Center of Drug Absorption and Transport, Department of Biopharmaceutics and Pharmaceutical Technology, University of Greifswald, Germany

Metabolism of baicalin by different microbiota determined by MimiCol³

D.-S. Seradj, L. Neubauer, S. Senekowitsch, W. Weitschies, P. Schick[1,*]

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**Corresponding author: Philipp Schick, Center of Drug Absorption and Transport, Department of Biopharmaceutics and Pharmaceutical Technology, University of Greifswald, Felix-Hausdorff-Str. 3, D-17489 Greifswald, Germany philipp.schick@uni-greifswald.de*

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Substances metabolised by the intestinal microbiota can be used as colon markers and are gaining importance. The flavonoid glycoside baicalin has been described in the literature to be metabolised by the intestinal microbiota. The aim of this work was to investigate how the biotransformation of baicalin to baicalein is related to the intestinal microbiota. For this purpose, stool samples from healthy volunteers with different dietary habits were used. From the pre-cultured stool samples, different standard microbiota were obtained which were used for the subsequent metabolism studies in the *in vitro* model MimiCol³. MimiCol³ represents the ascending section of the colon, the *colon ascendens*, in terms of available volume, pH-value, redox potential and bacterial abundance. While during the experiments with added standard microbiota a metabolism of baicalin to baicalein could be detected, this was not the case in a series of experiments without added microbiota. This confirmed the hypothesis that the metabolism of baicalin relies on the bacterial species that are present in the colon. The data collected in the MimiCol³ therefore support the use of baicalin as a potential marker for the determination of the colon arrival. This can be explained by the fact that baicalin in its native form is poorly absorbed from the gastrointestinal tract. Enzymes of the colonic microbiota, namely β-glucuronidases, hydrolyze baicalin to the aglycone baicalein. The resulting aglycone can be absorbed through the intestinal mucosa and detected in blood plasma. This potentially enables the use of baicalin as a marker to determine the time of arrival in the colon.

1. Introduction

The awareness of the gut microbiota and its metabolic activity is increasing. In addition to its importance for human health, the gut microbiota plays a decisive role in the modulation of drug metabolism and other biotransformation. Especially for substances which are poorly absorbed during transit through the small intestine, biotransformation by the intestinal microbiota might play a crucial role. Subsequent absorption in more distal sections of the gastrointestinal tract (GIT) can typically only occur through transformation after reaching the colon. This applies to glucuronides and sulphated substances, among others (Koppel et al. 2017). Due to their high hydrophilicity and the lack of necessary transporters in the small intestine, direct absorption is not possible. Once they reach the colon, they can be metabolised by enzymes secreted by the colonic microbiota. The resulting metabolite can then be absorbed and eventually enter the systemic circulation. The enzymes secreted by the gut microbiota can either metabolically activate or inactivate compounds (Swanson 2015).

With its great variety of enzymes, the microbiota makes a decisive contribution to this process. Mainly hydrolytic and reductive reactions are used to metabolise substances. Specifically, hydrolases, lyases, oxidoreductases and transferases should be mentioned for this metabolism (Koppel et al. 2017). The intestinal microbiome is of great importance as it contains most of the human microbiota. This is illustrated by the estimated 1.000 to 1.500 different bacterial species found there, which contain approximately 3.3 million non-redundant genes (Koontz et al. 2019). In addition, the composition of the microbiota can be significantly influenced by factors such as diet, diseases, pharmaceutical interventions and genetic factors, as well as personal parameters (Voreades et al. 2014; Zhernakova et al. 2016). Consequently, there may be both inter- and intra-individual differences in the composition of the microbiota (Doestzada et al. 2018). Colon colonization ranges from aerobes, such as eukaryotic microorganisms like yeasts and fungi, to obligate anaerobes and anaerobes. Obligate anaerobic bacteria make up the largest proportion at > 95%. A division into 6 – 7 phyla can be made, with the genera *Bacteroidetes* and *Firmicutes* being the dominant ones (Blaut 2016). In recent years, the importance of the microbiota has become increasingly important. The relationship between the bacterial colonization of the gut and the health status of an individual, the occurrence of certain diseases, and the influence on the metabolism of drugs and other substances has been further investigated. The same applies to the reverse influence, i.e., what influence the intake of certain substances has on the composition of the gut microbiota (Gnatzy et al. 2023).

The metabolic activity of the colonic microbiota is also exploited in the application of prodrugs, for example in colon targeting. One example is sulfasalazine, which, after oral administration, passes unchanged through the GIT into the colon. There, it is cleaved by azoreductases into its pharmacologically active metabolites, sulfapyridine and 5-aminosalicylic acid (McCoubrey et al. 2023). Such substances can also be used to determine the colon arrival time. The delayed measurable flooding of the drug into the systemic circulation can be considered as a characteristic of the transit time to the colon. Another potential colonic marker could be the flavone glycoside baicalin (7-D-glucuronic acid-5,6-dihydroxyflavone) from the root of *Scutellaria baicalensis* Georgi.

In the 1980s, Hattori et al. proved the influence of the intestinal microbiota on the metabolism of phytochemicals such as anthranoids and triterpene saponins (Hattori et al. 1983; Hattori et al. 1982). Kim et al. (1998) and Feng et al. (2005) reported that this was the same for the flavone glucuronide baicalin. After oral ingestion, baicalin is metabolised by intestinal bacteria to its aglycone baicalein before it can be absorbed. Flavonoids are attributed to a low oral bioavailability (Manach and Donovan 2004; Zhang et al. 2007). This is due to a low absorption rate, rapid degradation by the intestinal microbiota, and significant first-pass metabolism. The bioavailability range is usually around 2.5 – 18.5%. Ingestion is usually followed by cleavage of the glycoside in the large intestine, followed by absorption and metabolism of the resulting

aglycone (Rechner et al. 2002). This depends in part on the extent to which they are metabolised by enzymes, which depends on their structure (Srinivas 2010; Swanson 2015; Zhang et al. 2007). The xenobiotic baicalin also shows poor oral bioavailability. It is hydrolyzed by the secreted β-glucuronidases of the intestinal microbiota to the aglycone baicalein, which is subsequently absorbed by the gut (Ganguly et al. 2022; Kang et al. 2013; Kim et al. 2008). Studies have shown that baicalein rapidly enters the systemic circulation, which is not the case with baicalin taken orally. This could hardly be detected in plasma. Here, first-pass metabolism in the small intestine plays a crucial role in the low oral bioavailability of baicalein (Lai et al. 2010; Zhang et al. 2005). Baicalein formed from baicalin can be converted back to baicalin in the systemic circulation (Psimadas et al. 2012), which is due to uridine 5'-diphospho (UDP)-glucuronosyltransferases in phase II metabolism in the intestine and liver (Noh et al. 2016; Zhang et al. 2007). In addition to further biotransformation, it can also pass back into the intestinal lumen *via* the bile duct for excretion, or through the kidney into the urine. The metabolites that return to the intestinal lumen can either be excreted in the faeces *via* the colon or reabsorbed in the small intestine (Swanson 2015). The glucuronic acid component of baicalin is essential for this conversion. Since glucuronic acid is used in intestinal glucuronidation, baicalin can be formed in tissues even if it has been previously metabolised to baicalein by microorganisms in the gut (Noh et al. 2016).

