Time-Dependent Effects of Cooking on Antioxidant Capacity and Phytochemical Retention in Green Leafy Vegetables

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Abstract

Green leafy vegetables (GLVs) are major dietary sources of phenolic compounds, flavonoids, vitamin C and pigments, yet these constituents respond very differently to domestic heat treatment. Most previous work contrasts only raw versus cooked material and relies on a single antioxidant assay, leaving the time course of change poorly resolved. This study quantified the time-dependent effect of atmospheric boiling (0, 2, 5, 10, 15 and 20 min; 1:10 w/v in distilled water, timed from the return to a rolling boil) on five commonly consumed GLVs spinach, kale, Swiss chard, molokhia and amaranth using three complementary in vitro antioxidant assays (DPPH, ABTS, FRAP), total phenolic (TPC) and flavonoid content, vitamin C, chlorophylls, carotenoids and HPLC-DAD quantification of six identified phenolic markers, with mass-corrected true-retention (TR) analysis and multivariate statistics. Short boiling (2–5 min) increased Folin–Ciocalteu-measured TPC by 12–16% in the more robust species and was accompanied by higher measured antioxidant capacity; this apparent gain is interpreted as increased extractability and release of bound phenolics rather than net phenolic synthesis. Prolonged boiling (≥15 min) reduced every measure. Vitamin C was the most labile constituent (TR 11–22% after 20 min), while carotenoids were the most stable. A significant vegetable×time interaction confirmed species-specific thermal behaviour, with kale most resilient and Swiss chard least. Among the cooked levels tested, an equal-weight integrated retention index was highest at 2 min for all five species, and the ranking was unchanged on a mass-corrected TR basis; however, once mass loss was accounted for no cooked level retained the full raw complement of the measured constituents, and 2 and 5 min did not differ statistically for phenolics or antioxidant capacity in four of the five species. The data therefore support brief boiling (2–5 min, toward the shorter end where vitamin C is the priority) as a retention-favourable window for the species and boiling conditions studied. The assays quantify chemical antioxidant capacity of the drained edible portion only; bioaccessibility, constituents leached into the discarded cooking water, sensory quality and other cooking methods were not assessed, and the recommendations are bounded accordingly.

Keywords: *green leafy vegetables; boiling; antioxidant capacity; phenolic compounds; vitamin C; true retention; species-specific response; thermal processing.*

1. Introduction

Green leafy vegetables (GLVs) are one of the most nutritious parts of the human diet, providing dietary fibre, minerals and a wide range of health-promoting phytochemicals such as phenolic acids, flavonoids, vitamin C, chlorophylls and carotenoids [1], [2], [42]. These constituents account for a large part of the antioxidant activity in the plant matrix and by their ability to scavenge reactive oxygen species (ROS) and chelate transition metals, have been associated in epidemiological and mechanistic studies with protection against oxidative stress, a

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process implicated in cardiovascular disease, several cancers and other chronic conditions [2], [3]. It should be recognised, however, that antioxidant capacity measured in vitro is an index of the redox-active constituents present in an extract and does not translate directly into a physiological effect, which additionally depends on digestion, absorption and metabolism [36], [37]. The nutritional value that a GLV actually delivers therefore depends not only on its raw composition but on the fraction of these constituents that survives preparation and, subsequently, on the fraction that becomes bioaccessible.

In practice, however, GLVs are seldom eaten raw: they are boiled, steamed, stir-fried or blanched for widely different lengths of time depending on household and cuisine. Heat processing acts on plant phytochemicals through several competing mechanisms. It may cause destruction of thermolabile compounds like ascorbic acid, [4-6]. Because these processes occur simultaneously and at different rates, cooking can either increase or decrease the measured antioxidant capacity of a vegetable, depending on the vegetable species, the phytochemical composition, the cooking medium and, critically, the cooking time [7, 8, 9], [42], [43]. Boiling in particular combines high temperature with a large volume of water, so that both thermal degradation and leaching into the cooking medium operate, and it is the method for which duration is most consequential [10, 11], [43].

1.1 Research gap

The effect of cooking on vegetable antioxidants has been researched for 20 years, but a number of gaps need to be addressed to convert this research into practical advice. First, most investigations focus on comparing between one cooked state and the raw material and do not provide the full time dependent response, and thus the path between initial and final states, where any optimum must lie, is hidden [7], [12], [44], [45]. Second, many studies use a single antioxidant assay, for example DPPH, although antioxidant assays probe different reaction chemistries and endpoints; using complementary assays provides a broader assessment of antioxidant capacity [20-22], [51]. Third, there has been little comparison of the responses of various species of leaves to the same cooking treatment, which is complicated by procedural differences [15], [43]. Fourth, the antioxidant capacity, total phenolics and flavonoids, thermolabile nutrients and individual phenolics are not commonly included in a single experimental design, and therefore the compositional drivers for any changes in activity are inferred rather than measured [16]. Fifth, apparent changes in concentration are reported without accounting for mass changes that occur during boiling which may be the appearance of a gain [17, 18], [48], [49]. Last but not least, an increase in Folin–Ciocalteu total phenolic content after a short cooking time is often reported as a “gain in phenolics” but this could be due to better extraction or release of phenols that were bound in the raw material and may not actually represent an increase in the phenolic mass [38], [50].

These constraints make it difficult to define, for common GLVs, a boiling time window that both preserves the phytochemicals and results in a desired level of cooking. To compare species, quantify species-specific effects and to find the retention optima with respect to duration, a systematic, time-resolved comparison where several antioxidant assays, phytochemical and pigment measurements and targeted quantification of phenolic compounds are

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performed on the same material under identical, fully specified cooking conditions and corrected for the mass change is required.

1.2 Aim, objectives and hypotheses

The objective of the present study was to characterize the time-dependent effect of boiling on the antioxidant capacity, phytochemical composition and retention of five selected GLVs, in order to identify, within the boiling conditions tested, the cooked level associated with the highest retention of the measured antioxidant and phytochemical variables. The study was deliberately restricted to a single cooking method atmospheric boiling so that the time dimension could be resolved finely under otherwise constant conditions; comparison among cooking methods was outside its scope. The specific objectives were: (i) to determine the values of antioxidant capacity and phytochemical composition of the raw vegetables; (ii) to evaluate the changes in antioxidant capacity in six cooking times with three complementary assays; (iii) to quantify the effects of cooking time on the total phenolic content, total flavonoid content, vitamin C content, chlorophylls and carotenoids; (iv) to identify and quantify six marker phenolic compounds by HPLC-DAD against authentic standards and to resolve their retention patterns; and (v) to relate cooking time, composition and antioxidant capacity and thereby estimate a retention-based optimal cooked level for each species.

The hypothesis was that the antioxidant capacity and phytochemical content of GLVs can significantly change as a function of cooking time, with the direction and intensity of the change varying by species (H1). Specifically, it was hypothesized that longer cooking time would lead to changes in DPPH values, ABTS and FRAP values, and the extractability of some phenolics and therefore measured antioxidant activity (H1a); longer boiling periods would result in greater losses of thermolabile constituents like vitamin C (H1b); shorter to moderate boiling time would increase extractability of some phenolics and therefore measured antioxidant activity (H1c); changes in antioxidant activity would significantly differ among species (H1d); and that antioxidant activity would correlate with the concentration of some phenolic and related constituents (H1e). The null hypothesis (H0) was that there was no significant difference in antioxidant activity or phytochemical content of the vegetables studied due to the cooking time.

