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Dereplication of Natural Extracts Diluted in Propylene Glycol, 1,3-Propanediol and Glycerin. Comparison of *Leontopodium alpinum* Cass. (Edelweiss) Extracts as a Case Study

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Abstract: Many natural extracts used as cosmetic ingredients are available as solutions prepared in high-boiling-point solvents, called carrier solvents, such as propylene glycol (1,2-propanediol), propanediol (1,3-propanediol) and glycerin. The upstream chemical profiling of these extracts represents a major asset for the cosmetic industry, because it accelerates product development. A new workflow for the rapid characterization of the main metabolites present in natural extracts diluted in propylene glycol and 1,3-propanediol is presented here as an extension of previous works on glycerin-containing extracts. This method is an optimized version of a well-established dereplication procedure and consists of a fractionation by centrifugal partition chromatography followed by ^{13}C nuclear magnetic resonance analysis and dedicated data processing. The concentration by evaporation under reduced pressure was considered as a pertinent preliminary step, particularly adapted to the analysis of highly diluted extracts. A dried hydro-ethanolic extract of *Leontopodium alpinum* Cass. was prepared at laboratory scale and used for method validation. Three solutions at 5% wt. of dry extract were prepared with propylene glycol/water (1:1), 1,3-propanediol/water (1:1) and glycerin/water (1:1) as carrier solvents. The dereplication workflow was applied to the three resulting *L. alpinum* extracts. Each study led to the quick identification of 26 metabolites including five flavonoids (luteolin and its derivatives), five hydroxycinnamic acids (among which are leontopodic acids), sugars and organic acids.

Keywords: natural extract analysis; dereplication; propylene glycol; 1,3-propanediol; glycerin; *Leontopodium alpinum*; mixture analysis; centrifugal partition chromatography; NMR

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1. Introduction

The early chemical profiling of natural extracts incorporated in commercial cosmetic formulations is of prime importance for scientific, regulatory or safety reasons and, thus, accelerates the commercial product development process. In addition to gaining a thorough knowledge of extracts, it facilitates the detection of compounds that conflict with cosmetic regulations such as the Cosmetics Directive by the European Union and the US Federal Food, Drug and Cosmetic Act focused on consumer safety [1–3]. An increasing number of natural extracts are proposed to the market as solutions in high-boiling-point solvents, often called carrier solvents, such as glycerin or glycols. According to the Coptis ingredients database [4], which references more than 15,000 cosmetic ingredients, approximately one out of two commercial natural extracts is diluted in glycerin or in a glycol. The most represented glycols are 1,2- and 1,3-propanediol, known in International Nomenclature of Cosmetic Ingredients (INCI) as propylene glycol and propanediol, respectively. These glycols are increasingly added to formulations or used directly as extraction solvents especially in the case of natural extracts, as a texturizing agent or humectant but also for

stabilization and preservation purposes. Kerdudo et al. demonstrated that propylene glycol and propanediol have preservative properties [5]. The presence of the hydroxyl group on the alkyl chain on the glycol would favor water fixation and hinder the growth of bacteria and fungi. In 2019, Herman cited the propylene glycol in his study on antimicrobial ingredients as an alternative to cosmetic preservatives [6].

This article presents an original and efficient workflow for the rapid identification of the main compounds in natural extracts diluted in propylene glycol and propanediol, which complements the recently published work with a glycerinated extract [7]. The original identification procedure, published in 2014 [8], has already been used to characterize hundreds of dry extracts [9–13] both for academic or industrial purposes. It starts with a fractionation step of the starting extract by centrifugal partition chromatography (CPC) followed by the ^{13}C nuclear magnetic resonance (NMR) analysis of each chemically simplified CPC fraction. Hierarchical clustering analysis (HCA) is performed after alignment of NMR resonance peak data. HCA allows the grouping of chemical shifts into clusters based on the pattern recognition of their varying intensities in each of the CPC fractions, thereby enabling to relate the clusters to constituents of the extract. These chemical shift clusters are then used as search keys in a locally developed database containing metabolite structures and the corresponding predicted NMR spectral data. At the end, likely annotations of peak clusters are confirmed by additional analyses to obtain a global identification of the main constituents of the extract.

While this procedure is highly efficient for dry extracts, it cannot be directly applied to extracts diluted in glycerin or glycols, especially when dry matter content is low. A study of the Coptis ingredient database [4] shows that the average amount of dry plant material in the referenced glycerin extracts is 6.5 and 3.7% wt. in the extracts diluted in glycols. This low concentration of natural extracts in glycerin or glycols hampers the dereplication process for two main reasons. Firstly, the need to inject large volumes of carrier solvents in the CPC column to provide quantities of metabolites in the fractions compatible with ^{13}C NMR analysis can dramatically disrupt both the hydrodynamic behavior and the thermodynamic equilibrium of the CPC biphasic solvent system. Secondly, it makes NMR analysis more complex due to the very intense solvent peaks and due to the presence of numerous artefacts they induce in the spectra. These issues were recently overcome for glycerinated extracts by optimizing the original procedure through a combination of a physical suppression of glycerin by CPC and of a spectroscopic suppression of glycerin signals by presaturation in ^{13}C NMR [7]. Moreover, the presaturation in ^{13}C NMR solvent signals was already reported for the signals of the two propanediols, leading to an intensity decrease by at least 94% and to the suppression of the related artefacts [14].

The physical suppression of the solvent was investigated via two approaches. Firstly, a sample pretreatment for solvent evaporation was considered. Secondly, a separation by gradient elution CPC without prior sample pretreatment was considered. These results and those previously published on glycerinated extracts were combined to develop a general workflow dedicated to the dereplication of extracts diluted in propylene glycol, propanediol or glycerin, hereafter illustrated by the chemical characterization of an edelweiss extract diluted in propylene glycol, propanediol or glycerin.

Edelweiss (*Leontopodium alpinum* Cass.) is a rare alpine plant that grows naturally between 1000 and 3400 m above sea level [15]. It has a small size, and its leaves bear hairs to provide thermal regulation in harsh climatic conditions. Edelweiss naturally synthesizes numerous antioxidant molecules as protection agents against UV radiation exposure, which is particularly intense in its high-altitude biotope. Among them, phenolic acids, flavonoids, sesquiterpenes, coumarins and benzofurans were reported [15–18].

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and Reagents

Ethyl acetate (EtOAc), acetonitrile (CH_3CN), *n*-butanol (*n*-BuOH), methanol (MeOH), ethanol (EtOH), methyl *tert*-butyl ether (M*t*BE) and formic acid (HCOOH) were purchased from Carlo Erba reagents (Val de Reuil, France). Acetic acid (CH_3COOH) and 1,2-propanediol (99%) were purchased from VWR (Fontenay-sous-bois, France) and 1,3-propanediol from Alfa Aesar (Kandel, Germany). Glycerin was kindly provided by Pierre Fabre Dermo-Cosmétique (Toulouse, France).