In a study by Xing et al. (2005) baicalin was found to have an oral bioavailability of just 2.2 ± 0.2% after oral administration to rats. In contrast, a bioavailability of 27.8 ± 5.6% could be determined for the ingested baicalein. Similar results were obtained in another animal study by Lai et al. It was shown that with an oral administration of baicalin and baicalein with an equivalent dose of 224 µmol/kg, the aglycone reaches a maximum concentration with 13.6 ± 9 nmol/mL after 10 min, compared to baicalin with a maximum concentration of 2.9 ± 1.3 nmol/mL after 396 ± 438.8 min (Lai et al. 2010). This supports the assumption that baicalin is first hydrolysed by bacteria which is the rate-limiting factor of absorption. During absorption of baicalein by the intestinal epithelium, it undergoes renewed biotransformation, especially in the form of glucuronidation and sulfation (Feng et al. 2005; Zhang et al. 2005). Transport *via* the portal vein to the liver occurs. The high abundance of enzymes leads to further metabolisation possibilities before the metabolites enter the systemic circulation. Baicalein, for example, is re-conjugated to baicalin (Akao et al. 2010). During the extensive first-pass glucuronidation of baicalein in the liver and intestine, other glucuronide conjugates can be formed in addition to baicalin (Kang et al. 2014). These include, for example, oroxylin A and wogonin.

The aim of this study was to investigate glucuronide cleavage in the ascending colon as a function of colon-relevant bacteria. Therefore, the metabolism of the flavonoid glycoside baicalin to its aglycone baicalein was investigated. The focus of the work was on the metabolism that takes place after oral ingestion of baicalin when it enters the colon, thus highlighting the potential of baicalin as a colon marker. For this purpose, the *in vitro* model MimiCol[3] was used, which represents the physiological conditions of the colon ascendens (Beeck et al. 2022). The metabolisation of baicalin to the aglycone baicalein was investigated using different standard microbiota cultured from human fecal samples. In addition, a reference series without microbiota was performed to assess the necessity of the enzymes.

2. Investigations and results

2.1. Validation

The pre-developed method was partially validated with the following results.

2.1.1. Linearity, accuracy and precision

The calibration curves of the peak areas *versus* the concentration of baicalin and baicalein were all linear, with a 1/x-weighted linearization. The concentrations measured were in the range of 1.75 – 35 µg/mL and the correlation coefficients were above the acceptable value of $R^2 > 0.995$.

Precision and accuracy were determined using the QC samples of the two compounds and are summarized in Table 1. The accuracy ranged from 94.56% to 100.26%, and the precision, expressed as relative standard deviation (RSD), of both analytes ranged from 1.953 to 10.411%.

Table 1: Precision and accuracy of baicalin and baicalein (n = 6)

Compounds	Theoretical concentration (*mean ± SD) (µg/mL)	Determined concentration (mean ± SD) (µg/mL)	Precision (% RSD)	Accuracy (% recovery)
Baicalin	1.969 13.125 23.625	1.905 ± 0.090 12.411 ± 0.301 22.438 ± 0.438	4.701 2.429 1.953	96.78 94.56 94.98
Baicalein (*)	1.969 ± 0.001 13.130 ± 0.006 23.634 ± 0.010	1.986 ± 0.207 12.654 ± 0.678 22.948 ± 1.132	10.411 5.360 4.932	100.86 96.37 97.10

2.1.2. Stability

The results of stability evaluation in Fig. 1 indicated that baicalin and baicalein were stable under the anticipated conditions. The prepared samples were stored for 7, 14 and 63 d at −20 °C, thawed and measured. In addition, an aliquot was measured immediately on the day of preparation, which was used as a reference.

Table 2: Freeze-thaw-stability of baicalin and baicalein

Compounds	Fresh	Cycle 1		Cycle 2	
	Concentration (µg/mL)	Concentration (µg/mL)	Increase / Decrease (%)	Concentration (µg/mL)	Increase / Decrease (%)
Baicalin	2.626 17.5 31.5	2.500 17.026 31.244	− 4.802 − 2.711 − 0.812	2.611 17.276 30.965	− 0.586 − 1.282 − 1.698
Baicalein	2.626 17.5 31.5	2.600 17.127 31.118	− 0.996 − 2.132 − 1.213	2.705 17.299 31.319	+ 3.015 − 1.149 − 0.574

Additionally, the samples were tested for freeze-thaw stability. The freshly prepared samples were measured as a reference followed by freezing and thawing the entire set of samples twice, whereby the concentration was measured after each thawing process. The data obtained can be seen in the Table 2.

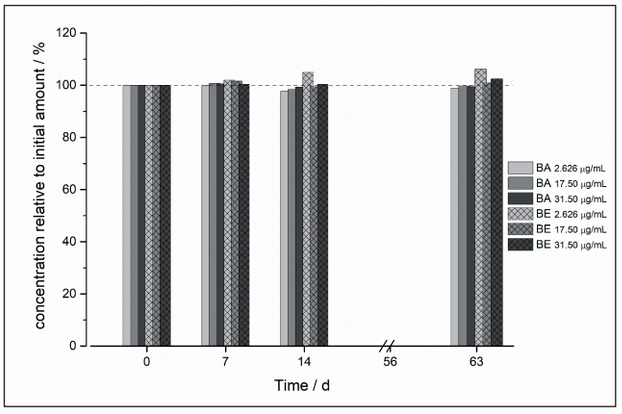

Fig. 1: Long-term storage stability of baicalin (BA) and baicalein (BE) over a period of 63 days.

