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The kinetic of key phytochemical compounds of non heading and heading leafy

***Brassica oleracea* landraces as affected by traditional cooking methods**

Running title: Kale phytochemical and colour profile during cooking

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ABSTRACT

BACKGROUND: Kales are often a key ingredient of traditional foods, containing high amount of indolic glucosinolates (precursors of indole-3-carbinol and ascorbigen), carotenoids and phenolics. The present trend to associate traditional foods crops with health promoting properties, suggested to investigate the degradation kinetic of three *Brassica oleracea* landraces' phytochemicals, subjected to boiling, steaming and stir frying.

RESULTS: Boiling determined substantial losses, due to leaching. Glucosinolates followed a second order degradation kinetic (20% of their initial values after 10 minutes in Nero di Toscana). Phenolic content in leaves + cooking water remained unchanged, whereas however, their antioxidant capacity was reduced. Carotenoid content increased during the first minutes of boiling. Steaming showed the highest retention of phytochemicals, with often zero order degradation kinetic, having however a strong effect on colour. Stir frying produced high losses for all measured compounds; also β -carotene reduced its content to 10-23%, independently on variety. Conversion values for indole-derived compounds ranged from non-detectable to 23.5%.

CONCLUSION: Variety strongly affected observed degradation rates, because of a different glucosinolate composition and leaf structure. With this research more information are gained on the kinetic degradation of *Brassica oleracea* landraces' phytochemical compounds upon cooking, highlighting the possibility of improving bioactive component retention.

Keywords:

Kale (*Brassica oleracea* ssp. *acephala*); glucosinolates; carotenoids; total phenolic compounds; cooking; retention.

INTRODUCTION

Brassica oleracea L. includes vegetables grown and consumed worldwide. Among this family kale (*Brassica oleracea* ssp. *acephala*) represent a leafy vegetable, generally grown in several European countries.

Brassica vegetables have been widely investigated in the last years for their richness in healthy phytochemicals. Kale, in particular, represent an important source of ascorbic acid, carotenoids, phenolics and glucosinolates (GLS);¹⁻³ moreover they show the highest antioxidant activity among other *Brassicaceae*.⁴

GLS are particularly important because of the anticancer properties of their metabolic products.⁵ Among putative health-promoting compounds, indolic GLS are found in relevant amounts in several *Brassica* vegetables,^{6,7} with kale representing particularly rich sources.⁸ Indole-3-carbinol (I3C) has been identified as the major breakdown product from GB degradation, after the enzymatic action of myrosinase (enzymic cleavage of the β -thioglucosidic bond), with plant tissue disruption; however other indole derivatives are generated, such as indole-3-acetonitrile and 3,3'-diindolylmethane.^{9,10} Thermal degradation of indolic GLS produces similar breakdown products. Also ascorbigen (indol-3-ylmethyl-ascorbate, ABG), the result of the reaction of I3C with ascorbic acid (AA),¹¹ possess cancer chemopreventive properties.¹² In a previous work Hrnčirik *et al.*¹³ calculated low conversion values of GB into ABG during homogenization of *Brassicaceae*, demonstrating that pH values of fresh *Brassica* vegetables differ from optimal values (pH 4.5-5.0) for ABG formation. In aqueous media a higher amount of I3C is produced, with respect to ABG; however I3C is unstable in aqueous solution and may condense to 3,3'-diindolylmethane.¹³ Kale is rich in ABG precursors so it could be interesting to evaluate their conversion in indole-derived compounds during processing.

However, other studies highlight the promotion of carcinogenesis by I3C; in fact indolo[3,2-*b*]carbazole (ICZ), produced after ingestion of I3C, in the acidic conditions of the stomach, show similar structure and biological effects with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), tumor promoter.¹⁴

Kale leaves represent a characteristic ingredient in the preparation of traditional dishes such as soups, gruels, side dishes and green filled pastries. The most common ways of kale preparation are water boiling, with or without a starchy matrix (e.g. potato, as it is for the Dutch dish “stamppot”), steaming and stewing in a pan with oil. In some cases, the preparation of traditional dishes involves cooking in water for very long times (e.g. the Italian “ribollita”).¹⁵

During heating of *Brassica* vegetables several mechanisms take place affecting the GLS content, such as thermal degradation, inactivation of enzyme myrosinase, and leaching of GLS and breakdown products.¹⁶ Mathematical models could represent a useful tool to predict relevant quality aspects of food, such as nutrient content.¹⁷ Several studies have investigated and modelled the trend of GLS during thermal treatment.¹⁸

Other works have evaluated the effect of thermal treatment or method of preservation on the content of bioactives in kale.¹⁹⁻²²

In this study three *Brassica* landraces, representative of Northern (the Dutch “Boerenkool”) and Southern Europe (the Italian “Nero di Toscana” and “Broccolo Lavagnino”) were studied. The main objective was to evaluate the fate and retention of various bioactive and antioxidant compounds (glucosinolates, I3C, ABG, carotenoids) and underlying mechanisms for three types of traditional prepared *Brassica* landraces. The applied modelling approach facilitates the comparison of the fate of these compounds between different cooking ways. Moreover, the effect of cooking on the formation of I3C, ABG and their precursors were investigated.

MATERIALS AND METHODS

Chemicals

Solvents used for chromatographic analyses were of high-performance liquid chromatography (HPLC) grade (Biosolve, Valkenswaard, The Netherlands). DEAE Sephadex A-25, indole-3-carbinol, lutein, β -carotene, gallic acid, ascorbic acid, tris-2-carboxyethyl phosphine, *meta*-phosphoric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and trolox were bought from Sigma-Aldrich (Zwijndrecht, The Netherlands). The internal standard glucotropaeolin was purchased from the Laboratory of Biochemistry, Plant Breeding and Acclimatization Institute (Radzikow, Błonie, Poland). *Tert*-butylhydroquinone and orthophosphoric acid were supplied by Merck Millipore (Amsterdam, The Netherlands).

Samples and experimental plan

The following material was used in this study: two kale varieties: the Dutch “Boerenkool”, and the Italian “Nero di Toscana” (*Brassica oleracea* L., var. *sabellica* L.); and an Italian heading form called “Broccolo Lavagnino”, that is however often used as leaves, like kales; this is a variable form, often classified as *Brassica oleracea* L., var. *capitata* L.. Boerenkool and Nero di Toscana seeds were purchased from seed firms, whereas Broccolo Lavagnino seeds were provided from a local Italian farmer. Sowing was done in cardboard alveolated trays on March 26, 2014; the seedlings were then transplanted in a field located in Wageningen (The Netherlands), on April 12. Harvest took place 7 weeks later; the leaves were selected, kept at 4 °C (~3 hours) and immediately processed.

The experimental treatments included three cooking systems: boiling, steaming and stir-frying, combined with four cooking times in the case of boiling and steaming (5, 10, 30 and 60 minutes), and three cooking times (5, 10 and 20 minutes) for stir-frying.

Sample processing

Kale leaves, at the same maturity grade and removed from damaged parts, were cut in stripes (2 cm width). A part of cut raw leaves was frozen overnight at -20 °C, subsequently freeze-dried (Christ Alpha 1-4 LD Freeze Dryer, SciQuip Ltd, Newton, United Kingdom) and considered as control. 50 g of kale leaves were used for each cooking trial. All the cooking experiments were conducted in triplicate.

In the boiling experiment raw leaves were immersed in 1 L of boiling water and occasionally stirred with a ladle during cooking; kale leaves and water cooking were sampled after 5, 10, 30 and 60 minutes. Steaming process was performed using a steam oven by Miele (The Netherlands), and putting 50 g of raw leaves in the specific tray; again kale leaves were sampled after 5, 10, 30 and 60 minutes. For stir-frying 10 mL of sunflower oil were pre-warmed in a pan; raw leaves were added after 2 minutes, when the oil had reached the temperature of 140 °C, spread in all the surface of the pan and stirred regularly; kale leaves were sampled after 5, 10 and 20 minutes. Cooked products, drained from the water in case of boiling, were cooled on ice for 2 minutes, and exactly weighed.

As already mentioned for the control ones, samples of kale were then stored overnight at -20 °C, and freeze-dried till constant weight. Freeze-dried material was ground to a fine powder by using a Waring blender (model 34BL99, Dynamics Corp. of America, New Hartford, CT, USA), and stored at -20 °C till further analyses.

The remained volume of water used for boiling was measured and a part was collected and stored at -20 °C till further analyses.