2.1.2. Plant Material and Preparation of the Extracts

The dry aerial parts of edelweiss, *Leontopodium alpinum* Cass. (Asteraceae), were cultivated in organic farming conditions in the Swiss Alps (Valais, 1500 m altitude) and were provided by DSM Nutritional Products LTD (Wurmisweg, Switzerland). The dried plant material (300.2 g) was macerated in 60% EtOH (4 L, 24 h) at room temperature. After evaporation at 40 °C under vacuum, 75.1 g of dried extract was obtained (25% yield). Three 5 g samples were dissolved in 95 g propylene glycol/water 1:1 (*w/w*), 95 g propanediol/water 1:1 (*w/w*) and 95 g glycerin/water 1:1 (*w/w*) to provide three edelweiss extracts diluted to 5% wt. in propylene glycol, propanediol and glycerin to mimic industry marketed extracts.

2.2. Ternary Diagrams

The stability of two biphasic systems upon addition of propylene glycol, propanediol and glycerin was evaluated by a method reported by Marchal et al. [19]. It relies on the drawing of a pseudo ternary diagram in which the mobile phase, the stationary phase and the putatively destabilizing compound intervene at the apexes. The carrier solvent was successively added to mobile and stationary phases present in predefined ratios: 0/1, 1/7, 1/3, 3/5, 1/1, 5/3, 3/1, 7/1 and 1/0 (*w/w*). The solvent mixture was shaken and then visually inspected for the presence of one or two phases. If the mixture became monophasic, the corresponding composition (*w/w/w*) of this mixture determined as amounts of upper phase, mobile phase and carrier solvent was reported on the phase diagram in its orthogonal representation. Then, the binodal curve was drawn by connecting the different demixing points. Propylene glycol, propanediol and glycerin were evaluated on EtOAc/CH_3CN/water 3:3:4 (*v/v/v*) and M*t*BE/*n*-BuOH/water 45:5:50 (*v/v/v*) biphasic systems.

2.3. Evaporation of Propanediols

The evaporation of propylene glycol and propanediol was undertaken on a Genevac HT Series (SP Scientific, Warminster, PA, USA). The lowest achievable pressure is less than 0.01 mbar (hereafter, "full vacuum"). Two set of operating conditions were used to evaporate each of these two high-boiling-point solvents at a 1 g scale. The temperature was set at 30 and 40 °C for propylene glycol and propanediol, respectively. The evaporation process started with a decrease in pressure from atmospheric pressure to full vacuum in 1 h, and then, full vacuum was maintained for 8 h and 14 h for propylene glycol and propanediol, respectively. These operating conditions were tested simultaneously on 72 one-gram samples and resulted in the complete evaporation of the carrier solvents in one run.

The concentration of the two edelweiss extracts diluted in propylene glycol/water 1:1 (*w/w*) and propanediol/water 1:1 (*w/w*) was performed by applying the evaporation conditions described above. The evaporation processes were repeated twice on 6 one-gram samples of the extract diluted in propylene glycol. This experiment was performed in triplicate. The first run concentrated the extract from 5 to 8.8 $\pm$ 0.1% wt. and the second run to 27.4 $\pm$ 3.1% wt. The same procedure was performed with the extract diluted in propanediol. The first run concentrated the extract from 5 to 8.6 $\pm$ 0.1% wt. and the second run to 9.6 $\pm$ 0.1% wt. Details as means and relative standard deviations of these experimental figures are reported in Tables S1 and S2.

In order to obtain an appropriate mass of concentrated extracts, 40 g of both edelweiss extracts diluted in propylene glycol and propanediol carrier solvents was concentrated by the selected method: two runs of the propylene glycol program and only a single run of the propanediol program as far as the second evaporation does not concentrate the sample significantly. These procedures resulted in 6.8 and 23.1 g of edelweiss extract diluted in propylene glycol and propanediol, respectively. Analysis on concentrated extracts was performed by LC-MS and NMR to ensure that extracts were not chemically transformed by the applied evaporation processes (Figures S1 and S2).

2.4. Centrifugal Partition Chromatography

CPC experiments were carried out using a lab-scale FCPE300® column of 303 mL inner volume (Kromaton Technology, Angers, France). The column was composed of 7 circular partition disks, each engraved with 33 twin-cells of 1.0 mL. The liquid phases were pumped by a preparative 1800 V7115 pump (Knauer, Berlin, Germany). Fractions of 20 mL were collected by a Labocol Vario 4000 (Labomatic Instruments, Allschwil, Switzerland).

Five CPC fractionations of edelweiss extracts were performed: one for each of the two extracts in propanediols with sample preparation by evaporation, one for each of the two extracts in propanediols without sample preparation and finally one with the glycerinated extract. Details of each fractionation are presented in Table 1. The method employed for the processing of the glycerinated extract has been previously described in [7]. First, solvent systems were prepared in a separatory funnel by mixing in the required amounts of pure solvents. After shaking and decantation, the stationary and mobile phases were separated. Each fractionation was performed in ascending mode, which means that the lower phase of the solvent system constitutes the stationary phase, and the upper phase is the mobile phase. The CPC column was filled with the stationary phase at a flow rate of 100 mL/min and at a rotation speed of 500 rpm. Then, the rotation speed was increased to 1200 rpm, and the column was equilibrated at 20 mL/min with the mobile phase. Measured stationary phase retentions were more than 70%. All samples, diluted in stationary and mobile phases or not, were injected through a 35 mL injection loop. The elution method was then started.

Table 1. Operating conditions of centrifugal partition chromatography (CPC) fractionations.

	Propanediols Extracts with Sample Preparation (a,b)	**Propanediols Extracts Without Sample Preparation (c,d)**	**Glycerin Extract (e)**
Biphasic solvent system	EtOAc/CH_3CN/water 3:3:4 (*v*/*v*/*v*)	M*t*BE/*n*-BuOH/water from Σ_i 45:5:50 to Σ_f 5:45:50 (*v*/*v*/*v*)	EtOAc/CH_3CN/water 3:3:4 (*v*/*v*/*v*)
Mode	Ascending	Ascending	Ascending
Elution mode	Isocratic	Gradient	Isocratic
Stationary phase retention	73%	72%	73%
Mass of sample injected (dilution in stationary/mobile phases)	6.6 g (14/4 mL) - 21.7 g (10/2 mL)	34.989 - 30.195 g	39.8 g
Elution	0 to 20 mL/min in 5 min; 20 mL/min for 35 min	Up_i 0 to 20 mL/min in 5 min; 100% Up_i for 10 min; from 100% Up_i to 100% Up_f in 1 h; 100% Up_f for 45 min	0 to 20 mL/min in 5 min; 20 mL/min for 65 min
Extrusion time	20 min	20 min	20 min
Number of pooled fractions	9	12	13
Recovery	89–89%	93–95%	110%

Performed on (a) propylene glycol edelweiss extract diluted at 27.4 ± 3.1% wt. after sample preparation by evaporation; (b) propanediol edelweiss extract diluted at 8.6 ± 0.1% wt. after sample preparation by evaporation; (c)/(d) edelweiss extract diluted at 5% wt. in propylene glycol/water and propanediol/water, respectively; (e) edelweiss extract diluted at 5% wt. in glycerin/water.

