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Versatile Green Processing for Recovery of Phenolic Compounds from Natural Product Extracts towards Bioeconomy and Cascade Utilization for Waste Valorization on the Example of Cocoa Bean Shell (CBS)

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Abstract: In the context of bioeconomic research approaches, a cascade use of plant raw materials makes sense in many cases for waste valorization. This not only guarantees that the raw material is used as completely as possible, but also offers the possibility of using its by-products and residual flows profitably. To make such cascade uses as efficient as possible, efficient and environmentally friendly processes are needed. To exemplify the versatile method, e.g., every year 675,000 metric tons of cocoa bean shell (CBS) accrues as a waste stream in the food processing industry worldwide. A novel green process reaches very high yields of up to 100% in one extraction stage, ensures low consumption of organic solvents due to double usage of ethanol as the only organic solvent, is adaptable enough to capture all kinds of secondary metabolites from hot water extracts and ensures the usage of structural carbohydrates from precipitation. A Design of Experiments (DoE) was conducted to optimize the influence of pH value and phase ratio on the yield and purity of the integrated ethanol/water/salt aqueous-two-phase extraction (ATPS) system.

Keywords: aqueous two-phase systems (ATPS); liquid–liquid extraction (LLE); pressurized hot water extraction (PHWE); solid–liquid extraction (SLE); natural products; cocoa bean shell (CBS); precipitation; bioeconomy; total phenolic content (TPC)

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1. Introduction

Products based on renewable resources, such as plants, represent a growing market and the associated industry is an important supplier of versatile products. Applications include pharmaceutical products, the food, health and nutrition sectors, as well as plant protection for ecological farming or construction materials, basic chemicals and energy resources [1–7]. In the context of bioeconomic research approaches, a cascade use of plant raw materials makes sense in many cases. This not only guarantees that the raw material is used as completely as possible, but also offers the possibility of using its by-products and residual flows profitably. To make such cascade uses as efficient as possible, efficient and environmentally friendly processes are needed.

Pressurized hot water extraction (PHWE) has been studied in the solid–liquid extraction community for a while and is well-established [8–11]. One of the main advantages is the utilization of water as a solvent instead of organic solvents, which can help to reduce the cost of goods (COGs) and global warming potential (GWP) and therefore help to reach climate neutrality goals [12]. Additionally, PHWE extracts consist of the whole spectrum of components in the plant material. Besides secondary metabolites, such as polyphenols and flavonoids, matrix components such as lignin, cellulose and proteins are extracted [13]. However, if there are processing steps after the solid–liquid extraction, they are often associated with the usage of different organic solvents. Possible steps are precipitation,

liquid–liquid extraction or chromatography, which usually come with organic solvents such as ethanol for precipitation, acetonitrile in chromatography or phase-forming solvents such as ethyl/butyl acetate in liquid–liquid extraction (LLE) [14,15].

Here, a promising approach is to reuse the ethanol, which is needed for the precipitation of glucans, in an ethanol/water/salt ATPS to recover the polyphenols from the precipitated extract [16,17]. The precipitate consists mainly of matrix components which can be further processed for usage in a variety of applications [18–22].

Every year, 675 kt of cocoa bean shell (CBS) accrues as a waste stream in the food processing industry worldwide [20] and has high research interest [20,23–31]. As a lignocellulosic biomass, CBS is a potential resource for β-glucans which can be utilized, for example, as bonding or binding agents. Besides β-glucans, CBS contains a high amount of polyphenols such as catechin and epicatechin [23,28] and methylxanthines such as theobromine and caffeine. The development of process routes for the use of co-products is essential for the economic viability of these processes [32,33]. To utilize the full potential of the CBS in the interest of bioeconomy, a process is developed using only minimal amounts ethanol as the only organic solvent. The process consists of an extraction with hot water, a precipitation with ethanol as an anti-solvent and a liquid–liquid extraction from the precipitation supernatant with salting-out of ethanol. The LLE is compared to a conventional LLE with ethyl acetate and butyl acetate. As the organic solvent LLE has to be conducted with an aqueous phase, the LLE has to be either conducted before precipitation or after precipitation and removal of the ethanol. This results in three possible process configurations, which are shown in Figure 1. The first process is the one with double utilization of ethanol from precipitation in an aqueous two-phase extraction (ATPE) with salt.

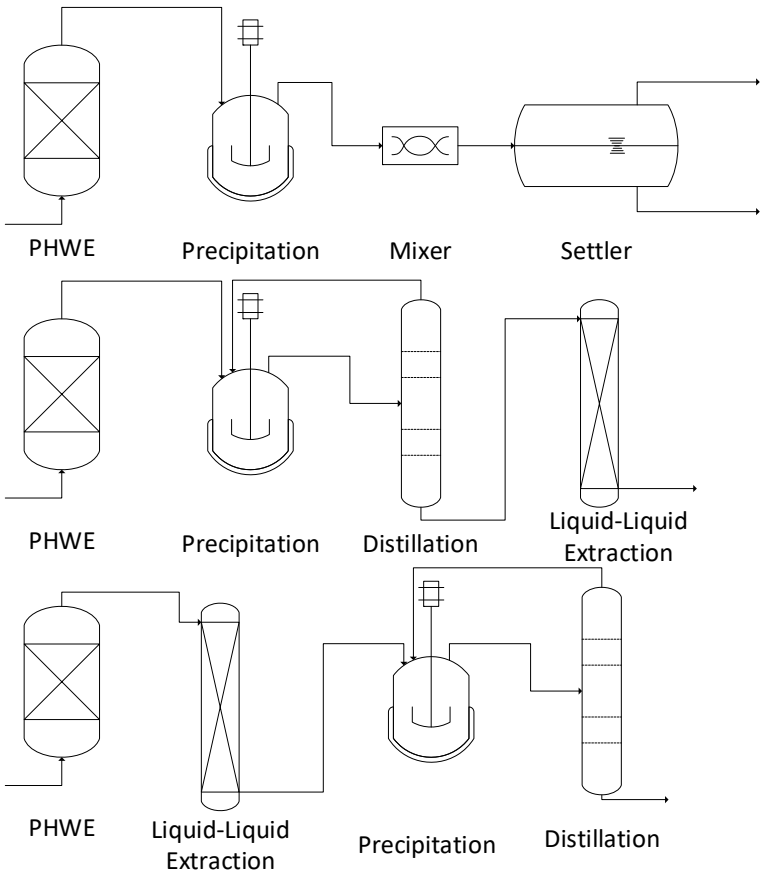


Figure 1. Overview of the three possible process configurations.

2. Materials and Methods

2.1. Extraction Setup

For temperature screening, 1 g of ground CBS was extracted in a 10 mL extraction column with a flow of 1 mL/min. The extraction plant consists of a pump, a GC oven for heating, a column for extraction and a water bath for cooling. For maximum yield, 120 mL of extract was collected as fractions.

For the solvent screening, 2 g of ground CBS is extracted with 40 mL of the respective solvent: 20–100% ethanol, methanol, iso-propanol, MTBE, butyl acetate, ethyl acetate, toluene and hexane. The vials were placed on a shaking device for 24 h to reach extraction equilibrium. All solvents were purchased at VWR International GmbH, 30163 Hannover, Germany.

The extraction was carried out as a pressurized hot water/liquid hot water/subcritical water extraction (PHWE). The extraction plant consists of an extraction column with a volume of 0.1 L, solvent vessel, heating unit with a heat exchanger, cooling unit with a heat exchanger, and an extract vessel. Extraction conditions were 140 °C at 1 L/h. The CBS was obtained as dried industrial waste from a project partner. The origin was not further specified. It was ground and sieved to 630–2000 µm, which is small enough to guarantee fast mass transfer but big enough to prevent blocking of the extraction column. The extraction column was filled with 20 g of ground plant material, and 500 mL of extract was collected. The extraction solvent was deionized water from an in-house deionization plant. The solvent ratio originates from the temperature screening, in which total yield was reached after a solvent ratio of 25.

