

Project Report

Study Acronym:

CuVi-16

Title:

Bioavailability of Vitamin B₉ (Folic Acid) from sprouted buckwheat

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1 EXECUTIVE SUMMARY

1.1 PROJECT BACKGROUND AND STUDY RATIONALE

The company Global Vital GesmbH germinates batches of buckwheat in vitamin enriched water. The germinated sprouted buckwheat is rich in the specifically added vitamin, enzymes, fibers, etc. The dried and milled (powdered) buckwheat is a raw material for various food manufacturers.

We investigated the bioavailability of folic acid (FA) and compared:

1. Cultavit®-FA, Vitamin enriched buckwheat powder
2. Powdered buckwheat powder (“empty” control),
3. Pure FA (Vitamin B₉, positive control).

1.2 ETHICAL AND LEGAL ASPECTS

The candidates provided their informed consent in written and agreed that their personal data including the study results are electronically stored and used for scientific purposes.

1.3 METHODS

We tested the feasibility of the study protocol with 5 test persons (TP) in a pilot phase, and optimized the study protocol for the main phase. At project start the available literature allowed to estimate the study power (cohort size). We aimed at a study power of 80% and at a significance level of $P = 0.05$, when we investigated the bioavailability of FA out of the three formulations: 1) Cultavit®-FA, 2) pure FA (Vitamin B₉), 3) buckwheat powder within a cross over design.

Children and young (< 18 years), diseased, and elderly (> 60 years) candidates were excluded from participation. The study was advertised at five university campus in the neighborhood of the study centre. For the pilot phase we recruited 6 TP. For the main phase 19 TP were recruited and completed their participation. Before and after ingestion of the capsules with the test substance hourly blood sampling and laboratory analyses revealed the FA serum levels. We computed the AUC (Area Under the Curve), for the different test items, and compared the FA bioavailability between them.

1.4 RESULTS

As expected the intake of milled buckwheat (negative control) did not cause distinguishable changes of the FA serum level.

After intake of 1000 µg pure FA (positive control) the serum levels were raised. The peak occurred after one hour, before the FA serum level decreased again and reached baseline levels after 6-7 hours.

After intake of 1000 µg FA in sprouted buckwheat powder (Cultavit®-FA) - compared to pure FA - the serum level raised slower, the peak occurred later and was higher. The AUC was larger, indicating superior resorption kinetics and a higher bioavailability. The AUC of the FA resorbed from the Cultavit® buckwheat matrix was 38% higher. The difference between the two FA formulations was statistically significant (p=0,049).

1.5 INTERPRETATION

Compared to the pure vitamin, the bioavailability of FA is significantly higher after the ingestion of Cultavit®-FA. The buckwheat grain and sprout matrix may protect the Vitamin from degradation before resorption and/or retards the resorption.

2 BACKGROUND

2.1 FOLATE, FOLIC ACID (FA)

FA is a Vitamin from the B-family. It supports adequate DNA replication. In case of deficiency the precursor-red-blood-cells do not divide properly, and give rise to megaloblastic anemia. FA deficiency may lead to mutations of cells with unrepaired or misrepaired DNA damage. Cancer may be a long term result, if critical genes (proto-oncogenes, tumor suppressor genes, etc.) are affected.

Due to a higher demand, FA deficiency is quite common during pregnancy. There is an association between the incidence of neural tube defects (e.g. spina bifida) and maternal folate deficiency, possibly attributable to hyperhomocysteinemia. Polymorphisms in genes encoding key enzymes in folate and homocysteine metabolism play a major role in occlusive vascular disease and neural tube defect (Ball, George F. *Vitamins In Foods*. CRC Press, 2006. VitalBook file). Figure 1 shows the chemical structure of FA.