1.3 Contribution and novelty

The novelty of this work is the combination of a time-resolved boiling experiment, three antioxidant mechanisms, phytochemical and pigment retention index, HPLC profiling of individual phenolics and multivariate statistics in a single design, applied to five species. Rather than a raw/cooked dichotomy, it provides full time courses, mass-corrected true-retention (TR) values, fully specified cooking conditions and an integrated retention index computed on botha concentration and a TR basis. The index is offered as a transparent, reproducible retention criterion that can inform though not by itself determine dietary guidance and food-service practice, since it does not incorporate sensory, safety or bioaccessibility considerations; the relation of the findings to previous studies is discussed in Section 4 [4], [6].

2. Materials and Methods

2.1 Experimental design

The experiment was conducted as a two factor design with level of vegetable species as one factor (5 levels) and boiling time as the other factor (6 levels: 0, 2, 5, 10, 15 and

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20 min), the raw sample (0 min) was used as a control. The preparation of each biological batch (n = 3) was repeated three times for each vegetable×time combination, and the results of each determination (nine per therefore not a controlled factor (Section 4.7). The final design, the samples and the cooking conditions are summarized in

2.2 Sample collection and preparation

Fresh, mature leaves were purchased from a local supplier on the day of each experiment, and were processed within 4 h of purchase. Non-edible parts, damaged leaves, petioles and thick midribs were removed so that the material consisted of leaf lamina; the edible parts were washed in cold running water, dried gently and cut into pieces of

All treatments were boiled under a single standardised protocol (Fig. 2). For each cooking time, 50 g of prepared tissue was added to 500 mL of distilled water (sample:water 1:10, w/v) that had been brought to a rolling boil in the same uncovered vessel on a temperature-controlled hotplate at a fixed setting; the setting was held constant for all runs so that the water was at atmospheric boiling (≈100 °C) throughout the cooking period. Nominal cooking time (2, 5, 10, 15 or 20 min) was counted from the moment the water returned to a rolling boil after sample addition, and the interval between addition and the return to the boil was not included in the nominal time. Because sample mass, water volume, vessel and hotplate setting were fixed, this interval was similar across treatments and species, so that the effective thermal exposure exceeds the nominal time by an

2.4 Moisture and weight change

Moisture content was found gravimetrically after oven drying to constant weight at 105

biological batch) were averaged within each batch and before statistical analysis. Each species was represented by the single market type available from the supplier; cultivar was

approximately uniform size (2–3 cm). Lamina thickness was not measured and differs inherently among the species (e.g., kale versus molokhia); it is therefore a source of between-species variation in heat penetration that could not be controlled. A pooled sample was then prepared for each batch and then split in half for the raw control and each cooking time.

2.3 Cooking procedure

approximately constant increment. At the end of each period the sample was drained in a sieve for 30 s, immediately cooled in an ice bath to arrest thermal reactions, blotted and weighed to obtain the drained cooked weight (W_cooked). The raw control (0 min) was carried through the same washing, draining, blotting and weighing sequence without heating. The cooking water was discarded without analysis, as is typical of household practice; consequently all retention values reported in this paper refer to the drained edible portion, and constituents transferred to the cooking water are counted as losses from that portion even though they were not necessarily destroyed (Section 2.10). Vitamin C content was determined per 100 g fresh weight (FW), while all other constituents were expressed on a dry-weight basis (DW).

°C and dry matter was calculated by difference. The ratio of the drained cooked mass to the initial raw mass of the same batch (W_cooked/W_raw) was recorded for

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every treatment to enable correction of concentration changes for the cooking mass change [18]; the ratio obtained for each species×time cell was used in all true-retention calculations (Section 2.10).

2.5 Extraction

The antioxidant and total phytochemical assays required 1 g of ground and freeze-dried tissue, which was shaken for 2 h at room temperature in 80% (v/v) aqueous methanol in the dark. The extracts were centrifuged (4000×g for 15 min) and the supernatant filtered and stored at −20 °C for analysis. Vitamin C extracts were made using aqueous extract and pigment extracts were acetone extracts.

2.6 Total phenolic and flavonoid content

The total phenolic content (TPC) was determined by Folin–Ciocalteu method using gallic acid as a standard and was expressed as mg of GAE/g DW [19]. Total flavonoid content (TFC) was measured by the aluminium chloride colorimetric method and expressed as mg quercetin equivalents (QE)/g DW. Because the Folin–Ciocalteu reagent responds to any readily oxidisable substance including ascorbic acid, reducing sugars and some amino acids and proteins [36], [37].

Vitamin C was quantified against an ascorbic acid standard and expressed per 100 g FW. This reporting basis facilitates evaluation of dietary contribution; vitamin C is also particularly sensitive to prolonged heating [11]. The chlorophyll a, chlorophyll b and total carotenoids content were spectrophotometrically analysed in acetone extract using the equations of Lichtenthaler and Buschmann and expressed as mg/g DW [23].

2.9 Individual phenolic compounds

Individual phenolics were separated by reversed-phase HPLC with diode-array detection (HPLC-DAD) on a C18 column using a water–acetonitrile–formic acid gradient (Appendix A.2) and monitored at 280, 320 and 360 nm. Six marker compounds the hydroxycinnamic acids chlorogenic, caffeic and ferulic acid, and the flavonols rutin, quercetin and kaempferol were selected on the basis of preliminary profiling of the raw material and of the phenolics reported for these leafy vegetables in the literature and in food-processing databases [16, 24, 25]. Peak identity was assigned by three concordant criteria [41]: (i) agreement of the retention time with that of the authentic standard analysed under

TPC is an operational estimate of extractable reducing capacity rather than a specific measure of phenolic mass [38], [50]. Changes in TPC were therefore interpreted alongside the HPLC quantification of identified phenolics (Section 2.9).

2.7 Antioxidant capacity

Three different assays were employed, and when possible, results are presented in a common unit of µmol Trolox equivalents (TE) per g DW to facilitate comparison. DPPH radical-scavenging activity was performed as described by Brand-Williams et al. [20]; ABTS radical-cation decolorisation was performed as described by Re et al. [21] and the ferric reducing antioxidant power (FRAP) assay was performed as described by Benzie and Strain [22]. Together, the three assays provide complementary measures of radical-scavenging and reducing capacity [20–22], [51]. All three are in vitro measurements of the chemical reducing or radical-scavenging capacity of a methanolic extract; they do not measure antioxidant action in a biological system and are interpreted in this paper solely as indices of extractable redox-active constituents

2.8 Vitamin C and pigments

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identical conditions (representative retention times in the standard mixture at 280 nm: chlorogenic acid 7.2 min, caffeic acid 12.5 min, ferulic acid 18.3 min, rutin 24.9 min, quercetin 32.1 min and kaempferol 38.0 min; the corresponding sample peaks fell within 0.1 min of these values, Fig. 7); (ii) agreement of the DAD absorption spectrum of the sample peak with that of the standard; and (iii) proportional growth of the peak, without splitting, when a sample extract was spiked with the standard. Quantification was by external calibration against the authentic standards at the wavelength of maximum absorbance of each compound, and results were expressed as mg per 100 g DW. All six methods, standards and units is given in Table 1.

markers were detected and quantified in all five species. Unassigned peaks present in the chromatograms (Fig. 7) were not quantified; the six markers therefore constitute a targeted, not an exhaustive, phenolic profile and cannot be expected to account fully for the aggregate TPC response. A summary of analytical

