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Influence of Maceration Solvent on Chemical Composition of Gemmotherapy Macerates—A Case Study on *Olea europaea* Young Shoots

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Abstract: Gemmotherapy, a natural therapy based on bud macerates, has recently gained importance in the field of food supplements. However, two coexisting extraction methods employ a glycerin-based solvent, either in a binary or ternary solvent mixture. The absence of an official method for bud preparation leads to non-standardized bud macerates. Given this context, this study aimed to (i) assess the influence of solvent composition on the chemical profile of olive young shoot macerates obtained using glycerin-based solvents or using different solvent extractions and (ii) to compare the two coexisting traditional bud extraction methods described by Dr Pol Henry and by the European Pharmacopoeia. A comprehensive phytochemical analysis of all macerates was conducted using HPLC-DAD-ELSD-MS^2, identifying 50 metabolites divided into 7 classes through dereplication. The extracts obtained with the solvent described by the European Pharmacopoeia (ethanol/glycerin) and by Dr Pol Henry (water/ethanol/glycerin) appeared to be the most diversified in terms of metabolite distribution and possessed higher rates of secondary metabolites. These observations reinforce the interest in a glycerin-based solvent mixture for bud extraction in gemmotherapy. In addition, the difference in composition between the two traditional solvents was highlighted. Indeed, iridoids were predominant in both macerates, representing about 50% of the chemical composition, but differences were observed from one macerate to another regarding the proportions of the other chemical classes. This emphasizes the necessity for standardized gemmotherapy macerates.

Keywords: gemmotherapy; bud; *Olea europaea*; phytochemical analysis

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1. Introduction

Phytoembryotherapy, also known as gemmotherapy, was introduced and defined by Dr Pol Henry as a therapy based on macerates obtained from meristematic tissues such as buds or young shoots [1,2]. The biological activities and phytochemical interest of buds have been reported [3]. Recently, the use of bud macerates has gained attention in other phytotherapy fields.

Although the main chemical classes of bud constitution are known (phytohormones, nucleic acids, amino acids, phenolic compounds, vitamins, etc.), few complete phytochemical analyses of bud macerates are available. However, deep knowledge of these extracts is needed for a better comprehension of their composition. Some studies were recently published on this. Blackcurrant (*Ribes nigrum*) is not only a well-known and commonly used bud but also the most studied for its chemical composition. As a result, many of its compounds have been identified, such as monoterpenes, catechins, cinnamic acids, flavonols,

benzoic acids, and vitamins [4–8]. The chemical compositions of chestnut (C*astanea* spp.), elmleaf blackberry (*Rubus ulmifolius*), raspberry (*Rubus idaeus*), lingonberry (*Vaccinium vitis idaea*), bilberry (*Vaccinium myrtillus*), dog rose (*Rosa canina*), rosemary (*Rosmarinus officinalis*), alder (*Alnus glutinosa*), grapevine (*Vitis vinifera*), hornbeam (*Carpinus betulus*), dogwood (*Cornus mas*), fig tree (*Ficus carica*), ash tree (*Fraxinus excelsior*), larch (*Larix decidua*), pine (*Pinus montana*), oak (*Quercus petraea*), and silver linden (*Tilia tomentosa*) have also been investigated [6–14].

Two main extraction protocols are described for bud maceration. Fresh material can either be macerated in an ethanol/glycerin mixture and then diluted with a solvent composed of water, ethanol, and glycerin (**w**/**e**/**g**), as described by the European Pharmacopoeia [15] or directly macerated in the tri-solvent **w**/**e**/**g** (1/1/1; *v*/*v*/*v*) mixture, which is not further diluted [2]. However, the maceration parameters, including the extraction solvent, raw material/solvent ratio, and extraction time, can vary from one production to another. Recently, Turrini et al. concluded that these parameters can affect the chemical composition of bud macerates, especially the polyphenol content [12].

The part of the plant that is used plays a major role in the chemical composition of macerates, as described by Tetau, who compared the chemical composition of *Ribes nigrum* macerates obtained from buds or leaves [1]. However, to the best of our knowledge, the effect of a glycerin-based solvent, either binary or ternary, has not yet been scientifically evaluated.

Therefore, this study focused on profile determination and the chemical characterization of macerates obtained from different solvent systems to investigate the impact of glycerin-based solvents on bud extraction. Macerates obtained with solvents of different polarities (ethanol, glycerin, water/glycerin, water/ethanol, ethanol/glycerin, or water/ethanol/glycerin) were compared in terms of extraction yields and chemical compositions. An additional comparison between frozen and fresh material was also made using the tri-solvent extraction system. Phytochemical analysis was conducted using the SPE-HPLC-DAD-ELSD-MS^2 method previously developed by the authors [8].

Olea europaea was selected as a case study due to its large chemical richness and diversity, with most of its phenolic compounds distributed in 5 main classes: oleuropeosides, flavones, flavonols, flavan-3-ols, and substituted phenols. Several biological properties have also been reported, such as antioxidant, antihypertensive, hypoglycemic, hypocholesterolemic, and cardioprotective activities [16].

Our study was carried out using a macerate obtained from young olive shoots, the part usually used in gemmotherapy and for which biological properties in the vascular and metabolic fields have been described [17]. However, the chemical composition of this specific part has never been reported.

2. Materials and Methods

2.1. Chemicals

Analytical grade solvents were used for SPE and HPLC analysis. Acetonitrile (CHROMASOLV gradient grade for HPLC) and formic acid (analytical reagent grade) were purchased from Fisher Scientific (Illkirch, France). Ultrapure water was obtained from a MilliQ advantage A10 purification system (Millipore, Molsheim, France). HyperSep C18 2000 mg cartridges were purchased from ThermoFisher Scientific (Courtaboeuf, France).

2.2. Samples

2.2.1. Plant Material

Young shoots of *Olea europaea* were collected in May 2020 in the Aude department (France). The raw material, which was organically certified, was collected in the wild. For technical reasons, maceration was mainly performed on frozen young olive shoots (−18 °C), but one part of these young shoots was macerated immediately after harvesting.

2.2.2. Bud Macerates

The extracts were obtained by maceration of young frozen or fresh *Olea europea* shoots for 21 days. All extracts were prepared from the same batch of raw material without any grinding at a concentration of 5% dry weight in the solvent mixture (*m*/*v*). To evaluate the dry weight, the humidity level was calculated according to the European Pharmacopoeia protocol [18]. A 21-day maceration in water alone could not be carried out due to its instability, with observed microbiological development. Table 1 details the solvent system used for each extraction.

Table 1. List of the analyzed extracts.

Extract Name	Extraction Solvent	Raw Material Treatment
w/e/g	water/ethanol/glycerin	frozen
e/g	ethanol/glycerin	frozen
w/g	water/glycerin	frozen
w/e	water/ethanol	frozen
e	ethanol	frozen
g	glycerin	frozen
Fresh w/e/g	water/ethanol/glycerin	fresh

2.2.3. Solid-Phase Extraction

A solid-phase extraction (SPE) procedure previously developed in the laboratory was applied to bud macerates containing glycerol [8]. Ethanol and/or water were first removed from the macerates by vacuum evaporation. The SPEs were performed out on a C18 cartridge, activated by methanol, and equilibrated with water. The samples were dissolved or diluted in water to reduce the viscosity of the glycerol to a final volume of 2 mL and then placed on the cartridge. Glycerol was first eluted with water (4 × 2 mL), then the adsorbed compounds were recovered by elution with methanol (4 × 2 mL). These eluates were then evaporated under vacuum and lyophilized to obtain the final dry extracts.

