Introduction of a new two-phase extraction method covering a broad spectrum of analytes in food and environmental samples and its miniaturization to determine the pesticide body burden of individual invertebrates

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Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel: „Introduction of a new two-phase extraction method covering a broad spectrum of analytes in food and environmental samples and its miniaturization to determine the pesticide body burden of individual invertebrates“ selbstständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe. Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen (Beschluss des Senats vom 25.5.2000) beachtet wurden. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe. Mir ist bekannt, dass die falsche Abgabe einer Versicherung an Eides statt mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft wird.

Tübingen, den

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Nadja Kalinke

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Author contribution

Erklärung nach §6 Abs. 2 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Tübingen.

Prof. Carolin Huhn supervised my thesis, contributed valuable scientific input and interpretations and reviewed the written thesis. Additionally, the experimental work for this thesis was supported by other researchers and interns. A transparent statement of individual contributions is provided below.

Abstract, Motivation, Introduction, Summary and Outlook

These parts were written by me and revised by Carolin Huhn.

Chapter 3: SWIEET — A salt-free alternative to QuEChERS

The first idea of using a two-phase system without membranes for extraction was developed by Pascal Stopper. Pascal Stopper, Florian Diehl, Luca Völkl, Jan Hanenberg, Jonas Caspers, Friederike Schön and Madita Stief partly conducted experiments and aided in data evaluation for the optimization and application of the SWIEET method during their internships under my supervision. Data visualization was done by me, except Fig. 2 which was created by Pascal Stopper. I wrote this chapter and Carolin Huhn revised it.

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Chapter 4: Understanding partitioning in two-phase systems with induced miscibility gaps – comparing SWIEET and QuEChERS

The idea of determining water and isopropanol contents as well as solvatochromic parameters to characterize the extraction phases was jointly developed with Carolin Huhn. Practical experiments were conducted mainly by me. Madita Stief and Emrullah Bahadir partly conducted experiments and aided in data evaluation during their internships under my supervision. NMR measurements were conducted by the NMR facility of the Institute of Organic Chemistry, University of Tübingen under the supervision of Markus Kramer. Water content determination by Karl Fischer titration was conducted by Tim Neumann under the supervision of Christoph Körber at Merck KGaA, Darmstadt. I wrote this part and Carolin Huhn revised it.

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Chapter 5: Miniaturization of the SWIEET extraction method enables the analysis of the pesticide carbendazim in *Chironomus riparius* midges after metamorphosis

The exposure experiment was conducted by Johanna Bock under the supervision of Jörg Oehlmann at the University of Frankfurt. They also aided in biological interpretation of the results. Experiments on the miniaturization of the method, as well as the adaptation of the LC-MS method were done by Lea Dovodja during her bachelor's thesis under my supervision. I conducted all experiments on the applicability of the method to midges, as

well as overall data evaluation and interpretation. I wrote this part, except for the Section “Exposure experiment” in the Materials and Methods section, which was written by Johanna Bock. Carolin Huhn, Johanna Bock and Jörg Oehlmann revised this section.

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Abbreviations

CIPS	cold-induced phase separation
DoE	design of experiment
dSPE	dispersive solid phase extraction
EtOAc	ethyl acetate
HILIC	hydrophilic liquid interaction chromatography
LOD	limit of detection
LOQ	limit of quantification
ME	matrix effects
NA	4-nitroanisole
NC	negative control
NP	4-nitrophenole
PEG	polyethylene glycol
QuEChERS(ER)	quick, easy, cheap, effective, rugged, safe (efficient, robust)
QuPPe	quick polar pesticide
RD	Reichardt's dye
Rec	recovery
Rpm	revolutions per minute
RSD	relative standard deviation
SC	solvent control
SPE	solid phase extraction
SWIEET	sugar water isopropanol ethyl nitrile extraction technique
WWTP	waste water treatment plant

Zusammenfassung

Für Lebensmittel, Umwelt- und Biota-Proben ist QuEChERS die Extraktionsmethode der Wahl für ein breites Spektrum von Probentypen und Analyten. Jedoch werden sehr polare und geladene Analyten mit dieser Methode kaum extrahiert. Alternative Methoden wie QuPPE hingegen wurden speziell für polare Analyten entwickelt, sind aber für unpolare Analyten nicht anwendbar. Darüber hinaus erschweren die in QuEChERS verwendeten Salze, die eine Phasentrennung zwischen Acetonitril und Wasser herbeiführen, die Handhabung und können bei der folgenden Analyse durch Ablagerungen auf den Analysegeräten Probleme verursachen. In dieser Arbeit habe ich eine salzfreie Extraktionsmethode entwickelt, die Zucker mit sehr hoher Löslichkeit in Wasser verwendet, um eine Phasentrennung in einem Acetonitril-Wasser-System zu bewirken. Während der Entwicklung der Methode wurde ein breites Spektrum an Additiven untersucht, von denen Zucker die stabilsten Phasentrennungen und die höchsten Wiederfindungsraten für einen Modellmix von Analyten mit breiter Polaritätsverteilung erzielten. Die Zugabe von Isopropanol als polares protisches Lösemittel erhöhte die Wiederfindungsraten für polare Analyten, während die Wiederfindungsraten für unpolare Analyten hoch blieben. Mit Hilfe eines Design of Experiment wurden alle Extraktionsparameter gemeinsam optimiert, um die Anzahl der Experimente zu minimieren und ein globales Optimum zu finden. Mit Doppelextraktionen konnten die Wiederfindungsraten für polare Analyten, insbesondere im zweiten Schritt, auf 69 % erhöht werden. Die optimierte Methode wurde auf feste und flüssige Lebensmittel- und Umweltproben angewandt und erzielte für alle Proben ähnliche oder höhere Wiederfindungsraten als die klassische QuEChERS-Methode.

Um eine einfache Anpassung der neuen SWIEET-Methode für die Anwendung auf verschiedene analytische Fragestellungen zu ermöglichen, habe ich die physikochemischen Aspekte des Extraktionsverfahrens untersucht. Hohe Phasenverhältnisse für bestimmte Additivkombinationen korrelierten während der Methodenentwicklung mit hohen Wiederfindungsraten und wurden detaillierter untersucht. Die Konzentrationen von Wasser, Isopropanol und Glukose wurden mittels NMR und Karl-Fischer-Titration bestimmt, um die Zusammensetzung der wässrigen und organischen Phasen von SWIEET zu charakterisieren. Der Wassergehalt konnte den Unterschied in der Wiederfindung der Analyten zwischen SWIEET und QuEChERS erklären, nicht jedoch zwischen den beiden organischen SWIEET-Phasen der Doppelextraktion mit deutlichen Unterschieden für polare und unpolare Analyten. Es wurde vermutet, dass dies auf die Bildung von H-Brückenbindungen mit Isopropanol zurückzuführen ist, was ich durch die Bestimmung von solvatochromen Parameter weiter untersucht habe. Aufgrund seiner hohen Empfindlichkeit war Reichardts Farbstoff in der Lage, signifikante Unterschiede zwischen der Polarität der organischen Phasen von SWIEET und QuEChERS zu detektieren und wir konnten die Parameter unbekannter Mischungen aus Extraktionen mit einer Reihe von Parametern von Mischungen mit bekannter Zusammensetzung vergleichen. Ich konnte nachweisen, dass durch eine Verringerung des Isopropanolgehalts und Erhöhung des Glukosegehalts die Wiederfindungsraten für unpolare Analyten verbessert werden können, und dass durch eine Erhöhung des Isopropanolgehalts polare Analyten besser extrahiert werden.

Die finale SWIEET-Methode wurde miniaturisiert und auf die Bestimmung von Carbendazim in adulten Mücken von *Chironomus riparius* angewandt, die diesem Pestizid in ihrem Larvenstadium ausgesetzt waren. Das Ziel war, das Verhältnis zwischen internen Konzentrationen und Expositionskonzentrationen zu untersuchen. Für die Analyse von kleinen Invertebraten, idealerweise auf der Ebene von Individuen, wurde die SWIEET-Extraktionsmethode so miniaturisiert, dass keine übermäßige Verdünnung stattfand. Bei SWIEET wurde dies im Vergleich zu QuEChERS durch den Verzicht auf Salze erleichtert. Ich konnte die erfolgreiche Miniaturisierung von SWIEET bis zu einem Gesamtvolumen von 0,5 mL, sowie die Anwendung auf die Extraktion von Carbendazim aus Mücken, sogar aus Individuen zeigen. Ich stellte fest, dass starke Matrixeffekte die Analyse dieser Biota-Proben erschwerten. Die Verwendung matrixangepasster Kalibrierungen entsprechend der Anzahl der Mücken in der Probe war entscheidend, um quantitativ präzise Ergebnisse zu erhalten. Ich konnte eine Dosis-Wirkungs-Beziehung für weibliche Mücken aus dem Carbendazim-Expositionsversuch nachweisen und zeigen, dass das Pestizid auch während der Metamorphose erhalten blieb.

Zusammenfassend wurde eine neue Extraktionsmethode mit vereinfachtem Handling entwickelt, die eine Automatisierung und Miniaturisierung erleichtert und zugleich das extrahierbare Analytspektrum auf stark polare bis ionischer Substanzen erweitert.

Abstract

For food, environmental and biota samples, QuEChERS is the extraction method of choice for a wide range of sample types and analytes, but very polar and charged analytes are hardly extracted with this method. Alternative methods like QuPPE on the other hand were developed especially for polar analytes, but are not applicable to nonpolar analytes. Furthermore, the salts used in QuEChERS to induce a phase separation between water and acetonitrile complicate handling and can cause problems in downstream analysis by deposits on the analytical instruments. In this work, I developed a salt-free extraction method, that uses highly soluble sugars to induce a phase separation in an acetonitrile-water system. Method development was done by screening a wide range of additives, from which sugars produced the most stable phase separations and highest recoveries for a model analyte mix with a broad polarity range. The addition of isopropanol as a polar protic solvent increased recoveries for polar analytes while maintaining high recoveries for nonpolar analytes. Using a design of experiment, all extraction parameters were optimized jointly to minimize the number of experiments and find a global optimum. Double extractions were found to further increase recoveries to 69%, for polar analytes especially in the second step. The optimized final method was applied to solid and liquid food and environmental samples and achieved similar or higher recoveries than the QuEChERS method for all samples.

To enable an easy adaptation of the new SWIEET method to various analytical tasks, I investigated the physicochemical aspects of the extraction procedure. High phase ratios for certain additive combinations correlated with high recoveries during method development, which I further investigated. Water, isopropanol and glucose concentrations were determined by NMR and Karl Fischer titration to characterize the composition of the aqueous and organic phases of SWIEET. The water content was able to explain the difference in analyte recoveries between SWIEET and QuEChERS, but not between the two SWIEET organic phases of the double extraction with different recoveries for polar and nonpolar analytes. This was hypothesized to be due to the H-bond formation with isopropanol, which I further investigated by the determination of solvatochromic parameters. Due to its high sensitivity, Reichardt's dye was able to sense significant differences between the polarity of the organic phases of SWIEET and QuEChERS and I was able to compare the parameters of unknown mixtures from extractions to a set of parameters of mixtures with known composition. I demonstrated that by lowering the isopropanol content and increasing the glucose content, recoveries for nonpolar analytes can be improved, and by increasing the isopropanol content, polar analytes are better extracted.

The final SWIEET method was miniaturized and applied to the determination of carbendazim in adult midges from *Chironomus riparius* exposed to this pesticide at their larval life stadium, to investigate the relation of internal concentrations to exposure concentrations. For the analysis of small invertebrates, ideally at the level of individuals, the SWIEET extraction method was miniaturized so that no excessive dilution took place. With SWIEET, this was facilitated compared to QuEChERS, due to omitting salts. I demonstrated the successful miniaturization of SWIEET down to total volumes of 0.5 mL, as well as the application to the extraction of carbendazim from midges down to individuals. I found that strong matrix effects complicated the analysis of these biota

samples and conducting matrix-matched calibrations according to the number of midges in the sample was crucial to gain quantitatively reliable results. I was able to show a dose-response curve for female midges from the carbendazim exposure experiment and that the pesticide was kept also during metamorphosis.

In summary, a new extraction method with simplified handling was developed, facilitating both automation and miniaturization while broadening the extractable analyte spectrum to highly polar and ionic compounds.

1 Motivation

The QuEChERS extraction method was developed for food samples, but has been adapted to address a broad range of sample types. It is a simple and easy method that works well for the extraction of nonpolar analytes. But although claiming to be a multimethod covering a broad polarity spectrum of analytes, extraction of polar analytes is still insufficient. Following adaptations of QuEChERS like QuEChERSER further broadened the extractable analyte spectrum and QuPPE was developed specifically for the extraction of polar analytes. Still, there is no method for the simultaneous extraction of polar and nonpolar analytes with high recoveries for all.

While the recently proposed QuEChERSER method is a simple solvent extraction method with acetonitrile and water, phase separation is crucial for an extraction method to separate the target analytes from the solid sample and avoid co-extraction of matrix effects. Furthermore, phase composition and therefore polarity can be better tuned for the analytical question in a system with a miscibility gap. In the original QuEChERS method, the salts NaCl and $MgSO_4$ are used to induce this phase separation between an aqueous and an acetonitrile rich phase. However, the addition of $MgSO_4$ beyond saturation introduces a new solid phase into the extraction mixture, which can cause adsorption. Furthermore, the weighing of the salts is tedious and increases the sample preparation time significantly. The high salt-load in the extract can not only deposit on the surfaces of the analytical instruments, but also cause matrix effects such as ion suppression in MS.

The adaptation of QuEChERS to a wide range of sample types and target analytes was mostly done by empirically testing different salts, buffers or compositions of extraction media. Theoretical aspects of salting-out are available in literature, but the physicochemical understanding of the method has rarely been considered for method adaptations. A firm understanding of two-phase systems is missing but would be needed for a more targeted and precise optimization of the extraction methods.

For the extraction of biota, specific adaptations of the extraction method are needed. Due to the often small sample sizes available, e.g. for invertebrates, the QuEChERS method was miniaturized. For the analysis of invertebrates, several individuals have to be pooled to increase sample size and therefore allow more reliable quantification of target analytes, although the analysis of single organisms would enable biologists to study the natural variance in the uptake of substances. For the analysis of e.g. pesticides in single organisms, the current extraction protocols need to be further miniaturized to prevent excessive dilution of the target analyte. With methods using salting-out however, miniaturization is limited. Salt amounts would have to be decreased according to the extraction solution and weighing of such small amounts increases errors in sample preparation.

The aim of this work is to develop a new salt-free two-phase extraction method, that extracts polar analytes well while keeping recoveries for nonpolar analytes high to extract a broad range of analytes from various types of samples. The method is envisaged to be easy in handling and use non-toxic, cheap and readily available chemicals, that are compatible with down-stream instrumental analysis, such as LC-MS. A deep physicochemical understanding of the extraction method is needed, to ensure easy and

precise adaptation of the method to address specific analytical needs. Miniaturization of the method is intended to be facilitated by omitting insoluble additives. The final protocol will be applied to the analysis of pesticides in single invertebrate organisms.

2 Introduction: Multiresidue extraction methods

If many different compounds including pesticides need to be analyzed in one sample, multiresidue methods (MRMs) are necessary. This is important for example in food safety control with a set of target analytes, but also for screening purposes. Multiresidue methods can be employed for sample preparation and extraction, as well as for subsequent separation and analysis. Multiresidue analysis methods often use LC-MS which can be applied to a broad range of pollutants in e.g. environmental samples [1-6]. Nevertheless, these analysis methods can only be applied successfully, if an adequate sample preparation method is used prior to analysis. Solid phase extraction (SPE) has often been chosen [1-4, 6], the stationary phase material of which defines the polarity range that can be covered. It can either be used for the extraction of liquid phases, or as a clean-up step after the extraction of solid or liquid samples. SPE and LC each operate within defined polarity ranges, which can limit the types of analytes that are separated and detected. To ensure high analyte recoveries across a wide range of polarities, it is crucial that the extraction step covers a broader polarity spectrum than the subsequent methods. The advantage of using MRMs in the sample preparation step is that ideally no analytes of interest are lost during this step. In sample preparation, extractions are conducted to separate the analytes from the matrix, concentrate the target analytes or partition them into another solvent to be compatible with downstream analysis. In the case of MRMs, this almost always is a compromise, since methods cannot distinguish between target analytes and matrix components and both are extracted and concentrated. Methods that extract a broad range of analytes thus often also extract unwanted matrix components with similar physicochemical properties. To combat this, additional clean-up steps to remove matrix components are often incorporated, which increases workload and cost of the method but can avoid or reduce matrix effects and decrease contamination of analytical instrumentation. The range of multiresidue extraction methods is broad, as reviewed by Lehotay and Schenk in 2000 [7]. Most MRMs described before 2003 were developed for the extraction of specific residue groups such as pesticides, fungicides or pharmaceuticals in specific types of food samples [8]. In order to cover a very broad range of analytes to ensure food safety, many substances need to be monitored at once. For this reason QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) was developed in 2003 and has since been the gold standard extraction method [9, 10].

2.1 Solvent extraction vs. liquid-liquid extraction

Multiresidue extraction methods can be divided into simple solvent extractions of solid samples and extracting analytes from solid or liquid samples into two-phase liquid-liquid systems. For solvent extraction, a solvent is simply added to the sample containing the target analytes. Depending on the solubility of the substances, they will remain in the sample or become dissolved in the extraction solvent. The main advantage of this type of extraction is that it does not require expensive chemicals or elaborate instrumentation and is therefore simple and cheap. The main disadvantage is the lack of selectivity, since it is only based on the analytes solubility and can hardly be tuned to fit the polarity of the target analytes other than changing the solvent or pH. Furthermore, matrix components can easily be co-extracted and subsequent clean-up steps are necessary.

2.1.1 Extraction from two-phase systems: QuEChERS

With extractions using two-phase systems with two immiscible or partly miscible liquid phases, many of the downsides of solvent extraction can be counteracted. For these methods, usually water and one or two solvents not fully miscible with water are used, so that phase separation occurs. Alternatively, if water-miscible solvents are used, a miscibility gap can be induced by adding a third component or changing of the temperature [11]. After phase separation, the organic phase is separated from the aqueous phase and possible solid sample components. These methods have the great advantage that the polarity of the phases can be tuned by either temperature or the amount and type of miscibility-inducing substance added, in addition to the choice of solvents and pH. The most widely known and applied method for an extraction with a two-phase system is QuEChERS method, which was first presented in 2003 by Anastassiades et al. [9]. Fig. 1 shows the workflow of the QuEChERS method: First, water and acetonitrile are added to the sample. Since these two solvents are fully miscible, NaCl is added to induce phase separation. Subsequently, $MgSO_4$ is added to bind water and thereby improve recoveries for polar analytes. Additional dispersive solid phase extraction (dSPE) steps may be added for further clean-up. QuEChERS has been used and adapted for a wide range of applications, starting with the analysis of food samples, but now extended also to environmental and biota samples [12]. The original QuEChERS publication also discussed more theoretical aspects of the extraction to improve understanding, e.g. analyte recoveries depending on the water content in the organic phase. In most applications, however, the adaptations made were based on a trial and error approach by varying different parameters like buffer pH, types of salts or clean-up sorbents used [12]. Only little attention has been given to the fundamentals of the QuEChERS extraction, which would aid in a more targeted adaptation and optimization for the specific analytical task.

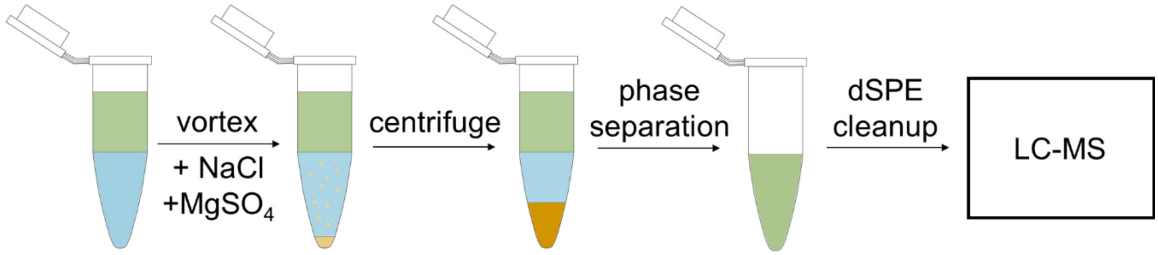

Fig. 1 Schematic representation of the workflow for the QuEChERS extraction.

QuEChERS extraction is based on a salting-out mechanism, which was already used in older MRMs, and also for different applications [13]. The theoretical background of salting-out was described entropically in the context of organic synthesis work-up by Hyde et al. [14]: Small ions with high charge density (kosmotropes) are introduced into a system of a solute in water and an organic solvent. The solute molecules aggregate due to the hydrophobic effect. The hydrophobic effect decreases due to increased electronic repulsion when kosmotropes are introduced and solute molecules are “ejected” into the organic phase. This mechanism explains the salting-out of nonpolar solutes well, but has difficulties in explaining it for polar solutes. Other theories about the underlying mechanisms of salting-out were summarized by Grover and Ryall in 2005 [13]: Internal pressure theory discusses salting-out in the context of volume contraction or expansion upon the addition of salts. A solute that causes the internal pressure in the solvent to increase causes salting-out for other solutes. This theory also only holds true for nonpolar

nonelectrolytes. Another explanation is based on the competition of the salt ions, especially the cations, with the solutes of interest for hydration. If the cations' attraction to water is stronger than the solutes, solutes become less hydrated and therefore more easily "ejected" into the organic phase. The water dipole theory states, that the water molecules in the hydration shell are oriented and therefore cause salting-out when a strong cation is introduced, since the water dipoles are then no longer preferentially oriented for solute hydration. The electrostatic theory uses the solvents' permittivity for the explanation of salting-out: If the permittivity increases upon the addition of a solute, salting-in occurs. If a different solute is added and the permittivity decreases below the value for pure water, salting-out occurs [13].

The type of salt added to a mixture plays a significant role in salting-out. As mentioned above, small highly charged cations have the greatest effect. Hyde et. al [14] correlated the strength of the salting-out effect to the Hofmeister series, since specific ion effects have a greater influence at the high salt concentrations used for salting-out. The Hofmeister series is empirically derived from the concentration of a salt needed to precipitate proteins. In QuEChERS, NaCl and $MgSO_4$ are used as salts [9]. NaCl is used to induce partitioning and described to increase recoveries of polar analytes, although too high concentrations of NaCl might cause the opposite by reducing the water content and therefore the polarity in the organic phase too much [9]. $MgSO_4$ is used as a drying agent and also described to increase recoveries of polar analytes [9]. This was explained by $MgSO_4$ binding large amounts of water, promoting partitioning of the analytes into the organic phase – as explained by the hydration competition described above. The effects of salts in a water-acetonitrile system were thoroughly investigated in 2021 by Li et al. [15]: Ternary phase diagrams of nine different salts in water-acetonitrile mixtures were created with cloud point measurements and component analysis, especially visualizing the size and position of the miscibility gaps in phase diagrams induced by the addition of salts. From phase diagrams like these, salt amounts needed for phase separation can be derived, preventing unnecessary overuse of salts which impairs sample handling. Furthermore, knowledge of the phase diagrams can aid in tuning the water content during extraction to better address the specific targets of the application. For example, for all salts used by Li et al. [15] except LiCl, a higher salt concentration was related to a more complete phase separation, synonymous with a lower water content and therefore lower polarity in the organic phase, which is commonly preferential for the extraction of nonpolar but not of polar or charged analytes.

Another liquid-liquid extraction method with a two-phase system introduced recently is based on ionic liquids [16, 17]. With this method, polar analytes are well extracted. Ionic liquids are composed of ions as is the case for salts, but the ionic liquids are liquid at lower temperatures, most of them at room temperature. In this case, ionic liquids chosen for extraction need to be stable to air and water and need to partly partition with water into the aqueous phase, so behave like organic solvents in two-phase systems. A downside of ionic liquids is their high viscosity, complicating handling and hindering automation of the protocol. Contrary to QuEChERS, ionic liquids have been extensively studied physicochemically by the determination of their solvatochromic parameters. Despite the good understanding of the physicochemical effects that underlie this approach, it is not yet routinely applied [17]. Major reasons are the limited commercial availability and the high price of ionic liquids.

2.1.2 Modern examples for solvent extraction

Extraction of solid samples is often accomplished by simply adding organic solvents or aqueous extraction media, evoking a solid-liquid partitioning of analytes. The polarity range covered is largely determined by the organic solvent, eventually supported by pH. To name a few examples, Murphy et al. [18] tested different solvents at different pH for the extraction of isoflavones from soy products. Daley et al. [19] used a DCM/hexane mixture to extract polychlorinated biphenyls from fish eggs. Two popular methods, that have been developed since QuEChERS are solvent extractions without phase separation: QuPPe (Quick Polar Pesticides) [20] and QuEChERSER (Quick, Easy, Cheap, Effective, Rugged, Safe, Efficient, Robust) [21].

A major goal of QuEChERS was to simplify and accelerate the extraction process and reduce the costs [9]. To avoid toxic organic solvents, acetonitrile is used instead of previously used halogenated solvents. This was achieved by omitting glassware, introducing a dSPE step instead of conventional SPE and using readily available and cheap chemicals. Regarding the broadness of the characteristics of extractable analytes, an improvement was also made for the research gap of the “most difficult analytes” (polar analytes) [9] compared to the traditional MRMs such as the Mills [22] or Luke [23] method, as well as other MRMs using nonpolar solvents for extraction. Polar analytes down to a $logD_{pH7}$ value of -0.5 could be included in the development of the QuEChERS method. More polar and even charged analytes like metformin ($logD_{pH7}$ = -5,7), however, would not be successfully extracted with this method.

To address the remaining challenge of the extraction of very polar analytes, the QuPPe method [20] was developed. As the name suggests, it was developed for the extraction of polar pesticides from food samples. As a simple extraction medium, an acidified methanolic solution is used. This method works well for the polar analytes that it is intended for, but not for mid- and nonpolar analytes and is therefore orthogonal to QuEChERS. If a broad range of analytes is to be extracted, both methods would need to be applied in parallel, which increases workload and costs.

Another variant of QuEChERS was introduced in 2020 and named QuEChERSER [21]. Again, the aim of this method was to increase the extractable analyte spectrum. An acetonitrile/water mixture (4/1) is added to the sample, subsequent LC-MS analysis is made without a phase separation, making it a simple solvent extraction. Compared to the original QuEChERS method, the extraction steps are similar in manual workload. Compared to QuEChERS, QuEChERSER uses a larger amount of acetonitrile/water mixture and buffers are omitted in most cases. A clear disadvantage is the need for two sample aliquots, as subsequent analysis is carried out with both LC-MS and GC-MS. For LC-MS, sample preparation is significantly easier than for the original QuEChERS method, since this simple extraction does not involve dSPE steps, which on the other hand is needed for GC sample preparation. The most important disadvantage of this method is the large dilution factor. Co-extraction of matrix component is expected, since no partitioning is induced.

The first aim of this thesis is to develop a straightforward method for the extraction of both polar and nonpolar analytes at the same time, achieving high recoveries for all analytes while maintaining easy handling.

2.2 Compatibility of multiresidue methods with LC-MS

A key factor of the applicability of an extraction method is its compatibility with downstream analysis. For non-target analysis, this is most often LC-MS. In the case of ionic liquids, they were tested as eluents in LC coupled to MS already in 1986 [24], up to more recent studies where ionic liquid extracts were analyzed directly with MS/MS [25]. In ESI, ionic liquids were even reported to lower the detection limits for anions via ion pairing which enabled analysis in the positive ionization mode [26]. The main issue of ionic liquids in mass spectrometry is that due to their low volatility, larger amounts of ionic liquids can deposit on the surfaces of the MS [27], as known for (inorganic) salts in mass spectrometry. The high amount of salts used in QuEChERS and other salting-out-based extractions can also cause pollution of the instruments, as well as strong matrix effects. Simple solvent extraction methods like QuPPE and QuEChERSER are at an advantage in this aspect, since they are usually salt-free and the solvents used are LC-MS compatible. However, in the original QuPPE publication, strong matrix effects were reported for this method [20] as well. Depending on the extraction solvent, polarity might be incompatible with direct injection for downstream LC analysis.

2.3 Application of QuEChERS to biota samples

QuEChERS has been developed for food samples, but it has been adapted to a wide range of sample types. One of the most challenging applications is in environmental science, especially biota samples such as insect larvae: For the extraction of small sample sizes, the extraction methods need to be miniaturized. Especially for the analysis of invertebrates, available amounts are only a few milligrams, even when several organisms are pooled. Important information for some biological questions can be gained when the analysis of single organisms becomes possible: By determining the body burden of pollutants in individual organisms, more detailed statements can be made about the natural variance in uptake and metabolism. These small sample sizes require adaptation of the volumes of the extraction medium used, so that dilution is minimized and concentrations in the sample are kept high enough for detection and quantification. In literature, only very few methods for the analysis of small numbers of individual invertebrates were published. In Table 1, miniaturized QuEChERS methods with sample amounts below 1 g are summarized. For almost all examples, several individuals were pooled to meet limits of detection (LODs). Miniaturizing the QuEChERS method, which is most often used for biota samples poses specific challenges: The amounts of salts added for phase separation in QuEChERS have to be reduced according to the reduction in solvents in the miniaturized QuEChERS variations, often called micro-QuEChERS. The amount of solid salt added, especially NaCl, was tuned carefully during QuEChERS method development [9], and changes in the salt content will lead to changes in the phase composition. Handling very small amounts of salts is necessary in miniaturization and will lead to greater errors than in larger scaled extractions. The ratio of solid added via the precipitate of insoluble salts to a solid sample is comparatively larger for smaller than for large sample sizes. Interactions of the analytes with this large solid salt phase, for example adsorption, can compromise the precision and recovery of the extraction. Another factor that needs to be addressed are matrix effects. For small sample sizes and low analyte concentrations near the LOD or LOQ, matrix effects can have a much greater impact on the quantification. Isotope labelled standards should be used when available to minimize these effects on quantitative precision. If isotope labelled standards are not available, matrix-matched calibration can be conducted alternatively. This quantification method

cannot always be applied, since the matrix for the calibration samples can be reproduced similarly to the sample matrix only if biological blank samples are available. This is not the case for most samples collected in the environment. Furthermore, the blank matrix can be reproduced similarly to the exposed sample, but will never be exactly the same, as weight and body composition of the individuals used in blank vs. loaded samples will naturally vary. This is especially relevant, if only a few or even single organisms are analyzed.

The second aim of this thesis is to develop a method, that enables the analysis of small sample sizes of invertebrate, down to single organisms. Omitting the salts used for QuEChERS/salting-out will facilitate not only miniaturization of the extraction method, but also decrease matrix effects and depositions on analytical instrument, which decrease the performance necessary for an analysis of low concentrations close to the LOD in biological extracts.

Table 1 Examples of miniaturized QuEChERS extractions from invertebrate samples, extended from Wicht [28] with sample sizes <1 g and recoveries. Dw: dry weight; ww: wet weight.

sample type	sample amount	analyte	recovery in %	ref.
bumble bees	98 ± 30 mg	pesticides	71-102	[29]
boluses	50 mg (dw)	pesticides	49-106	[30]
insects snails spiders	500 mg	pesticides	84-110	[31]
earthworms	250 mg (ww)	pharmaceuticals and hormones	44-98	[32]
daphnids	20 individuals	pesticides	95-111	[33]
invertebrates	12-20 mg	pollutants	40-98	[34]
chironomids	12 mg	biomarkers, pollutants	--	[35]
chironomids	25 mg	carbamazepine	95	[36]
gammarids	200 mg	pesticides and pollutants	48-127	[37]
gammarids	20 mg (ww)	pollutants	87-110	[38]
bivalves	500 mg	diclofenac	69-99	[39]
bivalves	100 mg	diclofenac and transformation products	78-117	[40]
gastropod	1 individuum	carbamazepine, fluoxetine	>85	[41]

3 SWIEET — A salt-free alternative to QuEChERS

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3.1 Abstract

The efficient extraction of various analytes from a wide spectrum of matrices with organic solvents is still a great challenge in analytical chemistry. Especially polar and charged compounds are hard to extract in combination with neutral analytes of intermediate to low polarity. The QuEChERS method is often chosen and has been adapted not only to the analysis of food samples, but also to environmental matrices (soil, wastewater) or biota. In this study, we overcome major drawbacks of QuEChERS such as low recoveries of charged analytes and impairment of downstream analysis by high salt loads. The new extraction method, applicable to liquid and solid samples, is called SWIEET (sugar water isopropanol ethyl nitrile extraction technique). Phase separation of the otherwise miscible extraction solvents water and acetonitrile is achieved by sugaring-out instead of salting-out. Extraction efficiencies were greatly improved by adding isopropanol to the acetonitrile phase. The concentrations of the additives glucose and isopropanol, as well as temperature were optimized by a design of experiment. Further improvement was achieved through electro- or double extractions. For all sample types tested (surface water, wastewater treatment plant effluent, tomato, soil and oats), recoveries and precision were higher with SWIEET than with the established QuEChERS method. From wastewater treatment plant effluent, 75% recovery on average were achieved with our SWIEET method compared to 37% with QuEChERS for a model analyte mixture with polarities of $\log D_{pH7}$ = −5.7 - 3.5. Higher recoveries and lower standard deviations compared to QuEChERS were achieved especially for polar and charged analytes such as metformin. Handling proved to be easy, since there was no additional solid phase and no tedious weighing of salts.

3.2 Introduction

The extraction method QuEChERS was first published in 2003 by Anastassiades et al. [9]. Since then, it has become the gold standard for the extraction of a broad spectrum of analytes from many different types of samples [12]. The method is applicable to solid and liquid samples and uses two liquid phases with their separation being induced by salts, often followed by a cleanup using dispersive solid phase extraction (dSPE). Parameters like pH, solvent composition and salt concentration were optimized for different target analytes and matrices, enabling a wide range of applications, as reviewed by Perestrelo et al. [42]. A major disadvantage of QuEChERS is the high amount of salt used during extraction, which can impair downstream analysis. Especially in ESI-MS, ion suppression can occur [43], as well as increased formation of sodium adducts. Furthermore, classical QuEChERS salts like NaCl and $MgSO_4$ are not volatile and therefore form deposits on surfaces of the analytical instrumentation [44]. The p-QuEChERS method uses potassium phosphates instead of the classical QuEChERS salts, so problems related to solid salt phase remain, but the formation of magnesium complexes is avoided and recoveries can

be increased [45]. The salts used for the extraction have to be weighed for each sample, which is time consuming. The other option is to buy QuEChERS kits, which significantly increases the costs of the sample preparation and limits possibilities to adapt the method to the analytical task under consideration. A further development of QuEChERS is QuEChERSER, which was introduced to further broaden the polarity range using acetonitrile/water for extraction first. A few µL are used for direct LC-MS analysis, then a salting-out step is used to create an extract for GC-MS analysis [21, 46, 47]. An orthogonal method to QuEChERS is QuPPe. Here, acidified methanol is used for the extraction of very polar analytes, while non-polar analytes have no recoveries [20].

Alternative strategies published so far replaced salts by sugars or organic solvents or lowered the temperature to reach the miscibility gap of water and acetonitrile.

