Phenolic content and potential bioactivity of apple juice as affected by thermal and ultrasound pasteurization

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Effect of thermal and ultrasound pasteurization on phenolic content and potential bioactivity of apple juice

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Complete List of Authors:	ALONGI, MARILISA; University of Udine, Department of Agricultural, Food, Environmental and Animal Sciences Verardo, Giancarlo; Univ Udine, Department of Agri-Food, Environment and Animal Sciences Gorassini, Andrea; Universita degli Studi di Udine Lemos, M.; University of Abertay Dundee, Food Science and Technology Hungerford, Graham; University of Strathclyde, Department of Physics Cortella, Giovanni; University of Udine, Polytechnic Department of Engineering and Architecture Anese, M.; Universita degli Studi di Udine, Di4A

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Dear Referees,

I would like to submit the manuscript entitled *"Effect of thermal and ultrasound pasteurization on phenolic content and potential bioactivity of apple juice"* by Marilisa Alongi, Giancarlo Verardo, Andrea Gorassini, M. Adilia Lemos, Graham Hungerford, Giovanni Cortella and Monica Anese for consideration for publication in *Food & Function*.

This study is part of a research project aimed at evaluating the effect of technological interventions, i.e. formulation and processing, on the bioactivity of some foods. To this regard, a previous paper (Alongi, M., Verardo, G., Gorassini, A., & Anese, M. (2018). Effect of pasteurization on *in vitro* α-glucosidase inhibitory activity of apple juice. *LWT - Food Science and Technology*, *98*, 366–371) demonstrated that the intensity of the pasteurizing thermal treatment applied to apple juice affected its phenolic content and ability to inhibit α-glucosidase and thus to exert an antidiabetic effect.

The present work further investigated on apple juice functional properties as affected by technological intervention, considering conventional (i.e. thermal) and unconventional (i.e. ultrasound) pasteurization. Results showed that no univocal indication on the best pasteurization process can be gathered and it is thus necessary to define the desired target to drive technological interventions by a customized approach.

Best regards,
Marilisa Alongi

ARTICLE

Effect of thermal and ultrasound pasteurization on phenolic content and potential bioactivity of apple juice

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Marilisa Alongi[a]†, Giancarlo Verardo[a], Andrea Gorassini[b], M. Adilia Lemos[c], Graham Hungerford[d,e], Giovanni Cortella[f], Monica Anese[a]

Thermal (T) and ultrasound (US) pasteurization processes were applied to apple juice and the phenolic compounds (TPC) were quantified before and after *in vitro* digestion by HPLC-DAD-ESI-MSn, with their bioaccessibility ascertained. Digested samples were analysed for their inhibitory capacity against α-glucosidase. Since some of the compounds exhibit fluorescence, both steady state and time-resolved fluorescence methods were used to investigate the binding to a blood transport protein, human serum albumin (HSA). It was found that processing induced an increase in the TPC content, which was more pronounced when US was applied. On the contrary, digestion reduced the TPC content, evening out the overall effect. Still T and US pasteurized juices presented a higher quantity of TPC upon digestion as compared to the raw sample. No correlation was found between TPC content and α-glucosidase inhibition, as the T and US pasteurized juices showed the highest and lowest inhibitory capacities against the enzyme, respectively. This is indicative that other compounds, such as those formed upon thermal treatment, may be involved in the antidiabetic effect of apple juice. The fluorescence study showed that binding occurred to HSA, at slightly different rates for different species present in the US treated extract. Considering energy consumption, US pasteurization was the most power consuming treatment despite its shorter duration. Overall, no univocal indication on the best pasteurization process can be gathered. Thus, it is necessary to define the desired target in order to drive technological interventions by a customized approach.

Introduction

During the last decade, a large part of food research has been driven by the increasing market demand for ready-to-eat, minimally processed vegetable derivatives.[1] Unconventional technologies, based on thermal or non-thermal processes, have been proposed to prevent processed food from losing its fresh-like features.[2–4] These represent one of the most important aspects affecting consumer choice and mainly include sensory attributes, of which appearance is the most critical.[5] Together with the demand for fresh-like foods, there is an increasing awareness of consumers towards the health benefits carried out by vegetable products.[6] Thus, the need to identify processing conditions able not only to preserve the sensory characteristics but also to maintain or even improve food nutritional properties is crucial to meet consumer expectations.

The nutritional functionality of vegetable derivatives is mainly attributed to phenolic compounds, which positively affect human health.[7] Extended literature review has already been provided about the different impact of conventional and unconventional technologies on the phenolic content of several vegetable matrices.[4,8] However, investigating the effect of technological interventions on phenolic content is not exhaustive when studying health-related properties. Therefore, it is necessary to also take into account the digestion process, considering the ratio of compounds able to reach the small intestine upon digestion, as compared to the amount present in the undigested food, i.e. their bioaccessibility.[9] Thus, *in vitro* digestion experiments are becoming popular, in part due to the growing interest in understanding the fate of bioactive compounds after food ingestion.[10] Despite the fact that both the technological and the digestion effects have been investigated on different vegetable foods, the harmonization between these two aspects is still weak. Therefore, it is increasingly important to consider the technological and the digestion effects concomitantly when evaluating the functionality of food, i.e. its ability to provide a health benefit for the prevention, management, or treatment of chronic disease.[11]

The biological activity of phenolic compounds has been attributed for a long time to their antioxidant properties.[12–14] However, in recent years several authors debunked the hypothesis of a direct connection between health effects and antioxidant activity, even when the latter is retained upon

[a] *Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, via Sondrio 2/A, 33100 Udine, Italy*
[b] *Department of Humanities and Cultural Heritage, University of Udine, vicolo Florio 2/B, 33100 Udine, Italy*
[c] *School of Applied Sciences, Division of Engineering and Food Science, Bell Street, DD1 1HG Dundee, UK*
[d] *Horiba Jobin Yvon IBH Ltd, 133 Finnieston Street, Glasgow G3 8HB, UK*
[e] *5 Department of Physics, SUPA, University of Strathclyde, 107 Rottenrow East, Glasgow G4 ONG, UK.*
[f] *Polytechnic Department of Engineering and Architecture, University of Udine, via delle Scienze 208, 33100 Udine, Italy*
† Corresponding author
Tel.: +39 0432 558137, e-mail address: alongi.marilisa@spes.uniud.it.

digestion.[15–17] Other mechanisms were suggested to explain the health-related properties of phenolic compounds. For instance, some of them were demonstrated to exert an antidiabetic effect through the ability to inhibit α-glucosidase, which is a key enzyme involved in carbohydrate digestion pathway[18,19] and thus targeted by several antidiabetic drugs.[20]

