Research article / Оригинальная статья

UDC 615.074

https://doi.org/10.33380/2305-2066-2026-15-2-2120

Polyphenolic combinations quantification through ABTS$^{\bullet+}$ radical cations accumulation kinetics measurement

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Received: 14.06.2025 **Accepted:** 09.09.2025 **Published:** 27.03.2026

Abstract

Introduction. Polyphenols are widely distributed in plant world and recognized for their high antioxidant activity. Traditional methods of polyphenolic compounds quantification, such as Folin – Ciocalteu and aluminum chloride assays, measure total phenolic or flavonoid content but fail to identify individual compounds in complex mixtures. Combining the kinetic profiling of antioxidant activity manifestation for a combination of antioxidants has certain potential for the development of inexpensive and simple quantitate analysis approaches.

Aim. To develop an approach for the quantitative determination of individual polyphenols in binary combinations through the measurement of ABTS$^{\bullet+}$ radical cations accumulation kinetics based on a lag-time experimental strategy.

Materials and methods. We have studied formulations of flavonoid dihydroquercetin (DHQ) and lignan secoisolariciresinol (SECO) and a combination of DHQ with essential antioxidant alpha-tocopherol (α-TOH). The antioxidants induced the inhibition of ABTS$^{\bullet+}$ radical cations accumulation initiated by potassium persulfate, which resulted in a lag-time formation and was monitored spectrophotometrically at 730–750 nm.

Results and discussion. Both combinations demonstrated two lag-time periods pattern typical for additive type interactions between its components. Lag-time period duration depends on concentration of the component. We matched every lag-time period with specific polyphenol which allowed us to determine the content of every component of studied combinations. Developed technique measured the content of polyphenols with value of relative standard deviation (RSD) being not higher than 2 % and value of relative measurement error (δ) not exceeding 3,7 %.

Conclusion. Our study confirmed that an antioxidant capacity measurement approach could be successfully applied for content determination of individual polyphenols in complex mixtures. Developed methodology demonstrated high reproducibility and acceptable accuracy levels for binary combinations constituents' quantification.

Keywords: polyphenols, ABTS, quantification, antioxidant capacity

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Konstantin S. Voronin – formal analysis, methodology, investigation, original draft preparation. Igor R. Ilyasov – methodology, formal analysis, investigation, review and editing. Vera V. Olicheva – investigation, visualization, review and editing. Anastasiya K. Zhevlakova – methodology review and editing. Vladimir L. Beloborodov – methodology review and editing. Irina A. Selivanova – conceptualization, supervision, review and editing.

Funding. This research was funded by Russian Science Foundation, grant № 25-25-00537, https://rscf.ru/en/project/25-25-00537/

For citation: Voronin K. S., Ilyasov I. R., Olicheva V. V., Zhevlakova A. K., Beloborodov V. L., Selivanova I. A. Polyphenolic combinations quantification through ABTS$^{\bullet+}$ radical cations accumulation kinetics measurement. *Drug development & registration.* 2026;15(2):37–42. https://doi.org/10.33380/2305-2066-2026-15-2-2120

Количественное определение компонентов полифенольных композиций посредством измерения кинетики накопления радикал-катионов ABTS$^{\bullet+}$

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Статья поступила: 14.06.2025 **Статья принята в печать:** 09.09.2025 **Статья опубликована:** 27.03.2026

Резюме

Введение. Для количественной оценки полифенольных соединений и их смесей широко используют спектрофотометрические методы исследования, в том числе Фолина – Чокалтеу, спектрофотометрию с хлоридом алюминия и другие. Они позволяют с высокой воспроизводимостью определять суммарное содержание полифенолов в различных образцах в пересчете на конкретный полифенол, но не количество отдельных компонентов. Спектрофотометрию в УФ и видимой области также применяют для исследования антирадикальной емкости полифенольных соединений, которая в том числе зависит от их количественного содержания. Соответственно, методы оценки антирадикальной активности теоретически могут быть использованы для количественного определения полифенолов.

Цель. Разработать методику количественного определения отдельных компонентов полифенольных композиций посредством измерения кинетики накопления радикал-катионов ABTS$^{\bullet+}$.

Материалы и методы. Исследовали бинарные композиции флавоноида дигидрокверцетина и лигнана секоизоларицирезинола, а также дигидрокверцетина и альфа-токоферола. Спектрофотометрически наблюдали кинетику накопления радикал-катионов 2,2'-азино-бис(3-этилбензотиазолин-6-сульфокислоты) диаммониевой соли (ABTS$^{\bullet+}$) в присутствии исследуемых образцов и стандартных смесей.