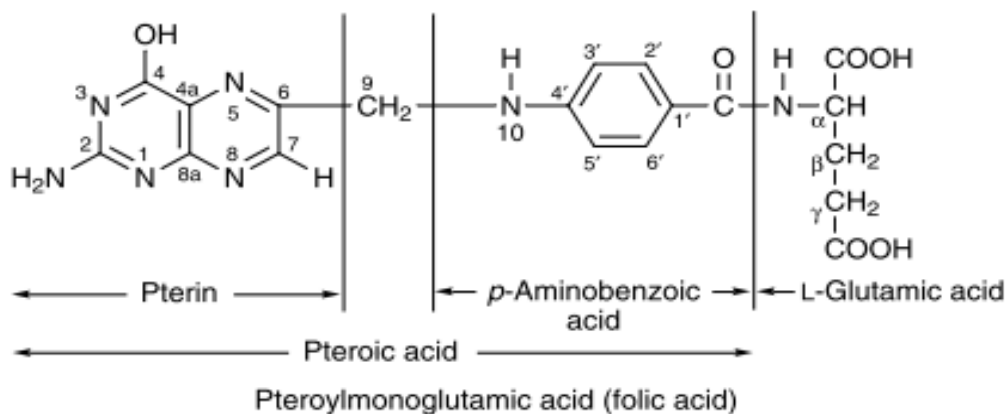


Figure 1 Chemical Structure of Folate, Raw green plants contain complexed **poly**-glutamyl-Folate, which is degraded to **mono**-glutamyl-Folate before absorption. (Figure modified from: George F.M. Ball; *Vitamins in Food, Analysis, Bioavailability, Stability*; PDF Books, CRC, Taylor an Francis New York)

2.2 FOLATE SOURCES

Most raw “green food” can be a source for FA. Raw spinach is very rich in FA, but cooking destroys ~ 80%. Full grain bread can be a source for FA, baking destroys ~ 20%. Other sources of FA are milk and beans.

2.3 FOLATE METABOLISM

Natural FA is a complex molecule with many glutamyl residues. After digestion in the intestinum the monoglutamyl form is absorbed by specific receptors. If the concentration

exceeds the transport capacity, this leads to unspecific uptake by diffusion through the intestinal lining. After the transfer from the small intestine to the blood compartment the FA is transported to the liver, chemically modified and stored. Some FA is secreted with the bile to the intestine and re-absorbed from there. High blood serum levels are regulated by the kidneys. Therefore, a retarded absorption, which delivers the ingested FA not in a “single short wave” but prolonged can increase the overall bioavailability of FA.

3 PROJECT RATIONALE

Because ingested FA is digested and degraded (in the stomach and small intestine), plant matrix constituents may protect the molecule and therefore improve the bioavailability of monoglutamyl-folate. To test whether or not buckwheat is such a protective plant constituent, we realized a clinical trial to investigate if FA integrated in buckwheat has a better bioavailability than the pure Vitamin.

4 PROJECT PLANNING

Literature Search

The survey of the available literature before the detailed planning of the study yielded over 500 peer reviewed publications on the topic “folate” & “bioavailability”. The search provided a basis for the detailed study planning.

Study Protocol development

The study protocol integrated the available literature and yielded the essential background information for the study team.

Randomisation

Before the start of each study phase we randomized the allocation of the recruited test persons to the specific group with the programme RandList V. 1,2 (Datinf GmbH, Germany).

Study advertisement

The study was announced on black boards at universities, and/or in medical practices. In view of the estimated study power we aimed to recruit 19 TP. Therefore, we opted against advertising the study in daily newspapers or similar media. We succeeded to recruit the planned test persons within two weeks and felt limited by the capacity of the study centre (seven test persons per week).

Ethical aspects

The study protocol complied with national legal regulations, GCP-guidelines (good clinical practice), and the Helsinki declaration (including later amendments) on studies with human subjects. Because the investigated substances are “food” and no “pharmaceutical or medical device”, we did not apply to any ethical committee.

Filling of Capsules

The substance of intent was filled into standard size capsules made of starch. Each capsule contained 500 µg FA, or the same volume of “empty” buckwheat powder.

The capsules - provided by the study sponsor - ensured that the dose between the different test days were identical. Capsules avoided the need of handling the powder on the test days.

5 CLINICAL STUDY MATERIAL AND METHODS

5.1 SUBSTANCES USED IN THE CLINICAL TRIAL

5.1.1 Test item 1, powdered buckwheat, negative control

Milled (powdered) buckwheat was taken as “empty” control. The ingredients are:

- Carbon hydrates (starch)
- Grain and germ vitamins
- Undigestible fibres
- Protein
- Enzymes
- Trace elements
- Essential fatty acids
- Anti-oxidants
- Secondary plant flavonoids

5.1.2 Test item 2, Cultavit®-FA (Vitamin B₉).

Cultavit®-FA was produced as follows:

