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Spruce, pine and fir needles as sustainable ingredients for whole wheat bread fortification: Enhancing nutritional and functional properties

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ABSTRACT

This study investigated green needles and fine twigs (NT) from spruce, pine, and fir, widely available forest by-products, as potential functional ingredients and natural preservatives for whole wheat bread fortification. The effects of replacing water with NT extracts at 0, 35, and 70% levels were assessed on bread's secondary metabolite profiling, bioactivity, nutrition, and quality. The NT-bread demonstrated good stability of compounds analyzed by HPLC-DAD after 24 and 72 h, with a notable increase in the content of several polyphenols after 72 h of storage. This increase was correlated closely with an over 80% enhancement in antioxidant activity during storage, suggesting extended shelf-life. A total of 115 compounds were identified by UHPLC-QTOF-MS/MS, including flavonoids, phenolic acids, alkaloids, stilbenes, lignans, resin acids, and gibberellins. Among the substitution levels, 35% , particularly pine NT-fortified bread, was sufficient to enhance functionality while preserving bread quality and sensory acceptability, making it a strong candidate for further development as a bioactive product. Overall, the findings demonstrate the valorization potential of underutilized NT as natural antioxidants and will help provide the industry with phytochemical compositional information. This study highlights the broader applicability of these side streams by employing a green extraction technique (hydrodynamic cavitation) that yields valuable compounds, promotes sustainability, and supports the circular economy through a cost-effective and efficient process.

1. Introduction

Softwood species, such as Norway spruce (*Picea abies*), Japanese red pine (*Pinus densiflora*) and silver fir (*Abies alba*), grow in different parts of the globe. Norway spruce is native to Northern, Central, and Eastern Europe while red pine is found in Japan, Korea, and Northeastern China. Silver fir is native to European mountains and widespread in southern and central-eastern Europe (Dobrowolska et al., 2017). During forest logging, trees are harvested for timber or pulpwood, often leaving their branches on-site to decompose.

Green needles are an underutilized reservoir of valuable polyphenols. For instance, Norway spruce needles are rich in hydroxycinnamic acids, flavonoids, and stilbenes, known for their biological and nutraceutical benefits (Mofikoya et al., 2022, 2023; Slimestad et al., 1992). A recent study found that adding pine (*P. densiflora)* needle powder extract as a feed supplement significantly reduced body weight, fat mass, plasma leptin levels while improving glucose metabolism in mice, regulating energy balance via the hypothalamus (Kim et al., 2021). Another research highlighted the antibacterial and antioxidant properties of 7-year-old self-fermented red pine needle extracts due to synergistic interactions among compounds during self-fermentation (Park et al., 2008). Other studies reported various beneficial effects of

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pine needle extracts, including antioxidant, antimicrobial, anti-diabetic, antimutagenic, antitumor, cytoprotective and antiapoptotic properties (Kwak et al., 2006; Park et al., 2021; Wu et al., 2015). Pine trees, with their global distribution, offer various consumable products based in needles in Asia, such as powder, tonic, wine, and tea (Kim & Chung, 2000). Japanese red pine needles also have a long history in herbal medicine, continuing to be used in Japan for nourishing and tonic preparations (Lee et al., 2021).

Recently, there has been a growing interest in using unconventional plants for new functional foods and medicine (Soltan et al., 2023). A study revealed that adding silver fir needle extracts to whole wheat bread increased its antioxidant capacity by 87% and improved dough and bread volume, highlighting their potential as beneficial ingredient for enhancing bread's functional and antioxidant properties (Parenti et al., 2022). Additionally, supplementing beer with a Scots pine needle extract enable the reduce oxidative stress in the brain under acute pathological conditions, suggesting its potential to mitigate alcohol's adverse effects (Penkina et al., 2017). A study on spruce sprouts and older needles showed higher antioxidant activity, energy, and calcium content in older needles, while sprouts were richer in vitamin C, magnesium, potassium, and phosphorus (Jyske et al., 2020). Furthermore, incorporating fermented pine (*P. densiflora*) needle extract into hen diets enhanced egg yolk antioxidants, color, and shell strength, demonstrating the potential of pine needles for improving food quality (Kothari et al., 2021).

Given the beneficial properties of conifer needles as supported by the literature, they could be effectively incorporated into cereal-based foods, particularly bread, which accounts for over 50% of energy intake in developed societies. Known for its high fiber content, whole wheat bread is linked to enhanced gut health and a lower risk of heart disease (Cappelli & Cini, 2021; Dziki et al., 2014; James et al., 1997). Even though bread is a promising vehicle for functional supplements, its potential in this area is yet to be fully explored.

Therefore, the aims of this study were (1) to provide a framework about the physico-chemical and bioactivity properties of aqueous extracts from needles and fine twigs (NT) of Norway spruce (*P. abies*), Japanese red pine (*P. densiflora*) and silver fir (*A. alba*), (2) develop bread models fortified with the most promising extracts at different incorporation levels, and (3) assess the secondary metabolites and their stability during storage, bioactivity, quality properties, and sensory characteristics of the fortified bread variants. This study highlights the potential of underutilized NT as a natural preservative and demonstrates how fortified bread models can improve nutrition and promote the use of these by-products. Importantly, utilizing a green, affordable, and efficient extraction method can contribute to building a sustainable economy for forestry by-products, serving as a new route to their valorization.

2. Material and methods

2.1. Chemicals and reagents

Folin-Ciocalteu reagent and phosphate buffer were purchased from Merck KgaA (Darmstadt, Germany). Chemical standards of gallic acid, catechin, epicatechin, gallocatechin, epigallocatechin, $FeSO_4 \bullet 7H_2O$, and DPPH free-radical were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Procyanidin B2 was purchased from Extrasynthese (Lyon, France). L(+)-ascorbic acid was purchased from VWR Chemicals (Germany). Unless otherwise specified, the chemicals were purchased from Merck KgaA (Darmstadt, Germany).

2.2. Collection and sorting of the plant materials

This study investigated needles and twigs of three tree species collected from different parts of the world: *Pinus densiflora* Siebold & Zuccarini (Japan), *Abies alba* Mill (Italy), and *Picea abies* (L.). H. Karst (Sweden). Table 1 summarizes the features of the sample collection. Twigs are a tree's smallest and thin branches to where leaves, needles, or buds are directly attached. Green needles and twigs samples were treated equally regarding harvesting, drying, and milling (Supplementary Fig. 1). Each sample was pre-dried in open air for around five days before shipping to Biomass Technology Centre in Sweden (BTC), where the samples were dried at 30 °C for 48 h in a cabinet before milling. The dried materials were milled in a Retch SM2000 laboratory mill (Haan, Germany) and at least 93% of milled material showed particle size below 1 mm (Supplementary Table 1).

2.3. Extraction process

Milled and dried green needles and twigs samples were extracted in water using hydrodynamic cavitation (HC) processes. The semi-industrial-scale (200 L) HC pilot device, optimized for food applications, included a closed hydraulic circuit with a centrifugal pump, in line with a circular Venturi-shaped reactor. Detail of all the components, and the assessment method of the cavitation number, which is a measure of the cavitation intensity, in turn regulating the extraction efficiency, were described in a previous study (Meneguzzo et al., 2019). No active heat dissipation method was applied. Power and energy consumption were measured by means of a three-phase digital power meter (IME model D4-Pd, Milan, Italy). Supplementary Table 2 shows the basic features of the extraction processes for the sample materials; in all processes, a practically constant cavitation number of 0.10–0.11 ensured optimal yield (Meneguzzo et al., 2021).

Extracts of green needles and twigs (NT) from spruce (SNT), pine (PNT), and fir (FNT) were collected at 10, 20, and 30 min, as well as at the point when the temperature reached 47 °C, which varied slightly (52–58 min) with the raw material and the solid-to-liquid ratio. The fresh biomass to water ratio was 16 kg/160 L for spruce, 9.1 kg/170 L for silver fir, and 7.9 kg/170 L for pine, corresponding to 100 g/L, 53.5 g/L, and 46.5 g/L, respectively. During the HC treatment, the temperature rose from the original room temperature (18–19 °C) to 47 °C. The rationale for the different quantities of raw materials was to work to the full potential of the HC pilot device, using all the available quantity of each biomass. After the extraction, samples were frozen and stored at −20 °C before further use.

2.4. Characterization of green needle and twig extracts

2.4.1. Physico-chemical analyzes

Crude extracts (1000 mL) were filtrated with a nylon (50 μm) filter bag (Eaton NMO-50-P03S-50S, Hyxo, Finland) and the solid material left in the filter bag was dried and weighed (i.e., part of total solids). The filtrate was further centrifuged 20 min at 4816 g to separate the remaining solid particles. The resulting supernatant was then used in the studies, and referred as follows only as an "extract". Total dissolved solids (TDS) were determined in aliquots of extracts after oven-drying at 105 °C. Additionally, pH of the extracts was measured at room temperature with a pH meter (Five easy, Mettler-Toledo, UK). The soluble sugars (glucose, fructose, sucrose) content was measured with a refractometer (Bellingham & Stanley Ltd, UK) using the °Brix scale.

2.4.2. Analyzes of secondary metabolites in extracts

For the determination and quantitation of phenolics and other UV-absorbing semi-polar compounds, freeze-dried extracts (50 mg) were extracted with 80% methanol (5 mL) in an ultrasonic bath for 30 min. An aliquot was then centrifuged (600 g, 10 min) and filtered for liquid chromatographic determination. Analysis of phenolic compounds was done by Agilent 1100-series high-performance liquid chromatography equipped with a diode array detector (HPLC-DAD, Agilent, Santa Clara, CA, USA). The HPLC pumps, autosampler, column oven, and diode array system were operated by the ChemStation computer program. The analytical column was Phenomenex Kinetex® C18 (150 × 3.0 mm; 5 μm

Table 1
Collection of experimental samples.