2.2. Investigation of baicalin degradation and bacterial growth using MimiCol[3]

2.2.1. Process parameters

Through the electrodes inserted *via* the lid of the vessel, the redox potential, the pH changes, and the temperature of the culture medium were recorded. At time 0 h, the tested bacterial micro-

biota was injected. Subsequently, a decrease of the redox potential occurred in the vessels. Immediately after the media changes at time points 3 and 5 h, an increase in both the redox potential and the pH value could be detected.

2.2.2. *Optical density*

Bacterial growth was measured by UV-VIS spectroscopy on samples of the optical density (OD) to monitor the growth of the bacteria during the experiments in the MimiCol[3]. The highest increase in bacterial density was recorded for all standard microbiota used in the interval from 0 to 3 h, i.e. until the first media change. In the two following intervals, from 3.083 to 5.00 h and from 5.083 to 7.00 h, the optical density increased again, but not as sharply as in the first interval. Also, in the series of experiments without added microbiota (SMx), which is the reference test, the growth of bacteria was measured using the OD. As can be seen in Fig. 2, no bacterial growth was detected over time.

Despite the re-addition of nutrients by media exchange, bacterial growth decreases over time in all microbiota. Growth is comparatively highest in SM_{pooled}, while it was lowest in SM_{omni_2}. The curves with the standard deviation shown indicate that there were marked fluctuations in growth over the duration of the experiments.

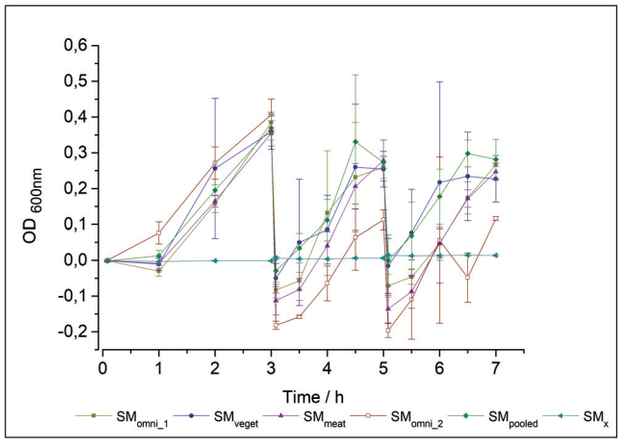

Fig. 2: Optical density in the MimiCol[3] over 7 h (150 mL Schaedler broth, 100 rpm, pH 6.2 (± 0.25), 37 °C, n = 3, (mean +/− SD))

2.2.3. *Metabolisation of baicalin to baicalein*

Figure 3 shows the results of the metabolisation of baicalin to baicalein by the different microbiota in the MimiCol[3]. After media change at 3.083 and 5.083 h, about 10 mg of baicalin was injected in each vessel. As can be seen from the figure, differences in metabolisation could be recorded. Variations occurred even within the different microbiota, as can be seen from the standard deviations. While SM_{meat} and SM_{omni_2} showed the highest degradation, SM_{veget} showed the lowest degradation in the series of experiments with microbiota. In the 1st interval of baicalin addition, the greatest degradation occurred at SM_{meat}, while in the 2nd interval after addition it was the case at SM_{omni_2}. Regarding the curves of the formation of baicalein, there can be said that it was equal to the degradation of baicalin. Thus, in the 1st interval the largest amount of baicalein was detected at SM_{meat} and in the 2nd interval at SM_{omni_2}. Likewise, the amount of baicalein produced was lowest in SM_{veget}. No baicalein was detected in the series of experiments without the addition of microbiota. Due to methodological problems, the values at time points 3.083 and 5.083 h were omitted from the experiment for further evaluation. Instead, an assumption of 100% was made for the calculation of the respective conversion, and the percentage share was determined based on this. Nevertheless, a decrease in the baicalin concentration could also be determined here.

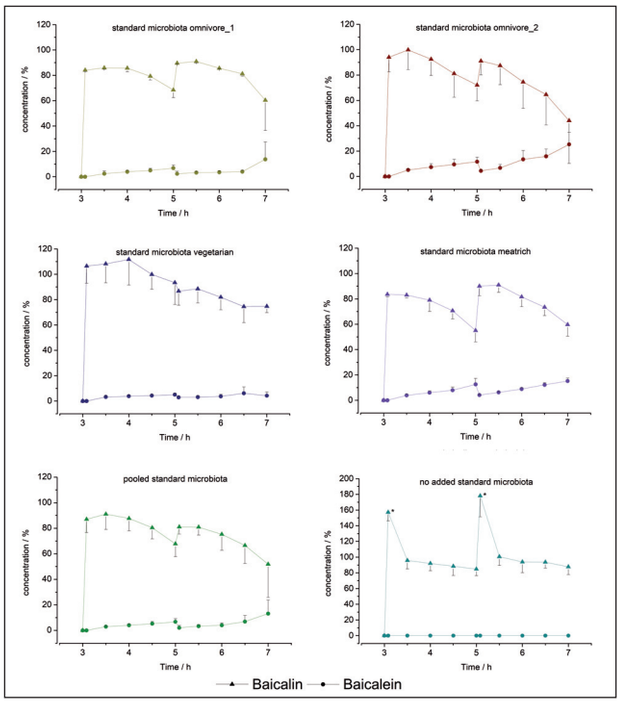

Fig. 3: Degradation of baicalin and formation of baicalein in the MimiCol[3] over 7 h (150 mL Schaedler broth, 100 rpm, pH 6.2 (± 0.25), 37 °C, n = 3, (mean +/− SD)). Shown are the degradation curves of the analysed standard microbiota and the reference experiment without added microbiota. [* Due to insufficient mixing, identified as outliers].

2.2.4. *Determination of different standard microbiota by using selective agar*

Besides the determination of bacterial growth during the ongoing experiments by optical density, as described in chapter 2.2.2, the bacterial composition of the individual microbiota in the MimiCol[3] was also characterized. Therefore, colony forming units (CFU) samples were plated out on selective agar at time points 0.083, 3, 3.083, 5, 5.083 and 7 h and characterized as described in 4.2.4. Figure 4 shows the results for each bacterial species over time from each experimental setup. To obtain comparable results, only the CFU samples from vessel 1 of each experimental series were plated out. In summary, the total CFU is also shown graphically.