Salting Out

The concept of salting-out has been known for centuries and used in organic chemistry for sample workup but also in larger scale industrial purification processes or smaller scale purification of proteins [13]. The solvent system in QuEChERS consists of acetonitrile and water. As acetonitrile and water are fully miscible, a miscibility gap has to be introduced, for which QuEChERS uses salts, mainly NaCl and $MgSO_4$. These salts are strongly bonded to water, so that a solvent layer forms around the salt ions [13]. This decreases the solubility first of all for acetonitrile in water until the miscibility gap is reached. The same process is relevant for many other organic compounds and can be used to enrich analytes in the organic acetonitrile phase for further analysis [14].

Temperature-Induced Phase Separation

For samples with a high fat content, lowered temperatures were used in addition to salt addition to evoke phase separation. At low temperature, lipids precipitate and can easily be removed [48]. As a QuEChERS alternative, salts may even be omitted and phase separation induced just by lowering temperatures as shown by Shao et al. [11] who named the method cold-induced aqueous acetonitrile phase separation (CIPS-QuEChERS). Similarly, to remove acetonitrile from otherwise aqueous protein solutions, Gu et al. [49] used low temperatures to induce a phase separation. The concept of adapting temperature to manipulate the miscibility gap was further demonstrated by Ullmann et al. [50], who extracted dyes in hexane-methanol and water-acetonitrile-toluene mixtures. The phase composition changed continuously by heating or cooling the mixtures. This is advantageous as no transfer across a sharp phase boundary is required upon the continuous formation of the two-phase system. A great advantage is, that less reagents are needed for this method. However, robustness of phase composition and thus repeatability of the extraction is compromised if the temperature cannot be fully controlled.

Sugaring-Out

Wang et al. [51] described the induction of a phase separation by addition of sugar to an acetonitrile-water system. Considering the influence of temperature on the ternary system, phase diagrams were also recorded to optimize acetonitrile retrieval after HPLC [52, 53]. Sugars neither interact with analytes nor change the sample solution in terms of pH contrary to the salts used in QuEChERS [42], which can facilitate robust analyte extraction. The influence of different sugars and polysaccharides on the distribution coefficients of various acidic compounds was investigated [51]. Their distribution coefficients between

the organic and aqueous phase ranged between 1.7-8.9. For the polar vanillin [54, 55], extraction efficiencies up to 95% were reported. Using sugar also enabled the use of electroextraction, which is not possible in samples with high salt loads due to the high conductivity. E.g. Mahdavi et al. [56] observed an improvement of the extraction efficiency, a stabilization of the current and lowered electrolytic reactions using sugars for the electromembrane extraction of basic drugs through a supported liquid membrane. Sugars, just like salts, are cheap and readily available. Since sugars are neutral, sugaring-out can be expected to be compatible with most downstream analytical methods which are sensitive to salt loads such as capillary electrophoresis and HILIC. Finally, the main advantage of sugar additives is their high solubility, resulting in facilitated method development and reduced workload, because pipetting highly concentrated sugar solutions is possible instead of weighing salts for each sample.

Organic solvents as additives

Another method for extractions based on phase separation was suggested by Gupta et al. [57] who induced phase separation adding an organic modifier to the mixture of acetonitrile and water. They used methyl isobutyl ketone as a modifier in addition to sodium chloride. Hydrophobic solvents were often used as modifiers: E.g. Liu et al. [58] used non-oxygenated solvents, such as dichloromethane (DCM), as modifiers in an acetonitrile-water system to extract flavonoids from plants, achieving higher recoveries than by using salts alone. They also applied their method to plasma samples spiked with three model drugs [59]. Hu et al. [60] tested ethyl acetate (EtOAc), ethyl ether and methyl tert-butyl ether as modifiers for the extraction of 17 organophosphate flame retardants and plasticizers from urine, achieving higher recoveries in comparison to solid-phase extraction. The main downside is that many of the modifiers used are toxic. To our knowledge, polar-protic solvents have not been used yet as modifiers in an acetonitrile-water system. Advantages of this method compared to classical salting out include facilitated and faster execution and the ease to adapt phase ratios and phase composition.

Electroextraction

Using electric fields, extraction of charged analytes can be enhanced from various liquid samples. This can be done across a solid or liquid membrane or directly across one or more phase boundaries [61-63] present in mixtures with miscibility gaps. However, this is only possible if salts are avoided to keep the conductivity low. Only a few of the strategies of inducing a phase separation described so far are compatible with electroextraction. As discussed previously, temperature plays a significant role for extraction, which may, however, be in conflict with temperature changes that occur during electroextraction due to Joule heating. Continuous cooling would be needed during the experiments to prevent disintegration of the phase boundary if the phase ratio and composition strongly depended on temperature. This would cause the need for a complex instrumental setup to ensure isothermal conditions. Furthermore, electrophoretic mobilities decrease at low temperatures.

With regard to the choice of organic solvents, their permittivity needs to be considered: If the permittivity is too low, no stable electric field can form and ion pair formation may prevent electromigration. In literature, next to 1-pentanol [64-66], EtOAc [67-71] is the most frequently used organic solvent for electroextraction, either as free liquid membrane or donor phase. It fulfils the requirement of immiscibility with water, but its permittivity is

relatively low, so acids or other electrolytes are added to increase conductivity [70]. Sugaring-out systems are a suitable option for electroextraction as sugars do not impair the electric field, but they may reduce electrophoretic mobilities by increasing the viscosity of solutions.

In this study, we developed a new extraction method for a wide range of analytes, which is applicable to a broad spectrum of sample types. Overcoming the downsides of salt used to induce a miscibility gap, we chose a sugaring-out approach. A focus of this work was to improve the recoveries of polar analytes by addition of polar solvents to the extraction mixture. To further improve the extraction of charged analytes, electroextraction and double-extractions were envisaged. QuEChERS extractions were made for comparison.

3.3 Materials and Methods

3.3.1 Chemicals

1-ethyl-3-methyl-imidazolium (EMI, ≥95%), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 5-amino-2-naphthalene sulfonic acid (ANSA, ≥95%), acesulfame (ACE, ≥99%), acridine (ACR, 97%), alpha-D-glucose (96%), carbon (>99%), clarithromycin (CLA, ≥98%), di-(2-ethylhexyl)phosphoric acid (DEHPA, ≥98%), dextran 450,000-650,000, diclofenac sodium salt (DIC, ≥98%), isopropanol (iPr, LC-MS grade), magnesium sulfate, methanol (MeOH, LC-MS grade), naphazoline (NAPHA, ≥98%), pindolol (PIN, 98%), polyethylene glycol 6,000, poly(4-styrensulfonic acid), poly(vinyl alcohol) 31,000-50,000, poly(vinyl alcohol) 89,000-98,000, poly(vinyl alcohol) 146,000-186,000, potassium phosphate monobasic (≥99.5%), saccharine (SAC, ≥98%) and tert-butyl alcohol (≥99%) were purchased from Sigma Aldrich (Steinheim, Germany). 4-hydroxybenzoic acid (HBA, ≥98%), chloroform (>99%), L-proline (≥99%), *N*-diethyl-m-toluamide (DEET, ≥98%), polyethylene glycol 35,000, poly(vinyl alcohol) 22,000, poly(vinyl alcohol) 72,000, poly(vinyl alcohol) 100,000 and sodium chloride (p.a.) were from Fluka (Buchs, Switzerland). Aliquat 336 TG (≥90%), dichloromethane (DCM, LC-MS grade), D(+)-galactose (≥99%), ethyl acetate (EtOAc, p.a.) and water (LC-MS grade) were provided by Thermo Fisher (Kandel, Germany). Acetonitrile (MeCN, LC-MS grade), D(+)-xylose (≥99%), formic acid (>99%), glycerol (≥98%), indigo carmine and tri-sodium citrate dihydrate (≥99%) were bought from Roth (Karlsruhe, Germany). Metformin (MET, 97%) was from Alfa Aesar (Haverhill, MA, USA), trehalose dihydrate (97%) from BLDpharm (Shanghai, China), dextran sodium sulfate 40,000 from ICN Biomedicals (Aurora, OH, USA), D-sorbitol (97%) from Acros Organics (NJ, USA), C18 and PSA from Agilent (Waldbronn, Germany) and polyethylene glycol 600 and 1,000 from Merck (Darmstadt, Germany). Doubly-distilled water was produced using a PURELAB Classic PL5241 (ELGA LabWater, Celle, Germany).

3.3.2 Model analyte mix

To cover a broad spectrum of analytes regarding size, polarity, charge and functional groups, 13 model analytes were chosen to optimize and judge the performance of the extraction protocol. Equal amounts of the analytes' methanolic stock solutions (1 g/L) were mixed to obtain the analyte mix at an individual analyte concentration of 77 mg/L each. For extraction experiments, the concentration of analytes in the aqueous mixture was 3 mg/L.

Table 2 Model analytes, their charge number at pH 7 and $logD_{pH7}$.

analyte	charge number$_{pH7}$	$logD_{pH7}$
metformin	+2	−5,7
1-ethyl-3-methyl-imidazolium	+1	−3,1
pindolol	+1	−0,5
naphazoline	+1	−0,2
clarithromycin	+1	1,2
acridine	0	3,5
5-amino-2-naphthalene sulfonic acid	0	1,7
N-diethyl-*m*-toluamide (DEET)	0	2,5
4-hydroxybenzoic acid	−1	−1,2
diclofenac	−1	1,4
acesulfame	−1	−1,5
MCPA	−1	-1
saccharine	−1	−0,5

3.3.3 Extraction procedure

3.3.3.1 Spiking of dry samples

For solid and dry samples, such as soil and oats, 0.98 mL of the analyte mix were added per 20 g sample, as well as 20 mL doubly distilled water to ensure proper distribution of the analytes on the sample. The mixture was shaken for 1 h using an overhead shaker. The water was evaporated from the sample at 60 °C, so analytes could sorb on the sample surface.

3.3.3.2 QuEChERS extraction

Aqueous samples: Analyte Mix was spiked to 2.5 mL aqueous sample such as surface water or wastewater treatment plant effluent to a final concentration of 3 mg/L. After adding 2.5 mL MeCN to the aqueous sample, the extraction mixture was mixed for 1 min using a vortexer. To the mixture, 0.25 mg NaCl and 1 mg $MgSO_4$ were added and the slurry was immediately shaken vigorously for another min. The extraction mixture was centrifuged at 3500 rpm for 10 min. Phases were separated by pipetting the upper phase into a different vial.

Dry samples: To 1.5 g of the spiked and dried sample, 2.5 mL doubly distilled water and 2.5 mL MeCN were added and the extraction mixture was mixed for 1 min using a vortexer. To the mixture, 0.25 mg NaCl and 1 mg $MgSO_4$ were added and immediately shaken vigorously for another min. The extraction mixture was centrifuged at 3500 rpm (1233 rcf) for 10 min. Phases were separated by pipetting the upper phase into a different vial.

For double-extractions, another 2 mL fresh MeCN were added to the residual aqueous phase, the extraction mixture was mixed for 1 min using a vortexer and centrifuged at 3500 rpm (1233 rcf) for 10 min. Phases were separated again by pipetting the upper phase into a different vial.

An aliquot of each or the combined organic phases were diluted for LC-MS analysis (see “3.3.7 Quantification”) and directly analyzed or stored at -20 °C.

3.3.3.3 Design of experiment

For the Design of Experiment (DoE) the software Develve Version 4.14.0.0 (Velp, The Netherlands) was used for design, calculation and plotting of the Box-Behnken-Design.

3.3.3.4 Optimization of the SWIEET extraction protocol

Various additives were added at different concentrations to an aqueous sample, containing 3 mg/L analyte mix: 2.5 mL of this spiked sample were mixed with the same volume of organic extraction mixture consisting of acetonitrile and 5-20 vol.% of either EtOAc, isopropanol, DCM or chloroform. The mixture was homogenized for 1 min using a vortexer. After a clear phase boundary was visible, usually within a min, phases were separated by pipetting.

For double-extraction, 1-2.5 mL fresh organic extraction mixture consisting of acetonitrile with either 10 or 20 vol.% isopropanol were added to the aqueous phase. After mixing for 1 min using a vortexer, the phases were allowed to separate again.

An aliquot of the organic phase was diluted with methanol for LC-MS analysis (see “3.3.7 Quantification”) and then stored at −20 °C or directly analyzed.

3.3.3.5 Final SWIEET protocol

Aqueous samples: For aqueous samples, glucose was added to the sample to a final concentration of 2 M, containing 3 mg/L analyte mix. A mixture of 2.5 mL of this spiked sample and the same volume of organic extraction mixture consisting of 80 vol.% acetonitrile and 20 vol.% isopropanol was prepared. The mixture was homogenized for 1 min using a vortexter. After a clear phase boundary was visible, usually within a min, phases were separated by pipetting.

Dry samples: To 1.5 g of the spiked and dried sample, 2.5 mL of a 2 M aqueous glucose solution and 2.5 mL organic extraction mixture, consisting of 80 vol.% acetonitrile and 20 vol.% isopropanol, were added. The mixture was homogenized for 1 min using a vortexer. After a clear phase boundary was visible, phases were separated by pipetting.

For double-extraction, 2.5 mL fresh organic extraction mixture consisting of 80 vol.% acetonitrile and 20 vol.% isopropanol were added to the residual aqueous phase from the first extraction step. After mixing for 1 min using a vortexer, the phases were allowed to separate again. Organic phases of the two extraction steps were combined prior to analysis.

An aliquot of the organic phase was diluted with methanol for LC-MS analysis (see “3.3.4 LC-MS sample preparation”) and then stored at −20 °C or directly analyzed.

3.3.4 LC-MS sample preparation

An aliquot of 10 µL of the organic extract was diluted with 40 µL MeOH for RPLC-MS analysis. Matrix-matched calibration was used to quantify the analytes. For this, the organic phase from a blank extraction (using doubly distilled water as a sample) was spiked at four concentration levels with all analytes.

3.3.5 Electroextraction

For electroextraction, a standard 5 mL plastic syringe was equipped with 0.5 mm thick and 5 mm long platinum electrodes at the outlet and the stamp, connected to a voltage source (Keithley 2290E5, Keithley, Cologne, Germany). The two-phase system was transferred to the syringe after mixing for 1 min using a vortexer. A constant current of 200 µA (chosen

after optimization) was applied for 10 min. The aqueous and organic phase were collected in separate tubes.

3.3.6 LC-MS method

The RPLC-MS method was adapted from Rösch et al. [72]. A 1260 Infinity LC system coupled to a 6550 iFunnel Q-TOF (Agilent Technologies, Waldbronn, Germany or Santa Clara, CA, USA) was used. An aliquot of 2 µL of the diluted sample was injected onto a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5 µm, Agilent Technologies) equipped with a Zorbax Eclipse Plus C18 guard column (2.1 x 12.5 mm, 5 µm, Agilent Technologies). The mobile phase consisted of water and acetonitrile with 0.1% formic acid. A gradient was used at a flow rate of 0.3 mL/min. Initially, a water content of 95% was used for 1 min, then it was decreased to 5% over 7 min and held for another 7 min. Finally, the water content was increased to 95% for 5 min.

For MS analysis, a jet-stream electrospray ionization source was used. The nebulizer pressure was 35 psig, drying gas temperature 160 °C, drying gas flow rate 16 L/min, fragmentor voltage 360 V, capillary voltage +/− 4000 V, skimmer voltage 65 V, nozzle voltage 500 V. The sheath gas had a temperature of 325 °C and was used at a flow rate of 11 L/min. Spectra were acquired at a rate of 1 spectrum/s in the mass range of 40-1000 m/z. Solutions of purine and HP0921 (Agilent Technologies) in methanol/water (95/5) were constantly infused into the ESI source through a reference sprayer for internal calibration.

Extracted ion chromatograms of the model analytes acquired with this method are shown in Fig. 4.

3.3.7 Quantification

The phase ratio was determined weighing the separated phases (m(org) and m(aq)). Analyte concentrations from the samples were calculated using the calibration curve resulting from matrix-matched calibration, taking dilution into account. Recoveries were calculated using the concentrations determined in the organic phase after extraction (c(org)) by LC-MS, the starting concentration in the aqueous sample (c(aq)), as well as the phase ratio after the extraction (m(org)/m(aq)):

$$rec\% = \frac{c(org)}{c(aq)} \cdot \frac{m(org)}{m(aq)} \cdot 100 \quad (1)$$

Average recoveries of extractions (n = 3 or n = 5) were determined for individual analytes. For an easier comparison of different extraction protocols, medians and averages over all 13 model analytes were calculated. In addition, analytes were grouped into polar ($logD_{pH7}<0$) and unpolar ($logD_{pH7} > 0$) substances.

For the determination of matrix effects (ME), the average peak area of five post-extraction spiked blank extracts (A) was compared to the average peak area of five spiked reference samples (B) of the analytes in methanol using Equation (2):

$$ME\% = 100 - \frac{A}{B} \cdot 100 \quad (2)$$

Positive values indicate ion suppression, negative values indicate ion enhancement.

3.4 Results and Discussion

3.4.1 Temperature-induced phase separation

Colour experiments were used in this study to monitor and visualize the robustness of the phase separation. Since the phase composition has a great influence on the extraction recovery, temperature has to be taken in account when optimizing extraction parameters. All liquid-liquid extractions across a phase boundary are based on miscibility gaps of a mixture of at least two solvents. The width of this miscibility gap defining the composition of the two (mixed) phases can vary with temperature. For example, in a water-acetonitrile-EtOAc mixture, Takahashi et al. [73] showed that the miscibility gap is broader at 0 °C than at 25 °C. This means, that at a fixed composition of the mixture, the organic phase contains less water and the aqueous phase less organic solvent at 0 °C. Thus, the polarity difference between the two phases is enhanced. To visualize this, we added indigo carmine as a model analyte to a two-phase system from water, acetonitrile and EtOAc. The dye is well soluble in water, but hardly in the (pure) organic solvents. It is thus mainly present in the aqueous phase but in the organic phase only when its water content is high. Indigo carmine can thus be used as a marker of the water content in the organic phase.

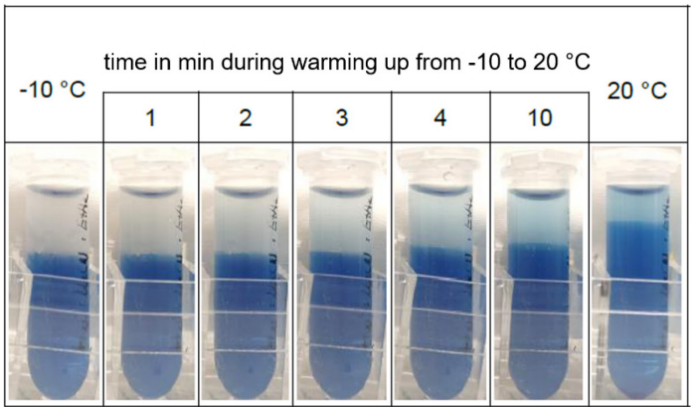


Fig. 2 Photos of a two-phase system from water-acetonitrile-EtOAc (50-40-10) mixture with 0.1 g/L indigo carmine at different time points while warming from −12 °C (t = 0 min) to 20 °C (t = 20 min).

Fig. 2 shows that at −12 °C, a stable two-phase system is present with EtOAc added as an organic modifier. The upper phase is almost colorless, indicating rather pure phases with a low water content in the organic phase. Upon warming the mixture, the phase boundary becomes more diffuse and the mole fraction of indigo carmine in the organic phase increases, turning it deeper blue whereas the aqueous phase lightens. This observation shows, that at 20 °C the phase composition chosen for the experiment must lie close to the upper critical point of the miscibility gap. Due to the enhanced mixing at higher temperatures, the water content in the organic phase, and therefore polarity, increases. The two phases become more similar in their characteristics. Additionally, the phase volume changes upon warming. The volume of the aqueous phase increases, indicating a higher content of organic solvent compared to the mixture at −10 °C. Since polarity and volume of the organic phase have a great influence on which analytes are preferentially extracted, extractions can be controlled by the water content in the organic phase and thus also by temperature.

3.4.2 Screening of additives to induce phase separation

To avoid the problems salts can cause in downstream analysis, we investigated a salt-free approach to induce a stable phase separation. Most two-phase systems rely on water and acetonitrile, since it is compatible with gas and liquid chromatography, as well as SPE, and because toxicity and environmental relevance are relatively low. Other frequently used solvents for extraction are acetone and EtOAc, but phase separation is easier with acetonitrile than with acetone, and polar analytes were better extracted than with EtOAc [9]. To support phase separation, 20% EtOAc was added to the acetonitrile-water system, though it does not induce a stable and complete phase separation at ambient temperature, as seen in Fig. 2. In a screening-approach, possible additives were chosen due to their previous use in liquid-liquid extractions or their number of OH-groups, which can bind water and therefore improve phase separation [56]. Molarities were chosen following Cray et al. [74], or based on own pre-studies and limiting factors like solubility. The additives were judged by their ability to induce a stable phase boundary and, when successful, by the median recovery for all model analytes. Some additives tested produced signals in LC-MS, that partially overlapped with analyte signals. This can lead to suppression of the analyte signal. To minimize the effect that this has on the evaluation of the additive during our screening approaches, we used *median* recoveries for the screening.

Table 3 Additives screened for phase separation at the molarity stated and the temperature, at which phase separation was observed. Median recoveries were determined for 13 model analytes (see Section "3.3.2 Model analyte mix") by LC-MS analysis of the organic phase after extraction. Extractions were conducted by adding 2.5 mL organic extraction mixture consisting of 20% EtOAc and 80% acetonitrile to 2.5 mL of doubly distilled water spiked with 3 mg/L analyte mix.

additive	molarity in mmol/L	phase separation temperature	median recovery in %
Aliquat 336TG	89.07	−10 °C	x
Dextran 450k-650k	0.00727	a.t.	33
Dextran 450k-650k	0.0726	a.t.	x
Dextran sodium sulfate 40k	0.994	a.t.	35
Di-(2-ethylhexyl)phosphoric acid	100.6	a.t.	46
D-Sorbitol	608.1	a.t.	28
Galactose*	1000	a.t.	27
Glucose*	1000	a.t.	28
Glycerol	92.1	a.t.	4
L-Proline	719.2	a.t.	24
Polyethylene glycol 600	56.2	−10 °C	41
Polyethylene glycol 1000	3.304	−10 °C	x
Polyethylene glycol 1000	148.0	−10 °C	x
Polyethylene glycol 6000	0.631	−10 °C	x
Polyethylene glycol 6000	24.67	−10 °C	x
Polyethylene glycol 35k	0.114	−10 °C	x
Poly(4-styrene sulfonic acid)	0.245	−10 °C	x
Poly(vinyl alcohol) 22k	0.183	−10 °C	x
Poly(vinyl alcohol) 31k-50k	0.0985	−10 °C	x
Poly(vinyl alcohol) 72k	0.0554	−10 °C	x
Poly(vinyl alcohol) 89k-98k	0.0427	−10 °C	x
Poly(vinyl alcohol) 100k	0.0400	−10 °C	x

Poly(vinyl alcohol) 146k-186k	0.0241	−10 °C	x
Potassium phosphate (monobasic)	102.6	a.t.	25
tert-Butyl alcohol	97.14	−10 °C	x
tert-Butyl alcohol	974.1	−10 °C	33
Trehalose*	1000	a.t.	41
tri-Sodium citrate dihydrate	99.78	a.t.	29
Urea	1000	a.t.	12
Xylose*	1000	a.t.	22

a.t. = ambient temperature x = not selected for detailed investigation

*An organic extraction mixture with 35% EtOAc and 65% acetonitrile was used to ensure stable phase separation without additive for better comparison.

With some of the additives, phase separation only occurred at lower temperatures. Since cooling during the extraction requires a more complex instrumental setup, these additives were not preferred for further optimization. Some of the additives that exhibited a phase separation at ambient temperature were chosen for further experiments. We included the liquids PEG600 and tert-butyl alcohol, as they promised easy handling, despite phase separation only occurring at −10 °C. In the second step, the additives were added to the extraction mixture and recoveries of the model analytes in the organic phase were determined. Lowest median recoveries were achieved using glycerol and urea. Contrary to the kosmotropic salts that are commonly used in QuEChERS, glycerol and urea are chaotropic. These chaotropes aided phase separation, but did not enhance extraction efficiencies. The addition of dextran 450k-650k resulted in relatively high median recoveries, but due to its low solubility, precipitation occurred already at lower molarities during extraction, resulting in a lower repeatability. High recoveries were also achieved using PEG600, but in LC-MS analysis, large amounts of the polymer were detected in the extract with a partial signal overlap with analyte signals.

The class of additives that consistently yielded high recoveries were sugars. All of them are highly soluble in water, are non-toxic and had no impact on LC-MS analysis. Therefore, glucose, galactose, trehalose and xylose were further investigated as additives to improve phase separation and analyte recovery. At the higher EtOAc contents of 35% used for these experiments, phase separation was possible without sugar addition, enabling a direct comparison of the effects of the different sugars. As shown in Fig. 3, all sugars improved the average recovery compared to an extraction using only EtOAc as a phase separating additive. Best overall recoveries were achieved using trehalose (40%), glucose (33%) and galactose (32%), the use of xylose resulted in slightly lower recoveries (29%).

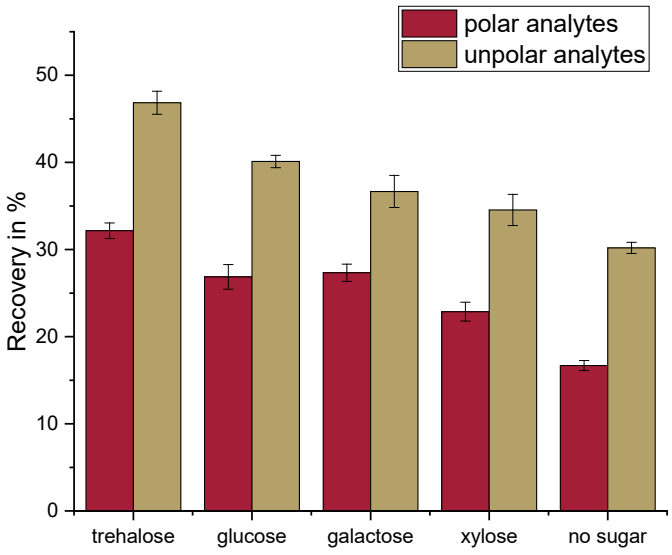


Fig. 3 Average recoveries of all model analytes (see "3.3.2 Model analyte mix") from three replicates depending on the type of sugar added to the aqueous phase at a concentration of 1 M. The organic solvent mixture consisted of 65% MeCN and 35% EtOAc. For details on the extraction procedure, see "3.3.3.4 Optimization of the SWIEET extraction protocol".

The recoveries for glucose and galactose, which have the same number of OH-groups, were very similar. Xylose has one OH-group less and so the lowest recoveries determined here corroborate findings by Mahdavi et al. [56], who hypothesized that the number of OH-groups in a molecule is essential for the effectivity of sugaring-out. If more water is required for carbohydrate solubilization, the solubility of the analytes in the aqueous phase decreases, which increases recoveries. The underlying mechanism is an excluded volume effect [75], meaning that H-bonds in water are bound by carbohydrates and cannot take part in the in solubilization of the analytes. The analyte concentration then increases in the organic phase. As can be expected from this mechanism, the improvement of recoveries by using sugars is more pronounced for polar analytes, which is favourable knowing that polar analytes are usually harder to extract from aqueous samples. The QuEChERS method for example is mainly used for analytes with low to medium polarity ($-1 < \log D_{pH7.4} < 7$) [76]. Solubility of the analytes is not only based on the excluded volume effect, but also on the permittivity of the phases, which changes when a cosolvent is added [77]. For aqueous glucose solutions, the permittivity decreases with increasing glucose concentration [78]. Thus, a decrease in solubility would be expected for polar solutes in the aqueous phase. The combination of fewer available H-bonds and reduced permittivity could explain the increased polar analyte concentration in the organic phase.

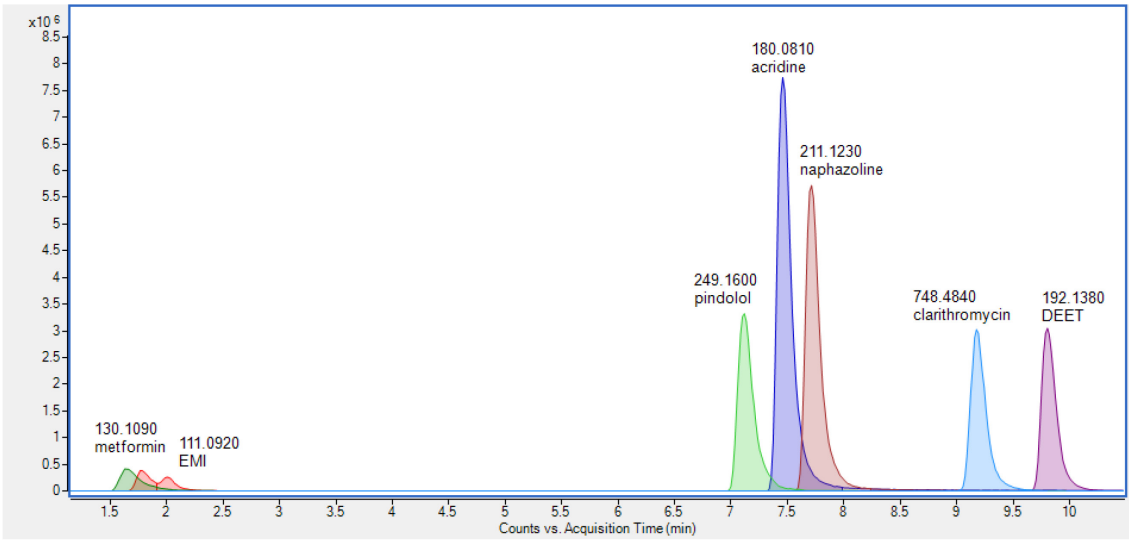
Higher sugar concentrations increased partition and distribution coefficients of three organic molecules in a study by Wang et al. [51]. Glucose addition yielded highest recoveries for their analytes in the $\log D_{pH7}$ range of -1.23 to -0.89. For our broad analyte spectrum, it ranked similar to galactose and second to trehalose. However, our recoveries were <50% necessitating further optimization. We preferred glucose, since it is significantly cheaper than trehalose while recoveries were acceptable. Its solubility is higher than that of galactose, which enables to use a broader range of additive concentrations to adapt phase separation and polarity for specific analytical tasks.

Interestingly, upon addition of glucose, EtOAc was no longer necessary to guarantee a stable phase separation and this solvent was omitted in further optimization steps.

Recovery not only depends on concentration, but also the volume ratio of the two phases. The addition of sugars to acetonitrile EtOAc mixtures increases the volume of the organic phase, which shifts the phase ratio and therefore recovery (see Equation (1)).

Using sugars, only the very polar analytes ANSA and acesulfame partially coeluted, which is why *average* recoveries were used in the following. Fig. 4 shows the extracted ion chromatograms of the model analytes after extraction with glucose. The glucose added in SWIEET extractions stays mainly in the aqueous phase [52]. Only a small amount is detected in the organic phases in ESI− mode, but it elutes in the dead volume and therefore does not affect ionization of the analytes significantly. In ESI+, only the most polar analytes may become affected, but it has to be noted, that RPLC is anyhow not optimal for the separation of these very polar analytes. Separation can be improved by using HILIC or SFC.

a) ESI+



b) ESI-

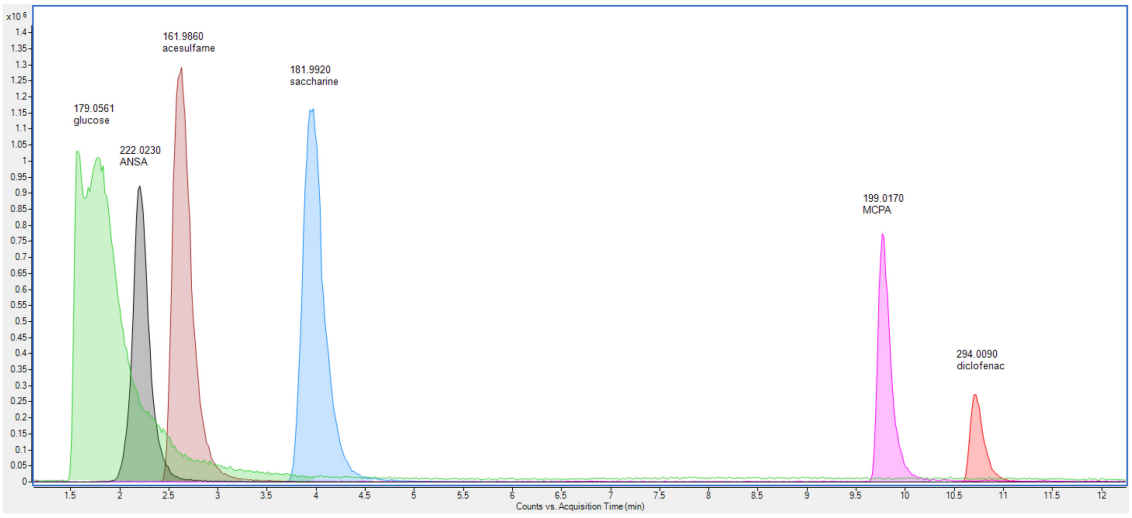


Fig. 4 Extracted ion chromatograms of glucose and analytes detected in a) ESI+ and b) ESI-. Measurement of the first organic phase from a SWIEET extraction of surface water, spiked post extraction with 0.42 mg/L model analyte mix. For the detailed extraction protocol, see “3.3.3.5 Final SWIEET protocol”. For LC-MS parameters, see “3.3.6 LC-MS method”. For details on the analyte mix, see “3.3.2 Model analyte mix”.

Unfortunately, recoveries were very low for the most polar analytes metformin and EMI (1% and 3%) and under 40% on average for all polar analytes, which shows that further optimization was necessary. For this, we chose to use sugars. Among them, glucose was preferred for its low price, good recovery and high solubility.

3.4.3 Choice of organic solvent

We tested several organic solvents chosen from literature studies, preliminary work or theoretical considerations. The halogenated solvents dichloromethane and chloroform were used by Liu et al [58] to induce phase separation of water and acetonitrile, the system yielded high recoveries of 100% for glycosides and aglycones. In our first experiments, EtOAc was used as an additive in the organic phase. However, in preliminary studies we observed, that especially polar ($logD_{pH7} < 3$) and charged compounds were hardly extracted using this solvent. To improve recoveries for these analytes, an elevated polarity and permittivity of the organic phase, as well as the ability to form H-bonds was envisaged adding the polar and protic isopropanol. In our study, we compared the extractions using chloroform, dichloromethane, EtOAc and isopropanol. We reached a stable phase separation when adding only sugars to water and acetonitrile. This allowed us to improve recoveries using solvents as additives, that are not able to induce phase separation when added alone.

A notably larger phase ratio of the organic:aqueous phase was observed after the addition of isopropanol. Among the organic solvent additives, the protic isopropanol is more strongly excluded from the aqueous phase. A larger organic phase volume indicated that the organic phase is more polar, which we would expect to result in higher recoveries especially for polar analytes, further aided by the large volume of the organic phase. The disadvantage of this large volume of the organic phase is the higher dilution.

