Effect of particle size upon the extent of extraction of antioxidant power from the plants *Agrimonia eupatoria*, *Salvia* sp. and *Satureja montana*

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ABSTRACT

The dependence of the extent of aqueous extraction of antioxidant compounds on particle size and contact time was studied for three important medicinal plants, that are commonly used in infusions: agrimony, sage and savoury. The effect of extraction time was dependent on the plant considered; however, ca. 5 min can be taken as the minimum period required to assure an acceptable degree of extraction of those compounds. As expected, a smaller particle size led to a higher extraction extents; a typical value of 0.2 mm is accordingly recommended. Chlorogenic acid was the dominant phenolic compound extracted from agrimony, whereas caffeic acid dominated in the case of sage or savoury. A mathematical model based on Fick's law was developed from first principles, and its two parameters were suitably fitted to the experimental data generated – in attempts to predict the evolution of antioxidant capacity extracted during contact time, for each plant and each particle size.

Introduction

Agrimony, sage and savoury (common English names for *Agrimonia eupatoria*, *Salvia* sp. and *Satureja montana*, respectively) are plants often used in traditional medicine in Portugal, and which grow in the poor soils of the Mediterranean basin. The former has been claimed to control uric acid, favour the respiratory system, function as an analgesic or a diuretic aid, treat wounds and provide a rich source of antioxidants (Venskutonis, Škėmaitė, & Ragažinskienė, 2007). Besides application as condiment, sage has been used as an anti-diarrhoea vector, and to help in digestion, contribute to heal wounds, play an anti-inflammatory role, fight insomnia and decrease blood pressure; some of these biological activities have been associated with its contents of rosmarinic acid (Petersen & Simmonds, 2003) and L-salvianolic acid (Lu & Foo, 2001), which are two antioxidant compounds. Finally, savoury is also used as condiment, and has been prescribed to combat diarrhoea, help digestion and heal wounds, as well as a disinfectant (Gião et al., 2007). Agrimony exhibits the highest antioxidant capacity and total phenolic content within the aforementioned three plants (Gião et al., 2007, 2008) – probably because of its rich contents of coumarins, flavonoids, tannins and terpenoids (Copland et al., 2003); savoury and sage come second (in this order) in those features.

Upstream of food (or beverage) production or formulation effective, harvesting antioxidants as nutraceuticals from the aforementioned plants depends on maximisation of their extraction. However, it is widely known that the efficiency of solid/liquid extraction processes is affected by critical processing parameters, such as temperature, nature of solvent, structure of solid matrix (mainly particle size) and extraction time (Franco, Pinelo, Sineiro, & Núñez, 2007). This means that each plant matrix/extraction solvent pair behaves in a unique way, so it should be studied as such. On the other hand, both the particle size of the plant matrix and the temperature of the extraction process are easily manipulated physical conditions. In general, a smaller size and a higher temperature facilitate mass transfer (Cacace & Mazza, 2003; Waterman & Sutton, 2003), but quantification of such heuristic rules for each plant source is required before optimisation efforts can be rationally developed.

The major goal of this research study was to model the influences of particle size and time of exposure on the efficiency of aqueous extraction of antioxidant power from solid dried material from given plants. A second goal was to characterise the chemical profiles of extracts in terms of phenolic compounds (which have been often associated with comparatively high antioxidant power).

Materials and methods

Sample preparation

Three plants were considered: agrimony, sage and savoury, all of which were a kind gift from ERVITAL (Castro Daire, Portugal). These plants had been cultivated as organic products, and were supplied in their commercial form of dried leaves: ca. 4 g was then

crushed (using a coffee mill) for 1 min, so as to obtain the corresponding powder. This powder was consecutively passed through bolters of 0.2, 0.25 and 0.3 mm mesh sizes (Haver and Boecker, Oeld, Germany), and the four different fractions thus collected were duly weighed.