Результаты и обсуждение. Для исследуемых композиций выявлено образование двух периодов плато в их кинетических кривых, продолжительность которых оказалась пропорциональна содержанию компонентов композиции, что дает возможность проводить их количественное определение при соотнесении периодов плато с конкретными компонентами. Разработанная методика позволяет определять количество полифенолов с относительной ошибкой (δ) не более 3,7 % и относительным стандартным отклонением (RSD) не более 2 %.

Заключение. Была показана принципиальная возможность применения метода оценки антирадикальной емкости для количественного определения полифенольных соединений в сложных смесях. Разработана методика количественного определения отдельных компонентов в бинарных смесях, которая характеризуется высокой воспроизводимостью результатов и приемлемой точностью.

Ключевые слова: полифенолы, ABTS, количественное определение, антирадикальная активность

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. К. С. Воронин – формальный анализ, методология, проведение исследования, подготовка черновика рукописи. И. Р. Ильясов – методология, формальный анализ, проведение исследования, рецензирование и редактирование. В. В. Оличева – проведение исследования, визуализация, рецензирование и редактирование. А. К. Жевлакова – рецензирование методологии и редактирование. В. Л. Белобородов – рецензирование методологии и редактирование. И. А. Селиванова – концептуализация, руководство, рецензирование и редактирование.

Финансирование. Данное исследование было профинансировано Российским научным фондом, грант № 25-25-00537, https://rscf.ru/en/project/25-25-00537/

Для цитирования: Воронин К. С., Ильясов И. Р., Оличева В. В., Жевлакова А. К., Белобородов В. Л., Селиванова И. А. Количественное определение компонентов полифенольных композиций посредством измерения кинетики накопления радикал-катионов ABTS$^{\bullet+}$. *Разработка и регистрация лекарственных средств*. 2026;15(2):37–42. https://doi.org/10.33380/2305-2066-2026-15-2-2120

INTRODUCTION

In recent decades, the role of polyphenolic compounds in the pharmaceutical, cosmetic, and food industries has attracted significant attention [1–3], and they are considered dietary supplements and promising therapeutic agents [4, 5]. Polyphenols are widely distributed in plants and all possess an aromatic ring with hydroxyl groups, but their structure can vary significantly. They are commonly divided into several classes such as flavonoids, lignans, phenolic acids, stilbenes, etc. [6, 7]. The potential health benefits of polyphenols are often associated with their high antioxidant activity [8, 9]. There are several well-known methods for the assessment of antioxidant capacity in vitro such as ABTS/PP, DPPH and others [10, 11]. They are inexpensive, easy to apply and give fast, reproducible results. However, these methods are not widely used for polyphenolic content determination. In this paper, we report a novel methodology for the quantitative determination of polyphenols by using antioxidant capacity measurement approach.

Several colorimetric methods are often employed to study antioxidant plant extracts and food materials such as total phenolic content determination by Folin – Ciocalteu method [12, 13] or total flavonoid content measurement with aluminium(III) chloride solution [14, 15]. However, these approaches do not allow us to determine the content of individual polyphenols in such complex mixtures, different HPLC techniques are typically used for their identification and quantification [16]. The main challenge in applying antioxidant capacity measurements for polyphenolic compounds quantification in complex mixtures lies in obtaining data for components without prior separation. We hypothesized that individual compounds content can be selectively calculated in polyphenolic formulations due to their different kinetics of interaction with $ABTS^{\bullet+}$ radical cations. We focused on binary polyphenolic combinations in this study. Our goal was to develop an approach for the quantitative determination of individual polyphenols in binary combinations through the measurement of $ABTS^{\bullet+}$ radical cations accumulation kinetics based on a lag-time experimental strategy.

MATERIALS AND METHODS

Materials

We have studied two binary polyphenolic formulations: manually derived combination of alpha-tocopherol (α-TOH) and dihydroquercetin (DHQ), as well as a polyphenolic extract of larch knotwood containing secoisolariciresinol (SECO) and dihydroquercetin (Figure 1).