The present paper aimed at investigating the effect of conventional (i.e. thermal) and unconventional (i.e. ultrasound) pasteurization of apple juice along with the effect of digestion on the phenolic content and bioaccessibility, the antioxidant activity, the inhibitory capacity against α-glucosidase and the binding ability to human serum albumin, chosen as a blood transport protein model. Apple juice was selected as a study case since it represents one of the most consumed vegetable products worldwide. Its intake is also associated with the prevention of one of the most alarming chronic metabolic diseases, type 2 diabetes,[21] which is linked to its phenolic content.[14]

Material and methods

Chemicals and materials

Methanol (MeOH), formic acid (HCOOH), acetic acid (CH3COOH), fructose, glucose, sucrose, (+)-catechin, (–)-epicatechin, chlorogenic acid, phloridzin, phloretin, 3-hydroxycinnamic acid (internal standard; I.S.), α-glucosidase, 4-nitrophenyl-α-D-glucopyranoside, acarbose, α-amylase from Bacillus sp., porcine pepsin, porcine pancreatin, porcine bile extract, HCl, NaOH, $CaCl_2(H_2O)_2$, Na_2CO_3, $NaHCO_3$, NaCl, KCl, K_2HPO_4, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, $MgSO_4$, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulfate, $FeCl_3$, 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) and Trolox were purchased from Sigma-Aldrich (Milan, Italy). Quercetin-3-O-galactoside, procyanidin B2, and epigallocatechin gallate were obtained from ExtraSynthese (Lyon, France). Quercetin-3-O-arabinoside and quercetin-3-O-rhamnoside were purchased from Carbosynth (Berkshire, UK). Milli-Q grade water was produced by Elgastat UHQ-PS system (ELGA, High Wycombe Bucks, UK). Solid phase extraction (SPE) columns ISOLUTE C18, 1 g, 6 mL were from Biotage (Milan, Italy). 10 kDa cut off sacks (Avg. flat width 35 mm) were purchased from Sigma-Aldrich (Milan, Italy). Human serum albumin (>99%) was obtained from Sigma.

Sample preparation

Golden delicious apples from the same production batch were purchased at the local market and stored at 4 °C until use. Apples were washed, wiped and the juice was extracted (Ariston Hotpoint Slow Juicer, Fabriano, Italy) at 4 °C to minimize enzymatic browning. The juice was centrifuged at 5000 ×*g* for 5 min at 4 °C (Beckman Avanti J-25 Beckman Instruments Inc., Palo Alto, CA, USA) and filtered through filter paper. The juice was kept refrigerated and was subjected to technological treatments within 10 min after preparation.

Thermal and ultrasound pasteurization

Thermal and ultrasound pasteurization conditions were chosen based on those previously applied by Alongi, Verardo, Gorassini, and Anese[22] and Saeeduddin *et al.*[23], which showed a complete inactivation of microbes (total plate count, yeasts, moulds).

For thermal pasteurization, 10 mL of freshly prepared apple juice was poured into 20 mL capacity glass vials, which were closed with screw caps. Thermal treatment was carried out in a silicone oil bath (Haake Phoenix B5, Thermo Electron Corporation, Karlsruhe, Germany) and provided a sterilizing effect equivalent to 14.8 min at 90 °C, able to reduce by 2 Log *Alicyclobacillus acidoterrestris* (D90=7.4 min and z=12 °C).[24]

For ultrasound pasteurization, freshly prepared apple juice (200 mL) was poured into a jacketed beaker connected with a thermostat (Thermo Scientific HAAKE PC 200), to maintain the juice temperature at 65 ± 2 °C during the ultrasound treatment. The latter was carried out for 10 min using an ultrasonic transducer (Hielscher ultrasound technology UP400S) equipped with 22 mm probe and working at 20 kHz. Immediately after treatments, juice samples were cooled in an ice bath and kept refrigerated until further analysis.

Temperature measurement

The temperature was recorded as a function of time using a copper-constantan thermocouple probe (Ellab, Denmark), connected to a data logger (CHY 502A1, Tersid, Milano, Italy).

Power and energy density computation

Instantaneous power density values (P_v, W/m^3) during the thermal treatment were estimated based on the temperature increase of the sample, according to Eq. (1):

$$P_v(T) = \rho c_p(\frac{\partial T}{\partial t}) \quad \text{Eq. (1)}$$

were ρ was the juice density (1040 kg m^{-3}), c_p was its heat capacity (3.86 kJ kg^{-1} K^{-1}), T was the temperature (K) and t (s) was time. Adiabaticity was assumed for the vessel, given that a possible increase in the power density due to heat loss would not affect the thermal treatment, because temperature would remain constant.

During ultrasound pasteurization, the sample was supplied with a significant power density, which lead to a major temperature increase thus requiring the use of a cooling device to control juice temperature. Due to cooling, it was not possible to estimate the power density based on the juice temperature values. The ultrasound power density was thus determined in a separate test performed at 65 °C, by recording the initial temperature (T, K) increase against time (t, s) and calculating its initial derivative at quasi-adiabatic conditions.[25]

In all cases, the energy density was then estimated by integration on the whole treatment time, according to Eq. (2):

$$E_v = \int P_v(T)dt \quad \text{Eq. (2)}$$

Electrical energy consumption

The measurement of electrical energy consumption was performed as reported by Bot *et al*.[26] The energy requirement was estimated by measuring the electrical consumption at the mains supply. Both the ultrasonic processor and the heater for the thermal treatment were supplied with single-phase 230 V

electrical power, and a power meter (PC-300, Lafayette, Taiwan) was connected to measure their electrical power and thus calculate the electrical energy density (MJ/m^3) for the whole treatment. The electrical energy use values must be considered typical of our laboratory apparatus. The cooling phase for both treatments was not taken into account, nor was the cooling applied during US treatment, as it could be performed with a variety of systems (e.g. chilled water, tap water, dedicated refrigeration system, ice bath).