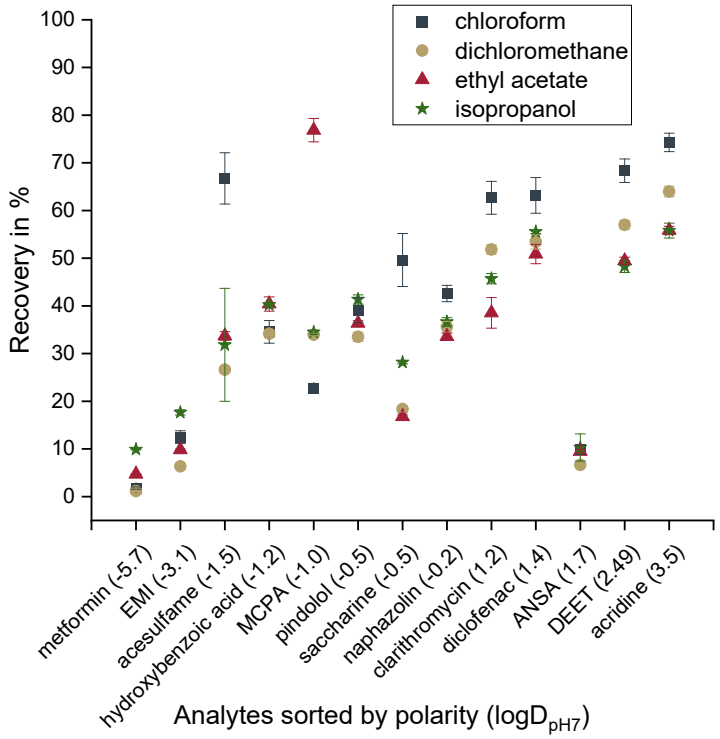


Fig. 5 Recoveries of the model analytes after extraction using 90-10 vol.% acetonitrile-solvent in the organic extraction mixture. Solvents chosen were chloroform, dichloromethane, EtOAc and isopropanol. The aqueous extraction mixture consisted of 3 mg/L analyte mix (see "3.3.2 Model analyte mix") and 2 M glucose in doubly-distilled water. For the detailed extraction procedure, see "3.3.3.4 Optimization of the SWIEET extraction protocol".

Looking at analyte recoveries in Fig. 5, especially for unpolar and medium polar analytes ($logD_{pH7} > 0$) the addition of chloroform resulted in the highest recoveries, followed by DCM. Recoveries using EtOAc and isopropanol were similar but lower for this class of analytes. For polar analytes, however, with $logD_{pH7} \leq -0.5$, the recoveries improved with isopropanol compared to chloroform, especially for the most polar analytes EMI and metformin, which were hardly extracted (only 12 and 1% for chloroform) when adding the aprotic, non-polar solvents. For MCPA, which is also polar, recoveries followed the order EtOAc (77%) >> isopropanol (34%) ~ dichloromethane (34%) > chloroform (23%). For acesulfame ($logD_{pH7} = -1.7$), the use of chloroform resulted in about 30% higher recoveries compared to the other solvents, but the reasons for this enhancement are not clear.

Isopropanol was the only solvent tested, that was not only polar, but also protic. We assume that it not only increased polarity in the organic phase, but also enabled better hydrogen bonding in the organic phase. The increase in recoveries for strongly polar and especially the charged analytes can be explained by enhanced solubilization in the organic phase aided by H-bond formation. Another factor, that has an influence on the extraction is the permittivity. Isopropanol has the highest permittivity of all the organic solvents tested as additives, expected to increase the solubility for polar and ionic solutes [78], which is in line with our observation.

Due to the higher recoveries, especially for polar and ionizable analytes while maintaining high recoveries for unpolar analytes, and the low toxicity resulting from the addition of

isopropanol, we chose this polar protic solvent for further optimization of the extraction method.

3.4.4 Optimization of extraction parameters

Further optimization was made with the addition of glucose and isopropanol to the water-acetonitrile mixture considering different temperatures. Conducting a design of experiment (DoE) allows to simultaneously vary multiple parameters in a manageable number of experiments. A Box-Behnken design was chosen because it avoids combinations of extreme values, that would lead to instable phase separations. The center point of the DoE was conducted three times to ensure statistical significance. The ranges of the parameters were 0-25 °C, 5-10% isopropanol in 2.5 mL organic extraction mixture (90-95% acetonitrile) and 1.5-2.5 M glucose in 2.5 mL aqueous extraction mixture, consisting of 3 mg/L analyte mix in doubly distilled water. The results are shown in hypersurface plots in Fig. 6.

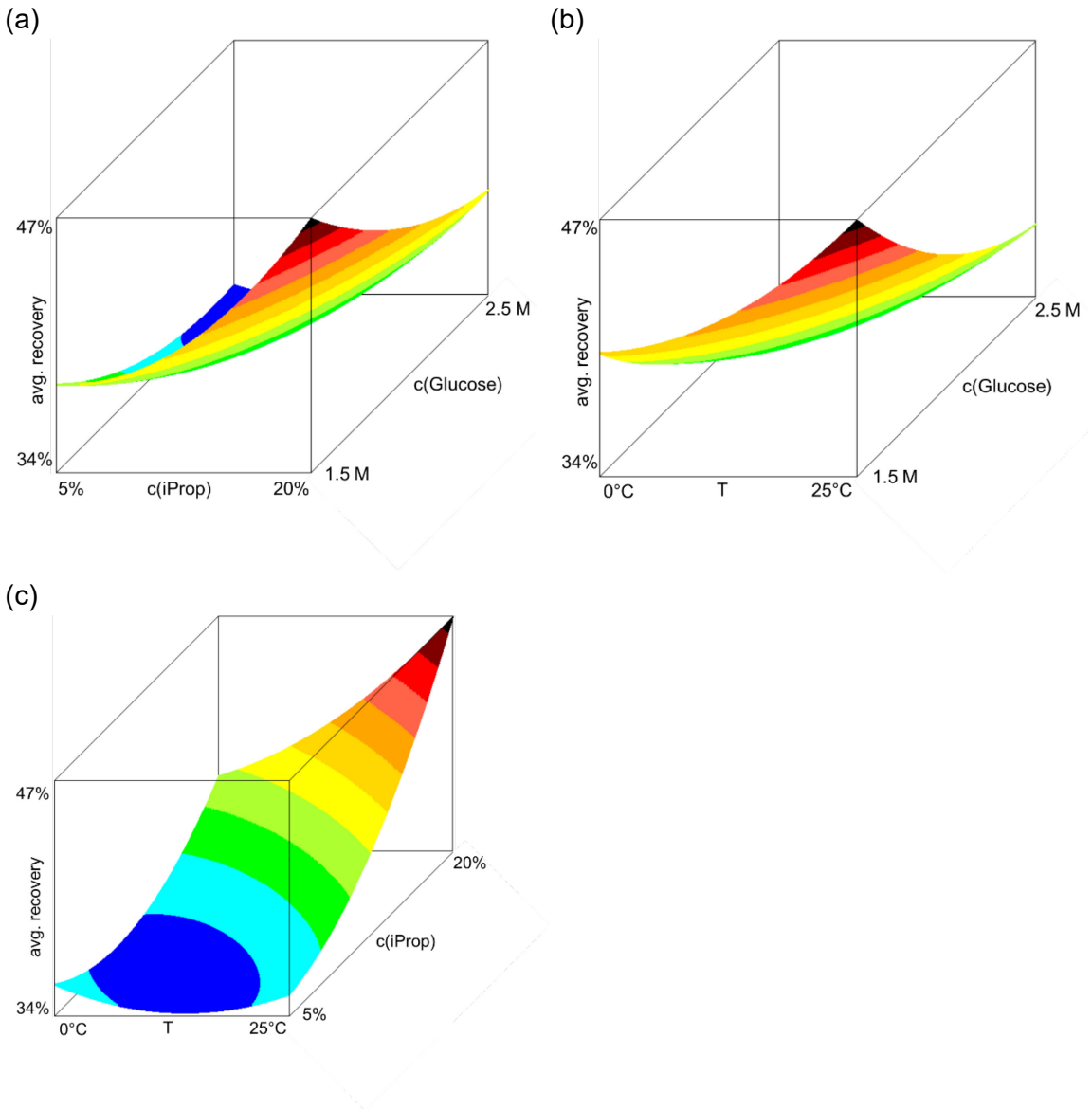


Fig. 6 Surface plots of the average recoveries of all analytes determined from the DoE (3.3.3.3 Design of experiment), in dependence of (a) isopropanol and glucose concentrations, (b) glucose concentrations and temperature and (c) temperature and isopropanol concentrations. Sample: aqueous extraction mixture.

Isopropanol concentration had the greatest effect on analyte recovery, whereas recoveries were least sensitive to temperature changes, indicating high robustness for possible differences in ambient temperature. In general, high isopropanol content, low glucose concentration and high temperatures revealed highest recoveries. The maxima of the hypersurfaces lie at the corners of the tested ranges, which indicates that an actual maximum would be found at more extreme parameters, where, however, phase separation will become instable or even impossible. For example, a high isopropanol content yielded high recoveries, but also compromised phase separation. This is due to isopropanol being a polar protic solvent like water, at high isopropanol content the two phases become too similar evoking miscibility. Even though the maximum of the surface plot was observed at a glucose concentration of 1.5 M, 2 M were chosen for further experiments, since this concentration provided a higher stability of the system and resulted in higher repeatability of recoveries, especially at high isopropanol contents of 20%. Decreasing the temperature facilitated phase separation and guaranteed a stable phase boundary. However, when adding 2 M glucose, phase separation was already very robust and cooling of the extraction system was not necessary. In addition, the surface plots show that lower temperatures were disadvantageous for recoveries. This might be due to the higher viscosity of the solvents and slower diffusion of the target analytes or differences in the width of the miscibility gap. A precise temperature control below room temperature would increase time and costs of the extraction, so further work was conducted at ambient temperature.

To achieve high recoveries while maintaining high repeatability, 20% isopropanol and 2 M glucose were chosen for extraction. The extraction process was clearly improved, but recoveries of <50% on average were still not convincing. Thus, two strategies were followed for further optimization: electroextraction and double extraction.

3.4.5 Electroextraction

Electroextraction can improve recoveries for charged analytes. To conduct these experiments, a syringe was modified as shown in Fig. 7. Two platinum electrodes were introduced into the syringe to enable the application of an electric field to the extraction mixture. Due to the clear plastic of the syringe, the phase boundary could be monitored during extraction, as well as possible bubble formation caused by electrolysis. The extraction mixture was drawn up into the syringe after mixing. After phase separation was visible, voltage was applied. The experiment was conducted in duplicates, using reversed polarities for 10 min each.

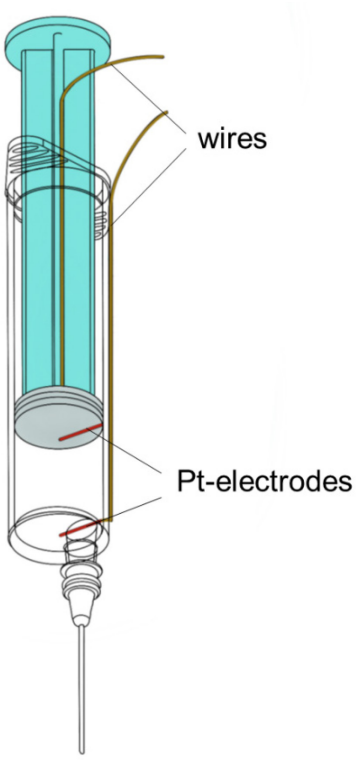


Fig. 7 Setup of the syringe modified for electroextraction. A regular plastic syringe was equipped with two platinum electrodes at the stamp and the outlet, which were then connected to a high voltage power supply.

Setting a constant current compared to a constant voltage proved to be more stable. Constant currents in the range of 100 – 2000 µA were tested and 200 µA were optimal regarding average analyte recoveries (data not shown). If the current was lower, the effect of the electric field was too small, but if the current was higher, side effects became prevalent: Electrolysis led to strong pH changes in the aqueous phase since it was not buffered, which can cause analytes to become neutral. For example, a pH of 3.6 instead of 7 was measured in the aqueous phase after the extraction with 2000 µA. Acesulfame and MCPA are negatively charged ($z = -1$) at pH 7, but at pH 3.6 the charge number decreases to $z = -0.28$ or $z = -0.81$ respectively. This led to lower recoveries compared to extractions with 200 µA.

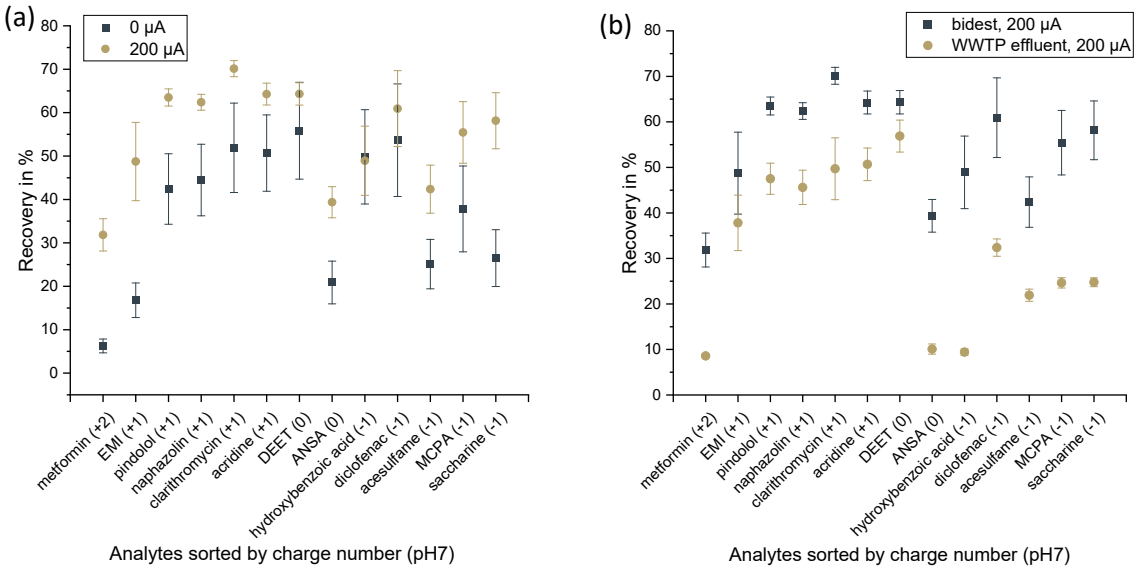


Fig. 8 Recoveries of all 13 model analytes (see Section "3.3.2 Model analyte mix") after 10 min extraction applying 200 µA or 0 µA. Aqueous phase: (a) doubly distilled water (b) doubly distilled water or WWTP effluent; organic phase: acetonitrile-isopropanol 80:20; 2 M glucose added. For the electroextraction protocol, see Section "3.3.5 Electroextraction". Charge number at pH 7 for analytes given in brackets.

Due to the addition of sugar, phase separation was stable despite elevated temperatures occurring due to Joule heating at high currents. A comparison of an extraction conducted with and without the application of an electric field at 200 µA showed an average increase of the recovery by 18% (see Fig. 8a). The increase was more pronounced for analytes with high charge numbers like metformin, EMI, and saccharine. Interestingly, however, an improvement of the recovery was observed for all analytes tested, charged analytes as well as neutral ones. An improved analyte migration cannot directly be caused by the electric field, but may be due to changes of the local environment of analytes. As previously explained, due to electrolysis of water, pH changes can occur. Some analytes may have gained charge, that were neutral at the starting pH of 7. Furthermore, if more analytes are transported through the phase boundary, viscosity of the aqueous solution might decrease and facilitate diffusion of neutral analytes. Due to Joule heating, temperature increases and thus viscosity and interfacial tension [79]. This likely facilitated phase transfer.

The same protocol was applied to a wastewater treatment plant effluent sample (see Fig. 8b). Due to the higher conductivity of the sample, the resulting voltage was about 10 times lower than in the doubly-distilled water sample, resulting in a weaker electric field. As a result, recoveries were lower, which could not be overcome by increasing the current to up to 2000 µA.

All in all, electroextraction proved beneficial to improve recoveries especially for charged analytes and recoveries increased to over 60% for many analytes. However, for real samples with higher ionic matrix loads, recoveries were still insufficient with under 60%.

3.4.6 Double-extraction with/without electroextraction

Another or additional strategy to improve recoveries is double-extraction. For this, fresh organic phase was added to the aqueous phase remaining after the first extraction, both for SWIEET and QuEChERS for comparison. To directly consider matrix effects, we

conducted these experiments with wastewater treatment plant effluent. Since hydroxybenzoic acid consistently yielded low recoveries (<30%) for SWIEET and QuEChERS extraction, we suspected a problem in downstream analysis. Therefore, we did not include this analyte into the average recovery calculations for the following experiments in real matrices.

For QuEChERS double-extractions (Fig. 9a), recoveries were already relatively high in the first step with 57% on average. In the second step, however, only further 9% were extracted additionally (total average recovery = 66%). The increase was largest for polar analytes, e.g. for EMI, the recovery increased by 26%. Salt contents in QuEChERS are commonly above saturation, therefore enough salt was left in the second step to induce a new phase separation, still with solid salt present as a third phase. No significant change in volume of the organic phase was observed between the two extraction steps. We thus assume that the compositions of the phases were similar in the second and first extraction step, explaining the low additional recoveries in the second step.

From the DoE (see Section “3.4.4 Optimization of extraction parameters”), we derived the optimized conditions for the SWIEET extraction, namely 2 M glucose and 20% isopropanol at room temperature. However, we decreased the isopropanol content to 10%, since this led to a more stable system. We observed, that the volumes of the newly formed two phases differed significantly from the first extraction step: volume of the organic phases increased significantly, which clearly indicates differences in the composition of the phases in the two extraction steps.

To understand the effects and to further increase recoveries from 47% on average, we varied the organic phase volume, organic phase composition as well as the application of an electric field and its polarity for the first and second extraction in different combinations, resulting in 15 different protocols for double extractions (see Fig. 10). Conditions and recoveries are summarized in Table 4.

Using an organic extraction mixture made of 90 vol.% acetonitrile and 10 vol.% isopropanol, we varied the relative volumes of the aqueous and organic phases. Adding the same amount of organic extraction mixture in both steps, 2.5 or 2 mL (experiments 12 and 13) resulted in lower recoveries of 44% and 45% in total for the two steps. This was lower compared to adding 2.5 mL in the first step and 2 mL in the second step, which yielded a total recovery of 55% (experiment 15). A reduction of the organic extraction mixture volume to 1 mL only in the second step was hypothesized to help enriching the analytes, but only 36% were recovered in total (experiment 14). It stands out that the robustness of the first extraction step is high, since the recoveries from the first step in experiments 7,8,11,12 and 15 varied only slightly at the same conditions (RSD = 5.7%). Only experiment 14 shows significantly lower recoveries and may be an outlier.

Varying the composition of the organic extraction mixture, highest average recoveries of 69% were achieved using an organic extraction mixture with 20% isopropanol and 80% acetonitrile for both extraction steps (experiment 9, Fig. 9b). After the addition of 2 mL of the organic extraction mixture in the second step, we observed an increase in the volume of the organic phase to about 3.5 mL. On a first glance, this increased volume was thought to be due to an increased water content in the organic phase in the second extraction step. However, experiments with indigo carmine revealed, that the water content was actually lower in the second organic phase. The overall water content in the total extraction mixture

must be lower in the second step, as a fraction of water was removed in the first step. To improve understanding of the fundamental physicochemical aspects of the SWIEET extraction, further experiments on the composition of the phases will be conducted in the future.

In the first step, average recoveries reached 37% using SWIEET. Notably, in the second step another 32% were recovered (experiment 9), but the increase in the second step was especially high for polar analytes. For metformin for example, 21% were recovered in the first step and additional 38% in the second step. It is important to state that for unpolar analytes like DEET and acridine ($2.5 < \log D_{pH7} < 3.5$), recoveries were already high in the first step with 50% each, while in the second step only an additional 20% were extracted. For the three most polar analytes ($-1.5 > \log D_{pH7} > -5.7$) metformin, EMI and acesulfame, recoveries were clearly higher in the second step (36-44% extracted) than in the first step (20-34%).

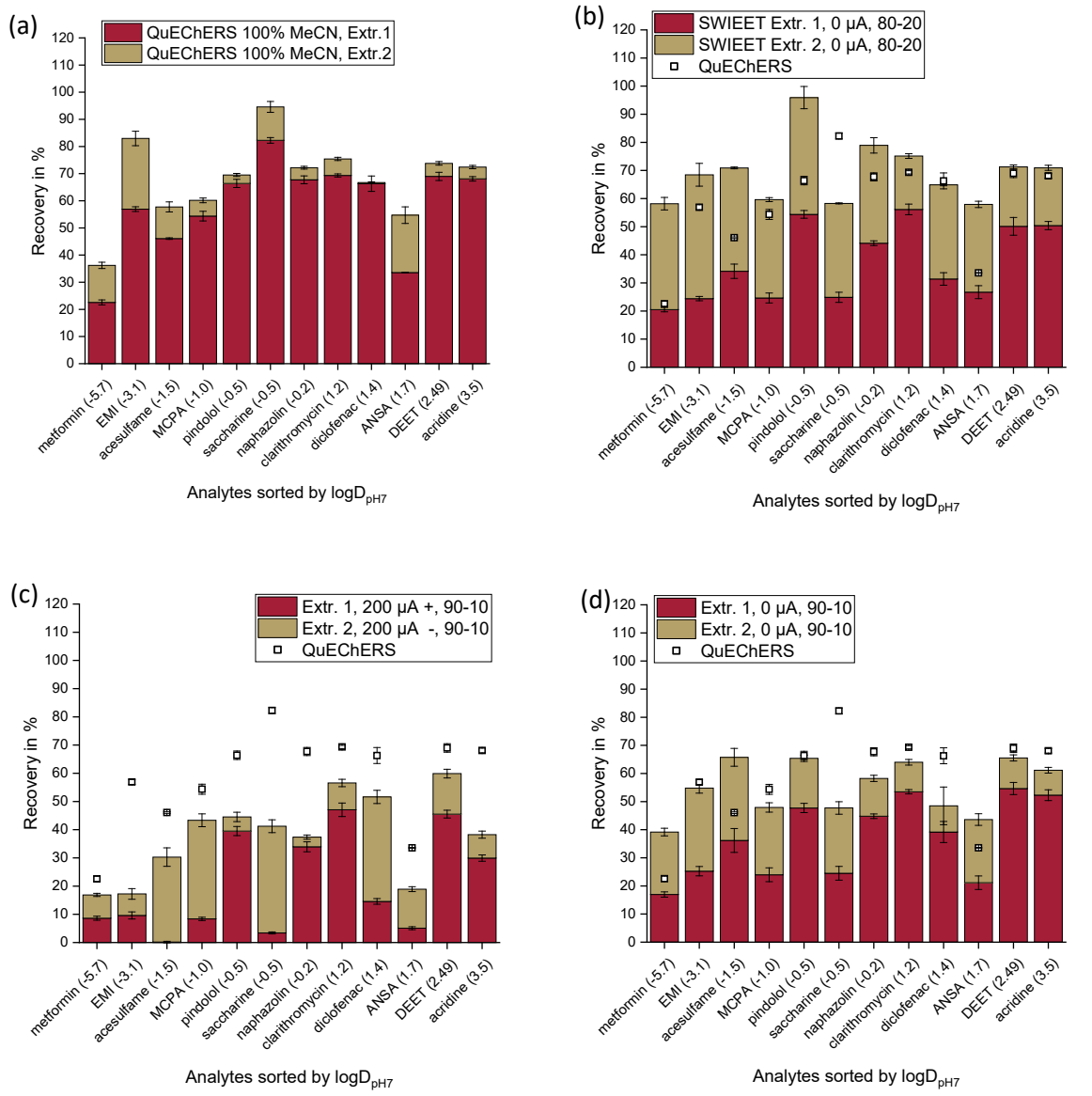


Fig. 9 Excerpt of results for double-extractions according to Table 4 for (a) Exp. QuEChERS, (b) Exp. 9, (c) Exp. 4 and d) Exp. 15. Recoveries of double-extractions of the analyte mix (see Section “3.3.2 Model analyte mix”) from 2.5 mL wastewater treatment plant effluent with (a) QuEChERS extraction (see “3.3.3.2 QuEChERS extraction”) and (b), (c) and (d) SWIEET extraction with 2 M glucose, (c) with 200 µA positive polarity in the aqueous phase in the first step and 200 µA negative polarity in the second step and (b) and (d) without the application of an electric field in both steps. Composition of the organic extraction mixture for SWIEET: (b) 80 vol.% acetonitrile, 20 vol.% isopropanol (80-20); c),d) 90 vol.% acetonitrile, 10 vol.% isopropanol (90-10). Recoveries from QuEChERS Extr.1 are plotted also in b) c) and d) for comparison. For detailed extraction protocols, see Section “3.3.3.4 Optimization of the SWIEET extraction protocol”, “3.3.3.2 QuEChERS extraction” and “3.3.5 Electroextraction” and Table 4.

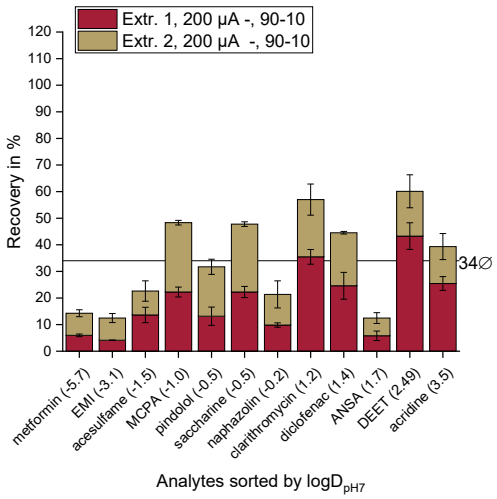
Table 4 Average recoveries of 12 analytes obtained for the double-extractions (see Section "3.3.2 Model analyte mix"). Wastewater treatment plant effluent (2.5 mL) was spiked and glucose was added at a final concentration of 2 M. The volume and the composition of the organic extraction mixture were varied. For electroextraction, different currents and polarities were used ("+" indicates positive polarity and "− " negative polarity applied at the electrode in the aqueous phase). QuEChERS extraction was conducted for comparison (Exp. Q). For the detailed extraction protocols, see Section "3.3.3.2 QuEChERS extraction", "3.3.3.4 Optimization of the SWIEET extraction protocol" and "3.3.5 Electroextraction".

Nr.	step 1				step 2				total recovery
	volume organic extraction mixture in mL	composition organic extraction mixture (vol.% MeCN-vol.% iPr)	current in µA and polarity	average recovery in %	volume organic extraction mixture in mL	composition organic extraction mixture (vol.% MeCN-vol.% iPr)	current in µA and polarity	average recovery in %	sum of average recoveries in step 1+2 in %
Q	2.5	100-0	x	57	2	100-0	x	9	66
1	2.5	90-10	200 −	19	2	90-10	200 −	16	34
2	2.5	90-10	200 −	21	2	90-10	200 +	19	40
3	2.5	90-10	200 −	21	2	90-10	x	30	52
4	2.5	90-10	200 +	20	2	90-10	200 −	18	38
5	2.5	90-10	200 +	23	2	90-10	200 +	10	33
6	2.5	90-10	200 +	20	2	90-10	x	28	48
7	2.5	90-10	x	35	2	90-10	200 −	13	48
8	2.5	90-10	x	32	2	90-10	200 +	10	43
9	2.5	80-20	x	37	2	80-20	x	32	69
10	2.5	80-20	x	31	2	90-10	x	31	62
11	2.5	90-10	x	33	2	80-20	x	26	60
12	2.5	90-10	x	35	2.5	80-20	x	9	44
13	2	90-10	x	13	2	90-10	x	32	45
14	2.5	90-10	x	22	1	90-10	x	14	36
15	2.5	90-10	x	37	2	90-10	x	18	55

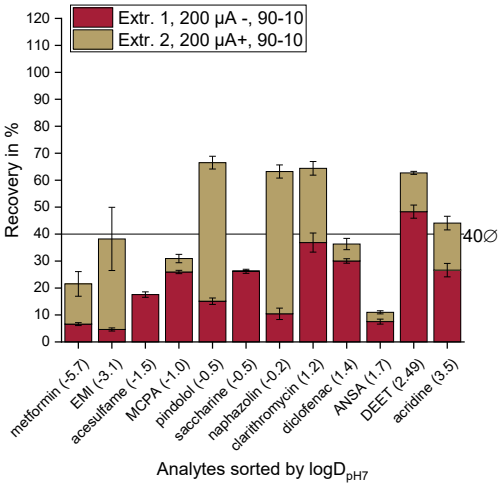
Interestingly, the application of an electric field did not yield highest recoveries, as shown in Fig. 9c and d, where we compared a double-extraction with and without 200 µA applied (experiment 4 vs. 15). We observed high recoveries for positively charged analytes and low recoveries for negatively charged analytes in the first step as would be expected due to the polarity applied. Nevertheless, recoveries achieved without electroextraction exceeded those by 17% in the first step. In the second step, the polarity was reversed, so negatively charged analytes became extracted primarily. On average, however, recoveries were similarly low in both steps (20% and 18%). Combining a regular extraction with electroextraction in the second step (experiment 7) increased overall recoveries to 48% as would be assumed from the previous observations (see Section "3.3.5 Electroextraction"), but still, total recoveries of 55% from diffusional extraction using the same organic extraction mixture were not reached. This might be due to the complex

matrix of wastewater effluent used in this set of experiments, as discussed in Section “3.3.5 Electroextraction”.

1


Extr. 1, 200 µA -, 90-10
Extr. 2, 200 µA -, 90-10
Recovery in %
34∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD pH7

2


Extr. 1, 200 µA -, 90-10
Extr. 2, 200 µA+, 90-10
Recovery in %
40∅
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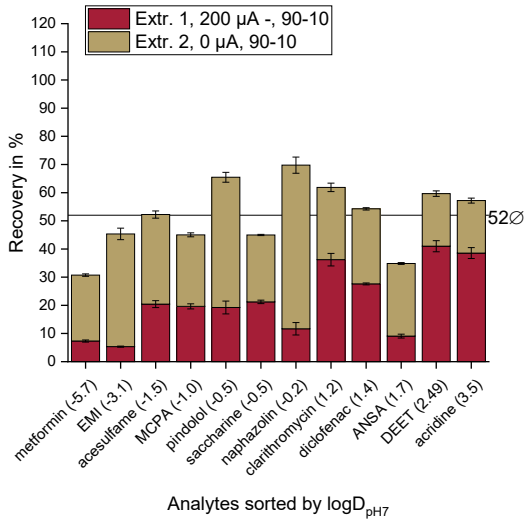
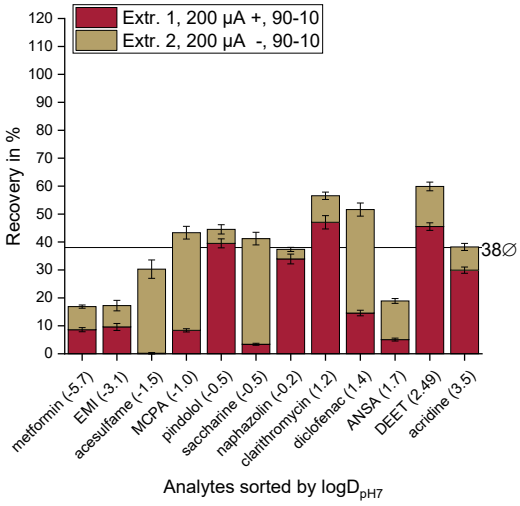
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD pH7

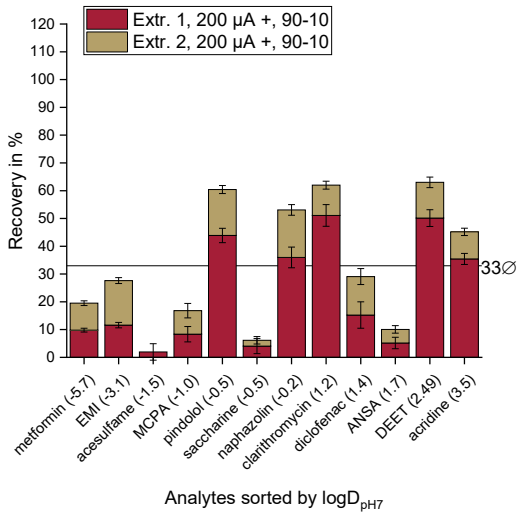
3


Extr. 1, 200 µA -, 90-10
Extr. 2, 0 µA, 90-10
Recovery in %
52∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD pH7

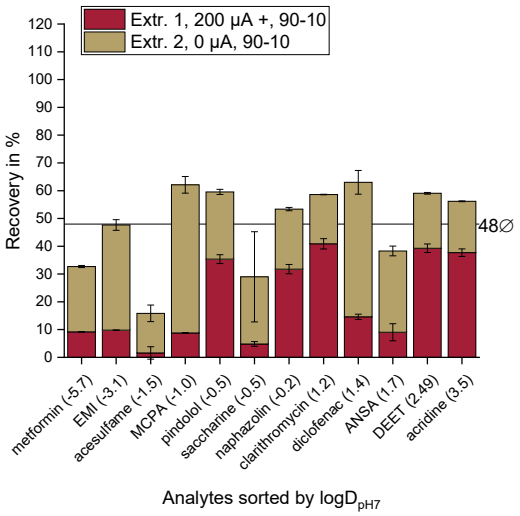
4


Extr. 1, 200 µA +, 90-10
Extr. 2, 200 µA -, 90-10
Recovery in %
38∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD pH7

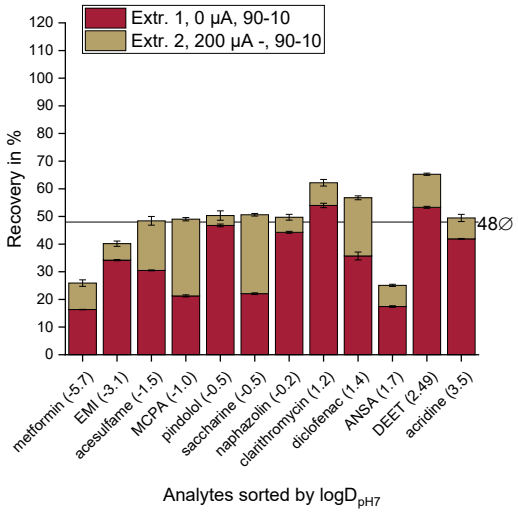
5


Extr. 1, 200 µA +, 90-10
Extr. 2, 200 µA +, 90-10
Recovery in %
33∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD pH7

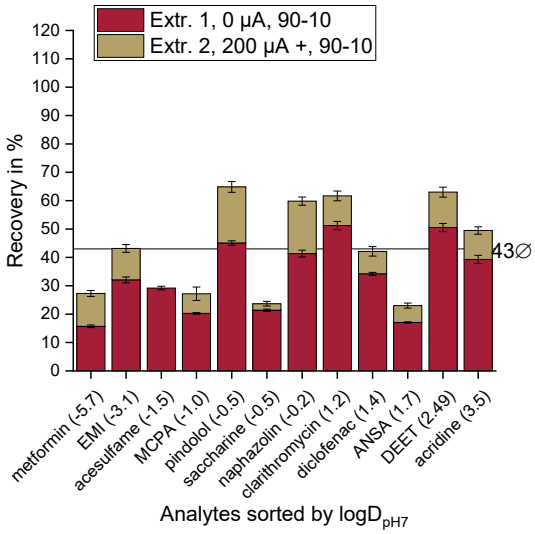
6


Extr. 1, 200 µA +, 90-10
Extr. 2, 0 µA, 90-10
Recovery in %
48∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD pH7

