

# OPTIMIZATION AND VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF POLYMETHOXYLATED FLAVONOIDS IN *CITRUS SINENSIS*

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## INTRODUCTION

*Citrus sinensis* is a fruit, commonly known as orange or sweet orange. It belongs to the family of the *Rutaceae*. Oranges contain high amounts of vitamin C and potassium and are low in fat. The interest in its biological activity is high, since *C. sinensis* has a wide range of compounds. One specific group of compounds is called the polymethoxylated flavonoids (PMFs). PMFs have a broad spectrum of activities, such as cardiovascular, anti-dementia and chemopreventive activities<sup>1</sup>. Many PMFs have already been identified, the two most common PMFs in *Citrus* are nobiletin and tangeretin. Since PMFs are often used for bio-activity studies, there was a need for an analytical method. We developed, optimized and validated an analytical method for the quantification of the polymethoxylated flavonoids nobiletin and tangeretin in dried orange peel powder.

## METHOD OPTIMIZATION

### Nobiletin

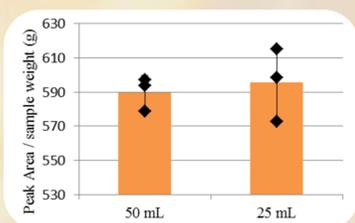


Figure 1: Extraction volume.

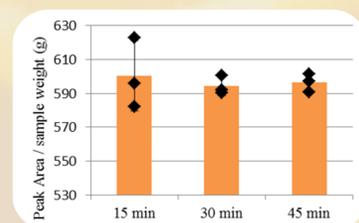


Figure 2: Extraction time.

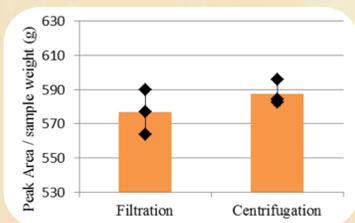


Figure 3: Sample clean up.

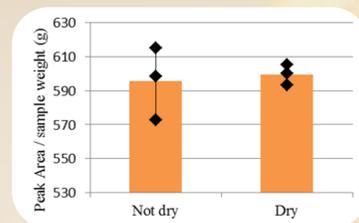


Figure 4: Evaporation grade.

### Tangeretin

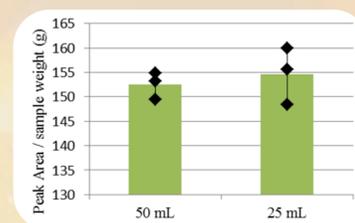


Figure 5: Extraction volume.

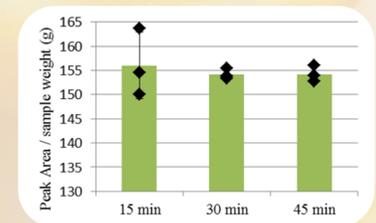


Figure 6: Extraction time.

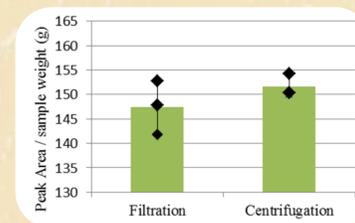


Figure 7: Sample clean up.

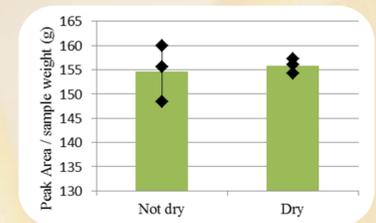


Figure 8: Evaporation grade.

