Distribution and metabolism of constituents and metabolites of a standardized maritime pine bark extract (Pycnogenol®) in human serum, blood cells and synovial fluid of patients with severe osteoarthritis

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naturwissenschaftlichen Doktorgrades
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Sensitive quantification of selected polyphenols and drugs in human complex biological matrices with LC-ESI/MS/MS
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A INTRODUCTION

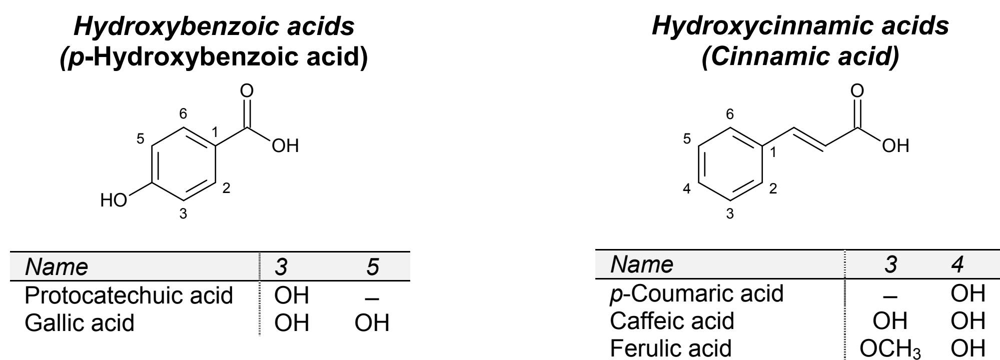
A Introduction

1 Polyphenols

1.1 Classification and occurence

A large number of polyphenols have been identified in plant foods for protecting the plants against UV light, parasites and predators. This large group of plant-derived compounds can be further subdivided into the classes of phenolic acids, flavonoids, stilbenoids and lignans [1-4]. In general, polyphenols occur as glycosides, partly as esters [1].

Phenolic acids can be divided into two classes: derivatives of hydroxybenzoic acid such as protocatechuic acid and gallic acid (see Figure 1, left) and derivatives of hydroxycinnamic acid like coumaric acid, caffeic acid and ferulic acid (see Figure 1, right) [1, 3].



Name	3	5
Protocatechuic acid	OH	–
Gallic acid	OH	OH

Name	3	4
p-Coumaric acid	–	OH
Caffeic acid	OH	OH
Ferulic acid	OCH_3	OH

Figure 1: Structure of hydroxybenzoic acids (left) and hydroxycinnamic acids (right) with examples of each.

In the group of hydroxycinnamic acids, esters of caffeic acid, coumaric acid and ferulic acid with D-glucose and D-quinic acid are most widespread in fruits and vegetables. Regarding derivatives of hydroxybenzoic acids, especially esters of p-hydroxybenzoic acid, salicylic acid (o-hydroxybenzoic acid), protocatechuic acid, gallic acid and ellagic acid have been found [1].

A particular variety of polyphenols is found among the flavonoids, which can be further subdivided into *anthocyanidins, flavones, flavanones, isoflavones, flavanols and flavonols*, depending on the oxidation state of the carbon atoms C-2, C-3 and C-4. The basic flavonoid structure consists of a flavan (= 2-phenylchroman) scaffold with overall 15 C-atoms (see Figure 2, middle) containing three rings (C-6-C-3-C-6), which are referred to as A, B and C [3]. The structural variation of the flavonoids depends on the degree and pattern of hydroxylation, methoxylation and other substitutions with different functional groups [2, 3]. Another subgroup of flavonoids is the *chalcones* with its open C-ring, representing a precursor of the *flavanones* in the biosynthesis of flavan derivatives. All higher plants are able to synthesize flavonoids

(*shikimate pathway*), whereas the animal and human organism are not known to build the *flavan* scaffold [5, 6].

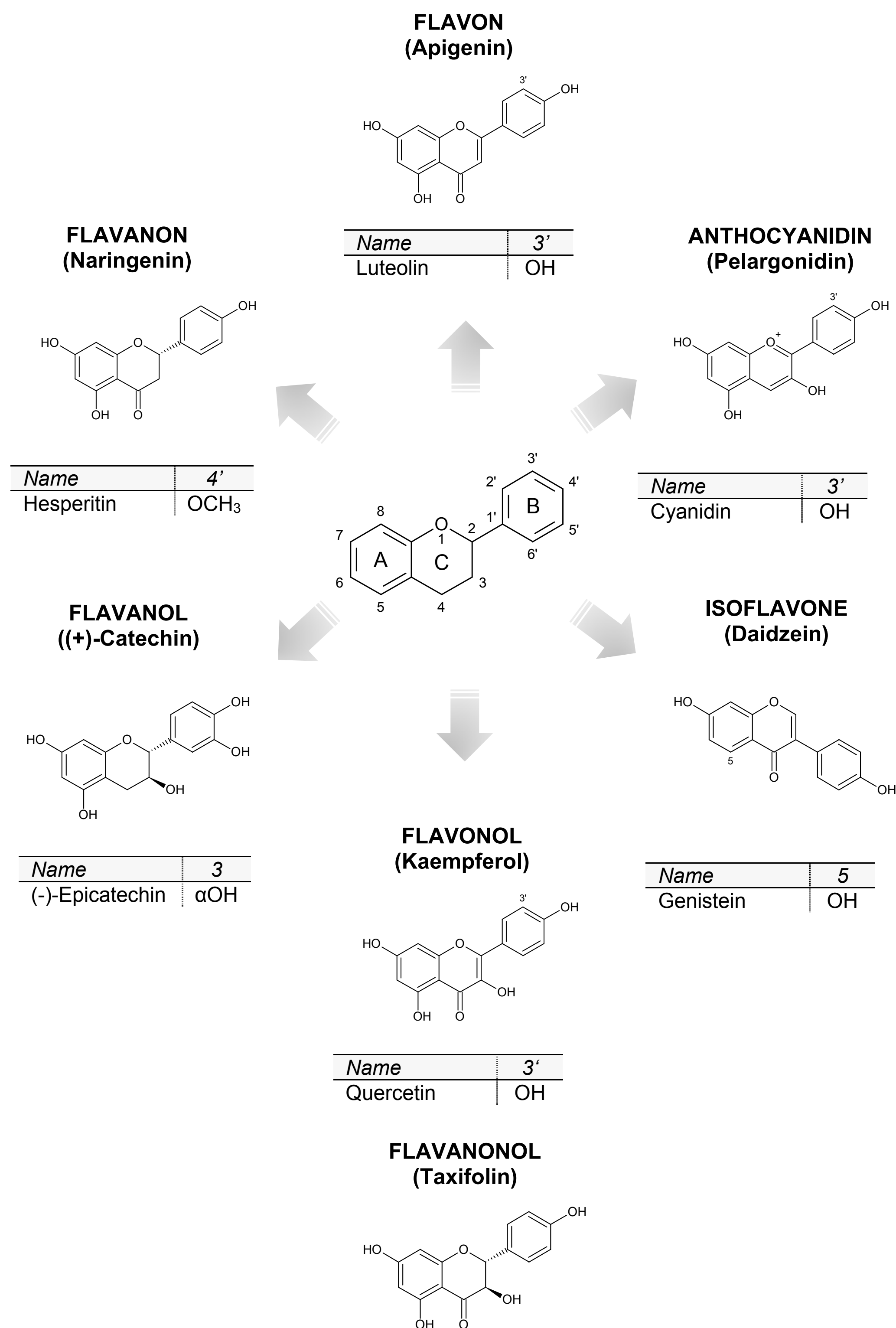


Figure 2: Overview of the different groups of flavonoids with prominent examples of each. In the middle the core structure of the flavonoids is shown.

Flavonoids are the most abundant dietary polyphenols. They are typically found in the shell and in the outer layers of fruits and vegetables. The flavonoid content is highly dependent on

the variety of the plant and the acting climate [7]. Naturally, they do not occur as unconjugated molecules (aglycones), but as *O*-glycosides, in which one or more hydroxyl group(s) of the aglycone is bound to a sugar [7, 8].

Kaempferol and quercetin are well-known representatives of the group of the *flavonols* (see Figure 2). They are both present in some domestic and tropical fruits especially as 3-*O*-glycosides. High concentrations of quercetin have been found e.g. in quince (180 mg/kg), cowberry (130 mg/kg), blueberry (70 mg/kg) and in apples (49 mg/kg) [1]. The source of the most abundant occurrence are onions with up to 347 mg/kg quercetin [7, 9]. In contrast to quercetin, the *flavanonol* taxifolin does not contain a double bound between the carbon atoms C-2 and C-3. Taxifolin shows a lower antioxidant effect compared to quercetin. Hence, the presence of an unsaturated bond therefore seems to be an essential feature of antioxidant flavonoids [10].

Flavones (see Figure 2) are less common in fruits and vegetables than *flavonols*. The main representatives of this group, luteolin and apigenin, mainly occur in celery leaves (200 mg/kg and 750 mg/kg) [7].

Citrus fruits are mainly used as a source of *flavanones* (see Figure 2) such as the aglycones naringenin in grapefruit and hesperitin in oranges. Their glycosides, naringin (naringenin-7-neohesperidosid) and neohesperidin (hesperitin-7-neohesperidosid), are connected to neohesperidose at position C-7 and thus are bitter-tasting compounds in grapefruits and bitter oranges. Once hesperitin is esterified with the rutinose, which is a dimer of rhamnose and glucose, the resulting flavonon-7-rutinosid is responsible for the sweetness in oranges (hesperidin) [1].

Daidzein and genistein are the main representatives of the *isoflavones* (see Figure 2). Due to their hydroxyl groups in position C-7 and C-4', the isoflavones show a structural similarity to estrogens and so they can bind to estrogen receptors as well [11, 12]. These phytoestrogens mainly occur in soybean and products thereof [13].

Anthocyanidins (see Figure 2) are the aglycones of the anthocyanins (glycosides), their common structural feature is a positive charge of the C-ring (flavylium cation), which is mostly compensated with chlorid ions in plants [2, 14]. The most important naturally occurring *anthocyanidins* are pelargonidin, cyanidin, delphinidin and malvidin, which differ in the hydroxyl- and methoxy- substitution pattern of the B ring. Increasing hydroxylation causes a shift to a blue color (pelargonidin -> cyanidin -> delphinidin; *bathochromic shift*), while a methylation take along a shift to a red color (delphinidin -> malvidin; *hypsochromic shift*) [1, 14]. The color of anthocyanins is highly dependent on the pH-value, since the flavylium cation is only stable at very low pH values (pH 1-3; red color). With increasing pH-value

(pH 4-5), the flavylium cation becomes an colorless compound (chromenol), which is further subjected to a color intensification by the formation of quinoid (purple color) and ionic anhydrobases (blue color) at a pH-value between 6-7. At a pH-value >7 it is transformed to a chalcone (yellow color) by ring opening [1]. The red, purple or blue colored flavylium salts occur in numerous domestic and tropical fruits in the form of their glycosides. Aubergines, blackberries, blueberries, black currants, cherries, strawberries, plums, grapes (black) and thus red wine may contain a high concentration up to 7.5 g/kg anthocyanins [15].

Flavan-3-ols (see Figure 2) belong to another structurally various subgroup of the flavonoids. They occur either as monomers (catechins), dimers, oligomers or polymers (*proanthocyanidins*). Catechin and epicatechin are the main representatives of the *flavan-3-ols* in fruits, which usually appear in free form or as 3-*O*-gallate [1, 15].
There exist four isomers of the monomers catechin and epicatechin, because of the two chiral centers of the C-atoms C-2 and C-3. (+)-Catechin (*2R,3S*) and (-)-epicatechin (*2R,3R*) are frequently found, while (-)-catechin (*2S,3R*) and (+)-epicatechin (*2S,3S*) occur rarely in nature. Apricots contain up to 50 mg/kg (+)-catechin, while on the other hand blackberries might contain up to 181 mg/kg (-)-epicatechin [1, 7]. However, the highest contents of *flavan-3-ols*, are found in red wine (80 to 300 mg/kg), chocolate (460 to 610 mg/kg) and in green tea (up to 800 mg/kg). On the contrary, black tea contains less monomer *flavanols* because of the formation of condensed polyphenols, which are known as theaflavins (dimers) and thearubigins (polymers), occuring during the fermentation process of the tea leaves. Gallocatechin, epigallocatechin, and epigallocatechin gallate (EGCG) are mainly found in grape seeds and especially in tea [15].

Proanthocyanidins (see Figure 3), which are also known as condensed tannins, consist of catechin monomers that are linked together between C-4 and C-8 (or C-6) [16]. Procyanidins are the most widespread group of the *proanthocyanidins* containing catechin and epicatechin monomers [1]. Procyanidin B1 (epicatechin-(4ß-8)-catechin) is an example for B-type procyanidins, with epicatechin and catechin being linked via an interflavan carbon bond between C-4 and C-8 (see Figure 3) [3, 15]. The name *proanthocyanidins* implies that they are colorless precursors of the *anthocyanidins*, which can be formed by oxidation reaction during heating in acidic solution [1, 3].
Condensed tannins are responsible for the astringency of fruits (e.g. apples, kakis and pears), beverages (e.g. wine, tea and beer) and for the bitterness of chocolate [1, 15]. Moreover, there also exist hydrolysable tannins consisting of a central core of glucose esterified with gallic acid (gallotannins) or with hexahydroxydiphenoic acid (ellagitannins) [3], which naturally can be found in fruits (e.g. pomegranate and strawberry), nuts and seeds [17].

Lignans are polyphenols containg two phenylpropane units (see Figure 3) and are present at rather low concentrations in foods. Linseed and sesame are the major sources of *lignans*.

Sesame contains up to 293 mg/kg pinoresinol, whereas secoisolariciresinol appears in higher concentrations in linseed (2932 mg/kg) [1]. *Lignans* are further metabolized by the intestinal flora to e.g. enterodiol and enterolactone [15, 18].

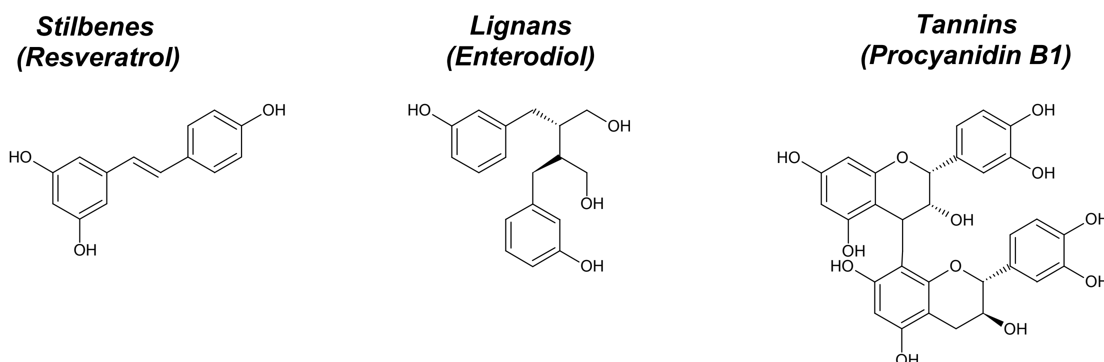


Figure 3: Other polyphenols: structure of stilbenoids (left), lignans (middle) and tannins (right) with a prominent example of each.

Stilbenes are another group of polyphenols and occur in low concentrations in human nutrition. The hydroxylated derivatives of *stilbenes* are called stilbenoids. The most prominent example is resveratrol (trans-3,4',5-trihydroxystilbene), which can especially be found in grapes and products thereof such as red wine [15, 19].

1.2 Daily dietary intake

The occurrence of polyphenols in human nutrition is ubiquitous [15, 20-23]. Herein the phenolic compounds are contributing to color and flavor [1, 3]. The daily total polyphenol intake is highly dependent on the individual nutritional habits.

In the year 1976, Kühnau determined a reference value for the total polyphenol intake of 1 g/day [24], which is still assumed to be valid. As part of the German National Food Consumption Survey (Nationale Verzehrsstudie, 1997) Linseisen et al. assessed the average intake of all flavonoids with 54 mg/day (calculated as agylcons) in a Bavarian subgroup of 119 adults. The flavanons were with 13 mg/day the most important source of flavonoids, followed by flavonols (12 mg/day), catechins (8 mg/day), proanthocyanins (4 mg/day) and anthocyanidins (3 mg/day). Fruits and their products were the main flavonoid source [25].

In recent years, more detailed studies were performed for the investigation of the polyphenol intake and food sources [26]. Ovaskainen et al. evaluated in the year 2008 the daily intake and the major polyphenol food sources of 2007 finnish adults and confirmed with a mean total intake of polyphenols of 863 ± 415 mg/day [27] the previously determined reference value of 1 g/day from Kühnau [24]. The dominant groups of the polyphenols were the phenolic acids (75%), followed by proanthocyanidins (14%), anthocyanidins (6%) and other flavonoids (4%).

The main sources that led to high concentrations of phenolic acids (especially caffeic-, ferulic- and gallic acid) were coffee and cereals (e.g. rye bread). Flavanones were the most abundant subgroup of the flavonoids, due to high intake of fruits. Berries and particularly bilberries were the major source for anthocyanidins (especially cyanidin and delphinidin). Chokeberry was the food item with the highest total concentrations of all polyphenols, followed by cocoa powder, rose hip and bilberry [27].

Studying the nutritional behaviour of 4942 french adults aged between 45-60 years Péréz-Jiménez et al. ascertained a daily polyphenol intake of 820 mg ± 335 mg/day (calculated as agylcons). In total 337 different polyphenols were consumed, among which the hydroxycinnamic acids and proanthocyanidins were the most important contributors. The food class with the highest contribution to the total polyphenol intake was nonalcoholic beverages (48%), coffee (79%), tea (17%) and orange juice (2%). Other important food classes were fruits (20%; apples and strawberries), cocoa products (11%; dark chocolate and cocoa powder), alcoholic beverages (10%; red wine), vegetables (6%; potatoes, green chicory and onions) and cereals (4%; refined wheat-flour products) [28].

1.3 Effects on health

Dietary polyphenols are associated with beneficial effects on human's health while they are represented ubiquitously in nutrition (see Chapter 1.2).

Their antioxidant activity depends on the number and the position of the OH group(s) and on the pH value of the poylphenols [1]. In addition to their clear antioxidant and antiinflammatory activities [3, 29-36] even more mechanism of action are discussed to explain the potential effects of polyphenols relating to several human diseases (see Table 1).

Due to their ability to scavenge free radicals, polyphenols inhibit the formation of reactive oxygen and/or nitrogen species (ROS/RNS) [3, 35] which plays an important role in the pathology of cardiovascular diseases (CVD), especially coronary heart disease (CHD) and stroke [37]. In this context, resveratrol, epigallocatechin gallate (EGCG) and curcumin were related to beneficial effects on cardiovascular health [35]. Epidemiological studies investigating the nutritional situation in France uncovered that a daily consumption of moderate doses of about 20-30 g alcohol reduced the risk of CHD by at least 40% despite of a simultaneously high intake of saturated fat. This phenomenon is nowadays known as "French Paradox" [38]. Alcohol consumption was beneficially associated with CHD predictive high-density-lipoprotein (HDL) cholesterol and an inhibition of platelet aggregation [38]. Moreover, it was found that alcohol consumed as wine was more protective than by beer and spirits [38]. These positive effects were mainly attributed to the polyphenol resveratrol occurring in high concentrations in

red wine [39]. Currently, several studies are balancing the effects of an intake of polyphenols vs. ethanol since the latter also has well-known toxic effects [40, 41].

Table 1: Overview of human diseases in which dietary polyphenols are showing beneficial effects and some related mechanism of action. *LDL: low-densitiy lipoprotein; NOS: nitric oxide synthase; ROS/RNS: reactive oxygen and/or nitrogen species; AGEs: advanced glycation end products; COX: Cyclooxygenase; MAPK: mitogen-activated protein kinases; ATPase: adenosine triphosphatase; BBB: blood-brain barrier; NF-κB: nuclear factor-Kappa B; IL: Interleukin; LOX: lipoxygenase; TNF: tumor necrose-factor.*

Disease	*Mechanism of action*
Cardiovascular disease	• Altering of lipid metabolism by inhibiting LDL oxidation [37, 42, 43] • Reduction of blood pressure [44] • Inhibiting platelet activation and aggregation [38, 45] • Improving endothelial function by modulation of eNOS [37, 46] • Inhibition of formation of ROS/RNS by scavenging free radicals and thus preventing oxidative stress [3, 35]
Neurodegenerative disease	• Prevention of the decrease of Na,K-ATPase [47] • Direct neuroprotective actions by BBB traversing polyphenols [37] • Protecting neurons against oxidative stress [36, 48] • Influencing the lipid kinase signaling pathways e.g. decrease of NF-κB [37]
Gastrointestinal disease	• Inhibition of LOX activity [49] • Reduction of colonic inflammation by decreasing of TNF-α and inhibition of the activation of NF-κB inflammatory mediators [50]
Diabetes mellitus (type 2)	• Protection of pancreatic ß-cells against glucose toxicity [51] • Inhibition of α-amylase and α-glucosidase [51, 52] • Reduction of inflammatory mediators e.g. IL-1ß, IL-8 and COX-2 [53]
Cancers	• Inducing tumor cell apoptosis, inhibition of cell growth and preventing tumor invasion and metastasis by influencing cell cycle regulatory and signaling proteins [33, 37, 39, 54-58] • Involving tumor cell proliferation by modulation of signaling pathways e.g. MAPK [37] • Influencing the carcinogenesis process by counteracting AGEs [37, 59] • Reduction of COX-2 expression [3, 37]

The “French Paradox” opened new research interest for examinating whether resveratrol or other polyphenols can provide beneficial effects on human health besides the reduction of CHD [60]. Sun et al. recognized a prevention of the decrease of Na,K-ATPase in rat synaptosomes under simultaneous intake of polyphenols extracted from grapes with ethanol showing neuroprotective effects [47]. Moreover, Bhullar et al. proposed strong evidence for polyphenols as therapeutic agents in neurodegenerative diseases such as alzheimer’s disease (AD), multiple sclerosis (MS), parkinson’s disease (PD) and huntington’s disease (HD) [36] by protecting neurons against oxidative stress [36, 48]. Polyphenols can exert direct

neuroprotective actions in the brain when they pass the blood-brain barrier (BBB) [37]. Additionally, some polyphenols have shown an improvement of memory and cognitive ability [33, 37].

Dryden et al. also suggested the therapeutically use of polyphenols in gastrointestinal diseases based on their ability to decrease colonic inflammation mediators [50]. Knaup et al. confirmed that anthocyanins such as delphinidin 3-*O*-glucoside and delphinidin 3-*O*-galactoside are able to inhibit lipoxygenase activity and hence may prevent inflammatory diseases in the intestinal tract [49].

The role of dietary polyphenols regarding chronic diseases e.g. diabetes mellitus type 2, in which inflammatory mediators are contributing the pathogenesis as well, is presently investigated [51-53, 61].

The flavan-3-ol glycoside EGCG is the main catechin in green tea. Because of the antioxidant properties [62] EGCG became interesting for prevention of several human diseases [63]. Besides this, EGCG is even attributed to chemopreventive effects and thus a potential use in cancer therapy might be possible [57]. It can e.g. counteract advanced glycation end products (AGEs), which arise due to the accelerated glycolysis of tumor cells and are known to be involved in cancer progression [37, 59]. Indeed, polyphenols are able to alter some steps of tumor cells like their invasion, growth, metastasis and apoptosis by influencing regulatory and signaling proteins [33, 37, 39, 54-58].

Because of all these positive aspects of polyphenols, many preparations of them are currently available as dietary supplements [64]. Some researchers are also investigating whether polyphenols exert negative effects as well [65]. As an example, Zeegers et al. found that coffee consumption might increase the risk of urinary tract cancer by about 20% [66].

1.4 Bioavailability

The health effects of polyphenols depend on their bioavailability influencing their fate in the human body [64]. After oral intake of polyphenols different hydrolytic processes take place according to the polyphenolic structure. While flavonoids (glycosides) are largely stable under acidic conditions, proanthocyanidins are degraded to monomers and dimers of catechin units. Polyphenols are mainly absorbed in the small intestine- and colon mucosa. In the small intestine first metabolic processes such as hydrolysis and phase-II-metabolism reactions take place due to the high metabolic activity of the enterocytes and of the intestinal microflora. Furthermore, the flavonoids glycosides can be hydrolyzed by ß-glucuronidase (GUSB, *EC 3.2.1.31*). Subsequently, after absorption through the intestinal epithelium the polyphenols, their conjugates and metabolites reach the liver via the portal vein, where they are subjected

to further phase-II-reactions, involving catechol-*O*-methyltransferase (COMT, *EC 2.1.1.6*), UDP-glucuronyltransferase (UGT, *EC 2.4.1.17*) and sulfotransferase (SULT, *EC 2.8.2.1*) among other enzymes [4, 15, 26, 67]. The polyphenols and their metabolites partially experience an enterohepatic circulation. Via the bloodstream they are distributed to tissues and organs such as lung [68], brain, pancreas or skin [69]. Serra et al. detected catechin-glucuronide, methyl catechin-glucuronide and methyl catechin-sulfate in thymus, intestine, lung, kidney, spleen and testicles of rats at nM levels after an intake of a hazelnut extract which was rich in procyanidins [70]. The polyphenols are mainly eliminated via the urine and faeces as glucuronides and sulfates [15].

The bioavailability of polyphenols is significantly affected by the metabolic activity of the gut microbiota [18, 71-76]. The human intestinal flora is highly individual and varies with lifestyle, diet and age [77, 78]. Some microbial metabolites have physiological effects. For example, equol has greater phytoestrogenic activity compared to its precursor, the soybean isoflavone daidzein [15, 18]. For the formation of daidzein to equol specific bacteria strains are necessary [79]. Furthermore, the metabolites enterolactone and enterodiol are formed by various bacteria after exposure with nutrition-derived lignans from linseed [15, 18]. These metabolites have also shown several *in vitro* bioactivities like anti-inflammatory effects [80]. Monagas et al. reviewed the microbial biotransformation of dietary flavan-3-ols and observed, that phenolic metabolites formed by the intestinal microbiota, might mainly be responsible for the beneficial effects on health [71].

Moreover, the bioavailability of polyphenols can also be influenced by their individual stability and possible degradation processes, which should be considered during all investigations. As an example, when examing anthoyanins it should be taken into account that the flavylium cation is only stable at very low pH values (pH 1-3) [1]. Catechins are stable at acidic pH-values, whereas they are subjected to an epimerization followed by a degradation at basic pH-values [81]. Moreover, some polyphenols can experience a significant decrease in their concentrations during cell culture conditions [82]. Sang et al. characterized the stability of tea catechin EGCG under cell culture conditions and found out that EGCG experienced an auto-oxidation and epimerization [83].

In the recent years, many studies have been investigating the bioavailability of polyphenols in humans [20, 84]. Plasma concentrations of polyphenols depend on the food source and the amount of intake and thus can be very low [15]. More knowledge about the *in vivo* pharmacokinetics and metabolism of polyphenols is required to assess their biological effects [64]. For this purpose ultrasensitive methods and a number of analytical techniques are necessary to determine them at low concentrations (nM-µM) in the related biological matrices (see Chapter 2.1) [8, 64].

2 Analytical Background

2.1 Analysis and quantification of polyphenols

The chemical structures of polyphenols reveal a large variability (see Chapter 1.1). The choice of the method for the analysis of polyphenols depends on numerous aspects: The analytical objective(s), e.g. quantification of targets and/or identification of untarget analytes, such as metabolites, as well as the type of sample matrix (e.g. plant, food or biological fluid) have to be taken into account. Furthermore, the required sensitivity of the analytes and the availability of the associated technique have to be considered as well [2]. Highly sensitive analytical methods might be necessary to study the pharmacokinetics of polyphenols in humans due to their low concentrations in the human body [4, 64].

2.1.1 Extraction methods

The selection of a suitable sample preparation technique is an important factor to achieve the highest sensitivity. The strategy for analysis in the respective matrix should be chosen based on the chemical properties of the target compound(s) and the complexity of the matrix (e.g. plasma, serum or more viscous matrix like blood cells) [2]. Phenolic compounds are typically found as glucuronide and sulfate conjugates in biological matrices (phase-II-metabolism). If quantification of the individual conjugates is not intended, an initial hydrolysis-digestion step with an enzyme mixture containing ß-glucuronidase and/or sulfatase prior to the sample preparation procedure has also to be considered [2, 8].

Liquid-liquid extractions (LLE) and solid-phase extractions (SPE) are the most commonly used techniques for isolation of the analytes of interest. The phenolic hydrogen posses a logarithmic acid dissociation constant (pK_a) of around 10, while the pK_a of the phenolic carboxylic acid is between 4 and 5 [2]. The fact that polyphenols are dissociated under basic conditions and undissociated at acidic pH-values governs the partition behavior during the sample preparation.

During LLE the recovery of the analytes is influenced by various aspects of the extraction procedure such as the number of individual extraction steps and the extraction solvent. The most frequently used extraction solvents are methanol, ethanol, acetone, ethylacetate, diethyl ether, mixtures of alcohols, water for very polar compounds and less polar solvents (e.g. dichloromethane) for nonpolar molecules. Besides the complexity of the matrix itself, e.g. its viscosity, the sample-solvent ratio, the pH-value before extraction, the extraction time and the temperature are other important factors [2, 3, 8]. Numerous scientists use LLE approaches as sample pretreatment for the quantification of polyphenols in biological matrices by liquid chromatography (LC)- mass spectrometry (MS) analysis [85, 86]. As an example, Sano et al.

successfully quantified the dimer procyanidin B1 in human serum after enzymatic hydrolysis using ß-glucuronidase/sulfatase and subsequent LLE with methanol/formic acid [87].

For SPE different commercially available cartridges using distinct separation strategies can be utilized. So, for example, reversed phase (RP) conditions at acidic pH-value capture the phenolic analytes in their protonated form, whereas strong anion exchange conditions at basic pH-value are used to grasp them in their deprotonated form. An advantage of SPE is the option for automation of the procedure. For example, Doerge et al. developed an on-line SPE LC-ESI-MS method for quantification of the isoflavones genistein and daidzein in rat serum [88]. Furthermore, Urpi-Sarda et al. described a LC-MS/MS method using an optimized 96-well plate SPE approach for the quantification of epicatechin, procyanidins and phenolic microbial metabolites in human and rat urine after cocoa intake [89]. Roura et al. also implemented a SPE clean up procedure to quantify catechin in human plasma via LC-MS/MS [90].

In some cases an additional protein precipitation step prior to the extraction procedure can be advantageous [8] since e.g. plasma contains 60-80 g/L proteins [91], thereof 60% albumin [92]. Other techniques for the isolation of polyphenols can be soxhlet extraction, supercritical fluid- and microwave-assisted extraction, but they are more likely applied in plant or food samples than in animal or human specimen [2, 3].

2.1.2 Separation and detection methods

Numerous analytical separation and detection techniques exist to evaluate the composition, content and pharmacokinetic aspects of dietary polyphenols.

2.1.2.1 General overview

Since the early 1960s thin-layer chromatography (TLC) has been performed and is still used for a simple and fast identification of phenolic compounds. They are detected by UV light or densitometry based on the light absorption of phenols in the ultraviolet (UV) range [2]. Quantitative analysis is not the main aim of TLC [2] due to the insufficient sensitivity and the selectivity of this method [93]. Recently, a few approaches for quantification of polyphenols with high-performance densitometric TLC have been reported [94, 95].

Besides UV absorption, various methods for the quantification of the total phenolic content exist such as colorimetry using iron salts and the Folin-Denis method, which is based on the reduction of phosphomolybdic-phosphotungstic acid. Furthermore, a complexation with aluminium ion (Al (III)), the vanillin method and the reaction with 2,4-dinitrophenylhydrazine can be used. These methods do not target all phenolic groups of polyphenols [2, 3]. Moreover, proanthocyanidins can be specifically detected when heating in a mixture of butanol/HCl (95/5; v/v) solution and iron (III) salts (Porter's reagents) [96] due to the fact that

they are transformed to anthocyanidins because of autoxidative depolymerization [97, 98]. As an example, Porter's reagents combined with high-performance liquid chromatography (HPLC)- UV analysis was used for the characterization of thearubigins and other non-proanthocyanidins [98].

Gas chromatography (GC) and HPLC provide advanced techniques for the quantification of polyphenols. GC is especially applied in the analysis of plant or food samples and was previously typically coupled with flame ionization detection (FID) until the detection with mass spectrometry (MS) became increasingly prevalent within the last few years [2]. Soleas et al. described an ultra-sensitive method for the determination of catechin, quercetin and resveratrol and their conjugates in biological fluids using GC-MS [99]. To achieve sufficient separation and sensitivity, a variety of reagents for derivatisation of phenols and carboxylic acids exist. Most commonly silylated derivatives or methyl esters, which are generated after reaction with diazomethane, are utilized for the quantification [2]. For the GC-MS analysis of bioflavonoids a derivatization of these non-volatile compounds is essential [8, 100]. As an example, Bell et al. quantified catechin in human plasma after ingestion of red wine via the trimethylsilyl derivatives of catechin after the processed samples reacted with *N,O-bis* (trimethylsilyl)trifluoroacetamide [101].

Whereas GC is limited to volatile compounds or to those that can be converted to volatile and thermally stable compounds by derivatization, HPLC offers a wider range of applications due to the accessibility to non volatile compounds [102].
Thus, since the 1990s HPLC techniques are dominating the quantification of polyphenols. The high variety of commercially available stationary phases and thus the ability to separate the analytes simultaneously is certainly an aspect for the extensive use of HPLC [2]. Reversed-phase (RP) columns and among these mainly modified C18-materials with diverse dimensions and particle sizes are used. For example, RP-embedded phases with pentaflourophenyl (PFP)-groups bound to C18-silica surface allow an adequate separation and selectivity of highly polar compounds [103]. Hydrophilic interaction chromatography (HILIC) offers a powerful technology for the analytical separation of small polar and hydrophilic molecules. It utilizes aqueous buffer systems as eluents while comprising a polar stationary phase as normally used in normal chromatography phase (NP) [104-109]. Nowadays several HILIC columns with different chemical properties are commercially available, for example a sulfobetaine type zwitterionic one (e.g. ZIC®-HILIC, SeQuant AB, Ulmeå, Sweden) [110] or silica surfaces covered with cross-linked diol groups [103]. Moreover, for the separation of enantiomeric compounds chiral columns like polysaccharid-derived and beta-cyclodextrin based polymer phases, which separate the stereoisomers by forming of inclusion complexes, are typically utilized [2, 111].

Regarding the mobile phases, acetonitrile and methanol are the most commonly used organic solvents in HPLC. Because of the pK_a values of the phenolics the optimal pH range of the mobile phase should be between 2 and 4 when using RP columns [2]. Acidification of the solvents with modifiers such as acetic acid, formic acid, phosphoric acid and trifluoroacetic acid (TFA) is often employed to reduce peak tailing [2, 3], whereas the ESI-MS compatibility is not provided by all mobile phase–modifiers [112]. By the addition of optically active molecules (e.g. cyclodextrins) directly in the mobile phase, the separation of chiral compounds such as flavonoids can be achieved [111].

Phenolic compounds display light absorption in the UV range of the electromagnetic spectrum with a maximum between 220 and 320 nm. Flavonoids feature chromophores yielding two maxima, the first ranges between 240 and 285 nm and the second between 300 and 550 nm (corresponding to the visible spectrum (VIS) range). A wavelength of 280 nm is most commonly used for the detection of phenolic acids. However, a dual recording of 254 and 280 nm can be beneficial for monitoring various polyphenols simultaneously [2]. Some UV-absorbing substances e.g. amino acids and proteins can interfere and thus affect the detection of phenolic compounds [2]. The use of a diode array detector (DAD) permits additional identification of further compounds next to the quantification of the target analytes by employing UV/VIS spectra [2].

Fluorescence detection (FD) is also applied in phenolics analysis [113] and provides more specificity and sensitivity than usual UV absorption [100]. The use is limited since not all phenolics can be induced to emit fluorescence light.

Moreover, electrochemical detection (ECD) might be a useful instrument to quantify compounds that can be oxidized or reduced, such as phenols [2]. Compared to UV detection, a higher analytical sensitivity can be achieved by ECD [100]. Nardini et al. successfully developed a HPLC-ECD method for the quantification of phenolic acids in food [114] and in human plasma [115]. A crucial disadvantage of the electrochemical coulometric detection is, that it is not compatible with a gradient elution, which is often necessary for achieving appropriate separation if multiple analytes are simultaneously quantified [2]. Developments in ECD technology, which counteract this fact, yielded multi-electrode array detection. This is currently employed for the quantification of phenolic compounds in food [116] and plasma samples [117-119]. Furthermore, a constant equilibrated baseline noise is essential for sensitivity [120].

Capillary electrophoresis (CE) coupled to an UV-, FD, ECD and MS detector represents a further possibility for the separation of polyphenols [2, 8, 100]. Lafont et al. and Huck et al. successfully applied CE-ESI-MS techniques for the quantification of phenolic compounds [121, 122].

2.1.2.2 LC-MS as advanced technology

In the last few years the application of HPLC coupled with MS has gained superior significance in bioanalytical and pharmaceutical research for the quantification of target analytes in complex biological samples [123]. For the ionization of the analytes diverse interfaces like an atmospheric pressure chemical ionization (APCI) or an electrospray ionization (ESI) are employed [2, 8, 100]. Besides of using e.g. an ion-trap (IT) for the LC-MS analysis, by which additional information can be obtained due of multi sequential fragmentation experiments (MS^n), a quadrupole mass spectrometer provides higher sensitivity and specificity, also compared with common HPLC-UV analysis. A triple quadrupole mass spectrometer operates by ionization and fragmentation of the analytes with e.g. collision induced dissociation (CID). After fragmentation of the precursor ion of the analyte, which is isolated by the first quadrupole (Q1), specific fragments (*product ions*) with associated *mass-to-charge-ratios* (*m/z*) are generated in the collosion cell (q2), which are selected with the third quadrupole (Q3). By monitoring these transitions via *multiple reaction monitoring* (MRM) highly sensitive methods are obtained for quantification (see Figure 4) [8, 100]. Finally, the actual detection takes place via an electron multiplier, which transforms the ions to electrons and amplifying their signal.

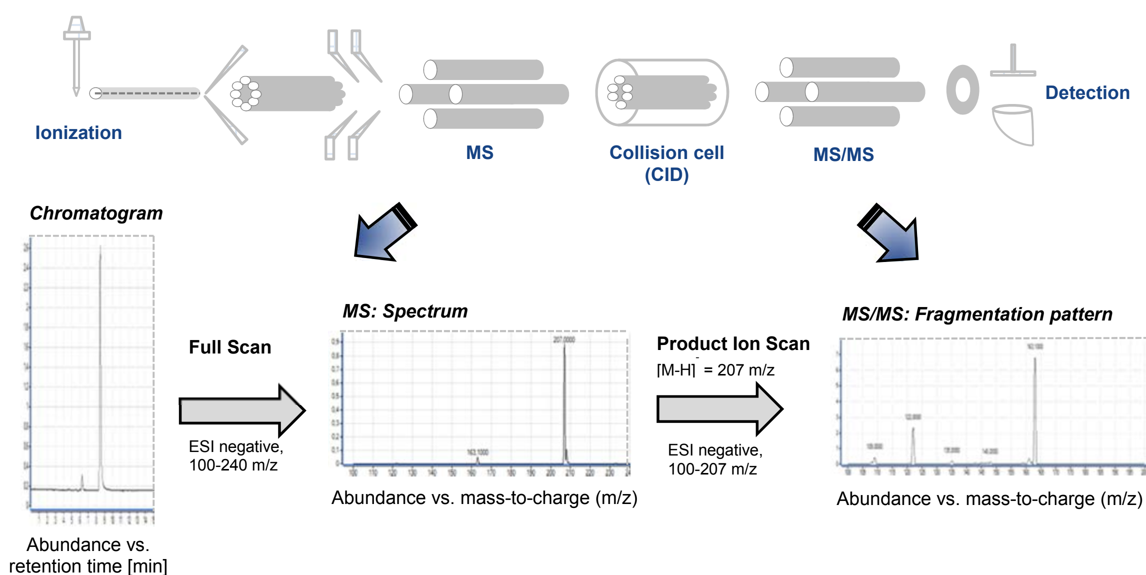


Figure 4: Development of a multiple reaction monitoring (MRM) method exemplified for the compound δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1; MW= 208 g/mol). *The most abundant peak of the full scan using ESI negative ionization leads to the single deprotonated precursor ion with 207 m/z. After fragmentation of the precursor specific product ions were formed with an associated mass-to-charge-ratio (m/z). The most abundant product ions were 163 m/z and 122 m/z. These transitions (207 > 163; 207 > 122) were monitored by MRM.*

A variety of applications for the quantification of phenolic acids and flavonoids via LC-ESI/MS-detection in food [14, 124-127] and biological matrices [2, 86, 128-132] have been described. The ESI ionization is most commonly used in negative mode for the quantification of phenolic compounds [86, 124-128, 130].

Satterfield and Brodbelt investigated metal complexations of flavonoids with diverse double charged transitions metals such as Co, Ni, Cu, Zn, Mn and Fe as alternative to the protonated or deprotonated form after ESI-MS ionization. Indeed, an enhanced detection of the flavonoid-metal complexes was described when using Cu (II) [133]. MS detection can be combined with other detection modes e.g. by UV absorption or fluorescence for the quantification of polyphenols in biological matrices [134-136].

Considering an appropriate sample preparation, LC-MS(/MS) analysis offers the opportunity for sensitive determination and quantification of polyphenols in very complex matrices like tissues [70, 137]. As an example, Aqil et al. performed bioavailability studies of anthocyanins and detected them and their metabolites in lung tissues of mice which were fed with blueberry powder [68].

By coupling LC with MS-detection via the ESI-interface matrix effects (ME) can occur [138]. Residual salts, endogenous phospholipids and other matrix constituents, which are not completely removed by the previous sample clean-up, can interact at ESI-ionization by charge competition with the analytes [139, 140]. Due to ME the analyte is subjected to a loss, so-called ion suppression, or to a gain, so-called ion enhancement, in MS-response [139, 141]. Because of the high individual variability of the matrix and thus different relative ME the quantification of low polyphenol concentrations in biological samples can be challenging [139, 141].

HILIC can be a very satisfactory solution to achieve high sensitivity of the analytes using ESI-MS detection because of the retaining of polar compounds with high organic concentration in the beginning of the analytical run and thus yield to higher intensity at ionization [142, 143]. In some cases basic pH-values (clearly above >7) of the sample or the mobile phase are necessary to obtain required retention of the phenolics on the HILIC phase. Polyphenols might be subjected to a significant decrease in their concentrations under experimental conditions [82, 83]. For example, catechin is known for degrading at basic pH-values [81]. Therefore, a possible stability loss should be considered and examined when using HILIC approach for the analysis of polyphenols.

Recent advances in LC-MS analysis support the use of ultra high pressure liquid chromatography (UHPLC) [2]. UHPLC operates at higher pressure than common HPLC (clearly above 400 bar) while providing the ability to use sub-2 µm columns for an increase in sensitivity, resolution and reproducibility. Due to the simultaneous decrease in analysis time, UHPLC offers a suitable opportunity for high-throughput analysis. Some adjustments regarding the LC-system might be necessary when using UHPLC, because of the analytical operation under high pressure [144]. Recently, some multi dimensions techniques like LC x LC

(e.g. HILIC x RP) have been applied for simultaneously separation and thus identification and/or quantification of complex polyphenol mixtures [145, 146].
For highly reliable identification purposes of phenolics with MS-detection a high mass accuracy is recommended, so quadrupole time-of flight (qTOF) detection or other techniques with high resolution (HR) can be used [8, 100].

2.1.3 Calibration

Different approaches exist for calibration when quantifying phenolic compounds in biological matrices with LC-MS/MS. According to EMA and FDA guidelines, the matrix for calibration should be the same as the samples to be analyzed. Known analyte concentrations are added to this matrix (calibration standards). Matrix-calibration with matrix-matched calibration standards added to a blank matrix is recommended for compensating ME due to their similar composition [147, 148]. Target-free blank matrix for preparing calibration standards is not always available. For analyte quantification in plasma Liao et al. used standard solutions in solvent for calibration and afterwards corrected the differences in ME using a matrix normalization factor (MNF) [149]. Particularly for the quantification of dietary polyphenols, analyte-free blank matrix such as serum can be difficult to obtain due to their ubiquitous presence in nutrition (see Chapter 1.2).
Therefore, scientists have been seeking reliable alternatives for calibration to overcome the lack of related blank matrices. Serra et al. and Rubio et al. obtained basal rat plasma after subjecting the animals to strict fasting conditions [137, 150]. However, for receiving human basal matrix the adherence of dietary restrictions under real life conditions would be more difficult compared to monitorable laboratory animals. Moreover, Serra et al. developed a SPE approach for dephenolizing plasma matrix employing a RP-SPE cartridge to obtain polyphenol-free blank matrix [132]. By performing this clean-up procedure it cannot be excluded that also other plasma constituents are removed which can possibly lead to interferences or deviations in the LC-MS analysis different from those of an untreated sample matrix. A matrix-matched calibration curve can be created even if the matrix already contains basal endogenous concentrations of the analyte of interest. In this case, the y-axis of the calibration curve of the analyte is shifted according the response of the analyte in the blank matrix [151, 152].

If the amount of the individual sample volume is not a limiting factor, the standard addition calibration (SAC) offers another effective opportunity for the quantification of phenolics in biological fluids. Here, the analytes are added at different concentrations to the same sample before they are processed and analyzed. The sample concentration is then calculated by a calibration function, in which the y-axes is intersected by a generated regression curve produced by the spiked samples [147, 148]. This procedure can be time-consuming if applying this approach for compensation of relative ME at each individual sample, e.g. as part of a

clinical trial. However, Frenich et al. used a single point SAC for minimizing the calibration efforts to a total of two injections for GC-MS analysis [153].

A novel alternative for quantification is the echo-peak technique. Briefly, the sample is injected immediately after the injection of the calibration standards, so that the analyte of the standard and the sample elute closely, but do not overlap. This approach has some essential advantages like the control of the chromatographic conditions and furthermore the calibration standard peak serves as internal standard (IS). So both, IS and analyte, are influenced by the matrix of the sample to a similar extent [147, 148, 154, 155]. Adversely, the echo-peak technique is technically more complex due to the rapid successively injections of the calibration standard and the sample and is less tolerant of retention time fluctuations [147].

Most techniques of calibration involve IS, which behave as the analyte during the complete analytical process and thus compensate for individual variations e.g. losses in the course of sample preparation [148, 156]. Principally, two different types of IS exist. Either a structural or chemical analog of the analyte with similar physicochemical properties can be used or a stable isotope labeled (SIL-) IS which comprises isotopes like ^{2}H, ^{13}C, ^{15}N and ^{18}O of the analyte [157]. ^{2}H labeled SIL-IS are the cheapest and thus the most common ones [157]. But, they are not as suitable as e.g. ^{13}C-labeled SIL-IS since they can cause unexpected changing peak area ratios analyte/IS during LC-MS/MS analysis [158, 159]. The use of SIL-IS is often associated with high costs because for some analytes, e.g. for endogenous metabolites, they are not commercially available and thus difficult to obtain [148]. Some scientists assume that SIL-IS yield better assay performance and thus more reliable quantification results [148, 160]. Within the last few years researchers have started to evaluate the use of a SIL-IS in their individual applications [157-159, 161-164]. As an example, Heideloff et al. described that both IS, respectively the structural analog (32-desmethoxyrapamyin) and the SIL-IS (everolimus-d4), provided acceptable performance for the quantification of everolimus using LC-MS/MS analysis [162]. Furthermore, Wu et al. compared analytical results using non- and labeled IS in pooled human plasma and in individual plasma samples. Both yielded satisfactory accuracy and precision in human pooled plasma, but only the SIL-IS was able to correct the interindividual variability in the recovery of the individual plasma samples [164]. Fernández-Peralbo et al. confirmed these results with similar investigations regarding the use of a SIL-IS and suggested a recovery correction factor to compensate ME in the quantitative analysis of hydroxyeicosatetraenoic acids in human serum by LC-MS/MS [157]. SIL-IS and analyte coelute at the same retention time during the analytical run and hence they are subjected to absolutely identical ME [158, 165]. However, the use of an SIL-IS can not completely overcome ME, especially relative ME, in biological matrices [158].
Besides using a SIL-IS to account for ME in LC-MS/MS analysis a thorough and focused development of an adequate sample preparation technique is essential for the accurate and sensitive quantification of polyphenols in biological matrices [141, 157, 166-169].

2.2 Validation of analytical methods

Validation is crucial for the quality assurance of an analytical method. It provides information about the ability of a method to meet a given specific task. Extent and implementation of a validation depend on the intended purpose [170].

Numerous committees and public authorities have dealt with the validation of analytical methods and have developed guidelines for their implementation [171-175]. The US-*Food and Drug Administration* (FDA) defines validation as a procedure ensuring that the performance of an analytical method is appropriate and reliable for the planned purpose. The published guideline of the FDA provides general recommendations for the validation of bioanalytical methods, which are important for HPLC, also in combination with mass spectrometric techniques such as LC-MS/MS. It includes requirements for the quantitative determination of analytes and metabolites in biological matrices such as blood, serum or plasma [176]. Furthermore, the *European Medicines Agency* (EMA) has published a guideline which recommendations are applicable for bioanalytical methods to determine e.g. concentrations of analytes in biological matrices as part of an toxicokinetic or clinical trial [177].

Based on the FDA and EMA guidelines more discussion papers and conference reports were created which offer more detailed explanations regarding their implementation [178, 179]. Additionally, there is an aid for interpretation which comments the EMA guideline and compares it with the recommendations of the FDA [180]. Moreover, numerous scientific and review articles deal with the validation of methods for the quantification of substances in biological matrices by LC-MS/MS and discuss individual validation parameters [181-183].

According to EMA and FDA different types and levels of validation are defined and characterized in detail, respectively *Full Validation, Partial Validation* and *Cross Validation* [176, 177]. A *Full Validation* is necessary when a method is developed and implemented the first time for this analytical purpose. After inclusion of an additional compound or metabolite the validation has to be revised. A *Partial Validation* is a modification of an already validated bioanalytical method. If the method has previously been completely validated (*Full Validation*) and afterwards small changes are made, such as storage conditions, concentration range, sample preparation, sample volume, alteration of the species providing the matrix (e.g. from rat to mouse plasma) or deviation of the matrix within a species (e.g. from human plasma to human urine), the method has to be partially validated. The extent of a *Partial Validation* can range from small efforts such as the determination of precision and accuracy to a nearly complete *Full Validation*, depending on the change. A *Cross Validation* is a comparison of validation parameters. This should be done as soon as more than two bioanalytical methods are used to produce data. For example, this is the case when samples within the same study

are investigated by two different laboratories or using different analytical techniques (e.g. LC-MS/MS vs. ELISA).

For the quantification of substances in biological matrices selective and sensitive analytical methods are essential for a correct analysis of clinical trial samples. A sufficient validation assures that the method is reliable and reproducible for the intended use [170]. The data of the validation results are often documented in scientific articles to prove the performance of the applied method [128, 130, 132, 137, 150, 184-193].

Validation parameters of a *Full Validation* were thoroughly investigated in this thesis for all the developed analytical methods for the specimen plasma, serum, blood cells and synovial fluid. This included: (1) selectivity/specificity, (2) linearity/calibration curve, (3) accuracy and precision, (4) limit of detection (LOD), (5) lower limit of quantification (LLOQ), (6) matrix effects (quantitative), (7) recovery and process efficiency, (8) carry-over, (9) crosstalk (10) robustness and (11) stability.

2.2.1 Selectivity/Specificity

Selectivity of a method is the ability to determine several compounds without mutual interference. A method is specific if the target analytes can be determined without adulteration by other components that are present in the sample [170].
According to the EMA and FDA guidelines, the specific and selective detection of an analyte in a biological matrix at the LLOQ should be investigated with at least six individual donors of the matrix [176, 177]. In case of matrix scarcity it is justified to use less than six individual donors [177]. Furthermore, possible interfering substances such as matrix components or metabolites, drugs and contaminants should be considered and studied for each analyte if the compounds are simultaneously determined in biological matrices. In this context, the *International Conference on Harmonization* (ICH) guideline recommends to perform tests with impurities [174]. There is no interference if the response of the co-eluting and thus interfering compound corresponds to less than 20% of the LLOQ for the analyte and 5% for the IS [176, 177].

2.2.2 Linearity/Calibration curve

In a defined concentration range linearity is given when the measured signal is directly proportional to the analyte concentration in the sample [170].
The calibration curve should be prepared in the same matrix as the samples by adding known analyte concentrations (calibration standards) according to the EMA and FDA guidelines. The number of the standards for a calibration curve should be appropriate and comply with the analytical requirements. Furthermore, the concentration range of the standards should be selected in such a way that they cover the expected range of the samples. A calibration curve

should consist of a matrix-blank (without IS), a zero-blank (with IS) and six to eight concentration levels (non-zero samples), including the LLOQ and the highest standard of the calibration curve (*Upper limit of quantification*, ULOQ). The simplest regression model that describes the relationship between the analyte concentration and the measured signal should be used. The back-calculated calibration standards within the calibration range should have an accuracy of 85-115% and the relative standard deviation (RSD) of the precision should be less than 15%, except at the LLOQ, at which an accuracy of 80-120% and a RSD of the precision of less than 20% is acceptable. At least six of the calibration standards (≥ 75% of the standards), including LLOQ and ULOQ, should meet these criteria. An exclusion of individual calibration standards which not comply with those criteria should not change the regression model [176, 177].
Additionally, the EMA recommends checking the linearity of each analyte in at least three calibration curves in the related matrix. Typically, the matrix- and zero-blank should not be included into the calculation of the calibration function [177], but this might be unavoidable in special applications (see Chapter 2.1).

2.2.3 Accuracy and precision

The accuracy of an analytical method is the deviation from the true value due to a systematic error [170] and can be calculated by generating the percent deviation of the determined concentration divided by the nominal concentration (Equation 1; [176, 177]).

$$\text{Accuracy [\%]} = \frac{\text{determined concentration}}{\text{nominal concentration}} \times 100 \qquad \textit{(Equation 1)}$$

The accuracy of an analytical method is typically evaluated by quality control standards (*QC samples*), which should be processed independent of the calibration standards. The concentration of each standard is determined using an additional calibration curve and the accuracy is calculated based on these results.
The precision of a method is the variance of repeated analytical runs. A measure of the precision is the relative standard deviation (RSD) of the determined values or the coefficient of variation (CV) if this is expressed as the percent deviation of the mean value [170].

The EMA advises the evaluation of the accuracy and precision during an analytical run (*within-run precision*) and between several analytical runs (*between-run precision*). The *within-run accuracy* and *-precision* should be determined for a minimum of four concentrations (LLOQ, low-QC (about 3 x LLOQ), medium-QC (about 50% of the geometric calibration range [180]), high-QC (at least 75% of the calibration range)) with a minimum of five samples per concentration. The *between-run accuracy* and *–precision* are determined by performing this approach with a minimum of three analytical runs, which should comply with the requirements of at least two days [177]. In the validation of a bioanalytical method the *within-run- and*

between-run accuracy and *–precision* are often expressed as *intra-* and *interday accuracy* and *–precision* [128, 130, 137, 150, 184-192].
Additonally, the FDA defines a *repeatability,* which reflects the precision over a longer period of time and accounts for various influences such as reagents, instruments and also the analyst itself [176]. The term *reproducibility* describes the precision between laboratories and should be evaluated as part of the standardization of an analytical method [174, 175].

In contrast to the EMA, the FDA requires a minimum of three concentrations within the calibration range (low-QC, medium-QC, high-QC) for assessing the precision and accuracy. The mean of the back-calculated calibration standards within the calibration range should have an accuracy of 85-115% and a precision, expressed as the RSD, of less than 15%, except at the LLOQ in which an accuracy of 80-120% and a precision of less than 20% is acceptable [176, 177].

2.2.4 Limit of detection (LOD)

The *limit of detection* (LOD) is the lowest concentration of the analyte in the sample, which can be qualitatively determined [170]. A differentiation between a method detection limit and an instrumental detection limit can be done [171]. In chromatography practice, an analyte response of at least three times the response compared to the blank response is described as LOD [170, 171, 194]. Various approaches exist for determining the LOD, e.g. by simplified calculation of the *signal-to-noise ratio* (SNR) [194, 195]. In principle, the presence of the analyte must be ensured visually [175].

2.2.5 Lower limit of quantification (LLOQ)

The *lower limit of quantification* (LLOQ) is the lowest concentration of the analyte in the sample, which can be quantitatively determined with a specified precision and accuracy [170]. Again, a method detection limit and an instrumental detection limit can be described [171].
In chromatography practice, an analyte response of at least ten times the response compared to the blank response or a threefold LOD is described as LLOQ [170, 194]. An experimental determination of the LLOQs for each compound in the related matrix is often not feasible without enormous efforts [195]. An analytical method should ensure that the LLOQ of the analyte covers the lowest expected concentrations of the examined samples. Furthermore, as previously described, the LLOQ represents the calibration standard with the lowest analyte concentration of the calibration curve [177].
The EMA and FDA guideline both recommend for the LLOQ a five-fold higher signal of the analyte in the LLOQ sample compared to the signal of the analyte in the blank sample. To summarize, the LLOQ is the lowest concentration of the analyte in the sample that can still be determined with a sufficient precision (expressed as RSD < 20%) and accuracy (80-120%) and has a SNR of higher than five [176, 177].

2.2.6 Recovery, matrix effects and process efficiency

According to the FDA the recovery rate of the analytes should be investigated for a minimum of three concentrations (low, medium, high) within the calibration range [176].

Furthermore, matrix effects (ME) should be examined to ensure that they do not affect the precision, selectivity and sensitivity of a bioanalytical method. For LC-MS/MS methods appropriate arrangements should ensure that the quantification result is not distorted by ME, especially if the nature of the sample matrix differs from the one used during method validation.

If possible the same biological matrix like that of the samples should be used for validation purposes. In case of limited availability of the matrix it can be replaced by an appropriate physiological matrix. Due to the physiologically heterogeneous nature of the sample, it may be important to analyze the interindividual variability of the matrix [176]. In this context, the EMA recommends the evaluation of ME using at least six lots of blank matrix from individual donors instead of pooled matrix. In case of matrix scarcity a deviation from this practise is justified. In spite of all, ME should essentially be examined in more detail [177].

For the quantitative evaluation of ME, the post-extraction spike method according to Matuszewski et al. [196] is frequently used. For this purpose, three sets of samples are processed (see Figure 5).

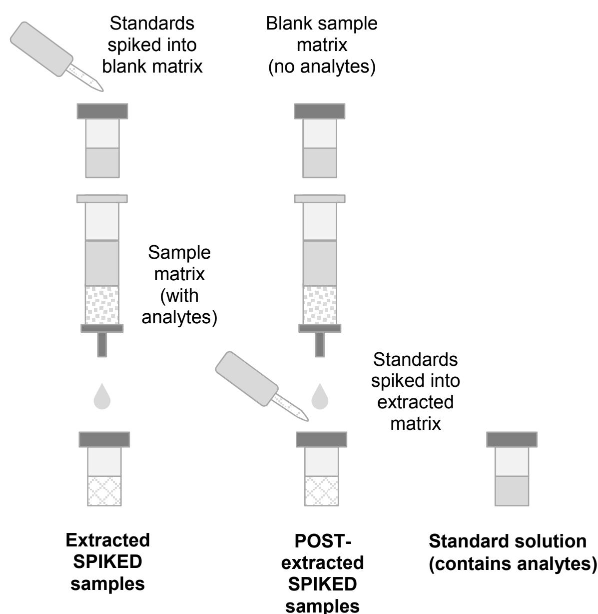


Figure 5: Post-extraction spike method to evaluate quantitative matrix effects (ME) proposed by Matuszewski et al. [196]. *Extracted spiked samples ("spiked") are prepared by adding standards into blank matrix followed by the subsequent sample preparation. Post-extracted spiked samples ("post-spiked") are prepared by extracting the blank samples matrix containing no analytes. After sample preparation the standards are spiked into the extracted matrix. Furthermore, the pure solvent containing the analytes is analyzed ("standard solution"). The figure is based on [139].*

Set 1: *Extracted spiked samples*: Standards are added into blank matrix. Then the samples are extracted and analyzed.
Set 2: *Post-extracted spiked samples*: The blank matrix is extracted containing no analytes. Afterwards the standards are spiked into the extracted samples and then are analyzed.
Set 3: *Standard solution:* Analytes in pure solvent are analyzed.
Using this approach, the recovery (RE; *Equation 2*; [196]), matrix effects (ME; *Equation 3*; [141]) and the process efficiency (PE; *Equation 4*; [196]) can be calculated as follows:

$$\text{Recovery [\%]} = \left(\frac{\text{Response}_{\text{extracted spiked sample}}}{\text{Response}_{\text{post-extracted spiked sample}}}\right) \times 100 \qquad \textit{(Equation 2)}$$

$$\text{Matrix effects [\%]} = \left(\frac{\text{Response}_{\text{post-extracted spiked sample}}}{\text{Response}_{\text{standard solution}}} - 1\right) \times 100 \qquad \textit{(Equation 3)}$$

$$\text{Process efficiency [\%]} = \left(\frac{\text{Response}_{\text{extracted spiked sample}}}{\text{Response}_{\text{standard solution}}}\right) \times 100 \qquad \textit{(Equation 4)}$$

Additionally, the matrix factor (MF) of the analytes and of the IS in each individual matrix should be calculated as the ratio of the peak area in presence of the matrix (*post-extracted spiked* samples) to the peak area in the absence of the matrix (*standard solution*).

The IS-normalised MF can be obtained by dividing the MF of an analyte by the MF of the IS as follows (Equation 5):

$$\text{IS-normalised matrix factor (MF)} = \left(\frac{ME_{\text{Analyte}}}{ME_{\text{IS}}}\right) \times 100 \qquad \textit{(Equation 5)}$$

To predict the variability of ME, the RSD of the IS-normalised MF for six lots of blank matrix from individual donors should not exceed 15% [177, 178].

The EMA advises the determination of the MF at a low (maximum of 3 x LLOQ) and at a high concentration level (close to the ULOQ) of the calibration curve. Moreover, besides of using “normal” matrix, it is recommended to assess ME also in treated test matrix, e.g. haemolysed or hyperlipidaemic, to investigate the method robustness [177]. Many ambiguities still exist about the interpretation of the assessment of ME [180].

2.2.7 Carry-over

A *carry-over* of analytes should be elucidated during method validation. Especially in samples from clinical trials with unknown expected concentrations an adsorption of the analyte on the stationary phase might occur after injection of a sample with a high analyte concentration before injection of the next sample. In order not to bias the quantification results of the study

samples by this effect, *carry-over* should be minimized from the beginning of the method development.
The extent can be evaluated by adding several processed samples of matrix-blanks after injection of a matrix sample containing the analyte at a high concentration (ULOQ). The *carry-over* in the first matrix-blank after injection of the ULOQ-spiked sample should not be higher than 20% or 5% of the analyte or IS in the related LLOQ sample. If the *carry-over* of the analyte exceeds this value, appropriate arrangements should reduce this effect, e.g. by introducing of matrix- or solvent-blanks after the injection of samples in which a high concentration can be expected.

Nowadays various types of autosamplers offer diverse possibilities for an automated reduction of *carry-over*, such as purging the sample loop and the needle seat with solvent(s) between two injections (*needle-wash*). In case that a high *carry-over* of the analyte cannot be counteracted, specific measurements within the validation have to ensure that the accuracy and precision are not influenced [177, 180].

2.2.8 Crosstalk

A *crosstalk* can occur when different compounds produce fragments (product ions) with similar *mass-to-charge ratio* (m/z). The dwell- and scan- times of the *multiple reaction monitoring* (MRM) are typically very short in LC-MS/MS analysis. For this reason it might be, that fragment ions of the first MRM-transition are still present in the collision cell and are recorded during the next transition [197]. Due to the incomplete "clearance" of the product ions after the transition of the first compound before the measurement of the transition of the second compound, this can lead to false positive results, particularly if the analytes have similar or identical product ions [198]. In addition to high sample concentrations this phenomenon can occur especially if using structurally similar compounds or stable isotope labeled (SIL-) IS [199].

Recently, the manufacturers of triple quadrupole mass spectrometers have been increasingly working to advance their instruments and measurement modes to reduce the appearance of a *crosstalk* [200, 201]. It is useful to thoroughly examine a possible *crosstalk* in the beginning of the method development and validation [202]. For this purpose, the analytes (and also the IS) are individually added in the associated matrix at high concentrations (ULOQ). Then the samples are prepared and analyzed. The response of each transition with the same retention time other than the transition of the examined compound should be less than 20% of the analyte or 5% of the IS in the related LLOQ sample [188].

2.2.9 Robustness

The robustness of an analytical method is a measure of its ability to remain unaffected by small but deliberate variations of parameters and is an indication of the reliability of the method [174]. A method is robust, if the end result is not or only insignificantly distorted by changes in the test conditions [170]. Consequently, the influence of critical parameters such as the stability of the analytes, temperature and pH values should be investigated [170, 171, 175]. It depends on the individual application which factors should be closer examined. For example, in the analysis of clinical trials samples, it may be useful to investigate an influence of haemolysed or hyperlipidaemic samples.

2.2.10 Stability

In a method validation the evaluation of the stability should be performed at a minimum of two concentrations (low (maximum 3 x LLOQ), high (close to ULOQ)) with at least three samples per concentration [176, 177]:

Freeze and thaw stability

The FDA recommends to investigate the stability of the analytes and the IS after three freeze-thaw cycles. For this purpose, the samples are frozen at the intended storage temperature for at least 24 hours and then thawed at room temperature (RT). After complete thawing, they are re-frozen for further 12 to 24 hours at the same storage temperature until the samples are subsequently analyzed after the third cycle. In contrast, the EMA equates the number of the cycles of the stability tests with those of the expected freeze/thaw cycles of the samples of the clinical trial.

Short-term stability

To assess the short-term stability the samples are kept for 4 to 24 hours at RT before they are processed and analyzed. The residence time depends on the expected duration that the clinical samples are processed and maintained at RT.

Long-term stability

The evaluation of the long-term stability should include the storage duration that the samples are subjected to real conditions, starting on the date of first sample collection to the date of the last sample analysis. The storage conditions should be consistent. A sufficient sample volume should be taken into account to prepare and analyze these samples for a total three times within this period. The concentration of the stability samples should be compared with those from the first measurement at the beginning of the stability studies.

Post-preparative stability

The stability of processed samples should also be considered, including the residence time in the autosampler, which should be consistent with the real queue time of the samples before analysis. The analyte concentrations of the autosampler stability samples should be determined using the corresponding calibration curve at the beginning of the measurements.

Stock solution stability

It is not necessary to investigate the stability of the analytes and the IS at all concentration levels. The stability of the stock solution (SL) at RT should be assessed for at least 6 hours. After completion of this time, the samples should be analyzed after appropriate dilution and compared with a freshly prepared SL. If the SL was frozen and thawed for a defined time, this should also be considered.

The detailed conditions of the stability studies should be adapted to the handling of the real samples, so that every step of them is taken into account during the sample preparation, analysis and storage [176, 177]. Furthermore, the stability of each analyte as well as the IS in the matrix is important. Stability studies should be performed especially if applications include multiple analytes [177]. The stability of an analyte in a defined matrix cannot be transferred to other matrices [176]. The determined concentration of the stability samples using a freshly prepared calibration curve is then compared to the nominal concentration and should not deviate by more than 15% [177]. Moreover, the FDA also accepts statistical approaches which allow an extrapolation of the stability of an analyte in a biological matrix [176].

3 Pycnogenol®

3.1 Standardized maritime pine bark extract

Since the year 2004, "*Maritime pine extract*" is monographed in the United States Pharmacopeia (USP) as a dietary supplement. A respective extract has to contain a 65-75% of procyanidins, consisting of catechin and epicatechin units [203]. A standardized extract of the French maritime pine *Pinus pinaster* Ait. complies with this monograph and is commercially available under the trade name Pycnogenol® (Horphag Research, Geneva, Switzerland).

The composition of this extract has been extensively investigated. Besides the high amount of procyanidins, the extract is rich in polyphenolic monomers, phenolic and cinnamic acids and their glycosides. Pirasteh identified the flavonoids (+)-catechin and taxifolin as well as some phenolic acids like protocatechuic acid, p-hydroxybenzoic acid, caffeic acid, gallic acid, ferulic acid, p-coumaric acid and vanillic acid. Moreover, procyanidin dimers such as procyanidin B1, B3, B6 and B7 and one procyanidin trimer were detected in the extract [204]. Chen et al. suggested a HPLC fingerprint analysis based on the USP monograph for the identification of the extract ingredients caffeic acid, ferulic acid, catechin and taxifolin to confirm Pycnogenol®-containing dietary supplements [205].

The extract can be separated in four fractions by size exclusion chromatography with Sephadex LH-20. The first fraction contains constituents with lower molecular weight such as the phenolic acids caffeic acid and ferulic acid, as well as the flavonoid monomers catechin and taxifolin. The second fraction consists of procyanidin dimers and trimers and the third fraction of procyanidin tetramers to hexamers. The last fraction includes the higher oligomeric procyanidins [206].

After ingestion of Pycnogenol® δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) has been found in human urine [206] and plasma [207]. This metabolite is not originally present in the pine bark extract, but generated *in vivo* from the procyanidins' catechin units through multiple step reactions by the intestinal microbiota [71, 208-210] (see Figure 6).
The human gut microbiota contributes significantly to the bioavailability of polyphenols (see Chapter 1.3). Certain bacteria strains (*Eggerthella lenta* and *Flavonifractor plautii*) are able to transform catechin units from procyanidins into γ-valerolactones, while cleavage of the C- and A ring with subsequent cyclic formation of esters [211]. Appeldoorn et al. identified δ-(3,4-dihydroxy-phenyl)-γ-valerolactone as one major metabolite of procyanidin dimers by the human microbiota [212]. So far it is not clear which main isomer (R or S) of M1 is generated *in vivo*.

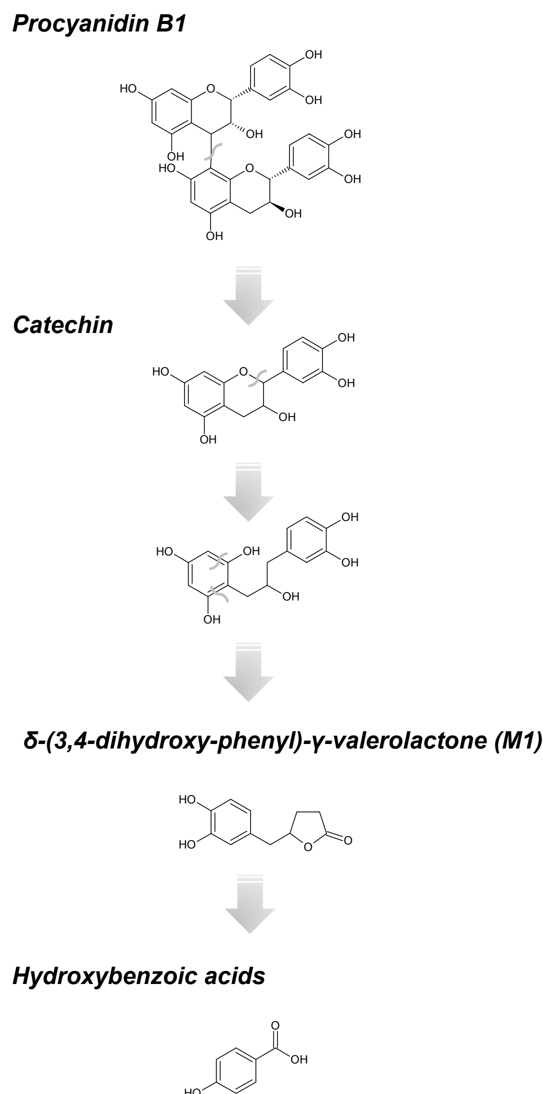


Figure 6: Biotransformation of dietary flavan-3-ols by the human intestinal microbiota [71, 208-210].

3.2 Pharmacokinetics

After oral ingestion of dietary polyphenols or plant extracts low concentrations of smaller constituents or *in vivo* formed components like aglycones and phenolic monomers are typically found in human plasma [15, 20].

Virgili et al. studied free and conjugated ferulic acid in human urine after oral administration of either a single dose (200 mg) or two doses (100 and 200 mg) of Pycnogenol® to eleven healthy adults using HPLC–diode array detection. The investigations revealed a conjugation of ferulic acid as glucuronide or sulfate in the individuals between 2 to 20%, also suggesting ferulic acid urinary excretion as a marker of consumption of Pycnogenol® [213]. Furthermore, Große Düweler detected the metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) in human urine samples after ingestion of Pycnogenol® [206].

In the course of a pharmacokinetic study with single and multiple doses of Pycnogenol® Grimm et al. analyzed plasma samples of human volunteers by HPLC with ion-pair reagents and simultaneous UV and electrochemical detection. Eleven volunteers received a single dose of 300 mg Pycnogenol® while keeping a flavonoid free diet the previous 24 hours. Plasma samples were collected before (t= 0 h) and at defined time points after intake of the extract (t= 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 14 h). In the plasma samples of the human volunteers the extract constituents (+)-catechin, taxifolin, caffeic acid, ferulic acid and the metabolite M1 were detected in the nanomolar range. Moreover, the plasma time courses of ten unknown compounds (U1-U10) were described. There were indications but no proof that the dimer procyanidin B1 was also present in plasma. The compounds were rapidly absorbed and some of them were present over the whole experimental period of 14 hours. (+)-Catechin (t_{max}= 4 h), caffeic acid (t_{max}= 4 h) and ferulic acid (t_{max}= 1 h) revealed early maximal plasma concentrations after a single dose of Pycnogenol®.
Furthermore, catechin showed increasing plasma concentrations to about 100 ng/mL at 4 hours and thereafter almost constant plasma levels from 6 to 14 hours (about 40 ng/mL). Taxifolin and the *in vivo* formed metabolite M1 from the procyanidins' catechin units were detectable rather late in plasma (2 h and 6 h) with t_{max}= 8 h for taxifolin and t_{max}= 10 h for M1 (mean concentration 3.59 ng/mL) [207, 214].

Moreover, 200 mg Pycnogenol® daily over the course of five days to reach steady state conditions were ingested by five volunteers. Plasma collection was performed after 4 hours of the last intake of Pycnogenol®. A flavonoid free diet for 24 hours before the blood sampling was followed. Mean total plasma concentrations of catechin (48.56 ± 16.66 ng/mL), ferulic acid (18.71 ± 4.50 ng/mL), caffeic acid (2.42 ± 1.80 ng/mL) and M1 (3.01 ± 0.38 ng/mL) were quantified. No taxifolin was found in these plasma samples. Additionally, free and total (free and conjugated) plasma concentrations were examined after incubation the samples with ß-glucuronidase/sulfatase prior to the liquid-liquid extraction for determing the individual conjugation degree in the steady state samples of the human volunteers (n= 5). The plasma samples revealed significant phase-II-metabolism. The conjugation with sulfate and glucuronic acid of the analytes were subjected to a high interindividual variability. The mean percentage of conjugation of catechin was 56.5% and ranged between 0% to almost 100% conjugated (n= 5). The mean conjugation degree of caffeic acid was 69.4%. Since no free concentrations of ferulic acid and M1 were detected, the conjugation degree was assumed to be close to 100% [207, 214].

During the previously described study, Grimm et al. also investigated the change of matrix metalloproteinase (MMP)-9 secretion *ex vivo* by the plasma samples of human volunteers after ingestion of Pycnogenol®. A statistically significant inhibition of MMP-9 release from human monocytes and NF-κB activation was observed. Thus, sufficient *in vivo* concentrations were obviously present in plasma for inhibiting mediators of inflammation [215].

In vitro experiments showed an inhibition of the MMP-9 secretion when using higher concentrations of M1. Other experiments elucidated the antioxidant and inhibitory effects upon various MMPs, which resulted in strong inhibitory effects of M1 towards the activity of MMP-1 and MMP-2 as well. Using a concentration of 1 µg/mL, the metabolite M1 was more effective in MMP inhibition than its precursor (+)-catechin and the whole extract. After the stimulation of freshly isolated human monocytes with 10 ng/mL bacterial lipopolysaccharide (LPS) for 48 hours the total MMP-9 release was reduced about 50% by 0.5 M M1 [216]. Further anti-inflammatory assays revealed that *in vivo* concentrations of bioactive compounds in human serum samples after ingestion of Pycnogenol® were sufficient to inhibit *ex vivo* enzymatic cyclooxygenase (COX)-1 and COX-2 activities [217].

Despite of very low plasma concentrations of constituents and metabolites after oral intake of Pycnogenol® [207] diverse pharmacodynamic effects of the plasma samples were recorded *ex vivo* [215, 217]. However, these low plasma concentrations of constituents and metabolites were not sufficient to induce any effects *in vitro* [216]. Thus, it was hypothesized that some bioactive compounds are present in human plasma which cause such pharmacodynamics effects *ex vivo* and *in vivo*. The bioactive compounds inducing previously described *ex vivo* effects have yet to be further elucidated.

Red blood cells (RBC) constitute more than 99% of the volume of the total human blood cell fraction and with a life span between 100 to 120 days [218] they represent a major reservoir for some drugs. The importance of the distribution of drugs between plasma and RBC and their consideration in pharmacokinetic and pharmacodynamic evaluations has been repeatedly described [218, 219] and may have a decisive role for the *in vivo* bioactivity [220]. As an example, the intracellular residence time of methotrexate can be up to 79 days [221], whereas its plasma half-life is considerably shorter [220]. Erythrocytes also represent a deep compartment for other drugs such as metformin, which is used therapeutically as an oral antidiabetic agent for type II diabetes mellitus [222]. Polyphenols are able to bind to RBC [223]. Significantly higher concentrations of quercetin and resveratrol were found in whole blood than in plasma after oral administration of polyphenols [224].

Hence, it is also possible that bioactive compounds derived from Pycnogenol® which are present at low concentrations in plasma, e.g. M1 [207], are distributed *in vivo* into the blood cells, contributing to the effects of the maritime pine bark extract Pycnogenol®. Some constituents of Pycnogenol® e.g. taxifolin and ferulic acid and particularly the metabolite M1 were able to bind to human red blood cells [225]. *In vitro* incubation of human erythrocytes with M1 yielded to an intracellular conjugation of M1, identified as glutathione adduct via LC-MS/MS [225]. Additional *in vitro* studies previously suggested a facilitated cellular uptake of M1 into human EA.hy926 endothelial cells, human monocytes and murine RAW264.7 macrophages [226].

3.3 Effects on health

3.3.1 Multiple effects

Early *in vitro* studies have shown strong free radical–scavenging activity of the procyanidin-rich nutritional supplement Pycnogenol® [227]. Several reviews highlighted the anti-oxidative and anti-inflammatority activity of Pycnogenol® and its pharmacological properties. The extract previously revealed beneficial effects in several human trials regarding chronic diseases (e.g. asthma, attention deficit hyperactivity disorder (ADHD), diabetes, chronic venous insufficiency (CVI)) and cardiovascular and neurological disorders [227-231]. Gulati described clinical effects of Pycnogenol® regarding chronic venous insufficiency (CVI) and related venous disorders such as deep vein thrombosis (DVT) [230]. Many more areas of application of Pycnogenol® are discussed in literature. For example, as anti-diabetic agent [228] due to the effective inhibition of α-glucosidase by oligomeric procyanidins of the pine bark extract [52] or in the prevention of cancer [228]. Furthermore, Pycnogenol® has also been studied in various degenerative neurological disorders like Alzheimer's disease (AD) and migraine [228]. Because of the low acute and chronic toxicity [231] Pycnogenol® can be used in a number of further disorders such as for skin care [228] e.g. psoriasis [232], metabolic syndrom [233], menopausal transition [234], acute hemorrhoidal episodes [235] and sexual disorders [228] e.g. erectile dysfunction [236].

In 2010, the American Botanical Council published a scientific and clinical monograph of Pycnogenol® with summarized clinical trials and related recommended daily doses of the extract, which ranged between 20-160 mg/day treating retinopathy and 150-360 mg/day for handling CVI [237].

Pycnogenol® has also been applied for nutritional effects e.g. as stabilizer in food storage because of the antioxidant nature of the extract [228]. Additionally, Frontela et al. described a higher detectable polyphenol level after enriching pineapple and red fruit juices with Pycnogenol® and subsequent *in vitro* gastrointestinal digestion, suggesting the extract can be used for the enrichment of fruit juices with polyphenols [238].

Because of the multifaceted therapeutic applications of Pycnogenol [239] the extract is also used in sports nutrition to improve the performance [240, 241]. Vinciguerra et al. evaluated the effects of a supplementation with Pycnogenol® on the fitness of normal individuals using the Army Physical Fitness Test (APFT) in athletes by performing a 100-minute triathlon. Before exercising the APFT 74 volunteers were subjected to a daily administration of 100 mg of the extract for eight weeks, which resulted in an improved physical fitness compared to the group without intervention (73 volunteers). Moreover, the Pycnogenol® group (32 males), which ingested 150 mg/day of the supplement for four weeks during training achieved a more

pronounced improvement in triathlon time compared to the control group (89 min 44 s vs. 96 min 5 s) [242].
Williamson and Manach compared the bioavailability and bioefficacy of polyphenols in 93 human interventions studies. They included the clinical application of e.g. the soybean isoflavones genistein and daidzein, the flavonol quercetin, monomeric catechins (like epigallocatechin gallate, EGCG) and oligomeric catechins, respectively procyanidins. The French pine bark extract Pycnogenol® as one example for a supplementation with procyanidins already demonstrated beneficial effects in diverse human trials [84].

3.3.2 Effects on osteoarthritis

Pycnogenol® has also displayed beneficial effects in osteoarthritis (OA) patients. The disease OA is a chronic and degenerative process which affects especially main joints like hips (coxarthrose) and knees (gonarthrose) [243]. The prevalence of suffering from OA increases with age. More than 50% of adults over 65 years have at least one affected joint [244]. Symptoms include stiffness of the joint(s), inflammation, deformations and pain [245]. Therapeutic approaches include analgesics (e.g. nonsteroidal anti-inflammatory drugs, NSAIDs) and specific cyclooxygenase (COX)-2 inhibitors for fewer gastrointestinal problems [243]. The movement restrictions and suffering accompanying gonarthrose often results in elective knee joint replacements [245, 246]. The expenses related to OA are huge. As an example, the knee replacement costs in the USA were determined with more than one billion dollars per year [247].

By using the so-called Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) the severity and the course of the disease can be evaluated in patients with OA of the hip or knee [248, 249]. The WOMAC questionnaire includes aspects for pain (WOMAC-A score), stiffness (WOMAC-B score) and daily activities (WOMAC-C score) and hence allows the monitoring of the disease and how OA influences individual life style [243, 250]. The degree of OA can also be assessed radiological by classification of five different grades (none (0), doubtful (1), minimal (2), moderate (3) and severe (4)) according to Kellgren and Lawrence [251].

On a cellular level, OA is caused by an imbalance between anabolic and catabolic pathways in the affected joints [245, 252]. In synovial fluid (SF) proinflammatory cytokines have been detected, e.g. interleukin (IL)-1ß and tumor necrosis factor alpha (TNFα), which are produced as an inflammatory response by the synovial cells, respectively chondrocytes. These mediators induce particularly the release of matrix metalloproteinasen (MMPs), which leads to a degradation of the extracellular matrix [253-255]. The digestion of the extracellular matrix by those proteolytic enzymes causes an irreversible loss of cartilage and thus joint dysfunction [243]. Nitric oxide (NO) is another mediator of cartilage destruction, which has also been found in OA patients [256, 257].

