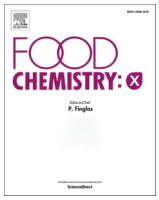
Food Chemistry: X 22 (2024) 101256

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Implementation of plant extracts for cheddar-type cheese production in conjunction with FTIR and Raman spectroscopy comparison

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ARTICLE INFO

Keywords:
Plant extracts
Cheddar-type cheese
Physicochemical properties
Antioxidant activity
Spectroscopy

ABSTRACT

Plant extracts have demonstrated the ability to act as coagulants for milk coagulation at an adequate concentration, wide temperatures and pH ranges. This research is focused on the use of different vegetative extracts such as Citrus aurantium flower extract (CAFE), bromelain, fig latex, and melon extract as economical and beneficial coagulants in the development of plant-based cheddar-type cheese. The cheddar-type cheese samples were subjected to physicochemical analysis in comparison to controlled cheese samples made from acetic acid and rennet. The fat, moisture, protein, and salt contents remained the same over the storage period, but a slight decline was observed in pH. The Ferric reducing antioxidant power (FRAP) increased with the passage of the ripening period. The FTIR and Raman spectra showed exponential changes and qualitative estimates in the binding and vibrational structure of lipids and protein in plant-based cheeses. The higher FTIR and Raman spectra bands were observed in acid, rennet, bromelain, and CAFE due to their firm and strong texture of cheese while lower spectra were observed in cheese made from melon extract due to weak curdling and textural properties. These plant extracts are economical and easily available alternative sources for cheese production with higher protein and nutritional contents.

1. Introduction

Cheese is considered the major fermented dairy product with a nutritional constituent of the dairy industry which serves as a tremendous source of milk protein and fat, which is essential for a healthy lifestyle (Cifelli, 2021). Chymosin is the main milk-coagulating protease in animal rennet, it has been a well-known source of milk clotting for centuries. However, due to higher rennet prices, religious restrictions (Halal and Haram), vegetarian diet concerns, or restrictions on recombinant animal rennet, other protease milk clotting substitutes were sought after (Ab Mutalib & Hakim, 2023). Additionally, to satisfy the requirements of the consumers, animal rennet must also provide safety regarding their usage (Zain et al., 2023). Furthermore, cheese and other dairy products must be safe for consumption so that the flow of zoonotic toxoplasmosis in food chain can be encountered efficiently. Also, even if it is raw milk, the animal must be checked for any history of infection of their outbreak reports (Almuzaini AM, 2023). However, the rate of transmission depends on diverse sources and the varying percentages found in dietary materials (Javed & Alkheraije, 2023). The programs for food safety should be put in place to guarantee food safety and inform the public of the benefits of implementing food safety precautions (Alshaikh et al., 2023; Kukina et al., 2024).

Plants and compounds of plant origin have been proven to be beneficial for the health and well-being of humans and animals as they also provide the bacterium for microbial production of rennet (Nkosi et al., 2023; Abbas and Alkheraije, 2023; Rehman et al., 2023). Plants can provide multiple health aspects in body as well as showed promising functional aspects when used in certain foods (Bangulzai et al., 2022; Al-Hoshani et al., 2023; Abduallah et al., 2023; Saleh et al., 2023). There are various plant species, each of which depends on an element that is a

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https://doi.org/10.1016/j.fochx.2024.101256
Received 21 December 2023; Received in revised form 13 February 2024; Accepted 25 February 2024
Available online 28 February 2024

Table 1
Concentrations of treatments for cheddar-type cheese production.

Trials	Plant extracts	Concentrations (mL/L)
T_1 (Control)	Acetic acid	10
T_2 (Control)	Rennet	0.5
T_3	CAFE	35
T_4	Fig latex	35
T_5	Bromelain	35
T_6	Melon extract	35

part of the same plant but has a distinct number and kind of enzymes. Many different plants have aspartic proteases (APs), which play a role in plant senescence, responses to pathogens and stress, protein storage mechanisms, and protein breakdown throughout plant development (Folgado & Abranches, 2020). The plants productivity could be possibly increased through the introduction of hybrids and cultivars deemed prolific from different regions. These kinds vary and provide higher yields of enzymes and other bioactive components in conjunction with dense planting with specific improvements in genetic aspects (Adelaide et al., 2023; Djulardi et al., 2024).

These plant extracts provide specific milk clotting activity (MCA), proteolytic activity (PA), and functional properties such as flavor and texture enhancement in food products (Gupta et al., 2022). Plant proteases provide an essential part in cheese ripening at an early stage concerning their primary activity in milk coagulation as well as antioxidant properties and significant control over lipid oxidation (Sharma et al., 2023). Cheese casein micelles are hydrolyzed by the remaining coagulant, creating vital substrates for bacterial microflora and their decomposition enables the production of tastes as the cheese ripens (Nadi et al., 2024).

Citrus aurnatium flower extract (CAFE) showed MCA/ PA over a wide temperature range (35–70 °C) in milk. The MCA / PA ratio was sufficient to cause milk coagulation like the commercial rennet (Khan et al., 2019). Fig latex contains ficin enzyme whose application, mechanism of action, and physical properties still need to be explored. The research interest in ficin is increasing due to its proteolytic extract to have active fragments production of antibiotics, milk clotting, promiscuous activity, and meat tenderization. The plant extracts of figs have gained importance related to the health perception of consumers (Shabani et al., 2018; Tahir et al., 2023). Bromelain has a higher MCA/PA ratio with an optimum pH range between 6 and 7 at 70 °C when used for milk coagulation. The milk coagulation activity of bromelain showed stability over a wide range of pH thus it is effective over the entire gastrointestinal tract (Singh et al., 2023). It is safe and non-toxic but there is a need to explore its potential in food products to have health advantages (Banerjee et al., 2018; Imran & Alsayeqh, 2022). The melon extract from the fresh sarcocarp portion showed a higher MCA/PA ratio at optimum pH and temperature but MCA declined at lower temperature and pH levels (Khan et al., 2023).

The MCA / PA activities of plant extracts depend on the coagulant type, amount of coagulant used, and specific enzymatic activity. These plant extracts demonstrated variation in their characterization which depends upon their hydrolyzing capacity against diverse substrates over a wide range of temperatures and pH, and these extracts contained several proteases at an adequate concentration that were used for milk coagulation over broad pH ranges. Some of the attributes of these vegetative coagulants were expressed as chymosin-like characteristics, and they represented MCA and PA properties that were fairly like those of rennet (Ferragina, 2015).

Thus, this study focused on the use of CAFE, *Cucumis melo* L. (melon) extract, *Ficus carica* (fig) latex, and bromelain from *Annanas comosus* (pineapple) in their optimized MCA / PA activity with optimum time and temperature treatments to develop cheddar-type cheese and to evaluate the plant extracts effects on antioxidant activity. Raman and FTIR spectroscopy of acetic acid and animal rennet coagulated cheese was carried out to have more information about the microstructure composition and interactions of cheese components such as casein, fat, and other molecules to have a better understanding of the texture and rheological aspects.

2. Materials and methods

2.1. Collection and preparation of plant extracts

The extraction of plant extracts was performed at the Department of Food Science, University of Massachusetts, Amherst, MA, USA. CAFE, melon extract, fig latex, and bromelain as natural plant extracts were extracted by using the hot continuous extraction methods (Soxhlet) according to the method described by Khan et al. (2023). CAFE was prepared by blending citrus flowers with a cold buffer of Tris-HCl (20 mmol/L; 7.2 pH). The blend was filtered and stored till further use. The fresh melon extract was prepared by homogenization and centrifugation of melon mesocarp slice in a Beckman centrifuge (ThermoScientific CL10, Centrifuge, USA) and the extract was maintained at 4 °C or frozen till usage. Fig latex was obtained from the fig plant and no further processing was applied to the crude fig latex. The crude bromelain extraction was carried out by peeling, cutting, and grounding pineapple fruit. The filtrate was mixed with 0.1 M phosphate buffer and centrifuged at 3500 rpm for 15 min in the Beckman centrifuge (ThermoScientific CL10, Centrifuge, USA). Then, it was filtered and stored at 4 °C till application. The bovine milk (10 L for each treatment) was procured from the Equine and Livestock Research and Education Farm of the University of Massachusetts, MA, USA. Acetic acid and animal rennet type II 9042–08-4 were purchased from Sigma Aldrich, MA, USA. The milk was standardized and homogenized to the standardized fat and solid-not-fat (SNF) contents typically required for cheddar-type cheese development. All other chemical reagents were purchased from Sigma Aldrich, USA. All treatments were analyzed in triplicates for standardized readings.