Each fraction (ca. 1 g) was finally contacted, under uniform stirring, with 110 ml of boiling distilled water – so as to mimick ready-to-drink infusion preparations; samples were collected every minute up to 10 min, and an extra two samples by 12 and 15 min. All those samples were kept in ice, so as to quench the extraction process prior to analysis. Samples were assayed for their total antioxidant capacity, as described below. All experiments were run in duplicate.

For chromatographic analyses, samples were taken of aqueous infusions after 5 min, and were filtered through a 0.45 μm filter before injection.

Antioxidant capacity assessment

Determination of the antioxidant capacity was as detailed previously by Gião et al. (2007). The ABTS$^{\bullet+}$ stock solution was prepared via addition, at 1:1 (v/v), of 7 mM ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic) acid) diammonium salt (Sigma–Aldrich, St. Louis, MO, USA) to a solution of 2.45 mM potassium persulphate (Merck, Damstadt, Germany); the developing reaction took place in the dark, for 16 h. In order to obtain an absorbance of 0.700 ± 0.020 at 734 nm, measured with an UV 1203 spectrophotometer (Shimadzu, Tokyo, Japan), the aforementioned stock solution was diluted in as much ultra-pure water as necessary. A 10 μl-aliquot of the sample was assayed for inhibition percentage (between 20% and 80%, so as to guarantee a linear response of the analytical method), after 6 min of reaction with 1 ml of diluted ABTS$^{\bullet+}$ solution; triplicates of each sample were averaged to generate each datum point (which implies a total of six replicates per plant). The final result was expressed as equivalent concentration of ascorbic acid (in g l^{-1}), using a calibration curve previously prepared with such a reference compound.

Phenolic compound profiling

The chromatographic system consisted of a Prostar 210 LC pump (Varian, Walnut Creek, CA, USA), coupled with a 1200 triple quadrupole mass spectrometer (Varian) with electrospray ionisation (ESI) in both positive and negative modes. A 5 μm C$_{18}$ column (4.6 mm × 100 mm, Merck) was used for separation, at a flow rate of eluant of 0.4 ml min^{-1}. The LC–MS/MS method was based on that followed by Politi, Rodrigues, Gião, Pintado, and Castro (2008). Chromatographic separation was performed within 33 min, using the following gradient pattern of eluant A (water with 0.1% (v/v) formic acid) and eluant B (methanol with 0.1% (v/v) formic acid): from 0 min with 90% A, to 12.05 min with 78% A, to 22.05 min with 50% A, to 27.05 min with 95% A, and to 30 min with 95% A. ESI–MS/MS detection used a capillary voltage of 55 V; for MS/MS fragmentation, Ar was used (under 1.20 mtorr, with a collision energy of 15 V). An in-house LC–MS/MS library

was created in advance, by injecting 33 chromatographic standards of phenolic compounds, under similar analytical conditions. Identification of the phenolic compounds in the samples was by direct injection and comparison with the spectra of the in-house library; for simplicity, the results of quantification were presented as percent abundance.

Data treatment

Starting from mechanistic modelling based on Fick's law (Çengel, 2007), the balance of the antioxidant concentration throughout contact time is expected to be given by

$$V_L \frac{dC_{AL}}{dt} = kA_S(C_{AS}^* - C_{AL}) \tag{1}$$
$$t = 0, \quad C_{AL} = C_{AL,0}$$

where V_L is the volume of the liquid phase, C_{AL} is the concentration of antioxidants in the liquid phase, t is the contact time, k is the mass transfer coefficient based on the liquid phase, A_S is the area of the solid phase, C_{AS}^* is the solubility of antioxidants in the liquid phase, and $C_{AL,0}$ is the initial value of C_{AL}. Defining a as the specific area of the powder, calculated as

$$a = \frac{A_S}{V_S} = \frac{6}{d} \tag{2}$$

where d is the average particle diameter, and defining R as the volume ratio of the solid to the liquid phases, namely

$$R = \frac{V_S}{V_L} \tag{3}$$

then Eq. (1) can be rewritten as

$$\frac{dC_{AL}}{dt} = kaR(C_{AS}^* - C_{AL}) \tag{4}$$

Assuming that there is an excess of solute in the solid phase, then C_{AS}^* can be taken as essentially constant; consequently, integration of Eq. (4) from the initial condition set forth in Eq. (1) leads finally to

$$C_{AL}(t) = C_{AS}^*(1 - e^{-kaRt}) \tag{5}$$

where (kaR) and C_{AS}^* accordingly become the only two adjustable parameters.