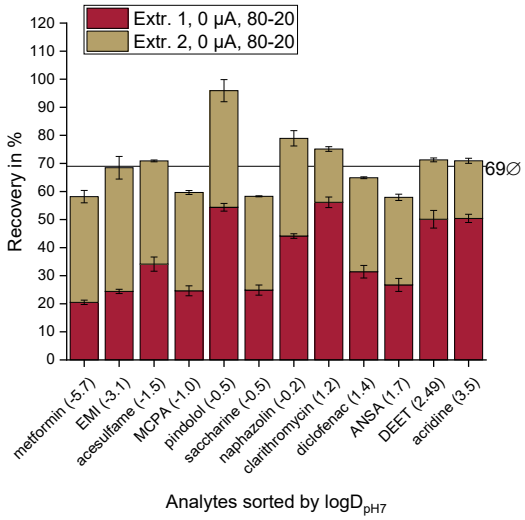
7


Extr. 1, 0 µA, 90-10
Extr. 2, 200 µA -, 90-10
Recovery in %
48∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD$_{pH7}$

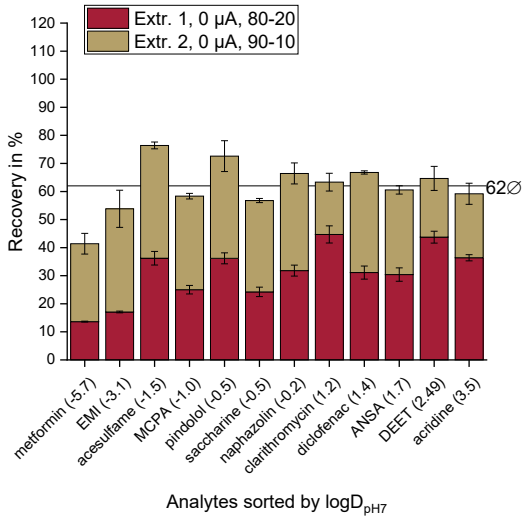
8


Extr. 1, 0 µA, 90-10
Extr. 2, 200 µA +, 90-10
Recovery in %
43∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD$_{pH7}$

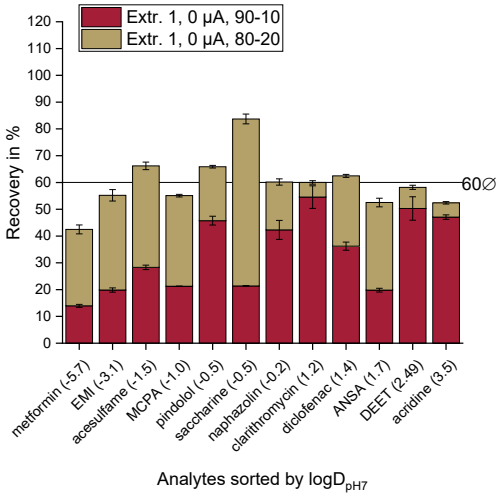
9


Extr. 1, 0 µA, 80-20
Extr. 2, 0 µA, 80-20
Recovery in %
69∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD$_{pH7}$

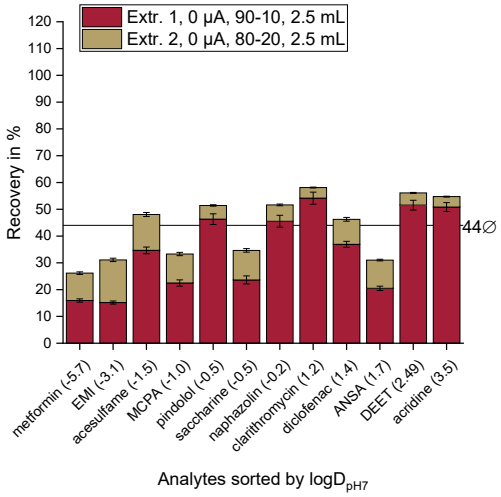
10


Extr. 1, 0 µA, 80-20
Extr. 2, 0 µA, 90-10
Recovery in %
62∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD$_{pH7}$

11


Extr. 1, 0 µA, 90-10
Extr. 1, 0 µA, 80-20
Recovery in %
60∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD$_{pH7}$

12


Extr. 1, 0 µA, 90-10, 2.5 mL
Extr. 2, 0 µA, 80-20, 2.5 mL
Recovery in %
44∅
metformin (-5.7)
EMI (-3.1)
acesulfame (-1.5)
MCPA (-1.0)
pindolol (-0.5)
saccharine (-0.5)
naphazolin (-0.2)
clarithromycin (1.2)
diclofenac (1.4)
ANSA (1.7)
DEET (2.49)
acridine (3.5)
Analytes sorted by logD$_{pH7}$

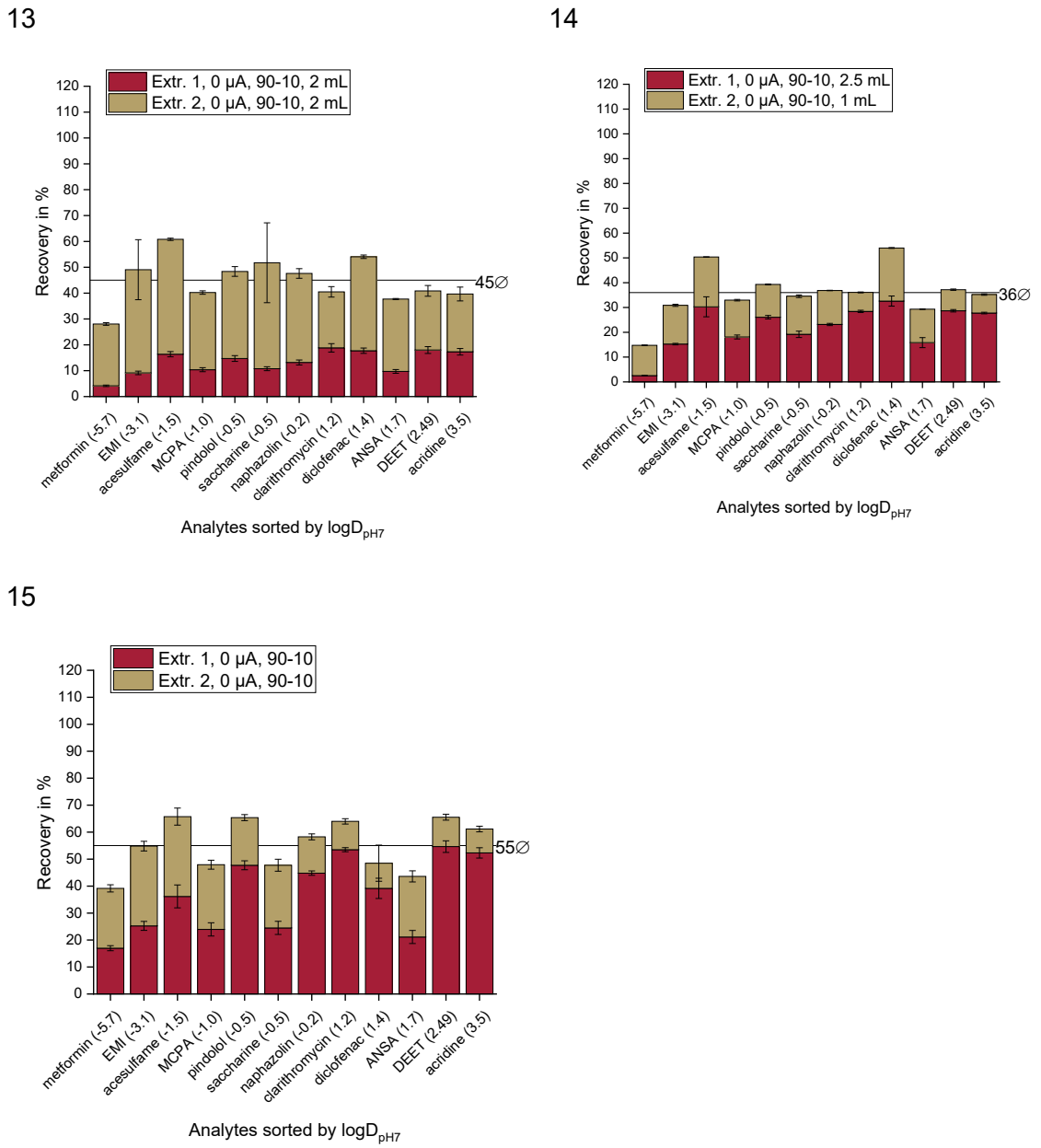


Fig. 10 Recoveries for the double-extraction experiments of the analyte mix (see Section “3.3.2 Model analyte mix”) in WWTP-effluent. Extraction parameters 1-15 are summarized in Table 4. The currents set for electroextraction as well as the organic extraction mixture composition acetonitrile-isopropanol are given in the panels. For the detailed extraction protocol see Section “3.3.3.4 Optimization of the SWIEET extraction protocol” and “3.3.5 Electroextraction”.

Overall, the double-extraction using 80-20 acetonitrile-isopropanol as the organic extraction mixture in both steps without the application of an electric field revealed the highest average recoveries (69%), which is similarly high as the QuEChERS extraction (66%). This protocol was chosen to be applied to different types of samples.

3.4.7 Comparison of SWIEET to QuEChERS for different types of samples

We compared the SWIEET double-extraction directly to the classical QuEChERS protocol using different liquid and solid samples: wastewater treatment plant effluent, river water, mashed tomato, an agricultural soil and oats. Both extraction methods were carried out without further clean-up steps. Results are shown in Fig. 11. For all samples, matrix effects were determined (see Section “3.3.7 Quantification”).

Liquid samples/samples with a high water content: For wastewater treatment plant effluent, recoveries increased significantly in the second extraction step for all model analytes. This was prevalent especially for positively charged and highly polar and charged analytes like metformin, EMI and acesulfame, for which a higher fraction was extracted in the second step than in the first step (see Fig. 11a-c). For all model analytes, the recoveries reached with the SWIEET double-extraction exceeded those reached using QuEChERS. Averaged over all analytes, SWIEET yielded 75%, QuEChERS only 37%. The same double-extraction was applied to surface water samples and tomato. On average, SWIEET yielded 81% and 57% and QuEChERS 54% and 47% for surface water and tomato, respectively. It is also notable that standard deviations were always lower using the SWIEET method, which indicates a higher repeatability compared to QuEChERS (e.g. for tomato, average standard deviation SWIEET 3.7%, QuEChERS 6%). We assume that this is due to the lack of the additional solid phase in the SWIEET method, which reduces possible sorption phenomena.

In river water and wastewater treatment plant effluent, matrix effects in SWIEET were similar to QuEChERS for most unpolar and medium polar analytes. Matrix effects were significantly higher for EMI, acesulfame and ANSA in SWIEET compared to QuEChERS and compared to less polar analytes. E.g. for metformin and EMI, matrix effects were 70% and 51% in wastewater treatment plant effluent for the first SWIEET extraction, compared to −6% and −2% for QuEChERS, but a direct comparison is hindered by the 2-4 times lower absolute concentrations in the QuEChERS extract. For acesulfame and diclofenac, matrix effects were also higher for SWIEET, but with ion suppression for SWIEET vs. ion enhancement for QuEChERS. For the tomato sample, differences between QuEChERS and SWIEET were more pronounced for unpolar and medium polar analytes, compared to wastewater treatment plant effluent and river water. For pindolol in wastewater treatment plant effluent, the matrix effect was 9% for QuEChERS and 5% for SWIEET, in tomato 5% and −5%, respectively. Since the SWIEET-method is superior in extracting polar analytes, more polar matrix compounds are likely to be coextracted causing these elevated matrix effects. Better separation, for example with HILIC, would minimize these effects.

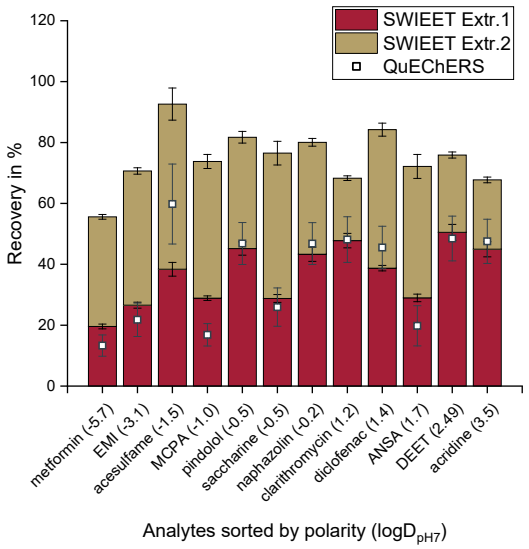
Dry samples: For the extraction from soil, SWIEET by far exceeded QuEChERS recoveries with 24% compared to 12%. Lowest recoveries were achieved for the extraction from oats with 20% and 18% both for SWIEET and QuEChERS. Higher recoveries than those achieved by us are possible with QuEChERS for similar samples, as shown by de Matos et al. [80] and Michel et al. [81]. Since we achieved low recoveries with both extraction methods, the problem is assumed to be due to the preparation of the solid samples. Since matrix effects were similar compared to tomato or aqueous sample extraction, a problem in LC-MS analysis is less likely. For sample preparation of the solid samples, water was added to the dry sample to create a slurry which was spiked with the analyte mix, mixed for 1 h using an overhead shaker and then dried in the oven at 60 °C. This was done to assure that the analytes are (partly) sorbed on the solid phase and not only dissolved in the added water, which would facilitate extraction. Possibly, the sorption of the analytes was not fully overcome by the extraction methods used. Alternatively, thermal degradation or evaporation may have occurred.

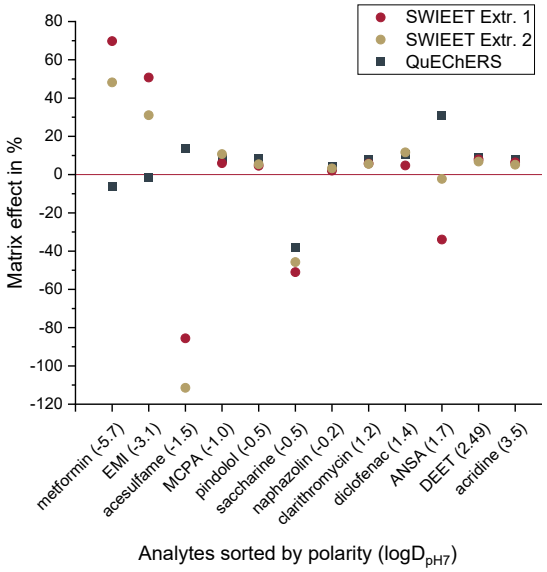
Combination of the extracts from double extraction: Since in SWIEET recoveries were similar in the first and second step for most analytes and sample types, extracts from both

steps were combined and analyzed, as no significant dilution occurs upon combining the extracts. Fig. 12 shows the direct comparison of the recoveries from the combined extracts to the individual extracts using oats as sample. Higher average recoveries were achieved with the analysis of the combined extract (29%) than with the sum of the individual extracts (10%+10% = 20%). This is likely due to a reduction of matrix effects, since only one sample has to be analyzed instead of two. This finding was confirmed with the extraction of a mashed tomato, where we achieved 46% with the combined extracts and 40% with the individual extracts. The combination of the extracts not only facilitates the extraction protocol and reduces analysis time, but it also increases the total analyte recovery. As seen in Fig. 12, extracts exceed those achieved with QuEChERS for oats and were similar for the extraction of a tomato.

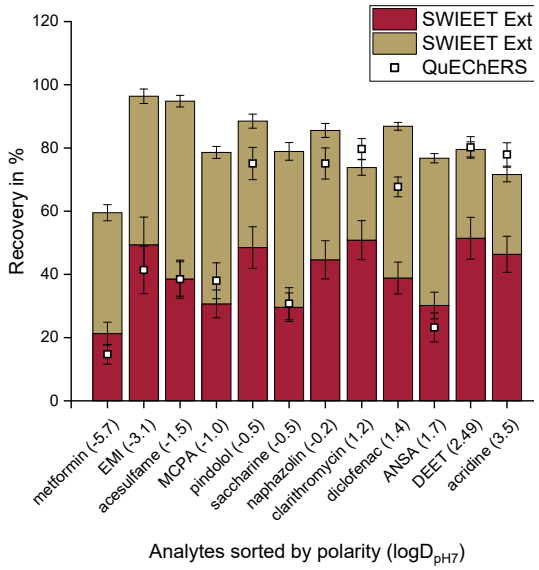
Overall, recoveries and matrix effects determined for SWIEET compared well or are better than those reached with QuEChERS for the matrices tested. Especially for polar analytes like metformin, EMI and acesulfame, SWIEET surpasses QuEChERS for all sample types. We saw matrix effects especially in case of polar analytes, presumably due to co-extraction of matrix components. Cleanup strategies, e.g. with dispersive solid phase extraction will have to be implemented in the future. SWIEET can be applied successfully to aqueous samples or samples with high water contents, but also to solid samples, where it yielded similar or higher recoveries as the QuEChERS extraction. Aqueous samples may require the addition of solid glucose instead of concentrated glucose solutions to reduce the volume of the extraction medium including the organic phase. We are not yet satisfied with the extraction efficiencies of solid samples, where further work is intended. In the future, the application of the method will be broadened to address further types of samples. With the simplified extraction protocol, SWIEET could improve sample preparation for analytical chemists working with environmental, food or biological samples up to bodyfluids. SWIEET is of particular interest in biota analysis as the reduced amount of solid phase may allow to use smaller extraction volumes compared to QuEChERS.

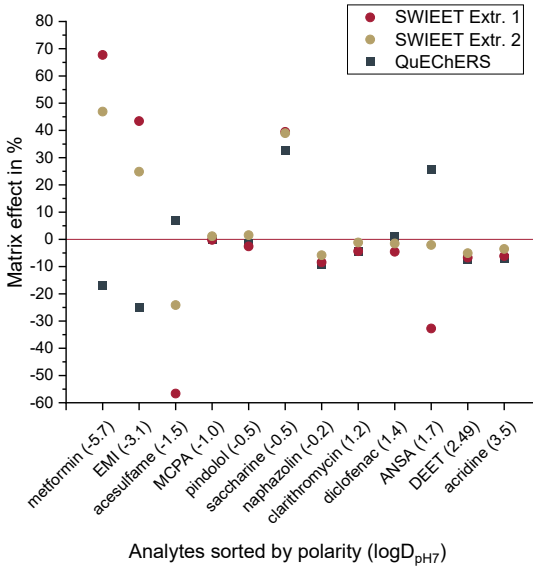
(a) wastewater treatment plant effluent



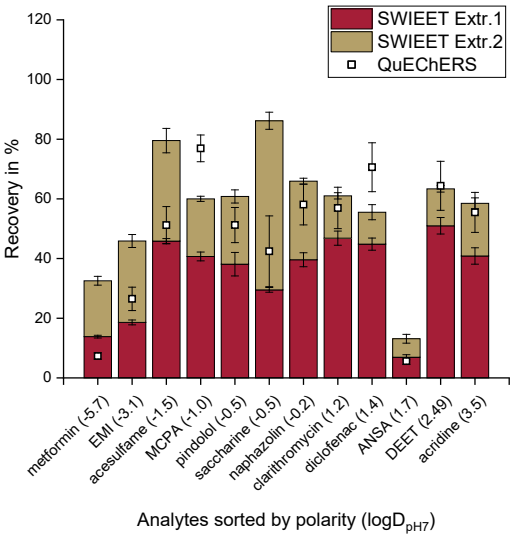


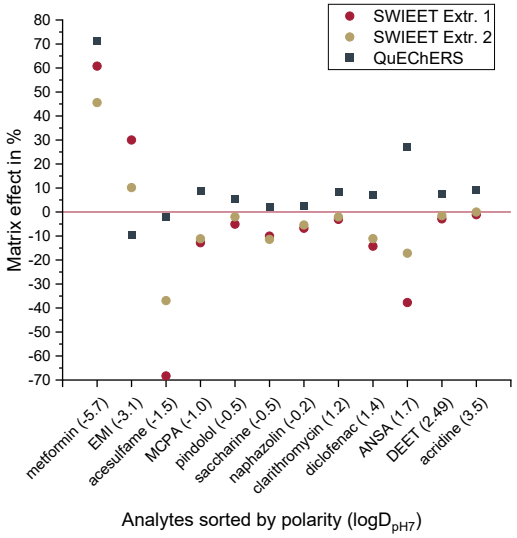
(b) river water



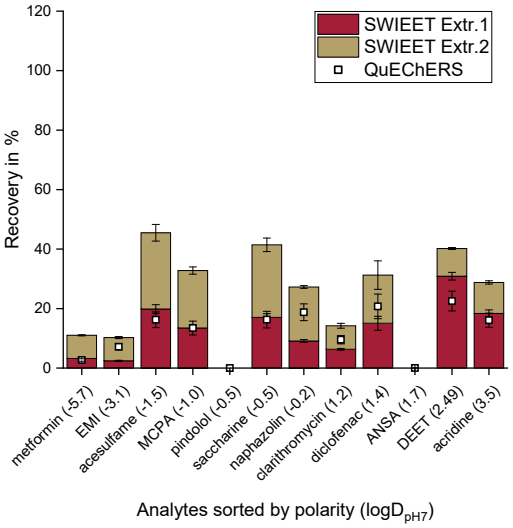


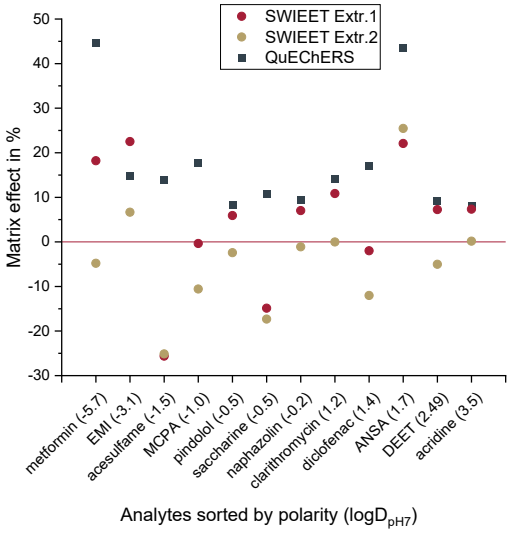
(c) tomato





(d) agricultural soil





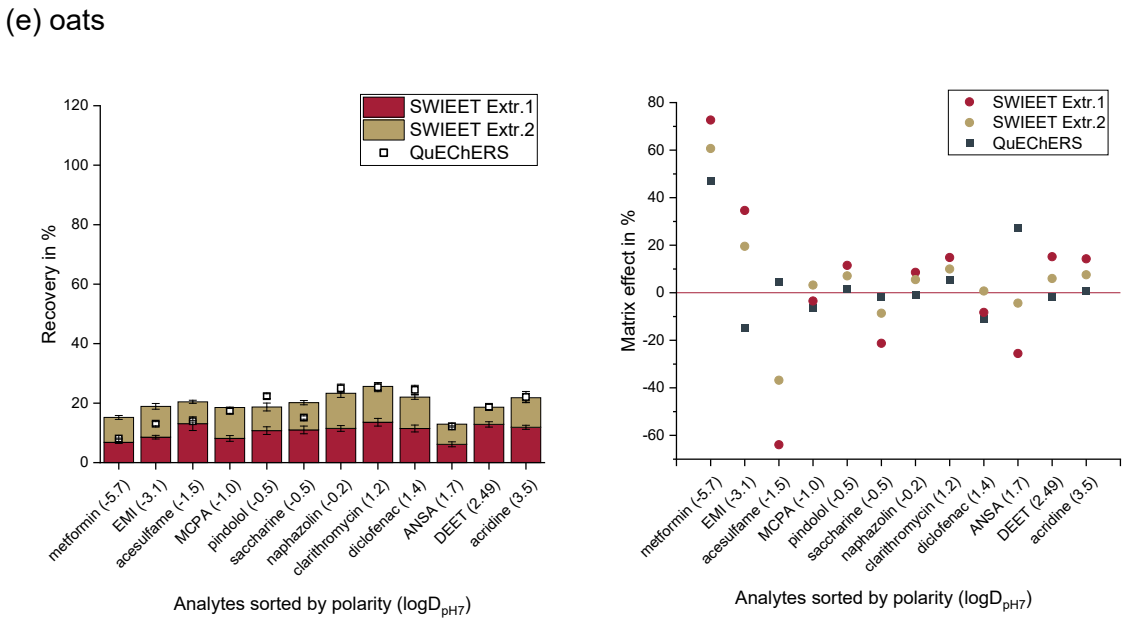


Fig. 11 Average recoveries (left) and matrix effects (right) (n = 5) for extractions of the analyte mix (3 mg/L) (see Section “3.3.2 Model analyte mix”) when spiked to (a) wastewater treatment plant effluent, (b) surface water, (c) mashed tomato, (d) soil and (e) oats using the SWIEET double-extraction compared to QuEChERS. For detailed extraction procedures, see Sections “3.3.3.5 Final SWIEET protocol” and “3.3.3.2 QuEChERS extraction”.

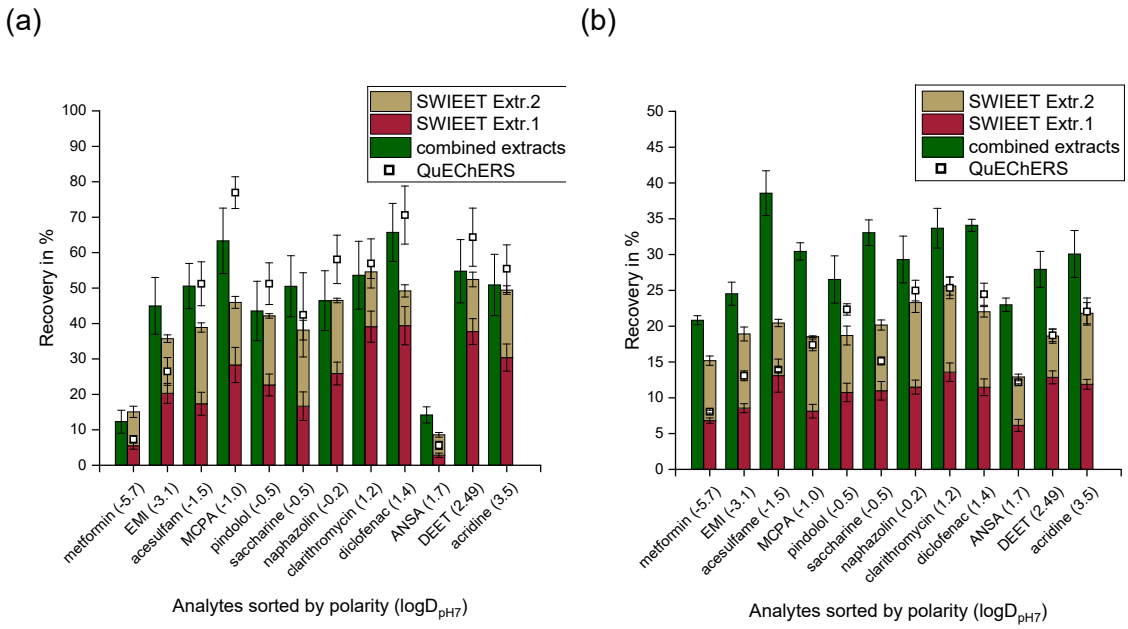


Fig. 12 Recoveries of the model analytes (see Section "3.3.2 Model analyte mix") after QuEChERS or SWIEET double-extraction from spiked (a) mashed tomato and (b) oats (see "3.3.3.1 Spiking of dry samples"). Red and gold bars indicate extracts that were analyzed individually, green bars that the analytes were quantified in the combined extracts. For detailed extraction procedures, see Sections “3.3.3.2 QuEChERS extraction” and “3.3.3.5 Final SWIEET protocol”.

3.5 Conclusion

We successfully developed a new extraction method combining sugaring-out with the addition of a protic organic solvent with a double-extraction. By screening a variety of possible additives for the extraction, we found that sugars helped to induce a very robust phase boundary over a wide temperature range and provided relatively high recoveries for our broad mixture of analytes. With glucose, we identified a cheap and readily available additive, which is highly soluble and therefore easy to handle and can simply be added by pipetting. Recoveries were improved from 23% to 34% comparing extractions with and without sugar.

To further improve recoveries, we investigated the influence of the volume and composition of the extraction mixture on the extraction recoveries. Adding isopropanol as a polar protic solvent resulted in the highest recoveries for polar analytes and also increased repeatability, as indicated by the lower standard deviations. It is also a great non-toxic alternative to halogenated solvents used in other studies.

Further improvements were based on a DoE including temperature, isopropanol and glucose content. Since recoveries did not exceed 50%, we tested electroextraction and double-extraction for further optimization. Electroextraction improved recoveries to over 60% for most analytes. Especially charged analytes like metformin, for which 32% were recovered, profited compared to 6% recovered without electroextraction. However, for real samples with higher salt loads, electroextraction was not successful.

Double-extraction significantly improved the recoveries of the SWIEET method. For QuEChERS, we only saw an improvement for single analytes like EMI and ANSA. Overall, using the SWIEET double-extraction, similar recoveries as with the regular QuEChERS extraction were often reached. For very polar substances, SWIEET clearly outperformed QuEChERS. Double-extraction is fast compared to weighing the salts and centrifuging the samples in QuEChERS. Also, handling is facilitated since all components are in solution when liquid samples are extracted and no solid phase impairs recoveries.

We applied the SWIEET method to aqueous and dry model samples. SWIEET proved to be similarly applicable as QuEChERS and yielded comparable or higher recoveries for the model analytes, while maintaining high repeatability. However, for wastewater treatment plant effluent, 75% recovery on average was achieved with SWIEET, compared to 37% with QuEChERS. Future work will address modifications of the original QuEChERS method such as QuEChERSER but also QuPPe.

Our results show that SWIEET is an interesting alternative to QuEChERS. All components used are cheap, readily available and toxicity is low. Handling is facilitated, since tedious weighing of salts is replaced with pipetting. We showed, that the SWIEET method, a salt-free extraction method, works for a broad range of sample matrices for the extraction of analytes widely differing in polarity, size, charge and functional groups. This makes the method an interesting alternative, that should be tested for further applications.

4 Understanding partitioning in two-phase systems with induced miscibility gaps – comparing SWIEET and QuEChERS

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4.1 Abstract

Our salt-free SWIEET extraction method recently developed uses a mixture of acetonitrile and isopropanol as an extraction solvent for extractions from aqueous solution. Glucose was added to induce a phase separation between an aqueous and an organic phase both of which contain water, acetonitrile and isopropanol but in different fractions. During method development, we observed that the amounts of isopropanol or glucose added greatly influenced analyte recoveries. This study aims to better understand the effects underlying especially the enhanced extraction of very polar and charged analytes. We varied the type and concentration of additives in SWIEET double-extractions and investigated their effects on the recoveries of a broad range of analytes. Furthermore, the organic and aqueous phases resulting from the extractions were characterized with NMR, solvatochromic dyes and Karl Fischer titration, to gain a deeper understanding of the effects of phase composition, the polarity and water content of the phases and the phase ratio. Significant differences between QuEChERS and SWIEET were observed regarding the water content of the organic phase. In contrast, differences in phase composition between SWIEET double extraction steps were minor and we discussed the high phase ratio of organic to aqueous phase to be a major reason for the enhanced recoveries of polar analytes in the second extraction step.

4.2 Introduction

The efficiency of an extraction in liquid-liquid extraction is based on the analytes' relative solubility in the donor and acceptor phase and thus the distribution coefficient. Conventionally, nonpolar solvents are used to extract nonpolar analytes and polar analytes are extracted with polar solvents [82], while mixtures can also be used to maximize solubility [83]. However, only a few methods evolved with a broad analyte coverage. Liquid-liquid extractions are based on the formation of a two-phase system, either through the use of two immiscible solvents or the induction of a miscibility gap as by sugaring-out in SWIEET (sugar water isopropanol ethyl nitrile extraction technique) [84] or salting out in QuEChERS extractions.

The SWIEET method uses sugars instead of salts to induce a phase separation in an acetonitrile-water mixture with isopropanol as a protic solvent present added to enhance recoveries. All method development steps, as well as the application of the SWIEET method to different sample types and a study of matrix effects can be found in Kalinke et al [84]. The optimized SWIEET method included two consecutive extraction steps to reach high recoveries especially for polar analytes. The best average recoveries were reached when the organic mixture consisted of 20% isopropanol and with 2 M glucose added to the aqueous sample. Overall, higher recoveries were achieved with SWIEET than with

QuEChERS in wastewater treatment plant effluent, with average recoveries of 69% compared to 66% with QuEChERS. Especially for polar analytes (logD < 0) recoveries were high in SWIEET extractions (70%) compared to QuEChERS (57%). In the first SWIEET extraction step, 37% were recovered on average for all analytes and 32% in the second. In comparison, recoveries from QuEChERS double extractions were already high in the first step (57%) while only 9% were additionally recovered in the second step [84].

The reason behind these differences between SWIEET and QuEChERS are further investigated in this work. The two phases resulting from the liquid-liquid extractions are never pure, the organic phase will always contain some water and the aqueous phase will contain a certain fraction of the organic solvent(s). The polarity of the phases therefore differs from the polarity of the neat solvents and the polarity may be tuned by the fractions of water and organic solvent. The water content in the QuEChERS organic phase is known to be low not only due to the addition of NaCl, but especially due to the addition of $MgSO_4$, which is added to dry the organic phase [9]. This decreases the polarity of the organic phase. Thus, target analytes of medium to low polarity can well be extracted from an aqueous phase and separation from polar matrix components, which remain in the aqueous phase, is well possible. The high fraction of organic solvents in the organic phase also ensures compatibility with further matrix removal by dispersive solid phase extraction. In the original QuEChERS publication [9], the water content in the organic phase was determined with NMR via the chemical shift of the proton signal of water, which depends on the amount of water in the sample. For example, the water content was up to 130 mg/mL in apple extracts. The rather low water content of the organic phase in QuEChERS limits the extraction efficiencies of polar and charged analytes. This was one of the main reasons to establish the new liquid-liquid extraction method SWIEET which was designed to enhance the extraction of polar analytes, while keeping high extraction efficiencies for analytes of medium to high polarity. A major aspect was the addition of the polar and protic solvent isopropanol to the extraction mixture intended to enhance the solubilization of charged compounds via hydrogen bond formation. Since salts were omitted, double extractions were conducted and shifted the phase equilibrium of analytes to result in overall higher recoveries.