Table 1
Analytical methods, reference standards and expression of results

Ref.	Unit	Standard	Method	Parameter
[19]	mg GAE/g DW	Gallic acid	Folin–Ciocalteu	Total phenolic content
	mg QE/g DW	Quercetin	$AlCl_3$ colorimetric	Total flavonoid content
[20]	µmol TE/g DW	Trolox	Radical scavenging	DPPH
[21]	µmol TE/g DW	Trolox	Radical cation decolorisation	ABTS
[22]	µmol TE/g DW	Trolox / Fe^{2+}	Ferric reducing power	FRAP
[11]	mg/100 g FW	Ascorbic acid	Titrimetric / spectrophotometric	Vitamin C
[23]	mg/g DW		Spectrophotometric	Chlorophyll a, b
[23]	mg/g DW		Spectrophotometric	Total carotenoids
[25]	mg/100 g DW	Authentic standards	HPLC-DAD	Individual phenolics

GAE, gallic acid equivalents; QE, quercetin equivalents; TE, Trolox equivalents; DW, dry weight; FW, fresh weight

2.10 True retention

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To distinguish genuine loss of a constituent from the concentration artefact created by the change in sample mass during cooking, true retention (TR) was calculated according to the formulation of Murphy et al. [18] as applied to cooked vegetables by Lee et al. [17] and Kim et al. [49]:

TR (%) = (C_cooked × W_cooked) / (C_raw × W_raw) × 100

where C_cooked and C_raw are the mean constituent concentrations in the cooked and raw samples on the reporting basis of that constituent (mg/100 g FW for vitamin C; per g DW for all others), and W_cooked and W_raw are the drained weight of the cooked portion and the initial weight of the raw portion of the same batch (Section 2.3). TR was computed for every biological replicate against its batch-matched raw control and then averaged (n = 3). A value below 100% denotes a real loss from the drained edible portion and a value above 100% a real gain per unit of starting material. Three points are essential to its interpretation. First, because the cooking water was not analysed, TR does not distinguish thermal destruction from leaching: both reduce the amount present in the drained tissue, and TR should be read as retention in the edible portion as consumed, not as chemical survival. Second, for constituents expressed on a dry-weight basis the correction uses the drained-mass ratio and therefore accounts for the change in the mass of the edible portion but not separately for changes in its dry-matter fraction; TR for these constituents is thus a mass-corrected retention index rather than a strict absolute-amount balance. Third, the raw control was processed through the same draining, blotting and weighing sequence as the cooked samples, so its TR is close to, but not identically, 100% (Fig. 6). TR values are presented for vitamin C, total chlorophyll and carotenoids (Fig. 6); the same formula was applied to TPC, TFC and the three antioxidant assays, and those values enter the mass-corrected version of the integrated retention index (Section 2.12). The cooking mass ratios W_cooked/W_raw recorded for every batch (Section 2.4) were used in every TR calculation.

2.11 Statistical analysis

Data are reported as mean ± standard deviation (SD) of three biological replicates. A two-way analysis of variance (ANOVA) was applied with vegetable species and cooking time as fixed factors, together with their interaction ($Y = \mu + V + T + V\times T + \varepsilon$). Where effects were significant, means were separated by Tukey's honestly significant difference (HSD) test at $p<0.05$, and compact-letter groupings are shown as superscripts within each species. Relationships among constituents were examined by Pearson correlation computed across all 90 samples, and the overall structure of the data by principal component analysis (PCA) of standardised variables. Because the pooled correlations are dominated by between-species differences in composition, they are reported as measures of covariation and are not interpreted as evidence of causal contribution (Section 4.4). Significance was set at $p<0.05$ throughout.

2.12 Integrated retention index

To summarise the response of all measured variables in a single criterion, an equal-weight integrated retention index was calculated for each cooked level as the arithmetic mean of six retention ratios (cooked/raw, expressed as %): TPC, TFC, vitamin C, total chlorophyll, total carotenoids and the mean of the three antioxidant assays. The index was computed on two bases: (a) a concentration basis, in which each ratio is the ratio of mean concentrations on the reporting basis of the variable (the version plotted in Fig. 10 and

used in Table 6); and (b) a mass-corrected basis, in which each ratio is replaced by the corresponding true retention (Section 2.10). The concentration-basis values are reported in Table 6 and the mass-corrected values are summarised in Section 3.7. The "optimal" cooked level was defined operationally as the cooked level (excluding raw) with the highest index. Three properties of this criterion must be kept in mind when interpreting it: it weights the six variables equally irrespective of their dietary significance; it is a retention criterion only and incorporates no sensory, textural, microbiological or anti-nutrient (e.g., oxalate, nitrate) considerations; and because the tested levels were 2, 5, 10, 15 and 20 min, the optimum it identifies is bounded below by the shortest level tested and is not a continuous estimate of the retention-maximising time.

3. Results

Vegetable species, cooking time and the interaction of these factors were found to have significant effect on the antioxidant and phytochemical variables for two-way ANOVA ($p<0.001$ for main effects). The species×time interaction was significant for TPC, TFC, vitamin C and all three antioxidant assays ($p<0.01$), whereas for the pigments the interaction term was weaker, indicating that boiling affected chlorophylls and carotenoids more uniformly across species than it affected the hydrophilic constituents and the antioxidant assays.

3.1 Baseline composition of the raw vegetables

The raw vegetables were quite different in composition (Table 2). Kale recorded the highest TPC, TFC, vitamin C and antioxidant capacity in all three assays with molokhia following and Swiss chard being the lowest in all three assays. These basic differences provide a standard against which all changes due to cooking are quantified.

Table 2

Baseline antioxidant capacity and phytochemical composition of the raw vegetables (mean ± SD, n = 3)

FRAP	ABTS	DPPH	Carot	Tot Chl	Vit C	TFC	TPC	Vegetable
48.9 ± 1.5	60.8 ± 3.1	41.3 ± 2.5	4.26 ± 0.10	8.86 ± 0.14	53.8 ± 1.4	8.5 ± 0.3	17.6 ± 0.4	Spinach
62.1 ± 0.4	75.6 ± 1.2	53.1 ± 1.7	6.76 ± 0.22	10.93 ± 0.35	89.1 ± 5.0	11.6 ± 0.4	23.8 ± 0.8	Kale
40.9 ± 1.1	47.4 ± 0.8	35.5 ± 0.3	3.62 ± 0.05	7.30 ± 0.05	32.0 ± 1.6	6.1 ± 0.3	14.9 ± 0.5	Swiss chard
54.1 ± 0.1	65.6 ± 1.5	48.1 ± 1.7	5.66 ± 0.22	9.59 ± 0.36	39.5 ± 0.9	11.0 ± 0.1	21.2 ± 0.4	Molokhia
45.3 ± 1.7	56.5 ± 0.3	39.8 ± 0.8	4.67 ± 0.13	8.08 ± 0.42	47.8 ± 1.8	8.7 ± 0.2	16.9 ± 0.4	Amaranth

Units: TPC mg GAE/g DW; TFC mg QE/g DW; vitamin C mg/100 g FW; chlorophyll and carotenoids mg/g DW; DPPH, ABTS, FRAP µmol TE/g DW.

