Pergamon

PII: S0031-9422(96)00568-7

Phytochemistry, Vol. 44, No. 8, pp. 1407–1413, 1997

0031-9422/97 $17.00+0.00

MELATONIN: OCCURRENCE AND DAILY RHYTHM IN *CHENOPODIUM RUBRUM*

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(*Received in revised form* 23 *July* 1996)

Key Word Index—*Chenopodium rubrum*; Chenopodiaceae; goosefoot; rhythmicity; indoles; melatonin.

Abstract—The occurrence of melatonin (5-methoxy-*N*-acetyltryptamine), a common animal hormone, in extracts of the above-ground parts of 15-day-old plants of *Chenopodium rubrum* was confirmed by liquid chromatography/tandem mass spectrometry. Using both this method and radioimmunoassay, changes in melatonin content during a 12 hr light/12 hr dark cycle were demonstrated. The melatonin concentration remained low or undetectable during the light period and increased during the darkness reaching a maximum at hours 4–6 of the dark period before rapidly decreasing. Both the nocturnal increase and the range of concentration are similar to those known in animals. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Melatonin, 5-methoxy-*N*-acetyltryptamine, has long been known as an animal hormone involved in the regulation of rhythmic behaviour and photoperiodic responses in a wide range of organisms, including planarians [1], molluscs [2], crustaceans [3], insects [4, 5] and vertebrates [6–10]. In vertebrates, melatonin is synthesized predominantly in the pineal gland and is excreted to, and transported in, the blood stream. In all species studied to date, a circadian rhythm in the level of melatonin occurs with the highest levels always being associated with the night. It is thought that the actual concentration of melatonin encodes information about the time of day and that the duration of the nocturnal melatonin signal contains information about the photoperiod, i.e. the time of year (see ref. [11] for review).

In 1991, melatonin was demonstrated in the unicellular alga *Gonyaulax polyedra*. A circadian rhythm in the level of melatonin is also observed in this alga, with the maximum occurring in the second hour of the dark period [12–14]. The occurrence of melatonin in higher plants has recently been reported [15]. Melatonin was detected by radioimmunoasay (RIA) in the fruits of tomato, banana and cucumber, in beetroot and in the leaves of tobacco plants. In the case of tomato fruits, the presence of melatonin was further verified by GC-mass spectrometry.

The widespread occurrence of melatonin, including representatives of both lower and higher plants, prompted us to investigate its presence and daily profile in the plant *Chenopodium rubrum* L. *Chenopodium rubrum* appears to be an ideal model for rhythmic and photoperiodic studies. It is an obligate short-day plant with very pronounced and precise photoperiodic responses and rhythmic behaviour. In addition to various other metabolic rhythms [16], rhythmicity in the flowering response has been extensively studied. An endogenous rhythm in the flowering response to both the duration of the dark period and in sensitivity to night break treatments are well documented (see ref. [17] for a review).

RESULTS

Extraction and purification

Compared with animal tissues, melatonin is extremely labile in disintegrated plant tissues. By use

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of a Tris-HCl/$HClO_4$ extraction mixture [14] incorporating high concentrations of antioxidants in combination with a solid phase extraction and HPLC purification we were able to extract melatonin from above-ground parts of *C. rubrum* plants with an efficiency of about 40%.

Identification of melatonin in extracts

The full ES^+ scan spectrum of a melatonin standard showed one major ion at *m/z* 233 corresponding to $[M]^+$. The daughter spectrum showed a major ion at *m/z* 174 (Fig. 1A). A comparable daughter spectrum was obtained from a purified extract of shoots sampled from plants maintained in the 12 hr light/12 hr dark cycle (LD 12:12) at the sixth hour of the dark period (Fig. 1B). Under the conditions employed, the compound in the extract displayed identical chromatographic characteristics to the melatonin standard (Fig. 2).