2.2. Treatments and experimental design

The cheddar-type cheese was produced at the food pilot plant of the Department of Food Science, University of Massachusetts, Amherst, MA, USA. The experiment included the use of CAFE, melon extract, *F. carica* extract (fig latex), and pineapple extract (bromelain) at their optimized higher milk MCA and PA ratios (2.50 MCU/mg; approximately 35 mL/L at 45 °C). The MCA / PA ratio depends on the ability of plant extracts to demonstrate coagulation at optimal time and temperature treatments, pH, type of plant, protein content and amino acid profiling. Therefore, concentrations of the plant extracts were chosen based on their optimized MCA / PA ratio to have higher milk coagulation according to the methods mentioned in the study of Khan et al. (2023). The developed cheddar-type cheese was compared with acetic acid and rennet-coagulated controlled cheddar-type cheese samples. A total of six cheddar-type cheese production trails were planned (Table 1).

The cheese samples were evaluated for their physicochemical parameter analysis, antioxidant activities (ferric reducing antioxidant power (FRAP) and free radical scavenging activity (2,2-Diphenyl-1-picrylhydrazyl commonly known as DPPH) and rheological aspects concerning Fourier Transform Infrared Spectroscopy (FTIR), and Raman spectroscopy techniques.

2.3. Development of cheddar-type cheese

The cheddar-type cheese was developed by slight modification in the cheese production methods described by Fox et al. (2017). Fresh buffalo milk (10 L for each treatment) was purchased from the Equine and Livestock Research and Education Farm of the University of Massachusetts, MA, USA for each trial. Five liters of buffalo milk was used in each treatment to make the cheddar-type (smaller scale) cheese at the food pilot plant of the Department of Food Science, University of

Table 2
Effect of plant extracts on physicochemical parameters of cheddar-type cheese.

Treatments	Fat content			Protein content			Moisture content			pH			Salt contents		
	Week 0	Week 4	Week 9	Week 0	Week 4	Week 9	Week 0	Week 4	Week 9	Week 0	Week 4	Week 9	Week 0	Week 4	Week 9
T_1	36.32 ± 0.14^{b}	33.56 ± 0.11^{b}	29.12 ± 0.11^{c}	25.70 ± 0.14^{d}	24.11 ± 0.09^{b}	20.39 ± 0.13^{b}	32.97 ± 0.13^{b}	31.66 ± 0.18^{a}	26.49 ± 0.17^{b}	5.52 ± 0.10^{c}	5.52 ± 0.11^{b}	5.49 ± 0.07^{c}	2.46 ± 0.19^{a}	2.41 ± 0.06^{c}	2.38 ± 0.19^{a}
T_2	37.01 ± 0.20^{a}	33.12 ± 0.10^{b}	30.10 ± 0.12^{b}	25.31 ± 0.11^{d}	23.89 ± 0.12^{c}	20.92 ± 0.16^{b}	33.56 ± 0.18^{a}	31.64 ± 0.14^{a}	27.50 ± 0.19^{a}	5.53 ± 0.11^{b}	5.52 ± 0.10^{b}	5.51 ± 0.13^{a}	2.43 ± 0.10^{d}	2.41 ± 0.12^{c}	2.38 ± 0.16^{a}
T_3	37.57 ± 0.19^{a}	34.12 ± 0.16^{a}	31.04 ± 0.17^{a}	27.53 ± 0.16^{b}	25.33 ± 0.13^{a}	25.41 ± 0.17^{a}	32.63 ± 0.13^{b}	30.42 ± 0.12^{b}	26.09 ± 0.16^{b}	5.52 ± 0.12^{d}	5.52 ± 0.12^{b}	5.50 ± 0.13^{b}	2.45 ± 0.13^{b}	2.43 ± 0.14^{a}	2.37 ± 0.17^{b}
T_4	36.34 ± 0.13^{b}	34.56 ± 0.15^{a}	30.13 ± 0.13^{b}	26.08 ± 0.10^{c}	23.91 ± 0.14^{c}	20.30 ± 0.14^{b}	33.42 ± 0.15^{a}	29.70 ± 0.11^{b}	25.50 ± 0.14^{c}	5.54 ± 0.15^{a}	5.53 ± 0.18^{a}	5.50 ± 0.11^{b}	2.44 ± 0.13^{c}	2.43 ± 0.13^{a}	2.34 ± 0.12^{e}
T_5	36.17 ± 0.11^{b}	34.87 ± 0.17^{a}	29.97 ± 0.12^{c}	29.84 ± 0.18^{a}	25.80 ± 0.18^{a}	18.80 ± 0.09^{d}	33.66 ± 0.19^{a}	29.54 ± 0.10^{b}	25.41 ± 0.13^{c}	5.53 ± 0.13^{b}	5.52 ± 0.13^{b}	5.49 ± 0.10^{c}	2.43 ± 0.11^{d}	2.42 ± 0.11^{b}	2.36 ± 0.13^{c}
T_6	36.02 ± 0.10^{b}	34.00 ± 0.15^{a}	30.67 ± 0.14^{b}	25.48 ± 0.12^{d}	23.38 ± 0.11^{c}	19.02 ± 0.11^{c}	32.77 ± 0.11^{b}	29.65 ± 0.11^{b}	24.02 ± 0.11^{d}	5.54 ± 0.17^{a}	5.53 ± 0.15^{a}	5.50 ± 0.12^{b}	2.44 ± 0.12^{c}	2.43 ± 0.15^{a}	2.35 ± 0.10^{d}

Different small alphabets show significant differences among the different treatments ($P < 0.05$).
T_1 = Controlled cheese with acid; **T_2** = Controlled cheese with rennet, **T_3** = Cheese prepared with CAFE; **T_4** = Cheese prepared with bromelain, **T_5** = Cheese prepared with fig latex; **T_6** = Cheese prepared with melon extract.

Massachusetts, Amherst, USA. Milk was first pasteurized at 65 °C for 30 min. Then it was inoculated with 2 % of starting cultures added to all treatments (*Lactococcus lactis* subsp. *Cremoris* and *Lactococcus lactis* subsp. *Lactis*). The controlled treatments were coagulated for 45 min at 33 °C with rennet (0.002 %) and acetic acid (2 %) at their optimum level until the milk was coagulated while the plant extracts were used at their optimum coagulation concentration levels (optimized MCA/PA ratio) for milk coagulation. Then the firmed curd was cut, and stirred, and the whey was separated from the curd. Then, to further separate the whey from the curd, it was heated and cooked at 38 °C for 15 to 20 min. The cheese blocks are then pressed to remove extra whey. The cheese blocks were waxed with food-grade wax made from microcrystalline wax to protect them from microbes and molds. First, the coating wax is heated in temperature-controlled containers. The coating wax is applied to the product using a straightforward dipping method that can be done manually or with the use of mechanical devices (overhead handlers/ conveyor belts). The cheese surface must be waxed in a very careful, clean and thorough way so that there is no chance of environmental contamination. Salting was done at the rate of 2.5 % then by pressing the cheese blocks whey was removed. The cheeses were then stored and ripened for two months at 10 °C.

2.4. Physicochemical properties of cheddar-type cheese

The physicochemical properties such as pH, moisture, salt, fat, and protein contents of plant extracts-based cheddar-type cheese samples were compared with the controlled cheese samples by the methods of AOAC (Poitevin, 2016).

2.5. Antioxidant activity of cheddar-type cheese

The antioxidant activity of cheddar-type cheese developed from CAFE, melon extract, fig latex, and bromelain were measured by modifications in the method explained by Fardet & Rock (2018). The water-soluble cheddar-type cheese extract was diluted in a phosphate buffer (0.1 M) of pH 7 and their antioxidant activity was tested using FRAP and DPPH assay (Himed-Idir et al., 2021).

