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Changes in the Concentrations of Bioactive Compounds in Plantain Leaves

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The plantain is used in herbal medicines and for pasturage. Two cultivars of plantain (*Plantago lanceolata* L.), Grasslands Lancelot and Ceres Tonic, were sown in spring. Changes in catalpol, aucubin, and acteoside concentrations in the leaves during the growing season and by drying after harvesting were quantitatively determined by high-performance liquid chromatography. The concentration of catalpol was relatively low, fluctuating between 1 and 2% of dry matter during the growing season, and there was no clear-cut seasonal change. From spring to midfall, the aucubin concentration increased from 2.1 to 4.8% in Grasslands Lancelot and from 1.0 to 2.7% in Ceres Tonic. These increases were gradual over the season, except for during midsummer, when aucubin concentrations were relatively constant. The acteoside concentration increased from 3.4 to 7.1% in Grasslands Lancelot and from 1.5 to 4.1% in Ceres Tonic over the course of the growing season, although in the summer it declined steadily to lows of 2.5% in Grasslands Lancelot and 1.9% in Ceres Tonic. Our data suggested that midfall was the appropriate time for harvesting plantain for medicinal use. The concentrations of the bioactive compounds steadily decreased in the initial stages of drying both under natural climatic conditions and at 60 °C. The development of processing methods to minimize the loss of bioactive compounds is imperative.

KEYWORDS: Acteoside; aucubin; catalpol; plantain; processing; seasonal change; HPLC

INTRODUCTION

The World Health Organization is aggressively banning feed grade antibiotic growth promoters. The plan in Europe, because of the risk of possible drug resistance in human pathogenic bacteria, was to leave only four such compounds (flavophospholipol, monensin, salinomycin, and avilamycin) in use from June 1999 onward. Not surprisingly, this EU statute is giving a huge boost in demand for nonpharmaceutical growth promoters. In response, feed manufactuers are refining their use of directly fed microbials, enzymes, and other additives. Moreover, they are adopting new forms of "all natural" feed additives, which are the products of modern science but which have their origins in traditional and even ancient medicine (*1*).

This new generation of growth enhancers includes blends of herbs; however, even one herb can contain many bioactive chemical compounds. Scientists are now working to identify the most useful herbs and to quantify any effects that they might have on animal production.

The plantain (*Plantago lanceolata* L.) has been used in herbal medicine in Europe (*2*, *3*) and is curretly being evaluated in New Zealand as a potential pasture species because of its medicinal value to animals (*4*, *5*). Recently, domestic cultivars of plantain have been selected for pasture production (*5*, *6*) and are now being used by organic farmers in New Zealand. The bioactive compounds in the plantain have been studied in depth (*3*). Of the iridoid glucosides, the major compounds studied have been catalpol and aucubin, and, of the phenylethanoid glucosides, acteoside. Aucubin stimulates the removal of uric acid from tissues to the blood, as well as the excretion of uric acid from the kidneys (*7*). Furthermore, it suppresses the inflammatory effect induced by the injection of carrageenan (*8*). Aucubin also exhibits significant protective activity against α-amanitin intoxication in mice (*9*). Catalpol is generally known to be the active diuretic principal of the fruit of *Catalpa ovata* G. Don. Acteoside is well-known for having a high level of antioxidative activity (*3*, *10*) and has shown inhibitory effects on arachidonic acid-induced edema of ears in mice (*11*).

We assume that feeding plantain to animals improves their physiological condition and diminishes the need for antibiotic growth promoters. So far, several experiments on the effects of plantain on animal health have been carried out. Deaker et al.

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(*4*) reported that lambs that grazed on plantain had relatively larger kidneys than lambs that grazed on either chicory, white clover, or perennial ryegrass, although serum urea and creatinine levels were within a normal range, indicating kidney function was enhanced. Sano et al. (*12*) reported that tissue responsiveness to insulin was enhanced by the plantain diet in sheep.

To produce a plantain with a higher concentration of bioactive compounds, it will be necessary to analyze the genetic, temporal, and spatial variations in the concentrations of the bioactive constituents. It will also be necessary to clarify the changes that occur in the concentrations of bioactive compounds as they are processed after harvest.

Therefore, in this study, changes in the concentrations of catalpol, aucubin, and acteoseide in plantain leaves were investigated during the growing season and as a result of drying after harvest.