2.2. Liquid–Liquid Extraction

The phase screenings for liquid–liquid extraction were conducted in 50 mL centrifugal vials, supplied by VWR International GmbH, 30163 Hannover, Germany. The extract/supernatant and solvent were measured into the centrifugal vials in the respective phase ratios. For the first LLE screenings for partition coefficients, the phase ratio was 50/50. The vials were placed on a shaking device for 2 h to reach extraction equilibrium.

The extracts used were collected as described in Section 2.1. For the ATPS, the extracts were precipitated with 60 wt.% ethanol, and supernatant was used for LLE. For the ATPS, a 40 wt.% citrate buffer and a 30 wt.% phosphate buffer were used at the respective pH values. Due to poor solubility properties at low pH values, phosphate buffers were only researched at pH 7 and pH 8. The salts were purchased at VWR International GmbH, 30163 Hannover, Germany.

2.3. Analytics

Offline analytics consists of three different analytic methods. For the target components theobromine, caffeine, catechin and epicatechin, reversed-phase high-performance liquid chromatography (RP-HPLC) analytics were conducted. The method is modified based on Rojo-Poveda et al. [28]. For detection, a diode array detector (DAD) is used, which detects theobromine and caffeine at 272 nm and catechin and epicatechin at 280 nm. For the separation, a Kinetex Phenyl-Hexyl C18 column (150 mm length × 4.6 mm internal diameter and 5 µm particle size; Phenomenex, Aschaffenburg, Germany) is used. Injection volume was 10 µL. The gradient consists of 0.1% formic acid as solvent A and methanol as solvent B with a flowrate of 1 mL/min. The elution program starts with 10% solvent B up to 12.5 min. The gradient reaches 80% solvent B at 37.5 min and a step up to 90% solvent B up to 42.5 min. For equilibration, the partition of solvent B is 10% from 42.5 to 45 min.

The column is heated to 35 °C. Methanol and formic acid in HPLC grade were bought from VWR International GmbH, 30163 Hannover, Germany. For calibration, theobromine, caffeine, catechin and epicatechin standards of the concentrations between 0.02 and 1 g/L from Sigma-Aldrich, St. Louis, MO, USA were used. The calibrations for HPLC analysis and Folin–Ciocalteu test are shown in Figure 2.

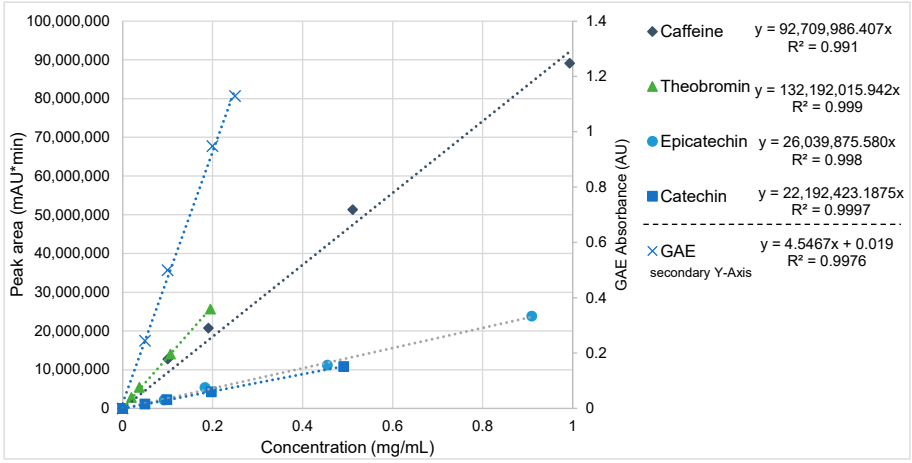


Figure 2. Calibration curves for caffeine, theobromine, epicatechin, catechin and gallic acid equivalents (GAE).

Determination of dry residue is conducted following the method described in the European pharmacopoeia (2.8.16 dry residue of extracts). An amount of 2 g per sample was dried in glass vials at 105 °C for 2 h, cooled down under a dry atmosphere and the residual mass was determined gravimetrically.

The total phenolic content of the samples is determined by UV/Vis spectroscopy using the Folin–Ciocalteu reagent. Gallic acid solutions of concentrations 0.05 g/L, 0.1 g/L, 0.2 g/L, and 0.25 g/L are utilized for the calibration. To prepare the calibration lines, 0.5 mL of gallic acid solution is mixed with 1.5 mL of Folin–Ciocalteu reagent, which is diluted to 10% of the original concentration beforehand and incubated for 5 min at room temperature. Then, 1.5 mL of 7% sodium carbonate solution is added, and everything is filled to 10 mL with HPLC grade water and incubated for 1.5 h. Measurement is conducted at 750 nm in triplicate. HPLC water is used as a blank sample instead of the gallic acid standards. The Folin–Ciocalteu reagent was supplied by VWR International, Hannover, Germany.

2.4. Statistical Analysis

The statistical analysis of the results from Design of Experiments (DoE) was conducted with JMP Statistical Discovery™ by SAS Institute, Cary, NC, USA.

2.5. Calculations

For the characterization of the solid–liquid extraction, the yield is calculated according to Equation (1), with m_{TC} as the extracted mass of the target component and m_{CBS} as the mass of ground CBS used in the extraction process.

$$\text{Yield} = \frac{m_{TC}}{m_{CBS}} \quad (1)$$

For characterization of the liquid–liquid extraction there are different target units. The partition coefficient describes the distribution of the target component in the two phases, the heavy phase and the light phase. The partition coefficient K is calculated according to Equation (2), with the concentration of the target component in the light phase $c_{TC,LP}$ and the concentration of the target component in the heavy phase $c_{TC,HP}$.

$$K = \frac{c_{TC,LP}}{c_{TC.HP}} \quad (2)$$

The yield in liquid–liquid extraction is calculated according to Equation (3), with the mass of the target component in the light phase $m_{TC,LP}$ and in the heavy phase $m_{TC,HP}$.

The purity of the liquid–liquid extraction for each component is calculated according to Equation (4), with the mass of the dry residue in the light phase $m_{DR,LP}$.

$$\text{Yield} = \frac{m_{TC,LP}}{m_{TC.HP} + m_{TC.LP}} \quad (3)$$

$$\text{Purity} = \frac{m_{TC,LP}}{m_{DR,LP}} \quad (4)$$

3. Results

3.1. Characterization of Solid–Liquid Extraction

The substance system of cocoa shells is first characterized according to a methodical procedure. Two different extraction methods are investigated. One is the pressurized hot water extraction (PHWE) and the other is a conventional extraction with organic or organic–aqueous extractants.

For the coupled glycan and polyphenol extraction based on prior knowledge, PHWE is the most suitable method, since the high temperature of the water and the associated slightly acidic properties of the water induce hydrolysis of the carbohydrate skeleton. The comparison with organic and organic–aqueous extraction agents provides, above all, a comparative overview of the substance properties of the polyphenols. Information on the solvents in which the polyphenols of the cocoa shells dissolve well can be informative with regard to the choice of extraction agent for the subsequent liquid–liquid extraction.

In the characterization, the experimental data are evaluated according to two target variables. The first is the dry residue. This describes the sum of all non-volatile components of the extract. In the present case, the main components of the dry residue are the glycans and the sum of the polyphenols.

The results for the solvent screening are shown in Figure 3. It can be seen that, in particular, ethanol/water mixtures between 20 and 80% ethanol show high solubilization properties for the polyphenols compared to the other extraction agents investigated, such as hexane, ethyl acetate or butyl acetate. All three would be suitable extractants for liquid–liquid extraction. The yield is calculated as the mass of the respective component in mg divided by the mass of CBS used in the extraction process.

