



Bioactive compounds extraction from malted quinoa using water-ethanol mixtures under subcritical conditions

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ABSTRACT

The extraction of bioactive compounds from malted quinoa flour using different water/ethanol mixtures under subcritical conditions was performed, and two mathematical models to describe the extraction kinetics were compared. The variables evaluated were extraction temperature, solvent mass flow rate and solvent composition, and the best conditions were selected using a screening design. The extract with the highest antioxidant activity with 95% inhibition of DPPH, 2084 mg GAE/100 g flour of total phenol content (TPC), and 2029 mg QCE/100 g flour of total flavonoid content (TFC) was obtained at 200 °C, 2.5 g/min and 25% ethanol. For these conditions, the two-site kinetic model had a better fit. When comparing these results with conventional extraction techniques, the extraction yield and antioxidant capacity enhanced. Hence this technique results promising since it also allows to obtain extracts with an antioxidant activity similar to commercial antioxidants.

1. Introduction

In recent years, an increasing interest in functional foods has been developed, since they provide not only nutritional and energy benefits but also additional physiological advantages, such as antihypertensive, antioxidant or anti-inflammatory effects (Alkhatib et al., 2017). These products are generally traditional foods enriched with ingredients capable of providing or promoting a beneficial action for human health. Functional ingredients are commonly extracted from natural sources, such as cereals and legumes, spices, aromatic plants, industrial by-products like cocoa and olive or even algae and microalgae (Gal-anakis, 2018; Herrero, Cifuentes, & Ibañez, 2006; Jokic, Gagic, Knez, Šubaric, & Škerget, 2018).

Quinoa (*Chenopodium quinoa* Willd) is an ancestral grain that has gained popularity due to its nutritional properties (Abugoch James, 2009), not only because of the high protein, fatty acid and mineral content, but also because of the presence of several bioactive compounds, such as saponins, flavonoids, polyphenols and anthocyanins (Liu, 2019). Many studies have focused on these compounds, as well as

their antioxidant activity, antimicrobial activity, and potential health-promoting and/or disease-preventive properties including anti-inflammatory, anti-cancer, and cardioprotective effects.

A method used to enhance the antioxidant capacity in cereals or pseudocereals is germination, an economical and effective process that also reduces antinutritional compounds while substantially increasing the bioavailability of micronutrients and improving the sensory properties of grains (Suárez-Estrella, Torri, Pagani, & Marti, 2018). In recent years, investigations have evaluated the content and potential of antioxidants in germinated pseudocereals, establishing the optimal germination time in seeds of quinoa, amaranth, cañihua and buckwheat (Abderrahim et al., 2012; Niro et al., 2019). It has also been studied how germination in quinoa grains had a positive influence on the functional (Carcioci, Galván-D'Alessandro, Vandendriessche, & Chollet, 2016; Atwaa et al., 2020), nutritional (Miranda-Villa, Mufari, Bergesse, & Calandri, 2019; Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010), technological (Mäkinen, Zannini, & Arendt, 2013) and sensory properties (Naga Sai Srujana, Anila Kumari, Uma Maheswari, Suneetha Devi, & Jessie Suneetha, 2017; Suárez-Estrella et al., 2018) in

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gluten-free food products.

One of the current challenges is the quantitative extraction of these bioactive compounds while maintaining their biological activity and chemical structure. This is a critical stage in order to correctly characterize them or to propose their incorporation as an ingredient in a functional food. The extraction techniques employed so far involve the use of organic solvents, which produce high extraction yields but have a low selectivity, resulting in an increase in time and cost of the process as subsequent purification stages may be required (Galanakis, 2012). In addition, the use of these solvents is prohibited for the food and pharmaceutical industry, due to their toxicity and residual effect. The solvents approved for extractions depend on the regulations of each country, although water and ethanol comply with the quality and safety requirements of products worldwide. However, it is not always possible to achieve high yields with traditional extraction processes under these conditions.

In this context, alternative extraction methods capable of overcoming the above-mentioned drawbacks are being studied: microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction and subcritical fluids extraction. These extraction techniques provide higher extraction yields and selectivity, shorter extraction times (increased diffusion and extraction rates) and do not use toxic organic solvents (Herrero, Cifuentes, & Ibáñez, 2006).

The subcritical extraction method uses mainly water or alcohol: water mixtures at temperatures above 100 °C and below 374 °C and at pressure above atmospheric (10–80 bar) to keep it in a liquid state, which provides particular characteristics to the solvent and emerges as a useful tool to replace traditional extraction methods. It is a green method and environmentally friendly, and a safe and fast extraction technique that achieves high extraction yields of medium and high polarity compounds contained in a solid matrix (Rodríguez-Meizoso et al., 2010).

Subcritical water could be an excellent alternative to extract all flavonoids and organic acids, including non-polar flavanones (M'hiri, Ioannou, Ghoul, & Mihoubi-Boudhrioua, 2014). This extraction methodology has been reported in several investigations on different food matrices, such as rice (Hata, Wiboonsirikul, Maeda, Kimura, & Adachi, 2008), pistachio (Bodoira et al., 2019), and peanut skins (Bodoira, Rossi, Montenegro, Maestri, & Velez, 2017). Along with the extraction, stages of purification, total quantification of compounds, individual characterization and analysis of the impact on the chemical characteristics and biological activity of the extraction process should be designed (M'hiri et al., 2014). Each matrix presents a different profile of phenolic compounds and therefore a particular behavior against the extraction parameters, hence many research works focus on finding the appropriate conditions for each matrix from which the bioactive compounds are extracted (Yamine, Delsart, Vitrac, Peuchot, & Ghidossi, 2020).

However, to our knowledge, there are no reports on the use of this methodology to extract antioxidant compounds from sprouted quinoa grains. Hence this study aims to evaluate the use of water:ethanol mixtures under subcritical conditions as an extraction technique and the effect of operating variables for the recovery of bioactive compounds from malted quinoa flour. These results can contribute to the selection of the optimal working conditions to obtain the highest extraction performance and the highest antioxidant activity in the extracts obtained from malted quinoa.

2. Materials and methods

2.1. Plant material

The quinoa seeds (Cica variety) were harvested in La Poma (24°43'08"South 66°12'00"West), Salta, Argentina, in 2016. The grains were sieved to remove impurities and then stored in sealed polyethylene bags until use.