Glucosinolate extraction and analyses

The method already described by Oliviero *et al.*²³ was used with some modifications. 0.1 g of freeze-dried sample was extracted using 2.4 mL methanol preheated at 75 °C,

in order to inactivate endogenous myrosinase. 200 μL of 3 mM glucotropaeolin solution were added as internal standard and samples were incubated in a water bath of 75 °C for 20 min. Samples were subsequently centrifuged, and supernatants were collected and re-extracted twice with 2 mL hot 70% methanol. The extracted glucosinolates were desulphated and the separation was conducted using a LiChrospher® 100 RP-18 column 5 μm (250 x 4.6 mm), furnished of a proper guard column. Elution from the HPLC column was performed by a gradient of water (A) and acetonitrile (B), as follows: from 0 to 2 min, 0% B; from 2 to 7,5 min, 0-8% B; from 7,5 to 14 min, 8-25% B; from 14 to 18 min, 25% B; from 18 to 20 min, 25-0% B; from 20 to 25 min, 0% B as post-run. The flow rate was 1 mL min⁻¹ and the injection volume was 20 μL . Detection of the desulphoglucosinolates was performed at $\lambda = 229$ nm. All the analyses were conducted in triplicate.

Determination of indole-GLS derived compounds: ascorbigen (ABG) and indole-3-carbinol (I3C)

ABG and I3C were simultaneously extracted as reported by Peñas *et al.*²⁴ Freeze-dried powders were analysed with slight modification of previously published methods.²⁵ 1 g of sample was hydrolysed in water ($c = 50$ mg mL⁻¹) for 4 hours in the dark and shaking periodically. Solutions were centrifuged at 3396 x *g* at 4 °C for 15 minutes and supernatants were extracted with dichloromethane. Extracts were filtered through a paper filter filled with sodium sulphate, dried in rotavapor and recovered in 1 mL of acetonitrile. Before injection in HPLC samples were filtered through a 0.20 μm RC membrane filter (Phenomenex) into an HPLC amber vial. Samples of cooking water were treated in the same way as reported above, without the hydrolysis step.

Separation was conducted using the same column and HPLC system already described for GLS extraction. Elution from the HPLC column was performed by a gradient of

water (A) and acetonitrile (B), as follows: from 0 to 15 min, 20-100% B; from 15 to 20 min, 100% B; from 20 to 25 min, 20% B as post-run. The flow rate was 1 mL min⁻¹ and the injection volume was 50 µL. Detection was performed at $\lambda = 280$ nm. All the analyses were conducted in triplicate.

Carotenoids

Carotenoids were extracted from freeze-dried samples according to Lefsrud *et al.*,³ and Ferioli *et al.*²⁶ 50 mg of lyophilized material were extracted using 2.5 mL of tetrahydrofuran (THF) stabilized with butylated hydroxytoluene (BHT; c = 25 mg mL⁻¹). The extraction with stabilized THF was repeated three times more; the collected supernatants were dried with rotavapor and recovered in THF-methanol, 1:1 (v/v). Before HPLC analyses, samples were centrifuged at 15000 x g (10 minutes, 10 °C). HPLC analyses were carried out on a UltiMate 3000 UHPLC system from Thermo Fisher Scientific equipped with a RD diode array detector. Separation was conducted using a Vydac RP C18 5.0 µm 250 x 4.6 mm column, maintained at 25 °C using a thermostatic column compartment. Eluents were A: 88% methanol, 10% Milli-Q water, 2% THF, and 0.1% triethylamine (TEA), and B: 92.5% methanol, 7.5% THF, and 0.1% TEA. The flow rate was 0.7 mL min⁻¹ and the gradient was 0% B for 12 minutes; from 12 to 15 minutes, 0-100% B; from 15 to 30 minutes, 100% B; from 30 to 40 minutes, 100-0% B; from 40 to 45 minutes, 0% B as post-run. Eluted carotenoids were determined at 452 nm. β -carotene and lutein stock solutions were prepared and test dilutions were prepared in the range 0.001-0.400 mg mL⁻¹. All the analyses were conducted in triplicate.

Total ascorbic acid determination

Total ascorbic acid concentration was calculated as the sum of ascorbic acid and dehydroascorbic acid, according to Wechtersbach and Cigic,²⁷ with some modification as reported by Jin *et al.*²⁸ 0.5 g of freeze-dried sample were extracted with 5 mL of *meta*-phosphoric acid (MPA) *tert*-butylhydroquinone (THBQ) solution (3 g 100 g⁻¹ MPA, 1 mmol L⁻¹ THBQ in Milli-Q water). The supernatant was collected in a new tube and the pellet was re-extracted with 5 mL of the MPA-THBQ solution, twice; the supernatants, collected in the same tube, was exactly weighed. To determine ascorbic acid, the extract was centrifuged at 12326 x g for 10 minutes at 4 °C, filtered and injected in HPLC. To determine the total ascorbic acid, 15 µL of tris-2-carboxyethyl phosphine solution (1 mol L⁻¹ in Milli-Q water) was added to 1.485 mL of the extract, incubated in the dark for 20 minutes and analysed by HPLC. Separation was conducted using a Varian Polaris C18-A column 5µm (150 x 4.6 mm), with a Varian ChromSep SS 10 x 3.0 mm guard column. The flow rate was 1 mL min⁻¹ and the injection volume was 20 µL. Elution was performed using 0.2% orthophosphoric acid in Milli-Q water, for a run time of 5.5 minutes. All the analyses were conducted in triplicate.

A stock standard solution of ascorbic acid in MPA-THBQ solution was prepared; test solutions were diluted in the range 1.56-200 µg mL⁻¹ in MPA-THBQ solution and used for the calibration curve.

Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method, as described by Heimler *et al.*²⁹ Phenolic compounds were extracted using the same hydro-alcoholic mixture obtained from GLS extraction. To 125 µL of the extract, 0.5 mL of distilled water and 125 µL of the Folin-Ciocalteu reagent were added. After mixing it was kept in the dark for 6 minutes; after that 1.25 mL of a 7% Na₂CO₃ solution was added and the final volume was adjusted to 4 mL with distilled water. The mixture was

kept in the dark for 90 minutes and then absorption at 760 nm was determined by UV-Vis spectrophotometer (Cary 50 BIO, Varian, Inc., Palo Alto, California, USA) against water as a blank. All the analyses were conducted in triplicate.

A gallic acid stock solution was prepared in methanol; test solutions were prepared in the range 0.020-0.500 mg mL⁻¹ in methanol and used for the calibration curve. Total phenolics were expressed as gallic acid equivalents (GAE, mg gallic acid kg⁻¹ of kale, dry weight (dw)).

Antioxidant activity

Total antioxidant activity was determined in the same hydro-alcoholic extract obtained from GLS extraction, as described above. The activity of cooked samples was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, according to Brand-Williams *et al.*³⁰ 0.1 mL of hydro-alcoholic extract was added to 3.9 mL of a 6 x 10⁻⁵ mol L⁻¹ methanolic DPPH[•] solution. Samples were incubated in a water bath at 25 °C, during 30 minutes, then absorption at 515 nm was determined by UV-Vis spectrophotometer (Cary 50 BIO, Varian, Inc., Palo Alto, California, USA). All the analyses were conducted in triplicate.

A trolox stock solution was prepared in methanol; test solutions were prepared in the range 0.020-0.200 mg mL⁻¹ in methanol and used for the calibration curve.

Values obtained for antioxidant activity were expressed as mg of trolox equivalents kg⁻¹ of kale (dw).

Colour determination

Colour of samples was determined as reported by Bongoni *et al.*³¹ Powder samples were placed into the cuvette of ColorFlex meter (Hunter Associates Laboratory, USA), with the lid closed and measured at two angles for the following parameters: L* (lightness),

a* (redness) and b* (yellowness). 6 replicates were measured for each sample. The following parameters were calculated, according to Mc Guire.³²

$$1) C^* = \sqrt{a^{*2} + b^{*2}} \text{ (colour saturation, or chroma)}$$

$$2) H = \left| \tan^{-1} \left(\frac{b^*}{a^*} \right) \right| \text{ (hue)}$$

Data processing

The data of all traits were subject to three-way ANOVA, including variety, way of cooking and cooking time, and their interactions, as experimental factors.

The patterns of individual traits as a function of cooking time, within variety and cooking way, were fitted by means of degradation kinetic equations, of different order, according to the scheme already illustrated elsewhere,³³ when applicable. Briefly models of order 0, 1, 2, and n, where order n was estimated as a parameter, were fitted to data and compared by means of the corrected Akaike information criterion.³⁴

For data fitting, the content of individual compound relative to time zero (raw material) were used, except for colour traits. In cases in which no adequate fitting was obtained by kinetic models, the significance of differences between varieties and cooking ways, within cooking time, were assessed by means of the protected Fisher's LSD test.

All the statistical analyses were carried out by means of the SYSTAT[®] package.

RESULTS AND DISCUSSION

Raw material profile

The values of phytochemical compounds contents and colour traits of raw materials are reported in Table 1.