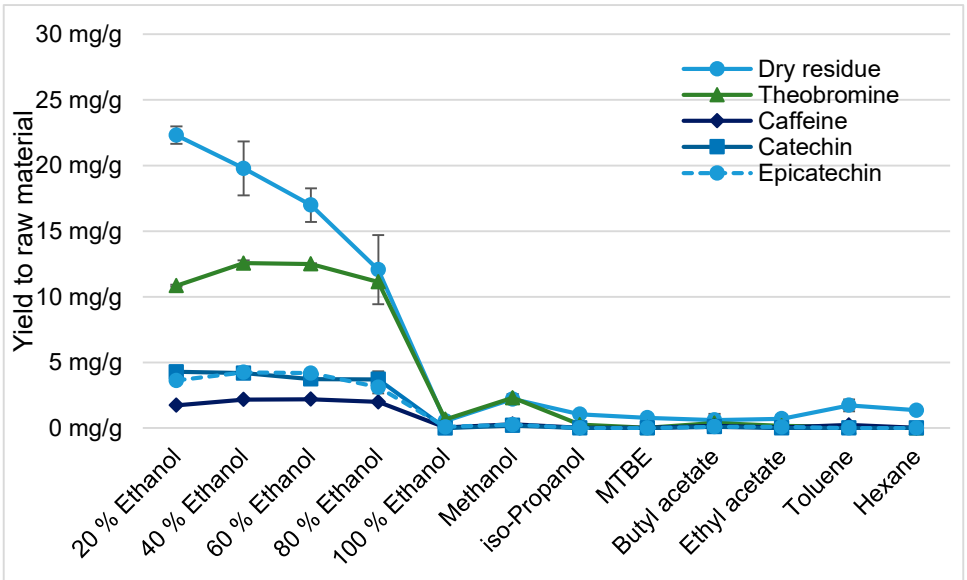


Figure 3. Yields for dry residue, methylxanthines and polyphenols in solvent screening with aqueous ethanol and organic solvents.

In addition to the extracted dry matter, the polyphenols of the cocoa shells are of interest. The yields achieved in milligrams of polyphenol per gram of extracted cocoa shell are shown in Figure 4. In addition to the investigated target components described at the beginning, where the two phenols catechin and epicatechin are considered, as well as the two methylxanthines theobromine and caffeine, a total phenol content appears here. These are remeasurements of old samples that were still available. The analysis by means of Folin–Ciocalteu was established additionally only in the later course of the project. Therefore, this analytical method has so far only been carried out in this series of measurements. It can be seen that in the temperature range between 120 °C and 160 °C, the yields obtained remain relatively constant. Accordingly, up to an extraction temperature of 160 °C, it can be assumed that no thermal decomposition processes of the investigated components take place. Based on the sum of extraction properties with respect to glycans and polyphenols, as well as operational and safety considerations, an extraction temperature of 140 °C is selected for the further extractions. Quercetin was not detected in any of the extracts from the present cocoa shells.

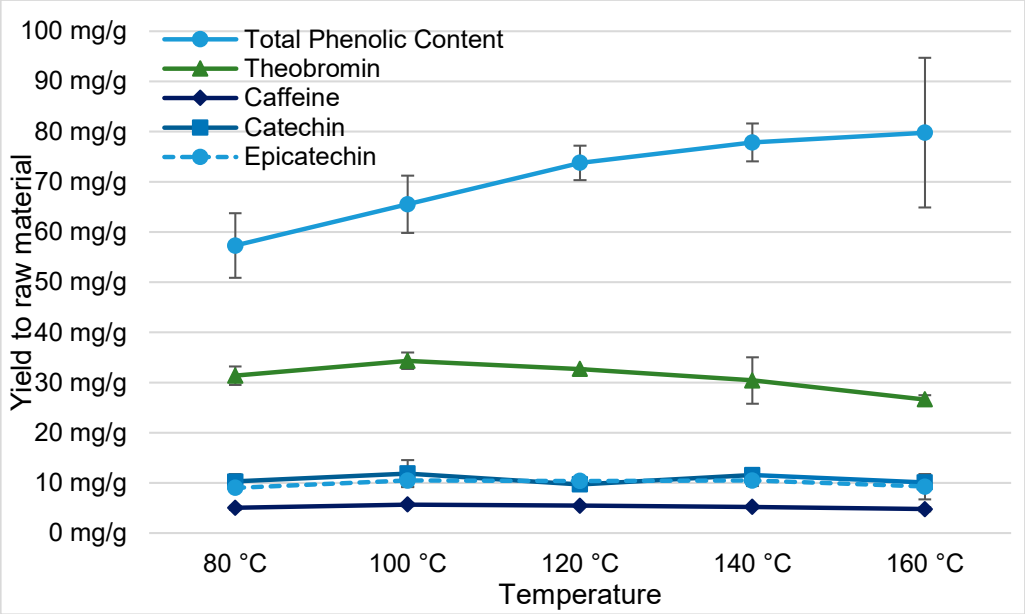


Figure 4. Yields for dry residue, methylxanthines and polyphenols in temperature screening.

3.2. Characterization of Liquid–Liquid Extraction

For the screening of suitable extraction solvents for liquid–liquid extraction, organic solvents that form a miscibility gap with water are required. Ethyl acetate, butyl acetate, methyl tert-butyl ether (MTBE), hexane and toluene are chosen for this purpose. The experiments are performed with volumetric phase ratios of 1:1 to determine the partition coefficients of the target components. Liquid–liquid extraction of the extracts with hexane and toluene resulted in emulsion formation in the organic phase with the fats from the cocoa shell. Accordingly, not only could these samples not be measured, but they also fall away for further processes considering that these processes could not be carried out. The further investigations are carried out with different starting extracts, which are based on considerations from the process synthesis. Here, there are different scenarios at which point in the process the LLE can be performed:

- Directly after extraction of the glycan–phenol mixture.
- After precipitation of the glycans with ethanol—an ethanol–water mixture is then present.
- After evaporation of the ethanol from the precipitation supernatant.

For LLE with ethanol–water mixtures, the above-mentioned organic extraction agents can only be used to a limited extent, if at all, because the ethanol content means that no more

mixture gaps are formed, which is a prerequisite for LLE. However, it is possible to carry out an aqueous two-phase extraction in an ethanol/water/salt system. In this case, the addition of salt to an ethanol–water mixture displaces ethanol with a lower water content from the salt-rich phase. In principle, a wide range of different salts are suitable for this purpose. In view of environmental compatibility and green extraction processes, a citrate salt is used. Comparatively, but less green, a phosphate salt is used. The partition coefficients for the target components are shown in Figure 5. The partition coefficient is calculated with the concentration in the light phase divided by the concentration in the heavy phase. Here, large values represent a preferential distribution of the target components in the light phase, which in all cases is the organic phase, or the ethanolic, low-salt phase. Here, only very low partition coefficients are obtained for the organic solvents in question. In contrast, large partition coefficients are achieved for the ethanol/water/salt systems. Here, the citrate system shows the best results in comparison. The use of this system also has the advantage that no additional organic extractant needs to be added to the process. Only the ethanol is used, which is required for precipitation anyway.

