ANTIOXIDANT CAPACITY OF (KLAKLAK)$_2$ BIOCONJUGATES ASSESSED BY THE ELECTRON-TRANSFER METHODS FRAP AND CUPRAC

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ABSTRACT

Oxidative stress and metal-driven redox processes are key contributors to the pathogenesis of chronic diseases and cancer, motivating the search for novel antioxidant molecules. In this study, the antioxidant potential of a series of synthetic peptides previously reported to possess antitumor and antibacterial properties was evaluated using two complementary electron-transfer assays: ferric reducing antioxidant power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC). Both assays were calibrated against caffeic acid, and results were expressed as caffeic acid equivalents (CAE).

The FRAP assay revealed substantial differences in reducing activity, with Si_8 exhibiting the highest value (0.558 ± 0.132), followed by Si_{12} (0.478 ± 0.0240), Si_{10} (0.293 ± 0.0220), and Si_{15} (0.250 ± 0.0200), whereas Si_1 (0.00439 ± 0.00240) and Si_{11} (0.00260 ± 0.000500) showed negligible responses. A comparable pattern was observed in the CUPRAC assay, where Si_8 again displayed the strongest reducing capacity (0.381 ± 0.0948), with Si_{12} (0.290 ± 0.0225), Si_{15} (0.262 ± 0.0223), and Si_{10} (0.224 ± 0.0290) also demonstrating appreciable activity, while Si_1 (0.001800 ± 0.000400) and Si_{11} (0.0132 ± 0.000500) remained inactive.

The combined application of FRAP and CUPRAC provided complementary and reproducible measures of peptide antioxidant capacity, establishing a framework for systematic characterization of redox-active peptides in relation to oxidative stress.

Keywords: antioxidant peptides, caffeic acid, FRAP, CUPRAC.

INTRODUCTION

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant defence systems, is a major contributor to cellular damage and the progression of chronic diseases (such as Diabetes, Cardiovascular diseases (CVDs), Autoimmune diseases, etc.) and cancer [1 - 6]. Transition metals such as iron and copper catalyse the conversion of hydrogen peroxide and superoxide into highly reactive hydroxyl radicals through Fenton and Haber-Weiss reactions, which represent key pathways in the generation of oxidative stress [7 - 9]. These reactions are fundamental to metal-driven oxidative stress and provide the rationale for evaluating the capacity of bioactive molecules to act as reducing agents or modulators of redox-active metals [10].

A wide variety of methods has been developed to evaluate antioxidant activity, ranging from radical-

scavenging assays to metal-centered reduction tests [11 - 15]. Among these, electron-transfer (ET) spectrophotometric assays are particularly prominent owing to their reproducibility and broad applicability [16]. Two of the best-established assays in this category are the Ferric Reducing Antioxidant Power (FRAP) and the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assays.

The FRAP assay, introduced by Benzie and Strain in 1996, quantifies the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions in the presence of 2,4,6-tripyridyl-*s*-triazine (TPTZ), yielding an intense blue Fe^{2+}-TPTZ complex with a maximal absorbance at 593 nm [17]. Since its development, FRAP has been widely applied to biological fluids, food matrices, plant extracts, and pure compounds, and has become one of the most standardized and reproducible tools for assessing total reducing capacity [18 - 22].

The CUPRAC assay was developed by Apak and colleagues in 2004 [23]. The principle of the assay involves the reduction of Cu^{2+} to Cu^{+} by antioxidants, followed by the formation of a stable yellow–orange complex with the chromogenic ligand neocuproine, which can be measured spectrophotometrically at 450 nm. CUPRAC has since been validated for a wide range of antioxidants, including dietary polyphenols, vitamins, proteins, and food extracts, and is recognized as one of the most versatile assays for antioxidant capacity [24 - 27]. FRAP and CUPRAC are complementary methods, as they differ in both the redox system assessed and the experimental conditions. FRAP reflects ferric ion reduction in acidic medium, while CUPRAC captures cupric ion reduction at near-neutral pH [24 - 26, 28].