Fractionations on extracts with sample preparation were performed in isocratic elution. The mobile phase was thus pumped at 20 mL/min for 40 min. For the fractionations with gradient elution method, the upper phase of the solvent system M*t*BE/*n*-BuOH/water 45:5:50 (*v/v/v*) was used as the initial mobile phase. After loading the sample solution, the flow rate was progressively increased from 0 to 20 mL/min in 5 min and maintained at 20 mL/min for 10 min. The gradient elution was then performed at 20 mL/min by pumping from 100% of the upper phase of the initial mobile phase system to 100% of the upper phase of the final mobile phase system (MtBE/*n*-BuOH/water 5:45:50 (*v/v/v*)). One hundred percent of the final mobile phase was finally introduced for 45 min.

At the end of the elution, the most hydrophilic compounds retained inside the column were extruded in each experiment by pumping fresh stationary phase for 20 min, maintaining the flow rate at 20 mL/min. A stationary phase loss was observed in the fractionations of propylene glycol and propanediol extracts with isocratic elution. This phenomenon reflected a disturbance in the biphasism of the solvent system used. This conclusion was confirmed by the measurement of the volume of retained stationary phase at the end of elution. Only 30 and 15 mL of stationary phase were recovered during the extrusion of fractionations on propylene glycol and propanediol extracts, respectively.

Collection tubes of 20 mL were spotted on Merck thin layer chromatography (TLC) plates coated with silica gel 60 F_{254} and developed with EtOAc/CH_3COOH/HCOOH/water 100:11:11:26 (*v/v/v/v*). After inspection at 254, 366 nm and under visible light and chemical revelation with Neu reagent (1.25 g of diphenylborinic acid aminoethylester dissolved in 250 mL of EtOAc), fractions were pooled according to their composition. The pooling details are available in Table S3.

2.5. NMR Analysis and Dereplication

NMR analyses were performed at 298 K on an Avance AVIII-600 spectrometer (Bruker, Karlsruhe, Germany) equipped with a cryoprobe optimized for ^{1}H detection and fitted with cooled ^{1}H, ^{13}C, and ^{2}H coils and preamplifiers. Dry fractions were dissolved in 600 µL of DMSO-d_6 and analyzed by ^{13}C NMR spectroscopy by means of the uniform driven equilibrium Fourier transform (UDEFT) pulse sequence with an acquisition time of 0.36 s and a relaxation delay of 3 s. A satisfactory signal-to-noise ratio was reached by accumulation of 1024 transients. The receiver gain was set to the highest possible value. The spectra were manually phased and baseline-corrected using the TOPSPIN v4.0.5 software (Bruker). The central resonance of DMSO-d_6 was set at 39.8 ppm for spectrum referencing. Additional homonuclear correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra were also recorded on dry fractions. For fractions in which propanediols and glycerin were still present in large proportion, the NMR samples were prepared with 200 mg of the fraction in 600 µL of DMSO-d_6. The presaturation sequence described in Canton et al. [14] was used to decrease the dominating ^{13}C NMR solvent signals and facilitate the identification of metabolites eluted in fractions still containing high amounts of carrier solvent. The presaturation field of intensity $\Omega 1/2\pi$ = 11.7 Hz was focused on the solvent resonances and applied for 3 s before the 90° pulse in a modified Bruker *zgpg* pulse sequence.

The dereplication method was the one developed by Hubert et al. [8]. After manual removal of residual solvent peaks from peak lists, all ^{13}C NMR signals were automatically collected on each spectrum. The resulting peak lists were stored in a text file from which a locally developed algorithm written in Python language aligned the NMR peak positions in regularly spaced chemical shift windows ($\Delta\delta$ = 0.3 ppm). The table of peak intensity values according to chemical shift bin index and fraction index was imported into the PermutMatrix v1.9.3 software (LIRMM, Montpellier, France) for hierarchical clustering analysis. The resulting ^{13}C NMR chemical shift clusters were visualized as dendrograms on a two-dimensional (2D) heat map. In order to identify compounds, the elements of each chemical shift cluster were used as a search key in a local database built using the

ACD/C+H NMR Predictors and DB software (Advanced Chemistry Development, Inc., Toronto, ON, Canada). This database contains more than 3050 compounds to date and associates structures to the predicted NMR chemical shifts of the hydrogen and carbon atoms, as calculated by the ACD/Labs predictors. A literature survey was carried out on the genus *Leontopodium*, resulting in 36 metabolites stored in the database [15–18,20–23]. A tolerated ^{13}C NMR chemical shift difference between the predicted database spectrum and the real spectrum was set at 2 ppm for spectra comparisons. Finally, each structure proposition issued by database query was confirmed by interpretation of 1D and 2D NMR data (^{1}H NMR, HSQC, HMBC, COSY) and LC-MS analyses.

2.6. Analyses by LC-MS and HPTLC

LC-MS analyses were performed with an Acquity UPLC H-Class (Waters, Manchester, UK) system coupled to a Synapt G2-Si (Waters) equipped with an electrospray (ESI) ion source. Chromatographic separation was achieved on a Kinetex C18 column (150 × 2.1 mm, 2.6 µm; Phenomenex, Le Pecq, France). The column temperature was regulated at 30 °C. Compounds were eluted with a gradient of water (Eluent A) and CH_3CN (Eluent B) with 0.1% formic acid in each mobile phase. The mobile phase flow rate was maintained at 0.6 mL/min, and the gradient was designed as follows: t = 0 min, 5% B; t = 1 min, 5% B; t = 9 min, 40% B; t = 15 min, 100% B; t = 17 min, 100% B; t = 17.1 min, 5% B until 19 min. Five microliters of crude edelweiss extracts diluted at 40 g/L in water were injected as well as 0.5 µL of CPC fractions diluted at 2 g/L in MeOH or water. MS analyses were performed in negative (ESI^-) ionization mode. The electrospray interface operated with the following parameters: capillary voltage 2 kV; desolvation temperature 450 °C; desolvation gas flow 950 L/h; source temperature 120 °C; cone voltage 40 V; cone gas flow 50 L/h; scanning range of *m/z* 50–2000.

High performance thin layer chromatography (HPTLC) analyses were carried out at the end of each CPC fractionation. They were performed on Merck HPTLC plates 10×20 coated with silica gel 60 F_{254}. Samples were laid on plates by an automatic TLC sampler (ATS 4) and eluted in an automatic development chamber (ADC 2) (CAMAG, Muttenz, Switzerland). Twelve micrograms of dry CPC fractions, or 30 to 240 µg of solvent-containing CPC fractions were deposited with a band length of 8 mm. The developing solvents were EtOAc/CH_3COOH/HCOOH/water 100:11:11:26 (*v/v/v/v*). First, the plates were visualized at 254, 366 nm and under visible light. Then, the plates were heated at 100 °C with a TLC plate heater 3 (CAMAG) for 3 min, immersed in a solution of Neu reagent and photographed under UV light illumination at 366 nm and under white light.