2.3. Phytochemical Analysis

2.3.1. HPLC-DAD-ELSD

The chromatographic analyses were carried out using a Shimadzu 2030C 3D-type device equipped with quaternary solvent and sample managers and a chromatographic column compartment coupled to a DAD and an ELSD. Samples were prepared at 10 mg·mL^{-1} in MeOH and filtered through a 0.45 µm nylon membrane before injection. A volume of sample, adapted to each extract, was injected into a Gemini C18 column (150 mm × 4.6 mm, 110 Å; 3 µm). The samples were stored at 10 °C, and the column was maintained at 25 °C with a flow rate of 0.75 mL·min^{-1}. The mobile phase consisted of 0.1% formic acid in both H_2O (A) and MeCN (B) used in gradient mode as follows: 0% B for 5 min, then 0 to 100% B from 5 to 55 min, then maintained at 100% B until 60 min. UV–Vis spectra were recorded between 190 and 600 nm. ELSD was heated to 40 °C, and the signal gain was adapted to each extract. Data were processed using LabSolutions software (version 5.87).

2.3.2. HPLC-UV-MS^2

The mass spectrometry analyses were carried out on a 2695 separation module coupled to a 2489 UV/Visible detector (Waters, Saint Quentin Yvelines, France) and an Esquire 3000 Plus ESI-IT-MS (Bruker, Marne-la-Vallée, France). Chromatographic conditions and sample preparation were the same as previously described for HPLC-DAD-ELSD. UV detection was carried out at 254 and 280 nm, and MS^2 detection was performed in positive and negative modes. Data were processed using DataAnalysis software (version 3.3).

2.3.3. Dereplication Analysis

The identification of compounds present in the analyzed extracts was based on four types of information: retention times, UV spectra, positive and negative MS^2 data (m/z and fragmentation pattern), and relevant data from the literature. Retention times and UV spectra provided information on the chemical class of the compounds. MS^2 and literature data allowed the identification of the compounds. If the data were not sufficient to identify a compound, it was referred to as follows: chemical class 1, chemical class 2, etc., with the number corresponding to the order of elution.

2.4. Determination of Extraction Yields and Individual Contents

2.4.1. Extraction Yields

The mass of the extracts obtained after the SPE procedure was used to calculate the extraction yields, expressed in mg/g of dry weight (DW) deduced from the starting ratio of 5 g of dried plant in 100 mL of solvent. Each bud macerate was processed in triplicate.

2.4.2. Determination of Individual Contents

The ELSD chromatograms of the final dry extracts (n = 3) were used to calculate the individual contents expressed in mg/g of DW. For this, all the chromatographic peaks were integrated, giving each compound a representative percentage relative to the total extract. Knowing the concentration and injection volume, as well as the yield of each extract, it was possible to calculate an individual content in mg/g of DW for each component.

3. Results and Discussion

3.1. Influence of the Extraction Solvent

To evaluate the choice of the solvent system—ethanol/glycerin (**e**/**g**) or water/ethanol/glycerin (**w**/**e**/**g**) as classic bud extraction systems—the chemical composition of the extracts made with the following systems—**w**/**e**/**g**, **e**/**g**, **w**/**g**, **w**/**e**, **e**, and **g** extracts—was compared. The individual effect of each solvent alone or in a mixture was thus evaluated by analyzing the extraction yields and the metabolite composition.

3.1.1. Effect on the Extraction Yield

Table 2 details the extraction yields of the different macerates obtained after the SPE process. Significant disparities could be observed between the extraction yield values. Indeed, the yields obtained for **w**/**e** (274 mg/g DW) and **e** (253 mg/g DW) were significantly higher than the yields obtained for **g** (175 mg/g DW) and **e**/**g** (164 mg/g DW). Finally, the extracts obtained from a solvent comprising both water and glycerin had the lowest extraction yields: 95 mg/g DW for **w**/**e**/**g** and 74 mg/g DW for **w**/**g**.

Table 2. Extraction yields (mg/g DW) of the different extracts.

Extract Name	Extraction Yield (mg/g DW)
w/**e**/**g**	94.8 ± 2.6
e/**g**	163.8 ± 2.6
w/**g**	73.8 ± 0.9
w/**e**	274.4 ± 2.4
e	253.0 ± 5.5
g	174.5 ± 14.2

3.1.2. Effect on the Metabolite Composition

First, the ratios between primary metabolites (sugars, organic acids) associated with chlorophylls and secondary metabolites (phenylethanoids, flavonoids, iridoids, and triterpenes) were compared (Figure 1). **g** and **w**/**e** extracts predominantly showed primary metabolites and chlorophylls (constituting 100% and 94% of the ELSD content, respectively). In contrast, **w**/**e**/**g** and **e**/**g** contained the highest levels of secondary metabolites (SMs)

(89 and 88% of ELSD content, respectively). Finally, **e** and **w**/**g** had intermediate profiles, with a ratio between primary metabolites associated with chlorophylls and secondary metabolites close to 2/3–1/3.

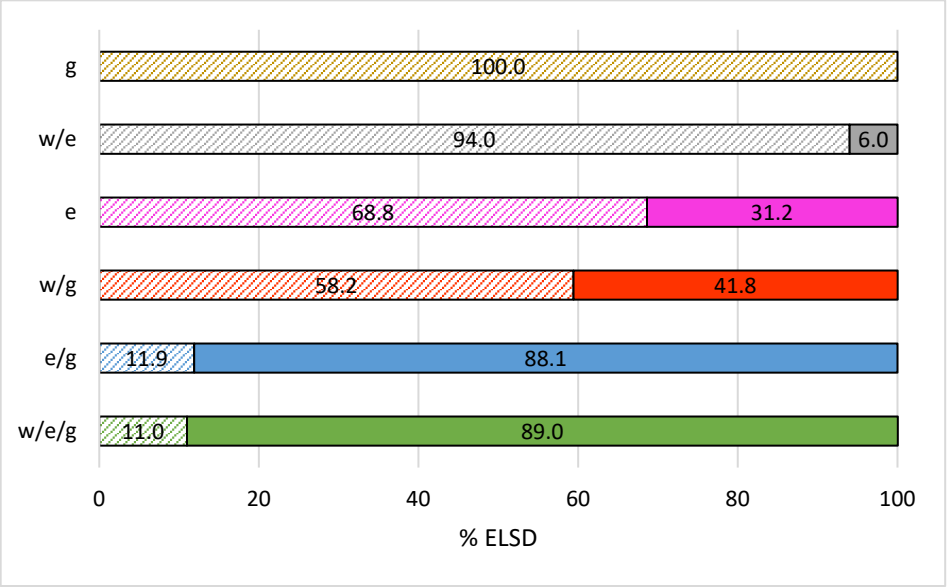

Figure 1. Metabolite repartition (% ELSD) of the different extracts. Primary metabolites and chlorophylls (hatched bar plots); secondary metabolites (full bar plots).

Solvent mixtures containing both glycerin and ethanol, as described by the European Pharmacopoeia or Pol Henry, were found to be the most effective in extracting SMs. Additionally, it is noteworthy that the sum of individual contents of the SMs does not reach the value of **e**/**g** and **w**/**e**/**g** extracts. For instance, the SMs content of **w**/**e** added to the SMs content of **g** (6%) is less than the SMs content of **w**/**g** added to the SMs content of **e** (42%), which is again less than the SMs content of **w**/**e**/**g** extract (89%). This observation emphasizes the significant interest in solvent synergy for the extraction of metabolites from buds.

