

**DETERMINATION OF KAEMPFEROL IN EXTRACTS  
FROM *LILIUM CANDIDUM* L. LILIACEAE BY MEANS  
OF LIQUID CHROMATOGRAPHY**

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There is an optimized, validated and implemented method for determination of kaempferol through liquid chromatography in terms of isocratic separation in the proportion: 0.05% phosphoric acid and acetonitril (50:50) v/v. The limits of detection (LOD) and quantification (LOQ) were determined. The content of kaempferol in samples from *Lilium candidum* L. with the use of different concentrations of extract agent and after releasing kaempferol from dermal semisolid medicaments was investigated. The samples were prepared by various producers. The results obtained proved that the implemented method shows good validated parameters and is favourable for determination of kaempferol in samples from *Lilium candidum* L. It was kaempferol that evaluated best from the samples that were prepared by extraction of 80% ethanol. The contents in the samples were determined using the method, after releasing kaempferol from dermal semisolid medicaments.

**Keywords:** kaempferol – *Lilium* – isocratic separation – liquid chromatography

**INTRODUCTION**

Flavonoids, characterized by the antioxidation activity depending on a position and a number of hydroxylic groups in a molecule and on its glycosylation belong to the group of vegetable phenols. Flavonoids have significant biochemical and pharmacologic effects. Among these substances we also include the flavonols (e.g. quercetin, myricetin and rutin) and kaempferol. This yellow coloured antioxidant is

resistant to oxidative damage of cells, lipids and DNA. It acts as prevention against arteriosclerosis since it inhibits oxidation of lipoproteins of low-density (Směla, 2009). A majority of works (Escarpa & Gonzales, 2000), (Ganzera et al., 2008) was aimed at determining phenolic substances including kaempferol in various vegetable materials. The method of simultaneous determination of quercetin, kaempferol and (E)-cinnamic acid in vegetative organs of *Schisandra chinensis* by means of liquid chromatography was published by Sladkovský et al., 2001.

Kaempferol belongs to the key content substances of *Lilium candidum* L., family Liliaceae, investigated by the team of research workers from the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University in Bratislava. So far from this plant species, many groups of secondary metabolites, such as organic acids, flavonoids, nitrogenous and steroid substances, have been isolated and identified (Eisenreichova et al., 2004). In recent years the anti-fungous and anti-tumorous activities of *Lilium candidum* L. have been proven (Mučaji et al., 2002), (Vachálková et al., 2000). Several works were focused on the determination of flavonoids by means of liquid chromatography (Sladkovský et al., 2001), (Crozier et al., 1997). The effectiveness of reverse phases of utilized columns in liquid chromatography at analyses of free and conjugated flavonoids has been investigated too.

## MATERIAL AND METHODS

The work is aimed at optimization and implementation of the method for determination of kaempferol by means of liquid chromatography as well as at comparison of samples of ethanol extracts from *Lilium candidum* L. obtained from various producers.

### 1. Preparation of samples

There were compared samples prepared by extraction from fresh blooms of *Lilium candidum* L. Liliaceae using different concentrations of ethanol and samples after releasing an amount of kaempferol from hydrogel. The samples were prepared both at the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University and in Calendula a.s., Nová Ľubovňa (Vítková et al., 2011).

Sample No. 1 – 80% ethanol extract *Lilium candidum* L.

Sample No. 2 – 80% ethanol extract *Lilium candidum* L. after releasing kaempferol from dermal semisolid medicaments.

Sample No. 3 – 60% ethanol extract *Lilium candidum* L.

Sample No. 4 – 60% ethanol extract *Lilium candidum* L. after releasing kaempferol from dermal semisolid medicaments.

Sample No. 5 – 80% ethanol extract *Lilium candidum* L. – a producer Calendula a.s.

Sample No. 6 – 80% ethanol extract *Lilium candidum* L. – a producer Calendula a.s. after releasing kaempferol from dermal semisolid medicaments.

Dry residue of the sample was quantitatively transferred into ependorf, rinsed three times by methanol and evaporated by means of nitrogen till dry. Then it was dissolved in methanol and centrifuged as needed at 4010 RPM for the period of 10 m. The supernatant was transferred into a microwave. The sample prepared was analysed in this way.

### 2. Standards and solvents

2.1 The standard of kaempferol from Aldrich company (Steinheim, Germany).

2.2 For liquid chromatography, acetonitril and methanol Chromasolv were supplied by the company Sigma Aldrich, Laborchemikalien Seelze, Germany; 85% phosphoric acid from Slavus s.r.o., Bratislava, Slovak republic.

2.2.1 Depot solution of kaempferol pure standards

A solution with the  $0.2 \text{ mg ml}^{-1}$  methanol concentration was prepared.