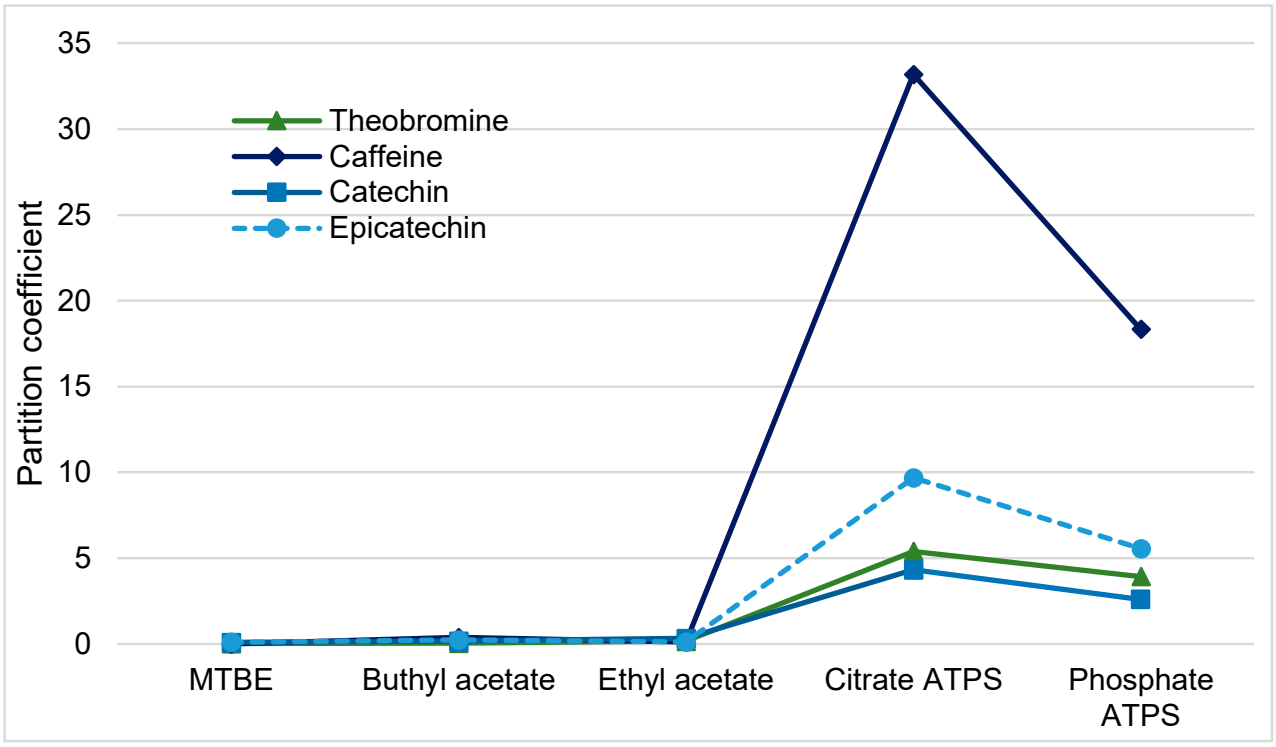


Figure 5. Partition coefficients for solvent screening for liquid–liquid extraction.

Therefore, the ethanol/water/salt systems will be characterized and investigated in more detail in further trials.

In Figure 6, the results of the phase screening with citrate ATPS at pH 6, 7 and 8, phosphate ATPS at pH 7 and 8, ethyl acetate and butyl acetate at phase ratios 30/70, 40/60, 50/50, 60/40 and 70/30 (m/m) expressed as feed/solvent are shown. The components researched are total phenolic content, theobromine, caffeine, catechin and epicatechin. The yield is calculated as the mass of the respective component in the light phase divided by the mass of the respective component which is brought into the system with the used CBS extract. The purity is calculated as the mass of the respective component divided by the mass of the dry residue within the light phase. The data show that, for all components, the aqueous two-phase systems reach exceptional high yields of up to 100% in one extraction stage, whereas the conventional extraction systems with ethyl acetate and butyl acetate reach only up to a maximum of 50% for caffeine and only up to 30% for the targeted polyphenols. The data show higher yields for the ATPS at a higher phase ratio, which

represents a lower consumption of solvent, whereas the organic solvents show a contrary behavior. So, for better yields in the ATPS, less solvent is used, and for better yields with organic solvents, a higher amount of solvent is needed. The pH value seems to have a negligible influence on the yield. Regarding purity, organic solvents reach significantly higher values. This is because of rather high salt contents in the ATPS in the light, phenol-rich phase. This can be optimized with an adaption of the extraction system, e.g., higher ethanol content in the feed. This will be researched in a follow-up study.