Collected material	Location	Date	Parts of the tree	Sampling
Norway spruce (*Picea abies* (L.) H. Karst)	Umeå, Västerbotten, Sweden (63°38′04.1"N; 19°59′41.2"E)	November 2022	Twigs were harvested from the lower branches of older trees, and the entire tree in younger specimens	4 large trees (around 70-year-old) and 6 small trees (around 20 years old)
Japanese red pine (*Pinus densiflora* Siebold & Zuccarini)	Minamiminowa Village, the forest near Ina city Nagano prefecture, about 930 m a.s.l., Japan (35°53′57"N; 137°54′58"E)	October 2022	Crown parts with green needles	3 trees (between 4 and 5 m)
Silver fir (*Abies alba* Mill.)	"Teso" Forest in Tuscany Apennines, about 1050 m a.s.l. (44°03′54"N; 10°48′34"E)	October 2022	Twigs from older trees were collected from lateral branches no more than 2 m above the soil, while for younger trees, they were collected from the crown branches	8 trees, around 4 high-rise trees (about 60 years old) and 4 low-rise trees (5–20 years old)

i. d.; 100 Å); the column oven was set at 35 °C. The mobile phase consisted of 0.05 M phosphate buffer (A) at pH 2.4 and methanol (B) with the following gradient: 5 − 60% B in 45 min; 60 − 98% B in 10 min, 98 − 100% in 10 min, and at 100% for 20 min. After the 85 min gradient, B was set to 5% in 5 min, and then a post-run time 15 min was applied. The flow rate was 0.6 mL/min. The chromatograms were obtained at 245, 280 and 350 nm wavelengths.

For identification purposes, UV-spectra were recorded at 190 − 600 nm. Based on the in-house UV spectral libraries and combined with the retention times, some compounds were identified, while the others were just putative identified. Quantitation of the compounds was performed using corresponding reference compounds, as in the case of ferulic acid, or by the compound with the closest resemblance by UV-spectrum. For SNT at 350 nm, the compound with UV spectrum resemblance of umbelliferone was expressed as peak area unit/g; otherwise, the results were given as mg/100 g dry weight (DW). The samples were prepared in triplicate. Further characterization of the compounds was conducted by UHPLC-QTOF-MS/MS according to the analytical conditions described by Karonen and Pihlava (2022) and Pihlava et al. (2018). Putative identification of the main peaks in the negative and positive base peak ion (BPI) chromatograms was performed. The LC-MS/MS-data was also screened using the targeted compound approach method (Pihlava et al., 2018).

Condensed tannins (proanthocyanins) were determined by a thiolytic degradation method according to Korkalo et al. (2020). Briefly, 20–30 mg of the sample was mixed with 1 mL of thiolysis reagent (methanolic cysteamine acidified by hydrochloric acid). After 60 min incubation at 65 °C, the thiolysis reaction was terminated, and the sample solutions were filtered into HPLC vials and analyzed by the UHPLC-DAD-FLD. The quantification was based on the external standards of catechin, epicatechin, gallocatechin, epigallocatechin, and thiolysed procyanidin B2. The aqueous extracts and the bread samples were freeze-dried prior to the analysis. The results were presented as mg/100 g per extract dry weight (DW) and fresh weight (FW).

2.4.3. Bioactive properties: Total phenolic content, antioxidant activity and antibacterial properties

All the extracts were diluted to 1, 0.5, and 0.25 g/L according to their dry weights (TDS) before analyses to make the results directly comparable. The total phenolic content (TPC) was determined following the method described previously (Ainsworth & Gillespie, 2007; Singleton et al., 1999; Singleton & Rossi, 1965), using the Folin-Ciocalteu reagent and gallic acid as the standard. The analysis was carried out in quintuplicate and the TPC was expressed as mg of gallic acid equivalents per g of extract. The ferric ion-reducing antioxidant power (FRAP) assay was conducted following the methodology previously outlined (Benzie & Strain, 1996; Välimaa et al., 2020). $FeSO_4 \bullet 7\ H_2O$ was used as a standard compound and L(+)-ascorbic acid (150 μM and 800 μM) as a positive control. The results were expressed as μmol/g of the extract (extract dry weight) Fe (II) equivalents. The oxygen radical absorbance capacity (ORAC) was determined following the method described by Huang et al. (2002) and Prior et al. (2003). ORAC assay was conducted to measure the capacity of the samples for quenching peroxyl-radicals, using AAPH (2, 2′-azobis(2-amidinopropane) dihydrochloride) as hydrophilic initiator. The results were expressed as Trolox equivalents (TE; μmol/g of extract DW).

The antibacterial activity of the extracts was evaluated using bacterial strains *Escherichia coli* K12+pcGLS11 and *Staphylococcus aureus* RN4220+pAT19, according to the procedure described by Vesterlund et al. (2004). The strains indicate the presence of antibacterial substances by a measurable decrease in the produced luminescent light signal. Bacterial cultivation and stock preparation were performed as previously described (Välimaa et al., 2020). The sterile-filtered extracts were diluted with double-distilled water to concentrations of 0.25, 0.5, and 1 mg/mL per microplate well. Ethanol concentrations of 8.75, and 17.5% per microplate well were used as positive controls and double-distilled water as a negative control. Samples and controls (50 μL each) were pipetted in triplicate into opaque white microplates, and 50 μL of bacterial inoculations were added to each well. The plate was shaken before each measurement and inserted into a Varioskan Flash Multilabel device to measure luminescence every 5 min for 60 min at room temperature. The results were expressed as inhibition percentages (inhibition%) at 50 min of measurement (Tienaho et al., 2015).

2.5. Processing and quality of bread fortified with green needle and twig extracts

2.5.1. Bread-making process and incorporation of extracts

The most promising NT extracts from pine, fir, and spruce in terms of bioactivity were selected for further supplementation in bread. Dough batches (500 g) were prepared using a standard bread formulation with modifications (Parenti et al., 2022). The ingredients consisted of whole wheat flour, fresh yeast, salt, water, and liquid extract (Supplementary Table 3). Kneading was performed with a mixer equipped with a dough hook (Hobart N50-110, Canada) at room temperature. The leavening phase was performed for 90 min at room conditions and then duplicate bread samples were baked for 50 min at 150 °C in an oven (Electrolux Professional Skyline Premium, Italy). Whole wheat bread samples were enriched with NT at 0% (control), 35% (S/F/PNT35), and 70% (S/F/PNT70) levels (w/w). Additions were constant for all the extracts. TDS for the added PNT, FNT, and SNT extracts were 5.74 g/L, 6.96 g/L, and 19.38 g/L corresponding to 46.5 g/L, 53.5 g/L, and 100 g/L of solid-to-liquid ratios used for the extractions, respectively. The extract additions were selected considering the minimum impact on the absolute threshold of needle extract taste perception reported in a previous study (Parenti et al., 2022). After baking, bread prototypes were allowed to cool to room conditions for subsequent analyses. Fresh samples were stored in plastic bags under the same room temperature for 24 and 72 h for evaluation of technological properties, antioxidant activity, and chemical composition.

2.5.2. Nutritional properties of bread

The proximate composition, sugar profile and mineral elements were analyzed using standard methods at accredited laboratories (Eurofins

Scientific Finland Oy) under SFS-EN ISO/IEC 17025:2017 (FINAS T089). The moisture, ash, fat, protein, total carbohydrate results were expressed as g/100 g FW. Sugar profile (fructose, galactose, glucose, lactose, maltose, and sucrose) consisted of an aqueous ethanol extraction of the sugars in the bread sample, followed by clarification with Carrez reagents. After Carrez treatment and filtration, the samples were diluted and analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection. Mineral elements, including Calcium (Ca), Copper (Cu), Iron (Fe), Magnesium (Mg), Phosphorus (P), Potassium (K), Sodium (Na), were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES).

2.5.3. Analyzes of secondary metabolites in bread

Freeze-dried bread samples (2 g) were extracted overnight in a magnetic stirrer with 50 mL of 100% methanol. The sample was then centrifuged (600 g, 10 min) to separate the solids, and the supernatant was transferred to an evaporation flask. The solid residue was mixed with 50 mL of 80% methanol and extracted in an ultrasonic bath for 30 min. The sample was then centrifuged, and the supernatant was combined with the first one. Supernatants were evaporated to dry in a rotary evaporator and resuspended into 2.0 mL 80% methanol. Control, SNT70, FNT70 and PNT70 bread samples (24 and 72 h) were analyzed by HPLC-DAD, as described in section *2.4.2*. Only control and NT-enriched breads stored for 24 h were analyzed by LC-MS/MS.

The condensed tannins content in breads was determined from freeze-dried bread samples, as described in section *2.4.1*. The results were presented as mg/100 g per bread dry weight (DW) and fresh weight (FW). Fresh and stored (24–72 h) bread slices were kept in plastic bags and stored at −21 °C before extracting antioxidant compounds. The samples were ground, and 10 g bread was dissolved in 20 mL of MeOH. The solutions were then mixed with a vortex and tube mixing machine for 10 min and centrifugated at 2500 rpm for 30 min.

2.5.4. Bioactive properties of bread during storage

Total phenolic content (Folin-Ciocalteu) was conducted as described in section *2.4.3*. The results were expressed as mg GAE per 100 g of bread DW. The antioxidant activity of bread samples during storage was measure by DPPH free-radical scavenging activity assay (Brand-Williams et al., 1995). The results were expressed as mg of ascorbic acid equivalents per 100 g of dry bread (mg AAE/100 g, DW).

