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The hydrophilic and lipophilic contribution to total antioxidant activity

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Abstract

The ABTS/H_2O_2/HRP decoloration method permits the evaluation of the antioxidant activity of complex food samples. This method, with slight modifications, is capable of determining both hydrophilic and lipophilic antioxidant properties, thus, it is possible estimate the antioxidant activity of both antioxidant types in the same sample. The method is easy, accurate and rapid to apply. Its application to three vegetable soups provided data on hydrophilic and lipophilic antioxidant activity, and the values reflect the contribution of the particular antioxidants (ascorbic acid and carotenoids) to the total antioxidant activity of the samples.

Keywords: ABTS; Antioxidant activity; Ascorbic acid; Carotenoids; Tomato juice; Vegetables

1. Introduction

Foods of plant origin not only provide our diet with certain antioxidant vitamins like vitamin C (ascorbic acid), vitamin E (α-tocopherol) and pro-vitamin A (β-carotene), but also a complex mixture of other natural substances with antioxidant capacity. An antioxidant is a compound that protects biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidation (Krinsky, 1989). According to numerous studies such antioxidant activity appears to be closely related with the prevention of degenerative illnesses, such as the different types of cancer, cardiovascular and neurological diseases, cataracts and oxidative stress dysfunctions (Frei, 1994; Gey, Paska, Jordan & Moser, 1991; Mackerras, 1995; Riemersma, 1994; Schwartz, 1996). Besides vitamins A, C and E, the most important naturally occurring plant substances showing antioxidant activity are carotenoids, flavonoids and other simple phenolic compounds which, in differing proportions and quantities, are to be found in cereals, fruits and vegetables (Duell, 1996; Mackerras, 1995). Of the above cited compounds, some are of a hydrophilic nature (e.g. ascorbic acid) and others are clearly lipophilic (e.g. carotenoids). It is of general interest to measure the antioxidant capacity (activity) of fruits, vegetables or foodstuff (Halliwell, 1996; Palozza & Krinsky, 1992; Stanley & Mogg, 1995).

The most widely used methods for measuring antioxidant activity are those that involve the generation of radical species, the presence of antioxidants determining the disappearance of these radicals (Arnao, Cano & Acosta, 1998, 1999; Arnao, Cano, Hernàndez-Ruiz, García-Cánovas & Acosta, 1996; Cano, Hernández-Ruiz, García-Cánovas, Acosta & Arnao, 1998; Cao, Alessio & Cutler, 1993; Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993; Miller & Rice-Evans, 1997; Rice-Evans & Miller, 1994). This approach has been applied to the estimation of antioxidant activity in aqueous systems, but in lesser extension for lipid-soluble antioxidants. In lipophilic systems, homogenous solutions have been used to study the protective effect of lipid-soluble antioxidants on lipids since this has the advantage of simplifying the assay; for example, carotenoids and lipids can be dissolved in organic solvents (Robards, Prenzler, Tucker, Swatsitang & Glover, 1999).

Recently, we have developed a decoloration method for measuring hydrophilic antioxidant activity using the enzymatic system ABTS/H_2O_2/HRP in an assay which measures the loss of absorbance (Cano et al., 1998). ABTS radical cation ($ABTS^{\bullet+}$) is pre-generated enzymatically and the antioxidant or sample to be analyzed is added to the reaction medium. This results in disappearance of

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the $ABTS^{\bullet+}$, which is measured by the decrease in absorbance (a wavelength between 400 and 750 nm can be selected to avoid exogenous absorption interference). This patented method, which is easy and rapid to perform, presents numerous advantages since it avoids unwanted reactions, high temperatures are not required to generate radicals and antioxidant activity can be studied over a wide range of pH values. The method also has the advantage that is avoids interference due to endogenous peroxidase activity in samples, so that the determination of hydrophilic antioxidant activity in plant and other extracts is more accurate and rigorous. A comparative discussion of methods to measure antioxidant activity can be consulted (Arnao et al., 1999). Different applications have determined antioxidant activity in plant materials such as citrus juices, fruits, soft drinks, beers and wines (Arnao et al., 1996, 1998).

In this study, we adapt our ABTS/H_2O_2/HRP decoloration method to measure both hydrophilic and lipophilic antioxidant activities in the same sample. We select three different vegetable soups for the study, which due to their diverse composition are of interest for the determination of the two types of antioxidants (hydrophilic and lipophilic) and their contribution to the total antioxidant activity. The correlation between ascorbic acid, carotenoid content and antioxidant activity was also examined.