MATERIALS AND METHODS

Seasonal Changes. Seeds of the two cultivars, Grasslands Lancelot and Ceres Tonic, obtained from New Zealand (Pyne Gould Guiness Ltd.), were sown on April 26, 2000 in an experimental field at the National Agricultural Research Center for the Tohoku Region, Morioka, Japan. The research center is located at 39° 45′ N at an altitude above sea level of 170 m. The moisture content of the soil was 30−40%, and the total nitrogen level was 50−70 mg kg^{-1} dry soil. The soil was andosol (black volcanic ash).

The seeding rate was 15 kg ha^{-1} for each cultivar. Fertilizer was initially applied at the rate of 70, 100, and 70 kg ha^{-1} of N, P_2O_5, and K_2O, respectively. The experiment was conducted in a randomized block design with three replications. Each plot was 5 m × 2 m, i.e., 10 m^2.

Thirty grams of leaves of each cultivar was harvested in each plot by cutting them at 5 cm above ground level at 2 week intervals from July 20 to October 24, 2000. The leaves were freeze-dried and ground to a fine powder using a mortar and pestle. The bioactive compounds were then analyzed. Plant height was measured before each harvest.

Effect of Drying. Leaves of the cultivar Grasslands Lancelot were harvested from each plot at 5 cm above ground level on August 12; 400 g of leaves was subsequently dried under natural climatic conditions outdoors for about 2 days, and 300 g of leaves was dried at 60 °C in a forced-draft oven for 8 h. The mean air temperature and solar radiation during the drying period were around 22 °C and 22 MJ m^{-2}, respectively. No rain was observed. Thirty grams of leaves drying under natural climatic conditions outdoors was sampled every 2 h from 8:00 am to 6:00 pm. Likewise, 30 g of leaves in the forced-draft oven was sampled at 1 h intervals, and the leaves were freeze-dried and ground. The experiment was conducted with three replications.

Analysis of the Bioactive Compounds. A 250 mg aliquot from each of the powdered freeze-dried leaf samples was taken for extraction of catalpol, aucubin, and acteoside. The aliquots were shaken in 25 mL of methanol for 2 h at room temperature. The solid plant material was filtered out using No. 5A quantitative filter papers (Toyo Inc., Japan), and 2 mL aliquots from each filtrate were further filtered using 0.45 μm syringe filters (Whatman Co., Ltd., England). The final filtrates were used for the quantitative determination of catalpol, aucubin, and acteoside by HPLC (high-performance liquid chromatography). For the simultaneous determination of catalpol and aucubin, 2 mL of the filtrate was diluted with 8 mL of distilled water and a 20 μL aliquot used for HPLC analysis, whereas for determination of acteoside, 10 μL of the undiluted filtrate was used (*13*−*15*). Commercially available catalpol and aucubin (Funakoshi, Tokyo, Japan) and acteoside that was isolated from *Forsythia viridissima* Lindl. (*16*) were used as standards. The standard solutions contained 2 mg each of catalpol and aucubin in 50 mL of 20% MeOH and 1 mg of acteoside in 5 mL of pure MeOH. The chemical structures of catalpol, aucubin, and acteoside are shown in **Figure 1**.

HPLC was performed at 40 °C using a 100 mm × 6.0 mm YMC-pack ODS-A column protected by a YMC guard pack. The mobile phase was 1% acetonitrile in water for catalpol and aucubin and 29% methanol in water (containing 5% acetic acid) for acteoside. The flow rate was 1 mL min^{-1}. For catalpol and aucubin, photodiode array detection was performed at $\lambda = 204$ nm. For acteoside, the detection was performed at $\lambda = 330$ nm (*13*−*15*). The HPLC system consisted of a Shimazu SPD-10A diode array detector, an SCL-10A autosampler, an LC-10AD pump, a CTO-10A column oven, a DGU-12A degasser, and Shimazu C−R7A software for data processing.

