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Simple and rapid silver nanoparticles based antioxidant capacity assays: Reactivity study for phenolic compounds

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ABSTRACT

A single-step, rapid (10 min), sensitive silver nanoparticles (AgNPs) based spectrophotometric method for antioxidant capacity (AOC) assay has been developed. The assay is based on the ability of natural polyphenols to reduce Ag(I) and stabilize the produced AgNPs(0) at room temperature. Localized surface plasmon resonance (LSPR) of AgNPs at ≈420 nm is then measured. Using different conditions of pH (8.4) and temperature (45 °C) a further assay based on the production of AgNPs with selectivity for flavonols was also developed. The reactivity of the two AgNPs based assays vs. 15 polyphenols belonging to different chemical classes and 9 different samples has been studied and compared with ABTS, Folin and AuNPs based methods for AOC. The proposed assays had good reproducibility (RSD ≤ 13) and are simple, sensitive and cost effective. Moreover, used in conjunction with the classical AOC assays, can improve the information on the polyphenolic pool of food samples.

1. Introduction

Polyphenols are ubiquitous secondary metabolites present in plant foods (El Gharras, 2009). The common structural feature of all polyphenols, the presence of phenolic hydroxyl group(s), is the basis of their antioxidant activity in vitro and in vivo. In food matrices, antioxidants prevent fat rancidity and decrease the adverse effects of reactive oxygen (ROS) and nitrogen species (RNS). In general, an antioxidant reacting with a free radical, yields an electron, is oxidized, and produce a weak, non-toxic free radical that is stable and unable to propagate the reaction. The antioxidant properties of polyphenols are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Pisoschi, Cimpeanu, & Predoi, 2015).

Since antioxidants are molecules able to slow down or prevent the oxidation of other molecules, a diet rich in antioxidants has been associated with a reduced risk of developing some pathologies; in particular, epidemiological studies and associated meta-analyses suggest prevention of development of cancer, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Del Rio et al., 2012; Lall, Syed, Adhami, & Khan, 2015; Pandey & Rizvi, 2009).

Polyphenols have been also demonstrated to be key modulators of signalling pathways and to influence micro-RNA expression (Lall et al., 2015; Zhang & Tsao, 2016).

Foods rich in polyphenols are widely used in dietary formulations and an increasing number of research papers have appeared in the literature on the discovery and application of natural antioxidants and their therapeutic and technological properties (El Gharras, 2009; Shahidi & Zhong, 2010).

The development of efficient procedures for the extraction, proper analysis and characterization of phenolic compounds from different sources is a challenging task due to the structural diversity of the compounds, complexity of natural sources and their interaction with other components of the matrix. Different analytical approaches have been attempted in recent years to evaluate antioxidant capacity (AOC) and the total polyphenols content (TP) in food samples (Della Pelle & Compagnone, 2018; Carocho & Ferreira, 2013). Particular emphasis has been given to assays having the advantage of sensitivity, rapidity, simplicity, cost effectiveness and reduced sample volume; this latter characteristic is related in particular to waste disposal. A key role for the development of novel AOC assays has been recently played by nanomaterials. Nanomaterial based AOC assays are valid alternatives to classical methods for polyphenols analysis, allowing rapid and smart assessment of food antioxidants. Recently, optical, electrochemical and bioelectrochemical nanomaterials-based approaches for AOC assay of food polyphenols have been reviewed by Della Pelle et al. (Della Pelle & Compagnone, 2018). Optical methods for AOC assays in food based on metal nanoparticles (MNPs) formation/aggregation mediated by reducing agents (Della Pelle & Compagnone, 2018; Vasilescu, Sharpe, & Andreescu, 2012; TułodzieckaSzyd & Szydłowska-Czerniak, 2016;

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Vilela, González, & Escarpa, 2012) as well as on quantum dots (Della Pelle & Compagnone, 2018; Vilela, González, & Escarpa, 2015) have emerged and successfully proposed.

The use of gold nanoparticles (AuNPs) was introduced as novel tool for reliable assessment of AOC (and for the evaluation of total polyphenolic content) in food and biological samples (Della Pelle et al., 2015; Della Pelle, Vilela, González, & Escarpa, 2015; Tułodziecka & Szydłowska-Czerniak, 2016; Vilela, González, & Escarpa, 2015). These methods are based on the reduction of metal ions (generally inorganic salts or metal complexes) to MNPs in colloidal dispersions form. Optical detection is achieved exploiting the MNPs localized surface plasmon resonance (LSPR), which refers to the collective oscillation of the conduction electrons of the metal (Della Pelle & Compagnone, 2018; Lopatynskyi, Lopatynska, Guo, & Chegel, 2011). The latter is one of the most remarkable features of MNPs; in fact, strong absorption band(s) or increased scattering intensity of the radiation occurs at certain wavelengths for the MNPs as a result of this phenomenon. LSPR of the MNPs is mainly related to the MNPs size, shape, composition, inter-particle distance, and dielectric constant (refractive index) of the surrounding medium (Della Pelle & Compagnone, 2018; Vilela et al., 2015; Lopatynskyi et al., 2011).