Both FRAP and CUPRAC quantify reducing capacity by measuring the ability of analytes to donate electrons to metal complexes. Although they do not directly determine metal chelation in the classical sense [29, 30], their outcomes are mechanistically linked to interactions between antioxidants and transition metals and can therefore be interpreted as indicators of the potential of molecules to modulate metal-driven oxidative processes [10, 28, 31]. Their successful use across diverse systems-including plant tissues, protein hydrolysates, and food matrices-demonstrates robustness and broad applicability [19, 21, 27, 31].

To facilitate comparability across structurally diverse samples, results from FRAP and CUPRAC are frequently expressed as caffeic acid equivalents (CAE). Caffeic acid, a hydroxycinnamic acid derivative, possesses well-documented radical-scavenging, reducing, and metal-interacting properties attributable to its ortho-dihydroxy substitution [32 - 34]. Its stability and dual functionality have made it a relevant natural calibration standard in antioxidant assays [24 - 26, 28]. Expressing activity as CAE provides a standardized quantitative framework that enables direct comparison of different analytes on a common reference scale.

Concurrently, growing attention has focused on antioxidant peptides, which are increasingly recognized as promising biomolecules for biomedical and nutritional applications [35 - 40]. Reviews synthesize advances in screening strategies, evaluation models, molecular mechanisms, stability, and bioavailability [35 - 38], while experimental studies confirm measurable antioxidant effects of peptide extracts from varied sources, often linked to specific sequences and structural modifications [39, 40].

Within this context, synthetic peptides with reported antitumor activity, including analogues of the $(KLAKLAK)_2$-NH_2 sequence, represent a timely class of molecules for study. Modified derivatives incorporating unnatural amino acids or secondary pharmacophores have demonstrated cytotoxic and antimicrobial properties [41 - 43]. Because oxidative stress is closely linked to tumor progression as well as to general cellular damage, evaluation of their antioxidant potential provides complementary insight into their biological relevance. Beyond these primary functions, their antioxidant capacity warrants systematic assessment. Since most of the analogues under investigation incorporate caffeic acid residues, analysing them with FRAP and CUPRAC and expressing the results as caffeic acid equivalents offers a logical and standardized framework for characterizing their reducing properties against a well-established natural reference.

EXPERIMENTAL

Materials

Absolute ethanol (≥ 99.8 % v/v), ammonium acetate (p.a.), sodium acetate trihydrate, hydrochloric acid and glacial acetic acid were purchased from Valerus Ltd. (Sofia, Bulgaria). Caffeic acid (CAS № 331-39-5), copper(II) chloride (CAS № 7447-39-4), neocuproine

(CAS № 484-11-7), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, CAS № 3682-35-7) and iron(III) chloride hexahydrate (CAS № 10025-77-1) were obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade and were used without further purification. Solutions were freshly prepared before each analysis.

The peptide samples investigated in this study were Si_1, Si_8, Si_{10}, Si_{11}, Si_{12}, and Si_{15}, and their general structures are presented in Table 1. The synthesis of these analogues was described previously by Jaber et al. [41 - 43]. For each experiment, stock solutions were prepared immediately before use and diluted to the required working concentrations.

Methods

FRAP method

The ferric reducing antioxidant power (FRAP) assay was performed following the procedure of Benzie and Strain with slight modifications [17]. Acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM in 40 mM HCl) and $FeCl_3 \cdot 6H_2O$ solution (20 mM in distilled water) were mixed in a volume ratio of 10:1:1 and the mixture was equilibrated at 37°C for 15 min before use. For each determination 50 µL of sample or caffeic acid standard solution was added to 1.5 mL of FRAP reagent in disposable polystyrene cuvettes with a 3 mL capacity and 10 mm optical path length. Measurements were performed using a T70 UV/Vis spectrophotometer (PG Instruments Ltd). The final volume in the cuvette was 1.55 mL. A reagent blank was prepared with solvent instead of sample. The mixtures were incubated at 37°C for 4 min and absorbance was recorded at 593 nm.