2.2.2. Working solution of kaempferol pure standards

For drawing up a calibration line the working solutions of kaempferol standards were used (Fig. 1).

2.3. Liquid chromatography

There is an optimized, validated and implemented method for determination of kaempferol (Sladkovský et al., 2001) samples of *Lilium candidum* L. extracts.

Determinations were done through the liquid chromatograph Hewlett Packard 1050 Series with MWD detector (Multiple Wavelength Detector) and an autosampler Waldbronn, Germany. The detection was realised at the wave length 370 nm. A chromatographic column was used – Zorbax Eclipse XDB- C 18, 150 x 4.6 mm i.d. 5µm with a first-stage column Zorbax XDB- C18, 12.5 x 4.6 mm i.d., 5µm, Agilent Technologies, Waldbronn, Germany. The conditions for the determination were a mobile phase, the use of the isocratic separation namely the 0.05% phosphoric acid (0.3 ml phosphoric acid in 500 ml deionised water) and acetonitril 50:50 (v/v). The flow of the mobile phase was  $0.3 \text{ ml.min}^{-1}$ , the feed 20 µl at the ambient temperature. The MWD detector signal was analysed via a chromatographic programme Agilent ChemStation.

## RESULTS AND DISCUSSION

The method of calibration and validation

Calibration was performed by means of kaempferol standards solutions at the total concentration range  $0.125 - 150 \text{ µg ml}^{-1}$ .

The regress coefficient value was 0.9995.

Sensitivity of the method was determined following the limit of detection (LOD) and quantification (LOQ). The quantification limit was determined experimentally in the standard sample. Concentration of the standard on the level of detection for HPLC ( $0.143 \text{ µg ml}^{-1}$ ) was prepared. The average value of signals and their decisive deviations were calculated following the obtained signals reflected in peaks' areas as follows:

$$Y(D) = X_0 + 10 \cdot S_x, \text{ where}$$

Y(D) is detection signal in mAU

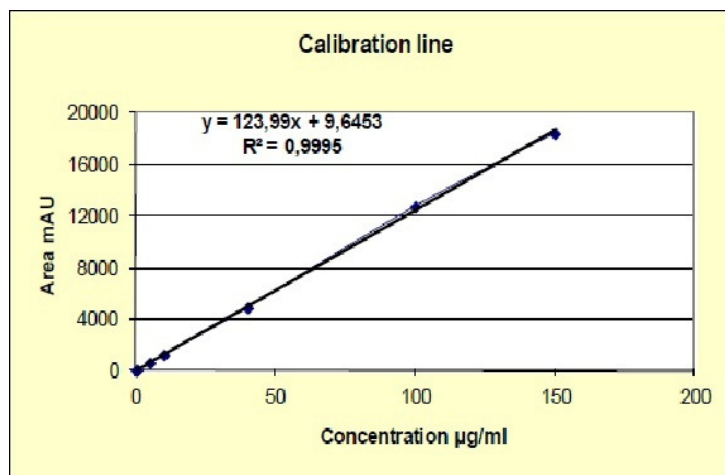
$X_0$  - average signal of blind samples in mAU

$S_x$  - decisive deviation of the signal of blind samples.

The value of quantification limit for kaempferol was  $0.198 \text{ µg ml}^{-1}$ .

In a similar way the detection limit was calculated, using the same relation and the decisive deviation multiplied thrice. From the MWD obtained signals, the average

value of the signal and its decisive deviation were calculated. The detection limit for kaempferol was fixed to be 0.143  $\mu\text{g ml}^{-1}$ . Concentration for LOD in  $\mu\text{g.ml}^{-1}$  for kaempferol was calculated from the calibration line (Figure 1).



**Figure 1. Calibration line of kaempferol**

The results of analysed samples are shown in the Table 1 and Figures (3–6). The Table 1 shows the required and investigated values of kaempferol (KE).

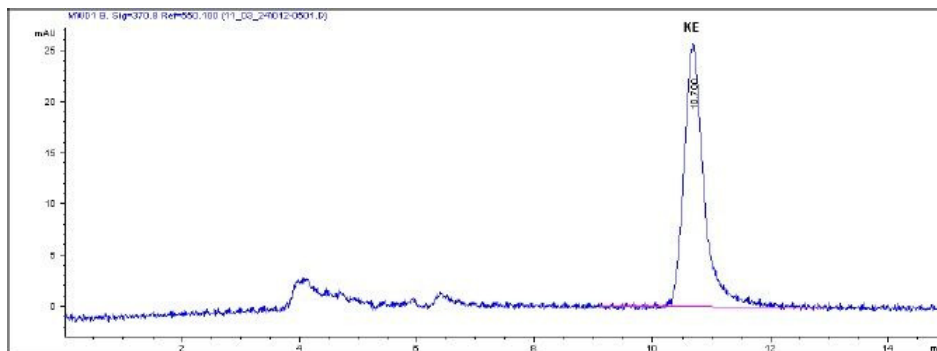
**Table 1. HPLC determination of kaempferol (KE) in the prepared extracts**

