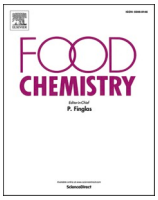
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Characterization and comparative analysis of saponins in various spinach (*Spinacia oleracea* L.) cultivars according to growing seasons

Su-Yeon Lee [a,b], Doo-Young Kim [a], Hyun-Jae Jang [a], Seon Min Oh [a], Hyoung-Geun Kim [a], Jongmin Ahn [a], In-Seo Heo [a], Heung Joo Yuk [c], Eun Kyoung Seo [b,*], Sei-Ryang Oh [a,*], Hyung Won Ryu [a,*]

[a] *Natural Product Research Center and Natural Product Central Bank, KRIBB, 30-Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungcheongbuk-do 28116, Republic of Korea*
[b] *College of Pharmacy, Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul 03760, Republic of Korea*
[c] *KM Science Research Division, Korea Institute of Oriental Medicine (KIOM), Daejeon 34054, Republic of Korea*

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ABSTRACT

Spinach is valued for its nutritional benefits, containing essential vitamins, minerals, and functional compounds that support health, particularly through anti-inflammatory and immunomodulatory properties. However, the specific metabolites responsible for these effects remain unidentified. This study quantified total saponin content in 15 spinach cultivars harvested in different seasons, expressed on a dry matter basis (mg/g D.M.), using gravimetric, spectrometric, and HPLC-CAD methods. Five new oleanane-type saponins, spinaciasaponin A, B, D, and E (**1**, **2**, and **4**–**6**), as well as the known compound celosin I (**3**), were isolated and quantitatively analyzed. The highest total saponin content was found in the spring, particularly in Luckyyou (3.2821 mg/g D.M.) and Shinwoldong (3.2214 mg/g D.M.) cultivars. Spinaciasaponin B (2) was most abundant in Luckyyou (1.1431 mg/g D.M.), while spinaciasaponin E (6) peaked in Shinwoldong (1.3668 mg/g D.M.). This study provides insights into saponin profiling and variation across spinach cultivars and seasons.

1. Introduction

Spinach (*Spinacia oleracea* L.) is a widely consumed vegetable providing vitamins (vitamin A, C, K and folate) and minerals (magnesium, potassium, and calcium), and regarded as a health-promoting food source of essential nutrients (Sarma & TR, 2024). These nutrients support various body functions, including immune system health (Mudondo, Happy, Gang, Ban, & Kang, 2025; Roberts & Moreau, 2016), bone health (Adhikary, Choudhary, Bordoloi, Jadeja, & Salunke, 2017), and red blood cell production (Natalia, Silalahi, Insani, & Sinambela, 2019). As interest in plant-based diets and functional foods grows, spinach continues to be an important subject of study because of its potential to increase overall health and well-being (Sarma & TR, 2024). However, the *Spinacia* genus exhibit a range of botanical and morphological characteristics (Fujito et al., 2015), and vary in leaf shape, size, and color and can have different growth habits depending on the environmental conditions and cultivar selection (Nešković & Ćulafić, 1988; Serpolay et al., 2011), and climate conditions affect their chemical composition and nutritional content significantly (Christopoulos & Ouzounidou, 2021; Kim et al., 2024; Kumarasinghe et al., 2024). As an easy-to-grow leafy vegetable, spinach thrives in various cultivars, climates and regions across the world (Deveci & Uzun, 2011; Panda & Shinde, 2016), and there are approximately 550 cultivated varieties of spinach in Korea according to the Korea Seed & Variety Service (https://www.seed.go.kr/sites/seed_eng/index.do).

The numerous effects of spinach have been reported including antioxidant, anti-inflammatory, anticancer (Roberts & Moreau, 2016), bone strengthening (Adhikary et al., 2017), eye nourishment (Olasupo, Aborisade, & Olagoke, 2018), anemia prevention (Mitchell & Miller, 1967), and hypertension prevention (Jovanovski et al., 2015) was known to be due to flavonoids and minerals, which are the main components. In contrast, the specific components of spinach that are responsible benefits to cognitive function (Jiraungkoorskul, 2016), the cardiovascular system (Panda, Bhandare, Mistry, Sudhamani, & Dande, 2022), the immune system (Ishida, Ose, Nishi, & Sugahara, 2016), skin health (Joshi, Kalambe, Jadhav, & Mana, 2023; Kim et al., 2024), anti-

* Corresponding authors.
E-mail addresses: yuny@ewha.ac.kr (E.K. Seo), seiryang@kribb.re.kr (S.-R. Oh), ryuhw@kribb.re.kr (H.W. Ryu).

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diabetic effects (Islam et al., 2018), and the liver (Flores-Estrada et al., 2023) have not yet fully been elucidated. According to previous studies (Elouafy et al., 2023; Liu et al., 2023; Ma et al., 2022; Park, Kim, & Choi, 2025; Wang, Chen, Lin, Feng, & Chen, 2022), these benefits may be related to the saponins suggesting that saponins in spinach may contribute the various biological effects of spinach. Previous studies have reported the anti-inflammatory and immunomodulatory pharmacological activities of spinach extracts (Mondal, Sinha, Maiti, Sathian, & Guha, 2015; Zhu et al., 2024), a potential role of spinach in regulating immune responses and reducing inflammation (Cartford, Gemma, & Bickford, 2002; Ishida et al., 2016; Lomnitski et al., 2000), inhibition of cancer cell proliferation and induce apoptosis (Kumar, Tripathi, Maurya, Nuwad, & Gautam, 2022) indicating that the cellular signaling pathways might be involved in immune regulation and inflammatory processes. These reports suggested that the saponins of spinach may offer therapeutic benefits in managing chronic inflammatory and immune-related diseases. Recent reports have indicated the presence of saponins in spinach (Ahmed, 2024; Cerulli, Polcaro, Masullo, & Piacente, 2024), which has led to increasing interest in their occurrence and potential nutritional relevance. However, further studies are needed to elucidate the precise mechanisms of saponins underlying these effects and explore their applications for immunomodulation and inflammation-related disorders.

Therefore, we investigated spinach saponins to offer new insights into their role in chronic inflammation and immune regulation besides well-known flavonoids and fatty acids. In this study, the total saponin contents of 15 spinach cultivars were examined, and major saponins were identified through NMR and UPLC-QTOF-MS techniques, the saponin composition and quantitation among the spinach cultivars and growing seasons were analyzed using UPLC-CAD to select cultivars showing high content of saponins as highly functional food materials.

2. Materials and methods

2.1. General experimental methods

High-performance liquid chromatography (HPLC) analysis was performed on a Thermo Ultimate 3000 system (Thermo Fisher Scientific, MA, USA) equipped with a photodiode array (PDA) detector and charged aerosol detector (CAD, Thermo). Ultra-high-performance liquid chromatography (UPLC) analysis was conducted using an ACQUITY UPLC I-Class system (Waters Corporation, Milford, MA, USA) coupled with PDA and CAD detectors. For tentative identification of extracts and compounds, the UPLC I-Class system was connected to a Vion IM-QTOF mass spectrometer (Waters Corporation) to obtain HRESIMS spectroscopic data. Fractionation was performed using multiple preparative HPLC (MPLC, k-prep LAB 100S, YMC, Kyoto, Japan) with a DAD-50-700S column (700 × 50.0 mm, 10 μm, YMC) packed with YMC ODS-AQ HG resin. Compound isolation and purification were performed using an MPLC system (LC-Forte/R series, YMC) equipped with an X-bridge column (250 × 20.0 mm, 5.0 μm, Waters Corporation) and a Kinetex Biphenyl column (250 × 20.0 mm, 5.0 μm, Phenomenex, Torrance, CA, USA). Additional purification was conducted via preparative HPLC (PLC 2020, Gilson, Middleton, WI, USA) using an Acclaim Polar Advantage II column (250 × 21.2 mm, 5.0 μm, Thermo) with a 172 diode array detector (Gilson). Nuclear magnetic resonance (NMR) spectra were recorded on an AVANCE II 900 MHz cryogenic NMR spectrometer (Bruker, Frankfurt, Germany). Method validation was performed using an ACQUITY UPLC I-Class system (Waters Corporation) with CAD (Thermo) and an ACQUITY BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters Corporation).

All solvents used were of analytical or HPLC grade. Prethanol A (95 % ethanol, C_2H_5OH) was obtained from Duksan Pure Chemicals Co. Ltd. (Ansan, Republic of Korea). *n*-Butanol (≥99 %) and sulfuric acid (95 %) were purchased from Daejung Chemicals & Metals (Siheung, Republic of Korea). *p*-Anisaldehyde (>98 %) was sourced from Sigma–Aldrich (St. Louis, USA). HPLC-grade methanol (≥99.9 %) and acetonitrile (≥99.8 %) were obtained from Honeywell Burdick & Jackson (Ulsan, Republic of Korea) and Merck Millipore (Darmstadt, Germany), respectively. Ultrapure water was produced using a Milli-Q Direct 16 System (Merck Millipore, Guyancourt, France). Formic acid for isolation and LC analysis (≥99.9 %) was purchased from Kanto Chemical, Inc. (Tokyo, Japan), whereas formic acid for LC–MS analysis (≥99 %) was obtained from Fisher Chemical (Pittsburgh, USA). The NMR solvents used included pyridine-d_5 (>99.96 atom% deuterium), deuterium oxide (>99.9 atom% deuterium), and trifluoroacetic acid-*d* (≥99.5 %), all of which were procured from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

2.2. Plant materials

The aerial parts of *S. oleracea* L. were harvested from an open field located at 1689–2 Namhae-daero, Idong-myeon, Namhae-gun, Gyeongsangnam-do, Republic of Korea. Fifteen spinach cultivars were cultivated for 50 days in spring, 73 days in fall, and 128 days in winter. Ten individual plants were collected per cultivar in each season, resulting in a total of 450 individual samples. The fifteen spinach cultivars harvested from each season are shown in Supplementary Fig. S1. The soil types (pH, organic matter, electrical conductivity, and soil classification) used for cultivation were consistent with those in the areas from which the plant materials were sourced and are listed in Supplementary Table S1 (Eco-Friendly Agriculture Center, Sunchon National University). The aerial parts of each group were pooled by combination, immediately freeze-dried, and ground into a fine powder for further analysis. The plant material was authenticated by Dr. Woo Duck Seo (National Institute of Crop Science, Rural Development Administration) and Dr. Seong Hun Jeong (Namhae Garlic Research Institute), and a voucher specimen (PB2613.2–2613.18) was deposited at the Korea Plant Extract Bank of Korea Research Institute of Bioscience and Biotechnology (KRIBB) in Daejeon, Korea. The extraction process was performed based on the findings of Kim, Murthy, Hahn, Lee, and Paek (2007), in which the highest ginsenoside content was obtained with 70 % ethanol. Therefore, 2.0 g of powdered spinach was extracted with 40 mL of 70 % ethanol. The extraction was performed using ultrasonic-assisted extraction (SD-350H, 40 kHz, sd-ultra, Seoul, Korea) at room temperature, with an ultrasonic power of 300 W for a duration of 1 h. After extraction, the resulting solution was filtered through Whatman No. 1 filter paper and concentrated to dryness under reduced pressure using a rotary evaporator at 45 °C. The dried residue was reconstituted in methanol to a final concentration of 5 mg/mL. The sample was then centrifuged at 13,000 rpm for 10 min, and the supernatant was collected for further analysis.

2.3. Gravimetric method

The total saponin content in spinach was determined using the gravimetric method, following the procedure described by Le Bot, Thibault, Pottier, Boisard, and Guilet (2022). A total of 100.0 mg of spinach extract was accurately weighed and dissolved in 50 mL of water. The solution was transferred to a separatory funnel, and 50 mL of *n*-butanol saturated with water was added. The mixture was shaken and allowed to stand for complete separation. The aqueous phase was recovered and fractionated twice more with *n*-butanol, and the organic phases were evaporated under reduced pressure to obtain a dry fraction. The total saponin content was calculated by measuring the mass of the *n*-butanol extract.

2.4. Spectrophotometric methods

The saponin content in spinach extract was measured using a spectrophotometric method. A volume of 100 μL of the supernatant obtained during sample preparation (Section 2.2) was transferred into an

Eppendorf tube (EP tube) with 100 μL of a 50 % *p*-anisaldehyde solution in methanol (*v/v*) and 2 mL of a 50 % aqueous sulfuric solution (*v/v*). The mixture was then immediately heated at 60 °C in a water bath for 20 min to facilitate the chromogenic reaction. The reaction was terminated by immersing the EP tube in an ice–water bath for 10 min. The resulting mixture was subsequently analyzed using a microplate reader (SpectraMax M4, Molecular Devices, California, USA), and the absorbance was measured at 600 nm, with a blank used for comparison. The total saponin content in the extract was determined using a calibration curve prepared with pure standards, specifically 95 % pure Rg3 (Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Soi-myeon, Eumseong-gun, Chungbuk, Republic of Korea). To minimize variability in absorbance between chromogenic reactions, both the standard solution and the samples were processed in the same batch.

2.5. UPLC-QTOF/MS^n characterization

To determine the fragmentation patterns and identify the structures of the six single compounds, UPLC–QTOF–MS analysis was performed in positive ion mode under the following conditions: capillary voltage, 2.3 kV; cone voltage, 50 V; flow rate of desolvation gas (N_2), 800 L/h; ion source temperature, 110 °C; desolvation temperature, 450 °C; mass scan range, 100–1500 Da; and scan time, 0.25 s. A sprayer with a reference compound of leucine-enkephalin [*m/z* 556.2771 in positive ion mode] was used as the lock mass. Aliquots (2.0 μL) of each extract sample were injected into an ACQUITY BEH C_{18} column (2.1 × 100 mm, 1.7 μm) eluted using a chromatographic gradient of two mobile phases (A: 0.1 % FA in H_2O and B: 0.1 % FA in ACN). The linear gradient was optimized as follows: 0.0–1.0 min, 5 % B; 1.0–20.0 min, 5–100 % B; 20.0–22.3 min, 100 % B; 22.3–22.4 min, 100–5 % B; 22.4–25.0 min, 5 % B. The flow rate was 0.4 mL/min, and the temperature of the column oven was maintained at 35 °C. The concentration of the extract sample was 10 mg/mL, the concentration of the isolated compounds was 0.1 mg/mL in 30 % methanol, and the elemental composition was calculated using the Waters Vion software.

2.6. UPLC–CAD method

The total saponin content in spinach extract was quantified using UPLC-CAD according to the following procedure. A Waters UPLC I-class instrument equipped with a PDA was coupled with a CAD system. A Waters ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm) was used for separation. The mobile phase was composed of two solvents: A, 0.1 % formic acid (*v/v*) in water, and B, 0.1 % formic acid in acetonitrile (ACN) (*v/v*). The linear gradient was optimized as follows: 0.0–1.0 min, 5 % B; 1.0–20.0 min, 5–100 % B; 20.0–22.3 min, 100 % B; 22.3–22.4 min, 100–5 % B; and 22.4–25.0 min, 5 % B. The flow rate was maintained at 0.4 mL/min, and the column temperature was set at 35 °C. The nebulizer temperature of the CAD system was also set at 35 °C, with a gas pressure of 59.9 psi. A volume of 100 μL of the supernatant obtained during sample preparation (2.2) was used as the analyte, and 2 μL of sample was injected. The areas corresponding to the saponins were integrated using the CAD system, and the total saponin content in the extract was quantified based on a standard calibration curve prepared with a pure Rg1 standard.

