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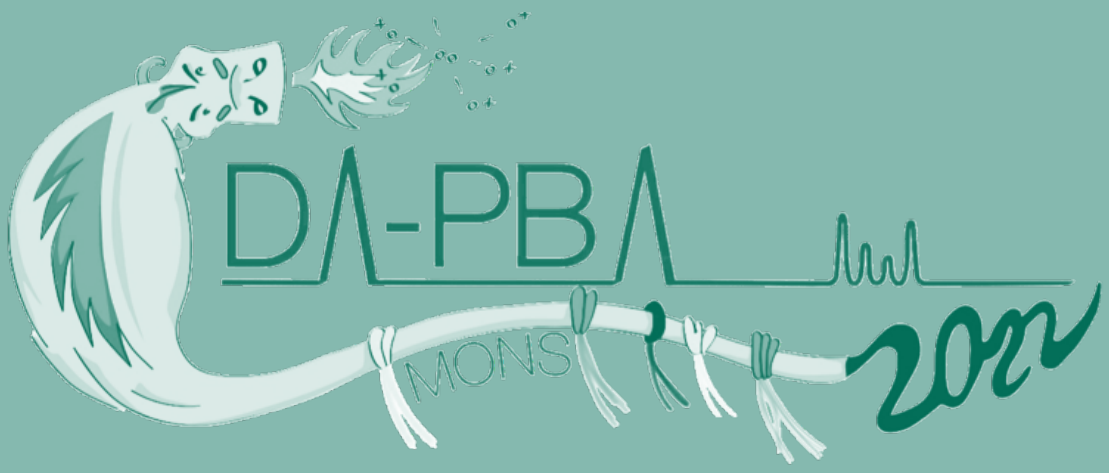

PLENARY LECTURES

The regulatory perspective on ATMP development

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Advanced Therapy Medicinal Products (ATMPs) comprise Somatic Cell Therapy, Tissue engineering and Gene Therapy medicinal products and are at the forefront of therapeutic innovation. The ATMP legislation is built on a risk-based approach to meet the needs of this heterogeneous class of products with various, often complex mechanisms of action and characteristics.

From a regulatory perspective additional complexity in ATMP development arises from the more general innovation trend of personalization, incorporating (integral) device components in medicinal products, association with (non-integral) delivery devices or co-development of companion diagnostics. Thus, developers need to navigate a complex regulatory environment in addition to building the analytical armamentarium.

As a consequence of the novel modalities and mechanisms of action the general knowledge base for the appropriate characterization and release is limited. Developing the analytical panel is therefore not as straightforward as picking amongst an established set of methods, but frequently involves establishing and testing entirely new approaches. In this setting of limited preexisting knowledge, applying multiple orthogonal methods is recommended be able to conclude on the best suited panel during development.

Clinical trials are the first procedural interaction between developers and regulators and guidance is compiled in EudraLex Volume 10. Legal requirements for approval of a clinical trial focus on patient safety and robustness of data, which, on the quality level, translates to the need to relate obtained clinical data to a specific (quality) product profile. The product profile is contingent on a consistent manufacturing process and appropriate and reliable analytics, including measures of potency. Developers are well-advised not only to consider phase-appropriate characterization for clinical trial approval but also take a wider view of the use of data for a potential Marketing Authorization. Experience gathered with clinical trial submissions and illustrative examples will be provided in the presentation.

Juggling analytes, column chemistry and charged aerosol detection – HPLC analysis of weak chromophore drugs

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Quality assessment of drugs is nowadays mainly performed by reversed phase column chromatography using aqueous buffers, acetonitrile and/or methanol as mobile phase, which do not interfere with the commonly used UV detection. However, many excipients and drugs do not contain a chromophore and can, therefore, not be identified and quantified by UV detection. In these cases, the charged aerosol detection can be an excellent alternative, because he is a kind of universal detector for almost all non-volatile compounds. However, the CAD´s response is dominated by the mobile phase composition and the mobile phase is dependent on the kind of chromatography performed. This holds especially true for ion pair chromatography (IPC), mixed mode (MMC) and hydrophilic interaction chromatography (HILIC), which are often employed for the analysis of analytes of different polarity. The juggling of satisfying separations with columns of different chemistry and the optimization of CAD response will be demonstrated for various non-chromophore analytes with diverse physicochemical properties, such as amino acids, vigabatrine, topiramate, ibandronate and some excipients [1-6].

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Biomolecules and their physicochemical properties: charge interactions, CIEF and affinity CE

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Today proteins are possibly the most important class of substances, e.g. as biopharmaceuticals. Yet new tasks for proteins are still often solved by trial-and-error approaches. However, a more strategic concepts are available: proteins can be grouped according to their physicochemical properties, including size, then charge and hydrophobicity as well as their patchinesses, and the degree of order. In addition, solubility, the content of (free) enthalpy, aromatic-amino-acid- and α/β-frequency as well as helix capping, and corresponding patchiness, the number of specific motifs and domains as well as the typical concentration range can be helpful to discriminate between different groups of proteins [1].

Antibody self-interaction including aggregation has been correlated to hydrophobic patches as well as the electrostatic potential distribution on a protein surface. These approaches rely on 3-D structures which may not always be available but can be predicted with an increasing precision from the sequence.

Collagen is a very important and highly abundant structural protein but hard to analyse due to its insolubility. In order to investigate collagen in a liquid environment and to maintain its biological function, we milled and suspended collagen in a phosphate buffer pH 7.4, 12.5 mM using a dual zentrifuge (ZentriMix 380R) to obtain particles with a size below 5 µm. Using these small collagen particles, affinity CE (ACE) was performed to study binding properties of Human Serum Albumin, Human Fibronectin and Collagenase Type I from *Clostridium histolyticum*.

The 2D-combination of strong-anion exchange chromatography and microchip capillary electrophoresis sodium dodecyl sulfate was employed for rapid two-dimensional separations of cell lysates from Sf9 cells, samples from Chinese hamster ovary cell culture before and after a purification and human plasma, showing the performance and broad applicability of this approach.

In order to contribute to the scientific research on the SARS-CoV-2, we have investigated the isoelectric points (pI) of several related proteins. Their theoretical pI values were calculated using the ProtParam tool from ExPASy. The proteins were then measured with the Maurice imaged CIEF system. Due to isoforms, it was not possible to determine exact pI values for those proteins, but pI ranges with several peaks: RBD/Fc 8.24-9.32 (theoretical pI: 8.55), RBD/His 7.36-9.88 (8.91), 7.30-8.37 (7.80) for the S1/His, 4.41-5.87 for S1/S2/His and 5.36-6.11 (5.60) for hACE2/His. These ranges always coincided with the calculated values, making the latter useful predictors for pIs of SARS-CoV-2 proteins [2].

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Vaccine characterization: from black box to well-defined biologics

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Vaccines are an extraordinary category of biopharmaceuticals. They work in low doses, they are mostly prophylactic and everybody on the planet is subject to vaccination. They are also among the oldest biopharmaceuticals and sometimes ill-defined. Antigens in vaccines are sometimes chemically inactivated, conjugated to carrier molecules, adsorbed to adjuvants and mixed with other antigens. Often antigens are produced in eukaryotic cells or in bacteria causing the introduction of host cell related impurities. These attributes make vaccines a challenge with respect to characterization and quality control.

In the not too distant past vaccines were often analyzed in vivo. Assays in mice, rats, and other experimental animals were needed to determine potency and safety of both experimental as well as routinely produced vaccines. The last decades the development of analytical techniques and strategies have enabled us to reduce the number of experimental animals in R&D as well as vaccine production substantially.

The analytical portfolio has expanded hugely and allows us to accurately characterize proteins, polysaccharides, bacteria, cells and viruses, both qualitatively as well as quantitatively. Spectroscopic techniques, immunoassays, chromatography and particle analysis address critical quality attributes of basically every vaccine family, from whole bacteria to mRNA vaccines. One particularly powerful technique is mass spectrometry. Mass spectrometry enables us to analyze complex proteomes both qualitatively as well as quantitatively in one run. Covalent modifications inflicted deliberately or ocurring during handling or storage, can be identified but also aspects of in vivo responses to vaccines can be analyzed by mass spectrometry based proteomics.

Several case studies will be discussed showing that even complex products such toxoid vaccines (formaldehyde inactivated bacterial toxins) can be characterized to great detail. Even if they are adsorbed to aluminum salt and in the presence of other antigens it is possible to assess the quality of these products.

Since vaccines are given to many, healthy and often very young children, great attention must be paid to impurities present in vaccines. Recent developments in this area will be discussed.

These developments in the analytical portfolio allow us not only to develop new vaccines but also to keep the old ones by keeping them up to today’s quality standards.

Protein biopharmaceuticals analysis with advanced chromatography and mass spectrometry approaches

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The therapeutic success of monoclonal antibodies (mAbs) in the treatment of cancer has contributed to their rise, ranking six mAbs and derived products among the 10 best-selling drugs in 2020. From production to patient administration, mAbs are subject to numerous chemical and enzymatic modifications that can alter their biological activity and pharmacological profile. A complete characterization of therapeutic proteins and their variants must then be performed by analytical methods, to ensure product safety and inter-batch reproducibility.

The aim of this presentation will be to detail some innovative chromatographic approaches recently developed in our laboratory, to improve speed of analysis, selectivity and MS compatibility when analyzing therapeutic proteins.

A first innovative approach is based on the use of ultra-short columns (only a few millimeters) in reversed phase liquid chromatography (RPLC) and ion exchange chromatography (IEX), to obtain separations as efficient as with standard size columns, but with significantly reduced analysis times [1,2]. Analyses of mAbs and immunoconjugates (ADCs) could thus be successfully performed in only a few tens of seconds.

The second approach is based on the use of special gradient conditions, allowing to significantly improve the selectivity between different protein isoforms. Based on the fact that the protein retention mechanism is on-off, it is possible to add one or more isocratic steps during the RPLC analysis to increase the distance between the chromatographic peaks [3]. Thanks to this principle, we were able to demonstrate infinite selectivity between protein chromatographic peaks simply by adding a suitable isocratic step.

The third approach is based on the use of volatile mobile phase components and innovative stationary phases (made with bioinert material) to make IEX and size exclusion chromatography (SEC) compatible with MS. Using this strategy, various biopharmaceutical products were successfully characterized using non denaturing chromatographic modes.

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Microextraction using artificial liquid membranes and electrical fields

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Electromembrane extraction (EME) is a green microextraction technique aimed for acidic and basic analytes [1]. Target analytes are extracted from an aqueous sample (biological fluid, environmental sample), across an artificial liquid membrane, and into acceptor solution. The liquid membrane is a few micro liters of organic solvent immiscible with water, which is immobilized by capillary forces in the pores of a polymeric filter. The acceptor is an aqueous solution of buffer or acid/base. Extraction is facilitated by a dc electrical potential applied across the liquid membrane. By such, EME is unique, and is based on electro-kinetic migration and electro-assisted partition. For extraction of basic analytes, the negative electrode is placed in the acceptor, and the positive electrode is in the sample. Sample and acceptor are kept neutral or acidic, and the target analytes are extracted as cations. For extraction of acids, the electrical field is reversed, and sample and acceptor are kept neutral or alkaline.

EME provides high selectivity due to the liquid membrane and the electrical field. Accordingly, the selectivity is controlled by multiple operational parameters including the chemical properties of the liquid membrane, the direction and magnitude of the electrical field, and the pH conditions in sample and acceptor. Sample volumes can range from 0.05 -10 mL, and EME can provide pre-concentration. Acceptors are aqueous, and can be injected directly in LC-MS without additional efforts. EME can been performed in 96-well format and in microfluidic systems, and has potential for automation and high-throughput applications. The consumption of organic solvent to establish the liquid membrane is minimal, and is typically in the range of 2-10 µL per sample. EME is therefore a green alternative for sample preparation.

Equipment for EME became commercially available during this summer (2022) [2]. The commercial products will most probably increase the awareness and interest for EME. This plenary lecture will discuss the principle and potential of EME, and will identify the different directions of current and future EME research.

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KEYNOTE LECTURES

Automated high-throughput oligonucleotide-lipid nanoparticle preparation and analytical characterization

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Lipid nanoparticles (LNPs) play to a critical role in the delivery of therapeutic nucleic acids, such as antisense oligonucleotides (ASOs) and mRNAs, which is clearly demonstrated by the rapid development of the mRNA-LNP COVID-19 vaccines. The selection of lipid composition, total lipid concentration and charge ratio directly impact the critical quality attributes of LNPs, such as size, drug loading, stability etc. The screening and optimization of the formulation is time consuming and tedious, and difficult to be systematic. We developed an automated high throughput approach to streamline the LNP formulation preparation by automated solvent mixing using robotic liquid hander, followed by particle size distribution and encapsulation efficiency measurement by dynamic light scattering (DLS) and UV spectroscopy, respectively, in the 96-well plate format.

Our results showed that PEGylated lipid was necessary for LNP formulation and its percentage significantly affected LNP size distribution; the charge ratio of ionizable lipid/oligonucleotide impacted the drug encapsulation efficiency of LNPs. We also demonstrated this automated HTS approach strongly correlated with the large scale formulation preparation. The presented approach reduced material usage by 10 folds and improved analytical outputs by 100 folds.

To further increase the speed of analytical characterization, we developed a machine learning method for high throughput quantification of nucleic acid loading in LNPs. This approach is based on locally weighted regression (LWR) of UV spectra of unpurified sample. No external standard is required for the quantitation. We successfully applied the model to different nucleic acid cargos, such as ASO, sgRNA and mRNA in different lipid matrices.

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The use of chemometrics to study natural products

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From the analytical point of view, natural products (NPs) may be considered complex samples. An extract is a mixture of metabolites, which can vary their chemical behavior depending on their polarity, structure, stability, etc. In this sense, the use of modern analytical methods, such as hyphenated techniques, is imperative in the contemporary pharmacognosy. As a consequence, a large chemical dataset is generated. In this context, it is fully justified the use of chemometric tools to provide maximum chemical information on these complex mixtures. Here, the use of chemometrics is demonstrated for three situations: (i) to identify new bioactive NPs, exemplified by the isolation of azepine-indole alkaloids from *Psychotria nemorosa*, able to interact in different targets related to neurodegenerative diseases; (ii) to identify markers for quality control of plant materials, exemplified by the selection of anti-inflammatory flavonoids from *Psidium cattleyanum* leaves, which can be used as biomarkers interchangeably for different morpho- and chemotypes of the plant; and (iii) to predict biological properties based on chemical features, exemplified by the prediction of oral bioavailability of tricyclic NPs with cytotoxic potential. In summary, the use of chemometrics for NPs study has been proven very promising. This is a growing area in pharmacognosy research and may lead to faster, more robust and more reliable bioactive compounds identification, reinforcing the renewal of the interest in natural product-based drug discovery.

Chromatographic-mass spectrometric approaches in sports drug testing - challenges and advances

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Analytical approaches in sports drug testing are continuously updated and expanded, exploiting new information on drug metabolism and disposition in humans as well as innovations in sample preparation and analysis, and also novel strategies focusing on marker-based test methods have been assessed, developed, and implemented. The resulting improved detection capability and retrospectivity of sports drug testing approaches has considerably limited the formerly available options of substances and methods of doping. In addition, however, and similar to the general population, elite athletes are exposed to a complex set of environmental factors including chemicals, biological and physical stressors, which constitute an exposome that is, unlike for the general population, subjected to specific scrutiny for athletes due to applicable anti-doping regulations and routine doping controls.
Test methods in sports drug testing, relying largely on chromatographic-mass spectrometric methods, were optimized and applied to newly identified challenges, including e.g. the detection and characterization of superior metabolic products of prohibited as well as non-prohibited substances, aiming at enhancing the analytical data available for decision-making processes in test result management. Additional information, resulting from controlled (microdosed) elimination studies and simulations of contamination scenarios, complements the dataset of routine doping controls.
Drug elimination profiles are an important aspect, contributing to the interpretation of analytical test results and supporting the assessment of drug exposure scenarios concerning their plausibility. By means of examples including ingredients of cosmetics, food potentially contaminated with doping agents such as anti-estrogens, and new anabolic agents (SARMs) contributing to continuously increasing numbers of adverse analytical findings, the particularly important role of chromatographic-mass spectrometric analyses in doping controls is illustrated. Optimized test methods allow for utmost retrospectivity and, at the same time, can offer critical information as to the time point of drug exposure and/or the source of the target analyte in athletes' doping control samples [1].

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Advances and Utility of Capillary Electrophoresis-Mass Spectrometry in Micro-Metabolomics

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In bioanalysis and metabolomics, there are plenty of biomedical and clinical questions dealing with inherently low sample amounts. For example, 3D (patient-derived) microfluidic cell culture models are more and more used to gain fundamental insights into cellular biological processes. These microfluidic cell culture systems often deal with low numbers of mammalian cells, i.e. typically in the range of hundreds to thousands of cells. To enable the study of material-restricted biomedical questions with metabolomics, i.e. micro-metabolomics, we have developed capillary zone electrophoresis-mass spectrometry (CE-MS) methods for the efficient and sensitive profiling of polar ionogenic metabolites in small-volume biological samples over the past years. In this presentation we reflect on the possibilities of CE-MS for micro-metabolomics.

Recent work from our lab revealed that CE-MS, regardless of utilizing a sheath-liquid or sheathless interface, is a strong analytical tool for probing polar and charged metabolites in volume-restricted biological samples with a good reproducibility. Moreover, in a simulated metabolomics study, CE-MS was able to find the right set of differential metabolites between controls and cases. These studies clearly indicate the value of CE-MS for comparative metabolomics studies and biomarker discovery.

Given our interest in volume-restricted biomedical questions, we show how CE-MS can be used for the direct and sensitive analysis of basic (endogenous) metabolites in rat brain microdialysis samples without compromising any analytical performance metrics. As only nanoliters of samples are consumed by a single CE-MS analysis, multiple injections/assays can be performed on the same valuable volume-limited sample allowing for technical replicates and/or probing different classes of polar ionogenic metabolites.

For so called “difficult/challenging” metabolites, such as sugar phosphates, nucleotides and small organic acids, detection limits in the low nanomolar range were obtained with sheathless CE-MS and structural isomers could be selectively analyzed without employing any derivatization procedure. Compounds such as ATP, ADP and AMP, can be analyzed in extracts from just a limited number of mammalian cells, opening up the possibility to assess the adenylate energy charge in studies dealing with microscale cell cultures. We also show for selected endogenous metabolites that a linear detector response was obtained when going from 10,000 to 500 HepG2 cells as starting amount, indicating the strong potential of CE-MS for quantitative metabolomics studies intrinsically dealing with limited sample amounts.

Importance of data pre-processing in accurate drug distribution profiling with LC-MS/MS and mass spectrometry imaging

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Mass spectrometry in imaging and in molecular profiling (generally coupled with liquid chromatography or capillary electrophoresis) settings provide complex data, which require sophisticated data pre-processing and quality control algorithms. This talk will focus on the various aspects data pre-processing of mass spectrometry imaging (MSI) and LC-MS/MS data acquired to profile drug distribution in biological samples. It will introduce the modules of Pipelines And Systems for Threshold Avoiding Quantification (PASTAQ) [1], which pipeline was design to capture all measured mass spectrometry signal, by avoiding to apply thresholds in early and intermediate signal processing steps such as peak detection in individual samples, while performing signal from noise discrimination at the end of the pipeline using all information available from the whole dataset. PASTAQ can capture all measured compound information both for proteomics and metabolomics LC-MS/MS datasets independently from identification status and provide accurate quantitative pre-processing for large datasets relying on precise automated Warp2D retention time alignment module [2].
MSI is an analytical platform, which can provide distribution of administrated intact drug and their metabolite in tissue section and enables to measure if a specific drug has reached the target location with sufficient concentration and allow to assess local tissue toxicity. The presentation will demonstrate the design of data structures, which allows fast automatic data driven ion image extraction from profile MSI data without data reduction [3] and shows the importance of an automated spectral alignment algorithm to correct m/z shift to enhance mass accuracy and improve quantification of compounds from extracted ion images [4].
Example applications to identify administrated Ochratoxin and its metabolite in rat urine using LC-MS/MS as well determination of tiotropium in rat lung and various drugs used for targeted cancer therapy in rat liver will be shown.

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Rapid screening by directly coupling sampling/sample preparation devices to mass spectrometry

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Recent trends in clinical and pharmaceutical sample preparation include shift towards automation, high-throughput, miniaturization, and extraction methodology with low or no solvent consumption facilitating on-site screening ("green chemistry") [1]. To accomplish this objective frequently microextraction approaches using sorbents combined with chromatography hyphenated with mass spectrometry or directly with mass spectrometry are being developed [1,2]. Further improvement in speed can be achieved by eliminating liquid chromatographic separation step and introducing the extracted sample directly to mass spectrometry. Reduction of even elimination of ion suppression effects in such ambient ionization approach is a result of an optimum extraction coating design, when using solid phase microextraction technique of sampling/sample preparation [1,3,4]. In the presentation we will discuss diverse SPME-based devices recently developed in our laboratory for the extraction/enrichment of analytes of interest from tissue and small volumes of complex sample matrices, which can be directly coupled with mass spectrometry instruments for rapid analysis. The approaches tested include Coated Blade Spray (CBS), SPME-transmission mode-direct-analysis-in-real-time (SPME-TM-DART-MS), SPME- microfluidic open interface (SPME-MOI). Total analysis time was typically 5 minutes, but did not exceed 15 minutes and sample volumes ranging between 1-100 µL to the whole organs were used. When 96 extractions are simultaneously conducted the optimized direct coupling workflow allows for rapid and high-throughput screening and quantitation of pharmaceutical drugs in range of matrices in both negative and positive ionization mode in one single run using a single CBS device with analysis times as short as 1 min per sample. Sampling/sample-preparation is performed either by spotting the sample onto the SPME-device, or by immersing the SPME-device in a vessel containing the sample or placed directly into tissue. Despite short extraction times, limits of detection in the sub-ng/mL range were obtained, while good accuracy, and linearity were attained for all the studied probes (e.g. therapeutic-drugs, drugs of abuse, and immunosuppressants) in the diverse matrices scrutinized (e.g. urine, plasma, blood, saliva, brain, muscle and other tissue). A new concept involving the extracted blood spots as replacement of the dry blood spots approach for rapid screening of the body fluid samples will be discussed.

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Electroanalytical Carbon Based Nanosensors: Their potential use in the life sciences and clinical medicine

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Nanomaterials have taken their place in many fields such as sensor development, biomedical, and environmental applications since they first appeared in the scientific literature. Thanks to carbon, an element that stands out with its versatility, carbon-based nanomaterials (graphene and derivatives, carbon nanotubes, nanodiamonds, etc.) are not only well-established nanomaterials that have been used for many years but are still a developing group [1, 2]. It is aimed to make the analysis more sensitive, selective, fast, and effective by using many different materials in the development of sensors, which are analytical devices that convert an analytical response into a measurable signal. In this context, carbon-based nanomaterials are preferred as modification agents in electrochemical sensors due to their exquisite physical, chemical, and electrical properties. Especially with their high active surface area, great electrical conductivity, and stability carbon nanomaterials-based electrochemical sensors offer lower limit of detection (LOD) values, higher sensitivity, repeatable and reproducible analysis with good stability for the determination of a wide variety of analytes [3].

When the studies in the literature are examined, we see that it is aimed to obtain a synergistic effect by using carbon nanomaterials together in order to increase the efficiency of the electrochemical sensor. In addition, hybrid platforms created by using carbon nanomaterials with other nanomaterials and materials such as composites and polymers are currently preferred. When all these advantages are added to the affordability, user-friendliness, environmental friendliness, and rapidness of electroanalytical methods, it can be said that superior analysis platforms are obtained for analytical determination [4].

Nowadays, the researchers focus on the development of novel sensor platforms that meet the need for portable, miniature, point-of-care, and practical analytical devices for the determination of numerous analytes, especially in clinical applications.

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Interest of unified chromatography (UC) to analyze polar biomolecules in pharmaceutical products

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Unified chromatography (UC) with pressurized carbon dioxide in the mobile phase is joining supercritical fluid chromatography (SFC) and liquid chromatography (LC) in a single experiment, with a wide gradient elution possibly ranging from 100% CO_2 to 100% liquid mobile phase. This allows to retain and separate analytes with a wide range of polarities, from the least polar (soluble in CO_2) to the most polar (soluble in hydro-organic liquids). Hyphenation to electrospray ionization - mass spectrometry (ESI-MS) is easily done and usually favored by CO_2 depressurization.

In this presentation, we will expose the fundamentals and technical constraints of UC methodology and illustrate with varied examples. In recent works, we have applied this strategy to analyze natural and synthetic biomolecules of pharmaceutical interest, namely flavonoids[1], amino acids[2], small and large peptides[3], nucleobases and nucleosides. In particular, the complementarity to more classical reversed-phase liquid chromatography (RPLC) methods will be discussed.

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Multi-dimensional HPLC analysis of chiral amino acids in complicated matrices including clinical, food and extraterrestrial samples

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All of the proteinogenic amino acids, except for Gly, have the chiral center and the enantiomers (L- and D-forms) are present chemically. In the living beings on the other hand, L-enantiomers are predominant and D-amino acids are long believed to be absent especially in higher animals, at least as the physiologically active molecules. Along with the progress of analytical technologies these days, several D-amino acids were found even in mammals including humans, and their physiological functions and diagnostic values were also reported. In order to evaluate their usefulness in health/medical areas and also to clarify their origins, chiral amino acid analysis in a variety of samples including clinical, food and extraterrestrial samples are expected. However, the amounts of D-amino acids in the real world samples are extremely low in most cases, and the real sample matrices contain uncountable interfering substances. Therefore, the development/utilization of highly sensitive and selective analytical method is practically essential. In the present study, amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) for the highly sensitive fluorescence detection, and the NBD-amino acids were analyzed using the multi-dimensional HPLC technique for the highly selective analysis. For the multi-dimensional HPLC analysis, several separation modes including reversed-phase, anion-exchange, mixed-mode and enantioselective separations were integrated. The first dimension is normally the reversed-phase mode, and the amino acids are separated as their scalemic mixtures by the hydrophobicity. The fractions containing the target analytes are collected, and are transferred to the next dimension. The final dimension is always the enantioselective mode, and the D- and L-amino acids are separately determined. For the higher selective analysis, MS or MS/MS systems are also able to be used for the determination. By using these multi-dimensional HPLC systems, various D-amino acids (D-Ser, Asp, Ala, Asn etc.) were found in the plasma/urine of mammals, and the relationships between their amounts and progression of the diseases were observed for some of the D-enantiomers. In the food/beverage samples, D-amino acids including D-Ala, Asp, Glu, Leu were observed especially in fermented products, and the amounts were altered depending on the fermentation/aging processes. In the extraterrestrial samples, various non-proteinogenic amino acids were found, and these non-proteinogenic amino acids were mostly racemic (D/L = 50/50). These results indicate that the D-amino acids are frequently present in our daily lives/environments, and further studies to clarify their physiological significance and diagnostic values are ongoing.

Peptide quantification at the picomolar level in volume-limited biological samples: obstacles to tackle

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In order to investigate the potential role of neuropeptides in neurological disorders, their endogenous concentrations are monitored in the rodent brain using *in vivo* sampling techniques such as microdialysis and miniaturized ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The low extracellular concentrations (low picomolar range) of these peptides, their non-specific binding, the small sample volumes (≤ 20 µL) and the complexity of the matrix make the quantification of these neuropeptides in microdialysis samples challenging. To achieve maximal sensitivity, the sample preparation, the UHPLC and the MS/MS parameters need to be optimized.

Several challenges when developing methods for the quantification of small neuropeptides (≤ 10-13 amino acids) in microdialysis samples were defined and some strategies to overcome these challenges were optimized. To start, a strategy to improve method sensitivity by intervening on all aspects of standard preparation, i.e. from dissolution of the powder until the injection of the sample, was developed [1]. Furthermore, the sample composition in the UHPLC vial was optimized by using design of experiments. Reduction of peptide adsorption and optimization of the injection solvent resulted in a clear and quantifiable signal for the peptides under investigation. In addition, gradient slope, flow rate and temperature of the analytical column were optimized to obtain the highest sensitivity and the lowest carry-over [2]. The optimized assay allowed for the accurate and precise quantification of low picomolar concentrations (0.5-3 pM) of these small neuropeptides in *in vivo* microdialysis samples [2]. Importantly, it was observed that the unique nature of every peptide analyte requires a tailored investigation.

In a next step, the quantification of two larger neuropeptides containing respectively 23 and 36 amino acids, neuromedin U (NmU) and neuromedin S (NmS), was envisaged using the strategies previously developed. A truncated form of NmU (NmU-8) was also included in the study. In contrast to small peptides such as NmU-8, the addition of adsorption competitors such as plasma, bovine serum albumin (BSA) or BSA digest is needed to allow detection of NmU and NmS, even at 1 nM level. Furthermore, the type of sample vial, dissolution and dilution solvents and the pipetting protocol were optimized. Next, some UHPLC parameters, including the stationary phase of the analytical column, the column temperature and the trapping conditions were investigated.

All optimizations clearly improved the method sensitivity for NmU and NmS, although further improvements are still required to allow the quantification of these neuropeptides in in vivo samples.

In conclusion, challenges are encountered when analysing larger neuropeptides, including non-specific binding, poor peak shape and low MS sensitivity. The addition of an adsorption competitor was found primordial to avoid peptide loss due to non-specific binding.

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Microscale strategies for Glycosylation analysis of Biopharmaceuticals and Disease Biomarkers

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Glycosylation is a quality attribute of many biopharmaceuticals and especially monoclonal antibodies. In addition, abnormal protein glycosylation is related to several pathologies, and the corresponding glycoproteins (GP) may constitute a source of disease biomarkers to be used for diagnostic assays. Glycan mapping is one of the most efficient approaches to detecting minor glycosylation modifications. This strategy nevertheless suffers from tedious, multistep and manual protocols that are time-consuming and hardly reproducible.. Capillary electrophoresis (CE) coupled with laser-induced fluorescent (LIF) detection has become an established technique for glycan analysis and mapping, mostly for N-glycans. This goes with heavy forefront sample treatment for enzymatic release of glycans from glycoproteins, their derivatization with a fluorophore (8-aminopyrene-1,3,6-trisulfonic acid, APTS), purification of labelled glycans from the excess of APTS and their elution for subsequent CE-LIF analysis.

We explored in our group and in parallel two different approaches to facilitate the glycan analysis and its sample pretreatment. The first one relies on operations performed in a capillary and the second on droplet microfluidic for releasing the N-glycans, labelling them and analysing the sample by CE-LIF.

