

LC–IMS–MS profiling of avocado acetogenins reveals tissue-dependent distribution and cultivar-specific metabolic signatures

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ABSTRACT

This study presents a comprehensive characterisation of acetogenin-related metabolites in avocado using an LC–IMS–MS workflow. A total of 26 metabolites were semi-quantified across peel, pulp and seed tissues from three cultivars (*Hass*, *Bacon* and *Fuerte*). The integration of ion mobility spectrometry enabled the generation of the first experimental database of collision cross section (CCS) values for avocado acetogenins, improving confidence in metabolite annotation.

Results revealed a pronounced tissue-dependent distribution, with seeds and pulp as the primary reservoir of several acetogenins, whereas the peel consistently exhibited lower concentrations. In contrast, acetogenin levels remained largely stable throughout ripening. Clear cultivar-dependent differences were observed, with *Hass* displaying a distinct metabolic profile compared to *Bacon* and *Fuerte*. Multivariate analysis confirmed these findings, showing tissue-dependent cultivar differentiation.

This study provides new insights into avocado chemical diversity and highlights the potential of avocado by-products as consistent and promising sources of bioactive acetogenins.

1. Introduction

Avocado (*Persea americana* Mill.) is an oil-rich fruit characterised by a high lipid content, predominantly composed of monounsaturated fatty acids associated with beneficial cardiovascular benefits (Lu et al., 2005). Beyond its lipid composition, avocado contains a diverse range of bioactive phytochemicals, including carotenoids, vitamins, terpenoids, phenolic compounds, and acetogenins, many of which have been linked to biological activity (Lu et al., 2005). Among these bioactive compounds, acetogenins represent a distinctive group of secondary metabolites belonging to a broader class of biologically active aliphatic compounds primarily found in plants of the *Annonaceae* and *Lauraceae* families (Silva-Platas et al., 2012).

Lauraceous acetogenins, which are the focus of this study, are

characterised by highly oxygenated long-chain structures containing acetylenic and olefinic moieties. These compounds are biosynthetically derived from fatty acid precursors such as oleic, linoleic, linolenic, or stearic acid (Rodríguez-Saona & Trumble, 2000b). They were first reported over fifty years ago by Kashman and colleagues, who isolated them from avocados, a species belonging to the *Lauraceae* family (Kashman et al., 1970, 1969). Structurally, acetogenins can be classified according to the number of carbon atoms in their structures, including avocadins, pahuatins, and persenins, which contain 17, 19, and 21 carbon atoms, respectively (Rodríguez-López et al., 2015).

Various studies suggest that acetogenins are synthesized in specialized idioblast oil cells during early stages of plant development (Rodríguez-López et al., 2017; Rodríguez-Saona & Trumble, 2000b). After biosynthesis, these metabolites can be transported to other tissues,

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where they are thought to contribute to plant defense and metabolism (Rodríguez-Saona & Trumble, 2000b). Evidence indicates that idioblast oil cells and their lipid contents may play an important role in protecting plants against pathogens, insect herbivores, and fungal infections (Kobiler et al., 1993; Rodríguez-Saona & Trumble, 2000a). Previous studies in the *Hass* cultivar showed that acetogenin levels in the pulp remain relatively stable during fruit maturation and postharvest ripening, whereas seed concentrations exhibit a pronounced increase during early maturation followed by minor changes during ripening (Rodríguez-López et al., 2017). However, information regarding acetogenin dynamics in the peel during fruit development and ripening remains scarce.

Beyond their ecological role in plants, acetogenins have attracted considerable interest due to their diverse biological activities, including anticancer, antimicrobial, antifungal, and insecticidal properties (Butt et al., 2006; D'Ambrosio et al., 2011; Kim et al., 2000; Lee et al., 2015). In addition, these compounds have shown promising antimicrobial activity against foodborne microorganisms such as *Clostridium sporogenes* and *Listeria monocytogenes*, highlighting their potential as natural food preservatives (Pacheco et al., 2017; Raymond Chia & Dykes, 2010; Rodríguez-Sánchez, Pacheco, et al., 2013; Salinas-Salazar et al., 2017). Their antifungal activity against *Colletotrichum gloeosporioides*, the causal agent of anthracnose disease, also suggests potential applications as natural fungicides in agriculture (Yasir et al., 2010). Consistent with their proposed roles in plant defense and metabolism, acetogenins have been reported in several avocado-derived matrices, including peel, pulp, and seed (Rodríguez-López et al., 2015) as well as in leaves (Carman & Handley, 1999; Colin-Oviedo et al., 2022; Rodríguez-Sánchez et al., 2019). This widespread occurrence suggests that these metabolites could potentially be recovered from avocado processing by-products, enabling the valorization of residual plant material (Salazar-López et al., 2020). Consequently, a comprehensive characterisation of the acetogenin profile in these tissues is necessary to better understand their distribution and potential value.

Although gas chromatography–mass spectrometry (GC–MS) following derivatization has been used for the determination of these compounds (Carman & Handley, 1999; Colin-Oviedo et al., 2022), liquid chromatography (LC) remains the most widely used technique for acetogenin analysis. Some studies have reported the use of evaporative light scattering detectors (ELSD) (Degenhardt & Hofmann, 2010; Rodríguez-Sánchez et al., 2019), whereas most investigations have relied on LC coupled with spectrophotometric detection for acetogenin quantification (Kobiler et al., 1993; Rodríguez-López et al., 2017; Silva-Platas et al., 2012). In many cases, mass spectrometry has been incorporated (LC–MS) to support compound identification (Pacheco et al., 2017; Rodríguez-López et al., 2015; Rodríguez-Sánchez et al., 2019; Rodríguez-Sánchez, Silva-Platas, et al., 2013; Salinas-Salazar et al., 2017), frequently complemented by nuclear magnetic resonance (NMR) analysis for structural elucidation (Rodríguez-Sánchez, Pacheco, et al., 2013). Owing to the limited availability of commercially accessible analytical standards, quantification strategies are often based either on in-house purified standards of the major acetogenins (expressed as mg g⁻¹ dry weight) (Rodríguez-López et al., 2017; Rodríguez-Sánchez et al., 2019; Salinas-Salazar et al., 2017) or on the expression of all detected compounds as persenone A equivalents (mmol persenone A g⁻¹ dry weight), with persenone A itself being isolated in-house from plant material (Rodríguez-Sánchez, Silva-Platas, et al., 2013; Silva-Platas et al., 2012).

Although the previously mentioned studies have successfully determined several acetogenins in different tissues, the inherent structural complexity and isomeric diversity of these metabolites suggest that their comprehensive characterisation would benefit from more advanced analytical strategies. In this context, ion mobility-mass spectrometry (IMS-MS) has emerged as a powerful complementary tool in metabolomic, lipidomic, and proteomic studies, as it provides ion structural information by introducing an extra dimension of separation beyond the

mass-to-charge ratio (*m/z*) (Chouinard et al., 2019). IMS separates ions based on their size, shape, and charge as they travel through a drift gas under the influence of an electric field (Chouinard et al., 2019). A distinctive feature of this technique is the generation of collision cross section (CCS) values, which serve as robust molecular descriptors that enhance confidence in compound annotation and facilitate the discrimination of isomeric species, particularly in natural products research and food metabolomics (Díaz-Galiano et al., 2024).

In this study, LC-IMS-MS was applied for the first time to comprehensively characterise the acetogenin profile in the pulp, peel, and seed of avocado fruits from three of the most commercially important cultivars grown in Spain (*Hass*, *Bacon*, and *Fuerte*) at different ripening stages. These three cultivars present complementary harvesting windows that enable near-continuous market supply throughout the season (Talavera et al., 2023), making them directly relevant from both agronomic and agri-food perspectives. Furthermore, although all three are inter-racial Mexican-Guatemalan hybrids, they represent distinct genetic backgrounds and phenotypic profiles (Chen et al., 2009; Talavera et al., 2023). Together, these criteria ensure that the cultivar selection is both commercially representative and genetically diverse, maximising the scope and relevance of the study.

Extraction, chromatographic separation and detection conditions were optimised to enable reliable determination of these metabolites. The incorporation of ion mobility separation introduced an additional analytical dimension compared with conventional LC–MS approaches, improving the resolution of structurally related compounds and facilitating the differentiation of potential acetogenin isomers. Furthermore, the determination of CCS values provided complementary structural information to support metabolite annotation. Therefore, the aims of this work were (i) to characterise the diversity of acetogenin-related metabolites in avocado tissues and assess the influence of genetic and physiological factors on their occurrence, and (ii) to explore the potential of underutilised fruit tissues, particularly peel and seed, as accessible sources of these bioactive compounds for value-added applications.

2. Materials and methods

2.1. Chemicals and reagents

High-purity water was produced using a Milli-Q water purification system (Millipore, Bedford, MA, USA). LC-MS grade acetonitrile (ACN) and methanol (MeOH) were supplied by VWR International EuroLab S.L. U. (Barcelona, Spain), and acetic acid (AcH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The analytical standard of avocadyne, with a purity of ≥98%, was obtained from Innovation Sciences Inc. (Pasadena, USA). J.T.Baker® 0.22 µm nylon syringe filters provided by VWR International (Radnor, USA) and Nylaflow™ 0.45 µm nylon membranes from Pall Corporation (Ann Arbor, MI, USA) were used for sample and mobile phase filtration, respectively.