The synthesis of silver nanoparticles (AgNPs) using different reducing compounds and natural extracts has been also reported because of the great interest into the “green” synthesis of AgNPs used in nanomedicine and microbiological applications (Ahmad et al., 2010; Marambio-Jones & Hoek, 2010; Moulton et al., 2010; Sharma, Yngard, & Lin, 2009). However, the formation of AgNPs by natural antioxidants as index of the AOC of the sample been proposed only in few papers (Chen, Zhang, Cao, & Huang, 2013; Özyürek, Güngör, Baki, Güçlü, & Apak, 2012; Teerasong, Jinnarak, Chaneam, Wilairat, & Nacapricha, 2017). In all these works the assay is not so straightforward; in fact, the antioxidant capacity is assayed as the ability to reduce Ag^+ ion in order to grow AgNPs already generated by another reducing agent. Thus, the approach requires multiple incubation steps. A AgNPs method for the evaluation of AOC of rapeseed and its products was also proposed by Szydłowska-Czerniak et al. (Szydlowska-Czerniak, Tulodziecka, & Szlyk, 2012), AgNPs formation using sinapic acid (as reference compound) and samples extraction influence on the method were investigated carrying out the measurement at pH 8.4 after 60 min of incubation.

In this work a simple and rapid colorimetric method for the detection of polyphenols based on the direct reduction of Ag^+ ions to produce AgNPs is proposed. The single step assay realised is simple, rapid and highly sensitive. The ability of different classes of polyphenols to form AuNPs and AgNPs in different conditions was studied using 15 polyphenols and 9 different samples. Data were compared with classical methods for AOC assay demonstrating the feasibility of the proposed approach. In order to understand the influence of the samples, endogenous polyphenols composition onto the MNPs formation UHPLC–MS/MS analysis of the phenolic content of the samples has been also carried out.

2. Materials and methods

2.1. Reagents, stock solutions and samples

All the chemicals were of analytical reagent grade. Epicatechin, catechin, epigallocatechin, catechol and phlorizin were purchased from Extrasynthese (Genay, France). Quercetin, gallic acid, kaempferol, rutin, p-cumaric acid, ferulic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), caffeic acid, myricetin, clorogenic acid, were purchased from Sigma-Aldrich (St Louis, MO, USA). Cetyltrimethylammonium chloride (CTAC; 25% in water), Cetyltrimethylammonium bromide (CTAB), polyethylene glicol (PEG), ethylenediaminetetraacetic acid (EDTA), hydrogen tetracholoroaurate ($HAuCl_4{\cdot}3H_2O$, 99.9%), silver nitrate ($AgNO_3$, > 99%), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich (St Louis, MO, USA). Folin–Ciocalteu reagent was obtained from Merck Schuchardt (Hohenbrun, Germany). Sodium phosphate monobasic monohydrate ACS reagent ($NaH_2PO_4{\cdot}H_2O$) sodium phosphate dibasic anhydrous (Na_2HPO_4) for buffer solution preparation, methanol, acetonitrile and formic acid were bought from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of polyphenol standards (in methanol) were prepared at a concentration of 1.0×10^{-2} mol L^{-1}, and stored at −20 °C in the dark. Different kinds of samples were purchased in local markets: digestive infusion (DIG), fennel infusion (IN), lemon tea (LT), pink forest infusion (RB), relax infused (RE), sogni d’oro camomile (SD), classic tea (TC), green tea (TG) and vanilla tea (VT). Samples were treated as follows: 500 mg were extracted using 10 mL of methanol under stirring for one hour in the dark at room temperature, samples were then centrifuged for 10 min (5000 rpm) and surnatants were collected, filtered (0.45 μm, PTFE syringe filter) and stored in the dark at −20 °C.

2.2. Apparatus

The centrifugation and stirring steps have been performed with a 4218 centrifuge from ALC International (Milano, Italy) and a sample orbital shaker SSL1 from Stuart equipment (Belfast, UK) respectively. For the assays the samples were heated in a water bath using a thermostat digital group 720 D (Asal, Italy). Absorbance measurements were carried out using a JENWAY 6400 Spectrophotometer from Barlworld Scientific (Staffordshire, UK,). The nanoparticles were characterized by transmission electron microscopy (TEM) using (TEM, S-2400 N, HITACHI, Japan). The samples for TEM characterization were prepared by placing a drop of the dilute sample solution on a carbon-coated copper grid and dried at room temperature before measurements. The samples were analyzed using an UHPLC Nexera LC20AD XR from Shimadzu (Kyoto, Japan) equipped with autosampler, vacuum degasser, and column oven. The UHPLC detection has been performed with a triple quadrupole mass spectrometer 4500 Qtrap from Sciex (Toronto, ON, Canada).

2.3. Silver nanoparticles antioxidant capacity based assay

Two different kind of assay were used for AgNPs: at room temperature (AgNPs-*RT*) and at 45 °C (AgNPs-*HT*).

2.3.1. AgNPs-RT

AgNPs were produced in 500 μL volume (reaction in eppendorf) with final concentrations of 8.0×10^{-6} mol L^{-1} of CTAC, 2.5×10^{-4} mol L^{-1} of $AgNO_3$ and 1.0×10^{-1} mol L^{-1} of NaOH (final pH = 13.0). 5 μL of CTAC solution (8.0×10^{-4} mol L^{-1}) was initially added to NaOH diluted in deionizer water, then 25 μL of $AgNO_3$ solution (5.0×10^{-3} mol L^{-1}) and appropriate dilutions of polyphenols standard or sample (reducing agent). All reagents need to be at room temperature. The reaction mix was stirred for 10 min in an orbital shaker and the reaction was blocked in ice for 10 min (this allows measurements in series, absorbance can be also taken immediately). Absorbance spectra were recorded in the 350–800 nm range against blank (all the reaction mix without polyphenols).

