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Antiproliferative Effects of Fresh and Thermal Processed Green and Red Cultivars of Curly Kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica*)

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Supporting Information

ABSTRACT: *Brassica* vegetables contain a diverse range of phytochemicals with biological properties such as antioxidant and anticancer activity. However, knowledge about how biological activities are affected by processing is lacking. A green cultivar and a red cultivar of curly kale were evaluated for water/methanol-soluble phytochemicals before and after processing involving blanching, freeze storage, and boil-in-bag heat treatment. In both kale cultivars, processing resulted in a significant decrease of total phenolics, antioxidant capacity, and content and distribution of flavonols, anthocyanins, hydroxycinnamic acids, glucosinolates, and vitamin C. Interestingly, the red curly kale cultivar had a higher capacity to withstand thermal loss of phytochemicals. The extracts of both green and red curly kale inhibited the cell proliferation of three human colon cancer cell lines (Caco-2, HT-29, and HCT 116). However, extracts from fresh plant material had a significantly stronger antiproliferative effect than extracts from processed plant material.

KEYWORDS: *curly kale, heat processing, phenolic compounds, flavonoids, anthocyanins, hydroxycinnamic acids, vitamin C, glucosinolates, isothiocyanates, antioxidant capacity, LC−MS, cell proliferation, Caco-2, HT-29, HCT 116, apoptosis*

INTRODUCTION

Vegetables from the *Brassica* family are among the most commonly grown vegetables worldwide. Studies have shown a relation between consumption of *Brassica* vegetables and cancer, particularly in the gastrointestinal system.[1−3] The anticancer effects have usually been dedicated to the glucosinolates and their degradation products,[4,5] often without a thorough investigation of other potential bioactive compounds found in *Brassica* vegetables.[6,7] Thus, it is not well understood which constituents are responsible for these anticancer effects or whether these effects are a consequence of synergistic action of several constituents.[8,9] *Brassica* vegetables are known to contain a complex mixture of health-related phytochemicals, including vitamin C, phenolic compounds, and glucosinolates, which differ in a species-specific manner.[10−12] The main phenolic compounds found in *Brassica* vegetables are flavonoids and phenolic acids, both of which predominantly exist as molecules in conjugated forms.[13−18] The most ubiquitous subclass of the flavonoids is the flavonols, with the most abundant aglycons quercetin and kaempferol. Another flavonoid subclass found in *Brassica* is the anthocyanins, responsible for the purple red color. In plant tissue, flavonols and anthocyanins are present as sugar conjugates and are often acylated with hydroxycinnamic acids.[19] Flavonoids are shown to possess a remarkable spectrum of biological and pharmacological activities at nontoxic concentrations, suggesting that they significantly affect basic cell functions, such as growth, differentiation, and/or programmed cell death (apoptosis).[12,20,21] However, although some epidemiological studies have provided evidence that a high dietary intake of flavonoids could be associated with low cancer prevalence in humans, others have not found such association.[21−25]

Curly kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica*) is a leafy green- or red/purple-colored vegetable belonging to the Brassicaceae family. The plant is robust and tolerates a broad range of agricultural and climatic conditions. It has a lively pungent flavor with bitter peppery qualities. However, a light frost will produce sweeter and more flavorful kale leaves. Among the *Brassica* vegetables, kale has been reported to exhibit the highest antioxidant capacity and high concentrations of vitamins, minerals, dietary fiber, glucosinolates, carotenoids, flavonoids, and phenolic acids.[11,26−30]

Brassica vegetables can be subjected to various forms of processing to make them more suitable for human consumption. Common processing steps include blanching, freezing, cooking, and, occasionally, warm-holding. Culinary processing affects the plant tissue and the phytochemicals present and may lead to alteration in health-related qualities. Processing may influence phytochemicals positively by releasing compounds and increasing their bioavailability or negatively by physical loss and chemical degradation of the phytochemicals. [31] Thus, it is crucial to gain more knowledge on the fate and final concentration of the phytochemicals before and after food processing to estimate the availability of

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the phytochemicals in the human diet. Some studies regarding thermal processing of curly kale have been conducted,[32,33] but without further investigation of the bioactive functions of the kale phytochemicals.

The aim of the present study was to determine the antiproliferative effects of both fresh and thermal processed green and red cultivars of curly kale, with characterized contents of phenolic compounds, ascorbic acid, glucosinolates, and isothiocyanates, as well as antioxidant capacity. The cooking method boil-in-bag was chosen to investigate the fate of selected health-related phytochemicals, as this pouch technology most likely will play an important role in culinary treatment of blanched/frozen vegetables in catering, professional kitchens, and the retail market in the future.

MATERIALS AND METHODS

Chemicals. Gallic acid, L-ascorbic acid, L-dehydroascorbic acid, quercetin, quercetin-3-rhamnosylglucoside (rutin), 1,2-benzenedithiole, 2-propenylglucosinolate (sinigrin), sulfatase, sodium tetraborate, staurosporine, MTT solution, and Folin−Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorogenic acid hemihydrate, 2,4,6-tripyridyl-*s*-trizine (TPTZ), and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Sodium carbonate, methanol, acetonitrile, and formic acid were obtained from Merck KGAa (Darmstadt, Germany). Cyanidin-3-glucoside was purchased from Polyphenols Laboratories AS (Sandnes, Norway). DEAE Sephadex A25 was purchased from Amersham Bioscience (Wien, Austria). All solvents were of HPLC grade, and the water used was of Milli-Q quality (Millipore Corp., Bedford, MA). Dulbecco's modified Eagle's medium (DMEM), McCoy's 5A medium, fetal calf serum (FCS), nonessential amino acids, and penicillin/streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA). Apo-ONE homogeneous caspase-3/7 assay was purchased from Promega (Madison, WI).

Plant Materials. The green and red cultivars of curly kale (*B. oleracea* L. ssp. *oleracea* convar. *acephala* (DC.), Alef. var. *sabellica* L., 'Reflex' and 'Redbor') were grown at an experimental field at the Norwegian University of Life Sciences (59°40′ N) in 2010. The curly kale plants were harvested approximately 140 days after transplantation into the field, in mid-September. The same day as harvested, five curly kale plants of the green and the red cultivars, respectively, were randomly picked and rinsed for stalks and ripped into pieces of ∼5 × 5 cm. Leaves from each of the five plants, approximately 1/4 of the curly kale material, were pooled and immersed in portions into a mortar filled with liquid nitrogen and ground until a homogeneous plant material was achieved. The samples of ground green and red curly kale were stored at −80 °C until extraction.