2.5.1. Ferric reducing antioxidant power assay (FRAP assay)

The antioxidant activity of cheddar-type cheeses (made from CAFE, *C. melo* L. (melon sarcocarp), and *F. carica* (Ficin)) was evaluated using FRAP assay. A ferric complex of 2,4,6-tris(2-pyridyl)-s-triazine and Fe3 + was used to conduct the assay (dissolving 0.31 g of TPTZ, 0.54 g Fe $Cl_3{\cdot}6H_2O$ in 100 mL of acetate buffer of 3.6 pH). After 4 min of incubation at room temperature in the dark, the 20 μL of cheese extracts were dissolved with 2 mL of ferric complex and then the absorbance was recorded at 593 nm with a spectrophotometer (Thermofisher Scientific Spectrophotometer, Evolution Series 201/220, USA) (Chapeau et al., 2016).

2.5.2. Free radical scavenging activity (DPPH assay)

The DPPH assay was evaluated by slight modification in the DPPH assay method as described by Vázquez-García et al. (2021). The extracts (50 μL) were dissolved in 2 mL of a methanol solution containing 0.06 mmol/L DPPH. The samples were incubated in the dark for 60 min, after which the reading from the spectrophotometer was at 517 nm. A Trolox calibration curve was prepared (0.02–1 mmol/L), and data were expressed in Trolox equivalent antioxidant capacity (mmol TEAC/kg). The below-mentioned equation was used to calculate the DPPH-scavenging activity.

$$\%\ \text{DPPH} - \text{scavenging activity} = \frac{\text{Absorbance (control)} - \text{absorbance (sample)}}{\text{Control absorbance}} \times 100$$

where Trolox was used for control absorbance and blank reading was recorded without DPPH addition.

2.6. Fourier Transform Infrared spectroscopy (FTIR) and Raman spectroscopy

The cheese sample analysis was done by IR Presige-21 Shimadzu FTIR, Massachusetts, USA with slight modifications in the method of Tarapoulouzi et al. (2020). While Raman spectroscopy was investigated by the method described by Yaman et al. (2022) and Zhang (2020).

2.6.1. FTIR spectroscopy

The fresh cheese samples were measured for 28 days of storage period. The direct measurement of the cheese sample was performed by taking slices of cheese (about 0.5 gm each sample) from different areas of the cheese samples. The cheddar-type cheese samples were then pressed with a high-pressure clamp to ensure good contact between the

Table 3
Effect of plant extracts on antioxidant activity of cheddar-type cheese.

Coagulants	Antioxidant activity							
	FRAP* (mmol Fe^{2+}/g)				DPPH* (Trolox equivalent μM/g)			
	0 day	20 days	40 days	60 days	0 day	20 days	40 days	60 days
Acid	3.12 ± 0.21^{f}	5.33 ± 0.43^{f}	8.30 ± 0.14^{f}	11.29 ± 0.18^{f}	421.03 ± 14.11^{f}	535.03 ± 13.12^{f}	686.02 ± 16.11^{f}	711.01 ± 19.10^{f}
Rennet	4.33 ± 0.45^{e}	9.32 ± 0.58^{e}	13.32 ± 0.30^{e}	16.28 ± 0.27^{e}	836.04 ± 15.12^{e}	956.03 ± 17.14^{e}	1098.03 ± 18.12^{e}	1131.02 ± 21.11^{e}
CAFE	7.38 ± 0.19^{ab}	14.45 ± 0.29^{c}	29.51 ± 0.23^{b}	38.55 ± 0.34^{b}	2143.08 ± 27.12^{b}	3304.07 ± 33.10^{b}	4421.05 ± 40.14^{b}	5013.03 ± 47.14^{b}
Bromelain	6.29 ± 0.17^{c}	11.37 ± 0.13^{d}	23.46 ± 0.59^{c}	29.51 ± 0.38^{c}	1054.06 ± 20.14^{c}	1164.05 ± 25.11^{d}	1267.04 ± 26.10^{c}	1376.02 ± 31.17^{c}
Fig latex	7.39 ± 0.20^{a}	17.47 ± 0.15^{a}	36.52 ± 0.29^{a}	42.57 ± 0.29^{a}	2243.09 ± 24.13^{a}	3406.06 ± 38.12^{a}	4674.04 ± 40.13^{a}	5219.03 ± 49.13^{a}
Melon extract	5.26 ± 0.64^{d}	13.30 ± 0.11^{b}	21.36 ± 0.40^{d}	28.44 ± 0.32 ^{cd}	931.04 ± 16.13^{d}	1042.03 ± 21.12^{c}	1189.03 ± 29.11^{d}	1256.02 ± 33.17^{d}

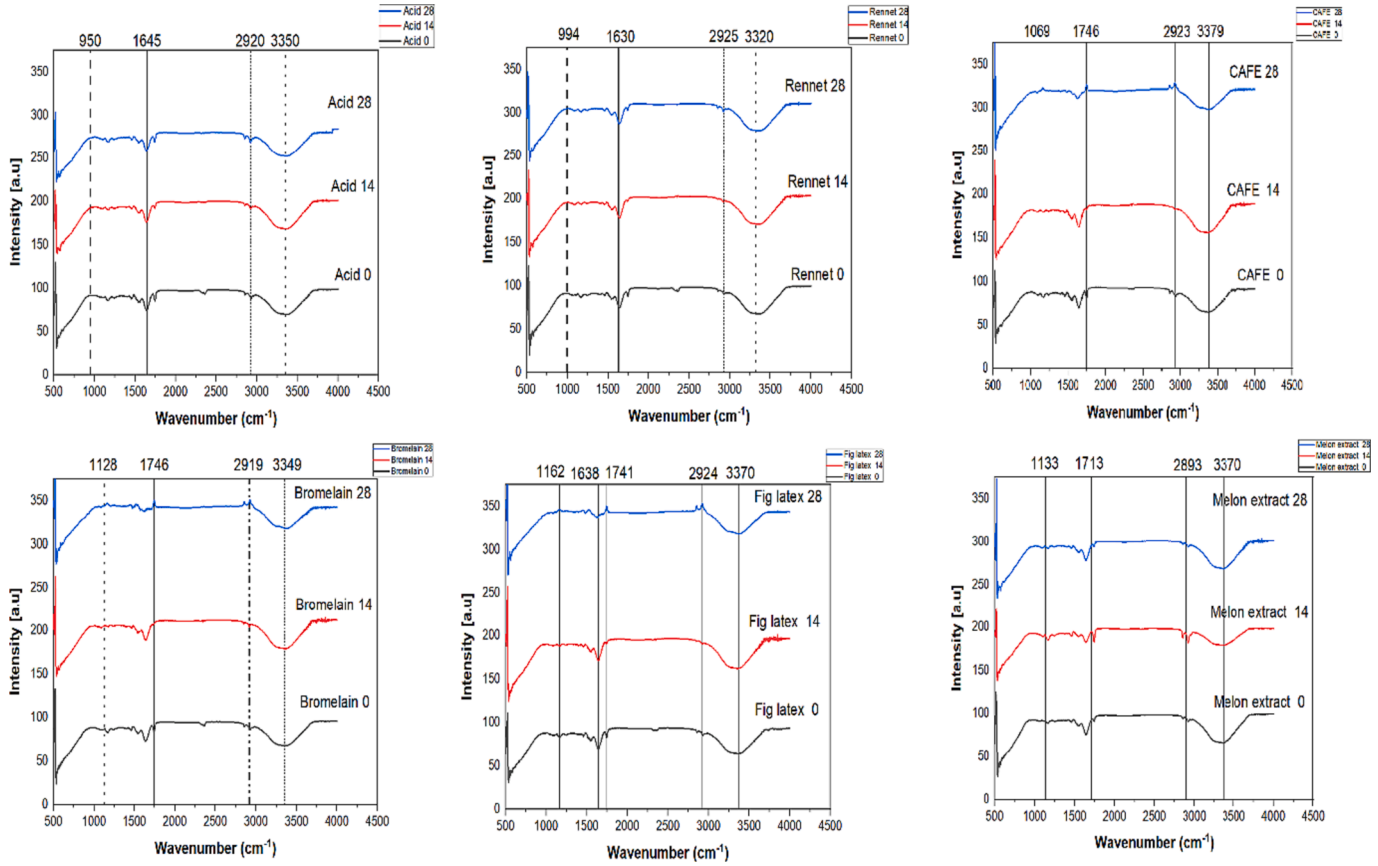


Fig. 1. FTIR spectra comparison of cheddar-type cheese developed from acetic acid, rennet, and plant extracts.

diamond crystal and the sample. The FTIR scanning process was performed on 400–4000 cm^{-1} wavenumber with the 4 cm^{-1} resolution. The signal was improved by adding 10 times measurements for each spectrum. A single laser beam was used to measure the absorption of the spectrum in comparison to the background level of air. Three spectra were collected for each sample of cheese to record precise observations. The wavenumber of the amide functional group of the samples was compared with the current standard to determine the functional group.

