EFFECT OF STEVIA SUPPLEMENTATION OF KALE JUICE SPHERES ON THEIR QUALITY CHANGES DURING REFRIGERATED SHELF LIFE

Running title: Stevia supplementation in kale juice spheres

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Abstract

BACKGROUND: Kale is a vegetable with high contents of health–promoting compounds although its consumption as a beverage is highly limited due to its bitter flavour. Nonetheless, the bitter flavour of *Brassicas* may be masked by sweetening. Then, the effects of different stevia extracts (CTRL, S0.5 (g L^{-1}), S1.25 and S2.5), added to an innovative kale beverage presentation, were studied on the kale juice spheres quality during 7 days at 5ºC. Kale juice spheres were produced with a double spherification technique, which allowed to obtain hydrogel spheres with high mechanical resistance.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.9445

RESULTS: The addition of the stevia extracts did not affect the physicochemical quality of spheres. Particularly, S2.5 spheres showed the lowest colour changes after 7 days. All spheres showed a good microbiological quality throughout storage, with loads <7 log CFU g^{-1}, regardless of the stevia concentration. The sulforaphane content of kale spheres was not affected by the stevia supplementation for 7 days.

CONCLUSION: Stevia addition to the kale juice spheres led to a better flavour without altering the product quality during refrigerated storage.

Keywords: *Brassica oleracea* var. *sabellica* L.; *Stevia rebaudiana bertoni*; ready–to–eat; spherification; glucosinolates; isothiocyanates.

1. INTRODUCTION

Kale (*Brassica oleracea* var. *sabellica* L.) is a vegetable rich in glucosinolates>isothiocyanates, phenolics, carotenoids, minerals (i.e. calcium), vitamins, etc.[1,2] Glucosinolates are sulphur–containing compounds that highly contribute to the characteristic bitter flavour of *Brassica* vegetables. Glucosinolates are principally stored in cell compartments separated from the enzyme myrosinase in *Brassica* vegetables. However, the myrosinase rapidly comes into contact with glucosinolates upon cell rupture (mastication, juicing, etc.) leading to an almost instantaneous glucosinolate conversion to multiple compounds. Among those compounds are isothiocyanates, thiocyanates, nitriles, oxazolidine–2–thiones and epithionitriles being such conversion influenced by several factors like specifier proteins, temperature, pH, ferrous ions (Fe^{2+}), etc.[3] However, isothiocyanates are preferably desired owing to their high health–promoting properties contrary to nitriles and epithionitriles, which have shown lower health benefits.[4] Hence, the glucosinolates

content from cruciferous vegetables has been recently recommended to be considered together with the isothiocyanates content as a glucosinolates/isothiocyanates system.[5] Glucoraphanin is one of the major studied glucosinolates owing to the reported high health–promoting properties of its cognate isothiocyanate: sulforaphane (4–(methylsulphinyl) butyl isothiocyanate). The kale glucoraphanin content has been reported to be very low.[6,7] Nevertheless, a recent screening of glucosinolates in several kale varieties from different parts of the world has found large differences depending on the variety, growing area, and even between individuals of the same variety and area.[8]

The bitterness of *Brassica* vegetables can be masked by sweetening.[9] Stevia (*Stevia rebaudiana bertoni*) is a plant widely known for its sweetening properties with a very low caloric content.[10] Furthermore, stevia contains several compounds that are responsible for its antimicrobial/antioxidant properties, among other health–promoting properties.[11,12] The stevia addition effect on the glucoraphanin>sulforaphane system of kale juice has not been studied yet. Furthermore, the consumption of kale juice may be even enhanced by innovative presentations such as spheres. Spherification is a culinary process that usually employs sodium alginate and calcium chloride to shape a liquid into spheres. In that sense, a double gelification technique has been recently modelled and optimized for vegetable juices showing the obtained spheres a high mechanical resistance.[13] Furthermore, the high calcium contents of kale allow a perfect spherification without the need to add calcium salts during the spherification process, as it is normally done with food having low calcium contents.

The aim of the present study was to evaluate the stevia supplementation effect on the quality and the glucoraphanin>sulforaphane system of kale juice spheres for 7 days at 5ºC.

2. MATERIAL AND METHODS

2.1. Preparation of ingredients

Kale (*Brassica oleracea* var. *sabellica* L.) was obtained from a local producer (Sacoje S.L., Lorca, Spain) in October 2017. It was grown under an open–air cultivation system in Lorca area (Murcia, Spain) using integrated pest management cultural practices. Kale was randomly hand–harvested at the commercial ripening in the first morning hours (≈8–9 h). Subsequently, kale was stored at 5ºC and 90–95 % relative humidity until the next day when it was processed. Lemon juice was obtained from lemon fruits, purchased in a local supermarket (Cartagena, Spain), using a hand−juicer. Stevia (*Stevia rebaudiana bertoni*) leaves (dried leaves) were purchased from a local store (Cartagena, Spain).

The stevia extract was obtained according to Ortíz-Viedma *et al.*[11] Briefly, 5 g of ground (with a domestic coffee grinder) stevia leaves was placed in a glass bottle (protected from light with aluminium foil) and 1 L of 80:20 (*v:v*) ethanol:water was added. Subsequently, the latter mixture was stirred for 3 h being then allowed to stand for 24 h at room temperature. The latter extract was filtered (4–layers cheesecloth), vacuum–dried at 40ºC (rotary evaporator) and then resuspended in 1 L of distilled water. The obtained concentrated stevia extract was stored at 4ºC in darkness until the next day when it was used.