2.2. Avocado samples

Avocados used in this study were provided by the Institute of Mediterranean and Subtropical Horticulture “La Mayora” (IHSM-CSIC-UMA), located in Málaga, Spain. This institution has a unique germplasm collection of several subtropical fruit species. It is located at latitude 36°45′ North, longitude 4°4′ West with an altitude of 35 m above sea level.

Bacon avocados were harvested at the end of October 2021, followed by *Fuerte* avocados in early November 2021, and *Hass* avocados in mid-March 2022. Cultivars were evaluated within their typical commercial harvest windows, and all fruits were sourced from the same orchards and geographical region to minimise spatial variability and ensure comparable preharvest conditions.

Dry matter (DM) content was analysed immediately after harvest

using the AOAC 920.15123 method (AOAC, 2019). Between 10 and 15 fruits per cultivar were analysed to calculate the mean DM value. Unripe *Bacon* fruits exhibited DM values of approximately $27 \pm 2\%$, whereas *Fuerte* and *Hass* avocados showed DM contents of $29 \pm 3\%$ and $28 \pm 2\%$, respectively. Following DM determination, 80 fruits per cultivar were selected and divided into batches of 20 for controlled ripening. Unripe fruits were processed immediately upon arrival at the laboratory to generate five biological replicates ($n = 5$), each consisting of four fruits. The remaining fruits were stored under well-ventilated ambient conditions ($22 \pm 2^\circ\text{C}$, natural relative humidity, without exogenous ethylene) for up to two weeks to simulate typical commercial ripening environments. Samples were collected at defined stages: intermediate-ripe (4–5 days after harvest), ripe (8–9 days), and overripe (12–14 days). To ensure comparability among cultivars with different ripening kinetics, stages were defined using consistent physiological and morphological criteria, including days after harvest, mesocarp colour, and fruit firmness. This sample set had previously been used in a separate study with a different analytical focus, targeting phenolic compounds and other metabolite classes (Serrano-García et al., 2024). Considering the three distinct tissue types (pulp, peel and seed), a total of 180 samples were obtained ($3 \text{ cultivars} \times 4 \text{ ripening stages} \times 3 \text{ tissue types} \times 5 \text{ replicates}$). Sample pre-processing consisted of peeling, cutting, freezing, lyophilisation, crushing, homogenisation, and storage at -23°C to ensure sample integrity.

2.3. Acetogenin extraction from avocado tissues

Target analytes were extracted by solid-liquid extraction, using 100 mg of lyophilized sample and 12 mL of MeOH. The mixture was vortexed for 3 min, sonicated in an ultrasonic bath for 30 min, and then centrifuged at 8600g for 5 min. The supernatant was carefully separated from the solid residue, filtered through a $0.22 \mu\text{m}$ nylon syringe filter and then transferred to an amber LC vial. A quality control (QC) sample was prepared by combining equal volumes of all extracts (including peel, pulp and seed) in the sample set.

2.4. LC–IMS–MS analysis

Sample analyses were performed using an Ultra-High Performance Liquid Chromatography (UHPLC) system from the Elute series coupled to a trapped ion mobility-time of flight (timsTOF) Pro 2 mass spectrometer equipped with a Vacuum Insulated Probe Heated ElectroSpray Ionisation source (VIP-HESI) (Bruker Datonics, Bremen, Germany).

Chromatographic separation was achieved with a Zorbax Eclipse Plus column ($2.1 \times 150 \text{ mm}$, $1.8 \mu\text{m}$ particle size) from Agilent Technologies (Waldbronn, Germany). The column temperature was kept at 25°C , and the autosampler maintained the extracts at 4°C during the analytical sequence. For each analysis, a sample volume of $2 \mu\text{L}$ was injected. The mobile phases consisted of water containing 0.5% acetic acid (phase A) and pure acetonitrile (phase B), with a flow rate of 0.35 mL/min. Gradient elution was applied as follows: 0 min, 80% B; 15 min, 85% B; 16 min, 99.9% B; 17 min, 99.9% B; and 18 min, 80% B.

The VIP-HESI source operated in positive ionisation mode. The source parameters were set as follows: capillary voltage at -4500 V , nebuliser pressure at 4.0 bar, drying gas at 8.0 L/min and 200°C , and sheath gas at 4.0 L/min and 450°C . Ion mobility separation was performed using nitrogen (N_2) as the drift gas, with 100 ms of accumulation and ramp times, and an ion mobility scan range from 0.10 to $1.80 \text{ V}\cdot\text{s}/\text{cm}^2$. Data were acquired both in Full Scan (m/z 20–1300) and parallel accumulation serial fragmentation (PASEF) modes. The collision cell was operated at 7.5 eV collision energy and 700 Vpp radiofrequency. Two MS/MS scans were performed per cycle (0.66 s total cycle time), with the number of valid precursors per PASEF scan set to 1. The target intensity and threshold were set to 10,000 and 1000 counts, respectively, and the exclusion release time was 0.1 min.

External calibration of both, the TIMS and TOF MS systems was

carried out using the ESI-L Low Concentration Tuning Mix solution (Agilent Technologies, Waldbronn, Germany), and sodium formate, respectively. In addition, internal calibration was carried out by infusing a mixture of the calibrant solutions at the beginning of each run to improve mass accuracy. Instrument stability and signal consistency were monitored by analysing a QC sample every 10 injections. An external calibration curve for analyte quantification was generated by serial dilution of the avocadyne standard dissolved in methanol, yielding 12 concentration levels from 0.5 to 1000 mg L^{-1} . The order of sample analysis was randomised throughout the analytical sequence to minimise potential analytical bias and to reduce the influence of instrumental drift and carryover effects over time. Instrument control and data acquisition were performed using Compass Hystar and timsControl software (Bruker Datonics, Bremen, Germany).

2.5. Data processing and metabolite annotation

Data processing was performed using MetaboScape 2023 and TASQ® software (Bruker Datonics, Bremen, Germany). MetaboScape was initially used to generate a preliminary list containing key information for the target analytes, including retention time (Rt), m/z values of the pseudo-molecular ions and main in-source fragments, and CCS values. Subsequently, quantification of the selected analytes was performed using TASQ®. The software automatically integrated the extracted ion chromatograms, considering those peaks with a signal-to-noise (S/N) ratio > 1 , using a mass window of $\pm 3 \text{ mDa}$ around the expected m/z values and a retention time tolerance of $\pm 0.1 \text{ min}$. An analyte was considered positively detected when the principal ion and at least one mandatory confirming ion were found within the expected retention time and mass tolerance. In the final step, only peaks exhibiting a S/N ratio higher than 10 were retained for quantification. In the absence of pure commercial standards, all analytes were quantified using avocadyne as the reference compound.

2.6. Assessment of the analytical parameters of the method

To evaluate the performance of the optimised method, both the avocadyne standard and the QC sample were used to assess the main analytical parameters, such as linearity, limits of detection (LOD) and quantification (LOQ), and method repeatability. Calibration curves were constructed by linear regression using the least squares method, with each calibration point representing the mean of two independent injections. LOD and LOQ were estimated from the lowest injected concentration producing S/N ratios of 3 and 10, respectively. Repeatability was expressed as the coefficient of variation (%CV) from replicate QC injections within a single day (intra-day repeatability) and across multiple days (inter-day repeatability). Recovery was assessed by subjecting samples to three consecutive extraction cycles. Results are expressed as the percentage of analyte recovered in the first extraction relative to the total amount extracted across all extraction cycles.

2.7. Statistical analyses

Statistical analysis of the data was performed using InfoStat v2020d (InfoStat Group, Córdoba, Argentina). One-way analysis of variance (ANOVA) was applied to evaluate differences in the concentration of each analyte across the four ripening stages for each tissue and cultivar. The significance of differences between means was determined at the 5% level ($p < 0.05$) using Tukey's test. Principal component analysis (PCA) was performed using SIMCA v14.1 software. Prior to modelling, the data matrix was standardised and scaled using unit variance. Model performance was evaluated based on the goodness of fit ($R^2\text{X}$) and predictive ability (Q^2) parameters.

Supervised partial least squares-discriminant analysis (PLS-DA) was additionally performed using SIMCA v14.1 to evaluate cultivar-related differences in acetogenin composition independently for each tissue.

Model performance was assessed using R^2X , R^2Y , and Q^2 parameters, and model validity was confirmed through permutation tests ($n = 200$ permutations). Variable importance in projection (VIP) scores were obtained from the PLS-DA models, and metabolites with $VIP > 1$ were considered relevant contributors to cultivar differentiation. Additionally, fold changes were calculated as the ratio of cultivar means averaged across ripening stages for each metabolite and tissue, an approach justified by the absence of statistically significant ripening-dependent variation in acetogenin concentrations demonstrated in this study.

3. Results and discussion

3.1. Optimisation of the analytical methodology

To establish a reliable and reproducible method for the extraction and analysis of acetogenins in avocado tissues, several experimental conditions related to sample preparation and chromatographic separation were systematically evaluated. The optimisation strategy was guided by an extensive review of previously reported methodologies for avocado acetogenin determination (Aguilar-Hernández et al., 2020; Colin-Oviedo et al., 2022; Rodríguez-López et al., 2015; Rodríguez-Sánchez et al., 2019; Rodríguez-Sánchez, Pacheco, et al., 2013; Rodríguez-Sánchez, Silva-Platas, et al., 2013; Salinas-Salazar et al., 2017).