2.6.2. Raman spectroscopy

The cheese samples were cut into 50 μm thick slices. A 96-well plate was filled with cheese samples. The Raman spectra of the cheese sample were recorded at 0 and 28 days of storage period. The instrument used to record Raman spectra was the Metrohm MIRA-M1 laser Raman spectrometer (ProttezRaman-d3; Enwave Optronics Inc., Massachusetts, USA) with modifications of the method described by Yaman et al. (2022) and Zhang (2020). The laser with an excitation wavelength of 785 nm and 450 mW with an integration time of 100 s was used implementing Orbital Raster Scan (ORS) technology. A spectral range of 300 to 3000 cm^{-1} with a resolution of 1 cm^{-1} was set to operate the spectrometer at 4.5 s. The spectrum was obtained by choosing eight points (located on the different corner or middle positions of the cheese sample). The average spectrum of each sample was used in the chemometric analysis. Raman spectroscopy technology was used to detect the spiral intensity on acquisition time and to examine the increased dispersion of replicated spectra. The Raman peaks were compared with the standard Raman peaks for a better understanding of the textural and rheological aspects of the microstructure composition of the cheddar-type cheese.

2.7. Statistical analysis

Statistical analysis was performed to determine the significance level of the data obtained from each parameter of cheddar-type cheese samples using a Completely Randomized Design (CRD) (Montgomery, 2017). The significant difference comparisons were performed by Duncan's Multiple Range (DMR) Test (SAS 9.1 Statistical Software).

Table 4
FTIR spectra comparison of cheddar-type cheeses with standard FTIR spectra ranges.

Observed Wavenumber (cm^{-1})	Standard wavenumber (cm^{-1})	Amide types	Specific marker groups
Acetic acid (3350 ≤ 3300) Rennet (3320 ≤ 3300) CAFE (3379 ≤ 3300) Bromelain (3349 ≤ 3300) Fig latex (3370 ≤ 3300) Melon extract (3370 ≤ 3300)	3300	Amide A	N—H
Acetic acid (2920 < 3100) Rennet (2925 < 3100) CAFE (2923 < 3100) Bromelain (2919 < 3100) Fig latex (2924 < 3100) Melon extract (2893 < 3100)	3100	Amide B	N—H
Acetic acid (1645) Rennet (1630) CAFE (1746 > 1690) Bromelain (1746 > 1690) Fig latex (1638) Fig latex (1741 > 1690) Melon extract (1713 ≥ 1690)	1600–1690	Amide I	C═O
–	1480–1575	Amide II	C—N, N—H
Bromelain (1128 < 1229) Fig latex (1162 ≤ 1229) Melon extract (1133 < 1229)	1229–1301	Amide III	C—N, N—H
–	625–767	Amide IV	O—C—N
Acetic acid (950 ≥ 800) Rennet (994 ≥ 800) Fig latex (1069 > 800)	640–800	Amide V	C═O
–	537–606	Amide VI	N—H
–	200	Amide VII	Skeleton torsion

3. Results and discussions

3.1. Physicochemical analysis of cheddar-type cheese

Cheddar-type cheese samples were analyzed for physicochemical properties such as pH, fat, moisture, protein, and salt contents at 63 days of storage. Results showed no significant differences ($P < 0.05$) among pH, moisture, fat, and salt contents but a significant difference was observed in the protein content of cheese made from plant extracts as compared to acid and rennet-based cheeses due to higher protein contents of plant extracts (Table 2).

The fat contents remained the same over the storage period and a slight decline was observed due to the lipolysis of fat during 9 weeks of storage period. The fat contents of control T_1 decreased from 36.32 ± 0.14 to 29.12 ± 0.11 while T_2 showed a slight decrease from 37.01 ± 0.20 to 30.10 ± 0.12 fat contents after 9 weeks of storage. There was a slight decline in the fat contents of T_3 from 37.57 ± 0.19 to 31.04 ± 0.17 while fat contents in T_4, T_5 and T_6 showed same decline from 36.34 ± 0.13 to 30.13 ± 0.13, 36.17 ± 0.11 to 29.97 ± 0.12 and 36.02 ± 0.10 to 30.67 ± 0.14 respectively, after 9 weeks of storage. The leakage of fat globules with moisture occurred on the outer surface of cheese during storage at room temperature (Alinovi & Mucchetti, 2020). At room temperature, the fat globules showed signs of leaking from the cheese matrix, making cheese hard and compact with less free space for fat globules to fill in the curd matrix. Moreover, the higher fat content led to a fatty texture of cheddar-type cheeses, with intense fatty aroma and flavor development but the decrease in fat may also be due to clotting time and if curd is not firm enough then it leads to a reduction in curd firmness and cheese yield (Cao & Mezzenga, 2020).

The protein content of plant extracts was higher and showed a significant difference between acid and rennet-coagulated cheddar-type cheeses due to the higher protein content of the plant extracts. The protein contents of controls T_1 and T_2 were 25.70 ± 0.14 and 25.31 ± 0.11 respectively, which were similar to protein content of T_6 (25.48 ± 0.12). The protein contents in T_3, T_4, and T_5 were 27.53 ± 0.16, 26.08 ± 0.10 and 29.84 ± 0.18 respectively, which were higher in protein content as compared to controlled ones (Table 2). The type of protease that is present in the plant extract determines its protein content, but the diversity and activation of these enzymes define the nature of proteolytic activity. Plant extracts showed properties to enhance the protein content, but it also depends on plant type, origin, and extraction methods used to extract these plant extracts (Khan et al., 2023; Shabani et al., 2018). The protein contents in all cheese samples tend to decline during storage due to proteolysis during cheese ripening and storage conditions. The starter cultures and other microorganisms also contribute to proteolysis of protein which leads to breakdown of protein into smaller peptides and amino acids (Feeney et al., 2021). Such peptides and amino acids further contribute to the flavor and maturation of the cheese and lead to a decline in the total nitrogen protein contents. Some other factors such as improper storage conditions and contamination aspects may contribute to the decline of the protein contents in certain conditions (Paximada et al., 2021).

The results showed that no significant difference was observed among different concentrations of moisture content in all cheese samples (Table 2). The processing, storage, and ripening conditions affect the moisture retention in cheese. The cheese is considered a viscoelastic solid with a casein protein network entrapped with moisture and fat. Thus, moisture contents also prevent fat from leaking outside of cheese boundaries and overcooking of curd closes the holes and makes the curd brittle which decreases the moisture retention capability of the curd, and continuous decreases were observed during storage (Lamichhane et al., 2018). The firmness is related to cheese moisture and if entrapped fat globules leak from the moisture which can make the cheese much harder to have elasticity. Moisture regulation contributed to restoring the textural properties upon a 50 % ratio in cheese samples, but other storage and processing condition factors were also important for the better quality of cheese (Alinovi & Mucchetti, 2020).

The plant extracts-based cheddar-type cheese samples showed the same trend in pH, which is related to controlled ones made by using rennet, and no significant difference was observed in all treatments (Table 2). There was a decrease in pH from 6 to 5.52 during the cheese production process, which is necessary to continuously monitor the pH during curd formation, whey drainage, and ripening for the proper maturation of cheese samples. These plant enzymes exhibit a drop in pH after 2 months of storage when cheese samples were added with additives to support texture firmness (Grossmann & McClements, 2021). The higher time temperature treatments during the curdling process may lead to acidic pH, which may cause a problem in the rheological properties of cheese during maturation (Yano & Fu, 2022).

The salt contents tend to remain constant in ripening but a slight decline in the salt contents was observed due to the leak out of moisture contents from cheese samples after pressing or withering in storage conditions. This decrease in the trend of salt contents in cheeses showed no changes in the composition or quality of cheese and it also masked the bitterness that occurred by the vegetative coagulants by higher addition of salts or brine solutions to the cheese. The increase in the salt content increases the firmness but decreases springiness and cohesiveness during the texture analysis of cheese analogs (Rocchetti et al., 2021).

