptake was demonstrated. Further study is warranted regarding the clinical effect of fruit consumption in people with age-related melatonin reduction problems such as sleeplessness and illnesses involving oxidative damage.

KEYWORDS: *melatonin, 6-sulfatoxymelatonin, fruit, antioxidant*

INTRODUCTION

Previous studies report that consuming fruits and vegetables can reduce the risk of chronic diseases, especially cardiovascular disease and cancer. Evidence has shown that this may be related to antioxidants such as vitamin C, vitamin E, carotenoids, flavonoids, and anthocyanins.[1,2] Other beneficial chemicals in fruits and vegetables, including vitamins, trace minerals, dietary fiber, and many biologically active compounds, are also expected.[3] One of these, tryptophan, an essential amino acid that can be obtained only in the diet, is the only precursor of the potent antioxidant melatonin, which also regulates the sleep−wake cycle and mood.[4] However, quantification of melatonin in tropical fruit and its uptake in humans after fruit consumption have been largely overlooked.

Melatonin (*N*-acetyl-5-methoxytryptamine) is also an endogenous neurohormone secreted primarily from the pineal gland in humans. It is a natural antioxidant and potent free radical scavenger.[5−8] Melatonin also controls circadian rhythms of the body; therefore, it is involved in the sleep−wake cycle, functions of the immune and cardiovascular systems, and cell regulation.[6,9] Age-related reduction of melatonin has been correlated with disturbance of sleep, deterioration of health, and chronic diseases related to oxidative damage, including cancer.[5,10−12] Melatonin is widely used in countries that consider it as a food supplement, such as the United States. However, in most countries this important neurohormone is categorized as a drug and often not even available. Alternative or natural sources of melatonin are therefore of interest.

Melatonin was first reported outside the animal kingdom in the dinoflagellate *Gonyaulax polyedra*[13,14] and subsequently identified in bacteria, algae, fungi, insects, and plants.[15] The concentrations of melatonin in different plants vary, ranging from picograms to micrograms per gram of tissue.[16−19] The highest levels are shown in medicinal plants such as feverfew, St. John's wort, and some Chinese herbs.[4,20,21] Interestingly, among 108 Chinese herbs tested, those with higher melatonin level were reported to treat diseases associated with free radicals and to slow the aging process.[21] High melatonin content is also associated with various healthy diets. For example, Mediterranean foods have high melatonin contents, for example, olive oil,[22] cherry,[23] and grape wine.[24] Melatonin has been found at a high level in walnuts, where its beneficial effect to reduce the risk of cardiovascular disease has been reported.[13]

Current evidence suggests that melatonin in plants may act as an antioxidant or growth promoter that protects plant tissues against oxidative stress and helps them to cope with harsh environments.[19] Alpine and Mediterranean plants exposed to high ultraviolet (UV) light contain more melatonin content than the same species living under low UV exposure.[25,26] Plants growing at high altitude or in a hot climate also have been reported to have high melatonin content.[27] With the sunny climate of the tropics, higher melatonin content is postulated. To date, there is no information concerning the effect of tropical fruit consumption on circulating melatonin.

There are several reports of increasing plasma melatonin level in animals after consumption of food that contains high melatonin content.[16,28] Also, increased 6-sulfatoxymelatonin (aMT6-s) in urine (a marker of circulating melatonin in humans) after vegetable[29] or cherry consumption[30] was observed. This supports such consumption being a potential

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Table 1. Concentration of Melatonin in Each Fruit[a]

	orange	pineapple	banana	mango	papaya	makmao
weight wet of fruit (g)	100	100	100	100	100	100
volume of juice extracted (mL)	64 ± 6	66.7 ± 0.6	67 ± 5	55 ± 2	62 ± 5	53 ± 3
% extract recovered from wet fruit	5.0 ± 0.2	5.8 ± 0.9	8.9 ± 0.6	7.5 ± 0.8	5.0 ± 0.3	3.3 ± 0.4
melatonin (pg/g) wet fruit	150 ± 6	302 ± 47	8.9 ± 0.6	699 ± 75	241 ± 15	*b*

[a]Note: ± is 1 standard deviation, $N = 3$. [b]Lower than limit of detection (LOD) of 0.5 ng/mL juice.

source of natural melatonin that can be used to provide associated health benefits.