Sample No.	Samples	Peak's area	KE concentrations in the feed ( $\mu\text{g.ml}^{-1}$ )	KE concentrations in the sample ( $\mu\text{g.ml}^{-1}$ )
1	80% ethanol extract <i>Lilium candidum</i> L.	2641	21.22	0.36
2	80% ethanol extract <i>Lilium candidum</i> L. after releasing kaempferol	ND	-	-
3	60% ethanol extract <i>Lilium candidum</i> L.	18130	146.14	2.63
4	60% ethanol extract <i>Lilium candidum</i> L. after releasing kaempferol	ND	-	-
5	80% ethanol extract <i>Lilium candidum</i> L. – prepared by Calendula a.s.	1089	8.10	0.15
6	80% ethanol extract <i>Lilium candidum</i> L. –	ND	-	-

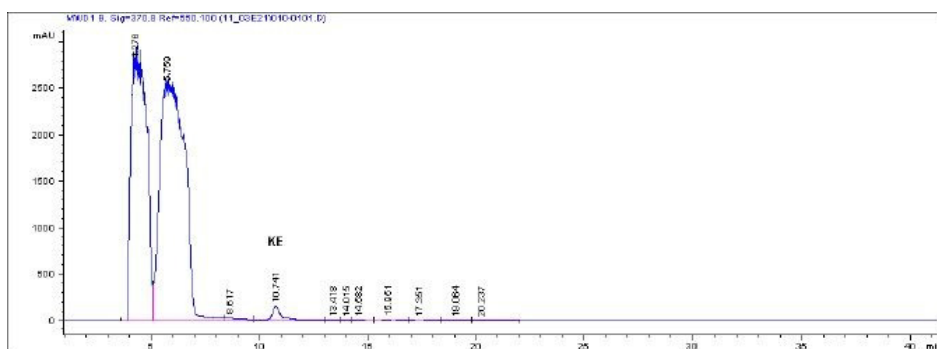
	a producer Calendula a.s. after releasing kaempferol prepared in Calendula a.s.			
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Table includes areas, KE concentrations in the feed and in the samples, ND - not-detected, in samples after releasing, kaempferol was found below the level of determinability.

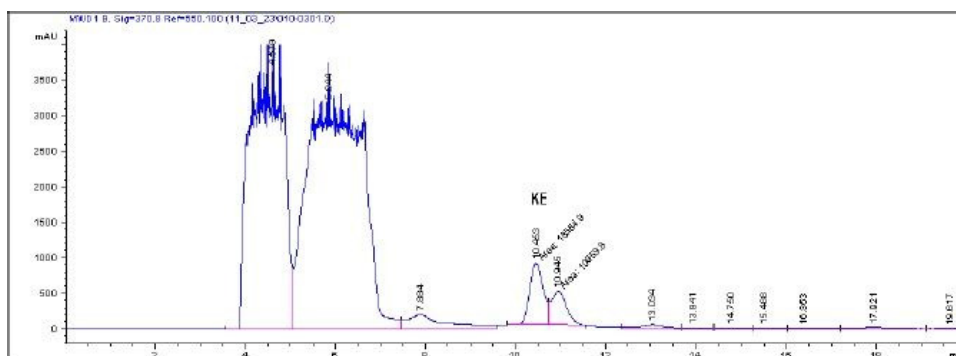
Chromatograms (Figures 2–6) represent a typical profile of separated kaempferol in the standard mixture and in the samples. Identification of kaempferol was performed on the basis of retention time periods and a standard addition.



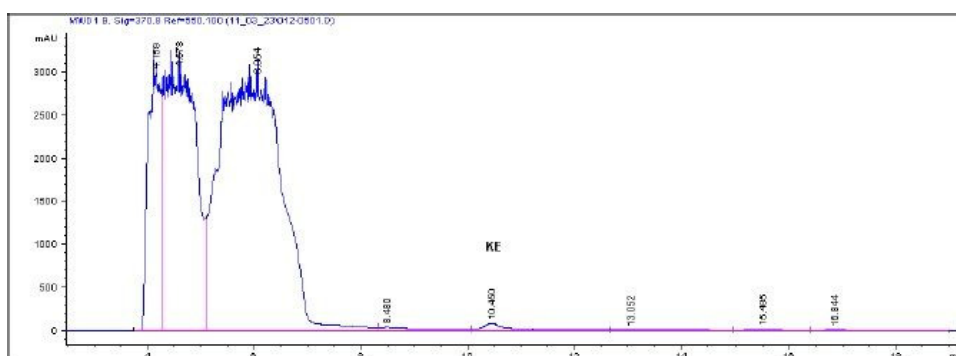
**Figure 2. HPLC chromatogram of standard kaempferol**