Processing. The remaining 3/4 of the green and red cultivars of curly kale material was blanched, freeze stored, and heat-treated using the boil-in-bag technology. Blanching of the kale material was carried out by immersing the leaves (∼3 kg) into boiling water (∼60 L) in a cooking vessel (18/10 stainless Hackman, Iittala Group OyAb, Helsinki, Finland) for 2 min with a blanching temperature ranging from 99 to 100 °C. The plant material was stirred gently during the blanching period. The blanched leaf pieces were cooled in ice−water until the temperature of the water reached 15 °C and centrifuged (Champion-Industrial-N, Eillert BV, Ulft, The Netherlands) to remove excess water from the kale material (∼1 min). Portions (300 g) of the the blanched kale were uniformly packed in vacuum pouches (15 × 20 × 2 cm, LINvac80N (75-8) PA/PE, Linpac Plastics, tolerant for heat up to 121 °C) and vacuumed. The vacuum-packed curly kale was stored at −40 °C for 1−3 weeks until further heat processing. The frozen pouches of kale were thawed at 4 °C in the dark overnight. The pouches with thawed kale were held on ice until they were placed in a steam oven holding at 100 °C (Air-OSteam, Electrolux Professional, Pordenone, Italy) with 100% steam. The boil-in-bags were heated for 17 min before the sealed pouches were left to cool in ice−water for 30 min. The heating time had been optimized in another study (not published) to have a core temperature above 72 °C for 10 min in the kale. The temperature was recorded using temperature loggers in the core of the boil-in-bags. After cooling, the processed plant material was removed from the bags, submerged in liquid nitrogen, ground to a coarse powder in a porcelain mortar, and stored at −80 °C until extraction. This heating process was repeated three times on different days.

Preparation of Curly Kale Extracts. Extraction with methanol was performed as previously described,[14] and TP, FRAP, TMA, vitamin C, flavonol, phenolic acid, anthocyanin, glucosinolate, and isothiocyanate contents of curly kale methanolic extracts were determined. Pooled extracts from fresh curly kale and pooled extracts from the three thermal experiments of each cultivar were concentrated ∼15 times using a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 30 °C. The concentrated curly kale extracts were used in the experiments where cell proliferation and apoptosis were studied. TP, FRAP, glucosinolate, and vitamin C contents were determined in the concentrated extracts.

Dry Matter. The dry matter content of fresh and processed kale material was determined by vacuum drying plant material (10 g) at 70 °C for 24 h in a vacuum oven.[34]

Total Phenolics (TP). The TP content of curly kale methanolic extracts was determined according to the Folin−Ciocalteu method as described by Waterhouse[35] with some modifications as previously described.[14] The TP content was determined with the use of an external standard curve and expressed as milligrams of gallic acid equivalents (GAE) per 100 g fresh weight of kale (mg of GAE/100 g fw) or milligrams of gallic acid equivalents per liter of extracts (mg of GAE/L). All extracts were analyzed in duplicate.

Total Monomeric Anthocyanins (TMA). The TMA content of curly kale methanolic extracts was determined according to the pH differential method[36] with some modification as previously described.[13] TMA was calculated as described in the method protocol[36] using a molar absorptivity of 26 900 L/(mol cm) and molar mass of 449.2 g/mol for cyanidin-3-glucoside and expressed as milligrams of cyanidin-3-glucoside equivalents (CGE) per 100 g fresh weight of kale (mg of CGE/100 g fw). All extracts were analyzed in duplicate.

FRAP Assay. The antioxidant capacity was determined using the FRAP assay as described by Benzie et al.[37] with some modifications. Briefly, 190 μL of freshly prepared FRAP reagent containing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 300 mM acetate buffer, pH 3.6, in a ratio of 1:1:10 (v/v/v) was mixed with 10 μL of the appropriate diluted sample. The mixture was allowed to stand for 60 min at room temperature before absorption was measured at 593 nm (FLUOstar OPTIMA, BMG Labtech GmbH, Offenburg, Germany). The FRAP values were determined with the use of an aqueous solution of Fe(II) ($FeSO_4 \cdot 6H_2O$) in the concentration range of 125−1000 μmol/L and expressed as millimoles of Fe equivalents per 100 g fresh weight of kale (mmol of Fe/100 g fw) or millimoles of Fe equivalents per liter of extract (mmol of Fe/L). Trolox was used as a control, and all extracts were diluted and analyzed in duplicate.

Vitamin C. The vitamin C content in the curly kale methanolic extracts was determined as L-ascorbic acid (AA) and L-dehydroascorbic acid (DHAA). The extracts were dried using N_2 gas, and the material was resolved to its original volume using 1% formic acid and further diluted (1:4) with Trizma−OPA−EDTA before L-ascorbic acid analysis. Reduction of DHAA in the sample was performed by mixing 50 μL of Triz−TCEP (800 mM Trizma base containing 5 mM tris(2-carboxyethyl)phosphine (TCEP), pH 9) with 100 μL extracts dissolved in 1% formic acid and incubated for 20 min in the dark at room temperature. Sodium phosphate buffer (0.2 M, pH 6.5) was added to the reduced extracts to a final volume of 500 μL. The samples were analyzed using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a Chromolite Performance RP-18e column (100 × 4.6 mm i.d., 5 μm particle size) from Merck (Darmstadt, Germany). The solvent consisted of 2.5 mM $Na_2H_2PO_4 \cdot H_2O$, 2.5 mM n-dodecyltrimethylammonium chloride, 1.25

mM disodium−EDTA in water, and 2% (v/v) acetonitrile. The column temperature was 25 °C, and the flow rate was 1 mL/min. The injection volume was 10 μL, and the run time was set to 10 min. L-Ascorbic acid was measured at 264 nm and quantified with the use of external standard (AA) in the concentration range 5−10 μg/mL. The concentration of DHAA was calculated by subtracting the AA concentration from the total AA content determined after reduction of the DHAA present. The content of ascorbic acids was expressed as milligrams of ascorbic acid per 100 g fresh weight of kale (mg of AA/100 g fw). All extracts were analyzed in duplicate.