Fifty compounds were identified, using dereplication based on HPLC-DAD-ELSD (Figure 2) and HPLC-UV-MS^2, belonging to seven chemical classes: sugars, organic acids, phenylethanoids, flavonoids, iridoids, triterpenes, and chlorophylls. The individual contents of each identified compound, calculated from the ELSD data of the different macerates, are presented in Table 3. Indeed, the ELSD is an evaporative aerosol detector based on the principle of nebulization of the eluent in the form of fine droplets followed by evaporation of the volatile mobile phases, producing the formation of dried particles. It directs a beam of light through the particles and measures the scattered light using a laser photometer. ELSD is generally considered to be a "universal" or mass-dependent detector for non-volatile compounds [19]. This method allows reliable quantification without the need for analytical quantification methods that are more cumbersome to implement and require numerous standards.

Figure 2 displays the HPLC-ELSD profile of each extract. The chromatograms of **g**, **w**/**g**, and **w**/**e** extracts display a small number of peaks, including sugar peaks (rt 1.7–2.8 min), consistent with the previously described high content of primary metabolites. The extracts obtained with solvents composed of ethanol (**w**/**e**/**g**, **e/g**, **w/e**, and **e**) present non-polar peaks, with **w**/**e** extract with less visible peaks due to a significant sugar peak at the beginning of the chromatogram. Extracts **w**/**e**/**g** and **e**/**g** have similar profiles in terms of metabolite distribution.

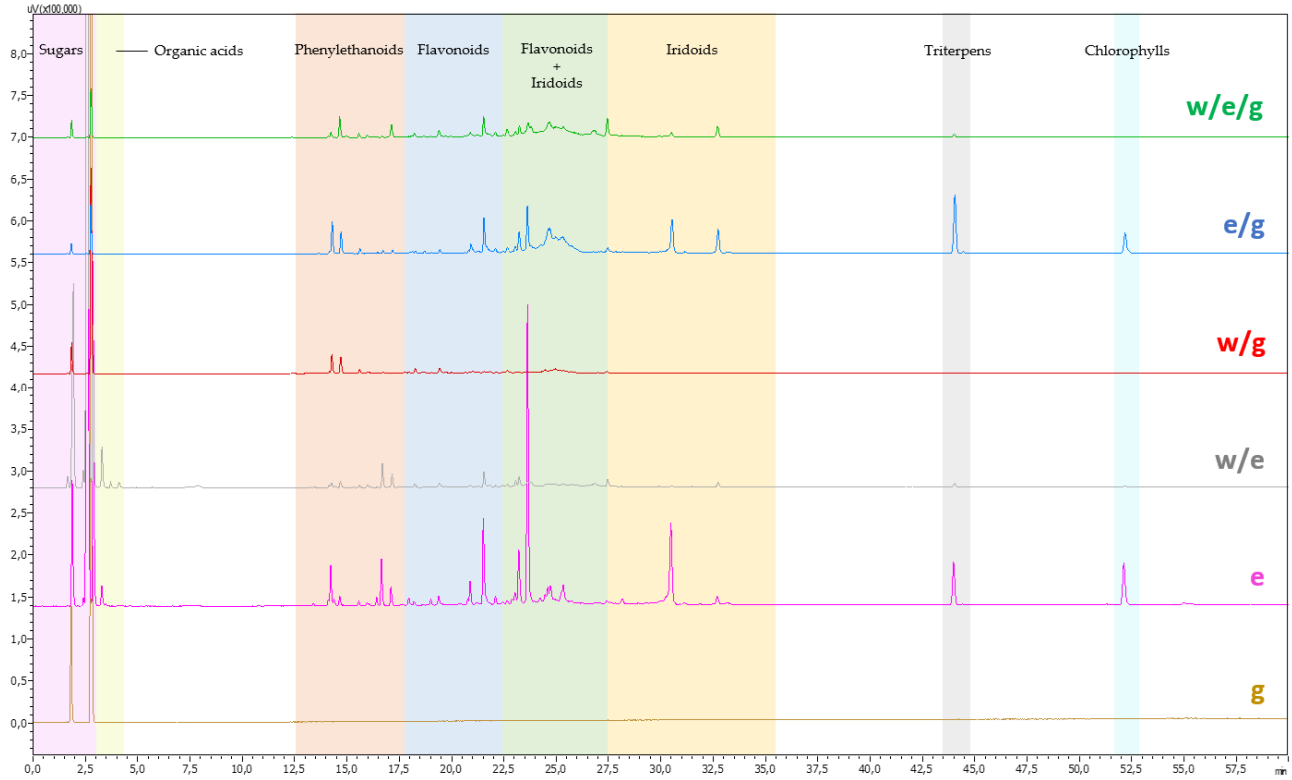

Figure 2. HPLC-ELSD profiles of the different extracts.

This was followed by a focus on the different chemical classes:

- Sugars, organic acids, triterpenes, and chlorophylls:

All solvent systems were able to extract sugars, but **w**/**e**, **e,** and **g** extracts were largely characterized by the presence of disaccharides with significant masses (199, 133, and 165 mg/g DW, respectively). These results were surprising, given the polarity of the different solvent systems. Indeed, solvent **e** should logically be less polar than **w**/**g**, but it was able to extract more disaccharides.

Only the **e**/**g** and **e** solvents were able to extract chlorophyll A and oleanic acid in significant amounts. Finally, organic acids were only identified in **w**/**e** and **e**. The presence of non-polar compounds (especially chlorophylls and triterpenes) could be explained by the high ethanol content (at least 50%) in **e**/**g** and **e**.

- Phenylethanoids

Ten phenylethanoids were quantified in the different macerates. Among them, 3 were identified according to their Rt, UV, and/or MS^2 data: secologanoside, calceolarioside, and loganin. In terms of diversity, **w**/**g** had the highest number of compounds with 7 different compounds. The contents vary from 0 for **g** to 13 mg/g DW for **e**/**g**. The range of contents was quite similar (~10 mg/g DW) for all solvents except for the **w**/**e** mixture, for which the amount is, on average, half that of the others. The presence of glycerol in the mixing system appears to be important.

- Flavonoids

Sixteen flavonoids or glycosylated flavonoids were quantified, with nine partially identified by dereplication: luteolin-*O*-rutinoside 1, rutin, luteolin-*O*-rutinoside 2, luteolin-*O*-glucoside 1, apigenin-*O*-rutinoside, taxifolin, apigenin-*O*-glucoside, luteolin-*O*-glucoside 2 and luteolin. The **w**/**e**/**g**, **w/g,** and **e**/**g** extracts were the most diversified, with 13, 13, and 8 compounds identified, respectively, but in the case of **w/g,** they are all present with individual contents lower than 2 mg/g DW. Luteolin glycosides or aglycones were predominant in these samples. Total flavonoid content was highest for **e**/**g** (33 mg/g DW)

and **w**/**e**/**g** (27 mg/g DW). The presence of ethanol and glycerol in the solvent system appeared to be crucial for flavonoid extraction, likely due to the average polarity of this chemical class.

- Iridoids

Sixteen iridoids were identified among the different macerates, including oleuropein, oleuroside, lucidumoside C, ligstroside, and oleuropein aglycone. **w**/**e**/**g**, e/**g**, and **w**/**g** showed the best distribution in terms of the number of metabolites, with 9, 7, and 6 iridoids identified, respectively. Higher yields were obtained with the solvent of **e**/**g** (79 mg/g DW), **e** (49 mg/g DW), and **w**/**e**/**g** (47 mg/g DW) macerates. A lower extraction yield of iridoids (relatively non-polar compounds) in the **w**/**g** and **w**/**e** mixtures could be explained by the high water content (50%). The iridoid content, depending on the extraction solvent used, is presented in Figure 3, selecting only iridoids with a content exceeding 1 mg/g DW.