Although measurement of drug or nutrient levels in serum is a gold standard, obtaining serum samples from human subjects for screening purposes can be problematic. First, as there is no previously published human study of melatonin in the blood after fruit or food consumption, it is difficult to estimate the pharmacokinetics of melatonin in the blood from food sources and, thus, when to draw blood samples. Generally, full pharmacokinetics of oral drug dosing would require 8−12 blood draws over 4 h to determine maximum concentration (C_{max}) and time (t_{max}). As melatonin absorption from a food source may be delayed compared to an oral drug, an even longer time may be needed, necessitating more blood draws. Second, if single-point blood draws are used, they provide only melatonin levels in the blood at fixed times, and the area under the curve (AUC) still needs to be extrapolated from these data; thus, C_{max} can be missed. Additionally, with the need to perform a crossover design to control for interindividual variation for multiple fruits, even more blood draws would be required. Therefore, a serum study could not be justified.

A noninvasive technique to measure aMT6-s, the major metabolite of melatonin found in urine, is a better method of choice. Collection of overnight urine will also capture the total amount of melatonin that was circulating in the blood from both endogenous and exogenous sources. This method has shown high correlation between urinary aMT6-s and serum melatonin and has been used previously for screening of dietary melatonin.[29,30]

Thus, the present study screened six common tropical fruits for melatonin and investigated whether such fruit intake could increase levels of melatonin in the body (as measured by its urinary metabolite aMT6-s, a marker of circulating melatonin).

MATERIALS AND METHODS

Chemicals. Methanol and acetonitrile (HPLC grade) were obtained from Lab-Scan Analytical Sciences (Poland). Melatonin standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). Organic free ultrapure water filtered with a 40 μm filter was from an Elga DV25 Purewater OptionQ system.

Methods. *Fruit Extraction and Quantification of Melatonin.* The levels of melatonin were investigated in six fruits grown in Thailand: banana (*Musa sapientum* Linn.), pineapple (*Ananus comosus* Merr.), orange (*Citrus reticulata*), papaya (*Carica papyya* L.), mango (*Mangifera indica* Linn.), and makmao or Thai berry (*Antidesma thwaiteaianun*). The fruit selection was based on their potentially high melatonin or tryptophan content.

Fruit was weighed (100 g, excluding skin and seed) and the juice extracted with a fruit extractor (Phillips model HR1821, Eindhoven, The Netherlands). The juice was extracted with methanol 1:2 by volume (juice/methanol) and mixed on a rotary shaker (UMAC model UM IS/20/R, Malvern, PA, USA) at 200 rpm at room temperature overnight and then filtered through folded muslin gauze. The resulting liquid was then filtered through filter paper (Whatman no. 1, Pittsburgh, PA, USA) twice, and then Whatman no. 42 twice, rinsing each time with a small amount of 100% methanol (1−2 mL each time). The filtrate was evaporated under vacuum (Eyela Rotary Evaporator, Tokyo Rikikiai Co. Ltd., Tokyo, Japan) in a weighted flask at 45 °C until dry. Extracts were frozen at −20 °C overnight and then freeze-dried (Flexi-Dry MP, FTS Systems, NY, USA) for 24 h or until the samples were dry. The amount of dry extract was determined and the percentage recovery calculated. Extracts were stored in light-protected, airtight plastic containers at 4 °C until analyzed.

High-performance liquid chromatography (HPLC) with fluorescent detection (FD) shows good specificity for indoleamines, with specific absorption and emission bands based on their substitution and electron delocalization. Previous studies have reported that melatonin was clearly separable from other indoleamines with HPLC-FD.[28,31−35] If an extraction/purification step with solid phase extraction (SPE) is used, it further reduces interfering compounds. Mass spectroscopic (MS) identification of phytomelatonin is also a widely used method; however, it will only aid identification of unknown peaks (components) if good separation has already been achieved by liquid chromatography (LC). With good HPLC separation, a specific detector (e.g., FD), and pure standards, MS analysis may not be necessary. Previous studies[31,32] also showed that the HPLC-FD method was validated as reliable for the quantitative analysis of melatonin and met Association of Official Agricultural Chemists (AOAC) requirements[36] compared to LC-MS. In this study, therefore, fruit samples (identical to those consumed by subjects) were analyzed for melatonin content by HPLC-FD.