2. Materials and methods

2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid; ABTS) in the crystallized diammonium salt form, L-ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), lycopene and β-carotene were purchased from Sigma Chem.Co (Madrid, Spain). H_2O_2 (30%, v/v) was obtained from Aldrich Chem. Co. (Madrid, Spain). The concentration of ABTS, hydrogen peroxide, lycopene and β-carotene was determined by measuring their absorbance using $\epsilon_{340nm} = 36\ mM^{-1}\ cm^{-1}$ for ABTS (Childs & Bardsley, 1975), $\epsilon_{240nm} = 43.6\ M^{-1}\ cm^{-1}$ for H_2O_2 (Bielski & Allen, 1977), $\epsilon_{474nm} = 185\ mM^{-1}\ cm^{-1}$ (in acetone) for lycopene and $\epsilon_{454nm} = 134.4\ mM^{-1}\ cm^{-1}$ (in acetone) for βcarotene (Britton, 1995). Horseradish peroxidase (HRP) type VI was obtained from Sigma ($\epsilon_{403nm} = 100\ mM^{-1}\ cm^{-1}$). Spectrophotometric measurements were recorded with a UV–VIS Perkin–Elmer Lambda-2S spectrophotometer interfaced on-line with a PC-computer. The temperature was controlled at 25°C using a Haake D1G circulating bath with a heater/cooler with a precision of ±0.1°C.

2.1. Biological material

Commercial vegetable soups containing only natural ingredients and slightly pasteurized were provided by Alvalle-Tropicana Pepsico Co. (Murcia, Spain). Three differents soups were analyzed: "Gazpacho", a traditional cold Mediterranean soup, containing blended fresh vegetables (90%) with ingredients: tomato, cucumber, onion, pepper, garlic, water, olive oil, wine vinegar, sea salt and lemon juice; "Tomato Soup", containing tomato blended with olive oil, sea salt and lemon juice; and "Seven-Vegetable Soup", a cold liquid-food, containing fresh vegetables (78%; tomato, carrot, celery, onion, pepper, cucumber, garlic), water, olive oil, vinegar, sea salt, lemon juice and spices. The refrigerated products (2–5°C) were opened in the laboratory and assayed immediately for antioxidant activity, ascorbic acid and carotenoid content.

2.2. Extraction and separation of components

Homogeneous soup samples (1 g), 2 ml 50 mM Na-phosphate buffer (pH 7.5) and 5 mL ethyl acetate were crushed in a Euroturrax T20 (IKA, Germany) for 2 min and transferred to a decantation funnel. The solid residue (totally colorless) was discarded. The aqueous phase was collected to measure hydrophilic antioxidant activity (HAA) and ascorbic acid content. The organic phase was collected to measure lipophilic antioxidant activity (LAA) and carotenoid content. All extraction procedures were carried out under subdued light at 4°C and analyzed as soon as possible.

2.3. Antioxidant activity

This was measured using our ABTS/HRP decoloration method (Cano et al., 1998) with some modifications. The method is based on the capacity of different components to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) compared to a standard antioxidant (ascorbic acid or Trolox) in a dose–response curve. For hydrophilic antioxidant activity (HAA), the reaction mixture contained 2 mM ABTS, 15 μM H_2O_2 and 0.25 μM HRP in 50 mM Na-phosphate buffer (pH 7.5) in a total volume of 1 ml. The assay temperature was 25°C. The reaction was monitored at 730 nm until stable absorbance was obtained. Then, 10 μl of the aqueous phase was added to the reaction medium and the decrease in absorbance, which is proportional to the $ABTS^{\bullet+}$ quenched, was determined after 5 min. For lipophilic antioxidant activity (LAA) the reaction mixture contained 1 mM ABTS, 15 μM H_2O_2 and 6 μM HRP in pure ethanol, in a total volume of 1 ml. In this case, 10 μl of the organic phase was added to the reaction medium and the decrease in absorbance at 730 nm was determined after 5 min. The total time needed to carry out each assay was approximately 6 min, including ABTS radical generation by peroxidase, the addition of antioxidant and acquisition of the final absorbance value. The absorbance decrease was determined from the difference between the A_{730}

values before and 5 min after sample addition. Antioxidant activity was calculated as moles of $ABTS^{\bullet+}$ quenched by 1 mol of Trolox. In both cases, the antioxidant activity was expressed as Trolox equivalents per fresh weight of soup (mg 100 g^{-1}). Trolox was dissolved by ultrasonication (as sodium salt) in phosphate buffer for HAA estimations and (as the free acid) in ethanol for LAA. Lycopene and β-carotene were dissolved in ethyl acetate for LAA. No effect of ethyl acetate on $ABTS^{\bullet+}$ was observed.