2.5.5. Instrumental evaluation of color

The ground inner part of fresh bread with and without the outer surface were analyzed for their color parameters in the visible light wavelength range 400–700 nm by a Minolta CM-508s spectrophotometer equipped with SpectraMagic software (Konica Minolta, Tokyo, Japan), which uses a CieLab method (International Commission on Illumination L*a*b* scale). The software calculates values for sample color lightness (L*, scale 100 … 0) and actual color in red-green (a*, scale +100 … −100) or yellow-blue (b*, scale +100 … −100) color spaces. Each sample was measured in 8 replicates. Color differences between the fortified and the control bread samples (CIE ΔE^*) were calculated based on the CIE L*, a* and b* values via Equation (1):

$$\Delta E^* = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (1)$$

where ΔL* = L*(NT)-L*(C), Δa* = a*(NT)-a*(C) and Δb* = b*(NT)-b*(C) are the differences calculated between the NT-rich bread (NT) and the control bread (C) from the average color coordinates.

2.5.6. Texture profile analysis (TPA)

Bread crumb hardness, fracturability, adhesiveness, springiness, cohesiveness, and chewiness were determined using a texture analyzer (TA.TX plus 100 Stable Microsystems®, London, England), using a radiused cylinder probe with a 12.7 mm diameter (hemispherical). Two control breads and SNT70 breads were sliced, and cubes were cut using a two-blade cutter set to 1–1.5 cm width. Each sample taken from a different slice was subjected to a double compress test with a 2 s waiting time between the two cycles, performing a 40% compression at 1 mm/s with a trigger force of 0.196 N.

2.5.7. Sensory evaluation

The appearance, smell/odor, taste, and texture of the coded bread samples were evaluated by a panel at a sensory evaluation laboratory (Luke, Jokioinen, Finland), consisting of 8 people involved in food product development activities weekly and consume bread on a daily basis. The laboratory meets the ISO 8589:2007 standard (Sensory analysis—General guidance for the design of test rooms). The samples were presented on cardboard plates labeled with three-digit characters to each evaluator in a randomized sequence. A seven-point hedonic scale was used, ranging from 0 (dislike extremely) to 6 (like extremely). The panelists were asked to rinse their mouths with still water between sample evaluations. The study also assessed participants' willingness to buy the product, measured on a scale from 1 (very low willingness to purchase) to 8 (very high willingness to purchase), represented by the average scores obtained.

2.6. Statistical analysis

The experimental data was expressed as means ± standard deviation. The Shapiro–Wilk test was used to assess the data's normality and the Brown-Forsythe test was used to assess the homogeneity of the data variance. A one-way analysis of variances (ANOVA) and the Tukey HSD post hoc test were used to compare the mean values. Differences reaching a confidence level of 95% ($p < 0.05$) were considered statistically significant. An unpaired Student's t-test was used to compare two different samples in terms of TPC, antioxidant activity, and TPA. Furthermore, correlation analyses were performed to examine the relationships between bioactivities and the contribution of phenolic compounds to bioactivities. Statistical analysis was carried out using TIBCO Statistica v.13.3 software.

3. Results and discussion

3.1. Extract characterization

3.1.1. Physico-chemical evaluation

Samples of conifer NT were collected at distinct extraction stages defined by varying temperatures and times: NT-1 (25 ± 3 °C; 10 min), NT-2 (30 ± 2 °C; 20 min), NT-3 (35 ± 2 °C; 30 min), and NT-4 (47 ± 2 °C; 55 ± 3 min). Results indicated that the spruce (SNT) and pine (PNT) extracts contained the highest TDS amounts at the fourth extraction stage (NT-4, 47 ± 2 °C/55 ± 3 min), while silver fir (FNT) extract exhibited the greatest extraction yield at the third extraction stage (NT-3, 35 ± 2 °C/30 min) (Supplementary Figs. 2a and 2b). These findings suggest that higher temperatures and prolonged extraction times could generally contribute to increased TDS, likely due to improved solubility, faster diffusion, enhanced mass transfer, the release of additional compounds, in particular high molecular weight compounds, and possibly balancing the loss of thermolabile compounds (Albanese et al., 2019). However, although significant, the absolute differences within each extraction test were relatively small, while differences across tests should be interpreted in the light of the different contents of plant material and liquid-to-solid ratio, as shown in *section 2.3*. Supplementary Figs. 2c and 2d shows the soluble sugar content on a Brix scale and the pH value of the extracts at four extraction stages. Hydrothermal biomass treatments release small organic acids, such as formic and acetic acids, that decrease the pH of the extract (Zhang et al., 2023). Overall, SNT samples had the highest Brix values, while FNT and PNT extracts showed the lowest pH. Based on TDS, Brix, and pH values, a short extraction time (10 min) might be enough to achieve expected outcomes.

3.1.2. Effects of HC extraction stages on total phenolic content and bioactivities

The aim was to examine the preliminary bioactivity of all liquid extracts and to select the extraction stage that provides the most bioactive extract from each plant material for further supplementation in bread. Preliminary results indicated that, while TPC levels yielded satisfactory outcomes for all extracts already in the initial liquid samples (NT-1), FRAP levels for SNT and ORAC levels for SNT and FNT appeared highest at the final stage (NT-4) of the extraction (Fig. 1). This is possibly due to the extraction of more potent antioxidants, such as tightly bound phenolics, at higher temperatures and/or extended extraction periods (Albanese et al., 2019). In a previous study, similar FRAP values were found for hot water extracts of spruce needles (around 900 μmol TE/g, DW), corroborating the outcomes. In contrast, comparatively lower antioxidant activity was found for ORAC (ca. 3500 μmol TE/g, DW) in a study conducted by Jyske et al. (2020).

The most inhibitory activity against *E. coli* and *S. aureus* strains were found in SNT and FNT extracts, specifically at the lowest temperature and shortest time (NT-1, 25 ± 1 °C/10 min). On the other hand, PNT extracted at higher temperatures and prolonged extraction times (PNT-3, 33 °C/30 min) led to higher inhibition of both strains (Fig. 1d and e).

The Pearson correlation analysis showed that TPC significantly correlated ($p < 0.05$) with FRAP ($r = 0.81$; $p = 0.0014$), ORAC ($r = 0.822$; $p = 0.0010$), and TDS ($r = 0.89$; $p = 0.0001$). It indicates that enhanced recovery of extractives may be associated with higher phenolic content in the extracts, which is generally associated with antioxidant properties (Fidelis et al., 2018). This relationship is reflected in a significant and positive correlation with ORAC assay. In contrast, no significant association was observed between *E. coli* and TPC ($r = 0.104$, $p = 0.748$), FRAP ($r = -0.121$, $p = 0.708$), ORAC ($r = -0.319$, $p = 0.312$), TDS ($r = -0.029$, $p = 0.928$), and *S. aureus* ($r = 0.433$, $p = 0.16$). Similarly, no significant correlation was found between *S. aureus* with TPC ($r = 0.245$, $p = 0.442$), FRAP ($r = -0.22$, $p = 0.492$), ORAC ($r = -0.016$, $p = 0.962$), and TDS ($r = 0.126$, $p = 0.697$), which indicates that other antimicrobial metabolites than soluble phenolic compounds would be responsible for the activities found in this study, possibly thermolabile and/or degraded by prolonged cavitation processes. One plausible option for the source of antibacterial activity is antimicrobial