2.2. Juice preparation

The ingredients proportions were: 200 g of kale, 10 mL of lemon juice and 30 mL of the correspondent stevia extract (S0.5/S1.25/S2.5; described below). All ingredients were then blended (MX2050 blender, Braun, Germany) and allowed to stand for 5 min to ensure the maximum glucoraphanin>sulforaphane conversion.[14] Finally, a slow juicer

(Silver Crest, Málaga, Spain) was used to obtain a clear juice from the latter blended mixture. The used lemon juice proportion was selected in order to obtain a final pH of 4.5. The maximum glucoraphanin>sulforaphane conversion has been reported to occur at pH=4.[3] Nevertheless, pH 4.5 was hereby selected in order to ensure an excellent spherification since too acidic conditions may limit the hydrogel formation during the spherification process.[15] Accordingly, the previously concentrated stevia extract was diluted with distilled water by 10:90 (*v:v*), 25:75 and 50:50 to reach the following final stevia concentrations in the juices: 0.5 (hereinafter: S0.5), 1.25 (S1.25) and 2.5 g L^{-1} (S2.5), respectively. A control juice (**CTRL**) was prepared with distilled water instead of stevia extract. The highest stevia concentration (S2.5) was selected based on the sensory scores (from a panel test conducted by 5 usual consumers of *Brassica* vegetables) that still led to the sensory acceptance of the kale juice with this maximum stevia concentration. On the other side, the minimum stevia concentration was selected, based on sensory scores, as the minimum stevia concentration that helped to smooth the strong bitter flavour of the kale juice.

2.3 Kale juice spherification and storage conditions

The kale juice spherification was conducted according to an optimized spherification method consisting of a double gelation tecnhique.[13] Briefly, the prepared kale juice was mixed with xanthan gum (Laguilhoat, Innovative Cooking S.L., Madrid, Spain) until a final concentration of 0.4 %. For the first gelation, 2.5 mL of juice was carefully dropped (distance spoon–solution=3 cm) in a 0.5 % alginate (Laguilhoat) solution and the first gelation was allowed to form for 24 min. Subsequently, the first gelation–spheres were carefully collected with a perforated spoon (Laguilhoat) and they were then sequentially washed with water followed by 95 % ethanol. For the second gelation,

the latter washed spheres were carefully dropped in a 1.27 % $CaCl_2$ (Laguilhoat) solution being the second gelation allowed to form for 6 min. Finally, the formed kale juice spheres were collected and washed as previously described.

Kale juice spheres (≈5 units) were stored in a 50–mL plastic bottle (approximately 10 spheres per bottle) containing 40 mL of a saline solution (0.5 % NaCl), namely 'covering liquid'. The NaCl concentration was selected in order to have the same electrical conductivity (conductivity meter GLP32, Crison, Barcelona, Spain) as the obtained kale juice to avoid electrolytes migrations. Samples were stored at 5ºC in darkness with sampling times at 0, 3, 5 and 7 days. Five replicates (5 bottles) were used for each treatment (stevia concentration) and sampling time. The juice from 5 spheres, of the same replicate (bottle), was obtained at each sampling time for the juice quality determinations.

2.4. Physical characterization of kale juice spheres

Stereomicroscopic analyses of the sphere's surface were accomplished using a binocular stereomicroscope SZ61 TR (Olympus, Center Valley, PA, USA).

Swelling capacity (SC) of the spheres was determined according to Tsai *et al.*[13] as described in Eq. 1. Firstly, water excess on the sphere's surface was gently eliminated with a paper towel. Then, the initial sphere's weight (W1) was registered. Subsequently, each sphere was placed in 10 mL of distilled water and it was allowed to stand for 10 min at room temperature. Then, water excess on the sphere's surface was newly removed and the final weight of this swollen sphere (W2) was registered.

$$SC\ (\%) = \left(\frac{W2-W1}{W1}\right) \times 100 \quad (1)$$

The firmness of spheres was determined with a texture analyser (Brookfield, Toronto, Canada). A compression test was used with a 3.5–cm Ø plate at a test speed of 10.0 mm

s^{-1} and compression distance of 4 mm. Firmness was expressed as the maximum force (N) required for compression. Five spheres were measured for each treatment. Diameter (averaged from largest and shortest diameter) of spheres was measured using a digital calliper (Mitutoyo Absolute, Guipúzcoa, Spain).

Electrolyte migration from the juice to the covering liquid was also estimated by measuring the electrical conductivity (Ec; µS cm^{-1}; conductivity meter Crison, Barcelona, Spain) of the juice (EcJ), contained in the spheres, and the covering liquid Ec (EcL). Then, Ec ratio (Eq. 2) was calculated to better observe the electrolyte migration through the sphere's structure during storage:

$$Ec\ ratio = \frac{EcJ}{EcL} \quad (2)$$

2.6. Physicochemical quality and colour of kale juice spheres

SSC, pH and TA were determined using the same devices and methodologies previously described.[28] Colour was determined with a colourimeter (Chroma Meter CR–400, Konica Minolta, Japan) using the illuminant D65, 2º observer, and an 8–mm viewing aperture. For the external colour of spheres, three colour readings on the sphere's surface were taken and then averaged. The external colour of five spheres per replicate (bottle) was measured. For the juice colour, kale juice was extracted from spheres and colour was measured with a special colourimeter device for liquids (CM–A131, Minolta, Japan). Hue index was calculated (Eq. 3) since it has been reported as the most appropriate colour index for dark green *Brassica* vegetables.[16]

$$Hue = tan^{-1}\left(\frac{b}{a}\right) \quad (3)$$

2.7. Microbial analyses

Standard enumeration methods were used to determine mesophilic, psychrophilic and yeast and moulds (Y+M) growth according to Klug *et al.*[17] Briefly, 10−fold dilution series were prepared starting from 3 spheres (approximately 10 g) together with 90 mL of sterile peptone saline solution (pH 7). All microbiological material was acquired from Scharlau Chemie SA (Barcelona, Spain). Mesophiles and psychrophiles were pour plated while Y+M were spread plated. Plate count agar was used for mesophiles and psychrophiles being then incubated for 48 h at 30ºC and 7 days at 5ºC, respectively. Rose Bengal agar was used for Y+M being incubated for 3–5 days at 25ºC. All microbial counts were reported as log colony forming units (CFU) per gram of product (log CFU g^{-1}). Each of the five replicates was analysed in duplicate.