Statistical analyses

Non-parametric tests were applied to each set of experimental data, owing to their intrinsic heteroschedasticity. Friedman and Wilcoxon tests were thus chosen to check whether time influenced the observed results. Kruskal–Wallis tests were applied to check whether, at each time, plant source and particle size influenced the results produced. Mann–Whitney tests were, in turn, applied to unfold possible differences between plant source and particle size pairs. Principal component analysis for categorical data (PCA) was used to ascertain differences between fractional mass distributions. Finally, Tukey's tests were considered to quantify dif-
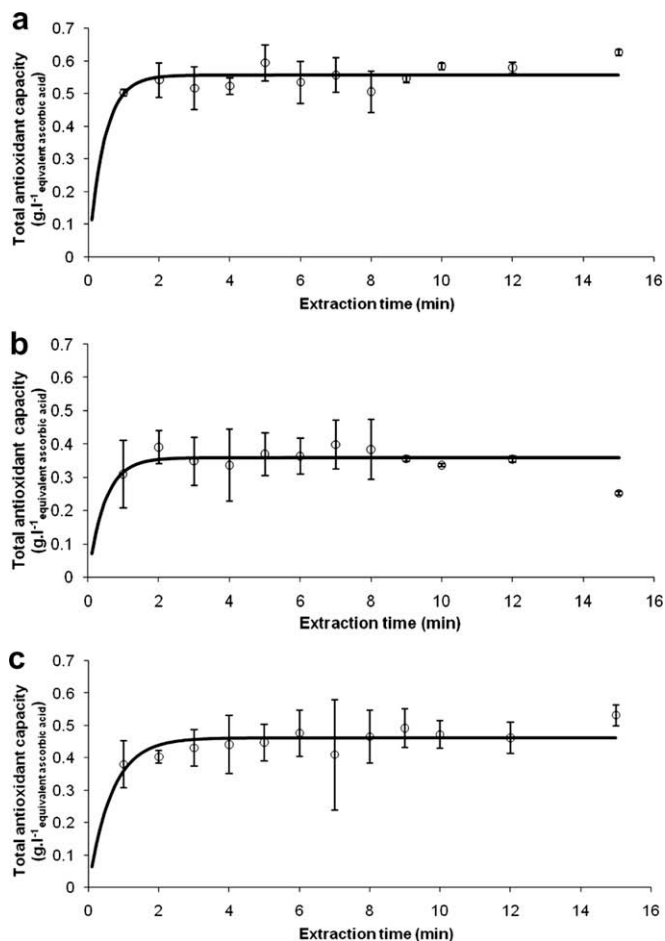
Fig. 1. Total antioxidant capacity, experimentally obtained (○, mean ± standard error) and theoretically predicted (—), of (a) agrimony, (b) sage and (c) savoury, as a function of extraction time, for particle sizes between 0.2 and 0.25 mm.

ferences between plants. All tests were used at a significance level of 0.05. The predictive model denoted as Eq. (5) was fitted by non-linear regression analysis to experimental data points, using the Levenberg–Marquardt estimation method. All statistical tests were performed using SPSS, v. 16.0.0 (Chicago, IL, USA).

Results and discussion

The total antioxidant capacities of the infusions obtained from the three plants, for various contact times and at particle sizes between 0.2 and 0.25 mm (for the sake of illustration), are depicted in Fig. 1. As expected (Waterman & Sutton, 2003), the antioxidant power increased with increasing extraction time and decreasing particle size; this means that an increase in the time and in the surface area available for molecular transport contribute to a more extensive mass transfer of solutes between phases – according to the general principles underlying Fick's law.