In this keynote lecture, I will first present some of our achievements regarding the development of a monolith-based microreactor photopolymerized inside a silica capillary to release O-glycans of glycoproteins [1]. In addition, an approach based on the transverse diffusion principle (TDLFP) to perform the in-capillary glycan labelling with APTS prior to their on-line separation by CE-LIF will be also presented [2]. By using an electrokinetic preconcentration based on a large volume sample stacking electrokinetic preconcentration, we could also significantly increase the detection sensitivity of the CE-LIF analysis of N-glycans from serum [3]. In a different piece of work, we developed a microfluidic droplet platform, for the multistep glycoprotein sample preparation to obtain labelled N-glycans. We demonstrated the superiority of this microfluidic approach by employing magnetic beads to transfer the analyte from one step to another one over the conventional batchwise protocol, with 10-fold less reagent consumption, 3-fold operating time reduction, and 2-fold improvement of glycan labelling yield [4].

The different reported approaches were applied to several glycoproteins (IgG and Rituximab) and for biomarker analysis in the case of the congenital disorder of glycosylation.

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ORAL CONTRIBUTIONS

LAQUINIMOD SODIUM CAPSULES, 0.6 MG: ANALYSIS OF A FOREIGN “SMALL CAPSULE” FOUND IN A CONTAINER OF THE BATCH FOR CLINICAL SUPPLY

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One "small capsule", different by its size and weight, was found among "bigger", regular capsules of Laquinimod, 0.6 mg, in one of the bulk containers at a Laquinimod clinical trials site in Europe. A real “detective story” occurred, to understand what it may be and how it came to the Teva’s bulk container. All we had – just 61 mg of “white powder” removed from this suspicious object. Comparative analysis of the content of a foreign "small capsule" and the regular capsule was performed, based on in-house HPLC methods using tandem UV and MS detection. It was found, that this capsule contained an unknown API, very similar by its structure and properties to Fingolimod (Gilenya® of Novartis). Chemical structure of this API has been established. Its possible manufacturer has also been uncovered.

Important results of this investigation:

A concern about batch mixing at Jerusalem facility of Teva (as the major GMP violation) has been removed

A new, earlier unknown to Teva, competitor in the field of oral MS treatment has been revealed

N-nitrosodimethylamine (NDMA) contamination of pharmaceuticals: ranitidine as a case study and the lessons learned

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Abstract

Nitrosamine molecules are considered carcinogenic and mutagenic chemicals by many safety and toxicology agencies. Unexpectedly, nitrosamine impurities have appeared in pharmaceuticals since 2018. Nitrosamines were firstly discovered in high blood lowering drugs “sartans” followed by ranitidine and then metformin. Unfortunately, additional cases of drug contaminations have been reported at least in the last year. N-nitrosodimethylamine (NDMA) is the most frequently encountered nitrosamine impurities. The analytical techniques utilized to measure this nitrosamine in sartans included High Performance Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectrometry (LC-MS) and Gas Chromatography Mass Spectrometry (GC-MS). However, not all those techniques were effective and accurate enough to analyse the same impurity in ranitidine medication due to intrinsic characteristics of ranitidine molecule. The case of ranitidine contamination by NDMA is interesting and in the current overview, I will critically analyze this topic in a narrative way according to our most recent works as well as the relevant literature data. Firstly, I will revise the literature information confirming ranitidine association with nitrosamines. Secondly, I will highlight the documented mechanisms for NDMA release from ranitidine, with their relevant analytical methods for detection. Thirdly, the stability issue for this medicine is briefly discussed. Fourthly, I will review and discuss the laboratory results released by regulatory authorities represented by the United States Food and Drug Administration (US FDA), Saudi Food & Drug Authority (SFDA) and the Australian Therapeutic Goods Administration (TGA) concerning testing of ranitidine products and the detected nitrosamine levels. Finally, the case of nitrosamine generation in Sartans is compared with that of ranitidine, in an attempt to address the conditions leading to the current contamination. Collectively, understanding analytical challenges encountered and the contamination circumstances might provide learning lessons to aid in mitigating drug-related impurities in order to protect consumers.

Evaluation of the delivered and the fine particle doses in different pharmaceutical formulations of dry powder inhaler containing formoterol

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Formoterol is known as a long-acting ß-agonist indicated in asthma and chronic obstructive pulmonary diseases treatments. Formoterol-based formulations are locally administrated in the lungs by oral inhalation.

A total of 8 capsule-based dry powder inhalers containing formoterol fumarate, marketed over the European and North African markets were involved in the present study, including the reference drug Foradil®. This work assessed and compared them in terms of aerodynamic performance, considering that only the fraction capable of reaching the lungs can provide therapeutic effects to patients. All studied medicinal products have a unit dose of 12 µg of formoterol fumarate and are all equipped with an Aerolizer®-like inhaler. However, they differ in the capsule composition and the packaging.

Three independent tests were performed for each evaluated drug. The assays were carried out using the standard procedures of the European Pharmacopoeia 0671 and 2.9.18 to determine respectively the delivered dose and the fine particle dose employing a multistage liquid impinger. After preparation, samples were further analyzed by a validated HPLC-UV method. Moreover, the current study also examined the impact of freezing-thawing cycles on the stability of samples concerning analytical purpose handling: investigation of the reanalysis capability in routine activities.

Among these studied pharmaceuticals, the aerodynamic profile varies from one product to another. Additionally, as expected, this work confirmed that the composition of hard capsules and the barrier properties of the primary packaging can affect the fine particle dose of capsule-based dry powder inhalers. A greater respirable fraction can be achieved using hydroxypropylmethylcellulose capsules and moisture-impermeable packaging as the primary barrier to water vapor.

Chemical profile of the marine sponge *Topsentia ophiraphidites* from Fernando de Noronha Archipelago (Brazil)

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Topsentia ophiraphidites is a marine sponge found on the Brazilian coast and in the Caribbean.[1] Organic extracts of this sessile organism have showed potential cytotoxic (HCT-116 cell line) activity and acetylcholinesterase inhibition. Few chemical studies deal with *T. ophiraphidites* collected from Brazil coast. Herein, we report the annotation of the chemical profile of samples collected in Fernando de Noronha Archipelago, PE, Brazil. To this end, the organic extracts were analyzed by LC-HRMS (positive ionization mode) and the data were upload to the Global Natural Products Social Molecular Networking (GNPS) platform [2]. The created classical molecular networking (Figure 1) had about 3142 nodes, distributed in 265 molecular families and 1768 self-loops. Dereplication process by comparison with MS/MS data from the GNPS platform allowed the annotation of 52 molecules, and the manual dereplication allowed the inference of further 99 molecules. The chemical profile of the sponge showed a diversity of classes of primary and secondary metabolites: amino acids, halogenated amino acids, alkaloids of indole, imidazole, quinolic, and phenazine classes, dipeptides, diketopiperazines, nucleosides, acylcarnitine, polyketides, phenolic derivatives and glycerophospholipids. Of the annotated metabolites, only two had been report in the literature for the genus *Topsentia*: 4-hydroxybenzaldehyde and 3-indole-carboxaldehyde. Therefore, the chemical information obtained for the non-target analysis of organic extract samples of *T. ophiraphidites* collect in Fernando de Noronha, Brazil, demonstrates a great structural diversity and the presence of several compounds that were not yet reported for *Topsentia*, contributing to increase the chemical knowledge of the genus.

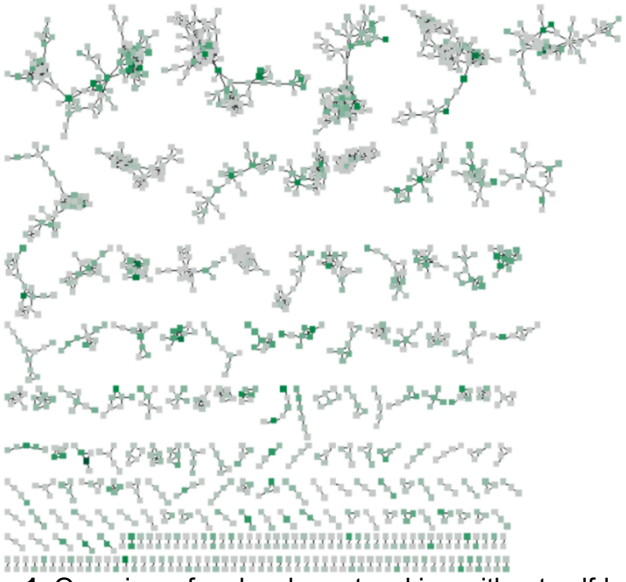

Figure 1. Overview of molecular networking without self-loops.

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Determination of Bisphenol A as contaminant in microalgae by GC-MS

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Microalgae are sources of valuable bioactive compounds such as vitamins, proteins, essential amino acids, polyunsaturated fatty acids, minerals, carotenoids and enzymes. [1] Thus, to date, microalgae are introduced in food, cosmetic, animal feed, pharmaceutical and biotechnological fields. [2] Microalgae, both in their natural habitat (fresh or salt water) or in artificial cultivation plants (photobioreactors and open ponds) [3], grow in contact with various micropollutants. [4] These include Bisphenol A (BPA), which may be released as a result of mechanical stress, thermal and UV-mediated degradation of polymeric plastics (polycarbonate and epoxy resins), which may compose bioreactors. BPA may be present in algal cells as a contaminant and it is classified as an endocrine disruptor due to its estrogenic and antiandrogenic properties. As an endocrine disruptor, BPA is responsible for toxic effects on the reproductive, central nervous, cardiovascular, immune, respiratory and renal systems. [5] Therefore, the pollution of the oceans and rivers caused by plastic waste and wastewater [4], as well as the potential deterioration of the artificial microalgae cultivation systems in polycarbonate, are the main causes of the presence of BPA inside algal cells.
According to the European legislation, the legal BPA limit within foods is 0.05 mg Kg^{-1} of food weight, therefore in this work, an efficient, sensitive and selective analytical method was developed for the determination and quantification of BPA in microalgae. [6] Commercial spirulina algae samples were analyzed for the determination of BPA. First, a fast and inexpensive liquid-solid extraction of BPA from the lyophilized algal powder was optimized, obtaining a quantitative recovery of BPA by analyzing spiked samples. Then, the extracted BPA, in presence of BPA-D16 as internal standard, was derivatized by modifying a procedure described by Ralph N. Mead et al. [7], and analyzed by GC-MS. The selectivity of the method was increased by employing the SIM mode.
The internal standard GC-MS method was validated by determining all the validation parameters: selectivity, linearity, precision, accuracy, recovery, LoD and LoQ. Indeed, LoD and LoQ values were respectively 2.1 ng mL^{-1} and 7 ng mL^{-1} and the calibration curved demonstrated a R^2= 0.9999.
The validated method demonstrated to be selective, sensitive and therefore applied to the determination of BPA in microalgal samples, also considering its use in the analysis of waste from microalgae processing.

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Evaluation of Cocoa Bean Shell Antimicrobial Activity and Tentative Active Compound Identification through Metabolomic Analysis

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The cocoa bean shell is the external tegument that covers the cocoa bean. It represents 10%-17% of the total cocoa bean weight and it is usually discarded after the cocoa bean roasting and husking, being one of the main by-products of chocolate manufacturing. Nevertheless, it possesses several compounds with biofunctionalities [1]. It can function as an antibacterial agent, and its action is mostly reported against *Streptococcus mutans*, a well-known bacterial strain associated with dental caries. However, only a few studies have investigated the cocoa bean shell compounds responsible for this activity. This study aimed to evaluate several extracts of cocoa bean shells from different geographical origins and cocoa varieties and to estimate their antimicrobial properties against different fungal and bacterial strains by determining their minimal inhibitory concentration. To do so, we employed the broth microdilution technique in 96-well plates [2,3]. The results demonstrated antimicrobial activity of cocoa bean shell against one of the tested strains, *S. mutans*. Contrariwise, the extracts seemed to boost the growth of *Saccharomyces cerevisiae*. Cocoa bean shell extracts were further analyzed via LC-HRMS for untargeted metabolomic analysis. LC-HRMS data were analyzed (preprocessing and statistical analyses) using the Workflow4Metabolomics platform, which is an online and freely available collaborative platform dedicated to metabolomics data processing [4]. The latter enabled us to identify possible compounds responsible for the detected antimicrobial activity by comparing the more and less active extracts.
Unexpectedly, the most active extracts were not the most abundant in polyphenols but rather contained higher concentrations of two metabolites. After tentative annotation of these metabolites, one of them was identified and confirmed to be 7-methylxanthine. When tested alone, 7-methylxanthine did not display antibacterial activity. However, a possible cocktail effect due to the synergistic activity of this molecule along with other compounds in the cocoa bean shell extracts cannot be neglected. Other tentative annotation possibilities were carried out for the second metabolite, but its identity must still be confirmed, and the molecule should then be tested against *S. mutans* to confirm its activity. In conclusion, cocoa bean shell could be a functional ingredient with benefits for human health as it exhibited antibacterial activity against *S. mutans*. However, the antimicrobial mechanisms still need to be confirmed.

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Applications of vibrational spectroscopy and hyperspectral imaging for the analysis of substandard and falsified medicines.

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Access to quality medicines is an essential right of the patients. However, in 2017, the World Health Organization estimated that 1 in 10 medical products circulating in low- and middle-income countries is either substandard or falsified. This reinforces the fact that post-marketing surveillance (PMS) of medical products by strong national regulatory authorities (NRA) is crucial. To achieve an efficient PMS, the NRA need analytical tools at the inspection, screening, confirmatory and forensics levels to control the physicochemical properties of the samples.

Because of their fast, non-destructive, and relatively affordable character, vibrational spectroscopy tools are unavoidably present at each step. Handheld devices are particularly useful during inspection and screening phases since these tools can identify and/or quantify active pharmaceutical ingredients (API) even through opaque packaging in seconds. However, they generally need exhaustive and up-to-date databases for each specific product. Another limitation is the work and time needed before going into the field to develop and validate the chemometric models. Indeed, this mandatory step requires highly skilled scientists and a prior collection of certified references of the medicines to analyse.

Benchtop systems and among them imaging systems are particularly useful in the confirmatory and forensic steps. Indeed, the imaging systems enable the visualization and identification of a large range of both organic and inorganic compounds used as API or excipients. In addition, thanks to the high spatial resolution, it allows the detection of trace contaminants. This information may be of particular interest during prosecutions and the clustering of criminal cases. Nevertheless, the extraction of the relevant information from the raw measurements requires once again intensive work by highly trained staff.

In conclusion, vibrational spectroscopy tools have particularly interesting features for the PMS of medicines, but research is still needed to make them easier to set up and use by NRA inspectors and non-specialists.

The national quality improvement program on compounded medicines by the Association of Pharmacists Belgium: how analytical sciences can promote patients' health.

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Pharmaceutical compounding is the most convenient way to produce a pharmaceutical product that fits the unique need of a patient. It is a routine activity for hospital and community pharmacists. Whereas an immense number of patients have already benefited from compounded preparations, their quality is sometimes questioned. This argues for the implementation of higher production standards and better quality management systems in pharmacies. [1]

A few years ago, the Belgian professional organizations of community pharmacies have initiated a quality program for compounded preparations. Formulations are selected based on the following criteria: frequency of prescription, preparation difficulty and risk for the patients. Up to one hundred community pharmacists can register voluntary for a cycle. After a thorough quality check of the preparation, an individual report is sent to each participant and the global results are communicated to the whole sector.

Since 2017, the quality of more than 3000 compounded preparations sent by more than a thousand different hospital and community pharmacies, has been assessed through this program. Depending on the galenical form, a careful selection of the critical analytical parameters to be tested is made for each cycle. The identification and the assay of the active ingredient is performed most of the time by HPLC-PDA but other techniques such as titration or atomic absorption can also be used. In general, a method needs to be developed and validated for each cycle, taking into account the different possible differences that can exist in the formulations for a given compounded preparation. This ensures that the developed method can be used for all the products that will be sent by the program participants, regardless of differences in the excipients they use. Where relevant, the microbiological quality of the preparations is also evaluated according to current pharmacopoeial requirements (enumeration, absence of specified microorganisms or sterility).

Several conclusions can be drawn about the quality of the compounded preparations in Belgium thanks to the large volume of analytical data generated by the program. These conclusions help the Association of Pharmacists Belgium to raise awareness amongst pharmacists on the importance of the quality of their compounded preparations, for the benefit of patients' health.

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Quality control of paracetamol generic tablets marketed in Benin and search of its two impurities p-aminophenol and p-nitrophenol

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In Benin, several studies confirm the circulation of substandard medicines. One of the most important therapeutic classes is represented by analgesics and antipyretics, among which paracetamol is one of the most consumed products in the world. It is low cost and very accessible. During chemical synthesis, it can be accompanied by impurities such as *p*-aminophenol (PAP) and *p*-nitrophenol (PNP), which have nephrotoxic and teratogenic effects.

In this work, we evaluated the quality of paracetamol generic tablets while looking for these two impurities. Several quality control (QC) tests were carried out on 94 samples collected by direct purchase at various levels of the medicine supply chain in Benin such as wholesaler distributors, hospital pharmacies, pharmacy dispensaries and village drug stores in the formal sector and in the black markets as well. Those QC tests included visual inspection according to WHO guidelines (focus on differences in packaging, labeling, and physical appearance of tablet characterized by specific size, shape and color), mass uniformity, disintegration test, dissolution test. The LC-DAD was applied for identification and assay of paracetamol, PAP and PNP, under the following conditions: column (250 mm x 4.0, mm i.d) containing LichroCart C18 (5µm d*p*); mobile phase (MeOH/ 10mM ammonium acetate buffer pH 6.8 (35/65, v/v) pumped at 1mL/min, detection at 245 nm.

As results, 77.7% of the samples did not comply with the visual inspection, 2.1% did not pass the mass uniformity, 24.3% didn't comply with the disintegration and the dissolution test as well. Paracetamol was identified in all the samples, but only 80.9% complied with the assay (90.0 – 110.0%; USP). None contained PNP (specification < 0.05%, w/w; BP), while 3 contained PAP but below the specification (PAP < 0.1%, w/w; BP). No statistical significant relationship was found between the non-conformities and sampling sites (*p*-value = 0.45).

Generally, the quality control of paracetamol in most African countries included Benin, is based on pharmacotechnical tests and paracetamol assay. This work highlighted the importance to search also other impurities which shall be done particularly for generic in a broader study.

Keywords: Quality control, paracetamol, p-aminophenol, p-nitrophenol, formal and illicit circuits, Benin

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Plasma Metabolomic Profiling of Patients with Primary Biliary Cholangitis

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Primary biliary cholangitis (PBC) is a chronic immune-mediated inflammatory liver disease characterized by the progressive destruction of interlobular bile ducts leading to bile duct injury, ductopenia, and cirrhosis [1]. The etiopathophysiology of PBC is still poorly understood, although the disease is more common in Caucasian middle-aged women and, without treatment, it progresses to end-stage liver disease demanding liver transplantation. In most cases, patients experience pruritus, fatigue, decreased bone mineral density, hyperlipidemia, and xanthelasma, which can be also associated with other immune diseases, including Sjogren's syndrome, Hashimoto's thyroiditis, and/or rheumatoid arthritis [2]. The serological hallmarks of PCB are the anti-mitochondrial antibodies (AMA), which are detected in 78-90% of patients [3]. Contradictorily, AMA can also be detected in 0.1-0.5% of supposedly healthy subjects or in patients with other autoimmune liver diseases, mostly autoimmune hepatitis (AIH). Moreover, it is well recognized that AMA-negative accounts for 5–15% of patients with PBC worldwide, leading to potential misdiagnosis and under-treatment of the actual disease [3]. Diagnosis of PBC uses a combination of clinical features, abnormal liver biochemical, autoantibody testing, and liver imaging to exclude extrahepatic biliary obstruction [4]. Therefore, because many autoimmune diseases are associated with PBC, improvement in the diagnosis for detection of early-stage disease is essential to reduce disease burden and complications. In the present study, we applied a liquid chromatography-high resolution mass spectrometry (LC-HRMS) metabolomics approach to identify distinctive signatures in plasma samples from PBC *versus* healthy patients (control group). Plasma samples of PCB patients (n=30) and healthy controls (n=20) were analyzed using an untargeted metabolomic profiling approach. The results showed the identification of 51 plasma metabolites that differed significantly ($p \leq 0.05$) from PBC and the control group. Classification analysis using supervised hierarchical clustering was able to distinguish the metabolic profile of patients with 95% accuracy, reflecting lipid metabolism as the main contributor. Metabolites related to the metabolism of glycerophospholipids, fatty acids biosynthesis, pyruvate metabolism, bile acids biosynthesis, porphyrin, and pyrimidine metabolism were identified as discriminating metabolites. As a result, metabolites related to bile acid metabolism, lipid metabolism, and amino acids were identified. These PBC-related metabolites could advance disease biomarkers and assist in understanding the potential pathogenic mechanism of PCB.

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Struggling to make it to the egg: metabolomics of seminal liquid to understand human fertility decline

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Over a half of humans live in regions where birth rates declined over the last decades below the levels needed to sustain populations [1]. One of the reasons for that is the steady drop in semen quality, paralleled by an increasing incidence of testicular cancer. Since ejaculate contains spermatozoa together with all the secretions from male genital accessory glands, it grants access to the direct phenotypic exploration of disorders affecting such organs.

A cross-sectional study was conducted on 2523 Swiss young males which provided seminal liquid, blood serum and urine samples [2]. Spermatozoa characteristics and concentration were analyzed using a computer assisted sperm analyzer (CASA). Since the link between male human fertility issues and steroidal endocrine disrupting chemicals has been well-stablished, an in-depth steroid profiling was seeked. For that purpose, we developed and validated a new SPE sample preparation protocol targeting steroids, conjugate steroid metabolites, and other low-to-medium polarity compounds from 100 µL-samples. LC separation was conducted under standard RP conditions and untargeted HRMS detection in positive ESI mode [3]. Annotation was done by matching features to accurate masses and experimental retention times (197 steroids, 29 acylcarnitines, 65 lipid mediators, and a collection of 700 other endogenous metabolites) using Progenesis QI.

A sub-set of 210 samples was created from extreme good or bad sperm quality groups (evaluated as concentration or morphology abnormalities). After annotation more than 200 metabolites, Uninformative Variable Elimination was used to remove irrelevant signals before building OPLS and random forest models. Our results highlighted the role of over 80 metabolites positively (acylcarnitines, retinol) or negatively (retinoic acid, oestrogens, prostaglandins) related to sperm count, in agreement with their biological relevance in sperm maturation, viability and survival. This study paves the way to using seminal liquid as a new diagnostic and mechanistic research tool in human male infertility.

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Development of a bioassay-guided fractionation protocol to unveil toad venoms as a new source of potential antimalarial candidates

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Malaria is among the deadliest parasitic diseases infecting humans worldwide. In 2020, the World Health Organization (WHO) reported 241 million cases and approximately 647,000 deaths. These figures have risen since 2019 partly due to the various disruptions caused by the COVID-19 pandemic. The fight against this disease is rendered more difficult by the emergence and spread of drug-resistant strains. The need for new therapeutic candidates is now greater than ever [1]. In this study, we investigated the antiplasmodial potential of compounds contained in toad venoms. The wide array of bioactive compounds present in *Bufonidae* venoms has allowed researchers to consider many potential therapeutic applications, especially for cancers and infectious diseases [2]. We focused on the small molecules, namely bufadienolides and alkaloids, found in the venoms of three species from the *Bufonidae* family: *Rhinella marina*, *Bufo bufo* and *Incillius alvarius*.

The present work aimed to develop a bioassay-guided fractionation process that would allow the identification of potentially antiplasmodial compounds in toad venoms. This approach combined various analytical and separative techniques along with a series of biological assays. The developed bio-guided fractionation process comprises a solvent-based extraction process. Fractionation techniques included flash chromatography and preparative thin-layer chromatography. This step allowed to generate fractions and, in some instances, sub-fractions. All samples were characterized using chromatographic, spectrometric, and spectroscopic techniques and then underwent testing on *in vitro Plasmodium falciparum* cultures. Two strains were considered: chloroquine-sensitive strain 3D7 and chloroquine-resistant strain W2. Samples displaying sufficient antiplasmodial activity that were then assessed for their toxicity on normal human cell lines and on human red blood cells. The whole process allowed to highlight several crude extracts and fractions displaying inhibitory activities against the laboratory-adapted strains. Within these samples, resibufogenin was identified as the most promising compound. Though its toxicity remains high, it could offer a solid basis as a pharmacophore for future antimalarial drug design. We also discussed the possibility of resibufogenin binding with the novel antimalarial drug target (*PfATP4*) through molecular modelling [3].

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Harnessing native MS and AS-MS to develop new therapeutics for challenging targets

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OMass Therapeutics uses different mass spectrometry (MS) techniques to develop new therapeutics for rare and challenging targets. We deploy native MS in synergy with LC-MS based techniques to identify small molecules that modulate the function of intricate biological system[1]. Recently, we have developed an affinity selection MS (AS-MS) platform that can identify binders with wide range of affinities from a mixture. Selectively, these compounds are analysed by LC-MS and their binding affinity is measured by the ratio bound/unbound intensities for target and control protein, respectively. AS-MS not only has enabled us to find hit candidates from our internal library, but also has helped to determine binding affinities (Kd), dissociation rates (Koff) and hint ligand localisation by competition assays. Here we present two examples where this platform played a major role: 1) KCC2 from hit finding to binding affinities; 2) MC2 binding kinetics for an insurmountable ligand.

KCC2 is a solute carrier membrane protein that regulates the potassium/chloride transport in neurons and keeps the chloride homeostasis. Therefore, downregulation of KCC2 disrupt the ion transport balance in nerve cells leading to Rett syndrome. We are aiming to find a small molecule potentiator of KCC2 in order to provide treatment for patients Rett syndrome patients. Using AS-MS and native MS have screened our internal drug library (>150k compounds) and have identified several ligands which bind specifically to KCC2, a key milestone in our discovery pipeline.

MC2 is a GPCR membrane protein that mediates the actions of ACTH in adrenal gland. Patients with congenital adrenal hyperplasia (CAH) have high secretion of ATCH, as a result finding small molecules that can antagonise MC2 at high concentration of ATCH would result in effective treatment for CAH. Using AS-MS we have identified small molecules that bind to MC2. Furthermore, we have developed two new methods: 1) a direct ligand competition assay to capture how our compounds compete with ACTH; 2) a kinetic binding assay to identify compounds with slow Koff that will perform better in antagonising ATCH binding to MC2. Taken together using LC-MS based methods and native MS we have been able to not only identify binders to difficult membrane protein targets, but also provide insights in their mode of action to enable the design of novel pharmaceuticals.

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Beta-secretase1 and Acetylcholinesterase co-immobilized enzyme reactors: Models for dual-ligand screening

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The β-secretase1 (BACE1) and acetylcholinesterase (AChE) are enzymes directly related to the development of neurological diseases such as Alzheimer's Disease (AD) and are relevant biological targets in the development of new drugs for AD. [1,2] The complexity and multifactorial nature of AD have led to an understanding of the need to identify inhibitors that act on multiple biological targets, which requires the development of a dual system assay to screen multi-target directed ligands that are more fitting to AD treatment. To contribute to this field, we developed two screening assay models based on AChE-hu and BACE1 enzymes co-immobilization: 1) the online activity screening assay by LC-MS system[3] and 2) the offline ligand-fishing approach. For the online approach the mixture of enzymes AChE-hu and BACE1 was covalently co-immobilized on the inner surface of a fused silica capillary. The procedure encompassed two steps and random immobilization. The resulting huAChE+BACE1-ICER was characterized by using acetylcholine (ACh) and JMV2236 as substrates. Analysis was performed in series for each enzyme. To validate the system, galantamine and a β-secretase inhibitor were employed as standard inhibitors, which confirmed that this assay was able to identify reference ligands and to provide quantitative parameters. The best conditions for the dual enzymatic system assay were evaluated and compared to the individual enzymatic system assays. 60 compounds (PQM codification) synthesized as AChE and BACE1 inhibitors were screened comparing the AChEhu inhibition activity using AChEhu-ICER-IND (AChEhu individually immobilized) and in the co-immobilized system (AChE in ICER-AChEhu+ BACE1). From that, 18 compounds were selected to screening BACE1 inhibition activity comparation by individually immobilized BACE1-ICER-IND and co-immobilized system ICER-AChEhu+BACE1. It was possible to observe differences in the percentages of inhibition. For example, the compound PQM 188, in BACE1-ICER-IND, showed 60.89% of inhibition. However, screening for BACE1 in ICER-AChEhu+BACE1 resulted in 11.99%. For AChEhu the inhibition was 24.44% and 31.80%, respectively. This result suggests that in the presence of two enzymes, it may have an affinity for both inhibiting them, and also a demonstrated preference for one decreasing the activity for other. For the offline approach the mixture of enzymes was covalently attached via amine-glutaraldehyde reaction on magnetic beads by random immobilization to produce the IMER-MB.[4] A mixture of caffeine (non-binding), donepezil (AChE -binding) and PQM185 (dual-target-binding) was used to validate the system. A mixture of PQM compounds and a natural extract from *Tabernaemontana litoralis* was evaluated as a proof of concept.

Ackowledgments: FAPESP, CNPq, CAPES

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Affinity capillary electrophoresis for fragment-based drug discovery projects: hit discovery and binding site characterization

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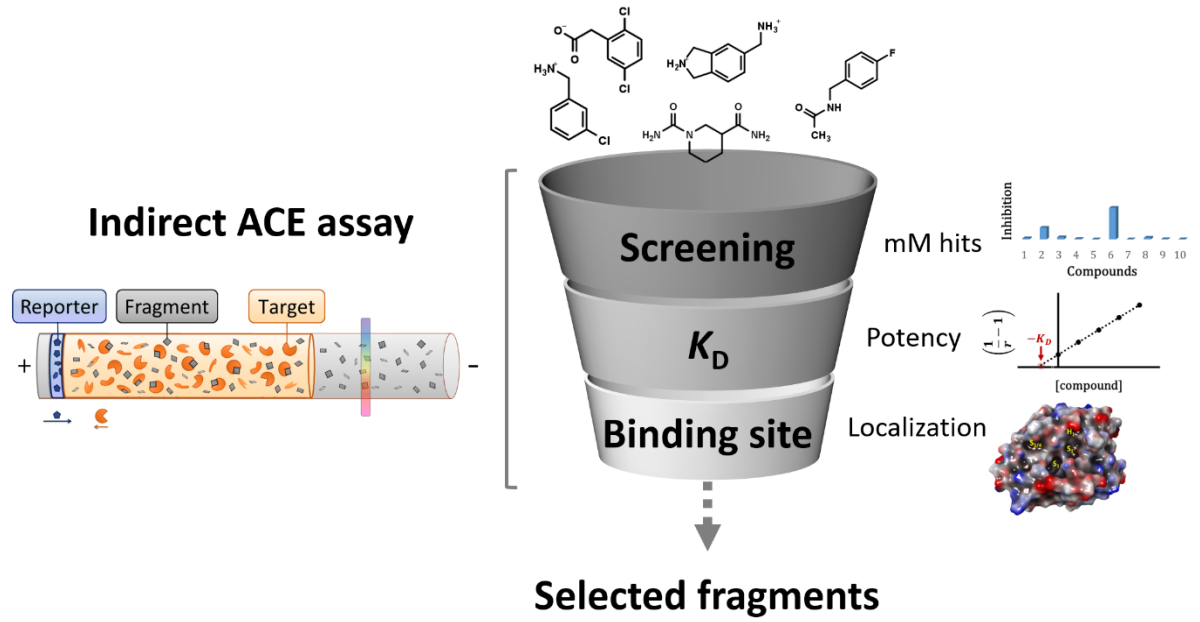
Fragment-based drug discovery is a usual approach to identify innovative lead compounds. This strategy relies on the screening of small-size compounds (< 300 g/mol). These fragments have a weak affinity for the target but explore more efficiently the protein chemical space. For successful applications, this strategy requires the detection of weak binders (µM–mM range) and the characterization of their binding site. NMR and X-ray crystallography can address this challenge and, in consequence, are commonly used in fragment-based drug discovery. However, their accessibility is limited due to their large protein consumption and the cost of equipment. Here, we present an affinity capillary electrophoresis methodology that detects mM binders, determines dissociation constants, and characterizes the fragment binding site. We use the multiple equilibrium theory to model the binding events occurring in the capillary. On the basis of the resulting mathematical models, we determine dissociation constants in the µM–mM range, and we propose an original methodology to establish graphically if two fragments bind the same protein pocket [1].



