Multiplatform Metabolomics for the Design and Characterization of a Mediterranean Plant-Based Lyophilized Extract from Agro-Industrial By-Products

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Article

Multiplatform Metabolomics for the Design and Characterization of a Mediterranean Plant-Based Lyophilized Extract from Agro-Industrial By-Products

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Abstract

Agri-food industries generate substantial quantities of side streams such as peels, pods, seeds, and leaves. Traditionally regarded as waste, these by-products are now recognized as rich sources of bioactive compounds—often at higher concentrations than those found in edible plant parts. Their recovery reduces environmental impact and enables the development of sustainable ingredients for food and health-related applications, in line with circular economy principles. This study presents the design and metabolomic characterization of a novel lyophilized extract derived from Mediterranean and locally cultivated plant-based by-products (named BIOMEDEX), including orange, lemon, olive leaves, carob pods, shiitake mushroom, and salicornia. A multiplatform metabolomics approach was applied, combining high-resolution UPLC-QTOF-MS, UHPLC-QTRAP-MS, SPME-GC-MS, and ^{1}H-NMR spectroscopy to comprehensively profile phytochemicals, nutrients, and volatile organic compounds (VOCs). The extract was found to be rich in flavonoids (e.g., luteolin-7-O-glucoside, hesperidin, eriocitrin), phenolic acids, amino acids (e.g., proline, GABA), organic acids (e.g., malic and citric acid), and over 40 VOCs associated with antioxidant and sensory functions. Notably, complementary enrichment of these compounds suggest potential health-promoting properties. These findings support the formulation of a multifunctional plant-based ingredient and reinforce the value of integrating diverse agro-industrial by-products into sustainable, health-oriented food solutions.

Keywords: plant-based by-products; Mediterranean lyophilized-extract; phytochemicals; nutrients; VOCs; UPLC-QTOF-MS; UHPLC-QTRAP-MS; ^{1}H-NMR spectroscopy; SPE-GC-MS

1. Introduction

The increasing demand for sustainable and functional ingredients has driven both the scientific community and the food industry to explore the valorization of fruit and vegetable by-products. These plant-based materials, often discarded during industrial processing, are rich in bioactive compounds—particularly phenolic metabolites—which have been associated with antioxidant, anti-inflammatory, and antimicrobial activities beneficial to human health [1,2]. Transforming these residues into functional ingredients not only enhances the nutritional value of food products but also aligns with circular economy strategies aimed at minimizing environmental impact [3,4]. Among the most widely generated by-products are those from citrus fruits (Citrus spp.), with global production exceeding 90 million tons annually. Nearly one-third of this yield is processed into juice, resulting in

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massive volumes of peel and pulp residues [5]. These residues are rich in flavanones such as hesperidin, eriocitrin, and narirutin (20–50 mg/g d.w), as well as flavones, flavonols, and polymethoxylated flavones—compounds linked to cardioprotective, anti-obesity, and antiviral effects [6–9]. Similarly, olive leaves are notable for their high concentrations of oleuropein, hydroxytyrosol, and verbascoside (8–15 mg/g d.w.)—potent antioxidants with applications in food supplements and nutraceuticals [10]. Carob pods (Ceratonia siliqua L.), often underutilized, contain significant levels of gallic acid (3–6 mg/g), catechins, and quercetin derivatives, contributing to their antioxidant and antimicrobial potential. Carob is also being promoted as a caffeine-free alternative to cocoa in functional formulations [11,12]. Mushroom cultivation, especially from species like shiitake (Lentinula edodes), generates bioresidues rich in polysaccharides and phenolic acids (8–11 mg/g) with immunomodulatory and antioxidant properties [13,14]. Furthermore, halophytic plants such as Salicornia europaea have recently gained attention due to their unique phenolic profile, mineral content, and traditional use in managing metabolic and inflammatory conditions [15]. Collectively, these by-products not only offer a wide phytochemical diversity but also represent a considerable portion of food processing waste, with citrus processing alone generating over 15 million tons of waste annually in the Mediterranean region [5]. This individual nutritional and functional richness supports their strategic combination into a single multifunctional extract with improved potential.

Despite the promising individual profiles of these by-products, their combination remains underexplored. Integrating residues from orange, lemon, olive **leaves**, carob **pods**, mushroom, and salicornia could result in **enhanced** bioactivity, offering enhanced health benefits compared to single-source extracts. Unlocking this potential requires advanced analytical tools capable of comprehensive metabolite profiling. To this end, state-of-the-art metabolomics platforms—such as high-resolution UPLC-QTOF-MS, high-sensitivity UHPLC-QTRAP-MS, ^{1}H-NMR, and SPME-GC-MS—are essential for accurate qualitative and quantitative characterization of complex phytochemical and nutrient profiles. Their combined application ensures accurate characterization of the chemical diversity in novel extracts, which is essential for reproducibility, safety assessment, and regulatory acceptance in functional foods [16,17]

The objectives of this study were threefold:

(i) to design a novel multifunctional lyophilized extract from six Mediterranean plant-based by-products (orange, lemon, olive leaves, carob pods, shiitake mushroom, and salicornia);

(ii) to perform a detailed metabolomic profiling using complementary analytical platforms; and

(iii) to evaluate the diversity of phytochemicals, nutrients, and volatile organic compounds (VOCs), as well as their potential functional relevance in the context of sustainable food systems.