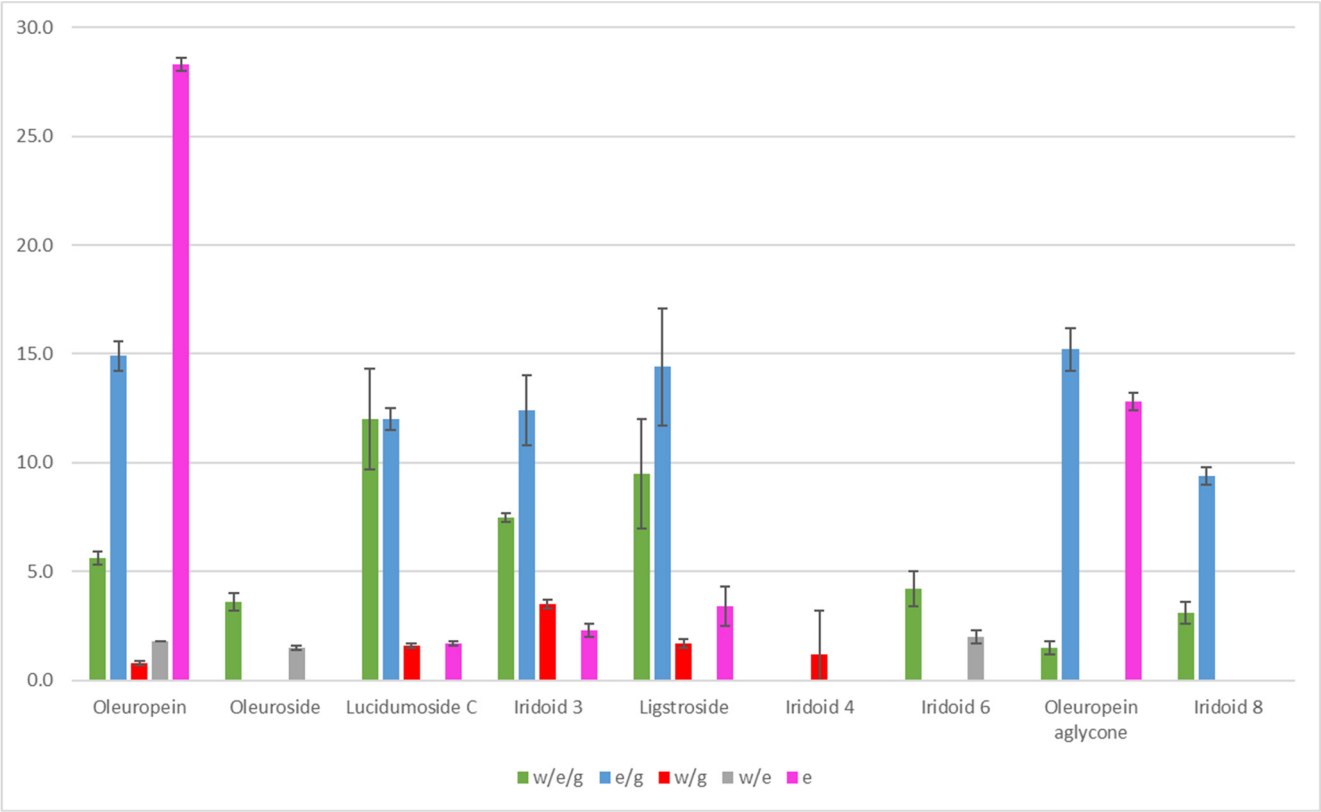

Figure 3. Principal iridoid contents (mg/g DW) in the different solvent systems extracts (sample **g** is not shown because it does not contain any iridoids).

Table 3. Retention times, UV/MS data, and contents (mg/g DW) of individual components identified in each macerate.

	Rt (min) [a]	λ_{max} (nm)	*m/z* for $[M-H]^-$ [b]	*m/z* for $[M+H]^+$ [b]	w/e/g	e/g	w/g	w/e	e	g	Fresh w/e/g
Sugar 1	1.7	-	-	-	-	-	-	1.2 ± 1.0	-	-	-
D-mannitol	1.8	-	181 (101, 85, 71)	-	2.3 ± 0.3	2.2 ± 0.6	5.8 ± 0.6	32.7 ± 0.5	9.0 ± 0.4	9.7 ± 5.8	2.2 ± 0.7
Disaccharide	2.8	-	341	343	7.9 ± 0.9	8.1 ± 1.4	38.0 ± 3.3	198.9 ± 2.3	132.9 ± 2.5	164.9 ± 17.9	13.4 ± 5.5
Total sugars					10.2 ± 0.6	10.2 ± 1.9	43.8 ± 3.2	232.7 ± 2.4	142.0 ± 2.9	174.5 ± 14.2	15.6 ± 6.2
Organic acid 1	2.9	258	-	-	-	-	-	19.5 ± 0.5	25.0 ± 0.8	-	-
Citric/Quinic acid	3.3	258	191 (173, 111, 87, 85/173, 127, 93, 85)	-	-	-	-	5.8 ± 0.3	2.2 ± 0.3	-	-
Total organic acids					-	-	-	25.3 ± 0.3	27.2 ± 0.8		-
Hydroxytyrosol-*O*-glucoside	14.2	230/280	315 (153, 135, 123)	317	-	-	0.4 ± 0.1	-	-	-	-
Secologanoside	14.3	220/277	389 (345, 227, 209, 183, 165, 121, 119)	391	1.1 ± 0.2	7.2 ± 0.3	4.1 ± 0.3	-	3.2 ± 0.1	-	0.9 ± 0.3
Calceolarioside	14.7	234/278	477 (323, 315, 179, 161, 135)	-	3.9 ± 0.4	5.1 ± 0.4	4.1 ± 0.4	-	0.7 ± 0.1	-	15.2 ± 1.7
Phenylethanoid 1	15.0	260	-	-	-	-	-	-	-	-	0.2 ± 0.3
Phenylethanoid 2	15.6	220/275	-	-	0.8 ± 0.1	0.5 ± 0.8	0.9 ± 0.5	-	-	-	-
Phenylethanoid 3	16.0	220/260/295	313	315	0.4 ± 0.4	-	0.8 ± 0.1	-	-	-	-
Loganin	16.7	234/274/325	389 (345, 209, 183, 165, 121, 119)	-	-	-	0.5 ± 0.1	3.0 ± 0.1	4.0 ± 0.2	-	-
Phenylethanoid 4	17.0	220/275	-	-	-	-	-	-	-	-	0.5 ± 0.1
Phenylethanoid 5	17.2	280	-	-	3.0 ± 0.2	-	-	1.9 ± 0.0	1.6 ± 0.1	-	0.9 ± 0.0
Phenylethanoid 6	17.8	225/280	-	-	-	-	0.5 ± 0.1	-	-	-	-
Total phenylethanoids					9.3 ± 1.1	12.8 ± 1.3	11.3 ± 1.5	4.9 ± 0.1	9.5 ± 0.4	-	17.7 ± 1.9
Flavonoid 1	18.0	220/285/325	-	-	-	-	0.5 ± 0.0	-	-	-	-
Flavonoid 2	18.3	220/275/325	-	-	1.0 ± 0.1	-	1.3 ± 0.1	-	-	-	-
Flavonoid 3	18.6	225/275/335	-	-	-	-	0.2 ± 0.3	-	-	-	-
Flavonoid 4	18.7	260/290/330	-	-	0.3 ± 0.4	-	0.2 ± 0.3	-	-	-	-
Flavonoid 5	19.4	220/275/320	-	-	1.9 ± 0.2	1.4 ± 0.3	1.8 ± 0.2	-	-	-	0.7 ± 0.1
Flavonoid 6	19.7	280	-	-	-	-	0.2 ± 0.3	-	-	-	-
Luteolin-*O*-rutinoside 1	20.8	220/280/300	593 (447, 285)	595 (449, 287)	0.6 ± 0.1	-	-	-	-	-	-
Rutin	20.9	254/357	609 (463, 343, 301, 300, 179)	611 (303)	1.7 ± 0.2	3.5 ± 0.4	1.0 ± 0.2	-	2.2 ± 0.1	-	-
Luteolin-*O*-rutinoside 2	21.3	290	593 (447, 285)	595 (449, 287)	1.2 ± 0.1	-	0.8 ± 0.1	-	-	-	0.7 ± 0.1
Luteolin-*O*-glucoside 1	21.5	250/265/350	447 (285)	449 (287)	4.9 ± 0.6	10.2 ± 2.0	0.8 ± 0.2	2.5 ± 0.0	8.4 ± 0.7	-	2.4 ± 0.3