- Dissolve FA in water
- Soak grains with the FA enriched water
- Grains take up vitamins dissolved in the soaking water, and integrate the vitamins in grain structures and growing sprouts
- The sprouting is stopped by drying
- The dried sprouted & vitamin enriched grain is milled (pulverized)

The ingredients of Cultavit® Folate:

- Added and integrated FA and
- Buckwheat ingredients, like
 - Carbon hydrates (starch)
 - Grain vitamins
 - Undigestible fibres
 - Protein
 - Enzymes
 - Trace elements
 - Essential fatty acids
 - Anti-oxidants
 - Secondary plant flavonoids

5.1.3 Test item 3, Pure monoglutamyl-folate, Vitamin B₉

The very same monoglutamyl-folate, which was dissolved in the soaking water for the buckwheat sprouting for the production of Cultavit®-FA in capsules served as positive control. The capsules of test item 3 contained the same amount and concentration of FA as the capsules with the Cultavit®-FA (test item 2).

5.2 STUDY DOCUMENTS

Study advertisement

We advertised the study with flyers and posters, distributed in the neighborhood of the study centre, at doctor offices or universities to invite preferentially young adults to participate.

Recruitment documents

To achieve homogeneous groups we systematically excluded persons with conditions that could introduce a bias to the study result. Table 1 shows the exclusion criteria.

Table 1, Exclusion criteria

Item	True	Not true
Age < 18 or >50 years		
Pregnancy?		
Acute, chronic, neurological or psychiatric condition		
Overweight > 35kg		
Disease of heart, kidney, liver, lung, _____		
Acute diarrhea during the last 4 weeks		
Cancer in the intestine, chronic inflammatory intestinal disease (Colitis ulcerosa, Morbus Crohn)		
Diagnosed buckwheat allergy		
Intake of Vitamin supplements in the last week		
Diagnosed intestinal condition associated with inhibited resorption		
Typ I diabetes or severe metabolic disease		
Severe gluten intolerance		
Infectious disease with fever in the last 4 weeks		
Cortison or antibiotic therapy in the last 4 weeks		
Participation at another study		

Healthy candidates with no exclusion criteria, who provided their informed consent to participate and consented to store their personal data electronically, and use their personal data anonymously for research purpose, were recruited.

We asked the candidates for their nutritional preferences. Vegetarians were advised to abstain from vitamin rich food on the day before their participation.

Documents on the observation day

During the test day the test persons documented every study specific event (capsule intake, blood sampling) on their “day-protocol”. This protocol contained the participant number, the name and date of birth, as well as the test item (test substance) and the exact time (hour, minute) of each event.

Case Report Form

After the participation we collected the “day protocol” and the blood serum parameters for each participant.

5.3 LABORATORY ANALYSES

Blood samples were analyzed for the FA content in a certified medical laboratory. From the first and last blood sample a full hemogramm was produced. It was announced as service for the test persons, but also served the study team as screening method for adverse conditions.

5.4 STUDY COURSE FOR THE RECRUITED TEST PERSONS

The testing of each item started in the morning and ended in the afternoon. With one exception (2 weeks pause), the volunteers paused one day between the test days.

The course on the specific test days was:

1. Baseline blood in the morning before the first meal.
2. Intake of 3 capsules (1500 µg, in the pilot phase), and 2 capsules (1000 µg, in the main phase) at start of the breakfast.
3. Hourly blood sampling for 7 hours.
4. Certified laboratory Vitamin B₉ serum analyses to:
 - a) Compare Cultavit®-FA intra-individually (cross over design) to „empty“ buckwheat.
 - b) Compare Cultavit®-FA intra-individually to chemically pure mono-glutamyl-Vitamin B₉.

6 CLINICAL STUDY REALIZATION

6.1 PILOT PHASE

Study team training

SCIgenia held responsible for the communication within the study team. The study team was trained before the first test persons arrived. As a result of the training the study centre dedicated a room entirely to this particular study.

Test days

The first blood sampling (baseline) was just before breakfast and intake of the capsules. The test persons received the substance of intent, breakfast and lunch. The time between the follow up blood samples was one hour.

Blood samples and CRF- monitoring

Every test person provided eight blood samples per test day. At the first and the last blood sampling a hemogram (full blood count) was made as a service for the test persons but also to check if the frequent blood sampling caused a recognizable blood loss.

Data entry

The laboratory data were entered into a spread sheet by the so called “double entry” method. The data were entered twice, independently; the first entry was subtracted from the second entry to receive “0”, if both entries were identical. Differences indicated obvious transcription errors which were corrected according to the original laboratory protocol.