2.8. Sensory analyses

Sensory analyses were executed according to international standards.[18] Sensory evaluation of kale spheres was carried out by a sensory panel composed of eight members (22−70 years) that were previously trained in discriminative quality attributes. Flavour and colour were scored on a 5−points hedonic scale (5: excellent, 4: good, 3: limit of acceptability (LA), 2: poor, 1: extremely bad). Overall quality was evaluated on a 9−points hedonic scale (9: extremely like, 7: moderately like, 5: LA, 3: moderately dislike, 1: extremely dislike). A 5−points scale of damage incidence and severity was used for off−flavours (5: none; 4: slight; 3: LA; 2: strong; 1: severe).

2.9. Glucoraphanin analysis

The kale juice glucoraphanin content was analysed as previously described.[19,20] Briefly, freeze–dried juice powder (0.2 g) was weighed into glass screw–cap tubes (10 mL) being heated at 80ºC for 10 min using a heating water bath. Then, 5 mL of

methanol:water (80:20, *v:v*) was added and heating was continued for 20 min (80ºC). Subsequently, 1 mL of hot (100ºC) nanopure water was added to each sample being then heated for additional 30 min (80ºC). The latter extracts were centrifuged (4,000×g/20 min/4ºC) and the obtained supernatants were decanted and stored on ice. The remaining pellets were re–extracted as previously described with 2.5 mL methanol:water (90:10, *v:v*). Both supernatants were combined and made up to a final volume of 5 mL with methanol:water (90:10, *v:v*). Purification of the latter extracts was conducted by solid phase extraction (SPE) using prepared DEAE Sephadex A25 (GE Healthcare, Uppsala, Sweden) mini−columns. For SPE, the methanolic extracts (1 mL) were loaded on the previous mini−columns and the unbound material was removed using two washings with nanopure water followed by two washings with 0.02 M sodium acetate (pH 5.0) per mini−column (0.5 mL each). Desulphation was done with purified sulfatase (75 µL; EC 10.000 units from *Helix pomatia*; Sigma−Aldrich, St Louis, MO, USA) overnight (12 h) at room temperature. Desulphoglucoraphanin was eluted with 1.5 mL of nanopure water and filtrated (0.22−µm polytetrafluoroethylene (PTFE) filter). The latter extracts were analysed using an ultra−high−performance liquid chromatography (UHPLC) instrument (Shimadzu, Kyoto, Japan) equipped with a DGU−20A degasser, LC−30AD quaternary pump, SIL−30AC autosampler, CTO−10AS column heater and SPDM−20A photodiode array detector. Chromatographic separation was done with a Gemini C18 column (250mm×4.6 mm, 5 µm; Phenomenex, Macclesfield, UK). The used mobile phases were water (A) and acetonitrile (B). Flow rate was set at 1.5 mL min^{-1} with a linear gradient starting with 2 % B to reach 20 % B at 28 min, and 2 % B at 32 min. The injection volume was 20 µL being the absorbance registered at 229 nm. Desulphoglucoraphanin was quantified using a desulphoglucoraphanin commercial standard (Toronto Research Chemicals Inc., North

York, Canada) being expressed as mmol kg^{-1} dry weight (dw). Each of the five replicates was analysed in duplicate.

2.10. Endogenous sulforaphane and sulforaphane nitrile analyses

Sulforaphane was analysed as previously described.[21] Briefly, 0.2 g of freeze−dried juice powder was mixed with 5 mL of acidic water (pH 6.0 using 0.1 M HCl). The latter mixture was then incubated in a heating bath at 45ºC for 2 h allowing the glucoraphanin>sulforaphane conversion. Then, 25 mL of methyl–tert–butyl ether (MTBE) was added to the mixture and it was sonicated for 1 min. The latter mixture was then dewatered (sodium sulphate), filtered (Whatman 41) and SPE purified (SI−1 silica cartridge; Phenomenex, Torrance CA, USA). Subsequently, the purified extract was vacuum−dried (rotary evaporator at 45ºC), resuspended in acetonitrile and filtrated (0.22−µm PTFE filter). The chromatographic separation was achieved using the previous Gemini C18 column and UHPLC system. The chromatographic conditions were→ mobile phase: 20 mM ammonium formate in water:acetonitrile (55:45, *v:v*); column temperature: 25ºC; injection volume: 5 µL; flow rate: 0.6 mL min^{-1}; λ=196 nm. Sulforaphane was quantified using a commercial DL−sulforaphane standard (Sigma–Aldrich, St. Louis MO, USA) being expressed as µmol kg^{-1} dw. Each of the five replicates was analysed in duplicate.

Sulforaphane nitrile was analysed by UHPLC−MS (6420 Waters Acquity Triple Quad, Waters Corp., Milford, USA) using the chromatographic conditions previously described.[22] Detection of the sulforaphane nitrile was conducted in the ESI positive mode through the detection of their characteristic product ions (55.0 and 81.9 m/z). Sulforaphane nitrile was quantified as sulforaphane equivalents due to the unavailability of a commercial standard.