Table 3. *Cont.*

	Rt (min) [a]	λ_{max} (nm)	*m*/*z* for [M-H]$^-$ [b]	*m*/*z* for [M+H]$^+$ [b]	w/e/g	e/g	w/g	w/e	e	g	Fresh w/e/g
Flavonoid 7	21.8	280	463	465	0.4 ± 0.6	-	0.8 ± 0.1	-	-	-	0.4 ± 0.4
Apigenin-*O*-rutinoside	22.1	220/265/332	577 (269)	579 (433, 271)	1.9 ± 0.2	2.4 ± 0.3	0.6 ± 0.1	-	-	-	0.6 ± 0.2
Taxifolin	22.7	220/290/330	303 (287, 285, 197, 177, 125)	-	2.5 ± 0.1	2.5 ± 0.3	1.4 ± 0.1	-	-	-	1.9 ± 0.1
Apigenin-*O*-glucoside	23.1	220/255/337	431 (269, 268)	433	2.0 ± 0.1	2.6 ± 0.1	-	-	-	-	1.2 ± 0.1
Luteolin-*O*-glucoside 2	23.2	240/265/340	447 (285)	449 (287)	3.2 ± 0.3	7.4 ± 0.6	-	2.1 ± 0.1	5.9 ± 0.3	-	1.6 ± 0.1
Luteolin	27.5	250/265/290/345	285	287	5.2 ± 0.3	3.4 ± 0.4	0.6 ± 0.2	1.7 ± 0.0	-	-	2.8 ± 0.3
Total flavonoids					26.9 ± 2.3	33.4 ± 3.2	10.1 ± 1.8	6.2 ± 0.1	16.5 ± 0.8	-	12.3 ± 1.4
Iridoid 1	22.5	220/290	377	379	0.3 ± 0.5	-	0.6 ± 0.1	-	-	-	-
Oleuropein	23.6	230/280	539 (403, 377, 307, 275, 345)	541	5.6 ± 0.3	14.9 ± 0.7	0.8 ± 0.1	1.8 ± 0.0	28.3 ± 0.3	-	3.7 ± 0.2
Oleuroside	23.8	230/290/330	539 (377, 345, 307, 275)	-	3.6 ± 0.4	-	-	1.5 ± 0.1	-	-	2.8 ± 0.8
Iridoid 2	24.0	220/290	-	-	-	-	-	-	-	-	2.1 ± 0.2
Lucidumoside C	24.6	234/282	583 (537, 403, 351, 223, 197, 179)	-	12.0 ± 2.3	12.0 ± 0.5	1.6 ± 0.1	-	1.7 ± 0.1	-	4.1 ± 0.1
Iridoid 3	24.7	225/280	-	-	7.5 ± 0.2	12.4 ± 1.6	3.5 ± 0.2	-	2.3 ± 0.3	-	-
Ligstroside	25.2	230/279	523 (361, 291, 259, 223)	-	9.5 ± 2.5	14.4 ± 2.7	1.7 ± 0.2	-	3.4 ± 0.9	-	1.9 ± 0.2
Iridoid 4	26.4	290	453	455	-	-	1.2 ± 2.0	-	-	-	1.4 ± 0.1
Iridoid 5	26.7	230/280	255	257	-	-	-	-	-	-	0.6 ± 1.0
Iridoid 6	26.9	250/290/350	533	535	4.2 ± 0.8	-	-	2.0 ± 0.3	-	-	5.0 ± 1.5
Oleuropein aglycone	30.5	230/280	377 (345, 307, 275, 241, 217, 197, 153, 149, 139, 111)	379	1.5 ± 0.3	15.2 ± 1.0	-	-	12.8 ± 0.4	-	7.4 ± 1.0
Iridoid 7	31.1	225/280	377	379	-	-	-	-	-	-	0.7 ± 0.7
Iridoid 8	32.7	225/280	391	393	3.1 ± 0.5	9.4 ± 0.4	-	-	-	-	15.4 ± 1.2
Iridoid 9	33.1	280	-	-	-	0.4 ± 0.7	-	-	-	-	0.2 ± 0.4
Iridoid 10	33.3	230/280	361	363	-	-	-	-	-	-	0.6 ± 0.1
Iridoid 11	35.3	225/275	-	-	-	-	-	-	-	-	0.2 ± 0.4
Total iridoids					47.0 ± 2.8	78.7 ± 2.1	8.8 ± 2.1	5.3 ± 0.4	48.5 ± 0.6	-	46.2 ± 3.3
Oleanolic acid	44.0	-	455	-	0.8 ± 0.0	22.1 ± 2.0	-	-	4.4 ± 0.7	-	-
Total triterpens					0.8 ± 0.0	22.1 ± 2.0	-	-	4.4 ± 0.7	-	-
Chlorophyll A	52.2	410/440/575	-	-	-	9.6 ± 2.6	-	-	4.4 ± 1.4	-	-
Total chlorophylls					-	9.6 ± 2.6	-	-	4.4 ± 1.4	-	-

[a] Retention time expressed in minutes; [b] *m*/*z* fragmentation values in brackets; individual contents (mg/g of DW) are presented as mean ± standard deviation (n = 3); -: not detected.

On the one hand, some of these results were rather expected and logical, such as the presence of chlorophylls and triterpenes obtained with an extraction solvent system containing 50% or more ethanol or the lower extraction yield of iridoids in a solvent with more than 50% water. On the other hand, the results concerning the two chemical classes, sugars and organic acids, were very surprising and difficult to explain. Furthermore, the individual distribution of the metabolites differed depending on the extraction solvent.

One hypothesis was that this study was designed to compare different solvent systems using a standardized protocol to produce macerates [15]. Since mass/volume ratio and extraction time are the same for all systems, it seems likely that some specific effects such as saturation, viscosity, or solubility would affect the different solvent systems differently and, therefore, their ability to extract different classes of compounds. Additionally, solvent mixtures are more complex than individual solvents in terms of extraction parameters (polarity and affinity).

All compounds identified here have been previously described in hydroalcoholic (methanol, ethanol, or glycerol) extracts of olive leaf extracts [20–25] and occasionally also in wood and/or flowers of olive trees hydroalcoholic (methanol, ethanol, or glycerol) or methanolic extracts [21,26]. Considering the distribution and diversity of the extracted metabolites, an additional comparison was made between the **w/e/g** and **e/g** extracts obtained with glycerol-based ternary and binary solvent mixes.