Statistics and analysis

The pilot phase data did not reveal significant results, but trends. The mean and standard deviation of the pilot phase data were used to confirm the study power calculation, and resulting cohort size for the main phase.

6.2 DISCONTINUITY

The first analysis of the pilot phase data revealed unpalatable - extremely high - values for test item 3 (pure vitamin). We consulted the protocols of the pharmacist, who filled the capsules and detected a serious error. One capsule did not contain 500 µg as expected, but 5.000 µg of FA. We called the six probands and inquired whether or not they noticed anything unusual. Specifically we asked for nausea, gastrointestinal symptoms, or any unusual sensation. Five test persons agreed to be tested again with the correct dose.

6.3 PROTOCOL ADAPTION BASED ON PILOT PHASE EXPERIENCE

After the pilot phase:

- The number of follow up blood samples remained “7”.
- The time between the blood samples remained one hour.
- The daily intake was reduced from 1500 µg FA (3 capsules) to 1000 µg (two capsules).
- During the test day the nutrition of the main phase TP was rigorously controlled. The test persons received food, which contained no or very little vitamin (mainly white flower products).
- We reduced the days for testing item 1 (empty buck wheat powder)

6.4 MAIN PHASE

SCIgenia trained the team in the study centre on the modified protocol, and recruited 19 test persons. On the first day they were randomly assigned to take the test item 2 or 3, and provided 8 blood samples every hour. Two days later the test persons ingested the alternative test item and again provided 8 blood samples. Four test persons volunteered to come in for an additional day to ingest test item 1 (empty control).

6.5 DATA PROCESSING AND STATISTICAL ANALYSES

Again we employed the double entry method to transfer the laboratory data to a spreadsheet as described for the pilot phase. After the data set was closed, we computed the AUC by the linear trapezoidal method for each test person per day. The intra individual statistical comparison of the AUCs were done with the software package IBM SPSS Statistics V.23. The specific test is mentioned in the table legends.

7 RESULTS

7.1 PARTICIPANTS (TEST PERSONS)

Table 2, Test persons age and gender in the pilot phase and main phase. In the pilot phase 6 TP were recruited, after 1 dropout 5 TP provided analysable data.

	pilot phase	main phase
Mean Age	27,0 ± 7,3	24,5 ± 2,2
Min Age (yrs)	22	20
Max Age (yrs)	43	30
Gender	pilot phase	main phase
M	2	4
F	(4-1) = 3	15
N Test Persons recruited and (completed)	(6-1) = 5	19

Table 3, test items and analyzed test days

test item	N, pilot phase	N, main phase
Cultavit®-FA	5	19
Pure FA	5	19
Buckwheat	5	4
Total	15	42

We observed no considerable blood loss or changes in the hemogramme in any of the test persons (data not shown).

7.2 PILOT PHASE

We enrolled 6 test persons into a test run (pilot phase). One test day had to be repeated with the correct dose (1500 µg). Figure 2 shows an example of the course after intake of the exaggerated and correct FA doses.

