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Folate determination in livers of different animal species

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Abstract: The liver is the main storage organ for folate. In the study the folate content in chicken, turkey, pig, and beef livers was evaluated with the use of the HPLC (high performance liquid chromatography) method which allowed various folate vitamers differentiation. The total folate content in the tested livers ranged from 419 to 1289 μg 100 g^{-1} in pork and chicken livers, respectively, and was several times higher than in folate rich vegetables, which are the most common folate sources in the human diet. Additionally, good stability of two folate abundant forms, tetrahydrofolate and 5-methyltetrahydrofolate, under frying was shown in the chicken liver samples. The obtained results contribute well to the general need of promoting the folate intake from various natural sources, not only of plant origin.

Keywords: folic acid; offals; frying; HPLC; food composition

The generic term "folate" refers to the group of B vitamin and includes naturally occurring folates and folic acid which is the synthetic form of folate used extensively in dietary supplements and for food fortification. Folate prevention role in macrocytic anemia, cancer, cardiovascular diseases, neurocognitive diseases (Ebara 2017) and neural tube defects (Czeizel et al. 2013) have been comprehensively reviewed in the recent literature. Despite folate importance for the proper functioning of the body, its deficiency in the diet is common worldwide. Since, mandatory fortification of selected food products with folic acid remains controversial, the consumption of food naturally rich in folate should be widely promoted (Fajardo et al. 2017).

According to the European Food Safety Authority, a Population Reference Intake for folate was established at the level of 330 μg day^{-1} (EFSA 2014). Folate are present in a wide range of foods, with high concentrations in green leafy vegetables, pulses, wheat germ, yeasts and some cereals (Delchier et al. 2016). Often neglected good dietary folate source is an animal liver, and liver derived products (e.g., paté), which is the main storage organ for folate in the body (Bailey 1992). Folate content in a liver is expected to be even several times higher than in the most folate abundant "green" sources, such as spinach and broccoli. In the Winkels et al. (2007) studies the aggregate bioavailability of folate from the liver was showed at the high level of approximately 80% and was equal to that from fruit and vegetables. Furthermore, the recent results overturned former reason to avoid liver consumption, showing bovine liver to be almost free of harmful substances such as heavy metals (Nohr & Biesalski 2007; DGE 2004). Meanwhile, little has been reported on the folate content in the livers of different animal species. Literature data are extremely variable, and presents only the sum of folate, without distinction between various folate vitamers which differs in bioavailability and stability in food products under changing chemical and physical conditions.

This study was designed to provide reliable data on the folate vitamers content in the livers of four animal species. Additionally, because of folate possible low stability during heating treatment of foods, the effect of frying on the folate content in the chicken liver was also evaluated.

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MATERIAL AND METHODS

Samples. All samples, pig, chicken, turkey and beef livers were obtained from three different retail shops and from two local Butcher shops (Table 1). Amount of 200 g of sub-sample was blended (Braun 600; Braun GmbH, Germany) and 3 g sample was taken for folate content determination. The additional amount of 1 kg of each chicken liver sample (n = 5) was prepared for frying. Livers were fried in a heated frying pan with rapeseed oil addition. Amount of 200 g of sub-sample was blended and 3 g sample was taken for the folate content determination.

Certified reference material, CRM 487 (a lyophilized pig liver sample), was obtained from the Institute for Reference Materials and Measurements (Belgium) with the certified total folate content of 13.3 ± 1.3 mg kg^{-1}, determined by microbiological assay, and the 5-CH_3-H_4PteGlu content of 2.6 mg kg^{-1}, determined with the HPLC method with the fluorescence detection.

Standards and enzymes. Folate standards, folic acid (PteGlu), 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu), 5-formyltetrahydrofolate (5-HCO-H_4PteGlu) and tetrahydrofolate (H_4PteGlu) were obtained from Sigma Aldrich (USA); 10-formylfolic acid (10-HCO-H_4PteGlu) and 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4PteGlu) were purchased from Schircks Laboratories (Switzerland). Standards were all prepared according to the method described by Konings (1999). 10-formyldihydrofolate (10-HCO-H_2PteGlu) was obtained from 5,10-methenyltetrahydrofolate as described by Pfeiffer et al. (1997). α-amylase (E.C.3.2.1.1) and protease (E.C.3.4.24.31) were purchased from Sigma Aldrich and fresh rat plasma (source of folate conjugase) from Europa Bioproducts Ltd. (UK).