HPLC-DAD−MS^n Analysis of Flavonols, Anthocyanins, and Phenolic Acids. Phenolic compounds in the methanolic extracts of curly kale were quantified using an Agilent 1100 series HPLC system (Agilent Technologies) equipped with a degasser, an autosampler cooled to 4 °C, a photodiode array detector (DAD), and an MSD XCT ion trap mass spectrometer (Agilent Technologies) with an ESI interface as previously described.[13] The phenolic compounds were analyzed in both negative and positive ionization modes. Fragmentation (MS^{2-5}) was carried out in the automatic mode; that is, the two most abundant ions in MS^{1-4} were fragmented. Naturally occurring flavonols, phenolic acids, and anthocyanins were quantified using external standards of rutin (at 330 nm), chlorogenic acid (at 330 nm), and cyanidin-3-glucoside (at 530 nm), respectively, in the concentration range of 5−150 μg/mL. The results are expressed as rutin equivalents, milligrams of RE per 100 g fresh weight of kale, chlorogenic acid equivalents, milligrams of CAE per 100 g fresh weight of kale, and cyanidin-3-glucoside equivalents, milligrams of CGE per 100 g fresh weight of kale, respectively.

HPLC-DAD−MS Analysis of Glucosinolates (GLS). The glucosinolate analysis of the methanolic extracts of curly kale was determined as previously described.[38] The curly kale extract was divided into two portions; one portion (2 mL) of the extract was mixed with 20 μL of sinigrin (16 mM) used as an internal standard, and the other portion (2 mL) was kept intact. Both samples (2 mL) were applied on in-house-prepared DEAE Sephadex A25 columns (130 mg), washed with 1 mL of water and 1 mL of 0.02 M sodium acetate. Purified sulfatase solution (75 μL) was added to the column and left overnight. The desulfoglucosinolates were eluted with 1.25 mL of water. The separation and detection were performed on an Agilent 1100 series HPLC system equipped with a DAD and a single-quadrupole mass spectrometer with an APCI interface (Agilent Technologies) using a Spherisorb ODS2 RP-C_{18} column (250 × 4.6 mm i.d., 5 μm particle size) with a matching guard column (10 × 4.6 mm i.d., 5 μm particle size) from Waters (Milford, MA). The column temperature was set to 30 °C, and the injection volume was 20 μL. Solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. The elution gradient used was 5% B in 2 min, 5−35% B in 8 min, 35−40% B in 5 min, 40−50% B in 1 min, 50% B in 4 min, 50−100% B in 0.5 min, and 4.5 min 100% B, with a flow rate of 1 mL/min. The column was allowed to equilibrate for 10 min with 5% B between the injections. Detection was carried out at 229 nm by the DAD and also by the single-quadrupole mass spectrometer. The mass spectrometer was used in positive ionization mode, with a nebulizer pressure of 60 psi, a flow rate of nitrogen dry gas of 13 L/min, a dry temperature of 350 °C, a capillary voltage of 4 kV, and a corona ampere of 4 μA. Analysis was carried out using a scan from m/z 50 to m/z 1000. The quadropole was used to facilitate the identification of the GLS based on the parent and fragmented ions. Quantifications were done by using absorbance at 229 nm by comparison with the internal standard (sinigrin) peak area ratio and with relative response factors for each of the glucosinolates. The results are reported as milligrams of GLS per 100 g fresh weight of kale (mg of GLS/100 g fw).

HPLC-DAD Analysis of Isothiocyanates. The analysis of isothiocyanates started with a cyclocondensation reaction between 1,2-benzenedithiole and the isothiocyanates present in the extracts. Curly kale extract, 0.096 M sodium tetraborate buffer (pH 9.25), and 20 mM 1,2-benzenedithiole (1:1:2, v/v/v) were mixed and incubated in a shaking water bath for 2 h at 65 °C. After incubation, the sample was centrifuged for 10 min at 13000 rpm and −4 °C, and the supernatant was ready for analysis. The analysis was carried out on an Agilent 1100 series HPLC system equipped with a DAD. Chromatographic separation was performed on an analytical Luna RP-C_{18} column (150 × 3.0 mm i.d., 3 μm particle size) from Phenomenex Inc. (Torrance, CA), with isocratic elution in 20 min with 20% solvent A consisting of 0.1% formic acid in water and 80% solvent B consisting of 0.1% formic acid in 80% acetonitrile. The column temperature was held at 40 °C, the injection volume was 100 μL, and the solvent flow rate was 0.3 mL/min. The isothiocyanates were quantified by an external standard of 1,3-benzodithiole-2-thione (prepared in-house) at 365 nm, and the concentration was expressed as micrograms of isothiocyanates per 100 g fresh weight of kale (μg/100 g fw).

Cell Lines and Culture Conditions. The human colon cancer cells lines Caco-2, HT-29, and HCT 116 were originally obtained from the American Type Culture Collection. Caco-2 and HT-29 cells were grown in DMEM medium supplemented with 20% and 10% FCS, respectively, 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. HCT 116 cells were grown in McCoy's 5A medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cell lines were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Measurement of Cell Proliferation. The Caco-2, HT-29, and HCT 116 cell lines were plated in 96-well plates at densities of 1.6×10^5, 1.9×10^5, and 1.6×10^5 cells/mL, respectively, in growth medium and incubated for 24 h. The growth medium in each well was then replaced with medium containing curly kale extracts with different concentrations of total phenolics (10, 50, 100, and 150 mg of GAE/L) or extracts containing different concentrations of curly kale (10, 20, 40, and 60 g fw of kale/L). Triplets of each concentration were added to three plates. Cell proliferation was determined using the colorimetric MTT assay, which measures the ability of metabolically active cells to cleave tetrazolium sodium salt to purple formazan crystal. [39] The resulting purple precipitate in the cells was dissolved in 120 μL of 2-propanol containing 0.04 M HCl and the absorbance measured at 562 nm using a Titertek Multiscan Plus MK II plate reader (Labsystems, Finland). Quercetin (200 μM), a flavonol known to have antiproliferative activity in colon cancer cells, was used as a positive control. The MTT experiments were repeated three independent times.