2.3.2. AgNPs-HT

AgNPs were obtained in phosphate buffer (pH 8.4; 1.0×10^{-2} mol L^{-1}) added with 8.0×10^{-6} mol L^{-1} of CTAC and 1.0×10^{-3} mo L^{-1} of $AgNO_3$. 5 μL of CTAC (8.0×10^{-4} mol L^{-1}), 25 μL of $AgNO_3$ solution (2.0×10^{-2} mol L^{-1}) and appropriate dilutions of polyphenols standard or sample reducing agent (final volume was 500 μL also in this case) were added to phosphate buffer (final pH = 8.4). The solution was stirred for 2 min and heated for 10 min at 45 °C in a water bath. The reaction was blocked in ice for 10 min (in this

case stopping of reaction time is always required) and, then, absorbance spectra in the 350 and 800 nm range were recorded against blank (all the reaction mix without polyphenols).

For each polyphenolic standard and sample extract, a dose-response curve was obtained by adding increasing amounts of standards/extract reading the absorbance at the LSPR maximum for both methods.

2.4. AuNPs assay

A slight modification of a previously published method (Della Pelle et al., 2015) was used. AuNPs synthesis was made with controlled reduction of $HAuCl_4$ mediated by polyphenols. 10 μL of CTAC (final concentration 1.6×10^{-5} mol L^{-1}), 25 μL of $HAuCl_4$ solution (final concentration 1.0×10^{-3} mol L^{-1}) were added with different amounts of polyphenols standard or sample extract in the reported order and brought up to a final volume of 500 μL with phosphate buffer (pH 8.0; 1.0×10^{-2} mol L^{-1}). The solution was stirred for 2 min and heated for 10 min at 45 °C in a water bath. Finally, the reaction was blocked in ice for 25 min and then absorbance spectra in the 350–800 nm range were recorded. For each polyphenolic standard and sample extract, a dose-response curve was obtained by adding increasing amounts of standards/extract reading the absorbance at the LSPR maximum.

2.5. Folin-Ciocalteu procedure

The total content of polyphenols was evaluated using the Folin-Ciocalteau (FC) reagent and by measurement of absorbance at 760 nm using gallic acid as the standard for the calibration curve. The method was adapted from Singleton and Rossi (Singleton, Rossi, & Jr, 1965). An appropriate volume of infused extract or polyphenolic standard was mixed with 20 μL of FC reagent (mixture of tungstophosphoric acid $H_3PW_{12}O_{40}$ and molybdatophosphoric acid $H_3PMo_{12}O_{40}$) and stirred for 3 min. 400 μL of sodium carbonate (Na_2CO_3, 7.5%) and deionized water up to the final volume of 1 mL were added and the sample was again stirred for 60 min, at room temperature, in the dark. Final absorbance at 760 nm was then taken. For each polyphenolic standard and sample extract, a calibration curve (in the linearity range) was also obtained by adding increasing amounts of standards/extract.

2.6. ABTS procedure

The AOC ABTS assay was run according to Re et al. (1999). Different volumes of extract or standards were mixed with the ABTS reagent to a final volume of 2 mL; after 5 min of reaction at room temperature in the dark, absorbance at 734 nm was measured. Each measurement was compared to a control sample prepared without adding phenolic compounds. The radical scavenging activity was expressed as GEAC (Gallic equivalent antioxidant capacity) based on calibration with gallic acid. For each polyphenolic standard and sample extract, a calibration curve (in the linearity range) was also obtained by adding increasing amounts of standards/extract.

2.7. UHPLC–MS/MS

The chromatographic separation was carried out using a ACE Excel 2 C18-PFP column (100 × 2.1 mm) packed with 2 μm particles; a guard column was also included. The mobile phases were as follows: ultrapure water with 0.1% HCOOH (phase A) and acetonitrile (phase B). A linear gradient was applied as follows: from 5% of phase B to 35% in 3 min, and then from 35 to 100% in 3.5 min, followed by a 3 min isocratic step, and then switched back to the initial 5% in 2 min. The flow rate of the mobile phase was 300 μL min^{-1}; all the mobile phase reached the ion source. Identification and quantification of the analytes were carried using a triple quadrupole mass spectrometer 4500 Qtrap from Sciex (Toronto, ON, Canada) equipped with a V-Spray source operating in negative ionization for all analytes. Nitrogen was used as the curtain gas (CUR = 20) and charged aerosol detection gas (medium setting), while air was used for the turbo gas (GS1 = 40) and nebulizer gas (GS2 = 40). The spray temperature was set at 500 °C and the capillary voltage to −4500 V. The quantitative analysis was carried in multi reaction monitoring (MRM), selecting two precursor ion/fragment ion transitions for each analyte (data not shown). The extracted ion currents (XICs) related to the most intense MRM transition for each selected analyte were employed to calibration curve construction; seven calibration levels between 1 ppb and 250 ppb in acetonitrile were employed for each polyphenol standard. All samples were prepared and analyzed in triplicate.

2.8. Multivariate analysis

The methods reactivity dataset (obtained analyzing the individual standard polyphenols) was analyzed by the unsupervised multivariate technique principal component analysis (PCA) using MatLab R2011 (Mathworks, Natick, MA, USA). Row normalized data were autoscaled (zero mean and unitary variance) before analysis. PCA was applied to inspect the multivariate data structure by decomposing a data matrix of eight rows (the standard compounds) and five columns (the different method response).

3. Results and discussion

3.1. Silver nanoparticles based antioxidant capacity assay: Optimization of the analytical strategy and setup

It is well-known that various salts containing metals, in particular conditions and in the presence of the proper reductive and/or stabilizing agent can generate MNPs (Della Pelle & Companone, 2018; Vasilescu et al., 2012; Vilela et al., 2012). The standard electrode potential value of the Ag/Ag^+ couple suggest a greater tendency to reduction, in the metallic state, compared to the most commonly used Au/Au^{+3} as MNPs precursor. Taking this into account, the ability of different polyphenols to form AgNPs has been investigated in order to assess the feasibility of AgNPs as basis for an AOC assay.