***In vitro* digestion**

In vitro digestion was carried out according to the protocol proposed by Minekus *et al.*[27] Briefly, the simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were prepared and stored at 4 °C. The fluids were preheated to 37 °C just before *in vitro* digestion. The oral phase was started by adding to the juice an α-amylase solution prepared in SSF and providing 75 U/mL activity in the final mixture, $CaCl^2(H^2O)^2$ (0.3 M) to achieve 0.75 mM in the final mixture and SSF. The final ratio of food to SSF was 50:50 (v/v). The sample was maintained at 37 °C under stirring for 2 min. The gastric phase was then started by mixing 5 parts of bolus with 4 parts of SGF, a pepsin solution prepared in SGF and providing 2,000 U/mL activity in the final mixture and $CaCl_2$ to achieve 0.075 mM in the final mixture. The pH was adjusted to 3.0 with HCl (1 M) and water was added to achieve a final ratio of bolus to SGF of 50:50 (v/v). The mix was stirred at 37 °C for up to 2 h. Five parts of chyme were mixed with 4 parts of SIF, a pancreatin solution prepared in SIF and providing 100 U/mL activity in the final mixture, bile salts prepared in SIF and providing 10 mM concentration in the final mixture and $CaCl_2$ to 0.3 mM in the final mixture. The pH was adjusted to 7.0 with NaOH (1 M) and water was added to achieve a final ratio of chyme to SIF of 50:50 (v/v). The mix was stirred at 37 °C for up to 2 h. At the end of the intestinal phase, samples were poured into 12 kDa cut-off sacks and phosphate buffer solution (0.1 M, pH 7) was added to achieve a ratio of sample to buffer of 1:6.25 (v/v). Dialysis was carried out at 37 °C for 4 h under stirring and the dialyzed sample was considered as the bioaccessible fraction.[28] The dialyzed sample was immediately frozen and stored for further analysis.

Total solid content and pH

The total solid content was measured by a gravimetric method (AOAC, 1995). pH was measured by a pHmeter (HANNA Instruments, pH 301, Padova, Italy).

Colour analysis

Colour analysis was carried out using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before measurement. Approximately 20 mL juice was poured in a plastic sample container (50 mm diameter) and the instrument measuring head was precisely placed 5 mm below the liquid surface. Colour was expressed in CIE units as L* (lightness/darkness), a* (redness/greenness) and b* (yellowness/blueness). The parameters a* and b* were used to compute the hue angle (arctan b*/a*).

Fluorescence measurements

The time-resolved fluorescence measurements were performed using a HORIBA Scientific DeltaFlex fluorescence lifetime system in a "T" format, similar to that described by Hungerford, Lemos, and Chu 29 for monitoring kinetics at two emission wavelengths simultaneously. Excitation was made using DeltaDiode DD-375L laser and detection was made using HPPD-860 and HPPD-890 hybrid detectors. The very low deadtime "FiPho" timing electronics were run in "photon streaming" mode in order to collect the kinetic binding data. These data were binned to produce histograms every 5 ms. For these studies the emission was monitored at both 415 nm and 510 nm simultaneously. 250 µL of (15 mg/mL) human serum albumin was added to 3 mL of US pasteurized and digested sample under stirring (300 rpm) at room temperature. All data were collected and analysed using EzTime software, which enabled the direct calculation of decay associated spectra from the global analysis of the time-resolved emission spectral measurements. Decays were reconvoluted with the instrumental response function (IRF) and fitted to a sum of exponentials. Excitation-emission matrices (EEM's) were collected on a HORIBA Scientific FluoroLog 3.

Phenolic composition

Phenolic compounds in the apple juice samples were analysed before and after *in vitro* digestion.[22] Samples were mixed with a methanolic solution of 3-hydroxycinnamic acid (50 µg/mL) as internal standard and loaded on a C18 SPE column. After loading, the column was washed with 10 mL of H_2O with 0.2% formic acid, and the phenolic fraction was eluted with 20 mL of H2O/MeOH 6:4 (v/v) with 0.2% of formic acid. After solvent removal, the residue was diluted in 1 mL of H_2O/MeOH 9:1 (v/v) and analysed by HPLC-DAD-ESI-MSn.

Chromatographic analysis was performed with a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) equipped with a thermostated autosampler and a column oven. The chromatographic separation was performed with a column Synergi Hydro, 4 µm, 250 x 2.0 mm (Phenomenex, Italy), thermostated at 30 °C. Elution was carried out at a flow rate of 0.3 mL/min, using as mobile phase a mixture of 0.2% formic acid in methanol (A) and 0.2% formic acid in water (B) with the following gradient: 0-6 min 10% A, 20 min 40% A, 40 min 40% A, 46 min 100% A, 52 min 100% A, 54 min 10% A, 54-60 min 10% A. The injection volume was 20 µL. The UPLC system was coupled with a diode array detector and an electrospray ionization mass detector (HPLC-DAD-ESI-MS^n) in parallel by splitting the mobile phase 1:1.

The acquisition was carried out in full scan (m/z 50 - 1500) and in full scan MS2 (m/z 50 - 600) selecting the precursor ion [M-H]– for each standard.[22] Phloretin-xyloglucoside and 4-p-coumaroylquinic acid were tentatively characterized, by comparison of their fragmentation pattern with those available in the literature.[30] The quantitative analysis was carried out

using an Ultimate 3000 RS Diode Array detector (Thermo Scientific, San Jose, CA, USA) controlled by Chromeleon software (version 6.80). Spectral data from all peaks were accumulated in the range 200-400 nm and chromatograms were recorded at 280 nm for (+)-catechin, (-)-epicatechin, procyanidin B2, epigallocatechine gallate, 3-hydroxycinnamic acid (I.S.), phloretin-xyloglucoside, phloridzin, phloretin, 314 nm for 4-p-coumaroylquinic acid, 328 nm for chlorogenic acid, 258 nm for quercetin-3-O-galactoside, quercetin-3-O-xyloside, quercetin-3-O-arabinoside and quercetin-3-O-rhamnoside, respectively. Calibration curves (R^2 > 0.99) were prepared by diluting a stock solution of each standard in H_2O/MeOH 9:1 (v/v) with 0.2% of formic acid in the range 12-3000 ng/mL with a constant concentration of the I.S. (500 ng/mL).

Antioxidant activity

The antioxidant capacity was measured based on the ABTS assay.[31] Briefly, 2.5 mL of ABTS 7 mM was added with 44 µL of 140 mM potassium persulfate and 1 mL of this solution was mixed with 0.1 mL sample. The absorbance at 734 nm was recorded after 90 s using an UV-VIS spectrophotometer (GENESYS™ 10S UV-Vis Spectrophotometer, ThermoFisher Scientific).

The FRAP (ferric reducing antioxidant power) assay was also used to measure the antioxidant capacity. 100 mL of acetate buffer (pH 3.6), 12 mL of distilled water, 10 mL of FeCl3 20 mM and 10 mL of TPTZ (10 mM) were mixed, and 3 ml of this solution was mixed with 0.9 mL of sample. The absorption at 593 nm was recorded after 4 min. Appropriate solvent blanks were also run. A standard curve (R^2 = 0.99) was plotted with different concentrations of Trolox (0-125 µM) and the antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC).