Moreover, it has been reported that the cytokine leptin, which is formed by the adipocytes, may enhance the release of MMPs e.g. MMP-1, MMP-3 and MMP-13 in chondrocytes as well [258, 259]. Ku et al. suggested a correlation of the leptin concentration in the SF with the radiographic severitiy of OA [260]. The leptin concentrations in the SF of 42 OA patients were highest when suffering grade 4 severity [260], according to the Kellgren–Lawrence grading scale [251].

Human intervention studies have already shown that an oral administration of 100-150 mg/Pycnogenol® over a course of three months resulted in a significant improvement in symptoms of knee OA, such as reducing the stiffness of the affected joint(s) while also reducing the pain and thus the need for anti-inflammatory drugs [243, 250, 256]. In all three placebo-controlled clinical trials (Belcaro et al.: n= 156 [250]; Farid et al.: n= 37 [256]; Cisar et al.: n= 100 [243]) the intervention group experienced a significant reduction in the WOMAC-score after three months of oral intake of Pycnogenol®.

Henrotin et al. assessed *in vitro* and *in vivo* scientific data on nutraceuticals and their benefit on OA considering five different classes of compounds, including avocado/soybean unsaponifiables, ω-3 polyunsaturated fatty acids, collagen hydrosylates, vitamin D and selected polyphenols like genistein, curcumin, epigallocatechin-3-gallate (EGCG), resveratrol and Pycnogenol®. They concluded that Pycnogenol® showed moderate evidence of efficacy in OA compared to the nutraceuticals vitamin D and collagen hydrolysate. With respect to dietary polyphenols and OA only Pycnogenol® has been investigated in clinical trials in more detail [261].

As previously described (see Chapter 3.2), it has been shown that Pycnogenol® is able to inhibit diverse MMPs *in vitro* [216]. Additional *ex vivo* investigations showed that the release of MMP-9, which has a significant influence of the cartilage damage [252], was inhibited by plasma from volunteers, who ingested multiple doses of the Pycnogenol® [215]. Furthermore, these plasma samples also inhibited the activation of the transcription factor NF-κB [215], which initiates the synthesis of various cytokines and thus plays a major role in inflammation [243]. Pycnogenol® also inhibited the enzymes COX-1 and COX-2 *ex vivo* [217], which are responsible for generating the pain-causing prostaglandins [250].

Currently 77 clinical trials involving the use of Pycnogenol® have been published. Still, it needs to be further elucidated which compound(s) of the complexe mixture of concentrated polyphenols is (are) mainly responsible for the documented bioefficacy. Particularly, the microbial metabolite M1 of Pycnogenol® has caught great interest because it showed higher bioactivity concerning the inhibition of e.g. MMPs (see Chapter 3.2) and nitrite generation as an indication for NO production [226] compared to the whole extract or its metabolic precursor (+)-catechin.

4 Aim of the thesis: Pycnogenol® administered to patients with severe knee osteoarthritis

Prospective intervention studies have already shown that an oral intake of the standardized maritime pine bark extract Pycnogenol® (see Chapter 3.1) resulted in a significant improvement in symptoms of knee osteoarthritis (OA), such as reducing the stiffness and the pain and thus led to a decreased consumption of analgesics (see Chapter 3.3.2).

The background of this doctoral thesis was a randomized controlled clinical trial in which Pycnogenol® was administered to patients suffering from severe osteoarthritis (OA) according to the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)-score (see Chapter 3.3.2) and therefore were scheduled for an elective knee replacement surgery. The study (reference number 248/11; reviewed by the local Ethics Committee of the Medical Faculty of the University Würzburg) was carried out in cooperation with an orthopedic hospital in Würzburg, specifically the Orthopädische Klinik König-Ludwig-Haus.

The purpose of the pharmacokinetic part of this study was the additional examination of far less investigated biological matrices, respectively blood cells and synovial fluid, besides common serum samples, for gaining deeper insights into the disposition of possibly bioactive constituents and metabolites of the extract after oral administration to OA patients.

In total, 30 study participants suffering from severe gonarthrose were recruited. On the one hand, 15 individuals (Pycnogenol® -group) who ingested 200 mg Pycnogenol® per day (twice daily two tablets with each 50 mg) over three weeks before the planned surgery to reach pharmacokinetic steady-state conditions. On the other hand, there were 15 patients receiving no treatment with Pycnogenol® served as a control group.

Blood samples were collected before the initiation of the oral intake of Pycnogenol® (V1, basal value); during the intake, approximately 1-2 days before the surgery (V2); and during or shortly before the knee arthroplasty (V3), respectively about 12 hours after the last ingestion of Pycnogenol®. From the patients of the control group the same number and volume of blood samplings were obtained. On the day of the knee replacement (V3), residual knee cartilage and synovial fluid were additionally obtained. Directly after the blood samplings the serum and blood cellular fraction were separated under sterile conditions and stored at -80 °C (see Figure 7).

Due to the ubiquitous presence of polyphenols in food and beverages (see Chapter 1.2) the patients were asked to comply with a polyphenol-free diet. For this purpose, they were provided with nutritional checklists for specifying food and beverages (e.g. coffee, green tea, chocolate, nuts and various fruits and vegetables) they should avoid.

They also recorded what they have ingested within the last two days (48 hours) before the blood samplings (V1, V2 and V3).

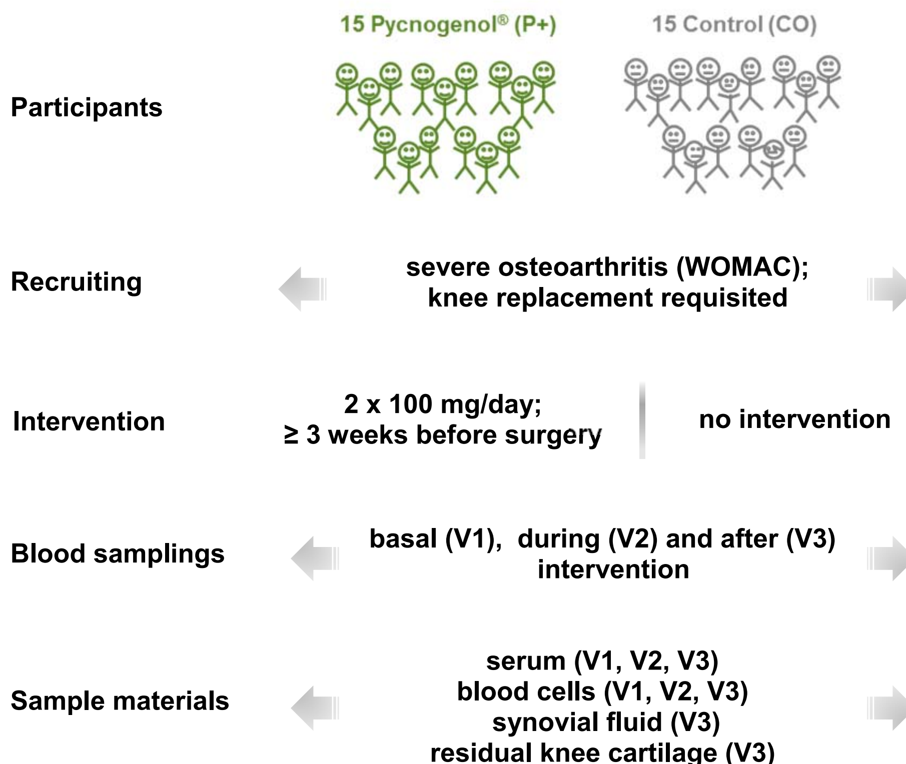


Figure 7: Overview of the randomized controlled clinical study: Pynogenol® administered to patients with severe knee osteoarthritis

The quantification of selected constituents and metabolites of Pycnogenol® was planned for the diverse biological specimen (serum, blood cells and synovial fluid) of the OA patients for all the three blood samplings (V1, V2 and V3). The expected range of the analytes in serum, blood cells and synovial fluid was to be considered very low (ng/mL concentrations in human plasma and no excisting data in the other body fluids; see Chapter 3.2).
Thus, for accurate and highly sensitive quantification of the analytes various analytical methods applying LC-ESI/MS/MS analysis needed to be developed, optimized and validated (see Chapter 2) in the course of this thesis.

B RESULTS

B Results

1 Facilitated uptake of a bioactive metabolite of maritime pine bark extract (Pycnogenol) into human erythrocytes

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PUBLICATION 1

ABSTRACT

Many plant secondary metabolites exhibit some degree of biological activity in humans. It is a common observation that individual plant-derived compounds in vivo are present in the nanomolar concentration range at which they usually fail to display measurable activity in vitro. While it is debatable that compounds detected in plasma are not the key effectors of bioactivity, an alternative hypothesis may take into consideration that measurable concentrations also reside in compartments other than plasma.

We analyzed the binding of constituents and the metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1), that had been previously detected in plasma samples of human consumers of pine bark extract Pycnogenol, to human erythrocytes. We found that caffeic acid, taxifolin, and ferulic acid passively bind to red blood cells, but only the bioactive metabolite M1 revealed pronounced accumulation. The partitioning of M1 into erythrocytes was significantly diminished at higher concentrations of M1 and in the presence of glucose, suggesting a facilitated transport of M1 via GLUT-1 transporter. This concept was further supported by structural similarities between the natural substrate α-D-glucose and the S-isomer of M1. After cellular uptake, M1 underwent further metabolism by conjugation with glutathione. We present strong indication for a transporter-mediated accumulation of a flavonoid metabolite in human erythrocytes and subsequent formation of a novel glutathione adduct. The physiologic role of the adduct remains to be elucidated

INTRODUCTION

Maritime pine bark extract is monographed in the United States Pharmacopeia (USP) as a dietary supplement [1]. A standardized pine bark extract that conforms with this monograph is derived from of Pinus pinaster, Ait., (Pycnogenol®, Horphag Research Ltd., UK). Procyanidins consisting of catechin and epicatechin moieties of varying chain lengths represent

approximately 65-75% of this extract [2,3]. Other constituents are polyphenolic monomers, phenolic or cinnamic acids and their glycosides. Pycnogenol® revealed diverse pharmacological actions in human trials, e.g. anti-inflammatory and cardiovascular effects [3,4]. So far there is still limited information on which compound(s) of the complex extract are mainly responsible for the documented bioefficacy.

One critical point with plant extracts is always the bioavailability of their constituents. Typically only low plasma concentrations are found after ingestion of dietary polyphenols or plant extracts [5]. In a pharmacokinetic study with single and multiple doses of Pycnogenol® we detected catechin, caffeic acid, ferulic acid, and taxifolin in the nanomolar range in the plasma of human volunteers [6]. We also found a maritime pine bark metabolite, δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1), in the plasma samples. This metabolite is no constituent of the extract, but is generated in vivo from the procyanidins' catechin units through multiple step reactions. This metabolite M1 has been also found in urine samples [7-10].

We previously investigated the bioactivity of M1 and discovered pronounced antioxidant activity as well as inhibitory effects upon various matrix metalloproteinases [11] which was consistent with the reported anti-inflammatory effects of the extract. However, again the plasma concentrations of M1 were only in the nanomolar range [6] which was too low to induce any effects in vitro. Though it is possible that M1 is not the main mediator of maritime pine bark extract's bioefficacy it is also conceivable that plasma is not the only compartment where M1 is present in vivo. Previously, significantly higher recoveries of quercetin and resveratrol were reported from whole blood compared to plasma which suggests that the polyphenols are also distributed into the cellular blood fraction [12]. Recently we observed pronounced uptake of M1 into endothelial cells and monocytes/macrophages in vitro. The uptake was decreased by phloretin, suggesting a facilitated transport mechanism [13].
Though the partitioning of compounds into red blood cells has received less attention than the plasma protein binding, erythrocytes constitute a significant compartment for distribution [14,15]. We recently analyzed the plasma protein binding of various maritime pine bark polyphenols and observed pronounced differences in the binding tendency [16]. While catechin and taxifolin displayed protein binding close to 100%, low binding around 30% was seen for M1 and its structurally related metabolite M2 (δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone). The purpose of the present investigation was to analyze the binding of selected Pycnogenol® constituents and the metabolite M1 to human erythrocytes to gain further insight into the disposition of these compounds.

MATERIALS AND METHODS

Chemicals and reagents

Ferulic acid, (±)-taxifolin, caffeic acid, p-coumaric acid, glutathione, glutathione-S-transferase (EC 2.5.1.18), phloretin, and 2,2'-azobis(2-amino propane (AAPH), cytochalasin B from

Drechslera dematioidea, D (+)-glucose, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), were all obtained from Sigma-Aldrich (Taufkirchen, Germany). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) was purchased from Gerbu (Wieblingen, Germany). The metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) was synthesized by M. Rappold as part of his diploma thesis [17]. Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany), acetonitrile (HPLC grade) was from Fisher Scientific (Schwerte, Germany). Ultrapure Milli-Q water was used for all aqueous solutions. All other chemicals were purchased from Sigma-Aldrich.

Buffers and human plasma / erythrocytes

The phosphate buffered saline (PBS, pH 7.4) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 and 1.5 mM KH2PO4. In case of incubation with erythrocytes the PBS buffer was supplemented with 0.1% (m/V) glucose. The buffer used in the AAPH assay (pH 7.4) consisted of 150 mM NaCl, 8.1 mM Na2HPO4 and 1.9 mM NaH2PO4 and 0.05% (m/V) glucose. Human plasma and packed red blood cells were obtained from the blood banks of the University Hospital of Würzburg and of the Bayerisches Rotes Kreuz, München, Germany.

Distribution of a polyphenol mixture between human plasma and erythrocytes

Packed red blood cells were washed twice with a threefold volume of cold PBS buffer (8 °C) and centrifuged for 5 min at 952 g (10 °C). Cells were weighted and assuming a density of 1.114 g/mL [18] 1.67 g were mixed with 2.0 mL plasma to obtain a hematocrit value of 0.43. The plasma contained a mixture of 1.3 µM caffeic acid, 80 µM ferulic acid, 6 µM taxifolin and 6 µM metabolite M1. The chosen concentrations were based on analytical considerations and previously also used for determination of plasma protein binding of these compounds [16]. In parallel a control was prepared containing the polyphenols in 3.5 mL plasma without erythrocytes. The tubes were incubated at 37 °C and samples of 250 µL erythrocytes/plasma or plasma, respectively, were drawn and centrifuged at 952 g for 5 min (10 °C). 100 µL of the supernatant was mixed with 10 µL of the internal standard p-cumaric acid, 40 µL 0.5 M hydrochloric acid and 130 µL methanol. After centrifugation at 14,000 g for 15 min (4 °C) 20 µL were directly injected into the HPLC. In case of inhibition experiments the erythrocytes were pre-incubated with 600 µM phloretin (445 mL of a stock solution of 20 mg phloretin in 10 mL PBS buffer containing 0.01% DMSO) for 15 min and the samples were subsequently treated as described above. The erythrocyte / plasma partitioning ratio of the compounds was determined based on the peak area ratios to the internal standard as described by Yu et al. [19]. To ensure the cell vitality the percentage of haemolysed erythrocytes was determined according to Salauze [20] by photometric measurement of haemoglobin in plasma at 450 nm. Plasma was used as blank and samples of the erythocytes / plasma incubation were compared to completely haemolysed erythrocytes obtained after one freeze-thaw cycle (-80 °C). The% haemolysis was calculated from the absorption of the cell supernatant in relation to

the absorption of the totally haemolysed sample. In all experiments the percentage of haemolysed erythrocytes was below 3% over the whole experimental period.

Uptake of M1 into human erythrocytes

Packed red blood cells were incubated with a threefold volume of PBS buffer with 100 mM D-glucose for 30 min at 37 °C and centrifuged for 5 min at 2,000 g temperated to 4 °C (Mikrofuge 22 R, Beckmann CoulterTM, Krefeld, Germany). Thereafter these cell pellets were washed twice with the threefold volume of cold PBS buffer (4 °C) containing 100 mM D-glucose and centrifuged for 5 min at 2,000 g (4 °C). 43 µL of these packed glucose-saturated cells were mixed with PBS buffer to obtain a hematocrit of 0.043. The cells were subsequently incubated with various concentrations of M1 (0.3 – 10 µM) for 1 min by rocking (Mini Rocker MR-1, Hartenstein, Würzburg, Germany) in closed reaction tubes (Eppendorf, Hamburg, Germany) at room temperature. In parallel control experiments were carried out accordingly for each variable without cells to monitor the stability of M1 during the experimental procedures. Similar to the procedure described by Leitch and Carruthers [21] the reaction was interrupted by adding a cold stop solution (4 °C) containing 150 mM KCl, 5 mM MgCl2, 5 mM EGTA, 5 mM HEPES, 20 µM cytochalasin B and 200 µM phloretin in PBS buffer (pH 7.4), followed by a centrifugation of the cell preparations and matched controls for 5 min at 2,000 g (4 °C). The supernatants were harvested and immediately analyzed by HPLC.

In case of competition experiments the erythrocytes were glucose-deprived. Cells were washed twice with the threefold volume of cold PBS buffer (4 °C) without D-glucose and centrifuged for 5 min at 2,000 g (4 °C). After incubating the cells with a threefold volume of PBS buffer (without D-glucose) for 30 min at 37 °C and centrifugation for 5 min at 2,000 g (4 °C), they were washed twice with the threefold amount of cold PBS buffer (4 °C; without D-glucose) again and centrifuged for 5 min at 2,000 g (4 °C). Subsequently, samples and controls were treated as described above, but this time in addition with 100 mM D-glucose to the various concentrations of M1 (0.3 – 10 µM). The erythrocyte / buffer partitioning ratio, or rather distribution coefficient, of M1 was determined based on the peak area ratios to the internal standard as described by Yu et al. [19].

In order to ensure equivalent cell counts in the experiments with glucose-saturated and glucose-deprived cells (competition experiments) the UV/VIS-absorption of free hemoglobin was measured in the supernatant after cell lysis. Therefore, the incubation mixtures with a hematocrit of 0.043 were prepared exactly as described above. In case of experiments with glucose-saturated erythrocytes (without D-glucose in the subsequent incubation) 43 µL of these cells were mixed with 957 µL PBS buffer. Simultaneously, 43 µL of glucose-deprived cells prepared for the competition experiments (with D-glucose in the subsequent incubation) were mixed with 100 mM D-glucose in PBS buffer to yield 1.0 mL. Then the samples were vortexed and snap frozen in liquid nitrogen for 2 min. After 15 minutes of thawing at 37 °C the

cells were centrifuged for 5 min at 2,000 g (4 °C). A defined volume of each supernatant was diluted and transferred into a 96-well plate (BD falcon™ clear 96-well microtest™ plate, Franklin Lakes, NJ, USA) for subsequent photometric measurement of hemoglobin. The absorption was measured at 450 nm (Multiskan Ascent® microplate-reader, Thermo Fisher Scientific, Waltham, MA, USA). We prepared and measured each six independent samples of both incubation conditions.

High performance liquid chromatography (HPLC)

High performance liquid chromatography was performed using a Waters HPLC (Milford, MA, USA) with a 1525 binary pump, a 717plus autosampler, a model 2487 UV/VIS dual wavelength absorbance detector set at the detection wavelength of 280 nm. Data collection and integration were accomplished using Breeze™ software version 3.30.

Method 1: The samples of the experiments elucidation the distribution of a polyphenol mixture between plasma and erythrocytes were analyzed by HPLC with a combination of electrochemical and UV detection. Analysis was performed on a Zorbax SB C8 column (150 × 4.6 mm I.D., 5 µm particle size, Agilent Technologies, Palo Alto, CA, USA). Caffeic acid, M1 and (±)-taxifolin were analyzed by electrochemical detection (CLC 100; Chromsystems, Munich, Germany) using oxidation voltage of 0.5 V. Ferulic acid was analyzed by UV detection (280 nm); this detector was connected to the control system by a satellite interface (Waters). The flow rate was 1 mL/min, the injection volume 20 µL. Isocratic elution was performed using 88% aqueous phase (containing 0.6 mM 1-octanesulfonic acid sodium salt, 0.27 mM ethylenediaminetetraacetic acid disodium salt, 0.04 M triethylamine; pH 2.95 adjusted with phosphoric acid) and 12% acetonitrile. The method was validated according ICH guidelines. The method fulfilled the quality criteria for linearity, selectivity and intra- and inter-day precision.

Method 2: The samples of the experiments elucidation the uptake of M1 into human erythrocytes were analyzed by HPLC with UV detection similar to the method described previously [13]. Therefore, samples were mixed with 0.6 µM p-coumaric acid as internal standard and 50 µL of 50% solution of trichloroacetic acid, vortexed for 10 s and centrifuged for 15 min at 18,000 g (4 °C). Afterwards, 200 µL of the supernatant was immediately subjected to HPLC analysis. Separations were carried out on a SunFire® C18 column (4.6 x 150 mm; 5 µm particle size) from Waters. The mobile phase consisted of 0.2% (v/v) acetic acid and acetonitrile. Isocratic elution of M1 and internal standard was performed using 85% aqueous phase and 15% acetonitrile at a flow rate of 1.5 mL/min followed by a short flush step for eluting remaining matrix components. M1 and internal standard absorption was monitored at 280 nm. Retention time for M1 was tR= 7.10 ± 0.08 min and for internal standard p-coumaric acid tR= 9.58 ± 0.09 min. Linearity was proven between 0.15 – 10 µM M1 in PBS buffer (r2= 0.9999; slope = 0.2708 ± 0.021; y-intercept =0.0189 ± 0.016) analyzing five concentration levels. The lower limit of quantification for M1 in PBS buffer was 0.15 µM M1 with VK (coefficients of variation) values for accuracy of 99.4% and precision of 24.3%.

Interday-accuracy and –precision VK-values for M1 were 100.2% and 10.8% and intraday-accuracy and –precision VK-values comprised 96.0% and 7.9%.

Computer-based structural comparison between glucose and M1

Calculations were made with the program SYBYL-X® (Tripos, version 1.0, August 2009). An energy field minimization was performed for the structures of glucose and M1 using the Powell method. Electrical charges and the resulting energy were calculated with MMFF94 taking various partial energies into account such as bond stretching, angle bending, torsional and Van der Waals energy. The energy-minimized molecules were used for alignments.

Screening of erythrocyte incubation mixtures for putative M1 metabolites

About 5 mL of packed red blood cells were washed twice with a threefold volume of cold PBS buffer (8 °C) centrifuged for 5 min at 952 g (10 °C). Cells were suspended in PBS buffer to yield a cell fraction of 40%. The metabolite M1 was added to yield a concentration of 15 mM and cells were incubated for one hour at 37 °C. In parallel a control was prepared containing M1 PBS buffer without erythrocytes. Cells were subsequently processed as described by Sana et al. [22]. Therefore, incubation vials were centrifuged at 1,000 g (4 °C) and erythrocytes were lysed by addition of 150 µL cold Millipore® water. Lysates were cooled on dry ice to -25 °C and 600 µL cold methanol was added. After vortexing and addition of 450 µL chloroform, samples were incubated for 30 min under frequent mixing. Another 150 µL cold Millipore® water was added and samples were frozen at -20 °C for at least 8 hours. Both the upper aqueous and lower organic phase were collected and evaporated to dryness. The residue was reconstituted in 50 µL mobile phase of which 5 µL were subjected to HPLC-MS/MS analysis.

Preparation of a M1-glutathione conjugate

Glutathione (10 µM) and the metabolite M1 (12 µM) were mixed with 1 U glutathione-S-transferase in 1 mL PBS buffer. The mixture was incubated for 30 min at 25 °C. The MS/MS spectrum of the reaction product was compared with the putative glutathione adduct found in erythrocytes.

HPLC-MS/MS conditions

High-performance liquid chromatography-MS/MS analysis was performed on an Agilent LC-MS 6460 triple-quadrupole mass spectrometer with an electrospray interface (Agilent, Böblingen, Germany). Chromatographic separations were carried out using an SunFire® C18 column (4.6 x 300 mm, 2.5 µm particle size with a guard column; Waters) at a flow rate of 0.5 mL/min using 0.1% formic acid in Millipore® water (solvent A) and acetonitrile / methanol 1:1 (solvent B) as mobile phase. A linear step gradient elution was performed: 95% to 10% solvent A in 40 min, followed by 100% B for 10 min. During screening, the electrospray interface source was operated in both the positive and negative ionization mode for later

measurements of metabolites only the positive ionization mode (ESI+) was used at a capillary voltage of 3.50 kV and a desolvation temperature of 300 °C. Detection was performed using multiple reaction monitoring (MRM) mode. The scan range used was 100-1000 m/z with a step size of 0.2 Da. Nitrogen was used as the desolvation and sheath gas with flow rates of 11 L/min, respectively. Nitrogen was used as the collision gas at a pressure of 45 psi. Data were analyzed using Agilent MassHunter data aquisition version B 02.01.

Analysis of protection against oxidative damage using the AAPH assay

About 3 mL of the packed red cells were washed twice with 10 mL of the AAPH buffer and subsequently mixed with the AAPH buffer to result in a 10% (V/V) suspension. 12.0 mL buffer containing 1 µM of the metabolite M1 were mixed with 1.5 mL of the erythrocyte cell suspension and incubated under gentle shaking for 10 min at 37 °C. 1.5 mL of AAPH solution (400 mM) was added either immediately or after pre-incubation of the cells with M1 for 60 min at 37 °C. Subsequently samples of 800 µL were drawn and centrifuged for 2 min at 10,000 g at 4 °C. The absorption of the supernatant was measured at 524 nm (uv-mini 1240, Shimadzu, Duisburg, Germany). For comparison a completely haemolysed sample was used. Therefore, 10 µL of the packed red cells were mixed with 990 µL of Millipore® water and subjected to one freeze-thaw cycle. The% haemolysis was calculated from the absorption of the cell supernatant in relation to the absorption of the totally haemolysed sample. The time lag for a 50% haemolysis occurred was determined.

Statistical and data analysis

Data sets were subjected to one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test using GraphPad Prism® 4 (GraphPad Software Inc., Dan Diego, CA). Results were considered statistically significant at $p \leq 0.05$. Data are shown as mean with standard deviation (SD) or as mean and mean deviation of the mean (MDM).

RESULTS

Distribution of polyphenols between human plasma and erythrocytes

The erythrocyte / plasma partitioning ratio of a mixture of caffeic acid, taxifolin, ferulic acid and the Pycnogenol® metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) was determined based on a previously described method [19]. While all compounds displayed some binding to the erythrocytes after 60 min this effect was no longer pronounced after 120 min for caffeic acid, taxifolin, and ferulic acid (Figure 1). In contrast, the binding of M1 to red blood cells increased further to result in an erythrocyte / plasma partition ratio of 32.83 ± 4.65 after 120 min and remained at 37.36 ± 10.13 until 350 min.

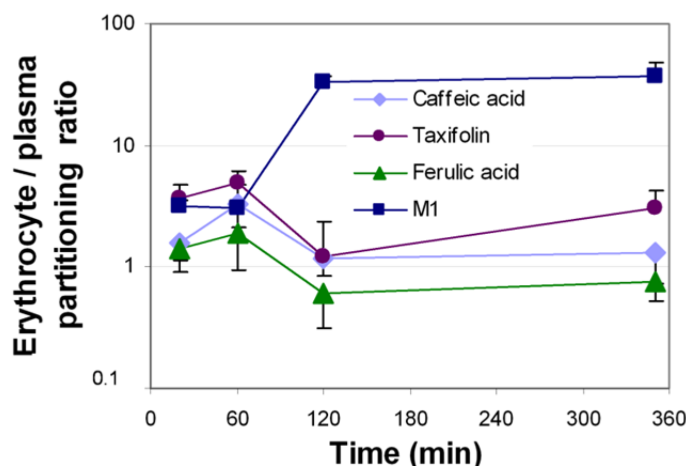

Figure 1. Erythrocyte / plasma partitioning ratios of polyphenols. *1.3 µM caffeic acid, 6 µM taxifolin, 80 µM ferulic acid and 6 µM of the Pycnogenol metabolite M1 were concomitantly incubated with a human blood mixture (hematocrit 0.43) at 37 °C. Each data point represents the mean and standard deviation of five replicates.*

To elucidate whether this high partition coefficient of M1 was not only related to an adsorption to erythrocytes' outer cell membrane and diffusion processes, but to an entry and accumulation in the cells we tested the influence of various inhibitors of transporters that facilitate the uptake of small molecules into red blood cells. While no significant effects were seen with modulators of the ABCB1 (P-glycoprotein) and amino acid transporters (data not shown) a statistically significant decrease ($p < 0.05$, one-way ANOVA with post-hoc Bonferroni test) of M1 uptake into erythrocytes was observed after 10 min in the presence of the inhibitor phloretin that e.g. inhibits the glucose transporters (GLUT-1) (Figure 2).

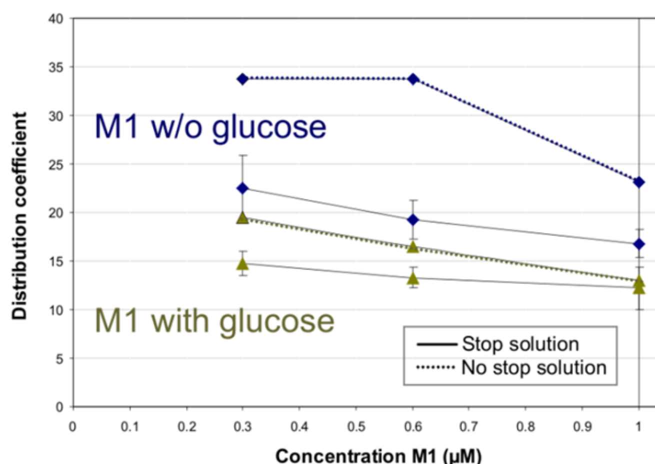

Figure 2. Influence of the stop solution on the uptake of M1 into human erythrocytes. *In an initial experiment the distribution of different concentrations of the metabolite M1 was analyzed in the absence and presence of glucose (100 mM) with and without addition of a stop solution containing phloretin (200 µM) and cytochalasin B (20 µM). Data points of the experiments with stop solution (solid lines) represent the mean and mean deviation of the mean of three replicates, the data points without stop solution (dashed lines) were single experiments.*

In the presence of phloretin the erythrocyte / plasma partitioning ratio of M1 displayed a mean value of 1 while the partition coefficient increased up to 2.47 ± 1.28 after 10 min in the absence of phloretin.

Uptake of M1 into human erythrocytes

To elucidate whether the high partition coefficient of M1 was solely due to an adsorption to erythrocytes' outer cell membrane, diffusion processes, or the presence of other polyphenolic compounds we determined the distribution of M1 in separate experimental series. In initial experiments we analyzed the uptake of increasing concentrations of M1 (0.3 to 1 µM) into red blood cells. When we added inhibitors of glucose transporters (200 µM phloretin and 20 µM cytochalasin B) to stop a potential facilitated uptake we observed clearly reduced distribution coefficients (Figure 2). Likewise, the concomitant addition of 100 mM glucose along with M1 resulted in reduced uptake of M1. In this case, the addition of the stop solution at the end of the incubation period again reduced the distribution coefficient.

Further experiments were performed in which the stop solution containing phloretin and cytochalasin B was always added to terminate any transporter-facilitated uptake. Erythrocytes of two different individuals (blood groups A and AB, respectively) were used for the experiments. The results differed only slightly, so that the data were pooled (Figure 3).

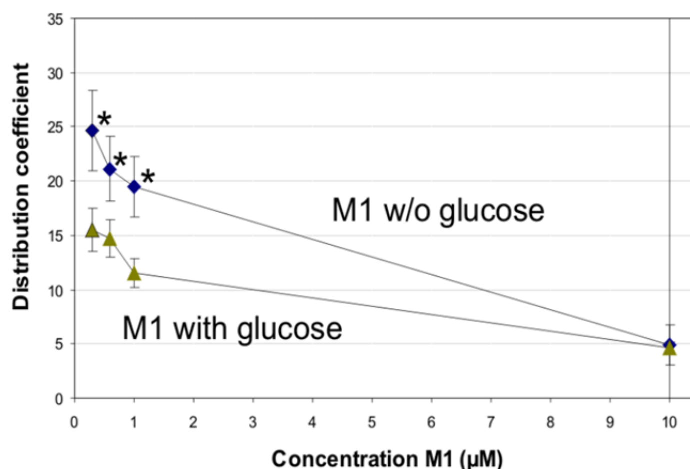

Figure 3. Distribution of M1 into human erythocytes. *Increasing concentrations of the metabolite M1 were incubated in the absence and presence of glucose (100 mM) with human erythrocytes (hematocrit 0.043) at 4 °C. The reaction was stopped after one minute with phloretin (200 µM) and cytochalasin B (20 µM). For 0.3 to 1 µM M1 the uptake into erythrocytes was statistically significant higher in absence of glucose compared to the respective uptake (0.3 to 1 µM M1) in the presence of glucose ($p < 0.05$) and also compared to the uptake of 10 µM M1 ($p < 0.001$; one-way ANOVA with Bonferroni post-hoc test). Each data point represents the mean and mean deviation of the mean of six replicates.*

In the absence of glucose, increasing concentrations of M1 resulted in decreasing distribution coefficients, from 24.68 ± 3.68 (0.3 µM M1) to 4.87 ± 1.97 (10 µM M1). Thereby, the distribution coefficients determined for 0.3, 0.6 and 1 µM M1 were statistically significant higher compared to that recorded for 10 µM M1 ($p< 0.001$; one-way ANOVA with Bonferroni post-hoc test). When 100 mM glucose was added to the red blood cells together with M1, the distribution coefficients were clearly lower, ranging from 15.48 ± 1.96 (0.3 µM M1) to 4.66 ± 0.57 (10 µM M1). For the concentrations of 0.3, 0.6 and 1 µM M1 the uptake into erythrocytes was statistically significant higher in absence of glucose compared to the respective M1 concentrations added simultaneously with glucose ($p< 0.05$; one-way ANOVA with Bonferroni

post-hoc test). At a concentration of 10 µM the distribution coefficient of M1 was not different in the absence or presence of glucose.

In order to exclude the possibility that the cells' exposure with high glucose concentrations altered the cell volume and thus the cell number that constituted the hematocrit, we prepared each six independent samples of both incubation conditions, lysed the erythrocytes and measured the absorption of the free hemoglobin in the supernatant (λ= 450 nm). We read absorptions of 0.8463 ± 0.036 (n= 6; mean and SD) and 0.7983 ± 0.083 (n= 6; mean and SD) which were not statistically significant different ($p > 0.05$, two-sided Student's t-test).

Structural comparison between M1 and glucose

Structural similarities between M1 and the natural GLUT-1 substrate α-D-glucose were analyzed using computer-based energy calculations. Molecule alignments showed good superimposing substructures between glucose and the S-isomer of M1 (Figure 4).

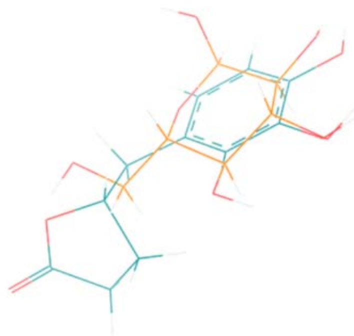

Figure 4. Structural alignments of M1 and glucose. *The S-isomer of the metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone; blue) and glucose (yellow). The calculations were performed with SYBYL-X® (Tripos, version 1.0).*

The hydroxyl groups of the benzene ring of M1 aligned well with the hydroxyl function of the pyranose ring and the hydroxymethyl moiety of glucose aligned close to the lactone structure of M1. Thus, functional groups such as OH-groups that might be critical for the transport through the GLUT uptake site can adopt similar positions in the three-dimensional space. Both molecules have similar space requirements, there are no obvious steric or volume hindrances that would suggest that M1 cannot pass through the GLUT transporter.

Screening of erythrocyte incubation mixtures for putative M1 metabolites

To screen for potential metabolites of M1 generated in human erythrocytes the compound was incubated with red blood cells and subjected to an extraction procedure that allowed the determination of both hydrophilic and lipophilic metabolites [22]. The extracts were scanned by LC-MS/MS in both the positive and negative ionisation mode over a range of 100-1000 m/z with a step size of 0.2 Da. For comparison an erythrocyte extract that was not exposed to M1 was used. During this screening procedure a new signal with $[M+H]^+$ m/z of 514 was detected

(Figure 5, A). This molecular mass was consisted with a glutathione adduct of M1. To obtain a reference compound M1 and glutathione were incubated in the presence of glutathione-S-transferase and the resulting MS/MS spectrum of the reaction product was analyzed (Figure 5, B). Besides the signal with [M+H]+ m/z of 514 fragments described to be characteristic for glutathione such as pyrroglutamic acid [MH+-129], cysteine [MH+-103] and glycine [MH+-76] [23,24] were detectable.

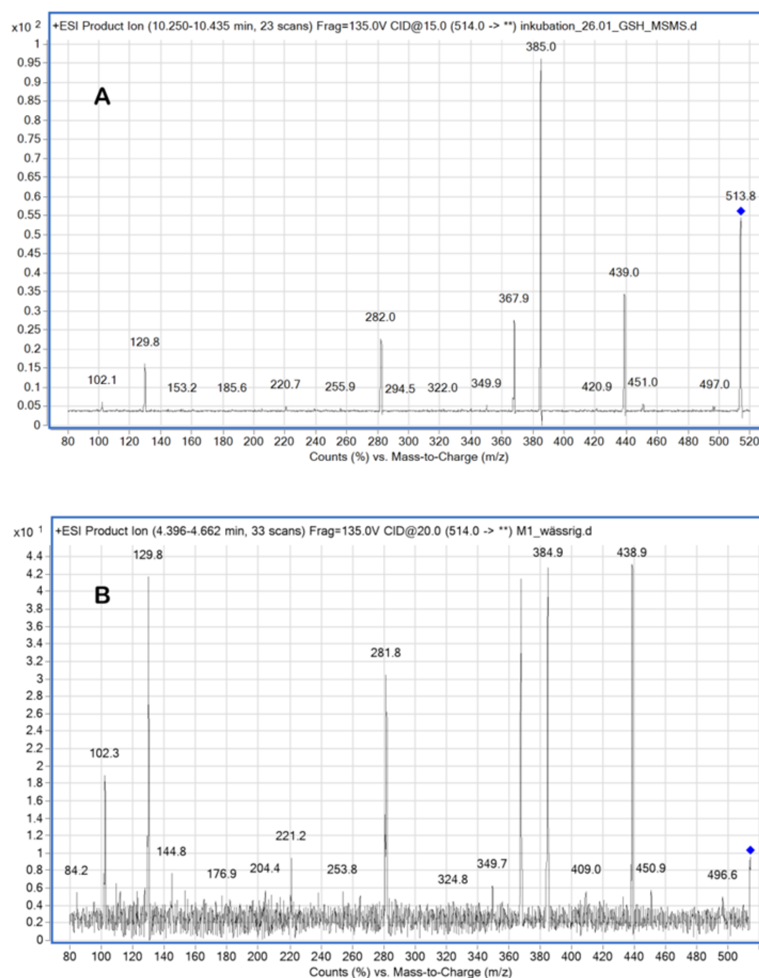

Figure 5. MS/MS spectra of the M1-glutathione adduct. A: *MS/MS spectrum of the putative M1-glutathione adduct with [M+H]+ m/z of 514 found in the erythrocyte lysate after incubation with the metabolite M1.* ***B****: MS/MS spectrum of the M1-glutathion adduct with [M+H]+ m/z of 514 obtained after incubation of the metabolite M1 with glutathione and glutathione-S-transferase. Characteristic fragments for glutathione are pyrroglutamic acid [MH+-129], cysteine [MH+-103] and glycine [MH+-76] are present.*

Analysis of protection against oxidative damage using the AAPH assay

To elucidate whether the red blood cell bound M1 or its glutathione adduct conferred a different degree of the erythrocytes' protection against oxidative damage an AAPH assay was performed. Therefore, erythrocytes M1 was either directly added to the incubation mixture or pre-incubated with the red blood cells for 60 min to allow for M1 uptake and metabolism. Subsequently the delay of 50% haemolysis was determined with reference to an incubation mixture without addition of M1 (Figure 6).

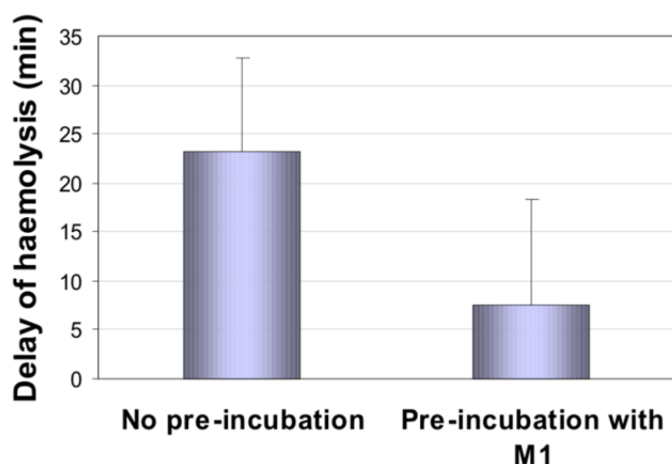

Figure 6. Protection of erythrocytes against oxidative haemolysis in the presence of M1. *Haemolysis of a 1% human erythrocytes suspension in the presence of the metabolite M1 (1 µM) was determined in an AAPH-assay. Erythrocytes were either co-incubated with M1 (left column) or pre-incubated with M1 for 60 min (right column), and delay of haemolysis was determined with reference to an incubation mixture without addition of M1. Columns represent the mean and standard deviation of three replicates.*

The more pronounced delay of induced haemolysis was seen when M1 was freshly added to the incubation mixture (Δt of 23.1 ± 9.6 min) compared the pre-incubation conditions (Δt of 7.47 ± 10.8 min).

DISCUSSION

In the present investigation we analyzed the distribution of polyphenols into human red blood cells and found a strong indication for a facilitated uptake and accumulation of the Pycnogenol metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) in erythrocytes. The partitioning of M1 into erythrocytes was significantly diminished at higher concentrations of M1, in the presence of glucose and upon the addition of a transporter-inhibiting stop solution containing phloretin and cytochalasin B. This is suggestive of a facilitated uptake of M1 into red blood cells, possibly via GLUT-1. This notion was further supported by structural similarities between the natural GLUT-1 substrate α-D-glucose and the S-isomer of M1. Erythrocytes metabolize M1 to form a novel glutathione adduct which role needs to be further investigated.

Many plant extracts used as phytotherapeutics or dietary supplements exhibit bioactivity [4,25] while plasma concentrations of individual compounds are typically in the nanomolar range [5,6]. However, these low concentrations are usually not sufficient to exert any measurable activity in in vitro cell culture assays [11,26]. It is possible that either the compounds detected in plasma are not the key effectors of bioactivity or that measurable concentrations also reside in compartments other than the plasma. It has been shown that the recoveries of resveratrol and quercetin were significantly higher from whole blood compared to plasma [12]. We recently found a pronounced binding of M1 to endothelial cells and monocytes/macrophages which was decreased in the presence of phloretin, suggesting a facilitated uptake [13].

Red blood cells represent more than 99% of the total cellular space of human blood and can thus constitute a significant compartment for distribution. Various drugs and endogenous compounds bind to erythrocytes [15]. Red blood cells were shown to bind polyphenols, and gallic acid, curcumin and resveratrol were most extensively bound [27]. Erythrocyte / plasma partitioning ratios higher than 0.25 indicate association of the respective compound with red blood cells, which could be either an uptake into the cells or binding to the surface membranes [15]. In our experiments with a polyphenol mixture all compounds revealed higher red blood cell / plasma partitioning ratios than 1.0 up to 60 min. Afterwards the partition coefficients of caffeic acid, taxifolin and ferulic acid decreased. In contrast, the erythrocyte / plasma partitioning ratio of M1 increased further to over 30 remained at that high level up to 350 min. This is suggestive of an accumulation of M1 within or on the surface of red blood cells.

It has been discussed that the compound’s lipophilicity is a major determinant for its distribution in the body [15]. Indeed, in an analysis of whole blood compared to analysis of plasma it appeared that the more lipophilic resveratrol was bound to a higher extent to blood cells compared to quercetin [12]. However, in the present study we did not observe any correlation between the compounds’ lipophilicity and the binding to erythrocytes, just as we previously did not find a correlation of the polyphenols’ lipophilicity or topological polar surface area with plasma protein binding or nonspecific binding to material surfaces [16]. Especially the high binding of M1 to erythrocytes was striking since its plasma protein binding is significantly lower compared to caffeic acid, taxifolin and ferulic acid. We suspected that the accumulation of M1 in erythrocytes was not solely driven by diffusion processes.

When we determined the binding of M1 alone we found high uptake into human red blood cells already after one minute and a statistically significant decrease of the distribution coefficient with increasing concentrations. The simultaneous addition of M1 and glucose to erythrocytes significantly reduced the uptake of M1 at lower concentrations (0.3 – 1 µM), but no further decrease was seen at the highest tested concentration of 10 µM M1. These results are consistent with a transporter-facilitated uptake and a substrate inhibition at higher M1 concentrations. Since erythrocyte glucose transport is facilitated by GLUT-1 transporter which is highly expressed in these cells, accounting for 10% of the total protein mass [28,29], it appears most likely that M1 is taken up via this transport system as well. Another indication for this notion is that the addition of a stop solution containing phloretin and cytochalasin B at the end of the incubation period clearly reduced the distribution coefficient of M1. Both phloretin and cytochalasin B are inhibitors of GLUT-1 transporters [30] although they are not highly selective. Phloretin for example interacts with various transporters such the monocarboxylate transporter [31], sodium glucose co-transporter SGLT-1 [32], volume-sensitive chloride channels [33], aquaporin water channels [34] or the red blood cell urea transporter [35]. Though phloretin also binds to other GLUT isoforms [36] it potently inhibits the GLUT-1-type glucose transporter [37].

Besides the facilitating the uptake of glucose into red blood cells GLUT-1 also transports other molecules such as galactose, mannose, L-dehydroascorbic acid (DHA) and tyrosine [38-40]. Interestingly, compounds such as DHA can be taken up into human erythrocytes although they are present at a significantly lower concentration in plasma compared to glucose. It has been suggested that the GLUT-1 uptake profile might be modulated by GLUT binding partners such as stomatin [41,42]. Association of GLUT-1 with stomatin was shown to decrease glucose uptake and enhance DHA uptake [41-43]. While we did not investigate any mechanistic background we also observed that M1 was taken up by human erythrocytes in the presence of an excess concentration of glucose.
Recently docking studies have shown that besides α-D-glucose also quercetin might slide through the GLUT-1 transporter [44], thus suggesting that this transporter accepts structurally variable molecules. Structural comparisons between α-D-glucose and the S-isomer of M1 revealed good alignment which further supports the notion that a facilitated uptake of M1 into erythrocytes might be possible since there are no obvious structural restrictions that make it unlikely that M1 can pass through the GLUT transporter. So far it is not clear yet which M1 isomer predominantly occurs in vivo. Though a preferred excretion of one isomer has been described [9,10] the designation as "–"isomer does not allow to deduce whether this is the R- or S-isomer according to CIP nomenclature.

The significance of partitioning of drugs into red blood cells has been detailed earlier [14,15]. The distribution into erythrocytes contributes to the storage, transport and metabolism of molecules and may affect their activity [45]. The elimination half-life of compounds from different blood constituents might vary, the discharge from erythrocytes is often faster than the loss from plasma proteins so that red blood cells constitute a transport system with high capacity and low affinity compared to plasma proteins [14]. However, it is also known that the half-life of a compound can be longer in erythrocytes compared to the plasma half-life, e.g. for methotrexate [45]. Due to an enhanced uptake of M1 into red blood cells the total presence of this compound in vivo might be overall higher than previously deduced from its plasma concentrations [6]. It can be speculated that an enhanced uptake of M1 will also seen in other tissues that express GLUT-1, such as the blood-brain barrier [46]. Furthermore it is possible that the transport in or on red blood cells facilitates an efficient exchange of the compound between the erythrocyte and the capillary endothelium [14].

After partitioning into red blood cells compounds might be subjected to intracellular metabolism. This has been described for many drugs and also for endogenous molecules [15,45]. Thus, after observing an accumulation of M1 in human erythrocytes we screened the cell lysates for potential metabolites and identified a M1 glutathione conjugate. Red blood cells contain 200-400 µg glutathione per mL blood [47] and possess a glutathione-S-transferase [48]. Formation of glutathione adducts has been described as part of detoxification of xenobiotics [49]. Recently it has been described that glutathione adducts with flavonoids, e.g.

quercetin, are formed after scavenging of free radicals and formation of electrophilic quinones [50,51]. M1 also displays structural features that allow oxidation under formation of an electrophilic benzoquinone that would be preferentially attacked at C4 by the nucleophilic thiol moiety of glutathione. However, this is not supported by the MS/MS spectrum of the M1-glutathione adduct with [M+H]+ m/z of 514 which is not consistent with formation of a quinone. Glutathione conjugation is a reversible process for certain compounds, e.g. for quercetin [50,52,53]. However, we did not investigate whether the M1 adduct formation is a reversible process and the precise role of the glutathione conjugate still needs to be clarified.
Quercetin and other polyphenols were reported to inhibit oxidative haemolysis of red blood cells [27,54]. We previously demonstrated in various assays that the Pycnogenol metabolite M1 is a potent radical scavenger [11]. We now analyzed whether a one hour pre-incubation and thus accumulation and conjugate formation of M1 in erythrocytes changed the resistibility of the cells against oxidative haemolysis. The protection against haemolysis was less pronounced after pre-incubation compared to direct addition of M1 to the erythrocyte incubation mixture. It can be concluded that M1 confers protection against oxidative stress primarily if present outside the cell. This is consistent with the results of Koren et al. [27] who found that the polyphenols bound the erythrocytes' surface form antioxidant depots and protect against oxidative stress.

Our study has a number of limitations. The initial experiments were done with mixtures of all polyphenols and it is possible that the partitioning behaviour of individual compounds influenced the partitioning of others, e.g. by inhibiting a relevant transporter system. However, we think that the significant decrease of M1 uptake into erythrocytes at higher concentrations of this metabolite as well as in the presence of glucose support our notion of an enhanced uptake of M1 into red blood cells. The intracellular presence of M1 was also confirmed by the detection of a glutathione conjugate. We did not elucidate the extent of glutathione adduct formation compared to M1 uptake into red blood cells or whether an enhanced outward transport of the glutathione conjugate or a reverse of the conjugation reaction occurred. Thus, we do not know whether the presence of M1 in erythrocytes is altered due to its metabolism. Finally, it is possible that M1 is taken up into erythrocytes by a transporter other than GLUT-1.

However, the high abundance of GLUT-1 transporters in red blood cells [28,29] and the structural similarity of M1 and the natural GLUT-1 substrate glucose suggest an involvement of GLUT-1. Yet it cannot be excluded that additional diffusion processes play a role as it was suggested by Sugano et al. that passive and carrier-mediated processes can coexist [55]. Finally, we did not investigate whether the glucose flux in erythrocytes was influenced by M1 or the precise type of interaction with the GLUT-1 transporter. Kinetic and mechanistic details of the erythrocyte glucose transport are still ascertained [21,56]. While GLUT-1 has binding sites for polyphenols such as quercetin or phloretin the type of interaction with the transporter appears to be complex as compounds can behave as competitive or noncompetitive inhibitors

regarding glucose uptake or exit [30]. Though we do not provide further details on the transport of M1 we uncovered a novel disposition site for this bioactive compound of plant origin.

To summarize, we found that caffeic acid, taxifolin, ferulic acid, and M1 all bind to human erythrocytes, but only the Pycnogenol metabolite M1 revealed accumulation within the cells. The more than 30-fold increase in the erythrocyte / plasma partitioning ratio indicates that red blood cells are a significant compartment for distribution of M1. M1 was previously shown to exert pronounced anti-inflammatory activities [11], but the plasma concentrations were rather low in the nanomolar range [6]. Our present results thus substantiate that low plasma concentrations do not necessarily reflect low presence of the compound in vivo. The uptake of M1 into erythrocytes was diminished in the presence of glucose and at higher concentrations of metabolite itself, suggesting a facilitated uptake of M1 into red blood cells, possibly via GLUT-1. In erythrocytes an intracellular conjugation of M1 yielding a glutathione adduct was detected, but the precise role of the reaction needs to be further investigated. Thus, we present novel data on the disposition of the bioactive maritime pine bark extract metabolite M1. This might help to further understand the in vivo behaviour of plant extract components.

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2 Highly sensitive analysis of polyphenols and their metabolites in human blood cells using dispersive SPE extraction and LC-MS/MS

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PUBLICATION 2

ABSTRACT

Blood cells, particularly erythrocytes, present a significant compartment for distribution of drugs and endogenous compounds and have been suggested to be factored in pharmacokinetic and pharmacodynamic evaluations. We previously detected binding of polyphenols to red blood cells and found indications for a facilitated uptake of the bioactive procyanidin metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) into human erythrocytes. The purpose of the present investigation was to develop an effective, sensitive and robust LC-MS/MS method to quantify low concentrations of polyphenols in human blood cells.

Various sample preparation methods including classic sample clean-up techniques and variations of the QuEChERS approach were compared regarding compound recovery, matrix effects and overall process efficiency.

The QuEChERS technique which involves a liquid-liquid extraction and clean-up by dispersive solid-phase extraction yielded best results. The method was fully validated for the six analytes (+)-catechin, ferulic acid, M1, taxifolin, caffeic acid and δ-3-methoxy-4-hydroxy-phenyl-γ-valerolacton (M2) in human blood cells with an optimized QuEChERS sample preparation and prior enzymatic hydrolysis of analyte conjugates. The lower limits of quantification for the analytes ranged from 0.12 ng/mL for M1, M2 and taxifolin to 48.40 ng/mL for caffeic acid. The application of the method to a blood cell sample of a volunteer ingesting 100 mg/day of the standardized pine bark extract Pycnogenol® over the course of three weeks revealed measurable steady-state concentrations of catechin, M1, taxifolin, ferulic acid and M2.

To our knowledge, this is the first report of using the QuEChERS approach for detection and quantification of plant-derived compounds in human blood cells. The method can be applied in pharmacokinetic studies to determine the distribution of polyphenols and their metabolites in human whole blood, blood cells or in erythrocytes. This might contribute in gaining deeper insights into the in vivo distribution of polyphenols and their metabolites.

INTRODUCTION

In a pharmacokinetic study with single and multiple doses of the standardized maritime pine bark extract Pycnogenol® we previously detected catechin, caffeic acid, ferulic acid, and taxifolin in the nanomolar range in the plasma of human volunteers [1]. Furthermore, we also found a maritime pine bark metabolite, δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1), in the plasma samples. The metabolite M1 originates from the procyanidins' catechin units which are metabolized by the human intestinal flora via multiple reaction steps [2]. M1 has been shown to exhibit a higher bioactivity compared to catechin. The methylated form of M1, δ-3-methoxy-4-hydroxy-phenyl- γ-valerolactone (M2), is less active in vitro compared to M1 [3].

Although very low plasma concentrations of constituents and metabolites have been found after ingestion of the extract, diverse pharmacological actions, e.g. anti-inflammatory and cardiovascular effects, have been reported for Pycnogenol® in human trials [4,5]. Therefore, we considered a potential uptake of compounds into other body compartments and recently investigated the distribution of pine bark constituents and metabolites into blood cells [6,7]. A facilitated cellular uptake of M1, possibly via GLUT-1, was detected. Additionally, an intracellular conjugation of M1 yielding a glutathione adduct was also identified in human erythrocytes after in vitro incubation [7]. Previously, significantly higher recoveries of quercetin and resveratrol were reported from whole blood compared to plasma which also suggested a distribution into the cellular blood fraction [8].

Further detailed investigations and human in vivo studies of polyphenol concentrations in blood cells require a powerful analytical method such as LC-MS/MS. Sample preparation is a challenge due to the viscous cell matrix and the high hemoglobin content. Residual co-eluting matrix components, e.g. endogenous phospholipids or salts affect ionization of the target analytes and might contribute to matrix effects (ME), resulting either ion suppression or ion enhancement. Classic sample clean-up techniques include protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Combining these techniques might be necessary, but is very material- and time consuming, and thus often expensive.

In 2003 Anastassiades et al. described a simple, fast and inexpensive method for determination of pesticide residues in fruits and vegetables which has been since known as QuEChERS (**qu**ick, **e**asy, **ch**eap, **e**ffective, **r**ugged and **s**afe) [9]. The original method involved a liquid-liquid extraction with acetonitrile and clean-up by dispersive solid-phase extraction (d-SPE) using anhydrous magnesium sulfate and a primary secondary amine (PSA) sorbent to remove residual water and various polar matrix components such as pigments. Since this initial publication various modifications of the original method have been developed and published [10-12], e.g. by the Association of Analytical Communities (AOAC) [10], the European Committee for Standardization (CEN) [13], and the Swedish National Food Administration (NFA) [14]. Subsequently, the QuEChERS approach has been also

successfully applied to biological fluids such as whole blood to determine drugs and toxins [15-20].

The purpose of the present investigation was to develop an effective, sensitive and robust analytical method to quantify low concentrations of polyphenols, especially M1, in human blood cells with LC-MS/MS. Therefore, we sought to compare various sample preparation methods including classic sample clean-up techniques and variations of the QuEChERS approach, account for matrix effects and apply the optimized and fully validated method to a human blood cell sample.

METHODS AND MATERIALS

Chemicals and reagents

Analytical standards (+)-catechin, taxifolin, ferulic acid, caffeic acid and the internal standard (IS) 3,4-dihydroxyhydrocinnamic acid (hydrocaffeic acid) were all obtained from Sigma-Aldrich (Taufkirchen, Germany). The metabolite M1 (δ-(3.4-dihydroxy-phenyl)-γ-valerolactone) and M2 (δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone) was synthesized by Matthias Rappold as part of his diploma thesis. Methanol (MeOH, LC-MS analyzed) from J.T.Baker Mallinckrodt, acetonitrile (ACN, CHROMASOLV® gradient grade, for HPLC, ≥99.9%) and water (HiPerSolv CHROMANORM® for LC-MS, VWR BDH Prolabo) were obtained from VWR (Darmstadt, Germany). Ammonium formate (AF), formic acid (FA) and acetic acid (HAC) were all purchased from Sigma-Aldrich. An enzymatic mixture of β-Glucuronidase/Sulfatase (ß-Gln/Sulfa) from Helix pomatia (Type HP-2; Sigma-Aldrich) was used for enzymatic hydrolysis prior to the sample preparation to determine free and conjugated analytes in human blood cells (phase-II-metabolism).

For comparing sample preparation techniques trichloroacetic acid (TCA), perchloric acid, phosphoric acid, ammonia and ammonium acetate (AA) were purchased from Merck (Darmstadt, Germany). Ethyl acetate (EA) and *tert*-butyl methyl ether (MTBE) were obtained from Sigma-Aldrich. Strong anion exchange cartridges Strata™-XL-A and reversed-phase cartridges Strata™-XL (both cartridges 1 mL, 30 mg sorbens mass, 100 µm) were purchased from Phenomenex (Aschaffenburg, Germany). Anhydrous magnesium sulfate, sodium chloride, sodium acetate, trisodium citrate dihydrate (Na3-citrate), disodium hydrogencitrate sesquihydrate (Na2-hydrogencitrate) were obtained from Sigma-Aldrich. The bulk sorbent Bondesil-PSA (primary secondary amine) for dispersive solid-phase extraction (d-SPE) was from Agilent Technologies (Santa Clara, CA, USA).

Buffer, standard solutions and standard substance mix

The phosphate buffered saline (PBS, pH 7.4) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 und 1.5 mM KH_2PO_4 (Sigma-Aldrich, Taufkirchen, Germany).

Stock solutions (1 mg/mL) of each standard substance ((+)-catechin, taxifolin, ferulic acid, caffeic acid, M1 and M2) and of the IS acid were prepared in 100% methanol. These stock solutions were then diluted further with methanol and the six analytes were mixed to give a series of working standards that were used for all further experiments. All prepared working solutions were aliquoted and stored at -20 °C and used up after one freeze-thaw cycle. Stock solutions were stored at -80 °C until use.

Source and handling of human erythrocytes / blood cells and clinical sample

Packed human erythrocytes and blood cells from blood donors were obtained from a blood transfusion service (Bayerisches Rotes Kreuz, München, Germany). Blood cells from three individual blood donors were pooled. An aliquot of each individual donor was retained for further investigations. Method development and optimization were performed with the packed erythrocytes. Method validation and quantification of samples was carried out with the pooled blood cells. For clinical sample handling, Sterican® cannulas with Luer-lock plastic neck, 1.2 x 0.5 mm (Braun, Melsungen, Germany) and disposable syringes with Luer-Lock (polypropylene), 2.5 mL (Hartenstein, Würzburg, Germany) were used.

Liquid chromatography (LC)

The analysis of human blood cell samples was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). For the LC analysis an Agilent 1200 system consisting of a pump model BinPump SL G1379B, a degasser model G1312B, an autosampler model HIP-ALS SL+ G1316B and a column oven model TCC SL G1316B were used. The chromatographic separation was carried out using a Pursuit PFP-C18 column (4.6 x 150 mm, particle size 3 µm) at 20 °C (all from Agilent Technologies, Santa Clara, CA, USA).

The mobile phase consisted of 5 mM ammonium formate with 0.065% (v/v) formic acid (pH =3.2; A) and methanol with 0.1% formic acid (B). The stock solution of 5 M ammonium formate for the mobile phase A was prepared monthly and stored at +4 °C. The flow rate was set to 0.6 mL/min, the sample injection volume was 5 µL. The gradient elution was conducted starting at 60% B (0 min) to 95% B (2.50 min) and maintained to 95% B to 5.50 min followed by re-equilibration at 60% B. The total run time was 10.00 min with a post time of 3 min.

Mass spectrometry (MS/MS)

The MS was equipped with a G 6460 TripleQuad LC/MS with turbo electrospray ionization (ESI) from Agilent Technologies, Santa Clara, CA, USA. The MS/MS detection was carried out in the dynamic multiple reaction monitoring (DMRM) mode employing positive and negative ESI modes. The negative ionization mode was used for the analytes (+)-catechin, taxifolin, ferulic acid, caffeic acid and M1 and the positive ionization mode for M2. The IS hydrocaffeic acid was monitored in both, ESI positive and negative, for internal quantification of the analytes.

In both cases the capillary voltage was set at 3500 V. The gas temperature and flow of the ESI source (drying gas, nitrogen) were set at 300 °C and 10 L/min. The nebulizer pressure was set at 50 psi, the nozzle voltage to 0 V. The gas temperature and flow of the turbo spray (sheath gas, nitrogen) were set at 400 ˚C and 12 L/min. The collision induced dissociation (CID) in the collision cell (q2) was operated with nitrogen as collision gas. Fragmentor voltage (Q1), collision energy (q2) and cell accelerator voltage (q2) were optimized by Flow Injection Analysis (FIA) and also by the MassHunter Optimizer TripleQuad B.04.01 software and confirmed subsequently by manual injections of a mixture of the standard substances (10 µg/mL) in methanol. The final conditions and optimizations were performed with processed samples of human blood cells.
In all cases, single deprotonated $[M-H]^-$ or protonated [M+H]+ ions were found to be the most abundant precursor ions. The MS/MS transitions of the analytes (+)-catechin, taxifolin, ferulic acid, caffeic acid, M1 and M2 and of the IS and the optimized mass spectrometric parameters are listed in Table S1 in the Electronic Supplementary Material. Two transitions (quantifier and qualifier) were monitored for each compound. The cycle time was 1000 ms. For getting maximum sensitivity the resolution of Q1 and Q3 were set to widest/widest for the analytes and wide/widest for the IS. The electron multiplier was set at + 500 V in negative mode and + 1000 V in positive mode. Data acquisition was performed with Mass Hunter Data Acquisition Version B 04.01. Qualitative and quantitative Analysis were achieved with Mass Hunter Qualitative and Quantitative Analysis Version B 05.00.

Quantitative assessment of matrix effects (ME)

For assessment of matrix effects (ME) the method proposed by Matuszewski et al. was performed [21]. Briefly, extracted spiked samples ("spiked") are prepared by adding standards into blank matrix. Subsequently, the sample matrix containing the analytes is extracted and analyzed. Post-extracted spiked samples ("post-spiked") are prepared by extracting the blank samples matrix containing no analytes. After extraction standards are spiked into the extracted matrix and the sample is analyzed. A third sample set is composed of the standard solution ("standard solution") containing the analytes in pure solvent.
By comparing the signal responses of extracted spiked samples (set 1), post-extracted spiked samples (set 2) and the standard solution (set 3) the recovery (RE; Equation 1; [21]), absolute matrix effect (ME; Equation 2; [22]) and the process efficiency (PE; Equation 3; [21]) can be calculated as follows:

$$\text{Recovery RE [\%]} = \left(\frac{\text{Response}_{\text{extracted spiked sample}}}{\text{Response}_{\text{post-extracted spiked sample}}} \right) \text{x } 100 \qquad \textit{(Equation 1)}$$

$$\text{Matrix effect ME [\%]} = \left(\frac{\text{Response}_{\text{post-extracted spiked sample}}}{\text{Response}_{\text{standard solution}}} - 1 \right) \text{x } 100 \qquad \textit{(Equation 2)}$$

$$\text{Process efficiency PE [\%]} = \left(\frac{\text{Response}_{\text{extracted spiked sample}}}{\text{Response}_{\text{standard solution}}}\right) \times 100 \qquad \textit{(Equation 3)}$$

The subtraction of 1 to the quotient in Equation 2 allows for interpretation of negative results as ion suppression and positive results as an enhancement of the analyte signal [21]. For calculation of relative ME, the ME between different lots of one biological matrix has to be compared [23]. To predict the variability of ME the IS-normalised matrix factor (MF; Equation 4; [24]) at six individual lots of plasma was calculated by dividing the ME of an analyte by the ME of the IS as follows:

$$\text{IS-normalised matrix factor (MF)} = \left(\frac{\text{ME}_{\text{analyte}}}{\text{ME}_{\text{IS}}}\right) \times 100 \qquad \textit{(Equation 4)}$$

The coefficient of variation of the IS-normalised MF at six individual lots of one biological fluid should not exceed 15% [24].

Method development: comparison of extraction procedures

For the development of a sensitive method for the quantification of selected polyphenols in blood cells, different sample preparation techniques were compared and analyte sensitivity evaluated regarding the recovery RE (Equation 1), matrix effect ME (Equation 2), process efficiency PE (Equation 3) and matrix factor MF (Equation 4). A cell volume of 0.5 mL human erythrocytes was subjected to protein precipitation (PPT), liquid-liquid extraction (LLE), solid phase extraction (SPE), one QuEChERS variation (EN 15662 method, [13]) and combinations thereof (Table 1). Extraction solvents were evaporated under a gentle stream of nitrogen. The respective residues were reconstituted in 100 µL of 100% MeOH and subjected to LC-MS/MS analysis.

For each extraction technique a sample set (set 1-3) [21] was prepared with the standard substance mix ((+)-catechin, taxifolin, ferulic acid, caffeic acid, M1 and M2):

Set 1: Extracted spiked samples containing each analyte at a concentration of 100 ng/mL. The residue was reconstituted with 100 µL 100% MeOH.

Set 2: Post-extracted spiked samples; the extracted matrix blank was reconstituted with 100 µL 100% MeOH, containing the analytes at a concentration of 500 ng/mL.

Set 3: Standard solution; analytes (500 ng/mL) in 100% MeOH.

***Table 1: Overview of the tested sample preparation techniques**. Various reagents were added to 0.5 mL human erythrocytes in different volume ratios. For some preparation methods (e.g. PPT 1) the erythrocytes were diluted before further reagent addition.Cold reagents were cooled to 4 °C.*
PPT: protein precipitation; LLE: liquid-liqid extraction; SPE: solid phase extraction; RT: room temperature

Sample preparation	***Process***
PPT 1 a	Addition of 50% TCA (1:1)
PPT 1 b	Dilution with PBS buffer (1:1); addition of 50% TCA (1:0.5)
PPT 2	Addition of 10% TCA (1:3)
PPT 3	Addition of H_3PO_4 (pH 3.2)/ACN_{cold} (1:3)
PPT 4 a	Addition of H_3PO_4 (pH 3.2)/$MeOH_{cold}$ (1:3)
PPT 4 b	Dilution with PBS buffer (1:1); addition of H_3PO_4 (pH 3.2)/$MeOH_{cold}$ (1:1.5)
PPT 4 c	Addition of H_3PO_4 (pH 3.2)/$MeOH_{cold}$ (1:4.5)
LLE 1 a	Addition of H_3PO_4 (pH 3.2)/MTBE:EA 50:50 (1:3)
LLE 1 b	Dilution with PBS buffer (1:1), H_3PO_4 (pH 3.2); addition of MTBE:EA 50:50 (1:1.5)
SPE 1 Strata™ XL, Reversed phase	Conditioning: 1 mL MeOH; Equilibration: 1 mL water Loading: 0.5 mL blood cells + 1.5 mL 4% H_3PO_4 Washing: 1 mL 20% MeOH in water (2 x 0.5 mL) Elution: 1 mL MeOH/ACN/water/FA 60:30:10:0.1 (2 x 0.5 mL)
SPE 2 Strata™ XL-A, Strong anion exchange	Conditioning: 1 mL MeOH; Equilibration: 1 mL water Loading: 0.5 mL blood cells + 1.5 mL 4% H_3PO_4 Washing 1: 1 mL 25 mM AA (2 x 0.5 mL) Washing 2: 1 mL MeOH (2 x 0.5 mL) Elution: 1 mL 5% FA in MeOH (2 x 0.5 mL)
Combi 1	LLE 1 a + PPT 1 a
Combi 2	LLE 1 a + PPT 4 a
Combi 3	PPT 1 b + LLE 1 a
Combi 4	PPT 1 a + LLE 1 a
Combi 5	PPT 1 a + LLE: addition of 100% MTBE (1:3)
Combi 6	PPT 4 a + LLE 1 a
QuEChERS according to EN-method [13]	Dilution with PBS buffer to 10 mL, + 5 mL 1% acetic acid in acetonitrile, vortex 1 min,+ 3 g $MgSO_4$ + 1 g NaCl + 1 g Na_3-citrate + 0.5 g Na_2-hydrogencitrate, vortex 1 min, centrifugation 5 min at 3,300 *g* (4 °C), + 100 mg PSA + 600 mg $MgSO_4$ to upper layer, vortex 1 min, centrifugation 5 min at 3,300 *g* (RT)

Method optimization: comparison of QuEChERS modifications

Optimization was performed by comparing modifications of the QuEChERS sample preparation technique to obtain best results for the analytes M1, taxifolin and ferulic acid. The following variations were used at the different steps:

1. Liquid -liquid extraction and phase partitioning

Human erythrocytes buffered to pH 7.4 were mixed with acetonitrile and shaken before a salt mixture was added. According to the original method [9], 4 g MgSO4 and 1 g NaCl is used while the AOAC 2007.01 method [10] applies 4 g MgSO4 and 1 g NaAc. The EN 15662 method [13] uses 4 g MgSO4, 1 g NaCl, 1 g Na3-citrate and 0.5 g Na2-hydrogencitrate. The three methods were compared employing a sample set [21] as described above with the alteration that set 2 and set 3 contained the analytes at a concentration of 1000 ng/mL.

2. Dispersive solid phase (d-SPE) clean-up

According to AOAC 2007.01 method 150 mg MgSO4 and 50 mg primary secondary amine (PSA) per mL extract is used for extract clean-up by d-SPE [10], whereas the original method suggests 25 mg PSA to be sufficient for 1 mL extract [9]. The required amount of the drying material MgSO4 and the bulk sorbent PSA was varied for method optimization. The influence of different variations of MgSO4 and PSA was investigated with 1 mL of human erythrocytes containing 5 ng/mL of M1, taxifolin and ferulic acid. Various combinations of PSA and MgSO4 were studied. The quantity of the sorbent material PSA ranged from 0 to 125 mg and MgSO4 between 0 to 900 mg.

Finally, the influence of different samples volumes (0.5, 1.0 and 2.0 mL) was analyzed and the overall method improvement of the optimization progress for the analytes of interest was evaluated by comparing sample preparation before and after optimization. Again, a sample set was assembled as described above. Set 1 containing each analyte at a concentration of 100 ng/mL, set 2 and -3 contained the analytes at concentrations of 500, 1000 and 2670 ng/mL for samples volumes of 0.5, 1.0 and 2.0 mL, respectively.

Sample preparation of human blood cell samples with optimized QuEChERS and prior enzymatic incubation

2.0 mL human blood cells were diluted with PBS buffer to 10 mL and 275 µL 4% o-phosphoric acid were added (pH 5.0). Subsequently, the samples were incubated with an enzyme mixture containing ß-Gln/Sulfa (625 U ß-Gln per mL blood cells) for 45 min at 37 °C on a horizontal shaker (100 rpm) to hydrolyse conjugated analytes [1]. Afterwards, 25 µL IS (= 25 ng/mL), 5 mL of 1% acetic acid in acetonitrile and a salt mixture comprising 4 ± 0.2 g magnesium sulfate (anhydrous) and 1 ± 0.05 g sodium acetate was added. The gemisch was vortexed for 1 min (Multi-Vortex, VWR, Darmstadt, Germany) and centrifuged for 5 min at 3,300 g (4 °C). Thereafter, 3.1 mL supernatant was transferred to a reaction tube (10 mL, Sarstedt, Nümbrecht, Germany) containing a mixture of 100 ± 5 mg PSA and 600 ± 25 mg magnesium sulfate (anhydrous) for further clean-up with d-SPE. The sample was vortexed for 1 min (Multi-Vortex) and centrifuged for 5 min at 3,300 g at room temperature. After d-SPE with the bulk sorbent PSA the analytes (polyphenols) were present in an alkaline acetonitrile extract, so 1.9 mL of the upper organic layer was removed and re-acidified with 20 µL 5% formic acid in acetonitrile to protect against loss of stability [9]. Subsequently, the extract was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 75 µl of 5% formic acid in methanol and centrifuged at 18,000 g for 15 min at 4 °C. Immediately, 5 µL of the supernatant was subjected to the LC-MS/MS analysis.

Method validation

A full validation was performed for the quantification of the six analytes (+)-catechin, ferulic acid, M1, taxifolin, caffeic acid and M2 in human blood cells with optimized QuEChERS sample preparation and prior enzymatic hydrolysis of analyte conjugates [1]. The validation

included the selectivity, linearity, lower limit of quantification (LLOQ), recovery, process efficiency, matrix effects (quantitative), carry over, cross talk and post-preparative stability according to EMA and FDA guidelines [24,25].

RESULTS

Method development

Eighteen different sample preparation techniques (Table 1) were compared by calculating the recovery (RE), matrix effects (ME) and process efficiency (PE) for all analytes with special focus on a sensitive detection of the metabolite M1 (Figure 1).

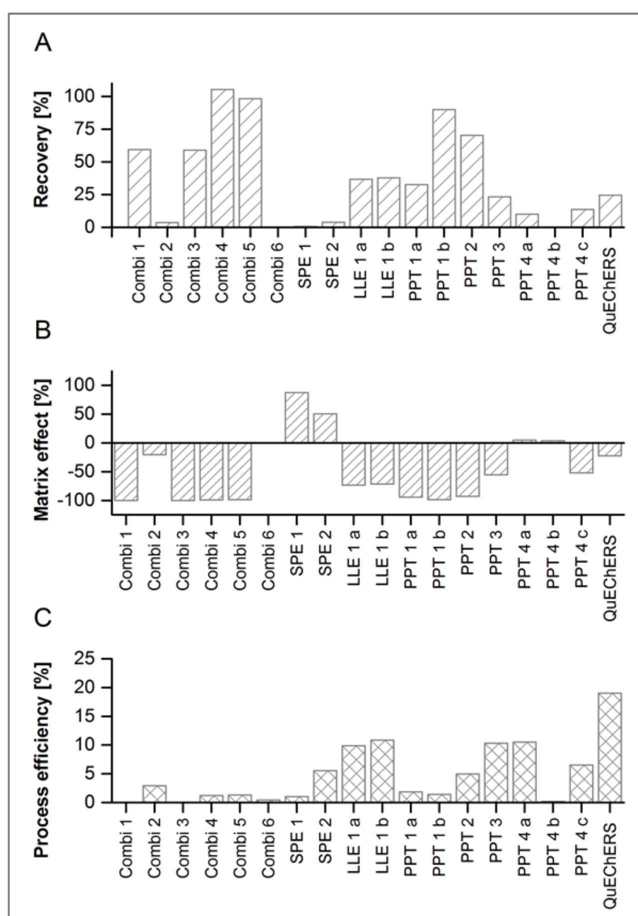

Figure 1. Comparison of different sample preparation techniques (Table 1) using 0.5 mL human erythrocytes exemplified for the metabolite M1 spiked at a concentration of 100 ng/mL (n= 1). A. *Recovery: Two protein precipitation (PPT) methods, PPT 1b and PPT 2, and two combination methods (Combi 4 and Combi 5) revealed highest recovery rates of > 70%.* ***B****. Matrix effect: Only the two solid phase extraction (SPE) methods and two PPT techniques (PPT 4a and PPT 4b) showed signal enhancement. M1 was subjected to different degrees of ion suppression with all other techniques.* ***C****. Process efficiency: One liquid-liquid extraction (LLE) method, LLE 1b, and two PPT techniques (PPT 3 and PPT 4a) resulted in total recovery rates of > 10% for M1. The QuEChERS approach (EN 15662 method [13]) achieved with almost 19% the highest total recovery of all investigated sample extraction methods.*

The RE of M1 ranged from 0.00% with Combi 6 to 105.3% by using Combi 4. Only protein precipitation PPT 1b and PPT 2 and two combinations (Combi 4 and Combi 5) achieved a high RE rate of > 70%. Except PPT 4b with 0.21%, all tested PPT methods showed a

moderate (PPT 1a/3/4a/4c; 10.00–32.62%) to high RE rate (PPT 1b/2; 70.31–90.00%). The two liquid-liquid extraction (LLE) techniques resulted in a RE of 36.80% and 37.86%, whereas the analyte M1 was hardly captured by the two solid phase extraction (SPE) methods (0.54% and 3.87%). Combined sample preparation techniques (Combi 1/3/4/5) increased the RE of M1 to 58.97–105.30%. QuEChERS (EN method, [13]) resulted in a RE rate of 24.44% (Figure 1 A).

Observed ME of M1 were between -99.97% with Combi 1 and +87.46% with SPE 1. Both SPE techniques resulted in positive values and thus ion enhancement (SPE 1: 87.46%; SPE 2: 50.55%), whereas the signal of M1 was suppressed to different degrees by most of the PPT methods. Using PPT 4a and PPT 4b, the analyte showed a slight signal increase of 4.04% and 4.85%, whereas an increased percentage of MeOH during extraction decreased the signal (PPT 4c; -52.03%). All other PPT methods (PPT 1a/1b/2) resulted in strong signal suppression of almost -100% (-94.35% / -92.90% / -98.41%). The LLE 1 and LLE 2 methods (-73.18% and 71.31%) and all combined techniques Combi 1–6 reduced the signal of M1 (-20.31% to -99.97%). The QuEChERS technique moderately attenuated the M1 signal by -22.32% (Figure 1 B.).

The PE of M1 ranged from 0.02% with Combi 1 to 18.99% by using QuEChERS. The techniques LLE 1b, PPT 3 and PPT 4a achieved a total recovery rate of > 10% for M1. With LLE 1a and LLE 1b, the total RE rate was 9.86% and 10.86%, whereas it was 1.02% and 5.55% by using the SPE techniques (SPE 1 and SPE 2). The PE of M1 was between 1.43% and 10.49% at all tested PPT. The applied combinations Combi 1–6 showed a PE of 0.02% to 2.92%. The QuEChERS method resulted in the highest total RE rate of 18.99% for the analyte M1 (Figure 1 C.).

Method optimization

Three QuEChERS variations were compared, the original method ("Original"; [9]), AOAC 2007.01 method ("AOAC"; [10]) and EN 15662 method ("EN"; [13]). The recovery (RE), matrix effects (ME) and process efficiency (PE) was calculated for all analytes with special focus on a sensitive detection of the metabolite M1 (Figure 2).

The RE of M1 ranged between 14.87% with the original method and 19.44% with the EN 15662 method (Figure 2 A). By using the AOAC 2007.01 method 19.08% of M1 were recovered. Furthermore, M1 was subjected to ion suppression with all QuEChERS variations, the ME were -31.49% with the original method, -40.01% with the EN 15662 and -38.72% when using the AOAC 2007.01 method (Figure 2 B). The PE of M1 was 10.19% with the original, 11.45% with EN 15662 and 11.69% with AOAC 2007.01 method (Figure 2 C).

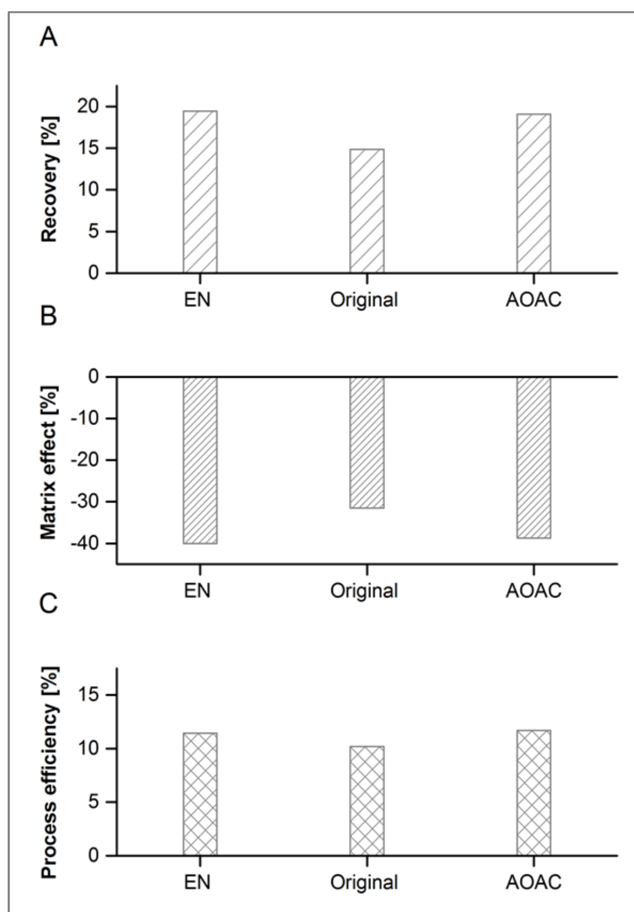

Figure 2. Optimization of the QuEChERS approach with 1.0 mL human erythrocytes containing 100 ng/mL M1, taxifolin and ferulic acid (n= 1). Three QuEChERS variations were compared, the original method ("Original"; [9]), AOAC 2007.01 method ("AOAC"; [10]) and EN 15662 method ("EN"; [13]). A. *Recovery: The recovery of M1 varied method dependent between 14.87% (Original) and 19.44% (EN).* ***B****. Matrix effect: M1 was subjected to ion suppression between -31.49% (Original) and -40.01% (EN).* ***C****. Process efficiency: The process efficiency of M1 ranged between 10.19% (Original) and 11.69% (AOAC). Thus, the AOAC method achieved the highest total recovery of M1 with human erythrocytes.*

Variations of the extraction and d-SPE process were analyzed. The first step of the QuEChERS approach involves sample extraction with acetonitrile (ACN) and optional acidification of the solvent. To analyze the influence of acids on the signal response, 1 mL of human erythrocytes containing 100 ng/mL of M1, taxifolin and ferulic acid were extracted without acidification and with addition of 0.5–2% acetic acid (HAC), 0.1–1% formic acid (FA) and 4% phosphoric acid (H3PO4) in acetonitrile using the EN 15662 extraction method (Figure 3). The addition of 1% HAC to the extraction solvent yielded the highest signal intensity (peak height) for M1, taxifolin and ferulic acid.