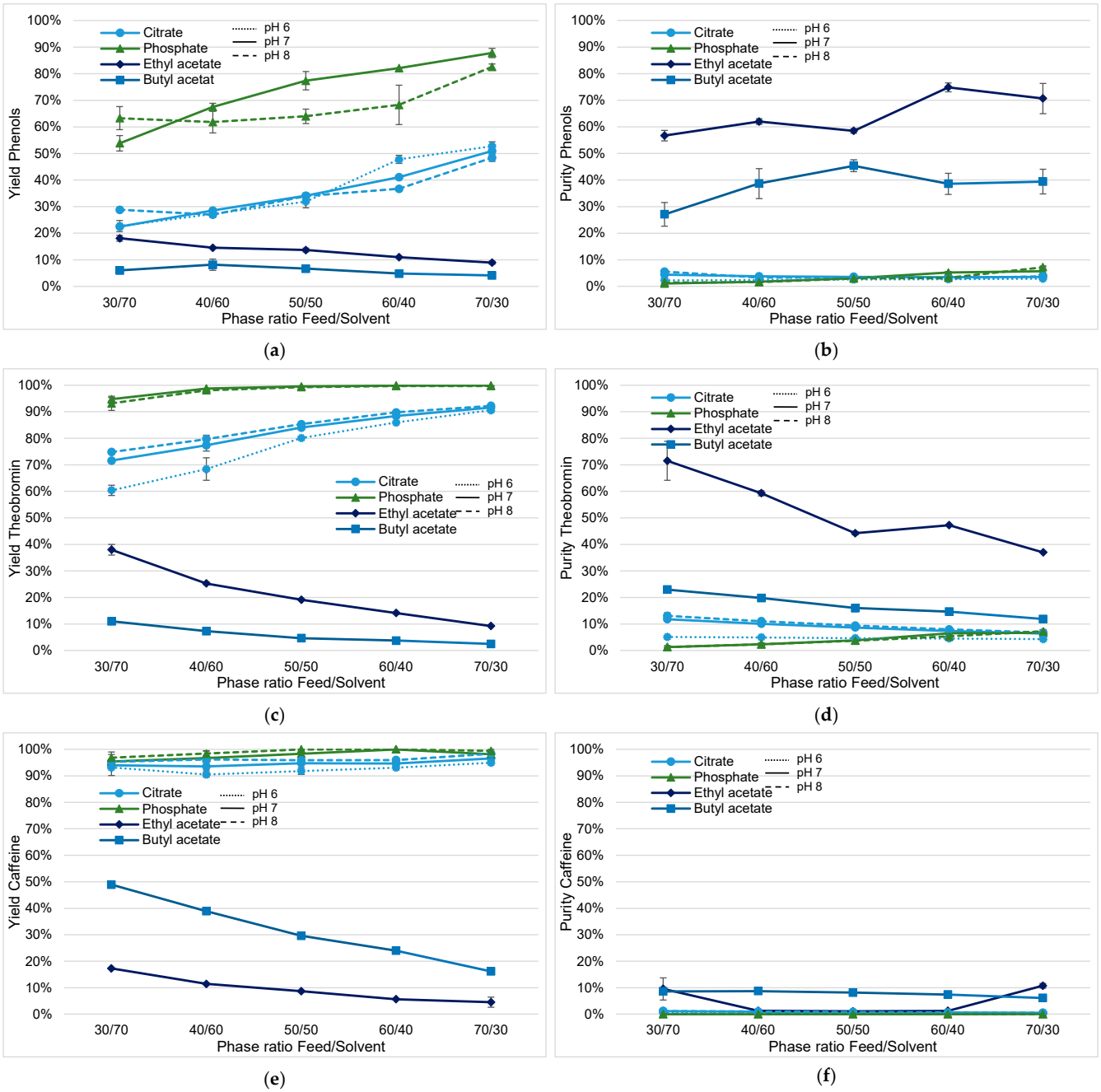


Figure 6. *Cont.*

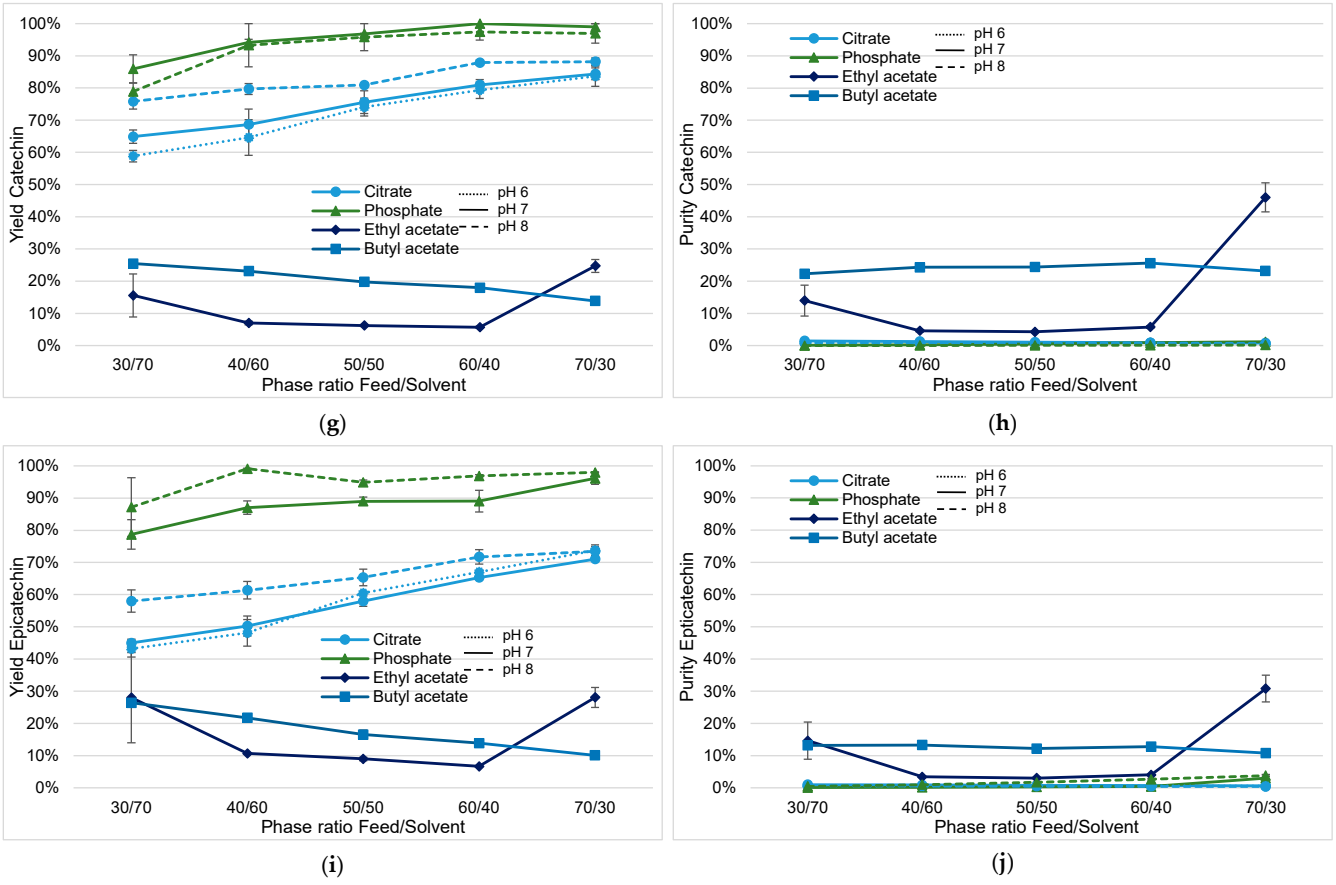
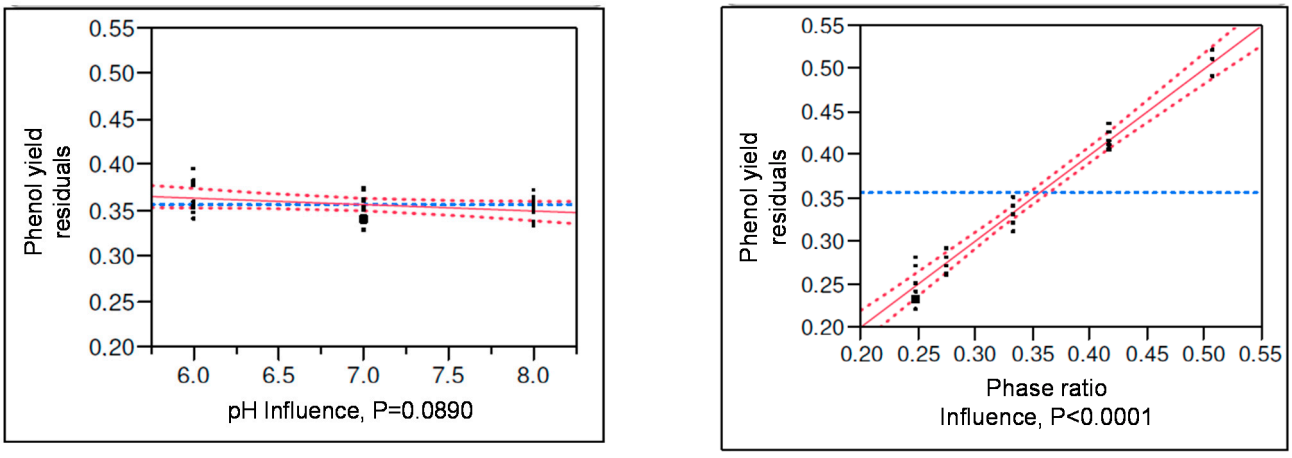


Figure 6. Yields and purities for total phenolic content (**a**,**b**), theobromine (**c**,**d**), caffeine (**e**,**f**), catechin (**g**,**h**) and epicatechin (**i**,**j**) in DoE with phase ratio and pH value including ethyl acetate and butyl acetate as reference.

In Figure 7, the statistical influence of pH value and phase ratio on the yield of phenol content, theobromine, caffeine, catechin and epicatechin are shown. The black squares in the plots represent the target values from the experimental data. These results are for the ATPS with a 40 wt.% citrate buffer. Due to low solubility of phosphate salts at pH 6, no full factorial DoE could be conducted.



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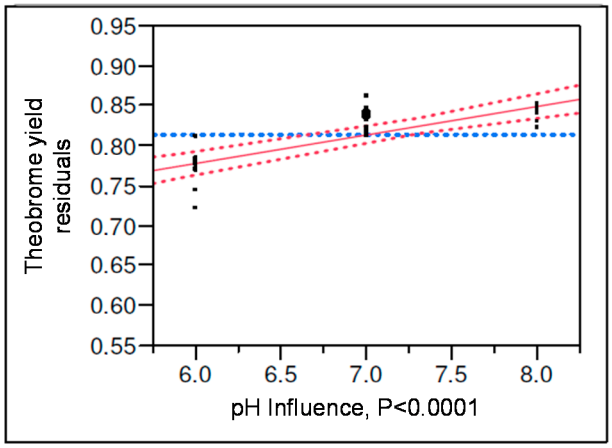
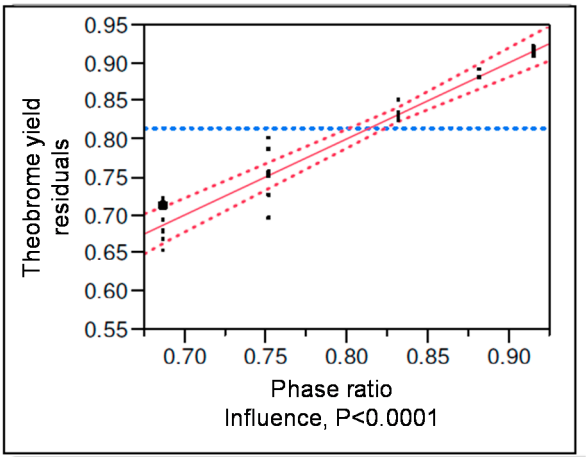