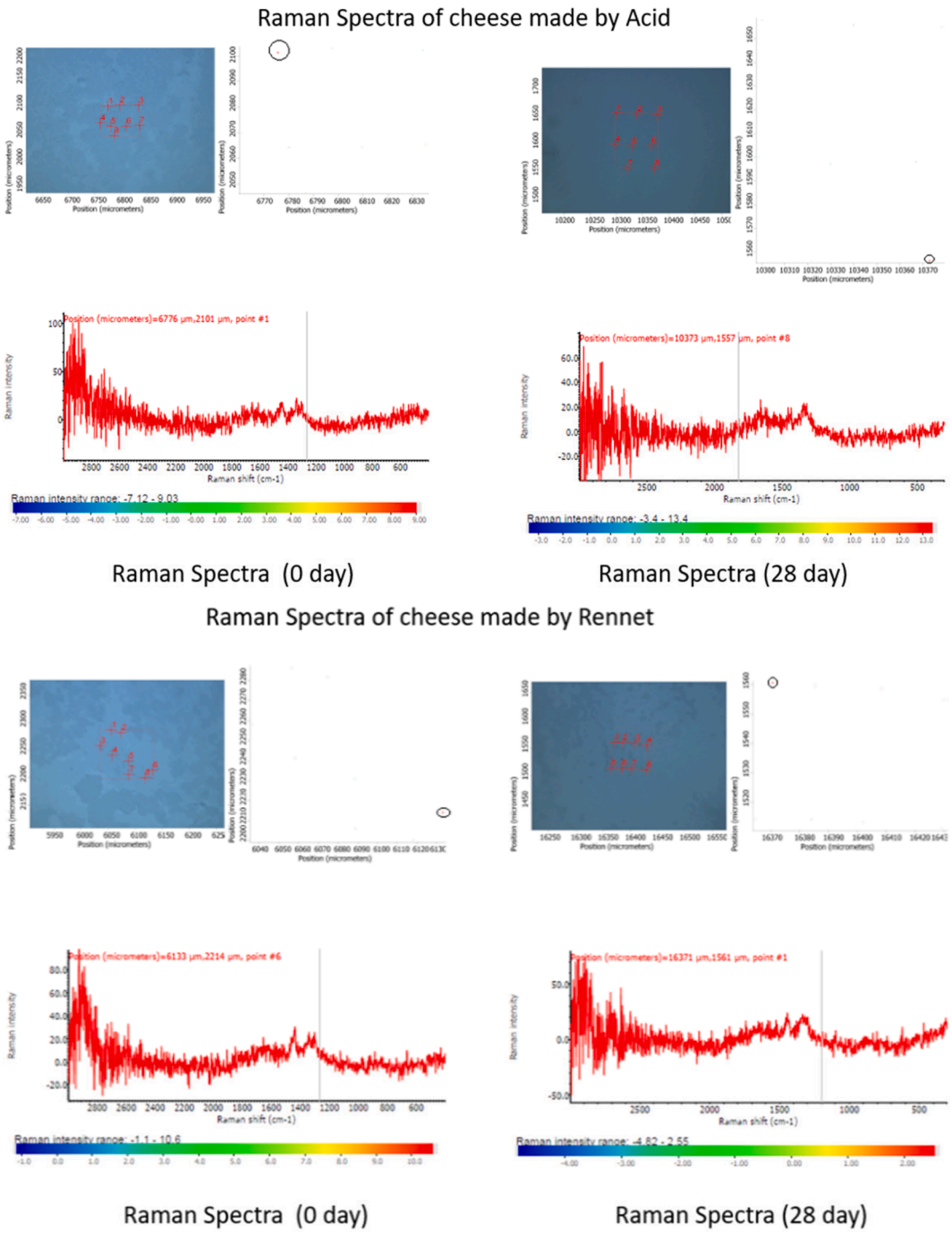


Fig. 2. Raman spectra comparison of cheddar-type cheese developed from acetic acid, rennet, and plant extracts.

3.2. Antioxidant activity

The plant coagulants expressed a higher antioxidant potential during ORAC analysis. Therefore, the plant-based cheese samples were explored for their antioxidant potential. The antioxidant activity of these extracts was evaluated by using 2 methods (FRAP and DPPH assay).

3.2.1. Ferric reducing antioxidant power (FRAP) assay

The results showed that FRAP values for fig latex (42.57 ± 0.29) and CAFE (38.55 ± 0.34) were higher than those of bromelain (29.51 ± 0.38) and melon (28.44 ± 0.32) after 60 days. The acid and rennet expressed the lowest FRAP activity values of 11.29 ± 0.18 and 16.28 ± 0.27 respectively, as compared to vegetative coagulants after 2 months of ripening (Table 3). The FRAP activity increased with the passage of the ripening period of cheeses while no significant effect was observed at 0 days, but it tended to increase during the ripening stage. The FRAP of fig latex and CAFE was higher due to the presence of phenolic compounds in their final form. The use of plant extracts in cheese enhanced the antioxidant activity due to the presence of higher total phenolic compounds (Asala et al., 2022). Thus, the increase in FRAP value was greater for ficin and CFE than the endogenous phenols of milk proteins while proteolysis was similar for each treatment, but it did not significantly affect the overall cheese composition. As a result, the increase in antioxidant activity of all cheddar-type cheeses led to chemical changes