CUPRAC method

The cupric reducing antioxidant capacity (CUPRAC) assay was carried out according to the method of Apak et al. [23, 24, 26], with minor methodological adjustments [44]. The CUPRAC reagent was prepared by mixing equal volumes of $CuCl_2$ solution (10 mM), neocuproine solution (7.5 mM in ethanol), and ammonium acetate buffer (1 M, pH 7.0). For each measurement 1.5 mL of CUPRAC reagent and 0.5 mL of distilled water were placed in a disposable polystyrene cuvette with a 3 mL capacity and 10 mm optical path length, followed by the addition of 50 µL of sample or standard solution to reach a final volume of 2.05 mL. A reagent blank was prepared in parallel. The mixtures were incubated at room temperature (25 ± 2°C) in the dark for 30 min and absorbance was measured at 450 nm using the same spectrophotometer.

Data processing

All determinations were performed in triplicate. Calibration curves were constructed with caffeic acid as a reference antioxidant, and linear regression equations were used for quantification. The analytical signal was expressed as presented in Eq. (1):

$$\Delta A = A_s - A_c \quad (1)$$

where A_s is the absorbance of the tested sample and A_c is the absorbance of the control solution (reagent mixture without antioxidant).

The antioxidant capacity was first expressed as CAE (µM) according to the Eq. (2):

$$CAE = \frac{\Delta A}{a}, \mu M \quad (2)$$

where a is the slope of the calibration curve obtained for caffeic acid.

To normalize for peptide concentration and provide a measure of intrinsic reducing capacity, FRAP/CUPRAC specific activity (SA) was calculated as presented in Eq. (3):

Table 1. General structures of the investigated peptides.

Code	Peptide structures	Code	Peptide structures
Si_1 (parent peptide)	$(KLAKLAK)_2$-NH_2	Si_{11}	Caf-KLβAKLβAK-NH_2
Si_8	Caf-KLAKLAK-NH_2	Si_{12}	Caf-(KLβ-AKLβ-AK)$_2$-NH_2
Si_{10}	Caf-$(KLAKLAK)_2$-NH_2	Si_{15}	Caf-KnLAKnLAK-NH_2

$$SA = \frac{CAE}{C_{cuvette}} = \frac{\Delta A}{a \times C_{cuvette}} \quad (3)$$

where $C_{cuvette}$ is the effective peptide concentration in the cuvette (μM). This parameter represents the number of caffeic acid equivalents corresponding to the reducing capacity of 1 μM peptide.

RESULTS AND DISCUSSION

FRAP Method

The ferric reducing antioxidant power (FRAP) assay was applied to determine the ferric-ion reducing capacity of the investigated peptides relative to caffeic acid (CA) as calibration standard. Calibration with CA was linear within the tested range (0.900 - 9.85 μM), yielding a slope *a* of 0.0568 Abs·μM^{-1} and a determination coefficient of R^2 = 0.997. The analytical signal was expressed as the absorbance difference (ΔAbs) according to Eq. (1), and CAE values were calculated from the calibration slope following Eq. (2).

Table 2 summarizes the experimental data, including peptide concentration in the cuvette, the corresponding absorbance change, and the calculated values expressed as CAE.

To allow direct comparison of their reducing capacities, the results were normalized to peptide concentration using Eq. (3). The SA_{FRAP} values are presented in Fig. 1.

As shown in Fig. 1, FRAP revealed substantial variability in the SA values of the peptides. Si_8 (0.558 ± 0.132) and Si_{12} (0.478 ± 0.0240) emerged as the most active peptides, followed by Si_{10} (0.293 ± 0.0220) and Si_{15} (0.250 ± 0.0200). In contrast, Si_1 (0.00439 ± 0.00240) and Si_{11} (0.00260 ± 0.000500) showed negligible activity under the assay conditions.

Overall, these results indicate that only a subset of the peptides demonstrate pronounced ferric ion reducing power under the conditions of the FRAP assay, while others contribute minimally to overall activity. The substantial variation across samples underscores the assay's ability to differentiate peptides with strong responses from those with little or no measurable effect.