Non-linear regression analysis was sequentially applied to fit Eq. (5) to the data pertaining to each plant and to each particle size range; typical curves are depicted also in Fig. 1, whereas estimates of the associated model parameters (coupled with relevant statistical information) are comprehensively tabulated in Table 1.

The correlation coefficient between parameters C_{AS}^* and kaR was low, so the parameter estimates were relatively independent of each other – and thus no further uncoupling was required for efficient estimation. Inspection of Table 1, reveals that the range of equilibrium antioxidant power in the liquid phase is 0.3–0.7 g l^{-1} of equivalent ascorbic acid min^{-1}, whereas the characteristic time scale for mass transfer ranges from 0.14 to 0.33 min; extraction was accordingly efficient and fast. The maximum rate of extraction, observed when $t = 0$, ranged in turn from 0.3 to 1.6 g l^{-1} of equivalent ascorbic acid min^{-1}, and increased when the particle size decreased; it was also higher for agrimony and savoury than for sage. On the other hand, the likelihood associated with the model initially postulated and later fitted is high – as concluded from visual inspection of the goodness of fit illustrated in Fig. 1.

Following statistical analysis of our data, several conclusions can be drawn. When considering all three plants together, Friedman's test indicated that extraction time influenced total antioxidant capacity. On the other hand, Wilcoxon's test allowed definition of six time groups, the first group including only 1 min, the second only 2 min, the third, 3 and 4 min, the fourth, 5 until 9 min, the fifth, 10 and 12 min, and the sixth only 15 min. The initial minutes are thus particularly important regarding extraction – as extraction rate slows down considerably between 5 and 10 min (Fig. 1). These results are consistent with the choice of 5 min for extraction time, as proposed elsewhere (Gião et al., 2007, 2008).

The total antioxidant capacity extracted in the first 2 min, for each of the three plants studied, can be seen in Fig. 2 for the various particle size ranges. For particle sizes below 0.2 mm, agrimony showed significantly higher total antioxidant capacity; this difference decreased in magnitude with increasing particle size, and essentially vanished for particles larger than 0.3 mm. A strong decrease (ca. 0.1 g l^{-1} of equivalent ascorbic acid) of the total antioxidant capacity can also be observed as the particle size increases (see Fig. 2a–d). Upon calculation of average values among the different particle size ranges, a value similar to that obtained in previous studies (Gião et al., 2007) could be obtained.

In terms of particle size, Kruskal–Wallis' test unfolded differences at all extraction times. In an attempt to discriminate which sizes led to better performance at each extraction time, Mann–Whitney's test was applied; no influence of particle size was ob-

Table 1
Parameter values (estimate ± standard deviation) entertained by the mathematical model labelled as Eq. (5), for each plant and granulometry.