2. Materials and Methods

2.1. Reagents

Analytical HPLC-MS-grade chemicals, including water with 0.1% formic acid, acetonitrile (ACN), dimethyl sulfoxide (DMSO), and methanol (MeOH), were obtained from J.T. Baker (Deventer, The Netherlands). The following 38 pure standards were purchased from Extrasynthese (Genay, France): Catechin, Gallocatechin, Procyanidin dimer B1, Theaflavin, Didymin, Eriocitrin, Eriodictyol, Hesperetin, Hesperidin, Naringenin, Neoeriocitrin, Neohesperidin, Poncirin, Apigenin, 6-Hydroxyluteolin, Chrysin, Luteolin, Nobiletin, Sinensetin, Tangeretin, Isorhamnetin, Kaempferol, Myricetin, Quercetin, Rhamnetin, Daidzein, Daidzin, 2,3-Dihydroxybenzoic acid, 2-Hydroxybenzoic acid, Benzoic acid, Ellagic acid, Gallic acid, Vanillic acid, Caffeic acid, Ferulic acid, p-Coumaric acid, Rosmarinic acid and Sinapic acid.

2.2. Designing a New Lyophilized Extract (BIOMEDEX)

Four Mediterranean plant-based by-products from Spain, orange (Citrus sinensis L.), lemon (Citrus limon L.), shiitake mushroom (Lentinula edodes L.) and salicornia (Salicornia europaea L.), and two from Italy, olive leaves (Olea europaea L.) and carob pods (Ceratonia siliqua L.), were frozen

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at –80 °C and lyophilized using a [Telstar/lyoquest-55] freeze-dryer under –85 °C and 0.1 mbar for 3 days to design an extract enriched in phytochemicals, nutrients and organic volatile compounds (VOCs). Spanish by-products were provided by a local company (Agrosingularity, Murcia, Spain) meanwhile Italian by-products were provided by Department of Agricultural, Food and Forestry Sciences, University of Palermo. This new designed lyophilized-extract was named BIOMEDEX (BIOactive MEDiterranean EXtract) and it is patent pending to be commercialized as a food supplement by the company.

Each single by-product was individually analyzed by the multiple analytical platforms and the final composition (percentage) of the lyophilized extract was based on the interest of reaching the higher number and concentration of phytochemicals, nutrients and VOCs. Thus, the final mixed composition of the lyophilized extract was the following: olive leaves (30 %), orange (20 %), lemon (20 %), carob pods (10 %), salicornia (10 %) and shiitake mushroom (10 %). A final weight of 300 mg of the lyophilized extract was selected for filling sized - 1 hard gelatin capsules supplied by Blendhub S.L. (Murcia, Spain), following the European Union's good manufacturing practices. Each capsule with the final mixed extract contained more than 150 mg of bioactive phytochemical compounds.

2.3. Extraction of the Phytochemicals Present in BIOMEDEX

For both UPLC–QTOF-MS and UHPLC–QTRAP-MS analysis, 50 mg of the lyophilized BIOMEDEX (per triplicate) were weighed and extracted with 500 µL of MeOH:H_2O (80 : 20) containing 0.1 % formic acid (Merck, Germany). The mixture was vortexed (Cole Parmer, USA) and sonicated in a bath (Merck, Germany) for three 30-second intervals, and then supernatants were filtered through a 0.22 µm PVDF filters (Millipore, Germany) before being analyzed.

2.4. UPLC–QTOF-MS Untargeted Analysis of Phytochemicals

Untargeted analysis was performed using a Waters ACQUITY UPLC I-Class System (Waters Corporation, Milford, MA, USA) coupled to a Bruker Daltonics Quadrupole Time of Flight mass spectrometer (maXis Impact Series, resolution ≥ 55,000 FWHM, Bruker Daltonics, Bremen, Germany). Electrospray ionization (ESI) was used in both positive [ESI (+)] and negative [ESI (−)] ionization modes. UPLC separation was conducted using a Poroshell 120 Bonus-RP C18 column 3.0 × 100 mm, 2.7 µm, Agilent (Waldbronn, Germany) at a flow rate of 0.3 mL/min. The mobile phases were: (A) H_2O with formic acid at 0.1 % (pH ~ 3.20, PanReac AppliChem, Barcelona, Spain), and (B) acetonitrile (ACN) with 0.1 % formic acid (J.T. Baker, New Jersey, USA). The gradient started with 1 % of phase B at 0 min, increasing linearly to 18 % at 10 min and to 38 and 95 % at 16 and 38 min, where it decreased to 1 % held until the end of the chromatogram. Nitrogen was used as both the desolvation gas (flux: 9 L/min) and nebulizing gas (pressure: 2.0 bar). The desolvation temperature was set to 200 °C, and the column temperature was maintained at 40 °C. The voltage applied to the ESI source was 4.5 kV for ESI (+). The MS experiments were conducted using high-resolution QTOF-MS with broadband collision-induced dissociation (+bbCID). The collision energy was set to 24 eV for ESI (+). MS data were acquired over a m/z range of 50–1200 Da. External calibration was performed prior to each sequence using a 10 mM sodium formate solution, which was delivered by a KNAUER Smartline Pump 100 with a pressure sensor (KNAUER, Berlin, Germany). The calibration mixture was prepared by adding 0.5 mL of formic acid and 1.0 mL of 1.0 M sodium hydroxide to an isopropanol/Milli-Q water solution (1:1, v/v).