α-Glucosidase inhibition assay

The inhibitory activity of digested samples against α-glucosidase was assessed spectrophotometrically (UV-2501PC, UV-VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan), as previously described.[32] Different aliquots of sample were introduced in 1 mL capacity cuvettes in the presence of 30 µL α-glucosidase solution (0.04 mg/mL in 0.1 M phosphate buffer, pH=7, corresponding to 1 U/mL), and phosphate buffer (100 mM, pH 7) to the volume of 900 µL, and mixed well. After incubation at 37 °C for 10 min, the reaction was started by adding 100 µL of 5 mM 4-nitrophenyl-α-D-glucopyranoside solution in 100 mM phosphate buffer (pH 7.0) as substrate. Absorbance was recorded at 405 nm during 10 min after every 30 s. Controls lacking inhibitors were run and defined the control activity in each experiment. The α-glucosidase inhibition carried out by digested samples was calculated using Eq. (3):

$$Inhibitory\ activity\ (\%) = 100 - \left(\frac{k_s}{k_c} \times 100\right) \quad \text{Eq. (3)}$$

where ks and kc were the kinetic constants in the presence and in the absence of the inhibitor (i.e. digested sample), respectively.

The inhibitory activity (%) against α-glucosidase was plotted vs digested sample concentration, and a logarithmic model was used to fit data (R^2 > 0.90) so that the half-maximal inhibitory concentrations (IC_{50}), i.e. the concentration of sample required to produce a 50% inhibition against α-glucosidase, was calculated. The same evaluation was applied to acarbose solutions, with increasing concentration in the range 0.02 – 0.80 mg/mL, as a standard indicator for rating the efficacy of juice samples in inhibiting α-glucosidase. The acarbose equivalent was calculated as IC_{50} acarbose/IC_{50} sample.[33]

Table 1 Colour parameters of apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization.

Sample	L*	Hue angle (arctan b*/a*)
Raw	29.1 ± 0.5^c	30.8 ± 1.2^c
T	35.5 ± 0.2^a	81.4 ± 0.4^a
US	30.3 ± 0.1^b	68.2 ± 0.5^b

Statistical analysis

Results are averages of at least three measurements carried out on two replicated samples and are reported as means ± standard deviation. Statistical analysis was performed using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$).

Results and discussion

Effect of thermal and ultrasound pasteurization on some physical and chemical properties of apple juice

Raw apple juice presented an average total solid content of 11.6 ± 0.1% and pH = 3.7 ± 0.1. As expected, no changes in these parameters were observed upon thermal and ultrasound pasteurization. By contrast, the different treatments led to significant changes in juice colour (Table 1). L* and hue angle values significantly increased upon thermal pasteurization, indicating sample bleaching. Thermal treatments are known to increase the concentration of low molecular weight, soluble compounds by inducing the hydrolysis of polymeric aggregates formed upon polyphenoloxidase activity.[34] When ultrasound pasteurization was applied, the reduced heat intensity allowed minimizing colour changes. The latter represents one of the most important intrinsic attributes determining consumer choice of vegetable derivatives.[5,35] Thus, by reducing changes in the appearance of apple juice, ultrasound pasteurization could positively affect the perceived quality of pasteurized apple juice as compared to the thermal one.

Effect of thermal and ultrasound pasteurization on the phenolic content of apple juice before and after *in vitro* digestion

As ultrasound pasteurization improved the fresh-like appearance of apple juice (Table 1), further research aimed at understanding its effect, as compared to the thermal pasteurization, on the potential functionality of apple juice. The latter is well known to contain consistent amounts of phenolic

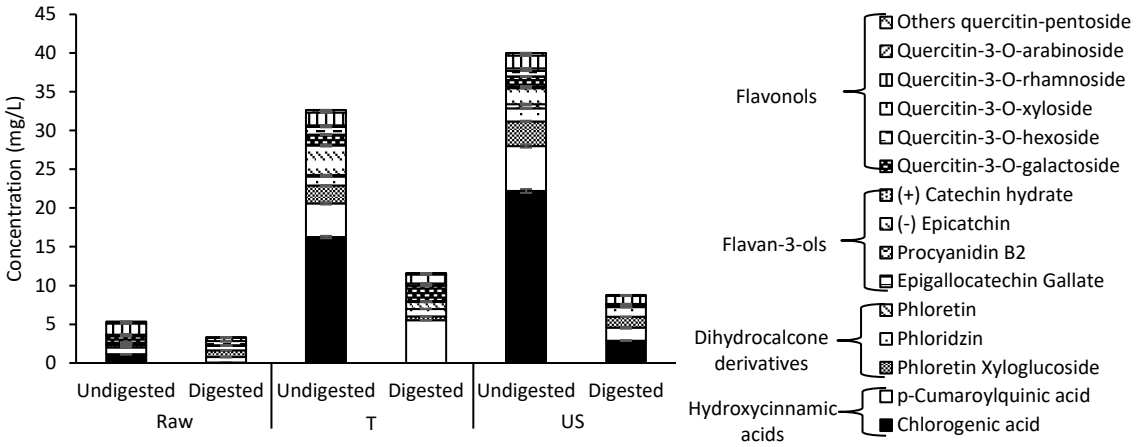

Figure 1. Phenolic compound concentrations of apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, before and after *in vitro* digestion.

compounds, which have been demonstrated to provide beneficial effects toward health.[14] Phenolic compounds were thus analysed in raw, and in thermal and ultrasound pasteurized apple juice (Figure 1), to investigate the effect of different technological interventions on their concentration. As previously reported,[22] the total phenolic concentration increased from 5.33 to 32.66 mg/L when raw apple juice was submitted to thermal pasteurization. This relates to polyphenol oxidase inactivation, as well as to the release of monomers and dimers upon thermally induced hydrolysis of heat-labile compounds.[36,37] When ultrasound pasteurization was used instead, the total phenolic content further increased to 40.03 mg/L, accounting for a 7.5-fold higher concentration than in the raw apple juice. This is in spite of using a lower temperature during ultrasound pasteurization as compared to the thermal one. The combination of cavitation with heat could have induced tissue disruption, producing not only bacterial and enzymatic inactivation, but also promoting the release of phenolic compounds from the vegetable matrix.[38,39]