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Screening of 54 pesticides by mass spectrometry and quantitative validation of 14 pesticides for the urine analysis of endometriosis patients

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The race for overproduction has led to the use of many pesticides with the view to increase crop yields but end up on our plates. Various studies have shown that these molecules are responsible for many metabolic disorders [1]. Exposure to pesticides can induce various diseases through the accumulation of these substances in the human body. In recent years, scientific interest in the potential adverse health effects that may result from human exposure to pesticides has increased. The relationship between exposure to certain pesticides used in agriculture and the risk of developing cancer has been studied, but also the risk of developing endometriosis [2].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are becoming increasingly popular. The advantage of multiple reaction monitoring (MRM) quantification methods is the rapid generation of quantitative data for many analytes in a single analysis [3].

The objectives are articulated in two points: (i) a bibliographical research work in relation to the authorization, their uses, their toxicity. Thanks to this, a selection of 54 molecules was established and their transitions (precursor product ions) were optimised on an LC 1290 coupled to a QqQ 6490 (Agilent Technologies, Palo Alto, CA, USA). (ii) We analysed by screening with the LC-MS/MS method the presence or absence of the pesticides in urine samples after extraction by the QuEChERS method [4], [5]. Twenty-one volunteers (14 people from the countryside, 7 people from the city), 30 women with endometriosis and 30 women without were enrolled.

Preliminary screening results revealed the presence of 14 pesticides in the urine samples of women while 12 were detected in the 21 volunteers. These results also showed that the number of pesticides present in the group of 30 women with endometriosis was significantly higher than in the control group ($p = 0.02$). On average, 7 ± 2 pesticides were determined in the endometriosis group and 5 ± 2 in the controls. In addition, 6 different pesticides were observed only in the diseased women. A validation of the method quantitative are carrying out on the 14 pesticides. The QuEChERS method allows a recovery between 80.8 and 110.9 % and an accuracy range between 5.6 and 20.5 %. A qualitative and quantitative answer can be given for these active substances in the biological samples. These encouraging results prompt us to carry out, a research project involving several hospitals in order to assess the role of those pesticide in endometriosis.

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Assessment of the ocular biodistribution profile of topically administered lutein by magnetic resonance spectroscopy

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The functionality of the eye is kept intact by efficient biological and physiological barriers that restrict intraocular penetration of xenobiotics, including most of the drugs intended to treat intraocular conditions. The choice of ocular drug depends on the targeted area, the bioactive compound's properties, its mode of action and the desired therapeutic effect. Efficiently and non-invasively delivering drugs to the inner segments of the eye continues to pose a challenge in ophthalmology and it is in the focus point of the most recent ocular drug development.
Magnetic resonance imaging (MRI) has evolved into a multifunctional, non-invasive bioimaging tool, serving both in preclinical and clinical environment. It may be used in a safe and non-invasive way for in situ, real-time visualization, high resolution localization, and quantification of ophthalmic drugs upon their administration, with or without contrast agents [1]. However, considering its rather limited sensitivity, MRI is rarely the first-choice analytical option in ocular pharmacokinetics studies [2]. Contrarily to the conventional anatomical imaging by MRI, magnetic resonance spectroscopy (MRS) measures chemical shift information of individual molecules within a living subject, enabling the monitoring of various biochemical and metabolic processes in comparison and upon the administration of a bioactive of interest.
Lutein, an oxygen-containing carotenoid, is present in the human retina and lens. As a natural antioxidant, it plays important roles in reducing the risk of eye diseases, such as age-related macular degeneration (AMD) and cataract, affecting hundreds of million people worldwide. The topical administration of lutein formulated in a bioadhesive hydrogel, significantly increases its chemical stability and ocular bioavailability. Our previous studies on rodent models showed that lutein could play an important role in the treatment and prevention of both cataract and AMD, through release on oxidative stress [3]. However, the time and dose-dependent biodistribution profile of lutein in different substructures of the eye, could not been consistently assessed due to limitations in size and number of biological samples available.
The present study, using a 7 T MRI Bruker Biospec 70/16 USR scanner, investigated the ocular biodistribution in adult rats of the topically-administered lutein within the hydrogel matrix using the MRS method. Intra- and interindividual comparative studies (treated vs. untreated eye; hydrogel with and without lutein; healthy vs. diseased - diabetic retinopathy; etc.) have been conducted to validate the applicability of this MRS technique in demonstrating the ability of topically-administered lutein in reaching the targeted retinal tissue.

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The on-line preconcentration in affinity capillary electrophoresis

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The capillary electrophoresis (CE) is an attractive technique for its rapid analyses, high efficiency, possible automation, and the ability to use a wide range of separation conditions, including the solutions with pH values from strongly acidic to highly basic.
Despite mentioned advantages of CE there is a certain limitation of the technique, i.e. the low concentration sensitivity associated with the conventional ultraviolet-visible (UV-VIS) detection. There are some possibilities of increasing the concentration sensitivity in CE. One of them is exchanging the detection device with a more sensitive one such are fluorescence, mass spectrometry or conductivity detectors. Another more elegant methods are the preconcentration techniques which allow the use of universal and less expensive UV-VIS detection and still enhance the concentration sensitivity. On-line preconcentration techniques provide automated analysis during separation where analytes are concentrated directly in the capillary. With these techniques there is no need of derivatization neither immobilization of the analytes [1]. CE not only serves as a tool for qualitative and quantitative sample analysis. It also enables to describe the interaction between binding partners in terms of binding strength and the number of binding sites. One of the established CE regimes for affinity interaction studies is capillary electrophoresis-frontal analysis (CE-FA) [2]. By choosing conditions close to physiological conditions, it is possible to study the pharmacokinetics between plasma proteins and drugs, thus obtaining a realistic picture of what is happening inside our bodies.
In this study was to optimize the new more sensitive CE-FA method by its combination with on-line preconcentration technique and the classical CE-FA method with similar conditions for data comparison was used.

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Fast and isomer-specific identification of new psychoactive substances by trapped ion mobility mass spectrometry

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New psychoactive substances (NPS) are derivatives of conventional (often illicit) drugs, which are designed to circumvent the law. NPS comprise a large group of closely-related chemical structures, including isomers. From a legal viewpoint, correct identification of individual NPS is of increasing concern. Current identification methods typically involve gas chromatography-mass spectrometry (GC-MS) supplemented with optical spectroscopic detection to achieve distinction of isomers. These methods are laborious, time consuming and relatively complex. We successfully employed the potential of trapped ion mobility mass spectrometry (TIMS-MS) - which separates ions based on their mass, charge, size and shape - for fast and consistent identification of NPS. In this contribution, new TIMS-MS strategies and dedicated data-handling tools will be presented which allow highly specific analysis of various NPS and their isomers as found in real cases. It will be shown that individual NPS can be discerned and unambiguously assigned based on their unique combination of accurate mass and IM profile, when applying optimized TIMS pressures and voltage gradients, and an internal standard for mobility calibration. Moreover, it will be demonstrated that addition of a crown ether to the sample provides unimodal IM profiles for each NPS, permitting efficient resolution of NPS mixtures and reliable annotation of the components, even when these are isomers. The developed TIMS-MS workflows were effectively used for the isomer-specific identification of NPS in confiscated forensic case samples, requiring total analysis times (incl. assignment) of only a few minutes per sample.

THE USE OF NITROUS OXIDE WHIPPETS AS A RECREATIONAL DRUG: HIDDEN HEALTH RISKS.

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Background:
Nitrous oxide whippets, also known as whipped cream canisters, are traditionally used in a culinary setting to prepare food foams. However, in the last couple of years, these gas canisters have been cracked open and inhaled to create a “legal” high.

Objective:
Users of these whippets have reported the presence of an oily residue containing metallic particles. This contamination was investigated using liquid chromatography-, gas chromatography- and inductively coupled plasma-mass and optical emission spectrometry. The particulate matter was also analyzed by scanning transmission electron microscopy (STEM) combined with energy-dispersive X-ray spectroscopy (EDX).

Results
The presence of cyclohexyl isothiocyanate was confirmed at a maximum concentration of 67µg per whippet. ICP-MS and OES analysis revealed the presence of mainly iron and zinc but trace amounts of aluminum, chromium, cobalt, nickel, and lead were also found. Using STEM-EDX analysis we could confirm the presence of nano-sized iron-containing particles and when simulating inhalation, using a multiple path particle dosimetry model it was confirmed that these nano-sized particles could reach the deeper parts of the lung.

Conclusion
Most users assume that inhalation of a food-safe nitrous oxide whippet entails no risks when misused as a “legal” high. This investigation shows however that those users will be exposed to cyclohexyl isothiocyanate a substance known to cause pulmonary injuries. Additionally, the presence of zinc and iron in the particulate matter could potentially be linked to pulmonary lesions previously reported in a case report.

Evaluation of different supercritical fluid chromatographic systems to model the skin permeability of pharmaceutical and cosmetic compounds

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Oral administration of drugs can entail certain disadvantages, such as a pronounced first-pass effect or adverse side effects. To avoid these problems, transdermal administration may be a good alternative. Therefore, there is an increased interest in the industry for skin permeability tests in the context of drug development and discovery, but also for dermal exposure and risk assessment evaluation. Quantitative retention-activity relationship (QRAR) models, including chromatographic retention as a molecular descriptor, could provide alternatives for the current in-vivo and in-vitro methods to predict the skin permeability coefficient, K_p.
Supercritical fluid chromatography (SFC) was explored as an alternative technique for liquid chromatography to predict the skin permeability of various pharmaceutical and cosmetic compounds. A test set of 58 compounds was applied to screen nine dissimilar stationary phases, including a cholesterol, silica, 2-ethylpyridine, cyanopropyl, pentafluorophenyl, amino, diol, phenyl and ethylene bridged hybrid silica column. The obtained retention factors (log k) along with two sets of molecular descriptors, determined with Vega ZZ and E-Dragon software, were used to model the skin permeability coefficients. Multiple linear regression (MLR) and partial least squares (PLS) regression modelling were applied as modelling approaches. In general, the MLR models performed better than the PLS models with either descriptor set. The best correlation with the skin permeability data was seen for the retention obtained on the cyanopropyl column. The retention factors obtained on this column were included in a simple MLR model, together with the octanol-water partition coefficient and the number of atoms as other descriptors. The best skin permeability models were selected based on their predictive capacities and fit, while maintaining an acceptable model complexity. These models were compared with those from previous studies. This study demonstrated that SFC may be a fast, suitable alternative for the methods previously applied in liquid chromatography to model skin permeability.

SFC-MS: New opportunities for pharmaceutical quality control

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Supercritical Fluid Chromatography (SFC) has known an impressive resurgence of interest since the 2010's. This analytical technique is generally promoted for its main advantages (i) versatility regarding the range of compounds polarity that could be analyzed; (ii) greenness by using mainly supercritical CO_2; (iii) high efficiency with short analysis time; (iv) easy hyphenation with MS detection thanks to the availability of dedicated interfaces and (v) easy transferability to preparative scale separation. Recent publications highlighted that SFC could be presented as a real challenger of gold-standard LC, with suitable quantitative performance and its easy implementation in several laboratories by means of inter-laboratory studies [1].
To extend the performances of SFC, hyphenation to MS is well currently one of the main research topics. Indeed, the combination of SFC advantages to MS specificity and sensitivity could be required for several applications such as complex matrices, bioanalysis or impurities tracking. In this context, our project was focused on the demonstration of SFC-MS quantitative performances for several applications. Firstly, the quality control of potentially counterfeited medicinal cannabis was successfully performed using generic SFC-MS conditions. Method validation was performed using one synthetic cannabinoid as model compound to demonstrate the suitable quantitative performances [3]. Secondly, the quality control of vitamin D3 oily formulations was deeply investigated. SFC-MS method was developed using AQbD strategy followed by method validation [3]. As this method was optimized for a dedicated formulation, a generic SFC-MS methodology was proposed for the analysis of oily medicines and food supplements [4]. This approach permitted to analyze using the same method a large panel of formulations with different oily matrices and a large range of vitamin D_3 nominal concentration. Matrix effect study and relevant control strategy highlighted the performances of this approach. Next to the control of API, the determination of impurities was investigated. This analysis requires high selectivity and sensitivity. Method development including sample preparation and validation are presented [5].

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Interaction of epilepsy drug Pregabalin with dsDNA

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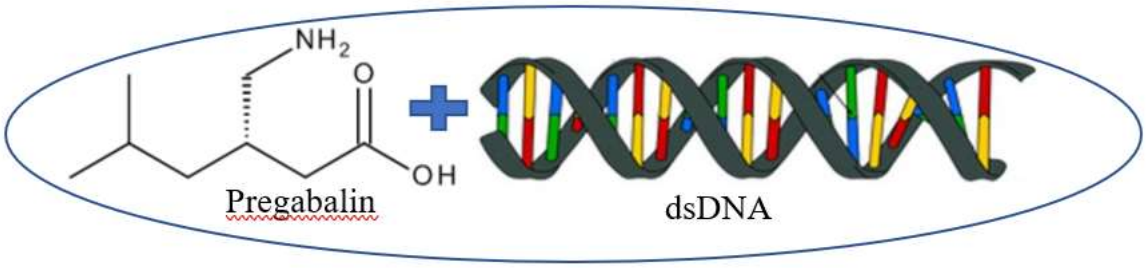
Pregabalin is a medication used to treat epilepsy, neuropathic pain, fibromyalgia, restless legs syndrome, and generalized anxiety disorder.



Scheme. Pregabalin and dsDNA interaction

Here, the binding mechanism and interactions of Pregabalin with double strand(ed) fish sperm deoxyribonucleic acid (dsDNA) (Scheme) were studied thoroughly both experimentally and theoretically, using multi-spectroscopic techniques and molecular docking simulations. dsDNA was purchased from Sigma company because it has a high purity value and has been widely used in previous studies. The purity value was confirmed in UV-Vis spectroscopy. Our ultimate goal is to better understand the potential of such epilepsy drugs and thus help design drugs with higher dsDNA binding affinity and fewer side effects. We employed several techniques yielding different but complementary results such as UV, fluorescence, thermal denaturation, viscosity, and molecular docking studies under physiological conditions. The absorption spectra were recorded in the range of 210–330 nm in a T80 + UV/VIS spectrophotometer, using the cells of a 1 cm light path. The competitive spectrofluorometric dsDNA binding studies of Pregabalin with EtBr and Hoechst-33258 bound dsDNA in Tris-HCl buffer solution were conducted to determine the binding character of Azacitidine. The viscosity measurements were conducted using a rheometer (Haake RheoStress1, Germany) equipped with a parallel plate sensor (d=35 mm, gap=1 mm) at room temperature in duplicate. The shear rate ramp was applied in the range of 0.01 and 200 s^{-1} in 60 s. And finally, the binding constant K_b, which indicates the binding strength of pregabalin to dsDNA, was calculated with intra-day and inter-day reproducibility [1].

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Disposable Rapid Magnetic Microbeads-based Immunoplatform for the Sensitive Amperometric Assessment of SOX2

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The first magnetic immunosensor was developed to determine the Sex-determining region Y-box 2 (SOX2) sensitively. SOX2 is a transcription factor involved in maintaining embryonic stem cell pluripotency and in multiple processes of progression, including lung branch morphogenesis. This work reports the first magnetic beads (MBs)-based electrochemical immunoassay for the determination of SOX2. The design involves the selective capture of sandwich immunocomplexes formed by capturing antibody-target SOX2-HRP-labeled detector antibodies onto carboxylic acid-functionalized magnetic beads (HOOC-MBs). The resulting magnetic bioconjugates were captured on the surface of disposable screen-printed carbon electrodes (SPCE) to perform amperometric detection at −0.20 V vs. the Ag pseudo-reference electrode in the presence of hydroquinone (HQ) and H_2O_2. The effect of key experimental variables affecting the immunosensor response was evaluated.

The analytical performance of the MBs-based immunosensor for the amperometric determination of SOX2 standards (calibration plot, LOD/LOQ values, and reproducibility) was performed. The potential interference compounds commonly found in serum samples at the concentration levels expected in healthy individuals were tested. The storage stability of the CAb-COOH-MBs immunoconjugates was assessed by storing them after their preparation in 0.01 mol L^{-1} PBS of pH 7.5 at 4 ºC.

Neurofilament light chain in the interstitial fluid of the mouse brain, a novel biomarker for epilepsy?

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Purpose: In the management of neurological diseases, there is a compelling need for reliable biomarkers that can improve the prognostic assessment as well as predict the response to treatments. According to the World Health Organization, ± 5 million people are diagnosed with epilepsy each year. Current drugs affect seizures, but no disease-modifying drugs are available that prevent or slow down epileptogenesis, emphasizing the need for new insights in the field. The interest in neurofilaments, especially the neurofilament light chain (NfL) subunit, and their role as neuronal disease biomarkers has grown tremendously in the last decades. NfL is a protein located in the neuronal cytoskeleton and released into the interstitial fluid (ISF) of the brain upon axonal injury and neurodegeneration. The aim of this study is to assess NfL levels in the intrahippocampal kainic acid (IHKA) mouse model for temporal lobe epilepsy. The greatest challenge is to chronically monitor NfL in the cerebral ISF, since there are only a few techniques available that allow for the direct sampling of macromolecules from the cerebral ISF.
Methods: To date, no methods have been described that permit analysis of ISF NfL. To this end, we implemented cerebral open flow microperfusion (cOFM) as technique to sample ISF NfL in freely moving C57BL/6J mice before and during status epilepticus (SE) and before and during the spontaneous recurrent seizure phase. cOFM probes are implanted in the ventral hippocampus. SE is induced by unilateral dorsal IHKA injection. The use of both a single molecule array and enzyme-linked immunosorbent assay was explored for sample analysis.
Results: Feasibility to sample ISF NfL using cOFM was first verified. Basal ISF NfL levels of 10-12-week C57Bl/6J mice are 3.02 ± 0.32 ng/mL (n = 5). IHKA experiments are currently ongoing. Preliminary results suggest that ISF NfL levels tend to increase during SE but not during the chronic phase with spontaneous recurrent seizures.
Conclusion: Assessment of the ISF pool of NfL may provide unique insights into the various phases of the IHKA disease model for temporal lobe epilepsy.

Characterisation of AAV gene therapies by chromatographic and electrophoretic techniques

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Adeno-associated virus (AAV)-based viral vectors have shown enormous potential in recent years for use in gene therapy as a therapeutic modality to treat a wide range of genetic diseases, with more than 150 clinical trials specific to AAV. AAV treatments first gained regulatory approval in the European Union, with alipogene tiparvovec's (Glybera, uniQure) approval in 2012 for lipoprotein lipase deficiency. The first US regulatory approval was in 2017 with voretigene neparvovec (Luxturna, Spark Therapeutics) for treatment of Leber's congenital amaurosis, a hereditary affection of the eye.

Adeno-associated viruses (AAVs) are small nonenveloped icosahedral viruses approximately 20 nm in diameter. The proteic capsid, built by the interaction of three different structural proteins (VP1, VP2, VP3) encloses a 4.7 kb ss DNA genome. While the wild type genome expresses structural and regulatory proteins needed for viral replication, the recombinant type, rendered replication defective, will code for specific therapeutic proteins.

As for all therapeutic products, AAVs should be thoroughly characterised using state-of-the-art analytical methods, and release tests should be implemented to assess product quality and safety. Separation techniques (liquid chromatography and capillary electrophoresis) are part of the analytical package used for the characterization of AAVs.

We will present recent developments in LC(/MS) and CE methods for the analysis of AAVs, with examples obtained in our lab on model compounds.

High performance analysis of N-glycans released from Alglucosidase Alfa

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Glycosylation is the most common and structurally diverse posttranslational modification of proteins; it plays key roles in different biochemical processes, including protein folding, stability, and localization. More in particular, the level of phosphorylated glycan species (glycans containing mannose-6-phosphate [M6P]) is an important attribute of alglucosidase alfa (Myozyme) since it targets the protein to the lysosomes. The level of sialylated glycans is also particularly closely-monitored as it directly correlates to the protein's circulating half-time once administrated to the patient.

At Sanofi, we developed a method where the oligosaccharide profile of the N-glycans released from myozyme is reported along with the molar ratio of M6P and sialic acids from a single analysis, without the need of introducing an internal standard. This method was developed in scope of replacing aging methods by state-of-the-art technology and as such, it replaces three methods: an already existing oligosaccharide profiling generating recurring lab events and two methods employing high-performance anion exchange chromatography with pulsed amperometric detection.

Separation is performed using a reversed-phase/anion exchange chromatography column which generate high peak capacity values (> 300 for sialylated glycans over 105 min run time) thereby allowing the comprehensive separation of N-linked glycans released from Myozyme.

In this presentation, the developments and the performance of the method will be discussed, as well as the results reporting methodology. The applicability of this method for other Sanofi products will also be discussed.

ANALYTICAL SETTINGS TO SUPPORT RECOMBINANT HUMAN COLLAGEN PRODUCTION BY FERMENTATION

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Collagen is a structural protein widely expressed among vertebrates. Its features such as the peculiar biosynthetic process and the complex extent of post translational modifications (PTMs) including proline hydroxylation, enable the formation of the triple helix, main collagen domain, and its further incorporation in fibrils of increasing complexity. These structures impart tensile strength and elasticity to many tissues in the body. As an alternative to extractive methods, the use of recombinant technology to produce animal-component-free collagen, overcomes the variability, potential immunogenicity, and risk of infection associated with the use of collagen for medical applications and offers traceable processes that provide products of consistent quality.
In this context, recombinant human collagen via fermentative processes in a green-production system have been considered. Critical steps are selection of the expression strain, optimization of the gene transfection method for protein and functional enzymes, selective isolation of the released protein, stability assessment in fermentation media, extensive structural and functional characterization.
The research project is focused on the development of ad hoc analytical methods supporting each production stage. Initially, the evaluation of the activity and selectivity of the prolyl-4-hydroxylase (P4H) has been assessed with both direct (in vitro assays) and indirect (search for hydroxyproline) evaluation. Secondly, immunoaffinity chromatographic methods have been set up using Protein-A magnetic particles with immobilized anti-procollagen mAb to selectively capture procollagen in fermentation broth and verify expression efficiency as well stability during fermentation and downstream.
SEC-UV analyses have also been developed to achieve preliminary information about collagen purity and to estimate molecular weight of both the entire molecule and the individual α-chains, as well as to detect the presence of aggregates. Moreover, complementary chromatographic techniques (HILIC, RP-HPLC) coupled to high resolution mass spectrometry (HRMS) have been applied at peptide level to assess the amino acid sequence along with the extension of proline hydroxylation.
For an extensive PTMs mapping, peptides obtained through different proteolytic enzymes, can be analyzed with LC-MS/MS leading to an accurate identification of cross-links and O-glycosylation degree and position.

Chromatographic approaches to address challenges related to polysorbate analyses in biopharmaceuticals.

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Polysorbates (commercialized as Tween®) are added in biopharmaceutical formulations where they act as stabilizing agents for therapeutic proteins present at high concentration. Their addition in such formulations prevents protein aggregation and denaturation. The application of polysorbates is quite common due to their efficacy and low toxicity.
Their analysis, on the other hand, can be challenging due to the complexity and polydispersity of the polysorbate and the presence of high concentrations of salts and protein in the formulation. Polysorbate analyses can involve analysis and characterization of the raw material, assay of the surfactant in intermediate and final drug products, monitoring polysorbate degradation, trace analysis in cleaning validation studies, etc.
During the presentation, an overview will be given on different approaches for the assay and characterization of polysorbates in standard and sample solution. Various chromatographic modes and detection techniques will be discussed together with their advantages and drawbacks and results obtained at the author's facilities will be shown to illustrate the above.

Metal-ion assisted molecularly imprinted electrochemical sensor for the selective determination of aclidinium bromide

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This work explains the development of a metal-ion assisted molecularly imprinted polymer (MIP)-based electrochemical sensor for the analysis of aclidinium bromide (ACL) for the first time. ACL is a long-acting muscarinic receptor antagonist used for the treatment of chronic obstructive pulmonary disease (COPD) [1,2]. Even though the routine clinical use of ACL is relatively new and the number of pharmaceutical preparations is limited, its place in COPD treatment has already reached an important point. Therefore, ACL analysis and sensitive determination in pharmaceutical and biological samples is significant. Nevertheless, there are no studies available for ACL determination in the literature. In this study, the MIP-based electrochemical sensor was developed using methacryloyl-L-cysteine-nickel (II) [MAC-Ni(II)] as the monomer in the presence of polyvinyl alcohol (PVA) as the pore-maker. The fabricated sensor is highly porous, hydrophilic and exhibits highly sensitive response toward template molecule, ACL. The developed sensor was successfully applied for ACL determination in standard solution, serum sample, and capsule formulation with high sensitivity and accuracy. The LOD values for standard solution and serum sample were found as 2.73×10^{-16} M and 1.76×10^{-16} M, respectively. Additionally, interference study with common interfering agents and imprinting factor calculations using oxitropium bromide, ipratropium bromide, glycopyronium bromide, and tiotropium bromide demonstrated the selectivity of the sensor. Finally, the non-imprinted polymer (NIP)-based sensor confirmed the performance of the MIP sensor. To the best of our knowledge this study is the first one that reports the electrochemical determination of ACL.

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Application of immobilized enzyme reactors for the determination of myeloperoxidase inhibition

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Myeloperoxidase (MPO), a hemo-protein present in neutrophils and monocytes significantly contributes to immune responses against microbial pathogens [1]. MPO is able to use chloride as a co-substrate with hydrogen peroxide to generate a powerful oxidizing agent, hypochlorous acid. Increased MPO activity promotes the production of highly oxidative molecules in extracellular fluids that can damage host tissue causing inflammatory events [2]. Therefore, MPO is a promising target for the development of new anti-inflammatory agents, and thus seeking the active compounds that can efficiently inhibit MPO activity and subsequently decrease inflammatory events can be of special interest.

Although several potent MPO inhibitors have been obtained in the last decade, there are no selective drugs to inhibit its unwilling activity. Apparently, a part of this weakness is due to the inadequate screening assays that mimic the enzymatic mechanisms of MPO [3].

In the first assay, an enzyme microreactor-detector has been developed in a thin layer flow-through configuration consisting of a platinum disk modified with gold nanoparticles for immobilization of MPO adjacent to a platinum disk as working electrode. Enzymatic substrate, hydrogen peroxide was oxidized and detected amperometrically at platinum electrode surface. Measurements were performed in 25 mM phosphate buffer, 375 mM sodium chloride pH 7.4 with an applied potential of +700 mV vs Ag/AgCl and a flow rate of 100 µL/min. The configuration was applied for the determination of the efficiency of inhibition for MPO inhibitors by using paroxetine as a model compound.

Subsequently, MPO was immobilized on the surface of pretreated silica gel. The enzymatic reaction and the inhibition were taken place in the centrifuge tube and the supernatant was used for the measurements of the remaining H_2O_2 as mentioned above. At the end of the reaction, the enzyme attached to silica gel was recovered. These results prompt us to validate the reactor model as another efficient tool for MPO inhibition screening.

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Sustainability in pharmaceutical (bio)analysis

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After the 2021 United Nations Climate Change Conference (COP26), sustainability is becoming a critical hot-topic issue across all industries, including pharma. As the ecological footprint of the pharmaceutical industry is enormous, including environmental impact and massive greenhouse gas production, strategies to reduce waste and climate change drivers are highly needed. This is not only an issue in commercial, large-scale API-syntheses and medicine-production, but also in the labor-intensive and time-consuming drug discovery and development phase [1].
As analytical activities are key in the pharmaceutical field, they are one of the hidden drivers next to production and use. Green analytics is proposed as one of the approaches to follow to reduce volumes, cost and ecotoxicity of analytically used chemicals and lowering energy usage. The emphasis is currently on the choice of the analytical techniques, including miniaturization and automation. Moreover, different options of chemicals and reagents in the analytical development process, including sample preparation, should be considered and judiciously selected to optimize sustainable analytics.
Different case studies will be presented, including the development of an eco-friendly anti-adsorption solution in bio-analytics as well as green analytical aspects of nitrosamine impurities in medicines.

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Enantiomeric profile of psychoactive drugs in wastewaters and river surface waters

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The misuse of psychoactive drugs (PAD) has been reported all over the world with notable health risk to human and wildlife [1]. Many controlled or prohibited PAD are chiral and depending on the manufacturing procedure, they are available either as racemate or as single enantiomers [2]. Though, human metabolism or biodegradation can further lead to racemization or enantiomeric enrichment causing changes in their enantiomeric fractions (EF) [2]. Consequently, the evaluation of enantiomeric profile of PAD and/or their metabolites in surface waters is crucial for environmental risk assessment, and determination of their EF in wastewaters can also allow discrimination between consumption, direct disposal, and synthesis pathways [2,3]. Indirect method by gas chromatography coupled to mass spectrometry (GC-MS) for enantiomeric quantification of PAD through derivatization using (*R*)-(-)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride as chiral derivatization reagent has been shown to be highly efficient for monitoring several classes of PAD in environmental matrices [4,5]. The quantification of drugs as amphetamine like substances, synthetic cathinones and achiral piperazines have been successfully achieved in surface and wastewaters, including the determination of EF for the chiral PAD [5]. This method allowed to evaluate the occurrence, spatial distribution, and the EF of target PAD in Portuguese surface waters and in effluents. OASIS® MCX cartridges were used for cleanup and preconcentration of samples for further derivatization and analysis by GC-MS. The potential of the analytical method to monitor consumption of the target drugs was recently demonstrated with the quantification of illicit synthetic cathinones in Douro River estuary along with other amphetamine derivatives [5], and it is been optimized for quantification of the selected PAD in suspended particulate matter. This communication aim to present the strategy, methodology and the importance of enantiomeric profile evaluation PAD as well as the most recent results.