Apoptosis Assay. The HT-29 and Caco-2 cell lines were screened for apoptosis using the Apo-ONE homogeneous caspase-3/7 assay. Active caspase enzymes cleave the substrate rhodamine 110 (bis(*N*-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide), Z-DEVD-R110), which causes a release of the luminescent rhodamine 110 group. The amount of luminescence is proportional to the amount of caspase-3 and -7 present in the cells. A high content of these enzymes indicates an activated apoptotic process. The HT-29 and Caco-2 cells were seeded out in white 96-well plates. After 24 h, the cells were exposed to curly kale extracts with different concentrations of total phenolics (10−75 mg of GAE/L), in parallel, and incubated for 4 h at 37 °C and 5% CO_2 in a humidified incubator. Following the incubation, the cells were washed with phosphate-buffered saline (PBS), and 100 μL of fresh growth medium and 100 μL of Caspase-Glo 3/7 reagent were added and the contents carefully mixed. The plate was then incubated for 1 h at room temperature before the generated signals were measured by the Glomax 96 microplate luminometer (Promega, WI) using the conditions set by the manufacturer. Staurosporine (1 μM), known to induce apoptosis in colon cancer cells, was included as a positive control. The experiments were repeated three independent times.

Statistics. The data from the chemical analysis and cell experiments were analyzed with one-way ANOVA using the statistical program MINITAB 16 (Minitab Inc., State College, PA). Significant differences ($p \leq 0.05$) between average responses were evaluated using Tukey's comparison test.

RESULTS AND DISCUSSION

Curly kale is representative of vegetables with a high level and broad spectrum of phytochemicals with potential positive

health effects in humans. In the present study, the antiproliferative effects of both fresh and heat-processed green and red curly kale cultivars were investigated. Thermal processing using the boil-in-bag principle was chosen in this study as it represents a promising thermal processing method for the food industry with an expected minimum physical loss of phytochemicals compared to boiling in water.[40] Extracts of fresh and processed curly kale used in the cellular response experiments were determined for their antioxidant capacity as well as characterized and quantified for their contents of specific methanol/water-soluble phytochemicals with potential cellular bioactivity, that is, vitamin C, phenolic compounds, glucosinolates, and isothiocyanates.

Contents of TP, TMA, FRAP, Vitamin C, and Dry Matter. TP in the methanolic extracts was determined in a colorimetric assay where the Folin−Ciocalteu reagent reacts with any reducing substances in the sample and gives the total reducing capacity, i.e., antioxidant capacity, of the sample.[35] The TP content of the fresh green cultivar was 424 mg of GAE/100 g fw (Table 1), which is of the same order of magnitude as previously reported.[14] Processing of the kale reduced the TP content by ∼40%, resulting in an average TP content of 249 mg of GAE/100 g fw in the green cultivar. The TP content in the fresh leaves of the red cultivar of curly kale was 562 mg of GAE/100 g fw, which is in the same range as found by Olsen et al.[13] During heat processing, the TP content in the red leaves was reduced by ∼20%, giving an average TP content in the methanolic extracts of 435 mg of GAE/100 g fw. The heat-processed red curly kale thus comprised the same total phenolics content as the fresh green kale cultivar.

Table 1. Determination of Dry Matter Content, TP, TMA, FRAP Values, and Vitamin C in Methanolic Extracts of Curly Kale Used for the Bioactivity Studies

	green cultivar[a]		red cultivar[a]	
	fresh[c]	processed[d]	fresh[c]	processed[d]
TP[e]	424 ± 18 a	249 ± 16 b	562 ± 16 a	435 ± 23 b
TMA[f]			120 ± 26 a	74 ± 18 b
FRAP[g]	5.1 ± 0.2 a	3.1 ± 0.1 b	9.6 ± 0.2 a	7.4 ± 0.8 b
vitamin C[h]	52 ± 5 a	28 ± 6 b	67 ± 10 a[b]	51 ± 12 a[b]
dry matter content (g/100 g fw)	18.2 ± 0.1 a	14.7 ± 0.4 b	16.7 ± 0.3 a	15.5 ± 0.7 b

[a]Different letters indicate significant differences ($p < 0.05$), calculated by Tukey's comparison test, between fresh and processed kale of the green and red cultivars. [b]$p = 0.115$. [c]Average of three extracts from a pooled curly kale sample from five plants ($n = 3$) ± standard deviation. [d]The results are from analyses of three extracts of each of three heat treatments ($n = 9$) ± standard deviation. [e]Analyzed by the Folin−Ciocalteu method, expressed as mg of GAE/100 g fw. [f]Analyzed by the pH-difference method, expressed as mg of CGE/100 g fw. [g]Analyzed by the FRAP assay, expressed as mmol of Fe/100 g fw. [h]Analyzed by HPLC, sum of *L*-ascorbic acid and *L*-dehydroascorbic acid, expressed as mg of ascorbic acid/100 g fw.

TMA in the red cultivar of curly kale was measured by the means of the inherent pH-dependent color characteristics. The TMA content in the fresh leaves of red curly kale was 120 mg of CGE/100 g fw (Table 1). In the processed material, the TMA level was reduced by ∼40% to 74 mg of CGE/100 g fw. The TMA level found in fresh leaves of red curly kale was of the same order of magnitude as previously reported in other fresh red-colored *B. oleracea* species.[13,41,42]

The antioxidant capacity was determined by the FRAP assay where the sample's ability to reduce a ferric complex (Fe^{3+}−TPTZ) to a blue-colored ferrous product (Fe^{2+}−TPTZ) at low pH is measured. In the fresh green kale the average FRAP value was 5.1 mmol of Fe/100 g fw (Table 1), which is higher compared to the previously reported results of 2.34 mmol of Fe/100 g of curly kale determined by Halvorsen et al.[28] This could be due to the longer reaction times used in the assay in this study. Processed green curly kale had an FRAP value of 3.1 mmol of Fe/100 g fw, which corresponds to a ∼40% decrease in antioxidant capacity. The FRAP value of the fresh red cultivar of curly kale was 9.6 mmol of Fe/100 g fw. During heat processing, the FRAP value decreased by ∼23%, resulting in an FRAP value of 7.4 mmol of Fe/100 g fw. The FRAP value and the total phenolics gave similar percentage reduction after processing of the kale.

