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Organic Acids of Plants and Mushrooms: Are they Antioxidants?

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ABSTRACT

In recent years, it has become evident that significant health risks and benefits are associated with dietary food choice. This association is often attributed to the antioxidants contained in fruits and vegetables. Among those phytochemicals, organic acids may contribute to the protection against various diseases involving oxidative processes, due to their antioxidant potential. In addition, these compounds are well-known for their determinant role in maintaining fruits and vegetables quality and organoleptic characteristics and have also been used in their quality control. Ascorbic acid is, probably, the most widely distributed water soluble antioxidant in plant foods, but other organic acids, like oxalic, tartaric, malic, citric and succinic, can also act as antioxidants. In this work, it will be highlighted the potential of organic acids from several matrices (fruits, vegetables, mushrooms and medicinal plants) as antioxidants.

Keywords: antioxidant activity, *Brassica* genus, *Cydonia oblonga*, edible mushrooms, organic acids, *Rumex induratus*
Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); **EDTA**, ethylenediaminetetraacetic acid; **HPLC-UV**, high performance liquid chromatography-ultraviolet detection; **DPPH**, 2,2-Diphenyl-1-picrylhydrazyl; **DNA**, deoxyribonucleic acid; **FRAP**, ferric reducing ability of plasma; **ROS**, reactive oxygen species; **RNS**, reactive nitrogen species; **X/XO**, xanthine/xanthine oxidase

CONTENTS

INTRODUCTION

Many of the beneficial effects in human health resulting from the consumption of vegetables are strongly associated with their content in antioxidants, which can protect from a number of diseases, from atherosclerosis to cancer.

Within phytochemicals that contribute to this activity, some groups, such as flavonoids, are, today, almost unequivocally regarded as strong antioxidants.

However, when it comes to organic acids, besides the well known case of ascorbic acid (vitamin C), the attribution to these compounds of antioxidative behaviour is sometimes doubted and looked down.

In this review, we intend to present some works in which organic acids content of many crops and relation with their antioxidant activity was pursued. Studies involving similar experimental conditions were preferred, thus allowing an easier comparison of results.

CHEMISTRY OF ORGANIC ACIDS

Organic acids are water-soluble, colourless liquids or solids with relatively low melting temperatures. With the exception of α-keto acids, that readily undergo decarboxylation, these compounds are generally stable. The chemical structures of the main organic acids in nature can be found in **Fig. 1**.

Ascorbic, shikimic and quinic acids are quite widespread through a number of species and ascorbic acid, a compound in lactone form, is universal in plants (Naidu 2003). Shikimic and quinic acids, two cyclohexane carboxylic acids, are very important as they are precursors of aromatic compounds in plants.

The simplest monocarboxylic acid is formic, followed by acetic. Acetic acid may be considered the most important one, once it is a universal precursor of lipids, fatty acids and other organic compounds, when as acetyl coenzyme A, together with malonyl coenzyme A, the active forms. In what concerns to dicarboxylic acids, the simplest one is oxalic, followed by succinic, whose unsaturated derivatives are fumaric and maleic acids, two geometrical isomers, with fumaric corresponding to the *trans* and maleic to the *cis* one.

Malic acid is equivalent to monohydroxysuccinic acid, while tartaric acid is the dihydroxylated form of succinic

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Invited Review

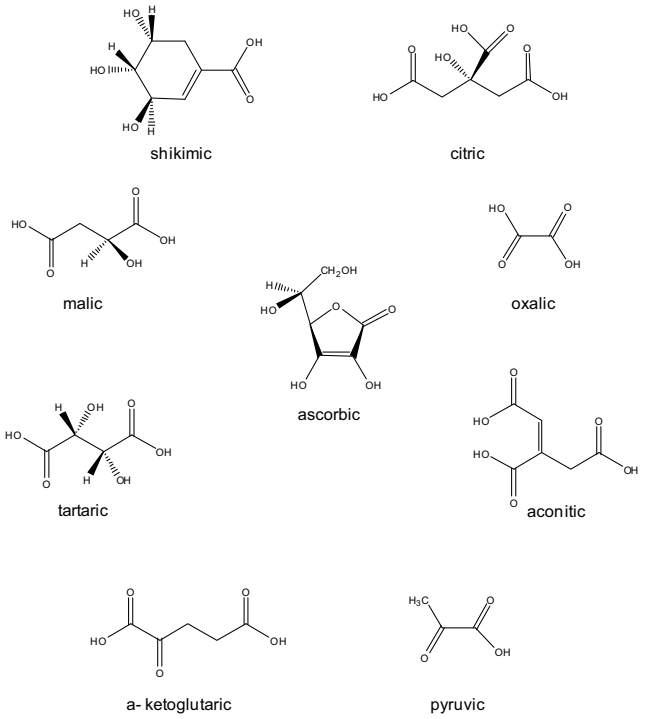


Fig. 1 Some of the main organic acids in plant crops.

acid. The keto acid corresponding to succinic is oxaloacetic acid.

One of the most widely known tricarboxylic acid is citric, which, together with its isomer, isocitric acid, and its dehydrated form, aconitic acid, participates in the tricarboxylic acid cycle. In citric acid, no center of asymmetry is found, while isocitric presents two, thus existing in four optical isomeric forms, from which only one is known to occur in nature.

Contrarily to animals or microorganisms, plants present a metabolism that allows them to accumulate organic acids in the cell vacuole, sometimes reaching considerable concentrations. In fact, the acidity of practically all edible fruits is due to this accumulation, a phenomenon which can be equally observed in the leaves of some species (Kluge and Ting 1978).

ANTIOXIDANTS

Nowadays there is increasing evidence that antioxidants in foods and beverages play an important role in the maintenance of health and prevention of disease. These antioxidants are believed to play a very important role in the body defence system against the various aggressions we encounter daily, such as the reactive oxidant species that are generated during several physiological and pathological processes (Cantuti-Castelvetri *et al.* 2000). From a food technology point of view, addition of antioxidants minimizes rancidity, retards the formation of toxic oxidation products, maintains nutritional quality and increases shelf-life (Jadhav *et al.* 1995).

A broad definition of an antioxidant may be "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate", with "oxidizable substrate" being almost every molecules found in foods and in living tissues, including proteins, lipids, carbohydrates and DNA (Halliwell *et al.* 1995).

A compound exerting antioxidant actions may do so either by inhibiting the generation of reactive species, or by directly scavenging free radicals. Additionally, an antioxidant might act indirectly by raising the levels of endogenous antioxidant defences *in vivo* (e.g. by upregulating the expression of the genes encoding superoxide dismutase, catalase or glutathione peroxidase) (Halliwell *et al*. 1995).

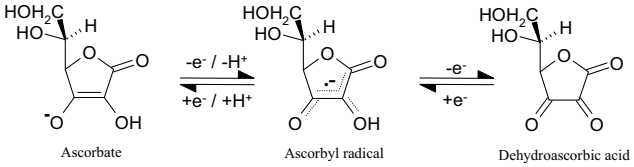


Fig. 2 Oxidation of ascorbate to give the ascorbyl radical and dehydroascorbic acid.

When reactive species, like those of oxygen or nitrogen (ROS or RNS), are produced in excess, some pathological conditions, related to the impairment of the oxidative/antioxidative balance in favour of the former, may occur as a consequence of the oxidative stress that takes place. Examples of ROS include the free radicals superoxide anion ($O_2^{\bullet-}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl ($HO^{\bullet}$) and the non radical species singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and lipid hydroperoxides. The RNS of primary concern are nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$), resultant from the combination of $O_2^{\bullet-}$ and $^{\bullet}NO$. Other potentially important RNS include nitrogen dioxide radical ($NO_2^{\bullet}$), nitrosonium (NO^+) and nitronium (NO_2^+) ions (Seabra *et al*. 2006).

The antioxidant activity of ascorbic acid is deeply related to its chemistry: when involved in radical scavenging it gives a single electron to the free radical species, thus originating the less reactive ascorbyl free radical. This ascorbyl radical can be reduced back to ascorbic acid or oxidized to dehydroascorbic acid (**Fig. 2**), which, in turn, can be reduced to ascorbic acid by reducing agents such as glutathione (Seabra *et al.* 2006).