The analytical strategy exploited was based on $AgNO_3$ reduction in an alkaline medium in the presence of a capping agent. The optimal setup for the formation of AgNPs was studied recording UV–Vis spectra in the 350–800 nm range and using different phenolic probes. Different parameters, potentially affecting the growth and stability of AgNPs, were considered: $AgNO_3$ amount, capping agent type and amount, reaction time and temperature, reaction pH and buffer. Using gallic acid (phenolic acid) and rutin (flavonol) as phenolic probes we observed that AgNPs are always formed in the $AgNO_3$ concentration range 0.25–0.75 mM; the best signal/noise ratio was attained at 0.25 mM $AgNO_3$.

Ethylenediaminetetraacetic acid (EDTA), polyethylene glicol (PEG), cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTAC) were tested as capping agents using quercetin, gallic acid, kaempferol and rutin. In the absence of capping agent no formation of AgNPs was observed, while using EDTA, PEG and CTAB at the concentration used in another work (Ajitha, Kumar Reddy, Reddy, Jeon, & Ahn, 2016) AgNPs were obtained but the suspensions were not stable enough to have a measurable (clear) solution with all the phenols tested. CTAC, on the other hand, gave measurable suspensions for all the compounds. Evaluation of different CTAC amounts tested (from 4.0×10^{-6} mol L^{-1} to 4.0×10^{-5} mol L^{-1} in water) led to the selection of 8.0×10^{-6} mol L^{-1} because of the best signal/noise ratio.

We found that pH of the reaction medium is a key parameter to drive the formation of a stable suspension of AgNPs. Ionization of the phenolic compounds influences their reactivity with the silver salt and the stability of AgNPs suspensions. For experiments carried out with gallic acid and rutin at room temperature, a measurable absorbance signal was observed only at pH 13.0 after 10 min of reaction (mixing).

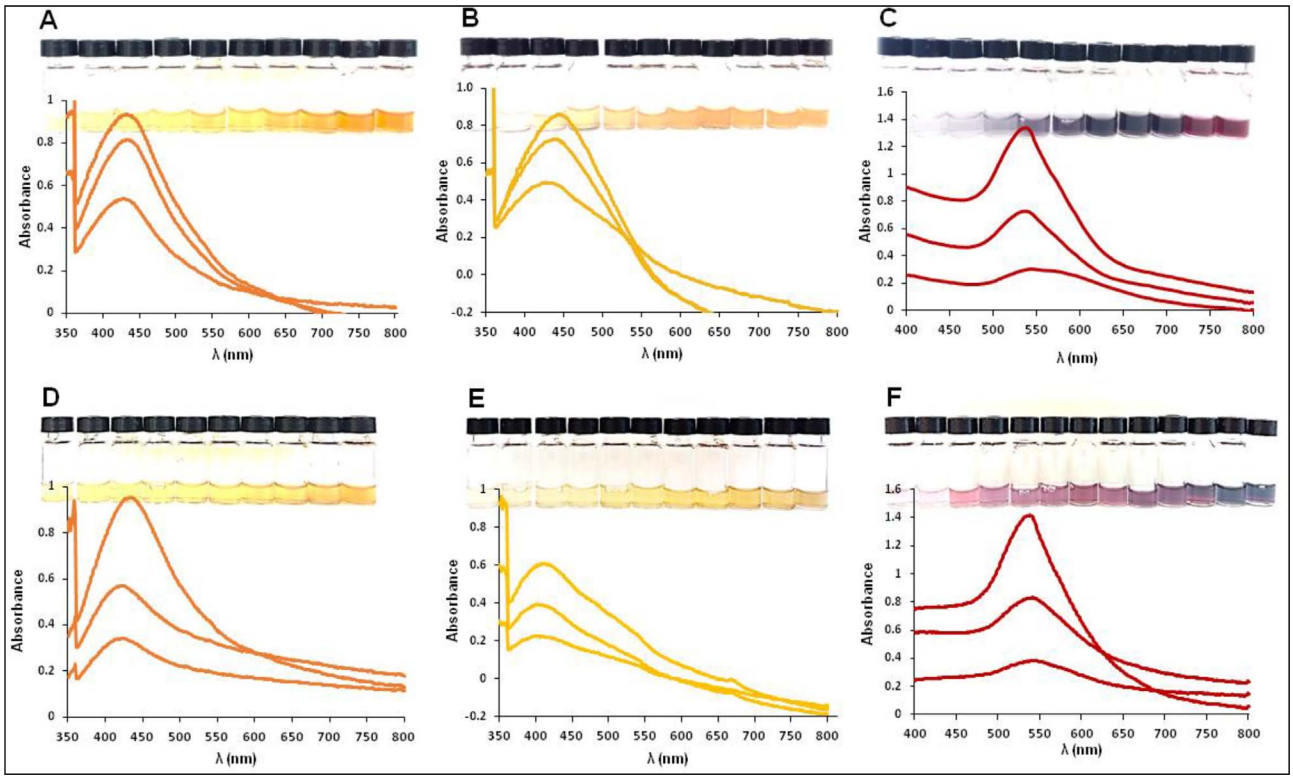

Fig. 1. (**A, B, C**) MNPs spectra obtained with AgNPs-*RT* (A), AgNPs-*HT* (B) and AuNPs (C) assays with increasing concentration of epicatechin. Epicatechin concentration: A = 2, 4 and 6 μM; B = 20, 50, 90 μM; C = 70, 90, 110 μM. On the top of each graph a picture of the entire series of suspensions used for the construction of the dose-response curve is shown. Epicatechin concentration: A = 1–7.5 μM; B = 20–90 μM; C = 70–150 μM. (**D, E, F**) MNPs spectra obtained with AgNPs-*RT* (D), AgNPs-*HT* (E) and AuNPs (F) assays using increasing volume of the sample 'RE' ('relax'). The pictures on the top report the formed MNPs employed for the construction of the dose response curve.