The QuEChERS AOAC 2007.01 method uses MgSO4 and NaAc for phase partitioning after ACN extraction. To analyze the influence of the addition of 0–2000 mg NaAc on the signal response, 1 mL of human erythrocytes containing 10 ng/mL of M1, taxifolin and ferulic acid were extracted the AOAC method (Figure S1 in the Electronic Supplementary Material). No addition of NaAc yielded the highest intensity (peak height) for the analytes M1 and ferulic acid. Taxifolin was detected most sensitively with 0.25 g NaAc and 4 g MgSO4. However, the

extract supernatant still displayed a reddish color and the evaporation times were long when no or 0.25 g NaAc were used. Therefore, a composition of the acetate buffer for phase partioning of 1 g NaAc and 4 g MgSO4 was selected.

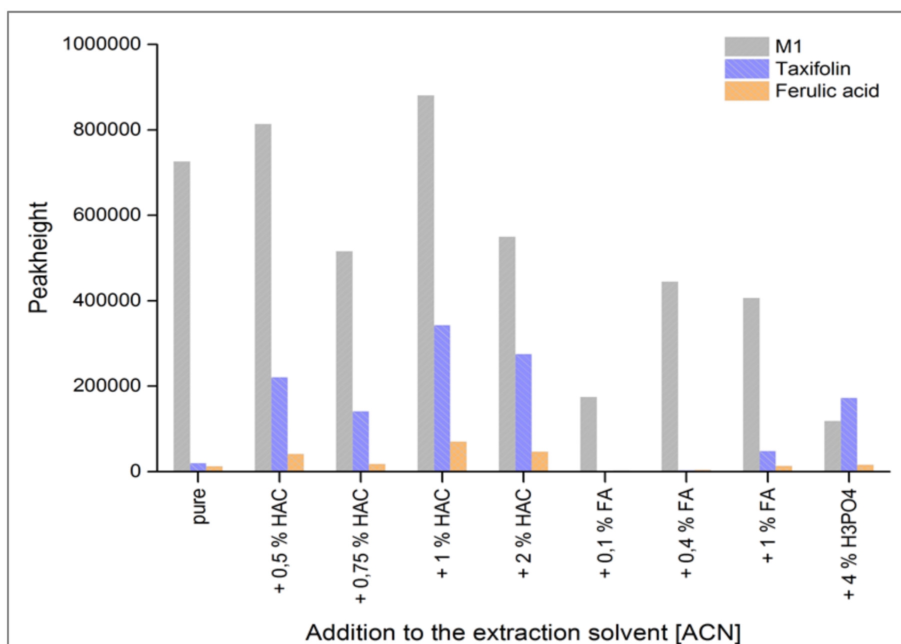

Figure 3. Influence of acidification of the extraction solvent actetonitrile (ACN) with 0.5–2% acetic acid (HAC), 0.1–1% formic acid (FA) and 4% phosphoric acid (H3PO4) on the extraction process. *Human erythrocytes (1.0 mL) containing 100 ng/mL M1, taxifolin and ferulic acid were extracted according to the EN 15662 method. The analytes were detected with the highest intensity (peak height) with 1% HAC in ACN.*

Within the d-SPE clean-up process of the AOAC 2007.01 method, the influence of variations of MgSO4 and PSA on the signal response was analyzed using 1 mL of human erythrocytes containing 5 ng/mL of M1, taxifolin and ferulic acid (Figure S2 in the Electronic Supplementary Material). No d-SPE was compared and also various combinations of 0–150 mg PSA and 450–900 mg of MgSO4. A composition of 100 mg PSA and 600 mg MgSO4 yielded the highest intensity (peak height) for the analytes M1 and taxifolin. Ferulic acid was detected most sensitively with 75 mg PSA and 450 or 500 mg MgSO4.

To compare the overall method improvement of the optimization progress for the analytes of interest in different samples volumes (0.5, 1.0 and 2.0 mL), the recovery (RE), matrix effects (ME) and process efficiency (PE) was analyzed before and after optimization (Figure 4). The QuEChERS EN 15662 extraction method with 0.5 mL cell material that was used during method development was compared with the AOAC 2007.01 method before optimization (1.0 mL cells) and the final optimized AOAC method with (2.0 mL cells). The final optimized AOAC 2007.01 method with 2.0 mL cells yielded the highest RE for M1 with 28.21% (Fig 4 a).

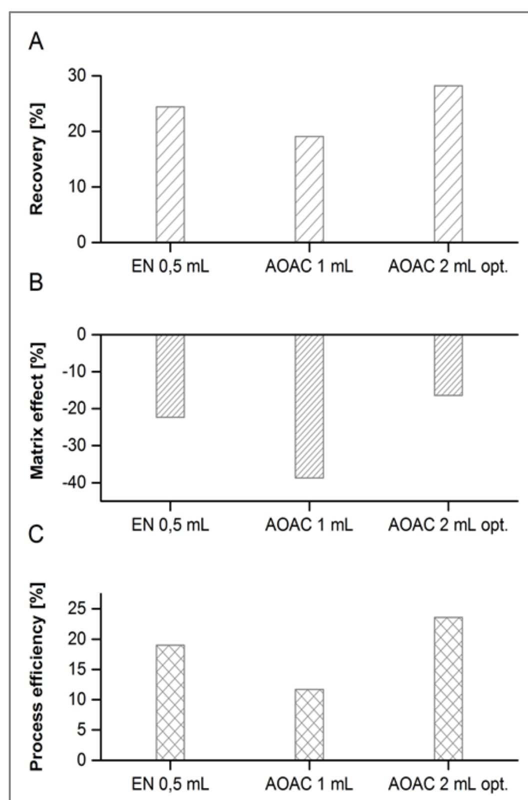

Figure 4. Overall method improvement of the optimization progress for 100 ng/mL M1, taxifolin and ferulic acid in different samples volumes (0.5, 1.0 and 2.0 mL). *The recovery, matrix effects and process efficiency were analyzed before and after optimization (n= 1).* ***A.*** *Recovery: The final optimized AOAC method with 2.0 mL cells yielded the highest recovery for M1 with 28.21%.* ***B****. Matrix effects: By using the EN method with 0.5 mL cells at the beginning of the method development M1 was subjected to ion suppression of -22.32%. By optimizing the AOAC method this was improved from -38.72% with 1.0 mL cells to -16.44% despite the higher cell volume of 2.0 mL.* ***C****. Process efficiency: The total recovery of M1 was initially 18.99% with 0.5 mL cells and EN method. This was improved by optimizing the AOAC method from 11.69% with 1.0 mL cells to 23.57% with 2.0 mL human erythrocytes.*

The initial EN 15662 method with 0.5 mL cells M1 was subjected to ion suppression of -22.32% (Fig 4 b). By optimizing of the AOAC 2007.01 method this was reduced from -38.72% with 1.0 mL cells to -16.44% despite the higher cell volume of 2.0 mL due to the high analyte concentration factor (f x 27 compared to f x 10 for AOAC before optimization and f x 5 for the EN method). The PE of M1 was initially 18.99% with 0.5 mL cells for the EN 15662 method. This was improved by optimizing the AOAC 2007.01 method from 11.69% with 1.0 mL cells to 23.57% with 2.0 mL human erythrocytes (Fig 4 c).

Method validation

Selectivity

To investigate whether endogenous compounds from the human blood cell matrix or other components in the sample interfered with the analytes of interest and IS, selectivity was analyzed with pooled blood cells and with blood cells of three individual donors by producing a blank sample (=matrix blank; containing neither analyte nor IS) and a matrix-matched LLOQ sample. Presence of interfering components was accepted where the response in the matrix blank was less than 20% of the LLOQ for the analytes and 5% for the IS.

The analytes and the IS were specifically and selectively determined without interference reciprocally and interference from the matrix.

Linearity

The calibration curve was prepared in human pooled blood cells by spiking the matrix with known concentrations of the analytes. The calibration curve consisted of a blank sample (containing neither analytes nor IS), a zero sample (containing IS) and 12 non-zero samples covering the expected range of each analyte, including the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). The criteria for acceptability of the back-calculated standard concentration included accuracy within ± 15% relative error of the nominal values and the precision within 15%, expressed as relative standard deviation (RSD), except for LLOQ at which both precision and accuracy were within 20%. At least 9 of these non-zero standards met the above criteria, including the LLOQ and ULOQ for the calibration curve. Excluding standards did not change the regression model used.
The ratios of the peak area analyte/IS were calculated at each concentration level. If the matrix already contained the compounds to be quantified, a matrix-matched calibration curve was applied by shifting this curve along the y-axes by the response of the non-spiked sample (zero sample) [26]. This unusual procedure was used because there was no alternative method [27]. Due to the presence of some analytes in the pooled human blood cell matrix the quotient of the peak area analyte/IS of the zero-blank was subsequently subtracted. For each analyte the resulting quotient (Y) was plotted against the spiked concentration (x) and the equation ($y = ax + b$; a: slope; b: intercept) was determined by linear regression (n= 3; Table S2 in the Electronic Supplementary Material). Correlation coefficients of ≥ 0.9960 revealed a linear relationship between analyte concentration and the quotient of the area ratios analyte/IS and hence the sample signal being directly proportional to the analyte concentration in the sample.

Lower limit of quantification (LLOQ)

The LLOQ was investigated by analyzing three replicates of spiked samples in low concentrations in human pooled blood cells. The signal-to-noise ratio (SNR; peak-to-peak height) of each analyte in the LLOQ samples was at least 5 times higher than the response in the related matrix blank (Figure 5).

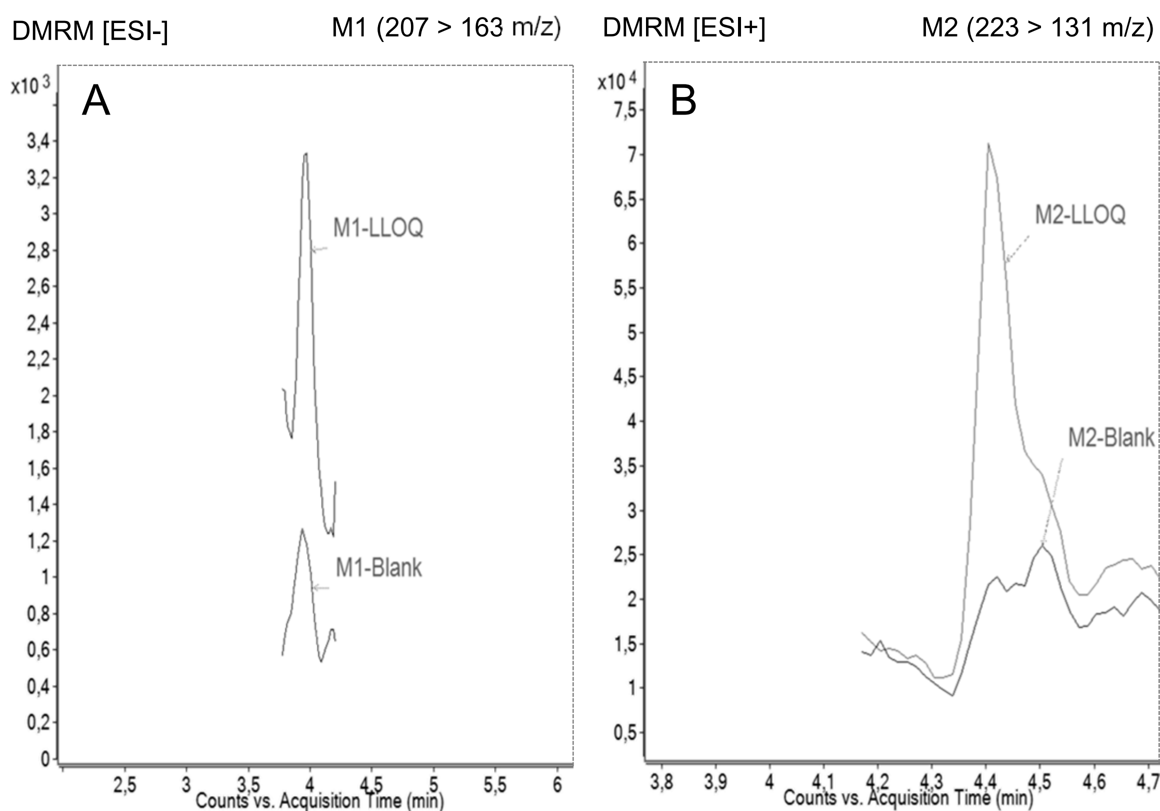

***Figure 5. Example chromatograms of the determination of the limits of quantification (LLOQ) in pooled human blood cells. A**. M1. The quantifier 207 > 163 of the zero-blank and a spiked LLOQ sample was overlaid. **B**. M2. Here, the quantifier 223 > 131 of the zero-blank and a spiked LLOQ sample was matched.*

The accuracy at the LLOQ ranged from 92.40 ± 18.07% (mean ± SD, n= 3) for ferulic acid to 111.47 ± 4.11% (mean ± SD, n= 3) for M2 (Table 2).

Table 2: Lower limit of quantification (LLOQ) and related accuracy of the six analytes extracted from human pooled blood cells (n= 3).

Analyt	LLOQ [ng/mL]	Accuracy $_{LLOQ}$ [%] Mean ± SD
Catechin	28.90	102.87 ± 8.41
Ferulic acid	0.97	92.40 ± 18.07
M1	0.12	105.41 ± 17.62
Taxifolin	0.12	100.36 ± 9.67
Caffeic acid	48.40	100.43 ± 16.91
M2	0.12	111.47 ± 4.11

Recovery (RE), process efficiency (PE) and matrix effects (ME)

The recovery (RE) of the analytes was determined at three concentrations (low-mid-high) in human pooled blood cells by comparing the analytical response of extracted spiked samples with those of post-extracted spiked samples (representing 100% RE; n= 3). The mean RE in human pooled blood cells was between 1.04 ± 0.15% for caffeic acid and 64.23 ± 10.48% for M2 (Table 3). The RE of the analytes was also determined in three individual lots of human blood cells at two concentrations (low-high) and ranged from 1.17 ± 0.04% for caffeic acid to 54.71 ± 7.14% for M2 (n= 3; data not shown). The maximum difference between the mean of the RE of the analytes in human pooled and in three individual lots of human blood cells was 14.87% for taxifolin. The process efficiency (PE) of the analytes was determined at three concentrations (low-mid-high) in human pooled blood cells by comparing the analytical response of extracted spiked samples with those of the analytes in standard solution (n= 3). The mean PE in human pooled blood cells varied between 0.78 ± 0.11% for catechin and 52.69 ± 3.56% for ferulic acid (Table 3). The PE of the analytes was also determined in three individual lots of human blood cells at two concentrations (low-high) and ranged from 1.00 ± 0.21% for catechin to 54.45 ± 5.61% for ferulic acid (n= 3; data not shown). The maximum difference between the PE of the analytes in human pooled and in three individual lots of human blood cells was 17.97% for catechin.

The absolute matrix effects (ME) were determined in human pooled blood cells at three concentrations (low-mid-high) by comparing the analytical response of post-extracted spiked samples with those of the analytes in standard solution (n= 3). The mean ME in human pooled blood cells ranged between -78.34 ± 2.03% for catechin to +66.42 ± 9.61% for ferulic acid (Table 3). The analytes catechin, M2 and taxifolin were subjected to different degrees of ion suppression, while an ion enhancement was observed for caffeic acid, M1 and ferulic acid. Furthermore, relative ME were investigated in three different lots of human blood cells at two concentrations (low-high) and ranged from -76.00 ± 2.00% for catechin to +75.80 ± 1.80% for ferulic acid (n=3; data not shown). The variability of the matrix factors (MF), as measured by the coefficient of variation of the IS-normalised MF, calculated from the three different lots of human blood cells was between 2.5% for ferulic acid and 14.3% for taxifolin. The maximum difference between the mean ME of the analytes in human pooled blood cells and in blood cells of three individual donors was 22.95% for M2. The deviation of the other analytes ranged from 0.51% for catechin to 21.53% for ferulic acid. While assessing the ME in pooled and in three individual lots of human blood cells, it was observed that higher (total) analyte concentrations were accompanied by lower ME.

Table 3: Recovery, process efficiency and matrix effects of the six analytes extracted from human pooled blood cells at three concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]			Matrix effect [%]		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin									
71.90	3.93	0.52	13.12	0.85	0.10	11.60	-78.34	2.03	-2.59
103.54	3.58	0.02	0.44	0.78	0.11	14.70	-78.22	2.35	-3.01
149.10	5.09	0.89	17.52	1.20	0.18	14.97	-76.37	1.31	-1.72
Taxifolin									
0.905	22.16	0.26	1.16	15.37	0.51	3.30	-30.64	6.34	-20.70
1.303	18.31	1.23	6.73	13.49	0.36	2.65	-26.31	4.86	-18.47
1.876	17.40	2.13	12.24	13.61	1.94	14.25	-21.76	3.54	-16.26
M1									
0.916	25.04	0.36	1.42	41.45	2.39	5.77	65.56	11.89	18.14
1.319	23.41	2.20	9.39	34.97	0.72	2.06	49.38	8.43	17.07
1.900	24.18	0.93	3.86	33.73	1.31	3.89	39.49	7.17	18.15
Ferulic acid									
7.21	31.51	1.49	4.73	52.44	4.03	7.68	66.42	9.61	14.47
10.39	27.37	0.10	0.37	44.73	3.08	6.89	63.40	7.47	11.78
14.96	32.45	1.21	3.74	52.69	3.56	6.75	62.37	7.02	11.26
Caffeic acid									
120.44	1.13	0.14	12.53	1.15	0.14	12.37	1.91	0.25	13.06
173.44	1.04	0.15	14.06	1.05	0.15	14.05	1.08	0.05	4.26
249.75	1.54	0.14	8.92	1.55	0.14	8.80	0.44	0.07	15.54
M2									
0.916	64.23	10.48	16.31	26.18	1.80	6.88	-59.23	5.74	-9.69
1.319	47.15	2.18	4.61	27.80	2.03	7.29	-41.04	1.56	-3.79
1.900	46.08	5.50	11.94	28.28	2.91	10.28	-38.62	0.99	-2.56

Sample carry-over

Carry-over was assessed by injecting two matrix blank samples of human pooled blood cells after an upper limit of quantification (ULOQ)-spiked sample of the calibration curve (n= 3). Because of the basal presence of some analytes in the matrix blank a carry-over in the first matrix blank after injection of the ULOQ spiked sample was accepted, when the response of the analyte and IS was not increased by more than 20% and 5%, compared to the response of a matrix blank before injection of the ULOQ-spiked sample. The analytes catechin, M1, taxifolin and caffeic acid showed no carry-over effects. Ferulic acid and M2 displayed light to moderate carry-over with an increase of the response of +3.98 ± 1.26% and +18.55 ± 3.95% (data not shown), respectively, which was still within the defined limits.

Post-preparative stability

The stability of the analytes in the processed samples, including the autosampler stability and the stability after one freeze and thaw cycle, was assessed with human pooled blood cells at four concentrations (LLOQ-low-mid-high) in triplicate. Samples were analyzed using a calibration curve obtained from freshly spiked calibration standards. The calculated concentrations were compared to the spiked concentrations.

Samples were kept in the autosampler after their first injection for 6 h and 12 h at room temperature before repetition of analysis. Processed samples placed in the autosampler for 6 h were stable between -13.75% for taxifolin and +18.73% for caffeic acid. With a longer residence time in the autosampler for 12 h these values ranged from -15.68% to +14.24% for catechin (Table 4).

Processed samples were frozen once at -20 °C for at least 12 hours before being thawed at room temperature for at least one hour prior to LC-MS/MS analysis. The freeze-thaw stability of the analytes ranged between -15.06% for catechin and +47.77% for taxifolin (Table 5). Notably, higher concentrations of M1 and taxifolin were recorded after one freeze-thaw cycle, especially at lower analyte concentrations. Therefore, immediate measurement after the sample preparation without freeze-thaw cycle was ascertained for application and human study samples.

Table 4: Post-preparative stability: autosampler stability of the analytes after 6 h and 12 h at room temperature after previous LC/MS/MS analysis (n= 3). *Conc.: concentration*

Analytes and spiked conc. [ng/mL]	Autosampler stability: 6 h - RT - in darkness			Autosampler stability: 12 h - RT - in darkness		
	Calculated conc. Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated conc. Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Catechin						
28.90	30.91 ± 5.19	16.79	6.97 ± 17.96	33.01 ± 2.55	7.72	14.24 ± 8.82
71.90	82.59 ± 5.46	6.62	14.87 ± 7.60	77.56 ± 2.59	3.34	7.87 ± 3.60
103.54	106.49 ± 12.78	12.00	2.85 ± 12.34	93.54 ± 14.26	15.24	-9.66 ± 13.77
149.10	133.87 ± 18.00	13.45	-10.22 ± 12.07	125.72 ± 18.53	14.74	-15.68 ± 12.43
Ferulic acid						
2.90	3.20 ± 0.48	14.86	10.50 ± 16.42	3.07 ± 0.77	25.00	6.09 ± 19.87
7.21	7.29 ± 0.80	10.95	1.10 ± 11.08	7.55 ± 0.85	11.22	4.65 ± 8.97
10.39	10.27 ± 1.82	17.68	-1.09 ± 17.49	10.35 ± 1.63	15.80	-0.36 ± 15.74
14.96	13.96 ± 0.21	1.51	-6.65 ± 1.41	13.99 ± 0.92	6.55	-6.45 ± 6.13
M1						
0.369	0.344 ± 0.019	5.55	-6.47 ± 5.19	0.368 ± 0.006	1.56	0.12 ± 7.29
0.916	0.774 ± 0.187	24.18	-15.53 ± 15.78	0.823 ± 0.189	22.99	-10.14 ± 15.37
1.319	1.246 ± 0.114	9.11	-5.54 ± 8.61	1.260 ± 0.171	13.61	-4.47± 13.00
1.900	1.721 ± 0.206	11.99	-9.43 ± 10.86	1.722 ± 0.121	7.04	-9.39 ± 6.38
Taxifolin						
0.364	0.400 ± 0.052	12.89	9.81 ± 14.15	0.394 ± 0.063	15.99	8.35 ± 17.32
0.905	0.990 ± 0.175	17.66	9.40 ± 19.33	0.975 ± 0.151	15.45	7.74 ± 16.65
1.303	1.248 ± 0.230	18.44	-4.23 ± 17.66	1.247 ± 0.206	16.51	-4.26 ± 15.81
1.876	1.618 ± 0.032	1.96	-13.75 ± 1.69	1.592 ± 0.047	2.97	-15.16 ± 2.52
Caffeic acid						
48.40	47.02 ± 5.88	12.51	-2.85 ± 12.16	46.00 ± 7.09	15.42	-4.97 ± 14.66
120.44	127.49 ± 25.58	20.06	5.85 ± 21.24	120.56 ± 21.43	17.77	0.10 ± 17.79
173.44	196.69 ± 17.45	8.87	13.41 ± 10.06	192.72 ± 16.17	8.39	11.11 ± 9.33
249.75	296.52 ± 17.08	5.76	18.73 ± 6.84	245.93 ± 26.61	10.82	-1.53 ± 10.65
M2						
0.368	0.365 ± 0.048	13.05	-0.76 ± 12.95	-[2]	-	-
0.916	0.844 ± 0.078	9.28	-7.89 ± 8.55	-	-	-
1.319	1.227 ± 0.140	11.45	-6.96 ± 10.65	-	-	-
1.900	1.942 ± 0.149	7.68	2.19 ± 7.85	-	-	-

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100
[2]: could not be determined due to low sample volume

Table 5: Post-preparative stability: stability of the analytes after one freeze-thaw cycle (n= 3).

Analytes and spiked concentration [ng/mL]	Freeze-thaw stability: 1 cycle -20 °C h/at least 12 h - RT/1h		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Catechin			
28.90	24.55 ± 3.61	14.69	-15.06 ± 12.48
71.90	63.26 ± 14.73	23.29	-12.03 ± 20.49
103.54	98.83 ± 5.33	5.39	- 4.55 ± 5.14
149.10	127.10 ± 7.55	5.94	-14.76 ± 5.06
Ferulic acid			
2.90	3.02 ± 0.10	3.47	4.21 ± 4.94
7.21	7.43 ± 0.71	9.57	3.03 ± 9.86
10.39	10.80 ± 1.12	10.35	3.96 ± 10.76
14.96	15.64 ± 1.06	6.76	4.57 ± 7.07
M1			
0.369	0.457 ± 0.049	10.68	24.19 ± 14.58
0.916	1.070 ± 0.203	18.99	16.86 ± 15.92
1.319	1.495 ± 0.293	19.61	13.34 ± 22.23
1.900	2.170 ± 0.214	9.87	14.20 ± 11.28
Taxifolin			
0.364	0.538 ± 0.052	9.75	47.77 ± 14.41
0.905	1.185 ± 0.046	3.89	30.95 ± 5.09
1.303	1.444 ± 0.142	9.85	10.85 ± 10.91
1.876	1.854 ± 0.316	17.05	-1.16 ± 16.85
Caffeic acid			
48.40	48.07 ± 3.14	6.52	-0.68 ± 6.48
120.44	118.26 ± 19.02	16.08	-1.81 ± 15.79
173.44	181.24 ± 12.85	7.09	4.50 ± 7.41
249.75	277.88 ± 35.80	12.88	11.26 ± 14.33
M2			
0.368	0.378 ± 0.011	2.93	2.61 ± 3.00
0.916	0.915 ± 0.112	12.29	-0.12 ± 12.28
1.319	1.285 ± 0.233	18.15	-2.61 ± 17.68
1.900	1.950 ± 0.224	11.50	2.62 ± 11.80

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Cross talk

Human pooled blood cells were processed with each of the analytes at the highest concentration of the calibration curve (mono-spiked-ULOQ-sample; n= 3; data not shown). Due to the basal presence of some analytes in the matrix blank a cross talk was accepted, in which the response of co-eluting other analytes or IS in the mono-ULOQ-spiked sample did not exceed 20% and 5%, respectively, compared to the response of the analyte or IS in the matrix blank. A cross talk of taxifolin-ULOQ-spiked samples and co-eluting ferulic acid of +18.66% was recorded as well as a cross talk of ferulic acid-ULOQ-spiked samples and co-eluting taxifolin of +16.09%. Both were within the acceptance limits. Other co-eluting analytes were not subjected to any cross talk.

Robustness

Since it could not be excluded that blood cell samples contained traces of serum, the robustness of the method was examined for two concentration levels (low-high) with pooled human blood cells, which were contaminated with 1% human pooled serum (n= 3; data not shown). The samples were analyzed against a calibration curve prepared in parallel. The mean accuracy of the back-calculated concentrations of the samples with serum contamination ranged between 90.06% for M1 and 115.68% for taxifolin and the precision, expressed as the RSD, was between 2.18% for taxifolin and 15.29% for catechin. Therefore, the method was regarded as robust.

Precision and accuracy

The intra- and interday precision and accuracy were determined with human pooled blood cells at four or five concentrations (LLOQ-(lower)-low-mid-high) in triplicate. The intraday accuracy was between 84.11% for M1 (LLOQ) and 112.41% for taxifolin with an intraday precision from 1.02% for catechin to 17.17% for M2 (LLOQ) (Table S3 in the Electronic Supplementary Material). The values of the interday accuracy ranged from 93.00% for M1 to 108.70% for M2 with a precision from 1.43% for caffeic acid to 16.89% for M1 (LLOQ) (Table S4 in the Electronic Supplementary Material).

Method application

The optimized sample preparation method was applied to an authentic blood cell sample of a human volunteer who ingested 100 mg/day Pycnogenol® over the course of three weeks to obtain steady-state conditions. The volunteer had steady-state concentrations of 202.8 ng/mL catechin, 0.26 ng/mL M1, 0.57 ng/mL taxifolin, 2.97 ng/mL ferulic acid and 0.23 ng/mL M2 (Figure 6).

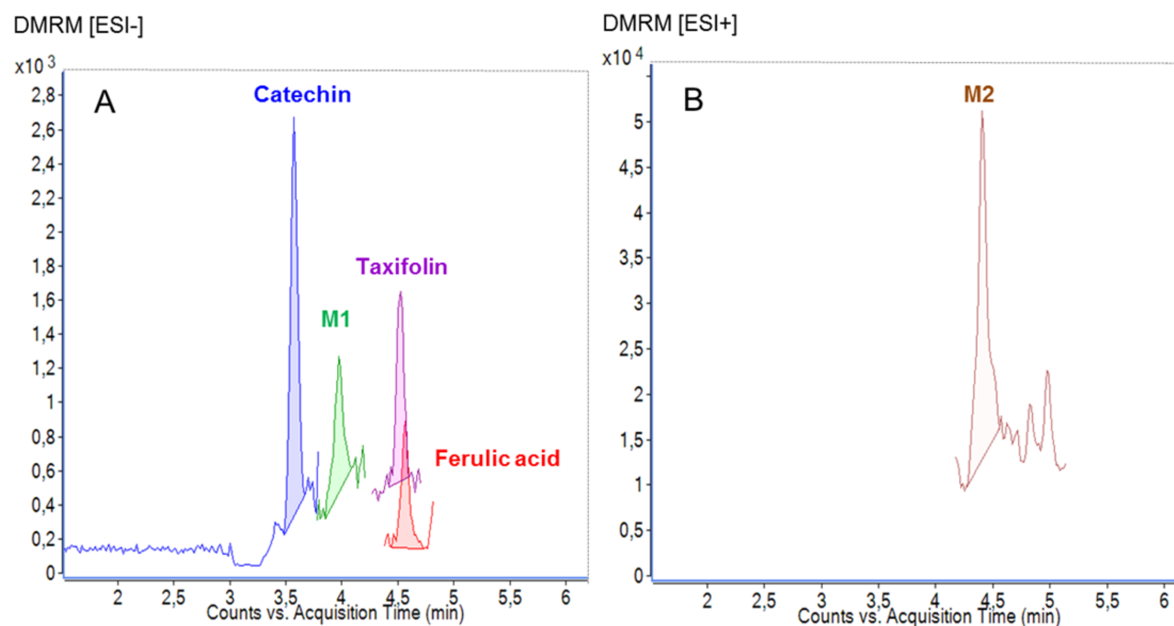

***Figure 6. Example chromatogram of an authentic human blood cell sample extracted with the optimized analytical method. A**. Quantifier of catechin (289 > 245), M1 (207 > 163), taxifolin (303 > 285) and ferulic acid (193 > 134) in ESI negative mode. The volunteer had a steady-state concentration of 202.82 ng/mL catechin, 0.26 ng/mL M1, 0.57 ng/mL taxifolin and 2.97 ng/mL ferulic acid after multiple dosing of Pycnogenol® of 100 mg/day over three weeks. **B**. Quantifier of M2 (223 > 131) in ESI positive mode. The steady-state concentration of M2 was 0.23 ng/mL.*

DISCUSSION

In the present study a sensitive method for analysis of selected polyphenols in human blood cells was successfully developed, optimized and validated. To our knowledge, this is the first report of using the QuEChERS approach for detection and quantification of plant-derived compounds in human blood cells.

Blood cells, particularly erythrocytes, present a significant compartment for distribution of drugs and endogenous compounds [28] and have been suggested to be factored in pharmacokinetic and pharmacodynamic evaluations [29]. It is well known that some drugs such as cyclosporine A or methotrexate significantly distribute into red blood cells [30,31]. Human erythrocytes also provide a deep compartment for other drugs such as metformin [32]. Furthermore, it has been observed that dietary polyphenols such as quercetin or resveratrol distribute into the cellular blood fraction [8] and it has been suggested that red blood cell analysis might be useful for determination of nutritional biomarkers [33]. We previously detected binding of polyphenols to red blood cells and found indications for a facilitated uptake of the bioactive procyanidin metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) into human erythrocytes [7]. Analysis of low compound concentrations in blood cells is a challenge due to the complex matrix and this might contribute to the fact that the uptake and presence of drugs or dietary compounds in the cellular blood fraction has received less attention than plasma or serum concentrations.

Various sample preparation techniques are frequently used before LC-MS/MS analysis and therefore we initially compared eighteen diverse approaches for sample extraction and clean-

up. Protein precipitation techniques (PPT) according to Polson et al. [34] achieved protein removal by addition of different concentrations of TCA (PPT 1 a and PPT 2), acetonitrile (PPT 3) or methanol (PPT 4 a). The methods were modified regarding the pH and dilution with PBS buffer because of the high viscosity of the blood cells (PPT 1 b and PPT 4 b). Sample preparation of human plasma and serum for LC-MS/MS involving liquid-liquid extraction (LLE) previously revealed best results for the relatively non-polar solvent mixture of *tert*-butyl methyl ether (MTBE; 50%) and ethyl acetate (EA; 50%) at an acidic pH of 3.2 (unpublished results), so these conditions were also used in the LLE approaches in the present study (LLE 1 a). Again, due to the viscosity of the blood cells a dilution was warranted (LLE 1 b). Various solid phase extraction (SPE) cartridges were tested and only those with a 100 µm pore size allowed working with the viscid blood cell matrix. Based on the polyphenolic nature of the analytes, SPE based on a reversed phase separation principle was used at an acidic pH (SPE 1) and strong anion exchange was used at a higher pH (SPE 2). However, best results, e.g. regarding complete removal of hemoglobin, were achieved with the QuEChERS approach which is a multi-method constituting of several purification steps.
Studying the catechin metabolite M1 as an example, it became obvious that the evaluated sample extraction methods differed considerably regarding their analyte recovery, matrix effects and overall process efficiency. Despite high recovery of M1 with methods involving protein precipitation the resulting overall process efficiency was insufficient because of the strong ion suppression. In contrast, although the QuEChERS approach resulted only in a moderate recovery of 24.44% for M1, it reached the highest process efficiency of 18.99% due to the lower ion suppression of -22.32% compared to the other techniques. Thus, during method development recovery, matrix effects and overall process efficiency should be simultaneously taken into consideration.
Several QuEChERS variations exist [10-12] and the extraction can be streamLined to the physicochemical properties of the analytes of interest. Using the standard protocols of three common QuEChERS methods only minor differences regarding recovery, matrix effects and overall process efficiency were observed in the present study. Therefore, the influence of protocol variations during the liquid-liquid extraction and dispersive solid phase clean-up for the three analytes M1, taxifolin and ferulic acid was investigated. An acidification of the extraction solvent acetonitrile had a pronounced effect on the signal response of the analytes depending on the kind and percentage of the acid. For phase partitioning, different salts are added to the extraction medium. It has been pointed out that the ratio of the buffer salts might affect the selectivity of the analyte detection [9]. Indeed it was shown that varying amounts of sodium acetate strongly influenced our analyte signals, but that this effect was not identical for the individual compounds. For optimal results, a sufficient analyte signal as well as practical aspects such as complete decoloration of the extract supernatant and short evaporation times were considered. After the liquid-liquid extraction step the QuEChERS approach specifies a dispersive solid phase extraction (d-SPE) for sample clean-up. It has been reported that the sorbent material(s) and their quantities might significantly influence the detection of the

analytes [15]. In this context, the influence of varying amounts of magnesium sulfate and primary secondary amines (PSA) on the signal response were evaluated in the present study. Again, these effects were pronounced while being distinct for individual analytes. After d-SPE with PSA, the extract has a basic pH-value, so optionally base-sensitive compounds can be stabilized by acidification. Then the extract can be immediately analyzed by LC- and/or GC-MS(/MS) [9].

After various optimization steps we found that the QuEChERS approach based on the AOAC protocol [35] yielded best results for our purpose. The method was fully validated for the six analytes (+)-catechin, ferulic acid, M1, taxifolin, caffeic acid and M2 in human blood cells with the developed QuEChERS sample preparation and prior enzymatic hydrolysis of analyte conjugates. Based on EMA and FDA guidelines [24,25] the method has proven to be selective, precise, accurate, robust and sensitive. The lower limits of quantification (LLOQ) were appropriate for the purpose of the method. For the analytes of highest interests the LLOQs were 0.12 ng/mL for M1, M2 and taxifolin and 0.97 ng/mL for ferulic acid. The method was less sensitive for catechin (LLOQ 28.90 ng/mL) and caffeic acid (LLOQ 48.40 ng/mL). However, since the expected catechin concentrations in authentic human blood samples was much higher, the LLOQ was suitable for the designated application. Indeed, the application of the method to a human blood cell sample of a volunteer who ingested 100 mg/day Pycnogenol® over the course of three weeks revealed that the LLOQ was sufficient for quantification of the analytes and measurable steady-state concentrations of catechin, M1, taxifolin, ferulic acid and M2 were detected. No caffeic acid was found in the human blood cell sample and it cannot be excluded that the concentrations were below the LLOQ. However, the herein described method can readily be modified and optimized to focus on caffeic acid if this was the analyte of highest interest.

The linearity of the method was appropriate. For analytes such as caffeic acid and catechin which were detected with less sensitivity on average 9 out of 12 non-zero standards were used for the calibration curves. For all other analytes, 10 to 12 out of 12 non-zero standards constituted the calibration curves. Thereby, this procedure met the criteria of acceptability of current EMA and FDA guidelines [24,25]. Moreover, correlation coefficients of ≥ 0.9960 demonstrated a linear relationship between the analyte concentration and the quotient of the area ratios of analyte and internal standard.

The recovery and precision of the method were within an acceptable range. The recovery was between 1.04 ± 0.15% for caffeic acid and 64.23 ± 10.48% for M2. Though the recoveries of catechin (3.95–5.09%) and caffeic acid (1.04–1.50%) were low, since the main focus was on other analytes such as M1, they were still sufficient for the expected concentration range in authentic human blood samples. According to the FDA guideline [25], the analyte recovery needs not to be 100%, but it should be consistent, precise and reproducible which was fulfilled by the method described herein. The method precision met the acceptance criteria of ± 15%

relative standard deviation for all analytes and concentrations except for the highest concentration of catechin (149.10 ng/mL) which slightly exceeded it with 17.52%.

The presented QuEChERS approach scores not only with the highest process efficiency compared to other sample preparation techniques, but also with a high purity of the extracted sample for LC-MS/MS analysis and a simple and rapid handling. Moreover, it is inexpensive, because it affords no special equipment for sample clean-up, only minimum amounts of solvents and salts are used. The QuEChERS method, which was originally developed for the food industry to determine pesticide residues in produce [9], was subsequently transferred to the clinical field for analysis of pharmaceuticals in human or animal whole blood [15-20].

In the present study, the QuEChERS approach has been utilized for the first time to detect and quantify polyphenols of a pine bark extract in human blood cells. After daily intake of 100 mg Pycnogenol® over three weeks steady state concentrations of 203 ng/mL catechin, 2.97 ng/mL ferulic acid, 0.57 ng/mL taxifolin, 0.26 ng/mL M1, and 0.23 ng/mL M2 were detected in the cell sample. No caffeic acid was discovered in the blood cells. In a previous pharmacokinetic study with five volunteers who took 200 mg Pycnogenol® over one week mean total plasma concentrations of 48.56 ± 16.66 ng/mL catechin, 18.71 ± 4.50 ng/mL ferulic acid, 2.42 ± 1.80 ng/mL caffeic acid, and 3.01 ± 0.38 ng/mL M1 were detected [1]. In this case, no taxifolin or M2 were discovered in plasma. It appears that catechin might be accumulating in blood cells since its concentration was clearly higher compared to plasma despite of the lower ingested dose. However, this needs to be investigated in more detail in a study analyzing blood cell as well as plasma or serum concentrations of the polyphenolic compounds. The method described herein can be applied in such pharmacokinetic studies to determine the distribution of polyphenols and their metabolites in human whole blood, blood cells or in erythrocytes. This might yield valuable information additional to plasma or serum concentrations. The QuEChERS method might also contribute in gaining deeper insights into the in vivo distribution of bioactive polyphenol metabolites produced by gut microbiota [36] or drugs and their metabolites.

CONCLUSION

By modifying the principles of the QuEChERS approach, an efficient method was established for the simultaneous and sensitive quantification of selected polyphenols in human blood cells. Due to its simple, cheap and rapid properties, this method can also serve as useful sample preparation of biological matrices in clinical fields, which are difficult to handle because of their high viscosity and strong coloration for example human whole blood or blood cells.

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ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1: Optimized transitions and parameters in dynamic multiple reaction monitoring (DMRM) mode

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	FV[a] [V]	CE[b] [V]	CAV[c] [V]	MS 1 Resolution	MS 2 Resolution	R_T[d] [min]	R_T Window *(DMRM)*	ESI mode
Catechin	289.1	245.0[e]	76	9	7	Widest	Widest	3.50	0.60	negative
	289.1	203.0	76	17	7	Widest	Widest	3.50	0.60	negative
Taxifolin	303.1	285.0[e]	95	9	4	Widest	Widest	4.50	0.45	negative
	303.1	125.0	95	21	4	Widest	Widest	4.50	0.45	negative
M1	207.0	163.1[e]	115	13	5	Widest	Widest	4.00	0.45	negative
	207.0	122.0	115	17	5	Widest	Widest	4.00	0.45	negative
Ferulic acid	193.1	134.1[e]	80	13	1	Widest	Widest	4.60	0.45	negative
	193.1	178.0	80	9	1	Widest	Widest	4.60	0.45	negative
Caffeic acid	179.0	89.0[e]	90	25	5	Widest	Widest	4.10	0.55	negative
Hydrocaffeic acid (IS)	181.2	137.0[e]	85	9	4	Wide	Widest	3.80	0.65	negative
	181.2	109.0	85	9	4	Wide	Widest	3.80	0.65	negative
M2	223.0	131.0[e]	100	10	5	Widest	Widest	4.40	0.45	positive
	223.0	101.0	100	20	5	Widest	Widest	4.40	0.45	positive
Hydrocaffeic acid (IS)	249.0	231.0[e]	155	12	1	Wide	Widest	3.80	0.65	positive
	249.0	155.0	155	12	5	Wide	Widest	3.80	0.65	positive

[a]FV. Fragmentor voltage. [b]CE. Collision energy. [c]CAV. Cell accelerator voltage. [d]R_T. Retention time. [e]Quantifier. transition for quantification

Table S2: Calibration range, calibration function and correlation coefficients of the six analytes extracted from human pooled blood cells (n= 3).

Analyt	Range [ng/mL]	Slope ± SD	y-intercept	Correlation coefficient R
Catechin	28.90 - 298.20	0.0014 ± 0.0003	0.0101	0.9972
Ferulic acid	0.97 - 29.91	0.0435 ± 0.0070	0.0223	0.9971
M1	0.12 - 3.80	0.6898 ± 0.0139	-0.0661	0.9985
Taxifolin	0.12 - 3.75	0.3690 ± 0.0308	-0.0290	0.9971
Caffeic acid	48.40 - 499.50	0.0001 ± 0.0000	0.0357	0.9960
M2	0.12 - 3.80	32.464 ± 2.0091	-2.7865	0.9982

Table S3: Intraday accuracy and precision of the analytes in human pooled blood cells (n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Catechin			
28.90	30.39 ± 3.77	105.16	12.40
71.90	62.38 ± 0.63	86.75	1.02
103.54	93.67 ± 5.90	90.47	6.30
149.10	128.80 ± 2.60	86.38	2.02
Ferulic acid			
0.97	0.83 ± 0.09	85.83	10.86
2.90	2.67 ± 0.30	91.97	11.14
7.21	6.66 ± 0.71	92.34	10.68
10.39	9.48 ± 1.06	91.31	11.21
14.96	13.98 ± 2.30	93.51	16.46
M1			
0.123	0.103 ± 0.006	84.11	5.83
0.369	0.319 ± 0.006	86.77	1.73
0.916	0.789 ± 0.043	86.14	5.50
1.319	1.290 ± 0.171	97.78	13.22
1.900	1.771 ± 0.180	93.22	10.14
Taxifolin			
0.121	0.136 ± 0.009	112.41	6.50
0.364	0.402 ± 0.028	110.43	7.02
0.905	0.813 ± 0.067	89.88	8.25
1.303	1.367 ± 0.138	104.89	10.12
1.876	1.842 ± 0.296	98.16	16.07
Caffeic acid			
48.40	47.05 ± 3.95	97.20	8.39
120.44	123.11 ± 7.25	102.21	5.89
173.44	163.27 ± 16.26	94.14	9.96
249.75	245.15 ± 37.86	98.16	15.44
M2			
0.123	0.121 ± 0.021	98.71	17.17
0.368	0.356 ± 0.061	96.80	17.08
0.916	0.811 ± 0.049	88.58	5.99
1.319	1.337 ± 0.102	101.38	7.62
1.900	1.727 ± 0.102	90.89	5.88

Table S4: Interday accuracy and precision of the analytes in human pooled blood cells (n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Catechin			
28.90	29.26 ± 0.98	101.26	3.34
71.90	69.05 ± 5.78	96.03	8.37
103.54	101.72 ± 7.92	98.24	7.79
149.10	145.14 ± 15.13	97.35	10.42
Ferulic acid			
0.97	0.92 ± 0.09	95.45	9.99
2.90	2.73 ± 0.24	94.26	8.73
7.21	7.04 ± 0.37	97.61	5.23
10.39	10.28 ± 1.03	98.94	9.97
14.96	15.17 ± 1.10	101.46	7.26
M1			
0.123	0.128 ± 0.022	104.40	16.89
0.369	0.342 ± 0.029	93.00	8.60
0.916	0.881 ± 0.084	96.22	9.57
1.319	1.260 ± 0.089	95.51	7.03
1.900	1.796 ± 0.137	94.53	7.61
Taxifolin			
0.121	0.131 ± 0.006	108.28	4.40
0.364	0.377 ± 0.026	103.71	7.00
0.905	0.916 ± 0.089	101.19	9.72
1.303	1.253 ± 0.100	96.17	8.02
1.876	1.803 ± 0.125	96.09	6.94
Caffeic acid			
48.40	46.80 ± 1.04	96.68	2.21
120.44	121.12 ± 1.73	100.56	1.43
173.44	169.23 ± 8.12	97.57	4.80
249.75	249.14 ± 8.51	99.75	3.42
M2			
0.123	0.134 ± 0.011	108.70	7.97
0.368	0.388 ± 0.030	105.54	7.70
0.916	0.921 ± 0.104	100.50	11.29
1.319	1.389 ± 0.071	105.31	5.12
1.900	1.935 ± 0.181	101.87	9.34

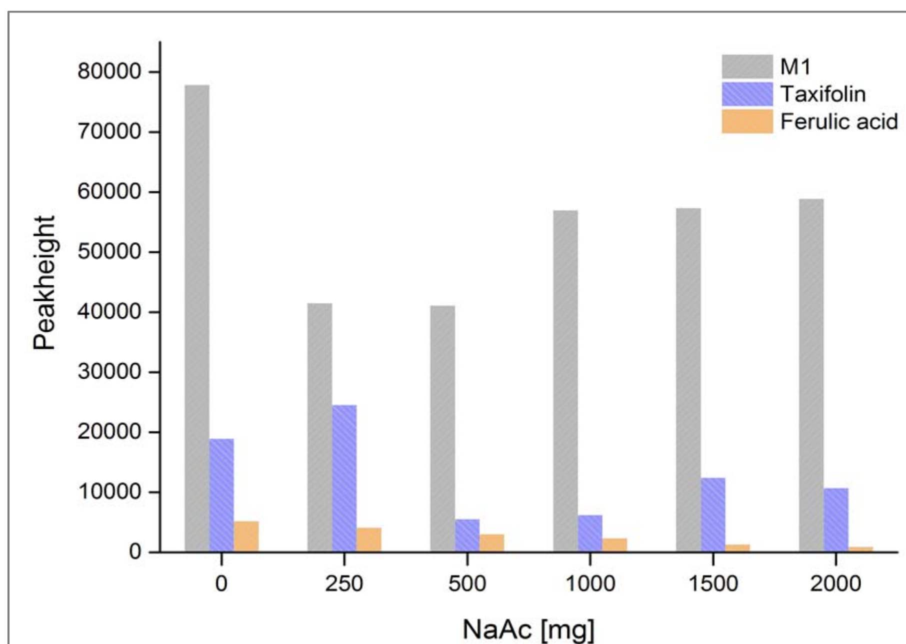

Figure S1. Influence of different quantities of NaAc with 4 g MgSO4 (constant amount) on the extraction process for phase partioning. *Human erythrocytes (1.0 mL) containing 10 ng/mL M1, taxifolin and ferulic acid were extracted according to the AOAC 2007.01 method.*

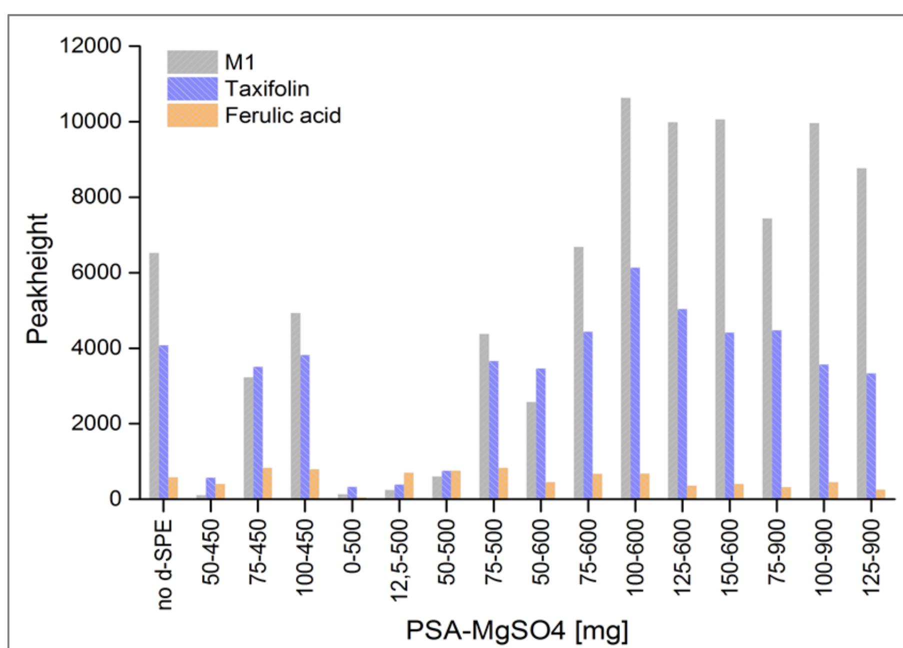

Figure S2. Influence of different quantities of PSA (0–150 mg) and MgSO4 (0–900 mg) on the d-SPE clean-up process. *Human erythrocytes (1.0 mL) containing 5 ng/mL M1, taxifolin and ferulic acid were extracted according to the AOAC 2007.01 method. A composition of 100 mg PSA and 600 mg MgSO4 yielded the highest intensity (peak height) for the analytes M1 and taxifolin. Ferulic acid was detected most sensitively with 75 mg PSA and 450 or 500 mg MgSO4.*

3 Profiling a gut microbiota-generated catechin metabolite's fate in human blood cells using a metabolomic approach

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PUBLICATION 3

ABSTRACT

The microbial catechin metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) has been found in human plasma samples after intake of maritime pine bark extract (Pycnogenol®). M1 has been previously shown to accumulate in endothelial and blood cells in vitro after facilitated uptake and to exhibit anti-inflammatory activity. The purpose of the present research approach was to systematically and comprehensively analyze the metabolism of M1 in human blood cells in vitro and in vivo.

A metabolomic approach that had been successfully applied for drug metabolite profiling was chosen to detected 19 metabolite peaks of M1 which were subsequently further analyzed and validated. The metabolites were categorized into three levels of identification according to the Metabolomics Standards Initiative with six compounds each confirmed at level 1 and 2 and seven putative metabolites at level 3.

The predominant metabolites were glutathione conjugates which were rapidly formed and revealed prolonged presence within the cells. Although a formation of an intracellular conjugate of M1 and glutathione (M1-GSH) was already known two GSH conjugate isomers, M1-S-GSH and M1-N-GSH were observed in the current study. Additionally detected organosulfur metabolites were conjugates with oxidized glutathione and cysteine. Other biotransformation products constituted the open-chained ester form of M1 and a methylated M1. Six of the metabolites determined in in vitro assays were also detected in blood cells in vivo after ingestion of the pine bark extract by two volunteers.

The present study provides the first evidence that multiple and structurally heterogeneous polyphenol metabolites can be generated in human blood cells. The bioactivity of the M1 metabolites and their contribution to the previously determined anti-inflammatory effects of M1 now need to be elucidated.

INTRODUCTION

Polyphenols are secondary plant metabolites and dietary components that attracted considerable research interests after epidemiological studies suggested that consumption of specific foods or dietary pattern might be associated with various beneficial health effects [1, 2]. Individual polyphenols or plant extracts have been widely tested in vitro to determine their mode of action on a cellular level. However, these assays do not take important in vivo aspects into consideration such as bioavailability, sites of distribution within the body and metabolism of the compounds.

In a previous study a standardized extract of the French maritime pine Pinus pinaster Ait. (Pycnogenol®) has been investigated [3, 4]. This extract is rich in procyanidins consisting of catechin and epicatechin units and it confers to the monograph “Maritime pine extract” in the United States Pharmacopoeia (USP) where it is specified as a food supplement. In a pharmacokinetic study with human volunteers various monomeric polyphenols such as catechin, taxifolin, ferulic and caffeic acid have been detected in plasma samples [5]. Additionally, δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) has been found, that is not originally present in the pine bark extract, but generated by intestinal microbia from catechin units [6-8]. This microbial metabolite M1 was also detectable in plasma samples after intake of Pycnogenol® [5] and attracted interest because it displayed a higher bioactivity, e.g. regarding the inhibition of nitrite generation as an index for NO production, than its metabolic precursor catechin [9]. Moreover, it was demonstrated that M1 accumulates in endothelial and blood cells in vitro after facilitated uptake [9, 10]. Recently, M1 was also quantified in human blood cells in vivo after intake of Pycnogenol® by a human volunteer [11]. To our knowledge the absolute stereochemistry of this bacteria-generated compound has not been clarified yet, although it can be assumed that only a single enantiomeric form is generated by the gut bacteria [11].

Similar to other polyphenols, M1 has functionalities that render the molecule as reactive. Although it has been pointed out that not all xenobiotics with carbon-carbon double bonds are readily subjected to a Michael reaction with e.g. glutathione [12] indeed a glutathione conjugate of M1 was detected in human blood cells [10]. Further degradation of M1 by metabolising gut microbiota, resulting in e.g. benzoic acids, has been already discussed [13, 14]. Besides gut microbiota mainly the liver and other tissues with high abundance of enzymes catalysing phase I / II metabolism have been in the focus of research activities [1]. In contrast, local metabolism in e.g. blood cells received little attention so far. However, differences in the metabolites of fatty acids and polyphenols found in plasma and blood cells have been recently described and it was suggested that the metabolites in diverse specimen provide complementary information [15].

It has been discussed that more a detailed knowledge of the metabolism of polyphenols might help understanding their health effects [2]. The purpose of the present study was to systematically and comprehensively analyze the metabolism of δ-(3,4-dihydroxy-phenyl)-γ-

valerolactone (M1) in human blood cells in vitro and in vivo. Therefore, a metabolomic approach that has been successfully applied for drug metabolite profiling was chosen [16].

METHODS AND MATERIALS

Chemicals, reagents and buffers

The reference materials δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) and δ-(3-methoxy-4-hydroxy-phenyl)-γ-valerolactone (M2) were synthesized according to M. Rappold [17]. Methanol (MeOH; LC-MS grade) was obtained from J.T.Baker Mallinckrodt (VWR, Darmstadt, Germany). Acetonitrile (ACN; LC-MS grade), water (MilliQ) and formic acid (LC-MS grade) for mobile phase were purchased from Biosolve (Valkenswaard, Netherland).

The phosphate buffered saline (PBS, pH 7.4) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 and 1.5 mM KH2PO4 (Sigma-Aldrich, Taufkirchen, Germany). A stock solution (2 mg/mL) of M1 was prepared freshly in PBS-buffer and diluted further with PBS-buffer for all incubation experiments.

Several reference compounds were synthesized (see 2.6.) for confirming the identification of M1 metabolites detected the in vitro incubation assays. For this purpose, glutathione-S-transferase from equine liver (Sigma-Aldrich) was used, which was dissolved in PBS-buffer to yield a stock solution of 100 U/mL. Esterase from Bacillus stearothermophilus recombinant from E. coli (Sigma-Aldrich) was dissolved in PBS-buffer to an activity of 293 U/mL. L-cysteine (CYS), reduced glutathione (GSH), oxidized glutathione (GSSG), ferulic acid and caffeic acid were also obtained from Sigma-Aldrich.

Source of human blood cells

Whole blood from individual donors (blood group A, Rh+, K+) were obtained freshly from a blood bank (Bayerisches Rotes Kreuz, München, Germany) and centrifuged for 25 min at 3,300 g (18 °C). Afterwards, the supernatants were discarded and the whole blood cells were used for the in vitro incubation assays.

Analysis of metabolites and data mining

For untargeted metabolite analysis, extracts were analyzed with an Acquity Ultra Performance Liquid ChromatographyTM system (UPLC) coupled to a Synapt G2 HDMSTM quadrupole time-of-flight hybrid mass spectrometer (MS) equipped with an electrospray ionization source (UPLC-ESI-qTOF-MS; Waters, Milford, MA, USA). Chromatographic separation was carried out on a BEH C18 column (2.1x100 mm I.D., 1.7 µm; Waters) with a linear binary solvent gradient of 0−30% eluent B over 6 min with a flow rate of 0.3 mL/min. The elution profile consisted a rinsing after gradient elution using 100% B for 4 min and the column was equilibrated afterwards with 0% B for 5 min. Eluent A consisted of water acidified with 0.1% formic acid and eluent B was acetonitrile. After chromatographic separation putative metabolites were ionized using ESI source operated in the positive or negative mode. The ESI capillary voltage was set to 0.8 kV and nitrogen (gas temperature: 350° C, flow rate of 800 L/h)

was used as desolvation gas. The quadrupole was operated in a wide band mass range, and data was acquired over the mass range of 50−1200 Da. Two discrete and independent interleaved acquisition functions were automatically created. The first function collected the low energy data while the second function collected the high energy data by using a collision energy ramp from 15 to 35 eV. Data acquisition was performed using MassLynx (Version 4.1; Waters). TransOmics (Version 1.0; Waters) data pre-processing software was used for the sample comparison data mining approach. For identification of expected metabolites extracted ion chromatogram (XIC) approach was applied using MetaboLynx and ChromaLynx software packages (XS Compare V4.1 SCN803; Waters). For calculation of elemental compositions in MS spectra and in silico fragments in MS/MS spectra, MassLynx embedded softwares, namely ‘Elemental Composition’ and ‘Mass Fragment’ (Version 1.1; Waters), were also applied. The setting parameters for ‘Elemental Composition’ were the following: mass tolerance= 3 mDa; range for double bond equivalent filter= -1.5 and 40; electron state= even electron only; number of isotope peaks to use= 3; element prediction filter= 9; element limits for C= 0-100, H= 0-500, N= 0-5, O= 0-40, P=0-1 and S= 0-2.

Incubation of M1 with human blood cells

Three in vitro experiments were performed by incubating human blood cells with M1. For this purpose, in an initial experiment 50 µL of fresh blood cells were incubated without (control) and with M1 (50 µM) for 0, 15, 90 min (each n= 3) and 4 hours (single experiment) at 37 °C and 300 rpm (Thermomixer®, Eppendorf, Hamburg, Germany). After addition 1 mL of methanol for protein precipitation the samples were vortexed and lysed for 20 min by ultrasonic treatment. Subsequently, the samples were centrifuged at 10,000 g for 15 min at 4 °C before the supernatants were evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 75 µL of methanol and centrifuged at 18,000 g for 15 min at 4 °C. The samples were stored in amber vials at -80 °C until UPLC-ESI-qTOF-MS analysis. The resulting putative metabolites from the two initial experiments were confirmed by repeating the whole experiment as described above. Therefore, 50 µL of human blood cells were incubated without (control) and with M1 (50 µM) for 15 min (n= 3) and 4 hours (n= 3) at 37 °C. In parallel, stability control samples with M1 in PBS-buffer were prepared and treated exactly the same.

Detection of M1 metabolites *in vivo*

Blood cells of two human volunteers who ingested a multiple oral doses of Pycnogenol® (200 and 300 mg, respectively, per day; Horphag Research) were analyzed for putative M1-metabolites. Blood cell samples either were extracted by protein precipitation with methanol (see 2.4.) or were subjected to sample preparation using the QuEChERS approach as described previously [11]. Briefly, 2.0 mL human blood cells were diluted with PBS buffer to 10 mL and 275 µL 4% o-phosphoric acid were added (pH 5.0). Afterwards, 5 mL of 1% acetic acid in acetonitrile and a salt mixture comprising 4 ± 0.2 g magnesium sulfate (anhydrous) and 1 ± 0.05 g sodium acetate was added, vortexed for 1 min (Multi-Vortex, VWR, Darmstadt,

Germany) and centrifuged for 5 min at 3,300 g (4 °C). Thereafter, 3.1 mL supernatant was transferred to a reaction tube (10 mL, Sarstedt, Nümbrecht, Germany) containing a mixture of 100 ± 5 mg PSA and 600 ± 25 mg magnesium sulfate (anhydrous) for further clean-up with dispersive solid phase extraction (d-SPE). The sample was vortexed for 1 min (Multi-Vortex) and centrifuged for 5 min at 3,300 g at room temperature. Thereafter, 1.9 mL of the upper organic layer was removed and re-acidified with 20 µL 5% formic acid in acetonitrile to protect against loss due to compound instability. Subsequently, the extract was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 75 µL of 5% formic acid in methanol and centrifuged at 18,000 g for 15 min at 4 °C. The supernatants were stored in amber vials at -80 °C until UPLC-ESI-qTOF-MS analysis.

Synthesis of reference compounds

For the synthesis of the glutathione conjugate of M1 (M1-GSH) 6 mM M1 was incubated with 5 mM GSH and 87 U glutathione-S-transferase (final concentration: 44 U/mL) in PBS-buffer for 6 hours at 37 °C on a horizontal shaker (Unimax 1010, Heidolph, Schwabach, Germany). Afterwards, the sample was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 µL of methanol. For purification and a higher yield of the M1-GSH adduct, the sample was fractionated analytically using a Waters HPLC system (Milford, MA, USA) with a 1525 binary pump, a 717plus autosampler, a model 2487 UV/VIS dual wavelength absorbance detector set at the detection wavelength of 280 nm. Data collection and integration were accomplished using Breeze TM software version 3.30. Separations were carried out on a SunFire® C18 column (4.6 x 150 µm; 5 mm particle size) from Waters. Isocratic elution was performed using 85% water acidified with 0.2% (v/ v) acetic acid and 15% acetonitrile at a flow rate of 1.5 mL/min. Retention time (tR) for M1 was 7.10 min and 4.40 min for M1-GSH. The identity and purity of M1-GSH in the fraction at tR of 4.40 min was determined by LC-MS/MS according to [17] and NMR before UPLC-ESI-qTOF-MS analysis.
For the synthesis of the oxidised glutathione conjugate of M1 (M1-GSSG) 6 mM M1 was incubated with 2.5 mM oxidized glutathione (GSSG) in PBS-buffer for 6 hours at 37 °C on a horizontal shaker (Unimax 1010, Heidolph, Schwabach, Germany). Subsequently, the sample was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 µl methanol and then diluted further 1:20 with methanol for direct injection in the UPLC-ESI-qTOF-MS system.

For the synthesis of the cysteine conjugate of M1 (M1-CYS) 6 mM M1 was incubated with 12 mM L-CYS and 30 U glutathione-S-transferase (final concentration: 21 U/mL) in PBS-buffer for 6 hours at 37 °C on a horizontal shaker (Unimax 1010, Heidolph, Schwabach, Germany). The sample was also evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 µl of methanol and then diluted further 1:100 with methanol for analysis.
The open-chained ester form of M1 (M1-COOH) was synthesized by enzymatic opening of the lactone ring of M1. Therefore, 3 mM M1 was incubated with 88 U esterase (final concentration:

147 U/mL) in PBS-buffer for 8 hours at 37 °C and 300 rpm (Thermomixer®, Eppendorf, Hamburg, Germany). The sample was diluted 1:100 with methanol and the solution was injected directly into the UPLC-ESI-qTOF-MS system.

^{1}H NMR of M1 glutathione adduct

NMR measurements were performed on a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) with a BBI 1H/D/19F Z-GRD probehead and data processing with TopSpin 3.0 software. The sample was dissolved in MeOH-d4 from Deutero GmbH (Kastellaun, Germany) and filled in standard 5 mm NMR tubes (ST 500) from Norell (Landisville, USA). The spectrum was recorded with a number of scans of 2048, spectral width 20.55 ppm, transmitter offset 6.175 ppm, flip angle 30°, acquisition time 3.985 s and a relaxation delay of 1 s at 300 Kelvin. Processing parameters were set to an exponential line broadening window function of 0.3 Hz, an automatic baseline correction and manual phasing. The spectrum was referenced to the residual solvent signal of MeOH-d4 (3.31 ppm). Because of the small amount of synthesized M1-GSH adduct a 1H NMR and a H,H-COSY experiment were measured only.

RESULTS AND DISCUSSION

Identification of in vitro biotransformation products of M1 metabolised by human blood cells

An untargeted metabolomics approach was applied in order to characterise the biotransformation products of M1 in vitro (Figure 1). To generate and identify cellular metabolites, M1 was incubated with human blood cells at 37 °C up to four hours. Two control incubations were required, one consisting of blood cells without M1 to recognize endogenous cellular compounds being formed over the course of incubation and one stability control of M1 in buffer to account for degradation products. Stability of polyphenols under cell culture conditions has to be carefully controlled even over short incubation periods to avoid erroneous conclusions [18]. Test incubation samples and control samples were analyzed by UPLC-ESI-qTOF-MS using 'all-in-one' acquisition (MSE). Due to the high peak capacity of the applied technique, improved resolution of the sample components, including endogenous metabolites and biotransformation products of M1 was achieved. In the MSE acquisition, full-scan experiments at alternating low and high collision energies (CE) were applied resulting in collection of two datasets [19] enabling simultaneous separation and structure elucidation of the biotransformation products of M1. The full-scan MS datasets recorded at low CE displayed mainly molecular ions (MS spectra), whereas the full-scan MS datasets recorded at high CE exhibited mainly the fragment ions without pre-selection of the precursor ions (MS/MS spectra). Thus, all ionisable compounds were detected with no assumptions made about the kind of metabolites that were expected to occur. Two different post-acquisition approaches, respective control sample comparison and extracted ion chromatogram (XIC) were applied to identify biotransformation products of M1 metabolised by human blood cells. The

chromatographic peaks identified as biotransformation products of M1 were then structurally characterised utilising isotope abundance distribution of the molecular ions and their fragmentation patterns [20]. The structure elucidation of the identified M1 metabolites was confirmed by comparing the retention times (tR), the exact mass and fragmentation patterns of synthesised reference materials.

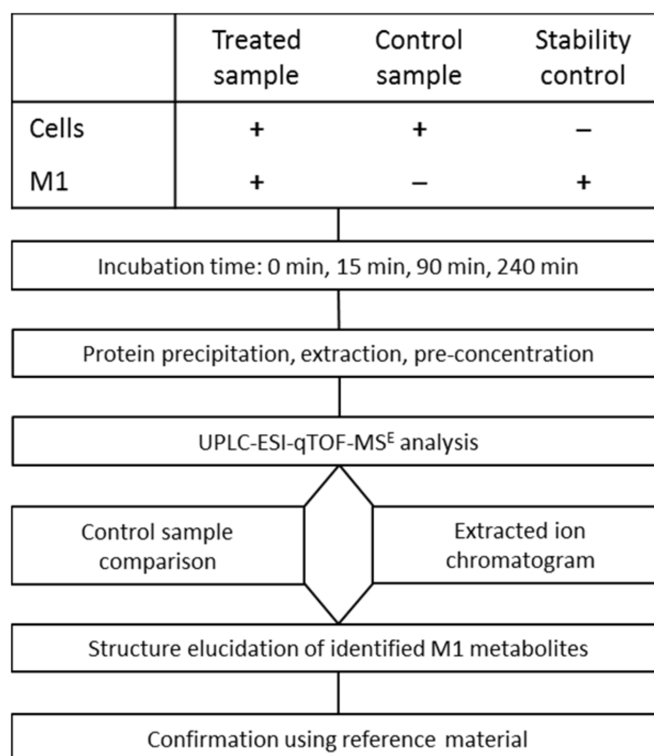

Figure 1. Workflow of the identification strategy of in vitro biotransformation products of M1 metabolized by human blood cells.

A representative base peak ion (BPI) chromatogram of test incubations and control samples measured in the positive and negative ESI mode are shown in Figure 2. A peak representing M1 (tR 5.24 min) along with other additional peaks were apparent in the incubation test samples in comparison to the control samples. The whole experiment was repeated two times independently, using different batches of human blood cells. M1 and the additional peaks were detected in the incubation test samples of each of the replicates.

However, in one experiment, in which the human blood cells were slightly beyond their expiration-date, the abundances of the putative M1 metabolite peaks were only ~10-30% compared to the other two experiments indicating that the biotransformation rate of M1 is likely to be dependent on the physiological activity of the human blood cell preparation.

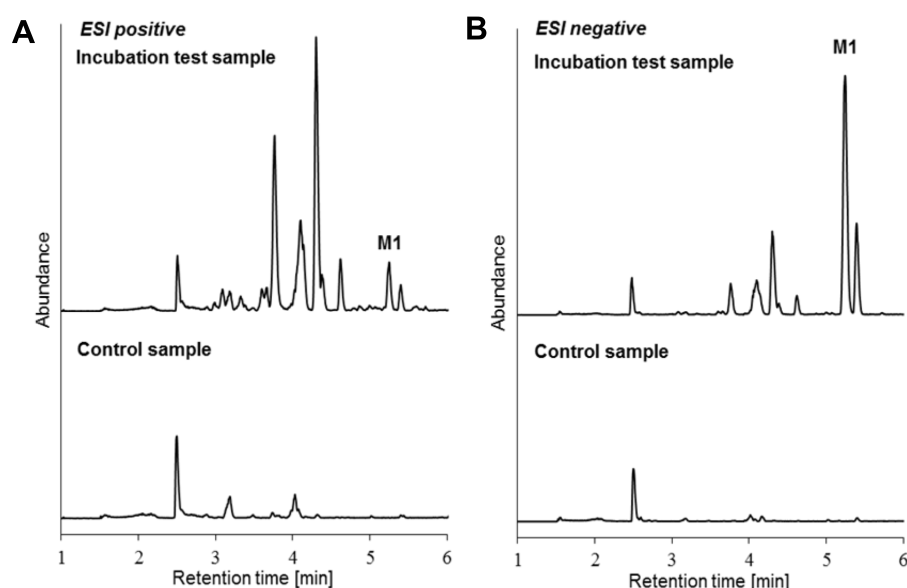

***Figure 2. Base peak ion chromatograms (BPI) revealed several biotransformation products of M1 after incubation of 15 min. A**. BPI of samples measured in the positive ESI mode. **B**. BPI of samples measured in the negative ESI mode.*

Since manual inspection of the BPIs in the positive and negative ESI mode revealed at least 14 biotransformation products of M1, acquired data sets were further processed. Initially, a control sample comparison approach was applied, which filtered peaks that were not present in control samples to identify all detectable biotransformation products of M1. After peak selection and manual inspection, 12 out of 53 in negative ESI mode and 54 out of 140 in positive ESI mode were identified as unique chromatographic peaks in the incubation test samples. All 66 putative M1 metabolites could be confirmed in the other independent in vitro experiments.

In addition to control sample comparison, expected biotransformation products of M1 were profiled using the XIC approach. Therefore, an extensive list of possible biotransformation reactions, like desaturation, hydroxylation, methylation, acetylation, glucuronide and glutathione conjugation, in conjunction with the elemental composition of M1 was generated. XICs that corresponded to ion currents that fell within a 10 mDa window around m/z values of the expected metabolites then were compared between the test and control samples in order to filter out those peaks that were unique for the incubation test samples. In total, 12 chromatographic peaks appearing in negative ESI mode and 49 peaks in the positive mode were annotated as additional 61 biotransformation products of M1.

After post-acquisition of the measured data sets using sample comparison and XIC approach, peaks identified as further biotransformation products were manually inspected by (i) determining the elemental compositions from isotope abundance distributions of the molecule ions in the MS spectra and (ii) looking for in silico fragments of the putative M1 metabolites in the MS/MS spectra. The total number of elucidated M1 metabolite peaks was thus further reduced from 24 to 10 in negative ESI and from 103 to 9 in positive ESI. The 19 metabolite

peaks were categorized into three levels of identification with six compounds each confirmed at level 1 and 2 and seven putative metabolites at level 3 (Tables 1 and 2). The first level describes identified compounds that were confirmed with chemical reference standards, level 2 are putatively annotated compounds that were identified based on the measured exact mass, specific MS/MS fragments and elemental composition and level 3 are putatively characterized compounds based on the measured exact mass and elemental comparison with spectral libraries [20].

Elucidation of the sulphur containing biotransformation products of M1

The formation of an intracellular conjugate of M1 and glutathione (GSH) was already known [10] and therefore anticipated to be found in the present investigation as well. However, two novel insights were gained regarding this glutathione conjugate.

Firstly, two isomers were detected in the human blood cells. Two peaks at tR of 4.10 min and 4.30 min corresponding to M1-GSH were detected in the negative ESI (m/z of 512.134) as well as in the positive ESI mode (m/z of 514.1495; Figures 3 and 4 A; Table 1).

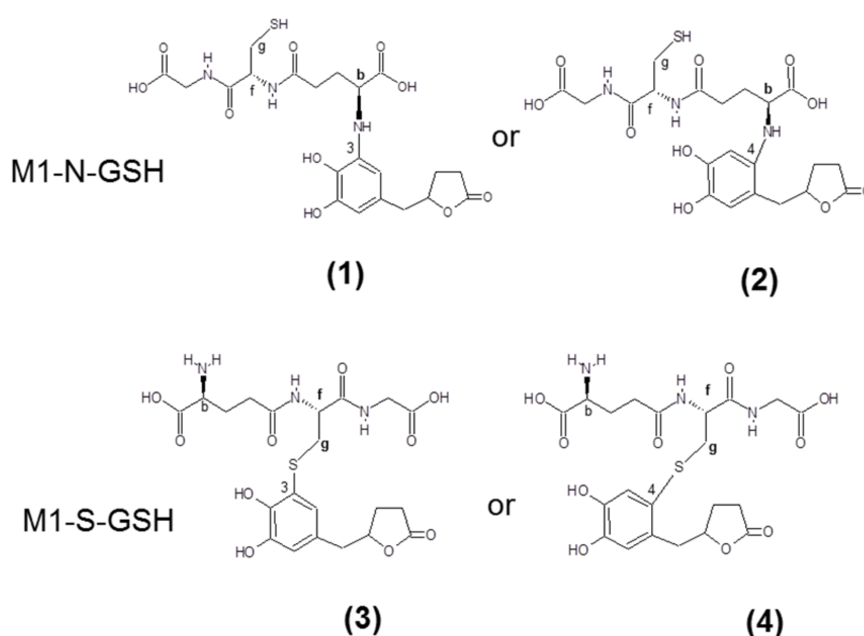

Figure 3. Structural formulas of the conjugation of M1 and glutathione (M1-GSH) yielding two isomers which bind either via the α–amino group of the glutamic acid of glutathione (M1-N-GSH; upper panel) or with the thiol group of the cysteine (M1-S-GSH; lower panel).

The calculated elemental compositions for both peaks were $C_{21}H_{28}N_3O_{10}S$ in positive ESI which corresponded to a molecular formula of $C_{21}H_{27}N_3O_{10}S$. The peak area at tR of 4.30 min was almost two times higher than the peak at tR of 4.10 min. The MS/MS spectra of the two isomer peaks of glutathione conjugates of M1 revealed differences in their fragmentations (Figure 4 B). In addition to the common fragment ions at m/z of 130.050, 439.118 and 385.107, unique fragment ions at m/z of 145.061 and 84.045 at tR of 4.10 min and m/z of 282.080, 265.053, 368.080, 205.032 and 112.039 at tR of 4.30 min were observed. The

conjugation of M1 with glutathione was confirmed by a reference compound (synthesis see 2.6.). A peak with high intensity at tR of 4.30 min and a small peak at tR of 4.10 min were detected after injection of the reference solution. The measured exact masses and calculated elemental compositions of both isomers were identical. Moreover, the fragment patterns of the two isomers from the reference compound and from the in vitro experiments were consistent with each other. For the synthesis of the reference material glutathione-S-transferase was used, which typically catalyses the binding of glutathione via the thiol group of the cysteine. However, recently it was reported that besides the nucleophilic substrate attack via the cysteinyl thiol group glutathione might also bind via the α–amino group of the glutamic acid [21].

The present results confirm this observation and point out that this novel GSH conjugate can be naturally generated in human blood cells. It is proposed that the more intensive peak at tR of 4.30 min corresponds to the conjugation of M1 and glutathione via the thiol group of the cysteine (M1-S-GSH; (3) or (4)) and that the peak at tR of 4.10 min is a conjugate of M1 via the α–amino group of the glutamic acid of glutathione (M1-N-GSH; (1) or (2)). In silico fragments of M1-S-GSH and M1-N-GSH were generated and matched to the measured MS/MS spectra at tR 4.30 min and 4.10 min, respectively. Decisive fragments for M1-N-GSH and for M1-S-GSH could be matched.
To further elucidate the structure of the glutathione conjugates of M1, NMR analysis of the reference compound was performed. The 1H NMR spectrum of M1-GSH adduct indicated a content of reactant M1 of about 6% and also a binding of glutathione with M1. The glutathione 1H NMR signals were assigned according to [22]. After binding of M1 the glutathione signals f, b and g displayed a different chemical shift compared to the glutathione spectrum (Figures 3 and 4 C). The signals g and f were influenced due binding over the sulfur of glutathione to M1. Signal g was low field shifted and signal f high field shifted, strongly indicating a binding via sulfur. However, the M1-GSH-adduct spectrum showed a signal for b shifted to low field compared to glutathione. This was likely due to an additionally binding via the nitrogen of the glutamine acid moiety as previously described [21]. The NMR spectrum of the reference compound showed bonding of glutathione via the thiol group of cysteine and additional binding via the α–amino group of the glutamic acid moiety of glutathione (Figure 4 C). Unfortunately, a confirmation of this assumption by NMR was hindered because of the small amount of available M1-GSH adduct, the high molecular mass and the impurity M1 reactant, therefore further investigations are recommended. The confirmation of the signal assignment was done by a H,H-COSY experiment (Figure 4 D). The coupling constant of the aromatic signals of 2.3 Hz and 2.0 Hz suggested a binding at position 3 of M1 with glutathione. A meta coupling constant is usually between 1-3 Hz, while a para coupling is smaller than 1 Hz. Furthermore a nucleophilic substitution could be preferred in this position, because of steric reasons and the leading effect of the –OH group in ortho position. For both isomers we suggest two possible structures, because it is not clear yet whether the binding of glutathione occurs at the position

C3 or C4 of M1. Further clarification of the structures could be achieved by a more sensitive 13C-NMR.

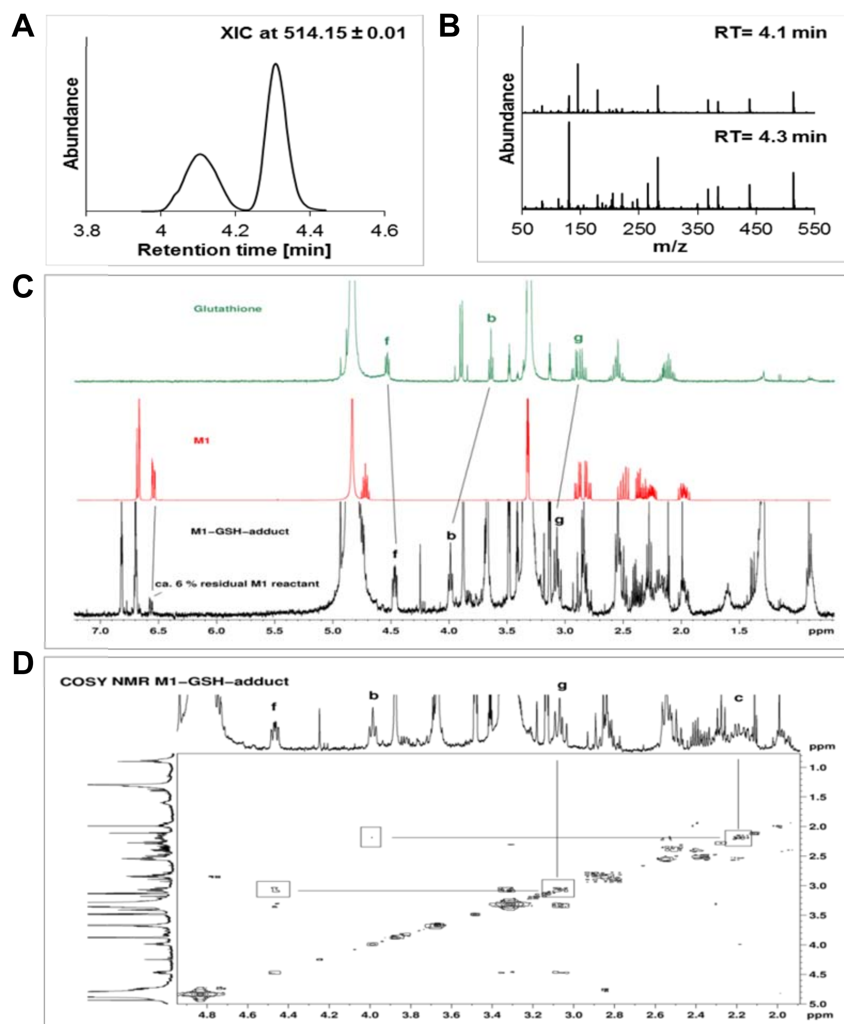

Figure 4. Data supporting the conjugation of M1 and glutathione (M1-GSH) yielding the two isomers M1-N-GSH and M1-S-GSH. A*. Extracted ion chromatogram (XIC) of 514.1489 m/z in the positive ESI mode.* ***B****. MS/MS spectra of the two isomers (tR of 4.10 min and 4.30 min) of the M1-GSH adducts.* ***C****. 1H NMR spectra of glutathione (top), M1 (middle) and M1-GSH adduct (bottom) in MeOD-d4.* ***D****. H.H-COSY spectrum of M1-GSH adduct in MeOD-d4*

To further confirm the presence of M1-N-GSH, profiling for the conjugated form of M1 with oxidized glutathione (M1-GSSG, (5); Figure 5) was performed since oxidized glutathione lacks a free sulfhydryl functionality that could react with M1. A peak at tR of 3.79 min in the XIC of 817.202 ± 0.01 in the negative ESI and XIC of 819.217 ± 0.01 in the positive ESI mode was detected. M1-GSSG ionized nearly tenfold better in positive ESI than in the negative ESI mode. The calculated elemental composition at m/z of 817.202 was assigned C31H41N6O16S2 at high confidence level (99.80%). The MS/MS fragmentation pattern yielded several specific fragments (m/z of 544.105, 306.077, 271.009, 254.078 and 143.046) that were fitted to the fragments generated in silico. The structure elucidation was confirmed with reference material by comparing the retention times, exact masses, calculated elemental

compositions and fragment patterns of the reference peak with the peak measured in test incubation samples.