3. Results and Discussion

3.1. Development of a Strategy for the Physical Suppression of Propylene Glycol and Propanediol in Natural Extracts

The aim of this research work was to develop an original and efficient workflow for the rapid analysis of the major compounds in natural extracts diluted in propylene glycol or propanediol, two carrier solvents largely present in marketed cosmetic ingredients. The adopted strategy is based on the one used in our previous works on the dereplication of glycerinated natural extracts [7]. Nevertheless, the physical removal of carrier solvent by CPC fractionation using the biphasic solvent system EtOAc/CH_3CN/water 3:3:4 (*v/v/v*), as used for glycerinated extracts, cannot be applied as such to extracts prepared in propanediols. Indeed, the injection of a large amount of propylene glycol or propanediol dramatically disturbs both the thermodynamic equilibrium of the CPC biphasic solvent system and the hydrodynamic behavior of the latter, making any CPC experience impossible. The effect of the addition of propanediol or propylene glycol compared to glycerin can be highlighted by means of the size of the biphasic zone of pseudo ternary phase diagrams "glycerin or propanediol or propylene glycol/upper organic phase of (EtOAc/CH_3CN/water 3:3:4)/lower aqueous phase of (EtOAc/CH_3CN/water 3:3:4)" (Figure 1) [19]. Whereas in Figure 1a, the system remained biphasic even with 90% wt.

glycerin added to the solvent system; the addition of propanediol or propylene glycol quickly led to a monophasic mixture as shown by the area of the biphasic zone, delimited by the binodal curve. As little as 16% wt. of propylene glycol (Figure 1b) and 27% wt. of propanediol (Figure 1c) added to the upper and lower phases in proportion 1:1 (*w/w*) caused the initial biphasic system EtOAc/CH_3CN/water 3:3:4 (*v/v/v*) to become monophasic. Such extracts cannot be directly fractionated with the solvent system EtOAc/CH_3CN/water 3:3:4 (*v/v/v*) in contrast to the fractionation of glycerinated extracts for which the ternary diagram showed a high stability.

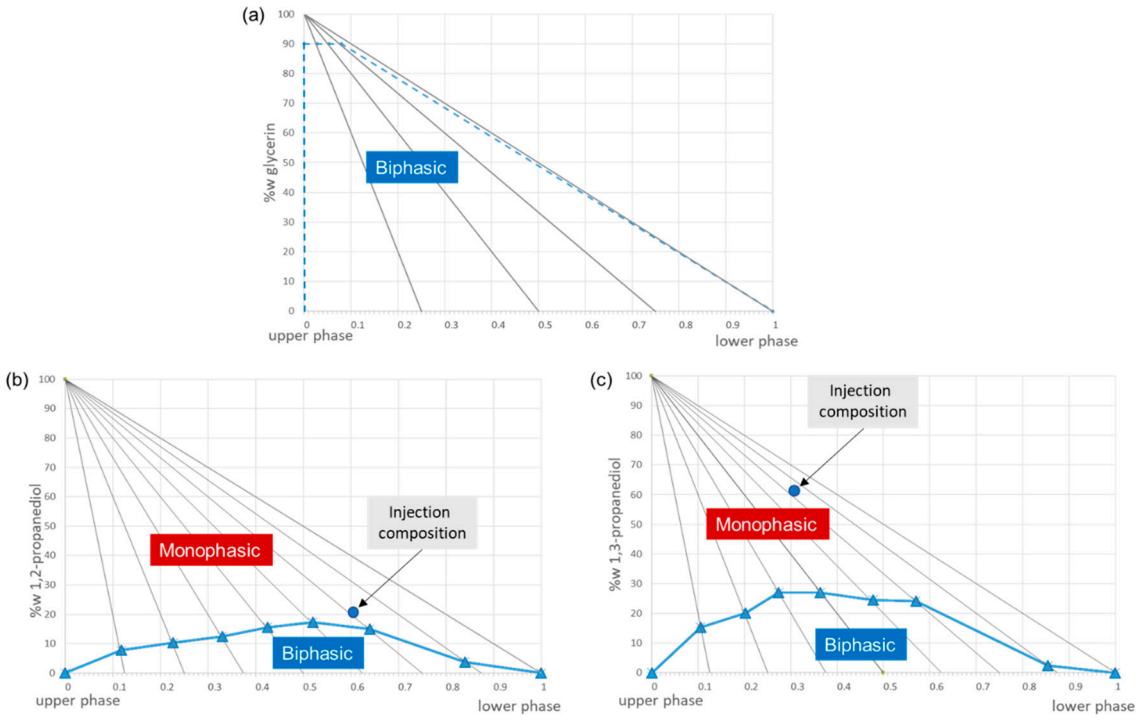

Figure 1. Ternary phase diagram of upper phase, lower phase and (**a**) glycerin, (**b**) propylene glycol, (**c**) propanediol (*w/w/w*) for the EtOAc/CH_3CN/water 3:3:4 (*v/v/v*) solvents system.

The binodal curve was drawn by connecting the demixing points symbolized by triangles in (b) and (c), while in (a), none were observed before the addition of 90% by weight of glycerin (maximum points studied). Experiments were performed at 20 °C.

Therefore, a preliminary step of sample preparation by evaporation was proposed for the removal of propylene glycol and propanediol from the extracts. Indeed, although the boiling point of the two carrier solvents is high, 187 °C for propylene glycol, 213 °C for propanediol at atmospheric pressure, their vapor pressures, about 0.1 mbar at 20 °C, with a slightly lower value for propanediol, are attainable. Solvent evaporation was thus attempted on a high-performance centrifugal vacuum evaporator, allowing parallel evaporation. A low temperature was selected in order to avoid artefact formation during sample preparation. Evaporation parameters were optimized and led to propose a 9-h program at 30 °C and a 15-h program at 40 °C to evaporate pure propylene glycol and propanediol, respectively.

These evaporation conditions were then tested on a dried hydro-ethanolic extract of aerial parts of *Leontopodium alpinum* Cass. (edelweiss) diluted at 5% wt. in propylene glycol/water 1:1 (*w/w*) or in propanediol/water 1:1 (*w/w*).

The results, summarized in Tables S1 and S2, were not a total evaporation of the carrier solvent as expected but rather a concentration of the plant material in the extracts from 5 to 8.8 ± 0.1% wt. and to 8.6 ± 0.1% wt. after one run of the propylene glycol and propanediol dedicated evaporation conditions, respectively. If a second run led to

a significant concentration of 27.4 ± 3.1% wt. for propylene glycol, its usefulness was much lower for propanediol (from 8.6 ± 0.1% wt. to 9.7 ± 0.1% wt.). With regards to ^{1}H NMR spectra recorded before and after evaporation (Figure S3), the water from the carrier solvents was fully evaporated in all cases. The evaporation method dedicated to propylene glycol was carried out on an aqueous extract of poplar bark extract diluted at 2% in propylene glycol/water 1:1 (*w/w*) (data not shown). It led to the concentration of the extract from 2 to 21% in a single run demonstrating that the evaporation level of the studied carrier solvents seemed to depend on extract composition.

To summarize the results of the study of carrier solvent evaporation, 40 g of edelweiss extract diluted at 5% wt. in propylene glycol, and water was reduced in two runs to 6.8 g of an extract concentrated at 27.4 ± 3.1% wt. In addition, the same mass of extract diluted in propanediol and water was reduced in only one run, the second one bringing no significant improvement, leading to 23.1 g of extract concentrated at 8.6 ± 0.1% wt. The two concentrated extracts were compared to the initial extracts by LC-MS and NMR. Chemical profiles on chromatograms and spectra, present in Figures S1 and S2, were similar, thus confirming the chemical stability of the metabolites under the selected solvent evaporation conditions.