Although ascorbic acid is the most studied organic acid, other compounds of this group can also be regarded as antioxidants. As stated earlier, oxalic acid is the simplest dicarboxylic acid. The most relevant chemical impact of this compound is related to its strong chelating ability for multivalent cations. Other carboxylic acids, such as tartaric, malic, citric, succinic and hydroxyglutaric, behave as antioxidants because they also have the ability to chelate metals. They are, therefore, classified as "preventive" or synergistic (Seabra *et al.* 2006).

BRASSICA SPECIES

Brassica vegetables, including all cabbage-like ones, are widely consumed throughout the world and are very important in human nutrition. They are reported to reduce the risks of some cancers, especially due to its content of glucosinolates and their derived products (Park and Pezzuto 2002; Chun *et al*. 2004), although phenolic compounds are also considered to contribute to this capacity (Hollman *et al*. 1996; Galati and O'Brien 2004). Some *Brassica oleracea* varieties, namely cauliflower (Proteggente *et al.* 2002; Llorach *et al*. 2003), broccoli (Kurilich *et al*. 2002; Proteggente *et al.* 2002; Ninfali and Bacchiocca 2003; Lin and Chang 2005) and several cabbages (Vinson *et al.* 1998; Chu *et al*. 2002; Proteggente *et al.* 2002) have already been studied for their antioxidant capacity in different experimental models, although no conclusion about the organic acids contribution was postulated.

Brassica oleracea L. var. *costata* DC (tronchuda cabbage)

1. Internal leaves

Tronchuda cabbage internal leaves presented a chemical profile composed by seven identified organic acids: aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids (**Fig. 3A**). Quantitatively, the aqueous lyophilised extract exhibited a high content of organic acids (*ca*. 23 g/kg), with

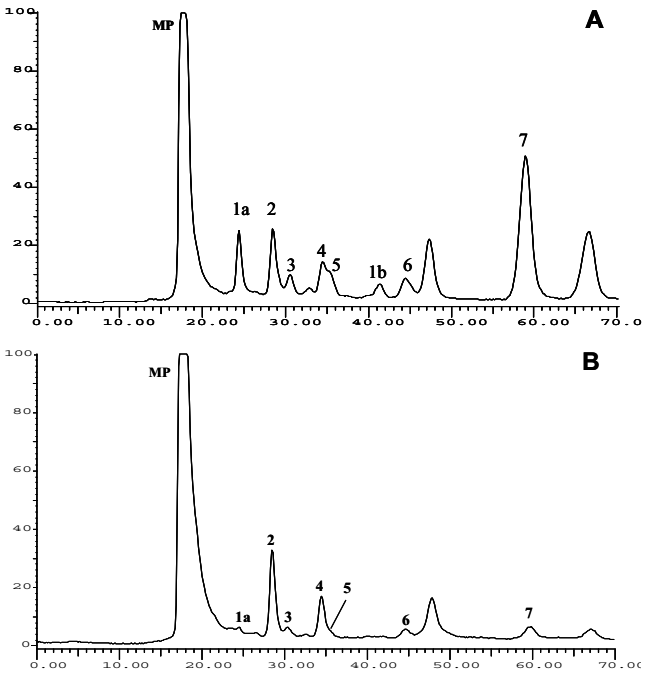


Fig. 3 HPLC-UV organic acid profile of tronchuda cabbage (**A**) internal and (**B**) external leaves aqueous extracts. Detection at 214 nm. Peaks: (MP) mobile phase; (1a and 1b) aconitic acid isomers; (2) citric acid; (3) ascorbic acid; (4) malic acid; (5) quinic acid; (6) shikimic acid; (7) fumaric acid. From **Ferreres F, Sousa C, Vrchovská V, Valentão P, Pereira JA, Seabra RM, Andrade PB** (2006a) Chemical composition and antioxidant activity of tronchuda cabbage internal leaves. *European Food Research and Technology* **222**, 88-98, ©2006, with kind permission of Springer Science+Business Media.

citric acid representing *ca*. 43% of total identified compounds, followed by the pair malic plus quinic acids (*ca*. 28% of total acids). Shikimic acid was the one present in minor amounts, accounting for ca. 0.2% of total acids (Ferreres *et al.* 2006a).

Regarding its antioxidant activity, this matrix was tested against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), $O_2^{\bullet -}$, and $HO^{\bullet}$ and also for its xanthine oxidase (XO) inhibitory activity.

In DPPH radical assay, internal leaves revealed an IC_{25} of 1192 μg/mL and a value for IC_{25} against $O_2^{\bullet -}$ equal to 101 μg/mL. The extract was also tested against $HO^{\bullet}$, where it revealed an IC_{25} of 27 μg/mL, and weak inhibition of XO with an IC_{10} of 273 μg/mL (Ferreres *et al*. 2006a) (**Table 1**). The IC_{25} values found against superoxide and hydroxyl radicals were quite low, which could be the result of the presence of almost 50% of citric acid in samples composition. With just one sample, the correlation of organic acids with the antioxidant activity of the extract is a challenging exercise, which would make necessary to remove these compounds from the matrix and retesting the material, in order to search for a reduction of activity. However, the removal of organic acids and subsequent testing of these molecules would be equally troublesome, as their activity is highly affected by synergism and antagonism phenomena.

The existence of additional works, aiming the study of organic acids and antioxidant activity of different parts of this species, and even of other related ones, allows inferring about the influence of both the qualitative and quantitative profiles of organic acids in the antioxidant potential of matrices.

2. External leaves

External leaves of *B. oleracea* var. *costata* revealed the presence of the same organic acids already identified in internal leaves (**Fig. 3B**). However, when it comes to quantitative profile, some differences can be pointed. In internal leaves ascorbic acid represented 26% of total compounds (Ferreres *et al.* 2006a). In external leaves, however, ascorbic acid was the major compound, corresponding to 35% of the organic acids found in the matrix, followed by the pair malic/quinic acids (33.7%) and citric acid (31.8%) (Ferreres *et al*. 2006a). Given the well established role of ascorbic acid as an antioxidant, this could explain, at least partially, the stronger activity of external leaves.

In fact, external leaves revealed to be more active than internal ones in all assays performed: DPPH (440 μg/mL *vs* 1192 μg/mL), superoxide radical (43 μg/mL *vs* 101 μg/mL) and hydroxyl radical (10 μg/mL *vs* 27 μg/mL) (**Table 1**).

Some caution must be taken when extrapolating the influence of organic acids in the antioxidant activity of these samples, as the authors also identified a different profile of phenolic compounds in both internal and external leaves, with the first presenting mainly phenolic acids and the later, flavonoids, whose antioxidant activity is also well recognized.

3. Seeds

Tronchuda cabbage seeds presented an organic acids profile constituted by aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids, totalizing *ca*. 16 g/kg (Ferreres *et al*. 2007). This content is similar to that previously found in the leaves (Ferreres *et al*. 2006a; Vrchovska *et al.* 2006). However, seeds exhibit a distinct quantitative profile, with ascorbic acid being the main compound, representing about 52% of total identified organic acids, followed by citric acid (28% of compounds).

These quantitative differences seem to exert a strong influence in the antioxidant activity displayed, as seeds were considerably stronger in all assays besides superoxide, for which the IC_{25} found was similar to external leaves (**Table 1**). Comparatively with the leaves of the same species, both internal and external, seeds revealed to be the material with the strongest antioxidative properties *in vitro* and, simultaneously, the one with higher amounts of ascorbic acid (Ferreres *et al.* 2007).

These results make sense as seeds constitute a reserve for many storage compounds, namely lipids, which need to be protected from oxidative stress, contributing to the viability of seeds and their rapid germination when oxygen demand is high (Andarwulan and Wattimena 1999; Randhir and Shetty 2003; Sattler *et al.* 2004).

4. Sprouts

The screening of organic acids showed the presence of oxalic, fumaric, citric, malic, pyruvic, shikimic and aconitic acids (Sousa *et al.* 2007). With the exception of oxalic and pyruvic acids, described for the first time in sprouts of *B. olearacea* var. *costata*, the remaining compounds had already been identified in leaves and seeds (Ferreres *et al.* 2006a, 2007).

