

Technical Note

Qualitative and Quantitative Comparison of Liquid–Liquid Phase Extraction Using Ethyl Acetate and Liquid–Solid Phase Extraction Using Poly-Benzyl-Resin for Natural Products

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Abstract: A key step in the process of isolating microbial natural products is the preparation of an extract from a culture. This step determines which molecules will be available for detection in the subsequent chemical and biological analysis of a biodiscovery pipeline. In the present study we wanted to document potential differences in performance between liquid–liquid extraction using ethyl acetate and liquid–solid extraction using a poly-benzyl-resin. For the comparison of the two extraction protocols, we spiked a culture of *Flavobacterium* sp. with a diverse selection of natural products of microbial and plant origin to investigate whether the methods were comparable with respect to selectivity. We also investigated the efficiency of the two extraction methods quantitatively, using water spiked with a selection of natural products, and studied the quantitative effect of different pH levels of the aqueous solutions on the extraction yields of the two methods. The same compounds were extracted by the two methods, but the solid-phase extract contained more media components compared with the liquid-phase extract. Quantitatively, the two extraction methods varied in their recovery rates. We conclude that practical aspects could be more important when selecting one of the extraction protocols, as their efficiencies in extracting specific compounds were quite similar.

Keywords: natural products; bacteria; downstream processing; antibiotics; isolation; extraction; secondary metabolites; pharmacognosy; bioprospecting

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1. Introduction

Natural products and their derivatives have successfully been developed as medicines that have enabled the treatment of various diseases [1]. Natural products differ from compounds that can be found in synthetic screening libraries by occupying a different chemical space that makes them a valuable source for new chemical scaffolds [2]. Microorganisms in general and bacteria in particular are promising sources for new bioactive natural products that can be developed as drug leads [3,4]. Natural product drug discovery commonly starts with the preparation of an extract, and this extract maybe pre-fractionated or directly investigated for bioactivity using bioassays such as anti-microbial or anti-cancer assays [5,6]. If bioactivity is detected, extracts or fractions are subsequently investigated for the presence of known active metabolites, which will then be removed from the pipeline [7]. This process is called dereplication and usually relies on hyphenated mass spectrometry techniques such as HPLC-MS [8,9]. An extract may also be investigated for novel metabolites without preceding bioactivity testing [10]. Potential new compounds will be isolated and subjected to structural elucidation and bioactivity profiling.

Extraction is a crucial step in the search for new active compounds. If a compound is not successfully extracted from the biomass or fermentation broth, it can obviously not be

detected in downstream bioassays or chemical analysis. For bacterial fermentations, many possible extraction techniques and protocols exist, but as it is usually not feasible to apply more than one of them when screening larger collections of bacterial isolates, it is important to be aware of the limitations of the selected protocol. While we have used liquid–solid phase extraction of fermentation broths in previous studies [11,12], there are other relevant techniques such as liquid–liquid partition with ethyl acetate (EtOAc) [13] or extraction of the bacterial mycelium with ethanol in case of actinobacteria [14]. Sample pre-treatment and solvent selection are also dependent on whether intracellular or secreted metabolites are of interest [15].

To assess the suitability of our standard method for extraction of bacterial and fungal fermentations (liquid–solid phase extraction), we conducted a small study to compare it with liquid–liquid partition using EtOAc. We investigated the qualitative difference when extracting a bacterial culture spiked with seven different natural products. So-called spike and recovery tests are an established method for verifying the suitability and detection limits of an analytical method or sample preparation, including extraction procedures [16,17].

In addition, the two methods were compared quantitatively by extraction of four of the natural products from aqueous solution. We also investigated a potential effect of the pH level of the aqueous solution on the extraction yields.

2. Materials and Methods

The chemicals used were of appropriate purity, as indicated by the supplier, and pure water (pH₂O) was produced using an in-house MilliQ system. For vacuum filtrations, a Büchner funnel with Whatman No. 3 filter paper (Whatman plc., Maidstone, UK) was used.

2.1. Preparation of the Spiked Culture

A *Flavobacteria* sp. isolate was cultured over 7 d at 10 °C in a 450 mL shaking bottle culture, shaking at 300 rpm. A DVR1 medium was used for cultivation, consisting of 6.0 g malt extract (Sigma-Aldrich, St. Louis, MO, US), 10.0 g Peptone from casein (Sigma-Aldrich), 6.0 g yeast extract (Sigma-Aldrich), 450 mL filtrated seawater and 450 mL pH₂O [11]. The media was sterilized by autoclaving at 120 °C for 30 min under pressure and inoculated using an inoculation loop. After cultivation, the fermentation broth was centrifuged at 4000 rpm for 10 min to remove the cells using a Multifuge 3 S-R equipped with a SORVALL 75,006,445 rotor (Heraeus GmbH & Co. KG, Hanau, Germany).