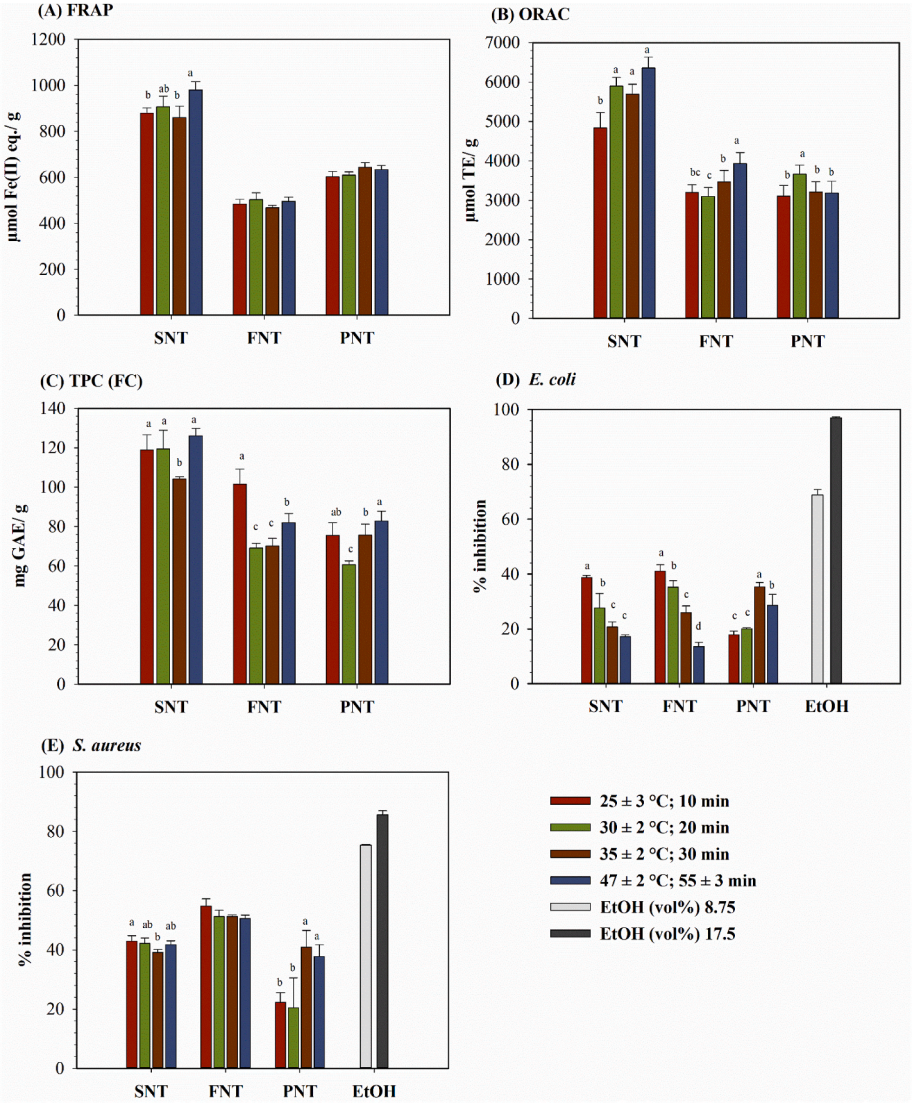

Fig. 1. Effect of varying extraction conditions on the antioxidant activities and antibacterial properties of green needle and twig extracts from spruce (SNT), pine (PNT) and fir (FNT), including (A) FRAP, (B) ORAC, (C) TPC (FC), (D) *E. coli* (1 g/L), and (E) *S. aureus* (1 g/L). Different lowercase letters in each sample represent statistically different results between extraction stages ($p < 0.05$). Extraction conditions at different extraction points: 25 ± 3 °C/ 10 min (NT-1), 30 ± 2 °C/ 20 min (NT-2), 35 ± 2 °C/ 30 min (NT-3), and 47 ± 2 °C/ 55 ± 3 min (NT-4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

peptides and the hypothesis is supported by previous literature. For example, a study found antimicrobial peptides from *P. densiflora* needles with activities against *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* (Lee et al., 2021). Another possible explanation for the source of antibacterial activity could include terpenes, as reviewed by Antonelli et al. (2020). Previous research found that conifer monoterpenes exhibited significant antibacterial effects but limited protection against hydrogen peroxide (Muilu-Mäkelä et al., 2022). This could explain why, unlike with the antioxidant activity, where the values generally increased with prolonged extraction time and elevated temperatures, the antibacterial activity was negatively correlated with time and temperature against *E. coli*, whereas mainly remained the same against *S. aureus*, except for PNT.

3.1.2.1. Secondary metabolites in extracts analyzed by HPLC-DAD, UHPLC-DAD-FLD, and UHPLC-QTOF-MS/MS. The content of individual compounds in the most antioxidative NT sample (NT-4) is shown in Table 2. UV spectra of the main peaks at 245, 280, and 350 nm on the HPLC chromatograms were evaluated and compared (Supplementary Figs. 3–19). While not all major compounds identified by HPLC-DAD could be confirmed by UHPLC-QTOF-MS/MS, several compounds, initially undetected due to low concentrations or lack of UV response,

Table 2
Quantification of compounds by HPLC-DAD and of condensed tannins by UHPLC-DAD-FLD in SNT-4, FNT-4, and PNT-4.

Compounds	RT (min)	SNT-4 (mg/100 g, DW)	SNT-4 extract (mg/100 g, FW)	Quantitated as
245 nm				
4-hydroxybenzoic acid	8.57	14.9 ± 1.0	2.7 ± 0.2	Benzoic acid
Unknown 3	25.51	8.1 ± 0.5	1.5 ± 0.1	Benzoic acid
Unknown 4	27.67	6.2 ± 0.7	1.1 ± 0.1	Benzoic acid
Piceatannol derivative	28.56	2.8 ± 0.1	0.5 ± 0.0	Piceatannol
280 nm				
Unknown 1	10.00	143.8 ± 1.0	26.0 ± 0.2	p-Coumaric acid
Lignan derivative 1	10.67	69.9 ± 0.6	12.7 ± 0.1	Secoisolariciresinol
Piceol derivative	13.38	137.9 ± 2.0	25.0 ± 0.4	Picein
Coumaric acid conjugate	15.84	6.1 ± 0.1	1.1 ± 0.0	p-Coumaric acid
p-Coumaric acid	17.31	10.1 ± 0.1	1.8 ± 0.0	p-Coumaric acid
Lignan derivative 2	18.94	47.3 ± 1.7	8.6 ± 0.3	Secoisolariciresinol
Ferulic acid	19.92	6.5 ± 0.2	1.2 ± 0.0	Ferulic acid
Lignan derivative 3	23.06	5.0 ± 0.1	0.9 ± 0.0	Secoisolariciresinol
350 nm				
Umbelliferone derivative*	16.53	91.8 ± 1.1	16.61 ± 0.20	Umbelliferone derivative
Sum		**458.6 ± 8.3**	**83.0 ± 1.5**	
Condensed tannins		2530.0 ± 30.0	45.8 ± 0.6	–
DP		2.7 ± 0.0	2.7 ± 0.0	–
PC/PD		91/9	91/9	–
Compounds	**RT (min)**	**FNT-4 (mg/100 g, DW)**	**FNT-4 extract (mg/100 g, FW)**	**Quantitated as**
245 nm				
4-OH benzoic acid	8.60	57.0 ± 0.7	0.4 ± 0.0	Benzoic acid
Unknown	15.29	583.5 ± 10.7	4.3 ± 0.1	Loganic acid
280 nm				
p-Coumaric acid	17.42	117.3 ± 1.2	0.9 ± 0.0	p-Coumaric acid
Ferulic acid	19.99	65.4 ± 0.7	0.5 ± 0.0	Ferulic acid
Lignan derivative 3	22.88	1739.0 ± 15.9	12.7 ± 0.1	Secoisolariciresinol
Sum		**2562.2 ± 29.2**	**18.8 ± 0.2**	
Condensed tannins		1620.0 ± 20.0	11.9 ± 0.1	–
DP		2.6 ± 0.0	2.6 ± 0.0	–
PC/PD		77/23	77/23	–
Compounds	**RT (min)**	**PNT-4 (mg/100 g, DW)**	**PNT-4 extract (mg/100 g, FW)**	**Quantitated as**
245 nm				
4-OH benzoic acid	8.47	54.0 ± 2.2	0.3 ± 0.0	Benzoic acid
280 nm				
Cinnamate	9.95	16.1 ± 0.5	0.1 ± 0.0	Cinnamic acid
Tryptamine derivative	16.12	111.4 ± 9.0	0.6 ± 0.0	Tryptamine
p-Coumaric acid	17.46	41.8 ± 5.2	0.2 ± 0.0	p-Coumaric acid
Unknown	18.12	225.7 ± 15.3	1.2 ± 0.1	Methoxyhydroquinone
Ferulic acid	20.03	101.5 ± 5.8	0.5 ± 0.0	Ferulic acid
Lignan-like	24.09	1084.3 ± 80.5	5.8 ± 0.4	Secoisolariciresinol
Lignan-like	28.62	322.9 ± 27.5	1.7 ± 0.1	Secoisolariciresinol
Lignan-like	28.85	1071.7 ± 89.7	5.7 ± 0.5	Secoisolariciresinol
Sum		**3029.5 ± 235.7**	**16.1 ± 1.3**	
Condensed tannins		1130.0 ± 30.0	6.02 ± 0.2	–
DP		3.4 ± 0.0	3.4 ± 0.0	–
PC/PD		100/0	100/0	–

Note: The data is expressed as mean ± sd (n = 3). All extracts were freeze-dried to ensure that the results were independent of the biomass concentration. Dry weight (DW): 1,81 % (SNT), 0.732% (FNT), 0.531% (PNT). *The compound with UV spectrum resemblance of umbelliferone was expressed as peak area unit/g; otherwise, the results were given as mg/100 g dry weight (DW). Abbreviations: RT: retention time, S/F/PNT-4: Norway spruce/silver fir/ pine NT obtained at the fourth extraction stage (47 ± 2 °C/ 55 ± 3 min), FW: fresh weight, n/d: not detected, DP: average degree of polymerization, PC/PD: PD refers to prodelphidinins (i.e., (epi)gallocatechin subunits) and PC refers to procyanidins (i.e., (epi)catechin subunits), n/d: not detected (i.e., below the detection level).

were tentatively identified through MS and MS/MS data.

A total of 115 compounds were identified or tentatively identified from the spruce, pine and fir NT, including flavonoids (12 compounds), hydroxycinnamic acids (6), hydroxybenzoic acids (3), alkaloids (10), stilbenes (10), lignans (14), resin acids (11), gibberellins (4), and others (29). In the control bread, 16 identified compounds could have originated from the wheat, potentially arisen from the yeast or its activity, or resulted from the heat treatment during the baking phase. These compounds included ferulic acid, tryptophan, pinellic acid, N1,N10-Bis(4-hydroxycinnamoyl)spermidine, apigenin-C-pentosyl-C-hexoside, apigenin diC-pent, luteolin-CC (carlinoside-5), and four unknown compounds. The complete list of the putative compounds and their elemental formulae are given in Supplementary Table 8.

Alkaloids, including epihydropinidine, euphococcine, dehydropinidinol, pinidinol were found in all extracts, while 1,6-dehydropinidine, pinidine, and bonvalotidine A was found only in SNT-4. The presence of alkaloids in spruce needles has been reported previously (Virjamo & Julkunen-Tiitto, 2014).