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3.2 Total phenolic and flavonoid content

Folin–Ciocalteu TPC responded biphasically to boiling (Fig. 3 and Table 3). In the more robust species (kale and molokhia) the measured TPC rose during the first 2–5 min to 12–16% above the raw value and then declined; kale peaked at 27.6 mg GAE/g DW at 5 min compared with 23.8 mg GAE/g DW in the raw leaves, and molokhia at 24.3 versus 21.2 mg GAE/g DW. In spinach, kale, molokhia and amaranth the 2- and 5-min means shared the same Tukey grouping. Because the assay measures reducing capacity rather than phenolic mass, and because raw and cooked material of each batch were extracted identically, this early rise is interpreted as increased extractability or release of bound phenolics into the extractable fraction rather than as de novo formation; this interpretation is examined against the HPLC data in Section 3.5. Amaranth and spinach were virtually unchanged in the early period, while Swiss chard was down almost from the start. After 20 minutes, all species were below their raw value with Swiss chard having the lowest. A similar but less dramatic trend (Fig. 4) is observed for TFC, the initial increase being less and the decrease at longer times being more rapid, which is indicative of the greater extraction rate of flavonoids compared to the broader phenolic class.

Table 3
Effect of cooking time on total phenolic and total flavonoid content (mean ± SD, n = 3)

TFC (mg QE/g DW)	TPC (mg GAE/g DW)	Time	Vegetable
8.55 ± 0.32^{a}	17.58 ± 0.41^{b}	0	Spinach
8.39 ± 0.15^{a}	19.40 ± 0.56^{a}	2	
8.55 ± 0.34^{a}	19.05 ± 0.98^{a}	5	
7.50 ± 0.40^{b}	17.13 ± 0.30^{b}	10	
6.42 ± 0.11^{c}	15.58 ± 0.25^{c}	15	
5.54 ± 0.16^{d}	13.74 ± 0.42^{d}	20	
11.62 ± 0.39^{b}	23.78 ± 0.80^{c}	0	Kale
12.69 ± 0.40^{a}	25.92 ± 0.20^{a}	2	
13.16 ± 0.35^{a}	27.57 ± 0.43^{a}	5	
11.67 ± 0.27^{b}	25.70 ± 0.79^{b}	10	
10.17 ± 0.32^{c}	23.30 ± 0.94^{c}	15	
9.21 ± 0.31^{d}	21.19 ± 0.02^{d}	20	
6.09 ± 0.29^{a}	14.93 ± 0.47^{a}	0	Swiss chard
6.41 ± 0.15^{a}	15.87 ± 0.47^{a}	2	
6.07 ± 0.22^{a}	14.82 ± 0.50^{b}	5	
5.10 ± 0.16^{b}	13.26 ± 0.16^{c}	10	
4.40 ± 0.17^{c}	10.92 ± 0.14^{d}	15	
3.56 ± 0.11^{d}	9.27 ± 0.34^{e}	20	
11.00 ± 0.14^{a}	21.21 ± 0.39^{b}	0	Molokhia
11.27 ± 0.32^{a}	23.73 ± 0.47^{a}	2	

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TFC (mg QE/g DW)	TPC (mg GAE/g DW)	Time	Vegetable
11.14 ± 0.64[a]	24.27 ± 0.38[a]	5	
10.21 ± 0.39[b]	22.28 ± 0.91[b]	10	
8.86 ± 0.40[c]	19.48 ± 0.29[c]	15	
7.65 ± 0.21[d]	17.80 ± 0.42[d]	20	
8.71 ± 0.20[a]	16.86 ± 0.40[a]	0	Amaranth
9.23 ± 0.21[a]	17.31 ± 0.38[a]	2	
9.20 ± 0.44[a]	17.60 ± 0.35[a]	5	
7.55 ± 0.23[b]	16.27 ± 0.63[a]	10	
6.97 ± 0.22[b]	14.12 ± 0.84[b]	15	
5.85 ± 0.22[c]	11.93 ± 0.14[c]	20	

Within a vegetable, means with no common superscript letter differ significantly (Tukey HSD, p<0.05). Time in minutes.

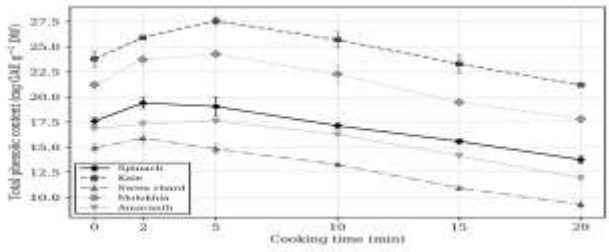


Fig. 3. Effect of cooking time on the total phenolic content of five green leafy vegetables. Points are mean ± SD (n = 3).

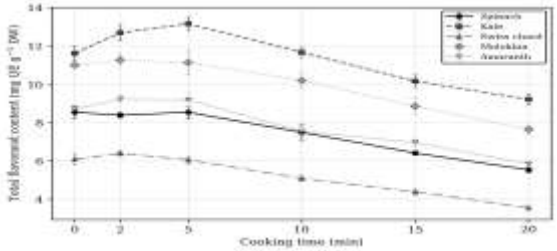


Fig. 4. Effect of cooking time on the total flavonoid content of five green leafy vegetables. Points are mean ± SD (n = 3).

3.3 Antioxidant capacity

The three antioxidant assays paralleled the TPC response (Fig. 5 and Table 4). All three assays increased upon short boiling and then decreased in kale and molokhia while Swiss chard decreased continuously. The longer times showed slightly higher retention of the ABTS response than that of DPPH or FRAP, in line with a contribution from lipophilic components (like carotenoids) that the ABTS assay is able to detect. Thermal response varied by species: after 20 min kale still retained 85–93% of its raw values across the three assays, whereas Swiss chard retained only 56–64%.

Table 4

Effect of cooking time and species on antioxidant capacity by DPPH, ABTS and FRAP (mean ± SD, n = 3)

FRAP	ABTS	DPPH	Time	Vegetable
48.9 ± 1.5[a]	**60.8 ± 3.1[a]**	**41.3 ± 2.5[b]**	**0**	**Spinach**
49.5 ± 0.6[a]	**59.0 ± 2.6[a]**	**44.4 ± 0.7[a]**	**2**	

FRAP	ABTS	DPPH	Time	Vegetable
51.1 ± 0.9[a]	63.8 ± 3.4[a]	45.4 ± 2.1[a]	5	
45.2 ± 2.3[b]	58.0 ± 0.6[a]	41.3 ± 0.8[b]	10	
39.1 ± 0.9[c]	50.0 ± 1.8[b]	35.3 ± 0.7[c]	15	
33.4 ± 1.5[d]	45.4 ± 2.9[b]	30.5 ± 0.3[d]	20	
62.1 ± 0.4[b]	75.6 ± 1.2[b]	53.1 ± 1.7[b]	0	Kale
69.6 ± 0.4[a]	85.4 ± 1.9[a]	61.0 ± 0.5[a]	2	
69.9 ± 1.0[a]	85.9 ± 2.6[a]	62.7 ± 0.2[a]	5	
66.6 ± 3.0[a]	80.5 ± 1.9[a]	60.3 ± 2.1[a]	10	
61.0 ± 2.0[b]	73.4 ± 2.8[b]	52.1 ± 1.1[b]	15	
53.1 ± 1.2[c]	66.5 ± 1.0[c]	49.5 ± 1.1[c]	20	
40.9 ± 1.1[a]	47.4 ± 0.8[a]	35.5 ± 0.3[a]	0	Swiss chard
41.9 ± 1.1[a]	49.5 ± 2.0[a]	35.1 ± 0.3[a]	2	
38.4 ± 0.3[b]	46.7 ± 0.6[b]	34.5 ± 0.8[a]	5	
33.2 ± 0.3[c]	41.8 ± 0.1[c]	29.9 ± 1.4[b]	10	
28.1 ± 1.0[d]	35.4 ± 0.5[d]	24.9 ± 1.5[c]	15	
23.1 ± 1.1[e]	30.2 ± 0.9[e]	21.2 ± 0.4[d]	20	
54.1 ± 0.1[c]	65.6 ± 1.5[b]	48.1 ± 1.7[b]	0	Molokhia
59.9 ± 0.9[a]	70.7 ± 0.7[a]	52.9 ± 1.0[a]	2	
62.3 ± 0.7[a]	73.8 ± 2.8[a]	56.1 ± 1.8[a]	5	
57.8 ± 2.2[b]	69.1 ± 3.3[a]	50.1 ± 2.2[b]	10	