2.2. Obtaining malted quinoa flour

Quinoa seeds were washed and moisturized in distilled water (1:10 ratio), with agitation, for 2 h at 20 °C and then drained using a cloth filter. The controlled germination was carried out in a closed plastic container, inside which the grains were arranged in the form of a monolayer on a grid of the same material, for 24 h, with a temperature of 25 °C and 60% relative humidity, protected from light. Finally, they were dried with hot air flow at 50 °C for approximately 30 min (11% humidity) and were ground in a hammer mill with 0.25 mm mesh to obtain the malted quinoa flour (MQF) (Miranda-Villa et al., 2019).

2.3. Extraction procedure and optimization of working conditions

The extraction was carried in an in-house developed apparatus according to the scheme shown in Supplementary Figure. It consists on a stainless-steel cell of 18.5 mL volume, equipped with an aluminum heating jacket and electrical resistances connected to a temperature controller, a HPLC type pump (ELDEX, model OPTOS 2SM, California, USA) for the regulation of the extraction solvent mass flow rate; a pre-heater with temperature control and a back-pressure regulator for pressure control. In order to maintain the temperature of the extraction cell, it was located in a thermally insulated box. Likewise, at the end of the equipment, the extracts were cooled down before decompression by means of 1/8-inch diameter stainless steel pipes immersed in a cold bath.

The extraction variables analyzed were temperature (T: °C), extraction solvent mass flow rate (F: g/min) and extraction solvent composition (S: % w/w ethanol). In order to know the effect of these variables on the extracts, an experimental screening design was applied in which each variable was evaluated independently, keeping the others at level 0, as shown in Supplementary Table.

For each extraction, 3 g of MQF were loaded into a stainless steel cartridge that was later placed inside the extraction cell. Seven extract fractions were collected: the first three fractions corresponded to 30 g and the following four of 70 g. A total of 370 g of extract for each combination of variables were collected. All extract fractions were stored in amber glass bottles and placed in freezer until further analysis. Each combination of variables (10 trials) was performed in duplicate.

In order to improve the extraction method, a final experimental run was carried out using the combination of the maximum extraction conditions of each independent variable (extract selected as optimal).

A conventional extraction by reflux using alcohol:water 75% (v/v) as solvent, at 60 °C and atmospheric pressure for 1 h was also carried out.

2.4. Chemical analysis of the extracts

Total solid, phenols and flavonoid content, antioxidant capacity and melanoidin contents were determined in the extracts obtained in each experimental run and in the conventional extract.

The total solids content (TS) or dry matter was determined by technical gravimetry 934.01 (AOAC, 1999). For this purpose, approximately 5 g of extract were placed in a previously weighed crucible and placed in an oven at 105 °C to constant weight, recording the dry mass obtained.

The total phenol content (TPC) was determined using the Folin-Ciocalteu method in microplate (Leos-Rivas, Rivas-Morales, & García-Hernández, 2016). For this purpose, 10 µL of sample, 10 µL of Folin reagent, 150 µL of distilled water and 30 µL of a saturated solution of sodium carbonate were placed. It was left to react in the dark for 1 h and the absorbance was determined at 765 nm in a UV-visible spectrophotometer (Spectrum SP-2100 UV-Visible, Zhejiang, China). A standard gallic acid (Sigma-Aldrich, Germany) curve was used to determine the total phenolic content. The results were expressed as mg GAE/ 100 g of dry matter.

The total flavonoid content (TFC) was determined using the AlCl₃

microplate method (Larrauri, Zunino, Zygadlo, Grosso, & Nepote, 2016). 150 μL of sample and 150 μL of AlCl_3 (2%, in ethanol) were placed in the microplate. It was left to react for 15 min and the absorbance was determined at 367 nm in a UV-visible spectrophotometer (Spectrum SP-2100 UV-Visible, Zhejiang, China). Calibration curves were made with a quercetin standard (Sigma-Aldrich, Germany). The results were expressed as mg QCE/ 100 g dry matter.

The radical scavenging capacity (RSC) of the first extracts obtained for each condition (initial 30 g), was determined using the 2,2-dyphenyl-1-picryl hydrazyl radical (DPPH \cdot) (Larrauri et al., 2016). 10 μL of sample, 150 μL of DPPH-methanolic solution (40 $\mu\text{g}/\text{mL}$) were added. The absorbance was measured after 30 min at 517 nm in a spectrophotometer (Spectro SP-2100 UV-Visible, Zhejiang, China). Radical scavenging capacity was calculated by the following equation:

$$\%RSC = \frac{1 - (Abs\ DPPH\cdot - Abs\ muestra)}{Abs\ DPPH\cdot} \times 100$$

where Abs: Absorbance.

Different concentrations of each extract were measured, following the methodology described above. Calibration curves were constructed, extract concentration (μg extract/ mL) vs. %RSC. IC_{50} was defined as the extract concentration at which the RSC is 50%. A low IC_{50} value corresponds to high antioxidant activity.

Melanoidin contents were qualitatively determined, estimated by means of browning intensity of the extracted samples. For this purpose, the absorption of the extracts was measured at 420 nm, using a microplate spectrophotometer (BMG Labtech GmbH, Germany).

All extractions in section 2.3 were performed in duplicate and each determination was performed in triplicate ($n = 6$).

2.5. Antioxidant activity of the extract selected as optimal

The analyses were performed on the first fraction of the seven collected. Each of the chemical determinations described above (TPC, TFC and DPPH assay) were analyzed. Further antioxidant activity assays were performed in order to evaluate the behavior of the extract considered as optimal against different antioxidant mechanisms of action: Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC), and an accelerated oxidation test performed by Rancimat.

TEAC assay: 990 μL of ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) reagent (Sigma®, St. Louis, USA) diluted in ethanol 95% (Abs 0.7 ± 0.02) was mixed with 10 μL of extract, and a standard Trolox (Sigma®, St. Louis, USA). The absorbance was measured after 25 min at 734 nm in a spectrophotometer (Spectro SP-2100 UV-Visible, Zhejiang, China). All measurements were conducted in triplicate and the results were expressed as mmol of Trolox equivalents/g of dry extract (Re et al., 1999).