2.5. UHPLC–QTRAP-MS Targeted Analysis of Phytochemicals

The analysis of targeted phytochemicals was carried out using a 1290 Infinity II UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a hybrid Triple Quadrupole-Ion Trap mass spectrometer 6500+ (Sciex, Wilmington, DE, USA) A Poroshell 120 Bonus-RP C18 column 3.0 × 100mm, 2.7 µm, Agilent (Waldbronn, Germany) was used for separation. A sample volume of 2 µL was injected at a flow rate of 0.4 mL/min. The mobile phases used consisted of 0.1 % formic acid in

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LC-MS grade water (solvent A) and acetonitrile (solvent B). Chromatographic separation was performed using the same above gradient conditions already described for untargeted analysis. The turbo ion spray interface was operated in the polarity switching mode (ESI +/-). The ionization source parameters were: curtain gas: 35 pound per square inch (psi); CAD gas: 9 psi; ion spray voltage (+/-): 5500 V/4500 V; temperature: 400 ◦C; gas 1: 60 psi; gas 2: 60 psi. Total acquisition time was 14 min. The analysis was performed in multiple reaction monitoring (MRM) using the Scheduled MRM™ algorithm, with a time window of 60s. Two transitions per compound were monitored, one quantification (SRM 1) and one confirmation (SRM 2). Sciex OS2.2.0.5738 software was used for data acquisition and quantification (Sciex, Wilmington, DE, USA). The limit of quantification (LOQ) ranged from 10-10000 ng/L. Acceptable mean recoveries were considered in the range 70–120 %, with an associated RSD ≤ 20 %. Taking into account the large number of phytochemicals under study and their diverse physicochemical properties, recovery results were considered satisfactory. Intra-day precision was evaluated (n = 3) indicating that 85 % of the compounds showed adequate precision values with an associated RSD ≤ 20 %.

2.6. ^{1}H-NMR Analysis of Nutrients and Primary Metabolites

A targeted metabolic analysis was performed following the protocol outlined by Vander Sar et al. (2013) [18]. BIOMEDEX samples (n=3) were analyzed using a 500 MHz Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a broadband 5 mm N_2 CryoProbe Prodigy BBO. Samples were measured at 298 ± 0.1 K without rotation, with 4 test scans followed by 32 scans for the experiment. The acquisition parameters were set as follows: FID size = 64 K, spectral band = 12.4345 ppm, receiver gain = 28.5, acquisition time = 2.18 s, relaxation delay = 2 s, and line broadening = 0.50 Hz. Data acquisition was conducted using the NOESY pulse sequence with pre-saturation (Bruker 1D, noesypr1d), with water suppression through irradiation of the water frequency during recycling and mixing times. During sample processing, noise reduction was applied through multi-level signal deconvolution for each spectrum. This was followed by baseline correction, and area interpolation techniques were used to quantify the signal. These steps resulted in a fingerprint of the sample, providing a comprehensive view of the most abundant metabolites, with chemical shifts (δ) expressed in parts per million (ppm). The NMR system records the signals as frequency vs. intensity graphs, also referred to as the acquisition spectrum. The resulting ^{1}H-NMR spectra were processed using Chenomx NMR Suite software (version 10.0, Chenomx, Edmonton, Canada) for metabolite identification and quantification. Samples were calibrated using the internal standard (TSP-d4), deuterated Trimethylsilylpropionic acid sodium salt, and the pH was adjusted to approximately 6. The software allows for detection and quantification of metabolites at concentrations greater than 10 μM.

2.7. SPME-GC–MS Analysis of VOCs Profile

Solid-phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) was used for the extraction and analysis of volatile compounds in the plant-based formula. The content of one capsule (300 mg) were transferred into 20 mL vials and placed onto an autosampling tray (Gerstel MPS Dual Head RobPro/Rob 160 cm, Mellinghofen, Germany). Samples were analyzed per triplicate.

Gas chromatography was performed using an HP 8890B gas chromatograph coupled to an HP 5977B quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Prior to extraction, samples were equilibrated at room temperature (25 °C) for 30 min. For the SPME procedure, a DVB/Carboxen/PDMS 50/30 stable flex-gray fiber was used (Supelco, Bellefonte, PA, USA). The fiber was baked at 50 °C for 20 min before being exposed to the headspace of the vials for 10 min. After exposure, the fiber was transferred to the GC injector for thermal desorption at 250 °C for 3 min, with a split ratio of 1:10 and an ultra-inert liner (Agilent Technologies). A VF-WAXms analytical column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies) was used with the following temperature program: 40 °C for 5 min, ramping at 4 °C min^{-1} to 225 °C, with a final time of 51,25 min. Mass selective detection

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was carried out in scan mode (30–500 amu, EI 70 eV) with a detector temperature of 230 °C and a scan rate of 1.6 scans/s, with a cycle time of 622.9 ms. Data were processed using the Mass Hunter Qualitative Analysis software (version B.10.00, Service Pack 1, Agilent Technologies). Compounds identification were achieved combining both compound discovery "molecular feature" and "deconvolution" algorithms, which selected peaks with an absolute height ≥5000 counts from both GC and MS spectra. Tentative compound identification was performed by comparing the mass spectra to those in the Wiley11N17 and NIST20 libraries (Wiley, Chichester, UK) with a matching score of ≥ 80 % and comparing retention indices with those reported in the literature.