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FRAP	ABTS	DPPH	Time	Vegetable
47.9 ± 0.5[d]	64.0 ± 3.4[b]	45.7 ± 1.1[c]	15	
44.4 ± 1.2[e]	58.3 ± 1.5[c]	41.7 ± 0.5[c]	20	
45.3 ± 1.7[a]	56.5 ± 0.3[a]	39.8 ± 0.8[b]	0	Amaranth
46.5 ± 2.6[a]	58.2 ± 2.6[a]	41.1 ± 0.5[a]	2	
47.9 ± 1.9[a]	62.0 ± 4.0[a]	43.0 ± 0.8[a]	5	
42.4 ± 0.8[b]	52.7 ± 1.8[b]	37.1 ± 1.4[c]	10	
36.3 ± 0.8[c]	46.3 ± 1.9[c]	33.5 ± 0.3[d]	15	
31.4 ± 1.0[d]	40.8 ± 0.8[c]	28.1 ± 0.7[e]	20	

Values in µmol Trolox equivalents per g DW. Within a vegetable, means with no common superscript letter differ significantly (Tukey HSD, p<0.05).

3.4 Vitamin C and pigment retention

Vitamin C was by far the most labile constituent (Fig. 6). After correction for mass change, true retention after 20 min ranged from 11.2% in Swiss chard to 21.8% in kale. Total chlorophyll true retention after 20 min ranged from 33.1% to 48.9%, whereas carotenoids were the most stable pigment class, with 56.6% to 71.1% true retention. Thus, the data show substantially greater retention of carotenoids than of vitamin C in the drained edible portion. These TR values include material transferred to the discarded cooking water

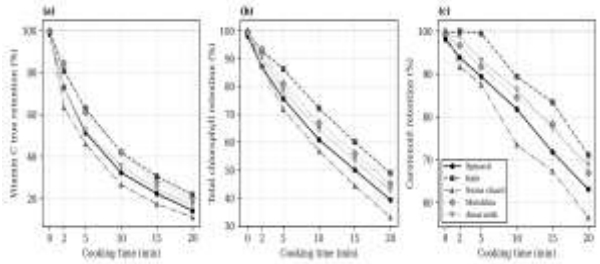


Fig. 6. True retention of (a) vitamin C, (b) total chlorophyll and (c) carotenoids as a function of cooking time. The dashed line marks 100% (raw).

3.5 Individual phenolic compounds

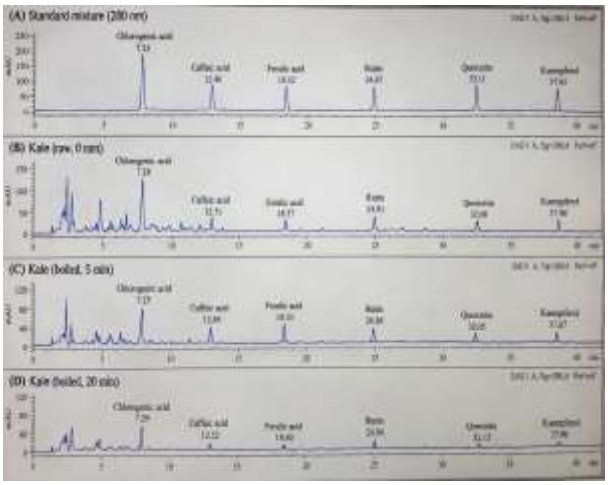


Fig. 7. Representative HPLC-DAD chromatograms at 280 nm: (A) standard mixture of the six marker phenolics with retention times (min); (B) kale, raw; (C) kale boiled for 5 min; (D) kale boiled for 20 min. Peak assignments were confirmed by agreement of retention time and DAD spectrum with the authentic standards and by spiking. Unlabelled early-eluting peaks were not quantified.

The HPLC-DAD data revealed compound-specific behaviour that is not visible in the aggregate TPC measure (Table 5, Fig. 7 and Fig. 8). The bound hydroxycinnamic acids, caffeic and ferulic acid, increased at short-to-moderate boiling, with caffeic acid reaching 115–130% of its raw value after 5 min, a pattern that is consistent with the liberation from cell-wall-associated complexes, and from ester-bound precursors including chlorogenic acid. The amount of rutin (a flavonol glycoside) was only slightly decreased throughout, while the amount of its aglycone, quercetin, and the related kaempferol, increased transiently but then decreased, as would be expected from partial hydrolysis of the glycosides to the aglycones in earlier stages of heat, followed by thermal degradation. Taken together, these compound-specific trajectories release of hydroxycinnamic acids and transient formation of aglycones alongside progressive loss of glycosides and of every marker at long times are consistent with the early rise in TPC being driven by redistribution among bound, glycosylated and free forms and by increased extractability, rather than by a net increase in phenolic mass. The two measures nevertheless capture different pools: the sum of the six markers rose by 8–12% at 5 min in every species, whereas TPC changed by −1% (Swiss chard) to +16% (kale) over the same interval. The targeted markers therefore neither fully explain the TPC increase in kale and molokhia nor the absence of one in Swiss chard, which is expected given that unassigned peaks (Fig. 7) and non-phenolic reductants also contribute to the Folin–Ciocalteu signal.

Table 5
Changes in major individual phenolic compounds during cooking (mg/100 g DW, mean ± SD, n = 3)

Kaempferol	Quercetin	Rutin	Ferulic	Caffeic	Chlorogenic	Time	Vegetable
28.4 ± 0.7^{b}	22.9 ± 0.5^{b}	14.4 ± 0.8^{a}	8.3 ± 0.5^{b}	3.6 ± 0.2^{b}	11.8 ± 0.4^{a}	0	Spinach
30.0 ± 0.6^{a}	23.8 ± 0.2^{a}	15.0 ± 0.2^{a}	8.8 ± 0.2^{a}	3.9 ± 0.1^{a}	13.0 ± 0.5^{a}	2	
32.1 ± 0.6^{a}	25.3 ± 1.0^{a}	14.0 ± 0.5^{a}	9.4 ± 0.3^{a}	4.2 ± 0.0^{a}	13.1 ± 0.3^{a}	5	