*3.2. Comparison between Classical Bud Extraction Methods (**w/e/g** and **e/g**)*

Both **w/e/g** and **e/g** extracts presented a large diversity of metabolites divided into 7 chemical classes (30 compounds for **w/e/g**, 22 for **e/g**), including fewer primary metabolites in favor of secondary compounds (Figure 2). These metabolites, including oleuropein, are known for their biological activities, further supporting the choice of a glycerin-based solvent for the extraction of buds or young shoots.

However, **w/e/g** and **e/g** extracts exhibited some differences in terms of chemical composition.

Additionally, the **e/g** extract possessed a higher extraction yield, approximatively two-fold higher than the **w/e/g** extract. It is important to note that the **e/g** macerate is supposed to be 10-fold diluted before use to comply with European Pharmacopoeia recommendations, resulting in a final quantity of secondary metabolites smaller than that of the **w/e/g** extract. The contents of the chemical classes in relation to the dosage are given in Appendix A.

Therefore, metabolite distribution (% ELSD, see Appendix A) was analyzed and discussed instead of expressing individual metabolite yields (Figure 4). Both **w/e/g** and **e/g** extracts were characterized by high levels of iridoids (≈50%) with 6 common compounds: oleuropein, lucidumoside C, ligstroside, oleuropein aglycone, and two other iridoids. **w/e/g** extract had a higher proportion of flavonoids than **e/g** (28 versus 20%), but their composition mainly consisted of luteolin and luteolin glycosides. The triterpene and chlorophyll classes were only represented by olealonic acid and chlorophyll A and were present in high quantities in **e/g** (respectively 13 and 6% of the composition), whereas they were barely noticeable in **w/e/g** (1 and 0%, respectively). The presence of water in the solvent for **w/e/g** makes it more polar than for the **e/g** mixture, explaining the absence of non-polar compounds. The levels of phenylethanoids were almost equivalent (~10%) in these two extracts, but the composition differed from one macerate to another. **e/g** was mainly characterized by secologanoside and calceaolarioside, while **w/e/g** was more diversified. The sugar content of the two macerates was close (6 to 11%). The sugars identified were *D*-mannitol and the disaccharide.

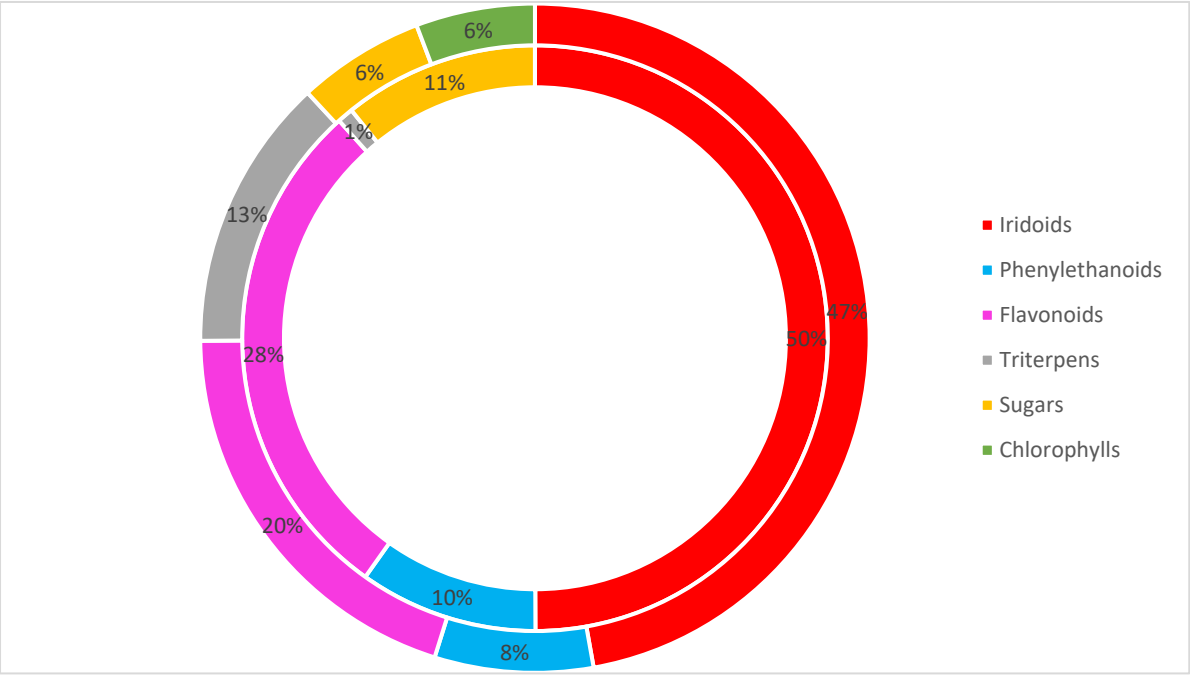

Figure 4. Metabolites repartition (% ELSD) of **w/e/g** (internal circle) and **e/g** (external circle) extracts (organic acids were not present in these extracts).

3.3. Influence of the Raw Material Treatment

Bud extracts are normally produced from fresh raw material. However, due to technical constraints, the analysis of the influence of the extraction solvent was carried out on frozen material. The chemical composition of the extracts obtained from frozen and fresh materials was compared to evaluate the impact of the raw material treatment on metabolite distribution.

3.3.1. Effect on the Extraction Yield

Freezing does not seem to affect the extraction yield, as the extraction mass values of **w/e/g** (95 mg/g DW) for frozen and fresh material (**Fresh w/e/g**) (92 mg/g DW) were nearly equivalent (Table 4).

Table 4. Extraction yields (mg/g DW) of **w/e/g** and **fresh w/e/g** extracts.

Extract Name	Extraction Yield (mg/g DW)
w/e/g	94.8 ± 2.6
Fresh w/e/g	91.9 ± 6.5

3.3.2. Effect on the Composition

The phytochemical profiles of the extracts obtained with frozen (**w/e/g)** and fresh (**fresh w/e/g**) material were compared by HPLC-DAD-ELSD, and the compounds were identified by HPLC-UV-MS^2, thus allowing the identification of 39 compounds (Table 3). Figure 5 shows similar profiles for both extracts, but the relative distribution of metabolites differs. Both macerates were mainly characterized by 4 classes of metabolites (Figure 6): iridoids, flavonoids, phenylethanoids, and sugars. However, their distribution was quite different. In terms of ELSD percentages (Appendix A), frozen **w/e/g** and **fresh w/e/g** included equivalent iridoid contents (≈50%), but **fresh w/e/g** contained a higher content of flavonoids to the detriment of sugars and phenylethanoids, suggesting that freezing the raw material improved the extraction of these compounds.