Vitamin C, comprising L-ascorbic acid and L-dehydroascorbic acid, is a water-soluble compound found in large quantity in curly kale.[11] The content of vitamin C in the investigated green cultivar was 52 and 28 mg of AA/100 g fw for the fresh and processed curly kale, respectively (Table 1), which corresponded to a ∼45% decrease in the vitamin C content after processing. In the red cultivar the vitamin C content was 67 and 51 mg of AA/100 g fw for the fresh and processed curly kale, respectively, which corresponded to a ∼25% decrease in the vitamin C content. The concentration of vitamin C in the present study was lower than previously reported in curly kale,[11] probably because the method used for extraction of phytochemicals was not optimized for extraction of vitamin C. However, it appears that the observed reductions in vitamin C content in both cultivars corresponds well with the decrease in TP content and FRAP value in both green and red cultivars.

The average dry matter content in leaves of curly kale, shown in Table 1, was reduced in the thermally treated kale, from 18.2% to 14.7% in the green cultivar and from 16.7% to 15.5% in the red cultivar. This corresponds to 20% and 7% decrease in dry matter content in the green and red cultivars, respectively. The increase in water content (or decrease in dry matter content) was most likely due to the blanching step where the kale was immersed in boiling water. Increased moisture content during blanching has been reported previously and is postulated to be due to absorption of water into damaged cells, and leakage of dry matter into the water will also increase the moisture content in the vegetable.[40] Considering the decrease in dry matter content after thermal processing, the reduction of TP, FRAP, and vitamin C on a dry weight basis was ∼30% and ∼17% in the green and red cultivars, respectively.

Phenolic Compounds. Two main classes of phenolic compounds, i.e., flavonoids and phenolic acids, have previously been identified in extracts of fresh curly kale (*B. oleracea* L. var. *acephala*).[13−15,43] In this study the same extraction procedure as previously used[13,14] was applied on both fresh and processed kale leaves, and the same phenolic compounds were identified in the methanolic extracts. The identification and concentrations of the individual phenolic compounds are summarized in the Supporting Information. In the methanolic extracts of the green and red cultivars of curly kale, kaempferol and quercetin derivatives, occurring as mono-, di-, tri-, tetra-, and pentaglycosides, several of them acylated with different hydroxycinnamic acids, were identified. A total of 27 individual flavonols were identified and quantified. The total concentration of flavonols, determined as the sum of individual flavonols in fresh leaves from the green cultivar, was 705 mg of RE/100 g fw (Table 2),

Table 2. Concentration of the Flavonols, Phenolic Acids, Anthocyanins, Glucosinolates, and Isothiocyanates in Methanolic Extracts of Curly Kale Analyzed by HPLC-DAD

	green cultivar[a]		red cultivar[a]	
	fresh[c]	processed[d]	fresh[c]	processed[d]
flavonols[e]	705 ± 25 a	390 ± 26 b	388 ± 30 a	329 ± 19 b
phenolic acids[f]	166 ± 6 a	64 ± 10 b	102 ± 12 a	74 ± 6 b
anthocyanins[g]			263 ± 9 a	137 ± 15 b
glucosinolates[h]	164.7 a	100.6 b	78.6 a[b]	68.5 a[b]
isothiocyanates[i]	4.0 ± 0.5 a	3.6 ± 0.3 a	4.4 ± 0.2 a	4.0 ± 0.3 a

[a]Different letters indicate significant differences ($p < 0.05$), calculated by Tukey's comparison test, between fresh and processed kale of the green and red cultivars. [b]$p = 0.249$. [c]Average of three extracts from a pooled curly kale sample from five plants ($n = 3$, flavonols, phenolic acids, anthocyanins, and isothiocyanates; $n = 2$, glucosinolates) ± standard deviation. [d]The results are from analyses of three extracts of each of three heat treatments ($n = 9$, flavonols, phenolic acids, anthocyanins, and isothiocyanates; $n = 6$, glucosinolates) ± standard deviation. [e]Sum of individual flavonols, expressed as mg of RE/100 g fw. [f]Sum of individual phenolic acids, expressed as mg of CAE/100 g fw. [g]Sum of individual anthocyanins, expressed as mg of CGE/100 g fw. [h]Sum of individual glucosinolates, expressed as mg/100 g fw. [i]The sum of isothiocyanates, expressed as μg/100 g fw.

which is in accordance with the previous finding of 646 mg of GAE/100 g fw. [14] The total content of flavonols in the processed green curly kale was 390 mg of RE/100 g fw, which corresponds to a 45% decrease in the flavonol content. In leaves of fresh material of the red cultivar, the flavonol content was 388 mg of RE/100 g fw, which is in accordance with previous findings of 425 mg of RE/100 g fw in kale from 2007 to 2009. [13] Heat processing of the red cultivar gave a surprisingly low reduction in the flavonol content to 329 mg of RE/100 g fw, corresponding to a 15% decrease.

Eight hydroxycinnamic acid derivatives, one benzoic acid derivative, and one compound containing both groups were identified and quantified in methanolic extracts of curly kale. The native phenolic acids were individually quantified as CAE at 330 nm, and the total concentration is summarized in Table 2. The total concentration in the fresh green cultivar of curly kale was 166 mg of CAE/100 g fw. In the processed green curly kale material, the total phenolic acid content was 64 mg of CAE/100 g fw, which corresponds to a ∼60% reduction in phenolic acid content. The total content of phenolic acids in the red cultivar was 102 mg of CAE/100 g fw and 74 mg of CAE/100 g fw of fresh and processed plant material, respectively, corresponding to a ∼27% reduction.

