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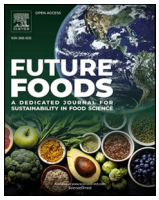

Exploiting vegetarian and vegan cheeses with *hibiscus* extract: Functional and nutritional perspectives

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ABSTRACT

An aqueous extract of *Hibiscus sabdariffa* L. was evaluated for dual functionality as a natural coagulant and a source of bioactive compounds in cheese and tofu production. Proteolytic activity and milk-clotting activity (MCA) assays, along with mini-curd preparation, demonstrated effective coagulation in both dairy and vegan-~~plant-based~~ matrices. Soy milk was prepared following standard procedures, and tofu was produced using the hibiscus aqueous extract as a coagulant. The chemical composition of the aqueous extract was characterized using UV–Vis spectroscopy, ATR–FTIR, and UHPLC—HRMS, revealing the presence of anthocyanins, flavonols, and phenolic acids. These bioactive compounds were partially incorporated into the cheese and tofu matrices, providing functional enrichment. Lipid profiles were analysed, showing beneficial modulation of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA), with an increase in MUFAs, maintenance of PUFAs, and improvement of lipid quality indices (AI, TI, and h/H ratio).

The study demonstrates that hibiscus aqueous extract effectively acts as a natural coagulant while enhancing the nutritional and functional properties of cheese and tofu. Incorporation of the extract enriched bioactive compounds and positively influenced lipid composition, supporting its potential as a multifunctional ingredient for the development of innovative, health-promoting foods.

1. Introduction

1.1. *Hibuscus sabdariffa L*

Hibiscus (*Hibiscus sabdariffa* L.) is a shrubby plant belonging to the Malvaceae family, widespread in tropical regions of Africa and appreciated for its hardiness and low water requirements (Ali et al., 2021). Among the numerous species, *H. sabdariffa* L. is the most relevant from a nutritional and technological perspective: its dried calyces and petals are used to prepare a deep-red beverage known as karkadè or roselle tea (Da Costa et al., 2014). This infusion, characterized by a slightly sour and refreshing taste, is naturally rich in bioactive compounds, including flavonoids, ascorbic acid, organic acids, anthocyanins, tannins, mucilages, and phytosterols, and is practically calorie-free (1 kcal/100 mL) (https://nutrientoptimiser.com/nutritional-value-beverages-tea-hibiscus-brewed/). Several studies have attributed to karkadè diuretic, digestive, hypotensive, hypolipidemic, and antioxidant properties (Mozaffari-Khosravi et al., 2009; McKay et al., 2010; Nguyen et al., 2019). Moreover, hibiscus flower extracts (dried calix) have shown antiproliferative and pro-apoptotic activity on breast and melanoma cancer cell lines, as well as a synergistic effect with conventional chemotherapeutic agents (Goldberg et al., 2017; Nguyen et al., 2019). These characteristics make hibiscus a natural source of bioactive molecules with potential interest for the development of functional foods. These latter are defined as foods that beneficially affect one or more target functions in the body, beyond providing adequate nutritional effects, in a way that contributes to improved health and well-being and/or reduces the risk of disease (Temple, 2022).

1.2. *Milk coagulation*

Milk coagulation, which underlies cheese production, is traditionally

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achieved through the use of animal rennet, an enzymatic complex mainly composed of chymosin (Caputo et al., 2022). However, in recent years, increasing attention has been devoted to the use of plant derived coagulants for ethical, religious, and sustainability reasons (Mohsin et al., 2024). Extracts from artichoke (*Cynara cardunculus*), fig (*Ficus carica*), pineapple (*Ananas comosus*), papaya (*Carica papaya*), and oyster mushroom (*Pleurotus ostreatus*) have demonstrated milk-clotting ability, while also modifying the lipid and aromatic profile of the resulting cheeses (Pacifico et al., 2024). At the same time proteolytic enzymes extracted from vegetal tissues such as cardoon flowers (*Cynara cardunculus*), artichoke (*Cynara scolymus*), and *Citrus aurantium* are capable of hydrolysing milk caseins across a broad range of pH and temperature conditions. These properties make them promising alternatives to animal rennet in cheese making (Nicosia et al., 2022).

Although the use of vegetable coagulants dates back to ancient times, their application declined after the nineteenth century following the industrial development of calf rennet. Interest in plant-based coagulants has since re-emerged due to the growing global demand for cheese and the limited availability of animal rennet. Most vegetable coagulants are applied as crude plant extracts containing multiple classes of proteases, which strongly influence their proteolytic and milk-clotting properties (Nicosia et al., 2022).

Research on vegetable coagulants has mainly focused on traditional cheeses, particularly those produced under artisanal conditions in Mediterranean regions. Notable examples include Warankashi, an unripened African cheese coagulated with *Calotropis procera* leaf extract (Husein et al., 2016), and several PDO- and PGI-certified Iberian cheeses manufactured using dried extracts of *Cynara cardunculus* flowers (Roseiro et al., 2003). These case studies clearly demonstrate that plant-derived proteases play a crucial role in casein hydrolysis, ripening kinetics, and the development of physicochemical and sensory properties, highlighting their technological relevance in both traditional and specialty cheese production.

1.3. Soy milk coagulation

The coagulation of soy milk in tofu production can be achieved using mineral salts ($CaSO_4$, $MgCl_2$) or natural organic acids such as glucono-δ-lactone and lemon juice, leading to products with high protein content, low fat levels, and favourable nutritional properties (Taha et al., 2024). A growing body of evidence points to the positive role of soy in the prevention and treatment of diseases such as cancer, cardiovascular disorders, kidney dysfunction, depressive symptoms, and skin damage. Soy is particularly rich in isoflavones, a class of flavonoids acting as phytoestrogens and estrogen receptor modulators, and the European Food Safety Authority has classified isoflavones as safe for human consumption (Messina, 2016).

This study aimed to develop innovative and sustainable functional foods naturally enriched with bioactive compounds, in response to the growing consumer demand for clean-label, health-promoting products capable of preventing chronic diseases and enhancing antioxidant and anti-inflammatory potential. The functional foods market continues to expand rapidly, with an estimated value of approximately $280 billion in 2021, projected to reach $340 billion in 2024, and expected to approach $580 billion by 2030 (https://www.grandviewresearch.com/industry-analysis/functional-food-market).

In this context, building on previously experience in the development of novel vegetable rennet s characterized by acidic and/or enzymatic coagulant activity (Pacifico et al., 2024), the aqueous extract of *H. sabdariffa* L. represents a promising candidate. This extract is characterized by a very low pH and a high concentration of phenolic compounds, anthocyanins, and several other bioactive molecules (Table 1), making it particularly attractive both as a natural coagulant and as a functional enrichment agent for dairy and soy-based products.

To evaluate its potential, an aqueous *H. sabdariffa* L. extract was prepared and characterized by ATR-FTIR and UV–Vis spectroscopy, along with UHPLC—HRMS/MS and HPLC-DAD analyses for the identification and quantification of its bioactive compounds. The extract was subsequently applied in the experimental production of cheese and tofu to assess its coagulating ability, the transfer of bioactive compounds to the final products, and its potential influence on their fatty acid profile.

2. Materials and methods

2.1. Chemicals and raw materials

All reagents were purchased from Merck–Sigma-Aldrich (Darmstadt, Germany). Calf rennet powder (175 International Milk Clotting Units, IMCU/mL) was supplied by Caglificio Clerici (Como, Italy). Pasteurized whole milk (3 % fat) and soy (Canadian origin) were purchased from a local market. Citric acid is organic and gluten free Spanish certified, and dried hibiscus calix were from Egypt, Austria Bio certified (AT BIO 301) (Figure S1).

Table 1
TOF-MS and TOF-MS/MS data of compounds tentatively identified in *H. sabdariffa* water extract.

n.	RT (min)	Tentative assignment	Formula	$[M-H]^-$ (*m/z*)	$[M-2H]^-$ (*m/z*)	$[M + H_2O-2H]^-$ (*m/z*)	RDB*	Error (ppm)
1	1.281	Dihydroxybenzoic acid hexoside	$C_{13}H_{16}O_9$	315.0737			6	4.9
2	2.492	3-CQA	$C_{16}H_{18}O_9$	353.0891			8	3.7
3	4.658	4-CQA	$C_{16}H_{18}O_9$	353.0895			8	4.8
4	4.922	Delphinidin 3-*O*-pentosylhexoside (e.g.., sambubioside)	$C_{26}H_{29}O_{16}^+$		595.1325	613.1429	n.d.	n.d.
5	4.987	5-CQA	$C_{16}H_{18}O_9$	353.0883			8	1.4
6	5.124	Cyanidin 3-*O*-pentosylhexoside (e.g.., sambubioside)	$C_{26}H_{29}O_{15}^+$		579.1378	597.1480	n.d.	n.d.
7	5.379	CSA	$C_{16}H_{16}O_8$	335.0775			9	0.8
8	5.538	Ethyl chlorogenate 1	$C_{18}H_{22}O_9$	381.1197			8	1.6
9	5.604	6-Hydroxydelphinidin hexoside	$C_{21}H_{21}O_{13}^+$		479.0850	497.0745	n.d.	n.d.
10	5.760	Quercetin 3-*O*-pentosylhexoside	$C_{26}H_{28}O_{16}$	595.1318			13	2.3
11	5.812	Ethyl chlorogenate 2	$C_{18}H_{22}O_9$	381.1197			8	1.6
12	5.885	Rutin	$C_{27}H_{30}O_{16}$	609.1483			13	3.6
13	5.959	Quercetin 3-*O*-hexoside	$C_{21}H_{20}O_{12}$	463.0904			12	4.8
14	6.041	Ethyl chlorogenate 3	$C_{18}H_{22}O_9$	381.1190			8	−0.3
15	6.330	6-Hydroxydelphinidin	$C_{15}H_{11}O_8^+$		317.0315		n.d.	n.d.
16	6.409	*N*-feruloyltyramine 1	$C_{18}H_{19}NO_4$	312.1247			10	1.8
17	6.699	*N*-feruloyltyramine 2	$C_{18}H_{19}NO_4$	312.1248			10	2.1
18	6.768	Quercetin *p*-coumaroyl-hexoside	$C_{30}H_{26}O_{14}$	609.1265			18	2.5
19	6.871	Delphinidin	$C_{15}H_{11}O_7^+$		301.0364		n.d.	n.d.
20	7.390	Cyanidin	$C_{15}H_{11}O_6^+$		285.0414		n.d.	n.d.

* RDB = Ring Double Bonds.

2.1.1. Chemical characterization of caw milk, soy milk, hibiscus extract and citric acid solution

All material used to produce vegetarian, vegan and control cheese were analyzed for their chemical properties. In particular, pH, titratable acidity, electrical conductivity and total dissolved solids (TDS, expressed in ppm) were measured. Conductivity and TDS were determined using a portable TDS & EC Meter (Bestgle, China) instrument. Titratable acidity was measured by pH-meter (Basic 20, Crison, Spain) and determined by titration with 1 N NaOH.