Dry matter ranged between 0.12-0.18 g g⁻¹, with the highest value for Nero di Toscana and the lowest for Boerenkool.

Total glucosinolate content ranged between 4.19-6.96 mmol kg⁻¹ dw, with Nero di Toscana showing the highest value, and Boerenkool the lowest.

The glucosinolate profile included 7 compounds: the aliphatic glucosinolates glucoiberin (GIB), sinigrin (SIN) and glucoraphanin (GR), and the indolic glucosinolates glucobrassicin (GB), 4-hydroxy-glucobrassicin (H4GB), 4-methoxy-glucobrassicin (M4GB), and neo-glucobrassicin (NGB). Indolic GLS accounted for 62-80%, with the highest percentage detected in Boerenkool. GB represented the most abundant GLS (47-51% on the total amount of GLS), except for Nero di Toscana (30%) where neo-glucobrassicin was prevailing (32%). Aliphatic GLS were more abundant in Nero di Toscana (38%) that also showed the highest sinigrin percentage (18%), while Broccolo Lavagnino and Boerenkool showed a higher glucoraphanin and glucoiberin content (about 13%).

Indole-3-carbinol (I3C) and ascorbigen (ABG) were identified and quantified as indole GLS breakdown products. I3C is formed upon hydrolysis of GB. ABG is formed by a condensation reaction of I3C with ascorbic acid (AA). Raw Broccolo Lavagnino had the highest initial contents for both compounds.

Total ascorbic acid (TAA), calculated as the sum of ascorbic (AA) and dehydro-ascorbic acid (DAA), ranged between 5.3-8.1 mg g⁻¹ dw, in raw leaves, with Nero di Toscana showing the highest value. Similar figures are reported in Becerra-Moreno *et al.*¹ The three landraces also differed for their AA and DAA relative contents: in Boerenkool DAA prevailed (76%), whereas AA represented 54 and 66% of TAA in Broccolo Lavagnino and Nero di Toscana, respectively.

Total carotenoid content was in the range 3.1-3.8 mg g⁻¹ dw, without significant differences among landraces. β -carotene and lutein were identified as the most abundant compounds, violaxanthin and neoxanthin as minor compounds. The relative lutein and

β -carotene contents were rather similar, without differences among landraces, as for minor compounds, that accounted for 13-18% of total carotenoids.

Total phenolic content (TPC) ranged between 17.5-22.3 mg gallic acid g^{-1} dw, with the highest value for Boerenkool. These values are comparable with previous results,³⁵ but lower compared with the amounts detected on kale by Ismail *et al.*³⁶ Likewise the highest antioxidant activity was observed in raw Boerenkool leaves (13.2 mg Trolox g^{-1} dw).

Colour parameters. Considering raw leaves the parameters a^* , b^* , and L^* ranged between -8.27- -9.09, 21.39-25.82, and 43.73-46.04, respectively, as reported in Table 5. Raw leaves of Broccolo Lavagnino showed the highest values for b^* (yellowness) and L^* (lightness), and the lowest for $-a^*$ (greenness). Hue values indicate a greyish-green colour for all landraces, with lower yellow component and lightness in Nero di Toscana.

Evolution of analytical and colour traits during cooking

Dry matter content of cooked leaves showed slight variations with respect to uncooked leaves. Boiling and steaming produced a loss of dry matter, with reduction ranging between 0.04-0.02 g g^{-1} and 0.02-0.01 g g^{-1} , respectively. Whereas stir frying determined an increase of dry matter (0.01-0.05), especially for Boerenkool.

Glucosinolates. Figures 1 and 2 report the patterns of total, aliphatic, indolic glucosinolates, and glucobrassicin, as a function of cooking time, together with the values of the best fit kinetic parameter (k), when applicable. In the case of boiling, two separate graphs report the patterns of compounds in the leaf matrix only and in leaf matrix+boiling water (highlight by a star *).

Data analysis indicated significant interactions between all the experimental factors, meaning that the evolution of compound content as a function of cooking time differed between both cooking systems and varieties.

Previous works^{18,23} have described GLS thermal degradation as a first order kinetic reaction. In the present study, following Akaike selection procedure total GLS content during boiling followed a second order kinetic. GLS reduction was faster in Nero di Toscana, as indicated by its significantly higher kinetic constant with respect to the other two landraces (Figure 1). An overall higher retention of glucosinolates was detected when referring to their content obtained from the sum of cooked leaves + cooking water (a lower 2nd order k_d in all situations); in this case, the difference between Nero di Toscana and the other two landraces was even higher than what detected for leaves only. As a result, the relative GLS content of Nero di Toscana decreased to less than 20% its initial values after 10 minutes boiling, compared to about 50% retention of the two other landraces.

Steaming determined a quite well characterised pattern. For Boerenkool no apparent decrease of GLS content was detected during the first 10 minutes, with a subsequent overall slightly significant decrease (22-38 %); this is perhaps due to a combination of the effect of leaf structure and GLS type, determining an overall low decline and better extractability, at shorter steaming times. The pattern of the other two landraces was represented by a second order kinetic, with again higher constant for Nero di Toscana. Overall, steaming determined a substantial lower GLS loss than boiling.

Stir frying also determined a different GLS trend, depending on landrace. In Boerenkool a moderate linear decrease was observed, whereas a second order kinetic represented GLS evolution of the other two landraces, with losses around 65-75%.

Indeed reductions were substantially higher for aliphatic glucosinolates. Non significant differences were detected among varieties, this fact likely depend on differences of leaf structure, so our data do not allow to identify the specific determinant traits.

Indole GLS followed trends similar to what explained for total GLS, with generally lower kinetic constants, except for Boerenkool during boiling, and lower differences between landraces. The structure of glucosinolates had an effect on their thermal stability, in accord with previous works,^{37,38} however they found a greater reduction for indolic GLS. On the contrary, in this study the reduction of aliphatic GLS appeared to occur quite rapidly, with very high kinetic constants for Nero di Toscana, especially for boiling and stir frying. Indeed this landrace had higher content of aliphatic GLS, less stable to cooking. Low amounts of aliphatic GLS were detected in cooking water, giving lower recovery factors with respect to the content detected in the corresponding raw leaves.

Glucobrassicin pattern did not differ among varieties for boiling, although best fit was given by a first order kinetic for Broccolo Lavagnino and Nero di Toscana and by a second order for Boerenkool.

Depending on the cooking methods different losses in GLS occurred, in accord with Xu *et al.*³⁹ In particular, steaming most retained GLS, whereas the difference between boiling and stir frying was strongly dependent on the characteristics of the variety.

It has been demonstrated that the food matrix plays an important role in GLS thermal degradation.³⁸ Also in our study the three landraces had different retention of GLS during cooking. In this respect, phenotypical features could play a role. Overall, thinner leaves of Boerenkool were associated with lower degradation. Nero di Toscana had very crinkled leaves that could contribute to a larger surface, increasing the exposure to cooking water and heating; moreover its fleshy texture could lead to a more drastic collapse of the structure during cooking. In a previous study, Rosa and Heaney⁴⁰ found

differences of GLS losses between cultivars, attributing this fact to differences in leaf characteristics, such as thickness and waxiness.

Indole-3-carbinol and ascorbigen formation. Figure 3 reports the values of I3C and ABG conversion factors from glucobrassicin, obtained relating I3C or ABG molar content to the glucobrassicin molar content in the same sample. The conversion values obtained were quite low (from non-detectable to 23.5%) probably caused by the inactivation of the myrosinase enzyme and the instability of these compounds during thermal treatment.

Indole-GLS derived compounds were not detectable in boiled leaves, independently on variety. I3C was however detected in cooking water. The absolute content of this compound does not indicate a clear pattern, since they derive from the balance of glucobrassicin conversion and external conditions. However, the pattern of conversion coefficient from glucobrassicin is much clearer. In boiling water, conversion linearly increased, with no significant differences among varieties, until 30 minutes cooking; after 60 minutes the conversion dramatically increased for Nero di Toscana and Boerenkool. Conversion was lower and linear, without a final increase in all varieties for steaming, with Broccolo Lavagnino having again the lowest increases as a function of cooking time. GB conversion into I3C was finally very low for stir fried leaves. A lack of literature data makes it difficult to compare our results. A previous study on fermented cabbage found an initial increase in I3C during the first 10 minutes of boiling, as a result of thermal hydrolysis of ABG; whereas for the rest of boiling I3C content was relatively stable, because compensated by I3C condensation.⁴¹

Also for ABG the absolute values of its content do not reveal a clear pattern, that is however detectable for conversion factors. Again a higher increase of the conversion value was noticed for longer cooking times, especially for Nero di Toscana upon steaming and boiling, and Broccolo Lavagnino during stir frying. The almost complete

absence of ABG following boiling can be related to the findings of Ciska *et al.*,⁴¹ showing a decrease of ABG in fermented cabbage as a function of boiling time.