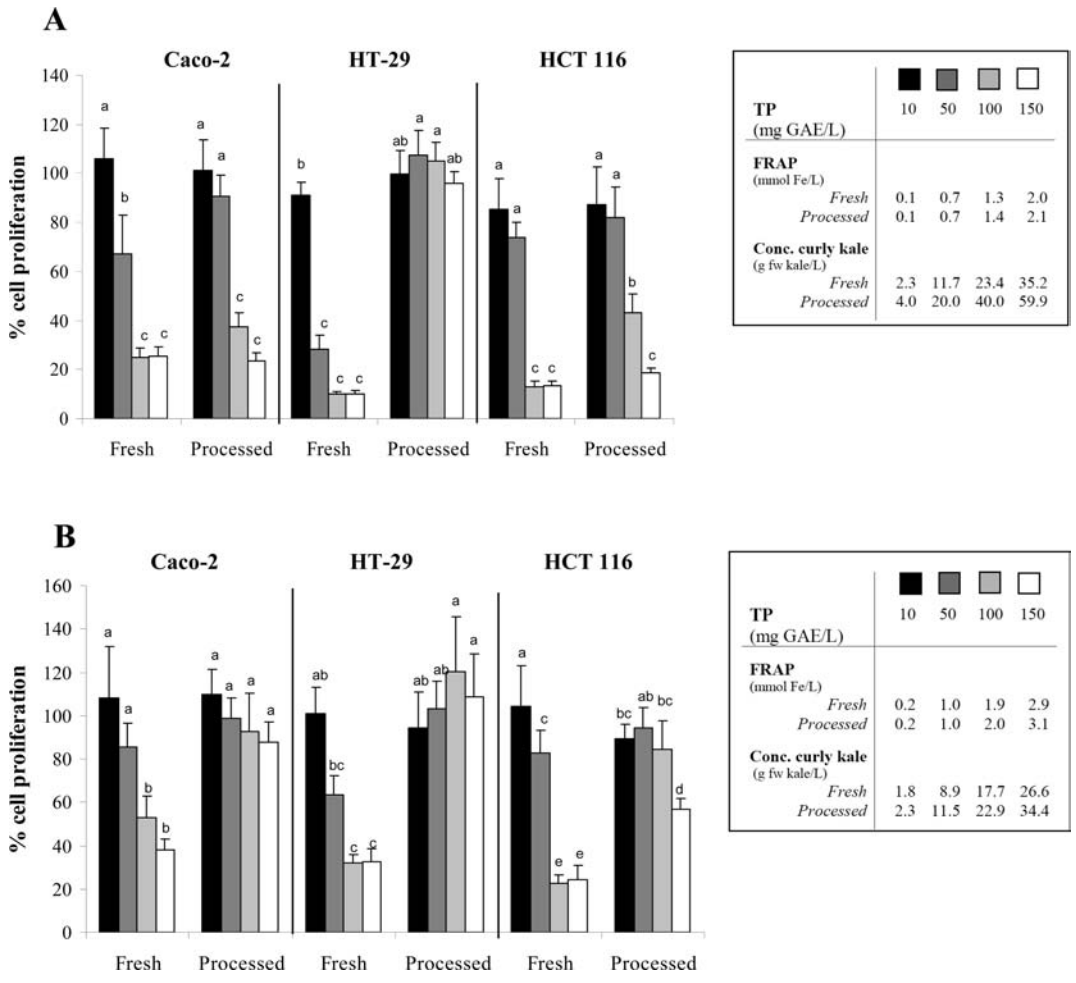
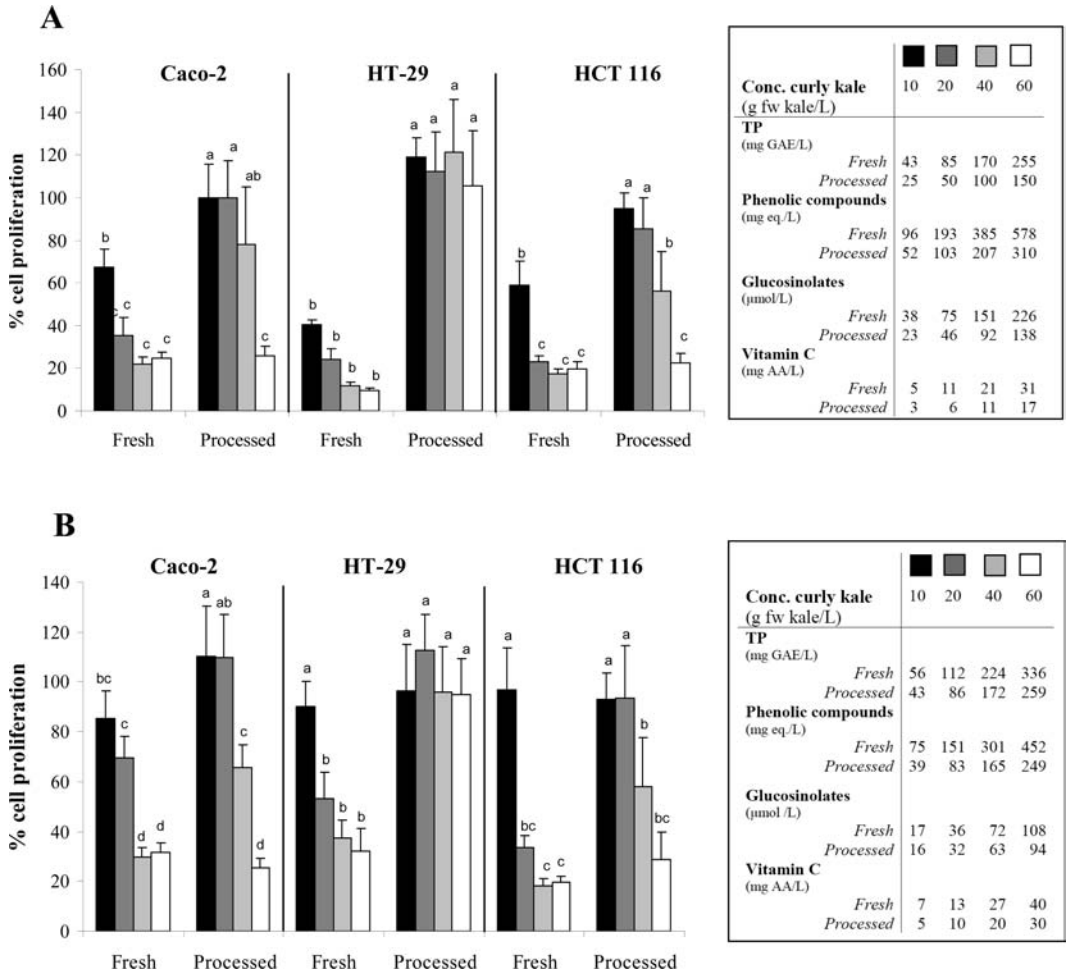


Figure 1. Effects on cell proliferation (MTT assay) of curly kale extracts from the (A) green cultivar and (B) red cultivar on Caco-2, HT-29, and HCT 116 cells. The cells were exposed to different TP concentrations (10, 50, 100, and 150 mg of GAE/L) of curly kale extracts in cell culture medium for 24 h before cell proliferation was measured at 562 nm. Data are expressed as absorbance (%) of cells treated with extracts normalized to the control (0 mg of GAE/L). The graphs represent the results of a typical experiment. Different letters indicate significant differences ($p < 0.05$), calculated by Tukey's comparison test, between fresh and processed kale of the green and red cultivars in one cell line.