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YOUNG SCIENTIST COMMUNICATIONS

Quality control of CBD based-products on the Belgian market

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Cannabis sativa L. is cultivated for multiple agricultural and industrial applications, though the cultivation of this plant was prohibited in many countries due to the presence of the psychoactive cannabinoid, Δ9-tetrahydrocannabinol (THC) [1]. For agricultural hemp, the GC-FID European Union method is available to check the content of THC according to the legislation fixed to a maximal content of 0.2% (*w/w*) [2]. *Cannabis sativa* L. is currently the most commonly used illicit drug available in Europe [3], but is also facing an exponential grow of new applications with the presence of new commercial products [4]. In addition to a growing number of administration modes of the psychoactive substance THC, some products as oils and herbs for smoking appear which contain cannabidiol (CBD) as main cannabinoid [5]. These new evolutions complicate the market surveillance since this molecule is not controlled in the European Union and so the quality of the products is not guaranteed [6]. Furthermore, the products must be limited in their Δ9-THC content at 0.2 % (*w/w*) according to European legislation. To circumvent the legislation, a lot of products are sold as e.g. “pot pourri” or cosmetics [7]. Quality control and state laboratories need analytical methods to perform a more intense surveillance. In this study, a procedure combining a sample cleanup by a QuEChERS technique and a validated method by GC-MS/MS was developed to quantify CBD in oily samples. The method was also evaluated to check the legality of THC and to detect seven other cannabinoids selected, based on their biosynthesis pathway and medical interest. In addition, the European method was also validated in our laboratory to analyze herbs for smoking next to agricultural hemp. The validation parameters of this method and a validated UHPLC-UV method were compared. Statistical tests were applied an showed significant differences between the means of total CBD and THC for samples analyzed with both methods.

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Targeted and untargeted mass spectrometry-based metabolomics for chemical profiling of coffee species

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While coffee beans have been studied for many years, researchers are showing growing interest in coffee leaves and by-products but few information are currently available on coffee species other than *C. arabica* and *C. canephora*. The aim of this work was to perform a targeted and untargeted metabolomics study on *C. arabica*, *C. canephora* and *C. anthonyi*. The application of the recent high resolution mass spectrometry-based metabolomics tools allowed getting clear overview of the main differences among coffee species [1]. LC-MS/MS analysis were performed and molecular networks were built to confirm the annotation. Results showed a different metabolite profile between *C. anthonyi* leaves and fruits and leaves and fruits from other species. In *C. anthonyi*, caffeine levels were found in lower concentrations while caffeoylquinic acid and mangiferin related compounds were found in higher concentrations. A large number of specialized metabolites can be found in *C. anthonyi* tissues, making this species a valid candidate for innovative healthcare products made with coffee extracts.

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Simultaneous quantitation of 11 major alkaloids for quality control of *Lepidium meyenii* (Maca) in food supplements

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On 31/08/2021, a Belgian Royal Decree was published stating the legislation for food supplements containing plant extracts, with 3 updated lists of plant species. Among the lists, list 3 contains an extensive series of plant species that require a notification before they can be marketed as a pre-dosed food supplement. For many of the plants in list 3 additional analytical documentation is required.[1]
Lepidium meyenii (Maca), also known as Peruvian ginseng, is a common ingredient in food supplements with several claimed health benefits, such as improved endurance, higher energy levels and sexual enhancing properties. According to list 3 of the Belgian Royal Decree, Maca roots are allowed in food supplements, but analysis should confirm that there are no detectable quantities of potentially toxic alkaloids.
The current study resulted in the first analytical method for quality control of Maca-containing food supplements, assessing the presence of 11 major alkaloids belonging to 3 different classes of alkaloids: imidazole, β-carboline and pyrrole. A fast and sensitive UPLC-MS/MS analysis was developed and is currently being validated according to the ICH guideline [2].

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Nephroprotective effect of a methanolic extract of two *Ganoderma* species and its association in an *in vitro* model of cisplatin induced tubulotoxicity

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Cisplatin is currently used as a first-line cancer treatment, such as testicular, ovarian or pulmonary cancers. Their nephrotoxicity remains a real problem. Acute kidney injury induced by cisplatin is located on proximal tubular cells, causing necrosis and possibly subsequent interstitial fibrosis and chronic dysfunction. These severe side effects can lead to a cessation of the patient's treatment. Currently, there is no effective prophylactic action to reduce cisplatin nephrotoxicity, beside hyperhydration of the patient [1].
The aim of the present work is therefore to identify new prophylactic therapy. For this, natural products can be studied, in this case, the interest of potential new medicinal mushrooms extracts. Among 13 mushroom extracts, the methanolic extracts of *Ganoderma parvigibbosum* Welti & Courtecuisse, *Ganoderma tuberculosum* Murrill and their association were selected to study their effects on human proximal tubular cells (HK-2) intoxicated with cisplatin.
HK-2 cells are grown in 75cm² sterile flasks using DMEM low glucose (1mg/mL), supplemented with FBS (10%), L-Glutamin and a mix of Penicillin/Streptomycin. Dried mushrooms were grounded and extracted 3 times by methanol, evaporated extracts are stored at -20°C. A viability assay allowed to determine the work concentration of extracts range have been done. After that, tests were performed after a pretreatment of 1h with the extracts before adding cisplatin at a concentration of 20 µM. Viability assays (CCK-8) and antioxidant activity (DPPH) were done in 96-well. The intracellular concentration of β-catenin and calcium, Caspase-3, p53, cytochrome C, IL-6, NFκB, the membranal expression of KIM-1 and finally the ROS production (H_2DCFDA) were studied by flow cytometry.
Tests have shown that methanolic extracts of *G. parvigibbosum* and *G. tuberculosum* (10 µg/mL) and their association (5 + 5 µg/mL) prevented the loss of viability after a 24h incubation. They also have prevented the apoptosis and the induction pathway after 24h. *G. parvigibbosum* and the association of the two mushrooms extracts have also prevented the increase of caspase-3 and intracellular β-catenin. Finally, *G. parvigibbosum* was the only to prevent the ROS overproduction and having a scavenger activity at work concentration. None of them showed a prevention in the increase of IL-6 and NFκB or the membrane expression of KIM-1.
Ganoderma parvigibbosum appears to be therefore more beneficial than *Ganoderma tuberculosum* and the association of the two mushrooms extracts by acting also on the ROS overproduction. In conclusion, in this study, the extracts have shown a significant activity on the prevention of the pro-apoptosis pathway rather than a pro-inflammatory prevention. Further investigation about metabolomic study and chemical composition (LC-UV/MS) are undergoing.

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Valorization of by-products from agro-foods for pharmaceutical, cosmetic, nutraceutical applications

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The agro-food by-products, whose annual volumes are estimated to be approximately 5 billion tons of biomass residues globally, when not further treated, are discarded as waste, generating several environmental problems[1]. These by-products (such as hulls, shells, peels, squeezing residues, seed residues etc.) have proved to be valorized in pharmaceutical, nutraceutical and cosmetic fields thanks to their content in bioactive molecules, such as polyphenols and proteins[2]. Therefore, in the present work some waste products of the Romagna agro-food industries: legumes (okara, peas and beans) and fruits (peaches, apricots and apples) were selected for further valorization. Mainly, the aim of the research was the chemical characterization of the by-products in terms of bioactive molecules. Then, in view of valorizing the waste products, normally discarded, we compared their content profile with the one resulted from their related final products commercially available (fruit juices and pulp, best-selected legumes, squeezed okara, etc.). This work concerned, primarily, the development and optimization of a high yield extraction, ultrasound based method to obtain polyphenols. Later, the extracted fractions were characterized applying two different UV-VIS spectrophotometric assays. First, the Total Phenolic Content (TPC)[3] was carried out in order to evaluate total polyphenolic content and, secondly, the Total Antioxidant Status (TAS)[4] was performed to determine their antioxidant activity. Then, a chromatographic HPLC-DAD method was optimized and validated with the purpose of defining the polyphenolic profile for each by-products[5]. Afterwards, a comparison of the polyphenolic profile in legume and fruit extracts was carried out, between that of the final products and the correspondent by-products. Moreover, a proteins extraction methodology was optimized and the extracted proteins were quantified by the Kjeldahl method. Fruit by-products resulted to have a greater antioxidant activity (in the range of 2,359 ± 0,401 to 4,019 ± 0,923 mmol trolox equivalents/100 g of dry powder) justified by an higher polyphenolic content, in comparison with legumes by-products (in the range of 0,813 ± 0,119 to 2,950 ± 0,398 mmol trolox equivalents/100 g of dry powder). The latter, on the contrary, demonstrated to have an higher content of proteins (34,5 ± 6,2 g/100 g of dry powder). Furthermore, the comparison between final products and correspondent by-products highlighted how all these residues still represent an important source of bioactive compounds, which has to be valorized, in order to be used in cosmetic, pharmaceutical and nutraceutical fields, in a green circular bioeconomy prospect.

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Effect of 3',6-dinonyl neamine on membrane remodelling in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium that is associated with urinary tract infections, burn wound infections, sepsis syndromes and cystic fibrosis. The asymmetric outer membrane (OM) of *P. aeruginosa* consists of phospholipids (PLs) in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet of the OM. OM creates a permeability barrier against the antibiotics (aminoglycosides, polymyxins, and fluoroquinolones) used in the clinics. The low susceptibility and increasing resistance are a worrisome characteristic of *P. aeruginosa.* Therefore, research for the discovery of new antibiotics against *P. aeruginosa* is needed.

Previously, it is already established that 3',6-dinonyl neamine (3',6-diNn), an amphiphilic aminoglycoside derivative has a MIC value of 2µg/ml against both wild-type (ATCC27853) and the clinical colistin-resistance *P. aeruginosa* strains. 3',6-diNn has the ability to bind LPS and disassemble cardiolipin domains. In this study, we tried to understand the mechanism action of 3',6-diNn on membrane remodelling. Scanning electron microscopy was used to visualize the morphological defects in the *P. aeruginosa* after the treatment with 3',6-diNn (2µg/ml). Further, the production of membrane vesicles (MVs) was quantified using nano-particle tracking analyser. Total PLs were quantified with FM-464 and the PLs composition of *P. aeruginosa* OM was analysed using LC-MS.

Exposure to the 3',6-diNn perturbed the membrane at the cell pole and produced small inward invaginations near the septum. *P. aeruginosa* treated with 3',6-diNn generated 5 times higher number of MVs. The size of these MVs was significantly larger compared to the non-treated condition. The OM extracted after the treatment showed accumulation of PLs. When PLs composition was analysed, it showed difference in the PE and CL composition but not in PG. Treatment with 3',6-diNn increased the detection of PE 36:0 (18:0-18:0), PE 36:0 (18:0-18:1), CL 72:0 and CL 72:1. An increase in the CL at the OM might change the membrane curvature, which induces the negative curvature and therefore generates MVs. In future, we will also explore the potential changes in the LPS and the membrane biophysical properties, which are also a contributor to the production of MVs.

Use of CE-IM-MS as a complementary tool to microfluidic-LC-IM-MS for cell surface antigen discovery

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Disease management of multiple myeloma is challenging owing to patients' multiple relapses and resistance to standard treatments. Therefore, the introduction of innovative cell therapies such as chimeric antigen receptor T-cells (CAR-T cells) opened new horizons in the outcome of severe refractory patients affected by aggressive forms of the disease. However, one of the limitations of the expansion of those treatments is the lack of specific MM tumor-associated antigens that could be targeted by current immunotherapies. Indeed, effectiveness of current CAR-T cells treatments could be hampered due to possible antigen-evasion strategies. Therefore, the discovery of new cell surface antigens could be an interesting approach to avoid occurrence of resistance that could lead to treatment failures.

For this purpose, the use of mass spectrometry (MS) proteomics-based methodologies was considered. Due to the high sample complexity, liquid chromatography (LC) is commonly used prior MS detection to maximize protein identifications. Similarly, capillary electrophoresis (CE) could be an alternative due to its ability to provide high efficiency and high throughput separation. In this study, the use of a neutral-coated capillary was considered in order to increase peak capacity leading to a higher number of identified entities compared to uncoated capillaries. Besides, different injected sample volumes were tested using dynamic pH junction to tackle the poor sample injection volume in CE without compromising separation efficiency.

Since CE separation principle is orthogonal to the mechanism that drives separation in LC, the use of both techniques to analyze the same sample allowed the increase of overall information in terms of number of identified proteins. As a matter of fact, despite the high number of proteins identified using LC-MS, more than a half of the proteins identified in CE could not be identified by LC. Moreover, the nature of uniquely identified peptides and orthogonality of both systems were investigated.

The capability of ion-mobility (IM) was also exploited in this study. Indeed, the addition of IM module between LC or CE and MS lead to better proteome coverage due to the additional dimension of separation. To the best of our knowledge, little attention has been paid to the potential orthogonality between CE and IM in proteomic studies to date. In this study the combination of CE with IM allowed the separation of isobaric and co-migrating peptides leading to the identification of a larger number of unique proteins, thus increasing the possibility of detecting new antigens.

Quorum Sensing Peptides in biofluids: a multifactorial "to be or not to be" approach.

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The question of the possible presence of quorum sensing peptides at low levels in biofluids is a critical medical issue in translationally unraveling the causal association between the microbiome and human diseases. In obtaining reliable conclusions of these peptide measurands, the different parts of the analytical process using LC-MS were investigated, with novel approaches recommended for each part.
The challenges presented here are:

1. **Sample collection**: obtaining and storing biofluids (plasma/serum, feces and saliva) from mice and humans up to the laboratory handling should ensure no degradation/metabolization of the peptide measurand (yielding false negatives) or of proteins (yielding false positives).
2. **Sample preparation**: to remove interfering compounds as well as to release peptides from the adsorbing matrix-components and to preconcentrate them, this is a crucial step towards analytical stability and adsorption-minimization.
3. **Chromatography**: not only the separation power and orthogonality of the complementary systems is critical, but adsorption losses leading to different detection limits need to be addressed as well.
4. **MS detection**: operational parameters such as duty cycle characteristics applied, often neglected, are however critical in obtaining optimal results.
5. **Data treatment**: while a tiered one-factor-at-a-time approach is currently most often used, as exemplified in the world anti-doping agency's criteria for identification of doping or the European Commission's guidance for mycotoxin identification in food and feed, other data-evaluations are required to minimize the loss of information.

Our work addresses aQbD solutions to these challenges, including sample stabilization measures, a novel anti-adsorption tool, judicious choice of injection solvent versus gradient system, optimal duty cycle parameters and multivariate data treatment [1,2]. Our recommendations will improve the bio-analytics of not only quorum sensing peptides, but also all relevant analytical challenges of measurands at low concentrations.

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Investigation of the interaction between HPV and host cell receptor by affinity capillary electrophoresis and bio-layer interferometry

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Human papillomavirus (HPV) infection is a necessary cause of cervical cancer. Specifically, HPV16 and HPV18 are together responsible for 70% of cases [1]. The receptor(s) implicated in HPV binding and entry in the host cells has (have) not been fully elucidated, even though many candidates have been identified. It was demonstrated that HPV16 might initially interact with laminin 332 (LN332), a key extracellular matrix component secreted by migrating and basal keratinocytes. Nevertheless, the dissociation constant (Kd) value was not determined [2]. Affinity capillary electrophoresis (ACE) is particularly interesting to study biomolecular interactions. It allows the analysis of interactions in solution. Therefore, each available site is accessible and dissociation constants reflect the native interactions between molecules [3, 4]. Because HPV *in vitro* production leads to low virus titers, HPV studies have used virus-like particles (VLP) as model. HPV VLP are formed by the self-assembly of viral structural protein L1 and are morphologically and immunologically similar to native virions [5].

To study the complex formation between HPV16 VLP and LN332 receptor, a method combining two ACE approaches was used. A constant amount of VLP was preincubated with various amounts of LN332 (molar ratio 1:5 to 1:66) and then the samples were analyzed by CE like in equilibrium mixture analysis. However, a variation in the electrophoretic mobility of the complex was observed as in mobility-shift analysis. The mobility of the complex decreases with increasing amount of LN332 in the sample. An equation derived from the mass action law was used to determine the dissociation constant Kd by nonlinear regression.

In order to validate the results obtained by ACE, the interaction was studied by bio-layer interferometry (BLI). This high-throughput label-free technique allows the measurement of VLP binding and release from immobilized receptor on the biosensor, allowing measurements of the kinetics of the interaction. It is noteworthy that for BLI measurements, the receptor has to be attached to the biosensor, contrary to ACE where the components move freely in solution.

The interaction between VLP and LN332 was also characterized in solution by flow induced dispersion analysis (FIDA). FIDA combines Taylor dispersion analysis and fluorescence detection to measure the change in apparent hydrodynamic radii of a fluorescent ligand as it interacts with its target allowing the determination of a Kd.

The use of these complementary techniques contributes to validate our approach based on ACE in order to gain a better understanding of the HPV binding mechanism.

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Analytical support to optimize outpatient parenteral antimicrobial therapy

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Outpatient parenteral antimicrobial therapy (OPAT) implies the use of portable elastomeric pumps which allow the patient to continue drug administration at home. This way intermittent injections or infusions in hospitals are avoided. Some examples of medicines that are administered this way are ceftazidime and piperacillin in combination with tazobactam. The elastomeric pumps are mostly worn around the hip where temperatures around 33 °C are reached. Currently, the elastomeric pumps are replaced every 6 to 12 h since no proper stability data over 24 h are available.
To find the best dosing regimen, the concentration of the antibiotics in plasma should clearly exceed the minimum inhibitory concentration (MIC), which varies depending on the type of bacteria.
In this study, LC-UV methods were developed and validated to follow up:
(1) the stability of ceftazidime and piperacillin/tazobactam in portable elastomeric pumps over 24 h at 33 °C.
(2) the concentrations of these antibiotics in plasma, including sample preparation and addition of an internal standard.
During degradation of ceftazidime, the formation of pyridine, with a toxicity profile, can not be avoided. So, besides the main compounds, this compound was also evaluated.
For all LC methods, a Kinetex® C18 (150 mm x 3 mm, 2.6 µm) column with gradient elution and acetonitrile as organic modifier was selected. After optimization, each method was fully validated in terms of selectivity, sensitivity, linearity, accuracy and precision.
It was found that ceftazidime degraded by more than 10% over 24 h at 33 °C in the elastomeric pumps, but this can be compensated by starting from a higher initial dose. The formation of pyridine amounted to 1%. However, the concentration of pyridine in plasma was low. With continuous administration over 24 h, the concentration of ceftazidime remained stable.
Tazobactam was found to be stable in the elastomeric pumps under the examined conditions while degradation of piperacillin was limited to an acceptable 5%. When analyzing plasma concentrations following continuous administration, the concentrations were quite stable, but care should be taken that the pumps are not empty before 24 h because plasma levels decay quickly when no piperacillin/tazobactam is administered due to fast elimination by the kidneys.

A novel internal calibration approach for LC-MS/MS-based monitoring of chronic kidney disease related metabolite concentrations

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Chronic kidney disease (CKD) is a widespread pathology associated to accumulation of metabolic products in plasma that exert detrimental biological activity: such small molecules are termed uremic toxins and are supposed to contribute to mortality. Recent metabolomics studies suggested, that metabolites of tryptophan and mineralocorticoid pathways along with several modified amino acids could be indicative for the disease progression [1,2]. Therefore, multitargeted approaches are desired to obtain a quantitative picture based on this data-driven hypothesis. We have thus developed a one-point internal calibration technique for a simultaneous quantification of more than 25 relevant analytes using a microsampling device (20 µL of plasma). This novel strategy relied on a response of stable isotope-labeled (SIL) standards added to the study samples to translate the endogenous metabolite concentrations [3].

First, an LC-MS/MS method for separation and analysis of all CKD-related metabolites, including mineralocorticoid isomers, was developed using a biphenyl column and linear gradient of acidified water and methanol (0.1% formic acid). A solution of ammonium fluoride (10mM in methanol) was infused post-column to increase ionization efficiency. Response functions of the analytes of interest were derived from the analysis of endogenous metabolite calibration curves spiked with increasing SIL concentrations. Chemical purities and concentrations of the respective SIL standards were confirmed to avoid quantitative bias due to the influence of impurities, isotopic interference, and ionization competition. The plasma metabolites were collected from EDTA-K tubes using a volumetric absorptive microsampling device (Mitra Clamshell from Neoteryx) and extracted by sonication in a water-acetonitrile mixture (10/90 *v/v*). After evaporation and reconstitution in water-methanol (95/5 *v/v*) the samples were injected into the LC-MS/MS system. The dynamic range of the method covered three orders of magnitude, with measured metabolite concentrations from 10 pg/mL to 20 µg/mL. The proposed quantification approach using SILs as surrogate calibrant with one level concentration is a promising technique for LC-MS/MS quantitation of multiple analytes. The simplicity and rapidity of the LC-MS/MS are compatible with applications in a clinical laboratory. Validation of the method is ongoing with a further application to individually monitor putative CKD biomarkers in a longitudinal follow-up clinical study.

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Identification of new therapeutic candidates targeting representative RNA probes of Steinert's disease by affinity capillary electrophoresis

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Myotonic dystrophy type 1 (DM1), also named Steinert's disease, is a genetic dominantly inherited degenerative disease. The pathology is caused by the expansion (>50) of a trinucleotide $[CTG]_n$ located on the 3'UTR non-coding region of the DMPK gene (Dystrophia Myotonica Protein Kinase, chromosome 19q13.3).

The pathogenesis of DM1 is not fully understood but most of the evidence indicate a RNA gain-of-function mechanism. The transcription of the expanded $[CTG]_n$ trinucleotide in a toxic $[CUG]_n$ RNA, forming hairpins, leads to RNA *foci* in the nucleus which can interact and sequestrate various molecules and proteins. That phenomenon leads to misfunction of some proteins and finally to the symptoms that are observed in the pathology. Currently, only symptomatic treatments are administrated even though several therapeutic strategies are considered. One of them hypothesis the use of small molecules that can selectively target the RNA sequence and release the sequestrating proteins [1].

The aim of this project is to demonstrate the suitability of an Affinity Capillary Electrophoresis (ACE) method to highlight ligands that bind specifically to DM1-representative RNA probes.

The first part of this study focuses on RNA probes generation. These are obtained with bacterial amplifications of suitable plasmids, containing 95 and 14 [CTG] repetitions, followed by linearization, purification, and finally *in vitro* transcription. The RNA $[CUG]_{95}$ and $[CUG]_{14}$ synthetized fragments are used as pathological and non-pathological probes, respectively.

The second part consists to the development of the ACE method. In ACE the affinity constants can be extracted from migration times which are modified due to the formation of complexes between ligands and analytes. The most important drawback of this mode of operation concerns the repeatability of data between analyses. The optimization of the method allows a good repeatability in terms of migration times. To achieve this goal, a dynamic coating process using polyethylene oxide as coating agent is performed.

Finally, the last part aims to test libraries of small molecules to identify their eventual affinity towards the pathological RNA probes, with the developed ACE method. Those interactions have been quantified by fitting the data by a non-linear regression (plotting $\Delta\mu$ as function of [ligand]) and compared to the ones obtained using the non-pathological RNA probes.

The optimized ACE method constitutes a simple and quick tool to highlight new therapeutic candidates for the clinical treatment of the Steinert's disease. The results will be corroborated by fluorescence microscopy assays to confirm the validity of the ACE method.

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Development and validation of a stability-indicating assay method for Gemcitabine

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Introduction: The preparation of therapeutic solutions of Gemcitabine in advance by a centralised intra-veinous admixture service [1] could optimise the production flow in the pharmacy department and reduce the waiting time of patients in day care units. To this end, stability studies must be conducted and require a specific stability-indicating assay method.
Objective: The objective of the study is to develop and validate a stability indicating assay method for Gemcitabine by ultra-high-performance liquid chromatography.
Materials and Method: A pharmaceutical product of Gemcitabine (Fresenius Kabi®, Sèvres, France) was used to prepare standard and control solutions. Gemcitabine is a polar molecule and present with a neutral form at a pH between 4 and 10 [2]. Gemcitabine was separated on a C18 Phenomenex Luna® Omega with an isocratic mobile phase compounded of phosphate buffer and acetonitrile (95% - 5%; pH 7). The injection volume was 1µl and the flow rate was set at 0.4 ml/min. Solutions were injected on the Acquity UPLC H-Class (Waters Association, Milford Massachusetts) coupled with a photodiode array detector. Linearity, precision, limits of detection and quantification were assessed to validate the method. A degradation test [3] was performed to evaluate the robustness of the method. It consisted in the exposure of the solutions to challenging conditions (acidic, alkaline and oxidative) at 60°C during four days.
Results: The method proved to be linear for a range from 0.152 to 14 mg/ml of Gemcitabine with a determination coefficient of 0.999. Relative standard deviations for intra- and inter-assay were employed to define the precision of the method and were both < 10%. The limit of detection was <0.151 mg/ml and the limit of quantification was <0.152 mg/ml of Gemcitabine. The degradation test showed a significant reduction of the peak of interest (>20%) that remained free from interfering degradation product.

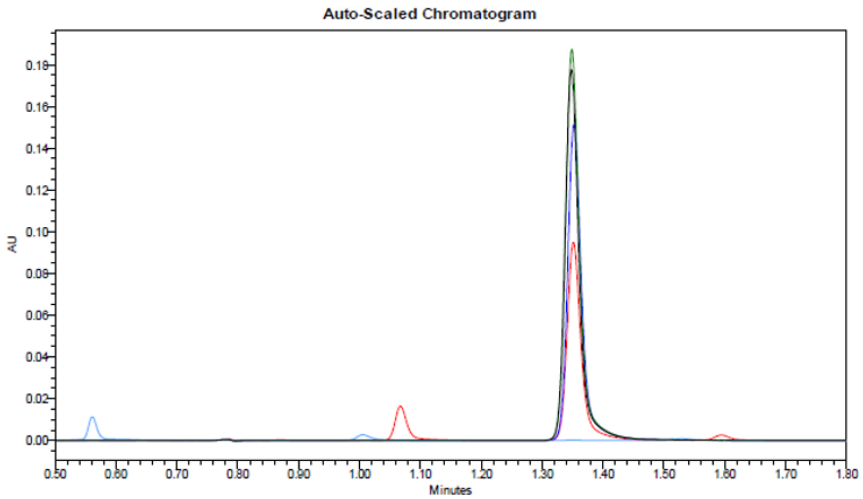

Fig.1: Degradation test. Black line: Natural t0, green line: natural t4/60°C, dark blue line: acidic t4/60°C, red line: alkaline t4/60°C, clear blue line: oxidative t4/60°C

Conclusion: This study led to the development of a stability-indicating chromatographic method to quantify Gemcitabine in order to conduct stability tests.

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The first electrochemical sensor for the determination of Ibrutinib in human serum in the presence of anionic surfactant

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Ibrutinib (IBR), is Bruton's tyrosine kinase (Btk) inhibitor for the chronic lymphocytic leukemia treatment, which is a highly potent, selective, and irreversible small molecule. The IBR was confirmed in 2013 for the treatment of chronic lymphocytic leukemia, and in 2015 as the first registered drug for Waldenström macroglobulinemia. IBR is eliminated via the feces (approximately 80% and only 1% as an unchanged drug) and in the urine is (less than 10%) in the metabolite forms [1].

Surfactants are amphiphilic molecules that can affect the electrode interface via changes in reaction rates and pathways [2] in electroanalytical methods, which are eco-friendly and time-saving. An adsorptive stripping square wave voltammetric technique has been developed for the quantification of IBR in an anionic surfactant medium. The effect of supporting electrolyte, pH, the surfactant concentration, and scan rate on the voltammetric peak responses of IBR were tested on a glassy carbon electrode. The model compounds were used for discussing the possible electrochemical mechanism. The electrochemical behavior of IBR demonstrates irreversible and diffusion-adsorption mix controlled oxidation processes depending on scan rate studies in 0.1 M H_2SO_4. The sensitivity of the method was increased by stripping conditions in the presence of surfactant. In the optimum conditions, a concentration range between 4.0×10^{-9} and 2.0×10^{-7} M was linear in 0.1 M H_2SO_4 containing 2.0×10^{-3} M sodium dodecyl sulfate. The limit of detection was calculated as 2.73×10^{-10} M. The proposed method was utilized for the quantification of IBR in biological samples. Besides that, this method was evaluated in the presence of some ions and potential interference compounds.

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Elaboration of a screening design for the optimization of silver nanoparticles synthesized by chemical reduction based on an Analytical Quality by Design approach

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Surface-enhanced Raman scattering (SERS) has shown great scientific interest all over the world, notably in the pharmaceutical and biomedical fields. Indeed, this vibrational technique presents several advantages like a high specificity and sensibility. This technique requires metallic nanoparticles (Nps) to enhance the Raman scattering signal, by a factor of 10^9 on average by the fact that the interested molecule will be near the surface of Nps. These Nps, also called SERS substrates can be synthesized by different methods. This study was focused on the Lee-Meisel chemical reduction protocol to synthesize silver nanoparticles (AgNps) [1]. This process has many advantages like the rapidity, simplicity of manufacturing but also for the low cost. However, Nps obtained based on this chemical reaction are not repeatable in terms of shape and size into a suspension but also from a synthesis to another one. That causes an issue, particularly for quantitative analyses because nature, size and shape of Nps influence the SERS response leading to unrepeatable signal. In this framework, an innovative approach based on an Analytical Quality by Design (AQbD) strategy was introduced by using design of experiments in order to optimize this synthesis protocol and thus to overcome this limitation [2].

For this first step, a “D-Optimal design” was selected to test several interactions with a limited number of experiments. Moreover, this design was separated into blocks taking into account that three syntheses can be made on the same day. Five parameters were selected, one as categorical variable, the reaction volume and the others as continuous variable, the reaction time and temperature, the pH and the concentration of the trisodium citrate solution. The responses in terms of critical quality attributes (CQAs) were the SERS intensity (to maximize), the relative standard deviation on SERS intensities (RSD% - to minimize) and the polydispersity index (PDI – to minimize).

These preliminary results were promising with PDI situated from 0.119 to 0.476. SERS analyses were made using a model molecule, the violet crystal. Interesting results were obtained with intensities up to 34 000 counts and RSD% of 3 % (n=9 per synthesis). Finally, the interpretation of this screening design showed parameters which are important to optimize to synthesize repeatable and homogeneous AgNps. Concentration and pH of citrate solution seemed to have an important impact on their formation while temperature and reaction time had a lower one.