An aliquot of 300 mL of the supernatant was spiked with eight natural products—namely, rifampicin (R3501, Sigma-Aldrich), vancomycin (Sigma-Aldrich), colchicine (C3915, Sigma-Aldrich), cyclosporine (32425, Fluka®, Honeywell, Charlotte, NC, US), paclitaxel (86346, Fluka®, Honeywell), ampicillin (A5354, Sigma-Aldrich) and gentamycin (A2712, Merck, Darmstadt, Germany). The concentrations of the respective natural products are given in Table 1 (structures in Figure 1). After the supernatant was spiked, it was thoroughly mixed and split into two samples of 150 mL each. The two samples were processed in parallel; one was extracted using liquid–liquid extraction and one using liquid–solid extraction.

Table 1. Final concentrations of the natural products in the bacterial culture.

Compound	Natural Product Class	Concentration (µg/L)
Rifampicin *	polyketide	2.0
Vancomycin †	glycopeptide	10.0
Colchicine †	alkaloid	5.0
Tetracycline *	polyketide	10.0
Cyclosporine *	cyclic peptide	10.0
Paclitaxel *	terpenoide	2.0

Ampicillin ‡	β-lactam antibiotic	10.0
Gentamycin ‡	aminoglycoside	10.0

* dissolved in ethanol; † dissolved in pH₂O; ‡ readymade solution.

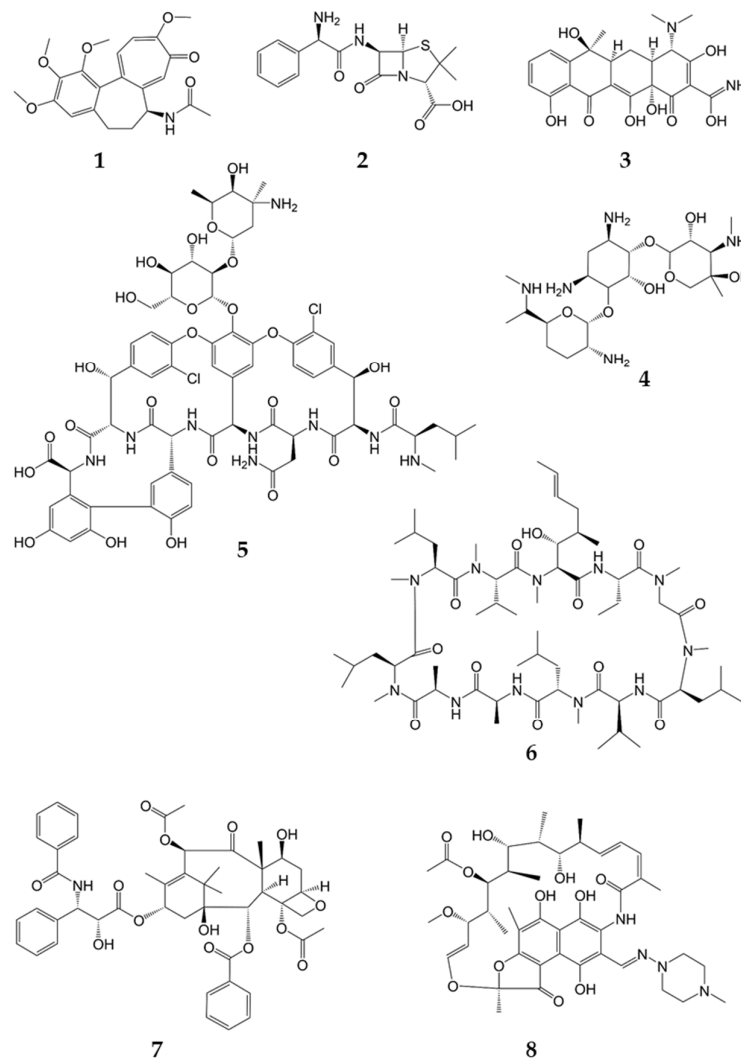


Figure 1. Structures of the natural products used for spiking within this study. (1) Colchicine; (2) Ampicillin; (3) Tetracycline; (4) Gentamycin; (5) Vancomycin; (6) Cyclosporine A; (7) Paclitaxel; (8) Rifampicin.