Phenolic acids were characterized mainly as hydroxycinnamic acid conjugates, such as 3-p-coumaroylquinic acid, p-coumaroyl-xyloside-hexoside, caffeic acid hexoside, caffeoylquinic acid and feruloylquinic acid. Identified flavonoids or flavonoid derivatives included catechin, epigallocatechin, procyanidin B1/2, luteolin, dihydrokaempferol, kaempferol glucoside, quercetin-galactoside, taxifolin, isorhamnetin and vitexin.

Additional compounds found from the negative MS data were stilbenes, including resveratrol, astringin, piceatannol, rhapontin and isorhapontigen, isorhapontin, and dimeric isorhapontin which were found in SNT-4 and piceid found in PNT-4. Lignans, such as secoisolariciresinol, lariciresinol and hydroxymatairesinol, along with their conjugates were identified in all samples. Resin acids primarily consisted of abietic acid and its derivatives (hydroxydehydroabietic acid and dehydroabietic acid). SNT-4 also contained smaller molecules like vanillin and piceol (4′-hydroxyacetophenone). The presence of umbelliferone in SNT-4, initially identified by its UV spectrum, was confirmed by both negative and positive MS data. In fact, umbelliferone-glucoside has been identified in spruce needles (Strack et al., 1989). The bioactivity profiles of flavonoids, phenolic acids, stilbenes, tannins, lignans, and alkaloids have been reported previously (Metsämuuronen & Siren, 2014; Metsämuuronen & Sirén, 2019).

The primary compounds contributing to the total amount in SNT were p-coumaric acid, picein, an umbelliferone derivative, and secoisolariciresinol; in FNT, they included secoisolariciresinol, loganic acid, p-coumaric acid, and ferulic acid (Table 2). In PNT, secoisolariciresinol, methoxyhydroquinone, and tryptamine appeared to contribute considerably to the overall sum content of compounds. The condensed tannins were essentially procyanidins in PNT extract; SNT and FNT extracts were mixtures of procyanidins and prodelphinidins. In all samples, tannins' average degree of polymerization (DP) was rather small.

3.2. Properties of NT-fortified bread

3.2.1. Phenolic content and antioxidant activity of bread samples after 24 h and 72 h of storage

Based on the TDS, TPC, and antioxidant activity levels, each aqueous extract obtained at the fourth extraction stage (NT-4, 47 ± 2 °C/55 ± 3 min) was selected to continue the study. It is worth noting that the initial solid-to-liquid ratios for the extractions were 100 g/L for SNT, 53.5 g/L for SFT, and 46.5 g/L for PNT, respectively (**see section 4.3**). Consequently, SNT35 results are more accurately comparable with PNT70 and FNT70 bread results. Overall, the addition of extracts as a partial water substitute in producing whole wheat bread at 35 and 70% levels significantly improved ($p < 0.05$) the antioxidant potential and TPC over time at room temperature, showing consistent trends across all NT-enriched bread samples (Fig. 2). Previous research assessed the antioxidant activity (DPPH) of bread enriched with 35% (22 mg TE/100 g) and 100% silver fir needles aqueous extract (29 mg TE/100 g), revealing higher values compared to the current study. Conversely, the authors reported reduced antioxidant activity over time (72 h) for stored samples (Parenti et al., 2022).

Incorporating NT into the recipe can enhance dough functionality due to the presence of bioactive compounds. It should be considered that the specific factors contributing to the to increased antioxidant activity in enriched bread may vary, depending on the extraction process, extract composition, and baking conditions. The improved availability of phenols may result from the stability of polyphenols in the extracts or the release of new bioactive compounds, influenced by thermal, enzymatic, microbial, or chemical reactions during or after baking (Meral & Köse, 2019). Additionally, this phenomenon may be influenced by factors such as synergistic effects, new interactions with other organic compounds during heat treatment, structural changes, enhanced stability (Larrosa & Otero, 2021), pro-oxidation, and nutrient degradation (Jensen et al., 2011).

3.2.2. Chemical composition of bread samples after 24 h and 72 h of storage and their potential contribution to the bioactivities

The results in Table 3 indicate that the selected compounds remain stable throughout the baking process and subsequent storage. The MS data for the compounds in breads are provided in Supplementary Table 8. Theoretical calculations for bread, considering the sum of solids added (flour, salt, yeast), were used for simpler comparison of compound stability during the bread-making process. Interestingly, while

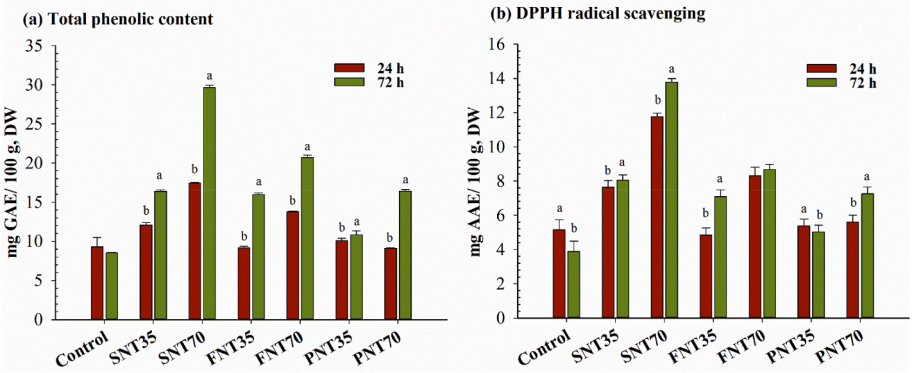

Fig. 2. Total phenolic content (a) and antioxidant activity (b) of control and whole wheat bread fortified with NT after 24 h and 72 h of storage. Data were expressed as mg/100 g DW of bread sample. After normalization of the extract additions according to the solid-to-liquid ratios of the extractions, SNT35 should be compared with FNT70 and PNT70. Different letters indicate statistically significant differences ($p < 0.05$). Control: dough sample with the addition of 0% NT, S/F/PNT35: dough sample with the addition of 35% spruce/pine/fir NT (w/w), S/F/PNT70: dough sample with the addition of 70% spruce/pine/fir NT (w/w).

Table 3
Secondary metabolites (mg/100 g) in control and SNT-, FNT-, and PNT-fortified breads putatively identified and quantitated by HPLC-DAD.