Ascorbic acid. Total ascorbic acid showed a decreasing pattern, which was not fitted by a model, allowing consistent comparisons between landraces and cooking systems on the basis of kinetic parameters (Figure 4): in fact, although some significance of overall data fitting was obtained, the initial pattern of ascorbic acid, that remained almost constant during the first minutes of cooking, is not consistent with a monotonously decreasing function. Between cooking methods, the differences between varieties were rather low and generally not significant, within cooking times; Broccolo Lavagnino showed a slower initial decrease, determining an overall lower order of degradation kinetic. Overall, steaming allowed a higher retention at intermediate cooking times (10-20 min), whereas the differences with boiling were not significant after 60 minutes cooking. Partially in agree with our results, Korus and Lisiewska¹⁹ reported drastic losses in vitamin C in leafy vegetables, regardless cooking methods.

This pattern of total ascorbic acid was generated by two rather different trends of DAA and AA. In fact, DAA decreased to only about 4-13% of DAA after five minutes cooking; this fact was clearly connected to its conversion into AA, as demonstrated by the strong increase of the latter after five minutes cooking and followed by degradation for longer cooking times. The consistency of this interpretation is confirmed by the fact that initial AA increase was particularly high in Boerenkool, in which DAA represented over 75% total ascorbic acid.

Carotenoids. Pigment profiles after cooking are represented in Figure 5. An asymptotic increase of total carotenes, β -carotene and lutein, for boiling, and of β -carotene for steaming was observed. In case of β -carotene, this increment was significantly higher for Broccolo Lavagnino and also for Boerenkool, in case of boiling. This pattern is likely due to a higher chemical extractability of these compounds, as a consequence of

thermally induced structural changes of membranes and denaturation of proteins.⁴² Dos Reis *et al.*⁴³ also observed that steam processing resulted in an increase of broccoli lutein and β -carotene extractability.

Lutein content, on the contrary, linearly decreased in steamed Boerenkool and Broccolo Lavagnino leaves, to about 54-42% the content of raw leaves, whereas no changes were observed in Nero di Toscana.

Stir frying caused a marked decrease of carotenoid content, with special respect to β -carotene, reducing its content to 10-23% of that of raw leaves, independently on variety. Partially in agreement with our results, Chang *et al.*⁴⁴ found large variations of carotenoids content after cooking: after boiling they observed lutein increase and β -carotene decrease, whereas stir-frying determined mainly a decrease of both compounds.

Different patterns of carotenoids after cooking are described; however in most cases, it was highlighted that water-cooking treatments better preserve these antioxidant compounds.⁴⁵ Boiling and steaming seem to enhance carotenoids bioavailability, probably following cell wall structure disruption of the leaf matrix.⁴⁶

Total phenolic content (TPC) and antioxidant activity (TEAC). The trends observed for TPC and TEAC are shown in Figure 6. TPC loss from the leaf matrix during boiling followed a second order degradation kinetic, with only slightly lower constant for Broccolo Lavagnino. However, high amounts of phenolic compounds were detected in boiling water, due to leaching; so when considering the sum of TPC in boiled leaves + cooking water no overall phenolic losses occurred for this cooking system. Steaming caused only a very limited linear TPC decrease (5-30%), as indicated by the zero order kinetic.

Finally, stir frying determined a second order kinetic loss of phenolics, with constants somewhat lower with respect to what observed for boiling on the leaves only, and no differences among varieties (losses around 35-41%).

The antioxidant activity relative to uncooked material followed different patterns. For boiled leaves and stir frying, its pattern was similar to that of TPC, with generally lower decrease rates. In boiling, the antioxidant activity remained indeed constant during the initial times of cooking. For steaming, an initial increase of relative antioxidant activity was detected, followed by a moderate decrease (15%) for longer cooking times, however resulting in an overall slightly significant linear decrease only for Nero di Toscana. In case of boiled leaves + cooking water, the pattern was substantially different from that observed on phenolics. In fact, a second order kinetic decrease represented it for all landraces, indicating that phenolics leached in water seemed to be subject to a substantial loss of antioxidant capacity, as represented in Figure 7. In fact the relation between TPC and antioxidant capacity, indicates a much higher antioxidant efficacy of phenolics retained in boiled leaves with respect to those leached in water. Besides this fact, the slope of the relation between TPC and antioxidant capacity was almost the same for raw and boiled leaves; however, boiled leaves reached comparable antioxidant effect at lower TPC concentration, supporting the idea of a lower antioxidant power of the phenolics leached in water.

Partially in agreement with our results, a similar work on kale cooking³⁵ determined no change in TPC and antioxidant activity, independent of the cooking time; in particular it was observed a variation in the flavonoid profile, but this simultaneous loss of antioxidants was compensated by neo-formed antioxidants during thermal treatment.

Whereas a previous work on red cabbage,⁴⁷ found a decrease of antioxidant activity following thermal treatment, except for steaming, confirming that this cooking way had a less marked effect on the compounds responsible of the antioxidant activity.

Colour traits. Figure 8 represents the pattern of hue and saturation, that were chosen as better suitable traits to represent colour variation,³² as affected by the experimental treatments.

Steaming and stir frying determined a more marked linear decrease of hue values, indicating a loss of green hue.

Colour saturation slightly increased during the first minutes of boiling; this trend can be connected to a change of surface-reflecting properties, as reported in the case of vegetable bleaching,⁴⁸ saturation then linearly decreased to slightly lower values than initial ones at longer boiling times. Saturation was not affected by steaming, whereas stir frying determined a decrease of colour saturation, represented by a second order degradation kinetic. This pattern, similar to the one detected on carotenoids, confirms that pigments are more sensitive to the more drastic thermal conditions occurring with stir frying.

Miglio *et al.*⁴⁵ reported a decrease of broccoli florets L^* values in all cooking treatments used (boiling, steaming, frying). Moreover these authors found steamed and fried broccoli parts (stem and florets) to be less green with respect to the raw and the boiled products. The loss of greenness observed in steaming was mainly due to the highest chlorophyll degradation, determined by a major exposure of kale leaves to air.

CONCLUSIONS

Variety plays an important role in determining the fate of bioactive compounds, with special respect for glucosinolates. We could assume that phenotypical traits of leaves (shape, texture, composition of external layers) and the kind of glucosinolate could have a role in determining the phenomena of leaching and thermal damage.

Also the cooking method had a significant impact in the final content of bioactives. Carotenoids appeared to be the most stable compounds, but were subject to decay in stir

frying. Total phenolic compounds and antioxidant activity were mainly affected by boiling (due in part to leaching) and stir-frying (major exposure to the oxygen associated with a higher temperature, due to the presence of oil). It is interesting to note that, total phenolics are apparently completely retained in traditional recipes in which the cooking water is conserved, as it occurs in some vegetable soups

Steaming showed the highest bioactive compound retention factors. The main determinant of this was the absence of direct contact with the water, reducing leaching of hydrophilic compounds. In many cases we also noticed an increase of bioactive compound contents, probably connected to membrane damage, increasing extractability of the compounds during the analysis. On the other hand physical traits, such as green colour of the leaves, were mostly negatively affected by this cooking method, due to the highest chlorophyll degradation. The results indicate that, although some ways of cooking are strictly inherent to traditional recipe preparation, some possibility of improving bioactive component retention may exist. The study of degradation kinetic allowed to calculate variety specific parameters, even if, the evolution of phytochemicals hardly follows simple models in open systems such the ones used in this experience.

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FIGURE CAPTIONS

Figure 1. Relative total glucosinolate and glucobrassicin contents as affected by cooking method and time.

Legend:

RTGLS: total glucosinolate content relative to time zero; RGB: glucobrassicin content relative to time zero.

(*): sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 2. Relative indolic and aliphatic glucosinolate contents as affected by cooking method and time.

Legend:

RIGLS: indolic glucosinolate content relative to time zero; RAGLS: aliphatic glucosinolate content relative to time zero.

(*): sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 3. Conversion value percentage (%Cv) of glucobrassicin into indole-3-carbinol and ascorbigen as affected by cooking method and time.

Legend:

I3C: indole-3-carbinol; ABG: ascorbigen.

(*): sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 4. Relative total, dehydro- and ascorbic acid contents as affected by cooking method and time.

Legend:

RTAA: total ascorbic acid content relative to time zero; RDAA: dehydro-ascorbic acid content relative to time zero; RAA: ascorbic acid content relative to time zero.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 5. Relative contents of total carotenoids, β -carotene and lutein as affected by cooking method and time.

Legend:

RTCAR: total carotenoid content relative to time zero; RbCAR: β -carotene content relative to time zero; RLUT: lutein content relative to time zero.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 6. Relative total phenolic content and relative trolox equivalent antioxidant activity as affected by cooking method and time.