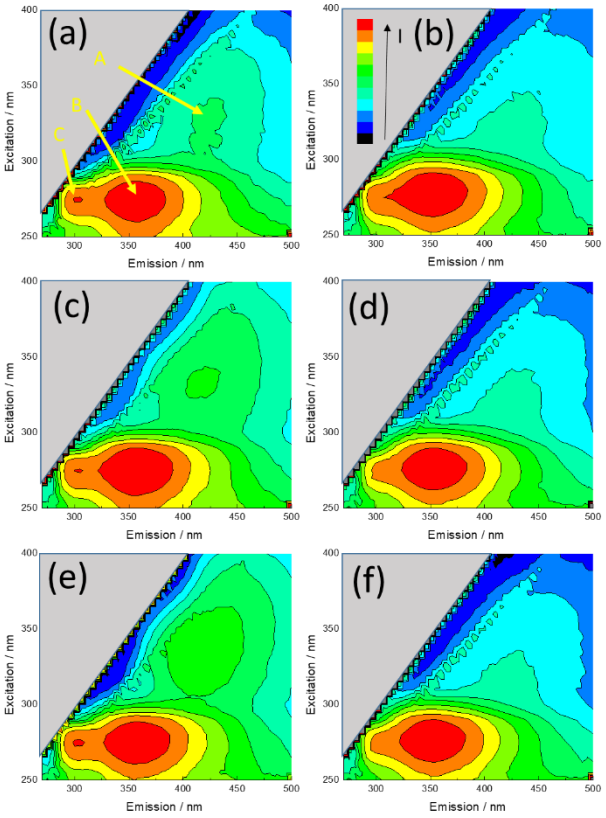

Figure 2. EEMs for (a) raw, (b) raw digested, (c) thermal, (d) thermal digested, (e) ultrasound and (f) ultrasound digested apple juice. The increasing intensity scale (blue to red, indicated in (b)) is logarithmic to clarify lower intensity emissions.

The effect of the treatments was also seen by studying the steady state fluorescence emission by use of an excitation-emission matrix (EEM). These are shown in Figure 2 for different treatments. The EEMs shown in Figure 2 are in keeping with those reported (using a larger wavelength range) by Włodarska et al.[40] Here differences in three spectral regions (A, B & C in Figure 2a) can be seen between the undigested and digested samples, as well as depending on the treatment. In the undigested samples, spectral regions C and B which can relate in part to emissions from tyrosine and flavanols,[40] are the dominant emissions and appear consistent for the raw and treated samples. The main spectral difference between the undigested samples is region A, which has principally been attributed to chlorogenic acid (CGA).[40] The relative intensity of the emission in this region appears to match changes in the concentration of this compound shown in Figure 1, with the highest emission in the US treated sample, followed by the thermal treated and then the raw sample. Upon digestion the greatest change is noted in region A, with the reduction in its relative emission, plus also a relative reduction is also noted in region C. Again, the result is in keeping with what we report in Figure 1.

When considering different components of the total phenolic compounds in raw apple juice, flavonols and hydroxycinnamic acids represent 51% and 38%, respectively. Even if an overall increase in the concentration was observed for both classes upon pasteurization, flavonols only accounted for 14%, while hydroxycinnamic acids represented the 63% of total phenolic compounds in thermal pasteurized apple juice. Such differences

Table 2 Bioaccessibility of phenolic compounds in apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization.

	Raw (%)	T (%)	US (%)
Chlorogenic acid	5.07 ± 0.43	0.00 ± 0.00	13.02 ± 0.00
p-Cumaroylquinic acid	78.96 ± 5.32	126.91 ± 0.28	28.61 ± 2.05
Hydroxycinnamic acids	**37.80 ± 2.59**	**26.78 ± 0.06**	**16.24 ± 0.43**
Phloretin Xyloglucoside	333.03 ± 9.13	20.90 ± 0.26	44.17 ± 0.46
Phloridzin	430.32 ± 23.24	82.79 ± 2.08	74.45 ± 3.21
Phloretin	n.d.	n.d.	n.d.
Dihydrocalcone derivatives	**365.50 ± 13.84**	**68.33 ± 1.84**	**54.74 ± 0.82**
Epigallocatechin Gallate	14.36 ± 1.71	n.d.	n.d.
Procyanidin B2	n.d.	0.00 ± 0.00	0.00 ± 0.00
(-) Epicatchin	n.d.	0.49 ± 0.03	0.00 ± 0.00
(+) Catechin	n.d.	n.d.	0.00 ± 0.00
Flavan-3-ols	**14.36 ± 1.71**	**0.47 ± 0.03**	**0.00 ± 0.00**
Quercetin-3-*O*-galactoside	2.58 ± 0.39	145.29 ± 4.41	15.81 ± 1.76
Quercetin-3-*O*-hexoside	654.87 ± 18.40	29.50 ± 0.92	19.64 ± 0.79
Quercetin-3-*O*-xyloside	0.00 ± 0.00	510.92 ± 21.78	16.80 ± 2.70
Quercetin-3-*O*-rhamnoside	29.65 ± 3.41	8.47 ± 0.30	62.59 ± 0.55
Quercetin-3-*O*-arabinoside	n.d.	n.d.	n.d.
Others quercetin-pentoside	32.54 ± 0.97	0.00 ± 0.00	33.90 ± 1.44
Flavonols	**38.78 ± 2.32**	**80.08 ± 2.80**	**35.67 ± 0.69**
Total phenolic compounds	**62.06 ± 3.27**	**35.47 ± 0.56**	**21.91 ± 0.27**

n.d.: not determined.

could be attributed to differences in degradation and formation pathways among apple juice phenolic subclasses.[36] The switch in the relative abundance of flavonols and hydroxycinnamic acids was even more pronounced when ultrasound pasteurization was applied, accounting for 10 and 70%, respectively. Although changes were observed in the composition of the phenolic pool upon thermal and ultrasound pasteurization, the latter induced a greater increase in the overall content of phenolic compounds in apple juice. To understand if the increase in phenolic compounds could actually result in an improved functionality of apple juice, the US treated sample was *in vitro* digested. The phenolic compounds were then quantified for the digested samples (Figure 1) and the results obtained were used to compute their bioaccessibility (Table 2). A decrease in total phenolic concentration was observed after *in vitro* digestion of all samples (Figure 1). Such a decrease was more pronounced in the order Raw<T<US, accounting for a 40, 65 and 78% decrease respectively. These results indicate a higher susceptibility of the phenolic compounds to degradation during the digestion process after exposure to technological interventions, resulting in a reduced bioaccessibility (Table 2). Since phenolic compounds were more abundant in pasteurized samples as compared to the raw juice (Figure 1), it can be inferred that they could be more easily available as a substrate for esterase activity at intestinal level.[41] This can be followed by oxidative degradation, polymerization and complexation with metal ions, proteins and fibres,[42] resulting in a more pronounced depletion. As already pointed out for the different pasteurization processes, besides producing an overall decrease in phenolic concentration, digestion also resulted in a prominent modification in their content profile. After digestion of raw apple juice, flavonols decreased from 51% to 32% of the total phenolic compounds and hydroxycinnamic acids from 38% to 23%, while dihydrochalcones increased from 7 to 44%.