In order to better understand the solubilization and enhanced extraction efficiencies of polar and charged analytes, this study intends to look at the physicochemical aspects of the extraction process. The polarity of solvents and especially solvent mixtures and the type and intensity of solvent-solute interactions can be studied via the use of solvatochromic dyes [85]. Solvatochromism describes the dependence of the absorption maximum of a probe molecule on solvent polarity and other aspects of solute-solvent interactions. This is based on the difference in stabilization of the ground or excited state of the probe molecule. If the molecule has a nonpolar ground state, the excited state will be better stabilized with increasing solvent polarity, decreasing the excitation energy. This is called a positive solvatochromism and can be observed by a red shift (bathochromic shift) of the absorption maximum. For negative solvatochromism on the other hand, a polar ground state will be better stabilized with increasing solvent polarity, leading to an increased excitation energy and a blue shift (hypsochromic shift) of the absorption maximum [85]. Based on this phenomenon, various empirical parameter scales were developed using single or multiple solvatochromic probes to describe solvent polarity [85]. The most popular approaches are the $E_T(30)$ scale and the Kamlet-Taft parameters, described below.

4.2.1 Reichardt's Dye – the $E_T(30)$ parameter

The $E_T(30)$ parameter is based on the absorption maximum of the betaine dye "Reichardt's Dye". This molecule was designed as a solvatochromic probe to cover a broad range of polarizability and to have an absorption in the visible range of 453-810 nm. The molecule's ground-state is better stabilized in environments of increasing solvent polarity, compared to the less dipolar excited state [86]. This results in a higher excitation energy in polar solvents and therefore a negative solvatochromism.

The empirical $E_T(30)$ parameter is based on the electronic excitation energies of Reichardt's Dye in the solvent of interest and can simply be calculated by converting the absorption maximum obtained from photometric detection in the solvent of interest to kcal/mol, according to Equation (3) [85] from the wavelength at the absorption maximum λ_{max}, a constant combining Planck's constant h, the frequency at the absorption maximum ν_{max}, propagation of light c and the Avogadro number N_A:

$$E_T30\left(\frac{kcal}{mol}\right) = hc\nu_{max}N_A = \frac{28591}{\lambda_{max}} \tag{3}$$

$E_T(30)$ values range from 63.1 kcal/mol in water to 30.7 kcal/mol in tetramethylsilane. According to these values, solvents can be classified as protic (47-63 kcal/mol), aprotic (40-47 kcal/mol) or apolar (30-40 kcal/mol) [85].

4.2.2 Kamlet-Taft parameters

Kamlet and Taft developed the parameters α, β and π*, that can be derived from UV/Vis spectra of solvatochromic probes in different solvents. The parameter α is a measure for the hydrogen-bond donating capabilities of a solvent [87] and β for the hydrogen-bond accepting capabilities [88]. The parameter π* is used to describe solvent polarity-polarizability [89]. Kamlet-Taft parameters were determined in literature for a large number of solvents and are often used in combination with a linear free energy relationship to compare solvent properties.

The method by González-Arjona et al.[90] was used for the determination of the Kamlet-Taft parameters α, β and π* in this work. The absorption maxima of Reichardt's Dye $E_T(30)$, p-nitroanisole and p-nitrophenol in the solvent mixtures of interest were converted to the unit Kilokaiser (kK, 1000 cm^{-1}) and used in Equations (4) and (5). For the determination of the polarity, 4-nitroanisole (NA) is used since it is not hydrogen-bond accepting, but sensitive to dipolar interactions with the solvent. 4-Nitrophenol (NP) is a good hydrogen-bond donor and can therefore assist in determining the hydrogen-bond accepting capabilities of a solvent.

$$\alpha = \frac{\nu_{max}(E_T30) + 1.873 \cdot \nu_{max}(NA) - 74.58}{6.24} \tag{4}$$

$$\beta = \frac{0.901 \cdot \nu_{max}(NA) - \nu_{max}(NP) + 4.16}{2.31} \tag{5}$$

The parameter π* was calculated from the absorption maximum (ν_{max}) of 4-nitroanisole in the organic phases of the extraction method according to Equation (6), with the literature

values for 4-nitroanisole in cyclohexane ν_0 = 34.12 kK and the empirical scaling factor s = -2.343 kK [90]. The π* values range from 0 for cyclohexane to 1 for dimethyl sulfoxide.

$$\pi^* = \frac{\nu_{max} - \nu_0}{s} \quad (6)$$

4.2.3 Application of solvatochromic parameters

$E_T(30)$ parameters are widely applied, e.g. for the characterization of surfaces [91, 92] and ionic liquids [93, 94], but also for the characterization of mobile phases in liquid chromatography as well as for the determination of the water content in organic solvents [86].

Solvatochromic parameters are often used in chromatography to understand retention through solute-solvent interactions [95]. Kamlet et al. [96] correlated solvation parameters to chromatographic capacity factors for a C18 stationary phase. For common eluents like acetonitrile/water and methanol/water, retention models based on Kamlet-Taft parameters were established [97]. Barbosa et al. [98] used such a model to select the optimal eluent composition for the separation of quinolones.

As intended in this work, solvatochromic parameters can be used to improve the understanding of extractions. Duchemin et al. [99] determined $E_T(30)$ values to determine changes in the extraction efficiencies for cesium from aqueous solution by the addition of alcohol modifiers to a crown-ether solution. Bednarz et al. [100] investigated the $E_T(30)$ and Kamlet-Taft parameters of binary two-phase systems made of deep eutectic solvents, which were used for the extraction of phenolic compounds. Tang et al. [101] derived Kamlet-Taft parameters for aqueous biphasic systems and correlated them to the yield of paeonol extracted from a herb in a linear solvation energy relationship to optimize the extraction. Deng et al. [102] used Kamlet-Taft parameters of deep eutectic solvents to understand the extraction mechanism of sulforaphane from broccoli.

The parameter $E_T(30)$ was often used to characterize binary mixtures as extensively reviewed by Spange [103]. However, describing the polarity of a binary solvent mixture remains complex, especially due to preferential solvation, which results in non-linear correlations of the $E_T(30)$ parameter and the composition of the mixture. Many theoretical approaches have been chosen to understand and proof preferential solvation of solvatochromic probes in binary mixtures [103]. Ternary mixtures have also been investigated in literature, for example by Jonquières et al. [104], who calculated $E_T(30)$ for a ternary alcohol/ether/polyurethaneimide system. Similarly, the $E_T(30)$ values of a methanol/acetonitrile/propanol system were investigated by Leitão et al. [105]. Kamlet-Taft and $E_T(30)$ parameters were calculated by Fletcher and Pandey [106] for an ionic liquid/ethanol/water mixture. Here, too, a non-linear correlation between the parameters and the composition of the mixture was observed. Nunes et al. [107] determined Kamlet-Taft and $E_T(30)$ values for methanol/formamide/acetonitrile mixtures corroborating studies with other binary mixture solvation models. It should be recognized that the solvation mechanisms are not fully understood yet and $E_T(30)$ values of mixtures should be interpreted carefully [103].

In this work, QuEChERS is used for comparison, since it is a liquid-liquid extraction technique using salts to induce a phase separation in an acetonitrile-water system, which is similar to SWIEET, which uses sugars. The aim of this study was to examine different

SWIEET extraction media and determine phase composition and solvatochromic parameters of SWIEET and QuEChERS organic phases to gain understanding of the physicochemical aspects that affect partitioning and analyte extraction in two-phase systems.

4.3 Materials and Methods

4.3.1 Chemicals

1-ethyl-3-methyl-imidazolium (EMI, ≥95%), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 5-amino-2-naphthalene sulfonic acid (ANSA, ≥95%), acesulfame (ACE, ≥99%), acetonitrile-d3 (>99.8%), acridine (ACR, 97%), alpha-D-glucose (96%), clarithromycin (CLA, ≥98%), dichloromethane (DCM, 99.9%), diclofenac sodium salt (DIC, ≥98 %), hexane (≥97%), isopropanol (iPr, LC-MS grade), maleic acid (>99%), methanol (MeOH, LC-MS grade), naphazoline (NAPHA, ≥98%), pindolol (PIN, 98%), saccharine (SAC, ≥98%) and toluene (99.9%) were purchased from Sigma Aldrich (Steinheim, Germany). 4-hydroxybenzoic acid (HBA, ≥98%) and *N*-diethyl-m-toluamide (DEET, ≥98%) were from Fluka (Buchs, Switzerland). 4-nitroanisol (4-NA, >99%), ethyl acetate (EtOAc, p.a.) and water (LC-MS grade) were provided by Thermo Fisher (Kandel, Germany). 4-nitrophenol (4-NP, ≥99%), acetonitrile (MeCN, LC-MS grade), dimethyl sulfoxide (DMSO, 99.5%), formic acid (>99%) and indigo carmine were bought from Roth (Karlsruhe, Germany). Metformin (MET, 97%) was from Alfa Aesar (Haverhill, MA, USA). Reichardt's Dye (RD) was purchased from Biosynth (Leicester, United Kingdom). Deuterium oxide (D_2O, 99.9%) was from deutero (Kastellaun, Germany). Purified water produced using a PURELAB Classic PL5241 (ELGA LabWater, Celle, Germany) was used throughout the study.

4.3.2 Extraction procedure

For SWIEET extractions, water was spiked with an analyte mix of 13 model analytes to a final concentration of 3 mg/L each for determination of recoveries. Glucose was added to a final concentration of 1.5, 2 or 2.5 M. For liquid-liquid extractions, 2.5 mL of this analyte mix were diluted with the same volume of an organic extraction mixture consisting of 80 or 90 vol.% acetonitrile and 20 or 10 vol.% isopropanol. The mixture was homogenized for 1 min using a vortexer. After a clear phase boundary was visible, usually within a minute, phases were separated by pipetting. For double-extraction, 2.5 mL fresh organic extraction mixture consisting of 80 or 90 vol.% acetonitrile and 20 or 10 vol.% isopropanol were added to the residual aqueous phase from the first extraction step. After mixing for 1 min using a vortexer, the phases were allowed to separate again.

QuEChERS extractions were conducted as described by Kalinke et al. [84]. Briefly, 2.5 mL acetonitrile as well as 0.25 mg NaCl and 1 mg $MgSO_4$ were added to 2.5 mL water with the analyte mix, to induce phase separation. For a modified QuEChERS extraction with isopropanol, an 80-20 acetonitrile-isopropanol mixture was used instead of acetonitrile.

To improve the visibility of the phase boundary and estimate the water content, indigo carmine only soluble in water or in water-rich mixed phases, was added to the aqueous phase for some experiments.

The organic extracts were analysed using a gradient elution RPLC coupled to a Q-ToF-MS. Details on the model analyte mix, RPLC-MS method and quantification can be found

in Kalinke et al. [84]. Recoveries were determined separately for the two extraction steps and data are reported for single analytes or average values for all 13 analytes.

4.3.3 Determination of solvatochromic parameters using UV-Vis spectroscopy

UV-Vis spectra of the dyes 4-nitroanisol, 4-nitrophenol and Reichardt's Dye in various solvents were recorded using a Lambda 19 UV/Vis spectrometer (PerkinElmer, Waltham, MA, USA) with LambdaSPX software (ascansis OHG, Überlingen, Germany). The spectra were recorded from 170-900 nm in 1 nm intervals at a scan rate of 480 nm/min, with the blank extracts as reference. The dyes were added to the separated phases after extraction without spiking the analyte mix, so that no bias was introduced due to partitioning.

4.3.4 Determination of the water, isopropanol and glucose content

For NMR analysis, 300 µL of the organic phase were combined with 200 µL acetonitrile-d3. For the analysis of the aqueous phase, D_2O was used. To quantify isopropanol, 5 mg maleic acid were added to the sample. Four replicates of each organic and aqueous phase were measured and integrals were averaged. The ^1H spectra were recorded with a Bruker Avance III HD 400 at a frequency of 400 MHz and evaluated using Topspin 4.1.4 (Bruker, MA, USA). The spectra were calibrated to the acetonitrile signal at 1.94 ppm. The amount of isopropanol was calculated relating the integrals of maleic acid (I_{MA}, 6.26 ppm, s) and isopropanol (I_{iPr}, 3.87 ppm, sept), considering the known amount of maleic acid (n_{MA}) and the number of protons causing the isopropanol signal ($p_{iPr} = 6$) according to Equation (7):

$$n_{iPr} = \frac{n_{MA}}{I_{MA}} \cdot \frac{I_{iPr}}{p_{iPr}} \tag{7}$$

In addition, the water content was determined by Karl Fischer titration at the analytical facilities at Merck, Darmstadt, Germany.

Glucose concentrations were determined by drying the organic and aqueous phases from SWIEET extractions conducted as described in Section "4.3.2 Extraction procedure" at 70 °C, so that the amount of glucose can be determined by weighing. For this experiment, extraction volumes were reduced by a factor of 5 to shorten the drying time.

4.4 Results

To better understand the reasons for the improved recoveries from SWIEET double extractions compared to QuEChERS and differences between the two SWIEET extraction steps, we first investigated the different SWIEET additive compositions regarding their abililty to extract the model analytes as well as the resulting phase ratio. For the most noticable phase composition, we determined water and isopropanol content, as well as solvatochromic parameters to gain insight into the phase compositions.

4.4.1 Influence of glucose and isopropanol content on analyte recoveries

During the optimization of the SWIEET extraction method [84], we observed that the volume of the organic phase increased significantly after the addition of the fresh organic mixture (a mixture of 20% isopropanol and 80% acetonitrile) in the second extraction step. The composition of the aqueous phase after the first extraction step is unknown: Due to the double-extraction and the addition of isopropanol, the increased glucose concentration is no longer simply correlated with increased recoveries, as described in literature [51, 55, 56]. To further investigate the roles of glucose concentration, isopropanol content and

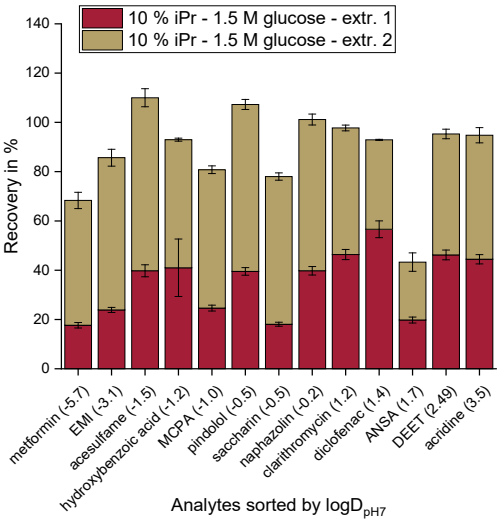
phase ratios on recoveries, further experiments with glucose concentrations in the aqueous sample ranging from 1.5 M to 2.5 M and 10 or 20% isopropanol in the organic mixture were conducted. The ranges were chosen during preliminary experiments, which showed that lower glucose concentrations do not induce a stable phase separation and higher glucose concentrations are above the solubility limit of 2.6 M in water. For the combination of 20% isopropanol content and a glucose concentration of 1.5 M, no phase separation was induced.

Considering first only the first extraction step with 10% isopropanol in the extraction mixture, we observed a slight increase in the average recovery from 35 to 42% when increasing the glucose concentration from 1.5 M to 2 M (Fig. 13 a vs. c), but no further improvement at 2.5 M (43%, Fig. 13e). The results for 2 M and 2.5 M glucose with 20% isopropanol added (45% and 47%, Fig. 13g and i) are similar to those at 10%, indicating a high robustness with regard to the isopropanol content in the first step. No combination tested significantly increased the recovery of a specific analyte or analyte class such as polar vs. nonpolar analytes in the first step.

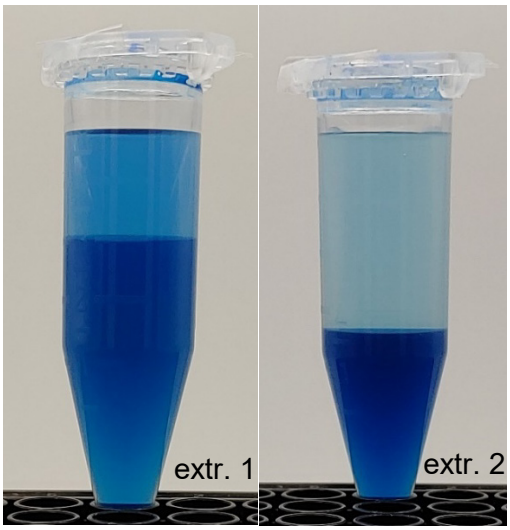
For the second step, an increased sugar concentration in the aqueous sample led to decreasing recoveries: At 10% isopropanol content in the organic mixture, average recoveries decreased from 53% to 23% to 9% with increasing glucose concentration, at 20% isopropanol from 45% to 9%. Average recoveries were similar for 1.5 M glucose at 10% isopropanol and 2 M glucose at 20% (44% and 45%) in the second step. The same held true for 2 M glucose at 10% isopropanol and 2.5 M glucose at 20% isopropanol (33% and 31%).

In order to visualize the effects of the double extraction on the recovery of different analytes depending on their polarity, we calculated the recovery ratio rec(step1)/rec(step2) between the two extraction steps for each analyte and for different compositions of the extraction medium. Values <1 indicate a higher recovery in the second organic phase, while values >1 indicate a higher recovery in the first organic phase. Fig. 14 shows that for all media, the second extraction step is more relevant to enhance the recoveries for polar analytes and especially for charged analytes, wheras nonpolar analytes are already well extracted in the first step. With 2.5 M glucose concentration, however, all ratios are >1 (except for metformin) demonstrating that the second step was not so efficient in enhancing the overall recoveries, which is especially the case for the least polar analytes with ratios >10. In contrast, using 1.5 and 2 M glucose, for polar analytes ($logD_{pH7}$ <0) ratios down to 0.2 were observed. For the final method chosen, the ratios were better balanced and remained in a range of 0.4 to 2.3 for all analytes. Values <1 for polar to charged analytes, values around 1 for analytes of intermediate polarity and of >1 for nonpolar analytes were observed leading to similar overall recoveries for all analytes with 90% on average. A QuEChERS extraction (see the Section “4.3.2 Extraction procedure”) was conducted for comparison (Fig. 13k), which yielded 35% analyte recovery on average. This percentage was the lowest of the tested combinations for the first extraction step, and similar to SWIEET with 10% isopropanol and 1.5 M glucose as additives.

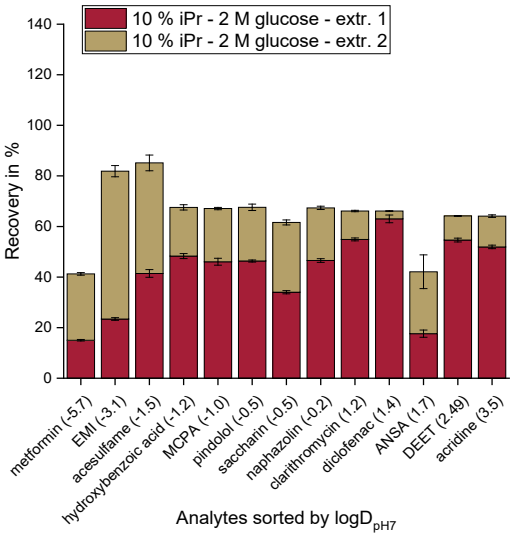
(a) 10% iPr, 1.5 M glucose



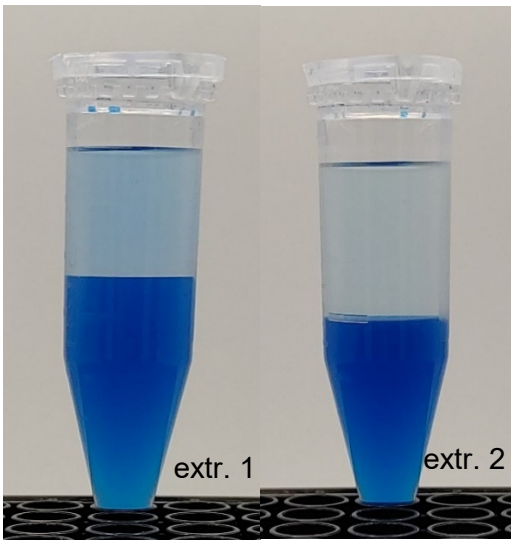
(b) 10% iPr, 1.5 M glucose



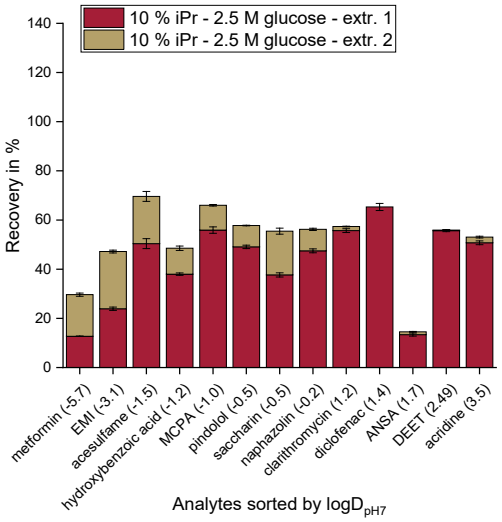
(c) 10% iPr, 2 M glucose



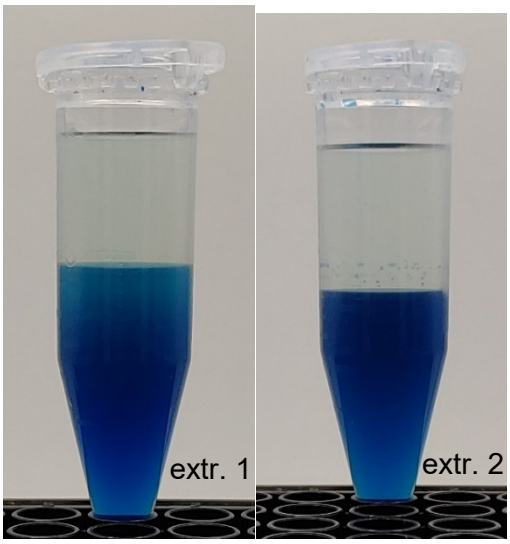
(d) 10% iPr, 2 M glucose



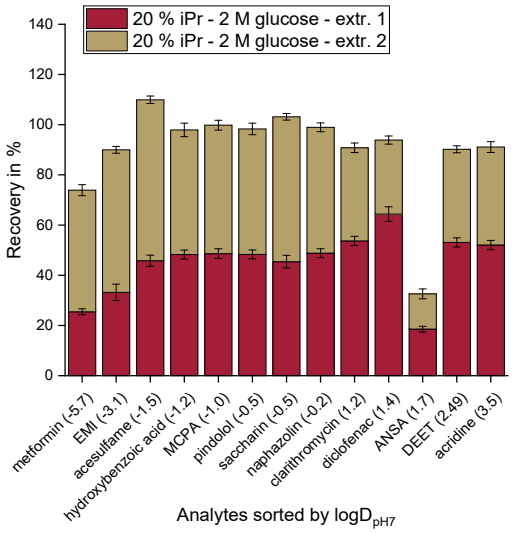
(e) 10% iPr, 2.5 M glucose



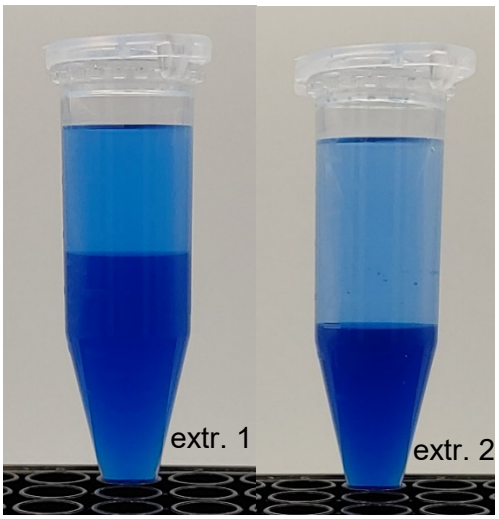
(f) 10% iPr, 2.5 M glucose



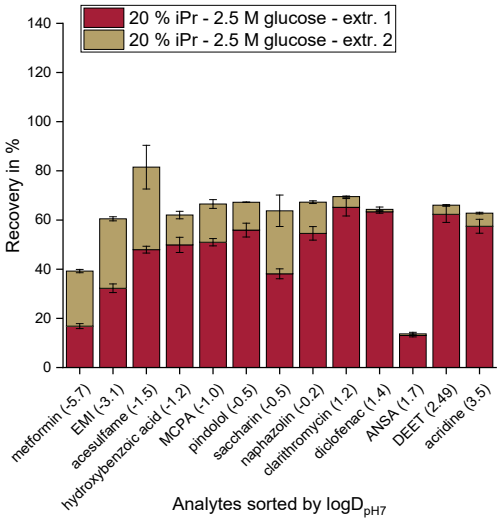
(g) 20% iPr, 2 M glucose



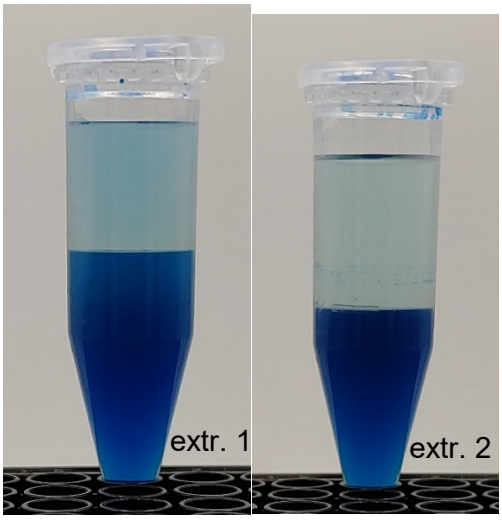
(h) 20% iPr, 2 M glucose



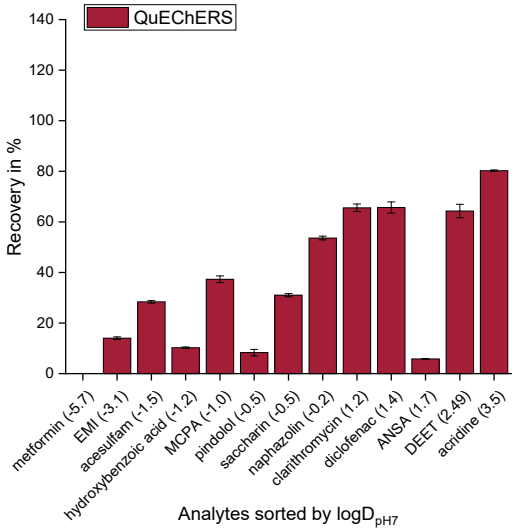
(i) 20% iPr, 2.5 M glucose



(j) 20% iPr, 2.5 M glucose



(k) QuEChERS



(l) QuEChERS

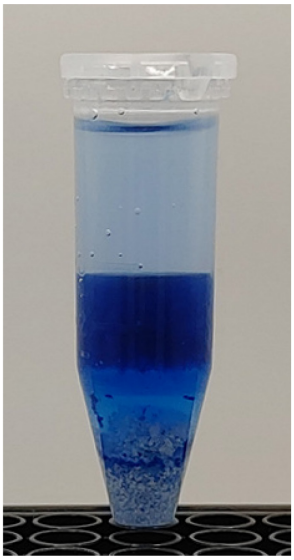


Fig. 13 (a-j) Recoveries of SWIEET double extractions from water with varying isopropanol content in the organic mixture (10-20%) and glucose concentration in the aqueous mixture (1.5-2.5 M) **(a, c, e, g, i)** and photographs of the extraction vials after phase separation of extraction steps 1 (left) and 2 (right). **(k,l)** Recoveries of QuEChERS extractions with the solid phase from excess salts visible. Phases were colored with indigo carmine **(b, d, f, h, j, l)** as a marker for the water content [84]. For all protocols, see Section "4.3.2 Extraction procedure".

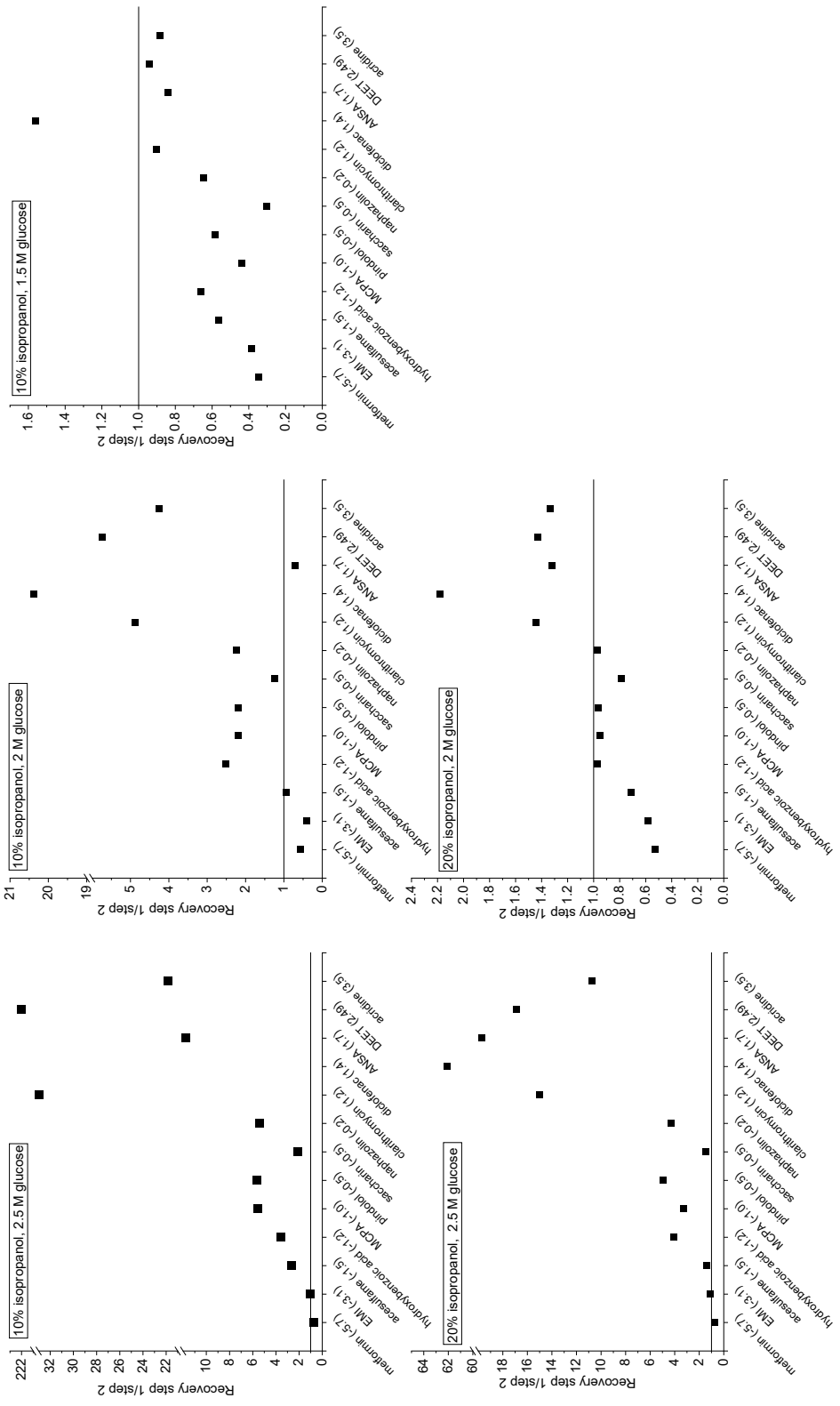


Fig. 14 Recovery ratios rec(step 1)/(step 2) for all 13 model analytes and all tested extraction mixtures calculated from recoveries of SWIEET double extractions (see Section "4.3.2 Extraction procedure") from water with varying isopropanol content in the organic mixture (10-20%) and glucose concentration in the aqueous mixture (1.5-2.5 M).

4.4.2 Influence of glucose and isopropanol content on phase ratios in SWIEET

In SWIEET extractions, recoveries for polar analytes were especially high in the second extraction step, when an organic mixture of 80% acetonitrile and 20% isopropanol was used for extraction. To better understand the reasons for this increase, we investigated the phase composition in SWIEET extractions. We presumed that a higher water content in the organic phase increases the polarity of the organic phase [108] and therefore the recoveries especially of polar analytes. A higher water content was also expected to increase the recoveries of polar and charged analytes by a better solvation through hydrogen bonding. The water content was first visualized adding indigo carmine. Indigo carmine is only soluble in water and solvent mixtures with water [84]. The water content can thus be estimated by the intensity of the blue color. As shown in Fig. 15, the first organic phase from SWIEET extraction has a deeper blue color than the second organic phase, indicating a higher water content in the organic phase during the first extraction. As expected, most of the indigo carmine remained in the aqueous phase.

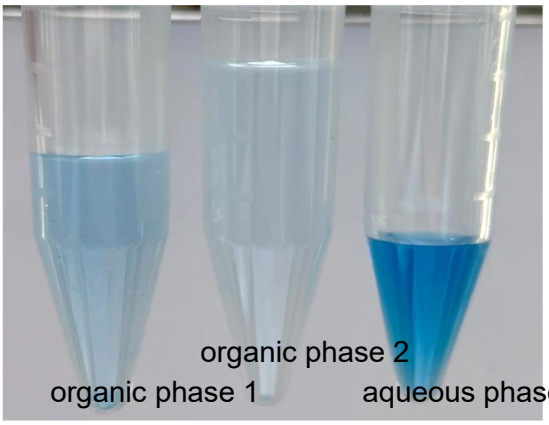


Fig. 15 Photograph of the organic phases 1 and 2 from the SWIEET double extraction and the aqueous phase (right) after SWIEET double-extraction from water with 2 M glucose and an organic mixture with 80-20 acetonitrile-isopropanol. Indigo carmine was added to the extraction mixture to visualize the water content. For the extraction procedure, see Section “4.3.2 Extraction procedure”.

Looking at the phase volumes for the different glucose and isopropanol concentrations (Fig. 13b, d, f, h, j), it is apparent that they were all very similar with about 2.5 mL organic phase in the first extraction and 3 mL in the second step, except when using 1.5 M glucose and 10% isopropanol, where only a small volume of the first organic phase (2 mL) formed in the first step compared to the other extractions, while the volume of the second organic phase was significantly larger (3.5 mL). The combination of using 2 M glucose and 20% isopropanol for extraction also exhibited a notably larger organic phase volume in the second extraction step (3.5 mL). With QuEChERS (Fig. 13l), the phase volume was the same as in most of the first SWIEET extraction steps, with 2.5 mL in the organic and aqueous phase.

Using the intensity of the blue color from indigo carmine a slight decrease of the water content in the second step vs. the first step was observed at all concentrations except 2.5 M glucose and 10% isopropanol, where almost no indigo carmine was visible in the organic phase in both steps. For extractions with 10% isopropanol and 1.5 M glucose (Fig. 13b) there was a similar intensity of the blue color in both phases in the first extraction step, indicating a similar water content in the organic and aqueous phase. In contrast, in

the second step, a clear difference in the water content was observed. Using 20% isopropanol and 2 M glucose, a similar color was visible after both extraction steps, see Fig. 13h. For this combination, the highest water content of all experiments was reached in the second step. For QuEChERS, the water content was comparable to the first SWIEET extractions with 10% isopropanol and 2 M glucose or 20% isopropanol and 2.5 M.

Clearly, the higher water content correlated well with higher recoveries for different extraction media as expected from an increase in the polarity of the organic phase. For metformin, 48% were recovered in the second step of the extraction with 20% isopropanol and 2 M glucose, were the highest water content was observed. At 10% isopropanol and 2.5 M glucose, where the water content was lowest in the second step, only 17% metformin were recovered. Especially the combination of 20% isopropanol and 2 M glucose proved interesting and further detailed experiments were conducted with this combination.