Legend:

RTPC: total phenolic content relative to time zero; RTEAC: trolox equivalent antioxidant activity relative to time zero.

(*): sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 7. Correlation between antioxidant activity and total phenolic content of raw and boiled leaves, and cooking water.

Legend:

TPC: total phenolic content; TEAC: trolox equivalent antioxidant activity.

Figure 8. Colour traits as affected by cooking method and time.

Legend:

H: hue; C*: saturation.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Table 1. Phytochemical compounds and colour traits in raw kale samples.

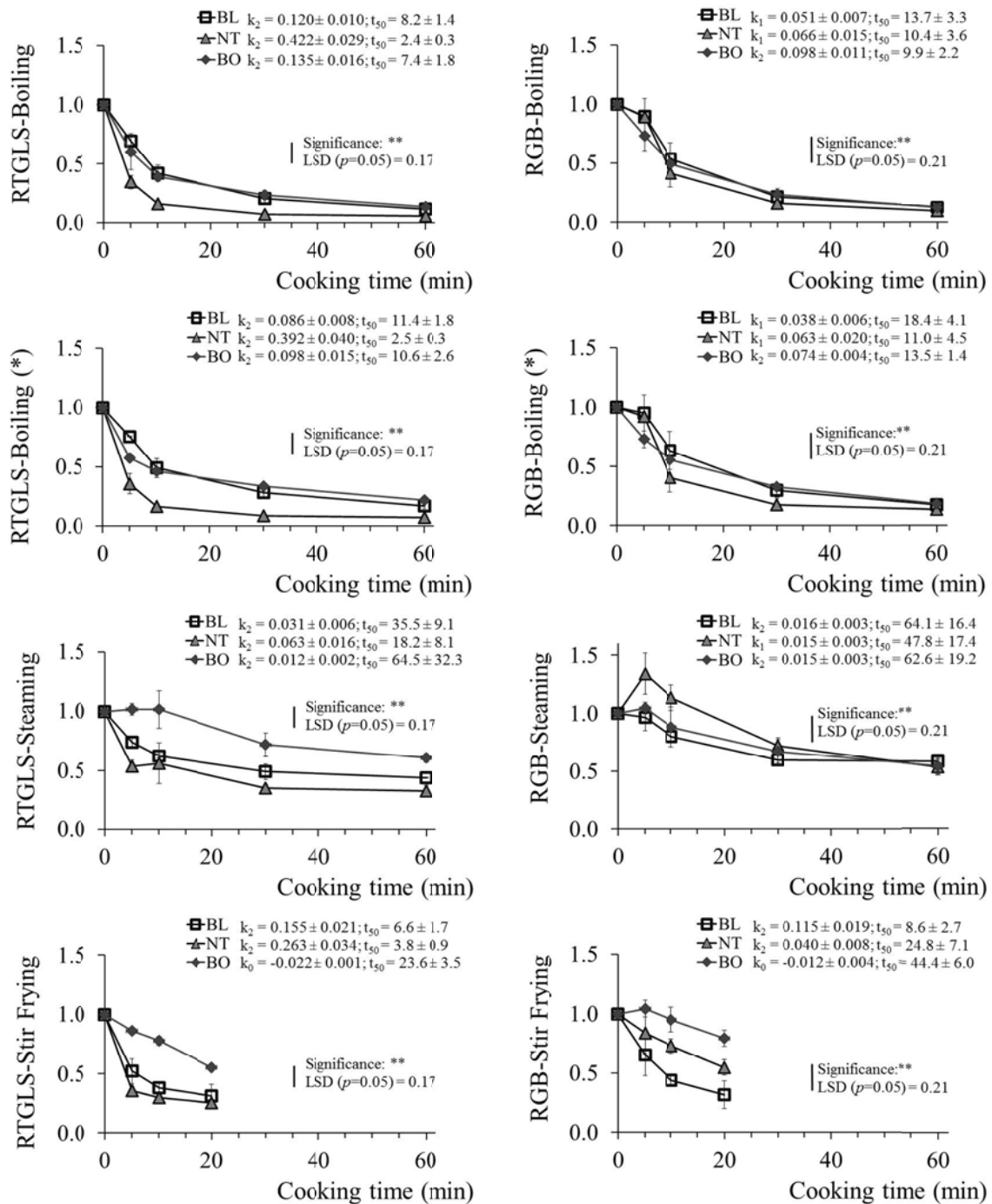
	Landraces			Significance ^a
	BL	NT mean ± SD ^b	BO	
Total Glucosinolates (mmol kg⁻¹)	5.30 ± 0.36 b	6.96 ± 0.26 a	4.19 ± 0.15 c	**
Relative contents (mmol mmol⁻¹ total glucosinolates)				
Glucoiberin	0.067 ± 0.003 c	0.104 ± 0.009 b	0.129 ± 0.008 a	**
Sinigrin	0.081 ± 0.007 b	0.176 ± 0.009 a	0.059 ± 0.004 b	**
Glucoraphanin	0.128 ± 0.007 a	0.100 ± 0.001 b	0.010 ± 0.002 c	**
4-hydroxy-glucobrassicin	0.006 ± 0.001 b	0.007 ± 0.001 ab	0.008 ± 0.001 a	**
Glucobrassicin	0.510 ± 0.019 a	0.294 ± 0.035 b	0.469 ± 0.015 a	**
4-methoxy-glucobrassicin	0.007 ± 0.001 b	0.002 ± 0.000 c	0.026 ± 0.001 a	**
Neo-glucobrassicin	0.201 ± 0.003 b	0.315 ± 0.034 a	0.298 ± 0.012 a	**
Aliphatic glucosinolates	0.276 ± 0.017 b	0.381 ± 0.001 a	0.199 ± 0.003 c	**
Indolic glucosinolates	0.724 ± 0.017 b	0.619 ± 0.001 c	0.801 ± 0.003 a	**
Indol-Glucosinolates derived compounds (mmol kg⁻¹)				
Ascorbigen	0.051 ± 0.001 a	0.020 ± 0.004 b	0.019 ± 0.004 b	**
Indole-3-carbinol	0.146 ± 0.012 a	0.034 ± 0.007 c	0.113 ± 0.014 b	**
Total Ascorbic acid (mg g⁻¹)	5.6 ± 1.5 b	8.1 ± 0.6 a	5.3 ± 0.1 b	**
Relative contents (mg mg⁻¹ total ascorbic acid)				
Ascorbic acid	0.544 ± 0.001 a	0.657 ± 0.029 a	0.237 ± 0.005 b	**
Dehydro-ascorbic acid	0.456 ± 0.001 b	0.343 ± 0.029 c	0.763 ± 0.005 a	**
Total Carotenoids (mg g⁻¹)	3.3 ± 0.3	3.8 ± 0.9	3.1 ± 0.4	ns
Relative contents (mg mg⁻¹ total carotenoids)				
Neoxanthin	0.153 ± 0.016	0.150 ± 0.008	0.132 ± 0.006	ns
Violaxanthin	0.186 ± 0.054	0.148 ± 0.011	0.179 ± 0.031	ns
Lutein	0.384 ± 0.003	0.386 ± 0.012	0.359 ± 0.021	ns
β-carotene	0.277 ± 0.073	0.316 ± 0.009	0.330 ± 0.004	ns
Total phenolic content (mg g⁻¹)	19.5 ± 0.5 ab	17.5 ± 0.3 b	22.3 ± 0.2 a	**
Trolox equivalent antioxidant capacity (mg kg⁻¹)	12.0 ± 0.1 ab	9.5 ± 0.1 b	13.2 ± 0.3 a	**
Colour traits				
L*	46.04 ± 0.34 a	45.00 ± 0.18 b	43.73 ± 0.10 c	**
a*	-9.09 ± 0.05 c	-8.52 ± 0.04 b	-8.27 ± 0.06 a	**
b*	25.82 ± 0.28 a	22.88 ± 0.04 b	21.39 ± 0.12 c	**
Hue	109.40 ± 0.09 b	110.43 ± 0.07 ab	111.14 ± 0.04 a	**
Saturation	27.38 ± 0.28 a	24.41 ± 0.05 b	22.93 ± 0.13 b	**

Legend:

^a Significance: **: p ≤ 0.01; ^b different letters indicate significantly different means for protected LSD, p ≤ 0.05.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 1. Relative total glucosinolate and glucobrassicin contents as affected by cooking method and time.



