A harmful effect of humidity was observed on the stability of the studied penicillin (**1**) in the solid state. It was found, e.g. that at 80 °C and 75% relative humidity (RH) the hydrolysis of the β-lactam bond in **1** occurred at a rate nearly 1000 times that found in the absence of humidity. The $t_{0.1}$ value was then from 4 (iodometric method) to 11 days (spectrophotomeric UV method) (Table 1).
The rate constant k was also analysed as a function of RH using the equation: $\log k = b + a \times RH$. Rectilinear plots were obtained by both of the method used, and their slopes (a) had similar values (Table 2). This shows that the β-lactam as well as lactone groups are sensitive to a similar degree to the action of changes in humidity.
The process of hydrolysis of the lactone groups at 20 °C in the presence of humidity occurs at a rate three times (Table 1) and at 80 °C about two times (Table 2) slower than hydrolysis of the β-lactam bond.

Experimental

1. Material

The experiments were conducted using bacampicillin hydrochloride (**1**) substance. The mean content of **1** in the preparation was 99.53 ± 0.35% $C_{24}H_{33}N_3O_6S \cdot HCl$ (iodometric method: $n = 7$, $s = 0.382$, $s^2 = 0.146$, $s_r = 0.38\%$), molecular mass 518.0, Yananouchi Pharmaceutical Co., Tokyo, batch N 24 A.

2. Methodology

Samples of **1** were kept at elevated temperature (60 °C–100 °C) in anhydrous conditions and also at raised humidity (RH 56% to 90% [5]), their colour changed through yellow to brown.
For spectrophotometric testing samples of 1.0 ml of 0.125% water – methanol [5 : 2(V/V)] solution of **1** were taken. The contents of a flask was made up to volume with 0.1 mol/l solution of sodium hydroxide (reference standard).
The method for kinetic studies and analytical procedures using iodometric and spectrophotometric methods have been described in detail in an earlier study [3].

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HPLC identification and determination of flavone aglycones in *Helichrysum plicatum* DC. (Asteraceae)

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Helichrysum plicatum DC. has been used in Macedonian folk medicine for a long time, for treatment of gastric and hepatic disorders, usually in combination with other plants with similar effects [1]. Another species that occurs in this climate is identified as *H. zivojinii* Cernjavski and Soska, representing an endemic, while *H. arenarium* has not been recognized in the flora of Macedonia [2].
The chemical composition of *H. plicatum* has been investigated in Turkey, where Mericli et al. isolated and identified chelipiron and 5-methoxy-7-hydroxy-phthalid from *H. plicatum* ssp. *plicatum* and chelipiron from ssp. *polyfyllum* [3]. Smirnov et al. showed that alcoholic extracts of *H. plicatum* possess bactericidal activity against *Corynebacterium michiganese, Xanthomonas malvacearum, Staphylococcus aureus* and *Bacillus subtilis* [4]. Among the extensive literature about the species of *Helichrysum*, flavonoids seem to be the most frequently investigated constituents. Thus, in three various subspecies of *H. arenarium* (ssp. *aucheri*, ssp. *erzincanicum* and ssp. *rubicundum*) apigenin, luteolin, naringenin, kaempferol and 3,5-dihydroxy-6,7,8-trimethoxy-flavone were identified as well as seven different glycosides [5]. Almost the same flavone aglycones were identified in few other *Helichrysum* species, in *H. pallasii* [6], *H. noeanum* [7], *H. stoechas* ssp. *barrelieri* [8], *H. graveolens* [9], *H. nitens* [10], *H. decumbens* [11], *H. armenium* ssp. *armenium* and ssp. *araxinum* [12], etc. Apigenin, naringenin, 3,5-dihydroxy-6,7,8-trimethoxy-flavone are mainly found in the flowers of *Helichrysum* sp. very often kaempferol and rarely luteolin and quercetin. The yellow colour of the flower is due to the halcone isosalipurposid [13]. Steams and leaves of *Helichrysum*, on the other hand, contain mainly quercetin and kaempferol glycosides, then naringenin and luteolin glycosides, and rarely astragalin [7, 9, 12], hispidulin, skutelarein and glycosides of gosipetin [14].
Up to now the chemical composition of *Helichrysum* species from Macedonia has not been investigated. In the present study flavone aglycones were identified and determined in different parts of *H. plicatum*. The plant material was collected during summer 1996, on the Golak Mountain in Eastern Macedonia. Extractions were performed using dried material previously separated into flowers, steams and leaves. The flavone aglycones in these extracts were analysed by HPLC. Identification was made according to the retention times and UV spectra of the components compared to those of authentic samples of available flavonoids (luteolin-7-glycoside, eriodictiol, qvercetin, luteolin, naringenin, apigenin, kaempferol and chrysoeriol). Chromatograms obtained using HPLC are presented in the Fig., where a stands for a mixture of authentic samples, b and c for ethylacetate extracts (glycosides previously hydrolised) from flowers and steams + leaves, respectively. Calibration curves for the flavones apigenin, naringenin, luteolin, quercetin and kaempferol were made for quantitative measurements. The results of the identification and determination of flavone aglycones in *H. plicatum* from Macedonia are presented in the Table.