2.2. Preparation and chemical characterization of the aqueous hibiscus sabdariffa extract

Dried *H. sabdariffa* L. flower calyces from certified organic cultivation (see Figure S1) were used. The aqueous extract was prepared according to Pacifico et al. (2024). Briefly, 10 g of dried flowers were incubated in 30 mL of tap water under stirring at room temperature for 6 h (Fig. 1). The mixture was centrifuged (9800 × *g*, 10 min, 4 °C), and the supernatant was collected and freshly used for enzymatic assays, mini-curd preparation, and tofu production. The total amount of compounds present in the extract was determined after drying the sample using a Speed-Vac apparatus (Savant, USA).

2.2.1. Spectroscopic characterization

Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR–FTIR) was employed to identify characteristic functional groups. Spectra were acquired using an IRXross spectrometer (Shimadzu, Tokyo, Japan) in the range of 4000 - 500 cm^{-1}, with a resolution of 4 cm^{-1} and 45 scans per sample. Data processing was performed using LabSolution IR (v.1.60). UV–Vis absorption spectra were recorded in the 200–800 nm range using a Cary 100 UV–Vis spectrophotometer (Agilent, Milano, Italy).

2.2.2. UHPLC–HRMS/MS metabolic profiling

The metabolic composition of the prepared flower extract was assessed through untargeted ultra-high-performance liquid chromatography coupled to high-resolution tandem mass spectrometry (UHPLC–HRMS/MS). Analyses were carried out using a NEXERA UHPLC system (Shimadzu, Kyoto, Japan) fitted with a Luna® Omega C18 column (50 × 2.1 mm, 1.6 μm). Separation was achieved using a binary solvent system composed of water (0.1 % formic acid, solvent A) and acetonitrile (0.1 % formic acid, solvent B). The gradient program started at 2 % B (0–1.5 min), increased to 5 % B at 3 min, to 17.5 % B at 8 min, then to 45 % B at 11 min, and finally reached 95 % B at 12 min, held for 1 min before returning to initial conditions (total runtime = 24 min). The flow rate was fixed at 0.5 mL min^{-1}, with 2 μL injections. Mass detection was performed on a hybrid quadrupole–time-of-flight (QqToF) mass spectrometer operated in negative electrospray ionization mode. Data were acquired in full-scan TOF-MS (200–1500 Da, 250 ms) and data-dependent MS/MS (IDA, 100–1000 Da, 100 ms) modes, applying a collision energy of 35 ± 5 V. Source conditions were: interface temperature 600 °C, curtain gas 35 psi, GS1 and GS2 at 60 psi, and capillary voltage equal to - 4500 V. Automatic calibration was performed before each run.

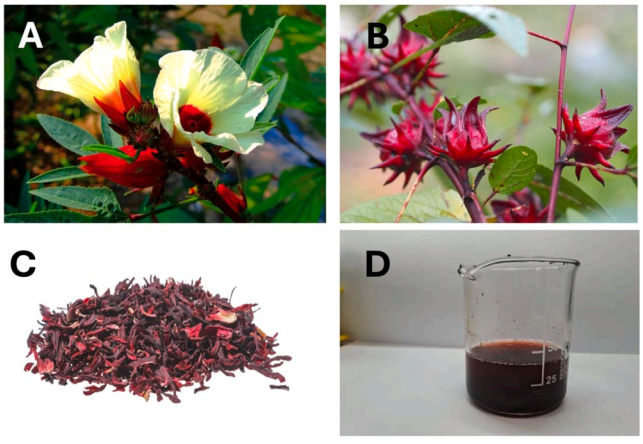

Fig. 1. *Hibiscus sabdariffa* L.; A) plant with flowers; B) calyces of hibiscus flower; C) dried flower calyces; D) the prepared aqueous hibiscus extract.

2.2.3. HPLC-UV-TQMS quantitative analysis

Quantitative analysis of the extract was carried out using a 1260 Infinity II system (Agilent Technologies, Santa Clara, CA, USA) equipped with a G7129A autosampler, a G7111A quaternary pump, and a G7115A UV–Vis/DAD detector set at 280 ± 8 nm, 325 ± 8 nm, and 525 ± 8 nm. The HPLC system was coupled to an AB Sciex TQ3500 triple quadrupole mass spectrometer operating in Multiple Reaction Monitoring (MRM) data acquisition mode. Chromatographic separation was achieved on a Kinetex® Evo C18 column (100 × 3.0 mm, 5 μm; Phenomenex, Torrance, CA, USA) using a binary mobile phase consisting of water and acetonitrile, both acidified with 1 % formic acid. The flow rate was maintained at 0.5 mL min^{-1}, and the column oven temperature was set at 40 °C. Quantification of the identified metabolite was performed using three external standards, representative of the main compound classes detected (i.e., chlorogenic acid for simple phenols, rutin for glycosylated flavonols, and kouromanin for anthocyanins/anthocyanidins). Results were expressed as mg equivalents of the corresponding reference standard *per* g of extract, based on calibration curves, constructed over the concentration range 0.1–10 ppm. The method showed a limit of detection (LOD) of 0.01 ppm and a coefficient of determination (R^2) ≥ 0.99. MRM transitions, derived from MS/MS spectra, are reported in Table S1. Data processing was performed using Sciex OS software.

2.3. Antioxidant activity: ABTS assay

Antioxidant activity of hibiscus extract, serum and final products, was evaluated by using the ABTS assay, as reported by van der Berg et al. (1999). Briefly, was prepared an 2,2′-azino-bis (3-ethylbenzothiazolino-6-sulfonic acid (ABTS) solution 0.5 mM, added with $K_2S_2O_8$, to obtain the free radical $ABTS^{\bullet+}$ of green/blue colour solution. By adding the sample and monitoring the decrease of absorbance at 734 nm, it is possible to establish the antioxidant capability of the sample. 50 μL) of hibiscus extract or water (blank) were added in a mix of 1 mL of ABTS- $K_2S_2O_8$ solution, and the As_{734} was measured at time t_0, and after 30 min (t_{30}). The antioxidant activity was expressed as percentage of inhibition and calculated according to the following formula: % inhibition = (1-As_{734}extract t_{1o}/As_{734} blank t_{30}) × 100 (van der Berg et al., 1999).

To determine whether antioxidant activity was detectable in whey and in the final products, aliquots of whey obtained from cheese and tofu production were collected and tested immediately. For each assay, 50 μl of whey were used. Freshly prepared cheese or tofu (5 g) as well as ripened cheese (see Section 2.7) were homogenized in 10 ml of distilled water. The mixtures were centrifuged (5 min, 12,000 rpm, 4 °C) and the supernatants were collected and analyzed for antioxidant activity; 100 μl of supernatant were used for each assay. All assays were performed in duplicate on two independent preparations.

2.4. Esterase activity

Esterase activity was spectrophotometrically determined as described by Pacifico et al. (2024). Assays were carried out in duplicate, and the results are expressed as the mean of two independent experiments. Enzymatic activity (U/ml) was defined as the amount of protein releasing 1 μmol of *p*-nitrophenoxide per min from *p*-NP-hexanoate.

2.5. Proteolytic activity

Proteolytic activity was assessed using bovine serum albumin (BSA) as substrate, following the procedure reported by Pacifico et al. (2024). In brief, 15 µl of 10 mM phosphate buffer (pH 7.0) containing 5 µg of BSA were incubated with 5 µl of hibiscus extract or 2 µl of calf rennet solution at 37 °C for 10 min. The reaction was stopped by adding 10 µl of O'Farrell solution. Samples were separated on 12.5 % SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. The remaining undigested BSA fraction was quantified using GelQuantNET software (Biochemlabsolutions, http://www.biochemlabsolutions.com/GelQuantNET.html). Analyses were performed in duplicate.

2.6. Milk-Clotting activity (MCA) assay

The MCA was determined as reported by Pacifico et al. (2024). Pasteurized whole milk (10 ml, 3 % fat) was adjusted to pH 6.0 with citric acid (0.5 M, pH 2.0). The sample was pre-incubated at 35 °C for 10 min, and 0.5 ml of hibiscus extract was added. Calf rennet served as the control. One Standard Unit (SU) of activity was defined as the amount of enzyme required to clot 1 ml of milk in 40 min. The MCA (SU/ml) was calculated as:

$$SU = \frac{2,400 \times 10 \times D}{0.5 \times T}$$

where D is the dilution factor and T is the clotting time (s).

2.7. Mini-curd preparation

Following optimization of the coagulant dose to maximize cheese yield, 50 ml of hibiscus extract were added to 500 ml of whole milk to produce experimental curds, according to the standard semi-hard cheese-making procedure described by Pacifico et al. (2024). Control cheese was obtained using 1 mL of diluted calf rennet solution (1 mg/ml in 20 mM sodium phosphate buffer, pH 5.5; 860 IMCU/g). As an acidic coagulant control, cheese was also prepared by adding 25 ml of 0.5 M citric acid (pH 2.2). Curdling tests were performed in duplicate. The resulting curds were molded and pressed for 20 h to promote whey expulsion, then brined in 20 % (w/v) NaCl for 1 h at 25 °C. After 2 h of draining, the cheeses were vacuum-sealed and stored at 4 °C for 30 days before analysis. All preparations were carried out in three independent experiments and the results were averaged.

2.7.1. Cheese melting point determination

The melting point of the cheeses was determined using an SMP10 Melting Point Apparatus (STUART, UK). Briefly, samples of control cheese or hibiscus-derived cheese were placed into capillary tubes and inserted into the apparatus. The analysis was performed using a starting plateau temperature of 25 °C and a final plateau temperature of 70 °C, with a heating rate of 2 °C/min. The melting temperature was monitored by visual observation of the samples within the block chamber. The measured values were consistent with those reported for semi-hard cheeses, typically ranging between 40 and 60 °C (Schenkel et al., 2013).

2.8. Soy milk preparation

Soy milk was prepared according to the method reported by Li et al. (2019) with slight modifications. Dried soybeans (100 g) were soaked in water overnight at room temperature, drained, and homogenized with 200 ml of tap water. An additional 800 ml of water was added (10:1 water/soybean ratio), and the mixture was boiled for 10 min. The resulting slurry was filtered and pressed to obtain approximately 900 ml of soy milk, which was freshly used for tofu production.

2.9. Tofu preparation

Tofu was prepared as described by Li et al. (2019). Fresh soy milk (500 ml) was heated to 80 °C, then mixed with 3 g of nigari ($MgCl_2$) dissolved in 10 mL of water. The mixture was stirred and incubated for 20 min at 80 °C to promote coagulation. The curd was transferred into a mold and pressed overnight (O/N) under a static weight of 800 g to remove whey. Hibiscus-derived tofu was prepared using the amount of extract defined by the optimization analysis, for 500 ml of soy milk were used 50 ml of extract. Experimental (hibiscus-derived) and control tofu samples were vacuum-sealed and stored at 4 °C until analysis within two days.