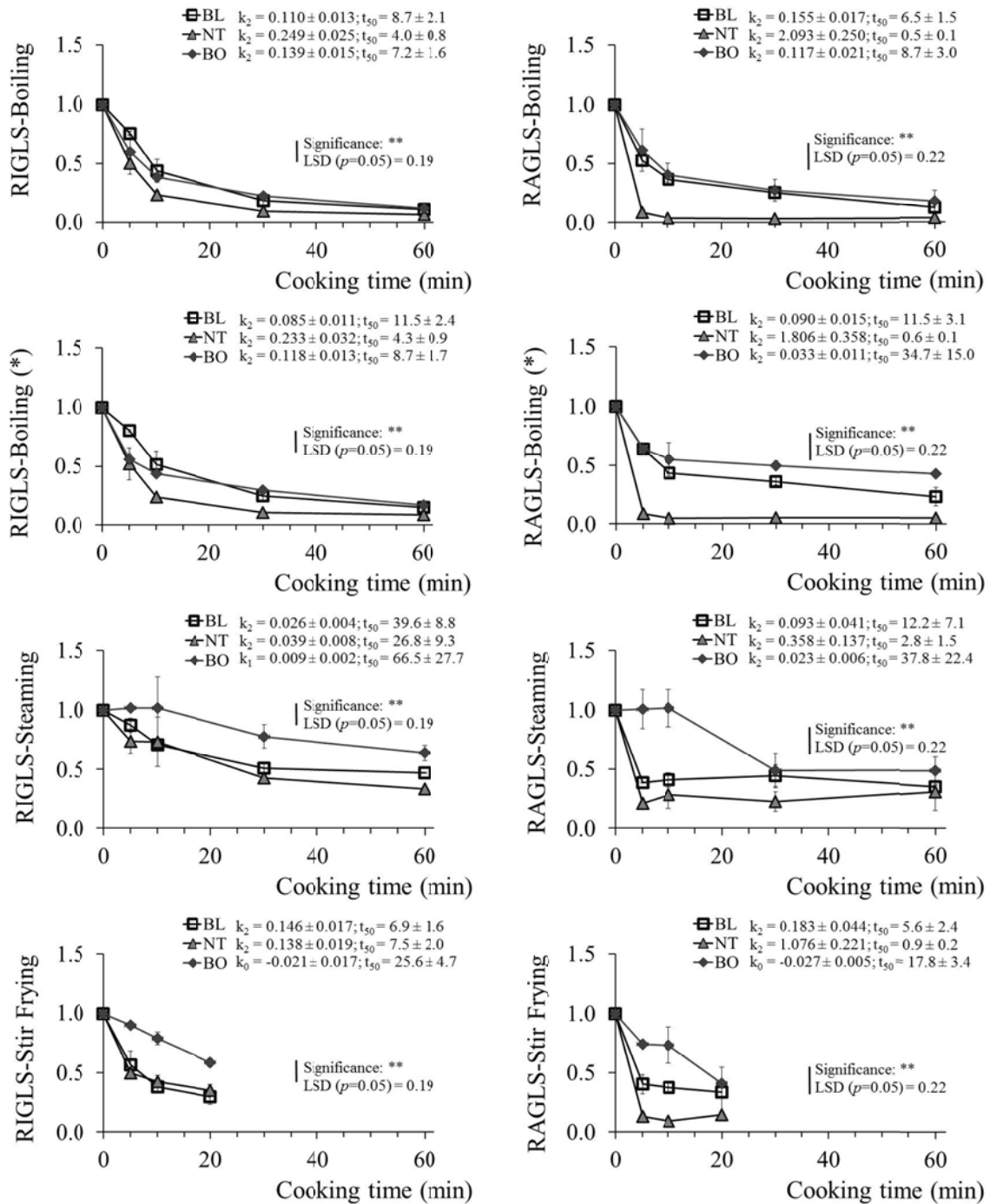
Legend:

RTGLS: total glucosinolate content relative to time zero; RGB: glucobrassicin content relative to time zero; t_{50} : time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

(*): sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 2. Relative indolic and aliphatic glucosinolate contents as affected by cooking method and time.



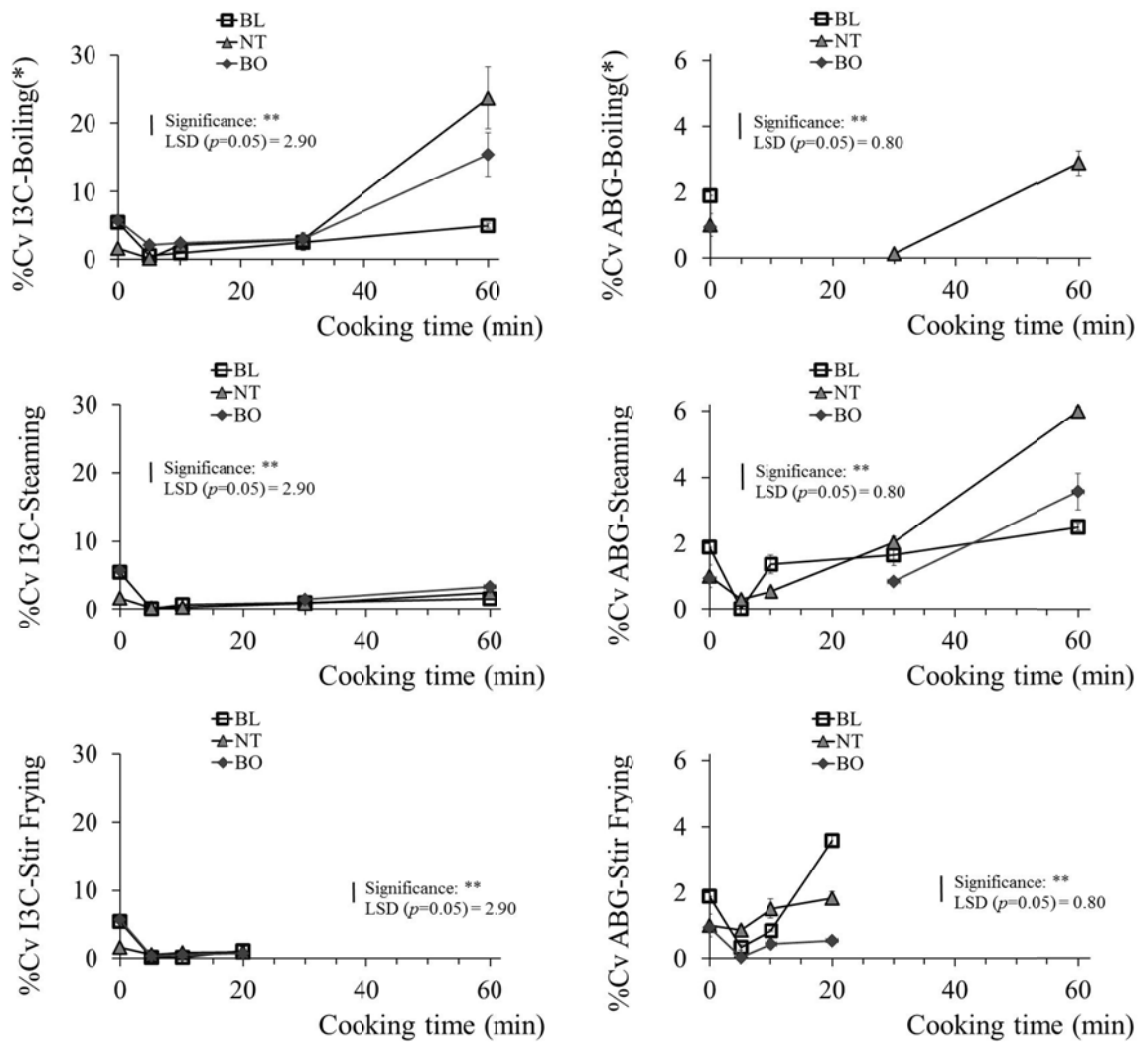
Legend:

RIGLS: indolic glucosinolate content relative to time zero; RAGLS: aliphatic glucosinolate content relative to time zero; t_{50} : time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

(*):sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 3. Conversion value percentage (%Cv) of glucobrassicin into indole-3-carbinol and ascorbigen as affected by cooking method and time.