Quantification of melatonin in extracts

Quantification by liquid chromatography/tandem mass spectrometry with electron spray interface (LC-MS/MS). The concentration of melatonin in samples from plants maintained in LD 12:12 and harvested after 4, 6 and 8 hr of darkness is depicted in Fig. 2. In another experiment, the melatonin content in samples taken at different time intervals during an LD 12:12 regime was measured (Fig. 3). In both experiments, a transient increase in melatonin content was observed with a maximum occurring at the sixth hour of darkness (780 and 233 pg g^{-1} fresh wt, respectively). Following the maxima, but still during the dark period, the content of melatonin very rapidly decreased again.

Quantification by radioimmunoassay. In order to obtain reproducible results in the melatonin RIA extracts from C-18 columns were further purified on HPLC. The HPLC step resulted in only a 5–10% loss of material. Samples were again harvested at various times during the LD 12:12 cycle. As in the case of LC-MS/MS, we observed a transient increase in melatonin content with the maxima occurring at the fourth hour (147 pg g^{-1} fresh wt) and sixth hour (583 pg g^{-1} fresh wt) of the dark period (Fig. 4).

DISCUSSION

Plants display rhythmic behaviour connected with daily light/dark cycles at both the metabolic and morphogenetic levels. A number of rhythms, mostly circadian in nature, have been described for enzymic activities [16] or even at the level of mRNAs [18]. Rhythms in leaf movements, sensitivity to photoperiod or red breaks in darkness in flower induction in short day plants serve as examples of rhythmicity at tissue and organism levels [19, 20]. Unfortunately, virtually nothing is known about the nature of the oscillator(s) governing these rhythms. Recent experiments with lower plants have suggested the existence of more than one oscillator [21]. The coupling of this oscillator(s) to individual rhythms may involve photoreceptors such as phytochrome [22] and/or second messengers like cAMP [23] or inositol-1,4,5-trisphosphate [24].

In mammals, the oscillator is localized in the suprachiasmatic nucleus of the brain and is entrained by the light period of the day. According to the oscillator programme, the pineal gland produces melatonin in a rhythmic fashion, with the maximum in the dark period [11]. The concentration of melatonin at any given stage of the light/dark cycle contains information about the time of day. Furthermore, as the duration of the melatonin peak is usually correlated to the length of night, information relating to seasonal changes in daylength (i.e. time of year) are also encoded [11].

Melatonin has also recently been found in the unicellular alga *Gonyaulax polyedra*. The rhythm in melatonin content in this alga is very similar to that observed in animals [14]. Our present results demonstrate the presence of melatonin in extracts from the above-ground parts of *C. rubrum* plants and, to our knowledge, for the first time a daily rhythm in melatonin concentration in higher plants, similar to that seen in animals and *G. polyedra*. As the melatonin content is observed to decrease before the end of the dark period it would appear to be controlled by an endogenous rhythmic process. This finding suggests the possibility of a similar function for melatonin in both plants and animals, i.e. the regulation of photoperiodic and rhythmic phenomena.

EXPERIMENTAL

Plant material. Seeds of *C. rubrum* (selection 374) were germinated for 12 hr in light at 30°, 12 hr in dark at 10° and 24 hr in light at 30° [25]. The plants were then grown in soil under continuous light provided by white fluorescent tubes (photosynthetic photon flux 184 μmol m^{-2} s^{-1}) at $20 \pm 1°$ until 19 days old. The plants were then transferred to LD 12:12. After 7 days under this regime, the above-ground parts of the plants were sampled at 2-hr intervals during the dark and subsequent light period. Sampling in the darkness was performed under dim green light. Plant material was immediately frozen in liquid N_2 and stored at −70° prior to analysis.

