Application Note
No. 417/2020

Determination of flavonoid content in calendula officinalis (marigold)

UniversalExtractor E-800
Extraction of marigold for the flavonoid determination using the UniversalExtractor E-800

1. Introduction

A simple and reliable procedure for determination of flavonoids content in calendula officinalis (marigold) is introduced. Flavonoids are polyphenolic secondary metabolites with a low bioavailability for the human body. Once absorbed, the flavonoids are rapidly metabolized, resulting in metabolites with anti-inflammatory, -oxidant, -thrombogenic, -diabetic and -cancer properties. Flavonoids are found in many plants and plant-based products that are commonly consumed, such as fruits, vegetables, wine or tea. One example represents the *calendula officinalis*, also known as marigold, which is described in this application note. Due to the low bioavailability of flavonoids, in combination to the rapid metabolization in the human body, even high intake of flavonoids is regarded to be safe. [1 - 3]

In the presented application, the sample is extracted with the UniversalExtractor E-800 using the method Soxhlet warm. The flavonoid content is determined by using UV/Vis-spectrophotometry. This application complies with the official method of 'Calendulae flos' from the European Pharmacopoeia. [4]

2. Equipment

- UniversalExtractor E-800 with chamber heater
- Analytical balance (accuracy ± 0.1 mg)
- UV/Vis spectrophotometer (Perkin Elmer Lamda 25)

3. Chemicals, Reagents and Materials

Chemicals:

- Acetone for analysis, Reag. Ph Eur (Scharlau, Order No. AC0314005P)
- Aluminium chloride hexahydrate Ph Eur (Roth, Order No. CP88.1)
- Acetic acid 99% (Sigma Aldrich, Order No. 27285)
- Hydrochloric acid 37% (Roth, Order No. 2607.1)
- Methanol supragradient HPLC grade (Scharlau, Order No. ME03062500)
- Hexamethylenetetramine puriss. p.a. (Sigma Aldrich, Order No. 33233)
- Sodium sulfate anhydrous (Merck, Order No. AM1160886814)
- Deionized water
- Cotton wool
- Cellulose thimbles, 25x150 mm, BUCHI (Order No. 11067445)

Reagents:

- Methenamine reagent: 5 g/l Hexamethylenetetramine in water
- Aluminium chloride reagent: 5 g aluminium chloride, solved in 1 l 5% acetic acid R[1] in methanol R[1] solution.

For a safe handling please pay attention to all corresponding MSDS!

Sample:

- Powder from dried marigold including the calyx, reference flavonoid content: 0.29% (determined according to Ph. Eur. monograph on *Calendula officinalis* L, flos)

The sample is a powder and therefore does not require additional homogenization.

[1] R indicates the preparation and/or quality to be found in Ph.Eur (Chapter 4 – Reagents)

4. Procedure

The determination of the flavonoid content includes the following steps:

- Simultaneous extraction and breaking up of flavonoid-glycoside with acetone and acid hydrolysis, respectively, forming flavonoid-aglycone.
- Liquid-liquid extraction of flavonoid-aglycone using ethylacetate
- Determination of flavonoid content using UV/Vis spectrophotometry

4.1. Sample preparation

1. Place the cellulose thimble into a thimble holder.
2. Weigh 0.8 g of homogeneous sample into the cellulose thimble.
3. Add 1.0 mL of methenamine reagent onto the sample
4. Use the cotton wool to cover the sample inside the cellulose thimble.
5. Add 7.0 mL hydrochloric acid (29.4 mL hydrochloric acid 37%, filled up to 50 mL with water) inside the cellulose thimble.

4.2. Extraction of the fat with the UniversalExtractor E-800 Soxhlet warm

4.2.1. Preparation of the beakers

Always use clean beakers for the Soxhlet extraction.

4.2.2. Soxhlet Extraction

6. Place the cellulose thimbles containing the sample into the extraction chamber and adjust the level sensor to the sample's height.
7. Fill the solvent into the beakers and place them on their corresponding heating plate.
8. Close the protection shield and lower the rack. Alternatively, fill the solvent through the condensers after lowering the rack. Activate the occupied positions and open the cooling water tap or switch on the connected chiller.
9. Start the Soxhlet warm extraction according to the parameters listed in Table 1.