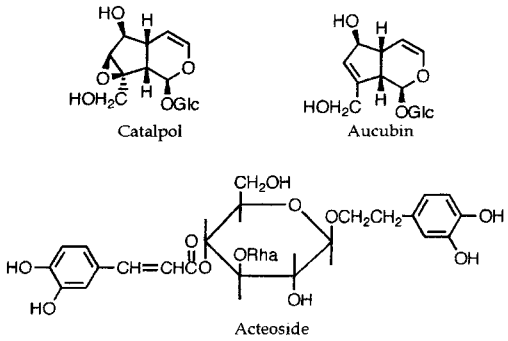

Figure 1. Structures of catalpol, aucubin, and acteoside. Glc, glucosyl; Rha, rhamnosyl.

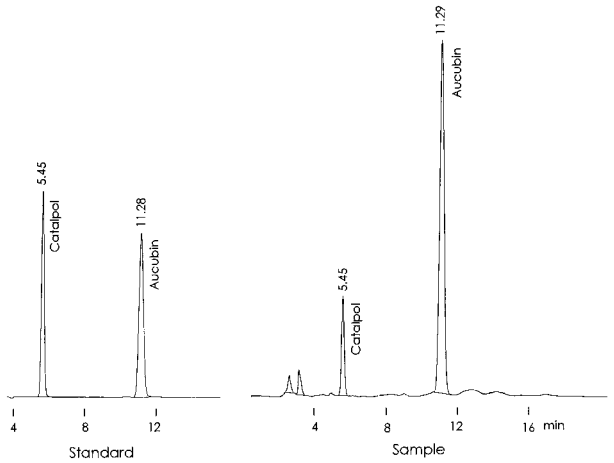

Figure 2. HPLC chromatograms of catalpol and aucubin for a standard and a sample from leaves of *Plantago lanceolata* L.

The peaks on chromatograms for catalpol and aucubin were observed around 5.45 and 11.28 min and for acteoside around 6.37 min after the injection of the samples as shown in **Figures 2** and **3**. The compounds were isolated, and their chemical structures were determined from their atomic spectra (using ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR) and identified by comparison with the spectra obtained for catalpol, aucubin, and acteoside standards.

Standard curves for catalpol, aucubin, and acteoside were prepared using concentrations ranging from 0 to 1000 μg mL^{-1} in steps of 10 μg mL^{-1} (0−100 μg mL^{-1}) and 100 μg mL^{-1} (100−1000 μg mL^{-1}). Calibration curves of the concentrations of bioactive compounds against HPLC areas were linear from 10 to 500 μg mL^{-1} solvent for catalpol and aucubin and from 50 to 800 μg mL^{-1} solvent for acteoside.

The recovery of the bioactive compounds by methanol extraction was investigated by repeating the extraction three times for representative 40 samples, and it was found that more than 99.3% of the compounds were extracted after the first extraction. Consequently, the extraction for all remaining samples was performed once only.

Climatological Data. Daily mean air temperature and solar radiation were obtained from monthly summaries of the Agro-meteorology Laboratory of the National Agricultural Research Center for the Tohoku Region.

Statistical Analysis. Standard deviation of means was calculated for all of the data obtained by HPLC analysis, and analysis of variance (ANOVA) was applied to clarify the differences in concentrations among bioactive compounds in each cultivar and those among time of sampling.

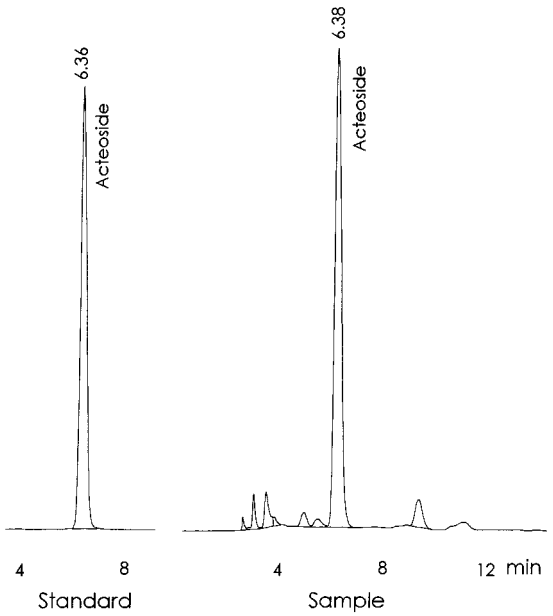

Figure 3. HPLC chromatograms of acteoside for a standard and a sample from leaves of *Plantago lanceolata* L.