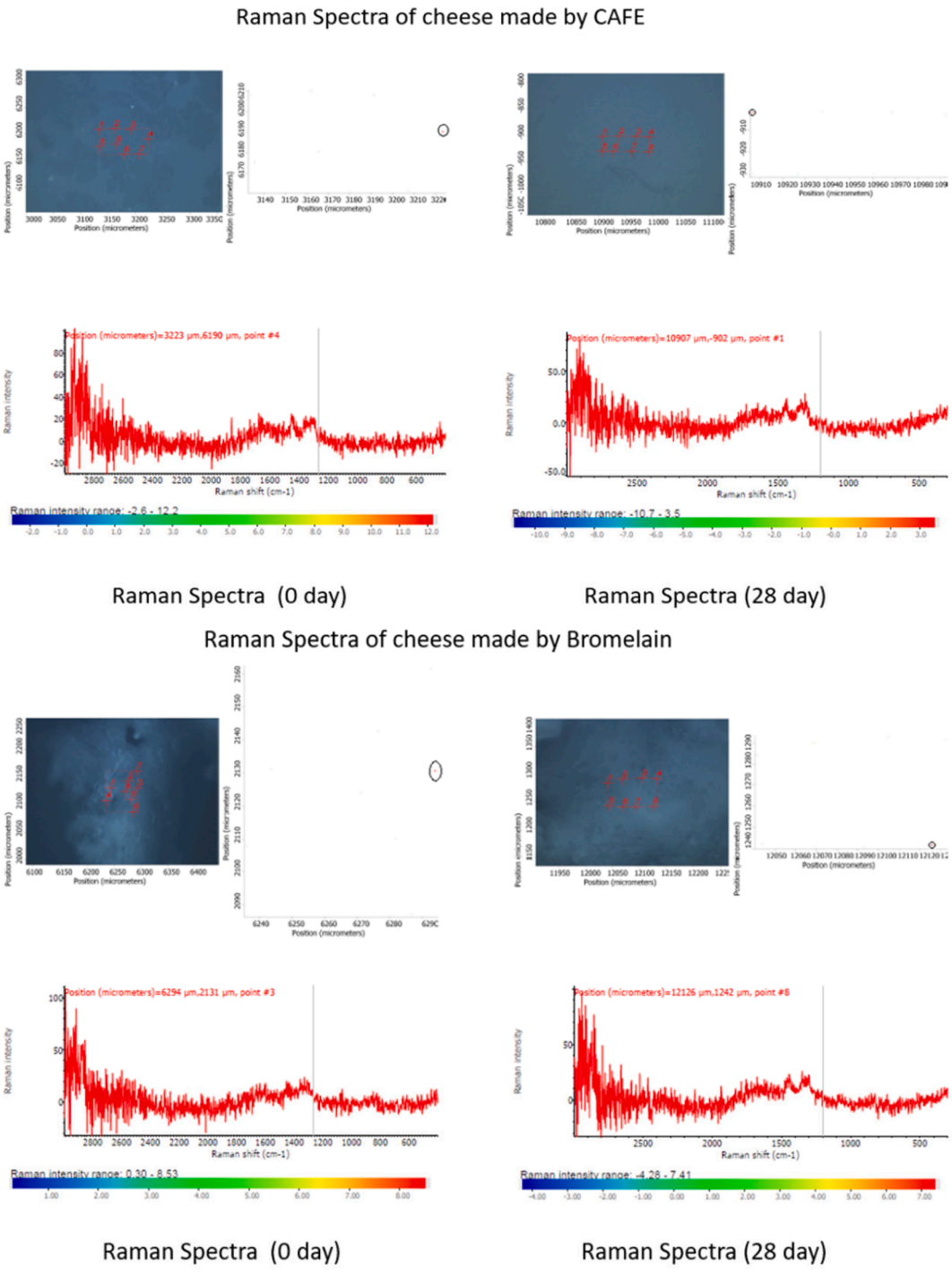


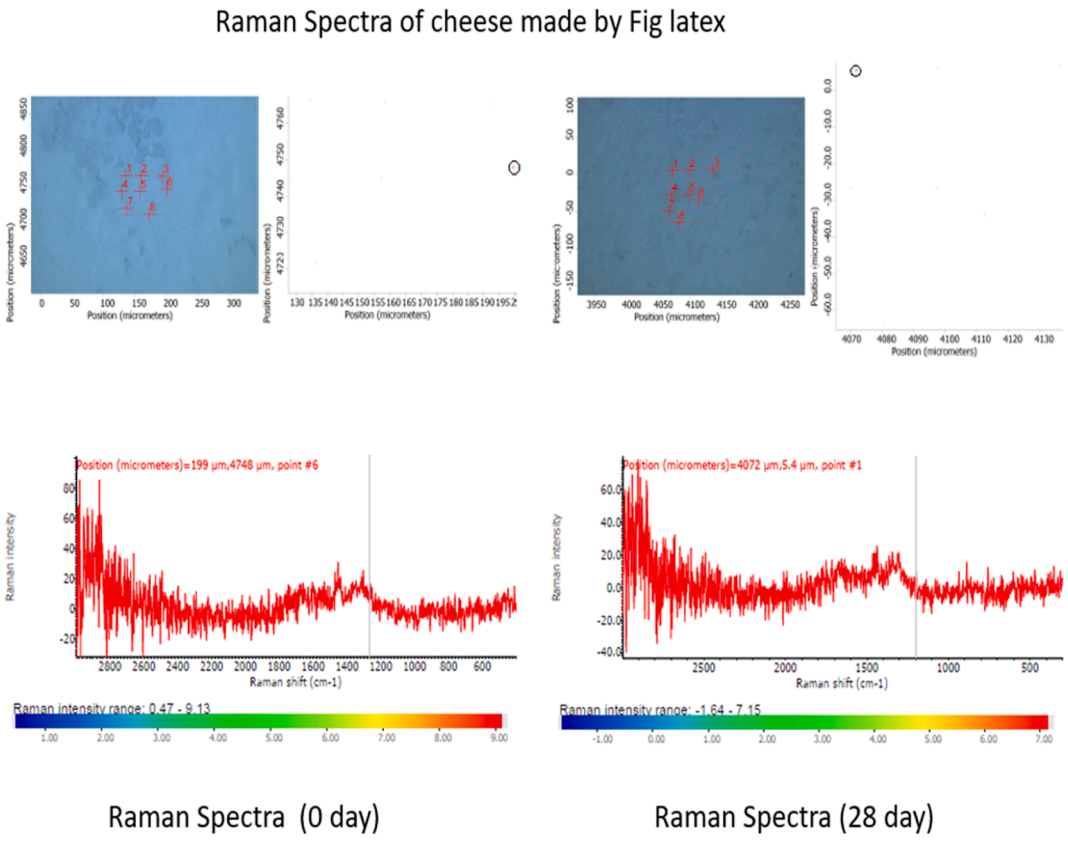
Fig. 2. (*continued*).

like proteolysis, which revealed the hidden antioxidant amino acid with sulfur (Granato et al., 2017). The FRAP ability of some amino acids did not show any antioxidant properties *in vivo* but contributed towards the antioxidant properties *in vitro* stage. This solemnly depends on the method of extraction of extracts and their use in the food product (Chávez-Servín et al., 2018).

3.2.2. Free radical scavenging activity (DPPH) assay

The antioxidant activity of cheddar-type cheeses made from CAFE, melon extract, fig latex, and bromelain was measured by DPPH assay. The higher DPPH activity values were observed with fig latex (5219.03 ± 49.13) and CAFE (5013.03 ± 47.14) while the bromelain (1376.02 ± 31.17) and melon extract (1256.02 ± 33.17) showed lower DPPH activity values. The rennet and acid-coagulated cheddar-type cheese had the lowest antioxidant activity values of 711.01 ± 19.10 and 1131.02 ± 21.11 respectively, after 60 days of ripening period. DPPH value expressed as Trolox equivalent μM/g, which is obtained from the Trolox solution with an antiradical capacity equivalent to that of the dilution of cheese extract (Table 3).

The antioxidant activity was decreased during cheese maturation in all cheese samples, which was dependent on the origin of free radicals and temperature used for different assays, but there was no significant effect on the overall composition of cheese samples (Melini et al., 2019). But this longer storage, different processing conditions, types of cheese,



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amount of extracts used, proteolysis and higher flavor compounds production of cheese affected their antioxidant activities which led to a decline in antioxidant activity after 2 month storage period (Lone et al., 2023; Yang et al., 2021). The antioxidant activity of ethanolic extracts, total phenolic content (TPC), and flavonoids varies among all plant extracts depending on the plant material, their solid-solvent ratio, and extraction methods of plant extracts. There is a dire need to evaluate the extract on their antioxidant potential in both *in vivo* and *in vitro* potential for a better future of the antioxidant potential of such vegetative coagulants as functional food ingredients (Latif et al., 2021).

Different small alphabets show significant differences among the different treatments ($P < 0.05$).

3.3. Fourier Transform Infrared spectroscopy (FTIR)

The amide types observed in spectra of cheese samples made from vegetative coagulants showed significant differences from acid and rennet amide types. This difference was due to the higher protein content and presence of vegetative protein with milk protein in the cheese samples which led to the shift of the spectra band to a higher place (Fig. 1).

The decrease in fat constituents and the slight increase in protein contents of cheese made from plant extract and thus amides showed higher wavenumbers than standards (Tarapoulouzi et al., 2020). The FTIR spectra showed the types of amides which are easy-to-identify protein group markers, which were detected by FTIR due to specific functional groups, and then comparing them with standard amide functional groups (Table 4).

The FTIR spectra of plant-based cheese show that proteins are present at 1700–1600 cm^{-1} and that lipids and carbohydrates are present at 1300–600, 1750, and 3000–2800 cm^{-1}. The amide I band spectra were observed with a range of 1600 to 1700 cm^{-1} for acid and rennet while plant extracts showed amide I band at a little higher range of 1700 to 1750 cm^{-1}. The water absorbs in the region of 3000 to 3600 cm^{-1} and strongly above 1650 cm^{-1}. The moisture affected the multiple N—H bonds (Amide A and Amide B) in 2500 to 3500 cm^{-1} regions. At the initial stage, spectra masking was predominant due to free water. The moisture of cheese affected the spectra of refrigerated cheese samples as it masked or modified strong broad brands (Pax et al., 2019). Strong bands were observed for all cheese samples from 2900 to 3500 cm^{-1} range of spectra. The secondary structure of the protein in cheese made with acid and rennet was reflected in the IR spectrum due to the region of amide I (1600–1690). The strong broad bands at 2700 to 3300 cm^{-1} and the amide I band in the region between 1600 and 1700 cm-1 were either masked or modified by the moisture in cheese, which had an impact on the spectra of microtome-frozen cheese samples. This outcome supported the findings by Alkhalf Maha and Mirghani (2017) that water absorbs strongly between bands at 3000 and 3600 cm^{-1} while bands at 1650 cm^{-1} lead to affect the textural properties of the cheese depending upon the storage conditions for ripening.

The next important aspect was whether the FTIR spectrum-based prediction of cheese total solids was a more precise estimation of fat and protein (as well as other components in cheese) retention in cheese curd or simply a different representation of the constant proportion of its fat and protein contents (Mota et al., 2022). Finally, the lower ability of FTIR calibration to forecast fat recovery than protein given higher energy value of fat as compared to protein which explains the decreased accuracy of recorded energy of FTIR spectrum compared to cheese solid (Fan et al., 2023).