The dereplication workflow was thus started on the concentrated edelweiss extracts using the previously described procedure involving among others a CPC fractionation step with the biphasic solvent system EtOAc/CH_3CN/water 3:3:4 (*v/v/v*) in the isocratic ascending elution mode [7]. Although the preliminary partition tests showed a good distribution of the sample compounds between the two phases of the biphasic solvent system (Figure S4), the fractionation by CPC was not satisfactory. Indeed, a significant loss of stationary phase occurred during the CPC run leading to a final stationary phase retention (Sf) lower than 10% (Sf was approximatively 70% before the injection step), resulting in a poor separation (Figure 4a,c). As shown on the pseudo ternary phase diagrams, the compositions of the two injected samples are in the monophasic zones (Figure 1). However, the situation seems to be less unfavorable for propylene glycol (due to the more efficient evaporation pretreatment). Nevertheless, the pumping of the mobile phase after the injection step and the dilution in the pre-equilibrated column with Sf = 70% did not allow the compensation of both the thermodynamic and hydrodynamic disturbances caused by the propylene glycol but also probably by the compounds contained in the edelweiss extract.

A new method to fractionate edelweiss extracts diluted in propanediols had to be developed. After having tested numerous biphasic solvent systems, the normal phase gradient elution mode in CPC using the "type 2" biphasic solvent system M*t*BE/*n*-BuOH/water was thus attempted. To the best of our knowledge, this solvent system is used for the first time in gradient solid-support free liquid–liquid chromatography, including CPC and countercurrent chromatography. Gradient elution mode in CPC implies that the composition of the stationary phase is not very highly affected by the change in composition of the mobile phase [24,25]. Thus, it is mainly applicable to type 2 biphasic solvent systems (two miscible solvents are not miscible with the third one), as the ternary phase diagram has converging tie lines. The M*t*BE/*n*-BuOH/water solvent system shows such a type 2 ternary diagram (Figure 2), allowing its use for an organic mobile phase polarity gradient (ascending mode) [26].

Pseudo ternary diagrams "glycerin or propanediol or propylene glycol/upper organic phase of M*t*BE/*n*-BuOH/water (45:5:50 *v/v/v*)/lower aqueous phase of M*t*BE/*n*-BuOH/water (45:5:50 *v/v/v*)" were drawn in order to anticipate the problems encountered with the EtOAc/CH_3CN/water solvent system and to evaluate the stability of the system during sample injection (Figure 3). A better stability of the biphasic system during the injection step is thus expected, as the pseudo ternary phase diagrams showed larger biphasic areas (ternary diagrams present maxima for the binodal curves of 53 and 78% wt. for propylene glycol and propanediol, respectively) than the ones previously observed for the EtOAc/CH_3CN/water solvent system.

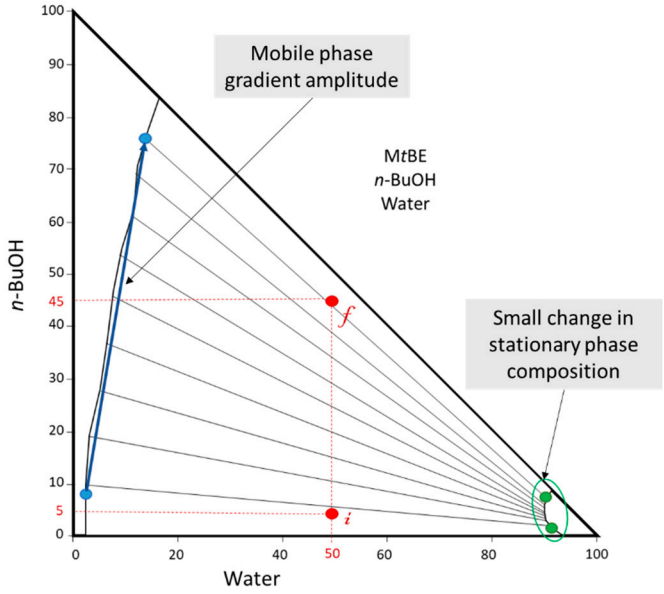

Figure 2. Ternary diagram of M*t*BE/*n*-BuOH/water: type 2 diagram allowing a fractionation by gradient elution CPC. Red markers refer to initial (i) and final (f) conditions. Green and blue markers refer to the composition of lower and upper phases, respectively. Compositions in terms of MtBE/n-BuOH/water for initial mobile phase 89/8.5/2.5 (*v*/*v*/*v*), final mobile phase 7/76/17 (*v*/*v*/*v*), initial stationary phase 7.5/1.5/91 (*v*/*v*/*v*) and final stationary phase 7/3/90 (*v*/*v*/*v*).

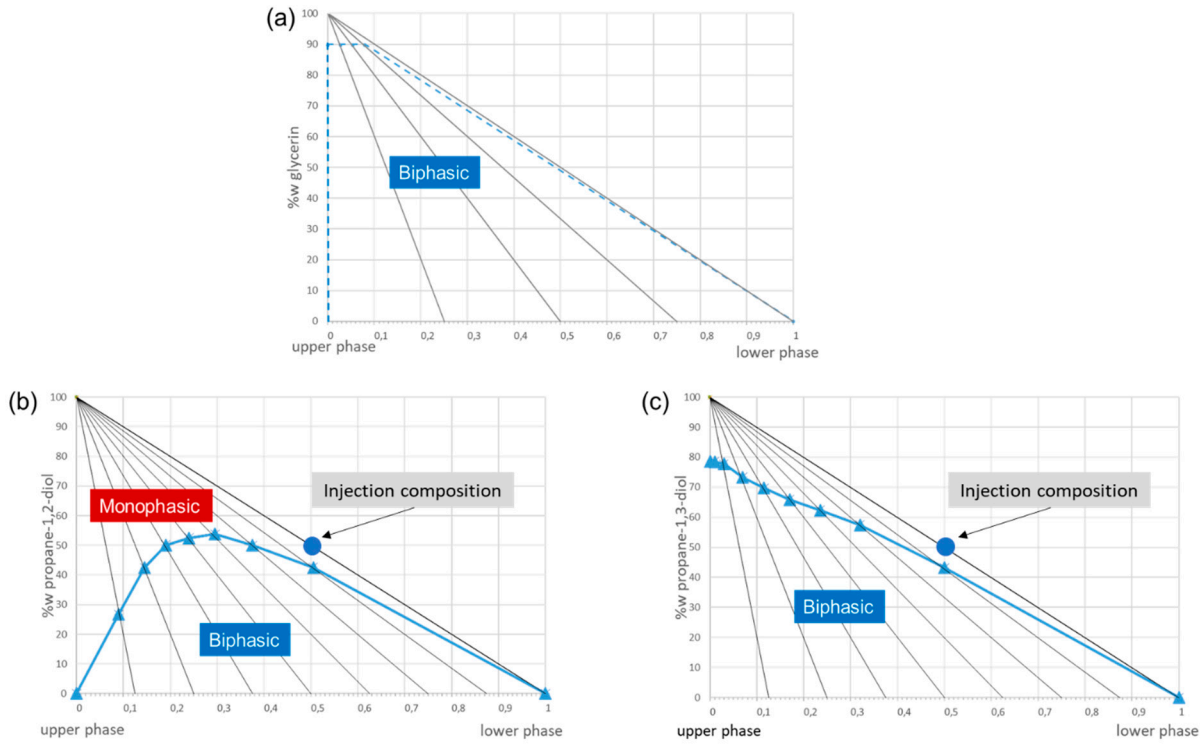

Figure 3. Ternary diagram of upper phase, lower phase and (**a**) glycerin, (**b**) propylene glycol and (**c**) propanediol for the M*t*BE/*n*-BuOH/water 45:5:50 (*v*/*v*/*v*) solvent system. Demixing points are symbolized by triangles in (**b**,**c**), while in (**a**), none were observed before the addition of 90% by weight of glycerin (maximum points studied).