In this work, besides the identification of the organic acids and its quantification, the authors tried to establish the qualitative and quantitative changes in sprouts' chemical composition through a twelve day germination period. The total organic acids content increased *ca*. 46% from 45.7 g/kg, after 2 days of germination, to 66.9 g/kg on day 12 (**Fig. 4**) and changes in individual compounds were also monitored (**Fig. 5**). This increase may be explained by the increased metabolic activity of the seeds, which rapidly resume the glycolytic and the tricarboxylic acid cycle and the β-oxidation of fatty acids after germination (Li *et al*. 2005; Pracharoenwattana *et al.* 2005).

Seeds had an amount of organic acids lower than that present in the sprouts. Ascorbic acid, one of the major compounds found in the seeds (*ca*. 13.3 g/kg) was present only in vestigial amounts in the sprouts, being greatly depleted since the beginning of germination, may be due to its antioxidant effect (Sousa *et al.* 2007).

Citric and malic acids were the major organic acids

Table 1 Organic acids composition and antioxidant activity of some *Brassica* species. [a] malic + quinic acids; [b] citric + ketoglutaric acids

	Organic Acids[1]									Aox. activity[2]	
	Aconitic	**Ascorbic**	**Citric**	**Fumaric**	**Malic**	**Oxalic**	**Pyruvic**	**Shikimic**	**Total**	**Assay**	**IC**
B. oleracea* var. *costata											
										DPPH•	IC_{25} = 1192
Internal leaves[3]	0.191	6.020	9.975	0.407	6.626	-	-	0.035	23.254	O_2•-	IC_{25} = 101
	(0.004)	(0.143)	(0.068)	(0.002)	(0.165)[a]			(0.001)		HO•	IC_{25} = 27
										DPPH•	IC_{25} = 440
External leaves[3]	0.022	8.754	8.131	0.014	8.605	-	-	0.020	25.545	O_2•-	IC_{25} = 43
	(0.008)	(0.518)	(0.421)	(0.000)	(0.975)[a]			(0.001)		HO•	IC_{25} = 10
										DPPH•	IC_{25} = 64
Seeds[4]	0.170	8.546	4.685	0.039	3.049	-	-	0.018	16.507	O_2•-	IC_{25} = 118
	(0.003)	(0.438)	(0.197)	(0.001)	(0.222)[a]			(0.000)		HO•	IC_{25} = 4
Sprouts[5]	0.081	-	12.471	0.820	55.205	1.386	2.012	0.158	72.133	-	-
	(0.008)		(0.595)	(0.010)	(1.377)	(0.085)	(0.092)	(0.010)			
***B. rapa* var. *rapa*[6]**											
Flower buds	6.608	-	19.647	1.232	19.504	-	-	0.065	47.055	-	IC_{25} = 470
	(0.359)		(2.087)[b]	(0.009)	(1.533)			(0.009)			
Leaves and stems	3.219	-	14.341	0.865	32.471	-	-	0.212	51.108	DPPH•	IC_{25} = 560
	(0.209)		(0.618)[b]	(0.011)	(0.827)			(0.022)			
Roots	0.642	-	5.200	0.945	29.255	-	-	0.065	36.106	-	IC_{25} = 1440
	(0.016)		(0.156)[b]	(0.019)	(0.270)			(0.003)			
***Brassica* inflorescences[7]**											
										DPPH•	IC_{50} = 754
B. oleracea var.	0.427	-	27.926	1.116	16.734	-	2.684	0.137	49.024	O_2•-	IC_{25} = 349
costata	(0.008)		(0.167)	(0.004)	(0.079)		(0.006)	(0.001)		HO•	IC_{25} = 172
										HOCl	IC_{10} = 639
										DPPH•	IC_{50} = 565
B. oleracea var.	0.097	-	48.373	0.018	108.159	-	5.687	0.765	163.098	O_2•-	IC_{25} = 281
acephala	(0.003)		(1.846)	(0.000)	(0.445)		(0.077)	(0.006)		HO•	IC_{25} = 10
										HOCl	IC_{10} = 1186
										DPPH•	IC_{50} = 774
B. rapa var. *rapa*	0.0423	-	13.177	1.261	22.350	-	1.123	0.069	38.022	O_2•-	IC_{25} = 363
	(0.000)		(0.075)	(0.002)	(0.021)		(0.001)	(0.001)		HO•	IC_{25} = 12

[1] mean (SD) of three determinations, in g/Kg (dry basis). [2] µg/mL. [3] from Ferreres *et al*. 2006a. [4] from Ferreres *et al*. 2007. [5] from Sousa *et al*. 2007.
[6] from Fernandes *et al*. 2007. [7] from Sousa *et al*. 2008.

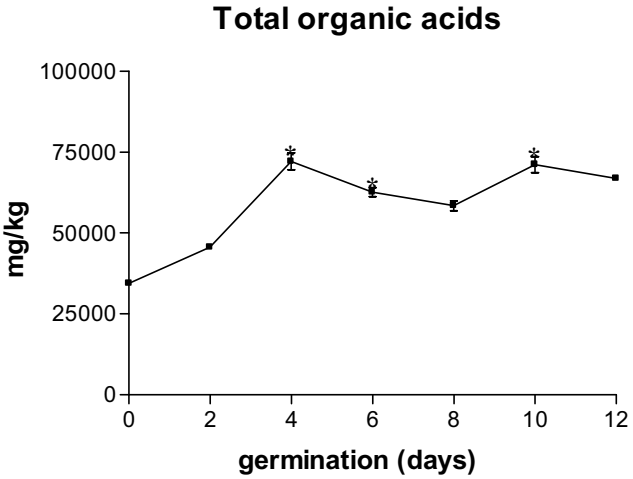


Fig. 4 Evolution in total organic acids content of tronchuda cabbage sprouts with germination time. * $p<0.05$, compared with the previous germination time. From **Sousa C, Lopes G, Pereira DM, Taveira M, Valentão P, Seabra RM, Pereira JA, Baptista P, Ferreres F, Andrade PB** (2007) Screening of antioxidant compounds during sprouting of *Brassica oleracea* L. var. *costata* DC. *Combinatorial Chemistry & High Throughput Screening* **10**, 377-386, ©2007, with kind permission of Bentham Science Publishers.

found in sprouts at all germination times. Citric acid, accounting for more than 15% of the total organic acids content, decreased ca. 37% from day 2 to day 12 (**Fig. 5**). However, despite the significant decrease between days 2 and 4 and increase between days 6 and 8, its variation during the germination period did not show a clear tendency. Malic acid increased from 27.9 g/kg (61% of the total organic acids) on day 2 to 51.6 g/kg (77% of the total organic acids) on day 12, which represents an increase of 85% along the studied germination period. This was the organic acid that registered the highest raise, which can indicate that besides oxidation, the glyoxylate cycle in which fatty acids are converted to sugars having malate as an intermediate product, was active (Eastmond and Graham 2001).

Differently from other tronchuda cabbage materials presented until now, in the referred work (Sousa *et al*. 2007) no antioxidant activity was evaluated. However, this parameter should be assessed in a near future as a deep knowledge about the variations on organic acids, at both qualitative and quantitative levels, was achieved with this work. Consequently, a connection with antioxidant activity could be established, given the great amount of information available, namely the behaviour of each organic acid through time.

Brassica rapa var. *rapa* L. (turnip)

In the work of Fernandes *et al*. (2007), three turnip edible parts (leaves and stems, flower buds and roots) were analysed, revealing similar organic acids composition. Generally, aconitic, citric, ketoglutaric, malic, shikimic and fumaric acids were detected in all parts.