2.2. Extraction

2.2.1. Liquid–Liquid Phase Extraction

For the liquid–liquid phase extraction, the 150 mL sample was extracted three times using 75 mL EtOAc (Sigma-Aldrich) and a separation funnel. The extracts were reduced to dryness at 40 °C under reduced pressure, and the yield was determined gravimetrically.

2.2.2. Liquid–Solid Phase Extraction

For the liquid–solid phase extraction, Diaion® HP-20 (SUPELCO, Sigma-Aldrich) resin was used. An amount of 6.75 g resin was soaked in methanol (MeOH, HiPerSolv, VWR, Radnor, PA, US) for 30 min for activation and transferred to pH₂O to wash out MeOH (~4.5 g resin was used for 100 mL sample or culture). After 20 min of washing in pH₂O, the resin was added to the spiked fermentation broth and shaken for 72 h at 10 °C

for extraction. The resin was filtered from the sample using a cheese-cloth filter (1057, Dansk Hjemmeproduktion, Ejstrupholm, Denmark) placed on a Büchner funnel and subsequently washed in pH₂O. Thereafter the resin was eluted twice using 50 mL MeOH. Extraction was done by eluting the resin in MeOH for 1 h and by subsequently removing the resin by vacuum filtration. The extracts were reduced to dryness at 40 °C under reduced pressure, and the yield was determined gravimetrically.

2.3. Preparation and Extraction of Spiked Water for Quantitative Comparison and Investigation of the pH Effect on Extraction Yields

Stock solutions of tetracycline (10 mg/mL aq.), cyclosporine (10 mg/mL in EtOH), colchicine (5 mg/mL in EtOH) and gentamicin (10 mg/mL aq.) were prepared to spike the five water samples. Five 200 mL samples of pH₂O were set to a pH of 4.0, 5.0, 6.0, 7.0 and 8.5, respectively, using 1 M HCl (EMSURE, Merck), 0.3 M NaOH (VWR) and a pH meter (WTW InoLab pH 720, Xylem Inc. Rye Brook, NY, USA) equipped with a SenTix®41 electrode (WTW). The five conditions were spiked with the natural products to reach a final concentration of 10 µg/mL for tetracycline, cyclosporine A and gentamicin and 5 µg/mL for colchicine. Thereafter the five conditions (each 200 mL) were split into two aliquots of 100 mL each for parallel extraction using liquid–liquid phase extraction and liquid–solid phase extraction. For liquid–liquid phase extraction, the 100 mL aliquots were extracted with 100 mL EtOAc using a separation funnel. After shaking the two phases for ~1 min, the funnel was left to rest until the two phases separated properly. The EtOAc fractions were reduced to dryness at 40 °C under reduced pressure. The other aliquot for each water sample for resin or liquid–solid phase extraction was extracted using resin as described above (using ~4.5 g resin/100 mL of sample).

2.4. Analysis of the Extracts

2.4.1. Gravimetric Analysis

The yields were determined gravimetrically using an analytical balance (Mettler Toledo AB204-S, Mettler Toledo, Columbus, OH, USA).

2.4.2. UHPLC-IMS-MS Analysis

For detection of the compounds, the extracts were re-dissolved in DMSO (Sigma-Aldrich) to a final concentration of 40 mg/mL and diluted 1:4 with 80% MeOH aq. to prepare an injection solution for UHPLC analysis.

For analysis of the presence of the respective natural products, UHPLC-IMS-MS was used. The analytical system consisted of an Acquity I-class UPLC (Waters, Milford, MA, USA) coupled to a PDA detector and a Vion IMS QToF (Waters). The chromatographic separation was performed using an Acquity BEH C18 UPLC column (1.7 µm, 2.1 mm × 100 mm) (Waters). Mobile phases consisted of acetonitrile (HiPerSolv, VWR) for mobile phase B and pH₂O produced by the in-house Milli-Q system as mobile phase A, both containing 0.1% formic acid (v/v) (Merck). The gradient was run linearly from 10% to 90% B over 12 min at a flow rate of 0.45 mL/min. Samples were run in ESI+ and ESI− ionization mode. The data were processed and analyzed using UNIFI 1.9.4 (Waters). Stock solutions of the respective natural products were injected as reference for unambiguous identification and to confirm that the compounds were detectable within the analytical setup.