For reaction media at pH 14 collapse of AgNPs occurred (grey/dark colour); pH values lower than 13 gave no detectable formation of AgNPs. Interestingly, increasing the temperature of the reaction medium to 45 °C, appreciable AgNPs spectra were obtained also at pH values lower than 13 with only 10 min of reaction time.

Therefore, further investigations were carried out using two different AgNPs assay (reported in Section 2.3): at room temperature, pH 13 (AgNPs-*RT*) and at 45 °C pH 8.4 (AgNPs-*HT*). Typical AgNPs spectra obtained for epicatechin at three different concentrations with the two AgNPs proposed methods are shown in Fig. 1A (AgNPs-*RT* assay) and Fig. 1B (AgNPs-*HT* assay). No AgNPs formation was observed in the absence of polyphenols. The absorbance maximum was in all cases between λ = 420–440 nm in agreement with the literature data (Chen et al., 2013; Özyürek et al., 2012; Teerasong et al., 2017). For comparison, spectra obtained for epicatechin using the AuNPs based assay are reported in Fig. 1C.

3.2. AgNPs-based assay (AgNPs-RT and AgNPs-HT): Food polyphenol standards reactivity

The tendency of a phenolic compound to be oxidised depends on the energy required to donate electrons. Thus, antioxidant activity can be considered as the ability to donate electrons; in order to compare different compounds the same scale must be used, that means, the same electron acceptor (Leopoldini, Marino, Russo, & Toscano, 2004). The case of MNPs based assays is not so straightforward; in fact, to have a readable signal, a stable colloidal suspension is required. Thus, the final output (absorbance) is related to a) reduction of the metal, b) growth of MNPs c) stabilisation of MNPs colloidal suspensions. A study on different phenolic structures potentially present in food samples was then carried out to better understand the performance of the assays. The analytical protocols reported in Section 2.3 were applied to 15 polyphenols, belonging to different chemical classes: epicatechin, catechin, epigallocatechin, catechol, phlorizin, quercetin, gallic acid, kaempferol, rutin, p-cumaric acid, ferulic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), caffeic acid, myricetin, clorogenic acid. A dose response curve was built taking the maximum of the absorbance peak; pictures of typical suspensions obtained for epicathechin in the whole range of concentration tested are reported at the top of Fig. 1A–C. The concentration dependent colorimetric response appears evident.

For all the polyphenols a linearity range was achieved with good determination coefficients ($R^2 \geq 0.991$) (Table 1) demonstrating that the relevant polyphenols found in food are driving the AgNPs synthesis and that max absorbance signal was linearly dependent of the polyphenol concentration. Both for the AgNPs methods the NPs average dimensions were evaluated by TEM; the diameter of the NPs formed was ≤15 nm.

Data in Table 1 proves that the AgNPs-*RT* assay is more sensitive than the AgNPs-*HT* (see linear range and slope for the reacting phenols). Despite the lower sensitivity, the AgNPs-*HT* seems to give information about the flavonols present; in fact, only flavonols, among all the polyphenols tested, were able to form AgNPs in the *HT* assay. The only non-flavonolic phenol that was able to successfully react with this method was gallic acid, but only at high concentrations. Since this behaviour is not correlated to the reducing ability of the phenols we can assume that in the conditions used (pH = 8.4 at 45 °C) the flavonols are able to stabilise the colloidal suspensions of AgNPs; this can be dependent by the chemical structure and the number of hydroxyl groups. Further studies, beyond the aim of this work, will be necessary to confirm this hypothesis.

All compounds tested are, instead, extremely reactive in the AgNPs-*RT* assay, apart from coumaric acid and phlorizin that, anyhow, have very poor antioxidant capacity (see Section 3.3). In order to normalise the data and facilitate the comparison of the reactivity of the phenols among the different methods, the ratio of the slope of the linear range

Table 1
Analytical parameters obtained by the polyphenol standards dose–response curve employing the two proposed AgNPs-based method.

Standard	AgNPs-*RT*			AgNPs-*HT*		
	Linear range μM	Equation	R^2	Linear range μM	Equation	R^2
Caffeic acid	1.5–12.5	y = 0.029x + 0.191	0.993			
Catechin	0.25–6	y = 0.056x + 0.331	0.991	10–100	y = 0.005x + 0.409	0.996
Catecol	1.5–30	y = 0.010x + 0.371	0.991			
Chlorogenic acid	3–25	y = 0.018x + 0.284	0.994			
Epicatechin	1–7.5	y = 0.041x + 0.286	0.991	20–90	y = 0.008x + 0.166	0.993
Epigallocatechin	0.5–7.5	y = 0.044x + 0.315	0.991	20–200	y = 0.003x + 0.365	0.996
Ferulic acid	10–125	y = 0.002x + 0.303	0.991			
Gallic acid	1–15	y = 0.019x + 0.280	0.991	200–600	y = 0.001x + 0.289	0.998
Kaempferol	5–65	y = 0.006x + 0.266	0.994	20–250	y = 0002x + 0522	0.999
Myricetin	1–6	y = 0.064x + 0.223	0.992	100–200	y = 0005x − 0280	0.991
Quercetin	1–12.5	y = 0.048x + 0.164	0.992	100–200	y = 0.005x − 0.115	0993
Rutin	2–20	y = 0.023x + 0.199	0.993	30–90	y = 0.007x + 0.289	0996
Trolox	1–20	y = 0.018x + 0.385	0.992			

Table 2
ABTS, FC, AuNPs and the proposed AgNPs-based methods single phenol compound reactivity. Slope/[A50%] values were obtained from dose-response curves.