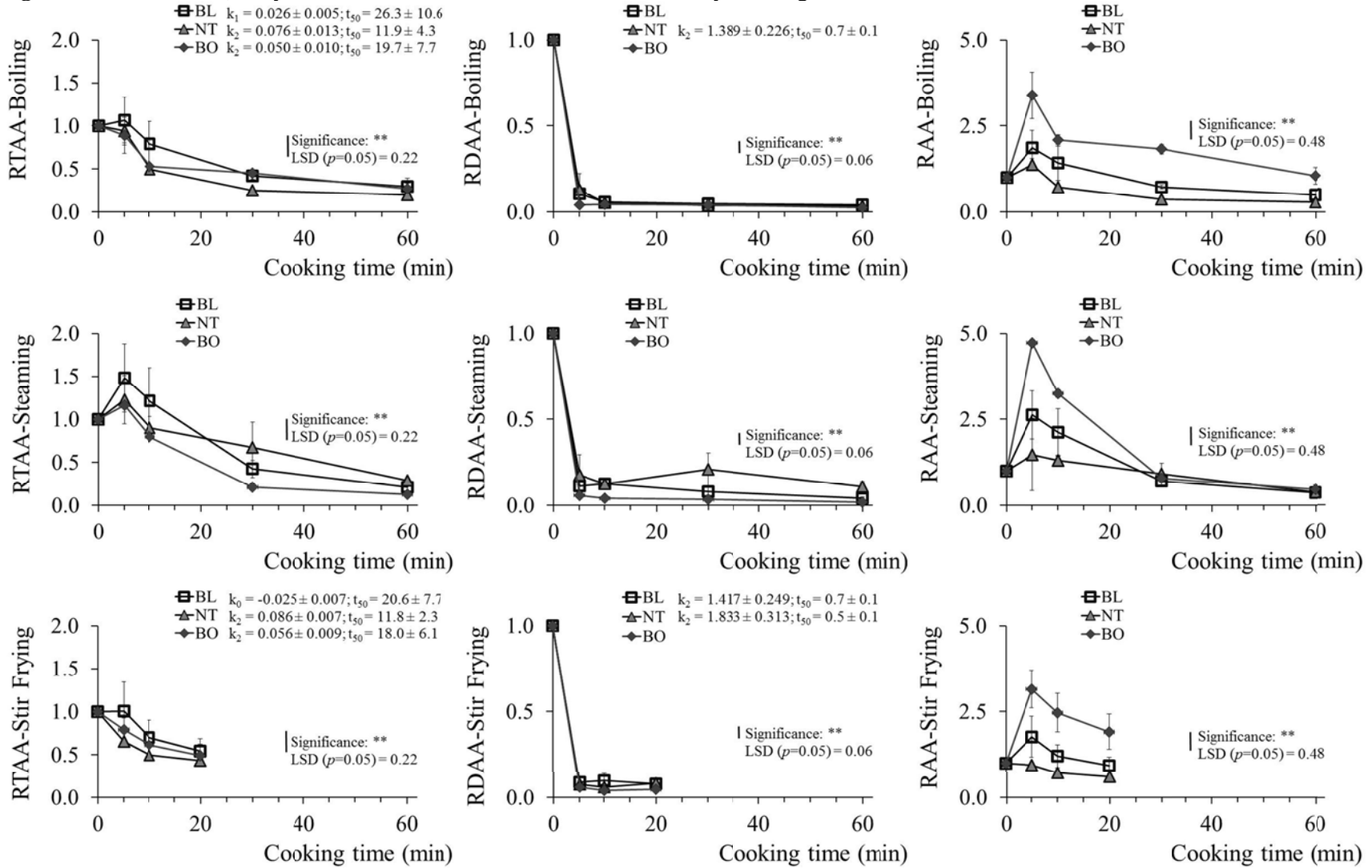
Legend:

I3C: indole-3-carbinol; ABG: ascorbigen; t_{50} : time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

(*):sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 4. Relative total, dehydro- and ascorbic acid contents as affected by cooking method and time.

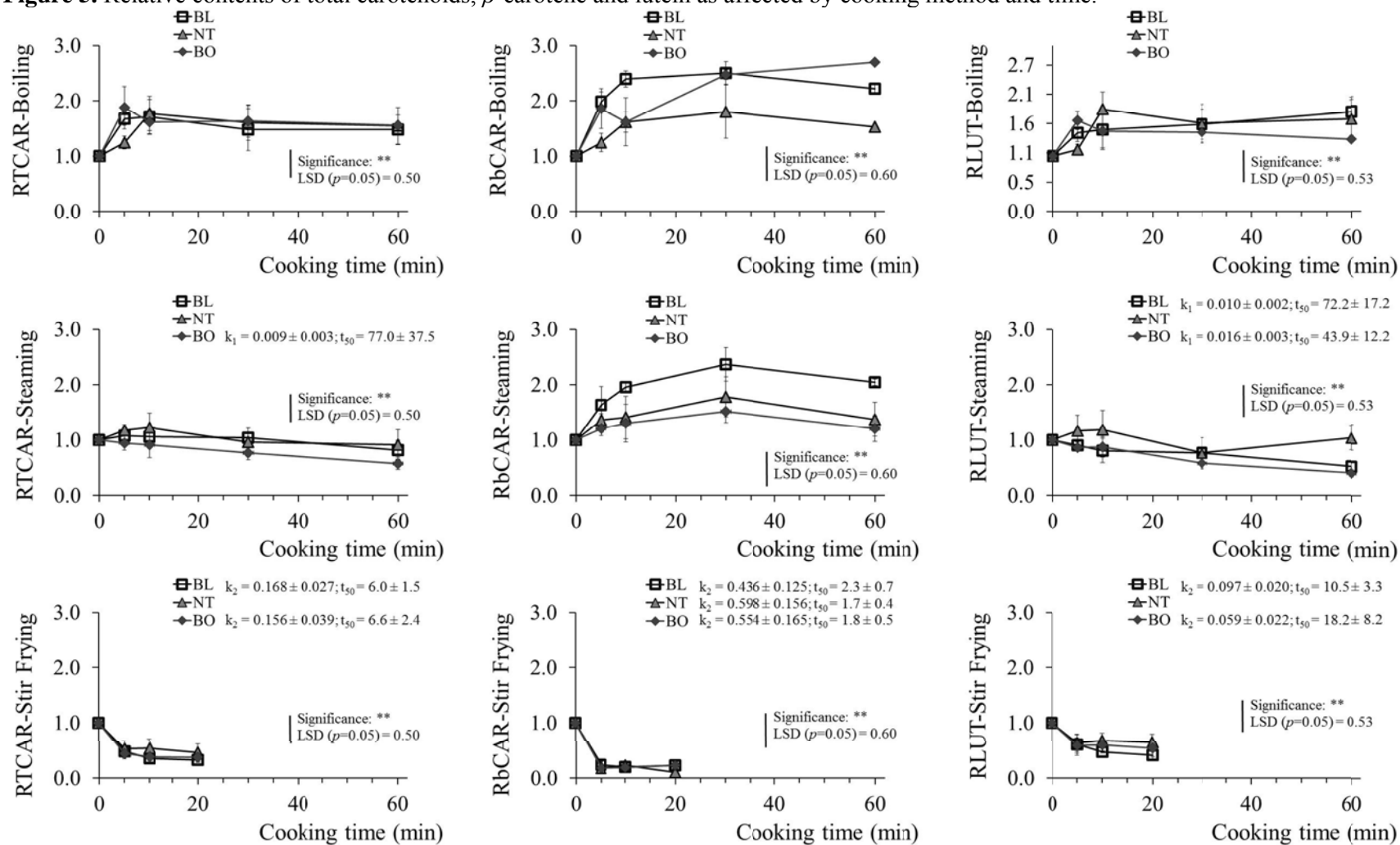


Legend:

RTAA: total ascorbic acid content relative to time zero; RDAA: dehydro-ascorbic acid content relative to time zero; RAA: ascorbic acid content relative to time zero; t_{50} : time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 5. Relative contents of total carotenoids, β -carotene and lutein as affected by cooking method and time.