2.11. Myrosinase activity

Myrosinase activity was analysed as previously described[23,24] with slight modifications. Frozen juice samples (-80ºC) were previously ground using a mill (IKA, A 11 Basic, Berlin, Germany) with liquid nitrogen. Then, 0.5 g of frozen ground sample was added to 10 mL of potassium phosphate buffer (50 mM; pH 7) being then homogenised (Ultra Turrax® model 18T, IKA−Werke GmbH & Co. KG, Staufen, Germany) for 5 min under an water-ice bed. Successively, the latter extracts were filtered (4–layers cheesecloth) and centrifuged (14,000×g/15 min/4ºC). Then, 3 mL of the latter supernatant was placed in a centrifugal filter tube (Amicon® Ultra–4 Ultracel® 30kD, Millipore, Cork, Ireland) and it was centrifuged (4,000×g/10 min/4ºC) to remove the compounds with a molecular weight lower than myrosinase (sugars, glucosinolates, etc.). The concentrated enzyme, contained inside the filter, was dissolved with 470 µL of phosphate buffer (50 mM, pH 7) being then used as the myrosinase extract. Myrosinase activity was determined by the glucose formation released during myrosinase−mediated hydrolysis of sinigrin using a coupled enzymatic procedure.[23] A commercial glucose determination kit (D–glucose–HK enzyme kit, Megazyme, Bray, Ireland) was used being the absorbance measured with a Multiscan plate reader (Tecan Infininte M200; Männedorf, Meilen, Switzerland) at 340 nm. Briefly for the coupled myrosinase enzymatic procedure, 208 µL of 0.525 mM $MgCl_2$ (containing 5.68 mM ascorbic acid) was placed in a flat−bottom polystyrene 96−wells UV−Star plate (Greiner Bio−One; Frickenhausen, Baden–Württemberg, Germany). Subsequently, 50 µL of the myrosinase extract, 14 µL of test kit solution R1 (buffer pH 7.6), 14 µL of test solution R2 (β–NADP plus ATP) and 5 µL of test solution R3 were sequentially added. Then, the latter reaction mixture was allowed to react for 5 min at room temperature.

Finally, 14 μL of sinigrin solution (30 mg mL^{-1}) was added and the absorbance increment at 340 nm was registered for 8 min. An external standard curve (0.2–1.2 enzymatic units (U) mL^{-1}) was prepared with commercial myrosinase (Thioglucosidase from *Sinapis alba* seed; Sigma Aldrich, Germany) being then the myrosinase U correlated with the registered absorbance (340 nm) increments for 8 min. Myrosinase activity was expressed as U kg^{-1} fresh weight.

2.12. Statistical analyses

The experiment had a two−factor (treatment×storage time) design subjected to analysis of variance (ANOVA) using the SPSS software (v.19 IBM, New York, USA). Statistical significance was assessed at p=0.05, and the Tukey's multiple range test was used to separate the means.

3. RESULTS AND DISCUSSION

3.1. Physical characteristics of spheres

Kale juice spheres showed an initial diameter of 20.5±2 mm with a visual homogeneous surface, as corroborated with the stereomicroscope captions (Figure 1A). No morphological differences on the sphere's surface were observed between the different treatments (Figure 2). Nevertheless, some protuberances were observed (Figures 1C, D) being probably owed to alginate or xanthan gum agglomerations resulted from undiluted particles. However, the latter protuberances could not be easily detected by the consumer due to their small size (0.2–0.3 mm). No influence (p>0.05) of the stevia supplementation was observed on the sphere's diameter neither on the stereomicroscope captions (Table 1).

The SC of a hydrogel sphere indicates the ionic strength decrease being associated a low SC with a higher stability of the hydrogel sphere, and consequently, a higher sphere

firmness.[25,26] A previous study with beads (4.5–4.7 mm) of radish leaves juice showed a SC of 5.1–6.0, which was correlated with a high beads mechanical resistance.[13] The spheres swelling was even lower with SC<1.3 for all samples on processing day, except for S1.25 spheres (SC=3.3) (Table 1). However, the S1.25 SC is still lower than values previously reported for other vegetable juices spheres.[13] All spheres showed an initial firmness ranging from 60.4 to 88.7 N, registering S1.25 samples the lowest firmness on processing day with a value of 34.6 N (Table 1). The latter low firmness of S1.25 spheres, and high SC, may be explained since the ionic concentration of the S1.25 juice led to a lower ionic strength during the second gelation step. Conclusively, a low SC is beneficial since it is also correlated with a low release of compounds from the hydrogel spheres due to their high mechanical resistance as it has been previously reported.[13]

The physical properties of spheres changed during storage. Particularly, firmness generally increased during the first 3 days of storage for all samples, except S2.5, with low variations (<21 N) from day 3 to the end of storage. Particularly, CTRL showed the highest firmness increase in the first 3 days while S0.5 and S1.25 showed increments of 30–45 N. Furthermore, CTRL registered the highest reduction of the electrolyte juice:covering liquid ratio after 3 days. The latter behaviour may be explained since the lower ionic concentration of CTRL juice (without stevia) probably led to the observed electrolyte migration from the juice to the covering liquid. Then, the ionic concentration from the covering liquid of CTRL samples was increasing during these first 3 days of storage possibly allowing a further gelification as deducted from the firmness increment. On the other side, the high ionic concentration of S2.5 juice did not lead to electrolyte migration as observed in the low variations of the electrolyte juice:covering liquid ratio of this treatment after 3 days. Nevertheless, the diameter of all samples was not significantly changed ($p>0.05$) during storage (data not shown).

It can be concluded that the production of kale juice spheres with the double gelation technique allowed to obtain hydrogel spheres with a uniform surface, high mechanical resistance (firmness), reduced swelling and unchanged size during storage. Among the different spheres, S2.5 showed the lowest firmness changes during storage together with a low electrolyte migration.