The quantification of the identified compounds revealed a high organic acids content (ranging from 36 to 51 g/kg, dry basis), with higher amounts of these compounds in flower buds and in leaves and stems than in the roots, in a general way. Three distinct quantitative organic acids profiles were obtained. Flower buds and leaves and steams presented an equivalent amount of organic acids (47.1 and 51.1 g/kg, respectively), with citric acid accounting for 41.8% in the first and 28.1% in the later. Roots were the sample presenting lower organic acids content, with citric acid constituting only 14.4% of the total amount. As it will be dis-

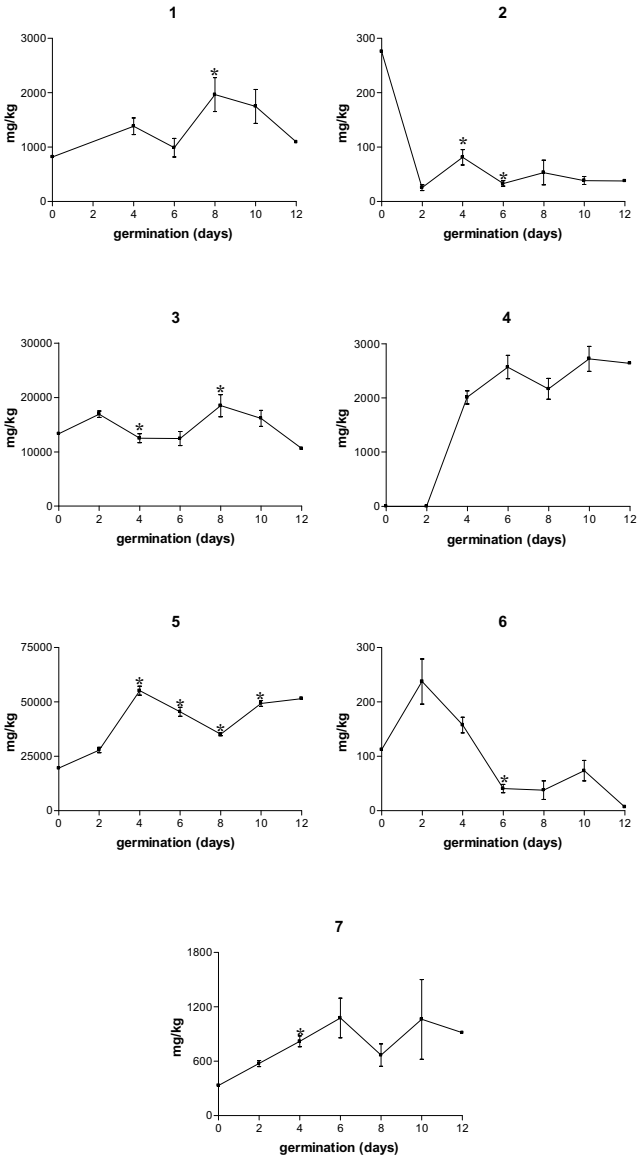


Fig. 5 Changes in individual organic acids of tronchuda cabbage sprouts with germination time. Numbers of panels refer to the number of compounds (1 - oxalic acid; 2 - aconitic acid; 3 - citric acid; 4 - pyruvic acid; 5 - malic acid; 6 - shikimic acid; 7 - fumaric acid). * $p<0.05$, compared with the previous germination time. From **Sousa C, Lopes G, Pereira DM, Taveira M, Valentão P, Seabra RM, Pereira JA, Baptista P, Ferreres F, Andrade PB** (2007) Screening of antioxidant compounds during sprouting of *Brassica oleracea* L. var. *costata* DC. *Combinatorial Chemistry and High Throughput Screening* **10**, 377-386, ©2007, with kind permission of Bentham Science Publishers.

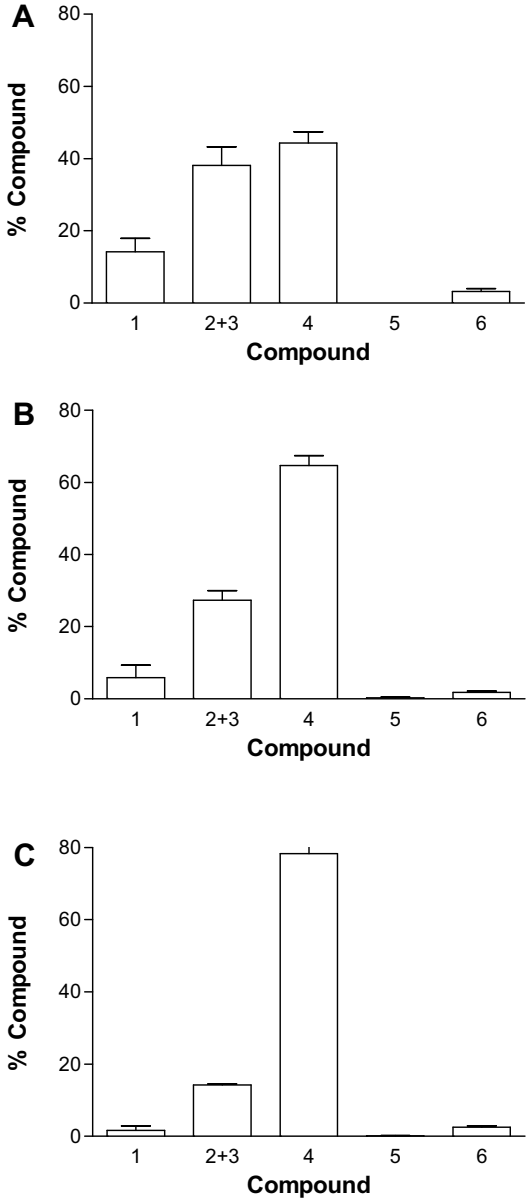


Fig. 6 Organic acids profile of turnip (**A**) flower buds, (**B**) leaves and stems and (**C**) roots. Values represent mean, and standard error bars are on the top of each column. (1) aconitic acid; (2) citric acid; (3) ketoglutaric acid; (4) malic acid; (5) shikimic acid; (6) fumaric acid. From **Fernandes F, Valentão P, Sousa C, Pereira JA, Seabra RM, Andrade PB** (2007) Chemical and antioxidative assessment of dietary turnip (*Brassica rapa* var. *rapa* L.). *Food Chemistry* **105**, 1003-1010, ©2007, with kind permission of Elsevier Ltd.

cussed later, this quantitative profile may be responsible, at least in part, for the results obtained in antioxidant activity assays (**Fig. 6**, **Table 1**).

Turnip edible parts displayed a scavenging activity against DPPH that was concentration-dependent. IC_{25} was determined in order to compare the results, once it was not possible to reach 50% scavenging activity with all samples and considering that it corresponds approximately to the middle activity of each curve. The flower buds revealed to be the most active part (mean IC_{25} of 470 µg/mL), followed by the leaves and stems (mean IC_{25} at 560 µg/mL). Turnip roots showed a significantly lower antioxidant capacity, with a mean IC_{25} of 1440 µg/mL (Fernandes *et al*. 2007).

The antioxidant potential exhibited by the different turnip edible parts is obviously determined by their composition and, in all samples, the values of IC_{25} in DPPH assay were correlated with citric acid content. The role of phenolics in the antioxidant activity displayed cannot be ignored: hydroxycinnamic acids and their derivatives (Plumb *et al.* 1997; Fukumoto and Mazza 2000) and flavonol glycosides (Tang *et al*. 2001; Braca *et al*. 2003) are known to exert antioxidant activity and they were also present in turnip.