For HPLC analysis, 6 g of extract was purified with 3 mL SepPac C18 SPE (Waters, Milford, MA, USA) cartridges, using 100% methanol as eluent. The eluent was dried under nitrogen, reconstituted with 500 μL of acetonitrile with sonication, of which 50 μL was autoinjected onto an HPLC column. Melatonin content was quantified by RP-C18 HPLC, using an Agilent Zorbax SB-C18 column (5 μm, 4.6 mm × 150 mm) at 25 °C on an Agilent 1100 (Santa Clara, CA, USA) with an FLD fluorescent detector (λ_{ex} = 285 nm, λ_{em} = 345 nm). The mobile phase was 15% acetonitrile in pH 7.0 phosphate buffer at a 1 mL/min flow rate. The elution time was 20.84 min for standard melatonin. Analysis used integrated peak areas compared to standard curves using pure melatonin standard. The quantification limit was 0.5 ng/mL (0.1 ng/g extract). To remove the possibility that melatonin was coeluted with other components with the same retention time, confirmation of the identity of melatonin in the fruit was performed using a nonextraction ELISA RE54041 kit (IBL International, Hamburg, Germany) as per the manufacturer's protocol using 0.1 mg of extract in 100 μL of deionized water, and 100 μL of standards and controls. The specificity of this ELISA kit for melatonin is high, with manufacturer quoted cross-reactivity for 5-methoxytryptamine of 2.5%; for *N*-acetylserotonin, 1.2%; for 5-methoxytryptophol, 1.2%; and for serotonin <0.02%. Functional sensitivity was 1 pg/mL.

Study Design and Participants for Human Uptake Study. A crossover trial was used to examine urine aMT6-s concentrations after fruit consumption. The study was approved by the Khon Kaen University Ethics Committee prior to initiation of the study. Informed consent was obtained from all 30 healthy volunteers (15 males and 15 females). Mean age was 22 years old, ranging from 18 to 25 years, with body mass index of 20 (male, 21; female, 19), body weight of 56 kg (male, 64 kg; female, 47 kg), and height of 167 cm (male, 176 cm; female, 157 cm). Participants had no chronic illness and did not use medication, normally ate fruits and vegetables at less than three portions per day, took no vitamin or mineral supplements, abstained

Table 2. Urinary aMT6-s after Adjustment with Urine Volume

type of fruit		*N*	median (ng)	interquartile range (ng)	mean difference[a] (%)	*p* value[b]
banana	control	28	6108	2339−14193		0.004
	banana	28	14108	6409−22540	266	
pineapple	control	28	10396	6820−14338		0.001
	pineapple	28	23099	17043−33748	180	
orange	control	29	10761	7257−15435		0.007
	orange	29	15894	10710−20748	47	
papaya	control	29	13273	6753−25702		0.256
	papaya	29	18807	11026−48890	271	
makmao	control	27	13273	5781−16500		0.962
	makmao	27	10417	6660−23161	113	
mango	control	26	13657	9698−25066		0.069
	mango	26	10336	5990−13764	−12	

[a]Mean difference of individuals from baseline (with 0% being the value obtained in the basal group, i.e., no change from baseline). [b]Wilcoxon signed-rank test.

from smoking or drinking alcohol, had no allergy to the studied fruits, and usually began sleeping prior to midnight.

It was desired that subjects consumed enough fruit to produce measurable change, based on melatonin content from prior studies; therefore, 1 kg was set as a basis for portions. However, this needed to be modified to take into account normal fruit consumption style and tolerable amounts of daily intake. Thus, in the case of orange, pineapple, papaya, and Thai berry, it was considered that trying to eat 1 kg of solid fruit was extreme, and people generally consume these fruits as juice; therefore, the juice was extracted from 1 kg of fruit (freshly squeezed for orange, fruit pulp minus skin from a blender for the others). Data for the amount of juice obtained from the fruit are shown in Table 1. For banana, 1 kg was also considered excessive to consume; the blended pulp is too difficult to eat, so two raw bananas (190 ± 9 g peeled) were consumed by each subject. The fresh pulp (peeled) of mango (0.5 kg) was consumed unprocessed. This preprocessing was necessary to make the foodstuffs acceptable and to reflect real daily intake of these fruits.

Volunteers were asked to consume one fruit or fruit juice on one evening, with a 1 week wash-out period before starting the next fruit, until they completed all six fruits. The amounts of the same fruit/fruit juice ingested by each subject were equivalent. Fruit was taken in the evening after a meal provided between 6:00 and 6:30 p.m. Only a supplied fried rice and crackers meal were allowed on the night of fruit consumption. Preparation of fruit and amount taken varied, following acceptable serving type and consumable amount. One kilogram of orange was squeezed and juice was taken within 5 min of preparation. Pineapple, papaya, and Thai berry were prepared by extraction of 1 kg of fruit (without skin) using a blender, and juice was consumed within 5 min. Half a kilogram without seed/skin of mango and two bananas were taken as fresh fruit. Participants were asked to keep a diary of food consumption and maintain the same eating habit (less than three portions of fruits/vegetable per day) throughout the study.