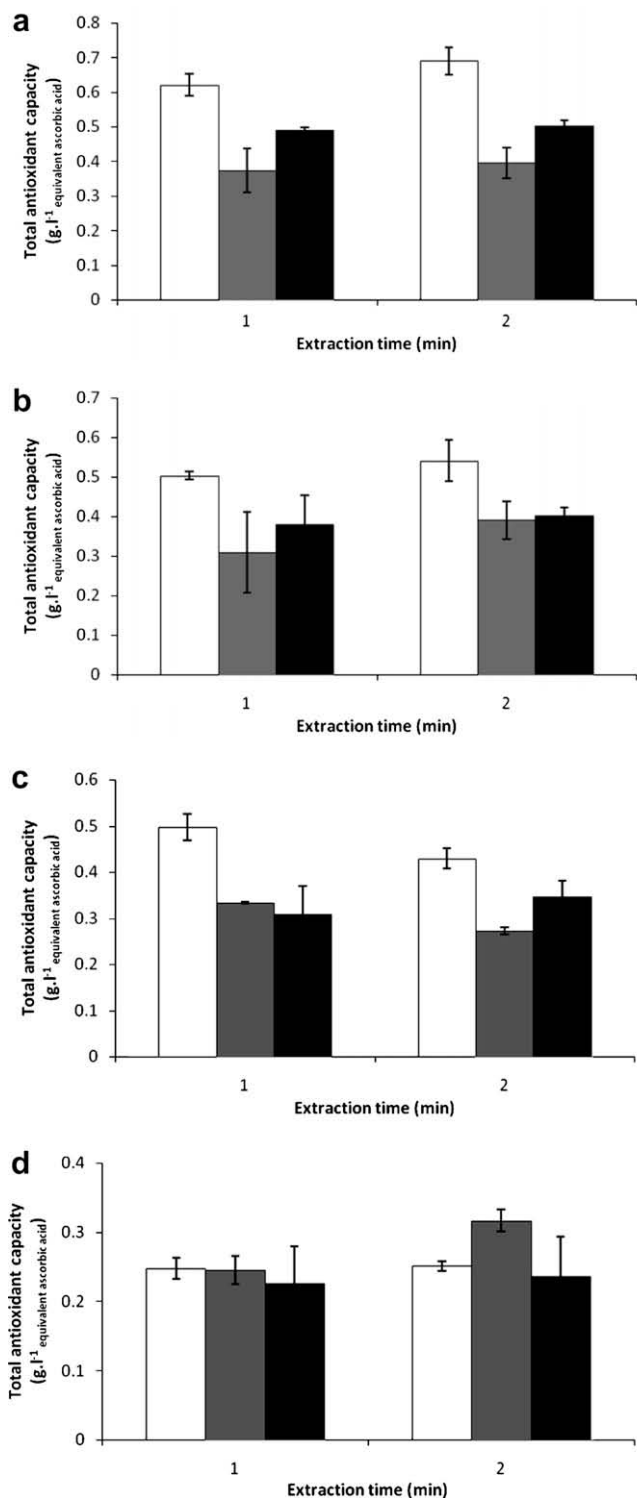| Plant	Granulometry (diameter: x)	C_{AS}^* (g l^{-1} equivalent ascorbic acid)	kaR (min^{-1})	Initial rate, $C_{AS}^* \cdot kaR$ (g l^{-1} equivalent ascorbic acid min^{-1})
Agrimony	$x < 0.2$ mm	0.694 ± 0.007	2.27 ± 0.31	1.58
	$0.2 < x < 0.25$ mm	0.557 ± 0.007	2.27 ± 0.38	1.26
	$0.25 < x < 0.3$ mm	0.582 ± 0.009	1.30 ± 0.17	0.76
	$x > 0.3$ mm	0.340 ± 0.010	0.84 ± 0.15	0.28
Sage	$x < 0.2$ mm	0.404 ± 0.009	2.56 ± 0.95	1.03
	$0.2 < x < 0.25$ mm	0.360 ± 0.010	2.13 ± 0.73	0.77
	$0.25 < x < 0.3$ mm	0.372 ± 0.013	1.51 ± 0.48	0.56
	$x > 0.3$ mm	0.388 ± 0.009	0.68 ± 0.09	0.26
Savoury	$x < 0.2$ mm	0.515 ± 0.008	2.97 ± 0.97	1.53
	$0.2 < x < 0.25$ mm	0.461 ± 0.010	1.51 ± 0.30	0.70
	$0.25 < x < 0.3$ mm	0.342 ± 0.008	2.37 ± 0.83	0.81
	$x > 0.3$ mm	0.301 ± 0.014	0.99 ± 0.30	0.30

Fig. 2. Total antioxidant capacity, experimentally obtained by 1 and 2 min of extraction, of (□) agrimony, (▨) sage and (■) savoury, for particle sizes (a) below 0.2 mm, (b) between 0.2 and 0.25 mm, (c) between 0.25 and 0.3 mm, and (d) above 0.3 mm.