B. oleracea L. var. *acephala* (kale)

In the work of Sousa and colleagues (2008), two *Brassica oleracea* varieties (*B. oleracea* L. var. *costata* DC and *B. oleracea* L. var. *acephala*) and *Brassica rapa* L. var. *rapa* inflorescences were studied for their chemical composition and antioxidant capacity. These three *Brassica* shared a profile composed by six organic acids (aconitic, citric, pyruvic, malic shikimic and fumaric acids), but *B. oleracea* L. var. *acephala* presented a considerably higher amount (163.1 g/kg against 49.0 g/kg and 38.0 g/kg in *B. oleracea* var. *costata* and *B. rapa* var. *rapa*, respectively). It should be emphasized that ascorbic acid, present in leaves and seeds of *B. oleracea* var. *costata* (Sousa *et al.* 2005; Ferreres *et al.* 2006a, 2007) was not detected in its inflorescences. As it

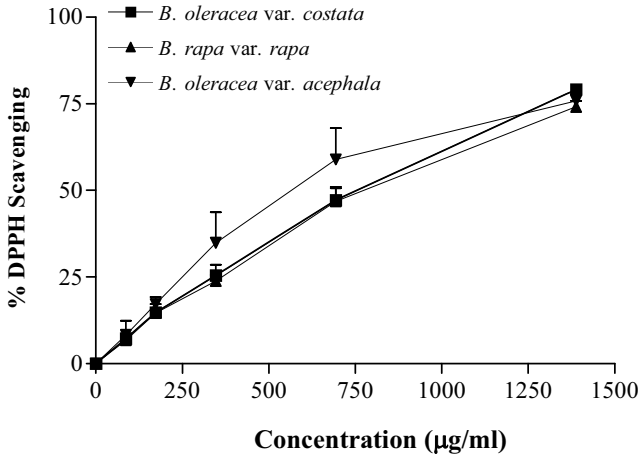
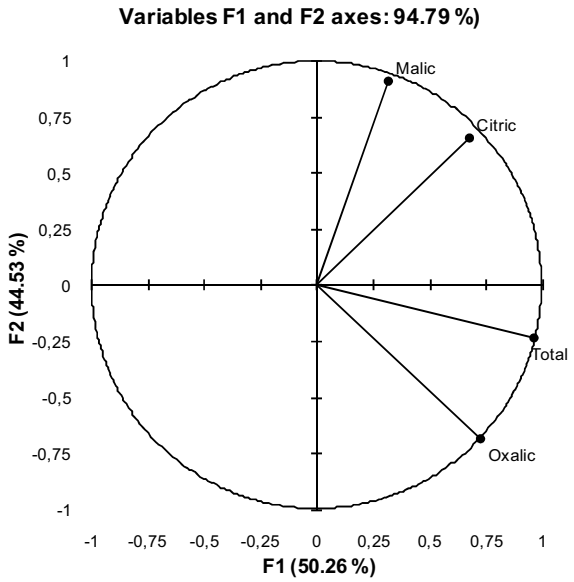


Fig. 7 Effect of *Brassica* inflorescences aqueous lyophilized extracts on DPPH reduction. Values show mean (SE) from 3 experiments performed in triplicate. From **Sousa C, Taveira M, Valentão P, Fernandes F, Pereira JA, Estevinho L, Bento A, Ferreres F, Seabra RM, Andrade PB** (2008) Inflorescences of Brassicacea species as source of bioactive compounds: A comparative study. *Food Chemistry* **110**, 953-961, ©2008, with kind permission of Elsevier Ltd.



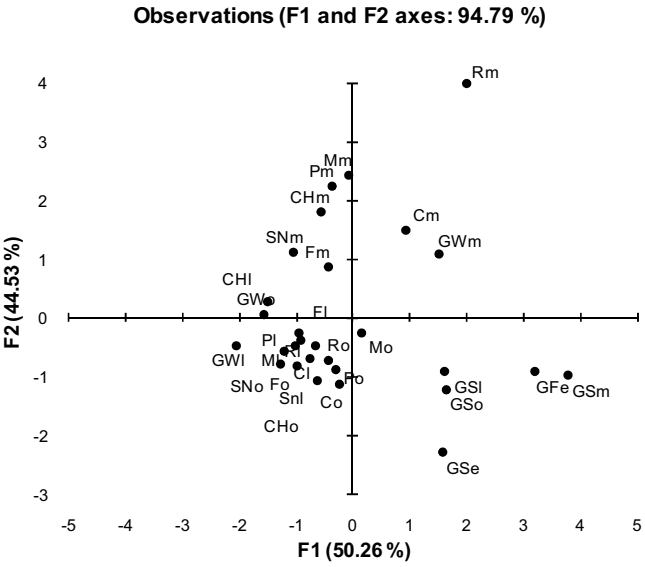


Fig. 8 Principal components diagram of the organic acids content in all analyzed samples of *Rumex induratus*: factor score plot 1-2. Components 1 and 2 accounts for 94.79 % of the total variance. Capital letters refer to origin (GF – Greenhouse Fall; GW – Greenhouse Winter; GS – Greenhouse Spring; F – Fervença; P – Pombares; M – Macedo; SN – Senhora das Neves; C – Cerejais; CH – Chãs). Lower cases refer to development stage (e – early stage; m – mid stage; o – optimal stage. From **Guerra L, Pereira C, Andrade PB, Rodrigues MA, Ferreres F, de Pinho PG, Seabra RM, Valentão P** (2008) Targeted metabolite analysis and antioxidant potential of *Rumex induratus*. *Journal of Agricultural and Food Chemistry* **56**, 8184-8194, ©2008, with kind permission of The American Chemical Society.

will be discussed later, this fact may explain some of the results found in antioxidant activity.

Each inflorescence was investigated for its capacity to act as a scavenger of DPPH radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid), exhibiting antioxidant capacity in a concentration dependent manner against all radicals.

Against DPPH, *B. oleracea* var. *acephala* revealed to have a stronger capacity (IC_{25} = 565 µg/mL) than *B. oleracea* var. *costata* (IC_{25} = 754 µg/mL) and *B. rapa* var. *rapa* (IC_{25} = 774 µg/mL), which exhibited a similar behavior (**Fig. 7, Table 1**). The fact that, from a quantitative point of view, *B. oleracea* var. *acephala* inflorescences showed the highest organic acids content (ca. 163 g/kg), corresponding to about three and four times the amount found for *costata* variety and *B. rapa* var. *rapa*, respectively, can explain, at least partially, its higher antioxidant activity.

In superoxide radical assay, the most active matrix was different when the radical was generated using a X/XO system (*B. rapa* var. *rapa* – IC_{25} = 244 µg/mL) or using a NADH/PMS one (*B. oleracea* var. *acephala* – IC_{25} = 281 µg/mL) (**Table 1**).

Against hydroxyl radical, as it had been determined for superoxide and DPPH radical, the most active matrix was the one presenting higher amounts of organic acids, *B. oleracea* var. *acephala* (IC_{25} = 10 µg/mL). When it comes to hypochlorous acid assay, the most active vegetable was *B. oleracea* var. *costata* (IC_{10} = 639 µg/mL), which was the sample with higher relative amount of citric acid (56.9%) (**Table 1**).

At this point, it should be highlighted that, in *B. oleracea* var. *costata,* ascorbic acid was present in leaves and seeds but absent in inflorescences. This may explain, in part, the weaker antioxidant activity displayed by inflorescences when compared with the other referred plant parts.

RUMEX INDURATUS

Several species of the *Rumex* (Polygonaceae) genus, namely its leaves and roots, have been used in traditional medicine for inflammation, blood purification and constipation (Medical Economics Co. 1998; Newall *et al.* 1996). However, oxalic acid intoxication, mainly in children, is a problem due to the high oxalic acid content of the species (Der Marderosian and Beutler 2002; Newall *et al.* 1996).

Guerra and colleagues (2008) proceeded to a targeted metabolite analysis, organic acids included, and evaluation of the antioxidant potential of *Rumex induratus* leaves. The HPLC-UV analysis of the aqueous lyophilized extracts revealed the presence of malic, oxalic, citric, ascorbic and shikimic acids, which were described for the first time, with the exception of oxalic acid (**Fig. 8**).

In addition, in this work the chemical composition of field samples was compared with that of greenhouse ones, both origins being tested to determinate the influence of maturation stage in the chemical composition. In a general way, field samples depicted a slight decrease in the total amount of organic acids during plant growth.

The sample chosen for antioxidant activity assays displayed a content of 182.2 g/kg of organic acids. In this sample, oxalic acid was the main organic acid (163.0 g/kg), followed by malic (18.3 g/kg) and shikimic acids (0.87 g/kg) (**Table 2**).

The lyophillized aqueous extract of *R. induratus* leaves exhibited a strong concentration-dependent antioxidant potential against DPPH (IC_{50} = 106.5 µg/mL).