Within the intervals between media addition and removal, an increase in CFU/mL was observed for the individual bacterial species, except for the genus *Bacteroides*. Here, an increase could only be determined in the interval from 0.083 to 3 h. In the following intervals there was no noticeable increase. Considering SM_{omni_1} in more detail, the bacterial density of *Bacteroides* was highest and despite an overall decrease of the genus within the intervals, an increase could still be determined. Due to the media changes and sample preparation, sterility could not be ensured, so the species of *Lactobacilli*, *Clostridia* and *Bifidobacteria* were also detected in the series of experiments without added microbiota. However, these could be detected in a significantly lower proportion than in the experimental series with microbiota. In the genus *Enterobacteria*, except for SM_{veget}, there is a decrease in density at the end of the 2nd and 3rd interval compared to the 1st interval. For the species of *Clostridia*, *Lactobacilli* and *Bifidobacteria*, the highest bacterial density was observed at 7 h for most of the microbiota.

2.3. *Conclusions*

In summary, the metabolism of baicalin to baicalein in the MimiCol[3] by the human colonic microbiota was successfully depicted. In addition to the degradation of the added substance baicalin, the formation of the main metabolite baicalein could also be quantitatively determined. While baicalein could be detected in

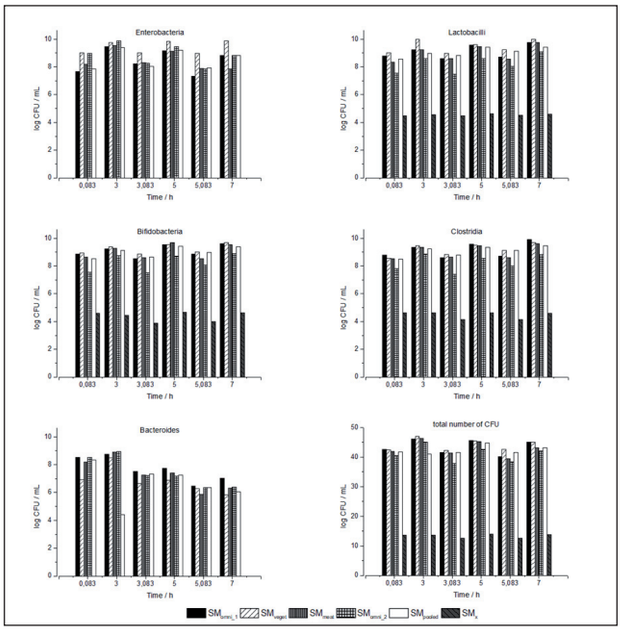

Fig. 4: Bacterial composition of the different bacterial culture and the total number of CFU in the MimiCol³ over 7 h (150 mL Schaedler broth, 100 rpm, pH 6.2 (± 0.25), 37 °C, n = 1).

the experiments with added microbiota, it could not be detected in the experiments without added microbiota. This supports the notion that the presence of colonic microbiota is necessary for metabolism. It also suggests that baicalin could be a suitable colon marker for determining the colon arrival time. Crucial to this are the enzymes in the colon. The metabolism of baicalin to baicalein is therefore mainly linked to the colon. By detecting the resulting metabolite baicalein in blood plasma, it is possible to determine the arrival of baicalin in the colon. In the future, other substances and their metabolism by the microbiota in stool samples should be investigated in the MimiCol³. This should provide new insights into possible metabolic pathways. It would also be interesting to study the influence of added substances on the composition of the microbiota.

3. Discussion

This study focused on the metabolism of the flavone baicalin to one of its major metabolites, baicalein, by colon bacteria. For this purpose, different microbiota were used to identify possible factors affecting the bacterial composition of metabolism. In addition, an experimental setup without added microbiota was performed. This was done to demonstrate that baicalin is not metabolised in the *in vitro* model MimiCol³ in the absence of bacteria under the test conditions.

High pressure liquid chromatography (HPLC) analysis was performed to analyse the samples. In this way, both the degradation of baicalin and the formation of baicalein could be determined quantitatively. To obtain qualitatively reliable results, a suitable sample preparation had to be found in advance. The Schaedler broth, which was used as the nutrient medium for the bacteria in the experiments, presented a challenge. Therefore, adequate preparation of the samples is essential. Liquid-liquid extraction with ethyl acetate, protein precipitation with acetonitrile and subsequent concentration under vacuum, and addition of acidified methanol to the samples were tested. Only the latter method showed good results. This can be explained by the acidic stability of baicalin. Feng et al. (2017) were able to provide stability data in their study depending on the pH value of the two substances. It was shown that baicalin exhibits better stability in a pH range of 2.0 – 4.5 than in higher pH ranges. This could be confirmed during the method development of the HPLC analysis in this study. For non-acidified samples, the peak area of baicalin in the chromatogram decreased over time (data not shown). Referring to the curves in Fig. 1, the concentration of both analytes in the acidified samples did not decrease noticeably over a period of nine weeks. Freeze-thaw stability was also determined, and the results are shown in Table 2. The parameters collected during the partial validation were all within the predefined limits, so that the HPLC method can be assumed to be suited for the analysis.

Different standard microbiota were used for the following experiments in the MimiCol³. To create an ideal environment for bacterial growth, the vessels were gassed with nitrogen for 1 h before the start of the experiment. The generated anaerobic atmosphere is mainly to serve the anaerobic bacteria. The redox potential can be monitored with an electrode during the experiment. For the proximal part of the colon, a redox potential of about –415 mV is assumed, whereas the collected data from the MimiCol³ were lower (Sousa et al. 2008). To provide sufficient nutrients, the Schaedler broth medium used is removed after 3 and 5 h to a remaining residual volume of 15 mL and supplemented with fresh, tempered medium. The addition of the new medium also leads to the increases in pH and redox potential shown in Figure 6. Bacterial growth during the experiments was monitored by measuring the optical density. As can be seen from Figure 2, bacterial growth was continuously kept in the exponential growth phase. In the series of experiments with standard microbiota, bacterial growth could be detected, even though there were partly large fluctuations in bacterial growth within a microbiota. It should be mentioned that the measurement of the OD is only a dimensionless determination of the turbidity of the samples. This entails a certain susceptibility to interference and can lead to discrepancies in the results. Nevertheless, the method is suitable for monitoring bacterial growth during the experiment in the MimiCol³.