Additionally, non-uniform cheese sample slices, voids in the cheese matrix, non-homogeneity in the fat and bound moisture in the protein matrix were the primary cause of the difference in the amide spectra from the standard spectra of all the cheese samples. Thus, it could be linked with β structure while the amide spectrum was higher in cheese made from plant extract due to higher vibrational stretching of the carbonyl groups and bands thus representing helical and random

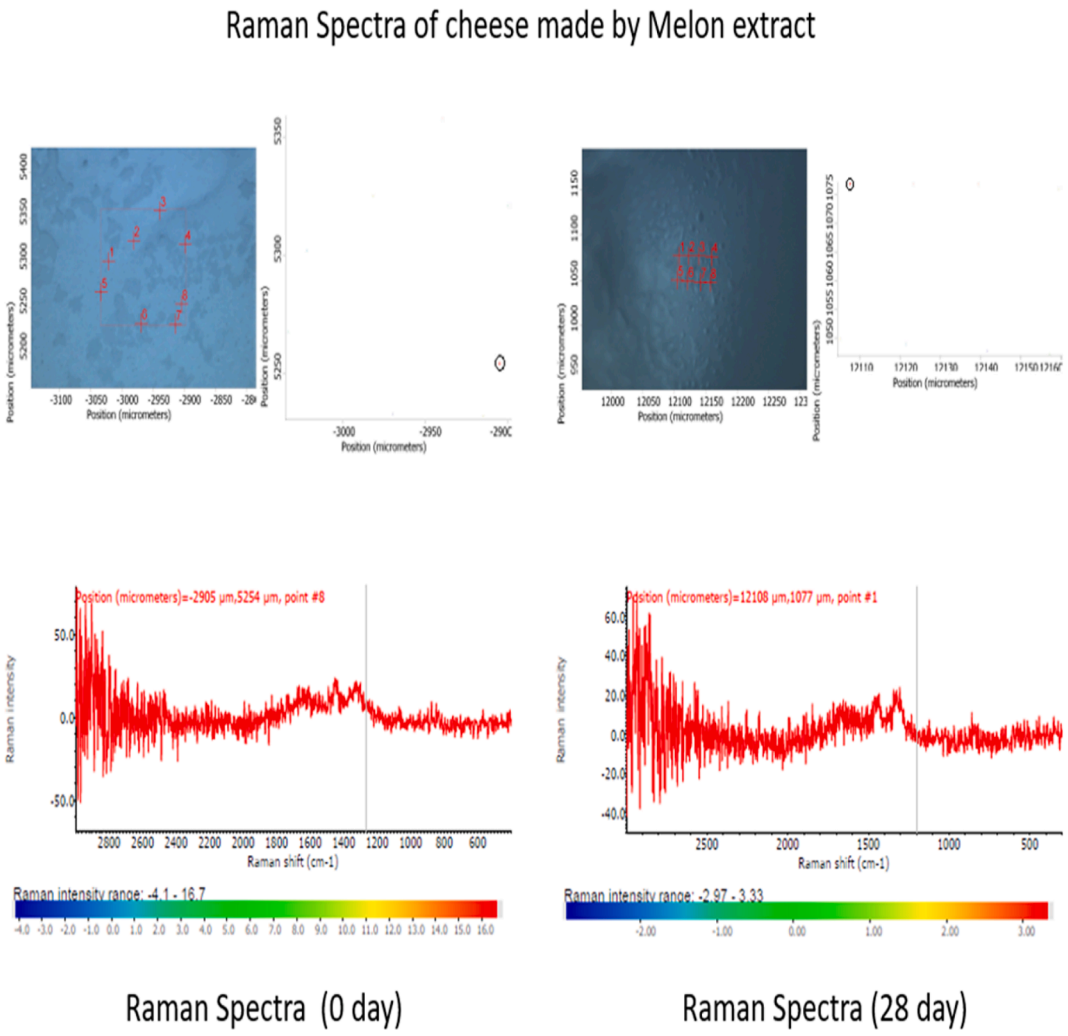

Fig. 2. (*continued*).

portions of proteins (Ferragina et al., 2013).

The numerous N—H bonds are also impacted by the moisture bands. Despite all efforts to obtain uniform samples, non-homogenized milk was used, which resulted in the non-homogeneity of the cheese samples used in FTIR tests. Another likely explanation could be the presence of holes in the cheese matrix as well as the non-homogeneity of fat and binding moisture in the protein matrix (Leite et al., 2019). The lower absorbance was at the start and higher bandwidths were due to the decrease in fat as a result of lipolysis during the storage of cheese. As a result, when the amount of fat in cheese was reduced, the absorbance at bands associated with fat also decreased whereas bands related to protein showed the opposite trend. FTIR results can be combined with electrophoresis results (SDSD-PAGE) to study the protein characteristics, chemical groups, and related compounds (Schreuders et al., 2021).

3.4. Raman spectroscopy

Raman spectroscopy revealed rich components and molecular vibration information of cheese samples. The higher bands were observed in bromelain and CAFE due to their firm and strong texture of cheese while the lowest spectra were observed in cheese made from melon extract due to weak curdling and textural properties (Fig. 2).

The spectra shift to the higher bands after 28 days of storage due to proteolysis and this activity of lower to sudden higher change of spectra bands still needs to be evaluated. The appearance of the cheese sample was similar with eight points of randomly selected data and the complexity and fluctuation in this data occurred, but high consistency was observed in overall spectra (Chawanji et al., 2022). The observed cheese sample spectra were compared with the standard spectra bands (Table 5).

The higher Raman above 1700 cm^{-1} peaks that attributed to the C═O ester stretching of fatty acid molecules. The Raman peaks above 1600 cm^{-1} showed the characteristics of amide I and unsaturated fatty acids with C═O stretching vibration. The weaker bands above 1300 cm^{-1} were attributed to the amino acid phenylalanine and the lowest bands were observed with CH_2 deformation, twisting, and vibration which was expressed as carbohydrates and lipids (Sha et al., 2020). Fig latex showed the spectra bands in a similar range of acid. Rennet expressed the spectra with medium to high bands after 28 days with continuous bands spectra. The vegetative extracts showed higher bands of spectra due to the higher protein content and presence of vegetative protein with milk protein in the cheese samples which led to the shift of the spectra band to a higher place. But it also depends upon the type of plant, extraction method, and processing conditions used for the extraction (Nasiri & Hanifian, 2022). Carbohydrate-related vibrational modes with distinctive characteristics can be seen in the spectral region between 1100 and 950 cm^{-1} where plant protease resides with the vibrational and rotational bonds of lipids and protein amides. These amides showed strong vibrational bonds thus comparable to each other these can be separated for identification of amid bond types and their contribution in the curd formation (Genis et al., 2021). The vibrational modes are related to the vibrational mode of the β-1–4 glycosidic bond

Table 5
Raman spectra comparison of cheddar-type cheese with standard Raman spectra bands.