2.10. Global composition of cheese and tofu samples

Experimental (hibiscus-derived) and control cheeses, as well as experimental and control tofu samples, were analysed in triplicate for pH, moisture, fat, and protein contents, according to the procedures described by Pacifico et al. (2024). In addition, stereomicroscopic images of all samples were acquired using a Zeiss Stemi 2000-C Stereo Microscope (Carl Zeiss, Thornwood, NY, USA) at three different magnification levels to assess surface morphology and microstructural features.

2.11. Analysis of bioactive metabolites in cheese and tofu

An aliquot (1.0 g) of each cheese and tofu sample was homogenized with 20 ml of 2-propanol (1:20, *w:v*). After vortexing for 3 min, samples were sonicated at 40 kHz for 30 min in an ultrasonic bath (Ultrasonics™ Bransonic™ M3800-E, Danbury, CT, USA). The suspensions were then centrifuged at 4800 rpm for 4 min (Beckman GS-15R, Beckman Coulter, Milano, Italy), and the supernatants collected. The entire extraction procedure was repeated three times, and the pooled extracts were subjected to subsequent analyses. The crude extracts were first characterized by ATR-FTIR and UV–Vis spectroscopy, as previously described in Section 2.2, to evaluate global compositional changes and the possible transfer of hibiscus-derived chromophores and functional groups into the matrices.

Deconvolution analysis was carried out by fitting Gaussian functions to resolve overlapping peaks, enabling the semi-quantitative evaluation of the main spectral bands and their relative contributions. For this purpose, the Multiple Peak Fit tool implemented in OriginPro 2015 software (OriginLab Corp., Northampton, MA, USA) was used.

Following spectroscopic assessment, the extracts underwent liquid–liquid separation to isolate the lipid and polyphenolic fractions. The nonpolar phase (*n*-hexane) was used for free fatty acid (FFA) determination, while the polar (hydroalcoholic) phase was dedicated to the analysis of polyphenols and anthocyanins (Pacifico et al., 2024). Both fractions were evaporated to dryness under vacuum (Heidolph Hei-VAP Advantage, Schwabach, Germany), reconstituted in LC-MS-grade solvents, and filtered through 0.2 µm RC membrane syringe filters (Millex, Phenomenex, Torrance, CA, USA) before UHPLC–HRMS/MS analysis.

2.11.1. UHPLC—HRMS and HPLC-UV-TQMS analysis of polyphenols and anthocyanins

The polar fractions were analyzed by UHPLC—HRMS/MS and HPLC-UV-TQMS under the same instrumental conditions described for the *Hibiscus sabdariffa* extract (see Section 2.2). Identification and quantification were carried out by comparison with reference standards and accurate mass data.

2.11.2. UHPLC—HRMS analysis of free fatty acids

FFA analyses were performed using a NEXERA UHPLC system (Shimadzu, Tokyo, Japan) coupled to an AB SCIEX TripleTOF® 4600 mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a DuoSpray™ ion source operating in negative electrospray ionization (ESI)

mode. Chromatographic separation was achieved on a Luna® Omega C18 column (50 × 2.1 mm, 1.6 μm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1 % formic acid.

A linear gradient was applied as follows: 55 % B (0–0.5 min), 55–90 % B (0.5–7 min), held for 1 min, and then re-equilibrated to initial conditions (total runtime 10 min). The flow rate was 0.5 ml min^{-1}, with 2 μL injections.

Mass spectra were acquired in full-scan TOF-MS (100–600 Da, 250 ms) and data-dependent MS/MS (80–500 Da, 100 ms) modes, with the following source parameters: curtain gas 35 psi, GS1 and GS2 60 psi, capillary voltage −4.5 kV, interface temperature 600 °C, declustering potential 60 V, and collision energy 45 ± 15 V. Quantification was based on calibration curves prepared using oleic, linoleic, and palmitic acids as standards for MUFAs, PUFAs, and SFAs, respectively. Results were expressed as mean weight percentage of total FFAs ± SD. Lipid quality indices were calculated according to Ulbricht and Southgate (1991), Osmari et al. (2011), Medeiros et al. (2014), and Ivanova and Hadzhinikolova (2015), using the following formulas:

1) Atherogenicity Index: AI = (C12:0 + (4 × C14:0) + C16:0)/(Σω−3 PUFA + Σω−6 PUFA + Σ MUFA)
2) Thrombogenicity Index: TI = (C14:0 + C16:0 + C18:0)/((0.5 × C18:1) + (0.5 × other MUFA) + (0.5 × Σω−6 PUFA) + (3 × Σω−3 PUFA) + Σω−3 PUFA/Σω−6 PUFA)
3) hypocholesterolemic/Hypercholesterolemic fatty acids ratio: h/*H* = (C18:1 ω-9 + C18:2 ω-6 + C18:3 ω-3 + C20:4 ω-6 + C20:5 ω-3 + C22:5 ω-3 + C22:6 ω−3)/(C14:0 + C16:0)

3. Results

With the aim of developing new strategies for the production of vegetarian and vegan cheeses, our attention focused on the aqueous extract of hibiscus calyces as it exhibited a low pH and distinct protease activity (Fig. 3). In agreement with previous studies on vegetable rennets (Pacifico et al., 2024), the hibiscus extract could potentially be used to coagulate cow and soy milk proteins. For this reason, two preliminary analyses were conducted to characterize its main bioactive compounds and to evaluate its antioxidant capacity, measured as ABTS radical scavenging activity. In addition, key chemical parameters-pH, titratable acidity, electrical conductivity, and total dissolved solids (ppm)) were determined for cow milk, soy milk, hibiscus extract, and citric acid solution (see Table S1). The cheese making performance of the hibiscus extract was evaluated and compared with that of calf rennet and citric acid coagulation. Tofu preparation by hibiscus extract was also compared with nigari and citric acid. Spectroscopic (UV–Vis and ATR-FTIR) and chromatographic (UHPLC—HRMS/MS and HPLC-DAD) analyses were performed to characterize the anthocyanins, phenolic acids, and other metabolites present in the extract. Identifying these compounds was crucial, since another objective of this study was to verify whether some of these bioactive molecules were preserved in the final products, thus determining whether the resulting cheese and tofu could be considered functional foods produced through alternative processing methods. .

3.1. Chemical composition of hibiscus aqueous extract

To gain insights into the anthocyanin composition and associated phenolic profile of the hibiscus extracts, UV–Vis and ATR-FTIR spectroscopic analyses were performed (Fig. 2).

In the UV region, the aqueous hibiscus extract exhibited a prominent absorption band centered at 283 nm, corresponding to π→π* electronic transitions typical of aromatic systems and phenolic compounds such as flavonoids and conjugated organic acids (Aleixsandre-Tudo et al., 2019). This band also reflects the presence of aromatic rings and extended π-conjugation, characteristic of anthocyanins and related polyphenols. A

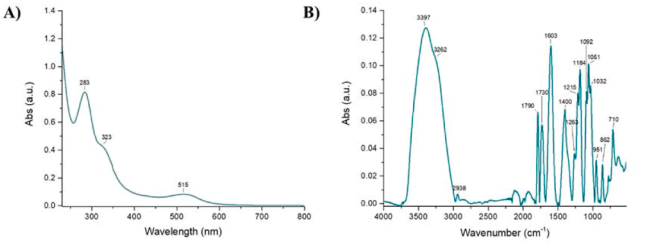

Fig. 2. (A) UV–Vis absorption spectrum and (B) ATR-FTIR spectrum of the *Hibiscus* extract.

secondary absorption band appeared at approximately 323 nm, which is commonly attributed to n→π* transitions of carbonyl and enolic groups present in flavonols and phenolic acids. In the visible region, the extract displayed a distinct absorption maximum at 515 nm, consistent with the characteristic visible absorption of anthocyanin chromophores (Dong et al., 2024), which are responsible for the red coloration of the hibiscus extract. The ATR FT-IR spectra corroborated the UV–Vis findings, further supporting the predominance of anthocyanins and phenolic compounds. A broad and intense band centered at 3397 cm^{-1}, with a shoulder at 3262 cm^{-1}, corresponded to O–H stretching vibrations associated with hydroxyl groups from polyphenols and sugars involved in hydrogen bonding. The weak band at 2938 cm^{-1} was assigned to aliphatic C–H stretching vibrations of methyl and methylene groups. Strong absorptions at 1790 and 1730 cm^{-1} indicated the presence of carbonyl (*C═O*) groups from esters and carboxylic acids, consistent with hydroxycinnamic acids and acylated flavonoids.

The prominent band at 1603 cm^{-1} corresponded to aromatic *C═C* stretching and conjugated *C═O* vibrations, typical of phenolic aromatic rings. The region between 1400 and 1200 cm^{-1} (1400, 1263, 1215, 1184 cm^{-1}) comprised O–H bending and C–O stretching modes of phenols, alcohols, and organic acids. The bands at 1092, 1061, and 1032 cm^{-1} can be attributed to C–O–C and C–O stretching vibrations from glycosidic linkages, whereas the bands at 951 and 862 cm^{-1} corresponded to out-of-plane bending of aromatic C–H bonds. Finally, the signal at 710 cm^{-1} was associated with out-of-plane aromatic ring deformations, confirming the presence of conjugated aromatic structures (Bhushan et al., 2023).