The influence of the colon microbiota on the metabolisation of baicalin to baicalein was proven in the MimiCol³. The data collected in the experiments indicate a correlation between bacterial growth and metabolisation. With the addition of the new culture medium before the sample time points of 3.083 and 5.083 h, 10 mg of baicalin was also added to the vessels. Degradation of baicalin was detected in all experiments. In contrast to previous studies on the metabolism of sulfasalazine in the MimiCol³, a different metabolism of baicalin by the different microbiota was found. During the cultivation of the different microbiota, based on different diets, there was a loss of pre-existing differences in bacterial diversity. This was determined by analysing the CFU samples. The subsequent experiments on the metabolism of sulfasalazine in the MimiCol³ with the different microbiota showed quite similar results due to the equalisation of the bacterial composition. The previously expected differences in the metabolism of sulfasalazine due to the different diets could therefore not be demonstrated in the MimiCol³ (Seradj et al. 2023). Although some of the microbiota used here were the same as those used in the sulfasalazine studies, slight differences were found in the metabolism of baicalin to baicalein. This may be explained by the different metabolic mechanisms of the two substances. While azoreductases are rather unspecific enzymes that are commonly found, β-glucuronidases are more characteristic of colon-relevant bacterial genera. Table 3 shows the different metabolisation behaviour of the different microbiota. While the highest metabolism was observed in SM_{meat} (with a rate of 5.5 µmol/h) in the 1st interval after baicalin addition, it was for SM_{omni_2} (with 7.5 µmol/h) in the 2nd interval.

As can be seen from the plotted standard deviations in Fig. 3, large differences in metabolism also occurred within a microbiota. Baicalin degradation in SM_{veget} differed from that of the other SM. Despite the addition of baicalin after 5 h, there is no renewed increase in the concentration in the vessel. Theoretically, the curve should have increased due to the new addition, but only a further decrease was determined. In SM_{veget}, there was the lowest conversion of baicalin and the lowest detection of baicalein. As expected, no baicalein was detected in the reference experiment without microbiota.

Table 3: Mean values of the percent degradation of baicalin relative to the total amount applied and formation of baicalein by the different standard microbiota in MimiCol³ (150 mL Schaedler broth, 100 rpm, pH 6.2 (± 0.25), 37 °C, n = 3).

Microbiota	Degradation of baicalin (%)		Formation of baicalein (%)	
	1. Interval	2. Interval	1. Interval	2. Interval
SMomni_1	15.66	29.22	6.86	11.44
SMveget	13.06	11.95	5.04	1.36
SMmeat	28.49	30.30	12.52	11.08
SMomni_2	21.98	47.04	11.63	20.88
SMpooled	19.25	29.23	6.74	11.07
SMx	15.23	12.34	0.00	0.00

The decrease in baicalin content suggests that other metabolites were formed, which were not further analysed in this work. The analysis of the series of experiments with added microbiota shows that the degradation of baicalin is accompanied by an increase in baicalein. The two outliers (* marked in Fig. 3) in the measured values at time points 3.083 and 5.083 h can be explained by the fact that the medium was not sufficiently mixed before the samples were drawn. Shortly before the medium change, the shaking speed of the water bath is reduced from 100 to 20 rpm to allow better removal of the vessel contents. During the addition of the substance and the new medium, the shaking speed is increased again. However, this was not carried out in this series of experiments before the next sampling, which is why the increased measured values of the concentration occurred. All other measured values of baicalin were in the expected range, although a slight drop in concentration was recorded. The formation of baicalein could not be detected, suggesting the formation of other metabolites. After oral administration of baicalin, other main metabolites were discovered in animal experiments. In addition to baicalein, wogonin and oroxylin A should be mentioned here (Feng et al. 2005; Liu et al. 2015). Different effects are attributed to them regarding to their mode of action. In the context of this work, only the formation of the main metabolite baicalein was investigated. The instability of baicalin and baicalein at neutral pH must also be considered, as the Schaedler broth used in the experiments is at neutral pH and baicalin degradation may occur.

Since the OD is just a measurement of the increase in bacterial density in general, the CFU samples were used for a more detailed analysis of the standard microbiota. The proportions of anaerobic and aerobic species in the individual microbiota were examined. This was followed by a detailed analysis of the composition of the genera *Enterobacteria*, *Lactobacilli*, *Clostridia*, *Bifidobacteria* and *Bacteroides*. In studies by Kim et al. baicalin and other flavonoids were incubated with human faeces and analysed for their major metabolites. Baicalin was found to be metabolised by β-glucuronidases to baicalein. This is followed by further transformations to the phenolic acids 3,4-dihydroxybenzoic acid, pyrogallol, and phenylacetic acid. However, these secondary metabolites were not analysed in detail in this study. The focus of this study was to quantify the formation of the aglycone baicalein by the intestinal microbiota after the addition of baicalin. These results were intended to be used to evaluate baicalin as a potential colon marker. The formation of other degradation products is therefore out of scope of the manuscript and will not be discussed further. The β-glucuronidases responsible for hydrolysis have been assigned to the bacteria *Escherichia coli*, *Eubacterium* and *Bacteroides*. *Streptococcus*, *Lactobacilli*, *Eubacterium* and *Bifidobacteria* were found to be involved in further conversion of baicalein to phenolic acids (Kim, 2002; Kim et al. 1998). Lim and Wang (2022) reported that glucuronidases from *Lactobacilli* are also involved in the biotransformation. Comparing this with the data (Table 3) from the series of experiments performed here, the following findings can be made.