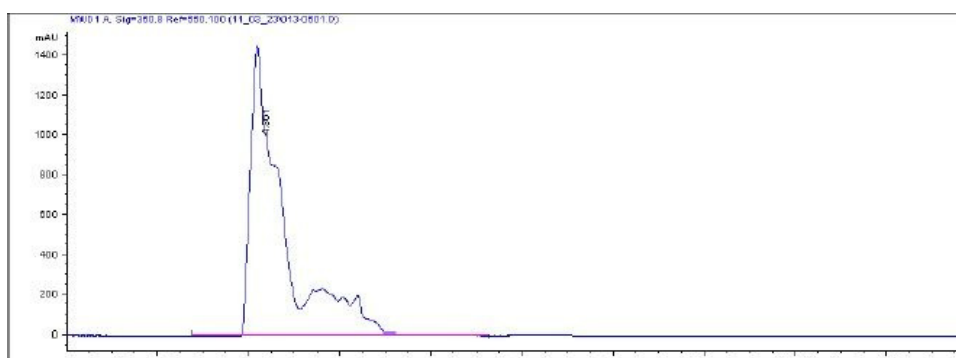
**Figure 3. HPLC chromatogram of extracts of *Lilium candidum* L. (Sample No. 1)**  
Sample No.1 – a 80 % ethanol extract *Lilium candidum* L.



**Figure 4. HPLC chromatogram of extracts of *Lilium candidum* L. (Sample No. 3)**  
Sample No.3 – a 60 % ethanol extract *Lilium candidum* L.



**Figure 5. HPLC chromatogram of extracts of *Lilium candidum* L. (Sample No. 5)**  
Sample No.5 – a 80 % ethanol extract *Lilium candidum* L. - the producer Calendula a.s.



**Figure 6. HPLC chromatogram of extract *Lilium candidum* L. (Sample No. 6)**  
The sample No.6 – a 80% ethanol extract *Lilium candidum* L. after releasing kaempferol, prepared in Calendula, a.s.

Chromatograms showed, that kaempferol in the described chromatographic system was evaluated independently. In the determined retention time, interferences did not occur. Small deviations in retention times in samples vs. standard were caused by influence of a matrix. The obtained results and validation parameters supported the adequacy of the implemented method for determining kaempferol in the prepared samples from *Lilium candidum* L. Comparison of the results obtained shows that kaempferol was evaluated best from the samples No. 1 and No. 5 for preparation of which the 80% ethanol was used.

The sample prepared by extraction of 60% ethanol did not show a distinct separation of kaempferol. The results obtained promote the availability of the use of extraction agent with higher concentration for preparing the samples from *Lilium candidum* L. with the aim to separate kaempferol better. In the samples after releasing from dermal semisolid topic medicaments the kaempferol was below the level of determinability.

## CONCLUSION

Main aim was to show use of analysis by evaluation of kaempferol in samples *Lilium candidum* L. According to the presented results we can conclude that this method is suitable for evaluation of kaempferol, is easy, practical and feasible with high accuracy.

## ACKNOWLEDGMENTS

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#### STANOVENIE KEMPFEROLU V EXTRAKTOCH Z *LILIUM CANDIDUM* L. LILIACEAE KVAPALINOVOU CHROMATOGRAPHIOU

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Bola optimalizovaná, validovaná a zavedená metóda na stanovenie kempferolu kvapalinovou chromatografiou v podmienkach izokraticej separácie v zložení: 0.05 % kyselina fosforečná a acetonitril (50:50) v:v. Stanovil sa limit detekcie (LOD) a kvantifikácie (LOQ). Touto metódou sa sledoval obsah kempferolu vo vzorkách z *Lilium candidum* L. za použitia rôznych koncentrácií extrakčného činidla a po uvoľnení kempferolu z dermálnych polotuhých liekov. Vzorky boli pripravené rôznymi výrobcami. Zo získaných výsledkov vyplynulo, že zavedená metóda vykazovala dobré validačné parametre a je vhodná na stanovenie kempferolu vo vzorkách z *Lilium candidum* L. Najlepšie sa eluoval kempferol zo vzoriek, ktoré boli pripravené extrakciou 80 %-ným etanolom. Vo vzorkách po uvoľnení kempferolu z dermálnych polotuhých liekov bol jeho obsah pod hladinou stanoviteľnosti metódy.

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