The second new insight in the context of the present study was that both M1-S-GSH and M1-N-GSH were formed exceedingly rapid with their concentrations subsequently remaining basically unchanged over an incubation time of 4 hours (Figure 6).
Besides catalysis by glutathione-S-transferases also non-enzymatic conjugation reactions with glutathione are well known. Apparently, non-enzymatic conjugation is preferred at high GSH concentrations [23]. Interestingly, rapid intracellular accumulation and formation of GSH adducts has been also reported for other dietary compounds such as isothiocyanates [24].
In search of other derivatives of M1-glutathione in the test incubation samples, profiling for reduction and hydroxylation reaction products of M1-GSH (Figure 5), here referred to as reduced M1-GSH and hydroxylated M1-GSH.
A peak at tR of 4.11 min in the negative ESI mode with an m/z of 510.1179 was detected as putative reduced M1-GSH. The calculated elemental composition was $C_{21}H_{24}N_3O_{10}S$. The intensity of the fragment ions was too low to investigate further the MS/MS in negative ESI. In addition, M1-N-GSH co-eluted that might have led to ion suppression of the reduced M1-GSH signal. It could be excluded that the peak was an in-source fragment of M1-N-GSH since no m/z of 510.118 was detected when precursor ion at m/z of 512.134 was selected with the quadrupole. A small but significant signal decrease over the incubation time was determined (peak areas were 34 ± 5 at 15 min and 23 ± 1 at 4 h). Profiling for the hydroxylated M1-GSH, a peak at tR of 3.70 min was detected in the XIC of 530.144 ± 0.01 using positive ESI. The calculated elemental composition was $C_{21}H_{28}N_3O_{11}S$ at a confidence level of 93.01%. Although the level of putative hydroxylated M1-GSH increased with the incubation time (peak areas were 21 ± 11 at 15 min and 198 ± 100 at 4 h), the peak areas were too low to investigate the fragmentation pattern for structure elucidation.

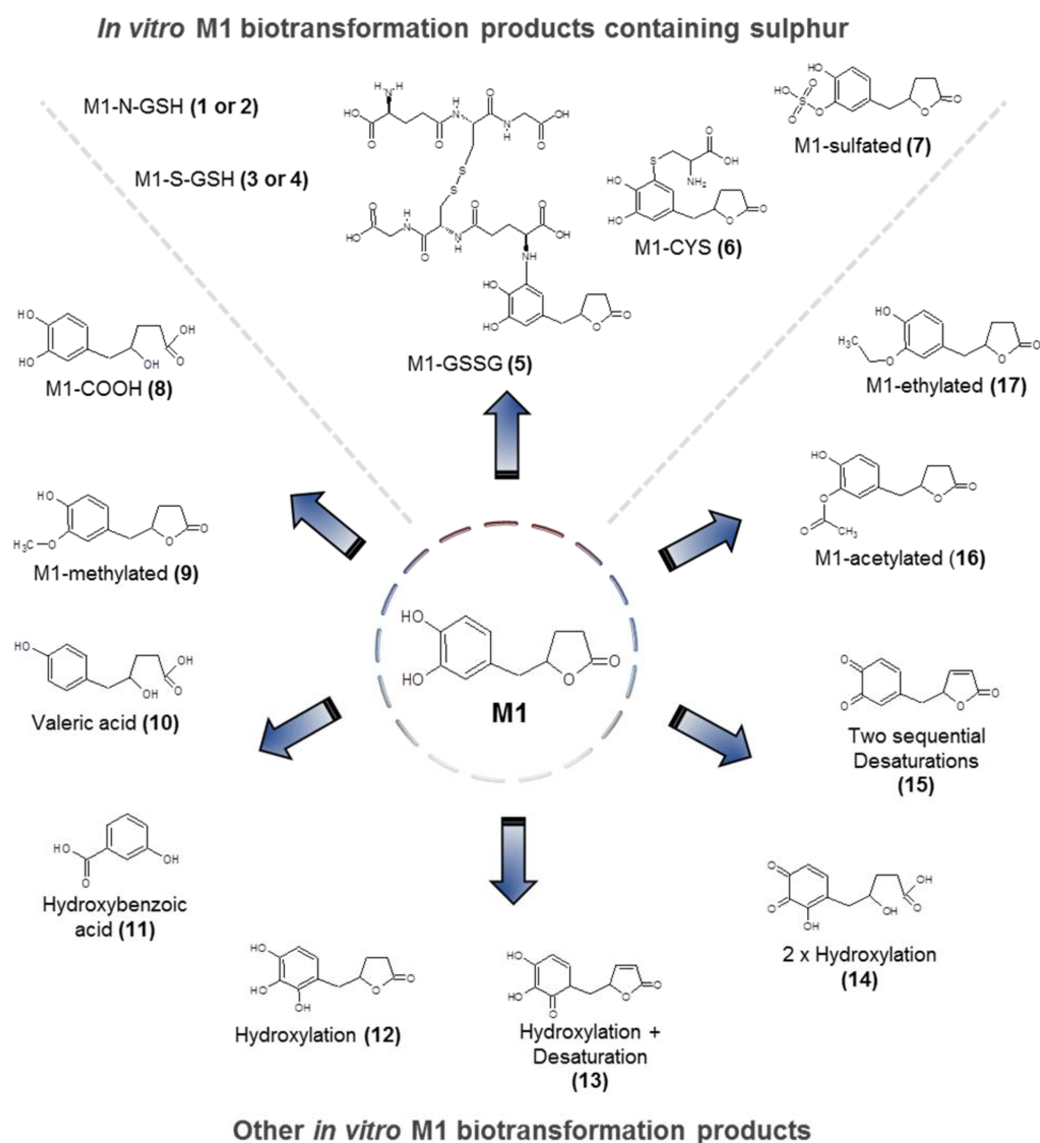

Figure 5. Structural formulas of putative in vitro M1 biotransformation products containing sulphur and other metabolites. *All listed compounds have been validated and categorized into three levels of identification according to the Metabolomics Standards Initiative (see Tables 1 and 2).*

Additionally to M1-GSH, the M1 conjugate with oxidized glutathione (M1-GSSG; (5)) was one of main in vitro biotransformation product of M1. In contrast to the GSH adducts, the presence this conjugate was decreasing over the incubation time with the level of M1-GSSG being half after 4 hours in comparison to incubation over 15 minutes (peak areas were 437 ± 258 at 4 h and 923 ± 111 at 15 min).

Moreover, a cysteine conjugate of M1 (M1-CYS, (6); Figure 5) was detected in the XIC of 328.085 ± 0.01 as a peak at tR of 3.94 min using positive ESI. The elemental composition was determined as C14H18NO6S at confidence level of 99.30%. The MS/MS spectrum yielded specific fragments such as m/z-s of 221.027 (C11H8O3S), 155.014 (C7H6O2S) and 85.029 (C4H4O2). The tR and fragment pattern of reference compound were identical to the peak at 3.94 min in the test incubation samples. The level of M1-Cys increased with the incubation

time (Figure 6). Although the role of the M1 metabolites still needs to be investigated in more detail, conjugates with GSH or cysteine have been reported to contribute to the bioactivity of dietary compounds, e.g. in case of thiosulfinates derived from Allium vegetables [25].

A sulfated derivative of M1 (M1-sulfated, (7); Figure 5) was identified at tR of 4.50 min in ESI negative mode with m/z of 287.023 (mass difference between measured and calculated m/z-s was 0.4 mDa). The calculated elemental composition was $C_{11}H_{11}O_7S$ (confidence level of 99.99%). The MS/MS spectrum revealed in specific fragments of 207.066, 163.076 and 122.037 corresponded to fragment ions of M1 and 79.957 corresponded to sulfate residue. After 4 h incubation time the level of M1-sulfated increased approximately tenfold compared with an incubation time of 15 min (peak areas: 48 ± 9 at 15 min and 451 ± 139 at 4 h; Figure 6).

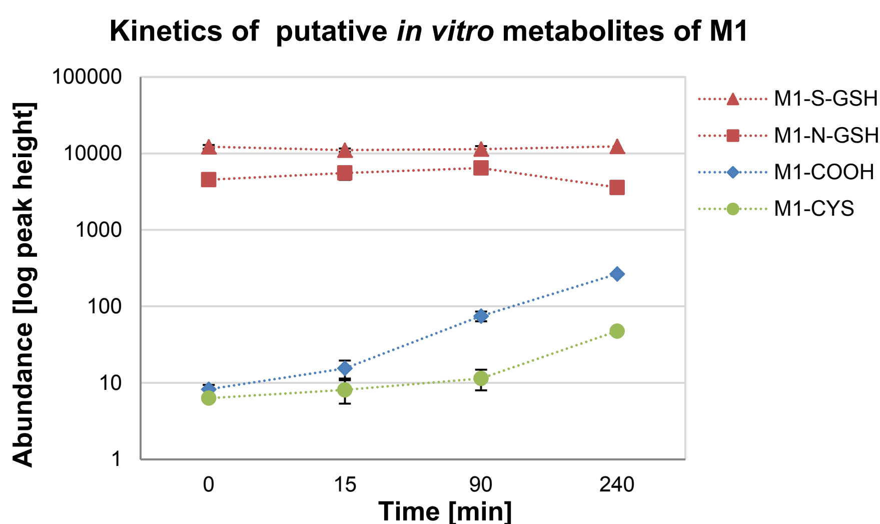

Figure 6. Kinetics of the in vitro formation of some putative M1 metabolites. *Human blood cells were incubated with 50 µM M1 for 0, 15, 90 min (each n= 3) and 4 hours (single experiment) at 37 °C. With increasing incubation time increasing concentrations of the M1-CYS adduct and M1-COOH were generated, whereas the formation of the two M1-GSH isomers (M1-N-GSH and M1-S-GSH) remained largely unaffected.*

Table 1: ***Interesting putative in vitro M1 biotransformation products containing sulphur.*** *The following metabolites were identified and classified as level 1, 2 or 3 according to Sumner et al. [20]. The molecular formula is the measured exact mass ± 1.0078 m/z depending on the ESI mode.*

Putative metabolite	**t_R [min]**	**ESI mode**	**Measured exact mass [m/z]**	**Molecular weight** (neutral)	**Molecular formula**	**Level**	**MS/MS spectrum: specific fragments**	**Proposed structure**
M1-N-GSH	4.10	positive	514.1489	513.1411	C21H27N3O10S	1	145.0613 m/z: C5H9N2O3; 130.0502 m/z; C5H8NO3; 439.1175 m/z: C19H23N2O8S; 385.1069 m/z: C16H21N2O7S; 84.0449 m/z: C4H6NO	**(1)** or **(2)** Figure 3
M1-S-GSH	4.30	positive	514.1489	513.1411	C21H27N3O10S	1	130.0504 m/z: C5H8NO3; 282.0802 m/z: C13H16NO4S; 265.0533 m/z: C13H13O4S; 439.1175 m/z: C19H23N2O8S; 385.1069 m/z: C16H21N2O7S; 368.0803 m/z: C16H18NO7S; 205.0320 m/z: C11H9O2S; 112.0394 m/z: C5H6NO2	**(3)** or **(4)** Figure 3
M1-GSSG	3.79	negative	817.2020	818.2098	C31H42N6O16S2	1	544.1055 m/z: C21H26N3O10S2; 306.0768 m/z: C10H16N3O6S; 271.0085 m/z: C9H9N3O3S2; 254.0777 m/z: C10H12N3O5; 143.0461 m/z: C5H7N2O3	**(5)** Figure 5
Reduced M1-GSH	4.11	negative	510.1179	511.1257	C21H25N3O10S	3	-	
Hydroxylated M1-GSH	3.70	positive	530.1443	529.1365	C21H27N3O11S	3	-	
M1-CYS	3.94	positive	328.0858	327.078	C14H17NO6S	1	221.0272 m/z: C11H8O3S; 155.0139 m/z: C7H6O2S; 85.0290 m/z: C4H4O2	**(6)** Figure 5

M1-sulfated	4.50	negative	287.0229	288.0307	C11H12O7S	2	207.0664 m/z: C11H11O4; 163.0762 m/z: C10H1102; 122.0370 m/z: C7H6O2; 79.9567 m/z: SO3 (0.1 mDa)	**(7)** Figure 5

Table 2: Interesting putative in vitro M1 biotransformation products that were expected and unexpected. *The following metabolites were identified and classified as level 1, 2 or 3 according to Sumner et al. [20]. The proposed structures of the metabolites hydroxybenzoic acid and valeric acid based on Monagas et al. [26]. The metabolite "hydroxylation" is according to Meng et al. [7] and the M1-metabolites with a glutathione conjugation (M1-GSSG, M1-N-GSH) are based on the publication of Mali'n et al. [21]. The molecular formula is the measured exact mass ± 1.0078 m/z depending on the ESI mode.*

Putative metabolite	**t_R [min]**	**ESI mode**	**Measured exact mass [m/z]**	**Molecular weight** (neutral)	**Molecular formula**	**Level**	**MS/MS spectrum: specific fragments**	**Proposed structure**
M1-COOH	4.01	negative	225.0758	226.0836	C11H14O5	1	207.0657 m/z: C11H11O4 (0.3 mDa); 123.0446 m/z: C7H7O2 (1.8 mDa); 101.0239 m/z: C4H5O3 (0.7 mDa)	**(8)** Figure 5
M1-methylated	6.40	positive	223.0973	222.0895	C12H14O4	1	163.0750 m/z: C10H11O2 (0.9 mDa); 137.0625 m/z: C8H9O2 (2.2 mDa); 131.0497 m/z: C9H7O (0 mDa); 103.0545 m/z: C8H7 (0.2 mDa)	**(9)** Figure 5
Valeric acid	5.70	negative	209.0813	210.0891	C11H14O4	2	181.0714 m/z: C6H13O6 (0.2 mDa); 133.0652 m/z: C9H9O (0.1 mDa); 177.052 m/z: C10H9O3 (1.4 mDa); 112.9863 m/z: C4HO4 (1.2 mDa)	**(10)** Figure 5
Hydroxybenzoic acid	5.45	negative	137.0239	138.0317	C7H6O3	3	-	**(11)** Figure 5

Hydroxylation	4.00	positive	225.0767	224.0689	$C_{11}H_{12}O_5$	3	-	**(12)** Figure 5
Hydroxylation +Desaturation	4.90	negative	221.0451	222.0529	$C_{11}H_{10}O_5$	2	177.0558 m/z: $C_{10}H_9O_3$ (0.6 mDa); 160.0529 m/z: $C_{10}H_8O_2$ (0.5 mDa); 133.065 m/z: C_9H_9O (0.3 mDa); 108.0213 m/z: $C_6H_4O_2$ (0.2 mDa); 82.0058 m/z: $C_4H_2O_2$ (0.3 mDa)	**(13)** Figure 5
2 x Hydroxylation	3.50	negative	239.0566	240.0644	$C_{11}H_{12}O_6$	2	195.0665 m/z; 143.0457 m/z; 128.0360 m/z; 96.9632 m/z	**(14)** Figure 5
Two Sequential Desaturations	5.03	positive	205.0501	204.0423	$C_{11}H_8O_4$	2	187.0398 m/z: $C_{11}H_7O_3$ (0.3 mDa); 97.0290 m/z: $C_5H_5O_2$ (0.8 mDa); 85.0287 m/z: $C_4H_5O_2$ (0.3 mDa)	**(15)** Figure 5
Demethylation	4.86	positive	195.0661	194.0583	$C_{10}H_{10}O_4$	3	153 .0544 m/z: $C_8H_9O_3$ (0.8 mDa); 89.0607 m/z: $C_4H_9O_2$ (0.4 mDa)	
M1-acetylated	2.99	positive	251.0919	250.0841	$C_{13}H_{14}O_5$	3	-	**(16)** Figure 5
Deethylation	5.54	positive	181.0501	180.0423	$C_9H_8O_4$	3		
M1-ethylated	6.40	positive	237.1127	236.1049	$C_{13}H_{16}O_4$	2	206.0879 m/z; 163.0781 m/z; 137.0612 m/z	**(17)** Figure 5

Elucidation of expected and unexpected biotransformation products of M1

Stability of M1 in PBS-buffer (pH 7.4) was controlled at each incubation time point. One additional peak at tR of 5.38 min and m/z of 239.093 was detected in the stability control samples that was identified as methylated and open-chained ester form of M1. It was found to be produced during sample preparation, specifically in the course of protein precipitation extraction with methanol. Profiling was also performed for the open-chained ester form of M1 (M1-COOH (8); Figure 5; Table 2). A peak at tR of 4.01 min in XIC of 225.076 ± 0.01 was detected using negative ESI ionization. The calculated elemental composition of 225.076 was $C_{11}H_{13}O_5$ and fragments at m/z of 207.066 (molecular ion of M1), 123.045, 101.024 were observed in the MS/MS spectra (negative ESI). The structure was confirmed by comparing the tR, the exact mass and fragments of the reference compound. Although M1-COOH was also detected in the stability control samples, the peak areas in the test incubation samples were always significantly higher.

Profiling was also done for the methylated M1 derivative ((9), Figure 5), also referred to as M2, which has been previously found in vivo [6]. It was detected at tR of 6.40 min and m/z of 223.0973 m/z using positive ESI. The calculated elemental composition was $C_{12}H_{15}O_4$ (confidence level of 99.99%). Fragments at m/z of 163.075, 137.063, 131.050 and 103.054 in the MS/MS spectra were identical to the fragments measured in the reference solution. Peak areas increased with the incubation time (9 ± 1 at 15 min and 104 ± 13 at 4 h).

Since valeric acid derivates have been proposed as biotransformation products of M1 in the literature [8, 26], profiling for 4-hydroxy-5-(3‘-hydroxyphenyl) valeric acid and 5-(3,4 dihydroxyphenyl)-valeric acid was performed in the measured data sets. A peak was detected at tR of 5.70 min in the XIC of 209.081 ± 0.01 using negative ESI. The calculated molecular formula was identical to the published one ($C_{11}H_{14}O_4$). The MS/MS spectra at tR of 5.70 min mean revealed fragments at m/z of 181.071, 133.065, 177.052 and 112.986. The most intensive fragment with a m/z of 133.0652 that was also predicted as a fragment of 4-hydroxy-5-(3‘-hydroxyphenyl) valeric acid (10), a molecule that has been suggested as possible metabolite of M1 [26]. The intensities decreased slightly but significantly over the incubation time (peak areas of 8.7 ± 1.0 at 15 min and 5.5 ± 0.5 at 4 h).

Another possible metabolite of M1 was hydroxybenzoic acid (11) that was proposed to be formed by the intestinal microbiota [26]. A peak at tR of 5.45 min and m/z of 137.024 was detected in the negative ESI mode. Although the elemental composition of the peak was calculated from isotope abundance distribution, specific fragments could not be determined due to the low intensity of the molecular ion peak.

To detect M1 transformation through hydroxylation reactions, profiling of peaks corresponding to hydroxylated, hydroxylated and desaturated as well as double hydroxylated derivatives of M1 was performed. For hydroxylated M1 (12), a small peak at tR of 4.00 min with elemental composition of $C_{11}H_{13}O_5$ (confidence level of 96.4%) was detected in the XIC of 225.077 ± 0.01 using positive ESI. Due to the low abundances of the peaks, the structure could not be further elucidated by inspecting the fragmentation pattern. XIC of hydroxylated and

desaturated M1 (13) revealed a peak at tR of 4.90 min and m/z of 221.045. In the MS/MS spectra fragments at m/z of 177.056 ($C_{10}H_9O_3$), 160.053 ($C_{10}H_8O_2$), 133.065 (C_9H_9O), 108.021 ($C_6H_4O_2$), 82.006 ($C_4H_2O_2$) were observed as possible fragment ions of 225.077. A peak corresponding to double hydroxylated form of M1 (14) was detected at tR of 3.50 min in the XIC of 239.057 ± 0.01 using negative ESI. The calculated elemental composition was $C_{11}H_{11}O_6$ at confidence level of 76%. The intensity of the fragments at tR of 3.50 min in the MS/MS spectra were low, but specific fragments at m/z of 195.066, 143.046, 128.036 and 96.963 could be identified. The peak intensities increased with the incubation (peak areas were 16.1 ± 1.5 at 15 min and 31.8 ± 8.7 at 4 h).

Desaturated or two sequential desaturated forms of M1 were sought in the test incubation samples. Although no peak was found for desaturated M1, a peak at tR of 5.03 min and m/z of 205.0501 was detected using positive ESI that could correspond to two sequential desaturated form of M1 (15). The calculated elemental composition in the MS spectra was $C_{11}H_9O_4$ at a confidence level of 99.99%. The intensity of the fragment pattern observed in the MS/MS spectra was low, but in silico calculation proposed matching fragments at m/z of 187.040 ($C_{11}H_7O_3$), 97.029 ($C_5H_5O_2$), 85.029 ($C_4H_5O_2$). The peak intensities increased slightly with the incubation time (peak areas were 54.9 ± 9.5 at 15 min and 104.1 ± 9.7 at 4 h). Other possible biotransformation products of M1 such as demethylation, deethylation, acetylation (16) or ethylation (17) metabolites are summarized in Table 2 and Figure 5.

Some of these compounds such as the open-chained ester form of M1 (M1-COOH; (8)) were expected since they have been previously described in the course of tentatively proposed metabolic pathways for the catabolism of polyphenols by gut microbia [8, 26] or as metabolites found in plasma or urine samples after catechin ingestion [7]. However, the present study provides the first evidence that multiple and structurally heterogeneous polyphenol metabolites can be also generated in human blood cells.

Determination of in vitro biotransformation products of M1 in authentic blood samples

Since metabolites found in in vitro assays might be of limited physiological relevance blood cells of two volunteers who ingested multiple oral high doses of the procyanidine-rich pine bark extract Pycnogenol® (200 and 300 mg, respectively, per day) were also analyzed. This has been previously shown to produce detectable plasma and also blood cell concentrations of M1 in vivo [5]. For M1 metabolite detection blood samples were subjected to two different sample preparation techniques, a simple protein precipitation with methanol and a dispersive solid phase extraction method known as QuEchERs [27]. In an earlier study the latter method was optimized for sensitive detection of M1 [11]. In contrast, in the present study both methods gave comparable results regarding detection of M1 metabolites, though the protein precipitation allowed the discovery of more metabolites than the QuEchERs approach. However, protein precipitation with methanol also generated a high number of methylated artefacts such as the methylated open-chained ester form of M1 (methylated M1-COOH), methylated M1-GSSH (tR 4.09 min and 849.213 m/z in ESI negative) or methylated M1-GSH

(tR 4.65 min and 544.151 m/z in ESI negative). Therefore, a protein precipitation with e.g. acetonitrile might be more advisable, though it is not clear how it would compare with the QuEchERs sample preparation. The biotransformation products of M1 identified in vitro were compared with those detected in vivo. M1-COOH (n= 2; (8)), M1-sulfated (n= 1; (7)), M1-S-GSH (n= 1; (3) or (4)), M1-methylated (n= 2; (9)), M1-acetylated (n= 5; (16)) and putative hydroxybenzoic acid (n= 1; (11)) were detected in vivo using protein precipitation extraction (Figure 7). In addition, the samples were prepared using QuEChERS approach [11]. This sample preparation technique detected M1-sulfated (7), M1-methylated (9) and M1-acetylated (16).

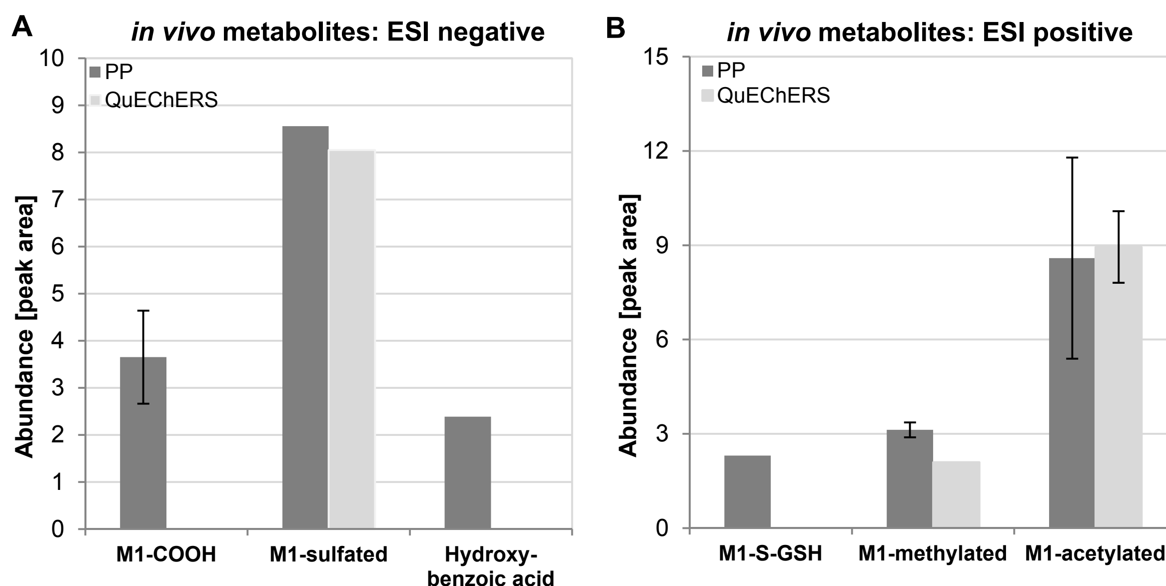

Figure 7. In vivo metabolites of M1 in blood cells of two human volunteers who ingested multiple oral doses of Pycnogenol® (200 and 300 mg, respectively, per day). *Blood cell samples were subjected to different sample preparation techniques: simple protein precipitation (PP) with methanol (see 2.4.) and QuEChERS approach (see 2.5.). Columns show mean and mean deviation of the mean values.* ***A****. In vivo metabolites detected in the ESI negative mode. The open-chained ester form of M1 (M1-COOH), the sulfated form of M1 (M1-sulfated) and putative hydroxybenzoic acid (exact mass of 137.0239 [m/z]- and molecular formula C7H6O3) were found.* ***B****. In vivo metabolites detected in the ESI positive mode. One isomer of the conjugation of M1 with glutathione (M1-S-GSH; tR = 4.30 min) and the methylated (M1-methylated) and acetylated (M1-acetylated) form of M1 were found.*

These in vivo results confirmed the validity of the in vitro experiments. Six of the same metabolites that were formed in blood cells ex vivo after incubation with a synthesized mixture of both M1 enantiomers were also found in vivo in the blood cells of volunteers after oral ingestion of the pine bark extract yielding the bacterial metabolite M1 which is possibly a single enantiomer. Thus, although the absolute stereochemistry of this bacteria-generated compound has not been clarified yet the fact that the main metabolites uncovered ex vivo are consistent with those detected in vivo suggests that metabolic processes have been most likely correctly characterized.

However, more research is underway to elucidate stereochemical aspects of M1 and its metabolites.

The extensive metabolism of M1 also contributes to understanding why rather low concentrations of the parent compound M1 previously found in blood cells (0.26 ng/mL M1 after intake of 100 mg Pycnogenol®; [11]) compared to plasma (3.01 ± 0.38 ng/mL M1 after ingestion of 200 mg Pycnogenol®; [5]) although it was demonstrated that M1 accumulates in endothelial and blood cells after facilitated uptake [9, 10].

CONCLUSION

In the present study the fate of the bioactive catechin metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) was systematically analyzed in vitro and in vivo. This is the first study that shows that the polyphenol M1 is comprehensively metabolized in human blood cells. The predominant metabolites were glutathione conjugates which were rapidly formed and revealed prolonged presence within the cells. Thereby, the formation of two isomers of glutathione conjugates, M1-S-GSH and M1-N-GSH, were observed. The bioactivity of the M1 metabolites and their contribution to the previously determined anti-inflammatory effects of M1 now need to be elucidated.

Conflict of interests

M.M, A.F., J.W., U.H., and M.J.M. declare no conflict of interests. P.H. received unrestricted research grants from Horphag Research, the producer of Pycnogenol®, within the past three years. However, the current work has not been funded by Horphag Research or any other organization.

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4 Development of LC-ESI/MS/MS methods for quantification of polyphenols in human plasma and serum with particular consideration of matrix effects

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ABSTRACT

The purpose of this study was to develop and validate a sensitive, robust and accurate LC-ESI/MS/MS method for quantification of selected polyphenolic constituents and a bioactive metabolite (M1) of maritime pine bark extract (Pycnogenol®) at trace levels in human plasma and serum. Thereby, particular consideration was given to absolute and relative matrix effects. Additionally, a comparison of the accuracy using two internal standard (IS) options, a structural and chemical analog, was exemplified for one analyte.

Various sample preparation techniques including solid phase and liquid-liquid extraction, protein precipitation and combined approaches were elucidated and the optimized sample clean-up procedure was used for method validation. Analyte recovery (RE), matrix effects (ME) and process efficiency (PE) were compared both between human plasma and serum as well as between pooled and individual lots of the respective matrices to account for interindividual variability between single patient samples. Furthermore, the IS hydrocaffeic acid and ferulic acid-1,2,3-13C3 were compared regarding the accuracy of the quantification of ferulic acid and the variability of the IS-normalized matrix factor.

In both matrices the validated method achieved low limits of detection ranging between 0.064 ng/mL for taxifolin and 8.22 ng/mL for caffeic acid, allowing a reliable and accurate quantification of the analytes. The RE was higher from pooled serum compared to plasma for most analytes while the PE was comparable for both matrices. The differences in the analyte RE between pooled matrix versus individual lots were clearly more pronounced for plasma as opposed to serum. The absolute as well as the relative ME were more pronounced in human plasma than in serum. Especially M1 and ferulic acid were subjected to a higher ion enhancement in plasma. The variability of the IS-normalized matrix factor calculated from different matrix lots was also higher in plasma compared to serum. Notably, the enzymatic hydrolysis of analyte conjugates prior to sample extraction significantly contributed to the ME, especially the IS hydrocaffeic acid was subjected to a strong ionization enhancement. No clear advantage of either IS was observed. The accuracy of the quantification of ferulic acid in six different lots of human plasma was in excellent agreement when using the structural analog

and the 13C-labeled IS. Finally the comprehensively analyzed and validated method was successfully applied to an authentic human serum sample and can now be used for further pharmacokinetic studies to gain more insight into the absorption, distribution and elimination of polyphenols in humans.

INTRODUCTION

In the last few years liquid chromatography coupled to electrospray-ionization tandem mass spectrometry (LC-ESI/MS/MS) has been increasingly applied in bioanalytical and pharmaceutical research for quantification of target analytes in complex human biological samples [1]. Besides numerous insightful articles on method development, optimization and validation [2-4] applications for quantification of phenolic acids and flavonoids via MS-detection in food samples have been described [5-7] as well as analysis in biological matrices like plasma [8-10].

For highly sensitive analyte detection special consideration should be paid to matrix effects (ME) which may occur especially when using electrospray-ionization [11-14]. Residual matrix components might affect the ionization process by charge competition of the target analyte with simultaneous co-eluting matrix components. Endogenous phospholipids were identified as main cause of ME in plasma [15, 16]. A presence of phospholipids can be monitored during method development e.g. by additional detection of a characteristic glycerophosphocholine fragment with a m/z of 184 in the positive ESI mode [17, 18]. Ghosh et al. observed that different glycerophosphocholines were responsible for ME, depending on the ionization source design [19].

ME might result in ion suppression or ion enhancement depending on whether the analyte is subjected to an attenuation or amplification of signal response intensity [20]. For assessment of matrix effects Bonfiglio et al. developed a post-column infusion method that allows a qualitative determination of ME [21]. Matuszewski et al. [22] proposed a post-extraction spike method which is commonly used for assessing quantitative ME during validation of LC-MS/MS methods for biological matrices [23, 24]. A high interindividual variability of a specific matrix, e.g. plasma, is frequently observed due to individual diet, protein concentrations and composition of plasma constituents [20] which might cause varying ME. Besides endogenous components exogenous sources for ME have been detected. Compounds leaching from sample container materials or anticoagulants such as Li-heparin have been shown to give rise to cause ME [25]. While exogenous ME can be controlled by proper selection of test tubes and the type of anticoagulant [25], relative ME arising from different lots of a given biological matrix have to be determined and considered during method development. Matuszewski demonstrated that the precision of standard line slopes in five different lots of plasma from the same species differed from each other [26]. Current FDA and EMA guidance documentation require ME to be evaluated in different lots of matrix as a part of quantitative LC/MS/MS method development and validation [27, 28].

Strategies to eliminate or reduce ME, particularly ion suppression, include an adequate sample preparation technique, adjustments of the chromatographic and mass spectrometric conditions and the use of an appropriate internal standard (IS) [20, 29-34]. Especially the use of stable isotopically labeled internal standards (SIL-IS) containing 2H, 13C, 15N or 18O is favored for controlling ME due to the fact that the analog displays almost identical physicochemical properties as the analyte of interest. Thus, the analyte and the co-eluting SIL-IS labeled should be subjected to the same degree of ionization suppression or enhancement [16]. However, several reports point out that deuterated IS do not necessarily co-elute with the analyte during reversed phase separations owing to a slightly altered lipophilicity and therefore displaying different ME [16, 35, 36]. Therefore, it has been suggested that 13C, 15N or 18O-labeled compounds should be preferred as SIL-IS if available and affordable [35, 36].

The purpose of this study was to develop a highly sensitive, robust and accurate mass spectrometric method for quantification of selected polyphenolic constituents and the bioactive metabolite M1 (δ-(3.4-dihydroxy-phenyl)-γ-valerolactone) of maritime pine bark extract (Pycnogenol®) at trace levels and thus advancing an earlier used HPLC method with UV and electrochemical detection [37] to gain deeper insights in the pharmacokinetics after oral ingestion of the extract. Additionally to the earlier detected analytes procyanidin B1 was included into the present method development since it has been discovered in human blood samples [38, 39] and its presence in plasma after intake of Pycnogenol® has previously been suspected [37]. Two frequently analyzed biological matrices, human plasma and serum, were considered and compared regarding various analytical aspects. In the course of method development and optimization attention was paid to recovery (RE), quantitative ME and process efficiency (PE) using the post-extraction spike method proposed by Matuszewski et al. [22]. Both the absolute ME in human pooled plasma/serum and the relative ME in six different lots of plasma/serum samples were determined with the optimized sample clean-up procedure. Furthermore, a comparison of the accuracy and thus the performance and reliability of the quantification using both a structural analog and a 13C-labeled IS was exemplified for one polyphenolic analyte.

METHODS AND MATERIALS

Chemicals and reagents

Analytical standards (+)-catechin, taxifolin, ferulic acid, caffeic acid and the internal standard (IS) 3,4-dihydroxyhydrocinnamic acid (hydrocaffeic acid) were all obtained from Sigma-Aldrich (Taufkirchen, Germany). Procyanidin B1 was purchased from TransMIT Project Devision for Plant Metabolites and Chemicals (Plant MetaChem, Gießen, Germany). The metabolite M1 (δ-(3.4-dihydroxy-phenyl)-γ-valerolactone) was synthesized by Matthias Rappold as part of his diploma thesis. Methanol (MeOH, LC-MS analyzed) from J.T.Baker Mallinckrodt, acetonitrile (ACN, CHROMASOLV® gradient grade, for HPLC, ≥99.9%) and water (HiPerSolv CHROMANORM® for LC-MS, VWR BDH Prolabo) were obtained from VWR (Darmstadt,

Germany). Ammonium formate (AF), ammonium acetate (AA), formic acid (FA), and acetic acid (HAC) as well as ethyl acetate (EA) and *tert*-butyl methyl ether (MTBE) were purchased from Sigma-Aldrich.
An enzymatic mixture of β-glucuronidase/sulfatase (ß-Gln/Sulfa) from Helix pomatia (Type HP-2; Sigma-Aldrich) was utilized for enzymatic hydrolysis prior to the liquid-liquid extraction (LLE) to determine free and conjugated analytes in human plasma (phase-II-metabolism).
For comparing different sample preparation techniques trichloroacetic acid (TCA), hydrochloric acid (HCl), phosphoric acid (H3PO4), ammonia (NH3), methylene chloride (CH2Cl2), sodium carbonate (Na2CO3) and monopotassium phosphate (KH2PO4) were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogensulfate (TBAS) was obtained from Sigma-Aldrich. Solid-phase extraction (SPE) cartridges with strong anion exchange character, respectively Strata™-X-A (33 µm, 30 mg sorbens, 1 mL) from Phenomenex (Aschaffenburg, Germany) and Oasis™ MAX (30 µm, 30 mg sorbens, 1 mL) from Waters (Eschborn, Germany) were tested as well as reversed phase cartridges, namely Strata™-X (33 µm, 30 mg sorbens, 1 mL) from Phenomenex and Oasis™ HLB (30 µm, 30 mg sorbens, 1 mL) from Waters. Supelclean™ ENVI-Carb™ with graphitized carbon material was purchased from Sigma-Aldrich and Telos®PPT cartridges for protein removal from Kinesis Abimed GmBH (Langenfeld, Germany).
For additional experiments assessing matrix effects (ME) the stable isotope labeled internal standard (SIL-IS) ferulic acid-1,2,3-13C3 was obtained from Sigma-Aldrich.

Standard solutions and standard substance mix

Stock solutions (1 mg/mL) of each standard compound ((+)-catechin, taxifolin, ferulic acid, caffeic acid, procyanidin B1 and M1) and of the internal standards (hydrocaffeic acid and ferulic acid-1,2,3-13C3) were prepared in 100% methanol. These stock solutions were then diluted further with methanol as required to yield working standards. All working solutions were aliquoted, stored at -20 °C and used after one freeze-thaw cycle. Stock solutions were stored at -80 °C.

Source of human plasma and serum

Human plasma and serum were obtained from a blood bank (Bayerisches Rotes Kreuz, München, Germany). Plasma (n= 6), respectively serum (n= 11) samples, from individual donors were pooled to single batches. Additionally, plasma and serum aliquots of the individual donors were retained for further investigations.

Liquid chromatography (LC)

For sample analysis liquid-liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used as described previously [40]. Briefly, LC analysis was performed using an Agilent 1260 system which consisted of a binary pump (G1379B), a degasser (G1312B), a high-performance autosampler (G1316B) and a thermostatic column oven (G1316B). The

chromatographic separation was carried out at 20 ˚C using a Pursuit PFP-C18 column (4.6 x 150 mm, particle size 3 µm; all from Agilent Technologies, Santa Clara, USA).
The mobile phase consisted of (A) 5 mM AF with 0.065% (v/v) FA (pH= 3.2) and (B) MeOH with 0.1% FA. The stock solution of 5 M AF for the mobile phase A was prepared freshly each month, aliquoted and stored at +4 °C until use. The gradient elution was conducted starting at 60% B (0 min) to 95% B (2.50 min) and maintained to 95% B to 5.50 min followed by re-equilibration with 60% B at an overall constant flow rate of 0.6 mL/min. The total run time was 10.00 min with a post time of 3 min. The injection volume was 5 µL.

Mass spectrometry (MS/MS)
The MS was equipped with a triple quadrupole (G6460) with turbo electrospray ionization (ESI) operating in dynamic multiple reaction monitoring (DMRM) employing negative ESI ionization mode (Agilent Technologies, Santa Clara, USA). The capillary voltage was set at – 3500 V. The gas temperature and flow rate of the ESI source (drying gas, nitrogen) were set at 300 °C and 10 L/min. The nebulizer pressure was set at 50 psi and the nozzle voltage to 0 V. The gas temperature and flow rate of the turbo spray (sheath gas, nitrogen) were set at 400 °C and 12 L/min. The collision induced dissociation (CID) in the collision cell (q2) was operated with nitrogen as collision gas.
In all cases, single deprotonated $[M-H]^-$ ions were found to be the most abundant precursor ions. The MS/MS transitions of the analytes (+)-catechin, (±)-taxifolin, ferulic acid, caffeic acid, M1 and of the IS hydrocaffeic acid and the optimized mass spectrometric parameters have been described before [40]. Additionally, procyanidin B1 and ferulic acid-1,2,3-13C3 have been included into the analysis using the product ions m/z 407.0 (procyanidin B1; precursor ion 577.1 m/z) and m/z 137.1 (ferulic acid-1,2,3-13C3 ; precursor ion 196.1) as quantifiers. Two transitions (quantifier and qualifier) were monitored for each compound. The used cycle time of 1000 ms led to a minimum dwell time of 163.2 ms and a maximum dwell time of 496.5 ms for the total 14 transitions. Resolution of Q1 and Q3 was set to widest/widest for the analytes and wide/widest for the IS for achieving maximum sensitivity. The electron multiplier was set at +500 V. Data acquisition was performed with Mass Hunter Data Acquisition Version B 04.01. Qualitative and Quantitative Analysis were achieved with Mass Hunter Qualitative Analysis Version B 05.00 and Mass Hunter Quantitative Analysis Version B 05.00.

Method development
Liquid Chromatography (LC)
Different chromatography columns from diverse manufacturers with various dimensions and particle sizes were tested to achieve optimal separation and highest analyte response. This included reversed phase (RP)-phases (C18 phases Atlantis, Symmetry, Sunfire and XTerra MS from Waters and one C8-phase, namely Zorbax SB, from Agilent), RP-embedded phases with Pentaflourophenyl (PFP)-groups bound to C18-silica surface (Luna PFP2 and Kinetex PFP, both from Phenomenex and Pursuit PFP from Agilent). Moreover, hydrophilic interaction

chromatography (HILIC)-phases (Kinetex HILIC from Phenomenex and SeQuant® ZIC® HILIC from Merck) were investigated for retention of the analytes.

For choice of an optimal mobile phase pure and acidified (with formic acid) water was investigated for eluent (A) with besides pure and acidified (with formic acid) water different ion strengths (5 – 10 – 100 mM) of AA and AF buffers and at various pH-values (3.2, 5.0, 6.8 and 7.4). For (B) the organic solvents MeOH, ACN and isopropanol in different compositions and with/without acidification were tested. Besides performing sufficient separation and optimal analyte response, special consideration was paid to short run times.

MS/MS Detection

The DMRM method was optimized for maximum analyte sensitivity. Therefore, full- and product ion scans were performed with each compound (10 µg/mL in MeOH) and for each transition the fragmentor voltage (FV, Q1), collision energy (CE; q2), cell accelerator voltage (CAV, q2) were optimized. Besides FIA (Flow Injection Analysis) and software-supported optimization by MassHunter Optimizer TripleQuad B.04.01, selected parameters were confirmed by manual injections of the standard compound mix (10 µg/mL in MeOH).

Furthermore, each parameter of the ESI source was optimized with extracted plasma/serum samples. The influences of changing single parameters or combinations thereof were investigated. Specifically, altering the capillary voltage (from -2000 to -6000 V), nebulizer pressure (from 35 to 60 psi), nozzle voltage (from 0 to 1000 V), gas temperature (from 300 °C to 350 °C) and flow rate (from 7 to 12 L/min) of the ESI source (drying gas, nitrogen) and the gas temperature (from 350 °C to 400 °C) and flow rate (from 10 to 12 L/min) of the additional turbo spray (sheath gas, nitrogen) were determined.

Sample preparation

Different sample preparation techniques were compared and the sensitivity of each analyte was evaluated regarding the recovery (RE), matrix effect (ME), process efficiency (PE) and matrix factor (MF) as described previously [40]. In each case, a sample volume of 0.5 mL plasma was subjected to protein precipitation (PPT), liquid-liquid extraction (LLE), solid phase extraction (SPE) and combinations thereof (Tables S1 and S2 in the electronic supplement). For each technique a sample set (set 1-3) according to Matuszewski et al. [22] was prepared with the standard compound mix (procyanidin B1, (+)-catechin, taxifolin, ferulic acid, caffeic acid and M1) as reported before [40].

Different protein precipitants were tested such as 10% TCA, 50% TCA, MeOH and ACN (PPT #1 - PPT #6;), suggested by Polson et al. [41], and one commercially available cartridge for protein removal, respective Telos®PPT (PPT #7 and PPT #8). For the quantification of some of the target analytes a previously developed a LLE for extracting plasma samples before HPLC-UV analysis was used (LLE #1; [37]). Furthermore, two extraction methods (ion-pair extraction and methanol extraction; LLE #3 and LLE #4; [42]) and one extraction procedure with different solvents in sequence were tested (LLE #5; [8]). Additionally, SPE cartridges with

two different separation principles (reversed phase or strong anion exchange) were tested according to the manufacturer's protocol (SPE #1 – SPE #10). Selected individually sample preparation techniques were combined (Comb #1 - Comb #6). After completion of the first sample preparation step (PPT or LLE) the complete supernatant was used subsequently for the second step (LLE or SPE), if not stated otherwise.

After adding various extraction reagents to 0.5 mL plasma, the samples were centrifuged for 5 min at 3,300 g (RT) for phase separation. Subsequently, the complete supernatants were evaporated under a gentle stream of nitrogen, if not mentioned otherwise. The residues were reconstituted in 100 µL of 100% MeOH, centrifuged at 18,000 g for 15 min at 4 °C before injection of 5 µL of the supernatant for LC-MS/MS analysis.

Method optimization

Sample preparation

The best liquid-liquid extraction approach (LLE #6) was further optimized to yield highest intensity for the analytes of primary interest, namely M1, taxifolin and ferulic acid. Therefore, 2.0 mL plasma was spiked with 10 ng/mL of each analyte. Following variations on the extraction procedure were investigated:

1. Extraction solvent(s) (MTBE or/and EA);
2. Extraction volume (3, 5, 6 and 8 mL);
3. Repetitions of the extraction process (once, twofold, threefold);
4. Extraction technique (vortex, mixing wheel and ultrasonic) and time (1, 10, 15 and 20 min);
5. pH-value before extraction (3.2, 3.0, 2.5, 2.0 and 1.7);
6. Reconstitution solvent (MeOH, water or mixtures of both in different compositions and without/with acidification with FA).

Enzymatic hydrolysis of conjugates

Enzymatic hydrolysis of polyphenol conjugates with a mixture of ß-Gln/Sulfa [37] prior to the LLE was optimized under two aspects:

1. Stability of free (= unconjugated) and conjugated analytes in plasma under different incubation conditions. Therefore, 2.0 mL human plasma was spiked with 100 ng/mL of the analytes. After enzymatic incubation at 37 °C with 500 U/mL ß-Gln at two pH-values (4.5 or 5.0) and different incubation times (30, 60, 90 and 120 min) the samples were extracted and analyzed.
2. Maximum release of conjugated analytes. Therefore, plasma from volunteers who ingested Pycnogenol® was used. Different incubation times (0, 15, 30, 45, 60 and 90 min) and added activities of the enzyme mixture from Helix pomatia with ß-Gln/Sulfa activity (+500, 1000, 2000 and 5000 U ß-Gln per 2 mL plasma) were compared regarding the analyte yield.

Optimized sample preparation protocol

150 µL 4% o-phosphoric acid was added to 1.5 mL human plasma (pH 5.0). The samples were incubated with an enzyme mixture containing ß-Gln/Sulfa (1000 U ß-Gln and 2 U

sulfatase per mL plasma) for 45 min at 37 °C on a horizontal shaker (100 rpm) to hydrolyze conjugated analytes. Subsequently, 255 µL 4% o-phosphoric acid (pH 3.2), 25 µL IS (= 20.8 ng/mL) and 4.5 mL extraction solvent containing EA/MTBE (1:1; V/V) were added, vortexed for 1 min (Multi-Vortex, VWR, Darmstadt, Germany) and centrifuged for 5 min at 3,300 g (4 °C). Thereafter, the upper organic layer was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 75 µL of 100% MeOH, centrifuged at 18,000 g for 15 min at 4 °C. 5 µL of the supernatant were used for LC-MS/MS analysis.

Method validation

For the optimized sample preparation method a full validation was performed for the quantification of the six analytes procyanidin B1, (+)-catechin, ferulic acid, taxifolin, caffeic acid and M1 in human plasma and serum according to EMA and FDA guidelines [27, 28]. The validation for plasma included the selectivity, linearity, lower limit of quantification (LLOQ), recovery, process efficiency, matrix effects (quantitative), carry over and post-preparative stability. The validation for serum additionally included intraday and interday accuracy and precision as well as robustness and cross-talk.

RESULTS AND DISCUSSION

Method development and optimization

The development of an analytical method for polyphenols primarily focused on the sensitive detection and quantification of the gut microbiota-generated catechin metabolite δ-(3.4-dihydroxy-phenyl)-γ-valerolactone (M1). Nanomolar concentrations of this metabolite had been previously found in human plasma samples after single and multiple intake of the maritime pine bark extract Pycnogenol® [37]. Similar to the method development approach described earlier [40] various parameters regarding chromatographic conditions and sample preparation were systematically tested to obtain optimal separation and highest analyte response in human plasma and serum.

In the present investigation different chromatography columns were compared regarding optimal separation and highest analyte response. Best results were obtained with a pentafluorophenyl RP-column. Methanol (MeOH) as organic solvent in the mobile phase allowed for a good separation, maximum response intensity of the analytes while retaining a short run time (< 10 min, Table S3 in the electronic supplement). Consistent with a previous report [43], significant differences in the LC-MS/MS response of the analytes were observed for different brands or grades of MeOH (data not shown). MeOH resulted in an increased sensitivity because the analyte response correlates with the organic composition of the mobile phase, especially when using ESI [44]. It also allowed starting with a higher organic percentage in the mobile phase at the beginning of the analytical run compared to using acetonitrile. It is known that mobile phase additives, e.g. ammonium formate, affect the sensitivity in the LC-MS/MS analysis [45-47]. In the present study the addition of 0.1% formic acid improved the signal to noise ratio (SNR; peak-to-peak height) of M1 by almost three times

(MeOH pure: 7229; MeOH +0.1% formic acid: 20738). By increasing the start percentage of MeOH in the mobile phase from 35% to 60% the analytes M1, ferulic acid and caffeic acid gained about twofold intensity (peak height, Figure S1 in the electronic supplement). Further optimization parameter included mobile phase additives at different ion strengths and pH conditions. The optimal SNR was obtained with 5 mM ammonium formate and pH 3.2 (Figure S2 in the electronic supplement).

In total 32 different sample preparation techniques (details in Tables S1 and S2 in the electronic supplement) involving solid phase extraction (SPE), liquid-liquid extraction (LLE), protein precipitation (PPT) and combination of methods (Combi) were compared by calculating the recovery (RE), matrix effects (ME) and process efficiency (PE) for all analytes [22] with special focus on a sensitive detection of the metabolite M1 (Figure 1). The RE of M1 ranged from 0% (Comb #2, Comb #4, PPT #4) to 74.29% (SPE #1; Figure 1 A). Of all evaluated methods only a small number resulted in RE rates of 50% or higher. Thereby, a higher number of liquid-liquid extraction, protein precipitation and solid phase extraction methods yielded RE ≥50% compared to combinations of different approaches. The ME of M1 with all tested techniques were between -99.79% with PPT #2 and +121.70% with Comb#3 (Figure 1 B). All protein precipitation methods and all but one combined sample preparation method led to ionization suppression and thus to a significant signal attenuation. This is consistent with the notion that particularly protein precipitation methods are associated with ion suppression [48]. Four out of ten solid phase extraction and three out of eight liquid-liquid extraction methods entailed ionization enhancement. The PE of M1 ranged from 0% with Comb#2 to 103.12% by using SPE #1 and 100.17% with LLE #6 (Figure 1 C). Although SPE #1 allowed for the best overall recovery of M1 from human plasma, the more cost-effective process LLE #6 was chosen and further optimized regarding pH adjustment before extraction and other extraction parameters (Figure S3 in the electronic supplement).

Also, the dynamic multiple reaction monitoring (DMRM) method was optimized for maximum sensitivity of analyte detection. The final transitions and DMRM parameters in are listed in Table S3 in the electronic supplement.

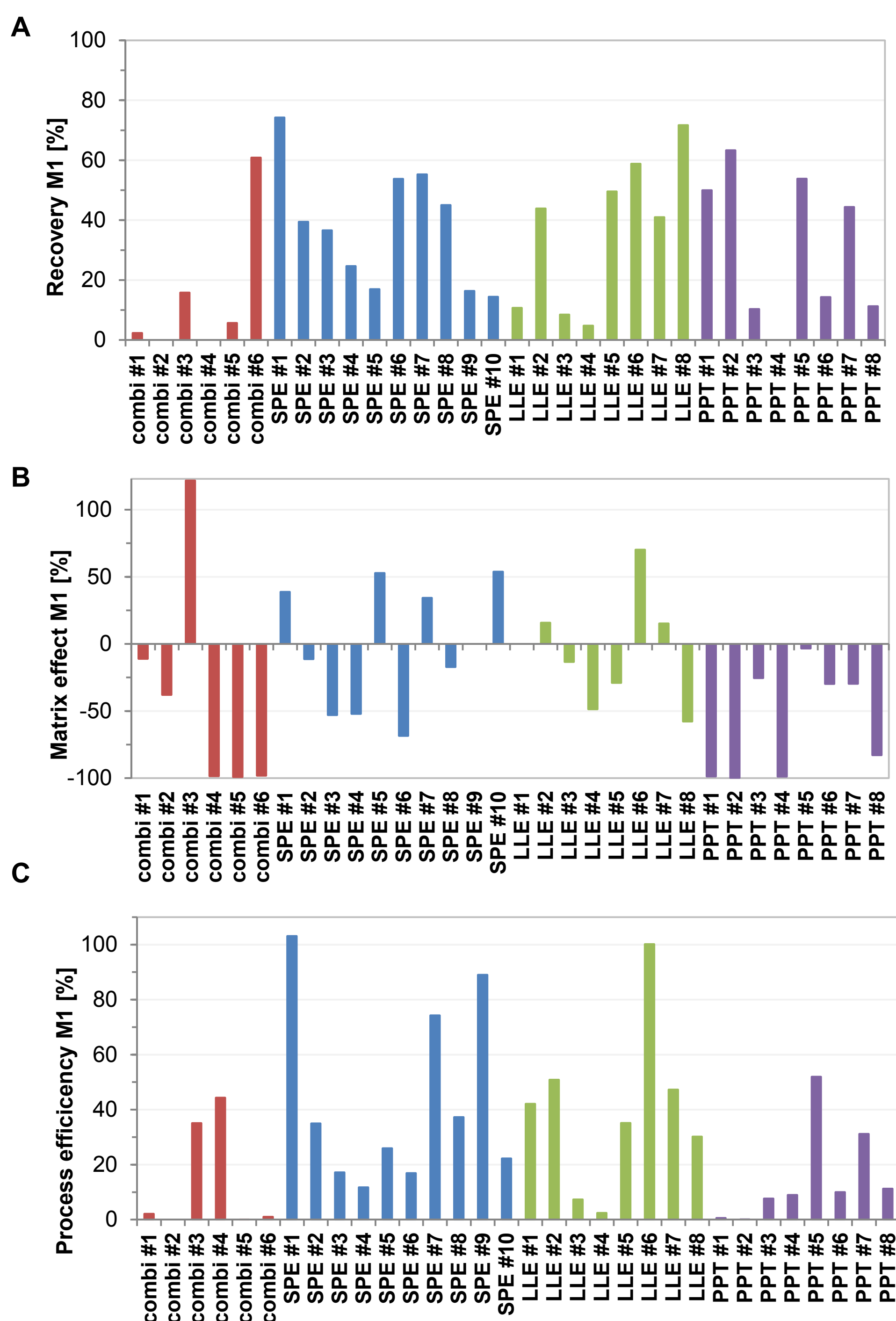

Figure 1. Comparison of different sample preparation techniques (see Table S1 and S2) using 0.5 mL human pooled plasma exemplified for the metabolite M1 spiked at a concentration of 100 ng/mL (n= 1). *Combi: combination of methods; SPE: solid phase extraction; LLE: liquid-liquid extraction; PPT: protein precipitation.* ***A****. Recovery (RE): The RE of M1 ranged from 0% (Combi#2, Combi#4 and PPT #4) to 74.29% (SPE #1).* ***B****. Matrix effect (ME): All protein precipitation techniques led to strong ion suppression. M1 was subjected to strong ion enhancement of 70.23% by using LLE #6 and 121.70% with Combi#3.* ***C****. Process efficiency (PE): The PE of M1 ranged from 0% (Combi#2) to 103.12% (SPE #1). The finally chosen method (LLE #6) allowed for a PE of 100.17%*

Optimization of the ESI source parameters with extracted plasma samples spiked with 100 ng/mL of M1, taxifolin, ferulic acid and caffeic acid resulted in a two- to sixfold (for M1) increase of intensity (peak height) compared to standard (default) values (Figure 2).

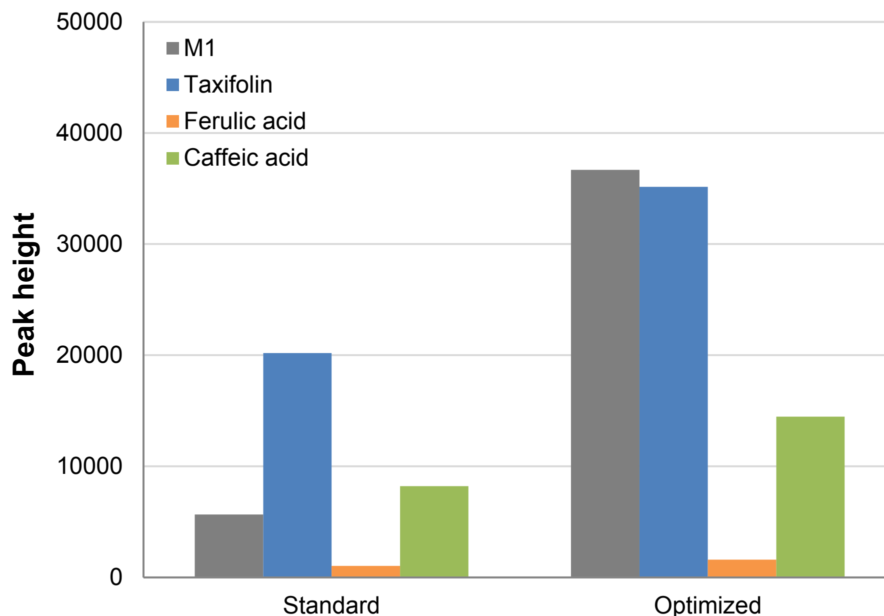

Figure 2. Optimization of the ESI source parameters with extracted plasma samples spiked with 100 ng/mL of M1, taxifolin, ferulic acid and caffeic acid. *Standard ESI conditions (each parameter set to default values) were compared with optimized ESI settings. By tuning each parameter the signals (peak height) of the analytes responses increased two- to sixfold (for M1) after optimization*

Pitfalls in conjugate hydrolysis

Prior to the sample clean-up with the optimized liquid-liquid extraction method an enzymatic hydrolysis with β-glucuronidase/sulfatase (ß-Gln/Sulfa) was performed to determine free and conjugated analytes in plasma. Initial experiments aimed to determine the maximal hydrolysis of conjugated analytes in plasma. Therefore, plasma samples from volunteer donors who ingested the maritime bark extract Pycnogenol® were employed since a previous pharmacokinetic study had revealed that the detected components and metabolites were all conjugated to various degrees ranging from 56.5% (catechin) to virtually 100% (e.g. ferulic acid) [37]. In the present study it was discovered that an incubation time of 45 min and 1000 U per mL plasma of the enzyme mix from Helix pomatia containing ß-Gln yielded best results. However, it was observed that this enzymatic step led to a strong ionization enhancement, especially for the IS hydrocaffeic acid. The influence of the resulting changes of the matrix was further investigated by subsequent addition of the analytes after incubation with different amounts of the enzyme mixture in human plasma (+0, 500, 1000 and 2000 U per mL; Figure 3).

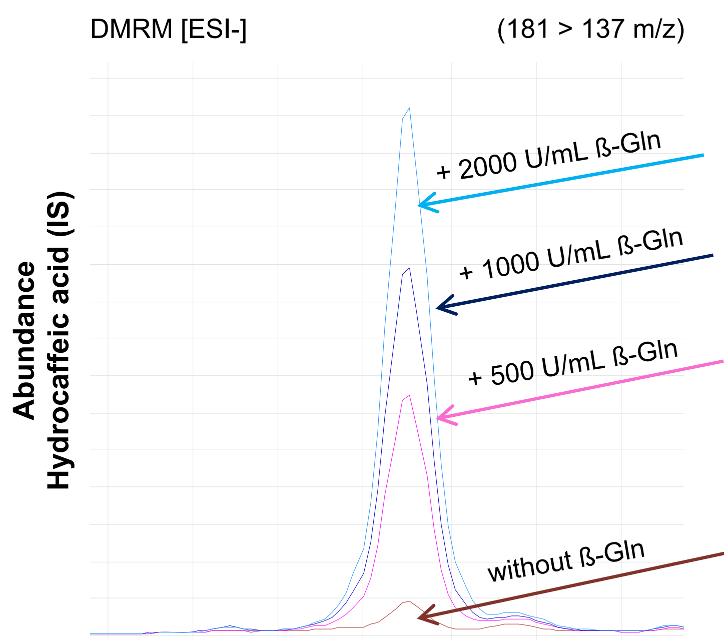

Figure 3. Influence of the enzymatic hydrolysis prior to the liquid-liquid extraction to determine free and conjugated analytes in plasma. *Analytes were spiked in plasma samples after incubation at 37 °C for 30 min with various additions of the enzyme mixture (without, 500, 1000 and 2000 U ß-Gln per 2 mL plasma). This led to a strong ion enhancement of the internal standard hydrocaffeic acid.*

Thus, the ß-Gln/Sulfa enzyme mixture contributed significantly to the matrix effect and emphasizes the need for carefully monitoring potential influences of analyte preparation measures. Other bioanalytical pretreatment procedures such as the addition of esterase inhibitors for drug stabilization have already been pointed out as potential source of matrix effects [49], but to the best of our knowledge this is the first report on the effects of a ß-Gln/Sulfa enzyme mixture. For not compromising accuracy in the present study, this effect was considered when preparing the calibration curve by spiking human matrix blank with the calibration standards after pre-incubated with ß-Gln/Sulfa under the same conditions as the samples.

Method validation: Linearity, sensitivity and selectivity

Linearity

The calibration curve consisted of a blank sample containing neither analytes nor internal standard (IS), a zero sample (containing IS) and 12 non-zero samples covering the expected range of each analyte, including the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). For determination of nutrition-derived polyphenols, target-free related blank matrix for spiking calibration standards might not always be available. Basal pooled matrix, e.g. rat plasma, can be obtained after subjecting the rats to strict fasting conditions [50, 51], but this is more difficult with human study participants. In the current study, the basal presence of some target analytes in the pooled matrix was factored in via a matrix-matched calibration curve prepared by shifting the curve along the y-axes by the response of the non-spiked sample (zero sample) [52]. The quotient of the peak area analyte/IS of the zero-blank

was subsequently subtracted. For each analyte the resulting quotient (y) was plotted against the spiked concentration (x) and subjected to a linear regression (y = ax + b; a: slope; b: intercept; Table 1).

Table 1: Calibration range, calibration function and correlation coefficients of the six analytes extracted from human pooled plasma (n= 3) and serum (n= 5). *P = plasma; S = serum.*

Analytes	Matrix	Range [ng/mL]	Slope ± SD	y-intercept	Correlation coefficient R
Procyanidin B1	P	2.00 - 24.28	0.0001 ± 0.0001	-0.0002	0.9878
	S		0.0035 ± 0.0010	-0.0125	0.9971
(+)-Catechin	P	3.91 - 71.25	0.0005 ± 0.0001	-0,0010	0.9870
	S	5.86 - 71.25	0.0119 ± 0.0043	-0.0461	0.9952
M1	P	0.156 - 2.850	0.5105 ± 0.0570	-0.0634	0.9892
	S		7.8515 ± 2.2566	-0.7380	0.9965
Caffeic acid	P	8.22 - 99.90	0.1952 ± 0.0260	-1,0878	0.9862
	S		3.4515 ± 0.5787	-9.6657	0.9947
Taxifolin	P	0.064 - 1.173	0.5028 ± 0.0380	-0,0165	0.9908
	S		11.9564 ± 1.8696	-0.4868	0.9974
Ferulic acid	P	2.74 - 49.85	0.0224 ± 0.0014	-0,0174	0.9939
	S		0.4991 ± 0.0666	0.0639	0.9966

Correlation coefficients of ≥ 0.9862 proved that the sample signal was directly proportional to the analyte concentration. Although calibration functions differed for plasma and serum and coefficients of correlation were slightly higher for serum compared to plasma linearity was given for both specimen.

Sensitivity: Lower limit of quantification (LLOQ)

The LLOQ was investigated by analyzing replicates of spiked samples at low concentrations in human pooled plasma and serum. The signal-to-noise ratio (SNR; peak-to-peak height) of each analyte in the LLOQ samples was at least 5 times higher than the response in the related matrix blank. In plasma the accuracy at the LLOQ of (+)-catechin, taxifolin, M1, ferulic acid and caffeic acid ranged from 95.66% for ferulic acid to 107.27% for M1 and hence fulfilled the criteria of accuracy of 80-120%, except for procyanidin B1 with an accuracy of 121.86% (Table 2).

Table 2: Lower limit of quantification (LLOQ) and related mean accuracy of the six analytes extracted from human pooled plasma (n= 3) and serum (n= 5). *P = plasma; S = serum.*

Analytes	Matrix	LLOQ [ng/mL]	Accuracy $_{LLOQ}$ [%]
Procyanidin B1	P	2.00	121.86
	S		100.05
(+)-Catechin	P	3.91	104.03
	S	5.86	99.94
M1	P	0.156	107.27
	S		101.97
Caffeic acid	P	8.22	106.64
	S		93.80
Taxifolin	P	0.064	99.28
	S		109.21
Ferulic acid	P	2.74	95.66
	S		87.23

In serum the accuracy at the LLOQ ranged from 87.23% for ferulic acid to 109.21% for taxifolin and therefore met the acceptance criteria for all analytes. With the exception of catechin the LLOQ of the analytes were the same in plasma and serum.

Selectivity

Selectivity was examined with human pooled plasma and with plasma from six individual donors using a blank sample (=matrix blank; containing neither analyte nor IS) and a related matrix-matched LLOQ sample. Presence of interfering components was accepted where the response in the matrix blank was less than 20% of the LLOQ for the analytes and 5% for the IS. No endogenous interfering peak with the analytes and the IS in human pooled plasma and in six different lots of plasma was observed (Figure 4).

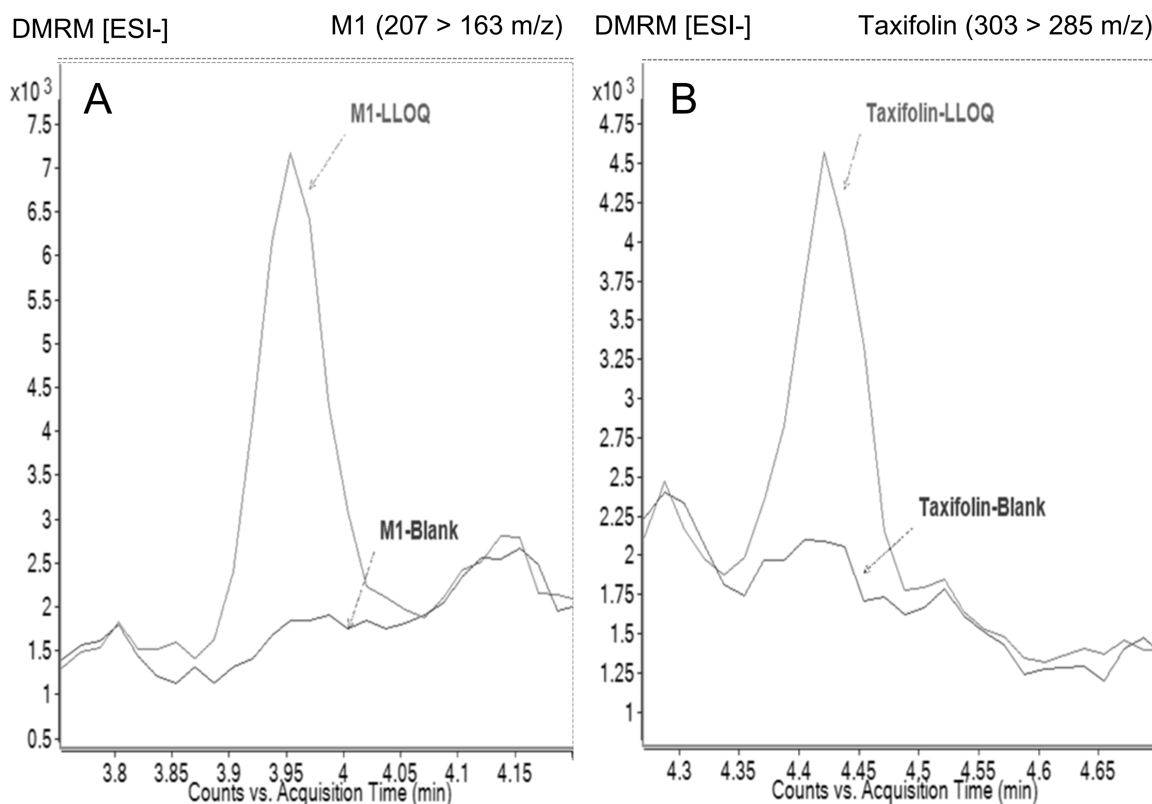

***Figure 4. Example chromatograms of the examination of the selectivity in six individual lots of plasma with the optimized method. A**. M1. The quantifier 207 > 163 of a spiked LLOQ sample (0.156 ng/mL) overlaid with a blank sample. **B**. Taxifolin. The quantifier 303 > 285 spiked LLOQ sample (0.064 ng/mL) was matched with a blank sample.*

Recovery and process efficiency: comparison of serum and plasma

The recovery (RE) of the analytes was determined at three concentrations (low-mid-high; n= 5 each) in human pooled plasma and serum by comparing the analytical response of extracted spiked samples with those of post-extracted spiked samples (representing 100% RE). The mean RE from pooled plasma ranged from 4.30 ± 0.21% for procyanidin B1 to 93.19 ± 3.68% for taxifolin (Figure 5 A; also see Table S4 in the electronic supplement). The mean RE from pooled serum was between 2.16 ± 0.04% for procyanidin B1 to 100.64 ± 2.04% for taxifolin (Figure 5 A; also see Table S5 in the electronic supplement). The RE was higher from serum compared to plasma for most analytes except for procyanidin B1 and catechin which had generally low RE. A higher analyte RE from serum has also been observed by others [24] and is most probably related to the different composition of plasma and serum.

The RE of the analytes was also evaluated with six individual lots of human plasma and serum at two concentrations (low-high). In plasma the RE ranged from 4.11 ± 0.77% for procyanidin B1 (high concentration) to 97.53 ± 8.70% for taxifolin (low concentration, n= 5; Table S6 in the electronic supplement). In six individual lots of serum the RE was between 1.60 ± 0.28% for procyanidin B1 (high concentration) and 122.34 ± 11.87% for ferulic acid (low concentration, n= 3; Table S7 in the electronic supplement).

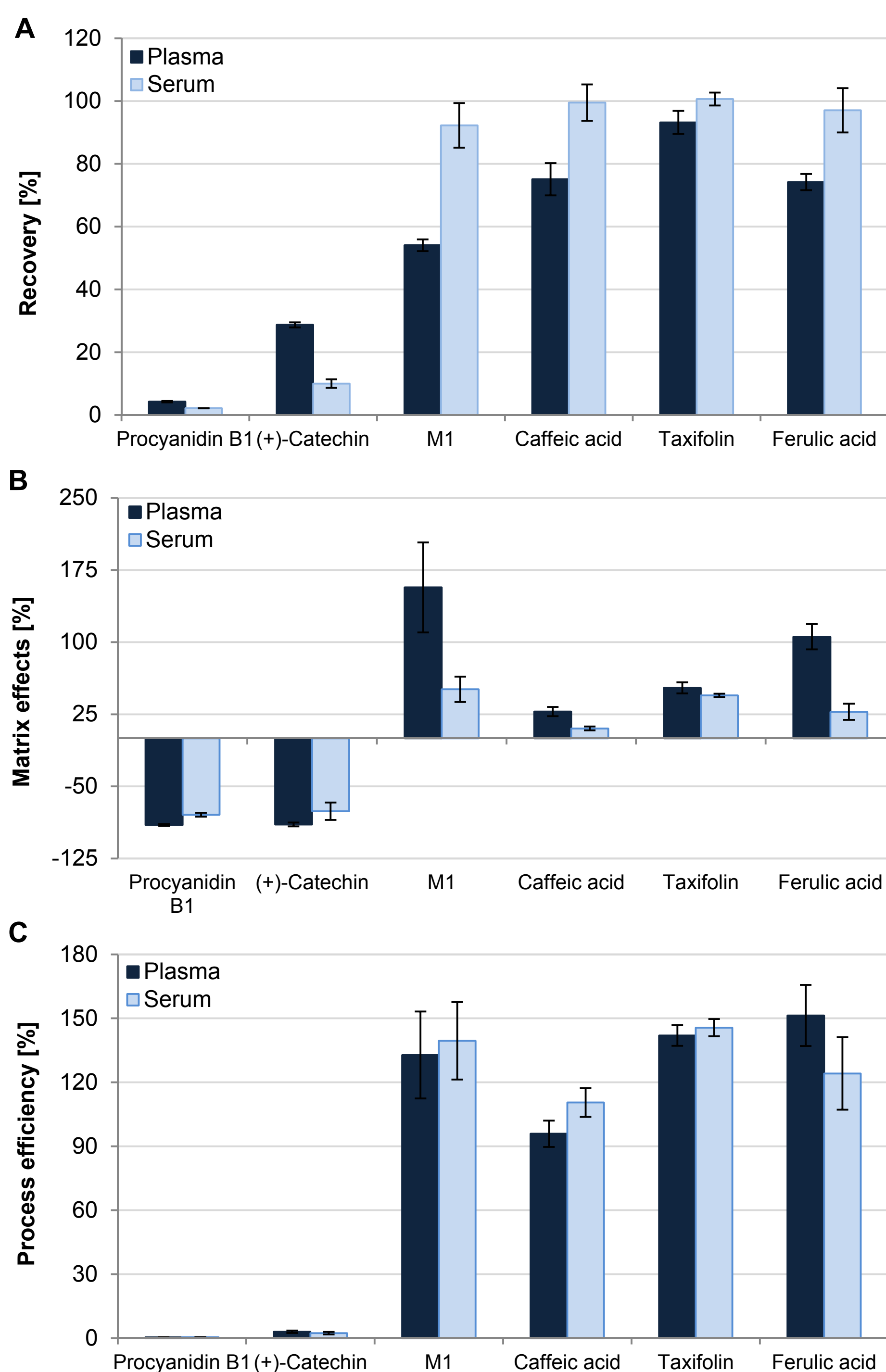

Figure 5. Comparison of the six analytes extracted from human pooled plasma and serum at three concentrations (low-mid-high; each n= 5). A. *Recovery: The RE in plasma ranged from 4.26 ± 0.21% (Mean ± SD) for the analyte with lowest interest, procyanidin B1, to 93.19 ± 3.68% for taxifolin, whereas it was in serum between 2.16 ± 0.04% (procyanidin B1) to 100.63 ± 2.04% for taxifolin.* ***B****. Matrix effect: All analytes were subjected to less ME in serum than plasma.* ***C****. Process efficiency: The PE ranged in plasma from 0.41 ± 0.05% (Mean ± SD) for procyanidin B1 to 151.35 ± 14.31% for ferulic acid, whereas it was in serum between 0.44 ± 0.04% (procyanidin B1) to 145.61 ± 4.04% for taxifolin.*

The differences in the analyte RE between pooled matrix versus individual lots were clearly more pronounced for plasma as opposed to serum. In plasma the absolute differences in the RE from pooled / individual matrices were below 1% only for one analyte (procyanidin B1), but higher than 10% for M1 (Tables S4/S6). In contrast, in serum the absolute differences were

below 1% for three analytes, namely procyanidin B1, catechin and M1 (Tables S5/S7). Thus, when analyzing individual serum samples using a calibration curve prepared with pooled matrix the results should be highly reliable.

The process efficiency (PE) of the analytes was determined at three concentrations (low-mid-high; n= 5 each) in pooled plasma and serum. The mean PE in human pooled plasma varied from 0.41 ± 0.05% for procyanidin B1 and 151.35 ± 14.31% for ferulic acid (Figure 5 C also see Table S4 in the electronic supplement). In pooled serum the PE ranged between 0.44 ± 0.04% for procyanidin B1 to 145.61 ± 4.04% for taxifolin (Figure 5 C; also see Table S5 in the electronic supplement). There was no distinct difference for any of the analytes regarding the PE comparing pooled plasma and serum.

The PE of the analytes was additionally explored using six individual lots of human plasma and serum at two concentrations (low-high). In plasma the PE ranged from 0.27% for procyanidin B1 (high concentration) to 170.78% for M1 (low concentration, n= 5; Table S6 in the electronic supplement). In six individual lots of serum the PE was between 0.39 ± 0.05% for procyanidin B1 (high concentration) and 163.89 ± 18.93% for ferulic acid (low concentration, n= 3; Table S7 in the electronic supplement). The differences in the analyte PE between pooled matrix versus individual lots were comparable plasma as opposed to serum.

When the results obtained for RE and PE at individual concentration levels (low-mid-high) were examined instead of the mean values a trend towards concentration dependency was uncovered (Tables S4/S6 and S5/S7 in the electronic supplement). Higher analyte concentrations, especially those of M1 and caffeic and ferulic acid, were accompanied by lower RE and PE. Similar observations regarding a concentration dependency of the RE of alkaloids from human plasma have been also reported by others [53].

Relative and absolute matrix effects: comparison of serum and plasma

The differentiation between absolute and relative matrix effects (ME) has been introduced by Matuszewski et al. [22]. Absolute ME are described by the relation between the analyte response of a post-extraction spiked sample and the standard solution and have been determined with pooled matrix samples in the present study. Relative ME account for the heterogeneous nature of individual samples [13] and are investigated with different lots of post-extraction spiked samples. Negative values for ME indicate a loss in signal (ionization suppression) and positive values as gain in analyte response (ionization enhancement) [20].

Absolute matrix effects in pooled matrix samples

Absolute ME of the analytes were determined at three concentrations (low-mid-high; n= 5 each) in pooled plasma and serum. The mean ME in human pooled plasma varied from -90.32 ± 0.94% for procyanidin B1 and 156.92 ± 46.80% for M1 (Figure 5 B; also see Table S8 in the electronic supplement). In pooled serum the ME ranged between -79.56 ± 2.02% for procyanidin B1 to 50.99 ± 13.20% for M1 (Figure 5 B; also see Table S9 in the electronic supplement). While both procyanidin B1 and (+)-catechin were subjected to different degrees

of ionization suppression an ionization enhancement was observed for caffeic acid, ferulic acid, taxifolin and M1. The latter analytes showed a higher positive matrix effect in plasma compared to serum. Diverging ME of the same analyte are related to the different matrix composition, e.g. the higher fibrinogen but lower phosphate and sodium ion concentrations in plasma compared to serum [24]. When the ME occurring at the respective concentration levels (low-mid-high) were examined instead of the mean ME it became obvious that the analyte concentration had a definite impact on the ME (Tables S8 and S9 in the electronic supplement). This is consistent with the observations of other researchers [54, 55]. For both plasma and serum higher analyte concentrations were accompanied with lower ME. This correlation was most pronounced for M1 and ferulic acid.

Furthermore, the IS-normalized matrix factor (MF) was calculated by dividing the ME of each analyte by the ME of the IS hydrocaffeic acid at each concentration level. Because of the strong ionization enhancement of the IS hydrocaffeic acid in human pooled plasma (see 3.2) a very low MF resulted. The MF ranged from -0.139 ± 0.015 for procyanidin B1 (high concentration) to 0.270 ± 0.034 for M1 (low concentration; Table S8 in the electronic supplement). The variability of the MF, expressed as the coefficient of variation of the IS-normalized MF should not be higher than 15% to meet the acceptance criteria [27]. All analytes met this criterion for all concentrations with the exception of caffeic acid at the high concentration (49.95 ng/mL). In pooled serum the MF varied from -1.024 ± 0.17 for M1 (low concentration) to 1.301 ± 0.06 for catechin (mid concentration; Table S10 in the electronic supplement). Thus, the MF were clearly higher in serum compared to plasma.

Relative matrix effects in individual matrix samples

The relative ME were investigated with six different lots of human plasma and serum at two concentrations (low-high; [31]). In plasma the mean relative ME ranged from -92.07 ± 1.64% for procyanidin B1 to 106.2 ± 31.61% for M1 (n= 5; Figure 6 A and Table 3, also see Table S11 in the electronic supplement).

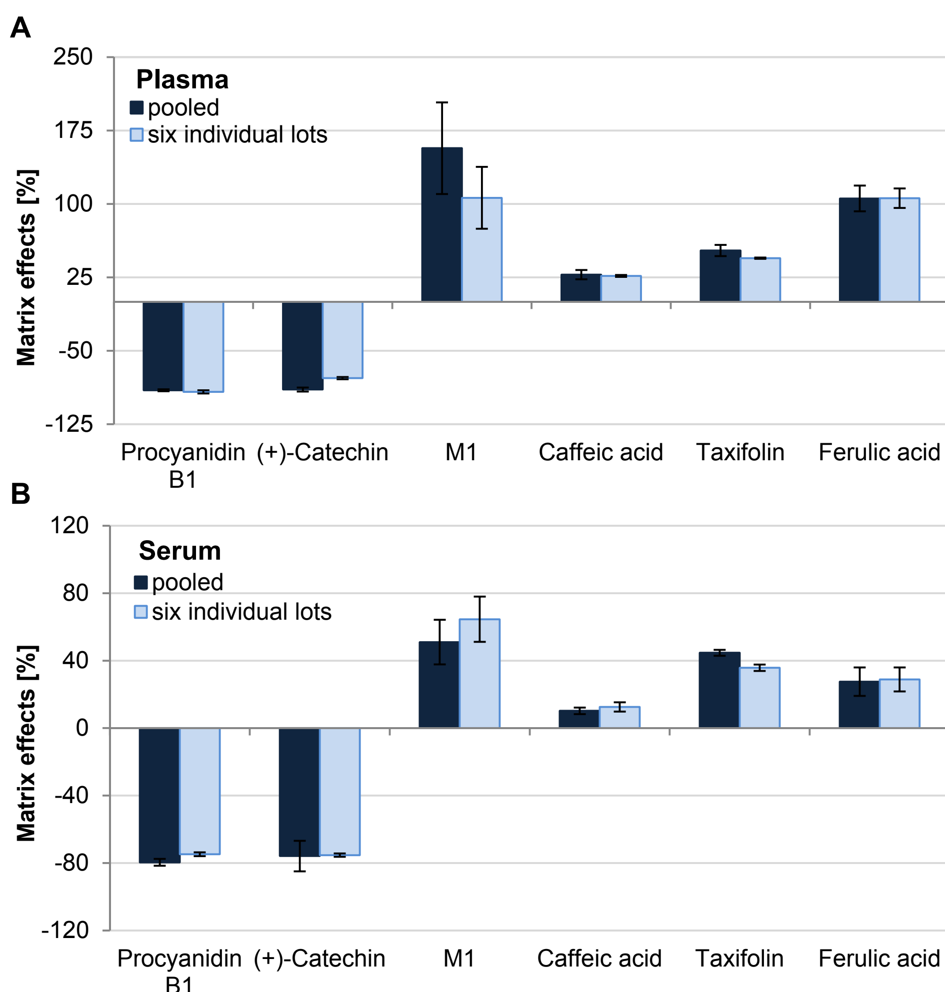

Figure 6. Comparison of absolute and relative matrix effects in human plasma and serum applying optimized sample preparation with enzymatic incubation. A. *Matrix effect: Absolute ME were determined in human pooled plasma at three concentrations (n= 5), whereas relative ME were evaluated at six individual lots of human plasma at two concentrations (n= 5).* ***B****. Matrix effect: Absolute ME were determined in human pooled serum at three concentrations (n= 5), whereas relative ME were evaluated at six individual lots of human serum at two concentrations (n= 3).*

Regarding the ME at individual concentration levels there was again a trend towards lower ME at higher concentrations, especially for M1 and ferulic acid (Table 3). There was no distinct difference to the ME observed with pooled plasma. Only for M1 the mean ME appeared to higher in pooled plasma as opposed to individual lots. However, the variability was high as well with widely overlapping standard deviations. In human serum the relative ME varied between -75.29 ± 0.98% for catechin to 64.56 ± 13.40% for M1 (n= 3; Figure 6 B, also see Table S9 in the electronic supplement). Again, no considerable differences were seen between ME in pooled serum compared to individual lots with the exception of M1 which displayed higher mean ME in individual serum lots and taxifolin which had slightly higher ME in the pooled matrix.