Compounds (mg/100 g)	RT (min)	Control 24h	SNT35-24h	SNT70-24h	Control 72h	SNT35-72h	SNT70-72h	Theoretical (SNT35)	Theoretical (SNT70)	Quantitated as
SNT-originating compounds										
245 nm										
4-hydroxybenzoic acid	8.64	0.7 ± 0.0	1.6 ± 0.0	2.4 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	1.9 ± 0.1	0.7	1.3	Benzoic acid
Unknown 3	25.61	n/d	0.4 ± 0.0	0.9 ± 0.0	n/d	0.3 ± 0.1	0.8 ± 0.0	0.4	0.7	Benzoic acid
Unknown 4	27.79	n/d	0.4 ± 0.0	0.7 ± 0.0	n/d	0.3 ± 0.0	0.8 ± 0.0	0.3	0.6	Benzoic acid
Piceatannol derivative	28.68	n/d	0.2 ± 0.0	0.5 ± 0.0	n/d	0.2 ± 0.0	0.5 ± 0.0	0.1	0.3	Piceatannol
280 nm										
Unknown 1	10.09	n/d	6.1 ± 0.2	12.3 ± 0.1	n/d	6.3 ± 0.2	13.2 ± 0.5	6.3	12.6	p-Coumaric acid
Lignan derivative 1	10.77	n/d	4.0 ± 0.9	6.8 ± 0.1	n/d	3.9 ± 0.2	7.4 ± 0.3	3.1	6.1	Secoisolariciresinol
Piceol derivative	13.47	n/d	9.2 ± 0.2	16.2 ± 0.1	n/d	9.6 ± 0.3	17.2 ± 0.4	6.0	12.1	Picein
Coumaric acid conjugate	15.96	n/d	1.9 ± 0.0	3.7 ± 0.0	n/d	2.0 ± 0.1	4.1 ± 0.1	0.3	0.5	p-Coumaric acid
p-Coumaric acid	17.44	n/d	1.2 ± 0.0	2.0 ± 0.0	n/d	1.3 ± 0.0	2.3 ± 0.1	0.4	0.9	p-Coumaric acid
Lignan derivative 2	18.94	n/d	2.2 ± 0.1	4.4 ± 0.1	n/d	2.5 ± 0.1	5.2 ± 0.2	2.1	4.2	Secoisolariciresinol
Ferulic acid	19.92									Ferulic acid
Lignan derivative 3	22.91	n/d	0.3 ± 0.0	0.5 ± 0.0	n/d	0.3 ± 0.0	0.6 ± 0.0	0.2	0.4	Secoisolariciresinol
350 nm										
Umbelliferone derivative (350 nm)	16.65	n/d	6.9 ± 0.2	13.4 ± 0.1	n/d	7.6 ± 0.2	16.2 ± 1.4	4.0	8.1	Peak area unit/g
Sum of SNT-originating compounds			**27.4 ± 1.5**	**50.2 ± 0.6**		**26.7 ± 1.1**	**53.9 ± 1.8**	**19.8**	**39.7**	
Wheat bread (WB) compounds										
245 nm										
4-hydroxybenzoic acid	8.6	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	Benzoic acid
280 nm										
Tryptophan	7.96	15.4 ± 0.4	17.0 ± 0.9	14.2 ± 0.1	14.9 ± 0.7	17.2 ± 0.9	15.3 ± 0.5	n/d	n/d	Tryptophan
WB unknown 1	13.53	0.2 ± 0.0	n/d	n/d	0.2 ± 0.0	n/d	n/d	n/d	n/d	Picein
Ferulic acid	20	1.0 ± 0.0	1.2 ± 0.0	1.7 ± 0.0	0.9 ± 0.0	1.3 ± 0.1	1.8 ± 0.1	0.3	0.6	Ferulic acid
WB Lignan-like	23.27	19.8 ± 1.6	26.5 ± 0.7	30.4 ± 0.4	18.3 ± 0.8	28.7 ± 1.6	33.3 ± 1.3	n/d	n/d	Secoisolariciresinol
WB unknown 2	24.83	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.8 ± 0.0	n/d	n/d	Ferulic acid
350 nm										
WB flavonoid 1	21.64	1.7 ± 0.1	1.8 ± 0.1	1.5 ± 0.0	1.6 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	n/d	n/d	Quercetin
WB flavonoid 2	21.86	1.7 ± 0.2	1.7 ± 0.2	1.4 ± 0.0	1.5 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	n/d	n/d	Quercetin
WB flavonoid 3	26.37	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 4	26.58	2.8 ± 0.1	2.9 ± 0.1	2.5 ± 0.0	2.8 ± 0.1	3.2 ± 0.1	2.9 ± 0.1	n/d	n/d	Quercetin
Sum of Wheat/yeast-originating compounds		**44.7 ± 2.5**	**52.5 ± 2.0**	**53.4 ± 0.6**	**42.4 ± 1.9**	**57.0 ± 3.0**	**58.9 ± 2.2**	**0.3**	**0.6**	
Sum total		**44.7 ± 2.5**	**80.0 ± 2.5**	**103.6 ± 1.2**	**42.4 ± 1.9**	**82.8 ± 3.3**	**112.8 ± 2.4**	**20.1**	**40.3**	
Compounds (mg/100 g)	**RT (min)**	**Control 24h**	**FNT35–24h**	**FNT70–24h**	**Control 72h**	**FNT35–72h**	**FNT70–72h**	**Theoretical (FNT35)**	**Theoretical (FNT70)**	**Quantitated as**
FNT-originating compounds										
245 nm										
4-OH benzoic acid	17.44	n/d	0.2 ± 0.0	0.4 ± 0.0	n/d	0.2 ± 0.0	0.4 ± 0.0	0.2	0.4	Benzoic acid
Unknown	15.29	n/d	1.0 ± 0.1	1.9 ± 0.0	n/d	0.9 ± 0.1	1.8 ± 0.1	1.0	2.1	Loganic acid
280 nm										
p-Coumaric acid	17.42	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	p-Coumaric acid
Ferulic acid	19.99	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	Ferulic acid
Lignan derivative 3	22.88	n/d	3.6 ± 0.0	6.4 ± 0.0	n/d	4.2 ± 0.3	7.0 ± 0.1	3.1	6.2	Secoisolariciresinol
Sum of FNT-originating compounds		**n/d**	**4.8 ± 0.1**	**8.7 ± 0.1**	**n/d**	**5.3 ± 0.5**	**9.2 ± 0.2**	**4.3**	**8.7**	
Wheat bread (WB) compounds										
245 nm										
4-hydroxybenzoic acid	8.6	0.7 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.1	0.6 ± 0.0	0.1	0.2	Benzoic acid
280 nm										
Tryptophan	7.96	15.4 ± 0.4	16.4 ± 0.4	12.6 ± 0.1	14.9 ± 0.7	16.9 ± 0.9	12.9 ± 0.6	n/d	n/d	Tryptophan
WB unknown 1	13.53	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n/d	n/d	Picein
Ferulic acid	20.00	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	1.0 ± 0.1	0.1	0.2	Ferulic acid

(*continued on next page*)

Table 3 (*continued*)

Compounds (mg/100 g)	RT (min)	Control 24h	FNT35–24h	FNT70–24h	Control 72h	FNT35–72h	FNT70–72h	Theoretical (FNT35)	Theoretical (FNT70)	Quantitated as
WB Lignan-like	23.27	19.8 ± 1.6	20.7 ± 0.3	16.4 ± 0.3	18.3 ± 0.8	20.6 ± 1.4	15.7 ± 0.2	n/d	n/d	Secoisolariciresinol
WB unknown 2	24.83	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	n/d	n/d	Ferulic acid
350 nm										
WB flavonoid 1	21.64	1.7 ± 0.1	1.7 ± 0.0	1.4 ± 0.0	1.6 ± 0.1	1.9 ± 0.1	1.5 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 2	21.86	1.7 ± 0.2	1.6 ± 0.0	1.3 ± 0.0	1.5 ± 0.1	2.0 ± 0.1	1.5 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 3	26.37	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	0.8 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 4	26.58	2.8 ± 0.1	2.8 ± 0.0	2.4 ± 0.0	2.8 ± 0.1	3.1 ± 0.2	2.4 ± 0.0	n/d	n/d	Quercetin
Sum of Wheat/yeast-originating compounds		**44.7 ± 2.5**	**46.8 ± 0.9**	**37.8 ± 0.5**	**42.4 ± 1.9**	**47.9 ± 3.1**	**37.3 ± 1.1**	**0.2**	**0.4**	
Sum total		**44.7 ± 2.5**	**51.6 ± 1.0**	**46.4 ± 0.6**	**42.4 ± 1.9**	**53.2 ± 3.5**	**46.5 ± 1.3**	**4.54**	**9.1**	
Compounds (mg/100 g)	**RT (min)**	**Control 24h**	**PNT35–24h**	**PNT70–24h**	**Control 72h**	**PNT35–72h**	**PNT70–72h**	**Theoretical (PNT35)**	**Theoretical (PNT70)**	**Quantitated as**
PNT-originating compounds										
245 nm										Benzoic acid
4-OH benzoic acid	8.6	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	
280 nm										
Cinnamate	9.95	n/d	0.1 ± 0.0	0.1 ± 0.0	n/d	0.2 ± 0.0	0.2 ± 0.0	0.0	0.0	Cinnamic acid
Tryptamine derivative	16.12	n/d	0.3 ± 0.0	0.2 ± 0.0	n/d	0.3 ± 0.0	0.4 ± 0.0	0.1	0.3	Tryptamine
p-Coumaric acid	17.46	n/d	0.1 ± 0.0	0.2 ± 0.0	n/d	0.2 ± 0.0	0.3 ± 0.0	0.1	0.1	p-Coumaric acid
Unknown	18.12	n/d	0.9 ± 0.0	0.9 ± 0.0	n/d	1.1 ± 0.2	1.4 ± 0.1	0.3	0.6	Methoxyhydroquinone
Ferulic acid	20.00	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	
Lignan-like	24.09	n/d	2.9 ± 0.3	4.2 ± 0.2	n/d	4.1 ± 0.2	4.8 ± 0.0	1.4	2.8	Secoisolariciresinol
Lignan-like	28.62	n/d	0.5 ± 0.0	1.0 ± 0.1	n/d	2.4 ± 0.1	1.1 ± 0.0	0.4	0.8	Secoisolariciresinol
Lignan-like	28.85	n/d	1.5 ± 0.0	2.8 ± 0.1	n/d	1.5 ± 0.4	2.9 ± 0.1	1.4	2.8	Secoisolariciresinol
Sum of PNT-originating compounds			**6.3 ± 0.4**	**9.5 ± 0.4**		**9.7 ± 0.9**	**11.1 ± 0.3**	**3.7**	**7.4**	
Wheat bread (WB) compounds										
245 nm										
4-hydroxybenzoic acid	8.6	0.7 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	1.0 ± 0.1	0.1	0.1	Benzoic acid
280 nm										
Tryptophan	7.96	15.4 ± 0.4	15.3 ± 0.1	11.8 ± 0.4	14.9 ± 0.7	15.5 ± 0.5	12.1 ± 0.2	n/d	n/d	Tryptophan
WB unknown 1	13.53	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	n/d	n/d	Picein
Ferulic acid	20	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	0.1	0.3	Ferulic acid
WB Lignan-like	23.27	19.8 ± 1.6	20.8 ± 0.4	16.8 ± 0.5	18.3 ± 0.8	21.8 ± 0.4	18.1 ± 0.4	n/d	n/d	Secoisolariciresinol
WB unknown 2	24.83	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	n/d	n/d	Ferulic acid
350 nm										
WB flavonoid 1	21.64	1.7 ± 0.1	1.6 ± 0.0	1.3 ± 0.0	1.6 ± 0.1	1.8 ± 0.0	1.3 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 2	21.86	1.7 ± 0.2	1.6 ± 0.0	1.2 ± 0.0	1.5 ± 0.1	1.9 ± 0.0	1.2 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 3	26.37	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	n/d	n/d	Quercetin
WB flavonoid 4	26.58	2.8 ± 0.1	2.9 ± 0.1	2.2 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.2 ± 0.1	n/d	n/d	Quercetin
Sum of Wheat/yeast-originating compounds		**44.7 ± 2.5**	**45.6 ± 0.7**	**36.8 ± 1.2**	**42.4 ± 1.9**	**47.5 ± 1.1**	**39.1 ± 0.9**	**0.2**	**0.4**	
Sum total		**44.7 ± 0.0**	**51.9 ± 1.1**	**46.3 ± 1.6**	**42.4 ± 1.9**	**57.3 ± 2.0**	**50.2 ± 1.2**	**3.9**	**7.8**	

Data (mg/100 g, DW) is presented as mean ± SD (n = 3). The theoretical amount of each compound in theoretical bread was calculated based on the amount of the extracts (w/w) added to the recipe to facilitate the comparison of stability during the bread-making process. Theoretical bread 35%: 77.56 g of extract added to the bread formulation and 70% indicates 155.4 g of extract addition. Initial solid-to-liquid ratios for the extractions were 100 g/L for SNT, 53.5 g/L for SFT, and 46.5 g/L for PNT, respectively. Abbreviations: RT, retention time, n/d: not detected (i.e., below the detection level).

the total compound content in control bread reduced over time, fortified breads generally showed a slight increase after 3 days of storage. The addition of 70% SNT and PNT led to a decrease in wheat/yeast-derived compounds compared to the control and 35% S/PNT breads, likely due to antagonistic interactions. Otherwise, concentrations of compounds originating from wheat flour and potentially yeast (WB) did not show considerable changes. As a result of the initial solid-to-liquid ratios in the extractions, a higher compound content was expected in SNT70 compared to the other extracts.