Observed wavenumber (cm^{-1})	Standard wavenumber (cm^{-1})	Marker group assignment
Acetic acid (0 days: 2545 ≥ 2500; 28 days: 2890 ≤ 3000) Rennet (0 days: 2590 ≥ 2500; 28 days: 2950 ≤ 3000) CAFE (0 days: 2625 ≥ 2500; 28 days: 2901 ≤ 3000) Bromelain (0 days: 2603 ≥ 2500; 28 days: 2880 ≤ 3000) Fig latex (0 days: 2545 ≥ 2500; 28 days: 2890 ≤ 3000) Melon extract (0 days: 2527 ≥ 2500; 28 days: 2981 ≤ 3000)	2500–3000	$\nu_{ass}(CH_2)$
Acetic acid (0 day: 1935 ≤ 2000; 28 days: 2490 ≤ 2500) Rennet (0 days: 1899 ≤ 2000; 28 days: 2401) CAFE (0 days: 1995 ≤ 2000; 28 days: 2345) Bromelain (0 days: 2010; 28 days: 2487) Fig latex (0 days: 2234; 28 days: 2445) Melon extract (0 days: 1874 ≤ 2000; 28 days: 2401)	2000–2500	$\nu_S(CH_3)$
–	1850–2000	$\nu_S(CH_2)$
Acetic acid (0 days: 1754; 28 days: 1825) Rennet (0 days: 1764; 28 days: 1848) CAFE (0 days: 1763; 28 days: 1803) Bromelain (0 days: 1790; 28 days: 1843) Fig latex (0 days: 1782; 28 days: 1833) Melon extract (0 days: 1753; 28 days: 1822)	1750–1850	$\nu(C{=}O)_{ester}$
Acetic acid (0 days: 1660; 28 days: 1734) Rennet (0 days: 1678; 28 days: 1741) CAFE (0 days: 1690; 28 days: 1735) Bromelain (0 days: 1677; 28 days: 1746) Fig latex (0 days: 2234; 28 days: 1732) Melon extract (0 days: 1664; 28 days: 1712)	1650–1750	$\nu(C{=}O)$ amide I; ν (C═C)
Acetic acid (0 days: 1610; 28 days: 1632) Rennet (0 days: 1603; 28 days: 1638) CAFE (0 days: 1611; 28 days: 1628) Bromelain (0 days: 1601; 28 days: 1619) Fig latex (0 days: 1611; 28 days: 1635) Melon extract (0 days: 1620; 28 days: 1631)	1600–1650	$\nu(C{-}C)_{ring}$
–	1450–1600	$\delta(CH_2)$
Acetic acid (0 days: 1321; 28 days: 1425) Rennet (0 days: 1310; 28 days: 1432) CAFE (0 days: 1307; 28 days: 1434) Bromelain (0 days: 1309; 28 days: 1427) Fig latex (0 days: 1313; 28 days: 1436) Melon extract (0 days: 1305; 28 days: 1446)	1300–1450	$\tau(CH_2)$
Acetic acid (0 days: 1132; 28 days: 1298) Rennet (0 days: 1135; 28 days: 1294) CAFE (0 days: 1142; 28 days: 1287) Bromelain (0 days: 1155; 28 days: 1296) Fig latex (0 days: 1134; 28 days: 1277) Melon extract (0 days: 1195; 28 days: 1283)	1130–1300	$\nu(C{-}O) + \nu$ (C—C) + δ (C—O—H)
Acetic acid (0 days: 1093; 28 days: 1123) Rennet (0 days: 1103; 28 days: 1127) CAFE (0 days: 1091; 28 days: 1120) Bromelain (0 days: 1094; 28 days: 1122) Fig latex (0 days: 1134; 28 days: 1277) Melon extract (0 days: 1195; 28 days: 1283)	1090–1130	$\nu(C{-}O) + \nu$ (C—C) + δ (C—O—H)
Fig latex (0 days: 1082; 28 days 1086) Melon extract (0 days: 1083; 28 days: 1089)	1080–1090	$\nu(C{-}O) + \nu$ (C—C) + δ (C—O—H)
Acetic acid (0 days: 1023; 28 days: 1063) Rennet (0 days: 1021; 28 days: 1068) CAFE (0 days: 1028; 28 days: 1043) Bromelain (0 days: 012 ≤ 1019; 28 days: 1076 ≥ 1070) Fig latex (0 days: 1026; 28 days: 1052) Melon extract (0 days: 1030; 28 days: 1055)	1019–1070	Ring-breathing (phenylalanine); ν (C—C) $_{ring}$
Acetic acid (0 days: 942; 28 days: 1001) Rennet (0 days: 957; 28 days: 1005) Fig latex (0 days: 992; 28 days: 1007) Melon extract (0 days: 928 ≤ 938; 28 days: 1003)	938–1010	δ(C—O—C) + δ (C—O—H) + ν (C—O)
–	850–925	δ(C—C—H) + δ (C—O—C)
–	620–825	δ(C—C—O)
–	450–600	Glucose
–	230–384	Lactose

and are ascribed to CO stretching. The CC and COH deformation modes (1120–1064 cm^{-1}), and COC deformation (950–870 cm^{-1}) showed no rotational vibrations for the plant protease neither the vibrations were observed for Acetic acid and rennet coagulated cheese samples. The difference is simple which is based on the presence of a band in 1700 to 1800 cm^{-1} that is characteristic of lipids that show lower variations in acetic acid and rennet coagulated one cheese (1600 to 1745 cm^{-1}). Using the correct marking by resampling approach, the uncertainty calculation during sample classification was carried out, allowing the building of a more reliable classification model and reducing the

likelihood of misclassification (de Sá Oliveira et al., 2016).

The bands at 1600 to 1650 cm^{-1} were assigned to ring vibration of aromatic amino acids and observed more clearly in the Raman spectra than in the FTIR spectra. The most prominent band were in the region of 1650 cm^{-1} and above 1800 cm^{-1} that is assigned to α-helix segments (Smith et al., 2017).

An increase in β-sheet structures was observed in the plant-based cheese during ripening that could be deduced from the intensity increase at 1650 cm^{-1}, continuing up to the end of the storage period (Zhang et al., 2023). The aromatic bands of the side chain also change in intensity over the entire ripening period. This increase in unordered structures, possible increase in turns, and changes in side-chain aromatic amino acid bands also took place during ripening in the cheese before being frozen when placed in the refrigeration temperature. The spectra (which are not included) also indicated that β-sheet structures increased over the first week of storage at the expense of a decrease in α-helices structure. Similar spectral changes were also observed when cheese was frozen under liquid nitrogen vapors (Li Vigni et al., 2020). During 3 months of ripening, the Raman spectra showed exponential changes in the binding and vibrational structure of lipids and protein in the plant-based cheeses that expressed significant changes in freezing processes and shelf life at the end of ripening (Li et al., 2022).

Conclusion

The plant extracts proved better, economical, and easily available commercial sources for milk coagulation as a replacement for animal and microbial rennet. These plant extracts showed antioxidant potential. The antioxidant potential depends upon the type of plant, extraction method, and time and temperature treatment during milk coagulation. The acetone mixtures performed better than methanol ones in the polyphenol extraction method. In most cases, the number of extraction stages had a statistically significant impact on both the phenolic extraction yield and the antioxidant capacity of plant extracts. The CAFE and bromelain showed higher coagulation activity and cheese with better textural properties. While higher antioxidant activity was observed in the cheese made from CAFE and fig latex. Further studies are needed to investigate the naturally derived plant extracts and their polyphenols to understand their physiological effects on the human body. The FTIR and Raman spectroscopy of cheddar-type cheese observed the qualitative estimates of protein and fat complex, and future study is needed to implement these spectroscopy techniques for better textural, rheological, and sensorial aspects. Future research must be focused on the involvement of amino acids in flavor development, and how to produce cheese with better flavor. Additionally, this study will provide new opportunities in the food science research field to characterize and modify plant extracts to obtain active bio-peptides for numerous health benefits such as anti-photo-aging, antioxidant, anti-cancer, anti-hypertensive and cholesterol-lowering effects.

Ethical approval

Not applicable.

Authors contributions

Usman Mir Khan: Conceptualization, methodology, software, and writing-original draft. **Aysha Sameen:** Supervision, Methodology, writing-review, and editing. **Eric Andrew Decker:** Resources, methodology, writing-review, and editing. **Muhammad Asim Shabbir:** Writing review, and editing. **Shahzad Hussain:** Writing review, and editing. **Anam Latif:** Writing review, and editing. Gholamreza Abdi: Funding, Conceptualization, writing review, and editing. **Rana Muhammad Aadil:** Supervision, methodology, conceptualization, writing review, and editing.

Funding

The authors received no funding to conduct this study and to assist with the preparation of this manuscript.

CRediT authorship contribution statement

Usman Mir Khan: Writing – review & editing, Writing – original draft, Software, Resources, Project administration, Methodology, Data curation, Conceptualization. **Aysha Sameen:** Supervision, Project administration, Methodology, Investigation, Conceptualization. **Eric Andrew Decker:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Muhammad Asim Shabbir:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Shahzad Hussain:** Visualization, Validation, Investigation, Funding acquisition, Conceptualization. **Anam Latif:** Writing – review & editing, Visualization, Data curation. **Gholamreza Abdi:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization. **Rana Muhammad Aadil:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Data curation, 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.

Data availability

No data was used for the research described in the article.

Acknowledgment

The authors are thankful to the National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan. The authors are grateful to the Higher Education Commission (HEC) of Pakistan for providing the HEC Indigenous scholarship and HEC International Research Support Initiative (IRSIP) opportunity to carry out the research in the USA. The authors are also thankful to the Department of Food Science, University of Massachusetts, Amherst, USA for facilitating this research. The authors also appreciate the support from the Researchers Supporting Project number (RSPD2024R1073), King Saud University, Riyadh, Saudi Arabia.

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