The optimisation of the extraction protocol involved the evaluation of several variables, including the choice of solvent, the effect of performing one or two extraction cycles, and the need for an additional clean-up step to remove potential interferences. Given the lipidic nature of acetogenins, the Folch method, a widely used lipid extraction technique (Saini et al., 2021), was also evaluated. Details of the extraction protocols assessed during method optimisation are summarised in Fig. S1 (Supplementary Material).

In an initial stage, protocols employing different solvent volumes were compared, using either 12 mL or 6 + 6 mL (volumes previously optimised in preliminary trials) of isopropanol, methanol, and a 50:50 (v/v) mixture of methanol and diethyl ether. The Folch method was also tested, with appropriate adjustment of the sample-to-solvent ratios. However, the results obtained using the Folch extraction were less satisfactory than those achieved with the solid-liquid extraction approaches. Fig. S2 presents a comparison of extraction efficiency, expressed as percentage recovery, for the different protocols tested using acetoxyl (AcO)-avocadene, persenone A, persenone B, and persin as representative compounds. Similar trends were observed for the remaining analytes; however, these compounds were selected due to their relatively high abundance in the samples. Methanol provided the best overall extraction performance, and a single extraction cycle yielded comparable results to two cycles. Based on these findings, the need for an additional clean-up step was further evaluated. Previous reports suggested that clean-up could be performed by evaporating the extract and reconstituting it in a water/dichloromethane mixture (50:50, v/v), enabling separation of the organic phase while the aqueous phase removes more polar components. The comparison between extracts processed with and without this clean-up step is shown in Fig. S2B, revealing that extraction efficiencies were essentially identical under both conditions.

The selected protocol, designed to ensure high recovery while minimising sample handling, consisted of a single-cycle solid-liquid extraction using methanol, with no additional clean-up step required. This choice was supported by the high extraction efficiency of methanol, the robustness of the method, and the consistent recovery obtained for the target compounds. The elimination of additional steps, such as fractionation or solvent evaporation, simplified the workflow, reducing processing time and potential sources of variability. Moreover, the exclusion of chlorinated solvents enhances the environmental sustainability of the analytical protocol. Overall, the resulting protocol provides a simple, reliable, and sustainable procedure for the extraction of

acetogenins from avocado tissues.

Chromatographic conditions, including column type, flow rate, and temperature, were examined to define the most suitable settings for acetogenin separation and detection. Three columns were evaluated: Zorbax Eclipse Plus (2.1×150 mm, particle size 1.8 μm), Bruker Intensity Solo 2 C₁₈ (2×100 mm, particle size 2 μm) and Thermo Acclaim RSLC 120 C₁₈ (2.1×100 mm, particle size 2.2 μm). Flow rates from 0.3 mL/min to 0.4 mL/min were compared, alongside column temperatures from 25 °C to 35 °C. Among the tested conditions, the Zorbax Eclipse Plus column, operated at a flow rate of 0.35 mL/min and a temperature of 25 °C, yielded the best performance in terms of separation efficiency and peak resolution, as illustrated in Fig. S3 and discussed in the following section. This combination was therefore selected as the optimal chromatographic setup for acetogenin analysis. A detailed description of the final extraction protocol and chromatographic method was provided in Sections 2.3 and 2.4, respectively.

3.2. Characterisation of the acetogenin profile of avocado tissues

First, the metabolic profiling of the three avocado tissues was performed using LC-IMS-MS, adopting an untargeted screening strategy. After the careful inspection of the obtained chromatograms, 26 metabolites were selected for a subsequent targeted analysis based on their prominence in the profiles. These metabolites included 26 acetogenins and related compounds. The analyses corroborated the presence of all previously reported acetogenins across seed, pulp and peel. In addition, several isomeric compounds were detected within different acetogenin classes, which, to our knowledge, have not been previously reported. Although not strictly classified as acetogenins, other fatty acid derivatives, such as polyhydroxylated fatty acids, were also considered due to their well-documented occurrence in avocado (Hashimura et al., 2001; Louis et al., 2023). Their inclusion was further supported by their consistent and prominent detection in the metabolic profiles.

High-resolution MS data, which enabled the prediction of the molecular formulas of the detected compounds with a mass error ≤ 0.5 mDa, along with relative elution order, were considered for compound annotation. The obtained information was compared with previously published reports (Hashimura et al., 2001; Louis et al., 2023; Oberlies et al., 1998; Ramos-Jerz, 2007; Rodríguez-Sánchez et al., 2019; Rodríguez-Sánchez, Pacheco, et al., 2013; Rodríguez-Sánchez, Silva-Platas, et al., 2013; Salinas-Salazar et al., 2017) and further supported by the detection of in-source fragments and MS/MS spectra consistent with in silico fragmentation predicted by the MetFrag tool in MetaboScape. As described by Rodríguez-Sánchez et al. (2019), characteristic fragmentation patterns of acetogenins were observed, including neutral losses of 18 amu (corresponding to water, $[\text{M}-\text{H}_2\text{O}]^+$), 60 amu (acetate, $[\text{M}-\text{CH}_3\text{COOH}]^+$), and 78 amu (combined loss of water and acetate, $[\text{M}-\text{CH}_3\text{COOH}-\text{H}_2\text{O}]^+$). In addition, the formation of sodium adduct dimers ($[2\text{M} + \text{Na}]^+$) was detected in some cases. Several consecutive neutral losses of 18 amu, consistent with successive water eliminations, were also observed for polyhydroxylated fatty acids during in-source fragmentation.

Fig. S3 presents an example of the base peak chromatogram (BPC) obtained from a QC sample together with the extracted ion chromatograms (EICs) of the 26 determined compounds. Each panel displays the accurate m/z signal (± 0.005) used for ion extraction, along with the corresponding analyte number (as in Table 1). Analyte numbers are shown in black when the monitored m/z corresponds to the most abundant ion and in grey when the extracted signal corresponds to a secondary in-source fragment or adduct. Table 1 provides a comprehensive overview of the targeted metabolites, arranged by elution order. It includes essential information such as molecular formula, retention time (Rt), most abundant adduct ion signal and corresponding m/z value, mass error, isotopic pattern fit (mSigma), $\text{TIMS}^\text{CCS}_{\text{N}_2}$ value, and additional detected signals (in-source fragments). CCS measurements showed excellent repeatability, with CV ranging from 0.2 to 0.4% for all

Table 1Comprehensive characterisation of acetogenins and related compounds detected in *Bacon*, *Fuerte* and *Hass* avocado tissues by LC-IMS-MS.