FRAP assay: 10 μL of extract, or distilled water as blank, and 990 μL of FRAP reagent (ferric chloride and TPTZ (2,4,6-Tris (2-pyridyl)-s-triazine) in acetate buffer (pH 3.6)) were placed in a reaction tube. The absorbance was measured after 5 min at 593 nm in a spectrophotometer Spectro SP-2100 UV-Visible, Zhejiang, China). A calibration curve with ascorbic acid (Biopack, Buenos Aires, Argentina) was used. All measurements were conducted in triplicate. The results were expressed as mg of ascorbic acid equivalents/g of dry extract (Benzie & Strain, 1996).

ORAC_{FL} assay: this assay was carried out according to Plaza, Amigo-Benavent, del Castillo, Ibáñez, and Herrero (2010) with slight modifications. Briefly, 100 μL of sample (with a concentration between 50 and 100 μg d.m./ mL), 125 μL of fluorescein (2 μM in 30 mM phosphate buffer, pH 7.5), the oxidation reaction was initiated by adding 100 μL of AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride) solution in a concentration of 590 mM. The fluorescence readings were recorded at $\lambda_{exc} = 493$ nm and $\lambda_{em} = 515$ nm every 5 min for 1 h, at 37 °C in a fluorometer (Synergy HT microplate reader, BioTek, Vermont,

United States). A blank was also analyzed in each run, calibration curves of Trolox were constructed and employed for quantification of the antioxidant capacity against peroxy radicals. Normalized antioxidant curves (fluorescence versus time) were obtained and the area under the fluorescence decay curve (AUC) was calculated. Regression equations between net AUC and antioxidant concentration were obtained and employed for calculation of the ORAC_{FL} values that were expressed as mg of Trolox equivalents/g of dry extract.

Rancimat analysis: the accelerated oxidation was determined using a Rancimat (Metrohm Model 743, Switzerland) equipment, under the following conditions: 100 °C and air flow of 20 L/h and. The extract was mixed with 2.5 g of degummed crude soybean oil provided by Trisoil S. A., at a concentration of 2000 ppm. The result was compared with soybean oil added with 200 ppm of BHT (Fluka, Sigma-Aldrich, St. Louis, USA). A control sample was also oxidized under the same conditions. The samples were analyzed according to Quiroga, Riveros, Zygadlo, Grosso, and Nepote (2011).

2.6. Statistical analysis

The results are expressed as mean \pm standard deviation of the replications. Data analysis was performed using InfoStat® professional version 2014 (InfoStat Group, FCA, National University of Córdoba, Argentina). The differences between the determinations were estimated using an analysis of variance (ANOVA). In those cases, where statistically significant differences were observed ($\alpha = 0.05$), a multiple comparison test was subsequently applied (DGC, DiRienzo, Guzmán and Casanoves).

2.7. Extraction kinetic models

To describe the extraction of the compounds of interest (total phenols and flavonoids) obtained under optimal conditions, two kinetic models were compared, known as one-site and two-site kinetic desorption models (Anekpankul, Goto, Sasaki, Pavasant, & Shotipruk, 2007).

The one-site kinetic model considers that initially the solute is uniformly distributed within the matrix, and that extraction is controlled by intraparticle diffusion. The mathematical expression for calculating the total mass of extracted solute (m_t) is as follows:

$$\frac{m_t}{m_0} = 1 - \exp(-kt)$$

where m_0 is the initial mass of solute in the plant matrix, k is the first order velocity constant and t is time.

The two-site kinetic model is a modification of the one-site model, and it considers that in plant matrices the compounds of interest are contained within cells. When the plant matrix is mechanically ground some of these cells are broken while others remain intact, so there is a part of the solute, known as free solute, which is more easily extracted since it was the one inside the damaged cells and is in direct contact with the solvent. On the other hand, the remaining solute fraction, known as bound solute, is found inside the cells that remain intact and is more difficult to extract due to the high mass transfer resistance that exists from inside the particle. According to the above, the two-site kinetic model is described by the following equation:

$$\frac{m_t}{m_0} = 1 - f \exp(-k_1 t) - (1 - f) \exp(-k_2 t)$$

where f is the fraction of solute that drains at a higher velocity defined by the first order constant k_1 , and $(1-f)$ is the remaining fraction that drains at a lower velocity, given by the first order constant k_2 , where t is the time.

The kinetic parameters of both kinetic models were obtained by adjusting the extraction curve under optimal conditions using Microsoft Excel Solver®, minimizing the "root mean square error (RMSE)"

function:

$$RMSE = \sqrt{\frac{\sum_{i=1}^n \left[\frac{(m_i/m_0)_{exp} - (m_i/m_0)_{model}}{n} \right]^2}{n}}$$

where m_i is the mass of analyte extracted in time “t” and “n” is the total number of samples analyzed.

To select the model that best predicts the extraction of the compounds of interest, the fit of each model was evaluated by comparing the average absolute relative deviation (AARD%) between the experimental and predicted yields:

$$(AARD\%) = \frac{1}{n} \sum_{i=1}^n \left| \frac{(m_i/m_0)_{exp} - (m_i/m_0)_{model}}{(m_i/m_0)_{exp}} \right| \times 100$$

3. Results and discussion

As mentioned above, different extraction conditions have been studied using subcritical fluids to obtain compounds with antioxidant activity contained in MQF. The extraction yield, the total phenol and flavonoid content and the antioxidant activity obtained for each combination of variables are very important when defining the best extraction conditions. Although there are many studies on the extraction of antioxidant compounds under subcritical conditions, the extraction yields are very sensitive to the characteristics of the matrix and the type of compounds that each matrix contains (Yamine et al., 2020).

Performing a statistical analysis by response variable and considering all the conditions tested (Table 1), significant differences are evident in all treatments for each of the responses studied.

Table 1
Extraction yield, composition and antioxidant activity of the different extracts.