Urine Collection and Urinary 6-Sulfatoxymelatonin. aMT6-s is the major urinary metabolite of melatonin. Urine collection is a noninvasive technique, and previous studies have shown strong correlation ($r = 0.86$; $p < 0.0001$) between urinary aMT6-s and serum melatonin.[37,38] Pooling total overnight urine will give the most accurate measure of total dietary and endogenously produced melatonin, whereas morning spot measurement with creatinine correction[39] may miss melatonin absorbed from food (as it is rapidly eliminated from the blood), due to overnight voids, and introduces more error.[40]

Therefore, total overnight urine was collected (7:00 p.m.−7:00 a.m.) on the night prior to each fruit consumption (baseline) and on the night of fruit consumption (trial). Total volume of overnight urine for each subject was recorded. Urinary aMT6-s was quantified using a Genway ELISA kit (catalog no. 40-371-25006, Genway Biotech Inc., USA). The concentration of aMT6-s was adjusted by total volume of urine to represent total amount of aMT6-s produced overnight. Urinary aMT6-s was expressed as percentage of increase from baseline (each participant served as their own control). Wilcoxon signed-rank test was used to compare the difference of urinary aMT6-s between each baseline and the night of fruit consumption. Statistical significance was set at $p < 0.05$.

RESULTS

Melatonin Content in Fruits. After correction for recovery rate, the calculated melatonin content from HPLC in study fruits (wet weight) showed the highest level in mango (699 ± 75 pg/g), followed by pineapple (302 ± 47 pg/g), papaya (241 ± 14 pg/g), orange (150 ± 6 pg/g), and banana (9 ± 0.6 pg/g), respectively (Table 1). Melatonin was not detected in makmao. The presence of melatonin in all samples was confirmed by ELISA (data not shown).

aMT6-s in Urine after Fruit Consumption. In the human study, there were significantly increased levels of urinary volume-adjusted aMT6-s concentrations after consumption of banana (266% increase, $p = 0.004$), pineapple (180% increase, $p < 0.001$), and orange (49% increase, $p = 0.007$). Consumption of papaya and makmao did not show significantly increased aMT6 levels from baseline despite having a large mean increase from baseline, due to a large variation in the data (Table 2; Figure 1).

Safety and Tolerability. There were few adverse events reported following fruit consumption. Dyspepsia was reported by a few subjects with orange consumption, whereas some nausea/vomiting and diarrhea were reported with makmao. All of these events were mild with self-remission without treatment.

DISCUSSION

As hypothesized, most of the tropical fruits used in the study contained moderate melatonin content except for banana (8.86 ± 0.6 pg/g) and makmao (lower than detection limit). Some differences were seen compared to other studies; for example,