In the first interval after addition of the substance, the greatest degradation of baicalin was detected in SM_{meat}, followed by SM_{omni_2}. The same applies to the amount of baicalein detected in the period from 3.083 to 5 h. Regarding these results with the evaluation of the CFU samples, the *Enterobacteria*, *Bacteroides* and *Clostridia* could be detected in SM_{meat} and SM_{omni_2}. However, the proportion detected there was lower than in other microbiota. Thus, during the period, the highest density of *Enterobacteria* was found in SM_{veget}, followed by SM_{omni_2}. For *Bacteroides*, this was the case in SM_{omni_1}, followed by SM_{meat}, and for *Clostridia*, the proportion was also highest in SM_{omni_1}, followed by SM_{veget} and SM_{meat}. Both, the degradation of baicalin and the detection of baicalein were lowest in SM_{veget} in the series of experiments with added SM. In the interval from 5.083 to 7 h, the greatest amount of baicalein was found in the experiments with SM_{omni_2}, followed by SM_{omni_1}. While the greatest amount of *Enterobacteria* was found in this interval in SM_{veget}, followed by SM_{omni_1}, which was the opposite for *Clostridia*. For *Bacteroides* this was the case in SM_{omni_1}, followed by SM_{omni_2}. The CFU samples were a single sample and only one determination was made in each case, so the results should be treated with caution. Nevertheless, the composition of the human gut microbiota plays a crucial role in the metabolism of phytochemicals (Murota et al. 2018). Studies have shown that demographic characteristics are not decisive for metabolic activity. Instead, this is associated with the gut microbiota (Plamada and Vodnar 2022; Yim et al. 2004). Studies on metabolic activity showed that the biotransformation of baicalin to baicalein predominated over oroxylin A. In the study by Trinh et al. (2010) for example, human faeces were incubated with 100 mg of baicalin for 20 h. The evaluation revealed that the metabolisation activity to the agylcone was significantly higher at 40.2 ± 26.2 nmol/h × mg weight of fecal bacteria, compared to oroxylin A at 1.2 ± 1.1 nmol/h × mg weight of fecal bacteria. Jung et al. (2012) also studied the enzyme activity in bacalin metabolism. They found that neither gender nor age had an influence on metabolic activity. Additionally, they also ran experiments with mice. The influence of antibiotic administration was investigated. It was shown that the β-glucuronidase activity in mice treated with antibiotics is significantly lower than in healthy mice (Jung et al. 2012). The same results were obtained in advance in a study on germ-free rats in the year 2000. No baicalein was found after administration of 20 mg/kg body weight orally administered baicalin. Compared to conventional rats, baicalein was found at a maximum concentration of 1.69 ± 0.65 µg/mL after approximately 1.50 ± 1.34 h. Also, the determined area under the curve from time 0 h to the last determination was only 12% in the germ-free rats compared to the conventional rats (Akao et al. 2010). This can be reconciled with the results from the series of experiments in the MimiCol³ without SM. As already described, a slight decrease of the baicalin concentration could be noted, but no baicalein could be detected in these samples. Looking at the results of the CFU sample evaluation shown in Figure 4, bacteria were also detected in this series of experiments. This can be explained by the fact that the experiments in the MimiCol³ do not take place under aseptic conditions and contamination can also occur during sample collection and preparation. However, neither representatives of the genera *Enterobacteria* nor *Bacteroides*, which are believed to be involved in the metabolism of baicalin to baicalein, could be detected. As shown in the graph of the total number of CFU in Figure 4, the bacterial density in this series of experiments was significantly lower than in the other series of experiments.

The results of these and other studies underline the importance of the gut microbiome. The influence of the microbiome on the effects of xenobiotics, drugs and other substances should further be studied. The series of experiments previously performed in the MimiCol³ with the drug sulfasalazine demonstrated the cleavage of the azo bond by the added standard microbiota. The present series of experiments investigated the metabolism of baicalin to baicalein by the gut microbiota. The biotransformation described in the literature, in which β-glucuronidases found in the large intestine are responsible for the cleavage, seems promising for the use of baicalin as a marker for colon arrival time. The experiments carried out in the MimiCol³ confirmed the described metabolism in the presence of colon-relevant bacterial genera.

4. Experimental

4.1. Materials

Baicalin was purchased from Cayman chemical company (Michigan, USA) and baicalein from Thermo Fisher Scientific (Lancashire, UK). Schaedler broth was purchased from Carl Roth (Karlsruhe, Germany) as dry substance and reconstituted according to the manufacturer's specifications. Glycerol Rotipuran 99.5% was purchased from Caesar & Loretz GmbH (Hilden, Germany) and diluted to obtain a concentration of 20%. The various agar media and the required supplements were obtained from Sigma-Aldrich/Fluka (St. Louis, USA) and Carl Roth. Peptone water was acquired as a dry substance from Carl Roth and reconstituted with sterile water for injection. Sodium chloride was purchased from Caesar & Loretz and diluted to obtain a concentration of 0.9%. Potassium dihydrogen phosphate for the buffer was purchased from neoFroxx GmbH (Einhausen, Germany), while hydrochloric acid 37% was used from Walter-CMP GmbH & Co. KG (Kiel, Germany) for pH adjustment. Formic acid was purchased from Sigma-Aldrich, methanol, and acetonitrile in HPLC grade were obtained from Fisher Scientific (Loughborough, UK). As solvent, only sterile water for injection (Ph. Eur. 11.2) was used in all cases.

4.2. Methods

4.2.1. High pressure liquid chromatography system and chromatographic conditions

HPLC was performed on a Shimadzu system equipped with an automatic injector (Shimadzu SIL-20ACXR) set at 10 °C, a column oven (Shimadzu CTO-20AC) whose temperature was maintained at 40 °C and a photodiode array detector (Shimadzu SPD-M20A). The eluates were monitored at 276 nm (baicalin) and 274 nm (baicalein). Other components of the system were two pumps (Shimadzu LC-20ADXR), a degassing unit (Shimadzu DGU-20A5R), and a control unit (Shimadzu CBM-20A). All components were obtained from the following manufacturer SHIMADZU Corp., Japan. Analyte separation was achieved on a Kinetex® 2.6 µm polar C18 column (150 x 2.1 mm; 100 Å) from Phenomenex (Aschaffenburg, Germany). Acetonitrile (B) and a phosphate buffer (A) (20 mM; pH 2.5) were used as mobile phases. A gradient elution program was carried out as follows: B: 20% between 0 – 5 min; B: 40% between 5 – 20 min; B: 40 – 80% between 20.01 – 22 min; B: 80% between 22 – 24 min; B: 80 – 20% between 24 – 25.50 min and B: 20% between 25.50 – 30 min. The flow rate was 0.4 mL/min and the injection volume was 5 µL. The total run time per sample was about 30 min. The chromatograms were recorded by LabSolutions software (SHIMADZU, Japan).

4.2.2. Preparation of calibration curve and samples

Calculation of the concentration of both analytes over the determined peak area was performed by preparing a stock solution of baicalin and baicalein in a methanol/formic acid mixture (95:5, v/v). The various concentrations were spiked with Schaedler broth, vortexed, and centrifuged at 14500 rpm for 6 min. The supernatant was measured by HPLC. The measured peak area was plotted against concentration in µg/mL and showed a good linear correlation between 1.13 – 26.25 µg/mL. A coefficient of determination of $R^2 > 0.995$ was deemed an acceptance criterion.
Samples collected from the MimiCol[3] were prepared in a similar way. 2000 µL sample was centrifuged at 14500 rpm for 6 min. 250 µL supernatant was collected and diluted with 750 µL of methanol/formic acid (95:5, v/v). The sample was vortexed, centrifuged at 14500 rpm for 6 min, and 500 µL supernatant was pipetted into a HPLC vial.