Chromatographic profiling coupled with high-resolution mass spectrometry (UHPLC-ESI-QqTOF-MS/MS) allowed the tentative identification of metabolites in HsF_W extract. Consistent with the UV–Vis and ATR FT-IR analyses, the DAD chromatograms recorded at 280, 325, and 525 nm (Figure S2) confirmed the co-occurrence of phenolic acids and their derivatives (280 nm), flavonols and conjugated hydroxycinnamic acids (325 nm), and anthocyanins (525 nm). Although only two anthocyanins were clearly detectable in the DAD chromatograms at 525 nm, the subsequent UHPLC—HR-MS analysis allowed the identification of six distinct anthocyanin compounds, demonstrating the higher sensitivity and selectivity of mass spectrometry for detailed metabolite profiling. Indeed, the TOF-MS and TOF-MS/MS data revealed a complex phenolic fingerprint dominated by hydroxycinnamic acids, quercetin derivatives, and anthocyanins (Figures S3-S5). Hydroxycinnamic acid derivatives included three monocaffeoylquinic acids (**2, 3** and **5**) and a monoshikimoylquinic acid (**7**), together with three ethyl chlorogenate isomers (**8, 11**, and **14**), reflecting an abundance of conjugated organic acids and esters. Monoshikimoylquinic acid (**7**) displayed a $[M-H]^-$ ion at *m/z* 335.0761, which generated the caffeate ion at *m/z* 179.0348 and the fragment at *m/z* 161.0244. These compounds are consistent with chlorogenic and caffeoylshikimic acids previously reported in hibiscus extracts (Mok et al., 2022; Kartinah et al., 2024). The additional ethyl chlorogenate isomers (*m/z* 381.12) exhibited a fragment at *m/z* 161.02, indicative of ethyl-ester cleavage. The occurrence of ethyl 3-, 4-, and 5-caffeoylquinates in *H. sabdariffa* L. has been previously documented (Borrás-Linares et al., 2015), confirming the diversity of naturally occurring hydroxycinnamate esters in roselle flowers. Among the

quercetin derivatives, quercetin 3-*O*-pentosylhexoside (**10**), rutin (**12**), isoquercitrin (**13**), and quercetin *p*-coumaroyl hexoside (**18**) were detected, indicating a wide diversity of glycosylation and acylation patterns that affect polarity and UV response. The $[M-H]^-$ ion at *m/z* 595.1329, attributed to quercetin 3-*O*-pentosylhexoside (**10**; likely quercetin 3-*O*-sambubioside; dos Santos Nascimento et al., 2021), yielded TOF-MS/MS fragments corresponding to the quercetin aglycone at *m/z* 301.0359 and its radical ion at *m/z* 300.0274. The deprotonated molecular ions of rutin (**12**) and quercetin 3-*O*-glucoside (**13**) were detected at *m/z* 609.1482 and 463.0901, respectively, while the neutral loss of 162.03 Da from the deprotonated ion at *m/z* 609.1273 of compound **18** allowed the identification of quercetin 3-*O*-(6″-*p*-coumaroyl) glucoside. Two main anthocyanins were tentatively identified as delphinidin 3-*O*-sambubioside (**4**; $[M-H]^-$ 595.1325) and cyanidin 3-*O*-sambubioside (**6**; $[M-H]^-$ 579.1378), respectively. Both the compounds were detected as $[M-2H]^-$ and $[M-2H+H_2O]^-$ ions, whose formation and fragmentation patterns were consistent with those described in the literature (Sun et al., 2012) and were previously reported in *H. sabdariffa* L. (Majdoub et al., 2019; Márquez-Rodríguez et al., 2021). Furthermore, the ion at *m/z* 479.0850 ($[M-H]^-$) was assigned to 6-hydroxydelphinidin hexoside (**9**). TOF-MS/MS analysis showed a neutral loss of 162.05 Da (hexose), producing the aglycone at *m/z* 317.0305, which was also detected as a constituent of the extract (compound **15**). In particular, the deprotonated molecular ion of the aglycone underwent dehydration (*m/z* 299.0179), loss of CO (*m/z* 271.0238) and ring-cleavage fragmentations yielding diagnostic ions at *m/z* 227.0362, 178.9983, 151.0039 and 137.0244 (Figure S4). Delphinidin (**19**) and cyanidin (**20**) aglycones were further identified. In addition, a hydroxybenzoic acid hexoside (**1**) and two *N*-feruloyl tyramine isomers were identified (**16** and **17**; Figure S5). *N*-feruloyl tyramine has been reported as one of the most abundant phenolic markers in white-calyx varieties of *H. sabdariffa* L. (Salinas-Moreno et al., 2023).

3.2. Esterase and protease activities

To establish reference values for calf rennet, the procedure described by Pacifico et al. (2024) was followed. Protease activity was assessed using bovine serum albumin (BSA) as the substrate, with the degree of BSA hydrolysis used as an indicator of enzymatic activity.

As shown in Fig. 3 and Table 2, the extent of BSA hydrolysis was approximately 55 % for the hibiscus extract, compared to 47 % for calf rennet. The measured values of milk-clotting activity (MCA) and esterase activity are also reported in Table 2. The hibiscus extract exhibited no detectable esterase activity, while its MCA was about 10 % of that recorded for calf rennet.

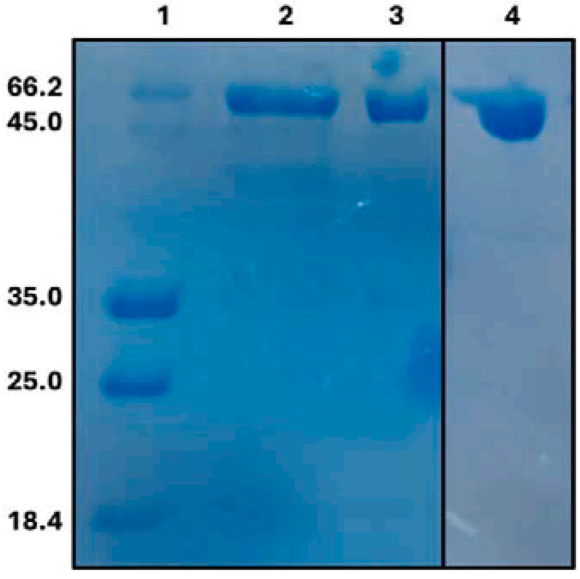

Fig. 3. Evaluation of the proteolytic activity level of hibiscus extract. The 12.5 % SDS-PAGE analysis: (1) molecular weight markers; (2) 5 μg BSA + 2 μl calf rennet; (3) 5 μg BSA+ 5 μl hibiscus extract; (4) 5 μg BSA (control).

Table 2
Main biochemical characteristics of the hibiscus aqueous extract compared with calf rennet. The extract was prepared by macerating dried *Hibiscus sabdariffa* L. flowers in tap water at room temperature. The pH, esterase and protease activities, and milk-clotting activity (MCA) were determined as described in the Materials and Methods section. Protease activity is expressed as the percentage of BSA hydrolysed, whereas milk-clotting activity (MCA) is reported in Standard Units (SU). Calf rennet was used as a reference. Data represent mean ± SD of two independent experiments.

	pH	Esterase activity (U/mL)	Protease activity[§] (% BSA digested)	MCA (SU[◊])
Calf rennet	5.57 ± 0.04	0.018 ± 0.002	47 ± 5	8000
Hibiscus extract	2.24 ± 0.02	n.d.	55 ± 5	727

[§] the incubation time was 10 min [◊]SU=Standard Unit, see M&M n.d.=not detectable.

Overall, the enzymatic and physicochemical characteristics of the hibiscus aqueous extract suggest that its coagulating capacity is mainly acid-driven rather than enzyme-mediated. Although its protease activity was comparable to that of calf rennet, the much lower MCA indicates that the proteolytic enzymes in the extract are likely non-specific or act under suboptimal conditions for casein cleavage. The strongly acidic pH of the extract (≈ 2.0) plays a major role in destabilizing casein micelles, promoting acid-induced coagulation similar to that observed in certain fresh cheeses and during tofu preparation. This behaviour highlights the dual functionality of the hibiscus extract, as both a mild proteolytic agent and a natural acid coagulant, supporting its use as a plant-based rennet substitute in dairy and soy protein systems.

3.3. Mini-curd making

The use of aqueous hibiscus extract, successfully, induced milk coagulation, producing cheeses with comparable yield and texture to control cheeses made with calf rennet. Preliminarily, we measured the amount of dried hibiscus present in the extract that was used, the concentration was measured on three different preparations of hibiscus extract, and the value obtained was 55.1 ± 4.3 mg/ml. It has been made an optimization of coagulant dose versus the yield in cheese, the best yield in cheese was obtained at the final concentration of hibiscus extract in milk at 8.5 mg/ml, obtaining about 52 g of cheese starting from 500 ml of milk, but also at 7.0 mg/ml of extract the yield was about 48 g, while at the final concentration of hibiscus at 10.5 mg/ml the yield was about 50 g, indicating that was reached the maximum of yield of cheese, starting from 500 ml of milk (see Figure S7).

To investigate wether milk coagulation was attributable to the organic acids present in the hibiscus extract and/or to its proteolytic enzymes, two control conditions were evaluated: coagulation induced by citric acid and coagulation induced by the hibiscus extract after thermal enzymes inactivation (20 min at 60 °C). A dose-response analysis was performed by monitoring the pH as a function of the volume of hibiscus extract added to milk. Regardless of the coagulant used (citric acid, crude hibiscus extract, or thermally inactivated extract), the pH reached a plateau value of approximately 4.5 (Figure S8). However, the thermally inactivated hibiscus extract was slightly less efficient. To achieve a coagulation level comparable to that obtained with the crude extract, 75 ml of inactivated extract were required, compared with 60 ml of the untreated extract (Figure S8). These findings suggest that both

acidity and proteolytic activity contribute to the coagulation process.

Another preliminary analysis was made to study the coagulant dose-response in term of yield of cheese. In particular, cheese yield was monitored as a function of the volume (mL) of hibiscus extract added. As control, cheese production by acidic coagulation with citric acid was also evaluated under the same conditions.

A standard protocol for semi-hard cheese making was followed. Under these conditions, 60 ml of hibiscus extract, corresponding to an extract concentration of approximately7.5–9.0 mg/ml, were added to the milk. Curd formation occurred within 30 min. The resulting cheeses showed a higher moisture content and slightly lower fat and protein levels than controls (Table 3). Cheese produced with hibiscus extract achieved a yield approximately 70 % of that obtained with calf rennet, and about 78 % of that obtained with citric acid coagulation.

The protein content remained comparable to that of the controls, while the hibiscus-derived cheeses exhibitedlower fat content and higher moisture levels. The reduced yield was not further investigated at this stage, as the primary goal was to assess the feasibility of hibiscus extract as a natural coagulant capable of producing a functional and sensorially distinctive cheese. The increased moisture and lower fat retention observed in hibiscus-derived cheeses compared with the control cheese (Table 3), are consistent with an acid-induced coagulation mechanism. Nevertheless, the resulting curd displayed good structural integrity, indicating that the extract was effective in forming a stable protein network.

The cheese-making protocol used was for semi-hard cheese, by using incubation temperature of 35 °C and 45 °C; the melting temperature of control cheese and hibiscus extract derived cheese was measured, obtaining the value of 55.9 ± 0.3 °C and 50.1 ± 1.2 °C, respectively.

3.4. Tofu making

The aqueous hibiscus extract efficiently induced soy protein coagulation, producing tofu with physico-chemical properties comparable to traditional $MgCl_2$ (nigari) coagulated tofu (Table 4). As further control, was used the acidic coagulation by citric acid.

A preliminary optimization of the coagulant dose was performed to evaluate tofu yield. The highest yield was obtained at a final hibiscus extract concentration of 5.5 mg/ml in soy milk at, obtaining approximately 72 g of tofu starting from 500 ml of soy milk. At 4.5 mg/ml, the yield was about 68 gr, while at 6.5 mg/ml the yield was approximately 73 g, indicating that the maximum of tofu yield had been reached (see Figure S9).