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POSTERS

The mechanism discovery of the binding interactions of Azacitidine with dsDNA via computational chemistry tools involving molecular docking, molecular dynamics simulation, and supporting analyses by multi-spectroscopic analyses

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Abstract

Azacitidine, a DNA hypermethylation antineoplastic medicinal drug, was studied comprehensively with a great variety of computational tools supported by experimental analyses to decipher the mode of binding and the mechanism behind its inhibition of cancerous DNAs. In terms of theoretical computational chemistry and genetics, molecular docking and molecular dynamics studies proved that Azacitidine is an intercalating agent that shuts down and perishes the helical structure of DNA via guanine and cytosine stack bases, supporting its behavior for RNAs stated in the scientific literature. To prove our point from the perspective of wet-lab, the analytical chemistry experiments including the multi-spectroscopic characterizations yield the data that Azacitidine is truly an intercalation agent towards DNA, it inserts itself between the stacked bases in double-stranded DNA [1]. Hence, this research article vindicates a brand-new discovery for the first time that this cytotoxic antineoplastic pyrimidine analog drug, Azacitidine, can be more useful in the fields of oncology, pharmacy, chemistry, and genetics if its detailed mechanism regarding its pharmaceutical chemical groups' affinities is known.

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Immobilization of cytochrome P450 enzymes: a new approach in drug metabolism and biocatalysis

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Cytochrome P450 enzymes (CYP450) present a wide range of applicability, mainly in drug metabolism studies and in biocatalysis, as a great alternative to synthesizing compounds. Traditionally, studies using CYP450 are carried out in solution, which requires a high number of tests, extensive sample preparation, and consumption of enzymes and cofactors. Thus, novel analytical strategies are required to minimize costs, promote higher stability, and enhance catalytic activity. As attractive alternatives to overcome these drawbacks, herein we demonstrated the development of two bioreactors: 1) immobilization of glucose-6-phosphate dehydrogenase onto magnetic beads (G6PDH-Mb) as an alternative to NADPH production; 2) immobilization of CYP450 enzymes to conduct metabolism studies and increase the production of metabolites.

First, the immobilization of G6PDH onto magnetic beads (G6PD-Mbs) was carried out according to the protocol reported [1]. A reversed-phase method was developed and qualified to monitor the production of NADPH. Then, the G6PD-Mbs kinetic parameters were determined for $NADP^+$, and a sigmoidal curve was obtained with a Km value of 12881 ± 5499 µmol/L and a Hill slope of 1.378 ± 0.0717. As proof of concept, the NADPH produced with G6PDH-Mb was applied in *in vitro* metabolism studies using albendazole.

After, the best immobilization condition was used for the immobilization of human liver microsomes (Hum) onto magnetic beads (Hum-Mb) and tested by the Dohelert experimental design [2]. The ability to metabolize albendazole to its major metabolite, albendazole sulfoxide (ABZ-SO), was used to monitor the activity of the produced Hum-Mb using a qualified LC-MS/MS method. Hum-Mbs was submitted to activity trials at three different temperatures and was verified a higher production of the ABZ-SO at 37 °C and higher stability of the bioreactor was achieved at 23 °C. The Hum-Mb was applied in stationary kinetic studies for albendazole, and a Michaelis-Menten curve was obtained with Km of 25.64 ± 4.90 nmol L^{-1} and Vmax of 121.0 ± 6.68 nmol L^{-1}. Furthermore, a selectivity chemical inhibition experiment for the CYP3A4 was carried out, and the production of ABZ-SO was lower in the presence of ketoconazole. Besides, the catalytic activity of supersomes expressing CYP2D6 and CYP2C9, were tested in Hum-Mbs, by the conversion of bufuralol into hidroxybufuralol and diclofenac into hidroxydiclofenac.

A single bioreactor composed of G6PGH and Hum was produced (G6PGH-Hum-Mbs) and applied in three consecutive metabolism assays. The development of a unique bioreactor demonstrated to be a great alternative for the production of metabolites and in drug metabolism studies.

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HILIC-MS/MS for the quantification of 29 non-derivatized amino acids in human urine

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The analysis of amino acids in biological fluids provides critical information on the understanding of metabolic regulation and serves as a sensitive indicator for the molecular mechanism of disease(s) and the discovery of new biomarkers. Common amino acid detection methods can be divided into derivatization and non-derivatization approaches. The derivatization step is mainly employed to increase amino acid detectability for ultraviolet absorbance, in case direct UV and fluorescence detection are not feasible due to the absence of an ultraviolet chromophore in most amino acids. Unfortunately, it can lead to drawbacks such as derivative instability, labor intensiveness, and poor reproducibility. Hence, this study aimed to develop an accurate and stable hydrophilic interaction liquid chromatography (HILIC) – tandem mass spectrometry (MS/MS) analytical method for the quantitative determination of 29 amino acids in human urine samples, which helps study the physiological roles of amino acids and predict related diseases. A new separation method is provided for the analysis of amino acids. The optimized method achieves a separation of the 29 amino acids in 24 minutes. All analytes have satisfactory linearity and sensitivity with limits of quantitation of 0.03-20 μM. The intraday and interday precision were within the range of 0.8-9.5% and 3.6-11.4%, respectively. Data on accuracy, recovery, matrix effects and stability will be reported. The method successfully analyzed 29 amino acids in human urine samples.

Method development for Lipophilicity determination of very hydrophobic pharmaceutical drugs via MEKC and MEEKC

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In pharmaceutical science, it is important to know the hydrophobicity of compounds, not only for drug (form) development, but also with regard to the LADME model. This means the liberation of the compound, followed by absorption in the body, distribution inside the vessels and the body, metabolism and finally excretion. Of particular interest is the distribution behaviour between the drug carrier and the blood, whereby plasma protein binding is neglected. The octanol-water coefficient (P O/W) is still the best-known method for determining hydrophobicity. However, this is not a good model for physiological conditions. Therefore, other methods such as micellar electrokinetic chromatography (MEKC) or microemulsion electrokinetic chromatography (MEEKC) are a better choice. The latter can be modified by different types of micelle/microemulsion composition to mimic drug-membrane interactions as much as possible [1]. MEKC has been found to be able to separate compounds up to a P O/W of about 5 and MEEKC up to about 7 [2]. However, this is a rough orientation and there is not yet an "one fits all" approach.
Therefore, we aim to develop a method to parallel determine the hydrophobicity of selected pharmaceutical compounds with a theoretical P O/W of up to 8.34, such as fenofibrate, orlistat and lumefantrine. Using the sought different retention time of these substances in our method, it is possible to calculate retention factors which can be similarly used as the P O/W and are comparable regarding the hydrophobicity classification. The highly hydrophobic nature of these compounds complicates the intended hydrophobicity determination in an ideal physiological system and requires the study of compositions of organic modifiers and surfactants, as they are not soluble in a pure aqueous solution. Several adjustments were made with regard to organic compounds such as ethanol, methanol, butanol, heptane, propanol and the surfactants SDS and CTAB.

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Application of multivariate analysis, linear regression and hierarchical modeling for evaluation of robustness in untargeted metabolomics

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Evaluation of robustness (ER) allows to identify critical parameters or state that the procedure remains unaffected by some variations in method parameters. ER is critical for analytical methods employed for targeted metabolomics. However, procedures applied for untargeted analysis may also be affected by small changes in method parameters (especially when the number of steps in a given procedure is high). The study aimed to evaluate robustness of urine sample preparation procedure for GC-MS metabolic fingerprinting. For this purpose, Placket-Burman design was used.

All controllable steps of sample preparation procedure might be tested in robustness studies. Selection of factors and their levels is crucial. ER was performed by shifts in 10 method parameters, *e.g.*: incubation time, or evaporation temperature. It seems that time-related factors might be especially important, since they may vary considerably, *e.g.* addition of reagent to subsequent samples may last even several dozen minutes.

Responses must provide significant information and be accurately measured. Metabolomics data are presented as hundreds of analytical signals. However, due to complexity of data matrix, variable reduction might be necessary during statistical analysis.

The obtained raw data were preprocessed. The missing data were replaced by the mean values of signals for each metabolite and the data were log-transformed to enhance additivity. Responses (PCScores for PCA or an average signal intensity across metabolites) were regressed with factors. Both approaches identified the overnight incubation time (16h±1h) as critical parameter. However, the limitations of these approaches are due to estimating only common effects of all metabolites (signals).

Another approach used Bayesian hierarchical modeling. The raw data were preprocessed, log-transformed, centered and scaled. The model assumed additive effect of all factors on the response and robustness to outliers. This approach enabled selection of the most influential factors affecting the obtained metabolic profiles at the population level (common for all metabolites) and at the individual level (metabolite specific).

Only 12 experiments provided useful information about the robustness of studied procedure. The results obtained for the overnight incubation time points to the necessity to limit the number of samples prepared in one batch. This finding will be implemented in future projects.

The presented approach might be useful *e.g*. for the evaluation of the number of samples that can be prepared or tested in a series (important for labile samples or ones with a complicated preparation procedure). Also, magnitude of change caused by altering conditions in the laboratory can be estimated [1].

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Development of a quantification method of serum amyloid A variants in plasma, synovial fluid and cell culture medium and a co-culture model to better understand rheumatoid arthritis

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In a first study, we developed and validated an UHPLC-MS/MS method for the quantification in plasma of five variants of serum amyloid A (SAA) protein, namely SAA-1α, SAA-1β, SAA-1γ, SAA-2α and SAA-2β. We showed that SAA-1α, SAA-1β and SAA-2α were upregulated in plasma of patients suffering from rheumatoid arthritis (RA) [1]. To better understand the physiopathological role of these SAA variants and the molecular pathways triggered by these variants in the context of rheumatoid arthritis, we decided to stimulate fibroblast-like synoviocytes (FLS) of patients suffering from RA with each of these three interesting SAA-variants. Since these variants are not commercially available, we made them produced by liver cells transfected with the appropriate plasmid. To validate this co-culture model, specific quantification of SAA variants using our UHPLC-MS/MS method were realized in plasma, synovial fluid and newly developed cell culture medium conditions. Concentrations obtained in the two biological fluids were found well correlated, but concentrations observed in plasma of RA patients were significantly higher than concentrations observed in synovial fluids of those same patients. For SAA-2α, concentrations observed in cell culture media were found in agreement with the SAA-2α concentrations detected in synovial fluid of RA patients. In the near future, we will focus on the role of SAA-2α performing an untargeted proteomics study of stimulated FLS cells.
In this project, we used a sensitive and specific MS/MS method to quantify SAA variants which provide additional value compared to ELISA, which is the gold standard used for SAA quantification but is unable to differentiate variants. Using our co-culture model, FLS could be stimulated with a specific SAA variant which constitutes an innovative approach to better understand the pathophysiological role of SAA variants in RA.

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A new convenient tool to analyse protein glycosylation based on FT-IR spectroscopy

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Glycosylation is the most common protein post-translational modification (PTM), especially in biopharmaceuticals. It is a critical quality attribute as it impacts product solubility, stability, half-life, pharmacokinetics and pharmacodynamics (PK/PD), bioactivity and safety (e.g. immunogenicity). Yet, current glycan analysis methods involve multiple and lengthy sample preparation steps which can affect the robustness of the analyses. The development of orthogonal, direct and simple method is therefore desirable.

We suggest the use of FT-IR spectroscopy as a suitable and powerful tool to analyse protein glycosylation. Three types of analyses can be realized using this tool:
- Comparative study in terms of global glycosylation level [1];
- Comparative study in terms of glycan composition [2];
- Prediction of the monosaccharide content using predictive models based on advanced statistical methods. [3]

The FT-IR-based method to analyse glycosylation offers three key advantages. Firstly, the analysis is performed on intact proteins, which represents a major asset. Indeed, all the existing methods for glycans and monosaccharides analysis involve several preparation steps: glycan release, labelling, separation and hydrolysis for monosaccharide analysis. Secondly, the processing time is extremely short (measurement in maximum 5 minutes and fully automated data analysis). Finally, FTIR spectroscopy can be used as a Multi-Attribute Methodology (MAM). Analysis of other critical parameters for therapeutic proteins (such as the protein structure or protein concentration) can be performed simultaneously.

The two major applications of this FTIR-based approach are:
1. Batch-to-batch consistency checks during development, optimization and verification of the production process;
2. In-process controls (at-line or on-line requiring the development of some devices) to assess the repeatability and the product quality during production processes.

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Production of SPMs by endothelial cells and vascular smooth muscle cells in presence of Mox-LDLs and nat-LDLs

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Myeloperoxidase-oxidized low-density lipoproteins (Mox-LDLs) play a role in inflammation and its resolution. Dufour et al. demonstrated that endothelial cells produced resolvin D1 (RvD1) after stimulation with Mox-LDLs and native LDLs (nat-LDLs).[1] RvD1 is one of the many specialized pro-resolving mediators (SPMs) which promotes inflammation resolution. We developed a LC-MS/MS screening method based on Dufour et al.[1] This screening detects a series of SPMs (lipoxin A4, maresin 1, resolvin E1, resolvin D1, D2, D3 and D5) and their precursors (eicosapentaenoic acid (EPA) and 18-HEPE, docosahexaenoic acid (DHA) and 17-Hydroxy-DHA). Analysis were conducted using an Agilent 1290 Infinity Binary – UHPLC system coupled to a mass spectrometer Agilent electrospray ionization source – Triple Quadrupole 6490. For each compound, we selected two transitions and the associated collision energy were optimized using Optimizer software from Agilent technologies. We tested the method on spiked plasma and interpreted the results by considering the signal-to-noise ratio (S/N ≥ 3), the retention time and the shape of the peaks observed for both transitions when compared to a mix of standards injected in the same run. We then measured DHA, 17-HDHA and RvD1 as Dufour et al. in cellular media in two different cell types: vascular smooth muscle cells (VSMC) and human umbilical vein endothelial cells (HUVEC). Each cell type was incubated with 100 µg/ml of Mox-LDLs and 1000 µg/ml of nat-LDLs as described in ref [1]. We used three controls: cell culture medium, medium supplemented with Mox-LDLs and nat-LDLs, and cells incubated without supplementation. We detected in the supernatants EPA, DHA, 17-HDHA and RvD1 in all conditions. In presence of Mox-LDLs and nat-LDLs, we noticed the apparition of 18-HEPE. Lipoxin A4, maresin 1, RvD2, RvD3 and RvD5 were not detected in any conditions. A quantitative method confirmed the conclusions of the screening regarding DHA, 17-HDHA and RvD1. We also observed that there is an increase in RvD1 concentration after stimulation by Mox-LDLs and nat-LDLs in both cell types (HUVEC: Ctrl cell: 0.003±0.003 ng/ml; Medium supplemented: 0.28±0.02 ng/ml; Cell supplemented: 1.4±0.7 ng/ml; VSMC: Ctrl cell: 0.049±0.007 ng/ml; Medium supplemented: 0.47±0.05 ng/ml; Cell supplemented: 0,67±0,05 ng/ml). The same trend was observed with 17-HDHA. Finally, we found that Mox-LDLs and nat-LDLs provide a large amount of DHA (520±37 ng/ml) to the cells which then probably consumed it. The reaction of the endothelial cells to Mox-LDLs and nat-LDLs seems to be specific to RvD1 as no other SPM was detected in the supernatants.

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Liquid chromatography tandem mass spectrometry determination method of bencycloquidium bromide: application to drug interaction study in human plasma

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ABSTRACT

Bencycloquidium bromide (BCQB) is a selective muscarinic M1/M3 receptor antagonist developed for the treatment of rhinitis, asthma and chronic obstructive pulmonary disease (COPD). This study was conducted to develop a sensitive and effective LC-MS/MS method for the determination of BCQB and its application in pharmacokinetic drug interaction study between BCQB and paroxetine.

The chromatographic separation was performed on Hedera ODS-2 C18 column (150 mm×2.1 mm i.d., 5 µm d.p, Hanbon Sci. And Tech) protected by a security guard C18 column (4 mm × 2.0 mm i.d., 5µm d.p, Phenomenex, Torrance, CA, USA) with a mobile phase consisted of acetonitrile-10 mmol/l ammonium acetate containing 0.2% acetic acid (33:67, v/v) at 550 µl/min, and the injection volume was 10 µl. The plasma samples were processed using weak cation-exchange solid-phase extraction (WCE-SPE). The LC system was coupled with an Agilent 6410B triple quadrupole mass spectrometry (Agilent Technologies, USA) equipped with an electrospray ionization source (model G1956B). The MS/MS transitions were *m/z* 330.2 → 142.0 for BCQB and *m/z* 344.2 → 156.1 for the ethyl-BCQB as internal standard (I.S) in positive ESI mode.

The validated method was linear over the concentration range of 2-1200 pg/ml with the correlation coefficient r^2>0.998. There was no endogenous interference observed at the retention times of BCQB and the IS, which were 3.35 and 4.25 min, respectively. The intra- and inter-batch precisions of the assay were lower than 8.2% and 9.1%, respectively. The lower limit of quantification (LLOQ) was 2 pg/ml. The stability data at different storage conditions of BCQB were within±5% RE. The mean $AUC_{0\text{-}36}$ of BCQB was increased by approximately 33% after co-administration with paroxetine during the drug interaction study compare to the administration of BCQB alone.

The LC-MS/MS method validated in this study was robust, reproducible, accurate, precise and reliable and was successfully applied in the pharmacokinetic drug interaction studies.

This method provided a higher mass spectrometric response and lower background noise; eliminated matrix effect and shortened the total analytical run time to 7.5 min instead of 12 min in previous methods. The calibration range was extended (2–1200 pg/ml) to fit into the drug drugs interaction study without further dilution step.

Keywords: Bencycloquidium bromide, LC-MS/MS, Pharmacokinetic drug interaction, Paroxetine

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Simultaneous determination of gentamicin and tacrolimus in rat whole blood by LC–MS/MS and its application to pharmacokinetic study

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The threat of bacterial infections has increased worldwide, not because of a pronounced rise in infections, but because of an increase in antibiotic resistance. Solid organ transplantation patients are becoming at high risk of infection, particularly with Carbapenem-Resistant Enterobacteriaceae (CRE) that pose globally a significant threat to patients and healthcare systems. Aminoglycosides (AGs) are being considered as a valuable effective alternative option in case of multidrug resistant pathogens despite their nephrotoxicity and ototoxicity. This is because of their chemical stability, little resistance, fast bactericidal effect and synergy with beta-lactam antibiotics. Gentamicin (GEN) is probably the most commonly used and studied of all aminoglycosides. Tacrolimus (TAC) is an immunosuppressive drug whose main use is after organ transplant to reduce the activity of the patient's immune system and so the risk of rejecting the donor organ. However, it shows considerable nephrotoxicity. Possible interactions between AGs and immunosuppressant make the treatment more complex in organ transplantation patients. The co-administration of these drugs changes the pharmacokinetic (PK) characteristics of both groups of drugs, having severe consequences.

In this work, a new UPLC-MS/MS method was developed and validated for the simultaneous determination of GEN and TAC in order to investigate the PK interaction between GEN and TAC in Wistar rats' whole blood. The analytes were extracted following a protein precipitation procedure, using clindamycin (CLN) as internal standard. The analytes were separated by means of a UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 µm) which was maintained at 50 °C. The mobile phases consisted of acetonitrile and water containing 2 mM ammonium acetate and 0.1% trifluoroacetic acid. Gradient elution was performed at a flow rate of 0.3 ml/min. The detection of the analytes was done on a triple quadrupole mass spectrometer operated in positive electrospray ionization mode (ESI) and quantified using multiple reaction monitoring (MRM). Calibration curves were linear over the range of 20–1250 ng/mL for GEN and 2–125 ng/mL for TAC ($r > 0.999$). The method was successfully applied in a PK study after intramuscular administration of GEN and oral administration of TAC in Wistar rats. Histopathology examination was performed on the kidney tissue of rats after euthanizing, to analyze any abnormal changes in the kidneys for each rat group.

Molecularly imprinted polymers via high internal phase emulsions polymerization for selective retention of Sartans

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Sartans are chemical compounds with antagonistic action towards the AT1 receptors of angiotensin II, a multifunctional peptide with vasoconstrictor action and one of the main peptides responsible for regulating blood pressure [1].
Their commercial success together with their low metabolic degradation and high environmental persistence has consecrated them as emerging pollutants in just 20 years. In fact, Sartans have been found with alarming frequency in all aquatic environments [2].
In this context, the aim of the study was to find effective methods to selectively extract Sartans from aqueous solutions.
Molecularly imprinted polymers (MIPs) are characterized by an excellent molecular recognition ability due to the use of a template molecule during their synthesis, a great physical strength, thermal stability and chemical resistance. For these reasons, they have been already largely studied in the field of water purification [3].
In this work, MIPs based on a polymerized high internal phase emulsion (polyHIPE) material were produced using irbesartan as the template molecule.
HIPEs are paste-like water in oil emulsions where the oil phase contains acrylates monomers and a crosslinker. PolyHIPEs have received increasing attention and wide applications in separation science due to their remarkable merits such as highly interconnected porosity, high permeability, good thermal and chemical stability, and tailorable chemistry [4-6]. Some applications of polyHIPE-based molecularly imprinted polymers (MIPs) have been already reported [7].
The polymerization of HIPE in the presence of targeted template molecules (irbesartan) was carried out at different template/monomers ratios (1:100, 1:30 and 1:15) and in various formats (disks, cartridges and columns) and materials were compared to the corresponding non-imprinted polymers (NIPs).
MIPs obtained in columns were investigated by HPLC frontal analysis and zonal elution experiments, while disks and cartridges were tested through spectrophotometric assays allowing to build binding isotherms and breakthrough curves. Experiments at different irbesartan concentrations and pHs were carried out together with specificity studies.
This pilot work paves the way to the use of MIPs as alternative purification methods to extract Sartans from wastewaters.

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Multidimensional Fingerprint Development for the Detection of *Tribulus terrestris* in Plant Food Supplements

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Tribulus terrestris is a popular Ayurvedic herb with potency enhancing properties. According to the Belgian Royal Decree of 1997, *Tribulus* is a regulated plant when used in plant food supplements. The dangers of self-medication and toxicity of such plant supplements is heightened by the ease with which they can be purchased from various illegal channels, necessitating an efficient screening approach.

This study aims to develop a multidimensional approach using chromatographic fingerprinting at different wavelengths for identification of *Tribulus terrestris* in plant food supplements. A specific, selective and fast fingerprinting method for *Tribulus* (reference material obtained from the American Herbal Pharmacopoeia) was developed using ultra high performance liquid chromatography coupled with a diode array detector. Next, a correlation analysis comparing the wavelengths was performed and 5 orthogonal wavelengths were selected. The developed method was tested for specificity in different triturations (1/20, 1/15, 1/10, 1/5 and 1/2). These were prepared using 10 blank matrices and lactose. In most triturations, *Tribulus* could visually be detected from concentration 1/10 onwards, by comparing the fingerprints with the reference plant.

A market study of a total of 20 samples (with potency enhancement as indication) seized by the Belgian Federal Agency for Medicines and Health Products (FAMHP) was carried out. Visual inspection was conducted to detect the presence of *Tribulus* in the real samples. This was done by overlaying the sample chromatograms with the reference plant and visually comparing the fingerprints. So, out of 20 samples, 11 samples were suspected to be positive, when analyzed at the 5 wavelengths, 254 nm and with the combined fingerprint. The multi and combined wavelengths approach provides a characteristic and specific fingerprint with more information at different wavelengths than only 254 nm, which is useful for visual inspection. In a second step, mass spectrometric (MS) measurements using a time of flight analyzer were performed. This was done in order to further confirm the presence of *Tribulus* in suspected samples. After obtaining MS fingerprints, the exact mass of the main peaks (peaks with the highest absorbance in the reference) were compared. It was concluded that out of 11 samples, 9 can be suspected to be positive for *Tribulus terrestris*. As future prospects, steps are being taken to combine chromatographic fingerprinting and chemometrics in order to use these multidimensional data and extract maximum information with the intention to create binary models for faster screening of plant food supplements.

Method development for studying protein binding properties by mobility shift affinity capillary electrophoresis with collagen as ligand

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Mobility shift affinity capillary electrophoresis is an approach to study interactions of drugs with biomaterials. This technology can help to better understand the function and role of proteins in the human body and improves the development of drugs and medical devices. Therefore, various concentrations of ligand are dissolved in the BGE and an analyte and a non-interacting mobility marker are injected into the capillary. During electrophoresis, the presence of a ligand can change the effective electrophoretic mobility of the analyte. Thus, the strength of the interaction can be estimated by calculating binding constants. [1]
The aim of this project is to investigate possible binding properties of proteins with a collagen product constructed for wound healing to find out about its biocompatibility. As a BGE, a phosphate buffer with pH = 7.4 was chosen to mimic natural conditions of the human body. Because of the poor solubility of collagen in neutral medium, a procedure was established to make parts of the collagen product suspendable which was achieved by grinding and freeze-drying. Subsequently, a robust method will be developed to measure different proteins reproducibly with and without the presence of collagen. The development of such a method is challenging, since differences in migration time and peak shape can occur due to adsorptive capillary effects. Therefore, an LPA coated capillary (75 µm ID) is used to reduce protein adhesion. Another important aspect is, that the BGE containing collagen is a suspension and this results in an increase in current strength. Thus we chose constant power as separation mode. In the future, mobility shift affinity capillary electrophoresis with collagen at various concentrations as ligand and the respective proteins as analytes will be carried out and evaluated.

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NIR and MIR spectroscopic fingerprints combined with chemometric tools for a successful geographical discrimination of Argan oil from four Moroccan regions

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Argan oil, a precious oil with many nutritional and cosmetic properties [1], has seen an increased national and international demand in recent years, and consequently an increase in falsifications. The determination of its geographical origin, which may be carried out using chromatographic fingerprints [2], has become a necessary task in the authentication and the quality control of this product. In this study, the capabilities of two spectroscopic techniques, NIR and MIR, which are faster and cheaper than chromatography, and non-destructive techniques were evaluated in combination with multivariate data analysis to discriminate 93 Argan oil samples from four Moroccan regions. The NIR and MIR spectra were preprocessed using mean centering and Generalized Least Squares Weighting (GLSW). The PCA score plots for NIR and MIR showed four distinct clusters. For discrimination purposes, PLS-DA and SIMCA models were constructed for each spectral data set. The recorded data was split into a training set (70%) and a test set (30%), while venetian blind cross-validation with six splits was used to select the best models. The constructed PLS-DA models had determination coefficients (R^2) above 99% and 96% for NIR and MIR, respectively. For both data sets, the PLS-DA models show 100% specificity and selectivity. For the SIMCA model, the spectral data was forced to be attributed to one of the four classes by choosing distance as assignation criterion. The accuracy was 96% and 94% for the training set, and 92% and 91% for the test set for NIR and MIR, respectively. The constructed models achieved a sensitivity of 87%-100% and 71%-100%, and a specificity of 88%-100% and 86%-100% for NIR and MIR, respectively. As a conclusion, applying PLS-DA and SIMCA good discriminative models were built with good sensitivities and specificities. However, when comparing the results of the NIR and MIR models, no significant differences were observed. Consequently, both techniques can be used for the authentication of Argan oils.

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Enantioseparation and quantification of amino acids with chiral capillary-electrophoresis mass spectrometry

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The comprehensive analysis of a metabolome requires the use of complementary separative techniques capable of addressing a large number of metabolites with diverse structures and properties. A remarkable example of that are the stereoisomers appearing when metabolites bear chiral centers. Opposite to diastereomers, which have quite dissimilar behaviours, enantiomers share most of their physicochemical properties but can exert dramatically different biological functions. Thus, the effective separation of enantiomers can play a major role in the understanding of the regulatory mechanisms of a biological system and the identification of biomarkers.

For a long time, it was considered that the D- form of amino acids had a minor role and that the only form naturally present in mammals was the L-. More recently, a growing number of studies have highlighted the importance of D- amino acids (D-AAs) and their involvement in stereoselective biological functions. For example, D-serine has been found to be a co-agonist of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors with glycine in brain [1]. D-aspartate has been evidenced to have hormonal regulatory functions in endocrine tissues [2]. D-aspartate and D-alanine were respectively downregulated in white matter and upregulated in gray matter of Alzheimer diseased brains compared to healthy ones [3]. More recently, the presence of D-AAs in altered concentration has been reported in patients with cancer [4].

From an analytical point of view, the resolution of amino acid enantiomers in biological samples has to address the challenge of resolving enantiomers present at very different concentrations, the L-form being physiologically much more abundant than the D-form. The potential of capillary electrophoresis for enantioseparation has been repeatedly demonstrated. The latter can be performed either in direct or indirect mode with the addition of a chiral selector to the background electrolyte or after a derivatization step.

Herein we describe the development of a direct chiral capillary-electrophoresis mass spectrometry method for the enantioseparation and quantification of amino acid enantiomers in biological matrices. The partial-filling technique was employed to enable mass spectrometry detection with non-volatile chiral selectors.

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Temporal monitoring of pharmaceutical consumption using a wastewater-based epidemiologic approach

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Introduction: Conventional epidemiologic datasets on pharmaceutical consumption include surveys, prescription, sales, and dispensing. However, prescribed medication may not always be dispensed, and surveys are affected by bias. Furthermore, data on pharmaceutical dispensing is limited to reimbursed medication and lacks coverage. Wastewater-based epidemiology (WBE) centres on the analysis of biomarkers, i.e., human metabolic excretion products of xenobiotics in influent wastewater. WBE complements existing drug utilisation approaches and provides objective, spatio-temporal information on the consumption of pharmaceuticals in the general population.
Methods: WBE was applied to 24-h composite influent wastewater from Leuven, Belgium. Daily samples were analysed from Sept 2019 to Dec 2019 (n=63), and on three weekdays (Mon, Wed, Sat) from Jan 2020 to Dec 2021 (n=165). The sampled period coincided with several governmental restrictions to contain the spread of SARS-CoV-2. Sample analysis consisted of solid-phase extraction and liquid chromatography coupled to tandem mass spectrometry. Measured concentrations (ng/L) of 21 pharmaceutical biomarkers were converted to population normalised mass loads (PNML) by considering the flowrate and catchment population. To better capture population movements, mobile phone data was used.
Results and discussion: Concentrations of bupropion, hydromorphone, melitracen, noroxycodone, oxycodone, and tilidine were negligible or below LOQ and therefore excluded from further analysis. Amitriptyline, hydroxy-bupropion, N-desmethylcitalopram, citalopram, N-desmethyl-mirtazapine, mirtazapine, trazodone, O-desmethylvenlafaxine, codeine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, methadone, morphine, nortilidine, O-desmethyltramadol, and tramadol were detected and included in the temporal assessment. The PNML of most psychoactive pharmaceuticals remained stable throughout the sampled period. Highest median PNML levels by pharmaceutical class were obtained for tramadol (opioid, median 508 mg/day/1000 people) and O-desmethylvenlafaxine (antidepressant median 392 mg/day/1000 people). Governmental measures appear to minimally impact consumption. Yet, there was an increase in the consumption of psychoactive pharmaceuticals during the lockdown phase, but this is probably caused by the changing demographics of the catchment area. This change may be explained due to a change in population demographics. Leuven has a large commuting student population (~45%), although the catchment area encompasses nearby areas. Otherwise, no dramatic changes occurred in the consumption of these prescription drugs. Furthermore, a small difference in week/weekend PNML is observed for all pharmaceuticals. Antidepressants and opioids are indicated to be used consistently, and on long-term in case of antidepressants.
Conclusion: WBE has the potential to monitor consumption trends of pharmaceuticals with high temporal resolution, that may not be captured by other data sources. Furthermore, care should be taken when interpreting WBE results in case of large population disruption.