2.4. Chromatographic analysis of ascorbic acid

HPLC determinations of the ascorbic acid content of the samples were carried out as in Cano et al. (1998) using a Beckman System Gold instrument with programmable injector and diode array detector. Briefly, samples of aqueous phase (20–60 μl) were injected into a RP-ODS-Spherisorb (5 μm particle size) column and the mobile phase (0.7 ml/min) was monitored at 250 nm. A single mobile phase consisting of 10 mM ammonium acetate plus 1 mM sodium EDTA (pH 3.0) was used. L-ascorbic acid was used as standard, eluting at 4.8 min. The diode array scanner was used for peak identification.

2.5. Chromatographic analysis of carotenoids

HPLC determinations of the carotenoid content were carried out as in Rivas, Abadia and Abadia (1989) with some modifications using the same equipment as above. Briefly, samples of organic phase containing carotenoids (20–60 μl) were injected into an RP-Ultrasphere (5 μm particle size) column and the mobile phase (1 ml/min) was monitored at 450 nm. Three mobile phases were used: mobile phase A (acetonitrile/methanol/water/ethyl acetate, 7:0.96:0.04:2) was pumped for 1 min. Then, mobile phase B (acetonitrile/methanol/water/ethyl acetate, 7:0.96:0.04:8) was pumped for 27 min; and lastly, mobile phase C (acetonitrile/methanol, 7:1) was used to re-equilibrate the column prior to injecting the next sample. Lycopene and β-carotene were used as standards, eluting at 18.2 min and 20.5 min, respectively. The precautions recommended by Hart and Scott (1995) for checking the standard solution and its manipulation were taken. Peaks were identified using the diode array scanner.

3. Results and discussion

As can be seen in Fig. 1 (Panel A), it is possible to make a dose–response curve with standard antioxidants (hydrophilic or lipophilic) to quantify the antioxidant activity. Here, Trolox (an analog of vitamin E) was used because it can be dissolved in aqueous (as a salt) or organic media (as an acid). In both reaction media, 1 mol Trolox quenches 2 moles of $ABTS^{\bullet+}$. In contrast, β-carotene in organic medium will quench 5 moles of $ABTS^{\bullet+}$ per mol (data not shown). No reaction appears with β-carotene in the aqueous medium. Thus, this assay can be used to evaluate the antioxidant activity of pure compounds, plant extracts or food samples in both aqueous and organic media.

The effect on $ABTS^{\bullet+}$ of aqueous and organic extracts of a Tomato soup sample is shown in Fig. 1 (Panels B and C). A biphasic response with $ABTS^{\bullet+}$ appears as has been reported (van den Berg, Haenen, van den Berg & Bast, 1999; van den Berg, Haenen, van den Berg, van der Vijgh & Bast, 2000), but the difference in Abs_{730nm} values (before and 5 min after sample addition) can be used to estimate hydrophilic and lipophilic antioxidant activity, depending on the reaction medium used. After sample addition, the Abs_{730nm} decreased in direct proportion to the increasing volumes of extract added, which is related to the $ABTS^{\bullet+}$ quenching capacity of the antioxidants in the sample. For comparison of samples, estimations of antioxidant activity must be performed at a fixed time-point (5 min in our case) to prevent differences in antioxidant activity of the same sample at different time-points.

Table 1 shows the values of hydrophilic (HAA) and lipophilic (LAA) antioxidant activity of different fresh vegetable soups. HAA follow the order: Tomato soup > Gazpacho > Seven-Vegetable soup. LAA follow the order: Gazpacho > Tomato soup > Seven-Vegetable soup. Total antioxidant activity (TAA = HAA + LAA) presents the order: Tomato soup > Gazpacho > Seven-Vegetable soup, but while in Gazpacho and Seven-Vegetable soup HAA represents 67 and 64% of TAA, respectively, in Tomato soup it represents nearly 80%. In Tomato soup, Gazpacho and Seven-Vegetable soup, LAA represent 21, 33 and 36%, respectively.