Standard	ABTS	RSD	FC	RSD	AuNPs	RSD	AgNPs-*RT*	RSD	AgNPs-*HT*	RSD
	(μmol^{-2})	(%)	(μmol^{-2})	(%)	(μmol^{-2})	(%)	(μmol^{-2})	(%)	(μmol^{2})	(%)
Caffeic acid	2.82E−01	8	5.44E−04	9	7.60E−05	5	8.54E−03	8		
Catechin	1.31E + 01	5	1.04E−03	5	2.82E−04	4	3.22E−02	5	9.69E−05	7
Catecol	3.40E−01	7	6.41E−04	8	1.44E−04	7	1.34E−03	9		
Chlorogenic acid	2.74E−01	5	5.48E−04	5	1.54E−04	6	3.37E−03	8		
Cumaric acid	2.58E−05	13	6.21E−04	13	1.03E−05	8				
Epicatechin	3.56E−01	9	5.42E−04	9	2.12E−04	6	2.06E−02	5	1.51E−04	9
Epigallocatechin	1.90E + 01	6	1.36E−03	5	2.73E−04	10	2.22E−02	4	3.05E−05	11
Ferulic acid	2.19E−04	14	3.80E−04	12	1.17E−05	9	6.67E−05	9		
Gallic acid	6.82E−01	5	9.55E−04	6	2.49E−04	5	4.59E−03	4	3.76E−06	13
Kaempferol	5.39E−01	13	6.51E−04	9	2.36E−04	9	3.14E−04	3	1.32E−05	4
Myricetin	5.97E + 00	8	8.81E−04	11	3.51E−04	7	3.64E−02	5	3.47E−05	8
Phlorizin	1.50E−01	13	3.86E−04	12	1.83E−04	11				
Quercetin	2.28E + 00	9	1.06E−03	11	3.18E−04	8	2.18E−02	7	4.03E−05	6
Rutin	1.38E−01	8	6.26E−04	5	9.46E−05	7	4.38E−03	2	1.29E−04	3
Trolox	3.58E−01	5	2.30E−04	13			3.73E−03	8		

(RSD%, n = 3).

and the concentration at 50% of the range (slope/[$A_{50\%}$]) was calculated and reported in Table 2. This allows to differentiate assays having the same slope and different linearity ranges (Della Pelle et al., 2015).

Looking at the reactivity of the AgNPs assays (Table 1 and Table 2) the compounds with ortho-diphenol functionalities were the most active in reducing Ag (I) to AgNPs (0). Among these ortho-compounds, flavonols exhibited the highest reactivity confirming the data obtained with the AgNPs-*HT* assay. An intermediate activity was recorded for the flavonol rutin; this behaviour can be attributed the conjugated sugar. Mono-phenols had a significantly lower activity than the other antioxidant compounds assayed (at least one order of magnitude). The higher reactivity for flavonols with ortho-phenolic structure was also reported in the literature for different assays (Del Carlo et al., 2012; Della Pelle et al., 2017; Enache & Oliveira-Brett, 2011). The highest electron density of OH groups in ortho position increases reactivity, reducing of the aromatic nature of the ring.

3.3. Comparison study with antioxidant capacity and total phenols assays

It is well known that responses of single phenols to the different assays can be significantly different due to different reactivity (Della Pelle et al., 2015). The ABTS method is directly linked to the radical scavenging activity of the compounds tested and is typically used for AOC assessment. Folin–Ciocalteu is a classical method for the total polyphenols quantification; being this method based on the reducing ability of polyphenols, can also be considered an AOC assay. Recently, our group and Prof. Escarpa group in Alcalà, have proposed (Della Pelle et al., 2015) an AuNPs-based method exploiting the polyphenols ability to reduce Au(III) to AuNPs(0), and proposed a new antioxidant capacity index (similar to the slope/[A50%] reported in the previous section). In this assay, the plot of absorbance obtained from AuNPs localized surface plasmon resonance (LSPR) at 540 nm is used as function of the concentration/antioxidant capacity of the polyphenols. Typical examples of the spectra and the pictures obtained for epicathechin are reported in Fig. 1C.

From the data reported in Table 2 it is clear that all the analytical methods used for the comparison of the standards antioxidant capacity exhibited good repeatability (RSD%, n = 3) that was the following: ABTS ≤ 14%, Folin–Ciocalteu (FC) ≤ 13% and AuNPs ≤ 11%, AgNPs-*RT* ≤ 9%, AgNPs-*HT* ≤ 13%. All the calibration curves had good linearity and excellent coefficients of determination ($R^2 \geq 0.990$). The ABTS method, as expected, exhibited higher reactivity towards flavonols, followed by the ortho compounds; significant lower reactivity was obtained for the simple mono-phenols (see coumaric acid and ferulic acid). The phenols reactivity with FC was also related to AOC since also in this case flavonols are the most reactive compounds.

AuNPs method confirmed to be more reactive towards ortho-phenols (Della Pelle et al., 2015) and, looking at the data (Table 2), the reactivity of the ortho-compound is not dependent on the structure. Indeed, ortho-phenols and flavonols have comparable reactivity. Thus, a similar trend in reactivity for AgNPs-*RT* and the ABTS method was found for the tested polyphenols.