Greener approaches in the extraction of the biological samples before the analysis of naltrexone and its major metabolite by HPLC

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Naltrexone (NTX) is an opioid antagonist that blocks the pharmacologic effect of opioids such as morphine and heroin etc. It has highly efficacious blocking capability with competitive antagonist activity at μ-opioid receptors. It also undergoes extensive hepatic metabolism, primarily via the reduction of its major metabolite, 6-β naltrexol (6BNTX) (Figure 1). 6BNTX is believed to be a major contributor to the pharmacological effect of naltrexone.

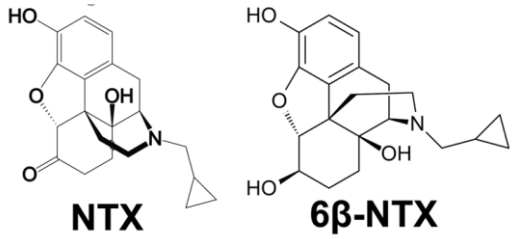

Figure 1. Molecular structures of NTX and 6BNTX

This study aims to develop extraction methodologies for greener, more sensitive, reliable, and precise determination of NTX and its metabolite by using high-performance liquid chromatography (HPLC) followed by UV detection. Based on the literature survey there is a gap in sample preparation methods for these compounds. In the literature, mostly used extraction techniques from biological matrices were liquid-liquid extraction and solid-phase extraction. However, greener approaches need to be developed to reduce organic waste consumption.

During the development of the dispersive liquid-liquid microextraction (DLLME) method, acetonitrile, acetone, and methanol were compared as dispersive solvents, chloroform, chlorobenzene, carbon tetrachloride, and carbon disulfide, 1,2 dichloroethane, and trichloroethylene were compared as extraction solvents. Furthermore, an investigation of the effects of ionic strength and pH on extraction efficiency was realized.

Optimized conditions for the extraction methodology were used to analyse both compounds simultaneously from biological media by using Kinetex EVO C18 (150 mm x 4.6 mm i.d., 2.6 μm) as an analytical column.

The optimized HPLC-UV methodology was validated according to the International Council for Harmonization Guidelines in terms of selectivity, linearity, range, the limit of detection and quantification, accuracy, precision, etc.

Eco-friendly extraction of bioactive metabolites from Ecuadorian quinoa (*Chenopodium quinoa* Willd.) by natural deep eutectic solvents (NADES)

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Quinoa has been widely cultivated in Ecuador to produce edible seeds. In the last 20 years, the interest in this crop has grown around the world due to its exceptional nutritional, health-promoting and/or disease-preventing properties.

In the first part of this study, we focused on the screening of α-amylase inhibitors and radical scavengers by high-performance thin-layer chromatography (HPTLC) bioautography of methanolic quinoa leaf extracts obtained from the sweet varieties INIAP-Tunkahuan and INIAP-Pata de Venado, and the bitter genotype, Chimborazo, harvested at different cultivation times (40, 60 and 80 days) and locations (Pichincha and Chimborazo Provinces in Ecuador). All samples of quinoa leaf methanolic extracts harbored high levels of free radical scavenging compounds, notably quercetin and kaempferol derivatives.

In the second part of this study, we studied a new generation of green solvents called natural deep eutectic solvents (NADES), which have emerged as an eco-friendly alternative to petrochemicals to dissolve and extract plant metabolites. An efficient "green" extraction method was developed by investigating eight different types of NADES based on choline chloride, sugars, amino acids, organic acids, and/or polyols. These NADES were characterized by rheological measurements and differential scanning calorimetry (DSC). The phytochemical compositions of NADES-extracts were analyzed by HPTLC-bioautography methods. From all NADES studied, the eutectic system based on choline chloride-glycerol-water at molar ratio 1:2:1 presented remarkable features: (i) a short time of preparation (20 min), relatively low viscosity, and high stability (storage up to 20 months without crystallization at room temperature); (ii) the best potential alternative to methanol for eco-friendly extraction of flavonoids, radical scavengers, and α-amylase inhibitors from quinoa leaves; and (iii) a higher capacity to stabilize the quinoa leaf radical scavengers for up to 4 months at 5°C when compared to methanol.

In the last part of this work, the retained eutectic system was used for the extraction and stabilization of quinoa saponins from 5 different samples of quinoa husks and seeds. Characterization and relative quantification of saponins were achieved by mass spectrometry. Regardless the type of sample, the major detected sapogenins were hederagenin and phytolaccagenic acid. We found that, compared to conventional solvents, the selected NADES offered a higher stabilization of quinoa saponins in liquid extracts for up to 2 months at 5°C.

In conclusion, our work opens new perspectives for the development of high added value products, based on natural extracts from quinoa leaves and husks using NADES, for pharmaceutical, nutraceutical and food applications.

Set up of an UPLC-MS quantification method for short chain fatty acids, branched chain fatty acids and citrate cycle metabolites

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Short chain fatty acids (SCFA) are mainly originating from dietary fiber fermentation by the gut microbiota whereas branched chain fatty acids (BCFA) are resulting from the fermentation of branched chain amino acids. The study of these small size metabolites gained interest due to the demonstration of the key role played by the microbiota in health and disease. Some of these microbiota-produced metabolites are an important source of energy for the host. Moreover, some of them (e.g. propionate and butyrate) play important regulatory roles notably by binding to G-protein-coupled receptors such as GPR43.

The metabolic pathways leading to their synthesis are complex and their levels can be affected by the diet and by alterations of the microbiota. Even if analytical methods are described for their quantification, usually focusing on the most abundant metabolites, there is a lack of specific and sensitive methods to characterize these metabolic pathways in their globality. Thus, we decided to develop and validate a quantification method allowing us to quantify the SCFA, the related BCFA, as well as the citrate cycle metabolites and the ketone bodies (that share some metabolic pathways with the SCFA and BCFA) in biological matrices such as feces, blood and liver tissue.

To avoid potential issues due to the volatility of the analytes and to take into account the presence of SCFA in most organic solvents, we decided to remove the proteins from the samples by protein precipitation and to apply a derivatization step in the resulting supernatant. After having tested several aqueous-compatible derivatization methods, we selected 3-nitrophenylhydrazine as reagent in presence of EDC and pyridine under mild conditions. Next, we optimized an UPLC method to analyze the derivatized products, using derivatized valproic acid and d_4-succinate as internal standards. The separation was achieved using an Hypersil-gold PFP column (100x2.1mm; 1,9µm) and a gradient system using H_2O-ACN and ACN (both acidified with 0,1% acetic acid). The derivatization step favored the separation of isomeric species and their detection in highly complex matrixes.

For the detection we used an LTQ-Orbitrap XL mass spectrometer which gave us access to both HRMS and fragmentation spectra. Both the mass precision and the MS/MS spectra support the specificity of our method.

In conclusion, this method will constitute a powerful tool to quantify SCFA, BCFA, citrate cycle metabolites and ketone bodies in preclinical and clinical samples.

Inhibition by pesticides of the main CYP450 forms involved in drug metabolism and prediction of in vivo inhibition

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Pesticides play an important role in food production since these chemicals help maintain the efficiency and quality of the crops by killing pests and preventing crop diseases. The use of pesticides has been increasing and the risks associated with their exposure may also affect the pharmacokinetics of drugs by interacting with the cytochrome P450 (CYP450) enzymes. The presence of pesticides in the body may change the plasmatic concentration of a drug which may lead to toxic effects. The implications of such interactions are especially important for elder people since polypharmacy in such a population and thus drug-drug interaction is more common. Based on that, our research group has been studying the inhibitory effect of pesticides on the main CYP450 forms involved in drug metabolism. This work describes the results regarding the inhibitory effect of fenamiphos, fipronil, myclobutanil, tebuconazole, and ethofumesate on CYP3A4/5, CYP2D6, CYP2C9, CYP2C19, CYP1A2, and CY2E1 forms. In addition, for the ethofumesate pesticide, a prediction of CYP2C19 in vivo inhibition was carried out. Among the evaluated pesticides, the ones that least influenced the enzyme activity of evaluated CYP forms were fipronil and ethofumesate, with just only one CYP form inhibited by these pesticides (CYP2D6 - fipronil; CYP2C19 - ethofumesate). Overall, most of the evaluated pesticides caused a decrease in the activity of important CYP forms that are involved in the metabolism of several drugs (myclobutanil and tebuconazole had IC50 values below 1 μM for CYP2C19, CYP3A4/5, and CYP2C19). To evaluate if an in vivo inhibition could happen, we carried out an in vitro-in vivo extrapolation for the ethofumesate pesticide according to the Food and Drug Administration's guideline on the assessment of drug-drug interactions used in the early stages of drug development. The correlation revealed that ethofumesate probably inhibits CYP2C19 in vivo for both chronic (oral) and occupational (dermal) exposure scenarios. In conclusion, our studies suggest another potential toxic effect of pesticides, since individuals who are taking medicines metabolized by the inhibited enzymes and were exposed to pesticides under study (either by ingestion of food or water contaminated) may suffer the toxic effects of drugs (medicines) due to their accumulation in the body.

Development and validation of an HPLC-PDA method to quantify methadone in syrups prepared by pharmacists

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Compounded preparations must meet certain quality criteria to be effective and not harm patients. This is why the Association of Pharmacists Belgium (APB) has initiated a quality program for compounded preparations for which Belgian pharmacists can participate on a voluntary basis.
In 2021, the laboratory of the Association of Pharmacists Belgium has analyzed not less than sixty syrups containing methadone hydrochloride at 1 mg/mL. To perform these analyses, an analytical method had to be developed and validated. The identification and the assay of the active substance was performed by HPLC-PDA. Chromatographic separation was achieved on a Symmetry Shield RP18 column (4.6 mm x 150 mm, 3.5 µm) maintained at 30°C. The mobile phase was composed of solvent A (ammonium acetate 5 µM in water, glacial acetic acid 0.03%) and solvent B (ammonium acetate 5µM in water/acetonitrile (1:9), glacial acetic acid 0.03%). An elution gradient was used to ensure good separation between methadone and the other constituents of the syrups. The flow rate was 1.0 mL/min. Samples were detected and quantified at 210 nm using a PDA detector.
The method was proven to be linear in the range of 20-80 µg/mL (r^2 = 0.99997). The samples were analyzed using a calibration curve in the range of 30-70 µg/mL. Repeatability and intermediate precision were determined on three different days on a spiked placebo, at three different levels in the range of 40-60 µg/mL. The within group variances were homogeneous, as evaluated by the Cochran test, and a repeatability standard deviation (RSDr) of 0.7% was obtained. The RSD value obtained for the intermediate precision (RSDR) was 0.7%. The measurement uncertainty was defined as 2.0%. The accuracy of the method was confirmed by calculating the 95% confidence interval, which was found to be 100.2-100.6%.
This analytical method allows to quantify methadone hydrochloride in two different formulations: the formulation described by the Magistral Therapeutic Form and the formulation which is generally used in prison. Sixty syrups were analyzed in the context of the quality program and 97% of the preparations met all analytical parameters. The average methadone hydrochloride content for all preparations was 99.9%. The minimum and the maximum assay value were respectively 91.6% and 115.8%.
The developed and validated method is suitable to evaluate the quality of syrups containing methadone hydrochloride made by pharmacists.

Neomycin (NEO) and kanamycin (KAN) as potential human serum albumin (HSA) antioxidant activity modulators – calorimetric and spectroscopic study

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Many diseases are associated with the disturbance of free radicals (RF) at normal level in the human body. The concept of modulating endogenous antioxidant mechanisms with medications provides a novel view on personalizing pharmacotherapy [1].
The aim of this study was to investigate the consequences and causes of neomycin (NEO) and kanamycin (KAN) effect on human serum albumin (HSA) antioxidant activity using spectroscopic (UV-Vis spectroscopy, CD spectroscopy) and calorimetric (nanoITC) techniques.
In the first part of the study, the interaction between ligands (KAN and NEO) and HSA was analysed. Based on the obtained data using nanoITC we concluded that both KAN and NEO have similar affinity. The binding reaction between KAN-HSA and NEO-HSA complexes, stabilized probably by ionic (KAN-HSA) and by ionic and hydrophobic bonds (NEO-HSA) was exoenergetic and endoenergetic, respectively [2]. The slight effect of interaction between ligands and HSA on protein secondary structure obtained based on the percentage change in the α-helix calculation (CD analysis), has also been proved. In the second part of the study, the antioxidant activity of tested samples (KAN, NEO, HSA) and their mixtures (KAN+HSA and NEO+HSA) was also analysed [3]. Based on the ABTS assay it was shown, that all tested samples as well as mixtures have antioxidant activity and longer incubation time increases the ability to scavenge radical cations formed by the reaction between 2,2`-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate ($K_2S_2O_8$). This probably means that both drugs act as modulators of HSA antioxidant activity.

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The use of a chitosan as a drug delivery system with prolonged release of hydrocortisone

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Hydrocortisone (HC) is a short-acting glucocorticosteroid, i.e. up to 12 hours from application, however the healing effect is maintained in the blood for approximately 6 hours. The use of polymeric carriers can improve the pharmacokinetic properties of hydrocortisone, which will lead to a more effective treatment process and reduce the frequency of dosing of the drug due to the sustained HC release [1,2].
Chitosan nanoparticles, both in presence and absence of HC, were obtained by gelation of low-molecular chitosan solution, where sodium tripolyphosphate (TPP) was used as a crosslinking agent. To mimic physiological conditions, drug releasing profile was investigated with dialysis method in 0.05 M phosphate buffer, pH 7.4 and constant stirring. Qualitative and quantitative analyzes were performed using UV-Vis spectroscopy (JASCO V-760, Hachioji, Tokyo, Japan).
Nanoparticles were obtained with encapsulation efficiency 94% ± 0.2. Spectroscopic studies confirmed the interaction of chitosan with HC and showed no changes in HC hydrophobicity after preparation process. Drug release was observed 30 minutes after the start of the experiment and remained constant for 24-72 hours. Obtained nanoparticles with drug are characterized by first order release mechanism.
Based on the obtained data we can concluded that encapsulation efficiency (EE) and drug release profile can be considered useful and valuable in pharmaceutical application.

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Spectroscopic study of 5-alkyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chlorides in terms of the in vitro interaction carrier plasma proteins

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Cancer diseases are the most frequent causes of death in the world. The numbers of them increase year to year. Due to this fact it becomes the motivation for exploration of innovative anticancer medicines, which are characterized by high efficiency and patient-safe [1].Synthesis of anticancer substances such as benzothiazine derivatives and testing their binding abilities to human serum proteins as carries are an important part of pharmaceutical and medical sciences development [2].
This study is a continuation of quinobenzothiazine derivatives binding with serum proteins [2]. The main aim of the study was spectroscopic analysis of one of quinobenzothiazine derivatives, 5-methyl-9-fluoro-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride (Salt2), interaction with human serum albumin (HSA) in terms of binding and protein secondary and tertiary structure changes using fluorescence, UV-Vis and CD spectroscopy. In order to mimic *in vivo* conditions, control normal serum (CNS) has been used. Using Klotz method [3], binding constants (K_a [M^{-1}]) and the number of binding classes (n) have been calculated. In addition, percentage of displacement of dansylated amino acids (binding site markers) from HSA molecule has been defined.
Based on the obtained data it can be concluded that Salt2 weakly binds with HSA, both in Sudlow's site I (subdomain IIA) and II (subdomain IIIA). Moreover, very slightly interacts with control normal serum (CNS).The analysis of CD spectra confirms not changes in secondary protein structure in the presence of Salt2. Because free fraction of drug is responsible for therapeutic effect, the therapy with Salt2, due to the low values of association constants (K_a), may be associated with a strong toxic effect and numerous side effects.

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Effect of palmitic acid on glycated human serum albumin tertiary structure

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Human serum albumin (HSA), transporting protein, is exposed to numerous *in vivo* structural modifications that affect its stability, activity and lead to disorders of biological functions. One of the processes causing the loss of HSA original properties is the increased glycation in a state of hyperglycemia. Heterogeneous, stable compounds formed at the end of this proces – Advanced Glycation End-Products (AGEs) – play a significant role in the development of chronic micro- and macroangiopathic diabetic complications as well as degenerative processes related to age. Molecular mechanics simulations suggest that binding of fatty acids (FAs) in two high-affinity sites (FA-4 and FA-5) leads to major conformational changes in albumin structure (Figure 1). Because palmitic acid (PA) is considered to be a factor in the development of heart disease and cancer, the influence of PA on glycated human serum albumin conformation has been studied (PA:gHSA 1.5:1 and 3:1 molar ratio).

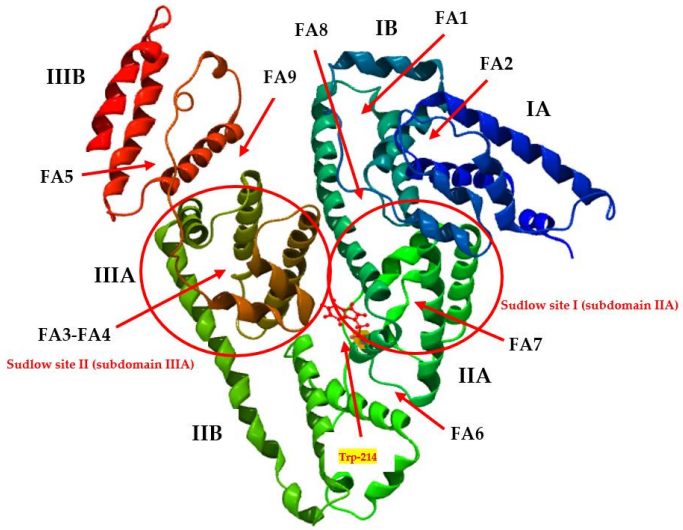

Figure 1. Human serum albumin (HSA) drug binding sites (Sudlow site I and II) with the location of main fatty acids in the HSA molecule (FA1-FA9) and the marked Trp-214 residue

Based on absorption and emission spectra and their second derivatives, synchronous and excitation spectra, Red Edge Excitation Shift (REES) analysis, palmitic acid has been found that cause the changes in the region of phenylalanine (Phe), tyrosyl (Tyrs) and tryptophanyl residues (Trp-214) of glycated by glucose-fructose syrup albumins. Moreover, the amount of free sulfhydryl groups –SH of glycated proteins in the presence of PA shows differences. Our study also proved the increase in the amount of AGEs in the presence of fatty acid. The presence of PA in experimental model that mimics metabolic disorders alters the structure of human serum albumin binding sites, influencing the pharmacokinetics of particles (including drugs) transported by albumin. It is therefore justified to limit the supply of palmitic acid in patients with diabetes and to reduce the consumption of products containing glucose-fructose syrup.

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Permeability and pharmacokinetic studies of a new amphotericin B formulation loaded in lipid nanoparticles to be orally administered

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Amphotericin B (AmB) has been the gold standard to treat systemic fungal infections. The use of AmB is restricted to hospitals because it poses several risks, mainly risks related to its high nephrotoxicity. Given the importance of this drug in medicine, new therapeutics and AmB formulations with nanotechnological improvements are required and could bring many benefits to patients. Therefore, a new drug formulation based on gastro-resistant coated granules of AmB Lipid Nanoparticles (AmB-LN-GR) has been proposed. The lipid-based system containing AmB was produced, next, the formulation suffered the granulation/coated process. In this context, firstly, this work evaluated the intestinal transport of both isolated and encapsulated AmB employing Caco-2 monolayer as in vitro model, as well as the influence of an absorption inducer (quercetin) in AmB absorption. In addition, the developed formulation was administered to rats in a single dose of 4.0 or 8.0 mg/kg and the pharmacokinetics was studied. Before the absorption and pharmacokinetic studies were conducted, bioanalytical methods were validated according to the official European Medicines Agency guideline and all evaluated parameters (linearity, selectivity, limit of quantification, accuracy, precision, matrix effect, residual effect and stability) were in agreement with the guidelines. During absorption experiments, after adding a known AmB amount in the apical chamber, the amount of AmB in the basolateral chambers was quantified in function of time and the absorption parameters were determined. The comparative study showed that AmB from the nanoparticle sample was better absorbed than isolated AmB by an increase of 38% in the apparent permeability coefficient. Quercetin even enhanced the absorption of AmB encapsulated in 52%. The pharmacokinetic results showed that Cmax was similar for both doses and that tmax was reached at 4−12 hours for a dose of 4.0 mg/kg and 4 hours for a dose of 8.0 mg/kg. The half-life related to the dose of 8.0 mg/kg increased significantly compared to the dose of 4.0 mg/kg (an increase of more than 3 times). In addition, the mean residence time related to the dose of 8.0 mg/kg was 4 times higher than for the lower dose. The clearance value showed to be higher for the lower dose. Together, these results provide important conclusions for the experimental design of other in vivo safety and efficacy studies of AmB-LN-GR.

Modelling the enantiorecognition of structurally diverse pharmaceuticals on O-substituted polysaccharide-based stationary phases

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This study aims to develop models to predict the retention, enantioseparation and elution sequence of structurally diverse enantiomers. Specifically, Quantitative Structure Retention Relationship (QSRR) models are built that describe the relationship between molecular descriptors and retention. Eighteen structurally diverse compounds were analyzed on two polysaccharide chiral stationary phases, Chiralcel OD-RH (cellulose tris(3,5-dimethylphenylcarbamate) selector) and Lux amylose-2 (amylose tris(5-chloro-2-methylphenylcarbamate) selector), using either a basic or an acidic mobile phase, and their retention times and elution sequences were determined. Achiral as well as in-house developed chiral descriptors were used as independent variables to build the models. Linear regression techniques, such as multiple linear regression (MLR) and partial least squares (PLS) regression, were applied to model the retention or separation as a function of the descriptors. In a first step, models were built with only achiral descriptors to model the global retention of the compounds. Subsequently, models were built with only chiral descriptors to predict the enantioseparation and finally models were considered with both chiral and achiral descriptors to predict the retention, the separation and the elution sequence of enantiomers. The general retention was predicted well by stepwise MLR models containing only achiral descriptors. The MLR models built with only chiral descriptors were found suitable to some extent for the prediction of the elution sequence. Finally, the models containing both chiral and achiral descriptors allowed predicting the retention time and elution sequence of the enantiomers, but the predictive abilities differed widely between chromatographic systems. Although the results of the latter models are promising concerning the prediction of the enantiomeric retention, more advanced chiral descriptors need to be developed to obtain models that can perform better predictions concerning enantiomeric retention and separation.

Fabrication and molecular modeling design of a highly selective and sensitive molecularly imprinted electrochemical sensor for ribavirin detection

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Ribavirin (RIB), an anti-viral drug, has been used to prevent clinical diseases caused by RNA and DNA viruses due to its broad-spectrum activity [1]. RIB (1-beta-d-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic purine analog of guanosine, various mechanisms have been proposed, including inhibition of inositol monophosphate dehydrogenase, mutagenesis, direct inhibition of RNA-dependent RNA polymerase, and immune-modulating effect [2]. The daily ribavirin dose is determined by body weight and HCV genotype. The usual dose ranges between 800 and 1200 mg/day, although further empirical dose adjustment is requested when the hemoglobin serum concentration falls below 10 mg/dL [3].

In this study, the electropolymerized functional monomer, p-aminophenyl boronic acid (p-APBA), and pyrrole (Py) were designed and used to fabricate a molecularly imprinted electrochemical sensor for RIB detection. The molecular imprinted thin film of the electrochemical sensor was formed by direct electropolymerization with p-APBA and Py in the presence of a template (RIB) on a glassy carbon electrode (GCE). After electropolymerization, the structure and morphology of the fabricated MIP sensor were characterized by methods such as Fourier-transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), contact angle, cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS). Furthermore, the interaction energies between p-AFBA, Py, and RIB were elucidated by molecular modeling. The limit of detection (LOD) and dynamic linear range were calculated at 0.863 pM and 10-500 pM, respectively. It was successfully applied for RIB determination in standard solution, serum sample, and capsule formulation with high sensitivity and accuracy using differential pulse voltammetry method and 5 mM solution of [Fe(CN)6]3−/4– as the redox probe. The current strategy for the electrochemical sensor has shown excellent recovery in synthetic serum samples and capsule form, with recoveries of 100.59% and 101.52%, respectively. The developed sensor exhibited an excellent electrochemical response for RIB due to the synergistic effect of the conductive polymer and molecular imprinting techniques.

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Development of molecularly imprinted electrochemical sensor for codeine detection in real samples

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Codeine is (7,8-didehydro-4,5-epoxy-3-methoxy-17methylmorphinan-6-ol monohydrate, COD) is an opioid analgesic naturally found in the poppy plant. Codeine is used to relieve mild to moderate pain and chronic cancer pain by providing a calming effect that helps to alleviate pain [1]. Codeine has been used as a pain reliever, for coughing, and diarrhea for many years. However, well-known hazards are connected with an opioid prescription, such as overuse, addiction (opioid use disorder), and overdose deaths. Codeine is a critical group of analytes in forensic chemistry, and developing quick, simple, and sensitive sensors is critical for their detection.

In this research, the special synthesis functional monomer, N-methacryloyl-L-tryptophan (MATrp), and 2-Hydroxyethyl methacrylate (HEMA), porous material agent (ZnO) were used to fabricate a polymeric film based electrochemical sensor for COD detection. The molecular imprinted thin film of the electrochemical sensor was formed by direct photopolymerization MATrp, HEMA, and template (COD) on a glassy carbon electrode (GCE). The structure and morphology of the fabricated MIP sensor were characterized by methods such as Fourier-transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), contact angle, cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS). The limit of detection (LOD) and dynamic linear range were calculated 1.49×10^{-14} M and $1 \times 10^{-13} - 1 \times 10^{-12}$ M, respectively. Finally, the developed method was used for the determination of COD in blood serum, urine and pharmaceutical compounds. The proposed electrochemical sensor has shown excellent recovery in synthetic serum samples and capsule form, with recoveries of 101.42% and 102.47%, respectively. This molecularly imprinted electrochemical sensor provides long-term stability, low detection limit, and good accuracy and precision results.

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Electrochemical sensor based on molecularly imprinted polymer coating for the selective and sensitive determination of tofacitinib citrate

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Tofacitinib citrate (TOF) is a new oral Janus Kinase (JAK) inhibitor developed by Pfizer for the treatment of rheumatoid arthritis (RA) [1]. It offers a promising target by blocking the Janus kinase/signal transducer and activator of transcription (JAK/STAT) interferon-dependent signaling pathway and inhibiting the production of inflammatory mediators in joint tissue [2]. TOF shows significant activity especially in patients who cannot tolerate or respond poorly to disease-modifying antirheumatic drugs and is used as monotherapy or in combination with these drugs [3]. This present study is based on the preparation of a highly sensitive electrochemical sensor for the first-time detection of tofacitinib citrate (TOF) using the molecularly imprinted polymer (MIP) method. A MIP-based electrochemical sensor was developed on the glassy carbon electrode (GCE) surface and acrylamide was used as a monomer. The resulting sensor exhibited great sensitivity and selectivity towards the template molecule TOF. The characterization of the sensor was performed by electrochemical (cyclic voltammetry, electrochemical impedance spectroscopy) and spectroscopic methods (scanning electron microscopy and Fourier transform infrared spectroscopy). Differential pulse voltammetry technique was successfully applied for the determination of TOF in standard solution, serum sample, and capsule formulation and with high sensitivity and accuracy using 5 mM $[Fe(CN)_6]^{3-/4-}$ as a redox probe.

Under optimized experimental conditions, the linearity range was obtained in the range between 10 and 100 pM, the limit of detection (LOD) and limit of quantitation (LOQ) values were calculated as 0.348 pM and 1.160 pM, respectively. Also, the selectivity of the sensor was carried out using common interference agents such as KNO_3, $MgCl_2$, Na_2SO_4, uric acid, ascorbic acid, dopamine, and parcetamol. The imprintic factor (IF) was calculated using active substances with similar chemical structures such as aripirazol, trimetazidine, and sildenafil citrate. The results demonstrated that the MIP could specifically recognize TOF compared to structurally related drugs and could be reliably applied to the direct determination of drugs from real samples.

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Sensitive determination of amoxicilline and ceftazidime in the microdialysate of diabetic foot by capillary electrophoresis with large volume sample stacking

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Determination of the antibiotics amoxicillin (AMX) and ceftazidime (CTZ) in human serum and in microdialysates of lower limb subcutaneous tissue is performed by capillary electrophoresis (CE) with contactless conductivity detection (C4D). The baseline separation of AMX from other biogenic substances is achieved in 0.5 M acetic acid (AcOH) and that of CTZ in 3.2 M AcOH with the addition of 13% MeOH. CE/C4D analysis is performed in a 25 µm capillary with suppressed EOF over an effective length of 18 cm and the migration times achieved are 4.2 min for AMX and 4.4 min for CTZ. The determination is performed from 20 µl of serum or 15 µl of microdialysate mixed with acetonitrile in a 1:3 ratio, and the treated sample is injected into the capillary using a large volume sample stacking technique. The LOQ achieved in microdialysate is 148 ng/ml for AMX and 339 ng/ml for CTZ and in serum 143 ng/ml for AMX and 318 ng/ml for CTZ. The developed CE/C4D method is used to monitor the transfer of AMX and CTZ from the bloodstream to the subcutaneous tissue at the sites of diabetic ulcerations in patients suffering from diabetic foot syndrome and also to measure the pharmacokinetics after intravenous administration of a single or continuous dose of antibiotic.

Separation of Different Types of Piperazine Antihistamines Drugs by Capillary Zone Electrophoresis

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Chirality plays a very important role in many fields of our lives, e.g., in the pharmaceutical industry. Capillary electrophoresis (CE) is one of the most employed analytical techniques for chiral analysis, owing to its high efficacy, short analysis time, low reagent consumption, and ease of automation. Piperazine antihistamines are a class of drugs that are represented by a wide number of chiral compounds. So, their enantiomers may exhibit different properties. The present study develops a method for the enantioseparation of piperazine antihistamines, including chlorcyclizine, norchlorcyclizine, and neobenodine, by CE with UV detection. The factors affecting separation, including type and concentration of the electrolyte, applied voltage, and buffer modifier were investigated. The baseline chiral separation of the studied piperazine compounds was obtained using a 25 cm long uncoated fused-silica capillary maintained at 20 °C, UV detection at 220 nm, 100 mM phosphoric acid buffer at a pH 6, 20 mM Sulfated-β-cyclodextrin (S-β-CD) and 8 kV applied voltage. Under these conditions, the analysis time was below 15 min with a resolution value of 4.12. The novel CE separation was tailored to detect these piperazine compounds.