Table 1: Parameters for the Soxhlet warm Extraction with the UniversalExtractor E-800.

Step	Value	Heating level
Extraction method	Soxhlet warm	
Solvent	Acetone	
Extraction	10 cycles	Extraction:11[2] Chamber: 3[1]
Rinse	5 min	11
Drying 1	☑ AP, 2 min	11
Solvent volume [mL]	100	

4.2.3. Liquid-liquid extraction

10. The extract is transferred into a 100 mL volumetric flask and filled up with acetone to the mark.
11. 20 mL of the obtained solution is transferred into a separating funnel. 20 mL of deionized water is added.
12. A liquid-liquid extraction is performed by washing the solution with 1x15 mL and 3x10 mL ethylacetate.
13. The collected organic phases are washed with 2x50 mL deionized water.
14. The organic phase is dried using 10 g sodium sulfate anhydrous.
15. The suspension is filtered using cotton wool. Hereby, the liquid is directly transferred in a 50 mL volumetric flask.

[2] Heating level proposed by the system depending on the selected solvent.

16. Residues are rinsed with additional ethylacetate before filling up the volumetric flask to the 50 mL mark.

4.2.4. UV/Vis spectrophotometry

17. Test solution: Take 10.0 mL of the stock solution, add 1 mL of aluminium chloride reagent R and dilute to 25.0 mL with 5 Vol.% glacial acetic acid R in methanol R.
18. The compensation liquid is prepared by diluting 10.0 mL of the stock solution to 25 mL with 5 Vol.% glacial acetic acid R in methanol R.
19. After 30 min, the absorbance of the test solution is determined and compared to the compensation liquid at 425 nm.

4.3. Calculation

The percentage content of flavonoids, expressed as hyperoside, are calculated according to the following equation (1).

$$\%\ Flavonoids = \frac{A * 1.25}{m_{sample}} \qquad (1)$$

% Flavonoids:	Percentage of flavonoids in the sample
A:	Absorption at 425 nm
m_{Sample}:	Sample weight [g]

The specific absorption of hyperosid is taken to be 500 (1 %, d=1 cm).

5. Results

The marigold sample was analysed in triplicates. The determined flavonoids content corresponds well with the reference value of 0.29%. Due to the low flavonoids content, a small deviation results in a high relative standard deviation. For this reason, an intern tolerance of 5% was defined. The results are shown in Table 2.

Table 2: Results for flavonoid determination of marigold extracts.

Sample	m_{sample} [g]	Absorption	Flavonoids [%]	Mean value
1	0.8006	0.2439	0.38	**0.36%** rsd : 4.18%
2	0.8006	0.2302	0.36	
3	0.8006	0.2251	0.35	

6. Conclusion

The determination of flavonoids content in marigold powder by use of the UniversalExtractor E-800 to prepare the extracts provides reliable and repeatable results. Compared to the method described in Ph. Eur., the laborious filtration steps during the extraction, including the rinsing of the cotton wool, are omitted. As a result, an even higher flavonoids content is obtained since no residues remain on the cotton wool. The automation of the flavonoids extraction is thus a complete success.

7. Acknowledgements

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8. References

[1] https://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/flavonoids#metabolism-bioavailability, 17.12.2020

[2] Chen A., Xiang W., Liu D., Liu C., Yang L., Determination of Total Flavonoids and Its Antioxidant Ability in Houttuynia cordata, Complementary medicine researchJournal of Materials Science and Chemical Engineering, 4, 131-136, 2016.

[3] Williams R. J., Spencer J. P, Rice-Evans C., Flavonoids: antioxidants or signaling molecules?, Free Radical Biology and Medicine, 36, 838-849, 2004.

[4] Ph. Eur. Monograph on *Caldendulae flos*, 07/09:3000, corrected 10.1

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Operation Manual of the UniversalExtractor E-800