Melatonin extraction and purification. The modified method of ref. [14] was used. Plant material (5 g for RIA, 15 g for LC-MS/MS determinations) was ground in liquid N_2 and 3 ml g^{-1} fresh wt each of 1 M TRIS-HCl buffer, pH 8.4, and 0.4 M perchloric acid (containing 0.1% EDTA, 0.05% sodium bisulphite and 10 mM ascorbic acid) added. At the beginning of the extraction procedure, 200 Bq of *O*-methyl-[^{3}H]-melatonin (Amersham, sp. act. 3.15 TBq $mmol^{-1}$) was added to enable calculation of recovery. Extraction was achieved by incubation on an orbital

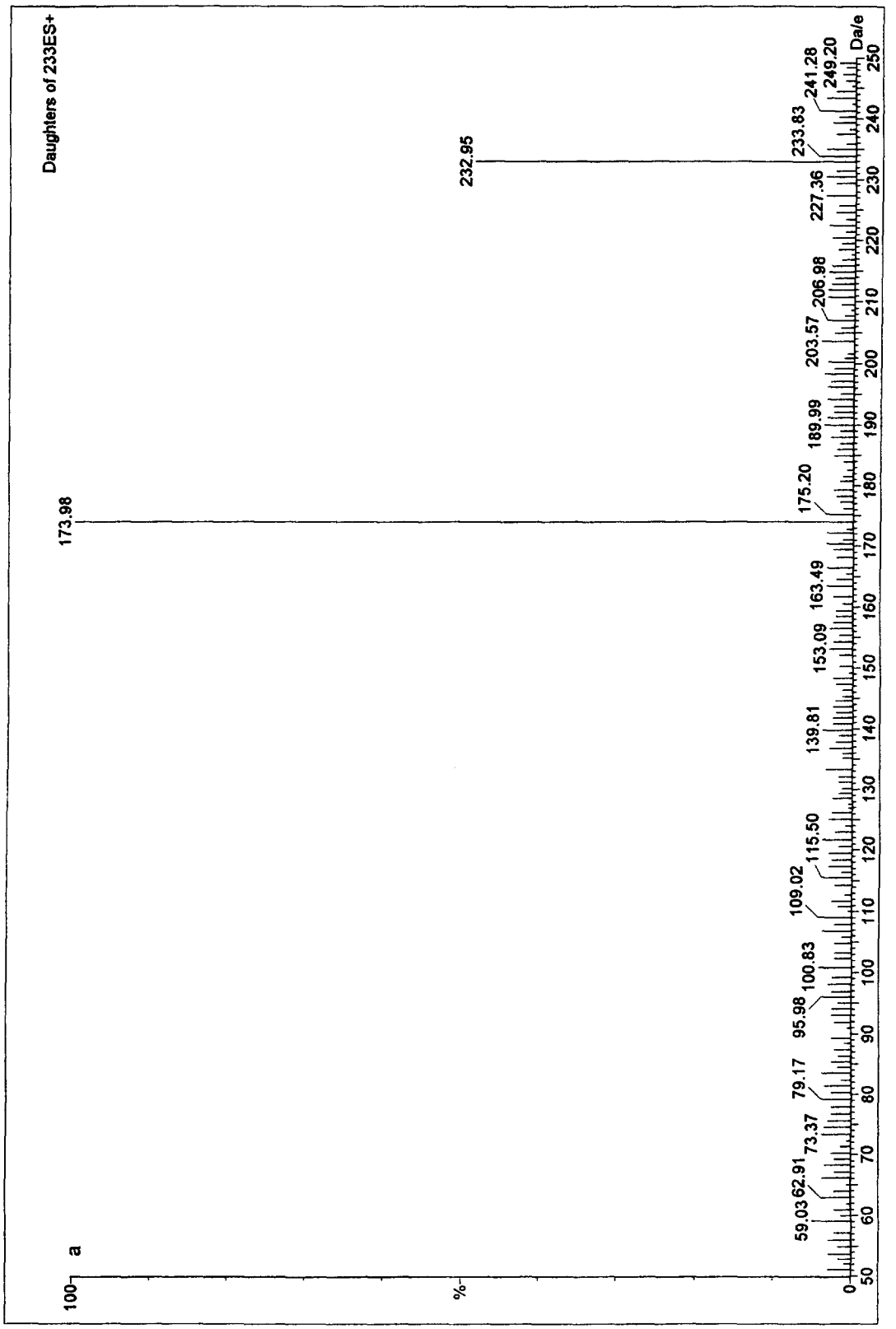

Fig. 1A. Tandem mass CAD-spectrum of $[MH]^+$ of melatonin standard.