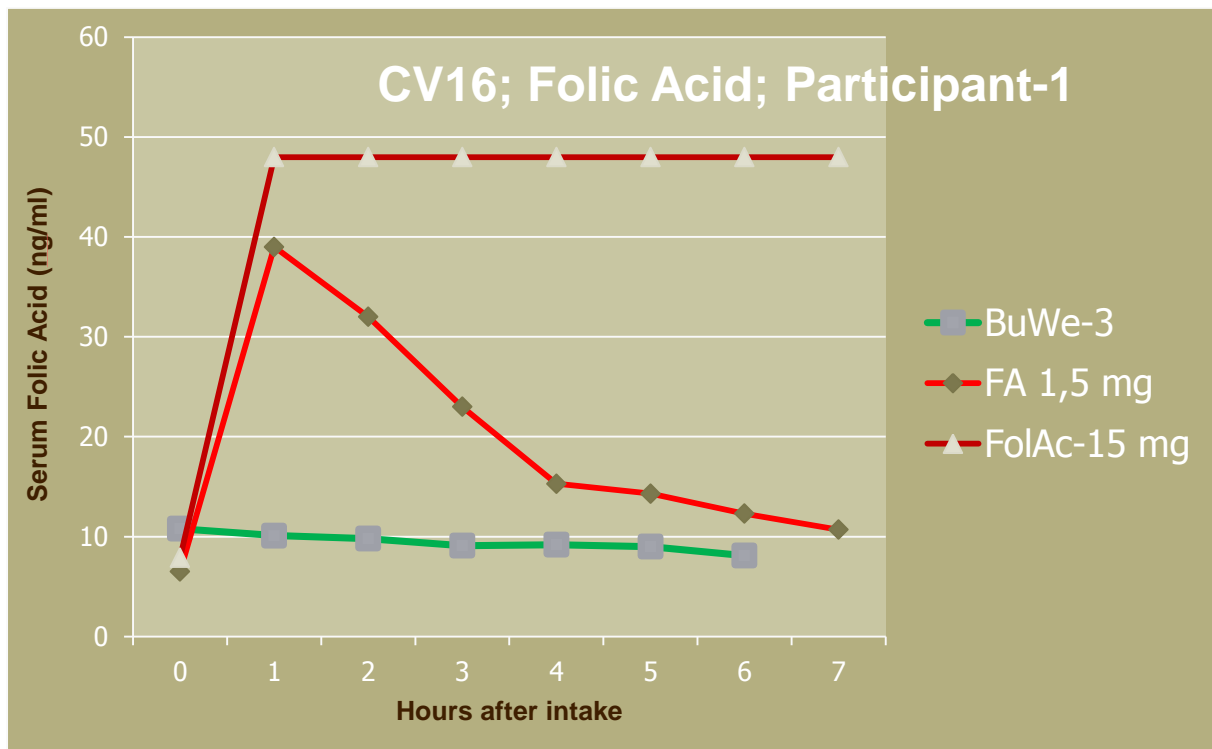


Figure 2, Comparison of two ingested doses FA; hourly monitoring of the serum levels. The high dose lead to an increase of FA serum levels beyond the range of the laboratory test, resulting in a plateau. When repeating the experiment two weeks later the low dose lead to an increase and decrease of the FA serum level within the measuring range of the laboratory test. The ingestion of buckwheat powder (BuWe-3, green line) did not cause particular changes of the FA serum level.