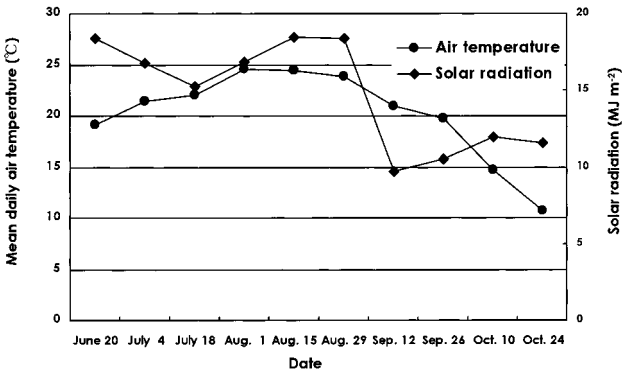

Figure 4. Changes in mean daily air temperature during the experimental period. Means of 14 days before each harvest date are shown.

RESULTS

HPLC Analysis. The HPLC analysis was highly accurate, as the calibration curves for catalpol and aucubin were linear ($r^2 > 0.989$) from 10 to 500 μg mL^{-1} solvent and from 50 to 800 μg mL^{-1} solvent for acteoside ($r^2 = 0.991$). The recovery of the compounds was over 99.3%.

Climatological Data. Changes in the 14 d mean daily air temperature and solar radiation before each harvest are shown in **Figure 4**. The mean daily air temperature in late spring (June 20) was about 19 °C. It increased to around 23 °C and stayed relatively constant for the summer (July 4 to September 12). It then steadily declined during the fall (September 26 to October 24) to about 10 °C at the last harvest. The solar radiation was higher in the late spring and summer than it was in the fall. We also observed that solar radiation decreased dramatically from August 30 to September 12.

Seasonal Changes. Plants of both cultivars increased from about 30 to 50 cm in height from June 20 to August 1. After that, plant height remained relatively constant at 45−55 cm for Grasslands Lancelot and at 40−50 cm for Ceres Tonic. Both cultivars achieved maximum growth in early August, and their growth remained relatively constant during the summer and fall (data not shown).

Table 1. *F* Values Obtained by ANOVA

		factors	*F* values
seasonal changes	Grasslands Lancelot	compds	41.1[a]
		time of sampling	2.8[b]
	Ceres Tonic	compds	21.5[a]
		time of sampling	4.6[b]
effect of drying	outdoor	compds	120.1[a]
		time of sampling	5.3[b]
	60 °C	compds	112.6[a]
		time of sampling	6.1[b]

[a,b] Significant at 1 and 5%, respectively.

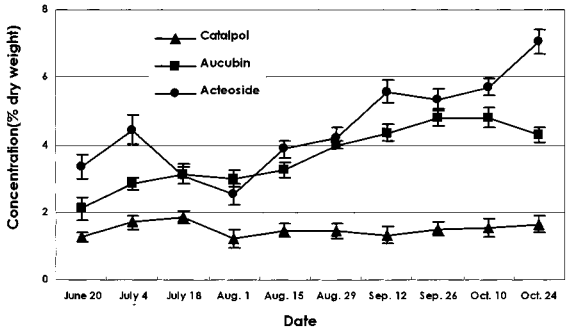

Figure 5. Changes in the concentrations of catalpol, aucubin, and acteoside in leaves of *Plantago lancelata* L. cv. Grasslands Lancelot during the experimental period. The vertical bars represent standard deviation of the mean.

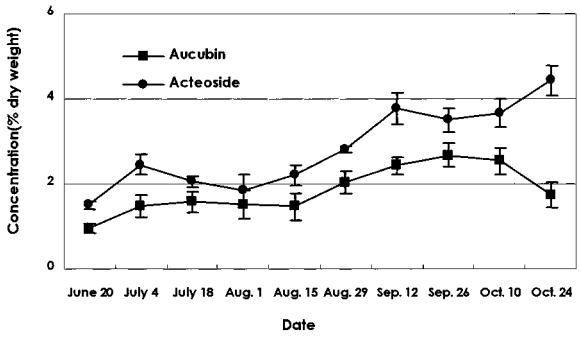

Figure 6. Changes in the concentrations of aucubin and acteoside in leaves of *Plantago lanceolata* L. cv. Ceres Tonic during the experimental period. The vertical bars represent standard deviation of the mean.