2.7. Extraction and isolation of compounds

The drug substances from the spinach extracts were produced via a processing method described at the International Conference on Harmonization and Ministry of Food and Drug Safety guidelines (http://www.mfds.go.kr/eng/wpge/m_17/de011008l001.do). For the isolation of the six saponins, 100.0 kg of the aerial parts of spinach were extracted twice for 3 h each with 30 % prethanol at 40 °C. The extract was filtered and concentrated under vacuum at 60 °C to obtain a dried extract weighing 14.6 kg, corresponding to a yield of 14.6 % (Korea Good Manufacturing Practice; Batch No. SW20190724–03). A portion of the extract (1.04 kg) was fractionated using a YMC K-prep LAB 100S system equipped with a DAD-50-700S column (50.0 × 700 mm) packed with YMC ODS-AQ HG resin (10 μm, 500 g). The fractions were eluted with a MeOH-H_2O gradient as follows: 0.0–60.0 min, 30 % B; 60.0–85.0 min, 30–50 % B; 85.0–105.0 min, 50–80 % B; 105.0–110.0 min, 80–100 % B; and 110.0–140.0 min, 100 % B, resulting in 8 fractions (SOA 1–SOA 8). The concentration of the extract was 250 mg/mL in 30 % MeOH, and the injection volume was 80 mL with an observance wavelength of 210 nm. Fraction 6 (SOA 6) was separated using an YMC LC-Forte/R series system equipped with a Waters X-Bridge column (250 × 20.0 mm, 5.0 μm, Waters), utilizing a gradient elution method with ACN and 0.1 % FA in H_2O. The elution gradient was as follows: 0.0–5.0 min, 15 % B; 5.0–34.0 min, 15–30 % B; 34.0–63.0 min, 30–35 % B; 63.0–77.0 min, 35–40 % B; 77.0–80.0 min, 40–100 % B; and 80.0–90.0 min, 100 % B, yielding five subfractions (SOA 6–1 to SOA 6–5). The concentration of the sample was 400 mg/mL in 30 % MeOH, with an injection volume of 1 mL. Fraction 7 (SOA 7) was fractionated using a YMC K-prep LAB 100S system equipped with a DAD-50-700S column (50.0 × 700 mm) packed with YMC ODS-AQ HG resin (10 μm, 500 g). A MeOH-H_2O gradient method was used, with the following elution conditions: 0.0–20.0 min, 25 % B; 20.0–40.0 min, 25–30 % B; 40.0–90.0 min, 30–35 % B; 90.0–120.0 min, 35–40 % B; 120.0–125.0 min, 40–100 % B; and 125.0–145.0 min, 100 % B, yielding subfractions (SOA 7–1 to SOA 7–4). The concentration of the sample was 150 mg/mL in 30 % MeOH, with an injection volume of 20 mL. Compound **1** (SOA 6–1–1-1, 25.1 mg) and Compound **2** (SOA 6–1–2-2, 29.4 mg) were isolated from SOA 6–1-2 (283.6 mg) using a recycling system of YMC LC-Forte/R series equipped with a Phenomenex Biphenyl prep column (250 × 20.0 mm, 5.0 μm). An isocratic solvent mixture of ACN and 0.1 % FA in H_2O (31:69, 15 mL/min) was utilized. The sample concentration was 25 mg/mL in 30 % MeOH, with an injection volume of 1 mL. The purity of Compound **1** was determined to be 99.3 %, and that of Compound **2** was 98.8 %, as assessed by HPLC analysis using a CAD system. Compound **3** (SOA 6–3-2, 26.4 mg) and Compound **4** (SOA 6–3-1, 20.5 mg) were obtained from SOA 6–3 (106.7 mg) through a Gilson prep-LC with a Phenomenex Polar Advantage II column (250 × 21.2 mm, 5.0 μm), using an isocratic method of ACN and 0.1 % FA in H_2O (34:67, 15 mL/min). The sample concentration was 10 mg/mL in 30 % MeOH, with an injection volume of 1 mL. The purities of both compounds exceeded 99.9 %, as determined by HPLC analysis using a CAD system. Compound **5** (SOA 7–4-2, 30.5 mg) and Compound **6** (SOA 7–4-1, 28.5 mg) were isolated from SOA 7–4 (439.6 mg) using a Gilson prep-LC with a Phenomenex Polar Advantage II column (250 × 21.2 mm, 5.0 μm) and an isocratic solvent system of ACN and 0.1 % FA in H_2O (35:65, 15 mL/min). The sample concentration was 15 mg/mL in 30 % MeOH, with an injection volume of 1 mL. The purity of Compound **5** was 99.9 %, whereas that of Compound **6** was 96.7 %, as determined by HPLC analysis using a CAD system.

2.8. Sample preparation

To optimize the recovery yield of saponins from the spinach aerial parts, the extraction process was performed based on the method described by Kim et al. (2007), with modifications. Freeze-dried spinach samples (100.0 mg) were weighed into clean 20 mL vials, and extraction was performed at room temperature for 1 h using a sonicator with 10 mL of 70 % ethanol. To determine the most effective solvent for saponin extraction, additional experiments were performed using 10 mL of distilled water and ethanol concentrations of 10 %, 30 %, 50 %, 70 %, and 100 %. After the optimal solvent was identified, the extraction was further optimized by testing different temperatures (25 °C, 40 °C, 60 °C, and 80 °C) and extraction durations (1, 2, 3, and 4 h). After extraction, the cooled extracts were filtered through two layers of Whatman No. 1 filter paper and evaporated under reduced pressure using the Biotage® V-10 Touch evaporation system (Uppsala, Sweden), maintaining the

temperature below 45 °C. This extraction process was repeated three times to obtain the total extract. The effectiveness of the methods was evaluated by comparing the extraction yield (mg/g) and the total area (pA) of saponins in the UPLC-CAD analysis, as detailed in Section 2.6.

2.9. Cultivar and seasonal quantification of six saponins in spinach

2.9.1. Validation of the quantitation method

The developed UPLC-CAD method for accurate analysis of six isolated saponins was validated by assessing various parameters, including the limits of detection (LODs) and quantification (LOQs), selectivity, linearity, accuracy, recovery, and specificity. For this purpose, a Waters UPLC I-class instrument equipped with a PDA was coupled with a CAD system, and chromatographic separation was performed using a Waters ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm). The mobile phase was composed of two solvents: A, 0.1 % formic acid (*v/v*) in water, and B, 0.1 % formic acid in ACN (*v/v*), with a linear gradient was optimized as follows: 0.0–10.0 min, 27 % B; 10.0–11.0 min, 27–30 % B; 11.0–16.0 min, 30–31 % B; 23.0–23.5 min, 34–100 % B; 23.5–26.0 min, 100–100 % B; 26.0–26.5 min, 100–27 % B and 26.5–28.0 min, 27 % B. The flow rate was set at 0.4 mL/min, and the column temperature was maintained at 35 °C. The CAD system was operated at a nebulizer temperature of 35 °C with a gas pressure of 59.9 psi. Linearity, as indicated by the coefficient of determination (R^2), was assessed using a calibration curve prepared at five concentrations ranging from 1.0 to 50.0 μg/mL for Compounds **1** to **4** and from 1.0 to 100.0 μg/mL for Compounds **5** and **6**, with all analyses performed in six replicates on the same day. The LOD and LOQ were calculated as 3.3 and 10 times the standard deviation divided by the slope, respectively. Repeatability was determined by performing at least five analyses for three concentrations (5.0, 6.0 and 7.0 mg/mL) and expressing the degree of closeness between the measured values as a relative standard deviation (RSD). To ensure interlaboratory reproducibility, method validation was performed in three different laboratories by analyzing the extract at a concentration of 1 mg/mL in six replicates. To assess accuracy, recovery tests were conducted by spiking three different concentrations (low, medium and high) of the six compounds into a 30 % prethanol extract of spinach. Recovery was estimated using the following formula: recovery (%) = (theoretical concentration/measured concentration) × 100.

2.9.2. Quantitative analysis of spinach

To quantitatively analyze spinach across various cultivars and cultivation seasons, 100.0 mg of the 45 pooled spinach samples was extracted with 5.0 mL of 30 % prethanol. The mixtures were centrifuged at 13,000 rpm at 10 °C for 30 min, and the supernatant were collected for further analysis using UPLC-CAD, following the method described in Section 2.9.1. All quantitative results were expressed as mean ± standard deviation (SD) of three independent replicates. Statistical analyses were performed using SigmaPlot 2001 (Systat Software Inc., Chicago, IL, USA). Differences among groups were assessed using appropriate statistical tests, and *p*-values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Total saponin content as determined via the gravimetric method

Spinach is a highly nutritious vegetable with various health benefits, including antioxidant, anti-inflammatory, anticancer, and bone-strengthening effects, primarily due to flavonoids and minerals. However, the specific components responsible for benefits to cognitive function, the cardiovascular system, immune system, skin health, anti-diabetic effects, and liver health are not fully understood. These benefits may be related to the saponins of natural products, suggesting that saponins may exist in spinach and that the above of health benefits mentioned effects are due to saponin components. Therefore, as the first step, we conducted an experiment to determine the presence of saponins in spinach. Following previous reports (Oleszek, 2002; Tenon, Feuillère, Roller, & Birtic, 2017), we selected the gravimetric method to measure the total saponin content. Unfortunately, this method is not accurate, quick, cost-effective, or universally applicable (Le Bot et al., 2022). Furthermore, these methods may result in inaccurate quantification of similar compounds, such as steroids and triterpenoids, due to the complex matrix of various metabolites present in the extract. However, in this study, to determine the presence or absence of saponins in spinach, the total saponin content of the aerial parts was first evaluated using a readily accessible and convenient gravimetric method.

According to some previously reported methods (Cheok, Salman, & Sulaiman, 2014), the maceration process using conventional ethanol or methanol generally results in saponin contents as high as 36 %. The effectiveness of the maceration process is determined by two main factors: solubility and efficient diffusion. Solubility follows the principle of similar dissolution, meaning that polar phytochemicals dissolve in polar solvents, whereas nonpolar phytochemicals dissolve in nonpolar solvents. Moreover, the combination of ethanol and methanol with water is beneficial for saponin extraction. Although the extraction temperature may increase the rate of phytochemical extraction, its content tends to decrease due to the degradation of phytochemicals by extraction at high temperatures (Cano-Lamadrid & Artés-Hernández, 2022). Therefore, we selected ethanol as the solvent to extract saponins from various spinach varieties at room temperature. The saponin content in the extract was measured by fractionating it with 70 % ethanol, as supported by findings reported in the literature. In particular, ultrasonic extraction is considered noteworthy because it is the most commonly chosen eco-friendly extraction technique for yield quantification experiments, and its effectiveness and efficiency are well established, making it an appropriate choice for investigating the total saponin yield in this study. A widely-used gravimetric method involves suspending the crude extract in distilled water and preparing a solvent fraction based on its affinity for butanol. From this fraction, saponins and other compounds are specifically extracted. The compound content was expressed as mg per g of dry matter (mg/g D.M.). The results presented in Table 1 represent total saponin contents of 5.8–58.5, 5.8–102.1, and 12.0–69.6 (mg/g D.M.) for the 15 spinach cultivars harvested in the spring, fall, and winter, respectively, and included in this study. Additionally, significant differences in total saponin content were observed, depending on the variety and harvest season. Previous studies have reported the contents and compositions of flavonoids in spinach leaves (Yuk et al., 2019; Kim B.-M., Lee, Song, & Kim, H.-J, 2021); however, cultivar variation in saponin content has not yet been studied. As an approach for the quality control of spinach, it is necessary to examine the variation in saponin contents according to cultivation conditions (cultivar, environmental, and growing season). In addition, this approach may be useful for addressing new problems to ensure continued quality control of spinach. A more comprehensive study was therefore conducted to investigate the differences in saponin content across different spinach varieties and harvest seasons and to provide new perspectives on this issue.

3.2. Characterization of new oleanane-type saponin derivatives via UPLC-Q-TOF-MS analysis

Saponins are glycosylated compounds that are widely found throughout the plant kingdom, and their name is based on the fact that when an aqueous solution containing saponins is stirred, a persistent foam is formed similar to soap. Therefore, like soap, it must have a nonpolar and polar part in the same molecule (Lee et al., 2008). Saponins are large molecules that include a hydrophobic part composed of a triterpenoid (30 carbon atoms) or steroid (27 carbon atoms with a 6-ring spirostane or a 5-ring furostane skeleton) backbone and a hydrophilic part consisting of several saccharide residues attached to the hydrophobic scaffold through glycoside bonds (Lee et al., 2019; Moghimipour & Handali, 2015). Saponins are classified as mono-, di-, or tridesmosidic

Table 1
Comparison of the results for total saponins obtained via the gravimetric, spectrophotometric, and UPLC-CAD methods.

No.	Cultivar[a]	Cultivation period by cultivar		Saponins content[b] (mg/g) by season								
				Spring[c]			Fall[c]			Winter[c]		
		Seeding (Month)	Harvesting (Month)	Gravimetric method	Spectro-photometric method	UPLC-CAD method	Gravimetric method	Spectro-photometric method	UPLC-CAD method	Gravimetric method	Spectro-photometric method	UPLC-CAD method
1	AD	October–December	January–March	9.9 ± 0.6	23.1 ± 0.6	22.4 ± 2.3	15.4 ± 0.2	31.1 ± 2.7	11.4 ± 0.2	20.9 ± 1.1	ND[d]	33.2 ± 0.9
2	DC	April–September	May–November	8.7 ± 0.3	208.5 ± 14.9	16.3 ± 1.4	16.8 ± 0.3	75.9 ± 2.0	13.3 ± 0.4	19.7 ± 0.2	1.1 ± 1.6	13.2 ± 0.2
3	FR	April–June	June–July	6.6 ± 0.2	57.4 ± 2.5	16.3 ± 1.4	37.6 ± 0.2	161.4 ± 1.3	12.9 ± 1.0	25.3 ± 0.4	26.4 ± 2.2	10.4 ± 0.8
4	HK	March–May	May–June	17.0 ± 0.6	45.2 ± 2.4	17.4 ± 3.7	19.0 ± 0.5	121.8 ± 0.6	10.8 ± 0.2	29.2 ± 0.5	ND	11.5 ± 0.7
5	LY	October–January	November–March	29.0 ± 0.3	72.0 ± 3.3	26.4 ± 3.1	18.6 ± 0.3	78.0 ± 1.3	16.9 ± 1.6	14.8 ± 0.2	26.8 ± 1.3	15.2 ± 1.7
6	MT	February–March	April–May	21.5 ± 1.1	ND	14.6 ± 3.1	14.3 ± 0.4	16.1 ± 2.5	13.1 ± 0.3	17.8 ± 0.2	65.7 ± 1.5	14.2 ± 1.4
7	PG	November–May	March–June	15.1 ± 0.4	36.7 ± 7.3	16.9 ± 3.7	34.5 ± 0.4	92.4 ± 1.9	16.7 ± 1.0	31.7 ± 0.4	ND	21.3 ± 1.0
8	PT	March–August	April–September	48.0 ± 0.1	ND	12.8 ± 0.4	19.5 ± 0.3	76.3 ± 1.5	11.0 ± 0.2	25.6 ± 0.8	65.8 ± 5.3	26.1 ± 0.9
9	SS	March–September	April–October	5.9 ± 0.1	ND	23.3 ± 1.8	27.2 ± 0.4	ND	16.8 ± 0.8	13.2 ± 0.2	69.6 ± 1.5	25.9 ± 1.2
10	SW	October	February	58.5 ± 0.2	36.3 ± 5.1	23.8 ± 2.0	11.1 ± 0.3	35.0 ± 2.9	15.7 ± 1.9	37.2 ± 0.2	ND	9.6 ± 0.7
11	SR	April–September	May–November	11.7 ± 0.2	1.1 ± 1.6	18.2 ± 0.2	102.1 ± 0.4	136.5 ± 1.5	20.0 ± 2.2	12.0 ± 0.3	51.8 ± 1.7	16.6 ± 1.3
12	TS	October	December–February	19.5 ± 0.2	68.2 ± 7.4	17.9 ± 0.8	5.8 ± 0.2	57.9 ± 1.1	10.2 ± 0.8	17.2 ± 0.2	6.0 ± 0.8	13.5 ± 0.2
13	VN	May–June	June–August	33.3 ± 0.3	12.5 ± 4.5	16.0 ± 0.8	21.2 ± 1.3	64.2 ± 1.5	14.0 ± 0.9	21.6 ± 0.2	ND	15.5 ± 0.9
14	WT	October–November	November–February	5.8 ± 0.3	71.8 ± 8.8	16.7 ± 1.5	13.3 ± 0.4	87.7 ± 0.7	17.0 ± 0.4	29.4 ± 0.3	ND	10.8 ± 0.7
15	YT	October–November	November–March	23.5 ± 0.2	26.9 ± 5.3	14.8 ± 2.2	38.3 ± 0.6	153.1 ± 1.8	9.0 ± 0.2	69.6 ± 0.4	ND	15.5 ± 1.0
Sum	–	–	–	–	659.7	273.8	–	1187.4	208.8	–	313.2	252.5
Average	–	–	–	–	43.98	18.25	–	79.16	13.92	–	20.88	16.83

[a] AD: Asiadongcho, DC: Daechan, FR: Friend, HK: Heukking, LY: Luckyyou, MT: Mustang, PG: Platon Gold, PT: Point, SS: Samsan, SW: Shinwoldong, SR: Sushiro, TS: Ttangshimyi, VN: Venus, WT: Woldong tobaki, YT: Younghonam teoppat.