To verify whether soy milk coagulation was due only to the acidic component of the hibiscus extract, a dose-response analysis was conducted by monitoring pH as a function of the volume of hibiscus extract added to soy milk. Acidic coagulation with citric acid and coagulation induced by thermally inactivated hibiscus extract (20 min at 60 °C) were used as controls. In all cases, the pH reached a plateau value between 4.2–4.4 (Figure S10). Notably, the thermally-inactivated hibiscus extract showed the same coagulation efficiency as the crude extract (Figure S10), suggesting that the acidification is the main mechanism driving soy protein coagulation.

Finally, the coagulant dose-response was studied in term of tofu yield by measuring the amount of tofu obtained as a function of the volume (ml) of hibiscus extract added, and comparing it with citric acid as the acidic coagulation control. The results are reported in Fig. 5.

Hibiscus-derived tofu was produced by using 50 ml of extract, corresponding to a final extract concentration of approximately 5–7 mg/ml. The yield obtained with hibiscus extract was comparable to that achieved using nigari ($MgCl_2$), whereas tofu produced with citric acid showed a yield of about 65 % relative to nigari (Table 4).

The tofu obtained with hibiscus extract exhibited a lower pH (4.14), reflecting the intrinsic acidity of the extract. In contrast, the pH value of tofu prepared with citric acid (4.71) was intermediate between that of nigari- and hibiscus-derived tofu.

Moisture, fat, and protein contents of hibiscus-derived tofu were similar to those of traditional tofu prepared with $MgCl_2$, while tofu produced with citric acid showed slightly higher moisture content compared with nigari.

Table 3
Chemical composition of cheeses, determined at the end of ripening (30 days). Moisture, fat and protein content are expressed as %. The results are mean of three independent experiments. The control cheese was obtained using calf rennet and citric acid. The curd yield was measured from 500 mL of milk.

	c.c.* yield from 500 mL milk (g)	pH	Moisture (%)	Fat (%)	Protein (%)
Calf rennet	75.2 ± 3.1	5.03 ± 0.08	44.5 ± 1.0	33.7 ± 0.6	23.0 ± 0.2
Citric acid	68.2 ± 2.6	4.61 ± 0.13	54.1 ± 2.5	27.8 ± 0.7	18.3 ± 1.1
Hibiscus extract	53.6 ± 0.5	4.60 ± 0.20	61.0 ± 3.0	21.5 ± 0.4	17.4 ± 0.7

* *c.c.= cheese curd.*

Table 4
Chemical composition of tofu determined one day from the preparation. Moisture, fat and protein content are expressed as %. Results are mean of two independent experiments. Traditional tofu was obtained by using $MgCl_2$, and as acidic coagulation citric acid.

	Yield from 500 mL soy milk (g)	pH	Moisture (%)	Fat (%)	Protein (%)
$MgCl_2$	88.2 ± 4.4	5.82 ± 0.07	70.9 ± 1.5	10.2 ± 0.5	15.8 ± 0.7
Citric acid	57.0 ± 3.0	4.71 ± 0.10	74.0 ± 1.1	8.9 ± 0.5	15.9 ± 1.2
Hibiscus extract	82.5 ± 5.8	4.14 ± 0.09	70.0 ± 1.3	9.8 ± 0.6	16.0 ± 1.0

3.5. Macroscopic and microstructural comparison of hibiscus-derived cheese and tofu

The visual comparison of the cheese and tofu matrices with their corresponding hibiscus-derived products is reported in Fig. 6.

The control cheese (top left) shows a compact, homogeneous matrix typical of rennet-induced coagulation, with a smooth surface and pale-yellow colour. In contrast, the hibiscus-derived cheese exhibits a softer, moister texture, consistent with the higher water content reported in Table 3. The tofu samples show structural differences between the control and the hibiscus-derived product. The control tofu, obtained using magnesium chloride, presents a smooth and uniform structure with well-formed protein aggregates. Conversely, the hibiscus-derived tofu appears darker and more granular, with a heterogeneous pore distribution. Our hypothesis is that, this morphology may result from the acidification promoted by the hibiscus extract, leading to a variable cross-linking of soy proteins. The visual observations suggest that hibiscus extract affects the colour of tofu.

3.6. ATR-FTIR characterization of cheese and tofu samples

To better understand the molecular interactions responsible for the observed structural modifications, ATR-FTIR analysis was performed on all samples to evaluate possible changes in the protein and polyphenol vibrational profiles (Fig. 7). All samples displayed the typical vibrational bands associated with protein-, lipid-, and carbohydrate-based matrices.

In the amide region (1700–1500 cm^{-1}), characteristic peaks at approximately 1639 cm^{-1} (amide I) and 1551 cm^{-1} (amide II) were observed, corresponding to *C═O* stretching and N–H bending vibrations of peptide bonds, respectively. These bands confirm the predominance of proteinaceous components in both cheese and tofu. The similarity of peak positions between control and hibiscus-derived samples indicates

that hibiscus extract did not significantly compromise the secondary protein structure during coagulation. In the O–H and N–H stretching region (3700–3000 cm^{-1}), broad absorption bands centered around 3377–3262 cm^{-1} were observed, reflecting hydrogen-bonded hydroxyl and amino groups.

The aliphatic C–H stretching region (2920–2853 cm^{-1}) exhibited typical bands corresponding to lipid methylene and methyl groups. These signals were slightly less intense in tofu samples compared to cheese, consistent with their lower fat content.

In hibiscus-derived cheese and tofu, minor shifts and intensity variations were detected in the 1500–1200 cm^{-1} region, which can be attributed to C–O and C–C stretching of phenolic rings and to C–O–C vibrations from carbohydrates and polyphenols originating from hibiscus extract.

Overall, the ATR-FTIR data confirm the successful incorporation of hibiscus-derived compounds into both protein matrices, without disrupting the overall protein structure. The observed spectral modifications, particularly in the hydroxyl and aromatic regions, support the formation of protein–polyphenol interactions, which may enhance the stability, antioxidant potential, and texture of the final products.

To support the qualitative interpretation of ATR-FTIR data, the relative areas (%) of five selected characteristic bands were calculated after baseline correction and deconvolution tools (Fig. 8A), providing semi-quantitative indicators of variations in protein- and hydroxyl-related vibrational contributions. Moreover, an exploratory principal component analysis (PCA) was performed (Fig. 8B) as a visualization tool to examine spectral patterns associated with matrix composition and hibiscus addition.

The score plot showed a clear separation along PC1 (62.3 % of explained variance) between control tofu and cheese samples, reflecting intrinsic compositional differences between dairy and soy protein matrices, likely related to differences in protein structure and organization. PC2 (37.4 % of explained variance) captured the effect of hibiscus extract addition in a matrix-dependent manner. *Hibiscus*-based cheese and tofu samples were displaced along PC2 in opposite directions relative to their respective controls, indicating that hibiscus-induced spectral modifications differ between casein and soy proteins. This behavior is consistent with the semi-quantitative band analysis, which revealed enhanced O–H contributions and reduced amide I–II bands in cheese, whereas an opposite trend was observed in tofu. These finding suggest different extents of protein–polyphenol interactions and hydration phenomena in the two matrices.

3.7. Metabolomic profiling and compound transfer

The metabolomic targeted analysis revealed that most of the characteristic phenolic acids, flavonoids, and anthocyanins from the *H. sabdariffa* L. aqueous extract were retained in both the hibiscus-derived cheese and tofu (Fig. 9). Data acquired revealed clear differences in metabolite distribution depending on the food matrix, reflecting the influence of protein source. Among the most abundant compounds in the free extract (HsF_W) were the chlorogenic acid derivatives 3-CQA (**2**), 4-CQA (**3**), and 5-CQA (**5**), together with the related ethyl chlorogenates (8, 11, 14). The 4-CQA (**3**) accounted for the highest relative proportion (approximately 30 % of total metabolites). In the cheese- and tofu-based formulations, ethyl chlorogenates were not detected (and were therefore not included in Fig. 9). Regarding the monocaffeoylquinic acids, a slight decrease in 3-CQA (**2**) and 4-CQA (**3**) was observed in the cheese formulation, whereas a slight increase was detected in tofu. Conversely, 5-CQA (**5**) increased in both matrices. These trends suggest matrix-dependent transformations, possibly influenced by protein and lipid interactions occurring during coagulation and heat treatment. Nonetheless, the consistent detection of monocaffeoyl quinic acids, together with caffeoyl shikimic acid (**7**) and *N*-feruloyltyramines (**16** and **17**), indicates that these relatively stable phenolic acids can withstand the processing conditions involved.

The decrease was more pronounced in the cheese matrix, whereas in tofu, 3-CQA (**2**) appeared relatively more stable, likely due to lower pH and milder protein–polyphenol interactions in the soy system.

The anthocyanins, including delphinidin 3-*O*-pentosylhexoside (**4**), cyanidin 3-*O*-pentosylhexoside (**6**), 6-hydroxydelphinidin hexoside (**9**), 6-hydroxydelphinidin (**15**), and delphinidin (**19**), exhibited the most pronounced losses following incorporation into the matrices. In the free extract, these pigments represented a considerable fraction of the phenolic profile, whereas in both the cheese- and tofu-based products their relative levels decreased dramatically, in some cases approaching zero. Interestingly, the cheese-based formulation retained slightly higher amounts, particularly of cyanidin (**20**), suggesting partial protection within the dairy protein-polyphenol matrix. Such interactions may help shield anthocyanins from degradation and contribute to maintaining colour stability and antioxidant capacity in the final product. The flavonols, specifically the quercetin derivatives quercetin 3-*O*-pentosylhexoside (**10**), rutin (**12**), quercetin 3-*O*-hexoside (**13**), and quercetin *p*-coumaroylhexoside (**18**), showed a more balanced distribution across the formulations. The tofu-based product displayed slightly higher relative levels of **10**, suggesting greater stability in the plant-derived matrix. In contrast, compounds **12** and **13** were slightly more abundant in the cheese-based product, indicating that these glycosylated flavonols are less susceptible to degradation during dairy processing.

Although cyanidin (**20**) and quercetin *p*-coumaroylhexoside (**18**) were not detected in tofu, likely due to pH- and temperature-related degradation during soy coagulation, the overall metabolite profile demonstrates effective transfer and retention of bioactive compounds from hibiscus in both matrices. The feruloyltyramine derivatives (**16, 17**) were better retained in the cheese-based. Thus, the retention and stability of phenolic compounds were highly compound- and matrix-dependent, reflecting the complex interactions and processing conditions in both dairy and soy-based systems.

To achieve a more accurate and selective quantification of specific hibiscus-derived phenolic compounds in the complex cheese and tofu matrices, Multiple Reaction Monitoring (MRM) was employed. Compared with full-scan acquisition, MRM offers enhanced sensitivity and selectivity by monitoring predefined precursor-to-product ion transitions (Cioffi et al., 2023), thereby reducing matrix interference and improving quantification reliability. This targeted approach is particularly well suited for food systems, where co-eluting compounds and matrix effects can significantly affect signal response and analytical accuracy.