In all samples, the average degree of polymerization (DP) of tannins was relatively low. The content of condensed tannins was below the limit of quantification (10 mg/100 g) in FNT- and PNT-fortified bread, detectable only in SNT-fortified breads (Supplementary Table 4). The baking process may have induced some polymerization reactions, as the tannins' average DP was slightly higher in SNT-fortified breads compared to the original extract. Prodelphinidins were undetectable in the samples due to their low content, and storage time had no significant impact on tannin levels.

Consistent with the TPC and antioxidant activity, a wide range of phenolic compounds were detected in fortified bread samples, including phenolic acids, flavonoids, stilbenes, and lignans. The contribution of phenolic composition to total phenolic content and antioxidant activity during bread storage was investigated (Supplementary Table 5). The total content of secondary metabolites quantified by HPLC-DAD in fortified bread significantly correlated ($p < 0.05$) with DPPH ($r = 0.816$) and TPC ($r = 0.647$). Similarly, a strong positive correlation was observed between the concentration of extractives in bread and DPPH after 24 h ($r = 0.813$) and 72 h ($r = 0.818$) of storage. Therefore, results suggest that incorporating NT extracts can enhance the bread's natural antioxidant properties, potentially providing synergistic effects that contribute to health benefits for consumers. These findings also indicate that NT extracts could serve as a biobased solution to replace fossil-based additives used for enhancing bread shelf-life.

3.2.3. *Proximate composition and mineral profile*

The present study attempted to perform a first investigation of the impact of replacing water with NT extracts on the stability of secondary metabolites, functionality, as well as the nutritional value (proximate composition and mineral profile) and technological quality (texture, color, and sensory evaluation) of bread. A representative sample was selected for certain analyses to provide a comprehensive examination of quality and technological factors, focusing on the formulation with the most promising functionality. Specifically, the SNT70 formulation was chosen for preliminary assessment of proximate composition, mineral profile, and texture profile, as it demonstrated the highest levels of bioactive compounds based on LC-MS analysis and showed considerable antioxidant activity.

In terms of proximate composition, the SNT70-fortified bread revealed comparable values to the control, with minor variations (Fig. 3). A slight increase in carbohydrate content of fortified bread, calculated by difference, can be attributed to the decreased moisture (41.7 for control vs 39.4 g/100 g for SNT70), likely due to solid content (NT extract), which does not hydrate as effectively as water. A similar reduction in moisture content has been reported in studies incorporating other plant-based ingredients, such as potato peel, into bread formulations (Soltan et al., 2023). Moreover, energy values between the control and fortified bread remained comparable, with a slight increase (245 kcal/100 g for SNT70 vs 236 kcal/100 g for control), indicating that fortification does not significantly alter the bread's caloric density, thereby maintaining its energy intake.

The higher levels of fructose and maltose in SNT bread (Supplementary Table 6) align with the superior Brix values in the SNT extract, which further contribute to the increased carbohydrate content in the fortified bread (Supplementary Fig. 2). Additionally, although there was a statistically significant reduction in protein content (10.3 g/100 g for control vs 10 g/100 g for SNT70), this difference remains minimal and within an adequate range for bread products. Overall, the minor alterations in protein, carbohydrate, and moisture levels reflect inherent trade-offs associated with incorporating plant-based ingredients into bread formulations, suggesting that SNT70 addition effectively supports the preservation of technological quality. Moreover, the data clearly indicate that incorporating SNT70 into the bread enhanced the levels of tested minerals, particularly Na (3700 mg/kg), Mg (670 mg/kg), Fe (25 mg/kg), and Ca (270 mg/kg), which is consistent with other studies that have identified spruce needles as a rich source of these minerals (Ivanov et al., 2022; Jyske et al., 2020). The presence of these components offers a functional and nutritional benefit by contributing to mineral intake, which is critical for health maintenance. Besides, conifer needles are known to be sources of vitamins, such as vitamin C, folates, and beta-carotene, adding further nutritional benefits to the product (Jyske et al., 2020). Future research should explore the impact of varying NT concentrations on additional nutrients, such as dietary fiber and vitamin content, to substantiate nutritional improvements of fortified whole wheat bread.

3.2.4. *Color properties*

As expected, bread supplemented with NT exhibited a variable intensity of color compared to the control, with significant differences in the L*, a*, and b* values (Fig. 4 and Table 4). A higher concentration of extracts (NT70) generally resulted in lower L*, a*, and b*, indicating darker breads. The total color difference (ΔE^*) values highlight that increasing the extract levels lead to enhanced color deviations from the control, with FNT70 showing the most pronounced change. Lower extract levels result in moderate color differences, with SNT35 being the closest to the control. Overall, PNT35 showed the lightest crumb color, with moderate levels of redness and yellowness, making it potentially

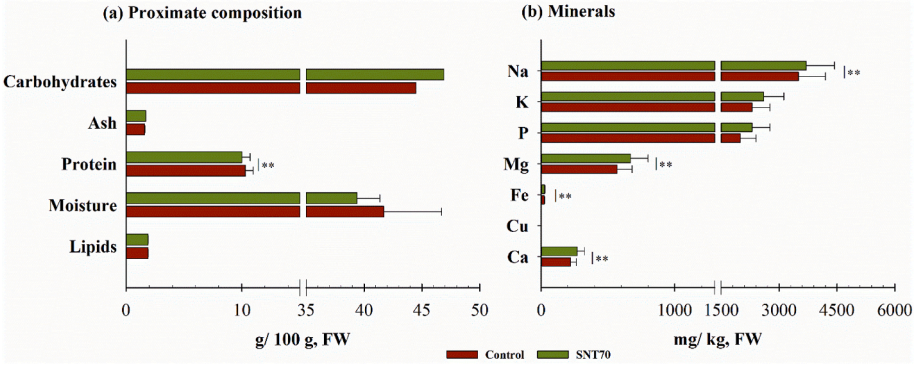

Fig. 3. Proximate composition (g/100 g bread, FW) (a) and mineral content (mg/kg, FW) (b) of fresh control bread and bread enriched with 70% spruce needle and twig extract (SNT70). Total carbohydrate was determined by calculation. The mean difference was compared using *t*-test ($p < 0.05$), ** indicates significant differences at $p < 0.01$.

the most visually appealing sample. This is further supported by its appearance attribute in the sensory evaluation (Fig. 4).

3.2.5. Texture profile analysis of bread crumb

As mentioned earlier, SNT70 was chosen as a representative bread sample to allow a comprehensive examination of quality and technological factors, focusing on the formulation that showed the most promising bioactivity and metabolite profile. According to TPA results, the incorporation of SNT70 significantly ($p < 0.05$) reduced the bread hardness by 16% and increased springiness by 19%, as shown in Table 5. The reduction in hardness, a crucial factor in determining bread quality, may be especially relevant due to its potential effects on dough handling and shelf-life stability. In contrast, fracturability, chewiness, cohesiveness, and resilience were similar to those of the control. Overall, preliminary assessment of TPA suggested that 70% replacement with NT extracts had little effect on textural properties, indicating that NT extracts could be used effectively at suitable levels to fortify whole wheat bread without compromising its textural quality.

These findings are consistent with previous research showing that incorporating polyphenol-rich extracts from pomegranate seeds decreased hardness and increased springines (Pamisetty et al., 2020). Similarly, another study found that the addition of vine tea extract and dihydromyricetin reduced bread hardness and increased springiness without significantly affecting other textural properties (Ma et al., 2020). Parenti et al. (2022) observed that a higher enrichment level (100% fir needle extract) maintained textural properties similar to those of non-enriched bread, likely due to an increase in bread volume compensating for changes in texture. Moreover, the observed changes in bread texture can be attributed to the interactions between phenolic compounds in NT extracts and gluten. Polyphenols are known to form covalent and non-covalent bonds with gluten, which strengthens the dough's gluten network and improves elasticity (springiness) while reducing hardness (Schefer et al., 2021; Xu et al., 2019). Phenolics may also delay starch gelatinization and retrogradation due to competition for water, which could help maintain the bread's softness over time. Nevertheless, previous research has reported conflicting effects on breadcrumb hardness when antioxidant compounds are added, likely due to variations in ingredients, their physical state, and processing conditions (Xu et al., 2019, 2024). Future research should explore the impact of varying NT levels on texture properties and assess their association with other quality changes over prolonged storage.