To directly investigate whether the sugar content has an influence on the phase ratio, the phases of two extractions were evaporated to reveal the sugar content in the organic and aqueous phases. We chose the final SWIEET additive composition (20% isopropanol, 2 M glucose) and for comparison a composition evoking low recoveries (10% isopropanol, 2.5 M glucose). The results are reported in Table 5. In the extraction with 10% isopropanol and 2.5 M glucose, only 4% of the glucose partitioned into the organic phase in the first and second extraction step. The extraction with 20% isopropanol and 2 M glucose has a higher concentration ratio of 0.2 in the first and 0.09 in the second step. However, it is interesting to note that the higher initial glucose concentration of 2.5 M (with 10% isopropanol) compared to 2 M (with 20% isopropanol) does not lead to a higher glucose concentration in the organic phase. In addition, the phase ratio does not correlate with the glucose concentration in the organic phase, e.g. a concentration of 0.31 mol/L was determined in step 1 vs. 0.18 mol/L in step 2, where the phase ratio is larger.

Table 5 Glucose masses, estimated concentrations and ratios between the organic and aqueous phases from both extraction steps of SWIEET double extractions with 10% isopropanol - 2.5 M glucose and 20% isopropanol – 2 M glucose (see Section “4.3.2 Extraction procedure” and “4.3.4 Determination of the water, isopropanol and glucose content”).

			glucose mass in mg	estimated glucose concentration in mol/L	glucose concentration ratio $c(glucose_{org})/c(glucose_{aq})$
10% isopropanol, 2.5 M glucose	Extr.1	org. 1	8.8	0.097	0.04
		aq. 1	209.1	2.3	
	Extr.2	org. 2	9.1	0.093	0.04
		aq. 2	175.4	2.1	
20% isopropanol, 2 M glucose	Extr.1	org. 1	28.2	0.31	0.2
		aq. 1	143.8	1.6	
	Extr.2	org. 2	22.8	0.18	0.09
		aq. 2	114.1	2.1	

4.4.3 Determination of the water and isopropanol content

To better understand the composition of the phases, NMR measurements were conducted with the organic and aqueous phases of SWIEET and of QuEChERS. By the shift of the water signal in reference to the CD_3CN signal in the ^{1}H-NMR, the water content can be estimated in the organic phase [9]. We recorded NMR-spectra of samples with known water content for calibration to estimate the water content in the samples. For the first organic phase, the water signal was detected at 3.67 ppm on average (n = 4), for the second organic phase at 3.59 ppm on average, which was equivalent to water contents of 24% and 20%. This confirms the lower water content in the second organic phase determined from visual inspection after addition of indigo carmine. The organic phase of a QuEChERS extraction had the lowest water content with an average shift of only 3.33 ppm which was equivalent to a fraction of water of only 14%. For a more precise investigation of the water content, Karl Fischer titrations of the three organic phases were conducted confirming the estimations made with NMR: the water content was 30.4 wt-% in the first SWIEET organic phase, 22.4 wt-% in the second SWIEET organic phase and 10.9 wt-% in the QuEChERS organic phase. For all polar analytes (logD < 0), recoveries are higher in the second extraction step in SWIEET than in the first (e.g. metformin extr. 1: 25% vs extr. 2: 48%), although the water content is lower. Water content therefore is not sufficient to explain the increased recoveries in the second SWIEET extraction step compared to the first.

Table 6 NMR shifts of the water signal and isopropanol concentrations in the organic phases of SWIEET and QuEChERS extractions (see Section “4.3.2 Extraction procedure”) determined using NMR (see Section “4.3.4 Determination of the water, isopropanol and glucose content”).

	average water signal shift in ppm (n = 4)	water content estimated from NMR in %	water content from Karl Fischer titration in %	average isopropanol concentration in mol/L (n = 4)
SWIEET organic phase 1	3.67 ± 0.01	24	30.4	0.82 ± 0.02
SWIEET organic phase 2	3.59 ± 0.02	20	22.4	1.02 ± 0.02
SWIEET aqueous phase 1	--	--	--	0.48 ± 0.02
SWIEET aqueous phase 2	--	--	--	0.40 ± 0.01
QuEChERS	3.33 ± 0.17	14	10.9	--

From the NMR measurements, we were also able to estimate the isopropanol content via the addition of maleic acid as an internal standard [109]. The isopropanol content proved to be higher in the second organic phase (1.02 mol/L on average) than in the first (0.82 mol/L on average). Isopropanol and acetonitrile contents were also estimated in the aqueous phases. The second aqueous phase contained a lower concentration of

acetonitrile and isopropanol (1.52 mol/L and 0.40 mol/L) than the first (2.34 mol/L and 0.48 mol/L). Due to the ability to form H-bonds, isopropanol can aid in the solvation and therefore extraction of polar analytes. This was further investigated via solvatochromic parameters.

4.4.4 Determination of solvatochromic parameters

For a deeper understanding of the influence of the phase properties on analyte extractions, solvatochromic parameters were determined. To calculate the solvatochromic parameters according to Kamlet & Taft [110], UV-Vis spectra of Reichardts dye, 4-nitroanisol and 4-nitrophenol dissolved in the organic phases resulting from QuEChERS and SWIEET extraction with 2 M glucose and 20% isopropanol were recorded. For a better comparison, we performed an additional modified QuEChERS extraction with the same 80-20 acetonitrile-isopropanol mixture as used for SWIEET extractions. From the wavelengths of the absorption maxima, the parameters α, β and π* were calculated using Equations (4-6) as described by Sindreu et al. [111] and Gonzalez-Arjona et al. [90]. The parameters for the extraction phases and mixtures of acetonitrile-water-isopropanol are summarized in Table 7.

To extend the interpretation of the solvatochromic parameters of our solvent mixtures, we conducted further measurements with known mixtures of isopropanol, acetonitrile and water. Isopropanol and water contents were in the range of the values determined in the "4.3.4 Determination of the water and isopropanol content" section. The resulting wavelengths and parameters are also listed in Table 7.

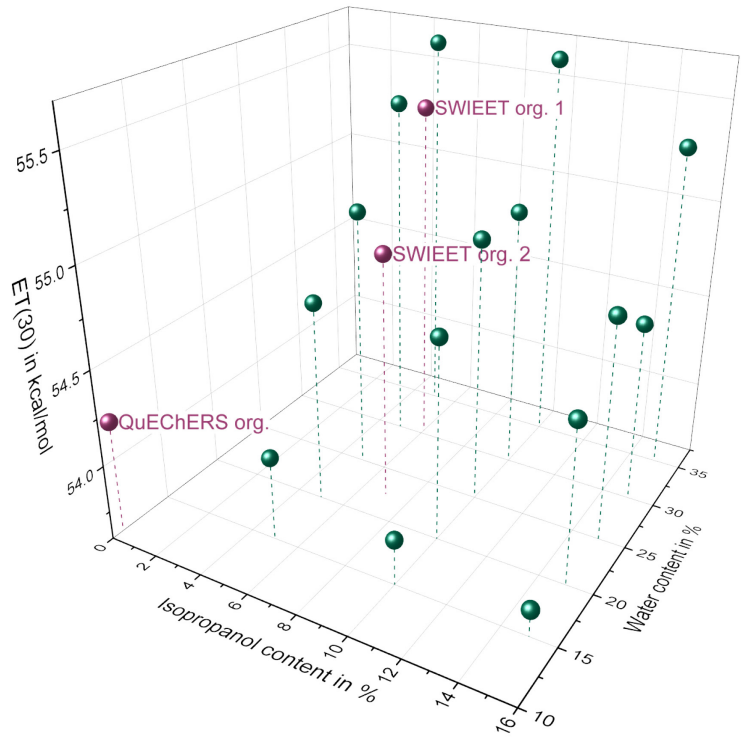


Fig. 16 $E_T(30)$ values for mixtures A-O of isopropanol and water in acetonitrile listed in Table 7, as well as values for SWIEET org. 1, SWIEET org. 2 and QuEChERS org. For details on the determination of the $E_T(30)$ value, see Section "4.3.3 Determination of solvatochromic parameters using UV-Vis".

The $E_T(30)$ value determined via the addition of Reichardt's Dye is a measure for the overall polarity of a solvent mixture. The $E_T(30)$ parameter is very sensitive to polarity changes, given the large shifts of the absorption maxima of Reichardt's Dye upon changes in the solvent composition. The overview of $E_T(30)$ values in Fig. 16 shows that with increasing isopropanol content in the mixture, $E_T(30)$ values decreased, whereas with increasing water content, $E_T(30)$ values increased. Comparing the slopes of the dependencies, it is obvious that the water content influenced the $E_T(30)$ parameter more strongly than isopropanol. This is also in line with the results from an analysis of variance of recoveries of individual analytes depending on glucose and isopropanol concentrations: Here we found that varying isopropanol concentration yielded fewer significant differences in recoveries for single analytes than varying glucose concentrations. The $E_T(30)$ values of our known mixtures varied between 53.7 and 55.6 kcal/mol and were between those for pure acetonitrile (45.6 kcal/mol, aprotic), isopropanol (48.4 kcal/mol, protic) and water (63.1 kcal/mol, protic) listed by Reichardt [85] as expected for mixtures. Of our unknown solvent mixtures from extractions, the $E_T(30)$ values of all mixtures except modified QuEChERS with isopropanol (56 kcal/mol) lay in the range of the known solvent mixtures. The mixture of 80-20 acetonitrile-isopropanol, which was added for SWIEET extraction, had an $E_T(30)$ value of 49.6 kcal/mol, which is higher than the neat solvents. $E_T(30)$ values were lower for the organic phase in QuEChERS (54.2 kcal/mol) compared to SWIEET (54.9-55.9 kcal/mol). As shown in Fig. 16, even small variations of the mixture composition led to large differences in the $E_T(30)$ parameter, especially when changing in the water content.

The Kamlet-Taft parameter α is a measure for the hydrogen-bond donating capacity of the solvent compared to methanol used as a reference. A similar trend as for the $E_T(30)$ values was observed along the solvent mixtures for α: a lower isopropanol content and higher water content increased α (the absorption maxima of Reichardt's Dye are included in the calculation of α). The values for α varied between 0.74 and 0.81 for the known mixtures A-O. For the extraction phases tested, the organic phase of QuEChERS had the lowest value of α with 0.76, meaning that it has a weaker hydrogen-bond donating capacity than the SWIEET organic phases ranging from 0.79 to 0.80. The modified QuEChERS extraction with isopropanol had the highest α value of 0.84 and was outside the values determined for the known solvent mixtures. Kamlet and Taft reported average α values of 0.69 for isopropanol, 0.29 for acetonitrile and 1.02 for water [87]. To obtain these values, they averaged the values resulting from six different calculation methods, based on different solvatochromic probes. For a better comparison, we determined the α values also in this study as described above. They were 0.35 for acetonitrile and 0.63 for isopropanol and differed slightly from the literature values. For comparison, we also determined the parameter α for the 80-20 acetonitrile-isopropanol mixture used for extraction. The α value was between the neat solvents (0.58) as can be expected. The parameter for water could not be determined, since the solubility of Reichardt's Dye was insufficient.

The parameter β, which describes the hydrogen-bond accepting properties of the solvent relative to DMSO, was lowest for the QuEChERS organic phase (0.56) compared to SWIEET (0.62-0.63). The isopropanol-QuEChERS organic phase revealed an absorption maximum between QuEChERS and SWIEET, which resulted in $\beta = 0.60$. For the neat solvents isopropanol, water and acetonitrile, literature values for the parameter β vary greatly [110, 112]: For example for water, Kamlet et al. [110] listed an average of $\beta = 0.13$ for water, acquired with different probes, while Marcus [112] listed $\beta = 0.47$. For

isopropanol, Kamlet and Taft reported β values in the range of 0.91 to 0.95 [88] and Marcus β = 0.84, for acetonitrile Marcus listed β = 0.4 [112]. In our experiments, we calculated β values of 0.76 and 0.34 isopropanol and acetonitrile, respectively, and 0.59 for the 80-20 mixture. Compared to the differences of the values of these parameters between neat solvents, the values of the organic mixtures tested in QuEChERS and SWIEET can be regarded to be similar in the two methods.

For the parameter π*, a measure for polarizability and dipolarity [85], similar values of 0.82-0.84 for SWIEET, 0.79 for QuEChERS and 0.84 for isopropanol-QuEChERS were obtained. The calculation of β and π* is based on the absorption maxima of 4-nitrophenol and 4-nitroanisol, which are not as sensitive to changes in the solvent composition as Reichardt's Dye. Sindreu et al. [111] determined Kamlet-Taft parameters for aqueous mixtures of tert-butanol and ethyleneglycol. Their values varied depending on the ratio of the solvents: For π* and α they differed by up to 0.6, for β up to 0.24. In the work of Cheong et al. [113], the values for π* of water-cosolvent mixtures differed by up to 0.67 depending on the volume fraction of the cosolvent. E.g. an increase of the volume fraction of methanol, acetonitrile, isopropanol or tetrahydrofuran by 10% changed π* by 0.01-0.15. In our experiments, π* of the QuEChERS organic phase (0.79) and SWIEET organic phase (0.82-0.84) differed by 0.05. The value for isopropanol-QuEChERS was identical to the SWIEET organic phase 1 (0.84). The π* value for the 80-20 acetonitrile-isopropanol mixture was higher compared to the one for the neat solvents, as opposed to α and β. The differences in beta values for the extraction phases were not regarded significant compared to the range of values for the mixtures of known composition.

To further broaden the understanding of the extractions, we also investigated the aqueous phases of the extraction methods. The solubility of Reichardt's Dye was too low to reach a sufficient concentration to record UV/Vis spectra. Thus, only the parameter π* could be determined for SWIEET. For QuEChERS, no absorption maxima were detected for 4-nitrophenol and 4-nitroanisol, possibly due to the low solubility of the dyes in water. The parameter π* of the first and second aqueous SWIEET phase did not differ significantly (1.03 vs. 1.01). However, the elevated organic content revealed a π* value significantly different from the one of pure water of 1.09 [89] in both steps.

All Kamlet-Taft parameters of the organic solvent mixtures determined lay between the literature values for the neat solvents. Comparing the SWIEET organic phases, the differences for the Kamlet-Taft parameters were within the standard deviation, so no significant differences were present. The largest differences were observed between QuEChERS and SWIEET and isopropanol-QuEChERS and SWIEET for all parameters.

Table 7 Average wavelengths λ_{max} (n = 3, *n = 1) of the dyes and resulting Kamlet-Taft parameters and $E_T(30)$ values determined in SWIEET and QuEChERS organic phases, as well as in known mixtures A to O with different fractions of isopropanol, water and acetonitrile (remainder to 100%), given as the percentages of these three solvents. For details, see "4.3.3 Determination of solvatochromic parameters using UV-Vis". RD: Reichardt's Dye, 4-NP: 4-nitrophenol, 4-NA: 4-nitroanisol

	λ_{max} (RD) in nm	λ_{max} (4-NP) in nm	λ_{max} (4-NA) in nm	α	β	π*	$E_T(30)$ in kcal/mol
QuEChERS	528 ± 5.4	313 ±0.5	310 ± 0	0.76 ± 0.04	0.56 ± 0.02	0.79 ± 0	54.2 ± 0.6
SWIEET organic phase 1	516 ± 1.5	316 ± 1	311 ± 0	0.80 ± 0.01	0.62 ± 0.04	0.84 ± 0	55.4 ± 0.16
SWIEET organic phase 2	521 ± 1	315 ± 1	311 ± 0.6	0.79 ± 0.02	0.63 ± 0.04	0.82 ± 0.03	54.9 ± 0.1
SWIEET combined	519 ± 0	315 ± 1	311 ± 0.5	0.80 ± 0.02	0.62 ± 0.06	0.83 ± 0	55.1 ± 0
QuEChERS with 20% isopropanol	510 ± 3	315 ± 0	311 ± 0	0.84 ± 0.03	0.60 ± 0	0.84 ± 0	56.0 ± 0.33
80-20 acetonitrile - isopropanol	576 ± 1.7	312 ± 0	308 ± 0	0.58 ± 0.01	0.59 ± 0	0.71 ± 0	49.6 ± 0.15
SWIEET aqueous phase 1	--	317 ± 0	315 ± 1.5	--	--	1.03 ± 0.07	--
SWIEET aqueous phase 2	--	318 ± 0	315 ± 0	--	--	1.01 ± 0	--
pure acetonitrile	626 ± 2.9	307 ± 1	308 ± 0	0.35 ± 0	0.34 ± 0.03	0.70 ± 0	45.6 ± 0.21
pure isopropanol	592 ± 1.5	312 ± 0	304 ± 0	0.63 ± 0.01	0.76 ± 0	0.52 ± 0	48.3 ± 0.12
Mix A*: 5% iPr, 20% H_2O	523	314	310	0.79	0.60	0.79	54.7
Mix B*: 10% iPr, 20% H_2O	523	314	310	0.79	0.60	0.79	54.7
Mix C*: 15% iPr, 20% H_2O	525	315	310	0.78	0.64	0.79	54.5
Mix D*: 5% iPr, 15% H_2O	529	314	310	0.76	0.60	0.79	54.0
Mix E*: 10% iPr, 15% H_2O	531	314	310	0.75	0.60	0.79	53.8
Mix F*: 15% iPr, 15% H_2O	532	314	310	0.74	0.60	0.79	53.7
Mix G*: 5% iPr, 25% H_2O	520	315	310	0.81	0.64	0.79	55.0

Mix H*: 10% iPr, 25% H_2O	520	315	311	0.78	0.60	0.84	55.0
Mix I*: 15% iPr, 25% H_2O	522	315	310	0.80	0.64	0.79	54.8
Mix J*: 5% iPr, 30% H_2O	516	315	311	0.81	0.60	0.84	55.4
Mix K*: 10% iPr, 30% H_2O	520	315	311	0.78	0.60	0.84	55.0
Mix L*: 15% iPr, 30% H_2O	524	316	312	0.73	0.60	0.88	54.6
Mix M*: 5% iPr, 35% H_2O	514	315	312	0.79	0.56	0.88	55.6
Mix N*: 10% iPr, 35% H_2O	514	315	311	0.82	0.60	0.84	55.6
Mix O*: 15% iPr, 35% H_2O	517	316	312	0.77	0.60	0.88	55.3

4.5 Discussion

In this study, we compared the phase composition and Kamlet-Taft parameters for organic phases in QuEChERS and SWIEET. In addition, we investigated different extraction media in SWIEET double extractions [84] varying glucose and isopropanol content to understand their relevance for analyte recoveries. For promising extraction media, the water content of the organic phases of both extraction steps was determined by Karl Fischer titration and the isopropanol content was determined by NMR spectroscopy to gain insight into the differences in the phase composition in the two extraction steps with their different recoveries especially for polar and charged analytes [84]. Furthermore, solvatochromic parameters of the phases are determined to provide information about polarity and specific solvent-solute interactions and are compared to the parameters of known mixtures.

The experiments with indigo carmine indicated a lower water content in the second organic phase in the SWIEET double extraction. For QuEChERS extracts, the water content was the lowest of all experiments. This was confirmed by NMR and Karl Fischer titration experiments, the latter with precise numbers [114]. These findings are in line with the lower values of the π* parameter in the second aqueous phase compared to the first. Comparing SWIEET extraction steps, no direct correlation of the recovery for single analytes, analyte groups (polar and nonpolar) or the overall average recovery to the water content was observed. In contrast, an elevated water content might play a role regarding the increased recovery with SWIEET extractions compared to QuEChERS extractions, but the database is not yet sufficient for a clear explanation. The recovery ratios of the first and second extraction step for single analytes with different extraction media show that isopropanol and glucose concentrations can be adjusted regarding the polarity of the target analyte with extraction media evoking higher phase ratios being of interest especially for polar analytes, whereas for nonpolar analytes, high glucose and lower isopropanol content seem advantageous and may allow to omit the second extraction step. The small ratios present for many analytes with 20% isopropanol and 2 M glucose and even more for 10%

isopropanol and 1.5 M glucose point to the relevance of the phase ratio in the second extraction step.

We regard the water content not to be sufficient as a sole reason for explaining the increased recoveries for polar analytes in SWIEET compared to QuEChERS. We therefore hypothesize that besides the difference in the water content, the addition of the protic solvent isopropanol enhanced the solubilization and thus recoveries of polar and charged analytes, especially when the water content was relatively low. This is due to the ability of isopropanol to form hydrogen bonds in contrast to the aprotic acetonitrile. The increased isopropanol content in the second organic phase supports this hypothesis. As a measure to quantify the hydrogen-bond donating and accepting capacities of the organic phase, Kamlet-Taft parameters were determined. Although the organic mixtures added to QuEChERS and SWIEET both mainly consist of acetonitrile (100% and 80%), the resulting organic phases exhibited different hydrogen-bond donating and accepting capabilities. In SWIEET, isopropanol is added to the organic extraction mixture, which increases the hydrogen-bond donating and accepting capabilities of the resulting organic phase after phase separation beside the effects exerted by the water content. However, the parameters α and β for the organic phases of both SWIEET extraction steps and QuEChERS did not differ significantly and no correlation to analyte recoveries was seen.

In contrast, strong differences between QuEChERS and SWIEET were observed in the parameter $E_T(30)$, as well as between the two extraction steps in SWIEET double extraction. Using Reichardt's Dye to determine $E_T(30)$ proved that the overall polarity was lower in the QuEChERS organic phase than in the two SWIEET organic phases. In addition, the overall polarity was highest in the first organic phase and slightly lower in the second in SWIEET. The higher isopropanol content did not significantly influence the Kamlet-Taft parameters. The value for the combined SWIEET organic phases was between the ones for the separate first and second, which is expected since it is a mixture of both. All mixtures of acetonitrile and water (plus isopropanol in case of SWIEET) investigated yielded higher $E_T(30)$ values than neat acetonitrile and would be classified as protic solvents (47-63 kcal/mol). Langhals [108] observed that a small amount of a polar solvent added to a nonpolar solvent has an unproportionally strong effect on the polarity of the mixture. $E_T(30)$ measurements of mixtures in literature also revealed that the parameter is not always linearly related to the solvent ratio due to selective solvation of the dye [85]. In ternary mixtures, this relation is even more complex and antagonistic effects may be present. This might explain, why the three different SWIEET organic phases (the combined and two separate phases) showed relatively similar $E_T(30)$ values despite their significantly different composition as determined by NMR and Karl Fischer titration.

Comparing the extractions with different additive compositions, it seems that higher initial glucose concentrations are not automatically linked to higher contents of glucose in the organic phase as with 2.5 M glucose in the extraction medium (10% isopropanol) the glucose concentration in the organic phase was 97 and 93 mM, whereas in the combination 2 M glucose with 20% isopropanol, we estimated a concentration of 310 and 180 mM of glucose in the first and second step. For glucose concentrations of 2 M, the isopropanol content caused significant differences (ANOVA/t-test) especially for polar and charged analytes like metformin, EMI and acesulfame. These analytes profited most from a higher isopropanol content due to its ability to form H-bonds. It thus seems that the

isopropanol content is decisive for the amount of glucose present in the organic phase, but not so much the amount of glucose for the isopropanol content. This would mean that the polar glucose just like polar analytes is better extracted at higher isopropanol content, but does not define the extractability of the analytes.

To better judge the differences in the $E_T(30)$ values, we established a scale for various ternary mixtures of the three solvents isopropanol, acetonitrile and water over a relatively wide range of compositions (see Table 7). $E_T(30)$ values covered a range of only 53.7 to 55.6. All organic phases revealed values within this range (SWIEET 1 and 2: 55.4 and 54.9 kcal/mol vs. QuEChERS 54.2 kcal/mol). Interestingly, the value for QuEChERS strongly increased to 56.0 kcal/mol when adding 20% isopropanol to the organic mixture prior to extraction indicating a relatively strong effect of isopropanol. From the mixtures of the solvents A to O (see Table 7), a clear increase of the $E_T(30)$ parameter upon a decreasing isopropanol and increasing water content was observed (see Fig. 16). This trend was also visible comparing the organic phases after extraction: From Karl Fischer titration we know the water content of the organic phases of SWIEET (organic phase 1: 30.4%, organic phase 2: 22.4%) and QuEChERS (10.9%), as well as the isopropanol content from NMR (organic phase 1: 6%, organic phase 2: 8%), which is in line with the trend for the parameter $E_T(30)$, albeit no linear correlation was observed.

This may be due to effects by the salts used in QuEChERS: For QuEChERS, the $E_T(30)$ parameter of the organic phase after extraction increased by 8.6 kcal/mol compared to neat acetonitrile. Similarly, a strong increase by 6.4 kcal/mol is seen between 80-20 acetonitrile-isopropanol vs. the QuEChERS organic phase with isopropanol. This might not only be due to the water content, but also halochromism: During QuEChERS extraction, salts are added to induce phase separation which will partly partition into the organic phase. Reichardt's Dye was reported to be cationic halochromic, meaning its absorption maximum is sensitive especially to cations in an electrolyte and a hypsochromic shift (blue shift, higher excitation energy) can be observed [86]. This shift was shown to be more intense after the addition of cations with high charge density [86], such as Mg^{2+}, which is also used in QuEChERS extractions. Summarizing, an increase in polarity/polarizability seems to be relevant for the differences between SWIEET and QuEChERS but not for the differences between the steps in SWIEET double extractions.

Overall, neither water content, nor isopropanol content, nor glucose concentration, nor polarity of the organic phases were able to satisfactorily explain the increased recoveries in the second step in SWIEET double extractions at 20% isopropanol and 2 M glucose, compared to the first step. The high recoveries of analytes obtained using this combination seems to be dominantly based on the very high phase ratio of the organic:aqueous phase in the second SWIEET extraction step in combination with a higher similarity between the organic and aqueous phase indicated by all parameters investigated. The increased phase volume of the second organic phase can be explained by the principle of the lever in miscibility gaps: Due to a fraction of water being removed with the organic phase in the first step and only organic solvent being added for the second extraction, the amount water available during phase separation in the second step is lower than in the first. This shift in the overall composition towards organic solvents lengthens the lever arm in the phase diagram to the phase rich in organics, and thus its volume increases and thus also the phase ratio. The phase ratio directly influences recoveries, as shown in [84]. Clearly, the second organic phase must contain a higher fraction (but not concentration) of the total

amount of water than the first, since the aqueous phase volume decreases from 2.5 to 1.5 during the second step and the organic phase volume increases to 3.5 mL, beyond the volume of extraction medium added (2.5 mL), see Fig. 13h. We presume that this shift in the phase ratio is crucial to improve the recoveries in the double extraction.

A reason why this effect on the phase ratio is only observed at the highest isopropanol content may lie in the structure of isopropanol-water clusters: When the isopropanol content in water is increased, a change in the hydrogen bonding network is observed at a mole fraction of 0.2 isopropanol and the conformation of the isopropanol-water clusters changes [115]. Guo et al. showed that isopropanol clusters with five water molecules form the most stable hydrogen bonds [116]. In the second SWIEET extraction step, the addition of organic mixtures led to an increased isopropanol content of 6 vs. 8% as determined by NMR. This might pull water into the organic phase to result in more stable isopropanol-water clusters. A similarly intense shift in phase volume was not observed at glucose concentrations higher than 2 M. It can be anticipated, that an increased glucose concentration leads to a competition with isopropanol for the water molecules, as they are required for solubilization, based on an excluded volume effect [75]. At high glucose concentrations in water, clustering of glucose molecules was observed on a microscopic level [117]. Glucose-glucose interactions increase when water-water interactions decreased, therefore less water will be available for the solvation of isopropanol in the organic phase. As we described previously [84], the permittivity decreases in higher concentrated glucose solutions [78], which also aids extraction of polar analytes due to a decreased solubility in the aqueous phase. A further increase of the isopropanol content is hypothesized to also increase recoveries, but would result in instable phase separations due to increased miscibility. Even if a phase separation may be reached, robustness will be lowered, especially in case of samples with a higher matrix load. Similarly, at glucose concentrations lower than 1 M, no stable phase separation was obtained with isopropanol present. Thus, a combination of 2 M glucose and 20% isopropanol proved best for extractions in SWIEET.

To conclude, we found that a complex counterplay between phase composition and phase ratio is decisive for the recoveries achievable in liquid-liquid extractions. For the difference in recoveries of polar analytes in SWIEET vs. QuEChERS, the water content and the addition of isopropanol were significant. The difference in recoveries between the first and second organic phase of SWIEET double-extractions can be explained by the higher phase ratio present in the second step. This understanding can be used to tune the SWIEET method parameters to fit specific applications if a specific group of analytes is of interest: Depending on the sample type and target analytes characteristics, phase polarity and ratio can be optimized by the amount of glucose and isopropanol added to improve recoveries and separation from matrix components. High isopropanol concentration can be expected to aid in the extraction of highly polar and also charged analytes, whereas a high glucose concentration will aid in reaching high extraction efficiencies for less polar analytes. A major conclusion from this fundamental study is that SWIEET is better suited for analytes solubilized via hydrogen bonding such as charged analytes than QuEChERS.

5 Miniaturization of the SWIEET extraction method enables the analysis of the pesticide carbendazim in *Chironomus riparius* midges after metamorphosis

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5.1 Abstract

For the analysis of biota samples, as for example organisms with aquatic life stages, miniaturization of common extraction methods is necessary. For QuEChERS extractions, miniaturization down to few individual organisms was reported for invertebrates. Our salt-free SWIEET extraction method recently developed as an alternative to QuEChERS was originally optimized for a total volume of 5 mL. To enable the determination of carbendazim loads in midges from exposure experiments, that weigh between 0.3 and 0.5 mg, the method required miniaturization. Using a model analyte mix, we investigated how reducing the total extraction volume affected analyte recoveries. Although recoveries declined significantly with decreasing extraction volume, the reduction was less pronounced for SWIEET extractions compared to QuEChERS extractions, which we conducted for comparison. The miniaturized SWIEET extraction was then applied to adult midges of *Chironomus riparius* from an exposure experiment at the larval stage to establish the analytical protocol for the determination of carbendazim. Since matrix effects played a significant role in the extraction of carbendazim, particularly for samples from the lower exposure concentrations, sample preparation and calibration modes had to be further optimized. With the final protocol, we were able to successfully extract carbendazim from single midges. The effects of pooling midges were investigated to better understand matrix effects and effects on robustness. With the optimized and miniaturized method, we achieved repeatable results, making it suitable for determining carbendazim loads in midges and correlating them to the exposure concentrations.

5.2 Introduction

For the analysis of micropollutants in biota with aquatic lifestages such as *Chironomus riparius* (Meigen), the main challenges arise from the impact of matrix components and the small sample size. The extraction of persistent trace organic contaminants from biota was extensively reviewed by Fidalgo-Used et al. [118]. They mainly discussed classical solid-liquid extraction techniques and additional clean-up steps for the removal of matrix components. Methods like Soxhlet extraction, supercritical fluid extraction or pressurized liquid extraction require special instrumentation and have a high energy consumption. Liquid-liquid extraction methods, which mostly do not require additional instrumentation, are a simple alternative for the extraction of biota samples, as reviewed by Knoll et al. [76]. The miniaturization of liquid-liquid extraction techniques was reviewed recently by Kannouma et al. [119] and da Silva Burato [120]. Techniques like single drop microextraction, hollow-fibre microextraction or dispersive liquid-liquid extraction can produce highly concentrated solutions of the analytes of interest in a small volume, but are mainly used for large volume liquid samples and therefore they are not suitable for the extraction of small biota samples.

An approach often chosen is to miniaturize regular liquid-liquid extraction techniques already established for larger sample amounts in food and environmental analysis. The

QuEChERS method and its variants, for example, have widely been adapted and often miniaturized to reduce food waste and chemical waste production. Andreu et al. [121] reviewed the analysis of pesticides in biota and compiled many applications of the miniaturized QuEChERS method. The lowest sample amount discussed in this review were 1.5 g of honey. More recent publications on the miniaturization of QuEChERS further decreased the sample size and demonstrated the extraction of 300 mg of baby food [122], 100 mg of food by-product ethanolic extracts [123] and 200 mg of herbs [124].

In biota analysis, often even smaller sample sizes are available, especially of invertebrates, necessitating miniaturization. Stöckelhuber et al. [31] had a comparably large sample size of 500-1000 mg of a mixture of insects, snails and spiders, from which they extracted pesticides with micro-QuEChERS. A similar sample size (500 mg) was used by Montemurro et al. [125], who compared different QuEChERS variations for the extraction of pollutants from earthworms. For some applications, further miniaturization in the µg range was necessary: Kurth et al. [126] conducted micro-QuEChERS with 10 zebrafish embryos exposed to 46 volatile hydrophobic organic pollutants with a total volume of the extraction medium of 140 µL, achieving recoveries in the range of 63-148%. For the analysis of fluoxetine and carbamazepin in snails, Berlioz-Barbier et al. [41] extracted a single gastropod using 550 µL extraction medium for micro-QuEChERS with recoveries of over 85%. They also developed a micro-QuEChERS workflow with a citrate buffer and hexane additionally to 500 µL water and 500 µL acetonitrile for the extraction of pollutants from single organisms of *P. antipodarum* and *G. fossarum*, as well as three to four pooled individuals of *C. riparius*, equivalent to 12 mg wet weight achieving 40 to 98% recovery [34]. Wicht et al. [36] also used QuEChERS for the extraction of carbamazepin from adult midges and larvae of *C. riparius* with sample sizes of about 25 mg, achieving recoveries of 95%. For the extraction of single midges, even smaller sample sizes are needed. However, QuEChERS is limited due to the obligatory use of salts to induce a phase separation between acetonitrile and water: One dried midge weighs between 0.3 and 0.5 mg. If the ratio of salts to solvent is to be kept, the very small amounts of salts are difficult to weigh and handle, likely inducing errors and therfore lower repeatability.

Carbendazim is a fungizide widely applied. Due to its application in agriculture for example, it can be detected not only in food samples, but also environmental samples [127]. Concentrations in surface waters were reported to be between 10 and 50 ng/L [128]. Its approval as plant protection agent was not renewed in 2014 in the EU due to its suspected carcinogenic properties. Other carbendazim sources include its use as a preservative in fiber and leather, as well as construction materials for facades [128]. For this application, carbendazim use was approved until the end of January 2025. The impact of carbendazim on aquatic invertebrates was already investigated by van Wijngaarden et al. in 1998 [129], as well as Cuppen et al. in 2000 [130]. For the determination of the body burden of various substances including carbendazim in gammarids, Könemann et al. [131] used the QuEChERS extraction method described by Inostroza et al. [37]. Up to 0.56 ng/g carbendazim per wet weight were found in gammarid tissue exposed to waste water treatment plant effluent.

In our previous work [84], we developed SWIEET (sugar water isopropanol ethyl nitrile extraction technique), a salt-free liquid-liquid extraction method. In this method, glucose is added to a water-acetonitrile mixture to induce phase separation instead of salts as in

QuEChERS. The addition of the protic solvent isopropanol aided in improving the recoveries of polar and charged analytes. The method proved to be applicable to a broad range of environmental and food samples and we achieved high recoveries especially for polar analytes, while keeping recoveries for nonpolar analytes high as well [84]. Since no solids are added to the extraction mixture and all extraction solvents can be pipetted, miniaturization to the µL scale was hypothesized to be possible and investigated in this work. *C. riparius* exposed to carbendazim at the larval stadium were chosen in this study to investigate the transfer of the pesticide to adult midges upon metamorphosis. For this, the fungizide is ideally analyzed on the level of single individuals. The aim of the study is thus to miniaturize SWIEET for small sample sizes down to individual organisms of 0.3-0.5 mg.