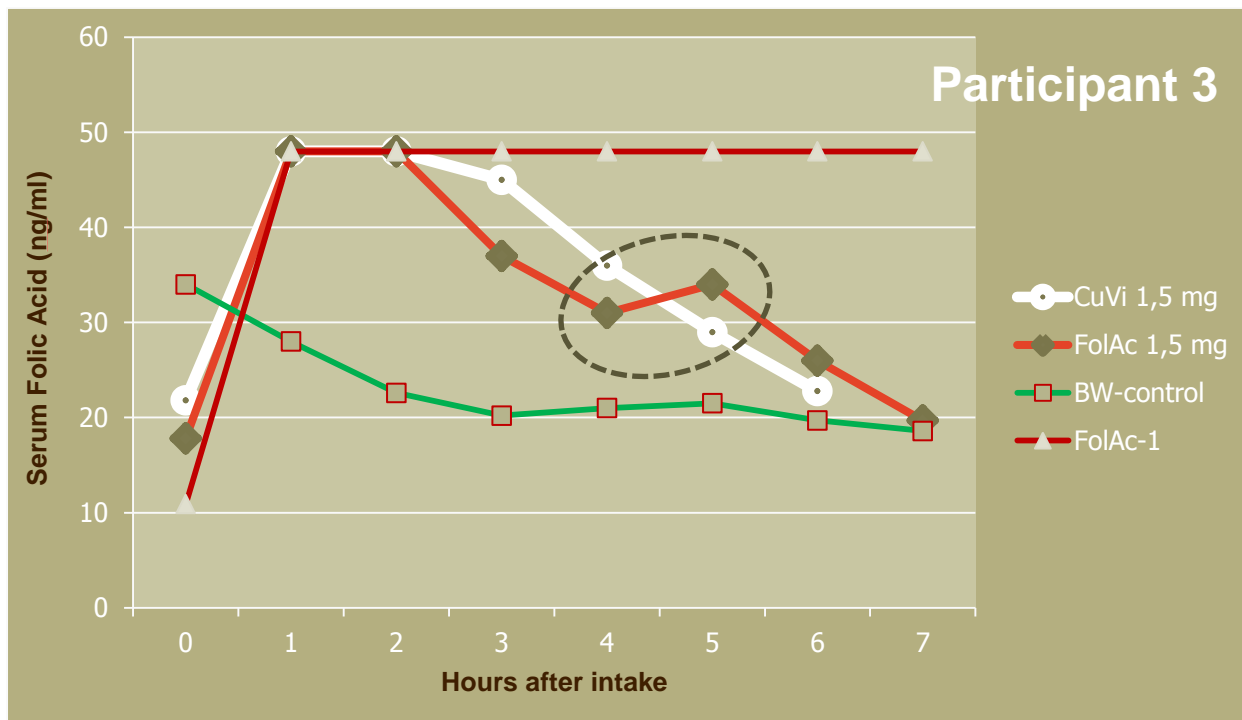


Figure 3, In test person 3 the exaggerated FA dose (FolAc-1) was given on the first day. As in participant 1 the serum levels raised quickly and remained above the measurable range for the rest of the day. On the next day (green line □) the baseline serum level (hour 0) was above 30ng/ml and after ingestion of “BW- control” dropped during the day, indicating that the initial high levels were a consequence of the overdose on the day before.

In this TP both, the Cultavit®-FA (CuVi) and the pure FA (FolAc) “hit the ceiling” of the test system at hour 1 and hour 2, after ingestion of 1500 mg. This observation prompted us to reduce the test dose from 1500 µg in the pilot phase to 1000 µg in the main phase.

Note that the curve to FolAc increases from hour 4 to hour 5 (circle), indicating that the test person had an un-authorized Vitamin B₉ uptake during the day. As consequence we increased the tightly monitoring of the test persons during the main phase, to avoid similar artefacts.

7.3 AMENDED PROTOCOL FOR THE MAIN PHASE

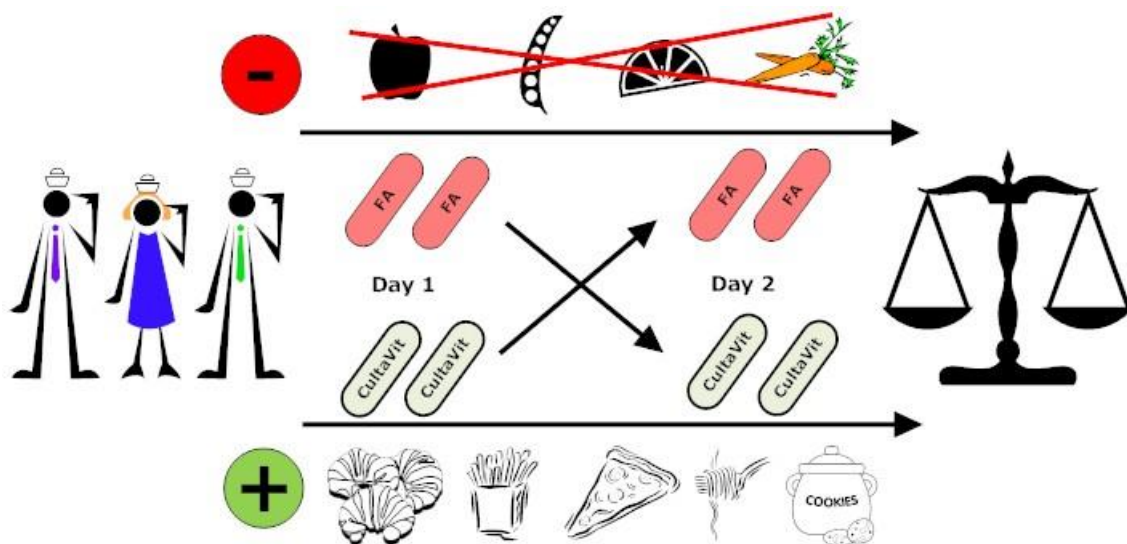


Figure 4, Scheme of the study course for the main phase. The probands were tightly monitored to exclude any consumption of FA containing food during the test day. The test items ingested on day 1, where randomly assigned, the test persons received either capsules with pure Folic Acid (FA, red) or Cultavit®-FA (white). After a pause of one day, the test persons appeared for day 2 to receive the alternative test item. The FA blood serum monitoring results after ingestion of both test items were compared per test person (cross over design).