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Kaempferol	Quercetin	Rutin	Ferulic	Caffeic	Chlorogenic	Time	Vegetable
29.9 ± 0.6[a]	24.9 ± 0.6[a]	12.0 ± 0.6[b]	9.3 ± 0.5[a]	4.1 ± 0.2[a]	12.2 ± 0.7[a]	10	
25.8 ± 1.8[b]	21.6 ± 1.0[b]	10.3 ± 0.2[c]	8.7 ± 0.2[a]	3.5 ± 0.2[b]	10.6 ± 0.3[b]	15	
22.0 ± 1.1[c]	18.0 ± 0.4[c]	8.7 ± 0.3[d]	7.8 ± 0.2[b]	3.1 ± 0.1[c]	9.0 ± 0.7[c]	20	
47.0 ± 2.1[a]	31.3 ± 0.5[b]	9.2 ± 0.3[a]	11.0 ± 0.1[b]	5.2 ± 0.2[a]	18.1 ± 0.6[a]	0	Kale
46.6 ± 2.6[a]	33.3 ± 2.1[b]	8.8 ± 0.6[a]	12.7 ± 0.3[a]	5.5 ± 0.3[a]	19.2 ± 1.8[a]	2	
47.2 ± 1.5[a]	38.5 ± 1.9[a]	8.2 ± 0.2[b]	12.8 ± 0.3[a]	6.0 ± 0.3[a]	20.3 ± 0.5[a]	5	
48.0 ± 1.7[a]	34.0 ± 0.5[b]	7.6 ± 0.2[b]	13.0 ± 0.4[a]	5.7 ± 0.2[a]	17.3 ± 0.4[b]	10	
40.2 ± 1.6[b]	32.0 ± 0.7[b]	6.3 ± 0.3[c]	12.0 ± 0.5[a]	5.0 ± 0.4[b]	15.9 ± 0.9[b]	15	
35.5 ± 2.4[b]	25.8 ± 1.7[c]	5.2 ± 0.3[d]	11.0 ± 1.0[b]	4.5 ± 0.3[b]	13.5 ± 0.4[c]	20	
12.1 ± 0.5[a]	14.8 ± 0.9[a]	10.7 ± 0.6[a]	6.1 ± 0.1[b]	2.8 ± 0.1[b]	8.7 ± 0.7[a]	0	Swiss chard
12.5 ± 0.5[a]	15.1 ± 1.0[a]	11.0 ± 0.2[a]	6.8 ± 0.1[a]	3.1 ± 0.0[b]	10.0 ± 0.2[a]	2	
13.3 ± 0.8[a]	15.7 ± 0.6[a]	10.6 ± 0.0[a]	7.1 ± 0.4[a]	3.5 ± 0.2[a]	9.6 ± 0.3[a]	5	
12.9 ± 0.7[a]	15.4 ± 0.3[a]	9.6 ± 0.3[b]	7.1 ± 0.4[a]	3.2 ± 0.2[a]	8.3 ± 0.7[b]	10	
11.5 ± 0.8[b]	13.6 ± 0.5[b]	7.7 ± 0.6[c]	6.6 ± 0.1[a]	2.9 ± 0.2[b]	7.7 ± 0.4[b]	15	
9.8 ± 0.1[c]	11.9 ± 0.5[b]	6.3 ± 0.2[d]	6.1 ± 0.7[b]	2.5 ± 0.1[c]	6.6 ± 0.4[c]	20	
29.2 ± 0.9[b]	27.0 ± 0.6[b]	19.7 ± 0.4[a]	10.5 ± 0.2[b]	4.6 ± 0.1[b]	15.7 ± 0.6[b]	0	Molokhia
32.3 ± 1.8[a]	27.3 ± 0.1[b]	19.6 ± 1.0[a]	11.2 ± 0.5[a]	4.9 ± 0.2[b]	17.1 ± 1.2[a]	2	
35.4 ± 1.4[a]	29.9 ± 1.3[a]	18.4 ± 0.4[a]	11.8 ± 0.1[a]	5.5 ± 0.5[a]	17.7 ± 0.2[a]	5	
30.5 ± 1.8[b]	28.2 ± 1.0[a]	16.4 ± 1.1[b]	10.9 ± 0.5[a]	5.5 ± 0.1[a]	15.5 ± 0.3[b]	10	
27.7 ± 1.7[b]	25.3 ± 0.8[b]	13.5 ± 0.9[c]	11.0 ± 0.7[a]	4.6 ± 0.2[b]	13.9 ± 1.0[b]	15	

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Kaempferol	Quercetin	Rutin	Ferulic	Caffeic	Chlorogenic	Time	Vegetable
24.9 ± 0.5^{c}	21.4 ± 0.7^{c}	11.6 ± 0.3^{c}	9.5 ± 0.1^{b}	4.1 ± 0.1^{c}	12.2 ± 0.3^{c}	20	
15.8 ± 0.3^{a}	18.0 ± 0.9^{a}	12.8 ± 0.5^{a}	7.9 ± 0.3^{b}	3.0 ± 0.2^{b}	10.2 ± 0.0^{a}	0	Amaranth
17.8 ± 0.9^{a}	19.5 ± 0.8^{a}	12.3 ± 0.7^{a}	8.1 ± 0.4^{a}	3.5 ± 0.2^{a}	10.3 ± 0.2^{a}	2	
18.3 ± 1.7^{a}	21.1 ± 0.5^{a}	12.1 ± 0.3^{a}	9.0 ± 0.4^{a}	3.9 ± 0.1^{a}	11.3 ± 0.5^{a}	5	
17.0 ± 1.0^{a}	19.5 ± 1.5^{a}	10.4 ± 0.3^{b}	8.5 ± 0.4^{a}	3.8 ± 0.0^{a}	10.1 ± 0.3^{a}	10	
14.2 ± 0.6^{b}	17.7 ± 1.7^{b}	9.1 ± 0.4^{c}	7.8 ± 0.4^{b}	3.2 ± 0.3^{b}	8.6 ± 0.7^{b}	15	
13.0 ± 0.6^{b}	14.8 ± 1.0^{b}	7.3 ± 0.3^{d}	7.6 ± 0.2^{b}	2.9 ± 0.1^{b}	7.3 ± 0.7^{b}	20	

Within a vegetable, means with no common superscript letter differ significantly (Tukey HSD, $p<0.05$).

3.6 Relationships and multivariate structure

The total phenolic content (TPC) correlated strongly and positively with DPPH, ABTS and FRAP ($r \geq 0.98$) and with TFC ($r = 0.96$) across all samples, indicating a strong association between Folin–Ciocalteu TPC and measured antioxidant capacity. Vitamin C showed weaker correlations with the antioxidant assays ($r = 0.69–0.74$). Because these coefficients are computed across a pooled set of 90 samples in which between-species differences in composition account for most of the variance, and because the Folin–Ciocalteu and antioxidant assays share a common redox chemistry, they quantify covariation and should not be read as a measure of the causal contribution of phenolics to antioxidant capacity (Section 4.4). This pattern was reflected in the principal component analysis (Fig. 9), which explained approximately 91% of the total variance and showed the samples arranged in a sequence with the raw state at one extreme, short-cooked samples in the middle and prolonged-cooked samples at the opposite, lower-capacity end.

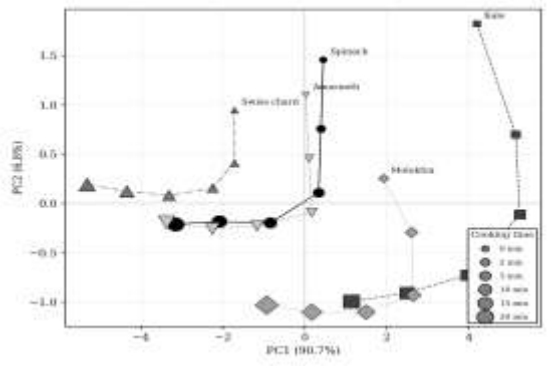


Fig. 9. Principal component analysis of phytochemical and antioxidant responses. Marker size increases with cooking time; lines trace each species' trajectory from raw to 20 min.