Fig. 2 shows the chromatographic separation of ascorbic acid in the aqueous extract of Tomato soup (Panel A) and carotenoids in the organic extract of the same soup (Panel B). Compounds in the extracts were identified by comparing their retention times (R_t) with the corresponding standard and by their spectra obtained with the diode array detector. Also, the L-ascorbic acid and carotenoids (lycopene and β-carotene) standards permit quantification of the respective compounds in the different soups analyzed. Table 1 shows the content of the compounds in each soup. The average content of these antioxidants in the soups was in accordance with the habitual content in the raw materials used to make them, and also with food analyzes by other authors (Granado, Olmedilla, Blanco & Rojas-Hidalgo, 1992; Hart & Scott 1995; Olmedilla, Granado, Blanco & Gil-Matínez, 1998) and the US Department of Agriculture Nutrient Database. Tomato soup presented the highest lycopene content, followed closely by Gazpacho. All three soups showed lycopene and β-carotene because all contained fresh tomato. Seven-Vegetable

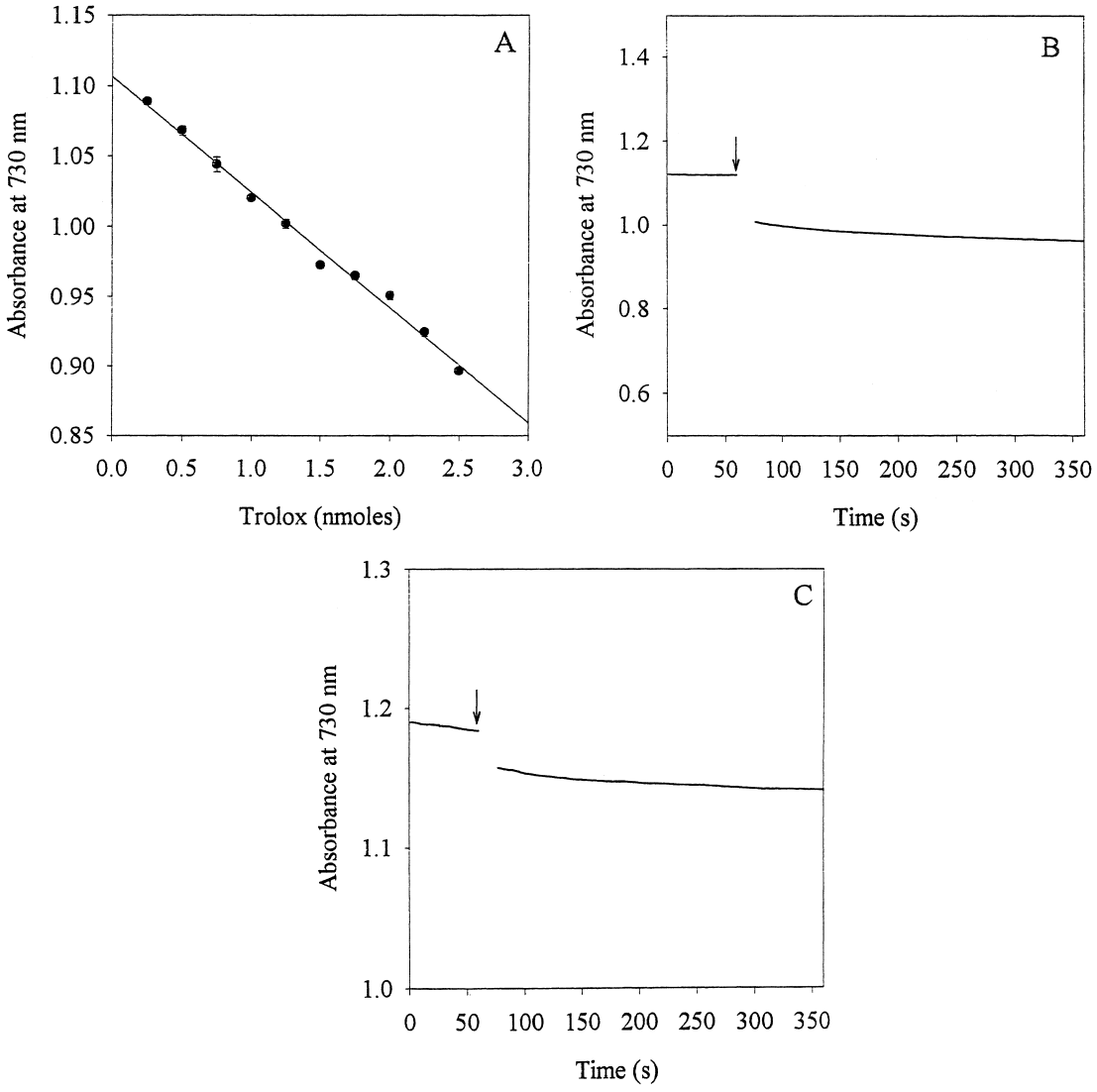

Fig. 1. Hydrophilic and lipophilic antioxidant activity estimated using the ABTS/HRP decoloration method. Panel A, Dose–response curve using Trolox as standard antioxidant. Plot of loss of absorbance at 730 nm against Trolox concentrations. A similar dose–response curve is obtained for Trolox in both buffered and ethanolic medium. Panel B, Hydrophilic antioxidant activity. Absorbance decrease at 730 nm when 10 μl of aqueous phase of "Tomato soup" extract was added to a buffered medium (pH 7.5) containing pre-formed $ABTS^{\bullet+}$ (70 μM). The arrow shows the addition of aqueous