Variable combinations	Extraction Yield (g dry extract/100 g MQF)	TS (g dry extract/100 g extract)	TPC (mg GAE/100 g dry matter)	TFC (mg QCE/100 g dry matter)	DPPH IC ₅₀ (µg extract/mL)
F0/T0/S0	64.3e ± 0.3	1.67e ± 0.02	474d ± 4	766c ± 2	1450.5h ± 0.7
F1/T0/S0	51.7c ± 0.3	1.54d ± 0.02	335b ± 9	666b ± 6	1604.0i ± 0.2
F2/T0/S0	54.4d ± 0.4	1.33c ± 0.01	357c ± 10	650a ± 7	2660.3j ± 0.4
T1/F0/S0	77.5h ± 0.3	2.27g ± 0.03	830h ± 10	999e ± 5	242.6d ± 0.7
T2/F0/S0	87.6i ± 0.1	2.73h ± 0.01	800g ± 4	1291h ± 2	109.8b ± 0.9
T3/F0/S0	75.2g ± 0.5	2.33g ± 0.01	1168i ± 7	1713i ± 5	96.3a ± 0.4
S1/F0/T0	70.5f ± 0.1	2.11f ± 0.03	807g ± 8	1155g ± 6	260.9c ± 0.6
S2/F0/T0	16.9b ± 0.1	0.54b ± 0.02	279a ± 8	1022f ± 6	1090.4g ± 0.5
S3/F0/T0	16.8b ± 0.1	0.53b ± 0.01	608e ± 5	1024f ± 4	264.9e ± 0.2
S4/F0/T0	9.8a ± 0.2	0.32a ± 0.01	660f ± 9	842d ± 4	448.4f ± 0.6
F0/T3/S1	73.2 ± 0.3	2.04 ± 0.02	2084 ± 11	2029 ± 2	29.6 ± 0.4
Conventional	50.1 ± 0.5	1.04 ± 0.04	837 ± 9	196 ± 7	3025.1 ± 0.6

The accumulated mass values for the 7 extracts are presented, mean ± standard deviation are reported for each combination of variables (n = 6). Different letters in the same column, denote statistically significant differences (ANOVA, Test DGC, p < 0.05). T: temperature, °C (T0:140, T1:160, T2:180, T3:200), F: solvent mass flow rate, g/min (F0:2.5, F1: 5, F2: 7.5), S: solvent mixture composition, % ethanol w/w (S0: 0, S1: 25, S2: 50, S3: 75, S4: 100), TS: total solids content, TPC: total phenolic content, TFC: total flavonoid content, DPPH IC₅₀: extract concentration at which the radical scavenging capacity is 50%.

The extraction yields were very variable, in a range between 10 y 87 g dry extract/100 g of MQF, TPC between 279 and 1178 mg GAE/ 100 g dry matter, TFC between 650 and 1713 mg QCE/ 100 g dry matter and IC₅₀ among 96 and 2660 µg extract/mL.

When analyzing the behavior trend of mass flow rate (Fig. 1), increases are observed for all response variables studied. However, when observing the final extraction values in Table 1, a significant raise in TS and IC₅₀ at 2.5 g/min could be detected; while no significant differences were found between TPC and TFC at any of the extraction solvent flows used.

Another variable evaluated was temperature (Fig. 2). This is the most important parameter to optimize in a subcritical extraction in the presence of water, because the physicochemical properties of the latter change with increasing temperature and this affects the extraction efficiency (Gilbert-López, Plaza, Mendiola, Ibáñez, & Herrero, 2017). As the temperature increases, the dielectric constant of water and its polarity decreases, favoring the solubilization of non-polar compounds such as polyphenols (Yan et al., 2020).

Polyphenols in plant matrices are usually linked to polysaccharides or proteins. As the temperature increases, these interactions are broken, leaving the polyphenols free, which facilitates their extraction. In addition, there is thermal damage to the cell walls which allows for better diffusion of the extracting solvent. Both effects produce an increase in the mass transfer coefficient, increasing the extraction of the compounds of interest (Gong, Zhang, He, Yan, Yuan & Gao, 2015).

The increase in extraction temperature resulted in a subsequent raise of TS, TPC and TFCs over 150% at 200 °C compared to lower extraction temperature (140 °C). However, specifically, TS content was higher at 180 °C, an increase in temperature above this value reduced the number of dissolved solids, since other co-extracted components of the matrix (amino acids, proteins, sugars and starch) are likely to be degraded, thus reducing their solubility in the extractive medium or causing gas loss during this degradation.

Most of the studies carried out in extraction with water in subcritical conditions highlight temperature as the key factor for increasing the extraction of antioxidant compounds, establishing between 200 and 220 °C as optimal temperatures, similar to the one found in this study. For instance, grape pomace by-products (Duba, Casazza, Mohamed, Perego, & Fiori, 2015), coriander seeds (Zeković et al., 2014), wild garlic (Tomšik et al., 2017) and *Phlomis umbrosa* Turcz (Ko, Lee, Nam, & Chung, 2017) were subjected to 200 °C; and pistachio nuts (Bodoira et al., 2019), sesame defatted seeds (Bodoira, Velez, Andreatta, Martínez, & Maestri, 2017). and peanut skins (Bodoira, Rossi, et al., 2017) were extracted at 220 °C. Other authors report extraction temperatures below 120 °C, to avoid degradation and polymerization of polyphenolic compounds and caramelization reactions that can occur at temperatures above 160 °C (Gong et al., 2015). As stated before, the optimal extraction temperature of bioactive compounds is highly dependent on the differences in the plant matrix and the extraction system.

In terms of extraction solvent composition, the addition of 25% ethanol generated the highest extraction of compounds TS, TPC and TFC, with markedly higher values compared to other compositions in the solvent mixture (Fig. 3). This indicates the presence of polar compounds and a fraction of compounds of lower polarity. However, phenolic compounds did not show the same trend. The addition of 25% ethanol produces a significant increase in the extraction of these compounds, marking a maximum for the studied conditions. Increasing the proportion of ethanol to 50% leads to a minimum recovery of these compounds, which then increases to a practically similar value for a solvent containing 75% ethanol and for pure ethanol.