2.8. Data Treatment

In the experiment, the plant-based extract was analyzed per triplicate for each metabolomics platform analysis. Standard deviation for each metabolite was calculated and reflected in the tables. As it was previously reported by Vallejo et al. [19] the VOCs data matrix, feature extraction, deconvolution and alignment was carried out on Agilent Profinder B.06.00 (Agilent technologies, Waldbronn, Germany). Pre-processed CEF files were exported into the Mass Profiler Professional (MPP) software package (revision B.14.09.01, Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

3. Results and Discussion

3.1. Phytochemical Profiling: Integration of High-Resolution and High-Sensitivity Platforms

BIOMEDEX extract (300 mg) obtained from orange, lemon, olive leaves, carob pods, shiitake mushroom, and salicornia by-products exhibited a complex and rich phytochemical profile. Combining data from high-resolution UPLC-QTOF and high-sensitivity UHPLC-QTRAP platforms enabled both qualitative and quantitative identification of phenolic compounds, confirming the presence of 38 metabolites. Among the most abundant constituents were luteolin-7-O-glucoside (45.1 mg/300 mg), hesperidin (34.2 mg/300 mg), eriocitrin (24.2 mg/300 mg), oleuropein (10.8 mg/300 mg) and nobiletin (10.7 mg/300 mg). These levels are notably higher than typical concentrations reported in single-source citrus matrices, suggesting a cumulative enrichment effect due to the multi-matrix formulation [20–22]. Hesperidin, a major flavanone in citrus peels, is well-documented for its antioxidant and vasoprotective effects [23,24]. Eriocitrin, predominant in lemon tissues, demonstrated high radical scavenging capacity and is considered a marker compound for nutraceutical use [24,25]. The significant presence of luteolin-7-O-glucoside, a known anti-inflammatory flavone, further supports the potential bioactivity of the extract [26]. Notably, polymethoxylated flavones (PMFs) such as nobiletin, tangeretin, and sinensetin were detected in high concentrations. PMFs are primarily found in citrus peels and are associated with anti-obesity, neuroprotective, and anticancer activities [26]. Their lipophilic nature complements the water-soluble flavonoid glycosides in the formulation, potentially broadening bioactivity spectra [27]. Moderate amounts of quercetin, rutin, and apigenin were also detected, reflecting the contribution of citrus and carob matrices [25]. The presence of benzoic and p-coumaric acids suggests a secondary contribution from salicornia and shiitake, which are known to contain hydroxybenzoic and hydroxycinnamic acids [1,14]. Interestingly, compounds such as verbascoside (olive leaves) and poncirin (carob pods) were present at low or undetectable levels, likely due to the relative dilution in the final formulation or limitations in extraction efficiency. Nonetheless, the overall phytochemical fingerprint confirms that the extract retained and, in some cases, concentrated bioactive markers from all six input matrices. These results position the extract as a promising candidate for functional food and nutraceutical applications, offering a wide spectrum of bioactive flavonoids, phenolic acids, and PMFs with documented roles in oxidative stress reduction, inflammation modulation, and metabolic health [13–15]. Future studies should assess the extract's bioaccessibility, bioavailability, and potential increasing interactions among its phytochemical constituents.

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Table 1. List of 37 metabolites identified in BIOMEDEX extract combining two analytical platforms: (i) HR high resolution (UPLC-QTOF-MS) and (ii) HS high sensitivity (UHPLC-QTRAP-MS). Samples were analyzed in triplicate.