N	Tentative name	Molecular formula	Rt (min)	Most abundant m/z	Most abundant adduct ion	Error (mDa)	mSigma	$^{TMS}_{CCS_{N_2}}$ ($\text{\AA}^2$) [◆]	Other signals m/z (in-source fragments/adducts); CCS ($\text{\AA}^2$) [◆]
1	Avocadyne*	C ₁₇ H ₃₂ O ₃	1.9	267.2319	[M+H-H ₂ O] ⁺	0.0	12.2	163.6	249.2221 ([M+H-2H ₂ O] ⁺); 159.8 231.2105 ([M+H-3H ₂ O] ⁺); 154.2 307.2240 ([M + Na] ⁺); 174.6 285.2416 ([M+H] ⁺); 169.8 265.2160 ([M+H-CH ₃ COOH] ⁺); 161.8 307.2268 ([M+H-H ₂ O] ⁺); 171.8, 187.4 347.2187 ([M + Na] ⁺); 181.9 249.2210 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 160.1 267.2317 ([M+H-CH ₃ COOH] ⁺); 161.5 349.2350 ([M + Na] ⁺); 185.9 249.2213 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 159.2 267.2320 ([M+H-CH ₃ COOH] ⁺); 162.1 349.2349 ([M + Na] ⁺); 183.7 265.2163 ([M+H-CH ₃ COOH] ⁺); 162.2 307.2269 ([M+H-H ₂ O] ⁺); 187.5 347.2187 ([M + Na] ⁺); 182.2 233.2265 ([M+H-3H ₂ O] ⁺); 165.1 251.2369 ([M+H-2H ₂ O] ⁺); 171.5 287.2580 ([M+H] ⁺); 263.6, 169.2, 178.9 249.2220 ([M+H-2H ₂ O] ⁺); 169.7160.2183.6 285.2422 ([M+H] ⁺); 183.4 349.2347 ([M + Na] ⁺); 185.0 249.2213 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 170.0, 157.4, 185.8 267.2319 ([M+H-CH ₃ COOH] ⁺); 178.8 351.2506 ([M + Na] ⁺); 191.5 251.2371 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 160.1, 174.3, 191.4 679.5117 ([2 M + Na] ⁺); 274.2 307.2268 ([M+H-H ₂ O] ⁺); 190.3 265.2162 ([M+H-CH ₃ COOH] ⁺); 176.7 347.2190 ([M + Na] ⁺); 183.4 351.2506 ([M + Na] ⁺); 183.4 251.2369 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 159.7, 191.4, 172.7 679.5118 ([2 M + Na] ⁺); 277.9 349.2349 ([M + Na] ⁺); 182.3 267.2321 ([M+H-CH ₃ COOH] ⁺); 174.9 361.2714 ([M + Na] ⁺); 190.0 339.2890 ([M+H] ⁺); 188.9 303.2680 ([M+H-2H ₂ O] ⁺); 182.5 285.2574 ([M+H-3H ₂ O] ⁺); 174.5, 188.7 313.2736 ([M+H] ⁺); 195.5 277.2526 ([M+H-2H ₂ O] ⁺); 183.0 335.2551 ([M + Na] ⁺); 190.2 259.2418 ([M+H-3H ₂ O] ⁺); 161.1, 195.9 353.2683 ([M + H] ⁺); 197.7 293.2475 ([M+H-CH ₃ COOH] ⁺); 183.4, 168.0, 200.1
2	AcO-Avocadenyne isomer I	C ₁₉ H ₃₂ O ₄	2.2	247.2054	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.3	12.1	156.3	
3	AcO-Avocadyne isomer I	C ₁₉ H ₃₄ O ₄	2.2	309.2422	[M+H-H ₂ O] ⁺	0.2	3.4	179.3	
4	AcO-Avocadyne	C ₁₉ H ₃₄ O ₄	2.5	309.2423	[M+H-H ₂ O] ⁺	0.1	2.4	179.4, 173.1	
5	AcO-Avocadenyne isomer II	C ₁₉ H ₃₂ O ₄	2.6	247.2058	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.2	10.7	164.0, 156.6	
6	Avocadene	C ₁₇ H ₃₄ O ₃	2.7	269.2472	[M+H-H ₂ O] ⁺	0.3	4.6	177.6	
7	Avocadenol A	C ₁₇ H ₃₂ O ₃	3.0	267.2316	[M+H-H ₂ O] ⁺	0.2	7.4	174.3	
8	AcO-Avocadyne isomer II	C ₁₉ H ₃₄ O ₄	3.3	309.2421	[M+H-H ₂ O] ⁺	0.3	5.1	182.6, 196.7	
9	AcO-Avocadene isomer	C ₁₉ H ₃₆ O ₄	3.3	311.2577	[M+H-H ₂ O] ⁺	0.4	6.7	186.8	
10	AcO-Avocadenyne	C ₁₉ H ₃₂ O ₄	3.7	247.2055	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.2	8.5	170.1	
11	AcO-Avocadene	C ₁₉ H ₃₆ O ₄	4.1	311.2577	[M+H-H ₂ O] ⁺	0.4	2.8	187.4, 174.3	
12	AcO-Avocadiene B	C ₁₉ H ₃₄ O ₄	4.4	249.2212	[M+H-CH ₃ COOH-H ₂ O] ⁺	-0.2	6.6	170.6, 158.1	
13	Trihydroxyheneicosatriene	C ₂₁ H ₃₈ O ₃	5.2	321.2788	[M+H-H ₂ O] ⁺	0.0	2.1	185.8	
14	Trihydroxynonadecadiene	C ₁₉ H ₃₆ O ₃	5.7	295.2633	[M+H-H ₂ O] ⁺	-0.1	15.5	186.2	
15	Persenone C isomer	C ₂₁ H ₃₆ O ₄	5.8	275.2367	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.2	9.1	179.3, 165.8	

(continued on next page)

Table 1 (continued)

N	Tentative name	Molecular formula	Rt (min)	Most abundant m/z	Most abundant adduct ion	Error (mDa)	mSigma	$^{TIMS}_{CCS_{N_2}}$ ($\text{\AA}^2$) [♦]	Other signals m/z (in-source fragments/adducts); CCS ($\text{\AA}^2$) [♦]
16	<u>Persediene</u>	C ₂₁ H ₃₆ O ₄	6.0	353.2685	[M+H] ⁺	0.1	2.0	196.4	375.2502 ([M + Na] ⁺); 193.2 727.5118 ([2 M + Na] ⁺); 282.2 275.2371 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 180.2 311.2579 ([M+H-H ₂ O] ⁺); 186.7, 204.6 293.2473 ([M+H-CH ₃ COOH] ⁺); 184.7 375.2502 ([M + Na] ⁺); 191.3 353.2680 ([M+H] ⁺); 197.7 293.2476 ([M+H-CH ₃ COOH] ⁺); 184.7 375.2504 ([M + Na] ⁺); 191.3 727.5119 ([2 M + Na] ⁺); 283.5 401.2664 ([M + Na] ⁺); 198.4 361.2738 ([M+H-H ₂ O] ⁺); 194.7 319.2632 ([M+H-CH ₃ COOH] ⁺); 186.9 379.2843 ([M+H] ⁺); 202.8 379.2843 ([M+H] ⁺); 200.6 401.2664 ([M + Na] ⁺); 197.7 361.2738 ([M+H-H ₂ O] ⁺); 195.7 319.2631 ([M+H-CH ₃ COOH] ⁺); 187.8 297.2787 ([M+H-CH ₃ COOH] ⁺); 191.2 279.2682 ([M+H-CH ₃ COOH-H ₂ O] ⁺); 185.4, 170.0, 198.0 357.3000 ([M+H] ⁺); 188.2, 201.4 313.2737 ([M+H-H ₂ O] ⁺); 192.2 403.2818 ([M + Na] ⁺); 195.7 363.2894 ([M+H-H ₂ O] ⁺); 201.7 321.2789 ([M+H-CH ₃ COOH] ⁺); 187.9 381.2999 ([M+H] ⁺); 202.2 403.2819 ([M + Na] ⁺); 196.8 321.2788 ([M+H-CH ₃ COOH] ⁺); 188.1 363.2895 ([M+H-H ₂ O] ⁺); 201.5 381.2999 ([M+H] ⁺); 202.8 377.2662 ([M + Na] ⁺); 201.0 295.2632 ([M+H-CH ₃ COOH] ⁺); 190.6 355.2840 ([M+H] ⁺); 203.0 377.2663 ([M + Na] ⁺); 203.5 295.2630 ([M+H-CH ₃ COOH] ⁺); 190.9 355.2843 ([M+H] ⁺); 202.9 381.2998 ([M+H] ⁺); 205.9 403.2817 ([M + Na] ⁺); 205.5 363.2895 ([M+H-H ₂ O] ⁺); 200.3 321.2790 ([M+H-CH ₃ COOH] ⁺); 192.8
17	<u>Persenone C</u>	C ₂₁ H ₃₆ O ₄	6.1	275.2369	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.0	14.4	179.0, 166.1	
18	Persenone A isomer	C ₂₃ H ₃₈ O ₄	6.3	301.2525	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.1	5.7	181.6, 196.6	
19	<u>Persenone A</u>	C ₂₃ H ₃₈ O ₄	6.6	301.2526	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.0	7.6	182.0, 196.2	
20	2,4-Dihydroxynonadecyl acetate	C ₂₁ H ₄₀ O ₄	7.0	339.2895	[M+H-H ₂ O] ⁺	-0.1	20.6	195.2, 183.1	
21	2,4-Dihydroxyheptadecyl acetate	C ₁₉ H ₃₈ O ₄	7.4	331.2840	[M+H] ⁺	0.3	1.0	195.7	
22	Persin isomer I	C ₂₃ H ₄₀ O ₄	7.8	303.2682	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.0	28.2	181.6, 203.4	
23	<u>Persin</u>	C ₂₃ H ₄₀ O ₄	8.1	303.2681	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.1	13.5	182.3, 202.5	
24	Persenone B isomer	C ₂₁ H ₃₈ O ₄	8.5	277.2527	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.1	11.6	184.6	
25	<u>Persenone B</u>	C ₂₁ H ₃₈ O ₄	8.9	277.2528	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.2	8.2	184.1	
26	Persin isomer II	C ₂₃ H ₄₀ O ₄	9.9	303.2680	[M+H-CH ₃ COOH-H ₂ O] ⁺	0.2	19.9	186.3, 202.8	

Abbreviations: Rt (retention time), CCS (collision cross section), AcO (acetoxy).

* Avocado identity was confirmed using a pure standard (confidence level 1: identified compound); underlined compounds, with confirmed relative retention times and NMR-established structures, are assigned a confidence level of 2 (putatively annotated compounds); the rest of compounds listed in the table are reported with a confidence level of 3 (putatively characterised compound classes) (Sumner et al., 2007).

♦ Multiple CCS values are reported for ions exhibiting multiple mobility peaks with comparable intensities (e.g., due to different charging positions); when a single peak dominated the mobilogram, only the predominant CCS value is provided.

annotated compounds across samples, demonstrating the high robustness and consistency of the obtained CCS values. The integration of TIMS in this study enabled the development of the first experimental CCS database for avocado acetogenins. CCS values constitute an orthogonal molecular descriptor that complements accurate mass, Rt, and MS/MS

fragmentation data, thereby increasing confidence in compound annotation. Consequently, the resulting CCS database constitutes a valuable resource for future studies on avocado acetogenins and is expected to enhance the reliability of avocado metabolomics research.