The flavonoid extraction yield presents a great increase when adding 25% of ethanol, reaching a maximum in the studied conditions. When using 50%, 75% and pure ethanol, the extraction yield of these compounds presents similar values, always above the yield of extraction with water, indicating the presence of compounds of average polarity. The addition of ethanol, modifies the dielectric constant and polarity of

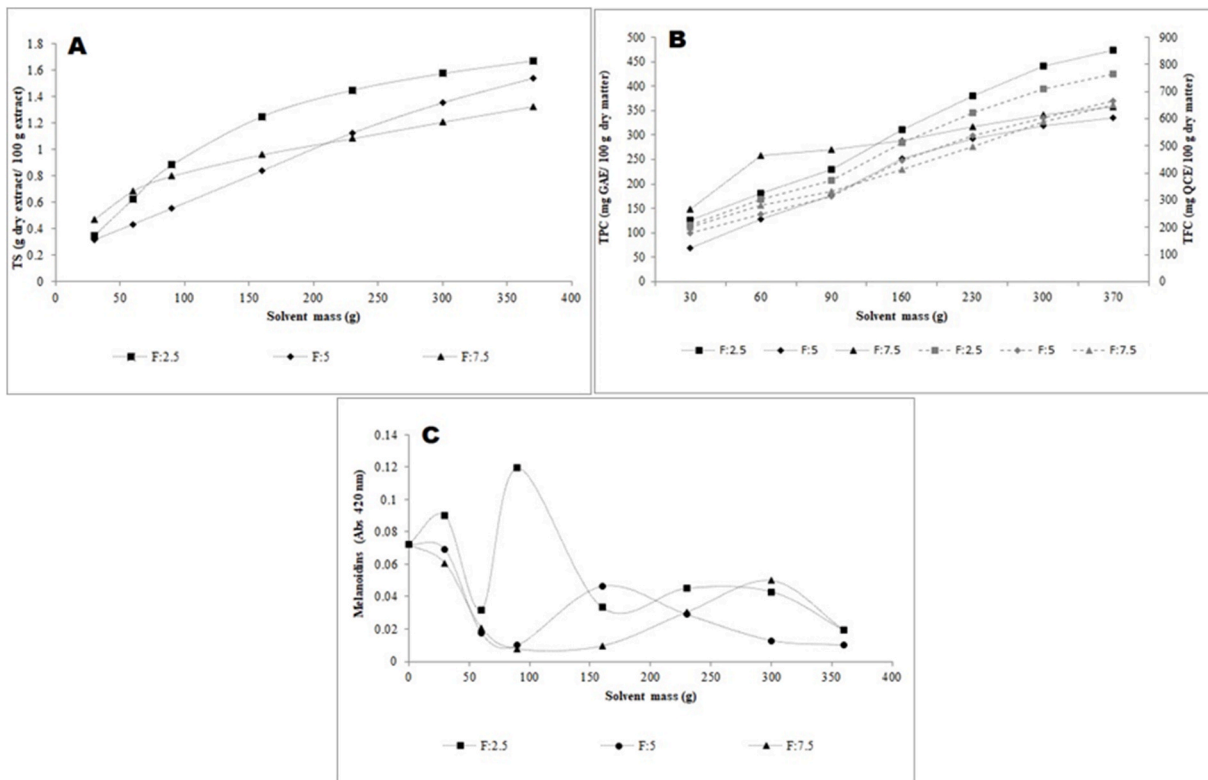


Fig. 1. Extraction kinetics with solvent flow variation. A) Total solids. B) Antioxidant compounds, black data: total phenolic compounds, grey data: total flavonoid compounds. C) Melanoidins.

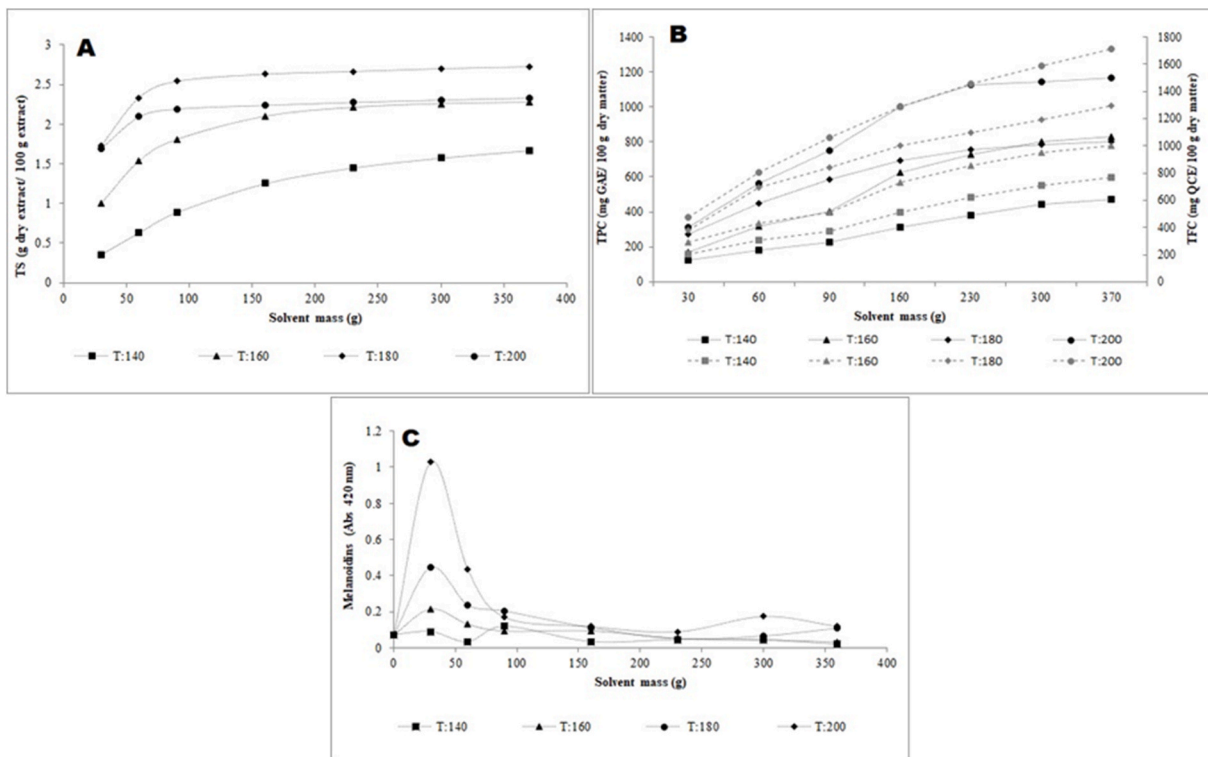


Fig. 2. Extraction kinetics with temperature variation. A) Total solids. B) Antioxidants compounds, black data: total phenolic compounds, grey data: total flavonoid compounds. C) Melanoidins.