4.2.3. Method validation

In accordance with the Food and Drug Administration and the European Medicine Agency harmonized guidelines for method validation, a partial validation of the HPLC method for the quantification of baicalin and baicalein was performed (European Medicines Agency 2019). Calibration curves consisting of 7 measured values of the two analytes were generated by plotting peak area ratios (y) against concentration in µg/mL (x) using a 1/x-weighted linear regression model. The lowest calibration standard (LLOQ) was 1.75 µg/mL while the highest calibration standard (ULOQ) was 35 µg/mL. After careful consideration and evaluation of preliminary analytical screening, no internal standard was added. In addition, three concentrations (high, medium, and low) were prepared as quality control (QC) standards. For validation, specificity, linearity, precision – expressed as RSD – and accuracy were evaluated. The allowed deviations were previously set to be in the range of ±15 %. Further, three QC samples were stored at −20 °C for 7 and 14 days, and 9 weeks. Stability was verified at different storage times. Also, the freeze-thaw stability of three QC samples was analyzed. For the HPLC analysis of the samples, purification of the samples was essential due to the complex matrix of the medium Schaedler broth. The preparation of the samples was carried out as described in 4.2.2. To increase the stability of baicalin, the samples were additionally acidified with formic acid. The selectivity of the method was tested by measuring a blank value of the medium Schaedler broth as a reference, and no interferences were found. The retention times of baicalin and baicalein were around 5 and 12 min, respectively.

4.2.4. Preparation and characterization of standard microbiota

The stool samples were taken from healthy volunteers (1 male, 3 females, age 26 – 30 years, body mass index 18 – 35 kg/m^2). The recruitment of volunteers and the collection of their feces was approved by the ethic committee of university medicine Greifswald (BB 009/22). Written informed consent was obtained from all volunteers prior to sample collection. A total of four different faecal samples were recruited and processed, which were used for the following experiments in the MimiCol[3]. Different diets were considered when selecting the volunteers, so that the following standard microbiota were cultured: SM_{omni_1} and SM_{omni_2}, which are based on an omnivorous diet; SM_{veget}, which is based on a vegetarian diet; and SM_{meat}, which is based on an omnivorous diet with high meat consumption.
Cultivation of the different standard microbiota were performed as follows. From the freshly obtained stool samples, 1.00 g was taken and suspended in 40 mL of sterilized peptone water. This was then allowed to rest for 5 min before the supernatant was removed for cultivation after the completion of sedimentation. Cultivations of the standard microbiota were performed in the Biostat® A plus batch fermenter (Sartorius stedim Biotech, Göttingen, Germany) under the following conditions.
A complex culture medium was used for cultivation, based on the composition published by Minekus et al. (Minekus et al. 1999). The volume of the fermenter vessel is 2000 mL and is equipped with an integrated stirring system. This was set to 200 rpm so that continuous mixing of the culture broth could be ensured. The complex culture medium was kept at a temperature of 37 °C and a pH of 6.2 (± 0.1) was set. Nitrogen was gassed into the fermenter continuously as well as 1 h before the start of cultivation to create optimal anaerobic conditions. Cultivation lasted for 24 h, with 5 mL of sample taken every 4 h. These samples were measured for OD to monitor bacterial growth during the cultivation. Therefore, 200 µL sample was diluted with 1800 µL of sterilized water for injection and was analyzed by UV/VIS-spectroscopy (Cary 50 Scan UV Visible Spectrophotometer, Varian Inc., Palo Alto, USA) at 600 nm in quartz cuvettes (path length = 1 cm). The sample at time 0.083 h served as a blank. After the cultivation was completed, all medium was removed and processed as follows. 50 mL tubes were loaded with 25 mL glycerol 20% and then refilled with 25 mL of bacterial culture. These were vortexed and then stored at −20 °C for 24 h before being transferred to −80 °C until further use.
To characterize the individual microbiota in terms of bacterial composition, the CFU samples were undergone the following preparation procedure. Therefore, 300 µL sample was diluted with 300 µL glycerol 20% and vortexed. For the first 24 h, the samples were stored at −20 °C before being kept at −80 °C until the time of characterization.
For the characterization of the CFU samples, different agar media were used. The composition of each microbiota in terms of bacterial species was analyzed at the following time points: 0.083, 3.00, 3.083, 5.00, 5.083, and 7.00 h. The samples were analyzed in terms of bacterial species. For this purpose, the cryo samples were diluted with sterilized peptone water. Therefore, a dilution series in steps of ten from 1 to 1 million was prepared. Then, 20 µL of each dilution was plated on each agar medium. Incubation of the inoculated agar plates was performed for 2 days under aerobic conditions (INB 400, Memmert GmbH, Schwabach, Germany) at 33 °C or for 5 days under anaerobic conditions (Whitley A35 Anaerobic Workstation, Don whitley scientific, Bingley, UK) at 37 °C. For the evaluation of bacterial growth, the plate sections with the highest growth were counted, whose colonies ranged from 3 to 90. The following Equation (1) was used for the evaluation. A sample volume of 20 µL was taken ('50'). This was mixed with glycerol in a ratio of 1:2 ('2').

$$\frac{CFU}{mL} = CFU \times dilution\ factor \times 5 \times 2 \qquad (1)$$

4.2.5. Experimental procedure

For the investigations of the metabolisation from baicalin to baicalein, the MimiCol[3], an *in vitro* model was used which simulates the conditions of the ascending colon. Detailed description of the methodology can be found in a previous publication by Beeck et al. (Beeck et al. 2022). Figure 5 is used to provide a schematic overview of the MimiCol[3].