Compared with previous reports focused on the isolation and

structural elucidation of acetogenins from avocado seed and pulp, the present results broaden the current understanding of the chemical diversity of this metabolite class. Earlier work by Hernández-Brenes' research group (Rodríguez-López et al., 2015; Rodríguez-Sánchez et al., 2019; Rodríguez-Sánchez, Pacheco, et al., 2013; Rodríguez-Sánchez, Silva-Platas, et al., 2013) provided detailed characterisation of several major acetogenins, including AcO-avocadyne, AcO-avocadenyne, AcO-avocadene, AcO-avocadiene B, persediene, persenones A–C, and persin, using targeted HPLC–PDA–MS and NMR-based approaches. Building upon this foundational work, the LC–IMS–MS profiling applied in the present study enabled the tentative annotation of 26 acetogenins and related metabolites across seed, pulp, and peel matrices, revealing a substantially broader spectrum of these lipophilic secondary metabolites than previously documented. Three C17 polyhydroxylated fatty acids (numbers 1, 6 and 7 in Table 1) previously reported in avocado were also observed (Louis et al., 2023; Oberlies et al., 1998). Their relative elution order in reversed-phase LC was in agreement with their expected hydrophobicity. Since all compounds share the same carbon chain length and degree of hydroxylation, the observed differences in retention are likely related to the number and type of unsaturations, with the alkynyl analogue (avocadyne) eluting earlier than the mono-unsaturated (avocadene) and diunsaturated (avocadenol A) compounds.

The newly detected compounds include several isomeric species that were primarily resolved by reversed-phase LC, as evidenced by their distinct retention times (*R_t*) in Table 1. In addition, ion mobility separation provided an extra dimension of discrimination, reflected in the different CCS values obtained for these species. As expected for long alkyl-chain molecules with considerable conformational flexibility, the CCS differences among positional or geometric isomers were relatively small. Isomers that differ only in the position of a double bond or in the configuration of hydroxyl or acetoxy groups often adopt similar overall geometries in the gas phase, limiting large CCS divergences (Camunas-Alberca et al., 2023; Díaz-Galiano et al., 2024). Consequently, the CCS differences observed among these compounds likely reflect subtle variations in double-bond position and chain bending rather than major rearrangements in overall molecular shape. This behaviour can be illustrated by persin (182.3 Å²) and its isomers I (181.6 Å²) and II (186.3 Å²). Overall, the detection of multiple isomeric species highlights the structural complexity of avocado acetogenins and suggests the presence of positional and geometric isomer diversity that has not been fully resolved in many previous LC-MS or NMR-based studies. Major acetogenins previously described in the literature, with confirmed relative retention times and NMR-established structures, are underlined in Table 1. Chemical structure and systematic nomenclature for these compounds are provided in Table S1. Compounds sharing the same molecular formula are designated as *isomers* and distinguished by Roman numerals in the table. Although unambiguous identities cannot be assigned to these isomers, several features are consistent with acetogenin-related structures previously isolated from avocado. For instance, AcO-avocadenyne isomers I and II may correspond to 1-acetoxy-2-hydroxy-4-oxo-heptadec-16-yne (AcO-avocadynone) or to 1-acetoxy-2-hydroxy-4-oxo-heptadec-5,16-diene (Ramos-Jerz, 2007). Likewise, the compounds tentatively annotated as AcO-avocadyne isomers may correspond to the structure proposed by the same author for 1-acetoxy-2,4-dihydroxy-heptadec-5,16-diene, a positional isomer of AcO-avocadiene B (Ramos-Jerz, 2007). Regarding persin isomers, two additional plausible structures have been reported in the literature: (12Z,15Z)-1-hydroxy-4-oxo-heneicosa-12,15-dien-2-yl acetate (isopersin) (Rodríguez-Saona et al., 1998) and 1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12-diene (Kawagishi et al., 2001). The latter showed good agreement with persin isomer II, reaching an intensity coverage of 87.9% using the MetFrag in-silico fragmentation prediction tool. Similarly, persenone B isomer could, based solely on exact mass, be tentatively associated with 1-O-linoleoyl-glycerol, previously isolated from thermally processed avocado pulp by Degenhardt and Hofmann (2010). However, this assignment is inconsistent with the MS/MS data. The

observed fragmentation pattern includes a characteristic neutral loss of 60 Da (CH₃COOH), which is not typical of monoacylglycerol fragmentation and instead indicates the presence of an acetoxy functional group. Interestingly, Degenhardt and Hofmann (2010) also described fully saturated acetogenins which, although scarcely reported in avocado, may likewise account for compounds 20 and 21. These were tentatively assigned as 2,4-dihydroxynonadecyl acetate and 2,4-dihydroxyheptadecyl acetate, representing the fully saturated analogues of persenone B and AcO-avocadene, respectively. Notably, the latter compound has previously been obtained by catalytic hydrogenation of AcO-avocadyne by Hashimura et al. (2001).

Additionally, compound 13 (C₂₁H₃₈O₃) and compound 14 (C₁₉H₃₆O₃) exhibited fragmentation patterns characterised by the sequential neutral loss of up to three water molecules, indicating the presence of three hydroxyl groups. Their elemental compositions and degrees of unsaturation (three and two double bond equivalents, respectively) are consistent with non-acetylated polyunsaturated acetogenin-type derivatives. In particular, compound 13 may tentatively correspond to a trihydroxylated C21 triene (trihydroxyheneicosatriene), whereas compound 14 is compatible with a trihydroxylated C19 diene analogue (trihydroxynonadecadiene). Although exact structural matches corresponding to these molecular formulas have not been explicitly reported, the accurate mass, elemental composition, and diagnostic fragmentation patterns observed are fully consistent with those previously described for oxygenated acetogenins in *Persea americana*. These convergent lines of evidence support their classification within this compound family.

For this subset of compounds, the absence of authentic standards precludes unequivocal structural confirmation. Accordingly, they were conservatively assigned a confidence level of 3 (putatively characterised compound classes) following the criteria proposed by Sumner et al. (2007).

3.3. Determination of the analytical parameters of the method

The analytical performance of the proposed methodology was evaluated according to the parameters described in Section 2.6. Three linear concentration ranges were established for metabolite quantification in all extracts, using avocadyne as the reference standard, in order to accommodate the variability in metabolite levels across tissues and cultivars. Linearity was satisfactory, with correlation coefficients (*r*²) ranging from 0.995 to 0.998. Additionally, avocadyne exhibited a LOD of 0.02 mg L⁻¹ and a LOQ of 0.08 mg L⁻¹. Intra-day repeatability, assessed from 7 repeated injections of QC sample within the same sequence (*n* = 7), showed CV ranging from 5.52% to 12.12% across all metabolites. Inter-day repeatability was determined from the analysis of the same QC sample over four consecutive days (*n* = 21), yielding CV values between 7.00% and 12.30%. The efficiency of the extraction protocol was also estimated, resulting in recovery rates ranging from 98% for persenone B to 100% for persin.

3.4. Comparative distribution of acetogenins in seed, peel, and pulp in the three avocado cultivars

After evaluation of the analytical performance of the method, quantitative analysis of 26 metabolites was performed on a total of 180 extracts. The experimental design comprised five biological replicates per ripening stage, three tissues per fruit (seed, peel and pulp), four ripening stages and three avocado cultivars. Quantitative results are expressed as mg g⁻¹ dry weight (mean ± standard deviation) and are reported in Table S2. To our knowledge, this dataset represents one of the most comprehensive comparative surveys of acetogenin distribution across avocado tissues, cultivars and ripening stages reported to date.

Due to the lack of commercially available standards, all analytes were quantified using avocadyne as reference compound following the semi-quantitative approach described in Section 2.6. The use of avocadyne as a reference compound provides a suitable basis for comparative

quantification, given the structural similarity among avocado acetogenins and their presumably comparable ionisation behaviour under the analytical conditions employed. The calibration was performed over a concentration range of 0.78 to 1000 ppm. Given the wide concentration range, three linear calibration ranges were established to ensure a linear

detector response across the entire working range: 0.78–25 ppm ($y = 132,610.362x + 63,853.342$; $R^2 = 0.995$), 25–200 ppm ($y = 71,951.347x + 1,978,436.239$; $R^2 = 0.988$) and 200–1000 ppm ($y = 48,889.547x + 6,827,616.038$; $R^2 = 0.998$).

To facilitate interpretation, the quantitative results are discussed by



Fig. 1. Tissue-specific partitioning of acetogenins among seed, peel and pulp in the avocado cultivars *Bacon*, *Fuerte* and *Hass*. Values represent the relative contribution (%) of each tissue to the total acetogenin pool within each fruit (normalised to 100%). Percentages were calculated from averaged concentrations across ripening stages.

first addressing tissue-dependent distribution patterns, followed by cultivar-related differences and, finally, the relative contribution of individual acetogenins to the overall metabolite profile.

3.4.1. Tissue-dependent distribution of acetogenins

Examination of the quantitative dataset (Table S2) reveals clear tissue-dependent trends in acetogenin abundance. No single distribution pattern was observed for all metabolites; rather, concentration profiles depended on both tissue type and cultivar. In general, peel showed the lowest concentrations, whereas seed and pulp contained the highest levels depending on the metabolite considered. In seed samples, several acetogenins reached concentrations in the order of tens of mg g^{-1} dry weight, with the most abundant compounds exceeding $70\text{--}80 \text{ mg g}^{-1}$ in specific cases. By comparison, concentrations in the peel generally remained below 40 mg g^{-1} (with the exception of persenone A in *Bacon* and persin in *Fuerte*), and were frequently below a few mg g^{-1} for several analytes. The pulp exhibited a broader concentration range, reflecting compound- and cultivar-dependent variability and varying between 0.04 and 100 mg g^{-1} , confirming that acetogenin accumulation in avocado is strongly tissue-dependent.