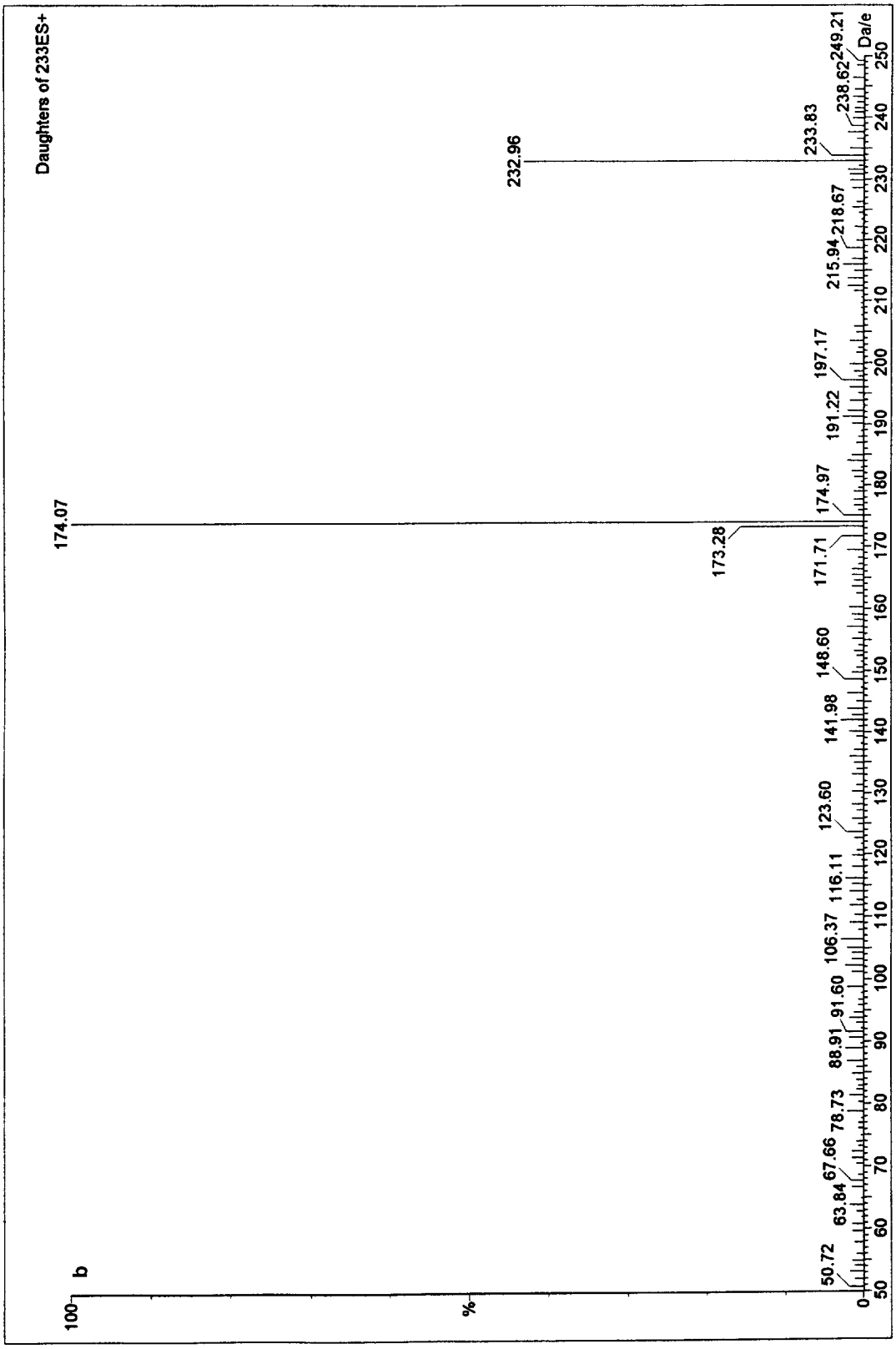

Fig. 1B. Tandem mass CAD-spectrum of the extract from plants after 6 hr of darkness in a 12 hr light/12 hr darkness regime. The extract was prepared from 100 g fresh weight (injected volume was 10 μl, corresponding to 20 g fresh weight).