5.3 Materials and Methods

5.3.1 Chemicals

1-Ethyl-3-methyl-imidazolium (EMI, ≥95%), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 5-amino-2-naphthalene sulfonic acid (ANSA, ≥95%), acesulfame (ACE, ≥99%), acridine (ACR, 97%), *α-D*-glucose (96%), carbendazim (97%), clarithromycin (CLA, ≥98%), dichloromethane (DCM, 99.9%), diclofenac sodium salt (DIC, ≥98 %), isopropanol (iPr, LC-MS grade), naphazoline (NAPHA, ≥98%), pindolol (PIN, 98%) and saccharine (SAC, ≥98%) were purchased from Sigma Aldrich (Steinheim, Germany). 4-Hydroxybenzoic acid (HBA, ≥98%) and *N*-diethyl-m-toluamide (DEET, ≥98%) were from Fluka (Buchs, Switzerland). Acetonitrile (MeCN, LC-MS grade) and formic acid (>99%) were bought from Roth (Karlsruhe, Germany). Metformin (MET, 97%) was from Alfa Aesar (Haverhill, MA, USA). Purified water produced using a PURELAB Classic PL5241 (ELGA LabWater, Celle, Germany) was used throughout the analytical workflows.

5.3.2 Exposure experiment

Exposure to carbendazim (CAS 10605-21-7; Sigma-Aldrich, St. Louis, USA; analytical standard purity) was conducted as a life-cycle test with *Chironomus riparius* (Meigen) according to the OECD guideline 233 [132] with a spiked sediment approach. Six concentrations, a negative control (NC) and solvent control (SC) were tested, with six replicates each. The nominal concentrations were corrected after quantification of carbendazim in sediment according to a detection measurement with LC-MS at the facilities in Frankfurt.

In brief, quartz sand was spiked with carbendazim diluted in acetone. The solvent was evaporated overnight and the spiked sand was mixed into the artificial sediment, which consisted of sand (Baumit, Bad Hindelang, Germany), clay (Hans Wolbring, Höhr-Grenzhausen, Germany), peat (SonnenMoor, Anthering, Austria) and $CaCO_3$ (VWR, Leuven, Belgium) according to the guideline standards. The sand for the NC was not spiked, SC sand was mixed with pure acetone and evaporated overnight. Sediment and M7 medium (composition according to the OECD guideline [132]) were inserted into 600 mL glass beakers and aerated for 48 h before introducing 20 first instar larvae each. Adult midges of three replicates of the same treatment were transferred to breeding cages for mating and oviposition. The dead midges were collected, dried in an oven at 40 °C for 2 h and stored in falcon tubes. Breeding of *C. riparius* was conducted under the same conditions as the experiment, at 21 °C room temperature, a light dark cycle of 12 hours and ground TetraMin (Tetra, Melle, Germany) as food.

To measure the exact concentrations of carbendazim in the experiment, the sediment was Soxhlet extracted with methanol for 24 h, based on the methods used in [133]. Ultrahigh-performance liquid chromatography (UHPLC) (Vanquish Flex Thermo Fisher Scientific, Waltham, MA, USA) and high-resolution hybrid quadrupole-Orbitrap mass spectrometry (Q-Exactive Orbitrap MS Thermo Fisher Scientific, Waltham, MA, USA) were used for detection with heated electrospray ionization in positive mode. A calibration curve was generated by dissolving carbendazim in acetonitrile and preparing a dilution in M7 medium.

The recovery rate for carbendazim from sediment was above 120% for all measured treatments: therefore, a mean recovery rate of 139.4% was used to calculate the actual exposure concentrations for the treatments that were not measured (Table 8).

Table 8 Nominal concentrations spiked to the substrate and measured/calculated concentrations for the different treatments detected with UHPLC-HRMS. NC = negative control, SC = solvent control.

Nominal concentration in treatment [mg/kg]	Detected (*calculated) concentration [mg/kg]
NC	0
SC	0*
0.25	0.36
0.5	0.7*
1	1.39*
2	2.79*
4	5.58*
8	10.94

5.3.3 Extraction procedure and LC-MS methods

5.3.3.1 Miniaturization

For the miniaturization of the SWIEET extractions, 2 M aqueous glucose solution was spiked with an analyte mix of 13 model analytes to a final concentration of 3 mg/L each to determinate recoveries. To 0.25, 0.5, 1.25 or 2.5 mL of this analyte mix, the same volume of an organic extraction mixture consisting of 80 vol.% acetonitrile and 20 vol.% isopropanol was added [84]. The mixture was homogenized for 1 min using a vortexer. After a clear phase boundary was visible, usually within a minute, phases were separated by pipetting. For double-extractions, 0.2, 0.4, 1 or 2 mL fresh organic extraction mixture consisting of 80 vol.% acetonitrile and 20 vol.% isopropanol were added to the residual aqueous phase from the first extraction step. After mixing for 1 min using a vortexer, the phases were allowed to separate again and the organic phase was sampled and combined with the organic phase of the first extraction step for analysis.

QuEChERS extractions were conducted as described by Kalinke et al. [84]. Briefly, 0.25, 0.5, 1.25 or 2.5 mL acetonitrile as well as 0.01, 0.125, 0.05 or 0.25 mg NaCl and 0.025, 0.2, 0.5 or 1 mg $MgSO_4$ were added to 0.25, 0.5, 1.25 or 2.5 mL water respectively, spiked with the model analytes, to induce phase separation.

The combined organic extracts were analyzed using a gradient elution RPLC coupled to a Q-ToF-MS. Details on the model analyte mix, RPLC-MS method and quantification can be found in Kalinke et al. [84]. Recoveries were determined for the combined extracts and data are reported for single analytes and average values for all 13 analytes.

5.3.3.2 Extraction of carbendazim from adult midges of C. riparius

For the extraction of midges, 1-9 midges were weighed and homogenized with a pestle. 0.25 mL of a 2 M glucose solution and 0.25 mL of an acetonitrile-isopropanol mixture (80-20 vol%) were added. The mixture was vortexed for one minute and then centrifuged at 13,000 rpm for 10 minutes. When both phases formed were clear, the organic phase was separated by pipetting and 0.2 mL fresh acetonitrile-isopropanol (80-20 vol%) mixture were added to the aqueous phase for the second extraction step. The mixture was vortexed for another minute and phases were separated after centrifugation. 100 µL of the combined and mixed organic extracts were transferred to an LC vial insert, evaporated to dryness under a stream of nitrogen and dissolved in 20 µL water prior to LC-MS analysis. For quantification, matrix-matched calibration was conducted: Extracts from midges of biological blanks were prepared as described above and spiked with carbendazim at concentrations of 0.01, 0.05, 0.1 and 0.25 µg/L. For the determination of carbendazim loads per mass in midges, a correction via the recoveries of 86% was made (see Section “5.4.2 Application to *Chironomus riparius* midges from exposure experiments”).

The LC-MS method was adapted for the analysis of carbendazim in midge extracts. The samples were analyzed by RPLC-Q-ToF-MS. 5 µL of the sample were injected onto a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5 µm, Agilent Technologies) equipped with a Zorbax Eclipse Plus C18 guard column (2.1 x 12.5 mm, 5 µm, Agilent Technologies) in a 1260 Infinity LC-system coupled to a Revident Q-ToF (Agilent Technologies, Waldbronn, Germany and Santa Clara, CA, USA). A gradient of water with 0.1% formic acid (Eluent A) and acetonitrile with 0.1% formic acid (Eluent B) was used for separation at a flow rate of 0.3 mL/min. The starting conditions of 95% A were decreased to 30% A over 1 min. This composition was held for 5 min and then increased back to 95% over 0.5 min. A jet-stream electrospray ionization source was used with the nebulizer pressure set to 35 psi, drying gas temperature to 160 °C, drying gas flow rate to 13 L/min, fragmentor voltage to 100 V, capillary voltage to 3000 V, sheath gas temperature to 325 °C and sheath gas flow rate to 11 L/min. Spectra were acquired at a rate of 2 spectra/s in the mass range of 80-1200 m/z. For internal calibration, acetonitrile/water (95/5) solutions of purine and HP0921 (Agilent Technologies) were introduced through a reference sprayer.

Quantification was achieved with matrix-matched calibration during method optimization and for biota samples as described in Kalinke et. al [84]. The use of a deuterated internal standard was not successful due to large differences in the peak areas observed. ^{13}C labelled standards for carbendazim were not commercially available.

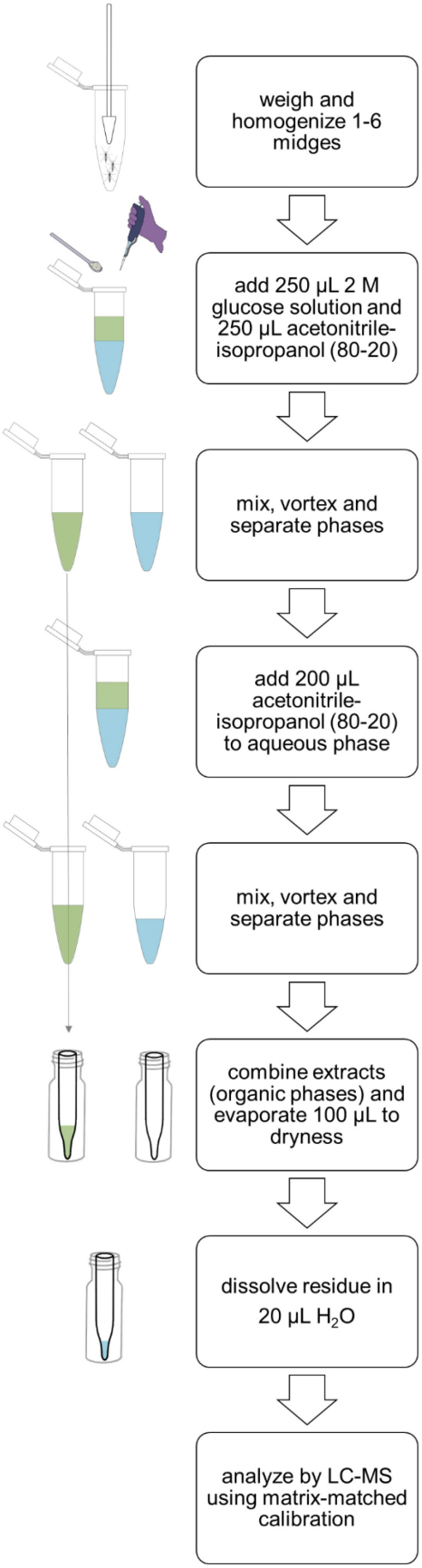

Fig. 17 Flow chart of the final miniaturized SWIEET extraction protocol for the analysis of carbendazim in 1-6 midges from *Chironomus riparius*.

5.4 Results and Discussion

5.4.1 Miniaturization of the SWIEET method

The original SWIEET extraction method [84] was developed with a total volume of 5 mL, consisting of equal volumes of an aqueous sample and an organic solvent mixture (acetonitrile-isopropanol 80-20 vol%). For midges as sample, miniaturization was necessary to prevent excessive dilution of the target analyte carbendazim. One midge weighs between 0.3 and 0.5 mg, so in order to keep solid:liquid ratios, the volume of the extraction medium needs to be decreased accordingly. Preliminary experiments showed that at total volumes of 1, 0.5 and 0.25 mL, phase separation and handling were well possible. However, at 0.25 mL total volume, errors were expected to be too large, due to difficulties in separating the phases by pipetting. Therefore, the effect of the extraction volume on recoveries was investigated with total volumes of 5, 2.5, 1 and 0.5 mL. Optimization was conducted with a mix of 13 analytes [84]. For comparison, classical QuEChERS extractions were conducted with the same total volumes.

Results are summarized in Fig. 18. With decreasing extraction volume, average recoveries decreased from 61% using 5 mL to 46% using 0.5 mL for SWIEET extractions. This might be due to difficulties and inaccuracies that come with handling smaller volumes. Since the phase volume is smaller compared to the contact surface of the vial, adsorption might also be more significant than at larger volumes. For QuEChERS extractions, the same volumes were tested and the same trend was visible, but with a stronger decrease of recoveries from 33% to 18%. With SWIEET, overall higher recoveries were achieved than with QuEChERS for all volumes, which corroborates our previous findings [84]. With both methods, recoveries decreased by 15 percentage points on average, but this means a relatively stronger decrease for QuEChERS (-45%) than for SWIEET (-25%). Losses for polar and nonpolar analytes were similar for QuEChERS. For SWIEET, polar analytes (logD<0) were slightly more (-27%) affected than nonpolar (logD>0) analytes (-24%). This indicates that no specific adsorption took place. The same reaction tubes were used for the total volumes 5 and 2.5 mL as well as 1 and 0.5 mL. For larger total volumes, a larger contact surface between the phases might also increase phase transfer rates and therefore recoveries.

A total volume of 0.5 mL was finally chosen for the extraction of midges. Higher extraction volumes promised higher recoveries, but for real samples with a very low expected analyte load as in the *C. riparius* samples in this study, dilution should be kept to a minimum to reach concentrations in the extracts above the LOQ.

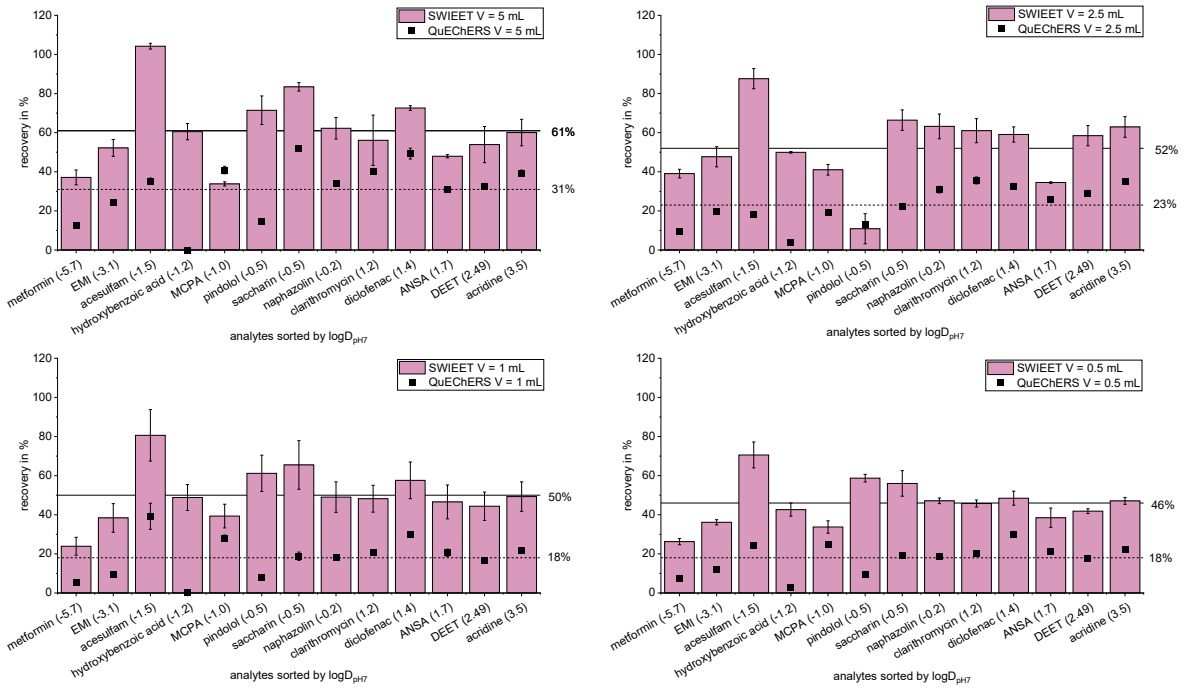

Fig. 18 Average recoveries (n = 2) of the 13 model analytes (3 mg/L) from SWIEET double-extractions from water at different total extraction volumes (5, 2.5, 1 and 0.5 mL), as well as QuEChERS extractions for comparison (for details, see Section “5.3.3 Extraction procedure and LC-MS methods”), determined by LC-MS (see Section “5.3.3 Extraction procedure and LC-MS methods”). Horizontal lines indicate the average recoveries of SWIEET (solid line) and QuEChERS (dashed line) extractions.

5.4.2 Application to *Chironomus riparius* midges from exposure experiments

To test the applicability of the miniaturized SWIEET method to midges, the 13 analytes of the mix were spiked to 3 individual biological blank midges. In each of the experiments, only male or female animals were used. Recoveries are shown in Fig. 19. Recoveries were significantly higher than in the previous extractions from ultrapure water. This indicated strong matrix effects, specifically, ion enhancement. Matrix-matched calibration was conducted, which was supposed to correct for matrix effects, however the samples will never be identical in composition to the calibration samples when using these few individuals.

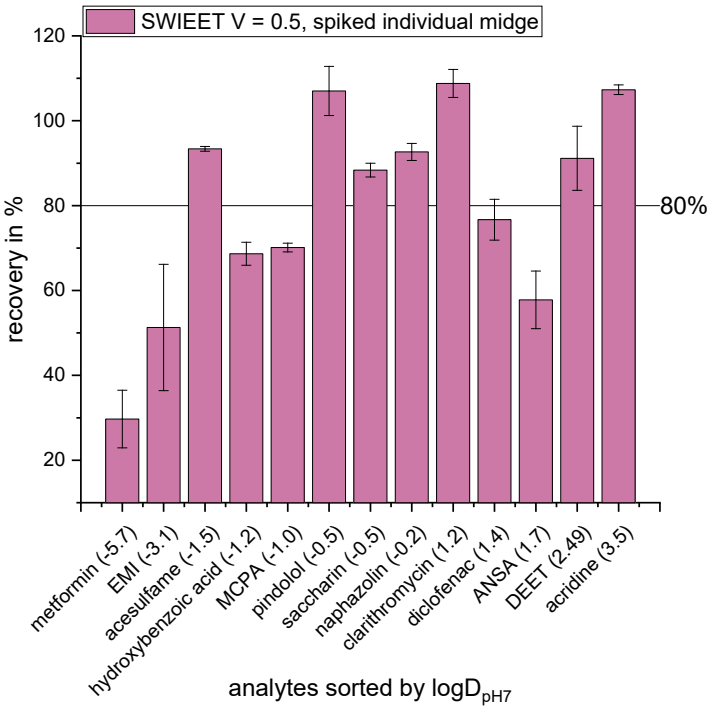

Fig. 19 Recoveries of the 13 model analytes extracted with SWIEET double-extractions with a total volume of 0.5 mL from spiked (3 mg/L) individual biological blank midges determined by LC-MS. The horizontal line indicates average recovery.

To investigate the applicability of the miniaturized SWIEET extraction method to the determination of carbendazim in midges of *Chironomus riparius* exposed to this pesticide during their larval life stage, recoveries and matrix effects were assessed using three pooled midges from biological blanks, spiked with 1 ng carbendazim. Average recoveries of 86% were reached for carbendazim. However, the sample preparation had to be adjusted to reduce dilution: Instead of diluting the extract with methanol for the sample to be better compatible with the eluent, which decreased the carbendazim concentration to values below the LOD, an aliquot of the extract was evaporated and the residue dissolved in a smaller volume of water to increase the concentration in the injection sample and enhance large volume injections.

In these pre-studies, we found that carbendazim spiked at 1 ng could be detected in extracts of single individuals of midges from an exposure experiment. The extraction of single midges being exposed at a concentration of 0.7 mg/kg can provide insight into the variation in carbendazim body burden between individual midges but concentrations were too close to the LOD and thus statistical variations were high. Comparative experiments were thus conducted with male midges that were exposed to 0.7 mg/kg carbendazim in sediment. Three replicate extractions were conducted with 3 pooled midges each and compared to nine parallel extractions of individuals. Each extract was measured three times with LC-MS. For quantification, best practice would be to use an isotope labelled internal standard. However, in the case of carbendazim, only deuterated standards were commercially available. Preliminary experiments showed, that this deuterated standard produced significantly smaller peak areas than the unlabelled substance at the same concentration, making it unsuitable as an internal standard. Therefore, matrix-matched

calibration had to be used for quantification. To account for the dependence of matrix effects on the number of individuals pooled, LOD and LOQ were determined for every calibration curve. In the first experiment, a matrix-matched calibration based on spiked extracts of three pooled midges not exposed to the pesticide (biological blank = negative/solvent control) was used for extracts of both pooled midges and individuals. As shown in Fig. 20a, the concentrations per mass between the pooled and individual midges differed by a factor of 2.5. On average, for 3 pooled midges the average carbendazim concentration was 4 ng/g and for individual midges 9.8 ng/g (see Fig. 20a). The main reason for this discrepancy were surely matrix effects as matrix components were concentrated in the evaporation step together with the analyte. The higher matrix load in pooled samples can impair the analyte signal and therefore decrease the response, which leads to a lower calculated concentration.

To better take into account these different matrix effects, measurements were repeated with adapted matrix-matched calibrations: For the extraction of individual midges, calibration samples were also prepared with an individual midge from biological blanks and for the pooled samples, three midges were pooled for calibration. As expected, the two calibration curves (Fig. 20c) varied in their slope and y-intercept, proving that adapted matrix-matched calibrations are necessary. Results for internal concentrations are shown in Fig. 20b.

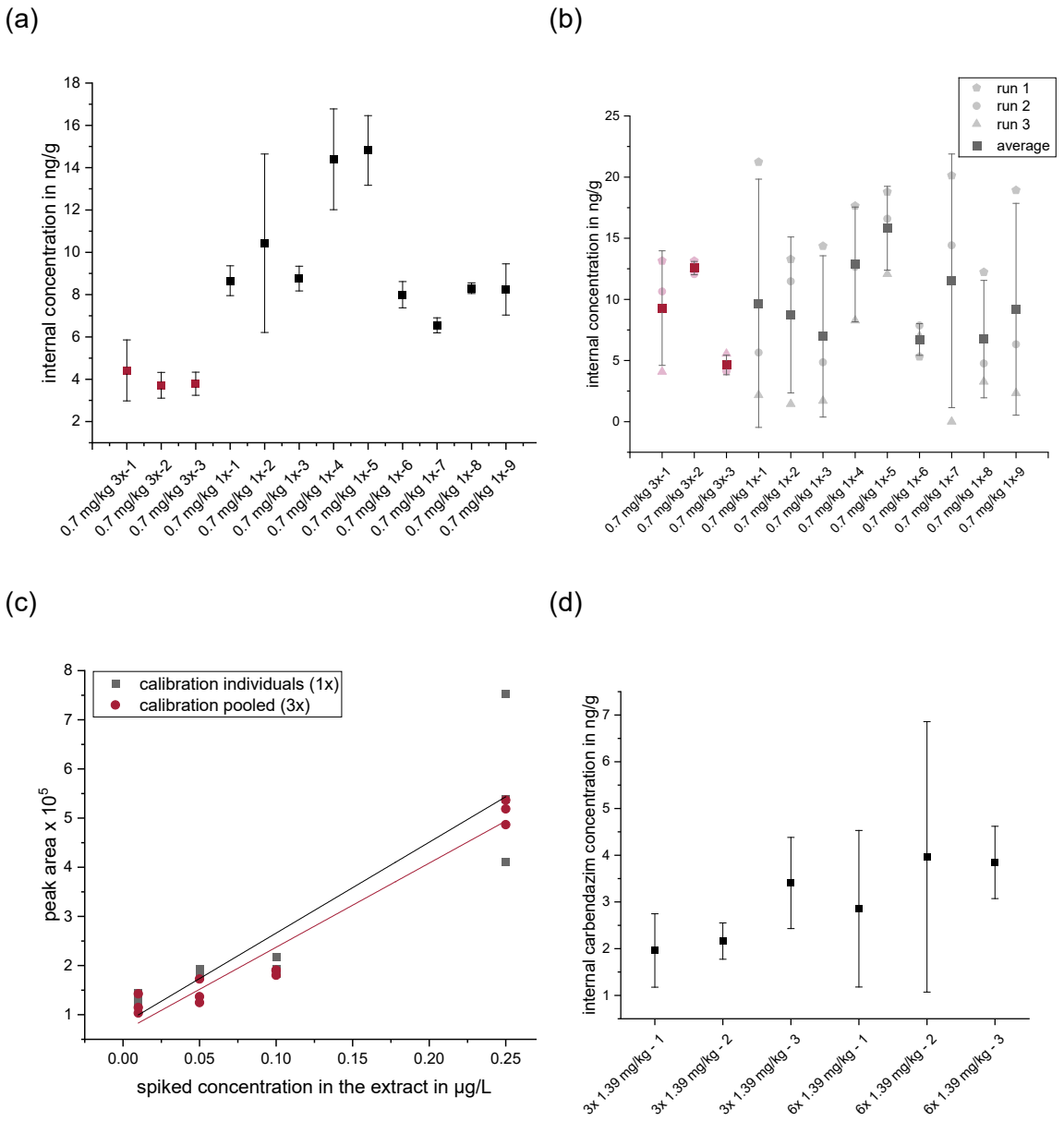

Fig. 20 Carbendazim concentrations in midges after SWIEET extraction (see Section "5.3.3 Extraction procedure and LC-MS methods") of (a) 3 pooled (red) vs. single (black) male midges exposed to a concentration of 0.7 mg/kg. Quantification was made with the same matrix-matched calibration, (b) 3 pooled (red) vs. single (black) midges exposed to 0.7 mg/kg with different matrix-matched calibrations shown in (c): linear fit for calibration with individual midges (black): y = 1849233 x + 81118; R^2 = 0.81, linear fit for calibration with pooled midges (red): y = 1711895 x + 66058; R^2 = 0.95. (d) 3 pooled vs. 6 pooled individuals exposed to a concentration of 1.39 mg/kg and quantified with different matrix-matched calibrations. Each sample was measured in triplicate with LC-MS (for details, see "5.3.3 Extraction procedure and LC-MS methods"), the results from individual runs are shown in lighter colours.

Internal concentrations in the midges (ng/g) were now in the same range regardless whether the midges were pooled (avg. (n = 3) 8.8 ± 4 ng/g) or individuals were extracted (avg. (n = 9) 9.8 ± 3 ng/g). Averages for the pooled extractions ranged from 4.6 to 12.6 ng/g and for the individual extractions from 6.7 - 15.8 ng/g. However, as shown in Fig. 20b, standard deviations were significantly larger for the extraction of individual midges than for the extracts from the pooled midges. This can be expected from the lower absolute carbendazim concentrations in the extracts: The concentration in the extracts

from the individual midge extractions were about three times lower than in the extracts from the pooled midges. With the low exposure concentration (the lowest in the biological experiment), concentrations were close to the LOD, so higher variations in the peak area are expected.

To test whether pooling more midges leads to more reliable results, we tested midges exposed to a concentration of carbendazim of 1.39 mg/kg. We compared results for 6 pooled midges vs. 3 pooled midges. Results are shown in Fig. 20d. In this experiment, two different matrix-matched calibrations based on the same number of individuals were used again. Average concentrations of the 3 pooled (2.5 ng/g) and 6 pooled (3.5 ng/g) still varied, but the differences were not significant (t-test) and within the standard deviation. The high variances are due to the low absolute concentrations in the sample being closer to the LOD of 0.07 µg/L on that day. Even with 6 pooled midges, the carbendazim concentrations were close to the LOQ (avg. S/N = 11), causing an elevated variance.

To quantify the matrix effects, 1, 3 and 6 biological blank midges were subjected to the extraction protocol in triplicates with the SWIEET method to obtain blank extracts. These were spiked to a concentration of 0.25 µg/L (5 pg) carbendazim. The same concentration was set to a reference sample consisting of pure methanol. LC-MS sample preparation was then continued as described above for all samples, including the reference. The matrix effects were calculated for carbendazim as described by Kalinke et al. [84]. The values for all samples tested were negative, indicating ion enhancement. The matrix effects were -264, -276 and -284% on average for 1, 3 and 6 midges respectively (triplicates measured in triplicates, n = 9), indicating very strong matrix effects. If more midges were pooled, no significant changes in peak areas from extractions from 1, 3 and 6 midges were observed (ANOVA). The high matrix effects supported the need for matrix-matched calibrations. Since the variances induced by the different number of midges pooled were insignificant regarding the intensity of the matrix effects, it is hypothesized that variances dure to low concentrations close to the LOD dominated high variances in the calculated internal concentrations. To ensure highest quantitative precision, however, we continued using matrix-matched calibration.

The final method was applied to midges exposed to different carbendazim concentrations during their larval stadium. Female and male midges were analyzed separately, here only the results for the female midges are shown. Results for male midges and generation 2, as well as the biological aspects of these results will be discussed in an upcoming publication.

For the lowest exposure concentration, 6 midges were pooled, for all other exposure concentrations, only 3 midges were pooled. Midges from each concentration were extracted in triplicates. To determine interday reproducibiity, each sample was measured 3 times on 3 consecutive days. The calibration samples were used for the calculation of standard deviations. As expected, repeatability was lowest at the lowest calibration concentration of 0.01 µg/L ($RSD_{interday}$ = 7.4%) and highest at the highest calibration concentration of 0.25 µg/L ($RSD_{interday}$ = 1.6%). As mentioned above, LODs and LOQs were determined from daily calibration curves adapted to the number of individuals pooled. LODs varied between 9 ng/L and 400 ng/L and were 80 ng/L on average, LOQs varied between 0.03 and 1.28 µg/L and were 0.25 µg/L on average. When a clear signal was

detected in replicates, half the LOD of the corresponding batch was used for the calculation of the internal carbendazim concentration.

The relation of the internal carbendazim concentrations of female midges from generation 1 to the concentration in the exposure experiments is shown in Fig. 21. The internal concentration increases with the exposure concentration as expected. Variances were large, due to the low absolute concentrations in the samples, but also due to natural variances in the individuals.

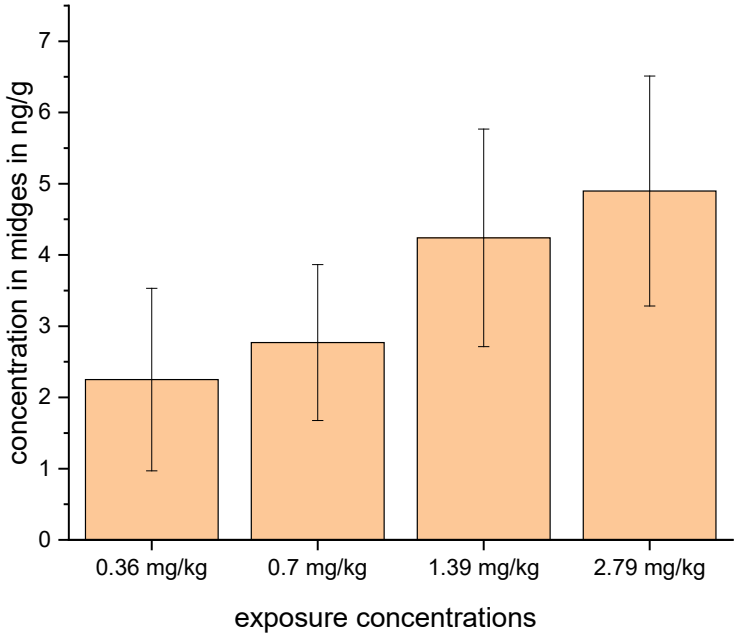

Fig. 21 Internal carbendazim concentration in female midges related to the exposure concentrations. 6 midges were pooled for an exposure concentration of 0.36 mg/kg, 3 midges for all higher concentrations. For the extraction protocol, see Section “5.3.3.2 Extraction of carbendazim from adult midges of *C. riparius*”.

6 Summary and Outlook

In this thesis, a salt-free extraction method was developed, which we called SWIEET. This method was shown to yield similar or higher recoveries for a broad range of analytes compared to QuEChERS. In a screening approach, we tested different possible additives to induce a stable phase separation in the acetonitrile-water system. Glucose yielded highest recoveries and induced a stable phase separation and was therefore used for further optimization. To further improve recoveries, isopropanol was chosen to be added to the mixture. Due to its ability to form H-bonds, recoveries of especially polar analytes increased. In a design of experiment, the content of isopropanol and glucose, as well as temperature were varied simultaneously to find the optimum extraction parameters. Additionally, a series of double-extractions were conducted to further improve recoveries by a second extraction step. Especially polar analytes profited from a second extraction. The final SWIEET method was applied to a wide range of sample types and compared well to or exceeded QuEChERS in recoveries. Matrix effects were similar in both methods. In the future, the application of SWIEET for more sample types can be tested. Especially for liquid samples, SWIEET achieved high recoveries for a broad range of analytes. For WWTP effluent, average recoveries of 75% were achieved, making it suitable for waste-water effluent monitoring. Furthermore, SWIEET should be easily automatable, as all components used for the extraction can be pipetted, and no vigorous shaking is required due to the omission of salts.

To gain a deeper understanding of the physicochemical aspects of the extraction from a two-phase system, the phase compositions of SWIEET and QuEChERS were investigated. Water, glucose and isopropanol concentrations were determined in the phases of QuEChERS and SWIEET double extractions, as well as phase ratios and solvatochromic parameters. We found that high phase ratios and isopropanol concentrations correlated with high recoveries for polar analytes, while unpolar analytes were extracted preferentially at low isopropanol and high glucose concentrations. With this firm understanding of the physicochemical aspects of SWIEET, the method is easily applicable and adaptable for specific analytical tasks.

For the extraction of biota, the SWIEET method was miniaturized. Since no salts are added for the phase separation, SWIEET is miniaturized more easily than QuEChERS, which was shown by the higher recoveries of the model analytes, as well as fewer losses with the decrease of the phase volume. The miniaturized method was applied for the extraction of adult midges from an exposure experiment with carbendazim. Strong matrix effects were observed for the real samples, which we counteracted using matrix-matched calibrations. With the optimized workflow, we were able to extract the pesticide carbendazim from single midges of 0.5 mg for the first time. With slightly larger sample sizes of 3-6 pooled midges, we were able to relate internal carbendazim concentrations in the midges to the exposure concentrations and prove, that carbendazim remains in individuals despite metamorphosis. This can aid in understanding the accumulation of pollutants in higher organisms, as well as the transfer of pollutants from the aquatic to the terrestrial environment. The analysis of single organisms can provide valuable insight into the natural variances in the uptake of pollutants of individuals.