Phenolic compound	M-1	Plants		
		Agrimony	Sage	Savoury
Protocatechuic acid	153–109	1.59	3.95	0.84
Coumaric acid	163–119	1.80	19.62	1.74
Gallic acid	169–125	3.98	0.17	–
Caffeic acid	179–135	–	64.96	72.94
Ferulic acid	193–134	–	–	–
Naringenin	271–151	–	–	1.86
Quercetin	301–151	–	–	–
Isorhamnetin	315–300	–	0.19	–
Chlorogenic acid	353–191	91.30	10.78	2.26
Prunin	433–271	–	0.13	–
Isoorientin	447–327	–	–	–
Quercitrin	447–301	1.32	–	–
Rutin	609–301	–	0.19	20.36
Total		100	100	100

Note: –, not detected.

sizes above 0.3 mm appeared to be statistically different from the others.

Setting a given extraction time, a clear trend of particle size as a function of fractional mass distribution is apparent. PCA was accordingly applied, and indicated that a total variance of 79.9% was associated with the three major groups: one accounted for particles below 0.2 mm in diameter, a second was characterised by particles between 0.2 and 0.3 mm, and another included particles above 0.3 mm. These three groups could be pinpointed for all three plants – and a similar performance was associated with each plant, concerning the mass distribution.

When plants were considered separately, Kruskal–Wallis' test indicated differences arising throughout extraction time: agrimony appeared as different from either sage or savoury at all times, whereas the latter two were similar throughout extraction time. Meanwhile, Friedman's test unfolded significant differences between extraction times; this is why Wilcoxon's test was applied, so as to understand which times accounted for that difference for each plant separately. In the case of agrimony, the effect of extraction time allowed identification of three major groups: a first one associated with 1 and 2 min, a second with 3 to 9 min, and a third with 10 to 15 min. For sage, only 1 min stood out, and both 1 and 15 min appeared different in the case of savoury.

The phenolic compositions of the extracts, by 2 min of extraction and irrespective of particle size range, as obtained by LC–MS/MS, are depicted in Table 2. The identification of such compounds was achieved by comparison with chromatographic grade standards, injected previously using the same separation and detection conditions. Our in-house library of phenols contains 33 compounds – 13 of which could be found and identified in the extracts (Table 2); chlorogenic and caffeic acids were the most abundant, in agrimony and sage/savoury, respectively. Note that the majority of phenolic compounds are not highly soluble in water at room temperature, yet they were found in our extracts – because high temperature aqueous extraction was employed. The aforementioned compounds were already found in aqueous extracts of plants such as green tea (Bastos et al., 2007), yerba maté (Bastos et al., 2007) and *Mentha cervina* (Politi et al., 2008).

Conclusions

In general, statistically significant differences exist, in terms of total antioxidant capacity, among the three plants at each specific particle size range. Regarding extraction of antioxidants from the powdered plant material via boiling water, an exposure period of

served only at 2 min (and this justifies the choice below of 2 min-extracted samples, for phenolic compound profiling). For 1 and 4 min, three different groups could in addition be pinpointed; one related to sizes below 0.2 mm, a second was associated with intermediate sizes (between 0.2 and 0.3 mm), and a third encompassed sizes above 0.3 mm; for 3 min, and from 5 to 15 min, only

5 min appears sufficient to assure that most antioxidant power is recovered. On the other hand, an indicative particle size of 0.2 mm, which is the smallest obtained via conventional coffee mill grinding, is sufficient to assure acceptable rates of extraction. The extraction phenomenon follows the typical asymptotic exponential behaviour predicted by Fick's law, with initial rates of extraction in the range of 0.3–1.6 g l^{-1} of equivalent ascorbic acid min^{-1}.

The data generated and the model proposed are innovative, and relevant to rational attempts to predict the antioxidant response of plant extracts, and how the degree of division of the plant material affects it.

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