Sample pretreatment. Folate content determination was carried out according to Czarnowska & Gujska (2012) with the modification of protease addition. 3 g (accurate to 0.001 g) of previously blended sample was inserted into 30 mL Oak Ridge PPCO (Nalgene, USA) centrifuge tube. All samples were prepared in triplicate as outlined in Figure 1. During sample preparation, folate were protected from oxidation by the use of nitrogen, being kept under subdued light and cooled in ice, each time after heating. Purification of sample extracts was carried out prior to HPLC analysis by Solid Phase Extraction (SPE) on Strong Anion Exchange (SAX) Bakerbond spe. JT cartridges (3 mL × 500 mg Solid Phase Extraction Column, PP (polypropylene), Quaternary Amine (N+) Anion Exchange, USA) as described by Jastrebova et al. (2003).

HPLC analysis. The chromatographic separation of folate with the use of HPLC (Shimadzu Series LC-10A; Shimadzu Co., Japan) was carried out according to Czarnowska & Gujska (2012). Briefly, folate were separated using Phenomenex column: Synergi 4u Hydro-RP 80A, 250 × 4.6 mm, 4 µm (Phenomenex, USA). The chromatographic condition for gradient elution were as follows: flow rate: 1 mL min^{-1}, volume injection 50 µl, column temperature 25 °C, UV detection: 290 nm, fluorescence detection: 290 nm excitation and 360 nm emission.

Table 1. The folate content in raw livers (µg 100 g^{-1} of fresh weight)

Liver	H_4PteGlu	5-CH_3-H_4PteGlu	Total folate	Sample pooled from:	Shop
Pig 1	343* ± 20	127 ± 6	463**± 5	10 pcs	butcher
Pig 2	640 ± 51	180 ± 13	808 ± 9	10 pcs	butcher
Pig 3	297 ± 14	261 ± 6	546 ± 9	10 pcs	retail
Pig 4	387 ± 27	154 ± 13	532 ± 6	10 pcs	retail
Pig 5	321 ± 20	110 ± 11	419 ± 9	10 pcs	retail
Chicken 1	269 ± 35	656 ± 34	925 ± 63	1 kg	butcher
Chicken 2	269 ± 19	602 ± 57	870 ± 66	1 kg	butcher
Chicken 3	319 ± 3	970 ± 96	1 289 ± 66	1 kg	retail
Chicken 4	402 ± 32	823 ± 80	1 225 ± 109	1 kg	retail
Chicken 5	433 ± 14	642 ± 32	1 075 ± 28	1 kg	retail
Turkey 1	183 ± 15	990 ± 39	1 137 ± 26	1.5 kg	butcher
Turkey 2	218 ± 17	874 ± 78	1 059 ± 23	1.5 kg	retail
Turkey 3	81 ± 3	991 ± 37	1037 ± 25	1.5 kg	retail
Beef 1	340 ± 27	177 ± 16	508 ± 7	8 livers as a piece	butcher

* The results are presented as the mean of three replicates ± standard deviation (SD); **the total folate content is the sum of H_4PteGlu and 5-CH_3-H_4PteGlu contents calculated to folic acid using molar absorption coefficient given by Blakley (1969)

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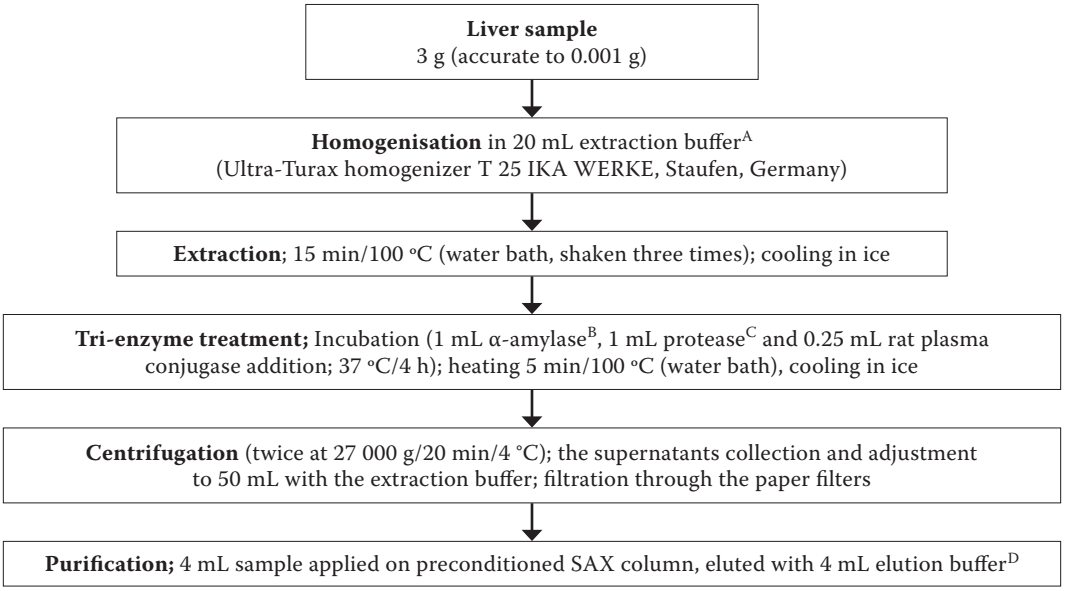