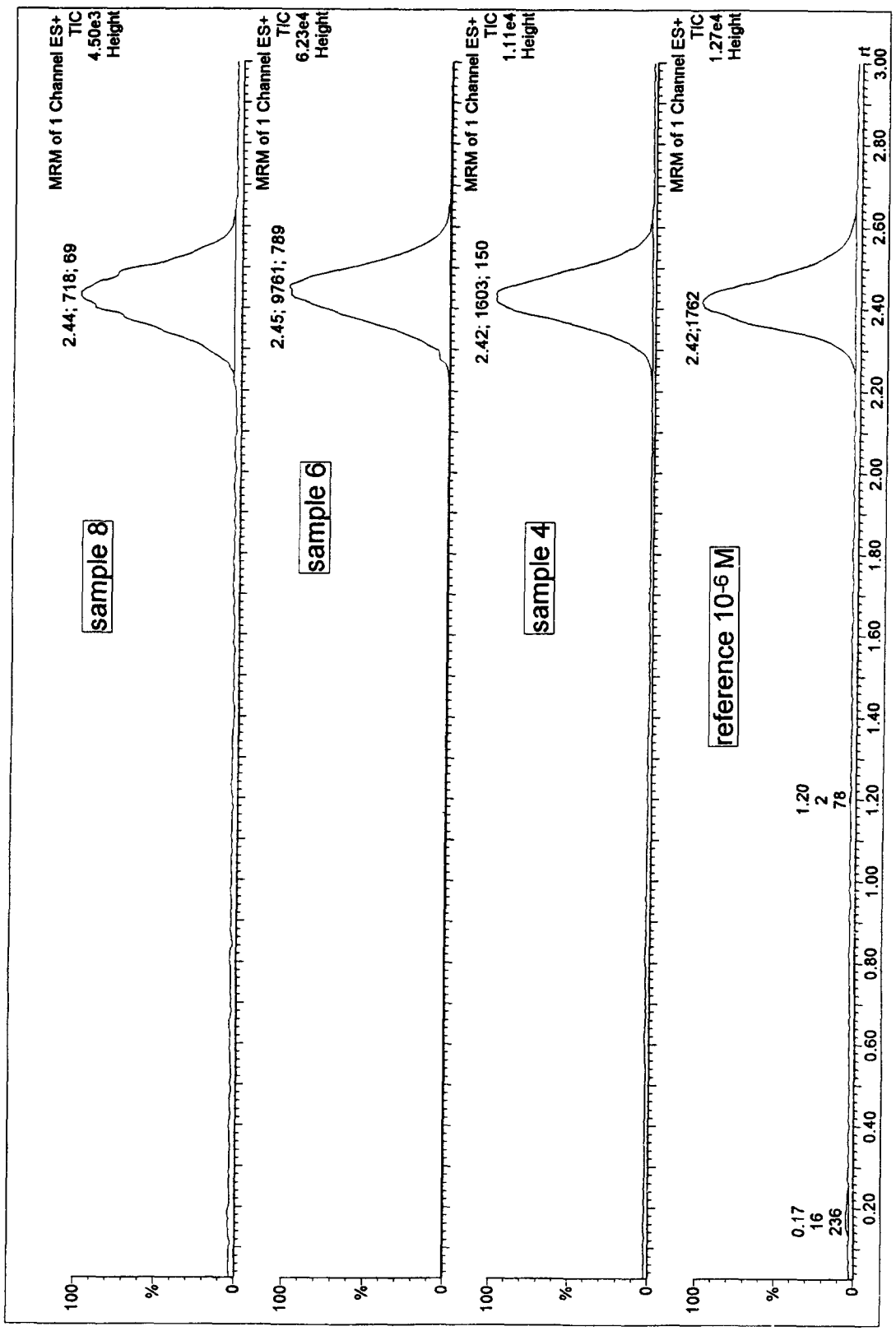

Fig. 2. Chromatograms of extracts from the above-ground parts of *Chenopodium rubrum* plants after 4, 6 and 8 hr of darkness in a 12 hr light/12 hr dark regime (extracts were prepared from 100 g fresh weight; injected volume was 5 μl, corresponding to 10 g fresh weight) and of melatonin standard (5 μl of 10^{-6} M solution injected, i.e. 1.16 ng). Melatonin was detected by liquid chromatography/tandem mass spectrometry with electron spray interface and multiple reactant monitoring ($233 \rightarrow 174$). Numbers above each peak indicate the R_t (min), peak area and melatonin concentration (pg g^{-1} fresh weight), respectively.

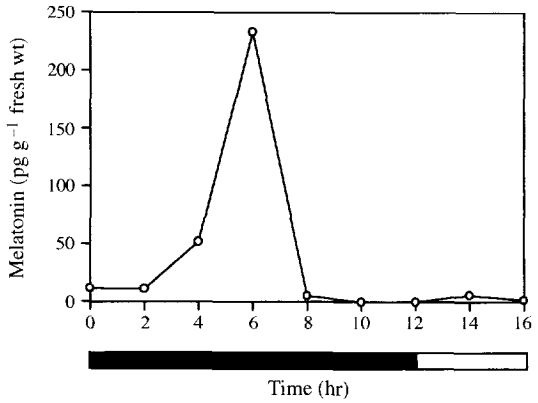

Fig. 3. The changes of melatonin contents in the above-ground parts of *Chenopodium rubrum* plants during a 12 hr light/12 hr dark cycle, as measured by liquid chromatography/tandem mass spectrometry with electron spray interface. The black bar at the bottom indicates the time of darkness.