In particular, chlorogenic acid represented the compound most susceptible to degradation, accounting for a 95% reduction in its concentration after digestion of raw apple juice. Such a decrease was even more marked when considering thermally pasteurized apple juice, in which chlorogenic acid, representing alone the 50% of the total phenolic content, completely disappeared upon digestion. Ultrasound pasteurization also produced an important decrease (i.e. 87%) of chlorogenic acid upon digestion (Figure 1). Overall, despite some exceptions (e.g. phloretin and quercetin-3-O-arabinoside only formed upon thermal pasteurization and digestion), carrying out the in vitro digestion after pasteurization produced a similar effect on all phenolic compounds, i.e. a more pronounced decrease in their concentration, resulting in a reduced bioaccessibility (Table 2). Nonetheless, it is important to highlight that upon in vitro digestion phenolic concentration in both thermal and ultrasound pasteurized apple juices was still higher as compared to the digested raw sample (Figure 1). In other words, the more pronounced loss of phenolic compounds observed upon digestion in pasteurized samples, as compared to the raw juice, was counterbalanced by the increase in their concentration produced during pasteurization. This resulted in an overall increase in the number of phenolic compounds potentially available for intestinal absorption when pasteurized juices were considered.

Effect of thermal and ultrasound pasteurization on the antioxidant activity of apple juice before and after *in vitro* digestion

Since changes in the concentration and bioaccessibility of phenolic compounds were observed in apple juices submitted to different pasteurization processes (Figure 1 and Table 2), samples were analysed for their antioxidant activity (Figure 3). As reported in Figure 3, the antioxidant activity measured by the ABTS assay increased by more than 6-fold after digestion, independently from the application of a pasteurization process,

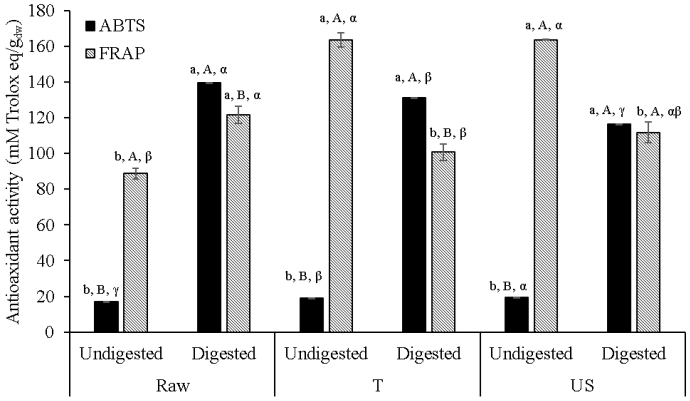

Figure 3. Antioxidant activity (ABTS and FRAP) of apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, before and after *in vitro* digestion. Different lowercase latin letters (a-b) indicate significant differences between Undigested and Digested juice subjected to the same treatment (Raw, T, US) and assayed with the same method (FRAP, ABTS). Different uppercase latin letters (A-B) indicate significant differences between FRAP and ABTS assay. Different lowercase greek letters (α-γ) indicate significant differences among Raw, and T and US treated juices, assayed with the same method (FRAP, ABTS).

with the highest value corresponding to the raw digested juice. An increase in the antioxidant activity upon digestion was also observed by other authors on other phenolic-rich juices.[43] These results diverge from those relevant to phenolic content (Figure 1), showing a decrease upon digestion in all cases and thus corroborating the lack of a direct relationship between the health effects attributed to phenolic compounds and the antioxidant activity.[16,17]

Overall, the results obtained with the FRAP assay significantly differed from those acquired by using the ABTS. In particular, after *in vitro* digestion the antioxidant activity of thermal (T) or ultrasound (US) pasteurized apple juice decreased when compared to that observed for undigested samples. The more pronounced differences were observed in the undigested samples, while digested ones presented closer antioxidant activity values. Still, no relation was found between such activity (Figure 3) and the phenolic content (Figure 1). The different results obtained from the two methods can rely on the different conditions underlying antioxidant activity measurement, including the oxidant/reductant used as well as the compositional and environmental variables.[12] In particular, the ABTS assay is based on the reduction of the antioxidant due to the loss of an electron by the nitrogen atom of ABTS. On the contrary, the FRAP method relies on the oxidation of the antioxidant leading to the reduction of the complex ferric ion-TPTZ.[44] As the response of antioxidants to different conditions differs, it is necessary to apply more than one technique to measure the antioxidant activity of a complex system, in order to avoid result misinterpretation.[45] Nonetheless, the antioxidant activity tested by *in vitro* assays does not provide biologically relevant information on antioxidants as it does not consider their behaviour under physiological conditions.[16,43]

Effect of thermal and ultrasound pasteurization on the α-glucosidase inhibitory capacity of digested apple juice

To understand if and how the different pasteurization processes affected the antidiabetic potential of apple juice, digested samples were analysed for their ability to inhibit α-glucosidase (Figure 4). All digested samples inhibited α-glucosidase in a concentration-dependent manner but to a different extent. To rate the efficacy of digested sample in inhibiting the enzyme, the IC_{50}, i.e. the half-maximal inhibitor concentration, was computed and compared to that of acarbose, which is one of the most commonly used drugs for the treatment of type 2 diabetes (Table 3). Thermal pasteurized apple juice was more effective than raw apple juice in inhibiting α-glucosidase upon *in vitro* digestion, while ultrasound pasteurization reduced the inhibitory capacity of apple juice (Figure 4 and Table 3). The ability of apple juice to inhibit α-glucosidase was previously assessed on undigested apple juice and was 6- to 40-fold lower as compared to the digested samples,[22] indicating that the digestion process plays a key role in determining food functionality. The most effective sample, i.e. thermal pasteurized apple juice, also contained the highest concentration of total phenolic compounds in the digested sample (Figure 1). However, no correlation ($p > 0.05$) was found

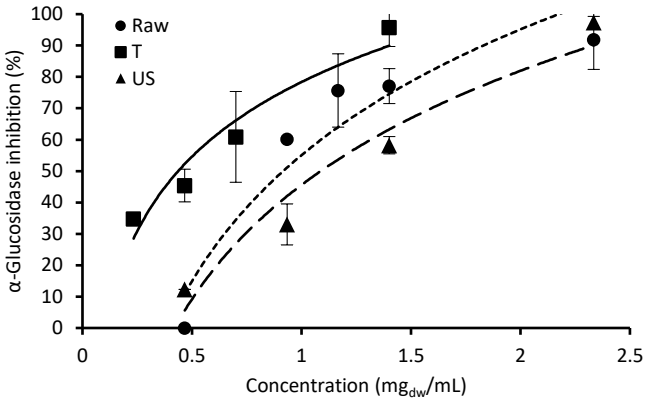

Figure 4. α-Glucosidase inhibitory activity of digested apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data.).