For the quantitative analysis, the extracts were dissolved in 1.0 mL 80% MeOH aq., and a 10-fold dilution with 80% MeOH aq. was prepared for injection. A sample of each of the four stock solutions was diluted to 0.1 mg/mL in 80% MeOH aq. to obtain a reference solution. An amount of 5.0 µL was injected and analyzed using the same UHPLC protocol as above. For the quantification, three injections per sample were made, and the response/ion count of the respective *m/z* signal of the pseudo-molecular ion was used for quantification. The results were analyzed using GraphPad Prism 8.1.0 for linear regression.

3. Results

3.1. Qualitative Comparison

The EtOAc extraction of the spiked bacterial ferment was done three times with the same spiked sample, and the yield was determined for each of the three extractions individually. The yields for the three extractions were 26.9, 11.7 and 15.0 mg, respectively. The combined yield of the extractions was 53.6 mg (dry weight). The liquid–solid extraction yielded 325.7 mg of extract. The first EtOAc extract and the solid-phase extract were analyzed using UHPLC-IMS-MS and investigated for the presence of the spiked natural products. A small quantity of the stock solutions was injected as reference to determine the retention time and mass spectra for the respective natural products. The extracts were investigated for the presence of the spiked compounds by comparison with the retention time and mass spectra of the references. The results for the qualitative comparison of the extraction methods are given in Table 2.

Table 2. Retention times and presence of the natural products in EtOAc and resin extracts.

Compound	Ret. Time (min)	Mw (u)	Present in Extracts:	
			EtOAc	Resin
Rifampicin	6.06	822.9	+	+
Vancomycin	0.97	1449.2	+	+
Tetracycline	2.09	444.4	+	+
Colchicine	3.30	399.4	+	+
Cyclosporine	9.60	1202.6	+	+
Paclitaxel	6.51	853.9	+	+
Ampicillin	1.51	349.4	-	-
Gentamycin	0.46	482.5	-	-

When comparing the chromatograms from the liquid–liquid and liquid–solid extractions, it appeared that many more polar metabolites and media components were extracted using the liquid–solid phase extraction compared with the liquid–solid phase extraction.

3.2. Quantitative Comparison

Water samples with pH values of 4.0, 5.0, 6.0, 7.0 and 8.5 were spiked with natural products, and an aliquot of each sample was extracted using EtOAc as well as resin extraction, as described above. For the quantitative comparison, the response of the protonated pseudo-molecular ions of the respective standard injections was used as reference to calculate the concentrations of the respective spike compounds. For determination of a calibration curve, LOQ and LOD, we injected 0.05, 0.25, 0.5 and 1 µg of cyclosporine, gentamycin, colchicine and tetracycline using the analytical protocol described above. We also injected 0.25 µg and 0.05 µg of ampicillin to determine the LOD of ampicillin that was not detected in the qualitative study. The calibration curves are shown in Figure 2. The LOD for ampicillin was <0.05 µg; LOD for tetracycline, cyclosporine and colchicine was <0.05 µg; LOD for gentamycin was <0.25 µg, and a proper quantification of gentamycin failed (see Figure 2). We also failed to detect gentamycin at 0.05 µg in negative electrospray. The extracts were dissolved in 1.0 mL of 80% MeOH aq., and the final yields were determined by multiplying the concentration with the volume; the final yields are given in Appendix A. Relative yields were calculated as ratios of the initial quantity of spike compound in the 100 mL of aqueous solution to be extracted. The relative yields are given in Figure 3.

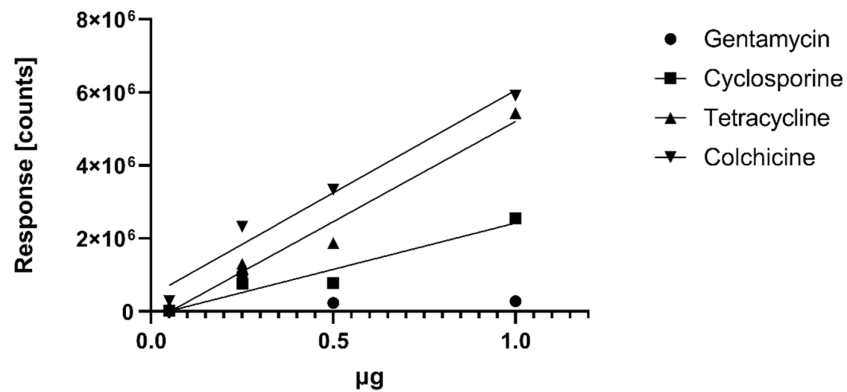


Figure 2. Calibration curves for the quantification of the selected natural products. Single injections were used for the calibration curves. The formulas and R^2 values for the calibration curves were tetracycline $y = 5487981x - 112802$, 0.97 ; cyclosporine $253601x - 11282$, $R^2 = 0.93$; colchicine $y = 5617051x + 438251$, $R^2 = 0.97$; with y being the response and x being the injected compound quantity in μg . We failed to generate a proper calibration curve for gentamycin.