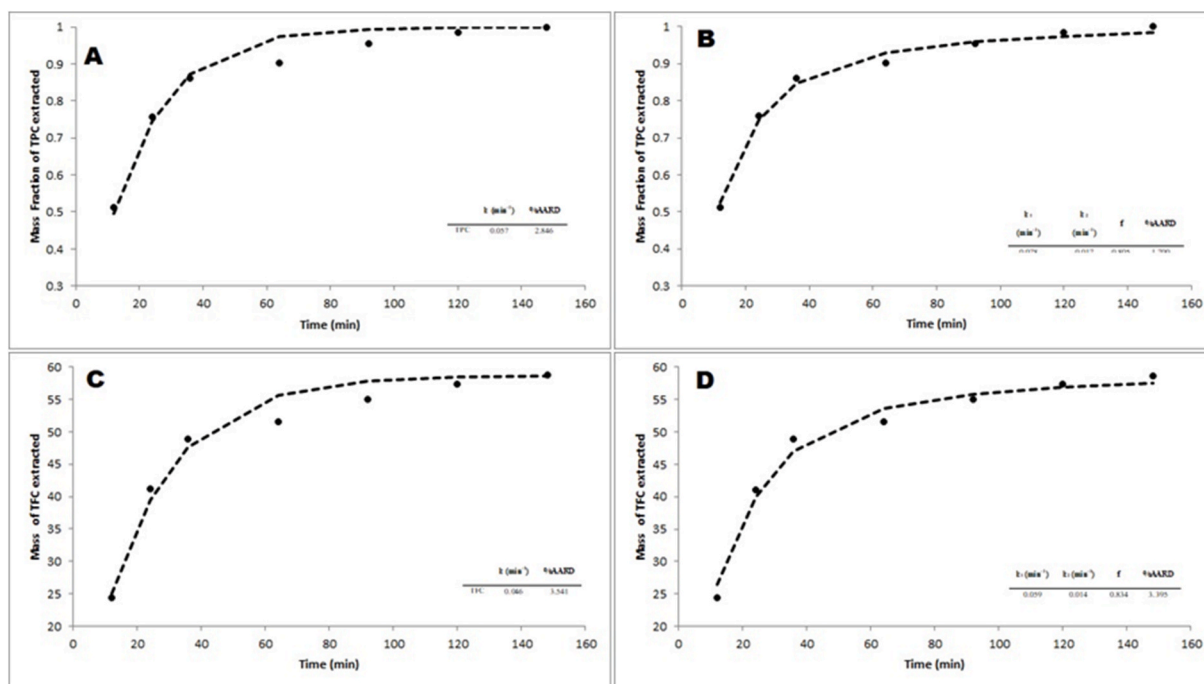


Fig. 3. Extraction kinetics with the variation of % alcohol in the extraction solvent. A) Total solids. B) Antioxidant compounds, black data: total phenolic compounds, grey data: total flavonoid compounds. C) Melanoidins.

the extraction solvent, which favors the extraction of a greater variety of biological compounds (Bodoira, Rossi, et al., 2017). As it is a complex matrix, it presents different antioxidants with varied physicochemical properties that in this case we grouped in two families (phenols and flavonoids), which is why they do not follow a trend of solubility in homogeneous working conditions and an integral behavior is described given the variation of the parameters under study.

The formation of brown pigments in the extracted solutions is an effective method to detect the occurrence of non-enzymatic browning reactions, including Maillard's and caramelization reactions (Plaza et al., 2010). In Figs. 1–3, the behavior observed during extractions under different conditions is presented.

As can be noted, the lower the mass flow rate, the longer the contact time between the sample and the solvent, thus favoring non-enzymatic browning reactions. As time passes, the absorbance units decrease because the concentration of all the extracted compounds is lower. However, a marked increase in brown coloring is observed after 40 min of extraction, probably during the first few minutes the cell matrix breaks down, proteins are denatured and amino acids and simple sugars are released, which then give rise to the non-enzymatic browning reactions.

As far as temperature is concerned, these reactions are favored at high temperatures (above 140 °C) because they are endothermic reactions (Hodge, 1953), so the higher the temperature the greater the formation of pigments, which in turn decrease in intensity by dilution of the pigments over time. Finally, the presence of 100% alcohol generates a higher initial intensity, probably due to the fact that water is a reaction product and its absence in the environment generates a displacement of the equilibrium towards the formation of products (Hodge, 1953). Also, alcohol at high temperature favors the dissolution of sugars and protein denaturation, which increases the availability of these products for Maillard's reactions to occur. In water, an increase in coloration is observed after 40 min of extraction, a behavior already described above.

Another aspect to highlight is that the formation of neo-antioxidants derived from Maillard's reactions and caramelization during extraction with subcritical water extraction has been proven (Gilbert-López et al., 2017; Plaza et al., 2010), which has an influence on the general

antioxidant activity of the natural extracts obtained.

According to the analysis carried out previously, it was found that the conditions of maximum general extraction of antioxidant compounds (without interaction between variables) correspond to F: 2.5 g/min, T: 200 °C and S: 25% w/w of ethanol. Under these conditions, it was possible to obtain an extract considered to be optimal.

A new extraction was carried out combining these conditions, obtaining significant increases in TPC, TFC and IC₅₀, although the phenolic compounds were those that presented the greatest impact. The extraction yield and TS were lower, indicating that the extract was further enriched in the compounds of interest (Table 1).

The extraction kinetics of TPC and TFC were favored at all times (Fig. 4), though the highest extraction rate occurred in the first 40 min approximately.

When comparing the values obtained when extracting under *conventional conditions* with those obtained by subcritical fluids, a very marked increase in the extraction capacity of phenolic compounds and flavonoids is observed, with the consequent increase in the antioxidant capacity of these extracts (Table 1). This behavior is observed since water in subcritical conditions reaches higher temperatures and lower polarity, facilitating the dissolution of the compounds of interest. In addition, water has lower viscosity and surface tension which increases the molecular diffusion speed (Yan et al., 2020).