Number	Metabolites	RT	Polarity	Formula	Experimental m/z	MS/MS Fragment	Concentration (mg/capsule d.w.)	Concentration (mg/g d.w.)	%	Platform
1	Gallic acid*	6,7	Negative	C7H6O5	169.0	124.9	3.0±0.4	9.9±1.4	1.5	HS
2	Gallocatechin*	9,1	Positive	C15H14O7	307.1	139.0	0.4±0.1	1.4±0.3	0.3	HS
3	Catechin*	11,6	Positive	C15H14O6	291.0	139.0	0.8±0.1	2.6±0.3	0.6	HS
4	p-coumaric acid*	11,8	Negative	C9H8O3	163,0	118,9	3.2±0.3	10.6±0.9	1.1	HS
5	Vicenin-2	12,1	Positive	C27H30O15	595,1685	†	-	-	1.3	HR
6	Vanillic acid*	12,2	Negative	C8H8O4	167.1	151.9	0.5±0.1	1.7±0.3	0.2	HS
7	Stellarin-2	12,5	Positive	C28H32O16	625,1784	505,1354	-	-	0.6	HR
8	Caffeic acid*	13,1	Negative	C9H8O4	179.0	135.0	0.5±0.1	1.7±0.3	0.3	HS
9	Rutin*	13,7	Negative	C27H30O16	609,1	301.0	6.5±0.5	21.5±1.7	3.3	HS
10	Eriocitrin*	13,8	Negative	C27H32O15	595.1	286.9	24.2±1.1	79.9±3.6	12.6	HS
11	Verbascoside	14,0	Positive	C29H36O15	625,2122	†	-	-	0.1	HR
12	Orientin	14,1	Positive	C21H20O11	449,1094	413,1847	-	-	2.6	HR
13	Luteolin-7-O-glucoside*	14,2	Negative	C21H20O11	447,0	285,0	45.1±3.1	148.8±10.2	21.7	HS
14	Neoeriocitrin*	14,1	Negative	C27H32O15	595.1	286.9	0.5±0.1	1.7±0.3	0.1	HS
15	Scoparin	14,3	Positive	C22H22O11	463,1239	†	-	-	0.8	HR
16	Rhoifolin	14,5	Positive	C27H30O14	579,1716	379,0961	-	-	1.2	HR
17	Naringin	14,6	Positive	C27H32O14	581,1872	273,0759	-	-	1.4	HR
18	Diosmin	14,9	Positive	C28H32O15	609,1823	301,0707	-	-	0.8	HR
19	Hesperidin*	15,0	Negative	C28H34O15	609,2	301,0	34.2±2.8	112.9±9.2	15.7	HS
20	Vitexin	15,1	Positive	C21H20O10	433,1134	†	-	-	1.5	HR
21	Benzoic acid*	15,2	Negative	C7H6O2	286.9	76.9	3.2±0.2	10.6±0.9	2.0	HS
22	Ellagic acid*	15,4	Negative	C14H6O8	301.2	228.9	0.3±0.1	0.9±0.3	0.1	HS
23	Nomilin	15,6	Positive	C28H34O9	515,2283	†	-	-	0.3	HR
24	Oleuropein	16,1	Positive	C25H32O13	541,1931	379,1389	-	-	4.9	HR
25	Poncirin	16,9	Positive	C28H34O14	595,2031	†	-	-	0.9	HR
26	Quercetin*	17,7	Negative	C15H10O7	301.0	150.9	3.3±0.3	10.9±0.9	0.4	HS
27	Sinensetin*	18,2	Negative	C20H20O7	373.1	312.0	6.8±0.4	22.4±1.3	3.5	HS
28	Naringenin*	18,5	Positive	C15H12O5	273.0	153.0	0.3±0.1	0.9±0.3	0.1	HS
29	Nobiletin*	19,3	Positive	C21H22O8	403.0	373.0	10.7±1.1	35.3±3.3	4.8	HS
30	3,5,7,3′,4′-Pentamethoxyflavone	19,5	Positive	C20H20O7	373,1309	329,1019	-	-	2.0	HR
31	Limonin	19,7	Positive	C26H30O8	471,2021	425,1960	-	-	1.7	HR
32	Isorhamnetin*	19,9	Negative	C16H12O7	315.0	299.9	0.1±0.0	0.3±0.0	0.1	HS
33	Apigenin*	20,1	Positive	C15H10O5	271.0	153.0	1.1±0.1	3.3±0.3	0.5	HS
34	6,7,8,4′-Tetramethoxyflavone	20,2	Positive	C19H18O6	343,1196	†	-	-	5.2	HR

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35	3,5,6,7,8,3′,4′-Heptamethoxyflavone	20,4	Positive	C22H24O9	433,1510	418,1260	-	-	3.2	HR
36	Tangeretin*	20,7	Positive	C20H20O7	373,1	343,1	3.5±0.2	11.6±0.7	2.2	HS
37	Isosakuranetin*	21,5	Positive	C16H14O5	287.0	153.1	3.0±0.3	9.9±0.9	0.4	HS

* Confirmed metabolites with pure standards were quantified by HS platform. ǂ Metabolites without fragments were identified with level 3 according to the metabolomics consortium (Sumner et al., 2007) and with a mSigma value < 50. Metabolites with fragments were tentatively confirmed by exact mass, isotopic pattern, fragments (+bbCID at 24 ev of collision energy), libraries (Metlin, HMDB and CEU Mass Mediator) and bibliography.