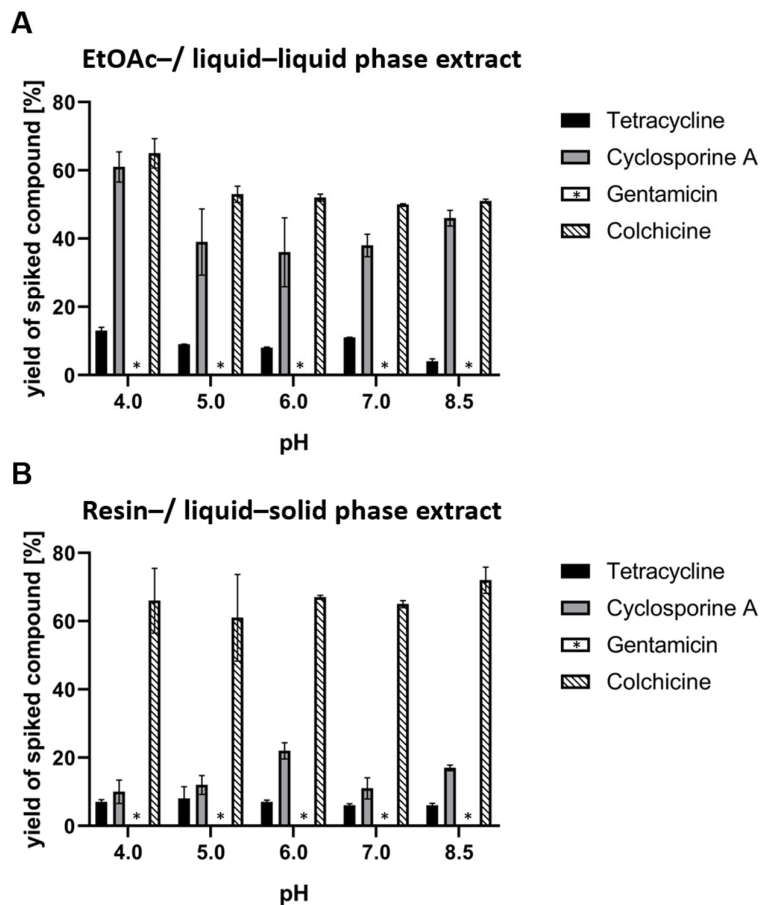


Figure 3. Yield of the natural products in percentage recovery from the spiked solution. A: Results for the liquid–liquid phase extraction. B: Results for the liquid–solid phase extraction. The different pH levels for the aqueous solutions/extraction conditions are given on the x -axis. Note that gentamycin (*) was not detected in any of the two extracts; however, the LOD for gentamycin would be around 5.0% of the initially spiked quantity; given the extract was dissolved in 1.0 mL of solvent, for all the other compounds LOD would equal <1.0% of the initially spiked compound quantity.

4. Discussion

The number of different protocols that can be used for extraction of microbial secondary metabolites is high, and they range from classical liquid–liquid extractions to more sophisticated applications, such as ultrasound assisted extraction and supercritical fluid extraction [18,19]. Furthermore, the selection of extraction solvents will have an impact on extracted metabolites, as observed in plant material [20]. However, we chose the two selected techniques in this investigation because they required no specialized equipment that went beyond the common equipment in a chemical laboratory, and they were widely used. When establishing the biodiscovery pipeline for bioactive secondary metabolites in our laboratory, we were not able to find a direct experimental comparison of the extraction methods using a spike and recovery test. To assess the potential difference of extraction efficacy and to make our data available to others were therefore the main objectives of this study.