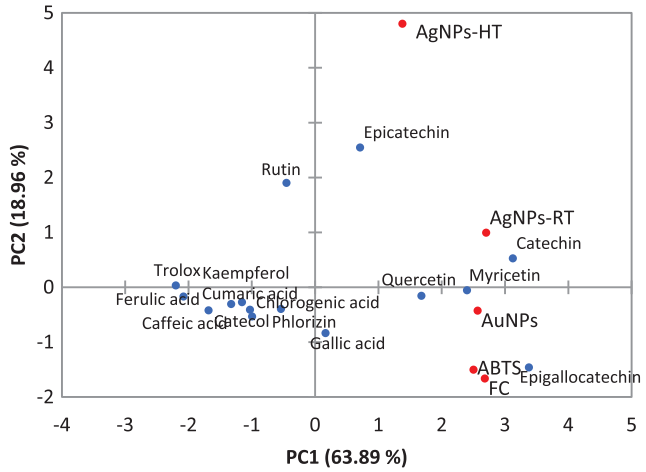

Fig. 2. PCA of the ABTS, FC, AuNPs and the proposed AgNPs-based methods reactivity vs. polyphenolic compounds. The biplot (Score and loading) of the first two principal components showed 82.85% of the cumulative variance. Rows normalization were applied to the dataset. Data were autoscaled before PCA.

Row reactivity normalized data (extrapolated by Table 2) were analyzed using PCA (Fig. 2). The biplot of the first two principal components is reported in Fig. 2. The first component represented 63.89% of the variance; the second 18.96% displaying together a cumulative variance of 82.85%. The changes of the phenols structural composition are well captured by the PCA; in particular PC1 contributed to a clear separation of (ortho-)flavonols from the others molecules, with the exception of gallic acid (probably because of the particular reactivity given by the trihydroxylic group). The PCA2 is important in the discrimination of rutin; the latter is the only sugar-conjugated molecule tested, being composed by quercetin and rutinose. Epicathechin and cathechin are separated from the other compounds, in the third quadrant. The contribution of the antioxidant capacity methods to the whole dataset can be evaluated considering the position of the loadings (the contribution of each variable to the principal components) with respect to the scores. The PC1 does not separate the methods since all the methods are able to give an evaluation of antioxidant capacity. On the other hand, observing the PC2 the AgNPs-based methods had an opposite performance with respect to the other three AOC assays confirming very high reactivity vs. flavonols. The other assays are reactive in a similar way towards all the phenolic compounds. It should be emphasised that the normalisation of the data carried in the PCA eliminated differences in sensitivity among the methods highlighting the selectivity of the different assays.

These data confirm the suitability of the proposed AgNPs-methods for the antioxidant capacity measurement of different polyphenolic compounds and underline the difference in assay selectivity with respect to the AuNPs-based method. Definitively, in the AgNPs assay an important component of reactivity is due to the molecular structure and the ionization state of the molecule (different reactivity with different pH: see Section 3.1).

3.4. Real samples analysis

In order evaluate the predominant class of phenolic compounds in the collected samples, the polyphenols tested previously were quantified using the UHPLC-MS/MS procedure reported in Section 2.7. The total phenols content was achieved by the sum of the polyphenols quantified by the procedure (Table S1). This surely represented an underestimation of the real content; however, the phenolic standards selected were those prevalently found in the samples and were representing over 90% of the total chromatographic area (the total ion current chromatogram); thus, the error was considered not significant. Phenols content of real samples and the relative amount as flavonols, o-diphenols and m-phenols are reported in supplementary material (Table S1).

Each compound was quantified via a calibration curve (linear range 1–250 ppb). A determination coefficient $R^2 \geq 0.99$ was satisfied for each of the analytes (data not shown). The samples were heterogeneous, in particular considering the single phenol type, flavonols amount and total phenols content (Table S1). This variability is correlated to different raw materials employed for the infuse (fruits, herb, etc.) and to the different technological production process.

Typical AgNPs spectra of the samples are reported in Fig. 1D-E (comparison with the AuNPs method is reported in Fig. 1F); the data demonstrate that the polyphenols in the medium can reduce Ag(I) to AgNPs(0) and that the LSPR maximum absorbance of the formed AgNPs is in the 420–440 nm range for all the extracts. For all samples a dose-response curve was obtained using A_{420} vs. different volumes of sample extracts (pictures of Fig. 1D–E).

Table 3 reports the samples AgNPs AOC index obtained using the AgNPs based assays for the samples. Gallic acid was used as reference phenol for antioxidant capacity evaluation in both AgNPs-based method. The calibration curves obtained with increasing concentrations of gallic acid taking the absorbance at 420 nm exhibited an excellent linearity. The limit of detections (LOD) calculated as 3 times the standard deviation of the blanks (n = 20) were for the AgNPs-*RT* and AgNPs-*HT* 0.4 and 58 μM, respectively. The limits of quantification, calculated as 10 times the standard deviation of the blanks (n = 20) were 1.5 and 192.8 μM, respectively. Samples were assayed also with AuNPs, ABTS and FC assays. Data expressed as gallic acid equivalents are also reported in Table 3. Gallic acid was used as reference phenolic compound since it is generally used for this purpose and also because it was reactive for all the AOC methods evaluated. Absolute reactivity for each of the sample tested with the different assays has been reported in supplementary material (Fig. S1). All the analytical methods used for the comparison of the antioxidant capacity exhibited good repeatability (RSD%, n = 3) that was the following: AgNPs-*RT* ≤ 9%, AgNPs-*HT* ≤ 14%, AuNPs ≤ 7%, ABTS ≤ 14%, Folin–Ciocalteu ≤ 11%. Samples were different in amount and composition of polyphenols; however, some considerations on the reactivity can be done. Sample TG has the highest content of polyphenols among all samples (Table S1) consisting almost exclusively of orthoflavonols (98%): the methods here proposed exhibit the highest reactivity with this sample (Table 3 and Fig. S1). The samples TC and VT have comparable amount of

Table 3
Data obtained using all the methods tested for samples expressed as gallic acid equivalent. (RSD%, n = 3).