CPC fractionations were then undertaken on edelweiss extracts diluted in propanediol or propylene glycol without any sample preparation. The injected sample masses were

35 g for the propylene glycol edelweiss extract (5 g of the edelweiss extract in 95 g of a 1:1 *w/w* propylene glycol/water mixture) and 30 g for the propanediol edelweiss extract (5 g of the edelweiss extract in 95 g of a 1:1 *w/w* propanediol/water mixture). The normal phase polarity gradients were carried out for 120 min from the initial mobile phase (M*t*BE/*n*-BuOH/water, 89:8.5:2.5 *v/v/v*) to the final mobile phase (M*t*BE/*n*-BuOH/water, 7:76:17 *v/v/v*), and they were followed by a stationary phase extrusion step [27] in order to recover the most polar compounds trapped in the aqueous stationary phase. No significant perturbation of the biphasic solvent system behavior was observed, and the fractionation could be successfully completed even though the injected sample, representing about 10% of the column volume, was mostly composed of carrier solvent. This result is in agreement with the conclusions made on the basis of the study of pseudo ternary phase diagrams. Indeed, assuming that the stationary phase of the biphasic solvent system M*t*BE/*n*-BuOH/water is mainly composed of water, one can plot the injection sample compositions. The corresponding points are very close to the biphasic zones, the latter covering a large surface, which is a guarantee of the robustness of the two-phase system when carrier solvents are added. In both cases, edelweiss metabolites were progressively eluted, leading to a fractionation profile compatible with the following steps of the dereplication workflow involving the recording of NMR data. The collected tubes were grouped into 12 fractions in both experiments and evaporated to dryness when possible. Carrier solvents were trapped in the seventh fraction predominantly. Figure 4 shows the HPTLC fractograms of the four CPC fractionations performed on the edelweiss extract diluted in carrier solvents (propanediol or propylene glycol as carrier solvent and with an isocratic or a gradient elution). These HPTLC fractograms confirm the best fractionation quality when using the gradient elution mode with the M*t*BE/*n*-BuOH/water biphasic solvent system together with the carrier solvent trapping in fractions 7. This protocol is, therefore, efficient for a physical suppression of both propylene glycol and propanediol without the need of sample pre-treatment.

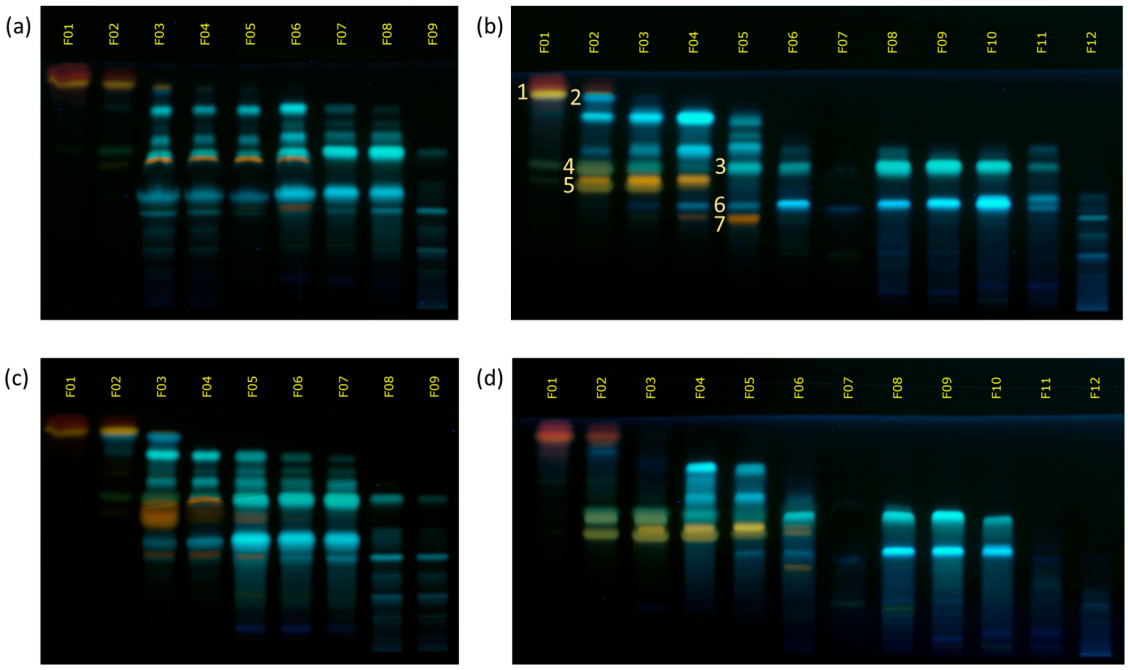

Figure 4. Summary HPTLC of CPC fractionations on edelweiss extracts diluted in: (**a**,**b**) propanediol and (**c**,**d**) propylene glycol. (**a**,**c**) refer to the fractionation performed after sample preparation by evaporation, (**b**,**d**) refer to the fractionation without sample preparation. Elution TLC solvents: EtOAc/CH_3COOH/HCOOH/water 100:11:11:26 (*v/v/v/v*); derivatization: neu reagent; visualization 366 nm. 1: luteolin; 2: caffeic acid; 3: leontopodic acid; 4: luteolin-3′-O-β-D-glucoside; 5: luteolin-4′-O-β-D-glucoside, luteolin-7-O-β-D-glucoside; 6: chlorogenic acid; 7: 6-hydroxy-luteolin-7-O-β-D-glucoside.

3.2. Workflow for the Dereplication of Natural Extracts Diluted in Glycerin, Propylene Glycol and Propanediol

The general workflow drawn in Figure 5 was proposed for the rapid identification of the main compounds in a natural extract diluted in glycerin, propylene glycol and propanediol, taking into account the results of the present study and of previous ones [7,14].