Nitric oxide and hypochlorous acid can be responsible for the formation of more reactive species, such as hydroxyl radical (Halliwell *et al*. 2005). The aqueous extract of *R. induratus* leaves showed a potent scavenging activity against nitric oxide in a concentration dependent manner, with an IC_{50} of 92.7 µg/mL. The extract exhibited a lower activity against hypochlorous acid. Nevertheless, a concentration-dependent antioxidant potential was observed (IC_{20} = 171.3 µg/mL). These findings, along with the fact that *R. induratus* leaves can act as superoxide radical scavenger and xanthine oxidase inhibitor (Ferreres *et al.* 2006b) are extremely valuable: the simultaneous scavenging capacity of superoxide radical and nitric oxide can prevent the formation of peroxynitrite and ultimately, hydroxyl radical (Halliwell *et al.* 2005).

Despite the considerable number of samples used for chemical characterization, when it comes to antioxidant activity assays, only one sample was tested. As so, it is difficult to associate the activity displayed only with organic acids composition, given the fact that phenolics were also present in samples, thus contributing to antioxidant capacity. However, the contribution of organic acids to this activity cannot be despised.

CYDONIA OBLONGA MILLER (QUINCE)

Quince fruit is a pome with numerous seeds. The fruits are big and exhibit a characteristic fragrance. The pulp is acidic and astringent and, so, it is not suitable for consumption when raw. The most important utilization of this fruit is in the production of jams and jellies, which are very appreciated in many countries.

Silva and colleagues (2004) studied the organic acids composition of both fruit (pulp, peel and seed) and jam and antiradical activity was assessed against DPPH.

Pulp, peel and jams extracts presented a similar profile composed by seven identified organic acids: ascorbic, oxalic, citric, quinic, malic, shikimic and fumaric acids, which had already been described by Silva *et al*. (2002). In seed extract, only oxalic acid could not be found.

In pulp, peel and jams extracts the sum of malic plus quinic acids always represented at least 95% of the total organic acid content, with all other acids present in small

Table 2 Organic acids composition and antioxidant activity of some mushrooms and vegetable foods.

	Organic Acids[1]											Aox. activity[2]	
	Aconitic	**Ascorbic**	**Citric**	**Fumaric**	**Ketoglutaric**	**Malic**	**Oxalic**	**Shikimic**	**Succinic**	**Quinic**	**Total**	**Assay**	**IC**
												DPPH$^{\bullet}$	IC_{50} = 107
Rumex induratus[3]	-	-	-	-		0.018 (0.000)	0.163 (0.002)	0.001 (0.000)	-	-	0.182	NO$^{\bullet}$	IC_{50} = 93
												HOCl	IC_{20} = 171
Cydonia oblonga[4]													
Jam A	-	0.028 (0.000)	0.054 (0.000)	nq	-	3.921 (0.273)[a]	0.007 (0.000)	0.004 (0.001)	-	-	4.014		IC_{50} = 23
Jam B	-	0.053 (0.000)	0.078 (0.006)	-	-	5.094 (0.215)[a]	nq	0.010 (0.000)	-	-	4.235		IC_{50} = 16
Peel	-	0.187 (0.007)	0.378 (0.015)	nq	-	13.818 (0.067)[a]	0.005 (0.000)	0.052 (0.000)	-	-	14.441	DPPH$^{\bullet}$	IC_{50} = 7
Pulp	-	0.109 (0.000)	0.159 (0.001)	nq	-	16.310 (0.177)[a]	nq	0.045 (0.000)	-	-	16.624		IC_{50} = 12
Seeds	-	0.568 (0.016)	0.670 (0.019)	0.004 (0.000)	-	0.611 (0.008)	-	0.004 (0.000)	-	-	1.858		IC_{50} = 13
Amanita rubescens[5]													
Entire mushroom	-	-	11.115 (0.383)	1.804 (0.027)	1.786 (0.112)	12.386 (0.313)	1.330 (0.673)	0.021 (0.001)	0.139 (0.003)	91.331 (2.100)	119.911		IC_{25} = 303
Stipe	-	-	3.596 (0.224)	2.919 (0.024)	2.204 (0.050)	10.453 (1.486)	1.061 (0.015)	0.037 (0.001)	0.032 (0.000)	114.902 (1.799)	135.204	DPPH$^{\bullet}$	IC_{25} = 527
Cap	-	-	15.293 (0.760)	2.245 (0.059)	1.357 (0.029)	11.117 (0.179)	0.634 (0.025)	0.067 (0.003)	0.281 (0.006)	89.075 (2.842)	120.070		IC_{25} = 990
Russula cyanoxantha[5]													
Entire mushroom	-	-	3.606 (0.125)	7.274 (0.168)	-	49.985 (1.340)	0.746 (0.094)	-	-	36.193 (2.662)	97.804		IC_{25} = 835
Stipe	-	-	2.192 (0.157)	6.681 (0.136)	-	39.887 (0.728)	0.809 (0.085)	-	-	41.964 (2.738)	91.533	DPPH$^{\bullet}$	IC_{25} = 936
Cap	-	-	4.801 (0.260)	6.543 (0.151)	-	33.737 (1.050)	1.060 (0.012)	-	-	50.498 (0.803)	96.638		IC_{25} = 760
Boletus edulis[5]													
Entire mushroom	-	-	0.817 (0.052)	0.031 (0.002)	-	8.822 (0.194)	0.659 (0.013)	-	0.064 (0.001)	-	10.393		IC_{25} = 184
Stipe	-	-	3.129 (0.106)	0.028 (0.000)	-	9.242 (0.022)	0.110 (0.005)	-	0.097 (0.003)	-	12.606	DPPH$^{\bullet}$	IC_{25} = 109
Cap	-	-	2.674 (0.031)	0.258 (0.004)	-	35.122 (1.263)	1.303 (0.016)	-	0.132 (0.006)	-	39.490		IC_{25} = 77
Suillus granulatus[5]													
Entire mushroom	-	-	13.231 (0.430)	7.931 (0.430)	-	7.157 (0.712)	6.255 (0.195)	nq	0.401 (0.011)	11.389 (0.001)	46.364		IC_{25} = 196
Stipe	-	-	6.416 (0.033)	10.210 (0.036)	-	0.912 (0.065)	1.849 (0.166)	nq	0.094 (0.002)	2.879 (0.433)	22.360	DPPH$^{\bullet}$	-
Cap	-	-	15.023 (0.719)	10.740 (0.227)	-	12.420 (1.275)	2.047 (0.102)	nq	0.258 (0.016)	84.443 (1.525)	124.931		IC_{25} = 184
												DPPH$^{\bullet}$	IC_{25} = 136
Fistulina hepatica[6]	0.044 (0.005)	1.222 (0.182)	1.251 (0.152)	0.552 (0.022)	-	5.956 (0.791)	0.041 (0.000)	-	-	-	9.066	$O_2^{\bullet-}$	IC_{50} = 105
												HOCl	IC_{25} = 1458

[1] mean (SD) of three determinations, in g/Kg (dry basis). [2] µg/mL. [3] from Guerra *et al*. 2008. [4] from Silva *et al.* 2004. [5] from Ribeiro *et al*. 2008.[6] from Ribeiro *et al*. 2007. [a] – concentration in mg/mL.