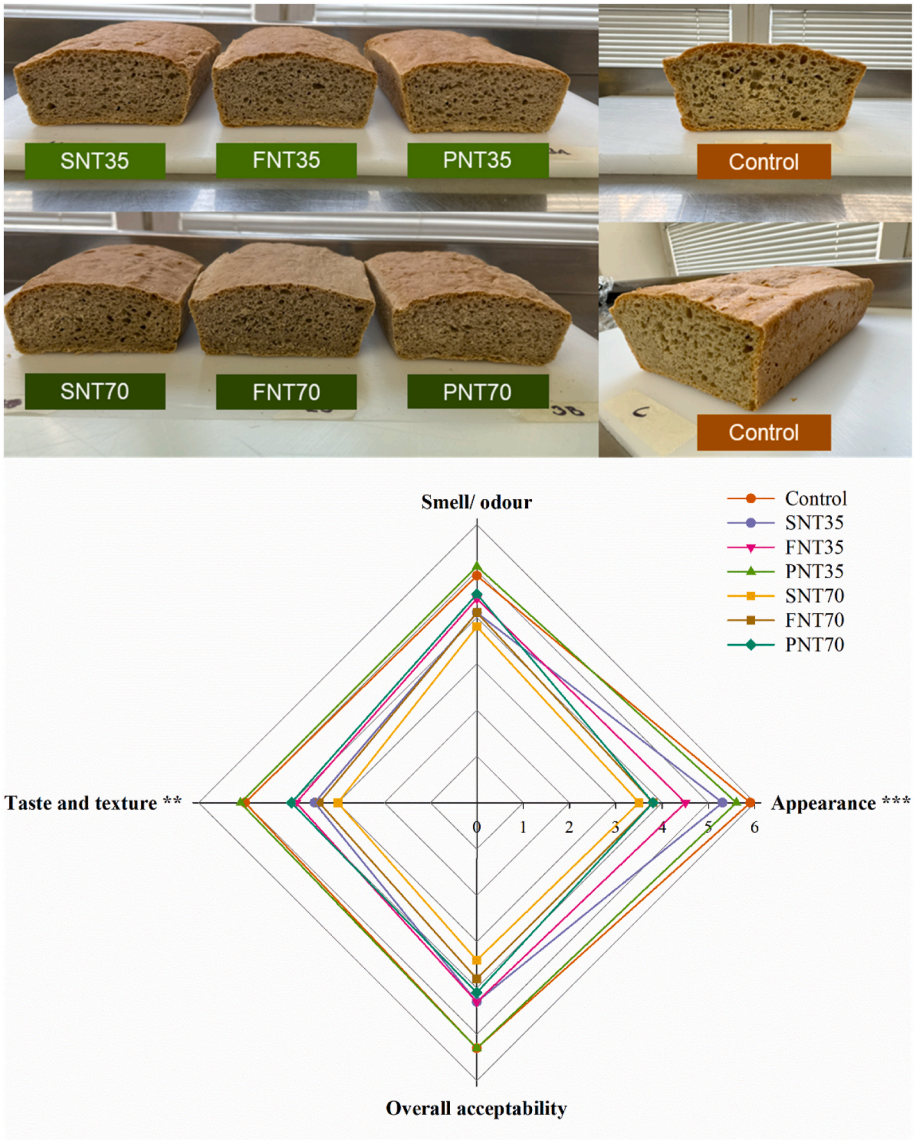

Fig. 4. Transversal cut of bread samples and sensory evaluation. Attributes marked with **, and *** indicate significant differences at $p < 0.01$, and $p < 0.001$, respectively. Abbreviations of bread samples can be found in Fig. 2.

Table 4
Effect of replacing water with 35% and 70% mass levels of NT extracts on whole wheat bread's color (crumb). Mass levels are respective to the mass of the added water in the control formulation.

Bread sample	Crumb			
	L*	a*	b*	Δ*E**
Control	54.7 ± 7.2^{bc}	6.6 ± 3.1^{a}	22.6 ± 3.7^{ab}	–
SNT35	55.6 ± 1.7^{c}	5.6 ± 0.8^{a}	21.3 ± 1.0^{ab}	1.82
FNT35	57.5 ± 0.9^{ab}	4.5 ± 0.5^{b}	20.5 ± 1.0^{bc}	4.01
PNT35	58.6 ± 1.0^{a}	6.0 ± 0.7^{a}	22.5 ± 1.1^{a}	3.90
SNT70	53.3 ± 0.6^{d}	4.2 ± 0.3^{b}	18.5 ± 1.2^{d}	4.97
FNT70	50.3 ± 2.1^{e}	4.6 ± 1.3^{b}	19.1 ± 2.3^{cd}	5.95
PNT70	56.9 ± 1.3^{bc}	4.3 ± 0.5^{b}	19.1 ± 1.2^{cd}	4.65
p-value (*one-way* ANOVA)	<0.001	<0.001	<0.001	–

Note: Data is presented as mean ± SD (n = 8). Values with different letters in the same column are significantly different ($p < 0.001$). Control: dough sample with the addition of 0% NT S/F/PNT35: dough sample with the addition of 35% spruce/pine/fir NT (w/w), S/F/PNT70: dough sample with the addition of 70% spruce/pine/fir NT (w/w).

Table 5
Effect of replacing water with 70% mass levels of Norway spruce NT (SNT70) on whole wheat bread's texture properties after 24h. Mass levels are respective to the mass of water.

Measurement	Control	SNT70
Hardness (N)	7.68 ± 0.22 ^{a}	6.43 ± 1.06 ^{b}
Fracturability (N)	6.04 ± 0.52	5.86 ± 1.32
Springiness (N)	0.46 ± 0.11 ^{b}	0.55 ± 0.06 ^{a}
Cohesiveness	0.44 ± 0.09	0.46 ± 0.06
Chewiness	1.69 ± 0.46	1.58 ± 0.29
Resilience	0.17 ± 0.04	0.19 ± 0.03

Note: Data are expressed as mean ± SD (n = 2). Different lowercase letters represent statistically different results between samples ($p < 0.05$).

3.2.6. Sensory evaluation

Fig. 4 and Supplementary Table 7 present the sensory assessment of control and fortified bread samples containing 35% and 70% levels of SNT, FNT, and PNT. For the appearance attribute, the scores increased in the following order: control > PNT35 > SNT35 > FNT35 > FNT70 and PNT70 > SNT70. In contrast, no significant differences were observed among the samples regarding the smell/odor. In the evaluation of taste and texture, the ratings increased in the following order: PNT35 > control > PNT70 > FNT35 > SNT35 > FNT70 > SNT70. As a result, overall acceptability rankings showed a similar trend: control and PNT35 > SNT35 and FNT35 > PNT70 > FNT70 > SNT70. Moreover, seven out of eight panelists expressed their willingness to purchase SNT35 and PNT35. Overall, panelists favored the PNT35 formula the most, as it provided an adequate forest-like flavor without compromising the bread's organoleptic properties.

The substitution of 70% water with NT extract, particularlly in SNT70, altered the bread's texture and sensory properties, resulting in lower hardness compared to the control bread despite having lower moisture content. As mentioned earlier, this distinction may beattributed to the interactions between NT polyphenols and gluten during dough development. The effects of polyphenols on gluten network depend on their concentration and their interactions with other components, such as polysaccharides, which could alter the the bread's structure and influence moisture distribution (Sivam et al., 2010; J. Xu et al., 2019; Zhu et al., 2016). Lower taste and texture scores for SNT70 could also be due to perceived dryness, chewiness, and off-flavors raised by the extract. Consequently, it was indeed confirmed that reducing the extract substitution level was already a sufficient change in a formulation that led to a more favorable overall acceptability. As supported by previous research, at 35% extract substitution, the bread retains its original sensory characteristics while offering nutritional improvements, making it a promising development in functional food production (Parenti et al., 2022).

4. Conclusion

Our study demonstrated that HC-based extraction in water effectively released a considerable amount of bioactive compounds from spruce, silver fir, and pine green needles and fine twigs. Higher extraction temperatures and prolonged times, particularly at 55 ± 3 min/47 ± 2 °C, enhanced the antioxidant properties of the extracts, while milder conditions were more effective in inhibiting of *E. coli* and *S. aureus* strains. A total of 115 compounds were identified in the NT extracts and bread samples, with selected compounds remaining stable throughout baking and storage. Incorporating these extracts into bread systems significantly increased antioxidant activity, total phenolic content, and the total sum of polyphenols after three days of storage. A 70% fortification (specifically SNT70) showed comparable proximate composition and textural quality, improved mineral profile, and enhanced functional properties. However, a 35% fortification was found to be sufficient to effectively increase functionality and shelf-life while also improving overall acceptability and purchase intent. Among all formulations, PNT35 exhibited the best overall balance of these factors, making it a promising option for further development as a bioactive, consumer-oriented product. Further studies should be conducted around the optimization of quality and technological aspects of NT incorporation in bread or other food matrices. In conclusion, this study provides a theoretical and practical foundation for product development, while promoting the innovative and value-added use of underutilized forest resources. The potential of using conifer needles as a source of natural antioxidants and functional ingredients in bread making deserves more investigation in the future. Yet, further toxicological studies are needed to ensure safety.

CRediT authorship contribution statement

Marina Fidelis: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jenni Tienaho:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Francesco Meneguzzo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Juha-Matti Pihlava:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Magnus Rudolfsson:** Writing – review & editing, Methodology, Investigation. **Eila Järvenpää:** Writing – review & editing, Methodology, Investigation. **Haruhiko Imao:** Writing – review & editing, Investigation. **Jarkko Hellström:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Jaana Liimatainen:** Writing – review & editing, Data curation. **Petri Kilpeläinen:** Writing – review & editing, Investigation. **Baoru Yang:** Writing – review & editing, Investigation. **Tuula Jyske:** Writing – review & editing, Resources, Project administration, Funding acquisition.

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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.lwt.2024.117055.

Data availability

Data will be made available on request.

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