Considering the presence of three main classes of compounds, one commercial standard representative of each class (chlorogenic acid, rutin, and kuromanin) was selected to construct calibration curves. The amounts of the identified metabolites were then estimated and expressed as mg/g of HsF_W and mg/100 g of the derived products. The quantitative results are reported in Table 5.

The quantitative data further support the matrix-dependent behavior of hibiscus-derived phenolics. Overall, the distribution pattern of metabolites in the two matrices was consistent with the previously reported relative data. Chlorogenic acids (**2, 3**, and **5**) were confirmed as the predominant phenols in the extract and remained the most abundant compounds in both HsF_W-based cheese and tofu. Their relatively high concentrations in both matrices suggest greater chemical stability and lower susceptibility to degradation compared with other phenolic constituents. In cheese, chlorogenic acids reached higher absolute levels

Table 5
Amount of identified metabolites in the aqueous *H. sabdariffa* extract (HsF_W) and in hibiscus-based cheese and tofu samples, determined by HPLC–MS/MS in Multiple Reaction Monitoring (MRM) mode using external calibration curves (* chlorogenic acid; ** rutin; § kouromanin). Values are reported as mean ± SD.

n.		Compound	mg/g HsF_W	mg/100 g cheese HsF_W-based	mg/100 g tofu HsF_W-based
1	*	Dihydroxybenzoic acid hexoside	0.06 ± 0.00	1.07 ± 0.05	0.05 ± 0.00
2	*	3-CQA	0.56 ± 0.03	10.19 ± 0.51	0.39 ± 0.02
3	*	4-CQA	0.97 ± 0.05	16.24 ± 0.81	0.62 ± 0.03
4	§	Delphinidin 3-*O*-pentosylhexoside (e.g.., sambubioside)	0.09 ± 0.01	n.q.	n.q.
5	*	5-CQA	0.09 ± 0.00	10.08 ± 0.47	0.32 ± 0.02
6	§	Cyanidin 3-*O*-pentosylhexoside (e.g.., sambubioside)	0.13 ± 0.01	0.33 ± 0.02	0.01 ± 0.00
7	*	CSA	0.05 ± 0.01	0.41 ± 0.06	0.01 ± 0.00
8	*	Ethyl chlorogenate 1	0.06 ± 0.00	n.q.	n.q.
9	§	6-Hydroxydelphinidin hexoside	0.20 ± 0.08	4.87 ± 0.11	0.13 ± 0.05
10	**	Quercetin 3-*O*-pentosylhexoside	n.q.	n.q.	n.q.
11	*	Ethyl chlorogenate 2	0.04 ± 0.00	n.q.	n.q.
12	**	Rutin	0.15 ± 0.01	6.14 ± 0.15	0.16 ± 0.01
13	**	Quercetin 3-*O*-hexoside	0.05 ± 0.00	1.83 ± 0.07	0.04 ± 0.00
14	*	Ethyl chlorogenate 3	0.03 ± 0.00	n.q.	n.q.
15	§	6-Hydroxydelphinidin	0.17 ± 0.02	0.69 ± 0.03	0.03 ± 0.00
16	*	*N*-feruloyltyramine 1	n.q.	n.q.	n.q.
17	*	*N*-feruloyltyramine 2	n.q.	n.q.	n.q.
18	**	Quercetin *p*-coumaroyl-hexoside	n.q.	n.q.	n.q.
19	§	Delphinidin	0.96 ± 0.12	7.87 ± 0.13	0.13 ± 0.02
20	§	Cyanidin	0.24 ± 0.01	7.81 ± 0.17	n.q.

(n.q.= not quantified).

(exceeding 10 mg *per* 100 g of product) than in tofu. This difference may be attributed to concentration effects occurring during curd formation and wey expulsion, as well as to potential interactions with the casein network that enhance their retention. In contrast, soy proteins may provide a less favorable environment for retaining high absolute levels of certain phenolics, despite showing a metabolomic profile that, in relative terms, more closely resembles that of the original extract.

Among flavonols, rutin (**12**) and quercetin 3-*O*-hexoside (**13**) showed the most consistent behavior across matrices. Their detection at quantifiable levels in both cheese and tofu, suggests a comparatively higher resistance to processing stresses, including heating, acidification, and protein aggregation. This stability may be attributed to their chemical structure, which promotes non-covalent interactions with proteins while limiting extensive degradation. These results support their suitability as reliable markers of phenolic transfer from hibiscus extract to the final products. On the contrary, anthocyanins and their aglycones displayed the most pronounced matrix-dependent differences. Although present in appreciable concentrations in the extract, their levels were markedly reduced in both cheese and tofu. Only cyanidin (**20**), delphinidin (**19**), and its glycosylated hydroxy-derivative (**9**) were partially retained in cheese, despite their known instability during processing in dairy matrices. Conversely, cyanidin could not be quantified with sufficient accuracy in the tofu matrix. Taken together, these results confirm that the stability and transfer of hibiscus-derived phenolics are strongly influenced by both the food matrix and the intrinsic properties of individual compounds. Chlorogenic acids emerged as the most robust class, exhibiting high retention in both cheese and tofu, with particularly elevated absolute concentrations in cheese, likely due to concentration effects during curd formation. Flavonols, such as rutin and quercetin 3-*O*-hexoside also demonstrated good stability, further supporting their suitability as reliable markers of phenolic transfer.

In contrast, anthocyanins and their aglycones were markedly reduced in both matrices, with only partial preservation observed in cheese, reflecting their intrinsic instability under processing conditions. Despite these losses, both systems successfully incorporated a meaningful fraction of bioactive compounds from the hibiscus extract. Overall, the findings demonstrate that *H. sabdariffa* L. extract can effectively enrich both dairy and vegan products with phenolic compounds, although retention levels vary depending on matrix composition and processing conditions. Cheese proved particularly effective in retaining higher absolute amounts of several phenolics, likely due to concentration phenomena during curd formation, whereas tofu maintained a metabolomic profile more closely resembling the original extract in relative terms. These complementary behaviors highlight the potential of both matrices as suitable carriers of hibiscus bioactives for the development of functional foods.

3.8. Free fatty acid (FFA) profile of cheese and tofu samples

In order to evaluate whether the addition of hibiscus extract could provide benefits not only as a coagulant but also due to its content of polyphenols and organic acids, the free fatty acid (FFA) profile and lipid quality of cheese and tofu were assessed, comparing control products with those prepared using hibiscus extract (Table 6). No FFAs with acyl chains between 10 and 14 carbon atoms were detected in either control or hibiscus-enriched tofu, consistent with the absence of these fatty acids in soy milk. In cheese samples, MUFAs represented approximately 50 % of total FFAs, while PUFAs accounted for <10 % (Table 6B). The addition of hibiscus extract caused a slight increase in MUFAs and a reduction in SFAs, while PUFAs remained largely unchanged. Oleic acid (18:1) was the most abundant FFA, followed by palmitic (16:0) and stearic (18:0) acids. In tofu, PUFAs comprised approximately 40 % of total FFAs, whereas SFAs and MUFAs each accounted for around 30 %; hibiscus addition induced a modest increase in SFAs (30.3 → 32.8 %) and a slight decrease in MUFAs (30.5 → 27.3 %), while PUFAs remained high (39.2 → 39.9 %). Taken together, these compositional changes are reflected in key lipid quality indices (Table 7). In cheese, the atherogenic index (AI) decreased from 0.94 to 0.77, as well as the thrombogenic index (TI) from 1.75 to 1.31, while the PUFA/SFA ratio increased from 0.12 to 0.16 and the hypocholesterolemic/hypercholesterolemic (h/H) ratio rose from 1.39 to 1.81, indicating an improvement in lipid profile and a potential reduction in cardiovascular risk. In tofu, already rich in PUFAs, AI remained low (0.36 → 0.40) and TI below 1 (0.87 → 0.98), the PUFA/SFA ratio remained high (1.29 → 1.22), and h/H was very high (2.81 → 2.51), confirming the favourable lipid quality even after hibiscus addition. The n-6/n-3 ratio, indicative of the balance between essential fatty acids, increased in cheese (4.62 → 5.70) and slightly decreased in tofu (9.33 → 8.79), reflecting the typical lipid composition of milk and soy.

These results also highlight the contribution of hibiscus polyphenols, such as anthocyanins and phenolic acids, which may provide antioxidant protection to unsaturated fatty acids, limiting lipid oxidation during processing and preserving nutritional quality. In cheese, the combination of reduced cardiovascular-risk SFAs and increased MUFAs suggests a significant protective effect, whereas in tofu, the extract maintained the already favourable lipid composition, with high PUFAs and good h/H and PUFA/SFA ratios.

Table 6
A) Retention time (RT, min) and mass-to-charge ratio (m/z) of individual free fatty acids (FFAs) together with their relative percentages (mean ± SD) in cheese and tofu samples; B) total content of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. Cheese ctrl: control cheese; Cheese HsF: cheese prepared with hibiscus extract; Tofu ctrl: control tofu; Tofu HsF: tofu prepared with hibiscus extract.

A)	RT (min)	$[M-H]^-$ (m/z)		FFA	Cheese ctrl	Cheese *Hs*F-based	Tofu ctrl	Tofu HsF-based
	1.124	171.1391	10:0	capric acid	0.39±0.02	0.22±0.01	-	-
	2.030	199.1704	12:0	lauric acid	0.75±0.03	0.66±0.02	-	-
	3.259	227.2017	14:0	myristic acid	3.89±0.10	4.28±0.12	-	-
	2.360	225.186	14:1	myristoleic acid	0.70±0.03	0.71±0.02	-	-
	4.642	255.2334	16:0	palmitic acid	33.40±0.80	28.28±0.75	24.78±0.60	26.73±0.62
	3.573	253.2179	16:1	palmitoleic acid	1.67±0.05	1.82±0.06	-	-
	5.986	283.2354	18:0	stearic acid	9.02±0.20	6.70±0.18	5.56±0.15	6.08±0.16
	4.913	281.2489	18:1	oleic acid	44.72±0.90	51.12±0.95	30.50±0.70	27.34±0.68
	3.986	279.2334	18:2	linoleic acid	4.81±0.12	5.47±0.14	35.38±0.85	35.79±0.88
	3.127	277.2178	18:3	α-linolenic acid	1.04±0.03	0.96±0.02	3.79±0.09	4.07±0.10
B)								
				SFA	47.5 ± 1.2	40.1 ± 1.0	30.3 ± 0.8	32.8 ± 0.9
				MUFA	47.1 ± 1.3	53.7 ± 1.2	30.5 ± 0.9	27.3 ± 0.8
				PUFA	5.8 ± 0.2	6.4 ± 0.2	39.2 ± 1.2	39.9 ± 1.0

Table 7
Lipid quality indices of cheese and tofu samples, with and without hibiscus extract (HsF). Values are expressed as mean ± SD of three independent experiments. AI: Atherogenic Index; TI: Thrombogenic Index; P/S: ratio of polyunsaturated to saturated fatty acids; h/H: hypocholesterolemic/hypercholesterolemic ratio; n-6/n-3: ratio of omega-6 to omega-3 fatty acids. Ctrl: control sample; HsF: sample prepared with hibiscus extract.