[b] Total content of six saponins in each cultivar.

[c] Spinach was cultivated for 50 days in spring, 73 days in fall, and 128 days in winter.

[d] ND = not detected.

depending on the number of sugar chains in their structure. Additionally, these compounds can be classified into 11 types, namely, dammarane, tirucallane, lupane, hopane, oleanane, taraxasterane, ursane, cucurbitane, cycloartane, lanostane, and steroids, depending on their chemical structure. Among them, oleanane-type saponins constitute the most common skeleton found in the plant kingdom. In oleanane-type structures, although methyl groups are frequently functionalized, those attached to the C-8 atom remain unmodified across all plant orders. The methyl group located at the C-14 atom undergoes moderate processing, whereas the groups at C-4, C-17, and C-20 are subjected to extensive processing and varying levels of oxidation. In the Fabales order, only the methyl group at the C-10 atom is functionalized; this is indicated by the presence of CH_2OH, CHO, and COOH groups (Vincken, Heng, Groot, & Gruppen, 2007). Furthermore, saponins are linked to monosaccharides such as D-glucose (Glc), D-galactose (Gal), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), L-rhamnose (Rha), L-arabinose (Ara), D-xylose (Xyl), and D-fucose (Fuc), which are bound mainly to C-3, 26, or 28 (Güçlü-Üstündağ & Mazza, 2007).

For the target components predicted to be saponins, experimental structural identification was conducted to select six components with greater contributions and variations depending on cultivar and harvest season, and the focus was on identifying plant chemicals (Fig. 1A). In preliminary experiments, we demonstrated that the MS and MS/MS patterns were indicative of six potential new triterpene saponins (Supplementary Table S2 and Fig. S2). Compound **1** was detected at a wavelength of 221 nm in the UV spectrum of UPLC-PDA-QTOF-MS. The positive HRESIMS (Supplementary Fig. S2) showed a protonated molecular ion peak at *m/z* 1133.5383 $[M + H]^+$, indicating a molecular formula of $C_{54}H_{84}O_{25}$ (calcd for $C_{54}H_{85}O_{25}$, 1133.5374). UPLC-QTOF-MS (Fig. 1B) revealed an ion at *m/z* 971.4841 with the loss of a hexose $[M + H\text{-}162]^+$. Other product ions at *m/z* 825.4282 and *m/z* 679.3714 suggested the neutral loss of two deoxyhexose units $[M + H\text{-}162\text{-}146\text{-}146]^+$. Additionally, the loss of a hexuronic acid unit (176 u) at *m/z* 503.3388 ($C_{30}H_{46}O_6$) $[M + H\text{-}162\text{-}146\text{-}146\text{-}176]^+$ was attributed to the loss of hexose, dideoxyhexose, and hexuronic acid units (630 u), and due to the loss of H_2O and CO_2 molecules, *m/z* 483.3142 ($C_{30}H_{43}O_5$) $[Agly+H\text{-}18]^+$ and 439.3208 ($C_{29}H_{43}O_3$) $[Agly+H\text{-}18\text{-}44]^+$ ions were detected, respectively, which are characteristic of medicagenic acid (Kozachok et al., 2020).

Compound **2** was detected at a wavelength of 221 nm in the UV spectrum of UPLC-PDA-QTOF-MS. The positive HRESIMS (Supplementary Fig. S2) showed a protonated molecular ion peak at *m/z* 1265.5810 $[M + H]^+$, indicating a molecular formula of $C_{59}H_{92}O_{29}$ (calcd for $C_{59}H_{93}O_{29}$, 1265.5797). UPLC-QTOF-MS (Fig. 1B) revealed an ion at *m/z* 1133.5383 with the loss of a pentose $[M + H\text{-}132]^+$, which was identical to the molecular formula of **1** ($C_{54}H_{84}O_{25}$). The MS spectrum revealed another ion at *m/z* 971.4880 corresponding to the loss of a hexose $[M + H\text{-}162]^+$. Other product ions at *m/z* 825.4282 and *m/z* 679.3691 suggested the neutral loss of two deoxyhexose units $[M + H\text{-}132\text{-}162\text{-}146\text{-}146]^+$. Furthermore, the loss of a hexuronic acid unit (176 U) at *m/z* 503.3360 ($C_{30}H_{46}O_6$) $[M + H\text{-}132\text{-}162\text{-}146\text{-}146\text{-}176]^+$ was attributed to the loss of a pentose, hexose, dideoxyhexose, or hexuronic acid unit (762 U).

Compound **3** was detected at a wavelength of 221 nm in the UV spectrum of UPLC-QTOF-MS. The positive HRESIMS (Supplementary Fig. S2) showed a protonated molecular ion peak at *m/z* 1103.5301 $[M + H]^+$, indicating a molecular formula of $C_{53}H_{82}O_{24}$ (calcd for $C_{53}H_{82}O_{24}$, 1103.5269). UPLC-QTOF-MS (Fig. 1B) revealed an ion at *m/z* 971.4880 due to the loss of a pentose $[M + H\text{-}132]^+$. Other product ions at *m/z* 825.4282 and *m/z* 679.3714 indicated the neutral loss of two deoxyhexose units $[M + H\text{-}132\text{-}146\text{-}146]^+$. Additionally, the loss of a hexuronic acid unit (176 U) at *m/z* 503.3388 ($C_{30}H_{46}O_6$) $[M + H\text{-}132\text{-}146\text{-}146\text{-}176]^+$ was attributed to the loss of a pentose, dideoxyhexose, and hexuronic acid unit (600 U), indicating the presence of an aglycone.

Compound **4** was detected at a wavelength of 221 nm in the UV spectrum of UPLC-QTOF-MS. The positive HRESIMS (Supplementary Fig. S2) showed a protonated molecular ion peak at *m/z* 1235.5725 $[M + H]^+$, indicating a molecular formula of $C_{58}H_{90}O_{28}$ (calcd for $C_{58}H_{91}O_{28}$, 1235.5691). UPLC-QTOF-MS (Fig. 1B) revealed an ion at *m/z* 1103.5301 with the loss of a pentose $[M + H\text{-}132]^+$, which was identical to the molecular formula of **3** ($C_{53}H_{82}O_{24}$). The MS spectrum revealed other ions at *m/z* 957.4706 corresponding to the loss of a deoxyhexose $[M + H\text{-}132\text{-}146]^+$. Other product ions at *m/z* 825.4282 and *m/z* 679.3700 suggested the neutral loss of pentose and deoxyhexose units $[M + H\text{-}132\text{-}146\text{-}132\text{-}146]^+$. Furthermore, the loss of a hexuronic acid unit (176 U) at *m/z* 503.3388 ($C_{30}H_{46}O_6$) $[M + H\text{-}132\text{-}146\text{-}132\text{-}146\text{-}176]^+$ was attributed to the loss of dipentose, dideoxyhexose, and the hexuronic acid unit (732 U), indicating an aglycone moiety.

Compound **5** was detected at a wavelength of 221 nm in the UV spectrum of UPLC-QTOF-MS. The positive HRESIMS (Supplementary Fig. S2) showed a protonated molecular ion peak at *m/z* 1175.5487 $[M + H]^+$, indicating a molecular formula of $C_{56}H_{86}O_{26}$ (calcd for $C_{56}H_{87}O_{26}$, 1175.5480). UPLC-QTOF-MS (Fig. 1B) revealed an ion at *m/z* 1013.4976 with the loss of a hexose $[M + H\text{-}162]^+$. The MS spectrum revealed other ions at *m/z* 867.4387, corresponding to the loss of a

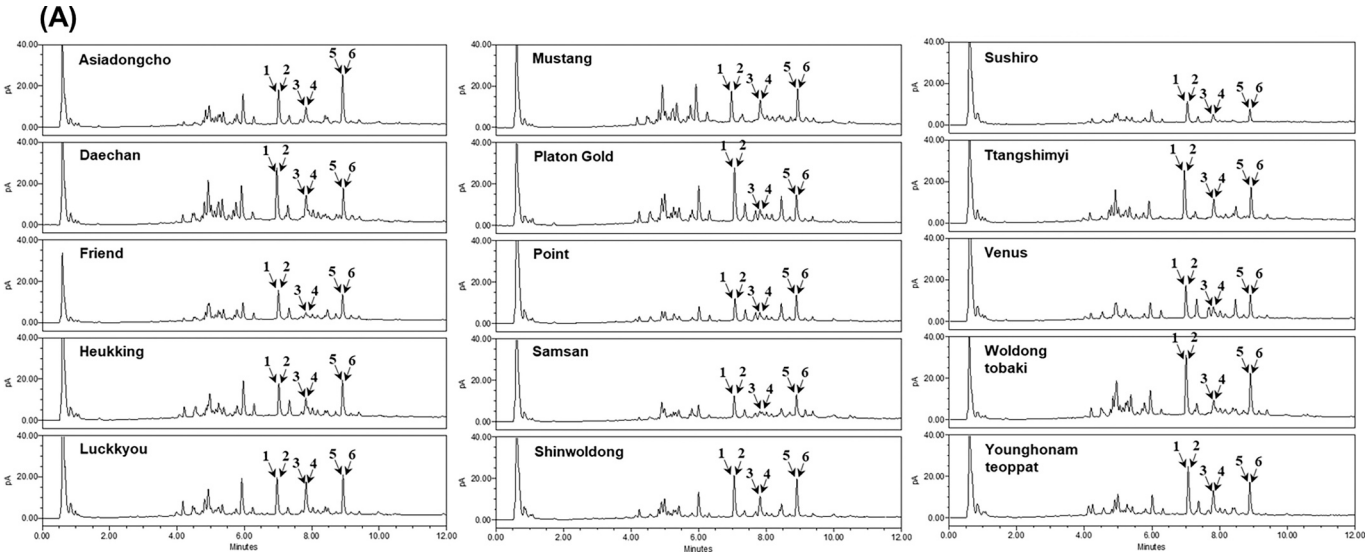


Fig. 1. (A) UPLC–CAD chromatograms of six triterpenoid saponins from 15 spinach cultivars. (B) UPLC–QTOF–MS/MS spectra of Compounds **1**–**6**.

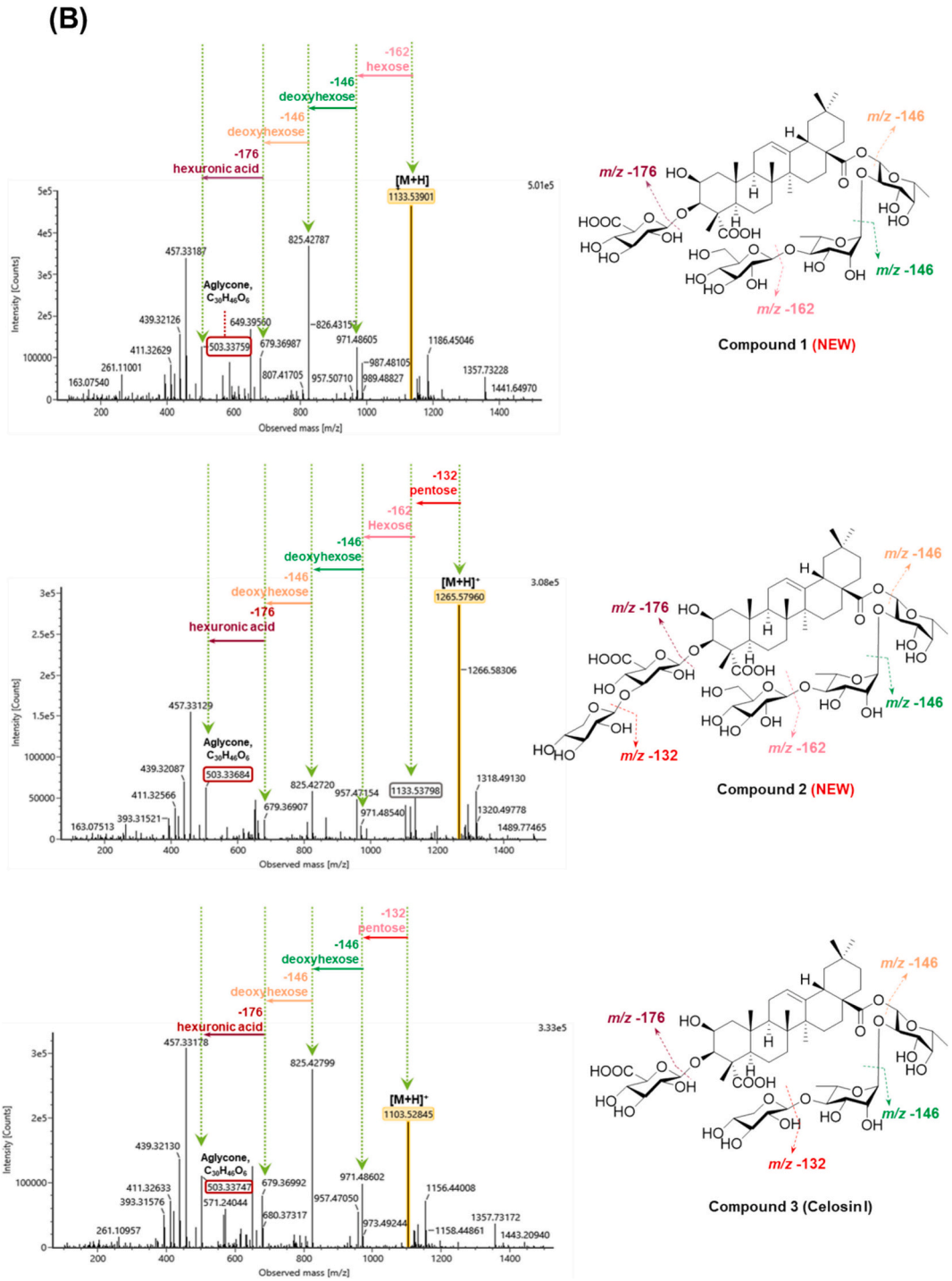


Fig. 1. (*continued*).

deoxyhexose [M + H-162-146]$^+$. Other product ions at *m/z* 679.3704 suggested the loss of an acetylated deoxyhexose unit [M + H-162-146-188]$^+$. Furthermore, the loss of a hexuronic acid unit (176 u) at *m/z* 503.3388 ($C_{30}H_{46}O_6$) [M + H-162-146-188-176]$^+$ was attributed to the loss of a hexose, deoxyhexose, acetylated deoxyhexose, and hexuronic acid unit (672 u), indicating an aglycone moiety.