There was a clear difference in concentrations among bioactive compounds in each cultivar and those among time of sampling (**Table 1**).

Changes in the concentrations in leaves of catalpol, aucubin, and acteoside are shown in **Figures 5** and **6** for Grasslands Lancelot and Ceres Tonic, respectively. Catalpol was detected only in Grasslands Lancelot, and aucubin and acteoseide concentrations were higher in Grasslands Lancelot than in Ceres Tonic.

The concentration of catalpol was very low as compared with that of aucubin and acteoseide and fluctuated between 1 and 2% of the dry matter. The seasonal change was less clear-cut, although it increased slightly from late spring to early summer, decreased at midsummer, and gradually increased during the fall.

The concentration of aucubin increased from late spring to early summer in both cultivars and remained relatively constant in midsummer. In early and midfall, it increased again until September 26; however, it decreased in late fall (from October 10−24) when the air temperature steadily declined from 15 to 10 °C.

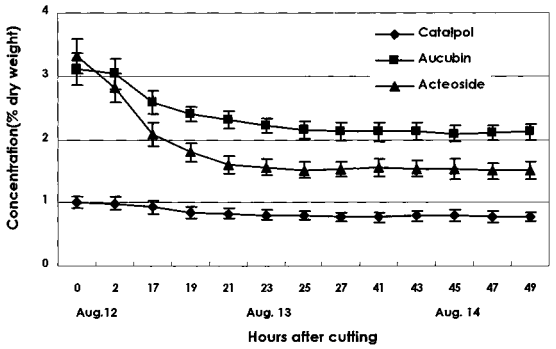

Figure 7. Changes in the concentrations of catalpol, aucubin, and acteoside in leaves of *Plantago lanceolata* L. cv. Grasslands Lancelot by drying under natural climatic conditions.

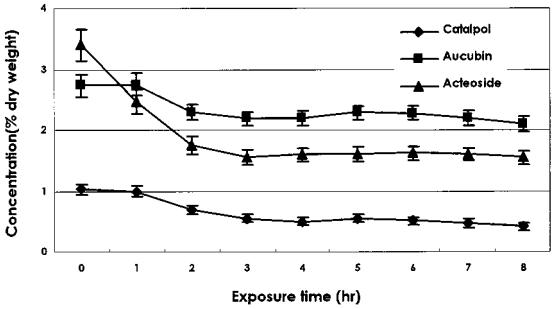

Figure 8. Changes in the concentrations of catalpol, aucubin, and acteoside in leaves of *Plantago lanceolata* L. cv. Grasslands Lancelot by drying at 60 °C.

Acteoside concentrations increased from 3.4% dry weight at the first harvest to 7.1% at the final harvest in Grasslands Lancelot and from 1.5 to 4.1% in Ceres Tonic. However, the increases were not strictly monotonic since leaves harvested in the summer had relatively low concentrations. The seasonal trend in the concentrations of acteoside was the same in both cultivars.

Effect of Drying. Changes in the concentrations in leaves of catalpol, aucubin, and acteoside during drying either under natural climatic conditions outdoors or at 60 °C in a forced draft oven are shown in **Figures 7** and **8**, respectively.

There was a clear difference in concentrations among bioactive compounds both drying outdoors and at 60 °C and those among time of sampling (**Table 1**).

Leaf catalpol, aucubin, and acteoside under natural climatic conditions steadily decreased for about 24 h after the beginning of drying and then remained relatively constant until the end of drying. The rates of decrease were higher in the order of acteoside, aucubin, and catalpol. Leaf catalpol, aucubin, and acteoside at 60 °C in the forced-draft oven steadily decreased for about 3 h after the beginning of drying and then stayed relatively constant until the end of drying. Similarly with natural drying, the rates of decrease were higher in the order of acteoside, aucubin, and catalpol.

DISCUSSION

HIPLC Analysis. Well-established methods for the application of HPLC analysis for the quantitative determination of catalpol, aucubin, and acteoside were used in this experiment. The calibration curves for each of the compounds were linear within the sample concentration range, and the recovery of the bioactive compounds was high, so we are confident about the accuracy of our measurements in both absolute and relative terms.