	AI	TI	P/S	h/H	n-6/n-3
Cheese ctrl	0.94 ± 0.03	1.75 ± 0.05	0.12 ± 0.01	1.39 ± 0.04	4.62 ± 0.15
HsF-based Cheese	0.77 ± 0.02	1.31 ± 0.04	0.16 ± 0.01	1.81 ± 0.05	5.70 ± 0.12
Tofu ctrl	0.36 ± 0.01	0.87 ± 0.03	1.29 ± 0.06	2.81 ± 0.06	9.33 ± 0.20
HsF-based tofu	0.40 ± 0.02	0.98 ± 0.03	1.22 ± 0.04	2.51 ± 0.05	8.79 ± 0.18

Collectively, the findings confirm the dual role of hibiscus extract as both a natural acidifying and coagulant agent and as a source of bioactive compounds, improving the lipid profile in dairy products and preserving the nutritional quality of soy-based foods. These characteristics highlight the potential of hibiscus extract in the production of functional foods, combining technological innovation and sustainability.

3.9. Antioxidant activity

Phenols, polyphenols, flavonols, anthocyanins, catechols identified in the hibiscus extract, have been previously described to exhibit antioxidant activity (Diep et al., 2020). Mass spectroscopy analysis (Table 5) quantified the total amount of these compounds in the hibiscus extract at approximately about 4.0 mg/g of extract. In the derived products, their concentration was about 70 mg/100 g of 100 in e hibiscus-enriched cheese and approximately 2 mg/100 gr in hibiscus-enriched tofu (Table 5). Given that both hibiscus-derived cheese and tofu retained a fraction of the phenolic compounds present in the original extract, we further investigated whether these compounds preserved their antioxidant activity after incorporation into the food matrices. Antioxidant activity was therefore evaluated in the raw extract, in the whey obtained during tofu and cheese production, in freshly prepared tofu and cheese, and exclusively for cheese, after 30 days of ripening.

Table 8
Antioxidant activity. The activity was measured by the ABTS assay, using 50 µl for liquid samples, whereas from cheese and tofu samples were extracted the soluble compounds (see paragraph 2.6) and tested (100 µl). Results are the mean of two different preparations.

Hibiscus extract (50 µl)	Whey (50 µl)	Fresh cheese (100 µl)	Ripened cheese (100 µl)
86.3 %	79.1 %	14.2 %	9.3 %
Hibiscus extract (50 µl)	Whey (50 µl)	Tofu (100 µl)	-
85.9 %	81.4 %	7.2 %	-

Table 8 reports the results of the antioxidant activity assays. The hibiscus extract exhibited a high antioxidant activity reaching approximately 85 %. Notably, the whey obtained during processing also maintained a high antioxidant level of, around 80 %. The most interesting data concern the antioxidant activity of fresh cheese and tofu. Both showed substantial antioxidant capacity, and after 30 days of ripening, cheese retained about 65 % of its initial antioxidant activity. This result indicates that the cheesemaking and ripening processes do not significantly inactivate the bioactive compounds derived from the hibiscus extract, allowing a considerable fraction of their antioxidant potential to be preserved over time.

4. Discussion

In recent decades, increasing attention has been directed toward the use of plant-derived coagulants in cheese making. This growing interest is attributable to multiple factors, including ethical and religious considerations, technological and production-related requirements, the pursuit of distinctive sensory attributes, and the preservation of local traditions. In several regions of Southern Europe, the use of vegetable rennet constitutes a long-standing cultural and technological practice. Rather than representing a modern substitution for animal rennet, it reflects an ancient cheesemaking tradition closely associated with the spontaneous plant species available in specific geographical areas.

From a technological and industrial perspective, the use of plant coagulants is partly driven by issues related to the availability and cost of animal rennet, particularly in the context of increasing global demand. Plant derived coagulants may offer advantages in terms of regional accessibility and seasonal availability, and in some contexts they represent a readily obtainable and sustainable alternative. Moreover, their adoption aligns with contemporary efforts to promote environmentally responsible production systems and to diversify raw material sources within the dairy sector.

Ethical considerations primarily relate to concerns regarding animal welfare. Religious requirements, such as kosher or halal certification, may impose restrictions on the origin and processing of animal-derived rennet; the use of plant-based coagulants eliminates these constraints

and facilitates compliance with certification standards. Dietary motivations also contribute to the adoption of vegetable rennets, particularly among vegetarian and vegan consumers, whose choices may be associated with ethical beliefs or health-related conditions. Furthermore, when plant-based milk substrates (e.g., soy milk) are employed, the resulting products are suitable for vegan consumption.

In addition, plant-derived coagulants significantly influence the sensory properties of cheese. Artisanal cheeses produced using extracts from thistle (*Cynara cardunculus*), artichoke, or fig latex are often characterized by specific organoleptic traits, including enhanced creaminess and a slight bitter note. Such attributes have been described in traditional Portuguese and Spanish ewe's milk cheeses (Delgado et al., 2010), where vegetable rennet contributes to the development of distinctive texture and flavour profiles. In these contexts, plant proteases not only induce milk coagulation but also play an active role during ripening, affecting proteolysis patterns and, consequently, the overall sensory complexity of the final product.

In this light, *H. sabdariffa* L. has been identified as potential source of vegetable rennet. This plant is recognized as a Generally Recognized as Safe (GRAS) ingredient by the U.S. Food and Drug Administration, and its use in foods does not require pre-market authorization (Drugs and Lactation Database (LactMed®) [Internet]. Bethesda (MD): National Institute of Child Health and Human Development; 2006-. Hibiscus. [Updated 2025 May 15]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK501882/). In this study, hibiscus extract demonstrated dual functionality: as a natural coagulant in both cheese and tofu production, primarily due to its intrinsic acidity (pH ≈ 2.2). In both cases, coagulated products were successfully obtained by exploiting the extract's acidic properties (Fig. 4, 5, S6 and S8). Furthermore, the hibiscus extract served as a source of bioactive compounds (Table 1), contributing to the enhancement of the functional quality of the final products. Both matrices showed effective coagulation, and physicochemical profiles comparable to those obtained using conventional coagulants. In particular, tofu prepared with hibiscus extract exhibited a yield comparable to that obtained using the traditional nigari method (Table 4). In contrast, the yield of hibiscus-derived cheese was approximately 70 % of that of the control cheese produced with calf rennet (Table 3).

These results are consistent with findings reported for other vegetable rennets. A previous study described cheese making by using novel plant-derived coagulants and reported yields, calculated from 500 ml of cow's milk, of approximately 95 % relative to calf rennet for extracts obtained from the internal bracts of artichoke, cardoon, papaya, and fig latex. Lower yields were observed for pineapple and oyster mushroom extracts, corresponding to 79 % and 71 % respectively (Pacifico et al., 2024). Notably, the cheesemaking conditions adopted in that study were comparable to those applied in the present work. Overall, these findings highlight the dual potential of hibiscus extract, as both, a vegetable rennet for cheesemaking and an effective coagulant for tofu production.

Beyond its technological role, hibiscus extract significantly influenced the chemical–nutritional profile of the products. While total lipid content remained largely unchanged, the distribution of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) was shifted in a beneficial direction (Table 6). The relative

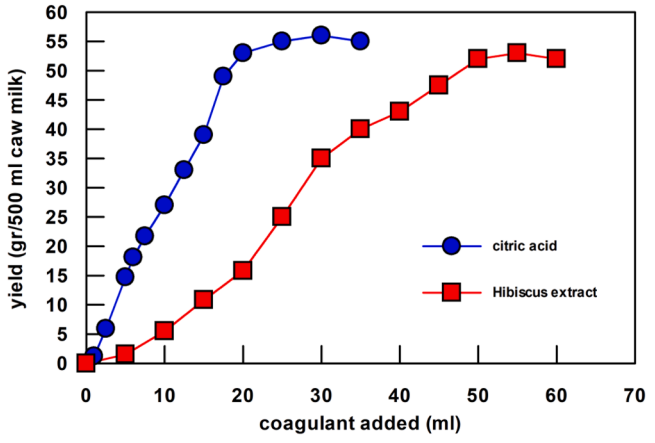

Fig. 4. Coagulant dose-response. The standard protocol for semi-hard cheese production was followed. For each experimental point, 500 mL of whole caw milk were used After incubation, the curd was collected and weighed to determine cheese yield. Citric acid was used as the acidic control for coagulation.

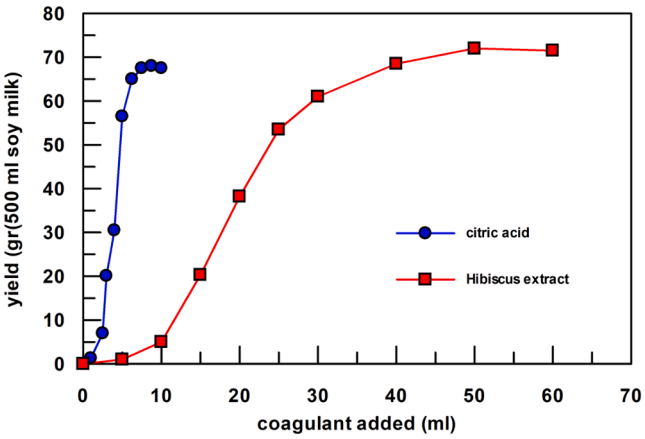

Fig. 5. Coagulant dose-response. For each point were used 500 ml of soy milk. After incubation the curd was recovered and weighed. As acidic control of coagulation was used citric acid.

Fig. 6. Representative images of control and hibiscus-derived cheese and tofu as visualized using a Zeiss Stemi 2000-C Stereo microscope with zoom magnification × 0.8, × 2, × 4 (Carl Zeiss, Thornwood, NY, USA).

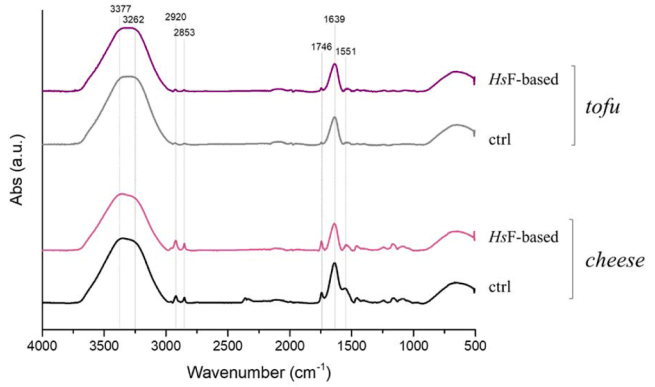

Fig. 7. ATR-FTIR spectra of HsF-based and control samples of tofu (upper panel) and cheese (lower panel).