In six different lots of plasma the IS-normalized MF ranged from -0.172 ± 0.027 for procyanidin B1 (high concentration) to 0.224 ± 0.052 for M1 (low concentration; Table 3).

Table 3: Relative matrix effects (ME) and IS (hydrocaffeic acid)-normalized matrix factor (MF) of the six analytes extracted from six different lots of plasma at two concentrations (n= 5). *For additional experiments with a stable isotopically labeled internal standard (SIL-IS) the IS (ferulic acid-1,2,3-13C3)-normalized matrix factor (MF) for ferulic acid was calculated at the same six different lots of plasma (n= 5).*

Analytes and spiked concentration [ng/mL]	ME [%]			IS (hydrocaffeic acid) normalised MF		
	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)
Procyanidin B1						
5.179	-90.91	2.82	3.10	-0.163	0.036	21.98
12.138	-93.23	3.11	3.34	-0.172	0.027	15.91
(+)-Catechin						
15.200	-77.12	7.44	9.64	-0.136	0.019	14.10
35.625	-78.81	9.09	11.53	-0.145	0.024	16.71
M1						
0.608	128.55	35.27	27.44	0.224	0.052	23.01
1.425	83.85	22.36	26.66	0.151	0.031	20.30
Caffeic acid						
21.312	27.19	9.29	34.17	0.047	0.010	21.17
49.950	25.83	8.61	24.04	0.065	0.013	20.08
Taxifolin						
0.250	44.10	8.11	18.40	0.078	0.017	21.25
0.586	45.11	5.50	12.19	0.083	0.014	16.95
Ferulic acid						
10.635	112.92	15.73	13.93	0.198	0.025	12.44
24.925	98.78	22.12	22.40	0.178	0.030	16.89

Analytes and spiked concentration [ng/mL]	ME [%]			IS (ferulic acid-1,2,3-^{13}C3) normalised MF		
	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)
Ferulic acid						
10.635	112.92	15.73	13.93	1.042	0.135	12.99
24.925	98.78	22.12	22.40	0.936	0.098	10.48

The coefficient of variation of the IS-normalized MF was between 12.44% for ferulic acid (low concentration) and 23.01% for M1 (low concentration) and thus exceeded the recommended value of < 15% relative standard deviation (RSD). In individual serum lots the MF varied from -1.089 ± 0.14 for M1 (low concentration) to 1.126 for catechin (high concentration; Table S10 in the electronic supplement). The variability of the MF ranged from 6.84% for procyanidin B1 (low concentration) to 15.53% for caffeic acid (low concentration).

To summarize, the results obtained with serum and plasma revealed that both, the absolute as well as the relative ME, were more pronounced in human plasma than in serum. Especially M1 and ferulic acid were subjected to a higher ionization enhancement in plasma at the same analyte concentration (Figures 5 and 6, Tables S8 and S9). In contrast, the ME for a single analyte was similar for pooled matrices and lots of individual donors. The variability of the

relative MF was higher in plasma than in serum. The evaluation of absolute and relative ME in human serum showed that higher (total) analyte concentrations were paralleled with lower ME which was consistent with the results obtained with human plasma.

Comparison of internal standard options: SIL versus structural analog

It is generally believed that stable isotopically labeled internal standards (SIL-IS) yield better assay performance [56] e.g. by improvement of the precision of standard line slopes [26]. Since the analyte and SIL-IS simultaneously elute during the analytical run they should be equally subjected to matrix-induced ionization suppression or enhancement effects [16, 57]. Comparisons of analyte quantification employing SIL-IS and a structural analog demonstrated superior results for the SIL-IS [58, 59]. Methods using a structural analog IS also allow accurate and precise analyte determination, but it has been argued that this might be to the fact that pooled matrix, e.g. plasma, was used in the validation process and that interindividual variability between single patient samples was not sufficiently accounted for [16, 59]. Therefore, in the present study six different lots of human plasma were employed to determine the variability of the IS-normalized MF of ferulic acid using ferulic acid-1,2,3-13C3 as SIL-IS in comparison with the structural analog hydrocaffeic acid as IS. The relative ME and the impact on the accuracy of the quantification of ferulic acid were elucidated at four concentrations (LLOQ-low-mid-high). Each concentration was determined by a concomitantly prepared calibration curve using pooled plasma. Subsequently, the calculated concentrations were compared with the spiked concentrations.

The IS-normalized MF of ferulic acid, calculated by division of the ME of ferulic acid by the ME of the structural IS hydrocaffeic acid, was 0.198 ± 0.025 (low concentration) and 0.178 ± 0.030 (high concentration) in six individual lots of human plasma (Table 3). By using the SIL-IS ferulic acid-1,2,3-13C3 with the same six individual lots of human plasma the IS-normalized MF was higher by approximately an order of magnitude, i.e. 1.042 ± 0.135 (low concentration) and 0.936 ± 0.009 (high concentration). At the low concentration the variability of the MF for ferulic acid, expressed as the coefficient of variation of the IS-normalized MF, was the same when using SIL-IS and structural analog IS (12.99% and 12.44% RSD). At the higher concentration level the variability of the MF was lower when employing the SIL-IS (10.48% RSD) compared to hydrocaffeic acid as IS (16.89% RSD). The latter variability of the IS-normalized MF thus slightly exceeded the recommended value of < 15% RSD [27].

Furthermore, the accuracy of analyte quantification in six individual lots of plasma employing the structural IS (hydrocaffeic acid) and SIL-IS (ferulic acid-1,2,3-13C3) were compared (Table 4; detailed data in Table S12 in the electronic supplement).

Table 4: Accuracy of quantification in six individual lots of plasma with structural analog internal standard (IS; hydrocaffeic acid) and stable isotopically labeled IS (SIL-IS; ferulic acid-1,2,3-13C3*). * IS (structural): Hydrocaffeic acid; ** SIL-IS: Ferulic acid-1,2,3-13C3*

Analytes	**Accuracy [%] in six individual lots of plasma**		
	Mean accuracy [%]	**± SD**	**RSD [%]**
Procyanidin B1*	98.74	16.32	16.53
(+)-Catechin*	85.85	5.91	6.89
M1*	91.07	7.21	7.92
Caffeic acid*	95.25	6.72	7.06
Taxifolin*	98.71	15.53	15.73
Ferulic acid *	**102.28**	**17.41**	**17.03**
Ferulic acid **	**101.40**	**26.78**	**26.41**

The quantification of the analytes procyanidin B1, catechin, taxifolin, caffeic acid, ferulic acid and M1 resulted in mean accuracy between 85.85 ± 5.91% for catechin and 102.28 ± 17.41% for ferulic acid using the structural analog IS. Thus, these results met the acceptance criteria for bioanalytical methods accuracy. Utilizing the SIL-IS for ferulic acid the mean accuracy at six different lots of human plasma was 101.40 ± 26.78%. Thus, the accuracy of the quantification of ferulic acid in six different lots of human plasma was in excellent agreement when using the SIL-IS and the structural analog IS. However, the RSD of the quantification results were higher when employing the SIL-IS as opposed to the structural IS (26.41% vs. 17.03%, Table 4). It can be concluded that the comparison of the accuracy and thus the performance of the quantification using both a structural analog and a 13C-labeled IS exemplified for ferulic acid did not reveal a clear advantage of either IS. This might be due to the rigorous evaluation and optimization of the sample preparation method which has been discussed to reduce ME [58].

Stability data

Stock solution stability

The stability of the stock solution (SL) of the analytes and the IS (1 mg/mL) in MeOH was investigated, which included the short-term stability comprising of a period over 4 and 8 h at room temperature (RT; n= 3) and the freeze-thaw stability after one cycle (n= 3). The peak areas were compared with a freshly prepared SL (Table S13 in the electronic supplement).

After 4 h at RT the analyte concentrations between 96.03% for ferulic acid and 102.03% for M1 were found. No major changes were seen for most analytes after another 4 h except for clearly reduced concentrations of catechin (89.82%) and taxifolin (68.10%). Freezing the analytes once and keeping them at -20 °C for at least 12 h before being thawed at RT uncovered a reduced stability of procyanidin B1 (82.10%) while the other compounds were stable. Consequently, for all experiments the SL of the analytes and IS were freshly prepared.

The diluted working solutions (with MeOH) were immediately aliquoted, stored at -20 °C until use and consumed after one freeze-thaw cycle.

Post-preparative stability

The stability of the analytes in the processed samples, including the autosampler stability and the stability after one freeze and thaw cycle, was assessed both with human pooled plasma and serum at four concentrations (LLOQ-low-mid-high; n= 3 for plasma and n= 5 for serum). Samples were analyzed using a calibration curve obtained from freshly spiked calibration standards and the calculated concentrations were compared to the spiked concentrations.
For processed plasma samples the most pronounced concentration decreases were seen for procyanidin B1 (up to -77.21% after one freeze-thaw cycle) and catechin (up to -23.33% after 12 h in the autosampler). There was a tendency towards higher compound loss at higher concentration levels (Tables S14 and S15 in the electronic supplement). In processed serum samples the analyte stability was higher and less compounds experienced a loss in concentrations. The strongest concentration declines were again recorded for procyanidin B1 (up to -35.66% after one freeze-thaw cycle) and catechin (up to -27.52% after one freeze-thaw cycle). Other analytes showed an apparent increase in concentration which lacks an evident explanation (Tables S16 and S17 in the electronic supplement).

Carry-over, robustness, accuracy and precision

Carry-over

Carry-over was assessed by injecting two matrix blank samples after an upper limit of quantification (ULOQ)-spiked sample of the calibration curve (n= 3). Because of the basal presence of some analytes in the matrix blank a carry-over in the first matrix blank after injection of the ULOQ spiked sample was accepted, when the response of the analyte and IS was not increased by more than 20% and 5%, compared to the response of a matrix blank before injection of the ULOQ-spiked sample. The analytes procyanidin B1, catechin, ferulic acid, caffeic acid and M1 showed no carry-over effects. The highest increase of the response in the first matrix blank after injection of the ULOQ spiked sample was +2.31 ± 15.24% for taxifolin (data not shown).

Robustness

Robustness of the method was investigated at two concentration levels (n= 5) with human pooled serum which was intentionally contaminated with 1% human whole blood. The mean accuracy ranged from 86.23% for the low concentration of ferulic acid to 114.69% for the high concentration of M1 (Table 5). The variability of the precision was between 2.53% for the low concentration of taxifolin and 11.37% for the low concentration of caffeic acid. Thus, all analytes still met the acceptance criteria for bioanalytical method validation.

Table 5: Robustness of the developed method at two concentration levels (n= 5) with human pooled serum which was intentionally contaminated with 1% human whole blood.

Analytes and spiked concentration [ng/mL]	**Calculated concentration Mean ± SD [ng/mL]**	**Accuracy Mean [%]**	**Precision [%]**
Procyanidin B1			
5.18	4.74 ± 0.36	91.46	7.59
12.14	10.83 ± 0.58	89.26	5.31
Catechin			
15.20	16.82 ± 1.15	110.65	6.86
35.63	37.80 ± 3.98	106.09	10.53
M1			
0.608	0.696 ± 0.02	114.47	2.72
1.425	1.634 ± 0.06	114.69	3.61
Caffeic acid			
21.31	21.73 ± 2.47	101.96	11.37
49.95	51.38 ± 3.12	102.87	6.07
Taxifolin			
0.250	0.239 ± 0.01	95.70	2.53
0.586	0.558 ± 0.03	95.20	5.71
Ferulic acid			
10.64	9.17 ± 0.25	86.23	2.77
24.93	24.44 ± 1.30	98.08	5.34

Accuracy and precision

The interday and intraday accuracy and precision was determined for the serum matrix only since a clinical study was planned with serum samples. All analytes met the acceptance criteria at all concentration levels (Tables S18 and S19 in the electronic supplement). Only caffeic acid displayed a slighter higher variation of the intraday precision of 17.08% at the lowest concentration level.

Application of the method

The optimized sample preparation method was applied to an authentic serum sample of a human volunteer who ingested 100 mg/day Pycnogenol® over the course of three weeks to obtain steady-state conditions. Serum concentrations of 43.66 ng/mL catechin, 23.27 ng/mL caffeic acid, 4.56 ng/mL ferulic acid, 2.83 ng/mL M1 and 0.27 ng/mL taxifolin were determined (Figure 7). The dimer procyanidin B1 was not detected in the sample. Thus, with the exception of procyanidin B1 the method was suitable for quantifying polyphenol in an authentic sample with the analytes being within the validated concentration range.

DMRM [ESI-]

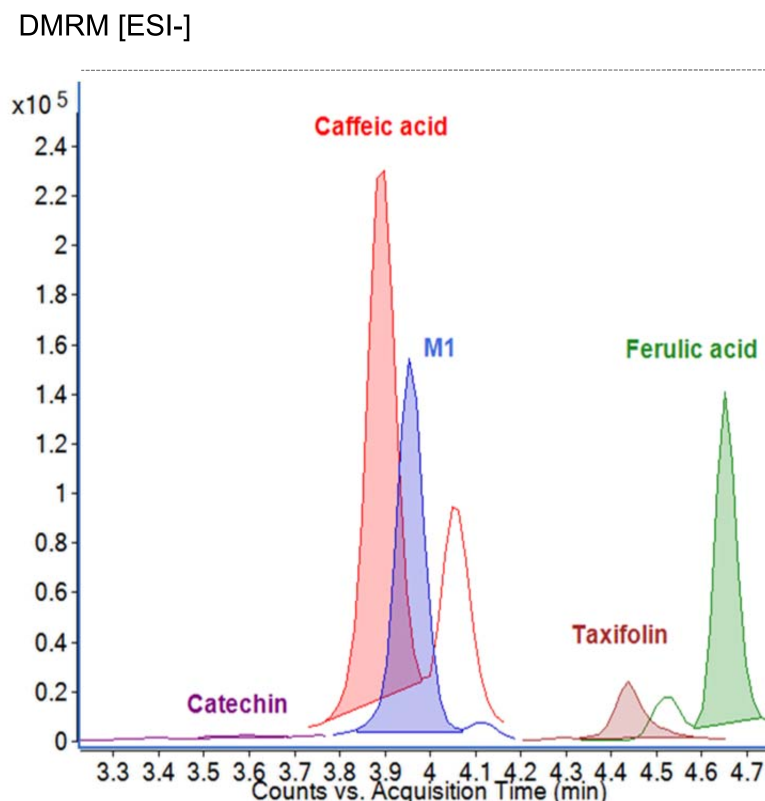

Figure 7. Example chromatogram of an authentic human serum sample extracted with the optimized analytical method. *Overlaid quantifier of catechin (289 > 245), M1 (207 > 163), taxifolin (303 > 285), ferulic acid (193 > 134) and caffeic acid (179 > 135) in the negative ESI mode. The volunteer had steady-state concentrations of 43.66 ng/mL catechin, 23.27 ng/mL caffeic acid, 4.56 ng/mL ferulic acid, 2.83 ng/mL M1 and 0.27 ng/mL taxifolin after multiple dosing of Pycnogenol® of 100 mg/day over three weeks.*

CONCLUSION

In the present study a sensitive LC-ESI-MS/MS method was developed for accurate, precise and robust quantification of selected polyphenols in human plasma and serum. All methodical steps were optimized for best analysis for M1 which turned out entailing a less sensitive detection of catechin and procyanidin B1. Since polyphenols are in vivo typically present as conjugates with β-glucuronic acid and/or sulfate an enzymatic hydrolysis was used to determine free and conjugated analytes. This enzymatic step led to a significant ionization enhancement, especially for the internal standard (IS) hydrocaffeic acid. This relevant but yet not described effect has to be considered, e.g. when preparing matrix-matched calibration standards, to ensure accuracy of the quantification results.

Two commonly used specimen, human plasma and serum, were systematically compared while deliberately taking into account the interindividual variability between single patient samples. Thus, analyte recovery (RE) and process efficiency (PE) was not only compared between plasma and serum, but also between pooled and individual matrices. The differences in the analyte RE between pooled matrix versus individual lots were clearly more pronounced for plasma as opposed to serum. The analyte RE was higher from serum for most analytes while PE was comparable for both matrices. Particular consideration was given to matrix

effects (ME) and both, the absolute as well as the relative ME, were more pronounced in human plasma than in serum. Especially M1 and ferulic acid were subjected to a higher ion enhancement in plasma. The variability of the IS-normalized matrix factor (MF) calculated from different matrix lots from human donors was also higher in plasma compared to serum.
Furthermore, two options for IS, the structural analog hydrocaffeic acid and chemical analog ferulic acid-1,2,3-13C3 were compared regarding the accuracy of the quantification of ferulic acid and the variability of the IS-normalized MF. No clear advantage of either IS was observed. At a higher concentration level the variability of the MF was lower when employing the stable isotopically labeled IS (SIL-IS) compared to hydrocaffeic acid. The accuracy of the quantification of ferulic acid in six different lots of human plasma was in excellent agreement when using the SIL-IS and the structural analog IS, though the variability of the quantification results were higher with the SIL-IS.
Finally the comprehensively analyzed and validated method was successfully applied to an authentic human serum sample and can now be used for further pharmacokinetic studies to gain more insight into the absorption, distribution and elimination of polyphenols in humans.

Conflict of interest

M.M. declares no conflict of interests. P.H. received unrestricted research grants from Horphag Research, the producer of Pycnogenol®, within the past three years.

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SUPPLEMENTARY TABLES AND FIGURES

Table S1: Overview of the tested sample preparation techniques, part I (PPT and LLE). *Various reagents were added to 0.5 mL human pooled plasma in different volume ratios. Cold reagents were cooled to 4 °C. PPT: protein precipitation; LLE: liquid-liquid extraction.*

Sample preparation	Process
PPT #1	Addition of 10% TCA (1:2)
PPT #2	Addition of 10% TCA (1:4)
PPT #3	Addition of ACN_{cold} (1:2)
PPT #4	Addition of ACN_{cold} (1:4)
PPT #5	Addition of 4% H_3PO_4 (pH 3.2); Addition of ACN_{cold} (1:2)
PPT #6	Addition of $MeOH_{cold}$ (1:4)
PPT #7 Telos®PPT	Addition of ACN (1:5)
PPT #8 Telos®PPT	Addition of 4% H_3PO_4 (1:1); addition of ACN (1:2.5)
LLE #1	Acidification with 1 M HCl; twofold extraction with 0.75 mL EA aliquots by shaking for 20 min with mixing wheel; combining of the two organic extracts
LLE #2	Acidification with 1 M HCl; twofold extraction with 0.75 mL MTBE aliquots by shaking for 20 min with mixing wheel; combining of the two organic extracts
LLE #3	Addition of 0.5 M TBAS (1:1); addition of 4 mL 0.25 M Na_2CO_3-buffer (pH 10); twofold extraction with 5 mL MTBE aliquots by shaking for 20 min with mixing wheel; combining of the two organic extracts
LLE #4	Threefold extraction with 2.5, 1.5 and 1.0 mL aliquots of methanol by shaking for 10 min with mixing wheel followed by ultrasonic for 10 min
LLE #5	Addition of 4% H_3PO_4 (pH 3.2); extraction with 1 mL methylene chloride by vortexing for 1 min; removing of the aqueous supernatant; extraction of the organic phase with 0.5 mL water and vortex for 1 min; after combining the two aqueous extracts they were extracted twice with 0.75 mL EA by vortexing for 1 min; final combining of this two organic extracts
LLE #6	Addition of 4% H_3PO_4 (pH 3.2); twofold extraction with 1.5 mL MTBE aliquots and vortex for 1 min; combining of the two organic extracts
LLE #7	Acidification with 1 M HCl; twofold extraction with 0.75 mL MTBE aliquots by vortexing for 1 min; combining of the two organic extracts
LLE #8	Twofold extraction with 1.5 mL 5% FA in MTBE aliquots by vortexing for 1 min; combining of the two organic extracts

Table S2: Overview of the tested sample preparation techniques, part II (SPE and Combis). *Various reagents were added to 0.5 mL human pooled plasma in different volume ratios. Cold reagents were cooled to 4 °C. SPE: solid phase extraction. Combi: combinated techniques.*

Sample preparation	Process
SPE #1 Oasis™ HLB, reversed phase	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 5% MeOH in water; Elution: 1 mL MeOH (2 x 0.5 mL)
SPE #2 Oasis™ HLB reversed phase	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing 1, 1 mL 5% MeOH in water; Washing 2, 1 mL 2% FA in 5% MeOH in water; Elution: 1 mL 2% NH_3 in 50% MeOH in water (2 x 0.5 mL)
SPE #3 Oasis™ MAX, strong anion exchange	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 5% NH_3 in water; Elution 1: 1 mL MeOH; Elution 2: 1 mL 2% FA in MeOH (2 x 0.5 mL)
SPE #4 Oasis™ MAX, strong anion exchange	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 5% NH_3 in water; Elution 1: 1 mL ACN; Elution 2: 1 mL 2% FA in MeOH (2 x 0.5 mL)
SPE #5 Oasis™ MAX, strong anion exchange	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 5% NH_3 in water; Elution 1: 1 mL MeOH; Elution 2: 1 mL 2% FA in 80% MeOH in ACN (2 x 0.5 mL)
SPE #6 Strata™ X, reversed phase	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 5% MeOH in water; Elution: 1 mL MeOH (2 x 0.5 mL)
SPE #7 Strata™ X, reversed phase	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 20% MeOH in water; Elution: 1 mL MeOH/ACN/water/FA 60:30:10:0.1 (2 x 0.5 mL)
SPE #8 Strata™ X, reversed phase	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing, 1 mL 2% FA in 5% MeOH in water; Elution: 1 mL 2% NH_3 in 50% MeOH in water (2 x 0.5 mL)
SPE #9 Strata™ X-A, strong anion exchange	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing 1, 1 mL 25 mM AA (pH 6-7); Washing 2, 1 mL MeOH; Elution: 1 mL 5% FA in MeOH (2 x 0.5 mL)
SPE #10 Strata™ X-A, strong anion exchange	Conditioning, 1 mL MeOH; Equilibration: 1 mL 100 mM K_2HPO_4 (pH 12); Loading, 0.5 mL plasma + 0.5 mL 100 mM K_2HPO_4 (pH 12); Washing 1, 1 mL 100 mM K_2HPO_4 (pH 12); Washing 2, 1 mL MeOH; Elution: 1 mL 5% FA in MeOH (2 x 0.5 mL)
Combi#1	LLE#3 + SPE (Supelclean™ ENVI-Carb™): Conditioning 1, 3 mL 0.1% NH_3 in MeOH; Conditioning 2, 3 mL water; Conditioning 3, 3 mL MeOH; Loading, 3 mL of LLE extract; Elution: 3 mL MeOH (2 x 1.5 mL)
Combi#2	LLE#4 + SPE (Supelclean™ ENVI-Carb™): Conditioning 1, 3 mL 0.1% NH_3 in MeOH; Conditioning 2, 3 mL water; Conditioning 3, 3 mL MeOH; Loading, 3 mL of LLE extract; Elution: 3 mL MeOH (2 x 1.5 mL)
Combi#3	PPT: Addition of ACN_{cold} (1:3) + SPE #10 (Loading, 1 mL supernatant from PPT + 1 mL 100 mM K_2HPO_4 (pH 12))
Combi#4	PPT: Addition of 10% TCA (1:3) + SPE #6
Combi#5	PPT #1 + LLE #1
Combi#6	PPT #1 + LLE #2

Table S3: Optimized transitions and parameters in dynamic multiple reaction monitoring (DMRM) employing negative ESI ionization mode for LC-MS/MS analysis of plasma and serum samples. *Electron multiplier voltage (EMV) was set to +500 V.*

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	FV[a] [V]	CE[b] [V]	CAV[c] [V]	MS 1 Resolution	MS 2 Resolution	R_T[d] [min]	R_T Window *(DMRM)*
Procyanidin B1	577.1	407.0[e]	21	135	2	Widest	Widest	3.35	0.30
	577.1	289.0	21	135	2	Widest	Widest	3.35	0.30
(+)-Catechin	289.1	245.0[e]	9	76	7	Widest	Widest	3.48	0.50
	289.1	203.0	17	76	7	Widest	Widest	3.48	0.50
M1	207.0	163.1[e]	13	115	5	Widest	Widest	4.00	0.40
	207.0	122.0	17	115	5	Widest	Widest	4.00	0.40
Caffeic acid	179.0	135.1[e]	13	90	5	Widest	Widest	3.95	0.45
	179.0	134.0	25	90	5	Widest	Widest	3.95	0.45
Taxifolin	303.1	285.0[e]	9	95	4	Widest	Widest	4.45	0.45
	303.1	125.0	21	95	4	Widest	Widest	4.45	0.45
Ferulic acid	193.1	134.1[e]	13	80	1	Widest	Widest	4.55	0.45
	193.1	178.0	9	80	1	Widest	Widest	4.55	0.45
Hydrocaffeic acid (IS)	181.2	137.0[e]	9	85	4	Wide	Widest	3.85	0.40
	181.2	109.0	9	85	4	Wide	Widest	3.85	0.40

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	FV[a] [V]	CE[b] [V]	CAV[c] [V]	MS 1 Resolution	MS 2 Resolution	R_T[d] [min]	R_T Window *(DMRM)*
Ferulic acid-1,2,3-$^{13}C_3$	196.1	137.1[e]	13	80	1	Widest	Widest	4.55	0.45
	196.1	181.0	9	80	1	Widest	Widest	4.55	0.45

[a]FV. Fragmentor voltage. [b]CE. Collision energy. [c]CAV. Cell accelerator voltage. [d]R_T. Retention time. [e]Quantifier. transition for quantification

Table S4: Recovery and process efficiency of the six analytes extracted from human pooled plasma at three concentrations (n= 5). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1						
5.179	4.43	1.44	32.48	0.46	0.13	28.75
8.092	4.33	1.20	27.62	0.37	0.06	16.88
12.138	4.03	0.62	15.40	0.40	0.01	3.46
(+)-Catechin						
15.200	29.40	1.80	5.00	3.56	0.14	4.02
23.750	28.94	3.49	13.95	2.37	0.13	5.33
35.625	27.84	1.53	5.48	3.04	0.15	5.00
M1						
0.608	55.97	3.86	6.89	150.80	13.12	7.67
0.950	53.96	3.83	7.09	137.00	10.70	7.81
1.425	52.25	5.25	10.04	110.62	7.85	7.10
Caffeic acid						
21.312	80.02	6.57	8.21	103.01	6.17	5.99
33.300	75.47	1.99	2.64	92.47	3.10	3.35
49.950	69.76	6.03	8.64	92.10	6.45	7.00
Taxifolin						
0.250	88.95	11.00	12.36	138.95	17.83	12.83
0.391	95.04	4.33	4.55	147.58	7.00	4.75
0.586	95.58	3.55	3.72	139.35	5.40	3.88
Ferulic acid						
10.635	75.87	7.61	10.03	167.33	12.23	7.31
16.617	75.49	4.93	6.71	147.03	5.67	3.85
24.925	71.21	2.43	3.41	139.70	3.02	2.16

Table S5: Recovery and process efficiency of the six analytes extracted from human pooled serum at three concentrations (n= 5). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1						
5.18	2.18	0.37	16.80	0.40	0.07	16.46
8.09	2.12	0.32	14.99	0.47	0.08	17.70
12.14	2.18	0.47	21.39	0.46	0.03	7.53
(+)-Catechin						
15.20	11.47	1.09	9.54	2.13	0.06	2.63
23.75	9.90	1.59	16.04	1.91	0.36	18.76
35.63	8.74	1.40	16.03	3.03	0.32	10.54
M1						
0.608	93.51	7.77	8.31	155.26	14.16	9.12
0.950	98.65	4.09	4.15	143.55	5.98	4.16
1.425	84.59	9.11	10.77	119.62	10.32	8.63
Caffeic acid						
21.31	102.03	5.23	5.12	114.30	5.82	5.09
33.30	103.57	9.78	9.44	114.54	10.91	9.52
49.95	92.87	6.08	6.55	102.71	8.12	7.91
Taxifolin						
0.250	102.72	8.14	7.92	148.59	11.97	8.06
0.391	100.54	4.79	4.76	147.22	10.91	7.41
0.586	98.65	6.92	7.02	141.01	5.76	4.08
Ferulic acid						
10.64	105.14	2.92	2.78	142.68	5.77	4.04
16.62	94.06	4.21	4.47	120.47	2.44	2.02
24.93	91.97	2.39	2.60	109.31	3.63	3.32

Table S6: Recovery and process efficiency of the six analytes extracted at six different lots of plasma at two concentrations (n= 5). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	**Recovery [%]**			**Process efficiency [%]**		
	Mean	**± SD**	**RSD (%)**	**Mean**	**± SD**	**RSD (%)**
Procyanidin B1						
5.179	4.42	1.49	33.67	0.38	0.14	37.39
12.138	4.11	0.77	18.73	0.27	0.12	42.17
(+)-Catechin						
15.200	20.92	6.54	31.25	4.48	1.18	26.25
35.625	20.24	5.91	29.20	3.92	1.18	30.09
M1						
0.608	74.85	6.63	8.85	170.78	36.73	21.50
1.425	64.04	7.47	11.67	117.21	14.78	12.61
Caffeic acid						
21.312	86.14	14.95	17.35	109.37	18.75	17.14
49.950	79.23	9.20	11.61	107.24	9.95	9.27
Taxifolin						
0.250	97.53	8.70	8.92	140.17	9.19	6.56
0.586	86.89	10.07	11.58	126.05	15.14	12.01
Ferulic acid						
10.635	68.63	10.54	15.36	145.09	15.14	10.14
24.925	60.12	8.87	14.75	118.28	13.09	11.07

Table S7: Recovery and process efficiency of the six analytes extracted from six different lots of serum at two concentrations (n= 3). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]		
	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)
Procyanidin B1						
5.18	1.70	0.30	17.54	0.43	0.06	14.18
12.14	1.60	0.28	17.66	0.39	0.05	14.16
(+)-Catechin						
15.20	10.73	0.85	7.90	2.72	0.59	21.58
35.63	9.23	1.22	13.24	2.20	0.31	14.12
M1						
0.608	92.64	16.83	18.16	161.91	33.99	20.99
1.425	84.68	12.05	14.23	131.18	17.71	13.50
Caffeic acid						
21.31	95.87	14.22	14.84	109.89	17.23	15.67
49.95	106.64	10.19	9.55	121.70	11.28	9.27
Taxifolin						
0.250	102.47	9.43	9.21	140.56	13.91	9.90
0.586	90.11	2.87	3.19	121.10	4.37	3.61
Ferulic acid						
10.64	122.34	11.87	9.71	163.89	18.93	11.55
24.93	94.67	9.12	9.64	117.01	8.83	7.55

Table S8: Absolute matrix effects (ME) and internal standard (IS; hydrocaffeic acid) normalised matrix factor (MF) of the six analytes extracted from human pooled plasma at three concentration levels (n= 5). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	ME [%]			IS (hydrocaffeic acid) normalised MF		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1						
5.179	-89.61	0.28	0.31	-0.118	0.006	4.75
8.092	-91.39	2.04	2.23	-0.118	0.007	6.32
12.138	-89.96	1.57	1.75	-0.139	0.015	10.53
(+)-Catechin						
15.200	-87.91	2.75	3.13	-0.116	0.009	8.07
23.750	-91.82	1.01	1.10	-0.118	0.006	4.90
35.625	-89.07	0.26	0.29	-0.137	0.016	11.80
M1						
0.608	205.17	18.65	9.09	0.270	0.034	12.74
0.950	153.87	3.27	2.12	0.198	0.007	3.48
1.425	111.71	7.48	6.69	0.172	0.018	10.71
Caffeic acid						
21.312	28.74	3.63	12.64	0.038	0.003	7.92
33.300	22.54	1.62	7.20	0.029	0.002	7.48
49.950	32.02	3.54	11.06	0.049	0.009	18.20
Taxifolin						
0.250	56.21	2.52	4.48	0.074	0.002	3.06
0.391	55.29	2.70	4.88	0.071	0.006	8.51
0.586	45.81	3.04	6.64	0.071	0.009	12.52
Ferulic acid						
10.635	120.56	6.88	5.71	0.159	0.016	10.18
16.617	100.06	12.08	12.07	0.129	0.011	8.70
24.925	96.18	4.00	4.16	0.148	0.018	11.95

Table S9: Absolute and relative matrix effects (ME) of the six analytes extracted after enzymatic incubation from human serum. Absolute ME were determined at three concentrations (n= 5), relative ME at two concentrations (n= 3). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	Absolute ME [%]			Relative ME [%]		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1						
5.179	-81.77	4.24	5.18	-73.92	4.98	6.74
8.092	-77.81	4.34	5.58			
12.138	-79.09	2.75	3.48	-75.52	3.03	4.01
(+)-Catechin						
15.200	-81.43	2.05	2.52	-74.60	5.20	6.98
23.750	-80.72	0.83	1.02			
35.625	-65.36	3.65	5.59	-75.98	3.69	4.86
M1						
0.608	66.04	11.47	17.37	74.03	9.25	12.50
0.950	45.52	2.35	5.15			
1.425	41.41	5.39	13.02	55.08	5.03	9.13
Caffeic acid						
21.312	12.03	1.83	15.25	14.53	2.10	14.42
33.300	10.59	1.07	10.12			
49.950	8.16	1.03	12.64	10.59	1.51	14.29
Taxifolin						
0.250	44.65	4.51	10.11	37.12	3.07	8.26
0.391	46.43	4.66	10.04			
0.586	42.94	5.50	12.81	34.42	4.07	11.83
Ferulic acid						
10.635	35.71	6.05	16.95	33.87	5.83	17.20
16.617	28.08	4.24	15.11			
24.925	18.86	1.91	10.15	23.86	4.52	18.93

Table S10: Internal standard (IS; hydrocaffeic acid) normalized MF in human pooled serum at three concentrations (n= 5) and in six lots of serum at two concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	IS (hydrocaffeic acid) normalized MF in human pooled serum			IS (hydrocaffeic acid) normalized MF in six lots of human serum		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1						
5.18	1.268	0.09	6.80	1.087	0.07	6.84
8.09	1.254	0.10	8.06			
12.14	1.171	0.07	6.03	1.123	0.12	11.07
(+)-Catechin						
15.20	1.263	0.05	3.90	1.097	0.08	7.40
23.75	1.301	0.06	4.93			
35.63	0.968	0.08	7.90	1.126	0.09	7.89
M1						
0.608	-1.024	0.17	16.39	-1.089	0.14	13.13
0.950	-0.734	0.06	7.54			
1.425	-0.613	0.07	11.33	-0.816	0.08	10.20
Caffeic acid						
21.31	-0.187	0.03	16.85	-0.214	0.03	15.53
33.30	-0.171	0.02	9.87			
49.95	-0.157	0.02	14.09	-0.209	0.02	8.25
Taxifolin						
0.250	-0.692	0.10	14.61	-0.548	0.07	12.08
0.391	-0.748	0.10	13.78			
0.586	-0.636	0.10	16.19	-0.510	0.07	13.19
Ferulic acid						
10.64	-0.554	0.11	19.56	-0.497	0.07	14.64
16.62	-0.453	0.06	13.87			
24.93	-0.279	0.03	11.70	-0.352	0.05	15.38

Table S11*: *Individual values of relative matrix effects (ME) of the six analytes extracted in six different lots of plasma at two concentrations (n= 5). *RSD: relative standard deviation.*

Analytes and spiked concentration [ng/mL]	ME [%]																	
	Individual donor #1			Individual donor #2			Individual donor #3			Individual donor #4			Individual donor #5			Individual donor #6		
	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)
Procyanidin B1																		
5.179	-94.73	0.75	0.79	-94.10	0.88	0.94	-87.88	1.88	2.14	-89.24	1.70	1.90	-89.36	1.56	1.74	-90.14	3.65	3.99
12.138	-95.23	0.46	0.48	-96.26	0.64	0.67	-93.76	0.79	0.84	-94.94	0.33	0.34	-91.24	0.48	0.53	87.94	0.34	0.38
(+)-Catechin																		
15.200	-78.74	1.84	2.33	-68.40	7.28	10.65	-87.95	0.88	1.00	-69.79	3.94	5.64	-75.76	1.59	2.10	-82.13	2.28	2.78
35.625	-69.91	2.78	3.98	-67.22	3.25	4.84	-84.84	0.92	1.08	-89.33	0.46	0.51	-76.07	2.79	3.66	-85.49	0.48	0.56
M1																		
0.608	131.24	9.25	7.05	117.21	4.78	4.08	110.32	15.58	15.07	89.33	7.34	8.21	129.74	15.95	12.30	193.45	12.57	6.50
1.425	102.52	5.05	4.92	83.42	9.00	10.79	59.31	2.43	4.09	58.02	3.49	6.02	86.44	2.95	3.41	113.42	5.56	4.90
Caffeic acid																		
21.312	28.78	8.98	31.20	19.25	3.76	19.53	23.27	9.34	40.11	23.06	5.58	24.18	23.68	2.59	10.95	45.11	10.87	24.10
49.950	36.16	4.34	11.99	28.02	2.29	8.19	39.43	11.72	29.72	23.22	4.37	18.83	43.13	5.03	11.66	45.02	9.30	20.66
Taxifolin																		
0.250	42.82	4.73	11.05	38.71	3.95	10.22	34.13	4.62	13.53	44.25	5.17	11.69	46.74	3.70	7.93	57.96	9.10	15.70
0.586	40.94	4.56	11.14	43.00	3.28	7.63	40.65	3.03	7.44	42.38	4.93	11.64	54.24	1.60	2.95	49.44	1.90	3.85
Ferulic acid																		
10.635	132.13	7.51	5.69	100.98	5.17	5.12	112.73	11.66	10.35	91.56	8.23	8.98	110.91	7.34	6.62	129.20	16.82	13.02
24.925	128.14	4.50	3.51	90.94	4.54	4.99	90.93	5.49	6.03	63.82	3.88	6.08	112.02	2.49	2.23	106.85	4.66	4.36

Table S12: Individual values of internal standards (IS; hydrocaffeic acid and ferulic acid-1,2,3-13C3) normalized matrix factor (MF) of the six analytes extracted in six different lots of plasma at two concentrations (*n*= 5).

Analytes and spiked concentration [ng/mL]	IS normalised MF																	
	Individual donor #1			Individual donor #2			Individual donor #3			Individual donor #4			Individual donor #5			Individual donor #6		
	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)
*Procyanidin B1**																		
5.179	-0.132	0.008	5.71	-0.222	0.011	4.74	-0.140	0.019	13.64	-0.175	0.017	9.89	-0.175	0.007	4.15	-0.131	0.011	8.36
12.138	-0.145	0.014	9.37	-0.212	0.011	5.27	-0.177	0.025	13.86	-0.189	0.018	9.45	-0.172	0.033	19.155	-0.138	0.028	20.37
*(+)-Catechin**																		
15.200	-0.109	0.007	6.65	-0.162	0.010	6.00	-0.141	0.021	14.56	-0.137	0.021	15.33	-0.149	0.010	7.01	-0.119	0.007	5.87
35.625	-0.106	0.014	13.47	-0.148	0.025	16.78	-0.161	0.024	15.07	-0.178	0.018	10.12	-0.143	0.025	17.36	-0.135	0.024	17.85
*M1**																		
0.608	0.182	0.015	8.40	0.277	0.017	6.28	0.176	0.040	24.00	0.175	0.022	12.41	0.254	0.038	14.81	0.280	0.030	10.65
1.425	0.156	0.012	7.54	0.183	0.039	21.31	0.112	0.014	12.13	0.116	0.016	13.45	0.163	0.029	17.87	0.178	0.033	18.50
*Caffeic acid**																		
21.312	0.040	0.011	27.41	0.046	0.011	23.13	0.037	0.012	32.68	0.045	0.010	21.68	0.046	0.007	14.29	0.065	0.013	19.40
49.950	0.055	0.006	10.12	0.062	0.009	14.67	0.075	0.014	18.93	0.046	0.010	21.14	0.081	0.017	20.40	0.071	0.003	4.70
*Taxifolin**																		
0.250	0.060	0.008	12.85	0.092	0.011	12.44	0.055	0.008	15.04	0.087	0.008	9.72	0.092	0.011	12.29	0.084	0.017	20.59
0.586	0.062	0.012	18.50	0.095	0.012	12.93	0.077	0.014	18.70	0.084	0.005	6.32	0.102	0.019	18.90	0.078	0.017	21.92
*Ferulic acid**																		
10.635	0.184	0.016	8.66	0.239	0.023	9.48	0.180	0.023	12.65	0.180	0.017	9.62	0.217	0.025	11.41	0.187	0.034	18.19
24.925	0.195	0.022	11.41	0.200	0.036	18.03	0.172	0.025	14.63	0.127	0.017	13.01	0.211	0.036	17.14	0.168	0.029	17.24

Table S13: Short-term stability of the stock solution (SL) of the analytes and the internal standard (IS; 1 mg/mL) in MeOH (n= 3) over 4 and 8 h at room temperature (RT) and freeze-thaw stability after one cycle (n= 3). *Samples were analyzed against a freshly prepared SL (100%) and the peak areas were compared.*

Analytes and spiked concentration [ng/mL]	Stability SL in MeOH [%]		
	short-term stability: 4 h - R_T	short-term stability: 8 h - R_T	freeze-thaw stability: 1 cycle; -20 °C for 12 h
Procyanidin B1	97.14	92.57	82.10
(+)-Catechin	98.17	89.82	97.72
M1	102.03	102.29	110.07
Caffeic acid	99.39	98.43	101.63
Taxifolin	97.83	68.10	101.22
Ferulic acid	96.03	97.24	104.07
Hydrocaffeic acid (IS)	100.51	98.56	95.59

Table S14: Post-preparative stability in plasma: autosampler stability of the analytes after 6 h and 12 h at room temperature (RT) after previous LC/MS/MS analysis (n= 3).

Analytes and spiked concentration [ng/mL]	Autosampler stability: 6 h - RT - in darkness			Autosampler stability: 12 h - RT - in darkness		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1						
1.998	1.95 ± 0.50	25.60	-2.55	1.83 ± 0.47	25.57	-8.40
5.179	3.92 ± 0.33	8.35	-24.36	3.69 ± 0.42	11.48	-28.76
8.092	6.02 ± 1.33	22.03	-25.56	5.55 ± 0.66	11.83	-31.41
12.138	7.67 ± 1.76	22.95	-36.81	7.00 ± 1.67	23.83	-42.36
(+)-Catechin						
5.864	6.38 ± 1.36	21.27	8.71	5.93 ± 0.55	9.32	1.16
15.200	13.97 ± 2.70	19.29	-8.07	12.35 ± 1.92	15.59	-18.77
23.750	20.55 ± 3.88	18.86	-13.46	18.21 ± 2.94	16.16	-23.33
35.625	32.51 ± 2.99	9.20	-8.76	30.95 ± 2.40	7.76	-13.12
M1						
0.235	0.256 ± 0.04	16.07	8.77	0.265 ± 0.05	17.80	12.79
0.608	0.618 ± 0.03	4.99	1.59	0.658 ± 0.09	13.61	8.22
0.950	0.941 ± 0.06	6.91	-0.99	0.940 ± 0.15	15.80	-1.04
1.425	1.272 ± 0.23	17.71	-10.74	1.340 ± 0.26	19.57	-5.94
Caffeic acid						
8.222	8.91 ± 1.46	16.41	8.38	8.92 ± 1.75	19.57	8.46
21.312	20.95 ± 3.02	14.42	-1.69	21.80 ± 2.67	12.27	2.29
33.300	35.62 ± 2.15	6.03	6.95	36.31 ± 2.84	7.83	9.04
49.950	48.89 ± 6.89	14.09	-2.12	50.33 ± 9.08	18.05	0.77
Taxifolin						
0.097	0.094 ± 0.01	9.76	-2.74	0.094 ± 0.01	14.12	-3.43
0.250	0.248 ± 0.02	7.79	-0.92	0.248 ± 0.01	4.41	-0.69
0.391	0.375 ± 0.02	5.51	-4.21	0.377 ± 0.02	5.84	-3.68
0.586	0.542 ± 0.08	14.89	-7.43	0.540 ± 0.08	14.54	-7.83
Ferulic acid						
4.103	4.50 ± 0.90	19.89	9.72	4.56 ± 0.92	20.07	11.23
10.635	10.85 ± 0.31	2.83	2.00	11.13 ± 0.38	3.37	4.69
16.617	16.12 ± 0.58	3.61	-3.02	16.80 ± 1.16	6.89	1.10
24.925	22.49 ± 4.18	18.58	-9.75	22.11 ± 3.65	16.49	-11.29

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Table S15: Post-preparative stability in plasma: stability of the analytes after one freeze-thaw cycle (n= 3).

Analytes and spiked concentration [ng/mL]	**Freeze-thaw stability: 1 cycle -20 °C h/at least 12 h - RT/1h**		
	Calculated concentration Mean ± SD [ng/mL]	**RSD [%]**	**Δ [%][1] Mean ± SD**
Procyanidin B1			
1.998	1.72 ± 0.25	14.71	-13.70
5.179	2.64 ± 0.40	15.27	-48.96
8.092	2.91 ± 0.53	18.23	-63.99
12.138	2.77 ± 0.51	18.47	-77.21
(+)-Catechin			
5.864	4.79 ± 0.73	15.25	-18.29
15.200	12.54 ± 1.23	9.84	-17.60
23.750	21.11 ± 4.28	20.28	-11.12
35.625	32.90 ± 2.20	6.69	-7.65
M1			
0.235	0.416 ± 0.08	19.94	77.20
0.608	0.894 ± 0.14	16.17	47.09
0.950	1.135 ± 0.12	10.19	19.51
1.425	1.452 ± 0.24	16.41	1.90
Caffeic acid			
8.222	8.42 ± 1.05	12.44	2.46
21.312	21.29 ± 2.46	11.54	-0.09
33.300	36.69 ± 4.30	11.71	10.17
49.950	49.84 ± 6.48	13.00	-0.22
Taxifolin			
0.097	0.085 ± 0.00	1.69	-12.23
0.250	0.236 ± 0.06	2.63	-5.72
0.391	0.367 ± 0.03	8.76	-6.17
0.586	0.524 ± 0.08	15.77	-10.53
Ferulic acid			
4.103	4.79 ± 0.69	14.42	16.73
10.635	12.27 ± 0.54	4.42	15.40
16.617	18.54 ± 0.96	5.19	11.55
24.925	26.01 ± 3.65	14.05	4.36

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Table S16: Post-preparative stability in serum: autosampler stability of the analytes after 6 h and 12 h at room temperature subsequent to LC/MS/MS analysis (n= 5).

Analytes and spiked concentration [ng/mL]	Autosampler stability: 6 h - RT - in darkness			Autosampler stability: 12 h - RT - in darkness		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1						
2.00	1.941 ± 0.48	24.94	-2.86	1.508 ± 0.32	20.94	-24.55
5.18	5.521 ± 1.58	28.67	6.60	4.822 ± 1.80	37.30	-6.89
8.09	8.161 ± 1.39	17.00	0.86	7.018 ± 1.35	19.23	-13.27
12.14	10.048 ± 1.51	15.02	-17.22	9.506 ± 1.30	13.65	-21.68
(+)-Catechin						
5.86	5.024 ± 0.61	12.20	-14.32	4.540 ± 0.78	17.16	-22.58
15.20	12.678 ± 1.53	12.08	-16.59	12.28 ± 1.46	11.92	-19.20
23.75	19.534 ± 1.67	8.57	-17.75	19.085 ± 2.94	15.43	-19.64
35.63	27.65 ± 4.03	14.57	-22.39	28.348 ± 6.61	23.32	-20.43
M1						
0.235	0.210 ± 0.04	17.81	-10.68	0.224 ± 0.03	14.65	-4.63
0.608	0.627 ± 0.11	17.45	3.19	0.652 ± 0.11	16.20	7.32
0.950	1.049 ± 0.25	24.07	10.39	1.128 ± 0.27	24.31	18.75
1.425	1.401 ± 0.21	15.27	-1.37	1.495 ± 0.28	18.88	4.93
Caffeic acid						
8.22	7.762 ± 1.21	15.55	-5.59	8.244± 0.99	11.98	0.27
21.31	24.089 ± 3.63	15.08	13.03	25.230 ± 3.08	12.19	18.39
33.30	37.324 ± 6.09	16.33	12.08	40.336 ± 6.05	15.00	21.13
49.95	53.366 ± 5.13	9.62	6.84	57.160 ± 6.65	11.63	14.43
Taxifolin						
0.097	0.113 ± 0.02	14.47	16.08	0.103 ± 0.02	14.98	6.08
0.250	0.277 ± 0.02	8.43	10.63	0.272± 0.02	8.96	8.70
0.391	0.420 ± 0.03	8.31	7.36	0.424 ± 0.03	8.25	8.48
0.586	0.594 ± 0.03	5.27	1.36	0.617 ± 0.05	7.53	5.24
Ferulic acid						
4.10	3.509± 0.57	16.30	-14.47	3.946 ± 0.66	16.72	-3.82
10.64	12.130 ± 1.94	15.99	14.06	13.110 ± 1.14	8.75	23.28
16.62	18.451 ± 3.58	19.41	11.03	19.993 ± 2.68	13.39	20.31
24.93	27.080 ± 3.192	11.79	8.65	28.681 ± 2.67	10.00	15.07

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Table S17: Post-preparative stability in serum: stability of the analytes after one freeze-thaw cycle (n= 5).

Analytes and spiked concentration [ng/mL]	**Freeze-thaw stability: 1 cycle -20 °C h/at least 12 h - RT/1h**		
	Calculated concentration Mean ± SD [ng/mL]	**RSD [%]**	**Δ [%][1] Mean ± SD**
Procyanidin B1			
2.00	1.285 ± 0.21	15.98	-35.66
5.18	4.513 ± 1.30	28.76	-12.86
8.09	6.040 ± 0.69	11.39	-25.36
12.14	9.351 ± 1.21	12.93	-22.96
(+)-Catechin			
5.86	4.250 ± 0.49	11.60	-27.52
15.20	11.222 ± 2.00	17.85	-26.17
23.75	19.510 ± 4.53	23.23	-17.85
35.63	28.485 ± 7.36	25.85	-20.04
M1			
0.235	0.305 ± 0.50	15.19	29.98
0.608	0.802 ± 0.10	11.92	31.86
0.950	1.217 ± 0.30	24.31	28.15
1.425	1.432 ± 0.12	8.67	0.50
Caffeic acid			
8.22	9.762 ± 1.03	10.56	18.73
21.31	28.245 ± 1.21	4.27	32.53
33.30	46.653 ± 7.56	16.20	40.10
49.95	54.228 ± 8.88	16.37	8.56
Taxifolin			
0.097	0.121 ± 0.01	4.93	24.27
0.250	0.299 ± 0.01	3.62	19.42
0.391	0.463 ± 0.04	9.17	18.32
0.586	0.578 ± 0.09	15.54	-1.28
Ferulic acid			
4.103	4.149 ± 0.76	18.25	1.12
10.635	13.78 ± 0.30	2.14	29.58
16.617	21.31 ± 1.63	7.64	28.23
24.925	27.940 ± 3.19	11.40	12.10

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Table S18: Intraday accuracy and precision of the analytes in human pooled serum (n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Procyanidin B1			
2.00	1.73 ± 0.21	86.54	12.27
5.18	5.62 ± 0.59	108.44	10.45
8.09	9.01 ± 0.58	111.31	6.45
12.14	10.94 ± 1.21	90.10	11.11
Catechin			
5.86	6.33 ± 0.90	107.89	14.23
15.20	16.18 ± 1.54	106.43	9.49
23.75	23.36 ± 2.20	98.37	9.44
35.63	31.99 ± 2.63	89.80	8.22
M1			
0.156	0.164 ± 0.02	105.38	14.39
0.235	0.241 ± 0.03	102.56	10.71
0.608	0.656 ± 0.08	107.81	11.80
0.950	1.000 ± 0.11	105.30	10.86
1.425	1.548 ± 0.12	108.62	7.84
Caffeic acid			
8.22	7.48 ± 1.28	90.95	17.08
21.31	20.98 ± 2.82	98.42	13.44
33.30	33.89 ± 3.83	101.76	11.30
49.95	49.87 ± 6.90	99.84	13.84
Taxifolin			
0.064	0.061 ± 0.01	95.03	14.23
0.097	0.088 ± 0.01	90.85	10.80
0.250	0.242 ± 0.02	96.98	8.50
0.391	0.385 ± 0.03	98.48	6.93
0.586	0.590 ± 0.06	100.67	10.57
Ferulic acid			
2.74	2.73 ± 0.32	99.98	11.62
4.10	3.75 ± 0.29	91.32	7.69
10.64	10.54 ± 1.46	99.13	13.84
16.62	17.55 ± 2.01	105.59	11.48
24.93	26.48 ± 2.59	106.23	9.77

Table S19: Interday accuracy and precision of the analytes in human pooled serum (n= 5).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Procyanidin B1			
2.00	1.95 ± 0.24	97.94	12.85
5.18	5.22 ± 0.48	100.81	9.18
8.09	8.13 ± 0.69	100.29	8.80
12.14	12.15 ± 1.05	100.01	8.76
Catechin			
5.86	5.53 ± 0.50	94.28	8.99
15.20	14.64 ± 0.88	96.32	6.00
23.75	22.54 ± 0.95	94.90	4.21
35.63	32.66 ± 0.44	91.68	1.35
M1			
0.156	0.155 ± 0.02	105.45	7.51
0.235	0.241 ± 0.02	102.45	7.72
0.608	0.631 ± 0.05	103.81	8.03
0.950	0.993 ± 0.09	104.52	9.53
1.425	1.532 ± 0.11	107.50	7.34
Caffeic acid			
8.22	8.49 ± 0.79	103.20	9.30
21.31	22.55 ± 0.95	105.83	4.21
33.30	35.84 ± 1.25	107.64	3.49
49.95	53.31 ± 2.11	106.73	3.95
Taxifolin			
0.064	0.070 ± 0.01	110.14	7.73
0.097	0.103 ± 0.01	105.94	8.00
0.250	0.271 ± 0.02	108.57	6.02
0.391	0.414 ± 0.02	105.96	4.73
0.586	0.626 ± 0.03	106.85	4.45
Ferulic acid			
2.74	2.85 ± 0.19	104.08	6.78
4.10	4.12 ± 0.35	100.41	8.54
10.64	11.23 ± 0.66	105.59	5.83
16.62	17.51 ± 1.10	105.39	6.26
24.93	26.39 ± 1.15	105.89	4.35

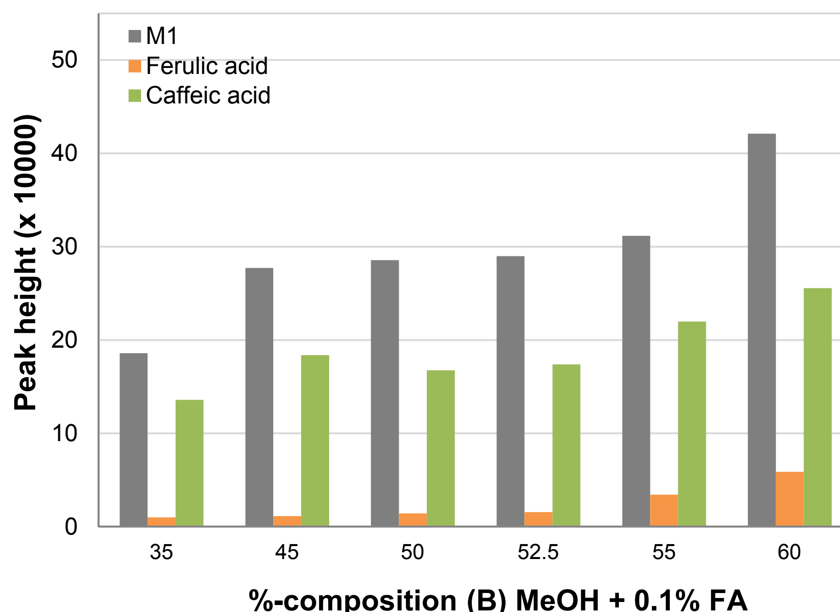

Figure S1. Dependence of analyte response from start%-composition of organic solvent at the beginning of the analytical run (B; MeOH +0.1% FA). *Solvent (A) was water with 0.1% FA. 10 µg/mL of M1, ferulic acid and caffeic acid in MeOH were analyzed with different%-composition of (B; 35 – 60%) at 0 min with the same gradient program. The analytes were detected with the highest intensity (peak height) with 60% MeOH +0.1% FA at the beginning of the analytical run (0 min).*

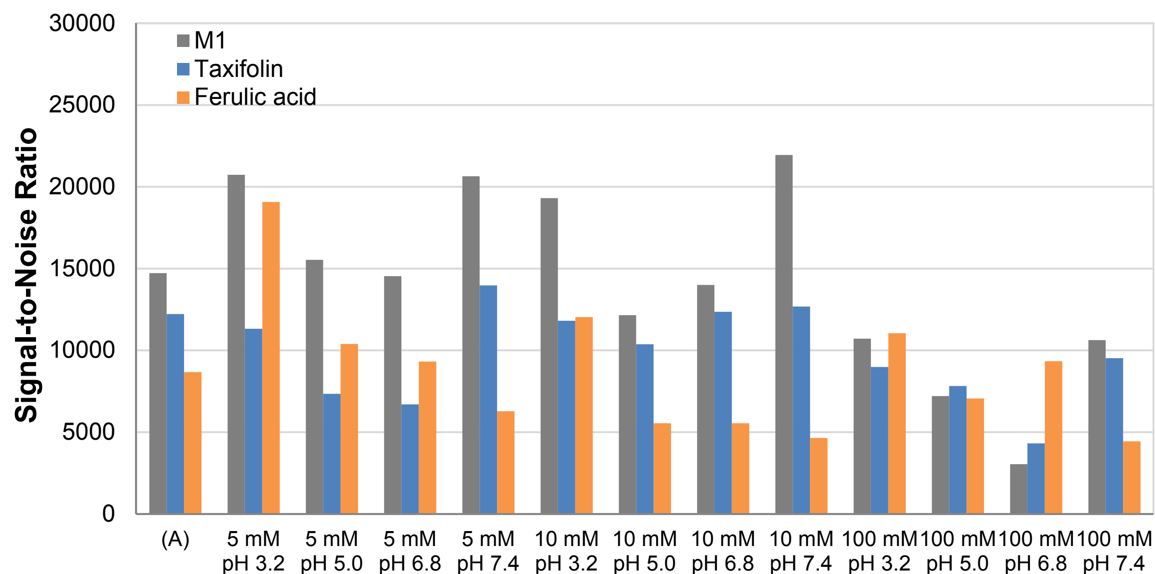

Figure S2. Optimization of the mobile phase (A; water +0.1% FA). Influence of the additive ammonium formiate (AF) with different ion strengths (5 -10-100 mM) and pH-values (3.2, 5.0, 6.8 and 7.4). *Solvent (B) was MeOH +0.1% FA. 10 µg/mL of M1, taxifolin and ferulic acid in MeOH were analyzed and the signal-to-noise ratio (SNR) of the quantifier was calculated (n= 1). The analytes were detected with the highest intensity (SNR, peak-to-peak height) with 5 mM AF, pH 3.2.*

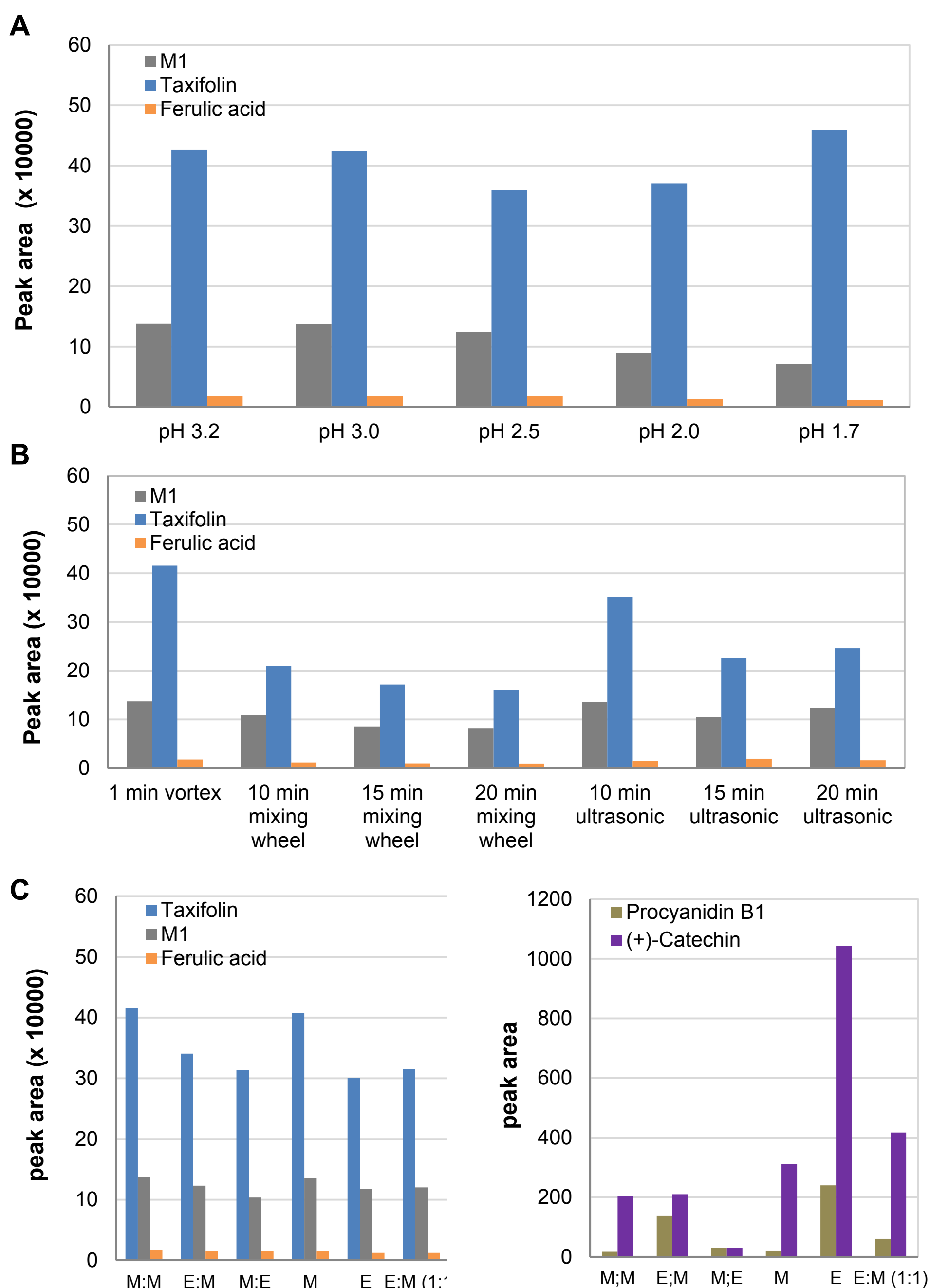

Figure S3. Optimization of the liquid-liquid extraction (LLE) for preparation of the plasma samples. *A sample volume of 2.0 mL plasma was spiked with 10 ng/mL per analyte and the influence of modifications of the extraction process on the intensity of the analyte signal was investigated.* ***A****. Influence pH-value of plasma before extraction with MTBE. Plasma was acidified with 4% H3PO4 to pH 3.2, 3.0, 2.5, 2.0, 1.7 before extraction of the analytes.* ***B****. Influence of the extraction technique and time. After acidification and addition of solvent the samples were vortexed (1 min), extracted using mixing wheel (10, 15 and 20 min) or an ultrasonic treatment (10, 15 and 20 min).* ***C****. Influence of the extraction solvent (M: MTBE; E: EA). The analytes of highest interest, respective ferulic acid, taxifolin and M1, were extracted best by MTBE (left) whereas other analytes with lower response, namely procyanidin B1 and catechin, were extracted with higher yields using EA (right). In addition to a mixture of these two solvents a twofold extraction was tested. A mixture of EA:MTBE (1:1, V/V) was chosen for extraction.*

5 Distribution of constituents and metabolites of maritime pine bark extract (Pycnogenol®) into serum, blood cells and synovial fluid of patients with severe osteoarthritis

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ABSTRACT

Scope: The present randomized controlled study aimed at investigating the in vivo distribution of constituents or metabolites of the dietary supplement Pycnogenol®.

Methods and results: Thirty patients with severe osteoarthritis scheduled for a knee arthroplasty received either 200 mg/day Pycnogenol® (P+) or no treatment (Co) over three weeks before surgery. Serum, blood cells and synovial fluid samples were analyzed by LC-ESI/MS/MS. Notably, highest polyphenol concentrations were not detected in serum. Catechin and taxifolin primarily resided within the blood cells while the metabolite M1, ferulic and caffeic acid were mainly present in synovial fluid samples. Taxifolin was detected in serum and synovial fluid exclusively in the P+ group. Likewise, no ferulic acid was found in serum samples of the Co group. Calculating ratios of analyte distribution in individual patients revealed a simultaneous presence of some polyphenols in serum, blood cells and/or synovial fluid only in the P+ group. Thus, it was possible to distinguish Pycnogenol® intake under real life conditions with occasional or regular consumption of foods or beverages rich in polyphenols.

Conclusion: This is the first evidence that polyphenols distribute into the synovial fluid of patients with osteoarthritis which supports rationalizing the results of clinical efficacy studies.

INTRODUCTION

Dietary polyphenols have been associated with numerous beneficial effects on humans' health. Studies investigating the absorption of polyphenols from the gastrointestinal tract revealed that blood concentrations of individual polyphenols are often very low [1]. Moreover, polyphenolic compounds are often subjected to an extensive metabolism [2]. Some metabolites generated by gut microbial metabolism obviously contribute to health effects [3].

One of those bioactive metabolites is δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) which is formed by the human intestinal flora from the procyanidins' catechin units [2]. It has been detected in urine and plasma samples after intake of Pycnogenol® [4, 5]. The dietary supplement Pycnogenol® is a standardized extract of the French maritime pine, which

conforms to the monograph “Maritime pine extract” in the United States Pharmacopeia (USP). It contains 65-75% oligomeric procyanidins and polyphenolic monomers, phenolic or cinnamic acids and their glycosides [6]. In numerous clinical studies Pycnogenol® demonstrated effects in different chronic diseases of e.g. inflammatory or cardiovascular origin [6, 7].

Another chronic disease with high pharmacoeconomic burden and significant impact on the patiens’ quality of life is osteoarthritis (OA). OA is a chronic degenerative joint disease which is characterized by progressive cartilage destruction and it is the leading cause of pain and disability [8]. Treatment of OA includes pharmacological and non-pharmacological interventions and aims at pain relief and improvement of function. Severe OA might also require surgical interventions such as knee or hip arthroplasty [9]. Dietary factors or supplements have been discussed as options in the management or prevention of OA [10].
In clinical studies OA symptoms such as pain and joint stiffness have been shown to improve upon intake of Pycnogenol® [11, 12]. While this clinical observation is consistent with a previously shown inhibition of NF-kB activation and inhibition of various matrix metalloproteinases by constituents or metabolites of this pine bark extract [13, 14] it is not clear yet whether bioactive polyphenols would actually be present at the site of disease, e.g. in the affected joints by OA. After an oral intake of multiple doses of Pycnogenol® concentrations in the nanomolar range of catechin, taxifolin, caffeic acid, ferulic acid and of a bioactive metabolite M1 have been detected in human plasma [5]. Moreover, an uptake of M1 into erythrocytes, monocytes and endothelial cells has been observed in vitro [15, 16]. The purpose of the current study was to investigate the in vivo distribution of constituents or metabolites of Pycnogenol® in serum, blood cells and synovial fluid of patients with severe OA scheduled for a knee replacement surgery.

PATIENTS, METHODS AND MATERIALS

Clinical study design

The present study was a randomized controlled clinical trial involving patients with severe osteoarthritis (OA) according to the Western Ontario and McMaster Universities Arthritis Index (WOMAC) score, who were scheduled for an elective knee replacement surgery. The study protocol was reviewed and approved (reference number 248/11) by the local Ethics Committee of the Medical Faculty of the University Würzburg.
A total of 30 OA patients were recruited for the study and gave informed consent. Patients were randomized into two groups using a computer-generated randomization list which was not accessible to the physicians and nurses who were involved in the patient care and management. Half of the study participants (n= 15) were assigned to the treatment group receiving 200 mg of the French maritime pine bark extract Pycnogenol® (Horphag Research Ltd.) per day (twice daily two capsules with each 50 mg) over three weeks prior to the planned surgery. The control group comprised of 15 patients who got no Pycnogenol®. All patients were asked to comply with a polyphenol-free nutrition, especially two days before each blood

sampling. For this purpose, they were provided with nutritional checklists specifying food/beverages they should avoid and for recording what they ingested within the last two days before blood sampling. Adherence to the study medication was estimated based on the number of returned Pycnogenol® capsules upon hospitalization for the knee replacement surgery. Blood samples from each study participant were collected (BD Vacutainer® SST II Advance; Becton Dickinson GmbH, Heidelberg, Germany) before oral intake of Pycnogenol® (V1, basal value); during the intake, approximately 1-2 days before the surgery (V2); and during or shortly before knee surgery (V3), about 12 h after the last dose of Pycnogenol®. Immediately after blood sampling the serum and cellular fraction were separated under sterile conditions. On the day of the surgery residual knee cartilage and synovial fluid were also collected. All samples were shock-frozen immediately and stored at -80 °C.

Chemicals, reagents and special materials

Analytical standards (+)-catechin, taxifolin, ferulic acid, caffeic acid and the internal standard (IS) 3,4-dihydroxyhydrocinnamic acid (hydrocaffeic acid) were all obtained from Sigma-Aldrich (Taufkirchen, Germany). The metabolite M1 (δ-(3.4-dihydroxy-phenyl)-γ-valerolactone) was synthesized by M. Rappold as part of his diploma thesis. Methanol (MeOH, LC-MS analyzed) from J.T.Baker Mallinckrodt and water (HiPerSolv CHROMANORM® for LC-MS) were obtained from VWR (Darmstadt, Germany). Ammonium formate (AF), and formic acid (FA) were purchased from Sigma-Aldrich. An enzymatic mixture of β-Glucuronidase/Sulfatase (ß-Gln/Sulfa) from Helix pomatia (Type HP-2; Sigma-Aldrich) was used for enzymatic hydrolysis. Ethyl acetate, *tert*-butyl methyl ether (MTBE) and phosphate buffered saline (PBS, pH 7.4) were obtained from Sigma-Aldrich.

Standard solutions

Stock solutions (1 mg/mL) of each standard substance ((+)-catechin, taxifolin, ferulic acid, caffeic acid and M1) and of the internal standard (IS; hydrocaffeic acid) were prepared in 100% methanol and stored at -80 °C. They were diluted with methanol to yield working standards which were aliquoted and stored at -20 °C.

Human specimen for calibration curves

Packed cells and serum were obtained from a blood bank (Bayerisches Rotes Kreuz (BRK), München, Germany) and handled as described before [17, 18]. Synovial fluid was collected from patients with intra-articular fluid accumulation who needed punctuation of the effusion for medical reasons. Synovial fluid samples were pooled to obtain a single batch for preparation of calibration standards for quantification of the clinical study samples.

Liquid chromatography (LC)

Details of the LC method have been reported before ([18] and unpublished data). Briefly, for the LC analysis an Agilent 1260 system was used. The chromatographic separation was

carried out using a Pursuit PFP-C18 column (4.6 x 150 mm, particle size 3 µm) at 20 °C (all from Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of 5 mM ammonium formate with 0.065% (v/v) formic acid (pH= 3.2; A) and methanol with 0.1% formic acid (B). The flow rate was set to 0.6 mL/min and the sample injection volume was 5 µL. The gradient elution was conducted starting at 60% B (0 min) to 95% B (2.50 min) and maintained to 95% B to 5.50 min followed by re-equilibration at 60% B. The total run time was 10.00 min with a post time of 3 min.

Mass spectrometry (MS/MS)

Details of the MS/MS method using a G 6460 TripleQuad LC/MS with turbo electrospray ionization (ESI; Agilent Technologies, Santa Clara, CA, USA) have been reported earlier [17, 18]. The optimized MS/MS transitions and mass spectrometric parameters of the compounds to be quantified in human blood cell and serum samples were recently reported ([18] and unpublished data), optimized parameters of additionally determined M1 metabolites in blood cells are listed in Table S1 in the electronic supplementary material. Optimized MS/MS transitions and mass spectrometric parameters of the compounds to be quantified in human synovial fluid samples are listed in Table S2.

Preparation of human serum samples

Serum samples (1.5 mL) were prepared by liquid-liquid extraction with prior enzymatic incubation containing ß-Gln/Sulfa to hydrolyze conjugated analytes [5] as previously described ([18] and unpublished data). Additionally, 1.5 mL of serum was analyzed without prior enzymatic hydrolysis to calculate the degree of conjugation with sulfate and glucuronic acid.

Preparation of human blood cell samples

Human blood cell samples were prepared as previously detailed [18]. Therefore, 2.0 mL blood cells of each study volunteer were processed with prior enzymatic hydrolysis step to determine the total concentration of the analytes.

Preparation of human synovial fluid samples

Method development and assessment of matrix effects

As described earlier ([18] and unpublished data), different sample preparation techniques were compared. Each analyte was evaluated regarding the recovery, quantitative matrix effect, process efficiency and matrix factor.

For this purpose, human synovial fluid samples were subjected to protein precipitation, liquid-liquid extraction, solid phase extraction, combined techniques and variations of dispersive solid phase extractions (Tables S3 and S4 in the electronic supplementary material). For each technique a sample set was prepared with the standard substance mix ((+)-catechin, taxifolin, ferulic acid, caffeic acid and M1) as described in detail previously [18].

Preparation of synovial fluid samples with the optimized liquid-liquid extraction

40 µL 4% o-phosphoric acid was added to 1.0 mL human synovial fluid (pH 5.0). Afterwards, the samples were incubated with an enzyme mixture containing ß-Gln/Sulfa (1500 U ß-Gln and 2 U Sulfatase per mL synovial fluid) for 45 min at 37 °C on a horizontal shaker (100 rpm) to hydrolyze conjugated analytes [5]. Then, 60 µL 4% o-phosphoric acid (pH 3.2), 25 µL IS (= 24.85 ng/mL) and 3.0 mL extraction solvent containing ethyl acetate and *tert*-butyl methyl ether (1:1; V/V) were added, vortexed for 1 min (Multi-Vortex, VWR, Darmstadt, Germany) and centrifuged for 5 min at 3,300 g (4 °C). Thereafter, 2.0 mL of the upper organic layer was evaporated to dryness under nitrogen. The residue was reconstituted in 75 µL of 100% MeOH, centrifuged at 18,000 g for 15 min at 4 °C before LC-MS/MS analysis.

Method validation

A full validation was performed for the quantification of the analytes in human synovial fluid with optimized liquid-liquid extraction method and prior enzymatic hydrolysis. The validation included the selectivity, linearity, lower limit of quantification (LLOQ), accuracy and precision (intra- and interday), recovery, process efficiency, matrix effects (quantitative), carry over, cross talk, and post-preparative stability. Also the freeze-and thaw-, short-term- and long- term stability of the analytes in human serum were investigated.

Quantification of the samples of the study participants

For each patient specimen human pooled matrix-matched calibration standards with an internal standard (structural) were used for quantification of the study samples. In case of a basal presence of an analyte in the blank matrix, the calibration curve was shifted along the y-axis by the response of the zero-sample (containing the IS) [19].

RESULTS AND DISCUSSION

Patients and protocol adherence

A total number of 30 patients (66.7% female) participated in the study and were randomized into a treatment (“P+”; 9 females, 6 males) and a control group (“Co”; 11 females, 4 males). There was no statistically significant difference between the groups in any of the basic demographic characteristics (Student’s T-test, $p > 0.05$), the mean age was 64.3 ± 8.2 years, height 1.69 ± 0.10 m, body weight 87.33 ± 15.66 kg (BMI 30.74 ± 5.29 kg/m2).

All study participants were requested to avoid polyphenol-rich food/beverages e.g. coffee, green tea, wine, chocolate, some fruits and vegetables, within the last two days before the blood samplings. Analysis of the nutrition protocols revealed that the nutritional advice was not followed well and dietary violations were admitted before collecting 42% of the blood samples. Thus, concentrations of common polyphenols such as catechin or caffeic acid from other sources than Pycnogenol® were to be expected in the blood samples.

In contrast, the adherence to the study medication was excellent based on the pill count-back on returned medication containers. In the Pycnogenol® group the average adherence was 99.4 ± 1.2% for all but one study participant who apparently took only 76% of the capsules.

Method for analysis polyphenols in human synovial fluid samples

To the best of our knowledge this is the first study describing the detection and quantification of polyphenols in human synovial fluid. Since concentrations in synovial fluid samples might be lower than in blood and based on the fact that a previous pharmacokinetic study revealed plasma concentrations of polyphenolic compounds in the nanomolar range after intake of Pycnogenol® [5] a highly sensitive method was required. In the course of method development the main focus was the optimal detection and quantification of the metabolite M1.

Analogous to previously developed methods for analysis of Pycnogenol® polyphenols in serum and blood cells ([18] and unpublished data) various sample preparation techniques (Tables S3 and S4 in the electronic supplemental material) were compared and a liquid-liquid extraction method (LLE #1; Figure S1) was chosen. The method was validated based on current EMA and FDA guidelines and complied with the requirements for selectivity, linearity (Table S5), precision and accuracy (Tables S6 and S7), robustness (Table S8), carry-over, cross-talk and post-preparative stability (Tables S9 and S10).

For the analytes of highest interests, the LLOQs in synovial fluid were 0.080 ng/mL for taxifolin and 0.117 ng/mL for M1 (Table 1 and Table S11). The method was slightly less sensitive for ferulic acid (LLOQ of 1.53 ng/mL), catechin (2.14 ng/mL) and caffeic acid (3.07 ng/mL). Thus, seen in the context of polyphenol detection in serum and blood cells ([18] and unpublished data), most sensitive analysis of M1 and taxifolin and least sensitive detection of catechin and caffeic acid was achieved in all specimen.

Table 1: Lower limits of quantification (LLOQs) of polyphenolic analytes of highest interest. *Data for serum and blood cells was derived from previous work ([18] and unpublished data).*

Analyte	LLOQ Synovial fluid [ng/mL]	LLOQ Serum [ng/mL]	LLOQ Blood cells [ng/mL]
Catechin	2.14	5.86	28.90
M1	0.12	0.16	0.12
Taxifolin	0.08	0.06	0.12
Caffeic acid	3.07	8.22	48.40
Ferulic acid	1.53	2.74	0.97

The mean recovery in human pooled synovial fluid was between 39.24% for catechin (8.18 ng/mL) and 87.63% for taxifolin (0.478 ng/mL; Table S12). The recovery of the analytes in three individual lots of synovial fluid reached from 43.46% for catechin and 69.25% for taxifolin (Table S13). The mean process efficiency in human pooled synovial fluid ranged from

29.03% for catechin and 192.04% for M1. In individual lots of synovial fluid the process efficiency varied from 31.23% for catechin and 200.23% for M1. The mean absolute matrix effects in human pooled synovial fluid ranged between -26.00% for (+)-catechin and 262.77% for M1. Relative matrix effects investigated in three different lots of human synovial ranged from –27.79% for catechin to 243.83% for M1.

Moreover, the IS-normalised matrix factor was calculated (Table S14) and ranged from -0.236 for catechin to 2.380 for M1 in pooled synovial fluid while it was between -0.252 for catechin to 2.207 for M1 in different lots of synovial fluid.

Thus, a novel and reliable analytical method for quantification of polyphenols in human synovial fluid has been successfully developed. Representative chromatograms of all three specimen of an individual study participant after multiple dosing of 200 mg/day Pycnogenol® over the course of three weeks (P+, V3) revealed total concentrations of 23.17 ng/mL catechin, 3.70 ng/mL ferulic acid, 0.19 ng/mL taxifolin, 0.16 ng/mL M1 in serum, 74.31 ng/mL catechin, 1.93 ng/mL ferulic acid, 0.57 ng/mL taxifolin in blood cells and 3.19 ng/mL ferulic acid, 0.18 ng/mL taxifolin, 0.17 ng/mL M1 in synovial fluid (Figure 1).

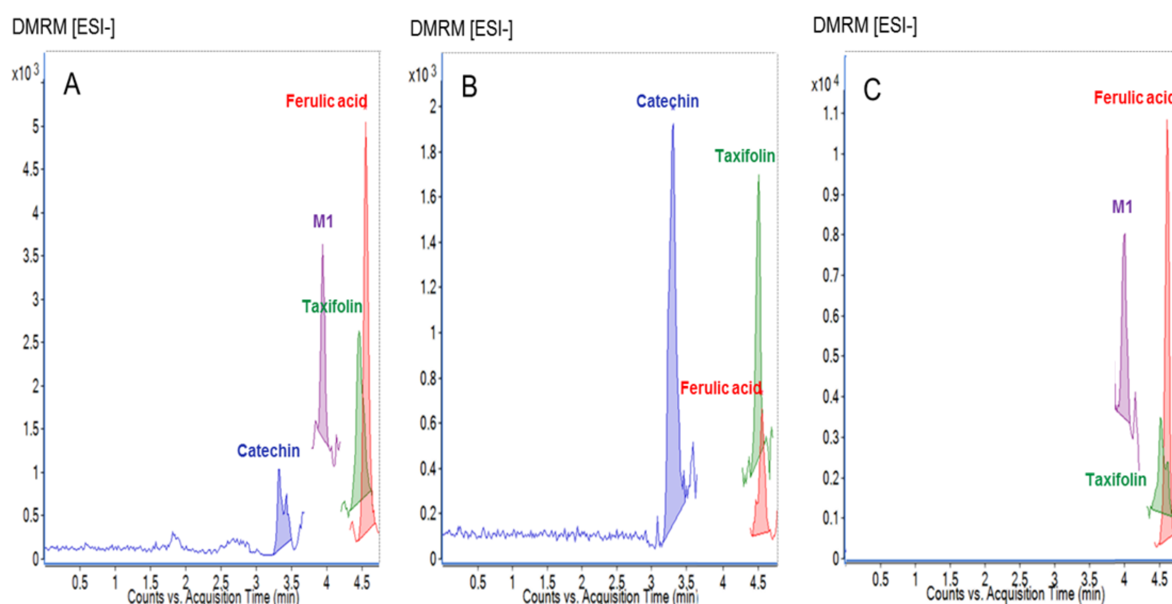

Figure 1. Example chromatograms for quantification in the three different sample matrices of one individual study participant after multiple dosing of 200 mg/day Pycnogenol® over the course of three weeks (P+, V3). A. *Serum. Quantifier of catechin (289 > 245), M1 (207 > 163), taxifolin (303 > 285) and ferulic acid (193 > 134) in ESI negative mode. The sample contained a total concentration of 23.17 ng/mL catechin, 3.70 ng/mL ferulic acid, 0.19 ng/mL taxifolin and 0.16 ng/mL M1.* ***B****. Blood cells. Quantifier of catechin (289 > 245), taxifolin (303 > 285) and ferulic acid (193 > 134) in ESI negative mode. The sample contained 74.31 ng/mL catechin, 1.93 ng/mL ferulic acid and 0.57 ng/mL taxifolin.* ***C****. Synovial fluid. Quantifier of M1 (207 > 163), taxifolin (303 > 285) and ferulic acid (193 > 134) in ESI negative mode. The sample contained 3.19 ng/mL ferulic acid, 0.18 ng/mL taxifolin and 0.17 ng/mL M1.*

Pycnogenol® constituents and metabolites in serum samples

In the basal serum samples (V1) the mean total concentrations (free and conjugated) of all study participants were 27.07 ± 16.39 ng/mL catechin (mean and standard deviation), 1.80 ± 2.63 ng/mL for M1, 0.07 ng/mL (n= 1) for taxifolin, 6.40 ± 2.58 ng/mL for ferulic acid and 18.58 ± 6.32 ng/mL for caffeic acid (data not shown). For example, catechin was detectable in 29 out of 30 V1 samples and in 24 samples the concentrations were above 10 ng/mL. Thereby, the catechin was primarily present as glucuronide-/sulfate-conjugate. When only the free concentrations were regarded, catechin was detectable in 20 out of 30 V1 samples and in 11 samples the concentrations were above 10 ng/mL. There were no differences in the basal concentrations between the participants assigned to the P+ or Co group. Even when disregarding those patients who admitted a violation of the dietary restrictions there were still considerable basal concentrations present in serum. These results clearly demonstrate that catechin and other polyphenols are ubiquitously present in human serum samples at measurable basal levels. Similar observations have been reported by others [20].