References

1. Nödler K, Licha T, Bester K, Sauter M. Development of a multi-residue analytical method, based on liquid chromatography–tandem mass spectrometry, for the simultaneous determination of 46 micro-contaminants in aqueous samples. J Chromatogr A. 2010;1217(42):6511-21. https://doi.org/10.1016/j.chroma.2010.08.048.
2. Grabic R, Fick J, Lindberg RH, Fedorova G, Tysklind M. Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta. 2012;100:183-95. https://doi.org/10.1016/j.talanta.2012.08.032.
3. Petrie B, Youdan J, Barden R, Kasprzyk-Hordern B. Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. J Chromatogr A. 2016;1431:64-78. https://doi.org/10.1016/j.chroma.2015.12.036.
4. Petrović M, Hernando MD, Díaz-Cruz MS, Barceló D. Liquid chromatography–tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. J Chromatogr A. 2005;1067(1):1-14. https://doi.org/10.1016/j.chroma.2004.10.110.
5. Leendert V, Van Langenhove H, Demeestere K. Trends in liquid chromatography coupled to high-resolution mass spectrometry for multi-residue analysis of organic micropollutants in aquatic environments. Trends Anal Chem. 2015;67:192-208. https://doi.org/10.1016/j.trac.2015.01.010.
6. Bourdat-Deschamps M, Leang S, Bernet N, Daudin J-J, Nélieu S. Multi-residue analysis of pharmaceuticals in aqueous environmental samples by online solid-phase extraction–ultra-high-performance liquid chromatography-tandem mass spectrometry: Optimisation and matrix effects reduction by quick, easy, cheap, effective, rugged and safe extraction. J Chromatogr A. 2014;1349:11-23. https://doi.org/10.1016/j.chroma.2014.05.006.
7. Lehotay S, Schenck F. Multi-residue methods: extraction. Encyclopedia of separation science. 2000:3409-15. https://doi.org/10.1016/B0-12-226770-2/05621-0.
8. Motohashi N, Nagashima H, Párkányi C, Subrahmanyam B, Zhang G-w. Official multiresidue methods of pesticide analysis in vegetables, fruits and soil. J Chromatogr A. 1996;754(1):333-46. https://doi.org/10.1016/S0021-9673(96)00576-6.
9. Anastassiades M, Lehotay SJ, Štajnbaher D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. J AOAC Int. 2003;86(2):412-31. https://doi.org/10.1093/jaoac/86.2.412.
10. Lehotay SJ, Anastassiades M, Majors RE. The QuEChERS Revolution. LCGC Europe. 2010;23(9):418-28.
11. Shao G, Agar J, Giese RW. Cold-induced aqueous acetonitrile phase separation: A salt-free way to begin quick, easy, cheap, effective, rugged, safe. J Chromatogr A. 2017;1506:128-33. https://doi.org/10.1016/j.chroma.2017.05.045.
12. González-Curbelo MÁ, Socas-Rodríguez B, Herrera-Herrera AV, González-Sálamo J, Hernández-Borges J, Rodríguez-Delgado MÁ. Evolution and applications of the QuEChERS method. Trends Anal Chem. 2015;71:169-85. https://doi.org/10.1016/j.trac.2015.04.012.

13. Grover PK, Ryall RL. Critical appraisal of salting-out and its implications for chemical and biological sciences. Chem Rev. 2005;105(1):1-10. https://doi.org/10.1021/cr030454p.
14. Hyde AM, Zultanski SL, Waldman JH, Zhong Y-L, Shevlin M, Peng F. General principles and strategies for salting-out informed by the Hofmeister series. Org Process Res Dev. 2017;21(9):1355-70. https://doi.org/10.1021/acs.oprd.7b00197.
15. Li M, Zhuang B, Lu Y, An L, Wang Z-G. Salt-induced liquid–liquid phase separation: Combined experimental and theoretical investigation of water–acetonitrile–salt mixtures. J Am Chem Soc. 2021;143(2):773-84. https://doi.org/10.1021/jacs.0c09420.
16. Huddleston JG, Willauer HD, Swatloski RP, Visser AE, Rogers RD. Room temperature ionic liquids as novel media for 'clean' liquid–liquid extraction. Chem Commun. 1998(16):1765-6. https://doi.org/10.1039/A803999B
17. Poole CF, Poole SK. Extraction of organic compounds with room temperature ionic liquids. J Chromatogr A. 2010;1217(16):2268-86. https://doi.org/10.1016/j.chroma.2009.09.011.
18. Murphy PA, Barua K, Hauck CC. Solvent extraction selection in the determination of isoflavones in soy foods. J Chromatogr B. 2002;777(1):129-38. https://doi.org/10.1016/S1570-0232(02)00342-2.
19. Daley JM, Leadley TA, Drouillard KG. Evidence for bioamplification of nine polychlorinated biphenyl (PCB) congeners in yellow perch (*Perca flavascens*) eggs during incubation. Chemosphere. 2009;75(11):1500-5. https://doi.org/10.1016/j.chemosphere.2009.02.013.
20. Anastassiades M, Kolberg D, Benkenstein A, Eichhorn E, Zechmann S, Mack D, et al. Quick method for the analysis of numerous highly polar pesticides in foods of plant origin via LC-MS/MS involving simultaneous extraction with methanol (QuPPe-method). EU reference laboratory for pesticides requiring single residue methods (EURL-SRM) CVUA, Stuttgart, Germany. 2015.
21. Lehotay S. The QuEChERSER Mega-Method. LCGC North America. 2022;40:13–9. https://doi.org/10.56530/lcgc.na.px2173m3.
22. Mills PA, Onley JH, Gaither RA. Rapid Method for Chlorinated Pesticide Residues in Nonfatty Foods. J AOAC Int. 2020;46(2):186-91. https://doi.org/10.1093/jaoac/46.2.186.
23. Luke MA, Froberg JE, Masumoto HT. Extraction and cleanup of organochlorine, organophosphate, organonitrogen, and hydrocarbon pesticides in produce for determination by gas-liquid chromatography. J AOAC Int. 1975;58(5):1020-6. https://doi.org/10.1093/jaoac/58.5.1020.
24. Poole CF, Kersten BR, Ho SSJ, Coddens ME, Furton KG. Organic salts, liquid at room temperature, as mobile phases in liquid chromatography. J Chromatogr A. 1986;352:407-25. https://doi.org/10.1016/S0021-9673(01)83397-5.
25. Calla-Quispe E, Robles J, Areche C, Sepulveda B. Are ionic liquids better extracting agents than toxic volatile organic solvents? A combination of ionic liquids, microwave and LC/MS/MS, applied to the lichen *Stereocaulon glareosum*. Front Chem. 2020;Volume 8 - 2020. https://doi.org/10.3389/fchem.2020.00450.
26. Joshi MD, Anderson JL. Recent advances of ionic liquids in separation science and mass spectrometry. RSC Adv. 2012;2(13):5470-84. https://doi.org/10.1039/C2RA20142A
27. Berthod A, Ruiz-Ángel MJ, Carda-Broch S. Ionic liquids in separation techniques. J Chromatogr A. 2008;1184(1):6-18. https://doi.org/10.1016/j.chroma.2007.11.109.
28. Wicht A-J. Development and application of new analytical methods based on chromatographic and electrophoretic separations to assess the environmental behavior of

anthropogenic pollutants and their uptake in fungi and chironomids: Universität Tübingen; 2018.
29. David A, Botías C, Abdul-Sada A, Goulson D, Hill EM. Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. Anal Bioanal Chem. 2015;407(26):8151-62. https://doi.org/10.1007/s00216-015-8986-6.
30. Haroune L, Cassoulet R, Lafontaine M-P, Bélisle M, Garant D, Pelletier F, et al. Liquid chromatography-tandem mass spectrometry determination for multiclass pesticides from insect samples by microwave-assisted solvent extraction followed by a salt-out effect and micro-dispersion purification. Anal Chim Acta. 2015;891:160-70. https://doi.org/10.1016/j.aca.2015.07.031.
31. Stöckelhuber M, Müller C, Vetter F, Mingo V, Lötters S, Wagner N, et al. Determination of pesticides adsorbed on arthropods and gastropods by a micro-QuEChERS approach and GC–MS/MS. Chromatographia. 2017;80(5):825-9. https://doi.org/10.1007/s10337-017-3280-8.
32. Bergé A, Vulliet E. Development of a method for the analysis of hormones and pharmaceuticals in earthworms by quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Anal Bioanal Chem. 2015;407(26):7995-8008. https://doi.org/10.1007/s00216-015-8972-z.
33. Kretschmann A, Cedergreen N, Christensen JH. Measuring internal azole and pyrethroid pesticide concentrations in *Daphnia magna* using QuEChERS and GC-ECD—method development with a focus on matrix effects. Anal Bioanal Chem. 2016;408(4):1055-66. https://doi.org/10.1007/s00216-015-9197-x.
34. Berlioz-Barbier A, Buleté A, Faburé J, Garric J, Cren-Olivé C, Vulliet E. Multi-residue analysis of emerging pollutants in benthic invertebrates by modified micro-quick-easy-cheap-efficient-rugged-safe extraction and nanoliquid chromatography–nanospray–tandem mass spectrometry analysis. J Chromatogr A. 2014;1367:16-32. https://doi.org/10.1016/j.chroma.2014.09.044.
35. Berlioz-Barbier A, Buleté A, Fildier A, Garric J, Vulliet E. Non-targeted investigation of benthic invertebrates (*Chironomus riparius*) exposed to wastewater treatment plant effluents using nanoliquid chromatography coupled to high-resolution mass spectrometry. Chemosphere. 2018;196:347-53. https://doi.org/10.1016/j.chemosphere.2018.01.001.
36. Wicht A-J, Heye K, Schmidt A, Oehlmann J, Huhn C. The wastewater micropollutant carbamazepine in insectivorous birds — an exposure estimate. Anal Bioanal Chem. 2022;414(17):4909-17. https://doi.org/10.1007/s00216-022-04117-0.
37. Inostroza PA, Wicht A-J, Huber T, Nagy C, Brack W, Krauss M. Body burden of pesticides and wastewater-derived pollutants on freshwater invertebrates: Method development and application in the Danube River. Environ Pollut. 2016;214:77-85. https://doi.org/10.1016/j.envpol.2016.03.064.
38. Sordet M, Berlioz-Barbier A, Buleté A, Garric J, Vulliet E. Quantification of emerging micropollutants in an amphipod crustacean by nanoliquid chromatography coupled to mass spectrometry using multiple reaction monitoring cubed mode. J Chromatogr A. 2016;1456:217-25. https://doi.org/10.1016/j.chroma.2016.06.022.
39. Cunha SC, Pena A, Fernandes JO. Mussels as bioindicators of diclofenac contamination in coastal environments. Environ Pollut. 2017;225:354-60. https://doi.org/10.1016/j.envpol.2017.02.061.

40. Daniele G, Fieu M, Joachim S, James-Casas A, Andres S, Baudoin P, et al. Development of a multi-residue analysis of diclofenac and some transformation products in bivalves using QuEChERS extraction and liquid chromatography-tandem mass spectrometry. Application to samples from mesocosm studies. Talanta. 2016;155:1-7. https://doi.org/10.1016/j.talanta.2016.04.016.
41. Berlioz-Barbier A, Baudot R, Wiest L, Gust M, Garric J, Cren-Olivé C, et al. MicroQuEChERS–nanoliquid chromatography–nanospray–tandem mass spectrometry for the detection and quantification of trace pharmaceuticals in benthic invertebrates. Talanta. 2015;132:796-802. https://doi.org/10.1016/j.talanta.2014.10.030.
42. Perestrelo R, Silva P, Porto-Figueira P, Pereira JAM, Silva C, Medina S, et al. QuEChERS - Fundamentals, relevant improvements, applications and future trends. Anal Chim Acta. 2019;1070:1-28. https://doi.org/10.1016/j.aca.2019.02.036.
43. Trufelli H, Palma P, Famiglini G, Cappiello A. An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrom Rev. 2011;30(3):491-509. https://doi.org/10.1002/mas.20298.
44. Gonzalez-Curbelo MA, Lehotay SJ, Hernandez-Borges J, Rodriguez-Delgado MA. Use of ammonium formate in QuEChERS for high-throughput analysis of pesticides in food by fast, low-pressure gas chromatography and liquid chromatography tandem mass spectrometry. J Chromatogr A. 2014;1358:75-84. https://doi.org/10.1016/j.chroma.2014.06.104.
45. Kaufmann A, Butcher P, Maden K, Walker S, Widmer M. Improving the QuEChERS liquid/liquid extraction of analytes with widely varying physicochemical properties: Example of 201 veterinary drugs in milk. J AOAC Int. 2022;105(4):1030-42. https://doi.org/10.1093/jaoacint/qsac013.
46. Monteiro SH, Lehotay SJ, Sapozhnikova Y, Ninga E, Lightfield AR. High-throughput mega-method for the analysis of pesticides, veterinary drugs, and environmental contaminants by ultra-high-performance liquid chromatography−tandem mass spectrometry and robotic mini-solid-phase extraction cleanup + low-pressure gas chromatography−tandem mass spectrometry, Part 1: Beef. J Agric Food Chem. 2021;69(4):1159-68. https://doi.org/10.1021/acs.jafc.0c00710.
47. Ninga E, Sapozhnikova Y, Lehotay SJ, Lightfield AR, Monteiro SH. High-throughput mega-method for the analysis of pesticides, veterinary drugs, and environmental contaminants by ultra-high-performance liquid chromatography−tandem mass spectrometry and robotic mini-solid-phase extraction cleanup + low-pressure gas chromatography−tandem mass spectrometry, Part 2: Catfish. J Agric Food Chem. 2021;69(4):1169-74. https://doi.org/10.1021/acs.jafc.0c00995.
48. Chen S, Yu X, He X, Xie D, Fan Y, Peng J. Simplified pesticide multiresidues analysis in fish by low-temperature cleanup and solid-phase extraction coupled with gas chromatography/mass spectrometry. Food Chem. 2009;113(4):1297-300. https://doi.org/10.1016/j.foodchem.2008.08.045.
49. Gu T, Gu Y, Zheng Y, Wiehl PE, Kopchick JJ. Phase separation of acetonitrile-water mixture in protein purification. Sep Technol. 1994;4(4):258-60. https://doi.org/10.1016/0956-9618(94)80031-6.
50. Ullmann A, Ludmer Z, Shinnar R. Phase transition extraction using solvent mixtures with critical point of miscibility. AIChE J. 1995;41(3):488-500. https://doi.org/10.1002/aic.690410307.

51. Wang B, Ezejias T, Feng H, Blaschek H. Sugaring-out: A novel phase separation and extraction system. Chem Eng Sci. 2008;63(9):2595-600. https://doi.org/10.1016/j.ces.2008.02.004.
52. Dhamole PB, Mahajan P, Feng H. Sugaring out: A new method for removal of acetonitrile from preparative RP-HPLC eluent for protein purification. Process Biochem. 2010;45(10):1672-6. https://doi.org/10.1016/j.procbio.2010.06.020.
53. Dhamole PB, Mahajan P, Feng H. Phase separation conditions for sugaring-out in acetonitrile−water systems. J Chem Eng Data. 2010;55(9):3803-6. https://doi.org/10.1021/je1003115.
54. Cardoso GdB, Souza IN, Pereira MM, Freire MG, Soares CMF, Lima ÁS. Aqueous two-phase systems formed by biocompatible and biodegradable polysaccharides and acetonitrile. Sep Purif Technol. 2014;136:74-80. https://doi.org/10.1016/j.seppur.2014.08.020.
55. de Brito Cardoso G, Mourão T, Pereira FM, Freire MG, Fricks AT, Soares CMF, et al. Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. Sep Purif Technol. 2013;104:106-13. https://doi.org/10.1016/j.seppur.2012.11.001.
56. Mahdavi P, Nojavan S, Asadi S. Sugaring-out assisted electromembrane extraction of basic drugs from biological fluids: Improving the efficiency and stability of extraction system. J Chromatogr A. 2019;1608:460411. https://doi.org/10.1016/j.chroma.2019.460411.
57. Gupta R, Mauri R, Shinnar R. Liquid−liquid extraction using the composition-induced phase separation process. Ind Eng Chem Res. 1996;35(7):2360-8. https://doi.org/10.1021/ie950362t.
58. Liu G, Yang H, Zhang M, Li S, Chen B, Yao S. Novel isolation of phytochemical compositions by phase transition extraction with acetonitrile. J Sep Sci. 2011;34(3):347-53. https://doi.org/10.1002/jssc.201000658.
59. Liu G, Zhou N, Zhang M, Li S, Tian Q, Chen J, et al. Hydrophobic solvent induced phase transition extraction to extract drugs from plasma for high performance liquid chromatography–mass spectrometric analysis. J Chromatogr A. 2010;1217(3):243-9. https://doi.org/10.1016/j.chroma.2009.11.037.
60. Hu L, Tao Y, Luo D, Feng J, Wang L, Yu M, et al. Simultaneous biomonitoring of 15 organophosphate flame retardants metabolites in urine samples by solvent induced phase transition extraction coupled with ultra-performance liquid chromatography-tandem mass spectrometry. Chemosphere. 2019;233:724-32. https://doi.org/10.1016/j.chemosphere.2019.05.242.
61. Pedersen-Bjergaard S, Huang C, Gjelstad A. Electromembrane extraction–Recent trends and where to go. J Pharma Anal. 2017;7(3):141-7. https://doi.org/10.1016/j.jpha.2017.04.002.
62. Yamini Y, Seidi S, Rezazadeh M. Electrical field-induced extraction and separation techniques: Promising trends in analytical chemistry – A review. Anal Chim Acta. 2014;814:1-22. https://doi.org/10.1016/j.aca.2013.12.019.
63. Oedit A, Ramautar R, Hankemeier T, Lindenburg PW. Electroextraction and electromembrane extraction: Advances in hyphenation to analytical techniques. Electrophoresis. 2016;37(9):1170-86. https://doi.org/10.1002/elps.201500530.
64. Kubáň P, Boček P. Simultaneous micro-electromembrane extractions of anions and cations using multiple free liquid membranes and acceptor solutions. Anal Chim Acta. 2016;908:113-20. https://doi.org/10.1016/j.aca.2016.01.007.

65. Kubáň P, Boček P. Preconcentration in micro-electromembrane extraction across free liquid membranes. Anal Chim Acta. 2014;848:43-50. https://doi.org/10.1016/j.aca.2014.07.037.
66. Kubáň P, Boček P. Micro-electromembrane extraction across free liquid membranes. Instrumentation and basic principles. J Chromatogr A. 2014;1346:25-33. https://doi.org/10.1016/j.chroma.2014.04.047.
67. He Y, Miggiels P, Wouters B, Drouin N, Guled F, Hankemeier T, et al. A high-throughput, ultrafast, and online three-phase electro-extraction method for analysis of trace level pharmaceuticals. Anal Chim Acta. 2021;1149:338204. https://doi.org/10.1016/j.aca.2021.338204.
68. Raterink R-J, Lindenburg PW, Vreeken RJ, Hankemeier T. Three-phase electroextraction: A new (online) sample purification and enrichment method for bioanalysis. Anal Chem. 2013;85(16):7762-8. https://doi.org/10.1021/ac4010716.
69. Lindenburg PW, Seitzinger R, Tempels FWA, Tjaden UR, van der Greef J, Hankemeier T. Online capillary liquid–liquid electroextraction of peptides as fast pre-concentration prior to LC-MS. Electrophoresis. 2010;31(23-24):3903-12. https://doi.org/10.1002/elps.201000347.
70. Lindenburg PW, Tempels FWA, Tjaden UR, van der Greef J, Hankemeier T. On-line large-volume electroextraction coupled to liquid chromatography–mass spectrometry to improve detection of peptides. J Chromatogr A. 2012;1249:17-24. https://doi.org/10.1016/j.chroma.2012.06.016.
71. Lindenburg PW, Tjaden UR, van der Greef J, Hankemeier T. Feasibility of electroextraction as versatile sample preconcentration for fast and sensitive analysis of urine metabolites, demonstrated on acylcarnitines. Electrophoresis. 2012;33(19-20):2987-95. https://doi.org/10.1002/elps.201200276.
72. Rösch T, Weber G, Bader T, Wicht A-J, Huhn C. Performance of free-flow field-step electrophoresis as cleanup step for the non-target analysis of environmental water samples. Anal Bioanal Chem. 2022;414(6):2189-204. https://doi.org/10.1007/s00216-021-03856-w.
73. Takahashi N, Hashimoto M, Tsukagoshi K. Two-phase extraction of metal ions using a water-acetonitrile-ethyl acetate ternary mixed-solvent separation system. Anal Sci. 2013;29(6):665-7. https://doi.org/10.2116/analsci.29.665.
74. Cray JA, Russell JT, Timson DJ, Singhal RS, Hallsworth JE. A universal measure of chaotropicity and kosmotropicity. Environ Microbiol. 2013;15(1):287-96. https://doi.org/10.1111/1462-2920.12018.
75. Atha DH, Ingham KC. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. J Biol Chem. 1981;256(23):12108-17. https://doi.org/10.1016/S0021-9258(18)43240-1.
76. Knoll S, Rösch T, Huhn C. Trends in sample preparation and separation methods for the analysis of very polar and ionic compounds in environmental water and biota samples. Anal Bioanal Chem. 2020;412(24):6149-65. https://doi.org/10.1007/s00216-020-02811-5.
77. Nayak AK, Panigrahi PP. Solubility enhancement of etoricoxib by cosolvency approach. ISRN Phys Chem. 2012;2012:820653. https://doi.org/10.5402/2012/820653.
78. Liao X, Raghavan VGS, Meda V, Yaylayan VA. Dielectric properties of supersaturated α-D-glucose aqueous solutions at 2450 MHz. J Microw Power Electromagn Energy. 2001;36(3):131-8. https://doi.org/10.1080/08327823.2001.11688455.

79. Docoslis A, Giese RF, van Oss CJ. Influence of the water–air interface on the apparent surface tension of aqueous solutions of hydrophilic solutes. Colloids Surf B. 2000;19(2):147-62. https://doi.org/10.1016/S0927-7765(00)00137-5.
80. de Matos EMC, Ribeiro LC, Prestes OD, da Silva JAG, de Farias BS, Pinto LAdA, et al. Multiclass method for the determination of pesticide residues in oat using modified QuEChERS with alternative sorbent and liquid chromatography with tandem mass spectrometry. Food Anal Methods. 2019;12(12):2835-44. https://doi.org/10.1007/s12161-019-01641-1.
81. Michel M, Pszczolinska K. The QuEChERS approach for the determination of pesticide residues in soil samples: An overview. J AOAC Int. 2016;99(6):1403-14. https://doi.org/10.7540/jaoacint.16-0274.
82. Lefebvre T, Destandau E, Lesellier E. Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. J Chromatogr A. 2021;1635:461770. https://doi.org/10.1016/j.chroma.2020.461770.
83. Barwick VJ. Strategies for solvent selection — a literature review. Trends Anal Chem. 1997;16(6):293-309. https://doi.org/10.1016/S0165-9936(97)00039-3.
84. Kalinke N, Stopper P, Völkl L, Diehl F, Huhn C. SWIEET — a salt-free alternative to QuEChERS. Anal Bioanal Chem. 2024;416(28):6387-403. https://doi.org/10.1007/s00216-024-05525-0.
85. Reichardt C. Solvatochromic dyes as solvent polarity indicators. Chem Rev. 1994;94(8):2319-58. https://doi.org/10.1021/cr00032a005.
86. Reichardt C. Solvatochromism, thermochromism, piezochromism, halochromism, and chiro-solvatochromism of pyridinium N-phenoxide betaine dyes. Chem Soc Rev. 1992;21(3):147-53. https://doi.org/10.1039/CS9922100147.
87. Taft RW, Kamlet MJ. The solvatochromic comparison method. 2. The α-scale of solvent hydrogen-bond donor (HBD) acidities. J Am Chem Soc. 1976;98(10):2886-94. https://doi.org/10.1021/ja00426a036.
88. Kamlet MJ, Taft RW. The solvatochromic comparison method. I. The β-scale of solvent hydrogen-bond acceptor (HBA) basicities. J Am Chem Soc. 1976;98(2):377-83. https://doi.org/10.1021/ja00418a009.
89. Kamlet MJ, Abboud JL, Taft RW. The solvatochromic comparison method. 6. The π* scale of solvent polarities. J Am Chem Soc. 1977;99(18):6027-38. https://doi.org/10.1021/ja00460a031.
90. González-Arjona D, López-Pérez G, Domínguez M, González A. Solvatochromism: A comprehensive project for the final year undergraduate chemistry laboratory. J Lab Chem Educ. 2016;2016:45-52. https://doi.org/10.5923/j.jlce.20160403.01.
91. Spange S, Reuter A, Lubda D. ET(30) surface polarity parameters of alkyl- and aryl-group-functionalized silica particles: Differentiating the surface environments by means of the application of differently substituted Reichardt's Dyes. Langmuir. 1999;15(6):2103-11. https://doi.org/10.1021/la980328u.
92. Michels JJ, Dorsey JG. Examination of the surface polarity of X-type alumina using ET-30 solvatochromism. Langmuir. 1990;6(2):414-9. https://doi.org/10.1021/la00092a022.
93. Baker SN, Baker GA, Bright FV. Temperature-dependent microscopic solvent properties of 'dry' and 'wet' 1-butyl-3-methylimidazolium hexafluorophosphate: correlation with (30) and Kamlet–Taft polarity scales. Green Chem. 2002;4(2):165-9. https://doi.org/10.1039/B111285F.

94. Spange S, Lienert C, Friebe N, Schreiter K. Complementary interpretation of ET(30) polarity parameters of ionic liquids. Phys Chem Chem Phys. 2020;22(18):9954-66. https://doi.org/10.1039/D0CP01480J.
95. Carr PW. Solvatochromism, linear solvation energy relationships, and chromatography. Microchem J. 1993;48(1):4-28. https://doi.org/10.1006/mchj.1993.1066.
96. Kamlet MJ, Abraham MH, Carr PW, Doherty RM, Taft RW. Solute–solvent interactions in chemistry and biology. Part 7. An analysis of mobile phase effects on high pressure liquid chromatography capacity factors and relationships of the latter with octanol–water partition coefficients. J Chem Soc, Perkin Trans 2. 1988(12):2087-92. https://doi.org/10.1039/P29880002087.
97. D'Archivio AA, Maggi MA, Ruggieri F. Quantitative structure/eluent–retention relationships in reversed-phase high-performance liquid chromatography based on the solvatochromic method. Anal Bioanal Chem. 2013;405(2):755-66. https://doi.org/10.1007/s00216-012-6191-4.
98. Barbosa J, Bergés R, Sanz-Nebot V. Linear solvation energy relationships in reversed-phase liquid chromatography. Prediction of retention of several quinolones. J Liq Chromatogr. 1995;18(17):3445-63. https://doi.org/10.1080/10826079508010462.
99. Duchemin CR, Engle NL, Bonnesen PV, Haverlock TJ, Delmau LH, Moyer BA. Solvatochromic solvent polarity measurements of alcohol solvent modifiers and correlation with cesium extraction strength Solvent Extr Ion Exch. 2001;19(6):1037-58. https://doi.org/10.1081/SEI-100107618.
100. Bednarz S, Mielczarek K, Wierzbicki S. Liquid–liquid phase separation in binary mixtures of quaternary alkylammonium chlorides with carboxylic acids. J Mol Liq. 2023;384:122231. https://doi.org/10.1016/j.molliq.2023.122231.
101. Tang M, Yi X, Huang H, Feng Y, Chen G, Mu X. Organic-NaH_2PO_4-H_2O aqueous biphasic system for extraction of paeonol from cortex moutan: Solvent selection and mechanism probing. J Mol Liq. 2023;370:121004. https://doi.org/10.1016/j.molliq.2022.121004.
102. Deng W-w, Mei X-p, Cheng Z-j, gan T-x, Tian X, Hu J-n, et al. Extraction of weak hydrophobic sulforaphane from broccoli by salting-out assisted hydrophobic deep eutectic solvent extraction. Food Chem. 2023;405:134817. https://doi.org/10.1016/j.foodchem.2022.134817.
103. Spange S. Polarity of organic solvent/water mixtures measured with Reichardt's B30 and related solvatochromic probes—A critical review. Liq. 2024;4(1):191-230. https://doi.org/10.3390/liquids4010010.
104. Jonquières A, Roizard D, Cuny J, Lochon P. Solubility and polarity parameters for assessing pervaporation and sorption properties. A critical comparison for ternary systems alcohol/ether/polyurethaneimide. J Membr Sci. 1996;121(1):117-33. https://doi.org/10.1016/0376-7388(96)00168-8.
105. Leitão RE, Martins F, Ventura MC, Nunes N. Structural characterization of the ternary solvent mixture methanol–acetonitrile–1-propanol. J Phys Org Chem. 2002;15(9):623-30. https://doi.org/10.1002/poc.520.
106. Fletcher KA, Pandey S. Solvatochromic probe behavior within ternary room-temperature ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate + ethanol + water solutions. J Phys Chem B. 2003;107(48):13532-9. https://doi.org/10.1021/jp0276754.

107. Nunes N, Elvas-Leitão R, Martins F. Study of the ternary mixture of methanol/formamide/acetonitrile via solvatochromic probes. Molecules. 2024;29(1):246. https://doi.org/10.3390/molecules29010246.
108. Langhals H. Polarity of binary liquid mixtures. Angew Chem, Int Ed Engl. 1982;21(10):724-33. https://doi.org/10.1002/anie.198207241.
109. Rundlöf T, Mathiasson M, Bekiroglu S, Hakkarainen B, Bowden T, Arvidsson T. Survey and qualification of internal standards for quantification by ^{1}H NMR spectroscopy. J Pharm Biomed Anal. 2010;52(5):645-51. https://doi.org/10.1016/j.jpba.2010.02.007.
110. Kamlet MJ, Abboud JLM, Abraham MH, Taft RW. Linear solvation energy relationships. 23. A comprehensive collection of the solvatochromic parameters, π*, α, and β, and some methods for simplifying the generalized solvatochromic equation. J Org Chem. 1983;48(17):2877-87. https://doi.org/10.1021/jo00165a018.
111. Sindreu RJ, Moyá ML, Sánchez Burgos F, González AG. Kamlet-Taft solvatochromic parameters of aqueous binary mixtures of tert-butyl alcohol and ethylene glycol. J Solut Chem. 1996;25(3):289-93. https://doi.org/10.1007/BF00972526.
112. Marcus Y. The properties of organic liquids that are relevant to their use as solvating solvents. Chem Soc Rev. 1993;22(6):409-16. https://doi.org/10.1039/CS9932200409.
113. Cheong WJ, Carr PW. Kamlet-Taft π* polarizability/dipolarity of mixtures of water with various organic solvents. Anal Chem. 1988;60(8):820-6. https://doi.org/10.1021/ac00159a018.
114. Rivera-Quintero P, Patience GS, Patience NA, Boffito DC, Banquy X, Schieppati D. Experimental methods in chemical engineering: Karl Fischer titration. Can J Chem Eng. 2024;102(9):2980-97. https://doi.org/10.1002/cjce.25295.
115. Yang B, Cao X, Wang S, Sun C. Exploring molecular association of isopropanol-water binary solution by Raman spectroscopy. Optik. 2020;204:163544. https://doi.org/10.1016/j.ijleo.2019.163544.
116. Guo S, Zhu C, Chen G, Gu J, Ma C, Gao H, et al. A theoretical study on intermolecular hydrogen bonds of isopropanol-water clusters. Theor Chem Acc. 2022;141(1):6. https://doi.org/10.1007/s00214-022-02865-x.
117. Chen C, Li WZ, Song YC, Weng LD, Zhang N. Formation of water and glucose clusters by hydrogen bonds in glucose aqueous solutions. Comput Theor Chem. 2012;984:85-92. https://doi.org/10.1016/j.comptc.2012.01.013.
118. Fidalgo-Used N, Blanco-González E, Sanz-Medel A. Sample handling strategies for the determination of persistent trace organic contaminants from biota samples. Anal Chim Acta. 2007;590(1):1-16. https://doi.org/10.1016/j.aca.2007.03.004.
119. Kannouma RE, Hammad MA, Kamal AH, Mansour FR. Miniaturization of liquid-liquid extraction; the barriers and the enablers. Microchem J. 2022;182:107863. https://doi.org/10.1016/j.microc.2022.107863.
120. Soares da Silva Burato J, Vargas Medina DA, de Toffoli AL, Vasconcelos Soares Maciel E, Mauro Lanças F. Recent advances and trends in miniaturized sample preparation techniques. J Sep Sci. 2020;43(1):202-25. https://doi.org/10.1002/jssc.201900776.
121. Andreu V, Picó Y. Determination of currently used pesticides in biota. Anal Bioanal Chem. 2012;404(9):2659-81. https://doi.org/10.1007/s00216-012-6331-x.
122. Casado N, Perestrelo R, Silva CL, Sierra I, Câmara JS. An improved and miniaturized analytical strategy based on μ-QuEChERS for isolation of polyphenols. A

powerful approach for quality control of baby foods. Microchem J. 2018;139:110-8. https://doi.org/10.1016/j.microc.2018.02.026.
123. Mateus ARS, Barros SC, Pena A, Sanches-Silva A. Miniaturized QuEChERS extraction (μQuEChERS) combined with HPLC-MS/MS as new analytical method for determination of 105 residues of pesticides in fruit by-products extracts. Food Chem. 2025;481:143898. https://doi.org/10.1016/j.foodchem.2025.143898.
124. Izcara S, Casado N, Morante-Zarcero S, Pérez-Quintanilla D, Sierra I. Miniaturized and modified QuEChERS method with mesostructured silica as clean-up sorbent for pyrrolizidine alkaloids determination in aromatic herbs. Food Chem. 2022;380:132189. https://doi.org/10.1016/j.foodchem.2022.132189.
125. Montemurro N, Joedicke J, Pérez S. Development and application of a QuEChERS method with liquid chromatography-quadrupole time of flight-mass spectrometry for the determination of 50 wastewater-borne pollutants in earthworms exposed through treated wastewater. Chemosphere. 2021;263:128222. https://doi.org/10.1016/j.chemosphere.2020.128222.
126. Kurth D, Krauss M, Schulze T, Brack W. Measuring the internal concentration of volatile organic compounds in small organisms using micro-QuEChERS coupled to LVI–GC–MS/MS. Anal Bioanal Chem. 2017;409(25):6041-52. https://doi.org/10.1007/s00216-017-0532-2.
127. Singh S, Singh N, Kumar V, Datta S, Wani AB, Singh D, et al. Toxicity, monitoring and biodegradation of the fungicide carbendazim. Environ Chem Lett. 2016;14(3):317-29. https://doi.org/10.1007/s10311-016-0566-2.
128. Merel S, Benzing S, Gleiser C, Di Napoli-Davis G, Zwiener C. Occurrence and overlooked sources of the biocide carbendazim in wastewater and surface water. Environ Pollut. 2018;239:512-21. https://doi.org/10.1016/j.envpol.2018.04.040.
129. van Wijngaarden RPA, Crum SJH, Decraene K, Hattink J, van Kammen A. Toxicicity of derosal (active ingredient carbendazim) to aquatic invertebrates. Chemosphere. 1998;37(4):673-83. https://doi.org/10.1016/S0045-6535(98)00083-6.
130. Cuppen JGM, Van den Brink PJ, Camps E, Uil KF, Brock TCM. Impact of the fungicide carbendazim in freshwater microcosms. I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates. Aquat Toxicol. 2000;48(2):233-50. https://doi.org/10.1016/S0166-445X(99)00036-3.
131. Könemann S, Müller Y, Tschentscher D, Krauss M, Inostroza PA, Brückner I, et al. Combination of in situ feeding rate experiments and chemical body burden analysis to assess the influence of micropollutants in wastewater on *Gammarus pulex*. Int J Environ Res Public Health. 2019;16(5). https://doi.org/10.3390/ijerph16050883.
132. OECD. Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing. 2010. https://doi.org/10.1787/9789264090910-en.
133. Liu K, Pan X, Han Y, Tang F, Yu Y. Estimating the toxicity of the weak base carbendazim to the earthworm (*Eisenia fetida*) using in situ pore water concentrations in different soils. Sci Total Environ 2012;438:26-32. https://doi.org/10.1016/j.scitotenv.2012.08.008.