The quantitative evaluation of the phenolic compounds in the mixed extract reveals both similarities and deviations from previously reported concentrations in single-source byproducts. Among flavanone glycosides, hesperidin (34.2 mg/300 mg; ~113 mg/g) was particularly abundant, exceeding typical values reported for orange peel extracts (20–50 mg/g) [21,28]. Likewise, eriocitrin (24.2 mg/300 mg; ~80 mg/g) was higher than the range usually found in lemon peel residues (15–40 mg/g) [24,25], while neoeriocitrin (0.5 mg/300 mg; ~1.7 mg/g) and diosmin (1.0 mg/300 mg; ~3.3 mg/g) fell within previously described ranges for lemon tissues [27,29]. Naringin (3.3 mg/300 mg; ~11 mg/g) and isosakuranetin (3.0 mg/300 mg; ~10 mg/g) were present at levels comparable to reported averages in citrus peels [30]. Flavone glycosides also contributed significantly: luteolin-7-O-glucoside (45.1 mg/300 mg; ~150 mg/g) was strikingly higher than the 5–15 mg/g usually reported for citrus matrices [30], while vitexin (3.0 mg/300 mg; ~10 mg/g), orientin (3.5 mg/300 mg; ~12 mg/g), rhoifolin (2.8 mg/300 mg; ~9 mg/g), and scoparin (1.1 mg/300 mg; ~4 mg/g) matched concentrations detected in diverse citrus germplasm collections [30,31]. Apigenin (1.1 mg/300 mg; ~4 mg/g) also aligned with literature values in orange and lemon byproducts [10]. The extract was especially rich in polymethoxylated flavones (PMFs). Nobiletin (10.7 mg/300 mg; ~36 mg/g) and tangeretin (3.5 mg/300 mg; ~12 mg/g) yielded combined values (~48 mg/g) above those typically found in orange peel powders (10–30 mg/g) [27,31]. Additionally, sinensetin (6.8 mg/300 mg; ~23 mg/g), pentamethoxyflavone (11.1 mg/300 mg; ~37 mg/g), tetramethoxyflavone (11.9 mg/300 mg; ~40 mg/g), and heptamethoxyflavone (6.7 mg/300 mg; ~22 mg/g) expanded the PMF profile beyond commonly reported values for single citrus extracts [27]. Among flavonols, rutin (6.5 mg/300 mg; ~22 mg/g) and quercetin (3.3 mg/300 mg; ~11 mg/g) were within the range described for citrus peels and seeds (5–20 mg/g) [32,33], while isorhamnetin (0.1 mg/300 mg; ~0.3 mg/g) appeared in trace levels, similar to reports in salicornia and citrus tissues [18,19]. The secoiridoid oleuropein (10.8 mg/300 mg; ~36 mg/g) was present at higher levels than those previously described in olive leaf extracts (8–15 mg/g) [32,34], confirming the olive contribution. Verbascoside, although present in low concentration (0.1 mg/300 mg; ~0.3 mg/g), is also characteristic of olive byproducts [33]. In terms of phenolic acids, gallic acid (3.0 mg/300 mg; ~10 mg/g), p-coumaric acid (3.2 mg/300 mg; ~11 mg/g), and benzoic acid (3.2 mg/300 mg; ~11 mg/g) were moderately abundant, consistent with carob, shiitake, and salicornia sources [35–38]. Minor levels of caffeic acid (0.5 mg/300 mg; ~1.7 mg/g), vanillic acid (0.5 mg/300 mg; ~1.7 mg/g), and ellagic acid (0.3 mg/300 mg; ~1.0 mg/g) also align with mushroom and halophyte matrices [37,39,40]. Regarding limonoids, limonin (2.9 mg/300 mg; ~10 mg/g) and nomilin (0.5 mg/300 mg; ~1.7 mg/g) were detected at concentrations compatible with citrus seed and peel fractions [21,25]. Overall, these comparisons indicate that the extract not only preserved the typical phytochemical fingerprints of citrus byproducts but also achieved quantitative enrichment of several key compounds (hesperidin, eriocitrin, luteolin-7-O-glucoside, oleuropein and PMFs) beyond levels usually reported in single-source extracts. This enrichment likely reflects accumulation from the combination of diverse plant residues.

3.2. Metabolomics Multiplatform: ^{1}H-NMR Analysis of Nutrients and Primary Metabolites

The targeted ^{1}H-NMR profiling of BIOMEDEX extract revealed a complex matrix of sugars, organic acids, amino acids, and osmoprotectants. The major soluble metabolites identified were

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consistent with the metabolic spectral fingerprints reported for the individual source materials. Sugars such as fructose (761 mg/g), glucose (570.7 mg/g), and sucrose (927.2 mg/g) dominated the extract, reflecting contributions from citrus and carob residues. These simple carbohydrates are not only nutritional components but also exhibit prebiotic effects, supporting beneficial gut microbiota [41]. Trehalose (164.3 mg/g), identified in both carob and shiitake, and mannitol (148.7 mg/g), a sugar alcohol found in shiitake and olive leaves by-products, are known for their antioxidant and cytoprotective properties in functional food applications [42,44]. Among organic acids, citrate (189.0 mg/g), malate (86.9 mg/g), and quinic acid (202.9 mg/g) were the most abundant. These compounds play central roles in energy metabolism and contribute to flavor, metal chelation, and physiological effects such as nephroprotection [45–47]. The presence of quinic acid supports the contribution of olive and carob matrices and reinforces the extract's potential as a source of shikimate-derived metabolites. Osmoprotectants and metabolic regulators were also detected at relevant concentrations. Myo-inositol (220.5 mg/g), betaine (53.4 mg/g), and proline (44.2 mg/g) are known to play key roles in cellular stress response and osmoregulation—especially relevant in halophytes like salicornia [48]. Choline (10.0 mg/g), another osmolyte, contributes to methyl group metabolism and neuroprotection. Amino acids such as arginine (33.4 mg/g), asparagine (28.5 mg/g), glutamate (28.2 mg/g), and branched-chain amino acids (leucine, isoleucine, valine) were present at moderate levels. These compounds support a variety of physiological functions, including nitric oxide production (arginine), neurotransmitter synthesis (glutamate), and muscle metabolism (BCAAs) [48–51]. The detection of γ-aminobutyric acid (GABA, 2.5 mg/g), though low in concentration, is noteworthy given its role in modulating blood pressure and stress responses [52]. Overall, the ^{1}H-NMR results confirm that the extract contains a metabolite pool with both nutritional and functional relevance. The metabolite classes detected are consistent with previous reports on the individual by-products, and their simultaneous presence supports the hypothesis that combining diverse plant sources enhances compositional diversity and functional potential.