The analysis of serum samples obtained after three weeks (V3) of Pycnogenol® intake revealed highest concentrations for catechin, followed by caffeic acid, ferulic acid, M1 and taxifolin (Table 2, panel A). Notably, there taxifolin and ferulic acid were only detectable in the P+, not in the Co group. In a previous pharmacokinetic study with healthy volunteers taxifolin was not detectable under steady state conditions which was most probably due to the less sensitive analytical method used [5]. This is consistent with the very low concentrations of taxifolin found in the present study. Ferulic acid has been suggested to be a marker of consumption of French maritime pine bark extract. In healthy volunteers adhering to a low polyphenol diet both free and conjugated ferulic acid were determined in urine samples [21]. The present results confirm that ferulic acid appears to be a suitable marker of Pycnogenol® intake since it was not discovered in serum samples of the control group.

Although there was a tendency of higher concentrations of catechin in the P+ compared to the control group the serum concentrations of M1 and caffeic acid in the control group exceeded those in the P+ group (Table 2, panel A). When patients who admitted intake of e.g. coffee, green tea or chocolate were excluded from the analysis the trend of higher catechin levels as well as clearly higher concentrations of M1 in the P+ group became obvious and caffeic acid was not even detectable in the control group (Table 2, panel B).

Table 2: Results of the sample analysis in serum, blood cells and synovial fluid of patients taking Pycnogenol® 200 mg/day (P+) and control patients (Co) receiving no supplement. A: *Total mean concentrations and standard deviations (SD) determined after three weeks (V3) without exclusion of patients who admitted violations of the dietary restrictions.* ***B:*** *Total mean concentrations and SD determined after three weeks (V3). Patients who admitted violations of the dietary restrictions in their nutritional protocols were excluded. n.d.: not detected; Conc.: concentration*

A

		Catechin		M1		Taxifolin		Ferulic acid		Caffeic acid	
		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD	
Serum	P+	52.53 ± 18.40	*n= 15*	0.54 ± 0.84	*n= 9*	0.20 ± 0.12	*n= 5*	3.02 ± 0.39	*n= 7*	9.28 ± 0.51	*n= 3*
	Co	45.85 ± 39.59	*n= 15*	1.07 ± 1.09	*n= 5*	n.d.		n.d.		14.84 ± 1.92	*n= 3*
Blood cells	P+	71.18 ± 27.34	*n= 14*	0.19 ± 0.07	*n= 12*	0.52 ± 0.23	*n= 15*	1.86 ± 0.36	*n= 10*	n.d.	
	Co	70.48 ± 36.22	*n= 13*	0.21 ± 0.05	*n= 5*	0.48 ± 0.32	*n= 15*	1.80 ± 0.85	*n= 7*	n.d.	
Synovial fluid	P+	2.99 ± 0.43	*n= 4*	0.62 ± 0.77	*n= 5*	0.21 ± 0.03	*n= 2*	4.29 ± 1.83	*n= 8*	10.32 ± 3.96	*n= 7*
	Co	3.94 ± 1.83	*n= 2*	0.78 ± 0.74	*n= 4*	n.d.		3.04 ± 0.79	*n= 6*	12.83 ± 8.95	*n= 10*

B

		Catechin		M1		Taxifolin		Ferulic acid		Caffeic acid	
		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD		Conc. [ng/mL] ± SD	
Serum	P+	48.41 ± 18.61	*n= 11*	0.70 ± 1.02	*n= 6*	0.20 ± 0.12	*n= 5*	3.09 ± 0.46	*n= 5*	9.78	*n= 1*
	Co	32.24 ± 17.38	*n= 8*	0.25 ± 0.05	*n= 2*	n.d.		n.d.		n.d.	-
Blood cells	P+	73.75 ± 29.25	*n= 11*	0.20 ± 0.07	*n= 9*	0.56 ± 0.19	*n= 11*	1.85 ± 0.38	*n= 9*	n.d.	
	Co	63.31 ± 31.28	*n= 7*	0.18 ± 0.05	*n= 2*	0.39 ± 0.16	*n= 8*	1.69 ± 0.10	*n= 3*	n.d.	
Synovial fluid	P+	3.00 ± 0.58	*n= 2*	0.92 ± 0.93	*n= 3*	0.21 ± 0.03	*n= 2*	4.31 ± 2.10	*n= 6*	10.63 ± 3.86	*n= 4*
	Co	2.65	*n= 1*	0.17 ± 0.03	*n= 2*	n.d.		3.16 ± 0.22	*n= 3*	10.99 ± 5.79	*n= 4*

The degree of analyte conjugation with sulfate and glucuronic acid in serum ranged from 54.29 ± 26.77% for catechin (n= 51) to 98.34 ± 4.40% for M1 (n= 30; Table 3). This is consistent with the data determined in a former investigation [5] which described a conjugation degree of 56.50 ± 27.90% for catechin (n= 5), 69.40 ± 11.80% for caffeic acid (n= 3) and no free concentrations of ferulic acid, taxifolin and M1.

Table 3: Mean conjugation degree in serum samples (both P+ and Co group; V1, V2 and V3 blood samples; in total n= 90 samples). *Results were compared with former investigations [5].*

Analytes	Conjugation degree [%]					
	Current study			Former investigation		
	Mean	± SD	Sample size	Mean	± SD	Sample size
Catechin	54.29	26.77	n= 51	56.50	27.90	n= 5
M1	98.34	4.40	n= 30	100 *		
Taxifolin	96.75	7.23	n= 11	100 *		
Ferulic acid	90.32	16.58	n= 24	100 *		
Caffeic acid	80.95	17.95	n= 10	69.40	11.80	n= 3

** A conjugation degree of 100% was assumed because no free concentrations were detectable.*

Due to the higher number of study participants and thus samples in the present study and the more sensitive analytical method, the conjugation degree of the analytes, especially ferulic acid, taxifolin and M1 can be now described more precisely. However, as observed before and reported by others [21] the interindividual variability of the conjugation degree of the analytes was high. Even in the individual person the degree of conjugation cannot be regarded as a constant since it apparently also depends on the current analyte concentration in the specimen [22].

Pycnogenol® constituents and metabolites in blood cell samples

Blood cells and erythrocytes represent a significant pharmacological compartment for distribution of xenobiotics [23, 24]. Individual polyphenols have been shown to accumulate in human blood cells, macrophage-derived foam or endothelial cells [15, 16, 24, 25].

As seen with the serum samples before, basal total concentrations of the analytes with the exception of caffeic acid were detectable at V1 with no differences between the participants assigned to the P+ or Co group. Mean concentrations of 61.38 ± 40.25 ng/mL catechin (mean ± SD), 1.68 ± 0.55 ng/mL ferulic acid, 0.40 ± 0.18 ng/mL taxifolin, and 0.19 ± 0.08 ng/mL M1 were determined (data not shown).

The analysis of blood cell samples obtained after three weeks (V3) of intake of Pycnogenol® revealed highest concentrations for catechin, followed by ferulic acid, M1 and taxifolin (Table 2, panel A). No caffeic acid was detectable in any of the samples. There were no clear differences in the concentrations determined in the P+ or Co group. When patients who admitted non-adherence to the dietary restrictions were excluded from the analysis there was a slight trend towards higher catechin, taxifolin and ferulic acid levels in the in the P+ group compared to the control group (Table 2, panel B).

Notably, only low concentrations of M1 were found. This apparently contradicts previous results showing an enhanced cellular uptake of M1, possibly via the GLUT-1 transporter, into human erythrocytes [16]. However, M1 is subsequently subjected to an extensive intracellular metabolism [17] which would explain the low remaining intracellular levels of M1 under steady state conditions. Consequently, the blood cells samples of the present study were also screened regarding the presence of any of the previously detected cellular M1 metabolites [17]. Indeed, in the V3 samples of the P+ group the open-chained ester form of M1 (M1-COOH; n= 5; Figure 2 A) was identified as well as the glutathione conjugate of M1 (M1-GSH; n= 1; Figure 2 B). In the V3 samples of the Co group only the M1-COOH was detected in one patient sample.

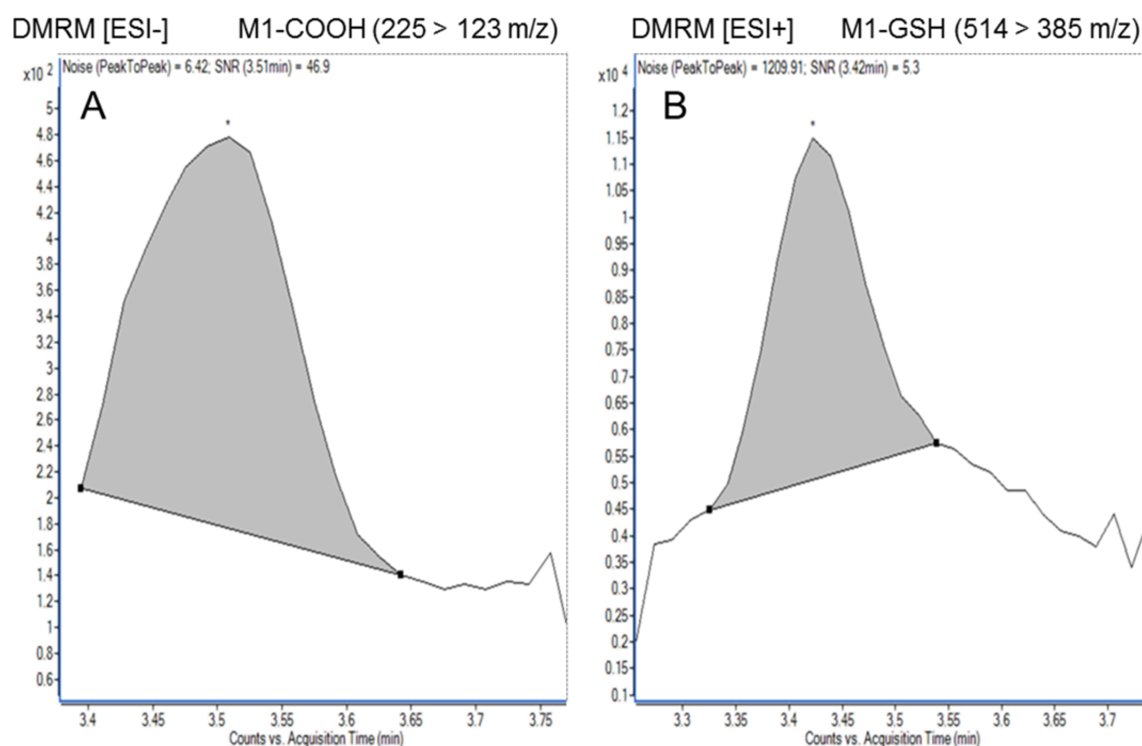

Figure 2. Example chromatograms for identification of intracellular metabolites of M1 in blood cells of study participants after multiple dosing of 200 mg/day Pycnogenol® over the course of three weeks (P+, V3). *After initially smoothing (function: Gaussian, 5 points) of the most abundant transition of the compound, the signal-to-noise ratio (SNR; peak-to-peak height) was calculated.* ***A****. M1-COOH. The SNR (Noise: 3.65 – 3.75 min) calculation of the transition 223 > 123 in ESI negative mode resulted in a value of 47.* ***B****. M1-GSH. Here, the SNR (Noise: 3.55 – 3.65 min) calculation of the transition 514 > 385 in ESI positive mode resulted in a value of 5.*

Pycnogenol® constituents and metabolites in synovial fluid samples

Sampling of synovial fluid is typically practiced for diagnostic reasons, e.g. for detection of a septic arthritis. In research, there is great interest in osteoarthritis biomarkers such as cytokines that might assist diagnosis or prognosis [26]. In contrast, drug concentrations are rarely reported for synovial fluid samples. To the best of our knowledge, this is the first study investigating polyphenol concentrations in human synovial fluid samples of patients with osteoarthritis. Prospective clinical studies have demonstrated that an oral administration of 100-150 mg Pycnogenol® over a course of three months resulted in a significant improvement of knee osteoarthritis symptoms, such as reduced pain and joint stiffness and diminished need for analgesics [11, 12]. However, so far it remained elusive whether any constituents or metabolites of the pine bark extract would actually be distributed into the knee synovial fluid and thereby contribute to the clinical effects.

In the present study synovial fluid samples were obtained at the time of knee surgery. The analysis of the patient samples revealed the presence of polyphenols with highest concentrations found for caffeic acid, followed by ferulic acid, catechin, M1 and taxifolin (Table 2, panel A). Similar to the results of serum analysis, taxifolin was only detectable in the P+, not in the Co group. Thus, it might be a marker of Pycnogenol® consumption. In contrast, there were no vast differences in the concentrations of the other polyphenols determined in the P+ or Co group. Excluding the results of those patients not adhering to the dietary restrictions exhibited a slight trend towards higher M1 and ferulic acid levels in the in the P+ group compared to the Co group (Table 2, panel B). For caffeic acid and catechin almost identical concentrations were found in the synovial fluid of both patient groups. This might be due to a lagged equilibrium time between serum and synovial fluid so that a diet low in polyphenols two days before specimen sampling might have been too short.

Distribution Pycnogenol® constituents and metabolites between specimen

An assessment of the mean concentrations of the constituents and metabolites in serum, blood cells and synovial fluid revealed that the individual compounds did not distribute equally between the specimen (Table 2). Notably, the highest concentrations of the polyphenols were not detected in serum. Catechin and taxifolin primarily resided within the blood cells while M1, ferulic and caffeic acid were mainly present in synovial fluid samples. Generally, data on distribution of polyphenols in humans is scarce. Although numerous investigations focus on absorption, metabolism and elimination of polyphenols [1, 27] only few studies investigate the distribution, e.g. into human tissues [28, 29]. Distribution into or accumulation in certain body compartments might help understanding the effects of polyphenols despite of the typically low plasma / serum concentrations that are usually observed [1, 28].

Although there were some trends towards higher analyte concentrations in the specimen of the patients who received Pycnogenol® in the present study, the mean concentrations were not statistically significantly different between the groups and were subject to high interindividual variability. It was possible that individual trends in distribution of the analytes

were overlooked if only group mean concentrations were considered. Therefore, the individual ratios of the analyte concentrations in the different specimen of single patients were calculated and summarized (Figure 3).

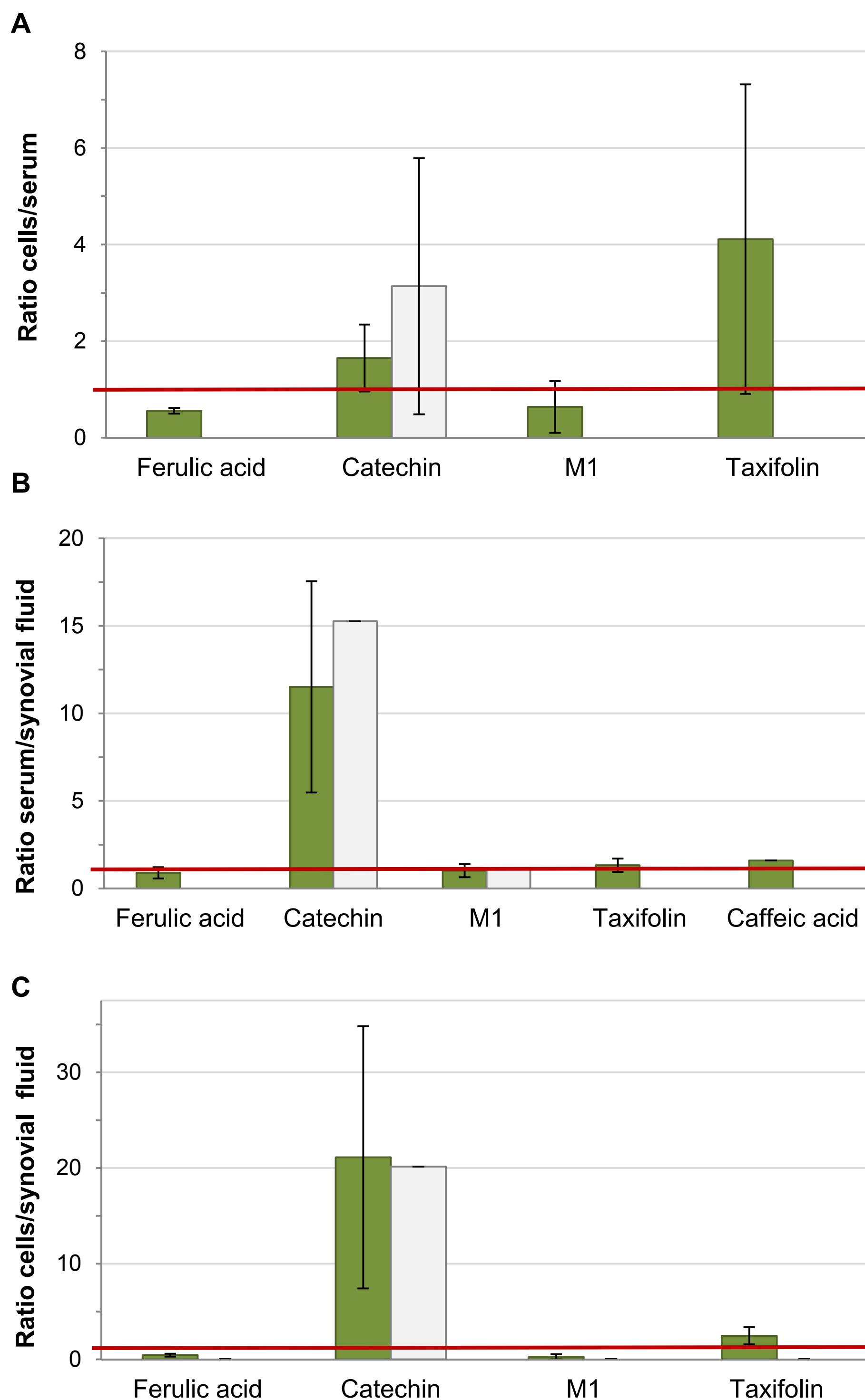

Figure 3. Summarized individual ratios of the analyte concentrations in different specimen of single study participant. *Columns of the intervention (P+; dark green) and control (Co; light grey) group represent the mean and standard deviation of the individual calculated ratios.* ***A****. Ratio cells/serum.* ***B****. Ratio serum/synovial fluid.* ***C****. Ratio cells/synovial fluid.*

The mean of the individual concentration ratios between blood cells and serum (total concentrations, V3) showed that ferulic acid (0.56 ± 0.06; n= 4), M1 (0.64 ± 0.54; n= 5) and taxifolin (4.11 ± 3.21; n= 5) were present in both matrices exclusively in the P+ group (Figure 3, panel A). While these results were expected for taxifolin and ferulic acid (see Table 2) it was a new observation that M1 was present both in blood cells and serum only when the participants received Pycnogenol®, but not when M1 originated from other dietary sources. Since ratios higher than 1 point out that the analyte is primarily distributed into blood cells compared to serum it can be concluded that higher concentrations of taxifolin were present in blood cells while ferulic acid and M1 preferentially resided in serum. Like taxifolin, catechin was clearly more present in blood cells compared to serum. In the patient group receiving 200 mg/day Pycnogenol® the catechin distribution into blood cells (1.65 ± 0.69; n= 11) was less pronounced than in the control group (3.14 ± 2.65; n= 7).

The mean of the individual concentration ratios between serum and synovial fluid showed that in both patient groups catechin was primarily distributed into serum compared to the synovial fluid (P+: 11.51 ± 6.04; n= 4 and Co: 15.27; n= 1; Figure 3, panel B). The analytes for caffeic acid (1.60; n= 1), taxifolin (1.33 ± 0.38; n= 2) and ferulic acid (0.89 ± 0.32; n= 4) were only present in both matrices after intake of Pycnogenol® and not in the control group. Ferulic acid preferentially resided in the synovial fluid, while taxifolin and caffeic acid showed the opposite tendency. The metabolite M1 was detected both in the P+ (1.01 ± 0.37; n= 3) and Co group (1.14; n= 1) and it appeared to be almost in equilibrium between serum and synovial fluid.

The mean of the individual concentration ratios between blood cells and synovial fluid revealed a strong tendency of catechin for localization within blood cells compared to the synovial fluid (Figure 3, panel C). This was observed in both groups of the study participants (P+: 21.11 ± 13.70; n= 2 and Co: 20.15; n= 1). Taxifolin (2.48 ± 0.90; n= 2), M1 (0.27 ± 0.27; n= 2) and ferulic acid (0.45 ± 0.15; n= 5)) were present in both matrices exclusively in the P+ group. Thereby, taxifolin was more present in blood cells compared to synovial fluid while M1 and ferulic acid preferentially resided in the synovial fluid.

CONCLUDING REMARKS

In the present study the in vivo distribution of constituents and metabolites of the French maritime pine extract Pycnogenol® between human serum, blood cells and synovial fluid was investigated for the first time. A newly developed and validated highly sensitive LC-ESI/MS/MS method allowed for the detection and quantification of various polyphenolic compounds in synovial fluid and thereby facilitated the first proof that polyphenols are actually distributed into joints. The comparison of compound concentrations in different specimen revealed that individual polyphenols displayed particular preference for sites of distribution other than serum. While catechin and taxifolin primarily resided within the blood cells, the metabolite M1, ferulic and caffeic acid were mainly present in synovial fluid samples. Although the mean polyphenol concentrations in serum, blood cells and synovial fluid were not statistically significantly different between the P+ and Co group due to non-adherence to the dietary

restrictions, distinctive observations were made in the P+ group. Taxifolin was detected in serum and synovial fluid exclusively after intake of the pine bark extract. Likewise, ferulic acid was only found in serum samples of the patients who received Pycnogenol®. A simultaneous presence of ferulic acid, M1 and taxifolin in blood cells and serum or cells and synovial fluid was only observed after intake of the pine bark extract. Also, ferulic acid, taxifolin and caffeic acid were only detected in both serum and synovial fluid in the P+ group. Thus, it was possible to determine markers of Pycnogenol® intake under real life conditions with people occasionally or regularly consuming foods or beverages rich in polyphenols.

Author contributions

M.M. planned and performed the experiments and contributed to drafting of the manuscript. L.S. and F.G. were involved in patient management and recording of clinical data. P.H. conceived of and planned the study and contributed to drafting of the manuscript.

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Conflict of interest statement

M.M., L.S. and F.G. declare no conflict of interests. P.H. received research grants from Horphag Research, the producer of Pycnogenol®, within the past five years.

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ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1: Additional optimized transitions and parameters in dynamic multiple reaction monitoring (DMRM) mode for identification of compounds in human blood cells. *Electron multiplier voltage (EMV) was set to +500 V in ESI negative and +1000 V in ESI positive mode. Following intracellular metabolites of M1 were monitored: Conjugation products of M1 and glutathione (M1-GSH), cysteine (M1-CYS) and oxidized glutathione (M1-GSSG); an acetylated form of M1 (M1-acetylated), the open-chained ester form of M1 (M1-COOH) and another metabolite, respectively hydroxybenzoic acid (see [28]).*

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	FV[a] [V]	CE[b] [V]	CAV[c] [V]	MS 1 Resolution	MS 2 Resolution	R_T[d] [min]	R_T Window *(DMRM)*	ESI mode
M1-GSH	514.0	514.0	95	0	1	Widest	Widest	3.50	0.50	positive
	514.0	385.0	95	13	1	Widest	Widest	3.50	0.50	positive
	514.0	130.0	95	23	1	Widest	Widest	3.50	0.50	positive
M1-CYS	328.0	328.0	100	0	1	Widest	Widest	3.60	0.50	positive
	328.0	155.0	70	15	7	Widest	Widest	3.60	0.50	positive
	328.0	85.0	100	25	7	Widest	Widest	3.60	0.50	positive
M1-GSSG	410.0	410.0	150	0	3	Widest	Widest	3.10	0.40	positive
	410.0	232.0	135	15	7	Widest	Widest	3.10	0.40	positive
M1-acetylated	251.0	251.0	100	0	7	Widest	Widest	5.40	0.50	positive
M1-COOH	225.0	123.0	100	10	3	Widest	Widest	3.60	0.40	negative
	225.0	101.0	100	5	3	Widest	Widest	3.60	0.40	negative
Hydroxybenzoic acid	137.0	137	70	0	7	Widest	Widest	3.90	0.40	negative
	137.0	123	150	7	7	Widest	Widest	3.90	0.40	negative

[a]FV. Fragmentor voltage. [b]CE. Collision energy. [c]CAV. Cell accelerator voltage. [d]R_T. Retention time.

Table S2: Optimized transitions and parameters in dynamic multiple reaction monitoring (DMRM) employing negative ESI ionization mode for LC-MS/MS analysis of prepared synovial fluid samples. *Electron multiplier voltage (EMV) was set to +750 V. Cycle time was 1000 ms.*

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	FV[a] [V]	CE[b] [V]	CAV[c] [V]	MS 1 Resolution	MS 2 Resolution	R_T[d] [min]	R_T Window *(DMRM)*
(+)-Catechin	289.1	245.0[e]	9	76	7	Widest	Widest	3.60	0.45
	289.1	203.0	17	76	7	Widest	Widest	3.60	0.45
M1	207.0	163.1[e]	13	115	5	Widest	Widest	4.00	0.45
	207.0	122.0	17	115	5	Widest	Widest	4.00	0.45
Caffeic acid	179.0	135.1[e]	13	90	5	Widest	Widest	4.00	0.50
	179.0	134.0	25	90	5	Widest	Widest	4.00	0.50
Taxifolin	303.1	285.0[e]	9	95	4	Widest	Widest	4.55	0.45
	303.1	125.0	21	95	4	Widest	Widest	4.55	0.45
Ferulic acid	193.1	134.1[e]	13	80	1	Widest	Widest	4.65	0.45
	193.1	178.0	9	80	1	Widest	Widest	4.65	0.45
Hydrocaffeic acid (IS)	181.2	137.0[e]	9	85	4	Wide	Widest	3.85	0.50
	181.2	109.0	9	85	4	Wide	Widest	3.85	0.50

[a]FV. Fragmentor voltage. [b]CE. Collision energy. [c]CAV. Cell accelerator voltage. [d]R_T. Retention time. [e]Quantifier. transition for quantification

Table S3: Overview of the tested sample preparation techniques, part I (PPT and LLE). *Various reagents were added to 0.25 mL human pooled synovial fluid in different volume ratios. Cold reagents were cooled to 4 °C. PPT: protein precipitation; LLE: liquid-liquid extraction.*

Sample preparation	Process
PPT #1	Addition of 4% H_3PO_4 (pH 2.5); Addition of ACN_{cold} (1:3)
PPT #2	Addition of 4% H_3PO_4 (pH 2.5); Addition of $MeOH_{cold}$ (1:3)
PPT #3	Addition of 4% H_3PO_4 (pH 1.5); Addition of $MeOH_{cold}$ (1:3)
PPT #4	Dilution with PBS buffer (1:1); Addition of 4% H_3PO_4 (pH 2.5); Addition of $MeOH_{cold}$ (1:6)
PPT #5	Addition of 4% H_3PO_4 (pH 2.5); Addition of $MeOH_{cold}$ (1:6)
LLE #1	Addition of 4% H_3PO_4 (pH 3.2); Addition of MTBE:EA 50:50 (1:3)
LLE #2	Dilution with PBS buffer (1:1); Addition of 4% H_3PO_4 (pH 3.2); Addition of MTBE:EA 50:50 (1:1.5)
LLE #3	Addition of 4% H_3PO_4 (pH 2.5); Addition of MTBE:EA 50:50 (1:3)
LLE #4	Dilution with PBS buffer (1:1); Addition of 4% H_3PO_4 (pH 2.45); Addition of MTBE:EA 50:50 (1:1.5)
LLE #5	Addition of 4% H_3PO_4 (pH 1.5); Addition of MTBE:EA 50:50 (1:3)
LLE #6	Dilution with PBS buffer (1:1); Addition of 4% H_3PO_4 (pH 1.5); Addition of MTBE:EA 50:50 (1:1.5)
LLE #7	Addition of 4% H_3PO_4 (pH 3.2); Addition of 100% MTBE (1:3)
LLE #8	Addition of 4% H_3PO_4 (pH 3.2); Addition of 100% EA (1:3)
LLE #9	Addition of 4% H_3PO_4 (pH 3.2); Addition of 100% isopropanol (1:3)
LLE #10	Addition of 4% H_3PO_4 (pH 3.5); Addition of MTBE:EA 50:50 (1:3)
LLE #11	Addition of 4% H_3PO_4 (pH 3.2); Addition of MTBE:EA 50:50 (1:6)
LLE #12	Dilution with PBS buffer (1:1); Addition of 4% H_3PO_4 (pH 3.2); Addition of MTBE:EA 50:50 (1:3)
LLE #13	Dilution with 0.1 M AA-buffer (pH 5;1:1); Addition of 100% EA (1:2.5)
LLE #14	Dilution with 0.1 M AA-buffer (pH 5;1:1); Addition of 100% MTBE (1:2.5)
LLE #15	Dilution with 0.1 M AF-buffer (pH 3.2;1:1); Addition of 100% MTBE (1:2.5)
LLE #16	Dilution with 0.1 M AF-buffer (pH 3.2;1:1); Addition of MTBE:EA 50:50 (1:2.5)

Table S4: Overview of the tested sample preparation techniques, part II (SPE, QuEChERS and Combis). *For QuEChERS variations and background also see [28].*
Various reagents were added to 0.25 mL human pooled synovial fluid in different volume ratios. Cold reagents were cooled to 4 °C. SPE: solid phase extraction. Combi: combined techniques.

Sample preparation	Process
SPE #1 Oasis™ HLB, reversed phase	Conditioning: 1 mL MeOH; Equilibration: 1 mL water; Loading: 0.25 mL synovial fluid + 0.25 mL PBS buffer + 50 µL 4% H_3PO_4; Washing: 1 mL 5% MeOH in water; Elution: 1 mL MeOH (2 x 0.5 mL)
SPE #2 Oasis™ MAX, strong anion exchange	Conditioning: 1 mL MeOH; Equilibration: 1 mL water; Loading: 0.25 mL synovial fluid + 0.25 mL PBS buffer + 50 µL 4% H_3PO_4; Washing: 1 mL 5% NH_3 in water; Elution 1: 1 mL MeOH; Elution 2: 1 mL 2% FA in MeOH (2 x 0.5 mL)
SPE #3 Strata™ X, reversed phase	Conditioning: 1 mL MeOH; Equilibration: 1 mL water; Loading: 0.25 mL synovial fluid + 0.25 mL PBS buffer + 50 µL 4% H_3PO_4; Washing: 1 mL 20% MeOH in water; Elution: 1 mL MeOH/ACN/water/FA 60:30:10:0.1 (2 x 0.5 mL)
SPE #4 Strata™ X-A, strong anion exchange	Conditioning, 1 mL MeOH; Equilibration: 1 mL water; Loading, 0.5 mL plasma + 0.5 mL 4% H_3PO_4; Washing 1, 1 mL 25 mM AA (pH 6-7); Washing 2, 1 mL MeOH; Elution: 1 mL 5% FA in MeOH (2 x 0.5 mL)
QuEChERS #1	Dilution with PBS buffer (1:10), + 5 mL 1% HAC in ACN;+ 4 g $MgSO_4$ + 1 g NaAc; vortex for 1 min, centrifugation 5 min at 3,300 g (4 °C), + 100 mg PSA + 600 mg $MgSO_4$ to upper layer, vortex for 1 min, centrifugation 5 min at 3,300 g (RT)
QuEChERS #2	Dilution with PBS buffer (1:10), + 5 mL 1% HAC in ACN;+ 3 g $MgSO_4$ + 1 g NaCl + 1 g Na_3-citrate + 0.5 g Na_2-hydrogencitrate, vortex for 1 min, centrifugation 5 min at 3,300 g (4 °C), + 100 mg PSA + 600 mg $MgSO_4$ to upper layer, vortex for 1 min, centrifugation 5 min at 3,300 g (RT)
QuEChERS #3	Dilution with PBS buffer (1:10), + 5 mL 1% HAC in ACN;+ 4 g $MgSO_4$ + 1 g NaCl; vortex for 1 min, centrifugation 5 min at 3,300 g (4 °C), + 100 mg PSA + 600 mg $MgSO_4$ to upper layer, vortex for 1 min, centrifugation 5 min at 3,300 g (RT)
QuEChERS #4	Dilution with PBS buffer (1:10), + 5 mL 100% EA;+ 4 g $MgSO_4$ + 1 g NaAc; vortex for 1 min, centrifugation 5 min at 3,300 g (4 °C), + 100 mg PSA + 600 mg $MgSO_4$ to upper layer, vortex for 1 min, centrifugation 5 min at 3,300 g (RT)
QuEChERS #5	Dilution with PBS buffer (1:10), + 5 mL 100% MTBE;+ 4 g $MgSO_4$ + 1 g NaAc; vortex for 1 min, centrifugation 5 min at 3,300 g (4 °C), + 100 mg PSA + 600 mg $MgSO_4$ to upper layer, vortex for 1 min, centrifugation 5 min at 3,300 g (RT)
Combi#1	PPT: Addition of 1 M HCl (1:0.5); Addition of ACN_{cold} (1:3.33) + LLE: Addition of 2.5 mL EA:MTBE 50:50

Table S5: Calibration range, calibration function and correlation coefficients of the five analytes extracted from human pooled synovial fluid (n= 3).

Analytes	Range [ng/mL]	Slope ± SD	y-intercept	Correlation coefficient R
(+)-Catechin	2.14 - 34.93	0.0195 ± 0.006	-0.0175	0.9977
Ferulic acid	1.53 - 24.93	0.1184 ± 0.024	0.0693	0.9979
M1	0.117 - 1.900	1.6016 ± 0.243	0.0330	0.9976
Taxifolin	0.080 - 1.307	0.7518 ± 0.010	-0.0388	0.9992
Caffeic acid	3.07 - 49.95	0.6752 ± 0.113	-0.8800	0.9988

Table S6: Intraday accuracy and precision of the analytes in human pooled synovial fluid (n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean ± SD [%]	Precision [%]
Catechin			
2.14	2.24 ± 0.15	104.55 ± 6.78	6.48
2.68	2.63 ± 0.43	98.24 ± 16.24	16.50
8.18	7.47 ± 0.79	91.40 ± 9.67	10.58
12.77	10.86 ± 0.41	85.04 ± 3.21	3.77
19.96	17.62 ± 0.71	88.25 ± 3.54	4.01
Ferulic acid			
1.53	1.70 ± 0.05	111.13 ± 3.45	3.10
1.91	1.98 ± 0.14	103.36 ± 7.18	6.95
5.83	5.13 ± 0.19	87.85 ± 3.28	3.73
9.12	8.60 ± 0.73	94.33 ± 7.97	8.45
14.24	13.27 ± 0.95	93.14 ± 6.68	7.17
M1			
0.117	0.129 ± 0.016	110.51 ± 13.43	12.16
0.146	0.152 ± 0.014	103.84 ± 9.37	9.02
0.445	0.457 ± 0.049	102.64 ± 10.97	10.69
0.695	0.770 ± 0.030	110.82 ± 4.25	3.84
1.086	1.014 ± 0.080	93.39 ± 7.37	7.89
Taxifolin			
0.080	0.084 ± 0.016	105.70 ± 19.48	18.43
0.100	0.096 ± 0.015	95.91 ± 15.48	16.14
0.306	0.270 ± 0.016	88.35 ± 5.11	5.78
0.478	0.427 ± 0.020	89.36 ± 4.27	4.77
0.747	0.653 ± 0.020	87.40 ± 2.72	3.12
Caffeic acid			
3.07	3.55 ± 0.18	115.73 ± 5.74	4.96
3.83	3.64 ± 0.13	95.11 ± 3.47	3.65
11.69	10.42 ± 0.70	89.15 ± 5.97	6.70
18.27	18.65 ± 1.99	102.10 ± 10.91	10.69
28.54	27.85 ± 2.17	97.57 ± 7.59	7.78

Table S7: Interday accuracy and precision of the analytes in human pooled synovial fluid (n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean ± SD [%]	Precision [%]
Catechin			
2.14	2.16 ± 0.33	100.72 ± 15.43	15.32
2.68	2.77 ± 0.16	103.53 ± 5.85	5.65
8.18	7.97 ± 0.53	97.51 ± 6.51	6.68
12.77	11.28 ± 0.40	88.34 ± 3.15	3.56
19.96	17.96 ± 0.39	89.98 ± 1.94	2.16
Ferulic acid			
1.53	1.51 ± 0.16	98.91 ± 10.71	10.82
1.91	1.88 ± 0.09	98.43 ± 4.63	4.71
5.83	5.60 ± 0.43	95.97 ± 7.31	7.62
9.12	8.76 ± 0.18	96.08 ± 2.02	2.11
14.24	13.04 ± 0.39	91.53 ± 2.71	2.97
M1			
0.117	0.119 ± 0.009	101.47 ± 7.86	7.75
0.146	0.143 ± 0.008	97.62 ± 5.59	5.73
0.445	0.446 ± 0.010	100.11 ± 2.32	2.32
0.695	0.682 ± 0.077	98.17 ± 11.03	11.23
1.086	0.975 ± 0.035	89.80 ± 3.21	3.58
Taxifolin			
0.080	0.080 ± 0.004	100.56 ± 4.92	4.89
0.100	0.096 ± 0.006	96.23 ± 6.29	6.53
0.306	0.286 ± 0.015	93.50 ± 4.93	5.28
0.478	0.441 ± 0.013	92.29 ± 2.64	2.86
0.747	0.675 ± 0.020	90.31 ± 2.66	2.94
Caffeic acid			
3.07	3.06 ± 0.42	99.90 ± 13.81	13.82
3.83	3.67 ± 0.16	95.89 ± 4.06	4.23
11.69	11.23 ± 0.86	96.08 ± 7.36	7.66
18.27	17.39 ± 1.30	95.19 ± 7.11	7.46
28.54	24.66 ± 4.35	93.19 ± 4.11	4.41

Table S8: Robustness of the developed method at two concentrations (n= 3) with human pooled synovial fluid which was intentionally contaminated with 1% human whole blood.

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Catechin			
8.18	8.68 ± 0.55	106.18	6.34
19.96	20.99 ± 1.98	105.16	9.42
M1			
0.445	0.478 ± 0.02	107.37	4.55
1.086	1.033 ± 0.08	95.12	8.17
Caffeic acid			
11.69	11.30 ± 1.40	96.65	12.37
28.54	24.69 ± 0.82	86.50	3.33
Taxifolin			
0.306	0.314 ± 0.04	102.77	13.50
0.747	0.776 ± 0.11	103.88	14.06
Ferulic acid			
5.83	6.50 ± 0.12	111.40	1.91
12.24	15.70 ± 0.49	110.21	3.11

Table S9: Post-preparative stability: autosampler stability of the analytes after 6 h and 12 h at room temperature (RT) after previous LC/MS/MS analysis (n= 3).

Analytes and spiked concentration [ng/mL]	Autosampler stability: 6 h - RT - in darkness			Autosampler stability: 12 h - RT - in darkness		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
(+)-Catechin						
2.68	2.72 ± 0.34	12.54	1.35	2.78 ± 0.34	12.32	3.71
8.18	7.84 ± 1.20	15.28	-4.16	7.21 ± 0.72	9.96	-11.87
12.77	10.72 ± 1.51	14.04	-16.04	10.92 ± 1.50	13.72	-14.50
19.96	17.71 ± 1.11	6.27	-11.25	16.10 ± 0.83	5.17	-19.36
M1						
0.146	0.151 ± 0.02	14.28	3.09	0.157 ± 0.01	7.21	7.24
0.445	0.457 ± 0.04	9.07	2.66	0.437 ± 0.01	3.06	-1.90
0.695	0.700 ± 0.08	11.06	0.73	0.749 ± 0.07	9.70	7.72
1.086	1.172 ± 0.06	4.70	7.96	1.097 ± 0.10	8.71	1.04
Caffeic acid						
3.83	3.73 ± 0.02	0.47	-2.76	3.71 ± 0.25	6.77	-3.14
11.69	11.26 ± 1.71	15.18	-3.66	10.15 ± 0.84	8.25	-13.17
18.27	15.86 ± 0.08	5.07	-13.16	15.71 ± 0.59	3.74	-13.99
28.54	26.80 ± 2.60	9.69	-6.10	25.24 ± 2.30	9.12	-11.58
Taxifolin						
0.100	0.107 ± 0.01	13.99	6.75	0.104 ± 0.01	12.53	4.06
0.306	0.268 ± 0.03	11.48	-12.57	0.277 ± 0.03	9.41	-9.46
0.478	0.398 ± 0.08	2.10	-16.70	0.430 ± 0.06	15.09	-10.06
0.747	0.744 ± 0.04	5.01	-0.46	0.666 ± 0.04	6.59	-10.80
Ferulic acid						
1.91	1.87 ± 0.29	15.63	-2.19	1.71 ± 0.19	10.86	-10.61
5.83	5.57 ± 0.79	14.20	-4.49	4.95 ± 0.25	5.02	-15.15
9.11	9.01 ± 0.51	5.68	-1.15	8.49 ± 0.83	9.80	-6.82
14.24	13.50 ± 0.83	6.12	-5.18	12.91 ± 0.26	2.00	-9.37

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Table S10: Post-preparative stability: stability of the analytes after one freeze-thaw cycle (n= 3).

Analytes and spiked concentration [ng/mL]	**Freeze-thaw stability: 1 cycle -20 °C h/at least 12 h - RT/1h**		
	Calculated concentration Mean ± SD [ng/mL]	**RSD [%]**	**Δ [%][1] Mean ± SD**
(+)-Catechin			
2.68	2.09 ± 0.16	7.81	-21.82
8.18	7.42 ± 0.59	7.52	-9.29
12.77	10.63 ± 0.34	3.21	-16.76
19.96	17.78 ± 2.66	14.96	-10.93
M1			
0.146	0.209 ± 0.00	0.99	42.87
0.445	0.564 ± 0.05	8.22	26.63
0.695	0.737 ± 0.06	8.64	6.02
1.086	1.180 ± 0.176	14.95	8.66
Caffeic acid			
3.83	4.04 ± 0.37	9.18	5.58
11.69	9.92 ± 0.43	4.38	-15.13
18.27	15.50 ± 0.39	2.51	-15.14
28.54	23.55 ± 1.85	7.87	-17.50
Taxifolin			
0.100	0.107 ± 0.01	5.56	7.45
0.306	0.352 ± 0.02	6.12	14.98
0.478	0.466 ± 0.07	14.48	-2.48
0.747	0.742 ± 0.12	15.95	-0.70
Ferulic acid			
1.91	2.24 ± 0.12	5.51	16.91
5.83	6.11 ± 0.59	9.64	4.80
9.11	9.94 ± 0.95	9.55	9.04
14.24	13.29 ± 1.89	14.20	-6.72

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Table S11: Lower limit of quantification (LLOQ) and related accuracy of the five analytes extracted from human pooled synovial fluid (n= 3).

Analytes	**LLOQ [ng/mL]**	**Accuracy $_{LLOQ}$ [%] Mean ± SD**
(+)-Catechin	2.14	101.41 ± 17.00
Ferulic acid	1.53	93.39 ± 14.38
M1	0.117	97.24 ± 17.38
Taxifolin	0.080	103.15 ± 12.49
Caffeic acid	3.07	106.85 ± 8.31

Table S12: Recovery, matrix effects and process efficiency of the five analytes extracted from human pooled synovial fluid at three concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	**Recovery [%]**			**Process efficiency [%]**			**Matrix effects [%]**		
	Mean	**± SD**	**RSD [%]**	**Mean**	**± SD**	**RSD [%]**	**Mean**	**± SD**	**RSD [%]**
Catechin									
8.18	39.24	3.04	7.75	29.03	2.09	7.21	-26.00	0.71	2.72
12.77	45.16	8.01	17.74	33.91	4.75	14.02	-24.91	2.91	11.70
19.96	40.81	2.41	5.90	32.37	2.62	8.10	-20.68	3.11	15.05
Taxifolin									
0.306	63.24	6.12	9.67	95.25	7.56	7.94	50.62	1.40	2.76
0.478	87.63	7.10	8.12	122.00	11.85	9.72	39.23	1.78	4.53
0.747	71.76	7.24	10.08	109.86	10.86	9.88	53.10	3.21	6.05
M1									
0.445	45.76	4.16	9.10	165.99	11.92	7.18	262.77	15.11	5.75
0.695	49.17	9.72	19.78	172.14	14.08	8.18	250.13	27.75	11.09
1.086	55.43	3.23	5.82	192.04	11.09	5.78	246.45	5.67	2.30
Ferulic acid									
5.83	52.47	7.32	13.94	149.25	19.75	13.23	184.42	2.45	1.33
9.11	58.14	8.00	13.75	155.37	17.05	10.97	167.22	5.40	3.23
14.24	57.54	1.35	2.34	148.15	3.44	2.32	157.48	0.42	0.27
Caffeic acid									
11.69	59.92	1.59	2.66	76.51	4.20	5.50	27.69	3.90	14.07
18.27	68.30	11.71	17.15	88.63	13.96	15.75	29.77	1.94	6.50
28.54	67.48	7.65	11.33	84.63	9.67	11.43	25.41	2.84	11.16

Table S13: Recovery, matrix effects and process efficiency of the five analytes extracted from three lots of synovial fluid at two concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]			Matrix effect [%]		
	Mean	**± SD**	**RSD [%]**	**Mean**	**± SD**	**RSD [%]**	**Mean**	**± SD**	**RSD [%]**
Catechin									
8.18	43.46	6.41	14.74	31.23	3.49	11.19	-27.79	4.97	17.87
19.96	48.17	3.24	6.72	36.92	1.97	5.34	-23.30	2.21	9.47
Taxifolin									
0.306	58.16	13.15	22.61	87.37	17.02	19.48	51.13	8.12	15.87
0.747	69.25	2.62	3.78	110.24	7.98	7.24	59.05	5.97	10.11
M1									
0.445	47.45	11.15	23.51	164.55	47.69	28.98	243.83	21.48	8.81
1.086	59.38	0.68	1.14	200.23	17.52	8.75	237.04	26.50	11.18
Ferulic acid									
5.83	46.82	7.58	16.19	130.57	17.40	13.33	180.61	27.99	15.49
14.24	48.47	3.45	7.13	128.48	9.16	7.13	165.07	3.01	1.82
Caffeic acid									
11.69	63.83	8.23	12.89	80.69	11.38	14.10	26.30	1.49	5.65
28.54	62.98	6.85	10.88	81.68	11.75	14.38	29.36	5.28	18.00

Table S14: Internal standard (IS; hydrocaffeic acid) normalised matrix factor (MF) at human pooled synovial fluid in three concentrations (n= 3) and in three lots of synovial fluid at two concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	IS-normalised MF in human pooled synovial fluid			IS-normalised MF in three lots of human synovial fluid		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin						
8.18	-0.236	0.01	3.08	-0.252	0.05	21.05
12.77	-0.229	0.04	18.11			
19.96	-0.195	0.03	16.68	-0.215	0.03	14.46
Taxifolin						
0.306	0.459	0.02	3.48	0.459	0.05	10.92
0.478	0.360	0.04	11.09			
0.747	0.501	0.05	9.66	0.540	0.03	6.18
M1						
0.45	2.380	0.21	8.61	2.207	0.28	12.74
0.70	2.297	0.28	12.29			
1.09	2.326	0.14	6.17	2.174	0.26	11.84
Ferulic acid						
5.830	1.671	0.08	4.97	1.638	0.32	19.45
9.110	1.536	0.09	5.82			
14.240	1.486	0.06	3.95	1.514	0.05	3.56
Caffeic acid						
11.69	0.251	0.03	11.11	0.237	0.00	1.41
18.27	0.273	0.01	2.60			
28.54	0.240	0.03	13.93	0.269	0.05	17.20

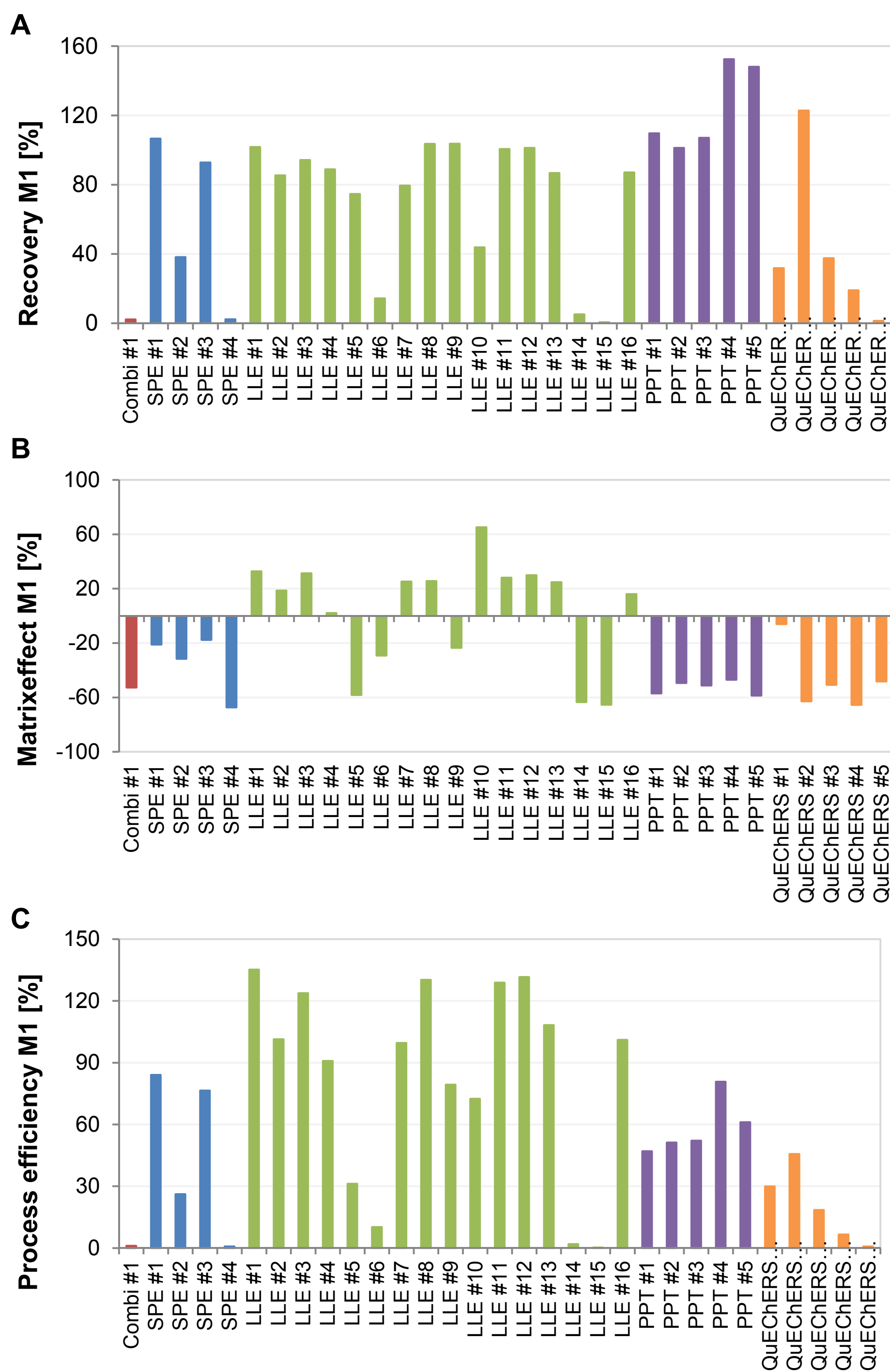


Figure S1: Comparison of different sample preparation techniques (Tables S3 and S4) using 0.25 mL human pooled synovial fluid exemplified for the metabolite M1 spiked at a concentration of 100 ng/mL (n= 1).

C FINAL DISCUSSION

C Final Discussion

The polyphenol-rich food supplement Pycnogenol® (see *Introduction*, Chapter 3.1) has already shown beneficial effects in human intervention studies investigating knee osteoarthritis [243, 250, 256] (see *Introduction,* Chapter 3.3.2).

Previously, very low concentrations in the nanomolar range of constituents and of the *in vivo* gut microbiota-generated metabolite M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) have been found in human plasma after intake of the standardized maritime pine bark [207]. These concentrations were too low to induce any effects *in vitro* [216], while simultaneously exerting diverse pharmacodynamics effects *ex vivo* [215]. It is not clear yet which compound(s) of the complex extract mixture is (are) the main contributor(s) to explain those effects. However, it appears to be possible that compounds present at low concentrations in plasma are distributed into other body compartments and accumulate therein (see *Introduction,* Chapter 3.2).

Metabolomic aspects

By performing initial uptake and inhibition experiments with glucose a significant decrease in the uptake of the pine bark extract metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1) into human erythrocytes was observed at lower concentrations (0.3–1 µM). Whereas there was no further decrease at the highest tested concentration of 10 µM M1, a facilitated cellular uptake of M1 was suggested, possibly via GLUT-1 transporter. Further details of the transport mechanism are not clear so far. Sugano et al. described a coexistence of passive and carrier mediated processes [262]. However, a contribution of the GLUT-1 transporter is highly probable, because of the high expression of this transporter in red blood cells [263, 264] and the structural similarity of M1 with its natural substrate glucose (***Publication 1***).

A distribution of M1 into the blood cellular fraction was supported by the evidence of an intracellular conjugation of M1 with glutathione (GSH) [225]. Further experiments by UPLC-ESI-qTOF-MS^E analysis showed an extensive and rapid intracellular metabolism of M1 in human blood cells, confirming the uptake of M1. The main metabolites were two forms of conjugation products with glutathione in which glutathione either bound via the α-amino group of the glutamic acid of glutathione (M1-N-GSH) or via the thiol group of the cysteine (M1-S-GSH). The binding position of glutathione at M1 (position C3 or C4) is not clarified yet and therefore two possible structures were proposed for each isomer (***Publication 3***). A more sensitive ^{13}C-NMR could bring here more enlightenment, but this was not successful yet due to the low synthesis yield of the reference material. Besides these compounds a variety of biotransformation products of M1 was formed *in vitro* within 4 hours, e.g. conjugates with oxidized glutathione (M1-GSSG) and cysteine (M1-CYS), the sulfated derivative of M1

(M1-sulfated), the open-chained ester form of M1 (M1-COOH), hydroxybenzoic acid and methylated (M1-methylated), acetylated (M1-acetylated), hydroxylated (M1-hydroxylated) and ethylated (M1-ethylated) derivatives of M1. After an oral intake of Pycnogenol®, the gut microbial metabolite M1 was previously detectable in plasma after 6 hours. A maximal plasma concentration (t_{max}) appeared after 10 hours [207] (see *Introduction*, Chapter 3.2). The present metabolomic experiments revealed that the uptake and intracellular metabolism of M1 in human blood cells occurred rapidly. Main metabolites (e.g. M1-COOH, M1-GSH and M1-CYS conjugates) were generated very fast (t= 0 hours), most of them increased within 4 hours. Few metabolites, as an example the acetylated derivative of M1, which was also found *in vivo*, could only be detected after incubations longer than 4 hours. The impact of a prolonged incubation time on the extent of metabolism products should be further studied considering the still low concentrations of M1 in human serum after multiple dosing of Pycnogenol® (see *distribution aspects*). Additionally, the *in vivo* significance of the intracellular M1 metabolism was confirmed with six of the *in vitro* metabolites, respectively M1-COOH, M1-sulfated, hydroxybenzoic acid, M1-S-GSH, M1-methylated and M1-acetylated, being identified in blood cells after ingestion of Pycnogenol® by volunteers (***Publication 3***).

Due to the unexpectedly high metabolic activity of human blood cells and based on their contribution as a significant compartment for distribution [218, 219] other constituents of the extract, e.g. ferulic acid or taxifolin, are probably also subjected to a metabolism in blood cells. This should be further investigated by using a similar metabolomic approach. In that course a more adequate sample preparation (e.g. protein precipitation with acetonitrile instead of methanol for avoiding methylated artefacts or QuEChERS approach; ***Publication 2***) with optional incubation times should be considered. Additionally, similar metabolic processes could also be investigated in other compartments of compound distribution such as the synovial fluid.

In summary, the intracellular metabolism of M1 in human blood cells yielded a high variety of possibly bioactive metabolites which might contribute to explain the beneficial effects of Pycnogenol®.

Distribution aspects

In the present randomized controlled clinical trial Pycnogenol® was administered to patients (in total 30) suffering from severe osteoarthritis. Doses of 200 mg/day were ingested over the course of three weeks before the planned knee replacement surgery. The adherence to the study medication, based on the pill count-back, was 99.4 ± 1.2% in the Pycnogenol® (P+)-group and thus excellent. By analysing the serum, blood cells and synovial fluid samples of the study participants with the highly sensitive developed analytical methods (see *analytical aspects*) novel pharmacokinetic insights regarding the distribution and bioactivity of the extract were sought to be gained.

The study participants were requested to avoid polyphenol-rich food and beverages within the last two days before the blood samplings. Due to the ubiquitious presence of polyphenols in food and beverages (see *Introduction*, Chapter 1.2) adhering to the recommended polyphenol-free diet was apparently very difficult for the participants. Thus, 38 of the 90 blood samples of the 30 osteoarthritis patients (V1, V2 and V3) also contained dietary-derived polyphenols which complicated the differentiation between intake of Pycnogenol® and common nutrition sources. The quantification results in the different biological specimen of the study participants uncovered the distribution of constituents and metabolites of Pycnogenol® into other sites than serum, respectively blood cells and synovial fluid. The measurable concentrations were all in the lower ng/mL range. After multiple doses of 200 mg/day Pycnogenol® over the course of three weeks the highest polyphenol concentrations were not detected in serum samples. However, in serum samples, catechin (48.41 ± 18.61 ng/mL; n= 11) and M1 (0.70 ± 1.02 ng/mL; n= 6) showed higher total concentrations in the intervention group (P+) than in the control (CO)-group. Measurable concentrations of caffeic acid (9.78 ng/mL; n= 1), ferulic acid (3.09 ± 0.46 ng/mL; n= 5) and taxifolin (0.20 ± 0.12 ng/m, n= 5) were also quantified in the P+-group. Free M1 (0.05 ng/mL; n= 1) and taxifolin (0.04 ng/mL; n= 1) were only determined in the intervention group. Moreover, useful data of the conjugation degree of the analytes in human serum were obtained. The mean conjugation degree of the analytes in all investigated serum samples of the osteoarthritis patients was 54.29 ± 26.77% for catechin (n= 51) and 80.95 ± 17.95% for caffeic acid (n= 10) and was hence in full agreement with a former investigation of Grimm et al. [207]. Due to a higher amount of the clinical samples (in total 90, including V1, V2 and V3 blood samplings) and the more sensitive analytical methods using LC-MS/MS compared to Grimm et al. [207], additional mean conjugation degrees were calculated, specifically 90.32 ± 16.58% for ferulic acid (n= 24), 96.75 ± 7.23% for taxifolin (n= 11) and 98.34 ± 4.40% for M1 (n= 30).

Regarding the synovial fluid samples from the P+-group, a higher mean concentration of M1 (0.92 ± 0.93 ng/mL; n= 3) was found compared to the CO-group, whereas the detected concentrations of caffeic acid (10.63 ± 3.86 ng/mL; n= 4), ferulic acid (4.31 ± 2.10 ng/mL; n= 6) and catechin (3.00 ± 0.58 ng/mL; n= 2) were similar in both groups. Taxifolin was only found in the P+-group (0.21 ± 0.03 ng/mL; n= 2). Based on the newly developed analytical method for the quantification of polyphenolic substances in human synovial fluid it was possible to determine constituents and the metabolite M1 of Pycnogenol® therein (***Publication 5***). Drug concentrations are rarely reported for human synovial fluid because it is not a commonly used matrix for clinical and diagnostic purposes. To the best of my knowledge this is the first study investigating polyphenols in human synovial fluid of osteoarthritis patients. Previous human intervention studies have already shown that an oral administration of 100-150 mg/Pycnogenol® over a course of three months resulted in a significant improvement of knee osteoarthritis symptoms, such as reduced pain and joint(s) stiffness [243, 250, 256].

Current results indicate that components and M1 of the pine bark extract are actually distributed into the knee synovial fluid, where they possibly contribute to clinical effects.

In the blood cell samples of the study participants the concentrations of catechin (73.75 ± 29.25 ng/mL; n= 11), ferulic acid (1.85 ± 0.38 ng/mL; n= 9), taxifolin (0.56 ± 0.19 ng/mL; n= 11) and M1 (0.20 ± 0.07 ng/mL; n= 9) were slightly higher in the P+-group than in the CO-group. Notably, the concentration of M1 in the blood cells was low which can be explained by the extensive and rapid intracellular metabolism (***Publication 3***). Indeed, the open-chained ester form of M1 (M1-COOH) and the glutathione conjugate of M1 (M1-GSH) were identified in the clinical samples of patients who received Pycnogenol®, suggesting that M1 does not accumulate in its unconjugated form *in vivo (see metabolomic aspects)*.

There were certain trends regarding the presence and distribution of some constituents and the metabolite M1 after oral intake of Pycnogenol®. Taxifolin and catechin resided preferentially in blood cells whereas the metabolite M1, ferulic and caffeic acid mainly occured in synovial fluid. Taxifolin showed a slight preference, whereas catechin was found to have a strong preference for the presence and distribution in blood cells compared to synovial fluid.

No statistically significant difference was observed between the intervention and the CO-group regarding the mean polyphenol concentrations in serum, blood cells and synovial fluid. However, it was possible to differentiate between Pycnogenol® intake under real life conditions with occasional or regular consumption of polyphenol-rich foods or beverages by checking the nutrition protocols of each study participant. Ferulic acid was found in serum samples exclusively after oral intake of Pycnogenol®, confirming that ferulic acid is a suitable marker of consumption of French maritime pine bark extract [213]. Similarly, taxifolin was only detected in serum and synovial fluid of the intervention group indicating a role as further marker of Pycnogenol® intake. Taxifolin, ferulic acid and caffeic acid were detected in both serum and synovial fluid only after multiple ingestion of Pycnogenol® over the course of three weeks. The metabolite M1, taxifolin and ferulic acid were only detected simultaneously in all matrices (serum, blood cells and synovial fluid) in the intervention group (***Publication 5***).

To summarize, the newly developed and highly sensitive analytical liquid chromatography tandem mass spectrometry (LC-MS/MS) methods (see *analytical aspects*) allowed further insights into the pharmacokinetics of Pycnogenol® suggesting that this process might be very complex *in vivo*. Further pharmacodynamic investigations, e.g. regarding osteoarthritis biomarkers such as cytokines [265] or mediators of cartilage degradation (matrix metalloproteinasen, see *Introduction*, Chapter 3.3.2) have to be carried out to elucidate which constituents and/or metabolites (primary and secondary as well) of the extract are mainly responsible for the described beneficial effects and how Pycnogenol® displays its effects in patients suffering from severe gonarthrose.

Analytical aspects

As the range of the analytes in the clinical samples was expected to be very low (ng/mL in plasma) or unknown (blood cells and synovial fluid), it was necessary to develop highly sensitive analytical methods for the quantification of selected constituents and metabolites of Pycnogenol® using liquid chromatography tandem mass spectrometry (LC-MS/MS) (see *Introduction*, Chapter 2).

For achieving maximum analytical sensitivity and reliability of the method different sample preparation techniques such as protein precipitation (PPT), liquid-liquid extraction (LLE), solid phase extraction (SPE) and useful combinations thereof were initially compared for each matrix. The post-extraction spike method proposed by Matuszewski et al. [196] (see *Introduction*, Chapter 2.2.6) was used which includes preparing a set of three samples ("spiked", "post-spiked" and "standard solution"). This method allows the quantitative assessment of the matrix effect of one analyte besides the determination of its recovery and process efficiency. When coupling LC with MS-detection via the electrospray (ESI)-ionization as interface, matrix effects can occur [138] and affect MS-response due to coelution of similarly charged components of the matrix e.g. residual salts, at ESI-ionization [139, 140] (see *Introduction,* Chapter 2.1.2). For all investigated matrices (serum, blood cells and synovial fluid) it was shown that the calculated recovery, matrix effects and overall process efficiency for the individual analytes differed strongly between the various sample clean-ups. Particularly, the majority of the performed PPT techniques were associated with strong ion suppression in all matrices which resulted in unsatisfying overall process efficiencies despite of the high recovery rates. Thus, sample preparation techniques yielding high recovery rates are not necessarily the best approach when using LC-MS/MS analysis due to matrix effects, especially when ion suppression is observed. For LC-MS/MS method development the post-extraction spike method has proved of value for simultaneously taken into consideration the matrix effects in addition to the recovery and process efficiency of an analyte. Thus, the selection of an appropriate sample clean-up is important for compensating matrix effects. SPE, LLE or combined techniques are frequently used for sensitive quantification using LC-MS/MS [141, 157, 166-169] (see *Introduction*, Chapter 2.1.1).

Preparation of the serum and synovial fluid samples of the osteoarthritis patients were performed using optimized LLE with ethyl acetate and *tert*-butyl methyl ether (***Publication 4 and 5***) whileas for the extraction of the analytes from the blood cell samples a multi-method with several purification steps, respectively QuEChERS (quick, easy, cheap, effective, rugged and safe) was used [266]. Using the QuEChERS approach to human blood cells it became obvious that this method which was originally developed for the food industry can be applied in clinical fields as well for cleaning-up more challenging biological matrices e.g. whole blood or red blood cells. This fast and low-cost method can now be used in pharmacokinetic studies to determine the absolute concentration and thus the distribution of compounds in whole blood, blood cells or erythrocytes (***Publication 2***).

After extensive optimization to achieve highly sensitive methods the obtained lower limits of quantification (LLOQs) varied depending on the respective matrix and were all in the lower ng/mL range (serum: 35 pg/mL for taxifolin to 8 ng/mL for caffeic acid; blood cells: 113 pg/mL for taxifolin to 48 ng/mL for caffeic acid; synovial fluid: 80 pg/mL for taxifolin to 3 ng/mL for caffeic acid). Most sensitive analysis of M1, taxifolin and ferulic acid and least sensitive detection of catechin and caffeic acid was achieved in all specimen although with different sample clean-up techniques. All methods suited the purpose for the quantification of the study samples (***Publication 5***).

Several decisions (e.g. selection of an adequate extraction solvent at LLE techniques) have been made during the method optimization since focussing on the most sensitive detection of the pine bark extract metabolite M1 and the constituents taxifolin and ferulic acid. This led to a negative impact on the sensitivity of detection of the other analytes which cannot be avoided when considering more than one analyte. Regarding blood cells as example, the LLOQs were 0.12 ng/mL for taxifolin, M1 and M2 and 0.97 ng/mL for ferulic acid, whereas the method was less sensitive for catechin (LLOQ 28.90 ng/mL) and caffeic acid (LLOQ 48.40 ng/mL). Undoubtedly, more sensitive LLOQs for the latter analytes can be achieved when they are the main focus during method optimization in blood cells.

Current guidelines recommend the investigation of matrix effects as part of the method validation for the quantification in biological matrices using LC-MS/MS to ensure that they do not affect the precision, selectivity and sensitivity of a bioanalytical method [176, 177] (see *Introduction*, Chapter 2.2.6). Particular attention should be paid to matrix effects when sensitive quantification in biological samples is required since the high interindividual variabiliy of the matrix leads to different matrix effects (relative matrix effects), especially when using ESI-ionization [139, 141] (see *Introduction*, Chapter 2.1.2). The RSD of the IS-normalised matrix factor at six lots of blank matrix from individual donors should not exceed 15% [177, 178] (see *Introduction*, Chapter 2.2.6). For avoiding incorrect quantification results in the current study a special focus was given to the assessment of matrix effects during all quantification approaches.

The evaluation of absolute and relative matrix effects revealed a correlation with the analyte concentration for both matrices plasma and serum. Thereby, higher (total) analyte concentrations were correlated with lower matrix effects. This agrees with the investigations of Wang et al. [267] and van Hout et al. [268]. Furthermore, by the comparison of the absolute and relative matrix effects between human plasma and serum with the same method, it became obvious that matrix effects were more pronounced in human plasma compared to serum. Most likely this is due to a higher concentration of residual proteins, respectively coagulation factors, of plasma samples. Plasma has higher fibrinogen and lower phosphate and sodium ion concentrations compared to serum [269] which may affect ESI-ionization

(***Publication 4***). To the best of my knowledge no comprehensive investigation comparing absolute and relative matrix effects in human plasma and serum existed so far. The results clearly emphasize that calibration curves should always be prepared in the same matrix as the clinical samples [176, 177] (see *Introduction*, Chapter 2.2) because even slight modifications of the matrix (human plasma instead of serum) can have a decisive impact on the accuracy of the quanfitication results due to the related matrix effect.

Further investigations revealed that a prior enzymatic hydrolysis of polyphenol conjugates with ß-glucuronidase/sulfatase before sample extraction caused a strong ion enhancement in human plasma, particularly for the internal standard (IS). Another bioanalytical pretreatment procedure is the incubation with esterase inhibitors for drug stabilization which has been already identified as potential source of matrix effects [270]. To the best of my knowledge, such effects of ß-glucuronidase/sulfatase incubation were not described yet. This observation was subsequently considered when preparing the calibration curves by spiking the calibration standards after pre-incubating the related human pooled matrix under the same conditions as the clinical samples to not comprise the accuracy in the quantification results (***Publication 4***).

In LC-MS/MS analysis one possibility for compensating matrix effects is the use of a stable isotope labeled (SIL-) IS like ^{2}H, ^{13}C, ^{15}N and ^{18}O [157] instead of using a structural or chemical analog of the analyte as IS [158, 165]. For many compounds such as for the pine bark extract metabolite M1 SIL-IS are not commercially available [148]. Some scientists assume, that SIL-IS yield better assay performance and thus more reliable quantification results [148, 160] based on the fact that the analyte and the SIL-IS elute simultaneously and thus are subjected to identical matrix effects [158, 165]. However, Heideloff et al. described that both options for IS provided useful performance in LC-MS/MS analysis considering the individual application [162]. The use of SIL-IS, especially of ^{13}C-labeled SIL-IS, can cause high costs (see *Introduction*, Chapter 2.1.3). In the present study two options for IS, the structural analog hydrocaffeic acid and the SIL-IS ferulic acid-1,2,3-$^{13}C3$, were compared regarding the accuracy of the quantification and the variability of the IS-normalized matrix factor in human plasma, exemplified for ferulic acid. The results showed a slight improvement of the variability of matrix effects in six different lots of human plasma using the SIL-IS ferulic acid-1,2,3-$^{13}C3$ (12.99% and 10.48% RSD; low-high concentration) as opposed to the structural IS hydrocaffeic acid (12.44% and 16.89% RSD; low-high concentration). The mean accuracy of the quantification with structural IS (102.28 ± 17.41%) in six different lots of plasma was similar compared when using the SIL-IS ferulic acid-1,2,3-$^{13}C3$ (101.40 ± 26.78%). Thus, an adequate sample clean-up can be an alternative option for dodging matrix effects in LC-MS/MS analysis instead of utilizing a SIL-IS (***Publication 4***). It can not be excluded that other analytes or other matrices yield different results. However, further investigations were beyond the scope of this thesis.

For quantification purposes the use of a calibration curve consisting of matrix-matched calibration standards added to the blank matrix has been suggested [147, 148] (see *Introduction*, Chapter 2.1.3). For the quantification of the clinical samples in the present study the same kind of human pooled matrix for preparing matrix-matched calibration standards was used. Because of the ubiquitary presence of polyphenols in human nutrition [15, 20-23] (see *Introduction*, Chapter 1.2) some analytes showed a basal presence in the related matrix. Some scientists obtained target-free blank matrix for calibration by subjecting their donors to strict fasting conditions [137, 150]. This procedure is more difficult when human basal matrix is required. In such cases, Cavaliere et al. proposed the shift of the calibration curve along the y-axes according to the response of the zero-sample (containing IS; see *Introduction*, Chapter 2.1.3) [151, 152]. Another effective option for analyte quantification while compensating relative matrix effects offers the standard addition calibration (see *Introduction*, Chapter 2.1.3). However, due to the limited sample volume this approach could not be used for the quantification of the clinical samples. Therefore, in the current study accurate and reliable quantification results were obtained using human pooled matrix-matched calibration standards with a structural IS and applying the calibration approach of Cavaliere et al. [151, 152] (***Publication 5***).

All analytical methods (serum, blood cells and synovial fluid with prior enzymatic inbubation and serum without enzymatic inbubation) for quantification of the clinical samples were subjected to a full validation according to current EMA and FDA guidelines and fulfilled those criteria showing excellent performance and reliability. The recovery of some analytes e.g. caffeic acid in blood cells was very low (1.04 ± 0.15%), since the main focus was on other analytes (M1, taxifolin and ferulic acid). Nevertheless, the methods were still sufficiently precise and reproducible and thus fully suitable for analyte quantification in the study samples. Based on the FDA guideline, the recovery of one analyte does not need to be 100% [176].

Because of the different LLOQs for one analyte in the different matrices, e.g. for caffeic acid 8.02 ng/mL in serum vs. 48.40 ng/mL in blood cells, it might be possible that this compound was present below the LLOQ in blood cells of the osteoarthrits patients. The evaluation of the results of the conjugation degree of the analytes in human serum should be considered in a similar way. The LLOQs of the analytes for quantification of the serum samples without prior enzymatic hydrolysis were in every case below the LLOQs with enzymatic incubation. Since no free concentration of the analyte was found it was concluded that these compounds were about 100% conjugated. However, it might be possible that the concentration was below the corresponding LLOQ affecting the results.

Enzymatic hydrolysis of polyphenol conjugates with a mixture of ß-glucuronidase/sulfatase [207] prior to the actual sample preparation of the serum, blood cells and synovial fluid samples of the study participants was optimized under two aspects.

One part was the stability of free (= not conjugated) analytes in the related matrix. The stability experiments were performed with free analytes spiked in related human pooled matrix. Indeed, the conjugated form of the analyte occured *in vivo*. For the evaluation of the stability of the conjugated forms in the related matrices, a variety of reference compounds would have been required and many of those were not commercially available. Hence, the stability of those conjugates has to be further clarified. The other aspect of the method optimization was the choice of the incubation conditions for maximum hydrolysis of conjugated analytes. Examined variations included incubation time, temperature, pH value and amount of enzyme mixture from *Helix pomatia* with ß-glucuronidase/sulfatase activity using samples from volunteer donors after oral ingestion of Pycnogenol®. An incubation time of 45 min at 37 °C and pH 5.0 was optimal for all matrices. The added enzyme mixture differed depending on the extent of conjugated analytes to be hydrolyzed (synovial fluid: 1500 U per mL; serum: 1000 U per mL; blood cells: 625 U per mL).

The individual stability of polyphenols can influence their bioavailability (see *Introduction*, Chapter 1.4). They can be subjected to diverse stability losses under experimental, e.g. cell culture conditions [82, 83]. Short term stability tests of the analytes in human pooled serum resulted in a significant decrease (-78.23%) of M1 stored for one hour at room temperature, which was consistent for two and four hours (-79.36% and -82.55%). Stability losses were slightly higher when storing at 37 °C (1 h: -78.13%; 2 h: -85.29%; 4 h: -89.65%). After one year at -80 °C (long-term stability) a significant decrease in compound stability was observed for human pooled serum which ranged between -16.00% for ferulic acid and -82.36% for taxifolin. The two investigated hydroxybenzoic acids ferulic acid and caffeic acid showed slightly lower stability losses compared to the other analytes, especially catechin (see *Appendix,* Chapter 5.3.9). Catechin is known for degrading at basic pH-values [81]. To avoid stability losses in the current clinical trial the transportation times of the samples were kept short, temperatures higher than room temperature were avoided, samples were rapidly cleaned-up and analysis was performed after freezing (-80 °C) and thawing once. Another option to prevent stability losses in human biological matrices like plasma and urine is the addition of stabilizers such as antioxidants, e.g. ascorbic acid, immediately after sample entry [271]. This was not done in the current clinical study to avoid an alteration of the matrix and thus an associated unpredictable impact on the results of all further investigations.

The concentration of M1 in the serum samples of the patients after previous intake of multiple oral doses of Pycnogenol® fluctuate considerably (0.16 – 2.77 ng/mL; see *Appendix,* Chapter 5.4.1.1) depending on the individual human gut microbiota (see *Introduction*, Chapter 3.1). However, due to stability losses it can be expected that the “real” *in vivo* concentrations of the analytes, especially the metabolite M1, were significantly higher in the serum samples than reported here. Especially the free serum concentration of the analytes (without prior enzymatic incubation) might be higher. A possible instability of conjugates may affect the total serum

concentration (free and conjugated) and should be investigated in further studies. It can be expected that conjugates are subjected to stability losses as well [82, 272, 273].

To conclude, the decrease in stability of M1 in human serum was high (-78.23% at one hour at room temperature) and probably affected the accuracy of the quantification results more than any analytical error. It can be assumed that the analytes were subjected to stability losses in the other matrices (blood cells and synovial fluid) as well. Hence, possibly higher *in vivo* concentrations occurred in the samples of the osteoarthritis patients.

D APPENDIX

D Appendix

1 Supporting Information for Publication 1

1.1 Initial experiments

Influence of the hematocrit (0.43, 4.3 and 43%), 0.6 µM M1, human erythrocytes (blood group AB; n= 3)

Ratios peak area M1 sample/control

Time [min]	***Hematocrit 0.43%***				
	I	II	III	Mean	MDM
0	1.02	1.00	1.01	1.01	0.01
15	1.02	0.99	1.01	1.01	0.01
30	0.99	0.99	0.96	0.98	0.01
60	0.91	0.95	0.92	0.93	0.02
Time [min]	***Hematocrit 4.3%***				
	I	II	III	Mean	MDM
0	0.51	0.53	0.50	0.51	0.01
15	0.47	0.45	0.47	0.46	0.01
30	0.44	0.44	0.42	0.43	0.01
60	0.43	0.45	0.47	0.45	0.01
Time [min]	***Hematocrit 43%***				
	I	II	III	Mean	MDM
0	0.26	0.30	0.27	0.28	0.02
15	0.25	0.28	0.25	0.26	0.01
30	0.24	0.28	0.28	0.27	0.02
60	0.27	0.29	0.26	0.27	0.01

Inhibition with glucose (+100 mg/dL) and phloretin (200 µM), 0.3 µM M1, 15 min incubation, human erythrocytes (hematocrit 0.43%, blood group AB; n= 3)

Ratios peak area M1 sample/control

	I	II	III	Mean	MDM
M1	0.97	0.99	0.93	0.96	0.02
M1+Glucose	1.02	1.02	0.96	1.00	0.03
M1+Phloretin	0.97	1.03	0.96	0.98	0.03

Influence of the concentration of M1 (0.15 – 0.6 µM) in pre- and absence of glucose (+100 mg/dL), 15 min incubation, human erythrocytes (hematocrit 0.43%, blood group AB; n= 3)

Ratios peak area M1 sample/control

Concentration M1		*M1*					*M1 + Glucose*				
[ng/mL]	µM	I	II	III	Mean	MDM	I	II	III	Mean	MDM
32	0.15	0.91	0.96	0.95	0.94	0.02	1.06	1.09	1.11	1.09	0.02
63	0.3	0.97	0.97	0.98	0.98	0.01	1.00	1.07	1.03	1.03	0.02
125	0.6	1.01	0.97	0.97	0.98	0.02	1.03	1.03	1.06	1.04	0.02

Influence of the concentration of M1 (0.15 – 0.6 µM) in pre- and absence of glucose (+100 mg/dL), 15 min incubation, human erythrocytes (hematocrit 4.3%, blood group AB; n= 3)

Ratios peak area M1 sample/control

Concentration M1		*M1*					*M1 + Glucose*				
[ng/mL]	µM	I	II	III	Mean	MDM	I	II	III	Mean	MDM
32	0.15	0.22	0.28	0.24	0.24	0.02	0.22	0.27	0.25	0.25	0.02
63	0.3	0.29	0.29	0.29	0.29	0.00	0.31	0.30	0.29	0.30	0.01
125	0.6	0.33	0.34	0.32	0.33	0.01	0.36	0.38	0.33	0.36	0.02

1.2 Influence of the stop solution

With stop solution: distribution coefficient K in human erythrocytes, blood group AB (n= 3);
without stop solution: distribution coefficient K in human erythrocytes, blood group AB (single experiment);
Hematocrit: 4.3%

Concentration M1		*with stop solution*										*without stop solution*	
		M1					***M1 + Glucose***					***M1***	***M1 + Glucose***
[ng/mL]	µM	I	II	III	Mean	MDM	I	II	III	Mean	MDM	I	I
63	0.3	27.56	20.92	19.03	22.51	3.37	16.65	13.49	14.24	14.79	1.24	33.78	19.50
125	0.6	22.20	18.93	16.51	19.21	1.99	11.76	13.30	14.84	13.30	1.03	33.79	16.51
208	1	14.68	17.60	18.14	16.81	1.42	11.35	15.43	9.79	12.19	2.16	23.14	12.96

1.3 Influence of D(+)-Glucose

Distribution coefficient K in human erythrocytes, blood group AB+B (n= 6), Hematocrit: 4.3%

Concentration M1		***M1***								***M1 + Glucose***							
[ng/mL]	µM	I (AB)	II (AB)	III (AB)	IV (B)	V (B)	VI (B)	Mean	MDM	I (AB)	II (AB)	III (AB)	IV (B)	V (B)	VI (B)	Mean	MDM
63	0.3	27.56	20.92	19.03	32.41	25.11	23.03	24.68	3.68	16.65	13.49	14.24	16.07	19.59	12.85	15.48	1.96
125	0.6	22.20	18.93	16.51	21.75	18.99	28.36	21.13	2.98	11.76	13.30	14.84	14.38	14.11	19.93	14.72	1.78
208	1	14.68	17.60	18.14	19.30	19.11	27.87	19.45	2.81	11.35	15.43	9.79	10.42	10.38	11.65	11.50	1.36
2080	10	6.18	4.88	5.04	3.13	9.01	0.99	4.87	1.87	5.42	5.46	4.20	4.72	3.42	4.76	4.66	0.57

Distribution coefficient K in human erythrocytes, blood group AB (n= 3), Hematocrit: 4.3%

Concentration M1		*M1*					*M1 + Glucose*				
[ng/mL]	µM	I	II	III	Mean	MDM	I	II	III	Mean	MDM
63	0.3	27.56	20.92	19.03	22.51	3.37	16.65	13.49	14.24	14.79	14.52
125	0.6	22.20	18.93	16.51	19.21	1.99	11.76	13.30	14.84	13.30	14.07
208	1	14.68	17.60	18.14	16.81	1.42	11.35	15.43	9.79	12.19	10.99
2080	10	6.18	4.88	5.04	5.37	0.54	5.42	5.46	4.20	5.03	4.61

Distribution coefficient K in human erythrocytes, blood group B (n= 3), Hematocrit: 4.3%

Concentration M1		*M1*					*M1 + Glucose*				
[ng/mL]	µM	I	II	III	Mean	MDM	I	II	III	Mean	MDM
3	0.3	32.41	25.11	23.03	26.85	3.71	16.07	19.59	12.85	16.17	2.28
125	0.6	21.75	18.99	28.36	23.04	3.55	14.38	14.11	19.93	16.14	2.52
208	1	19.30	19.11	27.87	22.09	3.85	10.42	10.38	11.65	10.82	0.56
2080	10	3.13	9.01	0.99	4.38	3.09	4.72	3.42	4.76	4.30	0.59

2 Supporting Information for Publication 2

2.1 Method development

Comparison of different sample preparation techniques: recovery, matrix effects and process efficiency using 0.5 mL of human erythrocytes exemplified for the metabolite M1 spiked at a concentration of 100 ng/mL (n=1).