3.2. Physicochemical and colour quality of kale spheres

The initial SSC of all samples ranged between 4.5 and 5ºBrix, although S1.25 registered the lowest value with 4.2ºBrix (Table 2). The lowest SSC of S1.25 may be explained by the previous high SC and low firmness of these samples, which could lead to a solids migration from the juice to the covering liquid through the hydrogel structure. Nevertheless, the latter slight SSC differences among initial values were decreased throughout storage. Furthermore, a SSC decrement was observed after 3 days. Then, SSC was not highly changed (<0.5 SSC units) from day 3 to day 7, registering SSC values of 3.1–3.4, without significant differences ($p>0.05$) among treatments. Besides, no high SSC variations have been observed in other beverages containing *Brassica* vegetables.[27] CTRL samples showed initial pH and TA of 5.2 and 0.17 g citric acid mL^{-1}, respectively (Table 2). The addition of stevia extracts to the kale juice did not ($p>0.05$) induce pH changes while TA was slightly increased to 0.20–0.25 g citric acid mL^{-1}. A general TA reduction was observed for all samples throughout storage reaching TA values of 0.08–0.12 g citric acid mL^{-1}, without significant ($p>0.05$) differences among treatments.

Colour changes of kale spheres may be expected due to colour changes of the juice itself or possible colour changes of the hydrogel structure. Hence, the sphere's surface colour, as well as the colour of the contained juice, were monitored during storage. Kale

spheres showed an initial dark colour with CIE L, a* and b* values of 24.4, –11.4 and 15.3, respectively (data not shown), and Hue angle of 144.8 (Table 2). Stevia addition to the kale juice did not induce colour changes either in the juice (data not shown) or in the formed spheres (Table 2). A Hue ratio ($Hue_{spheres}/Hue_{juice}$) was calculated for all samples during storage in order to deduct possible hydrogel colour changes that may affect the film transparency (data not shown). Nevertheless, the initial ratio (≈1) was not changed ($p>0.05$) during storage. Accordingly, Hue of sphere's surface is presented in Table 2 since the hydrogel film transparency was not apparently changed. Spheres Hue increased throughout storage, which means a decrease in the green colour of kale spheres. The latter green colour degradation may be due to a chlorophylls degradation.[28] The latter green colour degradation followed a zero order kinetic with R^2 of 0.94–0.99 (data not shown). S2.5 spheres showed the lowest Hue differences (6.9) after 7 days among the rest of samples with stevia. The lower green colour degradation of spheres with the highest stevia concentration may be owed to the high stevia antioxidant properties, which probably protected chlorophylls from enzymatic degradation.

In conclusion, the stevia extracts addition did not induce high changes in the physicochemical quality of samples neither on processing day nor during storage at 5ºC comparing to CTRL samples. Particularly, S2.5 spheres showed the lowest colour changes after 7 days.

3.3. Microbiological quality of kale spheres

The initial mesophilic, psychrophilic and Y+M counts of CTRL spheres on processing day were 3.7, 5.0 and 3.6 log CFU g^{-1}, respectively (Table 3). The latter microbial loads remained almost unchanged in the samples containing stevia extracts. Similarly, no immediate (on processing day) microbiological reductions were observed in other food

products when using stevia extracts obtained with the same extraction procedure.[11] As expected, mesophilic, psychrophilic and Y+M loads of samples increased throughout storage by 2.4–2.5, 1.4–1.5 and 2.8–3.0 log units, respectively, after 7 days. No antimicrobial effects were observed in the kale spheres due to the stevia incorporation, even showing S2.5 samples similar increments after 7 days compared to CTRL samples. Stevia extracts have shown antimicrobial properties due to their phenolic profile.[11] Nevertheless, the hereby used stevia concentrations were not probably enough to reach such antimicrobial activity. Furthermore, the antimicrobial effects achieved by the low juice pH and other antimicrobial compounds from kale, such as the formed isothiocyanates when juicing, could have masked the stevia antimicrobial effect. Nevertheless, microbial loads of all samples remained below 7 log units during storage.

3.4. Sensory quality of kale spheres

Flavour was the sensory parameter that achieved the lowest scores on processing day (Figure 3). Accordingly, flavour may be establish as the main sensory parameter that influences the consumer acceptance of kale. Nevertheless, the addition of stevia smoothed the bitter flavour being scored S1.25 and S2.5 samples with the maximum flavour scores (≈5). On the other side, colour scores remained unaffected on processing day regardless of the treatment. Furthermore, no off–odours were perceived on processing day for any of the samples. Flavour was highly affected during storage, contrary to colour and off-odours, and consequently, the overall quality of samples was also reduced. In that sense, S2.5 and S1.25 samples showed the highest overall quality after 7 days with scores of 7.5 and 7, respectively, while CTRL samples were scored with an overall quality score of 6.

3.5. Glucoraphanin/sulforaphane system catalysed by endogenous myrosinase

An initial glucoraphanin content of 2.33 mmol kg^{-1} was registered in CTRL spheres on processing day (Table 4). Sulforaphane content of CTRL spheres was 3.37 μmol kg^{-1} on processing day, with a myrosinase activity of 0.69 U kg^{-1} (Table 4). Sulforaphane nitrile content (the predominant glucoraphanin product apart from sulforaphane) of CTRL samples was 0.26 μmol kg^{-1}. The low sulforaphane nitrile formation may be explained by the adjusted (using lemon juice) low juice pH as previously reported.[3] CTRL samples registered the highest myrosinase activity on processing day, which was ≈0.4 U lower in the stevia–containing samples, without significant differences ($p>0.05$) among the stevia concentrations. The latter reduction of the myrosinase activity may be owed to the stevia antioxidant properties as similarly observed in other enzymes from fruit beverages, such as peroxidase and polyphenoloxidase.[29] In agreement with the latter finding, spheres containing high stevia concentrations showed a lower sulforaphane formation while the spheres with the lowest stevia content (S0.5) registered higher sulforaphane contents.