Seasonal Changes. Temporal and spatial environmental variation may influence the concentrations of bioactive compounds in plantain leaves in several ways. Air temperature, solar radiation, and nutrient availability are considered to be major factors in this respect. Tamura (*17*) reported that low light intensity and nitrogen supply strongly repressed the accumulation of acteoside and aucubin in plantain leaves, without affecting the concetration of catalpol. Tamura (*17*) also reported that relatively high air temperatures (20/18 °C, day/night) increased catalpol and aucubin concentrations (both iridoid glucosides) while lower temperatures (15/10 °C) increased the concentration of acteoside (a phenylethanoid glycoside). Furthermore, Tamura and Yoshida (*18*) and Tamura et al. (*19*) found significant genetic variation between and within plantain cultivars and ecotypes with respect to their accumulation of these bioactive compounds.

In the experiments reported here, seasonal changes in the concentrations of the bioactive compounds in plantain leaves were investigated. Catalpol concentration was very low relative to the concentrations of aucubin and acteoside. The seasonal changes were less clear-cut in catalpol concentrations than in those of aucubin and acteoside; however, catalpol concentration increased slightly during the growing season, with the exception of midsummer when the level of catalpol was low. Both catalpol and aucubin are iridoid glycoside compounds, and catalpol is biologically synthesized from aucubin (*20*). The less clear-cut seasonal changes observed in catalpol suggest a low basal rate of synthesis that is relatively unresponsive to environmental variations.

Aucubin concentrations generally increased in late spring to midfall (September 26), with the exception of midsummer, when the levels of aucubin were relatively constant. In late fall (October 10−24), aucubin levels steadily decreased by 0.13% $°C^{-1}$ in Grasslands Lancelot and by 0.20% $°C^{-1}$ in Ceres Tonic, relative to the decline in air temperature from 14.7 to 10.7 °C. The aucubin concentrations in the leaves of both cultivars were highest in midfall when the air temperature was around 20 °C. Tamura (*17*) reported that plants grown under 20/18 °C day/night temperatures accumulated more aucubin than plants grown under 15/10 °C. We assume that aucubin accumulates more when the air temperature is optimal for growth.

Bowers et al. (*21*) also investigated seasonal changes in the concentrations of the aucubin and catalpol in plantain leaves. Plants were harvested four times at approximately 2 week intervals from late spring to early fall, namely, July 26, August 10 and 23, and September 5. They found a significant increase in the concentrations of both iridoid glycosides over the growing season with the exception of the plants harvested in midsummer (August 10), which had reduced concentrations relative to those harvested on the other three sampling dates. Because the air temperature in midsummer was very high (around 25−30 °C), this result agrees well with our observations.

The mean acteoside concentration in plantain leaves generally increased during the growing season, especially when air temperatures declined in late fall. The plants harvested in the summer were an exception to this general increase. They had reduced levels of acteoside in their leaves relative to those harvested at other times. Tamura (*17*) reported that plants grown under 15/10 °C day/night temperatures accumulated more acteoside than plants grown under 20/18 °C. We assume that higher summer temperatures suppressed the accumulation of acteoside. As reported earlier (*17*), it is clear that acteoside, a phenylethanoide glycoside, and aucubin, an iridoid glycoside, accumulate at different rates depending on air temperature. Our

results suggest that midfall is an appropriate time for harvesting plantain sown in the spring because of the higher concentrations of bioactive compounds.

Effect of Drying. It is generally assumed that medicinal herbs for human beings should be dried at less than 60 °C to minimize loss of bioactive compounds. When animals feed on hay, they generally ingest medicinal herbs together with other pasture plants such as grasses and legumes. However, no precise investigation has been done to compare the changes in the concentrations of bioactive compounds in plantain leaves dried under natural climatic conditions or at 60 °C. It was apparent in both cases that the concentrations of bioactive compounds steadily declined in the initial stages of drying. There are several reports in the literature on the degradation of iridoid glycosides due to β-glucosidase activity and of phenylethanoid glycosides due to peroxidase (oxidation) and β-glucosidase activities (*22*−*24*). The reason for the decline in the concentrations of catalpol, aucubin, and acteoside might also be the existence of enzymes responsible for their degradation. The activities of these putative enzymes may have been neutralized after about 24 and 3 h after the beginning of drying under natural climatic conditions and at 60 °C, respectively, since the concentrations of the compounds remained constant after these times. The development of processing methods that will inhibit the activity of such enzymes to minimize the loss of bioactive compounds is imperative.

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