Method	Sample																	
	DIG (g kg^{-1})	RSD (%)	IN (g kg^{-1})	RSD (%)	LT (g kg^{-1})	RSD (%)	RB (g kg^{-1})	RSD (%)	RE (g kg^{-1})	RSD (%)	SD (g kg^{-1})	RSD (%)	TC (g kg^{-1})	RSD (%)	TG (g kg^{-1})	RSD (%)	VT (g kg^{-1})	RSD (%)
AgNPs-*RT*	8.66	4	1.20	9	9.91	5	5.31	3	9.12	8	9.62	7	49.50	8	143.01	3	52.19	4
AgNPs-*HT*	11.10	9	2.52	9	14.73	5			7.78	10	6.98	14	13.33	6	24.42	7	7.82	12
AuNPs	18.63	5	1.52	7	15.64	2	15.86	5	15.58	7	14.03	5	20.56	6	132.35	3	27.95	4
ABTS	3.03	12	1.12	14	11.26	7	0.70	14	2.13	9	2.21	12	11.55	8	54.57	5	8.92	7
FC	5.98	4	2.51	11	14.54	3	3.70	10	5.51	4	5.42	5	21.10	7	30.54	8	16.79	6

Table 4
Correlation matrix (Pearson coefficient) for all the antioxidant capacity methods tested.

	ABTS		FC		AgNPs-*HT*		AgNPs-*RT*		AuNPs	
ABTS	1	(p = 0)	0.876	(p = 0.002)	0.891	(p = 0.001)	0.956	(p = 0.000)	0.977	(p = 0.000)
FC	0.876	(p = 0.002)	1	(p = 0)	0.733	(p = 0.025)	0.913	(p = 0.001)	0.801	(p = 0.009)
AgNPs-*HT*	0.891	(p = 0.001)	0.733	(p = 0.025)	1	(p = 0)	0.770	(p = 0.015)	0.826	(p = 0.006)
AgNPs-*RT*	0.956	(p = < 0.0001)	0.913	(p = 0.001)	0.770	(p = 0.015)	1	(p = 0)	0.950	(p = < 0.0001)
AuNPs	0.977	(p = < 0.0001)	0.801	(P = 0.009)	0.826	(p = 0.006)	0.950	(p = < 0.0001)	1	(p = 0)

polyphenols (2.94 and 3.49 gKg^{-1}, respectively) and similar content of flavonols (64.4% and 72.9%); this is reflected by the amounts expressed as gallic acid equivalents using the two AgNPs assays. On the contrary, sample RB and IN have lower content of polyphenols (0.21 and 0.38 mg Kg^{-1}, respectively) and lower amounts of flavonols (23% and 0%) and are poorly detected by the AgNPs methods.

For a complete comparison of the result on the whole lot of samples the correlation matrix (Pearson coefficient) for all the methods used is reported in Table 4. The data demonstrate that the MNPs-based assays in all cases a had a nice correlation with the ABTS method ($r \geq 0.891$) confirming that these methods give information on the AOC. As expected, the MNPs-based assays exhibited a lower correlation with FC method, since this assay is more related to the total phenols amount (despite the reported lack of selectivity).

Considering the MNPs based assays, a higher correlation between the AgNPs-*RT* and AuNPs with respect to the AgNPs-*HT* was achieved. This evidence confirms the possibility to use AgNPs-*HT* assay to ascertain the presence of flavonols in real samples. Moreover, the p-values reported in Table 4 results confirmed that MNPs based protocols can be used as AOC assays having statistically highly significant correlation with ABTS ($p < 0.001$) and statistically significant correlation with FC ($p < 0.05$); only AgNPs-*RT* gave a $p < 0.001$ also with FC.

4. Conclusion

In this work, a simple AgNPs based colorimetric assay for the AOC assessment has been developed. The ability of polyphenols to form AgNPs has been proved and the polyphenols reactivity resulted related both to the intrinsic AOC of the compound and to the chemical structure. Two different approaches were proposed. In the first assay (AgNPs-*RT*), a single incubation of reagents run for 10 min at room temperature allowed sensitive (LOQ = 1.5 μM gallic acid) photometric assay of AOC. Using temperature control (45 °C) and a different pH (pH 8.4) a second AgNPs based assay with good selectivity for flavonols has been obtained (AgNPs-*HT*). For both the assays (AgNPs-*RT* and AgNPs-*HT*) the AgNPs formation is easily followed by the absorption band of localized surface plasmon resonance at 420–440 nm and the AgNPs synthesis is described by a linear dose-response curve. The performance of the developed assays has been evaluated vs. 15 different polyphenolic standards and vs 9 samples with different polyphenolic content. The polyphenols reactivity was related to the intrinsic polyphenols AOC, to the molecular structure and to the ionization state. The data, compared with Folin, ABTS, AuNPs and UHPL-MS/MS, demonstrates that the AgNPs-based assays in all cases showed the highest correlation with ABTS (AgNPs-*RT*: r = 0.956, p = < 0.0001; AgNPs-*HT*: r = 0.891, p = 0.001) and AuNPs (AgNPs-*RT*: r = 0.950, p = < 0.0001; AgNPs-*HT*: r = 0.826, p = 0.006) methods, confirming are useful AOC assay. All the proposed assays results to be reproducible (RSD ≤ 13), simple, sensitive, cost effective, rapid. The developed AgNPs assays can be used alternatively to classical methods for AOC or in conjunction with the aim of giving more complete information on the chemical nature of polyphenols present in the samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.02.141.

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