Although there are no similar studies in the literature applying subcritical water extraction techniques in MQF, the optimization of the extraction of antioxidant compounds by means of ultrasound-assisted extraction has been studied, finding that the most influential variables are temperature and alcohol content in the extracting solvent. Carciochi (2014) reported that the best extraction yields were found at the highest working temperature (60 °C) and using 80% of alcohol. Another aspect to highlight is that the author also reports increases in TPC, TFC and the formation of non-enzymatic browning products during the thermal treatment of the MQF (between 100 and 190 °C) (Carciochi, 2014).

In order to evaluate the free radical scavenging capacity of the extract obtained under optimal conditions with assays based on the electron transfer (ET) and hydrogen atom transfer mechanisms (HAT), the DPPH, TEAC and FRAP (ET) and ORAC (HAT) assays were

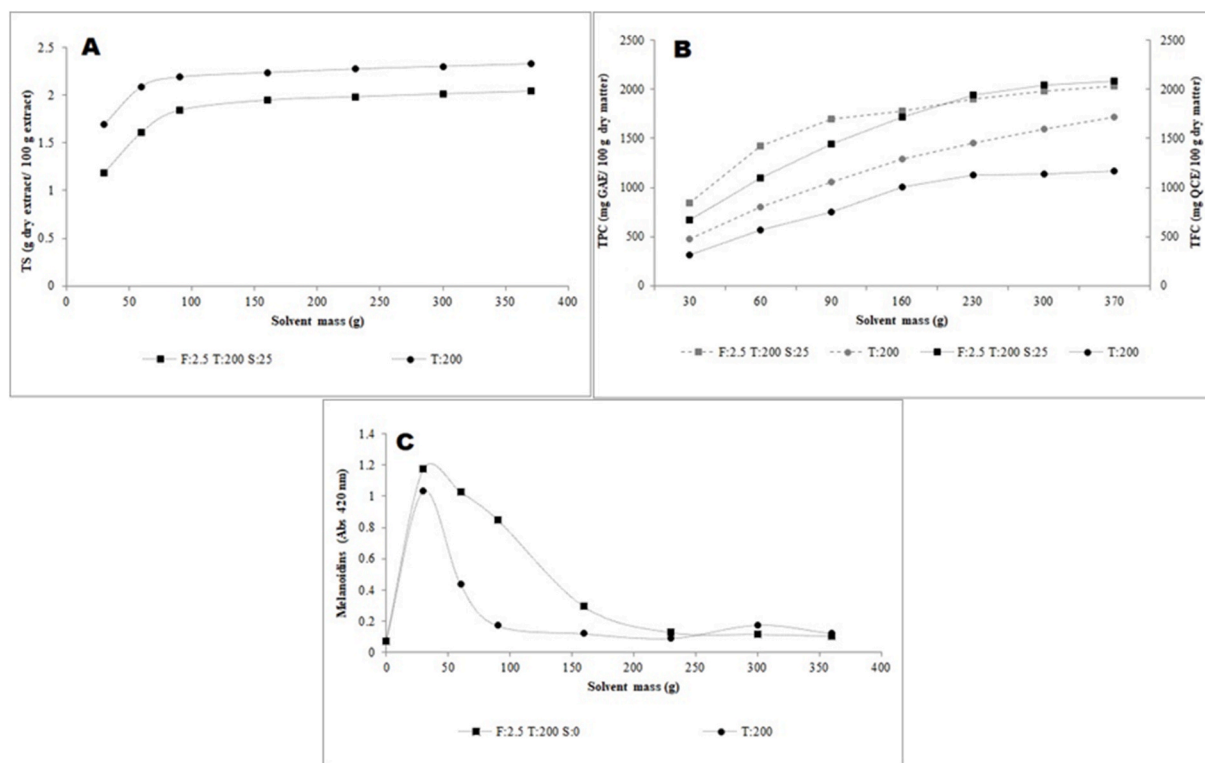


Fig. 4. Comparison of best extraction conditions of antioxidant compounds. A) Total solids. B) Antioxidant compounds, black data: total phenolic compounds, grey data: total flavonoid compounds. C) Melanoidins.

performed.

The extract presented a high scavenging capacity of DPPH[•] (IC₅₀) and ABTS^{•+} radicals (29.6 ± 0.4 µg/mL and 231.5 ± 0.2 µmol/g dry matter, respectively) compared to the values reported for different quinoa grain extracts. Nickel, Spanier, Botelho, Gularte, and Helbig (2016) described that quinoa grains samples subjected to various types of processing required from 2711 to 6178 µg/mL to inhibit 50% of DPPH radicals (IC₅₀). Regarding TEAC assay, Sobota, Świeca, Geśiński, Wirkijowska, and Bochnak (2020) reported ranges from 0.99 to 8.71 µmol TE/g dry matter, Escribano et al. (2017) between 6 and 47.4 µmol TE/g dry matter and Laus, Gagliardi, Soccio, Flagella, and Pastore (2012) ranges from 0.33 to 12.8 µmol TE/g dry matter. In turn, the extract displayed power to reduce ferric ions measured by the FRAP technique (39 ± 4 mg ascorbic acid eq./g dry extract). Other authors (Escribano et al., 2017; Nickel et al., 2016) reported high values of this activity but using different standards and expressing the results in different units of measurement, making numerical comparisons difficult. The ORAC protocol showed an antioxidant activity value (28.2 ± 0.9 mg Trolox eq./g dry extract) about 3-fold higher than that hydrophilic extract of quinoa grains reported by Laus et al. (2012). All of these differences found in the results of antioxidant assays with respect to literature may be due to improvements in the extraction method, as well as to changes produced during the germination process and the genotype of grain used.

The effect of the extract of germinated quinoa and BHT (synthetic antioxidant) enrichment on the oxidative stability of crude soybean oil was evaluated by Rancimat equipment. The induction time (h) was used as an indicator of the antioxidative potential of added antioxidants. The control sample displayed the lowest induction time (12.3 ± 0.5 h), followed by the quinoa extract and the BHT samples, with no significant differences (p > 0.05) between them (13.20 ± 0.03 h and 13.4 ± 0.2 h, respectively). This means that under the described working conditions, the quinoa extract was found to be effective in stabilizing soybean oil against oxidative deterioration, compared to a control sample. Atwaa

and El-Araby (2020) extracted antioxidant compounds from quinoa grains by a conventional method and reported an induction time of 12.7 h when adding the extract to fermented cream analogue (concentration of 200 ppm). The use of numerous assays based on different mechanisms, firmly support the bioactive potential of the germinated grain.