Dihydroquercetin (State Standard № 10766-2016, Ametis JSC, Russia), alpha-tocopherol (95 %, Acros organics BVBA, Belgium), secoisolariciresinol (Sigma-Aldrich, USA, 95.0 %), its combinations and polyphenolic extract of knotwood of *Larix dahurica* Turcz. (Ametis JSC, Russia) samples were dissolved in ethanol (99.9 %, Merck KGaA, Germany) – deionized water (95 : 5) before the spectrophotometric study. Potassium persulfate (99 %, PP, Sigma-Aldrich, USA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (98 %, ABTS, Sigma-Aldrich, USA). The components of phosphate buffer solution (PBS) were: potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride – Sigma-Aldrich.

Spectrophotometric assay

This approach is based on the ABTS/PP antioxidant capacity assessment method [17,18]. Spectrophotometric measurements were performed on a Cary 100 spect-

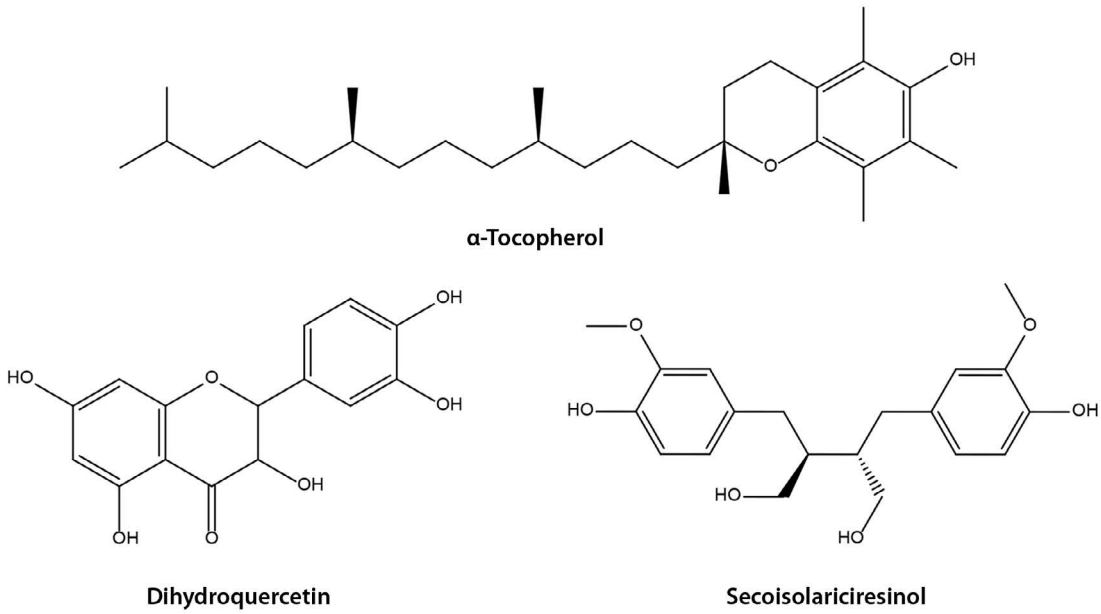

Figure 1. Chemical structure of studied compounds

rophotometer (Varian, USA) with a 1 cm path length Peltier temperature-controlled cuvette.

To record $ABTS^{\bullet+}$ radical cations formation, 10–50 μL of polyphenolic samples solution was added to ABTS dissolved in PBS (pH 7.4). To initiate the reaction, PP solution in PBS was applied with subsequent shaking for 15 s. The final concentrations of ABTS and PP were 1.21 mM and 0.43 mM, respectively. The kinetics of the $ABTS^{\bullet+}$ radical cations accumulation was monitored at maxima of 730–750 nm with an incubation temperature of 24 ± 1 °C until $ABTS^{\bullet+}$ radical cations accumulation reached a steady state. In the absence of antioxidants, the accumulation of $ABTS^{\bullet+}$ radical cations began immediately upon the addition of PP. However, when an antioxidant was introduced, a lag-time was typically observed before the accumulation of $ABTS^{\bullet+}$ radical cations commenced.

Calculations

We used the following equations to quantify concentrations of DHQ – α-TOH combination constituents (1) and content of polyphenolic larch knotwood extract components (2):

$$C_x = \frac{tx}{ts} \cdot C_{st}, \quad (1)$$

where C_x is concentration of an individual component in testing combination solution (M), C_{st} is concentration of an standard solution (M), *tx* is component lag-time duration in testing combination (min), *ts* is lag-time duration registered for standard solution (min).

$$X = \frac{tx}{ts} \cdot C_{st} \cdot \frac{Vx}{mx} \cdot 100\ \%, \quad (2)$$

where *X* is the content of individual component of extract (%), C_{st} is the concentration of DHQ or SECO in the standard sample (mg/mL), *tx* is component lag-time duration in extract (min), *ts* is lag-time duration registered for standard solution (min), *Vx* is a volume of the tested extract solution (mL), *mx* is a mass of extract sample (mg).