7.4 MAIN PHASE

7.4.1 Average FA serum levels

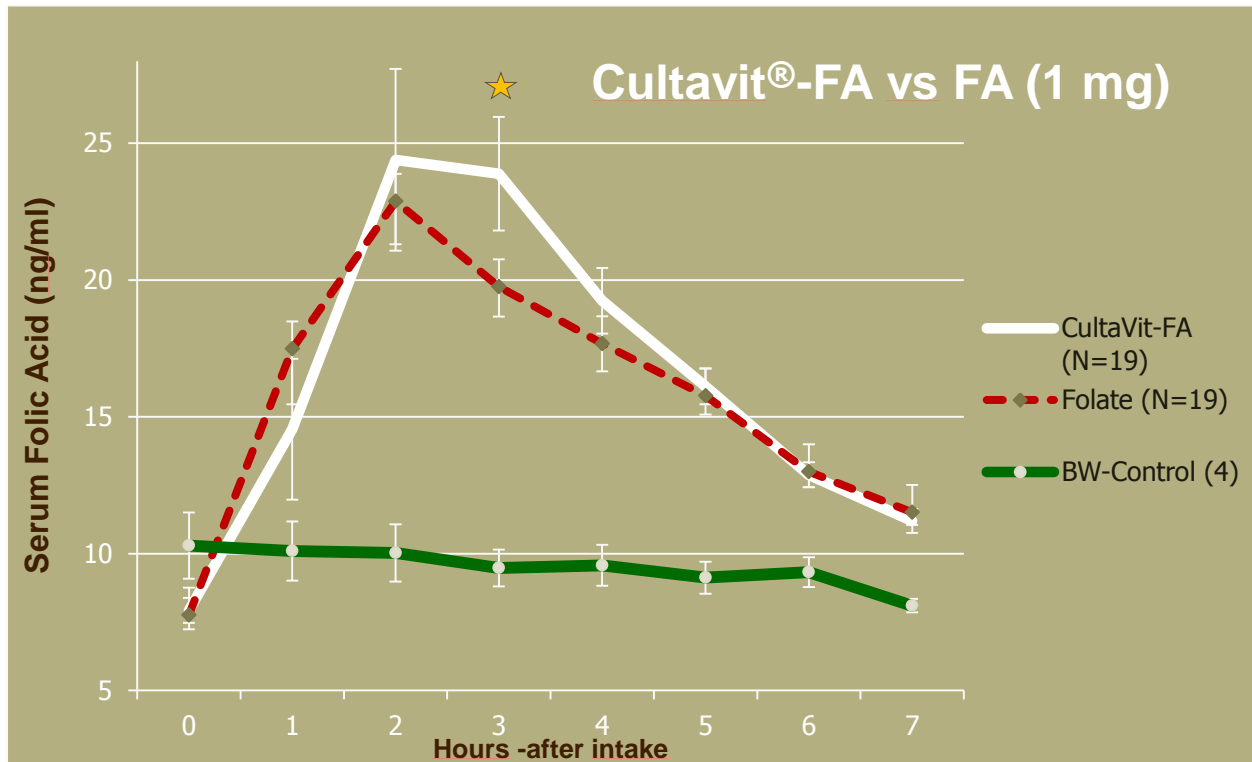


Figure 5, Average FA-serum levels before and after ingestion of the test items by 19 test persons or 4 test persons (control group). The mean baseline values (hour 0) ranged from ca. 7,5 ng/ml to 10 ng/ml. After ingestion of the FA containing test items the serum levels increased until hour 1 and 2. The increase was slightly slower for Cultavit®-FA, the peak after two hours was higher. Three hours after Cultavit®-FA ingestion the average serum FA level was significantly above the levels after ingestion of pure FA (Folate, red dotted line, ★ $p = 0,05$). The negative BW-control (empty buckwheat, green line) did not raise the FA-serum level.

7.4.2 Bioavailability (AUC, Area Under the Curve) analysis

To analyse the serum-level-change after intake the baseline levels (at hour 0) were subtracted from each obtained serum level, and the AUC was computed. Table 4 summarizes the AUCs for each test item. The bioavailability in five hours for pure FA was $14,2 \pm 1,27$ (\pm Standard Error of Means). The AUC after ingestion of Cultavit®-FA was significantly higher.