Among the quantified metabolites, persin and its isomers, together with several acetylated derivatives, accounted for a major fraction of the acetogenin pool in most tissues, underscoring their relevance within the overall metabolic profile of avocado fruits.

Inspection of the obtained quantitative data also reveals compositional differences among cultivars. For example, persenone B was the most abundant acetogenin in *Hass* and *Fuerte* seeds, whereas AcO-avocadene predominated in the seed of *Bacon*. These cultivar-dependent differences are discussed in greater detail in the following subsections.

3.4.2. Effect of ripening stage on acetogenin levels

To evaluate the potential influence of fruit ripening on acetogenin levels, a one-way ANOVA was applied to the quantitative data for each tissue and cultivar (Table S2). For the majority of metabolites, no statistically significant differences were observed among the four ripening stages ($p > 0.05$). The absence of pronounced variations in acetogenin concentrations throughout the ripening process represents a relevant observation. This compositional stability indicates that avocado by-products obtained from fruits at different commercial stages retain a relatively consistent profile of bioactive acetogenins. Such stability enhances their potential value for health-related applications or as functional food ingredients, since the presence of these compounds is not restricted to a specific ripening stage.

Based on this observation, metabolite concentrations were averaged across ripening stages to facilitate comparative analysis and streamline the discussion presented in this section. This approach allowed a clearer assessment of acetogenin partitioning among tissues within each cultivar and simplified the interpretation of their distribution patterns.

3.4.3. Cultivar-dependent differences in acetogenin distribution among tissues

Fig. 1 summarizes the distribution of acetogenins across the three analysed tissues (seed, peel and pulp) for each cultivar. Values are expressed as percentages of the total amount detected in the three tissues. In this way, the figure provides an integrated overview of acetogenin partitioning within the fruit and allows direct comparison among cultivars.

Overall, *Bacon* and *Fuerte* displayed highly similar acetogenin distribution patterns, suggesting comparable metabolic profiles. In contrast, *Hass* exhibited a markedly different distribution, indicating stronger cultivar-dependent variation in acetogenin partitioning.

In general, *Hass* was characterised by a higher proportion of most acetogenins in the seed and very low levels in the peel. Compounds predominantly detected in the pulp included compound 13 (tentatively assigned as a trihydroxyheneicosatriene), 2,4-dihydroxynonadecyl

acetate, and persin together with its isomer I, the latter also showing a moderate presence in the peel ($\sim 20\%$). Among the metabolites detected in the peel, 2,4-dihydroxyheptadecyl acetate exhibited the highest proportion in *Hass* (33.6%), a distribution pattern that closely resembles that observed in *Bacon* and *Fuerte*.

By contrast, *Bacon* and *Fuerte* fruits exhibited a more balanced acetogenin distribution across the three tissues. Nevertheless, several consistent predominances were observed. AcO-avocadenyne and its isomers were mainly located in the seeds of both cultivars, accounting for 71.5–88.9% of their total abundance in *Bacon* and 91.6–98.6% in *Fuerte*. Similarly, AcO-avocadiene B and avocadenol A showed comparable distribution profiles, reaching approximately 85% and 90–96%, respectively, in the seed.

Other metabolites showed preferential accumulation in the pulp. AcO-avocadene isomer and AcO-avocadyne isomer I displayed high relative abundances in the pulp of *Bacon* (71.1% and 66.8%, respectively) and *Fuerte* (78.2% and 56.7%), whereas in *Hass* these compounds were predominantly found in the seed (74.0% and 83.2%). Differences were also observed for persadiene, which was mainly located in the pulp in *Fuerte* (66.1%), while in *Bacon* its distribution was more evenly shared between seed (41.6%) and pulp (36.4%). The proportion detected in the peel remained comparable between both cultivars (22.0% in *Bacon* and 27.2% in *Fuerte*).

Regarding metabolites enriched in the peel, *Bacon* showed the highest proportions of persenone A and its isomer together with persin and its isomers I and II, with percentages ranging from 35.1% to 49.8%. In *Fuerte*, however, only persin and its isomer I were predominantly located in the peel (46.5% and 49.8%). Although persin and persin isomer I exhibited similar distribution patterns in both cultivars, persin isomer II followed a distinct trend. In *Bacon*, this compound was mainly distributed between pulp (47.7%) and peel (39.6%), with a lower proportion in the seed (12.6%). In contrast, in *Fuerte* it showed a higher proportion in the seed (37%) and a lower presence in the peel (25.5%). Compound 14 (tentatively characterised as a trihydroxynonadecadiene) also showed cultivar-dependent differences, being mainly located in the seed in *Fuerte* (59.9%), whereas in *Bacon* it was more abundant in the pulp (54.1%).

As noted above, *Hass* displayed a clearly distinct distribution pattern compared with *Bacon* and *Fuerte*. In this cultivar, most acetogenins were predominantly partitioned in the seed, representing approximately 60–100% of their total distribution across tissues (with the exceptions mentioned above). In particular, AcO-avocadenyne and its isomers were detected exclusively in the seed and were completely absent from the peel and pulp, a distribution previously reported in the literature (Rodríguez-López et al., 2015, 2017; Rodríguez-Sánchez et al., 2019; Salinas-Salazar et al., 2017). This distinctive distribution may therefore serve as a metabolic feature distinguishing *Hass* from the other cultivars.

In contrast, persin and its isomer I were predominantly located in the pulp of *Hass*, representing 76.5% and 60.0% of their total distribution, respectively. Although absolute concentrations are not directly comparable, this distribution pattern is consistent with previous studies (Rodríguez-López et al., 2015; Rodríguez-Sánchez et al., 2019; Salinas-Salazar et al., 2017). Similarly, 2,4-dihydroxynonadecyl acetate was mainly detected in the pulp of *Hass* (66.8%), following a distribution pattern comparable to that observed in *Bacon* (49.8%) and *Fuerte* (49.0%). Compound 13 (tentatively annotated as a trihydroxyheneicosatriene) also showed a strong predominance in the pulp, accounting for 70.2% of its total abundance.

Consistent with previous reports (Rodríguez-López et al., 2015), the peel represented the tissue with the lowest acetogenin abundance in *Hass*. Several metabolites were completely absent from this tissue, including AcO-avocadyne isomer I, AcO-avocadenyne and its isomers, AcO-avocadiene B, persenone C isomer, avocadyne and avocadenol A. The metabolites detected in the peel generally accounted for only 0.1–5% of their total distribution, with a few notable exceptions: persin isomer I (12.9%), persin (18.9%) and 2,4-dihydroxyheptadecyl acetate

(33.6%).

The preferential accumulation of acetogenins in avocado seeds may reflect their proposed defensive role in plant tissues. Acetogenins have been reported to exhibit antifungal, antibacterial and insecticidal activities (Kobiler et al., 1993; Rodríguez-Saona & Trumble, 2000a), suggesting that their presence in reproductive tissues may contribute to protecting the embryo against microbial infection or herbivory organisms during fruit development and seed dispersal. The lower concentrations observed in pulp and peel may therefore reflect differences in physiological function among fruit tissues, with seeds acting as a primary reservoir of these specialized metabolites.

From a biosynthetic perspective, avocado acetogenins are believed to derive from long-chain fatty acid precursors (primarily oleic, linoleic, linolenic, and stearic acids) through sequential oxidative modifications including hydroxylation and acetylation reactions (Rodríguez-Saona & Trumble, 2000b). This biosynthetic origin is consistent with the structural diversity observed in the present dataset, where compounds of varying chain lengths (C19–C23), degrees of unsaturation, and hydroxylation patterns likely reflect the broad substrate diversity of the fatty acid biosynthetic machinery involved. The preferential accumulation of acetogenins in seed tissue, particularly evident in the *Hass* cultivar, aligns with their proposed synthesis in idioblast oil cells during early stages of fruit development (Rodríguez-López et al., 2017; Rodríguez-Saona & Trumble, 2000b), contributing to the defensive function attributed to these compounds against pathogens and herbivores (Kobiler et al., 1993; Rodríguez-Saona & Trumble, 2000a). The cultivar-dependent differences in tissue distribution (most notably the near-exclusive accumulation of several acetylated derivatives in *Hass* seed compared to their broader distribution across tissues in *Bacon* and *Fuerte*) may potentially reflect genotype-specific differences in the regulation of biosynthetic pathways or in post-synthesis intercellular transport capacity, aspects that would require targeted transcriptomic and enzymatic studies to elucidate. Finally, the stability of acetogenin concentrations observed across the postharvest ripening stages analysed in this study suggests the absence of active metabolic turnover or ripening-induced regulation during this period, which is consistent with the view that the main accumulation of these metabolites occurs during pre-harvest stages of fruit development (Rodríguez-López et al., 2017).

Taken together, these results reveal a strong cultivar-dependent influence on the tissue-specific distribution of acetogenins in avocado fruits. *Bacon* and *Fuerte* exhibited closely aligned distribution profiles, suggesting a shared metabolic framework, whereas *Hass* displayed a markedly distinct pattern characterised by a pronounced accumulation of acetogenins in the seed and the exclusive occurrence of several compounds in this tissue. These findings not only expand current knowledge of chemical variability among avocado cultivars but also highlight the relevance of tissue selection when exploring avocado by-products as potential sources of bioactive acetogenins. In particular, the high concentrations observed in seeds reinforce their potential value as a promising raw material for the recovery and valorisation of these specialized metabolites.