## RESULTS

### Final Method

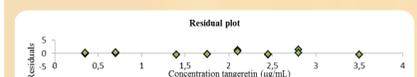
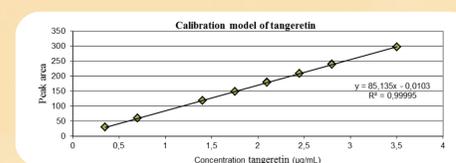
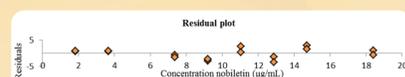
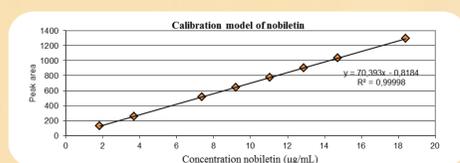
The concentration of nobiletin and tangeretin in the sample was determined on 1.0 g dried and powdered orange peel. The PMFs were extracted out of the matrix by adding 25 mL MeOH 70% and refluxing during 15 minutes. After cooling down, the sample was centrifuged. The supernatants were removed and the extraction was repeated twice on the residue. The extracts were combined and concentrated to less than 50 mL using a rotary evaporator. The concentrate was brought in a 50.0 mL flask and adjusted to the mark. The solution was filtered using a syringe-filter and brought into a vial for HPLC-DAD analysis. The HPLC conditions are written in table 1.

Agilent 1260 series, DAD  
 Column: Kinetex® EVO C18  
 Solvents: (A) 0.1% FA / (B) ACN  
 Gradient: 5 min 32% B, 5 - 15 min 41% B,  
 5 - 17 min 100% B, 17 - 22 min 100% B,  
 22 - 25 min 32% B, 25 - 30 min 32% B  
 Flow: 1 mL/min  
 UV: 330 nm

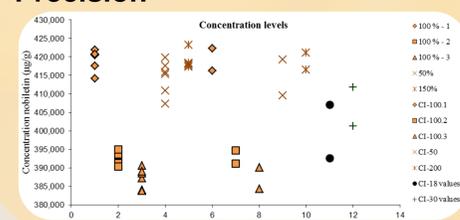
Table 1: HPLC conditions

## VALIDATION

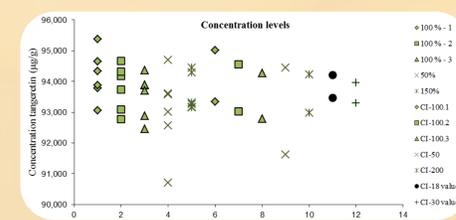
### Calibration model



### Precision



Nobiletin	Between days	Between levels
Mean (µg/g)	399.8	406.5
RSD <sub>between</sub>	4.3%	3.8%
Cochran	0.44 (C <sub>crit</sub> 0.71)	0.47 (C <sub>crit</sub> 0.51)
F-test	277.9 (F <sub>crit</sub> 3.68)	156.4 (F <sub>crit</sub> 2.76)
RSD <sub>max</sub>	4.33%	4.31%



Tangeretin	Between days	Between levels
Mean (µg/g)	93.8	93.6
RSD <sub>between</sub>	0.8%	0.9%
Cochran	0.38 (C <sub>crit</sub> 0.71)	0.47 (C <sub>crit</sub> 0.51)
F-test	1.14 (F <sub>crit</sub> 3.68)	1.37 (F <sub>crit</sub> 2.76)

## DISCUSSION

An analytical method for the quantification of the polymethoxylated flavonoids nobiletin and tangeretin in dried orange peel powder was developed, optimized and validated. The extraction was complete after three extraction steps when (1) the sample was refluxed for 15 minutes, (2) an extraction volume of 25 mL was used and (3) the different extracts were centrifuged before combining. A calibration model of both nobiletin and tangeretin standards was made in the concentration range from 1.8 µg/mL to 18.4 µg/mL and 0.4 µg/mL to 3.5 µg/mL respectively. The final method was validated and the concentrations of nobiletin and tangeretin were 406.5 µg/g and 93.6 µg/g dried orange peel powder respectively. The linearity of the method was proven for 0.5 till 1.5 g dried orange peel powder. The recovery levels of the method were determined as 101.2% for nobiletin and 99.8% for tangeretin.