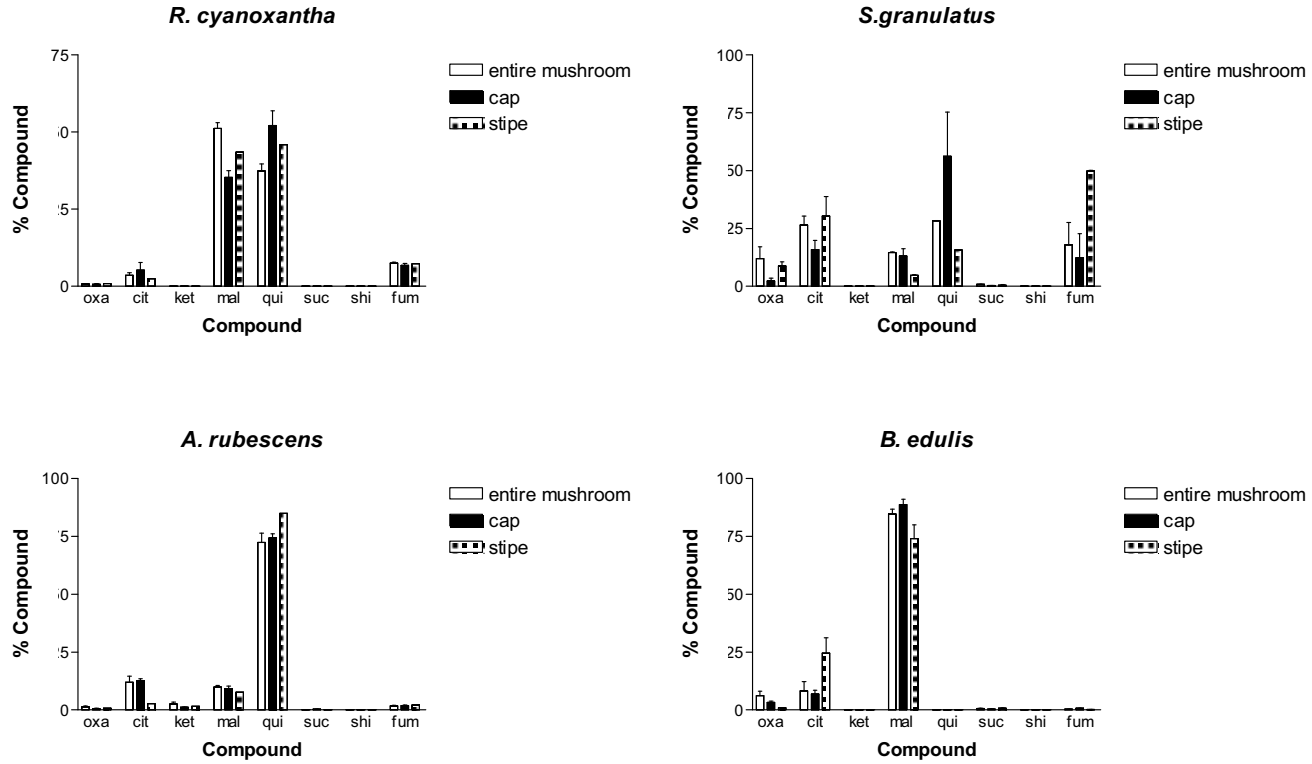


Fig. 9 Organic acids profile of edible mushrooms species. Values represent mean and standard error bars are on the top of each column. Abbreviations: (oxa) oxalic acid; (cit) citric acid; (ket) ketoglutaric acid; (mal) malic acid; (qui) quinic acid; (suc) succinic acid; (shi) shikimic acid; (fum) fumaric acid. From **Ribeiro B, Lopes R, Andrade PB, Seabra RM, Gonçalves R, Baptista P, Quelhas I, Valentão P** (2008) Comparative study of phytochemicals and antioxidant potential of wild edible mushrooms caps and stipes. *Food Chemistry* **110**, 47-56, ©2008, with kind permission of Elsevier Ltd.

amounts. Seed extract was very distinct from the others, with the sum of malic plus quinic acid representing only 33% of the total content. Citric and ascorbic acids were also present in high percentages (36 and 31%, respectively). The organic acid total content of seed extract was the lowest among all matrices (**Table 2**).

When considering the antioxidant activity displayed by these samples against DPPH radical, it is important to emphasize that in this work, both total methanolic extract and organic acids fraction were analysed, which may provide further data on the influence of organic acids in antioxidant activity.

Peel extract was the one that had the strongest antiradical activity (IC_{50} of 6.9 mg/mL), followed by pulp and seed extracts with very similar activities (IC_{50} of 11.6 and 12.9 mg/mL, respectively). The IC_{50} values of quince pulp, peel and jams organic acid extracts were correlated with the ascorbic acid content and citric acid contents (**Table 2**).

Due to the complex composition of both fruit and jam, interactions between different antioxidant components are likely important regarding the overall antioxidant activity of quince fruit and jam. The antioxidant activities of the analyzed samples cannot only be attributed to their phenolic and/or organic acid contents, but may result from the action of different compounds present in quince fruit and jam and to possible synergic and antagonist effects that take place. Additionally, the fact that the whole methanolic extract, in all samples, displayed an activity different from the sum of the analysed fractions, namely phenolics and organic acids, points to the occurrence of interactions between the present compounds in what concerns to antioxidant activity.

MUSHROOMS

Amanita rubescens; *Russula cyanoxantha*; *Boletus edulis*; *Suillus granulatus*

Mushrooms have been used as food and food-flavouring material in soups and sauces for centuries, due to their unique and characteristic flavour. Their high amounts of proteins, carbohydrates and fibres and low fat contents is often referred in literature in relation to their nutritional value. Furthermore, they contain significant levels of vitamins, namely thiamine, riboflavin, ascorbic acid and vitamin D2, as well as minerals (Mattila *et al.* 2000). Regarding their medicinal value, mushrooms revealed to be effective as antitumor, antibacterial, antiviral, haematological and in immunomodulating treatments (Wasser and Weis 1999; Yang *et al*. 2002).

Trás-os-Montes region (northeast of Portugal) is recognized as one of the richest regions of Europe in wild edible mushroom species, of considerable gastronomic relevance. *Russula cyanoxantha*, *Amanita rubescens*, *Suillus granulatus* and *Boletus edulis* are among the more common and eaten species.

In the work of Ribeiro *et al.* (2008), the four above mentioned mushrooms species were studied for their chemical composition, including organic acids. The organic acids profile showed that all of the species contained oxalic, citric, malic and fumaric acids. Some also exhibited ketoglutaric, quinic, succinic and shikimic acids. In a general way, the highest total organic acids content was found in *A. rubescens*, followed by *R. cyanoxantha*, *S. granulatus* and *B. edulis.*

In *A. rubescens*, citric, ketoglutaric, succinic, oxalic, malic, quinic, shikimic and fumaric acids were identified. The authors stated that organic acids weren't accumulated at a special part, since for each one of the three *A. rubescens* samples tested, the material presenting the highest organic acids content was different. In the cap, they were found significantly higher citric acid contents than in the stipe. On the other hand, quinic acid concentrations were significantly higher in the stipe (**Fig. 9, Table 2**).

R. cyanoxantha showed a profile composed by citric, oxalic, quinic, malic, and fumaric acids. The results indicated that these compounds accumulate mainly in the cap. In what concerns to the quantitative profile, the different mushroom parts were found to be similar, presenting quinic

and malic acids as the major compounds, with oxalic acid being the minor one. However, some differences were observed: the cap showed a tendency for higher concentrations of citric and quinic acids, but its malic acid content was significantly smaller than the one exhibited by the stipe (**Fig. 9, Table 2**).

The analysis of *S. granulatus* samples yielded the identification of citric, malic, quinic, succinic, shikimic, oxalic and fumaric acids, with clear evidence that the cap presented the highest contents of these compounds. The analysis of the mushrooms' quantitative profiles obtained made it possible to realize that succinic and shikimic acids were those appearing in lower quantities and that the major compounds differed according to the different mushroom parts (**Fig. 9, Table 2**): fumaric and citric acids in stipe and quinic acid in cap. Malic, oxalic and succinic acids presented important differences in their relative amounts in the different mushrooms materials.

B. edulis presented an organic acids profile composed by oxalic, citric, malic, succinic and fumaric acids. The total organic acids amount was higher in the cap, while stipe and entire mushrooms contained similar amounts of these compounds. These results indicate that, like in *R. cyanoxantha* and *S. granulatus*, organic acids of *B. edulis* species are preferably fixed in the cap. In a general way, the quantitative profile (**Fig. 9, Table 2**) showed malic acid as the major compound, while succinic and fumaric acids only appeared in low amounts. Moreover, cap and stipe had a tendency to concentrate malic and citric acids, respectively.

The antioxidant potential of the different mushrooms materials was evaluated by their DPPH scavenging effect, which was, in a general way, concentration-dependant. The IC_{25} values were used to compare their antioxidant properties.