Conc. curly kale (g fw kale/L)	10	20	40	60
TP (mg GAE/L)				
Fresh	43	85	170	255
Processed	25	50	100	150
Phenolic compounds (mg eq./L)				
Fresh	96	193	385	578
Processed	52	103	207	310
Glucosinolates (μmol/L)				
Fresh	38	75	151	226
Processed	23	46	92	138
Vitamin C (mg AA/L)				
Fresh	5	11	21	31
Processed	3	6	11	17

Conc. curly kale (g fw kale/L)	10	20	40	60
TP (mg GAE/L)				
Fresh	56	112	224	336
Processed	43	86	172	259
Phenolic compounds (mg eq./L)				
Fresh	75	151	301	452
Processed	39	83	165	249
Glucosinolates (μmol /L)				
Fresh	17	36	72	108
Processed	16	32	63	94
Vitamin C (mg AA/L)				
Fresh	7	13	27	40
Processed	5	10	20	30

Figure 2. Effects on cell proliferation (MTT assay) of curly kale extracts from the (A) green cultivar and (B) red cultivar on Caco-2, HT-29, and HCT 116 cells. The cells were incubated with increasing amounts of curly kale extracts (10, 20, 40, and 60 g fw of kale/L) in cell culture medium for 24 h before cell proliferation was measured at 562 nm. Data are expressed as absorbance (%) of cells treated with extracts normalized to the control (0 g). The graphs represent the results of a typical experiment. Different letters indicate significant differences ($p < 0.05$), calculated by Tukey's comparison test, between fresh and processed kale of the green and red cultivars in one cell line.

In the red cultivar of curly kale a total of 12 anthocyanins were identified and quantified. The anthocyanins found were mainly cyanidin-3-diglucoside-5-glucoside derivatives acylated with different hydroxycinnamic acids at the diglucosyl moiety in the 3-position. The total content of cyanidin derivatives in fresh red cultivar was 263 mg of CGE/100 g fw (Table 2), which is in accordance with the previous finding.[13] Processing reduced the concentration by 50% to 137 mg of CGE/100 g fw.

The total phenolic content calculated as the sum of all identified compounds in the green cultivar of curly kale was 871 and 454 mg of phenolic equiv/100 g fw in the fresh and the processed material, respectively, i.e., a ∼48% reduction in total phenolic compounds. In the fresh red cultivar of curly kale, the concentration of total phenolics quantified by HPLC and calculated as the sum of individual compounds was 753 mg of phenolic equiv/100 g fw, which corresponds to the previous finding in the red cultivar of curly kale, i.e., 872 mg of phenolic equiv/100 g fw. [13] The concentration in processed red kale leaves was 540 mg of phenolic equiv/100 g, which is a total reduction of phenolic compounds of ∼28%.

Glucosinolates and Isothiocyanates. A total of seven individual glucosinolates were identified and quantified in methanolic extracts of curly kale after desulfation (Supporting Information). Glucobrassicin (3-indolymethyl glucosinolate) was the major glucosinolate followed by glucoiberin (3-methylsulfinylpropyl glucosinolate) and neoglucobrassicin (*N*-methoxy-3-indolylmethyl glucosinolate) in both the green and red cultivars. In the fresh green cultivar, the total glucosinolate concentration was 164.7 mg/100 g fw (Table 2). In the processed green cultivar, the total glucosinolate concentration was 100.6 mg/100 g fw, corresponding to a ∼40% reduction in glucosinolate content. In the red cultivar of curly kale, the reduction after heat processing was only ∼15%, with a total glucosinolate concentration in fresh material of 78.6 mg/100 g fw and in processed material 68.5 mg/100 g fw. The glucosinolate contents detected in the two cultivars of curly kale were in the same range as reported previously.[27,44]

Breakdown products of glucosinolates include isothiocyanates, which are produced following exposure to the endogenous plant enzyme myrosinase.[4] However, heat treatment may also affect the breakdown of glucosinolates. Small amounts of isothiocyanates were detected, 3.6−4.4 μg/100 g fw (Table 2), with no significant differences in isothiocyanate content between the extracts of fresh and processed green and red cultivars of curly kale.

Statistical evaluation showed that there were significant differences between fresh and processed green kale in TP content, FRAP value, vitamin C content, dry matter content, and composition of phenolic compounds and glucosinolates (Tables 1 and 2). In the red cultivar a significant differences in TP content, TMA content, FRAP value, dry matter content, and composition of phenolic compounds, but not vitamin C and glucosinolate content, were observed between the fresh and processed material (Tables 1 and 2). Overall, the red cultivar was more resistant to losses of phytochemicals than the green cultivar, which might be due to a firmer and waxier epidermal surface of the red kale leaves compared to the leaves of the green cultivar.

Antiproliferative Effects on Colon Cancer Cells of Fresh and Heat-Treated Green and Red Curly Kale. Human colon cancer development is often characterized in an early stage by hyperproliferation of the epithelium leading to the formation of adenomas.[45] This is mainly a consequence of dysregulated cell cycle control and suppressed apoptosis. Protective effects against colon cancer development should consequently be associated with inhibition of cell proliferation and/or induction of the apoptotic pathway to delete cells carrying mutations and to maintain a normal cell population.

The antiproliferative effects of extracts of fresh and processed curly kale of the two cultivars were determined in preconfluent Caco-2, HT-29, and HCT 116 cells after 24 h of exposure to extracts of curly kale in the TP concentration range of 10−150 mg of GAE/L and with FRAP values of 0.1−3.1 mmol of Fe/L. Interestingly, a significantly stronger inhibition of cell proliferation was observed with extracts from fresh plant material than from processed plant material (Figure 1). This was especially prominent with the HT-29 cell line where processed curly kale extracts showed no antiproliferative effects. Furthermore, there was no significant difference between the antiproliferative effects of fresh green and red curly kale extracts (statistical data not shown), while processed red curly kale extract had a significantly less antiproliferative effect than processed green curly kale extract. Thus, the antiproliferative activity of the curly kale extracts could not alone be explained by the TP content in the extracts or by the antioxidant capacity of the extracts calculated as FRAP.