Table 3 IC_{50} (half-maximal inhibitory concentration) and acarbose equivalents of digested apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization.

	IC_{50} (mg/mL)	Acarbose equivalents ($IC_{50\ acarbose}/IC_{50\ inhibitor}$)
Raw	0.92	0.10
T	0.44	0.21
US	1.09	0.08
Acarbose	0.09	1

between the phenolic concentration and the IC_{50}. Even if several authors[14,46] attributed the antidiabetic effect of apple juice to the ability of phenolic compounds to inhibit α-glucosidase, the absence of a direct correlation between the phenolic content and the α-glucosidase inhibitory capacity was already pointed out in the undigested apple juice.[22] This might suggest that not only phenolic compounds but also other molecules, such as those formed upon thermal treatment, may be involved in the antidiabetic effect of apple juice.

Serum albumin binding ability

As already pointed out, T and US pasteurized juices still accounted for a considerable concentration of phenolic compounds even upon digestion, although their profile was different. In particular, chlorogenic acid survived gastrointestinal conditions only in the US pasteurized apple juice, whereas it was completely depleted in the thermally pasteurized sample (Figure 1). To understand whether chlorogenic acid could be effectively carried to other target sites to elicit its many other health-related benefits, among which are hepatoprotective, cardioprotective, neuroprotective, anti-inflammatory, antimicrobial, and anti-hypertension effects,[47] US pasteurized juice was analysed to assess the interactions occurring with a major transport protein in blood. As a model system human serum albumin (HSA) was used. This binding process was observed making use of the sensitivity of time-resolved fluorescence. The measurement of the fluorescence lifetime by its nature gives an absolute measure, which is concentration independent and thus not affected by photobleaching, sample dilution or changes in excitation intensity. Hence, this technique is highly suitable to monitor the

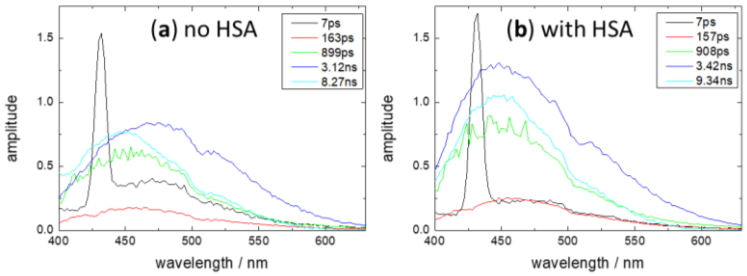

Figure 5. Decay associated spectra for US treated digested extract, both before (a) and after (b) the addition of HSA. The fluorescence lifetime associated with the spectra are shown. That of 7ps (half a time bin) was fixed in the decay analysis to account for scattered light and its associated spectra is dominated by a peak that can be attributed to Raman scattering from the solvent.

binding of fluorescent components in the apple juice extract to HSA. A methodology similar to that previously applied to the binding of butterfly pea extract to BSA was employed.[29] In order to excite the extract a longer wavelength (378 nm) was selected, as using a shorter wavelength light (e.g. <295 nm) would also excite fluorescent amino acids in the HSA (notably tyrosine and tryptophan) as well as the extract (Figure 2). Therefore, when considering the spectral regions indicated in Figure 2, it is be region "A", which is principally associated with chlorogenic acid, that was monitored. The emission around 450 nm with excitation at 378 nm was explored using decay associated spectra, both before and after the kinetic study. This type of measurement has previously been used to investigate blue-green fluorescence in artichoke leaves and was linked with chlorogenic acid.[48] The outcome of these measurements is shown in Figure 5. The results shown in Figure 5 indicate that the emission seen in the EEMs (Figure 2) is more complex and consists of several fluorescing species, with (at least) the presence of four principal emissions in addition to a short-lived one dominated by a Raman scattering feature (at 431 nm). This short-lived spectrum also appears to have an emission around 465 nm. Considering the extract prior to addition of HSA (Figure 5a) then an emission peaking ~458 nm is seen associated with a lifetime of 163 ps, this is a similar wavelength to the spectrum associated with a decay time of 899 ps. The spectra associated with longer lifetimes, 3.12 ns and 8.27 ns exhibit peak at ~468 nm and 450 nm respectively. These results are indicative of the presence of more than one fluorescing component.

After addition of HSA (Figure 5b) some notable changes in the decay associated spectra were observed. Overall, an increase in the emission intensity (close to 1.3 times) occurred and the lifetimes of the two longer-lived associated spectra increased. There were also significant changes in the peak emission wavelengths of the spectra associated with the 908 ps and 3.42 ns lifetimes (to 449 nm and 455 nm respectively). A hypsochromic shift is usually indicative of emission from a less polar environment[49] and in this case can relate to fluorescent compounds in the extract locating to the interior of the HSA and is corroborated by the increase in the fluorescence lifetime. These observations are in keeping with binding studies using fluorescent probes 50 and other work has indicated that the dominant binding site for both CGA and flavonoids is Sudlow's

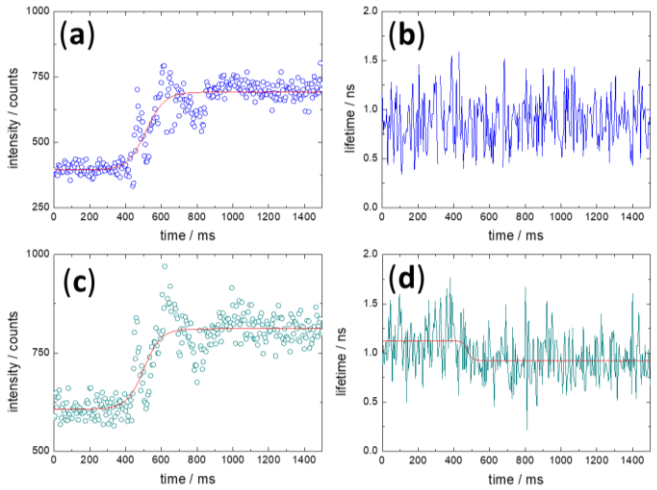

Figure 6. Time-resolved fluorescence data for the addition of HSA to US treated digested extract excited at 378 nm. The emission was monitored simultaneously at 415 nm (a & b) and 510 nm (c & d) with intensity data (a & c) and the lifetime obtained from a single exponential fitting model (b & d) shown.