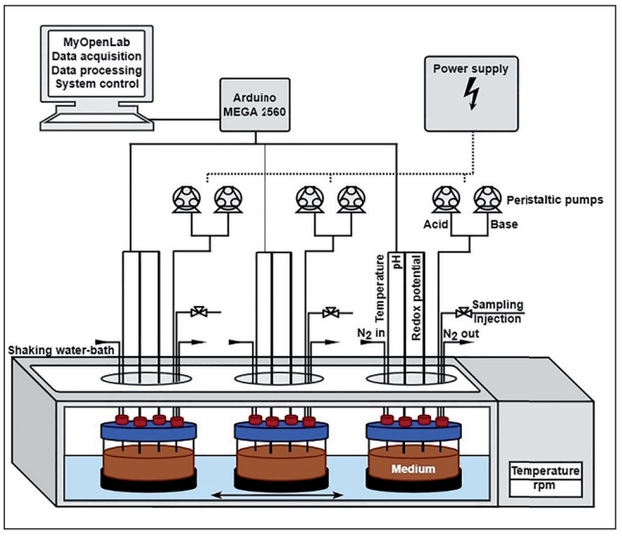

Fig. 5: Schematic representation of the MimiCol[3] by Beeck et al. (Beeck et al. 2022)

This *in vitro* model consists of three glass vessel, which hold 250 mL volume each. Schaedler broth was used as a culture medium. During the experiments, the pH of the medium was adjusted to 6.2 (± 0.25) whereby a pH value of 7.4 was set at the beginning. A pH drift over time can occur due to bacterial metabolic processes, therefore the model is equipped with programmable peristaltic pumps. This allows the required pH range to be maintained by titration with 1 M HCl and 1 M NaOH solution. The pH control is activated when the pH drops below 6.4 and is deactivated during the media change.

In a shaking water bath (SW22 model, JULABO GmbH, Seelbach, Germany) at 37 °C, constant mixing of the vessel volumes is ensured at a speed of 100 rpm. Nitrogen is continuously supplied to the vessels via an air supply system to create an anaerobic atmosphere in the vessels, which are already gassed 1 h before the start of the experiment. To monitor the process parameters during the experiment, a pH and redox electrode and a temperature sensor are inserted into each vessel and recorded using the software *MyOpenLab®*. While the data recording for vessel 1 is done digitally, the measured parameters for vessel 2 and 3 are recorded manually. Figure 6 illustrates an example of data recording in vessel 1.

The experimental conditions were based on those of the MimiCol by Beeck et al. (Beeck et al. 2021), whereas the experimental duration was reduced from 9 to 7 h. The pre-cultured and frozen microbiota were prepared on the experimental day as follows. The frozen fecal suspensions were completely thawed at 37 °C in a water bath. Thereafter, they were centrifuged at 6000 rpm for 6 min and the supernatant discarded. The bacterial pellet was resuspended in 10 mL Schaedler broth and injected into the appropriate vessel at the beginning of the experiment. For the experimental procedure with the pooled microbiota (SM_{pooled}), microbiota SM_{omni_1}, SM_{veget} and SM_{meat} were combined and homogenized in a flask after thawing. Afterwards, they were divided equally into tubes and centrifuged at 6000 rpm for 6 min. The bacterial pellets were dissolved in 10 mL Schaedler broth each and injected into the individual vessel at the start of the experiment.

At the start of the experiment, the microbiota was injected into the respective vessel and cultured for 3 h. The medium was changed after 3 and 5 h to ensure good bacterial growth by adding fresh nutrients. During the media changes, a residual volume of 15 mL remained in the vessel, so the change was performed at a ratio of 90:10. Besides, 10 mL of Schaedler broth containing 10 mg of baicalin was injected after addition of 125 mL of pre-tempered Schaedler broth. Samples of 5 mL were taken every 30 min and 5 min after media change to determine bacterial growth by OD and metabolisation of baicalin. The medium taken at this time was not replaced by new media. Samples for the analysis of the metabolisation of baicalin to baicalein were prepared as described in 4.2.2 and measured by HPLC.

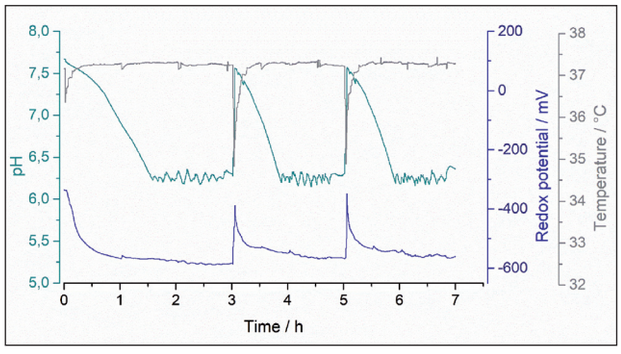

Fig. 6: Process parameters (temperature (grey), pH (cyan), redox potential (blue)) in the MimiCol[3] over 7 h (150 mL Schaedler broth, 100 rpm, pH 6.2 (± 0.25), 37 °C, n = 1) in vessel 1.

Optical density was determined photometrically, using 200 µL sample diluted with 1800 µL 0.9% NaCl solution. The sample at time 0.083 h served as a blank. To analyze the bacterial composition of the microbiota, CFU samples were taken hourly and 5 min after the media change. These were prepared and stored as described in 4.2.4 until the time of plating.

Table 4 lists the assignments of the microbiota to each vessel of the MimiCol[3]. This alternating distribution of standard microbiota was done to obtain representative results. A setup without added microbiota (SM_x) was performed as a reference experiment. Thereby, it should be determined whether a metabolisation of baicalin to baicalein also occurs in the absence of the microbiota.

Table 4: Different distribution of the standard microbiota in each of the three vessels of the MimiCol[3] when performing the experiments to minimise bias in the results

Experiment	Vessel 1	Vessel 2	Vessel 3
1	SMomni_1	SMveget	SMmeat
2	SMmeat	SMomni_1	SMveget
3	SMveget	SMmeat	SMomni_1
4	SMomni_2	SMomni_2	SMomni_2
5	SMpooled	SMpooled	SMpooled
6	SMx	SMx	SMx

4.2.6. Quantification of baicalin and baicalein by HPLC

The metabolisation of baicalin to baicalein was evaluated as follows. The molar mass of baicalin is 446.36 g/mol, while that of baicalein is 270.24 g/mol, leading to a lower result for the mass of baicalein. This results in the following calculation. When 10.00 mg of baicalin is injected into the vessel, a maximum of 6.054 mg of baicalein can be obtained. In the analysis of the HPLC data, the peak areas were plotted against the concentration (µg/mL) and the respective concentration at the time of the sample was calculated via the calibration curve. Thus, for baicalin, the concentration in the vessel could be determined by calculating the percentage of the amount of substance added at the time of addition. For the evaluation of baicalein, the conversion from the amount of baicalin used to the maximum resulting amount of baicalein still had to be calculated. To calculate the percentage of baicalein, it was assumed that 100% of the added baicalin can be converted.

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Institutional Review Board Statement: The study was approved by the ethics committee of University Medicine Greifswald (BB 009 / 22).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Subjects are not identifiable in these data; nonetheless, written informed consent has been obtained from the subjects for the publisher.

Data Availability Statement: The data presented in this study are available at the University of Greifswald's storage system.

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