Compound **6** was detected at a wavelength of 221 nm in the UV spectrum of UPLC-QTOF-MS. The positive HRESIMS (Supplementary Fig. S2) showed a protonated molecular ion peak at *m/z* 1307.5915 [M + H]$^+$, indicating a molecular formula of $C_{61}H_{94}O_{30}$ (calcd for $C_{61}H_{95}O_{30}$, 1307.5903). UPLC-QTOF-MS (Fig. 1B) revealed an ion at *m/z* 1175.5487 with the loss of a pentose [M + H-132]$^+$. The MS spectrum revealed another ion at *m/z* 1013.4976 corresponding to the loss of a hexose [M + H-132-162]$^+$. Other product ions at *m/z* 867.3343 and 673.2748 suggested the loss of a deoxyhexose unit and acetylated deoxyhexose, respectively [M + H-132-162-146-188]$^+$. Furthermore, the loss of a hexuronic acid unit (176 U) at *m/z* 503.3388 ($C_{30}H_{46}O_6$) [M + H-132-162-146-188-176]$^+$ was attributed to the loss of a pentose, hexose, deoxyhexose, acetylated deoxyhexose, and hexuronic acid unit (804 U), indicating an aglycone moiety.

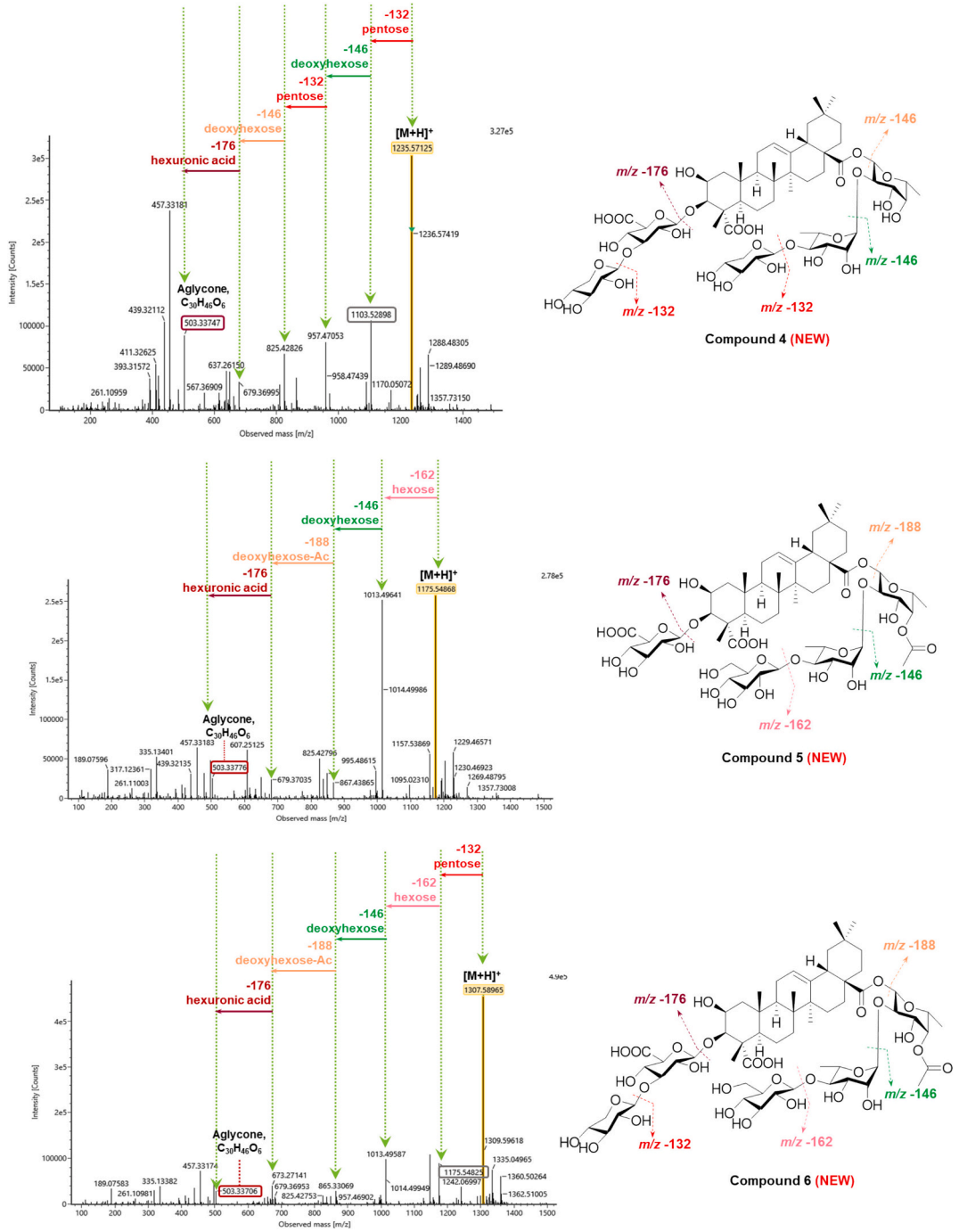


Fig. 1. (*continued*).

3.3. *Comparison of different methods for the extraction of saponins*

To develop a standardized method for saponin extraction from spinach, two of the three key extraction factors, ethanol concentration, extraction time, and extraction temperature, were kept constant, while the third variable was adjusted. This approach aimed to determine the optimal extraction conditions and assess the effects of the ethanol concentration, extraction time, and extraction temperature on the saponin content. The contents of the extracts were compared by examining the elution times of the target compounds. These compounds are part of a complex matrix primarily composed of flavonoids, saponins, and fatty acids. Preliminary extraction experiments were carried out to ensure the complete extraction of saponins from the aerial parts of spinach. The extraction performance was evaluated with the extraction yield (%) obtained as well as the integrated area in UPLC-CAD (pA) corresponding to the saponins. The major metabolite groups tended to increase with increasing ethanol concentration over a 1 h extraction period at room temperature with 10, 30, 50, 70 and 100 % EtOH concentrations; the concentration of 30 % EtOH resulted in the highest area (Supplementary Fig. S3a and Table S3). As mentioned earlier, the extraction rate of phytochemicals increased with increasing extraction temperature and duration. However, prolonged extraction at high temperatures led to a decrease in phytochemical content, likely due to their decomposition over time (Supplementary Table S4). The response area of the saponins did not significantly change with increasing bath temperature during the 1 h extraction period when 30 % ethanol was used. However, it reached

a maximum at 40 °C (Supplementary Table S5). The results showed that the use of 30 % ethanol at 40 °C for 1 h gave the best results in terms of extraction yield (34.1 %) and the integrated saponin ratio (24.75 %) for the spinach samples (Supplementary Fig. S3b).

3.4. Structural determination of new oleanane-type saponins

Using extracts obtained under optimal extraction conditions from spinach, we isolated six pure saponins via sequential chromatographic techniques (Fig. S4) and investigated their structural identification, with a focus on new compounds. The chromatographic details of the MS chromatograms of these six saponins are shown in Supplementary Fig. S5, and their chemical structures are depicted in Fig. 2A. A comparison of their spectroscopic data, including optical rotations, 1D and 2D NMR data (Supplementary Figs. S6–11), and HRMS data (Supplementary Fig. S2), with reported values (Pang et al., 2014) allowed us to identify the known compound celosin I (**3**).

Compound **1** was obtained as a white amorphous powder with $[\alpha]_{25}^{D}$–19.3 (c = 0.1, MeOH). The positive HRESI-MS data (Supplementary Fig. S2) revealed a protonated molecular ion peak at m/z 1133.5383 $[M + H]^+$, indicating a molecular formula of $C_{54}H_{84}O_{25}$ (calcd for $C_{54}H_{85}O_{25}$, 1133.5374). UPLC-Q-TOF/MS (Fig. 1B) revealed product ions at m/z 971.4841 with a loss of 162 Da, suggesting the presence of a hexose moiety. Two additional neutral losses of 146 Da were observed at m/z 825.4282 and m/z 679.3714, indicating the presence of two deoxy sugar moieties. Additionally, the ion at m/z 503.3388, resulting from the loss of 176 Da, which is typical of hexuronic acid, was tentatively interpreted as the residual mass of the triterpenoid aglycone after the complete removal of sugar moieties. In the ^{1}H NMR spectrum (Table 2), the proton signals of one olefinic methine (chemical shift, coupling pattern, J in Hz, proton number; δ_H 5.34, br s, H-12), two oxygenated methines (δ_H 4.72, br s, H-2; δ_H 4.65, d, 3.6, H-3), and eight tertiary methyls (δ_H 1.89, s, H-24; δ_H 1.64, d, 6.3, H-6‴′; δ_H 1.40, d, 4.3, H-6‴; δ_H 1.45, s, H-25; δ_H 1.03, s, H-26; δ_H 1.19, s, H-27; δ_H 0.76, s, H-29; δ_H 0.78, s, H-30) were observed. These spectral data were used to predict the presence of one oleanane-type triterpenoid. The ^{1}H NMR signals included four hemiacetal protons (δ_H 5.16, d, 8.1, H-1′; δ_H 5.15, d, 8.1, H-1″; δ_H 5.91, d, 8.1, H-1‴; δ_H 6.25, s, H-1⁗), 16 oxygenated methines (δ_H 4.76, dd, 2.7, 1.8, H-2‴; δ_H 4.61, dd, 9.0, 2.7, H-3‴; δ_H 4.54, t, 8.1, H-2″; δ_H 4.52, overlap, H-5′; δ_H 4.39, t, 9.0, H-4′; δ_H 4.19, t, 9.0, H-3′; δ_H 4.36, overlap, H-5‴; δ_H 4.30, t, 9.0, H-4‴, δ_H 4.09, overlap, H-3⁗; δ_H 4.09, overlap, H-3″; δ_H 4.06, t, 9.0, H-4⁗; δ_H 4.00, d, 7.2, H-2⁗; δ_H 3.99, d, 8.1, H-2′; δ_H 3.95, d, 3.6, H-4″; δ_H 3.83, q, 6.3, H-5″; δ_H 3.71, dq, 5.4, 2.7, H-5⁗), one oxygenated methylene (δ_H 4.32, dd, 11.7, 2.7, H-6a’‴, 4.16, 1H, dd, 11.7, 5.4, H-6b’‴), and two methyls (δ_H 1.64, d, 6.3, H-6‴; δ_H 1.40, d, 6.3, H-6″), which revealed the presence of two hexoses and two deoxyhexoses. Based on all the ^{1}H NMR data, the chemical structure of Compound **1** was inferred to be a saponin composed of an oleanane-type triterpenoid with two hexoses and two deoxyhexose moieties. The ^{13}C NMR spectrum of Compound **1** (Table 3), along with the DEPT spectra, included one ester (δ_C 176.6, C-28), a carboxyl group (δ_C 180.9, C-23), one olefinic quaternary group (δ_C 143.8, C-13), one olefinic methine (δ_C 122.4, C-12), two oxygenated methines (δ_C 85.7, C-3; δ_C 70.0, C-2), six quaternaries (δ_C 52.6, C-4; δ_C 46.8, C-17; δ_C 42.0, C-14; δ_C 40.1, C-8; δ_C 36.5, C-10; δ_C 30.4, C-20), three methines (δ_C 52.3, C-5; δ_C 48.4, C-9; δ_C 41.8, C-18), nine methylenes (δ_C 46.0, C-19; δ_C 44.1, C-1; δ_C 33.6, C-21; δ_C 32.7, C-7; δ_C 32.0, C-22; δ_C 28.0, C-15; δ_C 23.7, C-11; δ_C 23.1, C-16; δ_C 20.9, C-6), and six methyls (δ_C 32.8, C-29; δ_C 25.8, C-27; δ_C 23.5, C-30; δ_C 17.2, C-26; δ_C 16.6, C-25; δ_C 13.8, C-24), supporting the identification of the aglycone structure as a triterpenoid like medicagenic acid (Kozachok et al., 2020). The carbon signals due to one carboxyl group (δ_C 172.7, C-6′), two hemiacetals (δ_C 106.2, C-1⁗; δ_C 105.2, C-1′), eight oxygenated methines (δ_C 78.1, C-3⁗; δ_C 77.9, C-5⁗; δ_C 77.2, C-3′; δ_C 76.9, C-5′; δ_C 75.9, C-2⁗; δ_C 74.4, C-2′; δ_C 72.8, C-4′; δ_C 71.3, C-4⁗), and one oxygenated methylene (δ_C 62.4, C-6⁗) revealed that the two hexoses were β-glucuronic acid and β-glucopyranose. Two deoxyhexose moieties, including two hemiacetals (δ_C 101.1, C-1‴; δ_C 94.5, C-1″), eight oxygenated methines (δ_C 84.5, C-4‴; δ_C 75.8, C-3″; δ_C 74.1, C-2″; δ_C 72.6, C-4″; δ_C 72.1, C-5″; δ_C 71.9, C-3‴; δ_C 71.2, C-2‴; δ_C 68.2, C-5‴), and two methyls (δ_C 18.3, C-6‴; δ_C 16.5, C-6″), suggested that two deoxyhexoses were β-fucopyranose and α-rhamnopyranose. The anomer carbon from β-fucopyranose (C-1″) was increased at δ_C 94.5

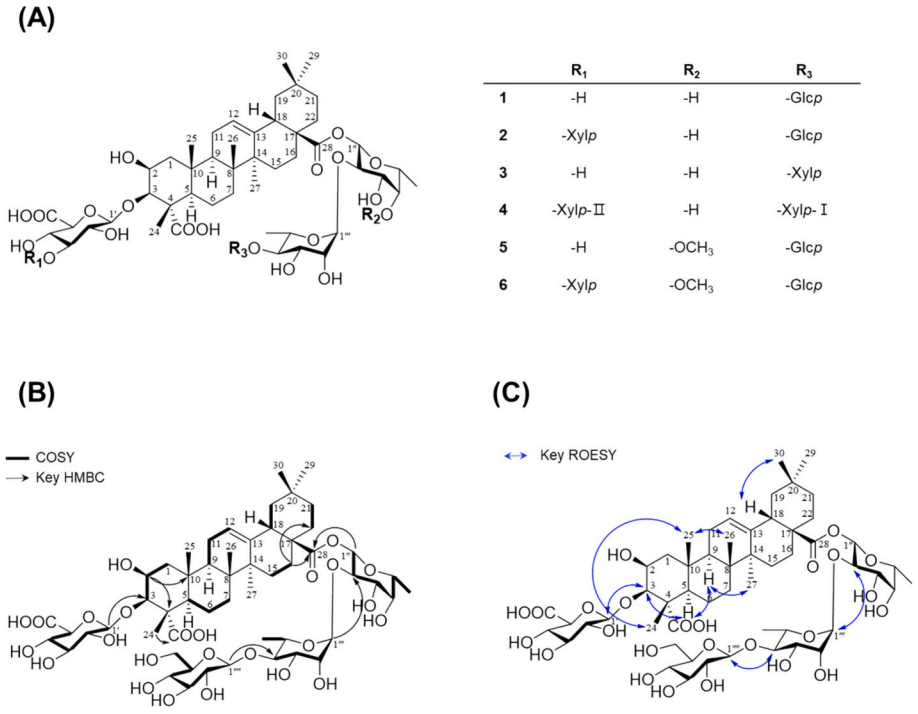


Fig. 2. Chemical structures of six saponins (**1**–**6**) isolated from the aerial parts of *S. oleracea* L. (A). The ^{1}H—^{1}H COSY and key HMBC correlations of Compound **1** (B). The key ROESY correlations of Compound **1** (C).

Table 2
^{1}H NMR data of celosin I and spinaciasaponin A–E.