3.5. Multivariate statistical analysis of tissue-specific metabolite profiles for avocado cultivar discrimination

Following the quantitative evaluation of acetogenin distribution across tissues and cultivars, multivariate statistics were applied to the semi-quantitative acetogenin concentration data to assess the potential of these metabolite profiles for cultivar discrimination. Because a large number of samples were included in the analysis, each tissue type (seed, peel and pulp) was analysed separately in order to facilitate interpretation and improve the visualisation of cultivar-related metabolic differences.

3.5.1. Unsupervised exploration of cultivar-related metabolic variation

PCA was firstly applied to explore the main sources of variance in the

dataset. PCA score and loading plots for each tissue are presented in Fig. 2. Score plots show sample clustering, while loading plots indicate the metabolites responsible for sample separation. Model robustness was evaluated by cross-validation, and the resulting Q^2 values supported the reliability of the observed clustering patterns.

3.5.1.1. Seed. For the seed dataset (Fig. 2A), the PCA model was constructed using six principal components and yielded a cumulative R^2X of 0.815 and a Q^2 value of 0.405, indicating a satisfactory model fit and acceptable model robustness. The first two principal components explained 42.6% and 10.2% of the total variance, respectively.

The score plot (Figure 2A1) showed a clear tendency towards separation between *Hass* and the other two cultivars along PC1, indicating that this component captures the primary metabolic differences distinguishing *Hass* seed samples. In contrast, *Bacon* and *Fuerte* exhibited partial overlap but were mainly differentiated along PC2, suggesting more subtle compositional differences between these cultivars.

The loading plot (Figure 2A2) supports this interpretation. Most metabolites were located in the left region of the plot, corresponding to the area where *Hass* samples clustered, indicating that their relative abundance characterizes the distinct metabolic profile of this cultivar. In contrast, AcO-avocadenyne isomer I, AcO-avocadenyne, and 2,4-dihydroxyheptadecyl acetate were positioned in the upper right quadrant, associated with *Bacon* samples. Meanwhile, avocadenol A, compound 14 (putatively annotated as a trihydroxynonadecadiene), and 2,4-dihydroxynonadecyl acetate were located in the lower right quadrant, corresponding to *Fuerte* samples. Additionally, AcO-avocadenyne isomer II and AcO-avocadiene B appeared in the overlapping region of the loading plot, consistent with their similar concentrations in *Bacon* and *Fuerte*. Overall, these results indicate that the metabolic profile of *Hass* seed is clearly distinct, whereas *Bacon* and *Fuerte* exhibit greater similarity with only a limited number of metabolites driving the differences between them.

3.5.1.2. Peel. The peel dataset produced the most clearly structured PCA model (Fig. 2B). The model, based on five principal components, showed excellent performance with $R^2X = 0.963$ and $Q^2 = 0.906$, indicating both strong model fit and high predictive relevance. The first two components explained 65.6% and 21.1% of the total variance, respectively.

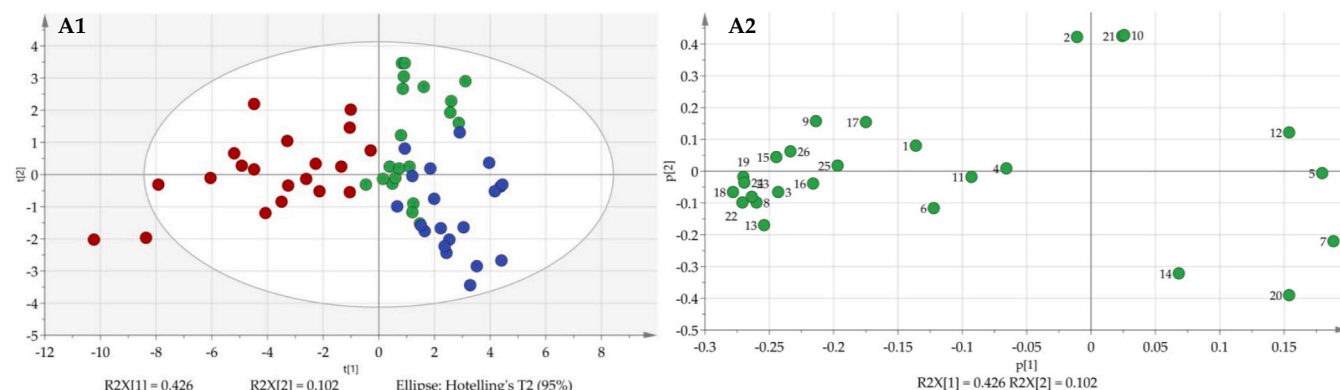
In the score plot (Fig. 2B1), *Hass* samples were predominantly located in the negative region of PC1, whereas *Bacon* and most *Fuerte* samples tended to lie in the positive region of this component. Furthermore, PC2 separated *Bacon* and *Fuerte* samples, with *Bacon* mainly located in the negative region and *Fuerte* in the positive quadrants of PC2. This pattern indicates that PC1 primarily drives the clustering of *Hass* away from the other cultivars, while PC2 captures the metabolic differences between *Bacon* and *Fuerte*.

The loading plot (Fig. 2B2) further clarifies the variables responsible for this separation. Most metabolites were distributed along PC2, suggesting that this component plays a key role in separating *Bacon* and *Fuerte* peel samples. In particular, AcO-avocadenyne, persenone C, and persenone A (together with their respective isomers) contributed strongly to the clustering of *Bacon*, whereas persadiene and persin isomer I were associated with the separation of *Fuerte* samples.

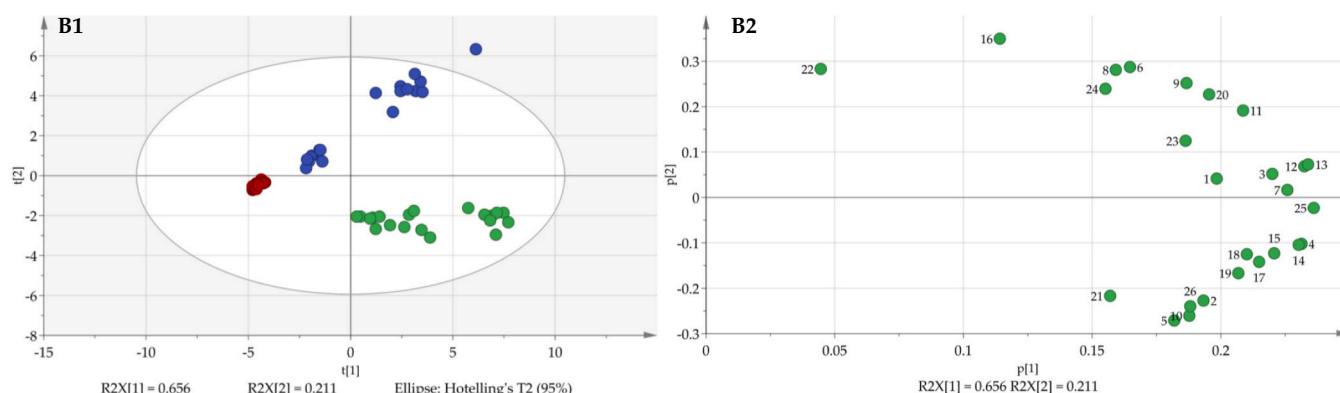
3.5.1.3. Pulp. For the pulp samples, PCA also revealed a clear cultivar-related pattern (Fig. 2C). The model was built with three principal components and achieved $R^2X = 0.857$ and $Q^2 = 0.782$, indicating good model robustness. The first two principal components explained 57.8% and 21.3% of the variance, respectively.

In the score plot (Fig. 2C1), *Bacon* samples were mainly distributed in the positive region of PC1, while *Hass* samples clustered in the negative region of both PC1 and PC2. *Fuerte* samples occupied intermediate positions but tended to appear in the positive region of PC2, indicating that

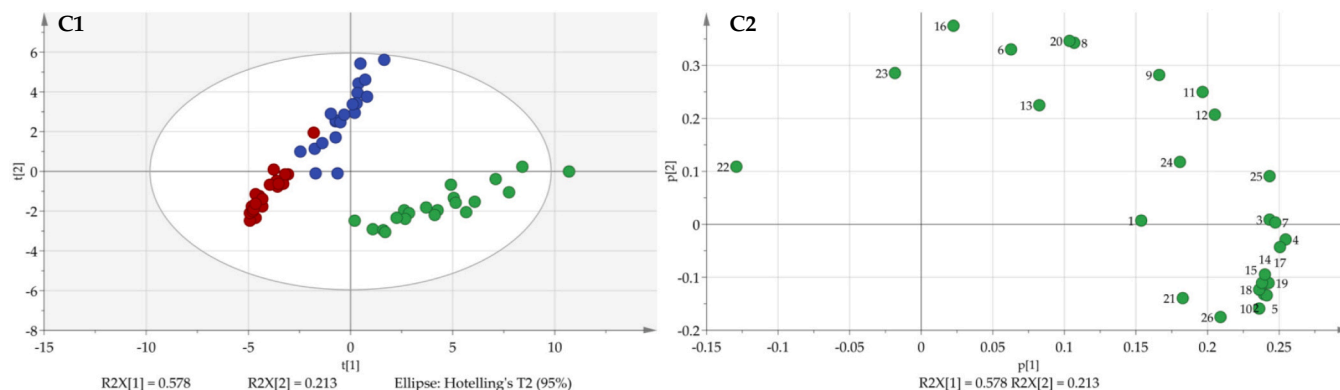
A) Seed



B) Peel



C) Pulp



■ Hass ■ Fuerte ■ Bacon

Fig. 2. PCA score plots (left) and loading plots (right) showing the first two principal components for each avocado tissue: (A) seed, (B) peel, and (C) pulp. Compound numbering corresponds to Table 1.

this component contributes to their separation from the other cultivars. These results suggest that PC1 mainly splits *Bacon* from the other cultivars, whereas PC2 contributes to distinguishing *Fuerte* from *Hass* and *Bacon*.