(**c**)



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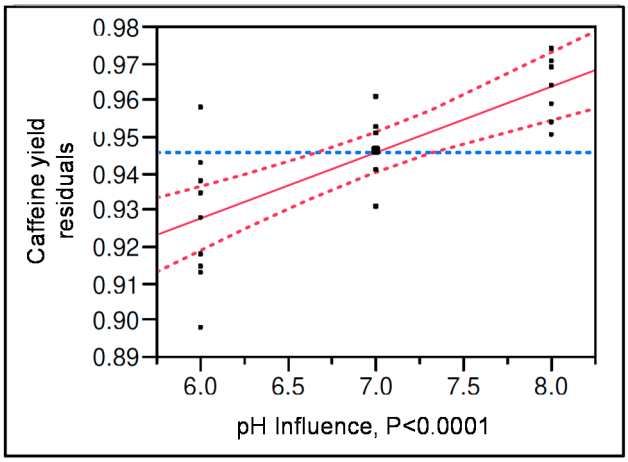

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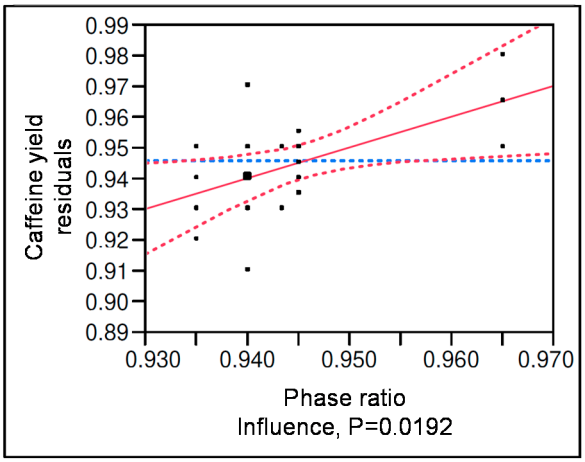

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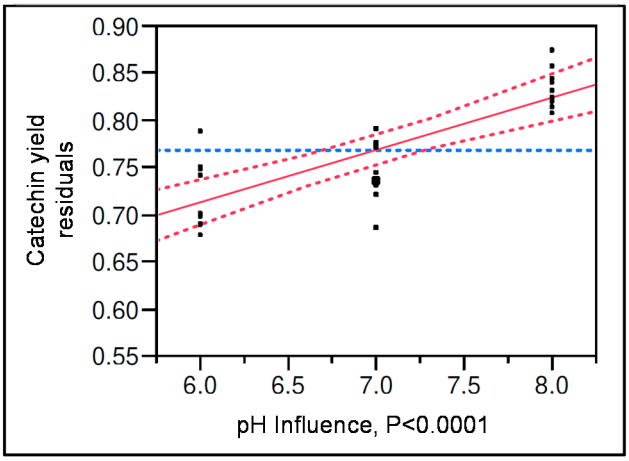

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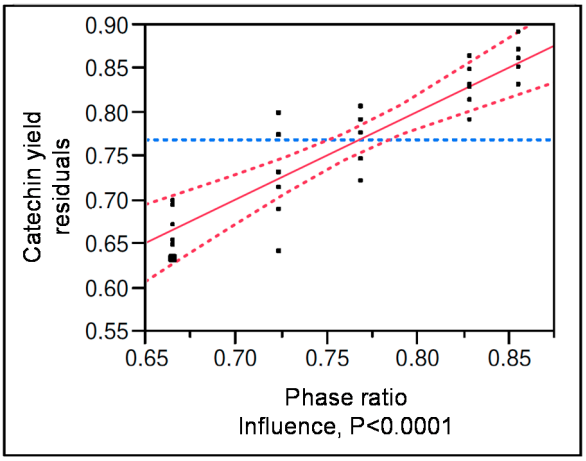

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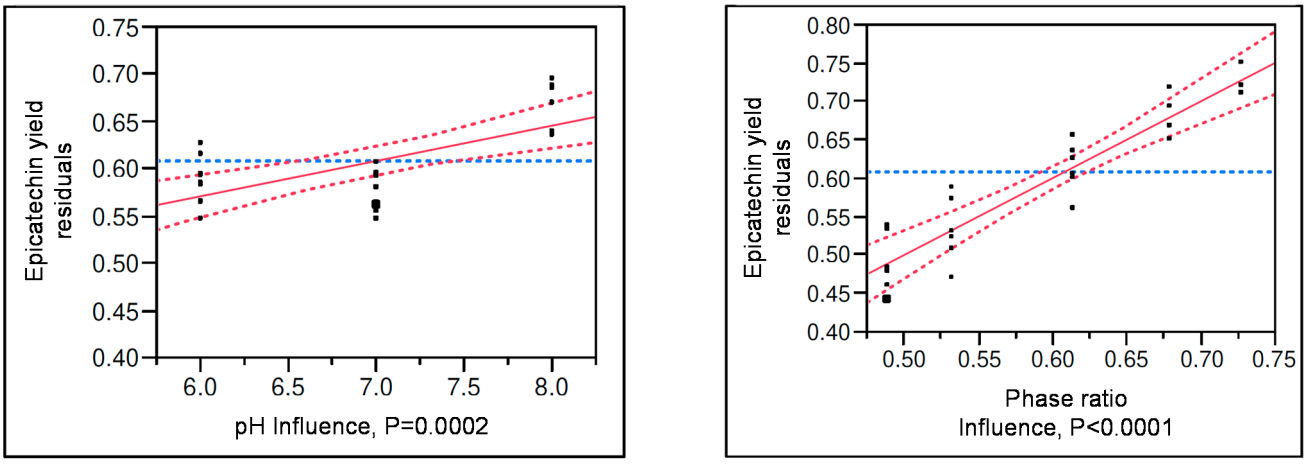


(**i**) (**j**)

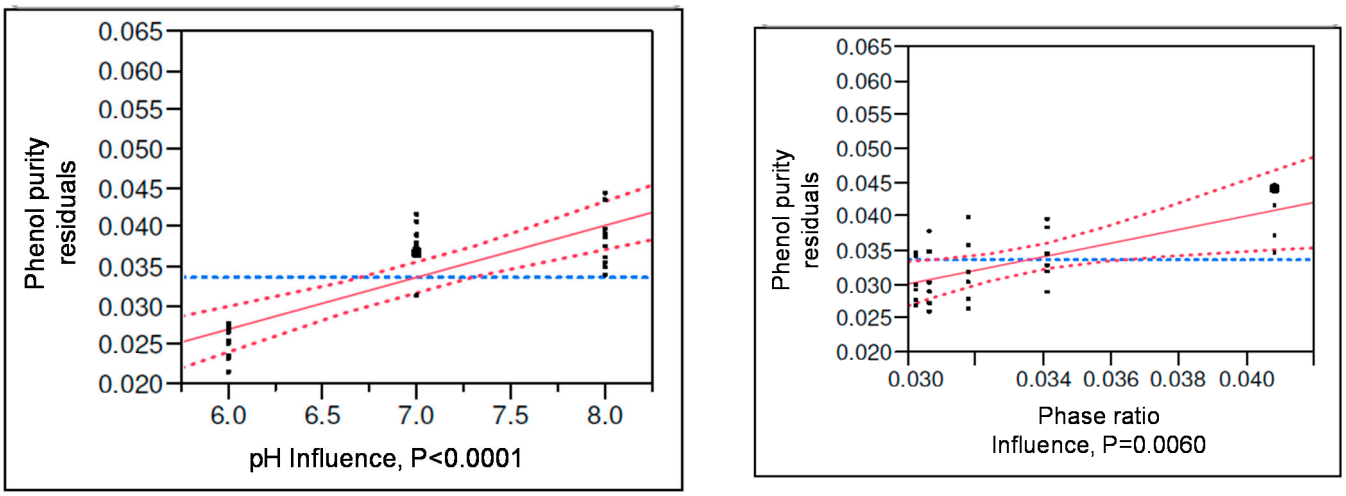
Figure 7. Influence of pH and phase ratio on yield for total phenolic content (**a**,**b**), theobromine (**c**,**d**), caffeine (**e**,**f**), catechin (**g**,**h**) and epicatechin (**i**,**j**).