Figure 1. The flow chart of the sample pretreatment

[A]extraction buffer: 0.1 M phosphate buffer pH 6.1 with 1% (w/v) sodium ascorbate and 0.1% (v/v) 2-mercaptoethanol; [B]1 mL of α-amylase solution (20 mg mL^{-1}); [C]1 mL of protease solution (4 mg mL^{-1}); [D]elution buffer: 0.1 M sodium acetate containing 10% (w/v) sodium chloride and 0.1% (v/v) 2-mercaptoethanol

The mobile phase was acetonitrile and 30 mM phosphoric acid buffer, pH 2.3. The gradient started at 5% acetonitrile and reminded so for the first 8 min before being raised to 17.5% within 17 minutes. The total separation time was 41 minutes. Peaks were identified based on retention times of standard. Peak purity and identity were confirmed by comparison of relative peak areas in both detectors. Quantification of individual folate derivatives was based on fluorescence detection using external multilevel (n = 8) calibration curves with the linear range of 0.3–66.3 ng mL^{-1} for 5-CH_3-H_4PteGlu (the correlation coefficient > 0.9994), 0.6–55.7 ng mL^{-1} for H_4PteGlu (> 0.9996) and 3–150 ng mL^{-1} for 5-HCO-H_4PteGlu (> 0.9997). The limit of quantification (LOQ) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 10 (Jastrebova et al., 2003) and were at the level of 0.3, 0.6 and 3.0 ng mL^{-1} for 5-CH_3-H_4PteGlu, H_4PteGlu and 5-HCO-H_4PteGlu, respectively. The coefficients of variation for folate vitamers contents determined with the HPLC method were lower than 10% (n = 10).

All results of folate vitamers content in liver samples are based on the fresh weight (FW) and were presented as means with standard deviations from triplicates.

Method validation. Recovery tests were performed by analysing spiked control samples of chicken and pig livers with known amounts of 5-CH_3-H_4PteGlu, H_4PteGlu and 5-HCO-H_4PteGlu before extraction. The spiked samples were then processed through the entire procedure. The recovery (R) was calculated as $R = (C_{found} - C_{sample})/C_{added}$, where C_{found} is concentration in spiked sample, C_{sample} is the concentration in the sample before spiking and C_{added} is the concentration of the added standard. The mean recovery (n = 10) was 95% ± 7% for 5-CH_3-H_4PteGlu, 91% ± 8% for H_4PteGlu and 88% ± 9% for 5-HCO-H_4PteGlu. The repeatability of the analytical procedure was checked by using a certified reference material on different extraction days. The obtained mean value of 5-CH_3-H_4PteGlu was 3.12 ± 0.43 mg kg^{-1} and the total folate was 12.76 ± 0.61 mg kg^{-1}. These results were well in line with the certified values of 2.6 mg kg^{-1} for 5-CH_3-H_4PteGlu and 13.3 ± 1.3 mg kg^{-1} for the total folate.

