Institute of Pharmaceutical Biology, University of Regensburg, Germany

Total phenolic and tannins determination: a modification of Ph. Eur. 2.8.14 for higher throughput

S. Wiesneth, G. Jürgenliemk*

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**Corresponding author: Dr. Guido Jürgenliemk, Institut für Pharmazeutische Biologie, Universität Regensburg, 93053 Regensburg*
Guido.juergenliemk@ur.de

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A principle mostly being used to quantify phenols and tannins is the Folin-Ciocalteu's reagent method described in the European Pharmacopeia's method 2.8.14. Aim of the present study was to simplify this procedure in order to handle larger sample numbers. By changing the wavelength, the amounts of reagents, miniaturizing the setting to microtiter scale and changing the incubation time before measurement, the Ph. Eur. method 2.8.14 was optimized for a larger number of experiments. Calibration curves and time kinetics with different phenolic compounds (catechol, procyanidin B1, gallic acid, (+)-catechin, tannic acid, salicylic acid, ferulic acid) were determined. In addition, the absorbance of a 1 M solution was calculated by extrapolation of each calibration curve, to examine correlations due to the reaction's stoichiometry. Furthermore, the repeatability concerning herbal samples was investigated using Quercus cortex. The method was validated concerning its repeatability, robustness, linearity and reproducibility. Using this method, at least 120 samples can be handled per day by one person to quantify the total phenolic and tannin content.

1. Introduction

It requires several days and a large number of glassware to quantify the total phenolic and tannins content of a large number of samples according to the Ph. Eur.'s method 2.8.14 (Ph. Eur. 2015). The aim of the present study was to modify this procedure to gain a method with a higher throughput. This new method should be validated due to the assay's repeatability, robustness, linearity and reproducibility. Investigations with several reference compounds should provide general information about the reaction's stoichiometry. Also the assay's functionality was proofed with *Quercus cortex*.

2. Investigations, results and discussion

Parameters of recent studies about the Folin Ciocalteus's reaction (Glasl 1983; Li et al. 2007) were adapted and further optimized to modify the Ph. Eur. method 2.8.14. Since this method is based on a time depending redox-reaction, the optimum time interval was determined by recording kinetic datasets of phenolic compounds. These investigations showed that the reaction should be completed after almost 150 min (Fig. 1). Thus, a reaction time of 180 min was chosen in order to get robust and reproducible results. Additionally, the linearity, repeatability and reproducibility were ensured by independent dilution series of reference compounds (Table) and exemplarily shown for catechol (Fig. 2). To get more information about the reaction's stoichiometry, the absorbance of a 1 M solution of each phenolic reference compound was calculated by extrapolation and correlated to the compounds' phenolic OH-groups (R^2 = 0.9936) and to their numbers of phenolic rings (R^2 = 0.9829) (Fig. 3). Finally, linearity for both parameters was observed with slight preferences to the phenolic OH-groups correlation. The total phenols and tannins quantification of Quercus cortex samples as tannic acid resulted in a good reproducibility (phenols: 5.1% ± 0.4%; tannins: 4.3% ± 0.3%).
The modifications of the Ph. Eur.'s method resulted in a much more simple procedure, enabling a throughput of at least 120 samples per day.

3. Experimental

1,2-Dihydroxybenzene (catechol) ≥ 99%, salicylic acid p. a. ≥ 99%, gallic acid ≥ 98% (Fluka) and (+)-catechin hydrate ≥ 98% were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Folin Ciocalteu's reagent and L-(+)-ascorbic acid p. a. (99.7%) were obtained from Merck Chemicals GmbH (Darmstadt, Germany). Tannic acid Ph. Eur. for biochemistry, ferulic acid ≥ 98% and sodium carbonate (Na_2CO_3) anh. were from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Procyanidin B1 was isolated from the bark of *Salix daphnoides* Vill. (Wiesneth et al. 2015) and the water being contained was determined by calculation after elemental analysis. The water for analysis was generated by an Astacus Reagent bench (MembraPure GmbH, Henningsdorf/Berlin, Germany).
Aliquots (25 mg to 28 mg) of *Quercus cortex* plv. (Caesar & Loretz GmbH, Hilden, Germany) were extracted in a supersonic bath with 1.00 ml water for 30 min in hexaplicates. Centrifugation (14000 rpm, 3 min) and dilution in water (1+9 [V/V])

Table: Summary of the parameters used for the linearity's validation and the gained results

	Catechol	Catechin	Procyanidin B1	Gallic acid	Ferulic acid	Tannic acid	Salicylic acid
Concentration range [µM]	15-157	7-74	4-40	15-158	15-154	1.4-15	15-153
Absorbance range [AU]	0.2012–1.7920	0.1690–1.2143	0.1533–1.1724	0.1679–1.4617	0.1709–1.1127	0.1536–1.4111	0.0688–0.2503
Correlation coefficient r^2 of Calibration curve	0.9979	0.9850	0.9867	0.9939	0.9930	0.9965	0.9899
Calculated 1 M absorbance	11100	14800	27100	9100	6400	87600	1300