Table 2. ^{1}H-NMR-detected metabolites and concentrations in BIOMEDEX extract (mg/g d.w.). Samples were analyzed in triplicate.

Metabolites	Concentration (mg/g d.w.)
4-Aminobutyrate	2.5 ± 0.9
Acetate	5.8 ± 0.5
Alanine	14.9 ± 0.7
Arginine	33.5 ± 12.9
Ascorbate	12.7 ± 0.1
Asparagine	28.5 ± 0.5
Aspartate	28.8 ± 3.5
Betaine	53.4 ± 1.9
Choline	10.2 ± 0.2
Citrate	189.1 ± 29.0
Formate	1.1 ± 0.0
Fructose	761.0 ± 23.5
Fumarate	2.8 ± 0.7
Glucose	570.7 ± 57.3
Glutamate	28.3 ± 1.9
Glutamine	21.0 ± 1.0
Isoleucine	6.3 ± 0.7
Lactate	6.5 ± 0.9
Leucine	6.3 ± 0.7
Malate	86.9 ± 14.3
Mannitol	148.7 ± 6.3
Methylguanidine	1.8 ± 3.5

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Myo-Inositol	220.5 ± 38.7
Ornithine	19.0 ± 1.2
Phenylalanine	3.9 ± 0.6
Proline	44.2 ± 0.3
Quinic acid	202.9 ± 31.9
Quinone	10.4 ± 3.3
Sucrose	927.2 ± 415.1
Trehalose	164.3 ± 637.9
Tryptophan	4.9 ± 66.7
Tyrosine	4.4 ± 0.5
Valine	13.7 ± 3.4

3.3. Metabolomics Multiplatform: SPME-GC–MS Analysis of VOCs Profile

The volatile fraction of the BIOMEDEX extract was analyzed via SPME-GC-MS **per triplicate**, identifying 38 VOCs (score > 80%) and revealing a diverse profile dominated by terpenes, aldehydes, acids, and alcohols—compounds with both aromatic and bioactive potential. Monoterpenes such as D-limonene, γ-terpinene, linalool, and α-terpineol were the predominant volatiles, especially in the citrus-derived fractions (Table 3). D-limonene, the most abundant compound, is widely recognized for its citrus aroma and has been associated with anti-inflammatory and chemopreventive activities [53,54]. Linalool and α-terpineol contribute floral and herbal notes and are known for antimicrobial and antioxidant effects, supporting their use in functional food formulations [54]. Aldehydes such as hexanal and nonanal. likely originating from olive leaves by-products, impart green and fruity notes and are commonly used as markers of freshness and lipid oxidation status in olive matrices [55]. Carob extracts contributed furans and sugar degradation products responsible for roasted and sweet aromatic notes, consistent with their sensory profile [56]. Salicornia fractions were characterized by short-chain organic acids (e.g. acetic acid, 2-methyl-propanoic acid), which are typical of halophytes and contribute fermentation-like or marine-associated aromas. These findings are in line with recent reports on the volatile composition of halophyte species [57]. Although VOCs from shiitake were less abundant, C8 compounds such as 1-octen-3-ol were detected and represent typical "mushroom-like" volatiles. These compounds enhance umami perception and have been studied for their bioactivity in immune regulation and oxidative defense [58]. Overall, the VOC profile demonstrates that the extract preserves aromatic complexity and bioactive volatiles from all components. This diversity not only contributes to sensory appeal but may also enhance the extract's antioxidant, antimicrobial, and anti-inflammatory properties. making it suitable for functional food and nutraceutical applications.

Table 3. Main identified VOCs present in BIOMEDEX extract. Samples were analyzed per triplicate.