No.	δ_H, Coupling Pattern, *J* in Hz[a]					
	A (1)	B (2)	Celosin I (3)	C (4)	D (5)	E (6)
1a	2.21, d, 12.6	2.26, q (12.0)	2.31, br s	2.18, d (12.6)	2.22, d (12.6)	2.19, d (12.6)
1b	1.20, overlap	1.27, overlap	1.30, overlap	1.17, overlap	1.21, overlap	1.17, overlap
2	4.74, d, 2.7	4.84, overlap	4.85, overlap	4.72, overlap	4.74, overlap	4.72, overlap
3	4.63, d, 2.7	4.77, overlap	4.75, overlap	4.65, d, 3.6	4.64, br s	4.65, br s
5	1.98, overlap	2.08, overlap	2.10, overlap	1.96, d, 11.7	1.99, overlap	1.96, overlap
6a	1.81, overlap	1.94, overlap	1.93, overlap	1.81, overlap	1.82, overlap	1.81, overlap
6b	1.65, overlap	1.80, overlap	1.79, overlap	1.68, overlap	1.66, overlap	1.67, overlap
7a	1.71, overlap	1.78, overlap	1.79, overlap	1.71, overlap	1.71, overlap	1.70, overlap
7b	1.57, overlap	1.66, overlap	1.69, overlap	1.60, overlap	1.56, d, 12.6	1.60, d,11.7
9	1.67, overlap	1.75, overlap	1.77, overlap	1.66, overlap	1.68, overlap	1.67, overlap
11a	2.03, overlap	2.11, overlap	2.12, overlap	2.02, overlap	2.05, overlap	2.03, overlap
11b	1.88, overlap	1.98, overlap	1.99, overlap	1.88, overlap	1.92, overlap	1.91, overlap
12	5.33, s	5.46, s	5.41, s	5.34, s	5.35, s	5.34, s
15a	2.00, overlap	2.11, overlap	2.10, overlap	1.99, overlap	1.97, overlap	1.96, overlap
15b	1.35, d, 10.8	1.40, d, 12.8	1.42, d, 10.8	1.36, d, 11.7	1.33, d, 12.6	1.33, d, 13.5
16a	2.00, overlap	2.07, overlap	2.07, overlap	2.00, overlap	2.02, overlap	2.01, overlap
16b	1.65, overlap	1.89, overlap	1.89, overlap	1.82, overlap	1.83, overlap	1.82, overlap
18	3.02, dd, 13.5, 3.6	3.12, dd, 13.6, 3.2	3.12, d, 12.6	3.02, dd, 13.5, 3.6	3.02, dd, 13.5, 3.6	3.02, dd, 13.5, 2.7
19a	1.65, overlap	1.73, overlap	1.73, overlap	1.65, overlap	1.65, overlap	1.65, overlap
19b	1.10, d, 10.8	1.18, overlap	1.19, overlap	1.10, d, 11.7	1.10, d, 10.8	1.10, d, 10.8
21a	1.18, overlap	2.12, overlap	2.18, overlap	1.19, overlap	1.19, overlap	1.19, overlap
21b	1.07, d, 12.6	1.15, overlap	1.17, overlap	1.08, d, 11.7	1.05, d, 11.7	1.04, d, 11.7
22a	1.92, dd, 12.6, 3.6	2.03, overlap	2.09, overlap	1.90, overlap	1.94, overlap	1.93, overlap
22b	1.59, overlap	1.68, overlap	1.71, overlap	1.62, overlap	1.62, overlap	1.62, overlap
24	1.89, s	2.01, s	2.00, s	1.88, s	1.90, s	1.89, s
25	1.45, s	1.58, s	1.57, s	1.44, s	1.46, s	1.46, s
26	1.03, s	1.14, s	1.14, s	1.03, s	1.02, s	1.02, s
27	1.19, s	1.26, s	1.26, s	1.19, s	1.20, s	1.20, s
29	0.77, s	0.85, s	0.84, s	0.76, s	0.77, s	0.77, s
30	0.79, s	0.90, s	0.89, s	0.78, s	0.78, s	0.81, s
OCH_3	–	–	–	–	1.95, s	1.94, s
1′	5.13, d, 8.1	5.28, d, 8.0	5.22, br s	5.16, d, 8.1	5.14, d, 7.2	5.16, d, 7.2
2′	3.99, d, 8.1	4.05, t, 8.8	4.00, br s	4.02, overlap	3.99, over1ap	4.02, t, 8.1
3′	4.19, t, 9.0	4.28, t, 8.8	4.23, overlap	4.16, overlap	4.09, t, 9.0	4.16, overlap
4′	4.39, t, 9.0	4.49, overlap	4.49, overlap	4.32, t, 9.0	4.39, overlap	4.30, overlap
5′	4.52, overlap	4.52, d, 9.6	4.59, br s	4.52, d, 9.0	4.52, m	4.58, m
1″	5.91, d, 8.1	6.04, d, 8.0	6.04, d, 8.1	5.91, d, 8.1	5.95, d, 8.1	5.94, d, 8.1
2″	4.54, t, 8.1	4.64, t, 8.8	4.66, t, 8.1	4.56, t, 8.1	4.44, t, 8.1	4.44, t, 8.1
3″	4.09, overlap	4.17, overlap	4.18, d, 8.1	4.09, overlap	4.31, overlap	4.31, overlap
4″	3.95, d, 3.6	3.95, d, 2.4	3.95, overlap	3.96, overlap	5.47, d, 3.6	5.47, d, 3.6
5″	3.83, q, 6.3	3.89, q, 6.4	3.89, q, 5.4	3.83, q, 6.3	3.99, overlap	3.99, overlap
6″	1.40, d, 6.3	1.48, d, 6.4	1.48, d, 5.4	1.40, d, 6.3	1.16, d, 6.3	1.16, d, 6.3
1‴	6.20, d, 1.8	6.36, br s	6.41, br s	6.25, s	6.13, s	6.13, s
2‴	4.76, dd, 2.7, 1.8	4.86, br s	4.84, br s	4.75, overlap	4.74, overlap	4.74, overlap
3‴	4.61, dd, 9.0, 2.7	4.74, dd, 9.6, 3.2	4.71, d, 9.0	4.59, dd, 9.0, 2.7	4.59, dd, 9.9, 2.7	4.58, dd, 9.0, 1.8
4‴	4.30, t, 9.0	4.39, t, 9.6	4.33, t, 9.0	4.28, overlap	4.31, overlap	4.31, overlap
5‴	4.36, m	4.47, overlap	4.47, overlap	4.36, dd, 9.0, 6.3	4.38, overlap	4.37, dd, 9.0, 6.3
6‴	1.64, d, 6.3	1.70, d, 5.6	1.70, d, 6.3	1.60, d, 6.3	1.72, d, 6.3	1.72, d, 6.3
1⁗	5.06, d, 7.2	5.17, d, 8.0	5.03, d, 7.2	4.94, d, 7.2	5.07, d, 8.1	5.06, d, 8.1
2⁗	4.00, d, 7.2	4.12, t, 8.0	4.07, overlap	3.95, overlap	3.99, overlap	3.99, overlap
3⁗	4.09, overlap	4.16, overlap	4.06, overlap	4.01, overlap	4.09, t, 9.0	4.09, t, 9.0
4⁗	4.06, t, 9.0	4.22, t, 8.8	4.21, overlap	4.12, overlap	4.07, t, 9.0	4.07, overlap
5⁗	3.71, dq, 5.4, 2.7	3.79, br s	4.25, overlap	4.15, overlap	3.71, qd, 5.4, 2.7	3.71, qd, 5.4, 2.7
6⁗	–	–	3.52, t, 10.8	3.44, overlap		4.33, overlap
	4.32, dd, 11.7, 2.7	4.44, dd, 12.0, 2.4	–	–	4.33, dd, 11.7, 2.7	4.17, overlap
	4.16, dd, 11.7, 5.4	4.32, dd, 12.0, 4.8	–	–	4.17, dd, 11.7, 5.4	–
1⁗′	–	5.30, d, 7.2	–	5.15, d, 8.1	–	5.15, d, 7.2
2⁗′	–	4.02, t, 8.8	–	3.92, t, 7.2	–	3.92, t, 7.2
3⁗′	–	4.15, overlap	–	4.07, overlap	–	4.06, overlap
4⁗′	–	4.18, overlap	–	4.08, overlap	–	4.08, overlap
5⁗′	–	4.35, dd, 11.2, 4.8	–	4.23, overlap	–	4.22, dd, 10.8, 4.5
		3.69, m	–	3.58, t, 9.9	–	3.58, t, 10.8

[a] ^{1}H NMR spectrum measured in pyridine-d_5 at 900 MHz.

owing to the esterification effect from its usual detection at δ_C 100.3 in *β*-D-fucopyranose (Eskander, Lavaud, & Harakat, 2011), suggesting that *β*-fucopyranose was attached to C23 or C28. In addition, the oxygenated methine carbon signal (C-3) was downshifted at δ_C 85.7 owing to the glycosidation effect from its usual detection at δ_C 76.3 in medicagenic acid (Kozachok et al., 2020), suggesting that one sugar was attached to C-3. The HMBC correlations (Fig. 2B) observed between H-2/C-4, C-10; H-24/C-23; and H-16/H-22, C-28 confirmed that the aglycone of Compound **1** was medicagenic acid. In addition, four anomer proton signals (δ_H 5.16, H-1′; δ_H 5.15, H-1″; δ_H 5.91, H-1‴; δ_H 6.25, H-1⁗) showed cross peaks with the carbon signals of oxygenated methine (δ_C 85.7, C-3), ester (δ_C 176.6, C-28), oxygenated methine in *β*-fucopyranose (δ_C 74.1, C-2″), and oxygenated methine in *α*-rhamnopyranose (δ_C 84.5, C-4‴), suggesting that *β*-glucuronic acid, *β*-fucopyranose, *α*-rhamnopyranose, and *β*-glucopyranose were attached to C-3, C-28, C-2″, and C-4‴, respectively. These results were further supported by the 2D T-ROESY (Fig. 2C) cross-peaks at δ_H/δ_C 5.13 (GlcA*p* H-1′)/4.63 (H-3), 6.20 (Rha*p* H-1‴)/4.54 (Fuc*p* H-2″), and 5.06 (Glc*p* H-1⁗)/4.30 (Rha*p* H-4‴). By comparing the spectroscopic data of Compound **1** with those of a similar known compound, celosin I, reported in *Celosia argentea* (Pang et al., 2014), the structure of Compound **1** was determined to be 3-*O*-*β*-D-glucuronopyranosyl-2*β*,3*β*-dihydroxyolean-12-ene-23,28-dioic acid-28-

Table 3
^{13}C NMR data of celosin I and spinaciasaponin A–E.

No.	δ_C[a]					
	A (1)	B (2)	Celosin I (3)	C (4)	D (5)	E (6)
1	44.1	44.9	44.9	44.1	44.1	44.1
2	70.0	71.0	70.7	70.1	70.0	70.1
3	85.7	86.8	86.7	85.7	85.7	85.7
4	52.6	53.3	53.3	52.6	52.6	52.6
5	52.3	53.1	52.9	52.4	52.3	52.4
6a	20.9	21.6	21.6	20.9	20.9	20.9
7	32.7	33.4	33.4	32.7	32.8	32.7
8	40.1	40.8	40.8	40.1	40.1	40.1
9	48.4	49.0	49.0	48.4	48.4	48.4
10	36.5	37.3	37.2	36.5	36.5	36.5
11	23.7	24.3	24.3	23.7	23.7	23.7
12	122.4	123.1	123.1	122.4	122.4	122.4
13	143.8	144.3	144.3	143.8	143.8	143.8
14	42.0	42.7	42.7	42.1	42.1	42.1
15	28.0	28.6	28.6	28.0	27.9	27.9
16	23.1	23.8	23.7	23.0	23.1	23.2
17	46.8	47.3	47.4	46.8	46.8	46.8
18	41.8	42.5	42.4	41.8	41.7	41.7
19	46.0	46.6	46.6	46.0	46.0	46.0
20	30.4	31.1	31.1	30.4	30.4	30.4
21	33.6	34.3	34.3	33.6	33.6	33.6
22	32.0	32.7	32.7	32.0	32.1	32.0
23	180.9	180.9	181.0	180.7	180.9	180.7
24	13.8	14.5	14.6	13.8	13.8	13.8
25	16.6	17.3	17.3	16.6	16.7	16.6
26	17.2	17.8	17.8	17.1	17.2	17.2
27	25.8	26.5	26.4	25.8	25.8	25.8
28	176.6	177.0	177.0	176.6	176.6	176.6
29	32.8	33.5	33.5	32.8	32.8	32.8
30	23.5	24.2	24.1	23.5	23.5	23.5
OAc	–	–	–	–	171.3	171.2
OCH_3	–	–	–	–	20.6	20.6
1′	105.2	106.3	106.0	104.9	105.2	105.0
2′	74.4	74.4	75.3	73.6	74.4	73.6
3′	77.2	86.1	78.2	85.5	77.1	85.6
4′	72.8	71.8	73.7	71.0	72.8	73.3
5′	76.9	77.0	77.6	76.4	77.1	75.7
6′	172.7	170.6	173.0	173.5	172.0	173.9
1″	94.5	95.1	95.1	94.5	94.3	94.3
2″	74.1	74.7	74.4	73.8	74.4	74.4
3″	75.8	76.8	77.0	76.0	73.3	73.6
4″	72.6	73.5	73.5	72.6	74.5	74.5
5″	72.1	72.7	72.7	72.1	70.2	70.2
6″	16.5	17.2	17.3	16.6	16.2	16.2
1‴	101.1	101.8	101.7	101.0	101.5	101.5
2‴	71.2	72.1	71.3	71.2	71.1	71.1
3‴	71.9	72.8	72.9	71.9	71.8	71.8
4‴	84.5	85.8	85.8	84.6	84.4	84.4
5‴	68.2	68.9	68.6	68.0	68.4	68.4
6‴	18.3	19.0	18.9	18.2	18.5	18.4
1⁗	106.2	107.4	108.0	107.0	106.2	106.2
2⁗	75.9	76.8	76.6	75.7	75.8	75.8
3⁗	78.1	79.2	79.2	78.0	78.1	78.0
4⁗	71.3	72.2	72.2	70.4	71.3	71.2
5⁗	77.9	78.8	67.9	67.0	78.1	77.9
6⁗	62.4	63.3	–	–	62.4	62.3
1⁗′	–	106.5	–	105.5	–	105.5
2⁗′	–	75.8	–	74.8	–	74.8
3⁗′	–	78.4	–	77.3	–	77.3
4⁗′	–	71.8	–	70.4	–	70.4
5⁗′	–	67.7	–	66.7	–	66.7

[a] ^{13}C NMR spectrum was measured in pyridine-d_5 at 225 MHz.

O-*β*-D-glucopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-fucopyranoside. This compound has not been previously reported, for which we propose the trivial name spinaciasaponin A (Supplementary Fig. S6).