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A new platform for enzymatic enantioselective biosensing of amino acids in human plasma using the D-amino acid oxidase enzyme encapsulated in dendritic silica

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Chiral amino acids (AAs) play a crucial role in chemical and biological systems. However, only L-AAs are translated to structural and functional proteins. D-AAs have been thought to be rarely present in nature. On the contrary, the presence of the D-AAs has been proved relevant to some diseases, like schizophrenia, epilepsy, Alzheimer's disease, etc. Thereupon, it is necessary to develop a fast and simple strategy for D-AAs detection. In this work, an innovative electrochemical biosensor for the sensitive enantioselective recognition of DL-tyrosine and DL-tryptophan in human plasma samples was prepared by the encapsulation of D-amino acid oxidase (DAAO) on amine-functionalized dendritic fibrous nano-silica, also known as KCC-1-NH_2. DAAO was used as a chiral selector. The KCC-1-NH_2/DAAO was cast onto a screen-printed carbon electrode surface and a specific biosensor for the chiral recognition of Tyr and Trp was fabricated. The prepared enzyme biosensor selectively detects AAs enantiomers at physiological pH. The results demonstrate that with a decrease in AAs concentration, the DPV oxidation peaks decreased gradually in standard samples with the linear range of 40 mM to 50 µM. It is shown that the prepared enzyme biosensor provides a sensitive and rapid strategy for the detection of AAs in plasma samples. The current response of the electrocatalytic oxidation of AAs by the developed biosensor was retained in an analytical solution up to 60 °C. Under natural conditions, the enzyme structure is denatured at higher temperatures and its activity diminishes, while encapsulating the enzyme inside silica fibers leads to increased stability and maintains the activity and natural form of the enzyme up to high temperatures. Therefore, due to the suitable function and high sensitivity of the prepared enzyme biosensor, this method can be used to encapsulate and increase the temperature stability of the enzyme as well as for the enantioselective detection of amino acids.

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New approach using Online SPE purification and procainamide for HILIC-FLR-MS N-glycan analysis

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Introduction: Glycosylation is one of the most prominent post-translational modification of proteins that plays a crucial role in maintenance of the structure and protein activity. In the last decades, monoclonal antibodies (mAbs) continue to reign supreme the biopharmaceutical approvals due to their successful treatment of a vast array of serious diseases, such as cancers, immune disorders, and infections. mAbs are glycoproteins and the modifications in the monosaccharide residues (galactose, fucose, mannose, and sialic acid) may alter their functioning such as their half-life, immunogenicity, toxicity, stability, and solubility. Therefore, special attention should be paid to the detection of changes in the glycosylation patterns.
Goal: The main goal of our study was to develop and validate a rapid sample preparation using procainamide labelling and online SPE purification to improve monitoring of *N*-glycans and compare the procedure with other commercial kits. This analytical approach will be used to monitor batch to batch sample of glycosylated biotherapeutics but also to investigate and to understand the impact of *N*-glycosylation alteration at several diseases like endometrioses and human immunodeficiency virus (HIV).
Materials and Methods: Briefly, *N*-glycans were (i) enzymatically released using PNGase F, (ii) labelled with procainamide or commercial kits: GlycoWorks RapiFluor-MS N-Glycan Kit[1] (RFMS kit) or Instant PC Kit, (iii) cleaned with µHILIC SPE plate or online HILIC SPE purification and (iiii) analyzed by HILIC coupled to a high-resolution mass spectrometer detector and a fluorescence detector.
Results and Discussion: Comparing the two approaches (online HILIC SPE purification and on the bench, purification using the µHILIC SPE plate) we proved that the online purification offers a higher sensitivity and abundance for the released *N*-glycans beside the better repeatability and the time saving. All these advantages came with accuracy and cost-effective. The validation of our method was based on different parameters: repeatability, stability, response function, comparison of our protocol to commercial procedures. Our protocol showed a higher sensitivity for the MS signals with a slight lower FLD signals comparing to the RFMS and the instant PC. We also tested the NIST mAb standard to compare our method to published results and we obtained the same observed ratio of galactosylation, sialylation, and fucosylation than described in literature. This protocol was actually used to characterize batch to batch variation of biopharmaceutical (Trixuma) and also applied to investigate *N*-glycans modification in endometrioses.
Conclusion:
The described approach offers a reliable and repeatable method for released *N*-glycans analysis with a high sensitivity and a cost-effective manner. It showed its efficiency in analysis of biosimilars and antibodies from clinical samples.

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Chemical fingerprinting of *Coffea canephora* in the Yangambi collection by LC-HRMS and untargeted metabolomics

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The importance of Robusta coffee (*Coffea canephora*) in the global market has increased in the latest years. However, Robusta coffee continues to be generally considered as a low-quality coffee with respect to Arabica coffee and the research on it is not as extended as the studies on the latter. The aim of this work was to study the chemical composition of several *Coffea canephora* genotypes which are present in the INERA (National Institute for Agronomic Study and Research) coffee collection at Yangambi (Democratic Republic of the Congo) to revalorize this coffee species.

Green leaves, green coffee beans, and roasted/brewed coffee of several genotypes in the collection were extracted for the analysis of their chemical fingerprints through LC-HRMS (liquid chromatography coupled to high resolution mass spectrometry) and the obtained data were studied with an untargeted metabolomic approach [1]. To do so, data from the LC-HRMS analyses were treated (preprocessing and statistical analyses) using the Workflow4Metabolomics platform, which is an online and freely available collaborative platform dedicated to metabolomics data processing [2]. Besides, some compounds of interest in the analysis of the quality of coffee (caffeine, chlorogenic acids, trigonelline, theobromine, theophylline, 7-methylxanthine) were quantified.
The obtained results were studied along with the genetic data of the different genotypes and the results from organoleptic analyses performed with the different genotypes.
Besides, metabolomic analyses also served to study the differences in composition according to the different post-harvest treatments followed by the coffee beans. A common link among the post-harvest treatment, organoleptic analysis and metabolomic analysis could serve to evaluate the genotype and treatment to be chosen in order to have the best possible quality Robusta coffee.

The metabolomic analysis will help to discover differences among Robusta coffee cultivars that allow for discrimination. Furthermore, the proper systematic analysis considering different factors such as the genetic properties, chemical properties, organoleptic attributes, and processing influence would allow for the selection of a promising high-quality Robusta coffee.

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Evaluation of relationships between metabolic changes and clinical parameters in plasma and urine samples of pulmonary arterial hypertensive patients.

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Pulmonary hypertension constitutes a rare disease characterized by a severe development and a high risk of premature death. One of its main clinical types is pulmonary arterial hypertension (PAH), in which the highest percentage of patients are affected by idiopathic PAH. The pathogenesis of this disease has not completely been discovered and elucidated so far, and non-specific clinical symptoms make the diagnosis of PAH a serious problem. Currently, PAH is confirmed with invasive examination based on right heart catheterization. Metabolomics, as a part of systems' biology, shows promise to expand knowledge about the underlying molecular mechanisms of PAH and to search for new and less invasive metabolic indicators of this disease. The main aim of the study was to evaluate and compare both plasma and urine fingerprints of PAH patients and control group with the use of an untargeted metabolomics approach. The study also focused on correlation analysis between the observed metabolic changes and the clinical parameters to select specific indicators of PAH disease.
An untargeted metabolomics approach was applied to the plasma and urine samples with the use of GC-QqQ/MS technique and advanced statistical tests were applied to evaluate the potential metabolic indicators of PAH. The PAH patients (n=40) and healthy controls (n=39) were matched for age, sex, BMI and included in the study. The obtained raw datasets were properly processed (data deconvolution, signal correction using QCSVR method and PQN normalization) and subsequently subjected to uni- and multivariate statistical tests (Student's t-test, Welch's test, U Mann-Whitney test, PCA,OPLS-DA, ROC analysis, Linear Discriminant Analysis). The statistically significant metabolites originate from various biochemical pathways associated with the carbohydrate, amino acid, lipid, fatty acid and pyrimidine metabolism. The metabolic changes observed in the urine samples of PAH patients (compared to the control group) included different concentrations in: threonic acid, hippuric acid, acetic acid, sorbitol, butanoic acid and propionic acid. The metabolic alterations in the plasma samples covered changes in the levels of valine, leucine, lactic acid, hydroxybutanoic acid, nonanoic acid, cholesterol and octadecanoic acid. Valine, propanoic acid and octadecanoic acid showed the most significant correlation with mean pulmonary arterial pressure.

On the formation of sodium taurocholate micelles in media that mimic the fasted and fed state intestinal fluid

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Bile salts, such as sodium taurocholate (NaTc), are synthetized in the liver and transported to the gastrointestinal fluids. This family of steroids is able to form micelles or mixed micelles, if combined with phospholipids, cholesterol, and/or others. The presence of these micelles in biological fluids enhances the solubility of low or non-polar nutrients and drugs, and has a great influence on their bioavailability. The affinity of molecules with the micelles not only will influence their solubility and transport through the gastrointestinal tract but also will determine their ability to cross the gut barrier. Hence, the critical micellar concentration (cmc), the concentration of surfactant above which micelles are formed, is one of the parameters to be included in the models that evaluate drug pharmacokinetics.

In this work, we have studied the ability of intestinal fluid components to form micelles by evaluating the cmc using isothermal titration calorimetry (ITC) at different temperatures (25, 32 and 37ºC). First, the cmc values of the surfactant alone (NaTc) have been measured in water, and next in buffers that simulate the pH and ion strength of intestinal fluids (hydrogenmaleate/maleate 19 mM, pH 6.5 I=121 mM for fasted state, hydrogenmaleate/maleate 55 mM, pH 5.8 I=242 mM for fed state). Finally, the ability of the components that mimic fasted and fed-state intestinal fluids to form micelles has been studied.

NaTc micellization in water is favored when increasing the temperature. When using specific buffers with higher salt content, cmc values decrease, aggregation occurs in a more synchronized way and the temperature influence is not so pronounced. Working with the components that simulate the fasted state (NaTc and the phospholipid lecithin) solved in the corresponding buffer, the micellization occurs gradually, probably due to the slow formation of NaTc-lecithin mixed micelles. Calculated cmc values are higher compared with the surfactant contents in commercial fasted state simulated intestinal fluid (FaSSIF-v2 from Biorelevant™), hence here the surfactant would not form micelles. In the case of the fed state experiments (NaTc, lecithin, sodium oleate, glycerol monooleate solved in the corresponding buffer), the incorporation of lecithin and fats into the NaTc mixed micelle is fast and organized, and cmc value is lower than the one observed for the fasted state conditions. Micelles will be present in commercial fed state simulated intestinal fluid (FeSSIF-v2 from Biorelevant™) and may promote nutrients and drugs solubility as real intestinal fluids do.

Determination of the binding parameters of flurbiprofen to human serum albumin by multiwavelength molecular fluorescence and computational analysis

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Flurbiprofen is a non-steroidal anti-inflammatory (NSAID) drug used to treat pain or inflammation caused by osteoarthritis or rheumatoid arthritis, between others. The bioavailability of this drug resides in its affinity for serum albumin, the main drug transport protein. Ideally, drug-albumin interaction must be strong enough for allowing the transport through the bloodstream and weak enough to release the drug in the target where the therapeutic effect is intended. One of the most widespread techniques for determining the binding parameters between drugs and albumin is molecular fluorescence. Usually, albumin fluorescence quenching is modeled by the double logarithm Stern Volmer (DLSV) equation and binding parameters (n, K_b) are obtained. However, this model assumes that the sole fluorophore is the protein.

In this study, fluorescence intensity spectra have been recorded after the addition of successive volumes of (*R*, *S*)-flurbiprofen to a cuvette that contains human serum albumin. For data treatment, the DLSV model cannot be used as flurbiprofen overlaps albumin spectrum. Chemometric data treatment of spectra using STAR program permitted dealing with the spectral interferences. Using a phosphate buffer (pH 7.4, I=150mM) as the solution medium and performing the experiments at three different temperatures (20, 25 and 37ºC), two binding events have been detected. Temperature has not a great influence on the results. At 37ºC, the first binding process occurs with K_b=4.98 (1:1 stoichiometry), and the second with K_b=5.22 (1:2 stoichiometry).

Computational analysis indicates that the two binding events detected by fluorescence may correspond to the interaction of (*R* and *S*)-flurbiprofen enantiomers to IIA or IIA-IIB sites. Modelization results were supported by crystallographic and computational analyses done using other NSAIDs such as ibuprofen, naproxen and diflunisal. Additionally, computational analysis indicated the presence of an additional binding site, IIIA, that would take place far from the albumin fluorescent residues, particularly Trp241, and hence should be detected by other complimentary techniques such as calorimetry.

Selective determination of D-amino acids in the rat plasma and urine using a two-dimensional LC-MS/MS system and their regulation by D-amino acid oxidase

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As newly recognized bioactive substances in mammals, the minor D-enantiomers of amino acids have been gathering high attention. These days, various D-amino acids besides well-studied D-serine (modulates the neurotransmission in the brain) were also found in the physiological fluids and tissues of mammals, and their distribution and regulation are expected to be clarified. Although several chiral discriminating analytical methods with high selectivity have been reported for the precise determination of trace levels of intrinsic D-amino acids, it is still challenging due to the presence of unknown/uncountable biological molecules *in vivo*. In the present study, a two-dimensional (2D) LC-MS/MS system having multiple separation modes (reversed-phase/enantioselective separations and the detection of selected ion pairs) was developed. For the application, plasma and urine of rats lacking D-amino acid oxidase (DAO, the enzyme known as one of the regulatory pathways of intrinsic D-amino acid) activity were analyzed.

For the target analytes, 10 amino acids (alanine, aspartate, glutamate, leucine, lysine, methionine, phenylalanine, proline, serine and valine) were selected considering the presence of their D-forms in mammals. These amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole and analyzed as the chiral carboxylic acids by using the 2D LC-MS/MS system. After investigating the LC conditions of the first dimension, target amino acids were separated by the difference in their hydrophobicity using a C18 column (Singularity RP18, 1.0 x 500 mm) and aqueous 10-30% acetonitrile containing trifluoroacetic acid for the stationary and mobile phases. For the second dimension, D and L-forms of acidic amino acids were separated by a Pirkle-type enantioselective column (Singularity CSP-011S, 1.5 x 150 mm) having L-*tert*-leucine in the chiral recognition site. Other amino acids were enantioselectively separated by a different column (Singularity CSP-001S) having L-leucine in the chiral recognition site. For the mobile phases, mixed solutions of methanol/acetonitrile containing formic acid were used. The detection was carried out by ESI-MS/MS (positive mode), and the conditions were optimized for all analytes. All analytical dimensions (LC/LC/MS/MS) were online connected, and the simultaneous/selective analysis of target analytes was performed by just one injection to the LC system. Using the developed system, chiral amino acids in plasma and urine of DAO deficient rats were determined. In the DAO deficient rats, D-alanine, leucine, methionine, proline and serine were increased in the plasma, and all D-enantiomers excluding acidic D-amino acids were increased in the urine compared with those in the control rats. Further investigations using various tissue samples are ongoing.

A molecular imprinted electrochemical sensor for selective and sensitive detection of tiotropium bromide

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Tiotropium bromide (TIO) is a long-acting bronchodilator used in the treatment of chronic obstructive pulmonary disease and asthma to prevent patients from worsening breathing difficulties [1]. This study includes the electrochemical analysis of TIO with a molecularly imprinted polymer (MIP) based sensor using different electroanalytical methods. In the design of MIPs, the choice of functional monomer is an important step in terms of the stability of the sensor. The molecular imprinted thin film of the electrochemical sensor was formed by direct photopolymerization with methacryloyl-L-histidine-cobalt (II) [MAH-Co(II)] has been used as a metal-chelating monomer for synthesizing selective molecularly imprinted polymer (MIP). MIP film has been developed on glassy carbon electrode using MAH-Co(II) as the functional monomer, 2-hydroxyethyl methacrylate (HEMA) as the basic monomer, and ethylene glycol dimethacrylate (EGDMA) as the crosslinker via performing photopolymerization method in the presence of a template (TIO) on a glassy carbon electrode (GCE). Morphological characterization of the developed MAH-Co(II)@MIP/GCE sensor was performed using Fourier-transform infrared spectroscopy (FT-IR), surface electron microscopy (SEM), and electrochemical techniques such as cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS). It was successfully applied for TIO determination in standard solution, serum sample, and capsule formulation with high sensitivity and accuracy using differential pulse voltammetry method and 5 mM solution of $[Fe(CN)_6]^{3-/4-}$ as the redox probe.
Under optimum conditions, the linear range and detection limit were found to be 10-100 fM and 2.73 fM, respectively. Satisfactory recovery results were obtained for the determination of TIO in serum sample and capsule form. In addition, MAH-Co(II)@MIP/GCE exhibited good repeatability and stability for TIO detection. The results demonstrated that the MIP could specifically recognize TIO compared to structurally related drugs and could be reliably applied to the direct determination of drugs from real samples.

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Two fatal cases of sodium nitrite poisoning - the use of Ion-Pair Reversed Phase HPLC with UV detection

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Sodium nitrite is a globally known food additive, commonly used as a preservative against bacteria responsible for botulism. What is more, it is one of the components of antifreeze mixtures, corrosion inhibitor and it is a cyanide intoxication antidote. If consumed in larger quantities may result in life-threatening health situations. Two individual cases of intentional sodium nitrite poisoning were presented. Both cases involve fatal intoxication in two men under the age of 40. They each acquired and ingested unknown amounts of a pure chemical substance which caused high methaemoglobinemia and thus inhibited the supply of oxygen to their tissues. Lethal dose of sodium nitrite is estimated to be 2.6 g, however, there are cases of non-lethal intoxications after much bigger dose administered.

Laboratory analysis of biological material collected during postmortem examination was conducted. For case 1 cardiac blood, gastric contents, liver, kidney and brain matter were collected, whereas in case 2 post mortem examination of the following materials had been harvested: blood (collected extravenously), vitreous humor, urine, cardiac blood, peripheral blood, bile, gastric contents, liver, kidney, and cerebellum.

The assay of both nitrate and nitrite levels was carried out with the use of Ion-Pair Reversed Phase HPLC with a direct nitrate and nitrite UV detection at low wavelength. The limit of detection for nitrite and nitrate assay was 200 ng/g. In both cases, the highest concentration of nitrite was detected in gastric content samples, as predicted.

What is more, bottled substances seized on the scenes of case 1 and 2, were delivered to the toxicology lab for confirmatory analysis. Both containers were labelled as sodium nitrite and the analysis confirmed that both substances found at the scenes contain nitrite of purity close to 100%.

Toxicological analysis confirmed the presence of nitrite in tested tissues acquired from both victims. Tissue nitrite levels have shown to be quite diverse between described cases of fatal nitrite ingestion, even in instances when approximately similar doses of the same substance have been ingested by the victims, making cases comparison problematic.

Ethnomycology, Sensory Analysis and Nutritional Composition of Eight Edible Mushrooms Consumed in the African Great Lakes Region

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The Great Lakes region is well known for its astonishing biodiversity, but also for its economic and food shortage. The protein sources are extremely low for local populations, as evidenced by various food security reports carried out in the area [1]. The mushroom resources found in the biodiversity of the mountain forests are recognized for their interesting nutritional qualities, in particular their presumed high protein content. The studies on mushroom nutritional values have been carried out in various African countries, including the Miombo woodlands of the R.D. Congo [2] but, until now, no complete nutritional analysis of edible mushrooms of the mountain forests has been achieved.

The overall objective of this study is to assess the nutritional quality of edible mushrooms consumed in the Great Lakes region. Their quality as food is also linked to food surveys carried out locally to assess the interest of mushroom consumption in the local diet.

The carpophores of eight edible mushrooms *Termitomyces microcarpus* (Berk. & Broome) R. Heim, *Termitomyces schimperi* (Pat.) R. Heim, *Termitomyces robustus* (Beeli) R. Heim, *Auricularia delicata* (Mont. ex Fr.) Henn., *Schizophyllum commune* Fr., *Hypholoma subviride* (Berk. & M.A. Curtis) Dennis, *Pleurotus ostreatus* (Jacq.) P. Kumm. and *Agaricus bisporus* (J.E. Lange) Imbach were collected, subjected to ethnomycological surveys, sensory analysis, and further dried, powdered and analysed for macronutrients and mineral elements compositions. For sensory analysis, the fresh carpophores were cooked and a nine-point hedonic scale method was used to establish food preferences. For proximate and mineral elements composition, data are calculated as % on dry matter basis.

The results showed that all mushroom samples contained interesting amounts of essential nutrients. The ash, crude protein, total lipids and crude fibre were in the ranges of 3.40-10.85%, 9.38-28,77%, 1.13-2.62% and 10.29-44.99%, respectively. Among the eight studied mushrooms, *Termitomyces* genus was the one containing the highest protein amounts. This specific property coincides with their use to treat children suffering from kwashiorkor as reported by the interviewed local populations. The analysed samples also contained abundant amounts of K, Na, Ca and Mg. The mushroom species subjected to sensory analysis were highly appreciated as they scored 8 on the nine-point hedonic scale.

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Olanzapine concentration in dried blood spot and plasma samples

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Olanzapine (OLA) is an atypical antipsychotic approved for the treatment of schizophrenia and bipolar disorder nearly 20 years ago. The antipsychotic treatment should be provided as a long-life treatment. However, in the long term perspective, some patients can become partially adherent or non-adherent to oral treatment. Moreover, therapeutic drug monitoring is strongly recommended according to the Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology, and the therapeutic range of 20 to 80 ng/mL in plasma has been proposed [1]. On the other hand, dried blood spot (DBS) represents mini-invasive microsampling method, but no reference range has been established yet.
Pilot procedure based on literature survey composed of RP-HPLC method (Kinetex C18 column, 100x3.0 mm, 2.6 µm, Phenomenex) hyphenated to mass spectrometry (MS, maXis impact qTOF, Bruker Daltonics) for the determination of OLA and its metabolite in plasma and DBS samples. Quantitation was evaluated as the ratio of analyte peak area to peak area of deuterated standard (OLA-d8, DM-OLA-d8, respectively).
Primarily, parameters of the separation and detection methods were optimized. Further, the OLA extraction procedure from different matrices was tested. A special attention was focused on DBS extraction procedure based on liquid-liquid extraction with the addition of internal standards before extraction solvents action. The highest extraction yield was obtained for the mixture of dichloromethane-isopropanol (9:1, v/v) for plasma and acetonitrile-methanol (9:1, v/v) for DBS samples. Finally, the LC-MS method was validated in basic parameters as intra-day repeatability and intermediate precision with satisfactory values and linearity was determined in the range from 10 (resp. 20 in DBS) up to 200 ng/ml. Limit of detection and limit of quantitation for OLA was 1 ng/ml and 2.9 ng/ml in plasma and 6.6 ng/ml and 19.9 ng/ml extracted from DBS. Matrix effects were evaluated and the ion suppression was more pronounced in the extracts from DBS.
The developed LC-MS method offers short analysis cycle time (12 min) with satisfactory sensitivity covering recommended OLA therapeutic concentration range in both types of samples. Primary patient plasma and DBS samples were acquired, quantified and preliminary comparison results were derived.

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The modern age of chemomometrics: What is the secret behind LC–ESI(+)/MS response generation?

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Accurate prediction of analyte response is fundamental for the efficient development of Liquid Chromatography–Electrospray Ionization/Mass Spectrometry (LC–ESI/MS) methods, but remains a demanding task regarding the gap in knowledge of factors governing response generation. In this research, we address the challenge of discovering the response's interactive relationship with structural properties, method parameters and solvent-related descriptors throughout approach featuring Quantitative Structure–Property Relationship (QSPR) methodology and Design of Experiments (DoE) principles.

The LC–ESI(+)/MS data were acquired for eight model compounds (atypical antipsychotic drug aripiprazole and related impurities) under 57 operating conditions corresponding to Box-Behnken DoE. The experiments differed in the methanol content (60–75%, v/v), the pH of the aqueous phase (3.0–8.2), the flow rate of the mobile phase (400–500 μL min^{-1}), the sheath gas pressure (12–52 AU), the auxiliary gas pressure (3–21 AU), the spray voltage (2.5–5.0 kV) and the capillary temperature (200–300°C). Several machine learning algorithms, namely, multiple linear regression (MLR), support vector regression (SVR), XGBoost, random forest (RF) and Bayesian regression were evaluated as QSPR model-building techniques. Due to highly skewed outcome distribution, the logarithmic, the square root and the cube root transformations were also investigated.

Inspection of models' performance on unseen data showed the coefficient of determination (R^2) in the range 0.770–0.820 and the relative root mean squared error of prediction (RMSEP) in the range 8.0–9.5%. Overall best performances were identified in SVR- and XGBoost-based models. Outcome transformations increased models' performance, with logarithmic and cube root transformations being the most common in the top-performing models. While SVR provided a low level of interpretability in the original feature space, XGBoost successfully identified 3D molecular descriptors (3D-MoRSE, RDF), the pH of the aqueous phase, the capillary temperature and the eluent conductivity as factors that most affect the LC–ESI(+)/MS responsiveness.

The proposed DoE-empowered XGBoost–QSPR model adds to the understanding of the given phenomenon and makes a potentially valuable tool in the LC–ESI(+)/MS method development, but the future compilation of a larger and structurally more diverse database is key to generalizing the findings.

Metabolomic response of four xenograft GIST models to imatinib treatment

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Gastrointestinal stromal tumour (GIST) is the most common gastrointestinal tract sarcoma with prevalence ranging between 1-10/100,000 population depending on a country [1] Despite being approved more than 20 years ago, imatinib is still the standard first-line treatment for advanced, inoperable and metastatic patients with vast majority of GIST molecular subtypes. Mutation type in *KIT* or *PDGFRA* genes determines the tumour responsiveness and the therapy outcome [2]. Unfortunately, 85%–90% of all patients experience disease progression within 20–24 months as the tumour develops secondary resistance [1]. A study on the effects of imatinib treatment on the GIST metabolome may help to better understand molecular processes during the therapy.

In this study, we applied metabolomic profiling of imatinib-treated (n=40) and nontreated (n=40) GIST tissue collected from patient-derived xenograft mouse models with four different mutations in KIT gene. A wide metabolome coverage was obtained with both gas chromatography as well as liquid chromatography in two complementary separation modes (RP and HILIC) coupled to mass spectrometry. Within each mutation type, treatment and control groups were compared using univariate (t-test with Benjamini Hochberg or Bonferroni correction) and multivariate (OPLS-DA) statistical analysis. The number of statistically significant features corresponded with the *a prori* known responsiveness status of the tumour. Afterwards, we identified biochemical pathways influenced by the administration of imatinib. The most common affected pathways included sphingolipid metabolism, fatty acids metabolism, or purine and pyrimidine pathway.

The metabolome seems to be a good reflection of the processes occurring in GIST tissue under the influence of imatinib. We propose that a deeper insight into the metabolic pattern characteristic for different *KIT* mutants may help explain the varied GIST response to imatinib as well as the emerging resistance.

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Mixed-mode RP/WCX chromatographic system evaluation using QSRR modelling approach

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Reversed-phase high-performance liquid chromatography poorly retains charged and highly polar molecules. Mixed-mode liquid chromatography (MMLC) can help overcome this problem. MMLC conjoins multiple separation mechanisms into a single column, expressing interactions dependent on mobile phase composition. Combination of hydrophobic and weak cation exchange (RP/WCX) interactions agrees with drug analysis, due to frequent basic nature of drug molecules [1]. Gaining insight into RP/WCX system is possible by developing quantitative structure-retention relationship (QSRR) models. QSRR mathematically relates molecular retention data with their structural properties (molecular descriptors, MDs). Including experimental parameter (EP) values varied according to Design of Experiments in QSRR, can provide a comprehensive insight into analytes' retention behaviour in a defined experimental space [2].

RP/WCX system obtained with Thermo Acclaim Mixed-Mode WCX-1 column was evaluated by retention properties of 34 drug molecules of different ionization abilities (cations, neutral, anions). Selected EPs were varied according to face-centred central composite design, encompassing mobile phase composition (acetonitrile content (ACN, 30-50 %(v/v)), buffer pH (3.8–5.6) and concentration (I, 20–40mM)) and column temperature (T, 30–38°C). During screening phase, EPs' expressed different influences on analytes' retention, dependent on their ionic form. ACN and T affected the retention of all analytes, pH regulated ionised analytes' retention, and I controlled retention of cations. Accordingly, EPs were considered during QSRR model development. Four QSRR models, developed upon different parts of the data set, were used for RP/WCX system evaluation. First QSRR employed the whole data set (global model), and other three QSRRs used data for similarly ionized analytes (local models). Models were built with gradient boosted trees algorithm, due to its ability to model complex retention patterns. QSRR models were validated by 5-fold cross-validation and externally, via test set. For global QSRR and local models for cations, neutral and anions, root mean squared error of test set were (0.135; 0.102; 0.042; 0.162) with coefficient of determination (0.954; 0.879; 0.996; 0.874) respectively. Influential MDs identified from global QSRR (totalcharge, CATS2D_00_DP, F10[C-N], CATS2D_05_DL, CATS2D_08_DL, SpMAD_B(m), HATS5s) indicated importance of charge, polarity and hydrogen bond donor ability, conjoined with their topological relation for molecular retention. From local models influential MDs for cations (nCb-, D/Dtr10, SpMAD_B(m), CATS2D_05_DL), neutrals (F01[C-O], piPC06, RDF090m) and anions (SHED_DL, Mor20s, CATS3D_07_NL) were identified. MDs indicated that ring structure and topology influenced cations' retention, intrinsic state and topological relation of lipophilic and negative center governed anions' retention, while for neutral molecules C-O ratio was the most influential.

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Development of an LC-MS/MS method for the combined quantification of oxysterols and bile acids

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Cholesterol can be oxidized through enzymatic processes, mainly involving cytochromes, or through the action of reactive oxygen species leading to the formation of oxysterols. Oxysterols play a role in many physiological and pathological processes such as metabolism, immunity or inflammation. They are also metabolic intermediates. Indeed, several oxysterols can be further metabolized into bile acids. Bile acids are also full-fledged bioactive lipids acting on specific receptors and regulating their own synthesis.

Due to their properties, there is a growing interest in the study of these lipid families. In this context, the quantification of these lipid mediators brings valuable information to the biological studies. While several methods to quantify oxysterols or bile acids have been reported, the development of a method allowing for their simultaneous quantification would have significant advantages. However, the development and validation of such a method comes with its share of challenges. The large number of analytes, the presence of isomers as well as the range of polarity between hydrophobic oxysterols and hydrophilic bile acids make complex every step of the analysis, from the extraction to the detection using the mass spectrometer.

We decided to work on the different steps of the analysis in parallel: the lipid extraction, the sample purification and the quantification. First, we investigated the potential of two different extraction methods: liquid-liquid extraction and protein precipitation. Then, we optimized the sample purification with the aim of removing the cholesterol and recovering our analytes along with a limited amount of other lipids (e.g., phospholipids). Analytes are finally detected using a Xevo TQ-S tandem quadrupole mass spectrometer (Waters). After investigating the use of an electrospray probe in negative mode, as well as an APCI probe in positive and negative mode, we opted for the use of an electrospray probe in positive mode. The chromatographic separation was performed in a reversed phase system with a gradient of acetonitrile, methanol and water.