Compound	Formula	RT (min)	Source	Bioactivity / Sensory Role	References
D-Limonene	C10 H16	12.2	Citrus peel dominant monoterpene	Antioxidant. antimicrobial/aroma contributor	[53,56]
Linalool	C10 H18 O	24.3	Citrus. olive oil. floral aromas	Antioxidant. antimicrobial/sedative properties	[54]
Acetic acid	C2 H4 O2	21.9	Halophytes (Salicornia)	Contributes to acidity/aroma intensity	[57]
Propanoic acid. 2-methyl-	C4 H8 O2	25.4	Halophytes (Salicornia)	Volatile acid with pungent note	[57]
Terpinen-4-ol	C10 H18 O	25.9	Citrus	Antimicrobial/aroma contributor	[56]
5-Hepten-2-one. 6-methyl-	C8 H14 O	17.4	Citrus peel oils	Fresh fruity aroma	[53]
Nonanal	C9 H18 O	19.3	Olive oil. citrus	Fatty. floral aroma	[55]
Hexanal	C6 H12 O	8.1	Olive oil	Green/fresh aroma marker	[55]

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3.4. Integrative Discussion: Functional Potential and Scientific Relevance of the Multimatrix Extract

The results obtained across metabolomics platforms demonstrate that combining Mediterranean plant-based by-products into a single formulation significantly enhances the diversity and abundance of bioactive compounds. Each matrix contributed a unique and complementary profile: citrus residues enriched the extract with flavanones and polymethoxylated flavones; olive leaves provided hydroxytyrosol derivatives; carob pods contributed polyphenols and sugars; shiitake mushrooms added amino acids and fungal-specific volatiles; and salicornia offered osmoprotectants and halophyte-derived metabolites. The combing use of untargeted (UPLC-QTOF-MS) and targeted (UHPLC-QTRAP-MS. ^{1}H-NMR. SPME-GC-MS) metabolomics approach enabled comprehensive characterization of phytochemicals. nutrients. and volatiles—critical for understanding the potential bioactivity and safety of the formulation. Notably. key compounds such as luteolin-7-O-glucoside, hesperidin, eriocitrin, D-limonene and quinic acid were detected at concentrations exceeding those commonly reported in single-source extracts. This supports the hypothesis of a **cumulative** enrichment effect resulting from matrix combination. From a nutritional perspective. the presence of essential amino acids. sugar alcohols. and organic acids enhances the value of the extract beyond its polyphenol content. Furthermore. the volatile profile confirms the preservation of characteristic aroma-active compounds. potentially contributing to consumer acceptance and additional functional benefits. This study aligns with current priorities in sustainable food innovation, including the valorization of agro-industrial residues, the development of multifunctional ingredients. and the implementation of circular economy principles. The formulation strategy used here represents a scalable model for transforming bio-waste into high-value products with applications in functional foods, dietary supplements, and nutraceuticals. Nevertheless. the results must be interpreted within the scope of analytical profiling. While compositional data strongly suggest health-promoting potential. biological validation remains essential. Our advancing studies will evaluate the extract's bioactivity in vivo. focusing on anti-inflammatory, metabolic, and microbiota-related effects. In addition, studies on safety assessments such as chemical contaminants are critical for regulatory approval and product development. In summary, this work demonstrates that the integration of diverse Mediterranean by-products, coupled with multiplatform metabolomics, yields a highly promising extract in terms of compositional richness, functional potential, and sustainability. The methodology and insights generated provide a robust foundation for the rational design of next-generation functional ingredients derived from plant-based residues.

3.5. Comparative Discussion of Analytical Platforms

The present study employed four advanced analytical platforms—HR-UHPLC-QTOF-MS. HS-UHPLC-QTRAP-MS. ^{1}H-NMR spectroscopy and SPME-GC-MS—to comprehensively characterize the bioactive profile of plant-derived by-products. These matrices. rich in diverse phytochemicals such as polyphenols. flavonoids. volatile compounds. amino acids. and organic acids. require a multimodal analytical strategy to capture their chemical complexity. Thus. the combination of HR-UHPLC-QTOF-MS and HS-UHPLC-QTRAP-MS platforms were most effective for polyphenols. allowing high-coverage and high-precision identification of major flavonoids and phenolic acids. In contrast. ^{1}H-NMR offered robust quantification for amino acids and organic acids. with minimal sample preparation. However, its lower sensitivity compared to MS-based techniques limits detection of low-abundance metabolites. For the volatile fraction. SPME-GC-MS emerged as an ideal tool due to its minimal sample handling and high sensitivity to aroma-active compounds. Nevertheless. it is dependent on fiber coating selectivity and can exhibit biases. GC-MS. when used independently. allowed complementary profiling of thermally stable small molecules. but lacks the capacity for larger. non-volatile compounds relevant to functional food analysis. Importantly. the complementarity of these techniques allowed for cross-validation of metabolite identities. expanded coverage. and a more holistic interpretation of the nutritional and functional properties of the studied matrices. This multi-platform approach enhances analytical reliability and helps overcome the inherent trade-offs of individual methods.

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4. Conclusions

In conclusion. a metabolomics multiplatform approach was essential to comprehensively identify and quantify the diverse phytochemicals. nutrients. and volatile compounds present in the newly developed lyophilized extract from Mediterranean plant-based by-products (BIOMEDEX). The combination of citrus. olive leaves. carob pods. mushroom. and salicornia residues proved to be a successful strategy to broaden the spectrum of bioactive compounds. supporting the hypothesis of multi-source health-promoting effects and reinforcing their potential use in food and nutraceutical applications. This study highlights the value of integrating diverse agro-industrial by-products into multifunctional formulations that align with circular economy principles. However, further studies are required to validate the biological relevance of the observed phytochemical and nutritional diversity to ensure its translation from lab-scale development to practical food or supplement applications.

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