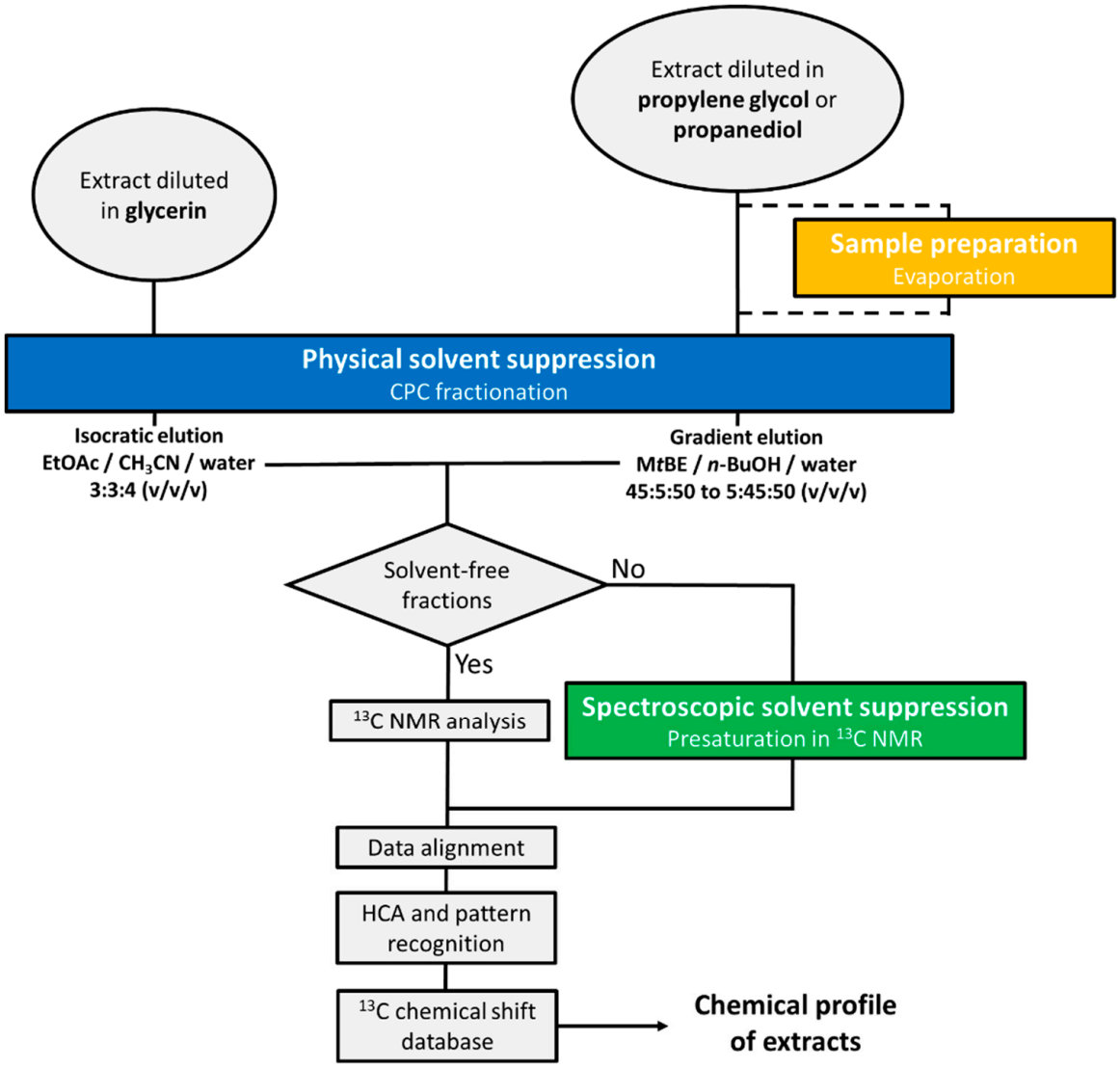

Figure 5. Workflow for the dereplication of extracts diluted in propylene glycol, propanediol and glycerin.

The workflow starts with the physical suppression of solvent by CPC fractionation. An optional sample preparation step by evaporation may also be extremely useful in the case of highly diluted extracts in propanediols. The methods developed for pure propanediols do not concentrate plant extracts in the same way. The evaporation time, or the temperature, should, therefore, be modulated according to the nature of the extracts.

Then, two solvent suppression methods by CPC are involved depending on the nature of the carrier solvent. A physical suppression performed using an isocratic elution with a biphasic solvent system such as EtOAc/CH_3CN/water 3:3:4 (*v*/*v*/*v*) for glycerinated extracts is proposed. For the extracts diluted in propylene glycol or propanediol, this step can be achieved by gradient elution with the system M*t*BE/*n*-BuOH/water from 45:5:50 to 5:45:50 (*v*/*v*/*v*). At the end of this step, 10 to 20 chemically simplified fractions are obtained. Some of them contain carrier solvent, while others can be evaporated to dryness.

The following part of the dereplication process consists of the ^{13}C NMR analysis of each CPC fraction. Dry fractions are directly analyzed by ^{13}C NMR. For fractions containing large amounts of carrier solvent, ^{13}C analysis is preferentially undertaken with a specific presaturation sequence targeted on the 1,2-propanediol (propylene glycol), 1,3-propanediol (propanediol) or glycerin signals [14]. NMR data (chemical shifts and intensities) are then aligned in regularly spaced chemical shift windows ($\Delta\delta$ = 0.3 ppm). The resulting table is visualized as a heat map where a hierarchical clustering analysis is carried out. It highlights clusters of chemical shifts showing the same behavior in terms of signal intensity during the fractionation process and thus which probably correspond to a metabolite or structurally related metabolites [8]. Finally, the comparison of the chemical shifts of the clusters with a local database containing predicted ^{13}C NMR spectra of metabolites allows for cluster to structure assignment and for the chemical profiling of the studied extract.

3.3. Implementation of the Dereplication Workflow on an Extract of L. alpinum Diluted in Propylene Glycol, Propanediol and Glycerin

The general dereplication workflow (Figure 5) of natural extracts diluted in propylene glycol, propanediol and glycerin was used on three edelweiss extracts diluted in each of these carrier solvents at a 5% wt. concentration. No sample pre-treatment by solvent evaporation was performed. First, a physical suppression of the solvent was carried out on the glycerinated extract by isocratic elution (EtOAc/CH_3CN/water 3:3:4 (*v/v/v*)) and on propanediol extracts by gradient elution (M*t*BE/*n*-BuOH/water from 45:5:50 to 5:45:50 (*v/v/v*)), as described in Section 3.1. It resulted in 13 simplified fractions for the glycerinated extract (Figure S5) and 12 simplified fractions for the propanediol extracts (Figure 3b,d). Sample recoveries of the three fractionations were about 90−95% wt. for propanediol extracts to 110% wt. for glycerinated extract. The hygroscopic character of glycerin explains the recovery of more than 100%. In each fractionation, the carrier solvent was concentrated in only three fractions. Due to its non-partitioning in the biphasic system, glycerin has been trapped in the stationary phase during fractionation and was finally recovered in the extrusion fractions F_{11} to F_{13}. For fractionation of edelweiss extracts diluted in propanediols, carrier solvents were eluted at approximatively the same time and were concentrated in fractions F_{06} to F_{08}, with a maximum concentration in F_{07}.