This outcome is in line with previous reports demonstrating that caffeic acid itself is a potent ferric-reducing antioxidant. In comparative FRAP assays, caffeic acid generated values around 476 μM Fe^{2+} equivalents at 100 μM concentration, ranking among the most active hydroxycinnamic acids together with gallic and rosmarinic acids [34, 45].

Table 2. FRAP assay data for the investigated peptides expressed as CAE, μM.

Code	$C_{cuvette}$, μM	ΔAbs	CAE, μM
Si_1	645	0.0992	1.75
	161	0.0300	0.528
	32.3	0.0132	0.232
Si_8	32.3	0.876	15.4
	16.1	0.445	7.83
	6.45	0.261	4.59
Si_{10}	32.3	0.496	8.73
	19.4	0.322	5.67
	12.9	0.231	4.06
Si_{11}	645	0.113	1.99
	322	0.050	0.872
	161	0.0183	0.323
Si_{12}	32.3	0.894	15.7
	25.8	0.660	11.6
	19.4	0.546	9.60
Si_{15}	64.5	0.951	16.7
	48.4	0.717	12.6
	42.0	0.524	9.22
	32.3	0.473	8.32

CUPRAC method

The reducing capacity of the peptides was evaluated using the CUPRAC assay with caffeic acid (CA) as

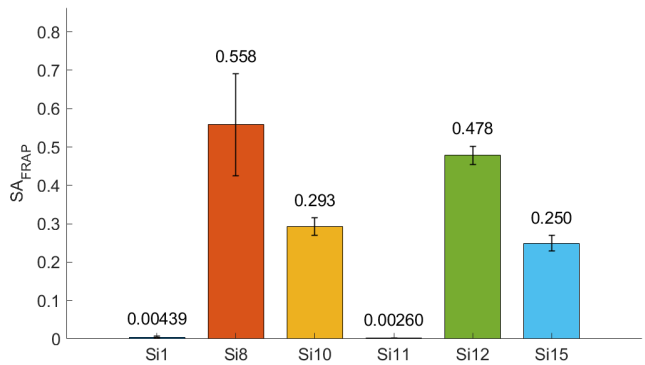

Fig. 1. SA profiles of the investigated peptides obtained by the FRAP assay.

reference standard. Calibration of CA was linear within the tested range (1.49 - 13.54 µM), with a slope *a* of 0.0622 Abs·µM^{-1} and a determination coefficient of R^2 = 0.9974. The analytical signal was expressed as the absorbance difference ΔAbs (Eq. (1)). These calibration parameters were then applied to calculate CAE for all peptide samples (Eq. (2)).

Table 3 presents the experimental data for the investigated peptides, including their effective concentration in the cuvette ($C_{cuvette}$), the measured absorbance difference (ΔAbs), and the corresponding CAE values.

After calculation of caffeic acid equivalents, the results were normalized to peptide concentration in the cuvette to obtain the parameter SA_{CUPRAC} (Eq. (3)), which represents the SA of a peptide at 1 µM, expressed relative to caffeic acid. The mean values with standard deviations are presented in Fig. 2.

Fig. 2 illustrates the differences in reducing capacities detected by the CUPRAC assay. The highest SA was observed for Si_8 (0.381 ± 0.0948), followed by Si_{12} (0.290 ± 0.0225) and Si_{15} (0.262 ± 0.0223), which exhibited similarly high values. Si_{10} displayed intermediate activity (0.224 ± 0.0290), whereas Si_{11} showed only minimal activity (0.0132 ± 0.000500). As expected, the parent peptide Si_1, which lacks caffeic acid residues, demonstrated negligible activity (0.001800 ± 0.000400).

These results are consistent with an enhanced electron-transfer capacity in peptides containing caffeic acid residues. The negligible activity of Si_1 further supports that the peptide backbone alone contributes little to the overall reducing power. These findings are consistent with previous studies that directly evaluated phenolic standards, including caffeic acid, and confirmed their strong reducing power in the CUPRAC assay [24, 25].