shaker for 1 hr at 4°. Samples were then centrifuged (10000 *g*, 10 min, 4°) and the supernatant filtered through a nylon cloth. Each sample was then loaded onto C18 Sep-Pak cartridges (two cartridges were connected in series) or, in the case of LC-MS/MS measurements, a single 10 ml column containing C-18 stationary phase. The cartridges (or columns) were then consecutively rinsed with 1 M Tris-HCl buffer, pH 8.4, and water before the retained melatonin was eluted with MeOH. The entire procedure was performed in a darkened room and all solns were kept on ice to minimize melatonin degradation. After evapn of MeOH *in vacuo* (temp. was maintained at below 35°), the melatonin content of samples was analysed by RIA or LC-MS/MS.

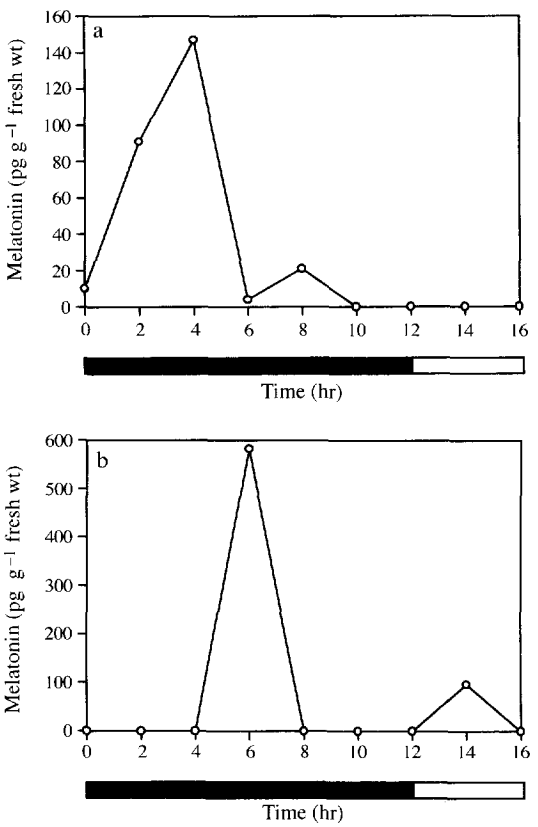

Fig. 4. The changes of melatonin contents in the above-ground parts of *Chenopodium rubrum* plants during a 12 hr light/12 hr dark cycle, as measured by radioimmunoassay (two independent experiments, a and b). The black bar at the bottom indicates the time of darkness.