Compound **2** was obtained as a white amorphous powder with $[\alpha]_{25}^{D}$–2.7 (c = 0.1, MeOH). The positive HRESI-MS data (Supplementary Fig. S2) revealed a protonated molecular ion peak at m/z 1265.5810 [M + H]$^+$, indicating a molecular formula of $C_{59}H_{92}O_{29}$ (calcd for $C_{59}H_{93}O_{29}$, 1265.5797). The ^{1}H NMR and ^{13}C NMR data of Compound **2** (Tables 2 and 3) were similar to those of Compound **1**, except for signals indicating additional monosaccharide moieties such as a hemiacetal (δ_H 5.30, d, 7.2, H-1⁗′; δ_C 106.5, C-1⁗′), three oxygenated methines [(δ_H 4.18, overlap, H-4⁗′; δ_C 71.8, C-4⁗′), (δ_H 4.15, overlap, H-3⁗′; δ_C 78.4, C-3⁗′), (δ_H 4.02, t, 8.8, H-2⁗′; δ_C 75.8, C-2⁗′)] and one oxygenated methylene (δ_H 4.35, dd, 11.2, 4.8, H-5a‘⁗′/ δ_H 3.69, overlap, H-5b’⁗′; δ_C 67.7, C-5⁗′). The chemical shifts of the ^{13}C NMR data and the coupling constant of the anomer proton signal (J = 8.0 Hz) revealed that the monosaccharide was a *β*-xylopyranose. In the HMBC data, an anomer proton signal in *β*-xylopyranose (δ_H 5.17, H-1⁗′) presented a cross peak with the carbon signals of oxygenated methine in *β*-glucuronic acid (δ_C 86.1, C-3′), suggesting that *β*-xylopyranose was attached at C-3′. Based on these spectroscopic data, the structure of Compound **2** was determined to be 3-*O*-*β*-D-xylopyranosyl-(1 → 3)-*β*-d-glucuronopyranosyl-2*β*,3*β*-dihydroxyole-an-12-ene-23,28-dioic acid-28-*O*-*β*-D-glucopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-fucopyranoside. This compound has not been previously reported, for which we propose the trivial name spinaciasaponin B (Supplementary Fig. S7).

Compound **3** was obtained as a white amorphous powder with $[\alpha]_{25}^{D}$ 5.5 (c = 0.1, MeOH). The positive HRESI-MS data (Supplementary Fig. S2) revealed a protonated molecular ion peak at m/z 1103.5285 [M + H]$^+$, indicating a molecular formula of $C_{53}H_{82}O_{24}$ (calcd for $C_{53}H_{83}O_{24}$, 1103.5267). The ^{1}H NMR and ^{13}C NMR data of Compound **3** (Tables 2 and 3) closely resembled those of Compound **1**, except for signals indicating a distinct monosaccharide moiety, such as a hemiacetal (δ_H 5.03, d, 7.2, H-1⁗′; δ_C 108.0, C-1⁗′), three oxygenated methines [(δ_H 4.21, overlap, H-4⁗′; δ_C 72.2, C-4⁗′), (δ_H 4.07, overlap, H-2⁗′; δ_C 76.6, C-2⁗′), (δ_H 4.06, overlap, H-3⁗′; δ_C 79.2, C-3⁗′)] and one oxygenated methylene (δ_H 4.25, overlap, H-5a‘⁗′/δ_H 3.52, t, 10.8H-5b’⁗′; δ_C 67.9, C-5⁗′). The chemical shifts of the ^{13}C NMR data and the coupling constant of the anomer proton signal (J = 7.2 Hz) revealed that the distinct monosaccharide was a *β*-xylopyranose, not the *β*-glucopyranose in Compound **1**. In the HMBC data, an anomer proton signal in *β*-xylopyranose (δ_H 5.03, H-1⁗′) showed a cross peak with the carbon signals of oxygenated methine in *α*-rhamnopyranose (δ_C 85.8, C-4‴), suggesting that *β*-xylopyranose was attached at C-4‴. Consequently, the structure of Compound **3** was identified to be 3-*O*-*β*-D-glucuronopyranosyl-2*β*,3*β*-dihydroxyolean-12-ene-23,28-dioic acid-28-*O*-*β*-D-xylopyranosy-l-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-fucopyranoside, celosin I (Pang et al., 2014), as shown in Supplementary Fig. S8.

Compound **4** was obtained as a white amorphous powder with $[\alpha]_{25}^{D}$–28.7 (c = 0.1, MeOH). The positive HRESI-MS data (Supplementary Fig. S2) revealed a protonated molecular ion peak at m/z 1235.5713 [M + H]$^+$, indicating a molecular formula of $C_{58}H_{90}O_{28}$ (calcd for $C_{58}H_{91}O_{28}$, 1235.5691). The ^{1}H NMR and ^{13}C NMR data of Compound **4** (Tables 2 and 3) were significantly similar to those of Compound **3**, except for signals indicating additional monosaccharide moieties, such as a hemiacetal (δ_H 5.15, d, 8.1, H-1⁗′; δ_C 105.5, C-1⁗′), three oxygenated methines [(δ_H 4.08, overlap, H-4⁗′; δ_C 70.4, C-4⁗′), (δ_H 4.07, overlap, H-3⁗′; δ_C 77.3, C-3⁗′), (δ_H 3.92, t, 7.2, H-2⁗′; δ_C 74.8, C-2⁗′)] and one oxygenated methylene (δ_H 4.23, overlap, H-5a‘⁗′/δ_H 3.58, t, 9.9H-5b’⁗′; δ_C 66.7, C-5⁗′). The chemical shifts of the ^{13}C NMR data and the coupling constant of the anomer proton signal (J = 8.1 Hz) revealed that the monosaccharide was a *β*-xylopyranose. In the HMBC data, an anomer proton signal in *β*-xylopyranose (δ_H 5.15, H-1⁗′) showed a cross peak with the carbon signals of oxygenated methine in *β*-glucuronic acid (δ_C 85.5, C-3′), suggesting that *β*-xylopyranose was attached at C-3′. Based on these spectroscopic data, the structure of Compound **4** was determined to be 3-*O*-*β*-D-xylopyranosyl-(1 → 3)-*β*-D-glucuronopyranosyl-2β,3β-dihydroxyolean-12-ene-23,28-dioic acid-28-*O*-*β*-D-xylopyranosyl-(1 → 4)-*α*-L-rhamnop-yranosyl-(1 → 2)-*β*-D-fucopyran-oside. This compound has not been previously reported, for which we propose the trivial name spinaciasaponin C (Supplementary Fig. S9).

Compound **5** was obtained as a white amorphous powder with $[\alpha]_{25}^{D}$ 1.3 (c = 0.1, MeOH). The positive HRESI-MS data (Supplementary Fig. S2) revealed a protonated molecular ion peak at m/z 1175.5487 [M

+ H]$^+$, indicating a molecular formula of $C_{56}H_{86}O_{26}$ (calcd for $C_{56}H_{87}O_{26}$, 1175.5480). The ^{1}H NMR and ^{13}C NMR data of Compound **5** (Tables 2 and 3) were similar to those of Compound **1**, except for signals indicating an additional acetyl group [(δ_C 171.3, OAc), (δ_H 1.95, s; δ_C 20.6, OAc-CH_3)]. In the HMBC data, the proton signal of the oxygenated methine in *β*-fucopyranose (δ_H 5.47, H-4″) showed a cross peak with an ester signal from the acetyl group (δ_C 171.3, OAc), suggesting that an acetyl group was attached at C-4″. Based on these spectroscopic data, the structure of Compound **5** was identified as 3-*O*-*β*-D-glucuronopyranosyl-2*β*,3*β*-dihydroxyolean-12-ene-23,28-dioicacid-28-*O*-*β*-D-glucopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-4-*O*-acetyl-*β*-D-fucopyranoside. This compound has not been previously reported, for which we propose the trivial name spinaciasaponin D (Supplementary Fig. S10).

Compound **6** was obtained as a white amorphous powder with $[\alpha]_{25}^{D}$–6.3 ($c = 0.1$, MeOH). The positive HRESI-MS data (Supplementary Fig. S2) revealed a protonated molecular ion peak at m/z 1307.5897 [M + H]$^+$, indicating a molecular formula of $C_{61}H_{94}O_{30}$ (calcd for $C_{61}H_{95}O_{30}$, 1307.5903). The ^{1}H NMR and ^{13}C NMR data of Compound **6** (Tables 2 and 3) were nearly identical to those of Compound **5**, except for signals indicating additional monosaccharide moieties such as a hemiacetal (δ_H 5.15, d, 7.2, H-1‴″; δ_C 105.5, C-1‴″), three oxygenated methines [(δ_H 4.08, overlap, H-4‴″; δ_C 70.4, C-4‴″), (δ_H 4.06, overlap, H-3‴″; δ_C 77.3, C-3‴″), (δ_H 3.92, t, 7.2, H-2‴″; δ_C 74.8, C-2‴″)] and one oxygenated methylene (δ_H 4.22, dd, 10.8, 4.5, H-5a‵‴″/δ_H 3.58, t, 10.8H-5b‵‴″; δ_C 66.7, C-5‴″). The chemical shifts in the ^{13}C NMR data and the coupling constant of the anomer proton signal ($J = 7.2$ Hz) revealed that the monosaccharide was a *β*-xylopyranose. In the HMBC data, an anomer proton signal in *β*-xylopyranose (δ_H 5.15, H-1‴″) showed a cross peak with the oxygenated methine in *β*-glucuronic acid (δ_C 85.6, C-3′), suggesting that *β*-xylopyranose was attached at C-3′. Based on these spectroscopic data, the structure of Compound **6** was determined to be 3-*O*-*β*-D-xylopyranosyl-(1 → 3)-*β*-D-glucuronopyranosyl-2*β*,3*β*-dihydroxyol-ean-12-ene-23,28-dioic acid-28-*O*-*β*-D-glucopyranosyl-(1 → 4)-*α*-L-rhamnopyranosy-l-(1 → 2)-4-*O*-acetyl-*β*-D-fucopyranoside. This compound has not been previously reported, for which we propose the trivial name spinaciasaponin E (Supplementary Fig. S11).

3.5. Comparative analysis of total saponins in samples: Spectrophotometric and UPLC-CAD

Several researchers have documented the presence and composition of flavonoids as key secondary metabolites in spinach leaves. Specifically, most studies have focused primarily on investigating the functionality of well-known flavonoids in spinach (Yuk et al., 2019; Kim B.-M., Lee, Song, & Kim, H.-J, 2021). However, changes in secondary metabolites, particularly those associated with chronic inflammation and immune regulation, have not been extensively studied. Despite the growing interest in the health benefits of spinach, there is a lack of research on how these bioactive compounds vary and contribute to its potential therapeutic effects. Understanding these changes could provide valuable insights into the role of spinach in managing inflammatory and immune-related conditions. Additionally, while direct studies on the immunomodulatory and anti-inflammatory effects of spinach extract are limited, its observed bioactive properties suggest a potential role in regulating immune responses and reducing inflammation (Cartford et al., 2002; Ishida et al., 2016; Lomnitski et al., 2000). Previous research has demonstrated that spinach extract can inhibit cancer cell proliferation and induce apoptosis (Kumar et al., 2022), indicating that it may influence the cellular signaling pathways involved in immune regulation and inflammatory processes. These findings suggest that spinach may offer therapeutic benefits in managing chronic inflammatory and immune-related diseases. However, further studies are needed to elucidate the precise mechanisms underlying these effects and explore their potential clinical applications in immune modulation and inflammation-related disorders.

The spectrophotometric method, as outlined in previous studies (Le Bot, Thibault, Pottier, Boisard, & Guiletm, 2022), utilizes a common saponin chromophore for both steroid and triterpenoid saponins at a wavelength of 600 nm. This allows for easy quantification of the total saponin content in samples containing both types of saponins. To validate the applicability of the proposed method, a total saponin quantification curve was first established using Rg3, a representative standard material from ginseng, as the reference. The saponin content in the spinach extracts was subsequently quantified using this curve, and the theoretical relative content of saponins in the extracts was analyzed, the results of which are presented in Table 1. Total area pA values (UPLC-CAD, 7–10 min) obtained were estimated to be more accurate than those obtained via the gravimetric method, and the total saponin contents of the 15 spinach cultivars harvested according to season were as follows: fall (avg.: 79.16, 16.1–153.1 mg/g D.M.) > spring (avg.: 43.98, 1.1–208.5 mg/g D.M.) > winter (avg.: 20.88, 1.1–69.6 mg/g D.M.). Furthermore, Daechan (208.5 mg/g D.M.) exhibited the highest total saponin content in spring, whereas Friend (161.4 mg/g D.M.), Heukking (121.8 mg/g D.M.), Sushiro (136.5 mg/g D.M.), and Younghonam teoppat (153.1 mg/g D.M.) demonstrated the highest total saponin content in autumn. In contrast, all varieties showed the lowest total saponin content during the winter season. Notably, the time required for spinach to reach full maturity (25–30 cm) varied considerably by season—approximately 50 days in spring, 73 days in autumn, and 128 days in winter. Additionally, significant differences in average saponin content were observed based on both the harvest season and the specific cultivar. This variation in growth duration appeared to contribute to qualitative differences in saponin content. Le Bot, Thibault, Pottier, Boisard, and Guiletm (2022) reported that spectrophotometric methods are unable to simultaneously quantify the total saponin content in mixtures of steroids and triterpenoids. In constrast, chromatographic analysis methods provide a more precise quantitative analysis, as they allow for the identification of individual saponin components; this is possible when the analysis time and parameters are specifically optimized for saponin elution through separation, which is typically coupled with high-cost mass spectrometry equipment. Additionally, chromatographic methods offer accurate peak resolution, further enhancing the precision of the analysis.

For this reason, the contents of newly identified saponins (**1**–**6**) in 15 spinach cultivars at each growing season were determined and verified via UPLC-Q-TOF-MS and UPLC-CAD. For saponin profiling analysis of the aerial parts of *S. oleracea* L., the UPLC–Q-TOF–MS method was used. This technique allowed for the identification and quantification of various saponins present in the sample (Fig. 1). In addition, the experimental procedure outlined in Section 2.9, which involves UPLC–CAD, was utilized specifically for the analysis of major saponin components **1**–**6**. This combined approach ensured accurate profiling of the saponin composition in the plant material. Validation was performed for the limit of detection, limit of quantification, analytical curve linearity, recovery, and selectivity of the developed UPLC–CAD method. The results are presented in Table 4 and Supplementary Table S6. All the calibration curves demonstrated excellent linearity within the test range, with correlation coefficients (r^2) greater than or equal to 0.991. The limits of detection (LODs) and limits of quantification (LOQs) for the six saponins were 0.201–0.966 μg/mL and 0.771–2.927 μg/mL, respectively. The aerial parts of *S. oleracea* L. were extracted with 30 % EtOH, and six saponins were spiked into the samples at three different concentrations to evaluate their accuracy through recovery tests. The relative deviation (RSD) of the six saponins was less than 2 %, confirming a recovery rate of 90.226–106.167 % (Supplementary Table S6). The validation analysis results confirmed that the method established by UPLC-CAD was accurate and quantified major saponins **1**–**6** in the aerial extract of *S. oleracea* L. As described above, the HPLC-CAD method developed in this study, which targets six saponin components and is coupled with mass spectrometry, was found to be highly accurate. The total saponin content in the 15 spinach cultivars harvested according to season was as follows: spring (avg.: 18.25 mg/g D.M., range: 12.8–26.4 mg/g D.M.) > winter

Table 4
Linearity, LODs and LOQs of the six saponins isolated via the developed UPLC–CAD analysis.

Comp.	Name	Calibration equation (y = area, x = μg/mL)[a]	R^{2}[b]	Linear range (μg/mL)	LOD[c] (mg/g) (*n* = 5)	LOQ[c] (mg/g) (n = 5)	Spiking level (mg/g)		Recovery (%) (*n* = 9)	RSD (%) (n = 9)
1	Spinaciasaponin A	y = 1,157,159.0259× + 268,018.3418	0.9999	1.000–50.000	0.966	2.927	Low	7.16	104.9	0.4
							Middle	10.85	106.2	0.4
							High	14.54	106.1	0.8
							Low	10.31	93.1	0.7
2	Spinaciasaponin B	y = 792,692.8466× + 204,462.1940	0.9998	1.000–50.000	0.255	0.771	Middle	15.60	93.0	0.1
							High	20.88	90.2	0.4
							Low	2.18	99.9	0.7
3	Celosin I	y = 898,189.3524× + 548,399.1643	0.9993	1.000–50.000	0.220	0.668	Middle	3.58	98.8	1.0
							High	4.97	101.8	1.7
							Low	1.73	98.2	1.1
4	Spinaciasaponin C	y = 740,511.3929× – 58,600.9288	0.9999	1.000–50.000	0.201	0.609	Middle	2.64	96.8	1.8
							High	3.55	98.0	1.4
							Low	3.83	99.7	0.4
5	Spinaciasaponin D	y = 913,844.0145× + 24,534.8877	1.0000	1.000–100.000	0.310	0.939	Middle	5.75	100.5	1.3
							High	7.68	103.7	1.0
							Low	5.59	103.8	0.7
6	Spinaciasaponin E	y = 735,383.4273× + 765,572.0348	0.9991	1.000–100.000	0.730	2.213	Middle	8.91	103.4	2.0
							High	12.22	99.8	0.8

For at least five points on the standard calibration curves, each concentration was injected six times with UPLC-CAD.
[a] y = peak area, x = concentration of standard.
[b] coefficient of determination (R_2) is the square of the correlation coefficient.
[c] LOD and LOQ are the limits of detection and quantitation, respectively.