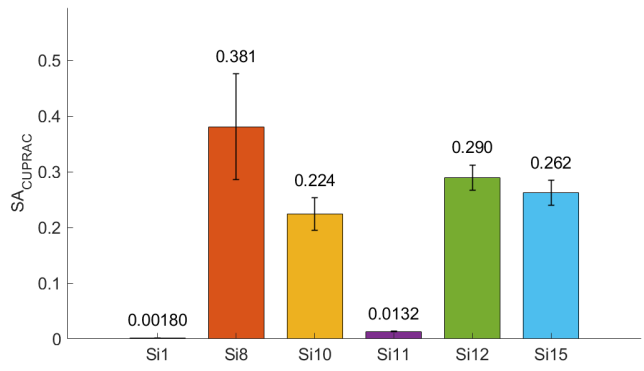

Fig. 2. SA values of peptides assessed by the CUPRAC method.

Table 3. CUPRAC assay data for the investigated peptides expressed as CAE, µM.

Code	$C_{cuvette}$, µM	ΔAbs	CAE, µM
Si_1	488	0.0660	1.06
	244	0.0284	0.457
	122	0.0102	0.164
Si_8	24.4	0.458	7.37
	9.76	0.215	3.46
	4.88	0.148	2.37
Si_{10}	48.8	0.591	9.51
	24.4	0.353	5.67
	14.6	0.190	3.06
	9.76	0.158	2.55
Si_{11}	488	0.401	6.44
	244	0.193	3.11
	122	0.104	1.68
Si_{12}	36.6	0.717	11.5
	19.5	0.361	5.81
	14.6	0.259	4.16
	9.76	0.159	2.55
Si_{15}	24.4	0.385	6.19
	19.5	0.349	5.61
	14.6	0.223	3.59

These observations highlight the usefulness of combining complementary assays for assessing antioxidant potential. Future studies should expand the evaluation to larger sets of peptides and explore additional antioxidant assays to confirm and extend the present findings.

CONCLUSIONS

This study provided a comparative evaluation of the antioxidant properties of synthetic peptides using two complementary electron-transfer assays, FRAP and CUPRAC, with results expressed as caffeic acid equivalents. Both assays revealed clear variability in reducing activity among the peptides: Si_8 emerged as the most active, whereas Si_1 and Si_{11} showed negligible responses.

The combined use of FRAP and CUPRAC effectively differentiated the reducing capacities of

the peptides under distinct redox conditions, offering a broader perspective than a single assay alone. By identifying both highly active and inactive analogues within the same framework, the results highlight the importance of caffeic acid residues in enhancing electron-transfer capacity.

Overall, this dual-assay strategy provides a reliable basis for characterizing antioxidant peptides and can be applied to broader peptide libraries. Future studies should extend the approach to larger sets of analogues, incorporate additional antioxidant assays, and investigate structure–activity relationships to advance the understanding of the determinants governing peptide redox performance.