RESULTS AND DISCUSSION

The total folate content. Three folate derivatives, 5-CH_3-H_4PteGlu, H_4PteGlu and 5-HCO-H_4PteGlu were identified in the analysed livers. In all samples H_4PteGlu and 5-CH_3-H_4PteGlu were found to be the predominant forms (Figure 2), which is in agreement with previously published data for livers (Müller 1993; Vahteristo et al. 1996; Konings 2001). Other identified folate form, 5-HCO-H_4PteGlu, was below the limit of

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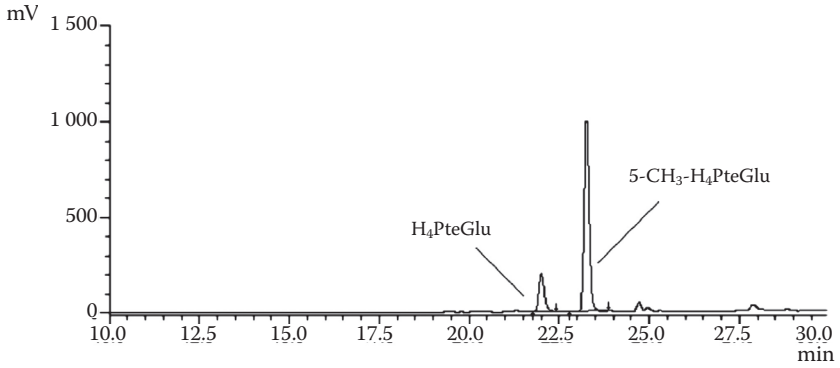

Figure 2. HPLC chromatogram of the raw chicken 3 liver sample (fluorescence detection: 290 nm excitation and 360 nm emission)

quantification (5 μg 100 g^{-1}). The highest mean folate content was found in poultry livers, 1 077 μg 100 g^{-1} in chicken liver and 1078 μg 100 g^{-1} in turkey liver (Table 1). All liver samples were characterized by much higher folate content than plant origin sources such as fresh spinach and broccoli, 180 and 159 μg 100 g^{-1}, respectively (Czarnowska & Gujska 2012). 5-CH_3-H_4PteGlu was the main vitamer in both poultry samples, whereas in pig and beef, H_4PteGlu constituted most of the folate activity (Table 1). This quantitative distribution of two main folate derivatives stays in agreement with previous Vahteristo et al. (1996) study. The author found domination of 5-CH_3-H_4PteGlu in frozen chicken livers and H_4PteGlu in raw pig and beef livers.

In Table 2 our results were compared with the literature data obtained mainly by liquid chromatography and microbiological methods. The results from previous years show high variability and make folate content comparison among livers from the same animal species almost impossible. Our results were closest to that of Vahteristo et al. (1996), when the HPLC method was used. Extremely different results of folate content in pig, chicken and beef livers were obtained in Holland et al. (1991) and Okholm-Hansen & Brogren (1991) studies when the microbiological method was applied. The lowest folate content in beef liver was determined with two less common methods, stable isotope dilution assay and optical biosensor (Rychlik 2004; Indyk & Woollard 2013).

Unfortunately, the problem with the variable folate data concerns all groups of food products. The existing folate data in the food composition tables are unreliable and need critical evaluation as this results from various procedures for folate extraction from food matrix and quantitative assays (Fajardo et al. 2017). Most commonly used microbiological method shows poor precision and fail to differentiate between several folate forms (Konings et al. 2001). In both microbiological and chromatographic analysis, extraction procedures for many products are incomplete, and according to some researchers, these differences can influence folate content greater than, for instance, the

Table 2. Comparison of the reported total folate content (μg 100 g^{-1} fresh weight) in livers of different animal species

Reference	Liver			
	pig	chicken	beef	turkey
Present study*	419–808	870–1 289	508	1 037–1 137
Vahteristo et al. (1996)	1 140–1 470	870–1 350	730–1 310	
Vahteristo et al. (1998)			690	
Müller (1993)	136		963	
Holland et al. (1991)	110	590	330	
Okholm-Hansen & Brogren (1991)	1 000	2 700	2 300	
Rychlik (2004)			298–770	
Indyk & Woollard (2013)			296	
USDA (2018)**		588	290	677

*The total folate content given as the range of the results presented in Table 1; **National Nutrient Database for Standard Reference Legacy Release

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variation in fruit/vegetable variety, season, climate and post harvested handling (Holasová et al. 2008). In case of the animal origin products, no data on the influence of animals breed, feeding, age or slaughtering traits on the folate content in meat and offal is available. Meanwhile, without such information it is difficult to conclude whether differences in folate content in the livers of the same animal species presented by different authors, are more due to the variety of the used analytical procedures or due to the factors related to the animal breeding.