The myrosinase activity of CTRL samples was highly reduced (by ≈0.6 U) after 3 days since enzymatic degradation in beverages is higher compared to solid food products, which have intact their plant cells. In that sense, the myrosinase activity of CTRL samples remained in the same range as for the remaining stevia–containing samples up to 7 days. Glucoraphanin content of samples remained with low variations from day 3 until the end of storage with levels ranging from 0.61 to 0.78 μmol kg^{-1}, regardless of the stevia concentration. Sulforaphane contents of samples also decreased reaching minimum levels after 5 days with 0.48–0.86 μmol kg^{-1}, without remarkable changes during all 7 days. On the other side, the observed low sulforaphane nitrile contents on processing day were maintained throughout storage. The latter findings may be

explained since sulforaphane, contrary to glucoraphanin, is a highly reactive compound being very unstable in aqueous solutions such as those of vegetable beverages.[27,30,31]

4. CONCLUSIONS

Kale juice sweetening with stevia is an excellent solution to reduce the strong bitter flavour of this vegetable that highly limits its consumer acceptance. Furthermore, the kale juice consumption may be even incremented if attractive presentations, such as spherifications, are used. The used innovative double spherification technique allowed to obtain kale juice spheres, preserved in a saline covering liquid, with excellent physicochemical and sensory quality during refrigerated storage at 5ºC. The use of stevia extracts in the kale juice spheres did not affect the physicochemical quality of this product being even improved he sensory quality of these samples. Furthermore, the sulforaphane content of kale spheres, one of the most interesting bioactive compounds of this *Brassica*, was not affected by the sweetening with the studied stevia concentrations throughout 7 days.

ACKNOWLEDGMENTS

The authors express their gratitude to CNPq (Council for Scientific and Technological Development, Brazil) for a doctoral grant (232758/2014−0) granted to Tâmmila Venzke Klug. The authors are grateful to S. G. Hasa for her skilful technical assistance. The authors are also grateful to Sakata Seeds Ibérica S.L.U. and to Sacoje SCL for providing the plant material.

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TABLES AND FRIGURES CAPTIONS

Table 1. Physical properties of kale juice spheres containing stevia for 7 days at 5ºC. Different capital letters for each parameter show significant differences between treatments for the same sampling time. Different lowercase letters for each parameter show significant differences between sampling times for the same treatment. NS: not significant; ***($p<0.001$); **($p<0.01$); *($p<0.05$).

Table 2. Physicochemical properties (soluble solids content, SSC; titratable acidity, TA; and Hue angle) of kale juice spheres containing stevia for 7 days at 5ºC. Different capital letters for each parameter show significant differences between treatments for the same sampling time. Different lowercase letters for each parameter show significant differences between sampling times for the same treatment. NS: not significant; ***($p<0.001$); **($p<0.01$); *($p<0.05$).

Table 3. Microbiological counts (log CFU g^{-1}) of kale juice spheres containing stevia for 7 days at 5ºC. Different capital letters for each parameter show significant differences between treatments for the same sampling time. Different lowercase letters for each parameter show significant differences between sampling times for the same treatment. NS: not significant; ***($p<0.001$); **($p<0.01$); *($p<0.05$).

Table 4. Glucoraphanin, sulforaphane, sulforaphane nitrile and myrosinase activity of kale juice spheres containing stevia for 7 days at 5ºC. Different capital letters for each parameter show significant differences between treatments for the same sampling time. Different lowercase letters for each parameter show significant differences between

sampling times for the same treatment. NS: not significant; ***($p<0.001$); **($p<0.01$); *($p<0.05$).

Figure 1. Surface of kale spheres on processing day (×20).

Figure 2. Kale juice spheres without (CTRL) and with different stevia concentrations on processing day.

Figure 3. Sensory quality of kale juice spheres containing stevia for 7 days at 5ºC. Different capital letters for each parameter show significant differences between treatments for the same sampling time. Different lowercase letters for each parameter show significant differences between sampling times for the same treatment. Missing letters denote absence ($p>0.05$) of significance.