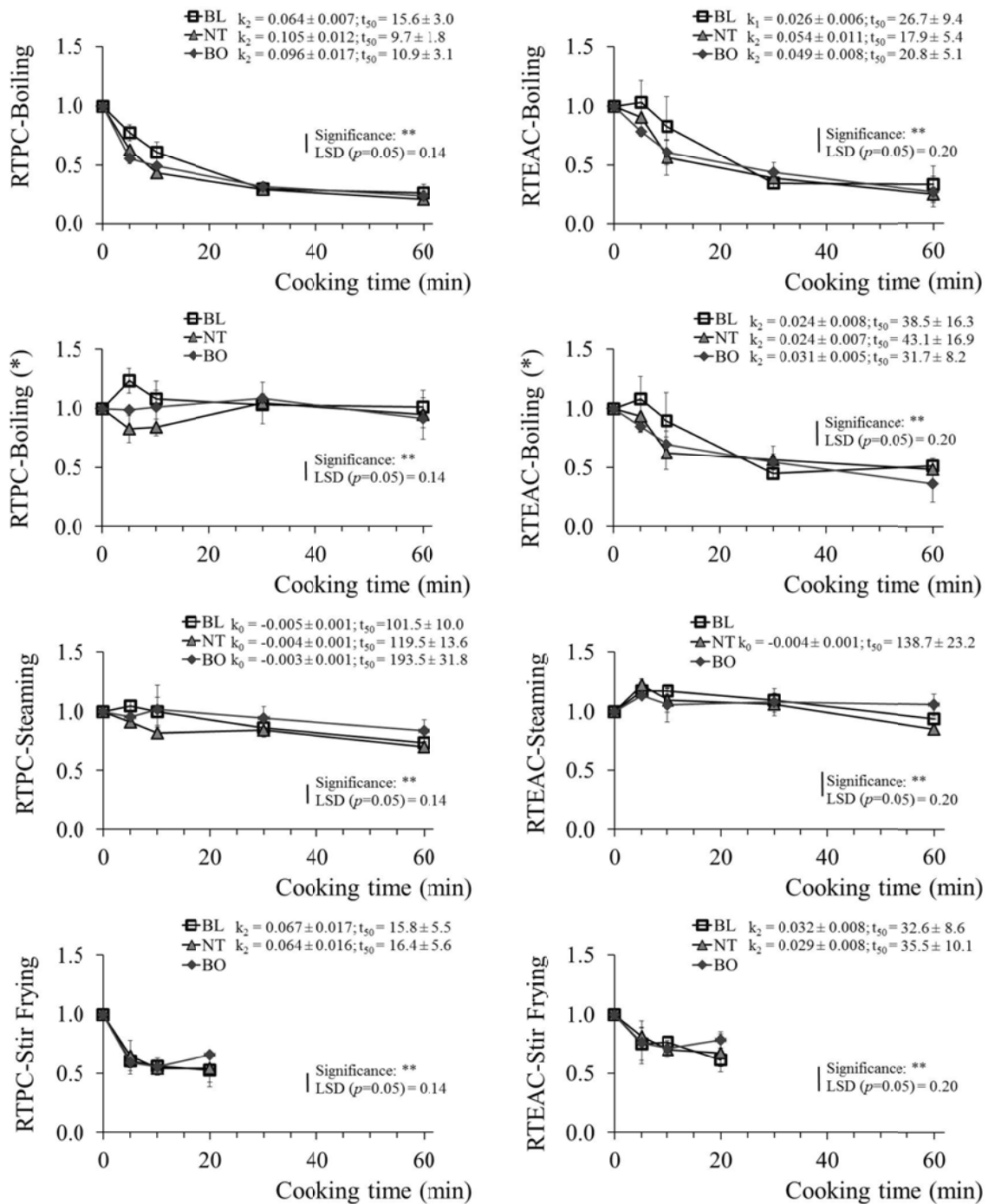


Legend:

RTCAR: total carotenoid content relative to time zero; RbCAR: β -carotene content relative to time zero; RLUT: lutein content relative to time zero; t_{50} : time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Figure 6. Relative total phenolic content and relative trolox equivalent antioxidant activity as affected by cooking method and time.



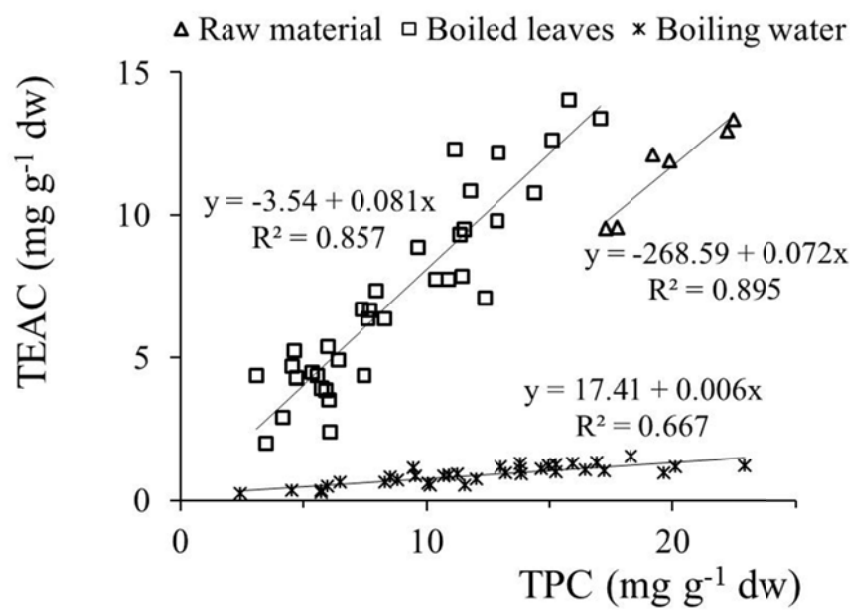
Legend:

RTPC: total phenolic content relative to time zero; RTEAC: trolox equivalent antioxidant activity relative to time zero; t_{50} : time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

(*):sum of the content detected in boiled leaves and cooking water.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

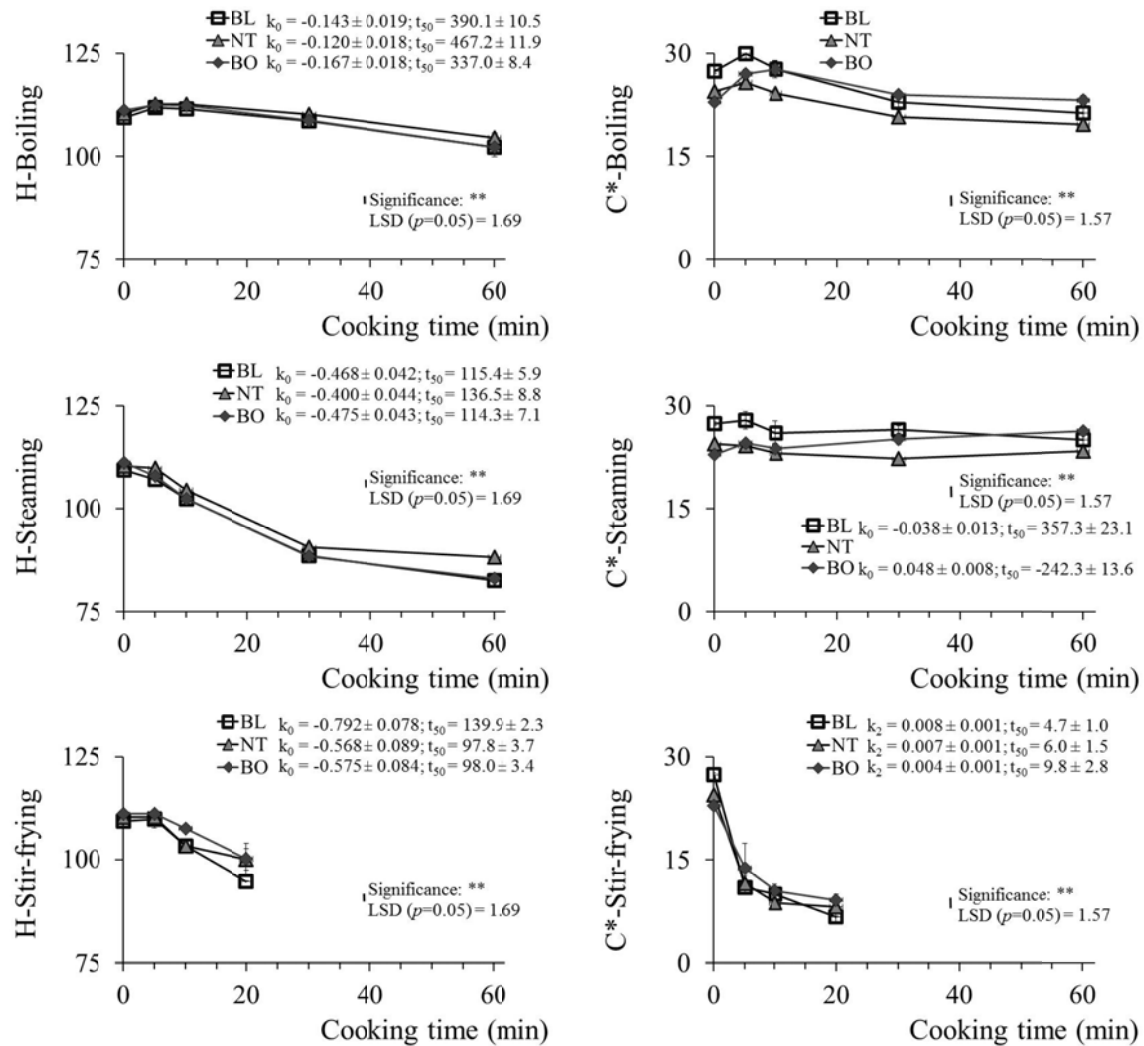
Figure 7. Correlation between antioxidant activity and total phenolic content of raw and boiled leaves, and cooking water.



Legend:

TPC: total phenolic content; TEAC: trolox equivalent antioxidant activity.

Figure 8. Colour traits as affected by cooking method and time.



Legend:

H: hue; C*: saturation; t₅₀: time (minutes) at which a 50% decrease of relative content is attained, on the basis of the calculated kinetic.

BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.