(avg.: 16.83 mg/g D.M., range: 9.6–33.2 mg/g D.M.) > fall (avg.: 13.92 mg/g D.M., range: 9.0–16.9 mg/g D.M.). In addition, the highest total saponin content in spring was found in Asiadongcho (22.4 mg/g D.M.), Luckyyou (26.4 mg/g D.M.), Samsan (23.3 mg/g D.M.), and Shinwoldong (23.8 mg/g D.M.). Sushiro (20.0 mg/g D.M.) exhibited the highest total saponin content in the fall. Moreover, in winter, Asiadongcho (33.2 mg/g D.M.), Platon Gold (21.3 mg/g D.M.), Point (26.1 mg/g D.M.), and Samsan (25.9 mg/g D.M.) showed high total saponin contents. Notably, the CAD method provided a more precise and reliable quantification of saponins than did the spectrophotometric approach, as it accurately measured the variations in saponin content across different spinach cultivars and harvest periods. These findings suggest that the CAD method offers a valuable tool for assessing saponin content, allowing for more detailed comparisons and a deeper understanding of how growing season and cultivar type influence saponin levels. We compared the results of saponin content analysis, which was both easily accessible and cost effective, across various spinach varieties to obtain more accurate content measurements. A detailed comparative analysis of the varieties, seasons, and components was conducted to identify the growing season, as well as to determine the most promising spinach varieties for saponin content.

3.6. Changes in the saponin contents of spinach cultivars during different harvest seasons

This study is the first to investigate the variations in key saponin compounds among different spinach cultivars in relation to growing season. The evaluation was based on the experimental methods for quality assurance and control conducted in the above analyses, ensuring the accuracy and reliability of the findings. This research provides valuable insights into how cultivation timing influences the composition of bioactive compounds in spinach. Therefore, we systematically cultivated various spinach cultivars, considering their known growing season, while accounting for the seasonal influences and environmental factors that impact the characteristics of each cultivar. We then conducted a quantitative analysis of their extracts to assess these variations. Tables 1 and 5 present the quantitative results for different sowing and harvesting stages, using a method that incorporates isolated and obtained saponins (**1**–**6**) reference substances to ensure accuracy and reliability. A comparison of the average contents of the six saponins revealed variations among the 15 spinach cultivars across three different seasons. The seasonal effects followed a distinct pattern, with the highest saponin levels observed in spring (1.1895–3.2821 mg/g D.M.) > fall (0.9167–2.3889 mg/g D.M.) ≥ winter (0.8103–2.7770 mg/g D.M.). The total saponin contents of compounds (**1**–**6**) significantly differed among the spring-harvested cultivars, and the cultivars with contents above 2 mg/g D.M. were as follows: Luckyyou (harvest period: November–March, 3.2821 mg/g D.M.) > Shinwoldong (harvest period: February, 3.2214 mg/g D.M.) > Daechan (harvest period: May–November, 2.2305 mg/g D.M.) > Sushiro (harvest period: May–November, 2.1112 mg/g D.M.) > Younghonam teoppat (harvest period: November–March, 2.0994 mg/g D.M.). The highest total content of standard saponins was obtained from the Luckyyou cultivar (3.2821 mg/g D.M.) when harvested in accordance with the guidelines suggested for harvesting in the spring season of March–May in Korea, whereas the Heukking cultivar (harvest period: May–June, 1.1895 mg/g D.M.) showed the lowest total content. The total saponin content of Compounds **1**–**6** was significantly different between autumn-harvested cultivars, with a lower content than in spring, and only one cultivar, Friend (harvest period: June–July, 2.3889 mg/g D.M.), was confirmed to have a content of more than 2 mg/g D.M. When the saponin content of isolated standard saponins was analyzed and compared with the recommended fall harvest period (September–November) in Korea, several cultivars appeared to align with the optimal harvest timing. These included Daechan (harvest period: May–November), Luckyyou (November–March), Point (April–September), Samsan (April–October), Sushiro (May–November), Woldong Tobaki (November–February), and Younghonam Teoppat (November–March). However, despite meeting the recommended harvest period, the actual saponin content analysis revealed relatively low levels, with an average range of 0.9167–1.7922 mg/g D.M. Notably, the Point cultivar, which was harvested from April to September, exhibited the lowest total saponin content, at 0.9167 mg/g D.M. When the saponin content of the isolated standard saponins was analyzed and compared with the recommended winter harvest period (December–February) in Korea, the results were similar to those observed in the fall. However, the overall saponin content tended to be lower. The cultivars Asiadongcho (harvest period: January–March), Shinwoldong (February), Ttangshimyi (December–February), Woldong Tobaki (November–February), and Younghonam Teoppat (November–March) did not align well with the optimal harvest timing.

Table 5
Results of the quantitative analysis of the contents of the six saponins (mg/g) in spinach by cultivar and cultivation season (continued).

Cultivar	Comp.	Spring	Fall	Winter	Cultivar	Comp.	Spring	Fall	Winter	Cultivar	Comp.	Spring	Fall	Winter
Asiadongcho	1	0.3325 ± 0.1322	0.1521 ± 0.0174	0.2406 ± 0.0270	Luckyyou	1	0.4291 ± 0.0167	0.1926 ± 0.0068	0.1872 ± 0.0047	Samsan	1	0.2080 ± 0.0337	0.2404 ± 0.0339	0.2766 ± 0.0440
	2	0.4271 ± 0.1887	0.4551 ± 0.0351	0.6040 ± 0.1004		2	1.1431 ± 0.0192	0.8769 ± 0.1014	0.7658 ± 0.0670		2	0.2441 ± 0.0433	0.2722 ± 0.0037	0.6700 ± 0.1857
	Sum	0.7596 ± 0.3161	0.6072 ± 0.0273	0.8446 ± 0.0919		Sum	1.5722 ± 0.0044	1.0695 ± 0.1076	0.9530 ± 0.0718		Sum	0.4521 ± 0.0483	0.5126 ± 0.0310	0.9466 ± 0.2172
	3	0.0312 ± 0.0055	0.1000 ± 0.0123	0.1373 ± 0.0123		3	0.1225 ± 0.0065	0.0993 ± 0.0226	0.1682 ± 0.0099		3	0.0889 ± 0.0217	0.1462 ± 0.0090	0.2161 ± 0.0508
	4	0.0808 ± 0.0285	0.1377 ± 0.0149	0.0992 ± 0.0623		4	0.1129 ± 0.0071	0.0827 ± 0.0146	0.1510 ± 0.0070		4	0.1163 ± 0.0014	0.1682 ± 0.0038	0.4149 ± 0.0781
	Sum	0.1120 ± 0.0286	0.2377 ± 0.0041	0.2365 ± 0.0591		Sum	0.2354 ± 0.0121	0.1820 ± 0.0107	0.3192 ± 0.0167		Sum	0.2052 ± 0.0230	0.3144 ± 0.0082	0.6310 ± 0.1273
	5	0.2128 ± 0.1146	0.0616 ± 0.0646	0.0829 ± 0.0204		5	0.2160 ± 0.0174	0.0604 ± 0.0052	0.1179 ± 0.0168		5	0.1487 ± 0.0145	0.1172 ± 0.0222	0.1586 ± 0.0056
	6	0.6485 ± 0.0717	0.3726 ± 0.0746	0.4142 ± 0.1129		6	1.2586 ± 0.0235	0.4803 ± 0.0302	0.7285 ± 0.2080		6	0.5551 ± 0.0389	0.4210 ± 0.0920	1.0407 ± 0.0466
	Sum	0.8613 ± 0.0432	0.4342 ± 0.1350	0.4971 ± 0.1276		Sum	1.4746 ± 0.0409	0.5407 ± 0.0349	0.8464 ± 0.2220		Sum	0.7038 ± 0.0532	0.5382 ± 0.1131	1.1993 ± 0.0503
	Grand total	1.7329 ± 0.3710	1.2791 ± 0.1390	1.5781 ± 0.2621		Grand total	3.2821 ± 0.0463	1.7922 ± 0.0717	2.1186 ± 0.2530		Grand total	1.3610 ± 0.0233	1.3652 ± 0.0765	2.7770 ± 0.2877
Daechan	1	0.3451 ± 0.0191	0.1621 ± 0.0060	0.1874 ± 0.0236	Mustang	1	0.2804 ± 0.0251	0.2004 ± 0.0184	0.1818 ± 0.0147	Shinwoldong	1	0.4190 ± 0.0285	0.1870 ± 0.0106	0.2171 ± 0.0080
	2	0.8353 ± 0.0546	0.8257 ± 0.1230	0.4022 ± 0.0525		2	0.5894 ± 0.0133	0.4315 ± 0.0812	0.3996 ± 0.0462		2	0.7202 ± 0.0765	0.6725 ± 0.1290	0.4577 ± 0.0620
	Sum	1.1804 ± 0.0478	0.9878 ± 0.1211	0.5896 ± 0.0463		Sum	0.8698 ± 0.0382	0.6319 ± 0.0979	0.5814 ± 0.0392		Sum	1.1392 ± 0.0544	0.8595 ± 0.1386	0.6748 ± 0.0698
	3	0.1402 ± 0.0054	0.1897 ± 0.0148	0.1561 ± 0.0055		3	0.0897 ± 0.0158	0.1387 ± 0.0090	0.1254 ± 0.0152		3	0.0468 ± 0.0020	0.1105 ± 0.0134	0.1617 ± 0.0079
	4	0.1656 ± 0.0080	0.2196 ± 0.0122	0.1696 ± 0.0172		4	0.0934 ± 0.0049	0.0842 ± 0.0036	0.1275 ± 0.0124		4	0.1790 ± 0.0064	0.1498 ± 0.0074	0.1392 ± 0.0057
	Sum	0.3058 ± 0.0105	0.4093 ± 0.0208	0.3257 ± 0.0131		Sum	0.1831 ± 0.0196	0.2229 ± 0.0122	0.2529 ± 0.0268		Sum	0.2258 ± 0.0072	0.2603 ± 0.0174	0.3009 ± 0.0136
	5	0.1415 ± 0.0138	0.0442 ± 0.0039	0.0504 ± 0.0004		5	0.1408 ± 0.0181	0.0354 ± 0.0010	0.0703 ± 0.088		5	0.4895 ± 0.0770	0.0485 ± 0.0040	0.1055 ± 0.0186
	6	0.6028 ± 0.0821	0.1831 ± 0.1481	0.2198 ± 0.0322		6	0.6227 ± 0.0624	0.1252 ± 0.1312	0.3431 ± 0.0165		6	1.3668 ± 0.0403	0.3911 ± 0.0761	0.2838 ± 0.0320
	Sum	0.7443 ± 0.0710	0.2273 ± 0.1451	0.2702 ± 0.0317		Sum	0.7635 ± 0.0804	0.1606 ± 0.1314	0.4134 ± 0.0240		Sum	1.8563 ± 0.0622	0.4396 ± 0.0746	0.3893 ± 0.0163
	Grand total	2.2305 ± 0.0734	1.6245 ± 0.0607	1.1855 ± 0.0659		Grand total	1.8163 ± 0.1345	1.0154 ± 0.1680	1.2477 ± 0.0442		Grand total	3.2214 ± 0.0506	1.5594 ± 0.2226	1.3650 ± 0.0712
Friend	1	0.2761 ± 0.0291	0.2870 ± 0.0047	0.1651 ± 0.0097	Platon Gold	1	0.4220 ± 0.0181	0.3836 ± 0.0608	0.4068 ± 0.0420	Sushiro	1	0.3133 ± 0.0170	0.1951 ± 0.0215	0.1768 ± 0.0074
	2	0.2957 ± 0.0351	0.1974 ± 0.0562	0.1440 ± 0.0177		2	0.2436 ± 0.0253	0.1733 ± 0.0011	0.7901 ± 0.1069		2	0.4705 ± 0.0768	0.7849 ± 0.1213	0.4002 ± 0.0141
	Sum	0.5718 ± 0.0601	0.4844 ± 0.0598	0.3091 ± 0.0213		Sum	0.6656 ± 0.0210	0.5569 ± 0.0606	1.1969 ± 0.0649		Sum	0.7838 ± 0.0919	0.9800 ± 0.1218	1.0991 ± 0.0074
	3	0.1087 ± 0.0112	0.3105 ± 0.0015	0.1650 ± 0.0072		3	0.1274 ± 0.0076	0.1535 ± 0.0058	0.1200 ± 0.0032		3	0.0966 ± 0.0081	0.1919 ± 0.0193	0.2317 ± 0.0017
	4	0.1273 ± 0.0160	0.9689 ± 0.1150	0.1790 ± 0.0200		4	0.1527 ± 0.0242	0.1119 ± 0.0164	0.2123 ± 0.0212		4	0.1198 ± 0.0265	0.1558 ± 0.0224	0.0999 ± 0.0206
	Sum	0.2360 ± 0.0176	1.2794 ± 0.1164	0.3440 ± 0.0264		Sum	0.2801 ± 0.0206	0.2654 ± 0.0220	0.3323 ± 0.0236		Sum	0.2164 ± 0.0325	0.3477 ± 0.0298	0.3316 ± 0.0215
	5	0.1673 ± 0.0284	0.1988 ± 0.0208	0.0362 ± 0.0010		5	0.1181 ± 0.0212	0.0345 ± 0.0037	0.0490 ± 0.0012		5	0.3662 ± 0.0179	0.0248 ± 0.0080	0.0532 ± 0.0086

(*continued on next page*)

Table 5 (*continued*)