site I.[51,52] This interaction process between the extract and HSA can also be followed making use of a "photo streaming" measurement. In this case, as the HSA is added to the US treated digested sample. Two wavelengths (415 nm and 510 nm) were selected either side of the main emission peak to elucidate differences in behaviour of the extract since it is known to be a mixture of different compounds. Both intensity and lifetime data were obtained (Figure 6) and a region 1.5 seconds in duration around the addition point was investigated. In order to obtain sufficient photons to analyse a resolution of 5 ms per point was used and the time-resolved decays were simply fitted to a single exponential decay model to elucidate differences.[53] Overall the results show an increase in intensity upon addition of the HSA (~1.75x at 415 nm and ~ 1.34x at 510 nm), which is in keeping with that observed in the decay associated spectra. At 410 nm no significant change in lifetime was seen, while a slight decrease was observed at 510 nm. This can be because of the hypsochromic shift noted in the decay associated spectra in some of the longer-lived (908ps, 3.42 ns) associated spectra. The rate at which the fluorescence intensity (counts) changes is slightly higher at 415 nm (52.2 ms-1) compared to that at 510 nm (48.1 ms-1). It should be noted that other work has shown that the binding constant to HSA of the probable main fluorescing species (CGA and quercetin) show that CGA has a higher binding constant than quercetin.[51,54] However, since the extract is complex in terms of having several fluorescing species it is difficult to make concrete comments in relation to the binding kinetics. It should also be kept in mind that as well as the species elucidated by their fluorescence in the wavelength range studied there are also compounds in the extract that can be binding, thus in fact competing with those fluorescent species, but because of their absorption and emission wavelengths go unnoticed in the study.

Energy density and electrical energy consumption

To estimate the efficiency of thermal and US pasteurization treatments the power and energy density for both the heating

Table 4 IC_{50} (half-maximal inhibitory concentration) and acarbose equivalents of digested apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization.

	Heating phase				Cooling phase			Final temperature (°C)
	Power density (kW/m^3)	Energy density (MJ/m^3)	Duration (s)	Electrical energy use (MJ/m^3)	Power density (kW/m^3)	Energy density (MJ/m^3)	Duration (s)	
Thermal pasteurization	max: 1588 average: 288	324	1120	520	max: -1084 average: -580	-174	310	51.6
US pasteurization	1160	696	600	4260	Max: -607 average: -437	-55.1	120	51.3

and cooling (to a final temperature of about 50 °C) phases were computed (Table 4). It appears that the US treatment required a much higher energy density despite its shorter treatment time. On the contrary, the energy density needed to cool down the US treated juice was lower, due to the lower temperature reached by the sample at the end of the heating phase. Table 4 also shows the electrical energy use of both treatments during the heating phase. US pasteurization resulted the most energy consuming treatment. Although its duration was lower, it must be born in mind that temperature control is needed during the whole US treatment, to mitigate the temperature increase that would occur.

Conclusions

To summarise, the results obtained highlight the fact that processing and digestion oppositely affected the phenolic content in apple juice: even if processing induced an increase in their concentration, they suffered a prominent decrease upon *in vitro* digestion. This was most pronounced in the US pasteurized sample. Still, after digestion both T and US pasteurized juices presented a higher concentration of phenolic compounds potentially available for intestinal absorption, as compared to the digested raw sample. Thus, since bioaccessibility was lower in pasteurized samples as compared to the raw one, it can be inferred that this functionality index alone is not able to explain the effect of both processing and digestion on the number of compounds actually available for gut absorption.

No correlation was found between phenolic content and α-glucosidase inhibition, as T and US pasteurized juices showed the highest and lowest enzymatic inhibitory capacities, respectively. It is also clear that components present in the extract after digestion can actively bind to the main blood transport protein (HSA). Finally, from the energy consumption point of view, US pasteurization was the most wasting treatment despite its shorter duration.

Based on the results obtained, no univocal indication on the best pasteurization process to be applied to boost apple juice functionality can be surmised. Evaluating the effect of a technological intervention on food functionality necessarily requires considering all available data to avoid result misinterpretation. When carrying out studies relevant to food enrichment with bioactive compounds it is crucial to bear in mind that increasing their bioaccessibility does not necessarily mean increasing the concentration of compounds available for intestinal absorption, nor their functionality. This includes transport efficiency and bioactivity. Defining the desired concentration at the intestinal level, as well as in the target organs and tissues, would be helpful in developing the proper technological intervention to drive functionality by a customized approach.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

M. Anese conceived the study in conjunction with M. Alongi. M. Alongi carried out all the experiments, except polyphenol content that was assessed by G. Verardo and A. Gorassini, and antioxidant activity and fluorescence analyses, carried out by A. Lemos and G. Hungerford. G. Cortella estimated energy density and consumption. All authors participated in manuscript revision and discussion, coordinated and critiqued by M. Anese and M. Alongi. Authors are grateful to Ms. Ludovica Dal Moro for contributing to analyses.

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1 Citations should appear here in the format A. Name, B. Name and C. Name, *Journal Title*, 2000, **35**, 3523; A. Name, B. Name and C. Name, *Journal Title*, 2000, **35**, 3523.

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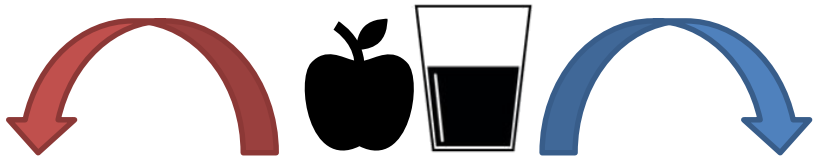

T	APPLE JUICE PASTEURIZATION	US
☹	Fresh-like appearance	☺
↑	Phenolic content before digestion	↑↑
≈	Phenolic content after digestion	≈
↑	Antidiabetic potential	↓
🪙	Cost	🪙🪙
⏱⏱	Time	⏱