To investigate which compounds influenced the antiproliferative effects of the curly kale extracts, the colon cancer cell lines were exposed to extracts containing increasing concentrations of curly kale leaf material, 10, 20, 40, and 60 g fw of kale/L (final concentration in the cell medium). The results are summarized in Figure 2, which also contains information about the TP concentration and content of phenolic compounds, glucosinolates, and vitamin C determined in the actual extracts presented for the colon cancer cell lines. Again, a significantly higher inhibition of cell proliferation following incubation with fresh curly kale than with processed plant material was observed, and there were no detectable antiproliferative effects on the HT-29 cells following incubation with processed green and red cultivars of curly kale. Interestingly, the amount of phenolic compounds, glucosinolates, and vitamin C in 20 g fw of kale/L of fresh green curly kale was almost equal to the amount found in extracts of 40 g fw of kale/L of processed green curly kale (Tables 1 and 2). However, the antiproliferative effects of extracts containing 20 g fw of kale/L of fresh green curly kale on Caco-2, HT-29, and HCT 116 were still ∼2-, 5-, and 2.5-fold higher, respectively, than those of extracts containing 40 g fw of kale/L of processed green curly kale (Figure 2). Furthermore, there was no significant difference between the antiproliferative effects of green and red curly kale extracts (statistical data not shown), although red curly kale extracts contained about 20% less phenolic compounds, 30−50% less glucosinolates, and 20−40% more vitamin C than the green cultivar. This indicates that the specific composition of compounds in an extract may be vital for the inhibitory effects on cell proliferation or that there are compounds not detected in the extracts which are degraded during thermal processing, which have antiproliferative effects on the cell lines. There is growing evidence that the antiproliferative capacity may arrive from the synergy between different compounds and compound groups.[46,47] Accordingly, Brandi et al. reported antiproliferative properties of *B. oleracea* juice in human cancer cell lines. [48] Also, Gorinstein et al. studied methanolic extracts of white cabbage and reported antioxidant and antiproliferative activities in human cancer cell lines. [49]

The induction of apoptosis of curly kale extracts (10−75 mg of GAE/L) was evaluated with the HT-29 cell line to ascertain whether the observed reduction in cell proliferation was due to induction of apoptosis. Increased concentrations of fresh green and red curly kale gave a significantly increased induction of apoptosis in HT-29 cells (Figure 3). At the highest concentration tested (75 mg of GAE/L), both green and red cultivars induced about 70% more apoptosis than untreated controls. However, increasing concentration of processed green and red curly kale extracts did not show any effect on apoptosis on HT-29 cells, which is in accordance with the results of cell proliferation. Similar results were obtained with the Caco-2 cell line (data not shown). These results indicate that the reduced cell proliferation observed in the colon cancer cell lines was due to induced apoptosis.

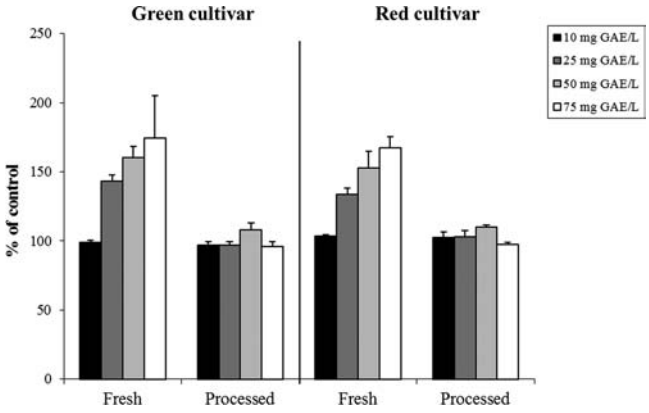


Figure 3. Effects on apoptosis of curly kale extracts on HT-29 cells. The cells were exposed to curly kale extracts of different TP concentrations (10, 25, 50, and 75 mg of GAE/L) in cell culture medium for 4 h before measurement of the activity of caspase enzymes which cleave rhodamine 110, causing a release of the luminescent rhodamine 110 group, which indicates an activated apoptotic process. Data are expressed as absorbance (%) of cells treated with extracts normalized to the control (0 mg of GAE/L). The graphs represent the results of a typical experiment. Different letters indicate significant differences ($p < 0.05$), calculated by Tukey's comparison test, between fresh and processed kale of the green and red cultivars in one cell line.

In conclusion, our study showed that heat processing significantly reduced the concentration of phytochemicals and antioxidant parameters in green and red cultivars of curly kale and that the processing affected the two kale cultivars

differently, with a greater loss of health-related phytochemicals in the green cultivar compared to the red kale cultivar. Furthermore, both green and red curly kale cultivars inhibited cell proliferation and induced apoptosis in colon cancer cells. Interestingly, the fresh curly kale extracts had a higher antiproliferative effect than the extracts from processed curly kale. To summarize, this study provides preliminary data about the ability of curly kale extracts to inhibit growth and induce apoptosis of different colon cancer cells in vitro. Further studies are required to clarify the link between results obtained in cell culture studies and the impact on human health before it can be determined that curly kale intake can affect colon cancer.

■ ASSOCIATED CONTENT

Supporting Information

Tables 3−5 giving the identification and concentrations of the individual phenolic compounds and glucosinolates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AA, L-ascorbic acid; ANOVA, analysis of variance; APCI, atmospheric pressure chemical ionization; CAE, chlorogenic acid equivalent; CGE, cyanidin-3-glucoside equivalents; DAD, diode array absorbance detector; DHAA, L-dehydroascorbic acid; DMEM, Dulbeccos's modified Eagle's medium; dw, dry weight; ESI, electrospray ionization; equiv, equivalents; FCS, fetal calf serum; Fe, iron(II) sulfate equivalents; FRAP, ferric reducing activity power; fw, fresh weight; GAE, gallic acid equivalents; GLS, glucosinolates; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; rutin, quercetin-3-rhamnosylglucoside; sinigrin, 1,2-benzenedithiole 2-propenylglucosinolate; RE, rutin equivalents; TMA, total monomeric anthocyanins; TP, total phenolics; TPTZ, 2,4,6-tripyridyl-*s*-trizine; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; UV−vis, ultraviolet−visible light

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