For total phenolic content, the regression coefficient of the statistical model is 0.98. The pH value has no significant influence on the extraction yield, where the phase ratio has a high positive influence on the extraction yield. This behaviour matches with the observations in Figure 6. For theobromine, the regression coefficient of the statistical model is 0.96. Both pH value and phase ratio have a significant positive influence on the extraction yield. The regression coefficient of the statistical model for caffeine yield is 0.72. According to the P-values, pH value has a significant positive effect, while phase ratio has no significant effect. However, due to the low R^2 and the high scattering of the measured values in Figure 7e,f, this statement is questionable.

For catechin, the regression coefficient of the statistical model 0.88. Both pH value and phase ratio have a significant positive influence on the yield of catechin.

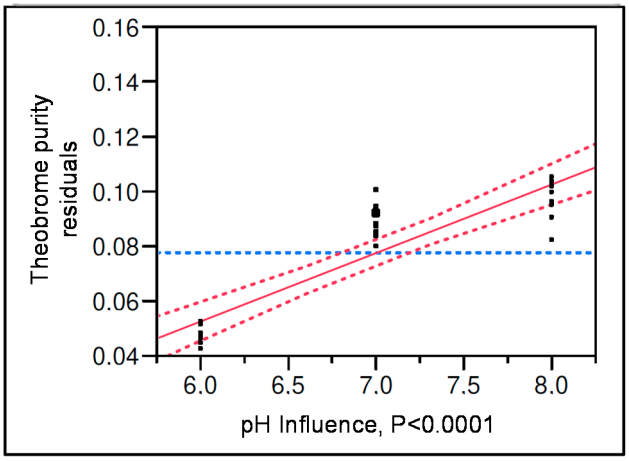
The regression coefficient of the statistical model for epicatechin is 0.91. The pH value has a medium significant positive influence on the yield, whereas phase ratio has a significant positive influence on the yield of epicatechin.

In Figure 8, the statistical influence of pH value and phase ratio on the purity of total phenolic content, theobromine, caffeine, catechin and epicatechin are shown.

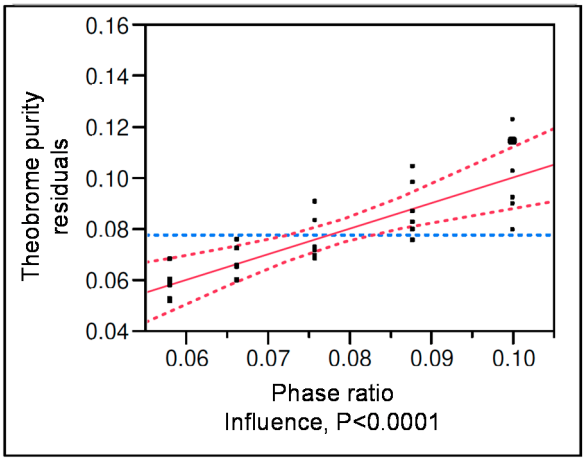


(**a**) (**b**)

Figure 8. *Cont.*



(**c**)



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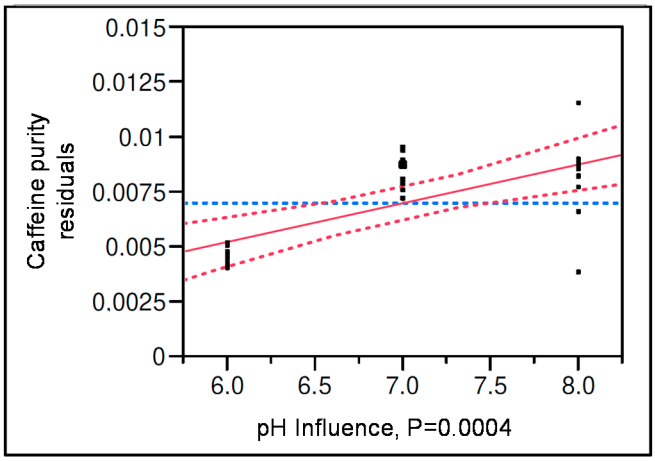
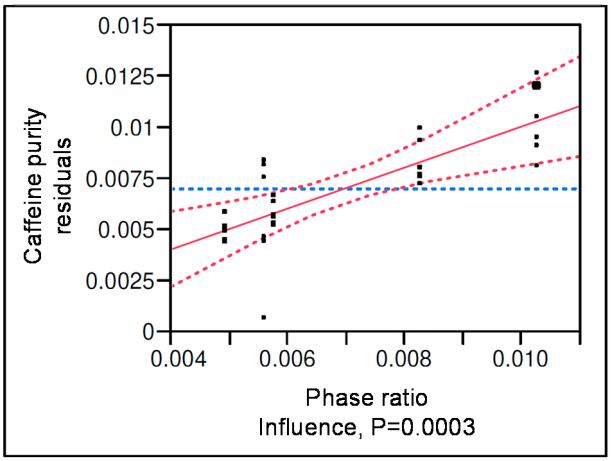
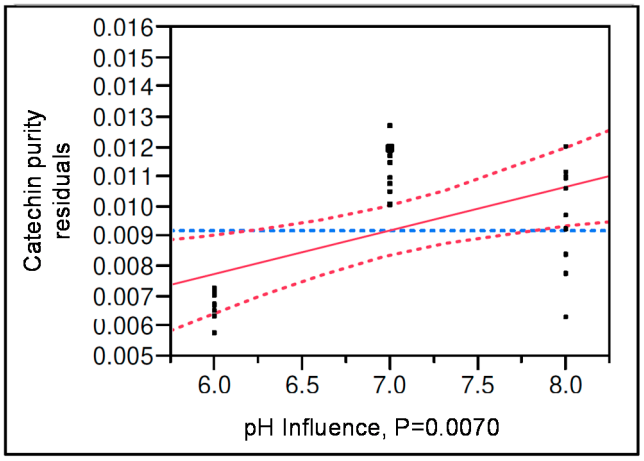


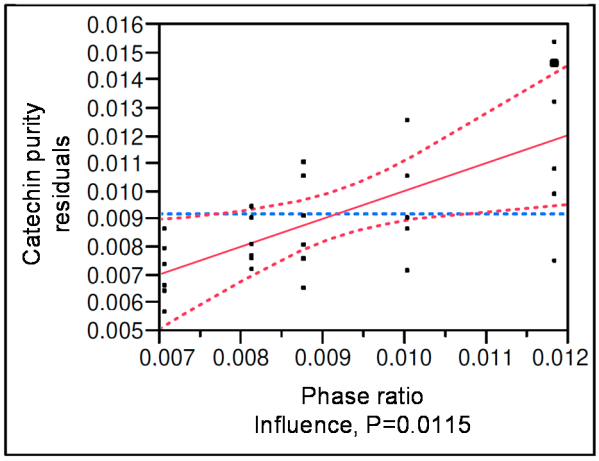
(**e**)



(**f**)



(**g**)



(**h**)