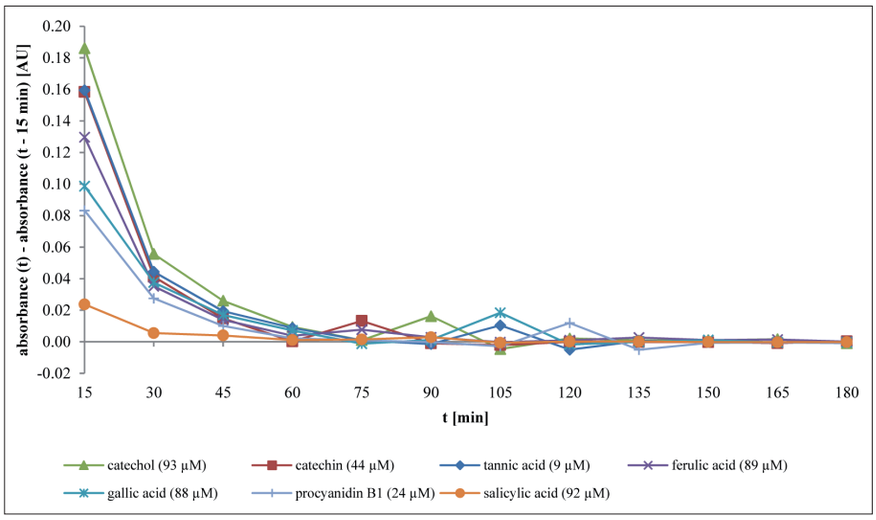

Fig. 1: Change in absorbance during the reaction between phenolic compounds (one exemplary concentration) and Folin Ciocalteu's reagent.

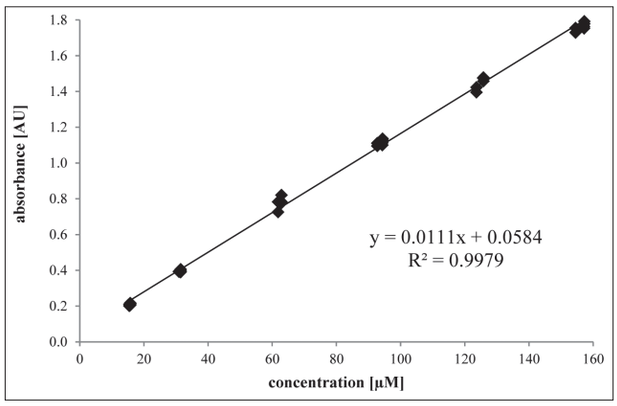

Fig. 2: Calibration curve of catechol.

96-well polystyrene F-bottom microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) were prepared with 200 µl water. To the other wells, 20 µl of TPS or NAPS were added, equally divided to two 96-well plates (reference compounds). The Folin-Ciocalteu's reagent was diluted in water (1+9 [V/V]) and 100 µl of this mix was added to the wells, followed by shaking without light for four minutes. Finally, 80 µl of a 10.6%-solution of Na_2CO_3 anh. in water was added to the samples. Measurement of absorbance at 690 nm at room temperature was achieved using a SpectraFLUOR PLUS® reader (Tecan Group Ltd., Männedorf, Switzerland). The first of the prepared 96-well plates was used to record the time kinetics in 15 min intervals for 180 min in triplicates. The second 96-well plate was measured after incubation for 180 min, while shaking without light. The calibration curves were generated of the 180 min values of both 96-well plates. The phenolic and tannins content of Quercus cortex samples were calculated by the tannic acid's calibration curve. The tannin's content resulted from subtraction of the NAPS's from the TPS's phenolic content.

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Conflicts of interest: None declared

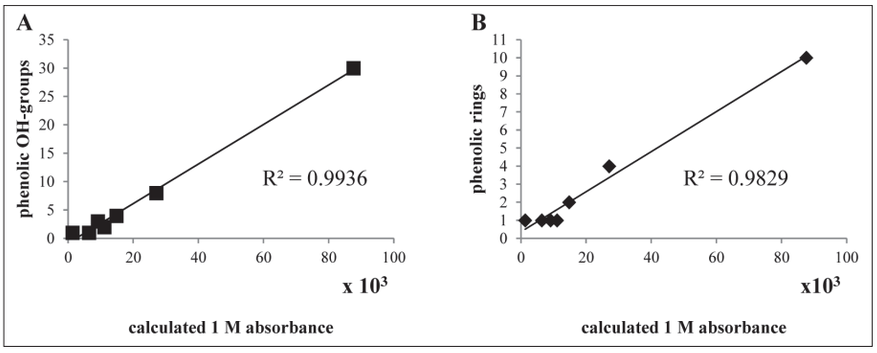

Fig. 3: Correlation of the calculated absorbance of a 1 M solution to the phenolic OH-groups (A) and to the phenolic rings (B).

yielded in the total phenol solutions (= TPS). The reference compounds were dissolved in triplicates in a defined volume of water to gain the stock solutions. These were used to produce dilution series of six different concentrations in duplicates of triplicates (TPS).

An aliquot of 0.5 ml of each TPS was added to a 1.5 ml SuperSpin™ microtube (VWR International GmbH, Darmstadt, Germany) prepared with 5-10 mg hide powder (Research Institute for Leather and Plastic Sheeting GmbH, Freiberg, Germany). These preparations were stirred and further incubated without light for 60 min while shaking. The supernatants after centrifugation (14000 rpm, 3 min) resulted in the solutions containing the phenols not absorbed by hide powder (NAPS). The outer wells of

References

Glasl H (1983) Zur Photometrie in der Drogenstandardisierung. Dt Apoth Ztg 123: 1979-1987.

Li H, Chenk K, Wong C, Fan K, Chen F, Jiang Y (2007) Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem 102: 771–776.

Ph. Eur. 2.8.14 (2015) Gerbstoffe in pflanzlichen Drogen. In: Europäisches Arzneibuch 8. Ausgabe Stuttgart, p. 383.

Wiesneth S, Petereit F, Jürgenliemk G (2015) *Salix daphnoides*: A screening for oligomeric and polymeric proanthocyanidins. Molecules 20: 13764-13779.