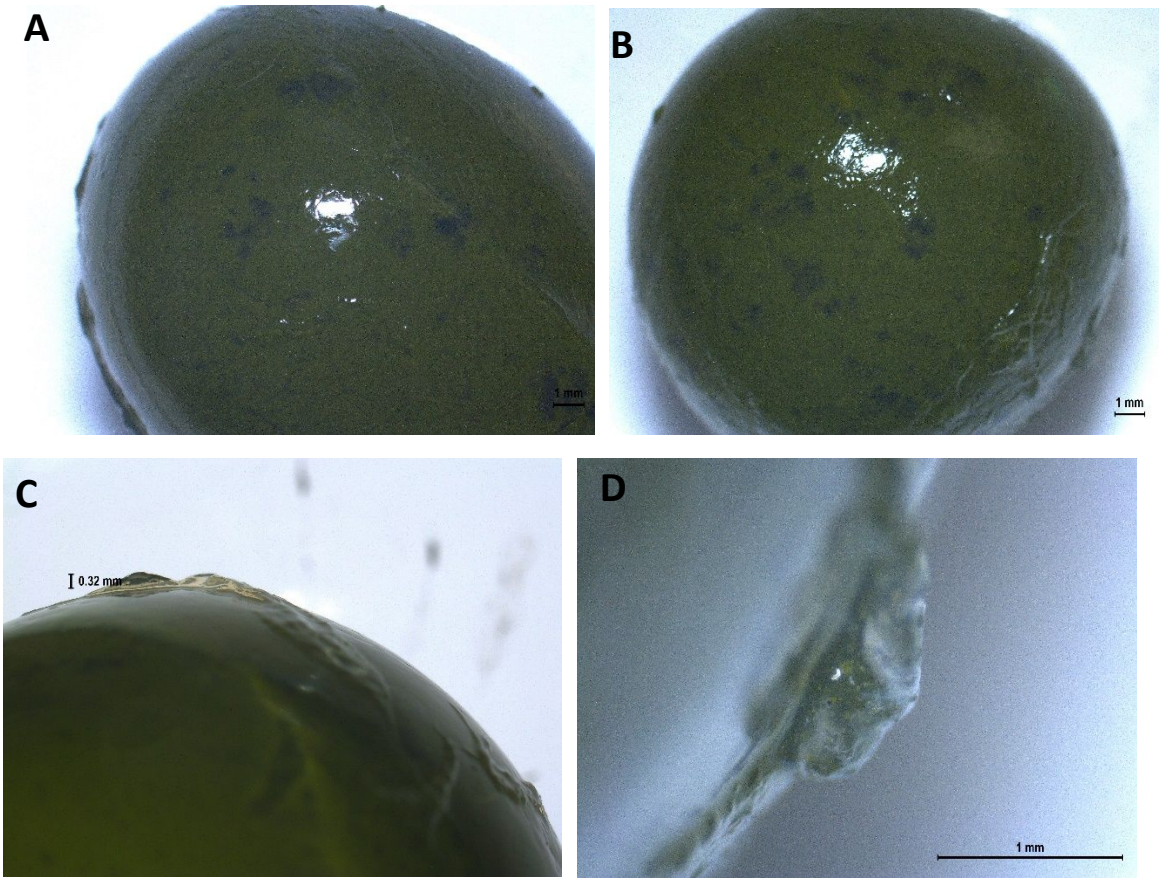
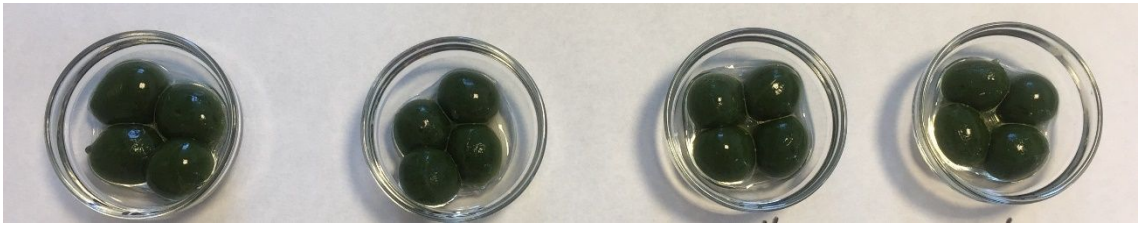
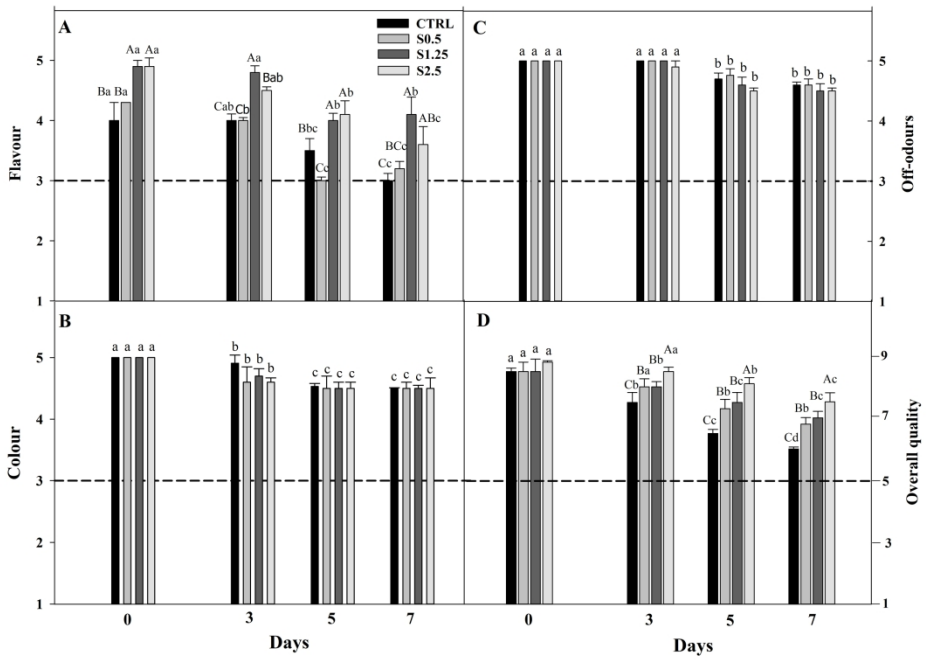


Figure 2



CTRL S0.5 S1.25 S2.5



258x196mm (300 x 300 DPI)

Table 1

Day	Treatment	Diameter (mm)	Swelling capacity (%)	Firmness (N)
0	CTRL	21.51±1.00	0.0±0.0 Bb	60.4±27.5 ABb
	S0.5	18.85±0.44	0.8±0.5 Bns	75.8±16.3 Ab
	S1.25	19.56±0.38	3.3±0.8 Aa	34.6±9.22 Bb
	S2.5	19.27±0.40	1.2±0.3 Bb	88.7±11.1 Ans
3	CTRL	19.76±1.79	0.7±0.1 Aa	119.1±28.3 Aa
	S0.5	21.08±0.92	0.1±0.1 Bns	105.3±19.3 Aba
	S1.25	21.21±1.71	0.0±0.0 Bc	81.4±9.25 Ba
	S2.5	19.92±1.29	0.8±0.2 Ab	100.6±20.3 ABns
5	CTRL	21.03±1.26	0.0±0.0 Bb	118.7±26.4 NSa
	S0.5	19.93±2.67	0.9±0.5 Bns	104.7±11.5 NSa
	S1.25	19.74±0.66	0.8±0.3 Bbc	91.8±16.6 NSa
	S2.5	17.58±0.90	2.0±0.2 Aa	97.7±18.8 NSns
7	CTRL	19.18±1.30	0.0±0.0 Bb	93.6±10.9 NSab
	S0.5	19.83±0.93	0.1±0.1 Bns	102.1±9.7 NSab
	S1.25	20.60±1.39	2.1±0.6 Aab	102.8±24.5 NSa
	S2.5	20.96±1.66	0.9±0.1 Bb	107.4±23.2 NSns
Treatments		NS	***	**
Days		NS	***	***
Days x Treatments		NS	***	*