3.7 Optimal cooking time

On the basis of concentration (Fig. 10) and on the basis of mass corrected relative to TR (Table 6), the shortest cooked level tested gave the highest integrated retention index for all five species, and these are compared with the other cooked levels in the table. In terms of concentration, however, mean values were at or above the raw values for all species except vitamin C, which was 69–90% of the raw value; index values exceeded 100% only in kale (103.3%) and molokhia (102.5%) and were slightly below 100% in spinach (97.4%), Swiss chard (96.5%) and amaranth (98.2%). After accounting for cooking loss (TR basis), the 2-min index was lower than 100% for all species (88.1–98.0%), which means that none of the species maintained the complete raw complement of the measured constituents after 2 min of cooking, and antioxidant TR at 2 min was higher than 100% only for kale (107%) and molokhia (103%). The margin separating 2 from 5 min was species-dependent: negligible in kale (103.3 versus 103.1%), small in amaranth (98.2 versus 96.1%), spinach (97.4 versus 93.9%) and molokhia (102.5 versus 99.0%), and largest in Swiss chard (96.5 versus 89.5%). The advantage of 2 min over 5 min in the index, for TPC, TFC and three antioxidant assays, appeared in the spinach, kale, molokhia and amaranth groups that shared the same Tukey grouping (Tables 3 and 4), suggesting that the advantage was not in the phenolic and antioxidant components but in the vitamin C and the pigment.

Table 6

Cooked level with the highest integrated retention index (concentration basis) and retention ratios at that level for each vegetable

Index (%)	Vitamin C ret. (%)	Phenolic ret. (%)	Antioxidant ret. (%)	Optimal time (min)	Vegetable
97.4	78.6	110.4	101.8	2	Spinach
103.3	85.0	109.0	113.3	2	Kale
96.5	69.4	106.3	102.0	2	Swiss chard
102.5	89.6	111.9	109.6	2	Molokhia
98.2	77.3	102.7	103.0	2	Amaranth

Retention ratios are concentration-based (mean cooked/mean raw concentration on the reporting basis of each variable, %); antioxidant retention is the mean of the DPPH, ABTS and FRAP ratios. Index is the equal-weight mean of TPC, TFC, vitamin C, total chlorophyll, carotenoid and mean-antioxidant retention (Section 2.12). Optimal time is the cooked level

(excluding raw) with the highest index; the same level is identified on the mass-corrected TR basis (see Section 3.7). Values do not incorporate sensory or safety criteria.

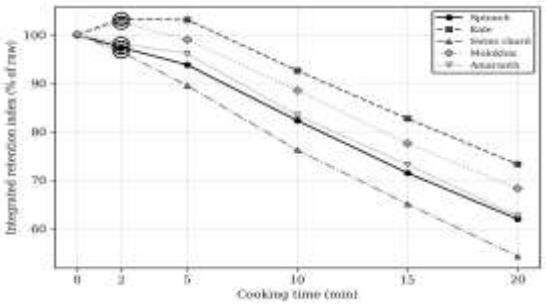


Fig. 10. Integrated antioxidant and phytochemical retention index (concentration basis, Section 2.12) as a function of cooking time; open circles mark the cooked level with the highest index for each species. The mass-corrected (TR-basis) values are summarised in Section 3.7.

4. Discussion

4.1 Overall effect of cooking time

The measured antioxidant variables of the five GLVs used did not all decrease after boiling, but instead had a species dependent biphasic response. Measured antioxidant capacity and extractable phenolics increased in the first minutes and then decreased in the more vigorous species, while the species which were more easily leached decreased from the beginning. This is the reason for the contradictory results obtained from the literature, in which the antioxidant activity of different systems has been found to increase, decrease or remain constant after cooking [7, 9, 12], [43], [44]. Once the final time course has been solved, these reports are not contradictory but merely a handful of points along a single curve, at various times and in various species. This supports hypotheses H1 and H1a and leads to rejection of the null hypothesis, within the boiling conditions tested.

4.2 Short versus prolonged boiling

The first rise in phenolics content and antioxidant activity are in agreement with the increased extractability in the early stages of heating. Heat can cause disruption of plant tissues and denaturation of oxidative enzymes resulting in more phenolics being available for extraction solvent and analytical methods [4], [26], [27], [47]. The HPLC data for caffeic acid and ferulic acid showed an increase following brief boiling, as did quercetin and kaempferol in the present data; this trend is consistent with the previous reports of processing related changes in extractability and stability of these compounds [16, 25, 27], [46], [47] and is consistent with H1c. These initial gains were more than offset by thermal degradation, oxidation and leaching into the discarded cooking water at longer cooking times as observed for other vegetables that have been cooked for extended periods or in water [14, 28, 34, 35]. The Folin–Ciocalteu assay does not differentiate phenolics from other reductants and can therefore not be used to indicate greater amounts of phenolics in the tissue if the TPC is high after short boiling. The present results suggest three nonexclusive explanations: (i) thermal destruction of cell walls and organelles and the inactivation of polyphenol oxidase that increases the amount of phenolics recovered by the extraction solvent [4]–[26]–[27]; (ii) the hydrolytic release of phenolics as esters and glycosides, as evidenced by an increased amount of free phenolics recovered with the extraction solvent and by the transient increase of free quercetin and kaempferol, which was accompanied by a decrease of rutin (Table 5); and (iii) a contribution of non-phenolic reductants to the Folin–Ciocalteu signal [38], [50]. The most obvious reductant is ascorbic acid which was lost during the time course and could not account for the early increase; however,

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its loss could account for the later decrease in TPC. The summed six HPLC markers and TPC did not agree in both directions between species (Section 3.5) suggesting that phenolics other than the six markers and other reductants also contributed. For this reason, the early TPC increase is also being interpreted in this paper as being due to an increase in extractable Folin-reactive material, and the corresponding increases in DPPH, ABTS and FRAP values as an increase in extractable reducing capacity.

4.3 Species-specific responses

The cooking-time response was different between species as shown by the significant vegetable×time interaction (H1d). The antioxidant activity of kale and molokhia increased following a short boiling period and then decreased with longer boiling times, while that of Swiss chard decreased earlier and more rapidly. Cooking effects also have been reported on species-specific basis for Brassica and tropical leafy vegetables [2, 15, 29], [43], [46]. Cooking-method- and duration-dependent changes in antioxidant activity have likewise been reported recently for spinach [44] and Swiss chard [45]. This relative resistance agrees with some cooking methods which are reported to preserve or enhance bioactive constituents in kale [27]. The differences make it a bad idea to cook times for leafy vegetables as a whole – the cooking-time recommendations in Section 4.6 are species-specific, as Swiss chard and to a lesser extent spinach and amaranth have a narrower range of tolerances to boiling than kale and molokhia.

4.4 Phytochemicals and antioxidant activity

TPC was highly correlated with all three antioxidant assays ($r \geq 0.98$) as reported earlier [9, 13] and also with H1e. This association should, however, be qualified by two things. First of all, a correlation computed across a pooled species data set where the between-species range of TPC (15 to 28mg GAE/g DW) is large compared to the within-species range of the capacity, does not imply that phenolics are directly responsible or the only responsible factor for the capacity measured; a strong correlation coefficient would be obtained even if part of the activity was due to co-varying components. Third, the Folin–Ciocalteu, DPPH, ABTS and FRAP assays all rely upon electron or hydrogen-atom transfer to an oxidant and are sensitive to similar subsets of reductants [36]–[38], [50], [51], which accounts for part of the covariance. The less correlation of vitamin C with the assays ($r = 0.69–0.74$) is probably because the amount of this decreased considerably compared to the amount of the assay responses; the relative stability of the ABTS activity at longer times also suggests that partly lipophilic compounds that are more stable than vitamin C, such as carotenoids, which responds to ABTS [21, 30, 31], may contribute to the activity. Lastly, all three assays measure the chemical reducing or radical scavenging activity of an in vitro methanolic extract. They do not simulate the physiological antioxidant network, the metabolic changes that phenolics undergo after consumption, or their bioavailability [36, 37], [51]. The present results thus do not provide evidence by themselves to draw conclusions about the antioxidant activity in vivo or the health effects.