A)

	O-H	N-H	C=O	Amide I	Amide II
Cheese ctrl	74.35	11.03	0.92	10.69	3.01
Cheese *Hs*F-based	84.13 ↑	6.78 ↓	0.74 ↓	7.36 ↓	1.00 ↓
Tofu ctrl	81.09	11.75	n.q.	7.15	n.q.
Tofu *Hs*F-based	78.04 ↓	14.18 ↑	n.q.	7.78 ↑	n.q.

B)

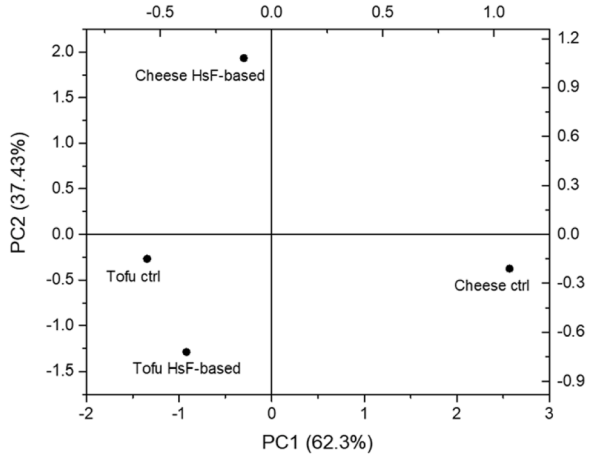

Fig. 8. A) Relative areas (%) of five selected characteristic ATR-FTIR bands in *hibiscus*-based cheese and tofu samples compared with their respective controls. Arrows indicate the direction of change relative to the control within the same matrix; n.q. = not quantifiable after deconvolution. B) Score plot of the principal component analysis (PCA) based on band areas obtained after spectra deconvolution.

increase in MUFAs and maintenance of PUFAs, particularly linoleic and α-linolenic acids, improved lipid quality indices, reducing atherogenic (AI) and thrombogenic (TI) indices while increasing the hypocholesterolemic/hypercholesterolemic (h/H) ratio (Table 7). These effects align with the recognized ability of phenolic-rich plant extracts to protect unsaturated fatty acids from oxidative degradation through radical scavenging and metal chelation (Al-Dhabi et al., 2017; Hainida et al., 2020). Metabolomic profiling confirmed the presence of diverse bioactive compounds in hibiscus extract, including anthocyanins (delphinidin-3-*O*-sambubioside, cyanidin-3-*O*-sambubioside), flavonols (quercetin derivatives), and phenolic acids (chlorogenic acids) (Fig. 9, Table 5). Many of these molecules were incorporated into the cheese and tofu matrices, demonstrating partial stability during processing (Table 5). The presence of active compounds in the hibiscus-derived product was confirmed by evaluating the antioxidant activity of the fresh total extract as well as of the resulting cheese and tofu. In the final products, antioxidant activity levels were consistent with the amount of bioactive compounds retained (Table 8). Notably, after 30 days of ripening, the hibiscus-derived cheese retained approximately 65 % of its initial antioxidant activity (Table 8), indicating a substantial persistence of functional properties over time.

These compounds are well-documented for their antioxidant, anti-inflammatory, hepatoprotective, and cardiometabolic effects (Borrás-Linares et al., 2015; Majdoub et al., 2019; Marquez-Rodriguez et al., 2021; Salinas-Moreno et al., 2023). The potential health benefits of hibiscus-derived products are further supported by studies on structural related metabolites from other plant sources. For istance, chlorogenic acids have been shown to exert strong antioxidant and anti-inflammatory activities, modulate glucose and lipid metabolism, and improve endothelial function (Nguyen et al., 2024; Wang et al., 2023).

Overall, these findings suggest that hibiscus extract not only functions as an effective coagulant but also enhances the nutraceutical value of the resulting dairy and plant-based products.

Rutin and quercetin derivatives prevent oxidative degradation of unsaturated fatty acids, protect cellular membranes, and display vaso protective, anti-hypertensive, and antiproliferative effects (Tobar-Delgado et al., 2023; Kostic et al., 2023). Anthocyanins, including delphinidin and cyanidin glycosides, contribute to colour stability and antioxidant protection in the food matrix, while modulating key pathways involved in inflammation, apoptosis, and lipid metabolism, thereby exerting anti-cancer, anti-obesity, and cardioprotective effects (Sharma et al., 2021; Safdar et al., 2023).

Importantly, these compounds can act synergistically with lipids and proteins in complex matrices such as cheese and tofu, enhancing lipid digestibility, slowing oxidation, and potentially improving the bioavailability of both polyunsaturated fatty acids and phenolics. The co-presence of multiple bioactive molecules may produce additive or synergistic effects, amplifying protection against oxidative stress, modulating gut microbiota, and supporting metabolic homeostasis (Torres-Gonzales and Rice Bradley, 2023; Paul et al., 2020). Thus, the inclusion of hibiscus extract not only ensures effective coagulation but also transforms cheese and tofu into functional foods, enriched with a spectrum of bioactive compounds that improve lipid quality, provide antioxidant protection, and support overall metabolic health. This dual technological and nutritional impact underscores the potential of hibiscus extract as a versatile ingredient for developing vegetarian and vegan, health-promoting foods in line with current clean-label and functional nutrition trends.

5. Conclusions

An aqueous hibiscus extract of *H. sabdariffa* L. demonstrated significant potential as a multifunctional ingredient in vegetarian and vegan food formulations. Its intrinsic acidity and proteolytic activity allowed it to function effectively as a natural coagulant in both cheese and tofu production. Beyond this technological role, the extract enriched the final products with a diverse range of bioactive compounds, including anthocyanins, flavonols, and phenolic acids, which were partially preserved during processing (Table 8) and contributed to improved chemical-nutritional profiles. The inclusion of hibiscus extract positively modulated lipid composition, favouring the maintenance of PUFAs and an increase in MUFAs, with a consequent improvement in lipid quality indices (reduced atherogenic index [AI] and thrombogenic index [TI], and increased hypocholesterolemic/hypercholesterolemic ratio [h/H]). These effects, coupled with the well-documented antioxidant, anti-inflammatory, cardioprotective, and anti-proliferative properties of hibiscus polyphenols (Maciel et al., 2018; Hamadjida et al., 2024), underscore the functional value of the derived cheese and tofu.

Overall, this study highlights the versatility of the prepared aqueous hibiscus extract as an ingredient capable of delivering dual benefits:

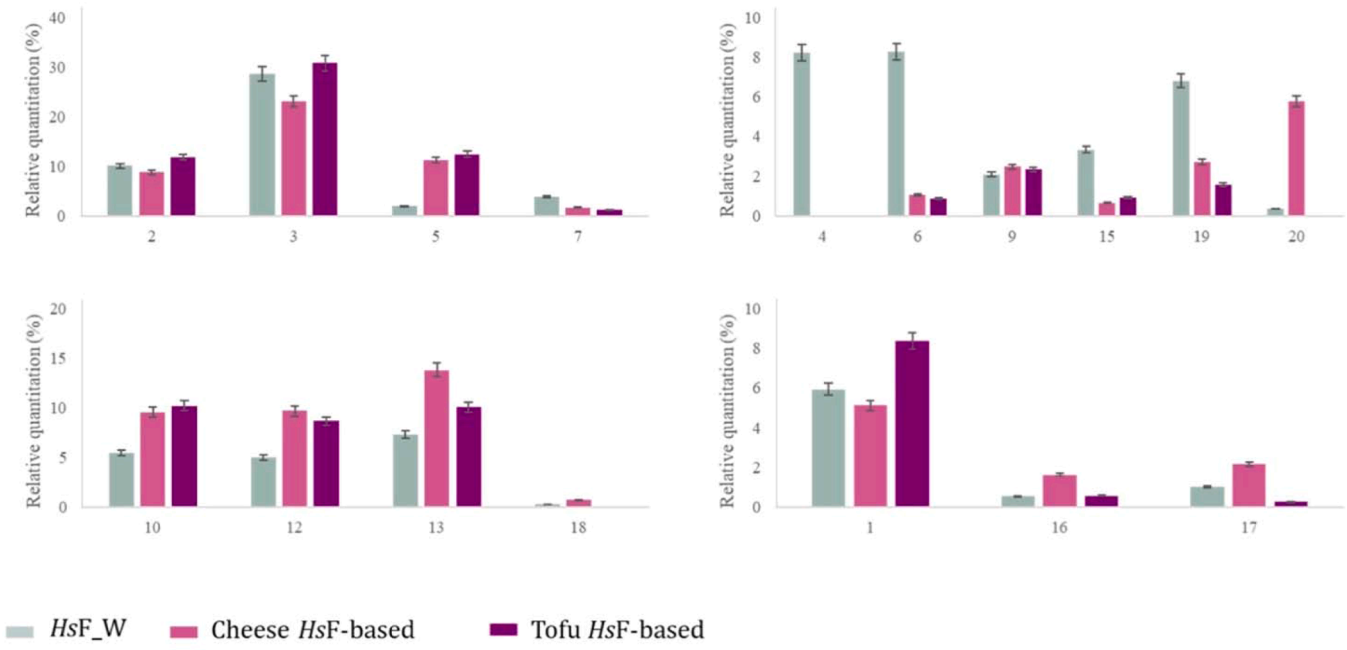

Fig. 9. Relative amount (%) of phenolic metabolites in the aqueous hibiscus extract (HsF_W) and in hibiscus-based cheese and tofu samples, expressed as mean ± standard deviation (SD) of the total sum of identified peak areas.

technological functionality through effective coagulation, and bioactive enrichment of the final products.

These findings provide mechanistic and compositional evidence supporting the formulation of plant-based functional foods enriched in specific bioactive compounds, such as polyphenols and antioxidant constituents. The observed biological activities further emphasize their potential role in modulating oxidative stress–related pathways, thereby contributing to the development of evidence-based, health-targeted food products.

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Ethical statement - studies in humans and animals

The authors declare that they have no use humans and/or animals or parts of them in this study

CRediT authorship contribution statement

Severina Pacifico: Writing – original draft, Resources, Methodology, Data curation. **Emilia Caputo:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Simona Piccolella:** Writing – original draft, Resources, Methodology, Data curation. **Carmen Diglio:** Investigation, Formal analysis. **Terenzio Zenone:** Investigation. **Teresa Bertolini:** Investigation. **Luigi Mandrich:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2026.100981.

Data availability

All the data are reported in the article and supplemental data

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