Effect of frying on the folate content. Folate are highly sensitive to light, air, heat and extreme pH conditions. Thus, the significant factors which affect their retention during cooking are leaching into the water, temperature, time, pH, presence of antioxidants, moisture content as well as folate polyglutamate chain length (Delchier et al. 2016). Most of the published studies on folate losses during different food preparation methods concern plant origin foods. Only limited literature data on folate retention from foods of animal origin is available. In beef liver samples after grilling and frying, the losses were reported at the high levels of 41 and 50%, respectively (Aramouni & Godber 1991). In our study chicken liver samples 1–5 (Table 1) were chosen for the determination of folate vitamers retention during frying. The total folate content losses ranged widely from negligible values of 2% up to 35% (Table 3). However, in three out of five samples losses did not exceed the level of 25%. Moreover, different stability of two dominant folate forms, 5-CH_3-H_4PteGlu and H_4PteGlu, in the liver samples was observed. Although raw chicken liver samples contained more 5-CH_3-H_4PteGlu, higher losses (from 24% to 63%) were observed in the H_4PteGlu content, which is known to be one of the most labile folate form. Significant losses of 21, 46, and 89% in H_4PteGlu content were also reported by Vahteristo et al. (1998) in chicken, rainbow trout and pollack meat after oven-baking, respectively. In our study, the 5-CH_3-H_4PteGlu losses were considerably lower and ranged from 8% to 29%. In two out of five samples, the 5-CH_3-H_4PteGlu content increase of 7 and 8% after frying was observed. This could be caused by enzymatic interconversions in raw livers during the sample preparation or the methylation reaction during frying (Vahteristo et al. 1998). The unexpected increase, even up to 136%, of the 5-CH_3-H_4PteGlu content was also reported by Vahteristo et al. (1998) in rainbow trout sample after heating.

CONCLUSION

The promotion of balanced diet with higher amounts of fruit and vegetables is the worldwide strategy to avoid nutrient deficiencies and reduce the risk of the diseases of civilization occurrence. However, it should be assumed that the moderate amounts of meat, including liver, cannot be ignored. Extremely high folate levels in animal livers, even up to 1.3 mg 100 g^{-1} in fresh poultry livers, together with folate high bioavailability and high content of other valuable compounds, is the sufficient argument to introduce liver based products to the diet. This can greatly contribute to the recommended folate and other nutrients daily intake.

Table 3. H_4PteGlu, 5-CH_3-H_4PteGlu and total folate contents in raw and fried chicken livers together with the total folate loss and changes in H_4PteGlu and 5-CH_3-H_4PteGlu contents after frying

Liver		H_4PteGlu (μg 100 g^{-1})	Changes (%)	5-CH_3-H_4PteGlu (μg 100 g^{-1})	Changes (%)	Total folate (μg 100 g^{-1})	Loss (%)
Chicken 1[a]	raw	269[b] ± 35	−24[c]	656 ± 34.1	−8	925[d] ± 63.4	13
	fried	204 ± 3		605 ± 21.8		809 ± 18.9	
Chicken 2	raw	269 ± 19	−32	602 ± 57.1	+11	870 ± 65.9	2
	fried	182 ± 12		667 ± 35.3		849 ± 48.4	
Chicken 3	raw	319 ± 3	−52	970 ± 96.4	−29	1 289 ± 65.7	35
	fried	152 ± 13		689 ± 35.7		842 ± 48.4	
Chicken 4	raw	402 ± 32	−52	823 ± 80.4	−19	1 225 ± 109.1	30
	fried	192 ± 17		666 ± 6.0		858 ± 23.2	
Chicken 5	raw	433 ± 14	−63	642 ± 31.6	+7	1 075 ± 27.8	21
	fried	159 ± 11		686 ± 32.6		845 ± 43.5	

[a]Chicken samples description given in Table 1; [b]results are presented as the mean of three replicates ± SD; [c](−) indicates folate derivative decrease; (+) indicates folate derivative increase; [d]sum of H_4PteGlu and 5-CH_3-H_4PteGlu contents calculated to folic acid using molar absorption coefficient given by Blakley (1969)

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The obtained results showing folate high stability under heat treatment are promising. In 3 out of five analysed chicken livers after frying, the losses in the total folate content did not exceed 22%. This encourage further research on the effect of various food preparation methods used in households on the folate content in order to provide practical advice on cooking to improve folate status.

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