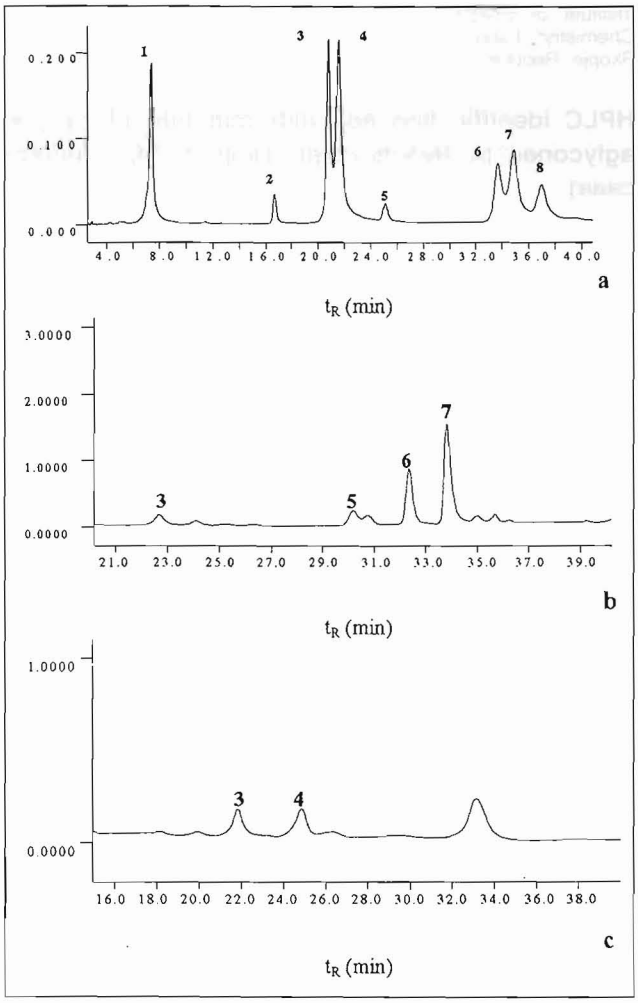

Fig.: HPLC: a. mixture of authentic samples (1-luteolin-glycoside; 2-eriodyctiol; 3-quercetin; 4-luteolin; 5-naringenin; 6-apigenin; 7-kaempferol; 8-chrysoeriol); b. ethylacetate extract of flowers, after hydrolysis; c.ethylacetate extract of steams and leaves after hydrolysis.