In the end, we were able to set up an innovative LC-MS/MS quantification method for oxysterols and bile acids. This method allows for the detection of 41 oxysterols and bile acids within a single 20-min chromatographic run. Once validated, this versatile method will facilitate the study of physiological processes as well as diseases involving these lipid mediators.

Characterization of the lipid content of extracellular vesicles isolated from human plasma

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Extracellular vesicles (EV) are vesicles of nanometric size consisting of a lipid bilayer surrounding an aqueous center. Their content in lipid, protein, and genetic material (e.g. miRNA) is highly dependent on the producing cell. Increasing evidence support a biological role for EV, notably as signaling elements in cell – cell communication. While proteins and miRNA are largely explored in the context of EV characterization, much less is known regarding the lipid content.

In the context of neuroinflammatory diseases, we aim at characterizing the lipid content of EV as this can play a role in cellular communication. Besides the well-known challenges of lipid analysis, analyzing EV adds a layer of complexity due to their small numbers in most biological samples. This is particularly the case in the cerebrospinal fluid, which is of particular interest in the context of neuroinflammatory diseases.

For the lipid analysis, we used analytical methods based on UHPLC separation of the analytes coupled to a tandem quadrupole (Waters Xevo Tq-s) for analysis and detection. For the analysis of lysophospholipids, phospholipids, sphingomyelins and sulfatides, we used a Waters Acquity HSS C18, 1.8µm, 2.1*100mm column, while for the analysis of ceramides we used a Waters Acquity BEH C18, 1.7µm, 2.1*50mm column.

As the available material is limited, an efficient extraction of the analytes is essential to ensure a large coverage of lipid species. Thus, using EV isolated from plasma samples, we first compared a commonly used extraction method in lipid analysis, i.e. the Folch method, to different protein precipitation protocols (i.e. using different organic solvents). Our data show that the Folch method allows a larger coverage of detected lipid species.

Next, we assessed, starting from 10^{10} EV and over 4 orders of magnitude, the minimal number of EV required to obtain a detection of the lipids. Several species of lysophospholipids, phospholipids, sphingomyelins and ceramides were detected when using the highest content of EVs and their detection decreased when decreasing the numbers of EV, according to their known abundance in mammalian cell membranes.

In conclusion, while these data support our ability to analyze lipid content of EV from plasma samples, further studies are necessary to assess lipid content in EV from cerebrospinal fluid.

Enantiomeric estimation of drugs consumption by gas chromatography

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The misuse of licit and illicit psychoactive drugs (PADs) is ubiquitous all over the world and is a serious public health problem [1]. Wastewater based epidemiology (WBE) combined with the evaluation of enantiomeric fractions (EF) complements the traditional drug monitoring methods to estimate drug consumption and gives insights about environmental risk, synthesis pathways, and discrimination between consumption and direct disposal [2,3]. The suspended particulate matter (SPM) of raw sewage plays an important role for an accurate determination of PADs by WBE because depending on their physico-chemical properties, the drugs may be adsorbed to SPM [4]. The aim of this study is to develop and validate an indirect method by gas chromatography coupled to mass spectrometry (GC–MS) based on chiral derivatization using (*R*)-(−)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride, for enantiomeric quantification and estimation of PADs consumption (amphetamine (AMP), methamphetamine (MAMP), 3,4-methylenedioxymethamphetamine (MDMA), buphedrone (BPD), butylone (BTL), 3,4-dimethylmethcathinone (3,4-DMMC), 3-methylmethcathinone (3-MMC)), in both raw sewage and SPM, for a better understanding on the behaviour and distribution of PADs. For that, raw sewage samples collected at the inlet of a wastewater treatment plant (WWTP) were filtered and the PADs were extracted from SPM by solid-liquid extraction, whereas the liquid phase was pre-concentrated by solid-phase extraction (SPE). Both extracts were derivatized and further analysed by GC–MS. For both matrices, the method was linear (R^2 > 0.99 and R^2>0.98, respectively) and limits of quantifications varied between 10 and 20 ppt. Inlet WWTP samples were analysed, collected weekly in a facility located in Portugal for 8 months. Both enantiomers of AMP, MDMA and 3,4-DMMC, (*S*)-MAMP and the first eluted enantiomer of BPD and 3-MMC (configuration not assigned) were found. The validated method will disclose the consumption patterns at a community level, as well as the occurrence, temporal variation, and EF of the target chiral PADs.

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Optimization and validation of an ultra-high-pressure liquid chromatography mass spectrometry method for metabolomics of low-volume plasma samples

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Metabolomics is the large-scale study of metabolites within a biological sample. Biomarker discovery through metabolic fingerprinting of biological samples holds great potential to improve diagnosis, to elucidate pathophysiology and to enable more precise prognosis of diseases. The combination of liquid chromatography and mass spectrometry is often used for metabolomics purposes. Gika et al. [1] proposed a general protocol for the “quality control” of analytical analyses for untargeted metabolomics, which should be applicable for different biofluids. It was based on an ultra-high-pressure liquid chromatography mass spectrometry (UHPLC-MS) metabolomics analysis of human urine samples. Our research group is analyzing low-volume plasma samples. Therefore, some adaptations were applied to the initial method. The UHPLC-MS method was optimized through a one-variable-at-a-time method by carefully selecting the wash solvent compositions and mobile phase composition along with the gradient profile. Good results were obtained with water/methanol 80/20 V/V as seal wash and purge solvent, water/acetonitrile 90/10 V/V as sample manager wash and water/acetonitrile 20/80 V/V as strong needle wash. Good performances were obtained with a water/acetonitrile mobile phase containing 0.1% formic acid by applying a linear gradient from 0 to 100% acetonitrile in 18 minutes. The sample preparation method will be evaluated, followed by method validation, evaluating precision, accuracy and linearity. Once the method is validated, metabolomic fingerprints of mice plasma samples can be acquired to investigate the plasma metabolomics profile related to brain diseases, such as epilepsy. Biomarkers can then be selected by multivariate data analysis, generating hypotheses for further specific investigation in the context of that condition.

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Semi-preparative enantioresolution of promethazine and its metabolites for enantiomeric profile in metabolic studies

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In recent decades, there has been an increase in the inappropriate use of pharmaceuticals [1]. However, the risks are considered greater when these substances are chiral and marketed as racemate, because enantiomers may have different behaviours in terms of pharmacodynamics, pharmacokinetics and toxicity [2]. For these reasons, enantiomers and their metabolites must be studied as different entities molecules, since one enantiomer may produce the desired therapeutic activity while the other may exhibit toxicity.
The antihistamines have been an example of the rise of inappropriate use of pharmaceuticals, used as drugs of abuse. For example, the famous hallucinogenic drink "Purple Drank" that combines codeine and/or promethazine (PMZ) with soda, has been winning new consumers around the world, mostly teenagers, being associated to serious health consequences and fatalities. PMZ is a chiral antihistaminic drug marketed as racemate that when used in high doses may cause severe toxicity effects.
The information about the enantioselectivity in toxicity of PMZ and its metabolites namely: promethazine sulfoxide (PMZSO), desmonomethyl promethazine (DMPMZ), desmonomethyl promethazine sulfoxide (DMPMZSO) and the hydroxylated metabolite (PMZOH) is scarce [3-4].
The aim of this work is to obtain the enantiomers of PMZ and its metabolites in their enantiomerically pure form in order to carry out *in vitro* metabolic studies. For this, a semi-preparative home-made column with *tris*-3,5-dimethylphenylcarbamate of amylose coated to aminopropyl silica nucleosil was used to enantioseparate the PMZ and its metabolites DMPMZ and PMZSO, under normal elution mode. The enantiomers of PMZ were obtained with an enantiomeric purity of 98.0% and 95.3% for the first enantiomer and second enantiomer eluted, respectively. The metabolite PMZSO had an enantiomeric purity of 98.6% for the first enantiomer and 96.4% for the second enantiomer eluted, and for the metabolite DMPMZ, the enantiomeric purity was 99.7% and 99.0% for the first and second enantiomer eluted, respectively. The *in vitro* metabolic studies will be performed in human liver microsomes.

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Uncovering the Antiproliferative potentiel of toad venoms on metastatic melanoma

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Melanoma is the most common cancer in young adults, with a constantly increasing incidence. Metastatic melanoma is a very aggressive cancer with a 5-year survival rate of about 22 – 25%. This is, in most cases, due to a lack of therapies which are effective on the long term. Hence, it is crucial to find new therapeutic agents to increase patient survival [1]. Toad venoms are a rich source of potentially pharmaceutically active compounds and studies have highlighted their possible effect on cancer cells [2]. We focused on the venoms of two different toad species: *Bufo bufo* and *Rhinella marina*.
We screened the venom crude extracts, the fractions from crude extracts and isolated biomolecules by studying their antiproliferative properties on melanoma cells aiming to determine the compound or the combination of compounds with the highest antiproliferative effect. The crude extracts were generated through sonication-assisted solvent extraction. The fractionation step was performed using flash chromatography. Four fractions were generated and characterized using chromatographic and spectrometric techniques. Both crude extracts and fractions were tested on several human melanoma cell lines that have developed resistance to targeted therapies (MM074(-R) mutated on the BRAF protein and sensitive or resistant to the BRAF inhibitor dabrafenib ; MM161(-R) mutated on the NRAS protein and sensitive or resistant to the MEK inhibitor pimasertib and HBL(-R) mutated on the cKIT protein and sensitive or resistant to the receptor tyrosine kinase inhibitor dasatinib). Our results indicated strong antiproliferative capacities of one particular fraction from *Rhinella marina (L.)*. The latter was mainly composed of four bufogenins. Among these, bufalin was shown to display promising properties against resistant melanoma cells with IC50 ranging from 0.01 to 0.5 nM. Bufogenins are cardiotonic steroids potentially acting on the Na^+/K^+-ATPase pump which is overexpressed in melanoma. We performed crystal violet and clonogenic assays to study the impact of bufalin on cell proliferation and clonogenic properties. Our boyden chambers assays also indicated that bufalin decreased melanoma cell migration and invasion. Finally, we performed a knock-down experiment to prove the implication of ATP1A1 on the anti-cancer effects induced by bufalin.

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Red yeast rice and related formulation quality control by a UHPLC-DAD method

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Nowadays, red yeast rice RYR, already popular in Chinese traditional medicine, is considered as the most effective food supplement able to reduce blood cholesterol level. [1] The demand of RYR is widely increased in western countries, where it is marked as "natural" alternative treatment to statins for hypercholesterolemia. Specifically in 2011, EFSA ascribed the beneficial effect of this product mainly to the presence of Monacolin K. Monacolin K has the same chemical structure of the commercial drug lovastatin. In the RYR extract both the lactone form and the hydroxy acid form could be present. Le lactone MK is biologically inactive but is characterized by a higher lipophilicity that allows its bioavailability, then *in vivo* the lactone ring is opened by the hydroxyl esterase and the active acid is obtained. The acid Monacolin K acts as a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, that is the rate-limiting enzyme in cholesterol synthesis. In addition, it improves the quantity and quality of LDL receptor on the cell membrane surface thus promoting the removal of serum cholesterol. Knowing the broad spectrum of statin activity, MK was studied for further uses as antiseptic against C. albicans, especially when used in combination with other antibacterial agents.[2] In this context, the purpose of this study was to develop and validate a RP-UHPLC-DAD method to characterize RYR in terms of lactone and acid forms of lovastatin amounts in the extract. Then, the extraction of monacolin K (acid and lactone) from red yeast rice was optimized using standard solvents. The following step regarded the preparation of a mouthwash containing Monacolin K extracted from red yeast rice that could be used in case of mouth stomatitis.[3] The method was validated in terms of linearity, precision, accuracy, LoD and LoQ and extraction recovery by analyzing extracts of red yeast rice samples spiked with low/medium/ high concentration levels of monacolin k acid and lactone. Knowing that the monacolin in lactone form is unstable and tends to hydrolyze during storage, this validated UHPLC-DAD method was useful to determine the quality control, stability of the RYR extract and formulation during storage conditions. Therefore, the present method resulted faster and more efficient than other reported methods [4] and could be applied for a quick RYR and RYR based formulations quality control.

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Quantitative and Enantioselective Bioanalysis of Propranolol and its Hydroxy Metabolites by Supercritical Fluid Chromatography Hyphenated to Mass Spectrometry: The Need for Sample Preparation

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Supercritical fluid chromatography (SFC) gained importance in the last decade since new and robust instruments entered the market. Known to be a valuable option for the separation of enantiomers, SFC may also be an alternative to conventional HPLC and GC methods. Chiral recognition of drugs and their metabolites plays an important role in the comprehension of pharmacodynamic effects and pharmacokinetic behaviour of chiral biological active compounds. Published by the FDA and EMA in 1992 and 1993, respectively, guidelines shall ensure to consider each enantiomer of pharmaceuticals as a single active compound. Chiral SFC techniques offer an opportunity for fast enantioselective discrimination of diverse types of analytes within minutes or even seconds in biological analysis [1]. Highly sensitive mass detectors, easily hyphenated to SFC, enable high-resolution mass detection for trace analysis in doping control, forensic investigation, and therapeutic drug monitoring.

The orthogonal chromatographic behaviour of analytes in SFC compared to HPLC was also observed for compounds of biological matrices [2]. Therefore, matrix effect (ME) in SFC hyphenated to electrospray ionization mass detection was also changing. In worst case, ion suppression can even lead to loss of signals. Especially in chiral SFC separation the possibilities to adjust chromatographic conditions to reduce ME are limited. Sample preparation by solid phase extraction can provide a viable way to reduce matrix and thus, ensures sensitivity and low limits of quantitation in bioanalysis.

This poster provides quantitative data on the excretion of (R)-propranolol and (S)-propranolol and their hydroxy metabolites in human urine. Furthermore, the results of an investigation on ME and optimization of sample preparation will be presented.

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Electrochemical Analysis of DNA Methylation using various carbon working electrodes

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The methylation of DNA is an epigenetic modification, which plays crucial roles in numerous biological processes [1]. This process is occurred by the addition of a methyl group of S-adenosyl-l-methionine in cytosine at the C5 position, in cytosine-guanine (CpG) dinucleotides. Global DNA methylation changes occur in carcinogenesis as well as early embryonic development. There are various approaches including immunofluorescence, enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry to analyze global DNA methylation. These techniques require sample preparation and expensive machines to measure the DNA methylation level.

Electrochemical techniques are the most important tools on studying redox chemistry of biomolecules using various working electrodes. The advantages of these techniques include low cost, simpler, short analysis time and environmental friendliness. Carbon working electrodes are commonly used due to their extensive potential window, low background current, chemical inertness and good reproducibility.

In the present study, the electrochemical behaviour of 5-methylcytosine was studied in the wide pH range of 2.0-10.0 by cyclic, square wave and differential pulse voltammetry using various carbon working electrodes including pyrolytic graphite, pencil graphite and boron doped diamond electrodes. The effect of supporting electrolyte, pH, and scan rate on the voltammetric peak responses of 5-methylcytosine were tested on these carbon electrodes. A simple and sensitive voltammetric technique has been developed for the detection of 5-methylcytosine. The proposed method was examined in the presence of some potentials interference substances.

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Low-pg/mL quantification of complex disulfide-rich peptides in rat plasma using microflow LC-MS/MS

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Cyclic peptides exhibit enhanced biological activity compared to traditional peptides, given their disulfide-rich composition, which confers structural stability and conformational rigidity. As a result,cyclic peptides have become crucial therapeutic candidates and successful therapeutic agents in cardio vascular diseases.With the current advancement of cyclic peptide therapeutics, there is an equivalent drive toward the development of highly robust and sensitive quantitative methods. Existing LC-MS based bioanalytical methods lack the sensitivity necessary to reliably quantify cyclic peptides. This is primarily due to their complex tertiary structure and high baseline interference with the application of single MS mode. In this study, low-pg/mL quantification for complex cyclic peptides was achieved at an LLOQ of 0.01 ng/mL using a microflow LC-MS/MS platform.

Assessment of reproducibility in high-throughput experiments by generating over 10,000 data points in an overnight run

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Abstract

Keeping up with high-volume sample demands has become a need in many types of laboratories. Plate readers are the traditional high-throughput approach for measuring biochemical assays, but some fluorescents or radiolabels can produce data artifacts, disrupt enzyme activity in enzymatic assays or require strict regulations for use. Acoustic Ejection Mass Spectrometry (AEMS) enables the introduction of nanoliter sample volumes with speeds of up to 1 sample per second while using native enzymes and substrates for measurement. With that sampling rate, reproducibility and robustness over large datasets could be a concern. Using the Echo® MS system, with the SCIEX Triple Quad 6500+ mass spectrometer, over 10,000 data points were collected in an 8.5-hour run to assess robustness. A second experiment was done over 4 days, collecting over 56,000 data points, to performance over multiple days.

Quantification of nitrosamines and related impurities in multiple drug products by LC-MS

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Nitrosamines have been a major point of concern in the pharmaceutical industry since they were detected in sartan medications. These compounds continue to be found in drug products, resulting in an analytical challenge to develop new or alter existing methods to suit new needs. The European medicines agency (EMA) recently released guidance permitting skip testing to be performed if sufficiently low LOQ values are achieved. This emphasizes a need for highly sensitive methods to detect nitrosamines, in addition to further chromatographic optimization. In addition to nitrosamines, the sartan impurity, 5-(4'-(azidomethyl)-[1,1'-biphenyl]-2yl)-1H-tetrazole (AZBT), provided positive AMES tests, suggesting it might be genotoxic. Therefore, a sensitive and robust method must be established to analyze this impurity.

Ultra-sensitive quantification of the low-level inhalant drugs fluticasone propionate and salmeterol xinafoate in human plasma

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Fluticasone propionate, an anti-inflammatory synthetic steroid and salmeterol xinafoate, a long-acting beta agonist, are often co-administered as inhaled medications for the treatment of asthma. These compounds are administered at levels less than 1 mg per dose. Therefore, methods used to detect the presence of these compounds must be very sensitive to generate relevant data. Here, we present a method for the analysis of fluticasone propionate and salmeterol xinafoate that achieved a lower limit of quantification of 0.050 pg/mL in 400 µL of human plasma.

Novel therapeutic strategy targeting the EGFR and inhibiting PI3K/AKT/mTOR pathway in anaplastic thyroid carcinoma

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The anaplastic thyroid carcinoma (ATC) is one of the most aggressive cancers and is characterized by a high mortality rate, occurring within 2-6 months from diagnosis. The current treatment is based on surgery and chemo-radiotherapies, but unfortunately do not allow to cure. New molecular targeted therapies are being investigated and are aimed to limit the systemic effects of the current therapies.
Our strategy consists of bringing a PIP3-targeted therapeutic peptide (TP) directly into the ATC cells thanks to a vector peptide (VP) targeted to EGFR and covalently coupled to TP. The TP and VP peptides were previously identified using the phage display technology. EGFR is overexpressed in ATC cells. The peptide complex (VP-TP) is expected to inhibit the PI3K/AKT/mTOR signaling pathway and induce apoptosis.
Our peptides were evaluated on human ATC (8505C; Cal62) and healthy (Nthy-ori 3-1) thyroid cell lines. We evaluated by sandwich ELISA the effect of peptides on pan and phosphorylated AKT. The protein Bad was evaluated by cellular ELISA. Indeed, once AKT is activated, it inhibits Bad by phosphorylation. Bad is a pro-apoptotic protein whose inactivation promotes cell survival in cancer cells. Apoptotic cell death was studied by the immunofluorescent (IF) detection of activated caspase 3 on ATC cells exposed to peptides at different concentrations and incubation times. EGFR-targeted endocytosis of PV and its effect on EGFR expression and phosphorylation were investigated by IF and colocalization studies.
Our results have shown that TP and VP-TP inhibit AKT expression and activation, activate the BAD protein, and induce apoptosis by activating caspase 3. A maximum apoptosis level is rapidly reached at 40 µM for TP and at 10 µM for VP-TP after 1h of incubation. No resistance phenomena were observed after 72 hours of incubation.
VP is able to decrease the expression and phosphorylation of EGFR. Concerning the colocalization of endocytosed VP coupled to rhodamine (VP-Rho) with EGFR, our results obtained by ImageJ software analysis show a good colocalization, with a Manders coefficient equal to 0.882 for the positive control (8505C cells incubated with 1 µM EGF) and to 0.977 for the negative control.
The use of targeted delivery of therapeutic peptides is promising in the research of ATC treatment. It is less immunogen and toxic, which is expected to limit the undesired side effects. Our findings need to be assessed *in vivo* to confirm the *in vitro* results and hopefully observe a limited delivery of VP-TP to healthy cells and tissues.

PCSK9 variants affect differently the risk of type 2 diabetes mellitus development. Potential impact on the therapy with PCSK9 inhibitors

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Introduction: Proprotein convertase subtilisin kexin 9 (PCSK9) inhibitors are powerful cholesterol-lowering drugs recently authorized by drug regulation agencies. However, there are persistent concerns about their association with the risk of developing type 2 diabetes mellitus (T2DM), since some loss-of-function PCSK9 variants present low serum levels of low density lipoprotein cholesterol (LDL-c) and are associated with T2DM, possibly due to cholesterol accumulation-induced β-cell dysfunction. We conducted a case-control study to explore the association of 3 PCSK9 variants with T2DM, namely 5'UTR variant rs28362201 (g.55039593G>T), synonymous variant rs926463199 (g.55039903C>G; p.Leu22=) and intronic variant rs2495482 (g.55040059A>G).

Methods: Socio-demographic data were collected from 60 T2DM patients and 141 non diabetic (ND) subjects living in the same region of Benin (West-Africa). Fasting glucose and lipid profile were determined by colorimetric method. Serum levels of PCSK9 and insulin were measured by ELISA. PCSK9 exons 1, 7 and the flanking sequences were sequenced using classical Sanger method. Homeostasis model of assessment of insulin resistance (HOMA2-IR) and β-cell function (HOMA2-β) were calculated. Data analyses were performed with SPSS 21 and expressed as means ± SEM or frequency (%).

Results: The mean age of the T2DM patients and ND were respectively 55.27 ± 1.13 and 44.61 ± 0.68 ($p<0.001$) among whom 49.2% were women. Variants rs28362201 and rs2495482 were present respectively in 20.40% (T2DM: 26.67%, ND: 17.73%; p=0,150) and 87.06% (T2DM: 91.49%, ND: 76.67%; p=0.004) of participants. Variant rs926463199 was detected in T2DM patients only (T2DM: 6.67%, ND: 0.00%; p=0.002). Serum level of PCSK9 was elevated in rs28362201 carriers (carriers: 85.57±12.46 ng/mL, non-carriers: 64.9±3.76 ng/mL; p=0.044) but not in rs926463199 (p=0.113) nor in rs2495482 (p=0.365) carriers. However, none of the 3 variants impacted significantly serum levels of LDL-c (all $p>0.05$). After adjusting for risk factors of interest (age, gender, residence, religion, ethnicity, alcohol excessive consumption, waist circumference, insulin resistance, β-cell function and LDL-c level), only rs2495482 [α=3.50, OR=33.08 (4.26-257.11), p=0.001)] remained significantly associated with T2DM, not rs926463199 [α=-23.30, OR=0.00 (0.00-), p=0.999)] nor rs28362201 [α=-1.44, OR=0.24 (0.04-1.41), p=0.113)]. Excepting rs28362201 that exhibited a worsened β-cell function as depicted by reduced HOMA2-β among T2DM (carriers: 65.17±13.49%, non-carriers: 26.45±9.23%; p=0.021), but not HOMA2-IR (carriers: 7.90±7.09, non-carriers: 1.16±0.16; p=0.113), other variants showed no significant difference in HOMA2-β (rs926463199: p=0.898; rs2495482: p=0.891) and HOMA2-IR (rs926463199: p=0.747; rs2495482: p=0.405).

Conclusion: Our study suggests that the association of PCSK9 variants with T2DM may involve different pathophysiological pathways, which should be considered when PCSK9 inhibition is envisaged.

Key-words: PCSK9, PCSK9 inhibitors, type 2 diabetes mellitus, HOMA, β-cell function.

Synthesis of new heterocyclic derivatives bearing benzimidazole moieties and evaluation of their biological activity

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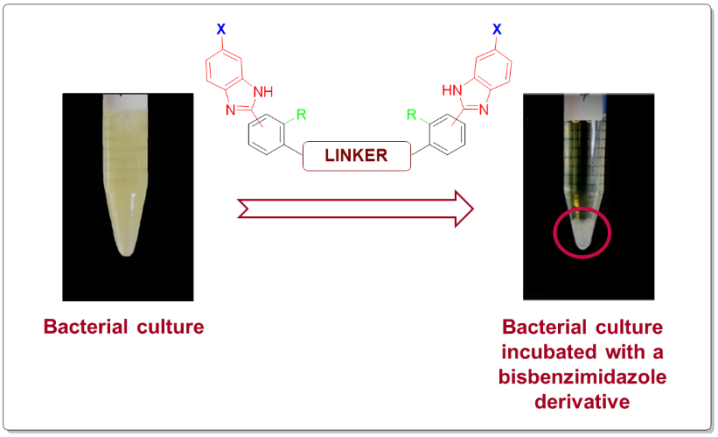
The benzimidazole moiety is an organic heterocycle found in many molecules exhibiting interesting and varied pharmacological properties (i.e. antiviral, antiparasitic or even antibacterial properties)[1]. Therefore, the development of new molecules bearing such units could lead to promising new drug candidates. For this reason, we decided to synthesize new heterocyclic derivatives bearing two benzimidazole units within their structure.

With the constant increase of antibiotic resistance and the resulting public health issues[2], we were particularly interested to evaluate the potential antibacterial properties of these new agents. By performing microbiological tests, we highlighted that some of these heterocyclic derivatives exhibit an important biological activity against both Gram(+) and Gram(-) bacterial strains. This activity is characterized by the rapid appearance (within a few minutes) of flocs when the bacterial suspension is incubated with a low concentration of bisbenzimidazole compound.



Although bacterial flocculation has already been referenced in the literature for other agents (particularly for cationic entities)[3], preliminary studies suggest a non-conventional mechanism of action for the evaluated structures. Moreover, the synthesis and evaluation of this chemical library allowed to highlight given structural criteria essential for the appearance of the flocculation activity. To go further into the comprehension of the observed phenomenon, we decided to carry out some additional studies including microscopic studies (fluorescence, scanning electron and atomic force microscopies) alongside with metabolomic experiments and other biological characterizations of these bacterial flocs.

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Update on technological developments and opportunities with Workflow4Metabolomics

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Introduction

Metabolomics data analysis is a complex and multistep process, which is constantly evolving with the development of new analytical technologies, mathematical methods, bioinformatics tools and databases. The Workflow4Metabolomics (W4M) infrastructure[1] provides tools in a single online web interface (through Galaxy[1]). During the last two years, W4M has evolved with new upgrades for LC-MS, LC-MSMS, GC-MS and NMR pipelines, including preprocessing, quality control, statistical analysis and annotation tools. W4M also proposes new community resources promoting open science in metabolomics.

Technological and methodological innovation

W4M major updates include: Specific Galaxy interactive tools with **NMRPro**[2] for NMR spectra visualization and **Xseeker** for visualization and annotation of LC-MSMS data (In collaboration with CHOPIN ANR project[3]); An improved MSMS pipeline; New annotation tools suite allowing connexion with PeakForest[4], a new spectral data manager infrastructure for laboratories; New functionalities regarding mixed model computation for repeated measure designs, and improvements in annotation of complex mixture bidimensional NMR spectra. New training resources through the Galaxy Training Network (GTN) are also proposed, and Training Infrastructure as a Service (TIass) is available through the new usegalaxy.fr host.

Results and impact

W4M's improvements increase raw data input management and LC/GC-MS(MS) workflow efficiency. We highlight how current advances, along with community training as through the yearly international school Workflow4Experimenters, contribute to open data analysis practices worldwide. In addition the W4M organization on Github repository aims to review code, annotate tools and propose a showcase for contributors from the metabolomics community.

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Untargeted Metabolomics Profiling of Patients with Progressive Familial Intrahepatic Cholestasis type 3 (PFIC3)

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Progressive familial intrahepatic cholestasis (PFIC) is a rare genetic disorder. There are three different types of cholestasis that have been correlated to mutations in hepatocellular transport system genes involved in bile formation. PFIC type 3 is caused by mutations in the ABCB4 gene, located on chromosome 7q21.1 [1,2], which is a translocator of phospholipids, also called ATP-binding cassette (ABC) transporter family, mainly phosphatidylcholine (PC). Phosphatidylcholine (PC) is secreted through the multi-drug resistance glycoprotein P3 in humans (MDR3) [3]. Loss of the canalicular protein MDR3 or loss of protein function results in deficiency of biliary secretion of phospholipids that result in damage to the biliary epithelium and biliary canalicular, leading to PFIC3, biliary cirrhosis, transient neonatal cholestasis, intrauterine cholestasis, hepatic pregnancy (ICP) and drug-induced cholestasis [4]. The absence of phospholipids in bile can also lead to low-phospholipid-associated cholelithiasis syndrome (LPAC) due to the destabilization of micelles that promotes the crystallization of cholesterol and consequently occurs in the formation of liver gallstones. This event increases the biliary lithogenicity and then liver damage. Bile acids, as a result of congenital enzyme mutations, are involved in the synthesis of cholic and chenodeoxycholic acids, which are considered markers for cholestasis. In the present study, we applied a liquid chromatography-high resolution mass spectrometry (LC-HRMS) metabolomics approach to identify distinctive signatures in plasma samples from PFIC3 versus healthy patients (control group). Plasma samples of PIFC3 patients (n=34) and healthy controls (n=35) were analyzed using an untargeted metabolomic profiling approach. The results showed the identification of 63 plasma metabolites that differed significantly ($p \leq 0.05$) from PFIC3 and the control group. Classification analysis using supervised hierarchical clustering was able to distinguish the metabolic profile of patients with 95% accuracy. As a result, metabolites related to bile acid, glycerophospholipid metabolism, fatty acids, and carnitine were identified as potential discriminating metabolites. Pathway analysis showed that metabolites related to fatty acid metabolism and carnitine (carnitine ($p<0.0001$), acetylcarnitine ($p<0.0001$), succinic acid ($p<0.001$), and lysine ($p<0.0001$)). As well as tryptophan ($p<0.01$) and glutamic acid ($p<0.0001$) metabolism were identified as discriminating metabolites. Bile acid biosynthesis, which include deoxycholic acid glycine conjugate ($p < 0.01$) and glycerophospholipid metabolism (1-acyl-sn-glycero-3-phosphocholine ($p < 0.01$), phosphatidylcholine PC (16:0/16:0) ($p <0.01$)) were also identified as discriminating metabolites. These PFIC-related metabolites may advance disease biomarkers and help in understanding the potential pathogenic mechanism of PFIC3.

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