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REFERENCES

1. N. Chandimali, S.G. Bak, E.H. Park, H.-J. Lim, Y.-S. Won, E.-K. Kim, S.-I. Park, S.J. Lee, Free radicals and their impact on health and antioxidant defenses: a review, Cell Death Discovery, 11, 1, 2025, 19. https://doi.org/10.1038/s41420-024-02278-8
2. E. Di Carlo, C. Sorrentino, Oxidative stress and age-related tumors, Antioxidants, 13, 9, 2024, 1109. https://doi.org/10.3390/antiox13091109
3. S.G. Tumilaar, A. Hardianto, H. Dohi, D. Kurnia, A comprehensive review of free radicals, oxidative stress, and antioxidants: overview, clinical applications, global perspectives, future directions, and mechanisms of antioxidant activity of flavonoid compounds, Journal of Chemistry, 2024, 1-21. https://doi.org/10.1155/2024/5594386
4. K. Li, Z. Deng, C. Lei, X. Ding, J. Li, C. Wang, The role of oxidative stress in tumorigenesis and progression, Cells, 13, 5, 2024, 441. https://doi.org/10.3390/cells13050441
5. N. Ghadiri, M. Javidan, S. Sheikhi, Ö. Taştan, A. Parodi, Z. Liao, M. Tayybi Azar, M. Ganjalıkhani-Hakemi, Bioactive peptides: an alternative therapeutic approach for cancer management, Frontiers in Immunology, 15, 2024, 1310443. https://doi.org/10.3389/fimmu.2024.1310443
6. A. Gholamian, H. MohebAlian, M. Heidarpour, Assessment of oxidative potential in culture medium conditioned by MCF7 cancer cells via FRAP and MDA assay, IJBMI, 2, 1, 2025, 1-8. https://doi.org/10.22034/ijbmi.2025.222384.
7. S. Puntarulo, Iron, oxidative stress and human health, Molecular Aspects of Medicine, 26, 4-5, 2005, 299-312. https://doi.org/10.1016/j.mam.2005.07.001
8. E. Birben, U.M. Sahiner, C. Sackesen, S. Erzurum, O. Kalayci, Oxidative stress and antioxidant defense, World Allergy Organization Journal, 5, 1, 2012, 9-19. https://doi.org/10.1097/WOX.0b013e3182439613
9. J.M.C. Gutteridge, B. Halliwell, Free radicals in biology and medicine, Oxford University Press, 2015. ISBN 9780191802133
10. İ. Gulcin, S. H. Alwasel, Metal ions, metal chelators and metal chelating assay as antioxidant method, Processes, 10, 1, 2022, 132. https://doi.org/10.3390/pr10010132
11. A. Shalaby, S.M.M. Shanab, Antioxidant compounds, assays of determination and mode of action, African Journal of Pharmacy and Pharmacology, 7, 10, 2013, 528-539. https://doi.org/10.5897/AJPP2013.3474
12. R. Apak, S. Gorinstein, V. Böhm, K. M. Schaich, M. Özyürek, K. Güçlü, Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report), Pure and Applied Chemistry, 85, 5, 2013, 957-998. https://doi.org/10.1351/PAC-REP-12-07-15
13. I.G. Munteanu, C. Apetrei, Analytical methods used in determining antioxidant activity: a review, International Journal of Molecular Sciences, 22, 7, 2021, 3380. https://doi.org/10.3390/ijms22073380
14. B. Xu, Q. Dong, C. Yu, H. Chen, Y. Zhao, B. Zhang, P. Yu, M. Chen, Advances in research on the activity evaluation, mechanism and structure-activity relationships of natural antioxidant