Table 4, AUC (Area Under the Curve), five hours monitoring after intake of pure FA or Cultavit®-FA. If the average bioavailability of pure FA ($14,2 \pm 1,27$ ng/ml*5h) is set 100%, the bioavailability of CultVit-FA is 38% higher. The difference is statistically significant (Students-t-Test for paired variables)

AUC after ingestion of the test item:	ng/ml*5h	SEM	N	Percent
Pure Folic Acid 1000 µg	14,2	1,27	19	100
Cultavit®-FA 1000 µg	19,6	2,50	19	138
Students' t-Test	P = 0,049			

Figure 6 shows the AUC course for the first 5 hours after ingestion. The ingestion of 1000 µg FA lead to a peak after one hour before the serum level decreased again. Cultavit®-FA raised the serum level slower, the peak occurred after 2 hours, and it was higher (Figure 6). Compared to pure FA the decrease was at a similar rate. Due to the higher peak the AUC after Cultavit®-FA ingestion was 38% higher. The difference was statistically significant ($p=0,049$).

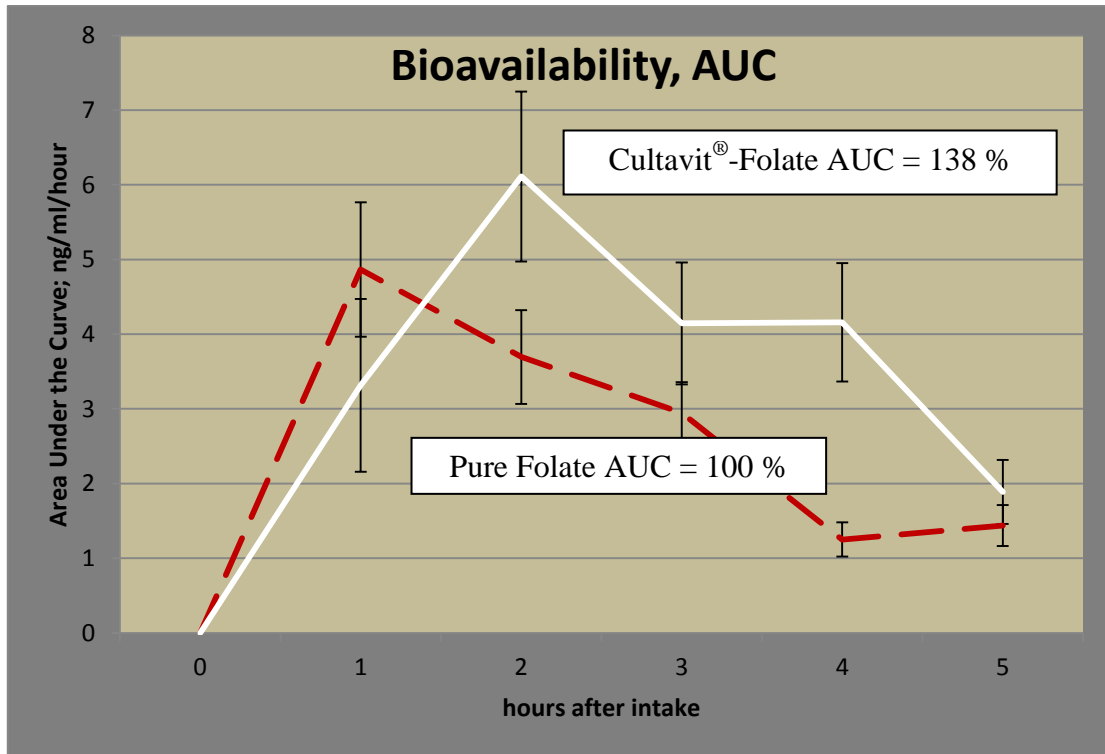


Figure 6, Bioavailability (change of serum levels) after ingestion of pure FA; the FA bioavailability after ingestion of Cultavit®-FA is 38% higher; the difference is statistically significant.

8 CONCLUSIONS

Monoglutamyl-folate from Cultavit®-FA reveals a significantly higher FA bioavailability than pure monoglutamyl-folate.

Both, the sprouting process and/or the FA presentation in a plant matrix may contribute to the superior bioavailability of Vitamin B₉ (Folic Acid) after Cultavit®-FA ingestion.

FA supplementation in healthy persons is safe. Overdosing does not cause any adverse effects. After ten fold overdosing, no adverse effects or unusual sensation (nausea, irritation, reduced wellbeing, etc.) were noticed.

The study CuVi-16 was planned and realized in Vienna in spring 2016

The responsible principal investigator and responsible for the report content is

Prof. Dr. Wilhelm Mosgoeller

Vienna, in June 2016