Sample preparation	Process	Recovery [%]	Matrix effects [%]	Process efficiency [%]
Combi 1	LLE #1a / PPT #1a	59.38	-99.97	0.02
Combi 2	LLE #1a / PPT #4a	3.67	-20.31	2.92
Combi 3	PPT #1b / LLE #1a	58.97	-99.86	0.08
Combi 4	PPT#1a / LLE #1a	105.30	-98.93	1.21
Combi 5	PPT #1a / LLE #1a	98.34	-98.70	1.30
Combi 6	PPT #4a / LLE #1a	0.00	0.00	0.47
SPE 1	Strata XA-L	0.54	87.46	1.02
SPE 2	Strata X-L	3.87	50.55	5.55
LLE 1	LLE #1a	36.80	-73.18	9.86
LLE 2	LLE #1b	37.86	-71.31	10.86
PPT 1	PPT #1b	32.62	-94.35	1.84
PPT 2	PPT #1a	90.00	-98.41	1.43
PPT 3	PPT #2	70.31	-92.90	4.99
PPT 4	PPT #3	23.21	-55.25	10.31
PPT 5	PPT #4a	10.00	4.85	10.49
PPT 6	PPT #4c	0.21	4.04	0.21
PPT 7	PPT #4b	13.62	-52.03	6.53
Quechers 1	Quechers (EN)	24.44	-22.32	18.99

Recovery, matrix effects and process efficiency using 0.5 mL of human erythrocytes for the intracellular M1-glutathione adduct spiked at a concentration of 100 ng/mL (n=1).

Sample preparation	Process	Recovery [%]	Matrix effects [%]	Process efficiency [%]
Quechers 1	Quechers (EN)	73.50	-42.43	42.31

2.2 Method optimization

Influence of acidification of the extraction solvent acetonitrile (ACN) with 0.5–2% acetic acid (HAC), 0.1–1% formic acid (FA) and 4% phosphoric acid (H_3PO_4) on the extraction process: Human erythrocytes (1.0 mL) containing 100 ng/mL M1, taxifolin, ferulic acid and M1-glutathione (GSH) adduct were extracted according to the QuEChERS EN 15662 method.

Extraction solvent ACN	Peakheight			
	M1	Taxifolin	Ferulic acid	M1-GSH Adduct
pur	726024	19535	12167	1552
+ 0.5% HAC	813672	220489	40785	10458
+ 0.75% HAC	515710	140601	17376	18829
+ 1% HAC	880739	342661	69999	12990
+ 2% HAC	549558	274887	46356	12589
+ 0.1% FA	174431	635	2075	1629
+ 0.4% FA	444679	2812	3791	3376
+ 1% FA	406339	47765	13061	5510
+ 4% H_3PO_4	118175	172298	15898	2939

Optimization of the QuEChERS approach with 1.0 mL of human erythrocytes containing 100 ng/mL M1 (n=1): It was compared three QuEChERS- (Original, AOAC 2007.01 and EN 15662 method) and SweEt- variations.

Variation	Recovery [%]	Matrix effects [%]	Process efficiency [%]
QuEChERS - EN 1.0 mL	19.44	-40.01	11.45
QuEChERS - Original 1.0 mL	14.87	-31.49	10.19
QuEChERS - AOAC 1.0 mL	19.08	-38.72	11.69
SweEt - EA 100% 1 .0 mL	21.64	-21.46	17.00
SweEt - EA:MTBE 50:50% 1.0 mL	10.15	-21.32	7.99

Optimization of the QuEChERS approach with 1.0 mL of human erythrocytes containing 100 ng/mL M1-glutathione adduct (n=1): It was compared three QuEChERS- (Original, AOAC 2007.01 and EN 15662 method) and SweEt- variations.

Variation	Recovery [%]	Matrix effects [%]	Process efficiency [%]
QuEChERS - EN 1.0 mL	97.8	-81.05	18.54
QuEChERS - Original 1.0 mL	74.65	-77.64	16.69
QuEChERS - AOAC 1.0 mL	114.88	-68.84	35.8
SweEt - EA 100% 1 .0 mL	35.2	-78.31	7.64
SweEt - EA:MTBE 50:50% 1.0 mL	111.2	-85.6	16.03

Influence of different quantities of NaAc with 4 g MgSO4 (constant amount) on the extraction process for phase portioning: Human erythrocytes (1.0 mL) containing 10 ng/mL M1, taxifolin, ferulic acid and M1-glutathione (GSH) adduct were extracted according to the AOAC 2007.01 method.

NaAc [g]	Peakheight			
	M1	Taxifolin	Ferulic acid	M1-GSH Adduct
0	77785	18866	5119	21670
0.25	41438	24477	4046	17938
0.5	41039	5469	2953	18972
1	56931	6159	2284	17521
1.5	57299	12353	1267	17921
2	58840	10644	850	18923

Influence of different quantities of PSA (0–150 mg) and MgSO4 (0–900 mg) on the d-SPE clean-up process: Human erythrocytes (1.0 mL) containing 5 ng/mL M1, taxifolin, ferulic acid and M1-glutathione (GSH) adduct were extracted according to the AOAC 2007.01 method.

PSA [mg]	$MgSO_4$ [mg]	Peakheight			
		M1	Taxifolin	Ferulic acid	M1-GSH Adduct
0	0	6516	4074	577	12598
50	450	103	572	402	27704
75	450	3226	3506	826	24184
100	450	4928	3817	792	27355
0	500	124	322	44	23927
12.5	500	240	386	695	26270
50	500	598	750	754	25071
75	500	4375	3658	826	26656
50	600	2573	3462	448	19396
75	600	6682	4438	671	22495
100	600	10627	6134	679	26656
125	600	9980	5033	354	15511
150	600	10056	4414	399	13402
75	900	7434	4474	315	15823
100	900	9961	3568	448	16262
125	900	8762	3331	250	12409

Efficiency of different sample volumes (0.5, 1.0 and 2.0 mL) of human erythrocytes: M1 (+100 ng/mL) was extracted according to AOAC 2007.01 method (n= 3).

Variation	Recovery [%] Mean ± SD	Matrix effects [%] Mean ± SD	Process efficiency [%] Mean ± SD
QuEChERS - AOAC 1.0 mL	28.10 ± 1.72	1.70 ± 0.25	28.58 ± 1.81
QuEChERS - AOAC 1.5 mL	23.42 ± 0.94	-1.96 ± 1.19	22.96 ± 1.02
QuEChERS - AOAC 2.0 mL	20.89 ± 1.53	-14.01 ± 1.83	17.98 ± 1.69

Overall method improvement of the optimization progress for 100 ng/mL M1 in different sample volumes (0.5, 1.0 and 2.0 mL) of human erythrocytes (n=1).

Variation	Recovery [%]	Matrix effects [%]	Process efficiency [%]
QuEChERS - EN 0.5 mL	24.44	-22.32	18.99
QuEChERS - AOAC 1.0 mL	19.08	-38.72	11.69
QuEChERS - AOAC 2.0 mL opt.	28.21	-16.44	23.57

Overall method improvement of the optimization progress for 100 ng/mL M1-glutathione adduct in different sample volumes (0.5, 1.0 and 2.0 mL) of human erythrocytes (n=1).

Variation	Recovery [%]	Matrix effects [%]	Process efficiency [%]
QuEChERS - EN 0.5 mL	73.50	-42.43	42.31
QuEChERS - AOAC 1.0 mL	114.88	-68.84	35.80
QuEChERS - AOAC 2.0 mL opt.	90.25	-78.84	19.10

2.3 Method validation blood cells with prior enzymatic incubation

2.3.1 Levels of calibration standards

Calibration curve in human blood cells

Analytes	Levels of calibration standards [ng/mL blood cells]*											
	L0	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Catechin	9.63	28.90	34.68	41.61	49.93	59.92	71.90	86.28	103.54	124.25	149.10	298.20
Taxifolin	0.121	0.364	0.436	0.524	0.628	0.754	0.905	1.086	1.303	1.563	1.876	3.752
M1	0.123	0.368	0.442	0.530	0.636	0.764	0.916	1.100	1.319	1.583	1.900	3.800
Ferulic acid	0.97	2.90	3.48	4.17	5.01	6.01	7.21	8.65	10.39	12.46	14.96	29.91
Caffeic acid	16.13	48.40	58.08	69.70	83.64	100.37	120.44	144.53	173.44	208.13	249.75	499.50
M2	0.123	0.368	0.442	0.530	0.636	0.764	0.916	1.100	1.319	1.583	1.900	3.800
Hydrocaffeic acid (IS)	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85

* including the purity of the used standard substances according to the Certificate of Analysis from the manufacturer's batch.
The synthesized compounds M1 and M2 were confirmed by NMR and LC/MS and equated with a purity of 95%.

	Level
LLOQ	L0 or L1
low	L6
mid	L8
high	L10

2.3.2 Recovery, matrix effects and process efficiency

Recovery [%] of the six analytes in three different lots of human blood cells at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Recovery [%]									Mean Recovery [%]		
	Single donor #1			Single donor #2			Single donor #3			#1-3		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
71.90	4.73	0.86	18.22	3.64	0.25	6.90	4.10	0.34	0.86	4.16	0.54	0.86
149.10	6.22	1.10	17.73	5.32	0.44	8.29	5.75	1.20	1.10	5.76	0.45	1.10
Taxifolin												
0.905	17.26	1.26	7.30	18.18	2.91	16.03	21.14	1.90	1.26	18.86	2.03	1.26
1.876	19.10	3.21	16.83	19.19	3.26	16.97	21.16	0.30	3.21	19.81	1.17	3.21
M1												
0.916	29.99	4.20	14.02	26.76	2.82	10.55	28.28	3.52	4.20	28.34	1.61	4.20
1.900	23.10	1.01	4.36	19.79	1.60	8.11	21.65	2.66	1.01	21.51	1.66	1.01
Ferulic acid												
7.21	29.56	3.80	12.84	31.39	5.55	17.67	28.15	3.01	3.80	29.70	1.63	3.80
14.96	30.94	3.33	10.77	34.20	7.92	23.16	27.80	2.19	3.33	30.98	3.20	3.33
Caffeic acid												
120.44	1.22	0.18	14.47	1.14	0.23	20.49	1.16	0.01	0.18	1.17	0.04	0.18
249.75	1.34	0.12	9.26	1.40	0.33	23.92	1.49	0.26	0.12	1.41	0.07	0.12
M2												
0.916	58.35	13.63	23.36	46.48	3.64	7.84	59.30	7.49	13.63	54.71	7.14	13.63
1.900	52.50	3.13	5.96	44.82	4.83	10.78	44.04	5.01	3.13	47.12	4.68	3.13

Matrix effects [%] of the six analytes in three different lots of human blood cells at two concentrations (low and high; n= 3). Listed are mean and SD of the ME of the analytes, prepared from 2 mL of blood cell matrix (n = 3) for each individual donor. The RSD of the mean of the three single donors represents the variability of the ME of an analyte in three different lots of human blood cells.

Analytes [ng/mL]	Matrix effects [%]									Mean Matrix effects [%]		
	Single donor #1			Single donor #2			Single donor #3			#1-3		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
71.90	-73.71	0.5	-0.6	-76.91	0.7	-0.9	-77.39	0.7	-1.0	-76.00	2.00	-2.6
149.10	-73.91	4.8	-6.5	-75.64	7.1	-9.4	-78.39	4.5	-5.7	-75.98	2.26	-3.0
Taxifolin												
0.905	-22.47	4.4	-19.3	-29.08	2.6	-9.0	-27.19	0.3	-1.0	-26.25	3.40	-13.0
1.876	-17.94	0.8	-4.5	-19.23	3.9	-20.3	-19.84	2.0	-10.2	-19.00	0.97	-5.1
M1												
0.916	75.36	6.8	9.0	69.87	14.1	20.2	65.23	7.6	11.7	70.15	5.07	7.2
1.900	41.46	5.6	13.6	37.55	5.4	14.5	40.94	2.5	6.2	39.98	2.13	5.3
Ferulic acid												
7.21	74.31	10.8	14.5	76.62	6.0	7.8	64.22	8.9	13.9	71.72	6.60	9.2
14.96	73.74	13.3	18.0	76.55	2.9	3.7	77.10	8.9	11.5	75.80	1.80	2.4
Caffeic acid												
120.44	1.82	0.4	20.0	1.65	0.3	16.5	1.60	0.1	5.1	1.69	0.12	6.8
249.75	0.49	0.1	18.3	0.45	0.1	15.7	0.48	0.1	16.1	0.48	0.02	4.5
M2												
0.916	-52.70	5.3	-10.0	-56.63	2.7	-4.7	-61.09	6.3	-10.4	-56.81	4.19	-7.4
1.900	-46.72	1.1	-2.3	-48.14	1.5	-3.1	-47.58	2.6	-5.5	-47.48	0.72	-1.5

IS-normalised matrix factor (MF) of the six analytes in three different lots of human blood cells at two concentrations (low and high; n= 3). Listed are mean and SD of the ME of the analytes, prepared from 2 mL of blood cell matrix (n = 3) for each individual donor. The RSD of the mean of the three single donors represents the variability of the IS-normalised MF of an analyte in three different lots of human blood cells.

Analytes [ng/mL]	IS-normalised MF									Mean IS-normalised MF		
	Single donor #1			Single donor #2			Single donor #3			#1-3		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
71.90	-0.67	0.04	5.8	-0.72	0.03	4.8	-0.72	0.05	7.1	-0.71	0.03	4.1
149.10	-0.72	0.05	7.4	-0.72	0.04	5.5	-0.76	0.06	7.2	-0.73	0.03	3.5
Taxifolin												
0.905	-0.20	0.05	25.2	-0.27	0.02	8.1	-0.25	0.02	6.8	-0.24	0.03	14.3
1.876	-0.17	0.01	7.7	-0.18	0.04	20.6	-0.19	0.02	9.3	-0.18	0.01	5.1
M1												
0.916	0.69	0.04	5.6	0.65	0.13	20.3	0.61	0.09	14.3	0.65	0.04	6.0
1.900	0.40	0.08	19.2	0.36	0.09	24.7	0.40	0.03	7.8	0.39	0.03	6.6
Ferulic acid												
7.21	0.68	0.06	8.2	0.72	0.02	3.2	0.60	0.04	6.8	0.66	0.06	9.0
14.96	0.72	0.13	17.7	0.73	0.11	14.8	0.75	0.08	10.3	0.73	0.02	2.4
Caffeic acid												
120.44	0.02	0.00	26.0	0.02	0.00	11.5	0.01	0.00	11.8	0.02	0.00	5.4
249.75	0.00	0.00	22.8	0.00	0.00	26.0	0.00	0.00	14.1	0.00	0.00	5.7
M2												
0.916	-0.14	0.01	8.9	-0.15	0.02	9.9	-0.16	0.02	12.9	-0.15	0.01	7.0
1.900	-0.12	0.01	9.8	-0.11	0.01	5.2	-0.12	0.01	11.7	-0.12	0.00	3.4

Comparison of IS-normalised matrix factor (MF) of the six analytes in human pooled blood cells at two concentrations (low, mid and high, n= 3) and in three different lots of blood cells at two concentrations (low and high; n= 3). The RSD of the mean of the three single donors represents the variability of the IS-normalised MF of an analyte in three different lots of human blood cells.

Analytes [ng/mL]	Human pooled blood cells			Three lots of human blood cells		
	Mean	± SD	RSD (%)	Mean	± SD	RSD (%)
(+)-Catechin						
71.90	-0.748	0.03	4.20	-0.705	0.03	4.08
103.54	-0.686	0.03	4.71			
149.10	-0.787	0.14	18.35	-0.732	0.03	3.49
Taxifolin						
0.905	-0.292	0.07	23.03	-0.244	0.03	14.32
1.303	-0.231	0.04	18.39			
1.876	-0.224	0.05	24.04	-0.183	0.01	5.12
M1						
0.916	0.626	0.12	19.77	0.650	0.04	5.98
1.319	0.433	0.07	15.66			
1.900	0.407	0.02	4.40	0.386	0.03	6.59
Ferulic acid						
7.21	0.634	0.09	13.69	0.665	0.06	8.98
10.39	0.556	0.07	11.82			
14.96	0.643	0.03	4.88	0.730	0.02	2.36
Caffeic acid						
120.44	0.018	0.00	15.87	0.016	0.00	5.40
173.44	0.009	0.00	8.04			
249.75	0.004	0.00	12.85	0.005	0.00	5.70
M2						
0.916	-0.160	0.03	16.11	-0.151	0.01	6.99
1.319	-0.101	0.00	3.66			
1.900	-0.098	0.01	13.65	-0.118	0.00	3.44

Process efficiency [%] of the six analytes in three different lots of human blood cells at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Process efficiency [%]									Mean Process efficiency [%]		
	Single donor #1			Single donor #2			Single donor #3			#1-3		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
71.90	1.24	0.24	19.2	0.84	0.04	4.8	0.93	0.04	4.7	1.00	0.21	21.1
149.10	1.62	0.23	14.2	1.3	0.15	11.5	1.24	0.04	3.5	1.39	0.21	14.9
Taxifolin												
0.905	13.4	0.15	1.1	12.9	1.76	13.6	15.4	1.11	7.2	13.89	1.33	9.5
1.876	15.7	1.14	7.3	15.5	1.04	6.7	17	0.55	3.3	16.04	0.80	5.0
M1												
0.916	52.6	5.33	10.1	45.5	2.41	5.3	46.7	6.58	14.1	48.26	3.80	7.9
1.900	32.7	0.45	1.4	27.2	1.78	6.5	30.5	1.25	4.1	30.14	2.75	9.1
Ferulic acid												
7.21	51.5	4.11	8.0	55.4	8.96	16.2	46.2	1.47	3.2	51.07	4.63	9.1
14.96	53.8	9.03	16.8	60.4	7.08	11.7	49.2	2.37	4.8	54.45	5.61	10.3
Caffeic acid												
120.44	1.24	0.14	11.2	1.16	0.11	9.7	1.18	0.01	1.0	1.19	0.04	3.6
249.75	1.35	0.08	6.0	1.41	0.13	9.4	1.49	0.12	7.8	1.42	0.07	5.1
M2												
0.916	27.6	4.61	16.7	20.2	0.24	1.2	23.1	0.41	1.8	23.61	3.75	15.9
1.900	28.0	2.06	7.4	23.2	1.85	8.0	23.1	1.09	4.7	24.77	2.78	11.2

2.3.3 Crosstalk

Crosstalk with developed method in human pooled blood cells (n= 3).

Mono-spiked analytes	Other analytes						
	Catechin	Ferulic acid	M1	Taxifolin	Caffeic acid	M2	IS
Catechin	-	114.00	37.62	103.15	n.d.	n.d.	n.d.
Ferulic acid	109.48	-	36.65	116.09	n.d.	n.d.	n.d.
M1	117.00	112.78	-	109.05	n.d.	n.d.	n.d.
Taxifolin	102.17	118.66	30.71	-	n.d.	n.d.	n.d.
Caffeic acid	113.34	109.83	32.29	112.00	-	n.d.	n.d.
M2	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.

n.d.: not detected

Calculation *Crosstalk* [%]:
(Mean (n= 3) peakarea analyt mono-spiked ULOQ-sample / Mean (n= 3) peakarea analyt matrix-blank)*100

Evaluation: ≤ 100% = no *Crosstalk*; > 100% = *Crosstalk, if co-elution*

2.3.4 Carry-over

Carry-over with developed method in human pooled blood cells (n= 3).

Analytes	I [%]	II [%]	III [%]	Mean ± SD [%]
Catechin	95.04	92.35	92.63	93.34 ± 1.48
Ferulic acid	103.58	102.98	105.39	103.98 ± 13.90
M1	77.03	108.21	89.87	91.70 ± 15.67
Taxifolin	84.59	110.13	87.84	94.19 ± 1.26
Caffeic acid	74.84	103.50	102.24	93.53 ± 16.20
M2	117.13	115.52	123.02	118.55 ± 3.95

Calculation c*arry-over* [%]:
(Peakarea 1st matrix-blank after ULOQ-sample / Peakarea matrix-blank before ULOQ-sample)*100

Evaluation: ≤ 100% = no c*arry-over*; > 100% = c*arry-over*

2.3.5 Robustness

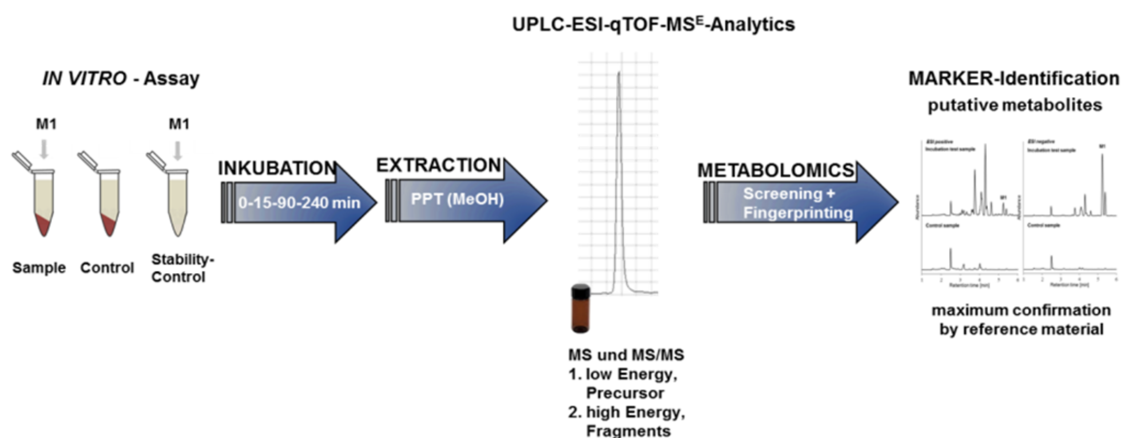
Robustness of the developed method in human pooled blood cells, which has been previously contaminated with 1% human serum, at two concentrations (low and high, n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Mean Accuracy [%]	Precision [%]
Catechin			
71.90	76.97 ± 4.70	107.05	6.10
149.10	145.63 ± 22.27	97.67	15.29
Ferulic acid			
7.21	7.58 ± 0.53	105.04	6.94
14.96	16.10 ± 0.90	107.65	5.59
M1			
0.916	0.825 ± 0.051	90.06	6.23
1.900	2.117 ± 0.080	111.43	3.76
Taxifolin			
0.905	0.997 ± 0.022	110.14	2.18
1.876	2.170 ± 0.059	115.68	2.74
Caffeic acid			
120.44	116.55 ± 6.97	96.77	5.98
249.75	257.30 ± 14.53	103.02	5.65
M2			
0.916	0.940 ± 0.042	102.60	4.43
1.900	2.080 ± 0.100	109.44	4.83

3 Supporting Information for Publication 3

3.1 *In vitro* metabolites

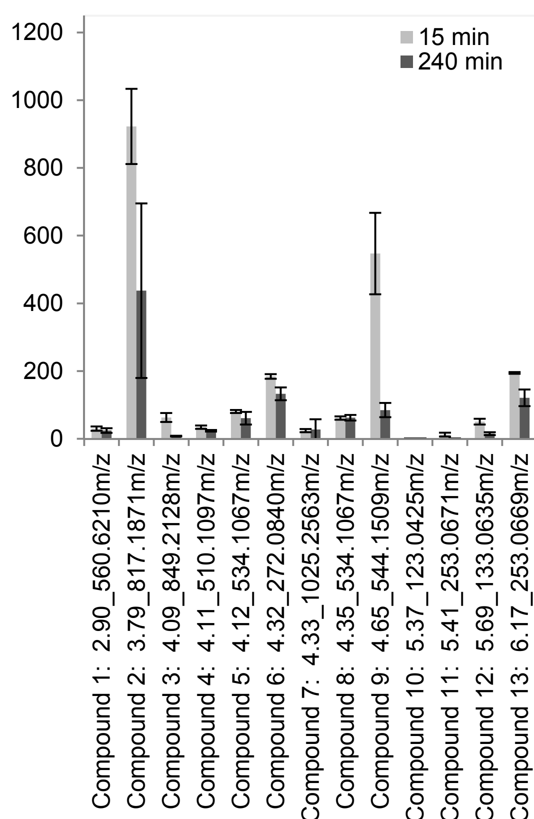
Strategy of *in vitro* assay for identification of putative metabolites of M1 in human blood cells by UPLC-ESI-qTOF-MS^E.



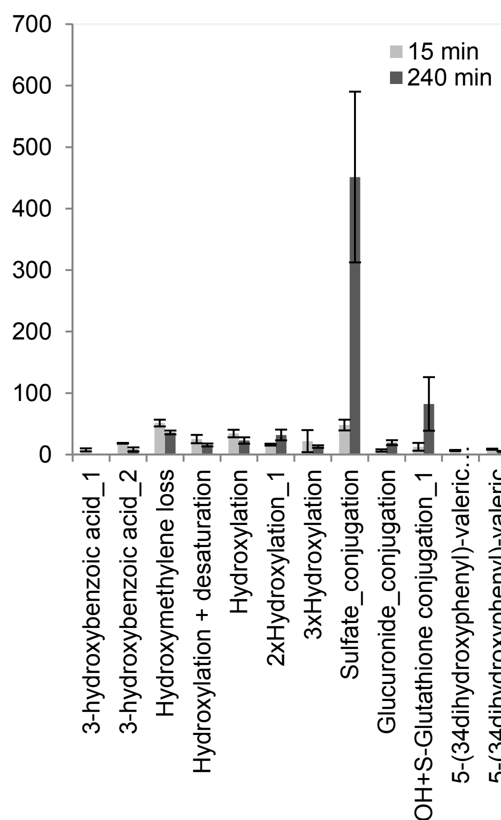
Putative *in vitro* metabolites, confirmed by experiment #2 (15 min and 240 min incubation time, mean ± SD; n= 3). Fingerprinted markers with associated retention time and measured exact mass were plotted against abundance (peakarea). Markers, which were detected by profiling were also plottet against related peakarea.

<u>ESI negative</u>

Fingerprinting: 12 markers

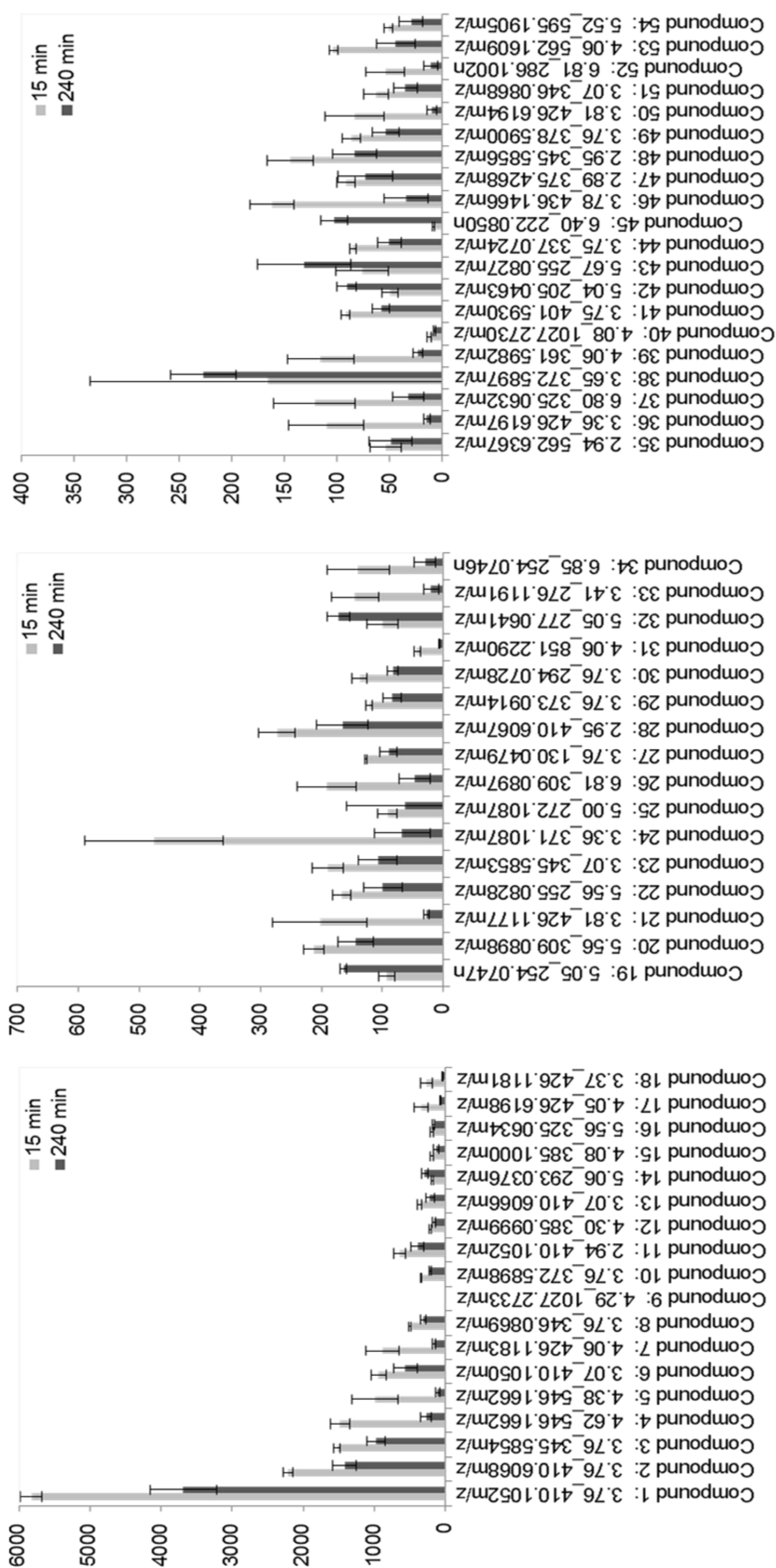


Profiling: 12 markers

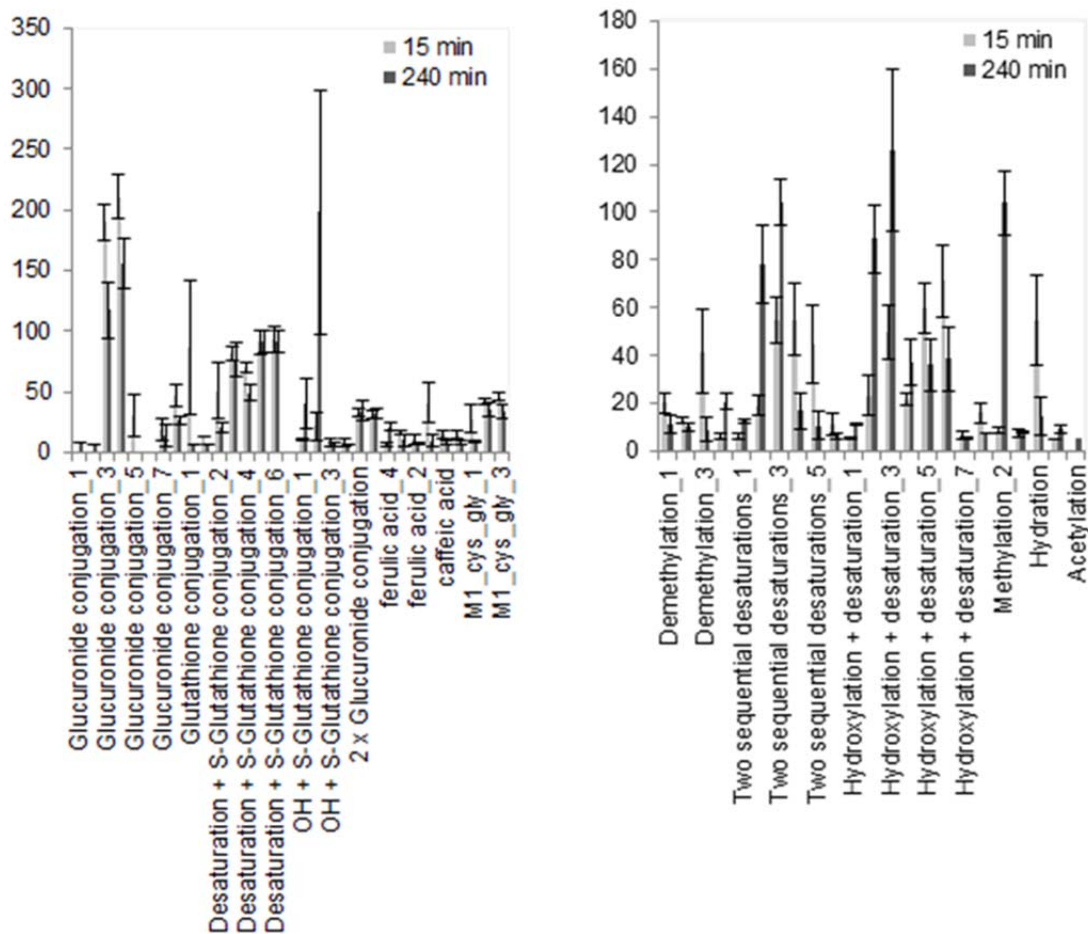


ESI positive

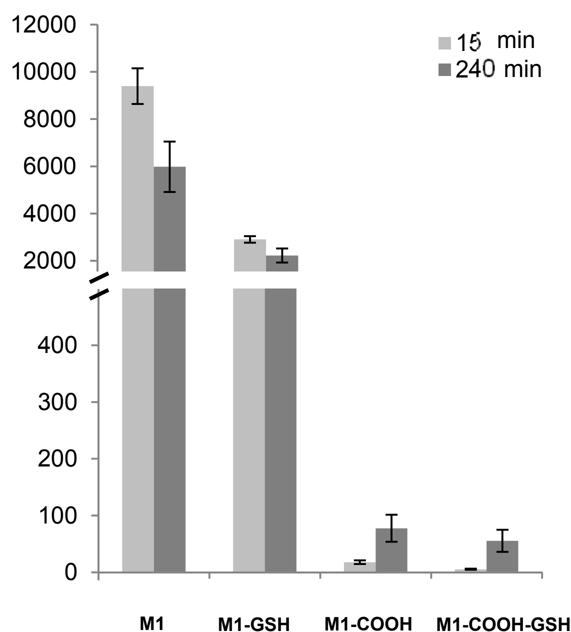
Fingerprinting: 54 markers



Profiling: 51 markers

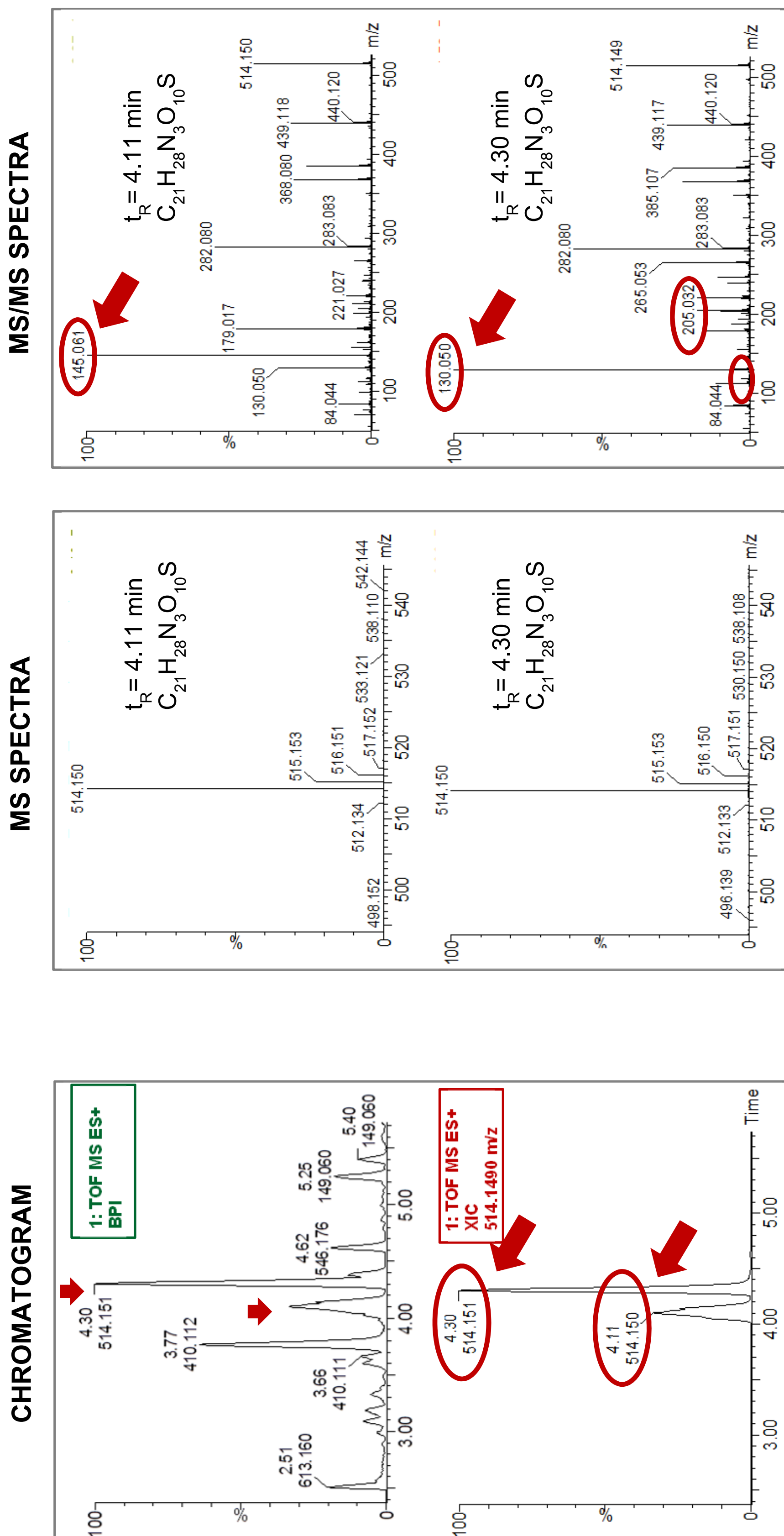


Semi-quantitative evaluation of M1 and its intracellular *in vitro* metabolites M1-GSH, M1-COOH and M1-COOH-GSH (530.144 *m/z*; ESI negative mode). Peakareas of related extracted ion chromatograms (XIC) were displayed (experiment #2; 15 min and 240 min incubation time, mean ± SD; n= 3).

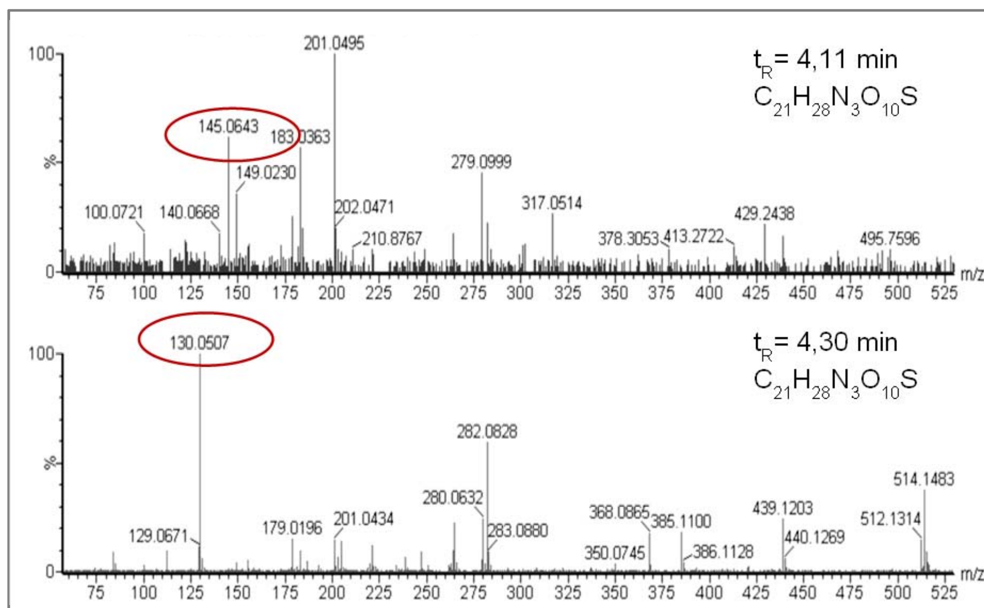


3.2 Two isomers of M1-glutathione adduct

Data supporting the *in vitro* conjugation of M1 and glutathione (M1-GSH) ielding two isomers, respectively M1-S-GSH (t_R= 4.30 min) and M1-N-GSH (t_R= 4.11 min). It was shown the extracted ion chromatogram (XIC) of 514.1490 *m/z* in the positive ESI mode, the MS and the MS/MS spectra of the two isomers.

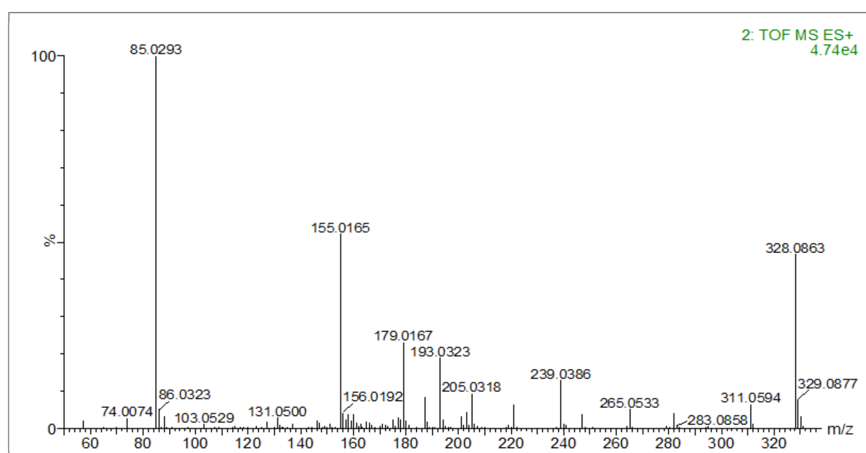


MS/MS spectra of two isomers (514.1490 *m/z* in the positive ESI mode) of the conjugation of M1 with GSH (M1-S-GSH at t_R= 4.30 min and M1-N-GSH at t_R= 4.11 min) of the self-synthesized reference material by enzymatic catalysis via GSH-S-transferase.

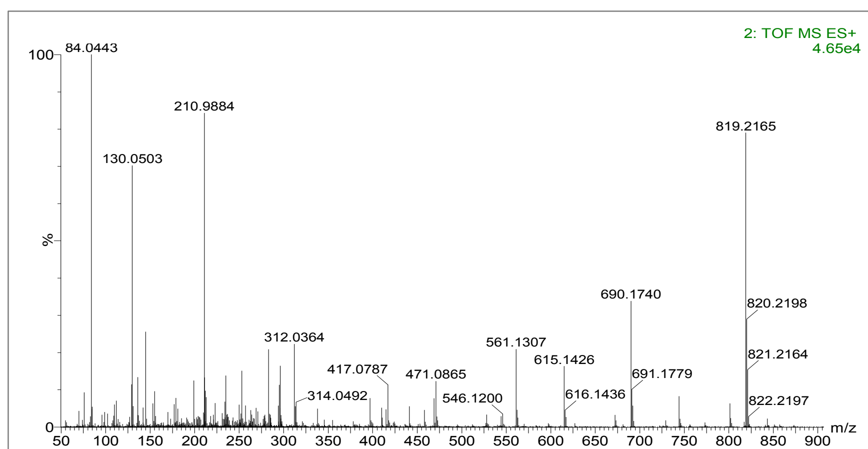


3.3 MS/MS spectra

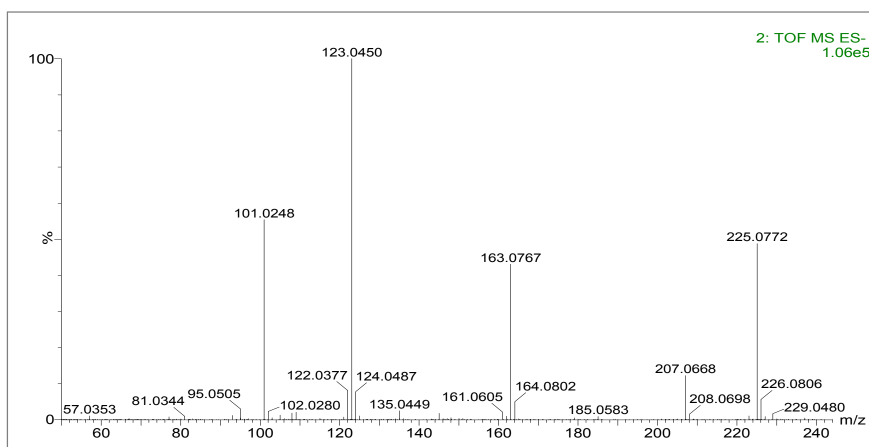
MS/MS spectra of the conjugation of M1 with L-cysteine, M1-CYS, at t_R= 3.94 min in ESI positive mode (328.0863 *m/z*).



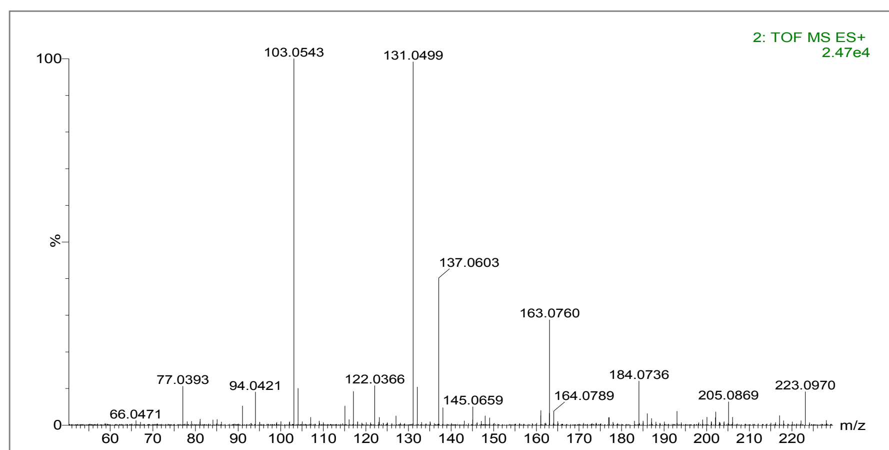
MS/MS spectra of the conjugation of M1 with oxidized glutathione, M1-GSSG, at t_R= 3.79 min in ESI positive mode (819.2165 *m/z*).



MS/MS spectra of the open-chained ester form of M1, M1-COOH, at t_R= 4.01 min in ESI negative mode (225.0772 *m/z*).



MS/MS spectra of the methylated form of M1, M1-methylated, at t_R= 6.40 min in ESI positive mode (223.0970 *m/z*).



3.4 Kinetics

Experiment #1:
Kinetics of the *in vitro* formation of some putative M1 metabolites. Human blood cells were incubated with 50 µM M1 for 0, 15, 90 min (each n= 3) and 4 hours (single experiment) at 37 °C. Mean and SD of the peakarea of M1, M1-COOH, M1-CYS adduct and the two M1-GSH isomers. M1, M1-COOH were measured in ESI negative mode; M1-CYS, M1-S-GSH (t_R= 4.30 min) and M1-N-GSH (t_R= 4.10 min) in ESI positive mode.

Incubation time	M1		M1-COOH		M1-CYS		M1-S-GSH		M1-N-GSH	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
0 min	12600	1397.62	8	1.16	6	0.05	12206	707.19	4526	87.58
15 min	12088	1950.42	15	4.06	8	2.73	11030	536.54	5542	1050.25
90 min	12307	561.57	74	10.96	11	3.41	11391	1085.62	6444	409.82
240 min	15869	-	265	-	47	-	12338	-	3581	-

Experiment #2:
Kinetics of the *in vitro* formation of some putative M1 metabolites. Human blood cells were incubated with 50 µM M1 for 15 and 240 min (each n= 3) at 37 °C. Mean and SD of the peakarea of M1, M1-COOH, M1-CYS adduct and the two M1-GSH isomers. M1, M1-COOH were measured in ESI negative mode; M1-CYS, M1-S-GSH (t_R= 4.30 min), M1-N-GSH (t_R= 4.10 min) and M1-GSSG in ESI positive mode.

Incubation time	M1		M1-COOH		M1-CYS		M1-S-GSH		M1-N-GSH	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
15 min	5499	477.07	18	3.21	7	-	8855	858.23	4779	307.99
240 min	3577	643.26	78	23.69	23	1.55	6623	1026.46	2784	604.05

Incubation time	M1-GSSG	
	Mean	± SD
15 min	5822	151.71
240 min	3687	470.53

3.5 *In vivo* metabolites

***In vivo* metabolites of M1 in blood cells of human volunteers who ingested multiple oral doses of Pycnogenol® (200, 300 and 500 mg, respectively, per day).** Blood cell samples were subjected to protein precipitation (PPT) with methanol and QuEChERS (Que) approach (without prior enzymatic incubation).

The bold written volunteers were involved in the manuscript (mean ± MDM).

In vivo metabolites detected in the ESI negative mode.

Sample	Intake Pycnogenol ® (per day)	Peakarea		
		M1-COOH	M1-sulfated	Hydroxybenzoic acid
PPT_MM1	500 mg	4.6	8.6	2.4
PPT_MSC	500 mg	4.3	-	2.0
PPT_MM2	200 mg	2.8	-	2.5
PPT_SJ	200 mg	-	-	-
PPT_PH	300 mg	2.7	-	-
Que_MSC	500 mg	-	12.7	-
Que_MM	200 mg	-	8.1	-
Que_SJ	200 mg	-	21.6	-
Que_PH	300 mg	-	-	-

In vivo metabolites detected in the ESI positive mode.

Sample	Intake Pycnogenol ® (per day)	Peakarea		
		M1-S-GSH	M1-methylated	M1-acetylated
PPT_MM1	500 mg	-	3.4	11.8
PPT_MSC	500 mg	-	5.0	18.2
PPT_MM2	200 mg	-	2.5	3.7
PPT_SJ	200 mg	-	-	3.6
PPT_PH	300 mg	2.3	2.9	5.4
Que_MSC	500 mg	-	-	8.6
Que_MM	200 mg	-	2.1	10.1
Que_SJ	200 mg	-	-	5.3
Que_PH	300 mg	-	-	7.8

4 Supporting Information for Publication 4

4.1 Method development

Comparison of different sample preparation techniques: recovery (RE), matrix effects (ME) and process efficiency (PE) using 0.5 mL human pooled plasma exemplified for the metabolite M1 spiked at a concentration of 100 ng/mL (n=1).

Sample preparation	Process	Recovery [%]	Matrix effects [%]	Process efficiency [%]
Combi #1	LLE #3 + ENVI-Carb™	2.39	-11.15	2.13
Combi #2	LLE #4 + ENVI-Carb™	n.d.	-37.99	0.00
Combi #3	PPT (ACN 1:3) + SPE #10	15.83	121.70	35.10
Combi #4	PPT (TCA 1:3) + SPE #6	0.00-	-98.57	44.38
Combi #5	PPT #1 + LLE #1	5.74	-99.23	0.04
Combi #6	PPT #1 + LLE #2	60.87	-98.25	1.06
SPE #1	Oasis HLB	74.29	38.81	103.12
SPE #2	Oasis HLB	39.48	-11.35	34.99
SPE #3	Oasis MAX	36.61	-53.05	17.19
SPE #4	Oasis MAX	24.68	-52.21	11.79
SPE #5	Oasis MAX	17.00	52.87	25.99
SPE #6	Strata X	53.79	-68.52	16.93
SPE #7	Strata X	55.30	34.35	74.29
SPE #8	Strata X	45.07	-17.23	37.30
SPE #9	Strata X-A	16.42	0.00	89.03
SPE #10	Strata X-A	14.47	53.87	22.27
LLE #1	EA	10.74	0.00	42.15
LLE #2	MTBE	43.93	15.84	50.90
LLE #3	IPM	8.51	-13.48	7.37
LLE #4	MEM	4.80	-48.72	2.46
LLE #5	MultiLM	49.63	-29.10	35.19
LLE #6	H+/MTBE	58.85	70.23	100.17
LLE #7	H+/MTBE	41.02	15.31	47.30
LLE #8	H+/MTBE	71.74	-57.85	30.24
PPT #1	TCA 1:2	50.00	-98.82	0.59
PPT #2	TCA 1:4	63.33	-99.79	0.13
PPT #3	ACN 1:2	10.36	-25.55	7.71
PPT #4	ACN 1:4	0.00	-98.87	9.01
PPT #5	H+/ACN 1:4	53.83	-3.44	51.98
PPT #6	MeOH 1:4	14.33	-29.89	10.05
PPT #7	Telos® PPT	44.40	-29.73	31.20
PPT #8	H+/Telos® PPT	11.29	-82.97	11.29

4.2 Method optimization

Dependence of analyte intensity from start%-composition of organic solvent (B; MeOH +0.1% FA). Solvent (A) was set to water +0.1% FA. 10 µg/mL of M1, ferulic acid and caffeic acid in MeOH were analyzed with different%-composition of (B; 35 – 60%) at 0 min with the same gradient program.

% composition (B) at 0 min	Peakheight		
	M1	Ferulic acid	Caffeic acid
35	185967	10056	135884
40	277260	11305	183787
50	285648	14229	167519
52.5	289816	15600	173914
55	311628	34313	219769
60	421105	58725	255562

Optimization of the mobile phase (A; water +0.1% FA). Influence of the additive ammonium formiate (AF) with different ion strengths (5 -10-100 mM) and pH-values (3.2, 5.0, 6.8 and 7.4). Solvent (B) was evaluated previously and set to MeOH +0.1% FA. 10 µg/mL of M1, taxifolin and ferulic acid in MeOH were analyzed and signal-to-noise ratio (SNR) of the quantifier was calculated (n= 1).

(A)	SNR (peak-to-peak height)		
	M1	Ferulic acid	Taxifolin
H20 + 0.1% FA	14725	8673	12217
5 mM AF pH 3.2	20738	19071	11326
5 mM AF pH 5.0	15530	10395	7346
5 mM AF pH 6.8	14537	9318	6696
5 mM AF pH 7.4	20645	6275	13973
10 mM AF pH 3.2	19309	12032	11808
10 mM AF pH 5.0	12149	5545	10374
10 mM AF pH 6.8	13996	5544	12357
10 mM AF pH 7.4	21942	4644	12682
100 mM AF pH 3.2	10718	11052	8989
100 mM AF pH 5.0	7207	7064	7821
100 mM AF pH 6.8	3041	9340	4312
100 mM AF pH 7.4	10629	4440	9521

Optimization of the ESI source parameters with extracted plasma samples spiked with 100 ng/mL of M1, taxifolin, ferulic acid and caffeic acid. Standard ESI conditions (each parameter was set to default values) were compared with previously optimized ESI settings.

ESI Source Parameter	Peakheight			
	M1	Ferulic acid	Taxifolin	Caffeic acid
Standard	31497	4583	97093	3558
Optimized	146713	7430	181361	8389

Optimization of the liquid-liquid extraction (LLE) for preparation of the plasma samples. A sample volume of 2.0 mL plasma was spiked with 10 ng/mL per analyte and the influence of changing selected steps of the extraction process on the intensity of the analyte signal was investigated.

Influence pH-value of plasma before extraction with MTBE. Plasma was acidified with 4% H_3PO_4 to pH 3.2, 3.0, 2.5, 2.0, 1.7 before extraction of the analytes.

pH-value	Peakarea		
	M1	Ferulic acid	Taxifolin
3.2	136954	415739	17515
3	137082	423561	17678
2.5	124707	359622	17658
2	89436	370707	13270
1.7	70749	459088	11346

Influence of the extraction technique and time. After acidification and adding solvent to the samples, they were vortexed (1 min), extracted by mixing wheel (10, 15 and 20 min) and by ultrasonic (10, 15 and 20 min).

Extraction technique and time	Peakarea		
	M1	Ferulic acid	Taxifolin
1 min vortex	136954	17515	415739
10 min mixing wheel	108031	11656	209599
15 min mixing wheel	85579	9676	171664
20 min mixing wheel	80860	9387	161054
10 min ultrasonic	136068	15169	351368
15 min ultrasonic	104586	19297	225337
20 min ultrasonic	123297	15949	245924

Influence of the extraction solvent (6 mL). In addition to a mixture of the two solvents *tert*-butyl methyl ether (MTBE) and ethyl acetate (EA), it was tested whether a twofold extraction with these both in sequence were more efficient.

Extracion solvent	Peakarea				
	M1	Ferulic acid	Taxifolin	Procyanidin B1	(+)-Catechin
2 x MTBE	136954	17515	415739	17	203
EA; MTBE	123137	15914	340733	137	210
MTBE; EA	103577	15455	313901	30	30
MTBE	135561	14663	407727	21	312
EA	117856	12565	300284	240	1043
EA + MTBE (1:1; V/V)	120247	12327	315428	61	417

4.3 Method validation plasma with prior enzymatic incubation

4.3.1 Levels of calibration standards

Calibration curve in human plasma

Analytes	Levels of calibration standards [ng/mL plasma and serum]*											
	L0	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Procyanidin B1	1.33	2.00	2.40	2.88	3.45	4.14	5.18	6.47	8.09	10.11	12.14	24.28
Catechin	4.09	6.14	7.36	8.84	10.60	12.72	15.90	19.88	24.85	31.06	37.28	74.55
Taxifolin	0.060	0.090	0.108	0.129	0.155	0.186	0.232	0.290	0.363	0.454	0.544	1.089
M1	0.156	0.235	0.281	0.338	0.405	0.486	0.608	0.760	0.950	1.188	1.425	2.850
Ferulic acid	2.74	4.10	4.92	5.91	7.09	8.51	10.63	13.29	16.62	20.77	24.93	49.85
Caffeic acid	5.48	8.22	9.87	11.84	14.21	17.05	21.31	26.64	33.30	41.63	49.95	99.90
Hydrocaffeic acid (IS)	20.80	20.80	20.80	20.80	20.80	20.80	20.80	20.80	20.80	20.80	20.80	20.80

* including the purity of the used standard substances according to the Certificate of Analysis from the manufacturer's batch.
The synthesized compounds M1 and M2 were confirmed by NMR and LC/MS and equated with a purity of 95%.

	Level
LLOQ	L0 or L1
low	L6
mid	L8
high	L10

4.3.2 Recovery and process efficiency

Individual values of recovery [%] of the six analytes in six different lots of human plasma at two concentrations (low and high; n= 5).

Analytes [ng/mL]	Recovery [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
5.179	5.33	0.50	4.06	0.39	4.35	1.01	2.53	0.11	3.45	0.23	6.79	1.22
12.138	5.07	0.56	3.44	0.61	5.10	1.26	3.50	0.52	3.84	0.61	3.70	0.26
Catechin												
15.200	23.85	2.94	11.42	2.13	30.74	2.20	19.75	1.55	22.60	3.11	17.15	3.28
35.625	19.26	2.00	10.35	1.71	26.77	0.88	25.92	6.63	19.67	3.77	19.47	1.73
M1												
0.608	64.48	1.91	74.03	13.7	80.90	6.73	74.97	1.46	71.77	4.67	82.93	1.10
1.425	60.43	4.49	71.26	6.47	75.53	3.95	58.48	1.89	57.61	4.37	60.91	3.79
Caffeic acid												
21.312	71.16	11.85	75.05	5.47	78.86	8.59	106.86	14.17	102.66	7.81	82.29	12.53
49.950	64.32	10.35	84.88	10.82	78.26	7.35	91.94	13.85	76.75	6.30	79.25	17.63
Taxifolin												
0.250	92.10	5.52	95.30	8.57	115.12	21.60	93.77	2.06	95.34	3.61	93.53	16.62
0.586	76.55	5.49	96.66	5.29	95.01	2.92	87.25	9.39	93.10	3.17	72.76	8.03
Ferulic acid												
10.635	56.49	11.80	63.06	3.09	66.09	6.53	87.11	6.12	73.22	5.59	65.79	10.13
24.925	45.51	5.88	63.74	2.08	69.39	3.02	67.11	4.49	54.46	1.61	60.51	7.05

Individual values of process efficiency [%] of the six analytes in six different lots of human plasma at two concentrations (low and high; n= 5).

Analytes [ng/mL]	Process efficiency [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
5.179	0.28	0.06	0.24	0.04	0.53	0.12	0.27	0.04	0.37	0.06	0.57	0.09
12.138	0.24	0.03	0.13	0.03	0.32	0.06	0.18	0.02	0.34	0.07	0.45	0.04
Catechin												
15.200	5.07	0.55	3.61	0.43	3.70	0.25	5.97	0.73	5.48	0.73	3.06	0.45
35.625	5.80	0.14	3.39	0.67	4.06	0.29	2.77	0.48	4.71	0.48	2.83	0.18
M1												
0.608	149.11	3.35	160.80	32.50	164.56	5.74	141.95	12.18	164.88	12.05	243.37	11.69
1.425	122.39	7.11	130.70	7.36	120.32	6.72	92.41	4.52	107.42	8.39	130.00	9.36
Caffeic acid												
21.312	91.64	18.30	89.50	5.50	97.21	9.17	131.50	18.62	126.97	7.84	119.41	14.62
49.950	87.58	13.57	108.66	14.82	109.12	5.68	113.29	17.42	109.85	8.25	114.93	19.09
Taxifolin												
0.250	131.54	4.78	132.18	10.73	154.41	9.16	135.27	5.84	139.91	2.12	147.73	19.51
0.586	107.89	7.85	138.22	7.67	133.65	5.45	124.23	9.22	143.59	3.95	108.73	12.80
Ferulic acid												
10.635	131.12	24.01	126.74	7.19	140.60	7.20	166.87	12.32	154.43	7.62	150.78	22.97
24.925	103.83	12.97	115.54	5.14	121.71	5.77	113.68	6.52	142.28	3.39	112.65	13.03

4.3.3 Carry-over

Carry-over with developed method in human pooled plasma (n= 3).

Analytes	I [%]	II [%]	III [%]	Mean ± SD [%]
Procyanidin B1	82.54	54.26	66.85	67.88 ± 14.17
Catechin	86.72	110.22	84.24	93.73 ± 14.34
Taxifolin	114.12	107.68	85.11	102.31 ± 15.24
M1	90.44	99.87	100.69	97.00 ± 5.70
Ferulic acid	98.45	99.27	100.42	99.38 ± 0.99
Caffeic acid	94.03	96.47	95.40	95.30 ± 1.22

Calculation c*arry-over* [%]:
(Peakarea 1st matrix-blank after ULOQ-sample / Peakarea matrix-blank before ULOQ-sample)*100

Evaluation: ≤ 100% = no c*arry-over*; > 100% = c*arry-over*

4.4 Method validation serum with prior enzymatic incubation

4.4.1 Recovery, matrix effects and process efficiency

Individual values of recovery [%] of the six analytes in six different lots of human serum at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Recovery [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
5.18	1.64	0.15	1.44	0.31	1.82	0.66	1.34	0.98	2.17	1.30	1.76	1.74
12.14	1.69	0.40	1.52	0.62	1.74	0.32	1.40	0.13	1.21	0.09	2.02	0.60
Catechin												
15.2	11.87	1.67	10.11	1.23	9.63	0.84	11.32	4.23	10.30	1.92	11.12	1.81
35.63	11.38	2.31	8.69	5.77	8.01	2.29	9.55	3.18	8.28	0.63	9.48	4.45
Taxifolin												
0.25	93.85	0.62	102.66	4.87	103.91	10.70	91.87	2.56	118.39	10.82	104.12	6.44
0.586	93.56	3.34	91.77	4.99	88.94	5.75	85.17	2.93	90.97	1.28	90.27	0.55
M1												
0.608	78.58	15.51	86.27	0.53	105.57	10.79	69.49	3.38	106.58	5.77	109.37	14.81
1.425	68.61	7.21	82.39	6.63	97.79	20.96	76.03	7.75	83.94	4.03	99.35	5.33
Ferulic acid												
10.64	105.86	7.80	134.24	60.28	132.59	7.58	112.20	7.03	130.65	16.15	118.50	10.30
24.93	95.48	10.11	85.14	1.14	106.91	5.21	87.05	6.92	89.35	4.68	104.08	2.96
Caffeic acid												
21.31	90.31	4.33	88.14	5.44	107.67	20.35	75.03	4.10	114.02	17.61	100.04	8.07
49.95	105.41	3.17	108.19	0.82	96.05	5.60	95.01	1.38	113.85	7.42	121.31	7.29

Individual values of relative matrix effects [%] of the six analytes in six different lots of human serum at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Matrix effects [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
5.18	-69.32	2.76	-71.37	3.75	-74.05	5.47	-72.34	8.03	-83.58	6.96	-73.33	5.03
12.14	-75.13	6.30	-72.41	9.97	-74.05	5.47	-76.08	1.27	-74.29	5.42	-81.18	4.74
Catechin												
15.2	-81.53	0.80	-77.78	1.43	-79.70	2.52	-70.51	2.55	-68.17	1.37	-71.72	2.33
35.63	-81.33	2.61	-73.76	11.55	-79.70	2.52	-73.42	4.04	-72.26	1.24	-75.41	1.51
Taxifolin												
0.25	34.79	1.16	41.49	0.21	33.84	2.49	36.35	2.00	37.36	3.94	39.58	9.84
0.586	29.14	4.08	39.91	1.76	33.84	2.49	36.19	1.43	30.53	2.86	36.94	6.23
M1												
0.608	66.37	7.84	84.51	4.46	50.86	11.22	63.47	13.93	67.39	5.57	81.72	13.99
1.425	54.91	4.70	63.95	7.95	50.86	11.22	56.56	7.83	49.88	2.54	54.32	6.21
Ferulic acid												
10.64	37.70	12.81	36.61	7.99	22.88	3.22	31.69	1.61	29.67	4.16	25.88	9.18
24.93	21.29	3.98	32.00	2.53	22.88	3.22	25.33	3.34	22.78	7.40	18.87	0.42
Caffeic acid												
21.31	14.66	5.45	12.65	5.98	16.06	4.42	14.07	8.30	17.58	6.84	16.19	4.90
49.95	12.26	14.41	14.46	18.61	16.06	4.42	14.30	2.47	13.66	5.59	14.21	3.75

Individual values of IS (hydrocaffeic acid) normalised matrix factor (MF) of the six analytes in six different lots of human serum at two concentrations (low and high; n= 3).

Analytes [ng/mL]	IS-normalised MF [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
5.18	1.01	0.06	1.08	0.13	1.00	0.11	1.14	0.14	1.18	0.09	1.12	0.10
12.14	1.13	0.13	0.98	0.12	0.99	0.07	1.21	0.03	1.12	0.15	1.30	0.04
Catechin												
15.2	1.19	0.04	1.17	0.06	1.05	0.03	1.11	0.06	0.97	0.04	1.10	0.05
35.63	1.23	0.03	1.00	0.14	1.07	0.02	1.16	0.04	1.09	0.05	1.21	0.04
Taxifolin												
0.25	-0.51	0.01	-0.63	0.04	-0.45	0.04	-0.57	0.05	-0.53	0.05	-0.61	0.16
0.586	-0.44	0.08	-0.54	0.02	-0.45	0.04	-0.57	0.03	-0.46	0.04	-0.59	0.12
M1												
0.608	-0.97	0.13	-1.28	0.16	-1.09	0.39	-1.00	0.26	-0.96	0.10	-1.25	0.21
1.425	-0.83	0.10	-0.87	0.11	-0.68	0.15	-0.90	0.14	-0.75	0.06	-0.87	0.11
Ferulic acid												
10.64	-0.55	0.17	-0.55	0.16	-0.56	0.08	-0.50	0.01	-0.42	0.07	-0.40	0.14
24.93	-0.32	0.05	-0.43	0.03	-0.31	0.05	-0.40	0.04	-0.34	0.09	-0.30	0.01
Caffeic acid												
21.31	-0.21	0.07	-0.19	0.08	-0.16	0.04	-0.22	0.14	-0.25	0.10	-0.25	0.07
49.95	-0.18	0.22	-0.20	0.25	-0.22	0.06	-0.23	0.04	-0.21	0.08	-0.23	0.06

Individual values of process efficiency [%] of the six analytes in six different lots of human serum at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Process efficiency [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
5.18	0.50	0.07	0.41	0.10	0.48	0.18	0.37	0.15	0.36	0.13	0.47	0.60
12.14	0.42	0.01	0.42	0.06	0.45	0.03	0.34	0.03	0.31	0.07	0.38	0.05
Catechin												
15.2	2.19	0.22	2.25	0.78	2.13	0.32	3.34	1.32	3.28	0.52	3.14	0.27
35.63	2.12	0.18	2.28	0.78	1.63	0.55	2.54	1.26	2.30	0.08	2.33	1.02
Taxifolin												
0.25	126.49	1.92	145.25	5.17	138.36	15.82	125.28	2.50	162.61	19.03	145.34	1.55
0.586	120.82	4.78	128.39	5.80	119.03	5.64	115.99	3.38	118.75	3.35	123.61	5.70
M1												
0.608	130.73	21.53	159.18	5.94	190.78	12.46	113.59	7.42	178.41	5.54	198.74	39.52
1.425	106.28	10.93	135.08	4.22	147.52	21.68	119.04	6.10	125.82	7.73	153.31	6.44
Ferulic acid												
10.64	145.76	6.54	183.38	60.76	187.87	8.68	147.75	8.20	169.41	16.26	149.17	4.26
24.93	115.81	11.48	112.38	3.12	131.38	6.35	109.10	6.23	109.71	8.81	123.71	3.84
Caffeic acid												
21.31	103.54	1.17	99.29	3.40	120.64	20.05	85.59	8.63	134.06	13.27	116.23	5.72
49.95	118.34	18.36	123.83	19.88	111.47	10.62	108.59	3.47	129.41	4.45	138.56	7.98

4.4.2 Crosstalk

Crosstalk with developed method in human pooled serum (n= 3).

Mono-spiked analytes	Other analytes						
	Catechin	Ferulic acid	M1	Taxifolin	Caffeic acid	Procyanidin B1	IS
Catechin	-	94.00	87.96	74.63	97.31	93.83	n.d.
Ferulic acid	45.23	-	79.25	73.09	105.73	93.55	n.d.
M1	44.08	86.62	-	68.73	100.60	71.26	n.d.
Taxifolin	56.83	95.37	65.06	-	107.11	88.37	n.d.
Caffeic acid	31.76	95.30	72.30	72.40	-	90.97	n.d.
Procyanidin B1	22.48	84.94	70.11	77.97	97.16	-	n.d.

n.d.: not detected

Calculation *Crosstalk* [%]:
(Mean (n= 3) peakarea analyt mono-spiked ULOQ-sample / Mean (n= 3) peakarea analyt matrix-blank)*100

Evaluation: ≤ 100% = no *Crosstalk*; > 100% = *Crosstalk*, *if co-elution*

4.4.3 Carry-over

Carry-over with developed method in human pooled serum (n= 5).

Analytes	I [%]	II [%]	III [%]	IV [%]	V [%]	Mean ± SD [%]
Catechin	78.40	91.53	106.85	104.28	127.11	101.64 ± 18.20
Ferulic acid	98.19	101.90	99.07	98.43	107.24	100.96 ± 3.81
M1	98.53	98.73	101.13	100.15	97.90	99.29 ± 1.32
Taxifolin	98.22	102.81	96.26	97.13	118.265	102.53 ± 9.14
Caffeic acid	100.18	99.33	98.74	100.41	114.11	102.56 ± 6.49
Procyanidin B1	103.78	110.603	97.09	105.22	101.69	103.67 ± 4.94

Calculation c*arry-over* [%]:
(Peakarea 1st matrix-blank after ULOQ-sample / Peakarea matrix-blank before ULOQ-sample)*100

Evaluation: ≤ 100% = no c*arry-over*; > 100% = c*arry-over*

4.5 Experiments with stable isotope labeled internal standard

Individual values of accuracy of quantification at six individual lots of plasma with structural IS (hydrocaffeic acid) and stable isotope labeld (SIL)-IS (ferulic acid-1,2,3-^{13}C3).

Analytes	Accuracy [%]					
	Single donor #1	Single donor #2	Single donor #3	Single donor #4	Single donor #5	Single donor #6
Procyanidin B1	118.97	84.62	110.14	110.95	85.12	82.66
(+)-Catechin	88.91	85.07	77.82	92.06	80.07	91.18
M1	95.35	101.59	81.72	93.73	85.69	88.31
Caffeic acid	102.19	91.70	90.09	94.57	104.69	88.26
Taxifolin	113.34	90.42	80.46	108.12	84.18	115.73
*Ferulic acid**	91.16	98.86	90.91	84.89	126.20	121.56
*Ferulic acid***	79.35	132.95	98.68	83.83	136.22	77.40

* IS (structural): Hydrocaffeic acid
** SIL-IS: Ferulic acid-1,2,3-^{13}C3

5 Supporting Information for Publication 5

5.1 Method development

Comparison of different sample preparation techniques: recovery, matrix effects and process efficiency using 0.25 mL human synovial fluid exemplified for the metabolite M1 spiked at a concentration of 100 ng/mL (n= 1).

Sample preparation	Process	Recovery [%]	Matrix effects [%]	Process efficiency [%]
Combi #1	Combi #1	2.30	-52.78	1.09
LLE #1	LLE #1	101.79	32.87	135.25
LLE #2	LLE #2	85.45	18.66	101.39
LLE #3	LLE #3	94.27	31.30	123.77
LLE #4	LLE #4	88.91	2.18	90.86
LLE #5	LLE #5	74.70	-58.25	31.19
LLE #6	LLE #6	14.45	-29.28	10.22
LLE #7	LLE #7	79.54	25.28	99.65
LLE #8	LLE #8	103.63	25.67	130.26
LLE #9	LLE #9	103.73	-23.51	79.34
LLE #10	LLE #10	43.88	65.18	72.48
LLE #11	LLE #11	100.61	28.16	128.94
LLE #12	LLE #12	101.27	29.99	131.64
LLE #13	LLE #13	86.73	24.85	108.29
LLE #14	LLE #14	5.20	-63.47	1.90
LLE #15	LLE #15	0.67	-65.38	0.23
LLE #16	LLE #16	87.16	16.02	101.12
PPT #1	PPT #1	109.70	-57.10	47.06
PPT #2	PPT #2	101.23	-49.37	51.26
PPT #3	PPT #3	107.10	-51.28	52.18
PPT #4	PPT #4	152.50	-47.02	80.82
PPT #5	PPT #5	148.05	-58.70	61.14
QuEChERS #1	QuEChERS #1	31.92	-6.08	29.98
QuEChERS #2	QuEChERS #2	122.83	-62.83	45.66
QuEChERS #3	QuEChERS #3	37.61	-50.74	18.53
QuEChERS #4	QuEChERS #4	19.12	-65.53	6.59
QuEChERS #5	QuEChERS #5	1.50	-48.32	0.77
SPE #1	SPE #1	106.65	-21.16	84.08
SPE #2	SPE #2	38.29	-31.65	26.17
SPE #3	SPE #3	92.89	-17.68	76.47
SPE #4	SPE #4	2.39	-67.38	0.79

5.2 Method validation synovial fluid with prior enzymatic incubation

5.2.1 Levels of calibration standards

Calibration curve in human synovial fluid

Analytes	Levels of calibration standards [ng/mL synovial fluid]*											
	L0	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Catechin	2.14	2.68	3.35	4.19	5.23	6.54	8.18	10.22	12.77	15.97	19.96	34.93
Taxifolin	0.080	0.100	0.125	0.157	0.196	0.245	0.306	0.382	0.478	0.597	0.747	1.307
M1	0.117	0.146	0.182	0.228	0.285	0.356	0.445	0.556	0.695	0.869	1.086	1.900
Ferulic acid	1.53	1.91	2.39	2.99	3.73	4.67	5.83	7.29	9.11	11.39	14.24	24.93
Caffeic acid	3.07	3.83	4.79	5.99	7.48	9.35	11.69	14.61	18.27	22.83	28.54	49.95
Hydrocaffeic acid (IS)	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85	24.85

* including the purity of the used standard substances according to the Certificate of Analysis from the manufacturer's batch.
The synthesized compound M1 were confirmed by NMR and LC/MS and equated with a purity of 95%.

	Level
LLOQ	L0 or L1
low	L6
mid	L8
high	L10

5.2.2 Recovery, matrix effects and process efficiency

Recovery [%] of the five analytes in three different lots of human synovial fluid at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Recovery [%]									Mean Recovery [%] #1-3		
	Single donor #1			Single donor #2			Single donor #3					
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
8.18	36.49	7.46	20.5	49.10	3.75	7.6	44.79	6.75	15.1	43.46	6.41	14.74
19.96	46.39	4.65	10.0	51.91	3.94	7.6	46.22	8.30	18.0	48.17	3.24	6.72
Taxifolin												
0.306	44.99	7.06	15.7	58.20	4.85	8.3	71.29	6.12	8.6	58.16	13.15	22.61
0.747	71.03	5.15	7.3	70.47	6.25	8.9	66.25	1.94	2.9	69.25	2.62	3.78
M1												
0.445	35.33	3.83	10.8	57.29	6.89	12.0	49.72	6.32	12.7	47.45	11.15	23.51
1.086	60.01	5.93	9.9	59.47	2.74	4.6	58.67	1.33	2.3	59.38	0.68	1.14
Ferulic acid												
5.83	47.19	8.07	17.1	39.75	3.47	8.7	54.83	6.07	11.1	46.82	7.58	16.19
14.24	60.01	5.93	9.9	45.84	3.22	7.0	52.38	6.24	11.9	48.47	3.45	7.13
Caffeic acid												
11.69	73.17	9.39	12.8	57.64	4.37	7.6	60.68	2.11	3.5	63.83	8.23	12.89
28.54	69.68	1.29	1.9	63.27	3.26	5.2	55.99	3.07	5.5	62.98	6.85	10.88

Matrix effects [%] of the five analytes in three different lots of human synovial fluid at two concentrations (low and high; n= 3). Listed are mean and SD of the ME of the analytes, prepared from 1 mL of synovial fluid matrix (n = 3) for each individual donor. The RSD of the mean of the three single donors represents the variability of the ME of an analyte in three different lots of human synovial fluid.

Analytes [ng/mL]	Matrix effects [%]									Mean Matrix effects [%] #1-3		
	Single donor #1			Single donor #2			Single donor #3					
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
8.18	-25.32	4.45	17.6	-33.50	1.23	3.7	-24.54	1.70	6.9	-27.79	4.97	17.87
19.96	-20.79	2.76	13.3	-24.93	2.71	10.9	-24.18	3.00	12.4	-23.30	2.21	9.47
Taxifolin												
0.306	60.28	3.00	5.0	44.79	4.03	9.0	48.31	6.27	13.0	51.13	8.12	15.87
0.747	65.33	5.72	8.8	58.39	3.93	6.7	53.44	6.3	11.8	59.05	5.97	10.11
M1												
0.445	227.15	17.73	7.8	268.07	9.08	3.4	236.27	3.91	1.7	243.83	21.48	8.81
1.086	246.29	15.73	6.4	257.68	3.74	1.5	207.15	7.51	3.6	237.04	26.50	11.18
Ferulic acid												
5.83	156.35	12.26	7.8	211.23	8.42	4.0	174.24	5.37	3.1	180.61	27.99	15.49
14.24	168.40	8.31	4.9	162.57	9.14	5.6	164.23	15.65	9.5	165.07	3.01	1.82
Caffeic acid												
11.69	28.01	1.49	5.3	25.58	1.70	6.7	25.32	2.27	9.0	26.30	1.49	5.65
28.54	32.32	4.43	13.7	32.50	2.20	6.8	23.26	2.62	11.3	29.36	5.28	18.00

IS-normalised matrix factor (MF) of the five analytes in three different lots of human synovial fluid at two concentrations (low and high; n= 3). Listed are mean and SD of the ME of the analytes, prepared from 1 mL of synovial fluid matrix (n = 3) for each individual donor. The RSD of the mean of the three single donors represents the variability of the IS-normalised MF of an analyte in three different lots of human synovial fluid.

Analytes [ng/mL]	IS-normalised MF									Mean IS-normalised MF #1-3		
	Single donor #1			Single donor #2			Single donor #3					
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
8.18	-0.216	0.03	14.8	-0.313	0.02	7.8	-0.226	0.01	6.0	-0.252	0.05	21.05
19.96	-0.179	0.03	15.9	-0.237	0.01	3.7	-0.227	0.02	10.9	-0.215	0.03	14.46
Taxifolin												
0.306	0.515	0.04	7.8	0.418	0.05	10.8	0.445	0.05	12.2	0.459	0.05	10.92
0.747	0.563	0.05	9.5	0.556	0.04	7.5	0.502	0.10	19.9	0.540	0.03	6.18
M1												
0.445	1.942	0.19	9.9	2.501	0.22	8.7	2.177	0.02	0.8	2.207	0.28	12.74
1.086	2.123	0.20	9.4	2.453	0.28	11.4	1.946	0.10	5.4	2.174	0.26	11.84
Ferulic acid												
5.83	1.336	0.13	9.9	1.971	0.07	3.4	1.606	0.07	4.1	1.638	0.32	19.45
14.24	1.452	0.07	4.9	1.547	0.19	12.3	1.542	0.11	6.9	1.514	0.05	3.56
Caffeic acid												
11.69	0.239	0.02	6.4	0.239	0.01	4.6	0.233	0.02	10.0	0.237	0.00	1.41
28.54	0.279	0.04	13.9	0.309	0.05	16.5	0.218	0.04	18.2	0.269	0.05	17.20

Process efficiency [%] of the five analytes in three different lots of human synovial fluid at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Process efficiency [%]									Mean Process efficiency [%]		
	Single donor #1			Single donor #2			Single donor #3			#1-3		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Catechin												
8.18	27.25	5.60	20.5	32.65	1.14	3.5	33.80	4.05	12.0	31.23	3.49	11.19
19.96	36.75	2.41	6.6	38.97	1.61	4.1	35.04	5.93	16.9	36.92	1.97	5.34
Taxifolin												
0.306	72.11	6.49	9.0	84.27	4.90	5.8	105.73	9.82	9.3	87.37	17.02	19.48
0.747	117.44	9.52	8.1	111.62	3.65	3.3	101.65	1.89	1.9	110.24	7.98	7.24
M1												
0.445	115.59	9.40	8.1	210.86	9.20	4.4	167.20	12.96	7.8	164.55	47.69	28.98
1.086	207.81	9.20	4.4	212.69	9.63	4.5	180.19	3.45	1.9	200.23	17.52	8.75
Ferulic acid												
5.83	117.64	3.21	2.7	123.72	5.25	4.2	150.36	5.78	3.8	130.57	17.40	13.33
14.24	126.66	6.68	5.3	120.36	6.66	5.5	138.41	10.88	7.9	128.48	9.16	7.13
Caffeic acid												
11.69	93.66	4.20	4.5	72.38	5.44	7.5	76.04	1.21	1.6	80.69	11.38	14.10
28.54	92.20	1.63	1.8	83.83	5.63	6.7	69.01	1.31	1.9	81.68	11.75	14.38

5.2.3 Crosstalk

Crosstalk with developed method in human pooled synovial fluid (n= 3)

Mono-spiked analytes	Other analytes					
	Catechin	Ferulic acid	M1	Taxifolin	Caffeic acid	IS
Catechin	-	108.77	122.17	121.41	123.70	n.d.
Taxifolin	80.99	88.60	96.63	-	86.77	n.d.
M1	117.47	120.25	-	119.33	119.61	n.d.
Ferulic acid	111.49	-	107.30	111.99	113.23	n.d.
Caffeic acid	47.13	92.32	90.19	80.01	-	n.d.