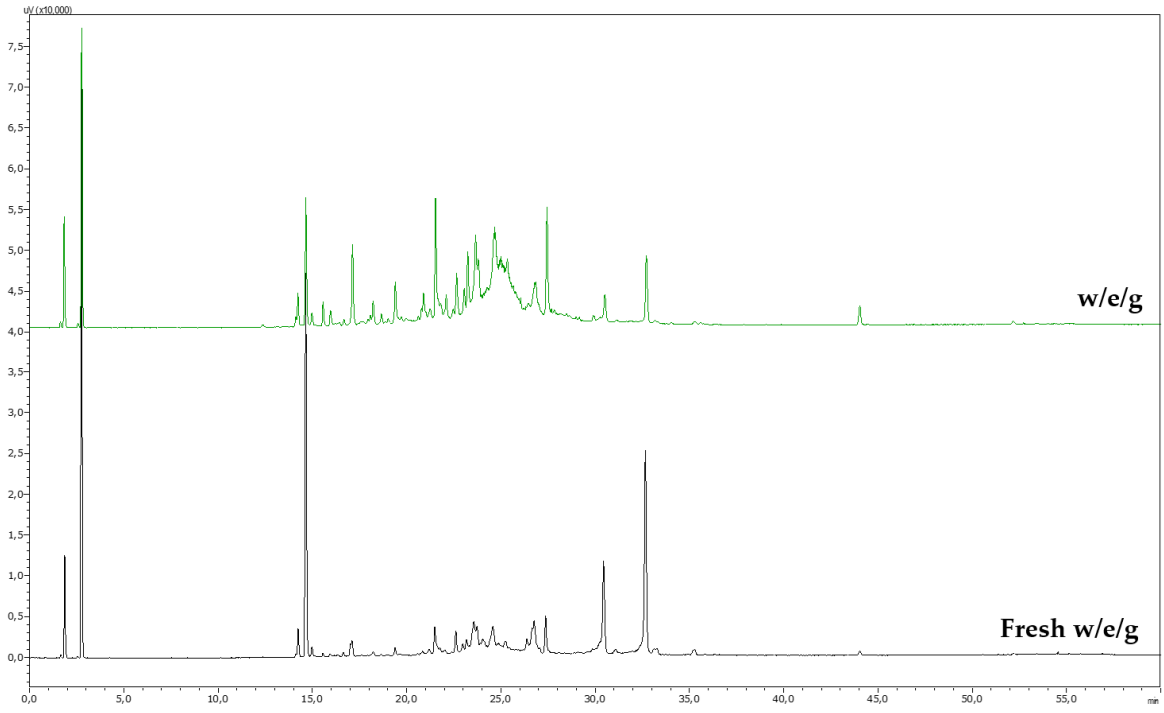

Figure 5. HPLC-ELSD profiles of **w/e/g** (green) and **fresh w/e/g** (black) extracts.

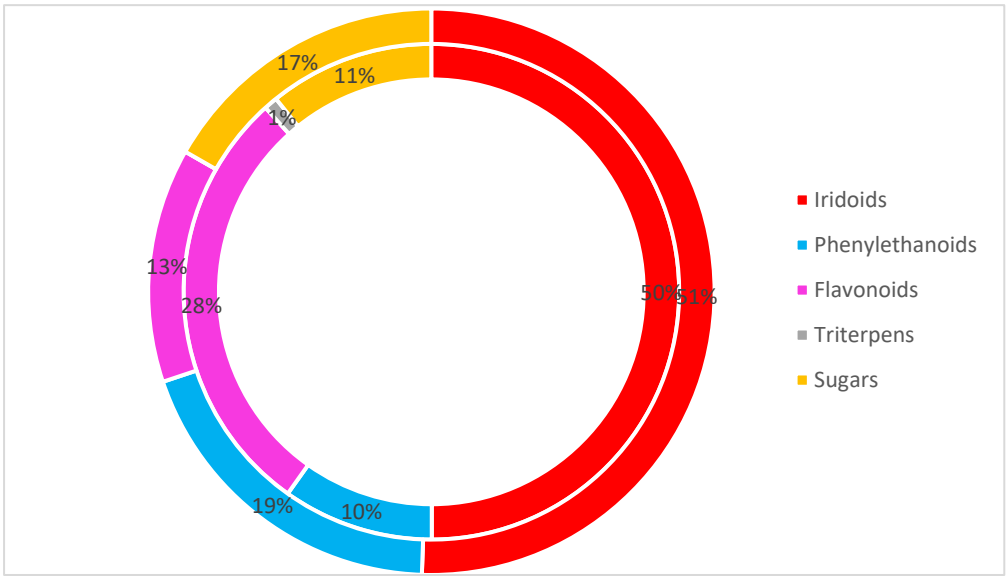

Figure 6. Metabolites repartition (% ELSD) of **w/e/g** (internal circle) and **fresh w/e/g** (external circle) extracts (organic acids and chlorophylls were not present in these extracts).

Considering the overall extraction yield and the percentage obtained by ELSD in the macerates, individual contents were calculated for each compound. A total of 39 compounds were identified, and Figure 7 shows the distribution of metabolites present in levels greater than 1 mg/g DW.

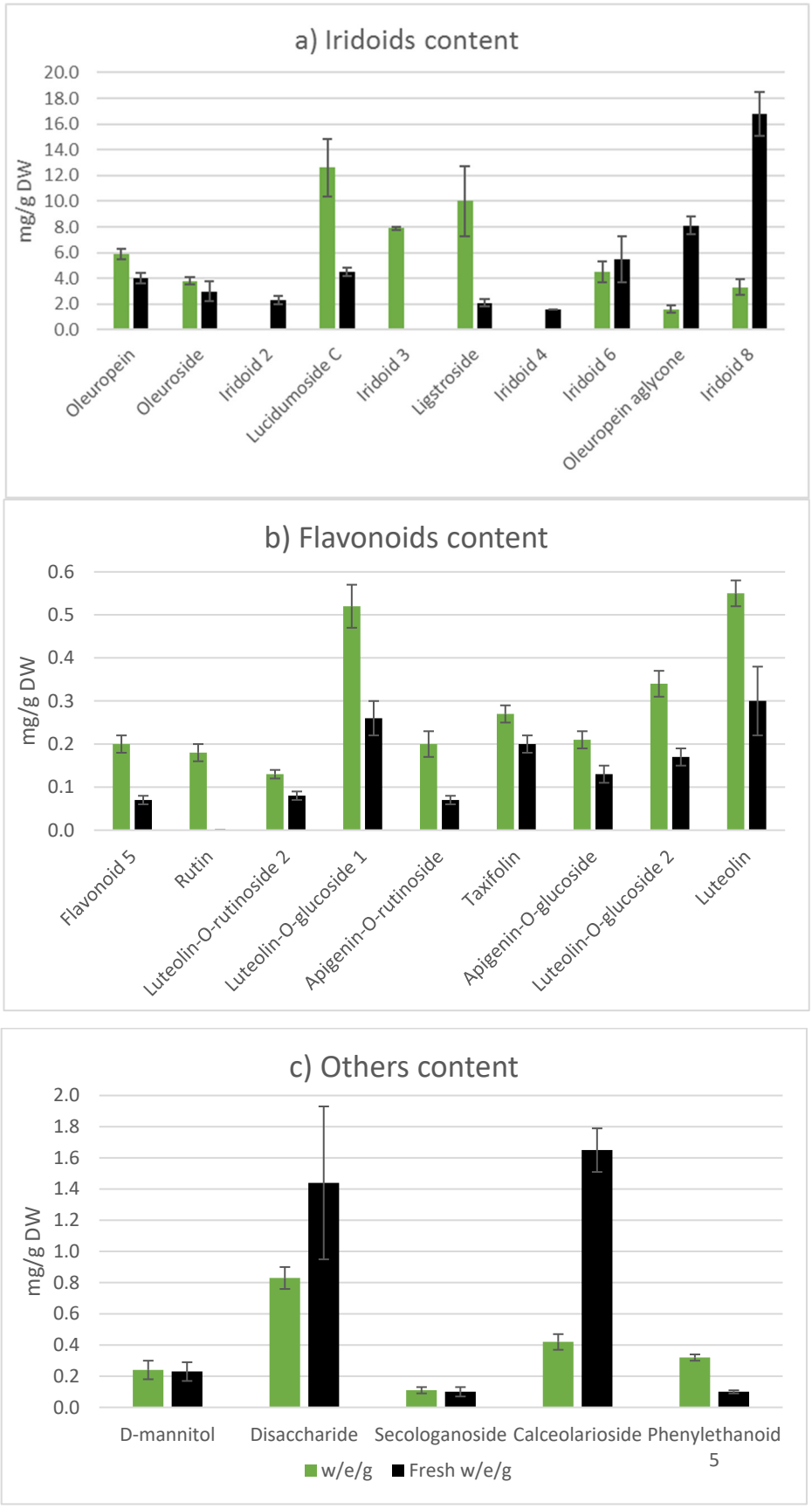

Figure 7. Iridoids (**a**), flavonoids (**b**), and other (sugar and phenylethanoid) (**c**) contents (mg/g DW) for **w/e/g** and **fresh w/e/g** extracts.