In *R. cyanoxantha* species, results found for antioxidative activity did not follow the total organic acids contents order. Nevertheless, citric acid concentration was considerably higher in the cap, with this material presenting higher antioxidant activity than the stipe, which contained the lowest amount of this acid. These data suggest that citric acid may be important for the antioxidant capacity of the species. The alkaloids could also have a relevant role, since their total contents followed the same order as that of the antioxidant capacity.

A. rubescens antiradical activity followed the order entire mushroom > stipe >> cap. Neither alkaloids nor organic acids contents displayed this sequence. However, the stipe contained higher organic acids content than cap which may give a major contribution for the antioxidant capacity.

S. granulatus antioxidant potential revealed the same sequence found for *R. cyanoxantha*: cap > entire mushroom > stipe. This order corresponded to that of the total organic acids amount in the sample. In addition, the content of the pair malic plus quinic acids followed the order referred above for the antioxidant activity, which strongly suggests that they may be important for the antioxidative properties of the species.

When the antioxidant potential of *B. edulis* species was assayed, the cap displayed the highest capacity, followed by the stipe and entire mushroom. These results are particularly relevant as this order suggests that some kind of antagonism could occur between some compounds of the cap and the stipe, in order to decrease the antioxidant potential of the entire mushroom. In addition, the organic acids profile could be responsible, in part, for the antioxidant activity of *B. edulis* species, given the fact that total organic acids content followed the sequence observed for the antioxidant activity. Malic acid accumulated preferentially in the cap which presented the highest antioxidant activity. Thus, it could give an important contribution to this capacity.

Fistulina hepatica

Fistulina hepatica mushroom, commonly known as beefsteak fungus, usually is a saprobic and sometimes a parasitic fungus that lives on the wood of hardwoods (especially oaks and chestnut). Its fruit body is annual, bracket-like to tongue-shaped, laterally attached. As the common name suggests, beefsteak fungus is remarkably similar in appearance to raw meat. In the past, it was often cooked and eaten as a substitute for meat. It is sold in several markets and can be eaten raw in salad or with a sauce of parsley and garlic.

The HPLC-UV analysis of beefsteak fungus yielded the identification of six organic acids: malic, oxalic, aconitic, ascorbic, citric, and fumaric (Ribeiro *et al.* 2007). The quantitative analysis revealed high organic acids content, in which malic acid was the main compound, representing ca. 60% of total acids. Oxalic and aconitic acids were the compounds present in lowest amounts.

Against DPPH, activity was concentration-dependent, with an IC_{25} of 136 μg/mL. The capacity of the lyophilized extract to scavenge superoxide radicals was confirmed and an IC_{50} of 105 μg/mL was determined. Additionally, the results demonstrate that the beefsteak fungus exerted some inhibitory effect on XO, which was concentration dependent (IC_{50} at 1444 (μg/mL) (**Table 2**).

Beefsteak fungus lyophilised extract showed little capacity to chelate iron ions for concentrations above 400 μg/mL.

Regarding HOCl scavenging ability, under the assayed conditions, lipoic acid was used as a reference compound and inhibited TNB oxidation in a concentration-dependent manner (IC_{25} at 21 μM). Beefsteak fungus lyophilized extract exhibited a weak antioxidant protective activity against damage by HOCl, with an IC_{15} at 1458 μg/mL.

VITAMIN C: A CASE STUDY

Undoubtedly, ascorbic acid (which, together with dehydroascorbic acid, constitutes what we call vitamin C) (Naidu 2003) is the most important organic acid when addressing antioxidant activity and its impact on human health.

This compound can act as a scavenger/neutralizer of a wide range of both oxygen and nitrogen reactive species, such as hydroxyl radical, alkoxyl radicals, peroxyl radicals, superoxide anion/hydroperoxyl radical, hypochlorous acid, ozone, singlet oxygen, nitrogen dioxide radical, peroxynitrite/peroxynitrous acid (Carr and Frei 1999).

Besides this direct action against oxidizing species, ascorbic acid amplifies the activity of other antioxidants, as it can regenerate α-tocopheroxyl, urate and β-carotene radical cation from their radical species (Niki *et al*. 1995; Niki 1987; Naidu 2003). We can, therefore, speak of synergism of several antioxidants.

The antioxidant properties of ascorbic acid are recognised as the most probable explanation for the role that it seems to play on atherosclerosis (Frei 1997; Martin and Frei 1997) and cancer prevention (Campbell *et al*. 1999; Naidu 2003).

Recently some concern has appeared in the literature given the fact that in *in vitro* experiments, ascorbic acid, when in low concentrations (0.2 M), was able to catalytically reduce active metal ions and therefore contribute to oxidative damage through the production of hydroxyl and alkoxyl radicals (Bast *et al*. 1991; Buettner and Jurkiewic 1996; Proteggente *et al.* 2000). Nevertheless, this data does not necessarily imply that ascorbic acid can be a pro-oxidant *in vivo* and many controversies are still unresolved in this area (Halliwell 1996; Carr and Frei 1999; Chen *et al*. 2000). Many studies indicate that, in fact, both actions can be linked with ascorbic acid, depending on the medium conditions.

In the work of Martínez-Sánches *et al.* (2008), a comparative study of flavonoid compounds, vitamin C and antioxidant properties (against DPPH and $ABTS^{\bullet+}$) of baby leaf Brassicaceae species was performed. The species involved were watercress (*Nasturtium officinale*), mizuna (*Brassica rapa* subsp. *nipposinica*), wild rocket (*Diplotaxis tenuifolia*) and salad rocket (*Eruca vesicaria*).

Besides important information regarding phenolic com-

position of these matrices, a correlation between phenolics and Vitamin C presence in leaves and antioxidant activity could be achieved, although Pearson's correlation coefficient was lower for Vitamin C than for flavonoids. The vitamin C content, measured as ascorbic acid and dehydroascorbic acid, ranged from 64 to 104 mg per 100 g of fresh weight. The highest content of Vitamin C was observed in watercress, followed by wild rocket and salad rocket and the lowest content of vitamin C was found in mizuna and antioxidant activity followed the same order. Ascorbic acid was the predominant form of Vitamin C in all of the studied species. Differences in ascorbic acid content can be related to both leaf age and the irradiance arriving at the leaf surface, among other factors (Foyer 1993).

Other study (Llorach *et al.* 2008) characterised polyphenols, Vitamin C and antioxidant properties of five varieties of lettuce (*Lactuca sativa* L.), three green varieties (iceberg, romaine, continental) and two red varieties (red oak leaf, lollo rosso) and one escarole one (*Cichorium endivia* var. *crispa*) "frissé". Vitamin C levels ranged between 2.8 (romaine) and 19.5 (continental) mg/100 g (fresh weight). The highest amount of Vitamin C was found in green lettuce, followed by red lettuce varieties. Antioxidant activity was also evaluated against DPPH and $ABTS^{\bullet+}$. In both screening tests, red varieties showed higher scavenging activity, a fact that was not in line with their content in Vitamin C, which was lower when compared with green varieties. However, it should be taken into account that the authors found anthocyanins in red varieties, which could contribute to the antioxidant activity in a higher degree than ascorbic acid, thus diminishing its influence in scavenging activity of samples.

CONCLUSION

After analysing several works dedicated to the influence of organic acids on antioxidant activity of vegetable matrices, one cannot state, without a doubt, their contribution, or not, to the activity showed. This happens due to many synergistic and antagonistic behaviour of compounds found in the plant matrices. These phenomena increase with matrix complexity, rendering almost impossible to clearly associate the presence of an acid(s) with the displayed antioxidant activity. In cases in which total organic acids content is correlated with antioxidant potential of the samples, ascorbic acid is, most of the times, a compound present in significant amounts. In fact, ascorbic acid is a well known antioxidant which is sold worldwide as a diet supplement given its beneficial effects.

However, some caution must be taken as an increasing number of studies show how this compound can act as a pro-oxidant in physiological conditions. In the end, the main factor contributing to antioxidant effects of organic acids is the complex chemical medium in which these compounds are included, which can dictate their neutral, deleterious or beneficial properties. Works on this theme, however, are rather scarce and therefore, more efforts should be put in this matter as a way to understand the complex biochemistry behind organic acids' biological activity.

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