Calculation *Crosstalk* [%]:
(Mean (n= 3) peakarea analyt mono-spiked ULOQ-sample / Mean (n= 3) peakarea analyt matrix-blank)*100

Evaluation: ≤ 100% = no *Crosstalk*; > 100% = *Crosstalk, if co-elution*

5.2.4 Carry-over

Carry-over with developed method in human pooled synovial fluid (n= 3)

Analytes	I [%]	II [%]	III [%]	Mean ± SD [%]
Catechin	103.41	120.13	113.55	112.36 ± 8.42
Taxifolin	96.84	80.63	93.05	90.18 ± 8.48
M1	94.23	96.27	114.71	101.74 ± 11.28
Ferulic acid	92.88	94.49	96.60	94.66 ± 1.87
Caffeic acid	103.28	100.99	99.91	101.39 ± 1.72

Calculation c*arry-over* [%]:
(Peakarea 1st matrix-blank after ULOQ-sample / Peakarea matrix-blank before ULOQ-sample)*100

Evaluation: ≤ 100% = no c*arry-over*; > 100% = c*arry-over*

5.3 Method validation serum without prior enzymatic incubation

5.3.1 Levels of calibration standards

Calibration curve in human serum (without enzymatic incubation)

Analytes	Levels of calibration standards [ng/mL serum]*											
	L0	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Procyanidin B1	0.84	1.27	1.52	1.82	2.19	2.62	3.15	3.78	4.53	5.66	8.50	16.99
Catechin	2.36	3.54	4.24	5.09	6.11	7.33	8.80	10.56	12.67	15.83	23.75	47.50
Taxifolin	0.035	0.052	0.063	0.075	0.090	0.109	0.130	0.156	0.188	0.235	0.352	0.704
M1	0.047	0.071	0.085	0.102	0.122	0.147	0.176	0.211	0.253	0.317	0.475	0.950
Ferulic acid	1.86	2.78	3.34	4.01	4.81	5.77	6.92	8.31	9.97	12.46	18.69	37.39
Caffeic acid	1.86	2.79	3.35	4.01	4.82	5.78	6.94	8.33	9.99	14.49	18.73	37.46
Hydrocaffeic acid (IS)	10.35	10.35	10.35	10.35	10.35	10.35	10.35	10.35	10.35	10.35	10.35	10.35

* including the purity of the used standard substances according to the Certificate of Analysis from the manufacturer's batch.
The synthesized compound M1 were confirmed by NMR and LC/MS and equated with a purity of 95%.

	Level
LLOQ	L0 or L1
low	L6
mid	L8
high	L10

5.3.2 Linearity

Calibration range, calibration function and correlation coefficients of the six analytes extracted from human pooled serum without enzymatic incubation (n= 5).

Analytes	Range [ng/mL]	Slope ± SD	y-intercept	Correlation coefficient R
Procyanidin B1	1.27 - 16.99	0.004 ± 0.000	0.016	0.9973
(+)-Catechin	3.54 - 47.50	0.035 ± 0.013	-0.041	0.9973
M1	0.047 - 0.95	16.691 ± 1.905	-0.256	0.9982
Taxifolin	0.035 - 0.70	24.995 ± 1.817	-0.154	0.9986
Caffeic acid	1.86 - 37.46	6.117 ± 0.409	0.255	0.9980
Ferulic acid	1.86 - 37.39	1.683 ± 0.144	0.341	0.9978

5.3.3 LLOQ

Lower limit of quantification (LLOQ) and related accuracy of the six analytes extracted from human pooled serum without enzymatic incubation (n= 5).

Analytes	LLOQ [ng/mL]	Accuracy $_{LLOQ}$ [%] Mean ± SD
Procyanidin B1	1.27	102.70 ± 9.97
(+)-Catechin	3.54	104.03 ± 12.48
Taxifolin	0.035	105.39 ± 9.95
M1	0.047	111.14 ± 2.46
Ferulic acid	1.86	95.32 ± 11.54
Caffeic acid	1.86	107.97 ± 11.44

5.3.4 Accuracy and precision

Intraday accuracy and precision of the six analytes in human pooled serum without enzymatic incubation (n= 3).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Procyanidin B1			
1.27	1.30 ± 0.17	102.75	13.27
3.15	3.19 ± 0.32	101.41	9.99
4.53	4.35 ± 0.42	95.99	9.67
8.50	8.19 ± 0.65	96.44	7.91
Catechin			
3.54	3.83 ± 0.33	108.23	8.53
8.80	8.83 ± 0.60	100.36	6.78
12.67	12.63 ± 1.47	99.74	11.65
23.75	22.18 ± 2.04	93.41	9.19
M1			
0.047	0.045 ± 0.00	95.06	10.73
0.071	0.063 ± 0.00	88.51	0.73
0.176	0.161 ± 0.01	91.74	6.36
0.253	0.251 ± 0.02	99.20	8.49
0.475	0.470 ± 0.03	99.02	7.27
Caffeic acid			
1.86	1.99 ± 0.20	107.30	9.85
2.79	2.89 ± 0.33	103.67	11.38
6.94	7.19 ± 0.66	103.70	9.16
9.99	10.81 ± 0.60	108.18	5.54
18.73	18.95 ± 1.86	101.15	9.84
Taxifolin			
0.035	0.039 ± 0.00	110.48	7.18
0.052	0.057 ± 0.00	110.80	4.57
0.130	0.131 ± 0.01	101.09	6.54
0.188	0.199 ± 0.02	105.66	7.65
0.352	0.377 ± 0.03	106.99	8.06
Ferulic acid			
1.86	1.83 ± 0.22	98.88	12.03
2.78	2.52 ± 0.18	90.46	7.04
6.92	6.28 ± 0.67	90.67	10.69
9.97	9.81 ± 0.88	98.35	8.97
18.69	18.26 ± 2.56	97.68	14.01

Interday accuracy and precision of the analytes in human pooled serum without enzymatic incubation (n= 5).

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Procyanidin B1			
1.27	1.33 ± 0.02	105.11	1.72
3.15	3.18 ± 0.18	101.03	5.66
4.53	4.55 ± 0.22	100.43	4.78
8.50	8.37 ± 0.31	98.52	3.75
Catechin			
3.54	3.53 ± 0.21	99.86	5.93
8.80	8.89 ± 0.33	101.07	3.72
12.67	12.89 ± 0.75	101.79	5.79
23.75	22.80 ± 0.84	95.98	3.67
M1			
0.047	0.043 ± 0.00	90.85	3.46
0.071	0.067 ± 0.00	93.70	3.82
0.176	0.165 ± 0.01	93.75	5.20
0.253	0.244 ± 0.01	96.28	3.83
0.475	0.444 ± 0.02	93.58	4.24
Caffeic acid			
1.86	1.87 ± 0.07	100.67	3.93
2.79	2.77 ± 0.07	99.51	2.49
6.94	7.24 ± 0.26	104.30	3.56
9.99	10.43 ± 0.56	104.43	5.37
18.73	19.40 ± 1.32	103.57	6.81
Taxifolin			
0.035	0.038 ± 0.00	109.82	3.88
0.052	0.054 ± 0.00	103.56	5.48
0.130	0.135 ± 0.01	104.14	4.71
0.188	0.199 ± 0.00	105.69	1.38
0.352	0.369 ± 0.03	104.95	7.09
Ferulic acid			
1.86	1.83 ± 0.07	98.51	4.01
2.78	2.83 ± 0.20	101.43	7.09
6.92	6.50 ± 0.20	93.85	3.06
9.97	9.84 ± 0.58	98.66	5.89
18.69	17.79 ± 0.94	95.21	5.30

5.3.5 Recovery, matrix effects and process efficiency

Recovery, matrix effects and process efficiency in human pooled serum without enzymatic incubation at three concentrations (n= 5).

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]			Matrix effects [%]		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1									
3.15	3.04	0.26	8.62	0.97	0.12	12.31	-67.94	3.38	4.98
4.53	2.16	0.36	16.49	0.77	0.11	13.96	-64.33	3.64	5.65
8.50	1.59	0.14	9.02	0.61	0.03	5.20	-61.82	4.06	6.56
Catechin									
8.80	6.26	0.88	14.12	12.77	1.76	13.79	104.11	15.52	14.91
12.67	5.72	0.78	13.61	10.45	1.15	11.03	82.61	6.23	7.55
23.75	5.95	0.86	14.46	8.98	1.10	12.23	50.90	4.56	8.96
Taxifolin									
0.130	95.06	3.42	3.60	258.39	9.85	3.81	171.82	12.65	7.36
0.188	107.86	13.80	12.79	306.07	28.29	9.24	183.77	11.57	6.30
0.352	100.17	10.42	10.40	303.52	29.23	9.63	203.02	7.59	3.74
M1									
0.176	86.90	8.33	9.59	395.60	17.32	4.38	355.22	35.64	10.03
0.253	80.05	7.83	9.79	325.61	35.59	10.93	306.76	8.94	2.92
0.475	86.72	11.64	13.42	342.99	54.19	15.80	295.51	12.25	4.15
Ferulic acid									
6.92	85.49	8.44	9.88	221.50	7.56	3.41	159.10	21.84	13.73
9.97	96.25	9.14	9.49	248.58	15.24	6.13	158.27	9.20	5.81
18.69	115.03	10.99	9.56	295.73	33.52	11.33	157.10	7.66	4.87
Caffeic acid									
6.94	103.24	2.75	2.66	86.51	2.15	2.48	-16.21	0.56	3.48
9.99	100.84	7.29	7.23	84.67	5.27	6.23	-16.03	1.31	8.14
18.73	103.94	11.88	11.43	95.77	11.94	12.47	-7.86	1.11	14.10

Recovery, matrix effects and process efficiency in six individual lots of human serum (without enzymatic incubation) at two concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	Recovery [%]			Process efficiency [%]			Matrix effects [%]		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1									
3.15	2.88	0.59	20.62	1.12	0.30	27.02	-61.39	4.86	7.91
8.50	2.01	0.59	29.16	0.84	0.30	35.87	-58.89	4.49	7.63
Catechin									
8.80	6.11	1.10	18.02	12.53	2.76	22.04	103.97	12.69	12.20
23.75	7.24	0.80	11.12	10.92	1.29	11.84	50.92	5.59	10.97
Taxifolin									
0.130	101.24	10.19	10.07	281.94	47.31	16.78	176.97	19.00	10.73
0.352	99.49	12.26	12.32	311.06	58.62	18.85	210.57	21.59	10.25
M1									
0.176	79.37	7.75	9.77	312.44	31.06	9.94	293.66	8.97	3.05
0.475	83.49	3.53	4.23	318.28	17.84	5.60	281.16	11.53	4.10
Ferulic acid									
6.92	93.32	12.59	13.50	259.40	30.76	11.86	178.47	5.87	3.29
18.69	113.47	6.50	5.73	310.45	21.30	6.86	173.50	6.38	3.68
Caffeic acid									
6.94	100.42	9.61	9.57	82.70	8.31	10.05	-17.67	0.73	4.16
18.73	105.18	5.68	5.40	97.01	5.63	5.80	-7.78	0.97	12.44

Individual values of recovery of the six analytes extracted in six different lots of human serum (without enzymatic incubation) at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Recovery [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
3.15	3.48	0.50	3.67	0.19	3.03	0.31	2.45	0.37	2.33	0.08	2.34	0.21
8.50	2.82	0.12	2.70	0.07	1.67	0.29	1.50	0.24	1.79	0.27	1.59	0.10
Catechin												
8.80	4.53	0.64	6.90	0.22	6.74	0.63	6.83	0.59	6.79	0.98	4.86	0.22
23.75	7.56	0.76	7.79	0.72	7.51	0.27	7.17	1.08	7.72	1.13	5.65	0.75
Taxifolin												
0.130	117.69	6.02	100.96	2.18	107.44	3.60	98.49	3.98	93.66	6.90	89.18	3.87
0.352	113.06	11.55	110.83	1.53	107.64	2.28	88.31	1.21	86.57	2.97	90.56	3.00
M1												
0.176	64.80	2.52	80.76	2.15	80.57	4.61	80.86	3.18	80.88	1.10	88.33	5.27
0.475	78.19	4.36	88.78	8.69	82.19	2.50	84.44	5.93	85.06	5.15	82.29	5.26
Ferulic acid												
6.92	116.65	5.76	96.32	0.60	90.67	4.88	88.33	13.89	88.06	4.12	79.90	0.32
18.69	121.46	4.48	107.00	2.89	110.80	4.93	110.84	4.97	121.86	9.70	108.85	6.09
Caffeic acid												
6.94	117.28	6.79	88.91	3.79	101.40	5.53	95.80	4.20	96.30	4.70	102.82	5.71
18.73	112.10	4.83	99.31	5.77	104.26	2.31	103.27	3.28	100.05	3.14	112.10	5.18

Individual values of relative matrix effects of the six analytes extracted in six different lots of human serum (without enzymatic incubation) at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Matrix effects [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
3.15	-58.76	3.17	-60.34	7.04	-59.79	6.52	-58.93	1.86	-59.27	1.87	-71.23	5.17
8.50	-54.86	6.99	-57.62	7.05	-56.78	5.90	-58.13	3.54	-58.26	3.54	-67.71	1.40
Catechin												
8.80	90.85	1.19	110.30	4.75	126.18	8.93	100.10	13.27	101.18	12.17	95.22	8.12
23.75	43.63	6.60	60.54	4.47	49.63	5.12	51.64	0.85	48.22	7.52	51.88	5.65
Taxifolin												
0.130	200.59	14.83	185.67	9.97	193.23	14.24	169.31	5.15	159.00	2.88	154.05	4.92
0.352	225.89	11.15	227.34	6.49	236.53	2.46	189.38	2.35	194.74	21.05	189.55	3.68
M1												
0.176	289.55	14.68	298.57	7.31	288.26	13.52	290.97	10.62	309.50	14.17	285.08	21.05
0.475	275.29	4.76	290.30	10.05	280.43	18.33	269.88	7.32	271.72	7.91	299.31	12.82
Ferulic acid												
6.92	172.33	8.00	175.35	9.61	174.45	11.57	183.83	2.80	177.50	2.87	187.36	3.14
18.69	177.46	9.64	179.75	4.47	176.61	5.02	166.92	2.66	176.19	11.99	164.09	5.11
Caffeic acid												
6.94	-16.72	0.43	-17.56	1.01	-18.31	1.15	-18.60	0.72	-16.98	1.25	-17.87	1.78
18.73	-7.65	1.05	-9.66	1.21	-7.40	0.96	-7.67	0.84	-6.85	0.85	-7.46	0.58

IS (Hydrocaffeic acid) normalised MF in human pooled serum (without enzymatic incubation) at three concentrations (n= 5) and in six individual lots of human serum at two concentrations (n= 3).

Analytes and spiked concentration [ng/mL]	IS (hydrocaffeic acid) normalised MF at human pooled serum			IS (hydrocaffeic acid) normalised MF at six individual lots of human serum		
	Mean	± SD	RSD [%]	Mean	± SD	RSD [%]
Procyanidin B1						
3.15	4.25	0.56	13.10	3.86	0.36	9.36
4.53	-35.17	6.09	17.32			
8.50	-12.61	1.50	11.91	-11.65	1.25	10.72
Catechin						
8.80	-6.51	1.28	19.65	-6.55	0.97	14.78
12.67	45.16	7.61	16.84			
23.75	10.38	0.93	8.93	10.08	1.32	13.06
Taxifolin						
0.130	-10.75	0.50	4.69	-11.18	1.78	15.88
0.188	100.46	18.42	18.34			
0.352	41.42	5.11	12.33	41.49	3.46	8.33
M1						
0.176	-22.22	2.89	13.03	-18.48	1.27	6.90
0.253	167.69	18.23	10.87			
0.475	60.29	5.80	9.62	55.55	3.55	6.39
Ferulic acid						
6.92	-9.95	1.61	16.18	-11.22	0.52	4.64
9.97	86.52	15.03	17.37			
18.69	32.05	3.03	9.45	34.24	1.08	3.15
Caffeic acid						
6.94	1.01	0.12	12.13	1.11	0.06	5.50
9.99	-8.77	1.20	13.67			
18.73	-1.60	0.25	15.88	-1.54	0.19	12.65

Individual values of IS-normalised matrix factor (MF) of the six analytes extracted in six different lots of human serum (without enzymatic incubation) at two concentrations (low and high; n= 3).

Analytes [ng/mL]	IS-normalised MF [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
3.15	3.93	3.17	4.08	7.04	3.82	6.52	3.36	1.86	3.59	1.87	4.39	5.17
8.50	-10.04	6.99	-11.24	7.05	-11.24	5.90	-12.20	3.54	-11.41	3.54	-13.77	1.40
Catechin												
8.80	-6.07	1.19	-7.46	4.75	-8.06	8.93	-5.71	13.27	-6.13	12.17	-5.86	8.12
23.75	7.98	6.60	11.81	4.47	9.83	5.12	10.84	0.85	9.45	7.52	10.55	5.65
Taxifolin												
0.130	-13.40	14.83	-12.55	9.97	-12.34	14.24	-9.66	5.15	-9.63	2.88	-9.49	4.92
0.352	41.33	11.15	44.35	6.49	46.84	2.46	39.75	2.35	38.14	21.05	38.55	3.68
M1												
0.176	-19.35	14.68	-20.18	7.31	-18.41	13.52	-16.60	10.62	-18.75	14.17	-17.56	21.05
0.475	50.36	4.76	56.64	10.05	55.53	18.33	56.65	7.32	53.22	7.91	60.88	12.82
Ferulic acid												
6.92	-11.52	8.00	-11.85	9.61	-11.14	11.57	-10.49	2.80	-10.75	2.87	-11.54	3.14
18.69	32.47	9.64	35.07	4.47	34.97	5.02	35.04	2.66	34.51	11.99	33.37	5.11
Caffeic acid												
6.94	1.12	0.43	1.19	1.01	1.17	1.15	1.06	0.72	1.03	1.25	1.10	1.78
18.73	-1.40	1.05	-1.88	1.21	-1.47	0.96	-1.61	0.84	-1.34	0.85	-1.52	0.58

Individual values of process efficiency of the six analytes extracted in six different lots of human serum (without enzymatic incubation) at two concentrations (low and high; n= 3).

Analytes [ng/mL]	Process efficiency [%]											
	single donor #1		single donor #2		single donor #3		single donor #4		single donor #5		single donor #6	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Procyanidin B1												
3.15	1.43	0.16	1.45	0.20	1.22	0.22	1.01	0.14	0.95	0.02	0.67	0.10
8.50	1.27	0.17	1.14	0.16	0.72	0.04	0.63	0.07	0.75	0.07	0.51	0.01
Catechin												
8.80	8.64	1.17	14.50	0.35	15.24	0.79	13.66	0.56	13.66	2.18	9.49	0.54
23.75	10.86	0.72	12.51	1.03	11.23	0.16	10.88	1.57	11.45	1.13	8.59	1.01
Taxifolin												
0.130	353.76	12.31	288.41	4.26	315.05	16.34	265.24	12.88	242.58	16.40	226.57	7.99
0.352	368.44	26.03	362.80	10.59	362.25	8.79	255.54	1.42	255.16	9.91	262.21	6.44
M1												
0.176	252.43	12.17	321.91	5.88	312.83	23.52	316.13	17.02	331.20	12.29	340.14	17.46
0.475	293.45	19.92	346.49	30.03	312.66	16.14	312.31	23.48	316.17	23.52	328.61	14.75
Ferulic acid												
6.92	317.68	7.66	265.23	8.03	248.84	23.34	250.70	39.57	244.36	12.16	229.61	2.92
18.69	337.01	23.27	299.33	11.14	306.48	8.39	295.86	13.90	336.57	13.83	287.47	11.20
Caffeic acid												
6.94	97.67	5.31	73.30	2.20	82.84	5.41	77.98	2.74	79.95	2.78	84.45	3.47
18.73	103.53	4.97	89.72	4.67	96.54	2.23	95.35	2.25	93.20	3.14	103.73	4.18

5.3.6 Crosstalk

Crosstalk with developed method in human pooled serum (n= 3).

Mono-spiked analytes	Other analytes						
	Catechin	Ferulic acid	M1	Taxifolin	Caffeic acid	Procyanidin B1	IS
Catechin	-	98.81	44.85	117.57	119.81	93.90	n.d.
Ferulic acid	115.76	-	38.29	115.59	117.81	114.44	n.d.
M1	110.87	95.88	-	116.35	115.71	95.05	n.d.
Taxifolin	77.98	102.54	46.80	-	119.33	107.39	n.d.
Caffeic acid	107.69	96.16	24.38	119.93	-	96.66	n.d.
Procyanidin B1	123.72	81.29	39.47	118.59	106.77	-	n.d.

n.d.: not detected

Calculation *Crosstalk* [%]:
(Mean (n= 3) peakarea analyt mono-spiked ULOQ-sample / Mean (n= 3) peakarea analyt matrix-blank)*100

Evaluation: ≤ 100% = no *Crosstalk*; > 100% = *Crosstalk, if co-elution*

5.3.7 Carry-over

Carry-over with developed method in human pooled serum (n= 3).

Analyt	I [%]	II [%]	III [%]	IV [%]	V [%]	Mean ± SD [%]
Procyanidin B1	103.33	106.45	108.95	77.20	92.66	97.72 ± 13.04
Catechin	89.13	53.41	113.66	90.82	69.35	83.27 ± 22.91
Taxifolin	80.96	89.63	83.45	109.43	79.78	88.65 ± 12.22
M1	96.13	67.34	104.70	67.96	114.65	90.16 ± 21.56
Ferulic acid	97.99	96.14	103.92	97.94	102.705	99.74 ± 3.37
Caffeic acid	70.52	100.16	100.16	95.92	31.525	79.66 ± 29.60

Calculation c*arry-over* [%]:
(Peakarea 1st matrix-blank after ULOQ-sample / Peakarea matrix-blank before ULOQ-sample)*100

Evaluation: ≤ 100% = no c*arry-over*; > 100% = c*arry-over*

5.3.8 Robustness

Robustness of the developed method at two concentrations (n= 5) with human pooled serum (without enzymatic incubation) which has been previously contaminated with 1% human whole blood.

Analytes and spiked concentration [ng/mL]	Calculated concentration Mean ± SD [ng/mL]	Accuracy Mean [%]	Precision [%]
Procyanidin B1			
3.15	2.84 ± 0.19	90.35	6.80
8.50	8.40 ± 1.04	98.82	12.44
(+)-Catechin			
8.80	8.55 ± 1.05	97.20	12.26
23.75	23.94 ± 1.27	100.82	5.30
Taxifolin			
0.130	0.148 ± 0.00	113.93	1.75
0.352	0.397 ± 0.02	112.78	4.24
M1			
0.176	0.152 ± 0.00	86.38	0.95
0.475	0.420 ± 0.03	88.41	6.45
Ferulic acid			
6.92	6.19 ± 0.27	89.38	4.41
18.69	18.03 ± 1.87	96.47	10.38
Caffeic acid			
6.94	7.68 ± 0.50	110.63	6.45
18.73	20.50 ± 1.18	109.46	5.73

5.3.9 Stability

5.3.9.1 Short-term stability

Short-term stability in serum: stability of the analytes for one, two and four hours at room temperature (RT; n= 3).

Analytes and spiked concentration [ng/mL]	Shortterm stability in serum: 1 h - RT			Shortterm stability in serum: 2 h - RT			Shortterm stability in serum: 4 h - RT		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1									
3.15	2.20 ± 0.11	5.15	-30.11	1.50 ± 0.14	9.30	-52.27	1.70 ± 0.38	22.25	-46.08
8.50	5.72 ± 0.74	13.01	-32.62	4.53 ± 0.38	8.36	-46.72	4.68 ± 0.55	11.70	-44.93
(+)-Catechin									
8.80	7.08 ± 0.41	5.84	-19.56	7.69 ± 0.21	2.69	-12.54	6.23 ± 0.75	12.03	-29.22
23.75	19.79 ± 2.34	11.85	-16.67	17.49 ± 1.30	7.45	-26.35	15.15 ± 1.70	11.23	-36.22
Taxifolin									
0.130	0.153 ± 0.01	7.98	17.65	0.125 ± 0.01	6.94	-4.13	0.142 ± 0.01	4.33	9.24
0.352	0.418 ± 0.03	6.33	18.82	0.378 ± 0.04	9.79	7.49	0.376 ± 0.01	2.01	6.82
M1									
0.176	0.062 ± 0.01	10.31	-64.82	0.055 ± 0.00	8.22	-68.47	0.049 ± 0.01	13.04	-72.12
0.475	0.103 ± 0.01	8.52	-78.23	0.098 ± 0.00	0.82	-79.36	0.083 ± 0.01	9.43	-82.55
Ferulic acid									
6.92	8.38 ± 0.22	2.66	21.09	6.78 ± 1.73	25.58	-2.10	7.25 ± 0.48	6.58	4.77
18.69	21.83 ± 0.88	4.05	16.76	20.94 ± 2.30	10.99	12.00	20.17 ± 0.51	2.52	7.89
Caffeic acid									
6.94	7.64 ± 0.37	4.91	10.07	7.13 ± 0.59	8.23	2.82	7.73 ± 0.56	7.25	11.47
18.73	22.07 ± 0.18	0.81	17.81	18.51 ± 1.71	9.26	-1.17	20.44 ± 1.01	4.92	9.14

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Short-term stability in serum: stability of the analytes for one, two and four hours at 37 °C (n= 3).

Analytes and spiked concentration [ng/mL]	Shortterm stability in serum: 1 h - 37 °C			Shortterm stability in serum: 2 h - 37 °C			Shortterm stability in serum: 4 h - 37 °C		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1									
3.15	1.91 ± 0.16	8.32	-39.26	1.69 ± 0.12	7.14	-46.24	1.32 ± 0.11	8.52	-58.14
8.50	4.27 ± 0.28	6.52	-49.74	3.91 ± 0.53	13.64	-53.94	3.69 ± 0.06	1.66	-56.53
(+)-Catechin									
8.80	6.22 ± 0.55	8.85	-29.23	5.89 ± 0.07	1.15	-33.08	5.21 ± 0.49	9.37	-40.73
23.75	16.73 ± 0.30	1.77	-29.54	12.90 ± 0.92	7.11	-45.70	11.48 ± 1.36	11.82	-51.66
Taxifolin									
0.130	0.119 ± 0.01	6.18	-8.34	0.118 ± 0.01	11.45	-8.99	0.112 ± 0.00	3.98	-13.95
0.352	0.334± 0.04	10.55	-4.99	0.325 ± 0.01	1.72	-7.70	0.295 ± 0.01	2.49	-16.26
M1									
0.176	0.056 ± 0.00	4.63	-68.29	0.044 ± 0.00	0.38	-74.83	0.038 ± 0.00	2.73	-78.13
0.475	0.104 ± 0.01	9.16	-78.13	0.070 ± 0.00	4.12	-85.29	0.049 ± 0.00	3.93	-89.65
Ferulic acid									
6.92	6.44 ± 0.59	9.18	-6.99	7.17 ± 0.99	13.87	3.52	6.74 ± 0.33	4.97	-2.60
18.69	18.61 ± 1.09	5.86	-0.43	18.79 ± 1.37	7.30	0.52	20.01 ± 1.28	6.42	7.04
Caffeic acid									
6.94	7.67 ± 0.56	7.32	10.57	7.86 ± 0.27	3.45	13.28	6.32 ± 0.22	3.50	-8.90
18.73	19.68 ± 2.19	11.10	5.09	17.71 ± 0.73	4.14	-5.43	16.97 ± 0.27	1.58	-9.38

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

5.3.9.2 Long-term stability

Long-term stability in serum: stability of the analytes after freezing at -80 °C for one year (n= 3).

Analytes and spiked concentration [ng/mL]	Longterm stability in serum: -80 °C h/1 year - RT/1h		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1			
3.15	0.66 ± 0.08	12.18	-78.87
8.50	1.99 ± 0.48	24.17	-76.61
(+)-Catechin			
8.80	3.20 ± 0.61	18.94	-63.62
23.75	6.94 ± 1.62	23.27	-70.77
Taxifolin			
0.130	0.023 ± 0.00	14.66	-82.36
0.352	0.066 ± 0.00	14.13	-81.19
M1			
0.176	0.054 ± 0.00	9.79	-69.16
0.475	0.130 ± 0.01	5.22	-72.65
Ferulic acid			
6.92	5.29 ± 0.48	9.04	-23.65
18.69	15.70 ± 0.21	1.31	-16.00
Caffeic acid			
6.94	4.41 ± 0.60	13.59	-36.40
18.73	9.56 ± 0.68	7.09	-48.95

5.3.9.3 Freeze-thaw stability

Freeze-thaw stability in serum: stability of the analytes after three freeze-thaw cycle (n= 3).

Analytes and spiked concentration [ng/mL]	**Freeze-thaw stability in serum: 3 cycles -20 °C h/at least 12 h - RT/1h**		
	Calculated concentration Mean ± SD [ng/mL]	**RSD [%]**	**Δ [%][1] Mean ± SD**
Procyanidin B1			
3.15	1.60 ± 0.26	15.98	-49.18
8.50	3.76 ± 0.10	2.67	-55.78
(+)-Catechin			
8.80	6.09 ± 0.02	0.27	-30.73
23.75	15.43 ± 0.58	3.76	-35.01
Taxifolin			
0.130	0.113 ± 0.00	2.46	-13.41
0.352	0.322 ± 0.01	3.08	-8.60
M1			
0.176	0.042 ± 0.00	1.80	-76.29
0.475	0.062 ± 0.00	1.26	-86.86
Ferulic acid			
6.92	5.72 ± 0.04	0.70	-17.39
18.69	18.43 ± 0.70	3.82	-1.40
Caffeic acid			
6.94	7.87 ± 0.25	3.22	13.39
18.73	19.60 ± 0.58	2.96	4.64

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

5.3.9.4 Post-preparative stability

Post-preparative stability: autosampler stability of the analytes after 6 h and 12 h at room temperature (RT) after previous LC/MS/MS analysis (n= 5).

Analytes and spiked concentration [ng/mL]	Autosampler stability: 6 h - RT - in darkness			Autosampler stability: 12 h - RT - in darkness		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1						
1.27	1.04 ± 0.08	7.61	-18.16	1.05 ± 0.04	3.39	-16.86
3.15	2.72 ± 0.25	9.13	-13.49	2.58 ± 0.15	5.79	-18.15
4.53	3.94 ± 0.21	5.42	-13.10	3.67 ± 0.21	5.80	-18.99
8.50	7.26 ± 0.41	5.69	-14.52	6.95 ± 0.33	4.72	-18.19
(+)-Catechin						
3.54	3.20 ± 0.13	4.09	-9.61	3.09 ± 0.30	9.83	-12.69
8.80	8.33 ± 0.84	10.11	-5.35	8.45 ± 0.66	7.81	-3.97
12.67	11.58 ± 0.66	5.72	-8.57	11.03 ± 0.72	6.54	-12.94
23.75	22.01 ± 1.45	6.59	-7.33	20.95 ± 1.37	6.53	-11.79
Taxifolin						
0.052	0.063 ± 0.01	17.50	21.87	0.060 ± 0.00	2.28	15.86
0.130	0.130 ± 0.01	7.87	0.14	0.132 ± 0.01	7.60	1.24
0.188	0.173 ± 0.01	4.15	-8.18	0.171 ± 0.01	3.61	-8.87
0.352	0.335 ± 0.03	10.24	-4.87	0.331 ± 0.03	8.72	-5.92
M1						
0.071	0.069 ± 0.01	15.46	-2.52	0.064 ± 0.01	11.11	-9.48
0.176	0.153 ± 0.00	1.92	-13.34	0.156 ± 0.01	4.40	-11.60
0.253	0.214 ± 0.01	5.38	-15.57	0.211 ± 0.00	1.10	-16.48
0.475	0.410 ± 0.03	7.74	-13.62	0.414 ± 0.03	7.90	-12.74
Ferulic acid						
2.78	2.46 ± 0.08	3.13	-11.69	2.68 ± 0.26	9.76	-3.53
6.92	6.81 ± 0.87	12.83	-1.65	6.67 ± 0.57	8.61	-3.65
9.97	8.53 ± 0.28	3.34	-14.44	8.88 ± 0.59	6.61	-10.98
18.69	16.49 ± 0.70	4.23	-11.81	15.86 ± 0.74	4.67	-15.14
Caffeic acid						
2.79	3.03 ± 0.29	9.45	8.68	2.83 ± 0.22	7.72	1.67
6.94	7.65 ± 0.36	4.65	10.20	7.44 ± 0.43	5.82	7.20
9.99	9.95 ± 0.68	6.82	-0.44	10.21 ± 0.58	5.70	2.21
18.73	19.31 ± 2.04	10.55	3.09	19.21 ± 1.43	7.42	2.56

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

Post-preparative stability: stability of the analytes after one freeze-thaw cycle (n= 5).

Analytes and spiked concentration [ng/mL]	Freeze-thaw stability: 1 cycle -20 °C h/at least 12 h - RT/1h		
	Calculated concentration Mean ± SD [ng/mL]	RSD [%]	Δ [%][1] Mean ± SD
Procyanidin B1			
1.27	0.96 ± 0.04	4.05	-24.02
3.15	2.58 ± 0.23	8.86	-18.13
4.53	3.65 ± 0.20	5.43	-19.41
8.50	6.68 ± 0.39	5.79	-21.32
(+)-Catechin			
3.54	3.01 ± 0.30	10.05	-14.76
8.80	7.32 ± 0.51	6.95	-16.80
12.67	10.49 ± 0.59	5.62	-17.19
23.75	18.46 ± 0.88	4.75	-22.29
Taxifolin			
0.052	0.066 ± 0.01	8.37	26.42
0.130	0.134 ± 0.02	11.45	2.89
0.188	0.184 ± 0.01	6.99	-2.25
0.352	0.1353 ± 0.03	7.85	0.19
M1			
0.071	0.083 ± 0.00	4.62	17.42
0.176	0.186 ± 0.01	7.65	5.91
0.253	0.263 ± 0.03	9.86	4.14
0.475	0.479 ± 0.06	12.83	0.84
Ferulic acid			
2.78	2.59 ± 0.27	10.41	-7.06
6.92	6.34 ± 0.71	11.22	-8.38
9.97	9.19 ± 0.66	7.19	-7.85
18.69	17.68 ± 1.59	9.02	-5.40
Caffeic acid			
2.79	2.78 ± 0.25	9.00	-0.23
6.94	7.82 ± 0.37	4.69	12.77
9.99	10.40 ± 1.23	11.87	4.09
18.73	18.49 ± 2.29	12.40	-1.29

[1]: (calculated concentration mean ± SD [ng/mL] / (spiked concentration [ng/mL])-1)*100

5.4 Current human trial: Pycnogenol® administered to patients with severe osteoarthritis (knee)

Intervention (P+; n= 15) group with a multiple dose of 200 mg Pycnogenol®/day for three weeks is highlighted in green. Blood samples were collected before ingestion (V1; basal value); during intake (about 1-2 days before the surgery, V2) and after the last oral dose of Pycnogenol®, during or shortly before knee surgery (V3). Control (CO; n= 15) group without intervention is marked in grey. Samples of patients, which have not observed a polyphenol-free nutrition within the last two days before blood samplings, are listed in red.

5.4.1 Serum samples of the study participants

5.4.1.1 Quantification results of serum samples with enzymatic incubation

Patient	Blood sampling	Concentration [ng/mL] in serum				
		Catechin	M1	Taxifolin	Ferulic acid	Caffeic acid
#101 P+	V1	49.19			4.34	
	V2	39.76		0.095	3.76	
	V3	23.17	0.157	0.193	3.70	
#102 CO	V1	27.30			10.09	27.46
	V2	24.48			3.87	
	V3	40.40				
#103 P+	V1	22.45	1.271		3.11	
	V2	21.25	2.335	0.105	4.60	8.72
	V3	73.12			2.86	9.32
#104 CO	V1	23.18	0.461		4.84	
	V2	20.32	0.479		6.01	21.39
	V3	55.47				
#105 P+	V1	52.04	1.082		4.13	18.60
	V2	20.28	0.898	0.062	2.79	8.25
	V3	70.43	0.327		2.81	8.76
#106 CO	V1	64.75	2.423		8.84	
	V2	31.64	1.935		5.79	21.33
	V3	25.07				
#107 CO	V1	47.25	5.545		3.28	
	V2	15.37	2.115		2.97	
	V3	53.56	0.215			
#108 P+	V1	36.82	0.343	0.068	5.13	
	V2	25.19	2.371	0.125	4.35	9.32
	V3	51.33	0.385	0.080	2.74	
#109 P+	V1	46.15			8.75	87.27
	V2	18.45		0.197	3.08	
	V3	53.86	0.430		3.46	
#110 P+	V1	17.40			5.28	14.65
	V2	20.11	0.304		2.74	
	V3	18.78				
#131 CO (for #111)	V1	28.25			7.88	
	V2	42.71	0.655		6.56	11.64
	V3	73.09				
#112 CO	V1	22.07	9.079			
	V2	14.82	0.673			
	V3	15.12	1.382			

#113 P+	V1	8.97	0.818			
	V2	8.90	2.732	0.157		12.00
	V3	32.25	2.767	0.368	2.76	9.78
#114 CO	V1	52.20	0.933		13.04	63.35
	V2	40.71	0.631		5.17	12.29
	V3	37.24				
#115 CO	V1	7.68				
	V2	17.49			5.19	16.68
	V3	167.92				13.04
#116 CO	V1	10.11	0.212			12.56
	V2	9.40	1.027			
	V3	44.26	2.838			
#117 P+	V1	21.24				
	V2	13.21				
	V3	73.50		0.260	2.78	
#118 P+	V1	27.86				
	V2	19.05		0.216		
	V3	51.64		0.080		
#119 CO	V1	15.36	0.696		3.70	
	V2	42.78	1.108			8.59
	V3	17.19				
#120 P+	V1	29.72			10.49	
	V2	39.19	1.692			
	V3	34.97	0.308			
#121 P+	V1	9.05			2.89	
	V2	18.11	0.156			
	V3	61.27	0.159			
#122 P+	V1	17.10			3.04	
	V2	6.72				
	V3	70.97				
#132 CO (for #123)	V1	53.82			9.91	20.46
	V2	55.97				10.26
	V3	11.81				14.61
#124 P+	V1	7.18		0.074	5.36	18.01
	V2	52.15	0.358	0.127	6.35	30.11
	V3	60.76				
#133 CO (for #125)	V1	10.72	0.243			
	V2	13.57	0.554			
	V3	22.28	0.290			
#126 CO	V1				4.48	
	V2	14.08	0.330			
	V3	47.54				
#127 P+	V1	20.12	0.231		6.98	10.41
	V2	19.37	0.293		4.39	12.22
	V3	68.60	0.156			
#128 CO	V1	10.03	0.491			
	V2	10.26				
	V3	6.71				
#129 CO	V1	16.23	8.607		7.55	19.90
	V2	6.93	0.215			
	V3	70.16	0.642			16.86
#130 P+	V1	7.95	0.161			22.56
	V2	15.14	0.249			
	V3	43.29	0.164			

Procyanidin B1 was not identified in any serum sample of the study participants (in total n= 90) with prior enzymatic incubation.

5.4.1.2 Quantification results of serum samples without enzymatic incubation

Patient	Blood sampling	Concentration [ng/mL] in serum				
		Catechin	M1	Taxifolin	Ferulic acid	Caffeic acid
#101 P+	V1	15.36				
	V2	12.39				
	V3	15.36		0.036		
#102 CO	V1	9.20			2.74	3.25
	V2	7.50				1.97
	V3	21.61				
#103 P+	V1	7.45				2.79
	V2	8.74	0.054			
	V3	53.34				2.10
#104 CO	V1	13.87			1.87	3.04
	V2	8.80			1.91	
	V3	40.95				
#105 P+	V1	16.02				5.24
	V2	7.79				
	V3	29.62				1.87
#106 CO	V1	17.77	0.059		2.54	4.15
	V2	22.70	0.058		1.88	8.63
	V3	18.21				4.55
#107 CO	V1	14.43	0.072			2.28
	V2	7.47				1.93
	V3	40.20				
#108 P+	V1	14.14			2.66	
	V2	18.46				1.87
	V3	41.66				1.96
#109 P+	V1	26.60			3.48	8.90
	V2	12.47		0.033		
	V3	23.83				2.07
#110 P+	V1	8.21			1.86	4.70
	V2	12.73				3.01
	V3	12.28				3.50
#131 CO (for #111)	V1	16.01			1.87	2.45
	V2	19.78			2.45	6.14
	V3	54.29				2.51
#112 CO	V1		0.273			
	V2		0.047			
	V3		0.099			
#113 P+	V1		0.138			
	V2		0.084			
	V3	22.69	0.047			
#114 CO	V1	23.43			4.48	
	V2	11.55				2.77
	V3	27.57				

#115 CO	V1					
	V2	5.64				6.02
	V3	127.11				
#116 CO	V1	4.37				6.30
	V2					2.42
	V3	31.75	0.097			
#117 P+	V1					
	V2					
	V3	48.61				
#118 P+	V1	5.85				
	V2	10.75				
	V3	40.53				
#119 CO	V1	7.84	0.052			2.02
	V2	26.83	0.072			2.09
	V3	14.16				
#120 P+	V1	8.98			1.94	3.46
	V2	9.24				2.03
	V3	21.50				
#121 P+	V1					3.34
	V2	11.35				2.50
	V3	48.77				
#122 P+	V1	5.88				2.17
	V2					3.77
	V3	47.66				1.98
#132 CO (for #123)	V1	26.40	0.056		1.97	3.20
	V2	34.94				
	V3	4.22				
#124 P+	V1					4.56
	V2	27.20	0.071		2.00	8.88
	V3	44.92				2.85
#133 CO (for #125)	V1	4.05				
	V2	4.93	0.050			
	V3	15.83				
#126 CO	V1					
	V2	9.19			4.71	
	V3	29.90				
#127 P+	V1					4.09
	V2	8.68	0.050			4.11
	V3	48.47				
#128 CO	V1					
	V2	5.56				
	V3					
#129 CO	V1	11.01	0.109		3.32	3.53
	V2					2.57
	V3	41.82	0.055			
#130 P+	V1					2.20
	V2	6.93				
	V3	30.88				

Procyanidin B1 was not identified in any serum sample of the study participants (in total n= 90) without prior enzymatic incubation.

5.4.1.3 Individual degree of conjugation with sulfate and glucuronic acid

Since no free concentrations (serum without prior enzymatic incubation) were found in the same serum sample, these analytes were set to 100% conjugation degree.

Patient	Blood sampling	Conjugation degree [%]				
		Catechin	M1	Taxifolin	Ferulic acid	Caffeic acid
#101 P+	V1	68.78			100	
	V2	68.84		100	100	
	V3	33.71	100	81.27	100	
#102 CO	V1	66.29			72.82	88.16
	V2	69.37			100	
	V3	46.51				
#103 P+	V1	66.83	100		100	
	V2	58.88	97.71	100	100	100
	V3	27.05			100	77.51
#104 CO	V1	40.15	100		61.48	
	V2	56.68	100		68.31	100
	V3	26.16				
#105 P+	V1	69.21	100		100	71.81
	V2	61.60	100	100	100	100
	V3	57.94	100		100	78.69
#106 CO	V1	72.56	97.58		71.30	
	V2	28.25	96.98		67.51	59.54
	V3	27.38				
#107 CO	V1	69.47	98.71		100	
	V2	51.39	100		100	
	V3	24.94	100			
#108 P+	V1	61.61	100	100	48.19	
	V2	26.74	100	100	100	79.91
	V3	18.84	100	100	100	
#109 P+	V1	42.35			60.21	89.80
	V2	32.41		83.03	100	
	V3	55.76	100		100	
#110 P+	V1	52.83			64.77	67.92
	V2	36.70	100		100	
	V3	34.62				
#131 CO (for #111)	V1	43.32			76.25	
	V2	53.68	100		62.64	47.22
	V3	25.73				
#112 CO	V1	100	96.99			
	V2	100	93.07			
	V3	100	92.84			
#113 P+	V1	100	83.12			
	V2	100	96.93	100		100
	V3	29.62	98.31	100	100	100
#114 CO	V1	55.12	100		65.63	100
	V2	71.62	100		100	77.45
	V3	25.95				

#115 CO	V1	100				
	V2	67.74			100	63.88
	V3	24.30				100
#116 CO	V1	56.76	100			49.86
	V2	100	100			
	V3	28.26	96.60			
#117 P+	V1	100				
	V2	100				
	V3	33.86		100	100	
#118 P+	V1	78.99				
	V2	43.55		100		
	V3	21.52		100		
#119 CO	V1	48.92	92.46		100	
	V2	37.29	93.50			75.69
	V3	17.59				
#120 P+	V1	69.78			81.52	
	V2	76.42	100			
	V3	38.50	100			
#121 P+	V1	100			100	
	V2	37.31	100			
	V3	20.41	100			
#122 P+	V1	65.62			100	
	V2	100				
	V3	32.85				
#132 CO (for #123)	V1	50.95			80.12	84.34
	V2	37.58				100
	V3	64.28				100
#124 P+	V1	100		100	100	74.70
	V2	47.85	80.13	100	68.56	70.52
	V3	26.07				
#133 CO (for #125)	V1	62.25	100			
	V2	63.68	91.04			
	V3	28.94	100			
#126 CO	V1				100	
	V2	34.73	100			
	V3	37.11				
#127 P+	V1	100	100		100	60.72
	V2	55.19	82.87		100	66.39
	V3	29.35	100			
#128 CO	V1	100	100			
	V2	45.81				
	V3	100				
#129 CO	V1	32.16	98.73		56.01	82.28
	V2	100	100			
	V3	40.39	91.44			100
#130 P+	V1	100	100			90.25
	V2	54.21	100			
	V3	28.66	100			

5.4.2 Blood cell samples of the study participants

5.4.2.1 Quantification results of blood cell samples with enzymatic incubation

Patient	Blood sampling	Concentration [ng/mL] in serum			
		Catechin	M1	Taxifolin	Ferulic acid
#101 P+	V1	69.92		0.487	1.87
	V2	70.06	0.131	0.467	1.77
	V3	74.31		0.570	1.93
#102 CO	V1	29.11		0.643	1.19
	V2	62.53	0.165	0.185	
	V3	53.30		0.253	
#103 P+	V1	32.92	0.186	0.254	
	V2	284.61	0.231	0.275	0.99
	V3	84.96	0.148	0.192	
#104 CO	V1		0.146	0.289	
	V2	39.24		0.598	2.59
	V3			0.274	
#105 P+	V1	43.79	0.148	0.707	1.48
	V2	45.33	0.166	0.564	0.97
	V3	47.28	0.194	0.457	
#106 CO	V1		0.149	0.331	1.21
	V2	65.36	0.164	0.242	
	V3	36.25	0.146	0.257	
#107 CO	V1	29.19	0.172	0.427	0.99
	V2		0.168	0.414	
	V3	53.18		0.436	
#108 P+	V1	38.08	0.139	0.370	1.18
	V2	39.59	0.153	0.427	1.13
	V3	49.10	0.145	0.378	
#109 P+	V1	43.94	0.153	0.423	1.18
	V2	77.33	0.148	0.565	1.56
	V3	105.09	0.289	0.697	1.97
#110 P+	V1	29.97	0.151	0.229	
	V2	43.23	0.140	0.355	0.98
	V3	29.61	0.141	0.424	
#131 CO (for #111)	V1		0.139	0.348	1.40
	V2		0.168	0.412	2.35
	V3	31.35		0.510	
#112 CO	V1	60.54	0.407	0.715	2.07
	V2	179.77	0.182	1.064	2.43
	V3	90.04	0.168	1.064	2.12
#113 P+	V1	92.49	0.144	0.518	1.82
	V2	234.85	0.243	1.011	2.17
	V3	64.21	0.157	0.424	1.75
#114 CO	V1	54.47		0.368	1.66
	V2	51.30		0.435	1.74
	V3	112.13		0.617	1.62

#115 CO	V1	93.31		0.373	2.28
	V2	39.72		0.191	1.38
	V3	92.14		0.864	2.13
#116 CO	V1	141.34		0.734	1.72
	V2	151.28	0.201	1.142	2.29
	V3	142.17	0.229	1.232	2.12
#117 P+	V1	126.11		1.308	1.96
	V2	102.50		0.757	1.75
	V3	84.29	0.144	0.615	1.41
#118 P+	V1	101.03	0.312	0.490	2.78
	V2	93.93	0.486	0.802	3.14
	V3	113.63	0.337	0.752	2.06
#119 CO	V1	65.48	0.312	0.648	2.61
	V2	325.77	0.235	0.297	1.41
	V3	102.73	0.216	0.424	1.65
#120 P+	V1	44.45	0.242	0.357	2.02
	V2	54.18	0.287	0.525	1.72
	V3	45.60	0.178	0.246	1.24
#121 P+	V1	56.48	0.237	0.526	1.81
	V2	135.25	0.274	0.608	2.24
	V3	112.73	0.240	0.562	1.67
#122 P+	V1	75.33	0.188	0.253	1.77
	V2	73.83	0.196	0.291	1.39
	V3	86.36	0.193	0.916	2.18
#132 CO (for #123)	V1	62.04	0.182	0.270	1.64
	V2	84.01	0.275	0.519	2.34
	V3	87.90	0.283	0.493	2.99
#124 P+	V1			0.186	1.92
	V2	35.06		0.379	3.12
	V3	46.28		0.588	2.48
#133 CO (for #125)	V1			0.511	2.20
	V2		0.159	0.513	1.89
	V3	33.94		0.624	1.80
#126 CO	V1			0.178	1.40
	V2				
	V3	29.44		0.185	
#127 P+	V1				
	V2		0.126		1.00
	V3			0.138	
#128 CO	V1			0.142	
	V2			0.222	1.66
	V3	51.63		0.236	
#129 CO	V1		0.396	0.198	2.20
	V2			0.170	1.15
	V3			0.218	
#130 P+	V1		0.193	0.383	2.44
	V2	36.17	0.140	0.710	1.95
	V3	53.11	0.130	0.857	1.91

Caffeic acid and M2 were not identified in any blood cell sample of the study participants (in total n= 90) with prior enzymatic incubation.

5.4.2.2 Identification results of blood cell samples with enzymatic incubation

The calculated signal-to noise ratio (SNR) of the monitored intracellular metabolites of M1 in the blood cell samples of the study participants are listed after initially smoothing (function: Gaussian, 5 points) of the most abundant transition of the compound (M1-COOH: 225 > 123; M1-GSH: 514 > 385).

Patient	Blood sampling	Identification of intracellular metabolites of M1					
		M1-COOH	Hydroxy-benzoic acid	M1-acetylated	M1-Cystein	M1-GSH	M1-GSSG
#101 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#102 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	**SNR 5**	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#103 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	**SNR 3**	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	**SNR 4**	n.d.
#104 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#105 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#106 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#107 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#108 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#109 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#110 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#131 CO (for #111)	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#112 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#113 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#114 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

#115 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	**SNR 6**	n.d.	n.d.	n.d.	n.d.	n.d.
#116 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#117 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#118 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	**SNR 7**	n.d.	n.d.	n.d.	n.d.	n.d.
#119 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#120 P+	V1	**SNR 4**	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#121 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#122 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	**SNR 47**	n.d.	n.d.	n.d.	n.d.	n.d.
#132 CO (for #123)	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#124 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	**SNR 7**	n.d.	n.d.	n.d.	n.d.	n.d.
#133 CO (for #125)	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#126 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#127 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	**SNR 7**	n.d.	n.d.	n.d.	n.d.	n.d.
#128 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#129 CO	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#130 P+	V1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

5.4.3 Synovial fluid samples of the study participants: Quantification results with enzymatic incubation

The synovial fluid of the study participants (n= 30) was collected on the day of the surgery for knee replacement. Because of lack of sample material at two patients, it was quantified synovial fluid from total 28 study participants.

Patient	Concentration [ng/mL] in synovial fluid				
	Catechin	M1	Taxifolin	Ferulic acid	Caffeic acid
#101 P+		0.167	0.183	3.19	
#102 CO	2.65			3.38	8.42
#103 P+	2.65	0.125		5.03	12.53
#104 CO		0.145		2.95	8.67
#105 P+	3.32	0.240		3.42	12.98
#107 CO		0.189		3.14	7.27
#108 P+					
#109 P+	3.41	0.628		6.28	13.80
#110 P+	2.59			2.55	8.72
#131 CO (for #111)	5.24			3.96	17.18
#112 CO		1.648		3.22	8.90
#113 P+		1.950	0.231	4.02	6.11
#114 CO					19.62
#115 CO					14.12
#116 CO		1.141			4.56
#117 P+				2.36	
#118 P+				7.47	13.87
#119 CO					
#121 P+					
#122 P+					
#132 CO (for #123)				1.59	34.02
#124 P+					
#133 CO (for #125)					
#126 CO					
#127 P+					
#128 CO					
#129 CO					5.52
#130 P+					4.21

5.4.4 Calculated ratios of quantification results of each study participant between two matrices

Individual ratios of the quantification results of each study participant (blood sampling V3) between two matrices (with enzymatic incubation) were formed.

5.4.4.1 Ratio blood cells/serum

Patient	Ratio blood cells/serum			
	Catechin	M1	Taxifolin	Ferulic acid
#101 P+	3.207	-	2.950	0.522
#102 CO	1.319	-	-	-
#103 P+	1.162	-	-	-
#104 CO	-	-	-	-
#105 P+	0.671	0.593	-	-
#106 CO	1.446	-	-	-
#107 CO	0.993	-	-	-
#108 P+	0.957	0.378	4.737	-
#109 P+	1.951	0.672	-	0.569
#110 P+	1.577	-	-	-
#131 CO (for #111)	0.429	-	-	-
#112 CO	5.954	0.121	-	-
#113 P+	1.991	0.057	1.152	0.636
#114 CO	3.011	-	-	-
#115 CO	0.549	-	-	-
#116 CO	3.212	0.081	-	-
#117 P+	1.147	-	2.363	0.506
#118 P+	2.200	-	9.363	-
#119 CO	5.977	-	-	-
#120 P+	1.304	0.578	-	-
#121 P+	1.840	1.505	-	-
#122 P+	1.217	-	-	-
#132 CO (for #123)	7.441	-	-	-
#124 P+	0.762	-	-	-
#133 CO (for #125)	1.524	-	-	-
#126 CO	0.619	-	-	-
#127 P+	-	-	-	-
#128 CO	7.692	-	-	-
#129 CO	-	-	-	-
#130 P+	1.227	0.793	-	-

5.4.4.2 Ratio serum/synovial fluid

Patient	Ratio serum/synovial fluid				
	Catechin	M1	Taxifolin	Ferulic acid	Caffeic acid
#101 P+	-	0.937	1.055	1.160	-
#102 CO	15.269	-	-	-	-
#103 P+	27.627	-	-	0.569	0.744
#104 CO	-	-	-	-	-
#105 P+	21.202	1.364	-	0.823	0.675
#106 CO	-	-	-	-	-
#107 CO	-	1.138	-	-	-
#108 P+	-	-	-	-	-
#109 P+	15.784	0.684	-	0.550	-
#110 P+	7.245	-	-	-	-
#131 CO (for #111)	13.959	-	-	-	-
#112 CO	-	0.839	-	-	-
#113 P+	-	1.419	1.595	0.686	1.599
#114 CO	-	-	-	-	-
#115 CO	-	-	-	-	0.924
#116 CO	-	2.487	-	-	-
#117 P+	-	-	-	1.180	-
#118 P+	-	-	-	-	-
#119 CO	-	-	-	-	-
#120 P+	-	-	-	-	-
#121 P+	-	-	-	-	-
#122 P+	-	-	-	-	-
#132 CO (for #123)	-	-	-	-	0.430
#124 P+	-	-	-	-	-
#133 CO (for #125)	-	-	-	-	-
#126 CO	-	-	-	-	-
#127 P+	-	-	-	-	-
#128 CO	-	-	-	-	-
#129 CO	-	-	-	-	3.054
#130 P+	-	-	-	-	-

5.4.4.3 Ratio blood cells/synovial fluid

Patient	Ratio blood cells/synovial fluid			
	Catechin	M1	Taxifolin	Ferulic acid
#101 P+	-	-	3.112	0.605
#102 CO	20.145	-	-	-
#103 P+	32.099	1.183	-	-
#104 CO	-	-	-	-
#105 P+	14.232	0.809	-	-
#106 CO	-	-	-	-
#107 CO	-	-	-	-
#108 P+	-	-	-	-
#109 P+	30.799	0.459	-	0.313
#110 P+	11.425	-	-	-
#131 CO (for #111)	5.987	-	-	-
#112 CO	-	0.102	-	0.659
#113 P+	-	0.080	1.837	0.436
#114 CO	-	-	-	-
#115 CO	-	-	-	-
#116 CO	-	0.200	-	-
#117 P+	-	-	-	0.597
#118 P+	-	-	-	0.275
#119 CO	-	-	-	-
#120 P+	-	-	-	-
#121 P+	-	-	-	-
#122 P+	-	-	-	-
#132 CO (for #123)	-	-	-	1.876
#124 P+	-	-	-	-
#133 CO (for #125)	-	-	-	-
#126 CO	-	-	-	-
#127 P+	-	-	-	-
#128 CO	-	-	-	-
#129 CO	-	-	-	-
#130 P+	-	-	-	-

E SUMMARY

E Summary

1 Summary

Dietary polyphenols have been related to beneficial effects on humans' health. Pycnogenol®, a dietary polyphenol-rich food supplement complies with the monograph "Maritime pine extract" in the United States Pharmacopeia (USP) and has demonstrated effects in different diseases. Several human trials concerning knee osteoarthritis have shown significant improvement of the symptoms like reducing the pain and the stiffness of the joint(s) upon intake of Pycnogenol®. After oral intake of multiple doses of Pycnogenol® previously low concentrations in the nanomolar range of monomeric extract constituents have been found in human plasma as well as a bioactive metabolite, δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1), which is formed by the human intestinal flora from the procyanidins' catechin units. It is not clear yet which compound(s) of the complex extract is (are) mainly responsible for the described clinical effects of Pycnogenol®. To gain deeper insights into the *in vivo* fate of the pine bark extract the distribution of its constitutents and metabolites was closer investigated in the present thesis.

Initial *in vitro* experiments suggested a facilitated cellular uptake of M1 into human erythrocytes, possibly via GLUT-1 transporter. For elucidating further the *in vitro* and *in vivo* metabolism of M1 in human blood cells, a metabolomic approach was performed using UPLC-ESI-qTOF-MS^E analysis, which revealed a comprehensive and rapid metabolism of M1 to a variety of biotransformation products in human blood cells. Predominant metabolites were found to be conjugates of glutathione (GSH) isomers, namely M1-S-GSH and M1-N-GSH. Further sulfur-containing biotransformation products of M1 were conjugates with oxidized glutathione (M1-GSSG) and cysteine (M1-CYS) and the sulfated derivative of M1 (M1-sulfated). Other *in vitro* biotransformation products constituted the open-chained ester form of M1 (M1-COOH), hydroxybenzoic acid and the methylated (M1-methylated), acetylated (M1-acetylated), hydroxylated (M1-hydroxylated) and ethylated (M1-ethylated) derivatives of M1. Indeed, six of these *in vitro* metabolites, respectively M1-COOH, M1-sulfated, hydroxybenzoic acid, M1-S-GSH, M1-methylated and M1-acetylated, were also identified *in vivo* in blood cells of human volunteers after ingestion of Pycnogenol®. Related reference material was synthesized for reliable confirmation of the metabolites M1-GSH, M1-GSSG, M1-CYS and M1-COOH.

In the course of a randomized controlled clinical trial patients suffering from severe osteoarthritis ingested multiple doses of 200 mg/day Pycnogenol® for three weeks before they were scheduled for an elective knee replacement surgery. Various biological specimen, respectively blood cells, synovial fluid and serum samples, were to be analyzed to investigate

the distribution and disposition of possibly bioactive constituents and metabolites. Therefore, highly sensitive methods were developed using liquid chromatography tandem mass spectrometry (LC-MS/MS)- technology because of the expected low concentrations of the analytes in the related matrices.

Initially, for each matrix different sample preparation techniques (protein precipitation, liquid-liquid extraction, solid phase extraction and useful combinations thereof) were compared to achieve maximum detection sensitivity of the analytes that were of highest interest, namely M1, ferulic acid and taxifolin. By comparing 32 various sample clean-up procedures in human serum, the highest recovery of the metabolite M1 was achieved using a liquid-liquid extraction with ethyl acetate and *tert*-butyl methyl ether at a serum pH-value of 3.2. A similar extraction method was also chosen for analyte detection in human synovial fluid after comparing 31 different sample preparation techniques. Whole blood or blood cells are difficult to handle because of their high viscosity and strong coloration. The QuEChERS (quick, easy, cheap, effective, rugged and safe) approach which was originally developed for the food safety and thus for the determination of pesticide residues in fruits and vegetables yielded the highest total recovery rate of M1 in human blood cells when assessing 18 different sample clean-up techniques. By applying the QuEChERS method for the first time for the simultaneous and highly sensitive quantification of selected polyphenols in human blood cells it was demonstrated that this fast and inexpensive technique can be applied in clinical fields for cleaning-up highly complex and thus challenging biological matrices. All developed methods for the different biological specimen were optimized to achieve maximum sensitivity of the target analytes. The determined lower limits of quantification (LLOQs) were sufficient for the quantification of the study samples. The LLOQs ranged from 113 pg/mL for taxifolin to 48 ng/mL for caffeic acid in blood cells and from 80 pg/mL for taxifolin to 3 ng/mL for caffeic acid in synovial fluid. In human serum the LLOQs even ranged down to 35 pg/mL for taxifolin and up to 8 ng/mL for caffeic acid. All analytical methods were subjected to a full validation according to current EMA and FDA guidelines and fulfilled those criteria, showing excellent performance and reliability of the developed and optimized methods.

Serum, blood cells and synovial fluid samples of the osteoarthritis patients were all processed with an enzymatic incubation with ß-glucuronidase/sulfatase to hydrolyse conjugates (phase-II-metabolism) prior the actual sample preparation. Additionally, serum samples of the osteoarthritis patients were prepared without enzymatic hydrolysis to determine the individual degree of conjugation with sulfate and glucuronic acid of the analytes.

All determined concentrations in the patients' samples were in the lower ng/mL range. Notably, highest total concentrations of the polyphenols were not detected in serum, in which the degree of analyte conjugation with sulfate and glucuronic acid ranged from 54.29 ± 26.77% for catechin to 98.34 ± 4.40% for M1. The flavonoids catechin and taxifolin mainly

partitioned into blood cells, whereas the metabolite M1, ferulic and caffeic acid primarily resided in the synovial fluid. The concentration of M1 in the blood cells was low, however, this could be explained by the previously observed extensive and rapid intracellular metabolism *in vitro*. This was now supported by the *in vivo* evidence in samples of patients who received Pycnogenol® in which the open-chained ester form of M1 (M1-COOH) as well as the glutathione conjugate of M1 (M1-GSH) were identified, indicating that M1 does not accumulate in its original form *in vivo*. Possibly, a variety of bioactive metabolites exist which might play an important role for the clinical effects of Pycnogenol®.

Although the study participants were requested to avoid polyphenol-rich food and beverages within the last two days before the blood samplings this was obviously difficult for most of the patients. Hence, no statistically significantly difference was observed in the mean polyphenol concentrations in serum, blood cells and synovial fluid between the intervention and the control group. Nevertheless, it was possible to identify marker compounds for Pycnogenol® intake under real life conditions with occasional or regular consumption of polyphenol-rich foods and beverages. Thereby, ferulic acid was found in serum samples exclusively after intake of Pycnogenol®, confirming that ferulic acid is a suitable marker of consumption of French maritime pine bark extract. Taxifolin was present in serum and synovial fluid exclusively in the intervention group indicating a role as further marker of Pycnogenol® intake. Taxifolin, ferulic acid and caffeic acid were detected in both serum and synovial fluid only in the intervention group. Moreover, the metabolite M1, taxifolin and ferulic acid were only detected simultaneously in all matrices (serum, blood cells and synovial fluid) after ingestion of Pycnogenol®.

Thus, deeper insights into the distribution of bioactive constituents and metabolites of Pycnogenol® into serum, blood cells and synovial fluid after oral administration to patients with severe osteoarthritis were gained. The present study provides the first evidence that polyphenols indeed distribute into the synovial fluid of patients with osteoarthritis where they might contribute to clinical effects.

2 Zusammenfassung

Polyphenole in Nahrungsmitteln werden mit positiven Wirkungen auf die menschliche Gesundheit in Verbindung gebracht. Pycnogenol®, ein polyphenolreiches Nahrungsergänzungsmittel, welches der Monographie "*Maritime Pine Extract*" im US-Amerikanischen Arzneibuch (*United States Pharmacopeia*, USP) entspricht, wurde bereits Effekte bei verschiedenen Krankheiten zugeschrieben. Eine orale Einnahme von Pycnogenol® hat in mehreren Humanstudien, welche sich mit Arthrose am Knie beschäftigt haben, eine signifikante Verbesserung der Symptome wie die Reduzierung von Schmerzen und der Steifheit des Gelenks gezeigt. Nach Mehrfacheinnahmen von Pycnogenol® wurden im menschlichen Plasma bereits niedrige Konzentrationen (im nanomolaren Bereich) von monomeren Extraktbestandteilen gefunden sowie ein bioaktiver Metabolit, δ-(3,4-Dihydroxyphenyl)-γ-Valerolacton (M1), welcher durch die menschliche Darmflora aus den Catechin-Einheiten der Procyanidine gebildet wird. Bis jetzt ist noch unklar, welche Verbindung(en) des komplexen Extraktes für die beschriebenen klinischen Wirkungen von Pycnogenol® hauptsächlich verantwortlich ist (sind). Um einen tieferen Einblick in das *in vivo* Verhalten des Kiefernrindenextraktes zu gewinnen, wurde in der vorliegenden Arbeit die Verteilung von Bestandteilen und Metaboliten des Extraktes näher untersucht.

Erste *in vitro* Experimente wiesen auf eine erleichterte zelluläre Aufnahme von M1 in menschliche Erythrozyten hin, möglicherweise vermittelt über den GLUT-1-Transporter. Um den *in vitro* und *in vivo* Metabolismus von M1 in menschlichen Blutzellen weiter aufzuklären, wurden metabolomische Untersuchungen mittels UPLC-ESI-qTOF-MS-Analyse durchgeführt, welche eine umfassende und schnelle Metabolisierung von M1 in menschlichen Blutzellen zu einer Vielzahl von Biotransformationsprodukten zeigten. Die Hauptmetabolite waren Konjugate von Glutathion(GSH)-Isomeren, nämlich M1-S-GSH und M1-N-GSH. Daneben entstanden schwefelhaltige Biotransformationsprodukte von M1, nämlich Konjugate mit oxidiertem Glutathion (M1-GSSG) und Cystein (M1-CYS) sowie ein Derivat von M1 mit Sulfat (M1-sulfatiert). Andere *in vitro* Biotransformationsprodukte waren die offenkettige Esterform von M1 (M1-COOH), Hydroxybenzoesäure, die methylierte (M1-methyliert), acetylierte (M1-acetyliert), hydroxylierte (M1-hydroxyliert) und ethylierte (M1-ethyliert) Form von M1. Sechs dieser *in vitro* Metabolite, nämlich M1-COOH, M1-sulfatiert, Hydroxybenzoesäure, M1-S-GSH, M1-methyliert und M1-acetyliert, wurden tatsächlich auch *in vivo* in humanen Blutzellen von freiwilligen Spendern identifiziert, welche zuvor Pycnogenol® oral eingenommen hatten. Für eine zuverlässige Bestätigung der Metaboliten M1-GSH, M1-GSSG, M1-CYS und M1-COOH wurde entsprechendes Referenzmaterial synthetisiert.

Im Rahmen einer randomisiert-kontrollierten Studie wurden Patienten, welche an einer schweren Arthrose litten, eine orale Mehrfachdosis von 200 mg Pycnogenol® pro Tag über drei Wochen hinweg verabreicht, bevor sich diese anschließend einer notwendigen

Kniegelenksersatz-Operation unterzogen. Um das Auftreten und die Verteilung von möglichen bioaktiven Bestandteilen und Metaboliten zu untersuchen, wurden verschiedene biologische Flüssigkeiten, nämlich Serum, Blutzellen und die Gelenkflüssigkeit analysiert. Da sehr geringe Konzentrationen der Analyten in den einzelnen Matrizes erwartet wurden, waren hochempfindliche Methoden erforderlich. Daher wurde Flüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie (LC-MS/MS) eingesetzt.

Zunächst wurden unterschiedliche Probenvorbereitungstechniken (Proteinfällung, Flüssig-Flüssig-Extraktion, Festphasenextraktion und sinnvolle Kombinationen davon) für jede Matrix verglichen, um eine maximal empfindliche Detektion der wichtigsten Analyten, nämlich M1, Ferulasäure und Taxifolin, zu erzielen. Durch den Vergleich von 32 verschiedenen Probenaufarbeitungen in humanem Serum wurde die höchste Wiederfindung des Metaboliten M1 unter Verwendung einer Flüssig-Flüssig-Extraktion mit Essigsäureethylester und Methyl-tert-butylether bei einem pH-Wert im Serum von 3,2 erreicht. Zum Nachweis der Analyten in der humanen Gelenkflüssigkeit wurde nach einem Vergleich von 31 verschiedenen Probenaufarbeitungen eine ähnliche Extraktion angewandt. Aufgrund der hohen Viskosität und der starken Färbung ist die Aufarbeitung von Vollblut oder Blutzellen sehr anspruchsvoll. Das QuEChERS (quick, easy, cheap, effective, rugged and safe) Verfahren, welches ursprünglich für die Lebensmittelüberwachung zur Bestimmung von Pestizidrückständen in Obst und Gemüse entwickelt wurde, ergab bei der Bewertung von 18 Probenaufarbeitungstechniken die höchste Gesamtwiederfindungsrate von M1 in menschlichen Blutzellen. Durch die erstmalige Anwendung von QuEChERS zur hochempfindlichen und simultanen Quantifizierung von ausgewählten Polyphenolen in menschlichen Blutzellen wurde gezeigt, dass diese schnelle und kostengünstige Methode durchaus auch in klinischen Bereichen zur Aufreinigung von sehr komplexen und anspruchsvollen biologischen Matrizes angewendet werden kann. Alle entwickelten Methoden wurden umfassend optimiert um eine maximal empfindliche Quantifizierung der Analyten zu erhalten. Die ermittelten unteren Bestimmungsgrenzen (*lower limit of quantification*, LLOQ) waren ausreichend für die Quantifizierung der Studienproben. Die LLOQs reichten in humanen Blutzellen von 113 pg/mL für Taxifolin bis 48 ng/mL für Kaffeesäure und in der menschlichen Gelenkflüssigkeit von 80 pg/mL für Taxifolin bis hin zu 3 ng/mL für Kaffeesäure. In humanem Serum bewegten sich die Bestimmungsgrenzen sogar bis zu 35 pg/mL für Taxifolin und bis zu 8 ng/mL für Kaffeesäure. Alle analytischen Methoden wurden einer *„Full Validation“* nach den gegenwärtigen EMA- und FDA-Richtlinien unterzogen und erfüllten deren Kriterien, was eine hervorragende Leistungsfähigkeit und Zuverlässigkeit der entwickelten und optimierten Methoden bewies.

Serum-, Blutzell- und Gelenkflüssigkeitsproben der Arthrose-Patienten wurden einer enzymatischen Inkubation mit ß-Glucuronidase/Sulfatase unterworfen, um die konjugierten (Phase-II-Metabolismus) Verbindungen vor der eigentlichen Probenvorbereitung zu hydrolysieren. Die Serumproben der Studienteilnehmer wurden zusätzlich noch ohne

enzymatische Hydrolyse aufgearbeitet, um den individuellen Grad der Analytkonjugation mit Sulfat und Glucuronsäure zu bestimmen.

Alle ermittelten Konzentrationen in den Patientenproben lagen im unteren ng/mL-Bereich. Bemerkenswerterweise wurden die höchsten Gesamtkonzentrationen der Polyphenole nicht in Serum, in welchem der Grad der Analytkonjugation mit Sulfat und Glucuronsäure von 54,29 ± 26,77 % für Catechin bis 98,34 ± 4,40 % für M1 reichte, bestimmt. Die beiden Flavonoide Catechin und Taxifolin verteilten sich vor allem in die Blutzellen, während der Metabolit M1, Ferulasäure und Kaffeesäure in erster Linie in Gelenkflüssigkeit zu finden war. Die Konzentration von M1 in den Blutzellen war gering, was durch den zuvor beobachteten umfangreichen und schnellen intrazellulären *in vitro* Metabolismus erklärt werden konnte. Durch den *in vivo* Nachweis der offenkettigen Esterform von M1 (M1-COOH) als auch des Glutathion-Konjugats von M1 (M1-GSH) in Proben von Patienten, welche zuvor Pycnogenol® eingenommen hatten, konnte dies bestätigt werden. Dies deutet darauf hin, dass M1 *in vivo* nicht in der ursprünglichen Form akkumuliert und möglicherweise eine Vielzahl von biologisch aktiven Metaboliten vorliegt, was für die klinische Wirkung von Pycnogenol® eine wichtige Rolle spielen könnte.

Obwohl die Studienteilnehmer darum gebeten wurden in den letzten zwei Tagen vor den Blutentnahmen weitestgehend auf polyphenolreiche Nahrungsmittel und Getränke zu verzichten, war die Umsetzung für die meisten der Patienten doch sehr schwierig. Daher wurden keine statistisch signifikanten Unterschiede zwischen der Interventions- und der Kontrollgruppe in den mittleren Polyphenolkonzentrationen im Serum, Blutzellen und in der Gelenkflüssigkeit beobachtet. Dennoch war es möglich, einige Markerverbindungen für eine Aufnahme von Pycnogenol® unter Alltagsbedingungen mit gelegentlichem oder regelmäßigem Konsum von polyphenolreichen Lebensmitteln und Getränken zu identifizieren. So wurde Ferulasäure nur in Serumproben nach der Einnahme von Pycnogenol® gefunden, was bestätigt, dass Ferulasäure ein geeigneter Marker für die Einnahme des Kiefernrindenextraktes ist. Taxifolin wurde ausschließlich im Serum und Gelenkflüssigkeit der Interventionsgruppe nachgewiesen, was auf einen weiteren Marker der Pycnogenol®-Einnahme hindeutet. Taxifolin, Ferulasäure und Kaffeesäure wurden nur in der Interventionsgruppe in den beiden Matrizes Serum und Gelenkflüssigkeit nachgewiesen. Darüber hinaus wurde das gleichzeitige Vorhandensein des Metaboliten M1, Taxifolin und Ferulasäure in allen Körperflüssigkeiten (Serum, Blutzellen und Gelenkflüssigkeit) nur nach einer Aufnahme von Pycnogenol® festgestellt.

Somit konnten tiefere Einblicke in die Verteilung von bioaktiven Inhaltsstoffen und Metaboliten von Pycnogenol® in Serum, Blutzellen und Gelenkflüssigkeit nach oraler Verabreichung an Patienten mit schwerer Arthrose gewonnen werden. Die vorliegende Studie liefert den ersten Beweis dafür, dass sich Polyphenole durchaus in die Gelenkflüssigkeit von Patienten mit Osteoarthritis verteilen, in welcher diese möglicherweise zu klinischen Effekten beitragen können.

F LIST OF ABBREVATIONS

F List of Abbreviations

AGEs	advanced glycation end products
APCI	atmospheric pressure chemical ionization
BBB	blood-brain barrier
CE	capillary electrophoresis
CHD	coronary heart disease
COX	cyclooxygenase
CV	coefficient of variation
CVD	cardiovascular disease
CVI	chronic venous insufficiency
DAD	diode array detector
ECD	electrochemical detection
EGCG	epigallocatechin gallate
EMA	*European Medicines Agency*
ESI	electrospray ionization
FD	fluorescence detection
FDA	*Food and Drug Administration*
GC	gas chromatography
GLUT	glucose transporter(s)
HDL	high-density-lipoprotein
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
HR	high resolution
IL	interleukin
IS	internal standard(s)
LC	liquid chromatography
LDL	low-density-lipoprotein
LLE	liquid-liquid-extraction
LLOQ	lower limit of quantification
LOD	limit of detection
LOX	lipoxygenase
LPS	lipopolysaccharide
m/z	mass-to-charge ratio
MAPK	mitogen-activated protein kinases
ME	matrix effect(s)
MF	matrix factor
MMP	matrix metalloproteinasen
MNF	matrix normalization factor
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NF-κB	nuclear factor-Kappa B
NOS	nitric oxide synthase

NP	normal phase
NSAIDs	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
PE	process efficiency
pKa	logarithmic acid dissociation constant
PPT	protein precipitation
QC	quality control (standard)
qTOF	quadrupole time-of-flight
QuEChERS	*Quick ,Easy, Cheap, Effective, Rugged and Safe*
RBC	red blood cells
RE	recovery
ROS/RNS	reactive oxygen and/or nitrogen species
RP	reversed phase
RSD	relative standard deviation
RT	retention time
SAC	standard addition calibration
SD	standard deviation
SF	synovial fluid
SIL-IS	stable isotope labeled internal standard
SL	stock solution
SNR	signal-to-noise-ratio
SPE	solid-phase-extraction
SweEt	*Swedish Ethyl acetate*
TLC	thin-layer chromatography
TNF	tumor necrose-factor
TOF	time-of flight
TQ	triple quadrupole
UHPLC	ultra (high) pressure liquid chromatography
ULOQ	upper limit of quantification
USP	*United States Pharmacopeia*
UV	ultraviolet
VIS	visible spectrum
WOMAC	*Western Ontario and McMaster Universities Osteoarthritis Index*

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Documentation of Authorship

This section contains a list of the individual contribution for each author to the publications reprinted in this thesis. Unpublished manuscripts are handled, accordingly.

P1	***Facilitated uptake of a bioactive metabolite of maritime pine bark extract (Pycnogenol) into human erythrocytes*** Kurlbaum, M., Mülek, M. and Högger P. *PLoS One*, 2013. 8: e63197 DOI: 10.1371/journal.pone.0063197

Author	**Kurlbaum**	**Mülek**	**Högger**
Distribution of polyphenols between human plasma and erythrocytes	x		
Structural comparison between M1 and glucose	x		
Screening of erythrocyte incubation mixtures for putative M1 metabolites	x		
Analysis of protection against oxidative damage using the AAPH assay	x		
Uptake of M1 into human erythrocytes		x	
Study design/concept development	x	x	x
Data analysis and interpretation	x	x	x
Manuscript planning			x
Manuscript writing	x	x	x
Correction of manuscript	x	x	x
Supervision			x

P2	***Highly sensitive analysis of polyphenols and their metabolites in human blood cells using dispersive SPE extraction and LC-MS/MS*** Mülek, M. and Högger, P. *Anal Bioanal Chem*, 2015. 407: 1885-1899. DOI 10.1007/s00216-014-8451-y.

Author	**Mülek**	**Högger**
Method development	x	
Method optimization	x	
Method validation	x	
Sample preparation of clinical samples	x	
Study design/concept development	x	x
Data analysis and interpretation	x	x
Manuscript planning	x	x
Manuscript writing	x	x
Correction of manuscript	x	x
Supervision		x

P3	***Profiling a gut microbiota-generated catechin metabolite's fate in human Blood cells using a metabolomic approach*** Mülek, M., Fekete, A., Wiest, J., Holzgrabe, U., Mueller, MJ. and Högger, P. *J Pharm Biomed Anal*, 2015. 114: 71-81 DOI: 10.1016/j.jpba.2015.04.042

Author	**Mülek**	**Fekete**	**Wiest**	**Holzgrabe**	**Mueller**	**Högger**
in vitro incubation experiments	x					
in vivo incubation experiments	x					
Performing and evaluation of UPLC-ESI-qTOF-MS analysis	x	x				
Synthesis of reference materials	x					
Performing and evaluation of ^{1}H NMR of M1-GSH adduct			x			
Study design/concept development	x	x			x	x
Data analysis and interpretation	x	x	x	x	x	x
Manuscript planning	x	x				x
Manuscript writing	x	x	x			x
Correction of manuscript	x	x	x	x	x	x
Supervision				x	x	x

P4	***Development of LC-ESI/MS/MS methods for quantification of polyphenols in human plasma and serum with particular consideration of matrix effects*** Mülek, M. and Högger, P. *in preparation (unpublished)*

Author	**Mülek**	**Högger**
Method development	x	
Method optimization	x	
Method validation	x	
Sample preparation of clinical samples	x	
Study design/concept development	x	x
Data analysis and interpretation	x	x
Manuscript planning	x	x
Manuscript writing	x	x
Correction of manuscript	x	x
Supervision		x

P5	***Distribution of constituents and metabolites of maritime pine bark extract (Pycnogenol®) into serum, blood cells and synovial fluid of patients with severe osteoarthritis*** Mülek, M., Seefried, L., Genest, F. and Högger, P. *in preparation (unpublished)*

Author	**Mülek**	**Seefried**	**Genest**	**Högger**
Development, optimization and validation of all analytical LC-MS/MS methods	x			
Sample preparation of all clinical samples	x			
Analysis of all study samples	x			
Patient management		x		
Patient coordination		x		
Collection and documentation of patient samples and data		x	x	
Study design/concept development		x		x
Data analysis and interpretation	x			x
Manuscript planning	x			x
Manuscript writing	x			x
Correction of manuscript	x	x		x
Supervision		x		x

ERKLÄRUNG ZU DEN EIGENANTEILEN DES DOKTORANDEN SOWIE DER WEITEREN DOKTORANDEN ALS KOAUTOREN AN PUBLIKATIONEN UND ZWEITPUBLIKATIONS-RECHTEN BEI EINER KUMULTIVEN DISSERTATION

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Die Beiträge der Mitautoren an den Publikationen sind in den vorausgehenden Tabellen aufgeführt.

Würzburg, den …… 2015

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Prof. Dr. Petra Högger

Würzburg, den …… 2015

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Melanie Mülek