Qualitative Comparison of the Extraction Methods

The first finding of the qualitative comparison of the extraction methods was the difference in yield when extracting the bacterial cultures. The yield of the resin extract was 325.7 mg, and this was about 6 times higher compared with the EtOAc extract, yielding 53.6 mg. Investigation of the chromatograms of the UHPLC-MS analysis revealed that the resin extract contained a higher share of polar compounds that originated from bacteria and media, as comparison with a media reference for DVR1 media confirmed. For the targeted natural products, the two extraction methods were quite comparable, as we detected the same spiked natural products in the resin and the EtOAc extracts (Table 2). Interestingly, ampicillin and gentamycin were not extracted by any protocols or at least they were not extracted in sufficient quantity for detection. When comparing the retention times in Table 2, it appears that ampicillin and gentamycin had rather short retention times (1.51 and 0.46 min, respectively) and vancomycin eluted between them (0.97 min). We speculate that both extraction methods are rather unsuitable for very polar and hydrophilic compounds. When comparing the structures of the analytes, it appears that vancomycin (Figure 1 5) is a relatively large molecule (1449.2 u) and consists of five benzylic rings, while ampicillin (2, 349.4 u) and gentamycin (4, 482.5 u) have substantially lower molecular weights.

For the quantitative comparison of the two extraction protocols, water was used as matrix to dissolve the natural products for reproducibility reasons since the media components and bacterial metabolites in a fermentation could affect the extraction (as we observed above that the solid-phase extract contained significantly more of those compounds). We intended to compare the extraction of the natural products in a simple way, from purified water, including the effect of the pH levels of the aqueous solutions on the extraction yield. Some bacteria prefer to grow or grow exclusively at acidic (acidophil bacteria) [21] or alkalic pH levels (alkaliphile bacteria) [22], and we also wanted to investigate if a post-fermentative change in pH by adding acid or alkali could be a strategy to increase yields. For the quantitative study, we selected four natural products (tetracycline, cyclosporine, colchicine and gentamycin), trying to cover a broad range of polarities and molecular weights. We investigated pH levels of 4.0, 5.0, 6.0, 7.0 and 8.5. In Figure 3, the yields of the respective natural products are given as percentage recovery or yield of the initially spiked compound quantity (1.0 mg for tetracycline, cyclosporine and gentamycin and 0.5 mg for colchicine). As expected, after the observations from the preceding qualitative comparison, gentamycin was not detected in the extracts of either extraction technique or at any pH-level. Generally, as visible in Figure 3, there were few variations among the pH levels within the same extraction. Generally, EtOAc extraction had a better recovery of the spiked compounds (except for gentamycin), tetracycline was recovered at about 10% using resin and 15% using EtOAc, colchicine was recovered at levels between 50% and 70%, depending on the condition, for colchicine; the resin extraction performed

better. The recovery of cyclosporine was, depending on the condition, at least around 2 times higher in the EtOAc extract samples compared with the resin extracts.

We conclude that the two extraction techniques are qualitatively comparable and that both are less suitable for polar compounds, which was expected given the chemical composition of the poly-benzyl resin. Quantitatively the EtOAc extraction showed higher yields for cyclosporine, while the other two compounds were comparable. One of the prominent advantages of EtOAc extracts is that the extracts contain fewer media-components and polar metabolites, which eases downstream purification of compounds from the extracts. Furthermore, technical considerations can play a decisive role when screening larger sample collections. The presented protocol of resin extraction appears to be rather impracticable for smaller culture volumes (> 5mL), but on the other hand, it can be more convenient when processing larger numbers of fermentations at an intermediate scale. Since we expect many of the microbial secondary metabolites to be excreted to the media in order to exert their biological effect [4], extraction of the media seems to be reasonable. However, extraction of a 10L culture using EtOAc would demand an equal volume of the organic solvent, while resin extraction would require just gram amounts of resin, but at the expense of extracting more polar molecules. It was important for us to document that the extraction methods were qualitatively comparable for the tested compounds; therefore, other technical considerations can be prioritized when deciding on one of the protocols.

5. Conclusions

The liquid–liquid and liquid–solid state methods are comparable when extracting the selected natural products from a microbial fermentation broth or water solutions at different pH levels. Both methods are less suitable for the extraction of polar metabolites. The liquid–liquid extract contains fewer media components, but this method is less suited when extracting larger cultivations due to high consumption of organic solvents.

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

Appendix A

Table A1. Table of calculated yields from the quantitative comparison.

Calc. Compound Yields in (µg)	EtOAc Extraction					Resin Extraction					
	pH	4.0	5.0	6.0	7.0	8.5	4.0	5.0	6.0	7.0	8.5
Colchicine		327	267	260	251	254	332	303	338	325	359
Cyclosporine		607	389	362	383	461	97	118	220	112	171
Tetracycline		134	88	78	106	44	70	77	69	58	59
Gentamycin		-	-	-	-	-	-	-	-	-	-

“-“ = not detected.

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