In agreement with the ELSD, the iridoid content of both macerates was quite similar (~50%, Figure 5), but their distribution differed depending on whether the raw material was frozen or not. Indeed, the **w/e/g** extract, obtained with frozen material, contained mainly lucidumoside C and ligstroside, while the **fresh w/e/g** was mainly characterized by an oleuropein aglycone and an unidentified iridoid (Table 3 and Figure 7a). **w/e/g** presented higher contents for each of the flavonoid-like compounds, and both macerates were characterized by high amounts of luteolin and luteolin glucosides (Figure 7b). The **w/e/g** extract (frozen material) showed a greater diversity of flavonoids, with 13 compounds identified compared to 9 for the **fresh w/e/g** extract.

The distribution of other metabolites differed mainly in the amounts of disaccharide and calceolarioside (Figure 7c).

The freezing step prior to maceration influenced the chemical composition of the macerate. Both macerates were composed of the same chemical classes, except for the iridoids, but their distribution was different.

4. Conclusions

The interest of a binary or ternary solvent containing glycerin for the maceration of buds was studied through a complete phytochemical analysis using HPLC-DAD-ELSD and HPLC-UV-MS^2. The choice of this type of extraction solvent is supported by a high chemical diversity and a high content of secondary metabolites. This study also revealed disparities in the chemical composition depending on whether the macerate was obtained with or without water in the ethanol–glycerin mixture (**w/e/g** and **w/g**, respectively). In fact, the presence of water in the solvent mixture modifies its polarity, which explains the qualitative and quantitative differences between the two macerates. The tri-solvent extraction mainly exhibited greater diversity in the composition of flavonoids and iridoids, while the binary system showed higher content of triterpenes and chlorophylls. In terms of extraction yield, the extract obtained with the **e/g** mixture was approximately two times higher than that obtained with **w/e/g** solvent. However, it should be considered that the commercial galenic formulation of **e/g** is consistently prepared with a 10-fold dilution, following the European Pharmacopoeia guidelines [15], resulting in a lower amount of secondary metabolites.

These observations show the importance of rigorous quality control for bud macerates in the market, as their composition is significantly influenced by the choice of the extraction solvent or solvent mixture.

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Appendix A

Table A1. Chemical class contents (mg/g DW) in **w/e/g** and **e/g** after virtual 10-fold dilution of **e/g** (in accordance with the European Pharmacopoeia recommendations).

	w/e/g	**e/g (10-Fold Diluted)**
Total sugars	10.2	1.0
Total organic acids	-	-
Total phenylethanoids	9.3	1.3
Total flavonoids	26.9	3.3
Total iridoids	47.0	7.9
Total triterpens	0.8	2.2
Total chlorophylls	-	1

Table A2. Individual metabolites repartition (% ELSD) of **w/e/g**, **e/g**, and fresh **w/e/g**.

	w/e/g	**e/g**	**Fresh w/e/g**
D-mannitol	2.4 ± 0.3	1.3 ± 0.4	2.3 ± 0.6
Disaccharide	8.3 ± 0.7	4.8 ± 0.8	14.4 ± 4.9
Total sugars	10.7 ± 1.1	6.1 ± 1.2	16.7 ± 5.5
Secologanoside	1.1 ± 0.2	4.3 ± 0.2	1.0 ± 0.3
Calceolarioside	4.2 ± 0.5	3.1 ± 0.2	16.5 ± 1.4
Phenylethanoid 1	-	-	0.2 ± 0.4
Phenylethanoid 2	0.8 ± 0.1	0.3 ± 0.5	-
Phenylethanoid 3	0.5 ± 0.4	-	-
Phenylethanoid 4	-	-	0.6 ± 0.1
Phenylethanoid 5	3.2 ± 0.2	-	1.0 ± 0.1
Total phenylethanoids	9.8 ± 1.3	7.7 ± 0.9	19.3 ± 2.2
Flavonoid 2	1.0 ± 0.2	-	-
Flavonoid 4	0.3 ± 0.5	-	-
Flavonoid 5	2.0 ± 0.2	0.8 ± 0.2	0.7 ± 0.1
Luteolin-O-rutinoside 1	0.6 ± 0.1	-	-
Rutin	1.8 ± 0.2	2.1 ± 0.2	-
Luteolin-O-rutinoside 2	1.3 ± 0.1	-	0.8 ± 0.1
Luteolin-O-glucoside 1	5.2 ± 0.5	6.1 ± 1.2	2.6 ± 0.4
Flavonoid 7	0.4 ± 0.6	-	0.5 ± 0.4
Apigenin-O-rutinoside	2.0 ± 0.3	1.5 ± 0.2	0.7 ± 0.1
Taxifolin	2.7 ± 0.2	1.5 ± 0.2	2.0 ± 0.2
Apigenin-O-glucoside	2.1 ± 0.2	1.5 ± 0.1	1.3 ± 0.2
Luteolin-O-glucoside 2	3.4 ± 0.3	4.4 ± 0.4	1.7 ± 0.2
Luteolin	5.5 ± 0.3	2.0 ± 0.2	3.0 ± 0.5
Total flavonoids	28.3 ± 3.7	19.9 ± 2.4	13.3 ± 1.7
Iridoid 1	0.3 ± 0.5	-	-
Oleuropein	5.9 ± 0.4	9.0 ± 0.4	4.0 ± 0.4
Oleuroside	3.8 ± 0.3	-	3.0 ± 0.8

Table A2. *Cont.*

	w/e/g	e/g	Fresh w/e/g
Iridoid 2	-	-	2.3 ± 0.3
Lucidumoside C	12.6 ± 2.2	7.2 ± 0.3	4.5 ± 0.3
Iridoid 3	7.9 ± 0.1	7.5 ± 1.0	-
Ligstroside	10.0 ± 2.7	8.6 ± 1.6	2.1 ± 0.3
Iridoid 4	-	-	1.6 ± 0.0
Iridoid 5	-	-	0.6 ± 1.0
Iridoid 6	4.5 ± 0.8	-	5.5 ± 1.8
Oleuropein aglycone	1.6 ± 0.3	9.1 ± 0.6	8.1 ± 0.7
Iridoid 7	-	-	0.8 ± 0.8
Iridoid 8	3.3 ± 0.6	5.6 ± 0.3	16.8 ± 1.7
Iridoid 9	-	0.2 ± 0.4	0.3 ± 0.5
Iridoid 10	-	-	0.7 ± 0.1
Iridoid 11	-	-	0.3 ± 0.5
Total iridoids	49.9 ± 8.0	47.2 ± 4.6	50.6 ± 9.1
Oleanolic acid	0.9 ± 0.0	13.2 ± 1.2	-
Total triterpens	0.9 ± 0.0	13.2 ± 1.2	-
Chlorophyll A	-	5.8 ± 1.6	-
Total chlorophylls	-	5.8 ± 1.6	-

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