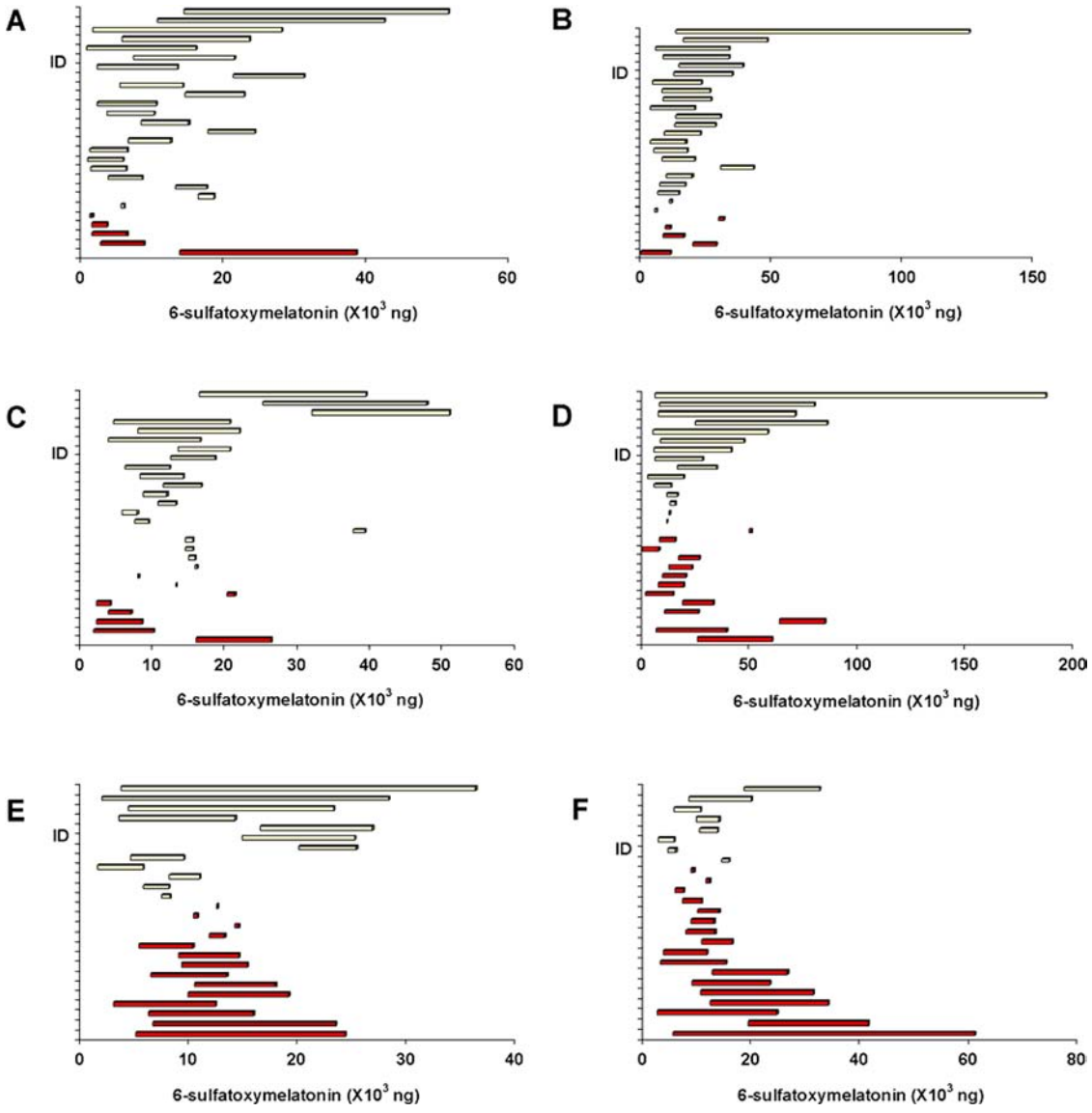

Figure 1. Concentration of urinary 6-sulfatoxymelatonin (aMT6-s) (ng) reported in each subject after consumption of banana (A), pineapple (B), orange (C), papaya (D), makmao (E), and mango (F). ID is each individual subject. Light gray bars represent increased level from baseline (left side of bar), and dark red bars represent decreased level from baseline (right side of bar).

the concentration of melatonin in pineapple was higher than previously reported.[16,18] This could be partly due to the different extraction and measurement methods or different exposure to UV irradiation during growth.[26]

Interestingly, the melatonin content in these tropical fruits or juices did not contribute to urinary aMT6-s after fruit consumption in the same way. For example, the low melatonin content reported in banana leads to >2-fold increase of urinary aMT6-s after consumption of two bananas compared with 1 kg of orange or pineapple juice. This could be partly explained by different metabolism/absorption rates of melatonin in each subject. Generally, oral melatonin is extensively metabolized in the liver before systemic distribution and excretion as aMT6-s in urine. However, there is a large interindividual variability in melatonin levels, partly due to CYP1A2 polymorphism, which leads to a >25 times difference in blood melatonin level after ingestion of oral melatonin drug.[38] It is possible that we would see even higher variation in melatonin from food sources. Additionally, different fruit preparation might also affect melatonin absorption and/or metabolism, which lead to different levels of circulating melatonin. Aldhous et al.[41] showed that the AUC of the plasma melatonin concentration increases more significantly when melatonin is taken with food compared with fasting. Thus, it is postulated that increased melatonin absorption might be found following consumption of raw fruit compared with fruit juice.