Determination of melatonin by RIA. Before RIA of melatonin, samples were further purified by HPLC using a Pye Unicam 4002 chromatograph equipped with a Separon SCX C18 6 μm (250 × 4.6 mm) column which was connected to a Varian Fluorichrom fluorescence detector (primary filter 220 nm, secondary filter 340 nm). Melatonin was eluted with a stepped MeOH gradient at a flow rate of 0.7 ml min^{-1} (40°): 0–4 min, 30–35% B, 4–8 min, 35–50% B, 8–36 min, 50–90% B, 36–37 min, 90% B, 37–40 min, 90–30% B; where, mobile phase A consisted of 5 mM citric acid, 5 mM sodium acetate, 10% (v/v) MeOH, pH 4.8, and mobile phase B contained 5 mM citric acid, 5 mM sodium acetate, 50% (v/v) MeOH, pH 5.35. Fractions eluting at the R_t corresponding to the melatonin standard were collected and dried. Melatonin was measured by a RIA developed by Stockgrand Ltd (Guilford, UK), with antiserum G/S 704-8483 [26]. *O*-Methyl-[^{3}H]-melatonin (sp. act. 3.15 TBq $mmol^{-1}$) was purchased from Amersham (the amount of labelled melatonin added to each of the samples for RIA was calculated on the basis of the remaining amount of [^{3}H] melatonin added at the beginning of extraction procedure). The limit of detection of the assay was 5 pg per tube. Samples of 12.5 and 40 pg per tube had interassay coefficients of variation 13% and 9%, respectively, and intra-assay ones of 10% and 6%, respectively. Purified evapd extract was dissolved in 2 ml of assay buffer and two portions (each 500 μl) were taken for RIA analysis.

Determination of melatonin by LC-MS/MS. Samples from C-18 columns were further purified by prep. HPLC on the same chromatograph as above but equipped with a Separon RP-C-18 column (7 μm, i.d. 8 mm) using mobile phase A (30% MeOH) and B (70% MeOH). A gradient was run: 0–30 min 30% B, 30–31 min 80% B, 31–41 min 30% B at 40°and at the flow rate 1 ml. min^{-1}. Frs containing melatonin (R_t 29.9 min) were collected and evapd *in vacuo*.

These evapd samples were analysed by HPLC linked to a Quatro II mass spectrometer with an electrospray interface (LC-MS/MS). The samples were dissolved in 50 μl 100% MeOH and 10 μl was injected on a C8 reversed phase column (Merck, Lichrosphere 60 RP Select B, 5 μm, 125 × 4 mm) and eluted with MeCN–H_2O (9:11) at a flow rate of 800 μl min^{-1}. Using a 1/20 postcolumn split, the effluent was introduced into the electrospray source (source temperature 80°, capillary voltage + 3.74 kV, cone voltage 25 V). Under these conditions, full-scan spectra of melatonin and plant extract were recorded (scan range

100–400 Da/e, scan speed 300 Da s^{-1}). Tandem mass CAD-spectra of $[M]^-$ were obtained at a collision energy of 15 eV (P_{Ar} 1.10-3 mbar). Quantification was done by multiple reactant monitoring (MRM) of $[M]^-$ (*m/z* 233) and *m/z* 174.

Acknowledgements—This work was supported by a grant 204/95/1576 to I.M. by the Grant Agency of the Czech Republic. E.P. is a research associate and H.V.O. a research director of the National Fund for Scientific Research, Belgium. The authors thank very much Dr V. Vlasáková of the Institute of Experimental Botany for HPLC purification of the samples for LC-MS/MS determinations and Mr M. Klicpera for excellent technical assistance.