These fractions were then subjected to ^{13}C NMR analysis with presaturation of the solvent signals, while dry fractions were analyzed with UDEFT ^{13}C NMR spectroscopy. After hierarchical clustering analysis of NMR data, three heat maps were obtained (Figure 6).

Dereplication of the glycerinated edelweiss extract led to the identification of 26 metabolites belonging to different classes: fatty acids, flavonoids, hydroxycinnamic acids, organic acids and sugars. In 2016, Stuppner et al. have referenced 14 secondary metabolites in the aerial parts of *L. alpinum* among which 11 were purified, including leontopodic acids, chlorogenic acid, 3,5-dicaffeoylquinic acid and flavonoids derived from luteolin such as luteolin-7-*O*-*β*-D-glucoside, luteolin-3′-*O*-*β*-D-glucoside and luteolin-4′-*O*-*β*-D-glucoside [16]. Eight of them were identified in the glycerinated extract of *L. alpinum* studied with the dereplication workflow developed in this paper.

The study of the two extracts diluted in propylene glycol or propanediol led to the identification of the same 26 metabolites. Comparatively, in both fractionations of the propanediol extracts, the elution of the metabolites was very close; for instance, linoleic acid was eluted in F_{01}, luteolin-3′-*O*-*β*-D-glucoside was mainly composed of fractions F_{02}-F_{03}, leontopodic acid presented a long elution around F_{06}-F_{09} and malic acid was present in the last fraction.

The workflow developed in this paper was, therefore, efficient on the three edelweiss extracts studied. Each compound structure assigned to a cluster was finally confirmed by further 2D NMR and LC-MS analyses (Table S4).

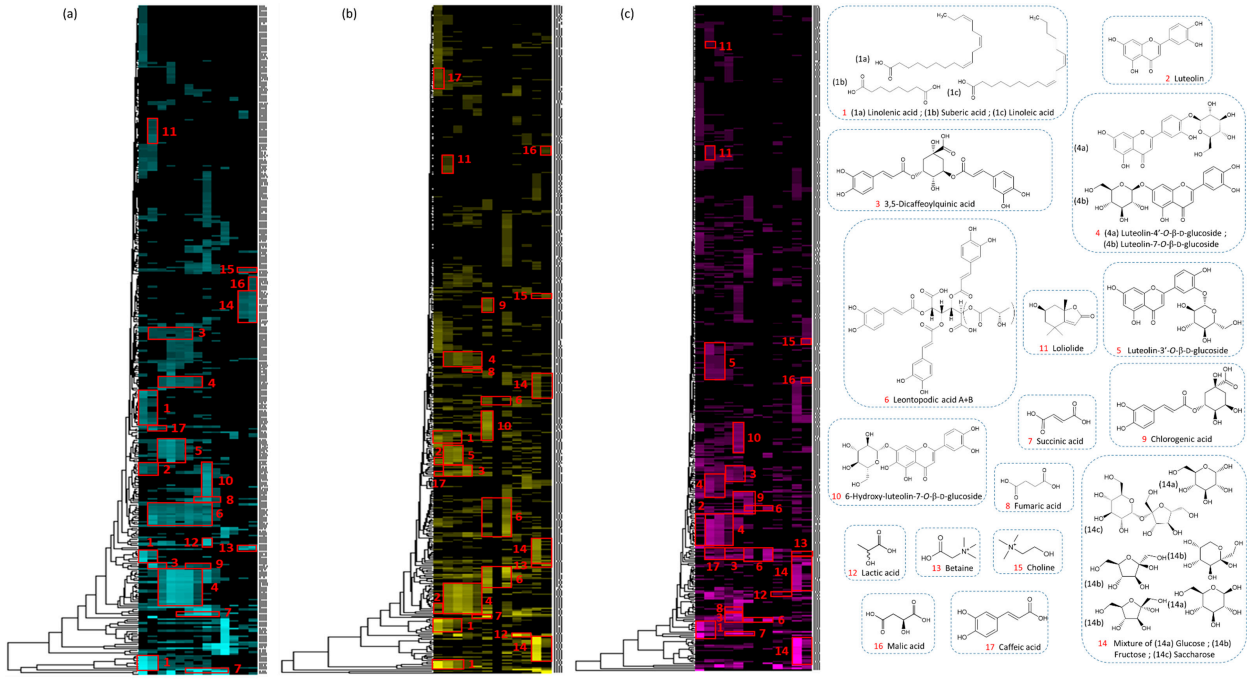

Figure 6. Comparative study of edelweiss extracts diluted in (**a**) glycerin; (**b**) propylene glycol; (**c**) propanediol.

4. Conclusions

A new and efficient workflow for the dereplication of natural extracts diluted in propylene glycol (1,2-propanediol), propanediol (1,3-propanediol) and glycerin was delineated. Their rapid chemical profiling will be very useful to actors of the cosmetic industry for where the use of these solvents is widespread. This workflow aims to overcome the problem of characterizing complex mixtures in high-boiling-point solvents to gain a greater knowledge of natural extracts for scientific, regulatory or safety reasons and thus facilitating the commercial product development process. As a proof of concept, a hydro-ethanolic extract of *Leontopodium alpinum* Cass. was successfully investigated. The same twenty-six metabolites of various polarities were identified whatever the carrier solvent. In perspective, the generalization of this workflow to other carrier solvents such as butylene glycol or natural deep eutectic solvents will be considered in future method developments. The quantitative aspect of the results as well as the identification of minority compounds also remain challenges for which solutions will have to be found.

Supplementary Materials: The following are available online at https://www.mdpi.com/2079-9284/8/1/10/s1, Figure S1: Confirmation of the non-degradation of the edelweiss extract metabolites diluted in 1,2-propanediol/water 1:1 by ^{13}C NMR, Figure S2: Confirmation of the non-degradation of the edelweiss extract metabolites diluted in 1,3-propanediol/water 1:1 by LC-MS and LC-DAD, Figure S3: Comparison of ^{1}H NMR spectra of edelweiss extract diluted in propanediol/water before and after a single evaporation run of 15 h at 40 °C, Figure S4: TLC summarizing shake flask assays to observe the partitioning of compounds in edelweiss extract diluted in 1,2-propanediol in 10 biphasic systems, Figure S5: Summary of CPC fractionation on edelweiss extract diluted in glycerin with EtOAc/CH_3CN/water 3:3:4 (*v/v/v*) solvent system, Table S1: Evaporation of edelweiss extract 5% wt. diluted in 1,2-propanediol/water 1:1, Table S2: Evaporation of edelweiss extract 5% wt. diluted in 1,3-propanediol/water 1:1, Table S3: Details of pooling after CPC fractionations, Table S4: Summary of compounds identified in *L. alpinum*.

Author Contributions: M.C. has performed experiments, data treatment and wrote the initial draft; J.-H.R. supervised the physical solvent suppression methodology by CPC; J.-M.N. supervised the spectroscopic suppression methodology of solvent signals by ^{13}C NMR; S.P. contributed to obtaining the edelweiss plant material; S.P., R.R., J.-H.R. and J.-M.N. reviewed the text and the figures of the paper. J.-H.R. and J.-M.N. are academic PhD thesis supervisors of M.C.; S.P. and R.R. are industrial PhD thesis supervisors of M.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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