RESULTS AND DISCUSSION

The lag-time spectrophotometric assay is based on recording the kinetic curves of $ABTS^{\bullet+}$ radical cations accumulation, initiated by potassium persulfate. The presence of an antioxidant results in $ABTS^{\bullet+}$ radical cations accumulation delay, lag-time duration depends on the antioxidant concentration. There are interactions typical for binary combinations of antioxidants potentially affecting the shape of kinetic curves: additive, synergistic, and antagonistic interactions. While synergistic and antagonistic effects refer to difference between the combination antioxidant effect and the total effect of individual components, the additive effect is equivalent to the sum of the effects of the individual components [19]. For additive type combinations, the formation of two lag-time periods of $ABTS^{\bullet+}$ radical cations accumulation is possible. This phenomenon was observed in a DHQ and α-TOH combination study (Figure 2).

Initially, we have tested two DHQ – α-TOH combinations with different component ratios – 1:1 and 1:6, both exhibited two lag-time periods: 4.54 min (τ_1) and 6.68 min (τ_2) for 1:1 ratio combination (Figure 2, A);

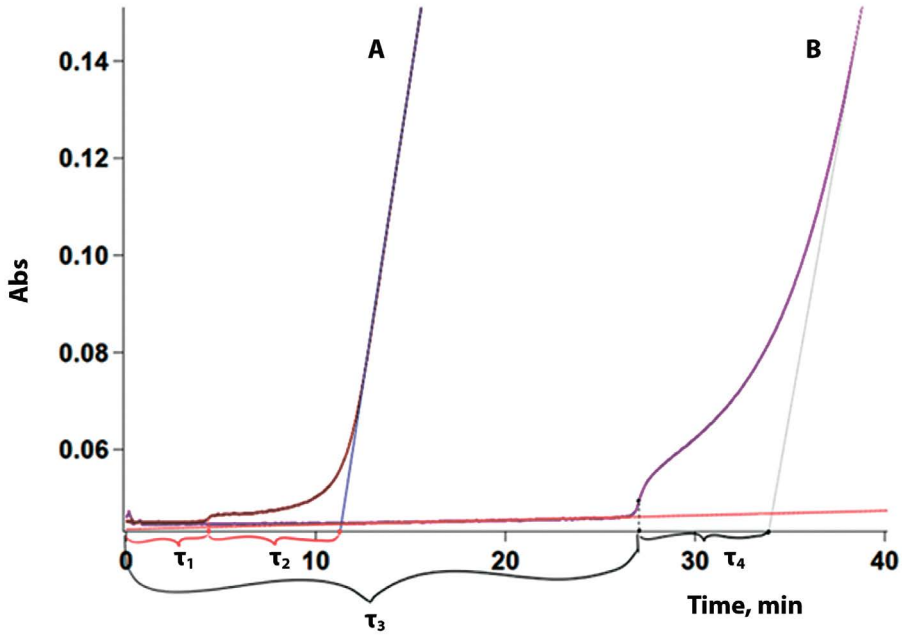

Figure 2. Kinetic curves of DHQ – α-TOH combinations:
A – molar ratio 1:1 (both 6 μM), τ_1, τ_2 – lag-time periods; B – molar ratio 1:6 (6 μM of DHQ and 36 μM of α-TOH), τ_3, τ_4 – lag-time periods

28.10 min (τ_3) and 6.43 min (τ_4) for 1:6 ratio combination (Figure 2, B). By comparing the lag-time durations of combinations with different component ratio, we can associate each lag-time period with a specific compound: τ_1 value is 6.1 times lower than τ_3, which correlates with the sixfold decrease in α-TOH concentration; τ_2 and τ_4 values are close to each other – DHQ concentration is the same in both combinations.

We propose that matching lag-time periods with components of combination makes possible their quantification using standard solutions of individual components. The content of a component was calculated using equation 1 (Materials and methods). To evaluate this procedure we performed a quantification assay of model DHQ – α-TOH combination (1:5 ratio) with known concentrations of components. The mean concentrations of components after six independent measurements (*C*), relative standard deviation (RSD) and the relative measurement error (δ) were calculated (Table 1). This technique demonstrated a high level of reproducibility with a relative measurement error not exceeding 3.6 %.