extract. Panel C, Lipophilic antioxidant activity. Absorbance decrease at 730 nm when 10 μl of organic phase of "Tomato soup" extract was added to a ethanolic medium containing pre-formed $ABTS^{\bullet+}$ (70 μM). The arrow shows the addition of organic extract.

Table 1
Antioxidant activity, ascorbic acid and carotenoid content of three vegetable soups

Type of soup	Lipophilic antioxidant activity (LAA)[a]	Hydrophilic antioxidant activity (HAA)[a]	Total antioxidant activity (TAA)[a]	Ascorbic acid[b]	Lycopene[b]	β-Carotene[b]
Tomato	7.9±0.3[c]	30.2±2.1	38.1±2.4	10.1±0.6	0.56±0.03	0.38±0.03
Gazpacho	9.1±0.7	18.9±0.9	28.0±1.6	9.0±0.4	0.48±0.02	0.57±0.05
Seven-Vegetable	7.2±0.4	12.9±0.6	20.1±1.0	0.9±0.1	0.26±0.01	1.37±0.09

[a] Antioxidant activity is expressed as mg equivalent of Trolox per 100 g of soup (mg · 100 g^{-1}).
[b] Ascorbic acid and carotenoid content is expressed as mg of compound per 100 g of soup (mg · 100 g^{-1}).
[c] Mean ($n=5$) ± standard error.

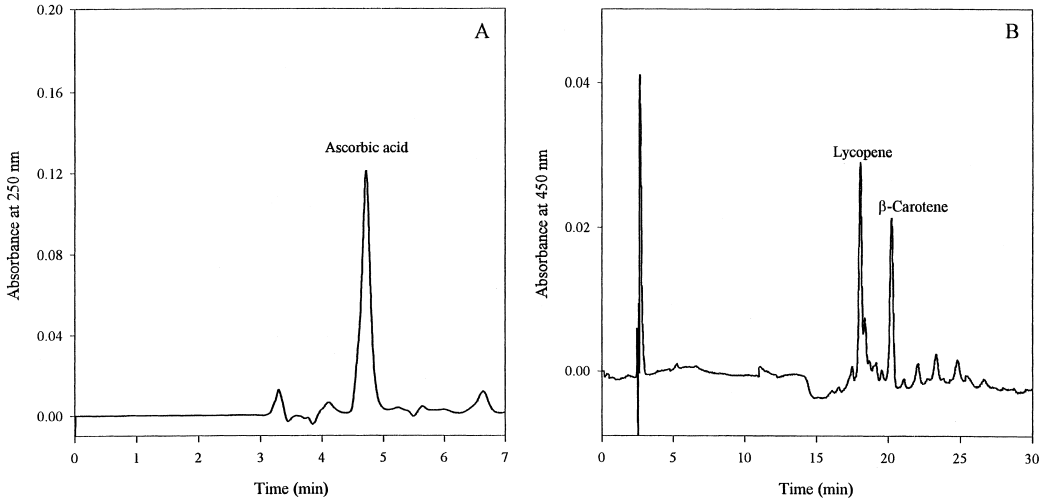

Fig. 2. Reversed-phase high pressure liquid chromatography of different antioxidants. Panel A, Separation of L-ascorbic acid in aqueous extract of "Tomato" soup. The R_t of ascorbic acid is 4.8 min. Panel B, Separation of the major carotenoids in organic extract of "Tomato soup". The R_t of the different peaks are: lycopene (18.2 min) and β-carotene (20.5 min).

soup showed the highest β-carotene content because carrot is an important component. With respect to ascorbic acid, Gazpacho and Tomato soup presented the highest content of this hydrophilic vitamin due to the contribution of fresh tomato. In addition, Seven-Vegetable soup had a higher water content, and so a lower ascorbic acid content was to be expected.