peptides, Antioxidants, 13, 4, 2024, 479. https://doi.org/10.3390/antiox13040479
15. O. Ishola, I.E. Ojo, E.T. Babatunde, T.I. Aanuoluwa, T.O. Fabiyi, A.A. Adeyanju, Determination of antioxidant capacity in aqueous extracts of Corymbia citriodora using DPPH, ABTS, FRAP, TPC, and hydrogen peroxide assays, Asian Journal of Research in Biochemistry, 15, 3, 2025, 171-178. https://doi.org/10.9734/ajrb/2025/v15i3398
16. M.N. Alam, N.J. Bristi, M. Rafiquzzaman, Review on in vivo and in vitro methods evaluation of antioxidant activity, Saudi Pharmaceutical Journal, 21, 2, 2013, 143-152. https://doi.org/10.1016/j.jsps.2012.05.002
17. I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay, Analytical Biochemistry, 239, 1, 1996, 70-76. https://doi.org/10.1006/abio.1996.0292
18. A.R. Gohari, H. Hajimehdipoor, S. Saeidnia, Y. Ajani, A. Hadjiakhoondi, Antioxidant Activity of some Medicinal Species using FRAP Assay, Journal of Medicinal Plants, 10, 37, 2011.
19. J. Carrasco-Castilla, A.J. Hernández-Álvarez, C. Jiménez-Martínez, C. Jacinto-Hernández, M. Alaiz, J. Girón-Calle, J. Vioque, G. Dávila-Ortiz, Antioxidant and metal chelating activities of *Phaseolus vulgaris* L. var. Jamapa protein isolates, phaseolin and lectin hydrolysates, Food Chemistry, 131, 4, 2012, 1157-1164. https://doi.org/10.1016/j.foodchem.2011.09.084
20. R. Sánchez-Vioque, M. F. Rodríguez-Conde, J. V. Reina-Ureña, M. A. Escolano-Tercero, D. Herraiz-Peñalver, O. Santana-Méridas, In vitro antioxidant and metal chelating properties of corm, tepal and leaf from saffron (Crocus sativus L.), Industrial Crops and Products, 39, 2012, 149-153. https://doi.org/10.1016/j.indcrop.2012.02.028
21. N. Liang, D. Kitts, Antioxidant property of coffee components: assessment of methods that define mechanisms of action, Molecules, 19, 11, 2014, 19180-19208. https://doi.org/10.3390/molecules191119180
22. I.F.F. Benzie, M. Devaki, The ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: concepts, procedures, limitations and applications, Measurement of Antioxidant Activity & Capacity, John Wiley & Sons, Chichester, UK, 2017, 77-106. https://doi.org/10.1002/9781119135388.ch5
23. R. Apak, K. Güçlü, M. Özyürek, S.E. Karademir, Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method, J. Agric. Food Chem., 52, 26, 2004, 7970-7981. https://doi.org/10.1021/jf048741x
24. R. Apak, K. Güçlü, B. Demirata, M. Özyürek, S.E. Çelik, B. Bektaşoğlu, K.I. Berker, D. Özyurt, Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay, Molecules, 12, 7, 2007, 1496-1547. https://doi.org/10.3390/12071496
25. M. Özyürek, K. Güçlü, E. Tütem, K.S. Başkan, E. Erçağ, S. Esin Çelik, S. Baki, L. Yıldız, Ş. Karaman, R. Apak, A comprehensive review of CUPRAC methodology, Analytical Methods, 3, 11, 2011, 2439. https://doi.org/10.1039/c1ay05320e
26. R. Apak, K. Güçlü, M. Özyürek, B. Bektas¸oğlu, M. Bener, Cupric ion reducing antioxidant capacity assay for food antioxidants: vitamins, polyphenolics, and flavonoids in food extracts, in: Advanced Protocols in Oxidative Stress I, Humana Press, Totowa, NJ, 477, 2008, 163-193. https://doi.org/10.1007/978-1-60327-517-0_14
27. S. D. Çekiç, K. S. Başkan, E. Tütem, R. Apak, Modified cupric reducing antioxidant capacity (CUPRAC) assay for measuring the antioxidant capacities of thiol-containing proteins in admixture with polyphenols, Talanta, 79, 2, 2009, 344-351. https://doi.org/10.1016/j.talanta.2009.03.061
28. H. Liu, J. Cao, W. Jiang, Evaluation and comparison of vitamin C, phenolic compounds, antioxidant properties and metal chelating activity of pulp and peel from selected peach cultivars, LWT - Food Science and Technology, 63, 2, 2015, 1042-1048. https://doi.org/10.1016/j.lwt.2015.04.052
29. J.S. Santos, V.R. Alvarenga Brizola, D. Granato, High-throughput assay comparison and standardization for metal chelating capacity screening: a proposal and application, Food Chemistry, 214, 2017, 515-522. https://doi.org/10.1016/j.foodchem.2016.07.091
30. G.L. Smith, A.A. Reutovich, A.K. Srivastava, R.E. Reichard, C.H. Welsh, A. Melman, F. Bou-Abdallah, Complexation of ferrous ions by ferrozine, 2,2′-bipyridine and 1,10-phenanthroline: implication

for the quantification of iron in biological systems, Journal of Inorganic Biochemistry, 220, 2021, 111460. https://doi.org/10.1016/j.jinorgbio.2021.111460