Table 1. Content determination of DHQ – α-TOH combination constituents

Component name	μ, μM	C, μM	RSD, %	δ, %
DHQ	10.0	9.7	2.0	3.0
α-TOH	50.0	51.8	1.8	3.6

Note. μ – value of concentration was taken as a true one.

To investigate the applicability of this methodology for studying natural products, a polyphenolic extract of larch knotwood with different contents of components were tested. They consist of two major polyphenols DHQ and SECO and are considered prospective antioxidant and antiproliferative pharmaceutical substances [20]. Model compositions of DHQ and SECO have shown kinetic curves with two lag-time periods formation (Figure 3). Testing combinations with the same DHQ (2 mg/mL) and different SECO (2 mg/mL and 3 mg/mL) revealed that the first lag-time period (τ_1) is the same for both combinations and the latter ones (τ_2 and τ_3) becomes longer for combination with higher SECO concentration. Thus, τ_1 could be attributed to DHQ and τ_2 and τ_3 – to SECO. The calculations of DHQ and SECO content were performed the same way as for DHQ – α-TOH combinations with minor modifications using equation 2 (Materials and methods).

Previously, polyphenolic extracts of larch knotwood were analyzed using a validated HPLC technique, and the obtained data on DHQ and SECO content were taken as true values (μ) in this assay [21]. The mean content ($n = 6$) of DHQ and SECO (*X*), relative standard deviation (RSD) and relative measurement error (δ) were calculated (Table 2).

Table 2. Content determination of DHQ – SECO combinations constituents

Extract	Component name	μ, %	*X*, %	RSD, %	δ, %
1	DHQ	59.9	60.8	1.4	1.5
	SECO	22.4	23.2	1.2	3.6
2	DHQ	58.8	59.6	1.4	1.4
	SECO	13.6	14.1	1.2	3.7

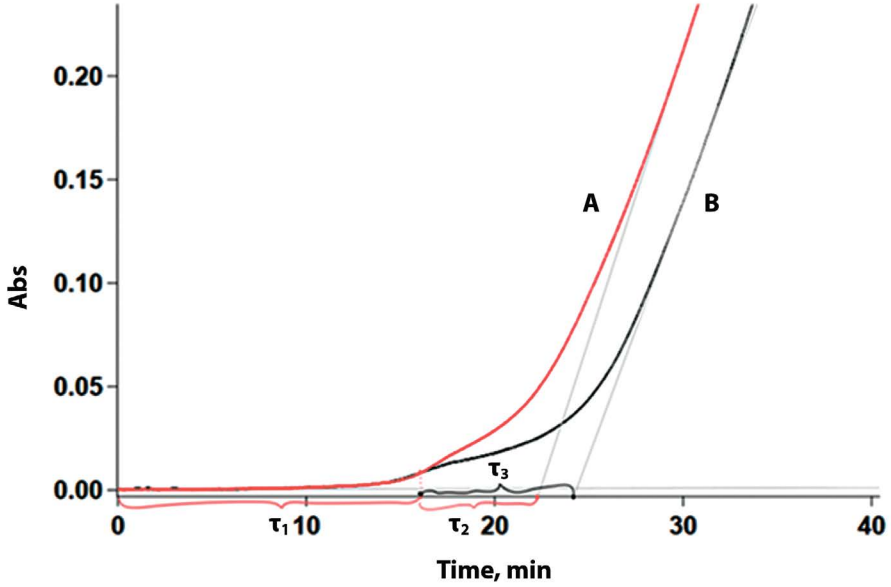

Figure 3. Kinetic curves of DHQ – SECO formulations:
A – 2 mg/mL DHQ, 2 mg/mL SECO; B – 2 mg/mL DHQ, 3 mg/mL SECO

The content calculations demonstrate the high reproducibility of the developed technique and yield results comparable to HPLC data results.

CONCLUSION

This is the first report of successfully applying an antioxidant capacity measurement approach for the quantification of individual constituents of polyphenolic compositions. Our study confirmed that the methodology based on the ABTS lag-time assay could be effectively implemented for determining the content of components in binary combinations. The quantification analysis of DHQ – α-TOH and DHQ – SECO combinations using this technique demonstrated high reproducibility (with RSD not exceeding 2 %) and acceptable accuracy levels (with δ not exceeding 3.7 %). As a result of applying the developed methodology, a patent was granted for the technique of analyzing polyphenolic compositions. We believe that our findings provide a foundation for future evaluations of this methodology, potentially extending its application to more complex combinations of polyphenols and a broader range of substances.

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