The loading plot (Fig. 2C2) shows that several metabolites contributing to the separation of *Bacon* and *Fuerte* in the peel also played an important role in clustering these cultivars in the pulp, highlighting their consistent contribution to cultivar differentiation across tissues. Interestingly, no variable was strongly associated with the negative region of PC1, where most *Hass* samples were located. This observation indicates

that the separation of *Hass* is not driven by a single dominant metabolite but rather by collective differences in the relative abundance of multiple acetogenins.

3.5.2. Supervised discrimination of avocado cultivars

To further evaluate the cultivar-related differences suggested by the unsupervised PCA models, supervised PLS-DA analyses were performed independently for seed, peel, and pulp samples. The corresponding score plots are presented in Fig. S4. Seed model showed satisfactory performance, with R^2X , R^2Y , and Q^2 values of 0.608, 0.803, and 0.764,

respectively. Similarly, peel model yielded R^2X , R^2Y , and Q^2 values of 0.590, 0.958, and 0.928, respectively, indicating strong explanatory and predictive capabilities. Pulp model showed the highest R^2X and Q^2 values, with R^2X , R^2Y , and Q^2 values of 0.891, 0.948, and 0.935, respectively. To further validate the models and rule out overfitting, permutation tests ($n = 200$ permutations) were performed for each tissue model. In all cases, the R^2Y and Q^2 values of the permuted models were substantially lower than those of the original models, and the Q^2 intercept values were consistently below zero [seed: R^2Y intercept = 0.123, Q^2 intercept = -0.314; peel: R^2Y intercept = 0.147, Q^2 intercept = -0.506; pulp: R^2Y intercept = 0.073, Q^2 intercept = -0.357], confirming that the observed cultivar-related patterns were not attributable to chance. The PLS-DA results were consistent with the trends observed in the PCA analysis, reinforcing the presence of cultivar-dependent differences in acetogenin composition across tissues.

Variable importance in projection (VIP) analysis was subsequently used to highlight the metabolites contributing most strongly to cultivar differentiation, with variables showing VIP values >1 considered relevant contributors (Table S3). In seed samples, the variables with the highest VIP scores were mainly represented by C19 metabolites, including AcO-avocadene, AcO-avocadyne, AcO-avocadenyne, avocadyne, and 2,4-dihydroxyheptadecyl acetate, together with persin isomer I, persenone A isomer and persin isomer II. The colour-scaled abundance patterns revealed cultivar-specific differences among these discriminant metabolites. Specifically, the acetylated C19 acetogenins were generally more abundant in *Bacon*, whereas 2,4-dihydroxyheptadecyl acetate showed higher levels in *Fuerte*. In contrast, *Hass* was characterised by higher relative abundances of avocadyne and C23-related metabolites. Regarding peel tissue, cultivar differentiation was determined by multiple discriminant acetogenins, including C21–C23 compounds and several acetylated derivatives. Specifically, *Fuerte* showed higher relative abundances of persin, persin isomer I, persediene, AcO-avocadyne isomer II, 2,4-dihydroxyheptadecyl acetate, avocadene, persenone B isomer, and AcO-avocadene isomer. In contrast, *Bacon* was mainly associated with higher levels of persenone A, AcO-avocadenyne and its isomer, persenone C, and persin isomer II, whereas *Hass* generally exhibited lower relative abundances for most peel VIP metabolites. For pulp samples, the variables with the highest VIP scores were predominantly C19 acetylated acetogenins, including AcO-avocadene, AcO-avocadene isomer, AcO-avocadyne isomer II, and AcO-avocadiene B, together with 2,4-dihydroxynonadecyl acetate and persediene. The colour-scaled abundance patterns revealed a remarkably consistent cultivar-dependent trend: most VIP compounds showed their highest relative abundances in *Fuerte*, intermediate levels in *Bacon*, and the lowest abundances in *Hass*, indicating that pulp differentiation was largely driven by quantitative differences in the accumulation of these acetogenins. These findings are consistent with the PCA loading plots and further support the contribution of tissue-specific acetogenin composition to cultivar differentiation.

To further characterise the magnitude of the observed inter-cultivar differences, fold changes were calculated for all 26 acetogenin-related metabolites and are reported in Supplementary Table S3 (Section B). In peel, the most pronounced differences were observed relative to *Hass*, with fold changes exceeding $60\times$ for several acetylated C19 derivatives and up to $293\times$ for persenone C (*Bacon/Hass*), consistent with the near-complete absence of these compounds in *Hass* peel. In pulp, AcO-avocadiene B showed the largest inter-cultivar differences among VIP metabolites, with fold changes of up to $66\times$ relative to *Hass*. The compound with the highest overall fold change in pulp was avocadenol A (up to $63\times$), although this metabolite did not reach the $VIP > 1$ threshold in this tissue. In seed, differences were comparatively more moderate, with persenone A isomer, persin isomer I, and persin isomer II showing the highest fold changes among the VIP metabolites, all three exhibiting higher concentrations in *Hass* relative to *Bacon* and *Fuerte* (up to $4.5\times$).

Taken together, these results indicate that acetogenin profiles constitute informative chemical descriptors for the classification of

avocado cultivars across all analysed tissues. However, the degree of cultivar-related separation varies among tissues. Peel samples provided the clearest cultivar separation, whereas seed samples showed partial overlap between *Bacon* and *Fuerte*, reflecting their more similar acetogenin composition. In contrast, *Hass* exhibited a consistently differentiated metabolic profile across all tissues. These findings suggest that cultivar genetic background exerts an important influence on acetogenin composition and indicate that tissue-specific metabolite profiles carry meaningful cultivar-discriminating information. Moreover, the observed tissue-dependent distribution patterns reinforce the relevance of avocado by-products, particularly seeds, as consistent and promising sources of acetogenins for future valorisation strategies.

4. Conclusion

This study provides a comprehensive characterisation of acetogenin-related metabolites in avocado fruits using an optimised LC–MS workflow complemented by IMS. The method enabled robust semi-quantitative analysis of 26 metabolites across peel, pulp and seed tissues from three cultivars. The incorporation of IMS allowed the generation of the first experimental database of CCS values for avocado acetogenins, improving confidence in metabolite annotation and providing complementary structural information for structurally related compounds.

The results revealed a pronounced tissue-dependent distribution of acetogenins, with seeds and pulp acting as the main reservoir of these metabolites, while peel exhibited lower concentrations. In contrast, acetogenin levels remained largely stable across ripening stages, indicating a stable acetogenin composition throughout the postharvest ripening period analysed. This stability supports the use of avocado by-products as a reliable source of acetogenins, independent of the processing stage.

Cultivar-dependent differences were also observed. *Hass* exhibited a distinct metabolic profile characterised by strong partitioning of acetogenins in the seed and specific tissue-dependent patterns, whereas *Bacon* and *Fuerte* showed broadly similar profiles with more balanced distributions. These compositional differences were supported by PCA and PLS-DA, with peel providing the clearest cultivar-related separation and seed showing partial overlap between *Bacon* and *Fuerte*.

Beyond cultivar characterisation, the results highlight the potential of tissue-specific acetogenin profiles as informative chemical descriptors for avocado cultivar characterisation, and reinforce the value of avocado by-products as sources of bioactive compounds. In particular, the high and stable concentrations observed in seeds emphasise their potential for the recovery and valorisation of these metabolites. Overall, this work expands current knowledge of avocado chemical diversity and demonstrates the applicability of LC–IMS–MS-based metabolite profiling for the study of structurally complex natural products in food matrices. The analytical and compositional insights provided here establish a solid basis for future research on acetogenin bioactivity and the sustainable exploitation of avocado-derived residues.

CRediT authorship contribution statement

María Gemma Beiro-Valenzuela: Writing – original draft, Methodology, Investigation, Formal analysis. **Lucía Olmo-García:** Writing – review & editing, Formal analysis, Conceptualization. **Sara Rodríguez-Rodríguez:** Investigation, Formal analysis. **Aiub Mohamed-Barara:** Investigation, Formal analysis. **Romina Monasterio:** Writing – review & editing, Methodology, Investigation. **Elena Hurtado-Fernández:** Methodology, Investigation. **Carmen M. Sánchez-Arévalo:** Writing – review & editing, Formal analysis. **Romina Pedreschi:** Funding acquisition, Conceptualization. **Alegría Carrasco-Pancorbo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2026.150516>.

Data availability

Data will be made available on request.

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