Table 2

Day	Treatment	SSC (ºBrix)	TA (g citric acid L^{-1})	Hueº
0	CTRL	4.97±0.15 Aa	0.173±0.025 a	144.82±0.80 Ad
	S0.5	4.46±0.35 ABa	0.197±0.014 a	142.33±0.38 BCd
	S1.25	4.20±0.10 Ba	0.249±0.001 a	141.49±0.29 Cd
	S2.5	4.53±0.23 ABa	0.209±0.056 a	143.92±0.92 ABd
3	CTRL	3.30±0.14 Bb	0.105±0.007 b	147.93±0.52 Ac
	S0.5	3.05±0.21 Bb	0.115±0.001 b	146.88±0.04 Bc
	S1.25	3.83±0.06 Ab	0.103±0.005 b	146.58±0.14 Bc
	S2.5	3.80±0.05 Ab	0.102±0.014 b	146.10±0.62 Bc
5	CTRL	3.40±0.26 ABb	0.173±0.076 b	149.46±0.06 Ab
	S0.5	3.53±0.06 Ab	0.181±0.065 b	147.73±0.50 Bb
	S1.25	3.40±0.01 ABc	0.107±0.011 b	148.86±0.57 Ab
	S2.5	3.10±0.03 Bc	0.098±0.011 b	149.03±0.11 Ab
7	CTRL	3.23±0.15 NSb	0.115±0.012 b	151.35±0.15 Aa
	S0.5	3.13±0.06 NSb	0.095±0.053 b	150.11±0.10 Ba
	S1.25	3.36±0.15 NSc	0.086±0.006 b	150.65±0.23 ABa
	S2.5	3.26±0.15 NSc	0.102±0.002 b	150.77±0.65 ABa
Treatments		*	NS	***
Days		**	***	***
Days x Treatments		***	NS	***

Table 3

Day	Treatment	Mesophiles	Psychrophiles	Y + M
0	CTRL	3.73±0.11 d	5.00±0.15 c	3.60±0.07 d
	S0.5	3.49±0.16 d	4.76±0.11 c	3.27±0.12 d
	S1.25	3.60±0.52 d	4.83±0.05 c	3.57±0.12 d
	S2.5	3.35±0.09 d	5.00±0.07 c	3.50±0.12 d
3	CTRL	4.98±0.06 c	4.91±0.14 c	4.06±0.19 c
	S0.5	4.98±0.17 c	4.96±0.01 c	4.16±0.04 c
	S1.25	4.84±0.03 c	5.02±0.08 c	3.98±0.35 c
	S2.5	4.60±0.09 c	5.01±0.12 c	3.52±0.34 c
5	CTRL	5.36±0.92 b	5.85±0.26 b	4.64±0.34 b
	S0.5	5.47±0.07 b	5.62±0.29 b	4.35±0.30 b
	S1.25	5.76±0.07 b	5.92±0.04 b	4.68±0.65 b
	S2.5	5.60±0.14 b	5.76±0,27 b	5.16±0.58 b
7	CTRL	6.28±0.15 a	6.50±0.57 a	6.50±0.56 a
	S0.5	5.88±0.49 a	6.20±0.74 a	6.24±0.66 a
	S1.25	7.00±0.36 a	6.32±0.31 a	6.33±0.29 a
	S2.5	5.89±0.50 a	6.46±0.13 a	6.47±0.13 a
Treatments		*	NS	NS
Days		***	***	***
Days x Treatments		NS	NS	NS

Table 4

Day	Treatment	Glucoraphanin (mmoles kg^{-1} dw)	Sulforaphane (µmoles kg^{-1} dw)	Myrosinase (U/kg fw)
0	CTRL	2.33±0.28 Aa	3.20±0.18 Ba	0.69±0.09 Aa
	S0.5	1.31±0.21 Ba	4.33±0.51 Aa	0.43±0.33 Ba
	S1.25	1.50±0.09 Ba	0.75±0.04 Db	0.26±0.03 Ba
	S2.5	1.56±0.08 Ba	2.19±0.35 Ca	0.34±0.01 Ba
3	CTRL	0.67±0.09 NSb	3.05±0.50 Aa	0.13±0.02 NSb
	S0.5	0.63±0.01 NSb	2.94±0.17 Ab	0.19±0.05 NSa
	S1.25	0.63±0.00 NSb	0.98±0.07 Ca	0.19±0.00 NSb
	S2.5	0.78±0.13 NSb	1.75±0.17 Ba	0.15±0.03 NSb
5	CTRL	0.62±0.05 Bb	0.86±0.22 Ab	0.11±0.01 Bb
	S0.5	0.61±0.04 Bb	0.68±0.08 ABc	0.06±0.01 Cb
	S1.25	0.74±0.04 Ab	0.61±0.09 ABb	0.18±0.01 Ab
	S2.5	0.75±0.01 Ab	0.48±0.05 Bb	0.11±0.04 Bb
7	CTRL	0.55±0.13 NSb	1.04±0.21 Ab	0.15±0.01 NSb
	S0.5	0.66±0.10 NSb	1.29±0.05 Ac	0.18±0.00 NSa
	S1.25	0.63±0.09 NSb	0.59±0.06 Bb	0.18±0.02 NSb
	S2.5	0.75±0.04 NSb	0.58±0.08 Bb	0.14±0.02 NSb
Treatments		***	***	***
Days		***	***	***
Days x Treatments		***	***	***