Moreover, other compounds in fruits may interfere with the analysis of melatonin content in the laboratory and/or melatonin absorption in humans. For example, in banana, glycopeptides may trap some melatonin during the extraction process, resulting in lower recovery from purification of thick banana pulp by SPE.[17,18] These procedures also usually involve the formation of high levels of oxidants.[42] Thus, the analysis method may report lower melatonin content in banana than the actual amount. In addition, other indoleamines such as tryptophan, 5-hydroxytryptophan, tryptamine, and serotonin may also be present in fruit. On the basis of the synthesis process of melatonin, and its subsequent sulfonation, it is

unlikely that any of these compounds could be converted directly to aMT6-s in human.[4] However, it is unknown if these compounds could be metabolized to melatonin and contribute to total systemic melatonin and appear as aMT6-s in the urine. Interestingly, previous studies have shown that consumption of foods with high tryptophan could enhance melatonin synthesis in the gastrointestinal tract and can also increase melatonin concentration in plasma.[43−46] Therefore, high tryptophan content as found in banana may enhance or up-regulate melatonin synthesis and lead to increased melatonin in the body.[47,48] The effect of other compounds in fruits warrants further study, because if proven, fruit contribution to systemic melatonin could be much greater than from the content of melatonin alone.

The immunoassay technique (ELISA) has been used for the identification and quantification of melatonin in plant materials,[49−52] and previous immunoassay works have also shown good correlation with HPLC.[35,53] The technique does have some cross-reactivity with structurally similar compounds and could cause an overestimation of melatonin content. However, cross-reactivity is low (a few percent), and extremely high amounts of cross-reactive compounds would be required to produce invalid results. Nevertheless, this should not affect the results from this study as there should be less phytochemical in fruits than in leaves (or fruit skins), where previous concerns about interferences have focused. The possibility of the coelution of another compound with the same retention time as melatonin when using HPLC was ruled out by the ELISA test, as reported by Dubbels et al.[54] In this study, ELISA was used only to confirm the presence of the samples in the absence of MS data, not to quantify melatonin content in the fruits or juices.

The current study shows that after consumption of banana, pineapple, and orange, aMT6-s in urine increased significantly up to 2-fold for some fruits. This amount may not be enough for pharmacological effects (e.g., for treating disease), but should be sufficient for health promotion, that is, to raise melatonin to the normal physiological level required to activate melatonin receptors. This would be of benefit to those with low melatonin production, for example, to supplement age-related melatonin reduction in the elderly, which can lead to insomnia.[5,12] Further study analyzing melatonin in the blood would be needed to confirm this.

There were some limitations of the study. First, urine samples of some volunteers were missing due to cumbersome urine collection overnight and were not included into the analysis. Second, the amount and administration of the studied fruits were not identical. This was adapted to meet with consumable amount and how the fruit could be eaten. Whereas two bananas and half a kilogram of mango were eaten, makmao, papaya, orange, and pineapple were drunk as juice. Different fruit texture/combination may affect melatonin absorption and/or metabolism, which may lead to different circulating melatonin levels. Therefore, how the fruit was eaten should be taken into consideration as different food forms might affect the uptake of melatonin in the body.

Furthermore, the study attempted to determine the dietary melatonin available for normal fruit or fruit juice consumption as part of a normal dietary intake, that is, as an addition to a standardized meal. As the baseline always consisted of the same standardized meal, additional melatonin can be attributed to the additional intake of fruit. To measure the absolute absorption of melatonin from fruit, measuring the level after a fasting period would be required.

It should be noted that the level of urinary aMT6-s at the baselines varied >2-fold between fruits. For this reason we used the baseline from the night before each fruit consumption, instead of the baseline before the beginning of the study, to reflect the potential change in baseline over time. We cannot explain the difference in baseline, but it is unlikely to be due to a carry-over effect as there is a 1 week washout period, and melatonin is rapidly cleared from the body. Subjects' intake of fruit and vegetables was also carefully controlled to be constant through the trial and their diary of fruit and vegetable intake checked (not more than three portions per day were allowed). The net effect of using an increasing baseline is that papaya, Thai berry, and mango did not give significant increase in aMT6-s. When we analyzed using a single baseline reference from before the trial for all fruits, they all produced statistically significant increases. We feel, however, that this is not methodologically sound, and we chose a more conservative analysis. It is hoped that this will also be of benefit to other trials of this kind to make researchers aware of possible changing baselines over time.

Melatonin was found in five of the six tropical fruits examined. Human consumption of pineapple, banana, and orange was found to significantly increase urinary 6-sulfatoxymelatonin. No relationship was found between fruit content of melatonin and increase in aMT6-s, highlighting the importance of human clinical trials to determine nutrient uptake in the body, in addition to content analysis of the food source.

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Notes

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ABBREVIATIONS USED

6-sulfatoxymelatonin, aMT6-s; HPLC, high-performance liquid chromatography with fluorescent detection; AUC, area under the curve; SPE, solid phase extraction

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