Apigenin and naringenin as free aglycones and glycosides of apigenin, naringenin, kaempferol and quercetin were found in the flowers of *H. plicatum*. The concentration of these components varied widely, depending on the extraction procedure. Primary extraction with ethanol/water (7:3, v/v) and secondary extraction of the water phase with ethylacetate provided lower concentrations of flavones compared to that obtained with methanol. After hydrolysis, almost the same quantities of apigenin (71.4 mg/100 g) and naringenin (71.0 mg/100 g) were determined. The content of quercetin was much lower (13.0 mg/100 g) while the most abundant was kaempferol (164.4 mg/100 g).

Steams and leaves of *H. plicatum* contained quercetin and luteolin glycosides and free luteolin. The concentration of luteolin after hydrolysis was 49.9 mg/100 g, whereas without hydrolysis it was two times lower (28.1 mg/100 g).

The results obtained showed that the flavone aglycones in *H. plicatum*, identified as free flavones before hydrolysis and aglycones from glycosides, after hydrolysis, were very similar to the flavones identified in some other *Helichrysum* sp. The results obtained are in good accordance with data of flavonoids in *H. arenarium* [3, 13]. It can also be concluded that the most abundant flavonoids in *H. plicatum* are the glycosides of kaempferol.

Table: Identification and determination of flavone aglycones in different extracts of *Helichrysum plicatum* (mg/100 g)

Extract	Apigenin	Luteolin	Naringenin	Kaempferol	Quercetin
Flowers					
a. Methanol	72.2	–	45.0	–	–
b. Ethylacetate	39.9	–	19.3	–	–
c. After hydrolysis	71.4	–	71.0	164.4	13.0
Steams and leaves					
a. Methanol	–	28.1	–	–	–
b. Ethylacetate	–	26.4	–	–	–
c. After hydrolysis	–	49.9	–	–	9.94

Experimental

1. Materials and instruments

The aerial parts of *Helichrysum plicatum* DC. were collected on the Golak Mountain, Eastern Macedonia, in the flowering period of the plant, air-dried; identified by Dr. V. Matevski, Department for Botany, Faculty of Science, Skopje, Republic of Macedonia.

All reagents used were of analytical grade. Authentic samples of apigenin, luteolin, naringenin, eriodyctiol, chrysoeriol, luteolin-7-glycoside (Extrasinthese, Lyon), quercetin dihydrate (Merck, Germany) were used.

A Varian HPLC system equipped with ternary pump model 9012 and UV diode array detector (UV-DAD) model 9065 was used.

2. Extraction procedures

Dried plant material (flowers, steams and leaves separately) was cut into small pieces and extracted in the ratio 1:10 (w/v) by three procedures: a. with methanol; b. with ethanol-water (7:3), then evaporated until water remains and extracted with ethylacetate; c. hydrolysis with conc. HCl, aglycones extracted with ethylacetate. All extracts were evaporated and the dried residues were dissolved in a small volume of methanol (2 ml).

3. HPLC analysis

A column (250 × 4.6 mm) filled with RP C-18 stationary phase with a particle diameter of 5 μm was used. Sample solutions were injected using a manual loop valve injector (20 μl). The separation was performed using $HCOOH/H_2O$ (0.1:99.9) as solvent A, acetonitrile (B) and methanol (C). The flow rate was 1.2 ml/min. The method was: at the beginning 70% A, 10% B and 20% C; then 1–5 min 65% A, 15% B and 20% C; 15–35 min 60% A, 20% B and 20% C; up to 45 min 55% A, 20 B and 20% C; up to 50 min 40% A, 40% B and 20% C. UV detection was carried out at 254, 286 and 360 nm. Data were compared with those for authentic samples.

Calibration curves of analysed flavones were the following (y-peak area, x-concentration in mg/ml): apigenin: $y = 5.8422 \times 10^6x$, $R^2 = 0.9955$; luteolin: $y = 6.1360 \times 10^6x$, $R^2 = 0.9965$; naringenin: $y = 1.7120 \times 10^6x$, $R^2 = 0.9989$; quercetin: $y = 1.1019 \times 10^7x$, $R^2 = 0.9985$; kaempferol: $y = 4.5938 \times 10^6x$; $R^2 = 0.9983$.

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