Cultivar	Comp.	Spring	Fall	Winter	Cultivar	Comp.	Spring	Fall	Winter	Cultivar	Comp.	Spring	Fall	Winter
Heukking	6	0.7014 ± 0.0346	0.4262 ± 0.0081	0.1211 ± 0.0152	Point	6	0.3819 ± 0.0587	0.1156 ± 0.0066	0.2618 ± 0.0582	Ttangshimyi	6	0.7448 ± 0.1102	0.2887 ± 0.0255	0.2449 ± 0.0220
	Sum	0.8687 ± 0.0613	0.6250 ± 0.0282	0.1573 ± 0.0161		Sum	0.5000 ± 0.0794	0.1501 ± 0.0038	0.3108 ± 0.0591		Sum	1.1110 ± 0.1015	0.3135 ± 0.0331	0.2981 ± 0.0282
	Grand total	1.6765 ± 0.0335	2.3889 ± 0.0925	0.8103 ± 0.0247		Grand total	1.4458 ± 0.0744	0.9724 ± 0.0365	1.8400 ± 0.1423		Grand total	2.1112 ± 0.2147	1.6411 ± 0.1535	1.7289 ± 0.0526
	1	0.2199 ± 0.2199	0.1134 ± 0.0086	0.1313 ± 0.0036		1	0.4296 ± 0.0429	0.3168 ± 0.0465	0.4626 ± 0.0989		1	0.2950 ± 0.0262	0.1899 ± 0.0241	0.1393 ± 0.0194
	2	0.3795 ± 0.0302	0.3745 ± 0.0555	0.4404 ± 0.0029		2	0.1851 ± 0.0526	0.1253 ± 0.0504	0.4825 ± 0.0835		2	0.6702 ± 0.1038	0.4678 ± 0.0277	0.5731 ± 0.0478
	Sum	0.5994 ± 0.1003	0.4879 ± 0.0509	0.5717 ± 0.0111		Sum	0.6147 ± 0.0444	0.4421 ± 0.0835	0.9451 ± 0.0808		Sum	0.9652 ± 0.1300	0.6577 ± 0.0157	0.7124 ± 0.0388
	3	0.1181 ± 0.0147	0.1306 ± 0.0168	0.1681 ± 0.0039		3	0.1408 ± 0.0190	0.1499 ± 0.0417	0.2172 ± 0.0171		3	0.0502 ± 0.0067	0.0704 ± 0.0029	0.0806 ± 0.0049
	4	0.1065 ± 0.0056	0.1628 ± 0.0155	0.1866 ± 0.0098		4	0.1122 ± 0.0202	0.1002 ± 0.0300	0.2584 ± 0.0789		4	0.1866 ± 0.0275	0.1458 ± 0.0151	0.1377 ± 0.0043
	Sum	0.2246 ± 0.0167	0.2934 ± 0.0200	0.3547 ± 0.0236		Sum	0.2530 ± 0.0383	0.2501 ± 0.0476	0.4756 ± 0.0726		Sum	0.2368 ± 0.0303	0.2162 ± 0.0123	0.2183 ± 0.0078
	5	0.0743 ± 0.0025	0.0722 ± 0.0140	0.0382 ± 0.0016		5	0.1418 ± 0.0012	0.0853 ± 0.0090	0.0756 ± 0.0098		5	0.1564 ± 0.0169	0.0946 ± 0.0073	0.1098 ± 0.0201
	6	0.2911 ± 0.0192	0.5429 ± 0.1386	0.1686 ± 0.0137		6	0.3516 ± 0.0341	0.1392 ± 0.0083	0.3947 ± 0.0382		6	0.5471 ± 0.0538	0.1351 ± 0.0069	0.5356 ± 0.0664
	Sum	0.3654 ± 0.0167	0.6151 ± 0.1523	0.2068 ± 0.0212		Sum	0.4934 ± 0.0329	0.2245 ± 0.0170	0.4703 ± 0.0460		Sum	0.7035 ± 0.0661	0.2297 ± 0.0140	0.6454 ± 0.0823
	Grand total	1.1895 ± 0.1029	1.3964 ± 0.1869	1.1333 ± 0.0368		Grand total	1.3610 ± 0.0515	0.9167 ± 0.1372	1.8910 ± 0.1466		Grand total	1.9055 ± 0.1776	1.1037 ± 0.0353	1.5761 ± 0.1162

Furthermore, the saponin content analysis revealed relatively low levels, with an average range of 0.9845–1.5781 mg/g D.M. Notably, the Friend cultivar, which was harvested from June to July, exhibited the lowest total saponin content, at 0.8102 mg/g D.M. The results revealed notable variations depending on the cultivar, with the spring season resulting in the most economically advantageous growing period. As a result, harvesting during spring may be optimal for maximizing spinach production. In particular, the seasonal growing period required for spinach to fully mature varied significantly, and there were notable differences in the average saponin content depending on both the harvest season and the specific cultivar. These findings suggest that multiple factors contribute to the qualitative variations in saponin content. The analysis of growing season and cultivar revealed that the Luckyyou (1.7922–3.2811 mg/g D.M.) and Sushiro (1.6411–2.1112 mg/g D.M.) cultivars exhibited consistently high total saponin contents. The saponin composition of spinach may be influenced by various factors beyond the recommended sowing and harvesting periods outlined in the guidelines. These factors include environmental stress, cultivar characteristics, and agricultural conditions. (See Table 5.)

In particular, the major saponins (**1** and **2**/**5** and **6**) found in the most viable spring-harvested spinach cultivars exhibited the following average concentrations: Asiadongcho (0.7596/0.8613 mg/g D.M.), Daechan (1.1804/0.7443 mg/g D.M.), Friend (0.5718/0.8687 mg/g D.M.), Heukking (0.5994/0.3654 mg/g D.M.), Luckyyou (1.5722/1.4746 mg/g D.M.), Mustang (0.8698/0.7635 mg/g D.M.), Platon Gold (0.6656/0.5000 mg/g D.M.), Point (0.6147/0.4934 mg/g D.M.), Samsan (0.4521/0.7038 mg/g D.M.), Shinwoldong (1.1392/1.8563 mg/g D.M.), Sushiro (0.7838/1.1110 mg/g D.M.), Ttangshimyi (0.9652/0.7035 mg/g D.M.), Venus (0.5883/0.4042 mg/g D.M.), Woldong Tobaki (0.7582/1.0060 mg/g D.M.), and Younghonam Teoppat (0.9332/0.9281 mg/g D.M.). The highest contents of major compounds (1 and 2/5 and 6) were obtained from the Luckyyou (1.5722/1.4746 mg/g D.M.) and Shinwoldong (1.1392/1.8563 mg/g D.M.) cultivars. The major compound spinaciasaponin B (**2**) showed the following content values: Luckyyou (1.1431 mg/g D.M.) > Daechan (0.8353 mg/g D.M.) > Shinwoldong (0.7202 mg/g D.M.) > Ttangshimyi (0.6702 mg/g D.M.) > Younghonam Teoppat (0.6353 mg/g D.M.) > Mustang (0.5894 mg/g D.M.) > Woldong Tobaki (0.5169 mg/g D.M.). Moreover, the major compounds spinaciasaponin E (**6**) presented the following contents: Luckyyou (1.2586 mg/g D.M.) > Shinwoldong (1.3668 mg/g D.M.) > Woldong Tobaki (0.8123 mg/g D.M.) > Younghonam Teoppat (0.7801 mg/g D.M.) > Sushiro (0.7448 mg/g D.M.) > Friend (0.7014 mg/g D.M.). Considering these comprehensive data, Luckyyou and Shinwoldong have emerged as the most promising cultivars in terms of saponin content. Luckyyou stands out as the top candidate owing to its consistently high concentrations across multiple saponin compounds, making it an excellent choice for maximizing bioactive saponin intake. Moreover, Shinwoldong also ranks highly and could be a viable alternative. These findings suggest that selecting cultivars with high saponin contents, such as Luckyyou and Shinwoldong, could be beneficial for enhancing the nutritional and functional properties of spinach, particularly for applications where saponins are valued for their potential health benefits.

Our results confirm that the saponin content in spinach interacts with both cultivar and harvest timing, revealing a correlation between specific cultivars and harvest periods. However, some cultivars were found to be independent of these factors, suggesting that the saponin content in those varieties is not significantly influenced by the timing of harvest or cultivar choice. However, this variation cannot be attributed solely to these factors. The differences in saponin content are likely the result of multiple interacting variables, including nutrient availability, plant physiological status, cultivation techniques, genetic modifications, phenotypic traits, and agricultural practices. In general, most studies analyze saponin content based on cultivar (Mroczek, Kapusta, Janda, & Janiszowska, 2012) and growth period (Zhang et al., 2019). However, our findings differ from these conventional results, suggesting that additional factors may play a significant role. Moreover, Szakiel,

Table 5
Results of the quantitative analysis of the contents of six saponins (mg/g) in spinach by cultivar and cultivation season.

Cultivar	Comp.	Spring	Fall	Winter	Cultivar	Comp.	Spring	Fall	Winter	Cultivar	Comp.	Spring	Fall	Winter
Venus	1	0.4113 ± 0.0532	0.4087 ± 0.0528	0.3001 ± 0.0430	Woldong tobaki	1	0.2413 ± 0.0097	0.2351 ± 0.0087	0.1699 ± 0.0115	Younghonam teoppat	1	0.2979 ± 0.0199	0.1765 ± 0.0026	0.1659 ± 0.0048
	2	0.1770 ± 0.0217	0.2587 ± 0.0271	0.3393 ± 0.0408		2	0.5169 ± 0.0646	0.5172 ± 0.0206	0.3006 ± 0.0071		2	0.6353 ± 0.0260	0.3858 ± 0.0077	0.3507 ± 0.0192
	Sum	0.5883 ± 0.0555	0.6674 ± 0.0320	0.6394 ± 0.0399		Sum	0.7582 ± 0.0672	0.7523 ± 0.0237	0.4705 ± 0.0167		Sum	0.9332 ± 0.0428	0.5623 ± 0.0052	0.5166 ± 0.0156
	3	0.1028 ± 0.0126	0.1678 ± 0.0088	0.2601 ± 0.0144		3	0.0655 ± 0.0046	0.1171 ± 0.0061	0.1613 ± 0.0085		3	0.1106 ± 0.0204	0.1722 ± 0.0103	0.0583 ± 0.0022
	4	0.1131 ± 0.0374	0.1102 ± 0.0302	0.2541 ± 0.0271		4	0.1271 ± 0.0136	0.1588 ± 0.0260	0.1547 ± 0.0305		4	0.1275 ± 0.0200	0.1897 ± 0.0205	0.0662 ± 0.0182
	Sum	0.2159 ± 0.0488	0.2780 ± 0.0390	0.5142 ± 0.0320		Sum	0.1926 ± 0.0172	0.2759 ± 0.0266	0.3160 ± 0.0237		Sum	0.2381 ± 0.0180	0.3619 ± 0.0300	0.1245 ± 0.0203
	5	0.1140 ± 0.0227	0.1451 ± 0.0204	0.0437 ± 0.0162		5	0.1937 ± 0.0152	0.0788 ± 0.0189	0.0671 ± 0.0054		5	0.1480 ± 0.0353	0.0653 ± 0.0127	0.0695 ± 0.0047
	6	0.2902 ± 0.0245	0.2521 ± 0.0126	0.1167 ± 0.0178		6	0.8123 ± 0.0242	0.2669 ± 0.0311	0.2653 ± 0.0331		6	0.7801 ± 0.1236	0.3571 ± 0.0502	0.2739 ± 0.0445
	Sum	0.4042 ± 0.0466	0.3972 ± 0.0329	0.1604 ± 0.0335		Sum	1.0060 ± 0.0257	0.3457 ± 0.0494	0.3324 ± 0.0310		Sum	0.9281 ± 0.1587	0.4224 ± 0.0613	0.3434 ± 0.0491
	Grand total	1.2085 ± 0.0599	1.3425 ± 0.0528	1.3140 ± 0.0484		Grand total	1.9568 ± 0.0340	1.3740 ± 0.0349	1.1190 ± 0.0595		Grand total	2.0994 ± 0.2025	1.3467 ± 0.0848	0.9845 ± 0.0440

Paczkowski, and Henry (2011) examined plant physiology and saponin synthesis, providing insights into these variations. However, they did not comprehensively explain the differences observed in our research. The findings of our study suggest that the differences in saponin content among spinach cultivars are not random but are instead linked to intrinsic genetic characteristics and environmental responses. These differences may result from variations in genetic lineage or differential reactions to seasonal growth conditions. Saponins are naturally occurring glycosides that play crucial roles in plant defense mechanisms (Morrissey & Osbourn, 1999), protecting plants from various environmental stresses, including pathogens, herbivores, and abiotic factors such as temperature fluctuations, drought, and soil composition. The biosynthesis and accumulation of saponins are highly responsive to environmental conditions, as stress factors can trigger metabolic pathways that increase their production (Szakiel, Paczkowski, & Henry, 2011). High temperatures (Phrompittayarat et al., 2011) and intense light exposure (Watcharatanon, Ingkaninan, & Putalun, 2019) have been reported to increase saponin concentrations in certain plant species. For example, research on *Medicago sativa* (alfalfa) by Pecetti, Tava, Romani, De Benedetto, and Corsi (2006) demonstrated that exposure to prolonged UV radiation led to a significant increase in saponin content, likely as a protective adaptation against oxidative stress. Similarly, studies of *Panax ginseng* by Zhang et al. (2021) revealed that low-temperature stress also enhances saponin biosynthesis as part of the plant's defense mechanism. Water availability is another critical factor influencing saponin levels. Drought stress has been shown to trigger increased saponin accumulation in several plant species. For example, research on *Quillaja saponaria* (soapbark tree) by Grandón Sa et al. (2013) indicated that reduced soil moisture conditions led to increased concentrations of triterpenoid saponins, likely in response to dehydration stress. Soil quality, including nutrient availability and pH, also affects saponin production. A study on spinach (Xu & Mou, 2016) revealed that nitrogen and phosphorus deficiencies influence the metabolic pathways involved in flavonoid and carotenoid synthesis, leading to altered concentrations of specific flavonoid and carotenoid compounds. Additionally, increased salinity has been linked to increased saponin contents in certain halophytes, as these compounds contribute to osmotic regulation and stress adaptation (Zare-maivan, Farahani, & Ghanati, 2016). Through our comparative analysis of seasonal and cultivar-specific spinach cultivation, we aimed to clarify the environmental and varietal influences on saponin composition. Additionally, our findings suggest that spinach leaves utilize ionic factors to regulate energy acquisition, highlighting the complex physiological mechanisms underlying saponin metabolism.

4. Conclusions

In the present study, the total saponin content and quantitative analysis of novel major saponin compounds isolated from 15 different spinach cultivars at different growing season was reported. In this study, simple, rapid, and inexpensive gravimetric, spectrophotometric, and HPLC-CAD methods were compared and analyzed for the total quantification of saponins in seasonal extracts of spinach cultivars, and the proposed CAD method was found to have excellent performance in terms of linearity, precision, and accuracy. In addition, the isolation and investigation of saponins identified from spinach were investigated using NMR spectroscopy and UPLC-QTOF-MS analysis. The six compounds isolated here were identified as spinaciasaponin A (**1**), spinaciasaponin B (**2**), celosin I (**3**), and spinaciasaponin C-E (**4**–**6**). The analysis of growing season and cultivar revealed that the Luckyyou and Sushiro cultivars exhibited consistently high total saponin contents. The major compound spinaciasaponin B (**2**) had the highest content in Luckyyou, followed by Daechan and Shinwoldong, with Woldong Tobaki having the lowest content. For spinaciasaponin E (**6**), the highest content was found in Shinwoldong, followed closely by Luckyyou, whereas Friend had the lowest content. These findings indicate that Luckyyou and Shinwoldong are notable cultivars with high levels of spinaciasaponin B and E, respectively. Considering these comprehensive data, Luckyyou and Shinwoldong have emerged as the most promising cultivars in terms of saponin content. Luckyyou stands out as the top candidate due to its consistently high concentrations across multiple saponin compounds, making it an excellent choice for maximizing

bioactive saponin intake. In addition, this study determined the optimal growing season and potential cultivars for the development of functional foods with high saponin contents from spinach, a diverse source of saponins. The results suggest that although growing season vary depending on the cultivar, certain varieties in spring could serve as excellent potential sources for functional food development. These findings are expected to contribute to the development of suitable cultivars that can be utilized as potential health-promoting functional foods.

CRediT authorship contribution statement

Su-Yeon Lee: Writing – original draft, Conceptualization, Formal analysis, Data curation. **Doo-Young Kim:** Data curation, Formal analysis, Writing – original draft, Resources. **Hyun-Jae Jang:** Formal analysis, Writing – original draft. **Seon Min Oh:** Methodology, Formal analysis, Data curation. **Hyoung-Geun Kim:** Methodology, Data curation, Formal analysis. **Jongmin Ahn:** Data curation, Formal analysis, Methodology. **In-Seo Heo:** Formal analysis, Validation. **Heung Joo Yuk:** Formal analysis. **Eun Kyoung Seo:** Conceptualization, Supervision. **Sei-Ryang Oh:** Writing – original draft, Supervision, Conceptualization. **Hyung Won Ryu:** Writing – original draft, Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.145741.

Data availability

Data will be made available on request.

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