3.1. Kinetic model

After the extraction in optimal conditions, the cumulative values of TPC and TFC were considered as m_t (Table 1). As shown in Fig. 5, both models can predict the experimental data with good accuracy, which is quantified and compared using AARD (%). However, according to the kinetic parameters and goodness-of-fit reported in Fig. 5, the two-site kinetic model presents a better fit. This model includes a fast and a slow rate constant of the extracted fractions, named k_1 and k_2 , related to the partition coefficients and the characteristics of the matrix (effective diffusivity), respectively. The model is simple and provides useful information practical for scale up and designing subcritical water extraction processes (Duba et al., 2015).

The minor differences between the predictions of the two models can be attributed to the high estimated value of the fraction of easily desorbed solute ($f = 0.805$ and 0.834 , for TPC and TFC, respectively), indicating that the predominance of rapidly desorbed solute.

In previous studies, the two-site kinetic model is applied for the extraction of polyphenols from wine by-products, with good results. The model is simple and provides useful information for process design at the industrial level (Duba et al., 2015).

4. Conclusion

A significant effect on the extraction of the antioxidant compounds was observed when the working conditions varied; an improvement in the extracts was noted when lower mass flow rates of extraction solvent were used, which favored longer contact times between the solvent and

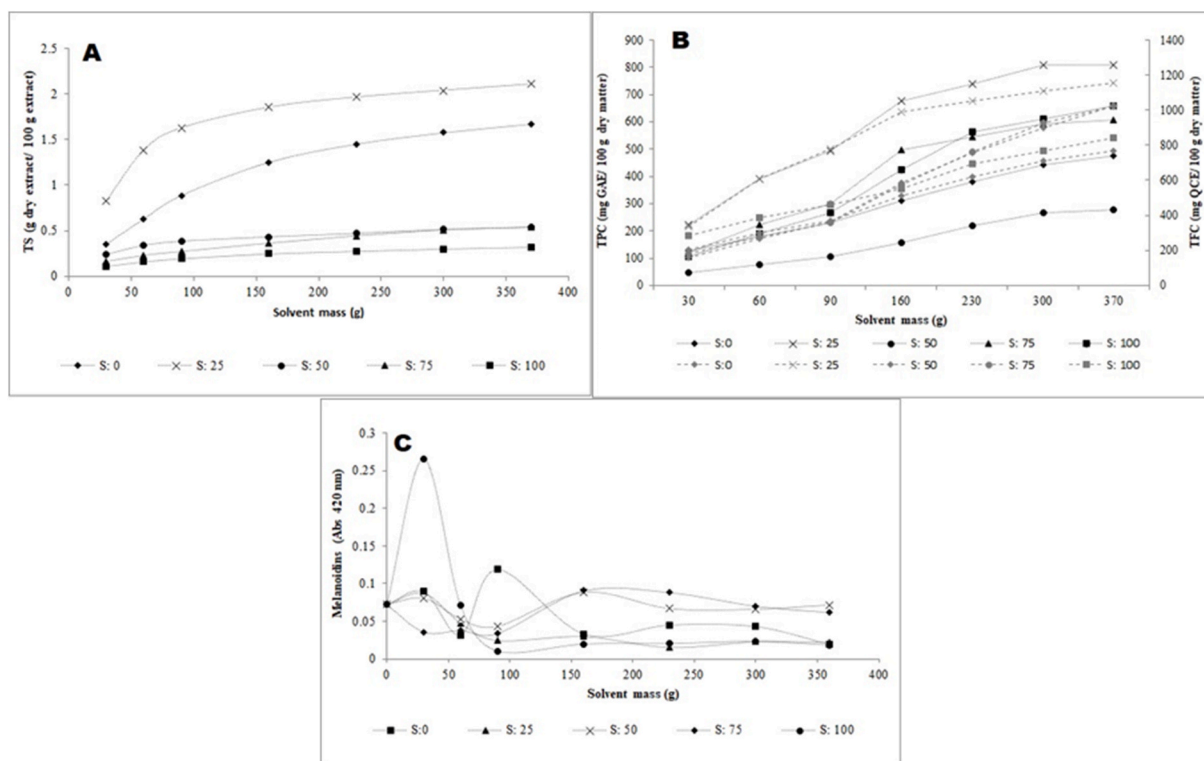


Fig. 5. Extraction yields as a function of time, curve predicted by the kinetic model.

Experimental values: points on the graph, values predicted by the kinetic model: dotted line. A- One-site model for phenols; B- Two-site model for phenols; C - One-site model for flavonoids; D- Two-site model for flavonoids.

the matrix to be extracted. The increase in temperature produced the greatest impact on the extraction and antioxidant activity of the extracts, not only because of the improvements in the diffusion of compounds in the matrix, but also because of the formation of new compounds with antioxidant activity derived from the non-enzymatic browning reactions (observed qualitatively by an increase in the brown color in the extracts). Finally, the content of alcohol as co-solvent is very dependent on the profile of antioxidant compounds in the matrix. In this case the best extracts were obtained with a 25% substitution, indicating a greater number of compounds of high and medium polarity. These data obtained at laboratory scale, could be used to extrapolate the process to industrial scale.

On the whole, the results of this study highlight an outstanding antioxidant potential of the subcritical fluid extraction of bioactive compounds from germinated quinoa grains, which ultimately encourages the use of these extracts for improving nutritive and health-beneficial properties of food, cosmetic and pharmaceutical products.

To better understand the dynamics of the subcritical water extraction technique, it would be necessary to perform more detailed characterizations of the extracts, to evaluate not only a general extraction behavior but also the performance of compounds of interest, to make a correlation with their chemical properties and to identify the newly formed antioxidant compounds.

CRedit authorship contribution statement

Jesica R. Mufari: Conceptualization, Formal analysis, Methodology, Writing - original draft. **Andrea C. Rodríguez-Ruiz:** Formal analysis, Methodology, Writing - original draft. **Antonella E. Bergesse:** Data curation, interpreted the results and wrote the manuscript. **Patricia P. Miranda-Villa:** designed the experiment, interpreted the results and wrote the manuscript. **Valeria Nepote:** Writing - review & editing. **Alexis R. Velez:** Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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