4.5 Vitamin C, pigments and true retention

The drastic reduction in vitamin C (H1b) corresponds to its sensitivity to leaching, oxidation and heat as ascorbic acid is water-soluble [8, 11], [52]. The effect of the true-retention correction in this case is a very evident one: some pigment concentrations appeared to remain constant by unit weight,

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but when the cooking loss of the weight is taken into account, the amount of pigment retained is quite low, as highlighted by the true-retention approaches [17, 18], [49]. Carotenoids were less susceptible and relative to the others, are lipophilic, which is in line with the low leaching susceptibility [32, 33], [48]. The true-retention framework can also be used to define the cooking-water question with precision: the "loss" of hydrophilic components (e.g. vitamin C, phenolics) can be viewed as a transfer to the cooking water, a loss that has been documented for boiling, and not necessarily as a breakdown reaction of the component [4], [10], [11]. Since the cooking water was not analysed, the two losses cannot be separated by the present design and the losses reported should be considered as losses accompanying the thermal degradation of the vegetable, after draining.

4.6 Practical implications

The results here contradict the idea of prolonged boiling: each of the measured variables was lower after 15-20 min of boiling than after 2-5 min for each species within the limits of this design. This was 2 minutes, as suggested by the optimum identified by the integrated index, but this should not be interpreted as a universal "best cooking time" for the following three reasons. First, 2 min was the shortest level tested and the optimal retention may be at or below this level. Second, the index only serves as a retention criterion; it does not take palatability, texture or microbial safety or the reduction of anti-nutrients like oxalate and nitrate (which increases with cooking time in spinach, Swiss chard and amaranth) into consideration, and it may even merit longer cooking time for some consumers [39]. Thirdly, none of the cooked levels contained the complete raw set of the measured constituents after mass loss is taken into account (Section 3.7). An alternative, species-sensitive interpretation of the data is as follows. Vitamin C decreased by the additional 15-21 percentage points between 2 and 5 min in all five species (Fig. 6) but TPC, TFC and antioxidant capacity did not significantly drop during the same period (only in Swiss chard for TPC and ABTS). The 2-5 min time window is therefore a plateau for phenolics and antioxidant capacity, but not vitamin C; hence the shorter part of the window should be preferred if vitamin C is the key parameter. (ii) The species-dependent nature of the responses to boiling for more than 5 min was clearly observed: There was no effect on kale and molokhia (elevated raw TPC and antioxidant values measured on a concentration basis); Up to 8% loss for spinach and amaranth; and 11–19% loss for Swiss chard, in line with the reported matrix- and species-dependent responses [6, 10], [43], [45]. Therefore, Swiss chard should be boiled for as little time as acceptable, while kale and molokhia can be boiled for a longer duration. Only a small proportion has been measured in these conditions, but if the leached hydrophilic constituents are retained in the cooking water of soups or sauces, as is often done in many cultures, then a proportion will be recovered, which is probably significant but was not measured here and is only a hypothesis for verification. None of these statements is applicable to steaming, microwaving, stir-frying or pressure cooking because these methods were not used for testing; they are also not applicable to the amount of the retained constituents which would be released during the digestion process.

4.7 Strengths and limitations

The advantages of the study are the time-resolved design which was standardised and

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fully specified for five species under identical experimental conditions, the three complementary antioxidant assays, the biological replication, the mass-corrected true-retention analysis based on recorded batch mass ratios, and the targeted HPLC-DAD quantification of identified phenolics by multivariate analysis. The results do not apply outside its scope of limitation and that should be clearly indicated. Only the water contact of 1:10 sample:water ratio of atmospheric boiling has been examined, the retention behaviour under other cooking methods (steaming, microwaving, stir-frying, or pressure cooking), which vary in water contact and heat, cannot be inferred from these data, and that is why recommendations are limited to atmospheric boiling. (2) Only a single market cultivar of unknown cultivar of each of the five species studied, and the results should not be extrapolated to green leafy vegetables as a class, and cultivar, maturity, season and growing conditions were not controlled as these are known to affect phytochemical content and thermal behaviour of food. It is important to note that none of the assays (DPPH, ABTS and FRAP) measure antioxidant activity in vivo, and the design does not consider the health relevance of the retained constituents, because the assays are in vitro chemical assays and a non-specific reducing-capacity assay, respectively. (4) Bioaccessibility was not evaluated: the proportion of a constituent that remains in the cooked tissue is not necessarily the proportion that will be released from the matrix for absorption during digestion, and it is known that cooking can either increase or decrease the proportion of the constituent released from the matrix; therefore, the next step is to couple the present retention design with a standardised in vitro digestion protocol [40]. Cooking water was not analysed and leaching was not separated from thermal destruction, and all retention values are for the drained portion - in dishes where the liquid is drunk the effective retention would be higher, but not measured here. (6) Targeted profile of 6 HPLC markers, unassigned peaks not quantified and markers do not represent 100% of TPC response. The time to return to the boil and the leaf lamina thickness were not measured but standardised by procedural procedures and the time to the optimum may vary between batches because of differences in effective thermal exposure, which is small but cannot be ruled out. Further research is required to compare cooking methods with the same time-resolved design, to analyse cooking water to close the mass balance, to include in vitro digestion to include bioaccessibility, to extend the range of species and cultivars, to include sensory evaluation and to test cooked levels of<2 min.

5. Conclusion

The antioxidant activity and phytochemical profile of green leafy vegetables changed significantly with time and in a species-specific manner following boiling. There appeared to be a gain in the more robust species with short boiling (2–5 min) due to increased extractability or release of bound phenolics (not a larger phenolic mass) as evidenced by the increases in Folin–Ciocalteu total phenolic content and measured antioxidant capacity, while longer boiling (15 min or longer) decreased all measured constituents. Vitamin C was most sensitive, retaining only about 11-22% while carotenoids were the least affected of the measured constituents. The strong correlation between TPC and all three antioxidant capacity assays is due to the

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composition of the powders, as well as the common redox chemistry of the three assays, and does not necessarily imply that the measured antioxidant capacity comes from antioxidants in the powders. When using either concentration or mass corrected, the integrated retention index was highest at the shortest cooked level tested (2 min) for all five vegetables; the mass corrected IRIs did not statistically differ for phenolics or antioxidant capacity for four of the five species at the two different cooked levels. Kale was the most resistant and Swiss chard the least resistant when boiled. Considering the species and boiling conditions examined, it is therefore recommended that the vegetables be boiled for a short period of time (2-5 min), preferably towards the lower end of the boiling range which is ideal for minimizing the loss of the investigated antioxidant and phytochemical parameters in the boiled portion of the vegetable, followed by prompt draining. The conclusions drawn in this context refers to atmospheric boiling of the five species and are based on in vitro chemical assays and do not consider other species that may be leached out into the cooking water nor do they account for the bioaccessibility of the constituent after digestion, and cannot be extrapolated to other cooking methods or to leafy vegetables in general without specific testing.

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