REFERENCES

1. Mortia, M. and Best, J. B., *Experientia*, 1993, **49**, 623.
2. Abran, D., Anctil, M. and Ali, M. A., *Gen. Comp. Endocrinology*, 1994, **96**, 215.
3. Vivien-Roels, B. and Pevet, P., *Experientia*, 1993, **49**, 642.
4. Finocchiaro, L. M. E., Callebert, J., Launay, J. M. and Jallon, J. M., *Journal of Neurochemistry*, 1988, **50**, 382.
5. Vivien-Roels, B., Pevet, P., Beck, O. and Fevre-Motagne, M., *Neuroscience Letters*, 1984, **49**, 153.
6. Brainard, G. C., Petterborg, L. J., Richardson, B. A. and Reiter, J., *Journal of Neuroendocrinology*, 1982, **35**, 342.
7. Gwinner, E., Schwabl-Benzinger, I., Schwabl, H. and Dittami, J., *Gen. Comp. Endocrinology*, 1993, **90**, 119.
8. Lerner, A. B., Chase, J. D., Takahashi, Y., Lee, T. H. and Mori, W., *J. Am. Chem. Soc.*, 1958, **81**, 2587.
9. Maywood, E. S., Hastings, M. H., Max, M., Ampleford, E., Menaker, M. and Loudon, A. S. I., *Journal of Endocrinology*, 1993, **136**, 65.
10. Rollag, M. D. and Niswender, G. D., *Endocrinology*, 1976, **98**, 482.
11. Reiter, J., *Experientia*, 1993, **49**, 654.
12. Balzer, I. and Hardeland, R., *Science*, 1991, **253**, 795.
13. Balzer, I. and Hardeland, R., *Chronobiol. Int.*, 1992, **9**, 260.
14. Poegeller, B., Balzer, I., Hardeland, R. and Lerchl, A., *Naturwissenschaft*, 1991, **78**, 268.
15. Dubbels, R., Reiter, R. J., Klenke, E., Goebel, A. Schnakenberg, E., Ehlers, C., Schiwara, H. W. and Schloot, W., *Journal of Pineal Research*, 1995, **18**, 28.
16. Wagner, E., in *Integration in the Higher Plant, Society for Experimental Biology*, Symposium 31, ed. D. H. Jennings, Cambridge: University Press, Cambridge, 1977, p. 33.
17. Cumming, B. C. and Seabrook, in *Handbook of Flowering*, Vol. 2, ed. A. H. Halevy. Boca Raton, Florida, 1985, p. 286.
18. Cremer, F., Dommes, J., Van de Walle, C., Bernier, G., *Plant Physiology*, 1990, **94**, 1590.
19. Vince-Prue, D., in *Hormonal Regulation of Development. III. Role of Environmental Factors, Encyclopedia of Plant Physiology, New Series*, Vol. 11, eds R. P. Pharis and D. M. Reid. Springer, Berlin, 1985, p. 308.
20. Satter, R. L. and Galston, A. W., *Annual Review of Plant Physiology*, 1981, **32**, 82.
21. Roenneberg, T. and Morse, D., *Nature*, 1993, **362**, 362.
22. Lumsden, P. J., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1991, **42**, 351.
23. Edmunds, L. N., in *Towards an Understanding of the Molecular Mechanism of Circadian Rhythms, Proceedings of the International Conference in Wohldenberg*, 1994.
24. Kallies, A., Gebauer, G., Fracello, F. and Rensing, L., in *Towards an Understanding of the Molecular Mechanism of Circadian Rhythms, Proceedings of the International Conference in Wohldenberg*, 1994.
25. Ullmann, J., Seidlová, F., Krekule, J. and Pavlová, L., *Biol. Plant.*, 1985, **27**, 367.
26. Illnerová, H., Burešová, M., Nedvídková, J., Dvořáková, M. and Zvolský, P., *Brain Research*, 1993, **626**, 322.