31. I. Gulcin, Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid), Toxicology, 217, 2-3, 2006, 213-220. https://doi.org/10.1016/j.tox.2005.09.011
32. A. Stasiłowicz-Krzemień, N. Rosiak, A. Miklaszewski, J. Cielecka-Piontek, Screening of the anti-neurodegenerative activity of caffeic acid after introduction into inorganic metal delivery systems to increase its solubility as the result of a mechanosynthetic approach, International Journal of Molecular Sciences, 24, 11, 2023, 9218. https://doi.org/10.3390/ijms24119218
33. D. Skroza, V. Šimat, L. Vrdoljak, N. Jolić, A. Skelin, M. Čagalj, R. Frleta, I. Generalić Mekinić, Investigation of antioxidant synergisms and antagonisms among phenolic acids in the model matrices using FRAP and ORAC methods, Antioxidants, 11, 9, 2022, 1784. https://doi.org/10.3390/antiox11091784
34. Y. Zhang, Y. Li, Z. Quan, P. Xiao, J.-A. Duan, New insights into antioxidant peptides: an overview of efficient screening, evaluation models, molecular mechanisms, and applications, Antioxidants, 13, 2, 2024, 203. https://doi.org/10.3390/antiox13020203
35. Y. Hu, C. Ni, Y. Wang, X. Yu, H. Wu, J. Tu, C. Li, Z. Xiao, L. Wen, Research progress on the preparation and function of antioxidant peptides from walnuts, International Journal of Molecular Sciences, 24, 19, 2023, 14853. https://doi.org/10.3390/ijms241914853
36. J. Lu, Y. Guo, A. Muhmood, B. Zeng, Y. Qiu, P. Wang, L. Ren, Probing the antioxidant activity of functional proteins and bioactive peptides in Hermetia illucens larvae fed with food wastes, Scientific Reports, 12, 1, 2022, 2799. https://doi.org/10.1038/s41598-022-06668-9
37. M. Pan, K. Liu, J. Yang, S. Liu, S. Wang, S. Wang, Advances on food-derived peptidic antioxidants - a review, Antioxidants, 9, 9, 2020, 799. https://doi.org/10.3390/antiox9090799
38. Y. Zhu, F. Lao, X. Pan, J. Wu, Food protein-derived antioxidant peptides: molecular mechanism, stability and bioavailability, Biomolecules, 12, 11, 2022, 1622. https://doi.org/10.3390/biom12111622
39. R. Liu, L. Xing, Q. Fu, G. Zhou, W. Zhang, A review of antioxidant peptides derived from meat muscle and by-products, Antioxidants, 5, 3, 2016, 32. https://doi.org/10.3390/antiox5030032
40. S. Jaber, I. Iliev, T. Angelova, V. Nemska, I. Sulikovska, E. Naydenova, N. Georgieva, I. Givechev, I. Grabchev, D. Danalev, Synthesis, antitumor and antibacterial studies of new shortened analogues of $(KLAKLAK)_2$-NH_2 and their conjugates containing unnatural amino acids, Molecules, 26, 4, 2021, 898. https://doi.org/10.3390/molecules26040898
41. S. Jaber, V. Nemska, I. Iliev, E. Ivanova, T. Foteva, N. Georgieva, I. Givechev, E. Naydenova, V. Karadjova, D. Danalev, Synthesis and biological studies on $(KLAKLAK)_2$-NH_2 analog containing unnatural amino acid β-Ala and conjugates with second pharmacophore, Molecules, 26, 23, 2021, 7321. https://doi.org/10.3390/molecules26237321
42. S. Jaber, V. Nemska, I. Iliev, E. Ivanova, T. Foteva, N. Georgieva, I. Givechev, D. Tanev, E. Naydenova, D. Danalev, Synthesis, antiproliferative and antimicrobial activities of (KLAKLAK)2-NH2 analogue containing nor-Leu and its conjugates with a second pharmacophore, Biotechnology & Biotechnological Equipment, 37, 1, 2023, 151-158. https://doi.org/10.1080/13102818.2022.2162965
43. R. Apak, M. Bener, S.E. Çelik, B. Bekdeşer, F.B. Şen, The CUPRAC method, its modifications and applications serving green chemistry, in: Green Analytical Chemistry, Elsevier, 2025, 357–383. https://doi.org/10.1016/B978-0-443-16122-3.00007-X
44. D. Zielińska, H. Zieliński, J.M. Laparra-Llopis, D. Szawara-Nowak, J. Honke, J.A. Giménez-Bastida, Caffeic acid modulates processes associated with intestinal inflammation, Nutrients, 13, 2, 2021, 554. https://doi.org/10.3390/nu13020554