To evaluate the contribution of each analyzed component to the antioxidant activity, it is necessary to know the relative antioxidant activity of each component. Thus, taking Trolox as reference, the relative antioxidant activity (RAA) of β-carotene ($RAA_{\beta\text{-carotene}}$) is 2.5. This means that, since 1 mol Trolox quenches 2 moles of $ABTS^{\bullet+}$, then 1 mol β-carotene quenches 5 moles $ABTS^{\bullet+}$. The RAA of ascorbic acid (RAA_{ASC}) is 1.0, which means that Trolox and ascorbic acid have the same antioxidant activity (Arnao et al., 1996, 1999). The parameter RAA corresponds to TEAC (Trolox Equivalent Antioxidant Capacity), using the nomenclature of Rice-Evans and Miller (1994).

The relative contribution of each component is shown in Fig. 3. The contribution of ascorbic acid to HAA differs in each soup: Tomato (33%), Gazpacho (47%) and Seven-Vegetable (7%). In a similar way, we can calculate the contribution of lycopene and β-carotene to LAA. Lycopene presents an RAA (with respect to Trolox) of 2.9 and β carotene of 2.5. Applying the respective RAA to each carotenoid content, the contribution of these components to the LAA of each soup was calculated as: Tomato (33%), Gazpacho (31%) and Seven-Vegetable (58%; Fig. 3).

In all cases, TAA was higher than the absolute contribution of the analyzed components. This means that other components also made a contribution to TAA. Probably, specific phenolic compounds, more than total phenols, make an important contribution to TAA. In the foods analyzed in this work, total phenols (estimated by the Folin Ciocalteu reagent) present values of 40–80 mg·100 g^{-1} of soup (using gallic acid as standard). These values are much higher than the measured values of antioxidant activity, indicating that only some phenolic compounds contribute, to various degrees, to TAA. Thus, food compositional data does not necessarily accurately reflect the predicted health effects due to matrix effects of foods and the bioactivity of unknown compounds.

In summary, the $ABTS/H_2O_2/HRP$ decoloration method permits the evaluation of the antioxidant activity of complex food samples in both aqueous and organic media. Thus, using the same sample, it is possible to estimate hydrophilic and lipophilic antioxidant activity. The method is easy, accurate and rapid to apply. Its

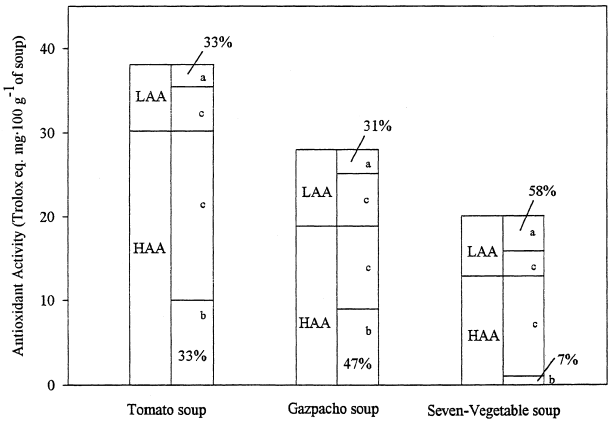

Fig. 3. Antioxidant activity of different vegetable soups and relative contributions of ascorbic acid and carotenoids to hydrophilic (HAA) and lipohilic antioxidant activity (LAA). *a*, contribution of carotenoids content (lycopene + β-carotene) to LAA; *b*, contribution of ascorbic acid content to HAA; *c*, antioxidant activity of unknown compounds. Values represent the percentages of HAA or LAA in each case. Absolute data from Table 1.

application to three vegetable soups provided data on antioxidant activity in the form of the relative contributions of hydrophilic and lipophilic components. The values of HAA and LAA reflect the contribution of the different antioxidants to the total antioxidant activity of the samples. The data obtained show the excellent properties of these Mediterranean soups from both the vitamin and antioxidant points of view. Thus, these popular recipes contribute many beneficial components to the diet, in the form of a refreshing liquid-food, which is both healthy and easy to drink.

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