Figure 8. *Cont.*

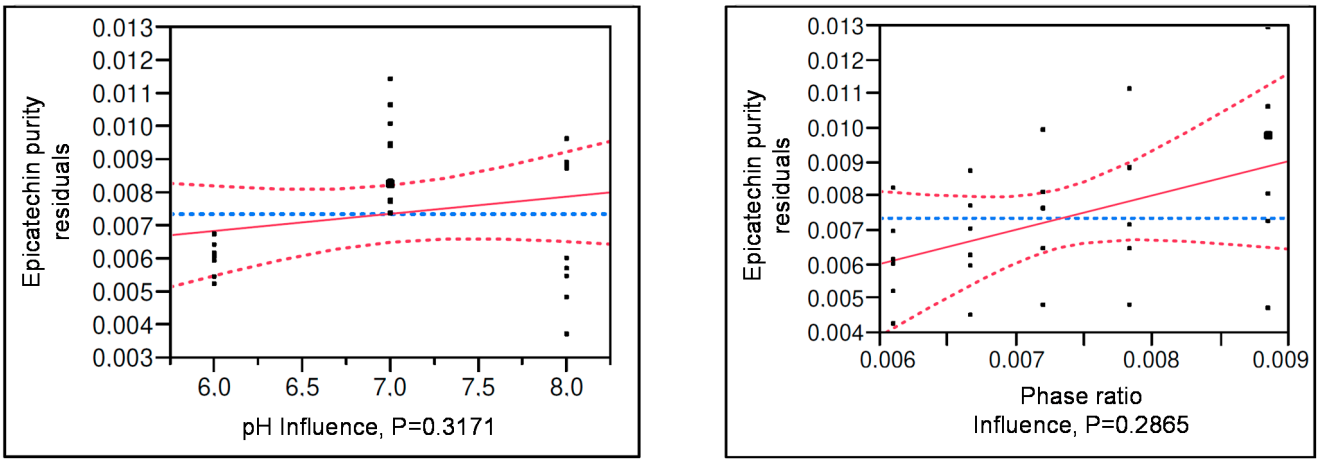


Figure 8. Influence of pH and phase ratio on purity for total phenolic content (**a**,**b**), theobromine (**c**,**d**), caffeine (**e**,**f**), catechin (**g**,**h**) and epicatechin (**i**,**j**).

For total phenolic content, the regression coefficient of the statistical model is 0.80. The pH value has a significant positive influence on the extraction yield, whereas the phase ratio has a low-to-no significant influence on the purity. This behaviour matches with the observations in Figure 6. For theobromine, the regression coefficient of the statistical model is 0.89. Both pH value and phase ratio have a significant positive influence on the purity of theobromine. The regression coefficient of the statistical model for caffeine purity is 0.76. According to the *p*-values, pH value and phase ratio have a medium significant positive effect on the purity of caffeine.

For catechin, the regression coefficient of the statistical model 0.58. Both pH value and phase ratio have no significant positive influence on the yield of catechin. However, due to the low R^2 and the high scattering of the measured values in Figure 8g,h, this statement has low confidence.

The regression coefficient of the statistical model for epicatechin is 0.28. Due to the low regression quality, there is no sophisticated statement to make.

In addition, for yield and purity, there is no significant influence of the interaction between pH value and phase ratio.

4. Discussion

In the present study, the extraction behaviour of the various target components in the CBS stock system was investigated. It was shown that the common organic solvents, with the exception of ethanol–water mixtures, give only poor extraction results. The comparatively good extraction properties of the ethanol–water mixtures already provided an indication, in the solvent screening of the SLE, that an ATPE with the ethanolic phase appears a promising method. Temperature screening showed very good extraction results for an extraction temperature of 140 °C, which is a common extraction temperature for the extraction of phenolic components.

In a first solvent screening for LLE, the three possible application points of LLE in the process alternatives are investigated. Whether LLE occurs before or after precipitation had no effect on the results in the present experiments. On the other hand, particularly strongly apolar solvents such as toluene or hexane are not suitable for fatty substance systems such as CBS. However, the potential of ATPE with ethanol, water and salt is confirmed, so that this process alternative is preferred. The only organic solvent required, ethanol, can even be used a second time for this purpose from the previous process step, precipitation. This saves resources and protects the climate; both points should be considered for processes in the bioeconomy. In the next step, the ATPE was investigated with two different salts. Here, pH of the salt buffer and the phase ratio were varied. From the statistical experimental

design, there is a positive influence on the yield for higher phase ratios, i.e., for a low proportion of the salt buffer. The pH value has a low influence on the yield and only for some components. The purity also increases for higher phase ratios. Increasing pH also has a positive effect on purity. For the alternative studies with ethyl acetate and butyl acetate from the crude extract, it was shown that a high organic phase content increases both the yield and purity. For the ATPE systems, yields between 80 and 100% are achieved for the different target components. The organic comparative tests only deliver yields between 20 and 40% in one extraction stage. The comparatively low purities of the ATPE systems are due to a transition of salt into the light phase. The salt content in the light phase can be reduced by optimizing the system point. While high phase ratios should be considered to maximize the yield, pH value can be used to influence the purity of some components.

The precipitation with 60 wt.% ethanol, which was defined in the present study by a project framework, will be investigated in more detail in follow-up studies. Due to the double utilization of ethanol in the ATPE, precipitation and LLE are directly linked. However, it is also conceivable to adjust the ethanol content to the optimum of the LLE after precipitation with an ethanol content that is optimal for this process step.

5. Conclusions

Following this study, a process is available which integrates the PHWE into an overall process for cascade utilization for waste valorization in an environmentally friendly, green and efficient economic manner. The process consists of an extraction with hot water, a precipitation with ethanol as an anti-solvent and a liquid–liquid extraction from the precipitation supernatant with salting-out of ethanol. In this way, both the matrix components and the secondary plant compounds can be fully utilized by integrating unit operations appropriately. The versatile green process for waste valorization is shown in Figure 9.

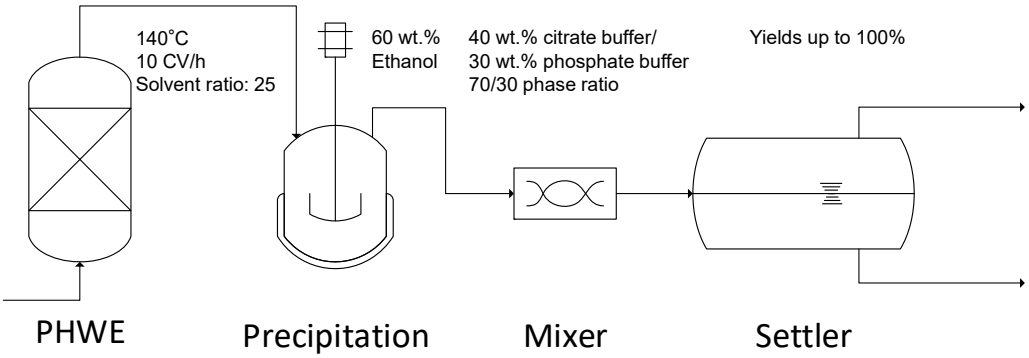


Figure 9. Overview of the novel process for the recovery of phenolic compounds from natural product extracts.

- The novel process reaches very high yields of up to 100% in one extraction stage.
- The novel process ensures low consumption of organic solvents due to double usage of ethanol as the only organic solvent.
- The process is adaptable enough to capture all kinds of secondary metabolites from hot water extracts and ensures usage of structural carbohydrates from precipitation. Ethanol is well-known as a precipitant for matrix components from hot water extracts. The ethanol content in the light phase is adaptable enough to match the solubility properties of the target component, usually between 50 and 80% ethanol [8,14].
- Follow-up studies will focus on process optimization, research on process analytical technology and complete dry residue characterization by component groups [13].

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